

Differential usage of alternate promoters of the human stress response gene *ATF3* in stress response and cancer cells

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ABSTRACT

Stress response gene *ATF3* plays a pleiotropic role in determining cell fate in response to mitogenic or stress stimuli. An alternate promoter of the human *ATF3* gene (designated P1 in this study) has recently been reported, which is located ~43.5 kb upstream of the previously reported P2 promoter. We showed here that the P1 promoter is highly conserved between human and mouse and is functional in response to various stimuli, whereas the P1 promoter was dominantly induced by serum and the P2 promoter was more efficiently activated in response to TGF- β and oncogenic *HRAS*. The P1 promoter contains multiple transcriptional start sites, and the different 5'-UTRs markedly affected their translation in response to stress. In human prostate and Hodgkin Reed–Sternberg cancer cells with elevated expression of *ATF3*, the P1 promoter was constitutively activated and its chromatin structure was modified into active configuration. The differential usage of alternate promoters of the *ATF3* gene at both transcriptional and translational level and the modification of chromatin structure may provide a novel mechanism for expressing *ATF3* in determining cell fate during stress response and cancer.

INTRODUCTION

An increasing number of studies demonstrate the existence of alternate promoters for human genes and their

differential usage is one of important source for generating protein and regulatory diversity (1,2). For example, transcripts from alternate promoters are translated into distinct protein isoforms when their variant 5' exons contain alternative ATGs, producing proteins with different N-termini or different proteins. For many genes with multiple promoters, however, no variation in the resulting proteins is generated, when a common downstream exon contains the translation initiation site and generates the same open reading frame (ORF). Some of these alternate promoters have different tissue specificity and developmental activity, such as the *CYP19* gene encoding the aromatase P450 (3) or glucokinase gene (4). Furthermore, mRNA variants transcribed from the alternate promoter cause difference of their translational efficiencies, since the variant 5'-UTRs regulate their secondary structure of mRNAs, thus affecting its stability and translation. The usage of alternate promoters of genes has also been reported to play role in stress response (5–8).

Activating transcription factor (ATF) 3 is a member of the ATF/CREB family of basic-leucine zipper (b-Zip) type transcription factors. Its mRNA level is low or undetectable in most cells, but is greatly induced by a variety of stress signal (9–11, and references therein). This response has dual effects on cell fate, such as cell cycle arrest and apoptosis (10–14), or cell survival and proliferation (15–21). We have recently reported that *ATF3* is induced downstream of c-myc in serum induction (22), and other laboratories showed that the *ATF3* expression is elevated in human prostate (23) and Hodgkin Reed–Sternberg cells (24), and more recently in human breast cancer cells (25). Furthermore, transgenic over-expression of *ATF3* in basal epithelial cells causes basal cell carcinoma in mouse (26). *ATF3* also plays role as a negative regulator of

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inflammation (27–29). These data support both physiological and pathological role of *ATF3* in regulating the stress response of cells to external stimuli.

The human *ATF3* gene is mapped to chromosome 1q32.3 region, and is comprised of five exons spanning ~16 kb (30). The putative gene promoter, designated P2 in this study, contains a TATA motif and many *cis*-elements for binding of transcription factors responsible for the regulated gene expression (11,21,30–34). More recently, a large scale and genome-wide analysis of 5'-end sequence of full-length cDNAs has identified a novel upstream alternate promoter of the human *ATF3* gene as deposited in Genbank (NM001030287) (35). We also identified the transcripts in human and mouse cells that contain various novel 5'-UTR (DDBJ/EMBL/GenBank with accession numbers AB291910, AB291911 and AB291912), but their functional and biological significance remains elusive.

In this report, we investigated the molecular and functional properties of a novel upstream promoter, designated P1, of the human *ATF3* gene. It is shown that the P1 promoter is highly conserved between human and mouse and is differentially used at both transcriptional and posttranscriptional level in stress response. More strikingly, the P1 promoter is constitutively activated in human cancer cells with elevated expression of *ATF3*. This study for the first time describes evidence for the differential usage of alternate promoters of the human *ATF3* gene, providing a novel insight into the molecular mechanism by which *ATF3* is induced in stress response and cancer.

MATERIALS AND METHODS

Plasmids, antibodies and reagents

Plasmids encoding human *HRAS* wild-type and oncogenic mutant 12V were purchased from Addgene, and expression plasmids for oncogenic Ras 61L mutant and HA-Suv39 were gifts from Dr Nevins and Dr Jenuwein, respectively. Antibodies used were as follow: rabbit anti-ATF3 (C19, sc-188), anti-H-Ras (F235, sc-29), anti-RNA-polymerase II (N-20, sc-899) from Santa Cruz, anti- β -actin (clone AC-74, A2228), anti- β -tubulin (clone TUB2.1, T4026), anti-HA (clone HA-7) and anti-Flag (clone M2) from Sigma, anti-Leo-1 (A300-175A) from Bethyl, anti-H3-trimethyl-K4 (H3K4me3, ab8580) and anti-H3-trimethyl-K9 (H3K9me3, ab8898) from abcam, anti-acetyl-histone H3 (06-599) from Upstate. Doxorubicin, thapsigargin, tunicamycin, etoposide were from Sigma, methyl methanesulfonate (MMS) and H₂O₂ were nachalai tesque, recombinant human TGF- β was from PeproTech. Other chemicals were reagents grade.

Cell culture and treatments

Cell lines used in this study are HCT116 human colon carcinoma cell, U2OS human osteosarcoma cell, LNCaP, DU145, PC3 human prostate carcinoma cell (ATCC), L428 and L540 Hodgkin Reed-Sternberg cell (DSMZ, Germany), DAUDI human B cell and MOLT4 human T cell (RIKEN, Japan), HaCaT human

keratinocyte (CLS, Germany). RAW264 is a mouse macrophage cell line (RIKEN, Japan). Cells were cultured in adequate media supplemented with 10% calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin in a 5% CO₂ atmosphere at 37°C. For induction by serum or various stimuli, cells were cultured in the presence of 0.5% serum for 48 h and induced by 20% serum, 100 μ g/ml MMS or various agents. For expression of oncogene *HRAS*, neomycin-resistant U2OS cells stably expressing mouse ecotropic retrovirus receptor were infected by pMX retroviral vector encoding oncogenic mutants 12V, 61L, or normal *HRAS* and selected with 2 μ g/ml puromycin. For transient expression, plasmid DNA was vortex-mixed with SuperFect (Qiagen) and transfected into cells according to manufacturer's instruction.

Cell extract preparation and western blot analysis

Cells treated as indicated were harvested, washed in PBS, and cell extracts were prepared as described (22). The amounts of protein were measured by Lowry method using bovine serum albumin as standard (36). Cell extracts (20 μ g protein) were separated on an SDS-PAGE, transferred onto a nitrocellulose membrane, and subjected to western blot using the protocol of ECL kit (Amersham).

RNA isolation and reverse transcription-PCR

Total RNA was isolated by acid-guanidinium method using a kit from Qiagen. For detecting transcripts from P1 or P2 promoters of *ATF3* gene, RT-PCR of 1 μ g total RNA was performed using a kit from TaKaRa. Primers used were as follow: human P1 transcript, 5'-AGGATGC TCTGCTGTTTCCT-3' (forward) and 5'-TTAGCTCTG CAATGTTCCCTC-3' (reverse); human P2 transcript, 5'-TGATGCAACGCTCTCCAAGC-3' (forward) and 5'-TTAGCTCTGCAATGTTCCCTC-3' (reverse); mouse P1 transcript, 5'-AACAGGATCTCCCACAGGGT-3' (forward) and 5'-GACAAAGGGTGTGTCAGGTTAG-3' (reverse); mouse P2 transcript, 5'-CAGTGGAGCCAAT CGGCTAA-3' (forward) and 5'-GACAAAGGGTGTCA GGTTAG-3' (reverse). Measurements of the *ATF3* transcripts from the P1 and P2 promoters or GAPDH were also performed by quantitative RT-PCR. To this end, the efficiency of PCR-amplification of mRNA from each promoter was determined by control reaction using the plasmid DNA for each transcript, and used for calibration (Supplementary Figure S1). The data obtained were further normalized with that of GAPDH. Primers used were as follow: *ATF3* (P1 transcript), 5'-AGGATGCTCTGCT GTTTCCT-3' (forward) and 5'-GACAAAGGGCGTCA GGTTAG-3' (reverse); *ATF3* (P2 transcript), 5'-TGATG CAACGCTCTCCAAGC-3' (forward) and 5'-CAGAAG CACTCACTTCCGAG-3' (reverse); GAPDH, 5'-TGAA GGTCGGAGTCAACGGATTTGGT-3' (forward) and 5'-TTGATTTTGGSGGGATCTCG-3' (reverse).

