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Estrogen Suppresses Transcription of Lipoprotein Lipase Gene

EXISTENCE OF A UNIQUE ESTROGEN RESPONSE ELEMENT ON THE LIPOPROTEIN LIPASE PROMOTER*

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Hiroaki Homma, Hirohisa Kurachi, Yukihiro Nishio‡, Takashi Takeda, Toshiya Yamamoto, Kazushige Adachi, Ken-ichirou Morishige, Masahide Ohmichi, Yuji Matsuzawa§, and Yuji Murata

From the Departments of Obstetrics and Gynecology and §Internal Medicine II, Faculty of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan

Estrogen exerts a variety of effects not only on female reproductive organs but also on nonreproductive organs, including adipose tissue. Estrogen inhibits obesity triggered by ovariectomy in rodents. We studied the mechanism underlying this estrogen-dependent inhibition of obesity. Estrogen markedly decreased the amounts of fat accumulation and lipoprotein lipase (LPL) mRNA as well as triglyceride accumulation in genetically manipulated 3T3-L1 adipocytes stably expressing the estrogen receptor (ER). A pLPL(1980)-CAT construct, along with an ER expression vector, was introduced into differentiated 3T3-L1 cells, and CAT activities were determined. ER, mostly ligand-dependently, inhibited the basal LPL promoter activity by 7-fold. We searched the LPL promoter for an estrogen-responsive suppressive element by employing a set of 5′-deletion mutants of the pLPL-CAT reporter. Although there was no classical estrogen response element, it was demonstrated that an AP-1-like TGAATTC sequence located at (−1856/−1850) was responsible for the suppression of the LPL gene transcription by estrogen. An electrophoretic mobility shift assay probed with the TGAATTC sequence demonstrated formation of a specific DNA-nuclear protein complex. Interestingly, this complex was not affected by the addition of any antibodies against ER, c-Jun, c-Fos, JunB, or JunD. Because this TGAATTC element responded to phorbol ester and overexpression of CREB-binding protein abrogated the suppressive effect of estrogen on the LPL promoter, we conclude that a unique protein that is related to the AP-1 transcription factor families may be involved in the complex that binds to the TGAATTC element.

Estrogen, a type of steroid hormone, evokes diverse effects in mammalian cells and tissues. Initially, estrogen was identified as an essential hormone for female reproduction (1, 2). Recent studies, however, have shown that estrogen works not only on the female reproductive organs but also on other target tissues including bone (3), the central nervous system (3, 4), the vascular system (3, 5), and adipose tissue (3, 6–8).

One of the findings demonstrating the action of estrogen on adipose tissue is that female rodents who undergo ovariectomy become obese (7, 8). One can imagine that lack of estrogen brought about by ovariectomy accounts for this change. In fact, this hypothesis was confirmed by the fact that replacement of estrogen in these ovariectomy animals abrogated the obesity. Administration of estrogen reduces food intake (9, 10) and increases ambulatory activity (11).

A line of evidence has been reported that strongly suggests the involvement of estrogen in lipid metabolism in the adipose tissue. Estrogen replacement to ovariectomy animals resulted in decreased lipoprotein lipase (LPL)[1] enzyme activity and mRNA in the adipose tissue (12–16). It has been also shown in humans that estrogen suppresses LPL activity in plasma (17) and the adipose tissue as well (18).

LPL is a key regulating enzyme for energy metabolism, catabolizing plasma triglycerides into free fatty acids and glycerol (19). The activity of this enzyme is mainly regulated at the transcriptional and translational levels (20). Translational LPL regulation has been intensively studied in the case of epinephrine and TPA-activated PKC (21, 22). Both regulators inhibit LPL translation through the production of an RNA-binding protein that binds to a region on the proximal 3′-untranslated region of the LPL mRNA (21, 22). We nevertheless were interested in transcriptional regulation because several hormones such as growth hormone activate the LPL gene at the transcriptional level.

Clinical observations have demonstrated that serum triglyceride levels increase in postmenopausal women and that the level of LPL activity is reduced by estrogen treatment (14). Despite these observations, it remains poorly understood how estrogen suppresses fat accumulation.

To investigate the cellular signaling pathway mediating the inhibitory effect of estrogen on adipogenesis, we have generated genetically manipulated 3T3-L1 mouse preadipocytes by permanently introducing the estrogen receptor. This variant, termed 3T3-L1-ER, was subjected to analyses of the phenotype as well as of the LPL mRNA profile in the presence or absence of estrogen. Next, we conducted chloramphenicol acetyltransferase (CAT)-promoter analyses to investigate how LPL gene transcription is regulated by estrogen aiming at identification of a responsive element for the suppressive action of estrogen. Finally, we employed an electrophoretic mobility shift assay (EMSA) to characterize an estrogen responsive nuclear factor that plays a role in regulating the LPL gene expression.

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‡ To whom correspondence should be addressed: Dept. of Obstetrics and Gynecology E5, Faculty of Medicine, Osaka University, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Tel.: 81-6-6879-3351; Fax: 81-6-6879-3359; E-mail: ynishio@gyne.med.osaka-u.ac.jp.

[1] The abbreviations used are: LPL, lipoprotein lipase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; bp, base pair(s); ER, estrogen receptor; DMEM, Dulbecco's modified Eagle's medium; ERE, estrogen response element; CBP, CREB-binding protein; C/EBPα, CCAAT enhancer-binding protein α.

