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**Research Article**

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# The Full Induction of Human Apoprotein A-I Gene Expression by the Experimental Nephrotic Syndrome in Transgenic Mice Depends on *cis*-acting Elements in the Proximal 256 base-pair Promoter Region and the *trans*-acting Factor Early Growth Response Factor 1

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## Abstract

To identify molecular factors regulating apo A-I production *in vivo*, we induced in transgenic mice the experimental nephrotic syndrome, which results in elevated levels of HDL cholesterol (HDL-C), plasma apo A-I, and hepatic apo A-I mRNA. Human (h) apo A-I transgenic mice with different length 5' flanking sequences (5.5 or 0.256 kb, the core promoter for hepatic-specific basal expression) were injected with nephrotoxic (NTS) or control serum. With nephrosis, there were comparable (greater than twofold) increases in both lines of HDL-C, h-apo A-I, and hepatic h-apo A-I mRNA, suggesting that *cis*-acting elements regulating induced apo A-I gene expression were within its core promoter. Hepatic nuclear extracts from control and nephrotic mice footprinted the core promoter similarly, implying that the same elements regulated basal and induced expression. Hepatic mRNA levels for hepatocyte nuclear factor (HNF) 4 and early growth response factor (EGR) 1, *trans*-acting factors that bind to the core promoter, were measured: HNF4 mRNA was not affected, but that of EGR-1 was elevated approximately fivefold in the nephrotic group. EGR-1 knockout (EGR1-KO) mice or mice expressing EGR-1 were injected with either NTS or control serum. Levels of HDL-C, apo A-I, and hepatic apo A-I mRNA were lowest in non-nephrotic EGR1-KO mice and highest in nephrotic mice expressing EGR-1. Although in EGR1-KO mice HDL-C, apo A-I, and apo A-I mRNA levels also increased after NTS injection, they were approximately half of those in the ne-

phrotic EGR-1-expressing mice. We conclude that in this model, basal and induced apo A-I gene expression *in vivo* are regulated by the *trans*-acting factor EGR-1 and require the same *cis*-acting elements in the core promoter. (*J. Clin. Invest.* 1998. 101:1699–1707.) Key words: high density lipoprotein • liver • kidney • gene expression • transcription

## Introduction

Epidemiological studies have established that HDL cholesterol (HDL-C)<sup>1</sup> levels are inversely correlated with the incidence of coronary heart disease (e.g., 1–3). The major structural protein of HDL is apoprotein A-I (apo A-I), and HDL-C levels are highly correlated with apo A-I levels. Expression of the human apo A-I (h-apo A-I) gene in transgenic mice has been shown to increase HDL-C levels, and when these transgenic mice are crossed with atherosclerosis-prone apo E-deficient mice, both lesion size and progression diminish (4). Therefore, the identification of mechanisms for increasing apo A-I synthesis to serve as possible modalities for increasing plasma apo A-I and HDL-C levels is of great interest and potential therapeutic importance.

In mammals, apo A-I is synthesized predominantly in liver and in intestine (e.g., 5–7). Several perturbations have been shown to increase plasma levels of apo A-I, including a high fat diet, alcohol, estrogen, and thyroxine. The increases are modest, and where studied, the molecular mechanisms involved have not been described in detail, but examples exist of both transcriptional and posttranscriptional regulation that increase hepatic apo A-I production. The strongest stimulation of hepatic apo A-I production has been reported in experimental nephrotic syndrome in rats, in which there are positive relationships among hepatic apo A-I mRNA levels, hepatic apo A-I secretion, and plasma apo A-I levels (for a review, see references 8 and 9). Furthermore, the increase in hepatic apo A-I mRNA abundance has been shown to result from increased transcription of the apo A-I gene (10, 11). Thus, the nephrotic syndrome presumably changes the amount or activity of transcription factors that interact with the apo A-I promoter.

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1. Abbreviations used in this paper: EAR, v-erb-related receptor; EGR, early growth response factor; EGR1-KO, EGR-1 knockout; h, human; HDL-C, HDL cholesterol; HNF, hepatocyte nuclear factor; m, mouse; NTS, nephrotoxic serum; PAN, puromycin aminonucleoside.

These studies were designed to identify the *cis*-acting sequences and *trans*-acting factors that increase apo A-I transcription in the experimental nephrotic syndrome. The *cis*-acting sequences were inferred by examining h-apo A-I transgene expression in two different lines of mice made nephrotic by injection of a nephrotoxic serum (NTS) containing antibodies to the glomerular basement membrane (GBM). The two transgenic lines expressed in liver h-apo A-I genes with different amounts of flanking sequences. They were examined to determine the minimal sequence necessary to give the same nephrotic syndrome-induced increase in h-apo A-I transgene expression as observed for the endogenous mouse apo A-I (m-apo A-I) gene.

In this study, we report that the complete induction of transgene expression was observed with the proximal 256 bases of the h-apo A-I promoter, the region that has been shown to confer liver-specific expression of h-apo A-I in transgenic mice (12). DNase I footprinting studies implicated *cis*-acting elements involved in apo A-I gene induction identical to those used for basal expression. Finally, studies of the transcription factor early growth response factor (EGR) 1, known to bind to this proximal promoter and induce h-apo A-I gene expression *in vitro* (13), indicated that this *trans*-acting factor was involved in basal apo A-I gene expression *in vivo* and was also required to achieve the maximal response of the apo A-I gene in the nephrotic syndrome.

## Methods

All animal procedures were approved by the institutional committees of Allegheny University of the Health Sciences, The Rockefeller University, University of Pennsylvania, and Washington University. All reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

### Animals

The h-apo A-I transgenic mouse lines used in this investigation have been described previously (12, 14) and are designated as 179 and 427. Mice of these lines expressed the human transgene only in liver. Both lines carry the 1.9-kb h-apo A-I gene, but with different amounts of 5' and 3' flanking sequences. In line 179, the DNA construct is 11.5 kb, extending from 5.5 kb 5' to 4.1 kb 3' of the gene. In line 427, the DNA construct is 2.2 kb, extending from 256 bp 5' to 80 bp 3' of the gene. As described previously (12), DNA was microinjected into fertilized eggs from superovulated (C57 BL/6J × CBA/J)F1 females that had been mated to males of the same genetic background. Founder animals were bred to (C57 BL/6J × CBA/J)F1 animals to establish transgenic lines.

Mice in which the EGR-1 gene was inactivated by homologous recombination (EGR-1 knockout [EGR1-KO] mice) were prepared in the laboratory of Dr. Jeffrey Milbrandt (Washington University, St. Louis, MO) and are described in detail elsewhere (15, 16).

