Identification of the cAMP-Responsive Enhancer of the Murine ABCA1 Gene
Requirement for CREB1 and STAT3/4 Elements

Wilfried Le Goff, Ping Zheng, Gregory Brubaker, Jonathan D. Smith

Objective—To determine the mechanism by which expression of the murine ABCA1 gene is highly induced by cAMP analogues.

Methods and Results—ABCA1 mRNA turnover cannot account for its induction by cAMP. Thus cAMP induction of ABCA1 mRNA occurs at a transcriptional level. Shotgun cloning DNA fragments from the murine ABCA1 locus identified a strong cAMP responsive enhancer located in the first intron, which led to 25- to 100-fold cAMP-mediated induction of reporter gene activity. Deletions and mutations of this enhancer led to the identification a cAMP-responsive element (CRE) that was essential for the cAMP induction. Furthermore, the capacity of this CRE site to mediate the cAMP induction required the presence of a STAT3/4 element located 81 bp away. A dominant-negative CREB expression vector inhibited the cAMP induction of ABCA1, demonstrating that CREB was required for cAMP induction of ABCA1 expression in RAW264.7 cells.

Conclusion—Phospho-CREB1 controls the cAMP-mediated induction of murine ABCA1 gene expression through a CRE site acting in cooperation with a nearby STAT element. This CRE site is not conserved in the human ABCA1 gene, explaining why human ABCA1 is not strongly stimulated by cAMP analogs. (Arterioscler Thromb Vasc Biol. 2006;26:527-533.)

Key Words: ABCA1 • cAMP response element • chromatin immunoprecipitation • gene expression • transcription factor

ATP-binding cassette transporter A1 (ABCA1) mediates cholesterol efflux to lipid-poor high-density lipoprotein (HDL) apolipoproteins and plays a key role in the elimination of cholesterol from macrophage-derived foam cells in the artery wall.1 Tangier disease, which is characterized by very low HDL levels, cholesterol deposition in macrophages, and premature atherosclerosis, is caused by mutations in ABCA1 gene.2-4 Macrophage ABCA1 has been shown to protect against atherosclerosis in mouse models, as transplantation of ABCA1-deficient marrow, compared with transplantation of wild-type marrow, resulted in an increase of atherosclerosis compared with transplantation of wild-type marrow.5,6 Thus, modulation of ABCA1 expression in macrophages constitutes a therapeutic target for the prevention of human atherosclerosis.

Although ABCA1 is highly regulated at a post-translational level,7-9 ABCA1 gene expression is also tightly regulated in macrophages. ABCA1 can be activated by the nuclear liver X receptor and retinoic X receptor in response to oxysterols or synthetic ligands,10,11 by PPAR-α and PPAR-γ activators,12 and by retinoic acid receptor ligands.13 ABCA1-mediated cholesterol efflux to apolipoprotein AI (apoAI) is stimulated by cAMP analogs in mouse macrophages14,15 as a result of 50- to 70-fold induction of ABCA1 gene expression.16,17 However, the response to cAMP differs between mouse and human ABCA1 genes,18 and among various cell lines tested, only mouse macrophages show a substantial induction of ABCA1 mRNA and cholesterol efflux by a cAMP treatment.19 In vitro promoter analysis suggests that the proximal promoter of the human ABCA1 gene contains element(s) responsible for 2-fold cAMP-mediated induction of luciferase reporter gene activity in RAW264.7 cells.20 However, the elements involved in this modest cAMP-mediated induction cannot account for the 50- to 70-fold increase of ABCA1 mRNA observed in RAW264.7 cells by a treatment with cAMP.17

In this study, we conclude that cAMP-mediated induction of ABCA1 occurs at a transcriptional level in the mouse macrophage RAW264.7 cell line. We identified a consensus cAMP response element (CRE) in the first intron of the murine ABCA1 gene that mediated cAMP induction of reporter gene constructions in cooperation with a nearby STAT3/4 element. We further report that the activated form of CRE-binding protein (CREB), ie, phospho-CREB, specifically binds the CRE in the first intron of ABCA1 gene, and...
that use of a dominant-negative CREB inhibits the cAMP induction of reporter transcripts mediated by this CRE. The dominant-negative CREB also reduced ABCA1 levels in cAMP-treated RAW264.7 cells, confirming that CREB is required for cAMP-mediated induction of murine ABCA1.

