Identification of a non-canonical E-box motif as a regulatory element in the proximal promoter region of the apolipoprotein E gene

Enrique SALERO, Cecilio GIMÉNEZ and Francisco ZAFRA[1]

Centro de Biología Molecular 'Severo Ochoa', Facultad de Ciencias, Universidad Autónoma de Madrid-CSIC, 28049 Madrid, Spain

We have used the yeast one-hybrid system to identify transcription factors with binding capability to specific sequences in proximal regions of the apolipoprotein E gene (*APOE*) promoter. The sequence between −113 and −80 nt, which contains regulatory elements in various cell types, was used as a bait to screen a human brain cDNA library. Four cDNA clones that encoded portions of the human upstream-stimulatory-factor (USF) transcription factor were isolated. Electrophoretic-mobility-shift assays ('EMSAs') using nuclear extracts from various human cell lines as well as from rat brain and liver revealed the formation of two DNA–protein complexes within the sequence CACCTCGTGAC (region −101/−91 of the *APOE* promoter) that show similarity to the E-box element. The retarded complexes contained USF1, as deduced from competition and supershift assays. Functional experiments using different *APOE* promoter–luciferase reporter constructs transiently transfected into U87, HepG2 or HeLa cell lines showed that mutations that precluded the formation of complexes decreased the basal activity of the promoter by about 50%. Overexpression of USF1 in U87 glioblastoma cells led to an increased activity of the promoter that was partially mediated by the atypical E-box. The stimulatory effect of USF1 was cell-type specific, as it was not observed in hepatoma HepG2 cells. Similarly, overexpression of a USF1 dominant-negative mutant decreased the basal activity of the promoter in glioblastoma, but not in hepatoma, cells. These data indicated that USF, and probably other related transcription factors, might be involved in the basal transcriptional machinery of *APOE* by binding to a non-canonical E-box motif within the proximal promoter.

Key words: Alzheimer's disease, apolipoprotein E, E-box, gene regulation, nervous system, upstream regulatory factor (USF).

INTRODUCTION

Apolipoprotein E (ApoE) has a key role in the metabolism of plasma lipoproteins by serving as a ligand for the low-density-lipoprotein-receptor family [1]. ApoE is a single-chain polypeptide of 299 amino acids in which the presence of either arginine or cysteine at amino acid position 112 and 158 [2] defines three human ApoE variants: E2, E3 and E4 [3,4]. A number of previous *in vitro* and *in vivo* studies, as well as recent experiments with ApoE-deficient mice and human ApoE-gene (*APOE*) transgenic mice, reveal that ApoE plays an important role in neuronal maintenance and repair [5–8]. Genetic studies have demonstrated that ApoE4 is an important risk factor for late-onset Alzheimer's disease [9,10]. This allele is also responsible for poor outcome after brain injury [11,12], stroke [13] or neurotoxic damage [8].

Although the major site of synthesis is the liver, the protein is also produced in extrahepatic tissues, such as the adrenals and the nervous system. In the human brain, astrocytes are the major site of immunoreactive ApoE [14], particularly the Bergmann glia of the cerebellum, but it is also found in subpopulations of neurons in the cortex and the hippocampus [15]. Several studies have demonstrated changes in astrocyte ApoE production in response to neurodegenerative insults in the hippocampus [16], or in response to inflammatory mediators [17]; however, the regulation of this gene remains largely unexplored in the brain, in spite of its importance in process of degeneration and regeneration of the nervous system. Previous studies demonstrated that transcriptional regulation of the human *APOE* gene is influenced by multiple regulatory elements in its promoter. Regulatory elements were identified at nucleotides −193 to −124 upstream of the first exon [named region URE1 (upstream regulatory element 1)], at nucleotides −366 to −246 (URE2) and a functional GC box at −59 to −45 [18,19]. In addition, a DNase I footprint was identified at −103 to −87 (named URE3) [18–20] by using nuclear extracts from various cell lines. However, the nature of the transcription factors that bind to these regions is poorly defined.

The importance of the regulatory region of the *APOE* gene in the production of ApoE in the brain is emphasized by the recent identification of a number of polymorphisms within the promoter [21,22]. Because some of these polymorphic sites are associated with increased risk of Alzheimer's disease [23,24], we have investigated the regulatory elements that specify its expression in neural cells. Previously we identified the transcription factor AP-2 as a mediator of the cAMP stimulation of ApoE synthesis in glial cells [25]; moreover, we have shown that transcription factors Zic1 and Zic2 bind and transactivate the apolipoprotein E gene promoter in glioblastoma cells [26].

Upstream stimulatory factor (USF) is a ubiquitous transcription factor that belongs to the class B proteins of the basic helix–loop–helix (bHLH) family. It also contains a leucine zipper that contributes to dimerization of the protein. USF was first identified as a stimulator of transcription from the adenovirus late promoter [27] and was purified as two polypeptides of 43 and 44 kDa termed USF1 and USF2 [28]. USF1 and USF2 bind to the E-box motif (CACGTG) as mono- and hetero-dimers [29]. Other transcription factors, including Myc, Max, transcription factor E3 (TFE3), TFEB, TFEC, as well as transcriptional repressors of the Mad family and Mnt, bind to the E-box motif.

