C/EBPα Regulates Human Adiponectin Gene Transcription Through an Intronic Enhancer

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Adiponectin is an adipose-derived hormone that enhances insulin sensitivity and plays an important role in regulating energy homeostasis. Here, we demonstrate that the DNA encoding the first intron of the human adiponectin gene contains an intronic enhancer that regulates adiponectin gene expression in an adipose tissue–specific manner. Insertion of the DNA encoding the first intron into reporter constructs containing the proximal adiponectin promoter (Pro-Int1-Luc) resulted in a 20-fold increase in activity relative to the promoter alone in 3T3-L1 adipocytes. Coexpression of CCAAT/enhancer-binding protein (C/EBP)α increased luciferase activity of the Pro-Int1-Luc construct ~75-fold but had no effect on the constructs containing the proximal adiponectin promoter alone. At least eight potential C/EBPα response elements are located between +3000 to +10000 nucleotides within the DNA encoding the first intron, including a 34-bp core sequence for the intronic enhancer that contains three tandem C/EBPα response elements. However, the intronic enhancer is not conserved between human and mouse. Overexpression or siRNA-mediated knockdown of endogenous C/EBPα significantly increased or decreased, respectively, adiponectin mRNA levels in differentiated human Chub-S7 adipocytes, while neither C/EBPβ nor C/EBPδ significantly affected adiponectin expression in mature adipocytes. Thus, C/EBPα is a key transcription factor for full activation of human adiponectin gene transcription in mature adipocytes through interaction with response elements in the intronic enhancer. *Diabetes* 54:1744–1754, 2005

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aP2, adipocyte-specific fatty acid–binding protein; C/EBP, CCAAT/enhancer-binding protein; EMSA, electrophoretic mobility shift assay; HEK, human embryonic kidney; PPAR, peroxisome proliferator–activated receptor; RXRα, retinoid X receptor α; TK, thymidine kinase; TZD, thiazolidinedione.

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Adiponectin is exclusively expressed in adipocytes (1–4) and has diverse roles that include regulation of insulin sensitivity and energy homeostasis, immunological responses, and the development of vascular disease (5–8). Expression and serum levels of adiponectin are diminished in humans and animals with insulin resistance, obesity, and type 2 diabetes (9–12). Although there has been rapid progress in understanding of the physiologic functions of adiponectin and the signaling pathways mediating adiponectin action (13–15), little information is available regarding the transcriptional regulation of adiponectin expression.

The human adiponectin gene spans 16 kb and is composed of three DNA sequences encoding exons from 18–4,277 bp in size with consensus splice sites (16,17). The two introns span 10 and 0.9 kb. Both human and mouse adiponectin promoters are TATA-less but contain a classical CCAAT box (16–18). Transient transfection studies indicate that a 1.3-kb fragment located upstream of the transcriptional start site of the human adiponectin gene, or 1.13 kb counterpart of mouse, confers basal transcriptional activity (17,18). However, promoter activity was increased only twofold in 3T3-L1 adipocytes compared with the activity in fibroblasts (17), suggesting that only modest adipocyte-specific regulatory elements are present in the proximal promoter region of the adiponectin gene. Transcriptional activity of basal promoters is often regulated by enhancer elements found at some distance from the start site of transcription, and the large DNA encoding the first intron often contains *cis*-acting regulatory elements (19). However, Das et al. (17) demonstrated that upstream sequences in the 5′ flanking region of the mouse adiponectin gene do not increase adiponectin promoter activity in adipocytes. Therefore, the current study was designed to explore whether sequences within the first intron functioned to enhance adiponectin transcription in adipocytes.

Peroxisome proliferator–activated receptors (PPAR)s and CCAAT/enhancer-binding proteins (C/EBPs) are the two major transcription factor families that transcriptionally transactivate adipose-specific genes, including adipocyte-specific fatty acid–binding protein (aP2), fatty acid synthase, leptin, and resistin (20). PPARγ may directly bind to the human adiponectin promoter by forming heterodimers with retinoid X receptor (RXR) (21,22). Moreover, PPARγ2 stimulates the adipocyte differentiation program in C/EBPα$^{-/-}$ fibroblasts and induces adi-

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ponectin expression at a modest level, but restoration of C/EBPα caused a remarkable increase in the expression of adiponectin (23,24). Therefore, full activation of adiponectin gene transcription requires both PPARγ and C/EBPα. Although sequence analysis revealed two putative C/EBPβ sites and a C/EBP consensus region in the adiponectin proximal promoter region, most available data suggest that no nuclear protein appears to bind to these sites, and ectopic expression of C/EBPα had no effect on adiponectin promoter activity (17,21,22,25,26). However, during the preparation of this manuscript, Park et al. (23) reported that both C/EBPα and C/EBPβ bind at the proximal promoter of mouse adiponectin gene. Therefore, whether C/EBPs regulate adiponectin gene transcription directly through the promoter or another region is controversial and warrants further investigation.

In this study, we show that the DNA encoding the first intron of the human adiponectin gene confers significant activity on the adiponectin promoter. We have identified adipocyte-specific regulatory elements in the DNA encoding the first intron of the human adiponectin gene. These elements are highly sensitive to the adipogenic transcription factor C/EBPα but not to PPARγ. Furthermore, in both human Chub-S7 or 3T3-L1 adipocytes, adiponectin mRNA levels were increased or decreased, respectively, by overexpressing or knocking down C/EBPα expression. In total, the results suggest that the DNA encoding the first intron of the human adiponectin gene contains an intronic enhancer and regulates adiponectin gene transcription in an adipose tissue–specific manner.

RESEARCH DESIGN AND METHODS

Cell culture. Chub-S7 cells are human preadipocytes immortalized by expression of human telomerase and papillomavirus E7 (27). 3T3-L1CARΔ1 cells stably express a truncated coxsackie-adenovirus receptor (28). Differentiated 3T3-L1CARΔ1 adipocytes are metabolically indistinguishable from their parental cells but demonstrate improved efficiency of adenoviral transduction for gain or loss of function studies (29). 3T3-L1 and human embryonic kidney (HEK) 293 cells were maintained at 37°C, 5% CO_2 in Dulbecco's modified Eagle's medium containing 10% FBS. Adipocyte differentiation of Chub-S7 or 3T3-L1 cells was induced as described previously (27,29).

Experimental animals. Male C57BLKS/J-*db/db* mice weighing 39–41 g and age-matched (8–10 weeks of age) wild-type littermates were purchased from The Jackson Laboratory (Bar Harbor, ME). Homozygous C/EBPβ gene knockout mice were obtained by cross-breeding female mice heterozygous for a null mutation of the C/EBPβ gene with homozygous male mice (30). After overnight fasting, the mice were killed and epididymal fat tissues collected and stored at −80°C for mRNA and nuclear protein isolation.

