

Tumor necrosis factor-induced *c-myc* expression in the absence of mitogenesis is associated with inhibition of adipocyte differentiation

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ABSTRACT Tumor necrosis factor (TNF) inhibits and reverses differentiation of mouse adipogenic TA1 cells. We have found that TNF induces *c-myc* in a sustained manner in both preadipocytes and adipocytes; in contrast, serum induces *c-myc* transiently and only in preadipocytes. This TNF-mediated *c-myc* induction is not coupled with cell proliferation but is correlated with TNF-mediated inhibition of adipocyte differentiation. We prepared an inducible *c-myc* transformatant of TA1 cells by transfection of the mouse *c-myc* gene under the control of the metallothionein-I promoter. These cells are unable to differentiate to adipocytes in the presence of Zn^{2+}/Cd^{2+} , and in differentiated TA1 cells, Zn^{2+}/Cd^{2+} causes reduction of adipocyte-specific gene expression as does TNF. Lastly, exposure of TA1 cells to antisense *c-myc* oligonucleotide partially blocked the TNF-mediated reduction of adipocyte-specific gene expression. Thus, TNF-mediated *c-myc* expression is distinct in character from that involved in mitogenic responses but appears to play an important role in inhibition and reversal of adipocyte differentiation.

Tumor necrosis factor (TNF) has a wide variety of biological activities depending on the cell type and the growth state of cells, including cytotoxicity and modulation of cell growth and differentiation (1). TNF initiates its functions through two distinct TNF receptors: type I (55–60 kDa) and type II (75–80 kDa) and stimulates intracellular events, including activation of protein kinase C (2) and release of arachidonic acid (3). Through activation of transcription factors AP1, NF κ B, and possibly other DNA-binding proteins, TNF induces expression of many genes including metalloproteases, cytokines (interleukin 1 and interleukin 6), and immediate-early genes of growth stimulation (*c-fos*, *c-jun*, and *c-myc*) (1). Despite extensive study of cellular responses to TNF, it remains uncertain which events are important for particular actions of TNF.

In mouse adipogenic cell lines such as TA1 and 3T3-L1, TNF completely inhibits the process of adipocyte differentiation and reverts the fully differentiated adipocyte to the preadipocyte (4, 5). Addition of TNF prevents expression of adipocyte-specific genes, such as *aP2*, *GPD1* (glycerol phosphate dehydrogenase), and *FSP27*, in differentiating adipogenic cells and reverses expression in fully differentiated cells (5, 6). It has recently been shown that TNF reduces the level of the CCAAT/enhancer-binding protein α (C/EBP- α) in fully differentiated adipocytes (7–10), which has been implicated as a major regulator of adipocyte-specific gene expression (11–14). Immediate-early genes such as *c-fos* and *c-jun* are transiently induced by TNF in TA1 cells; however, their induction has not been associated with inhibition of adipocyte differentiation (3).

Expression of *c-myc* is known to be important for promotion of cell growth (15). When resting cells are stimulated to

proliferate with serum or other mitogens, *c-myc* message increases rapidly and decreases 8–12 hr after stimulation (15, 16). In contrast, we have found that TNF induces *c-myc* message for at least 48 hr in TA1 cells but does not induce cell proliferation in either preadipocytes or fully differentiated adipocytes. Many studies have shown that ectopic expression of *c-myc* interferes with differentiation of various cell types, including adipogenesis in 3T3-L1 cells (17). Here we demonstrate that: (i) *c-myc* expression is correlated with TNF-mediated reduction of adipocyte-specific gene expression; (ii) *c-myc* expression under the control of the metallothionein-I promoter is able to mimic the effect of TNF; (iii) antisense oligonucleotides to *c-myc* partially block TNF action; (iv) the induction of *c-myc* by TNF, unlike that by serum, is independent of mitogenesis. These results strongly suggest that the unusual pattern of *c-myc* induction by TNF is central to its ability to interfere with adipocyte differentiation.

