# Physical and Functional Interactions between Liver X Receptor/Retinoid X Receptor and Sp1 Modulate the Transcriptional Induction of the Human ATP Binding Cassette Transporter A1 Gene by Oxysterols and Retinoids†

Efstathia Thymiakou,‡ Vassilis I. Zannis,§ and Dimitris Kardassis*,‡

*Laboratory of Biochemistry, Department of Basic Sciences, University of Crete Medical School, and Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology of Hellas, Heraklion, Crete 71110, Greece, and Molecular Genetics, Departments of Medicine and Biochemistry, Whitaker Cardiovascular Institute, Boston University School of Medicine, Boston, Massachusetts 02118*

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ABSTRACT: The lipid transporter ATP binding cassette transporter A1 (ABCA1) promotes the efflux of cellular phospholipids and cholesterol to lipid-free apolipoprotein A-I and thus initiates the biogenesis of high-density lipoprotein (HDL). The expression of the ABCA1 gene is controlled, coordinately with other genes of HDL metabolism, by liver X receptor/retinoid X receptor (LXR/RXR) heterodimers and their ligands oxysterols and retinoids. In the present study, we show that the oxysterol/retinoid-induced transcription of the ABCA1 gene is modulated by the ubiquitous transcription factor Sp1 that binds to the proximal ABCA1 promoter, adjacently to the LXR/RXR responsive element. The response of the ABCA1 gene to oxysterols/retinoids as well as the ligand-inducible recruitment of Sp1 and RXRα/LXRα heterodimers to the ABCA1 promoter was blocked by mithramycin A, a well-known Sp1 inhibitor. Using SL2 cells which lack endogenous Sp1, we showed that activation of the ABCA1 promoter by LXRα/RXRα heterodimers and their ligands requires Sp1. Functional interactions between these factors were demonstrated using the GAL4 transactivation system. Using both in vitro and in vivo assays, we show that physical interactions between Sp1 and LXRα require the N-terminal region of LXRα, which includes the AF1 and DNA binding domains and two different domains of Sp1: the transactivation domain B and the DNA binding domain. Overall, the present study revealed a novel mechanism of regulation of the human ABCA1 transporter which involves synergistic interactions between oxysterol/retinoid-inducible hormone nuclear receptors and the transcription factor Sp1.

ABCA1[1] is a member of the ATP binding cassette (ABC) family of transporters. It is a ubiquitous protein expressed abundantly in the liver, macrophages, brain, and various other tissues (*1*, *2*). ABCA1 promotes the efflux of cellular phospholipid and cholesterol to lipid-free apolipoprotein A-I (apoA-I) and other apolipoproteins but not to spherical high-density lipoprotein (HDL) particles (*3*−*5*). Cellular cholesterol efflux to apoA-I is severely reduced by inactivating mutations in ABCA1, both in fibroblasts of patients with Tangier disease (*6*−*9*) and in fibroblasts from ABCA1−/− mice (*9*). Tangier disease patients and ABCA1−/− mice fail to form discoidal or spherical HDL, have very low total plasma cholesterol, and exhibit abnormal lipid deposition in various tissues (*9*−*12*). The dramatic reduction of plasma HDL in mice, which have hepatocyte-specific ablation of ABCA1, combined with the data from studies in human populations, indicates that hepatic ABCA1 plays a major role in the formation of plasma HDL (*13*).

Overexpression of ABCA1 in liver and macrophages of transgenic mice raised plasma HDL levels and was atheroprotective when the animals were fed a high-cholesterol diet (*14*). Moreover, the selective inactivation of ABCA1 in macrophages enhanced atherosclerosis independently of changes in plasma lipids and HDL levels (*9*).

The transcription of the ABCA1 gene is regulated by the cellular cholesterol levels. One way cells "sense" cholesterol levels is through the liver X receptor (LXR), a member of the hormone nuclear receptor superfamily of transcription factors that is activated by a wide range of hydroxylated forms of cholesterol and by intermediates in the cholesterol

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* To whom correspondence should be addressed. Phone: +30-2810-394549. Fax: +30-2810-394530. E-mail: kardasis@imbb.forth.gr.

‡ University of Crete Medical School and Foundation of Research and Technology of Hellas.

§ Boston University School of Medicine.

[1] Abbreviations: ABCA1, ATP binding cassette transporter A1; apoA-I, apolipoprotein A-I; ChIP, chromatin immunoprecipitation; DBD, DNA binding domain; DR4, direct repeat with four nucleotides in the spacer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GST, glutathione S-transferase; HDL, high-density lipoprotein; HEK, human embryo kidney; HRE, hormone response element; HRP, horseradish peroxidase; LXR, liver X receptor; RXR, retinoid X receptor; LXRE, LXR responsive element; PMSF, phenylmethylsulfonyl fluoride; RAR, retinoic acid receptor; RT-PCR, reverse transcription polymerase chain reaction; Sp1, specificity protein 1; VDR, vitamin D receptor.

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biosynthetic pathway (*15*). Two isoforms of LXR, LXRα and LXR*β*, have been identified, and both have emerged as central regulators of genes that are involved in lipid metabolism including ABCA1, ABCG1, ABCG4, ABCG5, and ABCG8, cholesterol ester transfer protein (CEPT), phospholipid transfer protein (PLTP), and cholesterol 7α-hydroxylase (CYP7A) (*15*, *16*). The domain structure of LXRα and LXR*β* is typical of nuclear receptors and includes a DNA binding domain consisting of a zinc-finger module and a large ligand binding domain with a lipophilic core that binds specific small lipid molecules and is responsible for the dimerization with retinoid X receptor (RXR) and the transactivation of target genes (*15*). LXRα is primarly expressed in the liver, kidney, macrophages, and intestine, whereas LXR*β* is ubiquitously expressed (*15*, *16*). The LXRs form obligate heterodimers with RXR and bind to specific DNA response elements consisting of two hexanucleotide half-repeats having the consensus sequence 5′-AGGTCA-3′, separated by a 4-nt spacer (termed a direct repeat with four nucleotides in the spacer or DR4) (*15*, *17*, *18*). The ABCA1 gene contains a well-characterized LXR responsive element (LXRE) at position −62/−47 of the promoter upstream of exon 1 (*19*−*21*).

Recent findings suggest that the activity of nuclear receptors, such as the receptors for retinoids (RAR, RXR), vitamin D (VDR), and estrogens (ER), is modulated by interactions with the ubiquitous transcription factor Sp1 (*22*−*33*). Sp1 belongs to the Sp/XKLF family of transcription factors and binds to GC-rich or GT-rich DNA sequences present in a large number of genes (*34*, *35*). Sp1, Sp2, Sp3, and Sp4 form a subgroup based on their similar modular structure (*34*, *35*). This structure includes an N-terminal domain that consists of two homologous regions, designated A and B, which are rich in Gln and Ser/Thr residues and have strong transcriptional activation functions, region C, which is rich in charged amino acids and seems to play a regulatory role in various Sp1 functions, the highly conserved DNA binding domain (DBD), which consists of three zinc fingers, and the C-terminal domain D, which is important for cooperative interactions among Sp1 subunits when bound to promoters containing multiple GC boxes (*34*, *35*).

