

Macrophage-specific Up-regulation of Apolipoprotein E Gene Expression by STAT1 Is Achieved via Long Range Genomic Interactions^{*[5]}

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In atherogenesis, macrophage-derived apolipoprotein E (apoE) has an athero-protective role by a mechanism that is not fully understood. We investigated the regulatory mechanisms involved in the modulation of apoE expression in macrophages. The experiments showed that the promoters of all genes of the apoE/apoCI/apoCIV/apoCII gene cluster are enhanced by multienhancer 2 (ME.2), a regulatory region that is located 15.9 kb downstream of the apoE gene. ME.2 interacts with the apoE promoter in a macrophage-specific manner. Transient transfections in RAW 264.7 macrophages showed that the activity of ME.2 was strongly decreased by deletion of either 87 bp from the 5' end or 131 bp from the 3' end. We determined that the minimal fragment of this promoter that can be activated by ME.2 is the proximal -100/+73 region. The analysis of the deletion mutants of ME.2 revealed the importance of the 5' end of ME.2 in apoE promoter transactivation. Chromatin conformational capture assays demonstrated that both ME.2 and ME.1 physically interacted with the apoE promoter in macrophages. Our data showed that phorbol 12-myristate 13-acetate-induced differentiation of macrophages is accompanied by a robust induction of apoE and STAT1 expression. In macrophages (but not in hepatocytes), STAT1 up-regulated apoE gene expression via ME.2. The STAT1 binding site was located in the 174–182 region of ME.2. In conclusion, the specificity of the interactions between the two multienhancers (ME.1 and ME.2) and the apoE promoter indicates that these distal regulatory elements play an important role in the modulation of apoE gene expression in a cell-specific manner.

Apolipoprotein E (apoE), a glycoprotein of 35 kDa, is associated with the chylomicron remnants, very low density lipoproteins, low density lipoproteins (LDL), and high density lipoproteins (HDL) and plays an important role in lipid metabolism (1–6). Deficiency in apoE results in atherosclerosis in humans and in animal models (7–12). ApoE knock-out mice are the

best-characterized animal models of atherosclerosis (13). ApoE is a ligand for the LDL receptor found in the liver and other tissues and for the LDL receptor-related protein found in hepatocytes and as such it facilitates the clearance of lipoprotein remnants from the circulation (14–16). Malfunction of the mechanisms of cholesterol clearance leads to the accumulation of remnants in the plasma, a process associated with premature atherosclerosis (8, 11, 12). ApoE regulates plasma cholesterol levels, also having an important role in cholesterol efflux, as documented by studies in patients and animal models with apoE deficiency or mutated apoE genes (17–24). Recently, anti-oxidant and anti-inflammatory functions within the atherosclerotic plaque were attributed to apoE (23, 24).

ApoE is mainly synthesized by the liver and also by various cells and peripheral tissues (25). At the site of atherosclerotic lesion, apoE is provided by macrophages. Transgenic mice expressing apoE only in macrophages are protected against atherosclerosis even though the plasma levels of apoE are exceedingly low and the animals are hypercholesterolemic (26); by contrast, transgenic mice with normal levels of plasma apoE but not in macrophages are more susceptible to atherosclerosis (27). ApoE secreted by macrophages within the atherosclerotic plaque facilitates the cholesterol efflux to exogenous acceptors (such as HDL), thus, assisting the reverse cholesterol transport to the liver. We have previously reported that inflammatory conditions (similar to those found at the atherosclerotic site) lead to down-regulation of the endogenous apoE gene expression in macrophages (28), suggesting that the local beneficial effect of apoE may be impaired during the plaque development. As a result, despite the fact that macrophages are present in the lesion, their ability to regress atherosclerosis is seriously compromised.

Although apoE was initially identified decades ago (29), the knowledge related to the regulation of the apoE gene is very limited. The human apoE gene is located on chromosome 19 (30–32) in a cluster also containing apoCI, apoCIV, and apoCII genes. Even though these genes are closely located, these apolipoproteins are differentially regulated and have distinct functions in lipid metabolism. Regulation of apolipoprotein gene transcription is a highly complex process and requires the interaction of transcription factors with the proximal promoters but also with distal regulatory regions. Multiple positive and negative elements have been detected on the apoE promoter

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^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables 1 and 2.

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using *in vitro* systems (33–37); however, the promoter lacks the ability to direct gene transcription *in vivo* in any cells in the absence of distal enhancers (38). Cell-specific distal enhancers regulate the expression of the genes belonging to the apoE/apoCI/apoCI'/apoCIV/apoCII gene cluster in many tissues (38). Studies in transgenic mice established that the expression of the genes belonging to the above cluster in the liver is controlled by two homologous hepatic control regions, designated as HCR.1² and HCR.2, of ~320 bp each, located 15 and 27 kb downstream of the apoE gene, respectively (39–41). In addition, two homologous enhancers, designated multienhancer-1 (ME.1) and multienhancer-2 (ME.2), which contain 620 and 619 nucleotides respectively, were recently identified (38). The role of these enhancers, which are located at 3.3 and 15.9 kb downstream of the apoE gene, respectively (Fig. 1A), is not fully understood.

The regulatory machinery that promotes apoE expression during cell differentiation and the complete molecular mechanism of the cell-specific expression of apoE gene are unknown. Thus, the aim of this study was to elucidate the mechanisms that control apoE promoter activity in macrophages. We report here that in macrophages (and not in other cell lines) ME.2 physically interacts with the apoE promoter, and this interaction facilitates the transcriptional enhancement of the apoE promoter by the transcription factors STAT1 that bind on ME.2.

EXPERIMENTAL PROCEDURES

Chemicals—Restriction and modification enzymes (T4 DNA ligase, TaqDNA polymerase, M-MLV reverse transcriptase, calf intestinal alkaline phosphatase) and Lipofectamine 2000 were purchased from Invitrogen or Promega (Madison, WI); Pfu DNA polymerase and FastDigest Restriction Enzymes were from Fermentas; DMEM, RPMI 1640, fetal calf serum, and TRIzol reagent were from Invitrogen; ECL Western blotting kit was from Pierce; phorbol 12-myristate 13-acetate (PMA), Liga-Fast Rapid DNA Ligation System, and Luciferase assay system were from Promega; Dynabeads M-280 streptavidin was from Invitrogen Dynal (AS, Oslo, Norway), Halt Protease Inhibitor Single-Use mixture EDTA-free was from Thermo Scientific (Rockford, IL); FuGENE 6 Transfection Reagent was from Roche Applied Science; Histopaque-1077 and all other chemicals were from Sigma. The primers were obtained from Metabion Inc. (Martinsried, Germany), and all antibodies were from Santa Cruz Biotechnology, Inc. The expression vector for STAT1 (from Thermo Scientific/Open Biosystems) and JAK inhibitor AG-490 (tyrphostin B42) were kindly provided by Dr. Adrian Manea (Institute of Cellular Biology and Pathology “N. Simionescu,” Romania).

Plasmid Constructions—The apoE proximal promoter [–500/+73] was cloned into the pGL3 basic vector (Promega) that contains as a reporter the promoterless luciferase (luc) gene at the SacI/KpnI sites as previously described (28). To

construct the plasmid containing the –388/+18 human apoCII proximal promoter, the insert containing the apoCII promoter was excised from the plasmid described in Gafencu *et al.* (28) using Asp-718 and XhoI restriction enzymes and cloned in the pGL3 basic vector at the KpnI and XhoI restriction sites; the plasmid thus obtained was named [–388/+18]apoCII. ApoCI and apoCIV proximal promoters were amplified by PCR using the primers described in supplemental Table 1 and cloned at the XhoI/HindIII and KpnI/SacI cloning sites of the pGL3 basic vector, respectively, obtaining the following plasmids: [–523/+22]apoCI and [–650/+19]apoCIV. The construction of the plasmid containing the p22 proximal promoter ([–500/+66]p22) was previously described (42).

The sequence of the ME.2 was amplified by PCR using primers ME.2 + 19 forward and ME.2 + 619 reverse (described in supplemental Table 1) and was subsequently cloned at the KpnI site of the plasmids [–500/+73]apoE-luc, [–523/+22]apoCI-luc, [–650/+19]apoCIV-luc, [–388/+18]apoCII-luc, [–500/+66]p22-luc, and in pGL3 basic vector. The following plasmids were obtained: ME.2/[–500/+73]apoE-luc, ME.2/[–523/+22]apoCI-luc, ME.2/[–650/+19]apoCIV-luc, ME.2/[–388/+18]apoCII-luc, ME.2/[–500/+66]p22-luc, and ME.2-luc. ME.2 was also cloned in reporter plasmids containing the following fragments of the apoE promoter: [–450/+73], [397/+73], [–350/+73], [–300/+73], [–250/+73], [–193/+73], [–150/+73], [–100/+73], and [–55/+73]. The following plasmids were obtained: ME.2/[–450/+73]apoE-luc, ME.2/[–397/+73]apoE-luc, ME.2/[–350/+73]apoE-luc, ME.2/[–300/+73]apoE-luc, ME.2/[–250/+73]apoE-luc, ME.2/[–197/+73]apoE-luc, ME.2/[–150/+73]apoE-luc, ME.2/[–100/+73]apoE-luc, and ME.2/[–55/+73]apoE-luc. In addition, 5' and 3' deletion mutants of the ME.2 were cloned in the plasmid [–500/+73]apoE-luc, and the following plasmids were obtained: [19–141]ME.2/[–500/+73]apoE, [19–298]ME.2/[–500/+73]apoE, [19–386]ME.2/[–500/+73]apoE, [19–488]ME.2/[–500/+73]apoE, [19–619]ME.2/[–500/+73]apoE, [87–619]ME.2/[–500/+73]apoE, [165–619]ME.2/[–500/+73]apoE, [267–619]ME.2/[–500/+73]apoE. In all the above plasmids, ME.2 was cloned in 5' to 3' orientation. In addition, in the plasmid [–500/+73]apoE-luc, ME.2 sequence was cloned also in 3' to 5' orientation. Double-stranded oligonucleotides containing a STAT1 siRNA-expressing sequence or a scrambled sequence (described in supplemental Table 1) were ligated into BglII-HindIII sites of pSuper.GFP/neo vector (Oligoengine) to generate the sh-STAT1 and sh-control producing vectors.

