Transcription factors and age-related decline in apolipoprotein A-I expression

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Abstract **Apolipoprotein (apo)A-I alone or as a component of high density lipoprotein particles has antiatherogenic properties. The age-dependent decline in abundance of this protein may underlie the higher risk for developing occlusive coronary artery disease (CAD) in older individuals. Similar to humans, expression of rat apoA-I also declines with age. Results in rats showed that levels of serum apoA-I protein, hepatic mRNA, and transcription of the gene were decreased to 39%, 18%, and 38%, respectively, in 180-day-old animals compared to those of newborn rats. These findings suggest that a nuclear mechanism(s) may account for the decline in apoA-I expression. Accordingly, we examined hepatic nuclear binding activity to four specific *cis*-acting elements of the rat apoA-I promoter. There were age-dependent changes of binding activity to two proximal sites, B and C, but not to the more distal elements, IRCE and A. Decreased B-site binding activity correlated with lower mRNA levels encoding the activator, HNF-3β. The age-dependent change in the pattern of binding to site C was due to a switch from the activator, HNF-4, to the repressor, ARP-1. In summary, the age-related decline in apoA-I expression may arise from a reduction in the activity of both cis-acting elements, B and C.**—Nakamura, T., A. Fox-Robichaud, R. Kikkawa, A. Kashiwagi, H. Kojima, M. Fujimiya, and N. C. W. Wong. **Transcription factors and age-related decline in apolipoprotein A-I expression.** ***J. Lipid Res.*** **1999.** 40: **1709–1718.**

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Apolipoprotein A-I (apoA-I) is the major protein component of the high density lipoprotein (HDL) particles in serum. These antiatherogenic particles act as a shuttle to transport cholesterol from extrahepatic tissues to the liver for further processing and excretion in the form of bile salts (1, 2). Numerous epidemiologic studies show that the serum concentration of HDL correlates inversely with morbidity arising from coronary arterial disease (CAD) (3, 4). Additional observations demonstrate that apoA-I alone or as part of HDL have a protective effect which guards against the development of atherosclerosis (5, 6).

CAD is an acquired disease and the risk of developing CAD rises with an individual's age (7). CAD is currently the number one cause of premature death in modern affluent societies. Although poor dietary habits underlying hypercholesterolemia are an important risk factor for CAD, the age-related decline in the levels of HDL adds to the problem. This idea is supported by epidemiologic studies showing that serum concentrations of HDL are roughly 1.5-fold higher in infants and youths compared to those in adults (8, 9).

Similar to humans, the levels of HDL in rodents also decline with age (10, 11). Ontogeny studies showed that expression of apoA-I mRNA is highest around the time of birth (10–12). Soon after birth, there is an induction of apoA-I mRNA until day 19, followed by a steady decline. The observed induction and then decline in the levels of apoA-I mRNA correlate with weaning and the abundance of endogenous hormones such as glucocorticoid, insulin, and thyroxine (10, 12). Given the pivotal role of apoA-I protein in the protection against CAD, we have studied the mechanisms that regulate expression of this gene (13–16).

The goal of this study is to examine potential nuclear mechanism(s) that underlie the age-associated decline in apoA-I expression. It is expected that results arising from these experiments will provide important clues to finding new ways to raise levels of apoA-I and thus benefit those patients with CAD. In studies summarized below, we show that apoA-I expression decreases with age and this decline correlates with the changes in activity of two sets of nu-

Abbreviations: apo, apolipoprotein; ARP-1, apolipoprotein repressor protein-1; β-gal, β-galactosidase; CAD, coronary arterial disease; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; HDL, high density lipoprotein; HNF, hepatocyte nuclear factor; IGF, insulin like growth factor; IGFBP, insulin like growth factor binding protein; IRCE, insulin responsive core element; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR; SD, standard deviation; SDS, sodium dodecyl sulfate

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clear transcription factors that bind to different *cis*-acting sites which cooperate in a synergistic fashion to inhibit expression of the gene as the animal ages.

METHODS

Animals

Male Sprague-Dawley rats at the time of birth, and at 15, 30, 60, 120, and 180 days of age were purchased from Campus Breeders, University of Calgary. Newborn rats were killed 1 or 2 days after their birth. To eliminate the possibility of a circadian effect on the level of apoA-I expression, all animals were killed at 10:00 am. Blood was drawn using heparinized syringes and the livers were collected and snap-frozen in liquid nitrogen for analysis of parameters outlined below.

Western blot analysis

Relative amounts of apoA-I protein in serum were measured using Western blot analysis according to procedures described previously (17). In brief, 2 μg of serum protein was separated by standard SDS-polyacrylamide gel electrophoresis and then transferred to PVDF membrane. The antibody used to assay for the levels of apoA-I was created 'in house' (18) and the signal was detected using the ECL kit (Boehringer-Mannheim). The relative intensity of each signal was quantitated using computer-assisted videodensitometry (19).

Northern blotting

Relative amounts of hepatic apoA-I mRNA in rats of various ages were determined using Northern blot analysis. Hepatic RNA was extracted according to methods described previously (20). Five μg of total RNA from each rat was separated using a denaturing agarose gel electrophoresis (21) and transferred to a Zetabind membrane (Cuno, AMF) and apoA-I mRNA was detected by hybridization to a [^{32}P]-cDNA specific to the apoA-I probe. The signal was visualized by autoradiography and quantitated using a phosphoimager (Molecular Diagnostics).

Nuclear run-on transcription assay

Freshly isolated livers of two rats were pooled and hepatic nuclei were prepared as described (22). The run-on assays were performed according to procedures described by Skettering, Gjernes, and Prydz (23) with minor modifications. In brief, 3.7×10^7 nuclei were incubated in the presence of 25 mm Tris, pH 8.0, 2.5 mm $MgCl_2$, 150 mm KCl, 0.5 mm ATP, GTP and CTP, 10 mm creatinine phosphate, 100 mg creatinine phosphokinase, 1 unit/ml RNasin, 1 mm dithiothreitol, 0.5 mm phenylmethylsulfonylfluoride, 10% glycerol, and 100 μCi [^{32}P]-UTP at 30°C for 30 min. After addition of DNase I at 37°C for 10 min, the labeled RNA was purified by digestion of the reaction with proteinase K and then extracted with phenol–chloroform followed by precipitation with ammonium acetate–isopropanol. The labeled RNA product was hybridized to nylon membranes containing immobilized apoA-I cDNA at 65°C for 48 h. The ribosomal RNA and rat albumin cDNAs used as standards in the assay were generous gifts from M. Getz and H. Towle (University of Minnesota), respectively. Signals from the washed membranes were detected by autoradiography and quantitated by video-assisted densitometry. Intensity of the apoA-I signal was expressed relative to that of albumin.

