Enhanced Expression of the Human Vacuolar H\textsuperscript{+}-ATPase c subunit Gene (ATP6L) in Response to Anticancer Agents*  

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We have isolated two overlapping genomic clones that contain the 5′-terminal portion of the human vacuolar H\textsuperscript{+}-ATPase c subunit (ATP6L) gene. The sequence preceding the transcription initiation site, which is GC-rich, contains four GC boxes and one Oct1-binding site, but there is no TATA box or CCAAT box. In vivo footprint analysis in human cancer cells shows that two GC boxes and the Oct1-binding site are occupied by Sp1 and Oct1, respectively. We show here that treatment with anticancer agents enhances ATP6L expression. Although cisplatin did not induce ATP6L promoter activity, it altered ATP6L mRNA stability. On the other hand, the DNA topoisomerase II inhibitor, TAS-103, strongly induced promoter activity, and this effect was completely eradicated when a mutation was introduced into the Oct1-binding site. Treatment with TAS-103 increased the levels of both Sp1/Sp3 and Oct1 in nuclear extracts. Cooperative binding of Sp1 and Oct1 to the promoter is required for promoter activation by TAS-103. Incubation of a labeled oligonucleotide probe encompassing the −73/−68 GC box and −64/−57 Oct1-binding site with a nuclear extract from drug-treated KB cells yielded higher levels of the specific DNA-protein complex than an extract of untreated cells. Thus, the two transcription factors, Sp1 and Oct1 interact, in an adaptive response to DNA damage, by up-regulating expression of the vacuolar H\textsuperscript{+}-ATPase genes. Furthermore, combination of the vacuolar H\textsuperscript{+}-ATPase (V-ATPase) inhibitor, bafilomycin A1, with TAS-103 enhanced apoptosis of KB cells with an associated increase in caspase-3 activity. Our data suggest that the induction of V-ATPase expression is an anti-apoptotic defense, and V-ATPase inhibitors in combination with low-dose anticancer agents may provide a new therapeutic approach.

Tumor cells possess high glycolytic activity, and rapid growth produces acidic metabolites. Moreover, tumor cells often exist in an hypoxic microenvironment lower in pH than that of surrounding normal cells. Hence, proton extrusion may be up-regulated to protect tumor cells from acidosis. Four major types of pH regulators have been identified in tumor cells as follows: sodium-proton exchangers, bicarbonate transporters, proton-lactate symporters, and proton pumps. The vacuolar H\textsuperscript{+}-ATPase (V-ATPase)\textsuperscript{1} is ubiquitously expressed in eukaryotic cells (1–6), not only in vacuolar membranes but also in plasma membrane (7–9). It is a multisubunit enzyme composed of a membrane sector and a cytosolic catalytic sector (10); it pumps protons from the cytoplasm to the lumen of the vacuole and also regulates cytosolic pH. V-ATPase is active in the plasma membrane of human tumor cells (11), and V-ATPase genes are considered “housekeeping genes.” However, cytosolic pH is critical for the cytotoxicity of anticancer agents (12), and cellular acidosis is thought to be a trigger for apoptosis and to play a role in drug resistance. Therefore, understanding the mechanisms regulating tumor acidity is important for developing new approaches to cancer chemotherapy.

By using differential display, we have shown that one of the proton pump subunit genes, ATP6L (subunit c), is induced by cisplatin (13), and several V-ATPase subunit genes are up-regulated in drug-resistant cell lines (13, 14). Interaction of the V-ATPase c subunit with β3 integrin has been reported (15, 16), and β3 integrin-mediated signaling prevents lung cancer cells from drug-induced apoptosis. The level of the V-ATPase c subunit may be critical for V-ATPase activity. In order to study transcriptional regulation of the c subunit at the molecular level, we have identified its promoter sequences and characterized the transcription factors that regulate its expression in cancer cells. We hypothesized that V-ATPase expression is up-regulated in response to cellular acidosis and show that c subunit promoter activity is activated by treatment with anticancer agents, especially the DNA topoisomerase II inhibitor, TAS-103 (17, 18), which can induce cellular acidosis (19). We show also that the levels of two transcription factors, Sp1 and Oct1, increase in response to genotoxic stress and that V-ATPase inhibition strongly enhances TAS-103-induced apoptosis.

