Activation and centromeric localization of CCAAT/enhancer-binding proteins during the mitotic clonal expansion of adipocyte differentiation

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Hormonal induction of 3T3-L1 preadipocytes triggers a cascade of events that initiate differentiation into adipocytes. CCAAT/enhancer-binding proteins β and δ (C/EBPβ/δ) are expressed early in the differentiation program, but are not immediately active. After a long lag, C/EBPβ/δ become competent to bind to the C/EBP regulatory element in the C/EBPα gene promoter, C/EBPα being a transcriptional activator of numerous adipocyte genes. As C/EBPβ/δ acquire binding activity, they become localized to centromeres as preadipocytes synchronously enter S phase at the onset of mitotic clonal expansion. Localization to centromeres occurs through C/EBP consensus-binding sites in centromeric satellite DNA. C/EBPα, which is antimitotic, becomes centromere-associated much later in the differentiation program as mitotic clonal expansion ceases and the cells become terminally differentiated.

[Key Words: 3T3-L1 preadipocyte; cell cycle; C/EBP; satellite DNA; centromere]

Received May 17, 1999; revised version accepted July 15, 1999.

C/EBPα (CCAAT/enhancer-binding protein α) has a vital role in adipocyte differentiation [Cornelius et al. 1994; MacDougald and Lane 1995; Hwang et al. 1997]. C/EBPα is not only required for differentiation [Samuelsson et al. 1991; Lin and Lane 1992], but along with PPARγ [peroxisome proliferator-activated receptor-γ], is sufficient to activate the adipocyte differentiation program without the hormonal inducers normally required [Lin and Lane 1994; Brun et al. 1996]. C/EBPα serves as a pleiotropic transcriptional activator coordinately inducing expression of numerous adipocyte genes that promote acquisition of the adipocyte phenotype [Herrera et al. 1989; Kaestner et al. 1990; Cheneval et al. 1991; Christy et al. 1989, 1991; Hwang et al. 1996]. Two other members of the C/EBP family of transcription factors, namely C/EBPβ and C/EBPδ (C/EBPβ/δ), also function in the differentiation program [Cao et al. 1991; Yeh et al. 1995]. C/EBPβ/δ are expressed early in the program [Cao et al. 1991; Yeh et al. 1995], whereas C/EBPα is expressed much later [Cao et al. 1991; Christy et al. 1991; Yeh et al. 1995]. The proximal promoter of the C/EBPα gene contains a C/EBP regulatory element that mediates transactivation by C/EBPβ/δ [Christy et al. 1991]. Thus, the C/EBP family appears to function in a cascade [Yeh et al. 1995] in which C/EBPβ/δ initially activate transcription of the C/EBPα gene, after which C/EBPα coordinately activates the expression of adipocyte genes producing the terminally differentiated state [Lin et al. 1993]. Proof of the vital role played by the C/EBPs in adipogenesis in vivo was demonstrated by disruption of the C/EBPα gene (Wang et al. 1995) or both of the C/EBPβ and C/EBPδ genes [Tanaka et al. 1997], which prevented the normal development of adipose tissue.

Early in the adipocyte differentiation program preadipocytes undergo mitotic clonal expansion [Bernlohr et al. 1985; Cornelius et al. 1994; MacDougald and Lane 1995], which appears to be necessary for progression through subsequent steps in the differentiation program [Cornelius et al. 1994; MacDougald and Lane 1995]. Thus, treatment with the appropriate hormonal differentiation inducers causes confluent growth-arrested 3T3-L1 preadipocytes to reenter synchronously the cell cycle and undergo two to three rounds of mitosis [Bernlohr et al. 1985; Cornelius et al. 1994]. During these mitotic events preadipocytes express high levels of C/EBPβ/δ [Cao et al. 1991; Yeh et al. 1995]. These factors have been shown to activate transcriptionally the C/EBPα gene promoter through a C/EBP regulatory element in the proximal 5′ flanking region [Christy et al. 1991; Tang et al. 1999]. Expression of C/EBPα occurs later as the cells exit the cell cycle, begin to express adipocyte genes, and undergo terminal differentiation [Lin et al. 1993; MacDougald and Lane 1995]. Because C/EBPα is antimitotic [Umek et al. 1991; Lin et al. 1993; Timchenko et al. 1996, 1997], it is thought that this transcription factor may be responsible for terminating mitotic clonal expansion [MacDou-
By C/EBP