EXPERIMENTAL PROCEDURES

Chemicals—[α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from Amersham Pharmacia Biotech and [^{14}C]chloramphenicol (200 mCi/ml, CAT assay grade) from NEN Life Science Products. The restriction endonucleases, modifying enzymes, and DNA polymerases were purchased from Toyobo (Osaka, Japan). Synthetic oligonucleotides were obtained from Sawady Inc. (Tokyo, Japan). Chemicals and reagents were obtained from Sigma or Nacalai Tesque (Kyoto, Japan) unless otherwise stated. Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmids and Constructs—A plasmid containing the 3804 bp of the murine LPL promoter region (−1980/+1824) including the first exon and the first intron was kindly provided by Dr. J. M. Gimble (Oklahoma Medical Research Foundation, Oklahoma City, OK) (23). pLPL(−1980/+1824)-CAT was constructed as follows. Because the LPL gene fragment was flanked by *Sst*I restriction sites on both ends, a *Sac*I 8-mer linker (Takara) was inserted into the *Hin*dIII site of pCAT enhancer vector (Promega), because *Sst*I and *Sac*I generate compatible ends. The *Sst*I-*Sst*I (−1980/+1824) fragment from the LPL gene was fused to the *Sac*I site of the pCAT enhancer. pLPL(−1980/+1824)-CAT includes the first exon (+198/+285) and the first intron (+286/+1824). Therefore, the construct was digested by *Pst*I at +328 on the LPL fragment and the multicloning site of the pCAT gene and religated. The resultant pLPL(−1980/+328)-CAT construct was used in the following studies. Other deletion mutants were generated by polymerase chain reaction. The upstream primers for the −1980/−1573, −1898/−1573, and −1731/−1573 LPL gene fragments (see Fig. 4*A*) were 5′-GAGCTCATGTGAGCGTCTGC-3′, 5′-GAGCTCTACCTGCCCACCACTTGTCC-3′, and 5′-GAGCTCATTTATCTCTCTGGACTCTTGA-3′, respectively. The common downstream primer for these three fragments was 5′-AAGCTTACTCCAAACCGACCTGCACT-3′. Polymerase chain reaction products were linked to the minimal LPL promoter (−182/+328)-CAT. The internal deletion construct, pLPL(1898/1573)ΔAP-1-like)-CAT lacking four nucleotides (AATT at −1854/−1851) was generated by *Eco*RI digestion and mung bean nuclease treatment to make blunt ends and self-ligation of pLPL(1898/1573)-CAT (see Fig. 4*A*). All the constructs were sequenced using an ABI PRIZM Dye Terminator Cycle Sequencing Ready Reaction System (Perkin-Elmer). The human estrogen receptor (ER) expression vector, HEGO was a kind gift from Dr. P. Chambon (Institut de Chimie Biologique, Strasbourg, France) (24). The chimeric pERE-CAT reporter plasmid containing two estrogen response elements linked to the CAT reporter gene was a generous gift from Dr. Y. Miyashita (Osaka University Medical School, Osaka, Japan) (25). The CREB-binding protein expression vector was a kind gift from Dr. S. Ishii (Riken, Tsukuba, Japan) (26). pSV-β-galactosidase control vector and pCAT enhancer plasmids were purchased from Promega (Madison, WI).

Cell Culture and Differentiation Methods—3T3-L1 preadipocytes were obtained from the Riken Cell Bank (Ibaraki, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and induced to differentiate into adipocytes by switching culture medium to DMEM containing 0.5 mM methylisobutylxanthine, 0.25 μM dexamethasone, and 10 μg/ml insulin for 48 h (27). Using this protocol, >95% of the cells begin to acquire the adipocyte phenotype 3–4 days after initiating differentiation. Fully differentiated 3T3-L1 adipocytes were switched to fresh DMEM containing 10% fetal bovine serum.

Transfection and Generation of 3T3-L1 Variant Lines—Transient transfections were performed 96 h after the addition of differentiation medium, employing LipofectAMINE Plus Transfection System (Life Technologies, Inc.). Briefly, 0.7 μg of LPL promoter CAT plasmid was co-transfected with 0.7 μg of HEGO plasmid and 0.7 μg of pSV-β-galactosidase control vector into the cells in 60-mm tissue culture dishes. All other procedures were performed according to the manufacturer's instructions. 3 h after transfection, the medium containing DNA was replaced with growth medium containing 10% charcoal-dextran-treated fetal calf serum with different concentrations of estradiol. The cells were harvested for CAT assay 36 h after transfection.

For stable transfections, 50% confluent 3T3-L1 cells in 60-mm dishes were co-transfected with 1.8 μg of HEGO plasmid DNA and 0.2 μg of pSV2Neo plasmid. Stable transfectants were selected by adding G418 (250 μg/ml) to the medium.

RNA Extraction and Northern Blotting—Cellular RNA was isolated from 3T3-L1 cells and the variants using TRIZOL reagent (Life Technologies, Inc.) according to the manufacturer's instructions. The amount of murine LPL mRNA was assessed by Northern blot analysis (28). Poly(A)$^+$ RNA (2 μg) was separated by electrophoresis in horizontal 1.2% agarose gels containing 6.5% formaldehyde. RNA was transferred to nylon membranes (Hybond-N; Amersham Pharmacia Biotech) overnight and covalently bonded to the membrane by exposure to ultraviolet light (UV Stratalinker 1800; Stratagene). Blots were prehybridized for 16 h at 42 °C in a solution containing 50% formamide, 4× SSC, 5× Denhardt's solution, 100 μg/ml heat-denatured salmon sperm DNA, and 1% SDS. Hybridization was carried out for at least 16 h at 42 °C in an identical solution containing 4×10^6 dpm/ml of labeled mouse LPL cDNA probe (29). In general, hybridized blots were washed three times in a solution containing 0.5× SSC, 0.1% SDS at 60 °C. Autoradiography was performed at −80 °C with Hyperfilm MP (Amersham Pharmacia Biotech) and an intensifying screen for 48 h. Results were also quantified using a FUJIX BAS 2000 imaging system (Tokyo, Japan).

EMSA—Nuclear extracts were prepared according to the procedure of Dignam *et al.* (30). Briefly, cells were grown in the presence or absence of estrogen, washed twice with phosphate-buffered saline, scraped from the plates with a rubber policeman, and transferred to 15-ml conical Falcon tubes. The cells were centrifuged for 5 min at $500 \times g$, and the cell pellets were resuspended in 3 ml of hypotonic lysis buffer containing 10 mM HEPES (pH 7.6), 25 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 0.01% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, 10 μg of aprotinin/ml, 2 μg of leupeptin/ml, and 2 μg of pepstatin/ml. Nuclear pellets were resuspended with 300 μl of nuclear extraction buffer containing 50 mM Tris-HCl (pH 7.8), 420 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA (pH 8.0), 2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin/ml, 2 μg of leupeptin/ml, and 2 μg of pepstatin/ml and incubated for 30 min at 4 °C with gentle rocking. After centrifugation at $8.8 \times 10^4 \times g$ for 1 h at 4 °C, the supernatant fractions were recovered, and the protein content was determined by the Bradford assay. Equivalent amounts of nuclear lysates (5 μg of protein) or human recombinant c-Jun protein (Promega E3061) were incubated for 10 min at room temperature with 2 μg of poly(dI-dC)·(dI-dC) in a reaction mixture containing 10 mM HEPES (pH 7.8), 50 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol. A double-stranded radiolabeled (1×10^5 cpm) DNA probe was added, and the reaction mixture was incubated for 30 min at room temperature. To demonstrate DNA specificity, identical reactions were performed with the addition of unlabeled double-stranded oligonucleotides corresponding to AP-1-binding site (31) or consensus C/EBP DNA-binding site (32), as unlabeled competitors. To show AP-1 isoform-specific binding to the consensus AP-1 DNA-binding site, nuclear lysates were preincubated for 1 h at 4 °C with anti-c-Jun (SC-822X), anti-JunB (SC-46X), anti-JunD (SC-74X), anti-c-Fos (SC-52X), or anti-ER (SC-544X) antibodies prior to the addition of poly(dI-dC)·(dI-dC) and radiolabeled probe DNA. Protein-DNA complexes were resolved on a 5% neutral polyacrylamide gel containing 0.25× TAE (10 mM Tris acetate, 250 μM EDTA) and visualized by autoradiography.

