A mitochondrial ketogenic enzyme regulates its gene expression by association with the nuclear hormone receptor PPARα

Lisa M. Meertens, Kenji S. Miyata, Jonathan D. Cechetto, Richard A. Rachubinski1,2 and John P. Capone

Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5 and 1Department of Cell Biology and Anatomy, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

Corresponding authors
e-mail: caponej@fhs.csu.mcmaster.ca or rick.rachubinski@ualberta.ca

Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoAS) is a key enzyme in ketogenesis, catalyzing the condensation of acetyl-CoA and acetoacetyl-CoA to generate HMG-CoA, which is eventually converted to ketone bodies. Transcription of the nuclear-encoded gene for mHMG-CoAS is stimulated by peroxisome proliferator-activated receptor (PPAR) α, a fatty acid-activated nuclear hormone receptor. Here we show that the mHMG-CoAS protein physically interacts with PPARα in vitro, and potentiates PPARα-dependent transcriptional activation via the cognate PPAR response element of the mHMG-CoAS gene in vivo. Immunofluorescence of transiently transfected cells demonstrated that in the presence of PPARα, mHMG-CoAS is translocated into the nucleus. Binding to PPARα, stimulation of PPARα activity and nuclear penetration require the integrity of the sequence LXXLL in mHMG-CoAS, a motif known to mediate the interaction between nuclear hormone receptors and coactivators. These findings reveal a novel mechanism of gene regulation whereby the product of a PPARα-responsive gene, normally resident in the mitochondria, directly interacts with this nuclear hormone receptor to autoregulate its own nuclear transcription.

Keywords: cofactor/HMG-CoA synthase/mitochondria/nuclear hormone receptor/transcription

Introduction

Ketogenesis is a tightly regulated metabolic pathway carried out in the liver that supplies lipid-derived energy to the brain under normal conditions, and to peripheral tissues during starvation and sustained exercise and in disease states such as diabetes (McGarry and Foster, 1980). Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoAS) is the first and rate-limiting enzyme in this pathway, catalyzing the condensation of acetyl-CoA and acetoacetyl-CoA in the mitochondria to generate HMG-CoA and free CoA (Quant, 1994). HMG-CoA is eventually converted, through the actions of HMG-CoA lyase and Δ-3-hydroxybutyrate dehydrogenase, into the ketone bodies acetocacetate and β-hydroxybutyrate, which are used as sources of oxidative fuels in extrahepatic tissues.

Mitochondrial HMG-CoAS is regulated at both the transcriptional and post-transcriptional level by mechanisms that can increase both the amount and activity of the enzyme (Quant, 1994). Fatty acids have been shown to induce transcription of the nuclear-encoded gene for mHMG-CoAS through activation of the peroxisome proliferator-activated receptor (PPAR) α, a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors (Rodriguez et al., 1994). PPARα, and the highly related PPARβ and PPARγ subtypes, regulate the expression of a wide assortment of genes that are important in metabolic homeostasis, cellular differentiation and energy utilization, and the different subtypes have been variously implicated in molecular processes associated with diabetes, obesity, atherosclerosis, thermogenesis and inflammation (Devchand et al., 1996; Lemberger et al., 1996; Schoonjans et al., 1996; Latruffe and Vamecq, 1997; Jiang et al., 1998; Nagy et al., 1998; Puigserver et al., 1998). PPARα, which is expressed predominantly in liver (Braissant et al., 1996), controls the transcription of genes involved in fatty acid oxidation, fatty acid synthesis and ketogenesis, among other processes, in response to nutritional, hormonal and environmental stimuli. PPARα activates transcription by binding cooperatively with its obligate heterodimerization partner 9-cis retinoic acid receptor (RXRα) to characteristic PPAR response elements (PPREs) present in the promoter regions of target genes (Kliwer et al., 1992; Tugwood et al., 1992; Zhang et al., 1992; Marcus et al., 1993), including the PPRE identified upstream of the rat mHMG-CoAS gene (Rodriguez et al., 1994).

