Genetic Mechanisms of Age Regulation of Protein C and Blood Coagulation

Blood coagulation activity in humans increases with age. We previously identified two genetic elements, age-related stability element (ASE; GAGGAAG) and age-related increase element (AIE; unique stretch of dinucleotide repeats), which were responsible for age-related stable and increasing expression patterns, respectively, and together recapitulated normal age regulation of the human factor IX (hFIX) gene. Here we report the age-regulatory mechanisms of human anticoagulant protein C (hPC), which shows an age-stable pattern of circulatory levels. The murine protein C gene showed an age-related stable expression pattern in general agreement with that of the hPC. Through longitudinal analyses of transgenic mice carrying hPC minigenes, the hPC gene was found to have a functional age-related stability element (hPC ASE; CAGGAAG) in the 5′-upstream proximal region but was found to lack any age-related increase element. Three other ASE-like sequences present in the hPC gene, GAGGAAA and (G/C)AGGATG, also bound nuclear proteins but were not active in the age regulation of the hPC gene. Functional hPC ASE and hFIX ASE were apparently generated through convergent evolution, and hFIX ASE can fully substitute for the hPC ASE in conferring age-related stable expression pattern of the hPC gene. In the presence of the hPC ASE, hFIX ASE can convert the age-stable expression pattern of the hPC gene to a hFIX-like age-related increase pattern. These results support the universality of ASE and AIE functions across different genes. Clearance of hPC protein from the circulation was not significantly affected by age. We now have established the basic mechanisms responsible for the age-related increase of blood coagulation activity.

Blood coagulation activity in normal humans increases with age (1–3). This is apparently due to age-related increase in disparity between procoagulation and anticoagulation activities and may have critical contributions to age-related increases in frequency of thrombotic and cardiovascular diseases (4–7). Human PC is an important component of the anticoagulation pathway, while hFIX is a key blood procoagulation factor playing critical roles in blood coagulation (8, 9). Epidemiological studies showed that the activity and plasma levels of hFIX increased significantly with advancing age in normal human populations (3, 10). In comparison with hFIX, the plasma levels of hPC showed only marginal age-associated changes (3, 11). We previously identified two genetic elements, ASE and AIE (renamed from AE 5′ and AE 3′, respectively), which are required for age-related stability and increase of hFIX expression, respectively (12). ASE, originally identified as a footprint sequence between nucleotide (nt) 802 and nt 784 of the hFIX gene, has a core sequence, GAGGAAG, matching the transcriptional factor PEA-3 consensus sequence ((G/C)AG-GA(ATA/G)) (13, 14). AIE, present in the middle of the hFIX 3′-untranslated region (UTR), is composed of 102-bp dinucleotide repeats (AT, GT, and CA) and has the potential to form three distinct stem loop structures in its RNA form (15). Murine factor IX (mFIX) shows an age-related increase in both circulatory protein levels and gene expression in the liver (16, 17), and the mFIX gene also contains ASE-like sequences in the 3′-UTR and a stretch of 106-bp dinucleotide repeats in the 3′-UTR (18).

New critical questions have emerged, including whether the age-regulatory elements ASE and AIE can play roles in other genes, especially anticoagulation factor genes, and what are the fundamental genetic mechanisms responsible for the age-related increase of blood coagulation activity. To address these issues, we focused our efforts on the hPC gene. Human PC and hFIX share similarities in protein structure and gene coding region organization, but the 5′-flanking regions and 3′-UTRs of their genes are grossly dissimilar (15, 19–21). For example, the 5′-flanking region of the hFIX gene, from approximately beyond nt 350, where the functional ASE is located, was derived from retrotransposed LINE-1 sequences (22), while the corresponding 5′-flanking region of the hPC gene was not (20). Furthermore, the 3′-UTR of the hFIX gene is about 1.4 kb in length, whereas that of the hPC gene is only 295 bp, and no AIE-like element or dinucleotide repeats are present (15, 21). These differences between the hFIX and hPC genes may result in the substantial dissimilarity in their age-related expression patterns.

In this paper, we report the basic genetic mechanisms of age regulation of the hPC gene and the functional universality of the hFIX-derived ASE and AIE, thus laying the foundation for...
Age-related Regulation of Human Factor IX and Pseudocumarylation

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and DNA modification enzymes were purchased from Invitrogen and New England Biolabs. Radioactive nucleotides, [α-32P]dATP and [γ-32P]ATP, were obtained from Amer sham Biosciences. Mouse anti-hPC monoclonal antibody and rabbit polyclonal anti-hPC antibody were purchased from Celsus Laborato ries. Horseradish peroxidase-linked goat anti-rabbit IgG was purchased from Invitrogen. Antibodies used for hFIX-specific enzyme-linked immunosorbent assay (ELISA) were described previously (12). Medium, fetal calf serum, penicillin, and streptomycin for mammalian cell cultures were obtained from Invitrogen. Fugene-6 transfection reagent was purchased from Roche Molecular Biochemicals. HepG2 cells, a human hepatoma cell line, were obtained from ATCC. C57BL/6, SJL, and CD-1 mice used for generating transgenic animals were purchased from the Jackson Laboratory. All other reagents were of the highest quality commercially available.

