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REGULATION OF ADIPOCYTE DEVELOPMENT

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INTRODUCTION

The primary role of adipocytes is to store triacylglycerol during periods of nutritional caloric excess and to mobilize this reserve when caloric expenditure exceeds intake. Mature adipocytes are uniquely equipped to perform these functions. They possess the full complement of enzymes and regulatory proteins needed to carry out both de novo lipogenesis and lipolysis. The regulation of these processes in adipocytes is exquisitely responsive to hormones that regulate energy metabolism. For example, insulin promotes lipogenesis and inhibits lipolysis, whereas counterregulatory hormones [e.g. glucagon, epinephrine, and

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adrenocorticotropic hormone (ACTH)] promote lipolysis. The ability to carry out these functions is acquired during embryonic development in preparation for the postnatal period, when an adipose energy reserve becomes necessary. Although preadipocytes first appear late in embryonic life, major ex- pansion of the white adipocyte population is delayed until shortly after birth (35, 166). This increase in adipose tissue mass coincides with the need of the newborn to survive periods of fasting by mobilizing adipose triacylglycerol reserves.

During embryonic development, a single fertilized egg gives rise to the nearly 200 different cell types that comprise the mature animal (198). As the progeny of totipotent embryonic stem cells progress through the diverging pathways of development, they become increasingly committed to specific lineages, finally losing their potential to generate other cell types. The adipose lineage is derived from a multipotent stem cell of mesodermal origin that can also give rise to the muscle and cartilage lineages.

The availability of established cell lines that mimic cells arrested at different stages of adipocyte development has made it possible to characterize the differentiation program (Figure 1). For example, the multipotent 10T1/2 stem cell line represents an early stage in adipose development at which commitment to a specific lineage, including the preadipocyte lineage, has not yet occurred. The 3T3-L1 preadipocyte cell line represents a late stage of adipose development at which cells have undergone commitment to the adipocyte lineage and can only further differentiate into adipocytes. When treated with appropriate

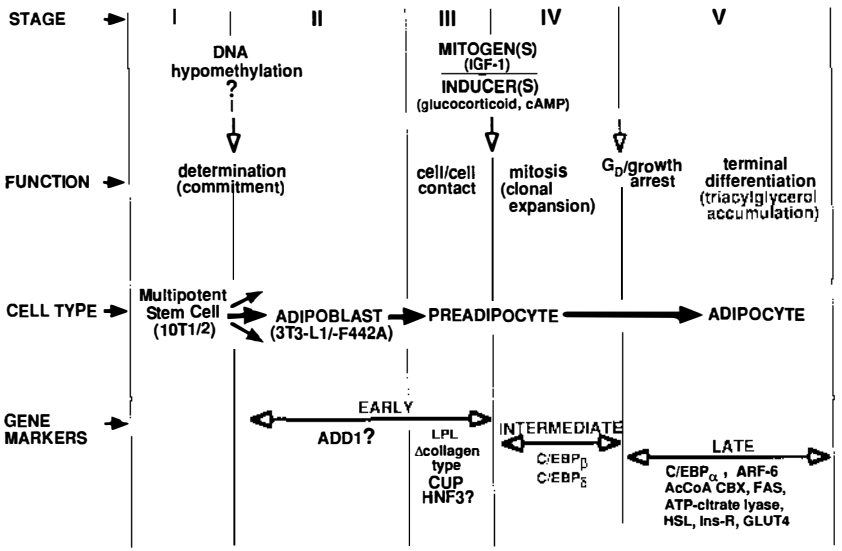

Figure 1 Stages in the adipocyte development program.

hormonal agents, 3T3-L1 preadipocytes differentiate into cells that possess the morphological and biochemical phenotype of mature adipocytes.

In this review we attempt to consolidate and interpret recent pertinent literature on adipocyte-forming cell lines, the adipocyte development program, and modulators (both external and genetic) that act at different stages of development. In many instances we cite recent references, realizing that the original and other references are contained therein.

CELL CULTURE MODELS FOR ADIPOCYTE DEVELOPMENT

Cell lines presently available fall into two classes: (*a*) multipotent stem-cell lines (e.g. 10T1/2, CHEF/18, and Balb/c 3T3) that can undergo determination (commitment) into adipocytes, myocytes, or chondrocytes and (*b*) preadipocyte cell lines that have already undergone commitment to the adipocyte lineage. As additional stage-specific markers become available, further division of these two classes into subtypes that have been arrested at other stages of development/differentiation should be possible.

Multipotent Stem-Cell Lines

10T1/2 cells, cloned from C3H mouse embryos (140), exhibit the characteristics of multipotent stem cells. Treatment of 10T1/2 cells with 5-azacytidine, an inhibitor of DNA methylation (185), generates several cell types, each of which can terminally differentiate along a specific lineage, i.e. either the adipocyte, myocyte, or chondrocyte lineage. Hypomethylation, and as a result the activation of a few regulatory genes, appears sufficient to commit these cells to a specific lineage (98). Transfection of 10T1/2 cells with hypomethylated genomic DNA leads to myogenic conversion at a frequency consistent with activation of a single gene and commitment to the myogenic lineage (105). The search for the gene responsible for myogenic determination led to the discovery of MyoD and subsequently other members of a gene family of *trans*-acting factors that activate transcription of muscle genes when transfected into 10T1/2 and certain other cell types (reviewed in 49). A similar approach to identifying a comparable regulatory gene(s) that commits 10T1/2 cells to the adipogenic lineage has not been successful.

Several other cell lines, including the CHEF/18 (153), Balb/c 3T3 (46, 170), RCJ 3.1 (76), and 1246 (42) lines, also exhibit stem-cell characteristics. Like 10T1/2 cells, many of these cells undergo determination upon treatment with 5-azacytidine. RCJ 3.1 cells should prove useful in future studies on modulators that provoke determination, since commitment to adipocyte and other lineages is dependent on glucocorticoid concentration (76, 77).

Preadipocyte Cell Lines

3T3-L1 AND 3T3-F442A CELLS The most extensively characterized and widely studied cell models for preadipocyte differentiation are the 3T3-L1 and 3T3-F442A cell lines. These lines were originally selected from disaggregated mouse embryo cells for their ability to accumulate cytoplasmic triacylglycerol (69–71). Considerable evidence has been amassed that indicates that these cell systems represent faithful models for preadipocyte differentiation. Perhaps the most compelling validation of the 3T3 preadipocyte model was derived from in vivo cell transplantation studies (72). When 3T3-F442A cells were injected subcutaneously into Balb-C athymic mice at an anatomical site devoid of adipose tissue, normal fat pads developed at the site of injection within five weeks. The new tissue had undergone vascularization, was histologically indistinguishable from normal adipose tissue, and did not produce malignancy.

