Identification of an Inverted CCAAT Box Motif in the Fatty-acid Synthase Gene as an Essential Element for Mediation of Transcriptional Regulation by cAMP*

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The antagonistic effect of cAMP on the insulin-induced expression of fatty acid synthase (FAS) in liver could be mimicked in vitro using H4IIE hepatoma cells, both by measuring the response of the endogenous FAS gene and by assaying expression of transfected reporter genes containing promoter elements of the FAS gene. 5′-Deletion analysis and replacement mutagenesis revealed that an essential element required for cAMP antagonism of the insulin effect is an inverted CCAAT box located between nucleotides −99 and −92. DNase I footprinting and gel shift analysis revealed that this region can bind a protein present in nuclei of liver and spleen, organs that express high and undetectable levels of FAS, respectively. This protein is not a CCAAT/enhancer-binding protein, C/EBP. Thus, the FAS gene appears unusual in that the sequence element required for transcriptional regulation by cAMP is neither a cAMP response element (CRE) nor a binding site for AP-1, AP-2, or C/EBP. These results suggest that essential to the regulation of FAS transcription by cAMP is the interaction of an inverted CCAAT box motif with a constitutively produced trans-acting factor that either itself undergoes modification in response to cAMP or associates with a protein that is produced or modified by cAMP exposure.

It has long been recognized that glycogen metabolism, gluconeogenesis, glycolysis, fatty acid oxidation, and synthesis are all coordinately regulated in the liver by short-term regulatory mechanisms involving primarily allosteric and covalent modulation of key enzymes (1). Critical to this short-term regulation are the opposing actions of insulin and glucagon. Glucagon, via its intracellular messenger, cAMP, activates protein kinases involved in the phosphorylation of key enzymes and transforms the liver from a glycogenic, glycolytic, lipogenic tissue to a glycogenolytic, gluconeogenic, and fatty acid-oxidizing tissue. Initially, when the food supply is stopped, serum insulin concentration falls and glucagon concentration rises, triggering this short-term regulatory mechanism. On refeeding, insulin concentration rises, glucagon falls, and these changes are reversed, and the liver reverts to its role as a glycogenic, glycolytic, lipogenic tissue. It seems likely that antagonism between insulin and glucagon may also serve in long-term regulation of these pathways by controlling transcription of key genes (2).

For example, on prolonged fasting, cAMP may eventually initiate the down-regulation of transcription of lipogenic enzymes. Indeed, it has been shown that injection of dibutyryl cAMP will prevent the activation of FAS1 transcription in the liver that normally accompanies refeeding (3).

All of the long-term changes in lipogenesis that occur in response to dietary, hormonal, and developmental cues appear to be accompanied by changes in the rate of transcription of the FAS gene in a tissue-specific manner. Elucidation of the sequence of the entire rat FAS gene, including 6.1 kilobases of the 5′-flanking region (4, 5), has made possible detailed exploration of the mechanisms regulating transcription of this gene, and, recently, sequences required for mediating the effect of insulin have been identified (6, 7). The objective of this study was to identify sequences in the FAS gene that are essential for mediation of the cAMP effect on transcription. Because cAMP has been reported to antagonize the insulin effect on transcription of the FAS gene in hepatocytes, we identified and utilized a hepatoma cell line that appears to be a good model system for this physiological process. Our strategy was to utilize 5′-deletion mutagenesis and replacement mutagenesis of chimeric FAS-reporter genes to narrow down the location of the sequences required for cAMP antagonism of insulin action and to look for trans-acting nuclear factors that might bind to the putative response element. The results of this study are presented in the following report.

