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c-Myc represses transcription *in vivo* by a novel mechanism dependent on the initiator element and Myc box II

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We show that c-Myc, in addition to activating transcription through E-box Myc binding sites (Ems), also represses transcription by a mechanism dependent on initiator (Inr) elements of the basal promoters of susceptible genes. Repression was first observed as a component of c-Myc biphasic regulation of the adenovirus-2 major late promoter (MLP), which contains both Inr and Ems sequences. Two differentiation-specific genes containing Inr, the C/EBPα and albumin genes, are repressed through their basal promoters by c-Myc, but are activated by the related B-HLH-LZ factor, USF. Repression requires both the B-HLH-LZ and Myc box II (MBII) domains. Significantly, a MBII deletion mutant which is deficient in repression, but transactivates normally, fails to cooperate with an activated *ras* gene to transform primary fibroblasts. Thus Myc-dependent transactivation is insufficient for Ras cooperation and the novel transcription repression function is implicated in Ras cooperation as well as the suppression of Inr-dependent genes.

Key words: adenovirus-2 MLP/c-Myc/C/EBPα/transcription/USF

Introduction

c-Myc protein can stimulate cell proliferation and its overexpression is associated with neoplasia and can block certain cell types from fully differentiating (reviewed by Cole, 1986; Luscher and Eisenman, 1990). c-Myc contains a basic region–helix–loop–helix–leucine zipper (B-HLH-LZ) DNA binding domain, a hallmark of a transcription factor. Indeed, several genes have been found to be activated by c-Myc, including the PAI-1 (Prendergast *et al.*, 1990), α-prothymosin (Eilers *et al.*, 1991), ECA39 (Benvennisty *et al.*, 1992) and ODC (Wagner *et al.*, 1993; Bello-Fernandez *et al.*, 1993) genes. c-Myc forms heterodimers with its partner Max (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991) and binds to DNA sequences with consensus CACGTG, called E Box Myc sites (Ems) (Blackwell *et al.*, 1990; Halazonetis and Kandil, 1991; Kerkhoff *et al.*, 1991; Prendergast and Ziff, 1991). The Myc N-terminus contains a transcriptional transactivation domain (Kato *et al.*, 1990) and c-Myc will transactivate a surrogate promoter containing multiple upstream Ems (Amati *et al.*, 1992; Gu *et al.*, 1992; Kretzner *et al.*, 1992). However, Myc expression parallels the down-regulation of a number of differentiation-specific genes, such as the N-CAM and LFA-1α genes (Akeson and Bernards, 1990; Inghirami *et al.*, 1990). The full mechanism of action of c-Myc and its primary genetic targets are ill defined. c-Myc may directly repress the expression of these genes, but Myc may also regulate other factors which are the direct regulators of differentiation-specific functions (Yang *et al.*, 1993).

The C-terminal B-HLH-LZ domain of c-Myc is necessary for DNA binding (Dang *et al.*, 1991; Prendergast *et al.*, 1991; Blackwood and Eisenman, 1992) as well as Myc-induced transformation, blockage of cell differentiation (Stone *et al.*, 1987; Dang *et al.*, 1989; Smith *et al.*, 1990) and autoregulation (Penn *et al.*, 1990). The N-terminus (amino acids 1–143) of c-Myc is required for cell transformation (Barrett *et al.*, 1992) and Myc-induced apoptosis (Evan *et al.*, 1992). Within the N-terminus, Myc box II (MBII, amino acids 122–143), one of the regions conserved amongst Myc family members, is required for cell transformation, the block of cell differentiation (Stone *et al.*, 1987; Freytag *et al.*, 1990) and autoregulation (Penn *et al.*, 1990).

In this report we demonstrate a new function of c-Myc, the ability to repress transcription *in vivo*. Repression was detected as a component of the effects of c-Myc on the adenovirus-2 major late promoter (MLP) (Bachenheimer and Darnell, 1975; Ziff and Evans, 1978), a naturally occurring promoter with two Ems that can be recognized by c-Myc (Prendergast and Ziff, 1991), as well as by the ubiquitous B-HLH-LZ transcription factor, USF (Carthew *et al.*, 1985; Sawadogo and Roeder, 1985; Lennard and Egly, 1987; Gregor *et al.*, 1990). The MLP also contains a specific transcription initiator element (Inr; Smale and Baltimore, 1989) which binds TFII-I, a transcription factor which recognizes a core sequence CTCA(+1)CTCTCT within the MLP (Roy *et al.*, 1991). We show that c-Myc has two MLP targets, the Ems, through which Myc stimulates transcription, and the Inr element, the target of the novel Myc repression function. Two differentiation-specific genes with Inr elements, the C/EBPα and serum albumin genes, are also repressed. USF activates transcription through specific cooperation with TFII-I, both *in vitro* and *in vivo* (Roy *et al.*, 1991; Du *et al.*, 1993) and we suggest that it potentially acts as an inducer of differentiation. Experiments with Myc mutants implicate repression as a Myc function necessary for blockage of cellular differentiation and cooperation with Ras in cellular transformation.