MATERIALS AND METHODS

Cells and Plasmids. TA1 cells (18) were cultured in Eagle's basal medium (BME Earle's salts medium) supplemented with 10% fetal calf serum (FCS) at 37°C and 5% CO₂. TA1 cells were differentiated spontaneously by the continuous maintenance of confluent cells; $\approx 70\%$ of the population was detected as adipocytes 6–10 days after reaching confluence. The developmental process was defined by referring to the day cells reach confluence as day 0. Purified recombinant TNF- α was provided by Cetus.

pHygMT-*c-myc* plasmid contains the *hyg^r* gene from pHyg (19) and mouse *c-myc* cDNA (provided by Naoko Arai, DNAX) under the control of metallothionein-I promoter. A 1.9-kb *EcoRI*-*Bgl* II fragment containing metallothionein-I promoter region (20) was subcloned into *EcoRI*-*Bam*HI sites of pBluescript II SK(+) (Stratagene) and recovered on a *Hind*III-*Xba* I fragment containing the promoter region. The SR α promoter was removed from pHygSR α vector (21, 22) by *Hind*III and *Xba* I digestion and replaced with the *Hind*III-*Xba* I metallothionein-I promoter fragment to yield pHygMT plasmid. The mouse *c-myc* cDNA was inserted into the *Bam*HI site downstream of the metallothionein-I promoter in pHygMT, and the plasmid containing *c-myc* cDNA in the sense orientation was used in this study.

Northern Blotting. Total cellular RNA was prepared by the method of Chirgwin *et al.* (23). Ten micrograms of total RNA was separated by electrophoresis on 1.1% agarose/6% formaldehyde gel, followed by blotting onto nylon filter (Hybond-N⁺, Amersham). The fragments of mouse *c-myc*, mouse *c-fos* (from Naoko Arai), human *c-JUN* (3), human ferritin light chain gene (24), mouse thymidine kinase gene (ATCC 37556), *aP2* cDNA (18), and *FSP27* cDNA (6) were labeled by using

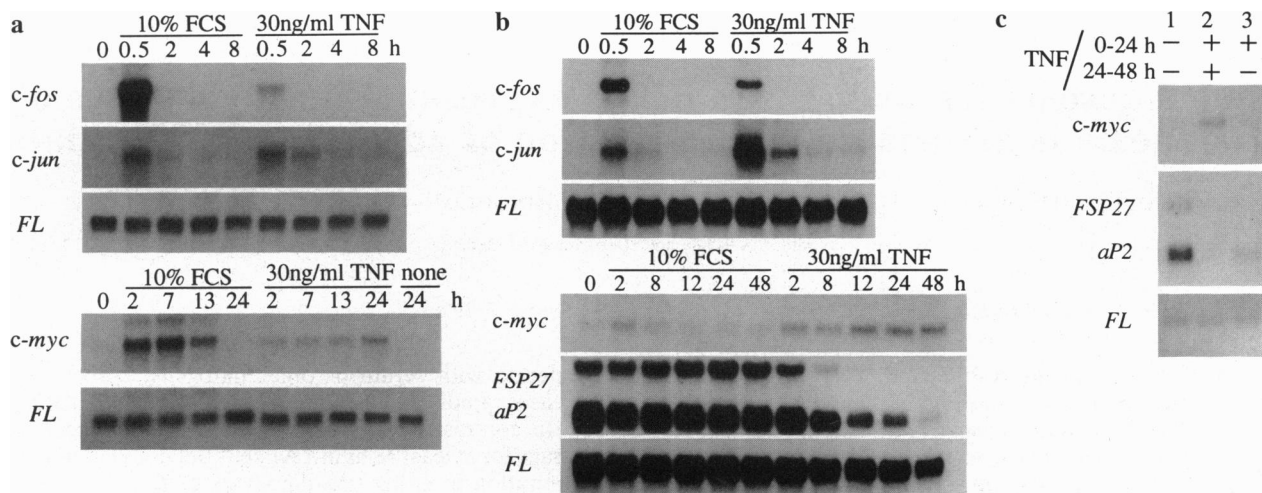


FIG. 1. Time course of *c-fos*, *c-jun*, and *c-myc* induction in TA1 preadipocytes and adipocytes. TA1 cells were plated and allowed to grow for 3–5 days without changing the culture medium (preadipocytes, *a*), or were cultured for 8 days after reaching confluence (adipocytes, *b* and *c*). (*a* and *b*) Cells were stimulated by adding fresh medium (10% FCS) or TNF (30 ng/ml) and harvested at the various time points thereafter. (*c*) Cells (day 8) were refed and incubated without (lanes 1) or with TNF at 30 ng/ml (lanes 2 and 3). Cells were washed in fresh medium twice after 24-hr incubation and maintained for an additional 24 hr in fresh medium without (lanes 1 and 3) or with TNF (lane 2). *c-fos*, *c-jun*, *c-myc*, *aP2*, and *FSP27* mRNAs were detected by Northern blotting, as described. The blots were reprobbed with ferritin light chain (FL) DNA to determine the evenness of RNA loading.

random primers to specific activity of 2×10^9 cpm/ μ g. Hybridization and washing was done as described (24).

