Journal of Cellular Biochemistry 49:219–224 (1992)

PROSPECTS

Identification of a Fat Cell Enhancer: Analysis of Requirements for Adipose Tissue-Specific Gene Expression

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Abstract The molecular basis for adipose-specific gene expression is not known. To approach the problem of adipocyte gene expression, we have analyzed in detail the capacity of the 5'-flanking region of the adipocyte P2 (aP2) gene to direct cell-type specific gene expression. Although the proximal promoter containing AP-1 and C/EBP binding sites is capable of directing differentiation-dependent gene expression in cultured adipocytes, these constructs are essentially inactive in the tissues of transgenic mice. We found that −5.4 kb of the 5'-flanking region were required to direct heterologous gene (chloramphenicol acetyl transferase; CAT) expression to the adipose tissue of transgenic mice. By deletion analysis, we identified a 520 bp enhancer at −5.4 kb of the aP2 gene. We show that this enhancer can direct high levels of gene expression specifically to the adipose tissue of transgenic mice. This enhancer also functions in a differentiation-dependent manner in cultured adipocytes and cannot be transactivated in preadipocytes by C/EBP. Molecular analysis indicates that several cis- and trans- acting acting elements, though not C/EBP, contribute to the specificity and potency of this enhancer.

Key words: differentiation, transgenic, transcription factor, C/EBP, obesity

We and others have utilized the adipocyte P2 (aP2) gene as a model system to analyze the molecular basis of adipocyte-specific transcriptional regulation (Distel et al., 1987; Cook et al., 1988). The murine aP2 gene encodes a member of the fatty acid binding protein family and is expressed specifically in adipose tissue (Zezulak and Green, 1985). The aP2 gene is regulated during the differentiation of preadipocytes in several cultured cell model systems (Bernlohr et al., 1984; Chapman et al., 1984; Doglio et al., 1986; Spiegelman et al., 1983). The proximal promoter of the aP2 gene is capable of directing heterologous gene expression (chloramphenicol acetyltransferase; CAT) in a differentiation-dependent fashion (Distel et al., 1987; Yang et al., 1989). Molecular studies of the proximal promoter have defined two DNA elements, an AP1 and a C/EBP binding site, that are important for this expression (Christy et al., 1989; Distel, et al., 1987; Herrera et al., 1989). Mutation of either site severely reduces promoter activity in adipocytes. The AP1 site of the aP2 gene, where binding of fos-related protein complexes was first demonstrated (Distel et al., 1987; Rauscher et al., 1988), is required for the cAMP responsiveness of the aP2 promoter (Herrera et al., 1989). Since the receptors for agents that increase the levels of cAMP are dramatically increased during the differentiation process, it seems reasonable to suggest that the apparent differentiation-dependent response of this element may merely reflect the increased responsiveness of adipocytes (compared with preadipocytes) to certain extracellular signals. The C/EBP binding site at −140 bp is also important for the differentiation-dependent activity of the proximal promoter (Christy et al., 1989; Herrera et al., 1989). The levels of mRNA for C/EBP are increased dramatically during differentiation and C/EBP has been shown to transactivate the promoters of several fat cell genes (e.g., glut 4, stearoyl CoA desaturase) (Christy et al., 1989; Herrera et al., 1989; Kaestner et al., 1990). Thus, C/EBP has been proposed to play a cen-

Received June 21, 1991; accepted March 5, 1992.

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tral role in regulation of genes involved in energy metabolism and also as a differentiation-dependent switch (McKnight et al., 1989). In this report, we review our recent results on the identification and characterization of a far upstream enhancer from the aP2 gene that directs high levels of adipose-specific gene expression in transgenic mice (Ross et al., 1990). Preliminary molecular analysis of the enhancer failed to identify a C/EBP responsive element in the enhancer (Graves et al., 1991b). We have previously shown that an NF-1 site was important for full enhancer function, but that there must be other cis-acting elements in the enhancer, since residual activity is observed when the NF-1 site is mutated (Graves et al., 1991a). Finally, we discuss the potential uses of this enhancer to target the expression of proteins to the adipose depot in transgenic animals.

