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Synergistic Transcriptional Activation of Human *ACAT-1* Gene by IFN-γ and ATRA in THP-1 cells

by

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Running Title: Activation of *ACAT-1* by IFN-γ in foam cell formation

SUMMARY

Acyl-coenzyme A: cholesterol acyltransferase (ACAT) is an intracellular enzyme involved in cellular cholesterol homeostasis and in atherosclerotic foam cell formation. Human ACAT-1 gene contains two promoters (P1 and P7), each located in a different chromosome (1 and 7) (Li *et al*., J. Biol Chem., (1999), 274: 11060-11071). Interferon-gamma (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-bp 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 (ATRA, a known differentiation agent) enhances the *ACAT-1* P1 promoter but not the P7 promoter. Additional experiments showed that ATRA 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.

INTRODUCTION

ACAT is an intracellular enzyme responsible for catalyzing the intracellular formation of cholesteryl 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 maybe 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 (NCEH) (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 NCEH (12-14). The main mode of sterol-specific regulation of ACAT-1 has been identified at the posttranslational 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 reported that IFN-γ increased the cellular cholesteryl ester content and reduced HDL-mediated cholesterol efflux; its cellular effects were attributed to its ability to increase ACAT-1 message (12) and to

induce downregulation 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 monocytic 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 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 one day. The cells were diluted (2:1, vol/vol) with cold phosphate-buffered saline (PBS), layered on an equal volume of Ficoll-Paque (Pharmacia), and centrifuged for 20 min at 2,500 rpm at room temp. Mononuclear cells were collected and washed three times at 4°C (to remove platelets) by adding 100 ml 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 0.2% NaCl for 45 s, followed by sequential additions of 10 ml 1.6% NaCl and 30 ml cold PBS. The pelleted cells were suspended in cold RPMI 1640 with 7% human type AB serum to a density of 5×10^6/ml, plated onto 60 mm tissue culture dishes that were precoated with 2ml /dish of FBS, and incubated for 90 min at 37°C. Next, the dishes were washed three times with warm RPMI 1640 (37°C) to remove unadhered cells. The adhered cells were judged to be more than 95% monocytes by α–naphthyl acetate esterase staining. The cells were cultured for up to16 days in RPMI 1640 medium supplemented with 7% human type AB serum, with a medium change every other day. Other cell lines were from ATCC. Cells were incubated 60-mm dishes in a 37°C incubator with 5% atmospheric CO_2. All media were supplemented with 100 μg/ml kanamycin, 50 U/ml streptomycin, 2 g/L sodium bicarbonate, plus 10% fetal bovine serum (FBS). THP-1 and U937 cells were grown in RPMI 1640 medium. HepG2 and Caco-2 cells were grown in DMEM medium. HEK293 cells

were grown in MEM medium. CHO cell lines AC29 and 25RA (22, 23) were grown in F12 medium.

Reagents. Human type AB serum was from Sigma. Fetal bovine serum was obtained from GIBCO-BRL (Life Technologies, Grand Island, NY). Purified recombinant human IFN-γ (1×10^7 U/mg protein) was a generous gift from professor Xin-yuan Liu (24) at Shanghai Institute of Biochemistry and Cell Biology. All-trans retinoic acid (ATRA) was from Sigma. Rabbit anti-Sp1 (PEP2, cat # sc-59-G, 200 μg/ml) and anti-STAT1 (C-111, cat # sc-417, 200 μg/ml) polyclonal antibodies were from Santa Cruz Biotechnology. [^{32}P-α]- and [^{32}P-γ]-dATP (6000 Ci/mmol) were from Amersham Corp. CHAPS, taurocholate, oleoyl-coenzyme A, egg PC, cholesteryl oleate, cholesterol and fatty acid-free bovine serum albumin were all from Sigma. Reagent-grade solvents were from Fisher. [^{3}H]-oleoyl-coenzyme A was chemically synthesized as described (25). Radioactive reagents were from Amersham.

Chimeric Plasmid Construction. The 632-bp DNA fragment containing the human *ACAT-1* P1 promoter (−598/+34) (26) was inserted into the multiple cloning sites of the luciferase reporter gene vector pGL2-E (Promega). This fragment was stepwise-deleted from both ends by various suitable restriction endonucleases to create plasmids that contained the −324/+34, −188/+34, −125/+34, −598/−126 and the −598/−189 fragments, respectively.

Deletion and Site-directed Mutagenesis of the Sp1 and GAS Elements in ACAT-1 P1 Promoter in pGL2-E. Deletions were achieved by PCR-mediated mutagenesis using the corresponding set of mutant primers that included a 6-bp KpnI or NheI linker flanking the primer

sequences on the vector. The primer sequences on the vector are: GLP1, 5'-TGTATCT TAT GGTACTGTAACTG-3'; GLP2, 5'-CTTTATGTTTTTGGCGTCTTCCA-3'. The primers used to make 5'- and 3'-deletion mutations (to generate the −110/+34, −100/ +34, −100/−7, −100/−17 and the −100/−27 fragments) were 5'-aaaggtaccGGTGGGCGGAAC-3', 5'-aaaggtaccACTGGCAACCTG-3', 5'-aaagctagCCGGCCCCTACGC-3', 5'-aaagctagCGCCCCCTGCCTC-3' and 5'-aaagctagCTCCGAGCACCGC-3', respectively. The PCR products were then digested with KpnI and NheI and subcloned into an empty pGL2-E vector. The fidelity of all these constructs was verified by sequencing. Site-directed mutagenesis was undertaken using a modification of the procedure described by Ho *et al*. (27). Briefly, two overlapping fragments of the promoter subcloned into the pGL2-E vector were amplified separately. The first reaction used a flanking primer that hybridized with the vector at the 5'- end of the inserted sequence, and an internal primer that hybridized at the site of the desired mutation and contained the mismatched base. The second reaction included one flanking primer that hybridized with the vector at the 3'-end of the inserted sequence, and an internal primer that overlapped with the site of desired mutation and also contained the mismatched base. The two overlapping fragments generated by PCR 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 KpnI and NheI and subcloned into the pGL2-E vector. To guard against PCR-associated nucleotide incorporation errors, the integrity of all the constructs generated was sequenced using an automated ABI Prism 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Canada Inc. Mississaug, 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'-CCTCCCCGTTCCGGTACCTCCCC-3'/5'-GGGGAGGTACCGGAACGGGGAGG-3', 5'-CAGTTCCGTTCACCTCCCCGCC-3'/5'-GGCGGGGAGGTGAACGGAACT G-3', 5'-GAGGCAGGAAGCGTAGGGGCCG-3'/5'-CGGCCCCTACGCTTCCTGCCTC-3', 5'-GGGCGTAGAAGCCGGGCTGTCC-3'/5'-GGACAGCCCGGCTTCTACGCCC-3', 5'-GTTGCCAGCCCCGCCCACCTCC-3'/5'-GGAGGTGGGCGGGGCTGGCAAC-3'. For additional mutagenesis work, we used Sp1-1m as the template and 5'-CAGTTCCGTTCACCTCCCCGTT-3'/5'-AACGGGGAGGTGAACGGAACTG-3' as the primer pair to generate Sp1-12m, used Sp1-12m as the template and 5'-GAGGCAGGAAGCGTAGGGGCCG-3'/5'-CGGCCCCTACGCTTCCTGCCTC-3' as the primer pair to generate Sp1-123m, used Sp1-123m as the template and 5'-AAGCGTAGAAGCCGGGCTGTCC-3'/5'-GGACAGCCCGGCTTCTACGCTT-3' as the primer pair to generate Sp1-1234m.

STAT1 Mutant Expression Plasmid. The human STAT1 cDNA (encoding the 750 amino acids of STAT1 protein) in pRC/CMV (pRC/CMV-STAT1) was a gift from Dr. Darnell (Rockefeller University). The mutant STAT1 expression plasmid was created using the site-directed mutagenesis procedure of Ho *et al*. (27). Two sets of primers 5'-GGAGAGAAGCTTCTTGGT-3'/5'-CTGAAGTCTAGAAGGGTG-3', 5'-AAGGAACTGGATTTATCAAGACTGA-3'/5'-TCAGTCTTGATAA

ATCCAGTTCCT T-3' were used to mutate STAT1 at amino acid 701 (the Jak1/2 phosphorylation site) from tyrosine to phenylalanine (28). The expression plasmid pRC/CMV-STAT1-Y701Fm was constructed by inserting the mutant PCR product digested with HindIII/XbaI into the same sites of pRC/CMV.