EXPERIMENTAL PROCEDURES

Materials—Bromoadenosine 3′,5′-cyclic monophosphate, 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate, insulin, theophylline, poly(dI–dC), and N-dimethyl sulfoxide were purchased from Sigma. Restriction enzymes were obtained from New England Biolabs or Boehringer Mannheim. α-thio-[dichloroacetyl-1-14C]chloramphenicol (50 Ci/mol) and [γ-32P]ATP (3000 Ci/mmol) were purchased from Amer sham Corp. Cell culture media, fetal bovine serum, bovine calf serum, penicillin, and trypsin were obtained from the tissue culture facility at the University of California, San Francisco. An oligonucleotide corresponding to FAS nt −106 to −85 (5′-GAGCAGCTCATTGGCCTGGGCGG-3′) was synthesized by Life Technologies, Inc. Polyclonal rabbit antibodies raised against the carboxyl-terminal region of rat C/EBPα (residues 253–265), the double-stranded consensus oligonucleotides for C/EBP and (5′-TGCGAGCTTGCACTCTCAATG-3′) CREB (5′-AGAGCTGCCAGCTCAGAGCTGAGG-3′), and purified recombinant CREB-1 (contains the DNA binding and dimerization domains of human CREB, residues 212–254) were purchased from Santa Cruz Biotechnology, Inc. The bacterial expression vector, pET-15b-C/EBPα, was obtained from Dr. Per Flodby at the Karolinska Institute, Huddinge, Sweden and used to produce extracts of Escherichia coli containing soluble C/EBPα.

Cell Cultures—Rat H4IIE hepatoma cells, purchased from the Tissue

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‡ The abbreviations used are: FAS, fatty-acid synthase; PBS, phosphate-buffered saline, pH 7.4 (in g/liter: NaCl, 8.0; KH2PO4, 0.2; Na2HPO4, 2.9; KCl, 0.2); CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT enhancer-binding protein; CRE, cAMP-responsive element; TK, thymidine kinase; nt, nucleotide(s).
Culture Facility at the University of California, San Francisco, were cultured in minimum essential medium containing Earle’s balanced salt solution and non-essential amino acids supplemented with 10% fetal bovine serum and 10% bovine calf serum. Cells were grown at 37°C under an atmosphere consisting of 5% CO₂, 95% air. Prior to hormonal treatment, cells at 60–70% confluence were maintained in serum-free media for 48 h. Cells were then supplemented with 10 μM fluoride (1 mM KCl, 1 mM sodium dithiothreitol, 0.5 mM EDTA, and 5% fetal bovine serum). Cells were then washed three times with PBS and lysed by three cycles of freezing and thawing. Cell debris was removed by centrifugation, and the supernatant was retained for analysis.

The transient transfections—H4IIE cells were grown on 60-mm² dishes to 60–70% confluency and transfected with 10 μg of plasmid DNA per plate by the calcium phosphate precipitation method (8). To permit normalization for variable transfection efficiency, 2.5–5 μg of a β-galactosidase expression plasmid, pHCl10 (Pharmacia LKB, Uppsala, Sweden) was co-transfected together with the DNA of interest. The calcium phosphate/DNA co-precipitate was added to the cells, and transfection was allowed to proceed for 20 h. In some early experiments, 100 μM chloroquine was included in the medium to increase transfection efficiency; in these experiments, exposure of the cells was reduced to 6 h (9). At the end of the transfection period, the medium was removed and the cells were shocked for 3 min with 20% dimethyl sulfoxide in PBS. Cells were then washed three times with PBS and were placed into the feeding medium.

For hormonal treatments, the transfected cells were maintained in the serum-supplemented media for 16 h, then switched to media containing 0.5% serum for at least 12 h prior to treatment with 20 μM insulin and/or 1 mM cAMP for 48 h. Cells were harvested, re-suspended in 0.2 ml of 0.25 M Tris-HCl buffer (pH 7.8), lysed by freezethawing, and soluble extract was prepared for β-galactosidase assay. The remainder of the supernatant was heated at 65°C for 10 min to destroy endogenous esterases. Heat-denatured proteins were removed by centrifugation, and portions of the soluble extract were assayed for CAT activity.

