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The C/EBP-Binding Region and Adjacent Sites Regulate Expression of the Adipose P2 Gene in Human Preadipocytes

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Human preadipocytes contain nuclear factors that specifically bind to the AE-1 sequence, previously demonstrated as an enhancer element in the regulation of adipose P2 gene expression during 3T3 adipose differentiation. By transient transfection and in vivo competition experiments, the *trans*-acting factors were found to bind either to the C/EBP recognition site in the AE-1 sequence and act as a negative regulator or to the adjacent site (termed 3'AE-1) and act as a positive regulator of adipose P2 gene activity in human preadipocytes.

Adipose differentiation features the transcriptional activation of several genes involved in triglyceride metabolism, including the adipose P2 (aP2 or 422) gene, which encodes the adipocyte lipid-binding protein (4, 5, 25, 29). The aP2 gene is widely conserved through evolution (19); thus, the adipocyte lipid-binding protein may have a critical function in the regulation of adipose metabolism. Recently, the human adipocyte lipid-binding protein has been purified and its cDNA has been cloned (3). This study has revealed that it is a member of a multigene family of intracellular lipid-binding proteins (3).

As reported elsewhere, the AE-1 sequence (nucleotides −159 to −125; Fig. 1) in the aP2 promoter region functions as an enhancer element in the regulation of murine aP2 gene expression (11, 18). Additionally, it has been determined that at least one protein, C/EBP (CCAAT/enhancer-binding protein; 12, 13, 20, 22, 23, 31), functions as a transcription factor to activate the aP2 gene during adipose differentiation (6, 18). From the results of methylation interference analysis performed with NIH 3T3 extracts, it was determined that guanine nucleotides located at −144, −141, and −139 were required for high-affinity binding of the 3T3 nuclear factors to the C/EBP-binding region in the AE-1 site (Fig. 1); further, binding of 3T3 factors to the AE-1 sequence was specifically inhibited by double point mutations at −144 and −139 (26a). Recently, it has been reported that disruption of a C/EBP-binding site in the clotting factor IX promoter is associated with hemophilia B (9), illustrating the importance of this transcription factor (the human analog of the rat protein C/EBP or another member of the C/EBP family) in humans.

Elucidation of the factors controlling aP2 gene expression in human preadipocytes would provide valuable information about both the process of adipose differentiation and the functional role of the product, the human adipocyte lipid-binding protein, in cells from both lean and obese subjects. Human preadipocytes are a fairly well defined reproducible fibroblastic system, capable of adipose differentiation under specific conditions (7, 15, 24, 28, 30). Our studies have defined the system in more detail and have demonstrated the usefulness of human preadipocytes.

We isolated and cultured omental cells from lean subjects as previously described and studied them in the absence of specific inducers of differentiation (7, 15, 16, 24, 30). We performed nuclear protein interaction studies (11, 18), using the synthetic oligonucleotides from the aP2 promoter region (Fig. 1). Consistent with previous results (18), experiments with nuclear extracts from 3T3 preadipocytes (Fig. 2a, lane 1) and differentiated adipose cells (Fig. 2a, lane 6) demonstrated sequence-specific interaction with the AE-1 sequence. Competition with excess unlabeled AE-1 sequence virtually eliminated the retarded bands (Fig. 2a, lanes 2 and 7). The consensus C/EBP-binding sequence (termed C/EBP sequence; see Fig. 1), which has been implicated as an enhancer element of several liver-specific genes (8, 14, 17, 22), was also able to compete with the AE-1-binding site (Fig. 2a, lanes 3 and 8). As reported earlier, purified rat C/EBP prepared as a C/EBP–β-galactosidase fusion protein could bind to the AE-1 site (6, 18).

