Regulation of human CETP gene expression: role of SP1 and SP3 transcription factors at promoter sites −690, −629, and −37

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Abstract **Cholesteryl ester transfer protein (CETP) is a key factor in plasma reverse cholesterol transport and is implicated in the pathophysiology of atherogenic dyslipidemia. Variations observed in plasma CETP mass and activity in both normolipidemic and dyslipidemic individuals may reflect differences in CETP gene expression. We evaluated the respective roles of the Sp1 and Sp3 transcription factors on the promoter activity of the human CETP gene at a new Sp1/Sp3 site identified at position −690, and at two previously described Sp1/Sp3 sites at positions −37 and −629. In transient transfection in HepG2 cells, site-directed mutagenesis using luciferase reporter constructs containing a promoter fragment from +32 to −745 indicated that the new −690 site acts as a repressive element in reducing CETP promoter activity (−22%; $P < 0.05$); equally, this site exerts an additive effect with the −629 site, inducing marked repression (−42%; $P < 0.005$). In contrast, in NCTC cells that display a 16-fold lower level of Sp3, the repressive effect at the −690 site was enhanced 2-fold (−45%; $P < 0.05$), whereas the −629 site exerted no effect. Cotransfection of Sp1 and/or Sp3 in SL2 insect cells lacking endogenous Sp factors demonstrated that Sp1 and Sp3 act as activators at the −690 and −37 sites, whereas Sp3 acts as a repressor at the −629 site. Taken together, our data demonstrate that Sp1 and Sp3 regulate human CETP promoter activity through three Sp1/Sp3 binding sites in a distinct manner, and that the Sp1/Sp3 ratio is a key factor in determining the relative contribution of these sites to total promoter activity.**—Le Goff, W., M. Guerin, L. Petit, M. J. Chapman, and J. Thillet. **Regulation of human CETP gene expression: role of SP1 and SP3 transcription factors at promoter sites −690, −629, and −37.** ***J. Lipid Res.*** **2003.** 44: **1322–1331.**

Supplementary key words reverse cholesterol transport • atherosclerosis • transcriptional repression

Plasma cholesteryl ester transfer protein (CETP) plays a key role in reverse cholesterol transport by mediating the transfer of cholesteryl esters (CEs) from HDL to atherogenic apolipoprotein B-containing lipoproteins, including VLDL, VLDL remnants, IDL, and LDL (1). The relationship between plasma CETP mass and activity on the one hand and coronary artery disease and cardiovascular risk on the other is indeterminate, however (2, 3). Nonetheless, it is established that variation in CETP mass and/or activity is closely associated with lipoprotein phenotype and, notably, HDL cholesterol levels, in both normolipidemic and dyslipidemic subjects (4–6). Moreover, therapeutic reduction in CETP mass and/or activity is associated with an increase of plasma HDL levels in humans (7) and with regression of atherosclerosis in rabbits (8).

Control of the expression of the CETP gene constitutes a major component in the regulation of plasma CETP mass in humans (9, 10). Major factors that influence CETP gene expression include dietary cholesterol (11), fatty acids (12, 13), and corticosteroids (14), all of which act directly on the promoter region.

The human CETP promoter is under the control of regulatory elements that modulate its transcriptional activity (15–22). Indeed, several *trans*-acting factors, including the orphan nuclear hormone receptor ARP-1 (18), the CCAAT/enhancer-binding protein (15) *trans* retinoic acid (20), the sterol-responsive binding protein (SREBP) (16, 19), and the liver receptor homolog-1 (22) are implicated in the regulation of the transcriptional activity of the CETP gene promoter through specific response elements.

Cellular cholesterol content can regulate CETP gene expression (11, 23, 24), and promoter elements implicated in such modulation have been identified (19, 21). The mechanism of sterol-mediated regulation of CETP expression is complex and requires interaction of LXR and SREBP transcription factors with their respective promoter response elements (19, 21), although the involvement of SREBP in the sterol-mediated up-regulation of

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CETP gene expression remains controversial (16). In addition to these transcription factors, two binding sites for Sp1 have been identified in the CETP gene promoter (17, 18); the first consists of a GC-box located at position −37 upstream of the transcriptional start site (18). Mutations at this site lead to marked reduction in the in vitro transcriptional activity of the CETP promoter, thereby indicating that Sp1 is a key factor in the activation of CETP gene expression. The second Sp1 site, at position −629 (C/A), exhibits a functional polymorphism that modulates CETP promoter activity (17). Indeed, the A allele binds both Sp1 and Sp3 transcriptional factors, leading to significant reduction in in vitro transcriptional activity as compared with the C allele, which does not bind Sp1 and Sp3.

Sp1 and Sp3 transcription factors are ubiquitous zinc-finger proteins and belong to the Sp family of transcription factors, which includes four proteins, i.e., Sp1, Sp2, Sp3, and Sp4 (25). Both Sp1 and Sp3 recognize G-rich elements such as the GC and GT boxes, through which these transcription factors contribute to the regulation of the expression of a wide spectrum of genes (26–30).

We have presently evaluated the respective roles of the Sp1 and Sp3 transcription factors in regulation of the promoter activity of the CETP gene. To this end, we analyzed the action of these transcription factors at a new Sp1 binding site identified at position −690 and at two Sp1 promoter sites described previously (−629 and −37 sites, respectively). Our data demonstrate that the nuclear Sp1/Sp3 ratio is a critical factor in the regulation of CETP gene expression, acting at three distinct promoter sites at positions −690, −629, and −37, respectively.