Primer extension analysis

Primer (5'-GGGCAAGGTGCTGAAAATCC-3') complementary to the region from +104 to +123 of the human *ATF3* mRNA with novel 5'-UTR sequence in database (NM001030287) was radiolabeled with

p³²- γ -ATP using T4 polynucleotide kinase, hybridized with PolyA mRNAs from serum-treated HCT116, and extended using AMV reverse transcriptase using a kit from Promega. Extended products were resolved on an 8% polyacrylamide sequencing gel. The sequence ladder was generated from the same primer using a template plasmid DNA containing upstream promoter region of the human ATF3 gene.

5'-Rapid amplification of cDNA ends analysis

Human HCT-116 and mouse RAW264 cells were treated with serum or MMS, and the 5'-ends of the ATF3 mRNA of the P1 promoter were determined by 5'-rapid amplification of cDNA end analysis (5'-RACE) using a kit from Invitrogen. ATF3 cDNA was amplified by PCR using a GeneRacer 5' primer or GeneRacer 5' nested primer and 3' primers specific for ATF3: human ATF3-3' reverse primer, 5'-CCTGGATGTTGAAGCATCATTTTGA-3'; mouse ATF3-3' reverse primer, 5'-TGGGATGAAGTGCTGGA ACTC-3'. Multiple transcription start sites (TSSs) of the P1 promoter of human and mouse ATF3 genes have been deposited in DDBJ/EMBL/GenBank with accession numbers AB291910 and AB291911, respectively.

RNAi experiments

Mixtures of siRNA oligos (final concentration 100 nM) specific for the 5'-UTR of P1 or P2 transcript isoforms were transfected into HCT116 or LNCaP cells using X-tremeGENE transfection reagent (Roche), or into L428 and L540 Hodgkin Reed-Sternberg cells by electroporation as in ref. (24). HCT116 cells were then cultured in medium with 0.5% serum for 24 h, followed by stimulation with 20% serum or 100 μ g/ml MMS. Sequences of siRNAs for the P1 5'-UTR were 5'-AGAGAAAUCCUC CUCUAUAUAGG-3' (83–105) and 5'-UUUCAGCACC UUGCCCCAAAUC-3' (129–151), and those for the P2 5'-UTR were 5'-GCCAGCCUGAGGGCUAUA AAGG-3' (46–58), and 5'-CUCGCCCGCCGCCAGA CAAACA-3' (190–212). Control RNAi (siCtl) was a silencer negative control #1 (AM4611) from Ambion.

Luciferase assay

Reporter plasmid, pLuc-P1ATF3 was constructed by subcloning a 2.1-kb fragment of the human ATF3 gene upstream promoter from -2091 to +9 (major TSS as +1) between KpnI and HindIII sites of pGL3 vector (Promega). pLuc-P2ATF3 reporter plasmid containing a 1850-bp region of P2 promoter was as described (11). Cells (5×10^4 cells) were transfected with 0.5 μ g each reporter plasmid. At 16 h posttransfection, cells were starved with 0.5% serum for 48 h and then stimulated with 20% serum, 50 μ g/ml MMS, 20 μ g/ml etoposide, 50 μ M H₂O₂, 1 μ M doxorubicin, 2 μ M thapsigargin, 1 μ M tunicamycin or 100 pM TGF- β . For treatment with H₂O₂, cells were exposed to 50 μ M H₂O₂ in PBS for 1 h, and then cultured in fresh medium containing serum. After cell culture for 16 h, extracts were assayed for the luciferase activity as described (11), using a Dual Luciferase Reporter Assay System according to the manufacturer's protocol (Promega).

Translation *in vitro* and *in vivo*

cDNAs for ATF3 transcripts with various UTRs were subcloned between NheI and BamHI sites of pcDNA3.1 expression vector. Each DNA (0.1 μ g) was transcribed along with 0.2 μ g vector encoding HA-Suv39 as an internal control in 10 μ l of *in vitro* TNT assay kit driven by T7 RNA polymerase, followed by translation. ATF3 protein generated was quantitated by western blot and densitometric measurement. For the *in vivo* translation, the plasmid above (1 μ g each) encoding the ATF3 protein tagged with Flag at the C-terminus was transfected into 293 cells and the expression of ATF3 protein was measured by western blot. Total RNA was also extracted, treated with RNase-free DNase (Sigma) and assayed for ATF3 mRNA generated from the transgene by quantitative RT-PCR. Primers used: 5'-CTGCAGAAAGAGTCGG AG-3' (forward, coding sequence of ATF3) and 5'-TAG AAGGCACAGTCGAGG-3' (reverse, BGH sequence of the vector).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described (22) according to the protocol supplied by Upstate. ChIP DNA was detected using standard PCR with the following primer pairs for the different regions of the ATF3 gene: -5-kb region, 5'-TGGACAC ACACACGGAAACT-3' (forward) and 5'-GTCACATC TTCCCATCTGATC-3' (reverse), the P1 promoter region, 5'-AGGATGATGGAAGGCTGTCA-3' (forward) and 5'-GTCACATCTTCCCATCTGATC-3' (reverse), +10-kb region, 5'-TATGGGCTTTTGTTCG GGTTC-3' (forward) and 5'-TCAAAATGCGTGTGTG TGTG-3' (reverse), +20-kb region, 5'-AATGCCCTCA CAGAAACACC-3' (forward) and 5'-GTTAGGCAGG AAGGGGAAAG-3' (reverse), +30-kb region, 5'-GGT GTGAATGTGCTTTGTGG-3' (forward) and 5'-AGAA TCGGAATGGCTGTGAG-3' (reverse), the P2 promoter region (+40 kb), 5'-CGAACTTGCATCACCAGTGC-3' (forward) and 5'-GGTCGTTTACTCCGTGTTGC-3' (reverse), +50-kb region, 5'-ACATGTCCATCAGCTTC CAG-3' (forward) and 5'-CCACTGCTTGTGAGATTAA GG-3' (reverse), +55-kb region, 5'-ACCTGTTCCCAT GGATGTA-3' (forward) and 5'-TGTGTCAGGGAGCC CAAATA-3' (reverse).

Statistical analysis

Multiple comparisons were evaluated by ANOVA followed by Scheffé's *post hoc* test. Data are presented as mean \pm SD. Statistical significance was assigned at the level of $P < 0.05$.

