Hepatic 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) protein and mRNA are substantially decreased in diabetic animals and rapidly restored by the administration of insulin. To begin to examine the underlying molecular mechanisms, measurements of transcription by nuclear run-on assays and an investigation of occupancy of the promoter were performed. The rate of transcription was substantially reduced in the diabetic rats and fully restored within 2 h after insulin treatment. In vivo footprinting revealed several areas of protein binding as shown by dimethyl sulfate protection or enhancement. The cAMP-response element was heavily protected in all conditions, including diabetes, feeding of dietary cholesterol, or statin treatment. Striking enhancements in footprinting revealed several areas of protein binding as shown by dimethyl sulfate protection or enhancement. The cAMP-response element was heavily protected in all conditions, including diabetes, feeding of dietary cholesterol, or statin treatment. Striking enhancements in footprinting from diabetic animals were visible at −142 and at −161 (in the sterol-response element). Protocols at a newly identified NF-Y site at −70/−71 were observed in normal animals and not in diabetics. This NF-Y site was found to be required for efficient HMGR transcription in luciferase assays. CREB-1 was able to bind the HMGR cAMP-response element in vitro and the promoter in vivo. This evidence supports an essential role for cAMP-response element-binding protein in transcription of hepatic HMGR and identifies at least two sites where in vivo occupancy is regulated by insulin.

Type I diabetes is associated with lower rates of cholesterol synthesis and increased absorption of dietary cholesterol in humans (1). These individuals are at high risk for the development of cardiovascular disease (2) and have higher total serum cholesterol levels. In rats, streptozotocin-induced diabetes also renders animals particularly susceptible to a dietary cholesterol insult (3). For reasons that are still unclear, this sensitivity correlates well with decreased expression of hepatic HMG-CoA reductase (4), the enzyme that catalyzes the rate-limiting reaction in cholesterol biosynthesis.

Hepatic HMGR-CoA reductase is responsible for the majority of the regulatable cholesterol synthesis in the body. The expression of this enzyme is affected by cholesterol, insulin, thyroid hormone, bile acids, fasting, and refeeding and also varies diurnally (5). HMGR-CoA reductase (HMGR) protein and mRNA levels are both decreased in diabetic animals and can be rapidly restored with insulin treatment (6), suggesting regulation at the transcriptional level. Previous experiments in H4IIE cells (rat hepatoma) showed that the proximal reductase promoter could be activated by insulin (7), at levels greater than or equal to those seen in live animals. Questions remain as to whether this mode of insulin activation mirrors the physiological regulation of the gene.

The hamster HMGR-CoA reductase gene requires about 300 bp of sequence upstream of the transcription start site for high level expression (8). This proximal promoter was found to contain sequences sufficient for sterol regulation in cultured cells (9) and shares about 90% sequence identity with the rat promoter (GenBank™ accession number S78687 (10)). The HMGR promoter contains a sterol-response element (SRE) that can be activated by SREBP-1 and SREBP-2 in cultured cells and in transgenic mice overexpressing these proteins (11–13). Although SREBP-1c appears to be insulin-responsive at the mRNA level, recent evidence suggests this factor is more closely tied to lipogenesis than cholesterol biosynthesis (14–16). SREBP-2 is a potent activator of the HMGR-CoA reductase gene, but insulin regulation of this protein has not been reported. There are several other important elements in the HMGR-CoA reductase promoter. These include possible binding sites for Sp1 and NF-Y, as well as a functional cyclic AMP-response element (CRE) (10). The CRE in particular was shown to be required for insulin activation of the HMGR promoter in rat hepatoma cells (7).

Because of the problems inherent in a cell culture model, especially for a gene that is sterol-sensitive, we decided to perform in vivo footprinting in rat liver. This approach allows for a complete unbiased survey of the HMGR promoter. Performing this technique in animals ensures that the footprint reflects physiological regulation of the gene, in the context of the many nutritional and hormonal stimuli that the liver receives. In vivo footprinting has been used successfully to map where transcription factors are bound to DNA in vivo (17, 18). Previous in vitro footprinting studies of the HMGR promoter were successful in identifying sterol-responsive elements (19). The only previous in vivo footprints of this promoter failed to detect changes in occupancy in response to insulin in HepG2 cells, although a 1.5-fold increase in mRNA was observed (20). In this report we show that diabetes alters the occupancy of the HMGR-CoA reductase promoter in live animals.

