# Tumour necrosis factor-$\alpha$ regulates expression of the CCAAT-enhancer-binding proteins (C/EBPs) $\alpha$ and $\beta$ and determines the occupation of the C/EBP site in the promoter of the insulin-responsive glucose-transporter gene in 3T3-L1 adipocytes

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We have demonstrated previously that treatment of 3T3-L1 adipocytes with tumour necrosis factor-$\alpha$ (TNF) results in a rapid (4 h) and significant (75–80 %) reduction in the rate of transcription of the *GLUT4* gene. Control of *GLUT4* gene transcription has been suggested at least in part to reside with the CCAAT-enhancer-binding protein (C/EBP) family ($\alpha$, $\beta$ and $\delta$ isoforms) of transcription factors. Using electrophoretic mobility shift assays, we have examined the ability of TNF to alter the occupation of the C/EBP site in the GLUT4 promoter. The data suggest that in fully differentiated adipocytes the C/EBP site is a ligand for predominantly $\alpha/\alpha$ homodimers; however, after exposure to TNF, a shift in occupancy of the site occurs and the ligands become $\alpha/\beta$ heterodimers and $\beta/\beta$ homodimers. Partner selection in dimer formation appears to be controlled by selective translocation of the $\beta$-isoform from the cytosol to the nucleus after exposure of the cells to TNF.

Key words: cytokine, differentiation, gene expression, glucose transport, transcription factor.

## INTRODUCTION

Recent studies examining the link between insulin resistance and the development of obesity and non-insulin-dependent diabetes mellitus are consistent with the involvement of tumour necrosis factor-$\alpha$ (TNF) as a central mediator [1–6]. Using an insulin-resistant obese-mouse model, Hotamisligil et al. [1] demonstrated that neutralization of the TNF resulted in restoration of insulin-mediated glucose uptake. Studies conducted on obese human patients demonstrated a correlation between levels of TNF and the extent of obesity, as well as with the level of hyperinsulinaemia observed [7]. Mechanistic studies using murine 3T3-L1 adipocytes were consistent with the ability of TNF to induce an insulin resistance through regulation of transporter synthesis [3,4,6,8] and potentially through interference with insulin signalling [2].

The 3T3-L1 preadipocytes differentiate in monolayer culture into cells with the morphological and biochemical properties of adipocytes. Critical to both the differentiation process and the maintenance of the adipocyte phenotype is the CCAAT-enhancer-binding protein (C/EBP) family ($\alpha$, $\beta$, $\delta$ and $\epsilon$ isoforms) of leucine zipper-type transcription factors [9–11]. C/EBP$\alpha$ is a critical factor in the differentiation process of the 3T3-L1 preadipocytes, as its expression leads to the transactivation of adipose-specific genes, such as the insulin-responsive glucose transporter (*GLUT4*), lipoprotein lipase and the adipocyte lipid-binding protein (422/aP2; [12]). Studies blocking the expression of this isoform have demonstrated the requirement of its expression for the differentiation process [13]. C/EBP$\alpha$ and C/EBP$\beta$ mRNAs possess alternative translation-initiation codons, which result in the formation of truncated forms of the two proteins. Translation of C/EBP$\alpha$ mRNA results in the production of a 42 kDa full-length protein and a 30 kDa truncated form of the transcription factor, both of which elicit similar transcriptional effects. In the case of the $\beta$ isoform, a truncated protein of 18 kDa (relative to the 30 kDa full-length protein that is known as LAP, or p30 C/EBP$\beta$ or liver-activating protein) lacking the transactivation domain is produced. The truncated protein, also known as LIP (p19 C/EBP$\beta$ or liver-inhibitory protein), can form homodimers or heterodimerize with other family members and, as it lacks the transactivation domain, can attenuate the transcriptional activation properties of the other isoforms [14–16].

Regulation of the adipocyte phenotype appears to occur through the selection of C/EBP dimer partners. Exposure of the adipocytes to glucocorticoids was shown to activate the transcription of C/EBP$\delta$, which in turn behaved as a transcriptional repressor of the $\alpha$-isoform [17]. Similarly, chronic insulin treatment established the role of LIP (C/EBP$\beta$) as a transcriptional repressor of $\alpha$-isoform expression [18]. In addition, TNF treatment of isolated hepatocytes has been shown to regulate the selection of dimer partners through alteration in the subcellular distribution of the $\beta$ and $\delta$ isoforms [19]. Extending this theme to non-family members, the C/EBPs have also been shown to dimerize with nuclear factor $\kappa$B (NF-$\kappa$B) [20] as well as activating transcription factor (ATF)-2 [21]. Clearly, the regulated selection of a dimer partner is a key mechanism for controlling gene transcription.

Our previous work has demonstrated that exposure of the fully differentiated adipocytes to TNF results in a co-ordinate transcriptional attenuation of both *GLUT4* and *C/EBPα* genes [3–5]. To address the mechanism of this observation, we have examined for TNF-induced alterations in the occupation of the C/EBP consensus site in the GLUT4 promoter. The results

Abbreviations used: C/EBP, CCAAT-enhancer-binding protein; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; LAP, p30 C/EBP$\beta$ or liver-activating protein; LIP, p19 C/EBP$\beta$ or liver-inhibitory protein; TNF, tumour necrosis factor-$\alpha$; TBS, Tris-buffered saline; NF-$\kappa$B, nuclear factor $\kappa$B; ATF, activating transcription factor; FBS, fetal bovine serum; DTT, dithiothreitol.

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demonstrate that prior to exposure to TNF, an $\alpha/\alpha$ homodimer occupied the C/EBP promoter site. However, after TNF treatment, a shift to predominantly $\alpha/\beta$ heterodimers was observed, which coincided on a temporal basis with the observed TNF-mediated transcriptional down-regulation of *GLUT4*.