EXPERIMENTAL PROCEDURES

DNase I footprinting assays

Nuclear extracts were prepared from confluent 150 mm dishes as previously described by Dignam et al. (31), and stored at −80°C before use. Two probes, *Nhe*I*-*Sty*I and *Nhe*I-StyI* (the asterisk indicates the ^{32}P-labeled extremity), were prepared as follows. The wild-type (WT) construct (see below "Plasmid constructs") was digested by either *Nhe*I or *Sty*I restriction enzymes (New England Biolabs, Saint Quentin en Yvelines, France). The linearised vector was then end-labeled by fill-in in a final volume of 30 μl containing 20 μCi of both [α^{32}P]dATP and [α^{32}P]dCTP (10 mCi/ml; 3,000 Ci/mmol; NEN Life, Paris, France), 10 units of Klenow fragment (New England Biolabs), and 8 mM of dGTP+dTTP at 20°C for 20 min. After additional incubation for 5 min with 8 mM of nonradiolabeled dATP+dCTP, and inactivation of the Klenow fragment for 10 min at 65°C, the labeled fragment was purified on MicroSpin™ G-25 columns (Amersham Biosciences, Saclay, France) and digested either by *Sty*I or *Nhe*I. The radiolabeled restriction fragment was purified by electrophoresis in a 6% polyacrylamide gel for 3 h at 150 V, excised from the gel, and incubated overnight at 42°C in an elution buffer containing 0.3 M sodium acetate, 2 mM EDTA, and 0.5% SDS. DNA was then precipitated in the presence of absolute ethanol and 80 μg of glycogen (Invitrogen, Cergy Pontoise, France) for 2 h at −20°C.

DNase I footprinting experiments were performed as follows: 1 μl (5 × 10^4 cpm) of radiolabeled probe (*Nhe*I-*Sty*I* or *Nhe*I-*Sty*I*) was incubated in a final volume of 50 μl with 5 μg poly(dI-dC), 2 mM spermidine, 14 μg of nuclear extracts (or BSA in control), and 5 μl of a binding solution [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM EDTA, 5 mM DTT, 25% glycerol] on ice for 15 min. After the addition of 5 μl of a solution containing 10 mM $MgCl_2$ and 5 mM $CaCl_2$ for 1 min at 20°C, the binding solution was treated with 1 μl of 1:10 to 1:2 (1:100 to 1:20 in control with BSA) dilution DNase I stock (10 U/μl, Amersham Biosciences) for 4 min. The reaction was stopped with a solution (140 μl) containing 190 mM sodium acetate, 30 mM EDTA, 0.15% SDS, 9 μg yeast tRNA, and 2 μg proteinase K for 30 min at 42°C. DNA fragments were subsequently extracted with phenol-chloroform and precipitated with NaCl 5 M-absolute ethanol before loading onto a 6% acrylamide sequencing gel (acrylamide-bis acrylamide, 19:1). Electrophoresis was carried out at room temperature at 60 W for 1 h and the gel was transferred to 3MM paper (Whatman, Ivry sur Seine, France), dried, and exposed to Hyperfilm MP (Amersham Biosciences) with intensifying screens at −80°C overnight.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assay (EMSA) was performed as follows: 25 bp synthetic oligonucleotides (Invitrogen) corresponding to the protected region (from −676 bp to −701 bp) in DNase I footprinting experiments [footprint (FP): 5′-CTGCTCCGCCCCTTTCCCCCGGATA-3′ and FPmut: 5′-CTGCTCCGaaCCTTTCCCCCGGATA-3′; the underlined and the lowercase letters indicating the Sp site and the mutation site respectively] were annealed with their respective complementary strands at 100°C for 3 min in a solution containing 100 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 13 mM EDTA, 13 mM spermidine, and 20 mM DTT. Double-strand probes were radiolabeled with 20 μCi of [γ^{32}P]ATP (5 mCi/ml, 3,000 Ci/mmol; NEN Life) by T4 polynucleotide kinase (Promega, Charbonnières, France) at 37°C for 30 min. Radiolabeled double-strand probes (0.25 pmol) were incubated for 15 min on ice in a final volume of 20 μl in the presence of 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 3 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 5% glycerol, 2 μg poly(dI-dC), 4 mM spermidine, 1 μg BSA, and 8 μg of nuclear extracts. In experiments that required the presence of an excess unlabeled competitor (100-fold excess), the latter was added to the mixture before the addition of radiolabeled probe. When indicated, 0.8 μg of rabbit affinity-purified polyclonal antibody raised against Sp1 or Sp3 (TEBU, Le-Perray-en-Yvelines, France) was incubated for 30 min before addition of radiolabeled probe. After incubation, samples were loaded on a 6% acrylamide gel (acrylamide-bis-acrylamide, 29:1). Electrophoresis was performed at room temperature at 200 V for 3 h, and the gels were transferred onto 3MM paper (Whatman), dried, and exposed to Hyperfilm MP (Amersham Biosciences) at −20°C overnight.

Plasmid constructs

Constructs used in this study have been previously described in detail by Dachet et al. (17). Briefly, a 777 bp DNA fragment corresponding to the region from +32 to −745 of the CETP promoter from individuals homozygous for either the −629A or −629C allele was amplified by PCR and digested by *Nhe*I and *Bgl*II restriction enzymes. The digested fragments were cloned between the *Nhe*I and *Bgl*II sites of the pGL3 basic luciferase expression vector (Promega) generating the WT (A allele) and pM1 (C allele) constructs. In both constructs, one or two point mutations were introduced either in the transcription factor binding site at position −690 or in the Sp1 binding site at position −37, or both, using the GeneEditor™ in vitro Site-Directed Mutagenesis System kit (Promega) according to the manufacturer's protocol in order to generate pM2 (−690 mutated site), pM3 (−37 mutated site), or pM2M3 (containing both −690 and

−37 mutated sites) constructs that derived from WT; and pM1M2 (−690 mutated site), pM1M3 (−37 mutated site), or pM1M2M3 (containing both −690 and −37 mutated sites) constructs that derived from pM1. Oligonucleotides used to create mutations in −690 and −37 binding sites were 5′-CTGCTC-CGaaCCTTTCCCCCGGATA-3′ and 5′-ATGTTCCGTGGGGGCT-GttCGGACATACATA-3′ (18), respectively, with the lowercase letters indicating the mutation site.

pPac, pPac-Sp1, and pPac-USp3 vectors were generous gifts from Guntram Suske (Klinikum der Philipps-Universität Marburg, Marburg, Germany). pAdh-LacZ vectors were a generous gift from Christine Vesque (Inserm U368, Ecole Normale Supérieure, Paris, France).