RESULTS

The upstream alternate promoter P1 is conserved between human and mouse

It has recently been demonstrated that the human ATF3 gene contains an alternate promoter (Genbank accession number NM001030287) (35). Figure 1B, upper panel, showed two different products were amplified from

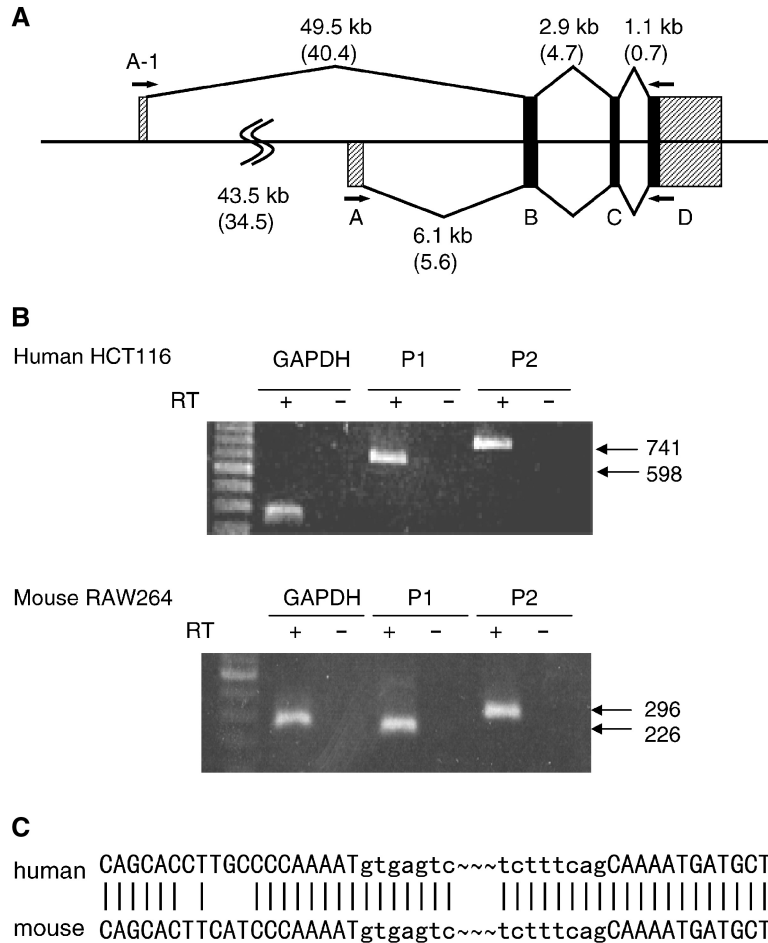


Figure 1. Conservation of the upstream alternate promoter of the *ATF3* gene in human and mouse. (A) Schematic representation of the structure of the human and mouse *ATF3* genes. Exon A is further divided into A-1 and A corresponding to the novel promoter recently identified (35) and that previously reported (27), respectively. (B) Total RNAs from serum-induced human HCT116 or mouse RAW264 cells were assayed for the expression of transcripts containing the exon A-1 or A by RT-PCR. (C) Specific bands in (B) were subcloned, sequenced and the nucleotide sequences of the junction between the exon A-1 and B of human and mouse are shown.

human cells by RT-PCR using two different 5' primers, which located at exon A-1 or canonical exon A (Figure 1A), respectively. These bands were only detected after the synthesis of the first strand cDNA using oligo-dT primer, indicating that they were derived from the matured mRNA. In Figure 1B, lower panel, a specific band was also amplified by RT-PCR of total RNA of mouse RAW264 cells using a 5' primer located at the ~34.5-kb upstream of the mouse exon A. Sequence analysis of these human and mouse transcripts revealed that the longer bands correspond to those previously reported (37,38), but the shorter ones contained novel 5'-UTRs representing the transcripts from the upstream promoter (DDBJ/EMBL/GenBank with accession number AB291912 for mouse *atf3*). In Figure 1C, the sequence comparison of the novel transcripts of human and mouse showed that the junction of the upstream exon A-1 and exon B are nearly identical. Moreover, the nucleotide sequence alignment of the human and mouse P1 promoter shows high homology (Figure 2B). Taken together, these data indicate that

the novel P1 promoter is highly conserved between human and mouse.

The upstream alternate promoter P1 contains multiple TSSs in human and mouse

To determine the TSSs from the P1 promoter of the human *ATF3* gene, we first performed a primer extension analysis using mRNA from the serum-stimulated human cells. As shown in Figure 2A, several extended products, one major and at least three minor bands were identified, indicating the presence of multiple TSSs from the P1 promoter. We further performed a 5'-RACE assay to determine the 5'-end(s) of *ATF3* transcripts. Figure 2B showed that multiple TSSs determined by 5'-RACE coincided with those by primer extension, indicating the isolation of intact mRNA and the complete reaction by reverse transcriptase. Multiple initiation sites were also observed in the mouse P1 promoter (Figure 2B). From the sequence homology of the P1 promoter and the multiple TSSs between human and mouse, it is speculated that ATAAA sequence at ~30-bp upstream from the major start site

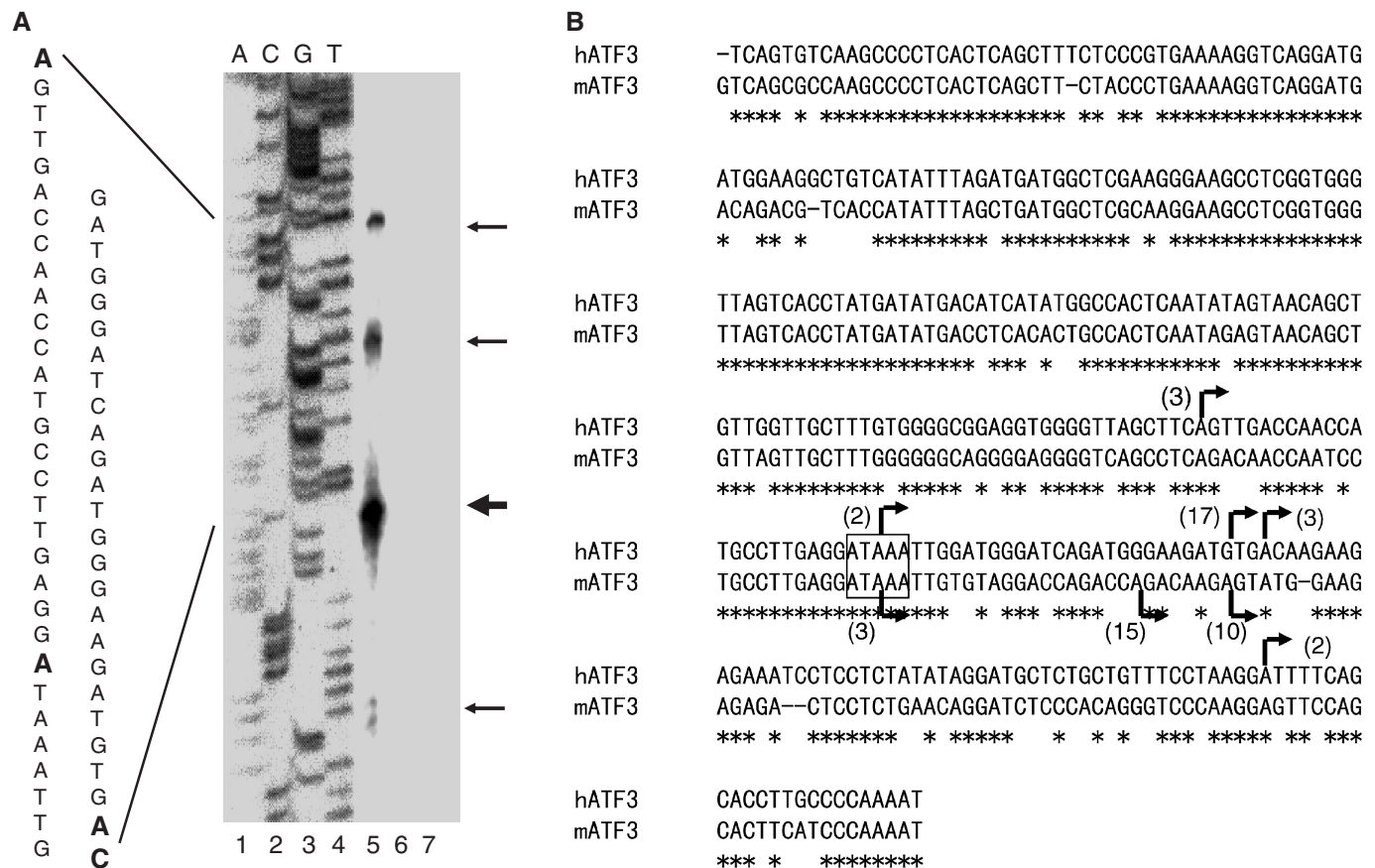


Figure 2. Structure of the upstream promoter P1 of the *ATF3* gene. (A) Primer extension analysis of mRNAs from the P1 promoter of the human *ATF3* gene. A primer specific for the P1 transcript was annealed to 1 µg of polyA mRNAs isolated from the serum-treated HCT116 cells (lane 5), 1 µg yeast tRNA (lane 6) or none (lane 7), and the TSSs were determined as in Materials and methods section. Lanes 1-4 represent a sequence ladder. The arrows on the right indicate the TSSs, and the sequence is shown on the left. Bold letters represent multiple TSSs identified. (B) Alignment of the nucleotide sequence of the P1 promoter of the human and mouse *ATF3* gene. Arrows indicate the multiple TSSs, and the numbers in parentheses represent the clones analyzed by 5'-RACE of the serum-stimulated cells. Putative TATA box is shown in the box. Multiple TSSs of the human and mouse genes were deposited in DDBJ with accession numbers AB291910 and AB291911, respectively.

is functional as putative TATA motif. Further, we observed other binding motifs for ATF/CRE, AP1, p53, E2F or NF-κB within the 2-kb upstream region of the P1 promoter (data not shown).

