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Participation of SRE4, an URE1 Enhancer Core Sequence, in the Sterol-Mediated Transcriptional Upregulation of the Human Apolipoprotein E Gene

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The expression of the endogenous human apolipoprotein (apo)E gene was significantly induced when HepG2 cells were treated with exogenous 25-hydroxycholesterol. This sterol-mediated apoE gene upregulation appears to require the participation of a positive element for the apoE gene transcription (PET) (−169/−140), a core sequence of upstream regulatory element (URE)1 enhancer of the human apoE gene. This PET was renamed as sterol regulatory element (SRE)4 based on its new role as a sensor for the level of intracellular sterol. Furthermore, a gel mobility shift analysis showed that binding activity of the SRE4 binding protein (BP) obtained from HepG2 cells was induced by sterol treatment, while that from either MCF7 or BT20 cells remained unchanged. Binding activity of SRE4BP was also induced in mouse macrophage cells, J774A.1, by sterol treatment, but it was drastically reduced when cells were subjected to treatment of AY-9944, a potent inhibitor for sterol synthesis. However, binding activity of Sp1, which is a co-binding protein to the SRE4 region, remained the same in either condition, suggesting that SRE4BP (formally known as PETBP) may be mainly responsible for the sterol-mediated regulation of the apoE gene expression. Deletion analysis of the core binding site of SRE4BP by gel mobility shift assays showed that the minimal sequence of the SRE4BP binding appears to reside between −157 and −140, confirming the identity of SRE4 with the previously determined core sequence of URE1.

Keywords: Apolipoprotein E, AY-9944, 25-Hydroxycholesterol, Sterol regulatory element.

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Introduction

It is well established that the apolipoprotein (apo)E, a common component of various classes of mammalian lipoproteins including chylomicron remnants and very low density lipoproteins (VLDL), mediates the cellular uptake and redistribution of cholesterol between peripheral tissues and the liver through the low density lipoprotein (LDL) receptor and the LDL receptor-related protein (Mahley, 1988).

In addition, apoE is believed to be involved in nerve cell growth (Weisgraber *et al.*, 1993), brain development (Mouchel *et al.*, 1995), formation of extracellular amyloid plaques as well as intracellular neurofibrillary tangles in the brains of individuals with a late onset of Alzheimer's disease (Strittmatter *et al.*, 1993; 1995; Berger and Harmon, 1997; Lambert *et al.*, 1997), and neuronal cell death (LaFerla *et al.*, 1997). The human apoE gene is 3.6 kb in length (Paik *et al.*, 1985), and it is linked closely to the apoC-I gene (Lauer *et al.*, 1987).

Previous studies have demonstrated that transcriptional and tissue-specific regulations of the human apoE gene are influenced by multiple regulatory sequences resided in its promoter (Paik *et al.*, 1988; Smith *et al.*, 1988) as well as its 3′-flanking region (Simonet *et al.*, 1991; 1993) in which hepatic control regions are located (Alan *et al.*, 1995; Dang *et al.*, 1995). For example, a strong nonspecific enhancer, termed upstream regulatory element (URE)1, was identified at nucleotides −193 to −124 upstream of the first exon. Another nonspecific enhancer was identified at nucleotides −366 to −246, and a GC-box motif that binds the Sp1 transcription factor was identified at nucleotides −59 to −45 (Paik *et al.*, 1988). Both cell-free transcription assays and expression studies in transfected cultured cells indicated that these elements promote gene expression (Paik *et al.*, 1988; Smith *et al.*, 1988; Chang *et al.*, 1990). In addition, using nuclear extracts from various cell lines, a DNase I footprint was identified at nucleotides −103 to

−87, termed URE3, a positive transcriptional element, and its binding protein was purified and characterized previously in this laboratory (Jo *et al.*, 1995). In contrast, it has been reported that part of the URE3 region (−94/−84) has been shown to be a transcriptional repressor binding site in HepG2 cells (Berg *et al.*, 1995) suggesting its dual role in apoE gene expresssion depending on the cell types.

