Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes

Fang-Tsyr Lin and M. Daniel Lane¹

Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 USA

Previous studies suggest that the CCAAT/enhancer-binding protein (C/EBP) functions in the coordinate expression of adipocyte genes during differentiation of 3T3-L1 preadipocytes. We sought to block expression of C/EBP selectively using a bovine papilloma virus (BPV) vector to direct transcription of a ~0.4-kb segment of C/EBP cDNA (in antisense orientation) containing translated sequence 5' to that encoding the basic and leucine zipper regions of the protein. Vector-directed expression of antisense C/EBP RNA in 3T3-L1 preadipocytes inhibited expression of C/EBP mRNA and protein, as well as several adipose-specific mRNAs, and also prevented cytoplasmic triglyceride accumulation. Rescue of the "adipocyte phenotype" was accomplished by transfection of cells expressing antisense RNA with a modified BPV vector that directs transcription of the complementary sense C/EBP RNA.

[*Key Words:* C/EBP; adipocyte genes; 3T3-L1 preadipocytes]

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With appropriate hormonal stimulation, confluent 3T3-L1 preadipocytes in cell culture differentiate into cells possessing the biochemical and morphological characteristics of adipocytes (Green and Kehinde 1974, 1975, 1976; Mackall et al. 1976; Coleman et al. 1978; Rosen et al. 1979; Reed and Lane 1980; Student et al. 1980). Acquisition of the adipocyte phenotype is preceded by a dramatic and coordinate rise in the transcription of a group of adipose-specific genes including the 422(aP2), SCD1, and GLUT4 genes (Bernlohr et al. 1985; Cook et al. 1985; Kaestner et al. 1990). In several laboratories considerable effort has been directed at defining the basis of the coordinate differentiation-linked transcriptional activation of such adipocyte-specific genes. The promoters of several of these genes have been analyzed (Phillips et al. 1986; Cook et al. 1988; Ntambi et al. 1988; Kaestner et al. 1990) and shown to drive the expression of reporter genes when subjected to agents that act as inducers of preadipocyte differentiation (Cook et al. 1988; Ntambi et al. 1988).

Recent evidence has implicated the CCAAT/enhancer-binding protein (C/EBP) in the transcriptional activation of adipocyte-specific genes during the differentiation of 3T3-L1 preadipocytes. C/EBP was identified as

a differentiation-induced nuclear factor that binds specifically to the promoters of three genes [the 422(aP2), SCD1, and GLUT4 genes] that are coordinately expressed when 3T3-L1 preadipocytes differentiate into adipocytes (Christy et al. 1989; Kaestner et al. 1990). Consistent with a role for C/EBP in coordinating adipocyte gene expression, the C/EBP gene is activated transcriptionally just before expression of 422(aP2), SCD1, and GLUT4 mRNAs (Birkenmeier et al. 1989; Christy et al. 1989). Both in intact 3T3-L1 cells (Christy et al. 1989; Kaestner et al. 1990) and with a cell-free transcription system, it was demonstrated further (Cheneval et al. 1991) that C/EBP can function as a *trans*-activator of the promoters of these genes. Mutation of the C/EBP-binding site within these gene promoters obliterates *trans*-activation (Christy et al. 1989; Herrera et al. 1989; Cheneval et al. 1991). Moreover, the premature activation (by estrogen) of a constitutively expressed, but inactive, C/EBP-estrogen receptor fusion protein caused premature expression of 422(aP2) mRNA relative to the normal differentiation program (Umek et al. 1991). These findings provide compelling, although circumstantial, evidence for the involvement of C/EBP in differentiation-induced gene expression in 3T3-L1 preadipocytes.

The aim of the present investigation was to block expression of C/EBP selectively during preadipocyte differ-

¹Corresponding author.

entiation with an antisense C/EBP RNA expression vector and to assess the effects of this blockade on the transcription of adipocyte genes. To obtain a high level of expression of the antisense or sense C/EBP RNA, we used a bovine papilloma virus (BPV) expression vector that is carried at a high copy number in animal cells. This vector, pBCMGNeo (Karasuyama et al. 1989), contains a 69% BPV subgenomic fragment (Lowy et al. 1980) that is maintained as a multicopy episome (Law et al. 1981; Sarver et al. 1981; DiMaio et al. 1982). The vector also contains a strong human cytomegalovirus (CMV) promoter with which to drive expression of inserted cDNAs (Boshart et al. 1985). In this paper we demonstrate that expression of antisense C/EBP RNA in 3T3-L1 preadipocytes blocks the expression of several adipocyte genes and prevents cytoplasmic triglyceride accumulation. Rescue of the "adipocyte phenotype" was accomplished by transfection with another vector that directs the transcription of sense C/EBP RNA in cells expressing the antisense RNA.

Results

Transfection of 3T3-L1 cells with expression vectors encoding partial antisense and sense C/EBP RNAs

Previous investigations have provided indirect evidence for the involvement of C/EBP in the transcriptional activation of the promoters of several adipocyte genes in 3T3-L1 preadipocytes (Christy et al. 1989; Kaestner et al. 1990). To test directly the hypothesis that C/EBP is necessary for coordinate expression of these genes during preadipocyte differentiation, we sought to block expression of C/EBP specifically with an antisense C/EBP RNA expression vector. Therefore, a BPV vector containing a segment of the C/EBP cDNA in the antisense orientation was constructed.

To construct the antisense C/EBP expression vector, pBCMGAS, the ~0.4-kb *HincII*–*SmaI* segment of the C/EBP gene containing translated sequence 5' to that encoding the basic and leucine zipper regions of the protein was inserted into the *XhoI* site of the pBCMGNeo expression vector (Karasuyama et al. 1989) in the antisense (or sense; see below) orientation placing it under the control of the human cytomegalovirus (CMV) gene promoter (Fig. 1). Because the nucleotide sequence of the ~0.4-kb segment of the C/EBP gene is not homologous to other members of the C/EBP gene family or to other proteins in the GenBank rodent data base, expression of the antisense RNA would be expected to interfere selectively with transcription of the C/EBP gene and the translation or stability of its mRNA. Importantly, the parental pBCMGNeo vector contains the necessary BPV sequences for extrachromosomal (nuclear episomal) replication of multiple copies of the vector in mammalian cells. The vector also contains a gene for selection of resistance to neomycin.

