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Analysis of a Tissue-Specific Enhancer: ARF6 Regulates Adipogenic Gene Expression

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The molecular basis of adipocyte-specific gene expression is not well understood. We have previously identified a 518-bp enhancer from the adipocyte P2 gene that stimulates adipose-specific gene expression in both cultured cells and transgenic mice. In this analysis of the enhancer, we have defined and characterized a 122-bp DNA fragment that directs differentiation-dependent gene expression in cultured preadipocytes and adipocytes. Several *cis*-acting elements have been identified and shown by mutational analysis to be important for full enhancer activity. One pair of sequences, ARE2 and ARE4, binds a nuclear factor (ARF2) present in extracts derived from many cell types. Multiple copies of these elements stimulate gene expression from a minimal promoter in preadipocytes, adipocytes, and several other cultured cell lines. A second pair of elements, ARE6 and ARE7, binds a separate factor (ARF6) that is detected only in nuclear extracts derived from adipocytes. The ability of multimers of ARE6 or ARE7 to stimulate promoter activity is strictly adipocyte specific. Mutations in the ARE6 sequence greatly reduce the activity of the 518-bp enhancer. These data demonstrate that several *cis*- and *trans*-acting components contribute to the activity of the adipocyte P2 enhancer and suggest that ARF6, a novel differentiation-dependent factor, may be a key regulator of adipogenic gene expression.

In the course of mammalian development, a single fertilized egg gives rise to all of the specialized cell and tissue types present in the adult organism. The development of morphologically and functionally distinct cell types results from the execution of an elaborate program of differential gene expression. Identification of the regulatory mechanisms that control differential gene expression is essential to an understanding of development. Cultured cells that differentiate in vitro into distinct cell types have been very useful in defining the molecular events that lead to terminal differentiation.

The differentiation of adipocytes can be studied in many rodent preadipocyte cell lines (5, 20, 25). Expression of several genes characteristic of adipose tissue is increased during differentiation (19, 31). Some of these genes are expressed in a differentiation-dependent manner in several cell types; for example, glycerol-3-phosphate dehydrogenase mRNA is found in differentiated myocytes as well as adipocytes (11, 23), and stearoyl coenzyme A desaturase mRNA is found in differentiated hepatocytes and adipocytes. Other genes, e.g., the serine protease adipsin and the lipid-binding protein adipocyte P2 (aP2) genes, are expressed only in adipose cells (2, 33).

The molecular basis of adipocyte-specific gene expression is not well understood. We have been studying the transcriptional regulation of the aP2 gene as a model system. Previous studies have shown that the proximal promoter region (168 or 247 bp of the 5′-flanking region) directs differentiation-dependent gene expression in cultured adipocytes (7, 10). This region contains binding sites for the transcription factors AP1 and C/EBP. In preadipocytes, the AP1 site at −120 is required for the responsiveness of this promoter to cyclic AMP analogs (21). Binding sites for C/EBP are present in the promoter regions of several genes that are expressed in a differentiation-dependent manner (in liver as well as fat), and the expression of C/EBP itself is differentiation dependent (6, 21, 22). C/EBP has therefore been proposed to play a central role in regulating differentiation-dependent gene expression (32). Both the C/EBP and AP1 binding sites are essential for expression from the aP2 proximal promoter (6, 21).

Studies using transgenic mice have demonstrated that the proximal promoter of the aP2 gene and the 5′-flanking DNA to −1.7 kb does not contain sufficient information to direct gene expression to adipose tissue (28). We searched further upstream and identified a 518-bp DNA fragment extending from −5.4 kb to −4.9 kb that can direct gene expression to the adipose tissue of transgenic mice as well as differentiation-dependent gene expression in vitro (17, 28). Previous studies in cultured adipocytes have shown that the enhancer does not appear to have an identifiable binding site for C/EBP, nor can cotransfection of a C/EBP expression vector stimulate enhancer activity in preadipocytes (18). Deletion studies revealed that a 183-bp fragment derived from the 5′ end of the 518-bp enhancer was capable of enhancing activity in adipocytes. DNA mobility shift binding assays established the presence of a functional NF-1 binding site in this 183-bp fragment (referred to as 190 bp in reference 17). In this study, we have attempted to define the molecular basis for the adipocyte specificity of this enhancer. We have identified an apparently novel differentiation-dependent nuclear factor that binds to the aP2 enhancer at two distinct sites. This factor, which we have termed ARF6 (for adipocyte regulatory factor), may play an important role as a regulator of adipocyte gene expression.