The activity of nuclear hormone receptors such as PPAR is modulated by complex interactive networks of auxiliary receptor-interacting factors that serve as coactivators and corepressors of hormone-responsive pathways (Glass et al., 1997; Shibata et al., 1997). We have been using the yeast two-hybrid system to attempt to identify novel factors that specifically modulate the function of PPARα. We report here that mHMG-CoAS interacts directly with PPARα in vitro and stimulates transactivation by this receptor in vivo. Stimulation of PPARα activity was only observed via the cognate PPRE present in the mHMG-CoAS gene but not with other PPREs. Moreover, mHMG-CoAS, which is located predominantly in mitochondria, was found to be present in the nucleus when coexpressed with PPARα. Finally, interaction with PPARα, stimulation of PPARα activity and PPARα-dependent nuclear accumulation were dependent upon a nuclear hormone receptor–cofactor interaction motif, LXXLL, which is present in mHMG-CoAS. Our findings demonstrate that the product of a PPARα target gene, which functions as a ketogenic enzyme in mitochondria, can also specifically autoregulate
of mHMG-CoAS showing the LXXLL motif (underlined; numbering cHMG-CoAS, L393V and mHMG-CoAS, respectively. (Figure 1A, lane 4). Addition of the PPARα ligand Wy-14,643 or the RXRα ligand 9-cis retinoic acid did not enhance further the binding of mHMG-CoAS to the respective receptors (Figure 1A, lanes 3 and 6). Similar results were obtained in immunoprecipitation experiments. As shown in Figure 1B, radiolabeled mHMG-CoAS, when coincubated with equivalent amounts of unlabeled PPARα or RXRα (Figure 1B, lanes 6 and 12, respectively), was immunoprecipitated with the corresponding receptor-specific antiserum, but not with control serum (Figure 1B, lanes 5 and 11).

As an additional control for specificity, we repeated the binding assays with the cytosolic (c) isozyme, cHMG-CoAS. cHMG-CoAS is encoded by a separate gene and shares ~67% sequence identity with the mitochondrial form (Ayte´ et al., 1990; Mascaro´ et al., 1995). cHMG-CoAS catalyzes an identical enzymatic reaction in the cytosol, where the HMG-CoA that is formed is used in the mevalonate biosynthetic pathway leading to the formation of cholesterol and isoprenoids. As shown in Figure 1B, cHMG-CoAS did not interact with PPARα or RXRα, as determined by coimmunoprecipitation (Figure 1B, lanes 2 and 8) or in GST pull-down assays (data not shown). This indicates that the interaction with PPARα is specific to the mitochondrial isozyme.

mHMG-CoAS contains a copy of the nuclear receptor-interacting signature motif LXXLL

Many nuclear receptor-interacting coactivators, including SRC-1, CBP and RIP-140, contain one or more copies of the sequence LXXLL, a motif that is necessary, and sometimes sufficient, to mediate their binding to nuclear receptors (Glass et al., 1997; Heery et al., 1997; Voegel et al., 1998). This element is thought to recognize determinants in the ligand-binding domain of nuclear hormone receptors and has been shown to be required in some cases for coactivator function. Strikingly, mHMG-CoAS contains one such consensus motif, LASLL, at amino acids 390–394 with respect to the precursor protein (Figure 1C). The homologous region in cHMG-CoAS contains an altered motif, LASYL, which is embedded in an otherwise conserved region. As shown in Figure 1B, replacement of Leu393 with valine (L393V) severely compromised the ability of mHMG-CoAS to bind to PPARα (Figure 1B, compare lane 4 with lane 6; binding efficiency was reduced by ~90%). Interaction with RXRα was also reduced, but to a lesser degree (Figure 1B, compare lane 10 with lane 12). L393V still bound weakly to PPARα (Figure 1B, lane 4), suggesting that other determinants in mHMG-CoAS also contribute to binding. This is consistent with the fact that the original mHMG-CoAS partial cDNA clone isolated in the two-hybrid screen lacked this region. Our findings indicate that mHMG-CoAS binds to PPARα, in part, by utilizing a recognition motif that is found commonly in well-characterized, nuclear receptor-interacting coregulators.

