A Critical Role for cAMP Response Element-binding Protein (CREB) as a Co-activator in Sterol-regulated Transcription of 3-Hydroxy-3-methylglutaryl Coenzyme A Synthase Promoter*

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3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase, a key regulatory enzyme in the pathway for endogenous cholesterol synthesis, is a target for negative feedback regulation by cholesterol. When cellular sterol levels are low, the sterol regulatory element-binding proteins (SREBPs) are released from the endoplasmic reticulum membrane, allowing them to translocate to the nucleus and activate SREBP target genes. However, in all SREBP-regulated promoters studied to date, additional co-regulatory transcription factors are required for sterol-regulated activation of transcription. We have previously shown that, in addition to SREBPs, NF-Y/CBF is required for sterol-regulated transcription of HMG-CoA synthase. This heterotrimeric transcription factor has recently been shown to function as a co-activator in several other SREBP-regulated promoters, as well. In addition to cis-acting sites for both SREBP and NF-Y/CBF, the sterol regulatory region of the synthase promoter also contains a consensus cAMP response element (CRE), an element that binds members of the CREB/ATF family of transcription factors. Here, we show that this consensus CRE is essential for sterol-regulated transcription of the synthase promoter. Using in vitro binding assays, we also demonstrate that CREB binds to this CRE, and mutations within the CRE that result in a loss of CREB binding also result in a loss of sterol-regulated transcription. We further show that efficient activation of the synthase promoter in Drosophila SL2 cells requires the simultaneous expression of all three factors: SREBPs, NF-Y/CBF, and CREB. To date this is the first promoter shown to require CREB for efficient sterol-regulated transcription, and to require two different co-regulatory factors in addition to SREBPs for maximal activation.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthesis is a key rate-limiting enzyme of cholesterol biosynthesis, converting acetoacetyl-CoA and acetyl-CoA into HMG-CoA. In order to achieve cellular cholesterol homeostasis, this and other genes of cholesterol metabolism are regulated by classical feedback repression: they are up-regulated when sterol levels fall and down-regulated when sterol levels rise (1–5). This control is exerted primarily at the transcriptional level through the action of the sterol regulatory element-binding proteins (SREBPs), a unique subfamily of basic helix-loop-helix zipper (bHLHZip) proteins. SREBPs are expressed as 125-kDa precursor proteins that are anchored to the endoplasmic reticulum and nuclear membranes. When cellular sterol levels fall, the precursor is released from the membrane by a two-step proteolytic mechanism, allowing the mature, transcriptionally active SREBPs to translocate to the nucleus (6). Once inside the nucleus, SREBPs activate cholesterogenic target genes through binding to sterol regulatory elements (SREs) present in their promoters. SREBPs also activate key genes of fatty acid metabolism (7–9) thus, they are central transcription factors of mammalian lipid metabolism.

SREBPs alone, however, are inefficient transcriptional activators, and in all SREBP-regulated promoters studied to date, additional transcription factors are necessary for maximal activation in response to sterol deprivation. Interestingly, the required co-regulator is not the same for all SREBP-regulated promoters. For example, in the case of the farnesyl diphosphate synthase promoter, the required co-regulator is NF-Y/CBF (10), whereas in the LDL receptor promoter, it is Sp1 (11).

The region of the HMG-CoA synthase promoter that is necessary for sterol-regulated transcription is contained within an approximate 100-base pair region, which includes two consensus SREs, a CAAT box, and a consensus cAMP response element (CRE) (12) (see Fig. 1). Previous studies have demonstrated that both SREs, termed SRE I 5’ and SRE I 3’, as well as the inverted CAAT box, are essential for sterol-regulated transcription. These elements recruit SREBP and NF-Y/CBF, respectively, to the promoter under conditions of sterol deprivation (10, 12). However, the potential involvement of the CRE has not previously been evaluated.

