

## Up-regulation of Na,K-ATPase $\beta_1$ Transcription by Hyperoxia Is Mediated by SP1/SP3 Binding\*

Received for publication, June 1, 2000, and in revised form, August 25, 2000  
Published, JBC Papers in Press, September 14, 2000, DOI 10.1074/jbc.M004759200

Christine H. Wendt<sup>‡§</sup>, Greg Gick<sup>¶</sup>, Renuka Sharma<sup>‡</sup>, Yong Zhuang<sup>¶</sup>, Wenlian Deng<sup>‡</sup>, and David H. Ingbar<sup>‡</sup>

From the <sup>‡</sup>University of Minnesota, Department of Medicine, Minneapolis, Minnesota 55455 and the <sup>¶</sup>Department of Biochemistry, State University of New York, Health Science Center, Brooklyn, New York 11203-2012

**The sodium pump, Na,K-ATPase, is an important protein for maintaining intracellular ion concentration, cellular volume, and ion transport and is regulated both transcriptionally and post-transcriptionally. We previously demonstrated that hyperoxia increased Na,K-ATPase  $\beta_1$  gene expression in Madin-Darby canine kidney (MDCK) cells. In this study, we identify a DNA element necessary for up-regulation of the Na,K-ATPase  $\beta_1$  transcription by hyperoxia and evaluate the nuclear proteins responsible for this up-regulation. Transient transfection experiments in MDCK cells using sequential 5'-deletions of the rat Na,K-ATPase  $\beta_1$  promoter-luciferase fusion gene demonstrated promoter activation by hyperoxia between -102 and +151. The hyperoxia response was localized to a 7-base pair region between -62 and -55, which contained a GC-rich region consistent with a consensus sequence for the SP1 family, that was sufficient for up-regulation by hyperoxia. This GC element exhibited both basal and hyperoxia-induced promoter activity and bound both transcription factors SP1 and SP3 in electrophoretic mobility shift assays. In addition, electrophoretic mobility shift assays demonstrated increased binding of SP1/SP3 in cells exposed to hyperoxia while mutation of this element eliminated protein binding. Other GC sites within the proximal promoter also demonstrated up-regulation of transcription by hyperoxia, however, the site at -55 had higher affinity for SP proteins.**

The Na,K-ATPase is a ubiquitous protein that plays an important role in maintaining cellular homeostasis. The Na,K-ATPase maintains homeostasis by regulating cellular volume, transepithelial ion transport, action potential propagation in nerve and muscle cells, and the sodium-coupled uptake of metabolic substrates such as glucose and amino acids (1, 2). The Na,K-ATPase is a transmembrane, heterodimer protein consisting of an  $\alpha$  and  $\beta$  subunit. The  $\alpha$  subunit contains the catalytic site responsible for ion translocation, whereas it is thought that the  $\beta$  subunit is responsible for plasma membrane targeting (3). In lung epithelial cells, evidence supports that the  $\beta_1$  subunit is the rate-limiting unit for functional pumps (4,

5). Several isoforms exist for each subunit, whereas the  $\alpha_1$  and  $\beta_1$  subunits predominate in epithelial cells. The Na,K-ATPase is regulated both transcriptionally, by glucocorticoids, thyroid hormone, and hyperoxia, and post-transcriptionally (5–10).

We, and others, have previously demonstrated that hyperoxia can up-regulate Na,K-ATPase gene expression in lung epithelial cells (5, 10–12). The amount of up-regulation ranges between 2- and 6-fold and varies according to the duration of exposure and oxygen concentration (5, 10–12). In addition, these relatively small changes in gene expression result in physiological responses with concomitant increases in protein levels and activity, which are also dependent on the dose and duration of oxygen exposure (10, 11). The up-regulation of the Na,K-ATPase by hyperoxia in the lung has physiological significance, because the lung is exposed to oxidant stress continuously, due to high oxygen tension. Oxidant stress can be exacerbated during lung injury and the therapeutic need for high oxygen delivery. We sought a model cell line to determine the molecular mechanism by which hyperoxia increased Na,K-ATPase gene expression, and screening of several epithelial cell lines revealed MDCK<sup>1</sup> cells to be an excellent model.

MDCK cells are a canine kidney epithelial cell line that is abundant in Na,K-ATPase. In culture, these cells form domes when exposed to hyperoxia, presumably due to increased ion transport (13). Although the kidney is not exposed to high oxygen tension, it is exposed to oxidant stress during ischemia and injury.