Antisense Oligonucleotide to *c-myc*. Oligodeoxynucleotides, antisense (a/s) *c-myc* CACGTTGAGGGGCAT and sense (s) *c-myc* ATGCCCTCAACGTG (25), were synthesized with an Applied Biosystems DNA synthesizer. Oligonucleotides were added to culture medium at 200 μ g/ml.

***c-Myc* Protein Analysis.** Cells were harvested 24 hr after addition of TNF and oligonucleotide and lysed in 1 mM phosphate buffer, pH 7.25/1% Triton X-100/0.5% deoxycholate/0.1% SDS/0.2% sodium azide. One milligram of cell lysate was preadsorbed with protein A-agarose and then incubated with 6E10 anti-*c-myc* antibody (Cambridge Research Biochemicals) (26) and protein A at 4°C overnight. Immunoprecipitated *c-Myc* protein was separated on SDS/polyacrylamide gels and electroblotted onto Immobilon-P (Millipore). The filter was incubated for 1 hr in phosphate-buffered saline/0.2% Tween 20 (PBS-T)/5% nonfat dried milk and then incubated for 1 hr in the same solution containing 6E10 antibody at 5 μ g/ml, washed three times for 5 min in PBS-T/1.8% Nonidet P-40/0.2% SDS and incubated for 45 min with anti-mouse IgG conjugated with peroxidase. p67-69^{c-myc} protein was visualized by the enhanced chemiluminescence system (Amersham).

RESULTS

Prolonged Induction of *c-myc* mRNA by TNF in TA1 Cells. As reported (3), TNF induces *c-fos* and *c-jun* mRNA transiently in preadipocyte TA1 cells at day -1 (Fig. 1*a*). The time course of induction of these mRNAs in TNF-treated

cells is very similar to that observed in serum-stimulated cells (Fig. 1*a*); this pattern of expression is also seen in fully differentiated cells (Fig. 1*b*). In contrast, *c-myc* mRNA is induced by TNF in both preadipocytes and adipocytes in a sustained manner, lasting at least 24–48 hr after stimulation, whereas serum stimulation results in transient induction of *c-myc* message only in preadipocytes (Fig. 1*a* and *b*). TNF does not induce cell division in either preadipocytes or adipocytes, whereas significant increase of mitotic cells is seen after serum stimulation in preadipocytes (Table 1). DNA synthesis, as monitored by incorporation of 5-bromodeoxyuridine (BrdUrd), is induced by serum in preadipocytes but is not induced by TNF (Table 1). In addition, thymidine kinase mRNA is induced in serum-stimulated preadipocytes, but no induction is observed in any TNF-stimulated cells or in serum-stimulated adipocytes (Fig. 2).

TNF treatment dramatically reduces both *aP2* and *FSP27* (adipocyte-specific genes) expression in adipocytes as reported (5), concomitant with increased abundance of *c-myc* mRNA (Fig. 1*b*). When TNF was removed from the culture medium, *c-myc* message decreased, and the levels of *aP2* and *FSP27* messages began to increase (Fig. 1*c*). These results suggest that prolonged induction of *c-myc* by TNF is not coupled with cell proliferation but is correlated with inhibition of adipocyte differentiation.

Exogenous *c-myc* Expression Mimics the Effect of TNF. Deregulated *c-myc* expression has been reported to abolish differentiation in 3T3-L1 adipogenic cells; however, morphological transformation of these cells may have contributed to the observed effects (17). To test whether *c-myc* is involved in the TNF signal-transduction pathway leading to inhibition

Table 1. DNA synthesis and cell division

	Preadipocytes (day -1)			Adipocytes (day 8)		
	Control	Serum	TNF	Control	Serum	TNF
BrdUrd positive, %	5.1	40.4	9.1	0.2	2.5	0.1
Mitotic index, %	0.40 \pm 0.06	7.18 \pm 0.43	0.65 \pm 0.17	0.07 \pm 0.04	0.23 \pm 0.11	0.06 \pm 0.04

TA1 cells (day -1 and day 8) were incubated with 150 μ M BrdUrd for 18–24 hr after stimulation with 10% serum or TNF at 30 ng/ml. The S-phase index was measured by staining cells with a monoclonal antibody to BrdUrd (Oncogene Science), according to the supplier. Results represent the percentage of BrdUrd-positive cells determined by counting \approx 1000 cells in a fluorescence microscope. Mitotic cells were counted (500–1000 total cells in each experiment) at 24 hr after stimulation. Results represent the average (\pm SEM) of four experiments.