MATERIALS AND METHODS
Isolation of V-ATPase Subunit c (ATP6L) Genomic Clones and DNA Sequencing—ATP6L genomic clones were isolated from a human placental genomic library in EMBL3 by screening with cDNA. All positive clones were also used as hybridization probes to confirm the overlapping regions. Two genomic DNA fragments around the first exon were subcloned into pUC18 (Fermentas AB, Lithuania) and sequenced with an Automated sequencer 377 (PE Applied Biosystems).

Primer Extension Analysis—The primer, 5′-GTCACATGACCCT

1 The abbreviations used are: V-ATPase, vacuolar H\textsuperscript{+}-ATPase; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; nt, nucleotide; PIPES, 1,4-piperazinediethanesulfonic acid; DMS, dimethyl sulfate.
ATP6L Gene Expression

GGGCCCGG-3', derived from the first exon of ATP6L, was labeled at its 5' end and hybridized with poly(A) RNA from KB cells in 80% formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.4), and 1 mM EDTA for 4 h at 52 °C. The primer-RNA hybrid was precipitated and resuspended in reverse transcriptase mixture (Invitrogen). After 1 h of incubation at 42 °C, the reaction was terminated by making the solution 20 mM in EDTA. The RNA was hydrolyzed with 0.125 M NaOH for 1 h at 65 °C, the reaction neutralized, and the extended DNA then precipitated with alcohol. The DNA was analyzed on a 7 M urea, 6% polyacrylamide gel to determine the size of the extended product. Sequencing reactions using the same primer were similarly analyzed.

Cell Culture and Antibody—Human epidermoid cancer KB cells (20), human prostate cancer PC3 cells (21), and human breast cancer MCF7 cells (22) were cultured in Eagle’s minimal essential medium (Nissui Seiyaku Co., Tokyo, Japan) or Dulbecco’s modified Eagle medium (Nissui Seiyaku Co., Tokyo, Japan) containing 10% fetal bovine serum, 0.292 mg/ml l-glutamine, 100 units/ml penicillin, and 100 mg/ml kanamycin. The anti-Sp1 (catalogue number sc-420 for supershift assay, sc-59 for chromatin immunoprecipitation assay, and Western blotting), anti-Sp3 (sc-644), anti-Oct1 (sc-232), and anti-Oct2 (sc-233) antibodies were purchased from Santa Cruz Biotechnology. Antiserum to V-ATPase subunit C was generated by immunization of a New Zealand White rabbit with synthetic peptides as described (23). The sequence of the synthetic peptides is ALFGANANRKFLD.

Northern—Total RNA was isolated using Sepasol reagent (Nacalai Tesque, Kyoto, Japan). RNA samples (20 μg/lane) were separated on a 1% formaldehyde-agarose gel and transferred to a Hybond N+ filter (Amer sham Biosciences) with 10% SSC. Prehybridization and hybridization were performed as described (24). For analysis of stability of V-ATPase subunit transcripts by cisplatin or TAS-103, KB cells were treated with ac tinomycin D (1 μg/ml) and cisplatin (10 μM) or TAS-103 (4 μM) for 6 h. Cisplatin was purchased from Sigma, and TAS-103 was kindly provided from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan).

Separation of Membrane Fractions—KB cells were treated with or without TAS-103 for 12 h. Briefly, cells were homogenized in 0.25 M sucrose, and the homogenates were centrifuged at 3,000 rpm for 10 min. The supernatant was centrifuged at 15,000 rpm for 30 min. The pellets were resuspended to 0.25 M sucrose. The resuspension was sonicated with 2.10 and 1.25 M sucrose cushions and centrifuged at 24,000 rpm for 12 h. The membrane fractions at the 0.25–1.25 M sucrose interface were collected and used for Western blotting.