## EXPERIMENTAL PROCEDURES

### Materials

Recombinant human TNF was generously provided by Biogen (Cambridge, MA, U.S.A.). The specific activity was $9.6\times10^6$ units/mg of protein, based on a cytotoxicity assay using L929 cells. The endotoxin contamination was 0.12 ng/mg of protein, based on a Limulus amoebocyte lysate assay (Sigma, St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) and bovine calf serum were purchased from HyClone (Logan, UT, U.S.A.) and used at a 1:10 dilution in Dulbecco's modified Eagle's medium (DMEM). Radiolabelled compounds were obtained from DuPont NEN (Boston, MA, U.S.A.). Hybond-N blotting membrane was purchased from Amersham Life Science (Arlington Heights, IL, U.S.A.). Protran nitrocellulose membrane was purchased from Schleicher and Schuell (Keene, NH, U.S.A.). Deoxyribonucleotides and double-stranded poly(dI–dC) were obtained from Pharmacia Biotechnology (Piscataway, NJ, U.S.A.). Klenow fragment and TRIzol Reagent were obtained from Gibco-BRL Life Technologies (Gaithersburg, MD, U.S.A.). Protogel and Sequagel were purchased from National Diagnostics (Atlanta, GA, U.S.A.). Bio-Rad reagent was purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). The C/EBP antibodies were graciously provided by Dr. D. Lane, The Johns Hopkins University, Baltimore, MD, U.S.A. All other chemicals, unless otherwise stated, were of molecular biological grade and purchased from Sigma.

### 3T3-L1 cell culture

Murine 3T3-L1 preadipocytes were cultured, maintained and differentiated as described previously [3]. Briefly, optimal differentiation was observed using a protocol that involved exposure of 3T3-L1 cells (day 0 = 2 days post confluence) to 10% FBS/10 $\mu$g/ml insulin/1 $\mu$M dexamethasone/0.5 mM 3-isobutyl-1-methylxanthine (MIX). The culture medium was changed 2 days later and MIX and dexamethasone were omitted. Day 8 adipocytes were serum starved by replacing the media with 0.5% FBS in DMEM for 48 h. The cells were refed with 0.5% FBS 2 h prior to stimulation with the appropriate agent. Recombinant human TNF was dissolved in Krebs/Ringer/Hepes buffer containing 0.1% BSA, and a 1:100 dilution was added to the cell-culture medium to produce a final concentration of 5.0 nM.

### cDNA probes

$\beta$-Actin, a 1.9 kb *Hin*dIII fragment was obtained from Dr. D. W. Cleveland, Johns Hopkins University [22]. C/EBP$\alpha$, a 1.7 kb *Eco*RI fragment was obtained from Dr. D. Lane [12]; C/EBP$\beta$, a 0.6 kb *Sma*I fragment was obtained from Dr. U. Schibler, University of Geneva, Geneva, Switzerland. C/EBP$\delta$, a 1.0 kb *Eco*RI/*Bam*HI fragment was obtained from Dr. P. Johnson, NCI-Frederick Cancer Research Facility, Frederick, MD, U.S.A.

### RNA isolation and Northern-blot analyses

Total RNA was isolated and Northern-blot analysis was performed as described [3] with the following modification. After UV crosslinking, the filters were prehybridized at 48 °C in Hybridsol (Oncor, Gaithersburg, MD, U.S.A.) for a minimum of 4 h. $^{32}$P-Labelled cDNA ($10\times10^6$ cpm/ml) was added and hybridization was carried out overnight at 48 °C. After hybridization the filters were washed with constant agitation in 0.1×SSC (where 1×SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS for two 15 min washes at room temperature, followed by one wash at 50 °C and the other at 60 °C. Autoradiography was performed using Kodak X-Omat AR film in DuPont Cronex Cassettes at −80 °C for various periods to ensure a linear film response.

### Preparation of nuclear extracts for C/EBP electrophoretic mobility-shift assay (EMSA) analysis

Approximately $100–150\times10^6$ cells (ten 10-cm petri dishes) from day 8 adipocyte cultures were serum starved for 48 h. On day 10, the plates were refed with 0.5% FBS containing DMEM, allowed to equilibrate, and then treated with 5.0 nM human TNF. The plates were then washed twice with cold PBS, and scraped in PBS. The cells were pelleted by centrifugation at 4 °C at 3000 ***g*** for 10 min. The supernatant was discarded and the floating fat removed with a cotton-tip applicator. The pellet was resuspended in five vols. of Buffer A (10 mM Hepes, pH 7.9/10 mM KCl/0.75 mM spermidine/0.15 mM spermine/0.1 mM EDTA/0.1 mM EGTA). The cells were allowed to swell in Buffer A for 10 min, followed by a low-speed centrifugation of 3000 ***g*** at 4 °C. The supernatant was discarded and the pellet resuspended in 2 vol. of Buffer A. The resuspended pellet was then homogenized by ten strokes in a ground-glass homogenizer. The lysate was centrifuged in a swing-bucket rotor at 3000 ***g*** at 4 °C. The supernatant was discarded, and careful aspiration of the top layer of unlysed cells from the top of the cell pellet was performed. The cell pellet was resuspended in 2 vol. of Buffer A and centrifuged. Analysis of the quality of the nuclei and the degree of contamination of cellular debris was conducted at this point by Trypan Blue staining. An excess of cellular debris led to another centrifugation step. The nuclei (pellet) were resuspended in 9 vols. of 1.1×NUN buffer [0.3 M NaCl/1% Nonidet P40/1 M urea/25 mM Hepes, pH 7.6/1 mM dithiothreitol (DTT)]. The pellet was then vortexed vigorously and the nuclei were allowed to swell on ice for 15 min. The chromatin was pelleted by centrifugation in a microfuge at 10000 ***g*** for 10 min at 4 °C. The fat–chromatin precipitate was removed and the nuclear extracts were flash-frozen at −80 °C. Determination of the protein concentration of the nuclear extracts was performed by use of the Bradford reagent.

