CCAAT/Enhancer Binding Protein and Nuclear Factor-Y Regulate Adiponectin Gene Expression in Adipose Tissue

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Adiponectin is one of the adipokines secreted by adipocytes and regulates energy homeostasis associated with insulin sensitivity, suggesting a possibility of nutritional regulation of adiponectin gene expression. In this study, we showed that the transcription of adiponectin gene was induced 4–6 h after refeeding of mice. Also, differentiated 3T3-L1 adipocytes that were treated with high glucose expressed significantly increased adiponectin mRNA. Promoter analysis using nuclear extracts from white adipose tissue revealed that CCAAT/enhancer binding protein (C/EBP) and nuclear factor-Y (NF-Y) bound on the −117/−73 region of the adiponectin promoter. This region was critical for the activity of the adiponectin promoter as the deletion or mutation of this region markedly diminished the promoter activity to a basal level. Furthermore, the C/EBP binding increased in both refed animal and high glucose–treated 3T3-L1 adipocytes in an electrophoretic mobility shift assay, suggesting that C/EBP is responsible for the dietary response of the adiponectin gene expression. Chromatin immunoprecipitation studies demonstrated the binding of C/EBP and NF-Y in both mouse and differentiated 3T3-L1 adipocytes and also that C/EBP binding increased in response to high glucose. These findings demonstrated that C/EBP and NF-Y are critical for the regulation of the adiponectin expression in response to nutrients and in the course of adipocyte differentiation. *Diabetes* 53:2757–2766, 2004

Adipose tissue, in addition to its ability to store extra energy as triglyceride, is now considered to be an endocrine organ performing metabolic regulation by releasing hormones in response to changes in metabolic status (1–3). These secreted molecules include leptin, tumor necrosis factor-α, adiponectin, and resistin, referred to collectively as adipokines (4). Because excess or lack of adipose tissue is associated with insulin resistance and type 2 diabetes, it has been postulated that adipokines are possible linkers between obesity and insulin resistance (5).

Adiponectin, also known as Acrp30, AdipoQ, apM1, and GBP28, is a recently found adipokine that is expressed exclusively in white adipose tissue (6–9). The plasma level of adiponectin declined at an early phase of obesity and a lower level was maintained after the development of type 2 diabetes in animals (10) and in humans (11,12), indicating a possible relationship with insulin resistance (13). It is reported that adiponectin decreases insulin resistance by increasing fatty acid oxidation and glucose transport in muscle (14,15). Furthermore, adiponectin lowers plasma glucose level by inhibiting endogenous glucose production from liver (16,17). Therefore, it is likely that adiponectin is a potent insulin enhancer linking adipose tissue and whole-body glucose metabolism.

Although the physiological effects of adiponectin have been investigated intensively, the molecular mechanisms that regulate the expression of the adiponectin gene are largely unknown. Several factors that regulate adiponectin expression were reported, including peroxisome proliferator–activated receptor-γ agonist (18), tumor necrosis factor-α (19), β-adrenergic stimulation (20), and insulin (9,21). These factors are considered to regulate adiponectin gene expression in response to a change in metabolic status; however, there is little correlation for the physiological regulators of adiponectin gene expression. In a clinical study of human subjects, inverse correlations were observed between plasma adiponectin and insulin in type 2 diabetic patients, suggesting that plasma insulin does not independently affect adiponectin secretion (11). Another report showed that plasma adiponectin increased postprandially in obese but not in lean subjects (22). In contrast, it was also reported that refeeding of animals led to increased expression of adiponectin along with leptin (23). It is not clear, therefore, how adiponectin expression and secretion are regulated by the metabolic stimulus either in a normal or in a pathological situation.

The family of CCAAT/enhancer binding protein (C/EBP) transcription factors are expressed in a number of tissues and are involved in the regulation of several biological processes, including control of energy metabolism (24–26). Importantly, C/EBP-α and -β are known to be key regulators for adipocyte differentiation (27–29) and play

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C/EBP, CCAAT/enhancer binding protein; ChIP, chromatin immunoprecipitation; CBP, cyclic AMP response element binding protein; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; NF-Y, nuclear factor-Y; PMSF, phenylmethylsulfonyl fluoride; SRE, sterol-response element.