Immunoprecipitation Assay—For metabolic labeling, KB cells in a 100-mm tissue culture dish were cultured in Dulbecco’s methionine and cysteine-free modified Eagle medium (Invitrogen) supplemented with 1% dialyzed fetal calf serum and were labeled with 50 μCi/ml [35S]methionine and -cysteine labeling mixture (Amer sham Biosciences) with or without 4 μM TAS-103 for 12 h. After washing the cells twice with ice-cold phosphate-buffered saline (PBS), cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM PMSF). After centrifugation at 9,000 rpm for 5 min, the supernatant (cellular fraction) were incubated with anti serum to V-ATPase subunit C or preimmune binding to 15 μg of protein A/G-agarose. The mixtures were incubated for 4 h at 4 °C and washed three times with RIPA buffer. Immunoprecipitation samples and 1% of preimmunoprecipitation samples (input) were simultaneously separated on a 15 or 10% polyacrylamide gel. Immunoprecipitated samples and 1% of input samples were separated on a 15 or 10% polyacrylamide gel. Immunoprecipitated samples and 1% of input samples were separated on a 15 or 10% polyacrylamide gel.

Construction of pV-ATPase c Luc luciferase reporter plasmid—KB cells were digested with XbaI and Smal (Nippon Gene, Tokyo) fragment (nt 715 to 194) of the V-ATPase c promoter primers are as follows: 5'-CGGCGAGACCGGTCCAACGCT-3' (primer 1); 5'-GCGCGGAACACCAATCGCAGACGACG-3' (primer 2); 5'-CCGGAACCACTGCGACAGACGACG-3' (primer 3); and 5'-AATGATCGTGTCGCACTTTGG-3' (primer 4). Amplification was performed by using primers in an optimized number of cycles. PCR products were separated by electrophoresis on 2% agarose gel, which were stained with ethidium bromide.

Expression of V-ATPase c promoter reporters were assayed using pCMV, pCMV 6×His, or pCMV 6×Myb. After transfection for 12 h, the cells were washed, incubated at 37 °C for 12 h in fresh medium or in medium containing either TAS-103 (4 μM) or cisplatin (10 μM), and then harvested. For co-transfection experiments with Sp1, Sp3, Oct1, and Oct2 expression plasmids, PC3 cells were transfected with 0.2 μg of luciferase reporter plasmid (pV-ATPase c Luc3) and 0.4 μg of expression plasmid. After transfection for 12 h, the cells were incubated at 37 °C for 24 h in fresh medium and then harvested.

Luciferase Assay—Lysed cells were assayed for luciferase activity using a Picogene kit (Toyoinoki, Tokyo, Japan); the light intensity was measured for 15 s with a luminometer (Dynatech ML1500, JEO, Japan). The β-galactosidase enzyme assay was performed according to the protocol of Promega.

Chromatin Immunoprecipitation—Protein-DNA cross-linking was performed by incubating KB cells with formaldehyde at a final concentration of 1% for 10 min at room temperature. Cells were washed with PBS and collected by centrifugation at 1,200 rpm for 5 min. Cells were then lysed in buffer X (50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 1 mM PMSF) for 15 min on ice. The lysate was sonicated with 10 pulses of 10 s each at 50% of maximum power. Tissue culture plates at a concentration of 106 KB cells were prepared as described (27). Briefly, 2×107 cells were seeded into 12-well tissue culture plates at a concentration of 4×104 KB cells, PC3 cells, and MCF7 cells. After the day, cells were transfected with 0.4 μg of luciferase reporter plasmid DNA using 2 μl of Superfect reagent (Qiagen, Germany) according to the manufacturer’s instructions. The β-galactosidase reporter gene (pSV-b-gal, Nippon Gene, Tokyo) was co-tran- sfect, targeting by cell fractionation. After incubation for 12 h, the cells were washed, incubated at 37 °C for 12 h in fresh medium or in medium containing either TAS-103 (4 μM) or cisplatin (10 μM), and then harvested for co-transfection experiments with Sp1, Sp3, Oct1, and Oct2 expression plasmids, PC3 cells were transfected with 0.2 μg of luciferase reporter plasmid (pV-ATPase c Luc3) and 0.4 μg of expression plasmid. After transfection for 12 h, the cells were incubated at 37 °C for 24 h in fresh medium and then harvested.