Enzyme Assays—FAS protein concentration was determined by an immunoprecipitation procedure using a rabbit anti-rat (FAS) IgG preparation as the primary antibody (10) and authentic rat FAS as standard; all assays were performed in duplicate using at least two different concentrations of cytostol. CAT activity was measured in a radiochemical assay. Reaction mixtures contained 0.25 μM Tris-HCl buffer (pH 7.8), 12 μM [14C]chloramphenicol and 1 μM acetyl-CoA in a final volume of 100 μL. The reaction mixture was incubated at 37°C for 1–2 h and then placed with 250 μL of ethyl acetate solvent was removed) and the organic phase in vacuo, and the reaction products were analyzed by thin layer chromatography. Product formation was quantitated either by direct phosphorimaging (Molecular Dynamics PhosphorImager SF 750) or by liquid scintillation counting.

Preparation of Nuclear Extracts—Nuclear extract from H4IIE hepatoma cells was prepared essentially as described by Dignam et al. (12) except that the protease inhibitors leupeptin (1 μg/mL) and pepstatin (1 μg/mL) were used in all buffers in addition to phenylmethylsulfonfluoride (1 mM). Male rats of the Long-Evans strain were obtained from Simonsen Inc. (Gilroy, CA) and either fasted for 48 h or fasted and refed a fat-free, high carbohydrate diet (“Fat Free Test Diet,” ICN Biomedical, Aurora, OH) and either fasted for 48 h or fasted and refed (where +1 is the start of transcription) cloned as a PsiI-BamHI fragment into the PsiI-XbaI sites of the pCAT-Basic plasmid (Promega). The second plasmid contained FAS sequences from −450 to +68 cloned in pCAT-Basic as a Sau3AI fragment. The resulting deletion constructs were sized by agarose gel electrophoresis, and the end points of the deletions were determined by nucleotide sequencing of the junction fragments. Subsequently, the plasmids ending at −656 (generated from the first series of deletions) were subcloned such that nucleotide −68 of the FAS sequence was positioned at the FAS-CAT junction of all chimeric genes.

Construction of Plasmid −249 + 68−D4−FAS-CAT—A plasmid carrying FAS sequences from −249 to +1272 cloned in pCAT-Basic (Promega) served as the substrate for the deletion construction as follows. The plasmid was digested with HindIII (cleaves the vector just upstream of −249) and KpnI (cleaves at FAS +400), the resulting −760 bp fragment was isolated and subsequently digested with AluI (cleavage site at FAS −75). At the same time, another plasmid containing FAS sequences −150 to +68 in the same vector was digested with SacI (cleaves at FAS −35). The site was made blunt-ended by treatment with mun glycan nucleotidase, and the plasmid was then cut with HindIII. The large fragment (−4600 base pairs) was ligated to the AluI digestion products of the first plasmid resulting in plasmid −249/+68 D−75−−−35.

Construction of the FAS-CAT Fusion Plasmid Carrying a Mutation in the Inverted CCAAT Box—Nucleotides −99 to −92 (CATTGGCC) were changed to GTTCCAGGG in the FAS-CAT fusion plasmids by in vitro site-directed mutagenesis (15) using the primer 5′-GCCAGGC- CCUCATGGTTCGAGGCGC-3′ and 5′-GCCAGGC-CCUCATGGTTCGAGGCGC-3′. The orientation of the FAS insert was determined by DNA nucleotide sequencing.

RESULTS

Characterization of a Hormonally Sensitive Hepatoma Cell Line as a Hepatocyte Model for Studying Transcriptional Regulation of the FAS Gene—Our first priority was to identify a cell line that would exhibit the modulated transcription of the endogenous FAS gene in response to insulin and cAMP that is typical of normal hepatocytes. We chose a rat hepatoma cell line, H4IIE, that previously has been used effectively for studying the regulation of phosphoenolpyruvate carboxykinase transcription by cAMP and insulin (16, 17). These cells normally are cultured in the presence of 20% serum, and, under these conditions, the induction of either insulin or cAMP to the medium had little effect on the content of endogenous FAS (Table I). However, when the cells were preconditioned to growth in a medium containing only 0.5% serum, addition of 20 μM insulin resulted in a substantial increase in the amount of FAS protein. Thus, the insulin stimulus effect observed in the presence of 20% serum likely resulted from the masking of the response by an unidentified agent(s) present in the serum. The maximum response to insulin, amounting to a 2.6-fold increase in FAS protein, was observed after 48 h. This response time is
similar to that observed in vivo in the livers of rats that are fed a high carbohydrate diet following a period of fasting (18).

