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A fat-specific enhancer is the primary determinant of gene expression for adipocyte P2 *in vivo*

(transgenic/lipid-binding protein)

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ABSTRACT **The murine gene for adipocyte P2 encodes an adipocyte-specific member of the family of intracellular lipid binding proteins. The region upstream from the start of transcription of this gene has been found to contain binding sites for the transcription factors c-jun/c-fos and C/EBP (CCAAT/enhancer binding protein) and several short sequence elements found in other adipocyte gene promoters, termed fat-specific elements. To identify DNA sequences that were responsible for the high level of transcription of the gene for adipocyte P2 *in vivo*, we made a series of transgenic mice containing 168 base pairs (bp), 247 bp, 1.7 kilobases (kb), and 5.4 kb of 5′ flanking sequence linked to the bacterial gene chloramphenicol acetyltransferase. Although plasmids containing only 168 bp of 5′ sequence including the C/EBP and AP-1 (activation protein 1) binding sites were expressed well in cultured adipocytes, high levels of chloramphenicol acetyltransferase activity in the adipose tissue of transgenic mice were not observed until the 5′ flanking region was extended to kb −5.4. An enhancer mapping between kb −4.9 and kb −5.4 upstream from the start of transcription was identified by transfection of further deletions into cultured adipocytes. This enhancer, when linked to a bp −63 promoter fragment from the gene for adipocyte P2, directed very high level chloramphenicol acetyltransferase expression specifically to adipose tissue in transgenic mice. These results identify a functional adipose-specific enhancer and indicate that it is the major determinant of tissue specificity of the gene for adipocyte P2. These results also demonstrate that the proximal-promoter binding sites for AP-1 and C/EBP are not sufficient or necessary to give adipose-tissue-specific expression *in vivo*, though they may play an important role in the response of this promoter to glucocorticoids.**

The major role of the adipocyte in higher eukaryotes is the storage of nutritional energy in the form of triglycerides. Disordered gene expression in the adipocyte may result in pathological conditions such as lipodystrophy and obesity, the latter of which contributes heavily to morbidity and mortality through associated cardiovascular disorders and diabetes. In addition, the ability to genetically alter the expression of adipocyte genes and thus control the fatness of feed animals remains an important goal of agricultural research. Thus, the regulation of adipocyte gene expression is of interest for biological, medical, and agricultural reasons.

A number of adipocyte-specific genes have been cloned from differentiating cultured adipocytes (1–4). The gene for adipocyte P2 (aP2), which encodes a very abundant lipid binding protein (1, 2, 5, 6), has been used as a model for understanding gene regulation in this cell type. Short constructs containing as few as 168 base pairs (bp) upstream from the transcription start site have been shown to be expressed in a differentiation-dependent fashion and, hence, considerable attention has been focused on putative regulatory sequences in this promoter. Several short DNA sequences that are common to genes expressed in fat cells, termed "fat-specific elements" have been noted (5) and fat-specific element 2 at bp −120 in the aP2 promoter was the first identified binding site for c-fos-containing protein complexes (7). This site, now recognized as an AP-1 (activator protein 1) sequence (8), acts as a positive regulator of aP2 promoter action in cultured adipocytes (9). Upstream of the AP-1 site at bp −140 is a binding site for the transcription factor C/EBP (CCAAT/enhancer binding protein) (9, 10). This element also plays a positive role in cultured adipocytes and is of particular interest because C/EBP mRNA and protein are expressed at high levels in adipose tissue (11) and are induced upon adipocyte differentiation (9, 10). Since C/EBP can bind to and transactivate several fat-cell gene promoters (10), it has been proposed as a general regulator of adipocyte gene expression as well as of other genes involved in energy balance (12).

In addition to its tissue specificity and regulation during differentiation, the aP2 promoter has been shown to respond to certain hormones in cell culture (9, 13, 14). cAMP analogues and the synthetic glucocorticoid dexamethasone induce this promoter in preadipocytes and this activation requires an intact AP-1 site (9).

