

# c-Myc Inhibits Ras-Mediated Differentiation of Pheochromocytoma Cells by Blocking c-Jun Up-Regulation

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## Abstract

Although mutant Ras proteins were originally described as transforming oncoproteins, they induce growth arrest, senescence, and/or differentiation in many cell types. c-Myc is an oncogenic transcription factor that cooperates with Ras in cellular transformation and oncogenesis. However, the Myc-Ras relationship in cellular differentiation is largely unknown. Here, we have analyzed the effects of c-Myc on PC12-derived cells (UR61 cell line), harboring an inducible *N-Ras* oncogene. In these cells, Ras activation induces neuronal-like differentiation by a process involving c-Jun activation. We found that c-Myc inhibited Ras-mediated differentiation by a mechanism that involves the blockade of c-Jun induction in response to Ras signal. Accordingly, ectopically expressed c-Jun could bypass c-Myc impediment of Ras-induced differentiation and activator protein 1 activation. Interestingly, it did not rescue the proliferative arrest elicited by Ras and did not enhance the differentiation-associated apoptosis. The blockade of Ras-mediated induction of c-Jun takes place at the level of *c-Jun* proximal promoter. Mutational analysis revealed that c-Myc regions involved in DNA binding and transactivation are required to block differentiation and c-Jun induction. c-Myc does not seem to require Miz-1 to inhibit differentiation and block c-Jun induction. Furthermore, Max is not required for c-Myc activity, as UR61 cells lack a functional *Max* gene. c-Myc-inhibitory effect on the Ras/c-Jun connection is not restricted to UR61 cells as it can occur in other cell types as K562 or HEK293. In conclusion, we

describe a novel interplay between c-Myc and c-Jun that controls the ability of Ras to trigger the differentiation program of pheochromocytoma cells. (Mol Cancer Res 2008;6(2):325–39)

## Introduction

c-Myc (Myc hereafter) is an oncogenic transcription factor of the basic-helix-loop-helix protein family. Myc forms heterodimers with the protein Max. Myc-Max dimers bind to specific DNA sequences (E-boxes) in the regulatory regions of target genes. Genes up-regulated by Myc include genes involved in carbohydrate metabolism, protein biosynthesis, cell cycle regulation, and other less represented functions. Also, an important fraction of the genes targeted by Myc (30-50% in different studies) are repressed by it (reviewed in refs. 1-3; a list of regulated genes can be found online<sup>4</sup>). Consistent with its effects on cultured cells and transgenic *in vivo* models, deregulated expression of Myc is found in a wide array of human cancers, in many cases associated to disease progression (4, 5).

Myc activity on its genomic targets translates into multiple biological effects. Importantly, Myc activity drives cells into proliferation by mechanisms impinging on G<sub>1</sub> cell cycle phase control (6, 7). Myc is also known to block differentiation in a number of model systems (5, 8). The mechanism(s) whereby Myc inhibits differentiation are unclear, but, as terminal differentiation is usually associated with cell cycle arrest, it has been proposed that Myc inhibits differentiation by stimulating cell cycle progression. Indeed, a significant number of Myc-induced genes encode positive regulators of the cell cycle (e.g., *cyclin D2*, *cyclin E1*, and *CDK4*), whereas cell cycle inhibitors such as p15<sup>INK4B</sup>, p21<sup>WAF1</sup>, and p27<sup>KIP1</sup> are repressed by Myc (7).

The Ras family of small GTPases includes three closely related proteins in mammals (H-, K-, and N-Ras). Ras proteins are activated by signals originated in surface receptors, acting as key components of signaling pathways by relaying signals downstream through diverse routes. Activated Ras interacts with diverse signaling effectors. One of the most relevant is the Raf kinase that signals through activation of mitogen-activated

Received 4/18/07; revised 9/3/07; accepted 10/9/07.

**Grant support:** Spanish Ministerio de Educación y Ciencia grant SAF05-0461, Fundación Marqués de Valdecilla AP05-17, and ISCIII-RETIC RD06/0020 (J. León) and Ministerio de Educación y Ciencia SAF05-0461CO2 (M.C. Marín). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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doi:10.1158/1541-7786.MCR-07-0180

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protein/extracellular signal-regulated kinase (ERK) kinase and ERK kinases (9, 10). *Ras* has been classically regarded as a transforming oncogene. In fact, activating point mutations in *Ras* genes that render a constitutively active protein have been found in 30% of all human cancers (11). However, the proliferative and transforming activity of *Ras* is cell context dependent. Thus, *Ras* exerts proliferation arrest and senescence in primary fibroblasts (12) and also exerts antiproliferative effects in some tumor cell lines as leukemia-derived K562 (13, 14). Also, *Ras* can induce differentiation of pheochromocytoma cells (15-17) and adipocytic precursors (18). Moreover, a systematic survey of the literature reveals that the most common response to *Ras* ectopic expression in cell lines derived from distinct tissues with differentiation potential is differentiation (19).

One of the earliest examples of oncogene cooperation was described for *Myc* and *Ras* in the transformation of rodent primary fibroblasts (20). However, to date, all the studies on *Myc* and *Ras* cross-talk have been undertaken in murine or human fibroblasts (21, 22) and in murine models (23-25), where *Myc*-*Ras* cooperation results in cellular transformation and oncogenesis (23-25). In sharp contrast, the *Myc*-*Ras* cross-talk in differentiation models has not been studied.

PC12 cells is a rat pheochromocytoma-derived cell line that, in response to nerve growth factor (NGF), differentiates into a sympathetic neuron-like phenotype, characterized by neurite outgrowth and induction of neuronal specific genes (26). NGF activates *Ras* and NGF-induced differentiation is dependent on *Ras*-ERK activation (27, 28). Consistently, *H-Ras* (15, 17), *K-Ras* (17), and *N-Ras* (16) oncogenes induce neuronal-like differentiation in PC12 cells.

c-Jun is a transcription factor of the leucine zipper family that dimerizes with another leucine zipper partner protein (c-Jun itself, ATF2 and members of the Fos and Fra families) to form active activator protein 1 (AP-1) complexes (29, 30). c-Jun expression is subjected to positive autoregulation, which depends on two AP-1 sites in the proximal promoter (30). In PC12, it has been shown that NGF induces c-Jun expression (31, 32) and that the concerted signaling through ERK and Jun NH<sub>2</sub>-terminal kinase converge at the expression and phosphorylation of c-Jun, an essential event for NGF-induced differentiation (33-35). Consistently, ectopic expression of constitutively active c-Jun is sufficient to induce neuronal-like differentiation of these cells (33).

Here, we investigated the effect of *Myc* constitutive expression in *Ras*-mediated differentiation. For this purpose, we used the UR61 cell line, a PC12-derived cell line with inducible expression of the *N-Ras* oncogene. Surprisingly, we found that *Myc* impairs the neuronal-like differentiation induced by *Ras* through a mechanism involving the repression of c-Jun up-regulation.

## Results

### *Myc* Inhibits *Ras*-Mediated Differentiation of UR61 Cells

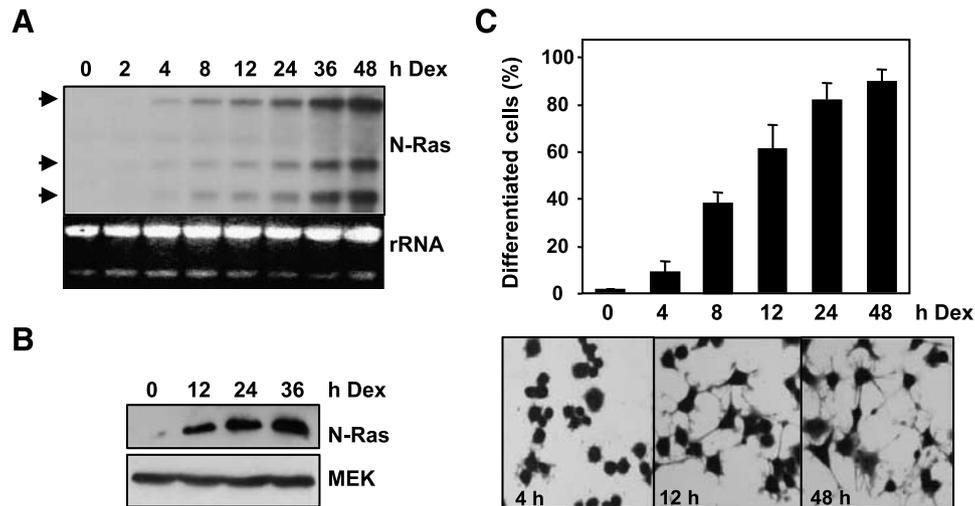
Treatment of UR61 cells with dexamethasone led to a progressive increase in *N-Ras* mRNA (Fig. 1A) and protein expression (Fig. 1B). This resulted in neuronal-like differentiation, as shown by morphologic changes, including the extension of

neurites (Fig. 1C, *bottom*) as well as nuclear reorganization and expression of neuronal markers (not shown). Differentiation of UR61 cells was maximal and irreversible after 24 h of exposure to dexamethasone (Fig. 1C).

To study *Ras*-*Myc* interaction in UR61 differentiation, we generated by retroviral infection a polyclonal cell line (termed UR61Myc) with constitutive expression of human *Myc* protein. As a control for potential *Ras*-independent effects of *Myc*, we also infected U7 cells (UR61 parental cells) with *Myc* viruses and obtained the cell line U7Myc. To assess the activity of *Myc* in these cells, we determined the transactivation of a reporter containing four *Myc*-responsive E-boxes directing the expression of the luciferase gene. The results (Fig. 2A) showed the transactivation of the E-box-containing promoter in UR61Myc. We also tested the expression of *PDK2* (pyruvate dehydrogenase kinase 2) and *LDHB* (lactate dehydrogenase B), two genes previously described as *Myc* target genes.<sup>4</sup> We chose these genes because they appeared up-regulated in UR61Myc cells in our preliminary microarray analysis. The mRNA levels was determined by quantitative reverse transcription-PCR (RT-PCR), and the results showed that both genes were up-regulated in UR61Myc cells compared with control cells in the absence or presence of *Ras* (i.e., dexamethasone; Fig. 2B).