Construction of hPC Minigenes—Human PC minigene construct, -1462hPC1m1, was first constructed and subsequently used to generate other hPC minigene constructs. The hPC gene nucleotide numbering system was used with the first transcriptional initiation site defined as nt +1 (20). A 2022-bp sequence spanning nt -1462 through nt +1560 of the hPC gene was amplified by polymerase chain reaction (PCR) using human genomic DNA as a template, and 5′ forward primer with SphiI linker (5′-CAAGCATGCGAATTCTGTAAGCATTTCCT-3′) and 3′ reverse primer with Mscl linker (5′-GAAATTCCCCAGGTGGC-GAC-3′). The amplified fragment was then inserted into pUC19-hPC (kindly provided by Dr. Francis Castellino, University of Notre Dame) to replace its 5′ portion released by SphiI/MscI double digestion. The 3′-end portion of the resultant intermediate construct, from the internal Sse8387I site at nt +10497 in the 3′-UTR to the EcoR1 site outside of the poly(A) attachment site (nt +10783), was released by Sse8387I/EcoR1 double digestion. This portion was then replaced by a PCR-amplified fragment with Sse8387I/EcoR1 sticky ends (612 bp in length, spanning nt +11108 through +11108 with a 325-bp extender into the 3′-flanking region sequence of the hPC gene), thus generating minigene -1462hPC1m1. By replacing the 5′ portion of -1462hPC1m1 (nt -1462 through +1560) with PCR-amplified SphiI/MscI fragments, spanning nt -849, -802, or -82 to nt +1560, new minigenes -498hPCm1, -802hPCm1, or -82hPCm1 were generated, respectively. ASE/-802hPCm1 or ASE/-82hPCm1 were similarly constructed by inserting a single 325-bp fragment containing nt -771 of the hFIX gene (with SphiI sticky ends into -802hPCm1 or -82hPCm1 at the 5′-end SphiI site, respectively. Mini gene pseudoASE/-802hPCm1 was generated by inserting a 23-bp fragment containing pseudo-ASE (nt -878 through -856 of the hPC gene) with SphiI sticky ends into -802hPCm1 at the 5′-end SphiI site. Minigenes pseudoASE/-1078hPCm1 or pseudoASE/-1462hPCm1 were constructed by inserting a 102-bp hFIX AFE fragment (nt +32142 through +32243 of the hFIX gene) with Sse8387I linker into the 3′-UTR of -1462hPC1m1 at the Sse8387I site in a normal or reversed orientation, respectively. ASE/-1462hPC1m1/AIE was generated by inserting above hFIX ASE into -1462hPC1m1/AIE at the 5′-end SphiI site. Human FIX minigenes, ASENDmin/-416FXm1 and AIE/-416FXm1/AIE, were constructed by inserting a single copy of the 17-bp minimum AIE sequence (ASEmin, nt -795 through -779 of the hFIX gene) or a copy of the above AIE into minigenes -416FXm1 and -416FXm1/AIE (12) at the 5′-end SphiI site, respectively. Minigenes -802FXm1 and -82FXm1 were constructed by inserting a 106-bp dinucleotide repeat sequence of the mFIX gene (nt +2018 through +2123 of the mFIX cDNA) (18) at the BamH1 site of -802FXm1. PCR-amplified sequences and ligation sites of all of the new constructs were confirmed by automated dideoxy sequencing.

Northern Blot Analysis of Mouse and Human PC mRNA—Northern blot analysis of mouse liver RNA was carried out as previously described (19). A murine PC (mPC) probe (223 bp, from nt +14609 to nt +14832 of the mPC gene) (23) was prepared by PCR amplification (5′ primer, 5′-TTTCTTTCGGGATCCTGG-3′; 3′ primer, 5′-GTCTCTGTTTGCATTACTG-3′). Two pairs of primer were used: one specific to the 746-bp hPC transgene fragment corresponding to exons 2–9 (nt +1577 through +9911 in the genomic nucleotide number (20, 21)) and one that produced a 494-bp mouse β-globin gene fragment (endogenous control) (nt +2590 through +3083). To be quantitative, 50 ng of genomic DNA from the tail tissue, 200 ng of hFIX primers, and 100 ng of mouse β-globin primers were applied. The transgene copy number was determined based on the relative ratio between the hFIX-specific band and that of the β-globin gene. Animals carrying ≥5–10 copies of the hPC transgene were selected for further analyses. Founders were back-crossed with nontransgenic mice (C57BL/6 X SJL) to generate F1 progeny animals. Homozygous F2 animals were generated by crossing among heterozygous F1 littermates. The zygosity status of animals was determined by quantitative multiplex PCR analysis as described above. Longitudinal blood collection via snipped tails was started at 1 month of age from individual animals of 4–8 representative founders for each minigene construct. Animals of subsequent generations of representative lines were similarly subjected to longitudinal analyses. Circulatory hPC or hFIX levels of transgenic mice at each age point were quantified by duplicated ELISA. Human FIX ELISA was applied as previously described (12). To minimize experimental fluctuations from assay to assay in the longitudinal analysis, over 100 serum samples from each animal were included in each assay. Transgene positional effect and copy number were carried out under the institutional guidelines for ethical animal use (Office for Protection from Research Risk No. A3114-01).