At present, there have been no reports of cis-acting regulatory sequences that can specifically direct gene expression to adipocytes *in vivo*. In this report, we have analyzed a variety of constructs from the aP2 5′ flanking region in transgenic mice from the perspective of tissue specificity of expression and hormone sensitivity. We find that the specific expression of the gene for aP2 in adipocytes derives from an enhancer element at kilobase (kb) −5.4 that directs linked chloramphenicol acetyltransferase (CAT) marker gene expression very strongly and specifically to fat tissue. This sequence appears to be the major determinant of expression of this gene. Furthermore, the previously described proximal-promoter elements (AP-1 and C/EBP binding sites) are neither necessary nor sufficient to get appropriate tissue-specific expression. However, this proximal-promoter region is necessary and sufficient to get a response to dexamethasone *in vivo*.

MATERIALS AND METHODS

Transgenic Animals and Cultured Cell Transfections. DNA injection into a Swiss Webster (SW) one-cell zygote and

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Abbreviations: aP2, adipocyte P2; C/EBP, CCAAT/enhancer binding protein; CAT, chloramphenicol acetyltransferase; GRE, glucocorticoid response element; AP-1, activator protein 1; SV40, simian virus 40.

implantation into a pseudopregnant foster mother were performed as described (15). SW mice were purchased from the National Cancer Institute Frederick Animal Production Facility. DNA fragments for microinjection were isolated away from the bulk of the plasmid sequences either by extraction from low-melting-point agarose gels and or by electroelution from standard agarose gels.

Positive transgenic animals with CAT-containing transgenes were identified by polymerase chain reaction, using primers that amplified a segment of the simian virus 40 (SV40) small tumor antigen splice/polyadenylylation sequences (data not shown). Quantitative DNA dot-blot analysis was carried out by hybridizing dot blots of genomic DNA to ^{32}P-labeled CAT probe and scintillation counting the hybridized spots. Copy number was estimated by comparing the cpm hybridized to the genomic DNA to the cpm hybridized to a known amount of plasmid DNA.

Cell culture and transfections of 3T3-F442A cells were carried out as described (16).

Construction of Transgenes. The plasmids used for the 168aP2CAT and 247aP2CAT transgenes have been described (7). For the 1.7aP2CAT transgene, an *Eco*RI (kb −1.7) to *Pst* I (bp +21) fragment was ligated upstream of CAT, and the 5.4aP2CAT transgene was generated by ligating an *Eco*RI fragment containing kb −1.7 to kb −5.4 into the 1.7aP2CAT transgene (see Fig. 1*A*). Deletion constructs of the fragment between kb −5.4 and kb −1.7 were made by blunt-end ligation of the various restriction fragments (see Fig. 1*A*) into *Hin*dIII-digested and filled-in −63aP2CAT (7).

CAT Assays. CAT assays were carried out according to Lopata *et al.* (17). For all of the 168aP2CAT, 247aP2CAT, and 1.7aP2CAT transgenic tissues and for the tissues from the 5.4aP2CAT and 540aP2CAT transgenic animals expressing low levels of CAT enzyme, 200–300 μg of protein was assayed for 3–5 hr. To be within the linear range of the assay (<50% conversion of chloramphenicol to its acetylated forms), assays on extracts prepared from the fat, spleen, and thymus of the 5.4aP2CAT and 540aP2CAT mice were performed with 0.5–1 μg of protein for 30 min to 1 hr. The acetylated spots were cut out of the TLC plate and radioactivity was measured. Specific activities are presented as cpm per μg of protein per min of reaction time.

RNA Isolation and Analysis. RNA was isolated from tissues according to the method of Chirgwin *et al.* (18). RNase T1 protection assays were carried out as described (19); the temperature of hybridization of the probes to the RNA were 37°C for all probes containing CAT sequences. Integrity of the RNA was checked by Northern blot analysis and hybridization to a housekeeping gene, glyceraldehyde phosphate dehydrogenase (20).