Abbreviations used: AD, Alzheimer's disease; ApoE, apolipoprotein E; *APOE*, human apolipoprotein E gene; bHLH, basic helix–loop–helix; DN-USF, dominant-negative form of USF1; EMSA, electrophoretic-mobility-shift assay; His-USF1, histidine-tagged USF1 protein; TFE, transcription factor E; URE, upstream regulatory element; USF, upstream stimulatory factor.

[1] To whom correspondence should be addressed (e-mail fzafra@cbm.uam.es).

The E-box motif is present in a large number of genes; however, how a given response element can discriminate among all these different transcription factors is currently unclear. In this respect, several mechanisms have been proposed and, in some instances, experimentally substantiated (for a review, see [30]). For instance, deviations from the consensus sequence found in non-canonical sites might allow preferential binding of a subset of complexes. Also the flanking sequences, chromatin structure, methylation status, relative position within the promoter or interaction with adjacent regulatory elements might contribute to selection of a particular complex [30]. USF has been involved in the basal transcription of a number of genes, including genes for rat γ-fibrinogen [31], murine metallothionein I [32], human CD2 [33], human β-globin [34] and rat amyloid β-protein precursor [35], among others. USF also plays a role in the signal-induced regulation of the L-pyruvate kinase gene [36] or the fatty acid synthase [37].

In the present study we searched for transcription factors with binding capability to the -113 to -80 sequence of the *APOE* gene, a region that contains the above-mentioned regulatory element URE3 [18–20,37,38]. We have localized a functional E-box-like element within this region, and identified transcription factor USF1 as a transcriptional activator for *APOE* gene in the astroglial cell line U87.

EXPERIMENTAL

Reporter constructs for library screen

The oligonucleotides TCGGGAGAACAGCCCACCTCGTGACTGGGGGCTGGC and CCGAGCCAGCCCCCAGTCACGAGGTGGGCTGTTCTC, containing the URE3 region [18–20] of the *APOE* promoter, were synthesized and annealed. The annealed oligonucleotide displayed overhanging ends (underlined) to promote oriented oligomerization upon ligation in the plasmid pAEA [39]. A fragment containing five tandem repeats was subcloned into the yeast reporter plasmids, pHIS-1 and pLacZi (Clontech, Palo Alto, CA, U.S.A.), yielding plasmids APOE3-pHIS-1 and APOE3-pLacZi respectively. The reporter constructs were subsequently linearized and sequentially integrated into the genome of the yeast strain YM4271. Yeast was transformed first with APOE3-pLacZi, followed by APOE3-pHIS-1. Transformants selected on Leu^-/Ura^- minimal medium (i.e. lacking leucine and uracil) were used as a dual reporter host yeast strain for the library screening.

Screening of the cDNA library

The host yeast strain was transformed with a MATCHMAKER human brain cDNA library constructed in the pGAD10 vector (Clontech) by the lithium acetate/poly(ethylene glycol) method (http://www.clontech.com/techinfo/manuals/PDF/PT3024-1.pdf). Approx. 5×10^4 transformants were plated per 150-mm-diameter dish containing His^-/Leu^- minimal selective medium supplemented with 15 mM 3-aminotriazole. Approx. 2.5×10^6 cDNA plasmids were screened. On the basis of large colony size and rapid growth, a total of eight positive clones were selected. These clones were tested for β-galactosidase activities using the filter replica method. Four clones showed a strong blue colour compared with the host strain. Plasmids were recovered from $His^+/LacZ^+$ colonies by transformation into DH5α cells. The plasmids were partially sequenced and the nucleotide sequences were compared with sequences in the GenBank®/EMBL databases using the BLAST program.

Purification of recombinant histidine-tagged USF1 protein (His-USF1)

To produce His-USF1 we prepared a bacterial expression construct by transferring cDNA inserts from the yeast pGAD10 vector into the *Bam*H1/*Bgl*II sites of the prokaryotic expression vector pTrcHisA (Invitrogen Inc., San Diego, CA, U.S.A.). Expression of His-USF was induced in the *Escherichia coli* strain BL21and purified with Ni^{2+}-nitrilotriacetate resins (Qiagen Inc, Valencia, CA, U.S.A.) using a batch protocol as recommended by the manufacturer.

Electrophoretic-mobility-shift assay (EMSA)

Oligonucleotides were 5′-end-labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Recombinant His-USF1 or nuclear extracts (0.5–1 μg) were incubated for 15 min at room temperature in 20 μl of binding buffer [Tris/HCl (pH 7.6)/100 mM NaCl/1 mM $MgCl_2$/0.5 mM EDTA/1 mM dithiothreitol, supplemented with 2 μg of poly(dI-dC) · poly(dI-dC)/assay]. Where indicated, competitor oligonucleotides were included during the preincubation period. In supershift assays, antibodies against USF1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) were also included during the preincubation, the length of which was extended to 40 min. Labelled oligonucleotide (1 ng/binding reaction, 100000–200000 c.p.m.) was then added, and the mixture was incubated for 30 min at room temperature. The incubation mixture was electrophoresed on 4% polyacrylamide gels containing 0.5 × TBE (45 mM Tris/borate/1 mM EDTA) buffer. Gels were dried and autoradiographed.