### Preparation of radiolabelled probe for C/EBP EMSA

An oligonucleotide was designed to the C/EBP site in the murine GLUT4 promoter as reported by Kaestner et al. [23]: 5′-AATTACTGCGAATTTCTGAAAGAATTG-3′. The double-stranded oligonucleotide was prepared by annealing 10 $\mu$g of the complementary oligonucleotides together at 70 °C for 15 min and then allowing the reaction to cool slowly to room temperature. Once annealed, the oligomer was stored at 4 °C. End labelling of 100 ng of the annealed oligonucleotide was performed using 0.4 $\mu$M [$\alpha$-$^{32}$P]ATP (3000 Ci/mmol; 10 mCi/ml) in a reaction containing 0.5 $\mu$M dGTP, dCTP and dTTP, and 10 units of the large fragment of DNA polymerase (Klenow, Gibco-BRL) at room temperature for 30 min. Unlabelled dATP (final concentration 1.2 $\mu$M) was added and the reaction was incubated for an additional 10 min. The un-incorporated dATP was removed by passing the labelling reaction over a React 2 buffer (0.05 M NaCl/0.025 M Tris, pH 8/0.01 M $MgCl_2$) equilibrated on a NAP5 (Pharmacia) column.

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### C/EBP EMSA

The binding reactions (30 $\mu$l) were performed with $2 \times 10^5$ c.p.m. $^{32}$P-labelled oligonucleotide and 7 $\mu$g of nuclear extracts in a reaction buffer containing 0.33 M urea/0.1 M NaCl/0.33 % Nonidet P40/25 mM Hepes, pH 7.9/10 % glycerol/5 $\mu$g of acetylated BSA, 3 $\mu$g of poly(dI–dC)/10 mM DTT, and were incubated at room temperature for 60 min. For supershifting reactions, 2 $\mu$l of the appropriate antiserum was also included in the reaction. The reaction mixture was then electrophoresed on a native 7 % polyacrylamide gel at 6 V/cm. In all cases, band shifts were competed by a 200-fold molar excess of the unlabelled C/EBP-binding-site oligonucleotide. The gels were dried and autoradiography was carried out at −80 °C with Kodak X-Omat AR film and an intensifying screen for approximately 12–16 h.

### Western-blot analysis

Western-blot analysis was performed using one day 10 adipocyte 10-cm petri dish (approximately $13 \times 10^6$ cells). The plate was washed with cold PBS and lysis of the cells was accomplished by addition of 400 $\mu$l of 1 × WSB (62.5 mM Tris/HCl, pH 6.8 at 25 °C/2 % SDS/10 % glycerol/50 mM DTT). The extracts were then sonicated for 10–30 s to shear the DNA and reduce sample viscosity. The samples were aliquoted and stored at −80 °C until ready for analysis. Prior to loading, the samples were heated to 95–100 °C for 5 min and cooled on ice. The samples were centrifuged briefly to pellet any insoluble material prior to loading on a 8 % SDS/PAGE gel. After transfer, the nitrocellulose membrane was incubated in 25 ml of blocking buffer [1 × Tris-buffered saline (TBS) containing 0.1 % Tween-20 with 5 % (w/v) non-fat dry milk] for 1–3 h at room temperature, or overnight at 4 °C. The membrane was then incubated in the appropriate dilution of the primary antibody in 10 ml of the antibody dilution buffer (1 × TBS, 0.05 % Tween-20 with 5 % BSA) with gentle agitation overnight at 4 °C. The membrane was then washed four times each with 15 ml of TBST (1 × TBS with 0.1 % Tween-20). The membrane was then incubated with a horseradish peroxidase-conjugated anti-rabbit secondary antibody in 10 ml of antibody dilution buffer with gentle agitation for 1 h at room temperature. The membrane was then washed again four times in TBST as described above. The detection of the proteins was conducted by incubating the membrane with the enhanced chemiluminescence detection reagent with gentle agitation for 1 min. Excess detection agent was drained and the membrane exposed to X-ray film.

## RESULTS

### Effect of TNF on the DNA-binding capacities of the C/EBP isoforms

To analyse alterations in protein binding at the C/EBP site in the GLUT4 promoter upon TNF treatment, EMSAs were conducted. An oligonucleotide probe corresponding to the C/EBP consensus site in the 3T3-L1 GLUT4 promoter, as reported by Kaestner et al. [23] (the exact sequence of the oligonucleotide is detailed in the Experimental procedures section), was synthesized. All three C/EBP isoforms ($\alpha$, $\beta$ and $\delta$) were capable of binding to this site [24,25]. Nuclear extracts were prepared from control and TNF-treated adipocytes. In preliminary experiments, extracts were prepared after periods of exposure to TNF ranging from 1.5 to 24 h and the EMSA assays were performed. The results demonstrated that maximal alterations in binding occurred after 4 h of exposure (results not shown).

