J. Biochem. **138**, 399–412 (2005)
doi:10.1093/jb/mvi136

Expression of Adipose Differentiation-Related Protein (ADRP) Is Conjointly Regulated by PU.1 and AP-1 in Macrophages

Ping Wei[1], Susumu Taniguchi[1], Yoshiyuki Sakai[1], Minako Imamura[2], Toyoshi Inoguchi[2], Hajime Nawata[2,3], Shinya Oda[4], Yusaku Nakabeppu[4], Junji Nishimura[1] and Shoichiro Ikuyama[1]

[1]Division of Clinical Immunology, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Beppu 874-0838; and [2]Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, [3]CREST, Japan Science and Technology Corporation, and [4]Division of Neurofunctional Genomics, Department of Immunobiology and Neuroscience, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582

Received March 19, 2005; accepted July 15, 2005

ADRP is associated with intracellular lipid droplets. We demonstrate the regulatory mechanism for ADRP expression in RAW264.7 macrophages. The ADRP mRNA expression was stimulated by PMA, and synergistically enhanced in association with its protein level in the presence of lipids. A proteasome inhibitor protected the protein from degradation under the lipid-free conditions. One of the possible sites of the PMA action was proved to be an Ets/AP-1 element in the promoter, since mutations of this site reduced the PMA-induced promoter activity, and ligation of this element led to a significant increase in the PMA-responsiveness of homologous or heterologous promoters. Mutations of this site diminished the synergistic effect on the promoter activity induced by PMA and oleic acid, suggesting a possible interaction between this site and the downstream PPARδ site. EMSA revealed that PU.1 and AP-1 conjointly bound to this site. The juxtaposition of the two sequences was requisite for full activity, since spacer sequences between them decreased the PMA-induced activity. PI3 kinase inhibitor was found to reduce the PMA-induced mRNA expression and promoter activity in parallel with PU.1/AP-1 complex formation on EMSA. From these results, we concluded that the Ets/AP-1 site is an important *cis*-acting element that regulates the ADRP gene expression in macrophages.

Key words: ADRP, AP-1, macrophage, PPARδ, PU.1.

Abbreviations: ADRP, adipose differentiation-related protein; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PI3 kinase, phosphoinositide 3-kinase; PMA, phorbol 12-myristate 13-acetate.

Most types of cells store lipid droplets in their cytoplasm. Adipocytes form lipid droplets during their differentiation and maturation, and these lipid droplets have been thought to function as reservoirs of neutral lipids, *i.e.*, energy storage (*1*). Such intracellular lipid droplet formation is, meanwhile, closely related to various pathological events. Liver cells store excessive lipid droplets, which leads to a fatty liver or non-alcoholic steatohepatitis (*2*). Macrophages, another example, accumulate lipid droplets through the uptake of modified low density lipoproteins and become foam cells, as found in atherosclerotic lesions (*3*). Nowadays, the intracellular lipid droplets are thought to be complex, metabolically more active organelles, since it has been disclosed that the protein profile of the droplets appears to include a molecular machinery to synthesize, store, utilize and degrade various lipids (*4*).

Although intracellular lipid droplets exist in the hydrophilic environment of the cytoplasm, little information about the interface of lipid droplets has been obtained yet. Perilipin was the first protein shown to exist on the surface of intracellular lipid droplets. Its expression is, however, almost completely limited to adipocytes and steroid-producing cells (*5–8*). Adipose differentiation-related protein (ADRP) is another dominant protein that is located on the surface of lipid droplets. ADRP was originally identified as a protein whose expression increased during mouse 1246 preadipocyte differentiation (*9*). In the early stage of adipocyte differentiation when lipid droplets are tiny or nascent, the lipid droplets are surrounded by ADRP. As adipocytes further differentiate and the lipid droplets grow in size, the proteins on the lipid droplet surface have been observed to almost exclusively comprise perilipin (*10, 11*). In contrast to perilipin, ADRP is ubiquitously expressed, not only in adipose and steroid-producing cells, but also in various cell types including liver, lung, macrophages, etc, as a major component of intracellular lipid droplets (*10, 12*).

Despite the wide distribution of ADRP, our knowledge about its physiological function and regulatory mechanism remains limited. ADRP expression was shown to accelerate the initial rate of uptake of long-chain, but not short-chain, fatty acids by COS-7 cells transfected with ADRP cDNA (*13*). ADRP has also been shown to bind cholesterol, which thus suggests that it plays an important role in moving cholesterol to the droplets for esterification (*14, 15*).

*To whom correspondence should be addressed. Phone: +81-977-27-1640, Fax: +81-977-27-1641, E-mail: ikuyama@tsurumi.beppu.kyushu-u.ac.jp

It has also been suggested that ADRP participates in cytoplasmic trafficking of newly synthesized lipids (*16*). Several physiological or pharmacological stimuli, such as long-chain fatty acids (*17*) and cyclooxygenase inhibitors (*18*), have been demonstrated to stimulate the ADRP expression in mouse 1,246 preadipocytes. Treatment of rats with etomoxir, an inhibitor of CPT-1 and mitochondrial fatty acid oxidation, results in accumulation of cytoplasmic lipids and induction of ADRP (*19*). A PPARδ-response element was recently identified between –2001 bp and –1889 bp [based on the transcription site reported by Eisinger and Serroro (*24*)] in the ADRP promoter, and shown to be involved in the regulation by VLDL and other PPAR ligands (*20*). In macrophages, PMA stimulates the ADRP protein expression and lipid accumulation in RAW264.7 cells in the presence of sterol esters (*21*). However, the molecular mechanism involved in PMA-induced activation of this gene remains to be clarified.

In the present study, we therefore focused on the regulatory mechanism for ADRP gene expression in PMA-induced RAW264.7 macrophage activation, and identified the *cis*-acting elements responsible for the PMA action in the promoter. We present herein evidence that an Ets/AP-1 composite element is one of the key elements in the ADRP promoter, and that it also possibly affects the PPARδ function. We suggest the possible interaction of transcription factors PU.1 and AP-1, both of which bind cojointly to the composite element, is the major determinant regulating the function of this element.

MATERIALS AND METHODS

Cell Culture—The mouse RAW264.7 monocyte/macrophage–like cell line was provided by the RIKEN Cell Bank (RCB0535, Ibaraki, Japan). The cells were routinely cultured in 10 cm tissue culture dishes (Falcon 3003; Becton Dickison Labware, Franklin Lakes, NJ) in α-MEM (GIBCO BRL, Rockville, MD) supplemented with 10% charcoal-treated fetal calf serum (FCS), 1% non-essential amino acids and appropriate antibiotics (designated as either the complete medium or lipid-containing medium). The FCS we used was proved to be the same in terms of the concentrations of fatty acids, triacylglyceride and total cholesterol with and without charcoal treatment. The cells were cultivated in the same medium containing 5% charcoal-treated lipid-free FCS (BioWest, Nuaille, France) instead of 10% FCS (designated as the lipid-free medium) in some experiments.

Cloning of Mouse ADRP cDNA and Genomic DNA—Full length mouse ADRP cDNA was cloned by PCR using appropriate primers, which were designed according to the published cDNA sequence (*9*), and first strand cDNA reverse-transcribed from mouse adipose tissue total RNA as a template. The cDNA was cloned into a pBluescript II KS+/– plasmid (Stratagene, La Jolla, CA), and then sequenced in its entirety to confirm the integrity of the sequence.

A mouse genomic DNA library derived from adult BALB/c male liver (Clontech, Palo Alto, CA) was screened using a full-length mouse ADRP cDNA as a ^{32}P-labeled probe, according to the method previously described (*22, 23*). We obtained seven positive clones on the secondary screening. During the tertiary screening, a pBluescript II KS+/– plasmid containing the 5′-flanking region of the mouse ADRP gene (named B4.EE3.3+EB1.8, and screened and constructed by S. Taniguchi and L. Chan) was provided to us (courtesy of Dr. Laurence Chan, Baylor College of Medicine, Houston, TX). Based on the information on the restriction enzyme sites and the sequence of the mouse ADRP 5′-flanking region (*24*), and that obtained on Southern hybridization using the 5′-flanking fragment provided, we found that one of our seven positive clones contained a 5′-flanking region of the gene identical to that involved in B4.EE3.3+EB1.8. We, therefore, sequenced the 5′-flanking region subcloned into B4.EE3.3+EB1.8. It contained a putative promoter region up to –2757 bp relative to the major transcriptional start site (*24*).

Northern Blot Analysis—RAW264.7 cells were plated at 1×10^6 cells/dish in the complete medium or the lipid-free medium and then left for 24 h, and then the medium was changed to the same medium containing a test reagent(s), followed by culturing for an appropriate time. Total RNA was purified using a commercially available kit (Isogen; Nippon GENE, Tokyo, Japan). Full-length mouse ADRP cDNA was [α-^{32}P] dCTP-labeled by a random priming method (Oligolabelling kit; Amersham Pharmacia Biotech, Buckinghamshire, England) and used as the probe. Hybridization was performed according to the standard method (*22*); final washing was carried out at 65°C in 1× SSPE (150 mM NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA, pH 7.4) containing 0.5% SDS for 30 min.

The intensity of the ADRP mRNA in autoradiographs and 28S rRNA on ethidium bromide staining was quantified with NIH Image (v.1.62). Although GAPDH mRNA was also detected on Northern blot analyses to compare the mRNA expression levels in the initial experiments, we found that the ADRP mRNA levels standardized as to GAPDH and the 28S rRNA level were similar. Therefore, the results for which the ADRP expression level was standardized as to 28S rRNA are shown.

Construction of ADRP Promoter Reporter Plasmids—To construct reporter plasmids containing the ADRP promoter region, we amplified the desired promoter region by PCR using appropriate forward and reverse primers that had an appropriate restriction site at each 5′-end. The PCR products were cloned into a pGL3 luciferase plasmid (Promega, Madison, WI). The name of each reporter plasmid reflects the position of the 5′-end relative to the transcriptional start site (+1 bp). The 3′-end of the promoter region of all chimeric constructs was –1 bp.

The introduction of site-directed mutations into the putative *cis*-acting elements was performed using a Quick Change site-directed mutagenesis kit (Stratagene) and a –2090 bp promoter plasmid as a template. Sense and antisense oligonucleotide primers (40–42 bp) containing the indicated mutations (Figs. 3B and Fig. 4A, legend) were synthesized and used for the PCR-based mutagenesis. Insertion of a spacer sequence into the Ets/AP-1 element of the –2090 bp promoter was also performed by PCR-based mutagenesis using appropriate oligonucleotide primers (Fig. 8A). To assess the enhancer activity of the Ets/AP-1 element, a double strand oligonucleotide containing a genomic sequence between –2052 bp and –2023 bp was ligated to a pGL3 promoter plasmid (Promega) or a homologous –248 bp promoter plasmid. For all these promoter constructs, the integrity of the total promoter region as

well as the mutations and the insertion were confirmed by sequencing.

