Temporal recruitment of CCAAT/enhancer-binding proteins to early and late adipogenic promoters *in vivo*

N Salma, H Xiao and A N Imbalzano

Department of Cell Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655, USA

(Requests for offprints should be addressed to A N Imbalzano; Email: anthony.imbalzano@umassmed.edu.)

Abstract

The CCAAT/enhancer-binding protein (C/EBP) family of transcriptional regulators is critically important for the activation of adipogenic genes during differentiation. The C/EBP β and δ isoforms are rapidly induced upon adipocyte differentiation and are responsible for activating the adipogenic regulators C/EBP α and peroxisome proliferator activated receptor (PPAR) γ 2, which together activate the majority of genes expressed in differentiating adipocytes. However, mitosis is required following the induction of adipogenesis, and the activation of C/EBP α and PPAR γ 2 gene expression is delayed until cell division is underway. Previous studies have used electromobility shift assays to suggest that this delay is due, at least in part, to a delay between the induction of C/EBP β protein levels and the acquisition of DNA binding capacity by C/EBP β . Here we used *in vivo* chromatin immunoprecipitation analysis of the C/EBP α , PPAR γ 2, resistin, adiponectin, and leptin promoters to examine the kinetics of C/EBP protein binding to adipogenic genes in differentiating cells. In contrast to prior studies, we determined that C/EBP β and δ were bound to endogenous regulatory sequences controlling the expression of these genes within 1–4 h of adipogenic induction. These results indicated that C/EBP β and δ bind not only to genes that are induced early in the adipogenic process but also to genes that are induced much later during differentiation, without a delay between induction of C/EBP protein levels and DNA binding by these proteins. We also showed that each of the genes examined undergoes a transition *in vivo* from early occupancy by C/EBP β and δ to occupancy by C/EBP α at times that correlate with the induction of C/EBP α protein levels, demonstrating the generality of the transition during adipogenesis and indicating that the binding of specific C/EBP isoforms does not correlate with timing of expression from each gene. We have concluded that C/EBP family members bind to adipogenic genes *in vivo* in a manner that follows the induction of C/EBP protein synthesis.

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Introduction

The major transcription factor families involved as key regulators of adipocyte differentiation include the nuclear hormone receptor peroxisome proliferator activated receptor (PPAR) γ and the CCAAT/enhancer-binding proteins (C/EBPs) (Darlington *et al.* 1998, Fajas *et al.* 1998, Lane *et al.* 1999, Morrison & Farmer 2000, Rangwala & Lazar 2000, Rosen *et al.* 2000, Camp *et al.* 2002, MacDougald & Mandrup 2002). The C/EBP family members belong to the basic leucine zipper (bZIP) class of transcription factors, and bind to specific DNA sequences as dimers with other C/EBPs (reviewed by Lékstrom-Himes & Xanthopoulos 1998, Ramji & Foka 2002). When adipocyte differentiation is induced in preadipocyte cell lines, C/EBP β and δ are rapidly and transiently induced (Cao *et al.* 1991, Yeh *et al.* 1995). These regulators act synergistically to modulate the expression of C/EBP α and PPAR γ 2 via interaction with the C/EBP regulatory elements present in the proximal promoters of these genes (Christy *et al.* 1991, Zhu *et al.* 1995, Clarke *et al.* 1997, Tang *et al.* 1999). Subsequently, C/EBP α and PPAR γ 2 play a prominent role regulating

the expression of adipocyte genes necessary for the development of functional, mature adipocytes (Lin & Lane 1994, Tontonoz *et al.* 1994, Fajas *et al.* 1998, Wu *et al.* 1999, Rosen *et al.* 2000).

The essential role of the C/EBP proteins in adipocyte differentiation has been established. Ectopic expression of C/EBP β or C/EBP α is able to force non-adipogenic cell lines to differentiate into adipocytes (Freytag *et al.* 1994, Wu *et al.* 1995, Yeh *et al.* 1995). In contrast, expression of antisense C/EBP α RNA in preadipocyte cell lines prevents the differentiation program (Lin & Lane 1992). Additionally, analysis of promoter regions of adipogenic genes as well as studies of knockout mice have demonstrated the involvement of this family of transcription factors in regulating adipogenesis and other important physiological processes (reviewed by Cornelius *et al.* 1994, MacDougald & Lane 1995, Tanaka *et al.* 1997, Darlington *et al.* 1998, Gregoire *et al.* 1998, Lane *et al.* 1999, Rangwala & Lazar 2000, Ramji & Foka 2002).