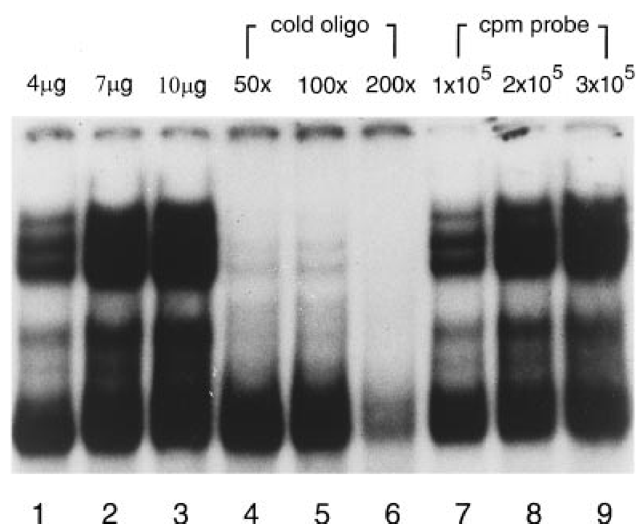


**Figure 1 Determination of binding conditions for the C/EBP EMSA**

Nuclear extracts were prepared from day 8 adipocytes that had been exposed to 5 nM TNF for 3 h. Nuclear extracts were incubated with a double-stranded radiolabelled oligonucleotide corresponding to the C/EBP site in the GLUT4 promoter. The binding reactions were incubated at room temperature for 1 h and then loaded on to a 7% native polyacrylamide gel. Lanes 1–3, increasing amounts of nuclear extract were added. Lanes 4–6, increasing amounts of unlabelled (cold) C/EBP-binding site oligonucleotide were added. Increasing amounts of radiolabelled probe were added in lanes 7–9 (shown as c.p.m./reaction). The data shown are representative of an experiment performed twice with similar results.

As shown in Figure 1 (lanes 1–3), binding of protein to the oligonucleotide increased with increasing amounts of nuclear protein extract. Examination of protein concentrations ranging from 4 to 10 $\mu$g indicated that maximum binding occurred when 7 $\mu$g of nuclear extract protein was used. Primary complexes were distinguished from non-specific binding through competition with 50–200 fold excess of unlabelled C/EBP double-stranded oligonucleotide, as shown in Figure 1, lanes 4–6. Full competition of the four labelled primary bands was observed. To determine conditions that ensured excess DNA probe in the binding reaction, increasing amounts of the radiolabelled probe (i.e. increasing c.p.m./reaction) were added, as shown in Figure 1, lanes 7–9. Based on the data shown in Figure 1, all subsequent gel-shift experiments were performed using 7 $\mu$g of nuclear extracts and $2 \times 10^5$ c.p.m. of radiolabelled probe per binding reaction at room temperature for 90 min.

TNF treatment for 4 h (Figure 2, lane 2) resulted in alterations of the intensities in the banding patterns of the four primary complexes, a–d, relative to the control (Figure 2, lane 1). Resolution of the composition of these four complexes was made possible by use of isoform-specific C-terminal antibodies. Potential occupants of the four primary complexes include: C/EBP$\alpha$, p42 and p30; C/EBP$\beta$, LAP and LIP; and C/EBP$\delta$ (no alternative translational products of C/EBP$\delta$ are currently known). Each antibody is isoform specific and does not cross-react with the others [17,18,26]. The presence of five C/EBP family members, all with the potential to form both homo- and heterodimers, some with near-identical molecular mass, results in a situation where there may be an alteration in the isoform occupancy of the promoter which may not alter the migration of the primary complex. Hence, resolution through use of isoform-specific C-terminal antibodies becomes crucial. However, based on the work of MacDougald and colleagues [17,18,26] and the similarity of the isoforms, we did use approximations of mol-

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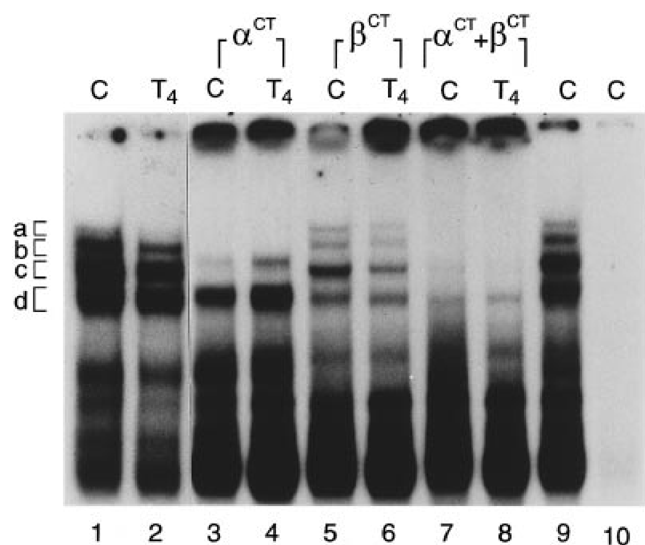


**Figure 2 TNF-induced alteration in C/EBP isoforms binding to the consensus binding site**

EMSA were conducted on day 8 nuclear extracts which were treated with 5 nM TNF for 4 h (T4) as described in the Experimental procedures section. Supershifting of bound complex was performed using 2 $\mu$l of antibody specific to the C-terminus (CT) of the C/EBP isoforms. Lanes 1 and 2 are control (C) and TNF-treated nuclear extracts with no antiserum added. Antisera directed against the C-terminus of the C/EBP$\alpha$ isoform (lanes 3 and 4) and antisera directed against the C-terminus of C/EBP$\beta$ (lanes 5 and 6) were added for supershifting reactions. A combination of the two antisera was used in the supershifting reactions as shown in lanes 7 and 8. Control extracts were incubated with 3 $\mu$l of preimmune serum (lane 9) and 100-fold excess of unlabelled oligonucleotide as shown in lane 10. The data shown are representative of an experiment performed five times with similar results.