MATERIALS AND METHODS

Animal Care and Treatment—Male Sprague-Dawley rats, 125–150 g (Harlan), were allowed free access to Harlan Teklad 22/5 rodent chow and water. Animals were kept on a reverse cycle lighting system and were sacrificed at 9:00–10:00 a.m., when HMGR-CoA reductase expression is at its diurnal high. Animals were rendered diabetic by a single subcutaneous injection of streptozotocin (Sigma), 65 mg/kg. Diabetes was verified by the presence of urinary glucose using Clinistix from Bayer. Where indicated, animals were injected subcutaneously with 3.0 units/100 g of recombinant human insulin (Novolin 70/30, Novo Nordisk) 2 h prior to sacrifice.
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**Nuclei Isolation**—Nuclei were prepared as described previously (21) by centrifugation through dense sucrose.

**Nuclear Run-on Assay**—Nuclear run-on assays were carried out essentially as described previously (22). After centrifugation, nuclei from 2 g of liver were resuspended in 100 μl of PBS with 3 mM MgCl$_2$. Next, 100 μl of 2X run-on buffer (160 mM Tris, pH 7.5, 20 mM MgCl$_2$, 2 mg/ml heparin, 1% Sarkosyl, 0.7 mM ammonium sulfate, 0.8 mM each of ATP, GTP, and UTP) and 250 μCi of [α-32P]CTP were added to each reaction. Samples were incubated at 37 °C to continue extension of RNA transcripts. Samples were then treated with 50 units of DNase I, 40 μg of proteinase K and lysed with the addition of 50 μl of 10% SDS and 25 μl of 0.2 M EDTA. After extraction and precipitation, equal counts of 32P-labeled RNA were added to each membrane at × 5 × 10^6 dpm. These membranes were spotted previously with 5 μg of cDNA encoding HMG-CoA reductase, catalase, or the pBluescript vector. Hybridizations were performed overnight at 57 °C. The next day membranes were washed with 2X SSC, 0.1% SDS at room temperature for 1 min, then 0.2X SSC, 0.1% SDS at 60 °C for 30 min, followed by 2X SSC containing 250 μg of RNase A at 37 °C for 30 min. Membranes were given a quick final rinse in 2X SSC and dried, followed by exposure to autoradiography film with an enhancing screen at −70 °C for 1−5 days.

**Sequencing**—The rat HMG-CoA reductase promoter was obtained by PCR of rat liver genomic DNA, using primers to the sequence published previously (10). PCR products were sequenced by Retrogen (San Diego).

**In Vivo Footprinting of Rat Liver**—Rat liver (2.2 g) was minced in 8 ml of ice-cold PBS. Liver pieces were homogenized 4−5 times on a drill press with a Teflon pestle in a glass vessel. A 5-ml portion of filtered homogenate was placed in a 50-ml polypropylene centrifuge tube. Each filtered homogenate was treated with 5 μl of dimethyl sulfate for 2 min at room temperature. The DMS reaction was slowed by rapid dilution with 40 ml of ice-cold PBS. Tubes were centrifuged at 1000 X g for 5 min at 4 °C. Pellets were resuspended in 20 ml of PBS and washed again. The pellet was then resuspended in 15 ml of lysis buffer (60 mM Tris, pH 7.5, 100 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K). Samples were rocked gently at room temperature for 3 h to completely lyse nuclei. Genomic DNA isolation and piperidine treatment were performed as described previously (17). Roughly 200−300 μg of DNA was obtained per sample.