The antisense C/EBP expression vector, pBCMGAS, or the pBCMGNeo vector that lacks the insert, was transfected into 3T3-L1 preadipocytes by electroporation after

which selection with G418 was performed. Three neomycin-resistant clonal cell lines (AS1, AS2, and AS3 cells) that harbored the pBCMGAS vector were selected for further analysis. Southern blots of *XhoI* digests of low-molecular-weight DNA isolated from AS1, AS2, and AS3 cells (Fig. 2A) indicated the presence of the extrachromosomal vector containing the ~0.4-kb insert of the C/EBP gene, with AS3 cells exhibiting the highest content of the antisense construct. The relative intensities on Southern blots (quantitated by laser densitometric scanning) of the 0.4 kb-band derived from low-molecular-weight cellular DNA were compared with that derived from a known quantity of pBCMGAS DNA. We estimate that AS1, AS2, and AS3 cells contain at least five, four, and eight copies, respectively, of extrachromosomal pBCMGAS per cell (Table 1). This is likely to be an underestimate in view of the typically low recovery (<50%) of extrachromosomal DNA.

In addition to being replicated extrachromosomally, after multiple passages of AS1, AS2, and AS3 cells, the antisense C/EBP expression vector also became integrated into chromosomal DNA (Table 1). Thus, digestion of high-molecular-weight chromosomal DNA with *XhoI*, which removes the ~0.4-kb antisense C/EBP insert from the vector, gave rise to the ~0.4-kb fragment, as well as an ~11-kb fragment derived from the endogenous C/EBP gene (Fig. 2B). Quantitation of the relative intensities of the two bands indicates that AS1, AS2, and AS3 cells contain ~4, 10, and 9 copies per cell, respectively, of an integrated form of the antisense C/EBP expression vector. In all experiments described below, cells that had been passaged 15–20 times were used.

Expression of antisense C/EBP RNA and its effect on the expression of C/EBP, 422(aP2), SCD1, and GLUT4

To ascertain whether 3T3-L1 cells harboring the pBCMGAS vector express antisense C/EBP RNA, RNA was isolated from both transfected and untransfected cells at various times (on days 0, 2, and 4) after the induction of differentiation. As indicated by RNase protection analysis (Fig. 3A), antisense C/EBP RNA was expressed in all three cell lines (i.e., AS1, AS2, and AS3 cells), the highest level of antisense C/EBP RNA occurring in AS3 cells followed by AS2 and AS1 cells. Northern blots confirmed that the antisense RNA expressed in AS3 was of the predicted size (results not shown).

Control cell lines transfected with the pBCMGNeo vector lacking an antisense or sense C/EBP insert expressed normal levels of C/EBP and 422(aP2) mRNA (Fig. 3B) and, as discussed below, differentiated into adipocytes. In contrast, cell lines harboring the pBCMGAS vector exhibited a reduced level of C/EBP mRNA that correlated closely with the level of expression of antisense C/EBP RNA (Fig. 3A). Thus, AS3 and AS2 cells that express the highest levels of antisense C/EBP RNA (as compared with AS1 cells) exhibited virtually complete suppression of endogenous C/EBP mRNA when compared with untransfected 3T3-L1 cells or cells transfected with the pBCMGNeo vector lacking an insert (Fig.

Figure 1. Construction of partial antisense and sense C/EBP RNA expression vectors. (A) Map of the murine C/EBP gene (from Christy et al. 1991). The open box corresponds to coding sequences; the hatched boxes correspond to the 5' or 3'-untranslated sequences. p6A-1 is a cDNA containing nucleotides +490 to the poly(A)⁺ tail of C/EBP cDNA (Christy et al. 1991), which was inserted into the *EcoRI* site of pBluescript. A ~0.4-kb C/EBP fragment corresponding to nucleotides +490 to +870 of C/EBP cDNA was isolated by digestion of p6A-1 at the *HincII* site in pBluescript and the *SmaI* site of the translated sequences 5' to that encoding basic and leucine zipper regions of C/EBP. (B) The parental 14.8-kb pBCMGSNeo expression vector contains a 69% BPV subgenomic fragment permitting extrachromosomal replication and contains a neomycin-resistance gene for selection. The ~0.4-kb C/EBP fragment was inserted into the *XhoI* site of pBCMGSNeo in the sense or antisense orientation, placing these sequences under the control of human CMV gene promoter. The antisense plasmid, pBCMGSAS, and the control plasmid, pBCMGSNeo, were transfected into 3T3-L1 preadipocytes. A hygromycin-resistance gene was inserted into the sense plasmid, which is designated pBCMGS. The control vector, pBCMGSHyg, was obtained by removing the ~0.4-kb C/EBP insert from pBCMGS.

3B). AS1 cells that expressed the lowest level of antisense C/EBP RNA expressed a reduced, but significant, level of C/EBP mRNA (Fig. 3A). It should be noted that although antisense RNA can lead to a reduced cellular level of its corresponding mRNA (Knecht and Loomis 1987; Yokoyama and Imamoto 1987), it need not, as formation of RNA-RNA hybrids does not guarantee decreased message stability. Nevertheless, hybridization of antisense RNA to pre-mRNA or to fully spliced mRNA would be expected to interfere with translation (Izant and Weintraub 1984; Kim and Wold 1985; Melton 1985).