CREs were originally identified as cis-acting elements that confer transcriptional activation in response to elevated cAMP levels (13–15). Using the somatostatin promoter, it was shown that this responsiveness was mediated through activation and recruitment of the basic leucine zipper containing transcription factor termed CAMP response element-binding protein (CREB) (16). CREB is phosphorylated in response to elevated cAMP, and this allows it to interact efficiently with the transcriptional co-activator protein called CREB-binding protein to stimulate transcription of CAMP target genes (17, 18). Subsequently,
numerous related CRE-binding proteins have been identified and cloned (19) and together they comprise the CREB/ATF family of transcription factors. Individual members of this family bind to CREs present in numerous eukaryotic promoters, and activate transcription in response to various cellular signals (20–22). In the current studies, we investigated the role of the putative CRE in sterol-regulated transcription of the HMG-CoA synthase gene. In a thorough mutational analysis of the promoter regulatory region, we noted that single point mutations that alter the consensus CRE resulted in a complete loss of sterol-regulated transcription. Using in vitro binding assays, we show that CREB binds to the wild type consensus CRE of the synthase promoter, and mutations within the CRE that abolish sterol-regulated transcription completely disrupt CREB binding to this element. Furthermore, using a Drosophila tissue culture cell line that does not express several mammalian transcription factors, we show that maximal activation of the synthase promoter requires the simultaneous expression of SREBP, NF-Y/CBF, and CREB. To date, this is the first case in which SREBP has been shown to require two different co-regulatory DNA-binding proteins to activate a promoter in response to sterol deprivation. Additionally, this is the first report demonstrating that a member of the CREB/ATF family can function together with the SREBP to mediate sterol-regulated transcription.

MATERIALS AND METHODS

Cells and Media—All of the cell culture lines used here have been described before (11). All cell culture materials were purchased from Life Technologies Inc. Lipoprotein-deficient serum was prepared by ultracentrifugation of newborn bovine serum as described previously (23). Cholesterol and 25-OH cholesterol were obtained from Steraloids (11). The point mutations were constructed using the “Altered Sites” mutagenesis kit (Promega Inc.) according to the manufacturer’s protocol. All mutants were confirmed by sequencing. pCMV2 β-Gal contains the cytomegalovirus early promoter linked to the E. coli β-galactosidase gene (11). The plasmid pPacSp1 was obtained from Al Inc., and stock solutions were dissolved in absolute ethanol. pSynSRE has been described elsewhere (12). Standard techniques were used in all cloning procedures (24). Construction of the plasmid p5SypSRE has been described elsewhere (12). pSynTlac is the same as the plasmid “TATA only” described previously (11). 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Expression and Purification of Fusion Proteins—The expression and purification of amino acids 1–490 of SREBP1a has been described previously (11). The glutathione S-transferase (GST)-CREB expression construct was made by excising the CREB coding sequence from the plasmid pPasCREB using HindIII and BamHI. The resulting fragment was then inserted in-frame with the GST coding region in the pGex2T plasmid. GST-CREB was expressed in E. coli strain DH5αF'. After harvesting the 200-ml cultures, pellets were resuspended in 4 ml of ice-cold saline solution (10 mM Tris, pH 8, 20% sucrose, 1 mM dithiothreitol), incubated on ice for 15 min in the presence of lysosome (50 mg/ml stock), then incubated on ice for 10 min in the presence of EDTA (final concentration of 1 mM). The extract (10% stock solution) was then added to a final concentration of 0.05% and the samples were sonicated 3 times for 10 s each. Cell debris was pelleted by centrifugation. The supernatant was then incubated with 1:1 slurry of glutathione-agarose (Sigma) on a rotating wheel at 4 °C for 3 h, followed by extensive washing with Wash Buffer (20 mM Tris, pH 7.5, 200 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin/potassium, 10% glycerol). Bound proteins were eluted with 5 mM glutathione.

