Brief Review

Genetic Mechanisms of Age Regulation of Blood Coagulation

Factor IX Model

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Abstract—Blood coagulation capacity increases with age in healthy individuals, apparently because of increases in the plasma concentration of most procoagulant factors. This phenomenon may play an important role in the advancing age–associated increase of cardiovascular diseases and thrombosis. Through longitudinal analyses of transgenic mice, we recently identified 2 critical age-regulatory elements, AE5′ and AE3′, which are together essential for age regulation of the normal human factor IX (hFIX) gene. AE5′, present in the long interspersed repetitive element–derived sequence of the 5′ upstream region, containing polyomavirus enhancer activator-3 or a closely related element, is responsible for age-stable expression of the gene and functions in a position-independent manner. AE3′, present in the middle of the 3′ untranslated region, is responsible for age-associated elevation of hFIX mRNA levels in the liver. Presence of both AE5′ and AE3′ is needed to recapitulate normal age regulation of the hFIX gene. Because factor IX clearance from the circulation is not significantly affected by age, age regulation of hFIX levels is achieved primarily by a combination of stabilization of gene transcription and age-dependent increases in the mRNA levels, which are presumably due to increasing mRNA stabilization. The stage is now set for further systematic studies of the genetic and molecular mechanisms of age regulation of other key coagulation and anticoagulation factors in hopes of understanding the overall age regulation of blood coagulation. (Arterioscler Thromb Vasc Biol. 2000;20:902-906.)

Key Words: aging ■ homeostasis ■ factor IX ■ hemophilia ■ cardiovascular disease ■ thrombosis

Blood coagulation plays a critical role not only in homeostasis but also in many physiological and pathological conditions. Blood coagulation potential in humans as well as other mammals reaches a young adult level around the time of weaning, followed by a gradual increase during young adulthood and an almost 2-fold increase by old age. This advancing age–associated increase in coagulation potential takes place in healthy centenarians, indicating that the increase is a normal age-associated phenomenon. However, it is conceivable that in the general human population, such increases in blood coagulation potential may substantially contribute to the development and progression of age-associated cardiovascular and thrombotic disorders. This increase in blood coagulation potential is apparently due to the collective effects of increases in the plasma level of procoagulant factors, combined with only marginal increases or even decreases in the plasma levels of anticoagulation factors (such as antithrombin III and protein C) or of factors involved in fibrinolysis. The age-associated increase in blood coagulation potential represents a net increase in the coagulation activity and appears to be a strictly controlled phenomenon rather than a consequence of general aging-associated dysregulation of hemostasis. Literally nothing was known about the genetic and molecular mechanisms responsible for this important phenomenon until the recent findings regarding the human (h) factor IX (FIX) gene. Why this controlled age-associated increase in the blood coagulation potential exists and how such age regulation of blood coagulation is maintained are two fascinating and intriguing questions.

Toward understanding of this phenomenon, we recently carried out systematic studies of the age regulation of hFIX, a key coagulation factor and the first model analyzed in depth. The transgenic mouse approach taken in these studies has proven to be highly effective in gaining critical insight into the mechanisms of age regulation of hFIX.

hFIX Gene and Age Regulation

FIX, a plasma protease precursor, is one of the key procoagulant factors. FIX occupies a key position in the blood coagulation cascade, where 2 major initiation pathways, intrinsic and extrinsic, merge. FIX is synthesized in the liver with high tissue specificity, and its deficiency results in the bleeding disorder hemophilia B. FIX is one of 2 blood coagulation factors, along with factor VIII, whose genes are on the X chromosome; thus, hemophilia B is primarily seen in males and only rarely in females. The hFIX gene is 40 kb in size and is composed of 8 exons (Figure 1). Its contiguous complete nucleotide sequence was reported in 1985, and >1500 abnormal genes have been analyzed to date to their...
molecular level.26 This enormous knowledge that has been accumulated regarding FIX, its genes, and their structure-function relation, physiology, and pathology makes hFIX one of the most extensively studied mammalian proteins and, thus, highly suited for studying the complex mechanisms of age-dependent regulation.

With advancing age, hFIX increases its circulatory levels to levels approximately twice those found at young ages, as does blood coagulation potential.27 Similarly, mouse FIX (mFIX) activity in the circulation also increases with age,22,28 which is directly correlated with an increase in the liver mFIX mRNA level.28 Mice are used as a well-established mammalian model system for various in vivo testing and have a blood coagulation system similar to that in humans. The transgenic mouse approach, therefore, is appropriate and is used in analyzing the molecular mechanisms of age regulation of the hFIX gene.22