ANALYSIS OF ADIPOSE-CELL SPECIFICITY USING TRANSGENIC MICE AND CULTURED CELLS

Since AP-1 and C/EBP binding sites are present in several genes whose expression is not restricted to adipose tissue, we wanted to determine if the aP2 promoter containing only these sites was sufficient for tissue-specific gene expression. Constructs containing different lengths of the aP2 5′-flanking region driving the CAT gene were evaluated for their ability to direct appropriate tissue-specific expression in transgenic mice. Four transgenes containing various segments of the aP2 promoter beginning from −5.4 kb to −168 bp and terminating at the 3′ end by the natural Pst I site at position +21 of the aP2 mRNA (Fig. 1 from Graves et al., 1991a). Promoter activity was assessed by quantitative CAT assay from various tissues of several strains of transgenic mice (Table I from Ross et al., 1990). Previous results had shown that the proximal promoter (either −168 or −247) was sufficient to confer differentiation-dependent expression in cultured mouse adipocytes (Cook et al., 1988; Distel, et al., 1987). Strikingly, those small constructs and a construct extending to 1.7 kb were uniformly inactive in adipose tissue of any of the transgenic mice. Only in an animal with a huge copy number of the 247aP2CAT transgene (strain 3_{1a}; 1,255 copies), was any significant gene expression observed. However, in this case, expression was an order of magnitude greater in spleen and thymus than in adipose tissue.

By contrast, the −5.4 kb aP2 promoter was able to direct high levels of expression specifically to the adipose tissue (Table I from Ross et al., 1990). In the adipose tissue of these strains, CAT activity directed by the 5400aP2CAT transgene was at least four orders of magnitude greater than transgene expression directed by any of the smaller constructs. The specificity of transgene expression for adipose tissue ranged from two-fold to greater than 20-fold in the different strains. It should be noted that the tissues (spleen and thymus) with the highest amounts of inappropriate transgene expression are also very likely to have been contaminated with fat when excised from the animal. Taken together, these results suggested that there is an element capable of conferring adipose-specific gene expression in the 3.7 kb fragment extending from −5.4 kb to −1.7 kb.

We next used cultured 3T3-F442A cells to rapidly scan this 3.7 kb DNA fragment for enhancing activity in adipocytes (Graves et al., 1991a). As a basal promoter we used either −63 aP2 CAT, which contains sequences from −63 to +21 of the aP2 gene and is deleted for the previously identified regulatory elements (C/EBP, AP1, CAAT box; Distel et al., 1987), or the enhancerless SV40 promoter (Celander and Haseltine, 1984). Both of these promoters are relatively inactive in preadipocytes and adipocytes. Several restriction fragments of the 3.7 kb fragment were joined to −63aP2CAT and assayed for enhancing activity following transient transfection into cultured adipocytes. From this analysis we identified a 520 bp "enhancer" extending from −5.4 kb to −4.9 kb (Fig. 1; from Graves et al., 1991b). This enhancer was capable of stimulating CAT gene expression from the −63 aP2 promoter when transiently transfected into differentiated adipocytes. By contrast, the same construct showed no stimulation when transiently transfected into preadipocytes. Finally, we compared enhancer activity in a cell line consisting of a pool of 36 colonies that were stably transfected. CAT activity increased 15-fold following adipose differentiation (Graves et al., 1991a).

To determine if this enhancer was capable of directing tissue-specific gene expression, we made transgenic mice containing this construct. The adipose specificity of the enhancer con-