The ABCA1 proximal promoter, in addition to the LXRE, contains binding sites for the transcription factor Sp1, which is a positive regulator of the ABCA1 gene expression (*21*). In the present work we show that Sp1 plays an important role in the transcriptional induction of the ABCA1 gene by oxysterols and retinoids via a mechanism that involves physical and functional interactions with the nuclear receptors LXR and RXR.

## MATERIALS AND METHODS

*Materials*. Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin for cell culture were purchased from Invitrogen/Life Technologies (Carlsbad, CA). Fetal bovine serum (FBS) was purchased from BioChrom Labs (Terre Haute, IN). Charcoal-stripped serum was prepared after treatment of FBS with charcoal and dextran. Restriction enzymes and modifying enzymes (T4 DNA ligase and calf intestinal alkaline phosphatase) were purchased from Minotech (Heraklion, Greece) or New England Biolabs (Beverly, MA). GoTaq DNA polymerase, dNTPs, the luciferase assay system, and the Wizard SV gel and PCR cleanup system were purchased from Promega (Madison, WI). Mithramycin A, 22(*R*)-hydroxycholesterol, 9-*cis*-retinoic acid (9-*cis*-RA), streptavidin−agarose, and streptavidin−HRP were purchased from Sigma-Aldrich (St. Louis, MI). Glutathione−Sepharose 4B and protein G Sepharose were purchased from Amersham Biosciences. Trizol reagent for RNA extraction and Super-Script RNAse H-reverse transcriptase were purchased from Invitrogen. The Super Signal West Pico chemiluminescent substrate was purchased from Pierce (Rockford, IL). Anti-Sp1 (H225) and anti-RXR (D-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-6myc was a kind gift from Dr. G. Mavrothalassitis (University of Crete, Heraklion, Greece). Anti-mouse peroxidase-conjugated secondary antibody was purchased from Chemicon International Inc. (Temecula, CA).

*Plasmid Constructions*. The ABCA1 promoter plasmids −668/+33 ABCA1-luc, −204/+205 ABCA1-luc, and −76/+205 ABCA1-luc were constructed by PCR amplification of the corresponding fragments of human ABCA1 promoter and subsequent cloning into the pGL3basic vector at the *Kpn*I−*Xho*I sites. To generate the expression vector pCDNA3-6myc-RXRα, the full-length human RXRα cDNA was excised from the plasmid pMT2-RXRα by *Eco*RI and subcloned into the pCDNA3-6myc vector in frame with the 6myc epitope tag at the N-terminus. The vectors expressing the full-length human LXRα (amino acids 1−447) or its truncated forms 1−163, 164−447, 1−97, 98−163, 1−127, 30−127, and 30−163 were constructed by PCR amplification of the corresponding fragments of human LXR cDNA and subsequent cloning into the expression vector pCDNA3-6myc. All oligonucleotides used as primers in PCR amplification were synthesized at the microchemical facility of the Institute of Molecular Biology and Biotechnology (IMBB) (Heraklion, Greece), and their sequence is shown in Table 1. The bacterial vectors expressing Sp1 (81−778) and different Sp1 domains (domains A, B, C, D, and DBD) fused with GST, the expression vector pCDNA3-Bio-Sp1, used for the in vivo biotinylation of Sp1, and the expression vector pCDNA3-BirA have been described previously (*36*−*38*). The hsp-lacZ expression vector used for normalization of transfections in *Drosophila* Schneider SL2 cells was a gift from Dr. C. Delidakis (Department of Biology, University of Crete, and IMBB). pG5B-luc and GAL4-Sp1 have been described previously (*39*). pCMX-LXRα was a generous gift from Dr. Ioannis Talianidis (IMBB).

*Cell Cultures, Transient Transfections, Treatments, and Luciferase and β-Galactosidase Assays*. Human embryonic kidney cells (HEK293T) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin at 37 °C in a 5% $CO_2$ atmosphere. *Drosophila* Schneider SL2 cells were cultured in Schneider's insect medium supplemented with 10% insect fetal bovine serum and penicillin/streptomycin at 25 °C. For the treatment of cells with 22(*R*)-hydroxycholesterol, 9-*cis*-retinoic acid, or mithramycin, 10% FBS was replaced by 5% charcoal-stripped serum (CSS), and the two ligands were added for 24 h at a final concentration of 1 μM. Mithramycin was also added for 24 h at a final concentration of 0.2 μM. Transient transfections were performed using the $Ca_3(PO_4)_2$ coprecipitation method. Luciferase assays were performed using the luciferase assay

Table 1: Primers Used in PCRs[a]

| name | sequence |
|---|---|
| ABCA1-F | 5′-GAAGCCACAAAAACATTGCTGCAT-3′ |
| ABCA1-R | 5′-CCTCATACCAGTTGAGAGACTTGAT-3′ |
| ABCA1-668 | 5′-CGG**GGTACC**GACCTGGGGAGCTCAGGCTGGGAAT-3′ |
| ABCA1+33 | 5′-CCG***CTCGAG***CCCCACTCACTCTCGCTCGCAATTA-3′ |
| ABCA1-204F | 5′-CGG**GGTACC**CCCAACTCCCTAGATGTGTCGTG-3′ |
| ABCA1-76F | 5′-CGG**GGTACC**GAGAGCACAGGCTTTGACCGATAG-3′ |
| ABCA1+205R | 5′-CCG***CTCGAG***GTCTCTTTCTCCTACCCCTTGACA-3′ |
| HLXR-F | 5′-CCG***CTCGAG***ATGTCCTTGTGGCTGG-3′ |
| HLXR-R | 5′-CGC***TCTAGA***TCATTCGTGCACATCC-3′ |
| hLXR-163-Rev | 5′-TGC***TCTAGA***TCACATGCCAGCCTGACGGCATTTGCG-3′ |
| hLXR-164-Fw | 5′-CCG***CTCGAG***CGGGAGGAGTGTGTCCTGTCAGAAG-3′ |
| hLXR-97-Rev | 5′-TGC***TCTAGA***TCATAGCTCGTTCCCCAGCATTTTG-3′ |
| hLXR-98-Fw | 5′-CCG***GAATTC***TGCAGCGTGTGTGGGGACAAG-3′ |
| hLXR-30-Fw | 5′-CCG***GAATTC***AGCCAGGCCCAGGGAGGCAGCAG-3′ |
| hLXR-127-Rev | 5′-TGC***TCTAGA***TCAGACGCTGCGGCGGAAGAATCCC-3′ |

[a] *Kpn*I (GGTACC), *Xho*I (CTCGAG), *Eco*RI (GAATTC), and *Xba*I (TCTAGA) sites are bold and italic.

kit from Promega Corp. according to the manufacturer's instructions. Normalization for transfection efficiency was performed by $\beta$-galactosidase assays. The statistical analysis of the data was performed using Origin v.6 software.

