Transcriptional Modulators Affect in Vivo Protein Binding to the Low Density Lipoprotein Receptor and 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Promoters*

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The low density lipoprotein receptor (LDLR)1 and the 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) proteins are responsible for regulating intracellular cholesterol homeostasis via extracellular uptake and biosynthesis (for review, see Ref. 1). The expression of these genes is highly regulated in order to provide the cell with sufficient cholesterol for normal growth and function. Because of the importance of these genes for a variety of cellular processes, both are subject to regulation by multiple signals. LDLR expression is primarily controlled transcriptionally (2), while HMGR expression is controlled transcriptionally, translationally, and post-translationally (3, 4). Effectors of LDLR and HMGR transcription include a variety of mitogenic and nonmitogenic signals including phorbol esters (5), insulin (6), oncostatin M (7), transforming growth factor β, interleukin-1β (8), tumor necrosis factor (9), and platelet-derived growth factor (10). In addition, there are a variety of sterols that act as feedback regulators of transcription. The manner in which this complex array of signals is integrated has not been elucidated.

Work on localization of the DNA elements responsible for regulation of the LDLR and HMGR genes has focused primarily on the role of sterols. There is a conserved octanucleotide sequence in these promoters as well as in other cholesterol regulated genes that has been termed the sterol regulatory element (SRE) (11, 12). However, single nucleotide mutations have revealed functional differences between the LDLR and HMGR sequences, suggesting that only part of the conserved octanucleotide is functional in the HMGR promoter and that additional sequences upstream are important (13, 14). Two related proteins, SREBP-1 and SREBP-2, bind specifically to the LDLR SRE and have been shown to be critically involved in sterol regulation (15-19).

In addition to the SRE, other DNA elements corresponding to protein binding sites have been identified in both promoters. The binding of multiple factors to these promoters has been shown via in vitro DNAase protection experiments and transfections using mutated promoters (2, 14, 20–23). In the HMGR promoter, a number of nuclear factor 1-like sites have been characterized by DNase protection while Sp1-like sites have been found flanking the SRE in the LDLR promoter (20, 24). The importance of the Sp1 consensus site is highlighted by the deleterious effects of a single base mutation that causes familial hypercholesterolemia (25). The sequence and previously characterized regulatory elements in the two promoter regions (26, 27) are indicated (Fig. 1).

The in vitro DNase protection data have yielded valuable insight into regulation of these genes, but these experiments suffer from nonphysiological protein and DNA concentrations, making in vivo relevance uncertain. To directly address these concerns, we have examined protein-DNA interactions in known regulatory regions of the LDLR and HMGR promoters in J urkat T cells and HepG2 cells. These experiments, coupled with those reported earlier in human fibroblasts and hepatocytes (28), provide insight into the regulation of cholesterol homeostasis in a variety of cell systems. By examining alterations in the DMS-induced DNA cleavage pattern (29), we are able to confirm and extend a number of in vitro observations as well as provide data regarding the effect of various physiological conditions on protein binding in the LDLR and HMGR promoters.

EXPERIMENTAL PROCEDURES

HepG2 and J urkat T cells (both obtained from the ATCC) were grown at 37 °C in 5% CO2. HepG2 cells were grown in DMEM medium (Life Technologies, Inc.) with 10% fetal calf serum (HyClone) and J urkat cells in RPMI 1640 medium with 10% fetal calf serum (HyClone). DMS treatment and polymerase chain reaction conditions for the LDLR promoter have been described previously (28). Cells were treated with 0.5% DMS...
In Vivo Protein Binding to LDLR and HMGR Promoters

**LDLR Promoter**

![LDLR Promoter Diagram](image)

**HMGR Promoter**

![HMGR Promoter Diagram](image)