The ME.2-mut/[–500/+73]apoE-luc and ME.2-mut/[–545/+18]apoCII-luc constructs, bearing mutations in the STAT1 binding site located on ME.2, were generated by overlapping PCR. For this, we used as external primers RV3 and GL2 oligonucleotides and internal primers containing five mutated nucleotides in the STAT1 binding site (described in supplemental Table 1) and the plasmid ME.2-luc as template. The ME.2-mut sequence obtained was subsequently cloned into the KpnI site in front of the [–500/+73]apoE or [–545/+18]apoCII promoter previously cloned in pGL3 basic vector.

Cell Culture—Human monocytes were isolated from healthy blood donor volunteers upon their informed consent. Briefly, the buffy coat obtained from 50 ml of whole blood was layered

² The abbreviations used are: HCR, hepatic control region; ME, multienhancer; luc, luciferase; 3C, Chromosome Conformation Capture; PMA, phorbol 12-myristate 13-acetate; STAT1, Signal Transducers and Activators of Transcription 1.

on 6 ml of Histopaque-1077. After centrifugation ($400 \times g$, 30 min at room temperature), the leukocyte band was collected, washed with RPMI, and resuspended in 5 ml of RPMI that was layered on top of a discontinuous Percoll gradient of 40, 50, and 60%. A monocyte-enriched fraction was collected at 1.060 g/ml density (determined using Density Marker Beads for calibration of gradients of Percoll; Sigma). The human monocytes and THP-1 cells were cultured in RPMI containing 5% inactivated fetal bovine serum and antibiotics at 37 °C in 5% CO₂. The differentiation of THP-1 and human blood monocytes into macrophages was induced by exposing the cells (plated at density 1.25×10^5 cells/cm²) to 1–100 nM PMA. RAW 264.7, HEK-293, and HepG2 cells were cultured in DMEM containing 1% glucose (RAW 264.7 and HEK-293) or 4.5% glucose (HepG2) supplemented with 10% fetal calf serum, 100 µg/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a 5% CO₂ incubator.

RT-PCR—Total RNA was extracted from THP-1 cells treated with 100 nM PMA for different periods using TRIzol reagent according to the manufacturer's instructions, and cDNA was synthesized from 1 µg of RNA using oligo(dT)_{12–18} and M-MLV reverse transcriptase. The specific primers used for PCR are described in [supplemental Table 1](#). The fragments obtained by RT-PCR were 584 bp for human apoE and 451 bp for GAPDH.

Transient transfections of RAW 264.7 and HepG2 cells were performed by the calcium phosphate co-precipitation method, and THP-1 cells were transfected by lipofection using FuGENE 6 transfection reagent or Lipofectamine 2000. Forty hours after transfection, the cells were harvested and subsequently lysed. The activity of reporter gene (luciferase) was measured with the Promega Luciferase Assay System and normalized to β-galactosidase activity (determined using *o*-nitrophenyl-β-D-galactopyranoside). All the transfections were done in triplicate, and the experiments were repeated at least three times.

DNA Pulldown Assay—DNA fragments of ME.2 were biotinylated by PCR. Briefly, to obtain 19–141 and 19–298 biotinylated ME.2 fragments, the ME.2/[–500/+73]apoE plasmid was used as template, and the primers were biotinylated RV3 forward and ME.2 141 and ME.2 298 reverse, respectively. To obtain the fragments 87–619, 165–619, and 267–619, the respective plasmids containing the deletion mutants of ME.2 cloned in pGL3 basic vector were used as templates along with the biotinylated RV3 forward and ME.2 619 reverse primers.

Biotinylated DNA was coupled to the Dynabeads M-280 streptavidin according to the manufacturer's instruction. Briefly, biotinylated DNA was incubated with Dynabeads M-280 streptavidin for 30 min at room temperature in B&W buffer (5 mM Tris-HCl, pH 7.5, with 0.5 mM EDTA and 1 M NaCl) in a slight excess of DNA. After coupling, the beads were washed twice with B&W buffer to remove the unbound DNA and once with the binding buffer (see below). Nuclear extracts were purified as previously described (28); these extracts were incubated with the biotinylated DNA immobilized on Dynabeads for 16 h at 4 °C in binding buffer (20 mM Tris, pH 7.5, with 50 mM KCl, 4 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, and protease inhibitor mixture EDTA-free). The complexes formed were isolated with Dynabeads M-280 Streptavidin, washed with binding buffer, and then subjected to Western blotting using

anti-STAT1 rabbit polyclonal antibody (sc-346, Santa Cruz Biotechnology). The blots were developed using an ECL kit from Pierce and a gel analyzer system (Chemiluminiscent Image Reader LAS-4000 from FUJIFILM Europe GmbH, Dusseldorf, Germany).

Immunoblotting—Cell homogenates obtained from human monocytes and THP-1 cells treated with various PMA concentrations were washed with PBS, harvested, solubilized, subjected to SDS-PAGE, and transferred onto nitrocellulose. The blots were tested with anti-apoE, anti-STAT1, and anti-actin antibodies followed by horseradish peroxidase-conjugated secondary antibodies. The protein bands were detected using chemiluminescence technique described above.

Chromatin Conformational Capture (3C)— 1×10^7 human monocytes treated with PMA and untreated cells were fixed with *p*-formaldehyde (1% final concentration) in the culture medium for 10 min at room temperature. Fixation was stopped by adding glycine to a final concentration of 0.125 M. Then cells were harvested by scraping and centrifuged at $1180 \times g$. The pellet was washed with PBS, and the cells were resuspended in 10 ml of cold lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 0.2% Nonidet P-40, and a mixture of protease inhibitors) and incubated on ice with mixing (75 rpm) for 15 min. The sediment of cell nuclei obtained by centrifugation at $425 \times g$ (12 min, 4 °C) was washed with 200 µl of 1× buffer O (Fermentas). After centrifugation at $300 \times g$ (5 min, 4 °C), the pellet was resuspended in 500 µl of buffer O (1×). 10% SDS was added to a final concentration of 0.2%, and the pellet was incubated for 15 min at 37 °C with mixing (100 rpm); then 20% Triton was added to 1% final concentration and incubated again at 37 °C for 15 min with mixing (100rpm). PstI was added to each sample to 0.4 units/µl final concentration and incubated at 37 °C for 18 h with mixing. To inactivate the enzyme, SDS was added to 2% final concentration, and the samples were incubated for 2 h at 80 °C. Samples were cooled at room temperature, and cold ligase buffer (40 mM Tris, pH 7.5, 10 mM MgCl₂, 10 mM DTT, 1 mM ATP, 1% Triton) was added to a final volume of 8 ml. Samples were transferred on ice, 250 units of T4 DNA ligase were added, and then incubated at 16 °C for 18 h. Proteinase K was added to 100 µg/ml final concentration, and the samples were incubated at 65 °C for 18 h. Then RNase was added to a final concentration of 0.5 µg/ml, and the DNA was purified using phenol/chloroform extraction followed by ethanol precipitation. DNA was resuspended in 240 µl of DNase RNase-free water. Samples of 10 µl were subjected to PCR using the primers apoE –1516, ME.1 –507, ME.1 +576, ME.2 –712, ME.2 +577, apoE –350, apoE +4, ME.2 +19 forward, and ME.2 +619 reverse, described in [supplemental Table 1](#).

Statistical Analysis of the Data—Statistical processing of the data was performed using one-way analysis variance between groups from OriginPro 7.5. All values were expressed as the means ± S.E. For *p* values less than 0.05, the population means are statistically different.

RESULTS

Multienhancer 2 Enhances the Activity of All Promoters of the ApoE/ApoCI/ApoCIV/ApoCII Gene Cluster in Macrophages—Initially, we investigated whether ME.2 has the capacity to