**Table 1 Potential dimers of the C/EBP isoforms**

The dimers of C/EBP$\alpha$, $\beta$ and $\delta$ are listed with respect to molecular mass. Bands identified potentially in DNA gel-shift analysis are denoted by letters (a–d), corresponding to bands shown in Figures 1–3. Note: whereas LIP was not identified specifically in the DNA gel-shift analysis, it has, based on the translocation data, the potential to form dimers of the indicated molecular masses and participate in complexes forming the indicated band.

| Dimer | Molecular mass (kDa) | Band |
|---|---|---|
| p42$\alpha$/42$\alpha$ | 84 | a |
| p42$\alpha$/30$\alpha$ | 72 | b |
| p30$\alpha$/30$\alpha$ | 60 | c |
| p30$\beta$/p30$\beta$ | 60 | c |
| p30$\alpha$/p30$\beta$ | 60 | c |
| p42$\alpha$/LIP | 60 | c |
| p30$\alpha$/p29$\delta$ | 59 | c |
| p29$\delta$/p29$\delta$ | 58 | d |
| p30$\alpha$/LIP | 48 | c and d |
| p30$\beta$/LIP | 48 | c and d |
| LIP/LIP | 36 | d |

ecular mass to support the immuno-identification of the various C/EBPs. The homo- and heterodimers potentially formed by the C/EBP family members are shown in Table 1.

TNF treatment leads to a decrease in the intensity of band a, as shown in lanes 1 (control) and 2 (TNF-treated) of Figure 2. In Figure 2, lanes 3 and 4, use of the C/EBP$\alpha$ C-terminal antibody resulted in a supershift of the primary complexes a and b. After TNF treatment, increases in two complexes were observed, which, based on the supershift data, do not contain the $\alpha$ isoform (Figure 2, lane 4). Note the increased intensities of bands c and d in lane 4 relative to lane 3. Band a is presumably a p42$\alpha$/p42$\alpha$ complex and band b is p30$\alpha$/p30$\alpha$ homodimer. A shift of a portion of primary complex c and a portion of d is observed on addition of C-terminal antibody directed to the $\beta$ isoform, as shown in Figure 2, lanes 5 and 6. The lack of shifting of bands a and b by the $\beta$ antibody is consistent with an $\alpha/\alpha$ homodimer composition of these complexes and the absence of involvement of the other isoforms in these two complexes. Band c appears to be a composite of more than one complex. As shown in Table 1, there are six potential dimers of approximately 60 kDa, which may migrate to the position of band c. There is an increase with TNF treatment in the intensity of the c complex left behind upon supershifting with the $\alpha$ antibody, supporting increased occupation by another isoform. When the reactions are incubated with the $\beta$ antibody, there is a decrease in the intensity of band c with TNF treatment and a major increase in the complex shifted to the top of the gel, indicative of a greater involvement of the $\beta$ isoform in complex c formation after TNF treatment (compare lanes 5 and 6, Figure 2).

Primary complexes a, b and c were observed to be totally supershifted when the binding reaction was exposed to a combination of C-terminal antibodies directed against C/EBP$\alpha$ and C/EBP$\beta$ (lanes 7 and 8). Whereas assignment of dimer partners in band c is equivocal due to multiple complexes, the complete shift of band c observed in lanes 7 and 8 indicates that either C/EBP$\alpha$ or C/EBP$\beta$ is involved in any complex formed at this band position.

With respect to band d, the data support multiple components with increased involvement of C/EBP$\beta$ with respect to TNF treatment (Figure 2, lanes 3–6). Whereas this band appears to be predominantly composed of dimers of the $\alpha$ and $\beta$ isoforms, a minor component remained unshifted when both antibodies were used for the supershift (Figure 2, lanes 7 and 8), consistent with multiple dimers responsible for band d formation and suggesting that C/EBP$\delta$ must be involved in dimer formation at this position.

Supershift analysis was then performed using antibodies against C/EBP$\delta$. Whereas antibody-induced alterations of the primary complexes were difficult to discern, long exposure times of the autoradiograms gave rise to a faint band of low mobility, consistent with a supershift; this also resulted in loss of resolution of bands a–d (results not shown). Thus, in an attempt to determine which complex C/EBP$\delta$ might be involved in, the $\delta$ antibody was used in combination with antibodies against the other C/EBP isoforms (Figure 3). The shifting pattern induced using a combination of antibodies directed against the $\alpha$ and $\delta$ isoforms (Figure 3, lanes 2 and 3) was reminiscent of that shown in Figure 2 using anti-C/EBP$\alpha$ alone. However, when the anti-C/EBP$\delta$ was used in combination with that directed against the $\beta$ isoform (Figure 3, lanes 4 and 5), band c was reduced in the control relative to the results displayed in Figure 2 (lanes 5 and 6). In addition, a total shift of the primary complexes was observed on addition of all three antibodies to the binding reaction, as shown in lanes 6 and 7 of Figure 3. Note the complete loss of band d (lanes 6 and 7) relative to the supershifts (Figure 2, lanes 7 and 8) with antibodies against only the $\alpha$ and $\beta$ isoforms. These data (Figures 2 and 3) support the presence of C/EBP$\delta$ as a minor component of band c in control extracts and suggest that its partner in dimer formation is most probably p30$\alpha$. In addition, the total shift of band d in Figure 3 (lanes 6 and 7) supports the presence of C/EBP$\delta$ homodimers in band d.