Protocols have been developed that can induce 3T3 preadipocytes to rapidly and synchronously progress through the differentiation program at high frequency (≥90%). The agents most widely used (often in combination) to differentiate 3T3-L1 preadipocytes and other preadipocyte cell lines include dexamethasone (a synthetic glucocorticoid agonist), high levels of insulin [which act through the insulin-like growth factor-1 (IGF-1) receptor], methylisobutylxanthine (MIX; a cAMP phosphodiesterase inhibitor), and fetal bovine serum (181). The agents most frequently used to differentiate 3T3-F442A cells are insulin and calf serum (71).

Prior to differentiation in cell culture 3T3-L1 and 3T3-F442A preadipocytes are morphologically similar to the fibroblastic preadipose cells in the stroma of adipose tissue. When induced to differentiate 3T3 preadipocytes lose their fibroblastic character, assume a rounded-up appearance, and acquire the morphological and biochemical characteristics of adipocytes. Detailed electron micrographic studies by Novikoff et al (124) revealed that mature 3T3-L1 adipocytes possess virtually all of the ultrastructural features of adipocytes in situ. Soon after the induction of differentiation, cytoplasmic triacylglycerol–containing vacuoles appear. After an extended period in culture, the vacuoles coalesce and become unilocular, causing the typical signet ring appearance of mature white adipocytes (73).

The coordinate expression of virtually every enzyme of the pathways of de novo fatty acid and triacylglycerol biosynthesis is correlated with the accumulation of cytoplasmic triacylglycerol (Table 1). The cells also acquire the proteins necessary for lipolysis of triacylglycerol, uptake, and intracellular translocation of fatty acids and responsiveness to the lipogenic and lipolytic hormones (12, 34, 36, 78, 139, 150, 176). Consistent with the transition from fibroblast to adipocyte morphology, a concomitant decrease occurs in the cellular content of the cytoskeletal proteins, actin and tubulin (12, 36, 172).

Table 1 Proteins differentially expressed during adipocyte development[a]

Hormone action and signaling	
▲insulin receptor (139, 150)	▼pref-1 (167)
▲pp160/IRS (141)	▲*rab*3D (8)
▲β1 adrenergic receptor (52)	▲estrogen synthetase (211)
▲β2 adrenergic receptor (78)	▲cGMP-inhibited cyclic nucleotide phosphodiesterase (183)
▲β3 adrenergic receptor (53)	▲$G_{s\alpha}$ (196)
▲glucocorticoid receptor (117)	▼$G_{i\alpha 2}$ (197)
▲phospholipase A_2 (61)	▼$G_{o\alpha}$ (197)
▲IGF-I (62)	▲retinoic acid receptor α (92)
▲IGF-IBP (123)	
▼CSF (189)	
Lipogenesis and lipolysis	
▲pyruvate carboxylase (12, 80, 113)	▲GLUT4 (8, 86–88)
▲ATP-citrate lyase (63, 114, 208)	▲aldolase (12, 36, 113)
▲acetyl CoA carboxylase (114, 131)	▲glyceraldehyde 3PDH (4)
▲fatty acid synthase (114, 118, 132, 181)	▲lactate dehydrogenase (113, 175)
▲422/aP2 (12, 173)	▲malic enzyme (63, 173, 208)
▲stearoyl CoA desaturase 1 (125)	▲perilipin (74)
▲stearoyl CoA desaturase 2 (90)	▲hormone-sensitive lipase (94)
▲PEPCK (9)	▲cholesterol esterase (94)
▲G3PDH (173)	▲lipoprotein lipase (37, 40, 176)
▲acyl-CoA binding protein (81)	▲fatty acid transporter (1, 2)
▲fatty acid CoA ligase (34)	▲S14 (108, 112)
▲glycerol-3-P acyltransferase (34)	▲glutamine synthetase (13)
▲diacylglycerol acyltransferase (34)	▲retinoid-binding proteins (216)
▲lysophosphatidic acid acyltransferase (34)	
Cytoskeletal and extracellular structure	
▼actin (12, 36, 173)	▲proteoglycan (cSPG-I; 20)
▼tubulin (8, 11, 12, 174)	▼annexin (209)
▲collagen α2, type VI (40, 41)	▲Na/K ATPase α2 subunits (151)
▲laminin (7)	▼collagen type I, III (201)
▼decorin (120)	▼entactin (7)
▲collagen α1 + α2, type IV (7)	
Secreted proteins	
▲angiotensinogen (115, 157)	▲apolipoprotein E (214)
▲adipsin (173)	▲lipoprotein lipase (37)
Proteins of unknown function[b]	
▲FSP27 (39)	▲gene 154 (ADRP; 85)
▲clone 5(AP27; 202)	

[a] ▲, increased expression; ▼, decreased expression.

[b] Numerous other, as yet uncharacterized, cDNAs have been cloned that encode mRNAs, which are differentially expressed during adipocyte conversion (26, 48, 132).

The coordinate changes in the cellular levels of proteins that give rise to the adipocyte phenotype are due almost entirely to changes in transcription rates of the corresponding genes (see section entitled "Transcriptional Control of Adipocyte Genes During Differentiation").

Ob17 AND Ob17-DERIVED CELL LINES The Ob17 cell line was derived from stromal cells of epididymal fat pads from C57BL/6J *ob/ob* mice (121) and thus has a defined tissue origin. Various subclones of Ob17 cells, e.g. the Ob1771 line, have been selected for their propensity to adiposity (5, 6, 48). Ob1771 preadipocytes can be induced to differentiate in serum-free media that contain insulin, triiodothyronine, growth hormone, and either glucocorticoid or agents, which increase cAMP concentration (24, 60, 191).

TA1 AND 30A5 CELLS The TA1 (26) and 30A5 (98) cell lines are both determined preadipocyte lines derived from 10T1/2 cells following treatment with 5-azacytidine. Treatment with dexamethasone or indomethacin, an inhibitor of prostaglandin synthesis (27, 97, 143), can initiate differentiation of TA1 cells. Differentiation of 30A5 cells can be induced with dexamethasone and insulin in media that contain fetal bovine serum (129, 131).

