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The CCAAT/enhancer binding protein and its role in adipocyte differentiation: evidence for direct involvement in terminal adipocyte development

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During the course of differentiation of preadipocytes into adipocytes, several differentiation-linked genes are activated synchronously with morphological changes. To follow this process we have used 3T3-F442A cells, known to undergo adipocyte conversion with high frequency. Accumulation of lipid droplets in the cytoplasm constitutes an easily visualized sign of the terminally differentiated phenotype. In this report we demonstrate that expression of the CCAAT/enhancer binding protein (C/EBP) is an important factor in determining the ability to accumulate lipid droplets in terminally differentiated adipocytes. In one experiment we can suppress C/EBP expression through administration of hydrocortisone to differentiating 3T3-F442A cells, which is accompanied by an inability of the cells to accumulate lipid. In another experiment a C/EBP antisense expression vector has been stably introduced into 3T3-F442A cells and as compared with control cells, a 62% decrease of C/EBP mRNA ($p < 0.01$) is demonstrated. This decrease of C/EBP mRNA is accompanied by a change in cellular morphology characterized by a reduced ability to form lipid droplets. We can also demonstrate a correlation between the degree of reduction of C/EBP mRNA and the amount of lipid present in the cells. These findings strongly support the view that C/EBP is a necessary component of terminal adipocyte differentiation.

Key words: adipocyte differentiation/antisense mRNA/glucocorticoids/C/EBP/3T3-F442A

Introduction

When 3T3-F442A cells are grown under appropriate conditions they will undergo a change from preadipocytes to adipocytes (Green and Kehinde, 1976). This *in vitro* model for adipocyte differentiation has been shown to closely resemble fat cell development *in vivo* (Green and Kehinde, 1979). The conversion of preadipocytes into adipocytes is characterized by transcriptional activation of several genes. During the early stage of adipocyte development, at the time point when the cells have reached confluence, the lipoprotein lipase (LPL) gene is transcriptionally activated (Amri *et al.*, 1986). As a result of its enzymatic action, free fatty acids are released. In the adipocyte, these are taken up by the cell, reesterified and stored as lipid droplets in the cytoplasm. This latter process is catalyzed by the action of 'adipocyte specific' proteins. As compared with LPL the 'adipocyte specific' genes are induced at the latter stage in adipocyte conversion, when the cells start to accumulate triglyceride (Spiegelman *et al.*, 1983). Glycerol-3-phosphate dehydrogenase (GPDH) is a member of this group of proteins, and is also used as an enzyme marker diagnostic of terminally differentiated adipoytes (Pairault and Green, 1979; Dobson *et al.*, 1987). The transcription factor C/EBP (Landschulz *et al.*, 1988) is induced at approximately the same time during adipocyte development as GPDH (Brinkenmeier *et al.*, 1989).

These observations suggest a key function of C/EBP as a regulator of terminal adipocyte development. This hypothesis is supported by the fact that three 'adipocyte specific' genes have been shown to be transactivated by C/EBP (Christy *et al.*, 1989; Herrera *et al.*, 1989; Kaestner *et al.*, 1989). The strongest argument for this case so far is found in the work by Umek *et al.* (1991) in which the coding sequence of C/EBP was fused to the C-terminus of the hormone binding domain of the glucocorticoid receptor gene or the estrogen receptor gene. These constructs were then used to conditionally express C/EBP prematurely in dividing adipoblasts. A direct cessation of mitotic growth was observed as a result of premature C/EBP expression. The authors conclude that C/EBP expression confers growth arrest to adipoblasts when prematurely expressed; a hallmark of the terminally differentiated adipocyte. In this paper we have investigated how different levels of C/EBP expression affect the cellular phenotype with special emphasis on the ability of the adipocytes to take up lipid. For this purpose we took advantage of the fact that glucocorticoids have been shown to block adipocyte conversion completely as judged by microscopic morphology (Pairault and Lasnier, 1987). In order to study the molecular mechanisms behind these findings we cultured 3T3-F442A cells under different conditions. Gene activation was monitored by measuring the cellular content of mRNA for LPL, GPDH and C/EBP respectively. In another experiment we stably introduced an antisense C/EBP expression vector into 3T3-F442A cells and measured the same mRNAs as described above. This has enabled us to correlate the pattern of gene activation with cellular morphology.