These results obtained using gel-shift analysis are consistent with a TNF-induced replacement of C/EBP$\alpha$ with C/EBP$\beta$ in

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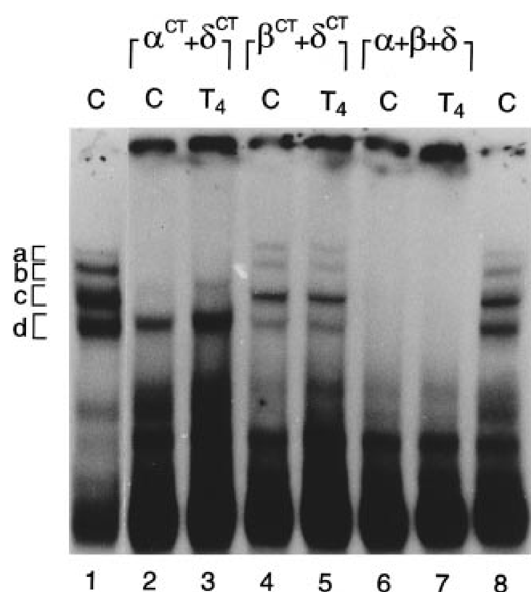


**Figure 3 Binding of C/EBP$\delta$ to the C/EBP site**

EMSAs were conducted on day 8 nuclear extracts, prepared from cells that had been exposed to 5 nM TNF for 4 h (T4) as described in the Experimental procedures section. Lane 1 contained no antisera. Antisera directed to the C-termini (CT) of C/EBP$\alpha$ and C/EBP$\delta$ were added in lanes 2 and 3. A combination of antisera against the C-termini of C/EBP$\beta$ and C/EBP was added in the reactions supershifted in lanes 4 and 5. A combination of antisera against all three isoforms was added in lanes 6 and 7. An equivalent amount of preimmune serum was added to control nuclear extracts in lane 8. The experiment was performed twice with similar results. C, control.

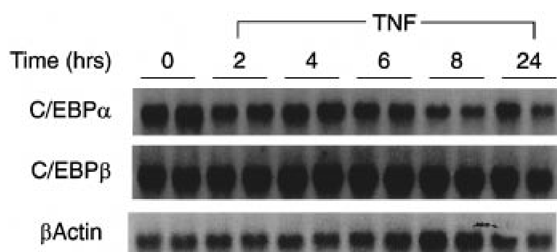


**Figure 4 TNF treatment leads to a decrease in C/EBP mRNA**

Fully differentiated adipocytes (day 8) were exposed to 5 nM TNF and total RNA prepared from two independent monolayers at the times indicated. Equal amounts of RNA (20 $\mu$g) were electrophoresed and analysed by Northern blotting using cDNAs complementary to C/EBP$\alpha$ and C/EBP$\beta$ as well as $\beta$-actin for normalization. The data shown are representative of an experiment performed three times with similar results.

the dimers that occupy the C/EBP site in the GLUT4 promoter. The lack of shifting of band c with C/EBP$\alpha$ antibodies and estimation of the molecular mass of the complex left behind support a model in which TNF treatment of the adipocytes results in $\alpha$ and $\beta$ homo- and heterodimer(s) representing the major components of the complex.

### Effect of TNF on the cellular content of the C/EBP protein and mRNA

To ensure that the decreased participation of C/EBP$\alpha$ in formation of transcription factor dimers described above was not secondary to decreased expression of the protein in this time frame, Northern- and Western-blot analyses were performed to

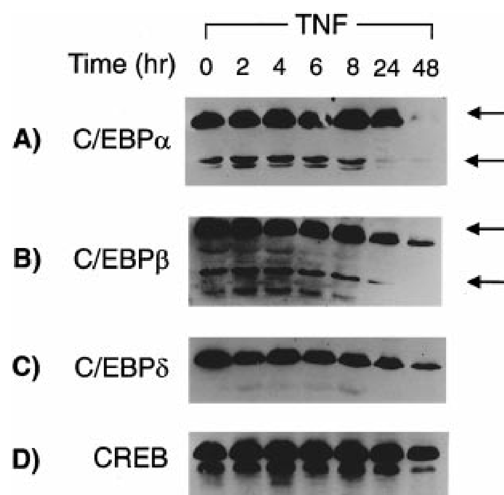


**Figure 5 Protein content of C/EBP$\alpha$ and C/EBP$\beta$ after exposure of the cells to TNF**

The protein content of cell lysates prepared from day 8 adipocytes was separated by SDS/PAGE and immunoblotted using antisera against the C-termini of (**A**) C/EBP$\alpha$ (upper arrow indicates p42; lower arrow, p30), (**B**) C/EBP $\beta$ (upper arrow indicates p30; lower arrow, p18), (**C**) C/EBP$\delta$ and (**D**) cAMP-response-element-binding protein (CREB). Data were normalized to the results for CREB. The data shown are representative of an experiment performed three times with similar results.

characterize cellular content of all three C/EBP family members. Fully differentiated adipocytes were serum starved for 48 h to minimize growth factor stimulation of the cells and then exposed to 5 nM TNF for a time course ranging from 0 to 24 h. Whereas a two-fold decrease in C/EBP$\alpha$ mRNA content was observed by the end of the time course, no substantial change had occurred at the 4 h time point. Thus, at the time when extracts were prepared for the DNA gel-shift assays described in Figures 1–3, C/EBP$\alpha$ mRNA synthesis was intact. No change was observed in the mRNA levels for C/EBP$\beta$ (Figure 4). Total mRNA loading was normalized to the hybridization signal generated by $\beta$-actin.

The effect of TNF on the expression of the cellular protein content of the three C/EBP isoforms was examined by Western-blot analysis. The results shown in Figure 5 demonstrate a greater than two-fold reduction in the levels of all three isoforms after 48 h of TNF treatment. The C/EBP$\alpha$ antibody used in this study was directed against the C-terminus of the $\alpha$ isoform and hence detected the full-length 42 kDa protein as well as the 30 kDa, truncated, alternative translation product (indicated by the lower arrow in Figure 5A). Similarly, the antibody used for determination of the cell content of the $\beta$ isoform was directed to the C-terminus and detected both LAP, the full translation product, as well as LIP, the truncated form of C/EBP$\beta$ (Figure 5B, upper and lower arrows, respectively). The total cellular protein content of the $\delta$ isoform also exhibited a marked decrease by 48 h of TNF treatment (Figure 5C). For quantitative analyses, the immunoblots were normalized to the content of cAMP-response-element-binding protein, a positive invariant control [11].