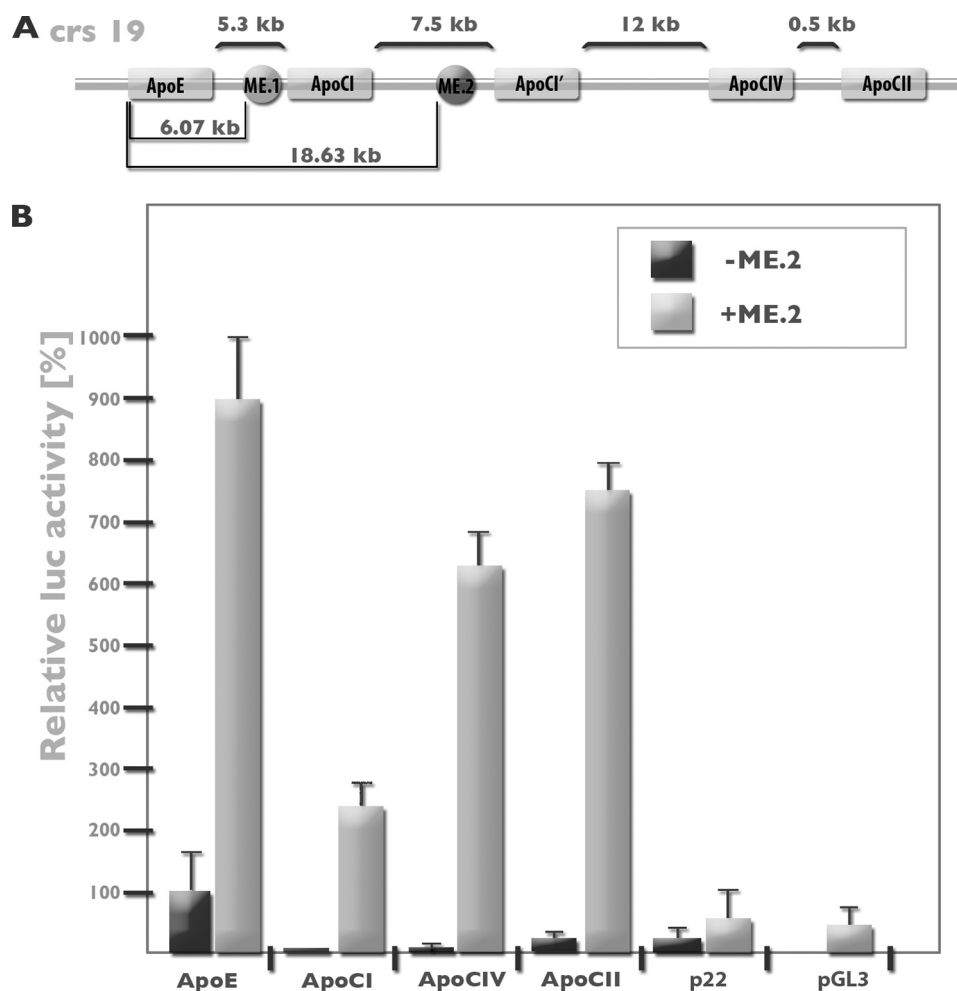


FIGURE 1. The effect of the multienhancer 2 on the activity of the apoE/apoCI/apoCIV/apoCII gene cluster promoters in macrophages. *Panel A*, the apoE/apoCI/apoCIV/apoCII gene cluster and multienhancer 1 and 2 (ME.1, ME.2) location is shown. *Panel B*, transient transfection experiments in RAW 264.7 cells using plasmids in which ME.2 was cloned in the proximal position of apoE, apoCI, apoCIV, and apoCII promoters in pGL3 basic vector is shown. Note that all four promoters of the genes found in the cluster are modulated by ME.2 (apoE, apoCI, apoCIV, and apoCII columns); the highest activity was found for apoE and apoCII. ME.2 did not significantly enhance the activity of p22 proximal promoter, used as control (p22 columns). The activity of ME.2 cloned directly in pGL3 basic vector in front of luciferase gene (pGL3 columns) represents ~50% of apoE promoter activity.

increase the activity of the promoters of the apoE/apoCI/apoCIV/apoCII gene cluster in RAW 264.7 macrophages. To achieve this goal, we placed ME.2 next to the proximal promoters of all genes of this cluster (Fig. 1A) in the pGL3 vector bearing the luciferase reporter gene, and we monitored the activity of these promoters in the absence and in the presence of ME.2 by transient transfections and luciferase assays. Our results showed that all four promoters of the gene cluster were modulated by ME.2 with the following order of enhancement: apoE > apoCII > apoCIV > apoCI (Fig. 1B). As a control, we used the proximal promoter of the p22 gene, which does not belong to the above cluster but is expressed in macrophages (43). The results showed that ME.2 did not significantly increase the activity of the p22 promoter (Fig. 1B). The activity of the ME.2/[−500/+66]p22 cluster was similar to the activity of ME.2 alone (Fig. 1B). In summary, the findings of Fig. 1 indicated that ME.2 is a transcriptional enhancer that can induce the activity of the promoters of all genes of the apoE/apoCI/apoCIV/apoCII cluster in macrophages.

The Effect of the Multienhancer 2 on the ApoE Promoter Activity in Monocytes and Macrophages—We investigated in more detail the effect of ME.2 on the activity of the apoE promoter as this promoter displayed the most prominent induction by ME.2 among all promoters tested. To this purpose we performed transient transfection experiments in RAW 264.7 macrophages, THP-1 monocytes, HepG2 hepatocytes, and HEK-293 fibroblasts using a plasmid containing apoE proximal promoter ([−500/+73]apoE) as well as a plasmid in which ME.2 was cloned in front of the proximal apoE promoter (ME.2/[−500/+73]apoE) or in the empty pGL3 basic vector (ME.2-luc) as a control. The results demonstrated that ME.2 induced (~9-fold, $p < 0.05$) the activity of the apoE promoter in macrophages (Fig. 2A) but not in monocytes or in the other cells tested (Fig. 2, B, C, and D). Moreover, only in macrophages (Fig. 2A), the enhancer alone (ME.2-luc) induced a certain luciferase activity, having ~50% of the potency of the apoE promoter. In monocytes and in other cell types tested (Fig. 2, B–D), the activity of luciferase produced by ME.2 alone (ME.2-luc) was less than 5% of the activity of the apoE proximal promoter

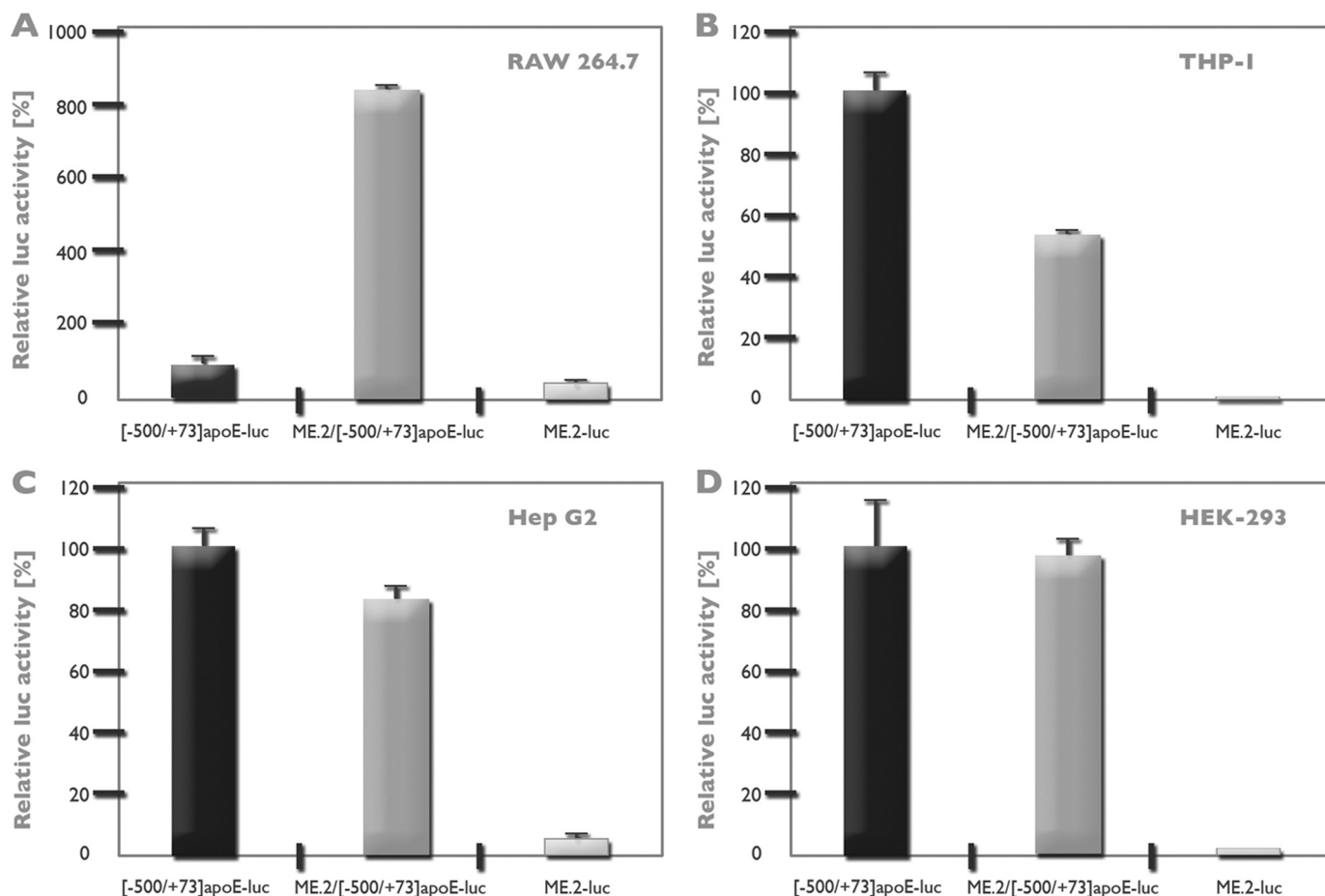


FIGURE 2. The effect of the multienhancer 2 on the apoE promoter activity in different cell types. The ME.2 sequence was cloned in the plasmid $[-500/+73]\text{apoE-luc}$, which contain apoE proximal promoter in pGL3 basic vector, as well as directly in pGL3 basic vector. The plasmids obtained (ME.2/ $[-500/+73]\text{apoE-luc}$ and ME.2-luc, respectively) as well as the plasmid $[-500/+73]\text{apoE-luc}$ were used in transient transfection experiments performed on RAW 264.7 macrophages (panel A), THP-1 monocytes (panel B), HepG2 hepatocytes (panel C), and HEK-293 cells (panel D). ME.2 induces an increase in the apoE promoter activity in macrophages but not in the other cell types tested (column ME.2/ $[-500/+73]\text{apoE-luc}$ versus column $[-500/+73]\text{apoE-luc}$). Moreover, only in macrophages the enhancer itself can induce luciferase activity, having ~50% of the potency of the apoE promoter; in the other cellular types tested, ME.2 alone induced an activity of luciferase less than 5% from the activity of the apoE proximal promoter (columns ME.2-luc).

($[-500/+73]\text{apoE-luc}$). These findings indicated that ME.2 contains regulatory elements that are functional in monocytes and macrophages and that these elements interact with the elements of the apoE promoter for optimal expression of this gene in these cell types.

Identification of the Minimal Region of the ApoE Promoter Activated by ME.2—To identify the minimal region of the apoE promoter that is enhanced by ME.2, a series of apoE promoter deletion mutants were tested for the capacity to interact with ME.2. Fig. 3A shows the results of the transient transfection experiments using plasmids containing the apoE proximal promoter ($[-500/+73]\text{apoE}$) and its mutants $[-100/+73]\text{apoE}$ and $[-55/+73]\text{apoE}$ (black columns) as well as the plasmids in which ME.2 was cloned in front of the above mutants (gray columns) or in the empty pGL3 vector (white column). The data showed that the activity of the $-500/+73$ apoE proximal promoter was increased about 9-fold ($p = 0.0032$) by ME.2 (plasmid ME.2/ $[-500/+73]\text{apoE}$). The same stimulatory effect of ME.2 was found for all deletion mutants of apoE promoter tested ($[-450/+73]$, $[-397/+73]$, $[-350/+73]$, $[-300/+73]$, $[-250/+73]$, $[-193/+73]$, and $[-150/+73]\text{apoE}$) (data not shown). The smallest apoE promoter fragment that was

enhanced by ME.2 was the $-100/+73$. As shown in the Fig. 3A, the activity of ME.2/ $[-100/+73]\text{apoE}$ was ~4 times higher than the activity of the $[-100/+73]\text{apoE}$ ($p = 0.0066$). By contrast, ME.2 did not significantly increase the activity of the $[-55/+73]\text{apoE}$ promoter fragment ($p = 0.2321$). The activity of ME.2/ $[-55/+73]\text{apoE}$ was similar to the activity of the ME.2 alone ($p = 0.176$). In summary, the data of Fig. 3A indicated that the minimal region of the apoE promoter that can be stimulated by ME.2 in macrophages is the $-100/+73$, suggesting that this region contains regulatory elements that functionally interact with the elements of ME.2.