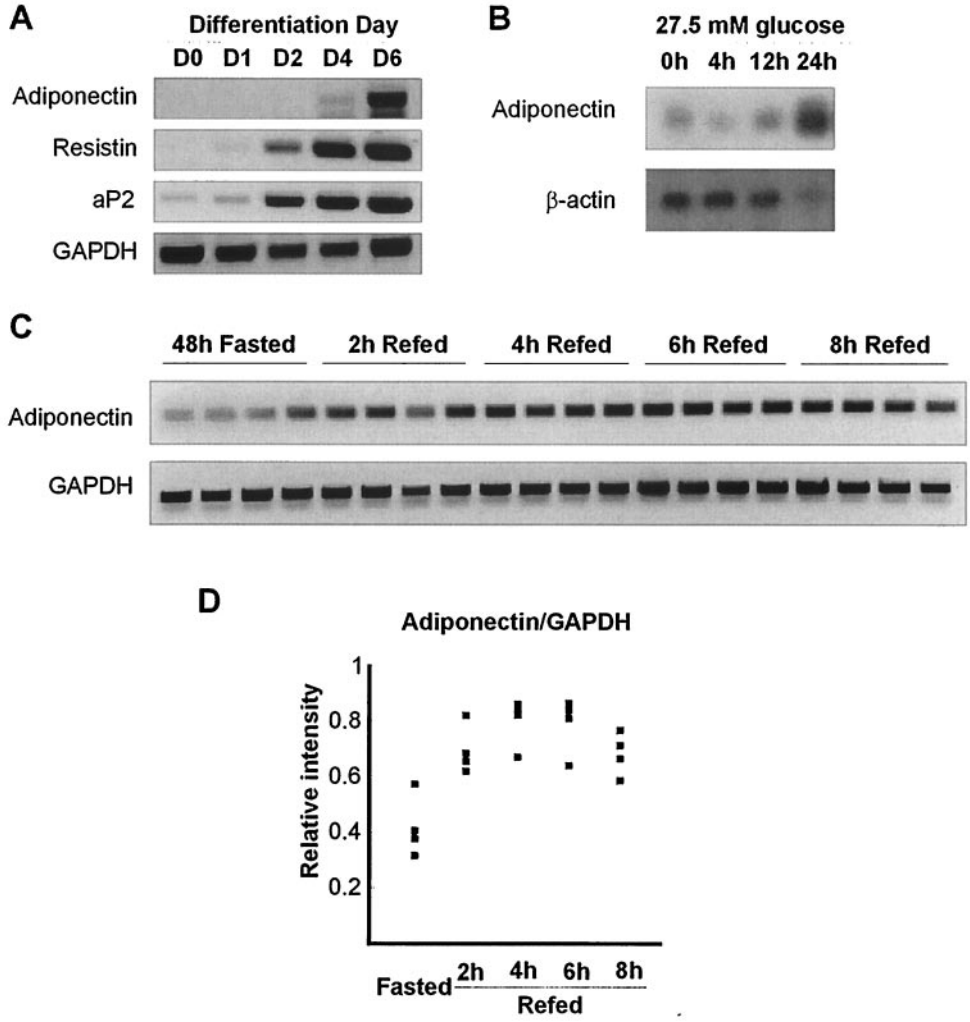

FIG. 1. Transcription of adiponectin is regulated by adipocyte differentiation and nutrient. *A*: Adiponectin expression in 3T3-L1 cells at different differentiation stages. 3T3-L1 preadipocytes at 2 days postconfluent state were induced to differentiation, and total RNA was isolated at 0, 1, 2, 4, and 6 days after the induction of differentiation. Glyceraldehyde-3-phosphate dehydrogenase as internal control. *B*: Northern blot of adiponectin mRNA in 3T3-L1 adipocytes in response to glucose. Fully differentiated 3T3-L1 adipocytes were treated with glucose (27.5 mmol/l) for 0, 4, 12, and 24 h after incubation in low glucose (5.5 mmol/l) for 24 h. Total RNA was prepared and subjected to Northern blot using specific cDNA probe for adiponectin and β-actin. *C*: Adiponectin expression in adipose tissues according to fasted and refed state. For each group, four mice were fasted for 48 h and refed with normal diet for indicated hours. Total RNA of adipose tissue was prepared and subjected to RT-PCR. *D*: Schematic representation of RT-PCR result shown in *C* by densitometry. The density of each band was quantified by densitometer and normalized by glyceraldehyde-3-phosphate dehydrogenase result.

roles in the expression of adipocyte-specific genes (30,31). In addition, C/EBPs control the synthesis of proteins that are necessary for lipid metabolism and gluconeogenesis (30,32), suggesting that this transcription factor may regulate the expression of genes related to energy metabolism in adipocytes.

In this study, we investigated the molecular mechanisms of adiponectin gene expression. The adiponectin promoter was analyzed by a DNA-protein binding assay, showing that the adiponectin promoter bound C/EBPs and nuclear factor-Y (NF-Y). We also showed that C/EBPs could transactivate the adiponectin promoter and that NF-Y also activated the adiponectin promoter regulating basal activity. Moreover, we revealed that adiponectin expression, along with C/EBP binding to its promoter, was regulated by dietary nutrients. These observations suggest a possible role of C/EBP and NF-Y in the control of adiponectin expression in response to diet as well as adipocyte differentiation.

RESEARCH DESIGN AND METHODS

Plasmids. Mouse adiponectin promoter spanning −1,138 to 30 bp was amplified by PCR and ligated with luciferase vector to generate pGLB-A-1138. 5′-Serial deletion constructs (pGLB-A-727, pGLB-A-502, pGLB-A-202, pGLB-A-134, and pGLB-A-57) were generated by deletion using restriction enzymes. Mutant constructs (pGLB-A-134m1, pGLB-A-134m2, pGLB-A-134m3, pGLB-A-134m4, pGLB-A-134m5, and pGLB-A-134m45) were produced by site-directed mutagenesis. Plasmid pmYA (Δ4YA13 m29), which encodes the dominant negative form of the A subunit of NF-Y, was provided by Dr. R. Mantovani (University of Milan, Milan, Italy) (33). Expression plasmids pCMV-rC/EBP-α and pCMV-mC/EBP-β were gifts from Dr. J.R. Cardinaux (Centre de Neurosciences Psychiatriques, Lausanne, Switzerland).

Adipocyte differentiation. 3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) that contained 10% FBS. For inducing differentiation, 2-day postconfluent cells (designated day 0) were fed DMEM that contained 10% FBS, 0.5 mmol/l 3-isobutyl-1-methyl-xanthine, 1 μmol/l dexamethasone, and 1.67 μmol/l insulin until day 2. Cells then were fed DMEM supplemented with FBS/insulin for 2 days, after which they were fed every other day with DMEM that contained FBS. For investigating the effect of glucose or fatty acid, day 6–8 cells were maintained with DMEM that contained low glucose (5.5 mmol/l) and 10% FBS 24 h before treatment.

Northern blot analysis and RT–PCR. Total RNA was extracted using TRIzol reagent. Ten micrograms of total RNA of each group was subjected to electrophoresis and transferred to nylon membrane for hybridization. For RT-PCR, 5 μg of total RNA was reverse transcribed, and the resulting cDNAs were used as a template for PCR. The sequences used for RT-PCR are as follows: adiponectin, 5′-AGCCT GGAGA AGCCG CTTAT-3′, 5′-TTGCA GT AGA ACTTG CCAGT GC; resistin, 5′-TCAAC TCCCT GTTTC CAAAT GC-3′, 5′-TCTTC ACGAA TGTCC CACGA; aP2, 5′-GATCG GATCC ACCAT GTGTG ATGCC TTTGT GGGAA CC-3′, 5′-GATCC TCGAG TCATG CCCTT TCATA AACTC TTGT-3′; glyceraldehyde-3-phosphate dehydrogenase, 5′-ACCAC AG TCC ATGCC ATCAC-3′, 5′-TCCAC CACCC TGTTG CTGTA-3′.

Nuclear extract preparation. Nuclear extract preparation protocol was modified from the description by Lavery et al. (34). Rat epididymal adipose tissues were homogenized with 0.3 mol/l sucrose buffer (15 mmol/l HEPES [pH 7.9], 60 mmol/l KCl, 15 mmol/l NaCl, 0.15 mmol/l spermine, 0.5 mmol/l spermidine, 0.3 mol/l sucrose, 0.5 mmol/l dithiothreitol [DTT], 0.5 mmol/l phenylmethylsulfonyl fluoride [PMSF], and 5 μg/ml protease inhibitor cocktail). The cell lysate was filtered through gauze and added on the 0.9-mol/l sucrose buffer for nuclear isolation. The nucleus was pelleted by centrifugation at 3,000 rpm for 5 min and resuspended in nuclear storage buffer (20

