

Regulation of Vacuolar H⁺-ATPase c Gene Expression by Oxidative Stress

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By using differential display, we identified one of the genes encoding the multi-subunit complex protein V-ATPase, c subunit gene (ATP6L), and showed alterations of the gene expression by oxidative stresses. Expression of the ATP6L gene in Neuro-2A cells was increased by the treatment with H₂O₂ and incubation in hypoxic chamber, implying that the expression of the ATP6L gene is regulated by oxidative stresses. To examine mechanisms involved in the regulation of the gene expression by oxidative stresses, the transcriptional activity of the rat ATP6L promoter was studied. Transcription initiation site was determined by primer extension analysis and DNA sequencing, and promoter of the rat ATP6L and its deletion clones were constructed in reporter assay vector. Significant changes of the promoter activities in Neuro-2A cells were observed in two regions within the proximal 1 kbp promoter, and one containing a suppressor was in -195 to -220, which contains GC box that is activated by binding of Sp1 protein. The suppression of promoter activity was lost in mutants of the GC box. We confirmed by electrophoretic mobility shift and supershift assays that Sp1 protein specifically binds to the GC box. The promoter activity was not changed by the H₂O₂ treatment and incubation in hypoxic chamber, however, H₂O₂ increased the stability of ATP6L mRNA. These data suggest that the expression of the ATP6L gene by oxidative stresses is regulated at posttranscriptional level, whereas the GC box is important in basal activities of the promoter.

Key Words: Vacuolar H⁺-ATPase, ATP6L, Oxidative, Promoter, GC box, Sp1, Hydrogen peroxide

INTRODUCTION

Cells have various transport proteins to control concentrations of intracellular ions. One important cellular parameter in regulation of metabolism is intracellular pH. Change of cellular pH influences the cell growth (Helmlinger et al, 1997), cell motility (Martinez-Zaguilan et al, 1998), apoptosis (Perona & Serrano, 1988), tumorigenesis (Gottlieb et al, 1995), and drug resistance (Martinez-Zaguilan et al, 1999). Cytoplasmic pH is regulated by transporters such as Na⁺/H⁺ exchanger, whereas the pH within intracellular compartments is regulated by vacuolar H⁺-ATPase (V-ATPase) (Forgac, 1999).

The V-ATPase resides within membranes of endosomes, lysosomes and secretory vesicles, and plays an important role in acidification of the compartments. The acidification by V-ATPase is important in the processes such as receptor-mediated endocytosis, protein targeting and processing, transport of small molecules (Forgac, 1999). V-ATPase, multisubunit complex protein, contains two distinct domains (Nishi & Forgac, 2002). The V₁ as a catalytic domain contains the site of ATP hydrolysis and consists of eight distinct polypeptide subunits. The V₀ as a membrane-span-

ning domain consists of five distinct polypeptide subunits that form pore for transporting protons.

Ischemia, one of oxidative stresses, increases reactive oxygen species. Induction of myocardial and hepatic ischemia delays the acidosis of intracellular compartments, which may contribute to the myocardial injury (Yellon et al, 1998; Carini et al, 2000). A short period of myocardial and hepatic ischemia (preconditioning) induces cytoprotection to the injury with a subsequent prolonged ischemia (Yellon et al, 1998; Carini et al, 2000). The preconditioning increases the V-ATPase activity through the activation of protein kinase C and p38 MAPK, which blocks delay of the intracellular acidosis (Carini et al, 2000; Carini et al, 2001).

By using differential display, we identified one of V-ATPase subunit genes, c subunit (ATP6L) and found decreased gene expression in rat ischemic kidney. The finding implies that the level of the ATP6L expression may be important for the V-ATPase activity, since earlier reports show close correlation of ischemic injury with the V-ATPase activity. In this study, we examined regulation mechanisms of the ATP6L expression by analyzing the gene promoter activities.

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ABBREVIATIONS: ATP6L, V-ATPase c subunit gene; EMSA, electrophoretic mobility shift assay; LUC, luciferase; Ac, actinomycin D.

METHODS

Promoter and cDNA isolation of V-ATPase c gene

5 kbp length promoter of V-ATPase c (ATP6L) was isolated from rat liver genomic DNA. Sequence information of rat genomic clone (NCBI, AC098626) was used to synthesize primer pair corresponding to the length of the promoter. The forward primer, 5'-TAGGACTTGGGAGTTAGAGCC-3', and reverse primer, 5'-GAGCGAGCAGACGAAA-GAAG-3', were used in PCR. The 5 kbp PCR product was cloned into pGEM T vector (Promega, Madison, WI) and confirmed by DNA sequencing. 271 bp length cDNA of the ATP6L gene was prepared from total RNA of rat brain using reverse transcription and PCR. The sequences of oligonucleotides used for PCR were as follows: forward, 5'-TCGCTCTCTCTCCCGT-3'; reverse, 5'-GTAGATGGCGATGATCCCA-3'. The ATP6L cDNA was used as a probe in Northern blot analysis.

