Identification of a novel distal enhancer in human adiponectin gene

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Abstract

Adiponectin is exclusively expressed in adipose tissue and secreted from adipocytes, and shows anti-diabetic and anti-atherogenic properties. However, the precise transcriptional mechanism of adiponectin remains elusive. In this study, the 5′ flanking promoter region of human adiponectin gene was analyzed using UCSC genome browser, and a 10 390-bp fragment, containing an evolutionally conserved region among species, was investigated. The luciferase reporter assay using this fragment identified a novel distal enhancer of human adiponectin gene. Promoter constructs with the distal enhancer exhibited high promoter activities in 3T3-L1 mature adipocytes. However, no such activity was observed in other types of cell lines. The distal enhancer is highly conserved, and contains two completely conserved CCAAT boxes. In 3T3-L1 mature adipocytes, deletion or each point mutation of these CCAAT boxes markedly reduced luciferase activity driven by adiponectin promoter. Knockdown of CCAAT/enhancer-binding protein α (CEBPA; also known as C/EBPα) using small interfering RNA diminished adiponectin mRNA expression and luciferase activity driven by adiponectin promoter with the distal enhancer. However, adiponectin promoter with each mutation of two CCAAT boxes in the distal enhancer did not respond to knockdown of CEBPA expression. Furthermore, CEBPA bound to the distal enhancer both *in vitro* and *in vivo*. We also identified a proximal promoter region responsible for transcriptional activation by the distal enhancer in human adiponectin gene. Our results indicate that CEBPA plays a pivotal role in the transcription of human adiponectin gene via the distal enhancer and proximal region in its promoter.

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Introduction

There is sufficient evidence that adipose tissue is not only an energy storage organ, but it produces and secretes a variety of bioactive molecules called adipocytokines, such as tumor necrosis factor α, leptin, adiponectin, plasminogen activator inhibitor type-I, resistin, and retinol-binding protein 4 (Hotamisligil & Spiegelman 1994, Zhang *et al*. 1994, Maeda *et al*. 1996, Shimomura *et al*. 1996, Steppan *et al*. 2001, Yang *et al*. 2005). Dysregulated production of adipocytokine is associated with obesity-related metabolic diseases (Matsuzawa *et al*. 1999). Adiponectin/acrp30/adippoQ is an adipocytokine specifically and abundantly expressed in adipose tissue (Scherer *et al*. 1995, Hu *et al*. 1996, Maeda *et al*. 1996). The normal plasma adiponectin level ranges between 5 and 20 μg, but the levels are lower in obese subjects, diabetics, and those with ischemic heart disease (Arita *et al*. 1999). Furthermore, many reports suggest that adiponectin is a key player in the regulation of insulin sensitivity, energy homeostasis, immunological reactions, cardiovascular events and appetite (Yamauchi *et al*. 2001, Berg *et al*. 2002, Maeda *et al*. 2002, Shibata *et al*. 2005, Kubota *et al*. 2007, Takemura *et al*. 2007).

Precise transcriptional mechanisms of adiponectin gene remain elusive. Previous studies reported that adiponectin is transcriptionally regulated by various transcription factors, such as peroxisome proliferator-activated receptor γ (PPARG, also known as PPARγ; Iwaki *et al*. 2003), CCAAT/enhancer-binding protein-α (CEBPA, also known as C/EBPα; Park *et al*. 2004, Qiao *et al*. 2005, Qiao *et al*. 2006), nuclear factor of activated T cells 4 and activating transcription factor 3 (ATF3; Kim *et al*. 2006), through cis-regulatory elements in its proximal promoter region, or 1st intron. However, the 5′ flanking distal region of adiponectin gene has not been fully investigated.

In this study, we analyzed a 10 390 bp fragment of the 5′ flanking promoter region of the human adiponectin gene, and found a novel distal enhancer located between −2667 bp and −2507 bp upstream from its transcription start site. Interestingly, transcriptional activation by this distal enhancer was observed in mature adipocytes but not other types of cell lines. CEBPA bound to this distal enhancer, and regulated the transcriptional

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activity of adiponectin gene. The results also showed that a proximal region in the human adiponectin promoter is necessary for full transcriptional activation by its distal enhancer. These results suggest that CEBPA plays a pivotal role in the transactivation of human adiponectin gene via a communication between the distal and proximal enhancers in its promoter.

Materials and Methods

Cloning of human adiponectin promoter

Human adiponectin promoter regions were cloned as reported previously (Segawa *et al.* 2006). Briefly, human adiponectin promoters (−10 390/+77, −4075/+77, and −2664/+77) were subcloned into pBSII vector using the *Escherichia coli*-based bacterial artificial chromosome (BAC) recombination system (Lee *et al.* 2001). Retrieval cassettes were generated by PCR amplification of pBSIIKS+ vector with primers corresponding to each promoter (these are listed in Supplementary Table 1, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol200/issue1/). The BAC RP23114P23, which contains human adiponectin gene, was obtained from BAC PAC Resources Center (Oakland, CA, USA).