**Ligation-mediated PCR**—Ligation-mediated PCR was performed according to the original method (17). The primers corresponding to the coding (top) strand of the HMGR promoter starting at −185 were as follows: primer 1, 5′-CAA TAG GAA GGA GCC CGC CAT GTC G-3′; primer 2, 5′-ATG CTG GGA CCC GCC GAC TAG CCA TTG-3′; and primer 3, 5′-ATG CTG GGA CCC GCC GAC TAG CCA TTG GTT G-3′. The primers to reveal the template (bottom) strand starting at −58 were as follows: primer 1, 5′-CCG AAG GAA CTG CGC TTA CGC CG-3′; primer 2, 5′-AAC CGG CCG CCA ATA AGG AAG GAC CCA TTG-3′; and primer 3, 5′-CCG CCC CCA ATA AGG AAG GAT CTT CCG ATC-3′. The following annealing temperatures were used for both primer sets: 58, 63, and 68 °C. All primers were ordered PAGE-purified from Integrated DNA Technologies. Products were resolved on a 6% polyacrylamide wedge gel with 7.75 m urea. Each PCR used 6 − 9 μg of DNA.

**Nuclear Extract**—Nuclei isolated from 2 g of rat liver were resuspended in 1 ml of PBS containing 3 mM MgCl$_2$ and were centrifuged at 3000 × g for 5 min at 4 °C. Nuclear pellets were resuspended in 0.5−1.0 ml of high salt buffer (420 mM NaCl, 20 mM HEPES, pH 8.1, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 20 mM NaF, 1 mM Na$_2$VO$_4$, 1 mM Na$_3$P$_2$O$_7$, 0.5 mM phenylmethylsulfonyl fluoride, 1X protease inhibitor mixture, Sigma). Nuclei were lysed by rotating slowly at 4 °C for 30 min. The lysates were then centrifuged at 15,700 × g for 15 min to pellet nuclear debris. The supernatant (nuclear extract) was collected and stored at −70 °C until needed. Protein concentrations were determined using the BCA assay (Pierce).

**EMSA**—Electrophoretic mobility shift assays were performed as described previously (23). Briefly, probes corresponding to the HMG-CoA reductase promoter footprinted regions were generated by annealing two complementary oligonucleotides (Integrated DNA Technologies, Inc.). The sequences are as follows: −59/−82, 5′-CAG CCT CCC GCC GAT TGG CTA GGG-3′ and 5′-CTG ACC CTA GGA AAT CGG CAG GAC GAG GCT G-3′; −115/−85, 5′-GCC ACC GTT CGT GCA GTA GGC CGT CAG GCT-3′ and 5′-AGC CTG ACG ACG TAC GTG AGC AAA CCG T-3′; −119/−152, 5′-GGG TGC CAG TGG GCC GTT GTT-3′ and 5′-CTG AAA CAA CAA CCG CCC ACT GCT CGC ACC C-3′ and −129/−152, 5′-TCC GCC CGG GTG CCA GCA GTG-3′ and 5′-CTG ACA CTG CTC GCA CCC CCC GGG CGG AGA A-3′. One pmol of probe was labeled by the Klenow fill in reaction using 20 μCi of [γ-32P]dCTP, along with cold 0.125 mM dATP, dGTP, and dTTP. Each probe (25 fmol) was incubated with 10 μg of rat liver nuclear extract in a binding buffer (10 mM HEPES, pH 7.9, 25 mM KCl, 0.5 mM EDTA, 50 μg/ml poly(dI-dC), 5% glycerol, 0.5 mM dithiothreitol, 125 μg/ml bovine serum albumin) for 20 min at room temperature. One to two μg of the following antisera were added to the binding reactions: NF-κB (sc-7711x), NF-κB (sc-13045x), NF-κB (sc-870), CREB-1 (sc-187x), and phospho-CREB (sc-187x), Sp1 (sc-59s), and Sp1 (sc-420x) from Santa Cruz Biotechnology. Binding reactions were run on a 6% polyacrylamide gel in 0.25× TBE.
Plasmid Construction—A PCR product from rat genomic DNA containing the HMG-CoA reductase promoter from −325 to +70 was cloned into the PGL3 basic vector (Promega) using standard molecular biology techniques. Briefly, the 5′ primer was designed to possess an overhang to introduce an MluI site by PCR, and the 3′ overhang added an XhoI site. The resulting PCR product was digested and cloned into the pGL3 basic backbone. Mutant HMGR promoter-luciferase plasmids were generated using the QuickChange kit from Stratagene. Both mutants were verified by sequencing at the core sequencing facility at the Moffitt Cancer Center, University of South Florida, Tampa, FL. pGL3-TK, a vector containing the Renilla luciferase gene driven by the thymidine kinase promoter was from Promega. (Primer sequences are available upon request.)

