

# Arsenite induces a cell stress-response gene, *RTP801*, through reactive oxygen species and transcription factors Elk-1 and CCAAT/enhancer-binding protein

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*RTP801* is a newly discovered stress-response gene that is induced by hypoxia and other cell stress signals. Arsenic is a heavy metal that is linked to carcinogenesis in humans. Here, we investigated the mechanism by which arsenic induces *RTP801* transcription. In HaCaT human keratinocytes, arsenite was able to induce a rapid rise in the *RTP801* mRNA level. Correspondingly, arsenite treatment was capable of stimulating a 2.5 kb human *RTP801* promoter. Such a stimulatory effect was inhibited by co-expression of superoxide dismutase or glutathione peroxidase, and was abrogated by *N*-acetylcysteine, implying that ROS (reactive oxygen species) were involved in transcriptional regulation of the *RTP801* gene. A series of deletion studies with the promoter revealed a critical arsenic-responsive region between –1057 and –981 bp of the promoter. Point mutations of the putative Elk-1 site and the C/EBP (CCAAT/enhancer-binding protein) site within this region were able to reduce the stimulatory effect of arsenite,

indicating that Elk-1 and C/EBP are involved in transcriptional regulation of the *RTP801* gene by arsenite. Furthermore, a gel mobility-shift assay demonstrated that arsenite was able to mount the rapid formation of a protein complex that bound the arsenic-responsive region as well as the C/EBP-containing sequence. The arsenite stimulation on *RTP801* transcription was partly mediated by the ERK (extracellular-signal-regulated kinase) pathway, since the effect of *RTP801* was inhibited by a selective ERK inhibitor. In addition, overexpression of Elk-1 and C/EBP $\beta$  was able to elevate the promoter activity. Therefore these studies indicate that *RTP801* is a transcriptional target of arsenic in human keratinocytes, and that arsenic and ROS production are linked to Elk-1 and C/EBP in the transcriptional control.

**Key words:** arsenite, CCAAT/enhancer-binding protein (C/EBP), reactive oxygen species (ROS), *RTP801* gene.

## INTRODUCTION

Metals are trace elements that are essential for life. However, certain metals, including arsenic, and their derivatives confer toxic effects on the body, which are manifested in carcinogenesis, neurotoxicity and immunotoxicity [1]. Two mechanisms are implicated in the toxicity of metals. One of them is through interruption of the normal physiology of cells by displacing the physiological metals from their natural binding sites. The second mechanism involves the ability of metals to serve as a catalytic centre for redox reactions that generate ROS (reactive oxygen species), including superoxide anion (O<sub>2</sub><sup>•-</sup>), hydroxyl radical (<sup>•</sup>OH) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). ROS are able to damage a variety of cellular macromolecules, as well as altering particular signalling pathways in the cell, leading to cancer and other toxic effects [2]. High concentrations of ROS impose oxidative damage on cellular components such as DNA, proteins and lipids. ROS-mediated DNA damage is probably the most biologically significant event that is implicated in cancer formation. For example, arsenic has been shown to function as a strong dose-dependent mutagen and to induce mostly large deletion mutations via ROS [3].

Arsenic has long been considered as a toxic metal [1]. Four major types of arsenic have been found in mammals, including arsenate [As(V)], arsenite [As(III)], monomethylarsonic acid (MMA) and dimethylarsenic acid (DMA). As(III) is the reduced

form of As(V). The form of arsenic that is more carcinogenic to humans remains controversial, an issue which is largely complicated by the fact that different forms of arsenic can be inter-converted into each other *in vivo*. Studies over the past decade have revealed that the reduction of arsenic is associated with *in vivo* and *in vitro* ROS production, which plays an important role in mediating arsenic-induced carcinogenesis [3]. The carcinogenic effect of arsenic is linked to activation of cell-proliferative signalling pathways. For example, arsenic is able to activate the MAPK (mitogen-activated protein kinase) cascade at concentrations ranging from 0.1 to 500  $\mu$ M [4]. Arsenic differentially activates ERKs (extracellular-signal-regulated kinases), p38 and JNKs (c-Jun N-terminal kinases) to mediate various effects on carcinogenesis, depending on the time, dose, chemical form and cell type [4]. Activation of ERKs by arsenic can induce cell proliferation and transformation, whereas p38 and JNK activation are associated with cell growth arrest and apoptosis. In addition to the mitogenic pathway, arsenic is also able to activate NF- $\kappa$ B (nuclear factor  $\kappa$ B), which plays a critical role in the inflammatory response as well as cancer formation [5]. It has been shown that low concentrations of As(III) (1–10  $\mu$ M) are able to activate NF- $\kappa$ B, and that arsenic-mediated generation of ROS is involved in NF- $\kappa$ B activation [6]. It has also been suggested that the SEK1 (stress-activated protein kinase 1/ERK1)–JNK pathway may partly contribute to the activation of NF- $\kappa$ B by arsenic [6].

Abbreviations used: C/EBP, CCAAT/enhancer-binding protein; EMSA, electrophoretic mobility-shift assay; ERK, extracellular-signal-regulated kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GPX1, glutathione peroxidase 1; HIF-1, hypoxia-inducible factor-1; HNF-4, hepatic nuclear factor-4; HRP, horseradish peroxidase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; NF- $\kappa$ B, nuclear factor  $\kappa$ B; ROS, reactive oxygen species; RT-PCR, reverse transcription-PCR; SOD1, superoxide dismutase 1.

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Studies over the past 3 years have indicated that *RTP801* (also referred to as REDD1 or dig2) is a novel stress-response gene [7–9]. *RTP801* was originally isolated as an HIF-1 (hypoxia-inducible factor-1)-responsive gene [7], and is likely to play a role in hypoxia-mediated cell survival downstream of phosphoinositide 3-kinase [10]. *RTP801* is strongly induced by hypoxia both *in vitro*, such as in mammalian cells, and *in vivo*, such as in an animal model of ischaemic stroke. In addition to hypoxia, *RTP801* has been found to be induced by several signals that mediate cell stress, apoptosis and DNA damage. For example, *RTP801* is induced by dexamethasone, thapsigargin, tunicamycin and heat shock in murine T-cell lymphoma cells [9]. It appears that *RTP801* is a transcriptional target of p63 and p53, and it is implicated in ROS regulation by these two tumour suppressor proteins [8]. In addition, recent studies have suggested that *RTP801* is implicated in the regulation of mTOR (mammalian target of rapamycin) that is also dependent on TSC1/TSC2 (tuberous sclerosis complex 1/2) [11–13]. Even though the biological function of *RTP801* remains to be defined, increasing evidence from recent studies has suggested that *RTP801* is a novel cell stress-responsive protein and plays a role in the regulation of cellular ROS and apoptosis.

Because arsenic is a cell stress signal that mediates ROS generation and carcinogenesis, we investigated the ability of arsenic to induce *RTP801* transcription. We found that arsenic is able to employ previously identified cellular pathways to activate the transcription of *RTP801*.

## MATERIALS AND METHODS

### Materials, cell culture and transfection

HaCaT cells, a spontaneously immortalized human epidermal keratinocyte cell line [14], were grown in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum, 1% non-essential amino acids (Gibco), 100 µg/ml streptomycin and 100 units/ml penicillin. Transient cell transfection was performed using SuperFect reagent (Qiagen). The three inhibitors of the MAPK cascade, including PD98059, SB203580 and JNK Inhibitor II, were purchased from Calbiochem. The trivalent arsenic(III) oxide and 30% H<sub>2</sub>O<sub>2</sub> used in the experiments were from Sigma. The anti-Flag antibody was purchased from Sigma. The antibodies against Elk-1, C/EBPβ (CCAAT/enhancer-binding protein β), ERK and phosphorylated ERK were from Santa Cruz Biotechnology.