Importantly, these data confirm that after 4 h of TNF treatment, the cellular content of all three C/EBP isoforms is relatively unaltered, indicating the potential for all three proteins to be involved in dimer formation and binding to the C/EBP consensus sequence in the GLUT4 promoter (Figures 2 and 3).

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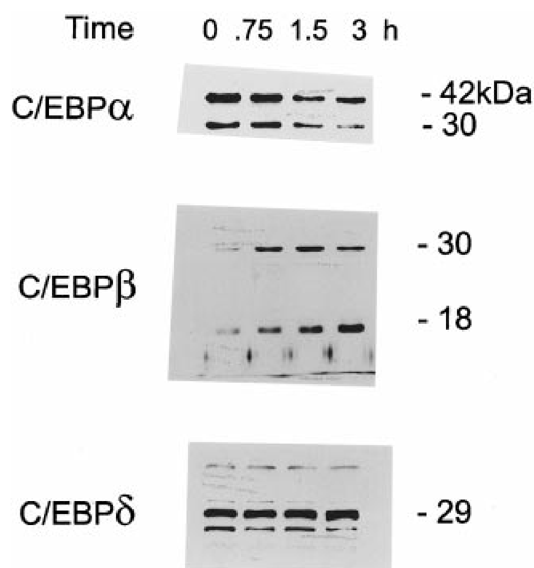


**Figure 6 Nuclear translocation of C/EBP$\beta$**

Nuclear extracts were prepared from day 8 adipocytes as described in the Experimental procedures section after 4 h of TNF treatment and the protein content of C/EBP$\alpha$, $\beta$ and $\delta$ was determined by Western-blot analysis. The blot was first probed for C/EBP$\alpha$, then stripped and probed for the $\beta$ and $\delta$ isoforms. The experiment was performed three times with similar results.

#### Effect of TNF on the localization of C/EBP$\beta$

The data described above indicate that occupation of the C/EBP-binding site changes with respect to TNF treatment. To begin to address the mechanism behind this observation we examined for changes in the nuclear residence of the isoforms. Nuclear extracts were prepared from 100–150 $\times 10^6$ day 8 adipocytes as described for the EMSA assay, and the proteins in 25 $\mu$g of the purified nuclear extract separated by SDS/PAGE and Western-blot analysis performed as described in the Experimental procedures section. As shown in Figure 6, the nuclear content of C/EBP$\alpha$ was maximal at 0 h in untreated cells and decreased by 60 % after 3 h of exposure to TNF. However, C/EBP$\beta$ was at its lowest concentration in the untreated cells (0 h) and during the 4 h experiment the nuclear content of both LAP and LIP increased markedly. No change was observed in the nuclear content of C/EBP$\delta$. As shown in Figure 5, the total cellular protein content of both C/EBP$\alpha$ and C/EBP$\beta$ remained constant during the 4 h. No changes were observed when the cytosol was examined for protein content of the three isoforms, indicating that only a small percentage of the total cytosolic content of the $\beta$ isoform translocated to the nucleus (results not shown). These data are consistent with a TNF-induced translocation of C/EBP$\beta$ from the cytosol to the nucleus on a temporal basis that is coincident with the down-regulation of *GLUT4* transcription [4]. The mechanism of this translocation is currently under investigation.

## DISCUSSION

Using DNA EMSAs, we have demonstrated that TNF treatment of the cells alters the composition of the proteins capable of binding to an oligonucleotide containing the C/EBP site from the GLUT4 promoter. The data are consistent with a shift from predominantly $\alpha/\alpha$ homodimers in control cells to a mixture of dimers of the $\alpha$ and $\beta$ isoforms after TNF treatment. The dynamic changes in occupation of the C/EBP site appear to be controlled by sequestration of C/EBP$\beta$; both LAP and LIP reside in the cytosol of control cells but, on exposure to TNF, translocate to the nucleus. The time frame for these changes is fairly rapid, with maximal response occurring within 4 h, long before the TNF-induced down-regulation of both C/EBP$\alpha$ and C/EBP$\beta$, which leads to decreased cellular content of both proteins (Figure 5).

Our previous work had demonstrated that exposure of the fully differentiated adipocytes to TNF resulted in a down-regulation of *GLUT4* gene transcription, leading to a decreased cellular content of the insulin-responsive glucose transporter, rendering the cells insulin resistant [2,4,6]. Whereas TNF-induced insulin resistance may also involve down-regulation of the insulin-signalling cascades [2], the focus of the current study was to elucidate the mechanism of TNF-induced *GLUT4* transcriptional control. The C/EBP family of transcription factors has been reported to play a critical role in gene regulation in the 3T3-L1 adipocytes (reviewed in [24,25]). Whereas occupation of the C/EBP site in the GLUT4 promoter by $\alpha/\alpha$ homodimers appears to activate the transcriptional process, alteration of the occupation and replacement by other family members may attenuate transcription. Variations in the C/EBP isoforms involved in formation of homo- and/or heterodimers have been shown to occur when the 3T3-L1 adipocytes are exposed chronically to either insulin or glucocorticoids [17,18,26]. In response to insulin treatment, a rapid dephosphorylation of the $\alpha$ isoform is observed with concomitant suppression of C/EBP$\alpha$ gene transcription and inactivation of C/EBP$\alpha$ through an increase in the cellular levels of the dominant negative C/EBP$\beta$ isoform, LIP [18]. When the adipocytes were exposed to glucocorticoids, a reciprocal regulation of C/EBP$\alpha$ and C/EBP$\delta$ was observed, characterized by a rapid decrease in the content of the $\alpha$ isoform with a simultaneous increase in the expression of the $\delta$ isoform [17]. Thus a pattern of cellular manipulation of C/EBP-isoform expression as a mechanism of regulation begins to emerge.

