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Opposite regulation of the rat and human cytosolic aspartate aminotransferase genes by fibrates

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Abstract

Fenofibrate, a peroxisome proliferator-activated receptor α (PPARα) activator, increases the expression of the cytosolic aspartate aminotransferase (cAspAT) gene in human liver cells, which may partially explain the increase of this enzyme in the serum of individuals undergoing fenofibrate treatment. Conversely, in rodents, fenofibrate represses the expression of the cAspAT gene. We compared the mechanisms of fenofibrate action in human and rat hepatoma cells. Transfection assays of the wild-type and mutated rat promoters in Fao and H4IIEC3 cells established a critical role for sequences similar to nuclear receptor responsive elements in the −404/−366 bp region. Nuclear proteins bound to these sequences and the amounts of protein bound were decreased by fenofibrate treatment, probably accounting for the decreased gene expression. Pharmacological studies confirmed the involvement of PPARα. However, this receptor did not bind directly to these sequences. The human promoter was cloned and the regulatory region localized between −2663/−706 bp. Co-transfection assays suggested that, in humans, the PPARα was also involved in the increase in expression of the cAspAT gene due to fibrates, without the presence of a canonical PPAR responsive element.

Keywords: PPARα; Transaminases; Fenofibrate; Human promoter sequencing; Peroxisome proliferators; Aspartate aminotransferase

1. Introduction

Fibrates, a class of drugs used in the treatment of atherogenic dyslipidemia act on plasma lipids by altering the transcription of genes involved in fatty acid and triglyceride metabolism [1]. Their effects are mediated by the PPARα [2]. After ligand activation and heterodimerization to the RXR, the heterodimer binds to a PPRE, generally a direct repeat of a hexamer AGG(T/A)CA, separated by one nucleotide.

One important property of fibrates is their species-specificity. Whereas they efficiently decrease plasma triglycerides in both man and rodents, their effects on the levels of HDL and its major protein, apolipoprotein AI, are opposite in the two species [3]. Their most striking species-specific effect is the peroxisome proliferation observed in rodents: long term administration of fibrates results in peroxisome proliferation, liver hypertrophy and hyperplasia, leading to non-genotoxic hepatocarcinogenesis [4]. PPs such as fibrates are generally believed to act through the PPARα. Indeed, mice homozygous for a disrupted PPARα gene show no hepatic enzyme induction, liver growth or peroxisome proliferation in response to clofibrate treatment [5]. Recently, it has been shown that highly selective PPARγ or dual PPARγ/PPARδ agonists also cause peroxisome proliferation in mice, independently of PPARα [6]. Thus, a functional overlap exists between

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Abbreviations: PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR response element; RXR, retinoid X receptor; PPs, peroxisome proliferators; cAspAT, cytosolic aspartate aminotransferase; PCR, polymerase chain reaction; NRRE, nuclear receptor response element; ACO, acyl-CoA oxydase; HNF-4, hepatocyte nuclear factor-4; EMSA, electrophoretic mobility shift assay; DR, direct repeat; GRE, glucocorticoid responsive element; AP1, activator protein-1; C/EBP, CCAAT enhancer binding protein; LXR, liver X receptor.

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members of the PPAR subfamily. In contrast, the fibrate hypolipidemic effect in humans and guinea pigs is accompanied neither by peroxisome proliferation nor by induction of peroxisomal beta-oxidation [7]. These differences have been accounted for by both quantitative and qualitative mechanisms, such as the amount of PPARα and the structure of the target promoters. However, the mechanisms underlying these differences remain controversial and generally poorly understood. AspAT, a ubiquitous pyridoxal phosphate-dependent transaminase (EC 2.6.1.1), exists in vertebrate cells as two isoenzymes, cytosolic and mitochondrial, which play major roles in amino acid metabolism and in the malate–aspartate shuttle [8]. In the liver, they also participate in ureogenesis and gluconeogenesis. The expression of the rat cAspAT gene is regulated by glucocorticoids, insulin and cAMP [9,10] and by a protein-rich diet or during prolonged fasting [11]. In contrast, human cAspAT gene regulation has not been well characterized to present because the promoter had not been cloned and because of the lack of an appropriate *in vitro* model for hormonal effects. In clinical studies, the serum AspAT level is widely used as a sensitive marker of possible tissue damage, particularly heart and liver toxicity. In man, whereas the hepatic activity is mostly due to the mitochondrial isoenzyme (80%), the serum activity is largely due to the cytosolic one [12]. This basal activity is partly the result of a poorly understood process of hepatocyte turnover and enzyme release. In the case of liver disease, the serum AspAT value increases up to 100-fold, indicative of hepatocyte cell lysis. In the case of a moderate increase, an alternative possibility is that the serum value could reflect expression of the liver gene. This hypothesis has not been supported to date because of the poor characterization of the liver gene regulation. In some patients treated with fenofibrate, the AspAT value increases 2- to 3-fold without any other clinical sign of hepatotoxicity. We hypothesized that, in some cases at least, the increase in serum cAspAT may be due to the induction of the liver gene by fibrates. In a previous study, we have shown that fibrates increase the expression of the human gene in HepG2 cells, while decreasing it in rodents by a PPARα-dependent mechanism [13]. It was the first demonstration that such a drug could regulate the level of the cAspAT by a non-toxic phenomenon at the gene level.

To determine how fenofibrate modifies expression of the cAspAT gene in opposite directions, we analyzed the mechanisms of the regulation by fibrates of the rat gene. This also involved cloning of 2.6 kb of the human promoter and comparison of the human and rat gene promoters.

2. Materials and methods

2.1. Cell culture

The closely related rat hepatoma cells Fao and H4IIEC3 [14] and the human HepG2 cells [15] were maintained as described [16]. Fenofibric acid and fenofibrate were from Fournier Laboratories. Fenofibric acid is the major circulating form of fenofibrate. In rat hepatoma cells, fenofibrate is more active than fenofibric acid whereas fenofibric acid is more efficient in HepG2 cells.

2.2. Isolation and sequencing of the 5′ region of the human cAspAT gene

The 5′ region of the human cAspAT gene was isolated using the GenomeWalker kit combined with an Advantage GC genomic PCR kit (Clontech). Two human cAspAT specific primers (Genset) were chosen near the translation start site: GSP1: 5′-GTGGCCTGCGGACCCTCGGCAAAGAC-3′ and GSP2: 5′-TGCCATCTCGAGAGACT AGGAATCAAGAG-3′, corresponding, respectively, to bases +41 to +16 and +6 to −23, +1 being the ATG used for the translation [17]. For the PCR cloning experiment, the PCR mix was prepared according to the Advantage GC Genomic PCR protocol. PCR cycles used were those described in the GenomeWalker protocol. As we expected to amplify long DNA fragments, an extension time of 5 min was used. A 2663 bp PCR product was obtained and subcloned into the pGL3 vector containing the firefly luciferase reporter gene (Promega) using *Mlu*I and *Xho*I restriction sites. Both strands of this plasmid p(2663/−6)hAspFL were sequenced on an ABI 310 Genetic Analyzer, using the Big Dye Terminator sequencing kit according to the manufacturer's instructions (Perkin-Elmer Applied Biosystems). To remove nonincorporated dye, a purification step on a multiscreen filtration system filled with Sephadex G50 was performed. The sequences were analyzed using the sequence Analysis 3.0 program (Perkin-Elmer Applied Biosystems).