Cell Culture—H4IE cells (rat hepatoma) were purchased from the American Type Culture Collection. Cells were grown in Eagle’s modified essential media supplemented with 10% fetal bovine serum, 100 units of penicillin/streptomycin per ml, and 1 mlx sodium pyruvate. Cells were kept at 37 °C and 5% CO₂ in a humidified incubator.

Transient Transfections—H4IE cells were plated to an initial density of 100,000 cells per well in 24-well plates the day before the experiment. The following day, the media were removed, and the cells were washed one time with PBS. Cells were transfected with 1 μg of DNA/well using Transfast reagent (Promega) in the recommended 2:1 ratio. Cells were co-transfected with reporter construct and pRL-TK in a 4:1 ratio. One h after transfection, the 200 μl of transfection mix in each well was diluted with 800 μl of growth media. 12–16 h later, cells were harvested in 100 μl of passive lysis buffer and assayed for luciferase activity using the dual luciferase assay kit (Promega). Data are shown as the average ratio of luciferase counts ± S.D. At least six independent yet identical transfections were performed per condition. All plasmid concentrations were checked by A₂₆₀ prior to transfection.

RESULTS
Nuclear run-on assays were performed to determine whether insulin acts to increase transcription of the HMG-CoA reductase gene. Inbred male Sprague-Dawley rats were injected with streptozotocin (65 mg/kg) to induce diabetes. Animals were sacrificed during the 3rd h of the dark cycle, at the diurnal high for hepatic HMG-CoA reductase expression. Nuclei were isolated from the livers of these animals, and nuclear run-on assays were performed as described under “Materials and Methods.” HMG-CoA reductase transcription was greatly diminished in the diabetic animals (Fig. 1A). It was also found that administration of insulin to diabetic animals restored HMG-CoA reductase transcription to normal in just 2 h (Fig. 1B).

We next carried out in vivo footprinting to examine the occupancy of the hepatic HMGR promoter. Livers from normal and diabetic animals were treated with dimethyl sulfate and subjected to footprinting by ligation-mediated PCR. A primer set designed to reveal the top strand begins by reading cytosines at −185. Another primer set reveals the bottom strand by reading guanines beginning at −58. It should be noted that these primers were designed against the rat HMGR promoter sequence, which varies slightly (about 10 bp) from the hamster (10). Our primers were designed against sequence from the inbred Sprague-Dawley rats used in our experiments. Minor differences from the published rat sequence included an extra G at −15 and a reversal of the CG at −3 and −4. Numbering is therefore −1 bp relative to the previously published sequence, based on the transcription start site (Fig. 2).

On the top strand, several areas of DMS protection or enhancement were detected (Fig. 3). The Naked lane refers to DNA that was first extracted and purified and then treated with DMS in vitro. This lane is a control that shows all the reactive G residues in the sequence. Bands that are absent or reduced in intensity in the in vivo samples (Fig. 3, lanes N and D) represent protections where protein binding shields the DNA from dimethyl sulfate attack. Bands that are significantly darker in the in vivo samples, or new bands that appear in these lanes, are known as enhancements. Enhanced DMS reactivity is indicative of protein binding in the nearby area, although generally not on that particular residue. In Fig. 3, the protections are noted with a filled triangle, and enhancements are marked with an open triangle. In both normal and diabetic samples, the CRE was completely protected at −101 and showed enhanced DMS reactivity at −104, −99, and −95/−94 (Fig. 3, right). This pattern was seen in all animals regardless of treatment. A significant protection seen only in normal animals occurred at −71, as shown in Fig. 3, right. Protections at −137 and −147 were not consistently observed.