mHMG-CoAS stimulates transactivation by PPARα specifically via the cognate PPRE of the mHMG-CoAS gene

To define a cellular role for the observed in vitro interaction between mHMG-CoAS and PPARα, we carried out transi-
was measured. The values shown are averages (± SEM) from at least two separate experiments that were carried out in duplicate, and were normalized to the activity of the reporter gene alone, which was set as 1. The inset is a representation of some results at a different scale normalized to the activity of the reporter gene by PPAR-α.

Two separate experiments that were carried out in duplicate, and were normalized to the activity of the reporter gene alone, which was set as 1. The inset is a representation of some results at a different scale normalized to the activity of the reporter gene by PPAR-α.

transfection assays with a reporter gene containing the AOX-PPRE. In this case, prominent nuclear staining of mHMG-CoAS-HA and L393V-HA gave a punctate pattern in each case (Figure 3C). Immunofluorescence of both species with an electrophoretic mobility corresponding to the predicted precursor form (57 kDa) was also present by immunoblot analysis (Figure 3C). A less abundant species with an electrophoretic mobility consistent with mitochondrial localization and, moreover, that this requires the integrity of the LXXLL motif was also present by immunoblot analysis (Figure 3C). A less abundant species with an electrophoretic mobility corresponding to the predicted precursor form (57 kDa) was also present by immunoblot analysis (Figure 3C). A less abundant species with an electrophoretic mobility corresponding to the predicted precursor form (57 kDa) was also present by immunoblot analysis (Figure 3C). A less abundant species with an electrophoretic mobility corresponding to the predicted precursor form (57 kDa) was also present by immunoblot analysis (Figure 3C). A less abundant species with an electrophoretic mobility corresponding to the predicted precursor form (57 kDa) was also present by immunoblot analysis (Figure 3C). A less abundant species with an electrophoretic mobility corresponding to the predicted precursor form (57 kDa) was also present by immunoblot analysis (Figure 3C). A less abundant species with an electrophoretic mobility corresponding to the predicted precursor form (57 kDa) was also present by immunoblot analysis (Figure 3C).

![Graph](https://via.placeholder.com/150)

**Fig. 2.** mHMG-CoAS stimulates transactivation by PPAR-α in vivo. (A) A luciferase reporter gene containing the rat mHMG-CoAS PPRE was cotransfected with expression vectors for PPAR-α (1.5 μg), mHMG-CoAS (1.25 or 2.5 μg) or L393V (1.25 or 2.5 μg), in the absence or presence of Wy-14,643, as indicated, and luciferase activity was measured. The values shown are averages (± SEM) from at least two separate experiments that were carried out in duplicate, and were normalized to the activity of the reporter gene alone, which was set as 1. The inset is a representation of some results at a different scale to show better the induction of the reporter gene by PPAR-α.

(B) Transfections were carried out as above using a reporter gene that contained the AOX-PPRE. In this case, an expression vector for RXR-α (1.5 μg) was included in the transfections.

ent transfection assays with a reporter gene containing the cognate PPRE from the promoter of the rat mHMG-CoAS gene (Rodriguez et al., 1994). As shown in Figure 2A, cotransfection with an expression vector expressing PPAR-α led to 2- to 4-fold increases in reporter gene activity over basal levels in the absence or presence of the PPAR-α activator Wy-14,643, respectively. This is in agreement with previous reports using a reporter gene that contained this PPRE (Rodriguez et al., 1994). Interestingly, coexpression of mHMG-CoAS led to a dramatic and dose-dependent increase in PPAR-α-mediated transactivation, resulting in 200- to 300-fold inductions over basal levels of the reporter gene alone. This mHMG-CoAS-mediated stimulation was dependent on the presence of PPAR-α and a PPRE-containing reporter gene. Stimulation was specific to mHMG-CoAS, as cHMG-CoAS had no effect on PPAR-α activity (Figure 2A). Significantly, the L393V mutant was unable to potentiate PPAR-α activity. Indeed, L393V actually served as a transdominant inhibitor, resulting in the abrogation of activation by PPAR-α.