We next asked whether constitutive expression of *Myc* modified the *Ras*-induced neuronal differentiation in these cells. Most of the UR61V cells (75-85% in our culture conditions) extended neurites upon *Ras* induction. In sharp contrast, the majority of UR61Myc cells (around 90%) either lacked neurites or these were much shorter (Fig. 2C), suggesting that *Myc* can inhibit the activation of the differentiation program activated by *Ras* in these cells. We sought to confirm the inhibition of differentiation by analyzing the expression of two neuronal differentiation markers known to be up-regulated by NGF in PC12 cells, namely GAP43 (neuromodulin, a neuronal plasticity-related protein; refs. 36, 37) and SNAP25 (synaptosomal-associated protein 25 kDa, a protein involved in synaptic exocytosis; ref. 38). Real-time RT-PCR revealed that *Myc* inhibited *Ras* mediated up-regulation of both markers (Fig. 2D). In contrast, treatment of U7V or U7Myc cells with dexamethasone did not result in morphologic differentiation (not shown) and did not induce the expression of these genes (Fig. 2D). The absence of up-regulation of GAP43 protein in UR61Myc cells by *Ras* was also confirmed by immunoblot (Fig. 2E).

To confirm that *Myc* overexpression was the cause for the block in differentiation of UR61Myc cells, we asked whether the suppression of *Myc* expression restored the differentiation-competent phenotype. To this purpose, we used a vector expressing a short hairpin RNA for human *Myc* (shMyc) previously shown to suppress human *Myc* expression (39). The ability of this vector to reduce *Myc* levels in UR61Myc cells was confirmed by immunoblot analysis (Fig. 3A). To test shMyc effect on differentiation, UR61 cells were cotransfected with a green fluorescent protein (GFP) vector together with the shMyc vector or the empty vector and the fraction of GFP-expressing differentiated cells was then scored. The result indicated that shMyc transfection significantly increased the fraction of differentiated cells upon treatment with dexamethasone (Fig. 3B). These data show that *Myc* antagonizes *Ras*-mediated differentiation.



**FIGURE 1.** Ras induces differentiation in UR61 cells. **A.** Northern analysis showing the induction of N-Ras in UR61 cells upon treatment with 200 nmol/L dexamethasone (Dex). Arrows, the three transcripts of the murine transgene. A picture of the filter stained with ethidium bromide is shown to assess RNA loading and integrity. **B.** N-Ras protein induction in UR61 cells. Cells were treated with 200 nmol/L dexamethasone for 12 to 36 h as indicated and whole-cell extracts were subjected to immunoblotting for N-Ras and (as protein loading control) MEK. **C.** Irreversibility of Ras-induced differentiation. UR61 cells were treated with 200 nmol/L dexamethasone for the indicated periods, washed twice with PBS, and the morphologically differentiated cells were scored after 48 h. Columns, mean of two independent experiments; bars, SE. Bottom, representative micrographs of cells corresponding to the indicated time points stained with crystal violet.

#### *Myc Does Not Inhibit Ras Induction and Activation*

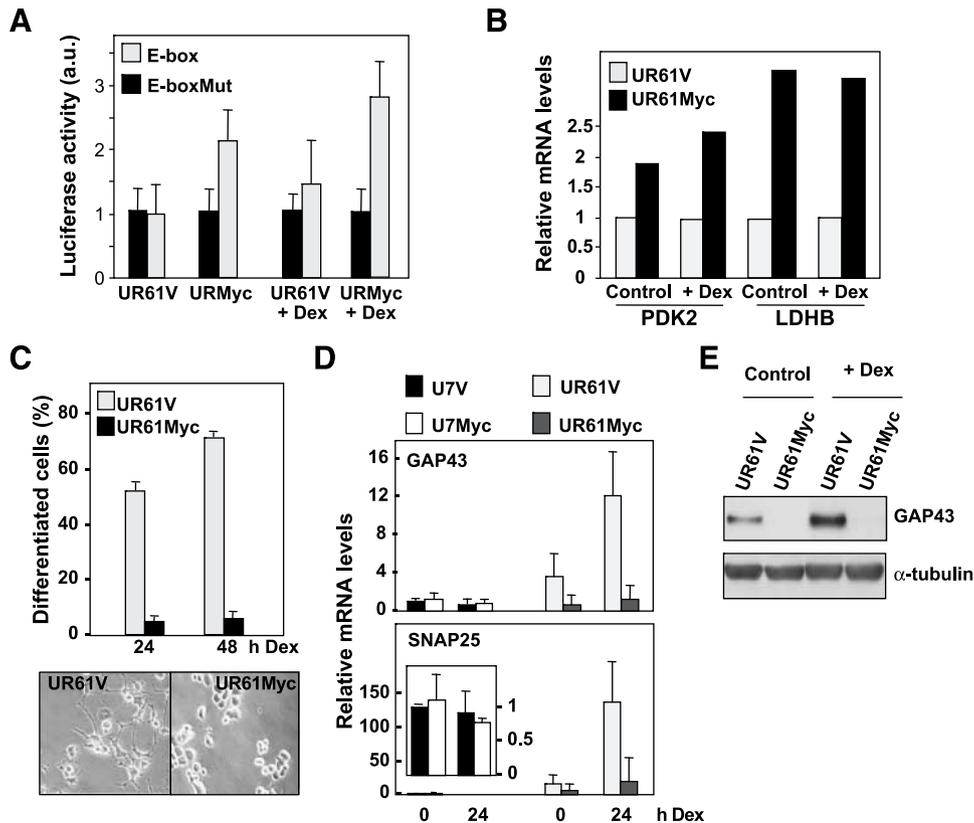
We next explored possible mechanisms by which Myc could impair Ras-induced differentiation. First, we wanted to rule out the possibility that Myc could hinder directly or indirectly the activity of mouse mammary tumor virus promoter, so as to inhibit the induction of Ras expression by dexamethasone. Northern blot analysis showed that N-Ras mRNA induction was similar in the parental UR61, UR61V, and the Myc-expressing UR61Myc cells (Fig. 4A). We also confirmed by immunoblot that N-Ras protein induction was similar in UR61V and in UR61Myc cells (Fig. 4B). In the immunoblot shown in Fig. 4B, as well in others described below (Figs. 5E and 7A), it is apparent that Myc protein levels were dramatically elevated in response to Ras. This effect is dependent on Ras, and not on the hormone, as shown by Myc immunoblot analysis of the U7Myc cells (which lack Ras ectopic expression; Fig. 4B). The Ras-dependent increase in protein levels is likely due to Myc stabilization, an effect previously reported in other systems (40), although concomitant increase in mRNA levels cannot be ruled out. Next, we tested the possibility that Myc impaired not the expression, but the activation of N-Ras oncoprotein in this system. Activation of Ras was analyzed by a Ras-GTP pull-down assay with the Ras-binding domain of Raf, which only binds GTP-bound Ras. The results showed that the fraction of active N-Ras was unchanged in UR61Myc cells compared with UR61V cells (Fig. 4C). We concluded that Ras activation was not impaired by Myc expression in this model, and thus Myc is acting at a level downstream of Ras activation.

#### *Myc Does Not Inhibit Proliferation Arrest of UR61 Cells Induced by Ras*

In view of the well-known effects of Myc as a stimulator of cell cycle progression and the growth arrest associated to Ras-

mediated differentiation in UR61 cells, it was conceivable that Myc would inhibit differentiation by maintaining the cells in a proliferative state. As such, we asked whether Myc-expressing cells continued proliferating despite the induction of Ras. To test this, we seeded similar numbers of UR61V and UR61Myc cells, and after 48 and 72 h the attached cells were counted. The results (Fig. 5A) showed that cells expressing Myc, despite their undifferentiated phenotype, remained growth arrested, similarly to differentiating UR61. To analyze the cell cycle distribution, DNA content was determined by flow cytometry. Ras induction resulted in a depletion of S-phase cells in both differentiating UR61V and nondifferentiated UR61Myc cells (Fig. 5B). In conclusion, Myc impaired Ras-mediated differentiation but did not reverse the proliferation arrest elicited by Ras. It has been previously reported that Myc sensitizes different cell types in response to growth factor deprivation and other stress stimuli (41). Myc-dependent apoptosis have also been reported in serum-deprived PC12 cells (42). Thus, we analyzed the effect of Myc on apoptosis of UR61 cells. Similarly to parental PC12 cells, serum deprivation resulted in higher rate of cell death in UR61Myc than UR61 cells (Fig. 5C). The results also confirmed the previous results (Fig. 5A and B) that Myc expression does not induce cell proliferation in Ras-induced cells.

It was conceivable that proliferation of UR61Myc cells in the presence of dexamethasone was limited by concomitant enhanced apoptosis mediated by Myc. We studied the apoptosis in response to Ras induction in both cell lines. The results showed that differentiation was accompanied with apoptosis, which increased after 24 h (i.e., once morphologic differentiation was already at its maximum levels). However, the extent of apoptosis was similar in UR61Myc cells and in control UR61V cells. This result was assessed by measuring apoptosis through the binding of Annexin V



**FIGURE 2.** Myc inhibits Ras-induced differentiation of UR61 cells. **A.** Transactivation of a Myc-responsive promoter by UR61V and UR61Myc cells. Cells were transiently transfected with a luciferase reporter containing four E-boxes or four mutated E-boxes (*E-boxMut*). Data are relative to the activity in cells transfected with the mutated E-box. Columns, mean of two independent experiments; bars, SE. **B.** mRNA expression of *PDK2* and *LDHB*. Cells were treated for 24 h with 200 nmol/L dexamethasone as indicated, total RNA was prepared, and relative mRNA levels were determined by quantitative RT-PCR with normalization against levels of RPS14 mRNA. **C.** Right graph, fraction of cells with neurites of the indicated cell lines was measured after 24 and 48 h of treatment with 200 nmol/L dexamethasone. Columns, mean of three independent experiments; bars, SE. Left, representative images from UR61V or UR61Myc cells treated for 24 h with 200 nmol/L dexamethasone. **D.** mRNA expression of the neuronal-specific genes *GAP43* and *SNAP25*. Cells were treated with 200 nmol/L dexamethasone as indicated and total RNA was prepared. Relative levels of *GAP43* and *SNAP25* transcripts were measured as in **A**. Inset, *SNAP25* expression in U7V and U7Myc cells. Data are relative to the mRNA levels of untreated U7V cells. Columns, mean of two measurements; bars, SE. **E.** UR61V and UR61Myc cells were treated with 200 nmol/L dexamethasone for 36 h as indicated and whole-cell extracts were subjected to immunoblotting for GAP43 and (as protein loading control)  $\alpha$ -tubulin.