Plasmids

pGL3 basic and pGL3 promoters were purchased from Promega. a908Luc, a2561Luc, and TK-Luc were previously reported or constructed in our laboratory (Iwaki *et al.* 2003, Kaneko *et al.* 2003). Each promoter fragment (−10 390/+77, −4075/+77, and −2664/+77) was subcloned into pGL3 basic vector (a10390Luc, a4075Luc, and a2664Luc). A serial deleted distal and proximal enhancer region (−2765/−2507, −2664/−2507, −2664/−2539, −2587/−2507, −563/+77, −540/+77, −513/+77, −449/+77, −232/+77, and −41/+77) was PCR-amplified using the primers listed in Supplementary Table 1. The generated proximal fragments were digested with *Mlu*I and *Nhe*I, and inserted into pGL3 basic plasmid (a563Luc, a540Luc, a513Luc, a449Luc, a232Luc, and a41Luc). A serial deleted distal enhancer region was excised with *Mlu*I and *Eco*RI, and inserted into a563Luc (a563Luc+Unit1 (158 bp), a563Luc+Unit2 (81 bp), a563Luc+Unit3 (126 bp), and a563Luc+Unit4 (256 bp)). The proximal fragments were excised with *Eco*RI and *Nhe*I and inserted into a563Luc+Unit1 (a540Luc+Unit1, a513Luc+Unit1, a449Luc+Unit1, a232Luc+Unit1, and a41Luc+Unit1). A2664LucMut1 and a2664LucMut2 were created by PCR mutagenesis using QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

RNA preparations from 3T3-L1 adipocytes

3T3-L1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS). For differentiation, cells (3–7 days after confluence) were cultured for 2 days with 10% FBS-supplemented DMEM containing 5 μg/ml insulin, 0·5 mmol/l 1-methyl-3-isobutyl-xanthine, and 1 μmol/l dexamethasone. The cells were further incubated with DMEM containing 10% FBS to differentiate into adipocytes. Total RNA was extracted from differentiated 3T3-L1 adipocytes (on day 8 after induction of differentiation) using RNA-STAT-60 (Tel-Test, Friendswood, TX, USA) using the protocol supplied by the manufacturer.

Quantitative real-time PCR

The cDNA was synthesized using the ThermoScript RT-PCR system (Invitrogen). Real-time PCR was performed on LightCycler system (FastStart DNA Master SYBR Green I, Roche) according to the protocol provided by the manufacturer. Sequences of primers used for real-time PCR are described in Supplementary Table 1.

RNA interference studies

3T3-L1 mature adipocytes on day 8 after the induction of differentiation were transfected with 20 nM control and CEBPA small interfering RNA (siRNA) oligonucleotides using DeliverX transfection reagent (Veritas, Tokyo, Japan) following the protocol recommended by the manufacturer. The siRNA oligonucleotides were purchased from Qiagen. The target sequences of CEBPA and negative control are described in Supplementary Table 1.

Luciferase reporter assay

On day 4 after the induction of differentiation, the medium of 3T3-L1 cells in six-well plates was changed to OPTI-MEM (Invitrogen), and the cells were transfected with luciferase reporter plasmids using Lipofectoamine 2000 reagent (Invitrogen) according to the protocol provided by the manufacturer. Transfection was performed using 1 μg pCMX-β-gal (internal standard) and 2 μg reporter plasmids, with or without 20 μl of 5 μM siRNA solution. Four hours later, the medium was changed to DMEM containing 10% FBS. After 40-h incubation, luciferase reporter assays were performed using Luciferase Assay system (Promega). Luciferase reporter assay with HEK293 cells was performed as previously described (Iwaki *et al.* 2003). Transfection efficiencies in different cell types were determined by transfection of green fluorescent protein (GFP)-expressing vector and counting the number of GFP-positive cells. Luciferase values were normalized by an internal β-galactosidase control and expressed as relative luciferase activity.

Electrophoretic mobility shift assay

Electrophoretic gel shift assay (EMSA) was performed as described previously (Iwaki *et al.* 2003) using the double-

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strand nucleotides described in Supplementary Table 1, and anti-CEBPA (#sc-61, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or non-immune rabbit IgG (#PP64B, Upstate Biotechnology, Lake Placid, NY, USA). CEBPA protein was synthesized from pcDNA3-HA-CEBPA expression plasmids by the T_NT T7 Quick Coupled Transcription/Translation Systems (Promega). The pcDNA3-HA-CEBPA expression plasmid (Kovacs *et al*. 2003) was a kind gift from Dr Cardinaux. Nuclear extracts were prepared from 3T3-L1 mature adipocytes on days 8–10 after the induction of differentiation using Nuclear Extract Kits (Active Motif, Tokyo, Japan).

Chromatin immunoprecipitation (ChIP) assay

ChIP assay of 3T3-L1 mature adipocytes on days 8–10 after the induction of differentiation was performed as described previously (Lee *et al*. 2006) with anti-CEBPA antibody (sc-61), or non-immune IgG (rabbit IgG: Upstate Biotechnology), and the primers described in Supplementary Table 1.