Protein-Protein Interaction Assay—The fusion proteins were expressed in E. coli strain DH5αF'. Cultures (100 ml) were grown to OD600 of 0.6 to 0.8, induced by adding isopropyl-1-thio-β-d-galactopyranoside to final concentration of 1 mM, and grown for an additional 3 h at 37 °C. Cells were then harvested by centrifugation. The cell pellets were resuspended in NETN (100 mM NaCl, 20 mM Tris, pH 8, 1 mM EDTA, 0.5% Nonidet P-40), lysed by sonication, and the cell debris was removed by ultracentrifugation. Glycerol was added to the supernatant to a final concentration of 10%, and extracts were stored in aliquots at −70 °C. The expression of the fusion protein was verified by SDS-PAGE analysis followed by staining with Coomassie Blue, as well as by an immunoprecipitation protocol with an anti-GST antibody and horseradish peroxidase-conjugated anti-rabbit IgG (Sigma). To 10 μl of glutathione-agarose beads, 100 μl of the induced crude GST fusion protein extract, and 300 μl of buffer (Z’0.1, 50 mM Hepes pH 7.5, 2 mM MgCl2, 100 mM KCl, 10% (v/v) glycerol, 0.1% (v/v) Nonidet P-40) + 0.5% non-fat dry milk + 5 mM dithiothreitol) were added followed by an incubation on a rotating wheel at 4 °C for 2 h. The bound GST fusion protein was pelleted in a microcentrifuge and nonspecifically bound proteins were washed off the beads with 3 times 1 ml of buffer. Then, 5 μl of purified SREBP-1a was added to the bound proteins, along with 300 μl of buffer, and incubated on a rotating wheel for an additional 2 h at 4 °C. The reactions were again washed 3 times with 1 ml of buffer, and specifically bound proteins were pelleted, resuspended in sample buffer, and analyzed by SDS-PAGE followed by Western blot analysis using the ECL kit (Pierce) and an anti-SREBP-1 primary antibody (6G02A4).

RESULTS

Mutations within the Consensus CRE Ablate Sterol-Regulated Transcription of HMG-CoA Synthase—Previous studies
have demonstrated that a 100-base pair region of the HMG-CoA synthase promoter is necessary and sufficient for efficient sterol-regulated transcription. Within this region, two binding sites for SREBP and one binding site for NF-Y/CBF were shown to be critical (12). To determine if there are other critical elements within this 100-bp interval, we introduced a series of single point mutations into the region and fused the resulting mutant promoters to a luciferase reporter gene. Each construct was then transiently transfected into CV-1 cells and assayed for sterol-regulated transcription by culturing the transfected cells either under induced (−sterols) or suppressed (+sterols) conditions. The results for constructs containing mutations within the region −277 to −260, relative to the transcription start site, are shown (Fig. 2A), and the ratio of reporter gene activity under induced to suppressed conditions is reported as fold regulation (Fig. 2B). SynSRE is the wild type promoter-reporter construct, and SynTLuc is a control promoter-reporter construct containing only the HMG-CoA synthase minimal TATA box.

Mutations in the 5′-half of the SRE-I 5′ resulted in a decrease in sterol-regulated transcription due to a loss in promoter activity under induced conditions, as expected. However, mutations in the 3′-half of the SRE-I 5′ did not result in a dramatic decrease in sterol regulation. In the case of −268T→G, this was due to an increase in activity under both induced and suppressed conditions. The reason for this is not clear. In the promoter constructs containing mutations corresponding to −268T→A, −267A→C, and −266C→A, an increase in sterol regulation was observed due to a decrease in the level of promoter activity under both induced and suppressed conditions. This effect may be due to disruption of binding of a CREB/ATF protein at the overlapping CRE, which normally contributes to both the induced and the suppressed activity (in this case equivalent to the basal activity) of the promoter. This possibility is further supported by the dramatic loss in sterol regulation for the mutants −264T→G and −265G→T, both of which mutate critical bases within the consensus SRE, while leaving the consensus CRE intact. Taken as a whole, these results indicate that a CREB/ATF family member is involved in sterol-regulated transcription of the HMG-CoA synthase promoter.