(Fig. 5D) and the fraction of cells with sub-G<sub>1</sub> content of DNA (Fig. 5E). In aggregate, the results indicate that in this model Myc is impairing differentiation without inducing cell proliferation.

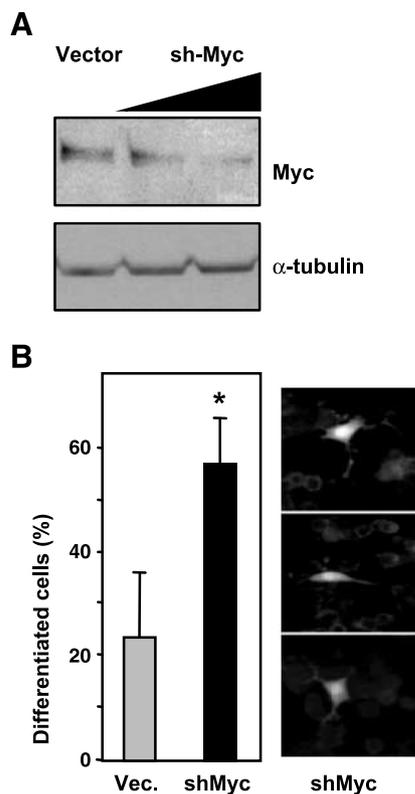
#### *Myc Inhibits Ras-Mediated AP-1 Activity and c-Jun Expression in UR61 Cells*

It is well established that in PC12 cells, sustained Ras-ERK activity induces c-Jun expression and that c-Jun up-regulation is critical for NGF-mediated differentiation (33). Thus, we asked whether this was also the case in the UR61 model. Similarly to PC12, the expression of c-Jun mRNA and protein increased upon Ras activation in UR61 cells (Fig. 6A and C). To assess the relevance of c-Jun in UR61 differentiation, we used a constitutively activated form of c-Jun (v-Jun). Cells were co-transfected with v-Jun expression vector (or the empty vector) and a GFP expression vector in a 10:1 ratio. The cells were treated with dexamethasone and the fraction of morphologically differentiated GFP-positive cells was scored 24 h later. It was found that ~55% of the v-Jun transfected cells appeared differentiated 24 h after transfection, versus <5% in cells

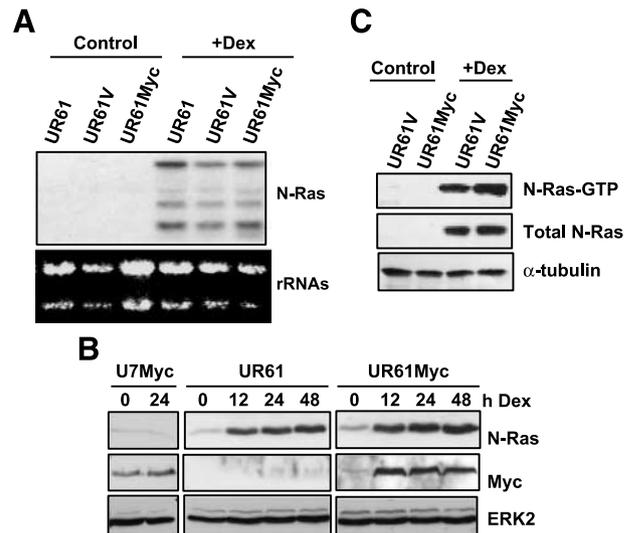
transfected with the empty vector. Thus, v-Jun was sufficient to induce differentiation in UR61 cells in the absence of Ras induction (Fig. 6B). The above results suggested that the Ras-Jun signal was sufficient for the differentiation of UR61 cells, similarly to what is described for NGF-induced differentiation in PC12 cells (33, 43, 44).

Given this relationship between c-Jun and differentiation in UR61 cells, we next sought to investigate whether Myc could affect the Ras-mediated up-regulation of *c-Jun* gene expression. To address this, we first analyzed c-Jun expression in UR61V and UR61Myc cells after Ras induction. In UR61V cells, c-Jun levels dramatically increased upon dexamethasone addition but this induction was abrogated in UR61Myc cells after 24 h of treatment, when differentiation is irreversible (Fig. 6C, left). It has been described that the protein ATF2 dimerizes with c-Jun to form the AP-1 factor that binds the c-Jun promoter (45) and that ATF2 is also activated during differentiation of PC12 cells (43). Thus, we studied ATF2 levels and phosphorylation in the UR61 model. We observed ATF2 phosphorylation in UR61 after Ras induction, but Myc did not modify the levels of phospho-ATF2 (Fig. 6C, right).

We next analyzed whether Myc overexpression also antagonized c-Jun binding to DNA in the UR61 model. We did electrophoretic mobility shift assays using as the probe an oligonucleotide encompassing the AP-1 (*jun1*) site of the rat *c-Jun* promoter mapping at -133/-126, conserved in human, mouse, and rat genes (46, 47). We tested nuclear extracts from UR61V and UR61Myc cells untreated or treated for 24 h with dexamethasone to induce Ras. The results (Fig. 6D) showed that Ras activation resulted in a dramatic retardation in control cells, but Myc completely inhibited this effect. We sought to confirm this result by testing the binding of c-Jun to its own promoter by chromatin immunoprecipitation (ChIP). c-Jun autoregulates its transcription via two conserved AP-1 binding sites (termed *jun1* and *jun2*, mapping at -124 and -243 from transcription start site), conserved in rat, mouse, and human promoters (46, 47). These sites are also critical for c-Jun induction during PC12 differentiation (48). The results of ChIP analysis (Fig. 6E) showed a dramatic increase in the amount of c-Jun bound to a promoter fragment containing *jun1* and *jun2* sites after Ras induction in control



**FIGURE 3.** Silencing of Myc expression in UR61Myc cells restores the differentiation response to Ras. **A.** Reduction of Myc protein levels in UR61Myc transfected with a shMyc expression vector (pRS/myc). Cells were transfected with 8  $\mu$ g of vector and 4 and 8  $\mu$ g of pRS/myc. Twenty-four hours after transfection, Myc levels were determined by immunoblot. Levels of  $\alpha$ -tubulin were also determined as protein loading control. **B.** Cells were cotransfected with vectors for GFP and shMyc (or the empty vector) in a 1:10 proportion (GFP/Myc) and incubated for 12 h. Dexamethasone was then added (200 nmol/L) and after 24 h the fraction of differentiated cells among those expressing GFP were counted. Columns, mean of two independent experiments; bars, SE. \*,  $P < 0.01$ , statistically significant difference from control. Right, three representative images of cells expressing Myc/GFP.

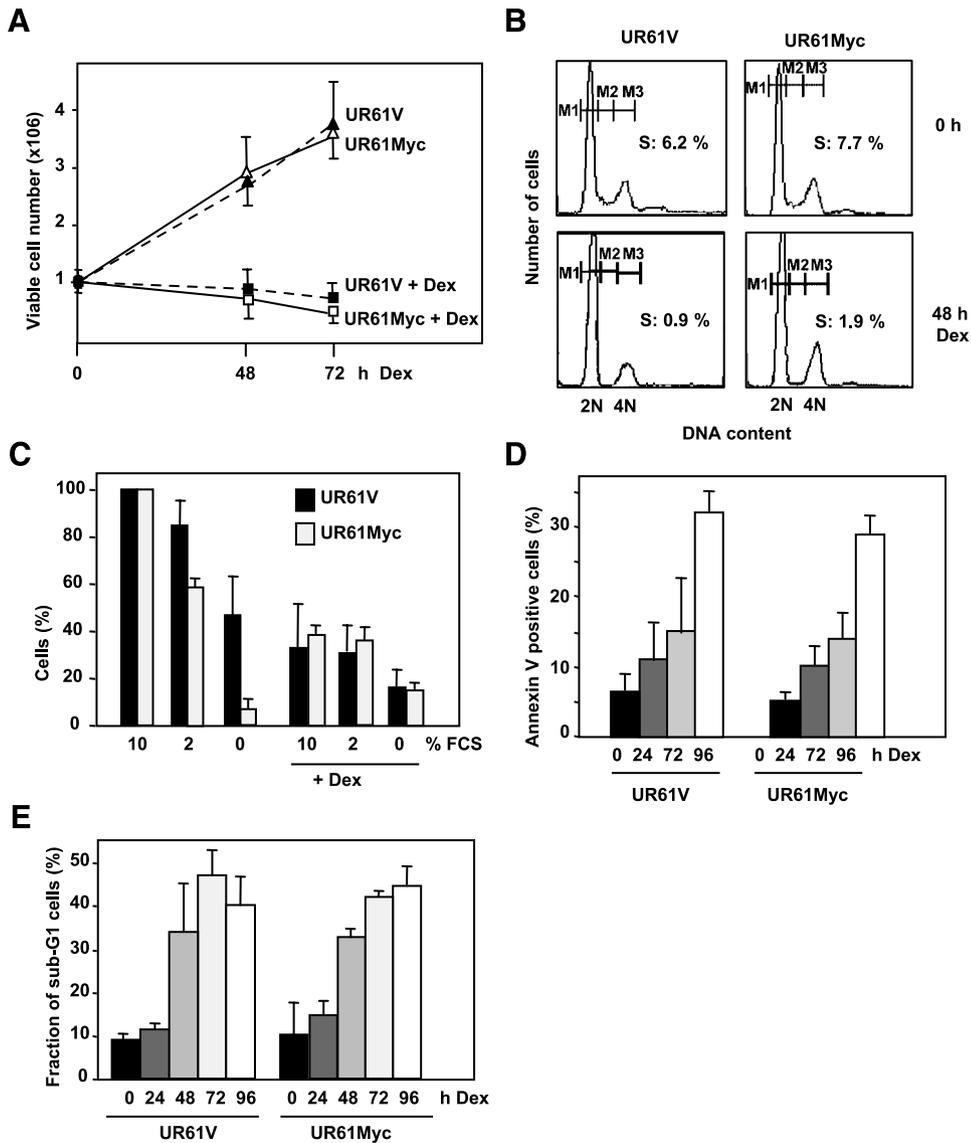


**FIGURE 4.** Myc does not impair N-Ras induction and activity in UR61Myc cells. **A.** Northern blot demonstrating N-Ras mRNA induction in the indicated cell lines untreated or treated for 24 h with 200 nmol/L dexamethasone. A picture of the filter stained with ethidium bromide is shown to assess RNA loading and integrity. **B.** Immunoblots showing the induction of N-Ras protein by 200 nmol/L dexamethasone. The same blot was successively incubated with antibodies against N-Ras, Myc, and (as loading control) ERK2. **C.** Ras-RBD pull-down analysis showing the fraction of active Ras (Ras-GTP) in UR61V and UR61Myc cells. Pulled-down material was subjected to immunoblot incubated with anti-N-Ras and (as protein loading control) anti- $\alpha$ -tubulin antibodies.