Statistical analysis

All data were expressed as mean ± S.D. Differences between groups were examined for statistical significance using the Student's *t*-test. A *P* value less than 0·05 denoted the presence of a statistically significant difference.

Results

Human adiponectin promoter exhibits high transcriptional activity in 3T3-L1 mature adipocytes

First, we analyzed the 5′ flanking distal region of human adiponectin gene with UCSC Genome Browser (http://genome.ucsc.edu). An uncharacterized gene next to adiponectin, AK094454, is located between 31 794 bp and 17 153 bp upstream from transcription start site of adiponectin gene. Analysis of this 17 153 bp fragment revealed the presence of evolutionally conserved regions within 10 000 bp upstream from the transcription start site of adiponectin gene (data not shown). Therefore, we cloned a 10 390 bp fragment of the 5′ flanking region of human adiponectin gene. Next, to analyze this fragment, luciferase plasmids driven by a serial deletion of adiponectin promoter were constructed, and transfected into 3T3-L1 mature adipocytes. As we have reported previously (Iwaki *et al*. 2003), a908Luc showed higher luciferase activity than control vectors (pGL3 basic vector containing no promoter and pGL3 promoter vector containing SV40 promoter). Surprisingly, constructs containing long fragments of adiponectin promoter, such as a10 390Luc, a4075Luc, and a2664Luc showed higher luciferase activities than a908Luc (Fig. 1A). Next, to investigate the adiponectin promoter activity in other cell lines, luciferase activities driven by control vectors and reporter constructs with human adiponectin promoters were measured in HEK293 cells. Luciferase activity driven by human adiponectin promoter was apparently lower than that driven by pGL3 promoter vector containing SV40 promoter, and similar to that driven by pGL3 basic vector containing no promoter (Fig. 1B). In other cell lines, such as 3T3-L1 preadipocytes, RAW 264.7 (macrophages), HepG2 (hepatoma) and N1E 115 (neuroblastoma), human adiponectin promoters showed very low promoter activity (Supplemental Figure 1, see Supplementary data in the online version of the Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol200/issue1/). Collectively, these data suggest that important elements for adipocyte-specific high expression exist between −2664 bp and −908 bp upstream from the transcription start site of human adiponectin gene.

Transcriptional activation of adiponectin via the distal enhancer

Next, to identify the cis-elements responsible for the transactivation in mature adipocytes, the 5′ flanking region of adiponectin gene was analyzed with the UCSC Genome Browser. The UCSC Genome Browser identified five evolutionally conserved regions within the −2664 bp upstream from the transcription start site of the human adiponectin gene (Fig. 1C). Two conserved elements are located between −2614 bp and −2517 bp (Element1 (E1): −2614/−2591 and Element2 (E2): −2524/−2517), and three conserved elements are in the proximal region of human adiponectin promoter (Element3 (E3): −177/−158, Element4 (E4): −105/−92, and Element5 (E5): −55/−43). To investigate the impact of these two distal conserved elements on the transactivation of human adiponectin promoter, luciferase activities driven by truncated constructs of human adiponectin promoter were measured in 3T3-L1 mature adipocytes. a2664Luc, including E1 and E2, exhibited high luciferase activity; however, the reporter construct deleted E1 (a2561Luc) showed apparently low luciferase activity similar to that driven by the proximal promoter construct with both deleted E1 and E2 (a563; Fig. 1D). This deletion suggested the significance of E1 for the transcriptional activation of human adiponectin promoter in 3T3-L1 mature adipocytes. To define the distal enhancer of the human adiponectin gene, reporter constructs of various truncated distal enhancers with proximal promoter were transfected into 3T3-L1 mature adipocytes. Although connection with the distal enhancer, including both E1 and E2 (a563+Unit1), potently increased the transcriptional activity of a563Luc, slight enhancement was observed by the distal enhancer deleted E1 or E2 (a563+Unit2 or a563+Unit3). Taken together, these results suggest that the distal enhancer of human adiponectin gene is composed of highly conserved E1 and E2, and that both are essential for transactivation in mature adipocytes.