Results

Effects of glucocorticoids on adipocyte differentiation

When 3T3-F442A cells are cultured in standard medium (for composition see legend to Figure 1), we found that the LPL gene is expressed soon after confluence and ~4 days later C/EBP and GPDH are induced (Figure 1A). In parallel with this the cells differentiate into adipocytes (Figure 2A). This is in agreement with the findings of other investigators (Brinkenmeier *et al.*, 1989). If cells are allowed to differentiate in the presence of hydrocortisone, they retain the preadipocyte phenotype (Figure 1B) as described by Pairault and Lasnier (1987). We discovered that in this case neither C/EBP nor GPDH genes were activated, whereas

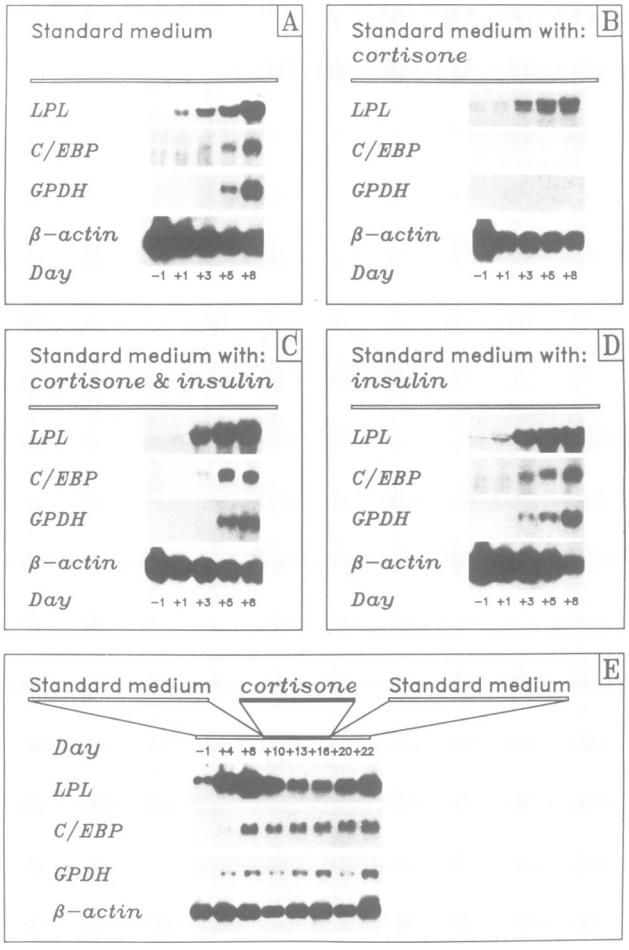

Fig. 1. Northern analysis of total RNA derived from 3T3-F442A cells. Cells were harvested at different time points, '−' denotes the number of days prior to and '+' denotes the number of days after confluence. Probes identifying transcripts from LPL (Enerbäck *et al.*, 1987), C/EBP (Landschultz *et al.*, 1988), GPDH (Kozak and Brinkenmeier, 1983) and β-actin (Cleveland *et al.*, 1980) were used. Standard medium is Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. In **B**−**E** standard medium was supplemented with cortisone (5 μg/ml of hydrocortisone) and/or insulin (10 μg/ml). In **A**−**D** cells were cultured in the same medium during the whole experiment. In **E** cells were cultured in standard medium until day +8 and from that time point they were grown in standard medium supplemented with cortisone; at day +16 they were again returned to standard medium. At least two independent RNA preparations were used; these were assayed in duplicate.