RESULTS

Adipose-Specific Expression Depends on Distal 5′ Flanking Sequences in Transgenic Mice. We first attempted to determine whether transgenes containing either 168 bp or 247 bp of the aP2 5′ flanking region were sufficient to drive transgene expression in mice, since both of these constructs had been shown to function in a differentiation-dependent manner in tissue culture cells (7). Inserts containing either bp −168 to bp +21 (168aP2CAT) or bp −247 to bp +21 (247aP2CAT) of the aP2 promoter and 5′ noncoding region (Fig. 1*A*) linked to CAT sequences and the SV40 small tumor antigen splice and polyadenylylation signals (Fig. 1*B*) were purified and microinjected into the nuclei of murine zygotes, and transgenic strains were generated from the founder animals that resulted from these injections. CAT activity was measured in extracts prepared from the tissues of offspring of the G_1 and subse-

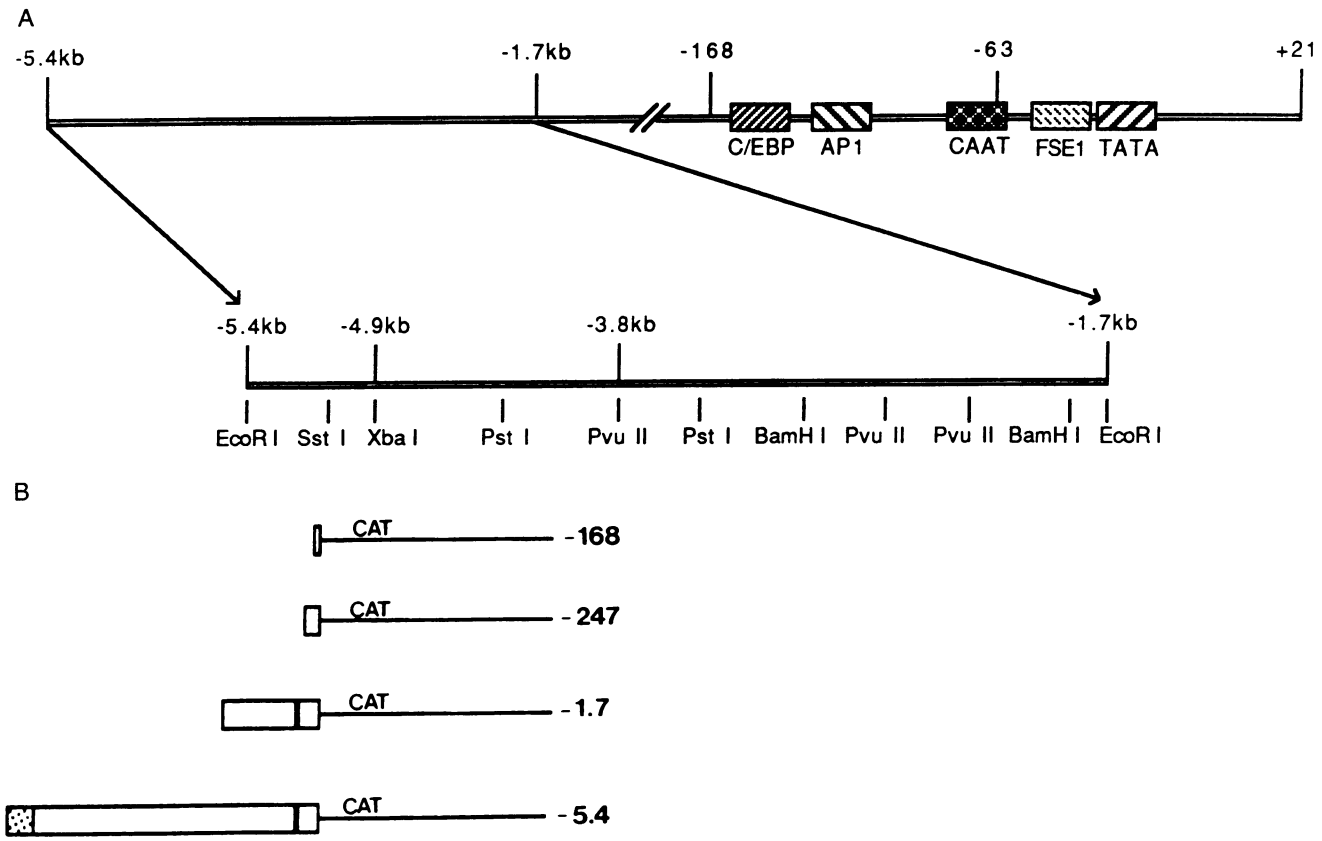

FIG. 1. (*A*) Map of the aP2 5′ flanking region used to construct transgenes. FSE1, fat-specific element 1. (*B*) Map of the −168aP2, −247aP2, −1.7aP2, and −5.4aP2 constructs used to make transgenic mice. Open box, aP2 sequences; line, CAT/SV40 sequences. The putative GRE at bp −363 is represented by a solid bar, and the 540-bp sequence containing the 5′ distal enhancer is represented by a stippled box.