Primer extension analysis and DNA sequencing

The antisense oligonucleotide primer, 5'-GCTTGCCCTC-TCCCGCTTGGTCTG-3', corresponding to the ATP6L gene base pair +181 to +195 from translation start site, was synthesized and end-labeled with [γ - 32 P]dATP using T4 polynucleotide kinase. Total RNA was prepared from rat brain using RNA STAT-60 (TEL-TEST, Friendswood, TX), following the manufacturers' instructions. Primer extension reactions were performed as described previously (Sambrook & Russell, 2001), and the products were analyzed on 6% polyacrylamide gel containing 1 \times TBE (0.09 M Tris borate, 2 mM EDTA, pH 8.3) with a DNA sequencing ladder as a molecular weight standard. For DNA sequencing, PCR reaction was performed under standard conditions in a 10 μ l of reaction mixture containing 20 ng of template DNA, 0.5 μ M each of primers, 0.2 μ M each of deoxynucleotide triphosphate, 1.5 mM MgCl₂, 0.4 unit of Taq polymerase, 0.5 μ Ci of [α - 32 P]dCTP (Amersham, Buckinghamshire, UK), and 1 μ l of 10 \times buffer. PCR products were then denatured and electrophoresed on 6% polyacrylamide gel containing 7 M urea. After electrophoresis, nucleic acids on the gels were transferred to 3MM Whatman paper, dried, and subjected to autoradiography using Kodak-OMAT film (Eastman Kodak, Rochester, NY).

Luciferase reporter constructs

Promoter fragments were subcloned into the firefly luciferase-encoding vector pGL3-basic (Promega). Deletions of the promoter were performed by PCR methods and confirmed by DNA sequencing. All fragments of deletion clones contained the entire 5'-untranslated region (up to bp +198) and were truncated at upstream sequences.

Cell culture and reporter gene assay

Neuro-2A cells, Neuroblastoma cell line, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 100 units penicillin/streptomycin (GIBCO, Grand Island, NY) at 37°C in a 5% CO₂ atmosphere. Cells in 24 well plates at 50% confluence were transiently transfected with 0.5 μ g of DNA using Lipofectamin 2000 reagent (Invitrogen, Carlsbad, CA). After incubation for 16 h, the cells were

seeded into 24 well plates at concentration of 1 \times 10⁵ cells per well. On the 2nd day of transfection, the cells were treated with H₂O₂ or incubated in hypoxic chamber (Thermo Forma, Marietta, OH) in serum free medium. The condition of hypoxic chamber incubation was in 85%/10%/5% of N₂/H₂/CO₂. Cell lysates for a reporter (luciferase) gene assay were prepared with lysis buffer kit (Promega). Promoter fragments in pGL3 were co-transfected at a ratio of 20:1 with the plasmid β -galactosidase (Promega) as an internal standard for transfection efficiency. The total concentration of plasmid added to the transfection mixture was kept constant in all experiments. Luciferase activity was quantified using the Dual-Luciferase Reporter Assay system (Promega) and luminometer (TD20/20, Turner Designs, Sunnyvale, CA), according to the manufacturers' recommendations. Data are presented as mean \pm SEM. Statistical significance of differences was determined by unpaired *t* test. Differences were considered significant at *P*<0.05.

Northern blot analysis

Total RNA was prepared from Neuro-2A cells treated with H₂O₂ or incubated in hypoxic chamber in serum free medium. In mRNA stability test, 6 μ g/ml actinomycin D (Sigma Chemical Co., St. Louis, MO) was co-treated with H₂O₂. Eight μ g of the total RNA sample was separated in a formaldehyde gel as previously described (Lee et al, 1995), and the RNA was transferred to Nylon Membranes (positively charged, Roche, Indianapolis, IN). The blots were probed with 11-DIG-UTP-labeled cDNA fragments of ATP6L as well as 11-DIG-UTP-labeled probe for β -actin. Final washing conditions for all probes were 0.2 \times SSC and 1% SDS at 55°C. Hybridization signals were visualized by chemiluminescent detection using anti-DIG antibody conjugated with alkaline phosphatase and substrate, CDP-STAR. β -actin probe or ribosomal RNA stained with ethidium bromide was used to confirm equal loading of total RNA.