delayed acquisition of DNA-binding function

Results

As a consequence of the above observations, we have considered whether C/EBPα is known to be transactivated by the C/EBPβ/δ gene and is expressed soon after (within 4 hr) the induction of differentiation, respectively. In this paper we show that although C/EBPβ and C/EBPδ are expressed soon after (within 4 hr) the induction of adipocyte differentiation, these transcription factors are unable to bind to the C/EBP regulatory element in the C/EBPα promoter. However, as the preadipocytes enter S phase at the inception of mitotic clonal expansion, C/EBPβ/δ begin to acquire the capacity to bind to the C/EBP regulatory element and concomitantly become centromere associated. Upon activation of the C/EBPα gene, C/EBPα becomes centromere associated and mitotic clonal expansion ceases.

Delayed acquisition of DNA-binding function by C/EBPβ/δ

During differentiation of 3T3-L1 preadipocytes C/EBPβ/δ transcriptionally activate the C/EBPα gene through a C/EBP-binding site in the promoter (see above). Kinetic analysis, however, revealed an unexpectedly long delay between expression of C/EBPβ/δ and the expression of C/EBPα. Immunoblots of cell extracts of 3T3-L1 preadipocytes [Fig. 1A] showed that expression of C/EBPβ/δ occurs rapidly after induction of differentiation, maximal levels being achieved within 4 hr and then maintained for ~48 hr. C/EBPβ/δ then begin to decline (results not shown). Given that C/EBPβ/δ are known to be transcriptional activators of the C/EBPα gene, a lag of ~30 hr before expression of C/EBPα seemed surprisingly long [Fig. 1A]. Once C/EBPα was expressed, expression of the 422/aP2 gene occurred almost immediately, a typical adipocyte gene known to be transactivated by C/EBPα (Christy et al. 1989; Cheneval et al. 1991) [Fig. 1A]. It should be noted that the kinetics of expression of C/EBPα mRNA closely correlates with the expression of C/EBPα protein (results not shown).

In view of the long lag between expression of C/EBPβ/δ and expression of C/EBPα, the possibility was considered that translocation of C/EBPβ/δ into the nucleus might be ratelimiting. Therefore, the intracellular localization of C/EBPβ/δ was assessed, both by cell fractionation and by in situ immunofluorescence, at

![Figure 1](genesdev.cshlp.org)
various times after the induction of differentiation. As shown in Figure 2A, virtually all C/EBPβ/δ was nuclear within 4 hr [and remained nuclear for 24 hr] after induction. Similar results were obtained with intact preadipocytes by in situ immunostaining with antibody against C/EBPβ/δ. As shown in Figure 2B virtually all C/EBPβ immunofluorescence was localized to the nuclei from 4 to 24 hr; similar results were obtained for C/EBPδ [results not shown]. It can be concluded that the reason for the delay in acquiring DNA-binding activity by C/EBPβ/δ in nuclear extracts is not a result of a lag in the translocating these transcription factors into the nucleus.

To ascertain whether there is a lag in the acquisition of intrinsic DNA-binding activity by C/EBPβ/δ, gel shift assays were performed. An oligonucleotide probe corresponding to the C/EBP-binding site in the C/EBPα gene promoter was used along with nuclear extracts from preadipocytes isolated every 4 hr [or 48 hr] after induction of differentiation. As shown in Figure 1A maximal expression of C/EBPβ/δ occurs within 4 hr after induction and remains high for 48 hr. However, DNA-binding activity, which begins to increase between 12 and 16 hr, requires more than 30 hr to reach a maximum [Fig. 1B and Fig. 3A]. It should be noted that the broadness of DNA–protein bands in the gel shift assays is due to homo- and heterodimer formation among the two isoforms of C/EBPβ and other C/EBPs [MacDougald et al. 1995]. A single C/EBPδ mRNA is known to give rise to two translation products of 38 and 18 kD, which can form homo- and heterodimers between themselves and other C/EBPs [Descombes and Schibler 1991]. That these DNA–protein complexes contain C/EBPβ or C/EBPδ is verified by the nearly complete supershift of the complexes with antibodies directed against C/EBPβ/δ [Fig. 3, cf. A with B, C, and D]. Comparison of the supershifts with antibodies against C/EBPβ/δ [Fig. 3 cf. A, B, and C] shows that C/EBPδ is dominant, relative to C/EBPβ, in nuclear extracts from preadipocytes induced to differentiate for 48 hr.

