# Functional Analysis of the Hepatic HMG-CoA Reductase Promoter by *In Vivo* Electroporation

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**HMG-CoA reductase (HMGR) catalyzes the rate-controlling step in cholesterol production. This enzyme is highly expressed in the liver, where it is subject to extensive hormonal and dietary regulation. Although much is known about the regulation of the HMGR promoter in cultured cells, this issue has not been directly addressed in liver. The technique of *in vivo* electroporation was utilized to perform the first functional analysis of the HMGR promoter in live animals. Analysis of a series of deletion constructs showed that deletion of the region containing the cyclic AMP response element (CRE) at −104 to −96 and an NF-Y site at −70 to −65 resulted in marked reduction of promoter activity. Sterol regulation of this promoter was investigated by raising tissue cholesterol levels by feeding cholesterol and by decreasing them through administration of a statin (lovastatin). Using this approach, we found that HMGR promoter constructs were sterol responsive in live animals, adding *in vivo* relevance to previous findings in cultured cells. We also conclude that *in vivo* electroporation is a convenient and powerful technique for the analysis of promoter elements in the livers of live animals.** Exp Biol Med 232:353–361, 2007

**Key words:** *in vivo* electroporation, HMGR promoter, liver

## Introduction

Investigation of the molecular mechanisms by which nutritional and hormonal cues regulate gene expression in the liver is critical to our understanding and treatment of metabolic diseases. A very important gene in this regard is HMG-CoA reductase (HMGR), the enzyme that catalyzes the rate-limiting step in cholesterol biosynthesis. HMGR is responsible for controlling the flux through the cholesterol biosynthetic pathway and is a key determinant in the maintenance of desirable serum cholesterol levels (1). This enzyme is the target of the popular "statin" drugs, which are currently being used to treat patients with hypercholesterolemia. Statins act by inhibiting HMGR activity and consequently have been shown to reduce serum cholesterol levels (2), improve survival rates for those at risk for coronary events (3), and even reduce the size of atherosclerotic lesions (4). A recent study has identified polymorphisms in the HMGR gene that are associated with decreased response to a statin (5). These findings highlight the importance of understanding the physiologic regulation of this key enzyme.

The liver expresses high levels of HMGR and is the major site of regulatable cholesterol synthesis. In the liver, HMGR is controlled at both transcriptional and post-transcriptional levels by several dietary and hormonal factors. Insulin, thyroid hormone, glucocorticoids, bile acids, and cholesterol all can have major impacts on HMGR expression. In addition, HMGR expression has a diurnal variation and dramatically varies with fasting and re-feeding (6). For these reasons, it is important to study the regulation of the HMGR gene in the context of the live animal.

The HMGR promoter has been well characterized in cell culture. There is about 90% sequence identity between the human, mouse, rat, and hamster promoters. The major regulatory elements are very highly conserved. Early experiments found that 277 bp of sequence upstream of the transcription start site was required for high-level expression of the hamster HMGR promoter both *in vitro* in HeLa cell extracts (7) and in mouse L cells (8). Further studies were successful in identifying sterol-responsive elements in the HMGR promoter (9). The HMGR promoter was found to contain a variant sterol response element (SRE) of the sequence GTGCGGTG (10), which is a 7/8-bp match to the consensus SRE found in the LDL receptor (11) and HMG-CoA synthase promoters (12). The HMGR promoter also contains binding sites for NF-Y, Sp1-like

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factors, and a functional cyclic AMP response element (CRE), among others (13, 14). Recently, an LRH-1/FTF site was identified that is proposed to mediate repression by bile acids on the human promoter (15).

Sterols repress HMGR transcription in cultured cells by decreasing the rate of processing of sterol response element binding proteins (SREBPs) to their mature forms. SREBPs are transcription factors that are synthesized as membrane bound precursors located in the endoplasmic reticulum (16). SREBPs normally associate with SREBP cleavage-activating protein (SCAP), which forms a tertiary complex with insulin-induced gene product (Insig) protein in the presence of sterols (17). When sterol levels fall sufficiently low, Insig dissociates from SCAP. SCAP then escorts SREBPs to the Golgi, where they are cleaved by two proteases to liberate their mature, active N-terminal domains (18). These N-terminal domains are basic helix-loop-helix-leucine-zipper transcription factors, which then migrate into the nucleus, dimerize, and bind to the SREs of target genes to activate transcription. There are three SREBP isoforms: 1a, 1c, and 2. SREBP-1a and -1c are splice variants that are involved in fatty acid metabolism (19). SREBP-2 is specific for genes involved in cholesterol metabolism and is a potent activator of HMGR in cultured cells and in transgenic mice that overexpress it (20).

Although cell culture model systems have been invaluable for the elucidation of molecular pathways and the mechanisms of transcriptional regulation, these systems should not be relied upon as the sole source of information about promoter function. This is particularly true when dealing with tissue-specific expression or nutritional and hormonal regulation. These effects are often lost or altered in immortalized cell lines. This limitation necessitates the development of new technology that allows for more effective promoter studies in the whole animal.

*In vivo* electroporation has recently been used to examine promoter function in the testes, epididymis, muscle, and liver (21–24). This technique uses electric pulses to deliver plasmid DNA, RNA, drugs, or protein directly to the tissue of interest. Electroporation is already a well-established method for the delivery of plasmid DNA to the liver (25). In at least two situations, it has been used successfully to map the response of a promoter to particular stimuli. The regions of the FABP promoter required for tissue specificity and PPAR alpha-responsiveness were identified using this technique (26). In another study, the PEPCK promoter was found to respond 13-fold to fasting, when electroporated into mouse liver (27). In many cases, the results obtained *in vivo* vary considerably from corresponding *in vitro* studies.

Electroporation of liver has several advantages over other existing methods for introducing reporter genes into animals. Transgenic mice are expensive and time consuming to produce, and only one or two reporter genes can be tested at a time, limiting the practicality of this otherwise powerful technique (28). Other methods for the introduction of DNA, such as adenoviral infection (29) and hydrodynamic tail vein injection, can primarily target the liver (30). Although these are very effective methods of gene delivery, they generally require a larger number of animals because each animal can receive only one promoter construct. Using *in vivo* electroporation, the expression of reporter genes can be limited to a particular anatomical area of liver where the plasmid is injected. This allows the testing of multiple promoter constructs in a single animal. This not only reduces the number of animals needed for a promoter study but also helps to control for changes in gene expression due to animal-to-animal variability.