MATERIALS AND METHODS

Cell culture, transfections, and CAT assays. 3T3F442A cells were cultured and transfected by the calcium phosphate method previously described (17). Adipocytes were transfected at 4 to 7 days postconfluence, while preadipocytes were transfected at 2 to 4 days postconfluence. The final

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DNA concentration in the transfection mix was 20 μg/ml, consisting of 5 μg of chloramphenicol acetyltransferase (CAT) construct plus 1 μg of human growth hormone construct (internal control) and 14 μg of calf thymus DNA (Sigma, St. Louis, Mo.). Each 100-mm dish of cells received 1 ml of this solution. All transfections were done in duplicate and repeated at least five times, and at least two different preparations of plasmids for each construct were tested. As an internal control, we transfected the human growth hormone gene driven by the mouse metallothionein-1 promoter (pXGH5) and measured the levels of human growth hormone by radioimmunoassay (Nichols Diagnostic Institute, San Juan Capistrano, Calif.). Only experiments in which the transfection efficiencies varied less than 15% are reported. CAT activity was determined as previously described (17). CAT assays were quantitated by using a phosphorimaging device (Molecular Dynamics).

Nuclear extracts and DNA mobility shift assays. Nuclear extracts were prepared as described by Dignam et al. (9) except that the extracts were not dialyzed but frozen immediately after salt extraction of the nuclei and used directly for DNA binding assays. DNA mobility shift assays were carried out as previously described (10) and modified by including 10 pm of nonspecific oligonucleotide in the binding reaction when the ARE6 (adipocyte regulatory element) containing oligonucleotide was used as a probe.

Plasmids and mutagenesis. The pSVKSI CAT vector was made by ligating the CAT gene and simian virus 40 (SV40) early promoter from pSVIBCAT (4) as a *Bam*HI-*Bgl*II fragment into the *Bam*HI site of pBluescriptKSII+ (Stratagene, La Jolla, Calif.). Multimerized oligonucleotides were inserted into the *Sma*I site of pSVKSI CAT. The sequence of the inserts was verified by DNA sequencing using the Sequenase kit (U.S. Biochemical, Cleveland, Ohio). The Amersham (Arlington Heights, Ill.) oligonucleotide-directed in vitro mutagenesis system was used to mutate the 518-bp enhancer. The single-stranded phagemid template for mutagenesis was isolated from DH5αF'IQ cells (Bethesda Research Laboratories, Bethesda, Md.) transformed with a pGEM11Zf+ plasmid (Promega, Madison, Wis.) containing the 518-bp enhancer inserted into the *Eco*RI and *Xba*I sites. The bacteria were infected with helper phage M13K07, and single-stranded phagemid was isolated as described by the manufacturer (Promega). The DNA sequence of the mutation was verified by DNA sequencing.

Nucleotide sequence accession number. The GenBank accession number for the sequence reported in this paper is M84651.

RESULTS

We have previously demonstrated that a 518-bp enhancer from −5.4 kb to −4.9 kb of the aP2 gene can direct adipocyte-specific gene expression in cultured cells or transgenic mice (17, 28). Figure 1 shows the sequence of this tissue-specific enhancer; the protein-binding sites that we will discuss are underlined. For the purpose of discussion, we have assigned coordinates to the enhancer beginning with the *Eco*RI site at −5.4 kb (bp 1) and numbered consecutively thereafter.

With the goal of identifying the *cis*- and *trans*-acting components that are central to the differentiation-dependent activity of the enhancer, we have extended our characterization of this region. In previous work, it was shown that a 183-bp fragment derived from the 5′ end of this enhancer (bp 1 to 183) stimulates promoter activity in cultured adipocytes;

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        10          20          30          40          50          60
GAATTCCAGC AGGAATCAGG TAGCTGGAGA ATCGCACAGA GCCATGCGAT TCTTGGCAAG
                                                      ARE1(NF-1)

        70          80          90         100         110         120
CCATGCGACA AAGGCAGAAA TGCACATTTC ACCCAGAGAG AAGGGATTGA TGTCAGCAGG
                                  ARE6

       130         140         150         160         170         180
AAGTCACCAC CCAGAGAGCA AATGGAGTTC CCAGATGCCT GACATTTGCC TTCTTACTGG
   ARE2                                                    ARE7

       190         200         210         220         230         240
ATCAGAGTTC ACTAGTGGAA GTGTCACAGC CCAAACACTC CCCCAAAGCT CAGCCCTTCC
                           ARE4

       250         260         270         280         290         300
TTGCCTTGTA ACAATCAAGC CGCTCCTGGA TGAACTGCTC CGCCCTCTGT CTCTTTGGCA

       310         320         330         340         350         360
GGGTTGGAGC CCACTGTGGC CTGAGCGACT TCTATGGCTC CCTTTTCTGT GATTTTCATG

       370         380         390         400         410         420
GTTTCTGAGC TCTTTTCCCC CGCTTTATGA TTTTCTCTTT TTGTCTCTCT CTTGCTAAAC

       430         440         450         460         470         480
CTCCTTCGTA TATATGCCCT CTCAGGTTTC ATTTCTGAAT CATCTACTGT GAACTATTCC