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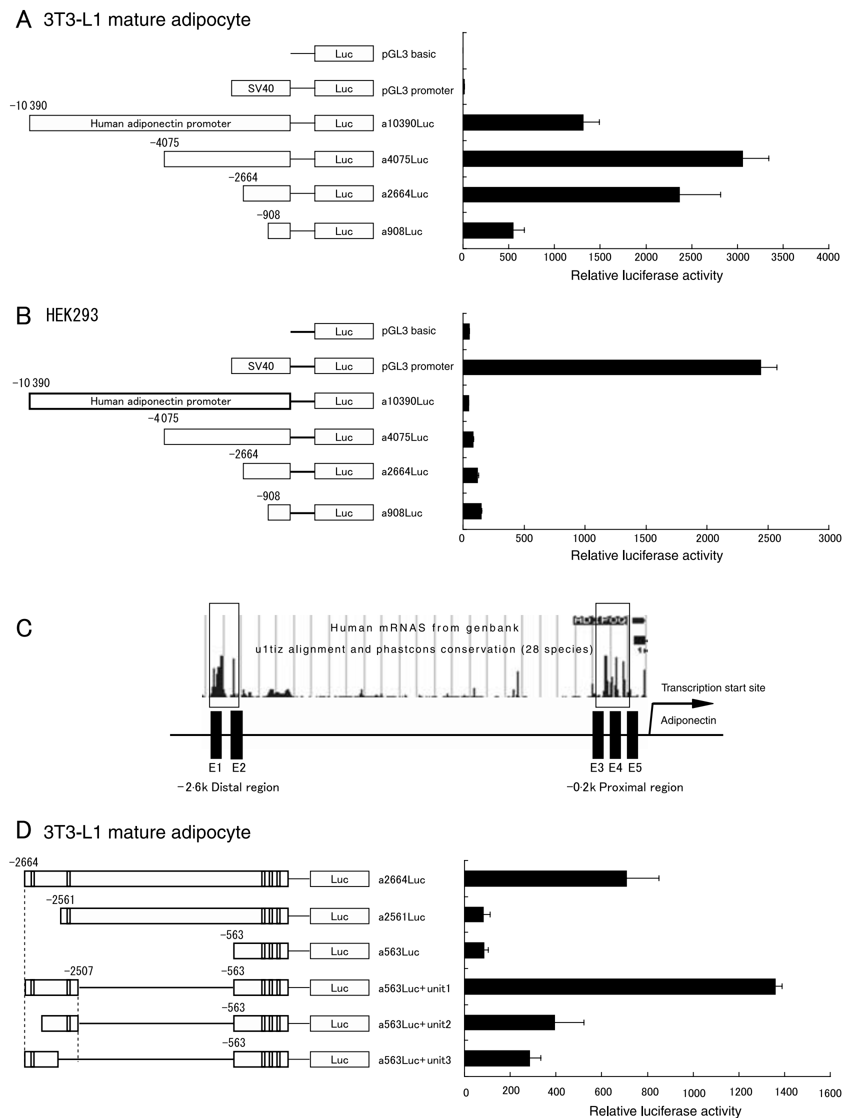

Figure 1 Impact of human adiponectin promoter activity in adipocytes. (A, B, and D) A series of fragments of the 5′-flanking region of the human adiponectin gene were subcloned upstream of the luciferase reporter gene as described in Materials and Methods. Each promoter/reporter construct was transfected into 3T3-L1 mature adipocytes or HEK293 cells, and 48 h later, luciferase activity was measured and normalized by β-galactosidase activity. Normalized luciferase activity is expressed as mean±S.D. (n=3). (C) The UCSC Genome Browser (http://genome.ucsc.edu) analysis. The histogram shows interspecies homology. Histograms in open squares indicate sites highly conserved between mammals. The arrow indicates the direction of transcription.

CEBPA functionally regulates adiponectin promoter activity through two conserved CCAAT boxes

As described above, the distal enhancer is composed of conserved E1 and E2 (Fig. 2A), and both are necessary for its transcriptional activation. To identify the transcription factor that binds to E1 and E2, the TRANSFAC database program (http://motif.genome.jp/) was employed. As shown in Fig. 2B, both E1 and E2 were predicted as a CEBPA-binding sites (CCAAT box) with over 85% identity to its consensus sequence. Furthermore, these putative CCAAT boxes were completely conserved among human, rhesus, mouse, dog, and horse (Fig. 2B). To investigate the importance of these CCAAT boxes in E1 and E2 (C1 and C2 respectively) in the region between −2507 bp and −2664 bp, mutations that disrupt CEBPA-binding of C1 and C2 were introduced into a2664Luc (Fig. 2C). a2664Luc showed higher luciferase activity than a2561Luc, and a mutation on either single C1 or