In this study, we have used the technique of *in vivo* electroporation (31, 32) to deliver HMGR promoter-luciferase reporter constructs directly into the livers of live rats. In doing so, we have performed the first functional analysis of the HMGR promoter in the liver. We have succeeded in defining the promoter regions required for high-level hepatic HMGR expression. We demonstrate that mutation of the proximal NF-Y site at –65 to –70 markedly decreases promoter activity. In addition, we have examined sterol regulation of the promoter under conditions of sterol depletion (lovastatin) or sterol loading (dietary cholesterol). We report that the HMGR promoter constructs are responsive to sterol depletion in live animals, adding *in vivo* relevance to previous findings in cultured cells. This is, to our knowledge, the first *direct* demonstration of sterol regulation of a promoter in live animals. We further affirm that electroporation is a convenient and powerful technique for the analysis of promoter function in the liver.

## Methods

**Plasmids.** The HMGR promoter was obtained by polymerase chain reaction (PCR) from rat genomic DNA. Primers were designed to introduce a *Mlu*I site on the 5′ end of the promoter fragments from –325, –228, –176, –123, and –58 and an *Xho*I site on the common 3′ end at +70. These were cloned into the *Mlu*I and *Xho*I sites of pGL3 basic (Promega, Madison, WI) using standard molecular biology techniques. The resulting plasmids encode the firefly luciferase gene driven by different fragments of the HMGR promoter, all ending at +70. An additional promoter fragment from –770 to +441 was also obtained using this strategy. All clones were confirmed by restriction analysis and then by DNA sequencing at either Retrogen (San Diego, CA) or the Moffitt Molecular Biology Core Facility (University of South Florida, Tampa, FL). Primer sequences were designed using the rat genome reference sequence: *-770* 5′-GCC GAC GCG TGC CAG AAG CAG AAG GTG TAA GCA C-3′, *-325* 5′-GCG CAC GCG TCT GCA GGT CAA ATT CTG AGT TCG-3′, *-228* 5′-GCG CAC GCG TAA TAG GAA GGC CGC GAT GCT-3′, *-176* 5′-GCG CAC GCG TTG GTG AGA GAT GGT GCG GT-3′, *-123* 5′ GCG CAC GCG TTT GTT AGG GCG ACC GTT CGT GA-3′, *-58* 5′GCG CAC GCG TGA TCG GAC GAT CCT

TCC TTA TTG-3′, *+70* GCG CCT CGA GAT CTC AAT GGA GGC CAC CAA GC-3′, *+441* 5′-GCG CCT CGA GGC CTG TAT CTG GCT CTT CTC CAT-3′. Plasmids harboring a mutation in the –70/–65 NF-Y site have been described previously (33). Renilla luciferase plasmids phRL-TK (thymidine kinase promoter) and phRL-CMV (cytomegalovirus promoter) were from Promega.

**Animals.** Male Sprague-Dawley rats, 150–200 g (Harlan, Madison, WI), were allowed free access to Harlan Teklad 22/5 rodent chow and water. Animals were kept in a reverse-cycle lighting room and were sacrificed at 0900–1000 hrs, corresponding to the third to fourth hour of the dark period when HMGR expression is at its diurnal high. Some animals were fed ground chow containing 1% cholesterol or 0.02–0.04% lovastatin. In one case, Wistar Furth rats weighing 150–200 g were used. All procedures were carried out according to the regulations and oversight of the USF Institutional Animal Care and Use Committee (IACUC), protocol 2317.

**Surgery.** Rats were anesthetized with 5% isofluorane in oxygen. The rats were then fitted with a standard rodent mask and kept under general anesthesia using 3% isofluorane. Next, the surgical site was trimmed with electric clippers and scrubbed with 70% isopropyl alcohol followed by betadine. The liver was surgically exposed by making a transverse incision starting from the mid-sagittal position, approximately 1 cm caudal to the xiphoid process, extending 3 to 4 cm toward the dorsal surface of the rat. The left, right, and median lobes of the liver were exposed by gently pushing them out of the incision over a piece of sterile gauze.

**Electroporation.** Electroporation was performed essentially as previously described (25). A subcapsular injection of approximately 16 μg of plasmid in 40 μl of sterile saline was performed using a 30-gauge, 3/8-in length needle. After injection the plasmid was visible below the capsular surface as a blanched out area. A circular six-node electrode (0.75-cm diameter) was placed on the liver such that the needle points lined the perimeter of the area where the plasmid was visible. Electrodes were sunk to a controlled depth of 2 mm using a rubber spacer. After placement of the electrodes, a BTX T830 square-wave electroporator was used to administer $6 \times 150$-msec pulses at 100 V/cm, with a 150-msec rest between pulses. These settings were found to be optimal for gene delivery to the liver with minimal tissue damage. Several HMGR promoter constructs linked to the luciferase reporter gene were tested at different locations in the liver (Fig. 1). To control for transfection efficiency, a plasmid-encoding renilla luciferase driven by either the thymidine kinase promoter (phRL-TK) or the CMV promoter (phRL-CMV) was coadministered. This was done using the following ratios of reporter construct to renilla vector: 4:1 with phRL-TK (Fig. 2); 500:1 with phRL-CMV (Fig. 3); experiments 1 and 2, 4:1 with phRL-TK, experiment 3, 50:1 with phRL-CMV (Fig. 4). After electroporation, the liver was placed back in the abdomen, and the wound was closed with surgical staples. At the time of surgery, animals were given a single subcutaneous injection of ketoprofen (5 mg/kg) for analgesia.

**Luciferase Assays.** Twenty-four hours after electroporation, the livers were harvested and tested for expression of the HMG-CoA reductase constructs by measuring luciferase activity. Livers were removed from the animal, and the electroporated region was extracted using a size 3 cork borer (0.6-cm diameter). Approximately 0.1 to 0.15 g of liver was placed in 600 μl of passive lysis buffer (Promega) and homogenized using a polytron tissue disruptor. The lysate was then centrifuged at 16,000 *g* for 5 mins, and the supernatant was assayed for luciferase activity using the dual luciferase assay kit from Promega and a Turner Designs 20/20 luminometer. For each animal, a nontreated area of liver was assayed for firefly and renilla luciferase background activity. Luciferase expression is localized within the field of the electrodes. Significant luciferase counts were not observed outside of the electroporated areas (data not shown).