### RNA isolation and RT-PCR (reverse transcription-PCR)

Total RNA was prepared from HaCaT cells with an RNA isolation kit (Ambion). The RNA was reverse-transcribed with oligo(dT) primer using the SuperScript First-Strand Synthesis System (Invitrogen) to generate first-strand cDNA. The products were diluted and used in PCR to detect the expression of *RTP801* and G3PDH (glyceraldehyde-3-phosphate dehydrogenase). The sequences of the PCR primers are as follows: 5'-TGGGCAAAGAAGACTACTGCGCCTGG-3' and 5'-TCAACA-CTCCTCAATGAGCAGCTG-3' for human *RTP801*, 5'-GTCTTCACCACCATGGAGAAGG-3' and 5'-TCGCTGTTGAAGTC-AGAGGAGA-3' for G3PDH. The PCR products were separated on a 2% agarose gel containing 0.1 µg/ml ethidium bromide. The band intensity was quantified using densitometric analysis by the NIH Image program.

### Plasmid construction

The 2.5 kb human *RTP801* promoter was cloned by PCR with the human genomic DNA as template, using two primers

[5'-CAGCCCTTCCCATTGAGACAAGTTGTT-3' (for the 5' end) and 5'-ATGAACTCAGAGTGCCGGAGCGTAGA-3' (for the 3' end)]. The resultant PCR fragment was first cloned into pCR4Blunt-TOPO (Invitrogen). The PCR insert was confirmed by DNA sequencing and then subcloned into the KpnI and SacI sites of pGL3-basic luciferase vector (Promega). All deletion constructs of the promoter were generated using PCR. The 5' primers for these constructs were: 5'-TGCAGTTC-CAGCTTCTCGGGA-3' (for ΔA); 5'-TGTCAAATGAGAGT-AGGGTGCA-3' (for ΔB); 5'-GTCTGGATCTTTTACACAAA-GCG-3' (for ΔC); 5'-CCCATATCCCG AGCCTTTGCAA-3' (for ΔD); 5'-CCTTGATCCTCCCCTGCCGCGA (for ΔE); 5'-CCTCTCCATCAGGGTCTCCT-3' (for ΔF); 5'-CCTCTG-AGCACTGCTGCCAGGC-3' (for ΔG); 5'-CCGCCTGAATGA-TGAAACACGG-3' (for ΔH); and 5'-CCTGGCTTTTCCAGA-GATCTC G-3' (for ΔI). The 3' primer used for these deletion constructs was the same as the one for the 2.5 kb promoter. The PCR products were cloned into pGL3-basic vector and confirmed by DNA sequencing. Point mutations of the *RTP801* promoter were introduced into the putative transcription factor-binding sites using PCR. In short, two PCR fragments were first generated with a point mutation introduced by the PCR primers. These two fragments were annealed and used as a template for PCR with two outside primers. The Elk-1 sequence ATTCTGTG was replaced by ATTggTGTG, the C/EBP sequence GATGAAACAC by GATGccACAC and the HNF-4 (hepatic nuclear factor-4) sequence ATGGCCATTGCA by ATGGatATTGCA (the mutated nucleotides are shown as lowercase letters). These mutant PCR products were cloned into pGL3-basic vector and confirmed by DNA sequencing. The FLAG-tagged *RTP801* protein was generated by PCR. The human *RTP801* cDNA coding region was obtained by PCR with primers 5'-TATCGATCCTAGCCTTTGGACCGCTTC-3' and 5'-CTTCTAGAAGTTCAACACTCC-TCAATGAGCAG-3' using human liver cDNA as template. The PCR product was cloned into a pRC/CMV (Invitrogen) that contains a FLAG tag (generated by ligation with a double-stranded oligonucleotide that contains a FLAG sequence) at the ClaI and XbaI restriction sites. A fusion protein of *RTP801* with green fluorescence protein (eGFP-C1, from ClonTech) was generated by ligation of a PCR fragment containing the whole coding region of *RTP801* with eGFP-C1 digested with EcoRI and XbaI. The subcloning was later confirmed by DNA sequencing. The full-length human Elk-1 and C/EBPβ cDNAs were cloned by RT-PCR with the RNA samples isolated from HaCaT cells. Primers of 5'-AGCGATGGACCCATCTGTGACG-3' and 5'-TGGAG-AGAGCATGGATGGAGTGACC-3' were used for cloning Elk-1, and primers of 5'-CGTTCATGCAACGCCTGGTGGCCTG-3' and 5'-CTA GCAGTGGCCGGAGGAGGCGA-3' were used for cloning of C/EBPβ. Both cDNA clones were cloned into pcDNA3 for mammalian cell expression and confirmed by DNA sequencing. To generate the minimal promoter construct, which contains –1057 to –981 bp of the As(III)-responsive region of the human *RTP801* promoter, the corresponding region of the promoter was cloned by PCR with primers of 5'-GCCGGTA-CCTCTTCCATCAGGGTCCCTCCT-3' and 5'-CTCGAGCTCG-AGATCTCTGGAAAAGCCAGG-3'. The PCR fragment was cloned into the KpnI and SacI sites of the pGL3-basic vector that contains an E1B TATA box previously described by us [15].

### Promoter assay

HaCaT cells were seeded in 24-well plates at a density of  $2 \times 10^5$  cells/ml and transfected by SuperFect (Qiagen). A *Renilla* luciferase vector phRL-SV40 (Promega) was co-transfected to serve as an internal control for transfection efficiency. The

cells were harvested at 24–48 h after transfection by lysis with 100  $\mu$ l of PBS with 0.1 % (v/v) Triton X-100 and 1 mM PMSF. Aliquots (10  $\mu$ l) of the lysate was used in a dual-luciferase assay (Promega). The samples were counted for 10 s in a FB12 luminometer (ZyLux). Data are represented in the present paper as relative light units/s. The Student's *t* test was used to compare the values of luciferase data from different groups.

### EMSA (electrophoretic mobility-shift assay)

EMSA was performed using nuclear extracts from HaCaT cells. The nuclear extracts were prepared as described previously [16]. Three probes were used in EMSA: (1) a PCR-generated DNA fragment spanning –1038 to –982 bp of the *RTP801* promoter; (2) a C/EBP-containing oligonucleotide probe of sequence AATGATGAAACACGGGAT; and (3) an Elk-1-containing oligonucleotide probe of sequence CCAGATTCCTGTGGCCC. All probes were labelled with [ $\gamma$ - $^{32}$ P]dATP by T4 polynucleotide kinase. The labelled probes (approx.  $5 \times 10^4$  c.p.m.) were incubated with 2  $\mu$ g of nuclear extract in a buffer containing a final concentration of 4 % (v/v) glycerol, 10 mM Tris/HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl and 0.1  $\mu$ g/ $\mu$ l poly(dI-dC). For supershift EMSA, antibodies against Elk-1 and C/EBP $\beta$  (0.2  $\mu$ g/reaction, from Santa Cruz Biotechnology) were added to the binding reaction. The reaction was incubated at 4°C for 30 min after addition of the probe, then separated by electrophoresis with a 5 % non-denaturing polyacrylamide gel [in 0.5  $\times$  TBE (Tris/borate/EDTA) buffer] and detected by autoradiography with the dried gel.

### Primer extension assay

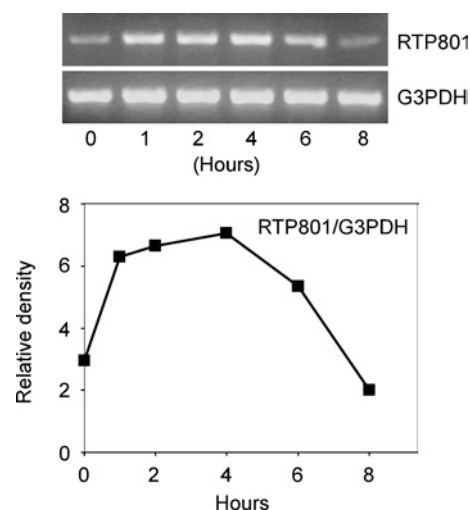
The primer extension assay was performed using the total RNA isolated from HaCaT cells with a method previously described by us [17]. The oligonucleotide primer 5'-ATGAACTC-AGAGTGCCGGAGCGTAGA-3' was used in this assay.