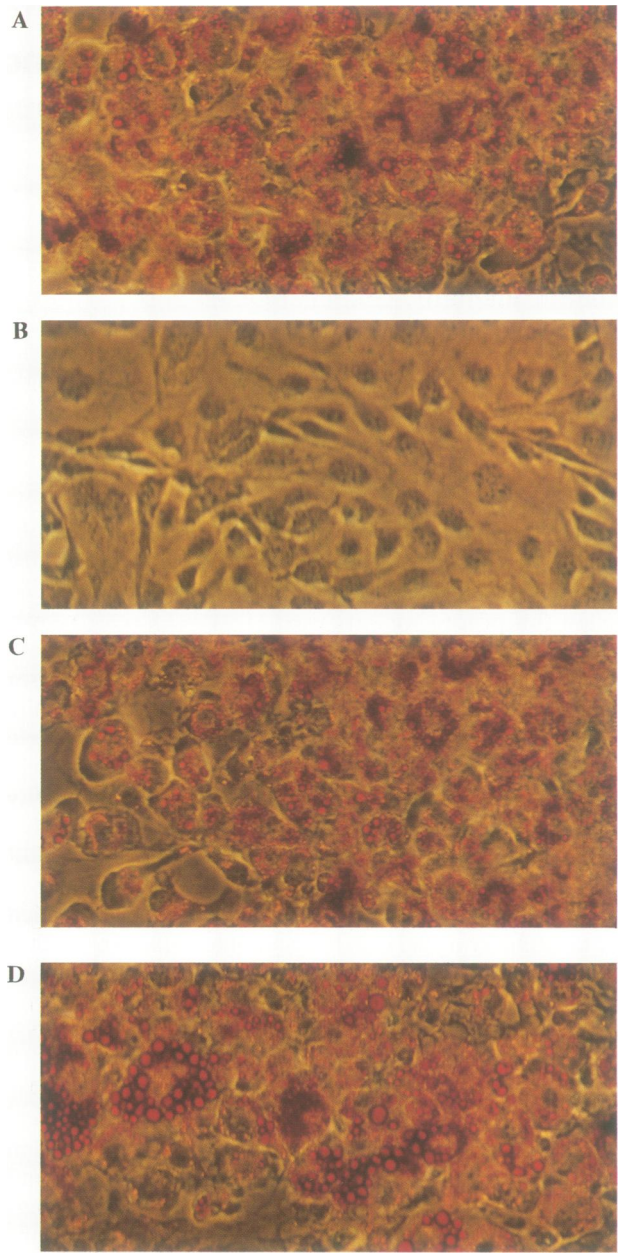

Fig. 2. Phase micrographs of Oil Red O stained 3T3-F442A cells cultured for 8 days after they had reached confluence. Culture medium: **A**, standard medium; **B**, standard medium supplemented with cortisone; **C**, standard medium supplemented with cortisone and insulin; **D**, standard medium supplemented with insulin (for exact formulae see Figure 1). In A, C and D, cells acquire adipocyte phenotype with typical lipid droplets in the cytoplasm; these are stained red by Oil Red O. In B, cells retain a preadipocyte phenotype: no lipid droplets can be visualized.

LPL was expressed (Figure 1B). In another experiment insulin was added to standard medium. No major differences from standard medium alone could be observed (Figures 1D and 2D). When cells differentiated in standard medium supplemented with both hydrocortisone and insulin, the C/EBP and GPDH genes were induced in a way closely resembling the case with standard medium only (Figure 1C). Such cells also develop into adipocytes (Figure 2C).

To investigate C/EBP expression and adipocyte differentiation further, we allowed 3T3-F442A cells to differentiate in standard medium for 8 days. Hydrocortisone was then added to the medium and the cells were kept under these conditions for another 8 days. Here it could be demonstrated that in terminally differentiated adipocytes the expression of C/EBP and GPDH genes was not influenced by hydrocortisone (Figure 1E). We would also like to point out that the decrease in LPL mRNA at days +13 and +16 is statistically significant (see Materials and methods), reflecting a hydrocortisone mediated regulation of LPL mRNA levels in terminaly differentiated 3T3-F442A adipocytes. β-Actin has been included as a control gene (Figure 1). The reduction of signal strength noted between days −1 and +3 is in accordance with the findings of Spiegelman and Farmer (1982).

With hormone treatment as a tool for regulating C/EBP expression we can show that: (i) C/EBP expression is subjected to regulation by insulin and hydrocortisone; (ii)

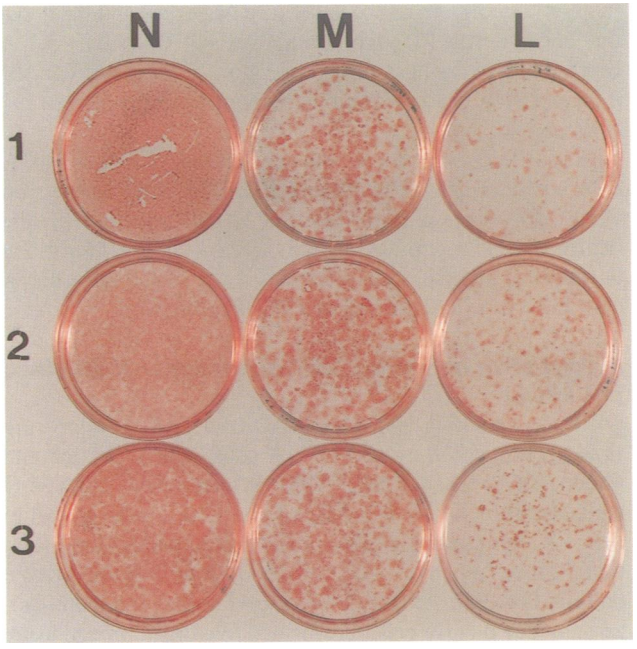

Fig. 3. A panel of Oil Red O stained 3T3-F442A cells, stained at day +8 after confluence. Cells were grown in standard medium supplemented with insulin as defined in Figure 1. These cells have been stably transfected with a pXTI plasmid, carrying the neomycin resistance marker gene. In group N1−3 a pUC plasmid accompanied the pXTI plasmid, in M1−3 and L1−3 a pmC/EBP(−) expression vector replaced the pUC plasmid. This vector directs the transcription of antisense C/EBP mRNA (for details see Materials and methods). As can be seen the amount of Oil Red O stainable lipid is reduced in cell lines M1−3 and L1−3 as compared with N1−3. These are representative results of independently processed duplicate dishes showing the same result.