**Treatment of Luciferase Data.** The backgrounds for firefly and renilla luciferase were defined as the counts obtained for an untreated area of liver, averaged for all the animals in a given experiment (i.e., those electroporated on the same day). Any sample with firefly or renilla luciferase counts below background was excluded from analysis. The firefly counts for each sample were calculated as the experimental value minus the background. The renilla were also calculated the same way. Promoter activity is reported as the mean ratio of firefly to renilla luciferase activity. For the mapping of the sterol response (Fig. 4), three separate experiments were performed. In the first experiment, animals were fed a diet containing 0.02% lovastatin ($n = 3$) or 1% cholesterol ($n = 3$) for 2 days prior to surgery. Each animal was electroporated with the five-promoter constructs, each comixed with phRL-TK. A second experiment was performed with conditions identical to the first experiment ($n = 2$ per group). In a third experiment, animals were fed 0.04% lovastatin ($n = 5$) or 1% cholesterol ($n = 5$) for 4 days prior to surgery. Each animal received the five promoter constructs with phRL-CMV. In each case, animals were maintained on the diets the day following surgery. The resulting pattern of promoter activity was the same for each of the three individual experiments, although none achieved statistical significance on their own, likely due to the low number of *n*. Because only a limited number of animals can be electroporated in a single day, it was necessary to pool the data from these three experiments. This was done by taking the average ratio (firefly: renilla luciferase) obtained for the shortest promoter construct (F) and setting this value equal to one for each experiment. Consequently, all the values for a given day were multiplied by a constant. The data from both feeding regimens were treated identically. Pooled data ($n = 10$ per group) was graphed as the mean ± standard error of the mean (Fig. 4). *P* values were determined using Student's *t* test. All calculations were performed using Microsoft Excel and Sigma Plot 8.0 software.

**Microsome Preparation.** After electroporated regions were removed with a cork borer, the surrounding liver was used to prepare microsomes as previously described (34). Approximately 2 g of liver was removed from the animal; this corresponded to roughly a lobe and a half of liver (left lobe, part of median lobe). The same liver sections were taken for all animals. Livers were minced in 10 volumes of ice-cold 0.25 *M* sucrose solution and homogenized using a drill press with a Teflon pestle in a glass homogenizing vessel. The homogenate was then centrifuged at 15,000 *g* for 15 mins at 4°C. The resulting supernatant was then centrifuged at 90,000 *g* for 1 hr at 4°C. The microsomal pellet was resuspended in 0.25 *M* sucrose and stored at −70°C. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions.

**Western Blot Analysis.** Thirty micrograms of microsomal protein was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 4%–15% gradient gels (BioRad, Hercules, CA) and transferred to a PVDF membrane. The membrane was then probed with an antibody to HMGR: A9 mouse monoclonal from ATCC (35) or β-actin as a loading control (Sigma, St. Louis, MO; catalog A-5441). Anti-mouse H.R.P. secondary antibody from Amersham (Piscataway, NJ) was used in a 1:5,000 dilution. Membranes were washed 3 × 10 min after each antibody incubation and developed using SuperSignal West Pico ECL reagent (Pierce) according to the manufacturer's instructions. Each lane represents a sample of liver microsomes from a separate animal.

**RNA Isolation and cDNA Synthesis.** A small, uninjected, untreated area of liver was taken from each electroporated animal. Total RNA was isolated from roughly 200 mg of tissue using Tri-Reagent (MRC) according to the manufacturer's instructions. Ethanol pellets were stored at −70°C until the day of the experiment. RNA concentrations were determined by measuring the absorbance of a 1:100 diluted sample at 260 nm. Prior to synthesis of cDNA for use in real-time PCR (RT-PCR), the isolated RNA was DNase treated using TURBO DNA-Free kit (Ambion, Austin, TX). Some modifications of the published protocol were made (36). Briefly, 23 μg of RNA was treated using 2 μl of Turbo DNase enzyme in a reaction volume of 30 μl. Next, 3 μg of the isolated and DNase-treated total RNA was used to generate cDNA for use in RT-PCR. The reverse transcriptase reaction was carried out with the Superscript II First Strand Synthesis system for RT-PCR (Invitrogen, Carlsbad, CA) according to the instructions.

**RT-PCR.** RT-PCR was conducted according to the protocol from iQ SYBR green supermix using an iCycler (Bio-Rad) with minor modifications. The total reaction volume was adjusted to 25 μl down from 50 μl. Two microliters of cDNA was used as template for reactions with the HMG-CoA reductase primers, which corresponded to regions of exon 2 of the rat mRNA sequence (GenBank accession # NM_013134). The primers were as follows: forward 5′-TGT GGG AAC GGT GAC ACT TA-3′ and reverse 5′-CTT CAA ATT TTG GGC ACT CA-3′. Two microliters of a 1:40 dilution of each cDNA was used for reactions with primers to the rat 18s ribosomal RNA sequences (GenBank accession # X01117), which were as follows: forward 5′-CCA TCC AAT CGG TAG TAG CG-3′ and reverse 5′-GTA ACC CGT TGA ACC CCA TT-3′. All primers were used at a final concentration of 100 n*M*. The annealing temperature was 61°C, and 40 amplification cycles were performed. Melting curves were done after each run, and a single distinct peak was obtained for each primer set. The data were then processed by iCycler IQ optical system software 3.0 (Bio-Rad) and analyzed by the ΔΔCT method using Microsoft Excel statistical programs and SigmaPlot (version 8.0). Data are shown as the mean ± standard error of the mean for five samples in each group.

## Results

The rat HMGR promoter was obtained by PCR of rat genomic DNA. A series of nested deletions of this sequence was cloned in front of firefly luciferase. These promoter constructs include a large piece extending from −770 to +441 relative to the transcription start site, as well as five smaller pieces from −325, −228, −176, −123, or −58 on the 5′ end to +70 on the 3′ end (Fig. 1). These promoter fragments contain all relevant transcription factor–binding sites previously identified by studies of this promoter in cultured cells. For the sake of brevity, only the major transcription factor–binding sites are displayed (Fig. 1, lower panel).

Next, we sought to determine the *in vivo* relevance of these promoter regions in driving HMGR transcription in the liver. To accomplish this, we used *in vivo* electroporation to deliver these reporter constructs to the livers of live animals. Animals were anesthetized and surgically opened to expose the liver. Plasmids were then delivered to the liver by subcapsular injection. A six-needle electrode array was used to administer electric pulses and drive the DNA into the hepatocytes (25). Each animal received all six of the promoter constructs at separate areas in the liver. A plasmid-encoding renilla luciferase was coadministered for normalization of transfection efficiency. After surgery, the animals were allowed to eat and recover overnight. The next day, livers were harvested and assayed for luciferase activity (Fig. 2). For each animal, an area of liver that was not electroporated or injected with DNA was taken as a control for luciferase background. This is represented by the minus sign in Figure 2. The pattern of promoter occupancy looked essentially the same in an individual rat (Fig. 2, Panel A) as it did for a group of normal rats (Fig. 2, Panel B).