BONE MARROW STROMAL VASCULAR PREADIPOCYTES Bone marrow adipocytes differ functionally from adipose tissue adipocytes in that they support hematopoiesis. Unlike adipocytes of adipose tissue, bone marrow adipocytes do not mobilize triacylglycerol in response to fasting, nor do they exhibit a lipogenic response to insulin (10). Rather, lipolysis is activated by stresses such as anemia (18). An important function of bone marrow stromal adipocytes is to release cytokines, which regulate clonal expansion and maturation of pluripotent hematopoietic stem cells that give rise to various blood cell types. Several marrow stromal vascular preadipocyte cell lines have been established (76, 189, 212), e.g. the +/+2.4 line, which supports the development of myeloid (but not lymphoid) cells, and the BMS2 line, which supports the development of both lineages (64–66, 134). Agents (i.e. glucocorticoid agonists, insulin, MIX, and indomethacin) that induce differentiation of other preadipocyte lines, e.g. 3T3-L1 cells, also induce differentiation of bone marrow stromal preadipocytes. As with 3T3-L1 preadipocytes, adipose conversion of +/+2.4 cells (66) is accompanied by the expression of adipocyte proteins such as CCAAT/enhancer-binding protein α (C/EBPα), 422/aP2 protein, adipsin, and lipoprotein lipase (LPL).

PRIMARY PREADIPOSE CELLS Although immortalized preadipose cell lines have been very useful for the study of adipocyte development, they suffer a disadvantage because they are aneuploid and therefore often possess charac-

teristics that differ from those of tissue preadipocytes. Where possible, it is important to verify findings obtained with immortalized cell lines by using diploid preadipose cells in primary culture. A limited number of studies were conducted recently with determined stromal-vascular preadipocytes isolated from adipose tissue derived from rodents, pigs, and avian species (19, 75, 83, 137, 138, 156, 163, 210). The same group of effectors (see sections entitled "Cell Culture Models for Adipocyte Development" and "External Modulators of Preadipocyte Differentiation") that induces immortalized preadipocytes to differentiate is also effective for adipose conversion of primary preadipose cells. Inhibitors of adipogenesis in established cell lines [e.g. transforming growth factor β (TGFβ), interleukin-β (IL-β), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)] also block expression of the adipocyte phenotype in primary culture of preadipose cells (75, 142, 163). More recent findings on the expression and apparent function of transcription factors during differentiation of immortalized preadipose cell lines must be confirmed with primary adipose cells (see section entitled "Transcriptional Control of Adipocyte Genes During Differentiation"). Another important approach for verification in a physiological context, which has been employed to a limited extent for adipose development, is the use of transgenic animals (67).

EXTERNAL MODULATORS OF PREADIPOCYTE DIFFERENTIATION

The conditions required for preadipocyte differentiation were initially evaluated in serum-containing media using the rate and extent of cytoplasmic triacylglycerol accumulation as criteria. Optimal conditions varied depending on the cell line and batch of serum employed. Most protocols that have been developed allow preadipocytes to proliferate to confluence in media that contain calf serum. This process is followed by treatment with one or more of the following inducers: MIX, dexamethasone, a high concentration of insulin, and fetal bovine serum (see 181). After undergoing several rounds of mitosis followed by growth arrest, adipocyte proteins are expressed, and cytoplasmic triacylglycerol accumulates (see Figure 1).

Smith et al determined (168) that IGF-1 rather than insulin (which interacts with the IGF-1 receptor at high concentrations) is the factor required for induction of differentiation. Interestingly, growth hormone, previously identified as a serum factor required for induction of adipocyte differentiation (215), stimulates the synthesis and secretion of IGF-1 by preadipocytes (47, 62, 123). Thus, growth hormone apparently plays at least a partial role in differentiation by initiating an autocrine/paracrine mechanism that leads to the secretion of IGF-1.

Several other agents either induce or inhibit adipocyte differentiation. Those

that induce differentiation include prostaglandin I_2 (PGI_2, or prostacyclin; 24, 191), thyroid hormone (63), sodium butyrate (187), ascorbic acid (127), aldosterone (148), progesterone (147), arachidonic acid (59), AD4743 (171), bezafibrate (16), pioglitazone (155), and 3-deazaadenosine (29). The most widely investigated inhibitory agents include tumor necrosis factor α (TNFα; 37, 54, 65, 129), TGFβ (65, 169), phorbol ester (46), and retinoic acid (180). Other inhibitory agents include endothelin-1 (184); dehydroepiandrosterone (165); epidermal growth factor (161, 164); dimethylsulfoxide (195); interleukins Iα (65), Iβ, and Iγ (43, 75); and PGF2α (162).

Conditions were developed recently for achieving adipocyte differentiation in the absence of serum using 3T3-L1, 3T3-F442A, 1246, Ob1771, and primary stromal preadipocytes (23, 79, 82, 83, 158, 162). This important advance has made it possible to reassess the requirements for differentiation with chemically defined cell culture media. Most of the agents identified previously as differentiation inducers in serum-containing media (insulin, IGF-1 or growth hormone, glucocorticoid, and cAMP) appear to be essential either for initiating or facilitating adipocyte conversion in the absence of serum. Taken together, these findings implicate at least three second messenger signaling pathways in the induction of adipocyte differentiation: the IGF-1-activated tyrosine kinase pathway, the glucocorticoid pathway, and the cAMP-dependent protein kinase pathway. Additionally, the insulin-activated tyrosine kinase pathway may be necessary to achieve the maximal rate of triacylglycerol accumulation that accompanies adipose conversion.

THE DEVELOPMENT PROGRAM

Information acquired in cell culture studies on adipocyte development has enabled investigators to identify key events that are likely to be important in this process. In this section, we incorporate this information into a working model for the program of adipocyte development, illustrated in Figure 1.

Determination

Treatment with 5-azacytidine causes multipotent 10T1/2 cells to undergo determination, which suggests that hypomethylation and activation of regulatory genes trigger commitment into one of several lineages, i.e. the adipocyte, myocyte, or chondrocyte lineage (98, 185). Specific external modulators likely activate such genes. Although none of these external modulators has been characterized, several genes that may be targeted by such a modulator(s) of myogenic determination have been identified. These genes encode transcription factors that coordinately activate muscle-specific gene expression

(49). Neither a modulator[1] nor a target regulatory gene that specifies the adipocyte lineage has been found. Available evidence suggests that once a given lineage, e.g. stem-cell conversion to adipoblast, has been determined, blast-cell proliferation occurs until cell-cell contact at confluent density inhibits mitosis (12). When this stage of development has been achieved, determination is apparently irreversible, barring external genetic manipulation.