The small amount of ‘unshifted’ protein–oligonucleotide complexes remaining when antibodies to both C/EBPβ and C/EBPδ are used [Fig. 3D] is due primarily to C/EBPα homo- and heterodimers, which begin to appear 28–32 hr after induction [Fig. 3D]. As with C/EBPδ, a single C/EBPα mRNA is known to give rise to two translation products of 42 and 30 kD [Lin et al. 1993], which can form homo- and heterodimers between themselves and with other C/EBPs [MacDougald et al. 1995]. This was confirmed with C/EBPα antibody, with which it was shown that the supershifted band [due to C/EBPα] exhibits similar kinetics [Fig. 3E] to that of the residual DNA–protein complexes remaining after removal of C/EBPβ/δ [Fig. 3D]. A graphic presentation of these results is given in Figure 1B. Taken together, these findings indicate that there is a long delay in the acquisition of binding activity by C/EBPβ/δ and suggest that this is responsible for the long delay in the transcriptional activation of the C/EBPα gene.

The acquisition of binding activity by C/EBPβ/δ, which begins between 12 and 16 hr after induction of differentiation [see Fig. 1B], coincides with entry of the preadipocytes into S phase and the onset of mitotic clonal expansion [Bernlohr et al. 1985]. Entry into S phase at this time is evidenced by the onset of phosphorylation of Retinoblastoma (Rb) [Fig. 4A] and of labeled thymidine incorporation into cellular DNA [Fig. 4B], both of which occur between 12 and 16 hr after the induction of differentiation. That the band shift of Rb to lower mobility at 12–16 hr [Fig. 4A] is due to phosphorylation is indicated by the fact that treatment with alkaline phosphatase caused a shift in mobility to that of the faster moving band [Fig. 4A]. These events are known to occur at the G1–S checkpoint [Hatakeyama and Weinberg 1995; Reed 1997]. It should be recalled that upon induction of the differentiation program, confluent preadipocytes reenter the cell cycle synchronously and undergo several rounds of mitotic clonal expansion (see

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**Figure 2.** Intracellular localization of C/EBPβ/δ early in the differentiation program. (A) At 4, 8, and 24 hr after induction of differentiation 3T3-L1 preadipocytes were lysed and resolved into nuclear and cytoplasmic fractions. The total lysate, cytoplasmic, and nuclear fractions [amounts equivalent to the same number of cells] were subjected to SDS-PAGE, then Western blotted with antibodies against C/EBPβ/δ. (B) Total cell lysate; (C) cytoplasmic fraction; (N) nuclear fraction. (B) 3T3-L1 preadipocytes on coverslips were induced to differentiate. At time 0 and 4, 8, 12, 16, and 24 hr after induction of differentiation the cell monolayers were fixed and subjected to immunofluorescence staining with antibody to C/EBPβ.
Figure 3. Changes in binding of C/EBPβ, C/EBPδ, and C/EBPα to the C/EBP regulatory element in the C/EBPα gene promoter during adipocyte differentiation. EMSA was performed on nuclear extract from 3T3-L1 preadipocytes after induction of differentiation. EMSA was conducted with 10 µg of nuclear extract and a labeled oligonucleotide probe corresponding to the C/EBP regulatory element in the C/EBPα gene promoter. (A) Day 0 post-confluent 3T3-L1 preadipocytes were induced to differentiation into adipocytes using the standard differentiation protocol. Every 4 hr after induction nuclear extracts were prepared and subjected to EMSA as described in Materials and Methods. Supershift experiments were performed as in A with antibodies directed against: C/EBPβ [B], C/EBPδ [C], C/EBPβ and C/EBPδ [D], and C/EBPα [E].
Centromeric localization of C/EBPβ/δ during mitotic clonal expansion