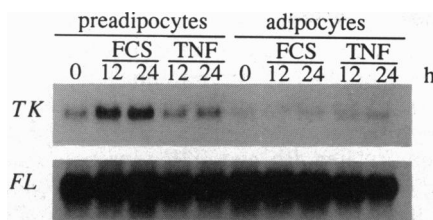


FIG. 2. Thymidine kinase gene expression. TA1 cells, preadipocyte (day -1) and adipocytes (day 8), were stimulated by fresh medium (10% FCS) or TNF (30 ng/ml). Cells were harvested at 12 and 24 hr after stimulation, and thymidine kinase (TK) mRNA was detected by RNA blotting. FL, ferritin light chain.

and reversal of adipocyte differentiation, we isolated inducible *c-myc* transformants of TA1 cells. TA1 cells were transfected with pHygMT-*c-myc* plasmid, which contains a mouse *c-myc* cDNA downstream of the metallothionein-I promoter. Inducibility of *c-myc* message was tested in 16 independent clones. Two clones initially showed good induction of exogenous *c-myc* mRNA by addition of Zn^{2+}/Cd^{2+} . One clone (MTmyc-2) that retained *c-myc* inducibility over 3 months was used for the following study. In MTmyc-2 cells, addition of Zn^{2+}/Cd^{2+} induces exogenous *c-myc* message within 2.5 hr, and the level of message remains elevated for at least 24–48 hr; removal of Zn^{2+}/Cd^{2+} from the culture medium reduces the level of *c-myc* mRNA within 24 hr (Fig. 3 *a* and *b*). This time course of exogenous *c-myc* induction with Zn^{2+}/Cd^{2+} is very similar to that of endogenous *c-myc* induction mediated by TNF (Fig. 1).

We examined whether regulated expression of *c-myc* in MTmyc-2 cells could mimic the effect of TNF on preadipocytes and adipocytes. First, three independent control transformants (vector alone) and MTmyc-2 cells were incubated with Zn^{2+}/Cd^{2+} or TNF from preconfluence (day -1) to day 5, and expression of adipocyte-specific genes was monitored. Consistent with prior experiments (6, 7), expression of aP2 and FSP27 was not detected in the presence of TNF in all clones (Fig. 4*a*, lanes 3). Expression of aP2 and FSP27 was reduced in the presence of Zn^{2+}/Cd^{2+} in MTmyc-2 cells but

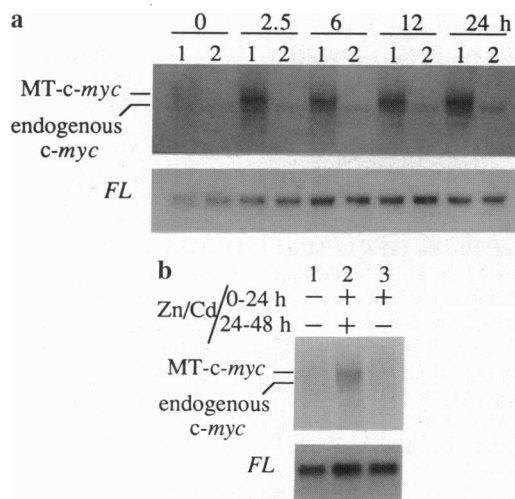


FIG. 3. *c-myc* induction by Zn^{2+}/Cd^{2+} in MTmyc-2 cells. (a) $ZnCl_2$ (40 μM) and $CdCl_2$ (4 μM) were added to MTmyc-2 (lanes 1) and vector transformant (MT-1) cells (lanes 2); RNAs were harvested at various time points after stimulation. (b) MTmyc-2 cells were incubated for 24 hr with fresh medium (lane 1) or with fresh medium containing 40 μM $ZnCl_2$ and 4 μM $CdCl_2$ (lanes 2 and 3); cells were then washed in fresh medium twice and incubated an additional 24 hr in the absence (lanes 1 and 3) or presence (lanes 2) of 40 μM $ZnCl_2$ and 4 μM $CdCl_2$ (lane 2). *c-myc* mRNA was detected by Northern blotting. MT-*c-myc*, exogenous *c-myc*; FL, ferritin light chain; h, hr.