Electrophoretic Mobility Shift Assay (EMSA)—EMASs of pseudo-ASE, hPC AIE, and other similar elements were carried out as previously described (26) with minor modifications. Nuclear extracts (NEs) were prepared from HepG2 cells or liver tissues of normal mice (C57BL/6) at 5 months of age. Single-stranded oligonucleotides were chemically synthesized and used to prepare double-stranded (ds; see Fig. 4) oligonucleotides (sequences shown in the figure legend). These oligonucleotides were labeled with 32P to a specific activity of ∼1.9 × 106 cpm. Aliquots (20,000 cpm) were incubated with 10 µg of NEs in the presence of 1 µg of double-stranded poly(dI-dC) in DNA binding buffer for 2 min at room temperature and subjected to vertical polyacrylamide gel electrophoresis under nonreducing conditions. For competitive binding assays, unlabeled oligonucleotides (double-stranded) in the amount of 100-fold excess were added to the labeled ones.

Human PC Clearance Assay—Half-clearance times of circulatory hPC were determined as previously described for hFIX (12) with appropriate modifications. Aliquots of plasma-derived hPC preparation (4 µg/0.1 ml of phosphate-buffered saline; Hematologic Technologies, Inc.)
were injected via tail vein into three separate groups of C57BL/6 × SJL mice of 2, 8, 10, and 18–20 months of age (n = 3 per group), respectively. Circulatory hPC levels were determined by ELISA using serum aliquots, which were prepared from blood samples (∼50-μl aliquots) collected via snipped tails at 10 min and at 2, 6, 12, 18, 24, 30, 36, and 48 h after protein injection.

RESULTS

Age-related Expression Patterns of the mPC Gene—To provide the rationale for utilizing transgenic mice for analyzing age regulation of hPC gene expression, we first examined the age-related expression pattern of the mPC gene (Fig. 1A). Mouse liver mPC mRNA levels rapidly increased during the perinatal stage (Fig. 1A, lanes 2–8), reaching the young adult age (∼3 weeks of age), followed by relatively stable levels through old age (20 months) (Fig. 1A, lanes 8–14 and B).

Genetic Control of Age-related Expression Patterns of hPC Minigenes—The first series of hPC minigenes were constructed to identify genetic elements responsible for age-related regulation of the hPC gene, specifically focusing on the PEA-3-like elements (Fig. 2A). There were two PEA-3-like elements present in the proximal 5′-upstream of the gene. The first element was located at nt −832 through −826 (CAGGAAG; designated as hPC ASE), which is consistent with the PEA-3 consensus motif and different from the hFIX ASE (GAGGAAG) by one nucleotide. The second element was present at nt −871 through −865 (GAGGAAA; designated as pseudo-ASE), differing from the PEA-3 consensus motif by one nucleotide. All of the hPC minigenes showed similar transient expression activities as assayed with HepG2 cells (Fig. 2A). The second series of hPC minigenes were constructed with hFIX-derived ASE or AIE (Fig. 2B). The presence of ASE did not have any significant effect on transient hPC expression in HepG2 cells, while the presence of AIE in hPC minigenes lowered their transient expression activities by ∼30% (Fig. 2B) in agreement with our previous observations on hFIX minigenes containing AIE (12). Transgenic mice carrying minigenes −1462hPCm1 (n = 40), −849hPCm1 (n = 31), −802hPCm1 (n = 35), or −82hPCm1
(n = 44) were constructed and subjected to longitudinal analyses of circulatory hPC. Age-related patterns of circulatory hPC levels in individual animals are shown in Fig. 3, A–D. Animals carrying either −1462hPCm1 or −802hPCm1 showed remarkable age-related stability in circulatory hPC at various levels as high as ∼3 μg/ml plasma, similar to natural hPC levels in humans (Fig. 3, A and B). The stability of circulatory hPC correlated with similar age-stable hPC mRNA levels in the liver (Fig. 3F), and was consistent with the absence of any AIE-like element in the hPC gene. These observations were reproducible in all animals under investigation, regardless of founder line, initial prepubertal hPC levels, sex, generation (including F2 animals), or zygosity status of the transgene. In contrast, circulatory hPC levels of animals carrying −802hPCm1 or −82hPCm1, which showed levels as high as ∼590 or ∼40 ng/ml serum, respectively, at 1 month of age, rapidly decreased through puberty to much lower or undetectable levels for the rest of their life spans (Fig. 3, C and D). This rapid age-related decline was observed in all animals, independent of founder line, initial prepubertal levels, sex, or zygosity status of the transgenes, and correlated with a similar decline in the steady state level of liver hPC mRNA (Fig. 3G). These results suggested that the region nt −849 through −803, where a PEA-3 element (CAGGAAAG; hPC ASE) located, was critically required for age-stable expression of the hPC gene.