### Western blotting analysis

HaCaT cells cultured on six-well plates were lysed with 2  $\times$  SDS/PAGE loading buffer. The DNA was sheared using a 22-gauge needle and equal volumes were loaded on to SDS/10 % PAGE gels. The protein was transferred on to PVDF and the membrane was blocked with 5 % non-fat dry milk in a buffer containing 10 mM Tris/HCl, pH 7.6, 0.9 % NaCl and 0.1 % (v/v) Tween 20. The membrane was then probed with an antibody specific for Tyr-205-phosphorylated ERK (sc-7383, 1:200 dilution; Santa Cruz Biotechnology), followed by incubation with a goat-anti-mouse HRP (horseradish peroxidase) antibody (1:2500 dilution; Bio-Rad) and ECL (enhanced chemiluminescence; PerkinElmer). The blots were stripped using Western blot stripping buffer from Pierce, and re-probed for total ERK (sc-94, 1:200 dilution; Santa Cruz Biotechnology) followed by incubation with goat-anti-rabbit HRP (1:5000; Bio-Rad) and ECL.

### Immunofluorescence staining of the cells

HaCaT cells were cultured at  $2 \times 10^5$  cells/ml on glass coverslips in six-well plates before transient transfection. Cell were cultured for the indicated times before fixation, which was performed with 3.7 % formaldehyde and 0.1 % Triton X-100. The cells were incubated with anti-FLAG antibody (1  $\mu$ g/ $\mu$ l; Sigma), washed, and then incubated with Alexa-546-conjugated anti-mouse antibody (Molecular Probes). Nuclear staining was performed with Hoechst 33342 (Sigma). The coverslips were



**Figure 1** Effect of As(III) on mRNA level of RTP801

HaCaT cells were treated with 10  $\mu$ M As(III) for various periods of time. The total RNAs were isolated and used for RT-PCR with specific primers for RTP801 and G3PDH. The PCR products were run on a 2 % agarose gel and stained with ethidium bromide (top panel). The relative density of the PCR product of RTP801 was normalized to G3PDH level and is shown in the lower panel.

mounted and the stained cells were analysed under a fluorescence microscope (DM5000B; Leica).

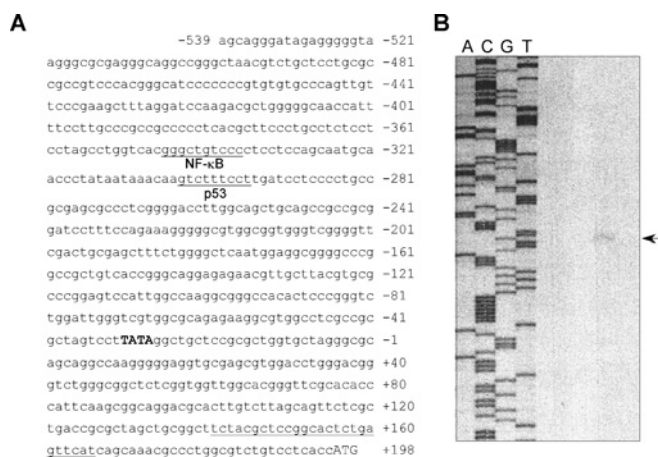
## RESULTS

### As(III) augments human RTP801 mRNA levels in HaCaT cells

Recent studies have indicated that *RTP801* is a novel stress-response gene that is induced by hypoxia and other cell stress signals [7–9]. As arsenic is able to elicit a stress response in the cells [1,4], we first determined whether or not the trivalent arsenic, arsenite [As(III)], is able to induce *RTP801* expression at the mRNA level. This was performed in HaCaT cells, which are from a spontaneously immortalized human epidermal keratinocyte cell line [14]. In addition, arsenic has been shown to be able to initiate ROS production and induce apoptosis in these cells, a phenomenon that is related to the carcinogenic effect of arsenic on skin [18]. In our experiment, HaCaT cells were treated with As(III) for various periods of time and the total RNA was isolated from the cells and used in RT-PCR. Primers specific for the human RTP801 cDNA were used in the PCR. The same samples were also subjected to RT-PCR with primers for the housekeeping gene, G3PDH, to monitor the relative RNA levels used in the experiment. As shown in Figure 1, As(III) was not able to cause discernible changes in the G3PDH level. However, As(III) was capable of inducing a rapid up-regulation of the RTP801 mRNA level. This stimulatory effect reached a maximal level at 4 h after As(III) treatment. These data provided the first evidence that As(III) is able to regulate RTP801 at the mRNA level in HaCaT keratinocytes.

### Determination of the transcription initiation site and isolation of a human RTP801 promoter

To identify the putative transcription initiation site, we performed a primer extension assay with total RNA isolated from HaCaT cells. As shown in Figure 2, a single extension band was detected at 166 bp upstream of the oligonucleotide primer, indicating that



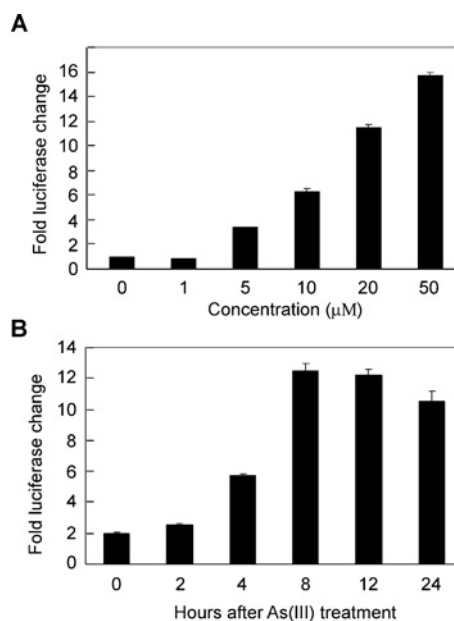
**Figure 2** The human *RTP801* promoter sequence and identification of the transcription initiation site

(A) A fragment of the human *RTP801* promoter sequence spanning the region  $-539$  to  $+198$  bp is shown. The translation initiation site (ATG) is shown in uppercase letters. The putative TATA box is shown in bold uppercase letters. The putative binding sites for transcription factors were determined through the internet TESS program (provided by the Computational Biology and Informatics Laboratory at the University of Pennsylvania Schools of Medicine Engineering and Applied Science). The putative NF- $\kappa$ B- and p53-binding sites are indicated. The position of the primer used for the primer extension assay is marked by an underline at the end of the sequence. (B) Results of the primer extension assay. Total RNA isolated from HaCaT cells was used in this assay. The position of the transcription initiation sites was determined by a DNA sequencing reaction (shown on left) and marked by an arrow on the right.

transcription starts 195 bp upstream of the translation initiation site, i.e. the first codon encoding ATG. With this information, we isolated a 2.5 kb human genomic *RTP801* fragment immediately in front of the coding region. This promoter region corresponds to  $-2548$  to  $+166$  bp relative to the transcription initiation site identified by the primer extension assay. Sequence analysis indicated that there is a putative TATA box at position  $-21$  to  $-18$  bp (Figure 2A).

### Regulation of the *RTP801* promoter by As(III)

To determine whether or not the isolated *RTP801* promoter is regulated by As(III), we analysed the transcriptional activity of the promoter. The 2.5 kb *RTP801* promoter ( $-2548$  to  $+166$  bp) was fused with a firefly luciferase reporter gene. This construct was transiently transfected into HaCaT cells and treated with various concentrations of As(III) for various periods of time. As shown in Figure 3(A), As(III) was able to stimulate *RTP801* promoter activity in a dose-dependent manner. At a concentration of  $10 \mu\text{M}$ , As(III) was able to activate the promoter to approx. 7-fold relative to the untreated samples. At  $50 \mu\text{M}$ , As(III) was capable of inducing the transcription to approx. 16-fold, even though most cells were dead at this high concentration (results not shown). In addition, we analysed the effect of various treatment times with As(III). We found that As(III) treatment for 8 h was able to activate the promoter to the maximal level (Figure 3B), equivalent to those treated for 12 and 24 h. Taken together, these data indicated that As(III) was indeed able to strongly activate the *RTP801* promoter, and that such stimulation took place rapidly upon treatment. These data, together with our findings that As(III) rapidly induced the *RTP801* mRNA level by RT-PCR assay (Figure 1), provided convincing evidence that *RTP801* is a transcriptional target regulated by arsenic in HaCaT keratinocytes.