Cell/Cell Contact at Confluence

Where tested, cell/cell contact at confluent cell density seems to be a prerequisite for subsequent preadipocyte differentiation. As preadipocytes arrest at the G_0/G_1 cell cycle boundary, they begin to express the early markers of adipocyte differentiation, including LPL (37) and the mouse equivalent of the human α2 chain of type VI collagen (designated A2Col6/pOb24; 41). Induction of the early markers is most likely mediated through an autocrine/paracrine mechanism(s) activated by cell-cell contact as adipoblasts reach confluence.

Induction of Differentiation

CLONAL EXPANSION Having ceased dividing at confluence, preadipocytes can be induced to differentiate by treatment with an appropriate combination of mitogenic and hormonal agents (see Figure 1 and section entitled "External Modulators of Preadipocyte Differentiation"). Immediately following exposure to these agents, the cells undergo several rounds of mitotic clonal expansion (12). DNA replication (during mitosis) presumably alters the accessibility of promoter control elements to *trans*-acting factors that transcriptionally activate (or derepress) regulatory genes that initiate differentiation. These mitotic events appear necessary in order for the cells to enter a unique growth-arrested stage of the cell cycle, referred to as G_D by Scott and colleagues (159, 160, 195), which is permissive for subsequent differentiation. Some mitogens, e.g. fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), stimulate postconfluent mitosis but do not induce differentiation, presumably because the requirements (e.g. glucocorticoid, IGF-1, and cAMP) for entry into G_D have not been met. Thus, DNA replication per se seems necessary but not sufficient for the induction of differentiation. Consistent with this view, studies by Freytag (56) suggest that expression of c-*myc* may prevent preadipocytes from entering G_D. Thus, when 3T3-L1 preadipocytes constitutively

[1]A recent study raises the possibility that a modulator of the *ras* signaling pathway may be involved (111).

expressing c-*myc* were treated with differentiation inducers, the cells underwent clonal expansion followed by growth arrest but failed to differentiate.

Clonal expansion has also been demonstrated during adipose development in vivo. Pulse-chase experiments using [^{3}H]thymidine were conducted with mice (135) to compare the mitotic activity of undifferentiated mesenchymal cells, partially differentiated preadipocytes, and fully differentiated adipocytes. Mitotic indices were highest in partially differentiated preadipocytes, a result consistent with findings in cell culture that clonal expansion precedes expression of the adipocyte phenotype (12). Cook & Kozak confirmed these findings with newborn mice (35). They observed a dramatic increase in expression of the adult isoform of glycerol-3-phosphate dehydrogenase (G3PDH) coincident with terminal differentiation of adipocytes and the appearance of cytoplasmic lipid droplets three days after birth. Only cells that had replicated their DNA expressed G3PDH, which verified that mitosis precedes adipose gene expression in vivo.

EXPRESSION OF ADIPOCYTE PROTEINS As clonal expansion slows and preadipocytes achieve growth-arrest (Stage V; Figure 1), expression of proteins that give rise to the adipocyte phenotype is initiated (Table 1). Among the first of these proteins to be expressed is C/EBPα, a nuclear DNA–binding protein that has been implicated in the coordinate transcriptional activation of adipose-specific genes (reviewed in 193; see section entitled "Transcriptional Control of Adipocyte Genes During Differentiation"). C/EBPα, which is antimitogenic, has also been implicated in the termination of clonal expansion and maintenance of the terminally differentiated state. The appearance of C/EBPα slightly precedes the expression of adipocyte proteins (30). Based on two-dimensional electrophoretic analysis of cell extracts before, during, and after adipose conversion, Sadowski et al estimated that the cellular levels of at least 100 proteins change within 5 h of initiating differentiation and that the levels of at least 200 additional proteins are altered by the time terminal differentiation has occurred (152). Although the levels of most of these proteins increase, the levels of some (e.g. actin and tubulin) decrease (12, 36, 172). In addition, a number of isoform/isozyme switches occur during differentiation, e.g. the embryonic isozyme of G3PDH is found in preadipocytes, whereas the adult isozyme is present in adipocytes (207). Many of the proteins differentially expressed during preadipocyte differentiation have been identified (Table 1).

Terminal Differentiation

For a period of time after differentiation has been initiated (i.e. early in Stage V; Figure 1), cells can apparently dedifferentiate and reenter mitosis. Dedifferentiation can be initiated by disrupting cell-cell contact or by exposing cells to certain agents, e.g. retinoic acid (84). Experiments with Balb/c 3T3 cells

(54, 195, 204) suggest that once cells have gone beyond a specific stage, they can no longer undergo dedifferentiation but rather are committed to terminal differentiation. Wier & Scott (203) purified a 45-kDa protein (referred to as aproliferin) from serum that can induce terminal differentiation. Certain other agents, e.g. TNFα, suppress induction of terminal differentiation of Balb/c 3T3 cells but do not cause rapid regression of the adipocyte phenotype (54). In 3T3-L1 and certain other cell types, however, TNFα causes regression of the adipocyte phenotype (179).

TRANSCRIPTIONAL CONTROL OF ADIPOCYTE GENES DURING DIFFERENTIATION

During differentiation there is a coordinate increase in the expression of most adipocyte proteins and a comcomitant decrease in the expression of some preadipocyte proteins (Table 1). Alteration of the rate of transcription of the corresponding genes accounts for almost all of these changes. In a few cases, however, altered mRNA stability is responsible for the changes in expression of the corresponding protein [e.g. fatty acid synthase (118) and actin and tubulin (12)] that take place during differentiation. Considerable effort has therefore been made to identify *cis*-promoter elements and cognate *trans*-acting proteins that control transcription of genes whose expression is altered during differentiation.

Although many *trans*-acting proteins have been identified that bind to specific elements within the promoters of adipocyte genes, a much smaller number [i.e. adipose regulatory factor-6 (ARF-6), C/EBPα, preadipocyte repressor element (PRE)-binding protein, C/EBPα undifferentiated protein (CUP), and ADD1] undergo differentiation-induced changes in expression. Members of the latter group of *trans*-acting factors regulate transcription of adipocyte genes by different mechanisms, including (*a*) interaction with a cell type–specific enhancer element, e.g. ARF-6 binds to an adipose-specific enhancer within an adipocyte gene(s), allowing transcription only in adipocytes (68); (*b*) *trans*-activation, e.g. C/EBPα serves as a pleiotropic transcriptional activator of a group of adipocyte genes, thereby coordinating their expression (32, 86); and (*c*) repression, e.g. the PRE-binding protein appears to maintain an adipocyte gene in the repressed state until differentiation is initiated (182).

The following section describes the characteristics of these *trans*-acting proteins and their presumptive roles in the program of transcriptional activation of genes expressed during adipocyte differentiation.