Transient Transfection and Luciferase Analysis—Reporter plasmids were introduced into RAW264.7 cells using Lipofectamine PLUS reagent (GIBCO BRL), basically according to the manufacturer's instructions. The cells (2×10^5/well) were seeded in a 12-well cell culture cluster (Costar 3513; Corning Incorporated, Corning, NY) and grown in the complete medium for 24 h. Then the medium was changed to serum-depleted α-MEM, and the transfection mixture (Lipofectamine 2 μl and PLUS reagent 5 μl in a total volume of 50 μl/well) containing 1 μg reporter plasmid and 0.4 μg pRL-TK plasmid (Promega) was added to the cells followed by incubation for 4 h. The cells were then washed with serum-depleted α-MEM, and cultured in α-MEM containing 1% FCS (lipid-containing) and a test reagent for an additional 24 h. When the promoter activity was examined under the lipid-free conditions, the lipid-free medium was used starting 24 h before and after the transfection procedure. Cell lysates were collected for a luciferase assay involving a Dual-Luciferase Reporter Assay System (Promega). The transfection efficiency was normalized as to the *Renilla* luciferase activity expressed by pRL-TK. Each transfection experiment was performed in triplicate and repeated at least three times.

Nuclear Protein Extracts—Nuclear protein extracts were prepared basically as previously described (*23, 25*). RAW264.7 cells were seeded at 1×10^7 cells/dish and then cultured in the complete medium for 24 h. The medium was then changed to the complete medium containing a test reagent or vehicle, followed by culturing for 1 h or 6 h. The cells were washed with PBS, harvested by gentle scraping, and then resuspended in 5 volumes of buffer A [10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$ and 0.1 mM EDTA] supplemented with 0.3 M sucrose and 0.5% NP-40. The cells were then homogenized by pipetting, and the suspension was layered onto 1 ml buffer A containing 1.5 M sucrose, followed by centrifugation for 10 min (4°C, 15,000 × *g*). After washing with buffer A, the precipitated nuclei were suspended in 50 μl buffer B [20 mM HEPES-KOH (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$ and 0.2 mM EDTA] and then left on ice for 20 min. The mixture was centrifuged for 20 min (4°C, 15,000 × *g*), and the resultant supernatant containing nuclear proteins was aliquoted, snap-frozen in liquid nitrogen and stored at –80°C until use (within 30 days). All solutions used were ice-cold and contained 0.5 mM DTT, 0.5 mM PMSF, 2 μg/ml pepstatin A and 2 μg/ml leupeptin. The protein concentration was determined with a commercially available reagent (BIO-RAD, Hercules, CA) using bovine serum albumin as a control.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed basically as previously described (*23*). A synthesized sense oligonucleotide containing the Ets/AP-1 composite element (from –2058 bp to –2023 bp relative to the transcription start site, Fig. 5A) was ^{32}P-labeled with T4 polynucleotide kinase (Takara, Shiga, Japan), double-stranded with unlabeled antisense-strand, and then purified using Chroma-Spin+TE–10 columns (Clontech, Palo Alto, CA). RAW264.7 nuclear extracts (10 μg) was first incubated in a 25 μl reaction volume for 20 min at 20°C with or without unlabeled competitor oligonucleotides (100-fold molar excess). The reaction buffer consisted of 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 12.5% (v/v) glycerol, 0.1% Triton X-100, 8 μg/ml calf thymus DNA and 50 mM PMSF. A radiolabeled probe (50,000 cpm, ~1.0 ng DNA) was then added followed by incubation for an additional 20 min at 20°C. DNA-protein complexes were analyzed on a 5% native polyacrylamide gel at 100 V for 3 h in TBE buffer. Thereafter, the gels were dried and subjected to autoradiography. To examine the inhibition of complex formation by antibodies, 2 μl of an appropriate antibody or control normal rabbit serum (NRS) was added to the mixture before adding the radiolabeled probe and incubation for 40 min at 20°C. The mixture was further incubated for 30 min after the addition of the probe.

Western Blot Analysis—Whole cell extracts (corresponding to 2×10^5 cells) were prepared by directly dissolving the cells in 2× SDS loading buffer [100 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 12% 2-mercaptoethanol and 2% bromophenol blue] at 1:1. The proteins were heated at 95°C for 5 min and then subjucted applied to 10% SDS-PAGE and electro-blotted onto a polyvinidene difluoride membrane (Millipore, Tokyo, Japan) for 1 h at 100 V with a wet blotting apparatus (BIO-RAD) in Tris-glycine transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol and 0.1% SDS]. The transfer was monitored with Kaleidoscope pre-stained standards (BIO-RAD). The membranes were blocked for 1 h at room temperature with 5% non-fat milk in PBS containing 0.1% Tween 20 (PBS-Tween 20). Next, the membranes were incubated with guinea pig ADRP antibodies (0.5 μg/ml in PBS-Tween 20) for 1 h. After washing 4 times (5 min each) with PBS-Tween 20, the membranes were reacted with an appropriate secondary IgG-horseradish peroxidase conjugate (Santa Cruz, CA) diluted in PBS-Tween 20 (0.08 μg/ml) for 1 h, and then washed as above. The blots were reacted with ECL Western blotting detection reagents (Amersham Bioscience) and then exposed to Hyperfilm ECL (Amersham Bioscience) for ~15 min. PU.1 and AP-1 proteins in the nuclear extracts were also analyzed using specific antibodies in the same way (data not shown). More than two independent experiments were carried out and a representative example is shown.

Materials and Other Assays—PMA, actinomycin D, lactacystin, LY294002 and calphostin C were purchased from Sigma (St Louis, MI) and dissolved in DMSO. Cycloheximide (Sigma) was dissolved in PBS. NH_4Cl was dissolved in water. Oleic acid (Wako Pure Chemicals, Osaka, Japan) was dissolved in water containing 0.5 mM bovine serum albumin. Antibodies to ADRP were purchased from PROGEN Biotechnik (Heidelberg, Germany). The preparation of specific antibodies against c-Jun and JunB has been described elsewhere (*26*). For the preparation of anti-JunD antibodies, TrpE-JunD (1–92 amino acids) fusion protein was used to immunize rabbits and the antiserum was affinity-purified as previously described (*27*). Other antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligonucleotides were synthesized by Phasmac Co (Atsugi, Japan). [α-^{32}P]CTP (5,000 Ci/mmol) and [γ-^{32}P]ATP (5,000 Ci/mmol) were purchased from Amersham Bioscience. DNA sequencing was performed with an Aloka DNA sequencing system (Tokyo, Japan) and a Thermo Sequenase Cycle Sequencing Kit (USB Corporation, Cleveland, OH).

RESULTS

PMA Stimulates Expression of the ADRP Gene in Macrophages—The expression of the ADRP gene in PMA-induced macrophage activation was examined using mouse RAW264.7 cells by Northern blot analyses (Fig. 1A). While detectable amounts of ADRP mRNA were constitutively expressed at the basal level (0 h), PMA at a concentration of 0.16 μM further stimulated the ADRP expression as a function of time. ADRP mRNA increased when the cells were cultivated even in the lipid-free medium, although the increase was less evident than that in the complete medium which contained lipids. The vehicle alone enhanced the ADRP mRNA expression very slightly at 24 h on cultivation in the complete medium, but not in the lipid-free medium (Fig. 1A). We presumed that lipids present in the complete medium possibly enhanced the ADRP mRNA expression, and that DMSO did not significantly influence the expression. This presumption was confirmed by the following experiment. After RAW264.7 cells had been cultured for 24 h in the lipid-free medium, they were exposed to PMA, 50 μM oleic acid, or both. While the ADRP mRNA expression was stimulated by PMA or oleic acid alone, but not by DMSO, the simultaneous addition of PMA and oleic acid synergistically increased the expression (Fig. 1B). All these findings were duplicated in human THP-1 cells, except that the mRNA expression reached the maximum at 12 h (data not shown).

In RAW264.7 cells pre-cultured for 24 h in the complete medium which contains lipids, ADRP protein existed at a detectable level and PMA further increased the protein level after an additional 12 h and 24 h (Fig. 1C). On the other hand, the ADRP protein level was very low and almost undetectable when the cells were pre-cultured for 24 h in the lipid-free medium, and PMA did not significantly increase its level at 24 h (Fig. 1D, second lane). This finding prompted us to clarify the mechanism underlying the possible degradation of ADRP protein. In the presence of lactacystin (20 μM), a proteasome inhibitor, PMA increased the ADRP protein level even in the lipid-free medium (Fig. 1D), while NH_4Cl (50 mM), a lysosome inhibitor, did not have such an effect, indicating that the ADRP protein is possibly degraded through the proteasome pathway in the absence of lipids.

Chen *et al.* (*21*) demonstrated that ADRP protein was increased by PMA in the presence of sterol ester, as we