2.3. Plasmids and oligonucleotides

The p(−682/−26)CAT, p(−553/−26)CAT, p(−286/−26)CAT and p(−225/−26)CAT plasmids containing various fragments of the rat cAspAT gene promoter in front of the CAT reporter gene and the pSV2neo plasmid have been described previously [9,16,18]. The mutations 1, 2 and 3 in probe II were created by double PCR experiments, as previously described [9]. Various 5′ deletions of the plasmid p(−2663/−6)hAspFL, p(−706/−6)hAspFL, p(−390/−6)hAspFL, p(−301/−6)hAspFL, p(−257/−6)hAspFL, p(−130/−6)hAspFL and p(−74/−6)hAspFL, were obtained by subcloning either *Eco*RI or *Kpn*I/*Xho*I cut PCR products into the corresponding sites of the pGL3 vector. The PPRE–pLa2FL plasmid, the RXRα and the PSG5 expression plasmids were kind gifts from Dr. Charbel Massaad (ESA-CNRS 7079). The human PPARα expression vector was a generous gift from Dr. B. Staels (INSERM-Unit-325). The 5′ to 3′ sequences of the top strand of the oligonucleotides used in EMSAs are shown in Fig. 3A.

2.4. Measurement of the cAspAT activity

The activities of the cytosolic and mitochondrial isoenzymes of AspAT and proteins were determined as described previously [10].

2.5. Primer extension assay

A 30 nucleotide primer 5′-CCTGCGGAACCTCGGCAAAGACTGACGGA-3′, corresponding to bases +8 to +38 of the human cAspAT gene 5′ region was end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and T4 polynucleotide kinase. The probe was purified using a Sephadex G50 spin column. HepG2 total RNA or yeast tRNA were hybridized overnight at 50° with 5×10^6 cpm of the labeled oligonucleotide in 0.01 M Tris–HCl, pH 8.3, 1 mM EDTA, 0.15 mM KCl buffer. After ethanol precipitation and resuspension in 11 μL H_2O, the oligonucleotide was extended for 90 min at 42° with 20 units of Avian Myeloblastosis Virus reverse transcriptase (Finnzymes) in 25 μL of 25 mM Tris–HCl, pH 8.3, 5 mM $MgCl_2$, 50 mM KCl, 2 mM DTT, 1 mM dNTP in the presence of 0.15 μg actinomycin D. Ten micrograms of RNAse A were added to the mixture to stop the reaction. After a phenol–chloroform extraction and ethanol precipitation, the extension products were fractionated on a 6% polyacrylamide 7 M urea gel and analyzed by autoradiography. The size of the extended fragments was determined by comparison with a cAspAT 5′ region sequence ladder obtained with a T7 sequencing kit (Amersham) run on the same gel.

2.6. RNA preparation and Northern blots

Unless indicated, total RNA preparation and Northern blots were performed as described by Barouki *et al.* [10]. The probes 4B21 [19] and 18S rRNA were labeled using the Megaprime DNA labeling system (Amersham) and hybridization was performed using Rapid-hyb buffer (Amersham) following the manufacturer's instructions. The membrane was washed 30 min at 65° with 2× SSC, 0.1% SDS and 30 min at 65° with 1× SSC, 0.1% SDS.

2.7. Nuclear run-on assay

Fao cells were treated with ethanol (0.1%) or dexamethasone (0.1 μM), with or without fenofibrate (200 μM), for 4 hr. Nuclei were then prepared and nascent RNA was labeled and purified as described [9]. The labeled RNA (80×10^6 cpm) was hybridized to the biodyne A 0.2 μm (PALL) nylon filters containing 10 μg of the following linearized plasmids: pGEM4Z, 4B21 rat cAspAT probe, mouse actin and 18S rRNA. The filters were hybridized for 72 hr at 42°, washed at 42° in 1× SSC, 0.1% SDS for 1 hr, then treated with RNAse A (10 μg/mL) for 30 min at 37° in 1× SCC, 0.1% SDS and analyzed by autoradiography.

2.8. Transfection experiments

HepG2 cells (0.4×10^6 cells per 6 cm dish) were transfected as previously described [20]. Two micrograms of the luciferase plasmid were introduced into the cells by the calcium phosphate co-precipitation technique followed by a 2 min glycerol shock, 4 hr later. Sixteen hours later, various agents were added to the culture medium. After a 24 hr incubation, the cells were homogenized in 200 μL of the following buffer; 100 mM potassium phosphate pH 7.8, 0.2% Triton X-100. The Firefly luciferase activities were determined as previously described [20].

The H4IIEC3 cells were transiently transfected as described by Beurton *et al.* [21] using Fugene™ 6 (Roche). Briefly, 3 μL of fugene were mixed with 97 μL of serum-free medium and 1 μg of plasmid was added. After standing 15 min at room temperature, the mixture was added to the cells. Six hours later, the medium was replaced by 4 mL of fresh medium containing serum. The cells were treated 16 hr later with various compounds and cultured for an additional 24 hr. CAT activities were determined as described by Beurton *et al.* [21].

Stable transfections in Fao cells were done as described [9]. HepG2 and H4IIEC3 cells were stably transfected as described below. One day prior to the transfection, the cells (1.5×10^6 cells/10 cm dish) were seeded into the usual medium containing fetal calf serum. The reporter plasmid (2 μg) and the pSV2neo plasmid (0.5 μg) were introduced into the cells using Fugene™ 6 as described above. Two days later, the cells were split 1:4 and allowed to grow for 24 hr prior to the addition of the neomycin analog, G418 (Invitrogen, 250–500 active μg/mL, depending on the batch). The medium was changed every 3 days. Two to four weeks later, the surviving cells were harvested and pooled for luciferase assay. Cells were continuously propagated in medium with G418. When needed, the cells were treated for 24 hr with the drugs, without G418.

2.9. Nuclear extracts and electrophoretic mobility shift assays

Nuclear extracts from H4IIEC3 cells (treated or not by 250 μM fenofibrate for 2 hr) were prepared as described by Morel and Barouki [22]. Mouse PPARα and RXRα proteins were synthesized, *in vitro*, using the rabbit reticulocyte lysate system (Promega).

For EMSAs, probes were end-labeled using the Klenow polymerase and purified on spin columns. Nuclear extracts were incubated at room temperature for 20 min in a final volume of 20 μL containing 10 mM HEPES, pH 7.9, 0.1 mM EDTA, 50 mM NaCl, 50 mM KCl, 5 mM $MgCl_2$,

4 mM spermidine, 2 mM DTT, 100 μg/mL albumin, 10% glycerol with or without a 50-fold molar excess of unlabeled probe or the antiserum (2 μL) before the addition of 1 ng of DNA probe and followed by another 20 min incubation.

In vitro translated PPARα and RXRα were preincubated in a total volume of 20 μL for 15 min on ice with 2.5 μg poly(dI–dC) and 1 μg salmon sperm DNA in 10 mM Tris–HCl, pH 8, 40 mM KCl, 10% glycerol, 0.05% NP-40 and 1 mM DTT before addition of the probe. Binding reactions were analyzed by electrophoresis at 4° on a 6% polyacrylamide gel in 0.25× TBE buffer. The anti-HNF-4 antibody was a generous gift from Dr. B. Viollet (INSERM-Unit-129).

