Analysis of a Novel Dexamethasone Response Element and a Putative C/EBPs *cis*-Motif: Controlling PAI-1 Gene Expression During Adipocyte Differentiation*

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Abstract It has been reported that there is a significant increase in PAI-1 expression level in obese subjects. To explore the linkage between PAI-1 gene expression and obesity, the restriction enzymes and DNA recombination technologies were used to construct the chimeric plasmids with luciferase and different lengths of PAI-1 promoter. After transfection of the chimeric plasmids into 3T3-L1 preadipocyte and detection of luciferase activity, the results indicated that a positive dexamethasone *cis*-acting element (bases − 690 to − 850) may be present in mouse PAI-1 promoter. In addition, computer analysis using Matchr-Search Software found that a new motif of DexRE (dexamethasone response element) 5′ GGTAACCTCTGTTCTCAT 3′ and a putative C/EBPs binding site (*cis*-motif) exist respectively in the fragment (nucleotides − 751 to − 770) of, and a sequence (bases − 720 to − 740) of, mouse PAI-1 promoter, and GMSA and competition assays identified that the *trans*-acting factors induced by dexamethasone can specifically bind to those *cis*-motifs. Meanwhile, the site-directed mutagenesis by PCR was performed to detect the influence of mutant DexRE and C/EBPs *cis*-motif on PAI-1 gene expression. Similarly, the chimeric plasmids containing luciferase as a reporter gene and a fragment of mouse PAI-1 promoter comprising the mutant *cis*-motifs were constructed, and then transfected into 3T3-L1 preadipocytes. The measurement revealed that the luciferase activities were markedly lowered by mutant DexRE and mutant C/EBPs *cis*-motif compared with their wild counterparts, implicating that the DexRE and C/EBPs *cis*-motif identified may control the expression of PAI-1 gene in 3T3-L1 adipocyte. The study is very helpful to elucidate a molecular mechanism through which the dexamethasone may regulate the expression of PAI-1 gene in 3T3-L1 adipocyte.
Key words PAI-1 promoter, dexamethasone, luciferase, *cis*-acting element, 3T3-L1 adipocyte

1 Introduction

In 1997, Alessi *et al*.[1] reported that adipose tissue can directly elevate the plasma level of PAI-1 (plasminogen activator inhibitor-1), that the PAI-1 protein synthesis and secretion in subjects with adiposity have been increased significantly, and that visceral adipose tissue may produce more PAI-1 protein than subcutaneous adipose tissue. Other studies indicated that adipose tissue in human is the key source that promotes the increase in plasma level of PAI-1 protein[2,3], and is also the pivotal organ that establishes the linkage between obesity and insulin resistance and the elevation in plasma level of PAI-1[4]. Based on the adipocyte differentiation being regulated by hormones, we have investigated the effect of insulin (Ins), dexamethasone (Dex) and methylisobutylxanthine(Mix) on expression of PAI-1 gene during adipocyte differentiation (published in another paper). In this research, to elucidate the molecular mechanism underlying the relationship between obesity and PAI-1 expression, we further analyzed and identified a new Dex *cis*-acting element (DexRE) and a putative C/EBPs *cis*-motif which regulate the expression of PAI-1 gene.

2 Materials and Methods

2.1 Materials

3T3-L1 cells lineage was a generous gift from Dr. Lane in Johns Hopkins University in USA. Ins, Dex and Mix were purchased from Sigma Company. 950 bp promoter of mouse PAI-1 gene was kindly provided by Prof. Cole in Princeton University in USA. Luciferase detection kit was bought from Promega Company. α-^{32}P-dATP was purchased from Yahui Company in Beijing. Poly(dI-dC)$_n$ was obtained from Pharmacia Company. DMEM was from GibcoBRL Company. FBS was bought from Clonetech Company. Klenow DNA polymerase was purchased from NEB Company.

2.2 Construction of chimeric plasmids

The restriction enzymes or PCR and DNA recombination methods were used to construct the chimeric plasmids with luciferase acting as a reporter gene and different lengths of PAI-1 promoter. Totally, six chimeric plasmids have been successfully produced(Figure 1).