Most regulatory sequences controlling the expression of adipocyte-specific genes contain at least one functional C/EBP binding site, from which transactivation

is mediated by members of the C/EBP family (reviewed by Hwang *et al.* 1997, Gregoire *et al.* 1998, Cowherd *et al.* 1999, Morrison & Farmer 2000, Rangwala & Lazar 2000). Work on the PPAR γ 2 and C/EBP α promoters has focused on the role of C/EBP β and δ as primary inducers of the expression of key regulators. PPAR γ 2 and C/EBP α are expressed by day 2 of the differentiation process, following one or two rounds of mitotic clonal expansion. The induction of C/EBP β and δ protein levels, however, occurs almost immediately after addition of differentiation inducers at the onset of differentiation (Cao *et al.* 1991, Yeh *et al.* 1995, Darlington *et al.* 1998, Tang & Lane 1999). Thus, even though both C/EBP β and δ are expressed at high levels at the beginning of the differentiation program, the target genes C/EBP α and PPAR γ 2 are not expressed until nearly 2 days later (Lane *et al.* 1999, Rosen *et al.* 2000). Previous work using electrophoretic mobility shift assay (EMSA) analysis (Tang & Lane 1999) determined that the lag in C/EBP α expression is due to a delay in acquisition of C/EBP β and δ DNA binding activity, therefore pausing the transcriptional activation of the gene. The need for such a delay fits well with the numerous observations that C/EBP α is anti-mitotic in both preadipocytes as well other cell types (Umek *et al.* 1991, Lin *et al.* 1993, Timchenko *et al.* 1996, Wang *et al.* 2001).

The transcriptional activity of the C/EBP β protein is regulated at several levels, including transcription, translation, association with other proteins, and post-translational modification, which includes the regulation of the phosphorylated state by multiple effectors. Multiple phosphorylation sites have been characterized on C/EBP β , some of which result in attenuation or enhancement of DNA binding and transactivation activity (Mahoney *et al.* 1992, Trautwein *et al.* 1993, 1994, Park *et al.* 2004a, Tang *et al.* 2005 and references therein). At least some of these phosphorylation events occur almost simultaneously with induction of C/EBP β levels, and it has been suggested that phosphorylation causes a conformational change in C/EBP β that transforms it from a repressor to an activator (Kowenz-Leutz *et al.* 1994).

We previously examined the temporal interactions of C/EBP family members, modified histones, and subunits of the SWI/SNF family of ATP-dependent chromatin remodeling enzymes at the PPAR γ 2 promoter as a function of adipocyte differentiation (Salma *et al.* 2004). In that study, we used chromatin immunoprecipitation (ChIP) assays to determine that C/EBP β was bound at the PPAR γ 2 promoter at 24 and 48 h following the initiation of the differentiation program in both differentiating 3T3-L1 cells and in fibroblasts forced to differentiation into adipocytes by ectopic expression of C/EBP α (Salma *et al.* 2004). Continuation of these ChIP studies at earlier time-points revealed that the C/EBP β

and δ isoforms were found on the regulatory regions of numerous adipocyte-specific genes in differentiating 3T3-L1 cells within a few hours of the onset of differentiation. These genes included PPAR γ 2, C/EBP α , and several genes expressed later in the differentiation process. Thus the binding of C/EBP β and δ to adipogenic genes in cells correlates with the kinetics of C/EBP β and δ expression. In addition, binding of C/EBP β and δ was replaced at each of the loci by the binding of C/EBP α . C/EBP α binding was noted on each promoter between 20 and 48 h post-differentiation, indicating that it was associating with promoters as soon as it was expressed and that binding did not strictly correlate with the time at which the locus became transcriptionally active. We have concluded that the binding of C/EBP transcription factors to regulatory sequences controlling the expression of adipogenic genes *in vivo* occurs rapidly and without significant delay following the induction of each isoform during adipogenic differentiation.

Materials and methods

Cell lines and differentiation methods

3T3-L1 preadipocytes were purchased from the American Type Culture Collection (Manassas, VA, USA), maintained in growth medium consisting of Dulbecco's minimum essential medium containing 10% calf serum, and induced to differentiate as described previously (Wu *et al.* 1995). Cells were collected at 0, 1, 2, 4, 8, 12, 16, 20, and 24 h and then at 24-h intervals for 7 days after addition of differentiation cocktail (Salma *et al.* 2004) for western blot, RT-PCR, and ChIP analysis. In experiments where 3T3-L1 cells overexpressed C/EBP β , cells were infected with pBABE retrovirus containing C/EBP β or empty vector as described previously (Tontonoz *et al.* 1994, Salma *et al.* 2004). The generation of the retrovirus has been described previously (Pear *et al.* 1993, Salma *et al.* 2004). Samples were collected at 0, 4, 24, 48, and 168 h in the presence or absence of differentiation cocktail for western blots, RT-PCR, and ChIP analysis.

EMSAs

Nuclear extracts isolated from 3T3-L1 cells differentiated in the presence or absence of cocktail were prepared as described (Hasegawa *et al.* 1997). The binding reaction contained 6 μ g nuclear extract and 5 fmol ^{32} P-labeled double-stranded oligonucleotide probe corresponding to the region from -343 to -306 bp from the mRNA start site in the mouse PPAR γ 2 promoter. This region contains a functional C/EBP binding site (Zhu *et al.* 1995, Clarke *et al.* 1997). Binding reactions contained 10 mM Tris-HCl (pH 7.5), 5%

glycerol, 50 mM NaCl, 0.5 mM dithiothreitol, 1 mM MgCl₂, and 0.5 mg/ml Poly (dI-dC) in a volume of 10 µl. Reactions were incubated for 30 min at room temperature and separated electrophoretically on 4% non-denaturing polyacrylamide gels made with 0.5 × Tris–borate–EDTA buffer. Some reactions were preincubated for 10 min with 1 µl IgG or anti-C/EBPβ antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; sc-7962) prior to addition of the labeled oligonucleotides. The sequence of the oligonucleotide probe was: 5'-TAAAAAGCAATCAATATTGAACAA TCTCTGCTCTGGTAA-3'.