To determine if the effects of mHMG-CoAS on PPAR-α activity are general, we tested a reporter gene that contained the PPRE from the rat acyl-CoA oxidase (AOX) gene (Tugwood et al., 1992; Zhang et al., 1993). In this case, an expression vector for RXR-α was included in the transfections, since endogenous levels of RXR-α in these cells are insufficient for strong activation from this PPRE (Marcus et al., 1993). As shown in Figure 2B, PPAR-α

**PPAR-α redirects mHMG-CoAS to the nucleus in transiently transfected cells**

For mHMG-CoAS to play a role in PPAR-α function, one would expect it to be present in the nucleus. Subcellular localization studies of human mHMG-CoAS have not been reported; however, the rat mHMG-CoAS has been localized by immunocytochemistry predominantly to mitochondria (Royo et al., 1995; Serra et al., 1996), although the presence of small amounts of mHMG-CoAS in other subcellular compartments has not been excluded. We examined the subcellular localization of mHMG-CoAS by attaching a hemagglutinin epitope tag (HA) to the C-terminus of the protein and carrying out immunofluorescent studies of transiently transfected cells. Addition of an HA tag to the C-terminus did not compromise activity, as mHMG-CoA-HA retained its ability to stimulate PPAR-α-mediated transactivation in vivo (Figure 2B) and to interact with PPAR-α in vitro (data not shown). Both the wild-type and mutant proteins were expressed in vivo and had electrophoretic mobilities consistent with that predicted for the mature 53 kDa protein, as determined by immunoblot analysis (Figure 3C). A less abundant species with an electrophoretic mobility corresponding to the predicted precursor form (57 kDa) was also present in each case (Figure 3C). Immunofluorescence of both mHMG-CoAS-HA and L393V-HA gave a punctate pattern of staining, consistent with mitochondrial localization (Figure 3A, panels 3 and 5, respectively). Nuclear staining over background was not evident, in agreement with previous findings on rat mHMG-CoAS (Serra et al., 1996).

However, when PPAR-α was coexpressed, the cellular distribution of mHMG-CoAS was dramatically altered. In this case, prominent nuclear staining of mHMG-CoAS-HA was observed, in addition to punctate staining (Figure 3A, panel 4; ~80% of transfected cells exhibited nuclear staining). In contrast, coexpression of PPAR-α did not alter the staining pattern of L393V-HA (Figure 3A, panel 6). Our findings suggest that, under certain conditions, PPAR-α is able to redirect mHMG-CoAS to the nucleus and, moreover, that this requires the integrity of the receptor-interacting LXXLL motif.

