



# Myc suppresses induction of the growth arrest genes *gadd34*, *gadd45*, and *gadd153* by DNA-damaging agents

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The growth arrest and DNA damage inducible (*gadd*) genes are induced by various genotoxic and non-genotoxic stresses such as serum starvation, ultraviolet irradiation and treatment with alkylating agents. Their coordinate induction is a growth arrest signal which may play an important role in the response of cells to DNA damage. Conversely, *c-myc* is a strong proliferative signal, and overexpression of Myc is frequently observed in cancer cells. We have found that ectopic expression of *v-myc* in RAT-1 cells results in an attenuated induction of the three major *gadd* transcripts by methyl methanesulfonate (MMS), and almost completely blocks the response to ultraviolet (UV) radiation. Myc acts in part by reducing the stress-responsiveness of the *gadd45* promoter, as a *c-myc* expression vector strongly suppressed activation of *gadd45*-reporter constructs. This activity of Myc localizes to a recently described GC-rich binding site within the *gadd45* promoter. These results indicate that a coordinate down-regulation of the *gadd* gene response is one mechanism by which Myc can circumvent growth arrest and contribute to the neoplastic phenotype.

**Keywords:** *gadd45*; *myc*; transcriptional repression

## Introduction

The five *gadd* genes were originally identified as transcripts induced in hamster cells by UV radiation (Fornace *et al.*, 1988; Fornace, 1992). Three of these original genes, *gadd34*, *gadd45* and *gadd153*, have been found to be upregulated in response to a variety of stresses, including treatment with DNA-damaging agents and starvation, in many different mammalian cell lines (Fornace *et al.*, 1989), and ectopic over-expression of the *gadd* genes has been shown to result in growth arrest (Zhan *et al.*, 1994).

Induction of *gadd45* in response to ionizing radiation (IR) is dependent on wild type p53. While the promoter is unresponsive to IR, a p53-binding element has been identified in the third intron of *gadd45* (Hollander *et al.*, 1993; Kastan *et al.*, 1992). This site is a likely mediator of the p53-dependent element of *gadd45* regulation. In contrast, other DNA-damaging stresses, including UV radiation and treatment with the alkylating agent MMS, induce

transcription of the *gadd* genes regardless of p53 status in all mammalian cells examined (Kastan *et al.*, 1992). Despite the inability of p53 to transactivate a *gadd45* promoter-reporter construct (Zhan *et al.*, 1993), p53 still appears to play some role in the stress-responsiveness of the promoter (Zhan *et al.*, 1998), consistent with the decreased response of *gadd45* and *gadd153* to UV radiation and MMS observed in p53-null mouse embryo fibroblasts and in human cancer cell lines with p53 function abrogated by dominant-negative vectors (Zhan *et al.*, 1996).

Over-expression and mutational activation of the proto-oncogene *c-Myc* are common changes found in many cancer cells, and can allow escape from growth arrest resulting in rapid proliferation. Although increases in p53 levels normally result in a decrease in *myc* transcription (Moberg *et al.*, 1992), *myc* activation leads to both increased transcription and accumulation of p53, but not to the usual cell cycle arrest (Hermeking and Eick, 1994). This may suggest that Myc can antagonize normal cellular growth arrest mechanisms by down-regulation of genes involved in cell-cycle control. Indeed, *CIP1/WAF1* (Hermeking *et al.*, 1995), *gas1* (Lee *et al.*, 1997), cyclin D1 (Philipp *et al.*, 1994), and *c-myc* itself (Facchini *et al.*, 1997) have been identified as targets of Myc down-regulation.

Using an inducible *mycER* system, we previously demonstrated that induction of *myc* resulted in a rapid decrease in basal levels of *gadd45* mRNA independent of cell cycle (Marhin *et al.*, 1997). As treatment with many DNA damaging agents normally results in coordinate induction of the *gadd* genes and cell cycle arrest, it was of interest to determine whether ectopic expression of *myc* would alter the damage responsiveness of these growth-arrest genes. We now show that over-expression of *myc* in RAT-1 cells sharply reduces the induction of the *gadd* genes by both MMS and UV radiation, and that the effect on UV radiation-induction can be mapped to a GC-rich motif within the *gadd45* promoter previously associated with regulation by WT1/Egr-1 binding.