CAT Assay—For CAT assays, cells were harvested by being washed twice in phosphate-buffered saline, resuspended in 100 mM Tris-HCl (pH 7.8), and lysed by 4 cycles of freeze-thawing (alternating between an ethanol dry ice bath and/or 37 °C water bath, 5 min/cycle). Cell lysates were heated at 68 °C for 15 min and centrifuged at 1.2×10^4 g for 10 min, and the supernatant fractions were recovered. CAT activity in the cell extracts containing 20–50 μg of lysate protein was measured by thin layer chromatography. The enzyme assay was carried out with 20 mM Tris-HCl (pH 8.0), 0.05 μCi of [^{14}C]chloramphenicol, 0.8 mM acetyl coenzyme A for the chromatographic assay. Reaction mixtures were applied to silica gels, and chloramphenicol species were separated by chromatography with 5% methanol, 95% chloroform. Chloramphenicol conversion to monoacetylated and diacetylated species was visualized and quantified using a FUJIX BAS-2000 imaging system. For each assay procedure, reaction mixtures were incubated at 37 °C for 12 h. Mock-transfected cells were used to establish the basal level of activity. Each CAT activity was normalized by the β-galactosidase activity. The β-galactosidase activity was determined by measuring the optical density at 420 nm using *o*-nitrophenol-β-D-galactopyranoside as a substrate (33).

Measurement of Intracellular Triglyceride and Oil Red-O Staining—The intracellular triglyceride levels were measured using a Determiner TG-S 555 kit (Kyowa Medicus, Tokyo, Japan). Differentiated 3T3-L1 adipocytes were stained with Oil Red-O (Sigma) by a method described previously (34).

RESULTS

Estrogen Suppressed Fat Accumulation in 3T3-L1-ER, a 3T3-L1 Mutant Expressing a Functional Estrogen Receptor, in

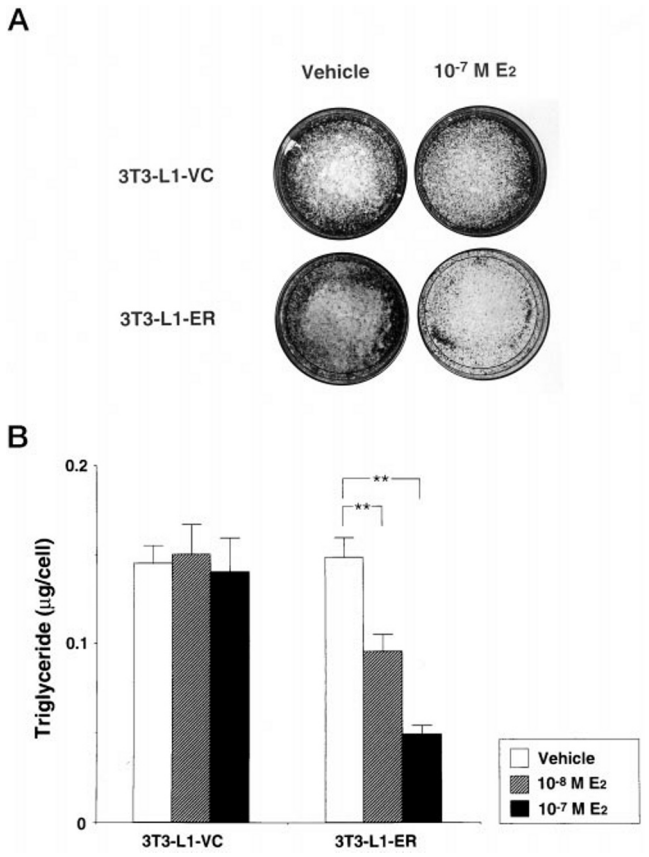

FIG. 1. **Estrogen decreases the amounts of fat deposit and intracellular triglyceride in adipocyte-differentiated 3T3-L1 cells stably expressing estrogen receptor (3T3-L1-ER).** 3T3-L1-ER and vector control (3T3-L1-VC) cells were induced to differentiate and then incubated in DMEM supplemented with 10% fetal calf serum in the presence or absence of 10^{-7} M estradiol (E_2) for 96 h. *A*, cultured cells were stained with Oil-Red-O and photographed (34). Note that staining was markedly reduced by estrogen in 3T3-L1-ER but not in 3T3-L1-VC cells. *B*, amounts of intracellular triglycerides in the cells after the same treatment as in *A*. Experiments were repeated three times with consistent results. Significant differences are indicated by ** ($p < 0.01$, $n = 5$ in each group).

