A CYP7A promoter binding factor site and *Alu* repeat in the distal promoter region are implicated in regulation of human CETP gene expression

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Abstract The cholesteryl ester transfer protein (CETP) plays a key role in reverse cholesterol transport in mediating the transfer of cholesteryl ester from HDL to atherogenic apolipoprotein B-containing lipoproteins (VLDL, IDL, and LDL). Variation in plasma CETP mass in both normolipidemic and dyslipidemic individuals may reflect differences in CETP gene expression. As the 5′ flanking sequence up to 3.4 kb of the human CETP gene contributes to transcriptional activity and tissue-specific gene expression, we evaluated the role of the distal promoter region in the modulation of CETP gene expression. In transfection experiments in HepG2 cells, we presently demonstrate that an Alu repeat (−2,153/−2,414) acts as a repressive element, whereas a binding site for the orphan nuclear receptor CYP7A promoter binding factor (CPF), at position −1,042, facilitates activation of human CETP promoter activity. Cotransfection of liver receptor homolog, the mouse homologue of CPF in HEK293 cells that lack CPF, indicated that the −1,042 CPF site is sufficient to induce CPF-mediated activation of CETP promoter activity. Taken together, our results indicate that the distal-promoter region is a major component in the modulation of human CETP promoter activity, and that it may contribute to the liver-specific expression of the CETP gene.—Le Goff, W., M. Guerin, M. J. Chapman, and J. Thillet. **A CYP7A promoter binding factor site and *Alu* repeat in the distal promoter region are implicated in regulation of human CETP gene expression. *J. Lipid Res.* 2003.** 44: **902–910.**

Supplementary key words cholesteryl esters • cholesteryl ester transfer protein • liver receptor homolog-1

In mediating the intravascular transfer of cholesteryl esters (CEs) from atheroprotective HDL to atherogenic apolipoprotein B-containing lipoproteins (VLDL, VLDL remnants, IDL, and LDL), the CE transfer protein (CETP) plays a key role in reverse cholesterol transport (1). The relevance of CETP to atherosclerosis risk remains controversial. Indeed, human CETP deficiency arising from mutations in the CETP gene is frequently associated with high HDL-C levels (2, 3), but potentially with elevated cardiovascular risk (4, 5). By contrast, transgenic mice overexpressing CETP tend to display reduction in HDL-C levels (6, 7) and to develop premature atherosclerosis (8).

In humans, CETP is expressed in a wide variety of tissues, among which the liver and adipose tissue are major sources (9, 10). Significant variation in both plasma CETP mass and activity has been demonstrated, however, in both normolipidemic individuals and in dyslipidemic subjects (11), possibly reflecting differences in CETP gene expression.

The human CETP gene contains about 25 kb and is composed of 16 exons (9, 12). The promoter region displays several regulatory elements implicated in control of its transcriptional activity (13–19). Among the *trans*-acting factors that contribute to regulation of human CETP promoter activity, nuclear receptors appear to play a key role. Indeed, induction of CETP gene transcription by dietary cholesterol (20, 21) was recently shown to require the liver X receptor (LXR) and involves its binding to a proximal direct repeat of a nuclear receptor site separated by 4 nucleotides (DR4 element, −384 to −399) (18). In addition, the liver receptor homolog-1 (LRH-1) was found to transactivate the human CETP promoter and potentiate sterol-mediated induction by LXR (16). LRH-1 is the mouse homolog of the human CYP7A promoter binding factor (CPF), and these two nuclear factors belong to the orphan nuclear receptor fushi tarazu Fi family (Ftz-F1) from *Drosophila*.

The proximal region of the human CETP promoter, up to position −629, has been extensively analyzed. Several studies indicate, however, that the distal promoter region

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equally contributes to the transcriptional activity of the human CETP gene (16, 19, 22, 23). Indeed, Oliveira et al. (23) reported that the human CETP promoter region upstream of position −570 contains positive elements determining the level of gene expression in the liver and spleen of transgenic mice expressing the human CETP gene. In addition, the expression of LXR-RXR in HEK293 cells led to a more robust sterol induction with the 3.4 kb promoter than that seen with the −570 bp construct (16), accompanied by a further increase of this induction by LRH. These data strongly suggest the presence of sterol-responsive element(s) upstream of position −570. Finally, whereas the proximal −138 bp construct in vitro induced maximal CETP promoter activity, it has been clearly demonstrated that extension of the promoter up to –4.7 kb results in marked repression of promoter activity (19), thereby indicating that repressive regulatory elements are present in the distal promoter region.

We presently evaluated the contribution of the distal region of the human CETP gene promoter to transcriptional activity and searched for potential regulatory elements. Two regulating regions were identified that acted either as an activator (−1,012/−1,398) or a repressor (−2,146/−2,680) of CETP promoter activity. This latter region is composed of numerous repeat elements, i.e., Sine and Line (Short and Long interspersed sequence). Here we identified a full-length *Alu* repeat sequence (−2,153/−2,414) as the repressive element and a functional −1,042 CPF site in the enhancer region that may be involved in liver-specific expression of the CETP gene.