Although construct A (−770/+441) contains the largest fragment of genomic sequence, it did not result in more activity than construct B (−325/+70). This is consistent with previous findings in the literature showing that 277 bp of the hamster promoter is required for high-level expression *in*

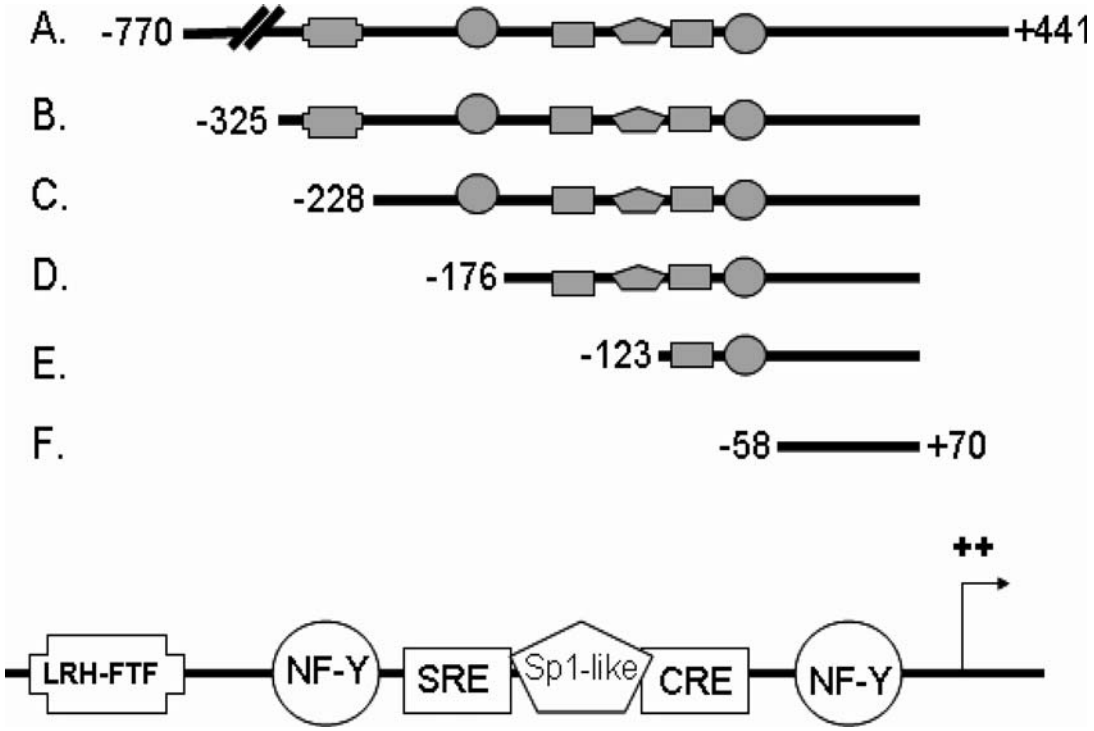


**Figure 1.** HMGR promoter-luciferase constructs. Top panel: Different fragments of the rat HMGR promoter were obtained by PCR and cloned into the pGL3 vector. The resulting plasmids encode firefly luciferase driven by the various fragments of the HMGR promoter: A –770 to +441, B –325 to +70, C –228 to +70, D –176 to +70, E –123 to +70, and F –58 to +70. All numbers are given relative to the transcription start site. Bottom panel: The location of the major previously identified transcription factor binding sites: LRH-1/FTF, NF-Y, SRE, Sp1-like, and the CRE.

*vitro* (7). A significant falloff in promoter activity was observed between constructs B and C (from –325 to –228). This region contains a recently identified LRH-1/FTF site, proposed to mediate a repressive effect of bile acids on HMGR promoter activity (15). Construct C (–228/+70) contains an NF-Y site that was previously shown to be constitutively occupied *in vivo* (33). Deletion of this site had little to no effect on promoter activity in normal rats. Construct D (–176/+70) contains the SRE, as well as a GC-rich, Sp1-like site. Although not statistically significant, this

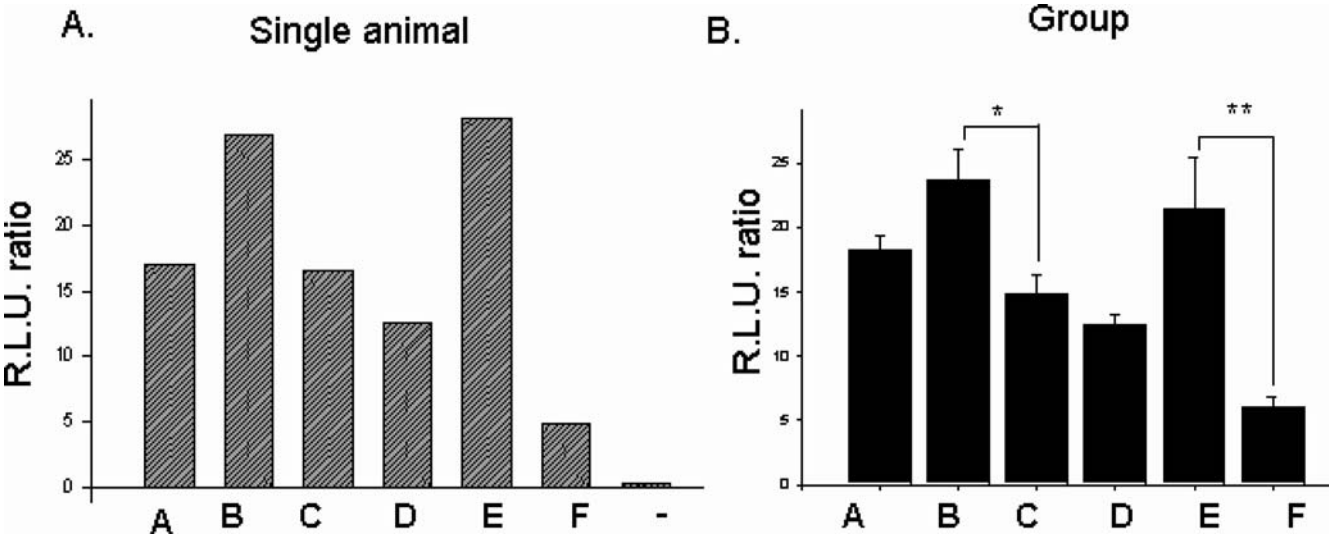


**Figure 2.** Functional mapping of the HMGR promoter in the livers of normal rats. Normal rats were electroporated with the HMGR-luciferase promoter constructs listed in Figure 1, along with renilla luciferase for normalization. Each animal received all six promoter-luciferase constructs A–F. Panel A. The pattern of HMGR promoter activity for a single animal. The minus sign "–" indicates an area of liver that was not electroporated but was assayed for background luciferase activity. Panel B. The pattern of HMGR promoter activity for a group of six animals. Data are reported as the mean ± standard error of the mean for each promoter construct. Statistically significant differences in promoter activity are noted as follows: $P < 0.05$ *, $P < 0.01$ **.