Detection of the Minimal Region of ME.2 Responsible for Transcriptional Enhancement of the ApoE Promoter—A series of deletion mutants of ME.2 was cloned in the plasmid $[-500/+73]\text{apoE-luc}$ to determine the importance of different regions of ME.2 for the interaction with the apoE promoter. The data obtained from transient transfection experiments (Fig. 3B) showed that ME.2 activity was decreased by ~1/3 ($p < 0.05$) by deletion of 87 bp from the 5' end of ME.2 (plasmid [87–619]ME.2/ $[-500/+73]\text{apoE}$) as well as when a fragment of 131 bp from the 3' end was deleted (plasmid (19–488)ME.2/ $[-500/+73]\text{apoE}$). The data suggested that the full-length

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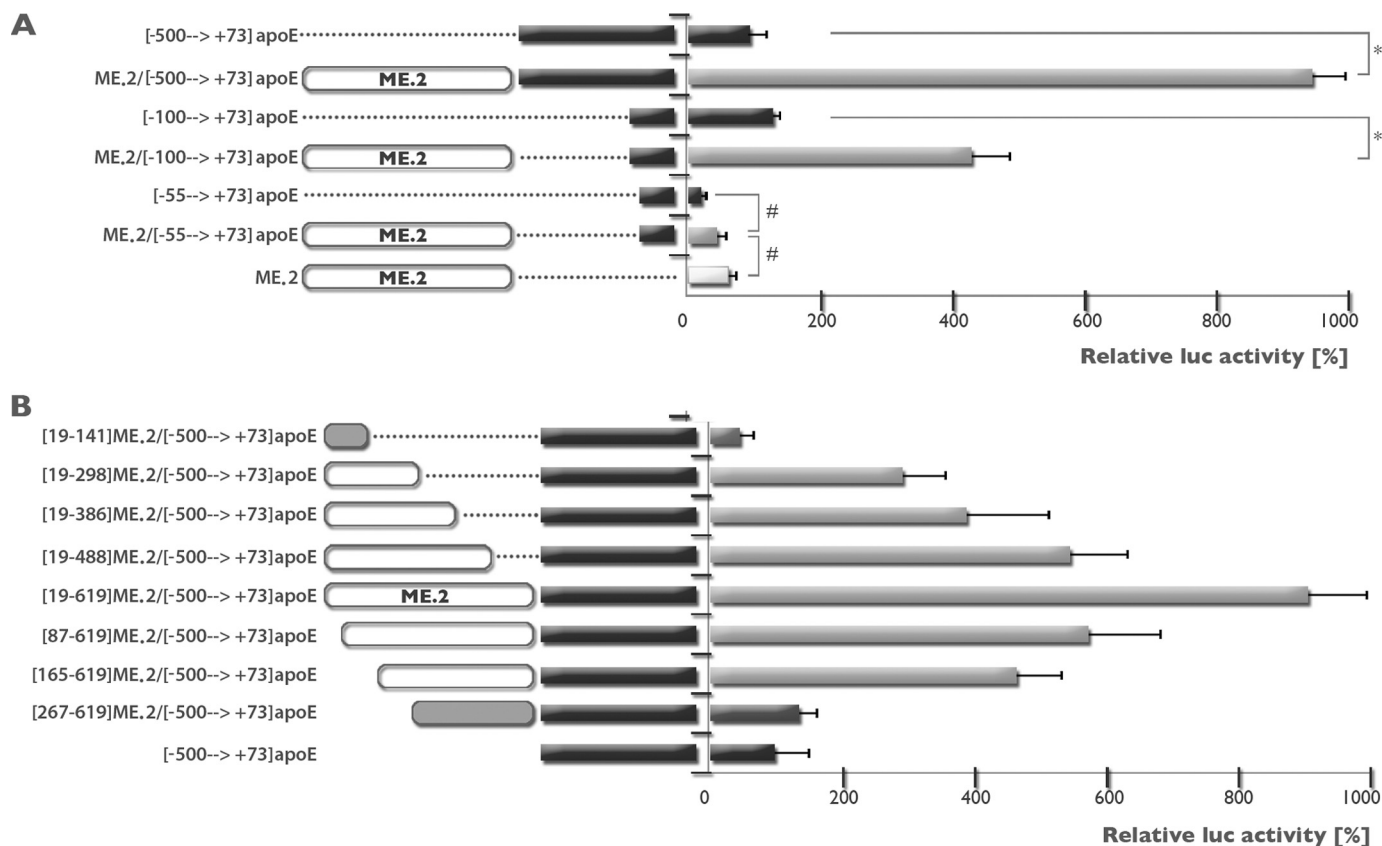


FIGURE 3. Detection of the minimal fragment of the apoE promoter transactivated by the ME.2 and the minimal regions of ME.2 responsible for transcriptional activity of apoE promoter in macrophages. Panel A, transient transfection experiments in RAW 264.7 cells show that the activities of apoE proximal promoter ([-500/+73]apoE) and its mutant [-100/+73]apoE are significantly increased (*, $p < 0.05$) when ME.2 was coupled in front of the these apoE promoter fragments (ME.2/[-500/+73]apoE and ME.2/[-100/+73]apoE). The activity of the smallest apoE promoter fragment tested ([-55/+73]apoE) is not significantly increased (#, $p > 0.05$) in the presence of ME.2 (ME.2/[-55/+73]apoE). Panel B, a series of deletion mutants of ME.2 cloned in front of the proximal apoE promoter ([-500/+73]apoE) in pGL3 basic vector was constructed to determine the role of different parts of the multienhancer in the interaction with apoE promoter. ME.2 activity is decreased with $\sim 1/3$ ($p < 0.05$) by deletion of 87 bp from 5' end of ME.2 (plasmid [87-619]ME.2/[-500/+73]apoE) as well as when a fragment of 131 bp from the 3' end was deleted (plasmid [19-488]ME.2/[-500/+73]apoE). Other deletion mutants of ME.2 ([19-386]ME.2/[-500/+73]apoE, [19-298]ME.2/[-500/+73]apoE, and [165-619]ME.2/[-500/+73]apoE) retain the potential to enhance the apoE promoter activity (light gray columns). Note that the 478 bp deleted from the 3' end of ME.2 (plasmid [19-141]ME.2/[-500/+73]apoE) as well as the 5' deletion mutant [267-619]ME.2/[-500/+73]apoE (dark gray columns) do not enhance the apoE promoter activity, having a similar activity with the apoE promoter (plasmid [-500/+73]apoE; black column).

ME.2 is necessary for an optimal interaction with the apoE promoter. The 19-298 and 165-619 deletion mutants of ME.2 also retained the potential to enhance the apoE promoter activity (light gray columns) albeit to lower levels than full-length ME.2. We also found that a deletion of 478 bp from the 3' end of ME.2 (plasmid [19-141]ME.2/[-500/+73]apoE) as well as a deletion of 266 bp from the 5' end of ME.2 (plasmid [267-619]ME.2/[-500/+73]apoE (Fig. 3B, dark gray columns) were not able to enhance apoE promoter activity, having a similar activity with the apoE promoter alone (plasmid [-500/+73]apoE; Fig. 3B, black column). Other smaller deletion mutants of ME.2 (fragments 341-619 and 427-619) were also ineffective in the activation of apoE promoter (data not shown). These results suggest that the 5' region of ME.2 is more important than the 3' region for enhancing the apoE promoter activity.

Induction of ApoE Gene Expression by PMA during Monocyte Differentiation—The PMA-induced differentiation of monocytes into macrophages, which is associated with a transcriptional induction of apoE expression, has been well established (44). Here we tested the minimal concentration of PMA that is

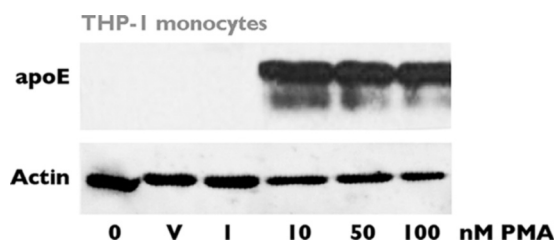


FIGURE 4. Modulation of apoE expression in PMA-treated monocytes. THP-1 cells were exposed to 1-100 nM PMA, and apoE expression was detected by Western blot. Note that control cells (0), monocytes treated with the DMSO for vehicle (V) or cells treated with 1 nM PMA do not express apoE; by contrast, treatment with 10, 50, or 100 nM PMA robustly induce apoE expression. Actin expression was tested as the control.

sufficient for the induction of apoE expression. As illustrated in Fig. 4, 10 nM PMA was the minimal concentration required for the induction of the apoE gene in THP-1 monocytes; higher concentrations of PMA (50-100 nM) produced the same effect. ApoE gene expression was not detectable in control cells or in cells treated with vehicle (DMSO) or with a very low concentration of PMA (1 nM). As a control for apoE induction in PMA-exposed monocytes, we tested actin expression (Fig. 4).

