Sterol regulatory element-binding proteins (SREBPs) activate promoters for key genes of metabolism to keep pace with the cellular demand for lipids. In each SREBP-regulated promoter, at least one ubiquitous co-regulatory factor that binds to a neighboring recognition site is also required for efficient gene induction. Some of these putative co-regulatory proteins are members of transcription factor families that all bind to the same DNA sequence elements in vitro and are often expressed in the same cells. These two observations have made it difficult to assign specific and redundant functions to the unique members of a specific gene family.

We have used the chromatin immunoprecipitation (ChIP) technique coupled with a transient complementation assay in Drosophila SL2 cells to directly compare the ability of two members of the CREB/ATF family to function as co-regulatory proteins for SREBP-dependent activation of the HMG-CoA reductase promoter. Results from both of these experimental systems demonstrate that CREB is an efficient SREBP co-regulator but ATF-2 is not.

The sterol regulatory element-binding proteins (SREBPs)1 are key metabolically regulated transcription factors. They are translated as large precursors, inserted into the membrane of the endoplasmic reticulum, and their amino-terminal domains are released into the cytosol when the cellular lipid level falls. The signaling pathway that results in SREBP release is not completely understood but requires two sequential proteolytic events, the first of which is actively regulated by sterols and once it is released from the membrane it enters the nucleus to increase expression of various genes that are important for cellular lipid homeostasis (2, 3).

SREBPs are weak activators of transcription by themselves, and they require co-regulatory transcription factors that bind nearby DNA sequences to efficiently stimulate gene expression (3). The identity and combinations of co-regulatory factors and the number and arrangements of the SREBP sites are promoter-specific. These differences likely provide a framework for gene-specific regulatory responses to the different SREBP isoforms and to specific cellular regulatory cues. For example, in the promoter for the low density lipoprotein (LDL) receptor, there is a single SREBP site, and Sp1 is the lone SREBP co-regulatory factor and binds to two separate sites (4). In the promoters for 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, farnesyl diphosphate (FPP) synthase, and squalene synthase there are multiple SREBP sites, and at least one of the co-regulators is the CCAAT-binding factor/nuclear factor-Y (CBF/NF-Y) (5–7). HMG-CoA synthase additionally requires a member of the CREB/ATF family (8).

In previous studies, we have shown separate DNA sites that bind CBF/NF-Y, and members of the CREB/ATF family are both required for expression from the HMG-CoA reductase promoter (9). CREB sites were originally identified as cis-acting elements that confer transcriptional regulation in response to elevated CAMP levels (10–12). In the somatostatin promoter it was shown that cAMP responsiveness was mediated through the basic-leucine zipper containing the transcription factor termed cAMP response element-binding protein or CREB (13). Protein kinase A phosphorylates CREB in response to increased cellular cAMP, which allows it to interact efficiently with the transcriptional co-activator protein called CREB-binding protein (CBP) to stimulate transcription of cAMP target genes (14, 15). Several related CRE-binding proteins have been identified and cloned (16), 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 (17). Major questions concerning this and any other related families of genes should determine how much overlap there is in function and identify specific physiological roles for the individual proteins.

In addition to mutagenesis studies that showed there is a CRE-like element in the HMG-CoA reductase promoter, we have used the chromatin immunoprecipitation technique (ChIP) to demonstrate that both CREB and CBF/NF-Y are both recruited to the HMG-CoA reductase promoter by SREBP when cells are deprived of exogenous cholesterol (18). Taken
together, our previous studies (9, 18) indicate that both CBF/NF-Y and CREB are important SREBP co-regulators for HMG-CoA reductase (9, 18). However, because CREB is a member of the CREB/ATF family, it was important to determine whether individual members of this family can substitute for CREB in the sterol regulatory response mediated by SREBP. In the current report we utilize the ChiP method to provide evidence that although CREB is recruited to the HMG-CoA reductase promoter efficiently by SREBP activation, binding of another member of the family, ATF-2, is not altered. We also provide evidence from direct promoter activation studies in Drosophila SL2 cells that CREB is an efficient SREBP co-regulator and efficiently stimulates the HMG-CoA reductase promoter along with SREBP NF-Y. In contrast, ATF-2 is unable to substitute for CREB in this independent assay system as well.

MATERIALS AND METHODS

Cells and Media—The CHO-7 and SL2 cell lines were cultured as described previously (4, 18). Lipoprotein-deficient serum was prepared by ultracentrifugation of newborn bovine serum as described previously (19). Cholesterol and 25-OH cholesterol were obtained from Steraloids Inc., and stock solutions were dissolved in absolute ethanol.