### Production and characterization of NTS

GBM was prepared from the kidneys of Sprague Dawley rats (to obtain significantly more material that could be conveniently isolated from mice) by a differential sieving technique as described previously (17). Total GBM protein (500 mg) was used to hyperimmunize sheep (initial injection and three monthly boosts, for a total of four doses). Preimmune and immune serum samples were tested by injecting 0.2 ml into the tail veins of mice (C57 BL/6J × CBA/J; The Jackson Laboratory, Bar Harbor, ME). The animals were examined 6 d later for hypercholesterolemia (see below for assay) and proteinuria (as in references 18 and 19). A few kidneys from control and nephrotic animals were taken for histological studies. In the nephrotic kidneys,

there was evidence of proliferative glomerulonephritis with deposition of immune complexes on the GBM (data not shown).

### Induction of nephrotic syndrome by NTS

Mice were housed in metabolic cages, maintained on 12-h light (7 a.m.–7 p.m.)–dark cycles, and fed water and standard rodent chow. Animals were typically studied at 4–7 mo of age. Experimental nephritis was induced by injection of NTS (0.2 ml) into the tail vein. Control group animals were injected with 0.2 ml of preimmune serum. 6 d after injection, animals were anesthetized with methoxyflurane, and blood was collected from the retroorbital plexus into heparinized microhematocrit capillary tubes containing EDTA. After centrifugation for 20 min at 4°C, plasma was separated and stored until analysis. Animals were then killed, and livers (transgenic and nontransgenic mice) and small intestines (nontransgenic mice) were removed and frozen immediately in liquid N<sub>2</sub>.

### Plasma lipid, lipoprotein, and apo analyses

Total plasma cholesterol and HDL-C were measured enzymatically using a colorimetric method with commercially available reagents (Boehringer Mannheim, Mannheim, Germany). Plasma h-apo A-I was measured by ELISA using a polyclonal goat anti-human apo A-I antibody (20). Plasma m-apo A-I was measured either by rocket immunoelectrophoresis (21) or by an ELISA assay (22) using monospecific polyclonal antibodies.

### Isolation of hepatic and intestinal RNA

Total RNA was extracted by the single-step guanidinium thiocyanate-phenol-chloroform method described previously (23). Approximately 400–500 mg of frozen tissue was homogenized in 5 ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Then 0.5 ml of 2 M sodium acetate (pH 4.0), 5 ml of phenol, and 1 ml chloroform/isoamyl alcohol (49:1) were added. The final suspension was shaken and then cooled on ice for 15 min. Samples were centrifuged at 10,000 g for 20 min at 4°C in a swinging bucket rotor, the RNA-containing aqueous phase was transferred to a new tube, and the RNA was precipitated by addition of 1 vol of isopropanol and incubation for 1 h at –20°C. After centrifugation at 10,000 g for 10 min, the RNA pellet was resuspended in 2 ml of denaturing solution, and the isopropanol precipitation was repeated. The RNA pellet was washed twice with cold ethanol/water (75% ethanol) and dissolved in RNase-free water. The integrity of the RNA and the absence of DNA contamination were verified by denaturing formaldehyde/agarose gel electrophoresis and ethidium bromide staining (24).

### Measurement of mRNA levels

#### NONTRANSGENIC MICE

The abundances of mouse m-apo A-I and β-actin mRNAs in 10 μg of total RNA were measured by Northern blotting analysis using <sup>32</sup>P-random primed-labeled cDNA probes, performed as described previously (25).

#### TRANSGENIC MICE

To quantitate separately changes in the abundances of m- and h-apo A-I mRNAs in transgenic mice, ribonuclease protection assays were performed. The riboprobes used for hybridization have been described previously and are species-specific (26). The m- and h-apo A-I riboprobes protected mRNA sequences with lengths of 188 and 230 nucleotides, respectively. Plasmid pTri-B-actin/mouse, containing a mouse β-actin cDNA fragment (nucleotides 660–905), was purchased from Ambion Inc. (Austin, TX) and was used as a control probe.

**Riboprobe preparation.** The antisense riboprobe synthesis was performed using the Maxiscript kit (Ambion Inc.) with protocols modified by Azrolan and Breslow (26). The reaction was carried out in a total volume of 50 μl containing 1 μg linearized plasmid (containing an insert of either m-apo A-I, human h-apo A-I, or mouse β-actin DNA), 1× transcription buffer, 8 μl ribonucleotide solution, 3.3 mM

each of ATP, CTP, and GTP (pH 7.0), 2  $\mu$ l 100 mM DTT, 10 U placental RNase inhibitor (Promega Corp., Madison, WI), 80  $\mu$ Ci [ $\alpha$ - $^{32}$ P]UTP (800 mCi/mmol; New England Nuclear, Boston, MA), 23  $\mu$ l RNase-free water, and 10 U T7 RNA polymerase. After incubation for 45 min at 37°C, 4 U of RNase-free DNase I (Boehringer Mannheim) and 2  $\mu$ l of RNasin were added, and the reaction was incubated for an additional 15 min at 37°C. The reaction was extracted with phenol/chloroform, and the aqueous phase was transferred to a fresh tube. 5  $\mu$ l of a solution containing 50  $\mu$ g yeast tRNA and 45  $\mu$ l of 6 M ammonium acetate was added, and the labeled riboprobe RNA was isolated by adding 275  $\mu$ l cold ethanol (95%) and centrifuging at 10,000 *g* for 10 min. The pelleted riboprobe was washed with 75% cold ethanol and resuspended in 100  $\mu$ l TE (10 mM Tris/1 mM EDTA, pH 7.0).