Cyclic AMP had no effect on the amount of endogenous FAS present in cells grown in medium containing either 20% or 0.5% serum. Nevertheless, in the presence of cAMP, insulin failed to induce an increase in the endogenous FAS content of H4IIE cells (Table I). Again, this result is similar to that observed in vivo, since administration of dibutyryl cAMP has failed to induce an increase in FAS expression in the liver that is normally seen following fasting (19). The FAS content of H4IIE cells (−0.7% of the cytosolic protein, by weight) cultured in the presence of insulin (Table I) is considerably less than that of normal liver (−6% of the cytosolic protein, by weight) derived from fasted rats that have been refed a high carbohydrate diet (19). Nevertheless, the observation that H4IIE cells modulate their FAS content in response to insulin and cAMP in a manner similar to that exhibited by normal hepatocytes encouraged us to adopt this cell line as a model system for identifying hormone response elements within the FAS gene.

Preliminary Localization of the cAMP Response Element by 5′ Deletion Analysis—Our objective was to identify sequences in the 5′-flanking region of the rat FAS that confer cAMP responsiveness to transcription of the gene. To this end, we engineered a series of plasmids each containing a promoterless CAT gene placed under the transcriptional control of different upstream sequences from the FAS gene (see "Experimental Procedures"). All of these constructs have a common 3′ end at +68 (where +1 is the transcriptional start site for FAS), but they carry sequences of different lengths, up to 1604 nucleotides, derived from the 5′-flanking region of the FAS gene. These plasmids were transfected into H4IIE cells, and their ability to confer hormonally modulated CAT expression was assayed in the presence of 0.5% serum. Expression of CAT was amplified from 2.6- to 4.1-fold in response to insulin with all of the chimeric genes except that containing only 62 nucleotides of upstream sequence from the FAS gene (Fig. 1); this observation was consistent with the finding by Moustaı¨d et al. (6) that the insulin response element is located between nucleotides −68 and −52.

The addition of cAMP alone to H4IIE cells transfected with various chimeric reporter genes and cultured in the presence of 0.5% serum had little effect on CAT expression. However, in the presence of cAMP, the ability of insulin to stimulate CAT expression was compromised. This muting of the insulin effect by cAMP was evident with all deletion constructs that exhibited an insulin effect (Fig. 1), suggesting that the sequence element responsible for sensitivity to cAMP is located between nucleotides −149 and +68 of the FAS gene. Similar results were obtained using two different cAMP analogs, 8-bromoadenosine 3′,5′-cyclic monophosphate and 8-(4-chlorophenylthio)-adenosine 3′,5′-cyclic monophosphate.

Evaluation of Candidate cAMP Response Elements within the −149 and +68 Region of the Gene—The consensus cAMP-responsive element (CRE) sequence, a well conserved 8-base pair palindrome TGACGTC (20) is absent from the FAS promoter region. The promoter region does not contain the consensus sequence for AP-1 (21), another well characterized factor that has been implicated as a mediator of the cAMP effect on transcription of some genes. Within the proximal promoter region of the FAS gene, at position −257/−250 is a stretch of eight nucleotides that perfectly matches the consensus AP-2 binding site CC(G/C)CC(A/G)GCG (22). This factor is believed to be involved in the cAMP-modulated transcription of a number of genes, including acetyl-CoA carboxylase (23). However, since this element is absent from the −249/+68 and −149/+68 FAS-CAT chimeric reporter genes that exhibit sensitivity to cAMP (Fig. 1) and is not footprinted by nuclear extracts from H4IIE cells (details not shown), it is unlikely to be required for tran-