Methods

Constructs

A 1131-bp DNA fragment corresponding to the −1104/+26 region of murine ABCA1 gene promoter was amplified by polymerase chain reaction (PCR) and subcloned using the TA overhang into the pCR2.1 vector (Invitrogen). The sequences of both upstream and downstream primers used for the generation of all constructs by PCR are presented in Table 1 (available online at http://atvb.ahajournals.org). Then, a 1189-bp HindIII fragment containing the ABCA1 promoter and a portion of the pCR2.1 polylinker was isolated and cloned into the HindIII-cutter pGL3-Basic vector (Promega), generating the pAABC1 construct (luciferase expression vector driven by the 1.1-kb murine ABCA1 promoter). The orientation and the integrity of the insert were verified by sequencing. Generation of the pABCA1 construct (luciferase expression vector driven by the 1.1-kb murine ABCA1 promoter and a portion of the pCR2.1 polylinker was isolated and cloned into the pCR2.1 vector (Invitrogen). The sequences of both upstream and downstream primers used for the generation of all constructs by PCR are presented in Table 1 (available online at http://atvb.ahajournals.org). Then, a 1189-bp HindIII fragment containing the ABCA1 promoter and a portion of the pCR2.1 polylinker was isolated and cloned into the HindIII-cutter pGL3-Basic vector (Promega), generating pABC1 construct (luciferase expression vector driven by the 1.1-kb murine ABCA1 promoter). The orientation and the integrity of the insert were verified by sequencing. Generation of constructs by shotgun cloning from the BAC RPCI 23-25 D17 are described in the supplementary materials (please see http://atvb.ahajournals.org).

The constructs pCMV500 and A-CREB, expressing a control plasmid and the dominant-negative inhibitor of CREB, respectively,21 were the generous gifts of Dr Charles Vinson (National Cancer Institute, NIH, Bethesda, Md).

Real-Time PCR

RAW264.7 cells were treated in the presence or absence of either 0.3 mmol/L 8Br-cAMP or 4 μg/mL 22(R)-hydroxycholesterol and 1 μmol/L 9-cis retinoic acid in DGGB for desired time, as indicated in the figure legends. Total RNA was extracted with TRIzol reagent (Gibco BRL) and 1 μg of RNA was reverse-transcribed into cDNA using Superscript II (Gibco BRL). PCR amplification and normalization of ABCA1 mRNA for 6 hours after the addition of actinomycin D indicated that ABCA1 mRNA turnover was faster in the 8Br-cAMP–treated cells with a half-life of 1.9 hours compared with 3.5 hours in 9cRA-treated cells (Figure 1C). Because the levels of ABCA1 mRNA were increased by 8Br-cAMP and ABCA1 mRNA was not stabilized by 8Br-cAMP, we conclude that 8Br-cAMP induced ABCA1 expression at a transcriptional level.

Western Blot Analysis

RAW264.7 cells were grown in 6-well plates to 80% confluence and incubated in DGGB in the presence or absence of 0.3 mmol/L 8Br-cAMP for 16 hours. Detection of ABCA1 by Western blot analysis was performed as previously described.7

Statistics

Data are shown as mean±SD. Comparisons of 2 groups were performed by a 2-tailed t test, and comparisons of 3 or more groups were performed by ANOVA with Dunnett’s postest. All statistics were performed using Prism software from GraphPad (San Diego, Calif).

Results

cAMP Induces ABCA1 Expression at a Transcriptional Level

To address the mechanism by which cAMP induces the expression of the murine ABCA1 gene, we analyzed the specificity of the 8Br-cAMP–mediated stimulation of ABCA1 mRNA by reverse-transcription PCR in the murine RAW264.7 macrophage cell line. As shown in Figure 1A, 8Br-cAMP is the most potent inducer of ABCA1 mRNA expression in RAW264.7 cells as compared with acetylated low-density lipoprotein (acLDL) or 22-hydroxycholesterol (22OHC) and 9-cis retinoic acid (9cRA) (55-fold versus 3.3- and 4.5-fold, respectively), and such an induction is rapidly observed after only a 2-hour treatment (≈30-fold) (Figure 1B). Addition of either acLDL or 22OHC and 9cRA to 8Br-cAMP was accompanied by a synergistic increase of ABCA1 mRNA (Figure 1A), indicating that those inducers, which act via the LxR transcription factor, and cAMP mediate ABCA1 induction by distinct mechanisms.

To determine whether cAMP-mediated ABCA1 mRNA induction results from an increase of mRNA stability, we analyzed the effects of 8Br-cAMP on ABCA1 mRNA turnover. Analysis of ABCA1 mRNA for 6 hours after the addition of actinomycin D indicated that ABCA1 mRNA turnover was faster in the 8Br-cAMP–treated cells with a half-life of 1.9 hours compared with 3.5 hours in 9cRA-treated cells (Figure 1C). Because the levels of ABCA1 mRNA were increased by 8Br-cAMP and ABCA1 mRNA was not stabilized by 8Br-cAMP, we conclude that 8Br-cAMP induced ABCA1 expression at a transcriptional level.
Identification of cAMP-Responsive Regions in the ABCA1 Gene