**Solution hybridization.** The ribonuclease (RNase) protection assay was performed by the method described previously (26). Briefly, 10  $\mu$ g of total RNA was combined with molar excesses of the labeled  $\beta$ -actin cRNA probe ( $3\text{--}5 \times 10^5$  cpm) and either the labeled h- or m-apo A-I riboprobe (also  $3\text{--}5 \times 10^5$  cpm of either). Then 25  $\mu$ l of hybridization buffer (80% formamide, 40 mM Hepes [pH 6.7], 0.4 M NaCl, 1 mM EDTA) was added. The mixture was overlaid with 25  $\mu$ l mineral oil. After an incubation at 90°C for 5 min for denaturation, the tube was placed at 65°C for 4 h for hybridization. RNA not protected by the probe is single-stranded, which was removed after hybridization by digestion with RNase solution (24  $\mu$ g RNase A and 3 U RNase T1 per  $\mu$ g total RNA, dissolved in 0.3 M NaCl, 10 mM Tris-HCl [pH 7.4], 5 mM EDTA [pH 8.0]) for 45 min at 34°C. Inactivation of the RNases was performed by adding 10  $\mu$ l 20% SDS, 50  $\mu$ g yeast tRNA, 30  $\mu$ l 1 M Tris-HCl, pH 8.0, 5  $\mu$ l proteinase K (10 mg/ml), and then incubating at 37°C for 20 min. The specific  $^{32}$ P-labeled RNA-RNA hybrids protected from digestion were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol-precipitated with 50  $\mu$ g yeast tRNA as carrier. The pellet was dissolved in 10  $\mu$ l loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, 2 mM EDTA), denatured at 95°C for 3 min, and separated electrophoretically on an 8 M urea/8% polyacrylamide gel. The gel was then dried and exposed at -70°C to XAR-5 film (Eastman Kodak Co., Rochester, NY) for 10 min with an intensifying screen.

Although equal amounts of hepatic RNA were used in the hybridization reactions, to control for nonspecific losses, as noted above, the abundance was measured in each sample of mouse  $\beta$ -actin RNA, a housekeeping gene not affected by nephrotic syndrome (see Table II).

To quantitate the abundance of h- and m-apo A-I and mouse  $\beta$ -actin mRNAs, after autoradiography the appropriate bands were excised from the gel and counted by liquid scintillation spectrometry. Results for the apo A-I mRNAs were normalized to the corresponding  $\beta$ -actin mRNA abundances.

#### MEASUREMENT OF MRNAS ENCODING *TRANS*-ACTING FACTORS

Total RNA samples from liver of nephrotic and control h-apo A-I transgenic mice were isolated as described above. Northern and slot blotting analyses were performed as described previously (25) using 10 and 20  $\mu$ g total RNA/sample, respectively. Hybridization probes included mouse-specific cDNAs for  $\beta$ -actin, the hepatic transcription factor hepatocyte nuclear factor (HNF) 4 (a gift of Dr. J. Darnell, The Rockefeller University), and a 3.1-kb EcoRI fragment of mouse cDNA for the transcription factor EGR-1, kindly provided by Dr. V.P. Sukhatme (Howard Hughes Medical Institute, Departments of Medicine, Molecular Genetics, and Cell Biology, University of Chicago). After hybridization, the filters were washed first at room temperature with  $0.2 \times$  SSC/0.1% SDS for 5 min, then at 42°C with  $0.2 \times$  SSC/0.1% SDS for 30 min, and twice at 50°C with  $0.2 \times$  SSC/0.1% SDS for 10 min. Washed filters were exposed to XAR-5 film (-70°C) for 24-48 h, and the autoradiographic signals were quantitated by densitometry using a Bio-Rad Laboratories (Hercules, CA) Gel Doc1000 system. Under these conditions, for each probe, a single band of the expected size was visualized on a Northern blot.

#### Plasmid construction

For DNase I footprinting analysis, a plasmid containing the liver-specific h-apo A-I promoter sequence was constructed by cloning an SmaI-StuI fragment, spanning the nucleotide region -252 to +69, into the SmaI site of pUC19. This plasmid was called pUC19/AI and was used as a template for the footprint assay.

#### Nuclei preparation

Isolation of hepatic nuclei and the preparation of nuclear extracts were performed as described (27).

All procedures were performed in the cold room, and all tubes, solutions, and centrifuges were prechilled and kept on ice. Minced mice livers (4-6 g) were resuspended in 60 ml of buffer (10 mM Hepes [pH 7.6], 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 10% glycerol, 10 mM NaF, 0.5 mM DTT, 0.5 mM PMSF, 1% Trasylol [aprotinin]) and homogenized using a motor-driven Teflon pestle and glass vessel set (Lawson Mardon Wheaton, Millville, NJ). By microscopy, > 90% of the cells were broken. The homogenate was then centrifuged at 75,000 *g* for 60 min at 0°C in a rotor (model SW27; Beckman Instruments, Inc., Palo Alto, CA). The supernatant was discarded, and the pellet of nuclei was stored at -70°C until use.

#### Nuclear extract preparation

The nuclear pellet was resuspended in 5 ml of nuclear lysis buffer (10 mM Hepes [pH 7.6], 100 mM KCl, 0.1 mM EDTA, 3 mM MgCl<sub>2</sub>, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, and 1% Trasylol). The nuclear suspension was diluted with lysis buffer for a DNA concentration of 0.5 mg/ml. One-tenth volume of 4 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added drop by drop, and the lysate was shaken gently on ice for 30 min. The lysate was then centrifuged for 20 min at 90,000 *g* and 0°C in a rotor (model Ti60; Beckman Instruments, Inc.) to pellet the chromatin. The supernatant was collected, and solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.3 g/ml) was added which was dissolved by shaking gently on ice for 60 min. The precipitated proteins were sedimented by a 30-min centrifugation at 90,000 *g* and 0°C in a Ti60 rotor. The protein pellet was resuspended in nuclear dialysis buffer (25 mM Hepes [pH 7.6], 40 mM KCl, 0.1 mM EDTA, 10% glycerol, and 1 mM DTT). The protein extract was dialyzed twice (2 h each time) in the cold against 100 vol dialysis buffer, then centrifuged at 4°C for 2 min in a microcentrifuge. Extracts were stored at -70°C until further use.

#### Footprint assay

The plasmid pUC19/AI, containing the liver-specific h-apo A-I promoter, was linearized with EcoRI and end-labeled by filling in the overhang with Klenow fragment (Promega Corp.) in the presence of dCTP, dGTP, dTTP, and [ $\alpha$ - $^{32}$ P]dATP (3,000 Ci/mmmole). The labeled promoter fragment was then excised completely from the plasmid by digestion with HindIII, gel-purified, and isolated by the Qiagex II gel extraction kit (QIAGEN, Inc., Chatsworth, CA). In pilot studies, the amount of extract, the concentration of DNase I, and the incubation time were optimized. The following final protocol was used.

