

## Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)–ATF composite site to regulate *Gadd153* expression during the stress response

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*Gadd153*, also known as *chop*, encodes a member of the CCAAT/enhancer-binding protein (C/EBP) transcription factor family and is transcriptionally activated by cellular stress signals. We recently demonstrated that arsenite treatment of rat pheochromocytoma PC12 cells results in the biphasic induction of *Gadd153* mRNA expression, controlled in part through binding of C/EBP $\beta$  and two uncharacterized protein complexes to the C/EBP–ATF (activating transcription factor) composite site in the *Gadd153* promoter. In this report, we identified components of these additional complexes as two ATF/CREB (cAMP-responsive-element-binding protein) transcription factors having differential binding activities dependent upon the time of arsenite exposure. During arsenite treatment of PC12 cells, we observed enhanced binding of ATF4 to the C/EBP–ATF site at 2 h as

*Gadd153* mRNA levels increased, and enhanced binding of ATF3 complexes at 6 h as *Gadd153* expression declined. We further demonstrated that ATF4 activates, while ATF3 represses, *Gadd153* promoter activity through the C/EBP–ATF site. ATF3 also repressed ATF4-mediated transactivation and arsenite-induced activation of the *Gadd153* promoter. Our results suggest that numerous members of the ATF/CREB family are involved in the cellular stress response, and that regulation of stress-induced biphasic *Gadd153* expression in PC12 cells involves the ordered, sequential binding of multiple transcription factor complexes to the C/EBP–ATF composite site.

**Key words:** GADD153 protein, gene regulation, stress response, transcription factors.

### INTRODUCTION

A critical feature of the cellular response to stress is the transcriptional activation of genes whose protein products function in the determination of cellular outcome. One such gene, *Gadd153* (also known as *chop*), encodes a member of the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors. Originally isolated based on its induction by UV-C irradiation [1], *Gadd153* has subsequently been shown to be highly induced in a variety of stress paradigms that result in growth arrest or DNA damage, including genotoxic agents [2–16], calcium ionophore [17], nutrient depletion [15,18–20], oxidative stress [21–23], reductive stress [24,25], endoplasmic reticulum stress [26–28] and the acute-phase response [29,30]. GADD153 (where GADD stands for growth arrest and DNA damage-inducible protein) has been implicated in the commitment to, or implementation of, growth arrest or cell death; microinjection of GADD153 induces 3T3 cells to arrest at the G<sub>1</sub>/S boundary [31], while ectopic expression of GADD153 causes M1 myeloblastic leukaemia cells to undergo apoptosis [32]. These effects require an intact basic leucine zipper (bZIP) domain through which GADD153 can form heterodimers with other bZIP family members, such as C/EBP proteins and

activating transcription factor-3 (ATF3) [19,31–34]. Because GADD153 contains several amino acid substitutions in its basic region [35], GADD153–C/EBP heterodimers are unable to bind to the C/EBP consensus site, so formation of these heterodimers consequently inhibits C/EBP-mediated transcriptional activation [35,36]. Similarly, GADD153–ATF3 heterodimers are unable to bind the ATF/CRE (cAMP-responsive element) consensus site, and such interactions relieve transcriptional repression by ATF3 homodimers [34]. Therefore GADD153 can negatively modulate the functions of C/EBPs and ATF3, but with opposing net effects: inhibition and de-repression (activation) respectively of transcriptional activity. GADD153–C/EBP complexes have also been shown to enhance transcriptional activity during stress through interactions at novel DNA-binding elements [37]. A novel signalling pathway dependent on both CHOP (C/EBP-homologous protein; another name for GADD153) and C/EBP $\beta$  has been implicated for the induction of specific DOC (downstream of CHOP) genes by endoplasmic reticulum stress [38].

Numerous *cis*-acting elements in the *Gadd153* promoter contribute to the rapid and dramatic increase in *Gadd153* transcription induced by stress; the C/EBP site constitutes one such element. We have previously shown that liver extracts derived from lipopolysaccharide-treated rats contain C/EBP $\beta$  complexes

Abbreviations used: AP-1, activator protein-1; ATF, activating transcription factor; bZIP, basic leucine zipper; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT-enhancer-binding protein; CHOP, C/EBP-homologous protein; CRE, cAMP responsive element; CREB, CRE-binding protein; GADD, growth arrest and DNA damage-inducible protein.