**Characteristics of hFIX Expression In Vitro From Minigenes**

A series of hFIX minigene expression vectors were constructed and first tested with the HepG2 cell (human hepatoma cell line) assay system22 (Figure 2). These minigenes contain a common hFIX coding region derived from cDNA with a shortened, but fully functional, first intron inserted at the natural position,29 which was linked with variously extended 5' promoter regions up to nucleotide −2231. They also carry the 3' untranslated region (UTR) with or without its middle portion deleted. The middle portion of the 3' UTR contains a 102-bp stretch of inverted AT, GT, and GC dinucleotide repeats, which can potentially form stem-loop structures in mRNA25 and are known to affect the stability of mRNA.30

Systematic testing of these minigenes with HepG2 cells unraveled unique characteristics of the hFIX gene. The HepG2 cell assay system with the transfection reagent Fugene 6 was invaluable for successful transient expression analysis,31 allowing all hFIX minigenes tested to produce recombinant hFIX at high levels (≈50 ng/10^6 cells per 48 hours).32 One of the unexpected findings was that no hFIX minigene carrying the 5' upstream region (nucleotide −802 up through nucleotide −1900) showed the significant silencer activity22 that was previously observed when a chloramphenicol acetyltransferase gene was used as a reporter.33 Use of such heterologous reporter genes to analyze unrelated genes, such as the FIX gene, may very likely give irrelevant observations, significantly skewed from the natural gene regulation. This may presumably be 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. Conversely, structural elements in the heterologous reporter genes may affect the transcriptional regulation of the studied gene. Irrelevant observations may also be due to the cell lines used.

All minigenes containing the entire 3' UTR reproducibly showed activity that was 25% to 30% lower than counterpart minigenes missing the middle portion of the 3' UTR.22 This moderately suppressive effect appears only in the presence of the middle portion of the 3' UTR. No mechanistic explanations for the suppressive activity are currently available, but this finding is intriguing when the in vivo observations discussed below are taken into account.

**Characteristics of hFIX Minigene Expression In Vivo: Observations Involving Transgenic Mice**

Critical insight into the regulatory mechanisms of the hFIX gene was obtained only from the longitudinal analysis of the entire life span of circulatory hFIX in hundreds of individual transgenic mice carrying hFIX minigenes22 (Figure 3). The prepuberty levels of systemic hFIX in these animals largely

![Figure 1. Organization of the hFIX gene. The gene is ~40 kb in size. Exons are shown as open boxes, and 5' and 3' flanking sequences and introns are shown by thick horizontal lines. LINE-1 sequences in a twintron-like organization are represented by dotted sequences and introns are shown by thick horizontal lines.](http://atvb.ahajournals.org/)

![Figure 2. hFIX minigene expression vectors. The structure is depicted with the promoter regions (solid thick line on left) with the 5' terminal nucleotide number. Transcribed hFIX regions (shaded rectangles, with thin lines representing the shortened first intron) are followed by 3' flanking sequence regions (solid thick line at right).](http://atvb.ahajournals.org/)
varied, primarily because of transgene positional effects and zygosity status, from very low levels (<10 ng/mL serum) to high levels similar to the plasma level of the natural gene (4 to 5 µg/mL serum). Regardless of the initial prepubertal hFIX levels in the circulation, comparison of age patterns of hFIX levels in the circulation among animals with different minigene transgenes provided the most valuable information and proved the robustness of the transgenic mouse approach for analyzing the complex age-regulatory mechanisms of the hFIX gene.

In the present review, we will focus our discussion on a few selected animal lines. At 1 month of age (prepubertal stage), transgenic animals carrying −416 FIXm1 minigenes, which contain the 5’ promoter region up to nucleotide −416 and the 3’ UTR with its middle portion deleted, produced hFIX at a wide range of levels, as mentioned above (Figure 3A). Importantly, independent of the initial prepubertal level, circulatory hFIX levels in these animals rapidly declined during puberty and during the subsequent 2 to 3 months to lower, stable levels. This rapid age-dependent decline in the circulatory hFIX level is observed regardless of the founder line, initial prepubertal hFIX level, generation, sex, or zygosity status of the transgenes and is directly related to a similar, though much less steep, age-dependent decline in the circulatory hFIX levels in animals with these minigenes beyond nucleotide −770, indicating that the essential element or elements required for normal age-associated regulation of the hFIX gene are not contained in these minigenes. The age-dependent decline in the circulatory hFIX in animals with these minigenes again correlates with the decline in liver hFIX mRNA, which is presumably due to a decline in promoter activity.22