*Chromatin Immunoprecipitation (ChIP) Assays.* HEK293T cells were grown in 5% CSS for 48 h and were subsequently treated with mithramycin A (0.2 $\mu$M), 9-*cis*-RA ($10^{-6}$ M), and 22(*R*)-hydroxycholesterol ($10^{-6}$ M) or left untreated for 24 h. The cells were cross-linked for 10 min at 37 °C with 1% formaldehyde, and cross-linking was stopped by adding glycine to a final concentration of 137.5 mM. The cells were then rinsed three times with phosphate-buffered saline (PBS) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), harvested in PBS containing 0.5% NP-40 and 0.5 mM PMSF, and centrifuged at 1000 rpm for 5 min at 4 °C. The pellets were resuspended in swelling buffer (25 mM Hepes, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5% NP-40, 1 mM DTT, 0.5 mM PMSF, 2 $\mu$g/mL aprotinin) and left on ice for 10 min. Subsequently, the cells were homogenized using a Dounce homogenizer and centrifuged at 2000 rpm for 5 min at 4 °C. The nuclei were resuspended in sonication buffer (50 mM Hepes, pH 7.8, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 2 $\mu$g/mL aprotinin) and sonicated six times for 30 s at 50% amplitude, leading to the generation of DNA fragments with an average size of 0.5 kb. The samples were cleared by centrifugation at 13 000 rpm for 15 min at 4 °C. The supernatant was precleared by rotation at 4 °C for 2 h with G-protein beads. One-tenth of the volume of supernatant was used as input, and the remaining volume was immunoprecipitated with a-Sp1 (H225) or a-RXR (D-20) O/N at 4 °C. The samples were subjected to extensive washings with buffer A (50 mM Hepes, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 2 $\mu$g/mL aprotinin), buffer B (50 mM Hepes, pH 7.9, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 2 $\mu$g/mL aprotinin), buffer C (20 mM Tris−Cl, pH 8, 1 mM EDTA, 250 mM LiCl, 0.5% sodium deoxycholate, 0.5 mM PMSF, 2 $\mu$g/mL aprotinin), and TE (10 mM Tris−Cl, pH 8, 1 mM EDTA, 0.5 mM PMSF, 2 $\mu$g/mL aprotinin). DNA was eluted in 300 $\mu$L of elution buffer (50 mM Tris−Cl, pH 8, 1 mM EDTA, 1% SDS, 50 mM $NaHCO_3$), and the cross-links were reversed by a 5 h incubation at 65 °C. The samples were subjected to RNAse A treatment for 1 h at 37 °C and to digestion of proteins by proteinase K for 2 h at 42 °C. DNA was extracted by a standard procedure, and the pellets were resuspended in 10 mM Tris−Cl, pH 7.5. Immunoprecipitated chromatin was analyzed by PCR using primers corresponding to the proximal region (−204/+205) of the human ABCA1 gene promoter. The sequence of the primers is given in Table 1. The products of the PCR amplifications were analyzed by agarose gel electrophoresis. The quantification of the results was performed by measuring the intensity of the bands using the Tinascan v.2 software. The statistical analysis of the data was performed using Origin v.6 software.

*Reverse Transcription and PCR Amplification.* RNA was isolated from HEK293T cells after treatment with mithramycin A, 9-*cis*-RA, and 22(*R*)-hydroxycholesterol, using Trizol reagent according to the manufacturer's instructions. A 1 $\mu$g portion of RNA was used to produce cDNA by reverse transcription. ABCA1 cDNA was amplified by PCR using primers corresponding to exons 8 and 9 (ABCA1-F and ABCA1-R, Table 1). For the normalization of the samples, the cDNA of the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was also amplified by PCR. The quantification of the results was performed by measuring the intensity of the bands using the Tinascan v.2 software. The statistical analysis of the data was performed using Origin v.6 software.

*GST Pull-Down Assays.* The GST fusion proteins of the wild-type Sp1 and of the different domains of Sp1 (A, B, C, D, and DBD) were expressed in *Escherichia coli* strain BL21pLys using standard protocols. Glutathione−Sepharose 4B beads were equilibrated in PBS and mixed with the bacterially expressed GST fusion proteins on a rotary shaker O/N at 4 °C. The beads were washed three times with 10 volumes of PBS containing 1% Triton X-100. Coupling efficiency was monitored by SDS−PAGE and Coomassie Blue staining.

For the binding reaction, a 1:1 bead slurry in 200 $\mu$L of 2× interaction buffer (40 mM Hepes, pH 7.9, 10 mM $MgCl_2$, 0.4% NP-40, 15% glycerol, 0.4% BSA, 2 mM PMSF) was combined with whole cell extracts from HEK293T cells overexpressing 6myc-LXR$\alpha$ (1−447 or its truncated forms 1−163, 164−447, 1−97, 98−163, 1−127, 30−127, and 30−163) or 6myc-RXR$\alpha$ after transient transfection, in a final volume of 400 $\mu$L on a rotary shaker O/N at 4 °C. The beads were then washed three times with washing buffer (20 mM