On the bottom strand, the CRE is heavily protected at −100, −105, −103, and −109 (Fig. 4, bottom right) in both normal and diabetic animals. The A at −102 showed up as an enhancement in both cases. A key difference in the footprints is a very obvious enhancement at −142 seen only in the diabetic samples (Fig. 4, middle right). This particularly dark band, indicating enhanced DMS reactivity, was seen in 4/5 diabetic footprints and 0/5 normal footprints. Conversely, the nearby enhancement at −138 of the normal lane was not seen in diabetic footprints, suggesting possible competition for a binding site in this region. Another obvious difference is an enhancement at −161 in the diabetic lane (Fig. 4, top right). This residue is in the middle of the SRE located between −164 and −155. The SRE appears unoccupied under normal conditions and enhanced in diabetic samples (4/5 animals). Protections at −189/−190 were seen in all groups, whereas those at −70 were not observed in the diabetics. Both of these areas contain potential NF-Y-binding sites, with the sequence ATTTGG.

Because there was an enhancement at −161 of the SRE in four of the five diabetic animals, we wondered if insulin activation could be a result of sterol regulation through the SRE. To investigate this possibility, we examined livers of rats fed lovastatin or cholesterol to alter liver cholesterol levels. Animals were fed 0.02% lovastatin or 1% cholesterol for 5 days. Previous research in our lab has shown that a similar dose of lovastatin elevates HMGR transcription 4–6-fold (22), whereas dietary
cholesterol reduces HMGR protein levels to about 1% of control. It should be noted that dietary cholesterol has only a minor repressive effect on the rate of HMGR transcription in these animals (26). We predicted that the lovastatin-fed animals would show strong protections at the SRE, because of elevated cleavage of SREBPs, induced by sterol deprivation. Curiously, no definitive protections or enhancements at the SRE were visible when animals were fed either lovastatin or cholesterol (Fig. 5, right). The footprints were the same for the two animals in each group. The CRE was also heavily protected in these animals but unchanged by either treatment. The enhancement at the CRE was also heavily protected in these animals. In addition, the NF-Y sites at the SRE and CRE have been enlarged as shown at right.

To identify some of the major factors bound to footprinted regions, we performed EMSAs with short oligonucleotide probes for these elements. Nuclear extracts prepared from normal and diabetic rats were used in this assay. By using the probe from normal larvae, we were able to supershift this band, nor was antibody to Egr-1, another GC-box binding protein (data not shown). The lower band in the 1st lane of Fig. 8 was found to be nonspecific, as it was present regardless of probe used.
Given the strong protection of the CRE seen in all the in vivo footprints, and the ability of CREB to bind in vitro, we wanted to find out if CREB was in fact bound in vivo to the hepatic HMGR promoter. To accomplish this, liver sections from normal and diabetic rats were cross-linked with formaldehyde and subjected to chromatin immunoprecipitation analysis. CREB-1 antibody was able to pull down the HMGR promoter from both normal and diabetic chromatin, whereas an iso-type-matched antibody to an irrelevant nuclear protein was not (Fig. 9A). The minor difference in intensity of this band between normal and diabetic samples was not reproducible, although CREB was clearly bound in both cases. This immunoprecipitation was not able to pull down exon 12 of the HMGR gene, confirming that DNA was sheared to an appropriate size. The inability of phospho-CREB to pull down the HMGR promoter could be a result of poor antibody-antigen interaction. We would expect that at least some of the endogenous CREB is phosphorylated. The data confirm reports that CREB is bound to the HMGR promoter in vivo and validates this observation in the context of the live animal.

Another point of interest in the in vivo footprints was the consistent protection of the NF-Y site identified at −70. Because this site was strongly protected in 4/5 of the normal footprints, and none of the diabetics, we hypothesized that this element might play a critical role in activation of transcription. In order to investigate a possible functional role for this site, we constructed luciferase reporter plasmids containing the full-length HMG-CoA reductase promoter starting at −325 and
endling at +70 of the 5′-untranslated region. Two identical plasmids harboring mutations in the NF-Y site were also made. These plasmids were transfected into H4IIE cells, a rat hepatoma line. The cells were harvested and assayed for luciferase activity (Renilla luciferase was co-transfected for normalization purposes). As seen in Fig. 10, the wild type promoter shows a high level of activity relative to the vector backbone. Both of the mutants significantly inhibited luciferase production, indicating that this NF-Y site is required for efficient HMGR transcription.