UR61V cells. In contrast, such binding was not observed in UR61Myc cells.

Although UR61Myc is a polyclonal cell line generated by viral transduction, to rule out the possibility that the inhibition of c-Jun expression was due to an artifact during the selection of the cell line, we followed two experimental approaches. First, we generated a UR61-derived cell line with conditional Myc activation. UR61 cells were infected with a retrovirus expressing the MycER fusion protein, which can be activated by 4-hydroxytamoxifen. The resultant polyclonal cell line was termed UR61MER. The functionality of the MycER chimera was confirmed by the transactivation assays using a luciferase reporter carrying four Myc-responsive E-boxes in the promoter. The results show that the treatment with 4-hydroxytamoxifen resulted in increased transactivation of the promoter (Fig. 7A). As expected, treatment with 4-hydroxytamoxifen partially inhibited the morphologic differentiation induced with dexamethasone (not shown). Immunoblot analysis revealed that Ras-mediated up-regulation of c-Jun decreased upon addition of 4-hydroxytamoxifen in a dose-dependent manner (Fig. 7B). The immunoblot also showed that Ras also dramatically stabilized the MycER protein, as already observed for Myc protein in UR61Myc cells (Fig. 4B). Overall, the results obtained with both cell lines (UR61Myc and UR61MER) show that Myc ablates Ras-mediated up-regulation of c-Jun.

The second approach to elucidate the effect of Myc on c-Jun regulation was to transiently express Myc in UR61 and analyze the expression of c-Jun, after Ras induction, in the transfected (GFP expressing) cells. The results indicated that in almost every Myc-expressing cell under observation, c-Jun was



**FIGURE 5.** Myc does not rescue Ras-mediated proliferation arrest of UR61 cells. **A.** Growth curves of UR61Myc and UR61V (control cells) treated with 200 nmol/L dexamethasone to induce Ras. At the indicated times, the number of viable cells was determined by trypan blue method. Points, mean of four independent experiments; bars, SE. **B.** Cell cycle distribution of UR61Myc and UR61V cells after 48 h of Ras induction. Cell nuclei were stained with propidium iodide and the cell cycle distribution was analyzed by flow cytometry. The fraction of cells in S phase is indicated in each case. **C.** Cells were seeded in six-well plates at a density of 150,000 per well. Twenty-four hours later, the medium was replaced with fresh medium containing 0%, 2%, and 10% FCS and 100 nmol/L dexamethasone and further incubated for up to 72 h. Cells were stained with crystal violet and the cell amount was measured as indicated in Materials and Methods. Columns, mean from two independent experiments; bars, SE. **D.** Apoptosis of UR61V and UR61Myc cells assessed by Annexin V binding. Cells were treated with 200 nmol/L dexamethasone for the indicated periods and the fraction of cells bound to Annexin V was analyzed by flow cytometry. Columns, mean of two independent experiments; bars, SE. **E.** Apoptosis of UR61V and UR61Myc cells analyzed by the fraction of subdiploid cells. Cells were stained with bromodeoxyuridine and analyzed by flow cytometry. Columns, mean of two independent experiments; bars, SE.

undetectable or very low when compared with neighboring untransfected cells (Fig. 7C). In turn, if the mechanism responsible for the inhibition of differentiation elicited by Myc was the down-regulation of c-Jun expression, it would be expected that c-Jun overexpression should counteract the Myc effect. We addressed this point by transfecting a c-Jun expression vector into UR61Myc cells and quantifying the morphologically differentiated cells upon Ras induction. The results showed that c-Jun expression resulted in a 3-fold increase on UR61Myc cells undergoing differentiation compared with cells transfected with the empty vector (Fig. 7D). Altogether, these data argue that Myc inhibits differentiation of UR61 via the down-regulation of c-Jun expression and activity.

#### *Myc Inhibits Ras-Mediated Transcriptional Activation of c-Jun Promoter*

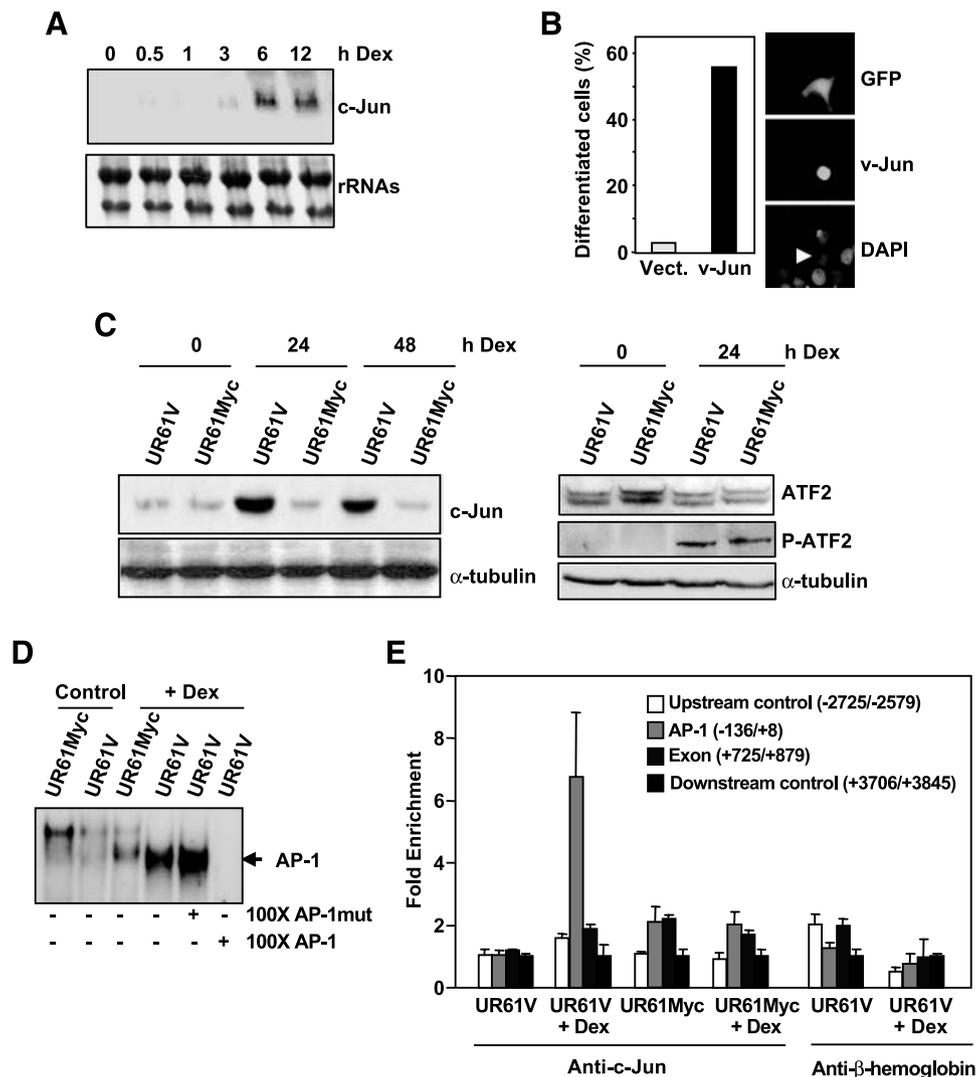
The aforementioned results indicated that Myc blocked the up-regulation of c-Jun expression induced by Ras. We next asked

whether Myc exerted this effect at a transcriptional level. To this end, we first analyzed c-Jun mRNA levels by quantitative RT-PCR. The results showed that 24 h after the induction of Ras by dexamethasone addition, c-Jun mRNA dramatically increased in control UR61V cells but not in UR61Myc cells (Fig. 8A). This result suggested that, in the UR61 system, Myc was impairing c-Jun expression at the transcriptional level. Thus, we next tested whether Myc inhibited the activity of the Jun promoter. We carried out luciferase reporter assays in UR61 cells with a luciferase reporter carrying the murine c-Jun proximal promoter. We found that Myc partially inhibited Ras-mediated activation of c-Jun promoter (Fig. 8B). However, Myc had no effect on the transactivation exerted by an activated c-Jun isoform (i.e., v-Jun), suggesting that Myc did not affect the transcriptional activity of c-Jun and its effect is likely limited to inhibit its induction in response to Ras.

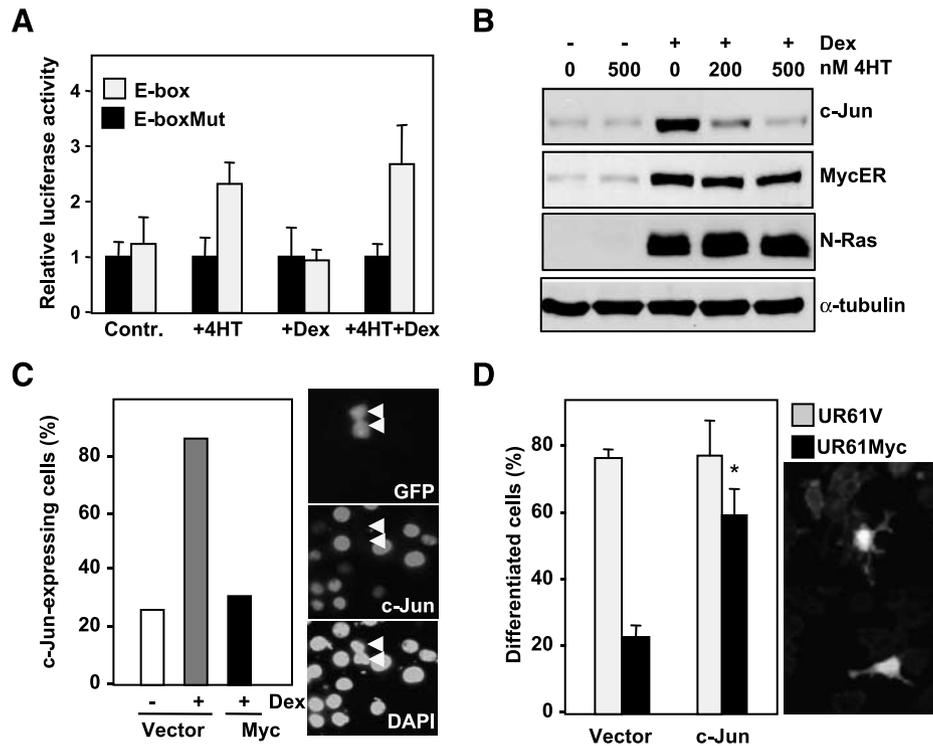
As noted above, two AP-1 response elements (*jun1* and *jun2*) in *c-Jun* promoter are critical for c-Jun autoregulation