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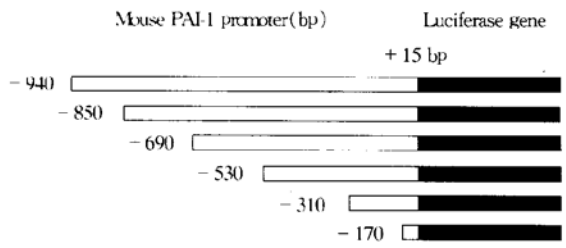

Fig. 1 Chimeric constructs of PAI-1 reporter plasmids
chimeric reporter plasmids consisted of luciferase gene and different lengths of mouse PAI-1 promoter.

2.3 Transfection of chimeric plasmids into 3T3-L1 cells

Six chimeric plasmids were constructed and PGEG plasmid containing β-galactosidase and GFP (green fluorescentprotein) were transfected together into 3T3-L1 preadipocytes(after cells grew to 60% ~ 80% volume of culture well), and the cocktail of Ins+ Dex+ Mix was applied to induce the preadipocyte differentiation. Simultaneously, the plasmids of SV40 promoter and SV40 promoterless were transfected with PGEG plasmid, respectively, into 3T3-L1 preadipocytes as positive and negative controls. The LipofectAmine transfection kit was used in this procedure.

2.4 Detection of luciferase activity

Transfected 3T3-L1 preadipocytes were stimulated by cocktail of Ins + Dex + Mix, and undergone differentiation for 8 days. In the 9th day, the differentiated adipocytes were treated with 1 μmol/L Dex for 24 h, with the group without Dex treatment as a control group. The cell lysates were prepared according to kit specification to assay the activities of luciferase and β-galactosidase, respectively. The detection of luciferase activity was referred to kit protocol; and the measurement of β-galactosidase activity was as following: 3 μl 100 × Mg solution, 66 μl 1 × O-nitrophenyl-β-D-galactosidase; 30 μl cell lysate; 0.1 mol/L Na_3PO_4 solution, these solutions were mixed and incubated at 37℃ for 1 h, then 500 μl Na_2CO_3 was added to terminate the reaction, the A_{420} value was read at Bio-Rad spectrometer.

2.5 Analysis of DexRE and C/EBPs *cis*-motif by computer

Match-Search Software was used to analyze the fragment bases - 690 to - 850 of PAI-1 promoter, and found that one DexRE and a C/EBPs binding site separately exist in the sequence (nucleotides - 750 to - 770) of, and in the fragment (bases - 720 to - 740) of, mouse PAI-1 promoter (Figure 2).

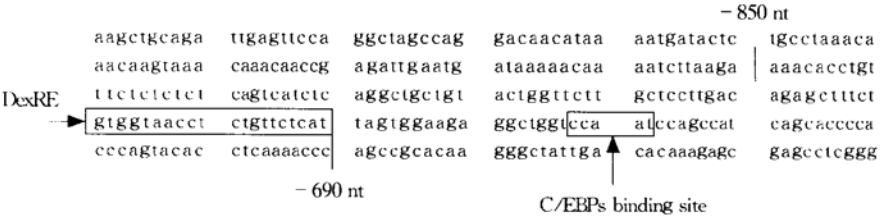

Fig. 2 Analysis of Dex response element(DexRE) and C/EBPs binding site in mouse PAI-1 promoter by Match-Search Software

2.6 Mutagenesis of site-directed DexRE and C/EBPs *cis*-motif

Site-directed mutagenesis of DexRE and C/EBPs *cis*-motif was performed by PCR, the mutated bases was introduced into the *cis*-elements by PCR primer, demonstrated by bold capitals in following. Wild type G: 5′ AATGAGAACAGAGGTTACCAC 3′; Mutant type Gm: 5′ AATGAGCGAAGAGGTTACCAC 3′; Wild type C/EBPs: 5′ GGATTGGACCAGCCTCT-TCCAC 3′; Mutant type C/EBPs m: 5′ GGATC-AGACCAGCCTCTTCCAC 3′.