EXPERIMENTAL PROCEDURES

Plasmid constructs

A plasmid containing a 3,488 bp fragment [nt −3,459/+29, relative to the transcription start site (23)] of the human CETP promoter was generously provided by Bayer Pharma (Germany). A 3,101 bp *Sac*I-*Xba*I fragment (nt −3,238/−137) of the human CETP promoter was cut out from this plasmid and cloned directionally into the previously described (24) *Sac*I-*Xba*I digested −971GG construct, which contains a 1,077 bp fragment of the human CETP promoter, to generate the p3242 construct. Deletions of this 3.2 kb promoter segment were performed by digestion between the *Sac*I restriction site at the 5′ end and the *Pml*I, *Apa*I, *Bsr*GI, *Tth111*I, and *Eco47*III restriction sites at the 3′ end in order to generate constructs that contain a 1,012, 1,398, 1,707, 2,146, and 2,680 bp fragment of the human CETP promoter (p1012, p1398, p1707, p2146, and p2680, respectively) after blunt-ending and ligation.

Constructs p138-*Alu* and p138-Rep were obtained as follows: 282 bp and 544 bp fragments were amplified by PCR from the p3242 construct and subcloned into a pGEM®-T vector (Promega, Charbonnières, France). The sequence of the downstream primer was 5′-ACAGAGGCTAGCTCTGTCG-3′; the sequences of the upstream primers were 5′-GACATAGGTACCGCATGGT-3′ and 5′-CCGAAGAGCTCAAATACTGG-3′, with the underlined letters indicating the restriction site. These primers contain sequences for the generation of a *Nhe*I restriction site in the downstream primer and *Kpn*I and *Sac*I restriction sites in the upstream primers, respectively. Subsequently, *Nhe*I-*Kpn*I 282 bp and *Nhe*I-*Sac*I 544 bp fragments were cut out from the pGEM®-T vectors and directionally cloned into the earlier described p138 constructs that contain a 138 bp fragment of the human CETP promoter (15), previously digested by *Nhe*I-*Kpn*I and *Nhe*I-*Xba*I, to generate the p138-*Alu* and p138-Rep constructs.

For mutational analysis, one or two point mutations were introduced either at the −1,042 site or at the −74 LRH site, or both, using the GeneEditor™ in vitro Site-Directed Mutagenesis System kit (Promega) according to the manufacturer's protocol in order to generate p1398M1 (−1,042 mutated site), p1398M2 (−74 mutated site), and p1398M1M2 (both −1,042 and −74 mutated sites). Oligonucleotides used to create mutations in the −1,042 and −74 binding sites were 5′-TGTCTCTGAGC-CaaGGGAAACAGT-3′ and 5′-GGCCAGGAAGAgCaTGCTGC-CCGG-3′ (16), respectively, with the lowercase letters indicating the mutation site.

The integrity of inserts and the presence of the mutations were verified by sequencing using the DNA Big Dye™ Terminator Cycle Sequencing kit (ABI Prism, Applera, France) and a Perkin Elmer 377 DNA sequencer.

The pCMX-mLRH1 expression vector was a generous gift from Dr. Kristina Schoonjans (UMR-CNRS 7034, Université Louis Pasteur de Strasbourg, Illkirch, France).

Cell culture and transfection experiments

The human hepatocellular carcinoma cell line HepG2 and the transformed human embryonic kidney cell line HEK293 (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% fetal calf serum, respectively (Invitrogen, Cergy Pontoise, France), and 2 mM L-glutamine and 40 μg/ml gentamycin. Cells were seeded on 6-well plates at 3×10^5 cells per well. After 48 h 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. In cotransfection experiments, HEK293 cells were transfected with 1 μg of each CETP promoter construct, 30 ng of pCMV-Sport-βgal (Invitrogen), and 0.5 μg of pCMX-mLRH1 expression vector. 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 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 (RLUs) or as *x*-fold induction after normalization for β-galactosidase activity. Experiments were performed in triplicate (duplicate in cotransfection experiments), and values correspond to the mean from at least three independent experiments.

Electrophoretic mobility shift assays

HepG2 nuclear extracts were prepared from confluent 150 mm dishes as previously described by Dignam et al. (25), and stored at −80°C before use. The protein concentration of nuclear extracts was determined using the bicinchoninic acid assay reagent (BCA:Pierce). The translated LRH-1 protein was obtained using the in vitro TNT Quick-Coupled Transcription/Translation System (Promega). The electrophoretic mobility shift assay (EMSA) was performed as follows: 24 bp synthetic oligonucleotides (Invit-

rogen) (CPF, 5′-TGTCTCTGAGCCTTGGGAAACAGT-3′ and CPFmut, 5′-TGTCTCTGAGCCaaGGGAAACAGT-3′; the underlined and the lowercase letters indicating the CPF site and the mutation site, respectively) were annealed with their respective complementary strand 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 dithiothreitol (DTT). Double-strand probes were radiolabeled with 20 μCi of [γ^{32}P]ATP (5 mCi/ml, 3,000 Ci/mmol; NEN Life, Paris, France) by T4 polynucleotide kinase (Promega) 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 or 10 μl of in vitro translated LRH-1 lysate. In experiments that required the presence of an excess of unlabeled competitor (100-fold excess), the latter was added to the mixture before the addition of radiolabeled probe. After incubation, samples were loaded onto 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, Ivry sur seine, France), dried, and exposed to Hyperfilm MP (Amersham Biosciences, Saclay, France) at −20°C overnight.