Consistent with *trans*-activation by C/EBP of the promoters of the 422(aP2), SCD1, and GLUT4 genes (Christy et al. 1989; Kaestner et al. 1990), blocking expression of the C/EBP gene appears to repress the differentiation-induced increases in the levels of 422(aP2), SCD1, and GLUT4 mRNAs. Cells harboring the an-

tisense C/EBP RNA expression vector exhibited markedly decreased levels of 422(aP2) mRNA, the level being lowest in cells expressing the highest level of antisense C/EBP RNA (Fig. 3A). Thus in the AS3 cell line in which antisense C/EBP RNA virtually eliminated expression of C/EBP mRNA, expression of 422(aP2) mRNA was almost completely suppressed (Fig. 3A).

Suppression of C/EBP and 422(aP2) mRNA levels caused by expression of C/EBP antisense RNA leads to a reduction of the cellular levels of the proteins that they encode. Thus, Western blot analysis of cell extracts revealed that cells harboring the antisense C/EBP RNA expression vector contained greatly reduced levels of C/EBP protein (Fig. 4A) and 422(aP2) protein (Fig. 4B) compared with wild-type 3T3-L1 adipocytes or 3T3-L1 adipocytes harboring the control BCMGSNeo vector. Of interest is the fact that expression of an apparent trun-

Figure 2. Southern blot analysis on DNA from the antisense (AS) cell lines. (A) Low-molecular-weight (extrachromosomal) DNA from two 100-mm dishes of confluent 3T3-L1 and AS cells and 1 ng of pBCMGAS were digested with *XhoI* and subjected to electrophoresis on 0.7% agarose gel. Southern blots were prepared and hybridized with the ^{32}P -labeled ~ 0.4 -kb C/EBP fragment obtained by digestion of pBCMGAS with *XhoI*. (B) High-molecular-weight (chromosomal) DNA (20 μg) prepared from 3T3-L1 and AS cells and 0.1 ng of pBCMGAS were digested with *XhoI* followed by Southern blot analysis using the same probe as in A.

cated ~ 30 -kD form of C/EBP is also markedly reduced in cells harboring the antisense RNA expression vector (Fig. 4A). We suggest that the 30-kD protein, which is recognized by antibodies against C/EBP, represents the product of translation initiated at a methionine that lies downstream from the methionine at which translation of "full-length" C/EBP is initiated. A similar situation in which more than a single translational initiation site is used has been demonstrated for the related liver-enriched transcriptional activator protein (LAP) (Descombes and Schibler 1991).

In addition to causing a decrease in the level of 422(aP2) mRNA, antisense C/EBP RNA—apparently by suppressing expression of C/EBP—also caused a decrease in the levels of SCD1 and GLUT4 mRNAs. As illustrated in Figure 5, AS1 and AS3 cells harboring the antisense C/EBP vector exhibited drastically reduced levels of SCD1 and GLUT4 mRNAs compared with control 3T3-L1 cells. Taken together, these results implicate C/EBP in the coordinate expression of a group of adipocyte genes during the differentiation of 3T3-L1 preadipocytes.

Effect of expression of antisense C/EBP RNA on the rates of nuclear run-on transcription of the C/EBP and 422(aP2) genes

To determine whether the decrease in message abundance caused by the expression of antisense C/EBP RNA was the result of a decrease in the rate of transcription of the C/EBP and 422(aP2) genes, nuclear run-on transcription experiments were performed. Accordingly, nuclei were isolated from control (i.e., untransfected and pBCMGNeo-transfected cells) and pBCMGAS-transfected 3T3-L1 cells on days 3 and 5 after the cells had

been subjected to the differentiation protocol. As shown in Figure 6, the amounts of both transcripts formed dur-

Table 1. Estimated copy number of chromosomal and extrachromosomal sense and antisense plasmids in transfected cell lines

Cell line	Low-molecular-weight (extrachromosomal) DNA		High-molecular-weight (chromosomal) DNA	
	antisense C/EBP	sense C/EBP	antisense C/EBP	sense C/EBP
AS1	≥ 5	—	4	—
Rev 1	≤ 5	≥ 20	1	3
Rev 2	≤ 5	≤ 5	0	1
Rev 3	≤ 5	≤ 5	2	4
AS2	≥ 4	—	10	—
AS3	≥ 8	—	9	—
Rev 4	≥ 8	≥ 8	9	21

Quantitation of the copy number of extrachromosomal and chromosomal pBCMGAS in AS cell lines was performed by densitometry of the autoradiograph shown in Fig. 2, using a known amount of pBCMGAS as standard. Similarly, low- and high-molecular-weight DNA from the revertant cell lines was digested either with *XhoI*, which revealed both the ~ 0.4 kb sense and antisense C/EBP fragments, or with *BamHI* and *HindIII*, which revealed a 0.96-kb fragment including ~ 0.4 kb of the sense C/EBP insert and 0.56 kb of the poly(A) $^{+}$ -containing segment of rabbit β -globin present in the vector. The copy number of pBCMGAS was calculated from the sum of the copy number of pBCMGAS and pBCMGAS (quantitated from the signals of the ~ 0.4 -kb *XhoI* fragments) minus the copy number of pBCMGAS (quantitated from the signals of the 0.96-kb *BamHI*–*HindIII* fragment).

Figure 3. Expression of antisense C/EBP RNA, C/EBP mRNA, and 422(aP2) mRNA in 3T3-L1 cells harboring the pBCMGS antisense expression vector. (A) The 3T3-L1, BCMGNeo (i.e., BCMGNeo control cell line; see Fig. 3B), and antisense (AS1, AS2, and AS3) cell lines were subjected to the differentiation protocol (see Materials and methods). Total RNA was isolated on day 0, 2, and 4 of induction of differentiation and subjected to RNase protection analysis. Total RNA (10 μ g) was hybridized with riboprobes specific for antisense C/EBP RNA, endogenous C/EBP mRNA, and 422(aP2) mRNA overnight at 54°C and treated with RNase A and T_1 . The protected radiolabeled fragments were subjected to electrophoresis on a sequencing gel. The protected segments of antisense C/EBP RNA, endogenous C/EBP mRNA, and 422(aP2) mRNA correspond to the 220-bp *HincII*-*MluI* fragment of C/EBP cDNA, the 280-bp *SmaI*-*SacI* fragment of C/EBP cDNA, and the 270-bp *HindIII*-*Sall* fragment of 422(aP2) cDNA, respectively. (B) Total RNA isolated from the control

