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Localization of a Pioglitazone Response Element in the Adipocyte Fatty Acid-Binding Protein Gene

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SUMMARY

The thiazolidinediones are a class of antidiabetic compounds that increase the sensitivity of target tissues to insulin. An earlier study has shown that these compounds enhance the insulin-stimulated differentiation of 3T3-L1 cells and up-regulate expression of differentiation-dependent genes. We have observed that the mRNA encoding the adipocyte fatty acid-binding protein (aFABP) increases shortly after incubation of cells with pioglitazone, a thiazolidinedione analogue. The drug was found to enhance, in a time- and dose-dependent fashion, the expression of a chimeric gene that was constructed by fusing the aFABP promoter upstream of the chloramphenicol acetyltransferase (CAT) gene. To localize the sequence within the promoter that is responsive to pioglitazone, a series of chimeric genes containing sections of the aFABP promoter fused to the CAT gene were analyzed after transfection of 3T3-L1 cells. A section of DNA located at −5.2 kilobases and known to encompass a tissue-specific and differentiation-dependent enhancer element was found to confer responsiveness to the drug. Analysis of sequences in this region of the aFABP promoter by DNA gel retardation assays revealed the presence of a protein in nuclear extracts from drug-treated cells that bound to a specific sequence (ARE-6). The presence of the protein could be demonstrated in differentiated adipocytes, but the protein was present at only low levels in preadipocytes. Treatment of preadipocytes with pioglitazone resulted in the precocious appearance of this protein in nuclear extracts. Multiple copies of the ARE-6 sequence inserted upstream of a heterologous promoter linked to the CAT gene conferred pioglitazone responsiveness. The experiments reported in this study establish that the insulin-sensitizing agent pioglitazone up-regulates expression of the aFABP gene through an element located within a region of DNA responsible for tissue-specific and differentiation-dependent expression of the gene.

In non-insulin-dependent diabetes, target tissues become unresponsive to insulin. Despite an adequate supply of the circulating hormone, adipose and muscle tissue underutilize glucose and the liver fails to suppress glucose output, resulting in hyperglycemia. The loss of carbohydrate homeostatic control by insulin-resistant individuals is often accompanied by defective lipid metabolism, which can lead to further complications such as hypertension and coronary disease (1).

The thiazolidinediones are a class of antidiabetic compounds that increase the sensitivity of target tissues to insulin in rodent models of non-insulin-dependent diabetes (2). Adipocytes from KKA^y diabetic mice demonstrated increases in insulin-stimulated glucose uptake after treatment with the thiazolidinedione analogue pioglitazone (3). Although the drug does not cause any alterations in the number or affinity of insulin receptor binding sites (2), increases in mRNA and protein levels of Glut-4 were observed (3). These data support the concept that the thiazolidinediones amplify postreceptor events in insulin signal transduction, perhaps through effects on gene expression. Our laboratory has shown that pioglitazone and other thiazolidinediones enhance the insulin- or IGF-1-regulated differentiation of 3T3-L1 cells (4). These cells, in the presence of the appropriate signals, undergo differentiation from fibroblastic adipoblasts to mature adipocytes (5). This process has been well characterized and provides an excellent *in vitro* system for studying hormonal regulation and signaling in adipose tissue.

Levels of mRNA encoding differentiation-dependent genes are increased in insulin-treated 3T3-L1 cells (6–8). The aFABP is expressed in a differentiation-dependent fashion and its gene has been studied extensively (9, 10). Although the cellular function of aFABP is unknown, it binds fatty acids (11) and is a substrate for the insulin receptor tyrosine kinase (12), leading to speculation that it may be partially responsible for facilitating insulin signaling in adipocytes. Previous studies have located elements within the aFABP gene promoter that permit its tissue-specific and differentiation-dependent expression (13). Earlier we showed that pioglitazone elicited a dose-dependent increase in expression of a chimeric gene containing 7

ABBREVIATIONS: Glut-4, facilitative glucose transporter; aFABP, adipocyte fatty acid-binding protein; CAT, chloramphenicol acetyltransferase; IBMX, isobutylmethylxanthine; DEX, dexamethasone; kb, kilobase(s); bp, base pair(s); PioRF, pioglitazone response factor; IGF-1, insulin-like growth factor-1; LPL, lipoprotein lipase; G6PDH, glucose-6-phosphate dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; SV40, simian virus 40. C/EBP, CAAT-box/enhancer-binding protein; ARE, adipocyte response element; ARF, adipocyte regulatory factor.

kb of the aFABP promoter fused to the CAT reporter gene, indicating a role for the drug in regulating transcription (14). In this study, we have delineated the region of the aFABP promoter that confers responsiveness to the thiazolidinediones.

Experimental Procedures

Materials

DMEM, calf serum, fetal calf serum, and the antibiotics G418 and gentamicin were purchased from Life Technologies (Grand Island, NY). DEX, IBMX, and acetyl-CoA were purchased from Sigma Chemical Co. (St. Louis, MO). Insulin was purchased from Boehringer-Mannheim (Indianapolis, IN). [^{14}C]Chloramphenicol, [α-^{32}P]dCTP, and [γ-^{32}P]ATP were purchased from Amersham (Chicago, IL). RNAzol was purchased from Cinna/Biotecx (Dallas, TX). *Thermus aquaticus* polymerase, used for polymerase chain reaction amplification, was purchased from Perkin-Elmer/Cetus (Norwalk, CT). All other enzymes were purchased from Life Technologies unless otherwise indicated.