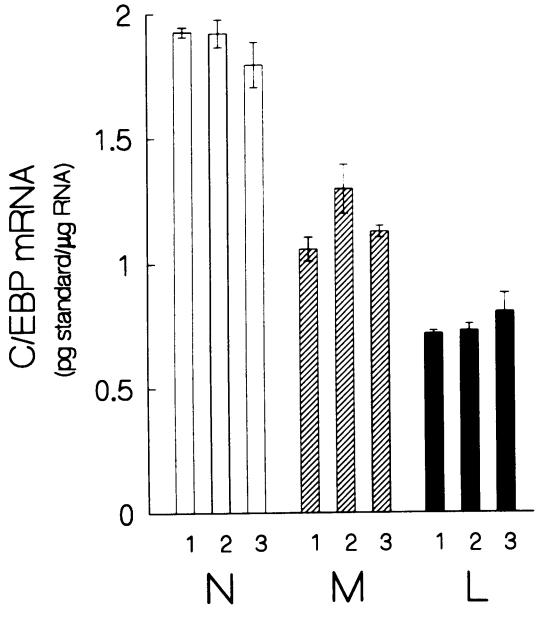

Fig. 4. Concentrations of C/EBP mRNA in cell lines N1−3, M1−3 and L1−3 (for details see Materials and methods). A solution hybridization assay was used. Values are ±SE. All groups differed from each other ($P < 0.01$) according to one-way analysis of variance followed by Student-Newman-Keul's test, between individual groups.

when C/EBP expression is abolished the cells do not develop into adipocytes; (iii) LPL does not require C/EBP induction for its expression; and (iv) only dividing adipoblasts are sensitive to this regulation whereas terminally differentiated adipocytes are inert.

Fig. 5. Northern analysis of total RNA derived from cell lines N1−3, M1−3 and L1−3. Cells were harvested at day +8 after confluence. Two independent RNA preparations were always used and representative results are shown. Probes identifying transcripts from LPL (Enerbäck *et al.*, 1987), GPDH (Kozak *et al.*, 1983) and β-actin (Cleveland *et al.*, 1980) were used. Cells were grown in standard medium supplemented with insulin as defined in Figure 1.

Effects of a C/EBP antisense construct on adipocyte differentiation

In order to elucidate the role of C/EBP expression during adipocyte development in greater detail, we initially tried to boost the endogenous C/EBP expression in 3T3-C2 cells, a cell line that does not develop into adipocytes although it was isolated together with 3T3-F442A cells and originated from the same founder cells (Green and Kehinde, 1976). Exceedingly low levels of C/EBP mRNA were found in these cells (data not shown). We used a C/EBP expression vector pmC/EBP(+) that was transfected into 3T3-C2 cells together with a pXTI construct encoding a neomycin-resistance marker gene (Neo^R; see Materials and methods). This was done with the aim of seeing if an increased C/EBP expression would make these cells develop into adipocytes. When the pmC/EBP(+) construct was included, we saw a drastic decrease in the number of neomycin resistant colonies, more than 30-fold less than cells transfected with the pXTI construct alone (data not shown). Thus the level of C/EBP expression induced by pmC/EBP(+) was not compatible with further cell growth. Similar data have been reported by Umek *et al.* (1991).

We then tried another approach, using the expression vector pmC/EBP(−) which had the coding part of the C/EBP gene in the opposite orientation, thus expressing an antisense C/EBP mRNA. pmC/EBP(−) and pXTI constructs were transfected into 3T3-F442A and resistant foci were cloned out (see Materials and methods). Here we could not detect any decrease in the number of foci when the pmC/EBP(−) construct was included. Initially 16 such foci were allowed to grow and ampoules were saved in liquid nitrogen. Cells were maintained to day +8 after confluence. They were then stained with Oil Red O (Kuri-Harcuch and Green, 1978), which stains intracellular lipid droplets red. These 16 clones showed a decreased ability to accumulate lipid droplets in their cytoplasm; the degree of inhibition varied markedly. Out of these 16 clones the six extremes with regard to inhibition of the ability to accumulate lipid droplets were selected. Clones named M1, M2 and M3 were the clones with the least inhibition whereas L1, L2 and L3 showed the greatest degree of inhibition. Three controls transfected with