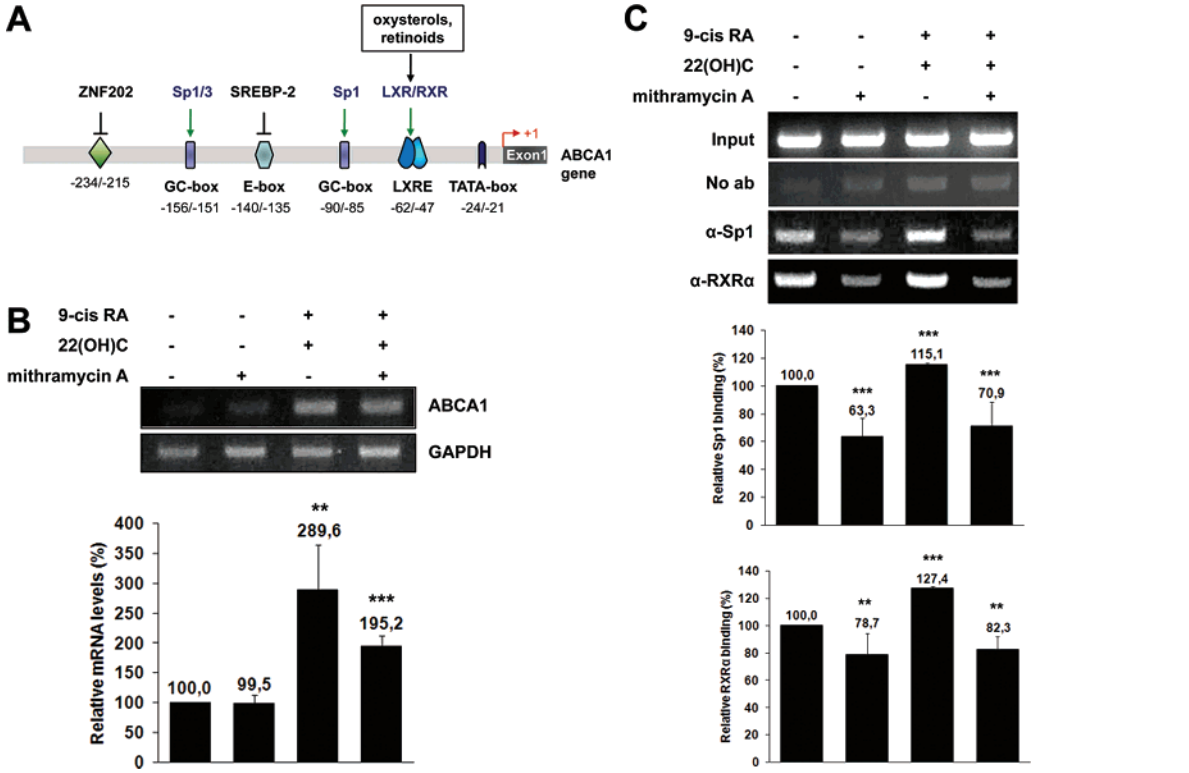


FIGURE 1: (A) Schematic representation of DNA elements and factors that regulate the activity of the human ABCA1 proximal promoter. Note that Sp1 and LXR/RXR heterodimers are positive regulators whereas ZNF202 and SREBP-2 are negative regulators of ABCA1 gene transcription. (B) Mithramycin A blocks the induction of the human ABCA1 gene transcription by oxysterols and retinoids. HEK293T cells were treated with 9-*cis*-RA and 22(*R*)-hydroxycholesterol [22(OH)C] ($10^{-6}$ M) for 24 h in the absence or in the presence of mithramycin A (0.2 $\mu$M) and were subjected to reverse transcription PCR analysis using primers that amplify the human ABCA1 cDNA. The mRNA levels of the GAPDH gene were determined for normalization purposes. The relative mRNA levels of the ABCA1 gene in the absence or in the presence of the ligands 9-*cis*-RA and 22(OH)C and mithramycin A were quantified and are shown at the bottom as a histograph. Each value represents the average from three independent RT-PCR experiments. In each experiment, the amplification of the PCR products was in the linear range. The quantification was performed by measuring the intensity of the bands using the Tinascan v2.0 software. (C) Mithramycin A blocks the recruitment of Sp1 and RXR to the proximal ABCA1 promoter. HEK293T cells were treated with 9-*cis*-RA and 22(OH)C for 24 h in the absence or in the presence of mithramycin A and were subjected to chromatin immunoprecipitation using antibodies for Sp1 or RXRα or no antibody as a negative control as described in the Materials and Methods. Each experiment was performed two times, and representative images are presented. The quantification was performed by measuring the intensity of the bands using the Tinascan v2.0 software. Key: **, $p < 0.05$; ***, $p < 0.001$.

Hepes, pH 7.9, 100 mM KCl, 5 mM $MgCl_2$, 0.2% NP-40, 2 mM PMSF), and the bound proteins were eluted by boiling in SDS−PAGE loading buffer and subjected to SDS−PAGE. Bound proteins as well as the starting material (input) were visualized by Western blot using anti-myc and visualized by enhanced chemiluminescence on an X-ray film.

*In Vivo Biotinylation and Protein−Protein Interaction.* For the in vivo biotinylation of Sp1, HEK293T cells ($7.5 \times 10^5$) were transfected in 10 cm dishes with 7.5 $\mu$g of pCDNA3-Bio-Sp1 expression vector in the presence or in the absence of 7.5 $\mu$g of pCDNA3-BirA vector expressing the bacterial biotin ligase BirA. For protein−protein interaction assays, HEK293T cells were cotransfected with the above plasmids along with 7.5 $\mu$g of expression vector pCDNA3-6myc-LXRα or pCDNA3-6myc-RXRα. The cells were lysed in lysis buffer (20 mM Tris−HCl, pH 7.5, 150 mM NaCl, 10% glycerol, and 1% Triton X-100) and allowed to interact with streptavidin−agarose beads on a rotary shaker for 3 h at 4 °C. The beads were washed three times with lysis buffer. Bound proteins as well as the starting material (input) were subjected to SDS−PAGE followed by immunoblotting using anti-6myc or streptavidin−HRP and visualized by enhanced chemiluminescence on an X-ray film.

## RESULTS AND DISCUSSION

*Mithramycin A Blocks ABCA1 Gene Induction by Oxysterols and Retinoids.* The proximal region of the human ABCA1 promoter contains an LXRE at position −62/−47 that binds heterodimers of RXR/LXR and mediates the activation of this promoter by oxysterols and retinoids (Figure 1A) (*19*−*21*). This LXRE is flanked by two GC boxes at positions −156/−151 and −90/−85, which are the binding sites for the transcription factors Sp1 and Sp3 (*21*).

In the present study, we investigated the role of the ubiquitous transcription factor Sp1 in ABCA1 gene regulation by the RXR/LXR pathway. For this purpose, we used the chemotherapeutic drug mithramycin A. This drug has been shown previously to globally inhibit the binding of Sp transcription factors to their cognate sites on the DNA and has been used extensively as a tool to study Sp-dependent transcriptional responses (*36*, *40*−*46*). Treatment of human embryonic kidney fibroblasts HEK293T with mithramycin A for 24 h did not affect the basal steady-state levels of ABCA1 mRNA (Figure 1B, top row, second lane). In contrast, treatment of HEK293T cells with 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid, which are the natural ligands of LXR and RXR nuclear receptors, respectively,

induced ABCA1 gene transcription 2.9-fold (Figure 1B, top row, third lane). Importantly, mithramycin A inhibited ABCA1 gene induction by the two nuclear receptor ligands by 35% (2.9-fold induction by the ligands alone vs 1.9-fold induction by the ligands in the presence of mithramycin A) (Figure 1B, top row, fourth lane). In a control RT-PCR experiment, it was shown that mithramycin A did not affect the mRNA levels of GAPDH gene in HEK293T cells, suggesting that this drug does not have a global deleterious effect on gene transcription. These findings suggested that Sp1 is required for the inducible transcription of the ABCA1 gene.

*Mithramycin A Blocks the Recruitment of Sp1 and RXRα to the Proximal ABCA1 Promoter.* We next investigated the effect of mithramycin A on the recruitment of nuclear receptors and Sp1 to the proximal ABCA1 promoter in vivo using the chromatin immunoprecipitation assay. For this purpose, we immunoprecipitated cross-linked chromatin from HEK293T cells using antibodies for Sp1 and RXRα and performed PCR using primers specific for the proximal region of the ABCA1 promoter. We found that both factors were bound to the proximal ABCA1 promoter under basal conditions in HEK293T cells (Figure 1C, third and fourth rows, first lane). As shown in Figure 1C, in the presence of mithramycin A, the binding of Sp1 to the ABCA1 promoter was not totally abolished (inhibition by 36.7%, third row, second lane). This finding suggested that (a) the binding of Sp1 to the ABCA1 promoter could be both direct and indirect (via physical interactions with other transcription factors bound to the proximal ABCA1 promoter) and (b) the Sp1 still bound to the ABCA1 promoter in the presence of mithramycin A could be sufficient for basal ABCA1 gene transcription (Figure 1B). Unexpectedly, however, mithramycin A inhibited the recruitment of RXRα to the proximal ABCA1 promoter by 21.3% (Figure 1C, second lane, fourth row). The addition of 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid enhanced the binding of both RXRα and Sp1 to the ABCA1 promoter (1.27- and 1.15-fold, respectively, Figure 1C, third and fourth rows, third lane). Finally, mithramycin A blocked the ligand-stimulated recruitment of RXRα and Sp1 to the promoter by 35.5% and 38.4%, respectively (Figure 1C, third and fourth rows, fourth lane).

To confirm that the decrease in ABCA1 mRNA levels by mithramycin A was due to transcriptional repression, we performed transient transactivation assays in HEK293T cells. As shown in Figure 2A, strong transactivation of the −668/+33 ABCA1 promoter was observed by overexpression of LXRα and RXRα in the presence of their corresponding ligands (16-fold), whereas this transactivation was inhibited by almost 50% in the presence of mithramycin A, in agreement with the data of Figure 1B.