Cell Culture Conditions

The 3T3-L1 cell line (ATCC CL 173; American Type Culture Collection, Rockville, MD) was maintained in DMEM (high glucose) containing 7.5% calf serum and 10 μg/ml gentamicin. Confluent 3T3-L1 cultures were staged for differentiation by treatment for 48 hr with DMEM containing 7.5% fetal calf serum, 1 μM DEX, and 0.5 mM IBMX. The cells were allowed to differentiate in DMEM containing 7.5% fetal calf serum and 150 nM insulin. Alternatively, stably transfected 3T3-L1 cells were grown to confluence and allowed to differentiate in DMEM containing 5% fetal calf serum, 1 μM DEX, and 150 nM insulin, as described previously (14). By eliminating IBMX from the differentiation medium, we were able to carry out transient expression studies using the chimeric aFABP genes. Pioglitazone and other thiazolidinediones were dissolved in dimethylsulfoxide (Sigma) and added to cells by 1000-fold dilution in medium. The 3T3-L1 cells stably transfected with the aP2(7kb)/CAT chimera were maintained in medium containing 400 μg/ml G418 as described previously (14). Other stable transformants were selected and maintained as described for cells expressing aP2(7kb)/CAT (14). A minimum of 50 colonies were pooled to initiate growth of each stably transformed line. Analysis of the gene copy number of the stably transformed lines revealed that those of aP2–8 and aP2(7kb)/CAT are nearly the same (within 10–15%), whereas that for aP2(−80b)/CAT is 2–2.5 times greater. The gene copy number was not determined for aP2–5. For transient expression analysis, plasmid DNA was precipitated and added to confluent cells (5 μg/60-mm dish) by a modification of the procedure of Graham and Van der Eb (15), using a calcium phosphate cell transfection kit (5′→3′, Boulder, CO). Transfection efficiency was monitored by co-transfection of a plasmid containing a β-galactosidase reporter gene or by transfection of sister cultures with a plasmid containing a Rous sarcoma virus long terminal repeat/CAT chimeric gene. Transfection efficiencies varied by <15% in the experiments reported here.

Molecular Biological Assays

RNA analysis. The relative abundance of mRNA was determined by isolating total RNA from cells using RNAzol (16), immobilizing this RNA on nylon membranes (Duralon UV; Stratagene) (17), and probing for the various messages by hybridization with either random-primed cDNAs (aFABP and G6PDH) or end-labeled antisense oligonucleotides (Glut-4 and LPL) (4, 14, 17).

CAT activity measurements. Stably and transiently transfected cells were harvested and their extracts were assayed for CAT activity by modifications of the procedure of Gorman *et al.* (18). Thin layer chromatography plates (Analtech, Newark, DE) containing the acetylated [^{14}C]chloramphenicol products were imaged and quantitated using an Ambis radioanalytical imaging system (Ambis Instruments, San Diego CA). CAT activity is reported in nmol of acetylated chloramphenicol produced per unit of time.

Plasmid constructs. All plasmid DNAs were prepared by modifications of the alkaline lysis procedure, as described by Sambrook *et al.* (17). Final separation of plasmid DNA from bacterial RNA and protein was performed by anion exchange chromatography using Nucleobond AX columns (The Nest Group, Southboro, MA). Plasmid aP2(7kb)/CAT has been described previously (14) (see Fig. 4). By digestion of this plasmid with *Bam*HI, followed by partial digestion with *Mbo*I and subsequent reclosure, plasmid aP2–5 was created. Plasmid aP2–8 was created by inserting the 770-bp *Ssp*I/*Pst*I aFABP promoter fragment (19) upstream of the CAT reporter gene in plasmid pBasic (Promega, Madison, WI). Plasmid 1–2 was made by inserting the *Eco*RI/*Xba*I, fat-specific, aFABP enhancer upstream of the SV40 promoter in plasmid pPromoter (Promega). Based upon the published aFABP enhancer sequence (13, 20), oligonucleotides were prepared and used to amplify plasmid 1–2 DNA (21). The two amplification products, D-2 and B-3, extended from 1 to 260 bp and from 240 to 518 bp of the aFABP enhancer sequence, respectively. These products were inserted into the *Bgl*II site of plasmid pPromoter. Duplex oligonucleotides GATCTGCACATTTCACCCAGAGAGAAGGGATTGA (ARE-6) and GATCTGCACATTTCAC TTGTAGAGAAGGGATTGA (ARE-6-mut) were cloned into the *Bgl*II site of plasmid pPromoter to make plasmids ARE-6/CAT (four copies of ARE-6 are present) and ARE-6-mut/CAT (four copies of ARE-6-mut are present).