Immunofluorescent staining with antibody against C/EBPβ shows that C/EBPβ is distributed diffusely within the nuclei of preadipocytes between 4 and 12 hr after the induction of differentiation [see Fig. 2B]. However, between 12 and 24 hr the immunofluorescence becomes punctate [Figs. 2B and 5A, top], a pattern that persists throughout the remainder of the cell cycle [see below]. A similar immunofluorescence staining pattern occurs with antibody against C/EBPδ [results not shown]. The shift from diffuse to punctate Immunofluorescence of C/EBPβ/δ antibody [see Fig. 2B] and acquisition of DNA-binding activity [see Fig. 1B] occur concomitantly and correlate well with the phosphorylation of Rb [see Fig. 4A] and the onset of labeled thymidine incorporation into cellular DNA [see Fig. 4B]. Both of the latter phenomena are known to begin at the G1–S checkpoint and to continue during S phase of the cell cycle [Hatakeyama and Weinberg 1995; Reed 1997]. These findings suggested that upon acquiring DNA-binding activity, C/EBPβ becomes associated with 'punctate' nuclear entities. Of interest, these nuclear sites of interaction with C/EBPβ/δ antibodies appeared to coincide with the bright punctate nuclear sites of DAPI fluorescence, which are evident both before and after induction of differentiation [Fig. 5A, bottom]. DAPI is known to interact strongly with the centromeric heterochromatin of mouse chromosomes. Comparison of the localization of DAPI and C/EBPβ antibody staining by confocal fluorescence imaging revealed that these sites are coincident as evident from their overlapping fluorescences [Fig. 5B]. It should be noted that the punctate nature of immunostaining of nuclei with antibody directed against C/EBPβ has been observed previously in macrophage cell lines [Baer et al. 1998]. Although it was not determined that these immunostained sites were centromeres, this seems likely as the same punctate-stained regions were also stained with DAPI.

After a long lag [i.e., 12–16 hr from the time of induction of differentiation] there is a shift from diffuse to punctuate nuclear immunofluorescence as preadipocytes enter S phase. Centromeric localization of C/EBPβ persists throughout the G1, M, and cleavage stages of the cell cycle as indicated by the series of images of selected cells 28 hr after induction of differentiation [Fig. 5C]. Although centromeric localization of C/EBPβ was suspected because of its coincidence with the sites of DAPI staining, this was verified with an antibody against centromere protein B [CENP-B], a bonafide centromeric protein [Earnshaw et al. 1989; Masumoto et al. 1989]. As shown in Fig. 5D, immunostaining of CENP-B colocalizes with C/EBPβ, although CENP-B staining is more focal being restricted to the edges of the centromeres. This is consistent with the fact that CENP-B associates with centromeres [Earnshaw et al. 1989; Masumoto et al. 1989]. Taken together these results indicate that C/EBPβ/δ [results not shown] become associated with centromeres at the point in the differentiation program when preadipocytes enter S phase at the onset of mitotic clonal expansion.

As C/EBPβ/δ achieve maximal DNA-binding activity [see C/EBPβ/δ achieve maximal DNA-binding activity] and the expression of C/EBPα is initiated [see Fig. 1A], an association of C/EBPα with centromeres begins [Fig. 5E, cf. 24 hr and 48 hr]. At 24 hr before the expression of C/EBPα no immunofluorescence is detected, however, by 48 hr when expression of C/EBPα has been initiated a significant percentage (~40–50%) of the cells exhibit immunofluorescence with antibody to C/EBPα. Although the expression of C/EBPα does not reach its maximum until >72 hr, immunostaining at times beyond 48 hr after induction of differentiation becomes uninterpretable because of the high levels of IgG-adsorbing glycosaminoglycans secreted by and adhering to the cells at that point in the differentiation pro-
Figure 5. Colocalization of C/EBPβ, C/EBPa, and CENP-B and DAPI staining during differentiation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were induced to differentiate using the standard protocol, fixed, treated with antibodies (and FITC-labeled anti-rabbit IgG), and DAPI at the times indicated. Fluorescence images were obtained by confocal microscopy. (A, top) Immunofluorescence with antibody against C/EBPβ and FITC-labeled anti-rabbit IgG. Similar results were obtained with antibody against C/EBPa [results not shown]. (Bottom) Immunofluorescence imaging of the same field as in A with DAPI. (B) Dual fluorescence imaging of C/EBPβ and DAPI by confocal microscopy of cells 24 hr after induction of differentiation. (C) Fluorescence imaging of 3T3-L1 preadipocytes treated with C/EBPβ antibody and DAPI during the first round of mitotic clonal expansion. These selected images of preadipocytes at 28 hr after induction of differentiation represent various stages of the cell cycle. (D) Fluorescence imaging of 3T3-L1 preadipocytes treated with antibodies against C/EBPβ and CENP-B during the first round of mitotic clonal expansion. These selected images of preadipocytes at 28 hr after induction of differentiation represent various stages of the cell cycle. The column on the right represents dual fluorescence images of the two columns of images to the left. (E) Colocalization of immunofluorescence of C/EBPa antibody and DAPI in the terminal stages of the differentiation program.
gram. Centromeric binding of C/EBPα begins as 3T3-L1 preadipocytes exit the cell cycle and become growth arrested. Previously, we showed that mitosis ceases by ~72 hr after induction of differentiation (Bernlohr et al. 1985). In view of the fact that C/EBPα is antimitotic (Umek et al. 1991), the question arises, does the interaction of C/EBPα with centromeres play a role in terminating mitotic clonal expansion?