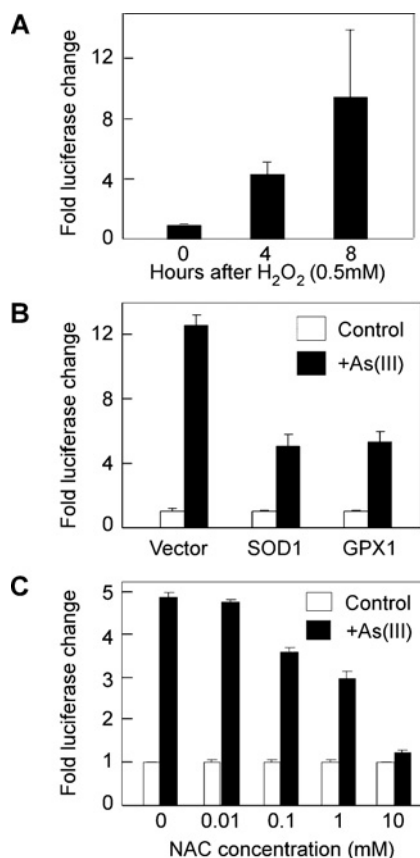


**Figure 3** Stimulation of the *RTP801* promoter by arsenite

(A) Effect of different As(III) concentrations. HaCaT cells were transiently transfected with *RTP801* promoter construct ( $-2548/+166$  bp). An SV40 (simian virus 40)-driven *Renilla* luciferase was used to delineate the transfection efficiency. At 24 h after transfection, cells were treated with As(III) for 8 h in various concentrations, as indicated. The whole cell lysate was used in a dual-luciferase assay. The fold change of luciferase activity normalized by *Renilla* luciferase activity is shown as the means  $\pm$  S.D. (B) Effect of various treatment times. The same promoter construct was used as above, but the cells were treated with  $10 \mu\text{M}$  As(III) for different periods of time. The fold changes in luciferase activity are shown as the means  $\pm$  S.D. At least three independent experiments were performed for the assays described above, and only one representative result is shown here.

### Activation of the *RTP801* promoter by As(III) is mediated by ROS production

The biological toxicity of arsenic is mainly caused by production of ROS catalysed by the redox reactions of the metal [1,4]. Since As(III) is able to activate *RTP801* transcription, we next examined if the effect of arsenic is mediated by ROS. We first analysed the effect of one of the ROS, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), on *RTP801* transcription. The 2.5 kb *RTP801* promoter construct was transiently transfected into HaCaT cells, and the cells were treated with  $\text{H}_2\text{O}_2$  for various periods of time. As shown in Figure 4(A),  $\text{H}_2\text{O}_2$  was able to rapidly activate the promoter activity, suggesting that  $\text{H}_2\text{O}_2$  itself is sufficient to activate *RTP801* transcription. We next analysed the activity of two ROS-reducing enzymes, SOD1 (superoxide dismutase 1) and GPX1 (glutathione peroxidase 1), in modulating the As(III)-mediated stimulation of *RTP801* transcription. SOD1 catalyses the reaction to reduce the cellular amount of superoxide anion ( $\text{O}_2^{\bullet-}$ ) [19]. GPX1 reduces  $\text{H}_2\text{O}_2$  by oxidizing glutathione to glutathione disulphide [2]. Therefore both SOD1 and GPX1 are able to protect the cells against oxidative damage by reducing the intracellular amount of ROS. When either SOD1 or GPX1 was co-transfected with the 2.5 kb *RTP801* promoter into HaCaT cells, the As(III)-induced *RTP801* promoter activity was inhibited by approx. 50% (Figure 4B). These data indicate that ROS production is at least partly implicated in the stimulation of *RTP801* transcription by arsenic. To further confirm that ROS generation is required for the effect of arsenic on the *RTP801* promoter, we employed an ROS scavenger, NAC (*N*-acetylcysteine), in the experiment. NAC is able to block ROS production and suppress the cytotoxic effects of ROS. As shown in Figure 4(C), treatment of HaCaT cells with NAC was able



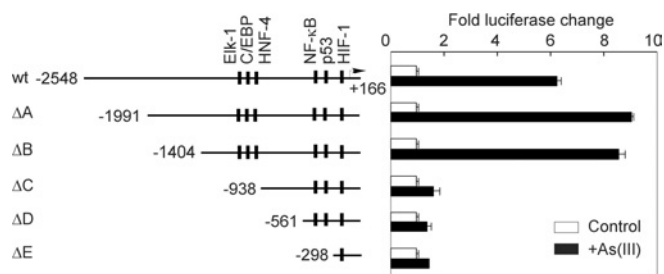
**Figure 4** Stimulation of the *RTP801* promoter by As(III) is mediated by ROS

(A) Activation of the *RTP801* promoter by H<sub>2</sub>O<sub>2</sub>. HaCaT cells were transiently transfected with *RTP801* promoter construct (–2548/+166 bp) and *Renilla* luciferase vector. The cells were treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for different periods of time, followed by a dual luciferase assay. The fold changes in luciferase activity are shown as the means  $\pm$  S.D. (B) Effect of SOD1 and GPX1 expression on As(III) stimulation of the *RTP801* promoter activity. HaCaT cells were transiently transfected with *RTP801* promoter construct together with SOD1 or GPX1 expression plasmids (0.2  $\mu$ g each), as indicated. At 24 h after transfection, the cells were treated with 10  $\mu$ M As(III) for 8 h before the luciferase assay. The fold changes in luciferase activity are shown as the means  $\pm$  S.D. (C) Effect of NAC on *RTP801* promoter activation by As(III). HaCaT cells were transiently transfected with *RTP801* promoter construct as described above. At 24 h after transfection, the cells were treated with 10  $\mu$ M As(III) for 8 h in the presence or absence of NAC at various concentrations. The fold changes of luciferase activity are shown as the means  $\pm$  S.D. At least three independent experiments were performed for the assays described above, and only one representative result is shown here.

to abrogate the stimulation of *RTP801* transcription by As(III) in a dose-dependent manner. Taken together, these experiments provided profound evidence that the arsenic-mediated activation of *RTP801* transcription is mediated by ROS production.

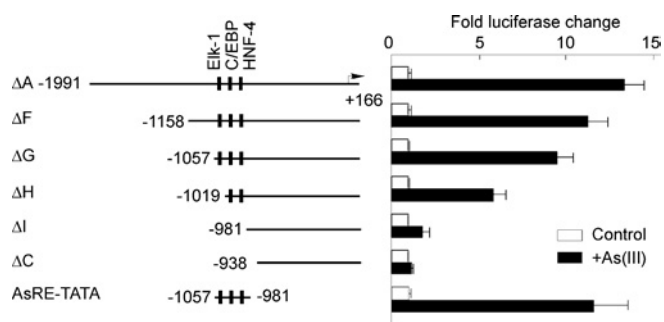
#### Identification of an arsenic-responsive region at the *RTP801* promoter

The human *RTP801* promoter contains multiple putative transcription factor-binding sites that are likely to be involved in the transcriptional regulation of the gene. For example, the promoter contains a putative NF- $\kappa$ B site around –350 bp and a p53 site around –300 bp (Figure 2A). It has been shown that arsenic is able to activate NF- $\kappa$ B to mediate its cellular effect [6]. In addition, a recent study also indicated that *RTP801* is a transcriptional target of p63 and p53 [8]. In order to delineate the region(s) of the *RTP801* promoter that is/are implicated in arsenic-mediated activation, we analysed the effect of As(III) treatment on a series of *RTP801* promoter deletion mutants which had been fused to



**Figure 5** Characterization of the human *RTP801* promoter

The left panel showed a graphic representation of the length of different promoter constructs and the relative location of putative binding sites for p53, NF- $\kappa$ B and HIF-1. HaCaT cells were transiently transfected with various deletion constructs of the *RTP801* promoter as indicated, together with *Renilla* luciferase vector. At 24 h after transfection, the cells were treated with 10  $\mu$ M As(III) for 8 h and the fold changes in luciferase activity are shown as the means  $\pm$  S.D. At least three independent experiments were performed for this assay, and only one representative result is shown here.