2.7 Nuclear proteins extraction and gel mobility shift assay (GMSA)

2.7.1 Nuclear extract[5]: nuclear proteins from 3T3-L1 cells were prepared using urea extraction method. 3T3-L1 cell monolayers were washed twice with cold PBS and once with hypotonic lysis buffer containing 20 mmol/L Tris-HCl, pH 7.5, 10 mmol/L NaCl, 3 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L sodium vanadate, 30 mmol/L β-glycerol phosphate and 2 μl/ml protease inhibitors cocktail 1 and 2(PIC 1 and PIC 2)[5]. Cells were then scraped from plates into the same hypotonic buffer and incubated on ice for 5 min. NP-40 was added to the cell suspension to a final concentration of 0.1% for preadipocytes and 0.15% for adipocytes. After homogenizing the cells, nuclei were pelleted by centrifuging at 500 *g* for 5 min. The nuclear pellet was then washed once with hypotonic buffer and once with nuclear storage buffer containing 40% glycerol, 50 mmol/L Tris-HCl, pH 8.0, 3 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L sodium vanadate, 30 mmol/L β-glycerol phosphate and 2 μl/ml PIC 1 and PIC 2. The washed nuclei were pelleted by centrifuging at 6 000 *g* for 20 s and resuspended in 10 pellet volumes of extraction buffer containing 1.1 mol/L urea, 0.33 mol/L NaCl, 1.1% NP-40, 17.5 mmol/L Hepes, pH 7.6, 1.1 mmol/L DTT, 1 mmol/L sodium vanadate, 30 mmol/L β-glycerol phosphate and 2 μl/ml PIC 1 and PIC 2. After incubation on ice for 30 min, the mixture was centrifuged at 12 000 *g* for 10 min. The supernatant was collected and glycerol was added to final concentration of 10%. The nuclear extract was

aliquoted, quick-frozen and stored at − 80 ℃. The protein concentration of the nuclear extract was determined by Lowry method.

2.7.2 Labeling of probes: *Hin*d Ⅲ restriction enzyme was used to cleave the fragment bases − 690 to − 850 of PAI-1 promoter (containing DexRE sequence and C/EBPs *cis*-motif) amplified by PCR. The wild type probe and mutant type probe were then labeled with α-^{32}P-dATP by Klenow enzyme. The labeled probes were purified under normal conditions.

2.7.3 Nuclear extract (10 μg) was incubated with 2 μg poly(dI-dC)$_n$ in 50 μl binding buffer containing 10 mmol/L Tris-HCl, pH 7.9, 50 mmol/L NaCl, 0.5 mmol/L EDTA, 1 mmol/L DTT, 10% glycerol and 5 mmol/L $MgCl_2$ on ice for 20 min. Approximately 2×10^4 ~ 6×10^4 cpm (1 ~ 5 ng) of ^{32}P end-labeled double-strand DNA fragment was added to the preincubated nuclear extract mixture and continued to incubate on ice for 50 min. DNA-protein complexes were resolved on 6% polyacrylamide gels in 0.5 × TBE buffer. The gel was autoradiographed. For competition experiment, usually 50- to 100-fold excess unlabeled competitor DNA was added to the reaction mixture at the same time that the labeled DNA fragment was added.

3 Results

3.1 Identification of chimeric plasmids

The six chimeric plasmids have been constructed by using PCR and DNA recombination methods. These plasmids comprised luciferase gene acting as reporter gene and different lengths of PAI-1 promoter, respectively, and were cleaved by two sets of restriction enzymes, electrophoresed by 2% agarose gel, confirming that they were successfully established (Figure 1).

3.2 Transfection effectiveness

The chimeric plasmids and control plasmids were then transfected into 3T3-L1 peadipocytes by lipofectamine after 2 days post-confluence of the cells. Transfected 3T3-L1 preadipocytes were stimulated by cocktail of Ins+ Dex+ Mix for 2 days, and undergone differentiation. Pictures suggest that EGF acting as a kind of indicator of transfection efficiency soundly expressed in transfected cells before and after differentiation(Figure 3).

3.3 Analysis of luciferase activity

The fully differentiated 3T3-L1 adipocytes monolayer(after 8 days differentiation) were collected, and cells lysates were made and stored. The luciferase activities were then detected according to Kit specification, suggesting that at least three positive *cis*-response elements may exist in three fragments (bases − 310 to − 530; − 690 to − 850; and − 850 to − 940) of mouse PAI-1 promoter, respectively. Meanwhile, at least a negative *cis*-element may be present in the fragment (nucleotides − 530 to − 690) of mouse PAI-1 promoter (Figure 4).