Competitive RT-PCR

This technique (24, 25) was used to measure the amounts of each of the HNF-3 isoforms, HNF-4, and ARP-1 mRNA. Materials required for this procedure include polyA RNA prepared from liver extracted using the polyA tract mRNA isolation kit (Qiagen). In addition, rat apoA-I cDNA (21 to 799) was obtained by RT-PCR from total liver RNA using the primer pairs apo 1 and apo 2 noted below. Upper case denotes sequences from HNF-3 isoforms, HNF-4 or ARP-1 and lower case from rat apoA-I cDNA. The three pairs of primers 3α1/3α2, 3β1/3β2 or 3γ1/3γ2 contain segments homologous to HNF-3 and apoA-I. The 4f and 4r contain sequences from HNF-4 and apoA-I. The sequences in Af and Ar come from ARP-1 and apoA-I. The three single primers; 3α, 3β, and 3γ were only homologous to HNF-3α, 3β, and 3γ isoforms, respectively. The 4s and As are the first strand primers for HNF-4 and ARP-1, respectively.

3α1.) TACCGCGGACGTCTCGGcacatccttcgggatgaa
3α2.) TTCTACGAGTGCGACTCttctttttggcctc
3β1.) GCGACCGGCGACGAGGTcacatccttcgggatgaa
3β2.) CCGCCGGCCCCGATACCttctttttggcctc
3γ1.) aagtagggcttcctacaGCGGCGAGGTGTATTCTCCAGT
3γ2.) tactccggtttttcttTGCCACCTTGACGAAGCA
3α.) GTCTCAGCGTGAGCATCTT
3β.) CCAGTTCATATTGGCGTA
3γ.) GCTGCCACCTTGACGAAGCA
4f) ACCTCCCATCCGACGcacatccttcggga
4r) CACAGGTAAGCGTAGGttctttttggcct
4s) CACAGGTAAGCGTAG
Af) GACCTTGTACATGcacatccttcggggatg
Ar) GTAGTGTATTCGTGGAttctttttggcct
As) GTAGTGTATTCGTG
apo 1) cacatccttcgggatgaa
apo 2) ttctttttggcctcat

The first reaction is reverse transcription of the HNF-3 isoforms, HNF-4 or ARP-1 mRNA. These reactions contained 1 μg of poly A^+ RNA, the antisense primer 3α, 3β, 3γ, 4s, or As specific for HNF-3α, -3β, -3γ, HNF-4 or ARP-1, respectively, and 200 units of reverse transcriptase (New England Biolabs). A fixed amount of the single-stranded DNA product from this reaction is mixed with the primer pair 3α1/3α2, 3β1/3β2, 3γ1/3γ2, 4f/r or Af/r, respectively comprised of sequences homologous to either HNF-3α, -3β, -3γ, HNF-4 or ARP-1 and rat apoA-I cDNA. In addition, also included in each reaction are varying amounts of competitor, rat apoA-I cDNA ranging from 19.2 to 5400 pg. The conditions for the PCR reaction are as follows: initial step of 3 min at 94°C and then 0.5 min at 94°C, 1 min at 57°C, 1.5 min at 72°C for 25 cycles with 10 min at 72°C final extension. These conditions ensured that the PCR reactions of the target or competitor DNA were still within the exponential range and that the ratios between the two products from PCR were the same (data not shown). The two cDNA products from this reaction were visualized by ethidium bromide staining after agarose gel separation. The quantities of HNF-3 isoforms, HNF-4 and ARP-1 mRNA were determined by equal intensity of the band arising from HNF-3 and the known concentration of the competitor, apoA-I cDNA added to each reaction.

In vitro transcription and translation of HNF-3 isoforms

The coding regions of the HNF-3 cDNAs were inserted separately into pTZ-18R or -18U vectors and these templates were transcribed in vitro with T7 RNA polymerase (Promega) according to the manufacturer's guidelines. The RNA product was translated with rabbit reticulocyte lysate system (Promega) in the presence of [^{35}S]methionone (NEN, 400 Ci/ mmol). The radiolabeled product from the reaction was analyzed by SDS-PAGE followed by autoradiography of the dried gel (26). Alternatively, an aliquot of the reaction was used in the EMSA studies noted in the next section.

Electrophoretic mobility shift assay

Synthetic oligonucleotide duplexes spanning the IRCE (−413 to −392, ACTTTGAGGCGGGGATGTGAGT) and site B (−172

to −143, CTTCCTGTTTGCCCACTCTGTTTGCCTAGCCT) from the rat apoA-I gene were radiolabeled by incubation with [γ-^{32}P]-ATP (NEN) plus T_4-polynucleotide kinase and [α-^{32}P]-dCTP (NEN) plus the Klenow fragment of DNA polymerase I, respectively. Oligomers spanning site A (−208 to −193, TGAACCCT TGATCCCA) and site C (−137 to −100, ACAGAGCTGATCCTT GAACTCTAAGTTCCACATCGCCA) was inserted into pTZ18R at the Sma I site and amplified before excising by digestion with Hind III plus Eco RI and Eco RI plus Xba I, respectively. These fragments were labeled by incubation with [α-^{32}P]-dCTP (NEN) plus the Klenow fragment of DNA polymerase I. Extracts from rat liver were prepared and EMSA studies were performed as described previously (27).