**Figure 6** Identification of a critical arsenic-responsive region

HaCaT cells were transiently transfected with various deletion constructs of the *RTP801* promoter, as indicated. The cells were treated with 10  $\mu$ M As(III) for 8 h. The activity of a minimal *RTP801* promoter was also analysed. The promoter region –1057 to –981 bp was fused to a TATA box (AsRE-TATA) and used in the luciferase assay. Data are shown as the fold changes in luciferase activities (means  $\pm$  S.D.). At least three independent experiments were performed for the assay, and only one representative result is shown here.

the luciferase reporter. As shown in Figure 5, the two deletion constructs, –1991/+166 bp ( $\Delta$ A) and –1404/+166 bp ( $\Delta$ B), displayed arsenic stimulation to a level similar to or even slightly higher than that of the 2.5 kb promoter (–2548/+166 bp). However, three other deletion mutants, –938/+166 bp ( $\Delta$ C), –561/+166 bp ( $\Delta$ D) and –298/+166 bp ( $\Delta$ E), demonstrated greatly reduced As(III)-induced stimulation. These data suggest that the promoter sequences between –1404 and –938 bp probably contain a major arsenic-responsive region (Figure 5). In addition, the putative NF- $\kappa$ B (around –350 bp) and p53 (around –300 bp) sites do not appear to be directly involved in the arsenic-mediated stimulation of *RTP801* transcription in these cells (Figure 5).

To characterize further the arsenic-responsive region of the *RTP801* promoter, we analysed a few more deletion mutants of the promoter, including –1158/+166 bp ( $\Delta$ F), –1057/+166 bp ( $\Delta$ G), –1019/+166 bp ( $\Delta$ H) and –981/+166 bp ( $\Delta$ I). These constructs were transfected into HaCaT cells, followed by treatment with As(III). We found that both the –1158/+166 bp ( $\Delta$ F) and –1057/+166 bp ( $\Delta$ G) constructs showed only a slight reduction in As(III)-mediated transcriptional stimulation (Figure 6). However, the construct –1019/+166 bp ( $\Delta$ H) markedly lost the ability to mediate arsenic activation of the promoter (Figure 6). Furthermore, the –981/+166 bp construct ( $\Delta$ I) only possessed a residual ability to mediate As(III) stimulation

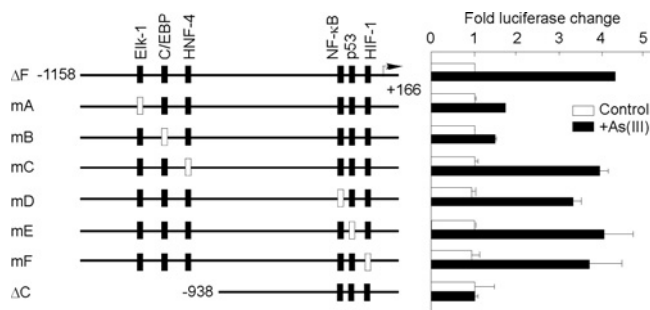
(Figure 6). These data therefore delineated further the arsenic-responsive region to  $-1057$  to  $-981$  bp of the *RTP801* promoter. In addition, we analysed the responsiveness of the identified arsenic-responsive region to arsenite by using a minimal promoter construct that contains a  $-1057$  to  $-981$  bp fragment of the promoter fused with a TATA box that was previously described by us [15]. As shown in Figure 6, this region (AsRE, standing for arsenic-responsive element) is able to mediate arsenite stimulation at a level even higher than the  $\Delta F$  construct. These data indicated that this region is not only required but also sufficient to mediate the stimulatory effect of arsenite on the human *RTP801* promoter.

#### Identification of an Elk-1 site and a C/EBP site that mediate arsenic activation of the *RTP801* promoter

As our experiments revealed a critical arsenic-responsive region between  $-1057$  and  $-981$  bp, we carefully analysed the putative transcription factor-binding sites in this area. There are three major putative transcription-factor binding sites that are well conserved in both human and mouse genomic sequences: an Elk-1 site (ATTCCTGTG at  $-1031/-1023$  bp), a C/EBP site (GATGAAACAC at  $-1009/-999$  bp) and an HNF-4 site (ATGGC-CATTGCA at  $-996/-985$  bp). Elk-1 is a regulator of the c-Fos proto-oncogene and belongs to the ETS-domain family of transcriptional factors [20] and functions as a substrate of MAPK [21]. C/EBP was originally identified as a heat-stable transcription factor in rat liver nuclei that was able to interact with the CCAAT motif present in several cellular gene promoters and certain viral enhancers [22] and plays a pivotal role in many cellular responses [23]. HNF-4 is a potent transcriptional activator that controls the expression of a wide variety of genes important for metabolism [24].

To analyse the potential contribution of these sites to the arsenic-mediated transcription of the *RTP801* gene, we introduced point mutations into these sites. The Elk-1 sequence ATTCCTGTG was replaced by ATTggTGTG, the C/EBP sequence GATGAAACAC by GATGccACAC and the HNF-4 sequence ATGGC-CATTGCA by ATGGatATTGCA (the mutated nucleotides are shown in lowercase letters). These mutations were incorporated into the  $-1158/+166$  bp region ( $\Delta F$ ) of the *RTP801* promoter and linked to a luciferase reporter. Transient transfection of these mutants was carried out in HaCaT cells, followed by As(III) treatment. As shown in Figure 7, mutation of the Elk-1 site was able to partially abrogate the arsenic-mediated stimulation of *RTP801* promoter. Meanwhile, mutation of the C/EBP site profoundly reduced the As(III)-mediated transcriptional activation. However, mutation of the putative HNF-4 site revealed no obvious effect on As(III)-initiated stimulation of the promoter. Taken together, these data provided significant evidence that the Elk-1 and C/EBP sites at the *RTP801* promoter are implicated in the transcriptional activation by arsenic.

In addition, we also analysed the contribution of other putative transcription factor-binding sites to the transactivation of the promoter upon arsenite stimulation, including NF- $\kappa$ B, p53 and HIF-1-responsive element. A conserved NF- $\kappa$ B site at  $-346/-337$  bp was mutated from gggctgtccc to aAActgtccc. A p53 site was found at  $-310/-301$  bp, and this sequence was previously identified to be responsible for p53 activation of the *RTP801* promoter [8]. The p53-responsive sequence aaacaagtct was replaced by aaaTaaAtct. Furthermore, we mutated a putative HIF-1-responsive site at  $-127/-122$  bp from acgtga to acAAgc, and such a site was shown to be responsive to hypoxia in a gel mobility-shift assay [7]. These mutations were integrated into the  $-1158/+166$  bp region ( $\Delta F$ ) of the *RTP801* promoter and used in luciferase assays in HaCaT cells. As shown in Figure 7, mutations



**Figure 7** Effect of different point mutations of *RTP801* promoter

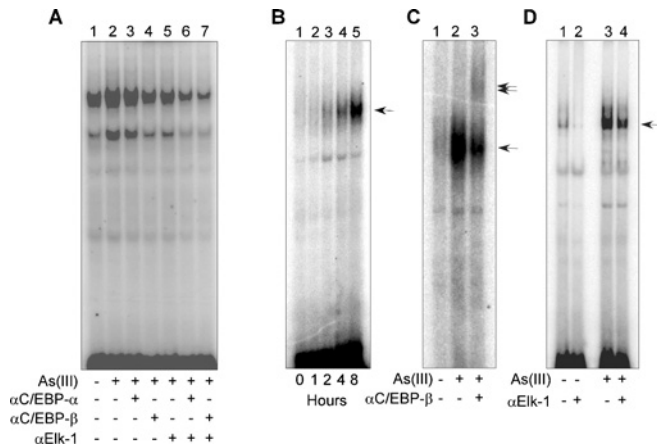
The left panel shows a graphic representation of the relative location of putative transcription factor-binding sites for Elk-1, C/EBP, HNF-4, NF- $\kappa$ B, p53 and HIF-1. The filled-in bars represent the wild-type sites and the open bars are for the mutated sites. HaCaT cells were transiently transfected with various mutation constructs of the *RTP801* promoter as indicated, together with *Renilla* luciferase vector. The  $\Delta C$  and  $\Delta F$  constructs were the same as shown in Figure 6. The cells were treated with  $10 \mu\text{M}$  As(III) for 8 h at 24 h after transfection. The fold changes in luciferase activity normalized by *Renilla* luciferase activity are shown as means  $\pm$  S.D. At least three independent experiments were performed for the assay, and only one representative result is shown here.

of these sites had no effect on As(III)-mediated stimulation of the *RTP801* promoter. Therefore these data suggest that the NF- $\kappa$ B-, p53- and HIF-1-responsive sites studied here are not directly involved in the promoter response to As(III) treatment in HaCaT cells.