We next showed that LXRα/RXRα heterodimers in the presence of their ligands 22(*R*)-hydroxycholesterol and 9-*cis*-retinoic acid transactivated strongly a short fragment of the human ABCA1 promoter that contains both Sp1/Sp3 binding elements and the LXRE (ABCA1 −204/+205) (Figure 2B). Deletion of the two Sp1/Sp3 binding motifs (ABCA1 −76/+205) did not have any significant effect on the activation of the ABCA1 promoter by LXRα/RXRα in the presence of their ligands (23-fold versus 18-fold). However, deletion of the Sp1 sites reduced the overall activity of the ABCA1 promoter by 72% (2304% versus 647% relative to the activity of the uninduced −204/+205 ABCA1 promoter, the activity of which was arbitrarily taken as 100%) (Figure 2B). These findings are in agreement with the findings of Figure 1B and support the participation of Sp transcription factors in the mechanism of transcriptional activation of the ABCA1 gene by oxysterols and retinoids.

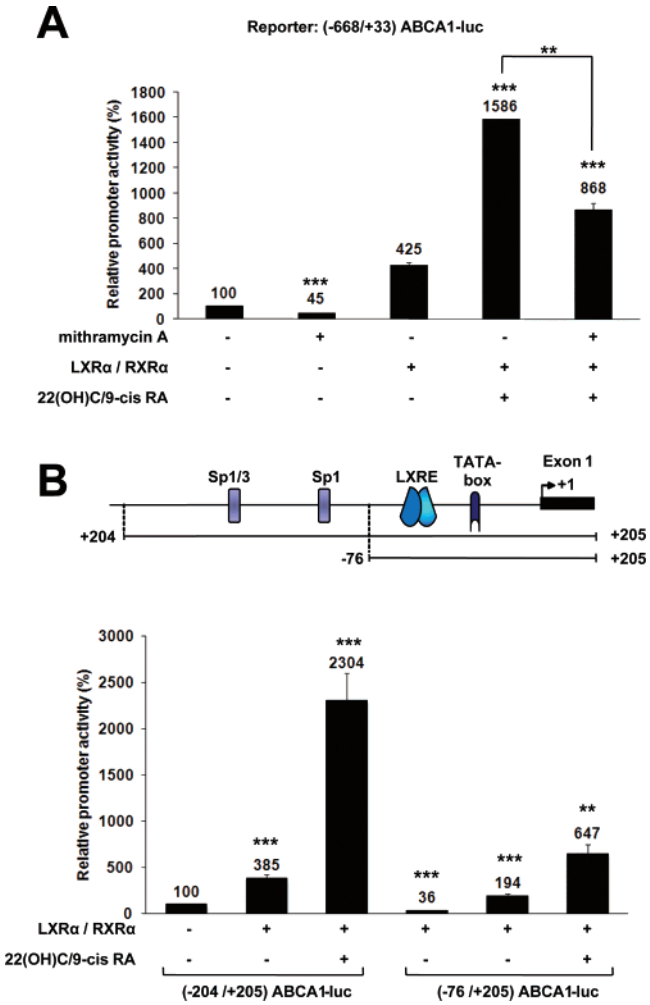


FIGURE 2: (A) Mithramycin A inhibits ABCA1 gene induction by LXR/RXR heterodimers and their ligands. HEK293T cells were transiently transfected with the −668/+33 ABCA1-Luc reporter plasmid (50 ng) along with expression vectors for LXRα and RXRα (0.5 μg each) and were either treated with 22(OH)C + 9-*cis*-RA ($10^{-6}$ M each) and mithramycin A (0.2 μM) for 24 h or left untreated as indicated. The normalized, relative luciferase activity (±SEM) calculated from two independent experiments performed in duplicate is presented with a histograph. (B) Top: Schematic representation of the two ABCA1 promoter fragments −204/+205 and −76/+205 that were used in transactivation experiments. Bottom: Deletion of the Sp1 sites reduced ABCA1 promoter induction by oxysterols and retinoids. HEK293T cells were transiently transfected with the −204/+205 ABCA1-Luc or −76/+205 ABCA1-Luc reporter plasmids (250 ng each) along with expression vectors for LXRα and RXRα (0.5 μg each) and were either treated with 22(OH)C + 9-*cis*-RA ($10^{-6}$ M each) for 24 h or left untreated as indicated. The normalized, relative luciferase activity (±SEM) calculated from two independent experiments performed in duplicate is presented with a histograph. Key: **, $p < 0.05$; ***, $p < 0.001$.

*Optimal ABCA1 Gene Induction by Oxysterols and Retinoids Requires Sp1.* To investigate further the requirement of Sp1 for the transcriptional induction of the ABCA1 gene by oxysterols and retinoids, we used *Drosophila* embryo-derived SL2 cells that lack endogenous Sp1 or related

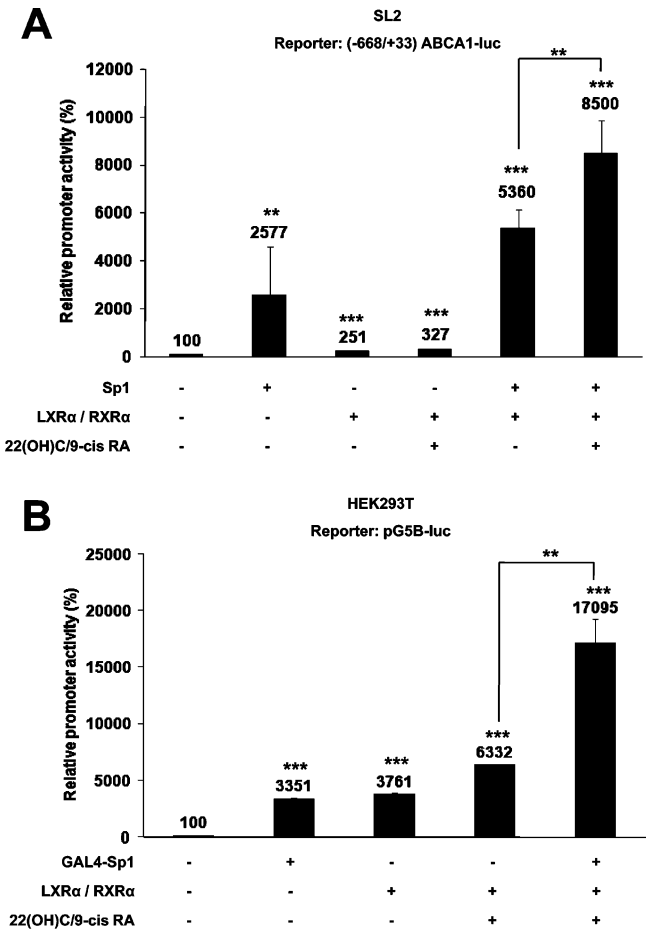


FIGURE 3: (A) Optimal ABCA1 gene induction by LXRα/RXRα and their ligands requires Sp1. Schneider's SL2 cells were transiently transfected with the −668/+33 ABCA1-Luc reporter plasmid (1 $\mu$g) along with expression vectors for Sp1, LXRα, and RXRα (0.5 $\mu$g each) and were either treated with 22(OH)C + 9-*cis*-RA ($10^{-6}$ M each) for 24 h or left untreated as indicated. The normalized, relative luciferase activity (±SEM) calculated from two independent experiments performed in duplicate is presented with a histograph. (B) LXRα/RXRα heterodimers in the presence of their ligands enhance the transcriptional activity of GAL4-Sp1. HEK293T cells were transiently transfected with the pG5B-Luc reporter plasmid (1 $\mu$g) along with expression vectors for GAL4-Sp1, LXRα, and RXRα (0.5 $\mu$g each) and were either treated with 22(OH)C + 9-*cis*-RA ($10^{-6}$ M each) for 24 h or left untreated as indicated. The normalized, relative luciferase activity (±SEM) calculated from two independent experiments performed in duplicate is presented with a histograph. Key: **, $p < 0.05$; ***, $p < 0.001$.