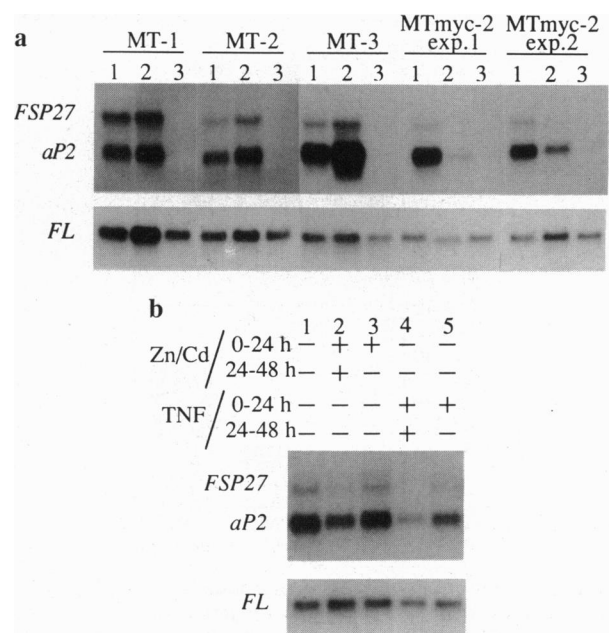


FIG. 4. Expression of adipocyte-specific genes in MTmyc-2 cells. (a) MTmyc-2 cells and three independent vector transformants (MT-1, -2, and -3) were refed at subconfluence (day -1) and left untreated (lane 1), incubated with 40 μM $ZnCl_2$ and 4 μM $CdCl_2$ (lane 2), or incubated with TNF at 30 ng/ml (lane 3). Cells were refed at day 2, and the same treatments were repeated. Cells were harvested at day 5. (b) MTmyc-2 cells (day 9) were refed and left untreated (lanes 1), incubated with 40 μM $ZnCl_2$ and 4 μM $CdCl_2$ (lanes 2 and 3), or incubated with TNF at 30 ng/ml (lanes 4 and 5). After 24 hr-incubation, cells were washed twice in fresh medium and incubated for 24 hr without $ZnCl_2$ and $CdCl_2$ (lanes 1, 3, and 5), with 40 μM $ZnCl_2$ and 4 μM $CdCl_2$ (lane 2), or with TNF at 30 ng/ml (lane 4). aP2 and FSP27 mRNAs were detected.

was not reduced in control transformants (Fig. 4*a*, lanes 2), suggesting that Zn^{2+}/Cd^{2+} inhibits differentiation of MTmyc-2 cells via exogenous *c-myc* expression. In fully differentiated (day 9) MTmyc-2 cells both Zn^{2+}/Cd^{2+} and TNF reduce levels of aP2 and FSP27 mRNA 48 hr after addition (Fig. 4*b*, lanes 1, 2, and 4). Removal of Zn^{2+}/Cd^{2+} or TNF from the culture medium results in recovery of aP2 and FSP27 mRNA (Fig. 4*b*, lanes 3 and 5). These results demonstrate that induction of *c-myc* expression can mimic TNF with respect to inhibition and reversal of adipocyte differentiation and that continued expression of *c-myc* is required to elicit these effects.

Antisense *c-myc* Oligonucleotide Interferes with TNF Action.

To test whether inhibition of *c-myc* expression blocks TNF action, we attempted to reduce c-Myc protein levels with a 15-base antisense oligodeoxynucleotide complementary to codons 1–5 of the mouse *c-myc* message. Fully differentiated TA1 and MTmyc-2 cells were incubated for 24 hr with TNF and the *c-myc* antisense oligonucleotide or with TNF and sense oligonucleotide as a control. As anticipated based on studies in other systems (27), the antisense *c-myc* oligonucleotide reduces the level of c-Myc protein significantly, although it was not reduced to control levels in TA1 cells (Fig. 5*a*). Fig. 5*b* shows that TNF-mediated reduction of aP2 expression was partially reversed by exposure of cells to the antisense but not to the sense *c-myc* oligonucleotide. These results suggest that *c-myc* expression is important for TNF-mediated reduction of expression of adipocyte-specific genes.