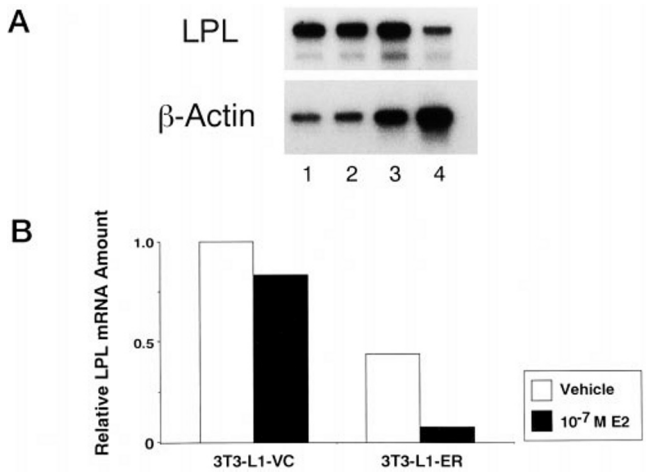

FIG. 2. **Estrogen reduces the amounts of LPL transcripts in 3T3-L1-ER cells.** *A*, poly(A)$^+$ RNA was isolated from the cultured cells 96 h after the addition of differentiation medium, separated on a 1.2% agarose gel, and subjected to Northern blot analysis using ^{32}P-labeled mouse LPL cDNA (29). The filter was reprobed with ^{32}P-labeled β-actin cDNA fragment after stripping. *Lane 1*, 3T3-L1-VC, estrogen untreated; *lane 2*, 3T3-L1-VC, treated with 10^{-7} M estradiol; *lane 3*, 3T3-L1-ER, estrogen untreated; *lane 4*, 3T3-L1-ER, treated with 10^{-7} M estradiol. *B*, relative LPL mRNA expression normalized by amounts of actin mRNA. The radioactivity of each band was quantified using a BAS2000 imaging system (Fuji Film, Tokyo, JAPAN). Similar results were obtained when total RNA samples were used.

an ER-dependent Manner—Although estrogen has been shown to inhibit fat accumulation in association with suppression of the LPL gene, it is still unclear whether estrogen causes similar effects in adipocytes *in vitro*. Because we believed that the lack of responsiveness to estrogen *in vitro* was due to a lack of ER in adipocyte cell lines (35), we generated a minimal deviation of 3T3-L1 mouse preadipocytes, termed 3T3-L1-ER, by stably introducing an ER-expression vector along with a drug selection plasmid. The blank vector control counterpart, 3T3-L1-VC, was also prepared, and these cell lines were subjected to the following phenotype analyses. First, the cells were induced to differentiate by standard methods using glucocorticoids, methylisobutylxanthine, and insulin, and we then observed whether estrogen suppressed fat accumulation in an estrogen-dependent manner. Lipid droplets were visualized by Oil-Red-O staining. Fig. 1*A* shows that estrogen, in 3T3-L1-ER cells but not in 3T3-L1-VC cells, markedly suppressed fat accumulation as indicated by the number and the size of lipid droplets. In addition, quantification of intracellular triglycerides indicated a decrease of intracellular triglyceride as well (Fig. 1*B*). These results indicate that estrogen ER-dependently acts as an inhibitor of fat accumulation in adipose tissue.

LPL mRNA Was Reduced in Parallel to Fat Accumulation in 3T3-L1-ER Cells—Because we observed a suppressive action of estrogen on lipid synthesis in 3T3-L1-ER cells, we next examined whether these changes were brought about by a decrease in LPL gene transcription. To address this question, we performed Northern blot analysis using the RNA samples obtained from the cells under each condition described in the previous section. Northern blots of total RNA as well as for poly(A)$^+$ RNA were probed with mouse LPL cDNA. The amounts of LPL transcripts was reduced in response to estrogen treatment in 3T3-L1-ER cells but not in 3T3-L1-VC cells (Fig. 2, *A* and *B*). This result is consistent with the observation in the previous section that estrogen suppressed fat accumulation in 3T3-L1-ER but not in 3T3-L1-VC cells (Fig. 1, *A* and *B*). Interestingly, 3T3-L1-ER cells not treated with estrogen showed a partial reduction of LPL transcripts to a level approximately 50% of that of untreated 3T3-L1-VC cells (Fig. 2, *A* and *B*). These results suggest that 1) estrogen reduces the level of LPL transcripts in an ER-dependent manner and 2) transcriptional suppression by the ER occurred even in the absence of estrogen. Combined with the phenotype analyses described above, these findings suggest that the reduction of LPL mRNA may contribute to the decrease in fat accumulation.

The Functional LPL Promoter Activity Is Suppressed by Estrogen in the Presence of Estrogen Receptor in 3T3-L1 Cells—Given the correlation of LPL mRNA and fat accumulation *in vitro*, we were prompted to ask if the functional LPL promoter, which contains up to 1980 bp upstream of the transcription start site, is regulated by estrogen in 3T3-L1 cells. Differentiated 3T3-L1 cells were transiently transfected with the CAT reporter plasmid, pLPL(1980)-CAT, along with an ER expression vector, HEGO, and then were treated with estrogen for 48 h. Fig. 3*A* shows the results of one of a representative CAT assay. The pLPL(1980)-CAT activity was not affected by estrogen in the cells transfected with a blank vector, pSG5. In the absence of estrogen, the activity of pLPL(1980)-CAT was suppressed to 60% of the level of the blank vector counterpart when the cells were transfected with HEGO. In the HEGO-transfected cells, estrogen suppressed pLPL(1980)-CAT activity about 6-fold. To rule out the possibility that estrogen nonspecifically suppressed the cell viability, 3T3-L1 cells were transfected with pERE-CAT plasmid, which contains two es-