Cell culture and transfection experiments

The human hepatocellular carcinoma cell line HepG2 and the mouse connective tissue cell line NCTC (American Type Culture Collection, Rockville, MD) were grown at 37°C in 5% CO_2 in Dulbecco's Modified Eagle's Medium containing 10% and 8% foetal calf serum, respectively (Invitrogen), 2 mM L-glutamine, and 40 μg/ml gentamycin. Cells were seeded on 6-well plates at 2.5×10^5 cells per well. After 48 h of growth, 3 μg of each CETP promoter construct was cotransfected with 0.5 μg of a β-galactosidase expression vector (pSV-βgal; Promega) using the Lipofectin Liposomal reagent (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after transfection, the medium was replaced by fresh medium and the cells were incubated for an additional period of 16 h. Cells were harvested with 150 μl of Cell Culture Lysis Reagent (Promega). The lysate was centrifuged for 10 min at 14,000 rpm in order to remove an excess of cellular fragments. Luciferase activity was measured on the supernatant using the Luciferase Assay System kit (Promega) in a 1420 VICTOR Multilabel counter (Wallac, EG and G Co.), and β-galactosidase activity was measured using the β-galactosidase Enzyme Assay System kit (Promega). Protein concentrations were determined using the bicinchoninic acid assay reagent (BCA; Pierce, Bezons, France). Transcriptional activity was expressed in relative luciferase units after normalisation for β-galactosidase activity; experiments were performed in triplicate and values correspond to the mean from five independent experiments.

SL2 cells, a *Drosophila* cell line obtained from the American Type Culture Collection, were grown at 25°C without CO_2 in Schneider's medium (Invitrogen) supplemented with 10% heat-inactivated foetal calf serum (Invitrogen) and 40 μg/ml gentamycin. Cells were seeded on 6-well plates at 2.5×10^6 cells per well. After 24 h incubation, SL2 cells were transfected by a calcium-phosphate method (32) with 2.5 μg of each CETP promoter construct, 1.5 μg of a pAdh-LacZ expression vector, and the indicated amount of pPac-Sp1 and/or pPac-USp3 expression vectors. The total amount of DNA was adjusted by the addition of pPac vector to obtain an equal quantity of DNA per well. Forty-eight hours after transfection, cells were harvested and lysates were assayed as described above. Results were expressed as *x*-fold induction relative to luciferase activities normalized for β-galactosidase activity obtained with pPac vector alone. Experiments were performed in duplicate and values correspond to the mean from at least three independent experiments.

Western blot analysis

Nuclear extracts, obtained as described above, were separated by 8% SDS-polyacrylamide gel electrophoresis and then transferred onto Hybond C-super nitrocellulose membranes (Amersham Biosciences). Membranes were blocked overnight at 4°C in 50 ml of PBS containing 0.05% Tween 20 (PBST) buffer (154 mM NaCl, 5 mM $Na_2HPO_4.12H_2O$, 5 mM $NaH_2PO_4.H_2O$, 0.3 mM EDTA, and 0.1% Tween 20) with 5% powdered milk and washed for 10 min at room temperature in 30 ml of TBST buffer. After incubation with the rabbit affinity-purified polyclonal antibody raised against Sp1 or Sp3 (final dilution 1:200) for 1 h at room temperature in 30 ml of PBST buffer with 1% powdered milk, membranes were washed three times in PBST for 10 min and incubated with mouse peroxidase-conjugated secondary anti-rabbit antibody (final dilution 1:15,000) for 30 min at room temperature. Membranes were then washed three times and bands were revealed using the enhanced chemiluminescence detection system (ECL-plus reagent, Amersham Biosciences). Quantification of Western blots was performed using a Kodak Image Station 440 CF with Kodak 1D Image Analysis Software (Perkin Elmer, Paris, France). To reprobe the blots, membranes were first washed in 30 ml of PBST buffer and stripped by shaking for 30 min at 50°C in a solution containing 62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM β-mercaptoethanol. Finally, membranes were washed, blocked, and rehybridized.

Statistical analyses

Statistical significance was determined by unpaired Student's *t*-test.

RESULTS

Identification of a new Sp1/Sp3 binding site in the CETP gene promoter

In order to identify potential binding sites for transcription factors located in the human CETP gene, we analyzed the promoter region from −550 to −745 bp upstream of the transcription start site by DNase I footprinting (**Fig. 1**). Experiments were performed using two probes (*Nhe*I*-*Sty*I and *Nhe*I-*Sty*I* radiolabeled at a different extremity as described in Experimental Procedures) in the presence of increased amounts of DNase I. As shown in Fig. 1, we identified a protected region (designated FP) between positions −677 and −702 bp with the *Nhe*I*-*Sty*I and *Nhe*I-*Sty*I* probes, suggesting the presence of transcription factor binding sites in this region.

The analysis of the FP protected region revealed a GC-box (5′-GCTCCGCCCC-3′) between positions −690 and −699 bp correspo nding to a consensus sequence for transcriptional factors of the Sp family [5′-(G/T)GGGCGGPu-PuPy-3′]. To verify whether this region binds transcriptional factors of the Sp family (Sp1/Sp3), we performed electrophoretic mobility shift assays (**Fig. 2**) using a radiolabeled synthetic probe corresponding to the FP protected sequence. Incubation of the radiolabeled FP probe with nuclear extracts from HepG2 cells resulted in the formation of three specific DNA protein complexes (FP1, FP2, and FP3; Fig. 2, lane 1). Both FP1 and FP2 complexes, but not the FP3 complex, were also obtained with a radiolabeled probe [specific protein (SP)] specific for the consensus sequence of the Sp-transcription factor family (Fig. 2, lane 8). The formation of FP1 and FP2 complexes was abolished in the presence of either an excess of nonradiolabeled FP probe (Fig. 2, lanes 2 and 10) or SP probe (Fig. 2, lanes 3 and 9), but not by an excess of a nonspecific competitor (Fig. 2, lanes 4 and 11). Finally, to determine whether Sp1 and Sp3 were involved in the formation of