Physical Interactions between the Distal and Proximal Regulatory Elements of ApoE during Monocyte Differentiation—Although different enhancers of apoE gene expression have been described, the existing literature does not explain how the interaction between the proximal and distal regulatory elements takes place *in vivo*. In this study we tested the physical interactions between the distal regulatory elements (MEs) and the apoE promoter elements by 3C experiments. Because apoE expression is induced during monocyte differentiation, we tested if the multienhancers interact with apoE promoter in normal human blood monocytes or PMA-treated cells. Briefly, DNA-protein complexes purified from the PFA-fixed cells were digested with PstI restriction enzyme, and the adjacent sticky ends produced were ligated using T4 DNA ligase. We took advantage of the fact that PstI sites flank the apoE gene (at positions -1790 and $+2846$) as well as ME.1 (at positions $-1033/+673$) and ME.2 (at positions $-1004/+677$). Moreover, PstI restriction enzyme does not cut either inside the apoE gene or inside the ME.1 or ME.2 sequences. We envisaged the four theoretical possibilities in which apoE promoter interacts with the multienhancers, as depicted in Fig. 5, A–D. After reverse cross-linking done with Proteinase K, the ligation products were detected by PCR. The primers used in PCR were designed to anneal on the apoE promoter and on different regions located on or adjacent to ME.1/ME.2. The PCR products obtained using these primers included the newly formed PstI site. Fig. 5 illustrates the possible interactions between apoE promoter and ME.1 (A and B) or ME.2 (C and D) in sense (A and C) or antisense (B and D) and the primers used to detect each type of interaction. The forward primer was common for all cases; it anneals in the region $-1516/-1537$ of apoE gene (primer named -1516). The reverse primers were specific for each case of interaction, $-507/-477$ before ME.1 (Fig. 5A), $+576/+551$ of ME.1 (Fig. 5B), $-712/-688$ in front of ME.2 (Fig. 5C), and $+577/+553$ of ME.2 (Fig. 5D). The calculated lengths of the ligated fragments were 800 and 371 bp for the sense and antisense interaction with ME.1, respectively, and 566 and 374 bp for the sense and antisense interaction with ME.2, respectively. The 3C experiments using monocytes isolated from blood as well as monocytes derived macrophages were repeated three times, and the results of a representative experiment are represented in Fig. 5E. The results obtained by PCR using -1516 and -507 ME.1 primers showed that ME.1 cannot interact with the apoE promoter in a sense orientation (the schematic is represented in Fig. 5A) either in control cells (C) or in treated (PMA) cells, as depicted in Fig. 5E, lanes $-1516/-507$ ME.1. By contrast, ME.1 interacts with the promoter in antisense orientation (Fig. 5B) only in PMA-treated cells when the predicted PCR product of 371 bp was obtained using -1516 and -576 ME.1 primers (Fig. 5E, lanes $-1516/-576$ ME.1). Concerning the ME.2-apoE promoter interactions, data showed that ME.2 cannot interact with the apoE promoter in sense orientation (Fig. 5C), because the putative fragment of 566 bp was not obtained either for untreated (C) or treated (PMA) cells as illustrated in the Fig. 5E, lanes $-1516/-712$ ME.2. The ME.2 interacted with the promoter in a similar

manner as ME.1, in antisense orientation as referred to apoE promoter (Fig. 5D) only in PMA-differentiated cells. The data shown in Fig. 5E (lanes $-1516/+577$ ME.2) showed that the predicted PCR product of 374 bp (marked with an *) was obtained using -1516 and $+577$ ME.2 primers only in PMA-treated cells (PMA) but not in the control cells (C). The 3C PCR products were checked by digestion with PstI restriction enzyme and by nested PCR (data not shown). As positive control for the DNA integrity and PCR, we amplified a 354-bp fragment of the apoE promoter and a 600-bp fragment of the ME.2, as revealed in Fig. 5E, lanes $-350/+4$ apoE and $+19/+619$ ME.2, labeled with control (354 bp) and control (600 bp), respectively.

The results obtained using the 3C technique were confirmed by transfection experiments using plasmids containing the apoE promoter alone or in the presence of ME.2 cloned in the sense (5' to 3') orientation ($[+]$ ME.2/ $[-500/+73]$ apoE-luc) or in the antisense (3' to 5') orientation ($[-]$ ME.2/ $[-500/+73]$ apoE-luc) relative to the apoE promoter. As shown in Fig. 6, ME.2 cloned in the sense orientation increased the apoE promoter activity about 9-fold. When ME.2 was cloned in antisense orientation in front of apoE promoter, the enhancement of apoE promoter activity was about 5-fold. These results indicate that the ME.2, when cloned in 5' to 3' orientation relative to the apoE promoter, is more potent than the ME.2 cloned in 3' to 5' orientation, most probably because in this case the folding of ME.2 on the apoE promoter leads to a more efficient interaction.

To understand the fine-tuning of the molecular mechanisms that modulate apoE gene expression during PMA-induced differentiation of macrophages, we performed an *in silico* analysis (45) to search for possible transcription factor binding sites on the distal enhancer ME.2, which may be involved in this process. We found that one of the most important transcription factors that bind to the ME.2, but not to ME.1, or to the apoE proximal promoter, is STAT1. TRANSFAC-MatInspector analysis predicted a binding site for STAT1 (5'-TTCCcctaaa) in the 182/174 region of ME.2 (on the 3' to 5' strand).

Kinetics of ApoE and STAT1 Induction by PMA Treatment during Monocyte Differentiation—To test the modulation of apoE and STAT1 gene expression by PMA exposure, THP-1 monocytes were stimulated with PMA for different time periods, and the levels of expression was assessed by RT-PCR (for mRNA levels of apoE) and by Western blot (for protein levels of STAT1). As illustrated in Fig. 7, the results showed that PMA rapidly induced apoE gene expression (even at 4 h of PMA treatment). During the course of differentiation of THP-1 cells, the levels of transcripts of the apoE gene increased; the maximal level of apoE mRNA was achieved after 24 h of exposure to PMA, when cells adhered to the culture plates. GAPDH expression during PMA treatment was analyzed as a housekeeping gene and was found to remain constant. It is worth mentioning that the control (untreated THP1- monocytes) does not express endogenous apoE, but expresses a basal level of endogenous STAT1 (Fig. 7). STAT1 expression was also increased during PMA treatment, reaching a high level at 4 h of PMA treatment. The same potent effect of PMA on apoE expression was noticed when the experiments were done using human blood monocytes, except that in this case the control cells did not express

ApoE Gene Regulation via Long Range Interactions in Macrophages

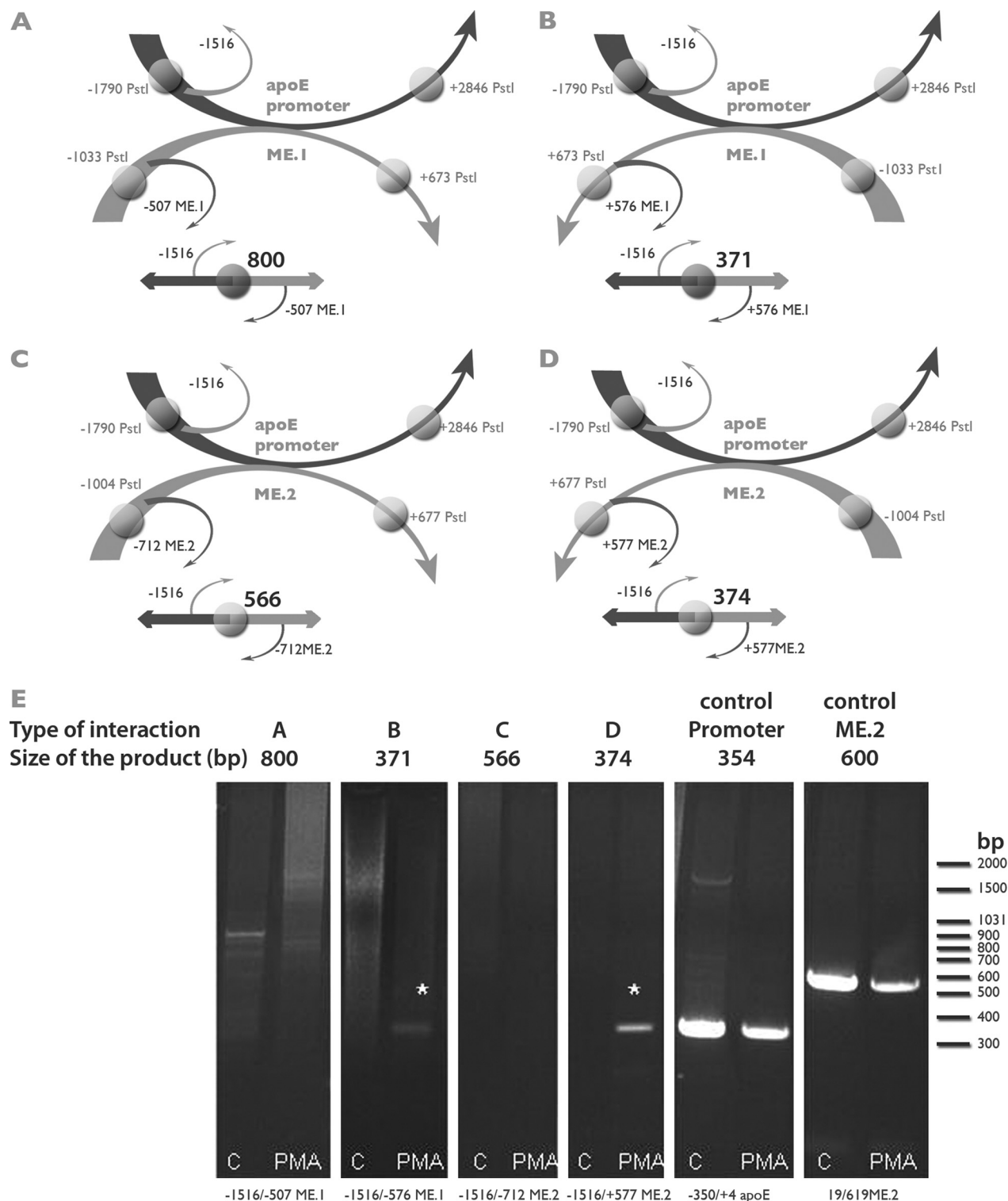


FIGURE 5. Physical interactions between the distal and proximal regulatory elements of apoE in monocytes and macrophages. Panel A–D, shown is a schematic illustration of the possible interaction between apoE promoter and ME.1 (A and B) or ME.2 (C and D) in sense (A and C) or antisense (B and D). PstI restriction sites are located at –1790 on apoE promoter, at +2846 in the apoE gene as well as at 1033 bp before ME.1 (–1033 PstI), at 53 bp after ME.1 (+673 PstI), at 1004 bp before ME.2 (–1004 PstI), and at 58 bp after ME.2 (+677 PstI). The location of the PCR primers used in the last step of 3C analysis is also shown. For all cases (A–D), the forward primer was common “–1516” (starting at –1516 on apoE promoter). The reverse primers were –507 ME.1, +576 ME.1, –712 ME.2, and +577 ME.2 (to test the interaction described in A and B and in C and D, respectively). The predicted lengths of the ligated fragments are 800 and 371 bp for sense and antisense interaction with ME.1 (A and B, respectively) and 566 and 374 bp for sense and antisense interaction with ME.2 (C and D, respectively). Panel E, the interactions between apoE promoter and ME.1/2 in normal human blood monocytes (C) or PMA-treated monocytes for 48h (PMA) as determined by 3C technique are shown. The type of interaction tested (A–D), the primers used and the expected size of the PCR fragments are pointed up. A band of 371 bp and a band of 374 bp (marked with *) were obtained, indicating that both ME.1 and ME.2 can interact with the apoE promoter only in macrophages, and the interaction takes place in antisense orientation of the two DNA fragments as depicted in the B and D. The positive control for the DNA integrity and for PCR are also shown; amplification is of a 354-bp fragment of apoE promoter (control promoter) and 600 bp of ME.2 (control ME.2).