3T3-L1 and BCMGNeo cell lines was isolated on day 0, 2, and 4 of differentiation. Total RNA (20 μ g) was denatured with glyoxal followed by electrophoresis on a 1% agarose gel. The Northern blot was prepared and hybridized with probes specific for C/EBP mRNA and 422(aP2) mRNA.

ing run-on transcription were markedly reduced in cells that express antisense C/EBP RNA (i.e., AS1, AS2, and AS3 cells) compared with control cells. As might be anticipated if C/EBP is a transcriptional activator of the 422(aP2) gene, the extent of suppression of run-on transcription of this gene was generally greater than that of the C/EBP gene. Consistent with results showing that the greatest decrease in the cellular abundance of the 422(aP2) message occurred in cells expressing the highest levels of antisense C/EBP RNA, that is, AS3 and AS2 cells (see Fig. 3A), the suppression of run-on transcription of the 422(aP2) gene was most marked in AS3 and AS2 cells (Fig. 6).

Effect of expression of antisense C/EBP RNA on the rate of cell proliferation and cytoplasmic triglyceride accumulation

After induction of differentiation, confluent 3T3-L1 preadipocytes undergo two to three rounds of mitosis, become quiescent, and then coordinately express the set of enzymes and proteins responsible for de novo cytoplasmic triglyceride synthesis (Mackall et al. 1976; Coleman et al. 1978; Bernlohr et al. 1985). Expression of C/EBP mRNA begins as preadipocytes reenter the quiescent state just before the onset of cytoplasmic triglyceride accumulation (Birkenmeier et al. 1989; Christy et al.

Figure 4. Expression of C/EBP and 422(aP2) proteins. 3T3-L1, BCMGNeo (i.e., BCMGNeo control cell line; see Fig. 3B), antisense (AS1 and AS3), and revertant (Rev 2 and Rev 4) cell lines were subjected to the differentiation protocol (see Materials and methods). After cell lysis, 250 μ g of cellular protein was subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis. (A) Western blot analysis of C/EBP with a primary antibody directed against a peptide corresponding to amino acid residues 253–265 of C/EBP. (B) Western blot analysis of 422(aP2) protein using an antibody directed against a peptide corresponding to the 14 carboxy-terminal amino acids of 422(aP2) protein.

Lin and Lane

Figure 5. Expression of GLUT4 and SCD1 mRNA. Total RNA (10 μ g) from the control 3T3-L1, AS1, AS3, and revertant cell lines (Rev 1, Rev 2, and Rev 4) was subjected to RNase protection analysis using sequence-specific riboprobes for GLUT4 and SCD1 mRNAs. The protected regions of GLUT4 and SCD1 mRNA correspond to a 180-bp *EcoRI*–*SmaI* fragment of GLUT4 cDNA and a 160-bp *EcoRI*–*PstI* fragment of SCD1 cDNA, respectively.

1989). Recent evidence has shown that C/EBP is anti-mitogenic (Umek et al. 1991) and, thus, may be responsible for entry of preadipocytes into the quiescent terminally differentiated state. Experiments were undertaken to determine whether inhibition of expression of C/EBP in 3T3-L1 cells harboring the antisense C/EBP expression vector affected their doubling time, morphology, and ability to accumulate cytoplasmic triglyceride when induced to differentiate.

Comparison of the rates of proliferation during logarithmic growth of control 3T3-L1 preadipocytes and of preadipocytes transfected with the pBCMGNeo or pBCMGAS (AS1, AS2, and AS3 cells) vectors revealed no significant changes. In several experiments the doubling times for these cell lines were indistinguishable, averaging 16–17 hr (results not shown). Perhaps this is not surprising, as proliferating 3T3-L1 preadipocytes do not express a detectable level of C/EBP mRNA (Birkenmeier et al. 1989; Christy et al. 1989). Upon achieving growth arrest $\sim$ 2 days after confluence (6×10^6 to 8×10^6 cells/10-cm culture dish); however, 3T3-L1 preadipocytes and pBCMGNeo-transfected 3T3-L1 preadipocytes express an extremely low, but detectable, level of C/EBP message (Christy et al. 1989 and data not shown). We have observed repeatedly that 3T3-L1 preadipocytes harboring the pBCMGAS vector, which do not express a detectable level of C/EBP mRNA, require slightly more time ($\sim$ 3 days after confluence) to achieve growth arrest and, upon doing so, exhibit a somewhat higher cell density ($\sim 10^7$ cells/10-cm culture dish). It should be noted, however, that, like 3T3-L1 preadipocytes, cell lines harboring the pBCMGNeo or pBCMGAS vectors exhibit density-dependent growth inhibition and are unable to proliferate in medium containing soft agar and, thus, have not acquired characteristics typical of transformed cells (results not shown).

Normally, the accumulation of cytoplasmic triglyceride droplets can be detected on day 3 after the induction of differentiation of 3T3-L1 preadipocytes (Student et al. 1980). Although in some pBCMGNeo-transfected con-

trol cell lines the onset of triglyceride accumulation was slightly delayed, by day 4 all lines exhibited the accumulation of cytoplasmic triglyceride and the spherical morphology typical of differentiating 3T3-L1 preadipocytes. In contrast, 3T3-L1 cell lines harboring the antisense C/EBP vector exhibited little or no cytoplasmic triglyceride accumulation. Figure 7 shows representative photomicrographs of control (untransfected and pBCMGNeo-transfected) and pBCMGAS-transfected 3T3-L1 cell lines 6 days after subjecting the cells to the differentiation protocol. Typically, preadipocytes transfected with the control pBCMGNeo vector exhibited a