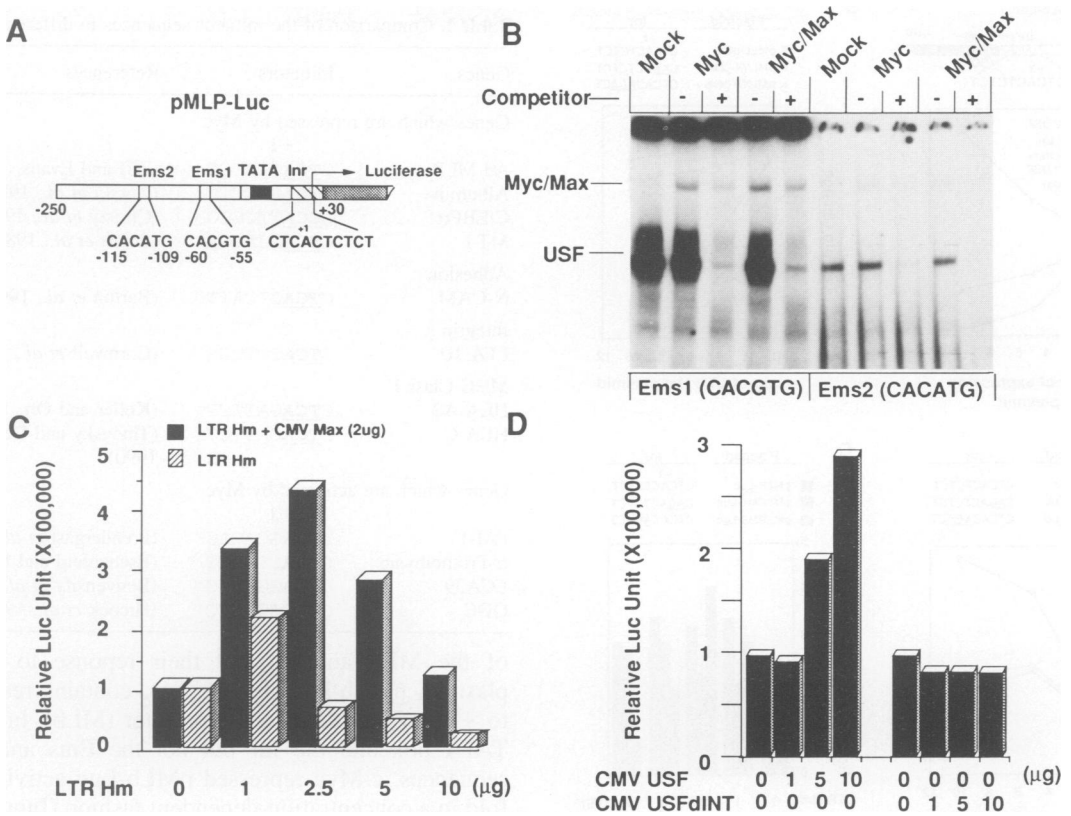

Fig. 1. Regulation of the adenovirus-2 major late promoter in pMLP-Luc by c-Myc and USF in NIH 3T3 cells. (**A**) Plasmid pMLP-Luc contains the adenovirus-2 major late promoter (from residue −250 to +30) linked to the firefly luciferase (Luc) gene. (**B**) Myc−Max and USF binding to the E-box Myc sites of the major late promoter was analyzed by the electrophoretic mobility shift assay. Myc and Max proteins were translated *in vitro* from CMV Hm and CMV Max respectively with rabbit reticulocyte lysate. USF is the endogenous protein present in the reticulocyte lysate. Competitors are the unlabeled oligonucleotides containing the Ems. (**C**) Myc exerts a biphasic effect on the activity of pMLP-Luc. NIH 3T3 cells were co-transfected with the indicated quantities of the Myc expression plasmid LTR Hm, with (■) or without (▨) 2 μg Max expression plasmid CMV Max plus 3 μg pMLP-Luc. (**D**) USF activates pMLP-Luc in a concentration-dependent manner. Co-transfection of USF expression plasmid CMV USF in increasing concentrations activates the reporter plasmid pMLP-Luc (bars 1–4). However, USF dlNT, which expresses an N-terminal truncated form (amino acids 1–200 are deleted) of USF, does not activate pMLP-Luc (bars 5–8). The assay was carried out by co-transfection of the indicated quantity of CMV USF or CMV USF dlNT plasmids with 3 μg pMLP-Luc in NIH3T3 cells.

Results

The c-Myc protein both positively and negatively regulates the MLP

We analyzed the effects of the human c-Myc, murine Max (Myn) and human USF proteins on the MLP contained in the reporter plasmid pMLP-Luc (Figure 1A). Previous reports have shown that the c-Myc−Max complex could specifically bind Ems (CACGTG) (Blackwood and Eisenman, 1991; Prendergast *et al.*, 1991; Kato, *et al.*, 1992). In agreement, of the two potential Myc binding sites within the MLP, the c-Myc−Max heterodimer produced by *in vitro* translation binds the proximal Ems1 (CACGTG) with higher affinity than the distal Ems2 (CACATG) (Figure 1B). The high basal level of c-Myc binding to Ems1 in the absence of exogenous Max is not seen when exogenous c-Myc is omitted and this binding most likely reflects exogenous c-Myc heterodimers with endogenous Max, which is present in the reticulocyte lysate. Endogenous USF, which is also present in the lysate, binds both Ems sequences, with a greater affinity for Ems1 than Ems2. This confirms that the c-Myc−Max complex binds *in vitro* to the Ems1 element of the MLP.

To determine the *in vivo* effects of protein binding to the Ems, we co-transfected NIH 3T3 cells with pMLP-Luc plus plasmids expressing c-Myc, Max or USF. Increasing levels of c-Myc, when co-expressed with an intermediate level (2 μg) of a Max expression plasmid, induced biphasic regulation of pMLP-Luc (Figure 1C). Low levels of c-Myc expression plasmid (up to 2.5 μg) stimulated 4- to 5-fold, but high levels repressed, pMLP-Luc activity. When c-Myc alone was expressed with pMLP-Luc we observed less activation of the MLP at low Myc levels, but greater repression (4- to 5-fold) at high Myc levels. We also found that c-Myc activated pMLP-Luc more strongly when the transfection assay was applied to cells near confluence (70–80% confluence) and more severe repression was seen when the transfection assay was performed with less confluent cells (30–40%) (data not shown). In contrast, increasing USF concentrations monotonically stimulated the MLP and stimulation was dependent upon the N-terminal 200 amino acids of USF (Figure 1D). The USF N-terminus has been shown to contain transactivation domains (Kirschbaum *et al.*, 1992). Together, these results suggested that biphasic regulation is a special property of c-Myc, in as much as the effects of USF were exclusively stimulatory under our assay conditions and cooperation with Max is required for transactivation by c-Myc.