DISCUSSION

Growth stimulation by various mitogens and cytokines, including TNF, has been reported to be accompanied by

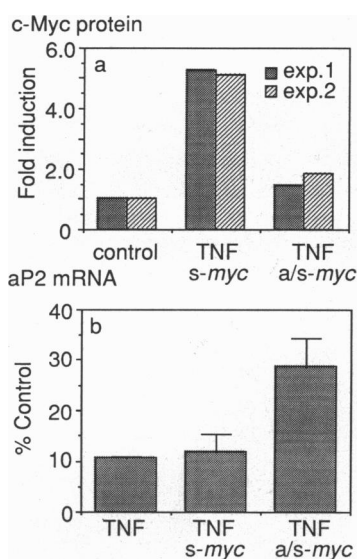


FIG. 5. Antisense *c-myc* oligonucleotide inhibits *c-Myc* protein and interferes with TNF-mediated reduction of aP2 expression. Differentiated TA1 cells or MTmyc-2 cells (days 7–10) were refed and left untreated (control) or incubated for 24 hr with TNF at 30 ng/ml and sense *c-myc* oligonucleotide (s-myc) or antisense *c-myc* oligonucleotide (a/s-myc) at 200 μ g/ml. (a) The *c-Myc* protein was immunoprecipitated by anti-*c-Myc* antibody. The intensity of the *c-Myc* protein bands in two independent experiments was measured by scanning densitometry. (b) The amount of aP2 mRNA was measured by scanning densitometry of RNA blots. The aP2 mRNA level from control cells (no TNF/no oligonucleotide) was defined as 100%. Results represent the average of five experiments \pm SEM.

induction of proto-oncogenes, such as *c-fos*, *c-jun*, and *c-myc* (16, 28–30). Their mRNAs are induced immediately after stimulation and decline to low or undetectable level by 60 min (*c-fos*), 4–12 hr (*c-jun*), or 10–24 hr (*c-myc*). In contrast to growth-coupled *c-myc* induction, TNF induces *c-myc* mRNA for prolonged periods of at least 24–48 hr in TA1 cells (Fig. 1 *a* and *b*) and, as described here, is not coupled with cell proliferation but, instead, is associated with TNF-mediated inhibition and reversal of adipocyte differentiation. The amount of *c-myc* mRNA in TNF-treated cells is lower than that at peak in growth-stimulated preadipocytes by serum (Fig. 1*a*), suggesting that a critical level of *c-myc* may be necessary to promote cell growth. Alternatively, transient rather than continuous increase of *c-myc* mRNA may be essential for progression in the cell cycle.

It remains likely that TNF-mediated inhibition of adipocyte differentiation is transduced through multiple pathways (including *c-myc* induction). Indeed, *c-myc* antisense DNA could block only a portion of the TNF-mediated reduction of adipocyte-specific gene expression. Furthermore, TNF exhibited more pronounced effects on adipocyte differentiation than did Zn^{2+}/Cd^{2+} in MTmyc-2 cells (Fig. 3), despite the fact that Zn^{2+}/Cd^{2+} induces *c-myc* to equivalent or even greater extent than does TNF in MTmyc-2 cells (data not shown).

TNF reduces the level of C/EBP- α (7–10), which acts as a transactivator of many adipocyte-specific genes and appears to play a critical role in differentiation of 3T3-L1 and TA1 cells (11–14). Because *c-myc* message increases within 30 min after TNF addition (data not shown) and a decrease in C/EBP- α mRNA is seen only after 3–6 hr in TA1 cells (10), it is possible that *c-myc* expression leads to inhibition of C/EBP- α gene expression; this hypothesis remains to be proven, however, by investigating the time course of TNF effects on C/EBP- α gene transcription. Because the promoter of C/EBP- α contains a consensus *c-myc*-binding se-

quence (31), we speculate that *c-myc* might participate directly to inhibit expression of the C/EBP- α gene.

The protein product of the retinoblastoma susceptibility gene, Rb, regulates *c-myc* transcription through an E2F (transcription factor)-binding site (32, 33). The complex of underphosphorylated Rb and E2F represses transcription, whereas phosphorylation of Rb by mitogens causes dissociation of the Rb-E2F complex, resulting in activation of E2F-mediated transcription (34, 35). We have recently obtained results indicating that TNF stimulates phosphorylation of Rb in TA1 cells (J.N.-T., personal observation) and may, therefore, activate *c-myc* transcription via an E2F-dependent pathway. These and future studies will help elucidate the details of this TNF-signaling pathway, which appears to involve a regulatory action for *c-myc* that is distinct from its well-described role in cell proliferation.

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