       490         500         510      518
CATTGTTTGC CAGAAGCCCC CTGGTTCTTC CTTCTAGA
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FIG. 1. DNA sequence of the aP2 enhancer from −5.4 kb (*Eco*RI; bp 1) to −4.9 kb (*Xba*I; bp 518). Underlined nucleotides represent protein recognition sites.

the cell type specificity of this activity was not explored (17). We have tested this fragment and two shorter fragments derived from it for their ability to stimulate CAT gene expression from the enhancerless SV40 early promoter (pSVKSI CAT) when transfected into cultured preadipocytes or adipocytes. All three fragments (183, 134, and 122 bp; Fig. 2) stimulated promoter activity in differentiated adipocytes but not in preadipocytes; all transfections presented in this report were performed in duplicate and normalized to an internal control of a cotransfected human growth hormone gene driven by the metallothionein promoter (pXGH5; see Materials and Methods). While deletion of bp 1 to 49 had no effect on overall enhancer activity (Fig. 2; 183 bp versus 134 bp), deletion of bp 49 to 61 significantly reduced (ca. 50%) the enhancing activity within this series of constructs (Fig. 2; 134 bp versus 122 bp). This region (bp 49 to 61) contains the ARE1 site that binds a member of the NF-1 family and that has previously been shown by mutational analysis to contribute to the enhancing activity of the 518-bp fragment (17). Nevertheless, the 122-bp fragment is effective in stimulating expression from the SV40 promoter in a tightly differentiation-dependent manner.

Preliminary analysis of protein binding to the 122-bp fragment by DNase I protection experiments revealed a prominent footprint extending from bp 115 to 135 (20a). We analyzed the binding of nuclear proteins to a double-stranded oligonucleotide spanning this region (ARE2; Fig. 3A) by DNA mobility shift binding assays (13, 15). A prominent DNA-protein complex is formed after incubation of ARE2 DNA with either preadipocyte or adipocyte nuclear extracts (indicated by the arrow; Fig. 3B; lanes 1 and 6). The sequence specificity of complex formation was assessed by using different oligonucleotides as competitors (Fig. 3A). The cognate oligonucleotide competed for the formation of the most prominent complex, whereas an oligonucleotide containing a five-base mutation in the ARE2 binding site (ARE2M1) as well as an oligonucleotide containing the consensus binding site for C/EBP (CAAT) did not compete (Fig. 3B, lanes 3 and 4). This factor was designated ARF2. In

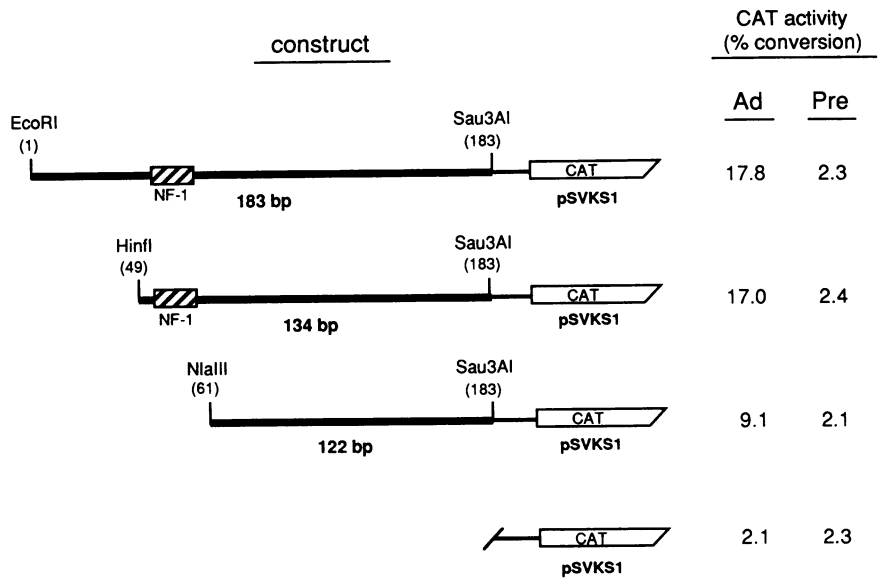


FIG. 2. Schematic maps and CAT activities of deletion constructs. Restriction fragments derived from the 518-bp aP2 enhancer (indicated by the heavy bar) were inserted into the *Sma*I site of the pSVKSI CAT reporter vector. The size of each fragment is indicated with the exact base pair coordinates in relation to the whole enhancer indicated in parentheses below the restriction site; see Fig. 1 for coordinate system. The NF-1 site is indicated by the hatched box. The CAT activities resulting from the transient transfection of constructs (5 μg of DNA) into either preadipocytes (Pre) or adipocytes (Ad) are indicated. The data reported represent numerical averages of duplicate experiments. See Materials and Methods for details of transfection procedures.