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incubated on ice for 15 min. The cells were lysed with a dropping of 0.6% Nonidet P-40, and the lysate was centrifuged at 3,000 rpm for 10 min. The resulting nuclear pellets were resuspended in 50 μl of ice-cold buffer S (20 mM HEPES-KOH (pH 7.9), 50 mM NaCl, 0.1 mM MgCl₂, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM DTT), and KCl was added to a final concentration of 0.5 M, incubated on ice for 15 min as with frequent gentle mixing. Following centrifugation for 5 min at 1°C in a microcentrifuge to remove insoluble material, the supernatant (nuclear extract) was stored at −70°C. Nuclear extracts using buffer C were also prepared as described (28). Briefly, 2 × 10⁷ cells were collected with PBS, suspended in 1 ml of ice-cold 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 0.5 mM PMSF, 1 mM DTT, and 0.1 mM EDTA, 0.1 mM EGTA, and incubated on ice for 15 min. The cells were lysed with a dropping of 0.6% Nonidet P-40, and the lysate was centrifuged at 3,000 rpm for 10 min. The resulting nuclear pellets were resuspended in 50 μl of ice-cold buffer C (20 mM HEPES-KOH (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSP, and 1 mM DTT) and incubated for 15 min on ice with frequent gentle mixing. Following centrifugation for 5 min at 4°C in a microcentrifuge, the supernatant was stored at −70°C. Its protein concentration was determined by the method of Bradford.

EMSAs—EMSAs were performed as described (29). Briefly, 4 μg of nuclear extract proteins prepared with buffer S were incubated for 30 min at room temperature in a final volume of 20 μl containing 20 mM HEPES-KOH (pH 7.9), 0.1 mM PMSP, 0.1 mM DTT, 57.5% glycerol, 0.5 μg of poly(dI-dC), and 1 × 10⁴ cpm (1 ng) of 32P-labeled oligonucleotide probe in the absence or presence of various competitors. The probe was synthesized using the primer CACCACACCGCCCCGCCCCG-3′ and TACGATTACTTCGTGTG-5′. EMSA samples were electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS and 32P-labeled oligonucleotide probe. Products were analyzed on nondenaturing 4% polyacrylamide gels using a bio-imaging analyzer (BAS 2000; Fuji Photo Film, Tokyo). The sequences of oligonucleotides used for EMSAs are as follows: Oligo1 (–118 to –89), 5′-CTCGACGACGCGACGCGAGAGGC-3′ and 5′-ACGTCTC-GTGGCCGCTGGCGCTCCGGC-5′; Oligo2 (–98 to –69), 5′-CAGACGCGACGCGACGCGAGAGGC-3′ and 5′-CTGCACGACGCGACGCGAGAGGC-5′; Oligo3 (–78 to –99), 5′-CCCGAGCCTCGCGCCGGGGG-3′; Oligo5 (–8 to –29), 5′-AGGCGC-CCCGGCTATGTAATAAGGACA-3′ and 5′-CCCGGGCCGGGCAAGGACGACTGTTG-5′; Oligo6 (–38 to –9), 5′-CCCCGGCGCCGGCCGGCCGGCCGCAGAGGC-3′ and 5′-ACTCCTGGTGTTGGTGAGGC-5′; Oligo6-1 (–38 to –9), 5′-CCCCGGCGCCGGCCGGCCGGCCGCAGAGGC-3′ and 5′-ACTCCTGGTGTTGGTGAGGC-5′; Oligo6-2 (–38 to –9), 5′-CCCCGGCGCCGGCCGGCCGGCCGCAGAGGC-3′ and 5′-ACTCCTGGTGTTGGTGAGGC-5′; Oligo6-3 (–38 to –9), 5′-CCCCGGCGCCGGCCGGCCGGCCGCAGAGGC-3′ and 5′-ACTCCTGGTGTTGGTGAGGC-5′; Oligo6-4 (–38 to –9), 5′-CCCCGGCGCCGGCCGGCCGGCCGCAGAGGC-3′ and 5′-ACTCCTGGTGTTGGTGAGGC-5′; Oligo6-5 (–38 to –9), 5′-CCCCGGCGCCGGCCGGCCGGCCGCAGAGGC-3′ and 5′-ACTCCTGGTGTTGGTGAGGC-5′. For supershift assay, nuclear extracts were incubated with probes and 2 μg of anti-Sp1, anti-Sp3, anti-Oct1, and anti-Oct2 antibodies, and the DNA-protein complexes were resolved on a 4% non-denaturing polyacrylamide gel.