The apoE gene expression has also been known to be modulated by many cellular factors including the thyroid hormone (Vandenbrouck *et al.*, 1994), the tumor necrosis factor α (Duan *et al.*, 1995), estrogen (Srivastava *et al.*, 1997), and cAMP (Andreani-Mangeney, 1996). Although it is well known that apoE gene expression is activated by cholesterol in *in vitro* tissue cultures (Mazzone *et al.*, 1987; 1989; Wyne *et al.*, 1989), there has been no report on the location of any *cis*-acting sequence and its binding proteins that may be responsible for its sterol response. Sterols are common regulators for cholesterogenic enzymes (e.g., 3β-hydroxy-3-methylglutaryl [HMG]-CoA reductase) and LDL receptor in which a sterol regulatory element (SRE) mediates sterol-mediated suppression of the corresponding genes (Sudof *et al.*, 1987). The action of the SRE requires cellular nuclear proteins such as the SREBP and SREBP cleavage activator protein (SCAP) (Brown and Goldstein, 1997). Recently, it was also reported that the secretion of macrophage-derived apoE was enhanced by 25-hydroxycholesterol suggesting the existence of an additional post-transcriptional regulatory system (Duan *et al.*, 1997).

The present study was undertaken to determine whether the human apoE gene contains SRE sequences, and to characterize the nature of these elements. The focus of the investigation is on the positive element for apoE gene transcription (PET), the core sequence of URE1 (−193/−124) which is one of the most dominant regulatory elements (Paik *et al.*, 1988; Chang *et al.*, 1990). URE1 has a very high homology to one of the SREs in the human LDL receptor gene (Paik *et al.*, 1988). In this paper, the additional role of URE1 as sterol sensor and one of its specific binding proteins (e.g., PETBP) in the light of sterol-mediated apoE gene expression, is reported. The results revealed that the core sequence of PET appears to be involved in sterol-mediated upregulation of human apoE gene expression. A new name for this PET and its binding protein as SRE4 and SRE4BP, respectively, should be suggested since each of them appears to have a unique function as a transcriptional enhancer and sterol-mediator in the regulation of the human apoE gene.

Materials and Methods

Materials All the isotopes including [α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from Amersham. The following chemicals were obtained from various sources as indicated. They are: nucleotide triphosphates, poly(dI-dC)·poly(dI-dC) (Pharmacia), RNasin ribonuclease inhibitor (Promega), reverse transcriptase of avian myeloblastosis virus (Life Sciences), DNA modifying enzymes including restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase and Klenow fragment of DNA polymerase I (New England Biolabs), protein assay dye (Bio-Rad Laboratories), lipoprotein-deficient serum, cholesterol, 25-hydroxycholesterol, $NaHCO_3$, gentamycin sulfate solution (Sigma), RPMI-1640 powder media, Dulbecco's Modified Eagle Medium, fetal bovine serum, LIPOFECTIN reagent, Trypsin-EDTA HEPES (GIBCO), tissue culture flask and culture dish (Nunc), and TRI Reagent (Molecular Research Center, INC). AY-9944, *trans*-1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride, was a gift from Dr. Dvornick (Whyth-Ayerst, Princeton, USA). Other chemicals and solvents were purchased from Sigma or Merck. All reagents were of the best grade available.

Synthesis of oligonucleotides The oligonucleotides were synthesized on an Applied Biosystems 392/394 DNA/RNA synthesizer, and they were purified by the Oligonucleotide Purification Cartridge system (Applied Biosystems, Foster City, USA). Sequences of synthetic oligonucleotides that were used for gel mobility shift assays are listed below.

SRE4 (or PET, Chang *et al.*, 1990):
(−169) 5′- AAAGACCTCTATGCCCCACCTCCTTCCTCC - 3′ (−140)
3′- TTTCTGGAGATACGGGGTGGAGGAAGGAGG- 5′

GC-box:
5′- GGGGCGGGACGGGGCGGGAC - 3′
3′- CCCCGCCCTGCCCCGCCCTG - 5′

For competition analysis in the gel mobility shift assay, the following nucleotides, in which partial seqeuences are deleted from the wild-type PET, were employed: they are, oligonucleotide **a** (PET, wild-type), −169/−140; **b**, $\Delta^{-161/-151}$; **c**, $\Delta^{-150/-140}$; **d**, $\Delta^{-169/-162}$; **e**, $\Delta^{-155/-140}$; and **f**, $\Delta^{-169/-157}$

Treatment of the cultured cells with sterols Human cell lines including HepG2, MCF7, and BT-20 or J774A.1 cells were seeded in 100-mm dishes and grown in 10% FBS-RPMI 1640 media in the presence of 5% CO_2, at 37°C for 24 h (Rih *et al.*, 1997). When the cells reached approximately 60% confluency, they were switched to growth in 10% LPDS-RPMI 1640 medium. An aliquot of 25-hydroxycholesterol (Sudof *et al.*, 1987) or AY-9944 (Kang *et al.*, 1995; Kim *et al.*, 1995; Cho *et al.*, 1998) was added to the cells to a final concentration of 1 μg/ml and cells were further grown for 48 h. The cells harvested at this point were subjected to RNA extraction for the primer extension analysis and to nuclear extract preparation.