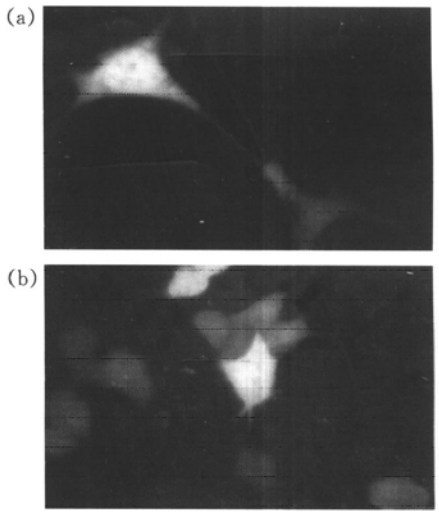

Fig. 3 Efficiency of transfection of chimeric plasmids into 3T3-L1 preadipocytes

(a) EGF acting as control expression after 2 days transfection but before stimulation; (b) EGF protein expression after 2 days stimulation by cocktail of Ins+ Dex+ Mix.

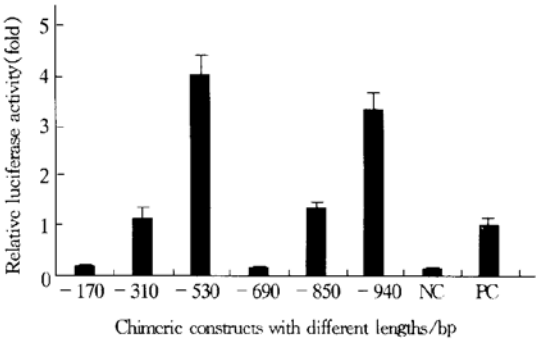

Fig. 4 Detection of reporter gene activity triggered by mouse PAI-1 promoter in normal differentiation

NC= negative control= promoterless; PC= positive control= SV40 promoter. Values are $\bar{x}\pm s$, $P<0.01$; $n=3$.

3.4 Determination of DexRE

To further determine where the Dex response element located, the transfected 3T3-L1 cells with

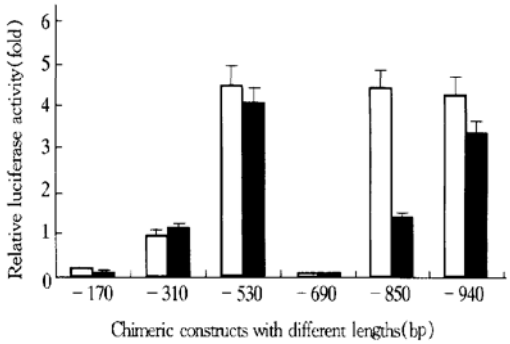

Fig. 5 Test of luciferase activity triggered by dexamthasone

■: represent normal differentiation; □: represent treatment by dexamethasone. Values are $\bar{x}\pm s$; $P<0.01$; $n=3$.

chimeric plasmids were induced and reach complete differentiation on the eighth day after removal of cocktail of Ins + Dex + Mix, and differentiated adipocytes were then triggered by 1 μmol/L Dex for 24 hours. The adipocytes lysates were collected and luciferase activity was assayed. The results demonstrated that at least one positive DexRE is present in the fragment (bases − 690 to − 850) of PAI-1 promoter (Figure 5).

3.5 The fine analysis of DexRE and C/EBPs *cis*-motif by computer

Although the rough position of DexRE has been uncovered by luciferase activity, the exact position and sequence of DexRE (Dex response element) in this fragment is still unknown. In this regard, Match-Search software was used in current experiment instead of footprint assay, and computer analysis indicated that the DexRE exists in the position nucleotides − 750 to − 770 of PAI-1 promoter, and that the DexRE sequence is 5′ GTGGTAACCTCTGTTCTCAT 3′. Meanwhile, a consensus sequence 5′ CCAAT 3′, a putative C/EBPs *cis*-motif, was also demonstrated (Method 2.5).