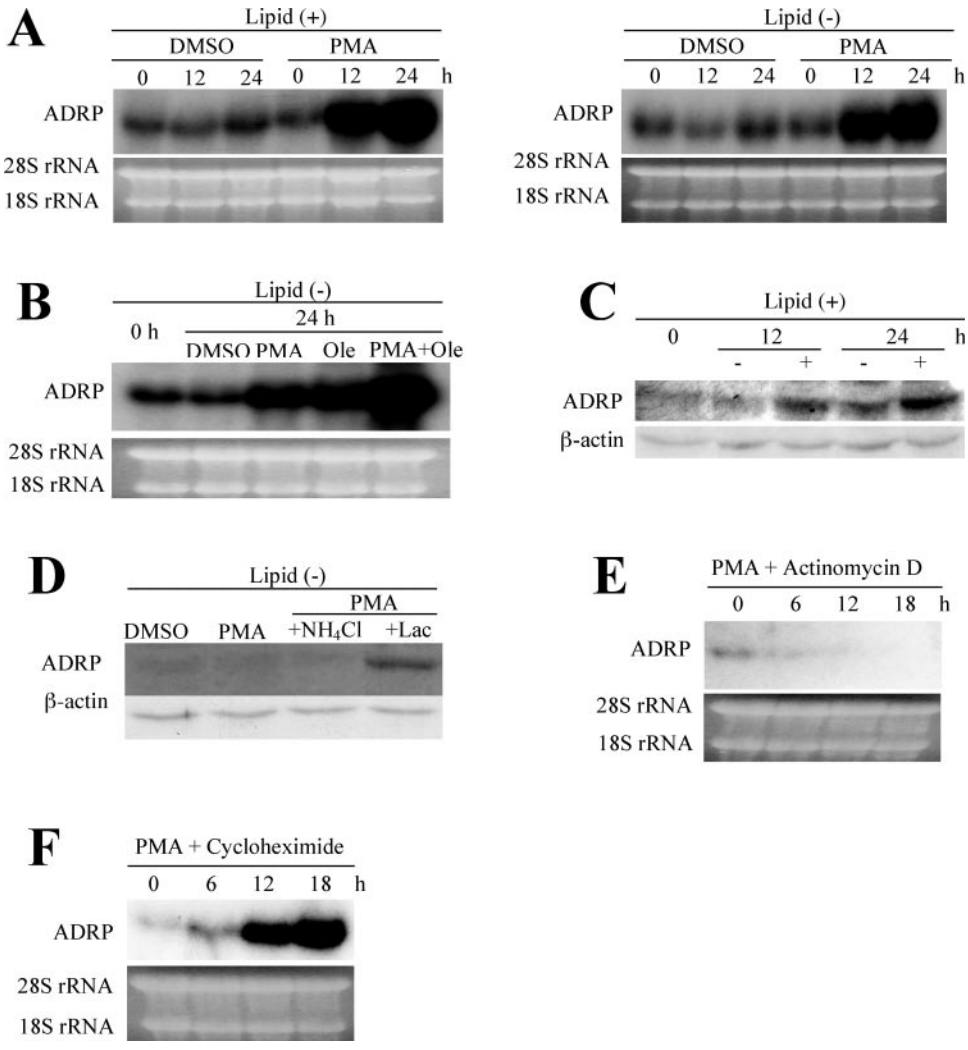

Fig. 1. **PMA enhances the expression of ADRP in RAW264.7 cells.** A: Effect of PMA (0.16 μM) on ADRP mRNA expression. RAW264.7 cells were cultured in the lipid-containing or lipid-free medium with the respective reagents. Twenty micrograms total RNA was analyzed. Ribosomal RNAs, quantified by ethidium bromide staining, showed that an equal amount of total RNA was applied in each lane. B: Effects of PMA (0.16 μM) and oleic acid (50 μM) and both on the ADRP mRNA expression in the lipid-free medium. C: Effects of PMA (0.16 μM) on the ADRP protein levels in the lipid-containing medium. D: Effects of PMA, and lysosome and proteasome inhibitors on the ADRP protein levels in the lipid-free medium. E: and F. Effects of actinomycin D (2.5 μg/ml) and cycloheximide (10 μg/ml) on the PMA-induced enhancement of ADRP mRNA expression in the complete medium. In the experiments under lipid-free conditions, RAW264.7 cells were cultured in the lipid-free medium for 24 h prior to the addition of the reagents.

demonstrated here. The precise molecular mechanisms involved in the gene regulation, however, remained to be disclosed. In the following experiments, therefore, we further pursued the mechanism underlying the PMA-induced ADRP gene regulation in RAW264.7 cells. When we examined the effect of the PMA concentration from 0.16 to 16 μM, 1.6 μM caused the highest ADRP mRNA induction at 24 h (data not shown). The expression of the ADRP gene was completely inhibited by actinomycin D (2.5 μg/ml) (Fig. 1E). Cycloheximide (10 μg/ml) did not inhibit the mRNA induction (Fig. 1F). These results collectively revealed that PMA enhanced the ADRP mRNA expression in both the presence and absence of lipids at the transcriptional level, and that its effect was further potentiated in the presence of lipids.

A Functional Ets/AP-1 Element Exists in the ADRP Promoter—To identify the promoter region responsible for the PMA action, we constructed chimeric plasmids containing the ADRP promoter with a luciferase gene, and then assessed the PMA-induced promoter activity in RAW264.7 cells. These experiments were performed using the lipid-containing (Fig. 2, A and B) or lipid-free medium (Fig. 2C). As shown in Fig. 2A, PMA (1.6 μM) stimulated the activity of all the promoter constructs. When the ratios of activity expressed in the presence and absence of PMA (relative fold induction) were calculated, however, promoters containing the region between –2090 bp and –2005 bp, *i.e.*, the –2752, –2308 and –2090 bp promoters, showed significantly higher inducibility than the shorter promoter constructs (Fig. 2B). This finding indicates that the region between –2090 bp and –2005 bp encompasses the element responsible for the PMA action. This finding was duplicated when the cells were incubated in the lipid-free medium, although the amplitude of the fold induction was reduced (Fig. 2C). In the lipid-containing medium, promoters shorter than –2005 bp (*i.e.*, –2005 to –248 bp) still exhibited significant PMA-inducibility in comparison to the promoter-less control (Fig. 2B), while this finding was not duplicated in the lipid-free medium (Fig. 2C). Therefore, the –248 bp promoter might possess an element(s) which could be stimulated by PMA in conjunction with certain serum factors including lipids. This possibility remains to be clarified. We are

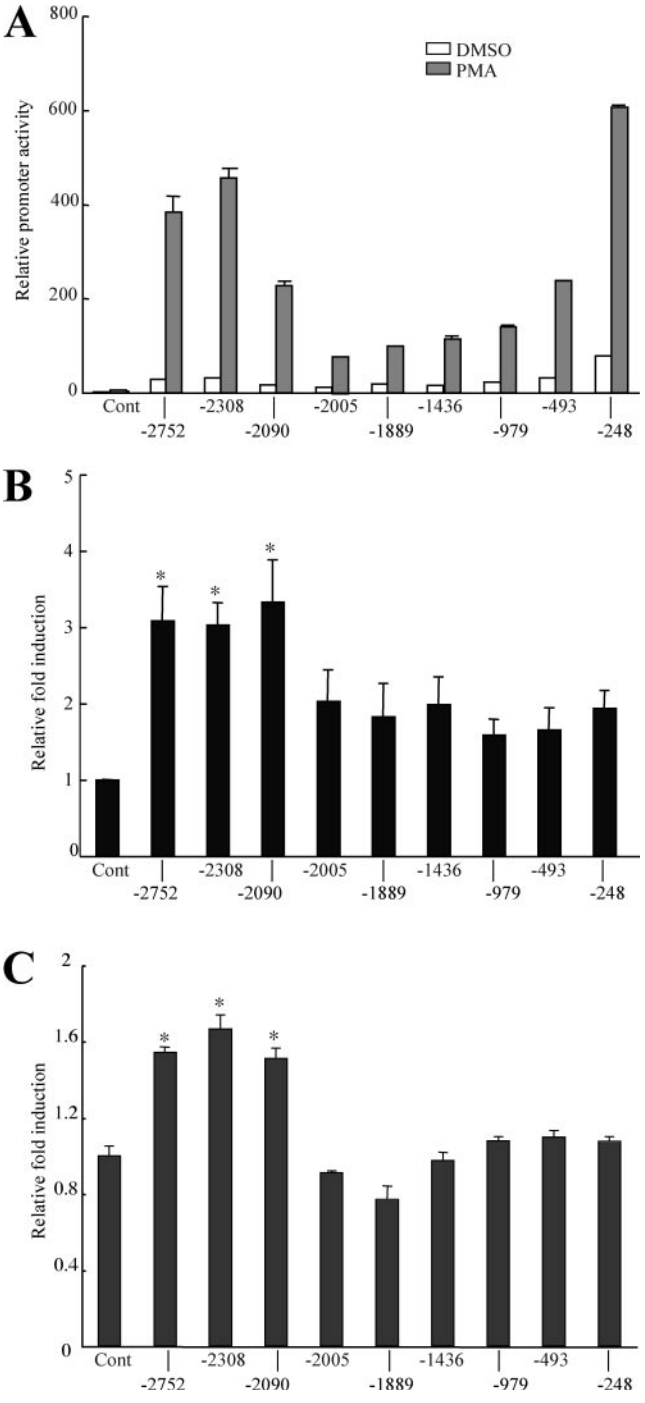

Fig. 2. **PMA stimulates the ADRP promoter activity.** Chimeric plasmids containing the ADRP promoter were transfected into RAW264.7 cells cultured in the lipid-containing (A and B) or lipid-free medium (C). Each plasmid contained a promoter region between the position designated and –1 bp in front of the luciferase gene. The luciferase activity was normalized as to the activity of co-transfected *Renilla* luciferase. A: PMA (1.6 μM) significantly enhanced the promoter activity of all chimeric plasmids as well as the promoter-less control ($p < 0.005$ *versus* DMSO, with Student's *t* test). The activity expressed by the control plasmid in the absence of PMA was arbitrarily designated as 1. The independent experiments were performed in triplicate and more than three times. Data are shown as means ± SEM. B: The inducible transcriptional activity of each promoter construct with PMA was compared as to relative fold induction. The value for the promoter-less control was arbitrarily designated as 1. Data are shown as means ± SEM. Relative fold induction of each promoter was compared with those of all the other promoters and evaluated by ANOVA (Scheffe). All promoters exhibited significantly higher PMA-inducibility than the promoter-less control ($p < 0.01$). The values for the –2752 bp, –2308 bp and –2090 bp promoters, respectively, were significantly higher than those of other shorter promoters (*$p < 0.01$). There are no significant differences exists among either the –2752, –2308, and –2090 bp promoters, or the –2005, –1889, –1436, –979, –493 and –248 bp ones. C: The cells were cultured in the lipid-free medium for 24 h prior to transfection. The data were analyzed in the same way as in panel B. The basal activity of all promoters was lower than that in the lipid-containing medium. The independent experiments were performed in triplicate and more than three times. The value for the promoter-less control was arbitrarily designated as 1. Data are shown as means ± SEM. The values for the –2752 bp, –2308 bp and –2090 bp promoters, respectively, were significantly higher than those of other shorter promoters including promoter-less control [*$p < 0.01$ by ANOVA (Scheffe)]. There were no significant differences among either the –2752, –2308, and –2090 bp promoters, or the promoter-less control, –2005, –1889, –1436, –979, –493 and –248 bp ones.

currently investigating this issue and thus it is outside of the scope of the present report.

We further focused on the region between –2090 bp and –2005 bp because the above results clearly indicated that PMA acts on this promoter region in both the presence and absence of lipids. Furthermore, a PPARδ response element, which was shown to mediate the effect of VLDL and PPAR ligands, exists just downstream of this region, starting at –2001 bp (*20*). We found a composite element starting at –2047 bp, which is formed from a canonical AP-1 site and a binding site for Ets family transcription factors adjacent to the AP-1 site, at its 5′-end (Fig. 3A). The PMA-induced activity of the –2005 bp promoter, which possesses the PPARδ site but not the Ets/AP-1 site, was significantly lower than that of the –2090 bp promoter, and was comparable to that of the –1889 bp promoter (Fig. 2, B and C). We, therefore, presumed that PMA could directly stimulate the promoter activity through the Ets/AP-1 site, but not through the PPARδ site.

In order to determine the significance of this composite element, we introduced mutations into the AP-1 site (A mut), Ets site (E mut), and both of them (AE mut) in the –2090 bp promoter (Fig. 3B), and assessed their activity in the lipid-containing medium. The basal activity of all these mutant promoters was not significantly different from that of the wild type –2090 bp promoter (data not shown). On comparison of the relative fold induction values (Fig. 3C), all three mutations exhibited significant reduction in the PMA-induced promoter activity in comparison to the –2090 bp promoter. The activity of the mutant promoters was comparable to that of the –2005 or –1889 bp promoters.