Nuclear extract (36  $\mu$ g), prepared as described above, was incubated at 4°C for 60 min with the end-labeled DNA (15,000 cpm) in a 20- $\mu$ l reaction containing 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 25 mM Hepes (pH 7.6), 34 mM KCl, 5 mM MgCl<sub>2</sub>, and 2  $\mu$ g double-stranded poly(dI:dC). DNase I (0.1 U) freshly diluted into 25 mM CaCl<sub>2</sub> was added, and the sample was incubated on ice for 4 min. The reaction was terminated by the addition of stop solution consisting of 125 mM Tris, pH 8.0, 125 mM EDTA, 3% SDS, 40  $\mu$ g proteinase K, and 5  $\mu$ g yeast tRNA. The mixture was incubated at 65°C for 30 min and extracted twice with phenol/chloroform, and the DNA was precipitated with cold ethanol. The pellet was rinsed with 70% ethanol and denatured at 95°C for 3 min before loading onto a 6% acrylamide/7 M urea sequencing gel. Marker lanes with G and G + A chemically sequenced DNA (28) were included. After electrophoresis, the gel was dried and exposed to XAR-5 film at -70°C for 12-24 h using intensifying screens.

**Table I. Relative Hepatic and Intestinal apo A-I mRNA Levels in Nontransgenic Mice Injected with Preimmune (Control) or NTS**

Treatment	Liver	Intestine
Control	1.0±0.1 (n = 8)	1.0±0.2 (n = 5)
NTS	2.1±0.2* (n = 18)	1.0±0.2 (n = 5)

RNA was extracted, blotted onto nylon membranes, and hybridized to <sup>32</sup>P-labeled cDNA probes (see Methods). The resulting autoradiograms were analyzed by densitometry. For each tissue, the apo A-I mRNA values were normalized to the corresponding signals for β-actin, and the results in the NTS group relative to the control group were calculated. The relative results are expressed as mean±SEM. \*P < 0.001, NTS liver vs. control.

### Statistical analysis

Typically, statistical analysis was performed using either one-way ANOVA or the Student's *t* test (InStat software package; Graph-PAD Software for Science, San Diego, CA). A *P* value ≤ 0.05 was considered significant.

## Results

**The effects of NTS on nontransgenic mice.** Before beginning studies in transgenic mice, to assess the efficacy of the NTS, experiments were performed in nontransgenic mice of the same strain used in the creation of the h-apo A-I transgenic lines.

6 d after the injection of 0.2 ml of either preimmune serum or NTS into the tail veins of mice, samples of serum, urine, and kidney were taken. Injection of only the NTS led to heavy proteinuria (typically > 30 mg/ml; data not shown), hypercholesterolemia (preimmune group: 105±4 mg/dl; NTS group: 398±19 [mean±SEM, n = 14 each group]), and histological evidence of proliferative glomerulonephritis (data not shown). In addition, as summarized in Table I, hepatic apo A-I mRNA levels increased twofold in nephrotic compared with control mice, in agreement with studies in rats with experimental nephrotic syndrome (e.g., 10, 11, 18, 29, 30). As in the rat (10), the induced apo A-I gene expression was tissue-specific, as demonstrated by the lack of significant change in intestinal apo A-I mRNA.

Overall, the studies in nontransgenic mice demonstrated that the NTS was effective in producing a nephrosis in mice

with the characteristic features of the syndrome in rat (for a review, see references 8 and 9). Studies of transgenic mouse lines expressing h-apo A-I were then begun.

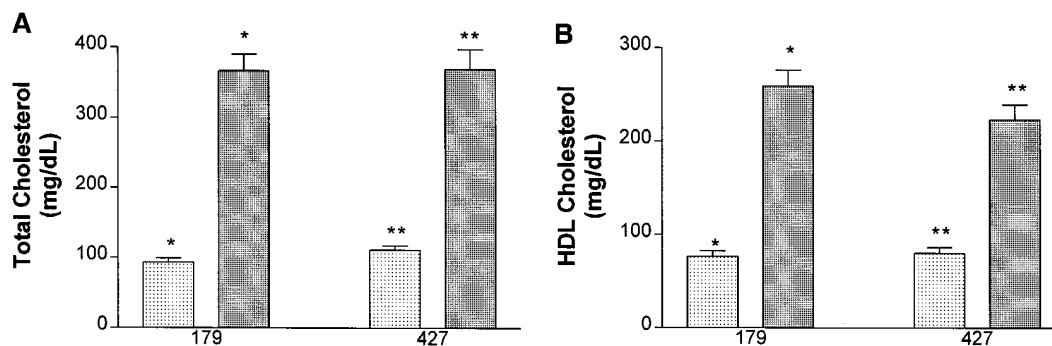
**Effects of NTS on the plasma levels of lipids and h-apo A-I in transgenic mice.** To investigate the response of the h-apo A-I gene to the nephrotic syndrome and the DNA elements regulating this response, two transgenic mice lines were used. Both expressed h-apo A-I in the liver, but the DNA constructs containing the h-apo A-I gene differed between the lines. In line 179, the DNA construct was 11.5 kb, extending from 5.5 kb 5' to the h-apo A-I gene to 4.0 kb 3'. Line 427 contained a 2.2-kb fragment extending from 256 bp 5' to 80 bp 3' to the gene (12, 14).

Mice of each line were injected with either preimmune or NTS and killed 6 d later. As shown in Fig. 1 A, with NTS, animals of both transgenic lines exhibited three to fourfold increases in plasma total cholesterol levels, similar to the results for nontransgenic mice. The changes in total cholesterol were paralleled by significant elevations of HDL-C (Fig. 1 B). Note that for both total and HDL-C, the corresponding control and experimental group values were comparable between lines 179 and 427. The data in Fig. 1 were obtained from 15 mice in each group, and for the comparison between nephrotic and control groups for any parameter displayed, the *P* value was < 0.001.

The HDL results suggested that the plasma levels of apo A-I were also raised. This was directly examined, and the results are shown in Fig. 2. Again, both lines exhibited similar results, with significant (approximately twofold; *P* < 0.001, n = 15) and comparable elevations in plasma h-apo A-I. More limited testing of plasma m-apo A-I also demonstrated approximately twofold increases (*P* < 0.02, n = 5 in each group) in animals of both lines injected with NTS (control: 20.2±4.3; NTS: 40.6±5.0; mean [mg/dl]±SEM).

In general, these data are consistent with the elevations of plasma cholesterol (total and HDL) and apo A-I observed in the classical rat model (8, 9). The results also demonstrated equivalent effects of expressing either of the two h-apo A-I DNA constructs on the plasma levels of cholesterol (total or HDL) and apo A-I (human or mouse) in the nephrotic mice. Furthermore, given the similarity of the plasma cholesterol results between the nontransgenic and transgenic mice, the expression of either form of the transgene did not change major features of lipid metabolism characteristic of the wild-type mouse.