and bound by c-Jun in UR61 cells (Fig. 6E). To investigate whether Myc effects on c-Jun transcription depended on these AP-1 sites, we analyzed the effect of the transient coexpression of H-RasG12V and Myc on the transcriptional activity of AP-1 using a luciferase reporter containing four AP-1 response elements (4×AP-1-Luc). The results (Fig. 8C) showed that Ras activated this promoter, but the coexpression of Myc inhibited the activation of AP-1. In contrast, a Myc mutant carrying a deletion of a conserved region required for

transactivation and transformation (MycD106-143) was unable to block Ras-mediated activation of c-Jun promoter (Fig. 8B) and to antagonize c-Jun transcriptional activity in UR61 cells using the 4×AP-1-Luc reporter (Fig. 8C). Similarly, a Myc mutant carrying and insertion in the basic region and unable to bind DNA (MycIn373; ref. 49) did not repress the Ras-induced activation of the AP-1 reporter (Fig. 8C). However, Myc did not impair the activation of the AP-1-Luc reporter induced by a constitutively activated c-Jun form as v-Jun



**FIGURE 6.** Myc impairs Ras-induced up-regulation of c-Jun. **A.** Expression of c-Jun mRNA during Ras-induced differentiation of UR61 cells. Cells were treated with 200 nmol/L dexamethasone for the indicated periods, and total RNA was analyzed by Northern blot. **B.** v-Jun induces differentiation of UR61 cells. Cells were transiently cotransfected with expression vectors for v-Jun and GFP in a proportion 1:10 (GFP/Jun). Twenty-four hours after the transfection, the fraction of morphologically differentiated cells was determined. The graph shows the results from 300 GFP-expressing cells. A representative image with a GFP/Jun-expressing differentiated cell is shown at the right. **C.** c-Jun up-regulation induced by Ras is impaired in UR61Myc cells. Cells were treated with dexamethasone for the indicated periods and whole-cell extracts were subjected to immunoblotting for c-Jun (left), ATF2, and phosphorylated ATF2 (P-ATF2; right).  $\alpha$ -Tubulin levels were also determined as loading control. **D.** Electrophoretic mobility shift assay showing the inhibition of protein bound to a Jun/ATF2-binding site in UR61Myc cells in response to Ras-mediated differentiation. UR61V extracts were incubated with the labeled probe plus a 100-fold excess of unlabeled probe or unlabeled mutated probe as specificity controls. **E.** ChIP of c-Jun on the promoter of rat c-Jun, showing that Myc inhibits binding of c-Jun to its promoter in UR61Myc cells upon induction of Ras. Cells were treated with 200 nmol/L dexamethasone for 24 h to induce Ras and the chromatin was immunoprecipitated with anti-c-Jun and (as a specificity control) anti- $\beta$ -hemoglobin antibodies. The immunoprecipitated DNA was analyzed by quantitative PCR was carried out with primers encompassing the *jun1* and *jun2* sites of rat c-Jun promoter (AP-1 amplicon) and other three control amplicons as indicated. Data are normalized to the corresponding inputs of chromatin before immunoprecipitation and are expressed as relative to the value of the downstream control amplicon. Columns, mean of two independent ChIP experiments; bars, SE.

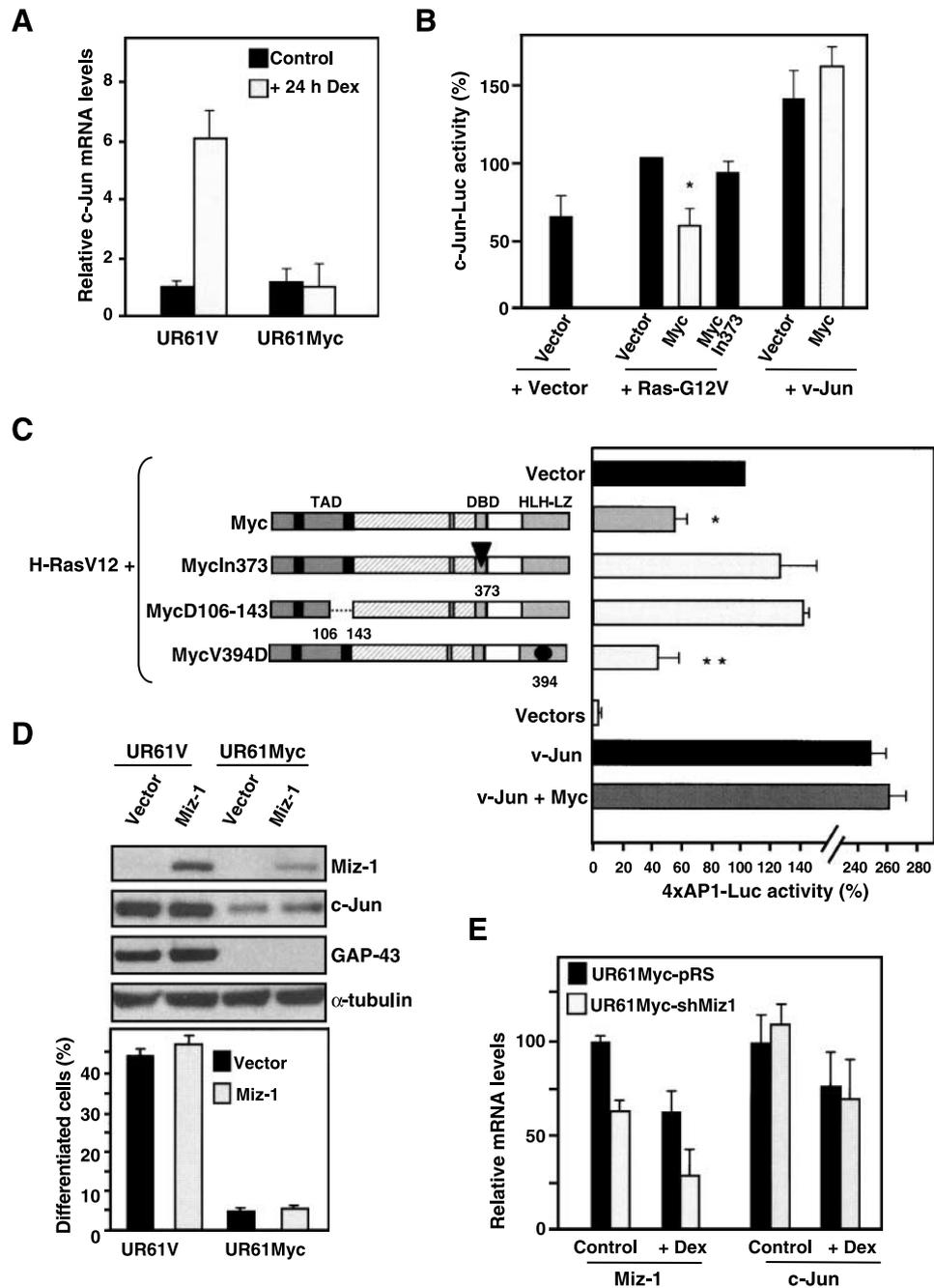


**FIGURE 7.** Myc-dependent inhibition of c-Jun up-regulation by Ras. **A.** Transactivation of a Myc-responsive promoter in UR61MER cells. Cells were transiently transfected with a luciferase reporter containing four E-boxes or four mutated E-boxes (*E-boxMut*). Following 24 h of transfection, 4-hydroxytamoxifen (4HT) was added to 500 nmol/L, and 1 h later dexamethasone was added to 200 nmol/L. The cells were further incubated for 24 h and the luciferase activity was measured in duplicates. Luciferase activity is expressed relative to the activity in cells transfected with the mutated E-box. Columns, mean of two independent transfection experiments; bars, SE. **B.** Myc down-regulated c-Jun in UR61MER cells. 4-Hydroxytamoxifen was added to the indicated concentrations 1 h before dexamethasone (final concentration, 100 nmol/L). Lysates were prepared 12 h later and analyzed by immunoblot with antibodies against c-Jun, Myc (detecting the MycER protein), N-Ras, and  $\alpha$ -tubulin. **C.** UR61 cells were cotransfected with an expression vector for human Myc and GFP in a 10:1 proportion. Twelve hours after transfection, the cells were treated with dexamethasone and 24 h later the expression of c-Jun was assayed by immunofluorescence. The results from 500 GFP-expressing cells are shown in the graph and a representative field is shown at the right. Arrowheads, two cells expressing Myc/GFP and lacking c-Jun. **D.** Ectopic c-Jun rescues the Myc-mediated inhibition of differentiation. Left graph, UR61V and UR61Myc were cotransfected with mixtures of expression vectors for GFP and c-Jun (or its empty vector) in a 1:10 proportion. Twelve hours after transfection, the cells were treated with dexamethasone, and 24 h later the fraction of differentiated cells was determined. The results from 400 GFP-expressing cells are shown in the graph. Columns, mean of three independent transfections; bars, SE. The difference between UR61Myc cells transfected with the empty vector and the c-Jun vector was significant (\*,  $P < 0.01$ ). Right, images of UR61Myc cells transfected with c-Jun and GFP and treated with dexamethasone showing two differentiating cells.

(Fig. 8C). Thus, although Myc antagonizes Ras-mediated induction of c-Jun, it did not impair c-Jun transcriptional activity once it is activated.