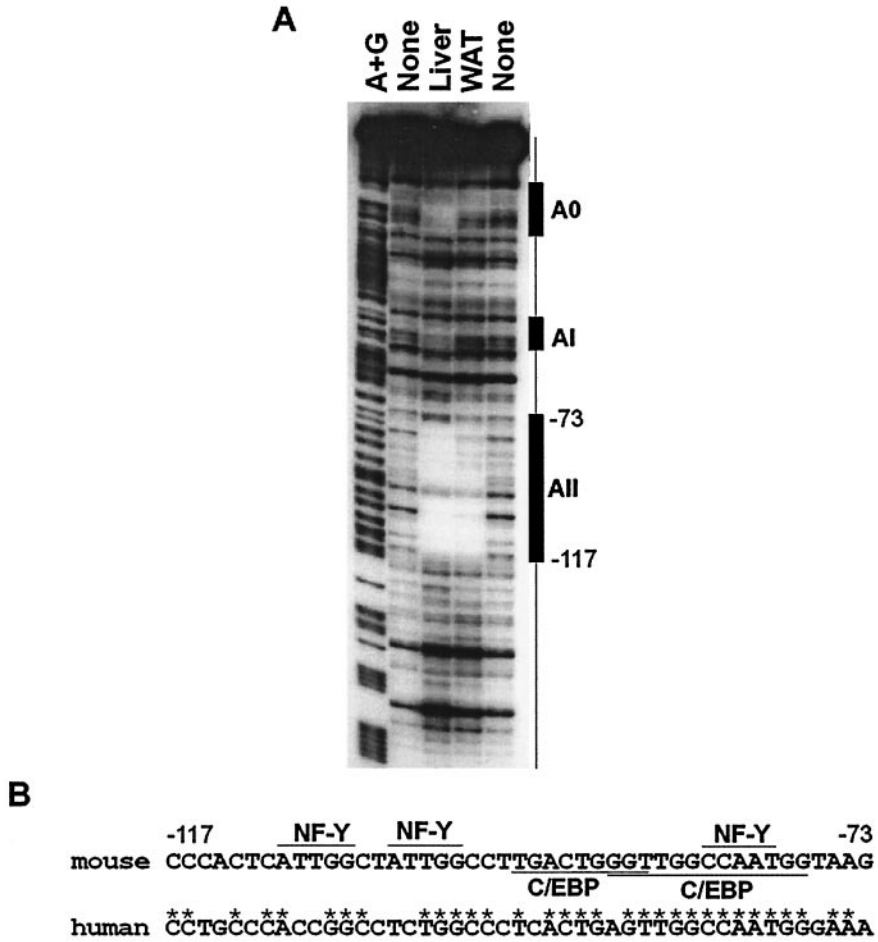

FIG. 2. Nuclear protein binding sites in the mouse adiponectin promoter. *A*: DNase I footprinting assay of the proximal adiponectin promoter. The mouse adiponectin promoter fragment from −256 to 21 bp was end labeled and used for DNase I footprint analysis with the nuclear extract of rat liver or white adipose tissue (WAT). Three regions (AO, AI, and AII) were protected by liver nuclear extracts, of which only region AII was protected by nuclear extract of white adipose tissue. *B*: Sequence analysis of region AII (−117/−73). One CCAAT box and two inverted Y-boxes are marked. Two possible C/EBP binding sites were indicated. For comparison, human DNA sequence of the same region was presented, and identical sequences were marked by ★.

mmol/l Tris-HCl [pH 7.9], 75 mmol/l NaCl, 0.5 mmol/l EDTA, 0.85 mmol/l DTT, and 0.125 mmol/l PMSF). After a brief centrifugation, the pellet was resuspended in 1× NUN buffer (1 mol/l urea, 0.3 mol/l NaCl, 1% Nonidet P-40, 25 mmol/l HEPES [pH 7.6], and 1 mmol/l DTT) and shaken for 30 min for extraction of nuclear protein. The protein concentration was determined by the Bradford method (35). For extracting nuclear proteins from cultured cells, the harvested cells were resuspended in Buffer A (10 mmol/l HEPES [pH 7.9], 10 mmol/l KCl, 0.1 mmol/l EDTA, 0.1 mmol/l EGTA, 1 mmol/l DTT, and 0.5 mmol/l PMSF). After 15 min of incubation on ice, 1:20 volume of 10% Nonidet P-40 was added, vortexed, and then centrifuged. The next steps were the same as those for adipose tissue.

DNase I footprinting assay. The promoter region of the adiponectin gene covering from −256 to 30 bp was labeled and bound with 60 μg of nuclear extract. After 30 min of incubation on ice, DNase I was treated for 2 min. The reactions were purified and analyzed on a 6% denatured polyacrylamide gel. A G+A ladder was prepared by using the same labeled fragment and was run on the same gel.

Electrophoretic mobility shift assay. The probes corresponding to region AII were generated as follows: 10 pmol of single-stranded sense oligonucleotides were labeled and annealed with 5 mol/l excess of antisense oligonucleotides. The probes encompassing −121 to −63 of promoter used in the mutation study were generated by PCR, using ^{32}P-labeled antisense primer (−80 to −63) and unlabeled sense primer (−121 to −104) and pGLB-A-134 and corresponding mutant constructs as templates. Probes were incubated with nuclear extract for 15 min on ice, and the DNA-protein complexes were resolved on 4% polyacrylamide gel. Competitive and supershift assays were performed by adding 100 mmol/l excess of unlabeled oligonucleotides or 2 μg of antibodies, respectively. The oligonucleotides used as competitors are as follows: GATA, 5′-CACTT GATAA CAGAA AGTGA TAACT CT-3′; LDL-R sterol-response element (SRE), 5′-TTTGA AAATC ACCCC ACTGC AAAC-3′; NF-Y, 5′-AGACC GTACG TGATT GGTTA ATCTC TT-3′; C/EBP, 5′-TGCAG ATTGC GCAAT CTGCA-3′; and AP-1, 5′-CGCTT GATGA GTCAG CCGGA A-3′.

Western blot analysis. Fifteen micrograms of nuclear extract was mixed in sample buffer, incubated at 95°C, and separated by 12% SDS–PAGE. The specific proteins were detected by enhanced chemiluminescence for 1 min. Equal protein loading was confirmed by staining with Coomassie Brilliant G-250.

Chromatin immunoprecipitation assay. The chromatin immunoprecipitation (ChIP) assay protocol was modified from the description by Latasa et al. (36). Briefly, mice were perfused and fixed with formaldehyde, and adipose tissues were homogenized and sonicated. The resulting supernatant was divided into aliquots for 10-fold dilution in ChIP dilution buffer and precleared with protein A–agarose that contained salmon sperm DNA for 1 h. The antibodies were added and incubated for 12–18 h at 4°C, and the complexes were collected with 50% slurry of protein A–agarose for 3 h. The beads were washed, and chromatin complexes were eluted from the beads. After reverse of the cross-linking, DNA was purified, and 4 μl of input control or ChIP samples was used as a template in PCR using the primer sets for region AII (5′-AGAAG CTCTA CTTGG CTTCC CAGAC CCAAG CTGGA-3′, 5′-GCAGA CCCCA GCTTA CCA-3′) or for exon 3 region (5′-AGCCT GGAGA AGCCG CTTAG-3′, 5′-TTGCA GTAGA ACTTG CCAGT GC-3′), respectively. In the experiments using 3T3-L1 cells, ~1×10^6 differentiated adipocytes were fixed with formaldehyde, harvested, and then sonicated as described above.

Transient transfection. Transient transfections were performed using Lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Luciferase activities were measured using 10 μl of cell extract and 50 μl of luciferase assay reagent. β-Galactosidase activity was measured for normalization.

RESULTS

Adiponectin gene expression during adipocyte differentiation and its regulation by nutrition. To investigate the regulation of adiponectin gene expression, we first examined the adiponectin mRNA level during adipocyte differentiation of 3T3-L1 fibroblasts. Total RNA was extracted from different stages of the cells after differentiation and subjected to RT-PCR. As shown in Fig. 1*A*,