the pXTI Neo^R construct and pUC plasmid alone were also included and were named N1, N2 and N3 (Figure 3). As can be seen in Figure 3, the N1, N2 and N3 cells are uniformly stained by Oil Red O, thus demonstrating a high degree of lipid accumulation, whereas L1, L2 and L3 only have few clusters of cells that contain lipid droplets. M1, M2 and M3 display an intermediate phenotype and fall between these two groups with regard to lipid accumulation. In order to rule out the possibility that the neomycin selection *per se* could favour a selection of cells incapable of developing into adipocytes, we cloned out 32 clones of cells that had been transfected with the Neo^R construct and pUC plasmid only. When compared with cells that had not been transfected, we found that all 32 clones did develop into adipocytes, displaying a phenotype identical to N1, N2 and N3 (data not shown).

To find out if there was a difference in the pattern of gene expression in the three groups N1−3, M1−3 and L1−3, we prepared RNA from parallel tissue culture dishes that had been grown to day +8 after confluence. As can be seen in Figure 4, a difference in the amount of C/EBP mRNA was found. These differences turned out to be statistically significant between groups N, M and L ($P < 0.01$). The other marker genes, LPL and GPDH, were also assayed for. Here no differences could be found (Figure 5). However, it should be pointed out that both LPL and GPDH mRNAs were present in similar amounts within and between groups N, M and L. To see if the difference in the amount of C/EBP mRNA also reflected a difference in the amount of C/EBP protein, we used a monospecific antiserum directed against C/EBP (Brinkenmeier *et al.*, 1989). The antiserum was used in an immunofluorescence assay (see Materials and methods) on cells derived from group N1−3 and L1−3. A representative result is shown in Figure 6. Here we can see a clear difference in immunoreactive material between N1 cells and L1 cells.

From these experiments we can say that: (i) 3T3-F442A cells stably transfected with an antisense C/EBP expression vector does have a lower level of C/EBP mRNA; (ii) the degree of lipid accumulation as judged by Oil Red O staining is reduced; and (iii) cells with low level of C/EBP mRNA also have a reduced level of C/EBP protein.

A correlation between C/EBP expression and cellular phenotype

Oil Red O staining is a well recognized method of scoring lipid accumulation in 3T3-F442A cells (Kuri-Harcuch and Green, 1978; Chen *et al.*, 1989). We examined Oil Red O stained dishes, of N1−3, M1−3 and L1−3 cells grown to day +8 after confluence. For this purpose we used a system that digitizes a colour image of the Oil Red O stained tissue culture dish. In brief (for details see Materials and methods), an analogue picture was transformed to a digital image using of a video camera and an ATVista card that was mounted in a pc/386. We used the HEIAB-MEDNET Color Picture Processing software. This enabled us to select a threshold of red colour-intensity corresponding to the colour of Oil Red O. The digital image was scanned and we could thus calculate the area of each tissue culture dish that had taken up Oil Red O stain. These measurements were always done on two parallel dishes in duplicate. For each measurement and cell group we had a maximum spread of the values of