Cell Culture—Stock flasks of CHO-7 cells (20) were grown in a 50/50 mixture of Hams F12 and Dulbecco’s modified essential medium (DMEM) (Irvin Scientific) containing 10% (v/v) fetal bovine serum at 37°C and 5% CO2. Tissue culture dishes (15 cm) were plated at 500,000 cells/dish on day 0 in the above medium. On day 1, the dishes were rinsed twice with 1x phosphate buffered saline, and half of the dishes were fed with either induced media (HamsF12/DMEM containing lipoprotein-depleted serum instead of FBS) or suppressed media (Hams F12/DMEM containing lipoprotein-depleted serum with 10 μg/ml cholesterol and 1 μg/ml of 25-OH cholesterol). Cells were processed for the ChiP procedure after an additional 24 h incubation.

Chromatin Immunoprecipitation Assay—We used a modification of the slot gel electrophoresis method and conditions (22) as described previously (18). Dishes of CHO-7 cells were placed in a fume hood and treated with formaldehyde (final concentration of 1% v/v) followed by a room temperature incubation for 8 min. The reaction was quenched by the addition of glycine (final concentration of 125 mM) and the dishes were incubated for an additional 5 min at room temperature. Medium was removed followed by 3 rinses with cold 1x PBS. Samples were then subjected to the protocol described in our previous report (18). The CREB and ATF-2 antibodies were from Santa Cruz Biotechnology (sc-186 and sc-187, respectively). After immunoprecipitation, DNA was extracted, and samples were ultimately resuspended in 50 μl of sterile H2O and 2–4 μl were used in each polymerase chain reaction (PCR).

Standard PCR reactions for hamster HMG-CoA reductase promoter were performed with 32P-kinased oligonucleotides and AmpliTaq Gold (PerkinElmer Life Sciences). The primers for HMG-CoA reductase were designed to hybridize and amplify a ~230-bp product encompassing the region displayed in Fig. 1A. To provide reactions that were in the linear dose response for the individual samples, we performed test PCR reactions and varied the number of cycles to obtain conditions where the signal intensity was linear with respect to amount of input as described previously (18).

Transient Transfection Assay in Drosophila SL2 Cells—Drosophila SL2 cells were cultured in Sheds and Sang insect medium (Sigma) containing 10% heat inactivated fetal bovine serum and were seeded at 480,000 cells/well in six-well dishes on day 0. On day 1, cells were transfected by the calcium phosphate co-precipitation method with each dish receiving 2 μg of each plasmid (HamsF12/DMEM containing lipoprotein-depleted serum instead of FBS) or suppressed media (Hams F12/DMEM containing lipoprotein-depleted serum with 10 μg/ml cholesterol and 1 μg/ml of 25-OH cholesterol). Cells were processed for the ChiP procedure after an additional 24 h incubation.

RESULTS

CREB is a member of a transcription factor family where individual proteins are all highly similar in their basic and leucine zipper DNA binding/dimerization domains. Even though they all bind the same cis-acting consensus sequence, the affinities of the different homo- and heterodimeric combinations vary for different CRE elements (23, 24). Additionally, they do not all respond identically to cellular signaling pathways. For example, ATF-2 activates transcription along with the adenovirus E1a protein (25), whereas both SREBP-1a (amino acids 1–490) was incubated with purified GST-CREB or GST-ATF-2, and the mixtures were bound to glutathione agarose beads that were subsequently washed and analyzed for specifically bound material by an immunoblotting protocol as described (8).

expression levels for CREB and ATF-2 proteins were normalized using the common HSV epitope for comparison. Briefly, transfection experiments were performed as above with differing amounts of the HSV-CREB and HSV-ATF-2 vectors and a constant amount of the pPAC β-galactosidase control plasmid. Protein extracts from the transfected cells were first normalized for transfection efficiency by measuring the β-galactosidase activity of individual extracts, and normalized amounts were analyzed as indicated. No primary antibody was used for the reactions in lanes 5–6.