Gel Mobility shifts and supershifts analysis

Nuclear extracts from Neuro-2A cells were isolated as described (Lee et al, 1998). Double-stranded oligonucleotide probes were 32P-labeled using T4 polynucleotide kinase and purified through Micro Bio-Spin 30 Chromatography Columns (Bio-RAD Laboratories, Hercules, CA). Ten micrograms of nuclear extract alone or premixed with 200 \times competitor oligonucleotide for 10 min were added to 30,000 cpm of labeled probe and incubated at room temperature for 20 min prior to gel electrophoresis. The sequences of oligonucleotides used for EMSA were as follows: Sp1 consensus, 5'-ATTCGATCGGGGCGGGGCGAGC-3' and 5'-GCTCGCCCCGCCCGATCGAAT-3'; Sp1 of c subunit (-211 to -191), 5'-TACGGCCCCGCCCTGGGGCC-3' and 5'-GGCCCCAGGGGCGGGGCCGTA-3'; Sp1 of c subunit mutant, 5'-TACGGCTTCGTTCTGGGGCC-3' and 5'-GGCCCCAGGAACGAAGCCGTA-3'. For supershifts, extracts were mixed with 30,000 cpm of probe for 30 min at 4°C, then 500 ng of purified goat antibody against Sp1 (NEB, Ipswich, MA) were added and incubated for an additional 30 min at 4°C. Antibody against Egr-1 (NEB), a transcription factor, was used as a control. Reactions were run in precast 1 \times TBE minigels containing 5% acrylamide. After electrophoresis, samples on the gels were transferred to 3MM Whatman paper, dried, and subjected to autoradio-

graphy using CP-BU NEW film (Agfa, Belgium).

Western blotting

Neuro-2A cells were treated with various concentrations of H₂O₂ for 8 h in serum free media. Nuclear extracts from the treated cells were prepared as described (Lee KA et al, 1998), and 200µg of extract were resolved on 7.5% SDS-PAGE and transferred to nitrocellulose transfer membrane (PROTRAN, Scheicher & Schuell Bioscience, Hahnestrasse, Dassel). Prestained protein marker (High Range, BIO-RAD) was used as a molecular weight standard. After blocking with 5% skim milk for 2 h, the membrane was incubated with goat antibody raised against Sp1 (NEB). Anti-goat IgG antibody conjugated with peroxidase was used as a secondary antibody, and protein signals were visualized using an enhanced chemiluminescence kit (Amersham, Cardiff, UK).

RESULTS

Isolation of a gene showing altered expression by oxidative stresses

It is well known that oxidative stresses induce apoptosis

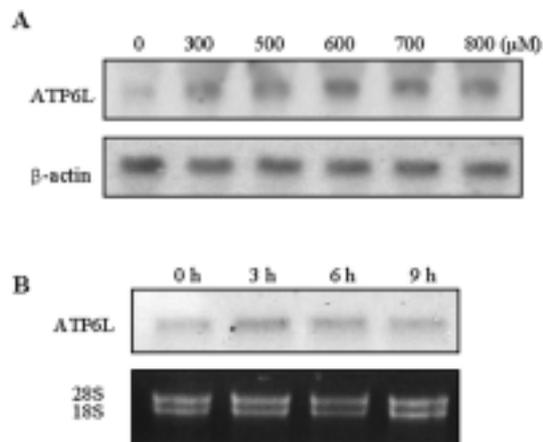


Fig. 1. Expression of rat vacuolar H⁺-ATPase c (ATP6L) gene. Total RNAs from H₂O₂-treated (A) or hypoxic chamber-incubated (B) Neuro-2A cells were separated on 1% agarose gel electrophoresis and transferred to nylon membranes. The membranes were incubated with a DIG-labeled ATP6L cDNA probe. Hybridization signals were visualized with chemiluminescent detection using anti-DIG antibody conjugated with alkaline phosphatase and substrate, CDP-STAR. β-actin probe or ribosomal RNA stained with ethidium bromide was used to confirm the equal loading of total RNA. Results are a representative of three independent experiments.