quent generations. In contrast to results previously obtained with cultured cells, the independently generated strains of transgenic mice containing either of these constructs showed little or no CAT activity in adipose tissue (Table 1). This lack of expression was independent of transgene copy number (even the adipose tissue from animals containing 1200 copies contained very low levels of CAT enzymatic activity; Table 1). Moreover, significant expression was seen in several nonadipose tissues, such as spleen, thymus, lung, and brain, although liver CAT levels were very low or undetectable (Table 1). Southern blot analysis of DNA from these animals revealed no gross rearrangement of the transgenes (data not shown).

The lack of aP2 promoter-directed CAT expression in adipose tissue of the 168- or 247aP2CAT transgenic animals implied that we were missing sequences necessary for expression in mice. We, therefore, introduced into mice two additional transgenes that contained more of the 5′ flanking region. Transgene 1.7aP2CAT contained the fragment from kb −1.7 to bp +21 and 5.4aP2CAT contained the fragment from kb −5.4 to bp +21 linked to the same CAT marker gene (Fig. 1*B*). Although the additional sequences in 1.7aP2CAT did not result in higher levels of CAT in adipose tissue, all three strains of transgenic animals containing the 5.4aP2CAT DNA expressed the transgene at very high levels in both white and brown adipose tissue (Table 1). The 5.4aP2CAT animals had little or no activity in most other tissues, including liver. The addition of the region from kb −1.7 to kb −5.4 not only increased the level of expression of the transgene in adipose tissue relative to other tissues but also boosted the overall level of expression in 5.4aP2CAT mice several orders of magnitude. Thus, sequences present in the gene for aP2 between kb −1.7 and kb −5.4 from the transcription start site appeared to be crucial for specific expression in adipose tissue.

A Fat-Specific Enhancer Maps to the 5′ Distal End of the 5.4-kb Fragment and Is the Primary Determinant of the Tissue-Specific Expression of this Gene. The results obtained thus far indicated the presence of an enhancer-like element in the gene for aP2 between kb −1.7 and kb −5.4. To more precisely map this enhancer, we carried out transient transfections with a variety of constructs into undifferentiated and differentiated 3T3-F442A cells (Fig. 2). As a basal promoter, we used −63aP2CAT (referred to as −64aP2CAT in ref. 7) because this construct is relatively inactive in both cell types and is deleted for the proximal regulatory elements (C/EBP, AP-1, and the CCAAT box). Transfected plasmids containing the fragment from kb −5.4 at the 5′ end to kb −1.7, kb −3.8, or kb −4.9 at the 3′ end of the fragment (Fig. 1*A*) ligated upstream of a fragment from bp −63 to bp +21 of the aP2 promoter stimulated higher levels of CAT expression in adipocytes relative to the expression seen from the basal 63aP2CAT construct (Fig. 2). It should be noted that the smallest construct (kb −5.4 to kb −4.9) gave significant stimulation in both orientations when linked to the −63aP2CAT vector (data not shown). A plasmid containing the region from kb −4.9 to kb −3.8 ligated upstream of this same promoter/CAT sequences showed no enhancement of expression. The 540-bp fragment mapping between kb −4.9 and kb −5.4, when linked to the minimal aP2 promoter, did not significantly enhance expression in preadipocytes (data not shown), indicating that its enhancing function was differentiation dependent.

To determine if the region between kb −4.9 and kb −5.4 could function as an enhancer in animals, we made transgenic mice containing this 540-bp fragment (linked upstream of the aP2 promoter at bp −63) and measured CAT activity in various tissues. As shown in Table 1, this construct (540aP2CAT) functioned in a fat-specific manner. Adipose tissue contained from 3- to 50-fold higher levels of CAT activity than any other tissue in the animal, depending on the transgenic strain. This degree of adipocyte specificity is generally consistent with the tissue-specific expression of the endogenous gene (21, 22). As was seen with 5.4aP2CAT mice, transgene expression was orders of magnitude higher in the 540aP2CAT animals relative to the levels seen in transgenic animals lacking the enhancer (Table 1).