Cell culture and transfection

Human hepatoma cells, HuH-7, were maintained in RPMI-ISE medium and COS-7 cells were maintained in DMEM with 10% FCS, as previously described (28). HuH-7 cells were transfected using the calcium phosphate co-precipitation method (29), with up to 5 μg of pAI 474.CAT vector along with 5 μg of HNF-3α, -β or -γ cDNA contained in the eukaryotic expression vector pCMV. COS-7 cells were also transfected with up to 5 μg of pAI 474.CAT plus 2.5 μg of HNF-3α, -3β or -3γ cDNA and the pMT2 expression vector that expressed either HNF-4 (F. Sladek, University of California Irvine) or ARP-1 (M. Tsai, Baylor University) (30). All transfections contained 2.5 μg of a β-Gal expression vector. Each transfection also contained 2.5 μg of pRSV-βgal plasmid to correct for DNA uptake by the cells. Total plasmid concentration was adjusted to 17.5 μg using a nonspecific plasmid, pTZ18R. The assay for CAT- and β-galactosidase activity has been described previously (31).

Statistics analysis

All results were expressed as the mean ± SD. Statistical significance was determined by one-way ANOVA analysis.

RESULTS

Age dependent decline in apoA-I gene activity

To determine whether levels of rat apoA-I declined with age, Western blot analysis was used to measure abundance

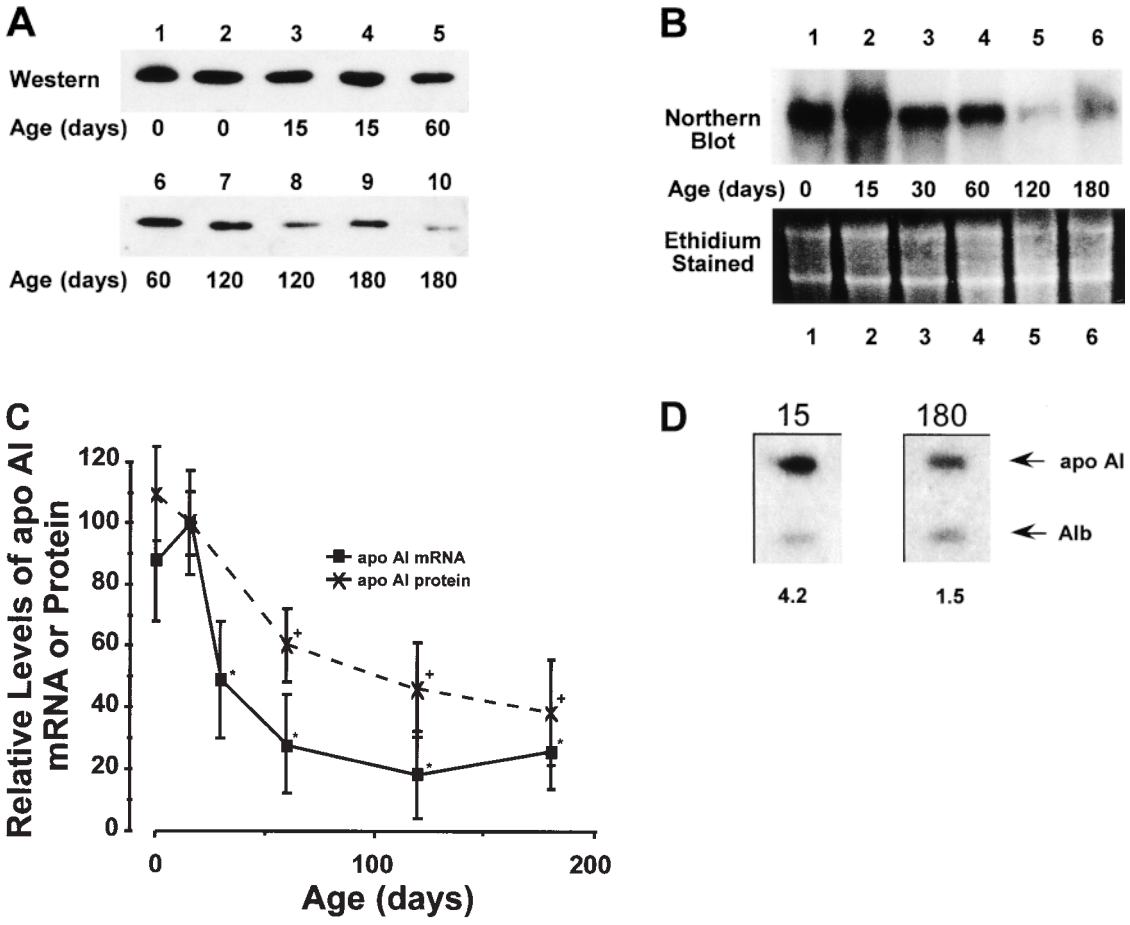

Fig. 1. Serum apoA-I protein, liver apoA-I mRNA, and gene transcription decline with age. Panel A shows a Western blot of the rat apoA-I signal contained in 2 μg of serum protein from rats at 0, 15, 60, 120, and 180 days after birth. The age of the animals is shown below each lane. Panel B shows an autoradiograph of a Northern blot probed with radiolabeled rat apoA-I cDNA. Each lane contains 5 μg of total RNA from 0, 15, 30, 60, 120, and 180-day-old animals. The lower part of this panel shows a photograph of ethidium bromide staining of the gel before transfer. Panel C shows the relative amounts of the protein and mRNA in a graphic form. Each analysis was presented as mean ± SD at each time point (* or + denotes $P < 0.01$ for protein and mRNA, respectively, n = 4). Panel D: Age-related transcription of rat apoA-I in vivo. Run-on transcription of the apoA-I gene in nuclei from 15- and 180-day-old rats is shown in this photograph. Radiolabeled RNA was hybridized to rat apoA-I and albumin cDNAs. Hybridized RNA detected by autoradiography are representative of experiments performed at least 3 times. The relative rates of apoA-I gene transcription expressed relative to that of albumin are shown below each autoradiograph.

of the protein in newborn (NB), and 15, 60, 120, and 180-day-old rats. Results of these studies (**Fig. 1A**) showed that relative amounts of the protein decreased with age. In the 180-day-old rats, the level of apoA-I was roughly 39% of that in the newborn animals. Next, Northern blot analysis was used to quantitate the relative amounts of hepatic apoA-I mRNA in the same animals (Figs. 1B and 1C). These findings confirmed previous observations that the levels of apoA-I mRNA were higher in 19-day-old rats (10, 12) followed by a progressive decrease such that at 180 days, the level of apoA-I mRNA was 18% of that in the newborn animals.