**RESULTS**

Close contacts between protein and DNA can be identified by using DMS to alkylate the N-7 of guanine or, at a slower rate, the N-3 of adenine. Proteins bound to DNA at or near these atoms in the major and minor grooves affect the DMS reactivity at these sites. Because DMS can penetrate the cell nucleus, information about protein binding in living cells can be obtained. Previously described conditions (29) were used as a starting point for the promoter sequences of interest. Conditions were chosen so that the correct sequence was readable from DNA treated with DMS in the absence of protein, and a low background for DNA not treated with DMS was obtained. The cleavage pattern obtained with DNA isolated from DMS-treated cells was compared with the cleavage pattern from DMS-treated, naked DNA. Differences in band intensity (either protection or hyperreactivity) are caused by protein-DNA interactions. The identity of the proteins binding in vivo can then be inferred from in vitro protection experiments with purified proteins or extracts.

Because of the pivotal role of the liver in maintaining cholesterol homeostasis, we chose the human hepatoma cell line, HepG2, as a model for our protein binding studies. To distinguish tissue-specific effects, we have also examined an unrelated cell line, J urkat T cells, for its basal response.

**In Vivo Footprinting under Normal Cell Culture Conditions**

**J urkat**—Prior to determining the effect of transcriptional modulators on protein binding, it was first necessary to characterize the basal pattern. In the LDLR promoter, there are multiple differences between the naked DNA (Fig. 2, lane 1) and in vivo DNA (Fig. 2, lane 2) from J urkat cells. The SRE region and both Sp1-like binding sites previously characterized in vitro are also occupied in vivo as strong protections are found throughout all three sites.

Detailed information about the TATA box region cannot be obtained because its AT richness provides few cleavage sites. Some protein binding can be detected, however, with protection at -27 frequently observed. In the transcriptional start region, there is hyperreactivity at -5 and protection at +2 and +3. Distal to the SRE, positions -107, -109, -110, -113, and -115 are minimized. LDLR primers were chosen in exons 13 and 14 to flank a 134-bp intron, generating fragments of 205 and 339 bp. For HMGR, primers were designed to flank short introns based on the hamster genomic structure. Primers homologous to 2614–2637 and 2881–2858 were found to generate appropriately sized fragments, 267 bp for the cDNA and about 390 bp for the genomic DNA. For each gene, one of the primers was labeled and used to amplify each gene for 25 cycles according to conditions provided by Perkin Elmer. Samples were electrophoresed on a 5% polyacrylamide gel and quantitated using a Fuji PhosphorImager. The ratio of cDNA to genomic DNA was measured for each gene. This method of quantitation has been found to give results similar to Northern but with less variability (data not shown).
are also protected even though no binding was observed at these positions in vitro, suggestive of proteins present in vivo that are not present in the nuclear extracts or cannot bind properly in that artificial environment. Weak protection of nucleotides 2131, 2138, 2139, and 2140 was also observed (data not shown), consistent with the in vitro data.

When the HMGR promoter is examined under the same conditions (Fig. 2, lanes 3 and 4), many regions of protection and hypermethylation are observed. In the SRE, positions 2168 and 2173 are protected, while 2172 shows hyperreactivity. In the hamster HMGR promoter, three nuclear factor 1 binding sites (24) have been identified in the region we have characterized by in vivo footprinting. In the hamster promoter, one of these sites is a perfect match to the consensus TGGN7CCA (30), while the other footprints were proposed to be half-sites involved in heterodimeric binding (24). In the human promoter, there are four TGG half-sites, but none of the sequences match the complete consensus perfectly. All four of these half sites (–217 to –215, –195 to –193, –184 to –182, and –174 to –172) share the same modification pattern with the upstream G protected and the downstream G hyperreactive. These four sequences are each separated by a helical turn, suggesting that the bound proteins are on the same face of the helix. The two downstream half-sites match the regions shown to be important in sterol regulation in the hamster gene. Additional sites of protection are located throughout the promoter

with especially strong sites at positions –113, –129, –133/–137/–139 (GC box), –144, –146, –178, and –185.