The role of oxidants and oxygen tension on gene regulation has been recognized in several systems. Several transcription factors have been identified that up-regulate eukaryotic gene expression in the presence of oxidizing conditions, such as the anti-oxidant responsive element, AP1, and NF $\kappa$ B (14–16). The mechanism by which oxidants up-regulate transcription has been most clearly defined for NF $\kappa$ B, however, for many genes the mechanism remains undefined. We previously demonstrated that hyperoxia up-regulated gene expression of the Na,K-ATPase  $\alpha_1$  and  $\beta_1$  subunits in MDCK cells (5, 9). In this cell system, hyperoxia increased the Na,K-ATPase  $\beta_1$  subunit transcription via a 61-bp region on the  $\beta_1$  subunit promoter. Although the Na,K-ATPase  $\beta_1$  promoter contains putative AP1 and NF $\kappa$ B sites, these sites were not located in the 61-bp region necessary for hyperoxia induction. Rather, this sequence contained a core GC element consistent with an SP family consensus site.

\* Some of the results contained in Table I were published in abstract form as a supplement to *Chest* 116, 87S–88S (Aspen Lung Conference, 1998). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>§</sup> To whom correspondence should be addressed: University of Minnesota, Dept. of Medicine, Box 276 Mayo Mail Code, 420 Delaware St. SE, Minneapolis, MN 55455. Tel.: 612-624-0999; Fax: 612-625-2174; E-mail: wendt005@tc.umn.edu.

<sup>1</sup> The abbreviations used are: MDCK, Madin-Darby canine kidney; EMSA, electrophoretic mobility shift assay; bp, base pair(s); FBS, fetal bovine serum; PCR, polymerase chain reaction; MMTV, mouse mammary tumor virus; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; WCE, whole cell extract; NE, nuclear extract; SM, single mutation; DM, double mutation.

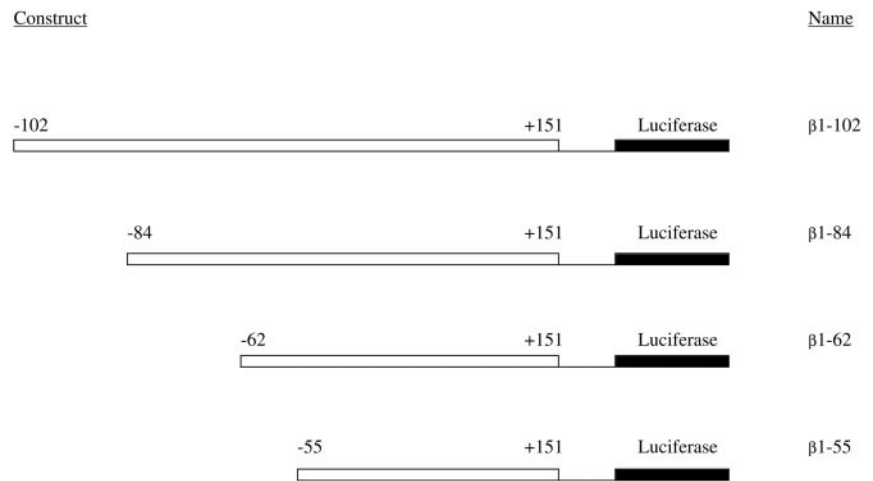


FIG. 1. Na,K-ATPase promoter-reporter constructs. The constructs consisted of the 5'-promoter plus 151 bp of the first exon linked in the promoterless luciferase expression vector (pXP1-Luc).

In this study, we demonstrated that an SP site is required for the up-regulation of the Na,K-ATPase  $\beta_1$  subunit transcription by hyperoxia in MDCK cells. Deletion of the GC element resulted in loss of both basal and hyperoxia-activated transcription. MDCK cells treated with hyperoxia demonstrated increased binding of SP1/SP3 in electrophoretic mobility shift assays (EMSA), which was eliminated with mutation of the GC consensus sequence. This represents a novel function for SP family transcription factors.