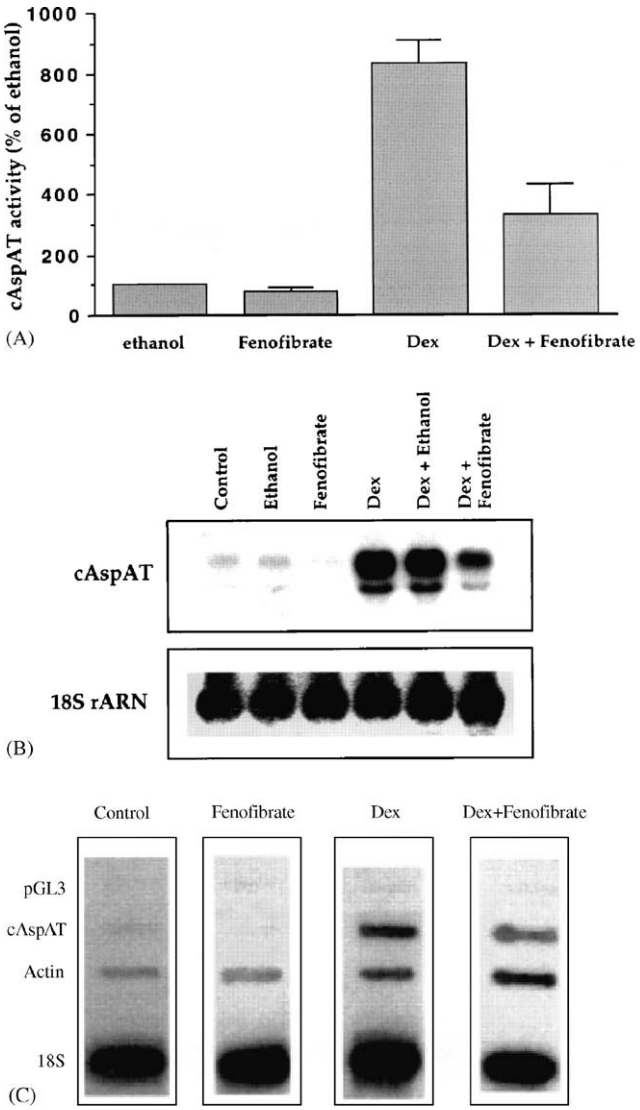

Fig. 1. Effect of fenofibrate on cAspAT gene expression in Fao cells. Fao cells were treated with 200 μM fenofibrate or 0.1% ethanol (vehicle), with or without 0.1 μM dexamethasone for 48 hr (for activity measurements), 24 hr (for mRNA levels) or 5 hr (run-on experiments). (A) cAspAT activity. The data represent the mean ± SEM of four independent experiments. (B) mRNA level. Twenty milligrams of total RNA were loaded in each lane. 18S rRNA was used as control. (C) The run-on assay was performed as described in Section 2.

3. Results

3.1. Fenofibrate decrease of the cAspAT expression in Fao cells

Treatment of rat Fao cells with fenofibrate resulted in a 22% decrease in basal cAspAT activity. However, the down-regulation of this gene was more readily observed following induction by dexamethasone. Fenofibrate provoked a 69% decrease of cAspAT activity induced by the glucocorticoid (Fig. 1A). This effect was maximal when the cells were treated for 48 hr with 200 μM fenofibrate. Within the dose range used, there was no sign of cytotoxicity as assessed by microscopic observation of the cells and measurement of released lactate dehydrogenase (data not shown).

Northern blot analysis indicated that the cAspAT mRNA level was also decreased by the fibrate (Fig. 1B). As most effects of fenofibrate on gene expression are transcriptional, we performed nuclear run-on experiments. The basal transcriptional activity was too low to be detected by this assay. In contrast, dexamethasone resulted in a large increase in cAspAT gene transcription, which was inhibited by fenofibrate (Fig. 1C). These results demonstrate that fenofibrate inhibition of cAspAT in Fao cells is due to decreased transcription of the gene.

3.2. Localization of the fenofibrate responsive element in the promoter of the rat cAspAT gene

In order to determine the fenofibrate responsive region in the rat promoter, we tested constructions in which serially

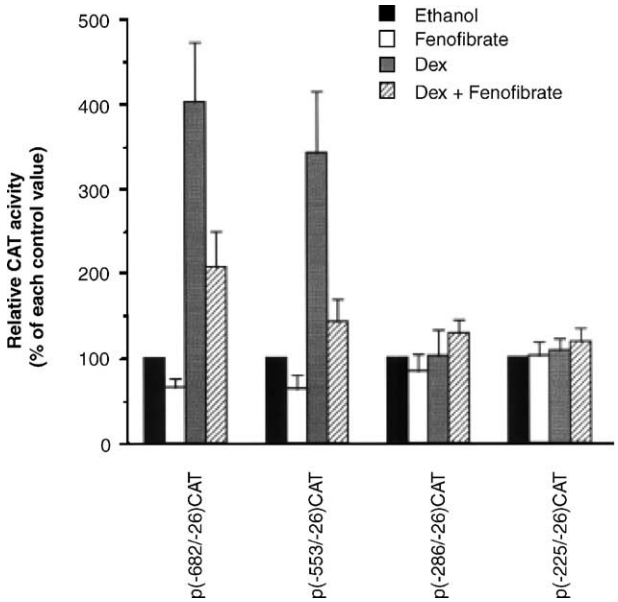

Fig. 2. Identification of the fenofibrate responsive region in the rat cAspAT promoter. Various constructs of the cAspAT promoter upstream of the CAT gene were permanently transfected in Fao cells. The cells were treated with ethanol (0.1%), fenofibrate (200 μM) or dexamethasone (0.1 μM) with or without fenofibrate for 24 hr and the CAT activity expressed as the mean ± SEM of four to nine independent experiments (100% corresponds to the value in the presence of ethanol).

truncated fragments of the 5′-flanking sequence of the rat cAspAT gene were linked to the CAT gene [16,18]. The 5′ ends of these constructs were at −682, −553, −286 and −225 bp, relative to the position of the translation start site. Fao cells, stably transfected with these constructs, were treated with fenofibrate or its vehicle (ethanol), with or without dexamethasone. Using the two longest constructs, p(−682/−26)CAT and p(−553/−26)CAT, glucocorticoids elicited a 4-fold increase in CAT activity and fenofibrate treatment led to a 40 and 60% decrease, respectively, of the basal and glucocorticoid-stimulated CAT activity (Fig. 2). Wy-14,643, bezafibrate and clofibrate also decreased the