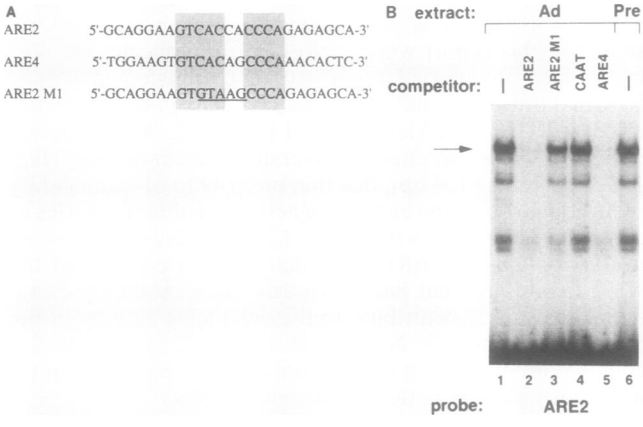


FIG. 3. Sequences and DNA mobility shift binding assays of ARE2-containing oligonucleotides. (A) Sequence comparison of the ARE2 and ARE4 motifs (shaded) and flanking regions from the aP2 enhancer. Changes in the DNA sequence that result in mutation of the protein-binding site are indicated by the underlining. (B) A double-stranded oligonucleotide containing an ARE2 binding site was radiolabeled and then incubated with nuclear proteins extracted from adipocytes (Ad) or preadipocytes (Pre), and the DNA-protein complexes were resolved from free DNA by electrophoresis on 4% polyacrylamide gels (see Materials and Methods). Sequence specificity of protein binding was assessed by inclusion of a 50-fold molar excess of various double-stranded oligonucleotides indicated above the lanes; a minus sign indicates that no competitor was added. Oligonucleotides used (only one strand is shown):

ARE2, 5'-GATCCAGCAGGAAGTCACCACCCAGAGAGCAAATGGA-3'
ARE2M1, 5'-GGGATTGATGTCAGCAGGAAGTGTAAGCCCAGAGAGCAAATGGAG-3'
ARE4, 5'-GATCCAAGTGTCACAGCCCAAACACTCCCCCAAAGCTCA-3'
CAAT, 5'-GATCCAATTGGGCAATCAGGA-3'

other experiments, the fastest-migrating complex was competed for by nonspecific oligonucleotides (data not shown). By DNA sequence comparison, we identified an additional region of the 518-bp enhancer as a potential binding site for ARF2. This sequence, referred to as ARE4, extends from bp 203 to 213 (Fig. 3A), and is an effective competitor for nuclear protein binding to ARE2 (Fig. 3B, lane 5).

The role of the binding sites (ARE2 and ARE4) for ARF2 in the aP2 enhancer was evaluated by two functional assays. First, ARE2-containing oligonucleotides were multimerized (three copies in tandem) and inserted into the pSVKSI CAT vector. Enhancing activity was then assayed following transient transfection into cultured cells. As shown in Fig. 4A, multiple copies of ARE2 oligomers were capable of significant stimulation of promoter function; however, this activity was not differentiation dependent, since equivalent stimulation was observed in both preadipocytes and adipocytes. In addition to the internal control of pXGH5, we also transfected a CAT construct driven by the Rous sarcoma virus (RS) long terminal repeat as a parallel control (RSVCAT; Fig. 4A, lanes 1 and 4). In a second type of analysis, the ARE2 and ARE4 sites were mutated independently in the context of the 518-bp enhancer, and the resulting constructs were assayed for activity by transient transfection. Although either mutation severely reduced enhancer function as indicated by the reduction in CAT activity (Fig. 4B), some residual enhancing activity was consistently observed. Moreover, the 122-bp fragment containing a mutation in the ARE2 site retained a low level of differentiation-dependent enhancer activity (data not shown).

While these data establish a role for the ARE2 and ARE4 sites in the full function of the aP2 enhancer, they also suggest that some other *cis*-acting element(s) must be present to account for the cell type specificity of the enhancer. To identify this element(s), we examined whether two additional oligonucleotides, X (bp 61 to 116) and Z (bp