3.6 Identification of DexRE and C/EBPs *cis*-motif by GMSA

To identify if the DexRE and C/EBPs *cis*-motif are unique and specific for the *trans*-factors induced

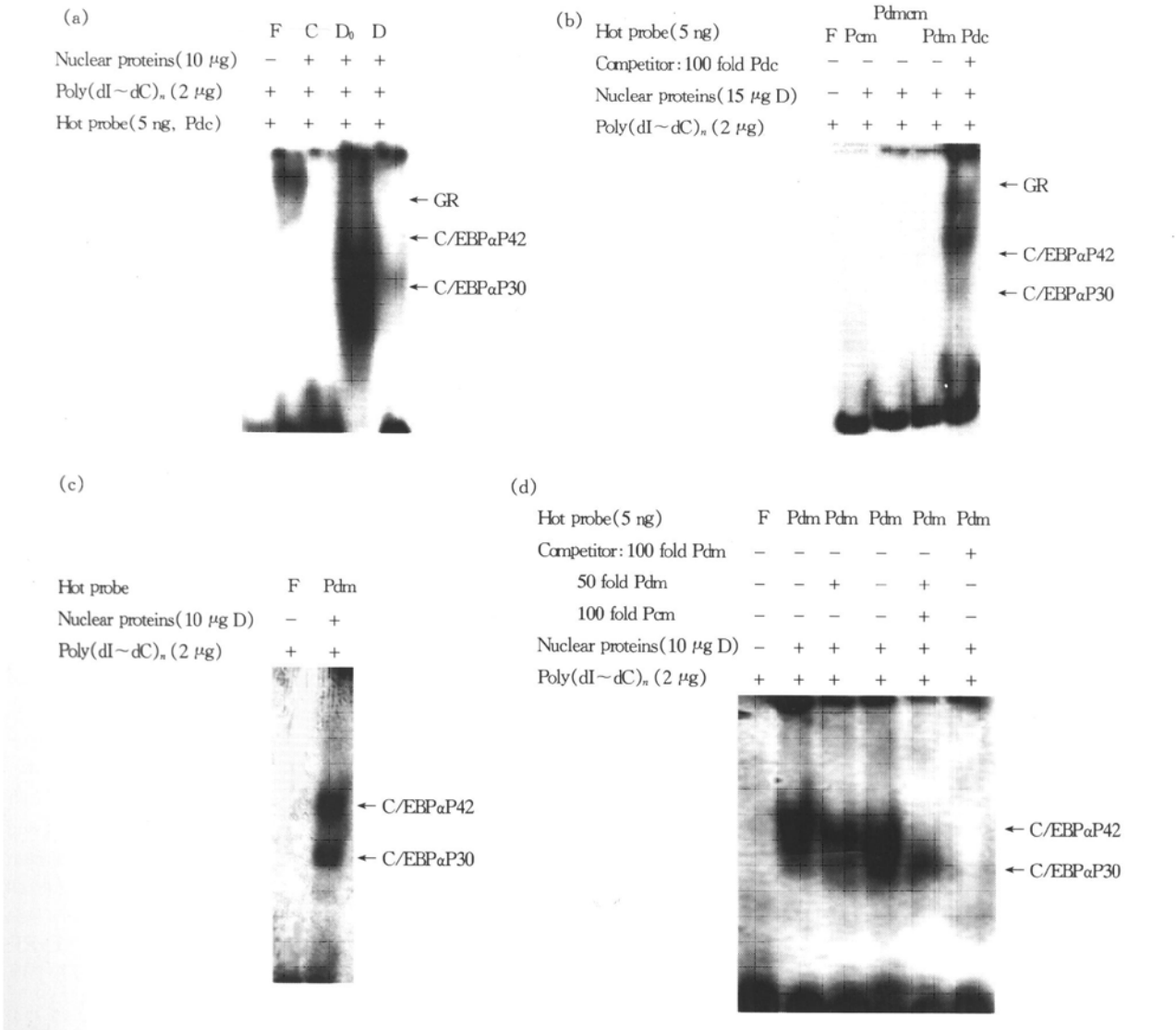

Fig. 6 Identification of DexRE and C/EBPs *cis*-motif in mouse PAI-1 promoter by GMSA and competitive GMSA