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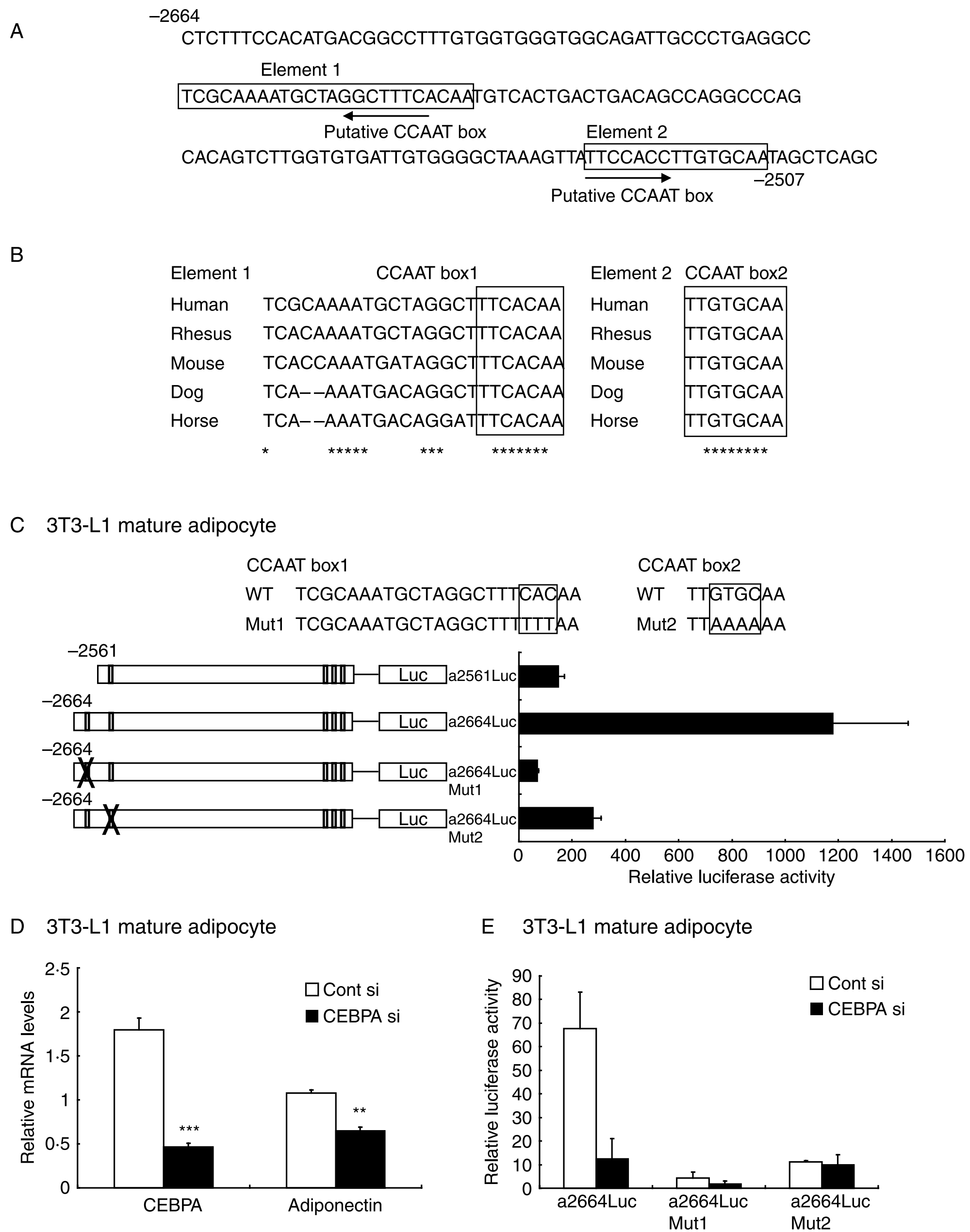

Figure 2 Functional transactivation by CEBPA through two CCAAT boxes in the distal enhancer of human adiponectin promoter. (A) Sequences of −2664/−2507 region of the human adiponectin promoter. Boxed sequences indicate conserved elements demonstrated by UCSC Genome Browser analysis. Arrows indicate the direction of putative CCAAT boxes. (B) Sequences of distal conserved elements in adiponectin gene. The sequences of mammals were obtained from UCSC Genome Browser. Putative CCAAT boxes are enclosed in boxes. Completely conserved nucleotides are represented with asterisks. (C) The indicated reporter constructs with or without mutations in CCAAT box were transfected in 3T3-L1 mature adipocytes. Normalized luciferase activity is expressed as mean±S.D. ($n=3$). Mutated sequences are enclosed in boxes. (D) 3T3-L1 cells on day 8 were transfected with the indicated siRNAs. The mRNA expression levels of CEBPA and adiponectin were measured by quantitative RT-PCR. Values are normalized to the level of 36B4 mRNA and expressed as mean±S.D. ($n=3$). $^{**}P<0{\cdot}01$, $^{***}P<0{\cdot}001$. (E) WT and mutated reporter constructs were transfected with the indicated siRNAs into 3T3-L1 adipocytes. Normalized luciferase activity is expressed as mean±S.D. ($n=3$–6).

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C2 attenuated the transcriptional activity driven by a2664Luc in 3T3-L1 mature adipocytes. These results indicate that both C1 and C2 are responsible for full-transcriptional activation by a2664Luc in 3T3-L1 mature adipocytes. Next, to ask whether the loss of transcriptional activity by mutations of C1 and C2 is a CCAAT box-specific effect, other mutant constructs with a random mutations around C1 or C2 were transfected into 3T3-L1 mature adipocytes (a2664Luc with a mutation located immediately upstream of C1 and a2664Luc with a mutation located immediately downstream of C2). However, these mutations did not affect the high transcriptional activities driven by a2664Luc (data not shown). Collectively, these data suggest that C1 and C2 are important sites in the distal enhancer of human adiponectin gene.