Differential response of the alternate promoters P1 and P2 to various stimuli

Stress response gene *ATF3* is activated by not only stress but also mitogenic stimuli such as serum, epidermal growth factor or fibroblast growth factor (16,17). As shown in Supplementary Figure S2, *ATF3* mRNA and protein was induced by serum and MMS treatment. Figure 3A showed that both the P1 and P2 transcript isoforms were induced to a similar extent by MMS treatment. In contrast, the P1 transcript was significantly induced by serum, whereas the induction of P2 transcript was more rapid but decreased earlier than the P1 transcript. To clarify the involvement of each promoter, we knocked down the P1 or P2 transcript by RNA interference-mediated gene silencing. As shown in Figure 3B, the knockdown of the P1 or P2 transcript partially suppressed the induction of *ATF3* protein in response to MMS,

demonstrating the contribution of both promoters. In contrast, in serum-stimulated cells, only the knockdown of the P1 transcript significantly reduced the *ATF3* induction, clearly defining the direct contribution of the P1 promoter in serum induction. Next, we addressed if the P1 and P2 transcripts were induced by other stimuli. Figure 3C showed that both transcripts were induced by etoposide, H₂O₂, doxorubicin, thapsigargin and tunicamycin. *ATF3* is also induced by TGF-β (13) or oncogenic *HRAS* (14), which mediates suppression of cell proliferation, tumor growth or cell senescence. Both the P1 and P2 isoforms were also induced in HaCaT human keratinocytes stimulated with TGF-β or in cells stably expressing oncogenic *HRAS* 12V or 61L, but not normal *HRAS*. The induction of the P2 transcript dominated that of the P1 transcript in these cells. Further, we examined the activation of the P1 and P2 promoter by these stimuli using a reporter assay. Figure 3D clearly showed both the P1 and P2 reporter activities were induced in response to these stimuli. Taken together, these data demonstrate that the upstream alternate promoter P1 of the *ATF3* gene is activated at the transcriptional level

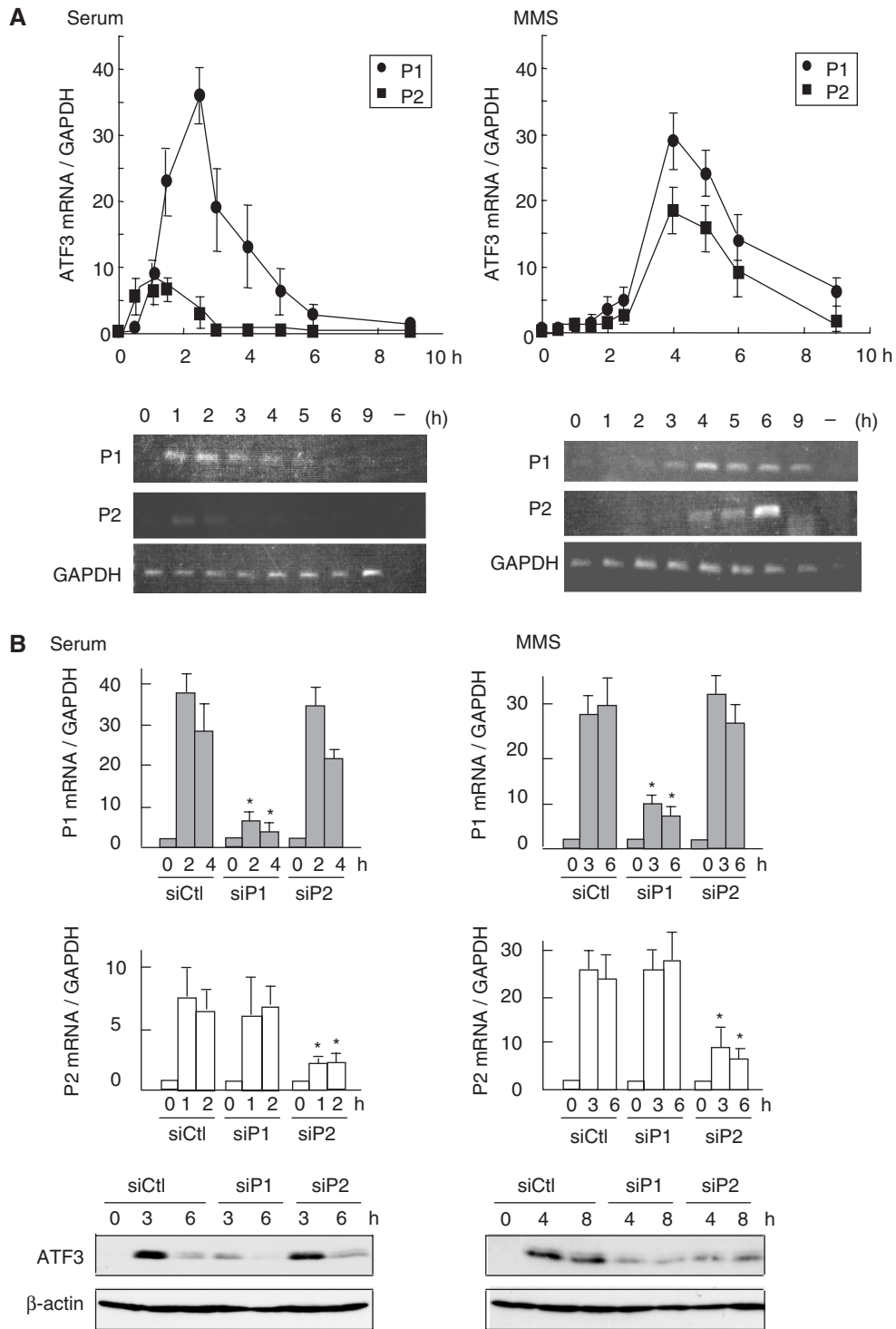


Figure 3. Differential response of the ATF3 alternate promoters to various stimuli. (A) HCT116 cells were serum-starved in the presence of 0.5% serum for 48 h, and then stimulated by 20% serum, or 100 μ g/ml MMS. At each time indicated, the ATF3 mRNA isoforms from the P1 (filled circle) or P2 promoter (filled square) was measured. Relative expression of these transcripts was normalized to GAPDH as in Materials and methods section and Supplementary Figure S1. Lower panel shows a regular RT-PCR gel of the representative experiment. (B) HCT116 cells transfected with siRNA oligos control (siCtl), specific for the P1 (siP1) or P2 transcript (siP2) were stimulated with serum or MMS as in Materials and method section. At each time indicated, the expression of the P1, P2 transcript or ATF3 protein was measured. * $P < 0.05$ compared with control siCtl. (C) HCT116 cells were treated with 20 μ g/ml etoposide (Etopo), 50 μ M H₂O₂, 1 μ M doxorubicin (Dox), 2 μ M thapsigargin (Thap), 1 μ M tunicamycin (Tuni) and HaCaT cells were treated with 100 pM TGF- β . At each time indicated, the expression of the P1, P2 transcript or ATF3 protein was measured. U2OS cells stably expressing the normal, oncogenic 12V or 61L mutants of *HRAS* were also assayed. (D) Cells transfected with pLuc-P1ATF3 (closed) or pLuc-P2ATF3 (open) were treated with various agents as in (C) for 16 h and assayed for luciferase activity. Data represent the fold induction compared to unstimulated cells. For U2OS cells expressing oncogenic *HRAS*, the fold induction of activity compared to the empty vector is shown. All the data from (A) through (D) represent means with standard error bars of three independent experiments.