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 washing twice with PBS, 1×10^6 cells were transfected with 1.5 μg *ACAT-1* promoter/Luc plasmid and 0.75 μg pCH110 as internal control in 1 ml of STBE (25 mM Tris-HCl, pH7.4, 5 mM KCl, 0.7 mM $CaCl_2$, 137 mM NaCl, 0.6 mM Na_2HPO_4, 0.5 mM $MgCl_2$) containing 150 μg DEAE-Dextran. The cells were incubated for 20 min. at 37°C, washed once with RPMI 1640 without FBS, then resuspended in 5 ml fresh RPMI 1640 with 10% FBS, and plated at 2×10^5 cells/ml/well in a 24-well plate for 40 hrs. HepG2, CaCo-2, HEK293 cells were transfected by the methods of calcium phosphate co-precipitation essentially as described by Liu J *et al*. (31). Briefly, cells were plated at 1×10^5 cells/well in 1 ml medium in 24-well tissue culture plates one day before transfection. One hr before transfection, cells were replaced with fresh medium. Calcium phosphate precipitates containing (per well) 0.3 μg *ACAT-1* promoter/Luc and 0.15 μg pCH110 were prepared. The DNA/calcium phosphate precipitates were incubated with the cells at 37°C for 8 hrs, after which time the cells were washed once with PBS, and replaced with DMEM or MEM containing 10% FBS. After incubation for 7 hrs, cells were treated with or without IFN-γ (100 U/ml), or ATRA (10^{-6} M), or IFN-γ (100 U/ml) plus ATRA (10^{-6} M). 40 hrs later, the cells were harvested and the cell pellets were lysed in 200 μl of lysis

buffer (Reporter lysis buffer, Promega, Cat. E397A), vortexed for 5 sec and spun at 2000 g for 5 min at room temp. 60 μl of the cell lysate was mixed with 60 μl luciferase assay buffer (Promega) for luciferase activity measurement (Promega Instruction Bulletin Part# TB101) in an Auto Lumat BG-P luminometer (MGM instrument Inc.). For β-galactosidase activity assay, the luminescent β-galactosidase detection Kit II was used (Clontech User Manual PT2106-1).

Reverse Transcription Polymerase Chain Rreaction. The total RNA (4 μg) prepared according to the single step acid guanidinium thiocyanate phenol chloroform method (Trizol Regent, GIBCO-BRL) was annealed with 1 μg oligo-dT (12~18 in length) in a total volume of 20 μl and reverse-transcribed with 5 U of avian myeloblastosis virus reverse transcriptase (GIBCO-BRL) at 42°C for 50 min, and then diluted to a volume of 80 μl as the ss-cDNA product. The 4 μl diluted ss-cDNA product was added to a reaction mixture in a final volume of 20 μl containing 10 mM Tris-HCl (PH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dNTP, 0.5 mM each pair of primers, and 1 U of *Taq* DNA polymerase (GIBCO-BRL). To serve as controls, GAPDH gene expression was assessed to ascertain that equal amounts of cDNA 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'-AAAGGAGTCCCTAGAG-3'/5'-GGATGAGAACTC TTGC-3' for *ACAT-1* P1 product (hACAT-1 cDNA K1 1486~2043, amplifying a 558-bp fragment), 5'-ACCCACCATTATCTAA-3'/5'-ACCCACCATTATCTAA-3' for human *ACAT-1* P7 product (hACAT-1 cDNA K1 982~1670, amplifying a 689-bp fragment) (2, 26),

5'-GCCCGACCCTATTACAAAAA-3'/5'-CTGCCAACTCAACACCTC TG-3' for STAT1 coding sequence (amplifying a 646-bp fragment) (32) and 5'-GAGTCAACGGATTTGGTCG-3'/5'-GAAGTGGTGGTACCTCTTC C-3' for GAPDH (amplifying a 291-bp fragment) (32).

Electrophoretic Mobility Shift Assays (EMSAs). 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 KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride), and stored on ice for 10 min, then vortexed for 10 sec. Nuclei were pelleted (10,000 × *g*, 10 sec), resuspended with ice-cold buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min. The mixture was subject 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 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 $MgCl_2$, 4% glycerol, 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, and 3 μg of poly (dI)-(dC) from Amersham Pharmacia Biotech Inc.). DNA probes were labeled using T_4 polynucleotide kinase (Promega) and [^{32}P-γ]-dATP. 1 ng of labeled probe (~1 × 10^4 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 non-denaturing, 4.5% acrylamide gel (29:1, mass:mass, acrylamide:*N*,*N*'-methylenebis- acrylamide), ran at 200 V for 3 hrs in

0.5× TBE buffer. The gel was dried and autoradiographed with phosphor-image scanning system.

RNA Preparation and Northern Blot Analysis. THP-1 cells were cultured at 2 $\times 10^5$/ml in 60-mm dishes. Human blood monocytes were cultured at 1.5×10^6/60-mm dishes. Cells were treated with IFN-γ as indicated for 40 hrs before harvest. The preparation of total RNA was according to the single step acid guanidinium thiocyanate phenol chloroform method (Trizol Regent, Gibco BRL). Total RNA, 20 μg per 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 cDNA (1486~2686, 1.2-kb) and human GAPDH cDNA (291-bp) were used as templates for labeling probes. Labeled probes were made with [^{32}P-α]-dATP by random primer method using a Random-labeling Kit (Promega). Blots were prehybridized and hybridized with labelled 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 labelled probe. The membrane was washed with 40 mM sodium phosphate buffer (pH 7.2), 0.1% SDS at room temp 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, 1mM EDTA (pH 7.5) with 25 mM DTT, and incubated at 37°C for 20 min., then sheared with a syringe fitted with an 18g 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 procedure described previously (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 hrs as indicated. The ACAT-1 deficient mutant cell line AC29, and its parental cell 25RA derived from Chinese hamster ovary (CHO) 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 scrapping, respectively, at room temp. 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 per sample, was added to cell pellet chilled on ice. The mixtures were left on ice for 5 min. Brief but vigorous vortexing (30 sec 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 transfected 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. 1A, the results indicated that the maximal transcriptional activity is located within the 159 base pairs from −125 to +34 (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. 2A and 2B) 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 electrophoresis mobility shift assays (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 (LANE 2, 4 and 5); the bindings were eliminated upon incubation with excess unlabeled probe (LANE 3), and were 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 (LANE 7~10).

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 monocytic cells to differentiate into mature macrophages within several days. Cells incubated for various time points were treated with or without IFN-γ for 40 hrs. 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. 3A and 3C). In contrast, the level of the human *ACAT-1* P7 promoter transcript was not significantly altered throughout the time course of the experiment (Fig 3 B).

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 towards 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 results (Fig. 4A) showed that neither IFN-γ, nor ATRA, nor their combination, had any detectable effect on the *ACAT-1* P7 or the SV40 promoter. We also tested the potential effect of ATRA and/or IFN-γ on *ACAT-1* P1 promoter in other human cell lines including HEK293, HepG2, CaCo-2 and U937. The results (Fig. 4B) demonstrated that the synergistic effect of ATRA and IFN-γ occurred only in the monocytic cell lines (e.g. U937, THP-1), but not in other cell types (e.g. HEK293, HepG2, or CaCo-2). Using THP-1 cells, we next investigated the time and dose requirements. For IFN-γ, the results showed that the enhancement exhibited a saturable process, with maximal enhancement seen when IFN-γ reached 500 U/ml (Fig. 5A). For ATRA, a non-saturable, linear relationship between concentration and effect was observed (Fig. 5B). The effect of IFN-γ and ATRA continued to increase within the time frame examined (60 hrs, Fig. 5C).

To ascertain that the activating effect of ATRA/IFN-γ on *ACAT-1* promoter bears biological relevance, we treated THP-1 cells for 40 hrs with either IFN-γ, or ATRA, or a combination of both, then examined the ACAT-1 at the levels of mRNAs, protein, and enzyme activities. Semi-quantitative RT-PCR analysis showed that the level of human *ACAT-1* P1 promoter transcripts in treated cells increased by about 3 folds (Fig. 6A), while the level of human *ACAT-1* P7 promoter

transcripts were not significantly altered (Fig. 6B). Consistent with these results, Northern blotting (Fig. 6C) showed that the amounts of 3.6-knt and 2.8-knt mRNAs (26) were significantly enhanced by the combination treatment of IFN-γ and ATRA. In addition, the ACAT-1 protein as analyzed by Western blotting (Fig. 7A), as well as by the ACAT enzyme activity, measured in cholesterol-independent manner, was all significantly and proportionally increased (Fig. 7B).

IFN-Gamma 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 IFN-gamma activated sequence (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. 8C and 8D, two specific bands, one migrating slower than the other, were detectable

(LANE 2). These two bands were abolished by pre-incubation 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 (LANE 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 (LANE 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 LANE 4~6 with LANE 2). This band was supershifted by adding anti-STAT1 antibody, but not by adding anti-Sp1 antibody (comparing LANE 4~6). When labeled probe containing mutant GAS was used, two bands moved faster (LANE 8); they were supershifted by anti-Sp1 antibody but not by anti-STAT1 antibody (comparing LANE 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. 9 A, STAT1 transcript was not detectable in control THP-1 cells (LANE 1). Treating 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). Treating 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 tyrosine701 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 individually into the IFN-γ and /or ATRA treated THP-1 cells, and measured *ACAT-1* P1 promoter activity. As shown in Fig. 9 B, 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. 9 C, the two GAS-specific bands were detectable in the nuclear extracts of cells transfected with wild-type 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 (LANE 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 12 and 13). These results showed that the GAS site, not the Sp1 sites, 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 ACAT-1 gene plays important roles in macrophage foam cell formation in atherosclerosis (42). In mouse macrophages, ACAT-1 message was found to be upregulated 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 (**TGGGCGGAA**, 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 trasfection studies, we demonstrated that 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 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 monocytes 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 pro-atherosclerogenic actions *in vitro*. It induces VCAM-1 on endothelial cells (45), decreases apoE secretion and increases uptake of hypertriblyderidemic VLDL on macrophages (46), induces MHC-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 knock-out (KO) mice crossed with the IFN-γ receptor KO 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 towards 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.