Plasmid constructs. A reporter plasmid encoding luciferase under the control of the human adiponectin promoter (Pro-Luc) was generated by excising the 1.3-kb promoter fragment and 18-bp DNA encoding exon 1 from human genomic DNA and insertion into the *Kpn*I and *Nhe*I sites of the pGL3 basic luciferase expression vector (Promega). To generate a gene reporter construct with the adiponectin promoter and DNA encoding exon 1 and intron 1, intron 1 encoding DNA was cloned by PCR from human genomic DNA, and the *Nhe*I-*Xho*I PCR fragment was inserted into the Pro-Luc plasmid (Pro-Int1-Luc). Serial deletion of the human promoter intron 1 luciferase plasmids was achieved by PCR. Site-directed mutations of the adiponectin Pro-Int1-Luc plasmids were constructed using a Quickchange mutagenesis kit following the instructions provided by the supplier (Stratagene). The mutations of the C/EBP consensus binding sites in intron 1 were confirmed by DNA sequencing. Heterologus gene reporter constructs were created using the human adiponectin promoter and the DNA encoding the intron from the adenovirus type 5 E1A 13S mRNA, intron 1 of the mouse phosphoinositide 3-kinase regulatory subunit p85α gene, or intron 1 of the mouse adiponectin gene. The diagrams of the constructs are illustrated in Fig. 1*B*.

The PPARγ-responsive luciferase reporter (PPRE-luc) contains six tandem repeats of the PPARγ response element in the pTAL-luc vector and was provided by Dr. Harvey F. Lodish (Massachusetts Institute of Technology, Cambridge, MA). The plasmid encoding human RXRα (CMX-hRXRα) was a gift from Dr. David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). The full-length human C/EBPα and PPARγ2 cDNAs were cloned and inserted into pcDNA3.1 to generate plasmids encoding C/EBPα (pcDNA-hC/EBPα) or PPARγ2 (pcDNA-hPPARγ2).

Generation of the siRNA expression cassette. The siRNA expression cassette was created by ligating the human U6 pol III promoter with sense and antisense oligonucleotides of self-complementary hairpin sequence. The human U6 pol III promoter was amplified by PCR using human genomic DNA as template. The PCR fragments were digested with *Kpn*I and *Hind*III and cloned into pBluescriptII SK+ (Stragagene, La Jolla, CA) to create pBluescriptII+U6. C/EBPα target sequence (human, rat, and mouse) for siRNA was selected according to the Ambion web-based criteria and further analyzed by BLAST search to avoid significant similarity with other genes. The sequences of the oligonucleotides are 5′-AGCTTGCGACGAGTTCCTGGCCGACTTCAAGAGAGTCGGCCAGGAACTCGTCGTTTTTTGGAAT-3′; and 5′-CTAGATTCCAAAAAACGACGAGTTCCTGGCCGACTCTCTTGAAGTCGGCCAGGAACTCGTCGCA-3′. The oligonucleotides contain the termination signal of five thymidines and cohesive ends for *Hind*III and *Xba*I at the 5′- and 3′- ends. The annealed oligonucleotides were inserted into the *Hind*III and *Xba*I sites of pBluescriptII+U6. The C/EBPα siRNA-expression cassette was digested with the enzymes *Kpn*I and *Xba*I and then cloned into the same sites in the adenoviral shuttle vector pAdTrack. Recombinant adenoviruses containing the cDNAs encoding mouse C/EBPβ, the human C/EBPα and PPARγ2 or siRNA against C/EBPα were generated using the AdEasy system (31) as previously modified (32).

Luciferase assay. Reporter constructs were introduced into cells by electroporation or chemical reagent. Differentiated (day 10) 3T3-L1 adipocytes were transfected by a modification of the electroporation method (33). For comparison, confluent 3T3-L1 fibroblasts or HEK293 cells were also transfected by electroporation. In some studies, the adiponectin promoter-luciferase gene reporter construct was cotransfected with pcDNA-hC/EBPα, pcDNA-hPPARγ2, and/or CMX-hRXRα into HEK293 cells using FuGENE 6 transfection reagent (Roche, Indianapolis, IN). A pCMV–β-galactosidase plasmid was cotransfected to control for transfection efficiency (Clontech Laboratories, Palo Alto, CA). Twenty-four hours after transfection, cells were lysed and luciferase activity measured. Luciferase data were normalized relative to β-galactosidase luminescence and expressed as the relative luciferase activity.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed using the LightShift Chemiluminescent EMSA kit following the protocol provided by manufacture (Pierce Biotechnology, Rockford, IL). Double-stranded DNA fragments corresponding to the C/EBP consensus sequences or mutant sequences and oligonucleotides from the DNA encoding the first intron of the human adiponectin gene were 5′ end-labeled with biotin (IDT, Coralville, IA). C/EBPα protein was synthesized in HEK293 cells transduced with the adenovirus encoding human C/EBPα. Double-stranded oligonucleotides composed of the following sequences were used for binding and competition assays: C/EBP consensus, 5′-TGCAGA*TTGCGCAA*TCTGCA-3′; C/EBP mutant, 5′-TGCAGA*GACTAGTC*TCTGCA-3′; C/EBPα binding sites in the DNA encoding the first intron of human adiponectin gene at +3062/+3082 (+1 nt refer to the transcription start site), 5′-GAAGATTAAGTAAAATAAATT-3′; at +3076/+3096, 5′-ATAAATTTTGAAAATGCCTTA-3′; AT +3089/+3109, 5′-ATGCCTTATGAAAATTACACT-3′; at +5913/+5931, 5′-ACTTATTTTACCTAATCTT-3′; at +6570/+6588, 5′-AGTTTTATTGCCAAAGCCA-3′; at +7872/+7890, 5′-TAATTTCTTACAGAATATA-3′; at +7922/+7940, 5′-AGTTTGATGAAATATAAAT-3′; at +9935/+9953, 5′-TGAATGGAGCAATCTGTGT-3′.

Nuclear protein extraction and Western blot analysis. Nuclear protein was extracted as described previously (34). Protein levels of C/EBPβ, C/EBPα, or PPARγ were measured by Western blot using antibody raised against the COOH-terminus of human C/EBPβ, C/EBPα, or PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA) (30).

RNA extraction and Northern blot analysis. Total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, PA). RNA was denatured, separated by electrophoresis on 1% agarose gels, and transferred to positively charged nylon membranes (Roche). The cDNA fragment of mouse or human adiponectin, aP2, and GAPDH were labeled with [α-^{32}P]dCTP using the Prime-It II Random Primer Labeling kit (Stratagene, Cedar Creek, TX). Membranes were blotted with labeled probes, and radioactive signals were quantified and analyzed with PhosphorImager and Imagequant software (Molecular Dynamics, Sunnyvale, CA).

Data analysis. Data are expressed as mean ± SE. Statistical analyses were performed using the Student's *t* test or ANOVA analyses followed by contrast test with Tukey or Dunnett error protection. Differences were considered significant at $P < 0.05$.