Plasmid constructions

The luciferase reporter plasmid pXP2 [40] was used to harbour different fragments of the *APOE* promoter. The fragments were generated by PCR using oligonucleotides from the desired regions as primers and the APOE-pCRII construct [25] as a template. Amplified fragments were ligated to the pCRII vector (Invitrogen), and the identity was confirmed by sequencing. Fragments were subcloned in the MCS of pXP2 in front of the luciferase reporter gene. Mutations were introduced by PCR by using mutant oligonucleotides as previously described [41]. A dominant-negative form of USF that contained an internal deletion of amino acids 200–212 [42] was derived from the wild-type USF1 by PCR, the method of Higuchi [41] being followed.

Cell culture and transfections

U87, HepG2 or HeLa cells were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal-bovine serum. Transfections were performed with 0.5 μg of DNA per well in 24-well tissue-culture plates by using the CalPhos™ Mammalian Transfection kit (Clontech) according to the instructions of the manufacturer.

Luciferase and β-galactosidase assays

Cells were harvested on day 2 following transfection with 150 μl of a lysis buffer containing 25 mM Tris/phosphate, 2 mM dithiothreitol, 2 mM EDTA, 10% (v/v) glycerol, 1% Triton X100. Luciferase was measured using the Luciferase Assay System (Promega, Madison, WI, U.S.A.) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA, U.S.A.) by incubation of 40 μl of cell extract with 90 μl of luciferase assay reagent as recommended by the manufacturer.

β-Galactosidase was determined in a 96-well microtitre plate by incubating 20 μl of cellular extract with 20 μl of a solution containing 3 mg/ml *o*-nitrophenyl β-D-galactopyranoside. Relative luciferase activity was calculated as the ratio between luciferase and β-galactosidase activities.

RESULTS

Isolation of cDNA clones encoding an *APOE*-promoter-binding protein

We were interested in identifying transcriptional factors that regulate the expression of the *APOE* gene. Since sequences in the proximal regions of the *APOE* promoter have been demonstrated to be functionally important for the regulation of the ApoE expression, we attempted to clone new transcription factors capable of binding the 5′-flanking region upstream of the TATA box of the *APOE* gene (Figure 1A). In the present study we selected the −113/−80 region, as deletion analyses and DNA footprinting experiments had previously revealed the presence of regulatory sequences and protein binding within this region of the promoter. We employed the yeast one-hybrid assay to screen a human brain cDNA library. Five tandem copies of the duplex DNA containing the sequence spanning −113 to −80 of the *APOE* promoter (region URE3) were linked to a minimal HIS3 promoter either in pHIS1 or in pLacZi reporter plasmids and integrated into the genome of the yeast strain YM4271 (*his3*, *leu2*) (HIS3 is the reporter gene conferring prototrophy on yeast). A hybrid expression library (MATCHMAKER) consisting of human brain cDNA fused to the GAL4 activation domain was then screened to identify proteins that bound to the *APOE* promoter fragment and activated HIS3 transcription from the reporter construct. Transformants growing on selective medium were assayed for β-galactosidase expression. After screening 2.5×10^6 independent colonies, eight colonies grew on His^-/Leu^- selective medium. Four of them produced strong blue colour on filter assay after 1 h of incubation in the presence of 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-Gal'). Plasmids of these transformants were then recovered and sequenced. Sequence analysis revealed that all four clones encoded to different portions of the same USF1 transcription factor and were in-frame with the activation domain of GAL4. The two longest clones encoded proteins missing the 34 N-terminal amino acids.

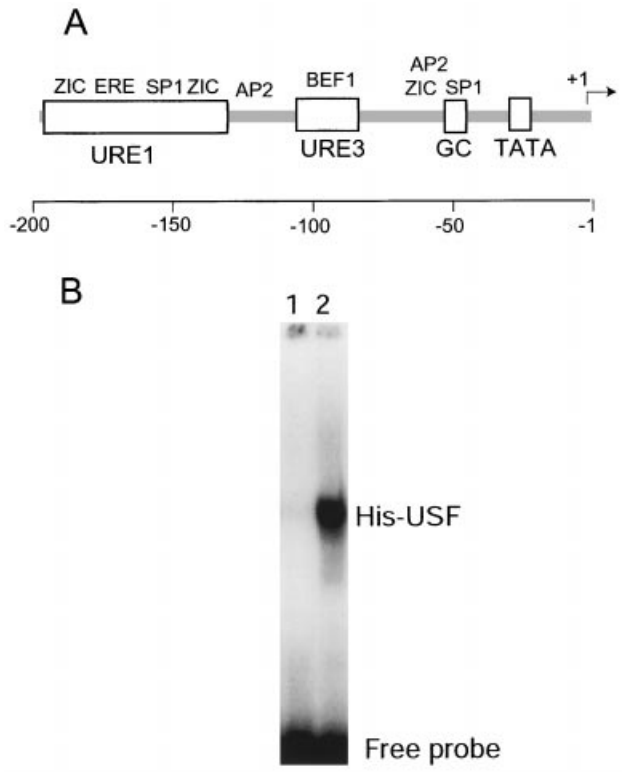

Figure 1 Binding of recombinant His-USF to the −113/−80 ApoE promoter sequence

(**A**) Diagram of the proximal *APOE* promoter showing the location of some putative regulatory elements. TATA, the TATA box element; GC, the GC box element; URE1 and URE3 have already been defined [18,20]; ERE, oestrogen response element. Binding sites for transcription factors SP1 (simian-virus-40 protein 1), AP2 (activator protein 2), ZIC (zinc finger of the cerebellum) and BEF-1 (BK virus enhancer factor-1) are indicated. (**B**) The gel-mobility-shift assay was performed with 1 ng of ^{32}P-labelled −113/−80 DNA fragment incubated with 500 ng of bacterial control extract (lane 1) or purified His-tagged USF (lane 2).

