Acyl-coenzyme Acylcoenzyme acyltransferase (ACAT) is an intracellular enzyme involved in cellular cholesterol homeostasis and in atherosclerotic foam cell formation. Human ACAT gene contains two promoters (P1 and P7), each located in a different chromosome (1 and 7) (Li, B. L., Li, X. L., Duan, Z. J., Lee, O., Lin, S., Ma, Z. M., Cheng, C. C., Yang, X. Y., Park, J. P., Mohandas, T. K., Noll, W., Chan, L., and Chang, T. Y. (1999) J. Biol Chem. 274, 11060–11071). Interferon-γ (IFN-γ), a cytokine that exerts many pro-atherosclerotic effects in vivo, causes up-regulation of ACAT-1 mRNA in human blood monocyte-derived macrophages and macrophage-like cells but not in other cell types. To examine the molecular nature of this observation, we identified within the ACAT-1 P1 promoter a 159-base pair core region. This region contains 4 Sp1 elements and an IFN-γ-activated sequence (GAS) that overlaps with the second Sp1 element. In the monocytic cell line THP-1 cell, the combination of IFN-γ and all-trans-retinoic acid (a known differentiation agent) enhances the ACAT-1 P1 promoter but not the P7 promoter. Additional experiments showed that all-trans-retinoic acid causes large induction of the transcription factor STAT1, while IFN-γ causes activation of STAT1 such that it binds to the GAS/Sp1 site in the ACAT-1 P1 promoter. Our work provides a molecular mechanism to account for the effect of IFN-γ in causing transcriptional activation of ACAT-1 in macrophage-like cells.

ACAT is an intracellular enzyme responsible for catalyzing the intracellular formation of cholesterol esters from cholesterol and long-chain fatty acyl-coenzyme A (1). In mammals, two ACAT genes have been identified (2–5). In adult human tissues, ACAT-1 is the major enzyme present in various tissues, including macrophages, liver (hepatocytes and Kupffer cells), and adrenal gland (6, 7). ACAT-1 is also present in the intestine; however, the major enzyme involved in the intestinal cholesterol absorption may be ACAT-2, which is mainly located in the apical region of the intestinal villi (7). The relative tissue distributions of ACAT-1 and ACAT-2 in mice and monkeys are not entirely consistent with those found in humans (8, 9) raising the possibility that the distribution of the two ACATs in various tissues may be species dependent. In macrophages and other cell types, a dynamic cholesterol-cholesteryl ester cycle exist; the formation of intracellular cholesteryl esters is catalyzed by ACAT-1, while the hydrolysis of cholesteryl esters is catalyzed by the enzyme neutral cholesteryl ester hydrolase (10, 11). The net accumulation of intracellular cholesteryl esters is affected at the substrate level, as well as at the levels of the enzymes ACAT and neutral cholesteryl ester hydrolase (12–14). The main mode of sterol-specific regulation of ACAT-1 has been identified at the post-translational level, involving allosteric regulation by its substrate cholesterol (1, 15). On the other hand, the cellular and molecular nature of non-sterol-mediated ACAT-1 regulation remains largely unknown. Recently, using mouse macrophage-derived foam cells, Panousis and Zuckerman (12) reported that IFN-γ increased the cellular cholesteryl ester content and reduced high density lipoprotein-mediated cholesteryl efflux; its cellular effects were attributed to its ability to increase ACAT-1 message (12) and to induce down-regulation of the Tangier Disease gene (the ABC1 transporter) (16). In the current work, we showed that IFN-γ increased ACAT-1 message and protein content in human monocyte-derived macrophages. To examine the molecular mechanism of IFN-γ action on ACAT-1 gene regulation in macrophages, we identified the important cis-acting elements in the human ACAT-1 P1 promoter. In order to perform transient transfection experiments, we used THP-1 cell, a monocyte human cell line as the cell model. Upon treatment with retinoids, including all-trans-retinoic acid (ATRA), THP-1 cells differentiate into macrophage-like cells (17–20). Our results show that ATRA and IFN-γ synergistically caused up-regulation of ACAT-1 gene expression. Additional experiments revealed that ATRA causes increased gene expression of the transcription factor STAT1, while IFN-γ is essential to cause reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; knt, kelonucleotides.
STAT1 to undergo phosphorylation dependent dimerization and to bind to the GAS site present in the ACAT-1 P1 promoter.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments—Human monocytes were isolated according to a published procedure (21) with slight modification: human leukocyte packs were obtained from Shanghai Blood Service Center and used within 1 day. The cells were diluted (2.1, v/v) with cold phosphate-buffered saline (PBS), layered on an equal volume of Ficoll-Paque (Amersham Pharmacia Biotech), and centrifuged for 20 min at 2,500 rpm at room temperature. Mononuclear cells were collected and washed three times at 4 °C (to remove platelets) by adding 100 ml of PBS followed by centrifugation at 1,000 rpm for 10 min. The remaining red blood cells in the pellet were lysed by treatment with 10 ml of 0.2% NaCl for 45 s, followed by sequential additions of 10 ml of 1.6% NaCl and 30 ml of cold PBS. The pelleted cells were suspended in cold RPMI 1640 with 7% human type AB serum, and 2 ml of Dulbecco’s modified Eagle’s medium was added. Cells were cultured for up to 15 h at 37 °C, and then diluted to a density of 5 × 10^5/ml, plated onto 60-mm tissue culture dishes that were precoated with 2 ml/dish of serum, with a medium change every other day. Other cell lines were three times with warm RPMI 1640 (37 °C) to remove unadhered cells.