Figure 6. Rates of run-on transcription of the C/EBP and 422(aP2) genes by nuclei from control and antisense cell lines. Control (3T3-L1 and BCMGNeo) and pBCMGAS-transfected cell lines (AS1, AS2, and AS3) were induced to differentiate. Nuclei from 3×10^7 cells, isolated 3 and 5 days after induction of differentiation, were subjected to nuclear run-on transcription assays using [α - 32 P]UTP and unlabeled NTPs. The 32 P-labeled transcripts were hybridized to 2 μ g of a 280-bp *SmaI*–*SacI* fragment of the C/EBP cDNA (solid bars), 2 μ g of a linearized plasmid containing 270-bp of the 422(aP2) [hatched bars] cDNA, and 100 ng of 3T3-L1 genomic DNA by slot blotting. Quantitation of the run-on transcripts was performed by densitometry of autoradiograms normalized to radiolabeled transcripts hybridized to 3T3-L1 genomic DNA.

somewhat reduced extent of adipose conversion than untransfected 3T3-L1 preadipocytes. This loss of potential to differentiate is typical of 3T3-L1 preadipocytes carried through the rigors of procedures used in selecting G418-resistant foci.

In contrast to control cells, cell lines harboring the pBCMGS vector, such as, AS1 and AS3 cells, which express antisense C/EBP RNA (see Fig. 3A), gave rise only to occasional cells that exhibited cytoplasmic triglyceride. This is true even when these cells were carried in culture for 2 weeks after the induction of differentiation. This finding supports the view that C/EBP is required for the expression of proteins involved in the process of triglyceride synthesis or deposition, or both, during preadipocyte differentiation. It was reported recently (Samuelsson et al. 1991) that transfection of an antisense C/EBP RNA expression vector into 3T3-F442A cells caused decreased expression of C/EBP mRNA and a reduced ability to accumulate cytoplasmic lipid droplets.

Reversal of antisense C/EBP RNA-suppressed gene expression and triglyceride accumulation by transfection with a sense C/EBP RNA expression vector

To verify that the process of transfecting 3T3-L1 preadipocytes with the antisense C/EBP expression vector had not irreversibly and nonspecifically damaged their capac-

ity to differentiate into adipocytes, we sought to reverse the loss of the "adipocyte phenotype" by transfecting AS cells with a sense C/EBP RNA expression vector. Therefore, a vector (pBCMGS) was constructed that directs the expression of a sense C/EBP RNA (~400 bases of C/EBP RNA complementary to those directed by pBCMGS) and would hybridize to the antisense RNA encoded by the pBCMGS vector. It was our expectation that transfection and overexpression of the vector-derived sense C/EBP RNA in cell lines harboring the pBCMGS vector would neutralize the antisense C/EBP RNA and possibly restore the adipocyte phenotype.

The pBCMGS vector (see Fig. 1B) contains both the ~400-bp *HincII-SmaI* segment of the C/EBP cDNA in the sense orientation and a hygromycin-B resistance gene (Gritz and Davies 1983) under the control of the HSV-1 thymidine kinase gene promoter (Sugden et al. 1985) permitting selection of stable cell lines. After transfection of the AS1, AS2, and AS3 cell lines (which already harbor the pBCMGS vector) with the pBCMGS vector and selection with hygromycin, resistant cell lines were subjected to the differentiation protocol to identify revertants that express substantial amounts of cytoplasmic triglyceride. Of eight revertant cell lines obtained, four (Rev 1, Rev 2, Rev 3, and Rev 4 cells) were propagated for further study. In addition, several control cell lines (BCMGS-Hyg 1, 2, and 3, derived from the AS1 and AS3 antisense cell lines), which harbor the vector

Figure 7. Cytoplasmic triglyceride accumulation. Six days after induction of differentiation, cells were photographed by phase-contrast microscopy. 3T3-L1 and BCMGNeo control cells (80–95%) expressed cytoplasmic triglyceride, whereas 15–20% and <5% of the antisense cell lines, AS1 and AS3, respectively, exhibited triglyceride accumulation. Revertant cell lines Rev 1 (70–80%) and Rev 5 (30–40%) harboring both the pBCMGS and the pBCMGS vectors, respectively, expressed cytoplasmic triglyceride. These findings are representative of results with several other AS and Rev cell lines.

containing the hygromycin resistance gene, but lack the sense C/EBP cDNA insert, were selected for study.

Southern blots (not shown) performed on low-molecular-weight DNA isolated from the revertant cell lines revealed that all four revertant lines possessed multiple, that is, 5–20, copies of the transfected ~400-bp sense C/EBP sequence in extrachromosomal vector DNA (see Table 1). As observed with the pBCMGS vector, integrated copies of the pBCMGS vector in chromosomal DNA were also detected after multiple passages of the cell lines. The revertant cell lines possessed from 1 to 21 integrated copies of the transfected ~400-bp sense C/EBP sequence per cell in addition to multiple copies of the ~400-bp antisense C/EBP sequence (see Table 1). The extent to which reversion to the adipocyte phenotype occurs would be expected to depend on the relative levels of expression of the sense and antisense RNAs that would differ for each cell line.

The four revertant cell lines (Rev 1, Rev 2, Rev 3, and Rev 4 cells), which accumulate cytoplasmic triglyceride when subjected to the differentiation protocol, were subjected to more detailed analysis. Each of these cell lines was found to express vector-encoded sense C/EBP RNA (Fig. 8A,B), which accounts for the marked reduction or virtual elimination of its complementary antisense C/EBP RNA (Fig. 8A,B). That the antisense C/EBP RNA had caused the decrease in level of endogenous C/EBP mRNA in the AS cell lines (see Fig. 3A) is indicated by the recovery of expression of this message and of 422(aP2) mRNA in the revertant cells (Rev 1, Rev 2, Rev 3, and Rev 4 cells), which express the exogenous sense C/EBP RNA (Fig. 8A,B). As with the mRNAs, decreased expression of C/EBP and 422(aP2) proteins caused by antisense C/EBP RNA was also reversed by overexpression of sense C/EBP RNA (see Fig. 4A,B). This interpretation is strengthened by the finding that the levels of two other messages (i.e., SCD1 and GLUT4 mRNAs), both of which were repressed by antisense C/EBP RNA, also return to near control levels in revertant cell lines expressing exogenous sense C/EBP RNA (see Fig. 5). Taken to-

gether, these findings are consistent with previous observations showing that C/EBP is capable of *trans*-activating reporter gene expression driven by the promoters of the 422(aP2), SCD1, and GLUT4 genes (Christy et al. 1989; Kaestner et al. 1990).