DNA retardation assays. Nuclear extracts from 3T3-L1 cells were prepared as described (22). Incubation of the extracts with labeled probes took place in a volume of 15 μl containing 1 μg of poly(dI·dC), 2 μg of bovine serum albumin, 80 mM KCl, 0.5 mM dithiothreitol, and 20 mM Tris, 7.4. After incubation for 15 min, samples were electrophoresed on 4.5% polyacrylamide gels at 27.5 mA. After electrophoresis, gels were dried and scanned for radioactivity using an Ambis radioanalytical imaging system. The sequences of the oligonucleotides used in these experiments are as follows (only one strand is shown): ARE-2, GCAGGAAGTCACCACCCAAGAGAGCAAAT; ARE-6, TCGGCGCCATTTCACCCAGAGAGAAGGGATTGA; ARE-6M10, TCGGCGCCATTTCACTTGTTTGTAGAGAAGGGATTGA (the underlined bases represent those mutated from ARE-6).

Results

Acquisition of the adipocyte phenotype by 3T3-L1 cells leads to a dramatic rise in cellular levels of glycolytic, lipogenic, and lipolytic enzymes (23–25). The thiazolidinediones enhance the insulin and IGF-1 regulation of 3T3-L1 differentiation (4), and extensive changes in the expression of differentiation-linked mRNAs accompany this process (8, 9). The experiment shown in Fig. 1 examines the relationship between treatment of cells with pioglitazone and/or insulin and changes in the level of mRNA encoding G6PDH, the insulin-responsive Glut-4, aFABP, and LPL in 3T3-L1 cells. Incubation of cultures with pioglitazone alone for 4 days resulted in little or no increase in the relative abundance of the mRNA encoding G6PDH, Glut-4, or LPL. Insulin caused a modest increase in these mRNA species, whereas the simultaneous presence of insulin and the drug elicited the largest increase. The pattern observed for aFABP mRNA expression under the same conditions was strikingly different. Pioglitazone by itself caused a substantial increase in aFABP mRNA, and the simultaneous presence of insulin failed to increase it further. Thus, aFABP appears to be a candidate gene for direct action of the drug, whereas the other genes required the simultaneous presence of insulin and the drug. The time course of aFABP mRNA induction by pioglitazone also supports the notion that the drug exerts an effect directly on the gene (Fig. 2). Cells treated with pioglitazone alone exhibited a nearly 10-fold increase in relative abundance of aFABP mRNA in 4 hr, whereas cells treated with

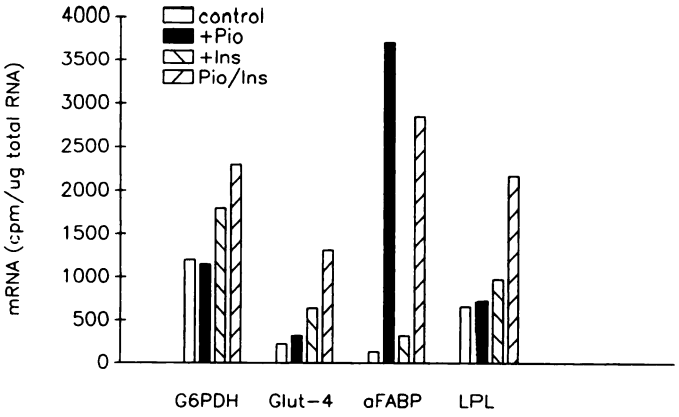

Fig. 1. G6PDH, Glut-4, aFABP, and LPL mRNA abundance in 3T3-L1 cells treated with insulin and pioglitazone. 3T3-L1 cells were grown to confluence in DMEM containing 7.5% calf serum. Confluent cultures were staged to differentiate in medium containing IBMX/DEX, as described in Experimental Procedures. The medium was changed to DMEM containing 5% fetal calf serum, with the additions of 150 nM insulin (*Ins*) and 25 μM pioglitazone (*Pio*) as indicated. After an additional 4 days, the cells were harvested and total RNA was prepared. The RNA was blotted onto Duralon membranes and hybridized to labeled probes for G6PDH, Glut-4, aFABP, or LPL as described in Experimental Procedures. The membranes were assayed for mRNA using an Ambis radioanalytical imaging system. Each *bar* represents the mean activity from three determinations, and the standard error was <10% for all values.

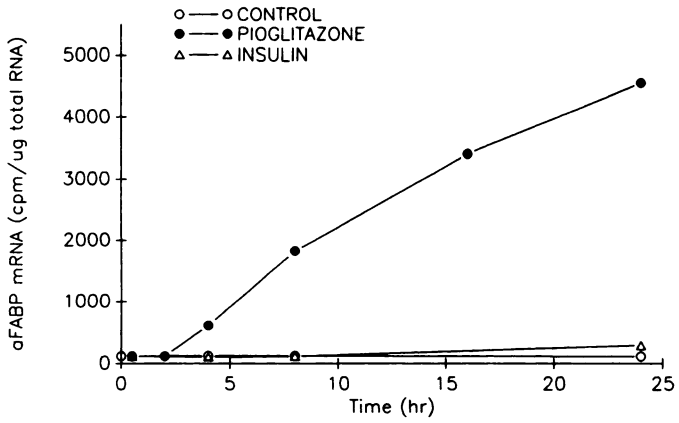

Fig. 2. Effect of pioglitazone on aFABP mRNA relative abundance as a function of time. Cells were grown to confluence and incubated for 48 hr in medium containing IBMX/DEX. Cells were then switched into DMEM containing 7.5% fetal calf serum, with the additions of insulin (150 nM) or pioglitazone (25 μM) as indicated. At the indicated times, cells were harvested and RNA was prepared and blotted. After hybridization to a radiolabeled aFABP probe, the radioactivity was quantified with an Ambis imaging system. Each *point* represents the mean activity from duplicate samples.