**Effect of NTS on h- and m-apo A-I mRNA expression in liver.** The increases observed in plasma levels of both h- and



**Figure 1.** Effect of NTS injection on plasma levels of total and HDL cholesterol in transgenic mice. 6 d after injection with either NTS (dark bars) or preimmune serum (light bars), plasma levels of total (A) and HDL (B) cholesterol were measured (see Methods) in transgenic mouse lines 179 and 427.

Data are expressed as mean±SEM. \* (for line 179) and \*\* (for line 427), Significant (*P* < 0.001) differences between nephrotic syndrome and control animals of the same line.

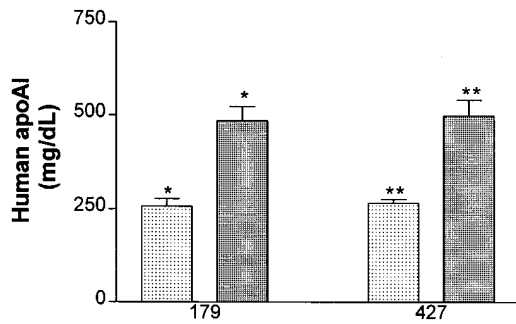


Figure 2. Effect of NTS injection on plasma levels of h-apo A-I in transgenic mice. Levels of h-apo A-I were measured in the plasma samples described in the citation to Fig. 1. The data are summarized as in Fig. 1.

m-apo A-I implied that hepatic production of apo A-I was increased in the nephrotic animals, especially considering that apo A-I can be lost in the urine of animals with the experimental nephrotic syndrome (18, 19). Hepatic apo A-I production is often correlated with apo A-I mRNA levels (31). This suggested that in nephrotic mice, there would be elevated hepatic h- and m-apo A-I mRNA levels. To measure the changes in these levels and to determine whether the different lengths of h-apo A-I 5' and 3' flanking sequences in the two transgenic lines influenced apo A-I gene induction, RNase protection assays were performed.

Mice from both transgenic lines were injected with either preimmune serum or NTS as above. 6 d later, they were killed, RNA was isolated from the livers, and the abundances of h- and m-apo A-I and mouse  $\beta$ -actin mRNAs were measured by RNase protection assay. The results for apo A-I mRNA were normalized to those for  $\beta$ -actin, whose expression did not change significantly with nephrosis (see Table II).

In Fig. 3 are shown the hepatic h-apo A-I mRNA levels in nephrotic and control mice of lines 179 and 427. There was an ~2–2.5-fold increase associated with nephrosis in both lines ( $P < 0.0001$ , nephrotic vs. control). Thus, the additional DNA sequences in line 179, particularly those 5' of the 256-bp liver-specific element present in line 427, did not impart a differential induction of the transgene.

Corresponding results for m-apo A-I mRNA are summarized in Fig. 4. As shown, there was a significant increase (2.5-

Table II. The Abundances of EGR-1, HNF4, and  $\beta$ -Actin mRNA Species in the Livers of Control and Nephrotic Transgenic Mice

mRNA species	Control	Nephrotic	P value
EGR-1	7.5 $\pm$ 1.3	34.9 $\pm$ 2.6	< 0.0001
HNF4	6.4 $\pm$ 0.2	6.8 $\pm$ 1.6	NS
$\beta$ -Actin	2.2 $\pm$ 1.1	2.1 $\pm$ 0.2	NS

From the livers of transgenic line 179 animals, RNA was extracted, slot-blotted onto nylon membranes, and hybridized to  $^{32}$ P-labeled mouse-specific probes (see Methods). After high-stringency washes, the membranes were exposed to x-ray film, which was then developed and analyzed by densitometry. The units shown are arbitrary signal intensities and are expressed as mean $\pm$ SEM.  $n = 7$  for each group.

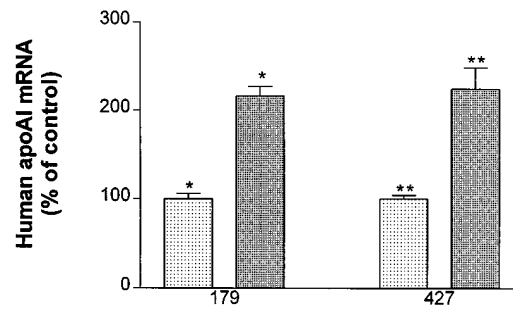


Figure 3. Effect of NTS injection on hepatic h-apo A-I mRNA levels in transgenic mice. Mice were treated as in the citation to Fig. 1, and hepatic RNA was isolated. The abundances of h-apo A-I and  $\beta$ -actin mRNAs were measured by RNase protection assays (see Methods). For each animal, the mRNA abundance for h-apo A-I was normalized to that for  $\beta$ -actin. For each transgenic line, the mean of the normalized values for the control (light bars) animals was set to 100%, and results for the NTS-injected group (dark bars) were then expressed as mean ( $\pm$ SEM) percentage of control. \* (for line 179) and \*\* (for line 427), Significant ( $P < 0.001$ ) differences between nephrotic syndrome and control animals of the same line.

fold,  $P < 0.0001$ ) in m-apo A-I RNA associated with nephrosis, similar to the increases observed for h-apo A-I mRNA in lines 179 and 427 (Fig. 3) and m-apo A-I in nontransgenic mice (Table I). These results demonstrate that the m-apo A-I gene, with all of the possible 5' and 3' regulatory sequences present, responded to the nephrotic syndrome similar to the human transgenes containing more limited flanking sequences. In addition, the similarity of the m-apo A-I mRNA data in the nontransgenic and transgenic animals indicates that relative response of endogenous apo A-I gene expression to NTS was not influenced significantly by the induction of either human transgene.