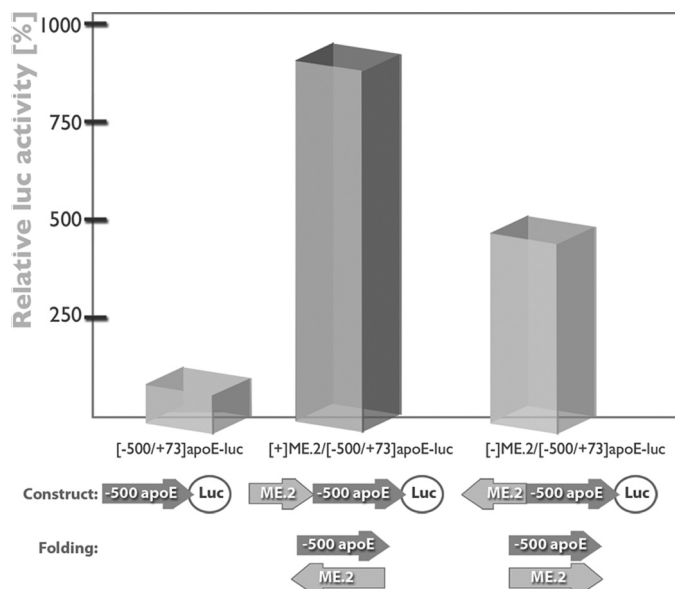


FIGURE 6. Assessment of the optimal orientation of apoE promoter and ME.2 during interaction in macrophages by transient transfection experiments. RAW 264.7 cells were transfected with plasmids containing apoE promoter alone ([−500/+73]apoE-luc) or in the presence of ME.2 cloned in sense ([+ME.2/−500/+73]apoE-luc) or antisense ([−ME.2/−500/+73]apoE-luc) orientation in pGL3 basic vector. The possible folding of cloned ME.2 on apoE promoter is also illustrated. The activity of the apoE promoter is better enhanced by ME.2 when it was cloned in sense (5′ to 3′) than in antisense (3′ to 5′) orientation, suggesting that the folding takes place with higher efficiency in antisense than in sense orientation.

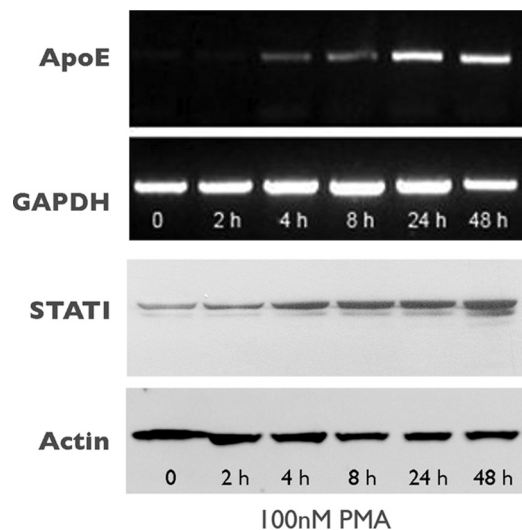


FIGURE 7. Kinetics of apoE and STAT1 induction by PMA in THP-1 monocytes. THP-1 monocytes were exposed to 100 nM PMA for different periods, and the level of expression was assessed by RT-PCR (for mRNA levels of apoE) or by Western blot (for protein levels of STAT1); GAPDH and actin expression were used as housekeeping genes. ApoE expression was not detected in untreated cells (0) and PMA-treated cells for 2 h. After 4 or 8 h of treatment, a small amount of apoE expression appeared. The PMA treatment for 24 or 48 h induces a significant apoE expression. THP1 cells express a basal level of STAT1, and its expression was also increased after 4 h of PMA treatment.

any STAT1 proteins. As expected, STAT1 was highly expressed in RAW 264.7 macrophages (data not shown).

Transactivation of apoE Promoter via Interaction with STAT1 Transcription Factor Acting on the Multienhancer 2 in Macrophages—To test whether the predicted STAT1 binding site is functional and serves as a specific modulator of apoE gene

expression in macrophages, we performed transient transfection experiments using plasmids [−500/+73]apoE-luc or ME.2/[−500/+73]apoE-luc and an expression vector for STAT1. We used RAW 264.7 macrophages in which apoE expression is controlled by the multienhancers ME.1 and ME.2 and hepatocytes (HepG2) in which apoE gene expression is control by the hepatic control regions 1 and 2 (HCR1 and -2) (46). As shown in Fig. 8, the activity of the apoE promoter −500/+73 alone was not affected by STAT1 overexpression in macrophages (A) or in hepatocytes (B). By contrast, overexpression of STAT1 increased 2-fold the activity of the −500/+73 apoE promoter/ME.2 cluster in macrophages but not in hepatocytes (Fig. 8, A and B), as ME.2 is not active in the latter cell type.

To verify the location of STAT1 binding site on ME.2, as predicted by TRANSFAC analysis, and to test whether this site is truly functional in macrophages, we performed DNA pull-down assay experiments. For this purpose, different fragments of ME.2 were biotinylated by PCR and incubated with nuclear extracts obtained from STAT1-overexpressing macrophages. The analysis of the 3′ deletion mutants of ME.2 revealed that the small 19–141 fragment did not bind STAT1 (lane 19–141), whereas the larger 19–298 fragment (lane 19–298) as well as the full-length ME.2 (lane 19–619) bound STAT1 efficiently, suggesting that the binding site of STAT1 is located in the 141/298 region of ME.2 (Fig. 8C). On the other hand, the analysis of the 5′ deletion mutants of ME.2 showed that STAT1 could bind to the 87–619 and 165–619 fragments of ME.2 (Fig. 8C, lanes 87–619 and 165–619) but could not bind to the 267–619 fragment (lane 267–619), indicating that the STAT1 binding site is located between 165/267 on ME.2 (schematically illustrated in Fig. 8D). As a negative control, we used biotin instead of biotinylated DNA, and we obtained no specific bands (Fig. 8C, no DNA).

Next, we focused on the 165–267 fragment of ME2 to identify the precise location of STAT1 binding site. For this purpose, we performed DNA pulldown assays using biotinylated, double-stranded oligonucleotides containing the sequence of wild type or mutated 167–189 ME.2 region (oligo wt/mut, described in supplemental Table 2). Our data showed that STAT1 binds to the native 167–189 ME2 region (Fig. 8E, lane oligo wt), and the binding was abrogated when the STAT1 binding site was mutated (Fig. 8E, lane oligo mut). As a positive control, illustrated in the lane ME2(19–619), the whole ME2 sequence was used; input represents the Western blot using the nuclear extract of RAW 264.7 cells (Fig. 8E). In summary, the data obtained from the DNA pulldown assays confirmed that the predicted STAT1 binding site located between 174 and 182 on ME.2, is biologically functional.

Next, we tested whether the inhibition of STAT1 binding on ME.2 influences apoE gene expression in macrophages. For this, we used a STAT1 shRNA-producing vector, a chemical inhibitor of JAK (AG-490), a protein inhibitor of STAT1 (PIAS1, protein inhibitor of activated STAT1) or we mutated the STAT1 binding site located in ME.2.

The results of transient transfections in RAW 264.7 cells using STAT1 shRNA-producing vector (sh-STAT1) or a scrambled shRNA-producing vector (sh-control) showed that