### Table I

Effect of insulin and cAMP on the amount of endogenous FAS protein present in cultured H4IIE cells

| Treatment          | Serum-free medium | With serum |
|--------------------|-------------------|------------|
|                    | 24 h  | 48 h  | 72 h  | 24 h  | 48 h  | 72 h  |
| None               | 2.8 ± 0.2 | 2.8 ± 0.1 | 2.8 ± 0.7 | 2.2 ± 0.3 | 2.8 ± 0.8 | 2.5 ± 0.6 |
| Insulin            | 3.8 ± 0.4 | 7.4 ± 0.6 | 5.5 ± 0.3 | 2.6 ± 0.1 | 3.9 ± 0.1 | 3.6 ± 0.6 |
| cAMP               | 3.5 ± 0.3 | 3.0 ± 0.4 | 2.5 ± 0.3 | 2.7 ± 0.3 | 2.7 ± 0.1 | 2.2 ± 0.1 |
| Insulin and cAMP   | ND*   | 2.3 ± 0.2 | ND    | ND    | ND    | ND    |

*ND, not determined.

FIG. 1. Effect of insulin and cAMP on transcription of the CAT reporter gene driven by the promoter and various flanking sequences from the FAS gene in H4IIE cells. H4IIE cells were co-transfected with a β-galactosidase expression plasmid and various chimeric FAS-CAT constructs containing different lengths of FAS upstream sequences. Cells were incubated for 48 h with no hormones, with 20 munits insulin, with 1 mM cAMP, or with both cAMP and insulin. The fold increase in CAT expression induced by hormone treatment was normalized to β-galactosidase expression. Full details are presented under "Experimental Procedures." ND, not determined. The data represent the means ± S.D. of 4 to 6 experiments. The range of observed CAT activities corresponded to acetylation of 1 to 6% of the chloramphenicol substrate per h.
cAMP Regulation of FAS Gene Expression

The first footprinted region, which contains an inverted CCAAT box motif at nt −99/−92, was evaluated by replacement mutagenesis in the context of the −249/+68 FAS-CAT construct. Although mutation of the inverted CCAAT box had no effect on the ability of the chimeric gene to direct the insulin-sensitive expression of CAT, responsiveness to cAMP was completely abolished (Fig. 3). The results of this experiment indicated that the insulin and cAMP effects on transcription of the FAS gene are mediated by different regions of the promoter and strongly implicated the inverted CCAAT box in the regulation by cAMP. To verify that the inverted CCAAT box motif specifically was responsible for the nuclear protein binding observed in the DNase I protection assay, we employed the electrophoretic mobility shift assay using a radiolabeled probe corresponding to nucleotides −106 to −85 (Fig. 4). Liver nuclear extracts from fasted and fasted/refed rats and spleen nuclear extracts from fasted/refed rats retarded the mobility of the probe to the same extent (slow migrating band, lanes 2, 8, and 5, respectively). Formation of these radiolabeled DNA-protein complexes was abolished in the presence of 100-fold molar excess of an unlabeled oligonucleotide corresponding exactly to nucleotides −106/−85 (lanes 4, 10, and 7), but not when the sequence between −99/−92 of the unlabeled probe was mutated (lanes 3, 9, and 6). When a radiolabeled −106/−85 probe containing the same mutated sequence between −92/−99 was used in the electrophoretic mobility shift assay in the presence of nuclear extracts from either liver or spleen, DNA-protein interactions were not observed (lanes 12–14). Faster migrating radiolabeled DNA-protein complexes were also formed with nuclear extracts from liver and spleen. However, formation of these radiolabeled complexes was reduced in the presence of unlabeled oligonucleotides containing either the intact or the mutated inverted CCAAT box sequence (lanes 2 and 3, 6 and 7, 9 and 10), and these complexes were formed from the radiolabeled mutated oligonucleotide probe (lanes 12–14). It would appear therefore that formation of these fast migrating complexes is not specifically dependent on the presence of the inverted CCAAT box sequence. However, from the properties of the slow migrating DNA-protein complex, one could conclude unequivocally that rat liver and spleen contain nuclear protein(s) that specifically interact with the inverted CCAAT box located at nt −99/−92 of FAS promoter.