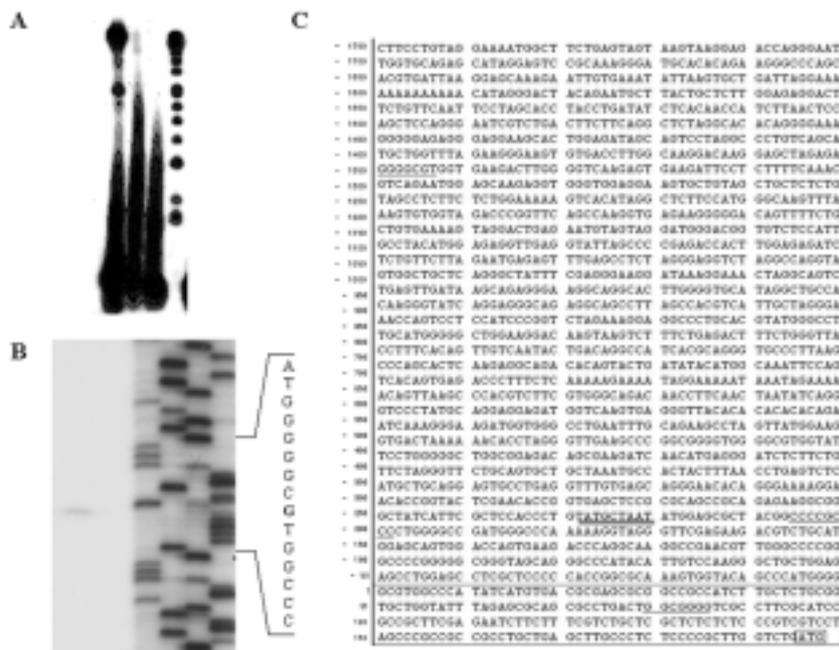


Fig. 2. Primer extension analysis and DNA sequencing of rat vacuolar H⁺-ATPase c (ATP6L) gene. (A) The antisense oligonucleotide primer corresponding to the 5' region of ATP6L cDNA was end-labeled with [γ-³²P]ATP using T4 polynucleotide kinase. Total RNA from Neuro-2A cells was used for primer extension reactions, and the products were analyzed on 6% polyacrylamide gel with a DNA ladder as a molecular weight standard. (B) For DNA sequencing, PCR reaction was performed including [α-³²P]dCTP. PCR products were electrophoresed on 6% polyacrylamide gel containing 7 M urea. After electrophoresis, nucleic acids on the gels were transferred to 3MM Whatman paper, dried, and subjected to autoradiography using Kodak-OMAT film (Eastman Kodak, Rochester, NY). (C) Transcription start site was determined by primer extension analysis and sequencing. Nucleotide sequence shows the 5' upstream region and first exon of the c gene. Nucleotides are numbered relative to the transcription initiation site. The sequences that serve as recognition sites for Sp1 (5'-GGGCGG-3') and Oct-1 (5'-ATGCAAAT-3') are underlined and double underlined, respectively. The translation start site (ATG) is in box.

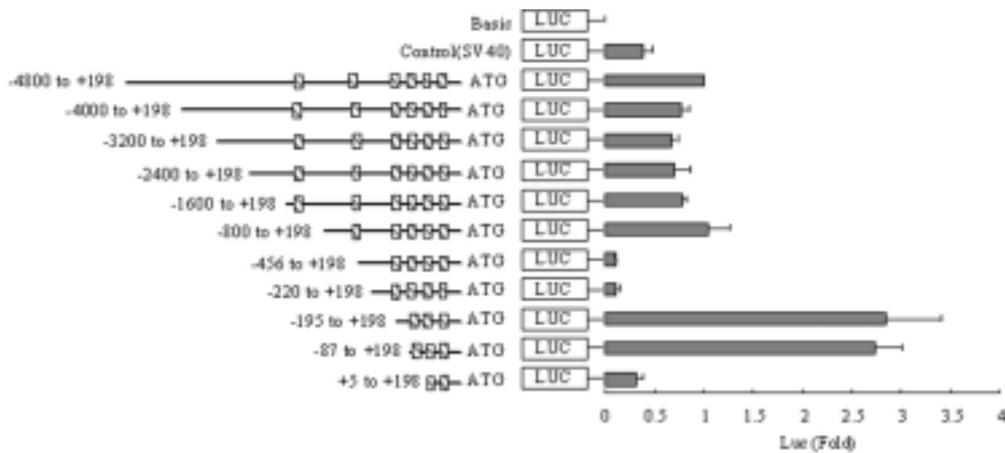


Fig. 3. Transcriptional activities of rat vacuolar H^+ -ATPase c (ATP6L) promoters. Deletion clones in vector pGL3-basic were transiently transfected into Neuro-2A cells for reporter gene analysis. The transfected cells were treated with H_2O_2 or incubated in hypoxic chamber in serum free medium. Cell lysates for a reporter (luciferase) gene assay were prepared with lysis buffer kit. Promoter fragments in pGL3 were co-transfected at a ratio of 20:1 with the plasmid β -galactosidase as an internal standard for transfection efficiency. Luciferase activity was quantified using the Dual-Luciferase Reporter Assay system. Relative promoter activities of the deletion clones were normalized against β -galactosidase activity. Nucleotide number is relative to the transcription start site with position of GC boxes. Two regions, enhancer and suppressor, were found in the promoter. Results are means \pm SEM from ten individual experiments.