The preceding observations suggested the possibility that changes in apoA-I gene transcription may underlie the decline in levels of the protein and mRNA. Therefore, the in vitro nuclear run-on assay was used to measure the rate of gene transcription. Results (Fig. 1D) showed that the older rats had a lower rate of apoA-I gene transcription, 39% compared to that in the younger animals. Data from 15- and 180-day-old animals were studied because the livers of newborn rats were too small and expression of apoA-I mRNA in the 60- and 120-day old animals was not different from that of 180 days. Together these findings show that apoA-I protein and mRNA decrease with age. This decline in apoA-I expression is due, in part, to a reduction in transcription of the gene.

Site B binding activity decreases along with ApoA-I expression

The age-related reduction of apoA-I gene transcription may arise from changes in the activity of specific hepatonuclear transcription factor(s). At least four candidate *cis*-acting elements bound by their respective factors are known to play important roles in regulating activity of the promoter (32). Each of the four sites, IRCE (−413 to −394), A (−208 to −193), B (−172 to −143), and C (−137 to −100), was used in EMSA studies to determine whether nuclear binding activity to these sites varied with age. Results (**Fig. 2**) showed that DNA binding activity from rat liver nuclear extract to the more distal sites, IRCE and A, were minimal and not significantly different amongst animals of various ages. In contrast, there were striking changes in the binding activity to the more proximal sites, B and C. Results arising from the study of site B (Fig. 2) revealed the presence of two complexes with different electrophoretic mobilities and relative abundance. Although the faster migrating complex was most abundant, both complexes had a marked and progressive decline in response to age such that site B binding activity in the 180-day rats was only a fraction of that in the newborn animals.

Site C binding activities changes with age

In contrast to the decreased binding activity to site B that to the C-motif had roughly the same intensity in all age groups examined. However, there was a slight but significant shift in the mobility of the protein:DNA complex (Fig. 2). The complex derived from extracts of the older animals migrated slightly faster compared to that in the young rats. The R_f values for the upper and lower complexes were 0.27 and 0.31, respectively. According to our previously published results, these values matched precisely

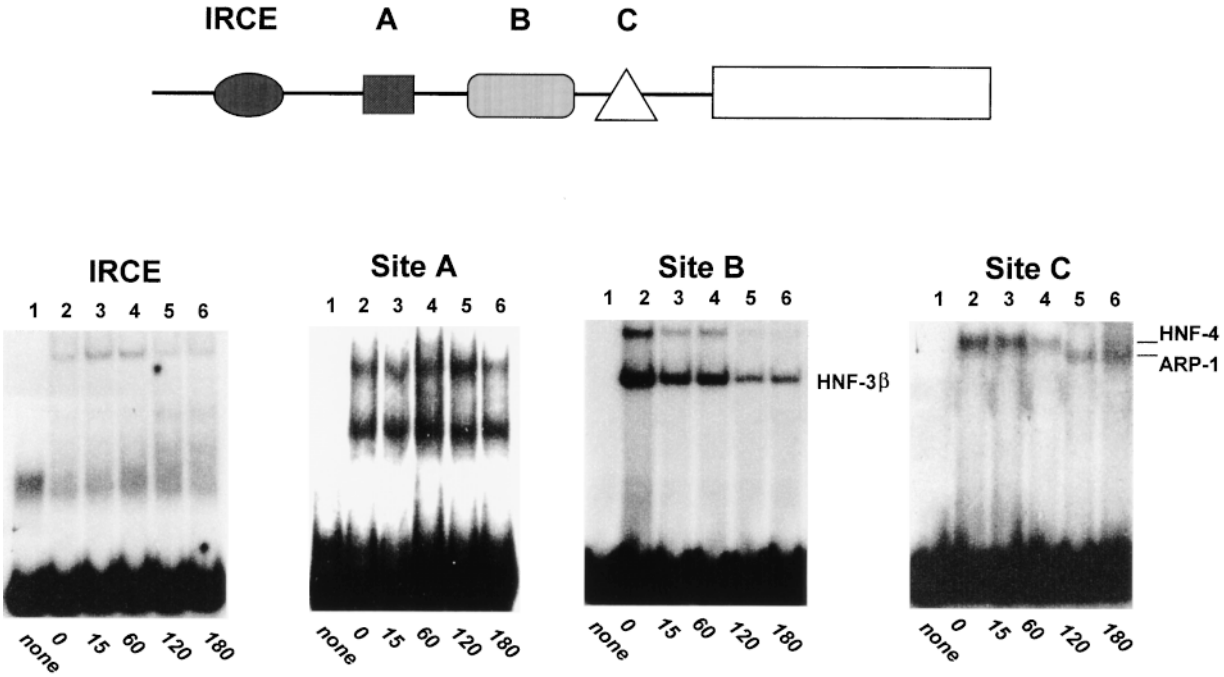

Fig. 2. Binding activity to IRCE, site A, site B, and site C of apoA-I promoter. The top portion shows a schematic map of the relative location of *cis*-acting sites in the rat apoA-I promoter. The lower portion shows autoradiographs of EMSA assays using hepatonuclear extract from pooled newborn rats, and single 15, 60, 120, and 180-day-old rats. Binding activity to radiolabeled IRCE, site A, site B, and site C is indicated above each panel. These results are representive of experiments performed in at least three different animals or pools of animals of each age.

with those of HNF-4 and ARP-1, respectively, bound to site C (13). The assignment of the identity of the proteins bound to site C was accomplished by matching the mobility of the protein–DNA complexes derived from liver extract with that from HNF-4 and ARP-1 expressed in vitro (13).