It was possible that the CAT activity that we detected in the adipose or other tissues of transgenic mice containing this construct was due to initiation at sites formed by the juxta-

Table 1. CAT activity in the tissues of aP2–CAT transgenic mice

Transgene	Copy number	Specific activity in tissues: Liver	Spleen	Thymus	Brain	Skeletal muscle	Lung	White adipose tissue
Progressive promoter deletion								
168aP2-14	50	0	0.033	0.017	0.001	ND	0.002	0
247aP2-3$_1$a	1255	0.012	1.351	3.295	0.305	0.107	0.051	0.07
247aP2-3$_2$	21	0	0.009	0.012	0.024	ND	0.011	0.006
247aP2-11	12	0.01	0.187	0.125	0.014	0.008	0.047	0.012
1.7aP2-3	5	0	0.456	0.241	ND	0	0.003	0.002
1.7aP2-6	45	0.004	0.814	0.402	0.006	0.003	0.007	0.034
1.7aP2-8	4	ND	0.484	0.001	0.425	0	0.019	0.008
5.4aP2-17	5	1	2200	4400	4	ND	1400	>11,000*
5.4aP2-18	5	8	450	360	29	43	73	860
5.4aP2-25	<5	27	93	130	0	ND	43	1,300
Upstream enhancer construct								
540aP2-2	10	26	1	17	0	4.2	1	1,235
540aP2-6	25	0.052	0	0.037	0	0.158	0	0.598
540aP2-11	5	0.025	0.166	0.195	0.057	0.178	0	9.065

CAT specific activities are presented as cpm of acetylated chloramphenicol per μg of protein per min of reaction time. Zero specific activity represents specific activities of <0.001. Each number represents the average of at least two independent assays on different mice. No gross differences were seen in activity levels between males and females. Transgene 168aP2 contains the *Ap2* gene promoter from bp −168; 247aP2 begins at bp −247, 1.7aP2 begins at kb −1.7 and 5.4aP2 begins at kb −5.4. All of these constructs extend to position +21 at the 3′ end. The approximate copy number was determined by quantitative dot-blot analysis. The 540aP2 construct contains from kb −5.4 to kb −4.9 in an inverted orientation ligated to the fragment from bp −63 to bp +21 of the gene for aP2. ND, not determined.
*CAT activity was in the nonlinear range of the assay, so that accurate specific activities could not be determined.

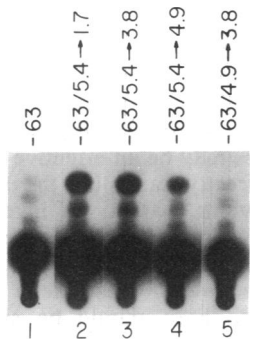

FIG. 2. Deletion analysis of the aP2 upstream regulatory region. CAT assays of extracts from 3T3-F442A adipocytes transiently transfected with 20 μg of plasmid DNA are shown. The origin and orientation of the DNA fragments inserted at the *Hin*dIII site of the basal −63aP2CAT vector is indicated above each lane (e.g., −63/5.4 → 1.7 has the −5.4-kb sequence fused to the −63-bp sequence and the −1.7-kb sequence at the 5′ end of the construct) (see Fig. 1*A*). −63 bp is the basal vector alone.

position of sequences within the 540-bp fragment and the promoter or within the CAT gene itself. We, therefore, carried out RNase T1 protection assays, using a probe that spanned the junction between the aP2 and CAT sequences (Fig. 3). As shown in Fig. 3, RNA transcription initiated at the normal aP2 start site in the adipose tissue of strain 540aP2CAT-2 transgenic mice, resulting in protection of a fragment of 294 nucleotides. CAT transcripts were detected only in the adipose tissue of the 540aP2CAT mice, which most likely reflects the lower levels of transgene expression in the other tissues.