RESULTS

To isolate genomic clones encoding the 5′ region of the ATP6L gene, a human genomic library was screened with a previously isolated ATP6L cDNA clone (13). Two clones containing non-identical inserts were characterized. The restriction map of these clones is shown in Fig. 1A, and sequence analysis confirmed that they encode ATP6L. To localize the first exon more accurately, the promoter proximal plasmid was digested with restriction enzymes and analyzed by Southern blotting using cDNA. In order to determine the nucleotide sequence of the primer region, a 1.9-kb EcoRI-SalI fragment of EMBL3 was subcloned into pUC18 (Fig. 1A), and the nucleotide sequence of the first exon and its 5′-flanking region was determined (Fig. 1B). This fragment contained exons with sequences identical to the 5′ portion previously determined from cDNA.

To define precisely the transcription initiation site, we performed primer extension. The cDNA products extended from the primer were analyzed by electrophoresis and sequenced using the same primer. Two major transcription initiation sites were observed (Fig. 2). About 20% of the transcripts initiated at +1 and 80% initiated at +75. The transcription initiation site of the human gene is located 236 bp upstream from that of the mouse gene (29). An additional 100-bp sequence of the 5′ untranslated region has been published. This indicates that the transcription initiation sites of the human gene are completely different from those of the mouse gene. The nucleotide sequences of the factor binding sites are also not the same, suggesting that the human gene differs from that of the mouse.

The analysis of the region upstream of the first exon failed to locate any sequence motifs such as TATA and CCAAT boxes. There were four GC boxes and one Oct-1-binding site in the proximal promoter region, with one GC box on its own in the promoter region. The GC content around the first exon was about 70%. To determine whether the region upstream of the first exon possesses promoter activity, the available restriction sites were utilized to construct a series of deletion reporter constructs. These constructs were tested by transient transfection in human cancer cell lines KB, PC3, and MCF7 cells. DNA extending only as far as −113 yielded full promoter activity, whereas the region between +57 and +194 retained 50% of maximum activity (data not shown).

We reported previously that V-ATPase gene expression is induced by cisplatin treatment (13) and examined further up-regulation of the V-ATPase genes by anticancer agents. As shown in Fig. 3A, the steady-state mRNA levels of two V-ATPase genes, ATP6L and -δE, increased 3–5-fold when cells were treated with cisplatin and TAS-103. To determine whether c and E subunit protein levels also increased when
The sequences that serve as recognition sites for Sp1 (5'-first exon and 5'-flanking sequence. numbered relative to the transcription initiation site, determined by upstream region and the first exon of the ATP6L gene. Restriction enzyme clones. V-ATPase c subunit ('A'), and dimerization site are as follows:

...isolated from a human placental genomic library. Restriction enzyme ATP6L genomic clones. V-ATPase c subunit ('A') and dimerization site... 

A C also increased (Fig. 3). We also analyzed the levels of the c (16 kDa), c (16 kDa), and D subunits (34 kDa) in KB cells. As expected, TAS-103 increased the expression of the c subunit. The levels of subunit a (100 kDa), subunit C (45 kDa), and subunit C (45 kDa) were increased after TAS-103 treatment (Fig. 3). These results indicate that TAS-103 may stimulate the expression of the V-ATPase complex as well as its subunits.