Trans-*Acting Factors*

THE C/EBP GENE FAMILY Members of the C/EBP family of transcription factors share amino acid sequence similarity within their C-terminal basic

region/leucine zipper (bZIP) domains and bind to the same *cis*-regulatory elements within the promoters of many adipocyte genes. C/EBP homologues are members of a larger class of eukaryotic proteins that contain the bZIP DNA-binding motif (100, 102-104). The bZIP element consists of 55–65 amino acids located near the C-terminus. Leucine zipper domains pack as parallel α helical coiled coils, allowing formation of homo- and heterodimers between C/EBP homologues as well as with certain other bZIP proteins. If one visualizes the leucine zipper dimerization interface as the stem of the letter Y, the basic DNA-binding region forms the bifurcating arms that pass through the major groove of each C/EBP half site and grip the DNA (50, 126, 194). The N-terminal region (50–75% of the molecule) appears to contain the domain(s) needed for *trans*-activation (58) and, in the case of C/EBPα, control of mitogenesis (110). This N-terminal region might be visualized as an extension of each bifurcating arm.

The characteristics of the C/EBP isoforms relevant to adipose development are summarized in Table 2. Three additional C/EBP homologues—D-site-binding protein (DBP; 119), immunoglobulin/enhancer-binding protein (Ig/EBP1; 145), and a1/EBP (15)—are not included because they have not been analyzed with respect to adipocyte development. We adopted the nomenclature proposed by Cao et al (21) and extended this usage to include a more recently discovered isoform.

C/EBPα C/EBPα has been implicated as a pleiotropic transcriptional activator of adipocyte genes during preadipocyte differentiation. Expression of C/EBPα is limited to tissues with high lipogenic capacity, including adipose, liver, intestine, and lung (14). The first indication that C/EBPα plays a regulatory role in adipocyte differentiation was its increased expression during adipose conversion of 3T3-L1 preadipocytes (14, 32) due to transcriptional activation of the C/EBPα gene (30). During the course of preadipocyte differentiation, C/EBPα expression is initiated as clonal expansion ceases (192), which occurs just prior to the coordinate expression of a group of adipocyte genes (i.e. 422/aP2, SCD1, GLUT4, insulin receptor, FSP27, angiotensinogen, and PEPCK; 17, 32, 39, 86, 116, 130; Table 1), many of which possess C/EBP binding sites. DNAseI protection studies revealed that nuclear extracts from differentiated 3T3-L1 adipocytes—but not from undifferentiated preadipocytes—footprinted sites within the promoters of three differentiation-induced genes (422/aP2, SCD1, and GLUT4) that possess a high degree of sequence identity (32, 86). The fact that C/EBPα footprinted these sites suggests that C/EBPα might coordinately regulate expression of these genes. Both cotransfection of chimeric promoter-reporter gene constructs into 3T3-L1 preadipocytes with C/EBPα expression vectors (32, 86) and cell-free transcription with recombinant C/EBPα (28) demonstrated that C/EBPα can *trans*-activate the

Table 2 Homologues of C/EBP implicated in adipocyte differentiation

C/EBP	Alternative translation product	Other names	Molecular mass[a] (kDa)	Dimerization	DNA binding	*Trans*-activation (▲) or *trans*-inhibition (▼)	References
α	$p42^{C/EBP\alpha}$	—	38	+	+	▲	102
	$p30^{C/EBP\alpha}$	—	30	+	+	▲,▼[b]	110, 128
β	LAP	NFIL6, AGP/EBP, CRP2, IL6DBP	31	+	+	▲	3, 21, 25, 44, 136, 206
	LIP	—	18	+	+	▼	45
δ		CRP3, NFIL6β, CELF	29	+	+	▲	21, 91, 96, 206
ϵ		CHOP-10 *gadd*153	19	+	–	▼	55, 146

[a] Predicted from the cDNA sequence.
[b] Effect appears to depend on gene promoter and cell type (see text).

promoters of all three genes. Moreover, mutation of the C/EBP binding site within these promoters blocks *trans*-activation.

Although compelling, the evidence implicating C/EBPα as an essential transcriptional activator for adipocyte differentiation was indirect. Definitive proof was obtained by transfecting C/EBPα antisense RNA expression vectors into 3T3-L1 preadipocytes to block expression of C/EBPα during differentiation (109, 154). Specifically inhibiting expression of C/EBPα blocked the transcription of several adipocyte genes (422/aP2, SCD1, and GLUT4), and the cells lost their ability to accumulate cytoplasmic triacylglycerol (109). However, blocking expression of C/EBPα did not prevent expression of an early adipocyte marker, LPL (154). Rescue of the adipocyte phenotype was accomplished by transfection of cells expressing C/EBPα antisense RNA with another vector that directs transcription of the complementary sense C/EBPα RNA (109). Taken together, these findings show that C/EBPα plays a vital role in the coordinate transcriptional activation of a family of adipocyte genes during differentiation of preadipocytes into adipocytes.

Although expression of C/EBPα is required for adipocyte differentiation, it does not appear sufficient to induce differentiation. Attempts to assess the effect of C/EBPα on differentiation (and other functions) by constitutively expressing C/EBPα in preadipocytes have been unsuccessful (RJ Christy, F-T Lin, MD Lane, unpublished data; 188) because C/EBPα is antimitogenic; such stable cell lines therefore cannot be propagated. However, Umek et al (188) obtained a single transfected 3T3-L1 subline that constitutively expressed a conditionally active C/EBPα-estrogen receptor fusion protein. Activation of the fusion protein by treatment of the cells with estrogen blocked mitosis but did not induce adipocyte differentiation or expression of 422/aP2 mRNA. When cells expressing the fusion protein were induced using a standard differentiation protocol supplemented with estrogen, expression of 422/aP2 mRNA was enhanced. Similar findings were obtained by Lin et al (110), who constitutively expressed a truncated isoform of C/EBPα (see below) that is not antimitogenic but that nonetheless retains the ability to *trans*-activate adipocyte genes. Surprisingly, Freytag & Geddes (57) were able to transfect 3T3-L1 preadipocytes and to select foci (8–200 cells per focus) that expressed C/EBPα. Although these foci could not be further propagated, they did undergo spontaneous conversion into adipocytes. How the cells escaped the antimitotic effect of C/EBPα during proliferation into foci remains unclear, but these findings are consistent with the large body of evidence that C/EBPα plays a key role in the differentiation process.