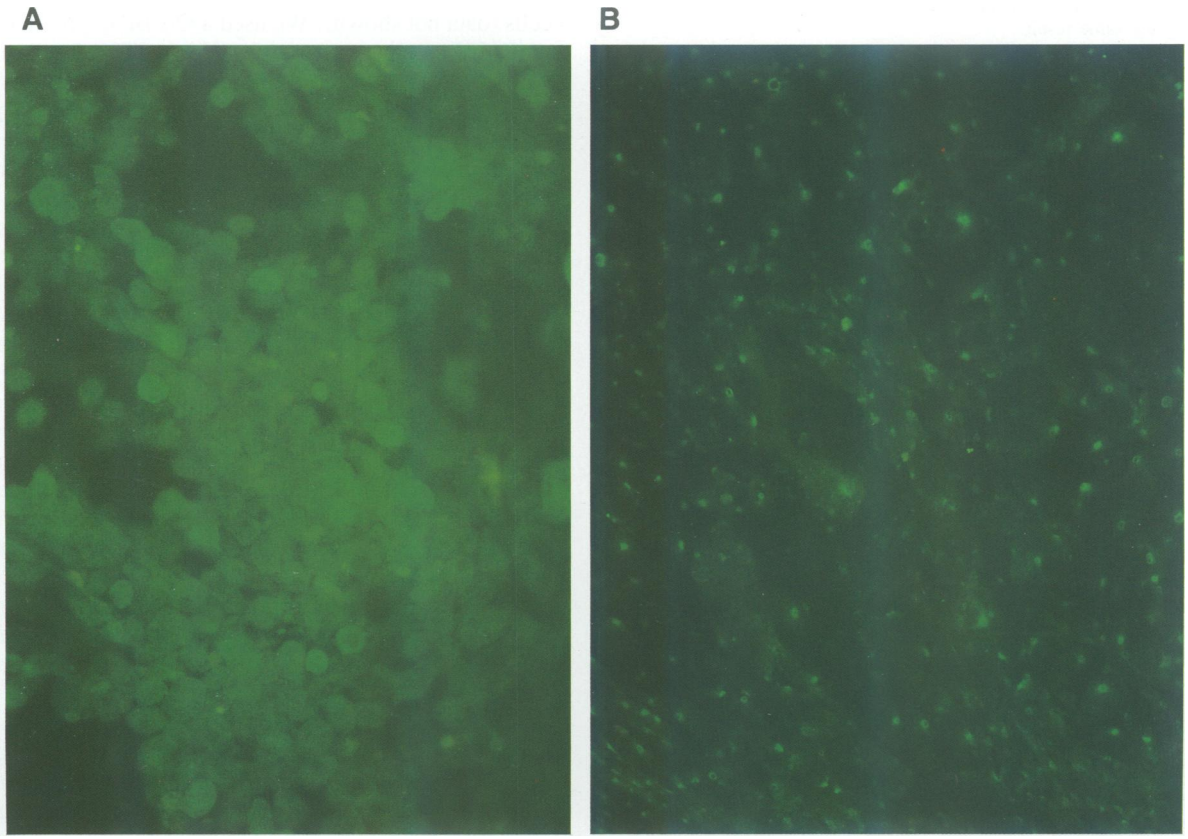

Fig. 6. Immunofluorescence assay. **Panel A**, cells from N1; **panel B**, cells from L1. Independent tissue culture dishes were assayed in duplicate and representative results are shown. A monospecific antiserum directed against C/EBP was used together with a FITC-conjugated secondary antibody as described in Materials and methods.

14%. In Figure 7 the mean of these values are plotted against the mean values of C/EBP mRNA levels, derived from Figure 4. As can be seen, there is a striking correlation between C/EBP mRNA level and Oil Red O stainable lipid.

Discussion

When growing nonconfluent 3T3-F442A cells are treated with hydrocortisone they do not start to accumulate lipid and the terminal phase of adipocyte differentiation is not induced (Pairault and Lasnier, 1987). We discovered that such cells did not express C/EBP or GPDH, whereas LPL expression was not affected. Insulin had a counteracting effect and restored the expression pattern of these genes (Figure 1). In a review article, McKnight *et al.* (1989) raise the question of whether C/EBP expression is regulated, either directly or indirectly, through a signal transduction system including insulin and glucocorticoids. The observations made in this report demonstrate that C/EBP is regulated either directly or indirectly by insulin and hydrocortisone *in vitro*. In this way C/EBP expression becomes closely linked to the action of these hormones, which may include a regulative role in adipocyte development *in vivo*.

In order to reduce the levels of C/EBP in 3T3-F442A cells selectively without interfering with other metabolic pathways, we chose to use an antisense construct (see Materials and methods; Green *et al.*, 1986). When an antisense C/EBP expression vector was stably transfected into 3T3-F442A cells, a reduction of C/EBP mRNA levels was demonstrated (Figure 4). In spite of several attempts we were not able to identify the antisense mRNA. This is in line with reports by others using antisense constructs (Paulssen *et al.*, 1990; Kim and Wold, 1985). These authors suggest that the formation of double-stranded RNA–RNA hybrids renders the RNA susceptible to double strand specific ribonucleases. This explanation seems likely since the reduction of C/EBP mRNA at most corresponds to a 62% decrease (Figure 4). Hence the amount of sense transcripts will always exceed that of antisense transcripts. The antisense transcripts will then be degraded as RNA–RNA hybrids, with a reduced level of sense transcripts as the only remaining C/EBP mRNA. It is interesting to note that GPDH levels were not influenced in the antisense experiment (Figure 5) in contrast to the hydrocortisone experiment (Figure 1B). This reveals an independent regulation of GPDH and C/EBP mediated by hydrocortisone. In the antisense experiment, GPDH and LPL mRNA levels are unaffected by the reduction of C/EBP mRNA. Still the accumulation of lipid is hampered in these cells. This mimics, in almost every detail, the metabolic situation in 3T3-F442A cells when cultured in a biotin deficient medium described by Kuri-Harcuch *et al.* (1978). They report that cells do not accumulate lipid in spite of normal levels of LPL and GPDH. These results make it less likely that C/EBP regulation of adipocyte development is mediated through GPDH as has been suggested (Brinkenmeier *et al.*, 1989). The decrease in C/EBP mRNA level is ~2.4-fold (Figure 4), and still this change is enough to alter the phenotype of the 3T3-F442A cells. Here we shall bear in mind that C/EBP, being a transcription factor, exerts its biological function by modulating the expression of other genes. In the *Notch* locus of *Drosophila*, a 2-fold difference in gene product yields an altered phenotype (Wright, 1970).