Preparation of RNA and primer extension analysis Total cellular RNA was isolated from HepG2 cells using TRI Reagent according to the method provided by the suppliers. Primer extension analysis was performed essentially as described (Walker *et al.*, 1985). A 40-mer single-stranded oligonucleotide primer that is complementary to nucleotides +120 to +160 of the coding sequence of the human apoE gene (5′-CTCGGGCTCC GGCTCTGTCTCCACCGCTTGCTCCACCTTG-3′) (Paik *et al.*, 1985) was end-labeled using [γ-^{32}P]ATP by T4 polynucleotide kinase. As a control, a 40-mer single-stranded oligonucleotide

primer that is complementary to the nucleotides +70 to +110 of rat β-actin gene (5'-CTTTGCCACTCGAGCTGATCTGTCAC CTCCGGCTCTCCCT-3') was used. Three ng of each [^{32}P]-labeled primer was hybridized to 20 μg of total cellular RNA and extended as described (Chang *et al.*, 1990). The extension products were analyzed on a 6% polyacrylamide gel containing 7 M urea. The relative amount of the primary transcripts produced in the gel were quantitated by the densitometric scanning method using the Pharmacia ImageMaster VDS.

Isolation and preparation of nuclear extracts Nuclear extracts were prepared from the cultured cells including HepG2, BT-20, MCF-7, and J774A.1 cells essentially as described by Dignam *et al.* (1983). Nuclei were suspended at a concentration of 2~3 × 10^8/ml in buffer B (20 mM Hepes, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 25% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride). The mixture was stirred gently for 30 min at 4°C. The supernatant fluid was dialyzed against 100 vol of buffer C (20 mM Hepes, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) for 4 h at 0°C with one change of buffer overnight. The dialysates were then centrifuged at 14,500 rpm for 20 min at 4°C, and the supernatant fluids stored in aliquots at −80°C. The protein concentration of the extract was determined by Bradford's method (1972) using bovine serum albumin as a standard.

Gel mobility shift assay of the nuclear extracts Gel mobility shift assays were performed essentially as described by Carthew *et al.* (1985). The standard reaction mixture containing 0.5 ng of [^{32}P]-5'-end-labeled DNA, 15 μg of nuclear extract, and 4 μg of poly(dI-dC)·poly(dI-dC) was incubated for 30 min at 30°C in 0.5× buffer C (10 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 0.25 mM dithiothreitol, 10% glycerol, and 0.25 mM phenylmethylsulfonyl fluoride). For the competition experiments, a 120-fold molar excess of the unlabeled competitor oligonucleotides were preincubated with the reaction mixture for 10 min prior to the addition of 5'-end-labeled DNA probe. The DNA–protein complex was examined by 5% polyacrylamide gel electrophoresis in 0.5× TBE buffer (44.5 mM Tris, pH 8.3, 44.5 mM boric acid, and 0.5 mM EDTA).

Results and Discussion

Effect of sterols on the expression of human apoE gene To determine whether human apoE gene expression can be regulated by exogeneous sterols, primer extension analyses were carried out using total cellular RNA extracted from HepG2 cells which had been treated with 1 μg/ml of 25-hydroxycholesterol. In the primer extension analysis, a probe for rat β-actin gene was also included as the internal control of transcription efficiency. As shown in Fig. 1, the relative intensity of the primary transcripts of the human apoE gene (about 160 nucleotide size) that was obtained from the sterol-treated HepG2 cells was significantly induced (e.g., about 3-fold increase when measured by densitometric scanning; 0.048 [control] vs 0.14 [+sterol] in OD after normalizing each value against that of the β-actin gene transcript). There was no change in

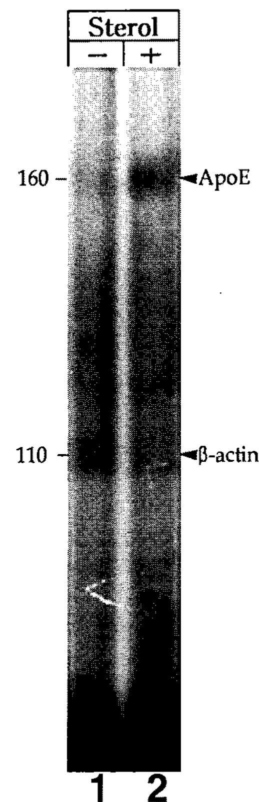

Fig. 1. Primer extension analysis for measurement of effects of sterols on the apoE gene expression in HepG2 cells. Total cellular RNA was extracted from the cells that had been grown in the presence or absence of 1 μg/ml 25-hydroxycholesterol for 48 h, and then subjected to the primer extension analysis as described in Materials and Methods. Lane 1, sterol-free HepG2 cell; lane 2, sterol-loaded HepG2 cell.