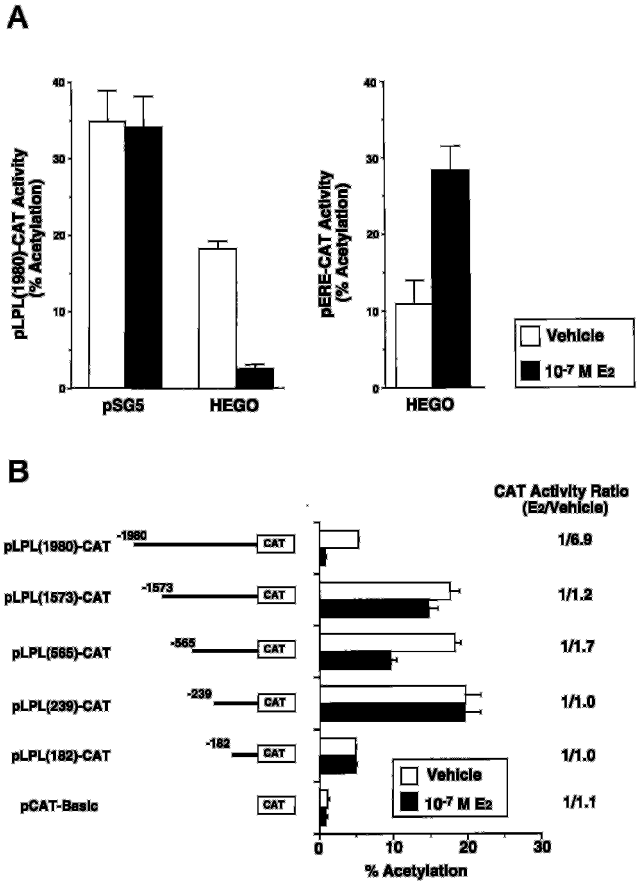

FIG. 3. **LPL promoter activity is suppressed by estrogen in 3T3-L1 cells.** *A*, the LPL gene promoter activity was suppressed by estrogen in the presence of ER in adipocyte-differentiated 3T3-L1 cells. pLPL(1980)-CAT reporter plasmid was transiently introduced into differentiated 3T3-L1 cells either with ER expression vector (HEGO) or blank vector (pSG5). Transfected cells were treated with or without 10^{-7} M estradiol for 48 h and assayed for CAT activity by a thin layer chromatography method (*left panel*). As a positive control experiment, pERE-CAT which contains two tandem ERE (25) was used instead of pLPL(1980)-CAT (right panel). All other experimental procedures were the same as those in the *left panel*. CAT activities are shown as percentages of acetylation normalized by β-galactosidase activities. The reported values are means of the results from five independent experiments with triplicate samples. *B*, activity of 5′-deletion mutants of pLPL(1980)-CAT reporter. pLPL(1980)-CAT was sequentially deleted to generate several 5′-deletion CAT reporter plasmids shown at the *left* of the *panel*. Adipocyte-differentiated 3T3-L1 cells were transiently transfected with each reporter plasmid and HEGO using the same procedures described in *A*. CAT activity ratio indicates the value obtained by dividing the percentage of acetylation in the estrogen-treated group by that in the vehicle-treated group. The reported values are the means of results from two independent experiments with triplicate samples.

trogen response elements upstream of the CAT reporter, and then treated with or without estrogen. The pERE-CAT activity was increased nearly 3-fold in response to estrogen treatment, suggesting that the suppressive effect of estrogen on the LPL promoter is not the consequence of the toxicity of estrogen (Fig. 3*A*, *right panel*). It was demonstrated that the suppressive effect of estrogen on the LPL promoter activity was ER dose-dependent as well as estrogen dose-dependent by using various amounts of each factor (data not shown).

A Single Negative Regulatory Element Was Identified in the LPL Promoter—Because we found that estrogen negatively regulates the LPL promoter activity, we next examined whether there were any suppressive element(s) in the LPL promoter region. To address this question, we systematically deleted the 1980-bp 5′-flanking fragment of the LPL promoter to generate several deletion mutant-CAT constructs and conducted CAT analyses of this array of constructs. It is shown in Fig. 3*B* that deletion to −1573 weakened the suppressive effect of estrogen to about one-seventh of that observed with pLPL(1980)-CAT. No constructs that contained shortened 5′-flanking fragments showed as much suppression as pLPL(1980)-CAT, although pLPL(1573)-CAT and pLPL(565)-CAT less reduction of transcriptional activities. The reason why these two constructs showed partial suppression is not known, but it is probably because some other transcriptional regulator(s) may cooperate with the factor that binds between −1980 and −1573.

We therefore concluded that a negative regulatory element is located in the region between −1980 and −1573. A data base search for transcription factor binding consensus sequences in this 407-bp region revealed that there was no ERE. Because some of the target genes of estrogen are regulated by AP-1 activity modulation by ER (36), we searched for an AP-1 binding consensus sequence throughout the 407-bp sequence and found an AP-1 sequence (TGACTAA) located at −1946/−1940 and an AP-1-like sequence (TGAATTC) at −1856/−1850. The former has been reported as an authentic AP-1 site (31, 37). To elucidate which of these sequences is responsible for the suppressive action of estrogen, we transferred the above 407-bp fragment in front of a minimal LPL promoter containing only the 5′ flanking sequence up to −182 (Fig. 4*A*). We deleted this 407-bp fragment so that each of the AP-1 sequences was sequentially deleted. To begin with, we tested whether these putative AP-1 sequences respond to TPA stimulation (Fig. 4*B*). The CAT reporter construct that contains the whole 407-bp [pLPL(1980/1573)-CAT] responded to TPA even at 0.1 μM. When the first AP-1 element was deleted (pLPL(1878/1573)-CAT), the TPA responsiveness became weaker but was still observed at 1 or 10 μM. When both of these elements were deleted (pLPL(1731/1573)-CAT), no TPA responsiveness was observed. Having thereby demonstrated the AP-1 activity of each putative AP-1 element, we tested whether these elements could account for the suppressive action of estrogen. As shown in Fig. 4*A*, deletion of the AP-1 sequence alone (TGACTCA, −1946/−1940) did not affect the suppression by estrogen. Deletion of both the AP-1 and AP-1-like sequences, however, resulted in a complete loss of suppression by estrogen. Because it seemed that the AP-1-like element was an estrogen-responsive negative regulatory element, we introduced a deletion into this AP-1-like sequence by removing four nucleotides (AATT). The CAT activity of this mutant construct was unaffected by estrogen, similarly to the construct in which the two putative AP-1 elements were missing (Fig. 4*A*). Taken together, these findings show that the negative regulatory element responsive to estrogen is the AP-1-like TGAATTC sequence located at −1856/−1850 in the LPL promoter.