HepG2—HepG2 cells were cultured in two different media commonly used when studying lipoprotein regulation. Cells grown in DMEM plus 10% calf serum were exposed to DMS. Protein binding to the LDLR promoter (Fig. 3, lane 3) and to the HMGR promoter (Fig. 3, lane 7) was characterized. With the LDLR promoter, the DMS protection pattern is qualitatively the same as that seen with Jurkat cells, although some-what less protection of the SRE and transcription start sites is seen. In contrast, the HMGR promoter contains many sites that are more strongly protected in the HepG2 cells than in the Jurkat cells.

When the cells are switched to a defined, serum-free medium, changes are seen in both promoters. While most of the LDLR footprint is unaffected by the inducing conditions (Fig. 3, lane 4), an increased hyperreactivity in the SRE at –59 and –61 was observed. Increased protection was observed over many areas of the HMGR promoter in the inducing conditions (Fig. 3, lane 8). Especially prominent is the hypermethylation at position –182, which changes to protection in serum-free medium.

Effect of PMA on Protein Binding

In order to better characterize the effect of transcription on protein binding, conditions known to induce changes in mRNA levels were examined. One of the most dramatic changes de-
scribed previously is induced by phorbol esters, which increase LDLR transcription severalfold over 2–4 h (5). In order to see what effect this increased transcription would have on protein binding, cells were exposed to 0.2 μM PMA for 1–24 h, and separate aliquots used for DMS protection and mRNA analysis. As shown in Fig. 4, mRNA levels increase by 4.6-fold at 2 h with the increase falling to 1.8-fold at 24 h, similar to previous results (5).

The only change in DMS protection that could be detected throughout the region examined was at position 259 in the SRE (Fig. 5). The extent of hypermethylation was observed to vary between experiments but could be observed as early as 1 h after the addition of PMA. Since this hypermethylation occurs concomitant with the increase in mRNA levels, it would appear that the change in protein binding indicated by hypermethylation is needed for the transcriptional increase. This is similar to the observation that fibroblasts exposed to cholesterol synthesis inhibitors or oxysterols change protein binding to the LDLR promoter prior to a change in transcription (28).

As a slight increase in the HMGR mRNA levels was observed in PMA-treated HepG2 cells at 1 and 2 h with a decrease at 24 h (Fig. 4). A 2.3-fold increase in HMGR was observed previously in THP-1 cells, but no data were provided for HepG2 cells (31). No effects on the HMGR promoter footprint pattern were observed in response to PMA (data not shown).

**Footprint Changes in Response to Insulin**

When HepG2 cells are incubated in a defined, serum-free medium (SSF) (32), addition of insulin stimulates an increase in LDLR mRNA levels (6, 33). We found an increase in LDLR mRNA levels of similar magnitude. HepG2 cells incubated for 2–6 h with insulin–containing serum-free media contained 1.4-fold more LDLR mRNA (data not shown). In these conditions, there is hypermethylation at position –59 in the SRE of the LDLR promoter relative to cells incubated in the absence of insulin (Fig. 6). These results indicate that the stimulatory effect of insulin on LDLR gene expression is mediated at least in part through alterations in protein binding to the SRE region. This is consistent with previous results that indicated the addition of lipoproteins could not effectively eliminate the insulin induction of LDLR (6). It is noteworthy that there is a 9 of 11 bp match between a region of the SRE sequence and a region in the phosphoenolpyruvate carboxykinase gene promoter shown to be involved in its insulin responsiveness (34, Fig. 1). Insulin induces no reproducible footprint changes in the HMGR promoter under the same conditions, even though a 1.5-fold increase in HMGR mRNA levels was observed (data not shown).