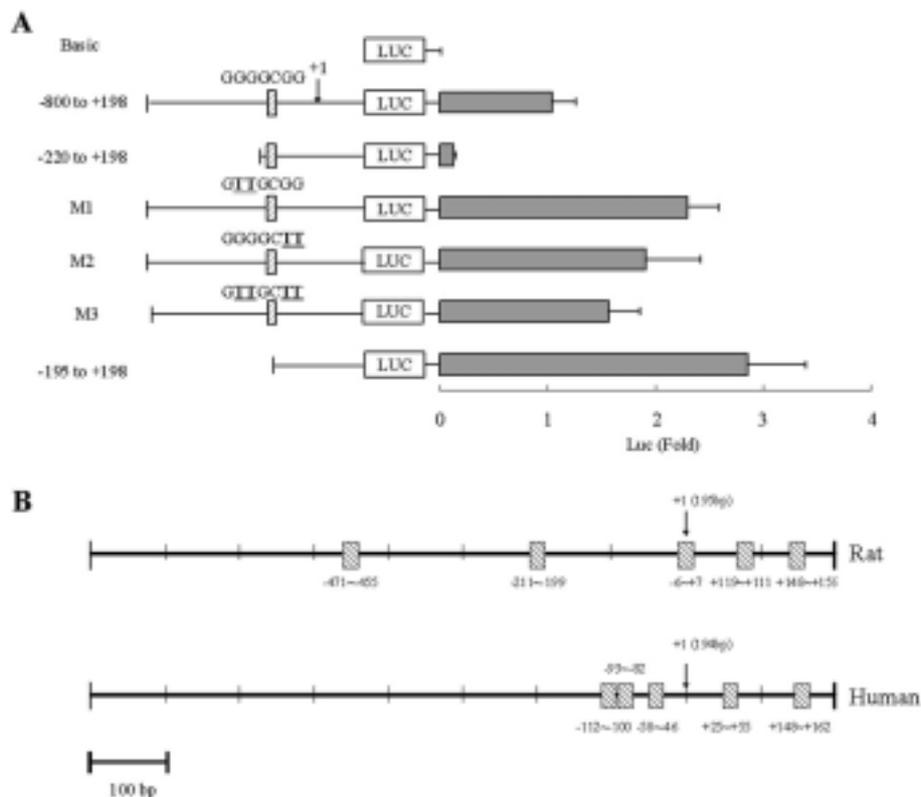


Fig. 4. Transcriptional controls of GC box in rat vacuolar H^+ -ATPase c (ATP6L) promoters. A. Three mutants of the GC box corresponding to -199 to -211 were constructed by in vitro mutagenesis using PCR. Transcriptional activities of the mutant ATP6L promoters were assayed as described in Fig. 3. Suppressive role of the Sp1 site was confirmed by reporter gene assay in three mutant clones. Results are means \pm SEM from ten individual experiments. B. Distribution patterns of GC boxes in ATP6L promoter are shown.

of cells *in vivo* and *in vitro*. Therefore, by using differential display, we screened the genes whose expressions were altered in rat ischemic kidney, and vacuolar hydrogen transporting ATPase c (ATP6L) gene was found as one of them. The expression of rat ATP6L gene in neuroblastoma cells (Neuro-2A) was increased by treatment with H₂O₂ (Fig. 1A) and incubation in hypoxic chamber (Fig. 1B). These data imply that the expression of ATP6L gene is regulated by oxidative stresses.

Isolation of rat ATP6L gene promoter and primer extension analysis

To examine mechanisms involved in the regulation of the ATP6L gene expression under oxidative stresses, the transcriptional activity of the rat ATP6L promoter was studied. First, 5 kbp length of ATP6L 5' upstream region (promoter) was isolated from rat genomic DNA. To precisely define the transcription initiation site, we performed primer extension analysis. As seen in Fig. 2A, the transcription initiation site

NE	-	+	+	+	+	+	+
Hot probe	+	+	+	+	+	+	+
Sp1 consensus	-	-	+	-	-	-	-
c-Sp1	-	-	-	+	-	-	-
m-Sp1	-	-	-	-	+	-	-
Sp1 Ab	-	-	-	-	-	+	-
Egr-1 Ab	-	-	-	-	-	-	+