Sterol Defective Mutants That Alter the CRE Element Decrease Binding by CREB and Not SREBPs—The mutations at −264 and −265 discussed above are adjacent to the consensus CRE element but are contained within the putative CRE. To determine if these mutations were defective for sterol regulation due to a loss in binding of either CREB or SREBP-1a, recombinant versions of each protein were used in an electrophoretic mobility shift assay with either the wild type or selective mutant HMG-CoA synthase probes (Fig. 3).

Using the wild type synthase probe, a shifted complex was observed in the presence of either SREBP-1a or CREB (Fig. 3, A and B, lanes 2 and 3). However, using probes corresponding to the single mutations in the consensus CRE at bases −264 or −265 (Mutant A or B, see Fig. 1) that were defective for sterol-regulated transcription (Fig. 2), the CREB-DNA complex was not formed (Fig. 3A, lanes 8–9 and 11–12). Using a probe with a single mutation in the SRE I 5′, but outside of the CRE, resulted in a decrease in the shifted complex observed in the presence of CREB (Fig. 3A, lanes 5 and 6), indicating bases outside the consensus CRE are important in recruiting CREB to the promoter. Similar results were observed when we used a recombinant version of the CREB family member ATF-3 (data not shown).

SREBP-1a bound to the wild type probe (Fig. 3B, lanes 2 and 3) and this was significantly decreased when a critical base in the SRE element was mutated (Fig. 3B, lanes 5 and 6). More importantly, SREBP bound efficiently to the probes containing either CRE mutation (Fig. 3B, lanes 8–9 and 11–12). Therefore, the mutations within the CRE of the HMG-CoA synthase promoter decrease the binding of CREB (and possibly other related family members), but not SREBP-1a. Taken together, the transfection results and the DNA binding assays suggest that the recruitment of CREB, or another related protein, is critical for sterol-regulated transcription of the HMG-CoA synthase promoter.

Maximal Activation of the HMG-CoA Synthase Promoter Requires SREBP, NF-Y/CBF, and CREB—To determine which transcription factors are simultaneously required for maximal activation of mammalian promoters, it is possible to take advantage of a Drosophila tissue culture cell line, SL2. This cell line does not express functional homologues for several mammalian proteins, including the transcription factors Sp1 (25) and NF-Y/CBF. Therefore, it is a useful cell-based assay system for the analysis of transcription factor requirements for promoter activation because it provides a negative background for such studies. For example, this assay system was used to directly show that SREBP and Sp1 function as co-activators of the LDL receptor promoter (11).

To directly evaluate the ability of SREBP, NF-Y/CBF, and CREB/ATF, or various combinations thereof, to activate the HMG-CoA synthase promoter, we used the SL2 assay system (Fig. 4). The wild type synthase promoter-reporter construct was co-transfected along with increasing amounts of a Drosophila expression construct for human CREB (Fig. 4A). CREB, either alone or together with expression constructs for all three subunits of NF-Y/CBF, was unable to activate the promoter. Expression of SREBP-1a and the NF-Y/CBF plasmids together resulted in only a very slight activation of the synthase promoter in the absence of CREB. However, addition of increasing amounts of CREB along with constant levels of both SREBP-1a

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and NF-Y/CBF resulted in a dramatic increase in promoter activity. Inclusion of expression constructs for all three sub-units of NF-Y/CBF was required for this activation.