To identify cAMP response elements (CRE) in the mouse ABCA1 gene, a bioinformatics approach was tried but was not successful (data not shown). Then a shotgun cloning strategy was performed by cloning restriction fragments derived from a mouse ABCA1 gene region BAC clone upstream of the 1.1-kb proximal mouse ABCA1 promoter driving a luciferase reporter gene. Among the 41 constructs tested in transient transfection experiments in RAW264.7 cells, luciferase activities of construct numbers 2, 6, 26, 31, and 37 were substantially induced by a 16-hour treatment with 0.3 mmol/L 8Br-cAMP compared with the control luciferase expression vector. After a 16-hour treatment in the presence or absence of 0.3 mmol/L 8Br-cAMP, luciferase and β-galactosidase activities were assayed. The ratio of luciferase activity to β-galactosidase activity was calculated and the fold induction by 8Br-cAMP for each test construct was normalized to 8Br-cAMP induction of the control pABCA1 construct (n=3 or 6 ±SD; **P<0.01 compared with control).

Restriction mapping with EcoRI and DNA sequencing of the insert ends revealed that the same 3.6-kb DNA fragment upstream of the ABCA1 gene (fragment A) was inserted in constructs 2 and 31, and that constructs 26 and 37 (the most highly induced constructs) shared the same 2.2-kb DNA fragment from intron 1 (fragment B). A larger 8.7-kb DNA fragment of intron 5 (fragment C) was the insert in construct 6 (Figure 2A). Thus the 5 active constructs were caused by only 3 different inserts, A, B, and C, each of which contain a DNA element that mediated induction by cAMP. On performing independent transfection experiments to compare the inducing strength of the A, B, and C fragments (Figure 2B), fragment B consistently yielded the highest induction of luciferase activity by 8Br-cAMP (74-fold, P<0.01 in Figure 2B), whereas fragments A and C mediated modest inductions of luciferase activity by 8Br-cAMP (∼4 and ∼7-fold, respectively). However, we noticed that the cAMP-mediated induction of fragment B varied from experiment to experiment and ranged from ∼25 to >100-fold. Thus fragment B, located in the first intron of ABCA1 gene, contained a strong cAMP-responsive element that may account for the majority of the ∼70-fold induction of ABCA1 mRNA by cAMP.

CRE on B Fragment Is Required for cAMP-Mediated Activation

To identify the specific cAMP-responsive element(s) in the B fragment, a series of deletion constructs were made and analyzed via transient transfection. As shown in Figure 3A, the construct that contains the full-length B fragment (the B sequence in Figures 3 and 4 is inverted relative to the coding strand of mouse ABCA1 gene, and thus corresponds to the noncoding strand) displayed a significant 36-fold induction of luciferase activity (P<0.01) by 8Br-cAMP as compared with the pABCA1 parent construct (the luciferase expression vector driven by the 1.1-kb murine ABCA1 promoter). Deletion of a 1.2-kb from the 5′ end of the B insert (B-2) completely abolished the 8Br-cAMP induction, whereas a 5′ 888 bp deletion (B-1) was still cAMP-inducible, suggesting that the active element resided within the 320-bp region at the 5′ end of the B-1 fragment (this 320-bp region is called fragment b). Surprisingly, a construct that only contained the b sequence was only weakly inducible by 8Br-cAMP. However, a longer fragment (b′), including a 46-bp extension at the 3′ end of the b fragment, restored most of the activity found in the B fragment (25-fold induction by 8Br-cAMP; P<0.01), suggesting that some additional elements in the b′ construct were required.

Sequence analysis of the B-1 and b′ constructs identified a consensus CRE (5′-TGACGTTCC-3′), within which was found the AatII restriction site that was used to generate the B-2 deletion fragment. Thus the B-2 fragment contained only a truncated CRE site. The b fragment also did not contain the intact CRE site. We hypothesized that a single CRE located at the AatII site in the B fragment was required for the cAMP-dependent enhancer activity of this fragment. To test this hypothesis, AatII digestion, overhang elimination, and blunt end ligation was used to create 4-bp deletions (5′-TGCC-C′) in the CRE of B and b′, (constructs B-Mut and b′-Mut, respectively). As expected, luciferase activities of the B-Mut and b′-Mut constructs were not significantly induced.
by 8Br-cAMP (Figure 3A), thus confirming that this CRE was necessary for cAMP induction.

**Phospho-CREB1 Specifically Binds the B Fragment CRE**

Gel mobility shift assays were performed to verify whether the activated form of CREB1, i.e., phospho-CREB1, binds to the CRE identified in the B fragment. As shown on Figure 3B, incubation of a biotinylated probe (CRE-b/H11032) with nuclear extracts of control-treated RAW264.7 cells did not lead to the formation of any DNA-protein complexes (Figure 3B, lanes 2 to 6), whereas 2 complexes (I and II) were observed with nuclear extracts of 8Br-cAMP–treated RAW264.7 cells (lane 7). Both complexes were also observed using a biotinylated probe specific for the consensus sequence of the CREB/ATF-transcription factor family (CREB, lane 15). A 4-bp deletion in the biotinylated probe (CRE-b’Mut), corresponding to the mutation that neutralized cAMP induction mediated by the B fragment (Figure 3B), prevented the formation of the 2 complexes with nuclear extracts of 8Br-cAMP–treated RAW264.7 cells (lane 14). Finally, the use of an antibody against phospho-CREB1 (phospho-Ser 133) confirmed that complex I was formed as a result of interactions with phospho-CREB1 (lanes 10 and 18).