USF1 binds to the *APOE* promoter

To confirm the presence of USF1-binding sites in the *APOE* gene promoter, we first produced His-tagged USF1 proteins by transferring the USF1-encoding inserts from the yeast vector pGAD10 to the prokaryotic expression vector pTrcHisA. The recombinant proteins were then purified on nickel-containing resins. The binding activity of the recombinant protein was assayed by EMSA using as a probe the radioactively labelled oligonucleotide covering bases −113/−80 (Figure 1B). No bands of retarded mobility were observed in the absence of recombinant protein, whereas a retarded band was detected in the presence of purified His-tagged USF1.

Next we analysed the presence of USF binding activity by EMSA in nuclear extracts derived from diverse cell lines and tissues, using as a probe the double-stranded bait oligonucleotide. When labelled −113/−80 was incubated with HeLa nuclear extracts, several retarded complexes were apparent (Figure 2, lane 2). The two upper bands (complex 1/2) were competed for by a molar excess of unlabelled double-stranded −113/−80 oligonucleotide (lane 3), as well as by the shorter −107/−88 oligonucleotide (lane 4) and by an oligonucleotide containing the consensus E-box sequence (lane 5), but not by comparable amounts of an oligonucleotide derived from the adjacent *APOE* promoter region (−163/−124) (Figure 2, lane 6). No displacement was observed by other unrelated oligonucleotides (results not shown). The nature of the lower bands observed with this probe (Figure 2, arrowheads) is unclear, as they were displaced by −113/−80 and −107/−88 oligonucleotides, but also by the unrelated oligonucleotide −163/−124. These bands were partially displaced by the USF consensus oligonucleotide.

When we used as a probe a labelled oligonucleotide containing the E-box consensus sequence (Figure 2, lanes 7–12), we observed the presence of two complexes with a pattern of displacement similar to that described above: both the −113/−80 and −107/−88 oligonucleotides displaced the binding and, as expected, these later complexes were also displaced by a molar excess of the unlabelled E-box oligonucleotide, but not by the unrelated one (Figure 2, lanes 9–12).

To confirm that USF1 is a component of the observed complexes, we performed supershift assays by incubation of probe −107/−88 and USF1 antibodies with nuclear extracts derived from HeLa cells, U87 glioblastoma cells, HepG2 hepatoma cells, rat brain or rat liver (Figure 3A). The two complexes observed in the absence of antibodies for all the three extracts

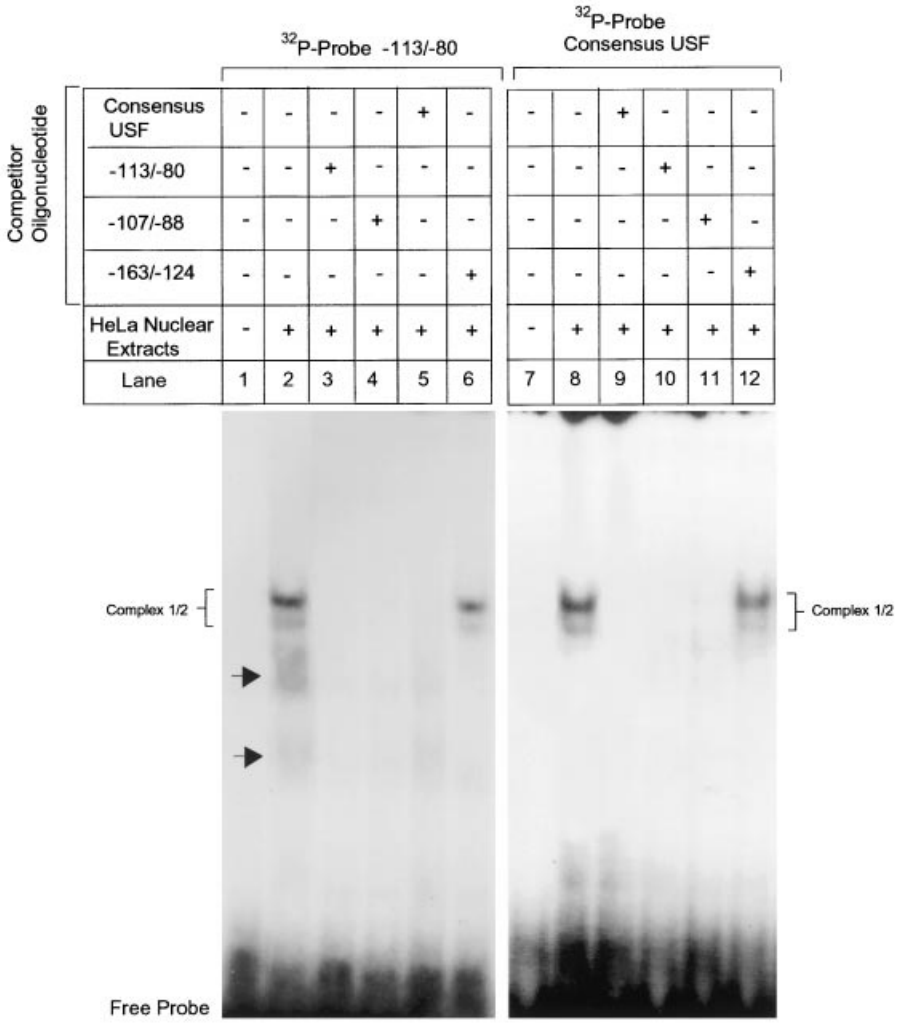

Figure 2 Interaction of nuclear extracts of HeLa cells with −107/−88 fragment of *APOE* promoter and with E-box consensus oligonucleotide