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FIG. 2. Immunoblot characterization of chromatin extracts. Equivalent amounts (A_{equiv}) of chromatin extracts from CHO-7 cells cultured in the absence (I) or presence (S) of cholesterol and 25-OH cholesterol were processed for immunoblotting using the indicated antibodies. The chromatin samples before immunoprecipitation (input) were analyzed for SREBP-2, CREB, and ATF-2 (lanes 1–2 of each panel). The chromatin was subjected to immunoprecipitation with an antibody to ATF-2, and the material remaining in the supernatant (panel C, Sup., lanes 3–4) and equal aliquots of the total immunoprecipitation pellets from both samples (panel C, IP, lanes 5–6) were analyzed. The migration positions for the precursor (P) and mature (M) forms of SREBP-2 are indicated in A. The migration positions for CREB and ATF-2 are indicated by arrows at the right in panels B and C, respectively. The dark staining band lower in the gel in C, lanes 5 and 6, corresponds to immunoreaction with a subunit of the antibody used for the immunoprecipitation reaction.

received LPDS with cholesterol and 25-OH cholesterol added back to keep SREBP s tethered to the endoplasmic reticulum membrane and sequestered away from their target genes.

The chromatin was then processed by our standard ChIP protocol followed by a PCR reaction with primers that amplify the HMG-CoA reductase promoter region encompassing the CRE site (Fig. 1A). As a control, we showed there were equivalent levels of HMG-CoA reductase promoter DNA in the starting chromatin samples (Fig. 1B, lanes 1 and 2). When equal amounts of chromatin from the two sets of dishes were incubated with an antibody against ATF-2 prior to the immunoprecipitation and PCR reaction, there were also equal levels of HMG-CoA reductase promoter DNA present in both samples (lanes 3 and 4). However, when the CREB antibody was used there was a significantly higher level of HMG-CoA reductase promoter DNA present in the sample prepared from cells cultured under sterol depleted conditions versus the sterol treated set (lanes 7 and 8).

An immunoblotting analysis demonstrated that the mature SREBP-2 transcription factor was properly regulated by the sterol depletion protocol (Fig. 2A). Additional immunoblotting experiments presented in Fig. 2, B and C, demonstrated that equal amounts of protein for both CREB and ATF-2 were present in the starting chromatin preparations (lanes 1 and 2 of Fig. 2, B and C). Also, the ATF-2 protein was quantitatively removed, and equal amounts were recovered by the immunoprecipitation protocol (Fig. 2C, compare lanes 3–6). We could not perform an immunoblot to determine whether CREB was not quantitatively precipitated because the CREB protein migrates too close to an immunoglobulin protein subunit from the immunoprecipitation reaction, which reacts with the secondary antibody and obscures the CREB band on the resulting gel.

These ChIP results along with the experiments from our previous study strongly suggest that CREB is an efficient coregulatory factor for SREBP in the HMG-CoA reductase promoter and that the ATF-2 protein does not participate in this response. To evaluate whether there is a difference in the ability of CREB and ATF-2 to directly stimulate transcription from the HMG-CoA reductase promoter, we used the transfection-complementation system in Drosophila SL2 cells that we have used extensively in previous reports. These cells do not express functional equivalents of several mammalian transcriptional regulatory proteins, including Sp1 (27). However, expression from mammalian promoters can be evaluated when expression plasmids for a critical missing regulatory protein(s) are included in the transfection protocol. Therefore, the SL2 transfection assay provides a background for evaluating mammalian promoters and their missing trans-acting regulatory proteins in an intact cell system (27). In fact, we have used SL2 cells to demonstrate that SREBP activation of the HMG-CoA synthase promoter requires both NF-Y and CREB (8).

When we transfected SL2 cells with the HMG-CoA reductase promoter reporter construct alone or with an SREBP expression construct, a low level of promoter activity was observed (Fig. 3, filled triangle at abscissa origin). This is consistent with previous studies (4), indicating that SREBP is a very weak activator by itself. When increasing amounts of either ATF or CREB expression vectors were included in addition to SREBP, a similar low level of activation was still observed (Fig. 3, open symbols). When expression constructs for the three subunits of NF-Y were included along with the SREBP expression construct the promoter was activated about 7-fold. When the CREB or ATF-2 constructs were included on top of the NF-Y plasmids, a robust activation was observed for CREB (Fig. 3, filled squares) but ATF-2 failed to induce expression above the level achieved by SREBP and NF-Y alone (Fig. 3, filled circles). When SREBP was omitted from the transfection experiment, there was no activation by NF-Y and CREB alone.2

To evaluate whether the low activation mediated by ATF-2 could be explained by a lower level of protein accumulation

2 T. Ngo and T. F. Osborne, unpublished data.
relative to CREB, we evaluated expression of the two proteins after transfection into SL2 cells. We had inserted the coding sequence for an HSV glycoprotein D epitope at the extreme amino terminus of the two expression vectors so that we could compare protein expression levels using the same antibody. When protein extracts from the transfected SL2 cells were analyzed, both CREB and ATF-2 were expressed at similar levels (Fig. 4).