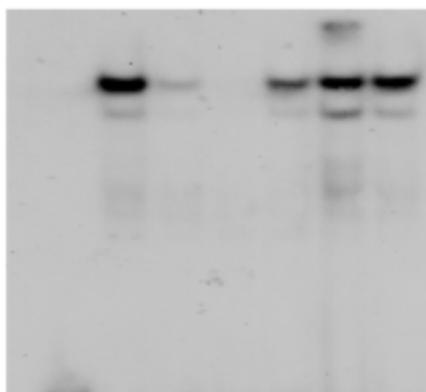


Fig. 5. Electrophoretic mobility shift assay of transcription factor Sp1 in rat vacuolar H⁺-ATPase c (ATP6L) promoter. Nuclear extracts from Neuro-2A cells were mixed with a radioisotope-labeled double-strand oligonucleotide probe corresponding to the GC box of the ATP6L promoter. Reactions were run in precast 1× TBE minigels containing 5% acrylamide. After electrophoresis, samples on the gels were transferred to 3MM Whatman paper, dried, and subjected to autoradiography. Band showing the specific binding of Sp1 protein to the hot probe was produced in lane 2. Competition with unlabeled oligonucleotide probe to the GC box resulted in loss of the band (lane 4), but mutated GC box did not compete with the labeled probe for the binding (lane 5). Addition of antibody against Sp1 to the reactions shifted the band (lane 6). Antibody against Egr protein was used as a control in supershift (lane 7). The competitors are as follows: consensus Sp1 (×200), c-Sp1 (×200), m-Sp1 (×200).

was observed 195 bp upstream from the translation start codon, sequence of the ATP6L clone was confirmed by DNA sequencing (Fig. 2B), and consensus sequences such as GC box and Oct-1 are shown in Fig. 2C.

Reporter gene analysis in deletion clones of ATP6L promoter gene

After determining the transcription initiation site and the DNA sequence of rat ATP6L gene, the sequence of the promoter region was analyzed by TFsearch program. However, the analysis failed to locate TATA and CAAT boxes, which is the same pattern as human ATP6L promoter. There were five GC boxes in proximal promoter region with two GC boxes on its own untranslated region, but the pattern was different from that in human ATP6L promoter. Deletion regions from the 5 kbp promoter of ATP6L gene were cloned in luciferase reporter expression vector (Fig. 3), and promoter activities were measured after transient transfection to Neuro-2A cells. As seen in Fig. 3, dramatic changes were observed within two regions of the proximal 1 kbp promoter: One containing enhancer element was in -456 to -800 from the transcription start site, and the other containing suppressor was in -195 to -220. Each of the two regions contained a GC box that was activated by binding of Sp1 protein, a transcription factor. The promoter activities in other cells (RAW264.7, HeLa, 293T, CHO-K1) were similar to that in Neuro-2A cells (data not shown).

Transcriptional control of GC box in rat ATP6L promoter

We identified two regions involved in control of the transcriptional activities of rat ATP6L promoter. The GC box in narrow range of sequence -195 to -220 could be a potential candidate for the suppressor in the transcriptional activity. Therefore, three mutants of the GC box were prepared to confirm the possible role as a suppressor (Fig. 4A). The nucleotide changes in three mutants were from 5'-GGGGCGG-3' to 5'-GTTGCGG-3', 5'-GGGGCTT-3', 5'-GTTGCTT-3'. The suppressed activity of the promoter clone was lost in all the mutants (Fig. 4A). Wang et al. (2002) suggested that the GC box in 5' upstream-untranslated region of human ATP6L promoter is a suppressor, which is different from our present observation on rat ATP6L

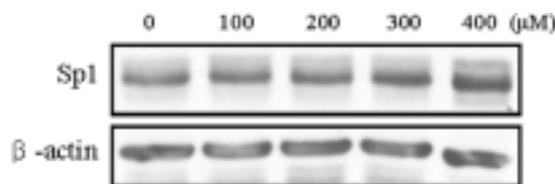


Fig. 6. Sp1 expression in Neuro-2A cells treated with H₂O₂. Neuro-2A cells were treated with various concentrations of H₂O₂ for 8 h, and protein lysates were prepared. The lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with primary antibody against Sp1 or β-actin, and IgG antibodies conjugated with peroxidase as a secondary antibody. Protein signals were visualized with an enhanced chemiluminescent kit. Results are a representative of two individual experiments.