*DNase I footprinting analysis.* Although the above results suggest that the 256-bp 5' sequence shown previously to confer constitutive liver-specific expression (12) also regulates the induction of the h-apo A-I gene in nephrosis, this does not necessarily mean that the responsible *cis*-acting elements are identical in the basal and induced states. To address this issue, DNase I footprinting analysis was performed. Nontransgenic mice of the same genetic background as the transgenic lines

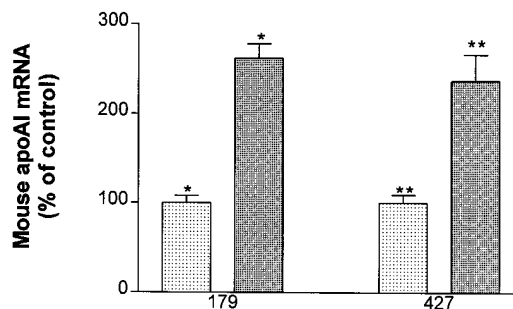
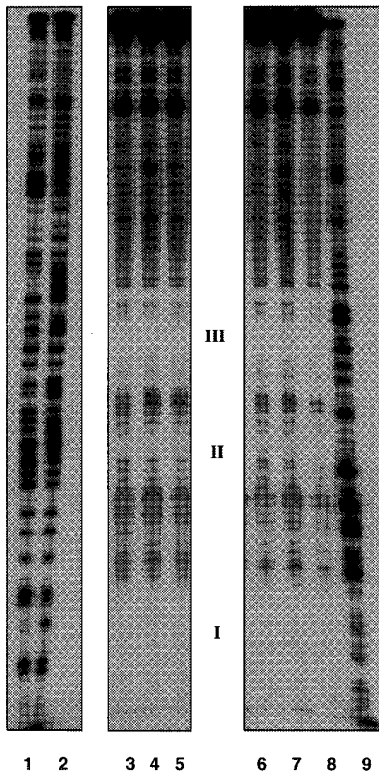


Figure 4. Effect of NTS injection on hepatic m-apo A-I mRNA levels in transgenic mice. The abundance of m-apo A-I mRNA in the hepatic RNA samples described in the citation to Fig. 3 was measured by RNase protection assay. The data were normalized and expressed as in Fig. 3.



**Figure 5.** DNase I footprint analysis of the h-*apo A-I* promoter using hepatic nuclear extracts from either control or nephrotic mice. A DNA fragment containing the h-*apo A-I* sequence from  $-252$  to  $+69$  was end-labeled with  $^{32}\text{P}$  and incubated with DNase I and nuclear extracts prepared from livers obtained 6 d after injection of mice with either NTS or pre-immune serum (see Methods). Controls included DNA incubated with DNase I but no nuclear extract (lane 9) and DNA subjected directly to chemical sequencing reactions specific for G or G + A (lanes 1 and 2). The DNA samples were purified and loaded onto a sequencing gel. After electrophoresis, the gel

was dried and exposed to x-ray film. Lane 1, G sequencing reaction; lane 2, G + A sequencing reaction; lanes 3–5, incubations with three different pools of hepatic nuclear extracts derived from control animals; lanes 6–8, incubations with three different pools of hepatic nuclear extracts derived from nephrotic animals; lane 9, DNA incubated with DNase I, but no nuclear extract. Each pool of nuclear extract represented material from two to three animals. I, II, and III, Protected regions that correspond, respectively, to sites called either A, B, and C (34), or D, C, and B (33), shown previously to be footprinted by nuclear extracts prepared from the human hepatocarcinoma cell line HepG2. Regions I–III span nucleotides  $-252$  (bottom) to  $-140$  (top) of the h-*apo A-I* promoter sequence.

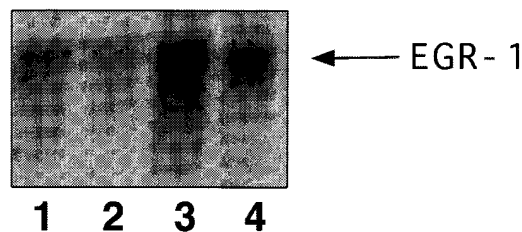
(C57 BL/6J  $\times$  CBA/J) were injected with either preimmune or NTS; 6 d later the animals were killed, and nuclear extracts were prepared from the livers.

Using an end-labeled fragment containing the 256-bp DNA element of the human gene, the protection patterns shown in Fig. 5 were obtained. Note that proteins contained in nuclear extracts from both control and nephrotic animals bound to similar regions of the DNA, indicating that the same *cis*-acting elements were occupied in both metabolic states. In addition, although to our knowledge there are no published reports displaying footprints of the h-*apo A-I* promoter by mouse nuclear proteins, the protected regions in Fig. 5 resemble the results using rat nuclear proteins that we (32) and others (33) have reported. Importantly, the three protected regions between nucleotides  $-140$  to  $-252$  in Fig. 5 correspond to three sites identified when HepG2 nuclear extracts were used in footprinting analysis and shown to regulate h-*apo A-I* transcription in vitro (e.g., see references 33 and 34). These sites are termed A, B, and C by some (34), or D, C, and B by others (33).

*Levels of trans-acting factor mRNA species in control and nephrotic livers.* The *cis*-acting elements used for h-*apo A-I* gene induction by nephrosis are likely to be part of the same set required for liver-specific expression, based on the above results. However, this does not explicitly identify the *trans*-acting factors involved in the increase in h-*apo A-I* mRNA. Several in vitro studies have shown that the  $-256$ -bp fragment contains binding sites for many transcription factors, such as apo-regulatory protein 1, HNF4, EGR-1, retinoic acid X receptor  $\alpha$ , CCAAT/enhancer binding protein, v-erb-related receptor (EAR) 2, and EAR-3 (see reference 35 for a recent review). The increased transcription could be the consequence of many changes related to *trans*-acting factors, such as increased abundance, altered activation state, new types of heterodimers, or the binding of a novel factor.

In a preliminary approach to this issue, we focused on whether changes in the abundances of the mRNAs encoding HNF4 and EGR-1 were associated with the apo A-I gene induction. These were chosen for initial study because both factors are required for basal apo A-I transcription in vitro (13). However, there are interesting differences between these two factors. (a) HNF4 also binds to the promoters of apo B and apo C-III, two genes that are not significantly transcriptionally activated by the experimental nephrotic syndrome (reference 10, and our unpublished results). Thus, it would not appear to be the most likely candidate to regulate induced apo A-I transcription. Consistent with this is the recent study of the human hepatocarcinoma-derived HepG2 and the intestinal cell line IEC-6 showing increased levels of HNF4 dramatically induced apo A-I gene expression in intestinal but not hepatic cells (36). (b) In contrast, EGR-1 is a member of a class of transcription factors known to be induced rapidly at the mRNA level after a variety of metabolic stresses (see reference 37 for a review) and has been directly implicated in regulating both basal and induced apo A-I transcription in vitro (13).