Surprisingly, animals carrying the minigene −802FIXm1 (Figure 4A), which has an extra 5’ stretch of 32 bp extended beyond nucleotide −770 (Figure 2) showed grossly different patterns of age-associated regulation of hFIX production.22 These animals invariably showed remarkably age-stable circulatory hFIX levels until death, usually for up to 20 to 24 months of age. Although age-stable plasma hFIX levels are also consistent with age-stable liver hFIX mRNA levels, interestingly, the circulatory hFIX protein turnover time does not change significantly in vivo with increasing age. These observations were further supported by the age-stable hFIX expression observed in mice carrying −2231FIXm1. Thus, a critical structural element required for age-stable expression of the hFIX gene was located in a small region spanning nucleotides −770 through −802, which was designated the “age-regulatory element in the 5’ end” (AE5’). This region contains a sequence element, GAGGAAG (nucleotides −784 to −790), which matches the consensus motif of polyomavirus enhancer activator-3 (PEA-3), a member of the Ets family of transcriptional factors.33 As demonstrated by footprint and bandshift analyses, a specific nuclear protein binds to this element in an age-dependent manner.22 Whether the protein is PEA-3 or a closely related protein, which can bind to the PEA-3 element, has yet to be determined. It is noteworthy that the presence of AE5’ is required for strict liver-specific expression of the hFIX gene, whereas in its absence, high, but incomplete, liver-specific expression was observed.
AE5', which contains the PEA-3 element identified, is located in the 5' flanking region derived from a long interspersed repetitive element (LINE-1 or L1, retrotransposable element). Interestingly, this region has a twintron-like LINE-1 organization (2 LINE-1-derived sequences overlapped). Apparently, this was generated by 2 successive retrotransposition with the second LINE-1 retrotransposed into the middle of the originally recruited LINE-1, thus dividing it into 2 parts. AE5' is present in the proximal region of the originally inserted LINE-1. Because the modern retrotransposable LINE-1 does not have an AE5' (PEA-3)-like structure at the corresponding region, AE5' of the hFIX gene must have been generated through substantial mutations of the inserted LINE-1 sequence. The mFIX gene, which has an age-regulation pattern similar to that of hFIX, also contains a LINE-1-derived sequence in its 5' upstream position similar to that in the hFIX gene and has multiple PEA-3 consensus elements. In evolution, therefore, retrotransposition of LINE-1 has played, and will continue to play, critical roles not only in inactivating functional genes but also in generating normal gene function.

The most critical observations were obtained from mice with −802FIXm1/1.4 as well as −2231FIXm1/1.4, both containing the complete 3' UTR. These mice showed an advancing age-associated increase in circulatory hFIX levels, very similar to those observed for the hFIX gene (Figure 4B). This age-associated increase in circulating hFIX levels was directly correlated with increased liver hFIX mRNA levels, resulting in discovery of the second age-regulatory element, designated AE3', located in the middle of the 3' UTR. Only with both AE5' and AE3' in the minigenes could the characteristic age regulation of the normal hFIX gene be recapitulated. The unique concerted actions of AE5' and AE3' are again independent of founder line, initial expression levels at 1 month of age, sex, generation, or zygosity of the animals. AE3' has been delineated to an ~300-bp middle region of the 3' UTR, where a stretch of dinucleotide repeats are present, although complete delineation will require further animal studies (S.K., K.K., unpublished data, 1999). AE3' does not function as a position-independent enhancer but raises the liver hFIX mRNA level in direct correlation with the age-dependent increase in circulatory hFIX levels (Figure 4B). Observations made to date suggest that the function of AE3' is to increase hFIX mRNA stability with age, although other possible mechanisms for AE3' action have yet to be explored. This in vivo observation appears to be contradictory to the moderate suppression observed in vitro with the HepG2 cell assay system and further suggests that use of in vitro assay systems alone in studying gene regulation may give skewed and misleading results.

Finally, it is noteworthy that animals expressing increasingly high levels of hFIX (~ > 1500 ng/mL serum) in addition to normal liver mFIX over some months die at much earlier ages than control animals or those producing lower levels of hFIX (see animals labeled “d” in Figure 4). This strongly suggests a possibility that substantially elevated levels of FIX may be a risk factor for thrombosis and/or cardiovascular diseases.

**Conclusion**

The basic genetic and molecular mechanisms of age regulation (homeostasis) of hFIX have now been established. In addition to the basic structures, such as the basal promoter, coding region, and introns, the mechanisms involve 2 critical elements (AE5' and AE3') that regulate hFIX gene expression at the transcription step and modulate hFIX mRNA stability (Figure 5). Success of the study of age regulation of hFIX is due to the systematic and longitudinal analyses of hFIX expression in a large number of individual transgenic animals during their life spans. This analysis was possible primarily because FIX is a plasma protein, thus allowing longitudinal analysis of many individual animals without euthanizing animals at various ages for analysis. If intracellular gene products ought to be used as a reporter for analyzing age regulation, frequent euthanization of animals would be required, making any quantitative study extremely
difficult, if not impossible. A stage is now set for investigating many other individual procoagulant and anticoagulant factors. It is of particular interest to determine whether AE5' and AE3' also function in other genes or whether each gene requires different mechanisms for age-dependent regulation. Eventually, we may be able to provide a rational explanation as to why such an increase exists in a healthy individual and its correlation with the aging-associated increase in thrombosis and/or cardiovascular diseases.

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References

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