Next, we used RT-PCR to measure the amounts of apoA-I, HNF-4, and ARP-1 mRNA in the livers of animals from the various ages. To be certain that the number of cycles used to amplify the products from the reverse transcription was within the linear range, we performed 15, 20, 23, and 27 cycles (**Fig. 3A and 3B**) of PCR using first strand product derived from a reaction primed with apo 2 oligomer. Results showed a linear increase in accumulation of the PCR products up to 27 cycles; we routinely used 25 cycles. In keeping with the findings from the EMSA studies, RT-PCR results (Fig. 3C) showed that abundance of the HNF-4 mRNA was high in the newborn rats and this value declined with age. The converse was true for the ARP-1 mRNA levels. The findings in this and the preceding section show that whereas sites IRCE (33) and A do not appear to participate in the age-dependent decline in apoA-I gene activity, the binding activities to sites B and C were associated with significant changes in response to aging.

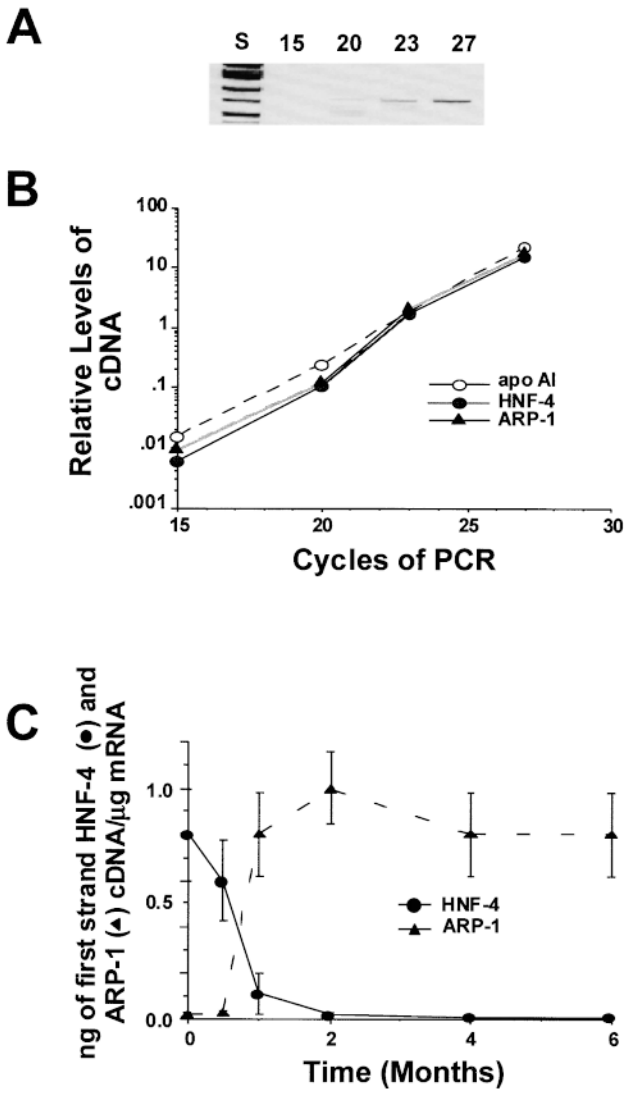

Fig. 3. Reciprocal changes in the levels of HNF-4 and ARP-1. Panel A shows an ethidium bromide stained gel of apoA-I cDNA derived from RT-PCR of total hepatic RNA from a newborn rat after 15, 20, 23, and 27 cycles (noted on top of each lane). Panel B shows relative levels of apoA-I (open circle), HNF-4 (closed circle), and ARP-1 (triangle) cDNA plotted as a function of PCR cycles. Panel C shows the concentration of first strand HNF-4 (circle) and ARP-1 (triangle) cDNA in hepatic RNA from rats of various ages; newborn, 15, 30, 60, 120, and 180 days. Each point represents the mean ± SD (* denotes $P < 0.01$, n = 4).

HNF-3β binding to site B declines with age

Despite the finding that site B binding activity paralleled the age-dependent reduction in apoA-I gene activity, the identity of this factor remained unknown. A previous report using a super-shift assay showed that site B was bound by HNF-3β (34). To determine whether other isoforms of HNF-3α, -3β or -3γ (35) could account for site B binding activity in hepatonuclear extract from rats of various age groups, we expressed each of the isoforms separately using in vitro transcription of the cDNA followed by translation of the mRNA product (**Fig. 4A**). These proteins were bound to radiolabeled site B and the resulting complexes were separated by gel electrophoresis (Fig. 4B). The protein:DNA complexes had the same eletrophoretic mobility as that of the slower migrating, most abundant site B binding activity found in rat hepatonuclear extracts. The identity of the minor band is not known. Unfortunately, all three of the dominant complexes arising from HNF-3α, -3β or -3γ bound to site B had the same electrophoretic mobility. The transcribed proteins bound specifically to the labeled site B, as indicated by the ability of unlabeled site B DNA to displace binding to the probe. These results provide two important pieces of information: *i*) site B binding activity in rat hepatonuclear extracts is an isoform of HNF-3 but *ii*) we could not tell which isoform changed with the animal's age because all three complexes had the same electrophoretic mobility. The translated material did not help us identify the protein that formed the fastest protein:DNA complex observed in Fig. 2, panel 3.

Abundance of HNF-3β mRNA matched with changes in binding to site B

The inability of EMSA to identify the specific HNF-3 isoform that changed with age prompted us to use an alternative approach. We postulated that perhaps a correlation between the levels of HNF-3α, -3β or -3γ mRNA in liver and site B binding activity might be useful. Northern blot analysis of the mRNAs failed because abundance of the transcripts was too low to be detected. Therefore, we turned to the use of competitive RT-PCR to measure the amount of each HNF-3 mRNA isoform. An example of the actual PCR products is shown in **Fig. 5A**. Quantitation of these results for each isoform (Fig. 5B) showed that changes in the amount of HNF-3β mRNA matched closely with the age-associated decline in site B binding activity. Additionally, neither HNF-3α nor -3γ changed significantly with age. Together these studies suggest that the de-