Reagents—Human type AB serum was from Sigma. Fetal bovine serum was obtained from Life Technologies, Inc. (Life Technologies, Grand Island, NY). Purified recombinant human IFN-γ (27) was produced in S. cerevisiae with a medium change every other day. Other cell lines were three times with warm RPMI 1640 (37 °C) to remove unadhered cells.

polyclonal antibodies were from Santa Cruz Biotechnology. [32P]-dATP (6000 Ci/mmol) were from Amersham Pharmacia Biotech. CHAPS, taurocholate, oleyl-coenzyme A, egg phosphatidylcholine, cholesterol, oleate, cholesteryl, and fatty acid-free bovine serum albumin (BSA, Sigma) were used as reagents. Rabbit anti-Sp1 (PEP2, catalog number sc-59-G, 200 units/mg of protein) was a generous gift from Professor Xin-yuan Liu (Beijing Normal University, Beijing, China). [32P]- and [α-32P]- and [35S]-labeled DNA fragments were used as probes. [α-32P]-labeled cDNA probes were used for the Northern blots. [35S]dATP (3000 Ci/mmol) was from Amersham.

Experimental procedures—Site-directed mutagenesis was performed using the corresponding set of mutant primers in ACAT-1 P1 Promoter in pGL2-E—

transfection with 1 μg of pGL2-E vector. To guard against PCR-associated nucleotide incorporation errors, the in vitro synthesis of all the constructs was sequenced. An automated ABI Prism 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Canada Inc., Mississauga, ON). To generate the fragments Sp1–1m, Sp1–2m, Sp1–3m, Sp1–4m, and GASm, respectively, the following sets of primer pairs were used: 5′-CCCTCCCGTTCGGCTACCCCC-3′/5′-GGGAGTATCCGGAAGCCGAGG-3′, 5′-GATCTCGGTCACTCCCTACGACG-3′/5′-GAGGGCAGGAAAGGTATGGCTACCTGCCTC-3′, 5′-GGCGGTAGAGGGGCTGCTGCC-3′/5′-GGAGGGGCTGCCTGCCTG-3′/5′-ACCGGAGGATGAAGCAACCTG-3′ as the primer pair to generate Sp1–120m, used Sp1–120m as the template and 5′-GAGGGCAGGAAGGTATGGCTACCTGCCTC-3′ as the primer pair to generate Sp1–123m, used Sp1–123m as the template and 5′-ACCGGAGGATGAAGCAACCTG-3′ as the primer pair to generate Sp1–1234m.

Transfection and Luciferase Assay—A series of ACAT-1 P1 promoter/luciferase reporter (Luc) constructs were transfected into THP-1 or U937 cells using the DEAE-dextran method (29, 30). After waiting 24 h, cells were washed with PBS, 1 × 10^6 cells were transfected with 1.5 μg of ACAT-1 promoter/Luc and 0.75 μg pCH110 as internal control in 1 ml of STBE (25 mM Tris-HCl, pH 7.4, 5 mM KCl, 0.7 mM CaCl2, 137 mM NaCl, 0.6 mM NaHPO4, 0.5 mM MgCl2) containing 150 μg of DEAE-dextran. The cells were incubated for 20 min at 37 °C, washed once with RPMI 1640 without FBS, then resuspended in 5 ml of fresh RPMI 1640 medium. Cells were seeded in 24-well plate for 40 h. HepG2, Caco-2, and HEK293 cells were transfected by the methods of calcium phosphate co-precipitation essentially as described by Liu et al. (31). Briefly, cells were plated at 1 × 10^5 cells/well in 1 ml of medium in 24-well tissue culture plates 1 day before transfection. One h before transfection, cells were replaced with fresh medium. Calcium phosphate precipitates containing (per well) 0.3 μg of ACAT-1 promoter and 0.15 μg of plasmid pRC/CMV-STAT1-Y701Fm were incubated with the cells at 37 °C for 8 h, after which the cell lysates were collected and assayed for luciferase activity using the luciferase assay system.

Transfection—Luciferase reporter gene (pGL2-E) containing (per well) 0.3 μg of ACAT-1 promoter and 0.15 μg of plasmid pRC/CMV-STAT1-Y701Fm was used for the transfection of cells. The total RNA (4 μg) prepared according to the single step acid guanidinium thiocyanate phenol chloroform method (Trizol Regent, Life Technologies, Inc.) was annealed with 1 μg of oligo(dT) (12–18 in length) in a total volume of 20 μl and reverse transcribed with 5 units of avian myeloblastosis virus reverse transcriptase (Life Technologies, Inc.) at 42 °C for 50 min, and then diluted to a volume of 50 μl as the ss-cDNA product. The 4 μl of diluted ss-cDNA product was added to a reaction mixture in a final volume of 20 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.5 mM dNTP, 0.5 mM each pair of primers, and 1 unit of Tag DNA polymerase (Life Technologies, Inc.). To serve as controls, GAPDH gene expression was assayed to ascertain that equal amounts of cDNA

are “fused” by denaturing and annealing in a subsequent primer extension reaction. Finally the “fusion” product was amplified by PCR using the primers GLP1 and GLP2. The product of the final PCR was digested with Kpn1 and Nhe1 and subcloned into the pGL2-E vector.
were added to each PCR. The PCR products (10 μl), taken at several different cycles (from 26 to 32), were separated in agarose gel and quantified by using the UVP Labwork Software (UVP Inc.). The sets of primers used are 5'-AAAAGGATCCCTAGGTTCC/5'-GGATGGAAGCTTGGTC-3' for the luciferase activity per transfected with pCH110 into THP-1 cells. The cells were harvested for 10 min on ice in 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride), and stored on ice for 10 min. The mixture was subjected to centrifugation (10,000 g, 4 °C, and the supernatant as nuclear extract was stored in aliquots at -80 °C.