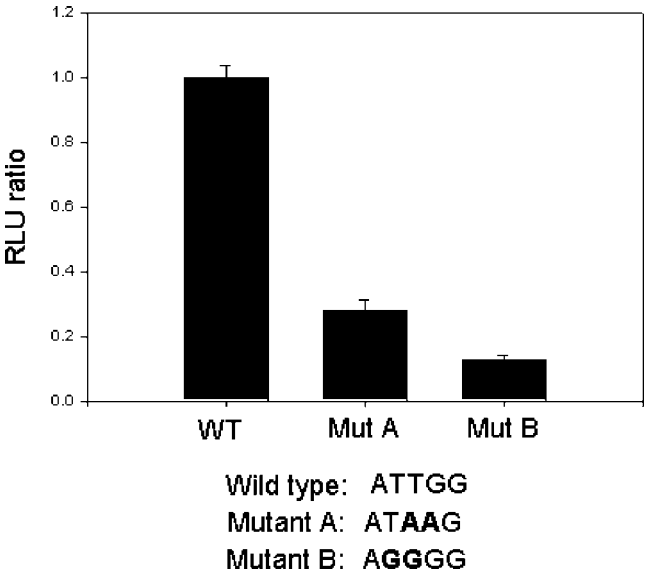


**Figure 3.** Effect of mutating the NF-Y site at –70/–65 on HMGR promoter activity in live animals. Normal Wistar-Furth rats were electroporated with plasmids containing the HMGR promoter from –325 to +70 along with renilla luciferase for normalization. Each animal received an injection of the wild-type construct and an injection of each construct harboring point mutations in the NF-Y site (A), (B). Data are reported as the mean ± standard error of the mean for each promoter construct. There were four animals in the group. The activity for construct B was significantly lower than the wild type ($P < 0.05$).

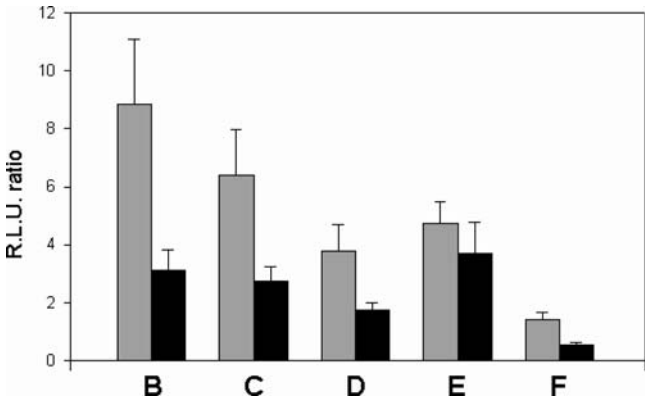


**Figure 4.** Response of the HMGR promoter to sterol depletion in rat liver. Rats were fed either 0.02–0.04% lovastatin (gray bars) or 1% cholesterol (black bars) for 3 days. Animals were then electroporated with constructs B–F, along with renilla luciferase. Animals were maintained on their respective diets overnight, and livers were harvested the next day for luciferase measurements. The data are represented as the mean ratio of firefly to renilla luciferase ± standard error of the mean. There were a total of 10 animals in each group.

construct gave consistently less activity than construct E (–123/+70). This is in agreement with previous *in vivo* footprinting studies, which found that this region was preferentially occupied in the diabetic state, where transcription is dramatically reduced (33). There is a major reduction in promoter activity when comparing construct E with construct F. The deleted region contains the CRE (–104 to –96) and an NF-Y site (–70 to –65). In a previous study, the CRE was shown to be required for insulin stimulation of the promoter in H4IIE cells, a rat hepatoma cell line (37). This site was also found to be occupied *in vivo* in rat liver. CREB is the major factor that binds to this element (38).

The most dramatic difference in promoter activity was observed between constructs E and F. This region contains both the CRE and the newly identified proximal NF-Y site. Interestingly, the NF-Y site at –70 was previously found to be preferentially occupied only in the presence of insulin (33). This site was found to be required for efficient transcription in H4IIE cells, as two different mutations inhibited luciferase production by about 80%. To determine the role of the NF-Y site in driving transcription in the liver, a similar experiment was performed by *in vivo* electroporation. Animals were electroporated with the wild-type construct B (–325 to +70) or two constructs harboring double-point mutations in the ATTGG core of this element. As shown in Figure 3, both mutants resulted in substantially less promoter activity (mut B, $P < 0.05$). This generally agrees with observations in H4IIE cells (37), although the relative reduction in activity was not as drastic in the liver.

To examine the sterol response, animals were fed diets containing either cholesterol or lovastatin for 3 days. Cholesterol feeding has been shown to drastically decrease HMGR protein levels while having little or no effect on mRNA levels in rats (39). Lovastatin feeding temporarily depletes the cell of cholesterol and mevalonate-derived metabolites, drastically increasing HMGR expression. This happens both transcriptionally (about a 4-fold effect) (40) and to a far greater extent post-transcriptionally (39). These two conditions are expected to cause the greatest possible difference in sterol levels, with lovastatin representing sterol depletion and cholesterol feeding resulting in sterol loading. The promoter activity in the cholesterol-fed animals followed a pattern similar to that observed for normal rats, while the animals fed lovastatin had considerably greater activity in constructs B, C, and D. Three separate experiments were performed, two with 0.02 % lovastatin and 1% cholesterol and another with 0.04% lovastatin and 1% cholesterol. All three experiments gave the same pattern for promoter activity and sterol response, although none achieved significance on their own due to the low number of animals. When the data sets are combined (Fig. 4), a clear picture of sterol regulation emerges. Constructs B, C, and D all contain the SRE and would be expected to respond to lovastatin feeding. These constructs responded 2.8-, 2.3-, and 2.2-fold to lovastatin treatment, with $P$ values of 0.02, 0.04, and 0.04, respectively. Construct E, which has had the SRE and Sp-like site deleted, did not respond to lovastatin (ratio = 1.3, $P = 0.44$). Unexpectedly, the shortest promoter construct (F), which extends from –58 to +70, had a significant sterol response (2.6 fold, $P = 0.003$), despite low overall activity. This region does not contain any previously identified response elements.