EMSAs were performed with 1 ng of double-stranded ^{32}P-labelled probe (−113/−80 *APOE* DNA fragment, or E-box consensus oligonucleotide) incubated with 500 ng of HeLa nuclear extracts in the absence (lane 2 and lane 8) or the presence of a 100 M excess of the indicated unlabelled competitor oligonucleotides. These oligonucleotides correspond to the consensus sequence for USF binding, or to the regions −113/−80, −107/−88 and −163/−124 of the *APOE* gene promoter (lanes 3–6 and 9–12). Lanes 1 and 7 correspond to the probes in the absence of nuclear extracts. Note that formation of Complex 1/2 was prevented by excess of oligonucleotides covering the URE3 region of *APOE* gene as well as by the E-box consensus oligonucleotide. The nature of the higher-mobility bands (arrowheads) is uncertain (see the text).

(lanes 1, 3, 5, 7 and 10, complexes 1/2) disappeared and, instead, new complexes were now evident (lanes 2, 4, 6, complex 3). The smeared appearance of these bands probably reflects the presence in the retarded complexes of both homo- and hetero-dimers containing USF1 that would bind different amounts of antibody. These supershifted complexes were not observed in the presence of an unrelated control antibody (Figure 3A, lanes 9 and 12). The band pattern observed with the APOE probe was identical with that observed using the E-box consensus sequence as a probe and nuclear extracts of HeLa, HepG2 or U87 cell lines (Figure 3B). Within this region, the sequence $^{-101}$CACCTCGTGAC^{-91} presents a single mismatch when compared with the USF binding sequence in the adenovirus major late promoter CACGTGAC. Alternatively, this APOE sequence can be view as identical with the consensus with the insertion of the nucleotides CTC between the CAC and GTG sequences of the consensus E-box.

Delineation of nucleotide sequences required for USF binding

To determine more precisely the nucleotide sequence required for binding of USF1 to the *APOE* promoter we tested the binding ability of cerebellar nuclear extracts to a series of mutant oligonucleotides derived from the −107/−88 sequence, as indicated in Figure 4. Mutation of $^{-101}$CAC^{-99} to AAA (mutant 1, lane 2) decreased considerably the binding of USF to the probe. Mutation of $^{-98}$CTC^{-96} to GTG did not affect the binding (mutant 2, lane 3), probably because this mutation created a new adjacent consensus E-box, and a mutation of this triplet $^{-98}$CTC^{-96} to TTT precluded the binding (mutant 3, lane 4). Binding was also impaired by mutation in 3′ end of this core sequence: mutants 4 and 5 (lanes 5 and 6). Together, these results indicate that USF binds to this atypical E-box located in the −101/−91 region of the *APOE* promoter.