Glucocorticoids Regulate the Transgenes for aP2 Through Promoter-Proximal Sequences. It has been reported that expression of aP2–CAT constructs can be elevated during transient transfection into preadipocytes by treatment of the cells with the synthetic glucocorticoid dexamethasone (9, 13, 14). A putative glucocorticoid response element (GRE) has been identified by sequence homology at position bp −393 (13). In addition, it has been shown in transient transfection assays that glucocorticoids enhance the cAMP-induced expression of aP2–CAT constructs and that the induction of these constructs by cAMP appears to be dependent on a functional AP-1 site at bp −120 (9). To determine whether dexamethasone could regulate the aP2 promoter *in vivo* and to ascertain the sequence requirements for this effect, age- and sex-matched pairs of 247aP2CAT (−GRE, +AP-1 site), 5.4aP2CAT (+GRE, +AP-1 site), and 540aP2CAT (−GRE, −AP-1 site) transgenic siblings were adrenalectomized. One of each pair was then injected with dexamethasone for 2 days and, on day 3, both animals were sacrificed and CAT assays performed on extracts made from adipose tissue. As can be seen in Table 2, glucocorticoid treatment caused an ≈5-fold increase in the level of CAT activity in the adipose tissue of both the 247aP2CAT and 5.4aP2CAT adrenalectomized animals treated with hormone, relative to adrenalectomized mice not treated with hormone. In contrast, no such effect of dexamethasone was observed with the 540aP2CAT transgene, which lacks the C/EBP and AP-1 sites as well as the GRE, as shown in Table 2 for one strain of these mice. Similar results were obtained with the other 540aP2CAT and 5.4aP2CAT transgenic strains (data not shown). Thus, as was seen in tissue culture, the aP2 promoter is responsive to glucocorticoids *in vivo*. Unlike the DNA sequence requirement for adipose tissue specificity, however, responsiveness to this hormone appears to require the proximal promoter region between bp −63 and bp −247 and does not require the upstream enhancer element or the classical GRE at bp −393.

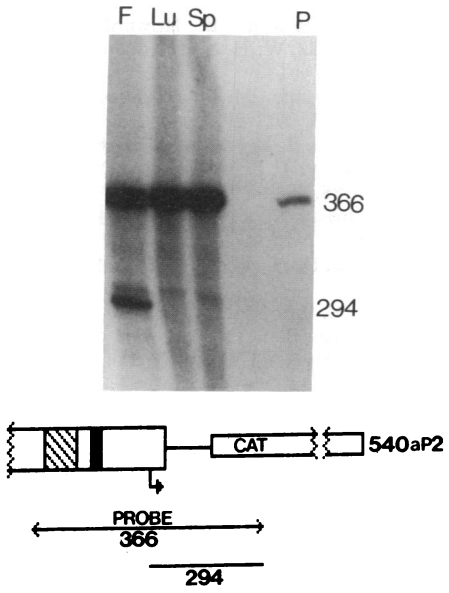

FIG. 3. RNase T1 protection assay of RNA isolated from the tissues of a 540aP2CAT-2 transgenic mouse. A fragment spanning the junction between the aP2 promoter and the CAT sequences from 540aP2CAT DNA was subcloned into a Gemini vector and used to make the probe of 366 nucleotides. Transcripts initiating from the aP2 sequences in the 540aP2CAT transgene protect a fragment of 294 nucleotides. Lanes: F, white fat; Lu, lung; Sp, spleen; P, probe alone.

DISCUSSION

In this study, we show that an enhancer element in the gene for aP2 can confer high levels of expression to a linked marker gene in the adipose tissue of transgenic animals. This element is located within a 540-bp fragment 5.4 kb upstream from the start of transcription and appears to be the major determinant of the fat-specific expression of the gene for aP2. We base this conclusion on the facts that (*i*) this upstream enhancer has a degree of specificity that is consistent with the tissue-specific expression of the endogenous gene and (*ii*) this enhancer appears to be the major positive-acting element found in the largest (5.4 kb) 5′ flanking region constructs studied. Previous studies had shown that only 168 bp upstream of the transcription initiation site was sufficient to confer differentiation-dependent expression of linked marker genes in cultured cells (7, 9, 13, 14). However, when we tested the same construct in transgenic animals, adipose tissue-specific expression was not observed. Instead, the 540-bp enhancer element, acting in concert with a promoter containing only the TATA box and one fat-specific element site 1, was found

Table 2. Dexamethasone-induced expression in the adipose tissue of 247aP2CAT, 5.4aP2CAT, and 540aP2CAT transgenic mice

Transgene	CAT specific activity		
	− Dex	+ Dex	Fold induction
247aP2CAT 3_1a	0.013	0.060	4.6
5.4aP2CAT 4	729	4286	5.9
540aP2CAT 2	6518	5886	—