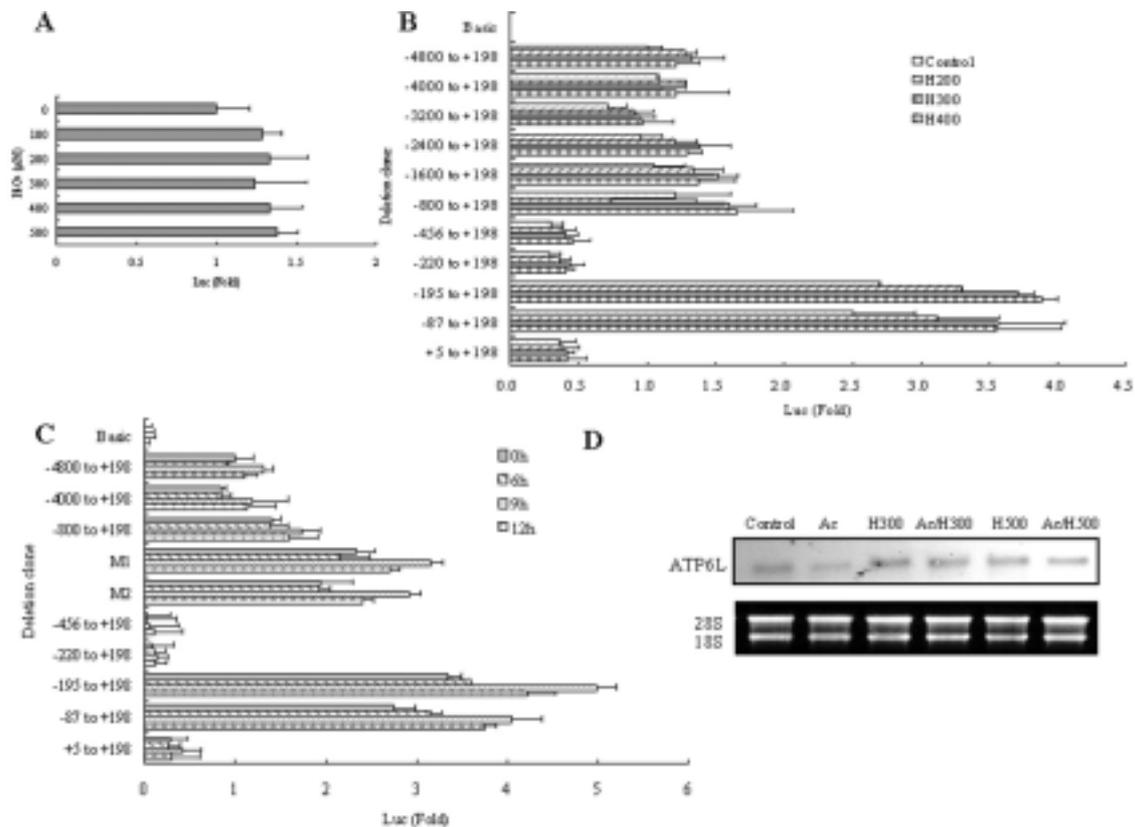


Fig. 7. Regulation of vacuolar H^+ -ATPase c (ATP6L) expression in Neuro-2A treated with oxidative stresses. Neuro-2A cells were transiently transfected with vector pGL3 containing 5 kb length of ATP6L promoter, and reporter gene analysis was performed after the treatment with various concentrations of H_2O_2 (A). The reporter gene analysis was done in deletion clones of ATP6L promoter after the treatment with various concentrations of H_2O_2 (B) and different durations of hypoxic chamber incubation (C). The oxidative stresses did not affect the transcriptional activities of the ATP6L promoter. Neuro-2A cells were co-treated with actinomycin D ($6\mu g/ml$) together with indicated concentrations of H_2O_2 for 6 h. Total RNAs were prepared from the cells, and Northern blot analysis was performed as described in Fig. 1 (D). H_2O_2 increases mRNA stability of the ATP6L gene in Neuro-2A cells. Results in A and B of the figures are means \pm SEM from ten individual experiments. Results in C are means \pm SEM from three individual experiments.

promoter. Distribution patterns of the GC boxes in rat and human were also different (Fig. 4B). Activity of the GC box is dependent on the binding of Sp1 proteins. Therefore, electrophoretic mobility shift assay (EMSA) was performed with Neuro-2A cells to confirm the Sp1 protein binding to the GC box. As seen in Fig. 5, strong band was seen in the radiolabeled oligonucleotide probe bound protein from nuclear extract. The band, however, was lost when the extract was preincubated with unlabeled competitor oligonucleotide probe, but not with the mutant form of unlabeled competitor. The band was supershifted by the addition of antibody against Sp1 protein, indicating that the protein bound to probe was Sp1. These data indicate that the GC box and its corresponding protein Sp1 may be important in regulation of ATP6L gene expression.

Effects of oxidative stresses on the transcriptional activities of rat ATP6L promoter

Oxidative stresses induced the changes of ATP6L gene expression (Fig. 1). We next examined the effects of oxidative stresses on the promoter activities in deletion clones

(Fig. 7). The promoter activities were, however, not changed by the treatment with H_2O_2 (Fig. 7A, B) and incubation in hypoxic chamber (Fig. 7C). Therefore, mRNA stability after H_2O_2 treatment was measured. The treatment combined with actinomycin D, a transcription inhibitor, showed that H_2O_2 increased the stability of ATP6L mRNA (Fig. 7D). Furthermore, the expression of Sp1 protein was not changed by the treatment with H_2O_2 (Fig. 6). These data suggest that the expression of the ATP6L gene by oxidative stresses is regulated at posttranscriptional level.