**Binding of the C/EBPs to centromeric satellite DNA**

It is well-known that the bright fluorescent spots in mouse nuclei stained with DAPI are due to heterochromatic satellite DNA (Miller et al. 1974; Hendrich and Bird 1998). Because the pattern of DAPI fluorescence was virtually identical to that of C/EBPβ (Fig. 5B) and C/EBP δ (results not shown) and mouse satellite DNA is located primarily in centromeres (Stephanova et al. 1988; Joseph et al. 1989), the possibility was considered that C/EBPβ/δ bind to sequences in centromeric satellite DNA. Inspection of the nucleotide sequence of the major species of mouse satellite DNA (Horz and Altenburger 1981) reveals eight repeats of a consensus C/EBP binding site (Fig. 6A). To ascertain whether C/EBP β can bind to satellite DNA, electro phoretic mobility shift analysis (EMSA) experiments were performed with rC/EBP β and a 234-bp segment of the major mouse satellite DNA that contains these consensus sequences. Remarkably, in gel shift experiments rC/EBP β gives rise to about eight DNA–protein complexes with mouse satellite DNA (Fig. 6B). As the concentration of rC/EBP β is increased, the fraction of lower mobility complexes increases, presumably reflecting increasing numbers of molecules of rC/EBP β bound per molecule of satellite DNA. Moreover, unlabeled competitor monomeric oligonucleotide (corresponding to the C/EBP-binding site in the C/EBPα gene promoter) causes a shift in the distribution of mobilities back to complexes of higher mobility. That all of the protein–DNA complexes contain rC/EBP β is indicated by the ability of anti-C/EBP β antibody to supershift all complexes (Fig. 6B). These findings provide convincing evidence that rC/EBP β binds to satellite DNA. Gel shift experiments with nuclear extracts from 3T3-L1 preadipocytes 4 or 24 hr after induction of differentiation were subjected to EMSA with a labeled oligonucleotide probe corresponding to C/EBP-binding site (sequence 7 in the major mouse satellite DNA; see A; Materials and Methods). Supershift experiments were performed with preimmune serum (Pi) or anti-serum to C/EBP β.

![Figure 6](https://www.cshlp.org/genesdev22/237/g06a.jpg)

**Figure 6.** Association of mouse satellite DNA with rC/EBP β or C/EBP δ in nuclear extract from 3T3-L1 preadipocytes induced to differentiate. (A) Nucleotide sequence of 234 bp of the major mouse satellite DNA. The underlined repetitive sequences represent probable C/EBP-binding sites based on their similarity to the consensus sequence for C/EBP-binding sites (shown at bottom) in numerous gene promoters. (B) EMSA with rC/EBP β and a 234-bp oligonucleotide probe corresponding to the major mouse satellite DNA. The unlabeled competitor oligonucleotide corresponds to the C/EBP regulatory element in the C/EBPα gene promoter. Similar results (not shown) were obtained with a competitor oligonucleotide corresponding to sequence 7 in A. (C) Nuclear extracts from 3T3-L1 preadipocytes 4 or 24 hr after induction of differentiation were subjected to EMSA with a labeled oligonucleotide probe corresponding to C/EBP-binding site (sequence 7 in the major mouse satellite DNA; see A; Materials and Methods). Supershift experiments were performed with preimmune serum (Pi) or anti-serum to C/EBP β.
experiments were also performed with C/EBPβ in nuclear extracts from 3T3-L1 preadipocytes to verify that ‘active’ C/EBPβ in preadipocytes induced to differentiate for 24 hr [a point at which C/EBPβ has acquired DNA-binding activity, see Fig. 1B] binds to a satellite DNA C/EBP-binding site probe [corresponding to sequence 7 in Figure 6A]. As shown in Figure 6C, C/EBPβ in 24-hr nuclear extract binds, whereas that in 4-hr nuclear extract (from cells in which C/EBPβ has not yet acquired DNA-binding activity; see Fig. 1B) does not. Supershift experiments with antibody to C/EBPβ verified that practically all of the DNA–protein complexes formed with the 24-hr nuclear extract are due to C/EBPβ. Similar results were obtained with C/EBPα in nuclear extracts from fully differentiated cells [results not shown]. Taken together these results provide compelling evidence that the C/EBPs interact with centromeres by binding to centromeric satellite DNA.