In order to confirm the existence of functional GC boxes and other transcription factor binding sites, we performed an in vivo footprint experiment as shown in Fig. 5. Because this experiment was performed using primers for the lower strand, transcription factor bindings to the complementary strand of the nucleotide sequence shown in Fig. 5 were detected. Protection of four 5'-guanines and hypersensitivity of the 3'-guanine of the consensus 5'-GGGCGG-3' which are typical Sp1 guanine binding sites, were observed at two potential GC box sequences (−73 to −68 and −36 to −31). In addition, there was slight protection of guanines at −84 and −61 on the lower strand surrounding the distal GC box (−73 to −68). Although the proximal GC box (−36 to −31) overlapped with an additional GC box (−41 to −36), a typical profile of Sp1 binding was not detected on this upstream GC box (−41 to −36). No signs of any binding to other tentative GC boxes located downstream of the transcription start site and GC box-like motif (−91 to −86) were seen in experiments with upper strand primers (data not shown).

In order to show that Sp1 (−73 to −68) and Oct1 (−36 to −31) bound specifically to the V-ATPase c promoter in vivo, we utilized the chromatin immunoprecipitation assay as shown in Fig. 6. PCR amplification of the V-ATPase c promoter was carried out with DNA extracted from the immunocomplex. Fig. 6A shows that significant levels of the V-ATPase c promoter sequence were detected as a 120-bp PCR product in the complexes immunoprecipitated with anti-Sp1 and anti-Oct1 antibody. The YB-1 promoter sequence was not detected, because there are no Sp1- and Oct1-binding sites in the YB-1 promoter, as shown in Fig. 6B. Furthermore, the V-ATPase c promoter sequence was not observed when normal rabbit IgG was used.
We next examined the effect of TAS-103 on luciferase activity induced by a series of 5'-H1032-deleted promoter constructs assayed 12 h after transfection (Fig. 7A). The luciferase activity of Luc1–3 was increased by about 3–6-fold compared with the control by 4/262M TAS-103. The activity of Luc4 was, however, not increased. These results suggest that an element responsible for V-ATPase c promoter activation by TAS-103 is located between 77 and 58. This region contains the GC box and the Oct1-binding site. To examine whether mutations of either the GC box or the Oct1-binding site affect the stimulation of V-

![Figure 3](http://www.jbc.org/)

**Fig. 3.** Induction of V-ATPase subunits in KB cells treated with anticancer agents. **A**, effect of anticancer agents on expression of ATP6L and -6E mRNA. KB cells were incubated with TAS-103 (4 μM) or cisplatin (10 μM) for the times indicated, and the steady-state levels of ATP6L and 6E mRNA were assayed by Northern blotting. 20 μg of total RNA was loaded per lane. **B**, induction of E subunit protein in KB cells treated with TAS-103. KB cells were treated with TAS-103 (4 μM) for 12 h. Cells were harvested and membrane fractions were prepared as described under "Materials and Methods." Forty μg of membrane fractions were loaded on a 15% SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. Immunoblot analysis was performed with antisera to the V-ATPase E subunit. **C** and **D**, immunoprecipitation with antisera to the V-ATPase E subunit. Anti sera to the V-ATPase E subunit or preimmune binding protein A/G-agarose were incubated for 12 h at 4 °C with 35S-labeled protein from KB cells with or without exposure to TAS-103 (4 μM). The mixtures were washed three times and separated on a 15% (C) or 10% SDS-PAGE gel (D). Molecular mass markers are indicated, as well as the positions of proteins that probably correspond to the low molecular weight V-ATPase subunits D and E, c', c (C), and the high molecular weight subunits a, A, C, E (D).