The deposition of cytoplasmic triglyceride is another phenotypic characteristic of adipocytes that is lost in AS cells expressing antisense C/EBP RNA (see above). Shown in Figure 7 are representative photomicrographs of control (3T3-L1 and pBCMGSNeo-transfected) cells, of cells (AS1 and AS3 cells) harboring the pBCMGS antisense C/EBP vector, and of revertant cells (Rev 1 and Rev 4 cells) harboring both the pBCMGS antisense C/EBP and pBCMGS sense C/EBP vectors. It is evident that the suppression of triglyceride accumulation in AS1 and AS3 cells caused by the pBCMGS (antisense) vector was reversed by subsequent transfection of these cells with the pBCMGS (sense) vector.

It should be noted that transfection of the AS1 and AS3 cell lines with the pBCMGS vector did not give rise to any "revertant" cell lines. Of 30 cell lines harboring both pBCMGS and pBCMGS vector, none exhibited the reverted adipocyte phenotype.

Discussion

This investigation shows unequivocally that expression of a partial (~400-bp) antisense C/EBP RNA can decrease cellular C/EBP mRNA level by 95–98%. The principal mechanism by which C/EBP message level is decreased is through a reduced rate of transcription of the C/EBP gene (Fig. 6). It is not obvious how expression of an antisense C/EBP RNA might inhibit the transcription of its own gene. Hybridization of antisense RNA to nascent or completed C/EBP pre-mRNA transcripts would not be expected *a priori* to block initiation of transcription or subsequent run-on transcription of the C/EBP gene. A possible explanation derives from our earlier finding (Christy et al. 1991) that C/EBP itself can bind to the promoter of the C/EBP gene, raising the possibility that

Figure 8. Expression of endogenous C/EBP and 422(aP2) mRNAs and exogenous sense and antisense C/EBP RNAs in revertant cell lines harboring both pBCMGS and pBCMGS. (A) Revertant cell lines Rev 1, Rev 2, and Rev 3 were derived by transfecting pBCMGS into the AS1 cell line. Six days after inducing differentiation, 10 μ g of total RNA from 3T3-L1, AS1, and Rev 1, Rev 2, and Rev 3 cells was subjected to RNase protection analysis. The protected segment of exogenous sense C/EBP RNA corresponds to the 160-bp *MluI*–*SmaI* fragment of C/EBP cDNA. The other protected fragments are the same as described in Fig. 3A. (B) Revertant cell line Rev 4 was derived by transfecting pBCMGS into the AS3 cell line. Six days after inducing differentiation, 10 μ g of total RNA from 3T3-L1, AS3, and Rev 4 cells was analyzed by RNase protection. The protected segments of the target RNAs were the same as described in A.

this transcription factor may autoactivate transcription of its own gene. Were this the case, inhibition by antisense C/EBP RNA of a post-transcriptional event (e.g., accelerated turnover of C/EBP mRNA or inhibition of translation of C/EBP mRNA) might indirectly reduce, but not completely block, transcription of the C/EBP gene. Supporting this hypothesis is the fact that run-on transcription of the C/EBP gene was repressed by 73% in AS3 cells expressing antisense C/EBP RNA (Fig. 6B), whereas run-on transcription of the 422(aP2) gene (Fig. 6B) and steady-state levels of 422(aP2) mRNA (Fig. 3B) were reduced to a much greater extent, that is, by 98.5%, in AS3 cells.

In support of the hypothesis that C/EBP (or another protein that interacts with the C/EBP-binding site in the promoter of the C/EBP gene) may regulate transcription of its own gene is suggested by DNase I footprinting of the promoter of the gene. The region between nucleotide -203 and -176 exhibits different, but overlapping, footprints by factors present in nuclei from differentiated and undifferentiated 3T3-L1 cells (Christy et al. 1991). We have observed that nuclear extracts from AS3 cells (subjected to the differentiation protocol), which express high levels of antisense C/EBP RNA (Fig. 3A), footprint the region between nucleotides -203 and -176 in a manner identical to extracts from wild-type 3T3-L1 preadipocytes, but differently from those of differentiated 3T3-L1 adipocytes (results not shown). It should be noted that LAP or C/EBP β (Descombes et al. 1990; Cao et al. 1991) and C/EBP δ (Cao et al. 1991) are also capable of binding at the same sites at which C/EBP binds (Descombes et al. 1990; Williams et al. 1991). Thus, at present, we cannot rule out the possibility that altering expression of C/EBP α using the antisense RNA approach alters the expression of other transcription factors which, in turn, interact with the promoters of the C/EBP α , 422(aP2), SCD1, and GLUT4 genes.

Specificity was a major consideration in the choice of a C/EBP cDNA sequence to incorporate into the antisense C/EBP expression vector. An attempt was made to avoid using sequences corresponding to the basic and leucine zipper domains of C/EBP, as these regions have a high degree of nucleotide sequence identity to several other transcription factors, namely LAP (Descombes et al. 1990; NF-IL6, Akira et al. 1990; IL-6 DBP, Poli et al. 1990; AGP/EBP, Chang et al. 1990; C/EBP β , Cao et al. 1991; CRP2, Williams et al. 1991), Ig/EBP-1 (Roman et al. 1990), CRP1 (Williams et al. 1991), CELF (Kageyama et al. 1991; CRP3, Williams et al. 1991; C/EBP δ , Cao et al. 1991). Hence, the ~400-bp segment of the C/EBP cDNA (between the *HincII* and *SmaI* sites in cDNA p6A-1, Fig. 1A) that lies 5' to the basic DNA-binding and leucine zipper dimerization domains was selected. A computer search of the GenBank rodent sequence library revealed no other nucleotide sequences with significant identity.