Discussion

Induction of differentiation of 3T3-L1 preadipocytes sets into motion a cascade of events beginning with the expression of C/EBPβ and C/EBPα [Fig. 1A,B] (Cao et al. 1991; Yeh et al. 1995), these factors being transcriptional activators of the C/EBPα gene (Tang et al. 1999). There is a delay, however, of nearly 30 hr before C/EBPα gene is expressed [Fig. 1]. Expression of C/EBPα is followed almost immediately by transcriptional activation of the adipocyte genes, such as the aP2 gene [Fig. 1], which are transcriptionally activated by C/EBPα (Christy et al. 1989; Cheneval et al. 1991). C/EBPα serves as a pleiotropic transactivator of numerous adipocyte genes that are coordinately expressed and contribute to the terminally differentiated phenotype (Christy et al. 1989, 1991; Herrera et al. 1989; Kaestner et al. 1990; Cheneval et al. 1991; Hwang et al. 1996).

The results presented in this paper indicate that the delayed expression of the C/EBPα gene [Fig. 1A] is due to the very slow acquisition of C/EBPβ/δ-binding activity [Figs. 1B and 3], hence delayed transcriptional activation of the C/EBPα gene. A question arises as to how C/EBPβ/δ acquire DNA-binding activity. Although the molecular basis for this acquisition of binding activity is not yet known, preliminary findings suggest that C/EBPβ undergoes critical phosphorylation events concomitant with acquisition of DNA-binding activity. As shown in Figure 7 exposure of nuclear extracts from 3T3-L1 preadipocytes [induced to differentiate for 4 or 24 hr] to alkaline phosphatase in vitro markedly [by 70–80%] reduced the DNA-binding activity of C/EBPβ. Although this finding suggests that acquisition of binding activity during mitotic clonal expansion involves phosphorylation of C/EBPβ, to prove this point definitively it will be necessary to identify the sites of phosphorylation responsible for the acquisition of DNA-binding activity in the context of clonal expansion. This will be a major undertaking, as C/EBPβ appears to be phosphorylated at multiple sites. Thus, C/EBPβ exhibits a complex pattern of apparent phosphorylated species on two-dimensional isoelectric focusing SDS–polyacrylamide gels (Q.-Q. Tang and M.D. Lane, unpubl.). In the isoelectric focusing dimension, we estimate that there are six to eight species of C/EBPβ/LAP (the 38-kD isoform of C/EBPβ) presumably reflecting different extents or combinations of phosphorylation. Other evidence consistent with the view that phosphorylation may play a role in the acquisition of the DNA-binding activity by C/EBPβ was reported by Williams et al. (1995). Thus, C/EBPβ possesses two regulatory regions. Mutations in a serine-rich sequence in one of these regions, which contains several presumptive protein kinase target sites, leads to increased DNA-binding activity by C/EBPβ. It was suggested that before phosphorylation, C/EBPβ assumes a tightly folded conformation in which the DNA-binding domain is obscured by interaction with the regulatory domain. Phosphorylation of the regulatory domain would be expected to disrupt this interaction rendering the binding domain accessible for binding to DNA (Williams et al. 1995). This hypothesis is consistent with the finding that C/EBPβ undergoes phosphorylation concomitant with the acquisition of DNA-binding activity and the initiation of mitotic clonal expansion during differentiation of 3T3-L1 preadipocytes. Studies are under way to test this hypothesis.