#### Characterization of C/EBP and Elk-1 binding by EMSA

We next employed a gel-shift assay to analyse further the association of the putative transcription factors with the critical arsenic-responsive region of the *RTP801* promoter. As a first step, we analysed whether or not As(III) treatment is able to induce protein binding to this critical region. HaCaT cells were treated with As(III) for various periods of time and the nuclear extracts were isolated for EMSA. A 57 bp DNA fragment spanning  $-1038$  to  $-982$  bp that contains both the C/EBP and Elk-1 sites was used as a probe in the experiment. We found that arsenite treatment in these cells was able to induce binding of nuclear proteins to this region at 4 and 8 h after treatment (results not shown). The C/EBP family is composed of at least six members [23]. Among these members, C/EBP $\beta$  has been reported to play an important role in keratinocyte differentiation involving growth arrest and keratin expression [25]. Therefore we analysed whether C/EBP $\beta$  is present in the protein complex that binds the C/EBP site-containing probe in HaCaT keratinocytes. As shown in Figure 8(A), addition of an anti-C/EBP $\beta$  antibody was able to reduce the binding of the protein complex to the 57 bp probe, indicating that C/EBP $\beta$  is indeed present in the complex (Figure 8A, lane 4). We also tested an anti-C/EBP $\alpha$  antibody and found that it did not affect the protein binding by itself (Figure 8A, lane 3). An anti-Elk-1 antibody was able to partially reduce the protein binding (Figure 8A, lane 5), indicating that Elk-1 is also present in the protein complex that binds the *RTP801* promoter. Interestingly, the anti-C/EBP $\alpha$  antibody or the anti-C/EBP $\beta$  antibody, when combined with the anti-Elk-1 antibody, was able to significantly reduce the protein binding to the 57 bp probe (Figure 8A, lanes 6 and 7), suggesting a synergistic effect between C/EBP and Elk-1 in binding the arsenic-responsive region of the *RTP801* promoter.

To analyse further the binding of the transcription factors to the *RTP801* promoter, we utilized an oligonucleotide probe (AATGATGAAACACGGAT) that contains the consensus



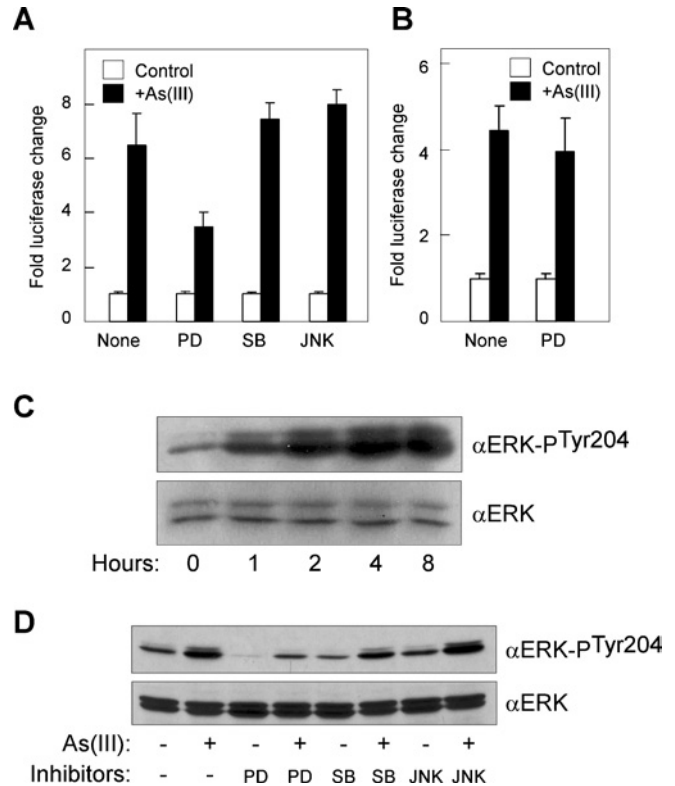
**Figure 8** EMSA using nuclear extracts

(A) HaCaT cells were treated with 10  $\mu$ M As(III) for 4 h, and the nuclear extracts were isolated from the cells and used in an EMSA with a 57 bp  $^{32}$ P-labelled probe that covers the critical As(III)-responsive region of *RTP801* promoter (from -1038 to -982 bp). Different anti-C/EBP or anti-Elk-1 antibodies were used in EMSA, as indicated. (B) The nuclear extracts isolated as described above were used in EMSA with an oligonucleotide probe (AATGATGAAACACGGGAT) that contains a C/EBP-binding site in the middle. The single arrow indicates the binding complex induced by As(III). (C) Supershift assay with an anti-C/EBP $\beta$  antibody. The nuclear extracts isolated from HaCaT cells treated with 10  $\mu$ M As(III) for 4 h were used in an EMSA with the oligonucleotide probe containing the C/EBP-binding site. The single arrow indicates the band induced by As(III), and the double arrows mark the band shifted by the anti-C/EBP $\beta$  antibody (lane 3). (D) Effect of an anti-Elk-1 antibody in EMSA. The nuclear extracts were isolated from HaCaT cells treated with 10  $\mu$ M As(III) for 4 h. An Elk-1-containing oligonucleotide probe (CCAGATTCCTGTGGCCC) was used in EMSA. The single arrow indicates the band induced by As(III). Note the reduction in the intensity of the band in the presence of the anti-Elk-1 antibody (lane 4).

C/EBP site at the *RTP801* promoter. Similarly, As(III) treatment was able to initiate a time-dependent binding of a protein complex to this sequence, with a maximal binding at 4 and 8 h after arsenite treatment (Figure 8B). Addition of an anti-C/EBP $\beta$  antibody was able to induce a 'supershift' in EMSA, indicating further that C/EBP $\beta$  is present in the complex (Figure 8C, lane 3). We later analysed the presence of Elk-1 protein in EMSA with an oligonucleotide probe (CCAGATTCCTGTGGCCC) that contains the consensus Elk-1 sequence at the *RTP801* promoter. Arsenite treatment was able to induce protein complex formation with this probe (Figure 8C, lane 3), and an anti-Elk-1 antibody was able to partially reduce such binding (lane 4). These data provided additional evidence that Elk-1 is present in the protein complex that binds the *RTP801* promoter upon arsenic treatment.