activities (*47*). This cell system has been utilized extensively in the past by us and other groups in studies of Sp1-dependent transcriptional mechanisms (*48*−*50*). As shown in Figure 3A, a significant transactivation of the −668/+33 ABCA1 promoter was observed by overexpression of Sp1 in SL2 cells (25.8-fold), confirming the role of this factor as a strong regulator of ABCA1 gene transcription. In contrast, a relatively weak transactivation of the ABCA1 promoter was observed by overexpression of RXRα and LXRα both in the absence and in the presence of their ligands (2−3-fold). Importantly, the simultaneous expression of Sp1 and the two nuclear receptors caused a strong synergistic transactivation of the ABCA1 promoter (53.6-fold), which was enhanced further by the addition of their ligands (85-fold), indicative of a functional cooperativity between these factors on the ABCA1 promoter.

The findings of Figures 2 and 3 suggested that optimal transactivation of the ABCA1 promoter by oxysterols and retinoids requires, in addition to the proximal LXRE, the presence of the Sp1 binding elements located at positions −90/−85 and −156/−151 and functional interactions between Sp1 and LXR/RXR heterodimers.

*Functional Interactions between Sp1 and LXRα/RXRα Heterodimers.* Functional interactions between Sp1 and LXRα/RXRα heterodimers were demonstrated using the GAL4 transactivation system. For this purpose, transient transfections were performed in HEK293T cells using Sp1 fused with the DNA binding domain of the yeast transactivator GAL4 and an artificial promoter consisting of the firefly luciferase gene under the control of five tandem GAL4 binding elements (pG5B-Luc). Using this system, we were able to show that LXRα/RXRα heterodimers in the presence of their ligands enhanced the transcriptional activity of GAL4-Sp1 5-fold (Figure 3B). Since the G5B artificial promoter does not contain any binding sites for LXR/RXR, we suggested that, in this system, the two nuclear receptors acted as superactivators of Sp1 (Figure 3B).

The ability of nuclear receptors to function as superactivators of Sp1 on promoters that do not contain hormone response elements have been documented in previous studies. In one of these studies, Suzuki et al. showed that retinoic acid induced the transcription of the urokinase gene, which lacks a canonical retinoic acid response element (RARE), in endothelial cells and that this induction required the presence of a GC-rich region that binds Sp1 (*23*). A similar finding was reported by Husmann et al. in the case of the interleukin 1$\beta$ promoter (*22*). Huang et al. and Cheng et al. showed that the response of the $p27^{Kip1}$ gene to vitamin $D_3$ is mediated by physical and functional interactions between the vitamin D receptor (VDR) and Sp1 bound to a GC-rich element on the $p27^{Kip1}$ gene promoter (*25*, *26*). In a different system, physical and functional interactions between estrogen receptors and Sp1 were found to mediate the induction of several GC-rich promoters by estrogens in breast cancer cells (*28*−*33*). Two models that could account for such interactions have been proposed. One model, which is supported by the retinoic acid receptor/Sp1 and estrogen receptor/Sp1 interaction studies, proposes that nuclear receptor/Sp1 interactions stabilize the binding of Sp1 to its cognate sites on the target promoters (*22*−*24*, *27*). The second model proposes that Sp1 serves as a plain anchor for nuclear receptor recruitment to target promoters that lack HREs (*25*, *26*). All these studies introduced a new concept in nuclear receptor function, namely, that nuclear receptors can regulate the expression of genes that lack canonical hormone response elements via physical and functional interactions with other promoter-bound transcription factors such as Sp1.

*Physical Interactions between Sp1 and Nuclear Receptors RXR and LXR.* To investigate potential physical interactions between the Sp1 transcription factor and the nuclear receptors LXRα and RXRα, we employed both in vivo and in vitro assays. First, we used a recently described protein−protein interaction assay based on the biotinylation of one of the interacting proteins in vivo (in this case Sp1) (*51*) to show that Sp1 specifically interacted with LXRα and RXRα. For this purpose, HEK293T cells were transiently transfected with expression vectors for 6myc-tagged RXRα or 6myc-tagged LXRα along with an expression vector for human

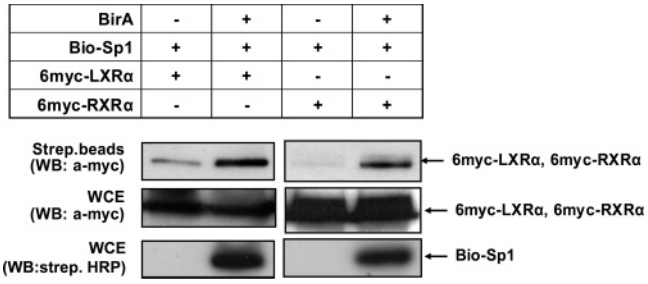


FIGURE 4: Physical interactions between Sp1 and nuclear receptors LXRα and RXRα in vivo. HEK293T cells in 10 cm Petri dishes were transiently transfected with expression vectors for Bio-Sp1, 6myc-LXRα, and 6myc-RXRα (7.5 μg each) and the biotin ligase BirA (7.5 μg) as indicated. Protein−protein interaction assays were performed as described in the Materials and Methods. The arrows on the right indicate the electrophoretic position of the corresponding proteins. Key: WCE, whole cell extract; WB, Western blotting; strep. beads, streptavidin−agarose beads; strep. HRP, straptavidin-conjugated horseradish peroxidase.

Sp1 bearing the Bio epitope tag at its N-terminus (*36*) in the absence and in the presence of the biotin ligase BirA. Extracts from the transfected cells were allowed to interact with Straptavidin agarose beads, and the bound proteins were analyzed by SDS−PAGE and immunoblotting using the anti-myc monoclonal antibody. As shown in Figure 4, top row, biotinylated Sp1 interacted strongly and specifically with both 6myc-tagged LXRα and 6myc-tagged RXRα in vivo. The expression levels of the 6myc-tagged RXRα and 6myc-tagged LXRα in the whole cell lysates are shown in the middle row. The bottom row shows the efficiency of biotinylation of Bio-Sp1 by the BirA ligase.

To characterize the domain(s) of Sp1 that is (are) responsible for the interaction with the two nuclear receptors, we performed GST pull-down assays in vitro. For this purpose, GST fusion proteins containing full-length Sp1 or different individual domains of Sp1 (domains A, B, C, D, and DBD) were constructed (Figure 5A). These proteins were expressed in *E. coli* (Figure 5C) and were examined for their ability to interact with LXRα and RXRα as described in the Materials and Methods. The results showed that full-length LXRα interacted with the B and DBD domains of Sp1 (Figure 5B, top row) while full-length RXRα interacted strongly with the DBD domain of Sp1 only (Figure 5B, bottom row).