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ATPase c promoter activity by TAS-103, we made three constructs with mutations in the promoter sequences of these binding elements (Fig. 7B). TAS-103 responsiveness was reduced when a mutation was introduced into the GC box, and mutation of the Oct1-binding site completely inhibited TAS-103-induced luciferase activity, suggesting that the Oct1-binding site has a role in the promotion of V-ATPase c expression by TAS-103. The Luc3m1, Luc3m2, and Luc3m3 mutations had no apparent effect on basal transcriptional activity in the absence of TAS-103 (Fig. 7B).

To investigate whether V-ATPase c gene transcription activated by TAS-103 was affected by an interplay between Sp1 and Oct1, we performed EMSA on KB cells following TAS-103 treatment to study the interaction between the promoter and transcription factors using nuclear extracts made in buffer S. Six probes were utilized covering the entire core promoter, as described under “Materials and Methods.” When Oligos 1, 5, and 6 were used as probes, we could not detect any retarded band (data not shown). However, a retarded band was observed when Oligos 2–4 were used, and its strength was increased by treatment with TAS-103 (Fig. 8A). GC boxes were present in each of these oligonucleotide probes, and the intensity of the retarded band was reduced by addition of unlabeled GC box DNA (data not shown). Furthermore, the retarded bands were supershifted by antibody to Sp1 but not by antibody to Sp3 or preimmune antibody (Fig. 8B).

We noted that the band retarded by Oligo3 probe was not completely shifted by the addition of an excess of Sp1 antibody (data not shown). Oligo3 contains a GC box and an Oct1-binding site. We used nuclear extracts made with two different buffers, as shown under “Materials and Methods,” to examine the specificity of the DNA-protein interaction by appropriate competition assays. The band retarded by the nuclear extract prepared in buffer S was also only completely eliminated by a 25-fold excess of unlabeled Oligo3 but not by Oligo3m1, Oligo3m2, and Oligo3m3. On the other hand, the band retarded by the nuclear extract prepared in buffer C was almost completely eliminated by a 25-fold excess of unlabeled Oligo3, as well as Oligo3 but not by Oligo3m2 and Oligo3m3 (Fig. 8C). These results suggest that only Oct1 binds to the Oligo3 probe when the nuclear extract is prepared in buffer C but that either Sp1 or Oct1 binds to Oligo3 in a mutually exclusive manner when the nuclear extract is prepared in buffer S. We performed supershift assays to confirm these results (Fig. 8D). The retarded band was completely shifted by the addition of the Oct1-specific antibody when the nuclear extract was prepared with buffer C, suggesting that only Oct1 can bind to the Oligo3 probe under these conditions. Furthermore, the retarded band was shifted by the addition of either Sp1- or Oct1-specific antibody when the nuclear extract was prepared with buffer S. This indicates that both Sp1 and Oct1 can bind to the Oligo3 but that Sp1 and Oct1 cannot bind simultaneously to the same oligonucleotide. However, the in vivo footprint clearly showed that both Sp1 and Oct1 could bind simultaneously in vivo.

We next analyzed the DNA-protein complex in more detail after prolonged electrophoresis. Two complexes (C3 and C6)
**DISCUSSION**

We have described the cloning and characterization of the human vacuolar H^+–ATPase subunit c (ATP6L) gene, and we have isolated overlapping genomic clones encompassing 10 kb of its 5' sequence and 14 kb of its 5'-flanking region (Fig. 1A). We determined the nucleotide sequence surrounding the 5' end of the gene that contains the initiation sites for transcription. The regulatory regions are highly GC-rich, and the CpG islands are located 5' to the first exon. Thus, the ATP6L promoter has structural features common to housekeeping genes. No typical TATA and CCAAT boxes exist in the region preceding the first exon (Fig. 1B), and this may account for the presence of two major transcription initiation sites (Fig. 2). Multiple GC boxes are found in the promoter and first exon. GC boxes are frequent DNA elements present in many promoters and are required for appropriate expression of many ubiquitous genes. This feature of the ATP6L 5' region is consistent with its ubiquitous expression in human tissues and suggests that it encodes a protein with an essential cellular function.