the three FP1, FP2, and FP3 complexes, supershift assays were carried out using polyclonal antibodies against either Sp1 or Sp3. We observed that anti-Sp1 antibody decreased and supershifted the FP1 complex in a similar manner to both the radiolabeled FP (Fig. 2, lane 5) and SP (Fig. 2, lane 12) probes, whereas anti-Sp3 antibody slightly decreased the intensity of the band corresponding to the FP1 complex and entirely abolished the formation of the FP2 complex, forming a supershift (Fig. 2, lanes 6 and 13). The addition of both anti-Sp1 and -Sp3 antibodies with the radiolabeled FP resulted in marked reduction in the band intensities corresponding to the two FP1 and FP2 complexes (Fig. 2, lane 7). These observations indicated that the FP1 complex was formed as a result of interactions with both Sp1 and Sp3 and that the FP2 complex was formed with Sp3 alone. However, we cannot exclude the possibility that other nuclear factors are implicated in the formation of both the FP1 and FP2 complexes. In addition, incubation of the radiolabeled FPmut probe, in which we introduced two point mutations into the consensus sequence for transcriptional factors of the Sp-family, prevented formation of the three specific DNA-protein complexes FP1, FP2, and FP3 (Fig. 2, lane 14). Furthermore, the incubation of an excess of nonradiolabeled FPmut probe with the radiolabed FP probe did not affect the formation of the FP1, FP2, or FP3 complexes (data not shown).

In order to identify the nuclear factor(s) implicated in the FP3 complex, we incubated the radiolabeled FP probe in the presence of nuclear extracts from HepG2 cells with a polyclonal antibody raised either against Sp2, a member of the Sp family, or against proteins that interact with Sp1, such as YY1 (33), or that recognize the GC-box motif, such as Krüppel-like factors (GKLF, EKLF, and LKLF) (34); however, none of these experiments led to the formation of a supershift. We also examined the possibility that Egr-1 or AP-2 was involved in the formation of the FP3 complex. The incubation of the FP probe with an excess of nonradiolabeled probe specific for the consensus sequence Egr-1 did not abolish formation of the FP3 complex. In addition, the FP3 complex was not formed when nuclear extracts from HepG2 cells were substituted by AP-2 proteins. Thus, these experiments did not permit the identification of nuclear protein(s) involved in the formation of the FP3 complex.

Taken together, these results indicated that the FP region binds both Sp1 and Sp3 with an additional as yet unidentified factor X.

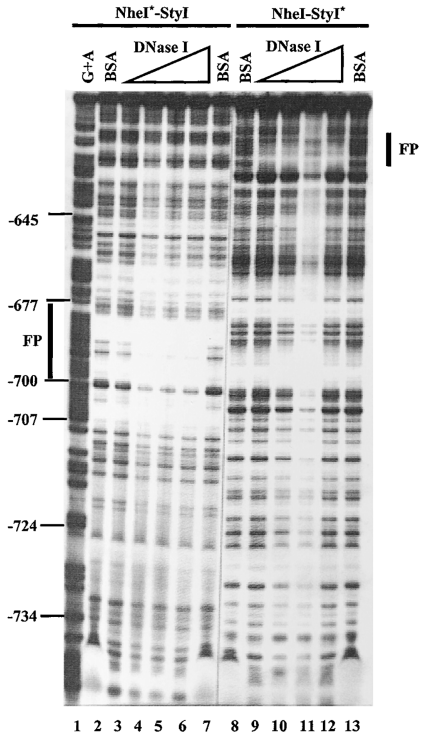

Fig. 1. DNase I footprinting analysis of the −550/−745 cholesteryl ester transfer protein (CETP) promoter region reveals a region protected by HepG2 nuclear extracts. Two radiolabeled *Nhe*I*-*Sty*I and *Nhe*I-*Sty*I* probes were incubated in the presence of HepG2 nuclear extracts [14 μg, except in slots 3 and 9 (7 μg)], or in the presence of BSA as a control, and increased amounts of DNase I [5 units (slots 3–4 and 9–10), 6 units (slots 5 and 11), and 7 units (slots 6 and 12)] for footprinting analysis as described in Experimental Procedures. The protected region (FP) is indicated by a vertical bar. G+A indicates Maxam and Gilbert sequencing reactions for the corresponding DNA fragment.

The −690 site represses the transcriptional activity of the human CETP promoter

To determine whether the −690 site is implicated in the regulation of CETP gene expression, we performed transient transfection experiments in HepG2 cells using several constructs. As shown in **Fig. 3A**, the WT construct displayed a significantly lower level of luciferase expression (−19%, $P < 0.05$) as compared with that of the pM2 construct in which we mutated the −690 Sp1/Sp3 site. This finding demonstrated that the −690 Sp1/Sp3 binding site repressed human CETP promoter activity. In a previous

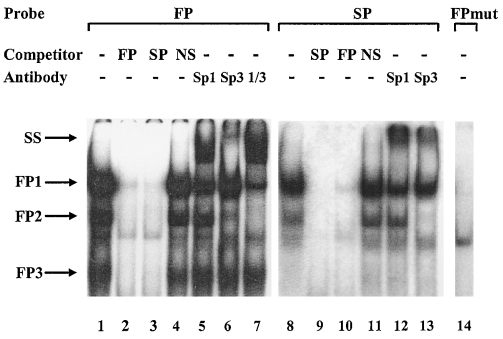

Fig. 2. The FP-protected region binds transcriptional factors Sp1 and Sp3 in the presence of nuclear extracts from HepG2 cells. Electrophoretic mobility shift assay experiments using radiolabeled FP (5′-CTGCTCCGCCCCTTTCCCCCGGATA-3′), SP (5′-ATTCGATCGGGGCGGGGCGAGC-3′), or FPmut (5′-CTGCTCCGaaCCTTTCCCCCGGATA-3′) probes with nuclear extracts from HepG2 cells (lanes 1, 8, and 14) and 100-fold excess of FP probe (lanes 2 and 10), SP probes (lanes 3 and 9), nonspecific (NS) probe (lanes 4 and 11), or 0.8 μg of antibody raised against Sp1 (lanes 5 and 12), Sp3 (lanes 6 and 13), or both (lane 7). The three DNA-protein complexes (FP1, FP2, and FP3) and supershifted bands (SS) are indicated by arrows.