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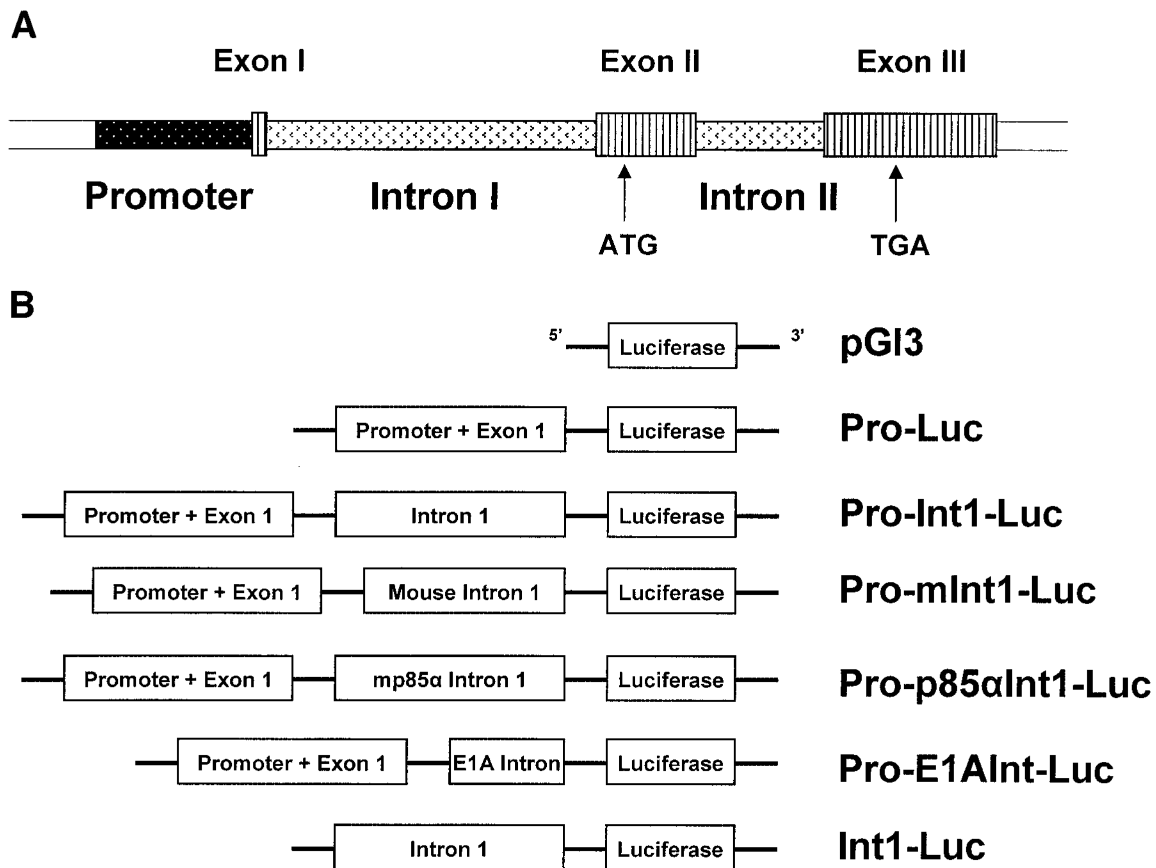

FIG. 1. The DNA encoding the first intron of the human adiponectin gene enhances the promoter activity only in adipocytes. ***A*****: Schematic representation of the human adiponectin gene.** ***B*****: 1.3-kb proximal promoter and DNA sequences encoding exon 1 and the intron were introduced upstream of the luciferase gene in pGl3-basic. Structures of reporter plasmids pGl3-basic vector (pGl3), Pro-Luc, Pro-Int1-Luc, Pro-mp85αInt1-Lu, Pro-E1AInt-Luc, and Pro-mInt1-Luc are indicated.** ***C*****: Gene reporter constructs were transfected into differentiated 3T3-L1 adipocytes, HEK293, and undifferentiated 3T3-L1 fibroblasts. The luciferase activities were normalized by β-galactosidase activity. The error bar indicates standard error for four independent experiments.** ***D*****: Gene reporter constructs were transiently transfected into differentiated 3T3-L1 adipocytes. The luciferase activities were normalized by cotransfected β-galactosidase activity. The error bar indicates standard error for six independent experiments.**

RESULTS

DNA encoding the first intron of the adiponectin gene enhances promoter activity in adipocytes. A schematic of the human adiponectin gene is shown in Fig. 1*A*. A series of gene reporter constructs with the 1.3-kb human adiponectin proximal promoter and 18-bp exon 1 coding region and DNA encoding the first intron of the human or mouse adiponectin genes, the intron from the adenovirus type 5 E1A 13S mRNA, or the mouse p85α intron 1 were created using pGl3 as vector (Fig. 1*B*). The gene reporter constructs were transiently transfected into HEK293, fibroblasts, and 3T3-L1 adipocytes. Luciferase activity directed by the Pro-Luc construct in 3T3-L1 adipocytes was nearly twofold higher than that in fibroblasts or HEK293 cells (Fig. 1*C*), suggesting the adiponectin promoter has modest adipocyte specificity. However, the insertion of the DNA encoding the first intron of the human adiponectin gene increased luciferase activity nearly 20-fold in adipocytes but had no effect in fibroblasts or HEK293 cells (Fig. 1*C*). The DNA encoding the first intron alone (human, Int1-Luc construct) also increased the luciferase activity in 3T3-L1 adipocytes compared with HEK293 or fibroblasts (Fig. 1*C*). These data suggest that the adiponectin proximal promoter is a basal promoter with modest adipocyte specificity and that the DNA encoding the first intron of the human adiponectin gene may be responsible for adipose tissue-specific expression.

One recent study reported that the heterologous Hbb intron enhances human adiponectin promoter-driven luciferase activity in adipocytes and has been used for tissue-specific gene expression (35). The human and rabbit β-globin (also named Hbb) genes have two introns. Both introns are essential for the accumulation of stable cytoplasmic mRNA and Hbb gene expression (36,37). However, there are no enhancer elements in the DNA encoding these introns, and regulation is exerted posttranscriptionally through increased efficiency of 3′-end formation (36,37). The finding of increased efficiency of adiponectin promoter-driven gene expression dependent on the Hbb intron raises the question of whether the enhancement of adiponectin promoter activity by intron 1 of the human adiponectin gene is gene-specific and whether there are enhancer elements in the DNA encoding the intron. To examine the mechanism(s), heterologous promoter constructs were created using the herpes simplex virus thymidine kinase (TK) promoter and the first intron of the human adiponectin gene. Transient transfection studies indicated that the presence of the DNA encoding intron 1 of the adiponectin gene increased TK promoter activity in response to C/EBPα (data not shown). Most importantly,