RNA analysis

RNA isolation and analysis by northern blotting have been described previously (de la Serna *et al.* 2001b). Probes were derived from plasmids containing PPARγ (provided by B Spiegelman, Dana Farber Cancer Institute, Boston, MA, USA) and the ribosomal phosphoprotein 36B4 (obtained by RT-PCR) and labeled by random priming. Washed blots were exposed to a PhosphorImager (GE Healthcare Chalfont St. Giles, UK). For RT-PCR, total RNA (3 µg) was reverse transcribed with Moloney murine leukemia virus RT (Invitrogen). cDNA was amplified by PCR with QIAGEN HotStart Taq master mix in the presence of 2 µCi [α -³²P]dATP. The sequences of the primers were as follows: 5'-CCG GCC GCC TTC AAC GAC-3' and 5'-CTC CTC GCG GGG CTC TTG TTT-3' for C/EBPα (288 bp product); 5'-GAA CTG AGT TGT GTC CTG CT-3' and 5'-TGC ACA CTG GCA GTG ACA-3' for resistin (340 bp product); 5'-GAT CAA TGA CAT TTC ACA CA-3' and 5'-GGA CGC CAT CCA GGC TCT CT-3' for leptin (281 bp product); 5'-CAG TGG ATC TGA CGA CAC CA-3' and 5'-CGA ATG GGT ACA TTG GGA AC-3' for adiponectin (433 bp product); and 5'-CTC CAA GCA GAT GCA GCA GA-3' and 5'-TCA ATG GTG CCT CTG GAG AT-3' for the ribosomal phosphoprotein 36B4 (351 bp). The PCR conditions for leptin and 36B4 were: 95 °C for 15 min, followed by 24 cycles of: 95 °C for 30 s; 62 °C for 40 s; 72 °C for 30 s, and a final round of extension for 5 min. The PCR conditions for adiponectin were the same, except that the number of cycles was 20. For resistin, the conditions were the same as for leptin except that the annealing temperature was 58 °C. For C/EBPα, the PCR conditions were: 95 °C for 15 min, followed by 25 cycles of: 95 °C for 50 s; 66 °C for 55 s; 72 °C for 50 s, and a final round of extension for 5 min.

Protein extracts and Western analysis

Isolation of protein and western blotting have been described (de la Serna *et al.* 2001a). Antibodies utilized

included the following from Santa Cruz Biotechnology: C/EBPα (sc-61), C/EBPβ (sc-7962), C/EBPδ (sc-151), and cyclin A (sc-596). Phosphatidylinositol 3-kinase (PI-3K) antibody (06-496) was obtained from Upstate (Charlottesville, VA, USA).

ChIP

The ChIP procedure was adopted from the Upstate protocol and was performed as described by Salma *et al.* (2004). One-tenth of the immunoprecipitated DNA and 1% of the input DNA were analyzed by PCR. Antibodies used included Santa Cruz antibodies: C/EBPα (sc-61), C/EBPβ (sc-7962), and C/EBPδ (sc-151). PCRs were performed with QIAGEN Hot Start Taq master mix in the presence of 2 µCi [α -³²P]dATP under the following conditions: a pre-heating at 94 °C for 15 min, followed by 24–30 cycles of 94 °C for 30 s, 62 °C for 40 s (except for PPARγ2 which was 49.5 °C), 72 °C for 30 s, followed by a 72 °C extension for 5 min. PCR products were resolved in 6–8% polyacrylamide–1 × Tris–borate–EDTA gels, dried, and exposed to a PhosphorImager. Primers used were the following: β-actin, 5'(+31) GCTTCTTTGCA GCTCCTTCGTTG-3' and 5' (+135) TTTGCACAT GCCGGAGCCGTTGT-3' (Rayman *et al.* 2002); PPARγ2 promoter, 5' (–413) TACGTTTATCGGT GTTTCAT-3' and 5' (–247) TCTCGCCAGTGA CCC-3'; upstream region of the PPARγ2 promoter, 5' (–1871) GGGCGTTAAAACACAATCCT-3' and 5' (–1707) TCTCTTCCTCCTTCCCTTCC-3'; C/EBPα promoter, 5' (–315) TGACTTAGAGGCTTAAA GGA-3' and 5' (–32) CGGGGACCGCTTTTATAG AG-3'; resistin promoter, 5' (–177) CACCATGGTC CCTGGTGTTA-3' and 5' (+26) CTCAGTTCTGGG TATTAGCTC-3'; adiponectin promoter, 5' (–272) ATTGTCCTTACCCTTGCCCC-3' and 5' (–15); and leptin promoter, 5' (–323) GCCTTCTGTAGCC TCTTGCT-3' and 5' (–22) GCTCCATGCCCTG CCTGC-3'. Representative experiments from at least three independent experiments are shown.