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that bind to the *Gadd153* C/EBP site during the acute-phase response [30]. We further demonstrated that overexpression of C/EBP $\beta$  transactivates the *Gadd153* promoter in HepG2 cells [30]. We recently began addressing the role of the C/EBP-binding element in the regulation of *Gadd153* expression in rat pheochromocytoma (PC12) cells following their treatment with the thiol reagent sodium arsenite. *Gadd153* mRNA expression was induced by arsenite in a biphasic manner: a rapid increase occurred during the first 4 h of treatment, followed by a transient decline in mRNA levels at 6–8 h, with a pronounced induction seen thereafter [21,36]. We showed that multiple protein complexes interact with the *Gadd153* C/EBP site following arsenite treatment, one of which contains C/EBP $\beta$  [36]. Additional experiments demonstrated that C/EBP $\beta$  transactivates the *Gadd153* promoter in PC12 cells, but that physical interaction between GADD153 and C/EBP $\beta$  proteins attenuates this activation, providing evidence for an autoregulatory mechanism whereby *Gadd153* gene transcription is controlled by its own protein product. The nature of the other protein complexes that interacted at the C/EBP site was not clear; they were not immunoreactive to antibodies specific for C/EBP family members ( $\alpha$ ,  $\beta$ ,  $\delta$ , GADD153), and nor did they co-immunoprecipitate with C/EBP $\beta$  (T. W. Fawcett and N. J. Holbrook, unpublished work).

In the present study, we sought to characterize these other protein complexes that show enhanced binding to the *Gadd153* C/EBP site during the early phase (the first 6 h) of the response of PC12 cells to arsenite treatment, and to elucidate their contributions to the regulation of *Gadd153* expression during stress. We provide evidence that ATF4 (also known as CREB2, where CREB stands for CRE-binding protein) complexes and ATF3 complexes bind sequentially to this site during the first 6 h of arsenite exposure in PC12 cells. ATF4 binding to the C/EBP-ATF site occurs as *Gadd153* mRNA levels are rising, while ATF3 interactions predominate as *Gadd153* expression decreases. We directly demonstrate that ATF4 activates, while ATF3 represses, *Gadd153* transcriptional activity under conditions of arsenite stress. These data highlight the complexity of *Gadd153* regulation during the stress response and provide insight into the mechanisms that control the convergence of multiple distinct transcription factor complexes at a single DNA-binding element.

## MATERIALS AND METHODS

### Plasmid and oligonucleotide construction

Double-stranded oligonucleotides (5'-GATCCGGTTGCCAA-ACATTGCATCATCCA-3') comprising the C/EBP-ATF-binding site (shown in bold) from the hamster *Gadd153* promoter were custom-synthesized (Integrated DNA Technologies, Coralville, IA, U.S.A.). The ATF4 (called CREB2 in this reference) expression plasmid (CMV-CREB2) was kindly provided by Dr. J. Leiden (Department of Medicine, University of Chicago, Chicago, IL, U.S.A.) [39]. The pCG-ATF3 expression vector was described previously [34]. The *Gadd153*CAT plasmid contains the *Gadd153* promoter region from -778 to +21 linked to the chloramphenicol acetyltransferase (CAT) reporter gene [40]. The mC/EBP-*Gadd153*CAT construct [41] contains the promoter region from -810 to +21 with the C/EBP-ATF site mutated from CATTGCATCATC to CCAGATCTCATC (underlined sequences represent a *Bgl*III restriction site that was introduced). The C/EBP-LUC construct was designed by inserting tandem repeats of an oligonucleotide encompassing the C/EBP-ATF-binding site (CATTGCATCATC) just upstream of the basal

*Gadd153* promoter (-36 to +21) to drive expression of a luciferase reporter gene.

### Cell culture and treatment conditions

PC12 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD, U.S.A.) supplemented with 10% (v/v) fetal bovine serum (HyClone Laboratories, Logan, UT, U.S.A.), 5% (v/v) horse serum and 50  $\mu$ g/ml gentamicin (Life Technologies, Inc.). At 48 h prior to arsenite treatment, cells were seeded at a density of  $1 \times 10^6$  cells/100-mm<sup>2</sup> dish. Because *Gadd153* expression has been shown to be induced by nutrient depletion [18], cell cultures were replenished with fresh medium 12–15 h prior to treatment. Sodium arsenite (Sigma, St. Louis, MO, U.S.A.) was added to the culture medium to a final concentration of 50  $\mu$ M.