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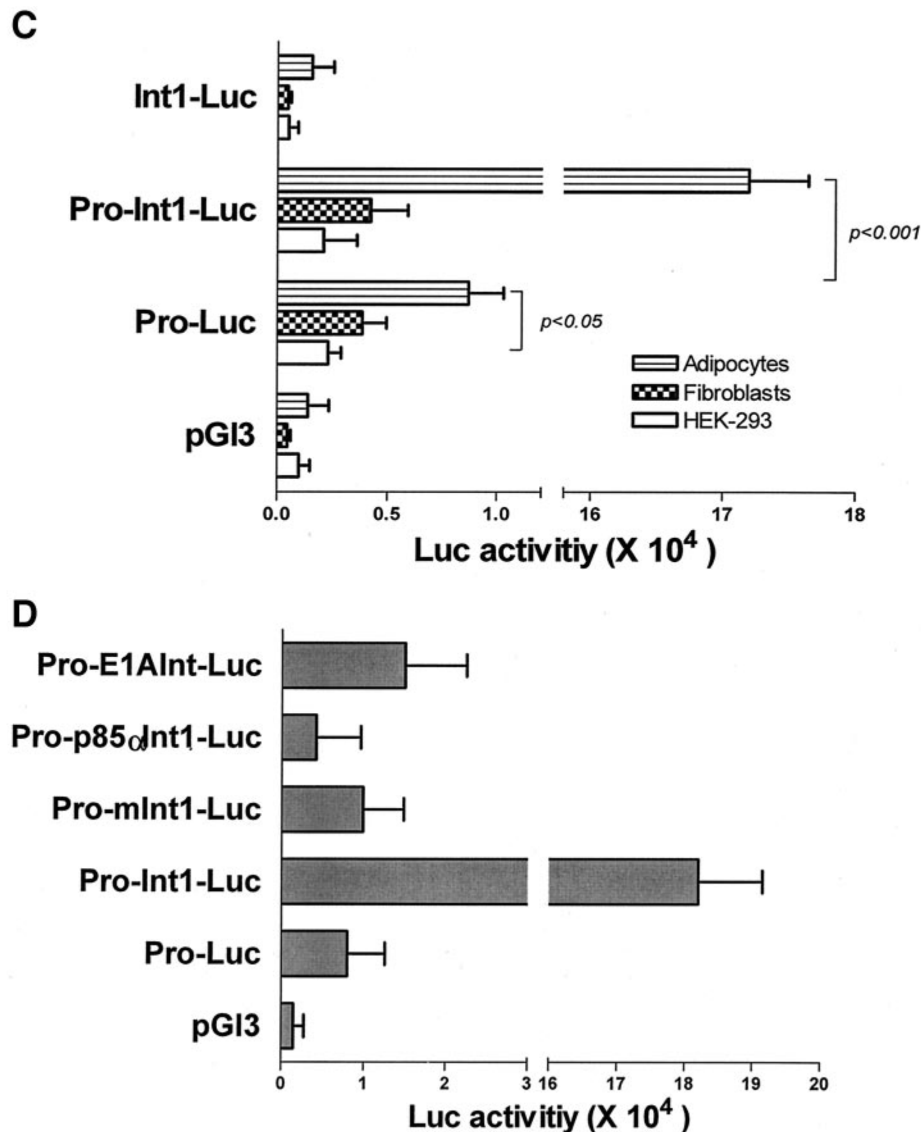

FIG. 1—*Continued*

the enhancement was not dependent on the location of the DNA encoding intron 1. However, upregulation of luciferase expression by the Hbb intron is position dependent (35). Generally, transcriptional enhancer elements exhibit a position-independent effect on gene expression. Therefore, these studies suggest that there are enhancer elements in the DNA encoding the first intron of the human adiponectin gene.

To test whether the adiponectin intron 1–dependent increased promoter activity occurs at the level of transcription or at a posttranscriptional step, two heterologous gene reporter constructs were created using the mouse p85α intron 1 and the 13S mRNA intron of adenovirus type 5 E1A. The first intron of the mouse p85α gene is 9.934 kb in length, similar to the size of intron 1 of the human adiponectin gene, and p85α is expressed in most tissues. To date, there are no reports indicating that the p85α intron 1 regulates gene expression. The DNA encoding the E1A 13S mRNA intron, which is only 116 bp in length, has no known function other than in splicing (35). As shown in Fig. 1*D*, the presence of the DNA encoding the E1A intron led to a slight increase, and the presence of the DNA encoding the p85α intron led to a slight decrease in adiponectin promoter-driven luciferase expression in adipocytes. However, neither intron had a statistically significant effect on the level of luciferase ($P > 0.05$). We also tested the functional similarity of the first intron of human and mouse adiponectin genes. Consistent with a previous study (17), the presence of mouse intron 1 did not increase the level of adiponectin promoter-directed luciferase activity in adipocytes (Fig. 1*D*). These data suggest that the enhancement of the human adiponectin promoter activity by the DNA encoding the first intron of the human adiponectin gene is dependent directly on the DNA sequence rather than on the presence of an intron in the primary transcript.

C/EBPα increases adiponectin promoter transactivation activity through the DNA encoding the first intron. C/EBPα and PPARγ are major adipogenic transcription factors that transactivate many adipocyte-specific genes. We tested the ability of these transcription factors to regulate adiponectin gene expression using

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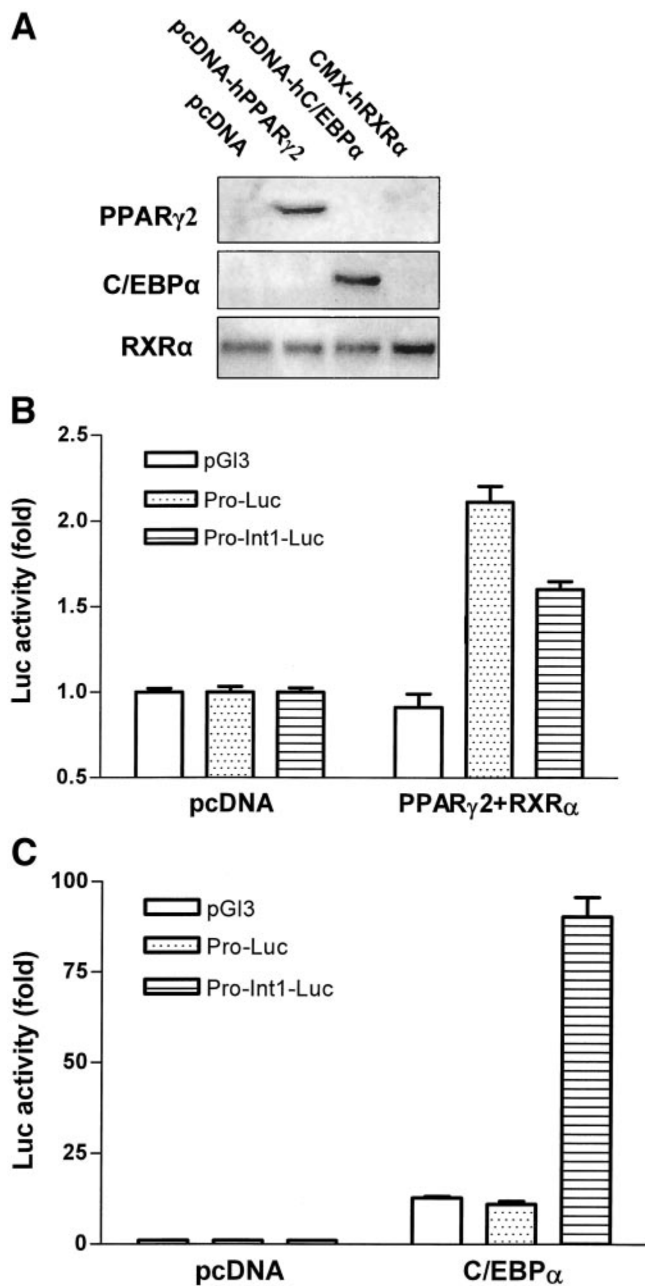