rat β-actin mRNA transcripts (110 nt size) that were obtained from the same HepG2 cells treated with sterol. This result indicates that the expression of the endogenous human apoE gene is transcriptionally upregulated in HepG2 cells by cellular oxysterol. This result is, in principle, in agreement with previous reports on sterol-mediated induction of the apoE gene in macrophage cells (Mazzone *et al.*, 1989).

ApoE enhancer, URE1, contains SRE which functions in a liver-specific manner Previous studies showed that there has been a striking homology (i.e., identity of 9 out of 12 nucleotides) between SRE42 repeat 3 of the human LDL receptor gene (Sudhof *et al.*, 1987) and the URE1 core sequence (−157/−146) (Paik *et al.*, 1988). This finding led us to investigate further the involvement of the core sequence of the URE region (−169/−140) (Chang *et al.*, 1990). To determine whether the core of the URE region, such as the PET sequence (−169/−140) (Chang *et al.*, 1990), can mediate this sterol-mediated induction of the apoE gene, gel mobility shift assays were carried out

using radiolabeled-PET in the presence of the nuclear proteins that had been obtained from three different human cell lines such as HepG2, BT20, and MCF7 cells. They had been treated with 25-hydroxycholesterol. Besides HepG2 cells, other human cell lines such as BT20 and MCF7 cells were chosen because they have been shown to be implicated in steroid hormone response (Cypriani *et al.*, 1988) as well as cholesterol synthesis regulation (Cho *et al.*, 1998). As shown in Fig. 2, the band intensity of PET binding protein (PETBP) that had been obtained from HepG2 cells were substantially stimulated by sterol treatment (Fig. 2, lane 7; e.g., about 14.8-fold, 0.013 [control] vs 0.198 [+sterol] by densitometric scanning). It was noticed that the size of the band which appeared in HepG2 cells was a little larger than those of MCF7 and BT20 cells. This might be due to the nature of the cell types of these cell lines. For example, MCF and BT20 cells are originated from the human breast cancer tissues (Cypriani *et al.*, 1988) while HepG2 cells are from the human hepatoma. However, there were no changes in the band intensities of Spl that were obtained from the same cells that had been subjected to the same sterol treatment. These results strongly suggest that there may be a factor that mediates the sterol response of the PET sequence in HepG2 cells. This PET sequence was renamed as SRE4 since it appears to confer a cellular response to the changes in the level of intracellular sterols. Accordingly, we believe that this factor binding to the SRE4 should be $M_r = 55{,}000$ protein (Chang *et al.*, 1990).

Expression of SRE4BP, not Spl, is regulated by the intracellular sterol level Previous results showed that efficient activation of transcription from the human apoE gene promoter required the proximal GC-box and its binding protein Spl (Chang *et al.*, 1990). That is, there are at least two different kinds of proteins that bind to the SRE4 (PET) sequence. They are $M_r = 55{,}000$ dalton protein (now renamed as SRE4BP) and Spl. In most SREBP-regulated promoters studied to date, additional co-regulatory transcription factors, such as Spl, are required (Wang *et al.*, 1993; Sanchez *et al.*, 1995; Yieh *et al.*, 1995). In the case of the human apoE gene promoter, it is not well understood whether or not there is a co-regulatory transcription factor for SREBP-mediated apoE gene expression. To determine whether binding intensities which could be reflected as the amounts of these two different proteins, SRE4BP and Spl, can be regulated by changes in the level of intracellular sterol, gel mobility shift assays were carried out using the nuclear extracts of J774A.1 cells that had been treated by either AY-9944, a potent inhibitor for cholesterol biosynthesis (Paik *et al.*, 1984; 1986; Kim *et al.*, 1995; Lee & Paik, 1997) or 25-hydroxycholesterol (Sudhof *et al.*, 1987; Wang *et al.*, 1995). As shown in Fig. 3, the intensity of SRE4BP was dramatically reduced when cells were subjected to AY-9944 treatment (lane 2) but it was significantly increased by sterol treatment (lane 3). However, the binding pattern of Spl that was obtained from the same cells essentially remained unchanged. These results strongly indicate that SRE4BP (formally known as PETBP) may be mainly responsible for the sterol-mediated regulation of the apoE gene expression. However, in contrast to the conventional SREBPs which usually downregulate the target genes (e.g., LDL receptor and HMG-CoA reductase) when cellular sterol levels are high, SRE4BP activates its target gene, human apoE. It is also noted that SRE4BP synthesis was