See legend of Table 1 for description of CAT specific activity. Animals were adrenalectomized, allowed to recover for 3–4 days, then injected twice with 10 μg of dexamethasone acetate (Dex) in saline, 24 hr apart. Twenty-four hours later the animals were sacrificed and CAT-specific activities in adipose tissue were measured. The numbers in this table represent the results of a single experiment. Essentially identical results were obtained in three experiments.

to direct high level transgene expression to adipose tissue. Although the adipocyte enhancer was ligated to its own minimal promoter in these transgenic studies, we have found (data not shown) that this enhancer can also function in a fat-cell-specific manner when linked to the SV40 promoter in cultured cell transfections. Thus these results suggest that the 540-bp enhancer element has a determinative role in the tissue-specific expression of the gene for aP2 independent of the promoter-proximal elements.

One of the promoter-proximal elements that has been hypothesized to play a role in the adipose-specific expression of this gene binds the transcription factor C/EBP (9, 10). Transient transfection studies showed that cotransfection of the gene coding for the C/EBP factor along with aP2 or stearoyl-CoA desaturase promoter constructs resulted in activation of these fat-specific promoters (9, 10). Several other genes, such as albumin, which is specifically expressed in liver, have also been shown to be transactivated by C/EBP in transient transfection assays (23). Studies of the tissue-specific expression of the C/EBP gene itself have shown that both liver and adipose tissue express the highest levels, tissues such as lung and kidney express intermediate levels, and lymphoid tissue and brain express little or none of this protein (11, 24). Because C/EBP tissue-specific expression and the ability of C/EBP to bind to and activate the promoters for several other genes expressed in fat and liver, it has been proposed that C/EBP may be a central regulator of at least some genes that are involved in energy metabolism (12). We show in this study that the presence of the C/EBP binding sites in the gene for aP2 at bp −140 and bp −65 (CCAAT box) are not sufficient to activate expression of this gene in adipose tissue; moreover, it appears that in the presence of the upstream enhancer, these C/EBP sites are not necessary to achieve adipose expression. Thus, C/EBP does not appear to determine the adipocyte-specific expression of the gene for aP2 in the animal.

The C/EBP transcription factor could, however, be involved in the modulation of expression of the gene for aP2, perhaps in response to metabolic regulation or hormonal stimulus. It has been reported that cAMP-inductive effects are observed in the C/EBP-mediated activation of several genes (12). We show here that glucocorticoid induction of the aP2 promoter is at least partially mediated through promoter-proximal elements between bp −247 and bp −63 that contain an AP-1 and C/EBP binding site but no classical GRE. Clearly, it will be important to determine whether these transcription factor recognition sequences can function as atypical GREs.

Surprisingly, animals lacking the upstream enhancer showed some transgene expression in tissues where endogenous aP2 is not expressed (e.g., spleen) (5, 21, 22), and some of these tissues from 5.4aP2CAT and 540aP2CAT mice also had measurable CAT activity. This low-level CAT activity could be due to transgene copy number or integration site effects or to the presence of adipocytes in these tissues. It is also possible that our transgenes lack a negative regulatory element that suppresses transcription in the inappropriate tissue. Some of the transcription factors that positively regulate expression from the gene for aP2 may be present in different tissues; for example, the AP-1 site in the promoter may function in different cell types in the absence of such a negative element. What is clear is that the positive-acting factors cannot function efficiently in adipose tissue without the 5′ distal enhancer.

The identification of a DNA sequence element that is able to direct high levels of expression to adipose tissue *in vivo* may be extremely useful in that it allows us a mechanism for altering the overall metabolism of this tissue and systemic energy balance in the context of the animal. As an example, this element can be used to alter the levels of endogenous genes that are thought to play key roles in the functioning of the adipocyte, thereby allowing us to better understand their roles in both adipose homeostasis and in disease states involving this tissue. Moreover, adipose-directed expression of exogenous genes may be an effective method to alter fat storage and thus directly manipulate the fatness of transgenic animals. The previous inability of investigators to direct expression specifically to adipose tissue has been a major problem that has hampered the use of transgenic technology to regulate the fatness of domestic feed animals. The distal enhancer described here should be specific and strong enough to permit initial experimentation toward this end.

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