FIG. 2. The effect of the DNA encoding the first intron on PPARγ- or C/EBPα-induced transactivation of the human adiponectin promoter. The gene reporter constructs Pro-Luc, Pro-Int1-Luc, and vector pGl3, as illustrated in Fig. 1*B*, were cotransfected into HEK293 cells with pcDNA-hPPARγ2, CMX-hRXRα, or pcDNA-hC/EBPα expression plasmids. *A*: The protein levels of PPARγ2, RXRα, and C/EBPα in HEK293 cells were significantly elevated after transfection of the genes encoding the proteins as measured by Western blot. *B*: The presence of the DNA encoding the first intron did not further increase PPARγ/RXRα-induced transactivation of the human adiponectin promoter. *C*: C/EBPα has no effect on the proximal promoter activity, however, robustly increases the activity when the DNA encoding the first intron is present. The control cells were cotransfected with pcDNA3.1 empty vector. The luciferase activities were normalized by β-galactosidase activity. The values are expressed as fold activities relative to the luciferase activities of the constructs cotransfected with pcDNA vector. The error bar indicates standard error for four independent experiments.

transient transfection assays in HEK293 cells, which are a well established transient transfection cellular model for studying transcriptional regulation of adipocyte gene expression (17,21,22). As shown in Fig. 2*A*, endogenous C/EBPα and PPARγ in HEK293 cells are barely detectable. Ectopic expression significantly increased C/EBPα, PPARγ2, or RXRα protein levels (Fig. 2*A*). Coexpression of PPARγ2 and RXRα increased adiponectin promoter activity twofold ($P < 0.05$, Fig. 2*B*). Although the luciferase activity directed by the Pro-Int1-Luc reporter construct was increased significantly in the presence of high levels of PPARγ and RXRα, the magnitude of the stimulation was less than that of the construct with the adiponectin promoter sequence alone (Fig. 2*B*). The reduced response from the Pro-Int1-Luc construct may be caused by the presence of the DNA encoding the first intron. These sequences may contain repressive elements (see Elements responsible for the C/EBPα-mediated transcriptional regulation of the adiponectin gene) that suppress adiponectin gene expression, especially in nonadipocytes. Nevertheless, the data suggest that PPARγ upregulation of human adiponectin gene transcription is mediated by the proximal basal promoter. In contrast, overexpression of C/EBPα did not increase luciferase activity directed by the Pro-Luc construct (after excluding the increment driven by the elements in the pGl3 vector, Fig. 2*C*). However, C/EBPα led to a robust increase in the activity of the adiponectin promoter when the DNA encoding the first intron was present in the construct (Fig. 2*C*).

Elements responsible for the C/EBPα-mediated transcriptional regulation of the adiponectin gene. Inspection of the sequence using MatInspector with Matrix Similarity >0.85 and Matrix Family Library, version 4.1 (available at http://www.genomatix.de), revealed 21 putative C/EBP binding sites scattered in the DNA encoding the first intron of the human adiponectin gene. EMSA analysis demonstrated that C/EBPα bound to 8 of the 21 putative binding sites in the first intron coding sequence (Fig. 3). These complexes could be supershifted specifically by anti-C/EBPα antibody and were effectively competed by competitor oligonucleotides (Fig. 3). Interestingly, the first three C/EBPα binding sites are in tandem within a 34-bp sequence (+3067 to +3101).

We next investigated the function and importance of these C/EBPα binding sites for adiponectin gene expression using 3′ serial deletions, truncations, and site mutations of the first intron coding sequence. As shown in Fig. 4*A*, the deletion of sequences from +10322 nt to +7586 nt or to +5993 nt resulted in significant reductions of luciferase activity. However, further deletion to +3175 nt led to recovery of luciferase reporter activity (Fig. 4*A*). Deletion to +1346 nt abolished C/EBPα-induced transactivation, indicating that the C/EBPα-responsive elements are scattered from +1346 nt to +10322 nt. There also appears to be a repressive element(s) between +3175 nt and +5993 nt. The mechanism leading to this repressive regulation is currently under investigation.

To further study the role of the C/EBPα binding sites, gene reporter constructs with mutations in the first intron–coding sequence were constructed and used with the C/EBPα expression plasmid to cotransfect HEK293 cells (Fig. 4*B*). Mutation of the C/EBPα binding sites individually reduced the transactivation activity from 5 to 12%, but mutation of the +5917/+5924 or +6574/+6581 consensus sites had no effect (Fig. 4*B*). Deletion of +3063/+3110, which contains three tandem C/EBPα binding sites, re-

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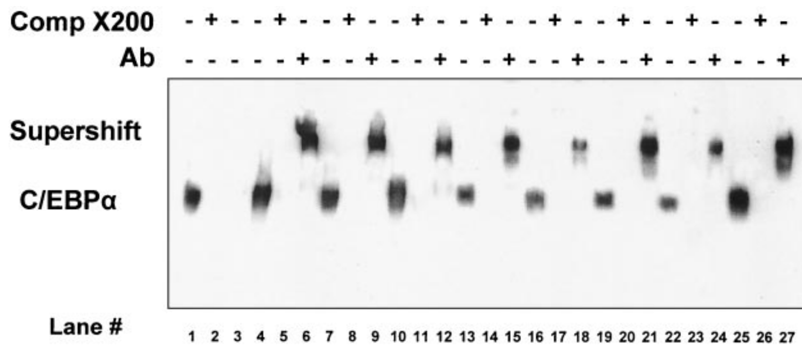

FIG. 3. Association of C/EBPα protein with intronic enhancer sequences in the human adiponectin gene by EMSA. Gel super-shift analyses using 21-bp oligonucleotides and C/EBPα synthesized in vitro were performed as described in RESEARCH DESIGN AND METHODS. *Lanes 1* and *2*, biotin-labeled C/EBP consensus; *lane 3*, mutated C/EBP consensus; *lanes 4–6*, biotin-labeled oligonucleotides corresponding to +3062/+3082 nt of the human adiponectin gene (+1 nt refers to the transcription start site); *lanes 7–9*, +3076/+3096 nt; *lanes 10–12*, +3089/+3109 nt; *lanes 13–15*, +5913/+5931 nt; *lanes 16–18*, +6570/+6588 nt; *lanes 19–21*, +7872/+7890 nt; *lanes 22–24*, +7922/+7940 nt; *lanes 25–27*, +9935/+9953 nt. Competition assays were performed using 200-fold excess of unlabeled oligonucleotides (comp ×200). The anti-C/EBPα antibody (Ab) was used for super shifting, and the C/EBPα complexes are indicated.

duced C/EBPα-stimulated transactivation nearly 46% compared with the construct with intact intron 1 coding sequence. These data suggest that the three C/EBPα responding elements form a core of the enhancer.