To map the domain(s) of LXRα which is (are) responsible for the interaction with Sp1, GST pull-down assays were performed using different myc-tagged truncated forms of LXRα (Figure 6A). All these LXRα forms were overexpressed in HEK293T cells, and extracts from the transfected cells were allowed to interact with GST- or GST−Sp1-coupled glutathione−Sepharose beads. As shown in Figure 6B, full-length Sp1 protein interacted strongly with the N-terminal 1−163 region of LXRα, which includes the AF1 and DNA binding domains (domains A/B and C, respectively). In contrast, no interaction was observed between Sp1 and LXRα 164−447, which includes the hinge region and the ligand binding domain (domains D and E, respectively).

We then showed that the physical interaction between LXRα and Sp1 was abolished when the 1−163 region of LXRα was truncated from either the C-terminus (LXRα 1−97) or the N-terminus (LXRα 98−163). Deletion of the AF-1 domain (LXRα 30−163) reduced significantly the

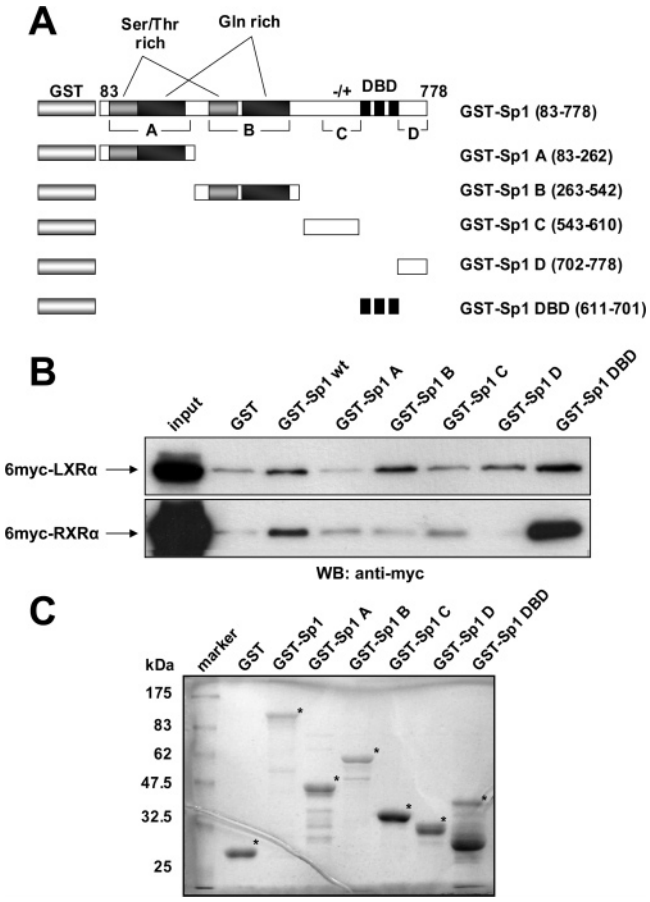


FIGURE 5: Characterization of domains in Sp1 that mediate physical interactions with LXRα and RXRα in vitro. (A) Schematic representation of the various GST−Sp1 fusion proteins that were used for the protein−protein interaction assays of panel B. Letters A−D and DBD indicate different functional domains in Sp1 protein. Key: Ser/Thr rich, domains in Sp1 rich in serine and theronine residues; Gln rich, domains in Sp1 rich in glutamine residues; DBD, DNA binding domain. (B) HEK293T cells were transiently transfected with an expression vector for LXRα or RXRα (20 μg each), and extracts from the transfected cells were allowed to interact with glutathione−Sepharose beads coupled with GST or the indicated GST−Sp1 fusion proteins as described in the Materials and Methods. Bound proteins were analyzed by SDS−PAGE and immunoblotting using the monoclonal anti-myc antibody. (C) The various GST−Sp1 fusion proteins shown on top were expressed in *E. coli*, and extracts of the transformed cells were coupled with glutathione−Sepharose beads as described in the Materials and Methods. A small aliquot of the coupled beads was subjected to SDS−PAGE and staining with Coomassie Brilliant Blue. The asterisks show the electrophoretic position of each GST−Sp1 fusion protein. Other visible bands correspond to degradation products.

interaction with Sp1, whereas deletion of the 128−163 region, which includes the second zinc finger of the DNA binding domain (LXRα 1−127), had a minimal effect on the interaction with LXRα. Interaction with Sp1 was abolished when both the 1−29 and 128−163 regions were removed (LXRα 30−127).

Finally, using GST pull-down assays in vitro (Figure 6C), we confirmed the direct physical interaction between the 1−163 domain of LXRα and the B and DBD domains of Sp1. A summary of the results from the protein−protein interaction experiments is presented in Figure 6A (on the right).

Direct evidence for physical interactions between Sp1 and nuclear receptors in vitro and in vivo as well as the