#### Arsenic-stimulated *RTP801* transcription is mediated by MAPK

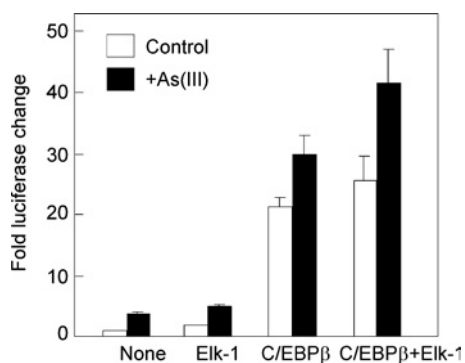
It has been reported that arsenic differentially activates ERKs, p38 and JNKs to mediate various effects on carcinogenesis [1,4]. Activation of ERKs by arsenic is able to induce cell proliferation and transformation, whereas p38 and JNK activation are associated with cell growth arrest and apoptosis. To elucidate a role for these kinases in the arsenic-mediated activation of the *RTP801* promoter, we examined the effects of various MAPK inhibitors. PD98059 is a selective inhibitor of ERK and MEK (MAPK/ERK kinase). SB203580 is able to selectively inhibit p38 MAPK, and JNK inhibitor II potently inhibits JNK. In the experiment, HaCaT cells were transfected with a -1158/+166 bp *RTP801* promoter construct and treated with these inhibitors. As shown in Figure 9(A), SB203580 and JNK inhibitor II at 10  $\mu$ M had no obvious effect on As(III)-mediated activation of the promoter. However, PD98059 at 10  $\mu$ M was able to



**Figure 9** Effect of MAPK and JNK inhibitors

(A) HaCaT cells were transiently transfected with *RTP801* promoter construct (-2548/+166bp) and *Renilla* luciferase vector. At 24 h after transfection, cells were treated with 10  $\mu$ M As(III) for 8 h in the presence of 10  $\mu$ M inhibitors, as indicated. PD, PD98059; SB, SB203580; JNK, JNK inhibitor II. The whole cell lysate was used in a dual luciferase assay. The fold changes in luciferase activity normalized by *Renilla* luciferase activity are shown as means  $\pm$  S.D. (B) The mA promoter construct used in Figure 7 with a mutation of the conserved Elk-1 site was used in the luciferase assay, together with PD98059 and As(III) treatment, as above. (C) Activation of ERK by arsenite. HaCaT cells were treated with 10  $\mu$ M As(III) for various periods of time, as indicated. The cell lysate was used in Western blotting analysis with an anti-ERK antibody and an antibody specific for phosphorylation of ERK at Tyr-204. (D) Inhibition of ERK activation by PD98059. HaCaT cells were treated for 4 h with or without 10  $\mu$ M As(III) and/or 10  $\mu$ M inhibitors, as indicated. The cell lysate was used in Western blotting analysis as above.

partially abrogate the arsenic effect, indicating that ERK is probably involved in arsenic stimulation of the *RTP801* promoter. Such an inhibitory effect of PD98059 could be observed at a concentration as low as 1  $\mu$ M (results not shown). To confirm that ERK is activated by arsenite in HaCaT cells, we analysed the phosphorylation status of ERK in these cells by Western blotting. As shown in Figure 9(C), arsenite treatment was able to induce a time-dependent elevation of ERK phosphorylation. In addition, we found that treatment of HaCaT cells with PD98059 was able to reduce the arsenite-mediated ERK phosphorylation (Figure 9D). However, the p38 inhibitor (SB203580) and JNK inhibitor (JNK inhibitor II) had a minimal effect on the phosphorylation of ERK upon arsenite exposure. Furthermore, we analysed the effect of the MAPK inhibitor PD98059 on the *RTP801* promoter with a point mutation of the putative Elk-1 site at the arsenite-responsive region, as described in Figure 7. As shown in Figure 9(B), such a mutant promoter construct was no longer inhibited by PD98059, indicating further that the conserved Elk-1 site in this region is implicated in MAPK-mediated As(III) stimulation of the promoter. Taken together, these data suggest that activation of ERK by arsenic is implicated in the signalling pathway that stimulates *RTP801* transcription. This is consistent with our



**Figure 10** Effect of overexpression of Elk-1 and C/EBP $\beta$  on *RTP801* transcription

The  $\Delta F$  promoter construct was co-transfected with Elk-1 or C/EBP $\beta$  (0.25  $\mu$ g of each), as indicated. The cells were treated with 10  $\mu$ M As(III) for 8 h at 24 h after the transfection. The fold changes in luciferase activity are shown as the means  $\pm$  S.D. At least two independent experiments were performed for this assay, and only one representative result is shown here.

findings that Elk-1, a transcription factor downstream of MAPK, is involved in arsenic stimulation of the *RTP801* promoter.

#### Effect of overexpression of Elk-1 and C/EBP $\beta$ on the *RTP801* promoter

To provide further evidence that Elk-1 and C/EBP sites are involved in the regulation of *RTP801* transcription upon arsenic treatment, we next analysed the effect of overexpression of Elk-1 or C/EBP $\beta$  on the human *RTP801* promoter. Both human Elk-1 and C/EBP $\beta$  were cloned and co-transfected with the human *RTP801* promoter in HaCaT cells. As shown in Figure 10, overexpression of Elk-1 has a small effect in raising the basal promoter activity (approx. 2-fold). Overexpression of C/EBP $\beta$  was able to dramatically elevate the promoter activity by more than 20-fold. Interestingly, co-expression of Elk-1 with C/EBP $\beta$  appeared to have a synergistic effect on the promoter activity.

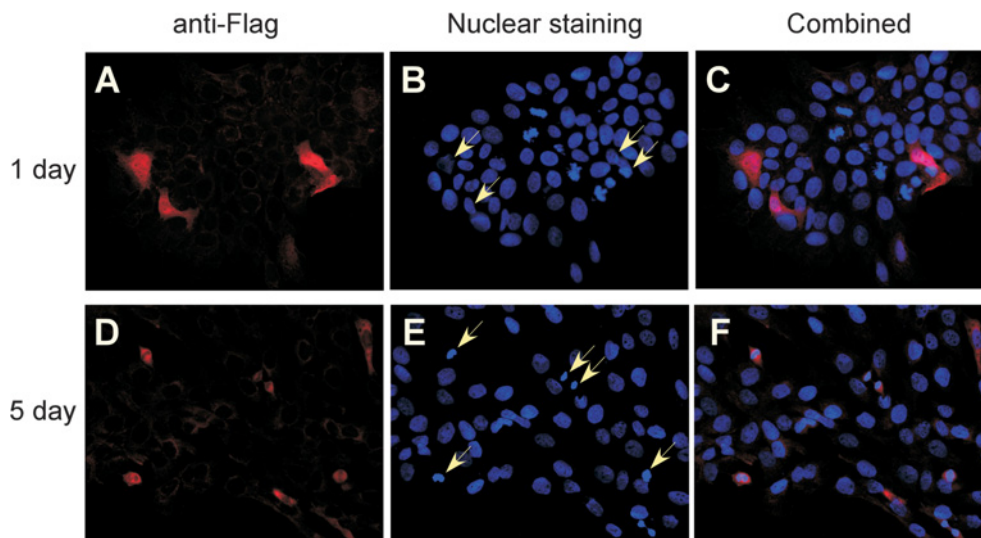
These data therefore provided additional evidence to implicate Elk-1 and C/EBP $\beta$  in the regulation of the human *RTP801* promoter.

#### Overexpression of *RTP801* induces apoptosis in HaCaT cells

*RTP801* was originally isolated as a target gene of HIF-1 [7]. It has been demonstrated that the biological activity of *RTP801* overexpression is dependent on the cellular context, and may promote or inhibit cell death [7]. To determine the biological effect of *RTP801* on HaCaT keratinocytes, we carried out an immunofluorescence assay to analyse the cellular effect of *RTP801* overexpression. A FLAG-tagged *RTP801* expression plasmid was transfected into HaCaT cells, and protein expression was analysed by immunofluorescence with an anti-FLAG antibody. Nuclear staining was also performed with these cells. At 24 h after transfection, *RTP801* was evenly expressed in both cytoplasm and nucleus (Figures 11A–11C). Interestingly, at 5 days after transfection, most *RTP801*-expressing cells (stained red) revealed condensed pycnotic nuclei (Figures 11D–11F), characteristic of apoptotic cells. Taken together, these data clearly indicate that overexpression of *RTP801* was able to rapidly induce apoptosis in HaCaT keratinocytes.