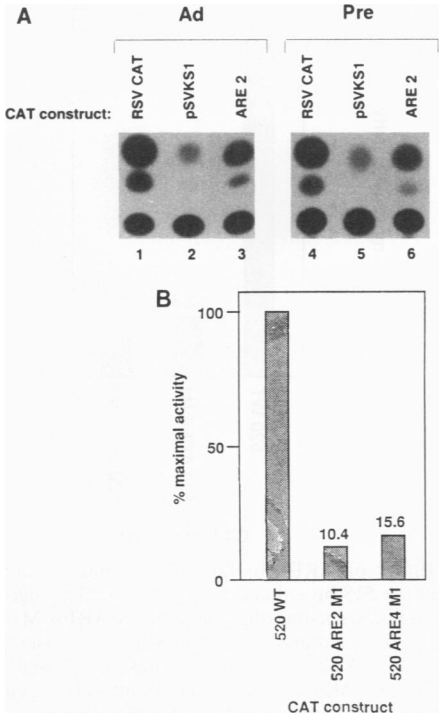


FIG. 4. Activities of ARE2 multimers and effects of ARE2 and ARE4 mutations on enhancer function. (A) The ARE2-containing double-stranded oligonucleotide shown in the legend to Fig. 3B was multimerized (three copies in tandem) and inserted 5′ to the SV40 early promoter in the *Sma*I site of pSVKSI CAT. The ARE2 sequences in this construct are inverted relative to their orientation in the endogenous gene. Samples (5 μg) of this construct (ARE2) and the parental construct (pSVKSI CAT) were transfected into preadipocytes (Pre; lanes 4 to 6) or adipocytes (Ad; lanes 1 to 3), and the level of CAT gene expression was determined by measuring CAT enzyme activity. We also transfected a construct containing the CAT gene under the control of the RSV long terminal repeat, (RSVCAT; lanes 1 and 3. (B) The ARE2 and ARE4 sites in the 518-bp enhancer were mutated by oligonucleotide-directed mutagenesis using oligonucleotides ARE2 M1 (Fig. 3A) and ARE4 M1 (5′-GAGTTCACTAGTGGAAGTGTAAGCTTCCAAACACTCCC CCAAAG-3′). The mutated enhancer was then inserted into the *Sma*I site of the pSVKSI CAT vector, 5-μg samples of these constructs (520 ARE2 M1 or 520 ARE4 M1) were transfected into adipocytes, and the level of CAT gene expression was determined by measuring CAT enzyme activity. These data represent numerical averages of duplicate experiments and are presented as percent maximal enhancer activity; maximal enhancer activity is defined as the stimulation of CAT activity above basal levels by the wild-type (520 WT) enhancer construct.

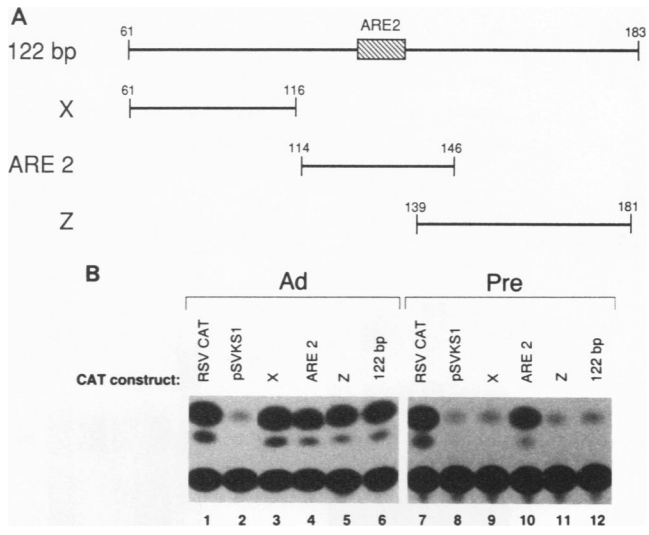


FIG. 5. Oligonucleotides spanning the 122-bp fragment and analysis of their activities. (A) The 122-bp fragment that has differentiation-dependent enhancing activity is schematically illustrated by the heavy line at the top. The coordinates of the fragment are indicated, and the location of the ARE2 site is shown by the hatched box. Three double-stranded oligonucleotides, X, ARE2, and Z, spanning this fragment are indicated with their coordinates. The sequences of the oligonucleotides (only one strand is shown) are as follows: X, 5′-CCATGCGACAAAGGCAGAAATGCACATTTCA CCCAGAGAGAAAGGGATTTGATGTCAG-3′; ARE2, same as in the legend to Fig. 3B; and Z, 5′-GATCCAAATGGAGTTCCCAGA TGCCTGACATTTGCCTTCTTACTGGA-3′. (B) Results of CAT assays from adipocytes (Ad; lanes 1 to 6) or preadipocytes (Pre; lanes 7 to 12) transfected with 5 μg of pSVKSI CAT (lanes 2 and 8) or of pSVKSI CAT constructs containing three copies of oligonucleotide X in an inverted orientation relative to its orientation in the endogenous gene (lanes 3 and 9), three copies of the ARE2 oligonucleotide (see the legend to Fig. 4A) (lanes 4 and 10), four copies of oligonucleotide Z in the same orientation as it is in the endogenous gene (lanes 5 and 11), and the 122-bp *Nla*III-to-*Sau*3AI fragment (see Fig. 2) (lanes 6 and 12). All inserts were cloned into the *Sma*I site of the pSVKSI CAT vector. The RSVCAT construct was also transfected (lanes 1 and 7). See Materials and Methods for details of transfection procedures.