RESULTS

The distal promoter region of the human CETP gene contains sequences that contribute to promoter activity

In order to determine whether the distal region of the human CETP promoter contributes to control of promoter activity, a set of constructs containing fragments of 1 kb, 1.4 kb, 1.7 kb, 2.1 kb, 2.7, and 3.2 kb of the human CETP promoter (p1012, p1398, p1707, p2146, p2680, and p3242, respectively) were made, and their transcriptional activities were compared in transient transfection experiments in HepG2 cells. As shown in **Fig. 1**, the p1398 construct displayed a 2-fold higher level of luciferase expression (+50%, $P < 0.05$) as compared with that of the p1012 construct. In addition, the promoter activity of both the p2680 and p3242 constructs was significantly lower than that of the p2146 construct (−26% and −21%, respectively, $P < 0.05$). There was no significant difference in luciferase activity, however, between both the p2146 and the p1707 constructs as compared with the 1,398 construct. Therefore, our results demonstrate that the promoter region from −1,012 to −1,398 induces marked activation of CETP promoter activity whereas that from −2,146 to 2,680 is responsible for repression.

Analysis of the human CETP promoter sequence from −1,012 to −3,242 using the Repeat Masker program, which screens DNA sequence against a library of repetitive elements, revealed that this promoter region contains multiple sequences of type Sine and Line as illustrated in **Fig. 2**. Thus, three *L1* sequences (305 bp, −1,635/−1,941; 66 bp, −2,491/−2,424; 87 bp, −2,908/−2,996), two previously described *Alu* sequences (290 bp, −2,129/−2,420; 250 bp, −2,996/−3,242) (22), and one *Mir* sequence (107 bp, −2,549/−2,657) were identified on the human CETP promoter and accounted for 46% of the promoter sequence from −1,012 to −3,242. Interestingly, the presently identified repressive region (−2,146/−2,680) is composed of a full-length *Alu* sequence and a fragment of both *Mir* and *L1* sequences that represent almost the totality of this region.

An *Alu* sequence is responsible for the repressive action of the −2,146/−2,680 promoter region

The *Alu* repetitive sequence is the most abundant of the human Sine and represents about 3–6% of the human genome, being present in about 500,000 to 1 million copies. Previous studies reported that interspersed sequences such as *Alu* repeats may be involved in regulation of the transcriptional activity of human genes (26–30). It was thus of interest to study the potential implication of the *Alu* sequence (−2,129/−2,420) in the repression exerted by the −2,146/−2,698 promoter region. To this end, we made two constructs containing either the complete repressive promoter region (−2,153/−2,680) or the *Alu* repeat (−2,153/−2,414) upstream to the proximal 138 bp (relative to the translation start site) of the human CETP promoter (p138-Rep and p138-*Alu*, respectively). Indeed, an earlier study demonstrated that this 138 bp fragment is sufficient to promote maximal activity of the CETP promoter (19). In transient transfections in HepG2 cells, the presence of the *Alu* sequence (p138-*Alu*) led to significant repression (−36%, $P < 0.05$; **Fig. 3**) of luciferase activity as compared with the construct containing only the proximal 138 bp promoter fragment (p138). Such repression is similar to that observed with the full-length repressive region (p138-Rep; −41%, $P < 0.05$; Fig. 3). This result confirmed that the region from −2,146 bp to −2,698 bp induces repression of CETP promoter activity and indicated that the *Alu* sequence −2,146/−2,414 accounts for this effect.

The 10 bp insertion in the −2,153/−2,414 bp *Alu/Sx* repeat of the human CETP promoter facilitates specific binding of nuclear factors

The −2,146/−2,414 *Alu* sequence belongs to the class II early *Alu* subfamily (type Sx) (31), which represents the majority of *Alu* repeats. The fact that this −2,146/−2,414 *Alu*/Sx element represses the promoter activity of the human CETP gene led us to suggest that this specific *Alu*/Sx sequence facilitates the binding of specific transcription factors that might be responsible for the repression of CETP promoter activity. As illustrated in **Fig. 4**, the comparison of the −2,153/−2,414 bp *Alu*/Sx with the human *Alu/Sx* consensus sequence (32) indicated that the two sequences were not identical. Interestingly, a 10 bp insertion was observed in the hormone-responsive element (HRE)-rich sequence generally present in such *AluSx* repeat elements (27). To determine whether such nucleotide insertion permits the binding of nuclear factors, we carried out EMSAs (**Fig. 5**) using two radiolabeled probes, *AluSx* and *Alu*-CETP (−2,357/−2,381), which differ only by the insertion of those 10 bp. In the presence of nuclear extracts of HepG2 cells, we observed