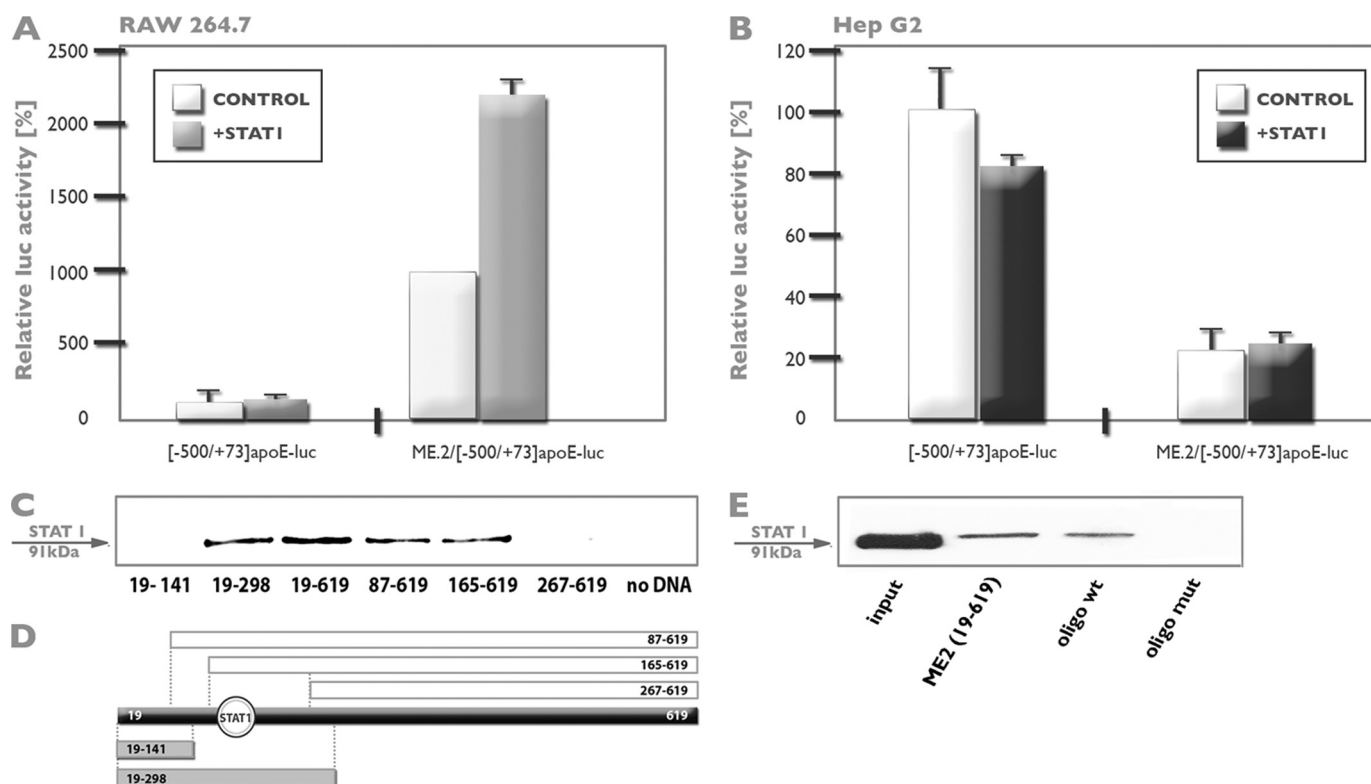


FIGURE 8. Transactivation of the apoE promoter in RAW 264.7 macrophages via interaction with STAT1 transcription factor acting on the multienhancer 2. Panel A and B, RAW 264.7 cells (A) or HepG2 cells (B) were transiently transfected with plasmids [-500/+73]apoE-luc or ME.2/[-500/+73]apoE-luc in the absence (Control) or in the presence (+STAT1) of an expression vector for STAT1. In RAW 264.7 cells, the overexpression of STAT1 did not increase the apoE promoter activity ([-500/+73]apoE-luc), but the activity of the apoE promoter in the presence of ME.2 (ME.2/[-500/+73]apoE-luc) was augmented by STAT1 overexpression. Overexpression of STAT1 in HepG2 cells did not increase the activity of apoE either in the absence or in the presence of ME.2. Panel C, D, and E, the STAT1 binding site on ME.2 is shown. DNA pull-down assays were performed using different fragments of the ME.2 (schematic illustrated in panel D) or with wild type or mutated 167–189 ME.2 region (oligo wt and oligo mut) and nuclear extract obtained from RAW 264.7 cells transfected with expression vectors for STAT1. Note that the whole ME.2 sequence (19–619) as well as the ME.2 fragments 19–298, 87–619, and 165–619 bind STAT1 transcription factor; by contrast, 19–141 as well as 267–619 fragments of ME.2 do not bind STAT1 (panel C). These results indicate a STAT1 binding site in the region 165–267 of ME.2. No bands appear in the negative control (“no DNA”) in which specific biotinylated DNA was replaced by biotin (panel C). STAT1 bound to the native 167–189 ME2 region (panel E, lane oligo wt), and the binding was abrogated when the STAT1 binding site was mutated (panel E, lane oligo mut). In the positive control, lane ME2 (19–619), the whole ME2 sequence was used; Input represents the Western blot using the nuclear extract of RAW 264.7 cells (panel E).

the activity of the ME.2/[-500/+73]apoE promoter enhancer cassette was decreased to ~64% in the presence of the sh-STAT1. By contrast, the activity of the apoE promoter alone ([-500/+73]apoE-luc) was not significantly affected by sh-STAT1 (Fig. 9A). The efficiency of STAT1 inhibition by sh-STAT1 was monitored (Fig. 9B). For this purpose, HEK293 cells (which express very low amounts of STAT1, data not shown) were transiently transfected with expression vectors for STAT1 in the presence of increasing concentrations of sh-STAT1, and the level of STAT1 expression was determined by Western blot. The data indicated that increasing concentration of sh-STAT1 induced a dose-dependent inhibition of STAT1 to ~36%.

To determine the role of STAT1 phosphorylation on apoE gene expression, RAW 264.7 cells transiently transfected with the plasmid ME.2/[-500/+73]apoE-luc were exposed to 5 μ M AG-490 for 24 h. The results showed that AG-490 induced a significant decrease (58%) of the apoE promoter activity in the presence of ME.2 (Fig. 9C). As expected, PIAS1 overexpression decreased the activity of the ME.2/[-500/+73]apoE-luc by ~45% (Fig. 9C), suggesting that the endogenous expression of STAT1 in macrophages is particularly important for apoE expression.

To test whether the STAT1 binding site located on ME.2 is critical for apoE promoter transactivation and if this mechanism is relevant for other genes of apoE/apoCI/apoCIV/apoCII cluster, mutagenesis of STAT1 binding site was performed. Thus, we introduced the mutations in the STAT1 binding site in ME.2 shown in Fig. 8 to abolish binding of STAT1 in plasmids ME.2/[-500/+73]apoE-luc and ME.2/[-545/+18]apoCII-luc. The results of the transient transfection experiments using the above plasmids revealed that the interaction between the apoE promoter and ME.2 was impaired when the STAT1 binding site was mutated (Fig. 9D). By contrast, apoCII promoter activity was increased by the mutations in the STAT1 binding site of ME.2, probably due to a different mechanism of gene regulation.

DISCUSSION

Apolipoprotein E is a major component of the lipoprotein transport system playing important roles in lipid metabolism and recognized with an atheroprotective role (1–6). Deficiency in apoE results in atherosclerosis (7–12). Restoring apoE levels only in macrophages by transplantation of normal bone marrow cells in apoE-deficient mice conferred virtually complete

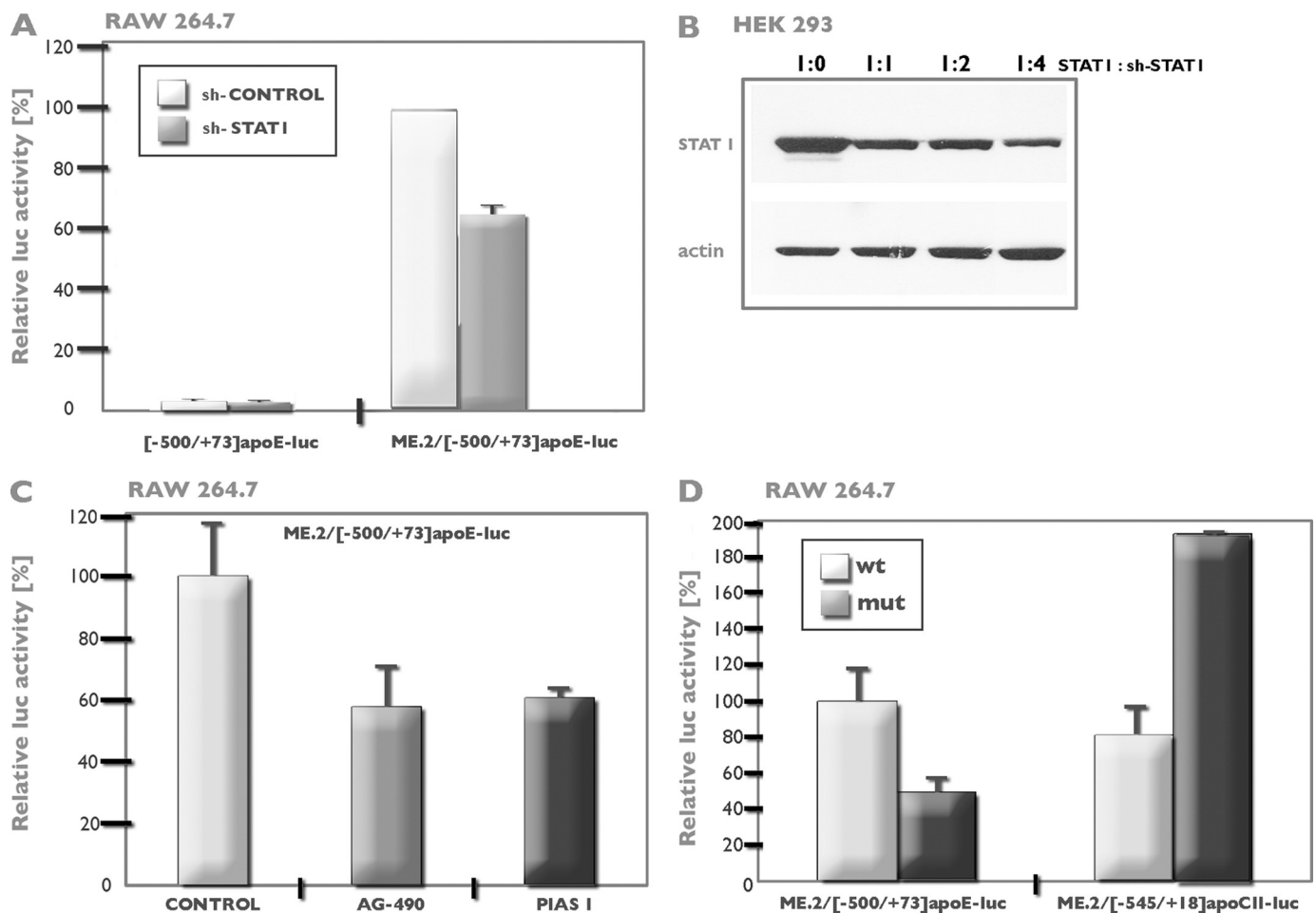


FIGURE 9. Importance of STAT1 for *apoE* gene expression in macrophages. *Panel A*, RAW 264.7 cells were transiently transfected with plasmids [-500/+73]apoE-luc or ME.2/[-500/+73]apoE-luc and STAT1 shRNA-producing vector (*sh-STAT1*) or a scrambled shRNA-producing vector (*sh-control*). The activity of ME.2/[-500/+73]apoE-luc was decreased to ~64% in the presence of the *sh-STAT1*, whereas the activity of [-500/+73]apoE-luc was not significantly affected by *sh-STAT1*. *Panel B*, the efficiency of STAT1 inhibition by *sh-STAT1* was tested by Western blot using HEK cells transiently transfected with expression vectors for STAT1 in the presence of increasing concentrations of *sh-STAT1*. Note that the level of STAT1 expression diminishes with increasing concentration of *sh-STAT1*. *Panel C*, RAW 264.7 cells were transiently transfected with plasmid ME.2/[-500/+73]apoE-luc and treated with 5 μ M AG-490 for 24 h or were cotransfected with an expression vector for PIAS1. AG-490 as well as PIAS1 overexpression induce a significant decrease (58%, 61% respectively) of the apoE promoter activity in the presence of ME.2. *Panel D*, RAW 264.7 cells were transiently transfected with plasmids ME.2/[-500/+73]apoE-luc and ME.2/[-545/+18]apoE-luc wild type (wt) or with a mutation in STAT1 binding site on ME.2 (*mut*). The interaction between the apoE promoter and ME.2 is impaired when STAT1 binding site is mutated; by contrast, apoCII promoter activity is increased after mutation within STAT1 binding site on ME.2.

protection from diet-induced atherosclerosis (23, 24). These findings demonstrate that the macrophages represent an important supplier of apoE in the atherosclerotic plaque. Therefore, in the current study we focused on apoE gene regulation in macrophages.