139 to 181) (Fig. 5A), could stimulate differentiation-dependent gene expression. As shown in Fig. 5B, multiple copies of either oligonucleotide X or Z stimulated CAT gene expression from the SV40 promoter in adipocytes but not in preadipocytes. By contrast, the ARE2-containing oligonucleotide again stimulated promoter activity equally in both cell types (Fig. 5B). As parallel controls, we transfected CAT constructs containing the 122-bp enhancer fragment (Fig. 2) or the RSV promoter (RSVCAT). As expected the 122-bp fragment stimulated promoter activity only in adipocytes, whereas the RSV construct produced similar levels of CAT activity in both cell types (Fig. 5B).

Since oligonucleotides X and Z both stimulated differentiation-dependent gene expression, we compared their sequences and found a motif (10- of 12-bp identity) that is present in both oligonucleotides. We will refer to these regions of X and Z as ARE6 (bp 93 to 104) and ARE7 (bp 180 to 169), respectively (Fig. 6A). It should be noted that in the enhancer, the two sites are inverted relative to each other. Nuclear protein binding to the ARE6 site was analyzed by DNA mobility shift binding assays. In Fig. 6B, we show that adipocyte nuclear extracts contain sequence-specific DNA-binding proteins that recognize the ARE6 motif. The most abundant complex (indicated by the arrow) was competed for by either the cognate oligonucleotide (ARE6) or an oligonucleotide containing an ARE7 site (lanes 2 and 7). The ARE2 oligonucleotide and oligonucleotides that contain mutations in the ARE6 site (ARE6 M1 and ARE6 M2; Fig. 6A) were not able to compete for binding (Fig. 6B, lanes 3 to 5). A complex of similar mobility and sequence specificity is formed when the ARE7 oligonucleotide is used as a probe (data not shown). These data suggest that the same protein recognizes the ARE6 and ARE7 sites and that mutations in the common motif disrupt protein binding. This factor was designated ARF6.

To evaluate the differentiation dependence of ARF6 bind-

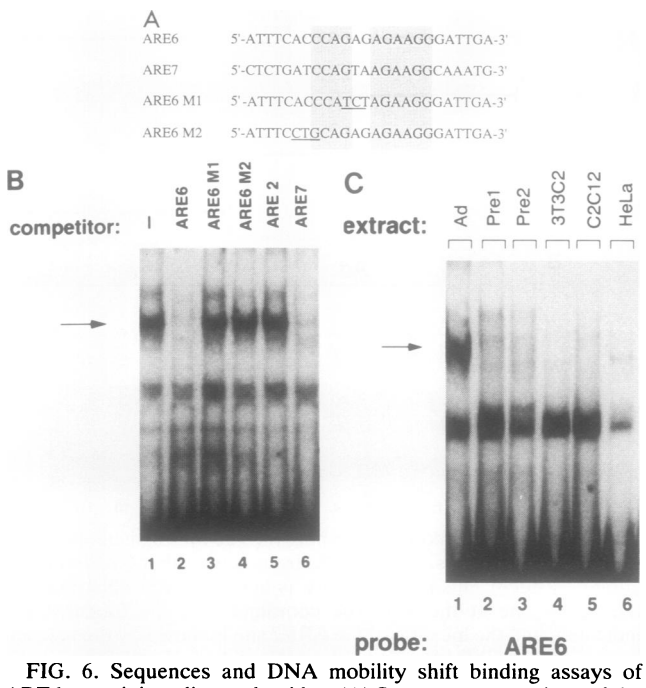


FIG. 6. Sequences and DNA mobility shift binding assays of ARE6-containing oligonucleotides. (A) Sequence comparison of the ARE6 and ARE7 motifs (shaded) and flanking regions from the aP2 enhancer. Mutations of the ARE6 motif (M1 and M2) that interfere with protein binding are indicated by underlining. (B) A double-stranded oligonucleotide containing an ARE6 binding site was radiolabeled and then incubated with nuclear proteins extracted from adipocytes, and the DNA-protein complexes were resolved from free DNA by electrophoresis on 4% polyacrylamide gels. Sequence specificity of protein binding was assessed by inclusion of a 50-fold molar excess of various double-stranded oligonucleotides indicated above the lanes; a minus sign indicates that no competitor was added. Oligonucleotides used (only one strand is shown):

ARE6, 5'-TCGGCGCCATTTCACCCAGAGAGAAGGGATTG-3'
ARE6M1, 5'-GCACATTTCACCCATCTAGAAGGGATTG-3'
ARE6M2, 5'-GCACATTTCCTGCAGAGAGAAGGG-3'
ARE2, same as in legend to Fig. 3
ARE7, same as oligonucleotide Z in Fig. 5