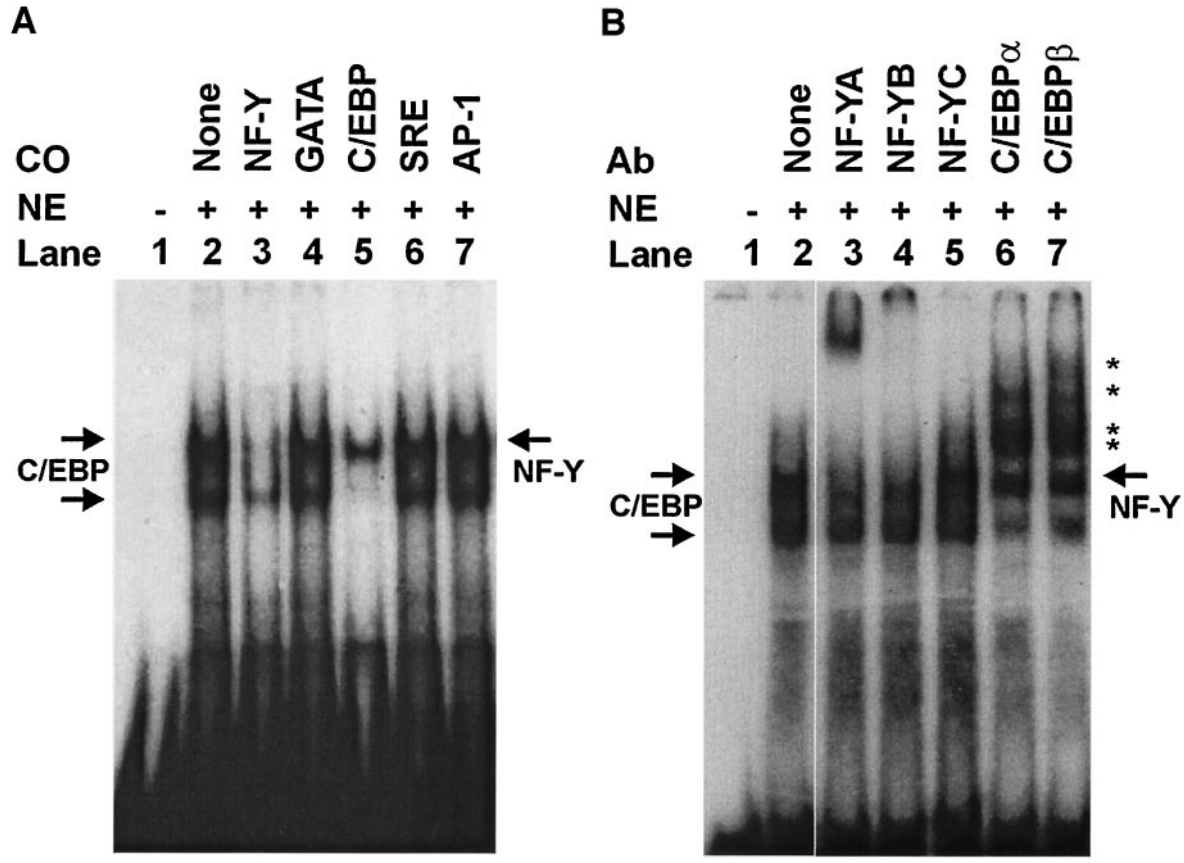

FIG. 3. NF-Y and C/EBP are bound to the adiponectin promoter in vitro. *A*: EMSA of region AII using nuclear extracts of rat white adipose tissue. The oligonucleotide covering region AII (−117/−73) was used as probe. ^{32}P-labeled double-stranded oligonucleotide (50,000 cpm) was incubated with nuclear extract. Unlabeled double-stranded oligonucleotides known as consensus sequences of NF-Y, GATA, C/EBP, SRE, and AP-1 were added to the reaction mixture in 100 mmol/l excess (*lanes 3–7*). *B*: Supershift assay of region AII. Two micrograms of antibodies against NF-YA, NF-YB, NF-YC, C/EBP-α, or C/EBP-β were incubated with reaction mixture before adding labeled probe.

adiponectin mRNA was not detected in preadipocyte but started to be induced at 4 days after induction of differentiation and elevated further until 6 days, which is consistent with the previous reports (6,9). Meanwhile, resistin appeared earlier than adiponectin and continued to increase until 6 days after differentiation. Another adipocyte-specific gene, aP2, was slightly expressed in the preadipocyte, and the expression level almost reached the maximum level at day 2. This indicates that adiponectin is expressed only in fully differentiated adipocytes compared with other adipocyte-specific genes, aP2 and resistin. On the basis of this result, we used differentiated adipocytes of at least day 6 for the next experiment to investigate nutritional regulation.

Next, we examined the effect of glucose on the expression of adiponectin. Differentiated 3T3-L1 adipocytes were maintained in media that contained 5.5 mmol/l glucose for 24 h and then were treated with 27.5 mmol/l glucose. Northern blot analysis showed that treatment with a high concentration of glucose for 24 h drastically upregulated adiponectin expression (Fig. 1*B*), suggesting that transcription of adiponectin is induced by glucose. This result led us to investigate whether adiponectin expression could be regulated in response to diet in living organisms. Mice were fasted and then refed a standard diet for 2–8 h, and the adiponectin expression was examined by RT-PCR. As shown in Fig. 1*C* and *D*, the expression was induced and peaked 4–6 h after refeeding, suggesting that the adiponectin expression is regulated by diet at the transcriptional level in the early phase of refeeding.

Localization of protein binding sites in adiponectin promoter. To identify *cis*-acting elements responsible for the regulation of adiponectin gene expression, we performed DNase I footprinting analysis using nuclear extract from white adipose tissues. The DNA fragments covering the proximal −500-bp region of the promoter were subcloned and used as probes. When liver nuclear extract was used as control because of its abundance of transcription factors, several protein binding site regions (AO, AI, and AII; Fig. 2*A*) were protected. However, when nuclear extract from adipose tissue was used, only region AII was clearly protected. Region AII has one CCAAT box and two inverted Y boxes, and, interestingly, potential C/EBP binding sites are located between them. Figure 2*B* shows the sequence of AII with potential nuclear protein binding sites. When it was compared with the human sequence, only the proximal CCAAT box and C/EBP site showed high sequence similarity between species.

Determination of the transcription factors bound to region AII. For investigating the binding of protein to region AII of the adiponectin promoter, a electrophoretic mobility shift assay (EMSA) was performed using nuclear extract from white adipose tissue. The oligonucleotide probe of the region AII sequence formed two major com-