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FOOTNOTES

* The abbreviations used are: ACAT, acyl-coenzyme A:cholesterol acyltransferase; kb, kilobase(s); bp, base pair(s); cDNA, complementary DNA; GAS, IFN-gamma activated sequence; JAK, Janus kinase; STAT, signal transducer and activator of transcription; IFN-γ, interferon-gamma; ATRA, all-trans-retinoic acid; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonil]-1-propanesulfonate; CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; ER, endoplasmic reticulum; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PC, phosphatidylcholine; EMSA, electrophoretic mobility shift assays; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

LEGENDS

Fig. 1 A 159-bp core region comprises the basal transcriptional activity of *ACAT-1* P1 promoter. **A.** Luc constructs (bars shown on the left panel) containing serial 5'- and 3'- deletions as indicated between −598 and +34 of the *ACAT-1* P1 promoter were cotransfected with pCH110 into THP-1 cells. The cells were harvested 48 hrs after transfection for activity assays. The luciferase activity per each cell extract was normalized by using the β-galactosidase value found in the same cell extract. The reporter construct 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 indicated the three major transcriptional initiation sites (Li et al., 1999). Sequence of Exon 1 was underlined.

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 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 symbol ×) of the159-bp core region were transfected into THP-1 cells. The luciferase activities shown on right panel were determined as described in Fig. 1A. **C**, Electrophoresis mobility shift assays (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 1×10^4 dpm of labeled probe was used for each binding reaction. LANE 1, [^{32}P]-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 non-specific DNA to the binding reaction described for LANE 2. LANE 5, supershift reaction by adding 1μl of anti-Sp1 antibody to the binding reaction described for LANE 2. LANE 6~10, the same conditions as described for LANE 1~5, except using the Sp1-1234-mutant DNA as the labeled probe.

Fig. 3 Effect of IFN-γ on the ACAT-1 gene expression during the human blood monocyte-macrophage differetiation process. Human blood monocytes were cultured at 1.5×10^6/60-mm dish for various days as indicated and then treated with or without IFN-γ (100 U/ml) for 40 hrs before harvested for preparation of total RNA and protein extract. **A**, **B**, Quantitation by RT-PCR (26 to 32 cycles). Appropriate primers described in 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 cycle 25 and 35, the *ACAT-1* P1 transcript and the *ACAT-1*

P7 transcript could be estimated semi-quantitatively by RT-PCR (data not shown). The ratio of DNA contents (shown at the bottom panels) was 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 U/ml) for 40 hrs. Protein extracts were prepared and immunoblotting were conducted as described in Experimental Procedures. Samples used (40 μg protein per 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 by 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.

Fig. 4 The synergistic effect of IFN-γ and ATRA on *ACAT-1* P1 promoter is promoter specific and cell type specific. **A**, The Lus constructs containing *ACAT-1* P1, or P7, or the SV40 promoters ligated to the luciferase reporter vector (pGL2-C) were transfected into THP-1 cells. 7 hrs after transfection, cells were treated with or without IFN-γ (100 U/ml), or with ATRA (10^{-6} M), or with IFN-γ (100 U/ml) plus ATRA (10^{-6} M), respectively. The luciferase activity was determined in lysates of THP-1 cells 40 hrs 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 in Experimental Procedures. The cells were then treated and assayed as described in **A**.

Fig. 5 The dose and time dependency of the IFN-γ and ATRA effects. The Luc constructs containing *ACAT-1* P1 promoter was transfected into THP-1 cells. 7 hrs after transfection, **A**, cells were treated with or without ATRA (10^{-6} M) alone, or with ATRA plus indicated concentrations of IFN-γ respectively for 40 hrs; **B**, cells were treated with or without IFN-γ (100 U/ml) alone, or with IFN-γ plus indicated concentrations of ATRA for 40 hrs; **C**, cells were treated with or without ATRA (10^{-6} M) plus IFN-γ (100 U/ml) for the indicated lengths of time, respectively. Afterwards, the luciferase activity was determined as described in Experimental Procedures.

Fig. 6 IFN-γ and ATRA synergistically increase ACAT-1 mRNA. Total RNAs were prepared from THP-1 cells treated for 40 hrs with or without IFN-γ (100 U/ml), or with ATRA (10^{-6} M), or with IFN-γ (100 U/ml) plus ATRA (10^{-6} M), respectively. **A**, **B**, Quantitation by RT-PCR (26 to 32 cycles). Appropriate primers described in 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 cycle 25 and 35, the *ACAT-1* P1 transcript and the *ACAT-1* P7 transcript could be estimated semi-quantitatively by RT-PCR (data not shown). The ratio of DNA

contents (shown at the bottom panels) was determined by using the UVP Labwork software (UVP. Inc.). **C**, Quantitation by Northern analysis. 20 μg total RNAs per lane from cells treated in various manners as indicated was employed, using a [^{32}P]-labeled ACAT-1 cDNA probe; the same filter was rehybridized with a [^{32}P]-labeled human GAPDH cDNA probe. After exposing with Phosphor-Imager, the intensities of the 2.8-knt and 3.6-knt ACAT-1 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-knt message and the 3.6-knt message from cells treated in various manners as indicated were shown on the right panel.

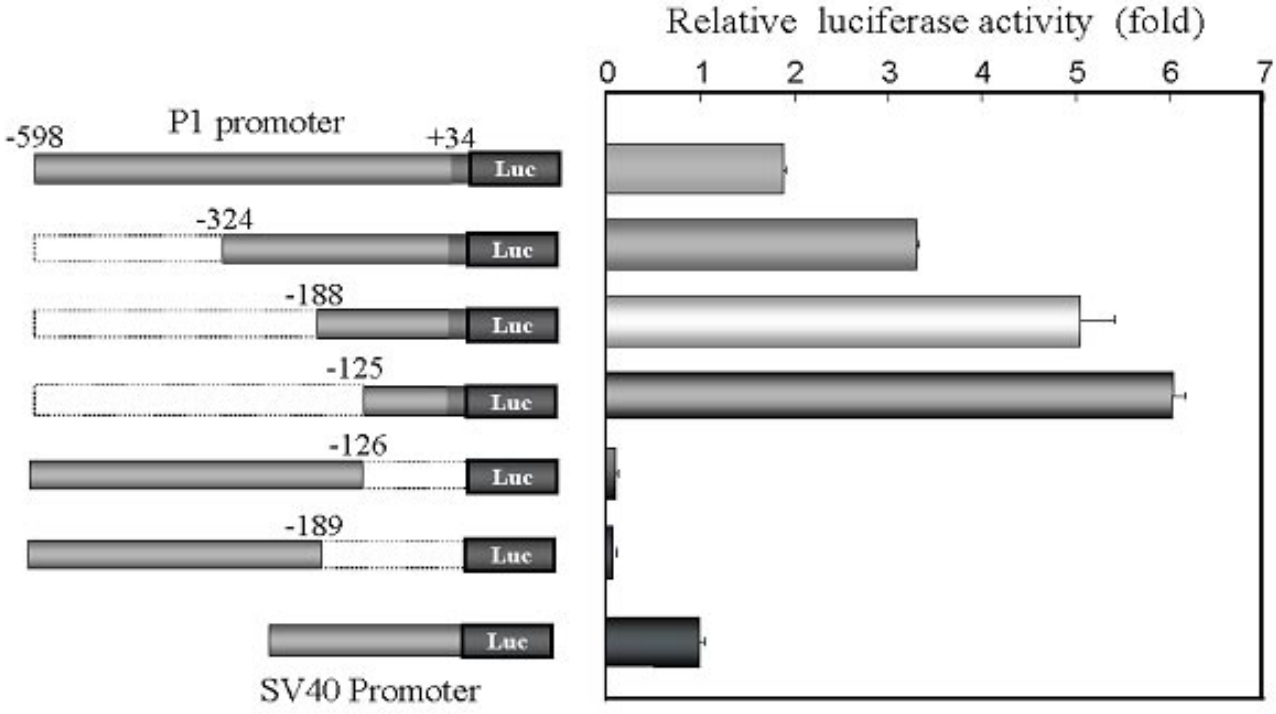
Fig. 7 IFN-γ and ATRA Synergistically increase ACAT-1 protein and enzyme activity. A, Immunoblotting of ACAT-1 protein from extracts of THP-1 cells were treated in various manners as indicated. Cells were seeded at 2 × 10^5/ml in 60-mm dishes with 7.5 ml medium, and treated for 40 hrs with or without IFN-γ (100 U/ml), or with ATRA (10^{-6} M), or with IFN-γ (100 U/ml) plus ATRA (10^{-6} M) as indicated. Cell extracts were prepared and immunoblotting were conducted as described in Experimental Procedures. Samples (40 μg protein per lane) freshly prepared with SDS. The membrane was incubated with DM10 (0.5 μg/ml) as the primary antibody. The immunoreactive proteins were visualized by 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 protein using the value in untreated THP-1 cells as 1.0. The ratios of the protein from cells treated in various manners as indicated were shown at the bottom panel. **B**, ACAT activity assayed *in vitro*. AC29, 25RA and THP-1 cells were cultured at 2 × 10^5/ml in 60-mm dishes with 7.5 ml medium and treated for 40 hrs with or without IFN-γ (100 U/ml), or with ATRA (10^{-6} M), or with IFN-γ (100 U/ml) plus ATRA (10^{-6} M) as indicated**.** ACAT activities were assayed as described in Experimental Procedures. The data are expressed as relative ACAT activity using the value in untreated THP-1 cells as 1.0. The ACAT specific activity in untreated cell extracts was 74 pmol/mg/min.