#### MATERIALS AND METHODS

**Cell Culture**—Three cell lines were tested to determine whether hyperoxia increased gene expression of the Na,K-ATPase. These cell lines included two lung epithelial cell lines (MP48, gift from G. Hunninghake; A549, ATCC) and one kidney epithelial cell line (Madin-Darby canine kidney (MDCK), low resistance, ATCC CCL 34). Only the MDCK cells had an increase in Na,K-ATPase gene expression by hyperoxia (data not shown) and were used for subsequent analysis. Cells were cultured on plastic tissue culture dishes and incubated in Eagle's minimum essential medium with Earle's salts (Life Technologies, Inc.). The media was supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc.) and 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2.5  $\mu$ g/ml amphotericin B (Life Technologies, Inc.). Cells were incubated in 5% CO<sub>2</sub>/95% air (normoxia) at 37 °C. Cells treated with hyperoxia were placed in a humidified, sealed chamber (Billups) that was flushed with 5% CO<sub>2</sub>/95% O<sub>2</sub> at 5 liters/min for 10 min each day of incubation under normobaric conditions. Cells treated with 0.2 mM diamide were placed in an incubator under normoxic, normobaric conditions. Cells were subconfluent at the start of the experiments and became confluent by 48 h of incubation, either in normoxic or hyperoxic conditions.

**Nuclear Run-on Assays**—Plasmids containing: pGEM empty plasmid (control), and Na,K-ATPase  $\beta_1$  subunit cDNA (gift from E. Benz, Johns Hopkins) were placed into wells of a slot blot (10  $\mu$ g/slot) on nitrocellulose filters and vacuum-dried. The Na,K-ATPase  $\beta_1$  subunit cDNA was contained in the pGEM plasmid, therefore, pGEM was chosen as a negative control to measure nonspecific binding. Nuclei were isolated from MDCK cells ( $5 \times 10^6$ ) by cell lysis using standard techniques (8). Nuclei were incubated with nucleotides and 0.5 mCi of [<sup>32</sup>P]UTP for 30 min at 37 °C before terminating the reaction with DNase I and extracting and precipitating the RNA. The RNA was resuspended in prehybridization solution, and 1 million counts of each sample was hybridized to the slot blots in the smallest possible volume for 48 h and then washed with 2 $\times$  SSC. The nitrocellulose filters were then autoradiographed. The RNA integrated optical density was determined using Densitometry Image software.

**Plasmid Construction**—The Na,K-ATPase  $\beta_1$  promoter-reporter constructs consisted of the 5'-promoter region plus 151 bp of the first exon linked to a promoterless firefly luciferase expression vector (pXP1-luc), as previously reported (9, 17). The deletion constructs designated  $\beta_1$ -102,  $\beta_1$ -84,  $\beta_1$ -62, and  $\beta_1$ -55 contained 102, 84, 62, and 55 bp, respectively, upstream from the transcription start site to +151 base pairs (Fig. 1). The  $\beta_1$ -102 construct was created by exonuclease III digestion of a  $\beta_1$ -817 construct as described previously (9, 17). Luciferase con-

structs that contained the  $\beta_1$ -84,  $\beta_1$ -62, and  $\beta_1$ -55 promoter sequences were synthesized using polymerase chain reaction (PCR) amplification. Oligonucleotides from -84 to +151, -62 to +151, and -55 to +151 were generated by PCR using  $\beta_1$ -102 as a template. The reactions to synthesize the -84 to +151 and -62 to +151 oligonucleotides were performed in a total volume of 100  $\mu$ l containing 1  $\mu$ g of template, 0.2 mM dNTPs, 2  $\mu$ M of each primer (5'-primers, GCGGATCCGATTGGCCTGCGGTGCGGATCCTAGGCGGAGCTAC; 3'-primer, GCAAGCTTGCATGCAGG), 2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, and 2.5 units of *Taq* polymerase (Promega). The run included 25 cycles, and each cycle consisted of: denaturation for 15 s (94 °C), annealing for 15 s (70 °C), and elongation for 1 min and 15 s (72 °C). The PCR fragments were digested with *Bam*HI and ligated into the *Bam*HI site of the luciferase vector pXP1. The PCR reaction to synthesize the -55 to +151 oligonucleotide was performed in a total volume of 50  $\mu$ l containing 30 ng of template, 0.1 mM dNTPs, 0.1 mM of each primer (5'-primers, AAAGAATCCGAGCTACGGATGGTGAGGC; 3'-primer, CTTGAATCCCCTGCTGCTTCAAG), 2 mM MgCl<sub>2</sub>, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, and 2.5 units of Pfu polymerase (ProStratagene). The run included 28 cycles, and each cycle included: denaturation for 1 min 30 s (96 °C), annealing for 40 s (60 °C), and elongation for 1 min and 30 s (68 °C). The PCR fragment was digested with *Bam*HI and ligated into the *Bam*HI site of the luciferase vector pXP1. Each clone was sequenced to confirm the appropriate DNA sequence. Transfection experiments with the empty vector did not demonstrate luciferase activity above background, either in normoxia or hyperoxia. For a control, cells were transfected with the  $\beta_1$ -41 construct, which contained 41 bp upstream from the transcription start site to +151 base pairs of the first exon, to test whether the vector or the 3'-region was responding to hyperoxia. This construct demonstrated less than 5% luciferase activity compared with the  $\beta_1$ -102 construct, similar to the empty vector (data not shown) and did not demonstrate increased activity in hyperoxia.