## Results

### *Constitutive overexpression of myc decreases the gadd gene response to MMS and UV radiation*

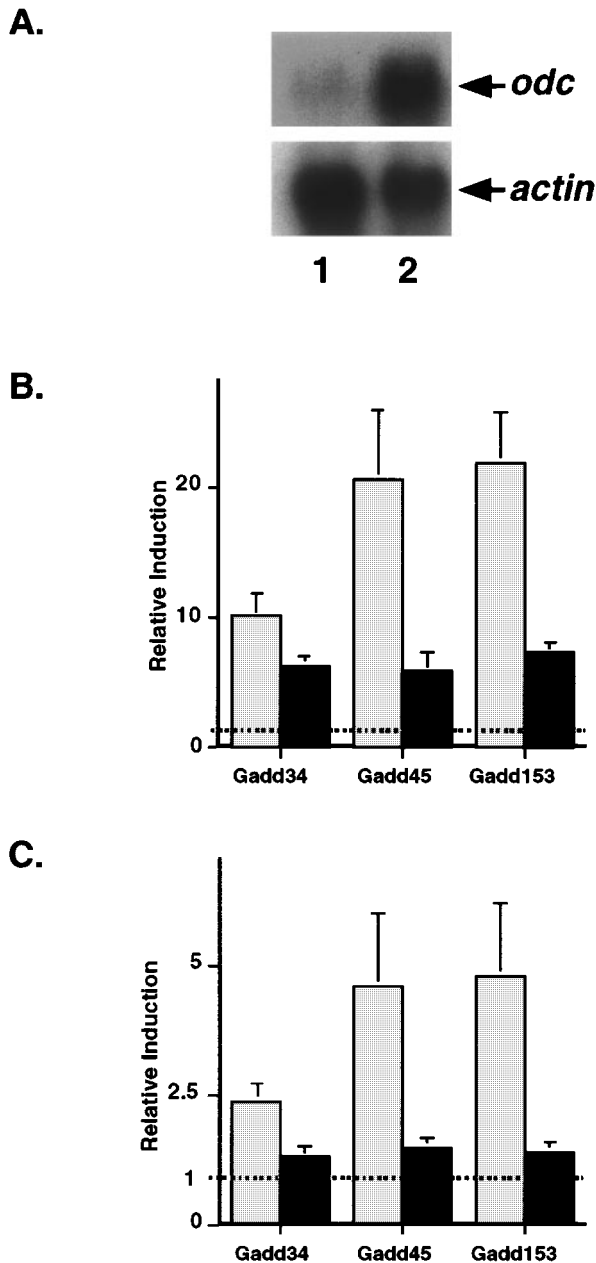
Rat-1 Neo and Rat-1 Myc cells were derived from the non-transformed, non-tumorigenic Rat-1 fibroblast line following infection with either a control retrovirus containing the neomycin resistance gene alone, or a retrovirus also encoding *v-myc*. Over-expression of functional *v-myc* in Rat-1 Myc cells was demonstrated previously (Marhin *et al.*, 1997) and

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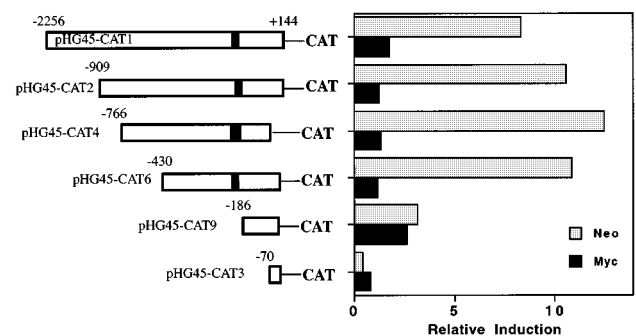
verified in these experiments by quantitative hybridization of *ornithine decarboxylase* (*odc*) mRNA in exponentially growing untreated cells. As elevated *odc* levels have been shown to be a marker for Myc up-regulation, expression of this gene was compared in Rat-1 Myc and Rat-1 Neo and found to be

markedly elevated in Rat-1 Myc cells. (Figure 1a). The basal levels of the *gadd* gene messages were also found to be reduced about twofold in Rat-1 Myc compared to Rat-1 Neo, consistent with our earlier findings (Marhin *et al.*, 1997). Through the course of the induction experiments discussed below, the average basal level of *gadd34* in Rat-1 Myc was  $0.51 \pm 0.06$  the level in Rat-1 Neo, *gadd45* was  $0.50 \pm 0.07$ , and *gadd153* was  $0.49 \pm 0.11$ .