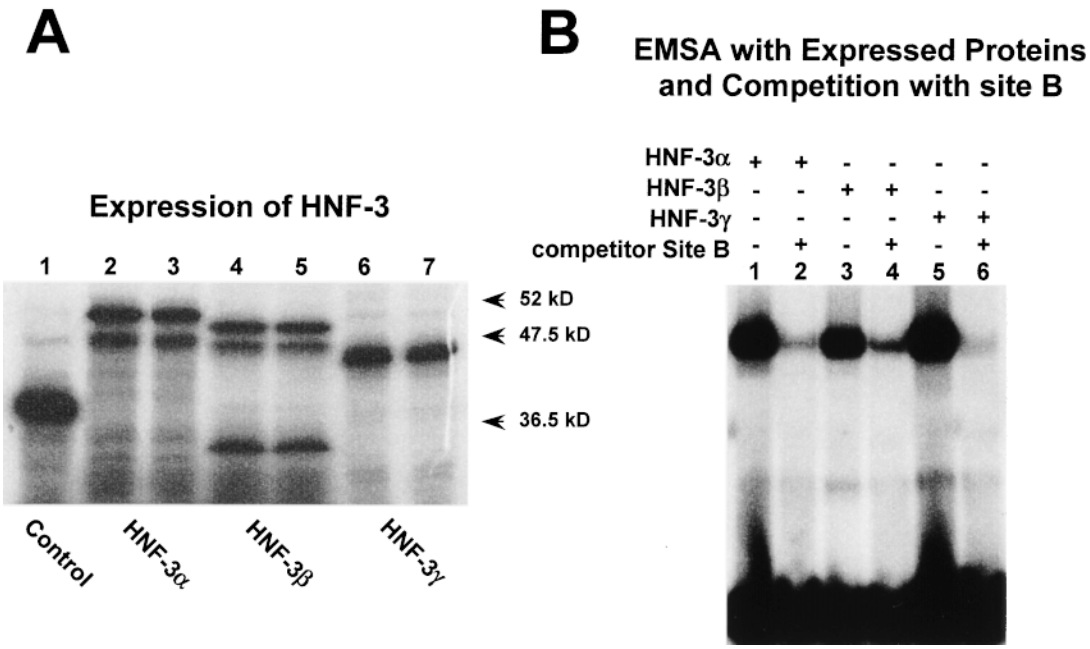

Fig. 4. HNF-3α, β, and γ protein binding to site B have the same mobility. Panel A shows an autoradiograph of translation products labeled with [^{35}S]methionine separated by SDS-PAGE. The products in lanes 2 and 3, 4 and 5, and 5 and 6 were programmed by HNF-3α, -3β, and -3γ mRNAs, respectively. A molecular mass marker of 36.5 kD appears in lane 1. The molecular masses of HNF-3α, -3β, and -3γ are 50, 47, and 42 kD, respectively. Panel B shows an autoradiograph of EMSA analysis of in vitro translated products bound to radiolabeled site B. The HNF-3α, -3β, and -3γ proteins bound to the probe appear in lanes 1, 3, and 5. Competition studies performed by adding 100-fold m excess of unlabeled site B DNA are shown in lanes 2, 4, and 6.

crease in the abundance of HNF-3β mRNA correlates with the age-related decline in binding activity to site B and apoA-I gene transcription.

HNF-3β activates but HNF-3α and -3γ exert a dominant negative effect on site B

Because site B has a positive effect on apoA-I promoter activity (32, 36) and isoforms of HNF-3 bind to this site, then these factors should enhance transcription of the gene. To test this possibility, we performed co-transfection studies to overexpress HNF-3α, -3β or -3γ in the presence of a reporter construct containing the B-motif. As predicted, HNF-3β enhanced the activity of the promoter compared to control in HuH-7 cells (**Fig. 6A**). However, both HNF-3α and -3γ had negative effects on site B. Additionally, when HNF-3β was co-transfected

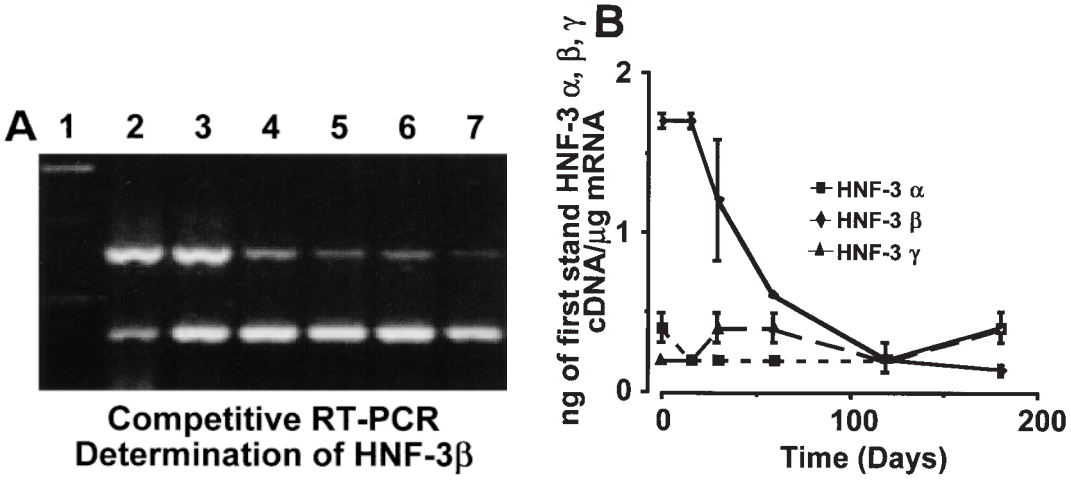

Fig. 5. HNF-3β mRNA decreases with age. Panel A shows an ethidium bromide-stained agarose gel of competitive RT-PCR products from hepatic mRNA of newborn animals primed with the 3β1/3β2 oligomer pair to measure HNF-3β mRNA. Additionally, a constant amount of apoA-I synthetic competitor cDNA was serially diluted to final concentrations of 5.4, 1.8, 1.2, 0.6, 0.2, and 0.07 ng/μl and then 1 μl was added to reactions shown in lanes 2–8, respectively. The contents of each lane are: 1, marker DNA; 2, 5.4 ng; 3, 1.8 ng; 4, 1.2 ng; 5, 0.6 ng; 6, 0.2 ng and 7, 0.07 ng of competitor apoA-I cDNA. Panel B shows concentration of first strand HNF-3α, -3β, or -3γ cDNA in hepatic RNA from rats of various ages; newborn, 15, 30, 60, 120, and 180 days. Each point represents the mean ± SD (* denotes $P < 0.01$, n = 4).