A Single Nuclear Protein Complex Was Formed with the TGAATTC Sequence on the LPL Promoter—Having identified the DNA sequence responsible for transcriptional suppression by estrogen, we next examined whether a nuclear factor(s) forms a complex with this negative regulatory element. For this purpose, we radiolabeled double-stranded oligonucleotides harboring the putative transcription factor binding sequence and subjected the fragments to an EMSA. Nuclear extracts were prepared from 3T3-L1-ER cells grown in the presence or absence of estrogen. Fig. 5*A* shows that a single DNA-protein complex was formed when the TGAATTC fragment was used as a probe. This experiment also indicated that this complex formation was completely impaired by the addition of a 100-fold excess of the unlabeled oligonucleotide used as a probe, sug-

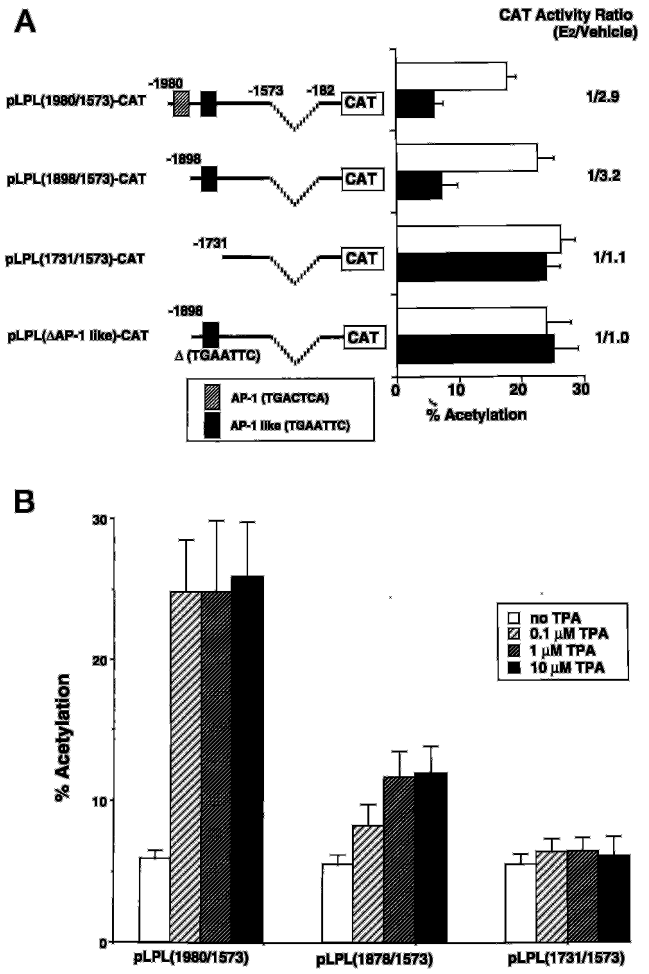

FIG. 4. **Identification of suppressive estrogen response element for LPL gene transcription.** *A*, characterization of estrogen-responsive suppressor element in the LPL promoter-proximal region. Three DNA fragments containing −1980/−1573, −1898/−1573, and −1731/−1573 were generated by polymerase chain reactions. Each fragment was fused to upstream of a minimal CAT reporter gene pLPL(182)-CAT. Note that these three fragments were designed so that the AP-1 sequence (TGACTAA, located at −1946/−1940) and AP-1-like sequence (TGAATTC, located at −1856/−1850) were sequentially deleted. Further, the construct that harbors the −1980/−1573 fragment was mutated so that the AATT sequence in the AP-1-like sequence was deleted (pLPL(1980/1573) ΔAP-1-CAT, shown at the *bottom* on the *left* side of the *panel*). Adipocyte-differentiated 3T3-L1 cells were transiently transfected with one of these four CAT-reporter plasmids and HEGO and then subjected to the same treatment as described in Fig. 3*A*. The reported values are the means of results from five independent experiments with triplicate samples. *B*, AP-1 and AP-1-like sequences in the LPL promoter behave as a TPA response element. The same deletion mutants described in *A* were subjected to a CAT assay in which the adipocyte-differentiated 3T3-L1 cells were treated with 0, 0.1, 1.0, and 10 nM of TPA for 48 h after transfection. The reported values are the means of results from five independent experiments with triplicate samples.

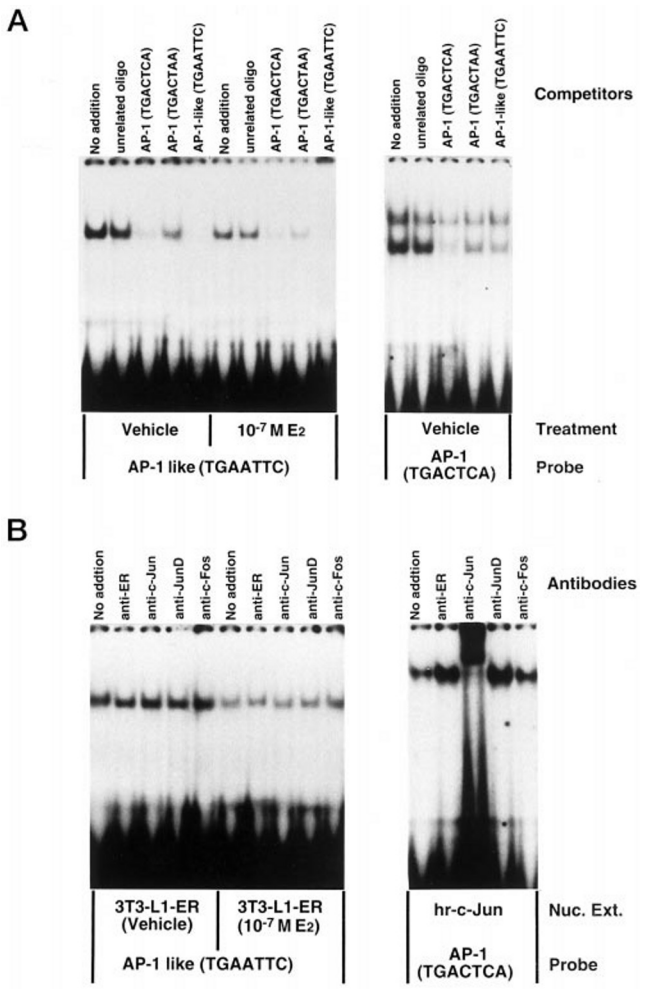

FIG. 5. **A single nuclear factor-DNA complex is formed with the TGAATTC sequence.** *A*, EMSA nuclear extracts prepared from adipocyte-differentiated 3T3-L1-ER cells were incubated with 1×10^5 cpm of radiolabeled double-stranded oligonucleotides containing the TGAATTC AP-1-like sequence (*left panel*) or TGACTCA authentic AP-1 sequence (*right panel*) and separated on a 5% nondenaturing polyacrylamide gel. To demonstrate DNA recognition specificity, unlabeled competitors were added to the reaction mixture. Unrelated oligo corresponds to consensus C/EBP-binding site, 5′-AGATTGCACAATCT-3′. AP-1 (TGACTCA), AP-1 (TGACTAA), and AP-1-like (TGAATTC) correspond to 5′-CTAGCGCTTGATGACTCAGCCGGAAGATC-3′, 5′-CTAGCTGGGGCACCTGACTAAGGCCAGATC-3′, and 5′-CTAGCCAGCGTGTCTGAATTCCGTGTTAGATC-3′, respectively. The gels were dried and then autoradiographed. *B*, antibody supershift analysis. Nuclear extracts prepared from adipocyte-differentiated 3T3-L1-ER cells used in *A* or purified recombinant human c-Jun protein were incubated with the same probes indicated in the *panel*. Antibodies were preincubated with the protein in the reaction for 1 h before radiolabeled probe was added. The reaction mixtures were subjected to electrophoresis as in *A*. The gels were dried and autoradiographed. Experiments were repeated three times with consistent results. *Nuc. Ext.*, nuclear extract; *hr-c-Jun*, human recombinant c-Jun.