Studies with human preadipose nuclear extracts also showed sequence-specific binding to the AE-1 sequence. The gel retardation experiments showed two retarded bands (Fig. 2b, lane 1), I (lower) II (upper). Neither factor seems related to the 3T3 factors, at least in size, since both bands migrated faster than the 3T3 factors (data not shown). Competition with the AE-1 sequence virtually eliminated both retarded bands (Fig. 2b, lane 2); however, competition with the C/EBP sequence eliminated only band II (Fig. 2b, lane 4). Thus, factor II may related to the 3T3 factors and C/EBP, at least with respect to the binding site. Comparison between the C/EBP consensus sequence and the AE-1 sequence suggests that C/EBP or C/EBP-like proteins (including factor II) may recognize the 11-mer (−145 to −135) sequence (Fig. 1b). Band I was eliminated by competition with the synthetic oligonucleotide spanning the sequence from −138 to −120 (termed 3'AE-1; see Fig. 1) of the aP2 gene (Fig. 2b, lane 3). Thus, the C/EBP binding and 3'AE-1 sites overlap. The 3T3 factors binding to the AE-1 sequence were not able to compete with the 3'AE-1 sequence (Fig. 2a, lanes 4 and 9). These results imply that factor I may be specific for human adipogenic cells. The gel retardation experiments with nuclear extracts of either human skin fibroblasts or HeLa cells demonstrated no significant sequence-specific binding to the AE-1 sequence (not shown). These results suggest that human preadipose factors bind to the AE-1 site with specificity for both sequence and cell type.

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a

AE-1

-159 -144 -139 -125

GATCCAGGGAGAACCAAA@TT@A@AAATTTCTATTAAA
GTCCCTCTTGGTTTCAACTCTTTAAAGATAATTTCTAG

3'AE-1

-138 -120

TCGACAAATTTCTATTAAAAACATC
GTTTAAAGATAATTTTTGTAGAGCT

C/EBP sequence

GATCCAATTGGGCAATCAGGA
GTTAACCCGTTAGTCCTCTAG

b

A@TT@A@AAAT AE-1
AATTGGGCAAT C/EBP

FIG. 1. (a) DNA sequences of the aP2 promoter region and C/EBP-binding site. The AE-1 sequence represents nucleotides −159 to −125 and the 3'AE-1 sequence represents nucleotides −138 to −120 relative to the transcription start site of the aP2 gene (19). Guanines whose methylation interferes with 3T3 nuclear factors binding to the AE-1 site are outlined. Double point mutations were made at nucleotides −139 and −144 (G-to-A transition) for plasmid paP2-CATm11 (see Fig. 4a and reference 18). The C/EBP sequence represents the consensus C/EBP-binding sequence (22). Each oligonucleotide was synthesized, made double stranded, and used in gel retardation and competition experiments. Double-stranded sequences were formed with 5' cohesive ends to facilitate subsequent cloning or 3'-end labeling of the oligonucleotide sequences. (b) Comparison of C/EBP-binding sites in the AE-1 sequence and the consensus C/EBP-binding sequence. The outlined guanines are as in panel a.

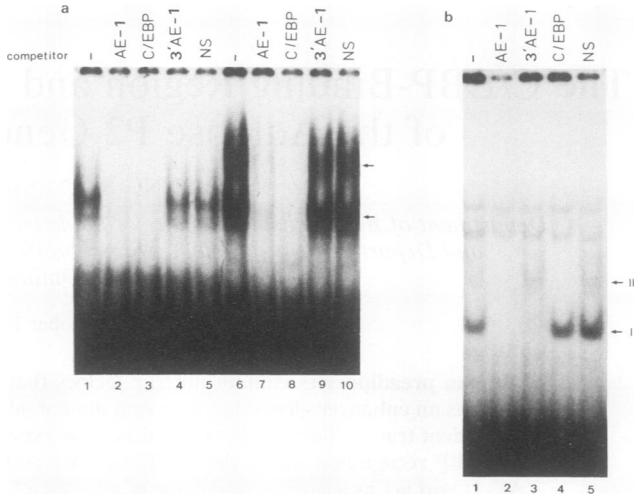

FIG. 2. Human preadipose factors binding to the aP2 gene enhancer. Nuclear extracts were made from preadipocytes and adipocytes essentially as described earlier (11). Oligonucleotides were labeled by fill-in with Klenow polymerase. Conditions of assays and gel electrophoresis were as described previously (11). (a) Gel retardation experiments with 3T3 nuclear extracts. Probe AE-1 (see Fig. 1) was used. About 200-fold molar excess was added for each competitor oligonucleotide. Lanes: 1 to 5, preadipose extracts; 6 to 10, adipose extracts. Arrows indicate the bound forms. NS, Nonspecific oligonucleotide. (b) Gel retardation experiments with human preadipocyte nuclear extracts. Probe AE-1 was used. About 200-fold molar excess was added for each competitor oligonucleotide. Specific bands I and II are indicated.