Another PEA-3 like element present in the region nt −871 through −865, GAGGAAA (pseudo-ASE), was also tested with transgenic mice carrying pseudoASE/−802hPCm1. Animals carrying pseudoASE/−802hPCm1 showed an age-related decline in hPC expression (n = 23) (Fig. 3E) similar to those carrying −802hPCm1, indicating that pseudo-ASE does not function as an age stability element.

In EMSA, pseudo-ASE and hPC ASE bound different liver nuclear proteins, as evidenced by the absence of cross-competition (Fig. 4, lanes 7 and 14) and a subtle difference in the apparent sizes between them (Fig. 4, lanes 3 and 10). Furthermore, hFIX ASE efficiently out-competed hPC ASE for its binding nuclear protein (Fig. 4, lane 13), indicating that hFIX ASE (CAGGAAAG) and hPC ASE (CAGGAAAG) bind the same nuclear protein. Protein binding to pseudo-ASE was present in HepG2 NEs at a significant concentration level (Fig. 4, lane 2), while the hPC ASE-binding protein was present in HepG2 NEs at a very low level, if at all (Fig. 4, lane 9).

Human PC minigenes used in this study had two additional consensus PEA-3 sequences. One is in intron 1 (nt +694 to +700; GAGGATG; designated as Intron1PEA-3), and the other is in the exon 9 coding region (nt +10286 to +10292; CAGGATG; designated as Exon9PEA-3) (21). Interestingly, they were not able to confer age-related stabilization effects on hPC gene expression as evidenced by age-unstable expression patterns of −802hPCm1 as well as −82hPCm1 in animals (Fig. 3, C and D). EMSA indicated that both elements bound nuclear proteins present in both liver and HepG2 cell NEs (Fig. 5A, lanes 2, 3, 8, and 9), but like in the case of pseudo-ASE above, these proteins were found to be different from those binding to functional hPC or hFIX ASE as shown by cross-competition assays (Fig. 5A, lanes 6 and 12). This was further supported by cross-competition assays using 32P-labeled hPC ASE, unlabeled Intron1PEA-3 (GAGGATG), or Exon9PEA-3 (CAGGATG) (Fig. 5B, lanes 8 and 9). Cross-competition assays also showed that Intron1PEA-3 and Exon9PEA-3 bound a common nuclear protein (Fig. 5B, lanes 10 and 11).

Functional Universality of ASE and AIE.—To address the possible functional universality of ASE elements, another series of hPC minigenes constructed with hFIX-derived ASE (Fig. 2B) were longitudinally analyzed for age-related expression in transgenic mice. Unlike animals carrying −82hPCm1 (Fig. 3D) or −802hPCm1 (Fig. 3C), transgenic animals carrying ASE/−82hPCm1 (n = 32) or ASE/−802hPCm1 (n = 30), which have the hFIX ASE inserted at the 5′-upstream position of hPC minigenes, reproducibly showed age-stable patterns of circulatory hPC at least up to 9 months of age (the last assay time points) (Fig. 6, A and B). The observed conversion in age-regulatory patterns was independent of founder animals, generation, initial pubertal levels, sex, or zygosity state. This age stability of the hPC circulatory levels paralleled similar age-stable levels of hPC mRNA in the liver (Fig. 6F). Thus, we conclude that the hFIX-derived ASE can functionally replace the hPC endogenous ASE. hPCMin−/−416FIXm1, a hFIX minigene with only a 17-bp nucleotide sequence containing the 7-bp essential PEA-3 motif sequence (GAGGAAAG) in the middle inserted at the 5′-end of minigene −416FIXm1, also fully converted the age-unstable hFIX expression of −416FIXm1 (12) to age-stable expression (supplemental Fig. A), further supporting the importance of the motif sequence in vivo.

The functional universality of AIE was then tested in transgenic mice with a new set of hPC minigenes, −1462hPCm1/AIE, ASE/−1462hPCm1/AIE, and −1462hPCm1/AIEr. These minigenes were constructed by inserting an hFIX-derived AIE, a minimal 102-bp core sequence of mostly dinucleotide repeats (15), in the middle of the 3′-UTR of −1462hPCm1 and ASE/−1462hPCm1, respectively. These minigenes contained one or two units of functional ASE as described above. Animals with −1462hPCm1/AIE or ASE/−1462hPCm1/AIEr clearly switched age patterns of circulatory hPC from the hPC age-stable pattern to an hFIX-like age-related increase pattern (Fig. 6, C and D). This switch in circulatory hPC patterns also correlated with a similar switch in the age-related liver mRNA levels (Fig. 6G) and was reproducibly observed with all animals carrying either −1462hPCm1/AIE (n = 30) or ASE/−1462hPCm1/AIEr (n = 28) regardless of founder line, initial pubertal hPC level, sex, generation, or zygosity. These results demonstrated that AIE can confer an age-related increase expression pattern on the hPC gene, which lacks any endogenous AIE-like element, and that the endogenous hPC ASE fully functioned in combination with AIE.