**Discussion**

Our findings reveal a novel and unexpected pathway of gene regulation in which the product of a PPAR-α target gene, predominantly found in mitochondria where its functions as a key ketogenic enzyme, can also specifically stimulate its own nuclear transcription by serving as a coregulator of PPAR-α. Evidence exists for similar autoregulatory mechanisms with other nuclear hormone receptors. For example, it has been shown recently that the product of the rat hairless gene, whose transcription
is induced by the thyroid hormone receptor, interacts with this receptor and acts as a transcriptional repressor, thereby modulating expression of its own gene (Thompson and Bottcher, 1997). Evidence presented herein indicates that mHMG-CoAS shares a number of properties with well-characterized nuclear hormone receptor coactivators (Glass et al., 1997; Shibata et al., 1997). First, mHMG-CoAS binds to PPARα in vitro and stimulates its activity in vivo. Secondly, mHMG-CoAS contains a nuclear receptor-interacting consensus motif (LXXLL) that is found commonly in many nuclear hormone receptor-interacting cofactors. The integrity of this motif is required for efficient binding to PPARα and stimulation of transactivation. Thirdly, a derivative of mHMG-CoAS, in which this motif is mutated, functions as a transdominant inhibitor of PPARα-mediated transactivation. Fourthly, mHMG-CoAS accumulates in the nucleus in the presence of coexpressed PPARα. Taken together, these findings suggest that mHMG-CoAS is a bona fide coregulatory factor for PPARα. The mechanism by which mHMG-CoA stimulates PPARα activity is not known at present. We have been unable to detect a higher order protein–DNA complex containing PPARα/RXRα/mHMG-CoAS using mobility shift analysis with the mHMG-CoA-PPRE and in vitro synthesized proteins (data not shown). However, this does not preclude the possibility of such a complex forming in vivo, where additional factors or events may be required for its formation and/or stability. The observation that the L393V derivative acts as a transdominant inhibitor of PPARα transactivation is consistent with the possibility that mHMG-CoAS interacts both with PPARα and with
a limiting downstream effector target(s) of PPARα. Similar findings were reported with the receptor coactivator SRC-1, in which mutation of LXXLL motifs resulted in dominant-negative effects, a result attributed to sequestration of downstream effector molecules such as p300/ CBP (Heery et al., 1997). Therefore, mHMG-CoAS may function through the recruitment of other coregulatory factors required for PPARα function. Nuclear hormone receptor cofactors function cooperatively in multimeric transcription complexes, and the recruitment, assembly, composition and function of cofactor complexes are differentially dependent upon the nature of the receptor, the response element and the availability of ligand (Kamei et al., 1996; Chen et al., 1997; DiRienzo et al., 1997; Korzus et al., 1998; Kurokawa et al., 1998). It is interesting to note in this context that mHMG-CoAS-mediated stimulation was observed with the mHMG-CoAS-PPRE but not with the AOx-PPRE, indicating that the nature of the PPRE plays a determining role in the recruitment and/or function of mHMG-CoAS in cells. For instance, structural differences in the PPREs may dictate conformational changes in bound PPAR–RXR heterodimers, which in turn preclude or promote access of cofactors. Indeed, corepressors NCoR and SMRT can access PPARα in solution but not when it is bound on the AOx-PPRE (DiRienzo et al., 1997). Finally, it is interesting to note that mHMG-CoAS is itself acetylated and acts as an acetyltransferase in catalyzing the synthesis of HMG-CoA. Recent findings have established the importance of the acetylation of histones and general transcription factors in nuclear receptor-mediated gene regulation, and many nuclear receptor coactivators contain intrinsic or associated protein acetyltransferase activity (Ogrzyzko et al., 1996; Chen et al., 1997; Imhof et al., 1997). We currently are investigating if the enzymatic activity and/or acetylation of mHMG-CoAS is required for the observed stimulation of PPARα transactivation in vivo.

The observation that mHMG-CoAS accumulates in the nucleus in the presence of PPARα supports a nuclear function for this protein. There are a growing number of examples of proteins that function in more than one subcellular compartment, and various mechanisms have been proposed to explain the differential targeting of proteins to different locations in the cell (Danpure, 1995). The mechanism by which a protein that contains a mitochondrial targeting sequence penetrates the nucleus in the presence of PPARα is unclear at present but appears to involve a direct association between the two proteins, since the subcellular distribution of the L393V mutant derivative was unaltered in the presence of PPARα. Nuclear translocation of mHMG-CoAS does not, however, appear to be due to PPAR-dependent differences in processing of the putative mHMG-CoAS mitochondrial targeting signal, since we observed no significant differences in the abundance and size of the steady-state levels of the processed and mature forms of mHMG-CoAS expressed in transiently transfected cells in the presence or absence of PPARα (Figure 3C). Our data at present do not allow us to distinguish whether the precursor or mature form of mHMG-CoAS is responsible for nuclear stimulation of PPAR transactivation. We are currently addressing this issue since it bears directly on the pathway by which mHMG-CoAS gains access to the nucleus. Nuclear hormone receptor-mediated translocation of receptor-binding proteins from one subcellular compartment to the nucleus is not without precedent. Rat deoxyuridine triphosphatase, which binds to PPARα and antagonizes its activity, is shuttled from the cytosol to the nucleus by PPARα (Chu et al., 1996). Moreover, calreticulin, a calcium-binding protein that resides almost exclusively in the endoplasmic reticulum, has been shown to bind to, and inhibit the transcriptional activity of, several steroid and nuclear hormone receptors (Burns et al., 1994). Consistent with our findings with mHMG-CoAS, it recently has been demonstrated that the glucocorticoid receptor enhances the nuclear accumulation of calreticulin (Roderick et al., 1997).