Next, to investigate whether CEBPA functionally regulates adiponectin transcriptional activity through C1 and C2, the endogenous expression of CEBPA was suppressed using siRNA in 3T3-L1 mature adipocytes. As reported previously (Qiao *et al*. 2005), adiponectin mRNA levels in 3T3-L1 mature adipocytes were significantly reduced following suppression of endogenous expression of CEBPA (Fig. 2D). Luciferase activity of a2664Luc was strongly reduced by CEBPA knockdown; however, those of a2664LucMut1 and a2664LucMut2 did not change significantly (Fig. 2E). Taken together, these data suggest that CEBPA regulates human adiponectin transcription through C1 and C2 in its conserved distal enhancer.

CEBPA directly binds to CCAAT box in the distal enhancer of adiponectin gene

To assess the direct binding of CEBPA to C1 and C2 in the human adiponectin gene, EMSA experiments using radiolabeled oligonucleotides including C1 or C2 were employed. Both C1 and C2 bound to *in vitro*-translated CEBPA, and the mobility of the labeled probes was shifted (Fig. 3A, lanes 2 and 8). The signal was attenuated by competition with excess homologous nucleotides (Fig. 3A, lanes 3 and 8), but not with a mutated derivative of these CCAAT boxes, which contains point mutations as described in Supplementary Table 1 (Fig. 3A, lanes 4 and 10). Although oligonucleotides containing consensus CCAAT box of mouse leptin gene were also an effective competitor (Fig. 3A, lanes 5 and 11); no competition was seen by non-functional CCAAT box mutant oligonucleotides reported previously (Hwang *et al*. 1996; Fig. 3A, lanes 6 and 12).

Next, to investigate whether endogenous nuclear complexes containing CEBPA bind to C1 and C2, we performed EMSA studies with nuclear extracts from 3T3-L1 mature adipocytes. As shown in Fig. 3B, formation of shifted bands was observed with radiolabeled oligonucleotides including C1 or C2 (lanes 1 and 8), which was abrogated by 50-fold molar excess of unlabeled probes (lanes 2 and 9) or a consensus CCAAT box of mouse leptin promoter (lanes 4 and 11), but not by that of unlabeled mutant CCAAT box probes (lanes 3 and 10) and mutant CCAAT box probes from

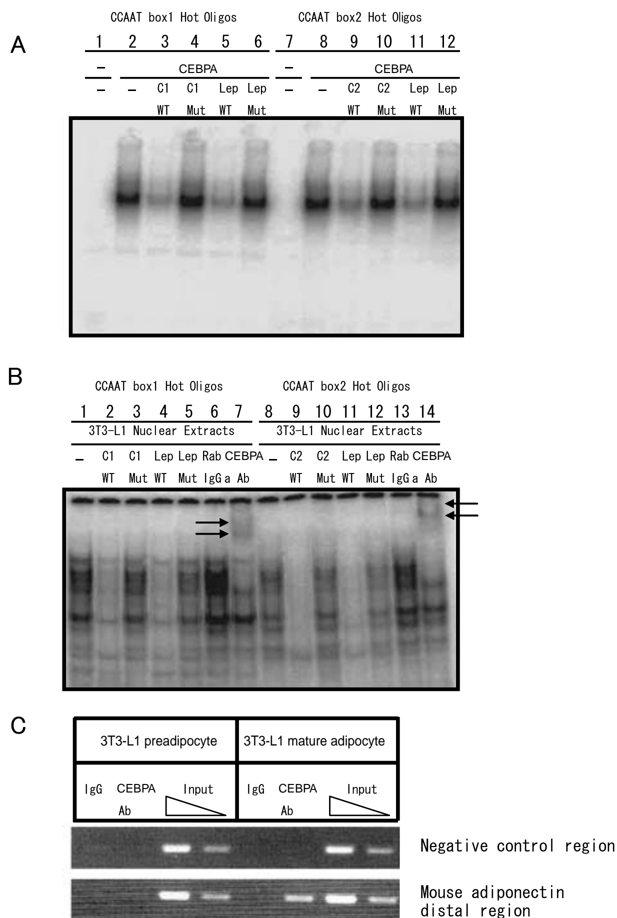

Figure 3 Specific binding of CEBPA to CCAAT boxes in adiponectin distal enhancer. (A) EMSA was performed with [^{32}P]-labeled oligonucleotides containing the CCAAT box in the distal enhancer region of human adiponectin gene. The indicated labeled probes were incubated with *in vitro* synthesized CEBPA. Competitive assays were performed using unlabeled oligonucleotides described in results as competitors by 50-fold molar excess. (B) EMSA was performed with the [^{32}P]-labeled oligonucleotides mentioned above. The indicated probes were incubated with nuclear extracts from 3T3-L1 mature adipocytes on days 8–10. Competition assays were performed by adding 50-fold molar excess of unlabeled probes described in results. Non-immune rabbit IgG (Rab IgG) or anti-CEBPA antibody (CEBPA Ab) was added to nuclear extracts from 3T3-L1 adipocytes. Arrows indicate shifted bands of the labeled oligonucleotides bound to CEBPA/antibody complex. (C) In ChIP, chromatin from pre- and mature 3T3-L1 adipocytes on days 8–10 was used. DNA was incubated with CEBPA antibody or non-immune IgG (IgG), and the recovered DNA was amplified using oligonucleotides flanking these CCAAT boxes or the negative control region. Aliquots of chromatin were analyzed before immunoprecipitation (input).

mouse leptin promoter (lanes 5 and 12). In addition, the specific bands were immunodepleted and supershifted using anti- CEBPA antibody (lanes 7 and 14), but not using non-immune rabbit IgG (lanes 6 and 13). Finally, *in vivo* binding of CEBPA to these CCAAT boxes was further verified by ChIP assay.