The effect of CREB was specific and dependent on the consensus CRE, as there was no activation observed for the CRE mutant promoter-reporter construct even when all three proteins were co-expressed (Fig. 4B). The results in Fig. 4C show that the CREB/ATF family member ATF-2 also functions with SREBP and NF-Y/CBF to activate transcription of the HMG-CoA synthase promoter, although the level of activation was not as high as that observed in the presence of CREB. This may be due to inherent differences in activation potential between the two proteins, or simply due to differences in expression levels of CREB and ATF-2 from the pPac expression vectors in SL2 cells. Again, this effect was specific, since the LDL receptor promoter-reporter construct, which does not contain NF-Y/CBF or CRE sites, was unaffected (Fig. 4D). These results support the hypothesis that efficient transcriptional activation of the HMG-CoA synthase promoter requires SREBP, NF-Y/CBF, and CREB (or other members of the CREB/ATF family) to be present simultaneously.

**SREBP Directly Interacts with CREB in Vitro—How do SREBP and CREB function, together with NF-Y/CBF, to achieve sterol-regulated transcription?** In an attempt to address this issue, we evaluated whether SREBP-1a and CREB could interact with each other in solution in the absence of DNA. CREB was expressed as a GST fusion protein, and immobilized on glutathione-agarose beads which was then incubated with purified, recombinant SREBP-1a. SREBP-1a was either loaded directly on an SDS-PAGE gel (Fig. 5, lane 1) or material eluted from GST-agarose beads after incubation with either the recombinant GST protein (Fig. 5, lane 2) or with the recombinant GST-CREB fusion protein (Fig. 5, lane 3). The SDS-PAGE gel was then analyzed by immunoblotting with an antibody directed against SREBP-1. Recombinant SREBP-1a bound specifically to the GST-CREB fusion protein. Thus, SREBP-1a and CREB are capable of interacting with each other in solution in the absence of a DNA fragment containing adjacent binding sites for the two proteins.

**DISCUSSION**

In response to cellular sterol deprivation, the HMG-CoA synthase gene is activated at the transcriptional level primarily through the action of SREBPs. However, in all SREBP target promoters studied to date, including the HMG-CoA synthase promoter, recruitment of additional, nonspecific transcriptional activators to the promoter is required to achieve this activation (10–12, 26, 27). The HMG-CoA synthase promoter has a complex array of cis-acting regulatory elements, each with the potential for involvement in sterol-regulated transcription. In the current studies, we show that the consensus CRE, located within the minimal regulatory region of the promoter, is essential for sterol-regulated transcription. In a previous report from our laboratory, it was demonstrated that this element mediated transcriptional activation of the synthase promoter in response to phorbol esters and the AP-1 transcription factor (28). Here, we have demonstrated that this element recruits CREB to the promoter, and mutations that abolish CREB binding also result in a loss of sterol-regulated transcription (Figs. 2 and 3). Although other cholesterogenic promoters contain consensus CREs, to date this is the first promoter shown to require a CREB/ATF factor for the regulation of transcription in response to cellular sterol levels.

We have previously shown that the two SREs and the CCAAT box are required for sterol-regulated transcription (12) of the HMG-CoA synthase promoter. However, as demon-
strated here, these elements are necessary but not sufficient for sterol-regulated transcription. This is based on two observations: 1) single point mutations in the CRE result in a loss of sterol-regulated transcription, even in the presence of the intact SREs and CCAAT box (Fig. 2); and 2) in a Drosophila SL2 assay system, maximal transcriptional activation is achieved only in the presence of all three proteins, SREBP, NF-Y/CFB, and CREB (Fig. 4A). This observation is further unique in that the HMG-CoA synthase promoter is the first in which three factors, SREBP and two different general factors, are necessary for efficient transcriptional activation in response to sterol deprivation.

CREB is a member of a large family of basic leucine zipper proteins called the CREB/ATF family that consists of at least 10 distinct members (22, 29). CREB, the first member to be cloned, was originally identified based on its ability to bind to a cis-acting element termed the CRE, which was known to confer cAMP responsiveness on corresponding promoters (16).