To address the question of whether the high and selective binding of human preadipose factors to the aP2 promoter region was a functional property of human preadipocytes, we performed transient expression analyses with a plasmid (paP2-CAT) containing sequence −168 to +21 of the aP2 promoter inserted upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene of plasmid pUC-CAT (11, 18). Human preadipocytes were isolated from human omental adipose tissue by our previously reported methods (7, 24). The preadipocytes and skin fibroblasts were grown in Dulbecco modified Eagle medium supplemented with 15% fetal bovine serum and transfected with plasmids, using Polybrene as the DNA adsorbent, as outlined by Kawai and Nishizawa (21). Cells were collected 48 h after transfection, and CAT activity was determined as described previously (11, 18). Transient transfections of paP2-CAT into 3T3 preadipocytes produced CAT activity similar to the background activity of pUC-CAT (11, 18). In contrast, in human preadipocytes, the activity was increased significantly (about fourfold) over the background level (Fig. 3). As was seen in 3T3 preadipocytes, in adult human skin fibroblasts, paP2-CAT expression was similar to the background expression of pUC-CAT (Fig. 3). Similar background expression was obtained in human neonate foreskin fibroblasts (not shown). In Northern (RNA) blot analyses with a human aP2-homolog cDNA probe, we were unable to detect any endogenous aP2 transcripts in human preadipocytes (unpublished results). These results suggest that a negative regulatory element(s) may be present outside the promoter region (−168 to +21).

To determine whether the AE-1 site imparts the functional regulation of CAT expression, we first transfected human preadipocytes with the mutant derivative of paP2-CAT

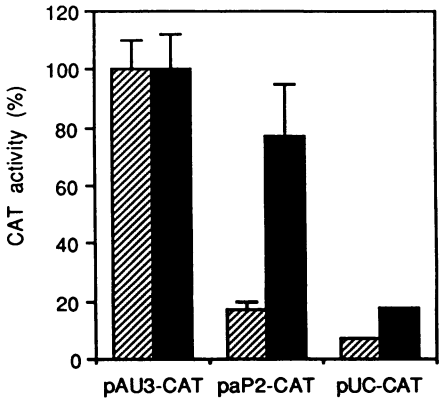

FIG. 3. Transient expression experiments. Either human skin fibroblasts (▨) or human preadipocytes (■) were used as host cells. The data are expressed as percentages of pAU3-CAT activity determined in parallel. Each value was converted to activity per microgram of plasmid. Values are averages of three transfection experiments except for pUC-CAT (one transfection). Plasmid pAU3-CAT represents a positive control plasmid driven by the AKV virus long terminal repeat. paP2-CAT contains nucleotides −168 to +21 of the promoter linked to the CAT gene of plasmid pUC-CAT (11, 18).