Minigene −1462hPCm1/AIEr, an hPC minigene with a reversed AIE sequence at the 3′-UTR, which is still capable of forming stem-loop structures in its RNA form, also showed an age-related increase expression pattern in transgenic animals (n = 18) (Fig. 6E), indicating that AIE functions independent of its orientation in the 3′-UTR. However, AIE/−416FIXm1/AIE, an hFIX minigene with the AIE inserted at the 5′-end, showed only age-stable, but not an age-associated increase in circulatory hFIX expression in transgenic animals (supplemental Fig. B), suggesting that AIE does not function as a transcriptional enhancer. A stretch of 106-bp dinucleotide repeats (mDR) present in the 3′-UTR of the mFIX gene was also tested for its possible age-related function. This dinucleotide repeat was composed of a GA repeat (H-DNA-forming sequence) and AT repeat (potential stem-loop-forming sequence in RNA form) (18). Transgenic mice carrying −802FIXm1/mDR, an hFIX minigene with one unit of mDR in place of the hFIX AIE in the 3′-UTR of −802FIXm1 (12), also showed substantial age-related increase in the circulatory levels (supplemental Fig. C).

Age-associated Effect on Circulatory hPC Clearance Time—Clearance of proteins from the circulation can also affect their circulatory levels. The clearance time of hPC in the circulation of mice was determined and summarized in Table I. Human PC clearance from the circulation was found to follow biphasic kinetics with rapid (α) and slow (β) phases. The clearance half-times (t1/2) of these two phases were −6 and 16 h, respectively.
FIG. 3. Longitudinal analyses of hPC levels in the circulation of representative transgenic mice carrying hPC minigenes. A, −1462hPCm1; B, −849hPCm1; C, −802hPCm1; D, −82hPCm1. E, pseudoASE/−802hPCm1. Animals are identified by the following format: F (for founder), identification number, sex, status (+, alive in good health; d, died; s, sacrificed). Longitudinal analyses of progeny mice (F1 and F2 generations) produced from founders carrying hPC minigenes showed age-regulatory patterns similar to their founders (supplemental Fig. D). F, Northern blot analysis of hPC mRNA levels in the liver tissues of 1-month-old (P758A; F1/f), 3-month-old (P758D; F1/f), and 5-month-old (P758G; F1/f) progeny mice carrying −1462hPCm1. Animals P758A, P758D, and P758G were from the same litter produced by founder F758 and had similar levels of circulatory hPC at 1 month of age (1592, 1610, and 1588 ng/ml serum, respectively). Animals P758D and P758G had 1622 and 1602 ng/ml serum at 3 and 5 months of age, respectively. Lane 1, nontransgenic mouse; lane 2, P758A (1 month old); lane 3, P758D (3 months old); lane 4, P758G (5 months old). Positions of hPC mRNA in the upper panel and 18 S rRNA bands in the lower panel are indicated by hPC and RNR18, respectively. Equivalent loading of total RNA samples was shown by 18 S rRNA bands. Relative mRNA levels quantified by PhosphorImager (Molecular Dynamics, Inc.). Ratios of hPC mRNA levels of 3- and 5-month versus 1-month-old animals were 1.03 and 0.98, respectively. G, Northern blot analysis of hPC mRNA levels in the liver tissues of 1-month-old (P41B; F1/m), 3-month-old (P41D; F1/m), and 5-month-old (P41F; F1/m) mice carrying −802hPCm1. P41B, P41D, and P41F animals were from the same litter produced by founder F41 animal and, at 1 month of age, had circulatory hPC of 698, 705, and 685 ng/ml serum, respectively. Animals P41D and P41F had circulatory hPC of 402 and 161 ng/ml serum at 3 and 5 months of age, respectively. Lane 1, nontransgenic mouse; lane 2, P41B (1 month old); lane 3, P41D (3 months old); lane 4, P41F (5 months old). Ratios of hPC mRNA levels of 3- and 5-month versus 1-month-old animals were 0.58 and 0.24, respectively. Positions of hPC mRNA and 18 S rRNA are indicated as in F.
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Fig. 4. Gel EMSA of pseudo-ASE and hPC ASE. Double-stranded (ds) pseudo-ASE oligonucleotide containing a PEA-3-like element sequence (GAGGAAA) spans nt −878 through −859 (5'-GGGCGAGGAGGAAAGGGGG-3') of the hPC gene (PEA-3-like element underlined). Human PC ASE oligonucleotide (double-stranded) containing a consensus PEA-3 element (CAGGAAG) spans nt −838 through −819 (5'-TGCGGACGGAAAGGGACCTTA-3') of the hPC gene. Mutated pseudo-ASE (pseudo-ASE'') and hPC ASE (hPC ASE'') used for competition assays were 5'-GGCGGTTTGGTGGGGGGG-3' and 5'-TGCGGTTTGGATAGCCTTA-3', respectively (mutated oligonucleotide is indicated by the underline). Human FIX ASE oligonucleotide (double-stranded) was 5'-TCGAGGGAAGGAGGATGG-3'. Lanes 1–7 contain 32P-labeled pseudo-ASE oligonucleotide. Lane 1, without NEs; lane 2, with HepG2 NEs; lane 3, with liver NEs; lane 4, with a 100-fold excess of unlabeled pseudo-ASE and liver NEs; lane 5, with a 100-fold excess of unlabeled mutated pseudo-ASE and liver NEs; lane 6, with a 100-fold excess of unlabeled hFIX ASE and liver NEs; lane 7, with a 100-fold excess of unlabeled hPC ASE and liver NEs. Lanes 8–14 contain 32P-labeled hPC ASE oligonucleotide. Lane 8, without NEs; lane 9, with HepG2 NEs; lane 10, with liver NEs; lane 11, with a 100-fold excess of unlabeled hPC ASE and liver NEs; lane 12, with a 100-fold excess of unlabeled mutated hPC ASE and liver NEs; lane 13, with a 100-fold excess of unlabeled hFIX ASE and liver NEs; lane 14, with a 100-fold excess of unlabeled pseudo-ASE and liver NEs. Shifted band positions are shown by a and b on the right.