The binding of phospho-CREB1 on the b’ CRE in situ was investigated by chromatin immunoprecipitation (ChIP) analysis using RAW264.7 cells treated for 16 hours with or without 0.3 mmol/L 8Br-cAMP. As shown on Figure 3C, overnight incubation of protein–DNA complexes with an antibody raised against either phospho-CREB1 (lanes 5, 10, and 18) or CBP (lanes 6 and 11). The 2 DNA-protein complexes (I and II) and supershifted bands (SS) are indicated by arrows. C, Chromatin-associated DNA from 1×10⁶ RAW264.7-cells treated (+) or not (−) with 0.3 mmol/L 8Br-cAMP for 16 hours was incubated overnight in the presence or absence of an antibody against phospho-CREB1 (p-CREB1). Immunoprecipitated DNA was subjected to PCR amplification along with specific primers flanking the B fragment CRE site and only the expected single PCR product was observed.

**A STAT3/4 Site Nearby the b’ CRE Is Required for cAMP-Mediated Activation**

To further characterize the mechanism by which the B fragment CRE can mediate the induction by cAMP, constructs containing a shortened b’ fragment were generated and analyzed via transfection in RAW264.7 cells. As shown in Figure 4A, the 366 bp b’ construct displayed a significant
55-fold cAMP induction of luciferase activity ($P<0.01$) as compared with the pABCA1 parent construct. However, this induction was no longer observed if the CRE site was mutated (b'-Mut), confirming the requirement of the CRE site for cAMP induction. Deletion of 234 bp from the 5' end (b'-1) did not affect the stimulation by 8Br-cAMP, whereas a 5' 246-bp deletion (b'-2) completely abolished this induction, indicating that the 12 bp region at the 5' end of the b'-1 construct was necessary for cAMP induction. Thus, although the CRE site was absolutely required for the cAMP-mediated activation, it was not sufficient and a 12-bp sequence located 81 bp away was also required for cAMP induction.

Mutations in this 12-bp sequence (1 to 9; Figure 4B) indicated that mutations 1, 2, 4, and 5 led to a complete loss of induction of luciferase activity by 8Br-cAMP (b'-1-M1, b'-1-M2, b'-1-M4, and b'-1-M5 constructs; Figure 4A), whereas mutations 3 and 6 had either no (b'-1-M3) or a weak effect (b'-1-M6). Additional mutations 7, 8, and 9 had no effect on the induction of the luciferase activity by cAMP (data not shown). Taken together, these results suggested that the core sequence element 5'-GGXAA-3' in the 12-bp sequence was required for cAMP induction.

This core sequence element matched the consensus binding sites for STAT3 and STAT4 (5'-GGGAA-3'). Using a double-stranded 28-bp biotinylated oligonucleotide probe (STAT-b'-1) spanning this region, gel shift assays were performed to test whether STAT3 or STAT4 binds to this fragment. Incubation of the biotinylated probe with nuclear extracts of either control or 8Br-cAMP–treated RAW264.7 cells led to the formation of 2 DNA–protein complexes (I and II) (Figure 4C, lanes 2, 3 and 10), which were not observed in the presence of an excess of nonbiotinylated probe (lanes 4 and 11), ISRE competitor (lane 5), STAT1 competitor (lane 12), STAT3 competitor (lane 13), STAT4 competitor (lane 14), and IRF-1 competitor (lane 15). The 2 DNA–protein complexes (I and II) are indicated by arrows.
complex I and II, respectively. Thus, the element 81 bp away from the B fragment CRE could bind both STAT3 and STAT4 in a cAMP-independent fashion.

**Dominant-Negative CREB Inhibits cAMP-Mediated Induction of ABCA1 in RAW264.7 Cells**

If the binding of CREB to the B fragment CRE is required for cAMP induction of ABCA1 expression in RAW264.7 cells, then we would expect that expression of a dominant-negative CREB (A-CREB) would prevent the cAMP dependent enhancer activity of the B fragment and abolish the cAMP-mediated stimulation of ABCA1 expression. The cAMP mediated induction of ABCA1 was drastically reduced (−71%, \( P<0.01 \)) with increasing amounts of A-CREB, demonstrating that expression of the dominant-negative CREB prevented the CREB-mediated activation through the B fragment in response to cAMP (Figure II, available online at http://atvb.ahajournals.org).