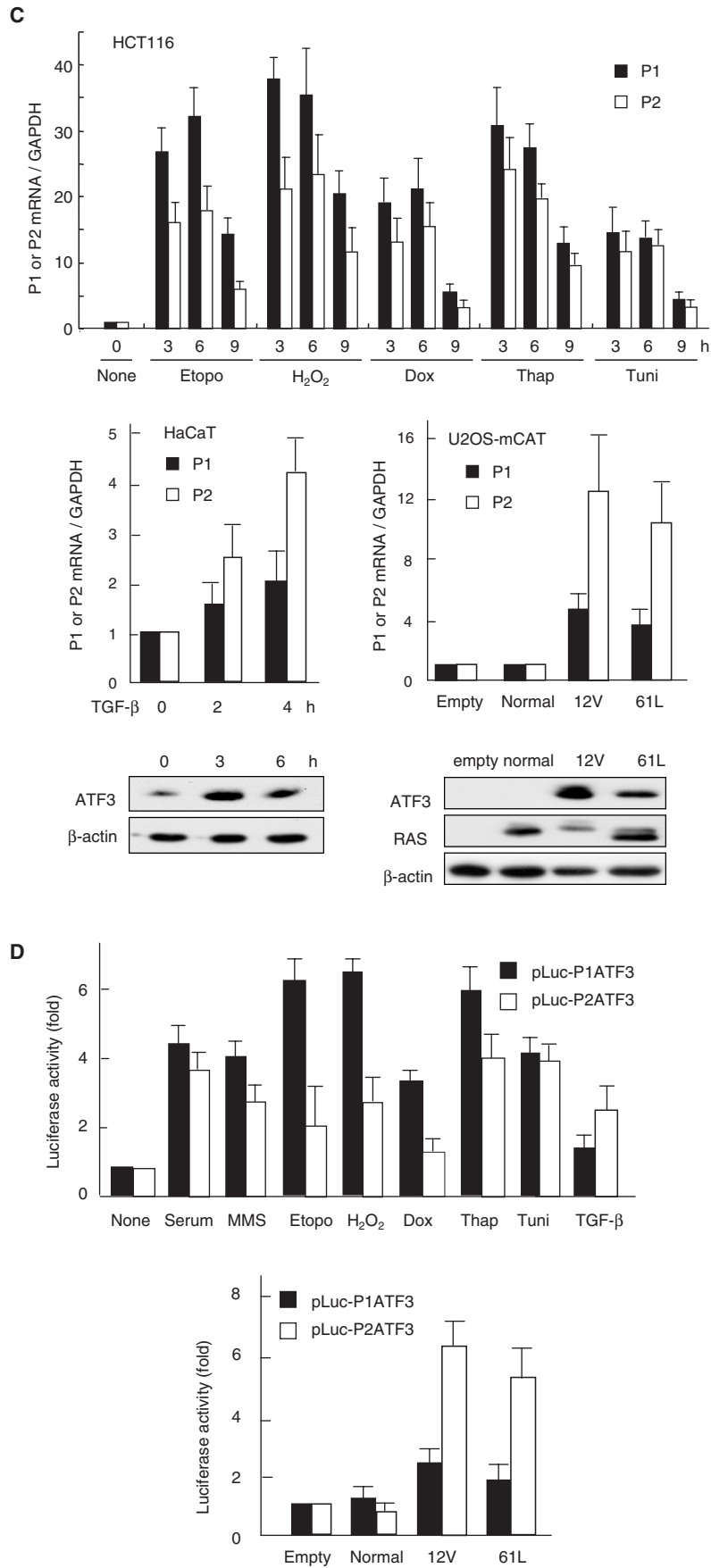


Figure 3. Continued.

by various stimuli, but differently from the canonical P2 promoter.

Regulation of translation by 5'-UTR of the transcripts from the P1 promoter

Figure 4A and Supplementary Figure S3 showed that the 5'-UTR of the P1 transcripts has unique structural properties; multiple TSSs, less score of GC content (~50%), and five upstream AUG (uAUGs) compared to the P2 transcripts; two TSSs, higher GC content (~70%), and one upstream AUG (uAUGs). Moreover, the 5'-RACE analysis revealed that multiple TSSs of the P1 promoter were differently used in MMS-treated HCT116 cells from serum-stimulated cells; the number ratio of clones obtained were 6:12:12:0 (b:c:d:e) in MMS-cells compared

to 3:2:20:2 in serum response as in Figure 2B. To explore the possible effect of different 5'-UTRs on the translational efficiency, we performed a translational reporter assay using the ATF3 expression plasmids containing different 5'-UTR of the P1 and P2 transcripts. Figure 4B showed that most of the P1 and P2 constructs generated the ATF3 protein *in vitro* with an apparently comparable efficiency, except for the long 5'-UTR P2 transcript. The relative efficiency of the *in vivo* translation of various reporters in unstimulated 293 cells appeared to be similar to those *in vitro* (Figure 4C). In contrast, in cells treated by serum or MMS, the translation of the reporters P1-c, d, e and P2-b were significantly increased, whereas the translation of the P1-a and P1-b constructs was barely activated (Figure 4D). This suggests the presence of not only the inhibitory sequence(s) between a and c but also the