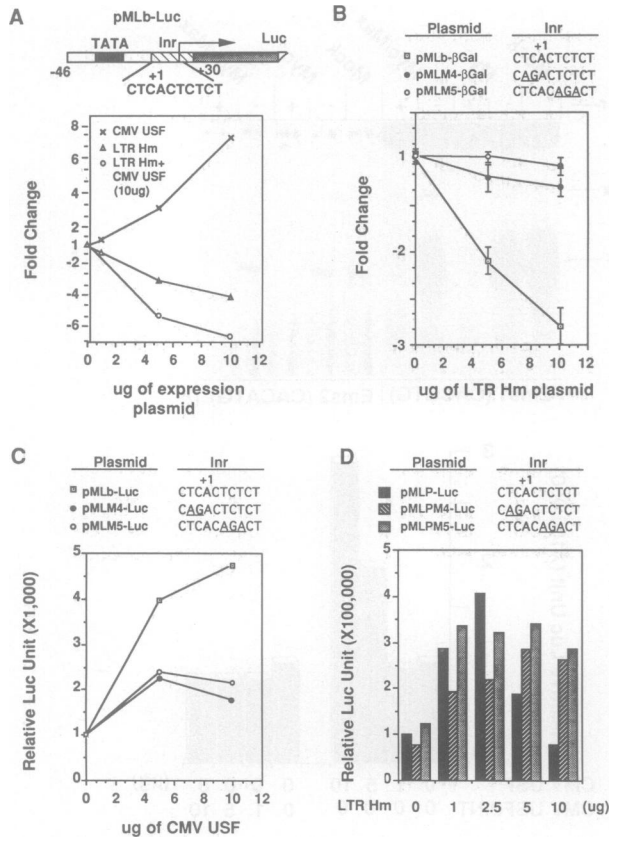

Fig. 2. Myc activates and represses the MLP through the upstream sequences and the basal promoter respectively. (**A**) Top: diagram showing the structure of pMLb-Luc, in which the adenovirus-2 major late basal promoter (MLb, from residue −46 to +30) is linked to the luciferase gene. The positions of the TATA box and initiator element (Inr) are indicated. Bottom: Myc represses, but USF activates, the adenovirus-2 major late basal promoter. The indicated quantities of LTR Hm (Δ) or CMV USF (×) were co-transfected with 5 μg pMLb-Luc in NIH3T3 cells. The basal activity (fold change = 1) was 1000 Luc units except for cells transfected with10 μg CMV USF (○). The basal activity (fold change = 1) was 5000 Luc units. This level is raised relative to the basal level with no expression plasmid additions (Δ) as a consequence of co-transfection of 10 μg CMV USF. (**B**). Top: the Inr of plasmid pMLb-βGal (□), in which the major late minimal promoter is linked to the *lacZ* gene and mutants of MLb-βGal, including MLM4 (●) and MLM5 (○), are shown. Bottom: mutations at the MLb Inr site block Myc repression. The indicated quantities of LTR-Hm were co-transfected with 5 μg pMLb-βGal, pMLM4-βGal, or pMLM5-βGal into NIH 3T3 cells. The absolute basal activity (fold change = 1) corresponds to 4000 β-Gal units. β-Gal activity is 4- to 5-fold higher than Luc activity assayed by using the Galacto-light™ chemiluminescent reporter assay system (see Material and methods) and therefore was employed for testing repression. (**C**). Top: the Inr of plasmid pMLb-Luc (□), in which the major late minimal promoter is linked to the luciferase gene, and mutants of MLb-Luc, including MLM4 (●) and MLM5 (○), are shown. Bottom: mutations at the Inr region reduced USF stimulation. The indicated quantities of CMV USF were co-transfected with 5 μg pMLb-Luc, pMLM4-Luc or pMLM5-Luc into NIH 3T3 cells. The absolute basal activity (fold change = 1) corresponds to 1000 Luc units. (**D**). Top: the Inr of plasmid pMLP-Luc (■), in which the major late promoter is linked to the luciferase gene, and mutants of MLP-Luc, including MLPM4 (▨) and MLPM5 (▪), are shown. Bottom: mutations at the Inr region reduced c-Myc repression. The indicated quantities of LTR Hm were co-transfected with 2 μg CMV Max and 2 μg pMLP-Luc, pMLPM4-Luc or pMLPM5-Luc into NIH 3T3 cells.

Targets for activation and repression by Myc.

To deduce the targets which provide the biphasic regulation, we separated upstream and core promoter components of the MLP and assayed their reponse to Myc. One plasmid, pMLb-Luc (Figure 2A), contains residues −46 to +30 of the MLP basal promoter (MLb), including the TATA box and the Inr but not the Ems and upstream sequences. c-Myc repressed pMLb-Luc activity up to 4-fold in a concentration-dependent fashion (Figure 2A) and no activation has been observed. In contrast to c-Myc, USF stimulated the MLb at all concentrations assayed (Figure 2A), in agreement with previous reports of USF stimulation through the major late basal promoter (Roy *et al.*, 1991; Du *et al.*, 1993). Strong repression of MLb-Luc by c-Myc was difficult to observe, due to the very low basal activity of this plasmid. However, a significantly greater repression (7-fold) was observed when increasing quantities of LTR Hm were co-transfected with a constant level of CMV USF, which raised basal activity of MLb-Luc 4- to 5-fold (Figure 2A). We conclude that the c-Myc repression target lies within the MLb, a promoter region which includes the TATA box and the initiator. Furthermore, c-Myc can exert an antagonistic effect on the USF-induced activity of MLb-Luc. This also raises the possibility that c-Myc and USF have overlapping targets within the major late basal promoter.

Table I. Comparison of the initiator sequences in different genes

Genes	Initiators	References
Genes which are repressed by Myc	+1	
Ad MLP	C**TCA**CTCTCT	(Ziff and Evans, 1978)
Albumin	A**TCA**CCTTTC	(Gorski *et al.*, 1986)
C/EBPα	G**CCA**TTCGCG	(Christy *et al.*, 1991)
MT-I	G**TCA**CCACGA	(Stuart *et al.*, 1984)
Adhesion		
N-CAM	C**TCA**CTCATT	(Barton *et al.*, 1990)
Integrin		
LFA-1α	A**TCA**TTTTCC	(Cornwell *et al.*, 1992)
MHC Class I		
HLA-A2	C**TCA**GATTCT	(Koller and Orr, 1985)
HLA-C	C**TCA**GATTCT	(Tibensky and Delovitch, 1990)
Genes which are activated by Myc	+1	
PAI-1	AGG**A**GCACAG	(Prendergast *et al.*, 1990)
α-Promothysin	CCA**A**CTGGCT	(Eschenfeld and Berger, 1986)
ECA39	CAG**A**GGTCGG	(Benvensity *et al.*, 1992)
ODC	GGG**C**TTTGTC	(Hicock *et al.*, 1990)

A comparison of the MLb with other promoters repressed by c-Myc, including the N-CAM and LFA-1α promoters (Table I), reveals a weak consensus sequence TCA(+1)YYYNY similar to the TdT Inr element (Smale and Baltimore, 1989). Mutations of the Inr at residues +3, +4 and +5 abolished the ability of USF to bind to the Inr and reduced the stimulation of MLb by USF (Roy *et al.*, 1991; Du *et al.*, 1993). To test the role of the Inr in repression by c-Myc, two mutants of the MLb Inr region (see Figure 2B for structures) were assayed for repression by c-Myc. Mutant MLM4 contains a double point mutation at conserved residues −1 and −2 and MLM5 bears a triple point mutation at residues +3, +4 and +5. The mutations did not substantially change the basal activity of the MLb (data not shown), perhaps because the TATA box dominates the Inr in establishing basal promoter activity (Smale *et al.*, 1990). Both MLM4 and MLM5 were insensitive to c-Myc (Figure 2B), while

the plasmid with the wild-type Inr was repressed. In parallel, the mutations in both MLM4 and MLM5 reduced USF stimulation (Figure 2C). These results indicate that residues −2, −1, +3, +4 and +5 within the Inr, which are required for USF stimulation of the Inr, are also required for Myc repression of the MLb, even though USF and c-Myc exert opposite effects on the MLb. Our observation that mutations in the Inr region at residues +3, +4 and +5 reduced USF stimulation is consistent with previous reports (Roy *et al.*, 1991; Du *et al.*, 1993). However, there has been controversy over the mutations at the 5′ end of the Inr (see Discussion). Taken together, these data indicate that the Inr is a target for Myc repression. However, additional MLb targets may also exist (see Discussion).

Given that the target for c-Myc repression lies within the major late basal promoter and repression is mediated by the Inr element, one would expect that the full-length MLP bearing Inr mutations should not be subject to c-Myc biphasic regulation. When we tested pMLPM4-Luc and pMLPM5-Luc, which contain the same mutations as described in MLM4 and MLM5 (see Figure 2B) in the background of the full-length promoter, we found that c-Myc can still activate, but cannot repress as strongly, as with the wild-type pMLP-Luc (Figure 2D). We also observed that activation of pMLPM4-Luc by low levels of c-Myc was partially reduced compared with the wild-type. Taken together, these results indicate that the upstream region of the MLP, which includes the Ems, is the target for c-Myc activation and that repression of MLP by c-Myc is Inr-dependent. However, cross-talk between the upstream region and basal promoter may be required for the full activation of MLP (see Discussion).

To further determine whether c-Myc activates through the upstream Ems1 element, the Ems1 element was isolated and linked to the SV40 early promoter in a tandem 3-fold repeat. The SV40 promoter is insensitive to Myc (Kaddurah-Daouk *et al.*, 1987), allowing Myc effects on this plasmid to be attributed to the Ems. When co-expressed with Max, c-Myc activated transcription of pEms/SV-Luc 3-fold and no repression was observed (data not shown). This result is consistent with previous reports that the c-Myc−Max complex activates through an Ems (Amati *et al.*, 1992; Gu *et al.*, 1992; Kretzner *et al.*, 1992).