In summary, we have shown that a mitochondrial protein that participates in an essential metabolic pathway can also be targeted to the nucleus where it functions in transcription, alluding to a potentially important pathway of nuclear–mitochondrial dialogue. This dual capacity suggests that mHMG-CoAS plays a multifunctional role in the cell; however, the physiological significance of the interaction between mHMG-CoAS and PPARα remains to be elucidated. Ketogenesis is crucial for the generation of lipid-derived energy during periods of carbohydrate deprivation and in diseases such as diabetes, and this pathway is subject to multiple levels of regulation, including the rate of delivery and β-oxidation of fatty acids in the liver, and the production and utilization of acetyl-CoA (McGarry and Foster, 1980). The simplest interpretation of our findings is that mHMG-CoAS is involved in a positive feedback loop that serves to amplify its own expression in response to various metabolic and physiological conditions that demand increased ketone body formation. The observation that mHMG-CoAS may also influence the expression of other PPAR-responsive genes, such as acyl-CoA oxidase, the rate-limiting enzyme in the peroxisomal β-oxidation pathway, suggests that mHMG-CoAS may play an integrative role along with PPARα in the maintenance and regulation of metabolic homeostasis.

Materials and methods

Plasmids

Plasmids were manipulated according to standard procedures (Ausubel et al., 1997). We previously isolated a partial cDNA clone of human mHMG-CoAS, encoding amino acids 1–345 with respect to the precursor protein, in a two-hybrid screen for PPARα-interacting factors (Miayata et al., 1996). A full-length, 2 kbp cDNA encoding the complete 508 amino acid human mHMG-CoAS, which includes the putative 37 amino acid mitochondrial targeting sequence (Mascaró et al., 1995), was constructed using a previously isolated 3′ fragment of human mHMG-CoAS cDNA (Boukafane et al., 1994) and cloned into the in vivo and in vitro expression plasmid pSG5 (Promega). The full-length cDNA clone for hamster (Mascaró et al., 1995) was also cloned into pSG5. pGST-RXRα and pGST-PPARα express the respective full-length receptors as GST fusion proteins and were cloned in the vectors pGEX-2T and pGEX-2TK (Pharmacia), respectively. In vivo and in vitro expression vectors for mPPARα, human RXRα and the luciferase reporter gene construct pAoxX2Luc, which contains two copies of the rat AOX-PPRE, have been described (Zhang et al., 1992). pHisGluc is a luciferase reporter gene construct containing a single copy of the rat mHMG-CoAS-PPRE, and was constructed by cloning a synthetic double-stranded oligonucleotide (upper strand: 5′-GATCC-AGACCTTTGGCCCAGTTTTTCTGAGGCAGAGGAGTTTCTGAGGCAGAGGA-3′) corresponding to the published PPRE sequence (Rodriguez et al., 1994) into the BamHI site of pCPSluc (Zhang et al., 1992). The L393V mutant of mHMG-CoA, cloned in pSG5, was created by site-directed
mutagenesis using the oligonucleotide 5′-GTTGCTCTGGCCCTCGGT-CTGTCCTACC-3′ (altered base underlined) and a commercially available kit (Stratagene). Versions of the wild-type mHMG-CoAS and the L393V mutant containing a single in-frame copy of the HA epitope tag at their C-termini were constructed in the mammalian expression vector pCDNA3 (Invitrogen). Briefly, a SacI site was generated by site-directed mutagenesis immediately upstream of the natural termination codon in the mHMG-CoAS cDNA, into which a double-stranded synthetic oligonucleotide encoding the HA epitope was inserted.