The involvement of CREB in the cAMP-mediated stimulation of ABCA1 expression was confirmed by Western blot after nucleofection of A-CREB in RAW264.7 cells (40% transfection efficiency). The level of ABCA1 protein was strongly induced in 8Br-cAMP–treated RAW264.7 cells after nucleofection with the control expression vector (Figure III, available online at http://atvb.ahajournals.org). However, nucleofection of the dominant-negative A-CREB expression vector led to a 49% reduction of the cAMP-mediated induction of ABCA1 (\( P<0.001 \)), providing evidence that activated CREB is required for the cAMP-mediated induction of ABCA1 in RAW264.7 cells.

**Protein Kinase A Mediates the cAMP Induction of ABCA1 in RAW264.7 Cells**

Activation of protein kinase A (PKA) by cAMP is the general mechanism by which CREB is phosphorylated and activated. Cholesterol efflux to apoAI was strongly induced by 0.3 mmol/L 8Br-cAMP; however, addition of the PKA inhibitor H-89 reduced, in a dose-dependent fashion, ABCA1-mediated cholesterol efflux to apoAI and the levels of ABCA1 protein (Figure IV, available online at http://atvb.ahajournals.org). Thus, the activation of PKA is absolutely required for the induction of ABCA1 by cAMP.

**Discussion**

Overall, our data support a model in which cAMP activates PKA leading to CREB1 activation that binds to a strong CRE in the first intron of the mouse ABCA1 gene and induces its transcription. The role of CREB1 in the cAMP-mediated induction of ABCA1 was confirmed by the use of a dominant-negative CREB that dimerizes with wild-type CREB and prevents binding to CRE.\(^{21}\) The dominant-negative CREB prevents the binding of CREB1 to a CRE without affecting the ability of others members of the ATF/CREB family to bind the CRE,\(^{21}\) suggesting that CREB1, and not other ATF factors, is required for the ABCA1 induction by cAMP.

We first used a bioinformatic approach, based on the finding consensus CRE sites in the mouse ABCA1 gene, to identify functional CREs, but in this case, this method was not efficient or successful. However, a shotgun cloning strategy was successful, and one of the reasons that this method was superior may have been that the strongest cAMP responsive enhancer required more than just a functional CRE consensus sequence. We determined that the strong CRE site in the first intron of the mouse ABCA1 gene could only mediate cAMP responsiveness of a reporter gene in transfection studies with the cooperation of a STAT element located 81 bp from the CRE site.

Gel mobility shift assays indicated that STAT3 and STAT4 could bind this STAT element, suggesting that STAT3 and/or STAT4 may interact with CREB1 on the enhancer in the first intron of the mouse ABCA1 gene. Recombinant CREB has previously been shown to bind to STAT1–3 in pull-down experiments,\(^{23}\) suggesting that STAT1–3 and CREB may form a transcriptional complex. Several studies also reported that the transcriptional coactivator CREB binding protein (CBP) can bind to various STATs.\(^{24-26}\) A recent study suggested that the association of phospho-CREB1 with a number of coactivators, such as CBP, is too weak for gene activation and that additional regulatory partners are needed for stable recruitment of such cofactors to the promoter.\(^{27}\) It is possible that the binding of STAT3 and/or STAT4 on the STAT element may help stabilize the recruitment of CBP by phospho-CREB1 on the CRE during cAMP induction of the murine ABCA1 gene. To our knowledge, this study is the first demonstration of the requirement for both a CREB and STAT3/4 element for the cAMP-mediated activation of gene expression.

Bortnick et al demonstrated that elevation of intracellular cAMP by prostaglandins \( E_1 \) and \( E_2 \) in mouse macrophages leads to an increase in ABCA1-mediated cholesterol efflux to apoAI, suggesting that G-protein–coupled prostanoid receptors could be involved in the stimulation of ABCA1 in this cell type.\(^{19}\) The multi-fold induction of ABCA1 gene expression by cAMP only occurs in mouse macrophages, whereas cAMP has little effect on ABCA1 expression in human cell lines.\(^{19,28}\) A weak induction (<2-fold) of both ABCA1 expression and cholesterol efflux to apoAI by cAMP has been observed in the human macrophage cell line THP-1.\(^{19,29}\) Furthermore, Cavelier et al reported that the human ABCA1 transgene is downregulated by a cAMP treatment whereas the endogenous murine ABCA1 gene is induced in peritoneal macrophages derived from transgenic mice expressing a human ABCA1 containing BAC.\(^{18}\)