insulin alone did not show an increase during the 24 hr of treatment (Fig. 2). With the protocol used here to cause cells to differentiate, the induction of aFABP was much more rapid than that which we reported earlier (Fig. 2) (14), for which longer times (days) of pioglitazone/insulin treatment were typically required to observe induction of aFABP.

We have previously shown that pioglitazone increases the expression of a chimeric gene containing the proximal 7-kb aFABP promoter fused upstream of the CAT reporter gene, aP2(7kb)/CAT (14). In stably transfected 3T3-L1 cells, we observed that the chimera was induced by pioglitazone in a dose-dependent manner, similarly to the endogenous FABP gene (ED_{50} = 0.66 μM and 0.73 μM, respectively) (14). The expression of the chimeric gene in the experiments reported earlier (14) was studied in cells that were staged to differentiate by incubation of confluent cells in the continuous presence of DEX and insulin. However, it is well established that differentiation of 3T3-L1 cells can be achieved by subjecting preadipocytes to several different treatment protocols (5, 8). The other protocol that we employed used a transient incubation (48 hr) of confluent cultures with IBMX/DEX followed by treatment of the cultures with insulin, alone or in combination with pioglitazone. In the experiment shown in Fig. 3, 3T3-L1 cells stably transfected with either aP2(7kb)/CAT or a chimeric gene containing only the proximal 80 bp of the aFABP promoter [aP2(−80b)/CAT] were staged to differentiate using transient incubation in IBMX/DEX. The presence of pioglitazone, added after IBMX/DEX treatment, enhanced the expression of the aP2(7kb)/CAT chimeric gene (Fig. 3), whereas the −80-bp construct was unaffected by this protocol. Thus, regardless of the method used to stage the cells to differentiate, the expression of the chimeric gene was clearly enhanced, provided that the appropriate sequences were present.

To determine the region within the aFABP promoter that is responsive to pioglitazone, 3T3-L1 cells were transfected with a series of chimeric genes encompassing regions of the promoter fused to the CAT reporter gene. In the first series of experiments, cells were stably transfected with plasmids that were composed of either the proximal 750 bp of the promoter fused to the CAT gene (aP2–8) or a distal upstream region of the promoter (−3.1 to −7.0 kb; aP2–5). Stable transformants expressing these constructs were compared with those reported above (Fig. 3) that expressed chimeric genes containing either the entire 7 kb of the promoter or just the proximal 80 bases (Table 1). Analysis of CAT activity revealed that pioglitazone failed to up-regulate expression of the plasmid construct containing the proximal 750 bp of the promoter. This result was surprising, because several important *cis* elements have been reported to be located in this region, including those for C/EBP, cAMP, glucocorticoid receptor, and *fos/jun* (19, 26, 27). The lack of regulation by pioglitazone was not the result of ineffective integration into the cellular genome, because both glucocorticoid and cAMP regulation of expression could be

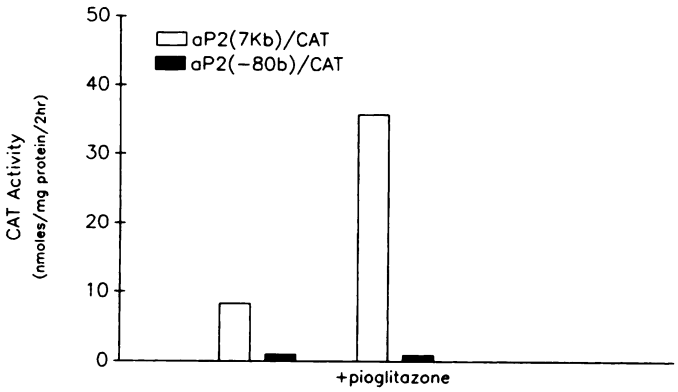

Fig. 3. Induction of aFABP chimeric genes by pioglitazone. Cells stably transfected with either aP2(7kb)/CAT or aP2(−80b)/CAT were grown to confluence and then incubated with IBMX/DEX for 48 hr. The culture medium was replaced with DMEM/7.5% fetal calf serum, containing 150 nM insulin or 5 μM pioglitazone where indicated, and the cells were harvested 4 days later. Values represent the means of triplicate determinations from a representative experiment.

TABLE 1

Influence of pioglitazone on expression of aFABP/CAT genes

Cells that were stably transfected with the indicated plasmids were analyzed for CAT activity after incubation for 4 days in the presence or absence of pioglitazone. Each value represents the mean of triplicate determinations from a representative experiment.