Results

In vitro binding of C/EBPβ at a C/EBP site in the PPARγ2 promoter occurs as early as 3 h post-differentiation

While investigating the role of C/EBP isoforms in the activation of adipogenic genes, we examined *in vitro* binding of C/EBPβ at a C/EBP regulatory element present in the PPARγ2 promoter by EMSA during a short time-course of 3T3-L1 preadipocytes induced to differentiate into adipocytes. We found that C/EBPβ present in nuclear extracts prepared from cells differentiated for 3, 18, and 24 h was able to bind to a

Figure 1 *In vitro* binding of C/EBP β to a C/EBP site in the PPAR γ 2 promoter occurs as early as 3 h following the induction of 3T3-L1 adipocyte differentiation. EMSA was performed using nuclear extracts prepared from preadipocytes (day 0) or from differentiating preadipocytes at 1, 3, 18, and 24 h after the addition of the differentiation cocktail (lanes 1–5). The double-stranded, ^{32} P end-labeled oligonucleotide probe encoded the C/EBP binding site between –343 and –306 relative to the start site of PPAR γ 2 transcription. Supershift experiments were performed by adding purified IgG from pre-immune serum (lane 6) or antibodies against C/EBP β (lane 7).

C/EBP site in the PPAR γ 2 promoter (Fig. 1, lanes 3–5). Confirmation that the shifted band in the EMSA was C/EBP β was demonstrated by the appearance of a supershifted band upon addition of C/EBP β antibody to the reaction while addition of purified IgG had no effect (Fig. 1, lanes 6 and 7). The results revealed that the C/EBP β regulator has the capacity to bind to DNA as early as 3 h following the induction of differentiation. In addition, we observed that the apparent level of C/EBP β binding under the reaction conditions used did not increase between 3 and 24 h post-differentiation (Fig. 1, lanes 3–5).

These results diverged somewhat from data published previously where the *in vitro* binding activity of C/EBP β was delayed until 12–16 h following the stimulation of adipogenic differentiation (Tang & Lane 1999). We note that the previous report demonstrated that C/EBP β binding could be observed at 4 h post-differentiation but that binding was significantly induced at the 12–16 h time-points. This dissimilarity in results is possibly due to differences in reaction conditions, or perhaps to the use of different oligonucleotide probes. In the previous study (Tang & Lane 1999), EMSA was performed using a C/EBP site from the C/EBP α promoter, while our

experiment utilized a probe that contained a C/EBP binding site from the PPAR γ 2 promoter. Nevertheless, the results presented in Fig. 1 raised a question about the timing of C/EBP β binding to adipogenic regulatory sequences. To better address this issue, we decided to perform ChIP experiments at different times following the induction of adipocyte differentiation in 3T3-L1 cells in order to examine the binding of C/EBP isoforms *in vivo*.

Kinetics of C/EBP expression in differentiating 3T3-L1

Since the EMSA data presented in Fig. 1 indicated a potential difference from previously published studies, we first performed a series of control experiments to analyze the expression levels of C/EBP β , δ , and α during adipocyte differentiation of 3T3-L1 cells by western blot in order to eliminate the possibility that our results might be due to differences in the experimental handling of the differentiating 3T3-L1 cells. It has been well established that initiating differentiation of 3T3-L1 preadipocytes activates a cascade of gene expression events. Among the initial events are the rapid induction of C/EBP β and δ , which are stimulated by components of the differentiation cocktail, followed by the induction of C/EBP α on the second day of differentiation (Yeh *et al.* 1995, Wu *et al.* 1996). Western blot analyses corroborated that induced protein levels of C/EBP β and δ were detectable at 1 h, reached a maximum at 4 h, and were declining at 48 and 120 h respectively (Fig. 2). Expression of C/EBP α occurred later; significant induction began at about 48 h post-differentiation and expression was maintained throughout the time-course (Fig. 2).

To corroborate that the cells began the mitotic expansion phase, we performed a western blot with antibodies directed to cyclin A (Fig. 2). As was expected, cyclin A levels were increased between 16–24 h post-differentiation, indicating that the cells had entered the cell cycle prior to 16 h and had exited by approximately 48 h. All of the data presented in Fig. 2 indicate that the differentiation of the 3T3-L1 cells occurred as expected and confirm previously published results (Morrison & Farmer 1999, Tang *et al.* 2003a,b). Thus we have demonstrated the integrity of the 3T3-L1 cells and the differentiation protocol used for this and subsequent experiments.