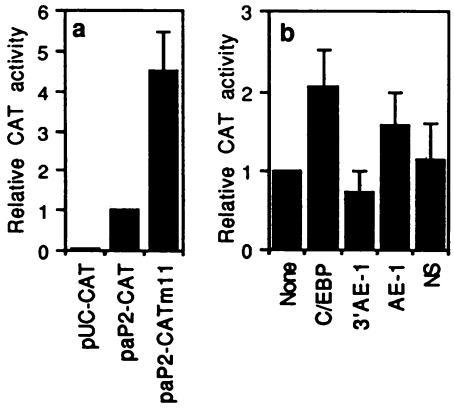

FIG. 4. Summary of CAT assays in human preadipocytes. The reported CAT activity values for all samples were made relative to the value for paP2-CAT, which was given the arbitrary value of 1.0. (a) Effect of the mutation at the C/EBP-binding site on transient transfection. Plasmid paP2-CATm11 is the same as paP2-CAT except that it contains double point mutations at −144 and −139 (G-to-A transition) in the promoter region (18). Values are averages of duplicate experiments. (b) Cotransfection of paP2-CAT with synthetic oligonucleotides. The oligonucleotides were ligated to make multimeric forms prior to transfection. None, Transfection without any oligonucleotide; NS, transfection with a nonspecific oligonucleotide. All oligonucleotides were made as described in the legend to Fig. 1. Values are averages of triplicate experiments.

(paP2-CATm11; 18), which contains double point mutations at −144 and −139 (high-affinity C/EBP-binding sites; see Fig. 1) in the AE-1 sequence. Such transfection resulted in expression at a level about fivefold greater than that occurring with regular paP2-CAT (Fig. 4a). No such effect was detected in 3T3 preadipocytes (18). These studies suggest that the C/EBP-binding site may act to either positively or negatively regulate aP2 gene expression in human preadipocytes. The mutation may increase or decrease the binding of a factor, specific for human preadipocytes (perhaps factor II), at the C/EBP-binding site. We also cannot rule out the possibility that the mutation results in binding of another positive factor at the C/EBP-binding site or in enhancement of the binding of a second factor (perhaps factor I) at the 3'AE-1 site.

To clarify the role of the C/EBP-binding site and factor II, in vivo competition assays were performed. Cotransfection of paP2-CAT with the C/EBP sequence (60-fold molar excess) resulted in higher stimulation (about twofold) than with paP2-CAT alone (Fig. 4b). These results clearly suggest that factor II and the C/EBP-binding site may function as negative regulators. In contrast, cotransfection of paP2-CAT with the 3'AE-1 sequence resulted in some lowering of expression (Fig. 4b), implicating factor I and its binding site as positive regulators. However, this effect (about a 25% reduction) was not as marked as that of cotransfection with the C/EBP sequence, suggesting the possible existence of another positive regulator site. Compatible with this suggestion, cotransfection with the AE-1 sequence resulted in significant stimulation (Fig. 4b). Thus, these results highlight the importance of binding of human preadipose factors to the AE-1 site in the regulation of aP2 gene expression in human preadipocytes.

Previous studies have suggested that human adipose tissue contains a pool of preadipocytes that can differentiate under appropriate conditions (7, 10, 15, 16, 24, 28). The regulation of aP2 gene expression and its role in human adipose differentiation or adipocyte function are unknown, but clues have come from this work. We have detected human preadipose factors that bind in a sequence- and cell-specific manner to the aP2 promoter site (Fig. 2) and demonstrated the high and selective expression of the aP2 chimeric plasmid in human preadipocytes (Fig. 3). We demonstrated that the C/EBP-binding site and its binding factor may function as negative regulators in human preadipocytes (Fig. 4). To our knowledge, there have been no reports directly indicating a repressor function for the C/EBP-binding site. There have been reports of other members of DNA-binding proteins that have considerable homology to C/EBP in the region of DNA binding and dimerization, each of which is expressed in a different tissue (2, 26, 27). These members of the C/EBP family apparently acted as positive factors.

The high expression of paP2-CAT in human preadipocytes is possibly due to a higher amount or stronger affinity of positive factor I than of negative factor II. They may compete with each other for binding at the AE-1 site. Removal of the negative factor, either by mutation at the C/EBP-binding site or titration with the C/EBP sequence, resulted in further stimulation of expression. In fact, this mechanism of interference between transcription factors has been demonstrated in the case of negative regulation by steroid receptors with a cyclic AMP-responsive enhancer (1) and certainly applies also to negative regulation in other systems. Characterization of these nuclear factors should extend our knowledge about the regulation of the aP2 gene during both adipose differentiation and in mature fat cells and about the metabolism of the lipid-binding protein product in lean and obese subjects.

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