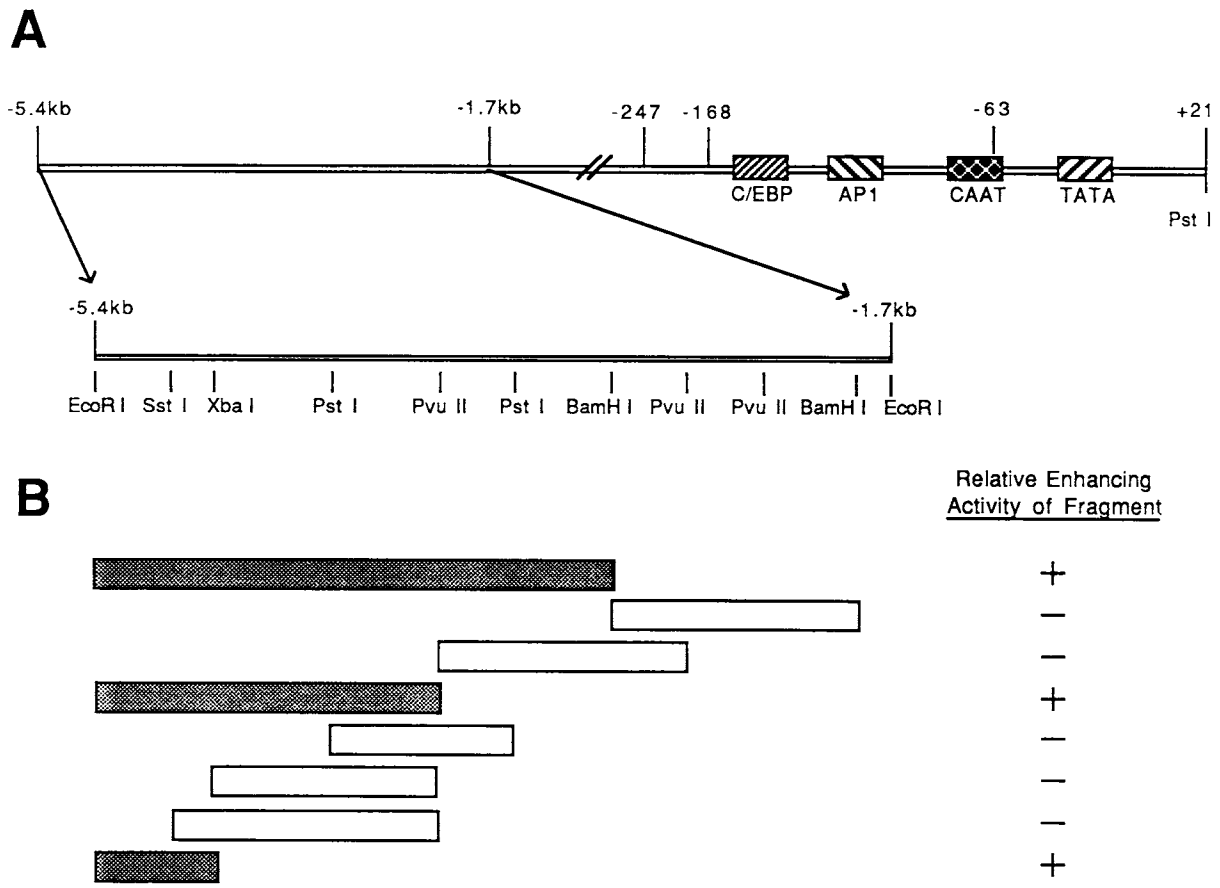

Fig. 1. Schematic map and enhancer activity analysis of the aP2 gene (Graves et al., 1991a). **A:** The −5.4 kb to +21 segment of the aP2 gene that was analyzed is shown. Previously identified binding sites for the transcription factors AP1 and C/EBP as well as the CCAAT and TATA box homologies are indicated as boxes. Note that the inverted CCAAT box is partially deleted in the −63aP2CAT construct. A detailed restriction map of the 3.7 kb EcoRI fragment (−5.4 kb to −1.7 kb) that has a tissue-specific activation function is shown in the lower portion of part A. The sites indicated were used to isolate the various restriction fragments indicated in part B. **B:** Various restriction fragments (indicated as boxes) were ligated to the −63aP2CAT vector and the resulting constructs were then transfected into adipocytes. The enhancing activity of the fragment is indicated by dark shading of the boxes as well as a ± in the right hand column.

structs (520/−63aP2 or 520/SV40) is equivalent or greater than the 5400aP2CAT construct. In Table I (part B; from Graves et al., 1991b), we present the results from a typical mouse; we have obtained similar results in three different strains (Ross et al., 1990). These results clearly demonstrate that this 520 bp enhancer is capable of directing high levels of adipocyte-specific gene expression in transgenic animals. Enhancer specificity (and activity) is independent of the aP2 promoter, since identical results in both cell culture and transgenic animals are obtained when the enhancer is joined to the enhancerless SV40 promoter (520/SV40; Table I, part B; from Graves et al., 1991b). These data clearly demonstrate that the AP-1 and C/EBP binding sites at −120 and −140 of the aP2 promoter are not necessary for adipose-specific gene expression. Apparently, gene expression in adipose cells in vivo has more stringent sequence requirements than is observed by transfection of cultured cells.

