Transcriptional regulation of apolipoprotein E expression by cyclic AMP

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Abstract Incubation of HepG2 cells in the presence of dibutyryl cAMP (db-cAMP), a cell permeable analogue of cyclic AMP, or forskolin, an agent which elevates intracellular cAMP, resulted in a 50% decrease in apoE mRNA levels within 24 h. Results of nuclear run-on transcription assays showed that db-cAMP down-regulates apoE gene expression at the transcriptional level. By transfection analysis with a plasmid containing the −614/+804 human apoE gene fused to the secreted placental alkaline phosphatase (SPAP) reporter gene, we showed that the SPAP activity was decreased by 50% when HepG2 cells were incubated in the presence of db-cAMP or forskolin, indicating that this promoter region mediated this negative effect. In contrast, when the smaller fragment −200/+1 of apoE promoter was linked to the CAT reporter gene, db-cAMP treatment of HepG2 cells resulted in a 2-fold increase in CAT activity, suggesting that positive cAMP-responsive elements were present in the proximal apoE promoter. These data indicate that transcriptional modulation of apoE gene expression by agents known to elevate the intracellular cAMP level is complex and involves several negative and positive elements located in the −614 to +804 region of the apoE gene whose global effect is negative on apoE gene transcription.

Key words: Apolipoprotein E; Gene expression; Cyclic AMP

1. Introduction

Apolipoprotein E (apoE) is a major structural component of various classes of mammalian lipoproteins, including chylomicron remnants, very low density lipoproteins, intermediate density lipoproteins, and high density lipoproteins. ApoE plays an important role in the redistribution of cholesterol and other lipids between peripheral tissues and the liver. It mediates the cellular uptake of specific lipoproteins, especially chylomicron remnants and intermediate density lipoproteins, via the interaction with low density lipoprotein receptors and distinct hepatic receptors [1]. Transgenic mice that overexpress apoE [2] or present apoE deficiency [3] have provided valuable information about the metabolic and anti-atherogenic role of apoE, indicating the importance of regulation of apoE synthesis. The major site of apoE synthesis is the liver [4]. However, unlike most apolipoproteins which are synthesized only in the liver and intestine, apoE is also synthesized in a number of peripheral tissues, such as adrenal, testis, ovary, spleen, kidney, and lung [4–7]. ApoE synthesis has been shown to be regulated in hepatic and steroidogenic cell types by nutritional or hormonal factors or during development [4,8–12], but the molecular mechanisms of apoE regulation by transduction of extracellular signals via second messengers remain obscure. A previous study in our laboratory has demonstrated that glucagon modulates apoE mRNA level in rat liver during development or when cyclic AMP (cAMP) was injected repeatedly into living rats [13]. Glucagon might modulate the expression of the apoE gene by the protein kinase A (PKA)-mediated, a process already described for other genes [14]. Since cAMP-dependent protein kinase has been implicated in lipid homeostasis [15,16] and lipoprotein metabolism [17,18], we are not certain whether changes in apoE gene expression observed in in vivo experiments are due to a direct effect of cAMP itself or to secondary changes in lipoprotein metabolism. The purpose of this study was to investigate the direct influence of cAMP on hepatic transcription of the apoE gene. We used the HepG2 cell line which provides a convenient model system to study the regulation of apolipoprotein gene expression in human liver [12,19,20].

In this paper, we demonstrate that cAMP exerts a repressive effect on apoE gene transcription. Using chimeric reporter gene constructs, we observed that the negative effect of cAMP is mediated by elements(s) located between −614 to +804 in the apoE gene and we provide evidence that the minimal promoter −200/+1 of the apoE gene contains element(s) which can respond positively to cAMP. Our results suggest that regulation of apoE gene transcription by cAMP is a result of a complex pattern of interactions between cAMP-sensitive nuclear factors, and positive and negative regulatory elements in the −614/+804 region of the apoE gene.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), L-glutamine, antibiotics, fetal calf serum and Dulbecco's phosphate-buffered saline (PBS) were obtained from Gibco BRL (European division). [α-^{32}P]dCTP, [γ-^{32}P]ATP, [α-^{32}P]UTP, [^{35}S]methionine (1000 Ci/mol) Nylon Hybond N+, Royal X-Omat films and multiprimer kits were purchased from Amersham International. We used β-galactosidase expression vector pCH110 from Pharmacia while dibutyryl cyclic AMP, *p*-nitrophenyl phosphate (alkaline phosphatase substrate) and all other products were purchased from Sigma. Anti-(human apoE) antiserum was purchased from Immuno France.