(a) In GMSA, α-^{32}P-ATP labeled Pdc harboring Dex response element and C/EBPα *cis*-motif was used to combine the nuclear extracts from 3T3-L1 preadipocytes before induction (lane C as a control). 3T3-L1 preadipocytes after 2 days induction (lane D_0), and 3T3-L1 terminally differentiated adipocytes treated with Dex (lane D), respectively. The combined complexes were resolved in 6% polyacrylamined gel at 150 V, for about 3 h. The two bands were shown in lane D. (b) α-^{32}P-ATP labeled mutant probes: Pcm, Pdm. Pdmcm were applied in GMSA, and an unlabeled probe Pdc as a competitor to labeled Pdc was added in compatitive GMSA. The GR and C/EBPα were identified in (a) and (b). (c) The specificity of binding of C/EBPα to their *cis*-motif was further confirmed, and P42 C/EBPα and its isoform P30 C/EBPα were revealed. (d) The unlabeled probe Pdm containing C/EBPα *cis*-motif and unlabeled Probe Pcm harboring Dex *cis*-element were used as competitors in competitive GMSA to detect the specific binding of C/EBPα isoforms to their *cis*-motif in mouse PAI-1 promoter. F: free probe, Pdc: a probe with DexRE and C/EBPs *cis*-motif, Pdm: a probe with mutant DexRE and native C/EBPs *cis*-motif, Pcm: a probe with mutant C/EBPs *cis*-motif and native DexRE, Pdmcm: a probe with mutant DexRE and mutant C/EBPs *cis*-motif.

by Dex, The mutagenesis of DexRE and C/EBPs *cis*-motif by PCR and GMSA(Gel Mobility Shift Assay) were conducted. In figure 6, picture (a) showed that the nuclear extract from 3T3-L1 adipocytes treated with Dex may contain the *trans*-acting factors that bind to the fragment bases - 690 to - 850 of PAI-1 promoter. Otherwise, the nuclear proteins from 3T3-L1 preadipocytes before induction (lane C) or from preadipocytes after only 2days induction (lane D_0) failed to demonstrate the ability to bind the labeled fragment. Meanwhile, two or three binding bands were revealed in test (lane D) that were indicated respectively as GR and C/EBPαP42 and C/EBPαP30 according to different molecular weights. In picture (b), the different labeled probes were used in GMSA and competitive-GMSA. The results demonstrated that probe Pcm with mutant C/EBPs *cis*-motif lost the ability to combine C/EBPα isoforms, that probe Pdm with mutant DexRE failed to bind the GR, and that the probe with mutant DexRE and mutant C/EBPs *cis*-motif was not indicated any binding band. Similarly, the specific combination of labeled Pdc with nuclear extract was totally inhibited by 100 fold excess unlabeled Pdc. The assay in picture (c) suggested that probe Pdm can specifically binds to two C/EBPα isoforms: C/EBPαP42 and C/EBPαP30, and the competitive-GMSA in picture (d) pointed out that this specificity of combination can be completely inhibited by 100 fold molar excess of unlabeled wild-type probe, and partly inhibited by 50 fold molar excess of unlabeled wild-type probe. These results unveiled that the bands showed in pictures may be the complex of DexRE sequence and related *trans*-acting factor (GR), and the complex of C/EBPs *cis*-motif and associated C/EBPα isoforms.

3.7 Effct of mutation in DexRE and C/EBPs *cis*-motif on transfected cells

The constructed chimeric plasmids with mutant sequence in DexRE or C/EBPs *cis*-motif, respectively, were transfected into 3T3-L1 preadipocytes, and related wild type plasmid was used as control. The protocol was just the same as the method **2.3**. The

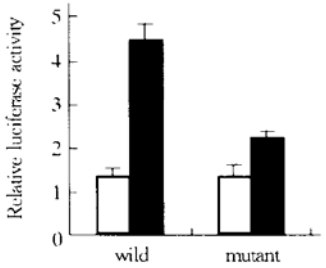

Fig. 7 Effect of mutation of DexRE in PAI-1 promoter on induction by Dex

3T3-L1 cells were transfected with chimeric plasmids containing wild or mutated DexRE sequence in PAI-1 promoter and luciferase gene, and treated with 1 μmol/L Dex. Values are $\bar{x} \pm s$, $P < 0.05$, $n = 3$. □: represent normal differentiation; ■: represent stimulation by Dex.

detection of luciferase activity indicated that the plasmid with mutant DexRE sequence dramatically decreased the response to Dex induction, and that the mutant C/EBPs *cis*-motif significantly lowered the report gene expression (Figure 7 and Figure 8).

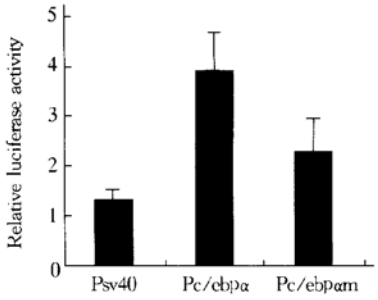

Fig. 8 Comparison of effect of mutagenesis in C/EBPα response element of mouse PAI-1 promoter on luciferase activity

Values are $\bar{x} \pm s$, $P < 0.01$, $n = 3$.