There are several genes whose transcription is regulated during the differentiation process (Bernlohr et al., 1984; Chapman et al., 1984; Cook et al., 1985; Spiegelman et al., 1983). Some of these genes are also expressed in a tissue-specific manner (e.g., adipsin, aP2). It is reasonable to suspect that there will be common cis-acting elements functioning in the enhancer/promoter of these genes. Some of these cis-acting elements may play roles in determining its tissue-specificity, as well as controlling differentiation-dependent gene expression. However,

TABLE I. CAT Activity in the Tissues of aP2CAT Transgenic Mice*

Part A	Copy	Specific activity in tissues (cpm/μg/min)						
Transgene	Number	Liver	Spleen	Thymus	Brain	Muscle	Lung	Adipose
168aP2CAT(14)	50	0	0.033	0.017	0.001	ND	0.002	0
247aP2CAT(3_1a)	1255	0.012	1.351	3.295	0.305	0.107	0.051	0.07
247aP2CAT(3_2)	21	0	0.009	0.012	0.024	ND	0.011	0.006
247aP2CAT(11)	12	0.01	0.187	0.125	0.014	0.008	0.047	0.012
1700aP2CAT(3)	5	0	0.456	0.241	ND	0	0.003	0.002
1700aP2CAT(6)	45	0.004	0.814	0.402	0.006	0.003	0.007	0.034
1700aP2CAT(8)	4	ND	0.484	0.001	0.425	0	0.019	0.008
5400aP2CAT(17)	5	1	2200	4400	4	ND	1400	>11,000[a]
5400aP2CAT(18)	5	8	450	360	29	43	73	860
5400aP2CAT(25)	<5	27	93	130	0	ND	43	1300
Part B		%CAT conversion						
Transgene	Kidney	Liver	Spleen	Thymus	Brain	Muscle	Lung	Adipose
520/−63aP2(2)	0.2	1.4	0.1	0.5	0.6	0.9	0.3	25.9
520/SV40(7)	0.7	1.0	0.8	1.5	0.8	0.8	0.8	16.3

*Transgenes in Part A, from Ross et al. (1990), contain from −168, −247, −1700 or −5400 bp to +21 bp of the aP2 gene promoter linked to CAT. In Part B, from Graves et al. (1991b), transgenes contained the 520 bp aP2 enhancer linked to either the −63 aP2 promoter or to the enhancerless SV40 promoter.

[a]CAT activity was in the nonlinear range of the assay, so that accurate specific activities could not be determined and this value represents a minimal specific activity. The strain of transgenic mouse is indicated in parenthesis after the transgene.

it is important to note that these phenomena may represent fundamentally different processes. The tissue-specific enhancer that we have identified also functions in a differentiation-dependent fashion in cultured cells. Identification of sites in the enhancer that contribute to both differentiation-dependent and tissue-specific gene expression will allow us to determine the role of differentiation and cell-type specific transcription factors in regulating the specificity of gene expression. Our immediate goal is to identify the elements that determine tissue specificity, since these elements are likely to play a role in the expression of many fat cell genes. Other differentiation-dependent genes are expressed in several tissues (e.g., glycerol-3-phosphate dehydrogenase) and it is important to determine whether or not these genes are regulated in a manner similar to the genes that are strictly adipose cell-specific. Finally, it will be important to determine whether or not there exists a "master gene" for the adipogenic lineage parallel to that observed for another mesodermal lineage, muscle (e.g., myoD) (Davis et al., 1987).

ROLE OF THE AP1 AND C/EBP BINDING SITES IN THE AP2 PROMOTER

Since C/EBP has been shown to transactivate the promoters of several adipose cell genes (including the aP2 gene) (Christy et al., 1989; Herrera et al., 1989), we wanted to determine whether or not the enhancer itself contained binding sites for C/EBP. We have not been able to identify a C/EBP binding site in the enhancer by either mobility-shift DNA binding or DNAase I footprinting assays (data not shown). We also performed transactivation experiments in preadipocytes by cotransfecting a C/EBP expression vector with the adipose enhancer construct. The results are shown in Figure 2. As was shown previously (Herrera et al., 1989), C/EBP was able to transactivate the −168aP2CAT construct which contains a bona fide C/EBP binding site (Fig. 2, lanes 1–3; from Graves et al., 1991b). However, there was no transactivation by the C/EBP vector of the 520 enhancer/SV40 promoter construct (Fig. 2, lanes 4–7; from Graves et al., 1991b). We conclude that C/EBP cannot activate the isolated aP2 enhancer in preadipocytes.