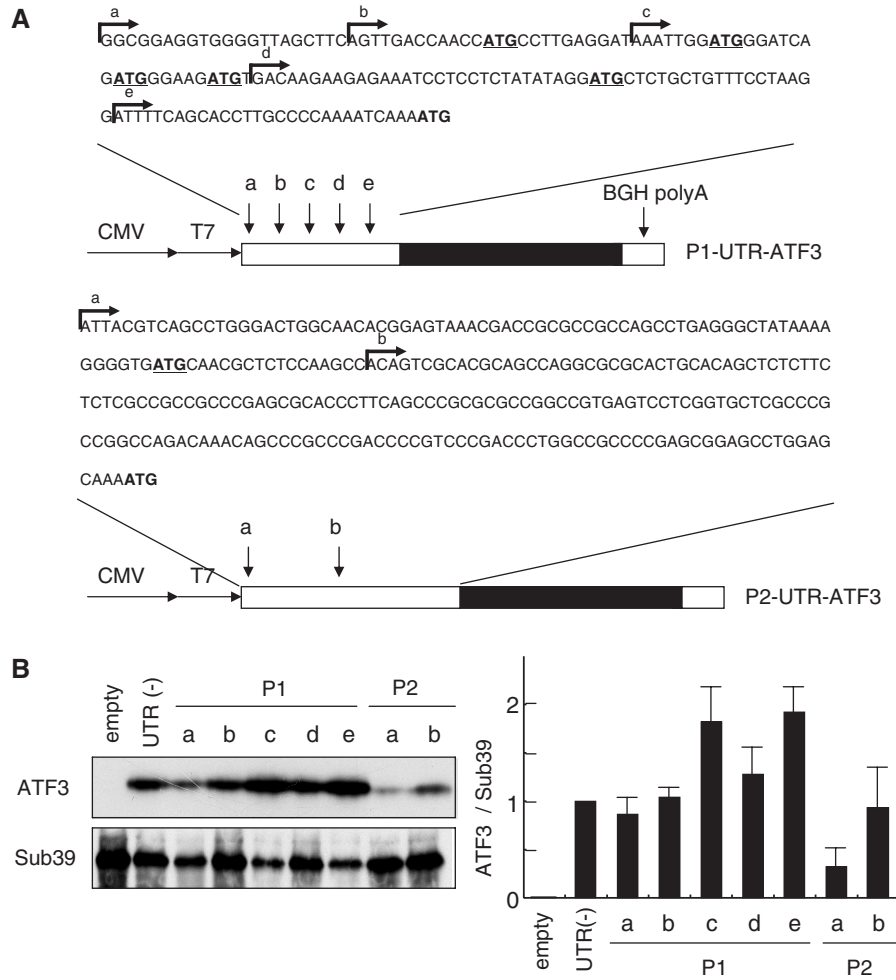


Figure 4. Efficiency of translation of the *ATF3* mRNA isoforms from the P1 and P2 promoters. (A) Expression reporter plasmids encoding the *ATF3* transcripts with various 5'-UTRs from the P1 (upper panel) and P2 promoters (lower panel) are shown. Arrows a through e indicate the 5'-ends of mRNAs corresponding multiple TSS of the P1 promoter in Figure 2B, and arrows a and b indicate the 5'-ends the P2 transcripts obtained from the database and reference (27). (B) Each plasmid was assayed for the *in vitro* translation as described in the Materials and methods section. The relative expression of *ATF3* to plasmid without UTR is shown. (C) 293 cells were transfected with each expression plasmid, cultured for 24 h, and the *ATF3* mRNA and protein generated from the transgene was measured as in the Materials and methods section. Relative efficiency of translation is the ratio of the *ATF3* protein normalized to mRNA compared to that without 5'-UTR. (D) 293 cells were transfected with each plasmid, starved with 0.5% serum, followed by stimulation with 20% serum for 24 h or 50 μg/ml MMS for 12 h. After measurement of both *ATF3* protein and mRNA generated from the transgene, relative efficiency of translation is shown as in (C). All the data from (B) through (D) represent the means with standard error bar of three independent experiments.

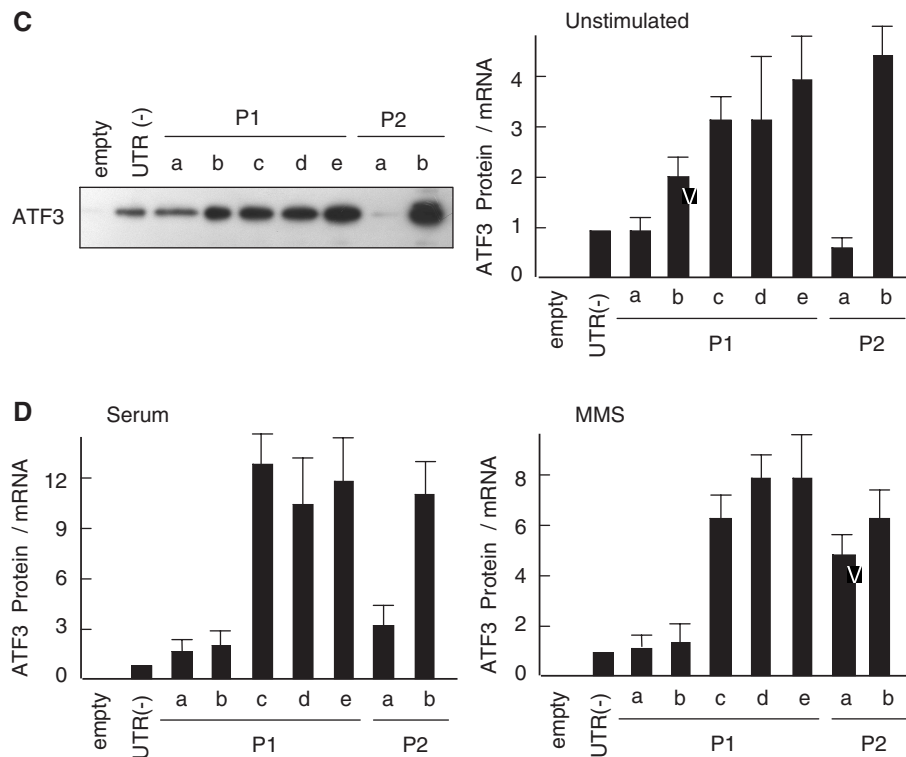


Figure 4. Continued.

stimulatory element(s) in the shorter UTR forms, c, d and e. Taken together, these data indicate that the structure of 5'-UTRs of the P1 transcripts as well as the differential use of TSSs significantly affects their translation in response to stress.

Constitutive activation of the P1 promoter in ATF3-expressing human cancer cells

ATF3 is constitutively overexpressed in several forms of human cancer cells such as prostate, Hodgkin disease and mammary tumors (23–25), and the elevated ATF3 expression correlates to the cell proliferation, progression and metastasis (23–25,39). To clarify the functional role of alternate promoters in these cells, we examined the expression of ATF3 from each promoter. Figure 5A showed that, in human prostate cancer LNCaP and Hodgkin Reed–Sternberg L428 and L540 cells, the amount of ATF3 protein was increased as reported (23,24). Most significantly, the P1 transcript was dominantly expressed in these cells compared to DU145 and PC3 or DAUDI and MOLT 4 cells, demonstrating that the P1 promoter is selectively activated. To further clarify the actual involvement of each promoter, we knocked down the P1 or P2 transcripts. Figure 5B clearly showed the knockdown of the P1 transcript drastically suppressed the expression of the ATF3 protein along with the P1 transcript in LNCaP, L428 and L540 cells, whereas the P2 knockdown had only marginally effect. The data strongly support that the P1 promoter has a direct contribution to the elevated expression of ATF3 in these cancer cells. We next performed ChIP

assay to examine the association of RNA polymerase II with the *ATF3* gene locus. Figure 5C showed that, in LNCaP cells, RNA polymerase II and Leo1, a component of PAF1 complex in the elongating RNA polymerase II (40), were clearly recruited through the P1 promoter to downstream regions, compared to –5-kb region and the control IgG, whereas the Leo1 signal peaked at around +10-kb region. In contrast, DU145 cells that had lower expression of ATF3 did not show significant recruitment of RNA polymerase II. The increased association of RNA polymerase II to the P1 promoter region was also observed in Hodgkin L428 cells (Supplementary Figure S4). These data unambiguously indicate the upstream P1 promoter of the *ATF3* gene is constitutively activated in human prostate and Hodgkin Reed–Sternberg cells.

Chromatin modification of the human ATF3 gene in cancer cells

Constitutive activation of the upstream P1 promoter in cancer cells prompted us to examine the chromatin modifications of the *ATF3* gene promoter. As shown in Figure 6A, antibodies against the pan-acetylated histone H3 and trimethylated H3K4me3 efficiently immunoprecipitated the P1 promoter region in LNCaP and Hodgkin 428 cells, compared to DU145 or DAUDI cells. In contrast, no significant H3K9 methylation was observed at the ATF3 P1 promoter region in LNCaP or L428 cells. As an inactivated gene control, the ChIP signal of H3K9 methylation at the β -globin gene promoter in L428 cells was also shown (Figure 6A).

Figure 6B and Supplementary Figure S5 further showed that the significant pan-acetylation and K4 trimethylation of H3 were detected from the P1 promoter to +10-kb region compared to other region in LNCaP and L428 cells, respectively. Taken together, the P1 promoter of the *ATF3* gene in LNCaP and Hodgkin L428 cells has an active chromatin configuration associated with the histone H3 acetylation and H3K4 trimethylation.

DISCUSSION

In the present report, we described the structural and functional properties of the upstream alternate promoter P1 of the human *ATF3* gene. The P1 promoter is used differently from the P2 promoter at the transcriptional and translational level in stress response. Moreover, it is constitutively activated in human cancer cells associated with the active chromatin configuration, as a consequence, followed by the elevated expression of ATF3.