The luciferase activities shown on the right panel were expressed as relative luciferase activities, using the value of the reporter activity driven by the SV40 promoter as one. Values were means of triplicate determinations. Sizes of error bars indicated 1 S.E. B, nucleotide sequence analysis of ACAT-1 P1 promoter core region. The four Sp1 elements were boxed. Asterisks indicate the three major transcriptional initiation sites (Li et al. (26)). Sequence of Exon 1 was underlined.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extract was prepared as described (33). THP-1 cells were harvested and washed twice with cold PBS at 4 °C, and resuspended gently in 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KC1, 1.5 mM MgCl2, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride), and stored on ice for 10 min, then vortexed for 10 s. Nuclei were pelleted (10,000 × g, 10 s), resuspended with ice-cold buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride), and incubated on ice for 20 min. The mixture was subjected to centrifugation (10,000 × g) for 2 min at 4 °C, and the supernatant as nuclear extract was stored in aliquots at -80 °C. For EMSA, 10 μg of protein of nuclear extract was incubated for 10 min on ice in 10 μl of binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, 4% glycerol, 50 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 3 μg of poly(dI-dC) from Amersham Pharmacia Biotech Inc.). DNA probes were labeled using T4 polynucleotide kinase (Promega) and [γ-32P]ATP. 1 ng of labeled probe (~1 × 106 dpm) was added to the binding reaction mixture and incubated at 25 °C for 30 min. For “supershift” analyses, 1 μl of each antibody as indicated was added and incubated 30 min at 25 °C before adding the probe. Binding reactions were size fractionated on a nondenaturing 4.5% acrylamide gel (29:1, mass:mass, acrylamide:N,N'-methylenebisacrylamide), run at 200 V for 3 h in 0.5 × TBE buffer. The gel was dried and autoradiographed with PhosphorImager scanning system.

**Fig. 2. Four Sp1 elements are functionally present in ACAT-1 P1 promoter.** A, Luc constructs containing serial 5' and 3' deletion (bars shown on the left) of the 159-bp core region were transfected into THP-1 cells. The luciferase activities (shown on right) were determined in the same manner as described in the legend to Fig. 1A. The promoterless plasmid pGL2-E was used as a negative control. B, Luc constructs containing single or multiple Sp1 mutations (marked by the x) of the 159-bp core region were transfected into THP-1 cells. The luciferase activities shown on right panel were determined as described in the legend to Fig. 1A, C, EMSAs using nuclear extracts of THP-1 cells. The wild-type and Sp1-1234-mutant DNA fragments of ACAT-1 P1 promoter (depicted at the left panel) were, respectively, labeled and 1 × 106 dpm of labeled probe was used for each binding reaction. Lane 1, 32P-labeled wild-type DNA as probe alone. Lane 2, binding reaction between labeled wild-type DNA probe and nuclear extracts. Lane 3, competition by adding 100-fold molar excess of cold probe to the binding reaction described for lane 2. Lane 4, competition by adding 100-fold molar excess of nonspecific DNA to the binding reaction described for lane 2. Lane 5, supershift reaction by adding 1 μl of anti-Sp1 antibody to the binding reaction described for lane 2. Lanes 6–10, the same conditions as described for lanes 1–5, except using the Sp1-1234-mutant DNA as the labeled probe.
treated or untreated cells were shown at level using the value in untreated cells as expressed as relative ACAT-1 protein work software (UVP Inc.). The data are determined by using the UVP Labwork software (UVP Inc.). C, immunoblotting of ACAT-1 protein from extracts of blood monocyte-derived macrophages treated with or without IFN-γ (100 units/ml) for 40 h. Protein extracts were prepared and immunoblotting were conducted as described under "Experimental Procedures." Samples used (40 μg of protein/lane) were freshly prepared with SDS. The membrane was incubated with DM10 (0.5 μg/ml) as the primary antibody. The immunoreactive proteins were visualized using the ECL detection system and autoradiography. The intensities of bands were determined by using the UVP Labwork software (UVP Inc.). The data are expressed as relative ACAT-1 protein level using the value in untreated cells as 1.0. The ratios of the ACAT-1 protein from treated or untreated cells were shown at the bottom panel.

according to the single step acid guanidinium thiocyanate phenol chloroform method (Trizol Regent, Life Technologies, Inc.). Total RNA, 20 μg/sample, were electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and transferred to a Nytran membrane (Schleicher and Schuell, Dassel, Germany) with 3.0 M sodium chloride, 0.3 M sodium citrate (20 × SSC) as the transfer buffer. The membrane was cross-linked by UV irradiation and incubated for 10 min at 65 °C in 0.5 M sodium phosphate buffer (pH 7.2), 7% SDS, 1 mM EDTA (prehybridization buffer). The polymerase chain reaction products of human ACAT-1 DNA (1486–2686, 1.2-kilobases) and human GAPDH cDNA (291-bp) were used as templates for labeling probes. Labeled probes were made with [α-32P]dATP by the random primer method using a Random-labeling Kit (Promega). Blots were prehybridized and hybridized with labeled probes and washed under high stringency conditions. Hybridization was carried out at 65 °C in the same solution as prehybridization, except for the addition of labeled probe. The membrane was washed with 40 μg sodium phosphate buffer (pH 7.2), 0.1% SDS at room temperature for 5 min three times, and at 65 °C for 20 min. After washing, the membrane was exposed and the intensity of the bands was quantified by densitometric analysis using the UVP Labwork software (UVP Inc.). To serve as control, rehybridization of the same blot with the human GAPDH probe was carried out. The sample mRNA expression levels were normalized by the intensity of the human GAPDH mRNA bands.