2.2. Cell culture

HepG2 cells were cultured at 37°C in a humidified atmosphere of 5% CO_2, 95% air. HepG2 cells were grown in DMEM containing 10% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics (penicillin, streptomycin). 24 h before hormonal treatment, when HepG2 cells were confluent, the cells were washed twice with PBS (pH 7.4) and further maintained in DMEM serum-free medium.

2.3. RNA extraction and analysis

After extraction according to the method of Chomczynski and Sac-

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Abbreviations: ApoE, apolipoprotein E; cAMP, cyclic AMP; CAT, chloramphenicol acetyltransferase; CRE, cAMP-responsive element; CREB, CRE-binding protein; db-cAMP, dibutyryl cyclic AMP; PKA, protein kinase A; SPAP, secreted placental alkaline phosphatase; ICER, inducible cAMP early repressor

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chi [21], total RNA was analyzed by Northern blotting as previously described [12]. The blots were hybridized with apoE cDNA probe labeled with [α-^{32}P]dCTP by random priming and rehybridized to a 28S rRNA probe labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP to standardize the amount of mRNA in each lane.

2.4. Protein labelling and immunoprecipitation

$HepG_2$ cells were cultured in DMEM with 2% (w/v) bovine serum albumin for 24 h. The cells were then incubated in DMEM plus albumin in the presence or in the absence of 10^{-4} M db-cAMP for 24 h. To study the synthesis of apolipoprotein E, the cells were washed three times with 2 ml methionine-free minimum essential medium and incubated for 3 h with the same medium containing [^{35}S]methionine (70 μCi/dish). Cell lysates and media were subjected to immunoprecipitation with anti-(human apoE) antiserum, followed by 10% SDS-PAGE. Autoradiograms show bands with the appropriate mobilities of apoE isoforms as previously described [12]. The bands of apoE isoforms were quantified by densitometric scanning.

2.5. Nuclear isolation and run-on assays

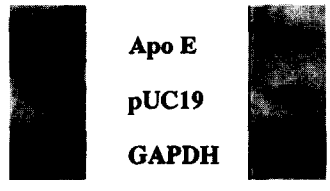
The rate of apoE gene transcription, 24 h after db-cAMP treatment, was measured using run-on assay. Nuclei were prepared from HepG2 cells according to Marzluff et al. [22]. Nuclear isolation and hybridization of nuclear transcripts to immobilized DNA were performed as described [12].

2.6. Plasmid constructs

The apoE reporter gene chimeric constructs were synthesized by inserting different fragments of the human apoE gene into a SPAP or a CAT reporter vector using standard molecular biology techniques. An *SpeI/Hin*dIII 1.4 kb fragment covering the −614 to +804 region of the apoE promoter was obtained by polymerase chain reaction (PCR) from human genomic DNA as template and cloned into *Spe*I and *Hin*dIII in the polylinker region of pSPAP vector (generously provided by Glaxo-Les Ulis), upstream to the secreted placental alkaline phosphatase (SPAP) gene.

The −200 to +1 region of the apoE promoter was obtained with a set of five double-stranded synthetic oligonucleotides (Applied Biosystems, model 380-B) named A–E after annealing between each oligonucleotide and its complement. Fragments A and E contained the *Hin*dIII and *Xho*I sites, respectively. The five fragments were then ligated into the pUC-SH-CAT vector [23] digested by *Xho*I and *Hin*dIII. All constructions were confirmed by DNA sequencing.

A.



B.

Treatment	CONTROL	db-cAMP
Apo E/GAPDH	0.80	0.42

Fig. 1. Effect of db-cAMP on the apoE gene transcription rate. HepG2 cells were maintained in serum-free medium for 24 h and were incubated in the presence or absence of 10^{-4} M db-cAMP for another 24 h. Nuclei were then prepared and used for the in vitro transcription assay as described in Section 2. Equal amounts (50×10^6 cpm) of ^{32}P-labeled RNA were hybridized to nitrocellulose filters containing 10 μg of linearized plasmids bearing apoE, GAPDH cDNA sequences, and pUC19 as a background control. (A) This autoradiogram is representative of two separate experiments. (B) The amount of each band was analyzed using a videocopy/Densylab system from Bioprobe and the data shown are the results of the apoE/GAPDH ratio after subtraction of background radioactivity hybridized to pUC19.