As shown in Fig. 6, there was an increase in EGR-1 mRNA levels in the livers of nephrotic transgenic mice. Table II summarizes results combined from seven control and seven nephrotic animals. For EGR-1, the increase over control was approximately fivefold; in contrast, there were no significant changes in the abundances of the mRNAs for HNF4 or  $\beta$ -actin. Therefore, these results are consistent with the roles in basal and induced apo A-I gene expression demonstrated in vitro for HNF4 and EGR-1. In addition, the changes in EGR-1 expression were tissue-specific, since its mRNA abundance in



**Figure 6.** Levels of EGR-1 mRNA in the livers of control and nephrotic mice. The abundance of EGR-1 mRNA was assayed by Northern blot analysis (see Methods) in some of the RNA samples described in the citation to Fig. 3. Lanes 1 and 2, Samples from two different control mice; lanes 3 and 4, samples from two different nephrotic mice. Comparison with size standards confirmed that the bands observed were of the expected size ( $\sim 3.1$  kb).

the kidney, an organ with significant levels of EGR-1 (37), did not increase in nephrotic mice (data not shown).

**Effects of NTS injection of EGR1-KO mice.** The availability of a mouse line in which the EGR-1 gene was inactivated by homologous recombination (15, 16) allowed the testing in vivo of the hypothesis that this *trans*-acting factor played a role in apo A-I gene induction by the nephrotic syndrome. Because there are identical phenotypes of mice with one or two wild-type alleles for EGR-1 (15), two groups of young adult mice (males and females) were studied, one group containing EGR1-KO mice (two inactive alleles) and one group containing mice with one or two wild-type EGR-1 alleles. NTS or preimmune serum was injected as in the transgenic mouse experiments, the animals were killed 6 d later, and plasma and liver samples were collected for analysis.

As seen in Table III, plasma HDL-C levels were highest ( $162 \pm 49$  mg/dl) in the EGR-1-expressing group injected with NTS and were lowest ( $41 \pm 8$  mg/dl) in the EGR1-KO group injected with preimmune serum. Although EGR1-KO mice injected with NTS had increased HDL-C ( $90 \pm 11$  mg/dl) compared with KO animals injected with preimmune serum, the absolute level achieved was somewhat below the nephrotic EGR-1-expressing group ( $P < 0.05$ , Mann-Whitney test). A similar pattern was seen in the levels of plasma apo A-I and hepatic apo A-I mRNA (Table III), implying that the changes in plasma HDL-C levels associated with EGR-1 status were due to effects on hepatic apo A-I production mediated by changes in mRNA abundance and not by changes in the fractional clearance rate of either apo A-I or HDL particles. Overall, then, EGR-1 appeared to participate in the regulated production of apo A-I and HDL in both basal and induced (nephrotic) states.

## Discussion

A standard method of inducing the nephrotic syndrome in rodents is to inject an NTS. By preparing an anti-GBM antiserum in sheep, we first confirmed that when administered to mice (wild-type and transgenic), the typical features of experimental nephrotic syndrome were obtained. This included proteinuria, hyperlipidemia, and linear deposits in GBM of immune complexes. Although these basic characteristics have

been reported for both rats and mice rendered nephrotic by NTS injection (e.g., see references 11, 38, and 39), studies of HDL metabolism in experimental nephrosis have been performed exclusively in rats. Major features related to HDL metabolism in nephrotic rats include increases in hepatic (but not intestinal) apo A-I mRNA, in the transcriptional activity of the hepatic apo A-I gene, in the hepatic synthesis of apo A-I, and in the plasma levels of HDL-C (for a review, see references 8 and 9). That mice resemble rats in their response to NTS was illustrated by increases in hepatic (but not intestinal) apo A-I mRNA and the plasma levels of the protein and lipid components of HDL (Table I, and Figs. 1–4). Since experimental nephrosis in rats has served as a valuable model of the human disease, the parallel results between the induced syndromes in the two species implied that studies in mice would also be relevant to human lipoprotein metabolism.

With these encouraging preliminary data, we were able to take advantage of transgenic mouse lines expressing h-apo A-I genes with different lengths of flanking sequences to investigate the molecular control of h-apo A-I and HDL production by transcriptional mechanisms. As noted in the Introduction and reviewed elsewhere (8, 9), the experimental nephrotic syndrome is one of the most potent known inducers of hepatic apo A-I mRNA and protein, and a large component of this induction is transcriptional (10, 11). Although the *cis*-acting factors for the liver-specific basal expression of the h-apo A-I gene had been shown in transgenic mice (12) to reside on the 256-bp fragment 5' to the transcriptional start site, there are currently no data concerning the metabolic regulation in liver of induced h-apo A-I gene expression.

In this report, we have determined that the 256-bp fragment that confers liver-specific expression of the h-apo A-I transgene in mice also contains the *cis*-acting regulatory information for the induction of apo A-I gene expression by the experimental nephrotic syndrome. This result was supported by finding comparable increases after NTS injection in the levels of hepatic h-apo A-I mRNA in both lines of transgenic mice, one containing the liver-specific promoter, the other containing 5 kb more 5' flanking sequence. Therefore, the additional sequence did not contain more *cis*-acting elements that enhanced h-apo A-I gene expression in the induced condition. Since there are examples of genes, such as  $\beta$ -globin (40), that require sequences quite distant from the transcription start site for maximal expression, an alternative explanation is that both h-apo A-I constructs did not include sequences required for maximal induction. This is not likely, since the induced expression of the endogenous m-apo A-I gene (Table I, and Fig. 4), with its full complement of 5' and 3' flanking sequences, was comparable to the transgenes.

It is interesting to compare the results in this report to those obtained in our previous studies of transgenic rats expressing h-apo A-I (18, 19). In both the rat and mouse studies, the h-apo A-I gene was responsive to nephrosis, but there are a number of notable differences between them: (a) the rat line was created with a construct containing 10 kb of 5' flanking sequence; (b) instead of NTS, the metabolic toxin puromycin aminonucleoside (PAN) was used to induce the syndrome in rats. Mice cannot be rendered nephrotic with PAN, presumably because they do not convert it to the active metabolite; and (c) the human gene in the high-expressor rat line was induced far in excess (65-fold) of the increases in either the hepatic production (4.6-fold) or plasma levels (4.6–5.1-fold) of

**Table III. Response to NTS of EGR1-KO (–/–) Mice and Mice with Either One (–/+) or Two (+/+) Wild-type EGR-1 Alleles**

EGR-1 genotype	Antiserum	Total cholesterol mg/dl	HDL-C mg/dl	apo A-I mg/ml	apo A-I mRNA* U
–/–	Preimmune	80±4	41±8	0.7±0.1	3.5±0.1
–/–	NTS	197±27	90±11	1.5±0.1	7.1±1.3
+/, +/–	Preimmune	108±15	73±10	1.2±0.1	5.0±0.3
+/, +/–	NTS	383±80	162±49	3.0±0.1	11.3±2.1

Mice were injected with either serum. The indicated assays were performed on plasma samples obtained at the time of killing 6 d later. There were three animals per group. Shown are the means±SEM. \*The abundances of hepatic m-apo A-I mRNA are given in arbitrary phosphorimager units.



h- $\alpha$ po A-I. In contrast, transgenic mice exhibited an increase in the level of hepatic h- $\alpha$ po A-I mRNA comparable to the increase in plasma h- $\alpha$ po A-I,  $\sim$  2–2.5-fold.