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 at the bottom) were transfected into THP-1 cells. 7 hrs after transfection, cells were treated with or without IFN-γ (100 U/ml), or with ATRA (10^{-6} M), or with IFN-γ (100 U/ml) plus ATRA (10^{-6} M) as indicated for 40 hrs. 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**, **D**, EMSAs using nuclear extracts of THP-1 cells treated for 40 hrs with IFN-γ (100 U/ml) plus ATRA (10^{-6} M). The wild-type and mutant GAS DNA fragments as indicated were labeled; 1 × 10^4 dpm of labeled probe was used in each binding reaction. **C**, LANE 1, [^{32}P]-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; LANE 4~6, supershift reactions by adding 1μl of anti-Sp1 antibody, or 1μl of anti-STAT1 antibody, or 1μl of anti-Sp1 antibody and 1μl of anti-STAT1 antibody

as indicated to the binding reaction; LANE 7~10, competition by adding 100-fold molar excess of probe containing mutation within the first, or second, or the first two, or all four Sp1 elements as indicated to the binding reaction; LANE 11 and 12, supershift reactions by adding 1μl of anti-Sp1 antibody or 1μl of anti-STAT1 antibody as indicated to the binding reaction described for LANE 10. **D**, LANE 1~3, the same conditions as described for Fig. 7C LANE 1~3 were employed; LANE 4, competition by adding 100-fold molar excess of probe containing the mutant GAS element; LANE 5 and 6, supershift reactions by adding 1μl of anti-Sp1 antibody or anti-STAT1 antibody as indicated to the binding reaction described for LANE 4; LANE 7~9, the same conditions as described for LANE 1~3 were employed, except the mutant GAS DNA fragment was used as the labeled probe; LANE 10 and 11, supershift reactions by adding 1μl of anti-STAT1 antibody or anti-Sp1 antibody as indicated to the binding reactions described in LANE 8.

Fig. 9 STAT1 is involved in the synergistic effect of IFN-γ and ATRA. Total RNAs were prepared from THP-1 cells treated for 40 hrs with or without IFN-γ (100 U/ml), or ATRA (10^{-6} M), or IFN-γ (100 U/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 in Experimental Procedures. Control experiments indicated that between cycle 25 and 35, the IFN-γ receptor transcripts and the STAT1 transcript could be estimated semi-quantitatively by RT-PCR (data not shown). **B**, THP-1 cells were cotransfected with the Luc construct containing the wild-type *ACAT-1* P1 promoter core region, and the wild-type STAT1 (indicated as STAT1-Y701), or the mutant STAT1 (indicated as STAT1-Y701Fm), or the empty vector (indicated as control). 7 hrs after transfection, the cells were treated in various manners as indicated for 40 hrs. The luciferase activities of treated cell extracts were then determined in the same way as described in Fig. 1A. **C**, EMSAs using nuclear extracts from the transfected THP-1 cells treated for 40 hrs with IFN-γ (100 U/ml). The wild-type 159-bp core region DNA was labeled as probe; 1×10^4 dpm of labeled probe was used for each binding reaction. LANE 1, [^{32}P]-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 1μl of anti-Sp1 antibody. LANE 5, supershift reaction by adding 1μl of anti-STAT1 antibody. For LANE 2~5, nuclear extracts were from cells transfected with the empty vector. LANE 6~9 and 10~13 are results using the same series of reaction conditions as described in LANE 2~5, but using the nucleic extracts from THP-1 cells transfected with wild-type STAT1 cDNA or with mutant STAT1 cDNA, respectively.

A



B

Sma I (-125)

CTGGCGCCGG GGACCCGCGC G**CCCGGG**AAG GGGCGGGGAG GTGGGCGGAA -100

Sp1 Sp1

CTGGCAACCT GGGGACCACC AATAGGATGC TCAGCCGGAG GTGGCCCTGC -50

GCGTGACGCA GCGGTGCTCG GAGGCAGGGG GCGTAGGGGC CGGGCTGTCC +1

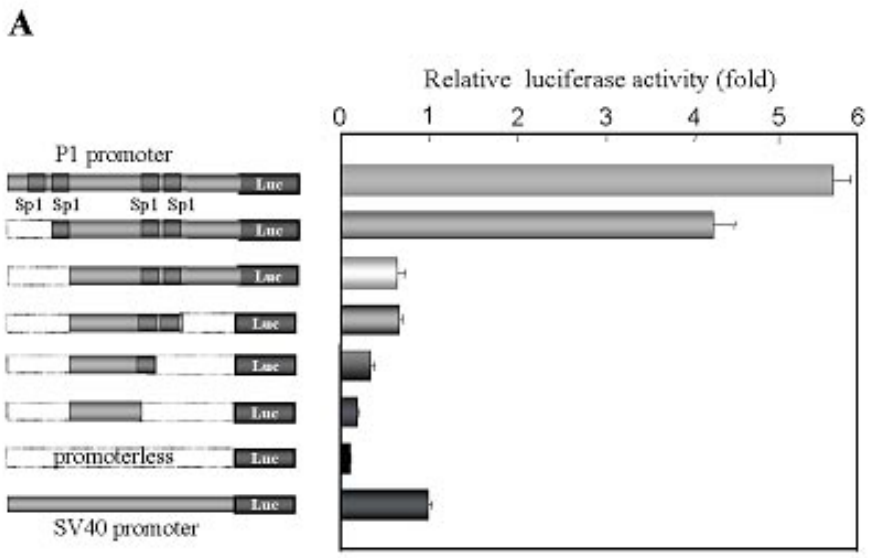
Sp1 Sp1 +1

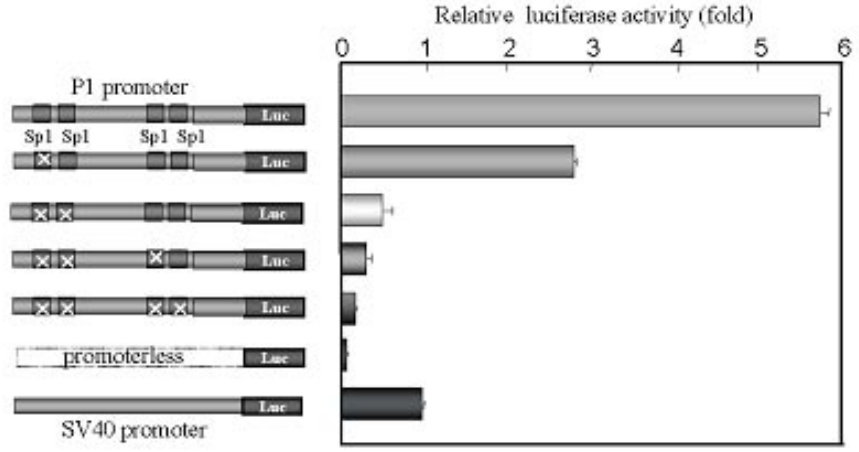
GGCGCTTTAG CTTAGCAGGC GACGTTGC**GG GCCC**TGGGCG CCAGGAGAGC +51

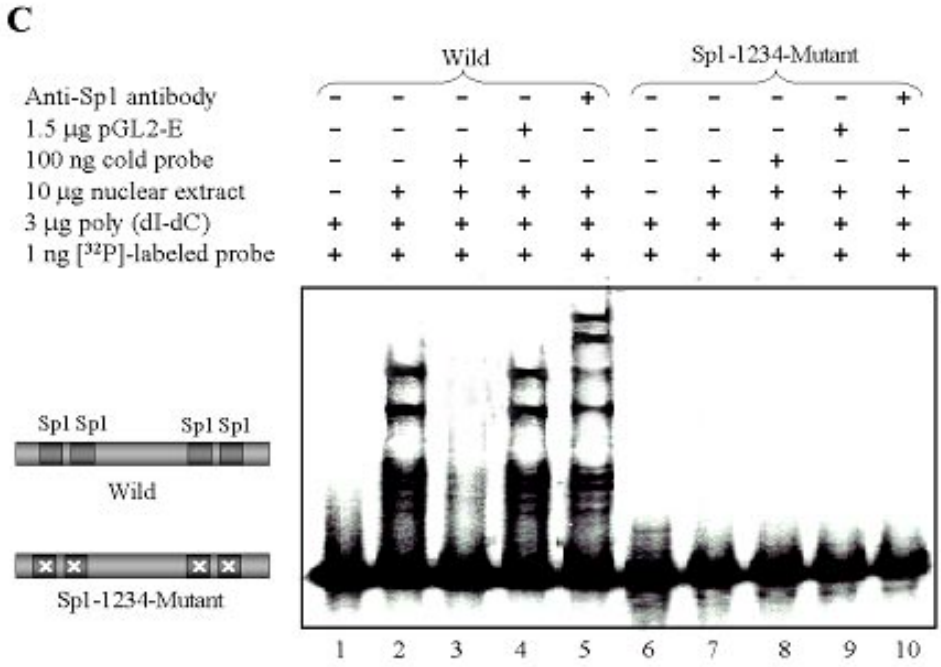
Apa I (+34)