It has been reported that the Ems1 element within the MLP is essential for USF stimulation (Hen *et al.*, 1982; Carthew *et al.*, 1985; Miyamoto *et al.*, 1985; Sawadogo and Roeder, 1985). Therefore, the monotonic stimulation by USF reflects positive action at both the Ems and the Inr targets. In contrast, the biphasic regulation of the MLP by c-Myc reflects its positive action at the Ems1 and negative action at the basal promoter which contains the Inr. We have also assayed the response of the MLb to Max under conditions of repression by Myc. Max reversed the repression by c-Myc, but neither Max mutRR nor Max dlZ (see Materials and methods) derepressed MLb activity (data not shown).

Repression requires C- and N-terminal domains of c-Myc and is required for cellular transformation

To identify the Myc domains required for repression, several Myc mutants were assayed. Mutant Hm dlZ, an LZ deletion which fails to dimerize with Max, and a basic region mutant, Hm mutRR (RR367, 368EE), whose heterodimer with Max fails to bind DNA (Prendergast *et al.*, 1991), were inefficient repressors of pMLb-Luc (Figures 3A). This suggests that repression at the Inr requires Myc binding to a protein partner and to DNA and reveals the same requirements for the DNA binding domain as c-Myc activation (Amati *et al.*, 1992; Gu *et al.*, 1992; Kretzner *et al.*, 1992). N-terminal domains of c-Myc are also required for MLb repression. While the MBI deletion mutant Hm dlMBI [Δamino acids (aa) 46–55] has partially lost its repression activity (data not shown), the MBII deletion mutant Hm dlMBII (Δaa 122–140) has lost essentially all repression activity (Figure 3A). Strikingly, although Hm dlMBII is inactive as a repressor, it still transactivates MLP and indeed is more active than the wild-type (Figure 3B). This suggests that MBII mediates a function essential for repression but not transactivation.

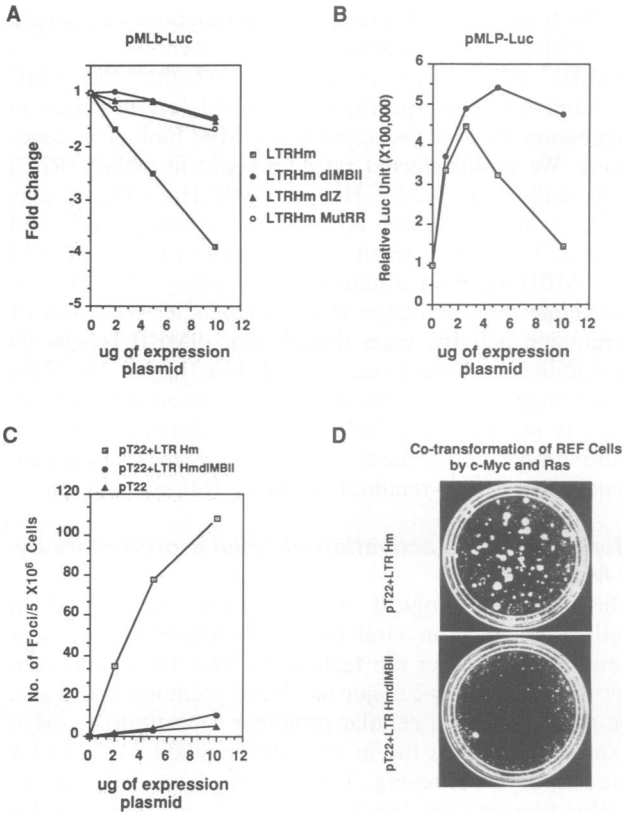

Fig. 3. Both the N-terminal and the C-terminal domains of c-Myc are required for Myc repression activity and Myc box II is required for Myc to co-transform REF cells. (**A**) c-Myc mutants (see Materials and methods) were assayed for their ability to repress the major late basal promoter in pMLb-Luc. The indicated quantities of LTR Hm or LTR Hm mutants were co-transfected with 5 μg pMLb-Luc into NIH3T3 cells. The absolute basal activity (fold change = 1) equals 1000 Luc units. (**B**). Mutant c-Myc dlMBII was tested for its ability to transactivate pMLP-Luc. The indicated quantities of LTR Hm or LTR HmdlMBII were co-transfected with 2 μg CMV Max and 2 μg pMLP-Luc into NIH3T3 cells. (**C**). c-Myc dlMBII was tested for its ability to co-transform REF cells with activated H-Ras controlled with the wild-type Myc. The indicated quantities of LTR Hm or LTR HmdlMBII (Δaa 120–140) were co-transfected with 5 μg pT22, an H-Ras expression plasmid, into REF cells. The no. of foci/5×10^{6}cells is the average number of foci from two experiments. (**D**) Photos showing the c-Myc/Ras co-transformation foci. Photos were taken after 2 weeks of transfection in REF cells, which were then fixed with methanol.

The transactivation and repression functions of Myc are separable, as demonstrated by the phenotype of Hm dlMBII. We wished to determine whether the MBII domain, which is specifically required for transcription repression by Myc, is required for Myc biological functions. We co-transfected rat embryonic fibroblast (REF) cells with either CMV Hm or CMV Hm dlMBII plus pT22, which expresses an activated H-*ras* protein, and assayed for focus formation. As shown in Figure 3C and 3D, MBII deletion eliminated the ability of c-Myc to cooperate with activated H-Ras in parallel with loss of repression activity, even though Hm dlMBII retains its full ability to transactivate via an E-box (Figure 3B). This result suggests that E-box dependent transactivation by c-Myc is not sufficient for c-Myc to cooperate with Ras. Furthermore, MBII, the domain necessary for transcription repression, is also required for Myc−Ras cooperation.