To confirm that the region from -59 to -55 was sufficient for hyperoxic induction independent of the Na,K-ATPase endogenous promoter, an oligonucleotide spanning this region was subcloned into a vector containing a minimal promoter of the mouse mammary tumor virus (MMTV-ATCC 37582) for transfection experiments. A 38-bp oligonucleotide was synthesized that spanned from -82 to -44 (5'-ATTGGCCTGCGGTGCGGCCCGGTAGGCGGAGCTACGGATG-3'; University of Minnesota Molecular Laboratory) and was subcloned into a luciferase vector containing a minimal promoter of the mouse mammary tumor virus. In addition, a 38-bp oligonucleotide was synthesized that spanned from -82 to -44, which contained a mutation of the GC site at -68 (5'-ATTGGCCTGCGGTGCTCTCCGGTAGGCGGAGCTACGGATG-3' University of Minnesota Molecular Laboratory) and was subcloned into the MMTV-luciferase plasmid. The minimal promoter consisted of 109 bp of the 5'-proximal promoter of the MMTV linked to the luciferase gene. The oligonucleotides were subcloned into *Hind*III/*Sac*I-digested MMTV-luc plasmid. Plasmids were transformed in *Escherichia coli* and isolated using the Qiagen maxi-prep and designated MMTV-82/44WT and MMTV-82/44SM.

To generate a construct with point mutations, we utilized a PCR-based method (Stratagene) using the double-stranded, supercoiled DNA vector,  $\beta_1$ -102, which was annealed to two synthetic complementary

oligonucleotides (–84 to –42) containing the desired mutation of the GC sites at –68 and –59 (5'-CGATTGGCCTGCGGTGCTCTCCGGT-GAGAGAGCTACGGATGGT-3'). The extension and incorporation of the mutant primers were accomplished by PCR. The PCR-generated mutant plasmid was treated with *DpnI*, which specifically digested the hemi-methylated wild type parental DNA plasmid and eliminated the non-mutant template from the PCR reaction. The plasmid containing the PCR-generated mutation was transformed and isolated as described above. The plasmid was sequenced to confirm the presence of the desired mutations and was designated  $\beta_1$ -102DM.

**DNA Transfection Experiments**—MDCK cells were plated at a density of  $8 \times 10^6$  cells/35-mm plate in media with 10% FBS. On the second day of culture, each well was transfected with 10  $\mu$ l of Superfect (Qiagen) and 2  $\mu$ g of the  $\beta_1$  promoter-reporter construct. Lipofection was carried out using the manufacturer's recommendation for a total of 4 h in serum-free and antibiotic-free media. After lipofection, the cells were incubated for 48 h in media plus 10% FBS in normoxia or hyperoxia. Cells were lysed and assayed for luciferase activity (Luciferase Assay System, Promega) in a luminometer (LB 9501, Berthold), and protein concentration was measured by the bicinchoninic acid system of Pierce. Luciferase activity normalized to either co-transfection with a CMV-LacZ plasmid or protein concentration was identical, therefore, all subsequent transfections were normalized to protein concentration as described previously (8). MDCK cells grew to confluency in both normoxic and hyperoxic conditions, however, overall cell number and total protein were less in the hyperoxia experiments. All normalized promoter activity was reported as a percentage activity over control (normoxia).