To examine the enhancer activity of the Ets/AP-1 site, this element was ligated to either a heterologous SV40 minimal promoter or a homologous –248 bp promoter. Ligation of the element stimulated both the basal (data not shown) and PMA-induced activity of the SV40 promoter as a function of the copy number in the lipid-containing medium (Fig. 3D). In addition, ligation of this element, in either the forward or reverse direction, enhanced the PMA-induced (Fig. 3E) but not the basal (data not shown) activity of the –248 bp promoter even in the lipid-free medium. These results clearly indicate that the Ets/AP-1 composite element is an enhancer which mediates the PMA action.

Synergistic Effect of PMA and Oleic Acid on the Promoter Activity Requires the Ets/AP-1 Function—As shown in Fig. 1, the ADRP mRNA expression is synergistically potentiated by PMA and oleic acid or lipids. We therefore examined the effects of oleic acid and/or PMA on the –2090 bp promoter and the mutant promoters in the lipid-free medium. For this analysis, we constructed additional mutant promoters named P mut and AEP mut whose PPARδ site was mutated (Fig. 4A). PMA enhanced the activity of the –2090 bp promoter, as shown in Fig. 2C, while 25 μM oleic acid increased the activity only slightly, *i.e.*, 1.27 ± 0.04 fold as compared to DMSO. Although this increase is not statistically significant at this moment, it is quite in contrast to in the case of other promoters whose activity is not changed or rather significantly decreased by oleic acid. When oleic acid and PMA were simultaneously added, however, the promoter activity was synergistically potentiated (Fig. 4B). The synergistic effect of oleic acid and PMA was abolished when the PPARδ site was mutated (P mut), although PMA alone still stimulated its activity (Fig. 4B). Interestingly, none of the promoters whose Ets/AP-1 site was mutated (A mut, E mut and AE mut) or deleted (–2005 bp) exhibited the effect of oleic acid or the synergistic effect of oleic acid and PMA, despite the existence of the intact PPARδ site. In separate experiments involving the co-expression of PPARα, we confirmed that activity of the promoters containing these Ets/AP-1 mutations was not stimulated by PPARα ligands in NMuLi mouse liver cells, which do not express PPARα (Ikuyama, S. *et al*., unpublished observation). This finding suggests a possible interaction between the Ets/AP-1 site and the PPARδ site, and further strengthens the idea that the Ets/AP-1 composite element is important for the ADRP gene regulation.

PU.1 and AP-1 Conjointly Bind to the Composite Element—To identify the transcription factors binding to the Ets/AP-1 element, we performed EMSA using nuclear extracts prepared from RAW264.7 cells and the oligonucleotide probes shown in Fig. 5A. The wild type sequence formed two major specific complexes (complexes A and B) when nuclear extracts from cells without PMA treatment were used, and the complex formation, that of complex A, in particular increased as a function of the duration of the PMA treatment (Fig. 5B). In addition, a faint but discrete band (complex C) became apparent when nuclear extracts from PMA-treated cells were used (Fig. 5B, lanes 2 and 3). These complexes were specific since a 100-fold molar excess of unlabelled oligonucleotide completely abolished all the complexes (Fig. 5C, lane 2), while the AE mut oligonucleotide failed to inhibit the complex formation at all (Fig. 5C, lane 5).

Complex B was completely inhibited by the A mut oligonucleotide, which contains a mutated AP-1 and an intact Ets site (Fig. 5C, lane 3). It was also inhibited by the PU.1 consensus oligonucleotide (Fig. 5D, lane 4) among the consensus sequences for Ets family proteins, indicating that complex B was formed by PU.1. On the other hand, complex A was partially, but not completely, inhibited by the E mut oligonucleotide, which encompasses the intact AP-1 and mutated Ets sites (Fig. 5C, lane 4). The partial inhibition of complex A was duplicated when the consensus AP-1 oligonucleotide was used as a competitor (Fig. 5D, lane 3). We presumed that complex A might involve PU.1 in addition to AP-1, since PU.1 could possibly function in conjunction with AP-1, as was suggested by the reporter assays described above. Our presumption was verified by the finding that the majority of complex A, as well as that of complexes B and C, was almost completely eliminated when the AP-1 consensus and PU.1 consensus oligonucleotides were added simultaneously to the reaction mixture (Fig. 5D, lane 5). We, therefore, deduced from these results that the dominant components of complex A were PU.1 and AP-1, while complex B consisted of PU.1 only. Complex C might be a higher order complex including AP-1, PU.1 and certain cofactors. We confirmed that complex A formed by nuclear extracts from the cells without PMA treatment (Fig. 5B, lane 1) also consisted of PU.1 and AP-1 (data not shown).

To further determine the factors binding to this composite element, we performed supershift analyses with specific antibodies. Among the antibodies against Jun and Fos

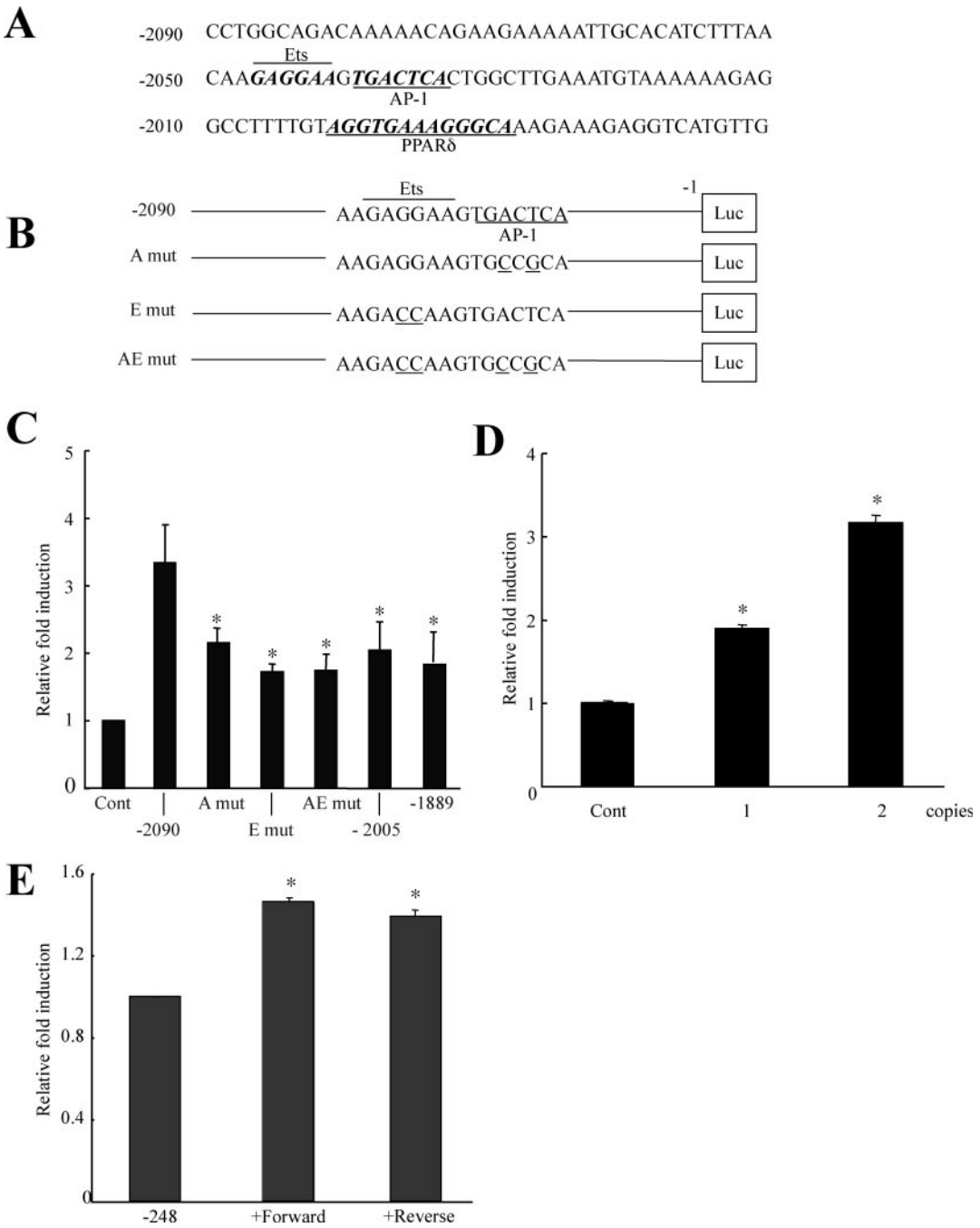

Fig. 3. **An Ets/AP-1 composite element mediates the PMA action.** A: The genomic sequence between –2090 bp and –1971 bp relative to the transcriptional start sites (based on Ref. *24*) is shown. A binding motif for Ets family transcription factor starts at –2047 bp and is adjacent to a canonical AP-1 site. A PPARδ site exists at –2001 bp (Ref. *20*). B: Site-directed mutations were introduced into the AP-1 site, Ets site, or both of them in the –2090 bp promoter. The mutated nucleotides are underlined. C: Relative fold induction of each promoter by 1.6 μM PMA in the lipid-containing medium is shown. The value for the promoter-less control plasmid was arbitrarily designated as 1. Relative fold induction of each promoter was compared with those of all the other promoters and evaluated by ANOVA (Scheffe). All promoters exhibited significantly higher PMA-inducibility than the promoter-less control ($p < 0.01$). Promoters containing specific mutations as well as the –2005 bp and –1889 bp promoters exhibited less PMA-inducibility than the wild type –2090 bp promoter (*$p < 0.01$). The independent experiments were performed in triplicate and more than three times. Data are shown as means ± SEM. D: Ligation of the Ets/AP-1 element to the SV40 core promoter increased the PMA-responsiveness in the lipid-containing medium as a function of the copy number. The value for the enhancer-less control promoter plasmid was arbitrarily designated as 1. Data are shown as means ± SEM. Relative fold induction of each promoter was compared with those of all the other promoters and evaluated by ANOVA (Scheffe) (*$p < 0.005$ *versus* control). E: Ligation of the Ets /AP-1 element to the –248 bp homologous promoter, both in either the forward or reverse direction, increased the PMA-responsiveness in the lipid-free medium. The cells were cultured in the lipid-free medium for 24 h prior to transfection. The value for the –248 bp promoter was arbitrarily designated as 1. Data are shown as means ± SEM. Relative fold induction of each promoter was compared with those of all the other promoters and evaluated by ANOVA (Scheffe) (*$p < 0.0001$ *versus* –248 bp promoter).