study (17), an Sp1/Sp3 site (−629A) was shown to regulate the transcriptional activity of the human CETP gene promoter. It was thus of interest to analyze the influence of a possible interaction between the −690 and −629 sites on the regulation of the transcriptional activity of the human CETP gene promoter. Use of WT, pM2 (−629A allele) and pM1, pM1M2 constructs (−629C allele) revealed that each Sp1/Sp3 site (pM1 or pM2) significantly decreased luciferase activity (22–23%, $P < 0.05$) as compared with a construct containing no Sp1/Sp3 site (pM1M2) (Fig. 3A). The presence of both Sp1/Sp3 sites (WT) resulted in significant repression of luciferase activity (−42%, $P < 0.0005$). We conclude that the −690 Sp1/Sp3 site induces repression of CETP promoter activity comparable to the −629 Sp1/Sp3 site, and that the two sites exert an additive repressive effect in HepG2 cells.

The −690 site-mediated repression of CETP promoter activity is modulated in a cell-specific manner

The repressive effect observed for the −690 Sp1/Sp3 site was obtained in HepG2 cells, a cell line that expresses CETP protein (35, 36). In order to determine whether the −690 site might regulate CETP gene expression in a tissue-specific manner, we evaluated the role of this Sp1/Sp3 site in murine fibroblasts that are known to lack CETP expression (18). Transient transfection experiments were performed in NCTC cells, a murine connective tissue cell line, using the constructs described above (Fig. 3B). In this cell line, the WT and pM1 constructs displayed significantly lower luciferase expression (−45%, $P < 0.05$) as compared with the pM2 and pM1M2 constructs, respectively. In addition, luciferase activity was equivalent between either the WT and pM1 or the pM2 and pM1M2 constructs. Thus, in NCTC cells, we observed that the −690 site strongly repressed CETP expression, whereas the −629 site had no effect, indicating therefore that the −690 and −629 sites are not equivalent.

In electrophoretic mobility shift assays using the radiolabeled FP probe, the three specific DNA-protein complexes FP1, FP2, and FP3, previously obtained with nuclear extracts from HepG2 cells, were also formed with nuclear extracts from NCTC cells (data not shown). However, the lower intensity of the FP2 band obtained in NCTC cells led us to propose that the concentration of Sp3 was lower in this cell line than in HepG2 cells.

To confirm this hypothesis, we carried out Western blot experiments using nuclear extracts from both HepG2 and NCTC cells (**Fig. 4**). The abundance of Sp1 was similar in both cell lines (Fig. 4A), whereas the three Sp3 isoforms were 16-fold less abundant in NCTC cells than in HepG2 cells (Fig. 4B). These data indicated that the Sp1/Sp3 ratio is 16-fold higher in NCTC cells than in HepG2 cells.

Sp1 and Sp3 regulate CETP promoter activity through both the −690 and −37 sites

To define the respective role of Sp1 and Sp3 in the regulation of human CETP promoter activity, we used the SL2 cell line, which is devoid of many ubiquitous mammalian transcription factors, such as those of the Sp family (37). Cells were cotransfected with either a pPac-Sp1 or a pPac-USp3 expression vector and a set of constructs described in Experimental Procedures. Briefly, WT and pM1M2 constructs, and three additional constructs (pM1M3, pM2M3, and pM1M2M3) in which the proximal Sp1 site at position −37 was mutated, were used.

As shown in **Fig. 5**, cotransfection of the WT construct, which contains the three intact Sp sites, with either a pPac-Sp1 or a pPac-Usp3 expression vector led to a significant induction of luciferase expression (20-fold, $P < 0.05$ and 4.3-fold, $P < 0.0005$, respectively, versus pPac vector). In addition, the presence of either the proximal −37 or the −690 site (pM1M2 or pM1M3, respectively) alone was responsible for a significant 9-fold induction of luciferase expression in response to Sp1 ($P < 0.0005$ and $P < 0.005$

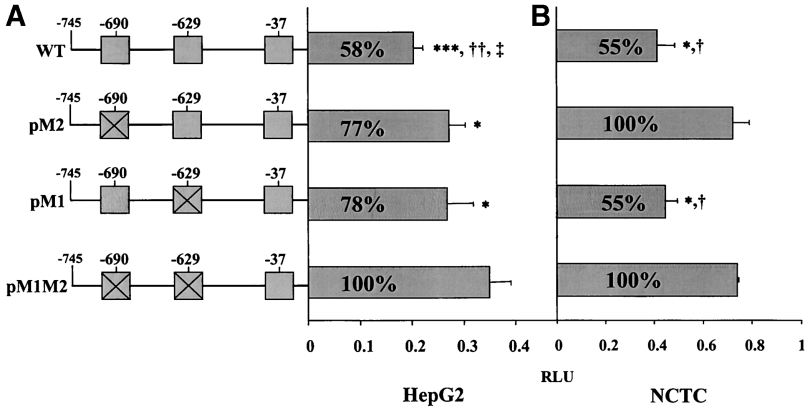

Fig. 3. The −690 site represses CETP promoter activity in a cell-specific manner. HepG2 (A) and NCTC cells (B) were transiently transfected with constructs containing 777 bp of the CETP gene promoter with either the A allele (WT) or the C allele (pM1) at position −629. Two point mutations were introduced in both WT and pM1 constructs in the Sp1/Sp3 binding site located at position −690, thereby generating the pM2 and pM1M2 constructs, respectively. Luciferase activity is expressed in relative luciferase units (RLUs) after standardisation for β-galactosidase activity. Experiments were performed in triplicate and values correspond to the mean ± SD from five independent experiments. *$P < 0.05$, ***$P < 0.0005$ versus pM1M2; †$P < 0.05$, ††$P < 0.005$ versus pM2; ‡$P < 0.05$ versus pM1.

versus pPac vector, respectively). To a lesser degree, Sp3 equally induced significant activation of both the pM1M2 and pM1M3 constructs (2.2-fold and 1.5-fold, respectively, $P < 0.05$ versus pPac vector). However, when the −629 site alone was intact (pM2M3), no effect on luciferase activity was found upon addition of Sp1 or Sp3. These results indicate that Sp1 and Sp3 act as activators at both the −690 and −37 sites, whereas they induce no effect at the −629 site.