Two isoforms of C/EBPα, a 42-kDa ($p42^{C/EBP\alpha}$) and a 30-kDa ($p30^{C/EBP\alpha}$) form, are expressed at similar levels in adipose tissue, liver, and 3T3-L1 adipocytes, all of which express a single species of C/EBPα mRNA (110). Mutational analysis of C/EBPα mRNA revealed that the full-length 42-kDa

and the 30-kDa proteins are alternative translation products initiated at the first and third methionine codons, respectively (110). Because the first four methionine codons in the message possess unfavorable Kozak consensus sequences for translational initiation, leaky ribosomal scanning likely leads to initiation at the third AUG codon. Moreover, because $p42^{C/EBP\alpha}$ and $p30^{C/EBP\alpha}$ possess identical bZIP domains, differential expression of these isoforms might serve a regulatory purpose. Similarly, alternative translation products—liver activator protein (LAP, or C/EBPβ) and liver inhibitory protein (LIP)—of the C/EBPβ message have been identified (44, 45; see below). Both LAP and LIP possess identical DNA-binding and leucine zipper dimerization domains and thus can form heterodimers; however, only LAP possesses a *trans*-activation domain. Hence, LIP acts as a dominant transcriptional repressor and can attenuate transcriptional activation by LAP.

Of interest in this context are the functional differences between the two C/EBPα isoforms. For example, $p42^{C/EBP\alpha}$ is antimitotic in 3T3-L1 preadipocytes (188), whereas $p30^{C/EBP\alpha}$ is not (110). Transfection of 3T3-L1 preadipocytes with a $p30^{C/EBP\alpha}$ expression vector did not interfere with cell proliferation, nor did it alter the doubling time of the cells. Moreover, although both isoforms appear to *trans*-activate certain adipocyte gene promoters (i.e. 422/aP2 and C/EBPα; 110), $p42^{C/EBP\alpha}$ *trans*-activates the hepatic albumin gene promoter, but $p30^{C/EBP\alpha}$ does not (128). These results suggest that different regions of the C/EBPα molecule are required for different biological functions. The N-terminal 12 kDa of $p42^{C/EBP\alpha}$ appears necessary for antimitotic activity of C/EBPα and its ability to *trans*-activate the albumin gene promoter but is not required for *trans*-activation of at least two differentially expressed adipocyte promoters (i.e. 422/aP2 and C/EBPα). This finding is notable because Friedman & McKnight (58) and Pei & Shih (133) identified two regions within the amino acid sequence of $p42^{C/EBP\alpha}$ that are required for *trans*-activation of the albumin gene promoter in hepatoma cells, i.e. residues 1–107, which are not found in $p30^{C/EBP\alpha}$, and residues 175–245, which are present in this isoform. This apparent discrepancy in the requirements for *trans*-activation could be due to differences between promoters or the cell types employed.

Homologues of C/EBP Following the discovery of C/EBPα, a number of C/EBP homologues were identified (see Table 2). A new gene family was thus defined whose members possess extensive amino acid sequence identity within the bZIP domain but little sequence similarity in other regions of the molecule that contain the regulatory domain(s) for *trans*-activation. C/EBP homologues have been found to heterodimerize with one another in vitro and in intact cells transfected with multiple C/EBP expression vectors (146, 206) or in liver nuclei (128). Certain C/EBP isoforms possess dimerization domains but lack either a *trans*-activation domain (i.e. C/EBPβ-LIP; 45) or a functional DNA-binding

domain [i.e. C/EBP homologous protein-10 (CHOP-10); 146]. Heterodimerization with either of these isoforms gives rise to dominant-negative inhibitors of transcription. Thus, C/EBPβ-LIP is an alternative translation product of C/EBPβ mRNA and lacks the amino-terminal half of the full-length translation product (C/EBPβ-LAP; 44) in which the regulatory domain resides. Cotransfection experiments indicate that although the LAP/LAP homodimer can *trans*-activate the albumin gene promoter, neither the LIP/LIP homodimer nor the LAP/LIP heterodimer is active (45). The LAP:LIP ratio in liver nuclei increases during development (45), and in 3T3-L1 cells the ratio increases during adipocyte differentiation (P Cornelius, MD Lane, unpublished data). The physiological significance of these changes remains to be determined.

Increasing evidence suggests that cross-talk occurs between C/EBP family members and other transcription factors, including members of the NF-κB/Rel (106, 177), the ATF/CREB (190), and the steroid hormone (glucocorticoid) receptor families (122). Although functional interactions of this type are important in some cell types, they have not been implicated in adipocyte development.

The C/EBPα Promoter Because transcription of the C/EBPα gene is activated during adipose conversion (30), regulatory elements within the promoter likely play a role in activating its transcription during differentiation. A number of potential binding sites for known transcription factors are present in the promoter of the C/EBPα gene, including binding sites for USF, NF1, Sp1, C/EBP, c-*myc*, Zif-268/Krox 20, basal transcription element (BTE), or any of their homologues (30, 107, 192), as well as a novel nuclear factor designated CUP (192). The c-*myc* site may contribute to suppression of the C/EBPα gene during mitosis, since enforced expression of c-*myc* inhibits expression of C/EBPα and adipose conversion (56). Two other sites, which bind nuclear factors expressed reciprocally during adipocyte conversion (i.e. C/EBPα and CUP), have been implicated in the differential expression of C/EBPα (30, 110, 192).

One of these *cis*-elements binds C/EBPα (30) and appears to mediate transcriptional autoactivation by its own gene product. Two lines of evidence support this view. First, blocking expression of C/EBPα posttranscriptionally with antisense C/EBPα RNA prevents transcription of the gene (109). Second, C/EBPα can *trans*-activate its own promoter (110). The C/EBPα promoter also contains a bipartite element with binding sites for CUP and an Sp1-like GT-box-binding protein (192). CUP has been purified extensively and seems to bind synergistically with the Sp1-like protein to the bipartite element. Preliminary evidence indicates that a protein complex containing CUP bridges between the CUP/Sp1-like GT-box element and a downstream element that

contains the C/EBPα-binding site. It has been suggested that bridging by this complex may maintain the C/EBPα gene in a repressed state (192, 193).