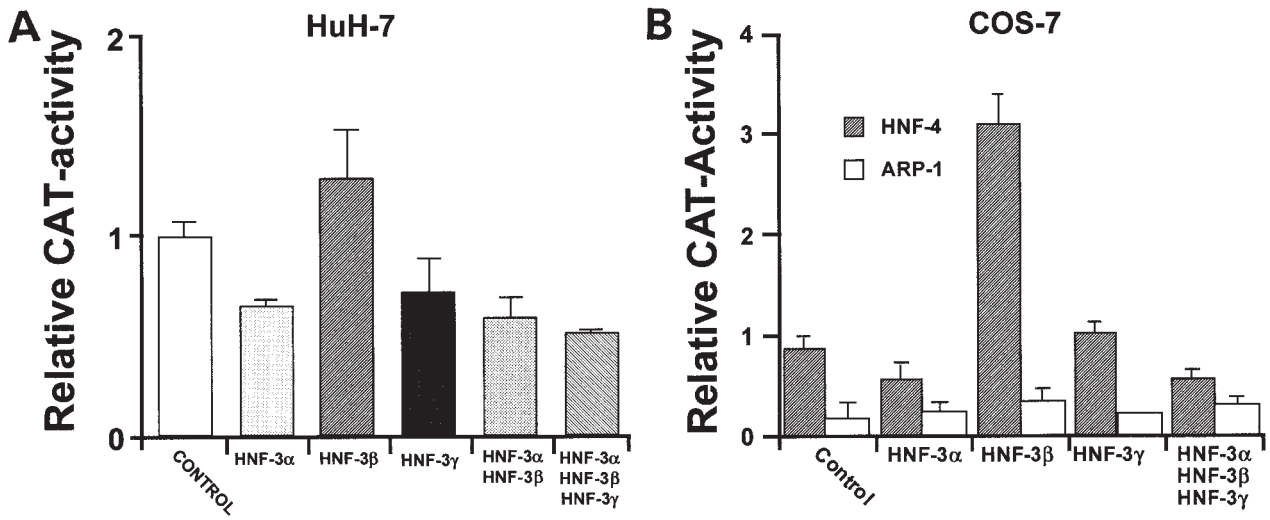

Fig. 6. Effect of HNF-3 isoforms on site B activity. Panel A shows relative CAT activity in HuH-7 cells transfected with pAI.474.CAT alone, with a vector that expresses HNF-3α, -3β, or -3γ or in combination as indicated below each column. Panel B shows similar studies in COS-7 cells co-transfected with pAI.474.CAT plus HNF-4 or ARP-1 and a vector that expresses HNF-3α, -3β, or -3γ or in combination as below each column. Each column represents the mean ± SD (* denotes $P < 0.01$, n = 3).

with -3α plus or minus -3γ these combinations inhibited promoter activity.

HuH-7 cells express endogenous HNF-3 which may make interpretation of above findings less clear. Therefore, COS-7 cells that do not express HNF-3 were used for additional transfection studies. For the apoA-I promoter to be active in these cells, the site C activator, HNF-4 is required. In the presence of HNF-4, the introduction of HNF-3β increased activity of the apoA-I reporter construct (Fig. 6B). However, HNF-3α inhibited activity of the reporter and the combination of HNF-3α and -3β also suppressed apoA-I promoter activity. As expected, when the site C suppressor ARP-1 is present, activity of the reporter is repressed in all transfections. Together these observations show clearly, in two different cell lines, that HNF-3β is an activator and HNF-3α has a dominant negative effect on site B activity. However, HNF-3γ also has a dominant negative effect in HuH-7 but not COS-7 cells.

DISCUSSION

The possibility that the higher risk of acquiring CAD in mature individuals arises from an age-dependent decline in levels of apoA-I prompted us to investigate the mechanism(s) underlying reduced expression of this protein. As the decline of apoA-I levels in rats mirrored that in humans, we used the rodent model for the present study. Results (Fig. 1) in 180-day-old rats showed that absolute values of apoA-I protein, hepatic mRNA, and transcription of the gene were lower compared to those in young animals. We have not expressed these parameters relative to total body or liver weight, which may reduce the differences of the absolute values. Nevertheless, these findings point to a nuclear site for the mechanism(s) that control the age-related decline in apoA-I gene activity. We postulated that differential activities of various transcription factors may serve as a potential mechanism. Therefore, hepatonuclear extracts prepared from rats of different age groups were surveyed for binding activity to four important *cis*-acting elements, IRCE, A, B, and C in the rat apoA-I promoter (Fig. 2). The binding activities to sites IRCE and A were not significantly affected by age. As the IRCE and site A mediate the effects of insulin (37) and thyroid hormone (38), respectively, the lack of binding differences at these motifs suggests that age-related changes are unlikely due to these hormones. In contrast, the binding activity to two proximal sites, B and C, changed with the age of the animal. The changes in binding activities between the two sites were different. Whereas binding activity to site B retained the same pattern (the amount of binding decreased with age), binding to site C formed two different complexes as the animals aged.

First, the studies dealing with site B binding activity in hepatonuclear extract from rats of various ages revealed the presence of two complexes with different electrophoretic mobilities and abundance. The faster migrating and more abundant species had the same electrophoretic mobility as HNF-3 bound to the B-motif. This binding activity decreased with age in parallel with expression of the apoA-I gene. However, there are at least three known isoforms HNF-3α, HNF-3β, and HNF-3γ. Unfortunately, the results of EMSA studies (Fig. 4) did not permit identification of the specific isoform(s) that declined with age. The inability of these findings to provide an answer prompted us to turn to the use of competitive RT-PCR (Fig. 5) to determine whether changes in the levels of mRNA for each isoform of HNF-3 matched with site B binding activity. The results using this technique showed that HNF-3β levels matched the decline in site B binding activity but HNF-3α and HNF-3γ did not change in response to age. Furthermore, the abundance of HNF-3β was much greater than that of either HNF-3α or

HNF-3γ. Together these findings suggest that decreased site B binding activity correlates with the age-dependent decline in the abundance of HNF-3β mRNA.