DISCUSSION

In this paper, we describe for the first time the genetic basis responsible for the age-related increase of blood coagulation activity and the mechanistic evidence for the functional universality of age-regulatory mechanisms identified in the hPC and hFIX genes.

General characteristics of mouse blood coagulation factors parallel their human counterparts (27). Our findings on the mPC levels in the liver (Fig. 1) indicate that the overall age-related expression pattern of the mPC gene correlates well with the known age-related pattern of circulatory hPC levels in humans (3, 4). Both mPC and hPC rapidly increase their expression during the perinatal stage, followed by a general age-stable expression pattern. This further supported the rationale of using a mouse model for studying age-related regulatory mechanisms of the hPC gene.

Age-related stable patterns of circulatory hPC levels observed for transgenic mice carrying hPC minigene −1462hPCm1 or −849hPCm1 recapitulated the natural patterns of mPC and hPC genes (Fig. 3, A and B). This suggests that these minigenes contained all of the necessary genetic elements required for age-stable expression of the hPC gene. Dramatic changes of age-related expression patterns of circulatory hPC in the animals carrying minigene −802hPCm1 or −82hPCm1 (Fig. 3, C and D) from those of −1462hPCm1 or −849hPCm1 allowed us to locate a critical element responsible for age-stable expression of the hPC gene to the region spanning nt −849 through −803. This region contains a single copy of the PEA-3 consensus element (CAGGAAG; hPC ASE) and no other known transcriptional elements, suggesting the critical role of hPC ASE in maintaining age-stable hPC expression. Although both hPC ASE and hFIX ASE are of the PEA-3 consensus elements (G/CAGGA/AT/G), it is important to find that both elements actually bind the same liver nuclear protein of the ETS superfamily, as shown by DNA-protein binding analyses (Fig. 4, lanes 10 and 13). Very low levels, if any, of hPC ASE-binding protein observed in HepG2 cell NEs (Fig. 4, lane 9) may correlate with virtually no effects of hPC or hFIX ASE on transient minigene expression activities in HepG2 cells (Fig. 2).

A nuclear protein that bound to pseudo-ASE (GAGGAAA) was different from that binding to functional ASE of hPC or hFIX (Fig. 4, lanes 6 and 7). Since transgenic animals carrying pseudoASE/−802hPC showed age-related decline expression patterns of circulatory hPC, similar to those of the animals carrying −802hPCm1, pseudo-ASE and its binding protein are not active in conferring age-related stabilization of the hPC gene expression. Furthermore, as shown by age-unstable hPC patterns observed in the animal carrying −802hPCm1 or −82hPCm1 (Fig. 3, C and D), the other two PEA-3 consensus elements present in these minigenes, Intron1PEA-3 (GAGGATG) and Exon9PEA-3 (CAGGAAG), do not function as age-related stabilization elements. This is consistent with the facts that these elements also bind a common nuclear protein (Fig. 5A), but the protein is different from that binding to functional hPC ASE as demonstrated by absence of cross-competition, size difference, and presence in both HepG2 cells and the liver (Fig. 5A, B). Together, these results indicate that specific single-base differences of PEA-3 motif (G/CAGGA/AT/G) facilitate highly selective binding of specific nuclear proteins of the large ETS super family (14, 28), resulting in distinctly different functions. Namely, not all of the known PEA-3 consensus sequences and binding proteins are active in age regulation of the genes. Efforts to identify the ASE-binding protein and to determine possible functions of GAGGAAA and (G/C)AGGAATG are in progress.

As demonstrated by the age stability of hPC expression in animals carrying ASE/−82 hPCm1 or ASE/−802hPCm1 (Fig. 6, A and B), hFIX ASE can functionally substitute for hPC ASE in conferring the age-related stable hPC expression, strongly suggesting the functional universality of ASE. This is particularly interesting because hFIX ASE and hPC ASE have different evolutionary origins. Human FIX ASE was derived from a retrotransposed LINE1 sequence (22), whereas hPC ASE was not (20). It is important to note that all animals carrying ASE/−82hPCm1 (Fig. 6A) showed age-stable hPC expression but maintained absolute hPC levels very similar to or only marginally higher than the prepuberal hPC levels of animals carrying −82hPCm1 (Fig. 3D), even with transgene positional effects taken into account. The minigene ASE/−802hPCm1 also showed a similar phenomenon (Fig. 3C; Fig. 6B). Together, these observations strongly suggest that ASE is a unique age-related transcription element essential for stabilizing gene expression at the prepu-
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**A**

|            | 32P-Intron1PEA-3 | 32P-Exon9PEA-3 |
|------------|------------------|----------------|
| Liver NEs  | - - + + + + - | - - + + + + + |
| HepG2 NEs | - + - - - - + |
| Intron1PEA-3 | - - - - + - - | - - - - - - - |
| Exon9PEA-3 | - - - - - + - |
| hPC ASE    | - - - - - - - |
| Intron1PEA-3m | - - - - - - | - - - - - - - |
| Exon9PEA-3m | - - - - - - | - - - - - - - |