Induction by genotoxic stress of the major *gadd* gene transcripts, *gadd34*, *gadd45* and *gadd153*, was measured by quantitative hybridization analysis. Treatment with 100  $\mu\text{g/ml}$  methyl methanesulfonate (MMS) resulted in a strong induction of all three *gadd* genes in Rat1 Neo cells by 4 h. In Rat1 Myc, however, the relative induction over basal levels of all three *gadd* transcripts was markedly attenuated (Figure 1b). Although all responses were of lower magnitude, the same pattern of induction was also seen with a lower dose of 50  $\mu\text{g/ml}$  MMS. Rat1 Neo cells treated with 14  $\text{J/m}^2$  UV radiation also induced *gadd34*, *gadd45* and *gadd153* by 4 h post-irradiation. In Rat1 Myc, however, the same dose of UV radiation did not result in a significant induction of any of the *gadd* genes (Figure 1c). A higher UV radiation dose of 20  $\text{J/m}^2$  also failed to induce significant levels of any of the *gadd* transcripts in Rat1 Myc cells (data not shown). The fact that both the induction and relative Myc suppression of *gadd34* was lower than that of the other two *gadd* genes for both UV radiation and MMS treatment may be an indication that *gadd34* plays a less important role in growth suppression in RAT1 cells, and is therefore a less prominent target for Myc suppression. Nonetheless, *gadd34* was reproducibly induced above the basal level by UV radiation in all experiments with RAT1 Neo and in none of the experiments with RAT1 Myc, consistent with the patterns of induction of the other two *gadd* genes.



**Figure 1** (a) Northern blot showing levels of *ornithine decarboxylase* message in exponentially growing untreated RAT-1 Neo (lane 1) compared with RAT-1 Myc (lane 2). (b) Relative induction over the basal mRNA level of the *gadd* genes by 100  $\mu\text{g/ml}$  MMS in RAT-1 Neo (light shaded bars) and RAT-1 Myc (solid bars). The induction levels shown are the average of three or more independent experiments and are relative to basal levels defined as one (indicated by the grey dotted line). Error bars are standard error of the mean. (c) Relative induction over the basal mRNA level of the *gadd* genes by 14  $\text{J/m}^2$  UV radiation in RAT-1 Neo (light shaded bars) and RAT-1 Myc (solid bars). The induction levels shown are the average of three or more independent experiments and are relative to basal levels defined as one (indicated by the grey dotted line). Error bars are standard error of the mean.