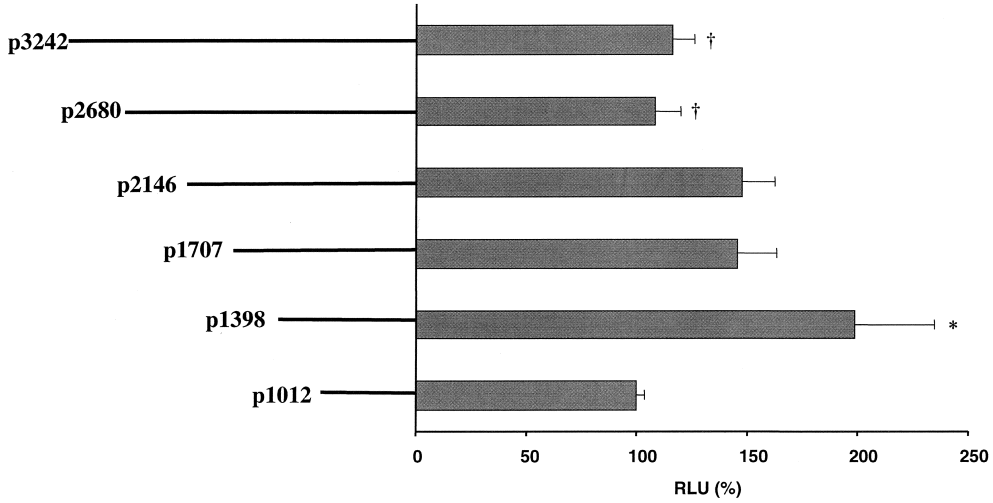

Fig. 1. The distal promoter region of the human cholesteryl ester transfer protein (CETP) gene contains transcriptional regulatory elements. HepG2 cells were transiently transfected with a set of constructs containing the luciferase reporter gene driven by a fragment of 1 kb, 1.4 kb, 1.7 kb, 2.1 kb, 2.7, and 3.2 kb of the human CETP promoter (p1012, p1398, p1707, p2146, p2680, and p3242, respectively). Luciferase activity is expressed as a percentage of relative luciferase activity (RLU) of the p1012 construct after standardization for β-galactosidase activity. Experiments were performed in triplicate, and values correspond to the mean ± SEM from four independent experiments. * $P < 0.05$ versus p1012; † $P < 0.05$ versus p2146.

that the *AluSx* probe, corresponding to the 50/64 bp region of the human *Alu/Sx* consensus sequence, did not lead to the formation of a DNA-complex (Fig. 5, lanes 1 to 4). By contrast, two specific DNA complexes (Fig. 5, Complex I and Complex II, lane 5) were observed with the *Alu*-CETP probe. The specificity of Complexes I and II was confirmed by the fact that an excess of nonradiolabeled probe for *Alu*-CETP (Fig. 5, lane 7), but not for *AluSx* (Fig. 5, lane 6) or a nonspecific competitor (Fig. 5, lane 8), abolished their formation. In conclusion, the 10 bp insertion in the −2,153/−2,414 bp *Alu/Sx* repeat of the human CETP promoter permits the specific binding of nuclear factors that might be implicated in the specific repressive action of this element.

Identification of a CPF binding site in the −1,012/−1,398 activating promoter region

Analysis of the sequence of the −1,012/−1,398 enhancer promoter region allowed the identification of a motif corresponding to the consensus DNA recognition sequence for the CPF (33) between positions −1,042 and −1,050 bp (5′-GAGCCTTGG-3′). CPF is the human homolog of the orphan nuclear receptor Ftz-F1 from *Drosophila* and of LRH-1 from the mouse. To verify whether this region binds the transcription factor CPF, we performed EMSAs (**Fig. 6**) using a radiolabeled synthetic probe (CPF) spanning the sequence of the CETP gene promoter from −1,034 to −1,058 bp. Incubation of the radiolabeled CPF probe with in vitro translated LRH-1 protein resulted in the formation of a specific DNA protein complex (Fig. 6, Complex I, lane 2) that is not observed with the control lysate (Fig. 6, lane 1). The Complex I is also obtained with the radiolabeled CL1 probe (33) corresponding to the CPF binding site of the cholesterol 7α-hydroxylase promoter gene (Fig. 6, lane 9), but not with the radiolabeled CPFmut probe (Fig. 6, lane 7), in which we introduced two point mutations known to abolish the binding of CPF (34). In addition, the formation of the Complex I is abolished in the presence of either an excess

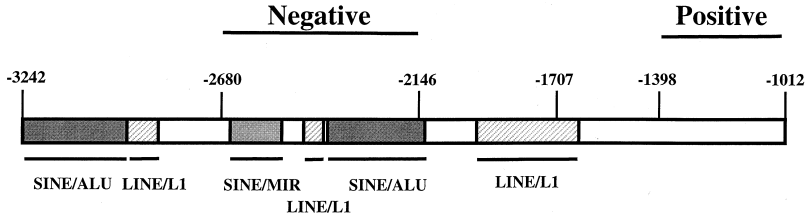

Fig. 2. The distal promoter region of the human CETP gene is composed of 46% of repeat elements of type Short and Long interspersed nucleotide element (Sine and Line). The human CETP promoter sequence from −1,012 bp to −3,242 bp was analyzed using the Repeat Masker program (http://ftp.genome.washington.edu/RM/RepeatMasker.html) that screens DNA sequence against a library of repetitive elements. Sine and Line for *Alu*, *L1*, and *Mir* repeats are underlined and shaded.