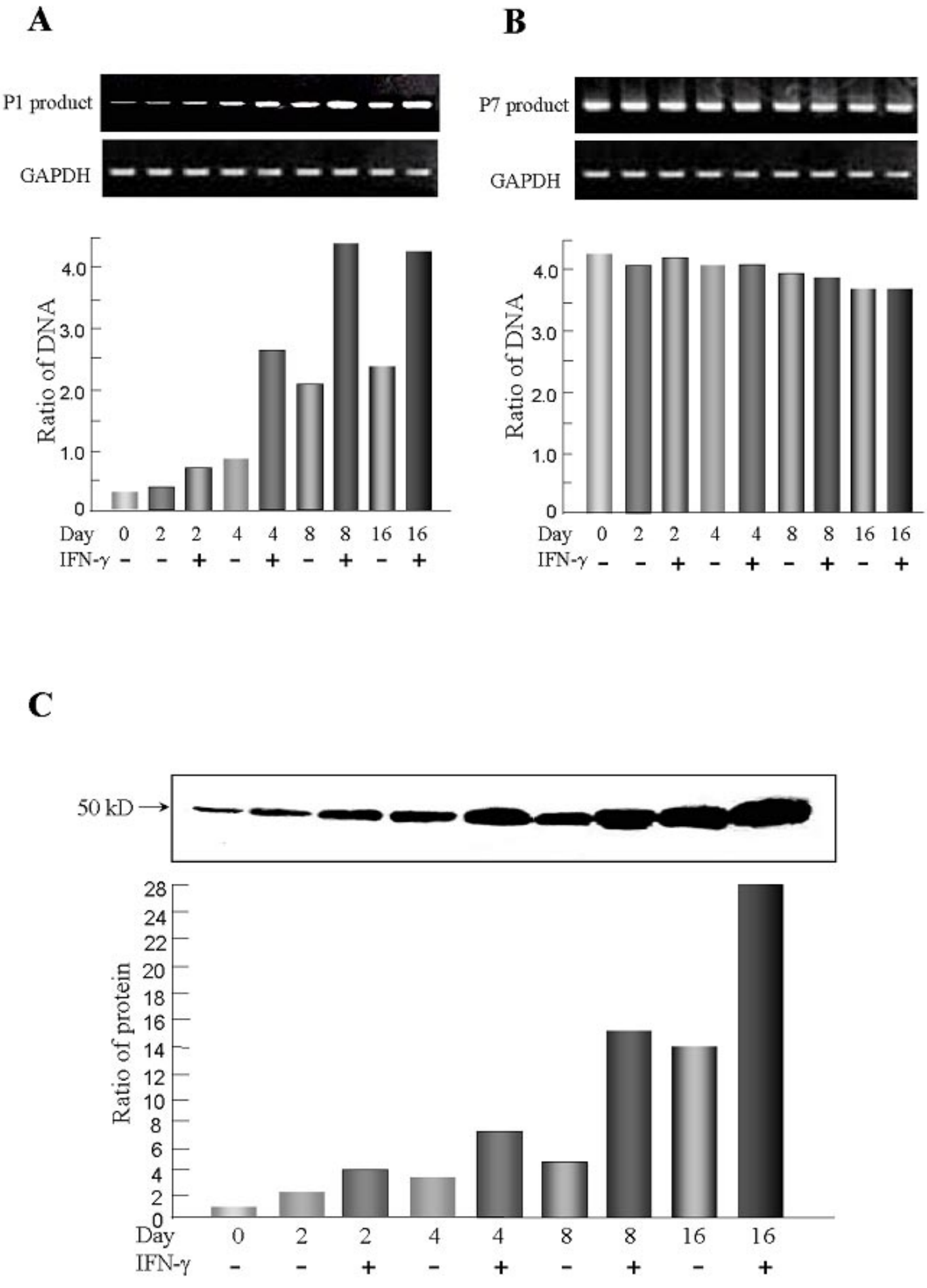
TTCCCGGAGT CGAC +65

Exon 1

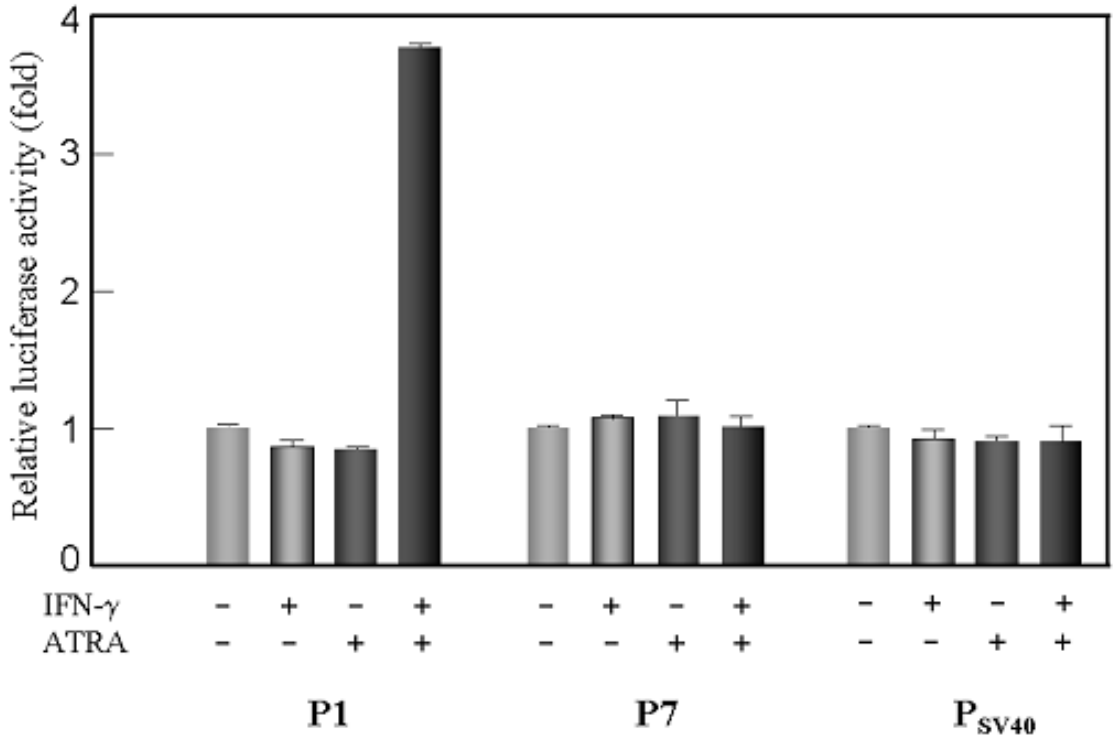

A
Relative luciferase activity (fold)
0 1 2 3 4 5 6
P1 promoter
Sp1 Sp1 Sp1 Sp1
Luc
promoterless
SV40 promoter


B
Relative luciferase activity (fold)
0 1 2 3 4 5 6
P1 promoter
Sp1 Sp1 Sp1 Sp1
Luc
promoterless
SV40 promoter

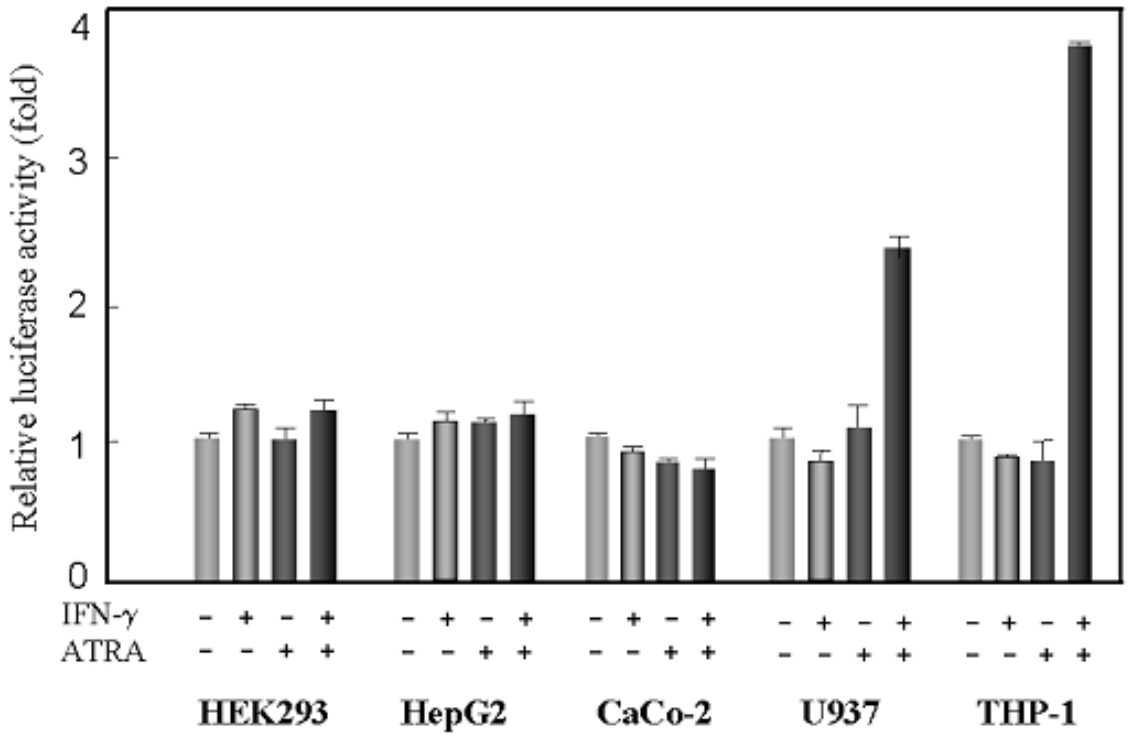

C
Wild
Sp1-1234-Mutant
Anti-Sp1 antibody
1.5 μg pGL2-E
100 ng cold probe
10 μg nuclear extract
3 μg poly (dI-dC)
1 ng [32P]-labeled probe
Sp1 Sp1 Sp1 Sp1
Wild
Sp1-1234-Mutant
1 2 3 4 5 6 7 8 9 10