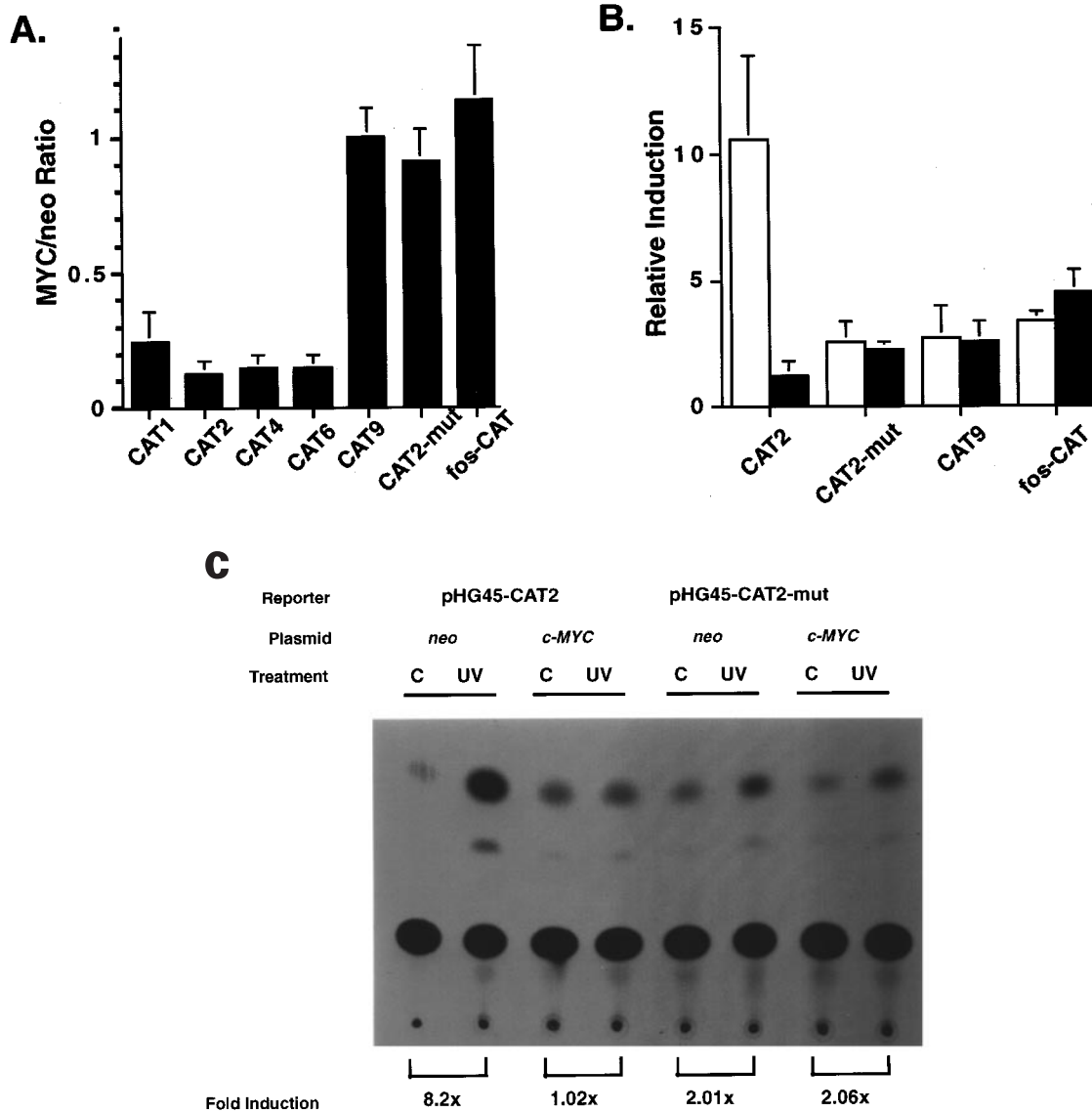


**Figure 2** Relative induction by UV radiation (14  $\text{J/m}^2$ ) of CAT reporter constructs driven by various 5' deletions of the *GADD45* promoter transfected into MCF7 cells. The numbers above each construct indicate the base position relative to the transcriptional start site. The black boxes in constructs one, two, four and six represent the position of the WT1/Egr-1 binding site. The bars on the right side of the figure illustrate the relative UV radiation-induction resulting from a typical experiment following co-transfection with either a c-MYC expression vector (dark bars) or a *neo* control vector (light bars). In repeated independent experiments, UV radiation-induction of pHG45-CAT1, pHG45-CAT2, pHG45-CAT4, and pHG45-CAT6 was significantly decreased by co-transfection of *MYC* ( $P < 0.001$ ; paired *t*-test) while *MYC* co-transfection had no significant effect on the induction of pHG45-CAT9 ( $P > 0.1$ ; paired *t*-test).

*Myc dampens the UV radiation-responsiveness of the GADD45 promoter in human cells*

*GADD45* promoter deletion reporter constructs were transiently co-transfected with either a *c-MYC* expression vector or a *neo* expression vector into the human breast carcinoma cell line MCF-7. Twenty-four hours after transfection, the cells were treated with UV radiation and incubated for another 24 h. Cellular lysates were then assayed for CAT activity. Co-transfection with *c-MYC* did not produce a consistent decrease in the basal levels of any of the *GADD45* CAT reporter constructs. However, the UV radiation-induced activity of the *GADD45*-promoter construct pHG45-CAT2 was effectively blocked by co-transfection with the *c-MYC* expression vector. This indicates that Myc can mediate repression of the *GADD* gene

stress response in human cells as well as rodent cells, and that Myc down-regulates *GADD45* transcription through action on the promoter. The activity of Myc on *GADD45* promoter deletion constructs clearly localizes the Myc repression effect to the proximal promoter (Figure 2). The ratio of induction in co-transfections with *MYC* to those with *neo* is consistently in the range of 0.1 to 0.2 for constructs containing greater than 186 bp of upstream promoter sequence, while for constructs containing less than this sequence or for the control *c-fos*-CAT construct, this ratio is essentially 1 (Figure 3a). The apparent loss of the *MYC* effect on pHG45-CAT9 coincides with the reported loss of the WT1/p53 effect on the *GADD45* promoter in constructs containing less than 186 bp of upstream sequence (Zhan *et al.*, 1998). This effect has recently been localized to a GC-rich motif in this