In vivo recruitment of C/EBP β , δ , and α to the PPAR γ 2 and C/EBP α promoters during adipogenesis

The results presented in Fig. 1 show that C/EBP β was able to bind to C/EBP binding sites on the PPAR γ 2 promoter at times that correlated with the induction of C/EBP β levels. To determine whether binding occurs

Figure 2 Western blot analysis of transcriptional and cell cycle regulators during differentiation of 3T3-L1 cells. Protein extracts were prepared from differentiating cells at the indicated times and the kinetics of protein expression for the indicated proteins were determined by western blot analysis. PI-3K was used as a loading control.

on endogenous adipocyte promoters at early times after the induction of adipocyte differentiation, we performed ChIP experiments and temporally analyzed binding, not only of C/EBPβ, but also of C/EBPδ and C/EBPα, to specific adipocyte promoters. First, we chose to evaluate binding at the C/EBPα and the PPARγ2 promoters, since these two essential adipogenic regulators are expressed early during differentiation, on day 2 (Fig. 3A and B).

Regulation of the C/EBPα and the PPARγ2 promoters by C/EBP family members has been characterized previously (Christy *et al.* 1991, Zhu *et al.* 1995, Clarke *et al.* 1997, Tang & Lane 1999, Tang *et al.* 1999, 2004, Elberg *et al.* 2000, Yang & Chow 2003). The C/EBPα proximal promoter contains a C/EBP regulatory element at -187 relative to the transcriptional start site that mediates transactivation by C/EBPs. The PPARγ2 promoter contains two previously characterized C/EBP recognition elements at -340 bp and -327 bp relative to the transcriptional start site in addition to other potential sites that diverge from the C/EBP consensus. As shown in Fig. 3A, recruitment of C/EBPβ as well as C/EBPδ at the C/EBP regulatory element on the PPARγ2 promoter was induced in a manner consistent with the protein expression patterns of both proteins (Fig. 2), with binding of both proteins apparent 2 h after the onset of differentiation. Subsequently, C/EBPβ and C/EBPδ were replaced by C/EBPα, which initiated binding to the promoter at 48 h and was maintained throughout the time-course. These results confirmed the transition of binding from C/EBPβ/δ to C/EBPα at this promoter *in vivo* (Salma *et al.* 2004, Tang *et al.* 2004).

Analysis of the C/EBPα promoter revealed the same pattern found at the PPARγ2 promoter, except that binding occurred even earlier following differentiation (Fig. 3B). C/EBPβ and C/EBPδ were bound as early as 1 h post-differentiation and remained present until 48 h

and 120 h respectively. Induction of C/EBPα binding to the C/EBPα promoter began at 48 h as reported previously (Tang *et al.* 2004). Therefore, the transition in binding of the C/EBP isoforms that was observed at the PPARγ2 promoter also occurred at the C/EBPα promoter.

The appearance and disappearance of binding of the different C/EBP isoforms at the PPARγ2 and C/EBPα promoters at different times post-differentiation indicate specificity of binding. No antibody controls provide further evidence for specific binding (Fig. 3A and B). As an additional control, we analyzed C/EBP factor interactions at the β-actin locus (Fig. 3C) and at sequences 1.8 kb upstream of the PPARγ2 start site (data not shown). Neither β-actin sequences nor sequences upstream of the PPARγ2 promoter were immunoprecipitated by any of the antibodies used in the ChIP procedure.

***In vivo* recruitment of C/EBPβ, δ, and α to additional adipogenic gene promoters during adipogenesis**

We next examined if C/EBPβ, δ, and α are recruited to the resistin, adiponectin, and leptin promoters. These adipocyte-secreted peptides, collectively referred to as adipocytokines, have generated considerable interest since they are important regulators of body mass and their misregulation may play a role in obesity (Miner 2004). The proximal promoters of these genes contain C/EBP binding sites that are necessary for expression. The resistin promoter contains a C/EBP site at -56 relative to the transcriptional start site, adiponectin contains two identified C/EBP sites at -775 and -264, and two potential binding sites at -117 and -73, and leptin has three consensus C/EBP binding sites at nucleotides -55, -211, and -292. The activity of these C/EBP sites has been confirmed by reporter assays

for each of these genes (de la Brousse *et al.* 1996, Hwang *et al.* 1996, Hartman *et al.* 2002, Park *et al.* 2004b).

Binding of C/EBP β and δ to the C/EBP site at the resistin proximal promoter was evident at 2–4 h post-differentiation and did not decline until after 48 h (Fig. 4A). A modest increase in binding of C/EBP α was observed from 20 to 48 h, and was further increased at 120 h (Fig. 4A). These results indicate that C/EBP β and δ bind early after differentiation to the resistin promoter and suggest that all three C/EBP isoforms play a role regulating this adipocyte gene. It was previously demonstrated that C/EBP α binds specifically to the C/EBP element on the resistin promoter that is essential for expression (Hartman *et al.* 2002); however, a transition in binding of these regulators has not been previously demonstrated.

The adiponectin promoter contains two C/EBP sites at –775 and –264 and two potential binding sites at –117 and –73; however, the C/EBP element at –264 and the potential C/EBP sites at –117 and –73 confer promoter activity as defined in transient promoter studies, EMSA, DNase I footprinting, and ChIP assays (Park *et al.* 2004b, Seo *et al.* 2004). Consequently, we performed ChIPs in the region of the proximal promoter that contains these C/EBP sites. The analysis of binding of C/EBP β , C/EBP δ , and C/EBP α to the adiponectin promoter showed a pattern nearly identical to that observed for the resistin promoter, despite the fact that adiponectin expression initiated later than resistin expression (Fig. 4B). C/EBP β and δ were bound to the promoter at 2 h and maintained until 48 h. Definitive binding of C/EBP α was present at 48 h, although a modest increase was noticed at 20–24 h.