It has been pointed out that in contrast to MyoD (Davis *et al.*, 1987), C/EBP is not able by itself to specify a distinctive differentiation program (Umek *et al.*, 1991). When C/EBP is expressed in cultured nonconfluent preadipocytes, C/EBP fails to induce terminal differentiation (Umek *et al.*, 1991). An explanation for this could be that the early phase of differentiation initiation, in our study represented by LPL, has not been induced. This is made likely since we have shown that LPL is not dependent on C/EBP for its induction or steady state expression (Figures 1B and 5), it is also not induced until the cells have reached confluence. The role of C/EBP as a key regulator of terminal adipocyte differentiation has been strengthened by the data presented in this report. The correlation between cellular levels of C/EBP and the degree of lipid content in cells directly demonstrates a master gene function of C/EBP in terminal adipocyte differentiation.

Materials and methods

Cell culture

3T3-F442A cells (Green and Kehinde, 1976) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco Ltd, UK). The cells were cultured at 37°C in 5% CO_2 and humidified air. In some experiments hydrocortisone (Sigma, USA) and/or insulin (Sigma, USA) were added, as indicated in the legend to each figure.

Histochemical procedures

Cells were stained with Oil Red O (Sigma, USA). A stock was prepared from 0.5 g Oil Red O dissolved in 100 ml of isopropanol, diluted 6:4 in distilled water prior to use and filtered through a Whatman no. 1 filter. Cultures fixed in 10% formalin in isotonic phosphate buffer were stained for 1 h and then washed with water (Kuri-Harchuch and Green, 1978). In all instances two or three independent dishes were stained in parallel experiments. Micrographs were taken using a Zeiss Axiovert 10, ×200.

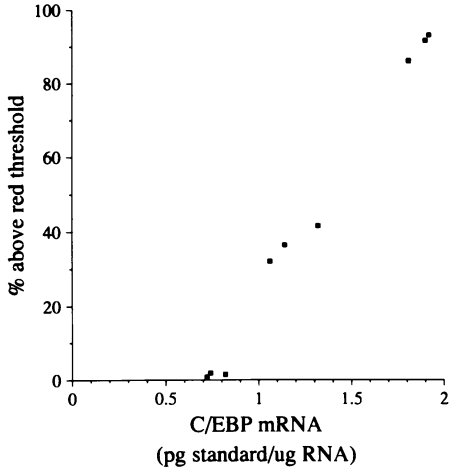

Fig. 7. Correlation between the amount of C/EBP mRNA in N1–3, M1–3 and L1–3 cells, as illustrated in Figure 4, and the relative area of cells stained by Oil Red O found in corresponding parallel experiments with cells derived from N1–3, M1–3 and L1–3, as shown in Figure 3. The relative area of Oil Red O stained cells on a tissue culture dish, is expressed as the percentage above the red threshold. For details see Materials and methods. These values represent the area corresponding to cells that have accumulated lipid and are derived from at least two independently stained tissue culture dishes originating from the cell lines N1–3, M1–3 and L1–3. These two parameters display a linear correlation, illustrating a linearity between the amount of C/EBP mRNA expressed and the amount of Oil Red O stainable lipid present in these cells.

Plasmids

In plasmids pmC/EBP(+) and pmC/EBP(−) a full-length copy of the intronless murine C/EBP gene, in either sense (+) or antisense (−) orientation, is driven by the human metallothionein promoter and the SV40 enhancer (Herrera *et al.*, 1989). p4ZmC/EBP contains a 700 bp *Bam*HI−*Pst*I fragment corresponding to the 5′-end of the coding sequence of the murine C/EBP gene [derived from pmC/EBP(+)], cloned into the *Bam*HI/*Pst*I sites of a pGEM4Z plasmid (Promega Inc., USA). In pXTI a selectable neomycin resistence gene (Neo^R) is under a viral LTR, derived from Moloney Murine Leukemia virus (Stratagene Inc., USA).