**Figure 3** (a) Comparison of the ratios of UV radiation-induction of CAT reporter constructs when co-transfected with *myc* to the same reporters co-transfected with a *neo* control. Each bar is the average of ratios from three or more independent experiments. Error bars are standard errors of the mean. (b) Induction of *gadd45* promoter-reporter constructs by UV radiation ( $14 \text{ J/m}^2$ ) in co-transfection with *neo* control vector (light bars) or *MYC* expression vector (dark bars). Each bar represents the mean of three or more independent experiments. Error bars are standard errors of the mean. (c) Example of CAT assay comparing the effect of *myc* co-transfection on reporter construct with the WT1/Egr-1 site intact (pHG45-CAT2) to the same reporter with a mutated WT1/Egr-1 site (pHG45-CAT2-mut)

CTTCCCTCTCGGCACCGCCCCCGCCCCCGCCCCCTCGGCTCGCCTCCCG	- 171	<i>GADD45</i>
-gc-g-gc-c-tc-c-----c-cc-c-cg-ct--c	- 88	<i>PDGF-A</i> (reverse compl)
gtc--gaggc-----g-cgg-cggg--c	- 362	<i>JUN-D</i>
gccg-ggc-----cac--g--t-cgg-c	- 102	<i>MDR3</i> (reverse compl)
		EGR1 site
		EGR1 site
		EGR1 site

CGCCCCCGC  
CGCCCCCGC  
CGCCCCCGC

**Figure 4** Sequence comparison of the *GADD45* WT1/EGR1 site with selected other human promoters. Sequence is shown with position of the last nucleotide on the right relative to the reported transcription start site. The core 20 bp motif found in all four sequences is underlined on the first line; nucleotides identical to the *GADD45* sequence have been replaced with dashes for the other three promoters. Non-identical nucleotides are shown in lower case. In the case of *PDGF-A* and *MDR3*, the reverse complement is shown. Sequence derived from GenBank accessions HUMPDGFA1, S69700 (*JUN-D*), HSMMDR3G

region which contains consensus sequences for multiple transcription factors, including WT1 and 3 overlapping Egr-1 sites (Zhan *et al.*, 1998). As the *MYC* effect mapped to the region of the *GADD45* promoter containing the GC-rich WT1/Egr-1 site, this was a possible candidate for the site of *MYC* regulation of *GADD45*. In order to test the role of this site in *MYC* regulation of the *GADD45* promoter, we used pHG45-CAT2-mut, a reporter construct consisting of 909 bp of upstream *GADD45* promoter sequence, the same as pHG45-CAT2, but with the GC-rich sequence of the WT1/Egr-1 binding site replaced with an AT sequence to eliminate specific binding. Unlike pHG45-CAT2, pHG45-CAT2-mut behaved exactly as pHG45-CAT9 in co-transfection experiments, showing no effect of *MYC* on the UV radiation responsiveness of the reporter (Figure 3b and c). As either deletion past the GC-rich site or mutation of the binding motif eliminated the induction-dampening effect of co-transfected *MYC*, this is an important site for regulation of the *GADD45* promoter by *MYC*.

## Discussion

We have used the Rat-1 Myc cell line to demonstrate the effect of Myc over-expression on induction of the *GADD* genes. Induction by MMS was significantly curtailed by ectopic *MYC* expression, while induction by UV radiation was almost completely blocked. Using *GADD45* promoter-reporter constructs, we demonstrated a similar effect in MCF7 human carcinoma cells and mapped the effect of Myc to a specific element within the *GADD45* promoter, indicating regulation at the level of RNA transcription. These findings elucidate another link in the complex interplay of regulatory factors governing the cellular response to DNA damage.

Deregulated expression of Myc is a strong proliferative signal which is observed in many human cancers. Myc contributes to cell transformation in part through formation of Myc:Max heterodimers which activate transcription in a fairly well defined mechanism. Several targets of Myc:Max transcriptional activation are known, and at least two of these, *ODC* and *cdc25A*, have themselves been associated with transforming properties (Auvinen *et al.*, 1992; Galaktionov *et al.*, 1995).