Both the mouse and human ABCA1 genes have 50 exons,\(^{20,30}\) and many large highly conserved noncoding elements have been identified.\(^{30}\) Although the sequence surrounding the functional CRE site in the first intron of mouse ABCA1 gene is 65% identical with the corresponding sequence in the first intron of the human gene, alignment of both sequences revealed that an 8-bp insertion disrupts the CRE site in the human sequence whereas the STAT element is conserved in both species (Figure V, available online at http://atvb.ahajournals.org). The absence of the functional CRE site in the first intron of the human ABCA1 gene could explain the lack of responsiveness of the human ABCA1 gene to cAMP. Because regulatory elements tend to be highly
conserved among mammals, the nonconservation of the functional CRE site in the first intron of the human gene as well as the loss of a substantial induction of human ABCA1 gene expression by cAMP suggests that this pathway is not essential for regulation of ABCA1 in humans. However, despite the lack of a role for cAMP in the induction of the human ABCA1 gene, it has been reported exogenous apoAI binding to cellular ABCA1 increases intracellular cAMP levels, activates PKA, and induces phosphorylation and post translational activation of ABCA1 in human fibroblasts.31,32 Thus, although human and mouse ABCA1 do not share transcriptional activation by cAMP, they are both responsive to cAMP, albeit by different mechanisms.

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References

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Supplemental Material.

Materials and Methods:

Constructs.

A BAC containing 118.1 kb of mouse ABCA1 locus (RPCI 23-25-D17, GenBank™ accession number AL807243), including 36 kb of the 5’ flanking region upstream from the transcription initiation start site and 82 kb in the gene including the first 19 exons was obtained from the Children’s Hospital Oakland Research Institute (CHORI). The BAC was digested with EcoRI and the resulting fragments were directly inserted by shotgun cloning into the EcoRI-digested pABCA1 construct, leading to insertion of these fragments upstream of the ABCA1 promoter. The constructs generated by shotgun cloning were screened by EcoRI digestion in order to confirm the insertion of only one ABCA1 fragment. The BAC contained 30 EcoRI sites in the murine ABCA1 locus and 1 EcoRI site in the backbone vector; thus, a total of 30 distinct insert fragments were expected by shotgun cloning. Deletions of construct #26, which contained the 2.2 kb “B” DNA fragment, were made by digestion with SmaI at the 5’ end and either BstXI or AatII at the 3’ end. The resulting vector plus shortened insert DNA was blunt-ended and self-ligated in order to generate constructs that contained a 1,353 or 1,033 bp fragment of the B sequence (B-1 and B-2, respectively). The 320 and 366 bp fragments in constructs b and b’ were obtained by PCR using construct #26 as the template (oligonucleotide primers for all constructions are shown in Table I). The PCR products were subcloned into the pCR2.1 vector, followed by digestion with EcoRI, yielding the 320 and 366 bp fragments that were isolated and cloned into pABCA1 to generate the b and b’ constructs, respectively. Shortening of the b’ construct by PCR led to the generation of the b’-1 and b’-2 constructs that contain a 132 and 120 bp fragment of the B
sequence, respectively. For mutational analysis, a 4 bp deletion was created in the CRE of the B and b’ constructs by AatII digestion, chewing back the 3’ overhang and creating a blunt-end with T4 DNA polymerase, and self-ligation to generate the B-Mut and b’-Mut constructs, respectively. Mutations in the b’-1 construct were generated by PCR leading to the b’-1-M1, b’-1-M2, b’-1-M3, b’-1-M4, b’-1-M5 and b’-1-M6 constructs, respectively.

Transfection experiments.

Murine macrophages RAW264.7 cells (ATCC) were seeded on 24-well plates at 2 X 10^5 cells per well. After 24 h growth, 2 µg of each ABCA1-luciferase reporter plasmid construct was cotransfected with 40 ng of a β-galactosidase expression vector (pCMV-βgal; Invitrogen) using the GenePorter® transfection reagent (Gene Therapy Systems) according to the manufacturer’s instructions with slight modifications. DNA plasmids and GenePorter reagent (10 µl) were diluted separately in 250 µl of serum-free medium, then mixed together rapidly, and incubated for 30 min at RT. The mixture was put onto the cells and after a 5-hour incubation at 37°C, 0.5 ml of fresh growth medium containing 20% FBS was added. Eighteen hours after transfection, the medium was aspirated, cells were washed twice with PBS and incubated for an additional period of 24 h in DMEM supplemented with 50 mM glucose, 2 mM glutamine, and 0.2% BSA (DGGB) in the presence or absence of 0.3 mM 8Br-cAMP. Cells were harvested with 35 µl of lysis solution (Applied Biosystems) and the lysate was centrifugated for 5 min at 14,000 rpm in order to remove cellular fragments. Luciferase and β-galactosidase activities were assayed on the supernatant (10 µl) using the Dual-Light® System (Applied Biosystems).
Transcriptional activity was expressed as fold induction by 8Br-cAMP after normalization for β-galactosidase activity. Experiments were performed in triplicate.