**DISCUSSION**

These studies address the regulation of the HMG-CoA reductase promoter by insulin in live animals. Here we present the first evidence that insulin acts to directly increase transcription of the HMG-CoA reductase gene in rat liver. Diabetic rats have lower rates of HMG-CoA reductase transcription than normal rats. With only 2 h of insulin treatment, transcription was restored to normal. Previous work from our lab showed that the corresponding increase in mRNA could be accomplished even in the presence of cycloheximide (6). Taken together, these results suggest that insulin acts rather directly to stimulate the HMG-CoA reductase promoter and does not require protein synthesis.

*In vivo* footprinting revealed numerous protections and enhancements throughout the HMGR promoter. The most pronounced of these was at the CRE, which was occupied under all conditions tested. EMSA analysis confirmed that CREB-1 present in nuclear extracts from normal or diabetic rat livers could bind to this element in vitro, in agreement with observations in FRTL-5 cells (10). Chromatin immunoprecipitation analysis of rat liver confirms the previous finding that CREB is bound to the HMGR promoter in vivo (25). Given the overwhelming and invariant occupancy of the CRE in vivo, it seems unlikely that CREB binding is the regulated event in insulin activation. This may differ from sterol regulation in which SREBP binding has been shown to selectively recruit CREB to the promoter in CHO cells (28). Although we previously showed that the CRE was required for insulin activation of this promoter in H4IIE cells (7), *in vivo* occupancy of this site did not vary in rat liver. It is possible that insulin regulation in cultured rat hepatoma cells differs from the physiological regulation of this gene seen in whole animals. It is also possible that the CRE is necessary but not sufficient for insulin activation, inasmuch as it is required for maintaining an appropriate level of basal transcriptional activity.

The enhancement at −138 in normal footprints was not seen in diabetic samples. Given the GC-rich content of the nearby sequence, it is likely that this may be due to binding of an Sp1-related factor. In fact, strong *in vitro* binding activity was observed with the probe from −119 to −142. The sequence "ggggcggctt" is a close match to the consensus binding sequence for Sp1. Although generally regarded as a more basal transcription factor, Sp1 has been invoked in the insulin regulation of several genes, including SREBP-1a (29).

Four of the five diabetic animals showed a particularly striking enhancement at −142. This enhancement was never seen under the other conditions examined, including cholesterol and lovastatin treatment. In addition, only diabetic samples showed a change in the DMS reactivity of the SRE. This enhancement at −161 of the SRE coincided with the enhancement at −142 and may be a result of binding of a repressive factor in the −161/−142 region. Binding of a factor in this region could distort the DNA in such a way that both −142 and −161 are more susceptible to dimethyl sulfate attack. In addition, this factor may preclude binding to the Sp1 site downstream of −138. This competition would explain why enhancements at −138 were only seen in the normal animals (due to binding at Sp1 sites) and the enhancement at −142 only in the diabetic animals.

When animals were fed either lovastatin or cholesterol, the footprint
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In summary, the areas of protection or enhancement identified in this study generally correspond with the large protected regions seen previously in DNase I footprinting studies of the hamster promoter (19). Previous in vivo footprinting of the human promoter in HepG2 cells did not find any differences with insulin treatment, despite a 1.5-fold increase in mRNA (20). These previous in vivo studies also identified the SRE as a protected region, something that was not observed in rat liver. These reports were useful in both helping us design the experiments and in allowing us to compare results from cultured tumor cells to rat liver. Our work represents the first examination of the in vivo occupancy of the hepatic HMG promoter in live animals. This is also the first demonstration that the CRE is occupied in vivo. We report a novel NF-Y site that is more often protected in normal animals and is required for efficient HMG transcription. Most importantly, we have identified a few key areas where occupancy varies with diabetes, particularly −138, −142, and −161. This work will help focus future studies of insulin activation of this promoter and further our understanding of the transcriptional regulation of genes in the liver. Additional studies are needed to address the functional roles of these footprinted elements in the context of the live animal. Such investigations into insulin regulation of cholesterol biosynthesis are of paramount importance if we are to understand the links between diabetes and cardiovascular disease.

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