In most cells with wild-type p53, DNA damage results in the rapid accumulation of p53 protein, and the down-regulation of endogenous Myc levels (Moberg *et al.*, 1992). While accumulation of p53 usually results in growth arrest, deregulation of *MYC*

expression can over-ride the usual DNA-damage-induced cell cycle arrest. In the presence of wild-type p53 this may lead to an enhancement of apoptotic cell death (Hermeking and Eick, 1994; Yu *et al.*, 1997). However, deregulated *MYC* may also act independently to regulate genes involved in cell cycle arrest. Several recent reports support this view. For instance, c-Myc deficient rat fibroblasts grew more slowly and had lengthened G1 and G2 phases, indicating a possible role for Myc in G2 progression as well as S-phase entry (Mateyak *et al.*, 1997). In addition, the over-expression of Myc could override p53-induced G1 arrest via destabilization of p21 protein (Hermeking *et al.*, 1995). Over-expression of *CIP1/WAF1* (p21), a major determinant of G1 arrest, could in turn restore p53-induced cell cycle arrest to cells over-expressing Myc. This identified down-regulation of p21 protein levels as another possible mechanism through which Myc can promote cell proliferation. More recently, induction of *myc* over-expression has been shown to repress basal transcription of another growth arrest gene, *gas1* (Lee *et al.*, 1997), while Miz-1, a transcription factor with strong growth arrest function, has been shown to be inactivated by specific binding with Myc (Peukert *et al.*, 1997). Thus, an emerging body of evidence suggests that down-regulation of growth inhibitory factors by multiple mechanisms may play an important role in the growth promoting and transforming potential of Myc.

Basal transcription of *gadd45* was previously shown to be suppressed by ectopic *myc* expression independent of p53 status, and the kinetics of *myc*-induction following serum stimulation mirrored those of *gadd45* suppression (Marhin *et al.*, 1997), indicating a role of *myc* in regulation of *gadd45*. Repression of basal and MMS-induced *gadd153* mRNA levels has also been reported in c-*myc* transfected rat fibroblasts (Chen *et al.*, 1996). We have now demonstrated a downregulation by *MYC* of the DNA damage-induced transcription of the *GADD* genes, a major class of growth-arrest genes.

Within the *GADD45* promoter, the effect of *MYC* is localized to the WT1/Egr-1 binding site. This site has been shown to contribute to the stress-responsiveness of the *GADD45* promoter through binding of a complex containing both WT1 and p53 (Zhan *et al.*, 1998). The GC-rich motif required for the WT1/p53 effect on the *GADD45* promoter contains consensus sequences for multiple transcription factors in addition to WT1. In particular, it contains three overlapping Egr-1 sites, two of which are perfect matches (Figure 4). This region of the *gadd45* promoter also contains a 20 bp match of a larger WT1 consensus site in the

*PDGF-A* promoter. While this site alone could induce transcription in transient assays with WT1, the combined effect of this site with a second site from the *PDGF-A* gene resulted in transcriptional repression by WT1 (Wang *et al.*, 1995). A BLAST search of this 20 bp consensus sequence against the GenBank database found 12 perfect matches and >40 matches with one mismatch. Figure 4 illustrates sequences from the *JUN-D* and *MDR3* promoters in which the region of perfect match extends to 25 bp and 26 bp respectively. In a genome of  $10^9$  bp the odds of such matches occurring by chance alone would be between  $10^{-12}$  and  $10^{-15}$ . When it is considered that all four of the sequences shown in Figure 4 occur in the proximal promoters of human genes, these odds would be further decreased. This would argue that the location of these conserved sequences in the proximal promoters has functional significance. The consensus sequence in *JUN-D* is in the same orientation as *GADD45* relative to the transcriptional start site, unlike the sites in *PDGF-A* and *MDR3*. Interestingly, *JUN-D* is an immediate-early gene with stress-induction similarities to *GADD45* (Prabhakar *et al.*, 1995). *JUN-D* may thus be another potential target for Myc regulation through this site in its promoter.

Myc may interact with the WT1 Erg-1 site either directly or indirectly through protein-protein interactions, or Myc may interact with the WT1-p53 complex to disrupt its activation of the *GADD45* promoter. These possibilities remain to be explored. It is becoming increasingly clear that activated or over-expressed *MYC* can function by different mechanisms at many levels to specifically over-ride the normal growth controls of the cell. Although deregulated Myc does sensitize cells to p53-dependent apoptosis in response to stress, in surviving cells *MYC*'s ability to diminish the response of *GADD34*, *GADD45*, and *GADD153* could drive cell-cycle progression. Such continuous proliferation, especially in the presence of unrepaired damage, could contribute to the transforming potential of Myc. The uncoupling of p53 effector gene regulation from the control of p53, as well as the repression of p53-independent mechanisms of growth arrest, may play a major role in the oncogenic function of activated Myc, as it represents a possible mechanism by which Myc may bypass both the tumor suppressive phenotype of wild-type p53 and other pathways for growth arrest.