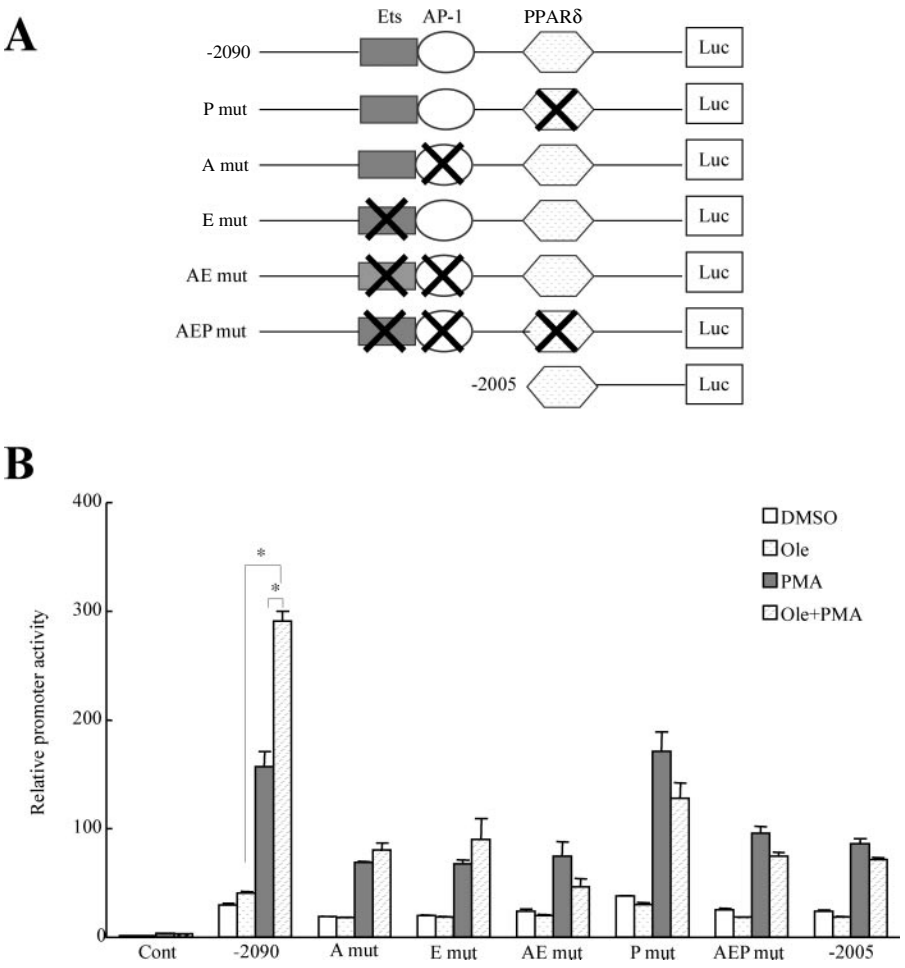

Fig. 4. **Synergistic effect of PMA and oleic acid on the promoter activity requires the Ets/AP-1 function.** A: Site-specific mutations were introduced into the AP-1 site, Ets site, PPARδ site, or all of them in the –2090 bp promoter. The mutations in the PPARδ site were as follows (underlined). AAGCTTAAGGGCA. B: The relative promoter activities induced by 1.6 μM PMA, 25 μM oleic acid or both of them in the lipid-free medium are shown. The activity of the –2090 bp promoter, which encompasses both the Ets/AP-1 site and the PPARδ site, was synergistically potentiated when oleic acid and PMA were simultaneously added (*$p < 0.005$ *versus* Ole or PMA). In contrast, although PMA or PMA plus oleic acid significantly increased the activity of all the promoters as compared to DMSO ($p < 0.001$), no significant difference was observed between PMA and Ole+PMA in all the mutant and –2005 bp promoters. Cells were cultured in the lipid-free medium for 24 h prior to transfection. The value for the promoter-less control treated with DMSO was arbitrarily designated as 1. The independent experiments were performed in triplicate three times. Data are shown as means ± SEM. Statistical evaluation was performed by ANOVA (Scheffe).

family proteins, antibodies to JunB and Fra-1 gave very faint supershifted bands (Fig. 6A, lanes 3 and 7). On the other hand, the PU.1 antibodies, but not antibodies against other Ets family proteins, specifically eliminated all the A, B and C complexes (Fig. 6A, lane 11).

Since the inhibitory effects of AP-1 antibodies on complex A formation were less evident than those of PU.1 antibodies, we assumed that the presence of PU.1 might prevent the AP-1 antibodies from gaining access to their target proteins. When the E mut oligonucleotide, to which AP-1 could but PU.1 could not bind, as evidenced by the absence of complex B, was used as a labeled probe, both Jun and Fos antibodies more effectively inhibited complex A formation (Fig. 6B, lanes 2 and 3). Therefore, we assumed that PU.1 might prevent the AP-1 antibodies from coming in contact with their target proteins in the ternary structures or, alternatively, the presence of PU.1 might induce an increase in the DNA binding affinity of AP-1. Nevertheless, these results, which were consistent with the above ones obtained with using competitor oligonucleotides, indicated that complex A consists of PU.1 and AP-1, and complex B is formed only from PU.1.

PMA Stimulates the Ets/AP-1 Element Mainly through the PI3 Kinase Pathway—To investigate the downstream signals stimulated by PMA, we examined the effects of a PI3 kinase inhibitor and a PKC inhibitor on the PMA-induced ADRP gene expression. The concentration of the inhibitors used was that which did not induce apoptosis of the cells. After being cultured in the lipid-free medium for 24 h, RAW264.7 cells were incubated with the inhibitors for 1 h prior to PMA (0.16 μM) addition. LY294002, a PI3 kinase inhibitor, suppressed the PMA-induced increase in the ADRP mRNA expression (Fig. 7A). Calphostin C, a PKC inhibitor, also suppressed the expression, but its effect was less evident than that of LY294002. When the cells were exposed to LY294002 (5 μM) for 1 h prior to PMA (0.16 μM) stimulation in the lipid-free medium, the PMA-induced enhancement of the promoter activity was almost completely abolished for the promoters whose Ets/AP-1 site is intact (–2090 bp, P mut) (Fig. 7B). Calphostin C (5 nM), on the other hand, suppressed the PMA-induced stimulation of all promoters weakly (data not shown), suggesting that PKC-mediated signals might act on the downstream element rather than this Ets/AP-1 site. Furthermore, PMA-induced enhancement of the complex A formation was found to be significantly reduced on EMSA, when nuclear extracts from cells treated with PMA (0.16 μM) and LY294002 (5 μM) simultaneously were used (Fig. 7C). When extracts from PMA plus calphostin C-treated cells were used, complex A formation was also reduced, but the reduction was less evident than with LY294002-treated cells.

All these results indicated that PMA activates the Ets/AP-1 element mainly through the PI3 kinase pathway by

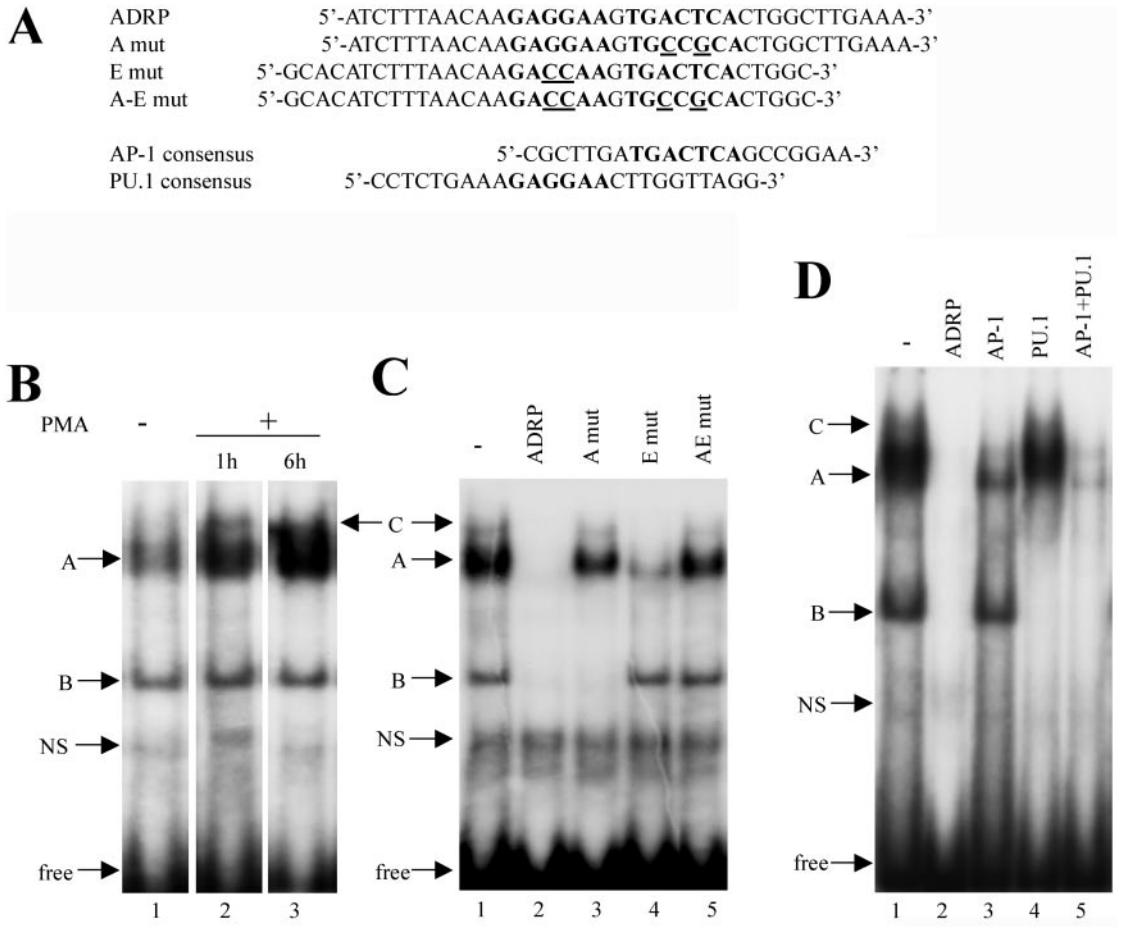

Fig. 5. **DNA-protein complex formation by RAW 264.7 nuclear extracts.** A: The sequences of the oligonucleotides used as a probe or competitors are shown. The Ets and AP-1 binding sites are shown in bold letters. A mut, E mut and AE mut are oligonucleotides into which mutations were introduced, as indicated by underlines (see also Fig. 3B). AP-1 and PU.1 are consensus oligonucleotides for each transcription factor. The ADRP oligonucleotide was used as a probe. B: Nuclear extracts from RAW264.7 cells treated (+) with 0.16 μM PMA for 1 h or 6 h, or non-treated cells (–) were used. The 6 h PMA sample was applied to a separate gel, although the experiment was performed at the same time. C: DNA-protein complexes were competed for with a 100-molar excess of oligonucleotides containing mutations. The nuclear extracts were prepared from cells treated with 0.16 μM PMA for 1 h. D. DNA-protein complexes were competed for with a 100-molar excess of consensus oligonucleotides. Nuclear extracts were prepared from cells treated with 0.16 μM PMA for 1 h. Representative results are shown. NS, non-specific band; free, unbound probe.

increasing the DNA binding of PU.1 and AP-1, although involvement of the PKC pathway cannot be completely ruled out.