Taken together with the results from the ChIP experiments of Fig. 1, these transfection results provide strong support for the conclusion that CREB is recruited to the native HMG-CoA reductase promoter and efficiently activates the isolated promoter in SL2 cells, and both are dependent on SREBP. However, ATF-2 is neither recruited to the native HMG-CoA reductase promoter by SREBP nor is it an efficient co-regulator for SREBP activation of the cloned promoter in the SL2 cell system.

In previous studies (8), we showed that CREB interacts with SREBP in solution and this interaction is likely part of the mechanism for the synergistic activation of transcription of the HMG-CoA synthase promoter by these two proteins (8). To evaluate whether ATF-2 was also capable of interacting with SREBP we compared the ability of GST fusion proteins of CREB and ATF-2 to bind to SREBP in solution (Fig. 5). The results demonstrate that under conditions where CREB binds SREBP efficiently, ATF-2 binding was minimal (compare lanes 3 and 4). Thus, the lack of efficient interaction between SREBP and ATF-2 is likely part of the reason why it is not recruited to the HMG-CoA reductase promoter by SREBP activation.

**DISCUSSION**

All CREB/ATF family proteins are highly similar in their basic and leucine zipper regions, and they bind the same cis-acting consensus sequence. Therefore, it is possible that individual members can have both overlapping and unique roles in specific and diverse biological processes. In the current studies, we tested the ability of ATF-2 to substitute for CREB in activation of the HMG-CoA reductase promoter by SREBPs. Using antibodies to each protein in chromatin immunoprecipitation studies, we showed that SREBP activation by sterol depletion resulted in efficient recruitment of CREB to the HMG-CoA reductase promoter, but ATF-2 binding was unaltered by this nutritional challenge. We did detect the HMG-CoA reductase promoter DNA in the chromatin samples that were precipitated with the ATF-2 antibody; however, the level was unaltered by the sterol manipulation protocol. These results indicate that ATF-2 may bind and activate the HMG-CoA reductase promoter at basal levels, but it is not recruited to the promoter by SREBP, and thus it cannot substitute for CREB as an important SREBP co-regulatory protein.

The result of our co-transfection studies in Drosophila SL2 cells supports and significantly extends this conclusion as well. The data in Fig. 3 shows that SREBP activates the HMG-CoA reductase promoter in cooperation with NF-Y and CREB, but when expressed at similar levels, ATF-2 cannot substitute for CREB.

Because CREB and ATF-2 bind to the same DNA sequence in vitro, it was important to investigate the mechanism for the differential recruitment of CREB to the HMG-CoA reductase promoter measured in the chromatin immunoprecipitation analysis. In Fig. 5, we show that CREB but not ATF-2 was capable of interacting with SREBP in solution in the absence of DNA. Thus, consistent results from three separate experimental approaches support our conclusions and provide at least a partial mechanistic understanding for the selectivity. It was previously shown (28) that the somatostatin promoter is stimulated in a cell type-specific manner by cAMP through the action of CREB. Importantly, these authors showed that ATF-2 was also unable to substitute for CREB in this response.

Our earlier mutational studies of the HMG-CoA reductase promoter showed that both the CRE and NF-Y sites were simultaneously required for normal sterol-dependent regulation (9). The data from Fig. 3 are also consistent with this conclusion because both CREB and NF-Y were required along with SREBP for efficient activation. These observations are similar to our previous findings for the HMG-CoA synthase promoter where both CREB and CBF/NF-Y were required for efficient activation by SREBP. Thus, two early genes that control simple carbon flux into the cholesterol/isoprenoid biosynthetic pathway require a similar set of SREBP co-regulatory proteins. This provides a molecular strategy to ensure the common early steps of the multivalent cholesterol/isoprenoid pathway are tightly co-regulated (29).

The chromatin immunoprecipitation method is a useful procedure for analyzing changes in the binding of specific regulatory proteins to their putative target elements in native chromatin in response to change in the intracellular environment. With the availability of antibodies with suitable specificity, ChIP can be used to effectively analyze the functional roles of highly similar proteins or even differentially modified versions of the same transcriptional regulatory protein that bind to very similar or identical DNA sites in vitro.

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