Chimeric gene	CAT activity[a]
	nmol/mg
aP2(−80b)/CAT	1.9
aP2(−80b)/CAT + pioglitazone	1.8
aP2-8	5.4
aP2-8 + pioglitazone	5.1
aP2-5	4.2
aP2-5 + pioglitazone	20.6
aP2(7kb)/CAT	9.1
aP2(7kb)/CAT + pioglitazone	37.2

[a] CAT activity is expressed in units of nmol of acetylated chloramphenicol produced per mg of cell protein.

demonstrated (data not shown). Additional studies with stable transformants containing the proximal 2.7 kb of the aFABP promoter failed to exhibit any pioglitazone regulation of expression (data not shown). In contrast, the promoter construct encompassing the enhancer region of the gene (aP2–5) was found to be strongly up-regulated by the presence of the drug, although overall expression was not as high as that observed for aP2(7kb)/CAT. Thus, the sequences conferring responsiveness to pioglitazone were upstream of −3.1 kb.

A series of experiments were carried out using transient expression of transfected chimeric genes to delineate the location of the pioglitazone response element within the region of the promoter defined by aP2–5 (−3.1 to −7.0 kb). Transient expression analysis of two plasmid constructs (aP2–5 and aP2–8) reconfirmed what was revealed above in stable transfection experiments; the construct encompassing the enhancer region of the promoter (aP2–5) was found to be up-regulated 8.3-fold by the drug (Fig. 4). However, cells transfected with aP2–8 showed that the proximal 800 bases of the promoter were not sufficient to cause up-regulation of CAT expression (pioglitazone elevated expression 1.15-fold). We then focused our attention on the enhancer located within aP2–5, at −4.9 to −5.4 kb, which has been shown in previous studies (13, 22) to contain the only tissue-specific, differentiation-dependent activity upstream of −800 bp. Two sets of chimeric genes were constructed that contained all or selected regions of the enhancer located at −4.9 to −5.4 kb. These constructs also utilized a heterologous promoter, the proximal SV40 promoter, which functions to permit correct and efficient loading of RNA polymerase II. The first set of plasmids, represented by 1–2, which contains the entire 518-bp enhancer element placed upstream of the SV40 promoter, exhibited higher CAT activity upon pioglitazone treatment (Fig. 4). The drug caused a 4.6-fold increase in CAT activity, strongly suggesting that most of the pioglitazone-responsive sequences are located within this 518-bp sequence. The second set of plasmids contained sequences from either the 5′ or 3′ half of the enhancer. Cells transfected with the plasmid containing the 5′ side of the enhancer, plasmid D-2, exhibited increased basal CAT activity, perhaps owing to the removal of negative elements (20, 22), and a 3.9-fold increase in response to pioglitazone, whereas the CAT activity in cells transfected with a plasmid containing the remaining sequences of the enhancer, B-3, was relatively unaffected by pioglitazone treatment (1.3-fold increase). Thus, a pioglitazone response element of the aFABP promoter is located in a region that is

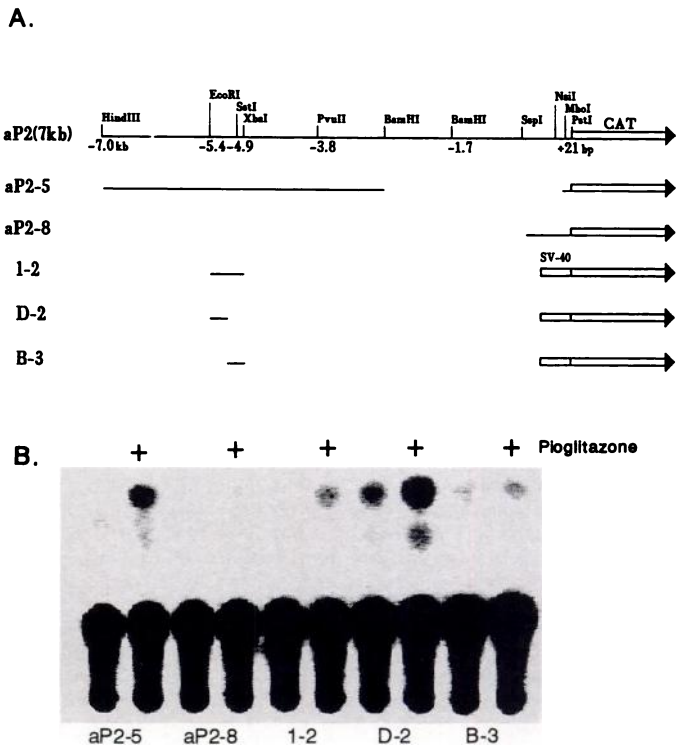

Fig. 4. Delineation of a pioglitazone-responsive element within the aFABP promoter. Confluent cultures of 3T3-L1 cells were transfected with the various plasmids described. Cultures were incubated for 4 days in DMEM containing 7.5% fetal calf serum, 1 μM DEX, and 150 nM insulin, with or without 5 μM pioglitazone. Cells were harvested, and extracts were prepared and assayed for CAT activity. A, Maps of the aFABP promoter/CAT chimeras used for transfection into 3T3-L1 cells. B, Radioanalytical image representing the CAT activity within extracts from transiently transfected 3T3-L1 cells.

known to be responsible for tissue-specific, differentiation-dependent expression of the aFABP gene (13, 20, 22), although these data do not rule out the existence of other, less potent, response elements in flanking regions of the promoter.