ADIPOSE TISSUE–SPECIFIC ENHANCER An adipose tissue–specific enhancer for the 422/aP2 gene has been identified and mapped to a region located ~ 5 kb 5′ to the transcriptional start site of the gene (149). When linked to a minimal promoter derived from the 422/aP2 gene, this 518-bp enhancer was sufficient to specifically direct reporter gene expression to adipose tissue in transgenic mice (149) and to activate differentiation-dependent reporter gene expression in 3T3-F442A preadipocytes (67). Further analysis identified 5 nuclear protein–binding sites, localized within a 183-bp segment at the 5′ end of the enhancer, that accounted for most of the enhancer activity (68). These sites have been designated as adipose regulatory elements ARE-1 (a NF-1 element), -2, -4, -6, and -7. Although mutation of the ARE-1 (NF-1) site reduced enhancer activity, only minimal differences were detected when preadipocyte and adipocyte nuclear protein binding at this site were compared (67). Two other nuclear factors have been identified: one that binds to ARE-2 and -4 and another, adipose regulatory factor-6 (ARF-6), that binds to ARE-6 and -7. ARE-6-binding activity was enriched in nuclear extracts from adipocytes compared with extracts from preadipocytes or nonadipogenic cell types (68). Consistent with these results, mutation of ARE-6 markedly reduces enhancer activity in adipocytes. Purification and further characterization of ARF-6 are necessary to ascertain the exact role of this factor. Whether the 422/aP2 adipose-specific enhancer is functionally associated with other adipocyte genes is also of interest.

ADD1 The gene encoding a novel basic region/helix-loop-helix domain (bHLH) protein, designated adipocyte determination and differentiation factor 1 (ADD1), was recently cloned from a rat adipocyte cDNA library (186). Members of the bHLH family of transcription factors, which includes MyoD (49), function as regulators of lineage-specific gene expression and as coordinators of mesoderm differentiation (49). ADD1 mRNA is expressed at low levels in several rat tissues, including white adipose tissue, liver, kidney, intestine, and thymus, but is expressed at high levels in brown adipose tissue. 3T3-F442A preadipocytes as well as nondetermined 10T1/2 cells express low levels of ADD1 mRNA. Upon differentiation of 3T3-F442A and determined 10T1/2 preadipocytes into adipocytes, expression of the ADD1 mRNA increased. However, confluent 3T3-C2 cells, which cannot differentiate into adipocytes, did not express ADD1 mRNA. On the basis of these findings, Tontonoz et al suggested that ADD1 may function both as a determination factor and a differentiation factor (186). Although a target gene for ADD1 has not been identified, a promoter element from the fatty acid synthase gene (an

adipocyte gene) can mediate *trans*-activation by ADD1. Further work is required to determine whether ADD1 plays a significant role either in determination or differentiation.

HNF3/*forkhead* Transcriptional activation of the LPL gene occurs earlier in the adipocyte differentiation program than that of C/EBPα (and most other adipocyte genes; see Figure 1) and therefore must be regulated by different transcriptional signals. Enerback et al (51) identified two potential regulatory regions, designated LP-α and LP-β, within the promoter of the LPL gene. Nucleotide sequence analysis of these regions revealed consensus-binding sites for members of the HNF3/*forkhead* family of transcription factors (33, 89, 199). Gel retardation studies with oligonucleotides corresponding to these sequences verified the presence of HNF3/*forkhead*-like factors in nuclear extracts from 3T3-F442A cells and confirmed that the binding activity of these nuclear factors increased during adipocyte differentiation (51). Furthermore, multimers of the LP-α or LP-β elements confer differentiation-dependent expression to heterologous promoter-reporter gene constructs transfected into 3T3-F442A cells (51). The probable importance of the HNF3/*forkhead* family of transcription factors in adipocyte differentiation is underscored by their role in regulating hepatic gene expression (99) and in embryonic development of tissues derived from the primitive gut in *Drosophila* (200).

REPRESSORS AND DEREPRESSION OF ADIPOCYTE GENE EXPRESSION Increasing evidence indicates that the timing of gene expression during adipocyte differentiation is regulated not only by transcriptional activators, but also by release from negative control. Two types of negative control factors for adipocyte genes have been identified: (*a*) repressors, which bind to a specific promoter element, thereby silencing gene expression and (*b*) dominant-negative factors such as C/EBPβ-LIP or CHOP-10, which bind competitively to a transcriptional activator (i.e. another C/EBP isoform), rendering it inactive.

A *cis*-acting preadipocyte repressor element (PRE) has been identified in the promoter of the stearoyl-CoA desaturase-2 (SCD2) gene that contributes to its repression prior to adipocyte differentiation (182). A 58-kDa nuclear protein expressed by 3T3-L1 preadipocytes but not present (or inactive) in adipocytes binds specifically to the PRE. When inserted 5′ to the SV40 enhancer/promoter of $_pSV_2CAT$, a single copy of the PRE element strongly represses transcription of the reporter gene in preadipocytes and HeLa cells, but not in adipocytes. These findings suggest that the PRE and its cognate-binding protein maintain the SCD2 gene—and possibly other genes—in a repressed state until adipocyte differentiation is initiated. The promoter of the SCD1 gene, whose expression is also induced during adipocyte differentiation (125), contains an element similar to the PRE (KT Landschulz, MD Lane,

unpublished data). The PRE has not been found in other adipocyte genes, although the core nucleotide sequence of the PRE resembles a repressor element in the β-interferon promoter, to which the repressor protein PRD1-BF1 binds (95).

Repression of transcription also appears to be involved in differentiation-induced expression of the 422/aP2 (144, 213) and adipsin (205) genes. Deletion analysis demonstrated (213) that the proximal promoter of the 422/aP2 gene contains overlapping negative and positive regulatory elements. The positive regulatory element contains a consensus activator protein 1 (AP-1)–binding sequence. The effect of the negative regulatory element is observed in preadipocytes but not in fully differentiated adipocytes, which suggests that it is an important component of the control mechanism that regulates expression of the 422/aP2 gene during differentiation. cAMP activates the 422/aP2 promoter in confluent preadipocytes but not in proliferating preadipocytes or fully differentiated adipocytes. Deletions that enter the negative element abolish the stimulatory effect of cAMP. This observation indicates that cAMP increases expression by relieving the inhibitory effect of the negative regulatory element. Because cAMP (MIX) induces adipocyte differentiation, this cAMP effect may contribute to expression of the 422/aP2 gene during adipose conversion.

Two members of the C/EBP gene family discussed above, C/EBPβ-LIP and CHOP-10, can heterodimerize with various other C/EBP family members (see section entitled "Transcriptional Control of Adipocyte Genes During Differentiation"). Because C/EBPβ-LIP (45) lacks a *trans*-activation domain and CHOP-10 (146) lacks a functional DNA-binding domain, heterodimers containing either of these homologues are inactive. One would expect a sufficiently high level of expression of either of these homologues prior to or during differentiation to exert a dominant-negative effect on transcription activated by another C/EBP isoform. Although expression of the C/EBPβ message (which encodes both the LAP and LIP alternative translation products) increases during adipocyte differentiation (21; P Cornelius, MD Lane, unpublished data), as does the LAP:LIP ratio (P Cornelius, MD Lane, unpublished data), investigators have not been able to ascribe a role for these changes in the differentiation process.