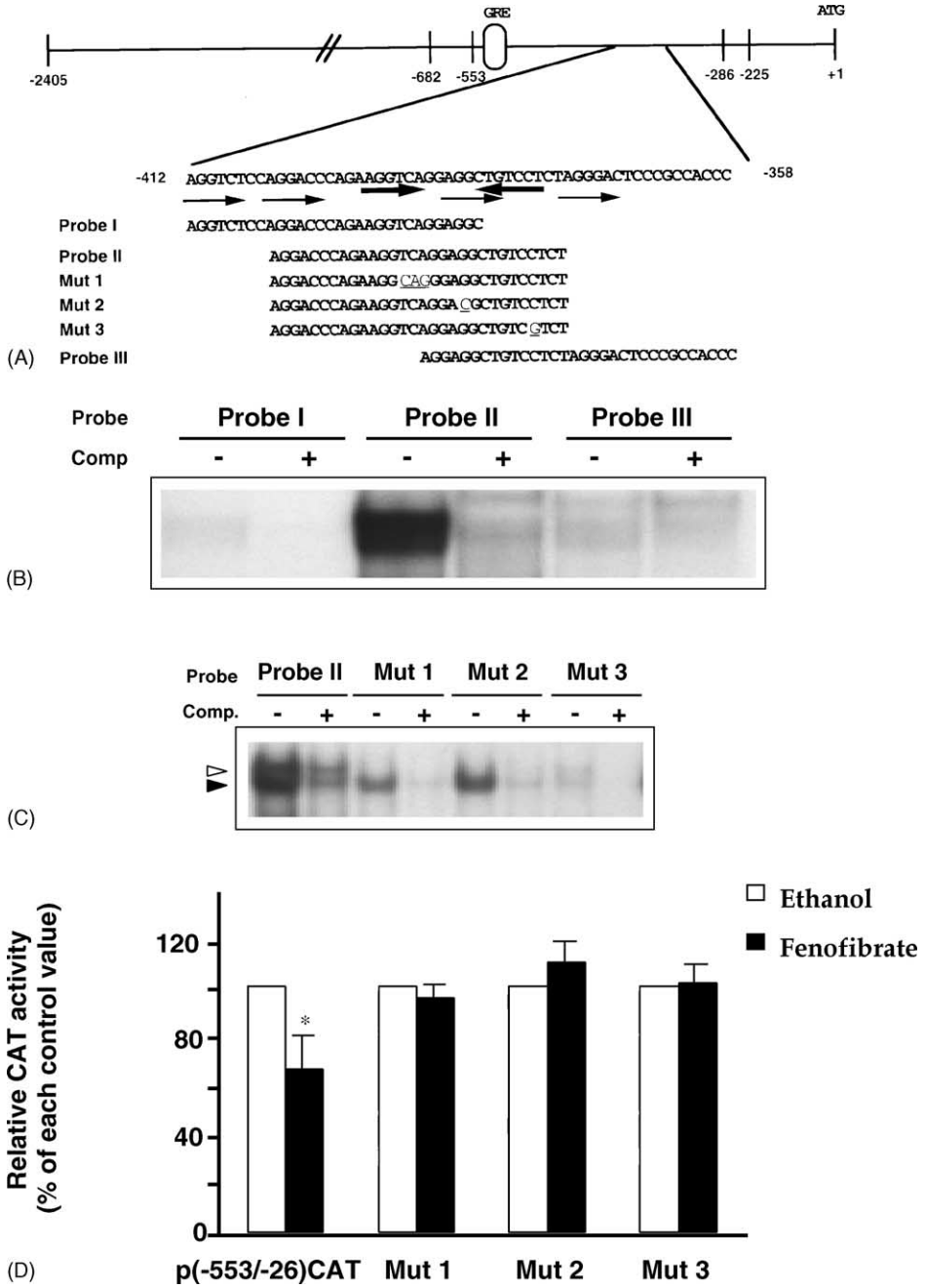

Fig. 3. Electrophoretic mobility shift analysis and mutations in the fenofibrate-responsive sequence of the cAspAT gene promoter. (A) The sequence of the (−412 to −358) fragment of the cAspAT gene promoter containing several NRREs and the sequences of the probes used in the EMSA experiments are shown. The mutations in the mutated probes are underlined. (B) EMSA: labeled Probe I, II and III were incubated with 5 μg of H4IIEC3 nuclear extract. In the indicated lane, a 100-fold molar excess of unlabeled oligonucleotide (comp) was added to the binding reaction. (C) Effects of mutations on the binding to nuclear extract. EMSA was performed using the same conditions as in Fig. 3B and D. Effects of mutations on the regulation of the gene activity by fenofibrate. Mutations 1, 2 and 3 were introduced in the construct p(−553/−26)CAT. Regulation by fenofibrate was studied after transfection into H4IIEC3 cells. The CAT activities are expressed as the mean ± SEM of five independent experiments (100% corresponds to the CAT value in the presence of ethanol). $^{*}P < 0.02$, relative to ethanol (Student's *t*-test for paired data).

glucocorticoid-stimulated CAT activity of the p(−553/−26)CAT construct by at least 67% (data not shown), suggesting the involvement of PPARα in these effects. No inhibition of the basal CAT activity by fenofibrate was detected with the shortest constructs p(−286/−26)CAT and p(−225/−26)CAT. The latter fragments were unresponsive to dexamethasone, in agreement with the absence of a functional GRE in these constructs. These findings suggest that fenofibrate acts primarily through the sequence between −553 and −286 bp.

The presence of putative PPREs in this sequence was next examined. The region −412/−358 bp contains several NRREs, including two perfect consensus sequences AGG(T/A)CA and four consensus-like motifs (Fig. 3A). However, none of these sequences are organized as a DR1, which typically corresponds to a functional PPRE [2]. Three probes encompassing this sequence (Fig. 3A) and H4IIEC3 cells extracts were used in EMSA experiments. With the distal probe I (−412 to −383) and the proximal probe III (−388 to −358), no specific binding was observed. With probe II (−404 to −366), one broad band (in fact, two very close bands, see below) could be detected which was competed by a 50× excess of cold probe (Fig. 3B).

To further characterize the sequences responsible for the band observed with probe II, mutations were introduced in the NRREs of this response region (Fig. 3A). With the three mutant probes, the slow migrating band disappears completely. The fast migrating band disappears completely with mutant 3 and partially with mutants 1 and 2 (Fig. 3C).

To further investigate whether these NRREs represent the functional responsive elements mediating the effects of fenofibrate on the rat cAspAT gene, the same mutations were introduced into the p(−553/−26)CAT plasmid and the activities of the mutants were compared to the activity of the wild-type construct transfected in H4IIEC3 cells. The three mutations led to a complete loss of the fibrate inhibitory effect in H4IIEC3 cells (Fig. 3D). Altogether, these experiments demonstrate that the functional responsive element mediating the inhibitory effect of fenofibrate on the cAspAT gene promoter is composed of NRREs organized as a DR2 with an inverted NRRE overlapping the 3′ half-site of the DR2.

We have previously reported that fenofibrate decreases cAspAT mRNA levels in mouse liver [13]. Using PPARα-null mice [5], we have shown that the absence of PPARα increases basal levels of cAspAT mRNA and that the inhibition by fenofibrate is prevented. In order to determine whether the complexes obtained with the H4IIEC3 cells correspond to the binding of the PPARα/RXRα dimer, experiments were performed with PPARα and RXRα transcribed and translated *in vitro* in rabbit reticulocytes. With probe II, no specific band was observed with *in vitro* PPARα and RXRα translated products (data not shown). Therefore, although the fenofibrate effect in rodents is PPARα-dependent, it does not seem to involve direct binding of this transcription factor. Since the HNF-4 transcription factor also binds DR1 and DR2 elements and is involved in some negative effects of fibrates [23,24], we tested an anti-HNF-4 antibody, which was unable to modify the complexes between H4IIEC3 nuclear extracts and probe II (data not shown). We verified that this antibody effectively displaced the binding of the same nuclear extracts to a consensus HNF-4 probe (data not shown). Therefore, another transcription factor is involved in the fenofibrate inhibition of the cAspAT gene.

We compared the binding to probe II of H4IIEC3 nuclear extracts from cells treated or not with fenofibrate. We consistently observed a 30% decrease in the intensity of the two complexes formed with the nuclear extracts from fenofibrate-treated cells (this effect was observed with three different preparations) (Fig. 4A and B). This decrease appears specific since no modification in the binding of the same nuclear extracts to nuclear factor 1 and HNF-3 probes was observed (data not shown).