Juxtaposition of the Ets and AP-1 Sites Is Requisite for Full Activity of the Ets/AP-1 Composite Element—The Ets family proteins are known to function cooperatively with AP-1 proteins. The functional elements for these two different kinds of transcription factors are located next to each other or, in a number of cases, more widely spaced. To examine the functional significance of the juxtaposition of the Ets and AP-1 sites, we introduced spacer sequences of different lengths between the core sequences of the two elements in the –2090 bp promoter (Fig. 8A). AP-1 and PU.1 could bind independently to their recognition sequences in the probes having insertions, as shown in Fig. 8B, which shows that AP-1 and PU.1 competitors inhibited complex A and complex B formation, respectively.

On investigation of the inhibition of complex A formation using a wild type sequence as a probe, a 6.25 to 25-fold molar excess of the self competitor efficiently prevented complex A formation (Fig. 8C, lanes 2–4). Oligonucleotide INS10, which has a 10 bp insertion, also inhibited complex A formation in a manner closely similar to the wild type competitor (Fig. 8C, lanes 8–10), while oligonucleotide INS8 having an 8 bp insertion showed apparently reduced competitive activity (Fig. 8C, lanes 5–7). Note that this result is in sharp contrast to the case of complex B formed by PU.1 only, which was inhibited in a similar manner by all of these different competitors (Fig. 8C, lanes 2–10). Since a 10 bp sequence inserted creates an additional one turn of a double helix between the Ets and AP-1 core elements, the two proteins binding to the respective elements could come to face each other on the same interface nearly as in the case of the wild type sequence. Therefore, the PU.1 and AP-1 proteins are thus suggested to possibly come into contact or face each other on some of their interfaces, which might increase their DNA binding affinity.

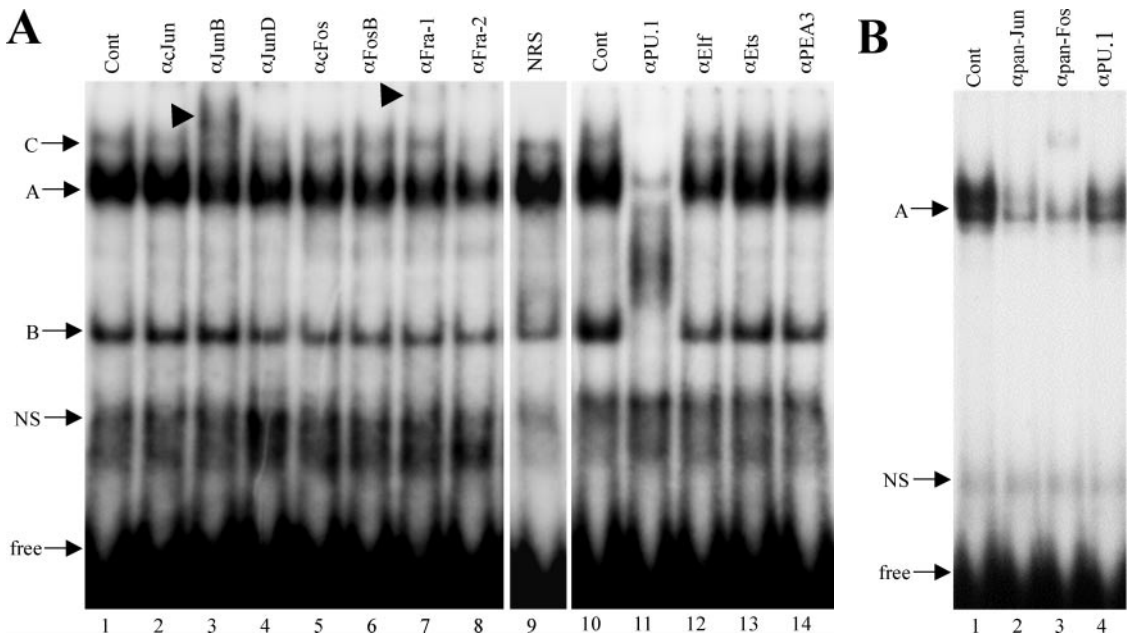

Fig. 6. **Identification of binding proteins with specific antibodies.** A: Specific antibodies against Jun, Fos and Ets family proteins were examined as to inhibition of complex formation. Very faint supershifted bands appeared when antibodies to JunB (lane 3) or Fra 1 (lane 7) were added (shown by arrowhead). PU.1 antibodies prevented the formation of complexes A, B and C (lane 11). The ADRP oligonucleotide (Fig. 5A) was used as a probe. B. Antibodies to either Jun or Fos family proteins effectively inhibited complex A formation (lanes 2 and 3) when the E mut oligonucleotide (Fig. 5A) was used as a probe. Complex B was not formed since PU.1 could not bind to the probe, and PU.1 antibodies had no effect on the complex A. Nuclear extracts were prepared from RAW264.7 cells treated with 0.16 μM PMA for 1 h. A representative result is shown in each panel. NS, non-specific band; free, unbound probe.

We next investigated the effect of the insertion on the promoter activity in the lipid-containing medium. While the insertion of a 4 bp nucleotide did not significantly influence the PMA-inducible activity, the insertion of an 8 bp or more nucleotide apparently reduced the response to PMA (Fig. 8D). Note that the PMA-inducibility of INS 10, which exhibited closely similar DNA binding affinity to the wild type sequence on EMSA, also decreased. These results clearly indicated that the two elements should be in close proximity to exert full enhancer activity, even though the PU.1 and AP-1 proteins could bind to the respective elements.

DISCUSSION

In the present study, we demonstrated that PMA enhanced the expression of ADRP in RAW264.7 macrophages. The PMA effect on mRNA expression was synergistically potentiated and associated with increases in protein levels in the presence of lipids or oleic acid. We identified an Ets/AP-1 composite site in the ADRP promoter as one of the elements responsible for the PMA action. In previous studies, PMA stimulated the ADRP expression in the presence of sterol ester, and it also stimulated lipid droplet accumulation in RAW264.7 cells (*21*) and in a human epidermal cell line (*21, 28*). Our present results have not only confirmed those in previous reports, but also, and more importantly, have provided a solid molecular basis for PMA-induced ADRP gene regulation.

The Ets/AP-1 element at –2047 bp consists of a canonical AP-1 site and a binding motif for Ets family transcription factors. Mutations in the Ets/AP-1 site, either in the Ets site or the AP-1 site, significantly decreased the PMA-induced activity. The combined action of Ets and AP-1 proteins has been demonstrated for several monocyte/macrophage-specific genes. The juxtaposition of Ets/AP-1 binding motifs has been detected in the granulocyte-macrophage colony-stimulating factor gene (*29*) and the urokinase-type plasminogen activator gene (*30*). These two motifs are more widely spaced in other genes such as matrix metalloproteinase genes (*31*) and the macrosialin gene (*32*). A variety of Ets family proteins, i.e., Ets-1, Ets-2, Elf-1, PEA3 and PU.1, have been demonstrated to interact cooperatively with AP-1 (*33*). The direct physical association between Ets family and Jun family proteins has been demonstrated, and the Ets/Jun dimer recruits the Fos protein to form a trimolecular complex (*33*).

Regarding the ADRP promoter, the factors binding to this element and forming a ternary complex are PU.1 and AP-1 proteins. We confirmed by Western blot analysis that PU.1, c-Fos and Fra-2 are constitutively expressed, and that PMA increases the protein levels of c-Jun, JunB and Fra-1 in RAW264.7 cells (data not shown). PU.1 is apparently far more abundant than AP-1 proteins. This might be the reason why only PU.1 was found to be able to form complex B alone in the absence of AP-1 on EMSA. On the other hand, it is difficult to identify each AP-1 protein involved in this complex formation, since EMSA involving antibodies specific for each Jun and Fos family protein failed to show a discernible supershifted band. We hypothesized that this might be because of the steric hindrance by PU.1. Based on the results of Western blot analyses, however, it is reasonable to regard c-Jun, JunB, c-Fos, Fra-1 and Fra-2 as probable candidates involved in the complex formation.

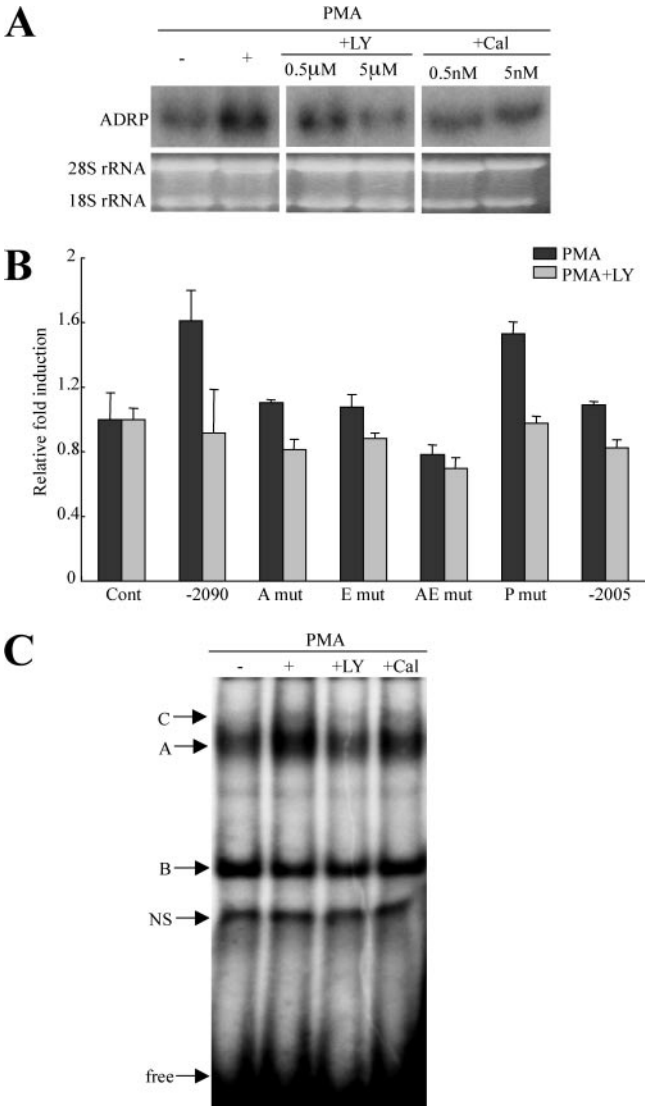

Fig. 7. **PMA effect on the Ets/AP-1 is eliminated mainly by PI3 kinase inhibitor.** A: RAW264.7 cells were cultured in the lipid-free medium for 24 h prior to PMA stimulation. After 24 h incubation with the reagents in lipid-free medium, total RNA was subjected to Northern blot analysis, as described in Fig. 1. The concentrations of LY294002 (LY) and calphostin C (Cal) were ones which do not induce apoptosis of the cells in the lipid-free medium. B: LY294002 (5 μM) abolished the PMA (0.16 μM)-induced enhancement of the –2090 bp and P mut promoter activity. Transfection of the reporter plasmids into RAW264.7 cells was performed in the lipid-free medium. Cells were cultured in the lipid-free medium for 24 h prior to transfection. Transfected cells were exposed to LY for 1 h prior to PMA stimulation. The value for the promoter-less control plasmid was arbitrarily designated as 1. The independent experiments were performed in triplicate three times. Data are shown as means ± SEM. C. EMSA performed using nuclear extracts from RAW264.7 cells treated for 1 h with 0.16 μM PMA, PMA plus LY294002 (5 μM), PMA plus calphostin C (5 nM), or the vehicle (DMSO). A representative result is shown. NS, non-specific band; free, unbound probe.