Transient transfections were performed by calcium phosphate precipitation [42] followed by a 90 s exposure to 15% (v/v) glycerol in Dulbecco's modified Eagle's medium 4 h after DNA addition. The total amount of transfected DNA was kept constant among experimental co-transfection groups by the addition of empty plasmid DNA. At 1 day after transfection, cells were treated with 50  $\mu$ M arsenite for 6 h, then replenished with fresh medium, and harvested 18 h later. A luciferase assay system kit (Promega, Madison, WI, U.S.A.) was utilized to measure luciferase activity, and CAT assays were performed as previously described [42].

### Northern and Western analyses

Total RNA was isolated from PC12 cells treated with 50  $\mu$ M arsenite using Stat-60 (Tel-Test 'B', Inc., Friendswood, TX, U.S.A.) according to the manufacturer's recommendations. Northern blot analysis was performed as previously described [36] using the human *Gadd153* cDNA probe. All hybridization signals were quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, U.S.A.), and normalized to values obtained after hybridization of the same blots with an end-labelled 24-base oligonucleotide complementary to 18 S rRNA so as to control for variations in loading and transfer between the samples.

Lysates from untreated and arsenite-treated PC12 cells were prepared for Western blot analyses using a lysis buffer containing 1% (v/v) Nonidet P-40 [36]. Protein concentrations of the crude extracts were determined by Bradford assay using Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA, U.S.A.). Antibodies for these studies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The specificities of the antibodies were verified using crude cellular extracts from PC12 cells transiently transfected with plasmids expressing the proteins of interest, followed by Western blot analysis.

### Gel mobility-shift assays

Nuclear extracts were prepared from untreated or arsenite-treated cells, and DNA-binding assays were performed as described [36]. Briefly, 5  $\mu$ g of nuclear extract was incubated with 10 fmol of double-stranded <sup>32</sup>P-labelled C/EBP-ATF oligonucleotide on ice for 30 min. For supershift analyses, the nuclear protein and radiolabelled probe were first incubated for 30 min on ice, after which the specified antibody was added for a further 30 min incubation period at room temperature. All reactions

were electrophoresed through 4% (w/v) polyacrylamide gels, which were then dried and exposed for analysis.

## RESULTS

### Time-dependent binding of two distinct transcription factor complexes to the C/EBP-ATF composite site in the *Gadd153* promoter

As we have previously reported [41], the C/EBP site in the *Gadd153* promoter appears to be a composite of one-half of each of the palindromic sequences that comprise an optimal C/EBP-binding motif and an ATF/CRE variant site (Figure 1). This ATF/CRE variant sequence has similar binding affinities as the consensus ATF/CRE site for CREB and ATF4 [44]. Functionally, the C/EBP-ATF composite site in the *Gadd153* promoter has been shown to bind several protein complex(es), including C/EBP $\beta$  [30,36] and ATF3 [41], *in vitro*.

The induction of *Gadd153* by arsenite in PC12 cells is characterized by a complicated biphasic pattern, as represented in Figure 2(A). This includes a prominent, early rise in mRNA

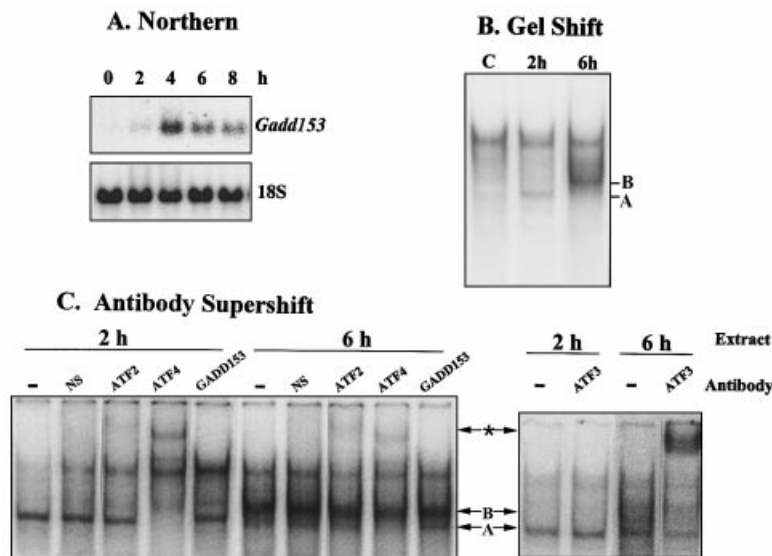
expression to approx. 20-fold over untreated control levels during the first 4 h of arsenite exposure. The mRNA levels then decline by 8 h, before rising again to as high as 50–100-fold above control levels by 24 h [21,36]. To explore the factors that function in the initial phases of *Gadd153* regulation by arsenite, we employed gel mobility-shift analyses to examine complexes that bind to the C/EBP-ATF element during arsenite exposure of PC12 cells at 2 h, when the *Gadd153* mRNA levels are rising, compared with 6 h, when the mRNA levels are declining. Consistent with our previous findings [36], we observed a constitutive binding factor in untreated and arsenite-treated extracts, as well as new DNA-binding protein complexes which appeared only after arsenite treatment (Figure 2B): a faster-migrating band (complex A) predominated after 2 h of treatment, while a slower-migrating band (complex B) was the major band at 6 h of arsenite treatment. Using antibody supershift analysis to identify components of these complexes, we observed changes in the binding patterns with antibodies specific for ATF4 and ATF3 (Figure 2C). The anti-ATF4 antibody completely supershifted complex A, while the anti-ATF3 antibody supershifted complex B, the major band present after 6 h of arsenite exposure. A variety of other antibodies specific for various transcription factors did not affect the binding patterns at any of the time points studied; these included antibodies against ATF2 and GADD153 (Figure 2C), as well as against other leucine zipper proteins, such as c-Jun and c-Fos (results not shown).