**Protein-binding assays**

GST pull-down assays were carried out as previously described (Miyata et al., 1996) using GST–PPARα or GST–RXRα immobilized to glutathione–Sepharose (Pharmacia) and [35S]methionine-labeled mHMG-CoAS, CHMG-CoAS or mutant L393V proteins synthesized in vitro by transcription–translation. Bound proteins were analyzed by SDS–PAGE on 10% gels and quantitated using a Molecular Dynamics Phosphor-Imager. Immunoprecipitations were carried out as described (Fujiki et al., 1984). Briefly, radiolabeled mHMG-CoAS, CHMG-CoAS or mutant L393V proteins were incubated with equivalent amounts of unlabeled PPARα or RXRα synthesized in vitro (normalization was determined in parallel experiments by [35S]methionine incorporation) and incubated with polyclonal antiserum to mouse PPARα or human RXRα, or with pre-immune serum, as appropriate (1:200 dilution). Complexes were recovered on protein A-conjugated agarose beads (Bio-Rad) and analyzed by SDS–PAGE.

**Transfection assays**

Transfection of BSC40 cells by the calcium phosphate method and measurement of luciferase activity were as described (Marcus et al., 1993; Miyata et al., 1993). Briefly, subconfluent BSC40 cells (2×10^5 cells/6 cm dish) were transfected with 1.25 μg of reporter gene plasmid, 1.5 μg of PPARα or RXRα expression plasmid, and various amounts of the different HMG-CoAS expression plasmids, as described in the figure legends. Plasmid DNA was normalized to 5 μg with sonicated salmon sperm DNA, and promoter dosage was kept constant by the addition of appropriate amounts of the corresponding vectors.

**Immunofluorescence**

Immunofluorescence was performed essentially as described (Ausubel et al., 1997). Cos-1 cells, grown on coverslips, were transfected with plasmids expressing HA-tagged proteins (0.5 μg), as above. Cells were fixed at room temperature with 4% paraformaldehyde in phosphate-buffered saline for 10 min, followed by blocking with 2% goat serum/0.2% Triton X-100 for 30 min. Permeabilized cells were incubated with mouse monoclonal anti-HA antibody (12CA5, Boehringer Mannheim, 1:15 dilution) for 1 h at 37°C and stained with Texas red-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Labs, 1:30 dilution). Nuclei were stained by treating cells at –70°C in methanol for 10 min, followed by incubation at 37°C for 4 h with 0.25 mg of Hoechst 33258/ml.

**Immunoblot analysis**

Immunoblot analysis was carried out using standard procedures (Ausubel et al., 1997). Briefly, extracts from Cos-1 cells, transfected as above but using 1.25 μg of the HA-tagged expression plasmids, were prepared using RIPA buffer [10% NP-40, 0.4% deoxycholate, 66 mM EDTA, 10 mM Tris–HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Proteins (75 μg) were resolved by SDS–PAGE on 10% gels, transferred to Hybond nitrocellulose membranes (Amersham), and incubated with mouse anti-HA antibody (1:500 dilution), followed by horseradish peroxidase-conjugated sheep anti-mouse IgG (1:1000 dilution). Proteins were detected by chemiluminescence using a commercially available kit (Amersham).

**Acknowledgements**

We thank G.M. Mitchell, M.S. Brown and J.A. Hassell for generously providing plasmids, and S. McCaw for constructing pGST-RXR. This work was supported by grants from the Medical Research Council (MRC) of Canada and the Heart and Stroke Foundation of Canada. L.M. and K.S.M. are recipients of Ontario Graduate Studentships. J.D.C. is the recipient of an MRC Studentship. R.A.R. is an International Research Scholar of the Howard Hughes Medical Institute and a Senior Scientist of the MRC. J.P.C. is a Senior Scientist of the National Cancer Institute of Canada.

**References**