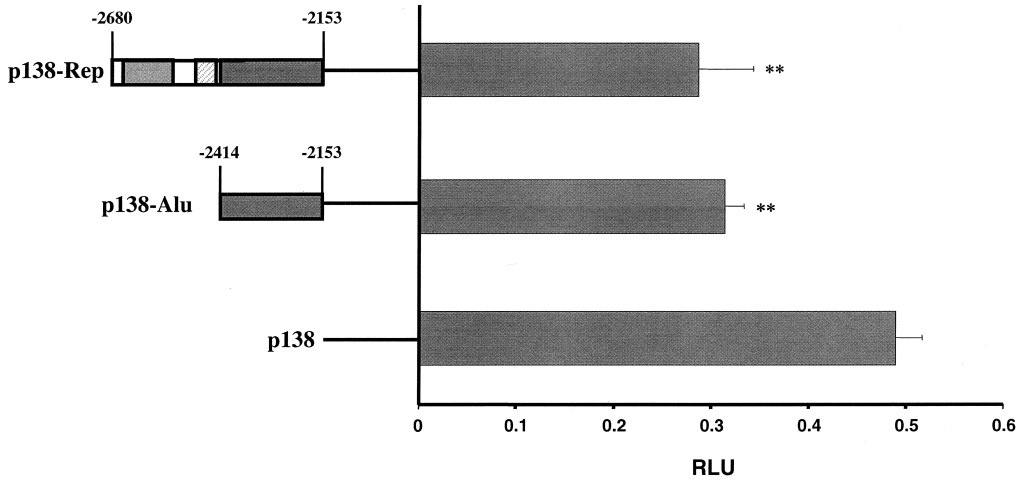

Fig. 3. The −2,153/−2,414 *Alu* repeat represses human CETP promoter activity. HepG2 cells were transiently transfected with constructs containing the proximal 138 bp (relative to the transcription start site) of the human CETP promoter alone or downstream to either the −2,153/−2,680 or the −2,153/−2,414 distal promoter region (p138, p138-Rep, and p138-*Alu*, respectively). Luciferase activity is expressed in RLUs after standardization for β-galactosidase activity. Experiments were performed in triplicate and values correspond to the mean ± SD from three independent experiments. ** $P < 0.005$ versus p138.

of nonradiolabeled CPF probe (Fig. 6, lane 3) or CL1 probe (Fig. 6, lanes 5 and 10), but not with an excess of CPFmut (Fig. 6, lane 4). Taken together, our results indicated that the Complex I was formed as a result of the interaction with LRH-1. In conclusion, we demonstrate that the DNA sequence from −1,042 to −1,050 bp permits the binding of the transcription factor LRH-1, the mouse homolog of CPF.