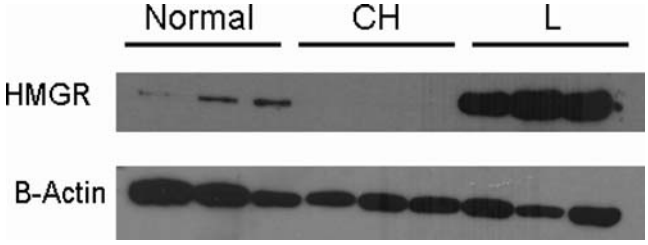


**Figure 5.** Effect of lovastatin or cholesterol feeding on HMGR immunoreactive protein and mRNA levels. Rats were fed either normal chow (N), 1% cholesterol (CH), or 0.02% lovastatin (L) for 3 days. After removal of the electroporated regions, approximately 2 g of surrounding liver was used to prepare microsomes. Western blot analylsis was performed for HMGR and β-actin. Each lane represents a separate animal.

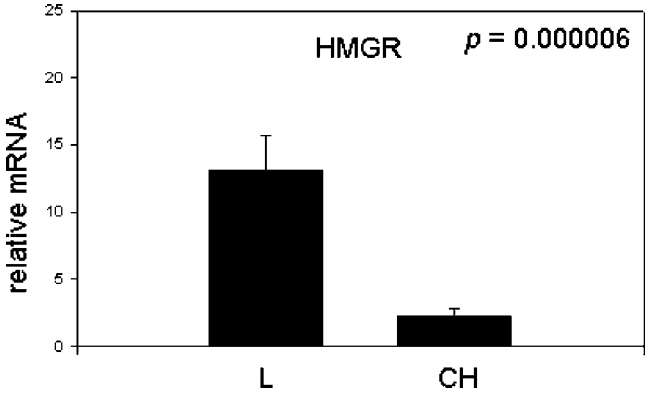


**Figure 6.** Effect of lovastatin or cholesterol feeding on HMGR mRNA levels. Rats were fed diets containing 0.04% lovastatin or 1% cholesterol. After electroporation, approximately 200 mg of liver was taken from a nontreated region and used to prepare total RNA. Relative mRNA levels were determined by RT-PCR using 18S ribosomal RNA for normalization. Data are expressed as the mean ± standard error of the mean for five animals per group.

To verify that lovastatin and cholesterol actually had an impact on the expression of endogenous HMGR, we isolated microsomes from the livers of electroporated animals fed a normal diet, 1% cholesterol, or 0.02 % lovastatin. These samples were subjected to Western blot analysis and probed with antibodies to HMGR and β-actin. Cholesterol drastically reduced HMGR protein levels, while lovastatin resulted in an equally astonishing induction of HMGR protein (Fig. 5). This is consistent with previous observations in rats (39) and shows that surgical manipulation of the animal does not adversely affect liver-wide expression or regulation of HMGR.

To determine if the feeding regimens had any effect on HMGR mRNA levels, we performed RT-PCR on RNA isolated from livers of electroporated animals. Animals were fed either 1% cholesterol or 0.04% lovastatin for 3 days (Fig. 6). Similar to previous nuclear run-on experiments (40), lovastatin feeding significantly upregulated HMGR mRNA levels greater than 6-fold ($P = 6.0 \times 10^{-6}$).

## Discussion

This study was undertaken to define the promoter elements required for expression and regulation of hepatic HMGR in live animals. It was found that about 300 bp of upstream sequence is needed for high-level expression in the liver. There was no benefit from additional flanking sequence extending back to –770 or to +441 past the transcription start site. This is consistent with previous studies in cultured cells (8). A significant falloff in activity was observed between –325 and –228. This region of the promoter contains a newly identified LRH-1/FTF site that is conserved in the rat promoter (15). In agreement with *in vivo* footprinting studies, including the region from –176 to –123 (contains SRE and Sp1-like sites) appeared to have a minor repressive effect on promoter activity. This area was previously found to have protein binding only in situations in which the gene was not transcriptionally active (33). The CRE and NF-Y sites close to –100 and –70, respectively, likely explain the increase in activity seen in construct E. Deletion of the region from –123 to –58 drastically reduced promoter activity. Furthermore, it was found that mutating the –70/–65 NF-Y site markedly reduced promoter activity for the construct from –325 to +70 (B). These findings support critical roles for CREB and NF-Y in activation of the hepatic HMGR gene (13, 14, 30).

The sterol response element is one of the key determinants of HMGR promoter activity in cultured cells (9). There has been a considerable amount of work in transgenic mouse models that point to its importance *in vivo*. For example, mice overexpressing the mature from of SREBP-2 have drastically elevated HMGR levels, as well as increased cholesterol synthesis (20). These animals rapidly develop fatty livers, showing that when overexpressed, SREBP-2 can activate the hepatic HMGR promoter. More recent evidence from the liver-specific SCAP knockout mouse points to the importance of SREBPs in maintaining appropriate levels of HMGR expression (41). These animals lack SCAP, the accessory protein required for processing of SREBPs to their mature forms. Thus, SREBPs can not be transported to the Golgi for processing, and consequently they can not activate target genes. These animals exhibit an 80% reduction in HMGR expression. In addition, both SREBP-2 and HMGR knockout mice are embryonic lethal (42, 43), suggesting that the lethal effect of SREBP-2 deletion might be ascribed to its inability to activate the HMGR gene during development. However, until now, the sterol responsiveness of the HMGR promoter has not been examined in the context of the liver.

We demonstrate that the HMGR promoter responds to sterol depletion in the livers of live animals. This is likely due to increased processing of SREBP-2 in response to lovastatin inhibition of cholesterol synthesis. All three constructs containing the HMGR SRE responded to lovastatin. The promoter construct from –123 to +70, which lacks the SRE, did not have a significant response to lovastatin. This finding is consistent with the predicted 2- to 3-fold response to sterol depletion seen for the HMGR

promoter in cultured cells (10). It is unclear why the shortest promoter construct from −58 to +70 responded to lovastatin, because this promoter construct lacks any of the previously identified enhancer elements and has less overall activity. Although the entire promoter is quite GC rich, there does not appear to be a good candidate for the consensus SRE sequence or an E-box, which is the preferred binding site for SREBP-1a and −1c in this region. It is worth noting that this construct extends out to +70, past the transcription start site. This represents the majority of the 100 bp of 5′ untranslated region present in rat liver HMGR mRNA. This sequence contains several predicted hairpin loops (44) and has been suggested to play a role in translational control of the gene either by a mevalonate-derived nonsterol product (45) or by dietary cholesterol (46).

The effect of lovastatin or cholesterol feeding on HMGR protein levels was greater than the observed increase in mRNA levels. This is consistent with the considerable amount of post-transcriptional regulation of HMGR by dietary cholesterol. The observed effect of sterol depletion on promoter activity was less than the 6-fold effect on mRNA seen by RT-PCR. There may be other regulatory elements that help enhance the promoter's response to sterol depletion that are not included in our promoter constructs. Alternatively, lovastatin treatment may have an effect on the stability of the HMGR mRNA.