Western Blot Analysis—Cells were harvested with 10% SDS in 50 mM Tris, 1 mM EDTA (pH 7.5) with 25 mM dithiothreitol, and incubated at 37 °C for 20 min, then sheared with a syringe fitted with an 18-gauge needle. Protein concentration of the cell extract was determined by a modified Lowry method (34). The affinity purified anti-ACAT-1 IgGs (designated as DM10) was used as the primary antibodies against ACAT-1 (35). Western blots, using freshly prepared cell extracts in SDS, were conducted according to a previously described procedure (35).

ACAT Activity Assay—The assay was performed essentially as described previously (15, 35). AC29, 25RA, and THP-1 cells were cultured at 2 × 10^5/ml in 60-mm dishes and then treated in various manners for 40 h as indicated. The ACAT-1-deficient mutant cell line AC29, and its parental cell 25RA derived from Chinese hamster ovary cells (2, 15, 35) were used to ensure that the ACAT activity assayed in vitro work properly. For THP-1 cells, the suspended and adherent cells were collected by direct centrifugation and scraping, respectively, at room temperature. The two groups of cells collected from the same dish were pooled together, washed with PBS once, and centrifuged to collect the cell pellets. Cold 1 mM Tris, 1 mM EDTA (pH 7.8), at 100 μl/sample, was added to cell pellet chilled on ice. The mixtures were left on ice for 5 min. Brief but vigorous vortexing (30 s to 1 min) was used to cause extensive cell lysis. The protein concentration of the cell homogenates was kept at 2–4 mg/ml in buffer A (50 mM Tris, 1 mM EDTA at pH 7.8 with protease inhibitors). The enzyme was solubilized and assayed in mixed micelle condition as previously described (35).

RESULTS

A 159-bp Core Region with 4 Sp1 Elements Is Responsible for Human ACAT-1 P1 Promoter Activity—Human ACAT-1 gene is located in two different chromosomes (1 and 7), each chromosome containing a separate ACAT-1 promoter (P1 and P7). Northern analyses have revealed the presence of four ACAT-1 mRNAs (7.0, 4.3, 3.6, and 2.8-knt) in all the human tissues and cell lines examined (3). The 2.8 and 3.6-knt messages are produced from the P1 promoter, while the 4.3-knt mRNA is produced from two different chromosomes by a novel RNA recombination event that presumably involves trans-splicing (26). The P1 promoter is contiguous with the coding sequence and spans from −598 to +65 of the ACAT-1 genomic DNA (26).

To determine the minimal region of the P1 promoter, we trans-
fected THP-1 cells with constructs containing various deleted fragments fused upstream to a luciferase reporter gene of the pGL2-enhancer vector (pGL2-E), and measured luciferase activities. As shown in the right panel of Fig. 1, the results indicated that the maximal transcriptional activity is located within the 159-base pair from 2125 to 134 (Fig. 1A). Sequence analysis by computer revealed 4 Sp1 elements are located in this core region (Fig. 1B). We next performed various specific deletion analyses to test the relative importance of these 4 Sp1 elements. The results (Fig. 2, A and B) showed that the most important basal transcription activity is present in the first two Sp1 elements from the 5'-end of the 159-bp core region.

To demonstrate the functional importance of the 4 Sp1 elements involved in basal transcription, we next performed EMSA. We used nuclear extracts of THP-1 cells and the 159-bp DNA fragments containing mutations in each of all the 4 Sp1 elements as labeled probes. The bindings of labeled probes were tested by competing with unlabeled wild-type or mutated probes in 100-fold molar excess. The results (Fig. 2C) illustrated that the wild-type DNA fragment formed several DNA-protein complexes (lanes 2, 4, and 5); the bindings were eliminated upon incubation with excess unlabeled probe (lane 3) and supershifted by incubation with the anti-Sp1 antibodies (lane 5). Additional control experiments showed that the fragment containing mutations in all 4 Sp1 elements had no specific binding (lanes 7–10).

**FIG. 4.** The synergistic effect of IFN-γ and ATRA on ACAT-1 P1 promoter is promoter specific and cell type specific. A, the Luc constructs containing ACAT-1 P1, P7, or the SV40 promoters ligated to the luciferase reporter vector (pGL2-C) were transfected into THP-1 cells. 7 h after transfection, cells were treated with or without IFN-γ (100 units/ml), or with ATRA (10⁻⁶ M) or IFN-γ (100 units/ml) plus ATRA (10⁻⁶ M), respectively. The luciferase activity was determined in lysates of THP-1 cells 40 h later, and normalized by using values of β-galactosidase. The data were expressed as luciferase activities relative to the value from untreated cells as one. Values represented the means from triplicate determinations. Sizes of error bars represented 1 S.E. B, the Luc construct containing ACAT-1 P1 promoter was transfected into THP-1, U937, HepG2, Caco-2, or HEK293 cells as indicated by methods described under “Experimental Procedures.” The cells were then treated and assayed as described in A.