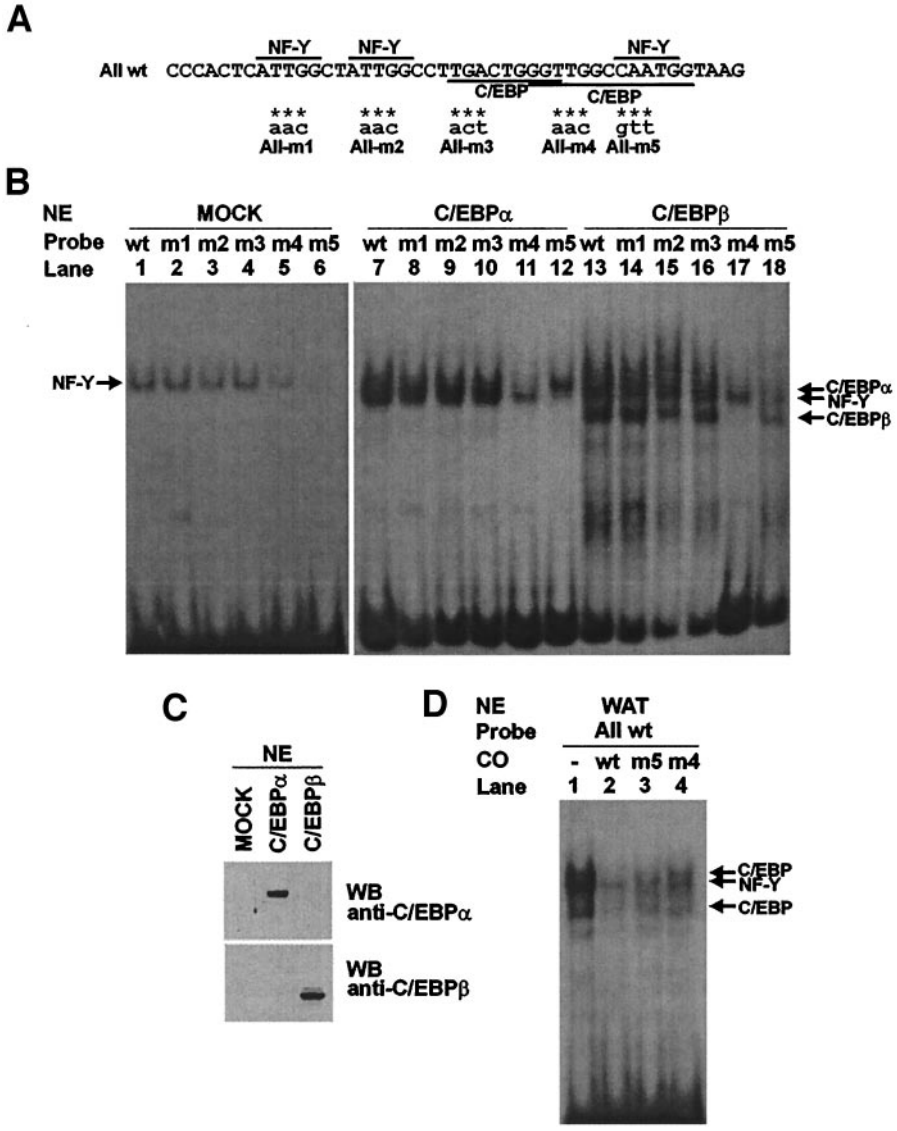

FIG. 4. Effect of site-specific mutations on C/EBP and NF-Y binding activity. Nuclear extracts were prepared from 293T cells overexpressing C/EBP-α or -β. For mock, the cells were transfected by pcDNA. All probes were generated by PCR with ^{32}P-labeled antisense primer and unlabeled sense primers using site-directed mutated plasmids as templates, and purified from the polyacrylamide gel, to equalize the specific activities between probes. *A*: DNA oligonucleotide sequence of wild and mutated probes used in EMSA. Mutated regions are indicated by lowercase with ★ with their name. *B*: EMSA using nuclear extracts of 293T cells overexpression C/EBP-α and C/EBP-β. Wild-type (wt) and mutant (m1, m2, m3, m4, and m5) probes were incubated with 4 μg of indicated nuclear extracts. The bands corresponding to NF-Y, C/EBP-α, and C/EBP-β are marked by arrows. *C*: Western blot (WB) analysis of C/EBP-α and -β for validating the expression of C/EBP-α and -β. 293T cells were transfected with expression vectors for C/EBP-α or -β and harvested 48 h after transfection, and nuclear extracts from the cells were subjected to SDS-PAGE. *D*: Competition assay of AII probe. Nuclear extracts prepared from rat white adipose tissue (WAT) were incubated with wild-type AII probe with or without indicated mutated oligonucleotides as cold competitors.

plexes, slow-migrating bands and a fast-migrating band (Fig. 3*A*). For determining which transcription factors are involved in these DNA-protein complexes, unlabeled competitor oligonucleotides for NF-Y, GATA, C/EBP, SRE, and AP-1 were added to the reaction. As shown in Fig. 3*A*, the addition of NF-Y and C/EBP competitors abolished the binding of slow- and fast-migrating bands, respectively, whereas unlabeled GATA, SRE, and AP-1 oligonucleotides did not affect the complex formation. The binding of NF-Y and C/EBP to region AII was further investigated by a supershift assay. As shown in Fig. 3*B*, the DNA-protein complexes were supershifted by anti–NF-YA, NF-YB, C/EBP-α, and C/EBP-β, indicating that both NF-Y and C/EBP bind to region AII of the adiponectin promoter.

Effect of site-specific mutations on C/EBP and NF-Y binding activity. For further investigation of precise binding sites of C/EBP and NF-Y, several mutant oligonucleotide probes were generated (Fig. 4*A*) and EMSA was carried out. Given that 293T cells express only a trace amount of endogenous C/EBPs, nuclear extracts from 293T cells that overexpress C/EBP-α or C/EBP-β were used to examine specific binding activities of C/EBP-α and C/EBP-β to adiponectin promoter. The overexpression of each transcription factor was confirmed by Western blot (Fig. 4*C*). As shown in Fig. 4*B*, C/EBP-α (*lane 7*) and C/EBP-β (*lane 13*) bound to the wild-type oligonucleotide probe. However, binding activities of C/EBP-α and C/EBP-β diminished when m4 mutated oligonucleotide was used as a probe (*lanes 11* and *17*). With nuclear extract from 293T cells, which do not express C/EBP, only NF-Y binding was observed (*lane 1*); however, this binding was almost diminished when the m5 probe was used (*lanes 6*, *12*, and *18*). It is interesting that the m4 mutation also caused the attenuated binding of NF-Y (*lane 5*), and, conversely, C/EBP binding was greatly affected by the mutation of the m5 region. It is acceptable because the potential C/EBP binding site expands the m4 and m5 regions, but it is not excluded that a certain form of the interaction is required for full binding of two transcription factor families from this result. When unlabeled mutated oligonucleotides were used as competitors, binding of C/EBP-α and C/EBP-β decreased by the addition of the m5 competitor (Fig. 4*D*, *lane 3*) as well as the m4 competitor (*lane 4*). These findings suggest that C/EBP-α and C/EBP-β bound to adiponectin promoter via the m4 and m5 regions, whereas

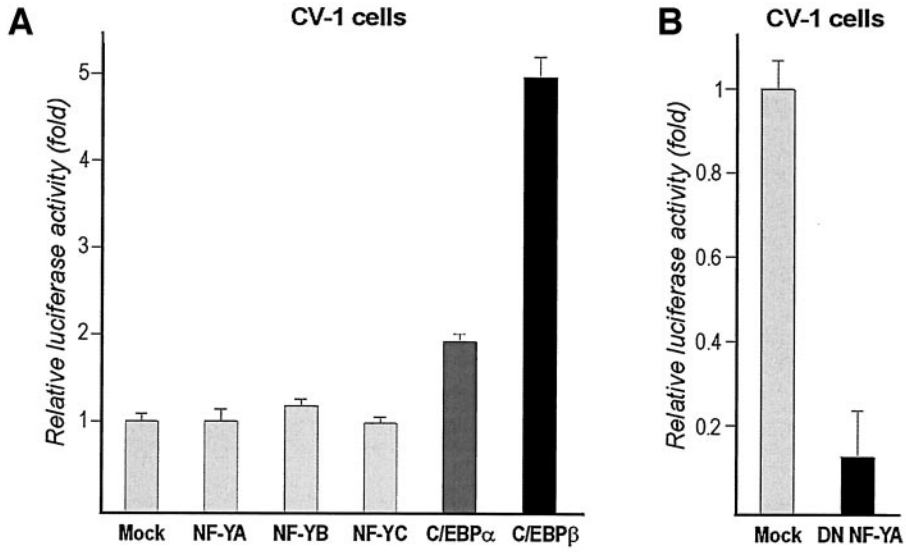

FIG. 5. Response of adiponectin promoter to NF-Ys and C/EBPs in CV-1 cells. *A*: A construct consisting of −1,138 bp of 5′-flanking sequences from the mouse adiponectin gene was inserted into luciferase reporter vector and transiently cotransfected into CV-1 cells in the presence or absence of expression vectors (0.1 μg) encoding NF-YA, NF-YB, NF-YC, C/EBP-α, and C/EBP-β, respectively. Luciferase activity was measured 48 h after transfection. Data represent relative luciferase activity (means ± SD) of three independent experiments. *B*: CV-1 cells were transfected with pGLB-A-1134 in the presence or absence of dominant negative mutant of NF-YA (0.1 μg). Luciferase activities were measured 48 h after transfection and normalized by β-galactosidase activity. Data represent means ± SD of three independent experiments.