Repression and activation of cellular promoters by c-Myc and USF

The primary biological effects of Myc are exerted on cellular rather than viral processes. Therefore, we next determined whether the features of Myc repression seen for the adenovirus-2 major late basal promoter could also be detected with a cellular promoter. Constitutive c-Myc expression inhibits the *in vitro* differentiation of 3T3-L1 pre-adipocytes (Freytag, 1988). With these cells, the C/EBPα transcription factor is necessary and sufficient for adipose conversion. In fact, expression of C/EBPα will overcome the c-Myc block of pre-adipocyte cell differentiation (Samuelson *et al.*, 1991; Freytag and Geddes, 1992; Lin and Lane, 1992) and down-regulation of C/EBPα expression by 2-fold is sufficient to block adipose conversion (Samuelson *et al.*, 1991). This suggests that c-Myc acts by directly or indirectly repressing the C/EBPα promoter, thereby preventing cellular differentiation. Indeed, c-Myc and C/EBPα mRNA levels are reciprocally regulated during differentiation (Freytag, 1988; Birkenmeier *et al.*, 1989). We sought to determine whether c-Myc expression was sufficient to block the activity of the C/EBPα promoter in 3T3-L1. In agreement with Freytag's observation (Freytag, 1988), wild-type 3T3-L1 cells subjected to the differentiation protocol acquired a characteristic morphology, which was not induced in two independent 3T3-L1 clones (clone 4-1 and clone 4-10) expressing a transfected mouse *c-myc* gene (data not shown). In wild-type 3T3-L1, c-Myc is induced early in differentiation and then down-regulated as C/EBPα mRNA is induced (Figure 4A). When clone 4-1 and 4-10 cells were subjected to the differentiation protocol, C/EBPα protein (Figure 4B) was repressed 10- to 20-fold relative to the parental cell line. Thus, expression of c-Myc is sufficient to repress the chromosomal C/EBPα gene and the differentiated phenotype of 3T3-L1 cells, as previously described (Freytag *et al.*, 1990). We next assayed the effect of c-Myc on the C/EBPα promoter. Since the C/EBPα promoter contains an Inr sequence (Table I) similar to that of the MLP, it may be regulated similarly to the MLb by c-Myc. In agreement with this, expression of c-Myc in 3T3-L1 cells provided concentration-dependent repression of the C/EBPα promoter (from residue −387 to +30) contained in pαP-Luc (Figure 5A). c-Myc also repressed pαMP-Luc, a reporter plasmid containing the

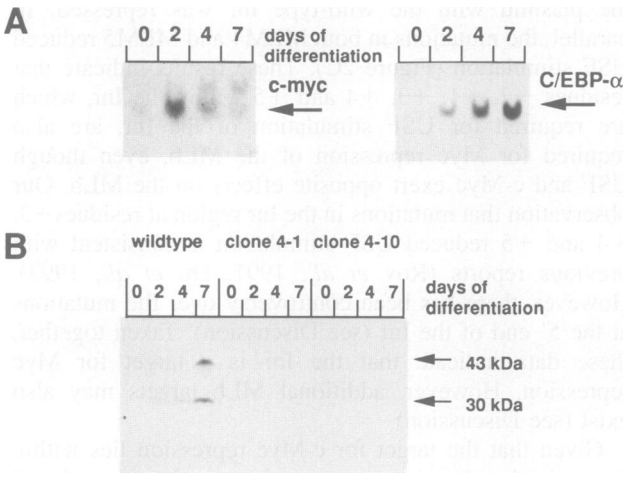

Fig. 4. Reciprocal regulation of C/EBPα and c-Myc mRNAs during 3T3-L1 differentiation. (**A**) 3T3-L1 cells were differentiated *in vitro* and RNA prepared after 0, 2, 4 and 7 days. Aliquots of total RNA (20 μg) were analyzed by Northern blotting for the presence of C/EBPα and c-Myc mRNA. (**B**) Total cellular protein was prepared from clones 4-1 and 4-10 and 3T3-L1 cells at days 0, 2, 4 and 7 after initiation of differentiation. Aliquots (20 μg) were separated by 15% SDS−PAGE and analyzed by Western blotting using anti-C/EBPα antibody.

C/EBPα basal promoter from which the upstream sequences, including the Ems, have been deleted, to a similar extent (Figure 5A), indicating that repression through the basal promoter by Myc dominates any activation which may occur through the Ems, as seen for the MLP.

To further investigate the target within the C/EBPα basal promoter that responds to c-Myc repression, we mutated the C/EBPα promoter Inr at positions equivalent to the residues mutated in the MLb Inr (Figure 5B). In a co-transfection assay, both pαM1P-Luc and pαM2P-Luc were refractory to c-Myc repression, while the wild-type pαMP-Luc was repressed by c-Myc. This result shows that c-Myc repression of the C/EBPα promoter requires the integrity of the Inr element. As shown in Figure 5C, the C-terminal Myc domain, including the basic region DNA binding domain and the LZ dimerization domain, and the N-terminal MBII domain are also all required for c-Myc repression of pαMP-Luc. Thus c-Myc repression of the C/EBPα promoter has the same requirements as repression of the MLP.

The mouse serum albumin promoter, a second differentiation-specific promoter which contains a TCA initiation sequence (Table 1), is repressed 4-fold by c-Myc in HeLa cells, but is activated by USF (Figure 5D), and thus responds in a way similar to the ML and the C/EBPα basal promoters (Figure 5A).

We have thus far shown that USF and c-Myc can antagonize each others' actions at the adenovirus-2 major late basal promoter and the C/EBPα and serum albumin basal promoters. Because USF is constitutively expressed in many cell types (Kirschbaum *et al.*, 1992), while c-Myc varies (reviewed by Luscher and Eisenman, 1990), we hypothesized that the full range of repression by Myc might be exerted when it reverses the USF-induced state. This possibility was also suggested by the experiment shown in Figure 2A, in which c-Myc repressed USF-

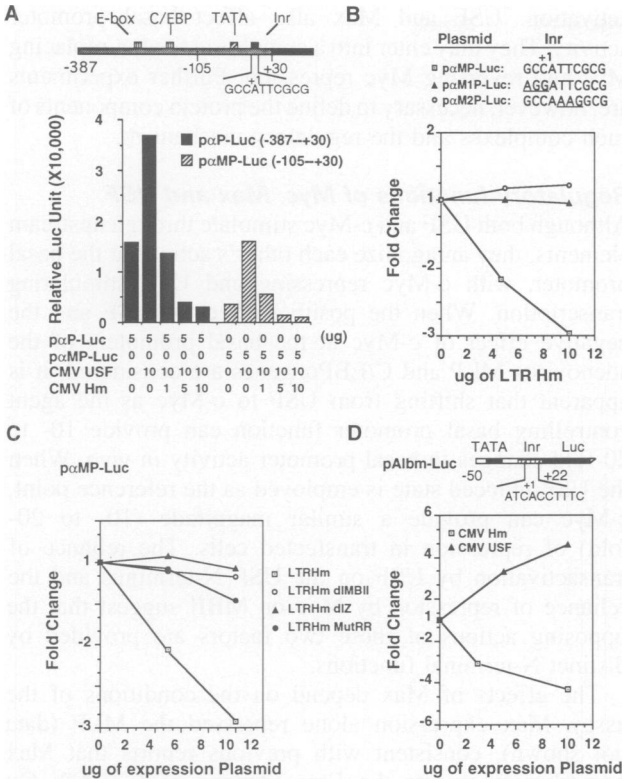

Fig. 5. Regulation of C/EBPα and albumin promoters by c-Myc and USF in 3T3-L1 and HeLa cells. (**A**) Top: the structure of the regulatory region of plasmid pαP-Luc in which the C/EBPα promoter (from residue −387 to +30) is linked to the luciferase gene. Bottom: c-Myc represses and USF activates respectively both the C/EBPα promoter and the C/EBPα minimal promoter in 3T3-L1 cells. The indicated quantities of CMV Hm or CMV USF were co-transfected with 5 μg pαP-Luc or pαMP-Luc into 3T3-L1 cells. (**B**) Top: the Inr sequences of plasmid pαMP-Luc and mutants of pαMP-Luc, including pαM1P-Luc and pαM2P-Luc, are shown. Bottom: mutations at the C/EBPα promoter Inr site block c-Myc repression. The indicated quantities of LTR-Hm were co-transfected with 10 μg pαMP-Luc, pαM1P-Luc or pαM2P-Luc into 3T3-L1 cells. The absolute basal level (fold change = 1) is 5000 Luc units. (**C**) Myc mutants were assayed for their ability to repress the C/EBPα basal promoter in pαMP-Luc. The indicated quantities of LTR Hm or LTR Hm mutants were co-transfected with 10 μg pαMP-Luc into 3T3-L1 cells. The absolute basal level (fold change = 1) corresponds to 5000 Luc units. (**D**) Top: the structure of the regulatory region of plasmid pAlbm-Luc, in which the mouse albumin minimal promoter (from residue −50 to +22) is linked to the luciferase gene, is shown. Bottom: c-Myc represses and USF activates the albumin minimal promoter in pAlbm-Luc in HeLa cells respectively. The indicated quantities of CMV Hm or CMV USF were co-transfected with 5 μg pAlbm-Luc into HeLa cells. The absolute basal level (fold change = 1) corresponds to 2000 Luc units.