To precisely define the sequence within the 5′ side of the enhancer that is responsible for pioglitazone responsiveness, gel retardation assays were carried out. Previous work (20) has shown that nuclear extracts from differentiated adipocytes contain proteins that bind to discrete regions of the DNA sequence defined by plasmid D-2, and this technology should permit the identification of *cis* sequences that may bind proteins induced by drug treatment. Using duplex oligomers representing regions of the enhancer, we identified one sequence that formed a complex with a protein found primarily in extracts from differentiated adipocytes (Fig. 5A). The labeled probe used in this experiment was previously designated as ARE-6 (20). A protein/DNA complex (Fig. 5A, *arrow*) was observed when nuclear extracts from differentiated adipocytes were incubated with the probe but not when extracts from fibroblasts were used. A minor band was also observed, which may reflect the mobility of a different complex or the same major protein/DNA complex lacking a minor component. Treatment of preadipocytes with pioglitazone for 48 or 96 hr enhanced the binding of the factor, compared with cultures that were staged to differentiate in the absence of the drug. Thus, the enhanced binding of the factor induced by the presence of pioglitazone is temporally correlated with the induction

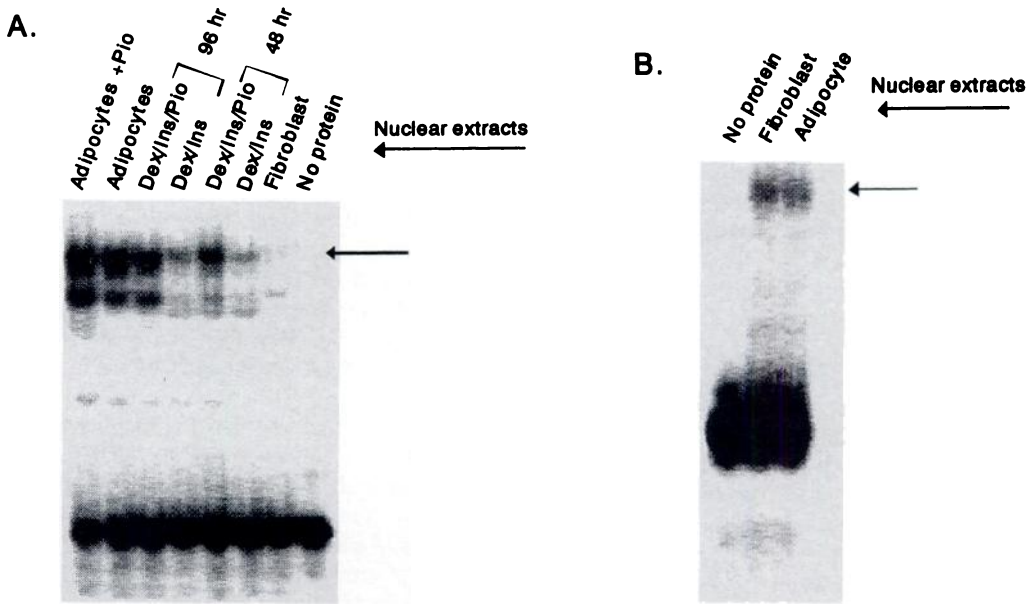

Fig. 5. Gel retardation assays using the ARE-6 and ARE-2 oligomers. Nuclear extracts were prepared from preadipocyte 3T3-L1 fibroblasts, fully differentiated adipocytes, and cultures that were undergoing differentiation after incubation with 1 μM DEX and 150 nM insulin (*DEX/Ins*) or DEX, insulin, and 5 μM pioglitazone (*DEX/Ins/Pio*) for the indicated length of time. Equivalent amounts of the nuclear protein extract from each condition were incubated with end-labeled ARE-6 (A) or ARE-2 (B) (10,000 cpm/reaction) for 15 min, as described in Experimental Procedures. Samples were then electrophoresed, and the gel was dried and scanned for radioactivity. *Arrows*, location of the major protein/oligomer complexes.

of the chimeric gene (14), suggesting that the factor is the PioRF. Another major protein factor, ARF-2, that binds to a sequence (ARE-2) of the enhancer in the pioglitazone-responsive region (20) was found not to be influenced by differentiation (Fig. 5B) or affected by drug treatment (data not presented).

If pioglitazone up-regulates the aFABP gene through the ARE-6 sequence, then the appearance of the PioRF should also be correlated temporally with that of the mRNA encoding aFABP. We demonstrated earlier that the drug can increase the relative abundance of the mRNA for aFABP in the absence of insulin in cells previously incubated with IBMX/DEX (Fig. 2). Nuclear extracts were prepared from cells treated with IBMX/DEX and incubated with DEX/insulin or pioglitazone alone for 6 hr. We observed the appearance of the factor in extracts from cells treated with the drug, whereas cells treated with DEX and insulin exhibited only a low level (Fig. 6). Thus, 6 hr of drug treatment are sufficient to cause up-regulation of endogenous aFABP expression and the presence of the factor that binds to ARE-6 in the nuclear extracts of treated cells, suggesting that pioglitazone induction of aFABP is mediated through the PioRF/ARE-6 complex. The binding specificity of the PioRF was also addressed in the experiment in Fig. 6. The ARE-6 probe was incubated with nuclear extracts from adipocytes, in competition with several other duplex oligomers. The cognate oligomer ARE-6 competed for binding, whereas ARE-2 was ineffective, establishing that the PioRF is specific for the ARE-6 sequence. Use of a competitor oligomer containing a 4-base mutation within the ARE-6 sequence resulted in complete lack of competition (Fig. 6). These results suggest that the PioRF is the ARF-6 factor described earlier (20), although definitive proof will require further experimentation.