C/EBPα regulates adiponectin gene expression in adipocytes. To further assess the role of C/EBPα, C/EBPβ, and PPARγ in adiponectin gene expression in adipocytes, we overexpressed C/EBPα, C/EBPβ, or PPARγ in mature Chub-S7 (day 17 after induction of differentiation) or 3T3-L1CARΔ1 adipocytes (day 10 after induction of differentiation) or knocked down endogenous C/EBPα expression. As shown in Fig. 5*A*, 24h after transduction with adenovirus vectors, C/EBPα, C/EBPβ, or PPARγ protein levels were significantly increased. Studies have suggested that during the adipocyte differentiation process, expression of these transcription factors can induce or regulate one another (20). Interestingly, in this study, over expression of C/EBPα, C/EBPβ, or PPARγ did not affect levels of the other proteins, neither did thiazolidinedione (TZD) treatment (Fig. 5*A*). The differences in results relative to the earlier studies may be explained by the relatively long period of treatment in this study and the fact that that the cells used here are differentiated mature human adipocytes. Surprisingly, in adipocytes transduced with adenovirus encoding C/EBPβ, C/EBPδ expression was significantly increased ($P < 0.001$, Fig. 5*A*). Expression of siRNA directed against C/EBPα knocked down endogenous C/EBPα protein levels by 78% in Chub-S7 adipocytes ($P < 0.001$, Fig. 5*B*), without any significant morphological change or change of lipid droplets (data not shown). The C/EBPα siRNA did not alter PPARγ2 protein level but slightly increased C/EBPβ and C/EBPδ protein levels without statistical significance (Fig. 5*B*).

Northern blotting showed that adiponectin mRNA was significantly increased in Chub-S7 adipocytes overexpressing C/EBPα but not C/EBPβ (Fig. 5*C*). Overexpression of PPARγ2 with TZD treatment also significantly elevated adiponectin mRNA in human adipocytes (Fig. 5*C*). C/EBPα siRNA suppressed adiponectin mRNA nearly 70% ($P < 0.001$, Fig. 5*C*). As a control, we measured mRNA levels of the C/EBPα and PPARγ-responsive aP2 gene. aP2 mRNA was significantly increased when C/EBPα was overexpressed. aP2 mRNA levels were suppressed by C/EBPα siRNA treatment.

Similar to the results from Chub-S7 adipocytes, overexpression or knocking down of C/EBPα in 3T3-L1CARΔ1 adipocytes resulted in a significant increase or decrease of adiponectin mRNA levels, respectively (Fig. 5*D*). Interestingly, consistent with a previous observation (38), overexpression of PPARγ2 and TZD treatment only slightly increased adiponectin mRNA levels ($P > 0.05$) in the 3T3-L1CARΔ1 adipocytes but markedly elevated aP2 mRNA ($P < 0.05$, Fig. 5*D*). The mechanism for the differences in the response to PPARγ2 and TZD treatment between Chub-S7 and 3T3-L1CARΔ1 adipocytes is currently unknown.

C/EBPβ gene deletion does not alter adiponectin mRNA and serum protein level in mice. Four members of the C/EBP family are involved in adipocyte differentiation (20). To determine the role of C/EBPβ in regulating adiponectin expression, we measured adiponectin mRNA levels and serum protein from C/EBPβ$^{-/-}$, *db/db* (leptin receptor gene mutation) diabetic and wild-type control mice. Consistent with former studies, adiponectin mRNA and serum protein levels were significantly reduced in *db/db* mice (Fig. 6). Surprisingly, although there is a significantly low fat mass in C/EBPβ$^{-/-}$ mice (data not shown), there were no significant differences in adiponectin gene expression and serum protein levels compared with wild-type control (Fig. 6).

DISCUSSION

Two independent studies recently demonstrated that there is a modest level of adiponectin expression in C/EBPα-deficient adipocytes with ectopic expression of PPARγ, whereas expression of both PPARγ and C/EBPα led to production of abundant quantities of adiponectin (23,24). These studies suggest that C/EBPα and PPARγ are required to fully activate adiponectin gene expression. Our study indicates that the regulation of adiponectin gene transcription by C/EBPα and PPARγ is through different sites of the human adiponectin gene. C/EBPα increased adiponectin promoter activity only when the DNA encoding the first intron was present (Fig. 2*C*). In contrast, overexpression of PPARγ2 and its partner RXRα increased the activity of the adiponectin proximal promoter, and this was not enhanced in the presence of the DNA encoding the first intron (Fig. 2*B*). These data are consistent with the identification of a PPARγ response element within both the human and mouse adiponectin proximal promoter (21,22). Thus, our study demonstrates that C/EBPα regulates the human adiponectin gene transcription through the enhancer in the DNA encoding the first intron.

Available data indicate that the coding sequences and adiponectin protein structure are well conserved between

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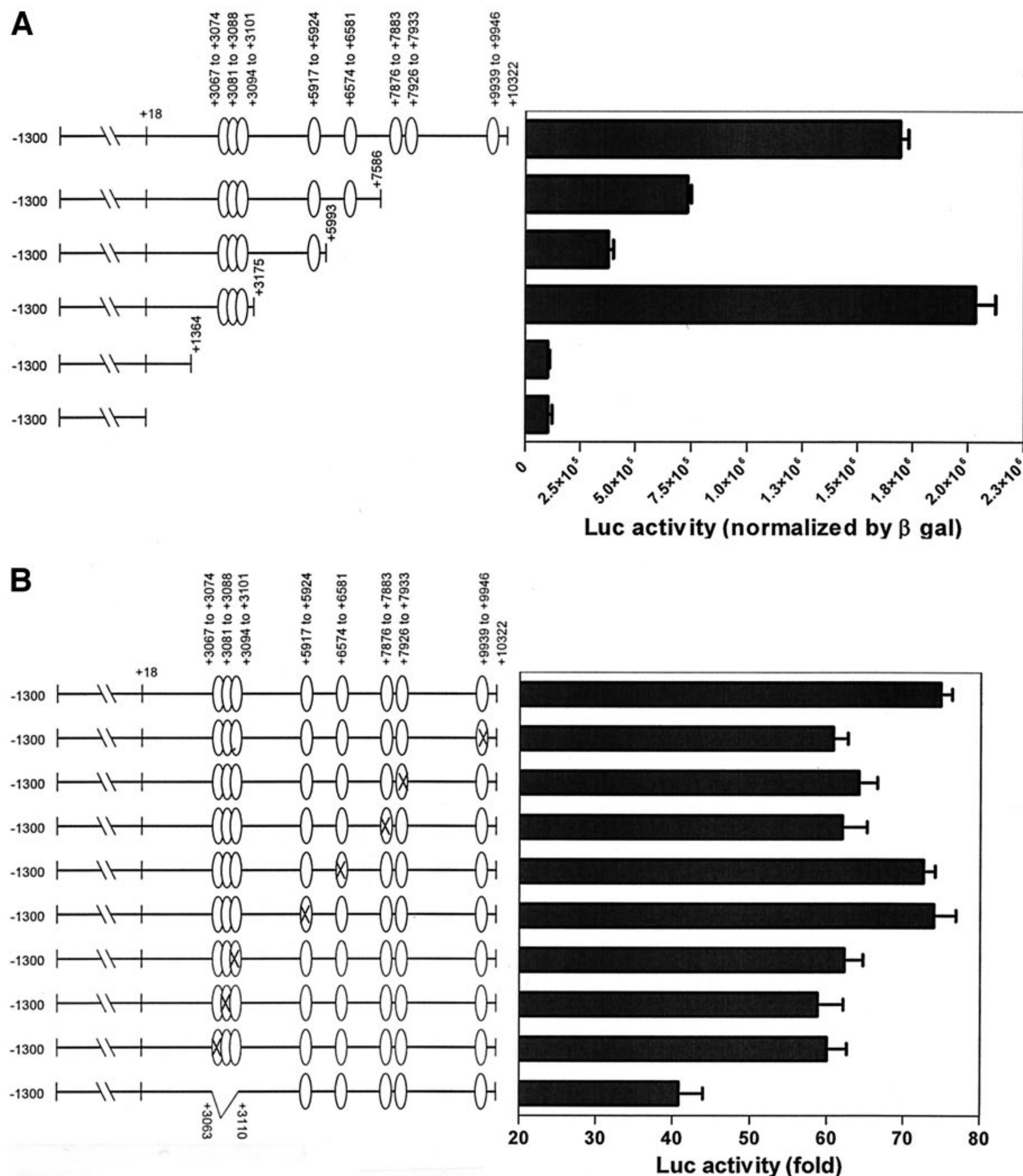