As one would predict, the effects of hormones such as insulin and glucocorticoids on C/EBP-isoform expression are rapid and transient [17,18]. However, exposure of the adipocytes to TNF leads to a down-regulation of the C/EBP family of transcription factors that, while rapid, is not transient, consistent with our previous observations that exposure of the adipocytes to TNF alters the phenotype of the cells [4].

Other regulatory events may be taking place: activation of transcription by C/EBP has been demonstrated to be influenced by other transcription factors. NF-$\kappa$B [20] and ATF-2 [21] are capable of binding to *cis*-elements in the proximity of a C/EBP element and of interacting with the C/EBP protein to alter transcription. The GLUT4 promoter contains consensus binding sites for both NF-$\kappa$B and ATF-2 as well as numerous other transcription factors, and we have demonstrated previously the activation of both ATF-2 and NF-$\kappa$B by TNF in these cells [27]. Thus the potential for interaction exists and is currently under investigation.

In summary, our data support a model for transcriptional control of *GLUT4* gene expression involving selective replacement of C/EBP$\alpha$ homodimers with $\alpha/\beta$ heterodimers and $\beta/\beta$ homodimers. Based on the data presented in Figure 6, there is potential for both LAP and LIP to participate in the contribution made by C/EBP$\beta$, suggesting that transcriptional control may be mediated by the dominant negative LIP isoform. Key to the regulation is the cytosolic localization of C/EBP$\beta$ prior to TNF treatment. It is the signalling initiated by TNF that promotes both translocation of C/EBP$\beta$ to the nucleus as well as the loss of C/EBP$\alpha$ binding to its cognate element in the GLUT4 promoter. Potentially, these events may all be regulated by

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phosphorylation/dephosphorylation [26,28,29] and that is currently under investigation.

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## REFERENCES

1 Hotamisligil, G. S., Shargill, N. S. and Spiegelman, B. M. (1993) Science **259**, 87–91
2 Hotamisligil, G. S., Peraldi, P., Budavari, A., Ellis, R., White, M. F. and Spiegelman, B. M. (1996) Science **271**, 665–668
3 Stephens, J. M. and Pekala, P. H. (1991) J. Biol. Chem. **266**, 21839–21845
4 Stephens, J. M. and Pekala, P. H. (1992) J. Biol. Chem. **267**, 13580–13584
5 Long, S. D. and Pekala, P. H. (1996) Biochem. J. **319**, 179–184
6 Stephens, J. M., Lee, J. and Pilch, P. F. (1997) J. Biol. Chem. **272**, 971–976
7 Hotamisligil, G. S., Arner, P., Caro, J. F., Atkinson, R. L. and Spiegelman, B. M. (1995) J. Clin. Invest. **95**, 1543–1549
8 Cornelius, P., Marlowe, M., Lee, M. D. and Pekala, P. H. (1990) J. Biol. Chem. **265**, 20506–20516
9 Landshultz, W. H., Johnson, P. F. and McKnight, S. L. (1988) Science **240**, 1759–1764
10 Cao, Z., Umek, R. M. and McKnight, S. L. (1991) Genes Dev. **5**, 1538–1552
11 Ron, D. and Habener, J. F. (1992) Genes Dev. **6**, 439–453
12 Christy, R. J., Kaestner, K. H., Geiman, D. E. and Lane, M. D. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 2593–2597
13 Lin, F. and Lane, M. D. (1992) Genes Dev. **6**, 533–544

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14 Descombes, P. and Schibler, U. (1991) Cell **67**, 569–579
15 Ossipow, V., Descombes, P. and Schibler, U. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 8219–8223
16 An, M. R., Hsieh, C. C., Reisner, P. D., Rabek, J. P., Scott, S. G., Kuninger, D. T. and Papaconstantinou, J. (1996) Mol. Cell. Biol. **16**, 2295–2306
17 MacDougald, O. A., Cornelius, P., Lin, F. T., Chen, S. S. and Lane, M. D. (1994) J. Biol. Chem. **269**, 19041–19047
18 MacDougald, O. A., Cornelius, P., Liu, R. and Lane, M. D. (1995) J. Biol. Chem. **270**, 647–654
19 Yin, M., Yang, S. Q., Lin, H. Z., Lane, M. D., Chatterjee, S. and Diehl, A. M. (1996) J. Biol. Chem. **271**, 17974–17978
20 Ruocco, M. R., Chen, X., Ambrosino, C., Dragonetti, E., Liu, W., Mallardo, M., De, F. G., Palmieri, C., Franzoso, G., Quinto, I. et al. (1996) J. Biol. Chem. **271**, 22479–22486
21 Shuman, J. D., Cheong, J. and Coligan, J. E. (1997) J. Biol. Chem. **272**, 12793–12800
22 Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J. and Kirschner, M. W. (1980) Cell **20**, 95–105
23 Kaestner, K. H., Christy, R. J. and Lane, M. D. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 251–255
24 Cornelius, P., MacDougald, O. A. and Lane, M. D. (1994) Annu. Rev. Nutr. **14**, 99–129
25 MacDougald, O. A. and Lane, M. D. (1995) Annu. Rev. Biochem. **64**, 345–373
26 Hemati, N., Ross, S. E., Erickson, R. L., Groblewski, G. E. and MacDougald, O. A. (1997) J. Biol. Chem. **272**, 25913–25919
27 Jain, R. G., Phelps, K. and Pekala, P. H. (1999) J. Cell. Physiol. **179**, 58–66.
28 Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T. and Akira, S. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2207–2211
29 Metz, R. and Ziff, E. (1991) Genes Dev. **5**, 1754–1766

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