Based on its ability to interact with the leucine zipper domain of C/EBPβ, CHOP-10 was cloned from a 3T3-L1 adipocyte library (146). CHOP-10 possesses >85% amino acid sequence identity to *gadd*153 and probably represents the mouse equivalent of CHOP-10. The *gadd*153 cDNA was cloned from a Chinese hamster ovary cell library while screening for genes induced upon growth arrest or DNA damage (55). The presence of proline and glycine residues in the basic region distinguishes CHOP-10 from other C/EBP homologues and interferes with its ability to bind DNA but does not alter its capacity to form heterodimers (146). CHOP-10 acts as a dominant-

negative inhibitor of the binding of C/EBPα or C/EBPβ to the C/EBP site in the angiotensinogen promoter and of their ability to *trans*-activate this promoter. Although CHOP-10 mRNA is differentially expressed upon adipose conversion of 3T3-L1 cells, its relevance to adipocyte differentiation remains unclear. Carlson et al (22) recently examined the regulation of CHOP-10 expression in both 3T3-L1 and HeLa cells and observed that accumulation of CHOP-10 transcripts was inversely related to glucose concentration of the culture media. Addition of glucose to the medium of 3T3-L1 adipocytes (or HeLa cells) dramatically repressed CHOP-10 gene expression (22). Thus, expression of CHOP-10 could be dissociated from adipocyte differentiation by maintaining a high glucose concentration in the culture media. Whether CHOP-10 is expressed in adipose tissue is unknown, although it is expressed in many other rat tissues (146).

c-*myc* is involved in the regulation of cellular proliferation, and its overexpression promotes transformation (reviewed in 93). The forced expression of c-*myc* in 3T3-L1 preadipocytes blocks adipocyte differentiation without causing transformation (56). Because the C/EBPα promoter contains a consensus c-*myc*-binding site (30) that can bind c-*myc* in vitro (107), the inhibitory effect of c-*myc* on differentiation may be mediated by binding to its cognate site in the C/EBPα promoter.

Expression of Transcription Factors During Development

The temporal pattern of expression of the transcription factors relevant to adipocyte development is illustrated in Figure 2 and discussed below in the context of the development program and the known actions of these factors. Based on the 10T1/2 cell model for myogenic determination, expression of a unique transcription factor(s) likely commits presumptive stem cells to the adipogenic lineage (Stage I; Figure 2). Although an equivalent of the transcription factors (e.g. myogenin and MyoD) for myogenic determination has not been identified for the adipose lineage, unique members of the HNF3/*forkhead* family (51) or the bHLH family (ADD1) are possible candidates (186). Members of both of these gene families participate in early events required for normal organ/tissue development in *Drosophila.*

Confluent preadipocytes (Stage III; Figure 2) express low levels of several C/EBP homologues, i.e. C/EBPβ, C/EBPδ, and CHOP-10, as well as ADD1 and HNF3/*forkhead* homologues. During this period, the gene encoding C/EBPα is maintained in the repressed state (30), perhaps by CUP, whose activity is found only in adipoblasts and preadipocytes (192, 193).

When differentiation is induced at the Stage III/IV border (Figure 2), several events occur in rapid succession, causing entry into the cell cycle. A transient increase occurs in the expression of several immediate-early genes (e.g. c-*fos,* c-*jun,* and c-*myc;* 178, 180) that coincides with a dramatic increase in the

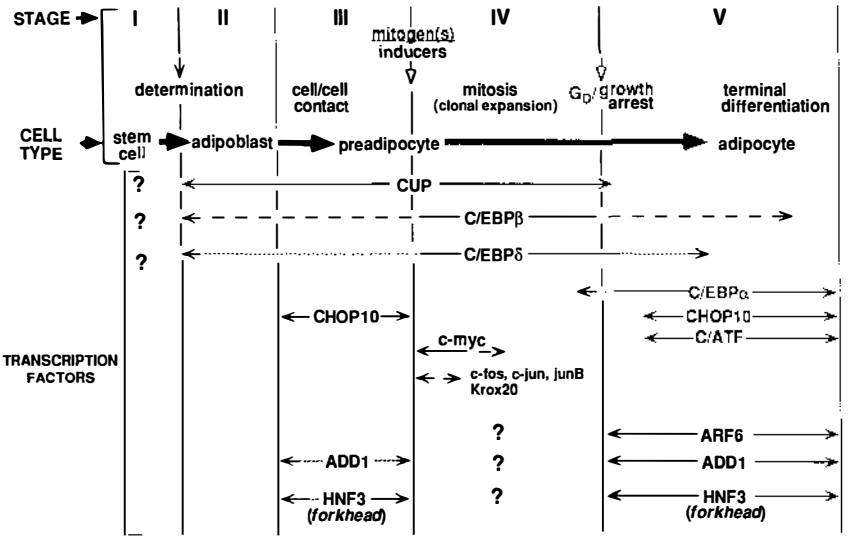

Figure 2 Expression of transcription factors during adipocyte development.

expression of C/EBPβ and C/EBPδ, which persists for several days (21; P Cornelius, MD Lane, unpublished data). During this period, preadipocytes undergo approximately two rounds of mitotic clonal expansion and then enter a growth-arrested state (G_D; 159, 160, 195) permissive for terminal differentiation. As the cells undergo growth arrest, CUP activity decreases (192, 193), concomitant with transcriptional activation of the C/EBPα gene (30). Considerable evidence indicates that C/EBPα performs dual functions during adipocyte differentiation: (*a*) termination of clonal expansion and (*b*) coordinate transcriptional activation of a group of adipocyte genes.

As discussed above, C/EBPα is antimitotic and thus may be responsible for inhibition of clonal expansion and maintenance of the quiescent state characteristic of terminally differentiated adipocytes. Following the expression of C/EBPα, transcriptional activation of a large number of adipocyte genes occurs (Table 1). Many of these genes (i.e. 422/aP2, SCD1, GLUT4, insulin receptor, FSP27, PEPCK, and angiotensinogen; 17, 32, 39, 86, 116, 130) are *trans*-activated by C/EBPα. The exact number of adipocyte genes regulated by C/EBPα during differentiation remains unknown but is estimated to be large. The C/EBPα promoter possesses a C/EBP binding site (30) that apparently mediates *trans*-activation by its own gene product (107, 110), which suggests that autoactivation may be responsible for the sustained expression of C/EBPα and thereby for maintenance of the quiescent adipocyte phenotype.

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