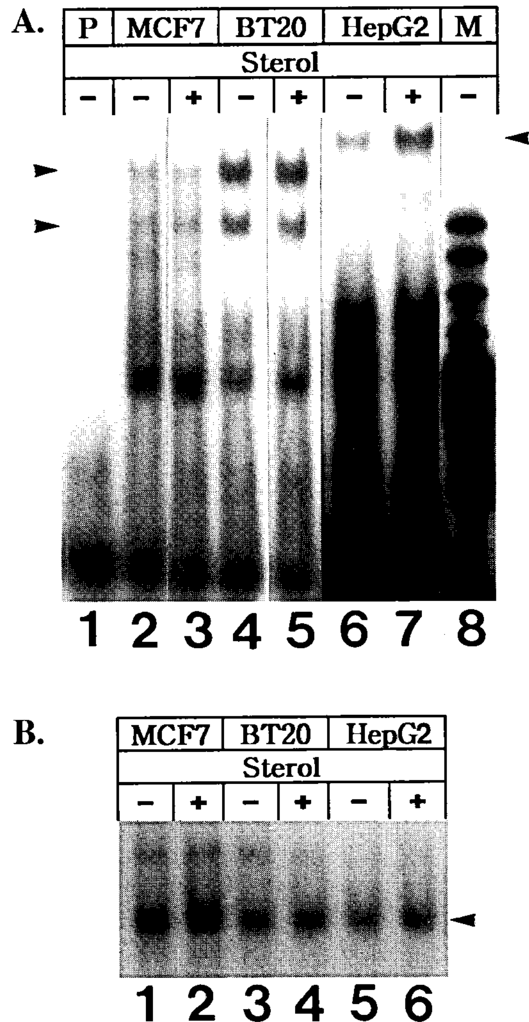

Fig. 2. Effects of sterols on the SRE4BP activity in various human cell lines. A. Lane 1, no nuclear extract; lanes 2–7 are either marked by '+' (sterol-loaded) or '–' (sterol-free); lane 8, molecular weight marker (pBR322-MspI digest). B. Same as panel A except for the probe (GC-box). Cells that had been maintained on 10% LPDS-RPMI medium were treated with 25-hydroxycholesterol (1 μg/ml) and further grown for 48 h prior to preparation of the nuclear extracts. Gel mobility shift assays were carried out using 15 μg of the nuclear extracts as described in Materials and Methods. The bands of SRE4BP shown here are indicated by the arrowheads.

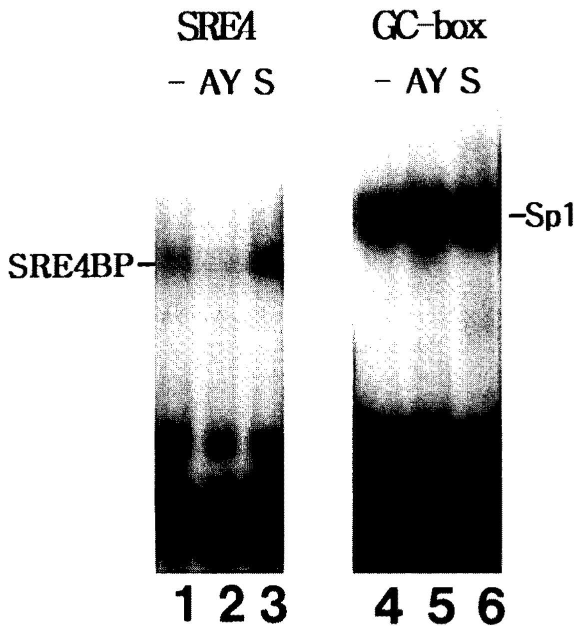

Fig. 3. Effects of sterols and sterol biosynthesis inhibitor (AY-9944) on the Sp1 and SRE4BP binding activity. Labeled oligonucleotides of the GC-box and SRE-4 (−169/−140) were incubated with nuclear extracts from sterol-free (lanes 1 and 4), AY-9944 treated (lanes 2 and 5), and sterol-loaded (lanes 3 and 6) J774A.1 cells and analyzed for gel mobility shift analysis.

repressed by AY-9944-mediated sterol depletion, but it was induced by the presence of oxysterol in this particular cell line. We used J774A.1 cells here because this cell seemed to be more stable than HepG2 cells when AY-9944 (1 μg/ml) was added into the cells.