**FIG. 5.** The dose and time dependence of the IFN-γ and ATRA effects. The Luc constructs containing ACAT-1 P1 promoter was transfected into THP-1 cells. 7 h after transfection, A, cells were treated with or without ATRA (10⁻⁶ M) alone, or with ATRA plus the indicated concentrations of IFN-γ, respectively; 40 h, B, cells were treated with or without IFN-γ (100 units/ml) alone, or with IFN-γ plus indicated concentrations of ATRA for 40 h; C, cells were treated with or without ATRA (10⁻⁶ M) plus IFN-γ (100 units/ml) for the indicated lengths of time, respectively. Afterward, the luciferase activity was determined as described under “Experimental Procedures.”
we tested the human ACAT-1 promoter toward IFN-γ, ATRA, or a combination of both. As shown in Fig. 4A, treating cells with IFN-γ and ATRA, but not with IFN-γ or with ATRA alone, synergistically enhanced the luciferase expression driven by the ACAT-1 P1 promoter. To investigate the cell type and promoter specificity of this effect, we tested the human ACAT-1 P1, P7, and SV40 promoters in THP-1 cells, using the luciferase reporter activity assays. The luciferase expression driven by the appropriate primers described under “Experimental Procedures” were used to obtain the ACAT-1 P1 promoter transcript (designated as the P1 product), the ACAT-1 P7 promoter transcript (designated as P7 product), and the transcript for the control gene (indicated as GAPDH) by RT-PCR. Control experiments indicated that between cycles 25 and 35, the ACAT-1 P1 transcript and the ACAT-1 P7 transcript could be estimated semiquantitatively by RT-PCR (data not shown). The ratio of DNA contents (shown at the bottom panels) was determined using the UVP Labwork software (UVP Inc.). C, quantitation by Northern analysis. 20 μg of total RNAs per lane from cells treated in various manners as indicated was employed, using a 32P-labeled ACAT-1 cDNA probe; the same filter was rehybridized with a 32P-labeled human GAPDH cDNA probe. After exposing with PhosphorImager, the intensities of the 2.8- and 3.6-knt ACAT-1 mRNAs were normalized to that of the GAPDH mRNA levels; the intensities of bands were determined by using the UVP Labwork software (UVP Inc.). The ratios of the 2.8- and 3.6-knt message from cells treated in various manners as indicated were shown on the right panel.

Interferon-γ Causes up-regulation of Human ACAT-1 Expression in Blood Monocyte-derived Macrophages—The human blood monocytes were incubated in culture for up to 16 days. This procedure causes monocytes to differentiate into mature macrophages within several days. Cells incubated for various time points were treated with or without IFN-γ for 40 h. The total RNAs and proteins of treated and untreated cells were extracted for RT-PCR and Western blot. The results show that both the ACAT-1 P1 promoter transcript and the ACAT-1 protein level increased during the monocyte differentiation process; and these increases were further augmented in cells treated with IFN-γ (Fig. 3, A and C). In contrast, the level of the human ACAT-1 P7 promoter transcript was not significantly altered throughout the time course of the experiment (Fig. 3B).

The Combination of IFN-γ and ATRA Is Needed to Enhance ACAT-1 P1 Promoter Activity in THP-1 Cell—Using THP-1 cell, we tested the functional responses of the ACAT-1 P1 promoter toward IFN-γ, ATRA, or a combination of both. As shown in Fig. 4A, treating cells with IFN-γ and ATRA, but not with IFN-γ or with ATRA alone, synergistically enhanced the luciferase expression driven by the ACAT-1 P1 promoter. To test its functional significance, a series of P1 promoter deletion and point mutation constructs were made, linked to a luciferase reporter gene, and used in transient transfection studies in THP-1 cells. The results indicated that the two constructs containing the GAS element (at the top of Fig. 8A) responded to IFN-γ and ATRA, while the shorter promoter lacking the GAS element and first two Sp1 elements (at the bottom of Fig. 8A) did not. Specific

---

**Fig. 6.** IFN-γ and ATRA synergistically increase ACAT-1 mRNA. Total RNAs were prepared from THP-1 cells treated for 40 h with or without IFN-γ (100 units/ml) or with ATRA (10−8 M) or with IFN-γ (100 units/ml) plus ATRA (10−8 M), respectively. A and B, quantitation by RT-PCR (26 to 32 cycles). Appropriate primers described under “Experimental Procedures” were used to obtain the ACAT-1 P1 promoter transcript (designated as the P1 product), the ACAT-1 P7 promoter transcript (designated as P7 product), and the transcript for the control gene (indicated as GAPDH) by RT-PCR. Control experiments indicated that between cycles 25 and 35, the ACAT-1 P1 transcript and the ACAT-1 P7 transcript could be estimated semiquantitatively by RT-PCR (data not shown). The ratio of DNA contents (shown at the bottom panels) was determined using the UVP Labwork software (UVP Inc.). C, quantitation by Northern analysis. 20 μg of total RNAs per lane from cells treated in various manners as indicated was employed, using a 32P-labeled ACAT-1 cDNA probe; the same filter was rehybridized with a 32P-labeled human GAPDH cDNA probe. After exposing with PhosphorImager, the intensities of the 2.8- and 3.6-knt ACAT-1 mRNAs were normalized to that of the GAPDH mRNA levels; the intensities of bands were determined by using the UVP Labwork software (UVP Inc.). The ratios of the 2.8- and 3.6-knt message from cells treated in various manners as indicated were shown on the right panel.

**IFN-γ Activated Sequence (GAS) Is Required for the Synergistic Effect by IFN-γ and ATRA**—Sequence analysis by computer showed that the core region of human ACAT-1 P1 promoter contained an GAS that overlaps exactly with the second Sp1 element from 5′-end (Fig. 8A). To test its functional significance, a series of P1 promoter deletion and point mutation constructs were made, linked to a luciferase reporter gene, and used in transient transfection studies in THP-1 cells. The results indicated that the two constructs containing the GAS element (at the top of Fig. 8A) responded to IFN-γ and ATRA, while the shorter promoter lacking the GAS element and first two Sp1 elements (at the bottom of Fig. 8A) did not. Specific
mutations in the GAS element, but not mutations in the Sp1 elements, abrogated the synergistic effect by IFN-γ and ATRA (Fig. 8B). Therefore, the GAS element, rather than the 4 Sp1 sites plays an important role in mediating the regulatory response to IFN-γ and ATRA.