Myc represses some promoters through the interaction with the zinc finger protein Miz-1 (reviewed in ref. 50). We explored the possibility of Miz-1 involvement in the UR61 model by using the MycV394D mutant, which is unable to bind Miz-1 (51). The results showed that MycV394D abolished Ras-mediated activation of the AP-1 reporter as efficiently as wild-type Myc (Fig. 8C), suggesting that the repression that Myc exerts on c-Jun promoter does not depend on its interaction with Miz-1. We also addressed the possible involvement of Miz-1 by ectopically expressing Miz-1 in UR61V and UR61Myc and asking whether the differentiation and/or c-Jun expression were affected in UR61Myc cells. Cells were transiently transfected by nucleofection with a Miz-1 expression vector, resulting in  $\geq 40\%$  efficiency of transfection. Morphologic differentiation, expression of the differentiation marker GAP43/Neuromodulin, and expression of c-Jun protein were determined in the transfected cells. The results indicated that Miz-1 overexpres-

sion did not modify the block in c-Jun expression of UR61Myc cells (Fig. 8D, top) and in morphologic differentiation (Fig. 8D, bottom). Finally, we asked whether the repression of Miz-1 blunted the effect of Myc on c-Jun regulation. For this purpose, we infected the UR61Myc cells with a retrovirus expressing a short-hairpin RNA for rat *Miz-1* gene (shMiz-1). This retroviral construct has been previously validated in rat cells (52). As we could not detect endogenous Miz-1 protein, we confirmed the repression of Miz-1 at the mRNA level, comparing the UR61Myc-shMiz1 with the cells transduced with the empty vector (UR61Myc-pRS). The results showed 40% to 50% reduction in Miz-1 expression (Fig. 8E). The results also show a down-regulation of Miz-1 by dexamethasone, a result also observed in parental UR61V and UR61Myc cells (not shown). We next asked whether the reduction in Miz-1 affected the block in Ras-mediated induction of c-Jun. The results showed that c-Jun was not induced by Ras in cells expressing shMiz-1. On the contrary, a modest decrease in c-Jun mRNA levels was detected, which was similar in control cells and shMiz-1 cells (Fig. 8E). Also, no induction of the



**FIGURE 8.** Myc impairs Ras-mediated activation of c-Jun promoter in UR61 cells. **A.** Inhibition of c-Jun mRNA up-regulation in UR61Myc cells after 24 h of treatment with dexamethasone. Levels of c-Jun transcripts in UR61Myc and UR61V cells were determined by quantitative RT-PCR. Columns, mean of four independent experiments (six measurements); bars, SE. **B.** Activity of murine c-Jun promoter. UR61 cells were cotransfected with the c-Jun-Luc reporter and H-Ras-G12V or v-Jun (2  $\mu$ g) and the Myc or its vector (4  $\mu$ g) as indicated. Luciferase activity was measured 24 h after transfection and normalized against the activity detected with RasG12V. Columns, mean of three independent transfections; bars, SE. The difference between values of cells transfected with wild-type Myc and vector were statistically significant ( $P < 0.01$ ). **C.** Activity of the 4xAP1-luciferase reporter. UR61 cells were cotransfected with the reporter and expression vectors for Ras-G12V or v-Jun (5  $\mu$ g) and Myc wild-type or mutants (5  $\mu$ g) as indicated at the right of the graph. Luciferase activity was measured 24 h after transfection and normalized against the RasG12V+vector value. Columns, mean of three independent transfections; bars, SE. Differences in luciferase activity between samples transfected with wild-type Myc, MycV394D, and vector were statistically significant (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ), whereas the difference between MycIn373 and vector was not significant. **D.** Top, cells were nucleofected with an expression vector for Miz-1 or the corresponding empty vector. Following 12 h of transfection, dexamethasone was added to 200 nmol/L, and 24 h later whole-cell extracts were subjected to immunoblotting for Miz-1, c-Jun, GAP-43 (as a control for differentiation), and  $\alpha$ -tubulin (as protein loading control). Bottom, fraction of morphologically differentiated cells from the above described transfections. Columns, mean of two independent transfections; bars, SE. **E.** Expression of Miz-1 and c-Jun mRNA in UR61Myc-shMiz1 and the control cell line UR61Myc-pRS (transduced with the empty vector). Cells were treated for 24 h with 200 nmol/L dexamethasone as indicated and RNA was prepared and analyzed by quantitative RT-PCR using RPS14 mRNA levels as internal control. The expression in untreated UR61Myc-pRS cells was defined as 100% for each gene and the other values were normalized accordingly.

neuronal marker SNAP25 and no morphologic differentiation was detected in cells expressing shMiz-1 treated with dexamethasone (not shown). Taken together, the results suggest that Myc blocks c-Jun induction by a mechanism independent from Miz-1.

As UR61 are deficient in Max, we considered the possibility that Myc impairs c-Jun induction through mechanisms unrelated to DNA binding activity of Myc. We first showed that a Myc mutant defective for DNA binding, MycIn373, failed to block Ras-mediated activation of c-Jun promoter-luciferase reporter (Fig. 8B) and the 4×AP-1-Luc reporter (Fig. 8C). Thus, we assayed the ability of MycIn373 to inhibit differentiation in transient transfection experiments. The results showed that MycIn373 was not able to inhibit Ras-mediated differentiation compared with wild-type Myc (Fig. 9A). These results suggest that Myc must bind to DNA targets to exert its effect as differentiation inhibitor in this model. Thus, we asked whether Myc could bind the promoter of rat c-Jun. Bioinformatic analysis revealed the presence of a noncanonical E-box (CATGCG) in the 5' region of rat c-Jun gene, mapping at -805/-799. ChIP analysis showed that Myc bound this E-box, and some binding was also detected to the amplicon containing the AP-1 sites. This could mean a low-affinity binding or, more likely, a residual binding to the proximity of the E-box-containing amplicon.

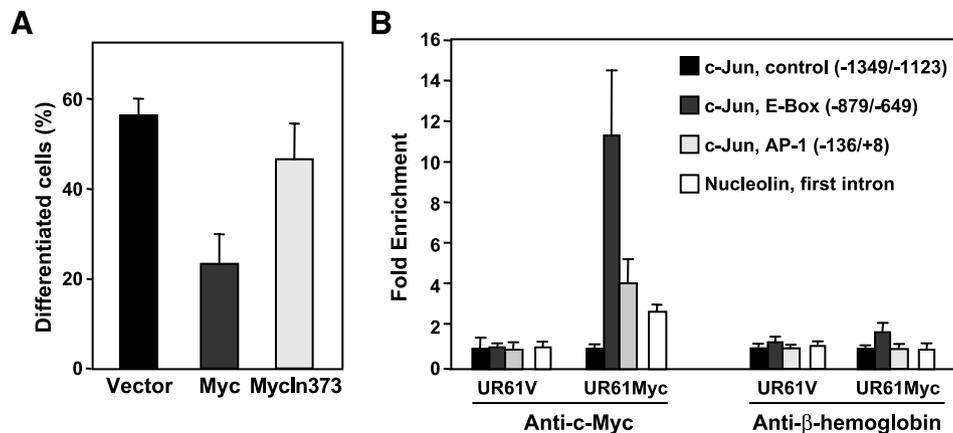
#### *Myc Inhibits Ras-Mediated Transcriptional Activation of c-Jun Promoter in Different Cell Types*

Finally, to address whether Myc repression of the Ras-mediated c-Jun induction is unique to the UR61 model, either because of its species or neuronal origin or its deficiency in Max (ref. 53; and data not shown), we analyzed four human cell lines derived from different tissues: HEK293T (kidney embryo cells), HeLa (cervical carcinoma cells), MEG05, and K562

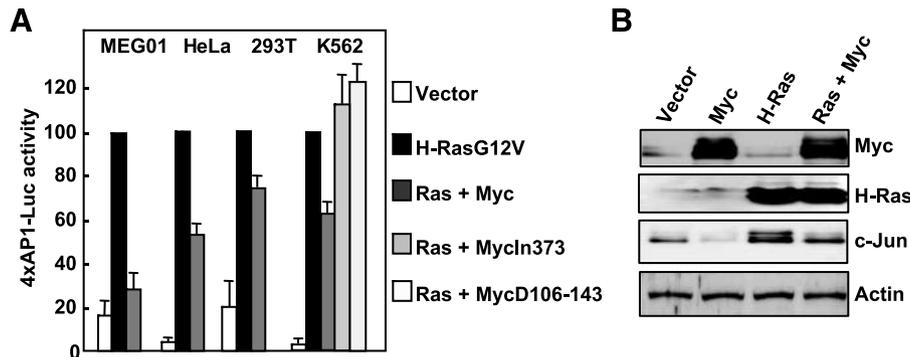
(both myeloid leukemia cells). In these cell lines, Ras did not induce differentiation; however, in K562 and HeLa, Ras provokes proliferation arrest (14). Expression vectors for oncogenic H-Ras and Myc were cotransfected in these cell lines along with the 4×AP-1-Luc reporter. The results showed that Ras activated this promoter and Myc antagonized this effect in all the cell types (Fig. 10A), confirming our previous results in UR61 cells. In K562 cells, similar results were obtained using a luciferase reporter for the proximal promoter of collagenase II, a typical AP-1-responsive promoter (not shown). We also tested the MycIn373 and MycD106-143 mutants in the K562 and we found that both were incapable of inhibiting AP-1 reporter (Fig. 10A), thus reproducing the results observed in UR61. Finally, we asked whether this effect correlated with down-regulation of c-Jun protein in HEK293T cells (which shows higher transfection efficiency). The immunoblot results revealed that Myc antagonized the up-regulation of c-Jun brought about by oncogenic Ras (Fig. 10B), thus confirming that impairment of c-Jun up-regulation by Myc was not unique to the UR61 model.

## Discussion

Whereas the cooperation between Myc and Ras in transformation has been explored in numerous studies, their functional interaction during cellular differentiation has been poorly investigated. This is surprising considering that the induction of differentiation is one of the most prominent effects of Ras (19) and that differentiation inhibition is one of the main and first biological effects described for Myc (reviewed in refs. 5, 8). In the present study, we show that Myc abrogates Ras-mediated neuronal differentiation of pheochromocytoma UR61 cells without reversing the proliferation arrest, and that the differentiation inhibition is mediated, at least in part, by blocking c-Jun up-regulation.