3.3. Human cAspAT gene promoter analysis: comparison with the rat gene promoter

In order to clone the promoter of the human cAspAT gene, we used the GenomeWalker kit. The first PCR reaction used an outer adaptor primer AP1 provided in the kit and an outer gene specific primer (GSP1) present in the first exon of the cAspAT gene. A second nested PCR was performed on the first PCR product, using the nested adaptor primer (AP2) from the kit and a nested primer from the human cAspAT cDNA (GSP2) (Fig. 5A). The two

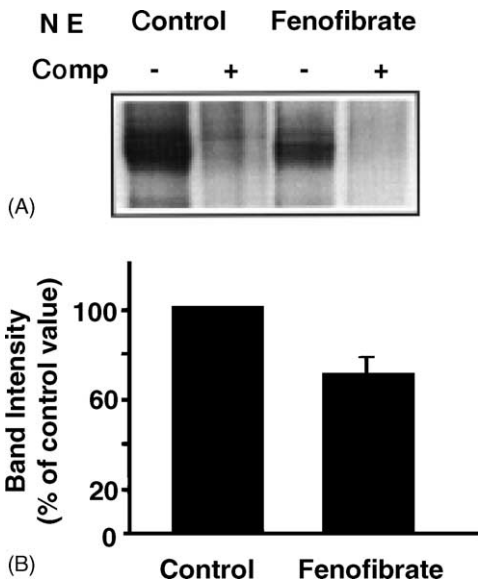

Fig. 4. Analysis of protein binding to probe II of the cAspAT gene promoter by EMSA. (A) Fenofibrate effects on the interaction of H4IIEC3 nuclear extracts with the rat cAspAT probe II. EMSA was performed by incubating labeled probe II with nuclear extracts obtained from cells incubated for 2 hr with ethanol or 250 μM fenofibrate. (B) Percentage of change in the intensity of the shifted band with respect to control values following treatment with fenofibrate. Each column represents the mean ± SEM of the results obtained with three independent preparations.

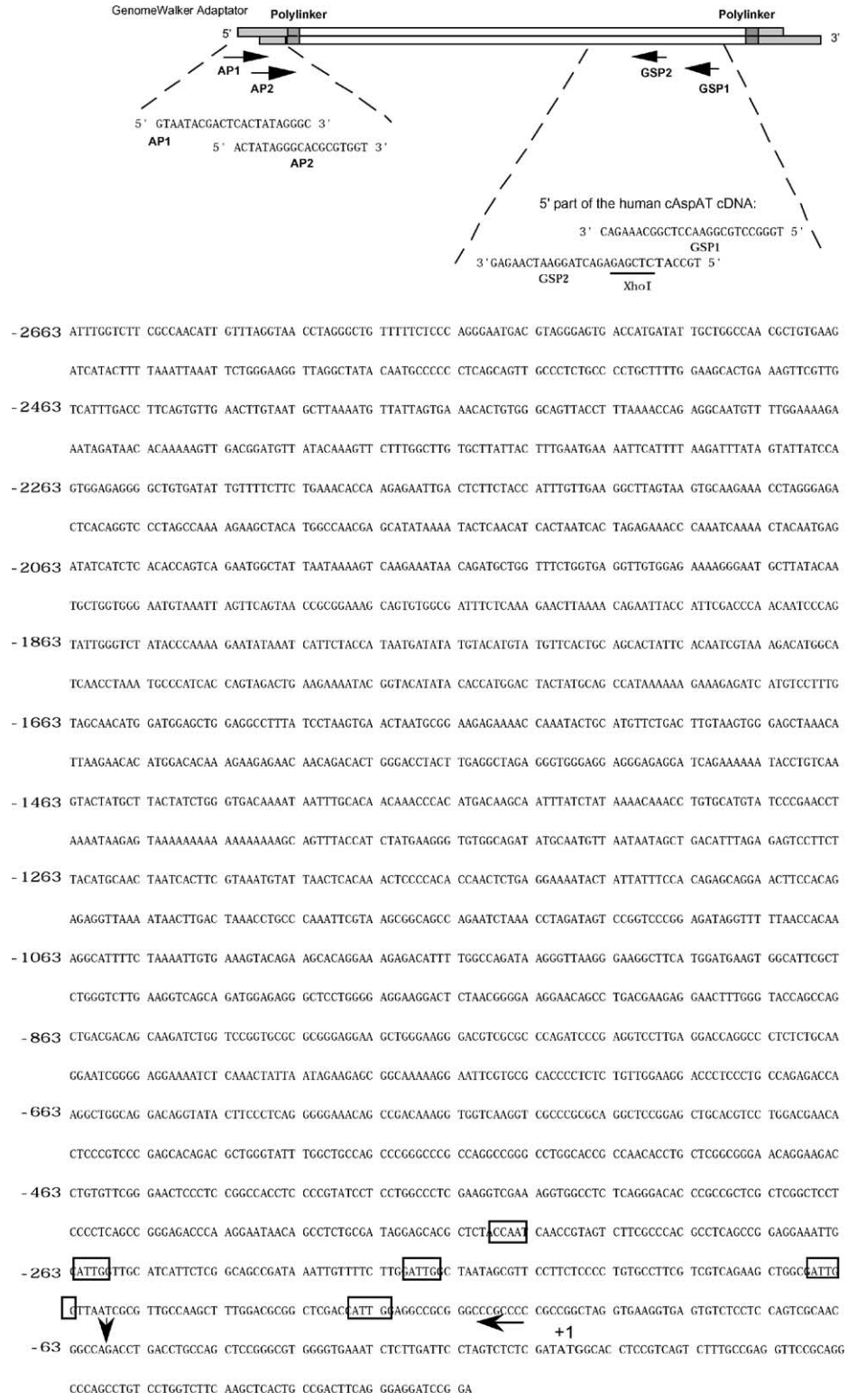

Fig. 5. The 5′ region of the human cAspAT gene. (A) Scheme of the cloning strategy. The specific primers used for PCR using GenomeWalker libraries are shown. GSP2 and AP2 contain, respectively *Xho*I and *Mlu*I restriction sites for subcloning the PCR product. (B) The sequence of the proximal 2663 bp of the promoter and of the 89 first bases after the ATG (bold letters) is shown. The transcription start site is indicated by a vertical arrow. The Sp1 site is underlined by an arrow and the CCAAT boxes are boxed. +1 corresponds to the ATG used for the translation.

nested primers AP2 and GSP2 contain restriction enzyme sites for the subsequent cloning of the PCR product. The longest PCR product of 2663 bp was subcloned into the *Mlu*I and *Xho*I sites of the pGL3 vector.