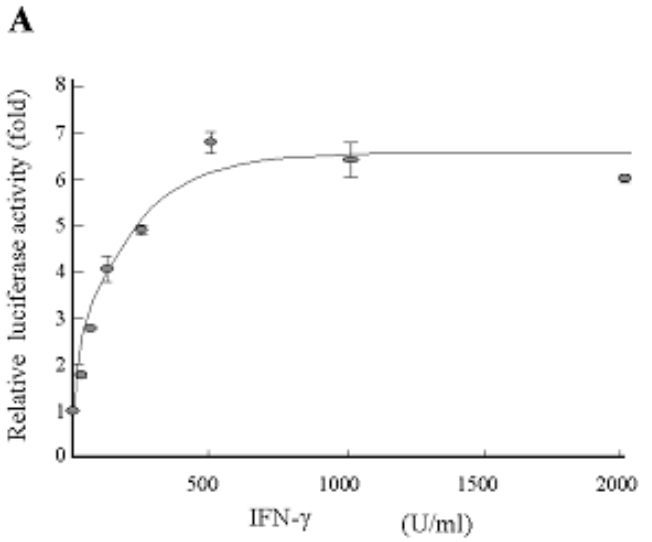

A
P1 product
GAPDH
Ratio of DNA
4.0
3.0
2.0
1.0
0
Day 0 2 2 4 4 8 8 16 16
IFN-γ - - + - + - + - +
B
P7 product
GAPDH
Ratio of DNA
4.0
3.0
2.0
1.0
0
Day 0 2 2 4 4 8 8 16 16
IFN-γ - - + - + - + - +
C
50 kD
Ratio of protein
28
24
22
20
18
16
14
12
10
8
6
4
2
0
Day 0 2 2 4 4 8 8 16 16
IFN-γ - - + - + - + - +