FIG. 4. C/EBPα transactivates the human adiponectin promoter through its consensus C/EBP sites in the DNA encoding the first intron. *A*: Reporter gene constructs with varying 3′ deletions of the intronic enhancer and the human adiponectin gene were prepared as illustrated (C/EBPα binding site were indicated by ovals). Constructs were cotransfected with the C/EBPα expression plasmid into HEK293 cells. After normalization of transfection efficiency using the activity of the pCMV–β-galactosidase plasmid, the relative luciferase activities of reporter constructs are shown as average values ± the SEs for four independent experiments. *B*: Reporter gene constructs with C/EBP consensus site point mutations (indicated by marked ovals) or deletion of three C/EBPα sites in the intronic enhancer were prepared. The constructs were cotransfected with pcDNA vector or pcDNA-hC/EBPα expression plasmid and pCMV–β-galactosidase into HEK293 cells. Luciferase activities were normalized with β-galactosidase activities for transfection efficiency, and the values expressed as fold activities relative to the control cotransfected with pcDNA vector.

human and mouse. However, alignment of the first intron (10,304 bp) of the human adiponectin gene with its mouse counterpart (8,584 bp) shows no significant homology. Consistent with a previous study (17), our study also showed that the presence of the DNA encoding the first intron of the mouse adiponectin gene did not increase reporter gene expression even in adipocytes (Fig. 1*D*). Thus, the regulatory elements in the first intron of the human adiponectin gene may not exist in mouse. However, the current study demonstrates that ectopic expression of C/EBPα indeed increases the adiponectin mRNA levels in both Chub-S7 and 3T3-L1 adipocytes, which are originally from mouse, suggesting that C/EBPα upregulates adiponectin gene expression in both human and mouse. It raises the possibility that C/EBPα upregulates adiponectin gene expression perhaps through a mecha-

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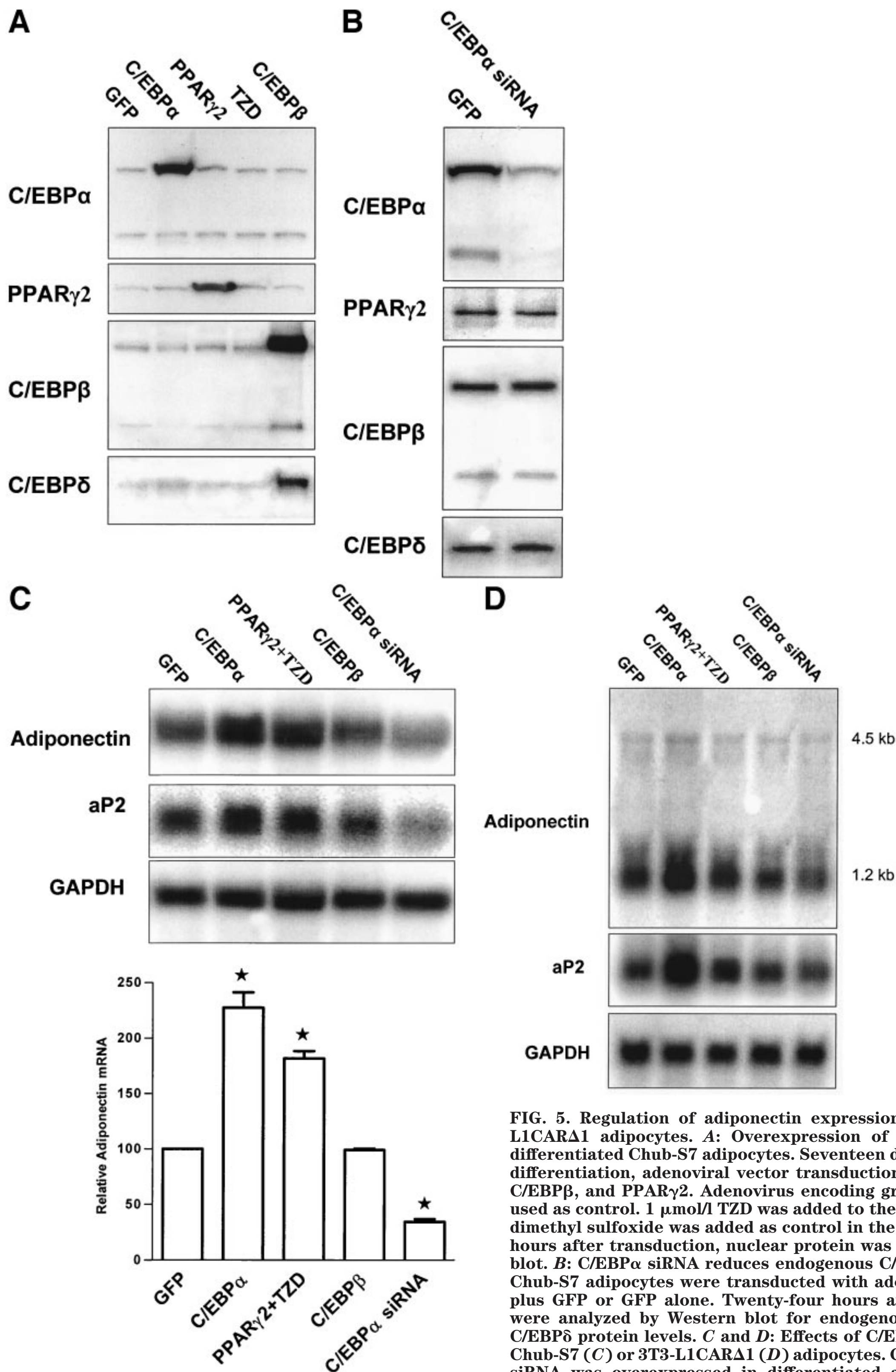