4 Discussion

3T3-L1 cells, an experimentally accessible system *in vitro*, is one of the most popular models to uncover the mechanism of adipogenesis, in spite of adipose tissue has also been the subject of intense scrutiny. A wealth of observations using a variety of biochemical and genetic means on the adipocytes differentiation have been well documented[6~8], and many transcription factors involved in this process have been identified that directly influence fat cell development or that tightly associate with cell adipogenesis. These include PPARγ, C/EBPs, ADD1/SREBP1c, GLUT4, 422/aP2 and PAI-1 etc[9~12]. Insulin, dexamethasone and methylisobutylxanthine are the essentially external factors that induce the differentiation of 3T3-L1 fibroblast-like preadipocytes into mature adipocytes. It was reported that insulin could increase the PAI-1 mRNA expression *in vitro* and *in vivo*[13]. However, the mechanism underlying this regulation is still elusive. In our study, we early demonstrated that insulin and dexamethasone both can boost the increase in PAI-1 mRNA level (see another paper). To further elucidate the mechanism through which Ins and Dex, particularly Dex, regulates the PAI-1 gene expression, current experiment was performed. The results revealed that one positive DexRE exists in the fragment (nucleotides - 751 to - 770) of PAI-1 promoter. It is 5′ AACCTCTGTCTCAT 3′, a kind of new *cis*-element compared with known related elements checked on internet. Simultaneously, a putative C/EBPs *cis*-motif 5′ CCAAT 3′ was found in the sequence (bases - 720 to - 740) of mouse PAI-1 promoter. In addition, gel mobility shift assay (GMSA) and competitive GMSA identified that the Dex-induced *trans*-activator (glucocorticoid receptor, GR) and C/EBPα isoforms: C/EBPαP42 and C/EBPαP30 may

specifically bind the DexRE and C/EBPs *cis*-motif, respectively, to regulate PAI-1 gene expression, and that the glucocorticoid receptor (GR) and the C/EBPα isoforms may competitively bind the fragment (nucleotides - 690 to - 850) of mouse PAI-1 promoter because of the DexRE and C/EBPs *cis*-motif being so close to each other.

Interestingly, a phenomenon appeared in the transfection experiment that luciferase activity decreased markedly (sixfold) when the fragment of PAI-1 promoter inserted in chimeric plasmid extended from bases - 530 to - 690, suggesting that at least one, even more negatively regulatory *cis*-motifs are present in this sequence. But these are neither IRE (data not shown) nor DexRE, whether these are Mix response elements or others is still to be further investigated.

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脂肪细胞分化过程中影响 PAI-1 基因转录表达的 Dex 和 C/EBPs 顺式调控元件的分析*

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摘要 在研究胰岛素 (Ins)、地塞米松 (Dex) 和甲基异丁基黄嘌呤 (Mix) 对脂肪细胞分化过程中 PAI-1 基因表达的影响基础上, 为进一步探讨 Ins、Dex 调控 PAI-1 基因转录表达的调控机制, 应用 DNA 重组技术, 构建含萤光素酶 (luciferase) 报告基因和 PAI-1 启动子不同长度片段的嵌合质粒, 转染 3T3-L1 前脂肪细胞并测定报告基因荧光素酶的活性. 结果表明, 小鼠 PAI-1 基因起动子- 690 至- 850 碱基序列之间有一个 Dex 的正调控元件. 用计算机软件进行分析发现: Dex 顺式元件位于 PAI-1 启动子的- 750 至- 770 碱基序列. 其组成为: 5′ GGTAACCTCTGTTCTCAT 3′. 同时还发现在 PAI-1 启动子的- 720 至- 740 碱基序列中, 存在一个 C/EBPs 的结合元件 5′CCAAT3′并用凝胶电泳迁移实验对这些元件进行了鉴定. 表明 Dex 正是通过激活转录因子 (糖皮质激素受体, GR) 和 C/EBPα 一起与各自的顺式元件结合来促进 PAI-1 基因的表达.

关键词 PAI-1 基因, 胰岛素, 地塞米松, 甲基异丁基黄嘌呤, 荧光素酶, 反应元件

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