ApoE is mainly synthesized by the liver but also by various cells and peripheral tissues including macrophages, kidney, lung, spleen, skin, and brain among others (25). For this differential expression, the apoE gene is regulated by versatile interactions between the promoter and different distal enhancers that facilitate the recruitment of various transcription factors on the regulatory elements of the promoter, switching the transcription on and off. For hepatic expression, previous reports revealed that the human apoE gene expression in the liver is regulated by specific enhancers, HCR.1 and HCR.2 (41, 46, 47). In the skin, apoE gene expression is controlled by a unique 1.0-kb enhancer domain located 1.7 kb downstream of the apoE gene (48). Two distal downstream enhancers, ME.1 and ME.2, direct the expression of the apoE gene in macrophages, adipose

tissue, and astrocytes (38, 49, 50). The molecular mechanisms underlying the roles played by the apoE enhancers are not entirely elucidated. Consequently, the aim of our experiments was to elucidate the regulatory mechanisms that control the expression of apoE in macrophages and the role of the distal enhancers in this regulation. Although ME.1 and ME.2 are 95% identical in sequence, Edwards and co-workers (51) found that ME.2 enhances apoCII promoter activity greater than ME.1. Thus, we focused on ME.2 to reveal its specific role on apoE gene regulation in macrophages.

First, we demonstrated that ME.2 has the ability to regulate all the apolipoproteins belonging to the apoE/apoCI/apoCIV/apoCII gene cluster in RAW 264.7 macrophages (Fig. 1). In hepatocytes, a similar modulation by long range interactions was found. Allan and co-workers showed that HCR.2 is able of directing the hepatic transcription of all four genes of apoE/apoCI/apoCIV/apoCII gene cluster (47). Our data showed that ME.2 activates the apoE gene in a cell-specific manner (Fig. 2), findings that are in agreement with previous results obtained

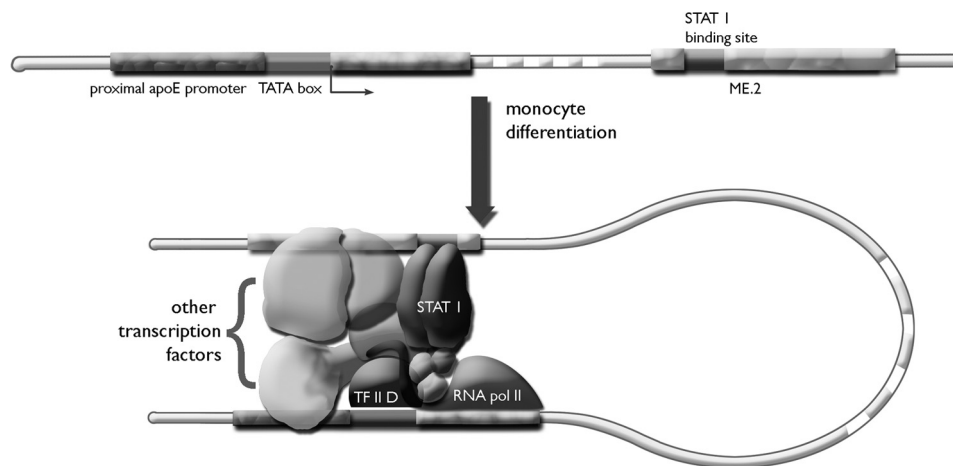


FIGURE 10. Schematic representation of the putative interaction of STAT1 with the transcription initiation machinery, leading to the modulation of apoE gene expression. Our model proposes that after DNA bending, which probably takes place during monocyte differentiation, STAT1 (bound to its site located in the 5' end of ME.2) interacts with the transcription initiation complex, leading to the activation of apoE expression. In addition, STAT1 can interact and cooperate with other transcription factors bound on the ME.2 or on the apoE promoter for the modulation of the apoE gene expression.

using transgenic mice, where the absence of the multienhancers abrogated apoE expression in macrophages but did not affect its expression in hepatocytes, where HCRs are involved (38).

Deciphering the regulatory system that is based on the long range genomic interactions is important for the development of novel strategies for the modulation of a particular group of genes. Thus, we investigated the mechanism by which ME.2 acts on the apoE promoter. We identified the region $-100/+73$ as the minimal region of the apoE promoter that is activated by the ME.2. Our data showed that the entire fragment of ME.2 is necessary for an optimal interaction with the apoE promoter, but the 5' region of ME.2 is more important than 3' region for enhancing the apoE promoter activity (Fig. 3). The involvement of 5' end of ME.2 in apoE promoter transactivation (determined by deletion mutants analysis) suggested the presence of important transcription factor binding sites in this region. Indeed, we identified and characterized a functional STAT1 binding site in the 174–182 region of ME.2.

It has been reported previously that PMA-mediated differentiation of THP-1 cells into mature macrophages is associated with a transcriptional induction of apoE expression (44). A proximal AP-1 binding site located in the region $-620/-583$ of the apoE promoter was found to contribute to the apoE gene induction during monocyte differentiation (52). Having established the optimal conditions for apoE gene induction by PMA (Fig. 4), we focused on the demonstration of physical contacts that may take place *in vivo* between distal and proximal regulatory elements during monocyte differentiation and that could affect apoE gene expression. For this, we performed 3C experiments in undifferentiated monocytes isolated from human blood as well as on PMA-differentiated macrophages. Our data showed that ME.1 as well as ME.2 physically interact with the apoE promoter only in an antisense orientation in PMA-differentiated macrophages but not in monocytes (Fig. 5E). Despite the fact that many enhancers were described for different apolipoproteins, this is the first evidence in the literature revealing long range physical contacts between distal and proximal regulatory elements involved in the modulation of an apolipoprotein

gene expression. In addition, we found that these interactions take place in a cell-specific manner.

The orientation of the interactions was also confirmed by transient transfections in which we used plasmids containing ME.2 cloned in sense (5' to 3') or antisense (3' to 5') orientation relative to the apoE promoter (Fig. 6). When ME.2 was cloned in the sense orientation, the folding of ME.2 on apoE promoter is in antisense direction and probably facilitates a better enhancer-promoter cooperation that leads to a higher expression.

Putative binding sites for transcription factors such as glucocorticoid receptor, C/EBP α and C/EBP β , were identified by *in silico* analysis in the multienhancers (38). In addition, it was experimentally demonstrated that the LXR/RXR heterodimers regulate apoE gene transcription through their interaction with a conserved LXR response element present in both ME.1 and ME.2 (48, 49). Because data showed that ME.2 is more potent than ME.1, we compared the TRANSFAC analysis of the apoE proximal promoter, ME1 and ME2, to search for transcription factor binding sites that are specific for ME.2. The comparative data showed that only four transcription factor binding sites are specific for ME.2: STAT1, IK3, VMYB, and FD2 (data not shown).

Recently, several data showed the involvement of STAT1 in atherosclerosis. It was demonstrated that the loss of STAT1 is associated with increased aortic rupture in an experimental model of aortic dissection and aneurysm formation (53). Thus, we questioned the role of STAT1 on apoE gene expression. Our experiments showed that differentiation of monocytes into macrophages upon PMA treatment is accompanied by a strong increase in STAT1 protein synthesis (Fig. 7). As a response to PMA, STAT1 proteins accumulate before apoE; thus, STAT1 could be one of the transcription factors that determines apoE gene induction during monocyte differentiation.

Our data showed that STAT1 increases apoE promoter activity only in the presence of ME.2 (Fig. 8). It is known that STAT1 potentially regulates gene expression after its phosphorylation by certain cytokines, among which the most important is the interferon γ (IFN γ). Although, IFN γ has been shown to be pro-

atherogenic (54), data from the literature showed that IFN γ inhibits macrophage apoE production by posttranslational mechanisms (55). In addition, IFN γ activates, beside STAT1, STAT3, and STAT5, other transcription factors, such as NF- κ B and AP-1 (56), which negatively modulate the apoE expression in macrophages (28). STAT1 might function as a transcription factor in the absence of tyrosine phosphorylation by IFN γ , being involved in other cellular processes, such as TNF-induced apoptosis (57). Here we provide evidence for another function of STAT1 in the cell-specific apoE gene regulation. Given the well characterized role of apoE as an anti-atherogenic factor, our results extend previous findings on the role of STAT1 in atherogenesis.

Based on the data obtained, the role of STAT1 in apoE gene regulation, including its putative interactions with the transcription initiation complex or with other transcription factors is schematically represented in Fig. 10. We assume that, after DNA bending that probably takes place during monocyte differentiation, STAT1 is bound to its site located in the 5' end of ME.2 and interacts with the transcription initiation complex, leading to the activation of apoE gene transcription. In addition, STAT1 can interact and cooperate with other transcription factors bound on the ME.2 or on the apoE promoter for the modulation of the apoE gene expression.

Taken together these data and those already reported contribute to the understanding of the specific mechanisms of apoE gene expression. These findings may help to selectively switch on the up-regulatory factors and switch off down-regulatory factors, which are key steps toward the development of successful therapies for atherosclerosis.

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