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Although 3T3-L1 is a cell line derived from mouse, the distal enhancer of the adiponectin gene is highly conserved in human and mouse (Figs 1C and 2B). Fragmented chromatin from formaldehyde cross-linked differentiated 3T3-L1 was subjected to immunoprecipitation with CEBPA antibody or with rabbit IgG as a control. To validate the data, we also performed ChIP with 3T3-L1 preadipocytes, in which CEBPA expression is extremely low (Ramji & Foka 2002). In 3T3-L1 mature adipocytes, endogenous CEBPA bound to the distal enhancer region of the mouse adiponectin gene, while in 3T3-L1 preadipocytes, CEBPA antibody barely immunoprecipitated the adiponectin distal enhancer (Fig. 3C). In contrast, CEBPA did not immunoprecipitate the sequences from the negative control region in both pre and 3T3-L1 mature adipocytes (Fig. 3C). Taken together, these data strongly suggest that CEBPA binds to the conserved distal enhancer of adiponectin gene *in vivo.*

Proximal regions of human adiponectin are required for transcriptional activation by the distal enhancer

During analysis of the distal enhancer of the human adiponectin gene, we found that it could activate its proximal promoter but not a minimal promoter as the herpes simplex virus thymidine kinase promoter (TK-Luc; Fig. 4A). These data led us to hypothesize that the distal enhancer could activate proximal promoter via functional communication in mature adipocytes. To define the communication between proximal and distal enhancers of human adiponectin gene, we performed luciferase assay with various sets of promoter-enhancer constructs in 3T3-L1 mature adipocytes. Luciferase activities driven by serial 5′ flanking deletion constructs of proximal adiponectin promoter with or without its distal enhancer were evaluated. As shown in Fig. 4B, the 563 bp and 540 bp proximal promoter constructs (a563Luc and a540Luc) clearly responded to the addition of the distal enhancer (17-fold and 17-fold respectively). However, constructs lacking the sequences between 540 bp and 513 bp apparently reduced the transcriptional activation by the distal enhancer (a513Luc, a449Luc, a232Luc, and a91Luc; 6-folds, 6-folds, 7-folds, and 4-folds respectively; Fig. 4B). These data suggest the importance of sequences between 540 bp and 513 bp of human adiponectin gene for full-transcriptional activation by its distal enhancer.

Discussion

Adiponectin is exclusively expressed in adipocytes of both rodents and human (Scherer *et al.* 1995, Hu *et al.* 1996, Maeda *et al.* 1996); however, little is known about the mechanisms underlying this adipocyte-specific expression. In the present study, we identified two highly conserved CCAAT boxes in the 5′ flanking distal region of human adiponectin gene. CEBPA bound to these CCAAT boxes and enhanced the transcriptional activities of human adiponectin promoter via a unique communication with its proximal promoter region. This proximal region located between 540 bp and 513 bp of human adiponectin gene, designated as 'the distal enhancer-response element', seems to play an important role.

The CEBP family is considered a key class of transcription factors in adipogenesis. Briefly, CEBPB and CEBPD are induced during the mitotic clonal expansion phase of preadipocytes in response to differentiation reagents. Then, the preadipocytes exit the cell cycle and start to express CEBPA. CEBPA has been implicated as a regulator of the integrity of adipocytes via induction of various adipose-marker genes (Mandrup & Lane 1997, Ramji & Foka 2002), including fatty acid-binding protein 4 (aP2), leptin, resistin, β3-adrenergeic receptor, and PPARG2 (Hwang *et al.* 1996, Mason *et al.* 1998, Elberg *et al.* 2000, Dixon *et al.* 2001, Hartman *et al.* 2002, Tuncman *et al.* 2006). Using siRNA of CEBPA, the present study highlighted the involvement of CEBPA in adiponectin expression. This result is in agreement with the findings of previous studies, which demonstrated that overexpression of CEBPA upregulates adiponectin mRNA levels in both human and mouse adipocytes, and that the knockdown of CEBPA expression reduces adiponectin mRNA levels both in human and mouse adipocytes (Qiao *et al.* 2005).