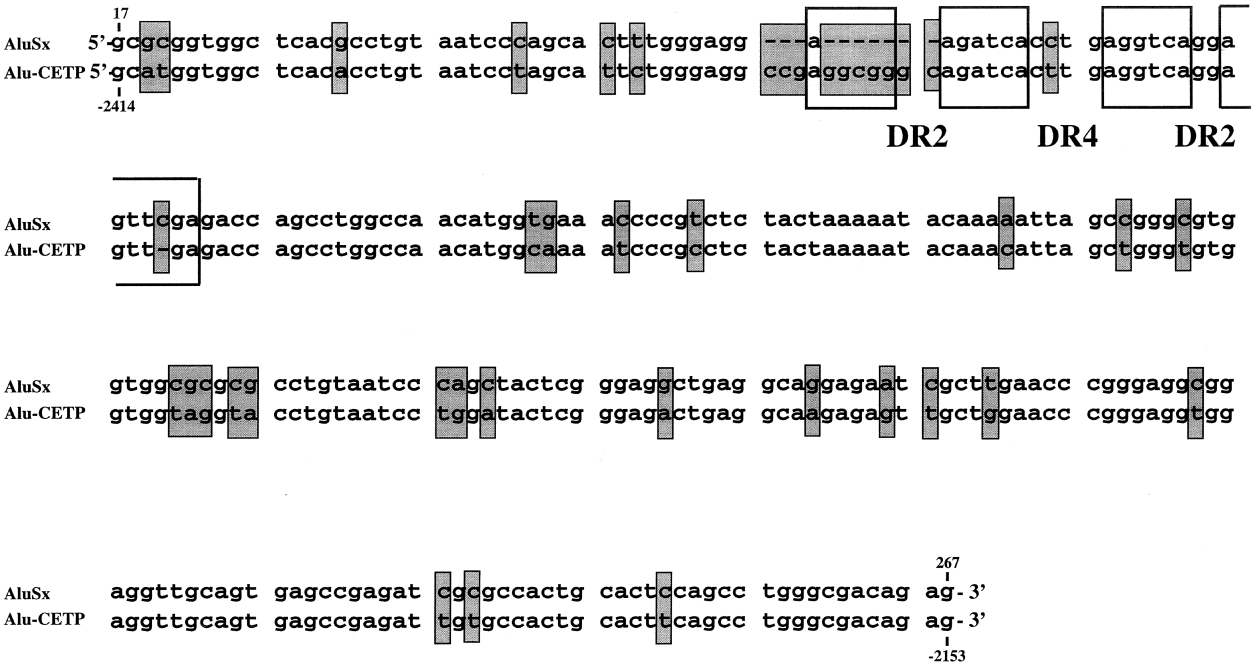

Fig. 4. Comparison between the nucleotide sequence of the *Alu/Sx* repeat of the human CETP promoter (GenBank™ accession number U71187) (23) and the human *Alu/Sx* consensus sequence (GenBank™ accession number U14574) (32) using the Clustal X Windows® interface (37). Filled boxes indicate the nucleotide differences between the two sequences. Frameworks correspond to potential hormone-responsive elements (HREs) that match the consensus hexamer half sites (A/G)G(G/T)T(C/G)(A/G). An insertion of 36 bp due to the previously described tetranucleotide repeat polymorphism (38) generates a shift in the position of the *Alu/Sx* repeat in the human CETP promoter sequence. Thus, the *Alu/Sx* repeat is located herein at positions −2,153/−2,414 bp, whereas the positions are −2,117/−2,378 bp in the sequence reported by Oliveira et al. (23).

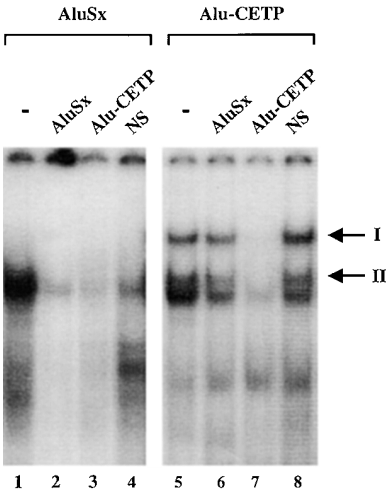

Fig. 5. The 10 bp insertion in the −2,153/−2,414 bp *Alu/Sx* repeat of the human CETP promoter allows the specific binding of nuclear factors. Electrophoretic mobility shift assay (EMSA) experiments were performed using the *AluSx* probe (5′-TGGGAGGAAGATCAC-3′) corresponding to the 50/64 bp region of the human *Alu/Sx* consensus sequence (32), and the *Alu*-CETP (5′-TGGGAGGCCGAGGCGGGCAGATCAC-3′) probe corresponding to the −2,357/−2,381 bp region of *Alu/Sx* repeat of the human CETP promoter. The underlined letters indicate the 10 bp insertion. Each radiolabeled probe was incubated with nuclear extracts from HepG2 cells (lanes 1 and 5) and 100-fold excess of *AluSx* probe (lanes 2 and 6), *Alu*-CETP probe (lanes 3 and 7), or nonspecific (NS) probe (lanes 4 and 8). Specific DNA-protein complexes are indicated by an arrow.

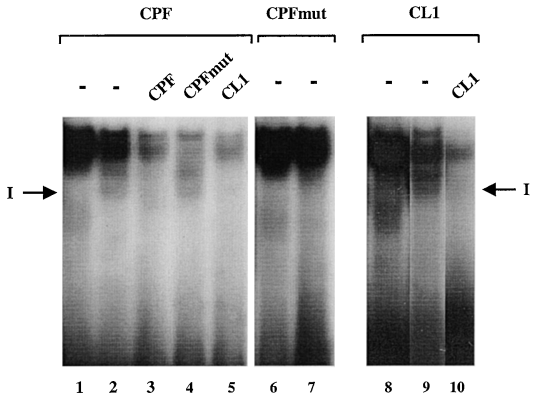

Fig. 6. The −1,012/−1,398 activating CETP promoter region contains a binding site for the CYP7A promoter binding factor (CPF). EMSA experiments using radiolabeled CPF (5′-TGTCTCTGAGCCTTGGGAAACAGT-3′), CPFmut (5′-TGTCTCTGAGCCaaGGGAAACAGT-3′), or CL1 (5′-TCTGATACCTGTGGACTTAGTTCAAGGCCAGTTA-3′) probes with control lysate (lanes 1, 6, and 8) or in vitro-translated liver receptor homolog-1 (LRH-1) lysate (lanes 2–5, 7, and 9–10), and 100-fold excess of CPF probe (lane 3), CPFmut probe (lane 4), or CL1 probe (lanes 5 and 10). The DNA-protein complex resulting from the binding of LRH-1 is indicated by the arrow.

The CPF site participates in the transcriptional activation mediated by the −1,012/−1,398 CETP promoter region

To determine whether the −1,042 CPF site is implicated in the transcriptional activation mediated by the 1,012/−1,398 promoter region, we performed transient transfection experiments in both HepG2 cells, a cell line which expresses CPF, and HEK293 cells that lack CPF (33). As shown in **Fig. 7**, the −1,398 construct displayed a significant 2-fold higher luciferase activity ($P < 0.0005$) than the p1012 construct in Hep G2 cells, as reported in Fig. 1; by contrast, the difference in luciferase activity between these two constructs in HEK293 cells was only 26% ($P < 0.005$). In addition, mutation of the CPF site (p1398M1) significantly reduced promoter activity (−24%, $P < 0.05$) in HepG2 cells, as compared with the construct containing the intact CPF site (p1398), whereas it induced no effect in HEK293 cells. We conclude that the −1,042 site accounts for a significant proportion (up to 50%) of the activation mediated by the −1,012/−1,398 promoter region in HepG2 cells.