	Consensus Binding Sequence	Reference
Palindromic C/EBP	GATTGCGCAATC	43
C/EBP-ATF composite ( <i>Gadd153</i> )	CATTGCATCATC	40
Variant ATF/CRE	GATGACATCATC	44
ATF/CRE	TGACGTCA	45

**Figure 1** The C/EBP-ATF composite site of the *Gadd153* promoter shows similarity to both palindromic C/EBP and ATF/CRE variant consensus sequences

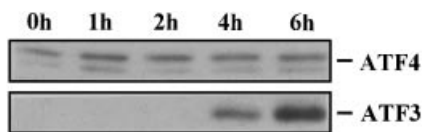
### Arsenite treatment has different effects on ATF4 and ATF3 protein levels

The above results suggest that ATF4 and ATF3 may play roles in regulating *Gadd153* expression in response to arsenite treat-



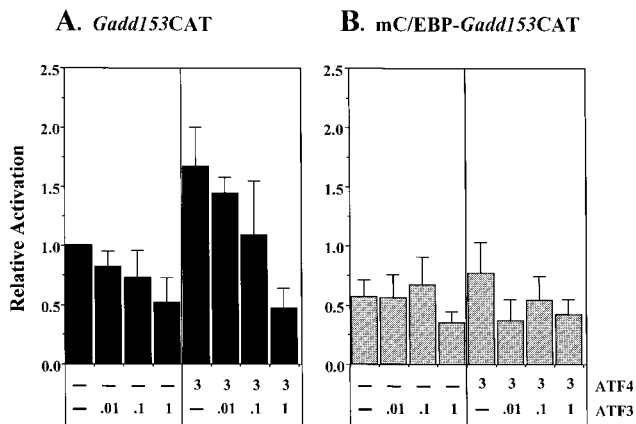
**Figure 2** Arsenite treatment stimulates *Gadd153* expression and the binding of two distinct nuclear complexes to the C/EBP-ATF composite site

(A) *Gadd153* mRNA expression is induced in a biphasic pattern during arsenite treatment. Northern blot analysis of total RNA extracted from PC12 cells treated for the indicated times with 50  $\mu$ M arsenite is shown. The blot was hybridized with the human *Gadd153* cDNA, then subsequently hybridized with an oligonucleotide complementary to 18 S rRNA. (B) DNA mobility-shift analysis using a  $^{32}$ P-labelled C/EBP-ATF oligonucleotide and crude nuclear extracts from PC12 cells which were untreated (lane C) or treated for 2 or 6 h with 50  $\mu$ M arsenite. The bands labelled A and B indicate complexes of differing electrophoretic mobility corresponding to discrete protein-DNA complexes. (C) Antibodies to ATF4 and ATF3 supershifted different complexes bound to the C/EBP-ATF site during arsenite exposure. Normal rabbit serum (NS) or the indicated rabbit antibodies were added to mixtures of the  $^{32}$ P-labelled C/EBP-ATF oligonucleotide and nuclear extracts of arsenite-treated PC12 cells prior to electrophoresis. Lanes without serum or antibody addition are labelled —. The bands labelled A and B indicate complexes of differing electrophoretic mobility corresponding to discrete protein-DNA complexes. The asterisk indicates the bands that were supershifted upon antibody addition. The radiolabelled probe was in excess in each of the lanes (not shown).