The rat mRNA results must be interpreted cautiously since the basal expression of the h- $\alpha$ po A-I gene was low (see Fig. 1 and Table 2 in reference 18), thereby exaggerating the relative increase in the nephrotic group. Nonetheless, there may have been a component of the increase due to a true enhanced responsiveness of the h- $\alpha$ po A-I gene in the rat compared with the mouse. This could be due, for example, to metabolic effects of PAN not obtained with NTS. Also, rat-derived *trans*-acting factors may be relatively more effective (versus those in the mouse) in stimulating transcription from the same or additional *cis*-acting sites within the promoter element, consistent with the similarity but not identity of the DNase I footprints of the human proximal promoter using hepatic nuclear extracts from mice (Fig. 5) or rats (32, 33).

Nevertheless, in the major focus of both mouse and rat studies, there is agreement: h- $\alpha$ po A-I gene expression can be induced significantly by the experimental nephrotic syndrome. Taken together, these and previous results imply that h- $\alpha$ po A-I gene induction in the nephrotic animals is regulated at the level of quantitative or qualitative changes in *trans*-acting factors interacting with the proximal promoter region. Numerous studies have examined the *trans*-acting factors that control apo A-I gene expression in vitro. Many factors are known to be involved, including apo-regulatory protein 1, HNF4, EGR-1, retinoic acid X receptor  $\alpha$ , CCAAT/enhancer binding protein, EAR-2, and EAR-3 (reviewed recently in reference 35). To approach this complex area, we chose for initial examination the potential roles in vivo for HNF4 and EGR-1 in h- $\alpha$ po A-I gene regulation in the experimental nephrotic syndrome, taking them as prototypes of factors involved in basal and induced gene expression, respectively. Although HNF4 is a positive regulator of basal apo A-I transcription, it is not likely to mediate induced expression, based not only on in vitro studies of Karathanasis and colleagues (13), but also on other considerations: (a) as reviewed recently (35, 41), HNF4 also has binding sites on the promoters of apo B and apo C-III, two genes whose expression are not induced significantly in nephrotic rats or mice (reference 10, and our unpublished results); (b) HNF4 is abundant in intestine, where the apo A-I gene does not respond to nephrosis (reference 10, and Table I); and (c) a recent study has shown that in vitro, increased expression of HNF4 induces primarily intestinal but not hepatic expression of the apo A-I gene (36).

On the other hand, based on studies on partially hepatectomized animals (42) as well as apo A-I transcription in vitro (13), EGR-1 is a *trans*-acting factor likely to be associated with a sustained increase in hepatic apo A-I production in response to injurious, stressful, or mitogenic stimulation. This would be consistent with its role in stimulating the induction of other genes under similar circumstances in a variety of tissues (see reference 37 for a review). Presumably, such induction is the result of activation either directly by EGR-1 or indirectly by its displacement of a less positive factor. EGR-1, which contains three zinc-finger motifs, binds to DNA apparently as a monomer, and in response to the appropriate stimuli, its expression is increased rapidly at the level of transcription so that there is an abrupt rise in the abundance of EGR-1 mRNA (37). Interestingly, EGR-1 is not expressed significantly in the intestine (15), which may be related to the unresponsiveness of the apo

A-I gene in that tissue in nephrotic animals (reference 10, and Table I).

The results in vivo for HNF4 and EGR-1 (no change and a large increase, respectively, in the mRNA levels in livers of nephrotic animals) were compatible with their effects in vitro on h- $\alpha$ po A-I gene expression. The existence of the EGR1-KO mouse model allowed us to test the role of this *trans*-acting factor in the expression of apo A-I expression in intact animals (unfortunately, inactivation of both HNF4 alleles is embryonic lethal [43], so a comparable study could not be performed). The results indicated (Table III) that EGR-1 is involved in maintaining the basal expression of the hepatic apo A-I gene in vivo. It should be noted that reductions in the basal expression of the apo A-I gene associated with deficiencies in two other *trans*-acting factors have been reported in recent studies that also used mouse models. Similar to our results for EGR-1, mice deficient in peroxisome proliferator-activated receptor  $\alpha$  had reduced levels of hepatic apo A-I mRNA, plasma apo A-I, and HDL (44). In the other study, mice deficient in the retinoic acid receptor-related orphan receptor  $\alpha$  had reduced levels of apo A-I mRNA in the intestine; however, effects on hepatic expression or levels of plasma apo A-I and HDL were not reported (45).

The results summarized in Table III also indicated that the maximal gene expression of hepatic apo A-I in response to the nephrotic syndrome required EGR-1. To our knowledge, this is the first demonstration of the regulation in vivo of induced apo A-I gene expression by a particular *trans*-acting factor. Given the equivalent relative induction via the core (–256 bp), extended (–5.5 kb), and endogenous apo A-I promoter sequences, this implies that the target (or targets) of EGR-1 resides on the core element and is most likely at least one of the two sites identified by in vitro studies (13). However, note that the relative increases in HDL-C and apo A-I plasma levels (approximately twofold) were independent of EGR-1 status. Thus, it is likely that other *trans*-acting factors, perhaps working in concert with EGR-1, are needed to achieve the highest absolute levels of apo A-I expression, or that another member of the EGR family of *trans*-acting factors (such as nerve growth factor inducible factor C [EGR-4], Krox-20 [EGR-2], or EGR-3 [15]) can serve as a partially effective substitute for EGR-1.

In summary, the h- $\alpha$ po A-I gene has been shown to respond to the experimental nephrotic syndrome in transgenic mice, and its induction led to significant elevations in the plasma levels of h- $\alpha$ po A-I and HDL cholesterol. Furthermore, the molecular regulation of induced h- $\alpha$ po A-I gene expression required the liver-specific core promoter element and used the *cis*-acting sites that also regulated basal expression. The potential involvement of specific *trans*-activators, such as EGR-1, in inducing h- $\alpha$ po A-I gene expression suggests a strategy by which an increase in the hepatic production of apo A-I with a resultant rise in the plasma level of HDL may ultimately be achieved.

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