Subsequently, CREB has been suggested to activate transcription in response to various other cellular signaling agents, as well, including insulin, growth factors, and calcium (30–32). In some cases, CREB alone is not sufficient, and additional transcription factors are required to achieve activation. For example, in the PEPCK promoter, the CRE functions together with a CCAAT box to achieve cAMP-mediated transcriptional activation (14), whereas in the T-cell receptor α gene, the T-cell specific factor TCF-1α must function together with a CRE-binding protein and the ETS-1 protein to achieve activation (33). Another example is the cell specific expression of the somatostatin promoter. Using the cell line Tu6, it was shown that this cell-restricted expression requires the concerted action of an islet cell-specific LIM family protein Isl-1, CREB (34), and a NF-Y/CFB-like protein (35).

The functional differences among the various CREB/ATF proteins are not completely clear at present. Although they are all highly similar in their basic and leucine zipper regions and...
they bind the same cis-acting consensus sequence, the affinities of the different homo- and heterodimeric combinations vary for different CREs (36, 37). Furthermore, they do not all respond to the same cellular signals. For example, ATF-2 mediates activation by the adenovirus E1a protein (38), whereas both CREB and ATF-1 mediate activation in response to elevated cAMP (18, 39). We tested the ability of ATF-2 to substitute for CREB in activation of the HMG-CoA synthase promoter. Although in an in vitro binding assay ATF-2 was able to bind to the CRE with the same specificity as we observed for CREB, it was unable to activate the HMG-CoA synthase promoter to the same level as that achieved with CREB (Fig. 4). It is possible that this result is due to a difference in expression levels of ATF-2 and CREB in SL2 cells, but this is unlikely since in similar experiments ATF-2 was more effective than CREB in activating transcription from the HMG-CoA reductase promoter. Interestingly, in the cAMP-independent cell type-specific expression of the somatostatin promoter mentioned above (34), ATF-2 was also unable to substitute for CREB. As more is understood about the functions of the various CREB/ATF proteins, the reason for this difference will become clearer.

In almost all eukaryotic promoters, multiple DNA binding transcription factors must assemble on the promoter, together forming a transcriptionally active complex. In many cases, this complex is composed of a combination of regulatory-specific transcriptional activators such as SREBP, together with more ubiquitous, generic, transcription factors. The functions of the individual components can include enhanced recruitment of other required factors (either by direct physical interaction or by alteration of DNA structure of nearby cis-acting elements), presentation of distinct and complimentary activation domains, and/or distinct interactions with essential components of the basal, RNA polymerase II transcription machinery. We previously reported that SREBP physically interacts with NF-Y/CBF (12), and another report demonstrated that NF-Y/CBF enhanced the binding of SREBP to the SRE in the promoter for farnesyl dipiphosphate synthase, another enzyme of cholesterol biosynthesis (40). Here we have shown that SREBP-1a physically interacts with CREB (Fig. 5). Interestingly, mutations that introduce insertions between the SRE I and the SRE I/CRE are defective for sterol-regulated transcription in transient transfection assays. The reason for this is unclear, as yet, but may reflect the importance of a requirement for this protein-protein interaction between SREBP and CREB when they are recruited to the promoter. Further experiments are required to determine the mechanistic significance of this interaction.

How does the SREBP family of transcription factors mediate the regulation of numerous genes involved in distinct but related metabolic processes such as fatty acid metabolism, cholesterol synthesis, and cholesterol uptake? Promoter specific effects of the different SREBP isoforms is likely to provide part of the basis for regulatory specificity (41). Additionally, the differences in promoter architecture result in a requirement for unique combinatorial arrangements of transcription factors that are required for activation of the various SREBP target genes. This is also likely to provide part of a mechanism by which coordinate, yet specific, regulation is achieved. The involvement of CREB in activation of the HMG-CoA synthase gene, for example, may add a further level of complexity to its regulation, relating the responsiveness to sterol deprivation with other cellular regulatory events.

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