Nucleofection experiments using the Nucleofector™ technology (Amaxa Biosystems) were performed according to the manufacturer’s protocol. For each experiment 2 x 10⁶ cells and 2 µg of DNA were diluted in 100 µl of T solution and processed with the program T-20. Five hours after transfection, the medium was removed and cells were treated as mentioned in the figure legends.

*Electrophoretic mobility shift assays.*

Nuclear extracts from RAW 264.7, with or without overnight pretreatment with 0.3 mM 8Br-cAMP, were prepared using the NE-PER™ Nuclear and Cytoplasmic Extraction Reagent (Pierce) and stored at -80°C before use. The electrophoretic mobility shift assay (EMSA) was performed as follows: 5’-biotinylated synthetic probes (Table II, Integrated DNA Technologies) were annealed with their respective complementary strand and binding reactions were performed using the LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer’s protocol. EMSA were carried out at 4°C with 1 pmole of double-stranded probes and 8 µg of nuclear extract. When indicated, 1.2 µg of goat polyclonal antibody raised against phospho-CREB1 (Ser-133) (Santa-Cruz Biotechnology) was incubated 20 min prior to the addition of biotinylated probe. The sequences of the double stranded STAT element competitors are presented in Table II.

*Chromatin Immunoprecipitation Analysis.*

Chromatin immunoprecipitation (ChIP) assays were carried out using the Chromatin Immunoprecipitation Assay kit (Upstate). RAW264.7 cells (5x10⁵
cells/ChIP assay) were grown on 6-well plates and treated for 16 hours in the presence or absence of 0.3 mM 8Br-cAMP. After cross-linking of histones and other bound protein to DNA with 1% formaldehyde for 10 min at 37°C, cells were washed twice with ice cold PBS containing protease inhibitors, scraped, and the cell pellets were resuspended in SDS lysis buffer for 10 min on ice. The cell lysates were sonicated eight times for 20 seconds, and after centrifugation the cell pellets were diluted in ChIP dilution buffer. In order to reduce nonspecific background, diluted samples were pre-cleared twice with salmon sperm DNA/Protein A Agarose-50% slurry for 1h at 4°C prior to incubation overnight at 4°C with a 1:250 dilution of rabbit anti phospho-CREB antibody (Cell Signaling). Immune complexes were collected with 30 µl of salmon sperm DNA/Protein A agarose-50% slurry for 2 hours at 4°C. Beads were sequentially washed with low salt, high salt, and TE buffer, and immune complexes were eluted with a solution containing 1% SDS and 0.1 M NaHCO₃. Protein-DNA crosslinks were reversed with 80 mM NaCl for 4 h at 65°C. After a treatment with proteinase K for 1 h at 45°C, DNA was recovered by using a QIAquick PCR purification kit (Qiagen) in 50 µl TE buffer. A 3 µl aliquot was used for PCR amplification of the murine ABCA1 first intron CRE for 25 cycles. The sequence of the upstream primer was 5'-TCAGCAAAGTGTTAGAGAC-3'; the sequence of the downstream primer was 5'-CCATAAGAGCACATTAATGAACAAC-3'.

Supplemental Figures

Supplemental Figure I. Screening for cAMP-responsive regions in the murine ABCA1 gene. RAW264.7 cells were transiently co-transfected with a β-galactosidase expression vector and plasmids with fragments of the mouse ABCA1 gene cloned upstream of the 1.1 kb proximal mouse ABCA1 promoter driving a luciferase reporter
gene. After a 16-hour treatment in the presence or absence of 0.3 mM 8Br-cAMP, luciferase and β-galactosidase activities were assayed. The ratio of luciferase activity to β-galactosidase activity was calculated and the fold induction by 8Br-cAMP for each test construct was normalized to 8Br-cAMP induction of the control pABCA1 construct (C). Screening of the 41 constructs was carried in multiple independent experiments (n=3 ± S.D.).

**Supplemental Figure II.** Dominant negative CREB represses the CREB-mediated activation through the B fragment in response to cAMP. RAW264.7 cells were transiently transfected with 0.5 µg of plasmid B, 60 ng of a β-galactosidase expression vector and increasing amounts of the dominant negative CREB vector. Total DNA was maintained at 2.06 µg in each well with the control pCMV500 plasmid. After a 16-hour treatment in the presence or absence of 0.3 mM 8Br-cAMP, luciferase activity was normalized to β-galactosidase activity and expressed as fold induction by 8Br-cAMP compared to non-treated cells (n=3 ± S.D.; *, p<0.01 compared to 8Br-cAMP treated cells in the absence of A-CREB).