**B**

|            | 32P-hPC ASE | 32P-Intron1PEA-3 | 32P-Exon9PEA-3 |
|------------|------------|------------------|----------------|
| Liver NEs  | - - - + + + + | - - - + + + + + | - - - + + + + + |
| 32P-hPC ASE | + - - - + - - | + - - - + - - |
| 32P-Intron1PEA-3 | - - - + + + + | - - - + + + + + |
| 32P-Exon9PEA-3 | - - - + + + + | - - - + + + + + |
| Intron1PEA-3 | - - - + + + + | - - - + + + + + |
| Exon9PEA-3 | - - - + + + + |
| hPC ASE    | - - - + + + + |

Fig. 5. EMSAs of PEA-3 consensus elements in the intron 1 and exon 9 of the hPC gene. Double-stranded oligonucleotides, **Intron1PEA-3** and **Exon9PEA-3**, corresponded to regions spanning nt +689 through +706 (5'-TCCTGGAGGATGGGGGAC-3') and nt +10280 through +10297 (5'-GACCGGCAAGATGGCTGC-3'), respectively, of the hPC gene (PEA-3 consensus element underlined). Mutated -dr double-stranded oligonucleotides, **Intron1PEA-3** and **Exon9PEA-3** used for competition assays were 5'-TCCTGGAGGATGGGGGAC-3' and 5'-GACCGGCAAGATGGCTGC-3', respectively (mutated oligonucleotide is labeled by the underline). AIE oligonucleotides of hPC and hFIX used were as described in the legend to Fig. 4. **A**, lanes 1–6 contain **32P-labeled** Intron1PEA-3 oligonucleotide. Lane 1, without NEs; lane 2, with liver NEs; lane 3, with liver NEs; lane 4, with a 100-fold excess of unlabeled Intron1PEA-3 and liver NEs; lane 5, with a 100-fold excess of unlabeled mutated Intron1PEA-3 and liver NEs; lane 6, with a 100-fold excess of unlabeled hPC ASE and liver NEs; lane 7, without NEs; lane 8, with HepG2 NEs; lane 9, with liver NEs; lane 10, with a 100-fold excess of unlabeled mutated Intron1PEA-3 and liver NEs; lane 11, with a 100-fold excess of unlabeled hPC ASE and liver NEs; lane 12, with a 100-fold excess of unlabeled mutated Intron1PEA-3 and liver NEs. **B**, lanes 7–12 contain **32P-labeled** Exon9PEA-3 oligonucleotide. Lane 7, without NEs; lane 8, with HepG2 NEs; lane 9, with liver NEs; lane 10, with a 100-fold excess of unlabeled Exon9PEA-3 and liver NEs; lane 11, with a 100-fold excess of unlabeled mutated Exon9PEA-3 and liver NEs; lane 12, with a 100-fold excess of unlabeled hPC ASE and liver NEs. Shifted band positions are indicated by c on the left. **B**, lane 1, labeled hPC ASE without NEs; lane 2, labeled Intron1PEA-3 without NEs; lane 3, labeled Exon9PEA-3 without NEs; lane 4, labeled hPC ASE with liver NEs; lane 5, labeled Intron1PEA-3 with liver NEs; lane 6, labeled Exon9PEA-3 with liver NEs; lane 7, labeled hPC ASE with a 100-fold excess of unlabeled Intron1PEA-3 and liver NEs; lane 8, labeled hPC ASE with a 100-fold excess of unlabeled Exon9PEA-3 and liver NEs; lane 9, labeled hPC ASE with a 100-fold excess of unlabeled Exon9PEA-3 and liver NEs; lane 10, labeled Intron1PEA-3 with a 100-fold excess of unlabeled Exon9PEA-3 and liver NEs; lane 11, labeled Exon9PEA-3 with a 100-fold excess of unlabeled Intron1PEA-3 and liver NEs. Shifted band positions are shown by b and c on the right.

The hPC gene lacks any AIE-like element. However, as demonstrated by the animals carrying −1462hPCm1/AIEr, an additional unit of hFIX AIE into the 3′-UTR dramatically changed the age-stable expression pattern of −1462hPCm1 to an age-related increase expression pattern, similar to that of the hFIX gene (Fig. 6C). A similar conversion was also obtained in animals carrying ASE/−1462hPCm1/AIEr, which has an additional ASE at the 5′-end of −1462hPCm1, further supporting the fact that presence of just one unit of ASE and AIE is sufficient to confer such a drastic change in age regulation of gene expression (Fig. 6D).