gesting that the complex formation is specific (Fig. 5*A*, *left panel*, *fifth* and *tenth lanes*). It is noteworthy that less complex was formed with the nuclear extract prepared from estrogen-treated cells than with that from untreated cells (Fig. 5*A*, *left panel*, *first* and *sixth lanes*). Interestingly, unlabeled consensus AP-1 fragment (TGACTCA) partially impaired the complex formation, suggesting that the complex may contain one of the AP-1 transcription factors. Contrary to our expectations, the AP-1 motif located on the LPL promoter at −1946/−1940 (TGACTAA) failed to inhibit the DNA-protein complex formation (Fig. 5*A*, *left panel*, *fourth* and *ninth lanes*). Similarly, when consensus AP-1 fragment was used as a probe, it was demonstrated that the AP-1-like TGAATTC unlabeled competitor partly inhibited the DNA-protein complex formation, whereas the TGACTAA fragment did not (Fig. 5*A*, *right panel*, *fourth* and *fifth lanes*). Next, we investigated whether this complex contains any known AP-1 component by adding a panel of supershifting antibodies to each reaction. No supershift or impairment of complex formation was observed with antibodies against c-Jun, c-Fos, JunD, or the ER (Fig. 5*B*, *left panel*). A separate experiment showed no supershift with anti-JunB antibody (data not shown). We could demonstrate a supershift in a control experiment in which recombinant human c-Jun protein was subjected to EMSA with consensus AP-1

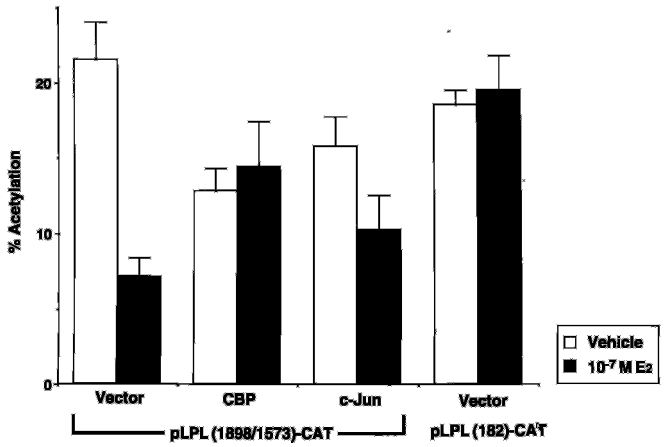

FIG. 6. **Abrogation of estrogen-dependent suppression of the LPL gene transcription by CBP.** Adipocyte-differentiated 3T3-L1 cells were transiently transfected with pLPL(1898/1573)-CAT or pLPL(182)-CAT and HEGO. Blank vector, CBP expression vector, or c-Jun expression vector was cotransfected. The cells were treated with or without estrogen and then harvested for CAT assay 48 h after transfection. pLPL(182)-CAT was used as a minimal promoter construct.

probe after preincubation with anti-c-Jun antibody (Fig. 5*B*, *right panel*). The rest of antibodies were also confirmed to supershift the protein-DNA complexes formed with the consensus AP-1 probe (data not shown). We could therefore conclude that the complex formed with the TGAATTC sequence contains none of the nuclear proteins tested with the antibodies above, although the results do not rule out the involvement of other AP-1 protein(s).

The Suppression of the LPL Gene Transcription by Estrogen Was Overcome by Overexpression of CREB-binding Protein (CBP)—The results described in the previous section demonstrated that the LPL gene expression is suppressed at the level of transcription through a unique negative regulatory element (TGAATTC). Although we could not demonstrate the existence of an AP-1-related protein in the complex, it is still likely that some other AP-1-related protein(s) is involved in this complex because this element responded to TPA stimulation (Fig. 4*B*). Because recent studies have revealed that an alternative mechanism of regulation of target gene transcriptional activity by the estrogen receptor occurs by the integration of more than two separate factors on a commonly shared protein termed a co-activator (38), we assumed that the suppressive action by the estrogen receptor might occur by utilization of one of these co-activators. Regarding the inhibitory actions of nuclear receptors, CBP has been reported to interact with estrogen receptor as well as AP-1 transcription factors (39). In a mechanism involving co-activation, AP-1 and ligand-activated estrogen receptor would compete for a limiting amount of CBP, leading to suppression of AP-1 transcriptional activity. We therefore tested whether overexpression of CBP abrogates the suppression of LPL gene expression by estrogen (Fig. 6). CBP coexpression abrogated the inhibitory effect by estrogen on the same reporter gene construct used in Fig. 4*A*, although the basal transcription level was suppressed approximately to 60% of vector control experiment.

In addition, overexpression of c-Jun partly overcame the suppression of LPL transcription by estrogen (Fig. 6). These results suggest that CBP is involved in the suppressive action by estrogen receptor. Overall, our findings suggest that a nuclear protein distinct from ER or known AP-1 proteins may be involved in a complex that binds to the TGAATTC element and that CBP may interact with this nuclear protein as well as with ER to regulate LPL gene suppression.

DISCUSSION

It is known that estrogen replacement inhibits obesity in ovariectomized rodents (8). LPL plays a key role in regulating lipid metabolism (19), and estrogen has been shown to decrease the levels of LPL enzymatic activity as well as mRNA (12–16). Although transcriptional regulation of the LPL gene has been proposed (20), few data are available regarding this issue. In this study, using a 3T3-L1 mutant cell line that expresses the ER, we found that estrogen suppresses fat accumulation and the amounts of LPL mRNA in adipocytes in a receptor-dependent manner. Using LPL promoter-CAT reporter analyses and EMSA, we found that 1) a single estrogen-responsive suppressive element, encoding TGAATTC, exists in the LPL promoter and 2) nuclear protein(s) form a single detectable complex with the above sequence.