It appeared, therefore, that the expression of antisense C/EBP RNA directed by the pBCMGS vector that contains the ~400-bp antisense sequence would specifically hybridize to endogenous C/EBP mRNA. RNase protec-

tion analysis revealed that pBCMGS-directed expression of antisense C/EBP RNA suppressed the level of endogenous C/EBP mRNA (Figs. 3A and 4) without lowering the level of LAP mRNA (results not shown).

Of interest is the fact that expression of antisense C/EBP RNA in 3T3-L1 preadipocytes had no detectable effect on cell proliferation rate. Hence, the doubling time of control (3T3-L1 and pBCMGSNeo-transfected cells) and pBCMGS-transfected cells was the same, that is, 16–17 hr. The laboratory of S. McKnight (Umek et al. 1991) has provided compelling evidence that C/EBP is antimitogenic. This finding is compatible with the fact that C/EBP mRNA (1) is not detected in logarithmically dividing 3T3-L1 preadipocytes, (2) is expressed at extremely low levels in confluent 3T3-L1 preadipocytes, and (3) is expressed at high levels only upon induction of differentiation of 3T3-L1 preadipocytes into adipocytes (Christy et al. 1989). Because expression of antisense C/EBP RNA, which effectively blocks expression of C/EBP mRNA and the acquisition of the adipocyte phenotype, has no detectable effect on mitosis, it is evident that C/EBP is not involved in the proliferative phase of the cell cycle. Rather, C/EBP may function by suppressing cell division as cells, such as 3T3-L1 preadipocytes, enter the quiescent terminally differentiated state.

The participation of C/EBP in differentiation-activated expression of the 422(aP2) gene in preadipocytes was questioned recently by B.M. Spiegelman and co-workers (Graves et al. 1991). These investigators discovered a strong upstream enhancer of the 422(aP2) gene that is capable of directing adipose-specific expression in transgenic mice. It was observed that a transgene containing the enhancer, but lacking the region of the 422(aP2) gene promoter containing the C/EBP-binding site and an overlapping negative regulatory element, retains the ability to direct adipose-specific expression. Investigations in our laboratory (Yang et al. 1989; Cheneval et al. 1991) suggest that C/EBP may be involved in the mechanism by which the negative regulatory element in the 422(aP2) gene promoter is derepressed during differentiation. Therefore, promoter constructs containing the enhancer, but lacking both the C/EBP-binding site and the overlapping negative regulatory element, might be expected to be constitutively active. Further work will be needed to determine whether and if so, by what mechanism, the negative regulatory element in the 422(aP2) gene promoter can override the effect of the adipose-specific enhancer before differentiation and the expression of C/EBP. We have observed (F.-T. Lin and M.D. Lane, unpubl.) that an extremely low level of 422(aP2) mRNA can be detected by RNase protection analysis in confluent, but not proliferating, 3T3-L1 preadipocytes before differentiation. This low level of 422(aP2) message correlates well with the extremely low level of C/EBP message present in confluent 3T3-L1 preadipocytes and its absence in dividing preadipocytes.

Materials and methods

Plasmid construction

The 14.8-kb pBCMGSNeo expression vector was derived by re-

moving the mouse IL-2 cDNA insert from the *XhoI* site of pBCMGNeo·mIL-2, which was a gift from Dr. Hajime Karasuyama [Karasuyama et al. 1989]. A segment of the C/EBP cDNA extending from nucleotide +490 to the poly(A)⁺ tail, previously designated p6A-1 [Christy et al. 1991], was subcloned into the *EcoRI* site of pBluescript. After digestion with *HincII* and *SmaI*, the gel-purified ~0.4-kb C/EBP fragment was ligated with *XhoI* linkers and inserted into the *XhoI* site of pBCMGNeo in the sense or antisense orientations, which was confirmed by DNA sequencing [Sanger et al. 1977]. The antisense C/EBP plasmid was designated pBCMGAS. A 2.5-kb hygromycin resistance gene from plasmid pBHPH, a gift from Dr. David Weng, was inserted into the *XbaI* site of the sense C/EBP plasmid, now designated pBCMGs, as a second marker for selecting stable cell lines harboring both pBCMGs and the antisense pBCMGAS construct. The control plasmid, pBCMGHyg, was derived by removing the ~0.4-kb sense C/EBP fragment from the *XhoI* site of pBCMGs.

Stable transfection by electroporation

Thirty percent confluent, low passage 3T3-L1 preadipocytes were transfected with 5 µg of the antisense C/EBP plasmid, pBCMGAS, or control vector, pBCMGNeo, by electroporation at 450 V with a capacitance of 25 µF, as described previously [Cook et al. 1988]. After a 24-hr recovery period, 350 µg/ml of G418 was added, and neomycin-resistant foci were isolated and propagated. Cells harboring the pBCMGAS vector were designated AS (antisense) cells.

Similarly, 20 µg of the sense C/EBP plasmid, pBCMGs, or control vector, pBCMGHyg, was electroporated into 30% confluent AS cells maintained under G418 selection pressure (300 µg/ml of G418 for 1 week). Twenty-four hours after transfection, 150 µg/ml of hygromycin B was added, and hygromycin-resistant foci were isolated and propagated.

Cell culture and differentiation

The 3T3-L1 preadipocytes were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% calf serum. Differentiation was induced by treating 2-day postconfluent cells (designated day 0) with 10% fetal bovine serum, 1 µg/ml of insulin, 1 µM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine as described [Reed and Lane 1980; Student et al. 1980]. The medium was then replaced, and cells were fed every other day with DMEM supplemented with only insulin and 10% FBS. The appearance of cytoplasmic triglyceride droplets as the cells differentiated into adipocytes was visualized by staining with Oil-Red-O [Preece 1972].