There are some important caveats when considering the use of *in vivo* electroporation. In some cases, tissue damage, stress-related effects, and inflammation must be taken into consideration. Tissue damage can be avoided by using shorter pulses or lower voltage. Alternatively, nonpiercing electrodes, such as plate electrodes, can also be used. In our experiments, the limited amount of tissue damage surrounding the needle points was beneficial in that it clearly marked the electroporated region for future extraction. Studies of promoters that are known to be affected by stress or inflammation may require a longer recovery period for the animals after surgery. This is limited by the longevity of luciferase expression. In our experiments, luciferase expression was maximal at 24 hrs to 48 hrs and declined to barely detectable levels by 4 days post treatment (data not shown). This technique is ideally suited to larger tissues, such as rat liver, which allow for the simultaneous examination of multiple promoter constructs. Nonetheless, *in vivo* electroporation has already been shown to be useful in other tissues, as well as smaller animals, such as mice (21–24, 26, 27). Smaller electrode arrays may improve the utility of this technique in other organs and species. It is our hope that this study will pave the way for the investigation of the mechanisms of transcriptional regulation for other important genes in the liver.

Evidence from transgenic and knockout mice have strongly suggested that the SRE is important for HMGR promoter function in the liver (41, 42, 47, 48). Yet until now there has not been any information regarding the specific functional elements of the *hepatic* HMGR promoter. Considering the large amount of post-transcriptional regulation in the liver, it is somewhat surprising that the HMGR promoter responds to sterol depletion. Although lovastatin feeding is a strong pharmacologic manipulation, it shows that the machinery needed for sterol regulation of the promoter is present. To our knowledge, this work represents the first *direct* demonstration of promoter regulation by sterols in live animals. These findings are of particular relevance to the millions of people worldwide currently taking statins. The ability to upregulate HMGR in response to statin treatment may be a key factor in determining the cholesterol-lowering capacity of these drugs. It will be interesting to see if polymorphisms in the human HMGR promoter, or in genes in the SREBP pathway, will be good predictors of an individual's response to statins.


1. Ness GC, Gertz KR. Hepatic HMG-CoA reductase expression and resistance to dietary cholesterol. Exp Biol Med 229:412–416, 2004.
2. Tobert JA, Hitzenberger G, Kukovetz WR, Holmes IB, Jones KH. Rapid and substantial lowering of human serum cholesterol by mevinolin (MK-803), an inhibitor of hydroxymethylglutaryl-coenzyme A reductase. Atherosclerosis 41:61–65, 1982.
3. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet 344:1383–1389, 1994.
4. Nissen SE, Tuzcu EM, Schoenhagen P, Brown BG, Ganz P, Vogel RA, Crowe T, Howard G, Cooper CJ, Brodie B, Grines CL, DeMaria AN; REVERSAL Investigators. Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis: a randomized controlled trial. JAMA 291:1071–1080, 2004.
5. Chasman DI, Posada D, Subrahmanyan L, Cook NR, Stanton VP, Jr., Ridker PM. Pharmacogenetic study of statin therapy and cholesterol reduction. JAMA 291:2821–2827, 2004.
6. Ness GC, Chambers CM. Feedback and hormonal regulation of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase: the concept of cholesterol buffering capacity. Proc Soc Exp Biol Med 224:8–19, 2000.
7. Osborne TF, Gil G, Brown MS, Kowal RC, Goldstein JL. Identification of promoter elements required for in vitro transcription of hamster 3-hydroxy-3-methylglutaryl coenzyme A reductase gene. Proc Natl Acad Sci U S A 84:3614–3618, 1987.
8. Osborne TF, Goldstein JL, Brown MS. 5′ end of HMG CoA reductase gene contains sequences responsible for cholesterol-mediated inhibition of transcription. Cell 42:203–212, 1985.
9. Osborne TF, Gil G, Goldstein JL, Brown MS. Operator constitutive mutation of 3-hydroxy-3-methylglutaryl coenzyme A reductase promoter abolishes protein binding to sterol regulatory element. J Biol Chem 263:3380–3387, 1988.
10. Osborne TF. Single nucleotide resolution of sterol regulatory region in promoter for 3-hydroxy-3-methylglutaryl coenzyme A reductase. J Biol Chem 266:13947–13951, 1991.
11. Sudhof TC, Russell DW, Brown MS, Goldstein JL. 42 bp element from LDL receptor gene confers end-product repression by sterols when inserted into viral TK promoter. Cell 48:1061–1069, 1987.
12. Metherall JE, Goldstein JL, Luskey KL, Brown MS. Loss of transcriptional repression of three sterol-regulated genes in mutant hamster cells. J Biol Chem 264:15634–15641, 1989.
13. Bennett MK, Osborne TF. Nutrient regulation of gene expression by the sterol regulatory element binding proteins: increased recruitment of gene-specific coregulatory factors and selective hyperacetylation of histone H3 in vivo. Proc Natl Acad Sci U S A 97:6340–6344, 2000.
14. Bifulco M, Perillo B, Saji M, Laezza C, Tedesco I, Kohn LD and Aloj

SM. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in FRTL-5 cells. I. Identification and characterization of a cyclic AMP-responsive element in the rat reductase promoter. J Biol Chem 270:15231–15236, 1995.