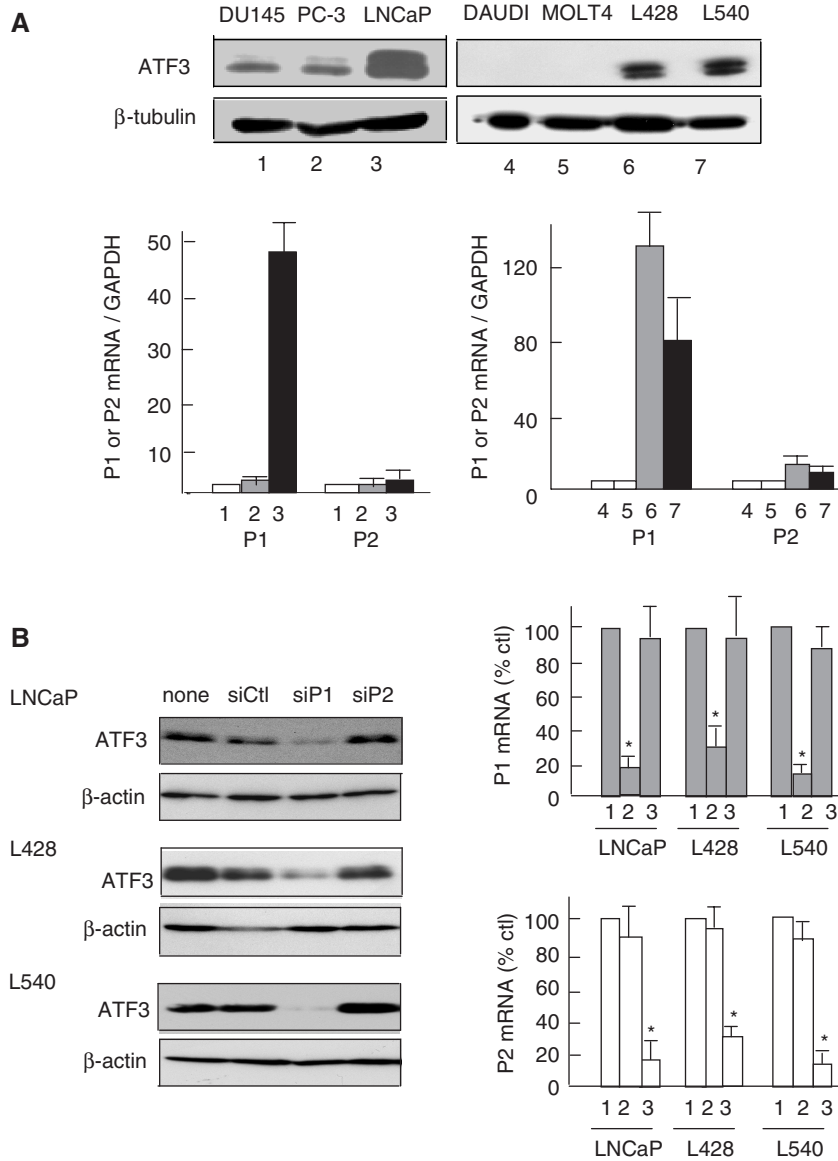


Figure 5. Constitutive activation of the upstream P1 promoter in human prostate and Hodgkin RS cancer cells. (A) The expression level of ATF3 protein in DU145 (lane 1), PC-3 (lane 2), LNCaP (lane 3), DAUDI (lane 4), MOLT4 (lane 5), Hodgkin RS L428 (lane 6) or L540 (lane 7) cells was measured by western blot (upper panel). In the lower panel, total RNAs from these cells were assayed for the P1 or P2 transcripts as in Figure 3A. Relative expression of the *ATF3* transcripts represents the ratio to that of *GAPDH*. (B) LNCaP, L428 or L540 cells were transfected with control siRNA oligos (siCtl, lane 1), or specific for the P1 (siP1, lane 2) or P2 transcript (siP2, lane 3), cultured for 48 h and assayed for the P1 and P2 transcripts or ATF3 protein. The relative expression of the P1 and P2 transcripts is shown as per cent of the control (siCtl). **P* < 0.05 compared with control siCtl. (C) LNCaP (filled circle, open circle) or DU145 (filled triangle, open triangle) cells were fixed with formaldehyde and immunoprecipitated using anti-RNA polymerase II (upper panel) or anti-Leo1 antibody (lower panel) as in Materials and methods section. Immunoprecipitated DNA was measured by quantitative PCR throughout the human *ATF3* gene locus and expressed as percent of the input DNA for the control IgG (open) and specific antibodies (closed), respectively. All the data from (A) through (C) represent the means of three independent experiments with standard error bars.

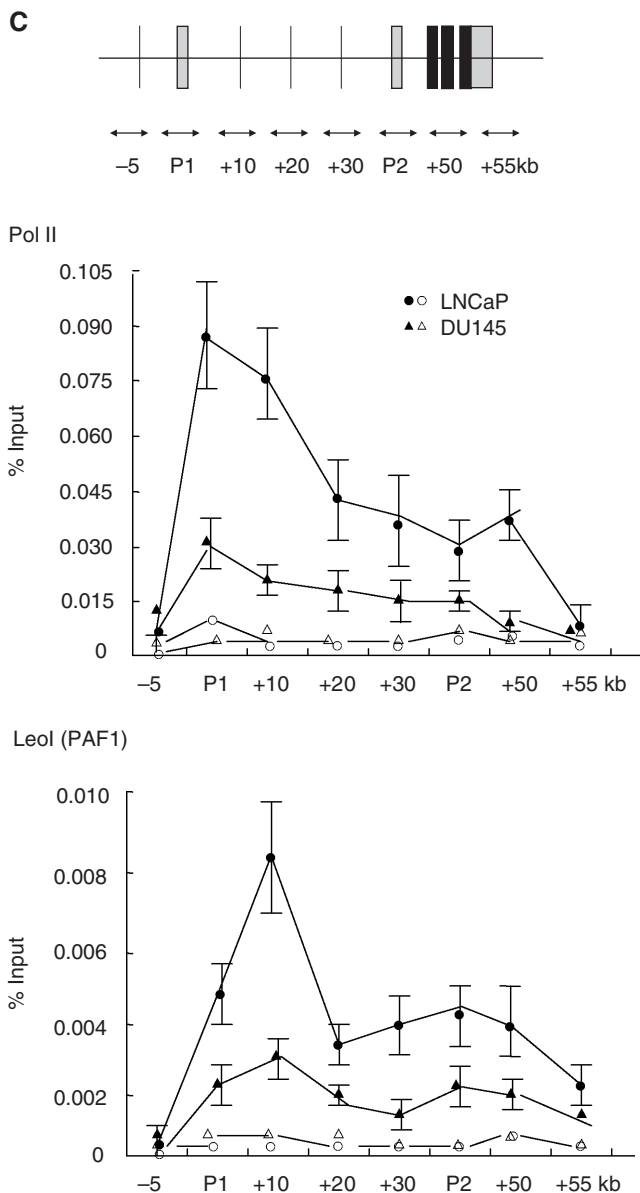


Figure 5. Continued.

Kimura *et al.* (35) reported that at least 52% of the human genes contain putative alternative promoters, with 3.1 alternative promoters per gene on average, and the genes subject to regulation by alternative promoters are enriched in those encoding signal transduction-related proteins. Indeed, the *ATF3* gene encodes a transcription factor that is induced and plays role in various stress response and signaling. The P1 promoter of the *ATF3* gene was highly conserved between human and mouse, which localizes ~43.5-kb and ~34.5-kb upstream of the previously reported canonical promoter P2 (30), respectively. The P1 promoter has several properties that are conserved between human and mouse. (i) Sequence of the promoter region as well as the junction of exon A-1 and B are highly conserved (Figure 1C and 2B). The putative TATA motif is localized ~30 base upstream from the

major TSS, and other binding motifs for AP1, ATF/CREB, NF- κ B, E2F, or p53 are conserved. (ii) Multiple TSSs are present in the human and mouse genes. (iii) The P1 promoter has less stretch of CG dinucleotides, whereas the P2 promoter contains at least three CpG islands within the 2-kb region of the promoter. This structural difference between the P1 and P2 promoters is of intrigue, because there is one CpG-island-containing promoter per 2.6 CpG-less alternate promoters (35). Overall, the structural properties of the P1 promoter strongly support that it is evolutionally conserved between human and mouse, and has distinct control mechanism.

The P1 promoter of the *ATF3* gene in human cells was activated by various stimuli including serum, DNA damage and oxidoreductive stress, and the extent of the P1 promoter activation was comparable to or exceeded that of the P2 promoter (Figure 3). Indeed, the 5'-flanking region of the P1 promoter contains several motifs for transcription factors involved in the signaling of stress stimuli, including the binding motifs for ATF/CRE, AP1, NF- κ B, E2F, c-Myc or p53. Whereas the significance and implication of these motifs in each stress response was not addressed in this report, it is also possible that a common motif(s) in the *ATF3* gene locus confers stress response on both the P1 and P2 promoters. This is an important issue and must await further study.