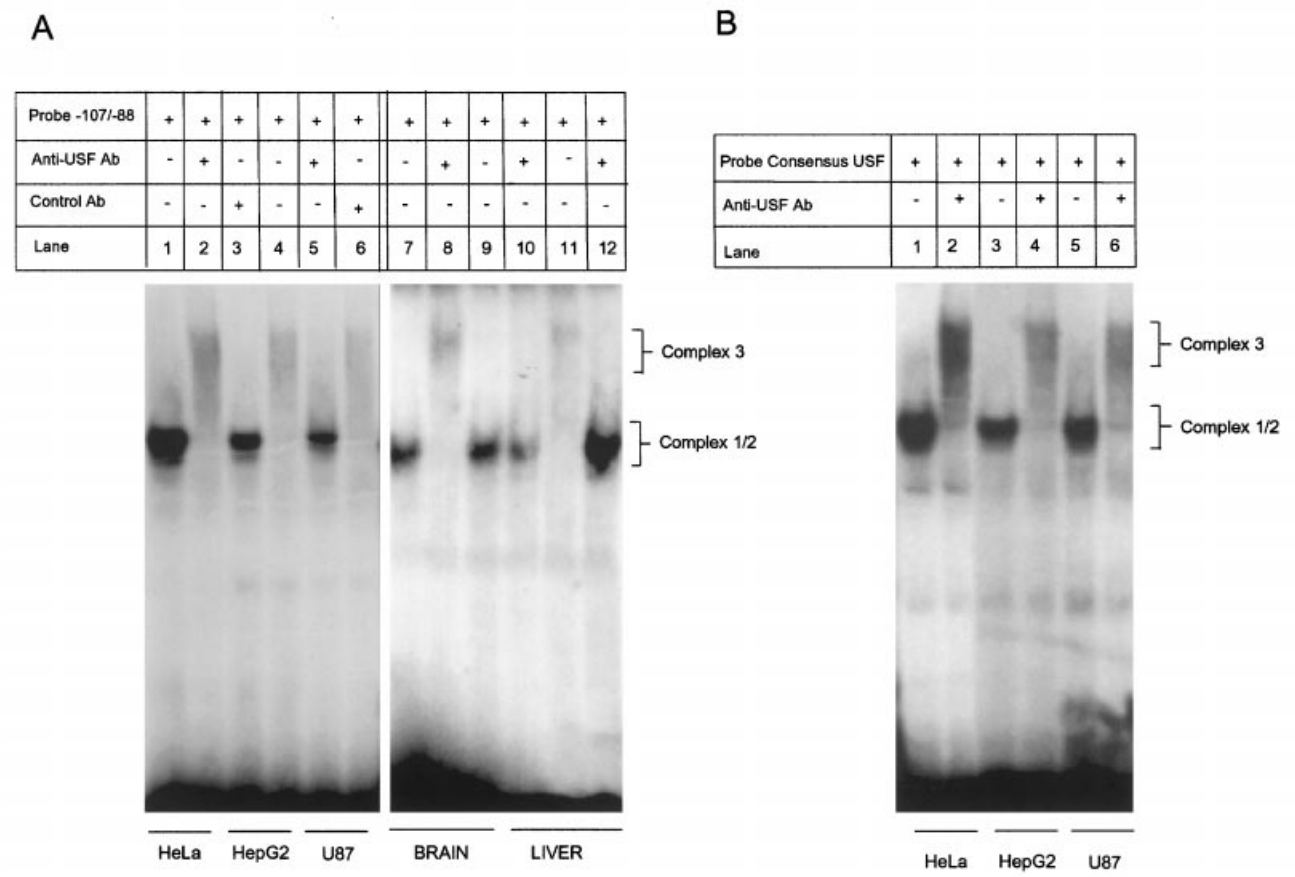

Figure 3 Supershift EMSAs of USF binding

Either double-stranded ^{32}P-labelled $-107/-88$ probe (**A**) or USF consensus (**B**) was incubated with nuclear extracts (500 ng) derived from HeLa cells, HepG2 cells, U87 cells, rat brain or rat liver, as indicated in the Figure, in the absence or the presence of antibodies (Ab) against USF. The specific complexes formed in the absence of antibody (lanes 1, 3, 5, 7 and 10, complex 1/2) disappeared in the presence of the antibody, and instead a supershifted smeared complex appeared (lanes 2, 4, 6, 8 and 11, complex 3). Where indicated, unrelated control antibody was included during the incubation (lanes 9 and 12).

Role of the E-box-like element and USF1 on the *APOE* promoter function

To analyse the functional role of the atypical E-box and that of USF1 on the *APOE* promoter, several *APOE* promoter–luciferase constructs were prepared and transiently transfected either in U87 human astrocytoma, or in HepG2 human hepatoma cells. Along with the test constructions, each plate was co-transfected with a β-galactosidase expression vector, which served as an internal reference for efficiency of transfection. Luciferase and β-galactosidase activities were determined 48 h later and the relative luciferase activity was calculated as the ratio between luciferase and β-galactosidase activities. Mutations of the identified E-box in the context of a $-189/+1$-luciferase construct reduced the basal activity of the promoter by 40 and 50 % in U87 and HepG2 respectively, as compared with the controls. These results indicate a role of the atypical E-box in the regulation of APOE in these cell types. When an expression vector for USF1 was co-transfected with different APOE constructs (Figure 5B) we observed an increase of 5.9-fold over the control value in the context of the $-189/+1$-luciferase construct. The stimulation was decreased to 2.7-, 2.2-, and 1.3-fold in constructs where the E-box was absent ($-70/+1$, $-60/+1$ and $-40/+1$-luciferase plasmids respectively). In the construct $-189/+1$m(E-box)-Luc, where only the E-box had been eliminated, overexpression of USF1 in U87 still increased the activity of the promoter 3.6-fold (Figure 6A), indicating that most of the effect of the overexpressed USF1 was independent of binding to the E-box-like motif. These effects of USF1 were cell-type-specific, as overexpression of USF1 in HepG2 cells did not increase the activity of the promoter (Figure 6B).

To clarify the role of USF and that of the atypical E-box on the activity of the *APOE* promoter, we used a dominant-negative (DN) form of USF1 (DN-USF). This mutant lacking the DNA-binding domain can dimerize with native USF and blocks its activity [41]. Overexpression of DN-USF in U87 cells decreased the luciferase activity of the $-189/+1$-Luc construct by to 61 % of the control (black bars in Figure 6A). Instead, when the transfection was performed with the construct mutated in the E-box [$-189/+1$m(E-box)-Luc] (white bars in Figure 6A), the overexpression of DN-USF produced a slight increase in luciferase activity. These results indicate that the endogenous USF1 regulates the activity of the promoter through the E-box-like motif. These effects were again cell-type-specific, as the DN form of USF1 did not affect the promoter activity in HepG2 cells (Figure 6B).