Impact of the Sp1/Sp3 ratio on Sp1/Sp3 promoter sites

To analyze the impact of the Sp1/Sp3 ratio on human CETP gene promoter activity, we performed transient cotransfection experiments in SL2 cells with 1,000 ng of pPac-Sp1 alone or in combination with 2,000 ng of pPac-USp3 (**Fig. 6**). Cotransfection with both Sp1 and Sp3 did not significantly modify luciferase activity in either the WT or the pM1M3 (active −690 site) constructs, whereas the transcriptional activity of the pM1M2 (active −37 site) construct was significantly increased (+12%, $P < 0.005$), and that of the pM2M3 (active −629 site) construct was significantly decreased (−37%, $P < 0.0005$) as compared with cotransfection with the pPac-Sp1 vector alone. Thus, we conclude that in addition to Sp1, high Sp3 expression acts as either a super-activator of Sp1-mediated activation at the −37 proximal site or as a repressor at the −629 site; by contrast, Sp3 does not influence Sp1-mediated activation at the −690 site. These results confirm that the combined effect of both Sp1 and Sp3, and subsequently of the Sp1/Sp3 ratio, is distinct at each site.

DISCUSSION

Our present studies demonstrate that the Sp1 and Sp3 transcription factors regulate human CETP promoter activity through three specific Sp1/Sp3 binding sites (−690, −629, and −37) in a distinct manner. In addition, the nuclear Sp1/Sp3 ratio, as well as the interaction of Sp1 and Sp3 with other nuclear proteins, is intimately implicated in the relative contribution of these Sp1/Sp3 sites to CETP promoter activity.

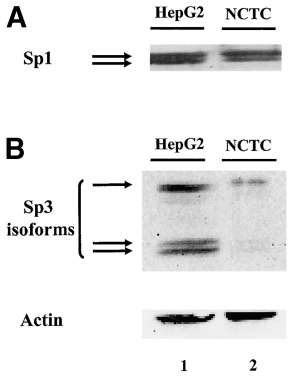

Fig. 4. Sp3 is 16-fold less abundant in NCTC cells than in HepG2 cells. Western blot analysis. Twenty-five micrograms of nuclear extracts from HepG2 and NCTC cells were loaded onto a 8% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, probed with a rabbit antibody against Sp1 (A) or Sp3 (B), and visualized using ECL.

Although Sp1 and Sp3 have similar domain structures, their action depends on promoter structure and cellular environment (38), as well as their potential interaction with other transcription factors (33, 39–41); therefore Sp1 and Sp3 can differ in their capacity to regulate transcription. Indeed, Sp1 acts mainly as an activator (42–46), whereas Sp3 can act either as an activator (44, 47–49) or as a repressor (50). A number of gene promoters have already been described to be differentially regulated by several Sp1 sites. Among them, the human transcobalamin II promoter is positively regulated by a distal Sp1 site and negatively by a proximal Sp1 site (30). The β_5 integrin gene (29) and the human glucagon-like peptide-1 receptor gene (51) are regulated by one repressive and two activating Sp1 sites. Two Sp1 sites have been previously identified in the human CETP promoter at positions −37 (18) and −629 (17). Here, we identified a new repressive binding site for Sp1 and Sp3 transcription factors located at position −690 in the human CETP gene promoter. The human CETP promoter is therefore regulated negatively by two distal repressive Sp1 sites (−690 and −629 sites) and positively by a proximal Sp1 site (−37 site). Clearly, transcriptional regulation of the promoter of the human CETP gene by the Sp1 and Sp3 transcription factors is mechanistically complex.

We demonstrate that Sp1 and Sp3 give rise to activation of CETP promoter activity in SL2 insect cells (Fig. 5). These findings are consistent with earlier studies that observed that Sp1 and Sp3 are required for activation of gene expression (44, 47, 48). We clearly confirmed the positive role of Sp1 at the −37 site observed by Gaudet et al. (18). Indeed, in SL2 cells, Sp1 and Sp3 act as activators at this site. Moreover, the combined action of both Sp1 and Sp3 led to super activation of expression, therefore providing evidence that Sp3 also binds the GC-box located at position −37.

The action of both the −690 and −629 sites appears to be more complex in modulating CETP promoter activity, because they behave distinctly in different cell types. Indeed, these two sites exert a comparable level of repression of promoter activity in HepG2 cells, whereas the −690 site induces a more pronounced repressive effect and the −629 site had no effect in NCTC cells (Fig. 3). These results suggest that the effect of the −690 and −629 Sp1 sites on CETP promoter activity may be modulated in a cell-specific manner. Campos-Caro et al. (27) have reported a comparable mechanism in regulation of the activity of the neuronal nicotinic receptor (nAChR) α5 subunit gene promoter. This promoter contains five GC boxes, all contributing to transcriptional activity in chromaffin cells. However, only the proximal GC box is implicated in the regulation of the neuronal nAChR α5 subunit gene promoter in SHSY-5Y neuroblastoma cells, whereas the other GC boxes are without effect. The authors suggest that variability in cellular Sp1 levels and/or