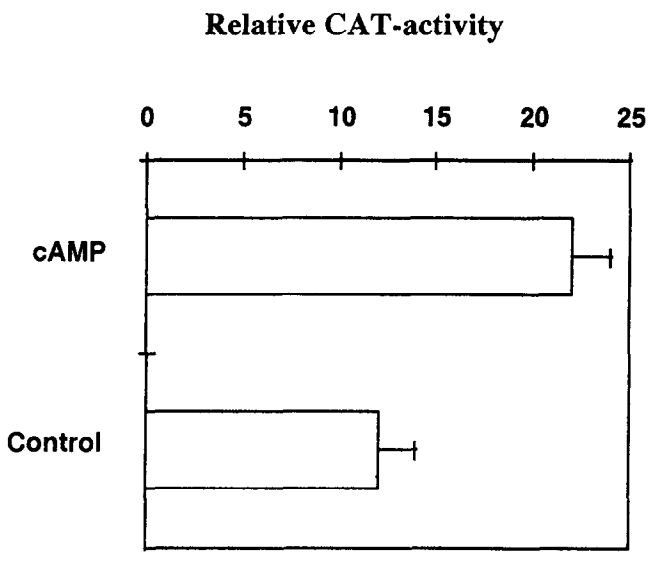


Fig. 2. Effect of db-cAMP on CAT activity in HepG2 cells transfected with apoE promoter (−200/+1)-CAT reporter gene construct. HepG2 cells transiently transfected with the apoE (−200/+1)-CAT construct were grown in serum-free medium 24 h and treated with or without db-cAMP 10^{-4} M for 24 h. CAT activity was measured as described in Section 2 and normalized to the β-galactosidase activity to correct for differences in transfection efficiency. Data represent mean ± S.D. from two separate experiments performed in triplicate.

2.7. Cell transfection, CAT and SPAP assays

Transient transfections of HepG2 cells were performed by the calcium phosphate co-precipitation method, as previously described [24]. Chloramphenicol acetyltransferase (CAT) activity was determined by acetylation of [^{14}C]chloramphenicol (Amersham) followed by thin-layer chromatography, as previously described [24]. For secreted placental alkaline phosphatase (SPAP) assays, on the day following transfection, HepG2 cells were trypsinized and seeded on well-titer microplates (microdish well-plate 96F). Secretion medium was used to measure SPAP activity and total cellular protein was determined. SPAP activity was measured as previously described [25].

3. Results and discussion

To assess whether cyclic AMP plays a role in apoE gene expression, we first examined the effects of two agents, forskolin and dibutyryl cAMP (db-cAMP), which increase the intracellular cAMP levels or mimic the action of cAMP in various cell types.

When HepG2 cells were exposed for 24 h to db-cAMP (10^{-4} M) or forskolin (10^{-6} M) after incubation in control medium, the apoE mRNA level was reduced by 40–50% (Table 1). The decrease in apoE mRNA induced by db-cAMP or forskolin was observed after a lag period and the effect was only significant after 24 h of treatment (data not shown). Apolipoprotein E synthesis was assessed by incubating HepG2 cells in the presence or absence of db-cAMP for 24 h then by performing a pulse of [^{35}S]methionine. Quantitation by laser scanning densitometry indicated that the amount of

Table 1
Effect of db-cAMP and forskolin on apoE mRNA in Hep G2 cells

	Control	db-cAMP	Forskolin
Relative apoE mRNA level	1.20 ± 0.03	0.72 ± 0.01 ($p < 0.01$)	0.68 ± 0.04 ($p < 0.01$)

HepG2 cells were maintained for 24 h in serum-free medium and then incubated for 24 h in the absence (control) or presence of 10^{-4} M db-cAMP or 10^{-6}M forskolin. Total RNA was extracted and analyzed by Northern blotting as described in Section 2. Data obtained by densitometric scanning was normalized for the 28S signal and given in arbitrary units. Results are the means ± S.D. of three independent experiments performed in duplicate.

Table 2
Incorporation of [^{35}S]methionine into cellular and extracellular immunoprecipitated apoE

	Control	db-cAMP	Inhibition (%)
Cells	17.1 ± 1.3	9.5 ± 1.2 ($p < 0.01$)	44
Media	62.9 ± 0.7	47.0 ± 4.5 ($p < 0.05$)	30

HepG2 cells were incubated in the presence or absence of db-cAMP for 24 h and then incubated with [^{35}S]methionine (70 μCi/dish) for 3 h. Media and cell lysates were immunoprecipitated with a specific anti-(human apoE) antiserum and analysed by 10% SDS-PAGE. The intensity of apoE bands was analysed by scanning densitometry. Values shown denote the amount of apoE (in arbitrary units, means ± S.D.). Two independent experiments were performed in duplicate.

Table 3
Effect of db-cAMP and forskolin on SPAP activity in HepG2 cells transiently transfected with apoE promoter (−614/+804)-SPAP reporter gene construct

	Control	db-cAMP	Forskolin
Relative pE-SPAP activity	0.59 ± 0.05	0.28 ± 0.06 ($p < 0.01$)	0.38 ± 0.03 ($p < 0.05$)

HepG2 cells transiently transfected with the apoE (−614/+804)-SPAP construct were trypsinized and seeded in microwell plates. The following day, cells were placed in serum-free medium for 24 h and incubated in the same medium with or without the addition of 10^{-4} M db-cAMP or 10^{-6} M forskolin. SPAP activity was determined as described in Section 2 and normalized to total cellular protein content. Data represent mean ± S.D. from three experiments performed in quadriplicate.

apoE secretion and cellular apoE were significantly reduced by 30 and 45%, respectively (Table 2).