NF-Y bound via the m5 region, and also suggest that NF-Y binding is required for the full binding activities of C/EBPs.

C/EBPs activate adiponectin promoter. For determining whether NF-Y and C/EBP are able to regulate adiponectin promoter activity, the adiponectin promoter (−1,138/30) linked to the luciferase reporter vector was constructed and its responsiveness to C/EBPs and NF-Ys was tested. The reporter construct was transiently transfected into CV-1 cells in the presence or absence of expression plasmids of NF-YA, NF-YB, NF-YC, C/EBP-α, or C/EBP-β. As shown in Fig. 5*A*, C/EBP-α and C/EBP-β activated adiponectin promoter activities, whereas NF-Ys could not. Because NF-Y is constitutively expressed in all eukaryotic cells and is composed of three different subunits, YA, YB, and YC, which are collectively required to bind to the sequence (37), it was thought that overexpression of individual NF-Y subunits probably did not affect the promoter activities. So we tested the response to the dominant negative mutant of NF-YA. The promoter activity was markedly inhibited to <20% of basal promoter activity when cotransfected with the dominant negative form. Taken together, these results suggest that C/EBPs upregulate adiponectin promoter, whereas NF-Y has a crucial role in maintaining basal adiponectin gene expression.

Binding of C/EBP and NF-Y at region AII plays a crucial role in adiponectin promoter activities. Next, we generated a series of plasmid constructs that contained 5′ serial deletion constructs shown in Fig. 6*A* and transfected into 3T3-L1 adipocytes. As shown in Fig. 6*B*, the adiponectin promoter activity decreased dramatically when up to −57 bp was deleted, demonstrating that the −134/−57 region is critical and sufficient for the promoter activity. Moreover, the C/EBP responsiveness was significantly decreased between −134 and −57 (Fig. 6*C*), so we concluded that the most proximal 134 bp of the promoter has a functional C/EBP site, taken together with the mutation EMSA study shown in Fig. 4. We also investigated the influence of the mutations used for the EMSA study on the C/EBP transactivation property. When the m4 or m5 mutation, which proved to be important for the C/EBP binding on the EMSA, was introduced into the −134 construct, C/EBP responsiveness was greatly reduced (Fig. 6*D*). This effect was more apparent when m4 and m5 mutations were introduced simultaneously, suggesting that the C/EBP and NF-Y sites on region AII are functional and necessary for the stimulation of the adiponectin promoter.

Binding of C/EBP and NF-Y is affected by dietary status. Because the adiponectin expression was regulated by diet as shown in Fig. 1, we examined whether the binding of NF-Y and C/EBP could be changed according to the dietary status. Nuclear extracts from white adipose tissues of fasted or refed rats were prepared and examined by EMSA. As shown in Fig. 7*A*, the dietary intakes increased the complex formation on the region AII probe. To confirm the binding activities of C/EBP, we incubated nuclear extracts at 72°C for 20 min before the binding reaction because C/EBP has heat-stable characteristics (38). The result clearly showed the increase of binding of the heat-stable factor, supposed to be C/EBP, to region AII (Fig. 7*A*). Therefore, it is likely that C/EBP is responsible, in part, for the increased expression of the adiponectin gene ~6 h after refeeding, as shown in Fig. 1 by the increased binding to region AII.

Next, we tested whether C/EBP binding is increased in response to glucose in differentiated 3T3-L1 adipocytes. Nuclear extracts were prepared from fully differentiated adipocytes that were maintained in media that contained 5.5 mmol/l glucose for 24 h and then treated with 27.5 mmol/l glucose. As shown in Fig. 7*B*, C/EBP binding was apparently increased 12 h after glucose treatment, similar to the result in refed animals (Fig. 7*A*). Thus, taken together with the result shown in Fig. 1*B*, it is concluded

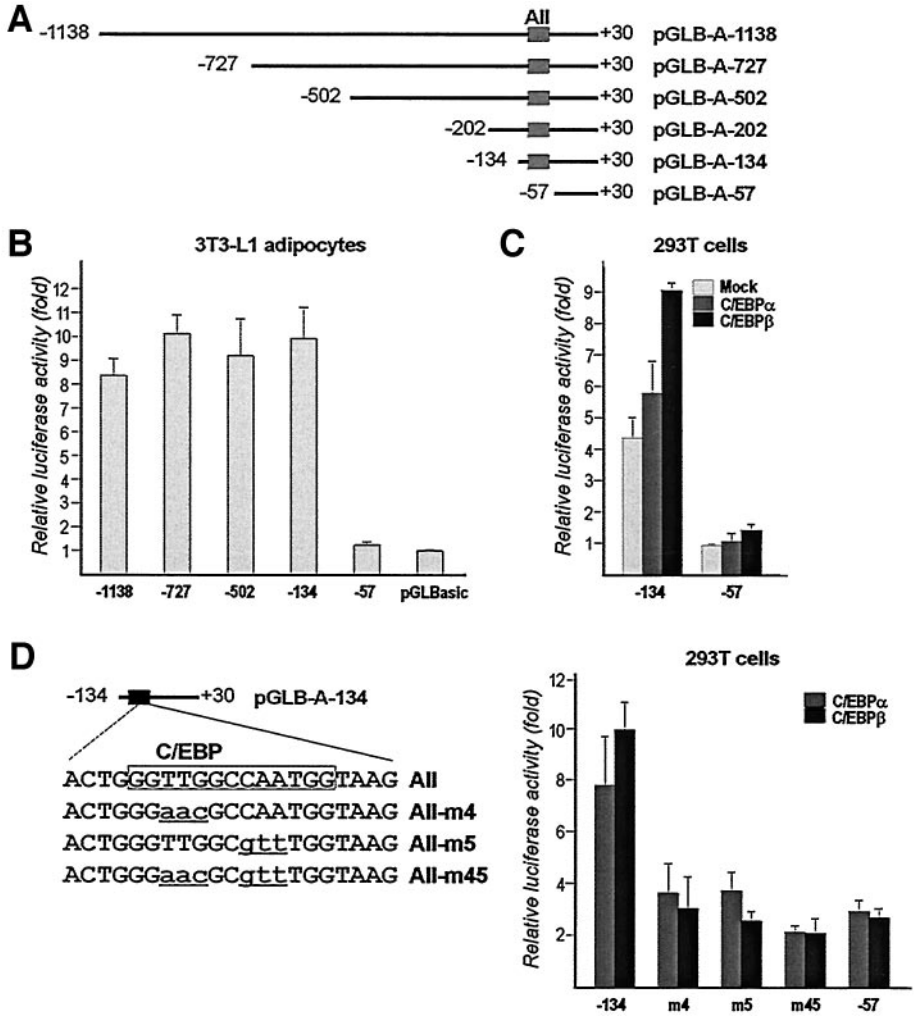

FIG. 6. 5′ Deletion and mutation study of the adiponectin promoter activity. *A*: Scheme for 5′-deletion construct of adiponectin promoter. *B*: 3T3-L1 cells were transiently transfected with indicated adiponectin promoter-luciferase construct (0.5 μg) and pCMV-β-galactosidase (0.1 μg) 2 days after reaching confluence. Cells were induced to differentiate into adipocyte 4 h after transfection and harvested 72 h after transfection for luciferase assay. *C*: C/EBP responsiveness of the deletion constructs. 293T cells were transfected with each construct in the presence or absence of C/EBP-α or C/EBP-β. *D*: Mutation study of the adiponectin promoter. Site-directed mutagenesis was performed to introduce the indicated mutation into pGLB-A-134 plasmid, and each construct was transfected into 293T cells with or without C/EBP. Luciferase activities were measured 48 h after transfection and normalized by β-galactosidase activity. Data represent means ± SD of three independent experiments.

that the C/EBP binding is responsible for the glucose induction of adiponectin in 3T3-L1 adipocytes.