**DISCUSSION**

The genes encoding LDL receptor and HMG CoA reductase are involved not only in regulating cholesterol homeostasis but also in other processes, many of which are specific for either LDLR or HMGR. Thus, there must be some sharing of regulatory signals while still allowing for independent inputs. Major
changes in protein binding to these promoters do not occur upon transcriptional induction or between cell types. The changes we do see are focused primarily on the SRE regions of both genes (data summarized in Fig. 8). Our data are consistent with previous in vitro data on the regulation of these genes. We are also able to extend previous observations and rule out certain proposed models.

In all of the conditions tested, there is virtually no change in binding to the Sp1-like sites flanking the SRE in the LDLR promoter, arguing against a major regulatory role for these sites in transcription. However, these sites are important for transcription based on both in vivo and in vitro data (2, 25). The constancy of binding that we observe suggests a structural role in assembling the appropriate protein-DNA complex needed for transcription. It seems likely that proteins bound to these sites may serve as a scaffold for the binding of other regulatory proteins rather than carry out that function themselves.

The clear in vitro demonstration that Sp1 is able to bind to the sites flanking the SRE and the similarity of these sequences to the Sp1 consensus sequence have led to the obvious conclusion that Sp1 is binding in vivo. However, the modification patterns we observe are not completely consistent with this interpretation and suggest that there may be Sp1-like proteins that are involved. When the modification patterns of the LDLR Sp1 sites are compared with 15 previously characterized Sp1 sites (including nine characterized in vivo, Refs. 36–40), differences are observed (Fig. 9). In all 15 sites, position seven is a guanine, and it is hypermethylated in all 13 non-LDLR sites. In contrast, this position is protected in the two LDLR sites. While this does not prove the presence of a novel protein, the cloning of a family of Sp1-related proteins (41, 42) makes it a distinct possibility, especially since the LDLR sites are not perfect matches with the consensus sequence.

Because of the central role that the liver plays in cholesterol homeostasis, the regulation of hepatic LDLR expression could potentially be very different than in other tissues. The basal protein binding pattern in the LDLR promoter is virtually identical in the Jurkat and HepG2 cells examined here and the primary fibroblasts and hepatocytes examined previously (28). While the basal protein binding pattern of the LDLR promoter does not change among these cell types, the DMS protection pattern of the SRE is altered upon transcriptional induction by ketoconazole, PMA, and insulin. In each case, the major alteration is at position −59. This could be caused either by new proteins binding to this region or by existing proteins being covalently modified or interacting with new partners. The recent cloning of proteins SREBP-1 and SREBP-2 (18) will provide reagents to aid in deciphering these possibilities.

While the cultured cells are all striking in their sameness of protein binding to the LDLR promoter, primary hepatocytes differ from other cell types in that −59 is not hypermethylated upon induction (28). This result is not readily interpreted based on the hamster SREBP expression results. While the expression of SREBP-1 and SREBP-2 is unchanged with transcription in cultured cells, the relative expression of SREBP-1 and SREBP-2 is affected by transcription in the intact liver (18). If the primary hepatocytes mirror the intact liver, SREBP-1 and SREBP-2 would have to yield the same protection pattern
when bound to the SRE. The hypermethylation of −59 in cultured cells would then be explained not by a new protein binding to the SRE but by DNA distortion caused by more long range interactions. Additional information about the expression and processing of the SREBP genes will be required to help resolve these questions.

Previous work has suggested that distinct proteins bind the SREs of the LDLR and HMGR genes (13, 14). Homologous positions in the SRE consensus sequences are differentially methylated in Jurkat cells, supporting this conclusion. In HepG2 cells, however, the same conclusion cannot be drawn because the HMGR SRE protection pattern is similar to that seen in the LDLR SRE. The DMS profiles of purified proteins may now be undertaken to clarify the in vivo binding patterns. These data suggest that the SRE and the protein(s) that bind to it are involved in regulating many, but not all, aspects of LDLR transcription. While the in vivo interactions identified here do not answer many of the questions about how these important genes are regulated, this information can be used to help focus future in vitro binding studies to provide a clearer picture of how these genes are regulated.

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