**Figure 3** Western blot analysis of ATF4 and ATF3 protein expression during the first 6 h of arsenite treatment

The identity of labelled bands was confirmed by Western blot analysis of lysates from PC12 cells transfected with plasmids overexpressing the proteins of interest.



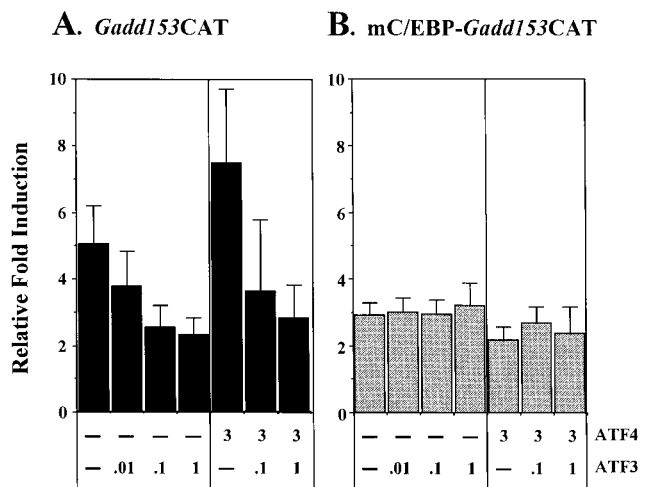
**Figure 4** Effects of ATF4 and ATF3 overexpression on the transcriptional activity of the *Gadd153* promoter

PC12 cells were co-transfected with 1  $\mu$ g of either *Gadd153*CAT (A) or mC/EBP-*Gadd153*CAT (B) and the indicated expression constructs: 0.01, 0.1 or 1  $\mu$ g of ATF3; 3  $\mu$ g of ATF4. CAT activity is expressed relative to co-transfection of *Gadd153*CAT with empty vector DNA. All values represent means  $\pm$  S.E.M. of 3–4 independent experiments. The quantity of co-transfected DNA was kept constant by the addition of empty plasmid DNA.

ment. To explore this possibility, we examined whether the levels of these proteins are affected by arsenite stress. As shown by Western blot analysis, ATF4 protein was present constitutively in PC12 extracts, and levels did not change significantly following arsenite treatment (Figure 3). In contrast, ATF3 levels were undetectable in control cells, but were greatly increased by 4–6 h of arsenite treatment (Figure 3); these levels declined again at later time points (results not shown).

#### ATF4 activates, while ATF3 represses, basal transcriptional activity of the *Gadd153* promoter

To elucidate how the binding of transcription factors to the C/EBP-ATF composite site regulates *Gadd153* transcriptional activity, we co-transfected a CAT reporter driven by the *Gadd153* promoter (spanning sequences from nt -778 to +21) with vectors that constitutively express ATF3 and ATF4. Consistent with previous findings [41], overexpression of ATF3 reduced the basal level of *Gadd153* promoter activity in a dose-dependent fashion (Figure 4A). We previously showed a more prominent repression (5–8-fold) of the *Gadd153* promoter by ATF3 in HeLa cells [34]; we attribute these differences in the magnitude of ATF3 repression of *Gadd153*CAT activity to cell-type specificity. In contrast with ATF3, ATF4 overexpression transactivated the *Gadd153* promoter construct approx. 2-fold. This activation by ATF4 was also repressed by ATF3 in a dose-dependent manner;



**Figure 5** The C/EBP-ATF site contributes to *Gadd153* promoter activity following arsenite stress and overexpression of ATF4 and ATF3