The transcription factor CPF activates CETP promoter activity at the −1,042 site

The orphan nuclear receptor LRH-1, the mouse homolog of CPF, is known to transactivate the human CETP promoter by binding to a proximal promoter element at position −75 (16). To test the implication of the distal −1,042 CPF site in the LRH-mediated activation of the human CETP promoter, transient cotransfection experiments were performed in the HEK293 cell line with a pCMX-mLRH1 expression vector and a set of constructs described in Experimental Procedures. Briefly, the p1398 and p1398M1 constructs, and two additional constructs (pM2 and pM1M2) in which the proximal CPF site at position −75 was mutated, were used.

As shown in **Fig. 8**, cotransfection of the p1398 construct, which contains the two intact CPF sites, with a pCMX-mLRH1 expression vector led to significant induction of luciferase expression (2-fold, $P < 0.05$) as compared with the p1398M1M2 construct mutated at both sites. In addition, whereas mutation at both CPF sites repressed the LRH-mediated activation of the human CETP promoter, mutation of either the proximal −75 or the −1,042 site alone (p1398M1 or p1398M2, respectively) did not affect this induction. These results indicated that the transcriptional factor CPF activates CETP promoter activity at the −1,042 site to the same extent as the −75 CPF site.

DISCUSSION

We report that the distal promoter region of the human CETP gene plays a key role in the regulation of promoter activity. Analysis of nucleotide sequences from −1,012 bp to −3,242 bp revealed that this promoter region is composed of multiple repeat elements, among which an *Alu* repeat sequence acts as a repressive regulatory element of CETP promoter activity. We equally describe a functional CPF site at position −1,042 bp, which may contribute to the hepatic expression of the human CETP gene.

We report that a full-length *Alu* repeat, located at positions −2,153/−2,414 bp in the distal promoter region,

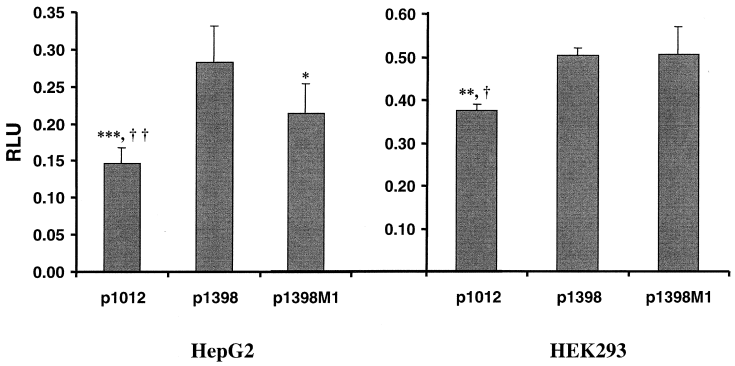

Fig. 7. The −1,042 CPF site contributes to the transcriptional activation mediated by the −1,012/−1,398 CETP promoter region in a hepato-specific manner. HepG2 (left panel) and HEK293 cells (right panel) were transiently transfected with constructs containing either 1,012 bp or 1,398 bp of the human CETP gene promoter (p1012 and p1398, respectively). Two point mutations were introduced in the p1398 construct in the CPF binding site located at position −1,042, thereby generating the p1398M1 construct. Luciferase activity is expressed in RLUs after standardization for β-galactosidase activity. Experiments were performed in triplicate and values correspond to the mean ± SD from at least three independent experiments. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ versus p1398; † $P < 0.05$, †† $P < 0.005$ versus p1398M1.

acts as a repressor of human CETP promoter activity. A transcriptional repressive role of the *Alu* element has already been demonstrated in several studies (29, 30). Thus, the Wilms' tumor gene WT1 contains a repressive regulatory element composed of a full-length *Alu* repeat in the third intron, located at 12 kb from the promoter, that represses WT1 gene expression (30). In addition, the *Alu* repetitive element found in the distal promoter region (−1,007/−1,330) of the human glycoprotein hormone α subunit acts as a negative transcriptional regulatory element (29). In these studies, transcription factor(s) involved in such an *Alu*-associated repression were not identified. Vansant and Reynolds (27) reported that the consensus sequence of *Alu* repeats contains a functional retinoic acid response element that is implicated in the regulation of expression of the keratin K18 gene expression. Indeed, the K18-associated *Alu* is a member of the evolutionarily more recent subfamilies (classes III and IV) and has four HREs (AGGTCA) separated by 2 bp (DR2), consistent with the binding specificities of retinoic acid receptors (RARs). The repressive *Alu* repeat found in the CETP promoter belongs to the class II early *Alu* subfamily (type Sx) (31), which represents the majority of *Alu* repeats. As illustrated in Fig. 4, this latter contains four HREs, separated