We suggest that the delayed acquisition of DNA-binding activity by C/EBPβ/δ [Fig. 1B] foretells the expression of C/EBPα to a point later in the differentiation program that does not interfere with mitotic clonal expansion. Because C/EBPα is antimitotic, this is an important consideration as mitotic clonal expansion is required for the events of terminal differentiation (Cornelius et al. 1994; MacDougald and Lane 1995). It has been suggested that DNA replication and the accompa-
nony remodeling of chromatin during mitotic clonal expansion may allow access of cis-acting elements to trans-acting factors that activate [or derepress] transcription of genes critical for completion of the differentiation program (MacDougald and Lane 1995). That the delayed acquisition of DNA-binding activity by C/EBPβ is demonstrated in vitro [by EMSA] also occurs in intact 3T3-L1 preadipocytes is indicated by the delayed interaction of C/EBPβ/δ with centromeres [Fig. 2B]. This interaction is due to the binding of C/EBPβ/δ (and later C/EPBα) to centromeric satellite DNA [Fig. 5B]. It should be emphasized that during differentiation, the acquisition of binding activity by C/EBPβ/δ (in nuclear extracts as measured by EMSA) to probes corresponding to the C/EBP-regulated mitotic clonal expansion. Conceivably, centromeres may have functional significance in the control of mitotic clonal expansion, a prerequisite for subsequent adipocyte differentiation. These interactions occur at different points in the differentiation program. C/EBPβ/δ become associated with centromeres as preadipocytes enter S phase at the inception of mitotic clonal expansion and maintain this association through about two to three rounds of mitosis. Later in the program, as cellular levels of C/EBPβ/δ begin to decline, expression of C/EPBα reaches a maximum and mitosis ceases, consistent with the fact that C/EPBα is antimitotic (Umek et al. 1991; Lin et al. 1993; Timchenko et al. 1994, 1995) with the following modifications. Reaction mixtures containing –0.25 ng of the appropriate 32P-labeled oligonucleotide probe (2.5 ng for the 234-bp satellite DNA, 2 µg of poly[dI–dC], and 10 µg of nuclear extract protein in 30 µl of buffer (10 mM HEPEs, 0.1 mM EDTA, 5% glycerol, 100 mM NaCl, 0.3 µg RNAase, 0.3% NP-40) were incubated on ice for 15 min, at room temperature for 15 min, and then separated electrophoretically on 5% polyacrylamide gels. For competition experiments, a 100-fold excess of unlabelled competitor oligonucleotide was added to reaction mixtures before the addition of labeled probe. For supershift experiments, 1 µl of antiserum (–5 µg of IgG protein) was added to the reaction mixture before the addition of labeled probe. Recombinant C/EBPβ was obtained from Steven McKnight (University of Texas Southwestern Medical Center, Dallas). The labeled oligonucleotide probes included double-stranded oligonucleotides corresponding to [1] the C/EBP regulatory element in the C/EPBα promoter, [-191]GGGTTGCAGCCACGATCTTCT (-172), and [2] one of the C/EBP-binding sites in mouse satellite DNA, [182] TGAAAAATGACGAAATCACTA(202).

The 234-bp mouse satellite DNA probe was prepared by cutting pBR 322-M.sat.4 (from Wolf Stratling, University-Krankenhaus Eppendorf, Hamburg, Germany) with BamHI.

Western blotting and phosphatase treatment
To follow changes in the level of C/EPBα, C/EPBβ, and C/EPBδ, Rb and 422/aP2 proteins after induction of differentiation, 2-day postconfluent [day 0] 3T3-L1 preadipocytes were treated with or without MIX and maintained in DMEM containing 10% FBS. In all cases, the following culture conditions were maintained: DMEM containing 10% FBS and 1 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (MIX) until day 2. Cells were then fed DMEM supplemented with 10% FBS and 1 µg/ml insulin for 2 days, after which they were fed every other day with DMEM containing 10% FBS. Adipocyte gene expression and acquisition of the adipocyte phenotype begins on day 3 and is maximal by day 8.