## Materials and methods

### Cell culture and treatment

Rat-1 fibroblasts were infected with retrovirus expressing either *neo* alone or *neo* plus *v-myc* and pooled isolates were characterized and maintained as previously described (Marhin *et al.*, 1997). Rat-1 cells were grown in Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS, Summit Biotechnology) and 100 U/ml penicillin (Sigma) and 100  $\mu$ g/ml streptomycin (Sigma). MCF7 human breast carcinoma cells were grown in RPMI 1640 medium supplemented with 10% FBS and 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. All cultures were

maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. Subconfluent Rat-1-Myc and Rat-1-Neo cultures were treated in exponential growth phase. For methyl methane-sulfonate (MMS) treatment, MMS (Aldrich) was added to the medium to a concentration of 50 or 100  $\mu$ g/ml and incubated for 4 h, when RNA was harvested. For UV radiation treatment, the cells were rinsed with buffered saline and irradiated with germicidal lamps at a dose rate of 2.41 J/m<sup>2</sup>/s to a dose of 14 J/m<sup>2</sup> or 20 J/m<sup>2</sup>. The medium was replaced and the cells were incubated at 37°C for 4 h before harvesting the RNA.

### Quantitative RNA hybridization analysis

mRNA was isolated by the guanidine thiocyanate method (Chomczynski and Sacchi, 1987), followed by poly(A)<sup>+</sup> purification using oligo dT cellulose as previously described (Hollander and Fornace, 1990). Rodent cDNA probes for *gadd34*, *gadd45* and *gadd153* were obtained by excising the inserts from xr34D (Jackman *et al.*, 1994), xr45M (Jackman *et al.*, 1994) and A5a4 (Luethy *et al.*, 1990) respectively, and *odc* was excised from pMK934 (ATCC #63075). The cDNA inserts were labeled with <sup>32</sup>P using the PrimeIt RT random primer system (Stratagene) according to the directions of the supplier. Serial dilutions of the mRNAs were dotted onto nylon membranes, hybridized with cDNA probes and washed under standard conditions as previously described (Hollander and Fornace, 1990). Hybridization was quantitated on a phosphorimager (Molecular Dynamics). Relative signal levels, normalized to the poly(A)<sup>+</sup> content of each sample, were determined using the RNA-Analysis program (Hollander and Fornace, 1990).

### Northern blot analysis

1  $\mu$ g polyA RNA was denatured and electrophoresed through a formaldehyde-containing 1.2% agarose gel. RNA was transferred to nylon membrane by capillary blotting, and the filters were hybridized to cDNA probes as described for the dot blots. Hybridization was detected by autoradiography.

### CAT assays

Transient co-transfections of MCF-7 cells were performed using the calcium-phosphate transfection system (Gibco-BRL) following the instructions of the supplier. Briefly,  $5 \times 10^5$  cells were seeded per 100 mm tissue culture dish and 16 h later refed with Ham's F12 medium supplemented with 10% FBS and 0.1% sodium bicarbonate. Four hours after refeeding, cells were cotransfected with 5  $\mu$ g of reporter plasmid (*gadd45* promoter reporter constructs previously described (Zhan *et al.*, 1998)) and 5  $\mu$ g of expression vector (either pCMV *neo* or pCMV *myc*). Twenty four hours after transfection, the cells were harvested, lysed in 0.25 M Tris (pH 7.8), and protein yield was measured using the BioRad protein assay following the directions of the supplier. CAT activity was measured as previously described (Zhan *et al.*, 1993). Briefly, 25  $\mu$ g cell protein was incubated at 37°C for 2 h with acetyl co-enzyme A and <sup>14</sup>C-labeled chloramphenicol. Acetylated forms were separated by thin layer chromatography and <sup>14</sup>C activity was measured with a phosphorimager (Molecular Dynamics). Specific CAT activity was determined as the fraction of chloramphenicol that had been acetylated, and relative induction of CAT activity was determined by normalizing activity in the treated samples to that in the untreated samples.

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