The possibility that the trans-acting factor binding to the inverted CCAAT box might be a member of the well character-
cAMP Regulation of FAS Gene Expression

The inverted CCAAT box motif is quite dissimilar from the consensus cAMP-responsive element (CRE), TGACCTCA, and an unlabeled oligonucleotide containing the CRE consensus sequence does not compete with the labeled inverted CCAAT box probe for binding to the liver nuclear protein (Fig. 5, lanes 2 and 5); neither does the labeled inverted CCAAT box probe bind to purified CREB protein (details not shown). Based on this evidence, CREB protein does not appear to be involved in mediating the cAMP effect.

Evaluation of Effectiveness of Putative Hormone Response Elements in the Context of a Heterologous Promoter—Finally, to determine whether cAMP antagonism of the insulin response in the context of the FAS promoter could be transferred to a heterologous promoter, we constructed a chimeric GAP 68 and 52 of the FAS gene can confer insulin responsiveness to a heterologous promoter, the inverted CCAAT box located at nt -99 to -92 is itself insufficient to confer cAMP responsiveness out of its normal promoter context.

Fig. 4. Demonstration that nuclear proteins bind to the the inverted CCAAT box region of the FAS gene. Electrophoretic mobility shift assays were performed using nuclear proteins from livers (L) or spleens (S) of fasted (F) or fasted/refed (R) rats. The end-labeled DNA probes comprising FAS promoter sequences from nucleotides -106 to -85 were either identical with the wild type sequence (Wt) or contained mutations (mu) in the region -92 to -99 (see Fig. 3). Some incubations also included 100-fold molar excess of unlabeled competitive oligonucleotides, representing either the wild type (Wt) sequence -106 to -85 or the mutated (mu) sequence.

Fig. 5. C/EBPα is not the protein involved in mediating the cAMP effect via the inverted CCAAT box. Two radiolabeled oligonucleotide probes were employed, one containing the FAS inverted CCAAT box, FAS nt -106 to -85 (lanes 1–8), the other containing the consensus binding site for C/EBP (lanes 9–13). Protein extracts were prepared either from liver nuclei of fasted or fasted/refed rats or from E. coli cells that had been transfected with a C/EBPα expression vector. Where indicated, a polyclonal antiserum raised against C/EBPα was included for “supershifting.” In some incubations, unlabeled oligonucleotides representing either the consensus C/EBP binding site, the FAS inverted CCAAT box, or the CAMP response element, CRE, were included at 100-fold molar excess as potential competitors for the radiolabeled probe. Lane 1, inverted CCAAT box probe alone; lane 2, inverted CCAAT box probe plus liver nuclear extract from fasted/refed rats; lane 3, same as lane 2, plus C/EBPα antibodies; lane 4, same as lane 2, plus unlabeled C/EBP competitive oligonucleotide; lane 5, same as lane 2, plus unlabeled CRE competitive oligonucleotide; lane 6, inverted CCAAT box probe plus liver nuclear extract from fasted rats; lane 7, inverted CCAAT box probe plus heat-treated liver nuclear extract from fasted/refed rats; lane 8, same as lane 1, plus E. coli extract; lane 9, consensus C/EBP binding site probe alone; lane 10, same as lane 9, plus E. coli extract; lane 11, same as lane 10, plus anti-C/EBPα antibodies; lane 12, same as lane 10, plus unlabeled C/EBP competitive oligonucleotide; lane 13, same as lane 10, plus inverted CCAAT box competitive oligonucleotide.
DISCUSSION