When injected in vivo, glucagon or db-cAMP increased hepatic apoE mRNA levels. The discrepancy between in vivo experiments and the observed data herein results from the secondary secretion of various hormones when the rats were treated with glucagon or db-cAMP. For example, we have previously shown that hepatic levels apoE mRNA are correlated with the glucagon/insulin ratio rather than plasma glucagon levels, itself [13].

In order to determine whether the observed changes in steady-state levels, of apoE mRNA were due to changes in the transcription rate, run-on assays were performed on HepG2 cells using nuclei from either control cells or cells treated with db-cAMP for 24 h. Quantitation of autoradiograms (Fig. 1A) by scanning densitometry after correction for GAPDH gene transcription showed that the apoE gene transcription rate was reduced 2-fold at 24 h (Fig. 1B). This result indicates that the decrease in the rate of apoE gene transcription could totally account for the change in steady-state level of apoE mRNA.

In eukaryotes, the signal transduction pathways that involve cAMP induce phosphorylation of transcription factors that interact promoter sequences termed cAMP responsive elements (CREs) [26]. Several regulatory sequences have been identified in the promoter region of apoE gene by nuclear footprint and electrophoretic mobility shift assays [27,28]. Inspection of these sequences by computer-aided analysis revealed three consensus CREs [TGACG (C/T)(C/A)(G/A)] as putative *cis*-acting elements in the promoter region localized at positions [−498 to 491], [−444 to −437] and [−95 to −88] [29]. Consequently, we investigated whether this region is involved in the cAMP effect. We first transfected HepG2 cells with a plasmid containing the −614/+804 fragment of the human apoE gene fused to the SPAP reporter gene (p[−614/+804]-apoE-SPAP). After 24 h of either db-cAMP or forskolin treatment, we observed a decrease in SPAP activity by 50 and 40% of the activity of untreated HepG2 cells, respectively (Table 3). This inhibition is of the same order of magnitude as that observed for the steady-state mRNA level (Table 1) or transcription rate (Fig. 1), suggesting that complete responsiveness to cAMP is mediated by the −614/+804 fragment of apoE gene.

In a second set of experiments, we transfected HepG2 cells with the plasmid containing the CAT-reporter gene under the control of the −200 to +1 region of the apoE gene promoter (p[−200;+1] apoE CAT). This construct was described to be necessary and sufficient for optimal expression in the HepG2 cell line [30]. Surprisingly, we observed a 2-fold increase in CAT activity in transfected cells treated for 24 h with db-cAMP compared with control transfected cells (Fig. 2). Since db-cAMP had no specific effect on the original cloning vector pUC-SH-CAT (data not shown), these results reveal the presence of one or several elements which respond positively to cAMP in −200/+1 region of apoE promoter. Moreover, these results suggest that sequences located upstream or downstream from this region, act to block or suppress the cAMP inducible activity in the proximal −200/+1 apoE promoter.

An increased intracellular cAMP level as a result of an extracellular signal activates protein kinase A (PKA), which in turn modifies the pattern of cellular protein phosphorylation [31]. The positive effect of db-cyclic AMP on (p[−200/+1] apoE-CAT) activity might be the result of transactivation by the nuclear factor CREB [32] after activation by phosphorylation [33,34]. The global negative effect of cAMP on either apoE mRNA steady-state, apoE gene transcription rate or

(p[−614/+804] apoE-SPAP) activity indicates that other cAMP-stimulated factors also counteracted the CREB effect on the −200/+1 apoE minimal promoter. Several CRE binding repressor families have been described. They include ICER proteins which could act as powerful repressors and CREM α, β and γ which are constitutionally present and whose activity can be modulated by phosphorylation [35]. These factors might either occupy CRE sites present in −614/+804 apoE promoter as homodimers in the active form or dimerize with activators present on the CRE site of the minimal −200/+1 promoter, blocking them by the formation of non-functional heterodimers [36].

In conclusion, complex regulation of apoE gene transcription by cAMP via PKA activation is likely to be determined by a combined action on the activator elements, present in the proximal region −200/+1 and repressor elements present upstream and downstream from this region with a dominant activity. The next step will be to identify the specific DNA sequence(s) and the regulatory proteins responsible for this complex effect of cAMP on apoE gene transcription.

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