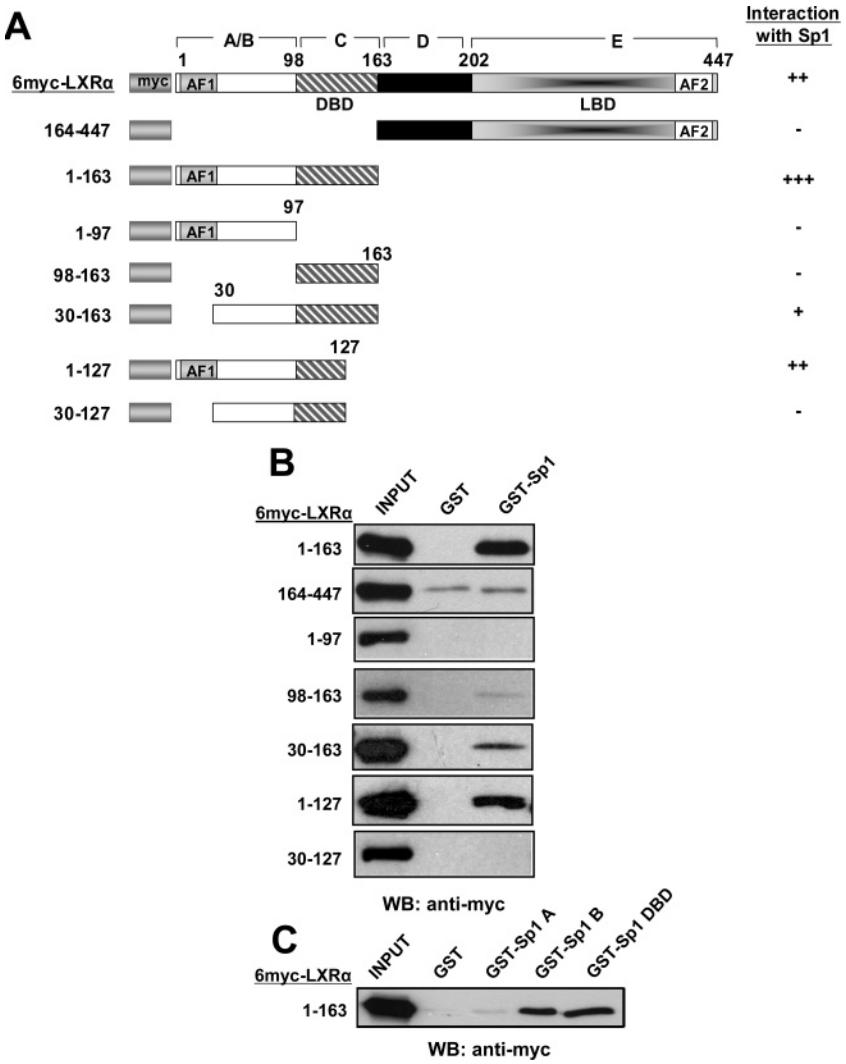


FIGURE 6: Domains in LXRα that mediate physical interactions with Sp1 in vitro. (A) Schematic representation of the various 6myc-LXRα wild-type and truncated forms that were used for the protein−protein interaction assays of panels B and C. Letters A−E indicate different functional domains in LXRα protein. Key: AF1, AF2, activation functions 1 and 2, respectively; DBD, DNA binding domain; LBD, ligand binding domain. A summary of the results of the protein−protein interaction experiments of panel B is shown on the right. The strength of the interactions is indicated with − and + (+++ being the strongest interaction). (B) HEK293T cells were transiently transfected with the various expression vectors for LXRα shown on the left (20 $\mu$g each), and extracts from the transfected cells were allowed to interact with GST- or GST−Sp1-coupled glutathione−Sepharose beads as described in the Materials and Methods. Bound proteins were analyzed by SDS−PAGE and immunoblotting using the monoclonal anti-myc antibody. (C) HEK293T cells were transiently transfected with an expression vector for LXRα (1−163) (20 $\mu$g), and extracts from the transfected cells were allowed to interact with glutathione−Sepharose beads coupled with GST or the indicated GST−Sp1 fusion proteins. Bound proteins were analyzed by SDS−PAGE and immunoblotting using the monoclonal anti-myc antibody.

interacting domains of these factors is very limited. Hussman et al. showed by pull-down assays in vitro that the C-terminal region of Sp1 between amino acids 622 and 788, which includes the DBD and D domains mediates interaction with VDR (*22*). In the same study, using gel electrophoretic mobility shift assays, this group showed that both the DNA and ligand binding domains of RAR are responsible for interaction with Sp1 in vitro (*22*). In another study by Suzuki et al., physical interactions between RAR and Sp1 in vivo were shown by coimmunoprecipitation assays, but the responsible domains were not characterized further (*23*). Finally, Porter et al. showed direct physical interactions between ER and Sp1 in vitro and in vivo (*33*). They also showed that, similarly to the VDR/Sp1 interaction, the ER/Sp1 interaction is mediated by the C-terminal 622−788 region of Sp1, which includes the DBD and D domains (*33*).

In the present study, we showed that the physical interaction between Sp1 and LXR requires the DNA binding domain and transactivation domain B of Sp1 whereas the interaction with RXR requires the DBD of Sp1 only (Figure 5). The involvement of the DNA binding domain of Sp1 in all interactions with nuclear receptors is of particular interest. The involvement of this domain may account for the observed modulation of the DNA binding properties of this transcription factor when it is complexed with other nuclear factors. It may also account for the enhanced recruitment of Sp1 to the ABCA1 promoter under conditions of ligand-induced transcriptional activation (Figure 1) (see model below). In a similar fashion, the interaction of Sp1 with the DNA binding domain of LXR (Figure 6) may induce a conformational change in the nuclear receptor that may result in the stabilization of the RXR/LXR/LXRE protein−DNA

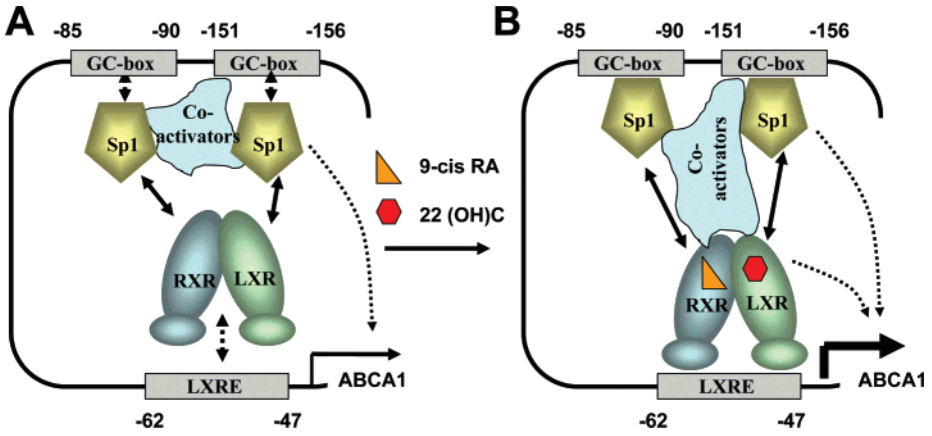


FIGURE 7: Proposed model for the role of Sp1 in the transcriptional induction of the human ABCA1 gene by oxysterols and retinoids. In the absence of ligands, Sp1 and LXR/RXR heterodimers bind to the ABCA1 promoter but cannot activate transcription due to the absence of coactivator recruitment (A). Administration of the two ligands causes the stabilization of the binding of Sp1 and LXR/RXR heterodimers to the proximal ABCA1 promoter and induces transcription possibly by enhancing the formation of a multiprotein complex consisting of Sp1 and LXR/RXR and their coactivators (B).

complex on the target promoters, but this hypothesis needs to be investigated further.

In conclusion, the findings of the present study support a mechanism of transcriptional regulation of the human ABCA1 gene which is based on the physical and functional interactions between the hormone nuclear receptors LXR and RXR and the ubiquitous transcription factor Sp1. According to the proposed model, which is shown schematically in Figure 7, in the absence of ligands, LXR/RXR heterodimers bind to the ABCA1 promoter but cannot activate transcription due to the absence of coactivator recruitment (*52*, *53*) (Figure 7A). Under these conditions, Sp1 also binds to the ABCA1 promoter and contributes to the basal ABCA1 gene expression. Administration of the two ligands induces the interaction of the receptors with their coactivators such as CBP/p300, which are also coactivators of Sp1 (*54*, *55*). As a result, the ligands could facilitate the formation of a multiprotein complex consisting of Sp1 and LXR/RXR and their coactivators and the stabilization of this complex on the ABCA1 promoter as suggested by the chromatin immunoprecipitation experiments of Figure 1C. Under these conditions, ABCA1 gene transcription is enhanced due to the coordinated action of Sp1 and the two nuclear receptors on the ABCA1 promoter (Figure 7B).

This model predicts that mutations in either the LXRE or the Sp1 binding site could equally desensitize the response of the ABCA1 promoter to oxysterols and retinoids. In support of this model are the data of Langman et al. (*20*), which showed that mutations in either the LXRE or the Sp1 binding site at position −90/−85 (but not of the Sp1 site at position −156/−151) reduced ABCA1 promoter induction by retinoic acid and oxysterols in HepG2 cells.

Of interest is the observation that combinations of LXREs and Sp1 binding elements are also found in other genes that are involved in reverse cholesterol transport. Specifically, the proximal promoter of the scavenger receptor BI gene (SR-BI), which encodes for the HDL receptor, contains several GC-rich boxes that have been shown to be important for its basal activity (*56*, *57*). The same promoter contains an LXRE that binds LXR/RXR heterodimers and regulates the induction of this gene by oxysterols (*58*). Similarly, a combination of LXRE and Sp1 sites has been identified and characterized on the promoter of the cholesterol ester transfer protein (CETP) gene (*59*, *60*). Thus, Sp1 or other members of the Sp1 family of transcription factors could be considered as general modulators of lipid metabolism and may influence the protection from or the pathogenesis of atherosclerosis.

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