The role of the ARE-6 sequence in pioglitazone induction of the aFABP gene was evaluated in a functional assay. Multiple copies of an oligonucleotide containing the ARE-6 sequence

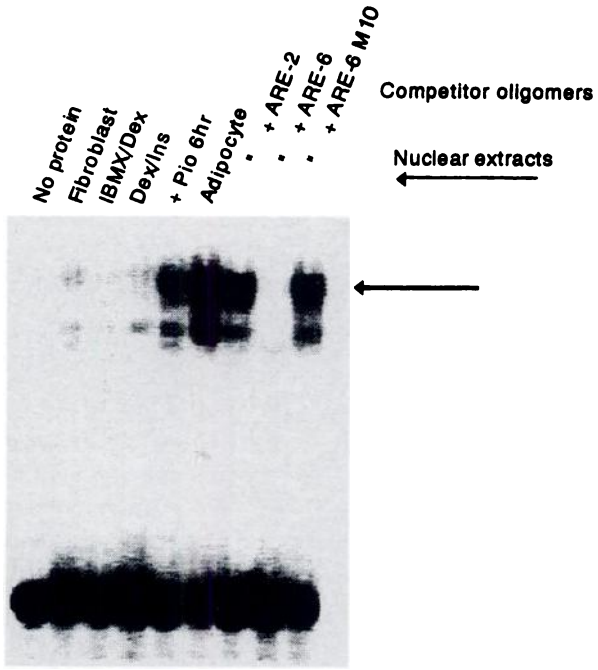

Fig. 6. Gel retardation assays with nuclear extracts from cells subjected to various treatment conditions. Nuclear extracts were prepared from preadipocyte fibroblasts or fully differentiated 3T3-L1 cells. Nuclear extracts were also prepared from cultures treated with IBMX/DEX for 48 hr, or treated with DEX/INS for 48 hr or with pioglitazone (*Pio*) for 6 hr (after a 48-hr incubation with IBMX/DEX). Equivalent amounts of nuclear protein were incubated with the end-labeled ARE-6 probe (10,000 cpm/reaction) for 15 min, as described in Experimental Procedures. Where indicated, the competitor unlabeled duplex oligomers (ARE-2, ARE-6, or ARE-6M10) were added to a final concentration 50-fold greater than that of the labeled oligomer. The ARE-2, ARE-6, and ARE-6M10 sequences (only one strand is shown) are given in Experimental Procedures. The samples were electrophoresed, and the gel was dried and scanned for radioactivity.

were inserted upstream of a minimal SV40 promoter linked to CAT. The ability of pioglitazone to induce expression of the ARE-6/CAT construct was determined after transfection and analysis of transient expression in 3T3-L1 cells. Drug treatment was observed to enhance expression of this chimeric gene, whereas the control plasmid (in which the ARE-6 sequence was not present) was not influenced by the presence of the drug (Fig. 7A). Insertion into this plasmid of multiple copies of a sequence containing a mutation of 4 bases in the ARE-6 sequence resulted in lack of up-regulation by pioglitazone, establishing the importance of the ARE-6 sequence in the control of gene expression by this drug. In addition, pioglitazone was found to modestly down-regulate expression of other chimeric genes, compared with the plasmid constructs containing the valid ARE-6 sequence (data not shown). Pioglitazone increased CAT activity in cells containing the ARE-6/CAT chimeric gene 5-fold, compared with the activity in untreated cells, whereas the activity of ARE-6-mut/CAT-containing extracts was unaffected by drug treatment (Fig. 7B). These results are similar to those observed for pioglitazone-treated cells stably transfected with the aP2(7kb)/CAT and aP2–5/CAT chimeric genes (Table 1). Thus, the ARE-6 sequence appears to be uniquely capable of conferring pioglitazone responsiveness upon a heterologous promoter.