PMA activates a number of downstream signal transduction molecules. We examined the involvement of the PI3 kinase pathway and the PKC pathway in the PMA-induced ADRP expression in RAW264.7 cells. Inhibitors of both pathways suppressed the ADRP mRNA expression. In the reporter assay, however, we could demonstrate more clearly that a PI3 kinase inhibitor abolished the PMA-induced enhancement of the promoter activity mediated by the Ets/AP-1 element. This effect was further evidenced by EMSA, which showed that complex A formation was significantly reduced when nuclear extracts from the LY294002-treated cells were used. Both PU.1 (*34*) and AP-1 (*35*) were shown to be activated through the PI3 kinase pathway. Involvement of the PKC pathway, however, cannot be completely ruled out, since complex A formation was somewhat reduced on calphostin C treatment. Moreover, PU.1, as well as AP-1, is known to be activated through the PKC pathway (*36, 37*). The activity of the Ets/AP-1 element might be, therefore, regulated differently and probably in a more complex way through a number of stimuli.

Furthermore, a more than eight nucleotide insertion between the Ets and AP-1 core sequences caused significant reduction in the promoter activity in response to PMA. These results indicated that PU.1 and AP-1, possibly Jun family proteins based on a previous report (*33*), should be in contact or in close proximity to exert full enhancer activity in this promoter. Taking these findings into consideration, we propose a possible mechanism for the function of the Ets/AP-1 element, as follows. In the wild type sequence in which intact Ets and AP-1 sites are adjacent, the binding of PU.1 and AP-1 to each element is enhanced by PMA stimulation, and the ternary complex can effectively recruit certain cofactors to form a higher order complex and thereby enhance promoter activity. In A mut or E mut promoters, on the other hand, such cofactors could not be efficiently recruited because of the absence of the PU.1/AP-1 interaction. A spacer sequence between the two sites might also prevent from the PU.1/AP-1 interaction and, therefore, recruitment of cofactors can not effectively take place. The complex C observed on EMSA might reflect a higher order complex including such cofactors. We presume that CBP/p300 is one of the possible candidates for the cofactors recruited to this element. CBP/p300 has been shown to interact with AP-1 (*38, 39*) and PU.1 (*40*). As we mentioned above, the Ets/AP-1 interaction occurs even though these two motifs are more widely spaced in other genes. In such cases, different cofactors might be recruited.

Interestingly, we found that the synergistic effect of oleic acid and PMA on the promoter activity was eliminated when the Ets/AP-1 site was mutated or deleted. Oleic acid seems to act on the PPARδ site identified by Chawla *et al*. (*20*), since the P mut promoter responded to PMA, and there was no synergistic effect of PMA plus oleic acid. We confirmed that RAW264.7 cells express PPARδ (data not shown). In addition, co-expression of PPARα, which could stimulate the –2090 bp but not the P mut promoter activity, also failed to stimulate the activity of promoters containing the Ets/AP-1 mutations in response to PPARα ligands in PPARα-negative NMuLi cells (data not shown). Therefore, it is strongly suggested that a functional Ets/AP-1 site is requisite for the PPAR-mediated activation of the ADRP promoter. In the experiments performed by Chawla *et al*. (*20*), their promoter (–2065 bp) contained this very element. The possible interaction between Ets/AP-1 and PPARδ (and α) might also be

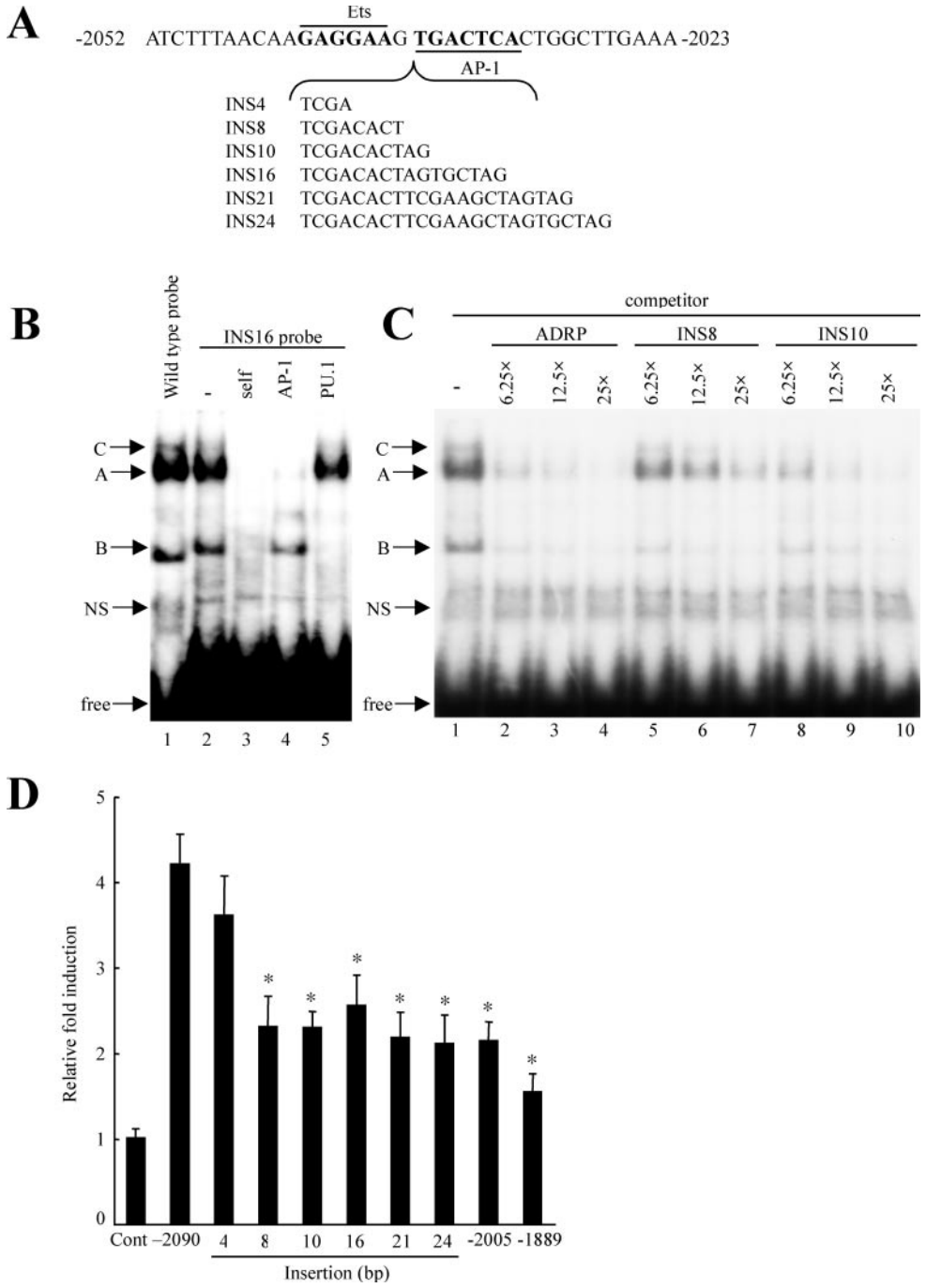

Fig. 8. **Juxtaposition of Ets and AP-1 elements is requisite for full enhancer activity.** A: Four to 24 bp intervening sequences were inserted between the Ets and AP-1 core elements in the –2090 bp promoter–luciferase construct. B: AP-1 and PU.1 independently can bind to each recognition site, respectively, even though these two elements are separated. A representative result with the INS16 oligonucleotide as a probe is shown. C: Inhibition of complex formation by serial dilution (6.25 to 25 fold molar excess) of the competitor oligonucleotide. The ADRP oligonucleotide was used as a probe. A representative result is shown. D: Relative fold induction of the wild type and the inserted promoters induced by PMA in the lipid-containing medium was compared. The value for the promoter-less control is arbitrarily designated as 1. The means ± SEM of six independent experiments are shown. Relative fold induction of each promoter was compared with those of all the other promoters and evaluated by ANOVA (Scheffe). All promoter constructs exhibited significantly higher PMA-inducibility than the promoter-less control ($p < 0.01$). Promoters with a more than 8 bp insertion exhibited significantly lower activity than the –2090 bp promoter as well as the 4 bp insertion one (*$p < 0.01$).

mediated by certain cofactors, such as CBP/p300 also in this case, since it has been shown that PPAR interacts with CBP/p300 (*41*). This is currently under investigation using our system.

From all these results collectively, we suggest the following scenario for ADRP regulation. Since the PI3 kinase and PKC pathways are involved in a number of basic cellular functions, including cell growth, differentiation and gene

expression (*42, 43*), the Ets/AP-1 function is constitutively active, thereby, ADRP mRNA is constitutively expressed. In the absence of lipids or fatty acids, however, the ADRP protein translated is rapidly degraded possibly through the proteasome pathway, as in the case of apoB, which is ubiquitinated and degraded by this machinery (*44*), although we have not yet obtained evidence as to whether or not ADRP is actually ubiquitinated. Once cells are exposed to fatty acids or lipids, these substances function as ligands for PPARδ to stimulate ADRP mRNA expression in one way, and the lipids incorporated or synthesized in the cells stabilize the ADRP protein by facilitating cytosolic lipid droplet formation in another way. For most cells, lipid droplet formation is important not only for energy storage but also for the detoxication of fatty acids, which are potentially harmful to cells (*16*). The Ets/AP-1 site in the ADRP promoter might be a molecular switch that keeps the PPARδ lipid sensor (*20*) "ON" at all times to incorporate or metabolize lipids.