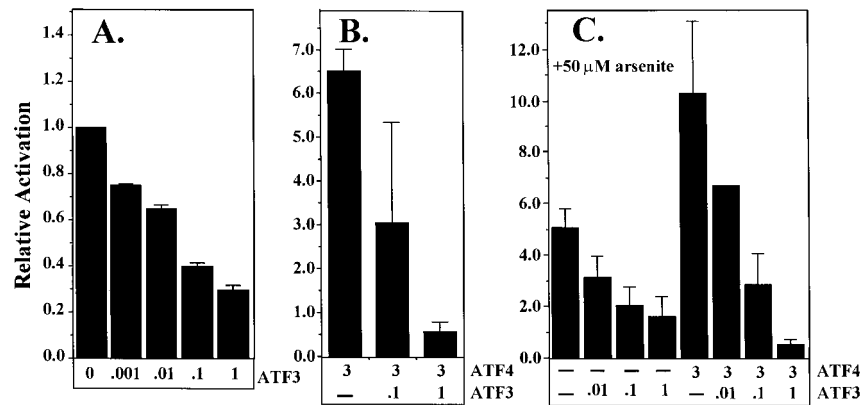
The indicated combinations of expression vectors were co-transfected with 1  $\mu$ g of *Gadd153*CAT (A) or mC/EBP-*Gadd153*CAT (B) constructs. The transfected cells were treated with arsenite (50  $\mu$ M for 6 h) as described in the Materials and methods section. The quantity of co-transfected DNA was kept constant by addition of empty plasmid DNA. CAT activity (mean  $\pm$  S.E.M. from three independent experiments) is expressed relative to activity seen with each reporter upon co-transfection with empty plasmid DNA in the absence of arsenite treatment.

with the highest amount of ATF3, transcription was repressed to levels similar to those seen in the absence of ATF4.

In order to assess the contribution of the C/EBP-ATF site to the regulation of the *Gadd153* promoter by the above proteins, we mutated the C/EBP-ATF element within the full-length *Gadd153* promoter construct (mC/EBP-*Gadd153*CAT). Mutation of this site alone lowered the basal *Gadd153* promoter activity by 40% (Figure 4B). ATF3 expression did not result in the same dose-dependent decrease in CAT activity as was observed with the wild-type promoter, although moderate repression was observed with the highest amount of ATF3. This finding indicates that the mutated promoter is less responsive to transcriptional repression by ATF3. It should be noted that, in our previous studies [41], ATF3 was found to bind and repress basal *Gadd153* transcription through the C/EBP site and the AP-1 (activator protein-1) site located 80 bases downstream in the *Gadd153* promoter. Therefore ATF3 binding at the intact AP-1 element in the C/EBP-mutated promoter could explain the moderate repression observed at the higher ATF3 doses. The C/EBP-mutated promoter was also less responsive to ATF4 transactivation. In summary, mutation of the C/EBP-ATF site in the *Gadd153* promoter rendered the promoter less responsive to both ATF3 and ATF4, indicating the functional significance of the C/EBP-ATF site in the regulation of the *Gadd153* promoter by these transcription factors.

#### Arsenite-induced activation of the *Gadd153* promoter is enhanced by ATF4 and repressed by ATF3

We next sought to determine the effects of the ATF4 and ATF3 transcription factors on arsenite-induced *Gadd153* promoter activity. In agreement with previous reports [36], a 6 h arsenite treatment activated the *Gadd153*CAT reporter in PC12 cells (Figure 5A). The presence of increasing amounts of ATF3 expression vector produced a dose-dependent repression of this



**Figure 6** ATF3 represses and ATF4 activates luciferase activity driven by tandem C/EBP-ATF elements

Shown is the relative fold expression of luciferase activity observed upon co-transfection of C/EBP-LUC (1  $\mu$ g) into PC12 cells with the indicated amounts of expression constructs: (A) ATF3 or (B) ATF4 combined with ATF3. (C) Relative induction of C/EBP-LUC activity following arsenite treatment of PC12 cells transfected with increasing amounts of ATF3 and ATF4. Luciferase activity is expressed relative to that obtained on co-transfection of C/EBP-LUC with empty plasmid DNA in the absence of arsenite. All values represent means  $\pm$  S.E.M. of 3–4 independent experiments. The quantity of co-transfected DNA was kept constant by addition of empty plasmid DNA.

arsenite-induced activation. Interestingly, the transcriptional activation by arsenite appeared to be further enhanced upon co-transfection with the ATF4 expression plasmid (from 5-fold to 7.5-fold); ATF3 could also repress this transactivation by ATF4 and arsenite. The significance of the C/EBP-ATF site in the arsenite-induced activation of the *Gadd153* promoter was examined using mC/EBP-*Gadd153*CAT as the reporter. Mutation of the C/EBP-ATF site reduced the overall arsenite induction seen with *Gadd153*CAT by approx. 40% (Figure 5B). ATF3 and ATF4 individually had little effect on the arsenite-induced mC/EBP-*Gadd153*CAT activity, indicating that mutation of the C/EBP-ATF site renders the *Gadd153* promoter less responsive to these proteins in the presence of arsenite.