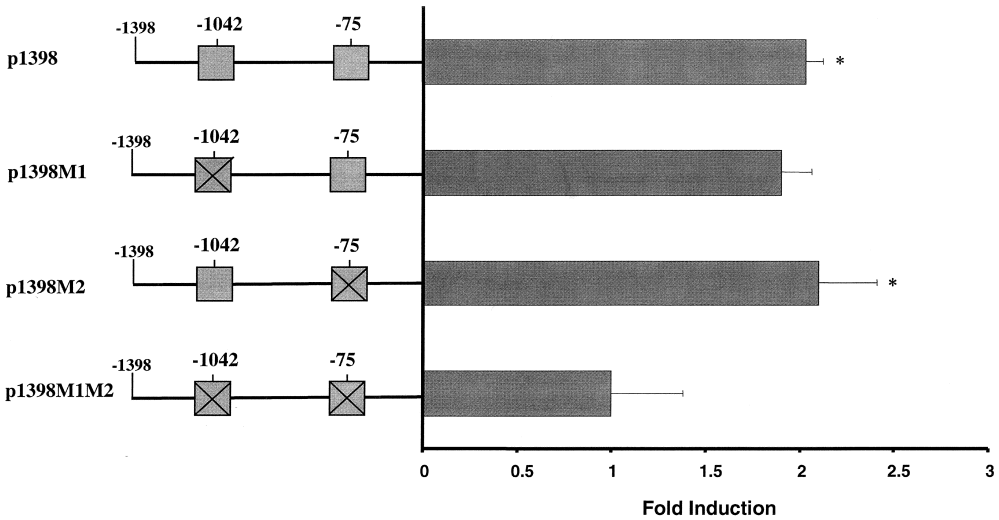

Fig. 8. The −1,042 CPF site is sufficient to induce the CPF-mediated activation of CETP promoter activity. One microgram of each CETP promoter construct was transiently cotransfected with 0.5 μg of pCMX-mLRH1 expression vector in HEK293 cells. Results were expressed as *x*-fold induction relative to luciferase activities normalized for β-galactosidase activity obtained without pCMX-mLRH1 expression vector. The marginal increment of promoter activity of the p1398M1M2 construct (mutated in both the −75 and −1,042 CPF sites) in response to CPF was subtracted from the promoter activity of all the constructs. Experiments were performed in duplicate, and values correspond to the mean ± SEM from three independent experiments. * $P < 0.05$ versus p1398M1M2.

by 2 bp and 4 bp, thereby generating two potential DR2 and one DR4 binding sites. A recent study reported that the DR4 found in this class of *Alu* repeat exhibits selective binding to the heterodimer complex of nuclear receptors LXRα-RXR, which is implicated in LXR induction by LXR ligands (35); however, the implication of RARs and/or LXRs in the repressive effect of the −2,153/−2,414 bp *Alu/Sx* region appears unlikely, since the transcriptional activity of a 3.4 kb promoter fragment of the human CETP gene was stimulated by *trans* retinoic acids (36) and LXR nuclear receptors (18).

It is unlikely that all *Alu* repeats function as regulatory elements due to their abundance; however, it is very interesting to note that the −2,153/−2,414 bp *Alu* repeat significantly decreases transcription activity from the CETP promoter when located upstream of the 138 bp minimal promoter region, indicating that the repressive action of this element is not a function of its position in the CETP promoter. We observed that the −2,153/−2,414 bp *AluSx* element displayed nucleotide differences with the *AluSx* consensus sequence (Fig. 4). We speculate that these nucleotide differences might be responsible for the specific *Alu*-mediated repression of the CETP promoter. Among them, we report that a 10 bp insertion located in the HRE-rich region, which led to the creation of a potential DR2 element, permits the specific binding of nuclear factors. Nevertheless, further investigations are required to identify these factors and to determine whether they may account for the specific repressive action of this element.

We identified a binding site for the orphan nuclear receptor CPF at position −1,042 bp in the promoter region, which transactivates the human CETP promoter. However, this site accounts for only half of the activation mediated by the −1,012/−1,398 promoter region in HepG2 cells, suggesting that other(s) positive regulatory element(s) are present in this promoter region. The presence of a positive regulatory element in the distal CETP promoter is consistent with a previous study, which reported that the 5.7 kb promoter region is responsible for a significant transcriptional activation in a conditionally transformed mouse hepatocyte line as compared with the proximal 137 bp promoter fragment (22). The orphan nuclear receptor CPF has already been described as being able to transactivate the CETP promoter by binding to a proximal promoter element at position −75 (16). We demonstrated that the new −1,042 CPF site is sufficient to induce the CPF-mediated activation of the human CETP promoter to the same extent as the proximal −75 CPF site. The liver-specific expression of this nuclear receptor has been reported to be a key regulator of human CYP7A gene expression in the liver (33). In agreement with the liver-specific expression of CPF, we observed that the −1,042 site participates in CETP promoter activity in HepG2 cells, whereas this site is not functional in nonhepatic HEK293 cells. Interestingly, Oliveira et al. (23) reported that the CETP promoter region between −570 bp and −3,400 bp confers predominant expression of the CETP gene in liver. The fact that the presently described CPF site is located in this promoter region led us to suggest that the binding of the orphan nuclear receptor CPF on the −1,042 site may contribute to the hepato-specific expression of the CETP gene.

In conclusion, our study provides evidence that the distal region of the human CETP promoter contains regulatory elements that contribute to the transcriptional activity of the CETP gene. Furthermore, it appears that, in the same manner as in the proximal region, nuclear receptors are intimately involved in the contribution of the distal promoter region to regulation of the transcriptional activity of the CETP gene, thereby confirming the crucial role of this family of transcription factors in the control of genes of lipid metabolism.

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