To correlate the change of C/EBP and NF-Y levels, we conducted Western blot analysis. As illustrated in Fig. 7*C* and *D*, C/EBP-α and C/EBP-β were markedly increased by diet or glucose, whereas NF-Y and other proteins were not changed significantly, suggesting that dietary status regulates the levels of C/EBP proteins, resulting in changes in their binding activities.

C/EBPs and NF-Ys bind to the proximal adiponectin promoter in vivo. To confirm whether NF-Y or C/EBP binds directly to the adiponectin promoter in vivo, we used chromatin immunoprecipitation analysis for the next step. The DNA encompassing region AII was amplified by PCR from chromatin immunoprecipitated with antibodies against C/EBP-α, C/EBP-β, NF-YA, NF-YB, and NF-YC, whereas exon 3, which is located 10 kb downstream of region AII, was not amplified (Fig. 8*B*). It is interesting that the cyclic AMP response element binding protein (CBP), known as one of the coactivators of C/EBP, also occupied the adiponectin promoter. These results indicate that NF-Y and C/EBP bind directly to region AII of the adiponectin proximal promoter in vivo and also suggest the involvement of CBP as a coactivator.

Previous reports and our results in Fig. 1*A* showed that adiponectin expression is induced 4 days after adipocyte differentiation. For investigating whether the binding of C/EBPs and NF-Y is adipocyte specific, a ChIP assay of preadipocyte and adipocyte was also performed. As shown in Fig. 8*C*, C/EBP-α and NF-YA occupied adiponectin promoter in adipocytes but not in preadipocytes. These results suggested that the drastic induction of adiponectin expression during adipocyte differentiation might be explained by the recruitment of C/EBPs and NF-Y into the adiponectin promoter. In addition, the glucose treatment of differentiated adipocytes gave rise to the increased C/EBP-α and C/EBP-β binding in the ChIP assay (Fig. 8*D*), also suggesting that these transcription factors are involved in the regulation of adiponectin gene expression in response to nutritional status as well.

DISCUSSION

For many years, a number of studies have reported the linkage between adiposity and insulin resistance. As it became apparent that adipose tissue plays special roles for body metabolism by secreting a set of molecules, recent research has been focused on investigating how these "adipokines" can act as link molecules between obesity and insulin resistance (4). Adiponectin is one of the adipokines secreted by fully differentiated adipocytes and thought to be associated with some metabolic disease because plasma adiponectin levels are decreased under conditions of obesity and insulin resistance (10–12). Moreover, the

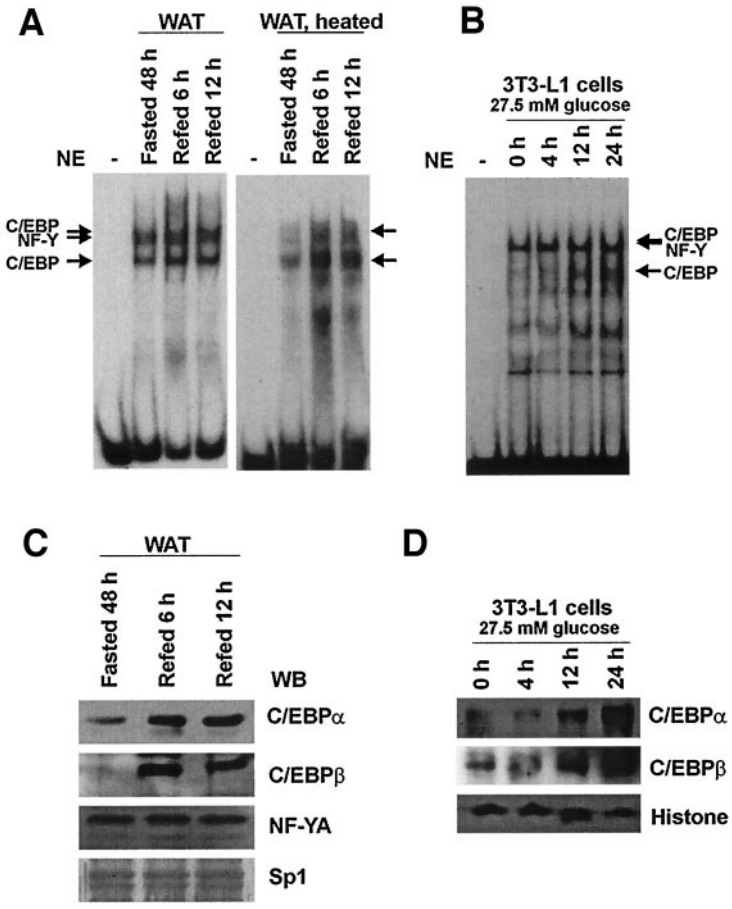

FIG. 7. Binding activities of C/EBP and NF-Y on region AII of adiponectin promoter were increased by diet intake. Rats were fasted for 48 h and refed for the indicated time, and the nuclear extracts of adipose tissue were prepared. *A*: EMSA of region AII. −120/−70 oligonucleotides were incubated with 5 μg of nuclear extract of adipose tissue (*left*) or heat-treated nuclear extract (*right*). Nuclear extracts were incubated at 72°C for 20 min for denaturation of heat-labile protein before incubation with the binding mixture. *B*: EMSA using nuclear extracts from glucose-treated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with glucose (27.5 mmol/l) for 0, 4, 12, and 24 h after incubation in low glucose (5.5 mmol/l) for 24 h, and nuclear extracts were prepared. *C*: Western blot (WB) analysis of nuclear extracts from adipose tissue was performed using antibodies against C/EBP-α, C/EBP-β, NF-YA, and Sp1. The same nuclear extracts (15 μg) used in EMSA were subjected to SDS-PAGE. *D*: Western blot analysis of nuclear extracts from 3T3-L1 adipocytes used in *C*, using antibodies against C/EBP-α, C/EBP-β, and histone H3. WAT, white adipose tissue.

administration of adiponectin causes glucose-lowering effects and improves insulin resistance in mice (17,39,40). Therefore, adiponectin is considered as an "insulin enhancer" linking adipose tissue and whole-body glucose metabolism. In this regard, if adiponectin plays a role as insulin enhancer, then it is likely that adiponectin could be regulated to control body homeostasis by the changes of metabolic stimulation such as the fasting-feeding cycle.