Core binding site of SRE4 for SRE4BP To determine the core nucleotide binding site for SRE4BP, gel mobility shift assays were carried out in the presence of 120-fold molar excess of different oligonucleotide competitors in which different portions of the SRE4 seqeunce (−169/−140) were deleted. As shown in Fig. 4, the minimal sequence of the SRE4 core appears to reside between −157 and −140, suggesting that this is essentially the same as the previously identified core sequence of PET. That is, this 18-nucleotide exactly matches the core sequence of the URE1 enhancer of the human apoE gene (Chang *et al.*, 1990). This SRE4BP binding was not affected by the presence of the GC-box (data not shown). It remains to be determined whether the action of SRE4BP requires the participation of Sp1, as in the cases of transcriptional regulation of other SREBPs (Sudhof *et al.*, 1987; Wang *et al.*, 1993; Sanchez *et al.*, 1995; Yieh *et al.*, 1995). Thus, sterol-regulated transcription of the gene for human apoE appears to be dependent in part on the binding of the SRE-4BP to a 18-bp PET sequence within the proximal promoter. In conclusion, the results here

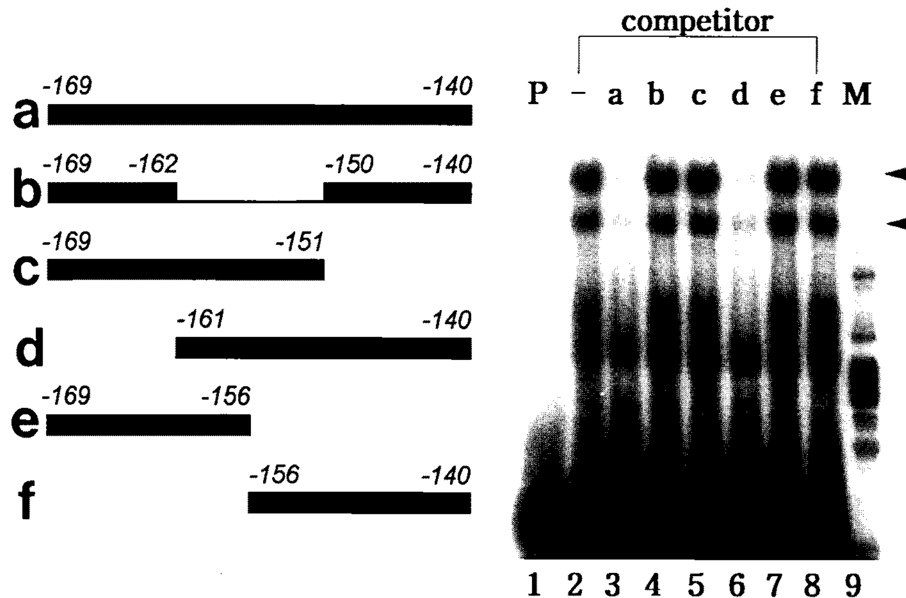

Fig. 4. Gel retardation analysis of SRE4 region. Gel mobility shift assays (right panel) were carried out using the PET oligonucleotide (a) as a probe and BT-20 cell nuclear extracts in the presence of a 120-fold molar excess of its deletion mutants (b–f) as competitors (left panel). Shown left are the oligonucleotides containing SRE4 sequence (−169/−140) or its mutant forms used in competition gel mobility shift analysis. The bars represent the sequences spanned by each oligonucleotide, and the numbers above the bars represent the beginning and end position of each oligonucleotide. Shown in the right panel are the gel retardation analysis of SRE-4 with oligonucleotide competitors. Lane 1, no nuclear extract; lane 2, no competitor; competitors used in lanes 3–8 are oligonucleotides **a**, **b**, **c**, **d**, **e**, and **f**, respectively; lane 9, molecular weight marker. The bands of SRE4BP shown here are indicated by the arrowheads.

indicate that the previously characterized URE1 enhancer (Paik *et al.*, 1988) also functions as a sterol sensor for regulating human apoE gene expression. Expression of SRE4BP (or URE1BP) seems to be greatly influenced by the presence of sterol in the hepatic cells as well as macrophage cells.

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