The hFIX-derived AIE and its reverse form have the potential to form distinct stem loop structures in its RNA form. A hPC minigene, −1462hPCm1/AIEr, which has a unit of AIE inserted in the 3′-UTR in the reverse orientation, showed an hFIX-like age-related increase expression pattern in animals (Fig. 6E), suggesting that the age-regulatory function of AIE is independent of its orientation in 3′-UTR of the hPC or hFIX gene and may correlate with its unique secondary stem loop structure. The failure of inducing any age-related hFIX expression increase in animals carrying minigene AIE/−416FIXm1/AIE, which has AIE inserted at the immediate 5′-end position of the hFIX promoter (supplemental Fig. B), indicates that AIE apparently does not function as a transcriptional enhancer. The age-related increase of circulatory hPC in the animals carrying −1462hPCm1/AIE was accompanied by a similar age-related increase pattern in the liver mRNA level (Fig. 6G), suggesting that AIE functions to induce age-associated elevation of mRNA levels, most likely through increasing mRNA stability. The underlying molecular mechanisms remain to be elucidated.

As demonstrated by animals carrying −802FIXm1/mDR, mDR actually functions as the murine counterpart of the hFIX AIE (supplemental Fig. C), supporting the possibility that the age-regulatory mechanisms involving ASE and AIE may be widely utilized across different animal species. Whether the GA or AT repeat alone or both are needed to confer the age-related increase in expression remains to be tested. Together, these results demonstrate that the dinucleotide repeats pres-
FIG. 6. Longitudinal analyses of circulatory hPC or hFIX levels in transgenic mice carrying hPC or hFIX minigenes. A, ASE/−82hPCm1; B, ASE/−802hPCm1; C, −1462hPCm1/AIE; D, ASE/−1462hPCm1/AIE; E, −1462hPCm1/AIEr. Animal lines are marked according to the format in Fig. 3. Longitudinal analyses of progeny mice (F1 and F2 generations) produced from founders carrying hPC minigenes showed age-regulatory patterns similar to their founders (supplemental Fig. E). F, Northern blot analysis of hPC mRNA levels in the liver of 1-month-old (P507A: F1/m), 3-month-old (P507C: F1/m), and 5-month-old (P507H: F1/m) progeny mice carrying ASE/−802hPCm1. Animals P507A, P507C, and P507H were from the same second generation produced by founder F507 and had similar levels of circulatory hPC at 1 month of age (567, 581, and 573 ng/ml serum, respectively). Animals P507C and P507H had circulatory hPC of 553 and 584 ng/ml serum at 3 and 5 months of age, respectively. Lane 1, nontransgenic mouse; lane 2, P507A (1 month old); lane 3, P507C (3 months old); lane 4, P507H (5 months old). Ratios of hPC mRNA levels of 3- and 5-month-old versus 1-month-old animals were 1.10 and 1.14, respectively. Positions of hPC mRNA in the upper panel and 18 S rRNA in the lower panel are indicated by hPC and RNR 18, respectively. G, Northern blot analysis of hPC mRNA levels in the livers of 1-month-old (P780C: F1/m), 3-month-old (P780E: F1/f), and 5-month-old (P780G: F1/o) progeny mice carrying −1462hPCm1/AIE. P780C, P780E, and P780G animals were from the same second generation produced by founder F780 and, at 1 month of age, had circulatory hPC of 358, 331, and 344 ng/ml serum, respectively. Animal P780E produced 659 ng/ml serum hPC at 3 months of age, and animal P780G had 1326 ng/ml serum hPC at 5 months of age. Lane 1, nontransgenic mouse; lane 2, P780C (1 month old); lane 3, P780E (3 months old); lane 4, P780G (5 months old). Ratios of hPC mRNA levels of 3- and 5-month-old over 1-month-old animals were 2.01 and 3.92, respectively. Positions of hPC mRNA and 18 S rRNA are indicated as in F.
Age-related Regulation

TABLE I
The clearance time of hPC in mice

| Age      | Clearance time (t1/2) |          |
|----------|-----------------------|----------|
|          | α-Phase               | β-Phase  |
| months   | h                     |          |
| 2        | 6.2 ± 0.2             | 16.6 ± 0.4|
| 8–10     | 5.8 ± 0.4             | 15.9 ± 0.4|
| 19–20    | 6.0 ± 0.3             | 16.1 ± 0.3|

critical age-related regulatory elements, ASE and AIE (Fig. 7). The hPC gene uses ASE only, resulting in its age-stable expression pattern, while the hFIX gene uses both ASE and AIE for its age-related increase pattern of expression.

We now have set a new stage toward comprehensive understanding of age-related regulation and homeostasis of the blood coagulation system. The regulatory mechanisms may also be involved in age-related regulation of genes of many other physiological systems. Genetic elements identified in the present studies may find their valuable utilities in modifying age regulation of various genes and in developing optimized gene transfer vector systems for gene therapy.

Acknowledgments—We thank Francis Castelino for providing human protein C cDNA and Akiko Kurachi, Myron Levine, John Moran, and Thomas Glover for critical reading of the manuscript.

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