Finally, we assessed the recruitment of the C/EBP members on the leptin promoter. The proximal promoter contains three consensus C/EBP binding sites. DNase I footprint analysis, reporter gene assays, and EMSA studies have demonstrated that one of these C/EBP sites, located at –53 relative to the transcriptional start site, is functional (de la Brousse *et al.* 1996, Hwang *et al.* 1996, Mason *et al.* 1998). However, because the C/EBP sites are near each other, we designed PCR primers to amplify a region of the proximal promoter containing all three sites. The recruitment of C/EBP β occurred at 4 h and remained relatively constant until 48 h (Fig. 4C). In contrast, C/EBP δ was detectable from 12 h to 48 h. Binding of C/EBP α was observed on the

promoter at 20 h, but was not robust until 120 h post-differentiation, which coincides with the start of leptin mRNA accumulation. These results indicate that C/EBP β and δ bind quickly after the induction of differentiation to adipogenic promoters that are not expressed until much later in the differentiation process. Furthermore, the transition from C/EBP β and δ to C/EBP α binding on these promoters also occurred prior to gene expression, suggesting that C/EBP factor interactions with adipogenic promoters is independent of the time at which gene expression is initiated.

Overexpression of C/EBP β is not sufficient to promote C/EBP protein binding to the PPAR γ 2 promoter in differentiating 3T3-L1 cells

The detection of C/EBP β on adipogenic promoters *in vivo* within a few hours of the onset of adipogenic stimulation caused us to evaluate whether overexpression of C/EBP β in 3T3-L1 preadipocytes would be sufficient to induce binding of C/EBP β to the regulatory sequences examined. 3T3-L1 preadipocytes were infected with a retroviral vector expressing C/EBP β and were allowed to reach confluence, but no differentiation cocktail was added. Instead, cells were maintained in 10% calf serum as a confluent plate, and C/EBP binding was assessed at 0, 4, 24, 48, and 168 h. Despite the ectopic expression of C/EBP β (Fig. 5A), no binding of C/EBP β , δ , or α was observed at the PPAR γ 2 promoter, whereas control plates treated with differentiation cocktail showed C/EBP β and δ binding to the PPAR γ 2 promoter within 4 h post-differentiation and C/EBP α binding by 48 h post-differentiation (Fig. 5B).

The data suggested that overexpression of C/EBP β in cells is not sufficient to promote C/EBP β binding to adipogenic promoters in the absence of differentiation cocktail. A potential caveat to this conclusion is that ectopic expression did not provide a high enough level of C/EBP β protein to surpass a threshold level of C/EBP β protein necessary to achieve binding. We note that the inoculum of pBABE-C/EBP β retrovirus used in these experiments is the same as we have previously used to trans-differentiate fibroblast lines into adipocyte-like cells; thus the levels of C/EBP β provided are sufficient to reprogram cells of a different lineage (Salma *et al.* 2004). However, to more directly address this concern, we compared the levels of C/EBP β protein present in

Figure 3 ChIP assays reveal that C/EBP β binding to the PPAR γ 2 and C/EBP α promoters occurs *in vivo* within 2 h of the induction of 3T3-L1 adipocyte differentiation. ChIP assays were performed at the indicated time-points using the indicated antibodies and amplified for the PPAR γ 2 promoter (A, top panel), the C/EBP α promoter (B, top panel), or the β -actin 5' untranslated region and coding region (C); 1% of each input is shown. A twofold titration (number of μ l indicated) of input from the 0 h sample is shown on the left to demonstrate linearity of the PCR reactions. (A, bottom panel) Duplicate samples were used to prepare RNA and a Northern blot showing the levels of PPAR γ and 36B4 mRNA at each time-point is shown. (B, bottom panel) C/EBP α and 36B4 mRNA levels were determined by RT-PCR. A twofold titration (number of μ l indicated) of input reverse transcribed RNA from the day 7 sample is shown on the left to demonstrate linearity of the PCR reactions. Ab, antibody.

Figure 5 Overexpression of C/EBPβ in undifferentiated 3T3-L1 preadipocytes is not sufficient to induce the binding of C/EBP factors at the PPARγ2 promoter. Subconfluent 3T3-L1 preadipocytes were infected with either pBABE retrovirus or pBABE retrovirus encoding C/EBPβ. For differentiation, cells were placed in fresh media lacking or containing the differentiation cocktail, and samples were harvested at the indicated times for (A and C) Western blot analysis or (B and D) ChIP analysis. Ab, antibody.