**FIGURE 9. A.** DNA binding domain of Myc is required for differentiation inhibition. UR61 cells were transiently transfected with expression vectors for GFP and wild-type Myc, MycIn373, and the empty vector (pMLV). Twelve hours after transfection, dexamethasone was added and the fraction of differentiated cells was determined after 48 h. Columns, means from two independent experiments; bars, SD. **B.** ChIP of Myc on the promoter of rat c-Jun. Cells were treated with 200 nmol/L dexamethasone for 24 h to induce Ras and the chromatin was immunoprecipitated with anti-c-Myc and (as a specificity control) anti-β-hemoglobin antibodies. The immunoprecipitated DNA was analyzed by quantitative PCR, which was carried out with primers encompassing the three amplicons of the rat c-Jun promoter and, as a control for Myc binding, the first intron of rat nucleolin. Data are normalized to the corresponding inputs of chromatin before immunoprecipitation and are expressed as relative to the value of the c-Jun gene upstream amplicon. Columns, mean of two independent ChIP experiments; bars, SE.



**FIGURE 10.** Myc impairs Ras-mediated activation of c-Jun in different cell lines. **A.** Luciferase assays with 4×AP-1-luciferase reporter in the indicated cell lines. Cells were electroporated with the reporter (2 μg), Ras expression vector (pCEFL-H-RasG12V, 2 μg), and Myc (pMLV-Myc wild-type, pMLV-MycIn373, and pMLV-MycD106-143, 5 μg). Luciferase activity was measured 36 h after transfection and normalized against the H-RasG12V value. Columns, mean of three independent transfections; bars, SE. **B.** Myc-mediated inhibition of c-Jun up-regulation in HEK293T cells. Cells were grown for 5 h in medium with 1% FCS and transfected with 1.5 μg of H-RasG12V expression vector and 4.5 μg of Myc expression vector (or 6 μg of empty vector) and 36 h later protein extracts were prepared and analyzed by immunoblot for the indicated proteins.

In the classic experiments on Ras and Myc cooperation in transformation of primary fibroblasts (20), Myc seems to antagonize Ras-mediated growth arrest and senescence, switching Ras into a growth-promoting protein (12). In contrast, in the UR61 model, Myc inhibits Ras-induced differentiation without affecting the growth inhibition associated to differentiation. Apoptosis ensues once the cells have achieved differentiation, but Myc does not enhance this apoptosis. Thus, the observed growth arrest is not due to apoptosis concomitant with increased-Myc induced proliferation. Inhibition of cell differentiation by Myc has been explained in some models as the result of the stimulation of cellular proliferation, thus antagonizing the cell cycle arrest associated to terminal differentiation (5, 6). However, our results in the UR61 system argue that unabated proliferation is not the only mechanism by which Myc can inhibit differentiation.

One of the first identified biochemical effects of Ras was the up-regulation of c-Jun, not only in fibroblasts (54, 55) but also in PC12 cells (31). However, the effect of Myc on the Ras-Jun interaction had not been explored thus far. This is probably because Ras cooperates with either c-Jun or Myc to induce transformation of fibroblasts, and therefore a major effect of Myc on the Ras-Jun pathway during transformation was not to be expected. However, in those models where the Ras-Jun signal results in cell differentiation, Myc could block the Ras-Jun effect. In UR61 cells, we found that Ras up-regulates c-Jun and that constitutively active c-Jun (i.e., v-Jun) is sufficient to induce differentiation, as previously described in parental PC12 cells (33, 56). Interestingly, Myc impairs this Ras-induced up-regulation of c-Jun in UR61 cells, and coexpression of Myc with c-Jun decreases the antagonistic effect of Myc on Ras-induced differentiation. Although alternative mechanisms could also contribute to Myc-mediated inhibition of differentiation (e.g., regulation of genes other than *c-Jun*), our results indicate that c-Jun suppression mediated by Myc plays an essential role for the inhibition of Ras-induced differentiation.

The data suggest that Myc antagonizes c-Jun up-regulation at the transcriptional level, as shown by the drop of c-Jun mRNA and the diminished c-Jun promoter activity. However, Myc could not reverse the transactivation activity of the

constitutively activated v-Jun and did not impair Jun NH<sub>2</sub>-terminal kinase activity as assessed by Jun NH<sub>2</sub>-terminal kinase phosphorylation.<sup>5</sup> These findings are consistent with a model in which Myc blunts Ras-mediated transcriptional activation of c-Jun by interfering with its positive autoregulation, but does not impair the transactivation activity of c-Jun once it is expressed and active.

The mechanism underlying this unexpected activity of Myc is unclear, but Myc impairment of Ras-mediated activation of c-Jun is not exclusive of PC12-derived cells, as it is also observed in other cell lines derived from different tissues. The mechanism seems to be independent of Miz-1, as (a) it is reproduced by a Myc mutant (MycV394D) unable to bind Miz-1, and (b) enforced Miz-1 expression and partial Miz-1 silencing do not modify the Myc-mediated inhibition of differentiation and the block in c-Jun up-regulation. Our observations are consistent with a recent report in human lymphoid cells that identifies *c-Jun* as a gene down-regulated by Myc (57). Interestingly, UR61 cells are deficient for Max,<sup>5</sup> like their parental cell line, PC12 (53). Max independence for Myc-mediated apoptosis of PC12 (upon serum deprivation) has already been described (42). We now show that Myc blocks c-Jun induction and neuronal differentiation in a Max-independent manner. However, Max deficiency is not required for inhibition of AP-1-dependent transcription by Myc, because such inhibition is not unique to UR61 but also occurs in Max-expressing cells (e.g., HeLa, HEK293T, and K562).

Interestingly, a DNA-binding defective Myc mutant is unable to block differentiation and to inhibit c-Jun induction, suggesting that DNA binding is required for Myc activity in this system. Also, Myc mutant lacking the conserved Myc box II region (amino acids 106-143, required for transactivation and transformation) failed to inhibit c-Jun promoter activity. ChIP assays show that Myc binds an E-box in the rat c-Jun promoter in UR61Myc cells, although further work is required to elucidate whether Myc binding is directly involved in the

<sup>5</sup> Unpublished results.

block of the promoter activation and which are the dimerization partners of Myc, if any, when bound to the E-box. Thus, the mechanism for the Myc effect may be indirect, that is, mediated through the up-regulation or repression of other Myc target genes. Further work in our laboratories is aimed to dissect the mechanism(s) by which Myc interferes with c-Jun autoregulation in UR61 without Max intervention.

Regardless of the molecular mechanisms involved, our results support the hypothesis that inhibition of differentiation by Myc occurs independently of its effects on proliferation and this may represent an important mechanism by which Myc exerts its oncogenic function. It is noteworthy that recent observations in conditional Myc-expressing mice indicate that redifferentiation upon discontinued expression of Myc is the mechanism responsible for tumor regression in some models (58).

In summary, our findings identified a novel activity of Myc, which is its ability to prevent cell differentiation provoked by Ras by inhibiting the expression of c-Jun. In turn, these observations suggest that the proliferative and the differentiation inhibitory activities of Myc can both contribute to promote the unregulated growth of Myc-induced neoplasias.

## Materials and Methods

### Cell Culture and Retroviral Infections

U7 cell line is a derivative of PC12 characterized by faster growth and a diminished differentiation capacity (16, 59). The UR61 cell line was generated by the stable transfection of U7 cells with an *N-ras* oncogene (*N-ras**Q61K*) under the control of the dexamethasone-inducible mouse mammary tumor virus promoter (60). Ecotropic retroviruses were generated in Phoenix-E cells. To generate U7 and UR61 cells with constitutive expression of Myc, cells were infected with pBSH2-h-Myc and pBSH2 retrovirus, in the presence of polybrene (4 µg/mL). Retroviral pBSH2 and pBSH2-h-c-Myc vectors have been described (61). Infected cells were selected with 100 µg/mL hygromycin-B (Roche Applied Science). To generate U7 and UR61 cells expressing the Myc-ER fusion protein, cells were infected with the pBabePuro and pBabePuro-MycER retroviruses (62) and selected with puromycin (1 µg/mL). To generate UR61Myc-pRS and UR61Myc-shMiz1, UR61Myc cells were infected with pRetroSuper and pRetroSuper-shMiz1 retroviruses (52) and selected with puromycin (1 µg/mL).

U7 and UR61 and their derivatives were grown in DMEM supplemented with 8% FCS, gentamicin (80 µg/mL), and ciprofloxacin (2 µg/mL). The media for UR61 and its derivatives were also supplemented with G418 (250 µg/mL). HeLa and HEK293T cells were grown in DMEM with 8% FCS; K562 and MEG01 (both derived from human chronic myeloid leukemia) were grown in RPMI 1640 with 8% FCS. Unless otherwise stated, Ras was induced with 200 nmol/L dexamethasone (Sigma Chemical Co.) and MycER was activated with 200 or 500 nmol/L 4-hydroxytamoxifen (Sigma Chemical).

### Cell Growth, Apoptosis, and Differentiation

Cell proliferation was assessed by counting trypsinized cell suspensions in hemocytometer and by crystal violet stain-

ing. For this, cells were washed with PBS, stained with 1% crystal violet in methanol for 15 min, dry, destained with 10% acetic acid for 2 h, and the color was measured in a colorimeter at  $A_{590\text{ nm}}$ . For apoptosis determination by Annexin V binding, cells were trypsinized, washed with PBS, and the Annexin V binding was determined by flow cytometry and Annexin V-phycoerythrin (BD PharMingen) following the manufacturer's instructions. For determination of subdiploid cells, the cells were stained with propidium iodide and analyzed as described below. Cell morphology was assessed by phase-contrast microscopy. Cells forming at least one neurite as long as the diameter of the cell soma was scored as differentiated. At least 300 cells were scored in each differentiation experiment.

### Cell Cycle Analysis

Cells ( $1 \times 10^6$ ) were collected and suspended in PBS, then fixed in ethanol 90% 4°C for 30 min. Cells were resuspended in PBS-sodium citrate buffer containing 10 µg/mL bovine serum albumin, 200 µg/mL RNase, 50 µg/mL propidium iodide (Sigma Chemical); incubated at 37°C in the darkness for 30 min; and analyzed by flow cytometry in an Excalibur cytometer (Becton Dickinson) and with the CellQuest software.

### Expression Vectors and Transient Transfections

The vectors used in transient transfections were as follows: pCEFL-H-RasG12V (14), pRS/myc (expressing short-hairpin RNA for human Myc; ref. 39), pMLV-Myc, pMLV-MycIn373 (49, 63), pCEFL-Myc (64), pCEFL-MycV394D (65), pRSV-v-Jun and pRSV-c-Jun (66), pcDNA3-Miz1 (provided by Martin Eilers, Marburg University, Marburg, Germany), pEGFP-N2 (expressing GFP; Clontech Laboratories), and the corresponding empty vectors (pCEFL, pMLV, pcDNA3, and pRetroSuper). For transient transfections for immunofluorescence or protein expression analysis, plasmids were transfected into UR61 and HEK293T cells with FuGene-6 transfection reagent (Roche Applied Science). Nucleofection was carried out in an Amaxa nucleofector using the kit V (Amaxa AG). Two million cells were transfected with 2.5 µg of the Miz-1 expression vector or the corresponding empty vector and 0.5 µg of the GFP-encoding vector pmaxGFP (Amaxa). At least 40% of the cells expressed GFP 24 h after each nucleofection.