**Supplemental Figure III.** Dominant negative CREB inhibits cAMP-mediated induction of ABCA1 in RAW264.7 cells. RAW264.7 cells were nucleofected with either the dominant negative CREB vector (A-CREB) or the control plasmid (control) and treated in the presence or absence of 0.3 mM 8Br-cAMP for 16 hours. Total ABCA1 in nucleofected cells was detected by Western blot analysis in the upper panel, and quantified in the lower panel after normalization to the Annexin 1 signal, (*, p=0.0007 compared with cells nucleofected with control plasmid, n = 3 ± S.D.).
**Supplemental Figure IV.** Inhibition of PKA abolishes cAMP-mediated induction of ABCA1 in RAW264.7 cells. RAW264.7 cells were treated for 16 hours with or without 0.3 mM 8Br-cAMP to induce ABCA1 in the presence or absence of increasing doses of the PKA inhibitor H-89. A, Cholesterol efflux to 3 µg/ml apoAI for 4 hours at 37°C from cholesterol-loaded cells. H-89 inhibited ABCA1-mediated cholesterol efflux to apoAI (n=3 + S.D., *, p<0.001 compared with cells treated in the presence of 8Br-cAMP alone). B, Total ABCA1 was detected by Western blot analysis. H-89 inhibited cAMP-mediated induction of ABCA1 protein levels.

**Supplemental Figure V.** Sequence alignment of the mouse sequence b’ containing the functional CRE site with the first intron of the human ABCA1 gene (GenBank™ accession number AF287262) using Clustal X ¹. Shaded areas indicate identity between both sequences. CRE and STAT binding sites are boxed. Both the mouse and human sequences are similarly oriented with the coding strand shown.
### Supplemental Table I. Oligonucleotides used for the generation of constructs.

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Upstream primer (5'-3')</th>
<th>Downstream primer (5'-3')</th>
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<tbody>
<tr>
<td>pABCA1</td>
<td>GCTGAATGCTTGCTGCTATGC-</td>
<td>GGAAGCTTGCTCGCCTCGGAGAATTACTGTTT</td>
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<tr>
<td>b</td>
<td>CATTAATGCATTTGGTGCGTGT</td>
<td>AGACTGTACCATTCTTACTCGCT</td>
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<tr>
<td>b'</td>
<td>TCACAGAAGTGTAGAGAC</td>
<td>GGCCCCCAAGTTTT</td>
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<td>b'-1</td>
<td>TCAGCAGAAGTGTAGAGAC</td>
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<td>b'-2</td>
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The underlined and the lowercase sequence indicating the enzyme restriction site and the mutations, respectively

### Supplemental Table II. Oligonucleotides used for the generation of EMSA probes and competitors.

<table>
<thead>
<tr>
<th>Probes</th>
<th>Sequences</th>
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<td>CREB</td>
<td>Bio-5'-AGAGATTGCTGACGCAGTAGAGCTAG-3'</td>
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<tr>
<td>CRE-b'</td>
<td>Bio-5'-AGATGCATTAATGACGTCCCCGCTGATC-3'</td>
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<tr>
<td>CRE-b'Mut</td>
<td>Bio-5'-AGATGCATTAAttgccGCCCCGTATC-3'</td>
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<td>STAT-b'-1</td>
<td>Bio-5'-CTCAGAAAAGGGAAATGGAACTGGGAGG-3'</td>
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<td>STAT-b'-1-M4</td>
<td>Bio-5'-CTCAGAAAAGGGAAATGGAACTGGGAGG-3'</td>
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<tr>
<td>STAT-b'-1-M5</td>
<td>Bio-5'-CTCAGAAAAGGGAAATGGAACTGGGAGG-3'</td>
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<tr>
<td>ISRE competitor 4</td>
<td>5'-AAGTACTTTCAGTTTCATTACTACTCTA-3'</td>
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<tr>
<td>STAT1 competitor 3</td>
<td>5'-CATGTTATGCATATTCCTGTGGAATGTG-3'</td>
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<td>STAT3 competitor 4</td>
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<td>STAT4 competitor 5</td>
<td>5'-GAGCCTGATTTCCCAGGAAATGATGAGC-3'</td>
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<td>IRF-1 competitor 6</td>
<td>5'-GGAAGCGAAAAATTTGAAACT-3'</td>
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The underlined and the lowercase sequence indicating the CREB site and the mutations, respectively

### References.


Supplemental Figure II

Normalized Luciferase Activity (cAMP mediated induction fold)

Ratio B:A-CREB

+8Br-cAMP
Supplemental Figure III

<table>
<thead>
<tr>
<th>Plasmid:</th>
<th>Control</th>
<th>A-CREB</th>
</tr>
</thead>
<tbody>
<tr>
<td>8Br-cAMP:</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

- **ABCA1**
- **Annexin 1**

**Graph:**
- **ABCA1/Annexin (%)**
- **Control**
- **A-CREB**

*Significant difference (*)
Supplemental Figure IV

A

[3H]Cholesterol Efflux to apoA1 (% of total)

+8Br-cAMP

[H-89] μM

0 0 10 15 20

B

H-89 (μM): - - 10 15 20
8Br-cAMP: - + + + + +

ABCA1

Annexin 1