uninfected cells and in cells infected with pBABE-C/EBPβ or with the pBABE empty retrovirus that were differentiated in the presence or absence of differentiation cocktail for 4 h. A western blot (Fig. 5C) demonstrates that cells infected with the C/EBPβ virus contained greater levels of C/EBPβ protein in both the presence and absence of differentiation cocktail (compare lane 2 with lane 3 and lane 5 with lane 6), as expected. We also observed that C/EBPβ levels in vector-infected cells that were differentiated in the presence of cocktail were lower than C/EBPβ levels in the pBABE-C/EBPβ-infected cells that were differentiated in the absence of cocktail (compare lanes 4 and 3). The levels of C/EBPβ protein in pBABE-infected cells treated with differentiation cocktail were sufficient to permit C/EBPβ binding to the PPARγ2 promoter, whereas higher levels of C/EBPβ in the C/EBPβ-infected cells differentiated in the absence of cocktail were not (Fig. 5D, compare lanes 4 and 3). The results exclude the possibility that insufficient levels of C/EBPβ were present in the cells not treated with differentiation cocktail. The C/EBPβ protein undergoes a number of post-translational modifications that are associated with

the induction of adipogenesis; it is likely that such modifications are induced by addition of the differentiation cocktail and are necessary to promote rapid binding to adipogenic gene regulatory sequences *in vivo*. Thus, simple overexpression of C/EBPβ is not sufficient to induce C/EBPβ binding in 3T3-L1 preadipocytes.

Discussion

The C/EBP family of transcription factors is widely expressed and is a key regulator of a variety of target genes important in physiological events, including energy metabolism, inflammation, hematopoiesis, cellular proliferation, and differentiation (Darlington *et al.* 1998, Lekstrom-Himes & Xanthopoulos 1998, Rosen *et al.* 2000, Ramji & Foka 2002, Kovacs *et al.* 2003). Of note is the essential role C/EBP family members play during adipogenesis (reviewed by Darlington *et al.* 1998, Lane *et al.* 1999). Almost immediately upon induction of adipogenesis, the C/EBP family members β and δ are induced in a manner dependent on several signal transduction cascades that result in phosphorylation of

Figure 4 ChIP assays show that C/EBPβ binding to the adipocytokine promoters occurs *in vivo* within 4 h of the induction of 3T3-L1 adipocyte differentiation. ChIP assays were performed at the indicated time-points using the indicated antibodies and amplified for the resistin promoter (A, top panel), the adiponectin promoter (B, top panel), or the leptin promoter (C, bottom panel); 1% of each input is shown. A twofold titration (number of μl indicated) of input from the day 0 sample is shown on the left to demonstrate linearity of the PCRs. Duplicate samples were used to prepare RNA at each time-point and mRNA levels for resistin (A, bottom panel), adiponectin (B, bottom panel), and leptin (C, bottom panel) as determined by RT-PCR are shown. 36B4 mRNA levels are shown as a control. A twofold titration (number of μl indicated) of input reverse transcribed RNA from the day 7 sample is shown on the left to demonstrate linearity of the PCRs. Ab, antibody.

these proteins (Park *et al.* 2004a, Bezy *et al.* 2005, Tang *et al.* 2005 and references therein). However, the activation of the early adipogenic regulators, PPAR γ 2 and C/EBP α , which are dependent on C/EBP β and δ , does not occur until day 2 of the differentiation process. During the first 2 days, cells undergo one or two rounds of mitosis, a process termed mitotic clonal expansion (Bernlohr *et al.* 1985, Cornelius *et al.* 1994, MacDougald & Lane 1995). Thus, the transcriptional activation potential of the C/EBP β and δ proteins are masked or repressed until clonal expansion commences.

Over the past several years data have accumulated that suggest that the binding capacity of C/EBP β for its cognate binding site is delayed 12–20 h post-induction of adipocyte differentiation (Lane *et al.* 1999, Tang & Lane 1999, Tang *et al.* 2003b, 2005). This observation fits well with the need to delay expression of C/EBP α , which has anti-mitotic properties (Umek *et al.* 1991, Lin *et al.* 1993, Timchenko *et al.* 1996, Wang *et al.* 2001), until clonal expansion occurs. Moreover, the kinetics of DNA binding activation fit well with the kinetics of other events that occur at this time, including the appearance of phosphorylated Rb and the localization of C/EBP β to pericentric heterochromatin, which contains numerous C/EBP binding sites in the satellite DNA sequences (Tang & Lane 1999). How this change in sub-nuclear distribution relates to gene expression has not been well established, but the observation raises the possibility that localization of proteins to specific nuclear compartments contributes to the complexity of adipocyte gene regulation.

In the course of examining the activation of the PPAR γ 2 promoter during adipogenesis, we noted that *in vitro* binding of C/EBP β to a C/EBP binding site in the PPAR γ 2 promoter did not appear to change significantly between 3 and 24 h post-differentiation (Fig. 1). Given the wide range of variable conditions that can affect protein binding in a gel shift assay, we initially did not view this as contradictory to the existing models explaining the delay in activation of C/EBP α and PPAR γ 2 gene expression. However, ChIP assays, which specifically detect *in vivo* protein:DNA interactions at endogenous loci, clearly demonstrated that C/EBP β and C/EBP δ were capable of binding to both the C/EBP α and the PPAR γ 2 promoter at very early times post-differentiation. Moreover, C/EBP β and δ could also bind at early times to adipocyte specific promoters that do not begin to transcribe until much later in the differentiation process. Our results do not alter the original conclusion that mechanisms exist during the time of mitotic clonal expansion to delay activation of C/EBP α and PPAR γ 2 gene expression and the target genes that they subsequently activate. Instead, they indicate that the rate-limiting step is not the interaction of the C/EBP β protein with binding sites at the endogenous target gene promoters.