dependent activity of the MLb. To test this possibility, we co-transfected pαMP-Luc, which contains the C/EBPα basal promoter, together with a constant amount of CMV USF to establish the USF-induced state of this promoter. The basal activity was elevated 4- to 5-fold under these conditions (Figure 5A), confirming that in the absence of co-transfected CMV USF, the exogenous C/EBPα promoter was at an intermediate level of activity. This intermediate level is presumably determined by the relative endogenous levels of c-Myc and USF. We then varied the quantity of co-transfected CMV Hm. As shown in Figure 5A, c-Myc repressed the USF-induced state of pαMP-Luc up to 14-fold. Thus, a range of regulation of C/EBPα activity was observed in transiently transfected cells which was similar to that previously seen for the chromosomal C/EBPα gene *in vivo* (Figure 4). This confirmed that the full range of regulation by c-Myc can be achieved by first establishing the USF-induced state and then repressing by increasing the level of Myc. We conclude that c-Myc and USF antagonize each other's action at the core regions of two differentiation-specific promoters and one viral promoter, all of which have Inr elements.

Discussion

In this paper we describe the ability of the c-myc protein to repress viral and cellular basal promoters through action at Inr elements by a mechanism dependent on MBII. This *in vivo* activity is likely to be related to the recently reported repression of the MLP *in vitro* by c-Myc (Roy *et al.*, 1993). It is striking that a biological activity of Myc, cooperation with an activated *ras* gene to transform rat embryo fibroblasts, is shown in the present report to be also highly dependent upon MBII, while transactivation is unaffected by MBII deletion. Because MBII is contained within a region of Myc involved in binding to TBP in an *in vitro* assay (C.Nerlov and E.Ziff, unpublished observations), c-Myc−TBP interaction is potentially the physical basis for the repression mechanism (see below). Detection of repression followed the finding that Myc biphasically regulates the adenovirus-2 MLP. Biphasic action reflects separate Myc targets within the MLP for activation and repression. This substantiates the conclusion that repression proceeds by a mechanism distinct from that of activation.

Structural requirements for core promoter repression by c-Myc

Mutagenesis identified three structures of c-Myc essential for repression, namely the LZ dimerization domain, the basic region of the DNA binding domain and MBII. Mutation or deletion of any of these severely affected the ability of Myc to repress. The integrity of the Inr elements of both the MLP and C/EBPα promoters was also essential for repression. However, we should note that activation of the Inr mutants of the MLP by c-Myc is also reduced, especially for MLPM4 (Figure 2D). This suggests that cross-talk occurs between the upstream elements and the basal promoter and that the Inr element, which is essential for Myc repression, may also be required for full activation of the MLP. Thus far, five Inr element families, corresponding to different Inr binding proteins, have been identified (reviewed by Weis and Reinberg, 1992). Amongst these are the CTCA-Inr, which is found in the MLP and which binds TFII-I (Roy *et al.*, 1991). The sequence of the 5′ end of the Inr element of the MLP and the albumin and C/EBPα promoters, TCAC or CCAT, resembles an optimal half-site for c-Myc DNA binding, namely CCAC (Halazonetis and Kandil, 1991). It would be interesting to determine whether the 5′ end of the initiator is involved in contact with the c-Myc basic region, a model which is consistent with the requirement for the c-Myc basic region in repression. Whether USF contacts the same DNA sequences as c-Myc is also an interesting question, because previous reports (Roy *et al.*, 1991; Du, *et al.*, 1993) and our observations suggest that USF and c-Myc may have

an overlapping binding site within the ML basal promoter. In agreement, mutations at residues −2 and −1 in the Inr of MLb reduced both c-Myc repression and USF activation (this report). This finding is consistent with Roy *et al.*'s (1991) observation that mutations at residues −2, −3 and −4 in the Inr reduced USF binding to the Inr. However, Du *et al.* (1993) found that mutations at residues −2, −3 and −5 in the Inr increased USF stimulation. The requirement for the leucine zipper suggests that protein−protein contact is involved, possibly with a protein binding to the 3′ part of the Inr. During the course of this work, Roy *et al.* reported that c-Myc can bind the MLP Inr as a heterodimer with TFII-I and can act as a transcription repressor *in vitro* (Roy *et al.*, 1993). The ability of c-Myc to interact with TFII-I at the Inr and to repress the MLP *in vitro* is in agreement with our *in vivo* observations and provides a physical basis for our findings.

The requirements for the Myc basic region and the LZ, as well as for the Inr, suggest that during repression Myc contacts DNA while associated with a second factor through the C-terminal domain. While TFII-I (Roy *et al.*, 1993) is an obvious candidate for a Myc-associated factor, we note that different Inr elements may interact with different Inr binding factors (Weis and Reinberg, 1992). Indeed, the Inr binding factor YY1 has been shown to interact with Myc (Shrivastava *et al.*, 1993).

Role of Myc box II

The ability of c-Myc to repress is dependent upon a Myc-specific amino terminal domain, MBII, a region previously shown by Freytag *et al.* (1990) to be required for c-Myc repression of differentiated functions *in vivo* and required for induction of cellular transformation (Stone *et al.*, 1987). We also found that Hm dlMBII exerts a dominant negative effect on Myc−Ras cooperation in the REF cell transformation assay (D.MacGregor and E.Ziff, unpublished observations), which emphasizes the importance of MBII in celluar transformation. This domain of c-Myc resides within a region of the protein shown to be involved in interaction with TBP *in vitro*, while, in contrast, Max and USF, which do not repress through the Inr, do not bind to TBP (C.Nerlov and E.Ziff, unpublished observations). Taken together, these observations suggest a model in which repression depends upon a direct interaction of c-Myc with TBP while c-Myc is in a protein complex with TFII-I (or other factor) at the Inr. In agreement, *in vitro* translated c-Myc dlMBII mutant protein is substantially reduced in GST−TBP binding relative to wild-type c-Myc protein (C.Nerlov and E.Ziff, unpublished observations). However, residual binding by this mutant suggests that a second c-Myc region can also contribute to TBP binding. Recently, Hateboer *et al.* (1993) have shown that the c-Myc N-terminus makes multiple contacts with TBP *in vitro* and, in agreement with the current work, a Myc−TBP complex has also been detected *in vivo* (Maheswaran *et al.*, 1994). We find that a c-Myc mutant with a deletion of MBII activates transcription via upstream E-boxes with the same efficiency as wild-type Myc. In agreement, a c-Myc mutant with MBII deletion was a better activator of the ODC promoter than wild-type Myc (Bello-Fernandez *et al.*, 1993). This strongly suggests that any MBII function which is required for promoter repression (such as direct MBII−TBP interaction) is not required for transcription activation. USF and Max also affect basal promoter activity. They may enter into a complex at the Inr, replacing Myc and reversing Myc repression. Further experiments are, however, necessary to define the protein components of such complexes and the regulatory mechanism.