#### DISCUSSION

In the present study, we investigated the activity of arsenic in regulating a newly discovered gene, *RTP801*, that is induced by hypoxia and other cell stress signals. In HaCaT human keratinocytes, we found that arsenic was able to induce a rapid rise of *RTP801* mRNA level. Similarly, arsenic treatment was capable of stimulating the human *RTP801* promoter in HaCaT cells, and such an effect was mediated by ROS production in these cells. Through studies with a series of deletion constructs of the promoter, we identified a critical arsenic-responsive region between –1057 and –981 bp of the promoter. When this critical arsenic-responsive region was linked to a TATA box, it was able to mediate arsenite stimulation, indicating that such a region is not only required, but also sufficient, for the transcriptional regulation of



**Figure 11** Induction of apoptosis by overexpression of *RTP801*

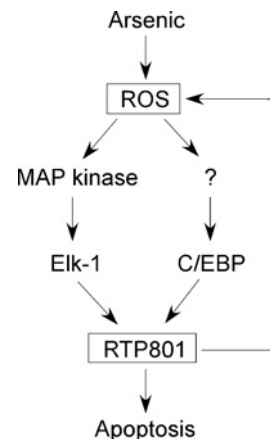
(A–C) HaCaT cells were transfected with FLAG-tagged human *RTP801* expression plasmid (2.0  $\mu$ g). Fixation and fluorescence staining of the cells were performed at various times after the transfection. An anti-FLAG antibody and a secondary Alexa-546-conjugated anti-mouse antibody were used to determine the expression of *RTP801* (shown by the red colour). Nuclear staining was performed with Hoechst 33342 (shown by the blue colour). The arrows indicate the cells expressing *RTP801*. Note the change in nuclear shapes in *RTP801*-expressing cells at 5 days after transfection (D–F).



the promoter by arsenite. Further point mutation studies with the putative Elk-1 site and C/EBP site within this region revealed that Elk-1 and C/EBP are involved in transcriptional regulation of the *RTP801* gene by arsenic. This was confirmed further by a gel mobility-shift assay. The involvement of the MAPK pathway in the effect of arsenic was supported by our findings that a selective ERK inhibitor was able to partially antagonize the arsenic effect on *RTP801* transcription. To elucidate further the effect of Elk-1 and C/EBP on *RTP801* transcription, we overexpressed these two genes in HaCaT cells and found that they were able to elevate the promoter activity. Therefore these studies provided the first evidence that *RTP801* is a transcriptional target of arsenic in keratinocytes, and that Elk-1 and C/EBP are involved in transcriptional regulation.

One of the interesting findings from this study is that C/EBP is involved in arsenic-mediated gene regulation. Our experiments revealed a critical region in the *RTP801* promoter that mediates transcriptional regulation by arsenic. Further deletion and mutation analyses of this region indicate that a consensus C/EBP site is implicated in the regulation. Furthermore, treatment of HaCaT cells with arsenic was able to rapidly induce a protein complex that binds the C/EBP consensus sequence, and such binding was partially 'supershifted' by an anti-C/EBP $\beta$  antibody. It is noteworthy that the arsenic-induced protein binding at the promoter reached a maximal level (Figure 8) later than the maximal induction of *RTP801* mRNA by arsenic (Figure 1), indicating that the transcription of the *RTP801* gene might be initiated at an earlier stage, before maximal C/EBP and Elk-1 complexes are formed at the promoter. C/EBPs are a family of transcription factors containing a highly conserved basic leucine-zipper domain at the C-terminus that participates in dimerization and DNA binding [23]. At least six C/EBP members have been characterized so far, including C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\gamma$ , C/EBP $\delta$ , C/EBP $\epsilon$  and C/EBP $\zeta$ . These factors play pivotal roles in the control of cell proliferation, cell differentiation, inflammation, metabolism and many other cellular responses. C/EBP has not been previously implicated in ROS-mediated gene regulation. Regulation of C/EBP could occur at the transcriptional, translational and post-translational levels [26]. It will be of great importance in the future to elucidate at which level C/EBP is involved in arsenic- and ROS-mediated transcriptional control.

*RTP801* has been shown to play a role in the regulation of ROS involved in the p53-dependent DNA damage response. Ellisen et al. [8] recently reported that *RTP801* is a transcriptional target of p63, a family member of p53. During mouse development, the expression pattern of *RTP801* closely correlates with that of p63. ROS production is largely abrogated in p63-deficient mouse embryonic fibroblasts [8]. Meanwhile, expression of *RTP801* is able to greatly induce H<sub>2</sub>O<sub>2</sub>-mediated ROS production in these cells [8]. It appears that *RTP801* is required for the production of ROS in a p63-dependent manner, and it may play a role in epithelial differentiation. However, the regulation of ROS by *RTP801* is probably dependent on the cellular context. In human breast cancer (MCF-7) cells and neuronal PC12 cells, overexpression of *RTP801* is not able to change the basal level of ROS [7]. On the contrary, *RTP801* can abrogate ROS production induced by H<sub>2</sub>O<sub>2</sub> or hypoxia, consistent with the ability of *RTP801* to protect these cells from apoptosis after treatment with these agents [7]. In the present study, we found that the transcription of *RTP801* itself is regulated by ROS following arsenite treatment in HaCaT keratinocytes. The arsenic-mediated *RTP801* transactivation is inhibited by overexpression of enzymes that reduce cellular ROS concentration, or by incubation with the ROS scavenger NAC. We propose that *RTP801* is implicated in the complex regulation of ROS production in the cells. On the



**Scheme 1** A hypothetical circuit that links arsenic and *RTP801* transcription to ROS production and apoptosis

one hand, *RTP801* is directly involved in the production of ROS, with an effect that varies from cell type to cell type. On the other hand, the transcription of *RTP801* is under the control of the ROS level inside the cell, which probably had a role in feedback regulation to further modulate the production and function of intracellular ROS. As the protein primary structure of *RTP801* does not share any similarity to the enzymes that regulate the production and conversion of ROS, it is unlikely that *RTP801* is directly involved in the generation of ROS in the cells. It remains to be determined whether *RTP801* serves as a modulator that functionally collaborates with or antagonizes enzymes that are directly involved in the regulation of ROS.

At present, the biological function of *RTP801* is largely unknown. One of the primary functions suggested by recent studies is its cell-type-dependent role in apoptosis. In some types of cells, *RTP801* has a protective effect. Overexpression of *RTP801* is able to protect MCF7 and PC12 cells from hypoxia- and H<sub>2</sub>O<sub>2</sub>-triggered apoptosis [7]. In addition, overexpression of *RTP801* in WEH17.2 murine T-lymphoma cells was able to protect the cells from dexamethasone-induced apoptosis [9]. However, *RTP801* may also function in promoting cell death in other types of cells. It has been shown that *RTP801* is able to increase the sensitivity of non-dividing PC12 cells to the insults of hypoxia and oxidative stress [7]. Concomitantly, liposomal delivery of *RTP801* to mouse lungs leads to massive cell death [7]. In our studies, overexpression of *RTP801* is able to induce nuclear condensation as well as apoptosis in HaCaT keratinocytes. Therefore the apoptotic effect of *RTP801* seems to be dependent on the cellular context. It is likely that *RTP801* interacts with the apoptotic machinery differently in a cell-dependent manner. Bearing this in mind, detailed biochemical analysis of *RTP801* function in the apoptosis pathway in various cell types will undoubtedly aid in clarifying this issue in the future.

Taken together, our studies have shown that arsenic is able to regulate *RTP801* transcription through ROS production and the transcription factors Elk-1 and C/EBP, as illustrated in Scheme 1. In this model, arsenic increases cellular ROS, which in turn activates Elk-1 via MAPK. Meanwhile, C/EBP is activated via an unknown mechanism. Both transcription factors then act on the promoter of *RTP801* and induce transcription of the gene. The change of intracellular level of *RTP801* will have an effect on the apoptotic potential of the cells, either enhancing or inhibiting apoptosis in a cell-type-dependent manner. In addition, *RTP801* may participate in the regulatory circuit that controls the production of intracellular ROS, which will modulate further

the transcription of *RTP801* as well as its effect on apoptosis in a cell-context-dependent manner.

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