Although the significance of CEBPA on adiponectin gene expression has been reported, the regulatory mechanism of adiponectin gene expression by CEBPA has been controversial. Park *et al.* (2004) demonstrated that CEBPA could bind to both mouse and human adiponectin proximal promoters and increase the activities of these promoters. On the other hand, others reported that ectopic expression of CEBPA could not upregulate luciferase activities driven by the proximal promoters of mouse and human adiponectin gene respectively (Seo *et al.* 2004, Qiao *et al.* 2005). Qiao *et al.* (2005) reported that an intronic enhancer of human adiponectin gene is important for this regulation, although no homology was seen in the first intron of human and mouse adiponectin gene. The present study demonstrated that other CEBPA-binding sites in the distal 5′ flanking region of the adiponectin gene are significant, and completely conserved among human, mouse, rhesus, dog, and horse. Furthermore, siRNA-mediated reporter assay showed that CEBPA functionally regulates human adiponectin transcriptional activity through CCAAT boxes in its distal enhancer. Collectively, CEBPA should be involved in adipose tissue expression of adiponectin mainly through this distal enhancer.

As demonstrated in Fig. 1A and B, the human adiponectin promoter, including its distal enhancer, exhibited high transcriptional activities only in mature adipocytes among various cell lines investigated in this study (Supplemental Figure 1). To investigate the mechanism underlying this cell line-specific induction, adiponectin promoter activity was measured in HEK293 cells overexpressing CEBPA. However, the transcriptional activity driven by any length of human adiponectin promoter did not significantly respond to ectopic

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Figure 4 Identification of the distal enhancer-response element in the proximal promoter of human adiponectin gene. (A) The indicated reporter constructs were transfected in 3T3-L1 mature adipocytes, and 48 h later, luciferase activity was measured and normalized by β-galactosidase activity. Normalized luciferase activity is expressed as mean±S.D. ($n=3$). (B) The indicated reporter constructs were transfected in 3T3-L1 mature adipocytes and 48 h later, luciferase activity was measured and normalized by β-galactosidase activity. Normalized luciferase activity is expressed as mean±S.D. ($n=3$). (C) Sequences of the distal enhancer-response element in the proximal promoter of human adiponectin gene. The underlined sequences represent putative binding sites of the indicated transcription factors.

CEBPA expression in HEK293 cells (data not shown). In fact, CEBPA is expressed at high levels in the liver and lung, in which no adiponectin expression is detected. These findings indicate that CEBPA *per se* is unable to increase adiponectin transcription, and not enough to attain adipocyte-specific expression of adiponectin. Exactly how the specificity of adiponectin gene is determined remains an unanswered question. Previously, we and others reported that PPARG regulates adiponectin mRNA level and promoter activity in mature adipocytes (Maeda *et al*. 2001, Iwaki *et al*. 2003, Qiao *et al*. 2005). PPARG is strongly expressed in mature adipocytes, and plays a pivotal role on differentiation of adipocytes (Rosen & Spiegelman 2001). In this regard, synergistic activation of adiponectin gene by both CEBPA and PPARG may, at least partly, explain its adipocyte-specific expression.

Interestingly, the distal enhancer in human adiponectin gene enhances the transcriptional activity driven by its proximal promoter, but not by minimal promoter like the TK promoter in 3T3-L1 mature adipocytes (Fig. 4A). Furthermore, our data indicated the presence of a 'distal enhancer-response element' in the proximal promoter, which should play a role in communication with the distal enhancer (Fig. 4B). A search for putative cis-elements in 'the distal enhancer-response element' with TRANSFAC database program identified putative binding sites of several transcription factors (PPAR/RXR-binding site, ATF-binding site, and GATA-binding site; Fig. 4C). Previously, we reported that PPARG regulates

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adiponectin mRNA levels and promoter activities in mature adipocytes (Iwaki *et al*. 2003). As mentioned above, PPARG is strongly expressed in mature adipocytes and plays a pivotal role in transactivation of adiponectin gene. In this regard, we focused on the putative PPAR/RXR-binding site among the predicted transcription factors. However, mutations on this site that should have disrupted PPARG/RXAα binding did not attenuate PPARG agonist-mediated transcriptional activity driven by a2664Luc in 3T3-L1 mature adipocytes (data not shown), indicating that this putative PPAR/RXR-binding site is not functional in terms of the transcriptional activation by PPARG/RXRα heterodimer. On the other hand, the functional PPARG response element, which was identified previously by our group (Iwaki *et al*. 2003), contributed to PPARG/RXRα-mediated transcriptional activation of human adiponectin gene. What factors and/or complexes bind to 'the distal enhancer-response element' in human adiponectin gene remain to be elucidated, and that may be a unique target to increase adiponectin as an anti-diabetic and anti-atherogenic adipocytokine.

In summary, the present study demonstrated that CEBPA plays a crucial role in the transcriptional regulation of human adiponectin gene via the distal enhancer region containing two functional CEBPA-binding sites. This distal enhancer region of adiponectin gene is highly conserved among species. We also identified a distal enhancer-response element in its proximal promoter region. Communication between the distal enhancer and distal enhancer-response proximal elements may play an important role in the adipocyte-specific expression of adiponectin gene.

Declaration of interest

The authors declare that they have no competing financial interests.

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