Regulatory functions of Myc, Max and USF

Although both USF and c-Myc stimulate through upstream elements, they antagonize each other's actions at the basal promoter, with c-Myc repressing and USF stimulating transcription. When the positive effect of USF and the negative effect of c-Myc at the basal promoters of the adenovirus MLP and C/EBPα genes are considered, it is apparent that shifting from USF to c-Myc as the agent controlling basal promoter function can provide 10- to 20-fold changes in basal promoter activity *in vivo*. When the USF-induced state is employed as the reference point, c-Myc can provide a similar magnitude (10- to 20-fold) of repression in transfected cells. The reliance of transactivation by USF on the USF N-terminus and the reliance of repression by Myc on MBII suggest that the opposing actions of these two factors are provided by distinct N-terminal functions.

The effects of Max depend on the conditions of the assay. Max expression alone repressed the MLP (data not shown), consistent with previous reports that Max homodimers repress the Ems (Amati *et al.*, 1992; Gu *et al.*, 1992; Kretzner *et al.*, 1992). However, Max activated the MLb in the presence of c-Myc and its effects were dependent upon the LZ and B region (L.Li, and E.Ziff, unpublished observations). Thus, Max may act primarily through displacement of other factors. Max displacement of USF, an activator, would be repressive and displacement of c-Myc, a repressor, would be stimulatory, as observed. This interpretation suggests that the level of Max can influence the level of c-Myc necessary for the biphasic transition of MLP activity, also as observed.

Cellular targets for Myc repression

Enforced Myc expression will prevent the differentiation of precursor cells, such as F9 embryonal carcinoma cells and leukemic cell lines, including HL-60, MEL, U937 and WEHI-3B, as well as 3T3-L1 pre-adipocytes (reviewed by Cole, 1986; Luscher and Eisenman, 1990; DePinho *et al.*, 1991). We have shown that the promoters of two genes, the hepatocyte-specific serum albumin gene and the C/EBPα gene (expressed primarily in hepatic and adipose cells; Birkenmeier *et al.*, 1989), which contain Inr elements similar to that of the MLP (Gorski *et al.*, 1986; Christy *et al.*, 1991), are repressed by c-Myc. In agreement, another group has also observed that c-Myc represses the C/EBPα basal promoter (G.Xanthopoulos, personal communication). The repression of the C/EBPα promoter provides a mechanism for the ability of c-Myc to block 3T3-L1 pre-adipocyte differentiation *in vitro* (Freytag, 1988). We find that c-Myc overexpression is sufficient to repress mRNA encoded by the chromosomal C/EBPα gene in 3T3-L1 cells. Experiments with antisense RNA (Samuelson *et al.*, 1991; Lin and Lane, 1992) show that C/EBPα repression is sufficient to inhibit adipose conversion. Thus, the c-Myc repression of C/EBPα seen here is strongly implicated as a basis for the c-Myc block to adipose differentiation. Both the block of conversion

(Freytag *et al.*, 1990) and C/EBPα repression (this report) depend on MBII. It would be interesting to determine whether USF, an antagonist of repression, can stimulate differentiation of 3T3-L1.

Induction of liver regeneration and the acute phase response lead to an increase in c-Myc mRNA levels in the liver which is concomitant with repression of albumin gene transcription (Milland *et al.*, 1990). Similarly, TNFα-induced de-differentiation of 3T3-L1 adipocytes involves up-regulation of c-Myc mRNA prior to down-regulation of C/EBPα mRNA levels (C.Nerlov and E.Ziff, unpublished observations). While a direct causative link is yet to be established, these correlations also support a role for c-Myc in repression of the albumin and C/EBPα genes *in vivo* and may account for other physiological de-differentiation events as well.

Biological role of repression of gene expression by c-Myc

c-Myc co-operation with activated Ras to transform primary cells (Land, *et al.*, 1983) depends on MBII and the Myc B, HLH and LZ motifs (reviewed by Prendergast and Ziff, 1992). One line of reasoning (see Prendergast *et al.*, 1992) suggests that the Myc−Max heterodimer is the active principle in Myc-dependent cellular transformation and that the primary function of this complex is activation of target genes whose expression is necessary for transformation. It is therefore a potentially important finding that deletion of MBII, which is detremental to the transforming activity of Myc, does not detectably affect the ability of c-Myc to transactivate via E-boxes. Although transactivation by the Myc−Max heterodimer may contribute to cellular transformation (Amati *et al.*, 1993), it is unlikely to be sufficient. Because MBII is essential for both core promoter repression and *ras* cooperation, Inr-dependent promoter repression is a strong candidate for a Myc function necessary for *ras* cooperation. If so, alterations that specifically abolish Myc actions at the core promoter via the Inr should inhibit the ability of Myc to induce cellular transformation.

It is noteworthy that several cellular genes which are subject to Myc negative control, including genes encoding integrins, cell adhesion molecules (Akeson and Bernards, 1990; Inghirami *et al.*, 1990) and MHC class I (Bernards *et al.*, 1986; Versteeg *et al.*,1988; Peltenburg *et al.*, 1993) possess C/TCA-Inr sequences (Table I), similar to the adenovirus MLP Inr. In contrast, genes activated by Myc, such as the PAI-1 (Prendergast *et al.*, 1990), α-prothymosin (Eschenfeld and Berger, 1986), ECA39 (Benvennisty *et al.*, 1992) and ODC (Hickok *et al.*,1990) genes, lack the consensus Inr (Table I). These observations suggest that changes in Myc levels may play a general role in the activation and repression of specific classes of genes during the shift from a proliferative to a fully differentiated phenotype.