To further examine the functional significance of the GAS element, we isolated nuclear extracts from THP-1 cells co-treated with IFN-γ and ATRA, and performed EMSA using the wild-type P1 promoter (the 159-bp DNA) as the labeled probe. As shown in Fig. 8, C and D, two specific bands, one migrating slower than the other, were detectable (lane 2). These two bands were abolished by preincubation with unlabeled competitors containing all the Sp1 and GAS elements (lane 3). As shown in Fig. 8C, gel supershift assays using either anti-Sp1 antibodies (lane 4) or anti-STAT1 antibodies (lane 5), or both antibodies (lane 6), indicated that these two bands were specific complexes formed between STAT1 and Sp1. When excess unlabeled probes containing either the first or the second Sp1 element were used as competitors, the two bands were also competed out (lanes 7 and 8). When unlabeled probe containing only the first two mutant or all four mutant Sp1 elements were used as competitors, both bands moved faster (lanes 9 and 10) than those in the control lane (lane 2). These two bands were supershifted by using the anti-Sp1 antibody (lane 11) but not by using the anti-STAT1 antibody (lane 12). Additional experiments (Fig. 8D) showed that when labeled wild-type DNA fragments were used as probe and unlabeled DNA fragment containing all four wild-type Sp1 elements and the mutant GAS (GASm) as competitors, one band was found to move faster than the control lane (comparing lanes 4–6 with lane 2). This band was supershifted by adding anti-STAT1 antibody, but not by adding anti-Sp1 antibody (comparing lanes 4–6). When labeled probe containing mutant GAS was used, two bands moved faster (lanes 8); they were supershifted by anti-Sp1 antibody but not by anti-STAT1 antibody (comparing lanes 10 and 11). Together, these results demonstrate that the first two Sp1 sites and the GAS site are functionally important, and that the second Sp1 site overlaps with a GAS site to form a novel overlapping GAS/Sp1 element. This GAS/Sp1 element is recognized by both STAT1 and Sp1 present in the nuclear extracts of treated THP-1 cells.

**ATRA Induces STAT1 Expression, While IFN-γ Causes the STAT1 to Dimerize and Bind to GAS Element in the ACAT-1 Promoter—**STAT1 is a key component of the IFN-γ-dependent transcriptional activation complex (36, 37). We examined the transcript level of STAT1 in control and treated THP-1 cells by RT-PCR. As shown in Fig. 9A, STAT1 transcript was not detectable in control THP-1 cells (lane 1). Treatment cells with ATRA gave rise to a remarkable increase (lane 3), while treating cells with IFN-γ caused only a modest increase in the STAT1 transcript (lane 2). Treatment cells with ATRA with or without IFN-γ caused large increases in similar fashion (lane 4). These results indicated that treating THP-1 cells with ATRA, with or without IFN-γ, increased significant gene expression of STAT1. It has been shown that STAT1 can be activated as a homodimer that moves into the nucleus and acts as a mature transcription factor by binding to the GAS element (39). The dimerization of STAT1 requires tyrosine phosphorylation of STAT1 in a manner triggered by IFN-γ (38). Mutant STAT1 (STAT1-Y701Fm, replacing tyrosine 701 with phenylalanine) is unable to undergo the tyrosine phosphorylation dependent dimerization process (40). To test the possibility that IFN-γ may be involved in activating STAT1 to up-regulate the ACAT-1 gene, we prepared wild-type STAT1 cDNA (STAT1-Y701) and the mutant STAT1 cDNA (STAT1-Y701Fm) in pRC/CMV vector. We then transfected these constructs individuially into the IFN-γ and/or ATRA-treated THP-1 cells, and measured ACAT-1 P1 promoter activity. As shown in Fig. 9B, when cells were treated with IFN-γ alone, a significant enhancement of the P1 promoter was seen when these cells were transfected with wild-type STAT1 cDNA. The enhancement was not seen when mutant STAT1 cDNA was used (comparing the sizes of the second bar in the STAT1-Y701 panel with the second bar in the STAT1-Y701Fm panel). These results imply that IFN-γ is involved in stimulating the phosphorylation of STAT1, causing the dimeric form of STAT1 to bind to the GAS element. To further test this interpretation, we treated THP-1 cells with IFN-γ alone, and transfected with or without the wild-type or the mutant STAT1 cDNAs, then prepared the nuclear extracts of these cells and performed EMSAs. As shown in Fig. 9C, the two GAS-specific bands were detectable in the nuclear extracts of cells transfected with wild-type