The nucleotide sequence of the human cAspAT gene promoter region up to the first 89 bp after the ATG is presented in Fig. 5B (GenBank accession number AF401279). The 200 bp proximal fragment upstream of

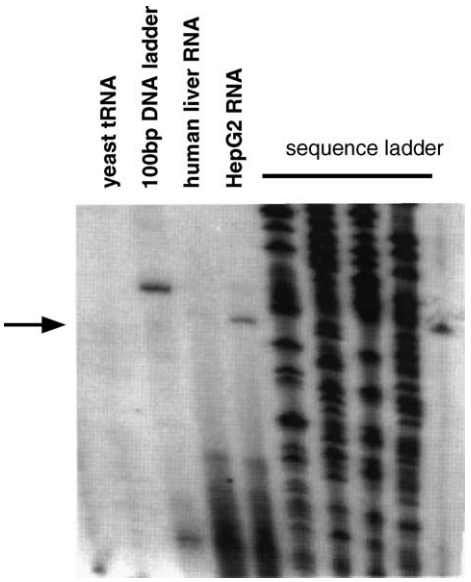

Fig. 6. Transcription initiation site of the human cAspAT gene. A primer extension experiment was performed on HepG2 cells and human liver total RNA, using an oligonucleotide complementary to the sequence +8 to +38. Yeast tRNA was used as negative control. The primer extended product size was determined by comparison to both a sequence ladder and a 100 bp ladder.

the ATG does not contain a TATA box; it is 63% GC-rich and contains a putative Sp1 site and five CCAAT boxes, four of them in the reverse orientation. In the region between −101 and −49 bp, several NRREs have also been identified (Fig. 5B).

To characterize the transcription initiation sites, we performed primer extension analysis using HepG2 RNA. The primer extension analysis assigns a predominant transcription start site to a G at position −58 bp from the ATG translation start site (Fig. 6).

The sequences of the human and rat cAspAT genes were aligned. Two regions show identity. There is a 67% sequence identity between the 537 bp distal region of the human gene (−2663/−2126) and the −1798/−1289 region of the rat gene. The other region displays a 61% identity between the −1139/−13 sequence of the human promoter and the −1134/−7 sequence of the rat gene (Fig. 7A). The proximal 500 bp of both promoters sequences show a 66% identity (Fig. 7B). Five out of the six CCAAT boxes, previously identified in the rat cAspAT gene, are conserved in the human gene, as well as the nuclear factor 1 binding site. The functional GRE of the rat gene [25] is fairly well conserved in the human promoter. However, only one of the four Sp1 sites present in the promoter of the rat gene is conserved in the human promoter. We also looked for putative NNREs in both sequences. Several consensus AGG(T/A)CA sites were found in the two promoters. Their locations and identities were compared (Table 1). Finally, unlike the rat promoter, which shows at least five major transcription start sites [16], the human cAspAT gene displays one major transcription site.

Table 1
Comparison of NNREs in the promoters of the rat and human cAspAT genes

Human NNRE	Location	Rat NNRE	Location	Homology
TGACCT	−2458/−2453	GGACCT	−1594/−1589	5/6
TGTCCT	−1672/−1667			No
AGGTCA	−952/−947	**AGGTCA**	−940/−935	6/6
AGGACA	−655/−650			NC
TGACCT	−54/−49			NC
		TGTCCT	−2348/−2343	No
		TGACCT	−2009/−2004	No
TGACGA	−893/−888	**TGACCT**	−881/−876	4/6
AGGTCG	−607/−602	**AGGTCA**	−601/−596	5/6
		AGGTCA	−394/−389	NC
		TGTCCT	−382/−377	NC

The consensus NNREs found in DR1 repeats known to bind PPARs are AGG(T/A)CA or, in the opposite orientation, TG(A/T)CCT. Five and seven perfect matches (in bold), respectively, were found in the promoters of the human and rat cAspAT genes. The corresponding sequences in the other species are indicated as well as the degree of homology between the rat and human NNREs. "NC" means that the homology was less than 4/6 and "no" means that were was no alignment between the two promoters in this region.

3.4. Localization of the fenofibrate responsive region in the promoter of the human cAspAT gene

In previous experiments, we have shown that fenofibric acid increases cAspAT activity and mRNA levels in HepG2 cells and that this effect is transcriptional [13]. To identify the DNA sequences involved in this regulation, we prepared deletion fragments of the 5′ upstream region of the promoter which were fused to the firefly luciferase reporter gene in the pGL3 vector. The plasmids were transiently transfected into the human hepatoma cell line HepG2. The basal activity of the various promoter fragments remained similar, except for the shortest construct (−74 to −6), the basal activity of which decreased by 50% (data not shown). Since HepG2 cells are known to contain little PPARα, we co-transfected the cells with the human PPARα expression plasmid or its empty vector pSG5 and analyzed the effect of fenofibric acid on the transcription of the various constructs. As a positive control, we used a pGL3 plasmid containing a consensus PPRE site upstream of the pLa2 promoter in front of the firefly luciferase gene (PPRE–pLa2FL). The HepG2 cells were treated, or not, with fenofibric acid for 24 hr and the luciferase activity was measured. For the PPRE–pLa2FL plasmid, the treatment with fenofibric acid resulted in 2- and 4-fold increases, respectively in luciferase activity with the pSG5 and the PPARα plasmids (the 2-fold increase with pSG5 is probably due to the presence of the endogenous PPARα) (Fig. 8A). When the various deletion constructs of the human cAspAT promoter were co-transfected with the empty vector pSG5, only a moderate increase of the reporter gene expression (1.55-fold at most) was observed after the fenofibric acid treatment. Co-transfection of the