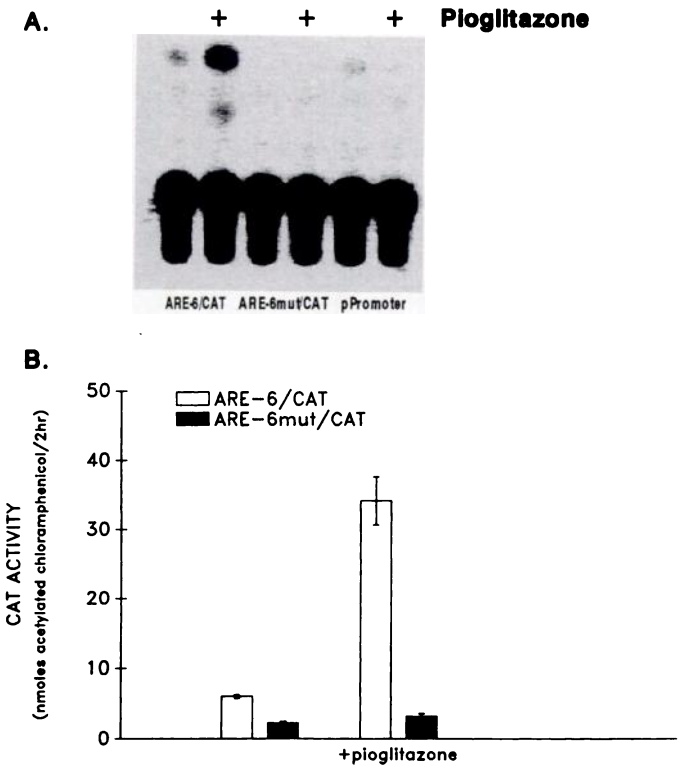

Fig. 7. Pioglitazone induction of a chimeric gene containing the ARE-6 element. Confluent 3T3-L1 cells maintained in 7.5% calf serum were transfected with either ARE-6/CAT, ARE-6-mut/CAT, or pPromoter plasmids. After 6 days in 7.5% fetal calf serum containing 1 μM DEX and 150 nM insulin, with or without 5 μM pioglitazone, cells were harvested and extracts were assayed for CAT activity as described in Experimental Procedures. A, Radioanalytical image of the CAT activity within extracts from a representative transfection. B, Comparison of the CAT activity within extracts from ARE-6/CAT and ARE-6-mut/CAT transfections (mean ± standard error from four determinations for the ARE-6/CAT plasmid and mean ± standard error from three determinations for the ARE-6-mut/CAT plasmids).

Discussion

Pioglitazone is an insulin/IGF-1-sensitizing agent that can enhance the differentiation of preadipocytes and influence gene expression in preadipocyte/adipocyte cell lines (4). Although many genes are indirectly influenced by the drug as a result of the change in cellular phenotype during differentiation, we are interested in identifying those genes that are causally related to the increased cellular sensitivity to insulin/IGF-1. In this regard, we focused our attention on aFABP, because it is an example of a gene that appeared to be directly regulated by the drug (see Fig. 1). We observed that aFABP could be up-regulated rapidly, independently of the onset of cellular differentiation. The work presented in this study establishes that ARE-6 is the DNA sequence through which pioglitazone responsiveness is conferred in the aFABP gene. Because the chimeric aFABP/CAT genes do not display the full range of responses to pioglitazone observed for the endogenous aFABP gene, we speculate that positional effects (location of *cis* elements regulating the endogenous gene within the chromosome) may provide additional controls on expression. Furthermore, increased mRNA stability could also be a major factor contributing to endogenous expression that would be absent in the chimeric gene, because the stability of the reporter mRNA would not be expected to be influenced by the drug.

The nuclear transcription factor C/EBP has been postulated to play a regulatory role in the differentiation of adipocytes (28) and in the differentiation-dependent expression of aFABP (27). However, we observed that pioglitazone failed to increase expression of C/EBP (data not shown) and the C/EBP complex within the aFABP promoter did not respond to the drug (Fig. 4; Table 1). In earlier studies (4), we showed that the thiazolidinediones primarily interact with adipose tissue. Sensitization of adipose cells to insulin may be accomplished by tissue-specific control by pioglitazone of gene expression. Therefore, our results support the idea of Graves *et al.* (20) that the ARF-6/ARE-6 complex is a key component in the tissue-specific, differentiation-dependent expression of aFABP.

A role for aFABP in mediating some aspects of insulin action in the adipose cell has been suggested by Lane and colleagues (12). We propose that pioglitazone can induce other genes (independently of differentiation) in the same time frame as induction of aFABP, which may also participate in mediating insulin/IGF-1 action in the cell. Thus, we envision that the coordinate action of this set of genes would be to enhance and amplify insulin/IGF-1 signaling, ultimately overcoming insulin resistance. Identification and characterization of the pioglitazone-inducible family of genes may provide a better understanding of the molecular mechanism of pioglitazone action.

The molecular mechanism by which pioglitazone generates signals to the cell nucleus is not clear at this time. If the drug exerts effects through a receptor of the steroid superfamily, then we would expect that a drug/receptor complex would be capable of recognizing and binding to the ARE-6 sequence. We have no evidence for this at the present time. Alternatively, if the drug interacts with a cell surface receptor, then we would expect that information would be transmitted to the nucleus through a signaling cascade, ultimately resulting in activation of the PioRF to recognize and bind to the ARE-6 sequence. The activation event could be represented either by the binding of nuclear inositol phosphates (29) by PioRF or by phosphorylation of the protein. In either case, the ability to recognize

ARE-6 could be lost upon dialysis, as has been observed by us and others (20). Considered less likely is the possibility that pioglitazone induces the synthesis of PioRF. Nevertheless, understanding how pioglitazone induces aFABP through the ARE-6/ARF-6 or PioRF/ARE-6 complex is central to delineating the molecular mechanism of drug action.

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