(C) The radiolabeled ARE6-containing oligonucleotide (see Fig. 3B) was incubated with equal amounts of nuclear protein prepared from various cultured cell lines: Ad, 3T3F442A adipocytes; Pre1, 3T3F442A preadipocyte preparation 1; Pre2, 3T3F442A preadipocyte preparation 2; 3T3C2, fibroblasts; C2C12, undifferentiated myoblasts; HeLa, HeLa cells.

ing to the ARE6 motif, nuclear extracts were prepared from several different cultured cell lines and used as the source of binding proteins in DNA mobility shift assays. Nuclear extracts from 3T3F442A preadipocytes (two different preparations), 3T3C2 fibroblasts, C2C12 myoblasts, or HeLa cells were not able to form sequence-specific DNA-protein complexes with the ARE6-containing oligonucleotide (Fig. 6C). This result is not due to the quality of the extracts, since the same extracts form sequence-specific DNA-protein complexes with the ARE2-containing oligonucleotide (Fig. 3; data not shown). Thus, the differentiation-dependent enhancing activity of the ARE6 site correlates with the presence of a factor (ARF6) found only in adipocyte nuclear extracts that recognizes this sequence.

Finally, to determine the functional significance of the

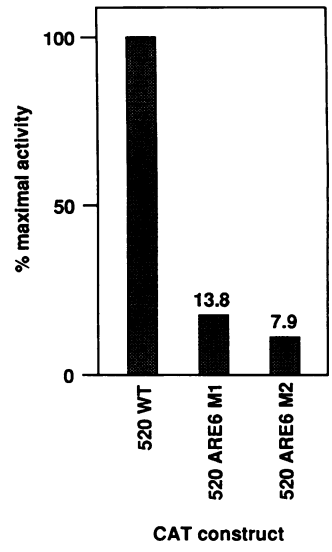
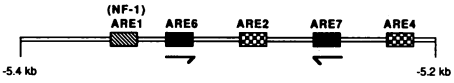


FIG. 7. Effect of ARE6 mutation on enhancer function. The ARE6 site in the 518-bp enhancer was mutated by oligonucleotide-directed mutagenesis using oligonucleotides ARE6 M1 and ARE6 M2 (Fig. 6A). The mutated enhancer was then inserted into the *Sma*I site of the pSVKSI CAT vector, these constructs (520 ARE6 M1 and 520 ARE6 M2) were transfected into adipocytes, and the level of CAT gene expression was determined by measuring CAT enzyme activity. These data represent numerical averages of duplicate experiments and are presented as percent maximal enhancer activity; maximal enhancer activity is defined as the stimulation of CAT activity above basal levels by the wild-type (520 WT) enhancer construct.

ARE6 site in the context of the 518-bp enhancer, we assayed the effects of two independent mutations that disrupt protein binding to the ARE6 site. Each of these mutations (ARE6 M1 and ARE6 M2; Fig. 6A) severely reduces enhancer activity in adipocytes (Fig. 7). Neither mutation had any effect on the minimal level of enhancer activity in preadipocytes.

DISCUSSION

The tissue-specific expression of genes often results from the interaction of several transcription factors that have binding sites clustered within a relatively short stretch of DNA (12, 14, 26). These regulatory segments of DNA (enhancers) can frequently function at considerable distances from the promoter and can usually function independently of orientation. We previously identified a tissue-specific enhancer from the aP2 gene that can confer cell type specificity on a heterologous promoter in both transgenic animals and cultured adipocytes. This 518-bp element, located at −5.4 to −4.9 kb of the aP2 gene, can function in a reversed orientation when inserted near a minimal promoter (17, 18, 28). Preliminary characterization of the enhancer demonstrated that a 183-bp fragment (bp 1 to 183) possessed the majority of the enhancing activity and that an NF-1 site at bp 53 to 58 was important for enhancer activity (17). In this study, we further define the molecular basis of this enhancer's cell type specificity. By the use of deletions, we show that a fragment of the enhancer from bp 61 to 183 (Fig. 1 and 2; 122 bp) can stimulate gene expression from the SV40 early promoter in a differentiation-dependent manner. We have assayed several other restriction fragments derived



autonomous enhancing activity	–	+	+	+	ND
differentiation-dependent activity	NA	+	–	+	ND
cell type-specific DNA binding	?	+	–	+	–
effect of mutation on enhancer function	+	+	+	ND	+

FIG. 8. Summary of the properties and locations of *cis*-acting elements in the 5′ 225 bp of the enhancer. The protein-binding sites are shaded, and sites with similar motifs are indicated by similar shading. The orientations of the ARE6 and ARE7 sites are indicated since they are inverted relative to each other. The properties of the elements are summarized at the bottom. NA, not applicable; ND, not determined; ?, unknown at present.

from the 518-bp enhancer and have not been able to detect any enhancing activity unless this 122 bp was included (data not shown).