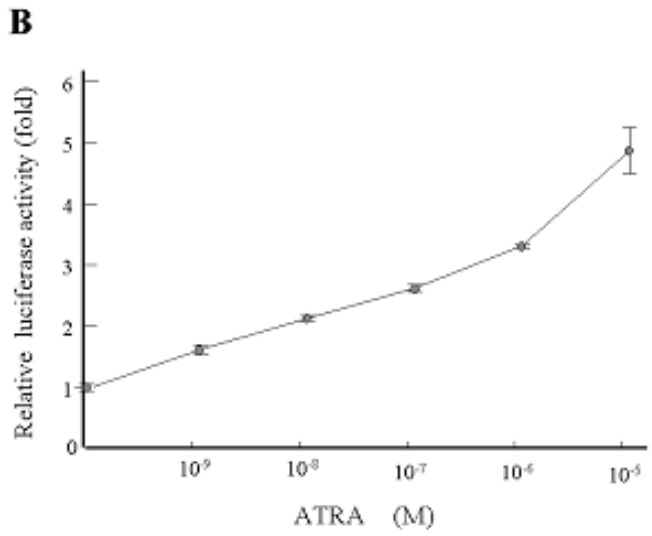
A

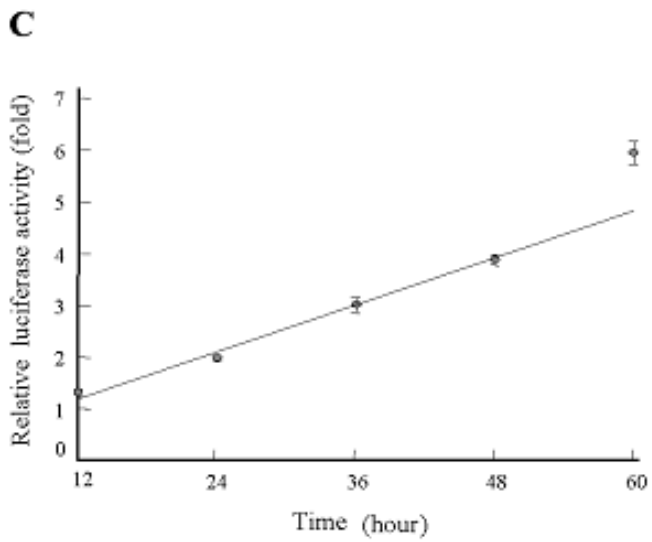

Relative luciferase activity (fold)
0
1
2
3
4
IFN-γ
ATRA
P1
P7
P_SV40

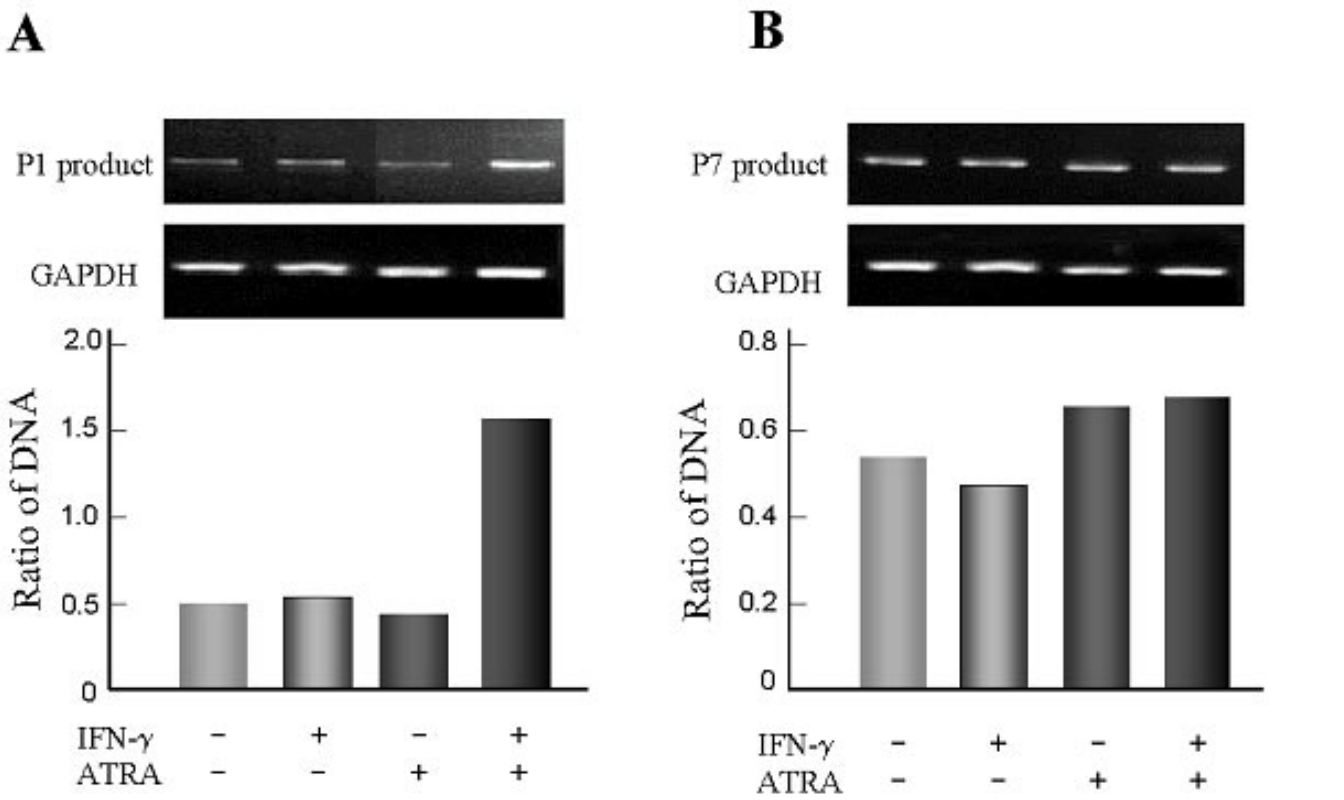
B

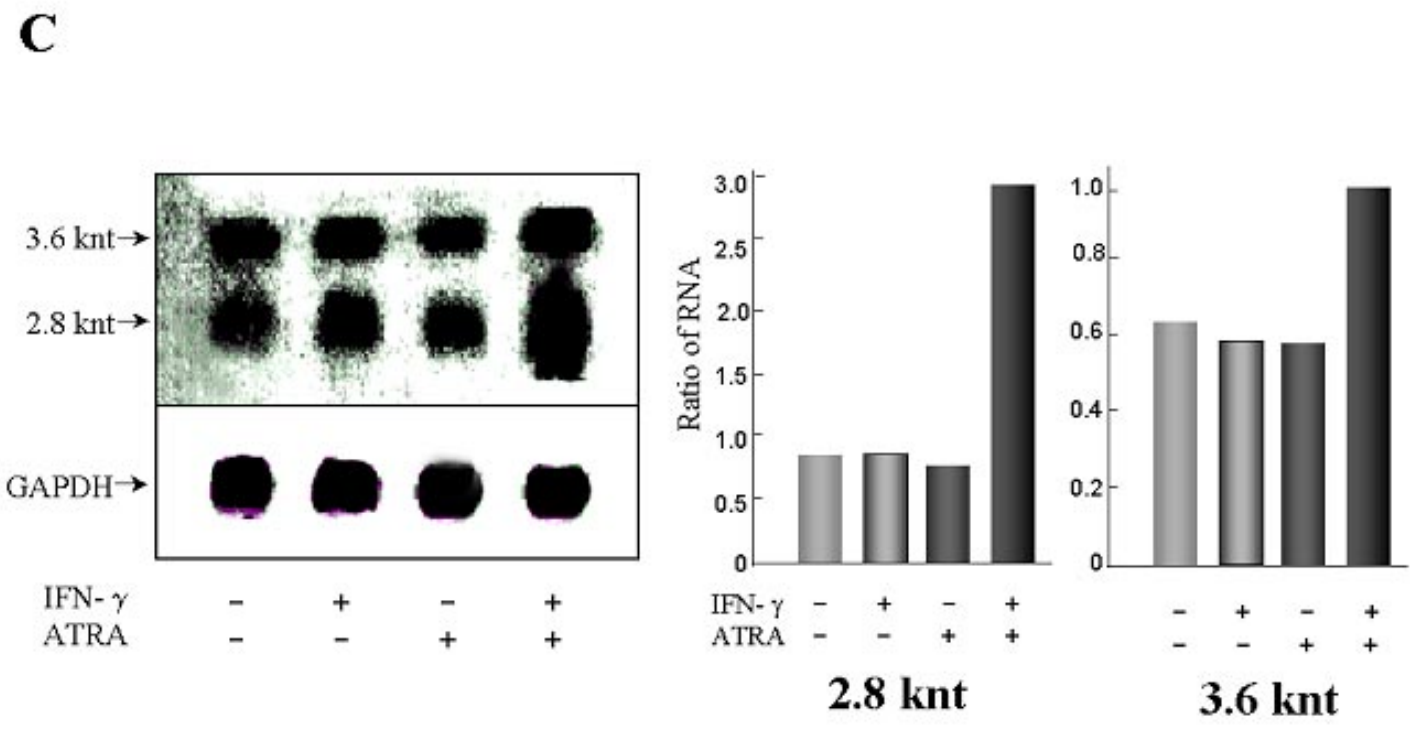

Relative luciferase activity (fold)
0
1
2
3
4
IFN-γ
ATRA
HEK293
HepG2
CaCo-2
U937
THP-1


A
Relative luciferase activity (fold)
0
1
2
3
4
5
6
7
8
500
1000
1500
2000
IFN-γ (U/ml)

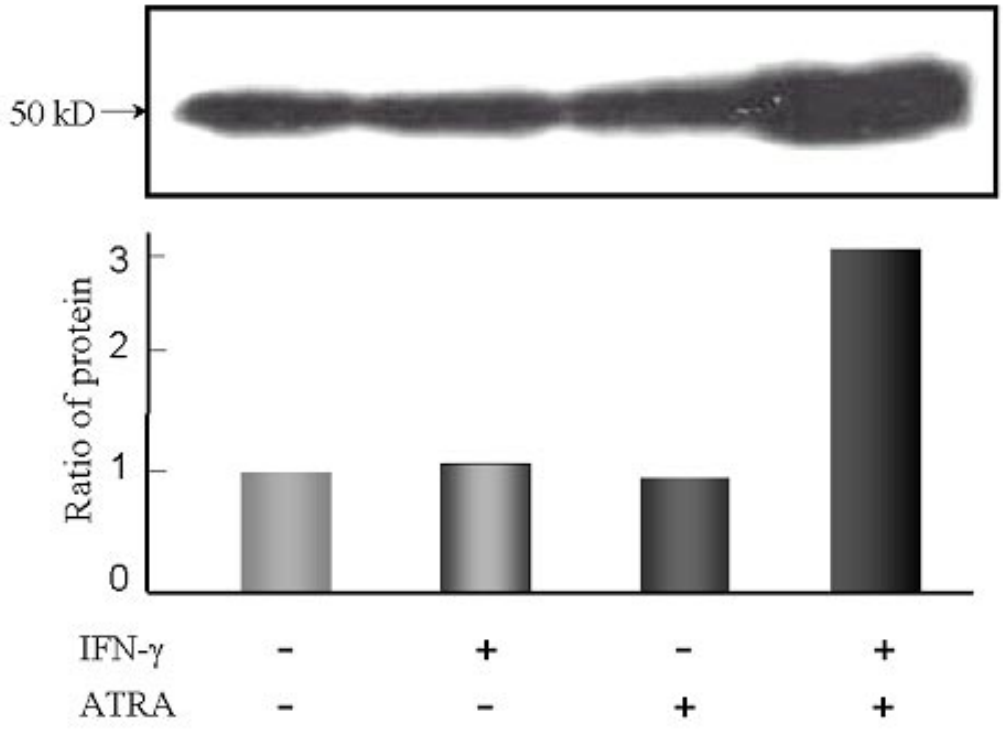

B
Relative luciferase activity (fold)
0
1
2
3
4
5
6
10-9
10-8
10-7
10-6
10-5
ATRA (M)


C
Relative luciferase activity (fold)
0
1
2
3
4
5
6
7
12
24
36
48
60
Time (hour)

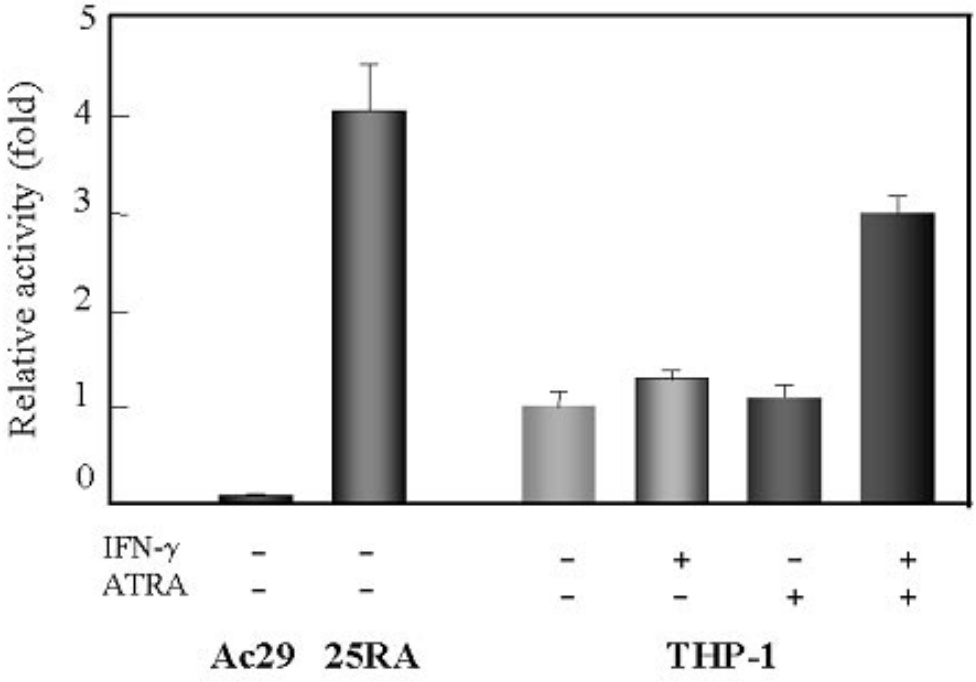

A
P1 product
GAPDH
Ratio of DNA
2.0
1.5
1.0
0.5
0
IFN-γ − + − +
ATRA − − + +
B
P7 product
GAPDH
Ratio of DNA
0.8
0.6
0.4
0.2
0
IFN-γ − + − +
ATRA − − + +