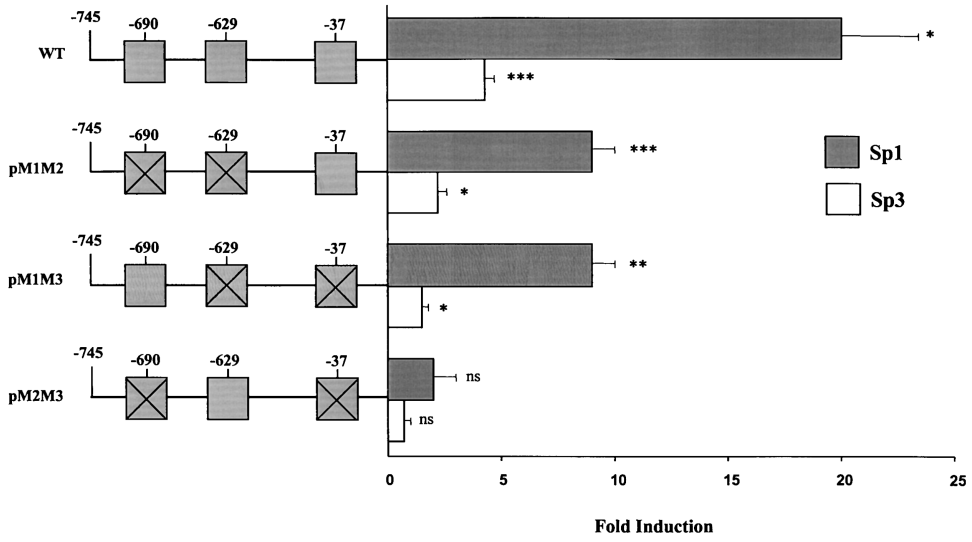

Fig. 5. Sp1 and Sp3 activate CETP promoter activity through both the −690 and −37 Sp1/Sp3 binding sites. Two and a half micrograms of each CETP promoter construct was transiently cotransfected with 2 μg of either a pPac-Sp1 (dark box) or a pPac-USp3 (light box) expression vector in SL2 cells. Results were expressed as *x*-fold induction relative to luciferase activities normalized for β-galactosidase activity obtained with pPac vector alone. The marginal increment of promoter activity of the pM1M2M3 construct (mutated in all of the three GC boxes) in response to Sp1 and Sp3 was subtracted from the promoter activity of the other constructs. Experiments were performed in duplicate and values correspond to the mean ± SEM from three independent experiments. Ns, non significant. $*P < 0.05$, $**P < 0.005$, $***P < 0.0005$ versus cotransfection with pPac vector.

competition of Sp1 with other members of the Sp-family for binding to the same elements may explain such tissue-specific regulation. Indeed, the abundance of Sp1 and Sp3 varies among different cell types (52–54), and variation in the Sp1/Sp3 ratio modulates gene activity, because these transcription factors compete for the same binding sites with an identical affinity (50, 53). Hata et al. (55) showed that the 10-fold higher Sp1/Sp3 ratio observed in

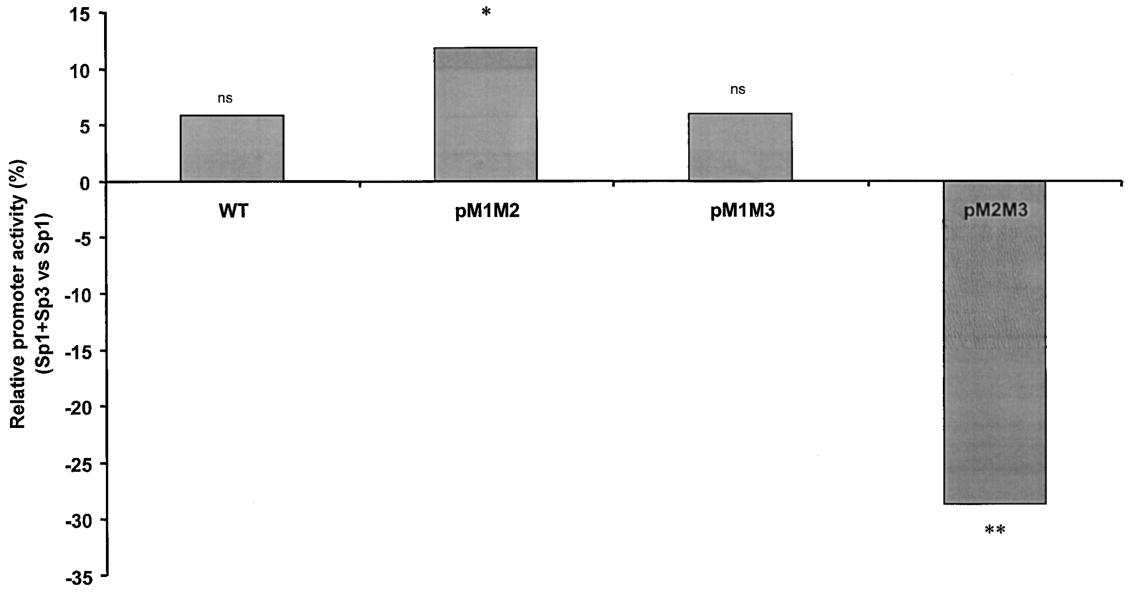

Fig. 6. Sp1/Sp3 ratio has a distinct effect on each Sp1/Sp3 binding site. Two and a half micrograms of each CETP promoter construct was transiently cotransfected with 1 μg of pPac-Sp1 alone or in combination with 2 μg of pPac-USp3 expression vector in SL2 cells. The bar graph shows the difference of promoter activity obtained with Sp1 and Sp3 together versus Sp1 alone. Experiments were performed in duplicate and values correspond to the mean ± SEM from four independent experiments. ns, non significant. $*P < 0.05$, $**P < 0.005$ versus cotransfection with pPac-Sp1 vector.