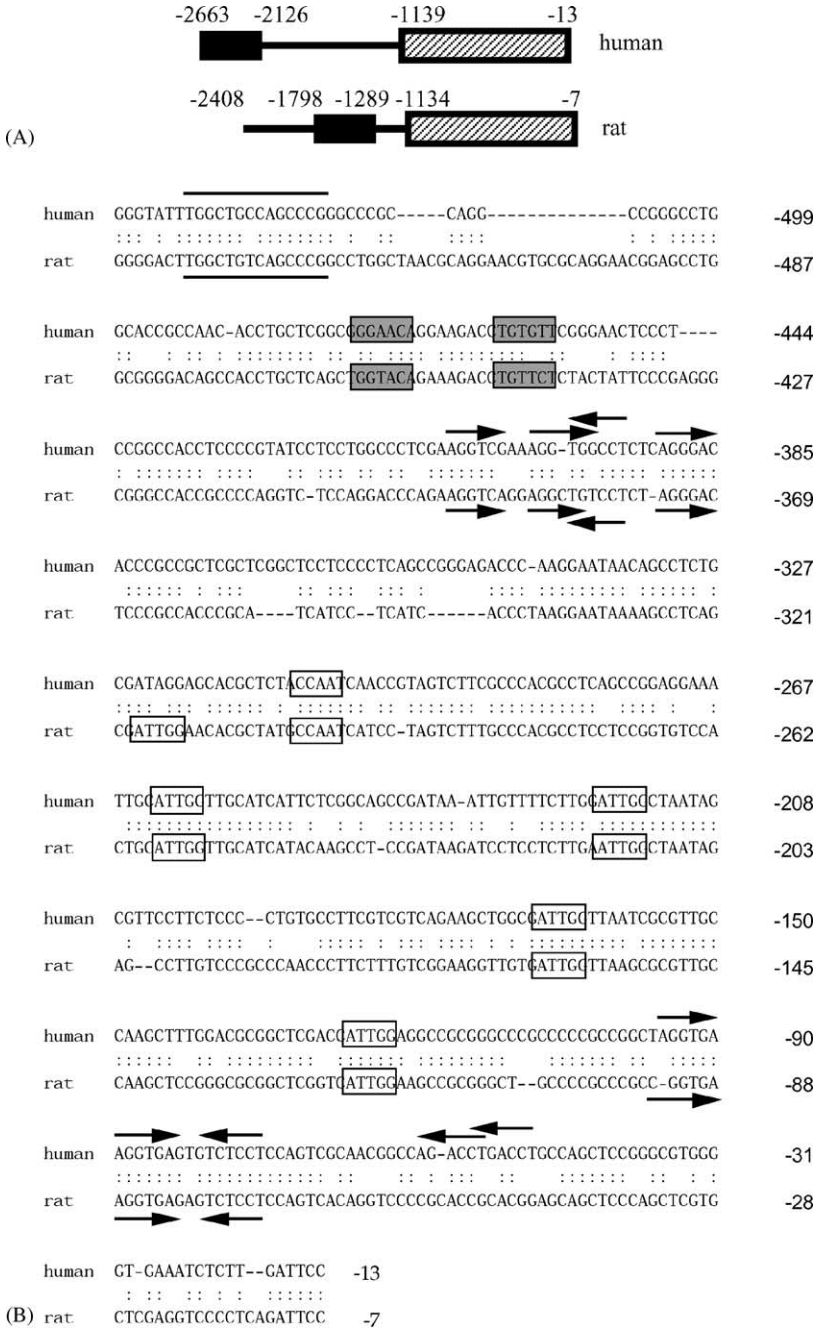

Fig. 7. Comparison of the proximal regions of the promoters of the human and rat cAspAT genes. (A) Schema of the homologies between the human and rat promoters (black box, 67%; striped box, 61%). (B) The nucleotide sequences of the 500 proximal bp of the 5′-flanking regions of the human and rat cAspAT gene were aligned. The CCAAT sequences are boxed, as well as the GRE half-sites (in gray). Putative NRREs (arrows) and the NF1 site (line) are indicated.

p(−2663/−6)hAspFL with the human PPARα expression plasmid led to a 2-fold increase in the luciferase activity after the fenofibric acid treatment (Fig. 8A and B). In contrast, when the other constructs were co-transfected with the human PPAR plasmid, none of them displayed an increased luciferase activity following the fenofibric acid treatment (Fig. 8B). Therefore, the fragment −2663/−706 of the promoter seems responsible for the fenofibric acid effect. Three perfect NNRE consensus AGG(T/A)CA are found at −952, −1672 and −2458.

3.5. Cell-type modulation of the fibrate effect

To assess whether the opposite effects of fenofibrate on the cAspAT gene in humans and rats are only due to the promoter sequences, we transfected either the rat or human cAspAT promoter into either H4IIEC3 or HepG2 cells. The rat p(−553/−26)CAT construct was transfected into the H4IIEC3 and HepG2 cells. Treatment of H4IIEC3 cells with fenofibrate reduced the CAT activity by 46%, whereas fenofibric acid treatment of HepG2 cells led to a 2-fold

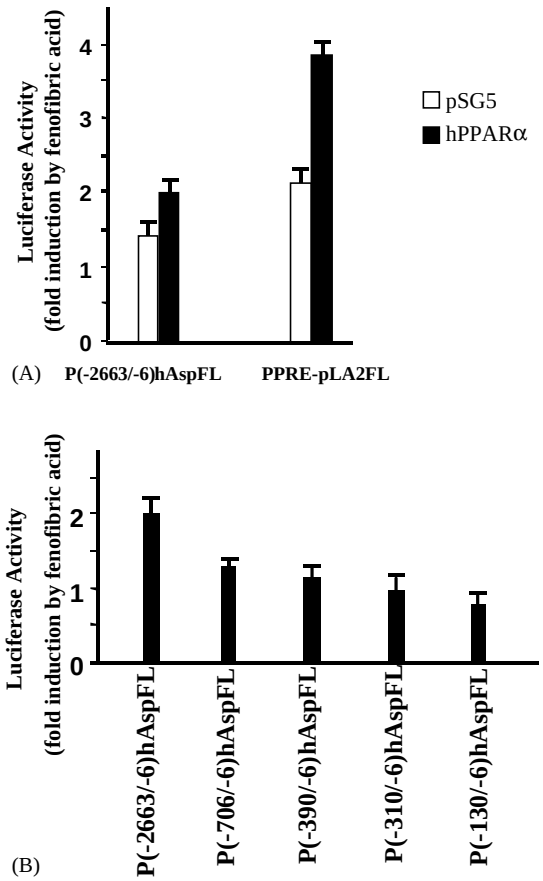

Fig. 8. Identification of the fenofibric acid responsive region in the human cAspAT gene promoter. (A) HepG2 cells were transiently co-transfected with PPRE–pLa2FL or p(−2663/−6)hAspFL plasmids (2 μg) in the presence of either the human PPARα expression plasmid or its empty vector pSG5 (250 ng) and treated with DMSO (0.1%) or fenofibric acid (200 mM) for 24 hr. The luciferase activity is the mean ± SEM of 2–13 independent experiments (100% corresponds to the luciferase activity in the presence of DMSO). (B) Constructs of the cAspAT promoter upstream of the luciferase gene were transiently co-transfected with either the human PPARα expression plasmid or its empty vector pSG5 into HepG2 cells and treated as in (A). The luciferase activity in the presence of the PPARα is the mean ± SEM of 5–14 different experiments.

increase in the CAT activity. When we transfected the human p(−2663/−6)pAspFL into HepG2 and H4IIEC3 cells, fenofibrate treatment of H4IIEC3 cells reduced the luciferase activity by 35% whereas fenofibric acid treatment of HepG2 cells led to a 2-fold increase in the luciferase activity (Fig. 9). Therefore, the nuclear receptors and/or co-effectors present in the cell contents may affect the regulation of the promoter by fibrates.

4. Discussion

In man, the serum levels of transaminases, such as AspAT, are widely used as markers of liver, heart and muscle disease. An increase in the serum level of AspAT is interpreted as reflecting its release from lysed cells. However, regulation of the expression of the cAspAT gene may alter cellular, and consequently, serum levels of this enzyme. This may account for some of the discrepancies between serum levels and the actual extent of cell damage. Few studies have addressed the regulation of cAspAT gene expression in man, particularly in liver cells. In the present study, we describe, for the first time, the cloning and the sequencing of the human cAspAT gene promoter, an essential step for understanding of its regulation and the opposing effects of fibrates in rats and men.

In the rat, the transcription of the cAspAT gene is regulated by various hormones and drugs [9,26]. It is not clear whether similar regulatory mechanisms are conserved in humans. In a previous study, we demonstrated that fibrates, used to treat hyperlipidemia in man, modify in opposite directions the levels of cAspAT mRNA in mice and humans, and that, in mice, the effect appears to be PPARα-dependent [13]. It is well known that, depending upon the species, fibrates may have opposite effects on the transcriptional regulation of several genes [27]. The most important species-dependent effects of fibrates are peroxisomal proliferation and, ultimately, the non-genotoxic hepatocarcinogenesis observed in rodents but not in humans.