The nucleotide sequence data reported are available in the DDBJ database under accession number AB207137. We wish to thank Ms Etsuko Kumagai and Misaki Kuwano for their excellent technical assistance, and Dr. Shoji Tokunaga, Dept. of Preventive Medicine, Graduate School of Medical Sciences, Kyushu University, for the helpful suggestions regarding statistical evaluation. This work was supported in part by research grants from The Ministry of Education, Culture, Sports, Science and Technology, Japan, and CREST, Japan Science and Technology Corporation.

REFERENCES

1. Londos, C., Brasaemule, D.L., Schultz, C.J., Segrest, J.P., and Kimmel, A.R. (1999) Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. *Cell Developmental Biol*. **10**, 51–58
2. Reid, A.E. (2001) Nonalcoholic steatohepatitis. *Gastroenterology* **121**, 710–723
3. Ross, R. (1995) Cell biology of atherosclerosis. *Annual Rev. Physiol*. **57**, 791–804
4. Liu, P., Ying, Y., Zhao, Y., Mundy, D.I., Zhu, M., and Anderson, R.G. (2004) Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic. *J. Biol. Chem.* **279**, 3787–92
5. Greenberg, A.S., Egan, J.J., Wek, S.A., Garty, N.B., Blanchette-Mackie, J., and Londos, C. (1991) Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J. Biol. Chem*. **266**, 11341–11346
6. Blanchette-Mackie, E.J., Dwyer, N.K., Coxey, R.A., Tekeda, T., Rondinone, C.M., Theodorakis, J.L., Greenberg, A.S., and Londos, C. (1995) Perilipin is located on the surface layer of intracellular lipid droplets in adipocytes. *J. Lipid Res.* **36**, 1211–1226
7. Servetnick, D.A., Brasaemle, D.L., Gruia-Gray, J., Kimmel, A.R., Wolff, J., and Londos, C. (1995) Perilipins are associated with cholesteryl ester droplets in steroidogenic adrenal cortical and leydig cells. *J. Biol. Chem.* **270**, 16970–16973
8. Wang, S.M. and Fong, T.H. (1995) A lipid droplet-specific capsule is present in rat adrenal cells: evidence from a monoclonal antibody. *Biochem. Biophys. Res. Commun.* **217**, 81–88
9. Jiang, H.P. and Serrero, G. (1992) Isolation and characterization of a full-length cDNA coding for an adipose differentiation-related protein. *Proc. Natl. Acad. Sci. USA* **89**, 7856–7860
10. Brasaemle, D.L., Barber, T., Wolins, N.E., Serrero, G., Blanchette-Mackie, E.J., and Londos, C. (1997) Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. *J. Lipid Res.* **38**, 2249–2263
11. Imamura, M., Inoguchi, T., Ikuyama, S., Taniguchi, S., Kobayashi, K., Nakashima, N., and Nawata, H. (2002) ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. *Am. J. Physiol. Endocrinol. Metab*. **283**, E775-E783
12. Heid, H.W., Moll, R., Schwetlick, I., Rackwitz, H-R., and Keenan, T.W. (1998) Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. *Cell Tissue Res.* **294**, 309–321
13. Gao, J. and Serrero, G. (1999) Adipose differentiation related protein (ADRP) expressed in transfected COS-7 cells selectively stimulates long chain fatty acid uptake. *J. Biol. Chem.* **274**, 16825–16830
14. Atshaves, B.P., Starodub, O., McIntosh, A., Petrescu, A., Roths, J.B., Kier, A.B., and Schroeder, F. (2000) Sterol carrier protein-2 alters high density lipoprotein-mediated cholesterol efflux. *J. Biol. Chem.* **275**, 36852–36861
15. Frolov, A., Petrescu, A., Atshaves, B.P., So, P.T.C., Gratton, E., Serrero, G., and Schroeder, F. (2000) High density lipoprotein-mediated cholesterol uptake and targeting to lipid droplets in intact L-cell fibroblasts. A single- and multi-photon fluorescence approach. *J. Biol. Chem.* **275**, 12769–12780
16. Murphy, D.J. (2001) The biogenesis and functions of lipid bodies in animals, plants and microorganisms. *Progress in Lipid Res.* **40**, 325–438
17. Gao, J., Ye, H., and Serrero, G. (2000) Stimulation of adipose differentiation related protein (ADRP) expression in adipocyte precursors by long-chain fatty acids. *J. Cell Physiol.* **182**, 297–302
18. Ye, H. and Serrero, G. (1998) Stimulation of adipose differentiation related protein (ADRP) expression by ibuprofen and indomethacin in adipose precursors and in adipocytes. *Biochem. J.* **330**, 803–809
19. Steiner, S., Wahl, D., mangold, B.L.K., Robison, R., Raymackers, J., meheus, L., Anderson, N.L., and Cordier, A. (1996) Induction of the adipose differentiation-related protein in liver of etomoxir-treated rats. *Biochem. Biophys. Res. Commun.* **218**, 777–782
20. Chawla, A., Lee, C-H., Barak, Y., He, W., Rosenfeld, J., Liao, D., Han, J., Kang, H., and Evans, R.M. (2003) PPARδ is a very low-density lipoprotein sensor in macrophages. *Proc. Natl. Acad. Sci.USA.* **100**, 1268–1273
21. Chen, J-S., Greenberg, A.S., Tseng, Y-Z.l, and Wang, S-M. (2001) Possible involvement of protein kinase C in the induction of adipose differentiation-related protein by sterol ester in RAW 264.7 macrophages. *J. Cell Biochem.* **83**, 187–199
22. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning—A Laboratory Manual*, 2nd ed, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
23. Ikuyama, S., Shimura, H., Hoeffler, J.P., and Kohn, L.D. (1992) Role of the cyclic adenosine 3′,5′-monophosphate response element in efficient expression of the rat thyrotropin receptor promoter. *Mol. Endocrinol.* **6**, 1701–1715
24. Eisinger, D.P. and Serrero, G. (1993) Structure of the gene encoding mouse adipose differentiation-related protein (ADRP). *Genomics* **16**, 638–644
25. Suzuki, K., Lavaron, S., Mori, A., Okajima, F., Kimura, S., Katoh, R., Kawaoi, A., and Kohn, L.D. (1998) Thyroid transcription factor 1 is calcium modulated and coordinately

regulates genes involved in calcium homeostasis in C cells. *Mol. Cell Biol.* **18**, 7410–7422
26. Nakabeppu, Y., Oda, S., and Sekiguchi, M. (1993) Proliferative activation of quiescent Rat-1A cells by ΔFosB. *Mol. Cell Biol.* **13**, 4157–4166
27. Nakabeppu, Y. and Nathans, D. (1991) A naturally occurring truncated form of FosB that inhibits Fos/Jun transcriptional activity. *Cell* **64**, 751–759
28. Corsini, E., Iviani, B., Zancanella, V.O., Lucchi, L., Visioli, F., Serrero, G., Bartesaghi, S., Galli, C.L., and Marinovich, M. (2003) Induction of adipose differentiation-related protein and neutral lipid droplet accumulation in keratinocytes by skin irritants *J. Invest. Dermatol*. **121**, 337–344
29. Wang, C-Y., Bassuk, A.G., Boise, L.H., Thompson, C.B., Bravo, R., and Leiden, J.M. (1994) Activation of the granulocyte-macrophage colony-stimulating factor promoter in T cells requires cooperative binding of Elf-1 and AP-1 transcription factors. *Mol. Cell. Biol.* **14**, 1153–1159
30. Stacey, K.J., Fowles, L.F., Colman, M.S., Ostrowsk, M.C., and Hume, D.A. (1995) Regulation of urokinase-type plasminogen activator gene transcription by macrophage colony-stimulating factor. *Mol. Cell. Biol.* **15**, 3430–3441
31. Benbow, U., and Brinckerhoff, C.E. (1997) The AP-1 site and MMP gene regulation: What is all the fuss about? *Matrix Biology* **15**, 519–526
32. Li, A.C., Guidez, F.R.B., Collier, J.G., and Glass, C.K. (1998) The macrosialin promoter directs high levels of transcriptional activity in macrophages dependent on combinatorial interactions between PU.1 and c-Jun. *J. Biol. Chem.* **273**, 5389–5399
33. Bassuk, A.G. and Leiden, J.M. (1995) A direct physical association between ETS and AP-1 transcription factors in normal human T cells. *Immunity* **3**, 223–237
34. Rieske, P., and Pongubala, J.M. (2001) AKT induces transcriptional activity of PU.1 through phosphorylation-mediated modifications within its transactivation domain. *J. Biol. Chem.* **276**, 8460–8468
35. Huang, C., Schmid, P.C., Ma, W-Y., Schmid, H.H.O., and Dong, Z. (1997) Phosphatidylinositol-3 kinase is necessary for 12-O-tetradecanoylphorbol-13acetate-induced cell transformation and activated protein-1 activation. *J. Biol. Chem.* **272**, 4187–4194
36. Pongubala, J., Van, Beveren, C., Nagulapalli, S., Klemsz, M., Mckercher, S., Maki, R., and Atchison, M. (1993) Effect of PU.1 phosphorylation on interaction with NF-EM5 and transcriptional activation. *Science* **259**, 1622–1625
37. Xue, Y., Alford, P., and Shackelford, E. (1999) Protein kinase C activation increases binding of transcription factor PU.1 in murine tissue macrophages. *Biochem. Biophys. Res. Commun.* **254**, 211–221
38. Arias, J., Alberts, A.S., Brindle, P., Claret, F.X., Smeal, T., Karin, M., Feramisco, J., and Montminy, M. (1994) Activation of camp and mitogen responsive genes relies on a common nuclear factor. *Nature* **370**, 226–229
39. Bannister, A.J., and Kouzarides, T. (1995) CBP-induced stimulation of c-Fos activity is abrogated by E1A. *EMBO J.* **14**, 4758–4762
40. Yamamoto, H., Kihara-Negishi, F., Yamada, T., Hashimoto, Y., and Oikawa, T. (1999) Physical and functional interactions between the transcription factor PU.1 and the coactivator CBP. *Oncogene* **18**, 1495–1501
41. Mizukami, J., and Taniguchi, T. (1997) The antidiabetic agent thiazolidine stimulates the interaction between PPARγ and CBP. *Biochem. Biophys. Res. Commun.* **240**, 61–64
42. Blumberg, P.M. (1991) Complexities of the protein kinase C pathway. *Mol. Carcinogenesis* **4**, 339–344
43. Cantley, L.C. (2002) The phosphoinositide 3-kinase pathway. *Science* **296**, 1655–1657
44. Yueng, S.J., Chen, S.H., and Chan, L. (1996) Ubiquitin-proteasome pathway mediates intracellular degradation of apolipoprotein B. *Biochemistry* **35**, 13843–13848