Numerous possibilities to restrict the transcriptional activating properties of C/EBP β exist even if the protein is DNA bound. The exact isoform of C/EBP β that is bound could influence the transcriptional potential. Interaction between C/EBP β and repressor proteins (Ron & Habener 1992, Tang *et al.* 1999) would not necessarily be restricted to solution interactions; repression could occur via interactions at promoter sequences as shown previously (Mo *et al.* 2004). DNA-bound C/EBP β could be and likely is still subject to post-translational modifications, including phosphorylation, acetylation, and sumoylation (Kim *et al.* 2002, Eaton & Sealy 2003, Xu *et al.* 2003, Park *et al.* 2004a, Tang *et al.* 2005), that may modulate transcriptional capacity. C/EBP β -bound loci could remain transcriptionally silent because other activators, RNA polymerase II (pol II)- or pol II-associated general transcription factors have not been synthesized, are spatially restricted, have not yet undergone the appropriate post-translational modification, or cannot bind in the absence of specific chromatin modifications or alterations. In support of this last possibility, Wiper-Bergeron *et al.* (2003) showed that histone deacetylase1 (HDAC1) could affect the acetylation status of the C/EBP α promoter in a manner regulated by glucocorticoids. Finally, C/EBP β -bound loci may change position within the nucleus, becoming associated or disassociated with specific sub-nuclear structures such as pericentric heterochromatin or splicing domains. None of these possibilities are mutually exclusive, and it is likely that multiple mechanisms are acting cooperatively to control the timing of expression for each individual target gene.

We also note that, in other cell types, C/EBP β -mediated activation of endogenous target genes can occur without induction of C/EBP β levels or induction of post-translational modifications. Lipopolysaccharide-mediated induction of C/EBP β target genes in B cell-derived lines lacking C/EBP proteins could be accomplished by constitutive expression of the bZIP domain of C/EBP β (Hu *et al.* 2000). More recently, C/EBP β binding to and subsequent activation of target genes in lipopolysaccharide-stimulated macrophages were shown to occur prior to induction of C/EBP β protein levels and in the absence of induced C/EBP β phosphorylation or nuclear translocation (Bradley *et al.* 2003). Thus, different mechanisms likely control C/EBP β function in different cell types.

The *in vivo* binding capacity of C/EBP β and δ to adipogenic genes as early as 1 h post-differentiation seems likely to be modulated by components of the differentiation cocktail. ChIP assays performed with 3T3-L1 over-expressing C/EBP β in the absence of differentiation inducers showed that this regulator is not recruited to the PPAR γ 2 (Fig. 5). This result shows that the physical presence of C/EBP β alone is insufficient to

be recruited to specific promoters. Further studies will be required to determine the nature of modifications required for both C/EBP β and δ to rapidly bind to adipogenic regulatory sequences following differentiation signaling as well as the relative importance of C/EBP β homodimers and C/EBP β and δ heterodimers.

Limited data on the binding of C/EBP β and δ to gene promoters other than C/EBP α and PPAR γ 2 exist. In differentiating 3T3-L1 cells, C/EBP β binds to the aP2 promoter between 24–72 h post-differentiation (Tang *et al.* 2004), and a recent report shows both C/EBP β and δ on the adiponectin promoter in mouse adipose tissue (Park *et al.* 2004b). These data raise the question of why C/EBP β and δ are bound to such promoters when evidence strongly suggests that the genes are not activated until later times when C/EBP α has replaced C/EBP β and δ at these loci. We speculate that the presence of C/EBP β may be part of the process by which C/EBP α is recruited to adipogenic promoters. Alternatively, or perhaps, additionally, all of the adipogenic loci undergo structural changes at the onset of differentiation, and the binding of C/EBP β to these loci serves as a mark for subsequent gene activation.

The data have also clearly demonstrated that the C/EBP binding sites on each of the genes undergo a transition in binding from C/EBP β and δ to C/EBP α *in vivo*. We and others have previously demonstrated that this occurred on the PPAR γ 2 promoter during adipogenesis (Salma *et al.* 2004, Tang *et al.* 2004). From the data presented here, we predict that this transition occurs on all adipocyte genes containing C/EBP binding sites with similar kinetics, indicating the importance of the transition and a role for C/EBP β and δ in both early and late events of the differentiation program. We note that the binding of C/EBP β and δ correlated with the induction of overall cellular levels of C/EBP β and δ . Similarly, C/EBP β and δ were replaced on each promoter between 24 and 48 h post-differentiation, the time at which C/EBP α protein levels begin to be induced. Thus the binding of the specific C/EBP proteins to adipocyte specific genes *in vivo* does not correlate with the time of target gene expression but instead occurs rapidly after the induction of C/EBP protein levels.

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