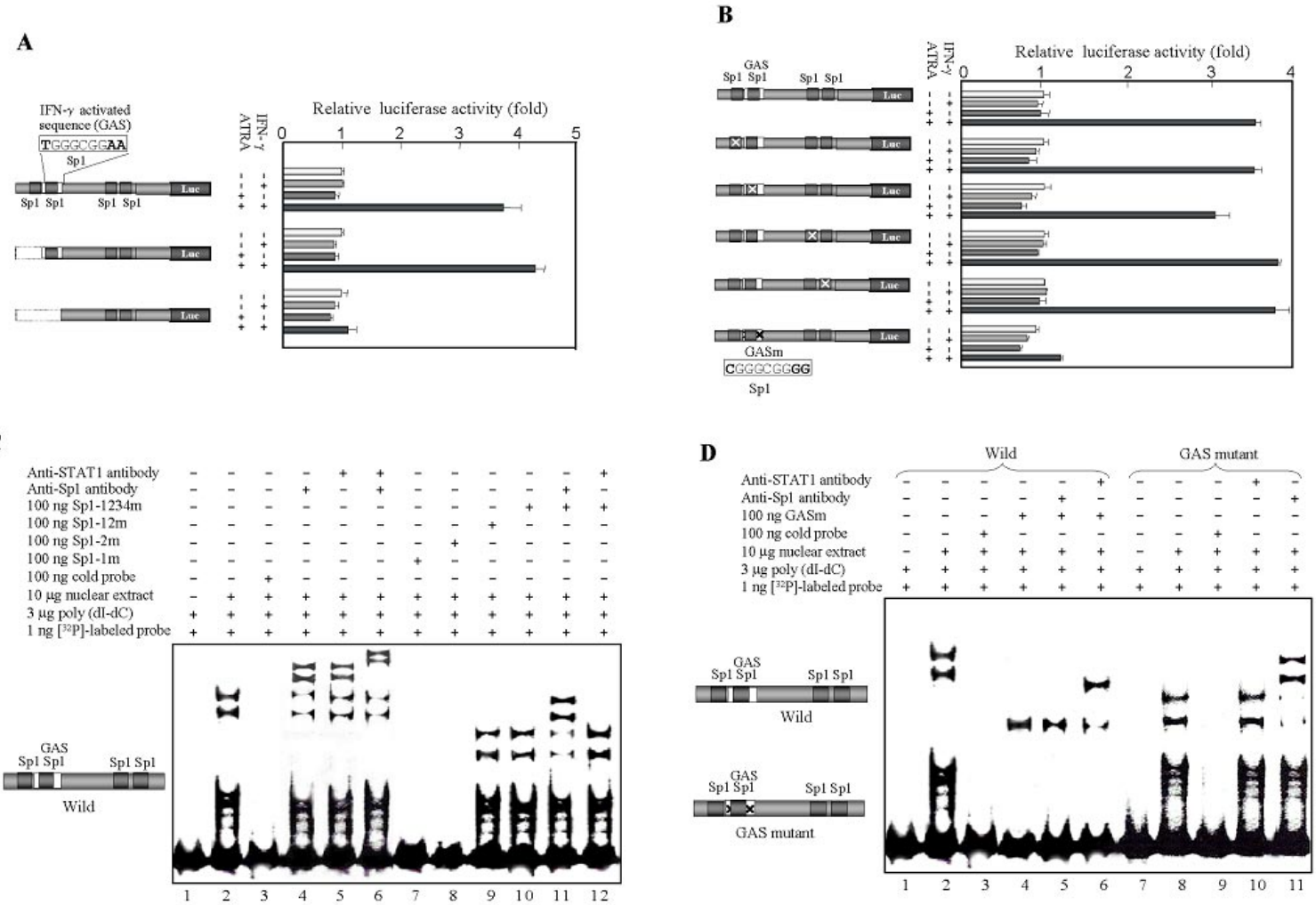

C
3.6 knt
2.8 knt
GAPDH
IFN-γ − + − +
ATRA − − + +
Ratio of RNA
3.0
2.5
2.0
1.5
1.0
0.5
0
IFN-γ − + − +
ATRA − − + +
2.8 knt
1.0
0.8
0.6
0.4
0.2
0
− + − +
− − + +
3.6 knt

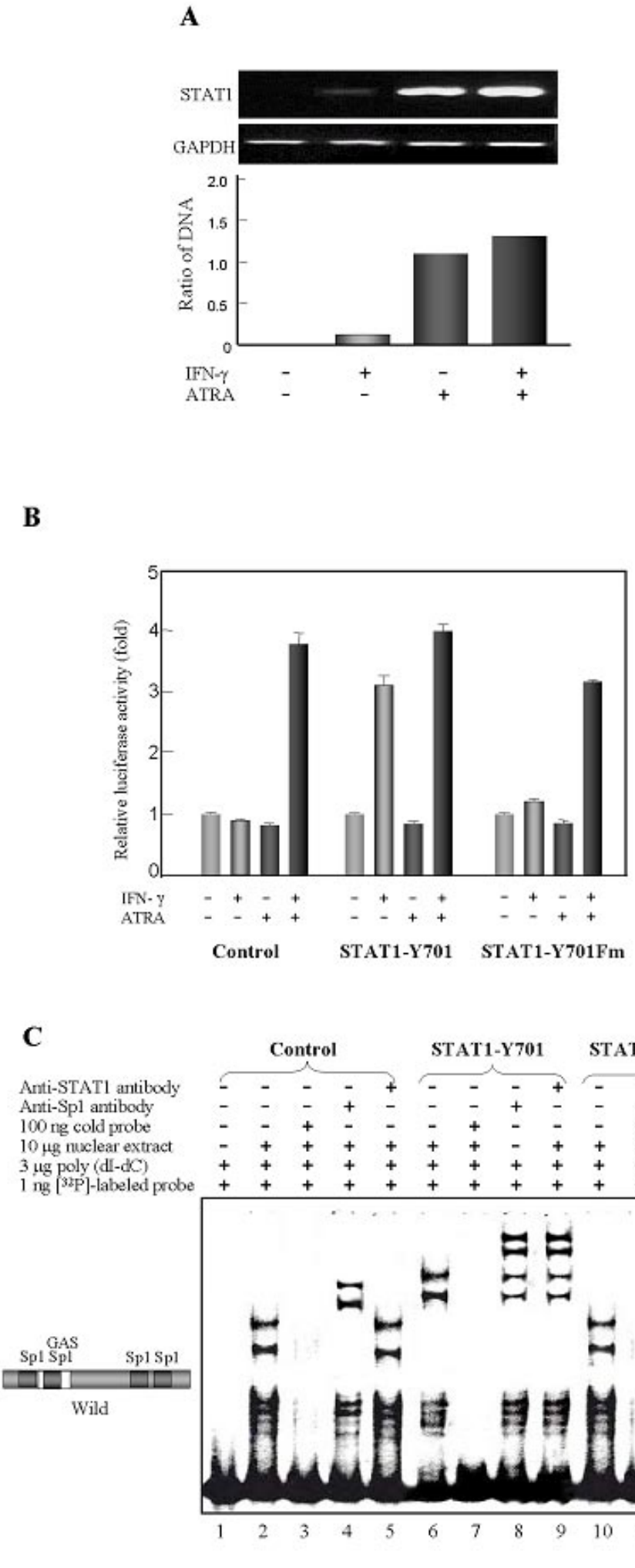
A


50 kD
Ratio of protein
0
1
2
3
IFN-γ - + - +
ATRA - - + +

B


Relative activity (fold)
0
1
2
3
4
5
IFN-γ - - - + - +
ATRA - - - - + +
Ac29
25RA
THP-1


A
IFN-γ activated sequence (GAS)
TGGGCGGAA
Sp1
Sp1 Sp1 Sp1 Sp1
Luc
IFN-γ
ATRA
Relative luciferase activity (fold)
0 1 2 3 4 5
B
GAS
Sp1 Sp1 Sp1 Sp1
Luc
GASm
CGGGCGGGG
Sp1
IFN-γ
ATRA
Relative luciferase activity (fold)
0 1 2 3 4
C
Anti-STAT1 antibody
Anti-Sp1 antibody
100 ng Sp1-1234m
100 ng Sp1-12m
100 ng Sp1-2m
100 ng Sp1-1m
100 ng cold probe
10 μg nuclear extract
3 μg poly (dI-dC)
1 ng [32P]-labeled probe
GAS
Sp1 Sp1 Sp1 Sp1
Wild
1 2 3 4 5 6 7 8 9 10 11 12
D
Wild
GAS mutant
Anti-STAT1 antibody
Anti-Sp1 antibody
100 ng GASm
100 ng cold probe
10 μg nuclear extract
3 μg poly (dI-dC)
1 ng [32P]-labeled probe
GAS
Sp1 Sp1 Sp1 Sp1
Wild
GAS
Sp1 Sp1 Sp1 Sp1
GAS mutant
1 2 3 4 5 6 7 8 9 10 11


A
STAT1
GAPDH
Ratio of DNA
2.0
1.5
1.0
0.5
0
IFN-γ - + - +
ATRA - - + +
B
Relative luciferase activity (fold)
5
4
3
2
1
0
IFN-γ - + - + - + - + - + - +
ATRA - - + + - - + + - - + +
Control
STAT1-Y701
STAT1-Y701Fm
C
Control
STAT1-Y701
STAT1-Y701Fm
Anti-STAT1 antibody - - - - + - - - + - - - +
Anti-Sp1 antibody - - - + - - - + - - - + -
100 ng cold probe - - + - - - + - - - + - -
10 μg nuclear extract - + + + + + + - + + + + +
3 μg poly (dI-dC) + + + + + + + + + + + + +
1 ng [32P]-labeled probe + + + + + + + + + + + + +
GAS
Sp1 Sp1
Sp1 Sp1
Wild
1 2 3 4 5 6 7 8 9 10 11 12 13

Synergistic transcriptional activation of human ACAT-1 Gene by IFN-gamma and ATRA in THP-1 cells

Jin-Bo Yang, Zhi-Jun Duan, Wei Yao, Oneil Lee, Li Yang, Xin-Ying Yang, Xia Sun, Catherine C.Y. Chang, Ta-Yuan Chang and Bo-Liang Li

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