---

**Fig. 7.** IFN-γ and ATRA synergistically increase ACAT-1 protein and enzyme activity. A, immunoblotting of ACAT-1 protein from extracts of THP-1 cells were treated in various manners as indicated. Cells were seeded at 2 × 10⁵/ml in 60-mm dishes with 7.5 ml of medium, and treated for 40 h with or without IFN-γ (100 units/ml), or with ATRA (10⁻⁶ M) or IFN-γ (100 units/ml) plus ATRA (10⁻⁶ M) as indicated. Cell extracts were prepared and immunoblotting were conducted as described under “Experimental Procedures.” The data are expressed as relative ACAT activity using the value in untreated THP-1 cells as 1.0. The ratios of the protein from treated cells in various manners as indicated were shown at the bottom panel. B, ACAT activity assayed in vitro. AC29, 25RA, and THP-1 cells were incubated with DM10 (0.5 mg/ml) as the primary antibody. The immunoreactive proteins were visualized using the ECL detection system and autoradiography. The intensities of bands were determined by using the UV Labwork software (UVP Inc.). The data are expressed as relative protein using the value in untreated THP-1 cells as 1.0. The ACAT specific activity in untreated cell extracts was 74 pmol/mg/min.
STAT1 cDNA (comparing lane 6 with lane 2). These two bands were not detectable in extracts of cells transfected with the mutant STAT1 cDNA (comparing lane 10 with lane 2). Results of the gel supershift assays using anti-STAT1 and anti-Sp1 antibodies confirmed that these two bands were complexes resulting from specific interactions of STAT1 and Sp1 with the ACAT-1 P1 promoter (lanes 8 and 9). Additional EMSAs, using the nuclear extracts from control (untransfected) THP-1 cells or from mutant STAT1 cDNA-transfected cells showed that the two bands described above migrated faster. These bands were not supershifted with anti-STAT1 antibodies, but were supershifted with anti-Sp1 antibodies (comparing lane 4 and 5 with lane 2 and 3). These results showed that the GAS site, not the Sp1 site, formed specific complexes with the wild-type STAT1 after activation by IFN-γ through the tyrosine-phosphorylation dependent mechanism.

DISCUSSION

ACAT-1 mRNAs and protein contents are significantly increased during the human monocyte-macrophage differentiation process in vitro (21, 41). Its protein content is amply present in macrophage-derived foam cells localized in the human atherosclerotic lesion, implying that up-regulation of the ACAT-1 gene plays important roles in macrophage foam cell formation in atherosclerosis (42). In mouse macrophages, ACAT-1 message was found to be up-regulated by cells with IFN-γ (12). The molecular basis of these findings has not been pursued at the gene transcription level. In our current work, we showed that IFN-γ increased ACAT-1 mRNAs and protein contents during the human blood monocyte-macrophage differentiation process. We then found that treating the human monocyte-like THP-1 cells with ATRA and IFN-γ caused up-regulation of ACAT-1 gene expression in cell-type specific manner. To elucidate the molecular basis of this finding, we identified a 159-bp core region with Sp1 elements that is responsible for the P1 promoter activity. This region also contains an IFN-γ activated sequence (GAS) that overlaps exactly with the second Sp1 element (TGGGCGGA, with the Sp1 site underlined). To our knowledge, this is the first example in literature describing an overlapping Sp1/GAS site. Using luciferase constructs in transient transfection studies, we demon-

FIG. 8. Identification of a functional GAS element in ACAT-1 P1 promoter. A, individual Luc constructs containing the 159-bp core region (shown on the left panel; with wild-type at the top, two different deletions as indicated in the middle or the bottom) were transfected into THP-1 cells. 7 h after transfection, cells were treated with or without IFN-γ (100 units/ml) or with ATRA (10⁻⁶ M) as indicated for 40 h. Results of luciferase activities are shown on the right panel. B, individual Luc constructs with or without various mutations in Sp1 or in GAS (as indicated on the left panel) were transfected into THP-1 cells. Cells were treated and assayed in the same way as described in A. C and D, EMSAs using nuclear extracts of THP-1 cells treated for 40 h with IFN-γ (100 units/ml) plus ATRA (10⁻⁶ M) were employed, except the mutant GAS DNA fragment was used as the labeled probe; lane 1, 3²P-labeled wild-type fragment as the probe alone serving as negative control. Lane 2, binding reaction between labeled probe and the nuclear extracts. Lane 3, competition by adding 100-fold molar excess of cold wild-type probe; lanes 4–6, supershift reactions by adding 1 µl of anti-Sp1 antibody, 1 µl of anti-STAT1 antibody, or 1 µl of anti-STAT1 antibody and 1 µl of anti-STAT1 antibody as indicated to the binding reaction; lanes 7–10, supershift reactions by adding 1 µl of anti-Sp1 antibody or 1 µl of anti-STAT1 antibody as indicated to the binding reaction described for lane 1, 3²P-labeled probe containing mutation within the first, or second, or the first two, or all four Sp1 elements as indicated; lanes 11, supershift reactions by adding 1 µl of anti-Sp1 antibody or 1 µl of anti-STAT1 antibody as indicated to the binding reactions described in lane 8.
Total RNAs were prepared from THP-1 cells treated for 40 h with or without IFN-γ (100 units/ml), or ATRA (10^{-6} M), or IFN-γ (100 units/ml) plus ATRA (10^{-6} M) as indicated. A, quantitation by RT-PCR (30 cycles). Primers were used for STAT1 and the GAPDH cDNAs as described under “Experimental Procedures.” Control experiments indicated that between cycles 25 and 35, the IFN-γ receptor transcripts and the STAT1 transcript could be estimated semiquantitatively by RT-PCR (data not shown). B, THP-1 cells were co-transfected with the Luc construct containing the wild-type ACAT-1 P1 promoter core region, and the wild-type STAT1 (indicated as STAT1-Y701Fm), or the mutant STAT1 (indicated as STAT1-Y701Fm), or the empty vector (indicated as control). 7 h after transfection, the cells were treated in various manners as indicated for 40 h. The luciferase activities of treated cell extracts were then determined in the same way as described in the legend to Fig. IA. C, EMSAs using nuclear extracts from the transfected THP-1 cells treated for 40 h with IFN-γ (100 units/ml). The wild-type 159-bp core region DNA was labeled as probe; 1 × 10^8 dpm of labeled probe was used for each binding reaction. Lane 1, control, 32P-labeled probe alone. Lane 2, binding reaction between labeled probe and the nuclear extracts. Lane 3, competition by adding 100-fold molar excess of cold probe. Lane 4, supershift reaction by adding 1 μl of anti-Sp1 antibody. Lanes 5–9, a series of reaction conditions as described in lanes 1–4, but using the nuclear extracts from THP-1 cells transfected with wild-type STAT1 cDNA or with mutant STAT1 cDNA, respectively.