It has been reported that the octamer sequence motif (ATTTGGAT) at position −46 and CCAAT enhancer located at −64 are sufficient for the basal promoter function of the LPL gene (23). Although the role of these elements has been intensively studied, the presence of other regulatory enhancers has been suggested (40, 41). Multiple glucocorticoid responsive elements are present in all species examined (23), and LP-α (−702 to −666) and LP-β (−468 to −430) elements are present in the human LPL gene (41). In addition to a report describing a silencer element for the LPL gene (42), further analysis of the 5′-flanking region of the LPL promoter suggests that additional regulatory elements exist in the 4-kb region upstream of the transcription start site (41).

Two transcription factors that play an important role in regulating adipogenesis have been studied utilizing the antisense DNA method: these are c-Fos, a component of AP-1 proteins (43), and C/EBPα (44). Introduction of antisense oligonucleotides for either c-Fos or C/EBPα blocked adipocyte differentiation. Interestingly, the amount of LPL transcripts was reduced by c-Fos antisense oligonucleotide (43) but not by C/EBPα oligonucleotide (41). Growth hormone, which plays an essential role in adipocyte differentiation (45), promotes LPL gene transcription by increasing the level of c-Fos, and antisense c-Fos abolishes the growth hormone-induced increase in LPL mRNA (43). Evidence showing the involvement of AP-1 proteins in LPL transcriptional regulation has been accumulated. For example, exposure of murine macrophages to high concentration of glucose results in an increase in LPL mRNA and LPL gene transcription (46). These results are associated with an increase in the c-Fos level, because antisense c-Fos or protein kinase C inhibitor blocked the increase in LPL mRNA level (46). It has also been reported that the AP-1 site located at (−1946/−1940) on the LPL promoter is a region responsible for c-Fos effects (46). By using several deletion mutants, we found that an AP-1-like sequence (TGAATTC) at −1856/−1850 is an estrogen-responsive suppressive element for LPL gene transcription. Mutational CAT analysis further supported the role of this sequence as a negative estrogen regulatory element.

The interaction between AP-1 proteins and members of the nuclear receptor family was first reported in the case of glucocorticoid receptor, which showed mutual inhibition of transcription because of direct protein-protein interaction (36). Several investigators have reported that estrogen receptor also interacts with AP-1 proteins (47–49), but in most cases, in contrast to the glucocorticoid receptor/AP-1 interaction, estrogen receptor showed a synergistic effect on the target gene transcription. Schmidt *et al.* (50) reported the existence of a negative regulatory effect on gene transcription through the AP-1 element in the case of transcription of the human choline acetyltransferase gene. Our data, which strongly suggest a negative regulatory action by estrogen on the LPL promoter

through putative AP-1 protein(s), are of significance because we have identified the unique TGAATTC sequence as a DNA element distinct from the classical AP-1 sequence.

We were particularly interested in the fact that it was not the previously reported AP-1 sequence (TGACTAA located at −1946/−1940) (31) but rather the AP-1-like sequence (TGAATTC located at −1856/−1850) that was responsible for the negative regulatory action by estrogen (Fig. 4*A*). Our EMSA data may give us a clue to this issue because a single specific DNA-protein complex was formed when the TGAATTC sequence was used as a probe (Fig. 5A). Competition EMSA with unlabeled fragments showed that this complex formation was blocked less effectively by the AP-1 sequence on the LPL promoter (−1946/−1940) than by authentic AP-1 sequence (TGACTCA), suggesting that the former sequence may not be involved in the suppressive action by estrogen (Fig. 5A). The fact that there was less complex formation on the TGAATTC sequence in extracts from cells grown in the absence of estrogen may indicate that the ligand-activated ER may have an inhibitory effect on the protein that is binding to the TGAATTC sequence. Although we could not demonstrate which (if any) AP-1-related protein is involved in the complex, we are strongly convinced that a transcriptional regulatory protein associated with the ER is participating in the complex for the following reasons. Firstly, the TGAATTC sequence showed TPA responsiveness, which is an essential feature of AP-1 transcription factors (Fig. 4B). Secondly, overexpression of CBP as well as c-Jun protein abrogated the suppressive action of estrogen (Fig. 6), suggesting that the ER and the factor binding to the TGAATTC sequence may share the same transcriptional platform. If this is the case, the suppression of LPL gene transcription probably occurs because multiple transcription factors are competing for limiting amount of CBP.

Recent studies report that CBP acts as an integrator of multiple signal transduction pathways including those of nuclear receptors (39), AP-1, and CREB. Lee *et al.* (38) recently reported that steroid receptor coactivator-1 can also bind to c-Fos and c-Jun. This finding also provides support for our proposal about the negative action of the ER because it demonstrates the existence of another competitive point in the sharing of a single transcription modulator by AP-1 and ER.

In humans, estrogen has a clinical impact on the lipid metabolism: estrogen replacement in postmenopausal women decreases serum total cholesterol (51, 52) and low density lipoprotein cholesterol (52, 53) levels, contributing to prevention of cardiovascular attack (52). Visceral fat accumulation occurs in postmenopausal women (51), suggesting that estrogen has an inhibitory effect on fat deposition, especially in the visceral adipose tissues in humans (51). Recently it has been reported that estrogen is a major suppressor of the fasting LPL activity in adipose tissue in women (14, 17). Comparison of the 5′-flanking regions between the human and murine LPL genes indicates that the 1,200 bp immediately upstream of exon 1 are conserved with homology over 80%. Therefore, it is conceivable that estrogen might also repress human LPL gene expression at the transcriptional level.

The biological interpretation derived from our experiments has intrinsic limitations because the ER was exogeneously expressed and the amounts of the ER might have been much higher than those expressed in adipose tissue *in vivo*. However, we believe that our study will contribute to better understanding of the *in vivo* actions of estrogen at the molecular level. Further characterization of a putative AP-1-related transcription factor binding to the TGAATTC sequence will give us new insight into the regulation of lipid metabolism by estrogen.

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