15. Datta S, Wang L, Moore DD, Osborne TF. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase promoter by nuclear receptors liver receptor homologue-1 and small heterodimer partner: a mechanism for differential regulation of cholesterol synthesis and uptake. J Biol Chem 281:807–812, 2006.
16. Hua X, Sakai J, Brown MS, Goldstein JL. Regulated cleavage of sterol regulatory element binding proteins requires sequences on both sides of the endoplasmic reticulum membrane. J Biol Chem 271:10379–10384, 1996.
17. Yang T, Espenshade PJ, Wright ME, Yabe D, Gong Y, Aebersold R, Goldstein JL, Brown MS. Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. Cell 110:489–500, 2002.
18. Dobrosotskaya IY, Goldstein JL, Brown MS, Rawson RB. Reconstitution of sterol-regulated endoplasmic reticulum-to-Golgi transport of SREBP-2 in insect cells by co-expression of mammalian SCAP and Insigs. J Biol Chem 278:35837–35843, 2003.
19. Shimano H. Sterol regulatory element-binding proteins (SREBPs): transcriptional regulators of lipid synthetic genes. Prog Lipid Res 40: 439–452, 2001.
20. Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H. Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. J Clin Invest 101:2331–2339, 1998.
21. Kirby JL, Yang L, Labus JC, Lye RJ, Hsia N, Day R, Cornwall GA, Hinton BT. Characterization of epididymal epithelial cell-specific gene promoters by in vivo electroporation. Biol Reprod 71:613–619, 2004.
22. Ike A, Ohta H, Onishi M, Iguchi N, Nishimune Y, Nozaki M. Transient expression analysis of the mouse ornithie decarboxylase antizyme haploid-specific promoter using in vivo electroporation. FEBS Lett 559:159–164, 2004.
23. Widlak W, Scieglinska D, Vydra N, Malusecka E, Krawczyk Z. In vivo electroporation of the testis versus transgenic mice model in functional studies of spermatocyte-specific hst70 gene promoter: a comparative study. Mol Reprod Dev 65:382–388, 2003.
24. Riquet FB, Rodriguez M, Guigal N, Dromaint S, Naime I, Boutin JA, Galizzi JP. In vivo characterisation of the human UCP3 gene minimal promoter in mice tibialis anterior muscles. Biochem Biophys Res Commun 311:583–591, 2003.
25. Heller R, Jaroszeski M, Atkin A, Moradpour D, Gilbert R, Wands J, Nicolau C. In vivo gene electroinjection and expression in rat liver. FEBS Lett 389:225–228, 1996.
26. Fujishiro K, Fukui Y, Sato O, Kawabe K, Seto K, Motojima K. Analysis of tissue-specific and PPARalpha-dependent induction of FABP gene expression in the mouse liver by an in vivo DNA electroporation method. Mol Cell Biochem 239:165–172, 2002.
27. Muramatsu T, Ito N, Tamaoki N, Oda H, Park HM. In vivo gene electroporation confers nutritionally-regulated foreign gene expression in the liver. Int J Mol Med 7:61–66, 2001.
28. Latasa MJ, Griffin MJ, Moon YS, Kang C, Sul HS. Occupancy and function of the –150 sterol regulatory element and –65 E-box in nutritional regulation of the fatty acid synthase gene in living animals. Mol Cell Biol 23:5896–5907, 2003.
29. Haviv YS, van Houdt WJ, Lu B, Curiel DT, Zhu ZB. Transcriptional targeting in renal cancer cell lines via the human CXCR4 promoter. Mol Cancer Ther 3:687–691, 2004.
30. Al-Dosari MS, Knapp JE, Liu D. Activation of human CYP2C9 promoter and regulation by CAR and PXR in mouse liver. Mol Pharm 3:322–328, 2006.
31. Heller LC, Ugen K, Heller R. Electroporation for targeted gene transfer. Expert Opin Drug Deliv 2:255–268, 2005.
32. Heller R. Delivery of plasmid DNA using in vivo electroporation. Preclinica 1:198–208, 2003.
33. Lagor WR, de Groh ED, Ness GC. Diabetes alters the occupancy of the hepatic 3-hydroxy-3-methylglutaryl-CoA reductase promoter. J Biol Chem 280:36601–36608, 2005.
34. Ness GC, Zhao Z. Thyroid hormone rapidly induces hepatic LDL receptor mRNA levels in hypophysectomized rats. Arch Biochem Biophys 315:199–202, 1994.
35. Liscum L, Luskey KL, Chin DJ, Ho YK, Goldstein JL, Brown MS. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase and its mRNA in rat liver as studied with a monoclonal antibody and a cDNA probe. J Biol Chem 258:8450–8455, 1983.
36. Ness GC, Holland RC, Lopez D. Selective compensatory induction of hepatic HMG-CoA reductase in response to inhibition of cholesterol absorption. Exp Biol Med (Maywood) 231:559–565, 2006.
37. Osborne AR, Pollock VV, Lagor WR, Ness GC. Identification of insulin-responsive regions in the HMG-CoA reductase promoter. Biochem Biophys Res Commun 318:814–818, 2004.
38. Ngo TT, Bennett MK, Bourgeois AL, Toth JI, Osborne TF. A role for cyclic AMP response element-binding protein (CREB) but not the highly similar ATF-2 protein in sterol regulation of the promoter for 3-hydroxy-3-methylglutaryl coenzyme A reductase. J Biol Chem 277: 33901–33905, 2002.
39. Ness GC, Eales S, Lopez D, Zhao Z. Regulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression by sterols and nonsterols in rat liver. Arch Biochem Biophys 308:420–425, 1994.
40. Lopez D, Chambers CM, Ness GC. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors unmask cryptic regulatory mechanisms. Arch Biochem Biophys 343:118–122, 1997.
41. Kuriyama H, Liang G, Engelking LJ, Horton JD, Goldstein JL, Brown MS. Compensatory increase in fatty acid synthesis in adipose tissue of mice with conditional deficiency of SCAP in liver. Cell Metab 1:41–51, 2005.
42. Shimano H, Shimomura I, Hammer RE, Herz J, Goldstein JL, Brown MS, Horton JD. Elevated levels of SREBP-2 and cholesterol synthesis in livers of mice homozygous for a targeted disruption of the SREBP-1 gene. J Clin Invest 100:2115–2124, 1997.
43. Ohashi K, Osuga J, Tozawa R, Kitamine T, Yagyu H, Sekiya M, Tomita S, Okazaki H, Tamura Y, Yahagi N, Iizuka Y, Harada K, Gotoda T, Shimano H, Yamada N, Ishibashi S. Early embryonic lethality caused by targeted disruption of the 3-hydroxy-3-methylglutaryl-CoA reductase gene. J Biol Chem 278:42936–42941, 2003.
44. Reynolds GA, Basu SK, Osborne TF, Chin DJ, Gil G, Brown MS, Goldstein JL, Luskey KL. HMG CoA reductase: a negatively regulated gene with unusual promoter and 5′ untranslated regions. Cell 38:275–285, 1984.
45. Nakanishi M, Goldstein JL, Brown MS. Multivalent control of 3-hydroxy-3-methylglutaryl coenzyme A reductase: mevalonate-derived product inhibits translation of mRNA and accelerates degradation of enzyme. J Biol Chem 263:8929–8937, 1988.
46. Chambers CM, Ness GC. Dietary cholesterol regulates hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in rats primarily at the level of translation. Arch Biochem Biophys 354: 317–322, 1998.
47. Korn BS, Shimomura I, Bashmakov Y, Hammer RE, Horton JD, Goldstein JL, Brown MS. Blunted feedback suppression of SREBP processing by dietary cholesterol in transgenic mice expressing sterol-resistant SCAP(D443N). J Clin Invest 102:2050–2060, 1998.
48. Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL. Overproduction of cholesterol and fatty acids causes massive liver enlargement in transgenic mice expressing truncated SREBP-1a. J Clin Invest 98:1575–1584, 1996.