To analyse more directly the effects of arsenite stress and ATF/CREB proteins on the C/EBP-ATF site, we used a luciferase reporter driven by two tandem repeats of the composite site (referred to as C/EBP-LUC). ATF3 significantly repressed the basal activity of the C/EBP-LUC construct in a dose-dependent manner (Figure 6A). Expression of ATF4 resulted in a 6.5-fold activation, which was attenuated upon addition of ATF3 (Figure 6B). Arsenite exposure resulted in a 5-fold increase in C/EBP-LUC activity, which was further enhanced to about 10-fold by the addition of ATF4 (Figure 6C). ATF3 repressed the arsenite-induced expression of C/EBP-LUC both in the absence and in the presence of ATF4. Therefore isolation of the C/EBP-ATF element produced transcriptional results consistent with those seen with the native *Gadd153* promoter, although the tandem C/EBP-ATF repeats were more sensitive than the full-length promoter to both transactivation by ATF4 and trans-repression by ATF3. Thus our findings demonstrate the functional significance of the C/EBP-ATF site for both positive (ATF4) and negative (ATF3) transcriptional regulation of *Gadd153* under conditions of stress.

## DISCUSSION

Taken together, our results suggest that the biphasic pattern of *Gadd153* induction following arsenite treatment reflects a series of ordered molecular events that function in the transcriptional regulation of this stress-responsive gene. We have provided direct evidence that members of the ATF/CREB family of

leucine-zipper transcription factors contribute to *Gadd153* gene regulation via interactions at the *Gadd153* C/EBP-ATF site. An initial binding of ATF4 to the C/EBP-ATF site is coincident with transcriptional activation of *Gadd153*, while binding of ATF3 complexes prevails at 6 h, during the decline in *Gadd153* mRNA expression. The control of transcription factor binding is likely to be mediated both by the differential affinities of these complexes for the C/EBP-ATF site and by the amounts of protein available for binding. For instance, ATF4 protein is constitutively available for early binding to the C/EBP-ATF site, whereas ATF3 protein is not present until 4–6 h after arsenite treatment, leading to a delay in the formation and binding of ATF3 complexes. The fact that ATF3 could repress transcription even in the presence of ATF4 also suggests that ATF3 has a greater affinity for the C/EBP-ATF site. Consistent with this notion, we observed that the intensity of complex A (composed of ATF4) was much weaker than that of complex B (composed of ATF3) after 6 h of arsenite treatment (Figure 2B), a time point at which both proteins are present (Figure 3).

## ATF4 and the stress response

A partial human cDNA clone (hATF4) was originally isolated by its ability to bind to the consensus ATF site [46]. The full-length human clone was later isolated and named TAXREB67 [47] and CREB2 [39], while the mouse homologues are designated mATF4 [48], mTR67 [49] and C/EBP-related ATF (C/ATF) [50]. ATF4 has been demonstrated to activate transcription through interactions at ATF/CRE sites [39,46,48], and also through the interleukin-2 CD28 response element [51]. While there are reports showing that ATF4 can suppress promoter activity [52], others describe 'apparent' transcriptional repression by ATF4 due to 'squenching' under certain conditions [46]. ATF4 can interact with several general transcription factors [46] and can dimerize with C/EBP family members, including C/EBP $\beta$  [50] and IgEBP [53,54]. Estes et al. [55] showed that ATF4 and C/EBP $\beta$  mRNA and protein levels increase upon exposure of cells to anoxic conditions. Interestingly, neither ATF4 nor C/EBP $\beta$  showed enhanced binding to their respective consensus sequences after anoxia exposure, but anoxia did lead to enhanced binding of ATF4 to an AP-1-C/EBP composite site

[55], suggestive of a unique function for the transcription factor during this condition of stress.