FIG. 5. Regulation of adiponectin expression by C/EBPα in Chub-S7 and 3T3-L1CARΔ1 adipocytes. *A*: Overexpression of C/EBPα, PPARγ2, and C/EBPβ in differentiated Chub-S7 adipocytes. Seventeen days after the induction of adipocyte differentiation, adenoviral vector transduction was used to overexpress C/EBPα, C/EBPβ, and PPARγ2. Adenovirus encoding green fluorescent protein (GFP) was used as control. 1 μmol/l TZD was added to the medium in one group of cells, while dimethyl sulfoxide was added as control in the medium of other cells. Twenty-four hours after transduction, nuclear protein was extracted and analyzed by Western blot. *B*: C/EBPα siRNA reduces endogenous C/EBPα protein levels. Differentiated Chub-S7 adipocytes were transducted with adenoviruses encoding C/EBPα siRNA plus GFP or GFP alone. Twenty-four hours after transduction, nuclear proteins were analyzed by Western blot for endogenous C/EBPα, PPARγ1, C/EBPβ, and C/EBPδ protein levels. *C* and *D*: Effects of C/EBPα on adiponectin mRNA levels in Chub-S7 (*C*) or 3T3-L1CARΔ1 (*D*) adipocytes. C/EBPα, C/EBPβ, PPARγ2 or C/EBPα siRNA was overexpressed in differentiated adipocytes using adenoviral vector transduction. 1 μmol/l TZD was added to the medium of the cells transduced by PPARγ2 encoding virus. Adiponectin and aP2 mRNA levels were measured by Northern blot 24-h after transduction and TZD treatment. As previously reported (17), there is a single adiponectin mRNA band of 4.5-kb in Chub-S7 human adipocytes (*C*), while two adiponectin mRNA bands of 1.2 and 4.5-kb are present in 3T3-L1CARΔ1 adipocytes (*D*). The bars of the lower panel of Fig. 5*C* depict the mean ± SE of at least four independent assays. ⋆$P < 0.05$ vs. cells transduced by GFP adenovirus.

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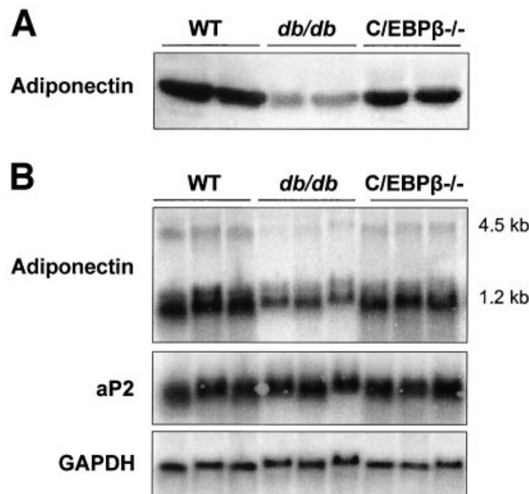

FIG. 6. Serum protein and mRNA profiles of adiponectin in white adipose tissue from wild-type, *db/db*, and C/EBPβ$^{-/-}$ mice. *A*: Serum proteins were separated using a 10% SDS-PAGE gel. After transferring the protein to a PVDF membrane, adiponectin were probed by anti-adiponectin antibody. *B*: Total mRNA was isolated from white adipose tissue and analyzed by Northern blot as described in RESEARCH DESIGN AND METHODS using [α-32P]-labeled probes.

nism that is conserved between human and mouse. Support for this hypothesis comes from a recent study demonstrating that both C/EBPα and C/EBPβ can bind to the mouse adiponectin proximal promoter and increase the promoter activity (39). This study also revealed significant sequence similarity for the C/EBP binding sites at the proximal promoter region between mouse and human (39). Our study demonstrated that cotransfection of C/EBPα significantly increased (12- to 16-fold) the human adiponectin promoter-driven luciferase expression. The vector we used for the luciferase gene reporter construct exhibits regulation by C/EBPα. Most likely, the C/EBPα-stimulated increase in luciferase activity (approximately twofold) (39) was obscured by the background vector response in our study. The discrepancy between our results and those of the previous study may also result from the use of different cell lines. Therefore, further studies are warranted to investigate whether C/EBPα binds on the proximal promoter region and regulates adiponectin gene transcription in humans. We also hypothesize that there might be an indirect pathway that mediates the regulatory effect of C/EBPα. The mechanistic difference between C/EBPα regulation of adiponectin gene expression in mice and humans is currently under investigation.

Because adiponectin is exclusively expressed in adipocytes, there must be a mechanism by which adipose tissue–specific transcription factors activate the gene in adipocytes. Although a PPARγ response element has been identified in the promoter of the human adiponectin gene and TZD treatment can increase the expression and secretion of adiponectin in both human subjects and *db/db* diabetic mice (21,38), the mechanism by which PPARγ activates adiponectin gene expression is not completely clear. Furthermore, the role by which PPARγ regulates adiponectin expression in an adipocyte-specific manner was not examined. The results from this study show that the presence of DNA encoding the first intron significantly increased the adiponectin promoter activity only in adipocytes (Fig. 1*C*), and the presence of this DNA fragment does not further increase the promoter activity in response to PPARγ2 (Fig. 2*B*). Thus, PPARγ is required but not totally responsible for adipocyte-specific activation of adiponectin gene transcription. It appears that there are elements in the DNA encoding the first intron of the human adiponectin gene responsible for adipocyte-specific expression (Fig. 1*C*). C/EBPα dramatically increased adiponectin promoter activity but in a manner dependent on the presence of the DNA encoding the first intron (Fig. 2*C*). Thus, C/EBPα, acting through the intronic enhancer, contributes to the tissue-specific expression of adiponectin. Although ectopic expression of either C/EBPα or PPARγ alone in preadipocytes can stimulate the adipogenic program, there are numerous lines of evidence to indicate that these two transcription factors act synergistically with one another (20,40,41). However, there is a subset of adipocyte-specific genes, including leptin and resistin, that rely on C/EBPα for expression (42,43). Furthermore, both leptin and resistin expression are not only dependent on C/EBPα, but are downregulated by liganded PPARγ (42,43).

C/EBPs are a family of leucine zipper transcription factors. During adipocyte differentiation, C/EBPβ and C/EBPδ increase transiently at the onset of adipocyte differentiation and decrease during terminal differentiation, but C/EBPα increases later and remains at a high level in mature adipocytes (20,44). Adiponectin expression parallels and occurs immediately after the rise of C/EBPα and is maintained at a relatively high level in mature adipocytes (2). However, C/EBPβ and C/EBPδ expression transiently increase at the beginning of adipocyte differentiation and drop rapidly after day 2 of differentiation (45). In contrast, C/EBPα and adiponectin expression are then activated, and protein levels are increased and maintained at a high level in the late stages of adipocyte differentiation and in differentiated mature adipocytes (2,45). Thus, during adipocyte differentiation, C/EBPβ and C/EBPδ protein levels correlate poorly with adiponectin expression. It is logical to speculate that C/EBPβ and C/EBPδ are not key regulators of adiponectin gene expression in differentiated mature adipocytes. Further support comes from the finding that there is a significant reduction of fat tissue mass in the C/EBPβ$^{-/-}$ mice relative to the wild-type mice (30), but there is no difference in serum adiponectin protein or adiponectin mRNA levels from the white adipose tissues (Fig. 6). However, in this study, the effects of ectopic expression of C/EBPβ and C/EBPδ were examined in differentiated mature adipocytes. Therefore, the current study cannot rule out roles for C/EBPβ or C/EBPδ in regulating adiponectin gene expression during differentiation. A recent study has reported that phosphorylation of C/EBPβ at a consensus ERK/GSK3 site is required for both C/EBPα and adiponectin gene expression during the differentiation of mouse fibroblasts into adipocytes (23).

In summary, this is the first report showing the DNA encoding the first intron of the human adiponectin gene acts as an enhancer in regulating adiponectin gene expression. The enhancer specifically responds to C/EBPα.

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