### Immunoblots

Cells were lysed in cold NP40 buffer and 50 µg of protein per lane were electrophoresed on SDS-10% polyacrylamide gels and transferred onto nitrocellulose membranes by standard procedures. The primary antibodies used were anti-c-Jun (Santa Cruz Biotechnology), anti-c-Myc (Santa Cruz Biotechnology), anti-MEK1/2 (Cell Signalling), anti-N-Ras (Santa Cruz Biotechnology), anti-ATF2 (Cell Signalling), anti-phospho-Thr<sup>69/71</sup>-ATF2 (Cell Signalling), anti-ERK2 (Santa Cruz Biotechnology), anti-GAP43 (BD Transduction Laboratories), anti-Miz-1 (Santa Cruz Biotechnology), anti-actin (Santa Cruz Biotechnology), and anti- $\alpha$ -tubulin (provided by Nicholas Cowan, New York University, New York, NY). Immunoblots were revealed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

### Immunofluorescence

Cells (grown at 50% of confluence on coverslips) were transfected with a mixture of the GFP vector pEGFP-N2 (Clontech) and the indicated expression vector (Myc, shMyc, v-Jun, c-Jun) in a 1:10 proportion and 2  $\mu$ g of total DNA. After transfection, cells were incubated for 12 h, treated with 200 nmol/L dexamethasone for 24 h, and then fixed in 3.7% paraformaldehyde-PBS. Immunofluorescence was done as described (13) using the anti-c-Jun antibody and Texas red-conjugated goat anti-rabbit IgG (The Jackson Laboratory). The statistical significance was determined using Student's *t* test.

### Electrophoretic Mobility Shift Assays

Nuclear extracts from UR61 cells were prepared as described (67). Gel retardation assays were done at room temperature in 20  $\mu$ L reactions containing 15  $\mu$ g of protein of nuclear extract and annealed oligonucleotides previously labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using Klenow fragment. DNA-protein and free complexes were resolved in 4% nondenaturing polyacrylamide gels. The oligonucleotides were 5'-GGA-AGGCCTTGGGGTGACATCATGGGCTATTTTAG-3' and its complementary 5'-CTTCCGGAACCCCACTGTAGTAC-CCGATAAAAATGGG-3'. The AP-1 site at -133 of rat promoter (Genbank accession no. NM021835) is underlined (46, 47). As specificity controls, we incubated extracts with the labeled probe and a 100-fold excess of cold probe and of a mutated probe with the underlined nucleotides (AP-1 binding site) mutated to ATCAGCAT.

### Chromatin Immunoprecipitation

Cells ( $5 \times 10^7$ ) were used for each ChIP assay. Cells were fixed in 1% formaldehyde, lysed, and sonicated essentially as described (68). The ChIP was done by using Dynabeads-Protein G (DynaL Biotech) coupled to anti-c-Myc, anti-c-Jun, and (as specificity control) anti- $\beta$ -hemoglobin antibodies (all rabbit polyclonal antibodies were from Santa Cruz Biotechnology). Dynabeads were incubated with lysates for 12 h at 4°C and washed several times with 50 mmol/L HEPES (pH 7.6), 1 mmol/L EDTA, 0.7% sodium deoxycholate, 1% NP40, and 0.5 mol/L LiCl. Chromatin was eluted with 500  $\mu$ L elution buffer [50 mmol/L Tris (pH 8), 10 mmol/L EDTA, 1% SDS], decrosslinked (8h at 65°C), and purified through Qiaquick columns (Qiagen). Real-time PCRs of the eluted DNA were done at 56°C as annealing temperature in a Bio-Rad iCycler apparatus. The PCR primers (in 5'-3' sense) are listed below. The sites of the rat *c-Jun* gene where the corresponding amplicons map (with respect to the transcription start site as in the NM021835 sequence) are indicated. For the distal upstream sequence (negative control), TTGGAAGACTGAGGGCAAAC and TGAAAGCTCGGTTGGTAAGG, which amplify between -2725 and -2579; for the proximal upstream sequence (negative control), CAAGCTCGCCACCTC-TTTAG and CTGGGAAAACAAGCCTTGAG, which amplify between -879 and -649; for E-box (-879/-799 from rat *c-Jun* transcription start site), ATTCCGAGCACAGCAAATCT and AATGATCTGGGCGATTGAAG, amplifying between -879 and -648; for the AP-1 sites (*jun1* and *jun2*), CATTACCTCATCCCGTGAGC and AGGCAGTCTCTGCA-

CACTCA, amplifying between -136 and +8; for the *c-jun* exon (negative control), CGCACGCTCCTAAACAAACT and CGTTTCCATCTTTGCAGTCA, amplifying between +725 and +3845; for the downstream sequence (negative control), GAGCCTGTGGTGGTAAATCC and GTGCCTCCACTCC-CAGTAAC, amplifying between +3706 and +3845; for nucleolin first intron (positive control for Myc binding; ref. 69), CGCGTCCGAGGCA GTG and TCCATCTACCGT-CACGGTCAG.

### RNA Analysis

Total RNA was isolated using Tri Reagent (Ambion, Inc.). For Northern blot analysis, total RNA (15  $\mu$ g per lane) was separated by electrophoresis through agarose-formaldehyde gel and transferred to nitrocellulose. Probe labeling with [ $\alpha$ -<sup>32</sup>P]dCTP and filter hybridization were carried out according to standard procedures. Probe for mouse *N-Ras* and *c-Jun* have been described (70, 71). The amount and integrity of the rRNAs were assessed by staining of the filter with ethidium bromide. cDNAs were obtained by reverse transcription using Script™ Kit (Bio-Rad Laboratories). For quantitative RT-PCR, cDNAs were amplified with SYBR-fluorescein PCR mix (ABgene) using an iCycler Bio-Rad apparatus. The primers for the indicated rat genes, written in the 5' to 3' sense, were as follows: for *c-Jun*, CGCACGCTCCTAAACAAACT and CGTTTCCATCTTTGCAGTCA; for *GAP43*, GTCAAACCGGAG-GATAAGG and CTTCTCCACACCA-TCAGCAA; for *SNAP25*, GAATCGCCAGATTGACAGG and CCATGAGA-GAAGCATGAAGGA; for *Miz-1*, ACCATCTCCTCCCA-TTCCTC and CCGCTGAATACTGCTTTGAAC-3'; for *LDHB*, TCTGGATTCTGCTCGGTTTC and GACTCCTGC-CACATTCACC; for *PDK2*, AGGAAGTCAATGCCAC-CAAC and TTGATGGGAGGGAGAGTGAG; for *ribosomal protein 14 (RPS14)*, 5'-CAAGGGGAAGGAA-AAGAAGG-3' and 5'-GAGGACTCATCTCG-GTCAGc-3'. The expression of the different genes was normalized against levels of *RPS14* mRNA.

### Luciferase Assays

The luciferase reporters used were as follows: 4 $\times$ AP-1-Luc, carrying four concatamers of a consensus AP-1-binding site (72); pJun-luc, carrying the mouse proximal promoter and part of the first exon (73); pGL3-E-box, carrying four E-box elements in the pGL3 vector; and pGL3-E-BoxMut, carrying four mutated E-boxes (74). For the assays with 4 $\times$ AP-1-Luc and pJun-Luc, cells ( $2 \times 10^6$ ) were electroporated at 260 V and 1 mFa (Bio-Rad Gene pulser). Luciferase assays were done using 2  $\mu$ g of the firefly luciferase reporter and 1  $\mu$ g of the plasmid for *Renilla* luciferase (pRL-Null, Promega), and, when indicated, 5  $\mu$ g of the expression vectors. Thirty-six hours after electroporation, the cells were lysed and luciferase activity was measured with the Dual Luciferase Assay kit (Promega). For the assays with pGL3-E-box and pGL3-E-boxMut, the cells were incubated for 24 h after transfection and further incubated for other 24 h in the presence or absence of 200 nmol/L dexamethasone and/or 500 nmol/L 4-hydroxytamoxifen. The cells were lysed and luciferase activity was measured as above.

The activity of firefly luciferase was normalized to that of *Renilla* luciferase used as internal control. Statistical significance of the differences between luciferase values was based on *P* values calculated using the Student's *t* test.

#### Ras Activation Assay

Ras-GTP was determined by the glutathione *S*-transferase-Ras-binding domain of Raf (amino acids 1-149) pull-down assay as described previously (75). Total and pulled-down proteins were analyzed by immunoblot antibodies.

#### Acknowledgments

We thank Angel Pellicer for U7 and UR61 cell lines; Bruno Amati (European Institute of Oncology, Milan, Italy), Thorsten Berg, (Department of Molecular Biology, Max Planck Institute, Martinsried, Germany), Carme Caelles (Institute for Biomedical Research, Barcelona, Spain), Angel Corbí (Centro de Investigaciones Biológicas, Madrid, Spain), Dirk Eick (Institute of Clinical Molecular Biological and Tumor Genetics, Munich, Germany), Jesus Gil (MRC Clinical Sciences, Imperial College, London, United Kingdom), Carla Grandori (Fred Hutchinson Cancer Research Center, Seattle, WA), Silvio Gutkind (National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD), Martin Eilers (Institute of Molecular Biology and Tumor Research, University of Marburg, Marburg, Germany), Trevor Littlewood (Nuffield Department of Clinical Laboratory Sciences, University of Oxford, Oxford, United Kingdom), and Steven McMahon (The Wistar Institute, Philadelphia, PA) for luciferase reporters, retroviral constructs, and expression vectors; Nicholas Cowan for anti-tubulin antibody; Miguel Lafarga and M. Teresa Berciano (Dpto. de Biología Celular, Universidad de Cantabria, Santander, Spain) for help with confocal microscopy; Rosa Blanco, Pilar Frade, and Silvia Gonzalez for technical assistance; and M. Dolores Delgado and Carme Caelles for helpful discussions.

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