**Electrophoretic Mobility Shift Assays (EMSA)**—To prepare nuclear extracts, cells were suspended in hypotonic solution (10 mM HEPES, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.2 mM PMSF, and 0.5 mM dithiothreitol (DTT)) to lyse the cells and homogenized, and the nuclei were collected by centrifugation. High salt buffer was added slowly to release soluble proteins from the nuclei. The nuclei were pelleted by centrifugation, and the supernatant was dialyzed into a moderate salt solution (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT; a reducing agent), and any precipitated protein was removed by centrifugation. Approximately  $2\text{--}5 \times 10^8$  cells were needed to obtain 100–150  $\mu$ g of nuclear extract.

Whole cell extracts (WCE) were obtained from MDCK cells incubated in media plus 10% FBS and incubated for 24 h in normoxia, hyperoxia, or 0.2 mM diamide. To prepare whole cell extracts, cells were suspended and washed in phosphate-buffered saline, followed by buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.2 mM PMSF, and 0.05 mM DTT). Next, cells were lysed in buffer B (0.1% Nonidet P-40, 20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM PMSF, 0.05 mM DTT) followed by centrifugation and collection of the supernatant. The supernatant was diluted to 100  $\mu$ l per  $10^7$  cells with modified buffer C (10 mM KCl, 20 mM HEPES, pH 7.9, 20% glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT). Protein concentration was determined by the bicinchoninic acid system.

The double-stranded oligonucleotide probes were generated by the University of Minnesota Molecular Laboratory and were gel-purified. The probes were as follows: –82/–44 (5'-ATTGGCCTGCGGTGCCG-CCGGTAGGCGGAGCTACGGATG-3'); –122/–84 (5'-CCGCCCTTCGGGCTCAGGCCCGCTTCTCCGGCACCGGC-3'); –84/–42DM (5'-CGATTGGCCTGCGGTGCTCTCCGGTAGAGAGAGCTACGGATGGT-3'); –84/–42SM (5'-ATTGGCCTGCGGTGCTCTCCGGTAGGCGGAGCTACGGATG-3'). The oligonucleotides were end-labeled with a random primer kit (Promega) using [<sup>32</sup>P]CTP and Klenow fragment. The probe was electrophoresed, eluted, and resuspended in TE buffer. The binding reaction included  $1 \times 10^4$  cpm of DNA probe (0.2–0.7 ng), bulk carrier DNA, and 6.5–8.0  $\mu$ g of extract in 24–36  $\mu$ l of  $10\times$  binding buffer (100 mM TrisCl (pH 7.5), 500 mM NaCl, 5 mM DTT, 50 mM MgCl<sub>2</sub>, 50% glycerol) incubated for 20 min at room temperature. The reaction mixture was placed in a 4% polyacrylamide gel and electrophoresed at 4 °C, blotted, and autoradiographed using standard methods (8). Competition experiments consisted of incubating the probe and extract with excess cold competitor, double-stranded oligonucleotides prior to electrophoresis. The SP1 and AP1 double-stranded oligonucleotides (Promega) used in competition assays contained 21 and 22 bp, respectively. Each oligonucleotide contained the core consensus sequence for either SP1 or AP1. Other oligonucleotides used in competition assays included: –86/–62 (5'-GGCGATTGGCCTGCGGTGCCG-3'); and –66/–44 (5'-GCCGGTAGGCGGAGCTACGGATG-3'). Supershift assays involved incubation of probe and extract with 2  $\mu$ l of antibodies (Santa Cruz Biotechnologies) to the SP1 and SP3 transcription factors prior to electrophoresis. The SP antibodies were polyclonal, rabbit IgG

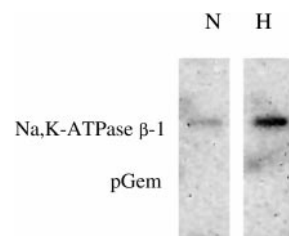


FIG. 2. Nuclear run-on assays of MDCK cells in normoxia and hyperoxia. Slot blots consisted of the following cDNAs (10  $\mu$ g/slot): Na,K-ATPase  $\beta_1$  subunit, pGEM (control). MDCK cells were incubated in normoxia or hyperoxia, and RNA was isolated from nuclei after treatment with 0.5 mCi of [<sup>32</sup>P]UTP. The blots were hybridized with 1 million counts of RNA, then autoradiographed. This blot is representative of two experiments. N, normoxia; H, hyperoxia.

antibodies that were not cross-reactive with other SP family members of different species, as reported by the manufacturer.