An analysis of the aP2 promoter function in cultured cells has suggested that the AP-1 and C/EBP binding sites function as positive acting elements. Furthermore, C/EBP has been suggested to act more generally in adipocyte-specific gene regulation (McKnight et al., 1989). However, it is clear from our data that a C/EBP binding site is apparently not required for the differentiation-dependent or adipose tissue-

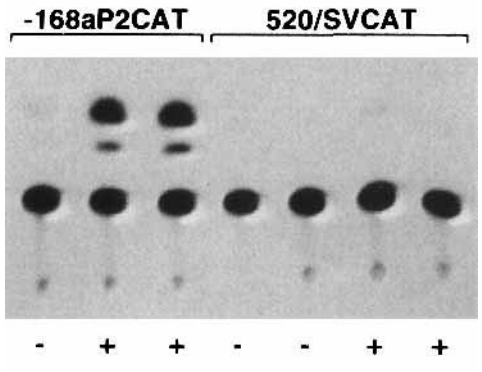

Fig. 2. C/EBP does not transactivate the aP2 enhancer (Graves et al., 1991b). 3T3F442A preadipocytes were transfected with either −168aP2CAT or 520/SVCAT (the 520 bp enhancer ligated to the enhancerless SV40 promoter). Cotransfection with the C/EBP expression vector is indicated by +.

specific gene expression derived from the aP2 gene. From our perspective and the data presented recently by our lab (Graves et al., 1991a,b; Ross et al., 1990), we believe that a likely role for C/EBP is as a qualitative modulator of certain genes expressed in tissues that express C/EBP, such as fat, liver, or kidney. Alternatively, C/EBP could be involved in a non-classical hormonal response. In this regard, it should be noted that we have demonstrated that constructs containing the proximal promoter of the aP2 gene (−247aP2CAT) can be activated by glucocorticoids in transgenic mice, despite the absence of a classical GRE sequence (Ross et al., 1990).

What factors bind to the enhancer? By DNaseI footprinting and mobility-shift DNA binding assays, we have identified at least 4 different proteins that bind to the enhancer. One of these proteins is a member of the NF-1 family (Graves et al., 1991a). Although mutation of the NF-1 site reduces enhancer function dramatically, it remains to be determined whether or not the NF-1 site plays a regulatory (i.e., tissue-specific or differentiation-dependent) role in enhancer function. It is clear, however, that NF-1 is not the only site in the enhancer, since the enhancer with the NF-1 mutation retains significant differentiation-dependent activity. Thus there must be other positive cis-acting elements in the enhancer. The regulation of all of these factors in cell differentiation and obesity will be important subjects for further studies and the aP2 enhancer can now provide a first look at the molecular requirements for fat-specific gene expression.

PROSPECTS FOR GENETICALLY ALTERED ADIPOSITY: USES OF THE AP2 ENHANCER

We have identified the first enhancer with specificity for adipocytes in culture and in transgenic animals (Graves et al., 1991a,b; Ross et al., 1990). The ability to target high level expression of genes with regulatory potential to adipose tissue will be of importance in the fields of biology, pathophysiology, and even agriculture. Pathological conditions that involve adipose tissue, such as obesity and lipodystrophy, are associated with a variety of significant health problems, especially cardiovascular disorders and diabetes (Coleman, 1982). New experimental models of obesity or lipodystrophy might be brought about, for example, by overexpression of the α- or β-adrenergic receptors. Alternatively, the relationship between obesity and diabetes could be probed in several obese/diabetic mouse models by directly suppressing adipose cell formation and/or function through the delivery of toxins or other agents that suppress lipid accumulation. In the agricultural field, there has been considerable interest in developing genetic methods to alter the balance between lean and fat body mass in feed animals and the enhancer described here could open the door to such methods.

ACKNOWLEDGMENTS

We thank Ms. Adah Levens for assistance in the preparation of this manuscript. We thank Dr. Greg Robinson for advice in the use of the C/EBP expression vector. We thank members of the Spiegelman and Ross laboratories for helpful discussions. This work was supported by grants from the March of Dimes (1-1126; S.R.R.) and the National Institutes of Health (DK31405; B.M.S.).

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