endothelial cells as compared with nonendothelial cells was responsible for the elevated expression of the kinase domain receptor promoter in the former, as Sp3 attenuated the Sp1-mediated activation of promoter activity. These studies lead us to suggest that the lesser abundance of Sp3 that we observed in NCTC cells as compared with HepG2 cells may be responsible for the distinct effect at both the −690 and −629 sites in those two cell lines. Based upon our present results, we propose a mechanism by which cellular Sp3 level may modulate the action of the two distal Sp1 sites on CETP promoter activity (**Fig. 7**). The combined effect of both Sp3 and Sp1 induced significant repression at the −629 site (pM2M3 construct, Fig. 6), whereas Sp1 or Sp3 alone did not exert a significant effect at this site (Fig. 5). Such repression involving Sp3 in SL2 cells is surprising since Majello et al. (38) reported that Sp3 cannot act as a repressor on this cellular background. It is for this reason that the repression of the Sp1-mediated activation by Sp3, resulting in competition with Sp1 for their common binding site, is frequently observed in SL2 cells (50); however, overexpression of Sp3 in NCTC cells confirmed that Sp3 exerts a repressive effect at the −629 site (data not shown). Thus, in HepG2 cells that display high Sp3 levels, Sp3 acts as a repressor at the −629 site. On the other hand, the abundance of Sp3 may not be sufficient in NCTC cells to repress CETP promoter activity at the −629 site, and may account for the absence of effect at this site.

The mechanism involved in the functionality of the −690 site seems to be distinct from that at the −629 site. The fact that on the one hand, Sp1 and Sp3 act as activators at the −690 site (pM1M3 construct, Fig. 5), and that on the other, Sp3 did not affect Sp1-mediated activation at this site (Fig. 6), suggests that another factor is responsible for the repressive effect observed at the −690 site. We speculate that the nonidentified nuclear factor(s) X involved in the formation of the FP3 complex in EMSA experiments performed with nuclear extracts from HepG2 and NCTC cells, but not from SL2 cells (data not shown), may explain this repression. The cellular abundance of Sp3 might influence the repressive effect mediated by this nuclear protein, probably by affecting the binding or the action of factor X at the −690 site. Thus, in HepG2 cells, the abundance of Sp3 permits only a weak repression (−22%) at the −690 site by factor X, whereas the lower level of Sp3 observed in NCTC cells allows a greater degree of repression (−45%) at this site. The hypothesis that an additional protein distinct from Sp1 and Sp3 might be responsible for the repression observed at the −690 site is consistent with previous studies that report that several other Sp1-like proteins, such as TIEG2 (56), BTEB3 (57), or members of the Krüppel-like factor family (58, 59), repress promoter activity through Sp1 motifs.

Our data, therefore, strongly suggest that the effect of both the −690 and −629 Sp1/Sp3 sites on CETP promoter activity might depend on the competitive binding of Sp1 and Sp3 as well as on the synergistic interactions of these two transcription factors, or on their interactions with other factors at each of these sites. However, as illustrated in our model, we cannot exclude the possibility that other mechanisms may modulate the contribution of these two Sp1/Sp3 binding sites to regulation of CETP promoter activity.

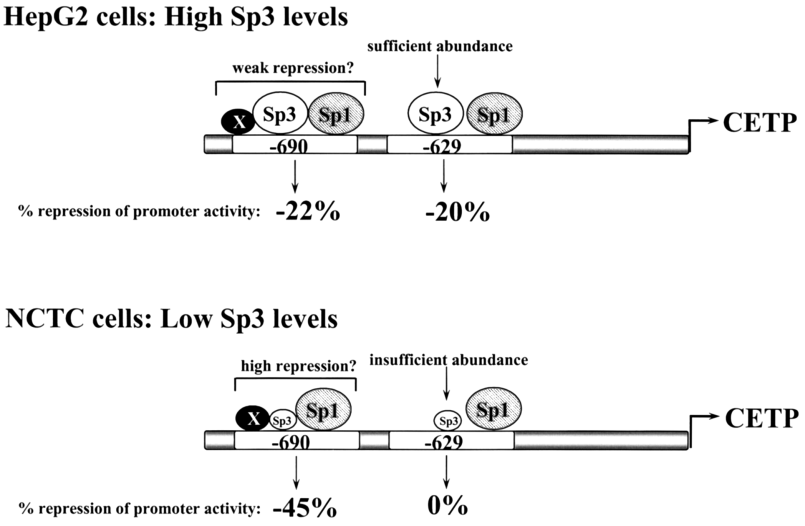

Fig. 7. Sp3 levels may modulate the repression of human CETP promoter activity observed through both the −690 and −629 sites. Both Sp1 and Sp3 are bound at the two −629 (17) and −690 sites located on the human CETP promoter, whereas factor X is only present at the −690 site. In HepG2 cells, the high abundance of Sp3 *i*) is sufficient to repress CETP promoter activity at the −629 site (−20%) because Sp3 acts as a repressor at this site when Sp1 is present, and *ii*) permits only a weak repression (−22%) by factor X at the −690 site. By contrast, the low level of Sp3 in NCTC cells is not sufficient to repress CETP promoter activity at the −629 site, whereas it allows a marked repression (−45%) at the −690 site. This model does not, however, account for total transcriptional promoter activity, because it does not include the contribution of the proximal −37 site.

In conclusion, we demonstrate that both the Sp1 and Sp3 transcription factors are required for basal expression of the human CETP gene and that they exert a dichotomous effect on promoter activity. Indeed, the binding of Sp1 and Sp3 leads to transcriptional repression at the two distal −690 and −629 sites, whereas activation occurs at the proximal −37 site, thereby illustrating the complex role of Sp1 and Sp3 in the transcription mechanism. Finally, it is of considerable interest that modulation of the transcriptional activity of the Sp1 or Sp3 factors by phosphorylation (60), acetylation (61), or inflammatory cytokines such as TNFα (62), or by the action of nitric oxide on such redox-sensitive proteins (63), may potentially result in regulation of CETP gene expression; it can be envisaged that such modulation of gene expression will be reflected in plasma CETP mass and/or activity, and thence, in HDL phenotype.

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