Recently, the nucleotide sequence of the mouse V-ATPase c subunit promoter has been reported (29). Although there is significant homology between the proximal promoter sequence of human and mouse, the GC boxes are not conserved in the mouse gene. Furthermore, it is noteworthy that the human transcription initiation site is completely different from that of the mouse. The human ATP6L cDNA sequence has been published, and based on a sequence alignment of human cDNA, human cDNA has an additional 100 bp compared with the mouse initiation site. This indicates that the human and the mouse ATP6L genes are differently regulated.

As shown in Fig. 3A, mRNA levels of two V-ATPase genes were significantly (3–5-fold) up-regulated when cells were treated with TAS-103. Quantitation by PhosphorImager indicates that the protein levels of V-ATPase subunits c and E increased only 1.5–2-fold compared with a dramatic increase of mRNA. This discrepancy is probably due to the translational or post-translational control of V-ATPase protein. Functional analysis of the promoter region in a transient expression system demonstrated significant promoter activity in human cancer cells, and this activity increased 3–6-fold in TAS-103-treated cells (Fig. 4A). Furthermore, the region between −77 and −58 was required for the transcriptional up-regulation (Fig. 7A). Protein DNA interaction on the proximal promoter region was investigated by in vivo DMS footprint experiments. We detected clear evidence of Sp1 binding to two GC boxes. As
shown in Fig. 5, the G residue in the octamer sequence was protected in the footprint. Also, the V-ATPase c promoter sequence that contains both Sp1- and Oct1-binding sites was recovered in complexes immunoprecipitated with either anti-

**Fig. 8. Characterization of proteins binding to regions of the human ATP6L promoter.** A, EMSA with the six core promoter oligonucleotides. Nuclear extracts from TAS-103 (4 μM) treated for 12 h and untreated KB cells, made using buffer S, were reacted with each of the oligonucleotides as described under “Materials and Methods.” The arrow indicates the principal retarded band, and F denotes free probe. B, analysis of GC box-binding proteins by supershift assay. Nuclear extracts from TAS-103 (4 μM)-treated KB cells, made using buffer S, were incubated with probes and 2 μl of anti-Sp1 or anti-Sp3 antibody (Ab) for 30 min at 4 °C. The position of the supershifted bands are indicated by the arrows. F is the free probe.
that both camptothecin and etoposide induce ATP6L promoter activity (data not shown). However, we could not detect promoter activation when cells were treated with cisplatin (Fig. 4A). To our knowledge, the present study is the first to demonstrate activation of Oct1 target genes after treatment with anticancer agents.

V-ATPase subunit genes are inducible by treatment of human cancer cells with cisplatin and are up-regulated in cisplatin-resistant cell lines (13). Transient transfection of a reporter plasmid showed that promoter activity is not activated by cisplatin treatment (Fig. 4A) and is not enhanced in resistant cell lines (data not shown). One possible explanation is that post-transcriptional mechanisms, such as mRNA stabilization, may be involved in the cisplatin induction and up-regulation of this gene in drug-resistant cells. Another possibility is that the pathway signaling DNA damage to transcription factors may differ between cisplatin and other anticancer agents. Because cisplatin can block degradation of the mRNA (Fig. 4B), certain pathways signaling DNA damage in human cancer cells may increase mRNA stability.

Our data indicate that drug-induced gene expression is regulated by both transcriptional and post-transcriptional mech-
Expression of V-ATPase could have significance for cell growth (43), cell motility (44), tumorigenesis (45), metastasis (46), and apoptosis (47). The reason for induction of V-ATPase by anticancer agents is unclear, but increased V-ATPase activity may represent a cellular anti-apoptotic response. Our results show that TAS-103 can induce apoptosis, especially in the presence of bafilomycin A1 (Fig. 11), suggesting that V-ATPase inhibits apoptosis of cancer cells by preventing cellular acido"
Enhanced Expression of the Human Vacuolar H\textsuperscript{+}-ATPase c subunit Gene (\textit{ATP6L}) in Response to Anticancer Agents
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