Our findings demonstrating both enhanced ATF4 binding activity to the *Gadd153* C/EBP-ATF composite site following arsenite exposure and the ability of ATF4 to transactivate the *Gadd153* promoter provides further support to the notion that ATF4 functions to regulate transcription during the response to stress. The presence of relatively high ATF4 protein levels in untreated PC12 cells, with little increase following arsenite treatment (Figure 3), suggests that the induction of ATF4 DNA-binding activity by this stress occurs primarily at the post-translational level. Perhaps these stress-induced modifications (e.g. phosphorylation) allow ATF4 to bind at sites other than its consensus ATF/CRE sequence, and would explain both anoxia- and arsenite-induced ATF4 binding to variant sites. In fact, ATF4 has been found to heterodimerize with another leucine-zipper kinase protein, ZIP kinase, and may act as a physiological inhibitor by preventing the formation of ZIP kinase homodimers, which would trigger apoptosis [56]. This allows for the possibility that, if ATF4 is normally and constitutively sequestered with ZIP kinase partners, cellular stress might promote homodimerization of these proteins, such that ATF4 dimers can transactivate transcription, while ZIP kinase dimers might promote stress-induced apoptosis. In any case, our findings have provided direct evidence for the role of ATF4 in the transcriptional regulation of at least one gene, *Gadd153*, during at least one condition of stress. Given its ubiquitous expression [39], ATF4 is likely to serve a broader function in the stress response of mammalian cells.

### Role of ATF3 in repressing *Gadd153* transcription

A potential role for ATF3 in the down-regulation of *Gadd153* transcription has been suggested by two studies. In the first, *Gadd153* and *ATF3* mRNA levels were found to be inversely correlated in the livers of control and carbon-tetrachloride-treated rats [34]. In the second, purified ATF3 was found to bind to the C/EBP and AP-1 sites of the *Gadd153* promoter, and exogenous ATF3 was able to suppress the basal (non-stress) transcriptional activity of the *Gadd153* promoter in HeLa cells [41]. Our present study extends these observations to another cell type, PC12. More importantly, however, we have provided the first biochemical and functional evidence to support a role for ATF3 in regulating *Gadd153* expression during conditions of stress. We found that exogenously expressed ATF3 potently inhibited both the basal and stress-induced transcriptional activity of the *Gadd153* promoter through the C/EBP-ATF site. In addition, we observed that endogenous ATF3 protein levels and binding activity were enhanced in our stress model system in PC12 cells: we found no significant binding of ATF3 to the *Gadd153* C/EBP-ATF element in nuclear extracts of untreated cells, but markedly increased ATF3 binding activity in extracts from arsenite-stressed cells. Indeed, maximum levels of endogenous ATF3 protein expression and binding activity coincided with a decrease in *Gadd153* mRNA expression at 6 h of arsenite exposure. Taken together, these findings strongly support a role for ATF3 as a stress-inducible transcription factor and, in particular, as a negative regulator of *Gadd153* transcription during the mammalian stress response.

However, it is worth noting that, while *Gadd153* induction occurs as a general response to stress, its regulation by ATF3 is more limited. This is due to the fact that *Gadd153* is expressed ubiquitously in mammalian cells, whereas ATF3 expression is restricted to certain cell types. For example, HeLa cells contain only low levels of ATF3.

### Contribution of other transcription factors and response elements in regulating *Gadd153* transcription

The interactions between C/EBP $\beta$  and components of the ATF/CREB family of transcription factors are also likely to play an important role in the regulation of *Gadd153* in response to stress signals. We have shown previously that binding of C/EBP $\beta$  to the C/EBP-ATF site exhibits a kinetic pattern similar to that seen for ATF3 binding during the first 6 h of arsenite treatment, but continues to increase thereafter [36]. Interestingly, GADD153 protein can dimerize with, and inhibit the functions of, both C/EBP $\beta$  [35,36] and ATF3 [34].

While the current studies have concentrated on the importance of the C/EBP-ATF composite site in regulating *Gadd153* transcription during arsenite treatment, it should be noted that other *cis*-acting elements may function in concert with, or in opposition to, the transcription factors interacting at the C/EBP-ATF site. In this regard, we have also previously shown that the AP-1 element in the *Gadd153* promoter contributes to its transcriptional activation by arsenite in HeLa cells [21]. Binding to this element was maximal at 2 h after arsenite treatment and declined thereafter; supershift analyses suggested that both c-Fos and c-Jun contributed to these complexes. This *Gadd153* AP-1 site has also been shown to bind purified ATF3 protein, resulting in transcriptional repression of *Gadd153* promoter activity in unstressed cells [41]. Thus AP-1-binding proteins are likely to function together with transcriptional factors that bind to the C/EBP-ATF site in the *Gadd153* stress response.

In summary, our studies exploring the factors that bind to the C/EBP-ATF element and affect the transcriptional regulation of *Gadd153* expression provide insight into the multiple levels through which positive and negative signals, mediated by numerous transcription factors, are integrated during the mammalian stress response.

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