Radiolabeled probes

DNA probes for Northern and Southern blot analyses were labeled by the random hexamer-priming method [Feinberg and Vogelstein 1983]. The 422(aP2) hybridization probe was the full-length cDNA [Bernlohr et al. 1984]. The hybridization probe for endogenous and exogenous sense or antisense C/EBP DNAs and RNAs was the ~0.4-kb C/EBP insert of pBCMGAS, corresponding to nucleotides +490 to +870 of C/EBP cDNA.

The plasmid templates for synthesis of riboprobes for RNase protection analysis of C/EBP mRNA and exogenous sense and antisense C/EBP RNA were constructed by removing partial C/EBP sequences from p6A-1 using appropriate restriction enzyme digestion and religation. The hybridization probes for 422(aP2), GLUT4, and SCD1 mRNAs were prepared by cloning a sequence-specific cDNA fragment into the polylinker cloning

sites of pGEM. The riboprobes were generated using 1 µg of linearized plasmid templates, [α -³²P]CTP, and NTPs transcribed with bacteriophage T7 or T3 RNA polymerase in the antisense orientation [Melton et al. 1984].

Isolation and analysis of DNA

Low-molecular-weight DNA (including episomal vector DNA) from two 100-mm dishes of confluent preadipocytes was prepared by the procedure of Hirt [1967]. DNase-free RNase A at 20 µg/ml was added to remove contaminating RNA followed by phenol/chloroform/isoamyl alcohol (25 : 24 : 1) extraction and ethanol precipitation. High-molecular-weight-chromosomal DNA from one 100-mm dish of confluent preadipocytes was isolated by the method of Wigler et al. [1979]. Low-molecular-weight DNA from two 100-mm dishes, 20 µg of high-molecular-weight DNA, and plasmid DNAs were digested overnight with *XhoI* followed by electrophoresis on 0.7% agarose gels. After denaturation, neutralization, and transfer to Hybond membranes [Southern 1975], hybridization was performed with 10⁶ cpm/ml of DNA probe as described [Ntambi et al. 1988]. The copy number of integrated DNA was quantitated by densitometry of the autoradiograms and was normalized to the signals of restriction fragments of endogenous C/EBP derived from genomic DNA.

Isolation and analysis of RNA

Total RNA was isolated from 2-day postconfluent preadipocytes (day 0 of differentiation) and adipocytes after 2, 4, or 6 days of differentiation by the method of Chirgwin et al. [1979]. For Northern blot analysis, 20 µg of total RNA was denatured with glyoxal and dimethylsulfoxide and analyzed by electrophoresis on 1% agarose gels as described [McMaster and Carmichael 1977; Thomas et al. 1980]. After transfer to Hybond membranes (Amersham) and UV cross-linking, the RNA blots were stained with methylene blue to locate 28S and 18S rRNAs and to verify loading. The blots were hybridized with 10⁶ cpm/ml of C/EBP and 422(aP2) DNA probes. Procedures for prehybridization and hybridization were the same as those for Southern analysis.

For RNase protection analysis, 2 × 10⁵ to 5 × 10⁵ cpm/sample of the riboprobe was hybridized overnight with 10 µg of total RNA in 80% formamide at 54°C. After RNase A and T₁ treatment, the protected probe fragments, annealed to complementary sequences of the target RNA, were precipitated and analyzed on 6% polyacrylamide/7 M urea sequencing gels [Melton et al. 1984].

Isolation of nuclei and nuclear run-on transcription analysis

Isolation of nuclei from day 3 and day 5 differentiated cells was performed as described [Marzluff 1978]. Nuclei from 3 × 10⁷ cells were collected for nuclear run-on transcription assays using the protocol modified by Groudine et al. [1981]. Transcripts from day 3 and day 5 differentiated cells (1.74 × 10⁷ and 6.6 × 10⁶ cpm/ml) were hybridized to filter-bound cDNA in slot blots. Two micrograms of the 280-bp *SmaI*-*SacI* fragment of C/EBP cDNA, 2 µg of linearized plasmid containing 270-bp *HindIII*-*SalI* fragment of 422(aP2) cDNA, and 100 ng of genomic DNA were denatured in 0.4 M NaOH and 10 mM EDTA at 100°C for 10 min followed by neutralization with cold 2 M ammonium acetate (pH 7), on ice. The denatured DNAs were applied to Hybond membranes (Amersham) using a Dot Blot apparatus. The procedures of prehybridization and hybridization were the same as those for Northern blots, except hybridization was for 4 days and treatment with RNase at 37°C for 30 min during

washing. Run-on transcription rates were quantitated by densitometry of autoradiograms, and the signals were normalized to radioactive transcripts hybridized to 3T3-L1 genomic DNA.

Western blot analysis of C/EBP and 422(aP2) proteins

Five days after exposure to the differentiation protocol, cells were harvested and lysed with $1 \times$ lysis buffer containing 60 mM Tris (pH 6.8) and 1% SDS. The whole cell lysate was boiled for 5 min. Cellular protein (250 μ g) was diluted in SDS-sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (Laemmli 1970). After electrophoretic transfer to nitrocellulose, Ponceau-S staining was performed to ensure equal loading of each sample. C/EBP and 422(aP2) proteins were detected using a rabbit antibody specific to an internal 14-amino-acid peptide (amino acid residues 253–265) of C/EBP (provided by Dr. P. Cornelius) or the carboxy-terminal 14 amino acids of 422(aP2) protein (Bernlohr et al. 1984; provided by Dr. R.D. Hoffman). The membrane was then incubated with horseradish peroxidase-conjugated second antibody. The immunoreactive polypeptides were visualized by the ECL detection system (Amersham), following the procedures recommended by the supplier.

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F T Lin and M D Lane

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