EMSA
Nuclei were isolated and nuclear extracts prepared using 1x NUN buffer (Lavery and Schiller 1993) containing 0.3 M NaCl, 1 M urea, 1% Nonidet P-40, 25 mM HEPEs (pH 7.9), and 1 mM DTT. Protein concentration was determined by the Bradford method [Bio-Rad]. EMSA was performed essentially as described (MacDougald et al. 1994, 1995) with the following modifications. Reaction mixtures containing ~0.25 ng of the appropriate 32P-labeled oligonucleotide probe [2.5 ng for the 234-bp satellite DNA] 2 µg of poly[dI–dC], and 10 µg of nuclear extract protein in 30 µl of buffer [10 mM HEPEs, 0.1 mM EDTA, 5% glycerol, 100 mM NaCl, 0.3 µg RNAase, 0.3% NP-40] were incubated on ice for 15 min, at room temperature for 15 min, and then separated electrophoretically on 5% polyacrylamide gels. For competition experiments, a 100-fold excess of unlabelled competitor oligonucleotide was added to reaction mixtures before the addition of labeled probe. For supershift experiments, 1 µl of antiserum (~5 µg of IgG protein) was added to the reaction mixture before the addition of labeled probe. Recombinant C/EBPβ was obtained from Steven McKnight (University of Texas Southwestern Medical Center, Dallas). The labeled oligonucleotide probes included double-stranded oligonucleotides corresponding to [1] the C/EBP regulatory element in the C/EPBα promoter, [-191]GGGTTGCAGCCACGATCTTCT (-172), and [2] one of the C/EBP-binding sites in mouse satellite DNA, [182] TGAAAAATGACGAAATCACTA(202).

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Central to understanding the control of adipocyte differentiation is the regulation of gene expression that results in the oncogene [or derepress] transcription aspects that lead to differentiation. 2-day postconfluent preadipocytes (designated day 0) were fed DMEM containing 10% FBS. In all cases, the following culture conditions were maintained: DMEM containing 10% FBS and 1 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (MIX) until day 2. Cells were then fed DMEM supplemented with 10% FBS and 1 µg/ml insulin for 2 days, after which they were fed every other day with DMEM containing 10% FBS. Adipocyte gene expression and acquisition of the adipocyte phenotype begins on day 3 and is maximal by day 8.

Materials and methods
Cell culture and induction of differentiation
3T3-L1 preadipocytes were propagated and maintained in DMEM containing 10% [vol/vol] calf serum as described [Stu-
ditions. The reaction mixtures were then subjected to immu-

no blotting with anti-Rb antibody.

$^{3}H$Thymidine incorporation

At various times after induction of differentiation, two 35-mm
cell monolayers were pulse-labeled with $^{3}H$thymidine (1 µCi/ml)
for 15 min, then washed twice with cold phosphate-buffered
saline (pH 7.5), extracted with 2 ml of 0.5 M KOH, and chromo-
somal DNA precipitated with 4 ml of 20% TCA on ice for 15
min. The DNA was filtered onto GF/C glass microfibre filters
(Whatman), washed with 95% ethanol, dried and counted.

Immunofluorescence microscopy

3T3-L1 preadipocytes were plated onto coverslips in 35-mm
dishes at the same cell density as usual and then induced to
differentiation as above. At various times thereafter, cell mono-
layers were washed with cold PBS, fixed with 4% formaldehyde
in PBS on room temperature for 20 min, permeabilized with
0.075% Triton X-100 in 2 mg/ml BSA in PBS for 30 min; and
blocked with 2 mg/ml BSA in PBS for 1–2 hr at room tempera-
ture. Cells were incubated with the first antibody (C/EBPa,
C/EBPβ, or C/EBPγ antibody at 1:1000 dilution) in 2 mg/ml
BSA in PBS for 1–2 hr and then incubated with FITC-labeled
second antibody in the same buffer for 1 hr. After each step, the
cells were washed with PBS three times. Antifade solution [Mo-

cular Probes] was added to the monolayers and mount on
slides. For DAPI staining, coverslips were incubated with DAPI
(Molecular Probe) for 5 min and then washed with PBS.

Acknowledgments

This work was supported by a research grant from the National
Institutes of Health [National Institute of Diabetes and Digestive
and Kidney Diseases [NIDDK]]. We thank Drs. Steven
McKnight for providing rC/EBPβ, William Earnshaw for sup-
plying CENP-B antibody, Adrian Bird and Wolf Stratling for
providing mouse satellite DNA vectors, Joseph Gall and Tom
Loftus for helpful discussions, and Derrick Robinson for assis-
tance with fluorescence microscopy.

The publication costs of this article were defrayed in part by
payment of page charges. This article must therefore be hereby
solely to indicate this fact.

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*Genes Dev.* 1999, 13:

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