Various 5'-UTRs of the P1 transcripts contain the features of poorly translated mRNA, that is relatively long 5'-UTR with multiple upstream AUGs (uAUGs) and upstream ORFs (Figures 2B and 4A). These features are often found in mRNAs encoding regulatory proteins like proto-oncogenes or growth factor (41), consistent with biological role of the *ATF3* gene. In our translational reporter assay, however, sequential mutations of uAUGs in the 5'-UTR of the P1 transcripts had no significant effect on their translation (data not shown). The assay revealed the presence of the inhibitory region in the longest 5'-UTR as well as the stimulatory element(s) in the shorter 5'-UTRs, implicating a role of *trans*-acting factor(s) interacting with these element(s) in the 5'-UTR in stress response. The identification of such factor(s) and the corresponding *cis*-element(s) is an important issue of further study. It should be noted that the present study employed the reporters containing only the different 5'-UTRs, since the P1 and P2 transcripts have the common sequence from the coding through the 3'-UTR (NM_001030287 and NM_001674). However, it is possible that the 3'-UTR may contribute to the regulation, since 3'-UTR is well known to affect the translational efficiency *in vivo*. More importantly, the 5'-RACE analysis revealed the differential usage of TSSs in MMS- and serum-treated cells. Thus, it is highly likely that the selection or shift of the TSSs within the P1 promoter also regulates the efficiency of translation *in vivo*.

Early studies showed that the *ATF3* induction correlates with cell-detrimental outcome such as cell cycle arrest or apoptosis, thus providing an idea that it is death factor (9–12). However, *ATF3* is also implicated in cell proliferation (13,15,18,22), transformation (19), cell survival (20,21) and tumorigenesis (14,23–26). Our present study demonstrated for the first time that the P1

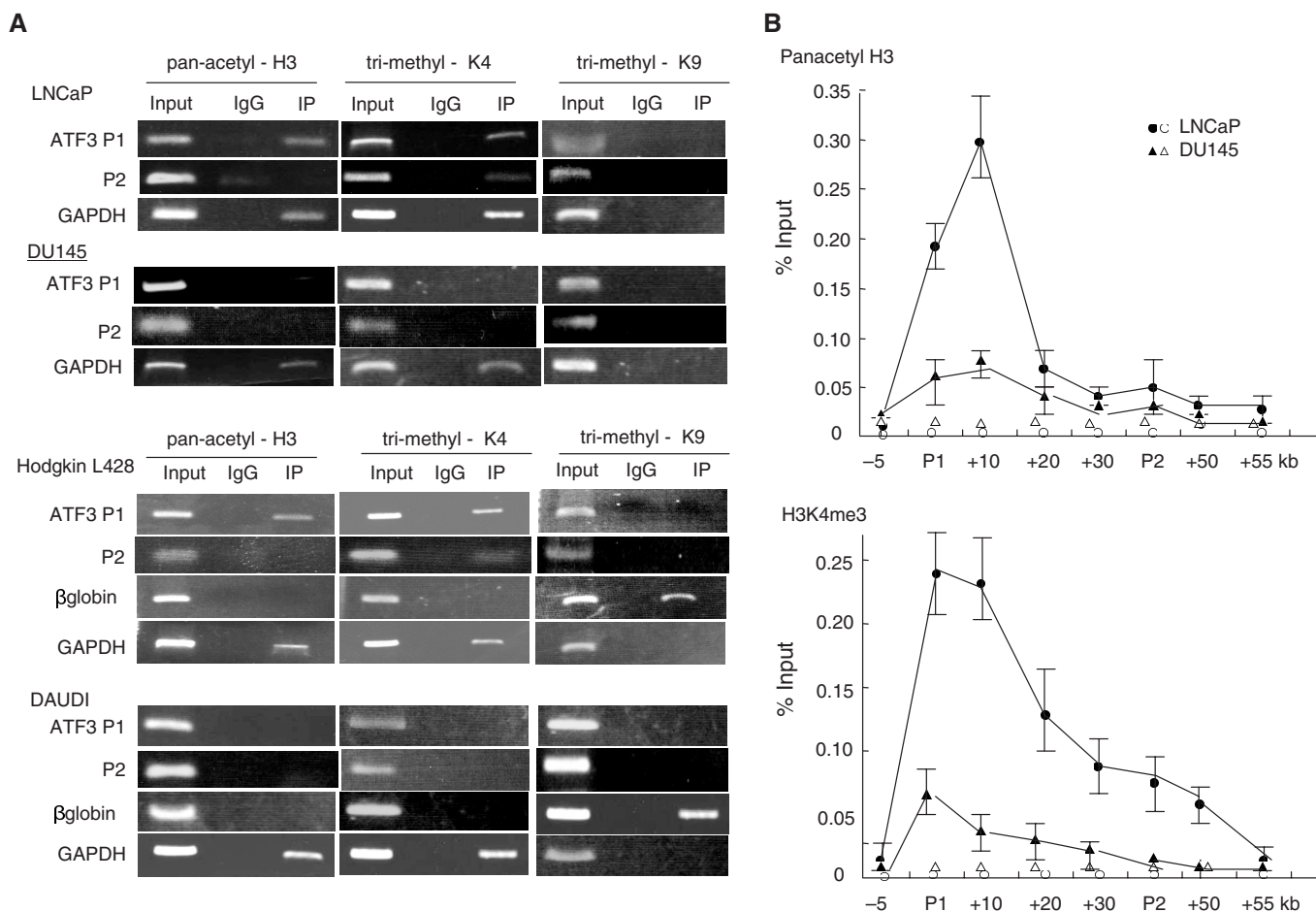


Figure 6. Histone modifications across the human *ATF3* gene locus in LNCaP and Hodgkin L428 cells. (A) ChIP assay was performed in LNCaP, DU145, L428, or DAUDI cells using anti-panacetyl H3, anti-trimethyl H3K4 or anti-trimethyl H3K9 antibodies as in Materials and Methods section. The P1 and P2 promoter region of the *ATF3* gene and the control *GAPDH* gene was amplified and analyzed by gel electrophoresis. In L428 and DAUDI cells, the promoter region of the human β -globin gene was also examined. (B) Immunoprecipitated DNA by anti-panacetyl H3 or anti-trimethyl H3K4 antibodies of LNCaP (filled circle, open circle), DU145 (filled triangle, open triangle) cells was measured by quantitative PCR throughout the human *ATF3* gene locus, and expressed as percent of the input. Data represent the means of three independent experiments with standard error bars for control IgG (open) and specific antibodies (closed), respectively.

promoter is constitutively activated in human cancer cells with the elevated expression of ATF3 protein. In LNCaP and Hodgkin cancer cells, the chromatin structure of the P1 promoter region is remodeled and fixed to an active open state through the histone modification, including histone H3 acetylation and H3K4 trimethylation (Figure 6). As in Figure 3A, the serum response of the P2 promoter was more rapid but suppressed earlier than the P1 promoter. It is speculated that the P2 promoter responds to serum, but is suppressed by the transcription from the upstream promoter P1 through a mechanism called delayed transcriptional interference (42), while it is also possible that the splicing of the transcripts is differently regulated. At this moment, however, it is not known to what extent these mechanisms could be applied to constitutive activation of the P1 promoter in LNCaP and Hodgkin cancer cells. It is also unlikely that *cis*-element(s) of the promoter responsible for serum response is the only mechanism by which the P1 promoter is constitutively activated in cancer cells, since the P2 promoter is induced

by serum in rat fibroblasts via ATF/CRE motif (22), and the reporter activity driven by the P2 promoter was activated by serum as efficiently as the P1 promoter (Figure 3D).

In conclusion, the present study for the first time described the molecular and functional properties of the upstream alternate promoter P1 of the stress response gene *ATF3*. Of intrigue, the expression from the P1 promoter is regulated at both the transcriptional and translational levels, and is constitutively activated with altered chromatin structure in human cancer cells. The differential usage and regulation of the alternate promoters of the human *ATF3* gene adds an important dimension to the control mechanism by which it plays dual role in determining cell fate during stress response and oncogenesis.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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