In response to insulin and cAMP, the hepatoma cell line H4IIE modulates its endogenous FAS concentration in a manner qualitatively similar to that exhibited by normal liver cells. Thus, the elevation of endogenous FAS protein concentration induced by exposure to insulin can be blocked by simultaneous exposure to cAMP. Using these cells as a model system, we transfected chimeric reporter genes containing various 5′-flanking sequences from the FAS gene and demonstrated that expression of the reporter gene could be enhanced by insulin and that this response could be antagonized by cAMP. In the course of this study, using 5′-deletion analysis of the FAS-reporter gene, we initially localized the insulin response element between nt −124 and −30 (details not shown) and subsequently identified within this region a sequence element between −74/−52 that was protected from DNase I action in the presence of H4IIE nuclear extract. Deletion of the footprint region from the chimeric FAS-reporter gene resulted in the loss of insulin responsiveness indicating that this region of the promoter is required for mediating the insulin response. While these studies were in progress, Moustaid et al. (6) independently reported that the insulin response element of the FAS gene is located between nucleotides −68 and −52. Thus, the results of our studies are in complete agreement with their findings.

By examining the effect of deletion and replacement mutations on the ability of CAMP to antagonize insulin action, we were able to identify a sequence element between nt −99/−92 that is essential for CAMP action. This region was also found to be essential for the binding of a nuclear factor present in the livers and spleens of fasted and fasted-refed rats. The electrophoretic mobilities of the DNA-protein complexes formed with these various nuclear extracts were indistinguishable. Since spleen does not express detectable levels of FAS (4) and expression in the liver is elevated on feeding but drastically lowered on fasting, there is clearly no correlation between the presence in a particular tissue of a nuclear protein binding to nt −99/−92 and the level of FAS expression. More likely then, this protein is a constitutive transcription factor that either itself undergoes covalent modification as a result of exposure of the cells to CAMP or associates with a protein that is either expressed or modified following CAMP treatment. Mutational disruption of the inverted CCAAT box also decreases activity of the FAS promoter approximately 2-fold, in the absence of insulin or CAMP (Fig. 3), indicating that the constitutive transcription factor binding to the inverted CCAAT box plays a role in supporting basal, as well as CAMP-modulated, transcription.

When placed in front of a heterologous promoter, nt −124 to −30 of the FAS gene confer responsiveness to insulin but not to cAMP. This observation indicates that the context of the FAS gene is important in mediating the CAMP effect and raises the possibility that a protein binding to another sequence element within nt −124 and −68 may also be required for mediating the CAMP effect, perhaps through interaction with the protein binding to the inverted CCAAT box.

The FAS appears to be one of a small group of genes transcriptionally regulated by cAMP that bear cAMP-responsive sequences distinct from the more common CREB, AP-1, and AP-2 binding sites. Other genes that fall into this category include the mouse renin gene (24), the human myelin basic protein gene (25), the bovine steroid hydroxylase (P-450c21) gene (26), the rat c-fos gene (27), the porcine G-protein αs subunit gene (28), and the human tryptophan hydroxylase gene (29). In all of these other examples, cAMP is an inducer of transcription, whereas in the case of the hepatic FAS it is a negative regulator. In contrast of these genes, CCAAT box-binding proteins have been implicated as mediators of the CAMP-regulated transcription, notably the c-fos gene which is regulated by the phosphorylated form of CREB (27), the G-protein αs subunit which is regulated by an unidentified CCAAT box-binding protein and the human tryptophan hydroxylase gene which, like the FAS gene, has an inverted CCAAT box that is essential for mediating the cAMP effect on transcription. In the case of the FAS, it appears highly unlikely that the protein binding to the inverted CCAAT box is a member of the CREB family of transcriptional factors, based on its heat lability and immunochemical properties. Clearly, the purification and characterization of the transcription factor(s) binding to the inverted CCAAT box motif will provide further insight into the mechanism by which cAMP regulates transcription of the FAS gene.

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