To identify the *cis*-acting element(s) in the enhancer that possess enhancing activity in the absence of other protein-binding sites, we have routinely multimerized oligonucleotides, inserted them upstream of the enhancerless SV40 early promoter linked to CAT, and assayed their function by transient transfection into cultured preadipocytes or adipocytes. This approach (27, 29) is very useful in that it allows a relatively clear examination of the cell type specificity of each site that has such autonomous enhancing activity. However, one limitation of this approach is that it would not allow us to identify any factor that does not function in the absence of other enhancer elements. A factor that functions only in the context of an additional *cis* element, either in a synergistic manner or as a repressor of activity, would have been overlooked. Indeed, the NF-1 site (bp 53 to 58) that we previously identified falls into this class. Multimers of this site have no activity on their own; nevertheless, when this site is mutated, there is a significant reduction in enhancer activity (3). It remains to be determined whether NF-1 is playing a regulatory role in the function of the enhancer or whether it acts only to potentiate the overall activity (see below).

A summary of our results is presented in Fig. 8. Within the 5′ 225 bp of the aP2 enhancer, we have identified five protein recognition sites. Multimers of ARE2 or ARE4 stimulate promoter activity in all cell lines examined, including NIH 3T3, C2C12, 3T3C2, and HeLa. Consistent with these data, we have detected sequence-specific binding proteins for these elements in nuclear extracts derived from all of the aforementioned cell lines. Moreover, the ARE2 and ARE4 sites share a common motif and appear to be recognized by the same protein(s) (ARF2), as judged by competition studies in DNA mobility shift binding assays. Both of these sites are important for enhancer activity; mutation of either site severely reduces (but does not abolish) the activity of the 518-bp enhancer. Thus, these sites contribute to the overall activity of the enhancer but probably do not explain the cell type specificity of the enhancer.

By contrast, multimers of ARE6 and ARE7 stimulate promoter activity only in adipocytes. Consistent with these activity data, we show by DNA mobility shift assays that (i) the ARE6 and ARE7 sites share a similar motif and appear to bind the same protein (ARF6), as judged by competition analysis, and (ii) protein binding to these sites is differentiation dependent. ARE6 and ARE7 binding activities can be detected in nuclear extracts from adipocytes but not from other cell types (e.g., preadipocytes, 3T3C2, C2C12, or HeLa cells). Thus, in undifferentiated cells, ARF6 is either absent or modified such that it is no longer able to bind to these sites. The ARE6 site is critical for enhancer activity, since mutation of this site severely reduces the activity of the 518-bp enhancer.

Both of the *trans*-acting factors and presumably all four binding sites identified here appear to be essential for enhancer activity, since mutation of a single site reduces enhancer activity by about 80% or more. However, it appears that only the ARE6 and ARE7 binding activities are differentially regulated. This finding suggests that the other important factors are already present in the preadipocyte and that the binding of ARF6 acts as a switch that allows expression of full enhancer activity in adipocytes.

It remains to be seen how the sites that we have identified are integrated to yield the potent enhancer activity that we observed in cells and transgenic animals. Several questions have yet to be answered. How is the positive activity of the ARE2 and ARE4 sites silenced in inappropriate cell types? Though synthetic multimers of ARE2 have autonomous enhancing activity, it is possible they are inactive in their native context. For example, they may function only if they can interact with factors bound at the ARE6 and ARE7 sites. It is also possible that the NF-1 site acts to silence the activity of the ARE2 and ARE4 elements in preadipocytes and synergizes with them in adipocytes. NF-1 is a large family of proteins (16), and it has been observed that several species of NF-1 mRNA change during adipocyte differentiation (20b).

There are many genes whose transcription is increased during adipose conversion (1, 8, 30). The expression of some of these genes is restricted to adipose tissue, while others are expressed in several tissues in a differentiation-dependent manner (2, 33). Although there may be some overlap, it is important to distinguish between tissue-specific and differentiation-dependent gene regulation. For example, C/EBP is expressed in a number of tissues, including liver, fat, intestine, and kidney, and may play a role in modulating differentiation-dependent gene expression in each of these tissues. Additional factors, however, must be required for tissue-specific expression. Recent data suggest that C/EBP may be involved in accelerating the execution of the differentiation program rather than determining the program per se (3). The nature of the program itself may be determined by factors displaying a narrow cell type specificity; these factors presumably initiate tissue-specific gene expression.

From a molecular analysis of the adipocyte-specific aP2 enhancer, we have identified an apparently novel regulatory factor, ARF6, that is present in differentiated adipocytes but not preadipocytes and is critical for enhancer activity. Could ARF6 be a master regulator of adipogenesis, analogous to the MyoD family of myogenic regulators? Members of the MyoD family are key regulators of muscle-specific enhancers; they are cell type specific and often bind at multiple sites within an enhancer. Cloning of the cDNA encoding ARF6 will allow us to examine the cell type specificity of this factor, and forced expression of the cDNA in a variety of cell types should determine whether this factor has broad significance in adipocyte development.

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