DISCUSSION

Various transport proteins control intracellular pH, which is important in cellular metabolism. Cytoplasmic pH is regulated by transporters such as Na^+/H^+ exchanger, while the pH within intracellular compartments is regulated by vacuolar V-ATPase (Forgac, 1999). Therefore, V-ATPase plays an important role in acidification of endosomes, lysosomes and secretory vesicles. V-ATPase, a multisubunit complex protein, is composed of 13 distinct polypeptides

that form two distinct domains, V₀ and V₁ (Nishi & Forgac, 2002).

Apoptosis has been proposed to be a mechanism for the cell death due to oxidative stresses such as hypoxia and ischemia. However, several studies implicated V-ATPase in the regulation of apoptotic processes (Crifer et al, 1994; Gottlieb et al, 1995). Preconditioning of hepatocytes and cardiomyocytes prevented apoptosis from ischemia via activation of V-ATPase (Yellon et al, 1998; Carini et al, 2000), and bafilomycin A1, a V-ATPase inhibitor, induced apoptosis in lymphoma cells (Nishihara et al, 1995).

By using differential display, we found that the expression of one of the V-ATPase subunit genes, c subunit (ATP6L), was decreased in rat ischemic kidney (data not shown). Previous studies showed that genes of V-ATPase, a multi-subunit complex protein, were coordinately and stoichiometrically regulated (Lee et al, 1995; Lee et al, 1999). These results suggest that the expressions of all subunit genes are regulated by a universal mechanism. Our finding implies that the level of ATP6L expression is important for the V-ATPase activity. Since earlier studies showed close correlation between ischemic injury and V-ATPase activity (Gottlieb et al, 1995; Yellon et al, 1998; Carini et al, 2000), we examined mechanisms involved in the regulation of ATP6L expression with analysis of the gene promoter activity.

Before studying the promoter activity, however, we examined the expression pattern of the ATP6L gene in H₂O₂- and hypoxic chamber-treated Neuro-2A cells. In our study, the oxidative stresses induced significant changes of the gene expression: The expression was increased by H₂O₂-treatment and hypoxic chamber-incubation of Neuro-2A cells. These data suggest that the regulation of the ATP6L gene is important in oxidative stress-induced apoptotic cell death.

We cloned 5 kbp promoter of ATP6L gene from rat genomic DNA and identified the transcription start site by primer extension analysis and DNA sequencing. The sequence showed TATA-less promoter that has also been reported in human ATP6L promoter (Wang et al, 2002). Consensus sequences such as GC box and Oct-1 were shown in the promoter. Nucleotide sequence of the proximal 1 kbp promoter showed low homology (34%) between rat and human.

Analysis of the ATP6L promoter activities in deletion clones showed two important regions containing enhancer or suppressor element, respectively. The GC box in bp -199 to -211 was identified as a suppressor by analysis of mutation clones. Wang et al. (2002) studied the regulation of ATP6L gene in human macrophage cells, and suggested that the GC box located in bp +55 to +25 of human ATP6L promoter was a suppressor, which is different from our data on rat. Furthermore, distribution patterns of the GC boxes in rat and human are also different. These data imply that the regulation mechanisms of ATP6L promoter may be different between rat and human.

In the present study, we showed that the GC box was important in ATP6L gene expression. However, promoter analysis with deletion clones showed no change of activity by treatments with different doses of oxidative stresses. Otherwise, H₂O₂ increased the mRNA stability of ATP6L gene. These data suggest that the GC box is critical for the basal level of the promoter activity with the interaction of Sp1 protein. It was reported that ATP6L expression is regulated by transcription or mRNA stability depending on stimuli (Torigoe et al, 2002). Camptothecin and etoposide

increase the transcription, whereas cisplatin decreases the mRNA stability of the gene. The greater expression of the gene in RAW cells than in 3T3 cells is primarily due to mRNA stability (Wang et al, 2002).

Regulation of V-ATPase expression has been reported to have significant role for apoptotic cell death by oxidative stresses as well as cell growth, tumorigenesis, and drug resistance. Our results suggest that oxidative stresses influence the expressions of V-ATPase proteins by regulating the mRNA stability, but not regulating transcription activity. Moreover, the GC box of the V-ATPase promoters might be important in the basal activity. Nevertheless, further studies are needed to confirm the regulation of other subunit genes, because we examined only an ATP6L of the multisubunit protein V-ATPase.

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