In the rat promoter, a 267 bp responsive region (−553 to −286) mediates the repression by fibrates. In this sequence, no canonical PPRE can be found. However, several NRRE-like motifs are present within this region and only the −404/−366 sequence which includes three such motifs bound H4IIEC3 nuclear proteins. Several observations suggest that this sequence is critical for the effect of fibrates. Firstly, the mutation of any one of the three NRRE motifs rendered the cAspAT gene promoter unresponsive to fenofibrate. Secondly, these mutants were no longer able to bind nuclear proteins. Finally, the binding of nuclear proteins prepared from fenofibrate-treated H4IIEC3 cells was consistently diminished as compared to the binding of extracts prepared from control cells. Previously, using PPARα$^{-/-}$ mice, we reported that the negative effect of fenofibrate on the cAspAT gene promoter was PPARα-dependent [13]. To confirm the PPARα-dependent effect, we showed that various fibrates (fenofibrate, clofibrate and bezafibrate) and one specific PPARα agonist (Wy-14,643) inhibit the rat promoter. However, no direct binding of the PPARα/RXRα heterodimer to the −404/−366 sequence was observed. Several genes are regulated by PPARα without direct binding to a PPRE. Transcription factors, such as HNF-4 [23,28] and Rev-erbα [29] have been implicated in some cases. Since neither an anti-HNF-4 nor an anti-RXRα antibody was able to supershift or decrease the complex bound to probe II, the inhibition of the rat cAspAT promoter by fibrate probably does not involve HNF-4. The role of Rev-erbα is linked to its increased expression [29]. Since we observed a decrease in the intensity of the retarded complexes when H4IIEC3 cells are treated with fenofibrate, Rev-erbα is unlikely to be the factor involved in the inhibition of the rat cAspAT gene.

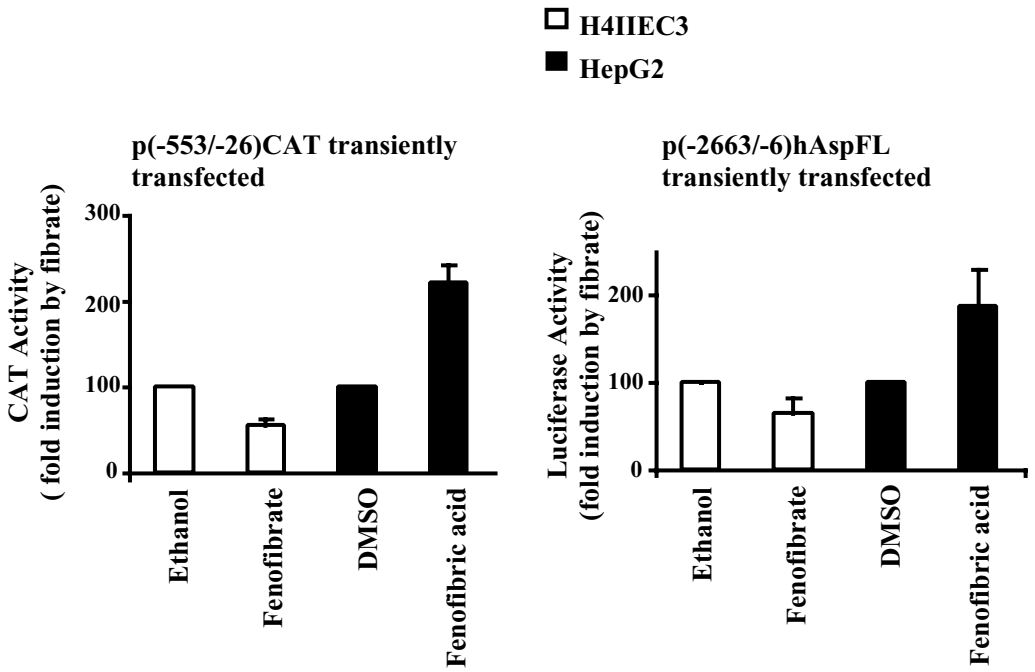

Fig. 9. The fenofibrate effect on the cAspAT gene is dependent upon the cell-type. The rat p(−553/−26)CAT and human p(−2663/−6)hAspFL plasmids were transiently transfected into H4IIEC3 and HepG2 cells. H4IIEC3 cells were treated for 24 hr with fenofibrate (200 μM) or ethanol (0.1%) whereas HepG2 cells were treated with fenofibric acid (250 μM) or DMSO (0.1%). The CAT and luciferase activities were expressed as the mean ± SEM of 3–12 independent experiments.

The inhibition of numerous liver genes after exposure to PPs suggests that their down-regulation could result from PPARα interaction with transcription factors or co-activators. Titration of c-*jun* and of GRIP/TIF2 by PPAR was correlated with the down-regulation of the glutathione transferase P and fibrinogen genes, respectively [30,31]. PPARα also interferes negatively with AP-1 and NFKB to inhibit the interleukine-6 gene expression [32]. In the case of the L-pyruvate kinase, although Wy-14,643 down-regulates the gene expression in a PPARα-dependent mechanism involving an HNF-4 binding site, the PPARα/RXRα does not bind to this site and, thus, an unknown factor is involved in this regulation [33]. Similarly, activation of the prolactin gene by PPARα is not dependent on DNA binding but rather on an interaction with other proteins [34]. Furthermore, PPs decrease the amount of endogenous LXR ligands by a mechanism not entirely PPARα-dependent which results in an alteration of the expression of LXR-controlled genes [35]. Our results concerning the repression of the rat cAspAT gene are in favor of an indirect PPARα-dependent effect, which may interfere with a liver transcription factor.

The activity of the human cAspAT gene promoter, in co-transfection assays with the PPARα expression plasmid, is increased 2-fold in response to fibrates, a result in agreement with the levels of cAspAT activity and mRNA in HepG2 cells [13]. The responsive region is localized at −2663/−706, which contains three putative NNREs. This region differs completely from the proximal region responsible for the inhibitory effect of fibrates in the promoter of the rat gene. More studies are needed to unravel the mechanism responsible for this fibrate effect on the human gene. The increase in human cAspAT gene expression by fibrates obtained in liver-derived cells may explain the observation that a moderate increase of serum transaminases is observed in some patients treated with such drugs, even in the absence of cell toxicity [36].

An important aspect of the effects of fibrates on cAspAT gene expression is the species specificity. The cellular environment and the promoter sequences appear to be key factors in these opposing actions. Therefore, quantitative and/or qualitative aspects may explain the species differences. In the case of the apolipoprotein A-I gene, fibrates have opposite effects, respectively decreased and increased apoA-I mRNA levels in the rat and man, due to differences in the *cis*-acting regions [29]. Another major difference is that liver PPARα is less abundant in poorly responsive species, such as man and the guinea pig, as compared to the mouse and the rat [37,38]. Thus, the low level of PPARα in man could account, in part, for the lack of peroxisome proliferation. It is also possible that the sensitivity of genes to PPARα is different and that the genes encoding the proteins involved in peroxisome proliferation may not respond when the receptor level is too low. However, several recent papers have demonstrated that increasing the amount of the human PPARα in HepG2 cells does not always result in concomitant increased sensitivity to the induction by PPs [39,40]. Moreover, Rodriguez *et al.* [41] have described that, in HepG2 cells treated with fibrates, the nuclear extract binding to the ACO–PPRE

is weak, in accordance with a lack of induction of the ACO mRNA. In this cell line, there is a high level of PPARβ which may heterodimerize with the RXRα and modify the activity of the PPARα.

In conclusion, the species specificity of the regulation by fibrates of cAspAT gene expression depends on several factors. Firstly, the drugs target different regions of the promoters, a proximal one in the rat gene promoter and a more distal one in the human promoter. Although the PPARα seems necessary for the regulation by fibrates of both promoters, it may act without binding directly to a PPRE in both cases. Secondly, the cell content of nuclear receptors and co-effectors, which are putative modulators of the PPARα activity, may also vary from one species to another and explain the regulation in opposing directions by the same class of compounds.

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