Stable transfectants

Preadipocytes were stably transfected using electroporation, with a 15:1 molar ratio of pmC/EBP(+/−) or pUCmp18 and pXTI, linearized plasmids. Approximately 2/3 confluent preadipocytes were trypsinized and washed twice in Opti-MEM (Gibco Ltd., UK), then resuspended in Opti-MEM at a concentration of $5-10\times10^6$ cells/ml. Plasmid DNA was added in 50 μl Opti-MEM to 0.45 ml of the cell suspension, mixed and placed in a sterile 1 ml disposable cuvette (Kartell, Italy). Electroporation was performed with a BTX Transfector-300 (BTX Inc., USA) with a 3.5 mm electrode gap and with settings of 220 V and 2200 μF. Cells were kept in the cuvette for 60 s after discharge and then transferred to a tissue culture dish. Medium was changed after 18−20 h, at which time point 1 mg/ml of G418 sulphate (Gibco Ltd, UK) was added to the medium (Southern and Berg, 1982). After 8−12 days, resistant foci appeared; at this time point all cells had died in a control experiment, only transfected with pUC plasmid. At this stage the G418 concentration was lowered to 0.8 mg/ml.

RNA analysis

Cells were harvested by scraping in 1×PBS. RNA was prepared according to Chirgwin *et al.* (1977), 20 μg of total RNA was denatured and electrophoresed through 1% agarose−formaldehyde gels (Lehrach *et al.*, 1977). DNA probes were radiolabeled with [^{32}P]dCTP (Feinberg and Vogelstein, 1983). Hybridization was carried out with 50% formamide at 44°C. Post-hybridization washes were performed in 0.2×SSC, 0.2% SDS at 50°C. Films were exposed overnight with intensifying screens at −70°C. In all experiments at least two, and in most cases three, independent RNA preparations were used. These RNA preparations were then blotted and hybridized twice in order to compensate for experimental errors. Films were scanned using a laser densitometer (data not shown); the only statistically significant differences obtained were between C/EBP and GPDH in Figure 1B, LPL in Figure 1E and C/EBP in M1−3 and L1−3 in this latter groups we performed solution hybridization assays. Probes were hybridized sequentially using the same blot.

Solution hybridization assay

C/EBP mRNA was quantified using a solution hybridization assay (Durnam and Palmiter, 1983). p4ZmC/EBP was linearized with either *Eco*RI or *Hin*dIII and labeled with [^{35}S]dUTP (Amersham Ltd, UK). cRNA probes were generated with T7 or SP6 polymerase (Promega Inc., USA) under the conditions indicated by the manufacturer. In brief, total RNA was incubated at 70°C for 24 h with 0.06 M NaCl, 20 mM Tris−HCl pH 7.5, 4 mM EDTA, 0.1% SDS, 10 mM dithiothreitol and 25% formamide with a ^{35}S-labeled cRNA probe in a volume of 40 μl. The samples were then treated with 40 μg RNase A and 2 μg RNase T1 (Boehringer, FRG) in the presence of 100 μg herring sperm DNA for 45 min at 37°C in a volume of 1 ml. Protected probe was precipitated with 100 μl 6 M trichloroacetic acid. The precipitates were then collected on GF/C filters (Whatman International Ltd, UK) and counted in a liquid scintillation counter. The hybridization signal was compared with that of an *in vitro* synthesized mRNA, complementary to the ^{35}S-labeled cRNA probe. The lowest concentration was well above the lowest point of the standard curve. Results are expressed as the amount of C/EBP mRNA per microgram of synthetic standard. At least two independent RNA samples were analyzed in triplicate. The within-assay coefficient of variation was <6.5%. The result is expressed as mean ± SE; for statistical evaluation of data, a one-way analysis of variance was performed followed by Student-Newman-Keul's test (Woolf, 1968).

Immunofluorescence assay

The antiserum used is a polyclonal monospecific one, directed against C/EBP (kindly provided by Steven L.McKnight), raised in rabbit. The immunofluorescence assay was performed essentially as previously described (Brinkenmeier *et al.*, 1989), except that a monolayer of cells rather than a tissue was used. A FITC-conjugated goat anti-rabbit antibody (Dakopatts A/S, Denmark) was used as secondary antibody. Control reactions were carried out using non-immune serum and secondary antibody alone. In these cases no apparent signal, differing from a control of non-C/EBP-expressing cells, could be detected. These reactions were always carried out in duplicate and with cells from two independent tissue culture dishes.

Digital image processing

We used a JVC TK870E CCD-RGB video camera, for registration of an analogue picture. The image was then digitized using an ATVista card mounted in a pc/386. Using the HEIAB-MEDNET Color Picture Processing system software, we set threshold values for estimations of relative areas with a certain colour intensity. We always worked with two independently Oil Red O stained tissue culture dishes, assayed in duplicate. A threshold value was selected that only registered Oil Red O stained lipid. This threshold value was based on estimates from cells that do not accumulate lipid such as HeLa cells and preadipocytes. The software also enabled us to point out the areas above and below the threshold value. These areas could be further analyzed in an inverted microscope and the threshold value could, based on these control experiments, be selected to reflect the relative area stained by Oil Red O.

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