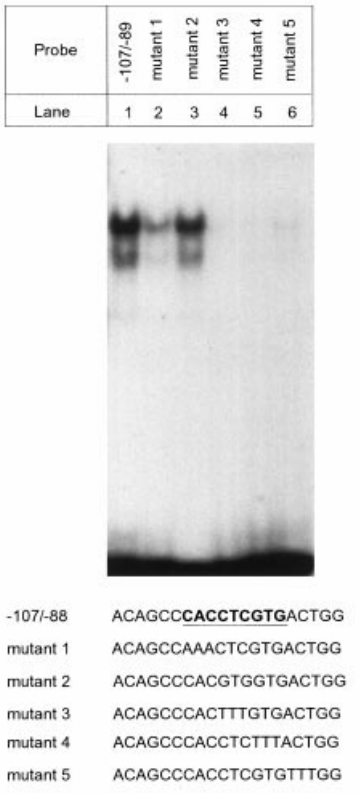

Figure 4 Mutational analysis of USF binding to the −107/−88 region of the *APOE* promoter

EMSAs were performed by incubation of 1 ng of the indicated ^{32}P-labelled DNA fragments with 500 ng of HeLa nuclear extract. Sequences of the −107/−88 probe and mutations introduced are shown. The sequence of the non-canonical E-box is underlined and shown in **bold**.

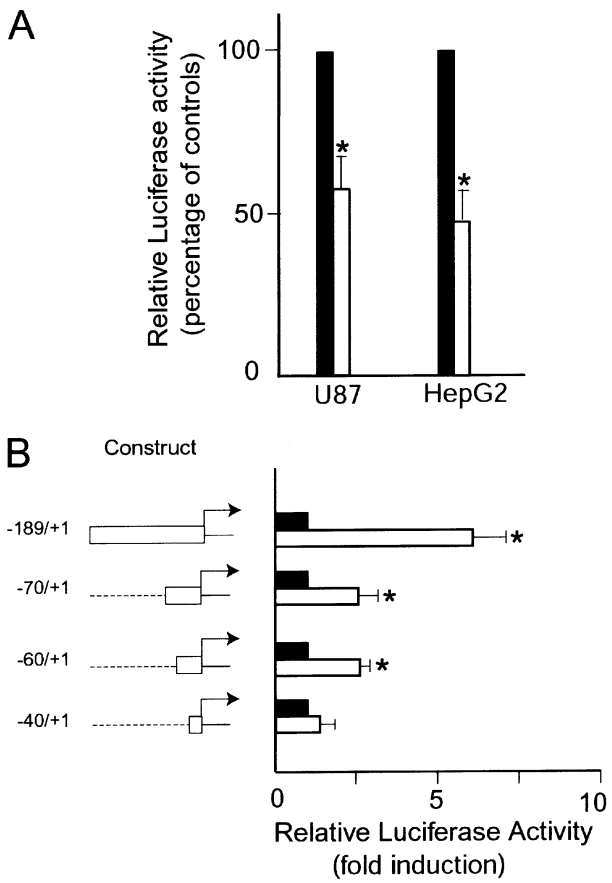

Figure 5 Role of the E-box-like element and USF on the *APOE* promoter activity

(**A**) U87 or HepG2 cells were transiently transfected with the wild-type −189/+1-luciferase construct (black bars) or with a −189/+1-luciferase construct mutated in the E-box-like element (^{95}GCG^{93} mutated to AAA) (white bars) and a β-galactosidase expression vector. Luciferase activity was determined 48 h later and normalized for transfection efficiency as measured by β-galactosidase activity assay. Normalized luciferase activity is presented as a percentage of the promoter activity of the wild-type construct. (**B**) U87 cells were transiently transfected with the indicated *APOE* promoter-luciferase constructs, a β-galactosidase expression vector and the empty pN3 vector (black bars) or the USF expression vector pN3-USF (white bars). Luciferase activity was determined 48 h later and normalized to β-galactosidase activity. For each construct the results are relative to values measured in pN3-transfected cells. Values represent means ± S.E.M. for four separate experiments each performed in triplicate. *$P < 0.05$.

DISCUSSION

In the present study we have identified the sequence CACCTCGTGAC, spanning nucleotides −91 to −101 of the *APOE* gene, as a functional promoter element in several cell types. This sequence is located within the formerly defined URE3 region of the promoter [34–37], and presents similarities to the consensus E-box element CACGTG located in a number of genes [43]. Indeed, by using the yeast one-hybrid genetic-selection approach to screen a human brain cDNA library for sequences encoding DNA-binding proteins that can recognize the URE3 region of the *APOE* gene, we isolated a human cDNA that encodes for the transcription factor USF. EMSA studies confirmed the binding to the above-mentioned core sequence of USF present in nuclear extracts derived from diverse human cell lines as well as from rat brain and liver. Mutations within this core sequence suppressed USF binding. USF is a member of the bHLH family of transcription factors that binds DNA *in vivo* as homo- or hetero-dimers of USF-1 and USF-2 to the E-box element [43]. In addition to the consensus E-box, USF proteins bind to other related sequences, including CGCGTG [28], CCCGTG [27], CAGCTG [44], CACCTG [30], CACATG [33], or CAGATG [45], all of them differing from the core CTCGTG that we have found in the *APOE* promoter. Our functional data indicate that mutations in this sequence of the *APOE* gene decrease the basal activity of the promoter in all the cell types assayed. However, in spite of the presence of a capacity of USF to bind to the *APOE* promoter in all the nuclear extracts assayed in the present study, our functional data indicate that the effect of USF is cell-type-specific. Thus a DN form of USF that dimerizes with native USF, precluding binding to DNA [41], decreased the basal activity of the promoter in U87 astrocytoma cells, but not in hepatoma HepG2 cells. These data suggest that, in HepG2 cells, a transcription factor other than USF must bind to the atypical E-box. Indeed, many other transcription factors of the bHLH family bind to E-box-like elements, including c-Myc, Max, TFE3, TFEB, or SREBP1 [43]. Previous studies indicate that other proteins might bind to this region of the promoter. Jo et al. [20] purified a 67 kDa URE3-binding protein from human placenta with affinity for the −101 to −89 region. The protein was not further characterized, but its molecular mass is clearly higher than that of USF. This region of the promoter is also partially overlapping the binding site for the transcriptional