The combination of IFN-γ and ATRA is needed to enhance ACAT-1 P1 promoter activity. Additional experiments using RT-PCR and EMSA showed that ATRA caused large induction of the transcription factor STAT1, while IFN-γ triggered the phosphorylation dependent activation of STAT1. The activated STAT1 then acts by binding to the overlapping GAS/Sp1 site in the ACAT-1 P1 promoter. Our work dissects the non-sterol-mediated ACAT-1 regulation at the transcriptional level, and provides a molecular mechanism to account for part of the effects of IFN-γ in causing macrophage foam cell formation in vitro.

In atherosclerosis, the infiltration of T-cells and monocyte-derived macrophages into the intimal layer of the artery is believed to lead to foam cell formation. Activated T-cells found in human atheroma secrete high levels of IFN-γ (43, 44). IFN-γ has been shown to exert certain proatherosclerotic actions in vitro. It induces VCAM-1 on endothelial cells (45), decreases apoE secretion, and increases uptake of hypertriglyceridemic very low density lipoprotein on macrophages (46), induces myosin heavy chain-II on macrophages and smooth muscle cells (47), and induces scavenger receptors on smooth muscle cells during atherogenesis (48). On the other hand, IFN-γ has also been shown to exert protective action against atherosclerosis in certain in vitro systems examined (49, 50). Recently, it has been shown that apoE knockout mice crossed with the IFN-γ receptor knockout mice display reductions in lesion size, lipid accumulation, and cellularity (51). In addition, in mice, post-transplant graft arteriosclerosis is associated with the presence of IFN-γ; the serological neutralization or the genetic absence of IFN-γ markedly reduces the extent of intimal expansion (52). These results support the notion that IFN-γ is pro-atherogenic in vivo. If this concept holds true, then our finding described here may explain some of the effects of IFN-γ on foam cell formation in vivo.

IFN-γ exhibits antigrowth or antiproliferation effects in various target cells. Its effects often occur synergistically with retinoids (53). To cite a few examples, in various myelogenous leukemic cell lines, Gianni et al. (54) showed that ATRA can bypass IFN/IFN receptors and induce the expression of IFN-regulated genes including STAT1; Matikainen et al. (55, 56) showed that ATRA causes up-regulation of several IFN-specific transcription factors and signal inducers including STAT, and enhances their responsiveness toward IFNs. The molecular mechanism(s) for the synergism observed between ATRA and IFNs in these studies remain to be elucidated. Our work described here may serve to explain some of the synergistic actions of ATRA and IFNs described in these studies.

Acknowledgments—We thank Dr. Darnell for wild-type STAT1 cDNA, and Dr. Xinyuan Liu for the gifts of recombinant IFN-γ. We also acknowledge Deng-Hong Zhang for participating in certain stages of this work, and thank Bao-Liang Song, Nian-Yi Zhang, and Wei Qi for helpful discussions.

REFERENCES

1. Chang, T. Y., Chang, C. C. Y., and Cheng, D. (1997) Annu. Rev. Biochem. 66, 613–638.
2. Chang, C. C. Y., Huh, H. Y., Cadigan, K. M., and Chang, T. Y. (1993) J. Biol. Chem. 268, 20747–20755.
3. Anderson, R. A., Joyce, C., Davis, M., Reagan, J. W., Clark, M., Shelness, G. S., and Rudel, L. L. (1998) J. Biol. Chem. 273, 26747–26754.
4. Cases, S., Novak, S., Zheng, Y. W., Myers, H. M., Lear, S. R., Sande, E., Welch, C. B., Cline, T. H., Spence, T. A., Krause, B. R., Erickson, S. K., and Farber, R. V., Jr. (1998) J. Biol. Chem. 273, 26755–26764.
5. Oelkers, P., Behari, A., Crowley, D., Billehmeier, J. T., and Turley, S. L. (1998) J. Biol. Chem. 273, 26765–26771.
6. Lee, O., Chang, C. C., Lee, W., and Chang, T. Y. (1998) J. Lipid Res. 39, 1722–1727.
7. Chang, C. C., Sakashita, N., Ornvold, K., Lee, O., Chang, E. T., Dong, R., Lin, S., Lee, C. Y., Strom, S. C., Kashyap, R., Fung, J. J., Farese, R. V., Jr., Patoiseau, J. F., Delhorn, A., and Chang, T. Y. (2000) J. Biol. Chem. 275, 28083–28092.
8. Buhman, K. K., Arcad, M., Novak, S., Choi, R. S., Wong, J. S., Hamilton, R. L., Turley, S., and Farese, R. V., Jr. (2000) Nat. Med. 6, 1341–1347.
9. Lee, R. G., Willingham, M. C., Davis, M. A., Skinner, K. A., and Rudel, L. L. (2000) J. Lipid Res. 41, 1991–2001.
10. Brown, M. S., and Goldstein, J. L. (1983) Annu. Rev. Biochem. 52, 223–261.
11. Fielding, C. J. (1992) FASEB J. 6, 3162–3168.
12. Panousis, C. G., and Zuckerman, S. H. (2000) J. Lipid Res. 41, 75–83.
13. Ross, R. (1993) Nature 363, 801–809.