The present study clearly suggests that adiponectin is regulated in response to nutrition. We showed that glucose seemed to regulate adiponectin gene expression at the transcriptional level (Fig. 1*B*). Most important, adiponectin gene expression was induced in a relatively short period (4–6 h) after refeeding of mice (Fig. 1*C* and *D*). This finding in refed mice is interesting because several studies have failed to show the relationship between the adiponectin expression and fasting-refeeding cycle. It was reported that adiponectin levels are relatively constant and do not dramatically change postprandially in human subjects (11). It is possible that the plasma adiponectin levels might not represent acute changes of transcriptional levels because adipocytokines may function as autocrine or paracrine or the regulation at the protein level might appear in the late period after diet. In addition, we cannot exclude the possibility that the secretion pathway of the adiponectin protein participates in another way of regulation when the animals are subjected to a fasting-refeeding cycle (41). Although the regulation observed in this study cannot fully explain adiponectin biology, it is noteworthy that the induction profile of the adiponectin gene transcription (Fig. 1*C*) seemed to be similar to that of DNA-protein binding on region AII on the promoter (Fig. 7). Thus, it is likely that the expression of adiponectin is induced shortly after feeding, at least at the transcription level.

The present study revealed that C/EBPs and NF-Y are involved in the regulation of adiponectin gene expression. C/EBP-α and C/EBP-β bound to the adiponectin gene in in vitro and in vivo assays (Figs. 4 and 8) and activated adiponectin promoter in vitro (Figs. 5 and 6). In addition, NF-Y binds to the adiponectin promoter and plays a vital role in regulation of adiponectin gene expression. It is interesting that although transient transfection assays showed that the adiponectin promoter was activated by both C/EBP-α and C/EBP-β, the fold increase by C/EBP-β was larger than that by C/EBP-α (Fig. 5), indicating that C/EBP-β had more crucial roles in regulation of adiponectin expression than C/EBP-α. In this regard, it should be noted that a recently published study failed to show C/EBP-α responsiveness in the adiponectin promoter (42). It is unclear why the C/EBP-α response is small compared with that of C/EBP-β, despite its increased protein expression by nutritional status (Fig. 7*C* and *D*). One possible explanation is that in our EMSA findings C/EBP-α protein alone could not lead to the fasting migrating band (Fig. 4*B*), which is apparent in natural nuclear extract from white adipose tissue (Fig. 3). Meanwhile, the addition of C/EBP-β formed the fasting migrating band (Fig. 4*B*), suggesting that C/EBP-β, in association with C/EBP-α, plays an important role in the physiological regulation of adiponectin expression. Moreover, we showed that both

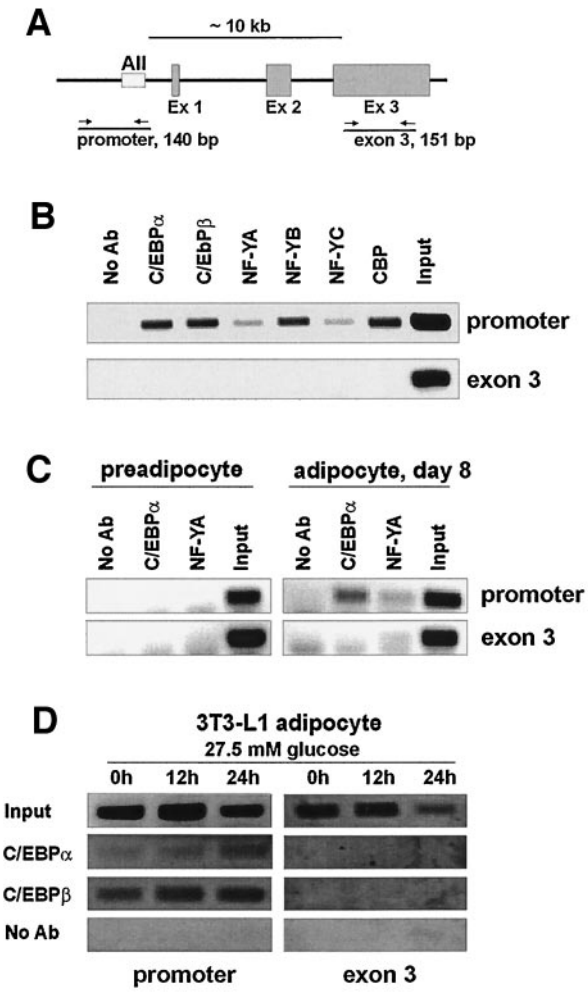

FIG. 8. C/EBP-α, C/EBP-β, NF-Ys, and CBP are bound to adiponectin promoter in vivo. *A*: Diagram indicating the oligonucleotides used to amplify the adiponectin promoter in the ChIP assay. The amplified regions were the adiponectin promoter from −202 to −60 and exon 3 as indicated in the figure. *B*: ChIP assay in mouse adipose tissue was performed with or without antibodies (Ab) against C/EBP-α, C/EBP-β, NF-YA, NF-YB, NF-YC, or CBP as indicated. DNA isolated from input chromatin was amplified as a control. *C*: ChIP assays with undifferentiated 3T3-L1 preadipocyte and differentiated 3T3-L1 adipocytes were subjected to the ChIP assay. *D*: ChIP assays with glucose-treated 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with glucose (27.5 mmol/l) for 0, 12, and 24 h after incubation in low glucose (5.5 mmol/l) for 24 h, and the ChIP assay was performed as described.

C/EBP-α and C/EBP-β are important for adiponectin expression as shown by in vitro (EMSA) and in vivo (ChIP) assays along with the overexpression study. Thus, it can be concluded that C/EBP-α, in association with C/EBP-β and/or NF-Y, has a role in the adiponectin gene expression.

As summarized in the introduction, C/EBP has been identified as one of the major adipogenic factors controlling the transcription of the adipocyte-specific gene. The regulation of C/EBP gene expression has been examined in adipose tissue and liver. In liver cells, glucocorticoids induced C/EBP-α and C/EBP-β at the level of transcription and translation (43,44). Conversely, in adipose tissue, glucocorticoids inhibited C/EBP-α expression (45). Meanwhile, the effects of insulin on C/EBP-α expression have generally been shown to be inhibitory in adipose tissue (46,47). In our study, C/EBP-α and C/EBP-β protein levels were elevated in the refed state; thus, it is within the realm of possibility that the elevated adiponectin mRNA level from dietary nutrients is mediated by C/EBP-α or C/EBP-β.

The interaction between NF-Y and other transcription factors on several gene promoters has been reported. NF-Y is required for thyroid hormone regulation of liver S14 gene transcription (48), and NF-Y also participated in the regulation of the ATP citrate-lyase gene under the control of SREBP1c (49). Although NF-Y is unlikely to be regulated by metabolic status as shown in this study, it is interesting to note that NF-Y is needed not only for basal promoter activity but also for C/EBP responsiveness (Figs. 5 and 6). The region AII of the adiponectin promoter has three potential binding sites for NF-Y (represented as m1, m2, and m5 in Fig. 4); only mutation m5 abolished the binding of NF-Y. Comparing this sequence with human adiponectin gene, most nucleotides, including m4 and m5 but not m1/m2, were highly conserved between species (Fig. 2). Therefore, we can conclude that NF-Y could be another important regulator for the proper expression of the adiponectin gene. In this regard, the binding of NF-Y along with C/EBP might be required for adiponectin gene expression during adipocyte differentiation as shown in the ChIP assay (Fig. 8).

In summary, we found that transcription of adiponectin is regulated by diet at the early stage of the refed state and that C/EBP and NF-Y play roles in this process. It remains to be clarified how the nutrients regulate these transcription factors in adipose tissue. Also, whether the binding of C/EBP or interaction with NF-Y is dysregulated in obesity or insulin resistance must be investigated; however, it is clear that C/EBP and NF-Y are important for the regulation of the expression of adiponectin in response to nutrients and in the course of adipocyte differentiation as well.

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