In previous studies, the deletion of site B reduced apoA-I promoter activity by 30-fold (13). Therefore, we wondered which of the HNF-3 isoforms is expected to have a positive effect on this motif. To address this question, the role of HNF-3 isoforms on site B activity was examined using co-transfection studies in both HuH-7 and COS-7 cell lines (Fig. 6). These results showed that HNF-3β did indeed enhance rat apoA-I site B activity. This finding is similar to that in the human gene (34, 36). Unexpectedly, both HNF-3α and -3γ isoforms had inhibitory effects in HuH-7 cells. Furthermore, combined expression of HNF-3β and -3α with or without -3γ also suppressed activity of the site B. This latter observation suggests that both HNF-3α and -3γ had dominant negative effects on the activity of site B and blocked the enhancing actions of HNF-3β.

The results of the EMSA studies uncovered not only an age-dependent change in binding activity to site B but also to the C-motif. In contrast to the reduction in site B binding activity with age, that to site C showed a shift in electrophoretic mobility in response to age (Fig. 2). Extracts from newborn animals and those up to 60 days of age had a factor that bound to site C with a slightly slower electrophoretic mobility compared to that of the older animals. Results of a previous study from our laboratory showed that the complex with slower mobility reflects binding of an activator, HNF-4 to site C (13). The faster migrating complex represents binding of a repressor, ARP-1 to the C-motif. Therefore, when apoA-I expression is high in the young animals, site C is bound by an activator HNF-4, but as age increases, low apoA-I expression is associated with the binding of a repressor ARP-1 to this motif. Changes in abundance of the mRNA levels encoding HNF-4 and ARP-1 with age mirror the changes in binding activity (Fig. 3). This switch in the age-dependent binding from an activator to the repressor is significant because this change also adds to lower activity of the apoA-I gene in response to age.

How do these observations help explain the age-dependent decline in apoA-I expression? First, there is a clear correlation between apoA-I expression and site B binding activity. As decreased site B binding activity is most likely attributed to reduced levels of the activator, HNF-3β, then this change is expected to lower transcription of the gene. Second, the age-dependent decrease of HNF-3β is not accompanied by changes in levels of other site B binding factors; HNF-3α and -3γ. Both HNF-3α and -3γ have dominant negative effects on site B in the human hepatoma cell line, HuH-7. Although levels of the mRNA encoding these factors remain unchanged as the activator HNF-3β decreases the actions of the inhibitors, HNF-3α and -3γ become more prominent. Recent studies show that the negative effects of HNF-3α are believed to arise from its ability to displace HNF-3β from a common binding site (39). The contribution of site B to the age-dependent decrease in apoA-I is 2-fold, *i*) decreased HNF-3β and *ii*) unmasking of the dominant negative actions of HNF-3α and -3γ both lead to decreased apoA-I gene transcription. A dominant negative effect of HNF-3α and -3γ is not unique because the human insulin-like growth factor I gene, IGF-1 is subject to similar regulatory mechanisms (39, 40).

Given the observed reduction in HNF-3β, two obvious questions arise: *i*) why does the -3β isoform but not -3α or -3γ mediate the age-dependent effects on apoA-I expression and *ii*) do other genes under the control of HNF-3β behave in a manner that parallels its reduction in binding activity. In response to the first question, the HNF-3 isoform has two unique structural features: *i*) it has a stretch of histidine residues within the activation domain of the protein and *ii*) it has 2-casein kinase I phosphorylation sites absent in both HNF-3α or -3γ (41–43). This stretch of histidine residues in HNF-3β may enhance its affinity for the B-motif. Evidence in support of this idea comes from a comparison of HNF-3α and -3β showing a difference in their binding affinities for the TTR promoter (26). The potential phosphorylation sites may allow HNF-3β to respond differentially to various signals (44). These features make HNF-3β different from both HNF-3α and -3γ. It is possible that these unique features enable HNF-3β to respond to the age-dependent effects. The second question deals with the other genes that are regulated by HNF-3β (45, 46). For example, the transthyretin gene is also regulated, in part, by HNF-3β. As predicted from our observation that HNF-3β decreases with age, the expression of transthyretin also declines with age (47). The fact that both transthyretin and apoA-I are somehow involved in amylodosis (48) and that overexpression of these proteins may underlie the onset of amylodosis raises the possibility that nature has purposefully designed the decline in expression of both genes to avoid acquiring such a problem. HNF-3β is known to regulate its own expression (41). If our observation is correct, expression of this gene should decline with age. In line with our hypothesis, we have shown that HNF-3β mRNA expression declines with age. IGFBP-1 promoter is another gene that is bound by HNF-3β (49) and the expression of this protein also declines with age (50).

It is apparent from previous studies that HNF-3β decrease is an important finding that extends to genes other than apoA-I and controls the age-related decline of this promoter. Now we must determine whether the same holds true for the changes predicted by site C. In this case, the switch from HNF-4 to ARP-1 also has important implications with respect to the expression of other genes. ApoC-III expression is also regulated by the same combination of transcription factors. If our predictions are correct, apoC-III expression should also decline with age, but this remains unknown.

Of what importance are our findings regarding previous theories concerning age-dependent changes in apoA-I gene activity? It was previously thought that levels of apoA-I decreased with age because the insulin levels in young animals were higher (51) and this hormone enhances apoA-I gene expression (12, 52). Our studies do not support this possibility. Further studies in this area should be undertaken to include or exclude this possibility.

In summary, the study reported here reveals three novel findings: *i*) the age-dependent decline in apoA-I expression may arise from changes in binding activities to two *cis*-acting sites B and C in the rat apoA-I promoter; *ii*) aging decreases the abundance of HNF-3β mRNA and this decrease matches the decline in binding to and may account for the reduction in the activity of the rat apoA-I site B; and *iii*) the abundance of HNF-4 bound to site C decreases but ARP-1 increases with age. The impact of such changes in HNF-3β, HNF-4, and ARP-1 extends to other genes regulated by these factors. These findings related to the apoA-I promoter open up several areas of investigation for age-dependent changes in transcription factors and the genes they regulate.

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