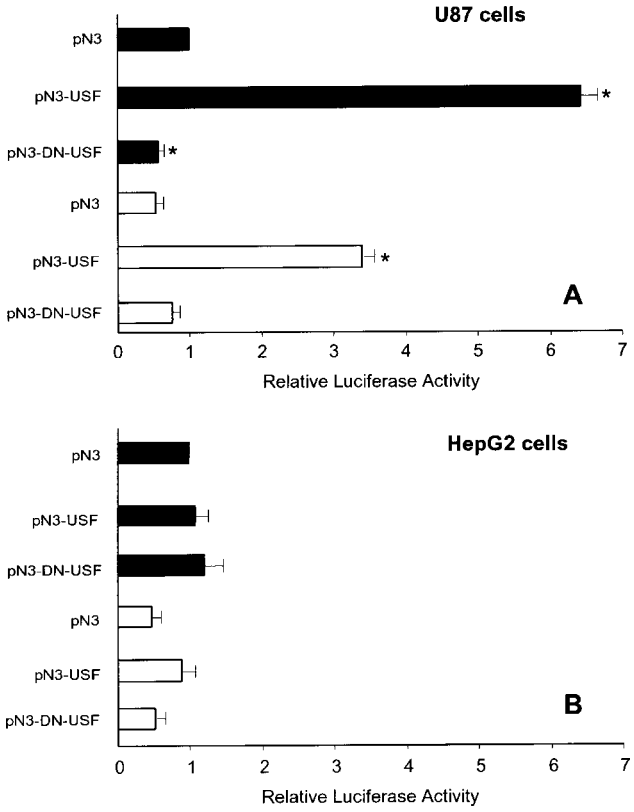

Figure 6 Effect of USF and a dominant negative mutant of USF on the *APOE* promoter activity in U87 and HepG2 cells

U87 cells (**A**) or HepG2 cells (**B**) were transiently transfected with the wild-type −189/+1-luciferase construct (black bars) or with the E-box-mutated 189/+1 construct (white bars), a β-galactosidase expression vector and the empty pN3 vector (pN3) or the USF expression vector pN3-USF (pN3-USF) or the DN expression vector pN3-DN-USF (pN3-DN-USF). Luciferase activity was determined 48 h later and normalized to β-galactosidase activity. For each construct the results are relative to values measured in cells transfected with the wild-type −189/+1-luc construct and empty pN3. Values represent means ± S.E.M. for four separate experiments each performed in triplicate. $^*P < 0.05$.

repressor BEF-1, a protein that binds to the −94 to −84 sequence in HepG2 cells that could mediate the regulation of the *APOE* gene by cytokines [46].

Although USF is a ubiquitous factor, it has been involved in the transcription of genes with tissue specificity. Thus it is conceivable that USF could regulate the *APOE* gene in astrocytoma cells either by heterodimerizing with other glioma-specific factors, or by being incorporated into a complex of transcription factors including co-activators, whose stereospecific assembly allows cell-restricted gene regulation, as has been proposed by Qyang et al. [47]. In this context, it has recently been shown that the glial-specific expression of ApoE is governed by two distal enhancer sequences, named ME-1 and ME-2, that are located 3.3 and 15 kb respectively downstream of the *APOE* gene [48]. It is noteworthy that, within this enhancer, there is a perfect consensus E-box sequence. Although the functionality of this sequence remains to be determined, it is reminiscent of that reported for the *APOCII* gene, where USF bound to the proximal promoter interacts with a second USF bound to a distal enhancer to promote transcription of this gene in a synergistic manner [49].

Exogenous overexpression of USF also increases the level of transcription from the *APOE* promoter in a cell-specific manner, being observed only in U87 cells, but not in HepG2 cells. Nevertheless, much of the effect of overexpressed USF was independent of binding to the atypical E-box and mapped upstream of nucleotide −40. USF has been shown to bind to the pyrimidine initiator sequence ('Inr') in a number of genes [50]. This is a quite loose sequence that would be compatible with the pyrimidine-rich sequence found in region −80 to −60 of the *APOE* promoter.

ApoE plays a crucial role in many physiological processes, including cholesterol transport in the peripheral circulation and in the central nervous system. ApoE is also involved in the response to neural injury, maintenance of dendritic arborizations and neuronal remodelling *in vitro*, as well as in Alzheimer's disease (AD) (see [1] for a review). The recent association of different polymorphisms in the promoter region with AD [20,21] strongly suggests that transcriptional regulation may play an important role in the development of this deleterious disease [18]. Thus identification and characterization of the transcriptional machinery involved in the regulation of ApoE may be relevant for the development of a rational pharmacology of AD.

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