Materials and methods

Plasmids and oligonucleotides

The human c-Myc (Hm) cDNA and mouse Max cDNA (previously described as Myn; see Prendergast *et al.*, 1991) were inserted into pcDNAI (Invitrogen) at *Hin*dIII and *Eco*RI sites and expressed from the cytomegalovirus (CMV) promoter, creating CMV Hm and CMV Max (Prendergast *et al.*, 1991). Max mutRR and Max dlZ, have been described previously (Prendergast *et al.*, 1991, 1992). LTR Hm was described by Kelekar and Cole (1986). LTR Hm dlMBI and LTR Hm dlMBII were generated by replacing the *Dra*III−*Bsu*36I DNA fragment within LTR Hm with PCR product fragments bearing in-frame deletion of nucleotides 4297–5504 corresponding to amino acids 46–55 and in-frame deletion of nucleotides 5705–5759 corresponding to amino acids 122–140 respectively. The oligonucleotides for making MBI and MBII in-frame deletions are 5′-GGACAGGGGCGGGGTGGGCAGGGGCGCCGGGGGCTGC-AGCTC and 5′-CTCTGAGACGAGCTTGGCGGCCTGGATGATG-ATGTTTTTGAT respectively. LTR Hm dlZ and LTR Hm mutRR were constructed by replacing the *Cla*I−*Eco*RI DNA fragment within LTR Hm with the DNA fragments derived from CMV Hm dlZ and CMV Hm mutRR respectively (Prendergast *et al.*, 1991). The human USF cDNA (kindly provided by Dr R.Roeder) was inserted into pcDNAI at *Bam*HI and *Xho*I sites creating CMV USF. USF dlNT was generated by inserting the polymerase chain reaction (PCR) product encoding the N-terminal truncated USF fragment and amplified with sequence-specific primers 5′-CGGGATCCAATGACTCGGGATGAGAAACGC and 5′-TATGAATCCTTAGTTGCTGTCATT into pcDNAI at *Bam*HI and *Eco*RI sites. pT22 has been described previously (Land *et al.*, 1983).

The adenovirus-2 MLP was derived from pMLP-CAT (kindly provided by Y.Kato) and inserted into pGL2-Basic (Promega) at *Xho*I/*Hin*dIII sites creating pMLP-Luc. The MLb promoter was constructed by PCR amplification with the sequence specific primers 5′-TTCTCGAGGTT-CCTGAAGGGGGG and 5′-TTAAGCTT CTTCACCCCAACAGCTGG, which introduced a *Xho*I and a *Hin*dIII site at the 5′ and the 3′ ends respectively. The oligonucleotides for making MLM4 and MLM5 are 5′-TTCGTCCAGACTCTCTTCCGCATC and 5′-TTCGTCCTCACAG-ACTTCCGCATC respectively (the antisense sequences are not shown). The mutations were generated by PCR, amplified by the 5′ (TTCTCG-AGGTTCCTGAAGGGGGG for MLM and CTGGGTACCGCGGT-CGGTGTCCAC for MLPM) and 3′ primers and inserted into pGL2-Basic at the *Xho*I/*Hin*dIII sites. pMLb-βGal, pMLM4-βGal and pMLM5-βGal were constructed by replacing the luciferase gene with the *lacZ* gene derived from pSV-βGal (Promega) at the *Hin*dIII/*Bam*HI sites.

The C/EBPα promoter sequence was isolated by PCR amplification of pPNC2.3 (a gift of P.Johnson, Frederick, MD) with the primers: 5′-GGGCTCGAGACGCTCCCCAACCTCCAC and 5′-TTTAAGCTTGC-TGACCCCGCGCAG and inserted into pGL2-Basic at *Xho*I and *Hin*dIII sites creating pαP-Luc. pαMP-Luc was made by similar PCR amplification with the oligonucleotides 5′-CCGCTCGAGCCCAGCAGGC-ACCAT and 5′-TTTAAGCTTGCTGACCCCGCGCAG and inserted into pGL2-Basic. The oligonucleotides for making pαM1P-Luc and pαM2P-Luc are: 5′-CGGGCCTGCGGATTCGCG and 5′-GCCTGGCCAAA-GGCGACC. pAlb-CAT was provided by Dr U.Schibler (Mueller *et al.*, 1990). pAlbMP-Luc is described elsewhere (Nerlov and Ziff, 1994).

The structure of all of the mutants used in the assays were checked by sequencing with the Sequenase system (USB).

Electrophoretic mobility shift assay

The oligonucleotides for the Myc−Max complex DNA binding assay were: Ems1, 5′-GTAGGCCACGTGACCGGGTGTTCCTGA and Ems2, 5′-TGAAGACACATGTCGCCCTCTTCGGCA. The procedure for electrophoretic mobility shift assay has been described previously (Prendergast and Ziff, 1991).

Transfection assay and transformation assay

NIH/3T3, HeLa and 3T3-L1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (GIBCO). Transfection of HeLa and NIH3T3 cells was carried out with the modified calcium phosphate precipitation method (Chen and Okayama, 1987). 3T3-L1 cells were transfected by the DOTAP lipofection method according to the manufacturer's instructions (Boehringer Mannheim). Transfections were performed when cells were 70–80% confluent. The experiments shown are representative experiments, except where error bars are given, which are average. The data are in the form of a titration, with repressive or inductive effects shown by multiple titration points. All the transfection experiments were repeated at least twice and are normalized with an internal control, pCMV-βGal. in each experimental repetition, the inductive and repressive curves were similar. In general, 20–25 μg DNA, consisting of 3–10 μg reporter, plus 0.1 μg pCMV-βGal (generated by inserting the *lacZ* gene into the pcDNAI vector) internal control plasmids, a variable amount of expression vector supplemented with parental plasmids to make up equal amounts of input expression plasmids in different dishes and pUC18 as carrier DNA (Sigma), were used. For stable transfections of 3T3-L1 cells, 18 μg

pSV2myc (Kelekar and Cole, 1986) and 2 μg pRSVneo (ATCC) were used. Stable transfectants were selected in the presence of 300 μg/ml G418 (Geneticin, GIBCO) and c-Myc-expressing clones identified by Northern blot analysis. Differentiation of 3T3-L1 cells was performed as previously described (Birkenmeier *et al.*, 1989).

Transformation assay procedure was as described previously (Prendergast *et al.*, 1992).

Reporter gene assays

We used the Galacto-light™ chemiluminescent reporter assay system (Tropix, Inc.) for β-galactosidase assay, carried out as per the manufacture's protocol with the modification that 100 μl lysis buffer were employed for lysing the cell pellet harvested from 10 cm dishes. Twenty microliters of the lysate were mixed with 100 μl reaction buffer and 100 μl accelerator buffer. The same lysate was also used for the luciferase assay. Luciferase activity was assayed by using a Berthold Lumat LB9501 luminometer with 50 μl cell lysate essentially as described previously (Matinez-Salas *et al.*, 1989).

Luciferase activity were normalized to the luciferase level in the absence of supplemental expression plasmid (basal value). In some figures, stimulation is shown as a positive fold change corresponding to the ratio of the experimental value to the basal value for luciferase activity. Repression is shown as negative change corresponding to the ratio of the basal value to the experimental luciferase value.

RNA preparation, Northern blotting and Western analysis

RNA was isolated from 3T3-L1 cells by the guanidinium isothiocyanate method as described (Chomczynski and Sacchi, 1987), fractionated on 1.2% formaldehyde−agarose gels and transferred to Duralon-UV™ membranes (Stratagene) by capillary blotting. Integrity and transfer of RNA was verified by ethidium bromide staining. After UV cross-linking, membranes were prehybridized and hybridized at 68°C in Quickhyb (Stratagene) to probes for mouse c-*myc* (*Xba*I−*Sac*I fragment from pSV2myc) and rat C/EBPα (*Pst*I fragments from pMSV-C/EBP; Friedman *et al.*, 1989) labeled with [α-^{32}P]dCTP by random priming (Boehringer). Stringent washing was in 0.1× SSC, 0.1% SDS at 68°C for 30 min. Western blotting has been previously described (Nerlov and Ziff, 1994).

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