**Western Analysis**—Whole cell extracts (50  $\mu$ g), as described above, were separated by 8% SDS-polyacrylamide gel electrophoresis and then transferred onto Hybond-nitrocellulose membrane. The blot was blocked overnight with 10 ml of TBST (Tris-buffered saline, 0.05% Tween 20, 0.02% NaN<sub>3</sub>) with 6% powdered milk. This was followed by incubation with the goat polyclonal SP1 antibody (final dilution 1/100; Santa Cruz) at room temperature for 1 h. After incubation, the blot was washed three times with TBST and then incubated with horseradish peroxidase-conjugated secondary anti-goat antibody (final dilution 1/2000) for 1 h. After washing with TBST, bands were visualized using the enhanced chemiluminescence method (Amersham Pharmacia Biotech). Probing for SP3 and actin was performed separately, after the blot was stripped with buffer (62.5 mM Tris, pH 6.8, 100 mM  $\beta$ -mercaptoethanol, 2% SDS) at 55 °C for 30 min and then washed with TSBT. The goat polyclonal SP3 antibody (Santa Cruz) was incubated at a final dilution of 1/300, and the rabbit polyclonal actin antibody was incubated at a final dilution of 1/100. Appropriate anti-goat or anti-rabbit secondary antibodies were used as described above.

**Statistics**—Numerical data are expressed as means  $\pm$  S.E., and *p* values were determined by Student's *t* test using Macintosh InStat 2.00 software (GraphPad, La Jolla, CA).

## RESULTS

**Nuclear Run-on Assays**—Our previous studies demonstrated that hyperoxia increased Na,K-ATPase  $\beta_1$  subunit mRNA levels 5-fold in MDCK cells without a change in mRNA stability (9). To demonstrate that hyperoxia increased Na,K-ATPase transcription, we performed nuclear run-on assays in nuclei isolated from MDCK cells treated with either normoxia or 12 h of hyperoxia. Nuclei were incubated with nucleotides and 0.5 mCi of [<sup>32</sup>P]UTP, then the newly synthesized RNA was hybridized to slot blots containing Na,K-ATPase  $\beta_1$  subunit and pGEM (control) cDNAs. There was no MDCK RNA nonspecific binding to the pGEM cDNA, whereas hyperoxia increased transcription of the Na,K-ATPase  $\beta_1$  gene 3.0-fold (Fig. 2).

**Identification of a Hyperoxia-responsive Element in the Na,K-ATPase Promoter**—In our previous studies we localized a 61-bp region, between –102 and –41, on the Na,K-ATPase  $\beta_1$  promoter that was necessary for induction by hyperoxia (9, 18). This region contained multiple GC elements, but no consensus sequences for transcription factors with known responsiveness to oxidizing conditions, such as AP1, anti-oxidant responsive element, or NF $\kappa$ B. To further define the area involved in hyperoxia up-regulation, we transfected MDCK cells with deletion constructs of the Na,K-ATPase  $\beta_1$  promoter between –102 and –41, designated  $\beta_1$ -84,  $\beta_1$ -62, and  $\beta_1$ -55 (Fig. 1). These constructs were generated via PCR, subcloned into the luciferase expression vector (pXP1), and transfected into MDCK cells. Transfected cells were treated with either 48 h of normoxia or hyperoxia after transfection (95% O<sub>2</sub>/5% CO<sub>2</sub>) under normobaric conditions, then lysed, and luciferase activity was measured. There were positive regulatory elements between –102 and –62, demonstrated by a decrease in basal promoter activ-

TABLE I  
Effect of hyperoxia on Na,K-ATPase  $\beta_1$  subunit promoter activity

| Construct      | Normoxia        | Hyperoxia        | Hyperoxia/<br>normoxia |
|----------------|-----------------|------------------|------------------------|
|                |                 | %                |                        |
| $\beta_1$ -102 | 100             | 194.3 $\pm$ 16.8 | 1.9 $\pm$ 0.2          |
| $\beta_1$ -82  | 86.2 $\pm$ 12.1 | 219.6 $\pm$ 56.5 | 2.5 $\pm$ 0.4          |
| $\beta_1$ -62  | 27.7 $\pm$ 5.1  | 60.9 $\pm$ 2.9   | 2.2 $\pm$ 0.5          |
| $\beta_1$ -41  | 4.3 $\pm$ 1.9   | 2.3 $\pm$ 0.7    | 0.5 $\pm$ 0.1          |

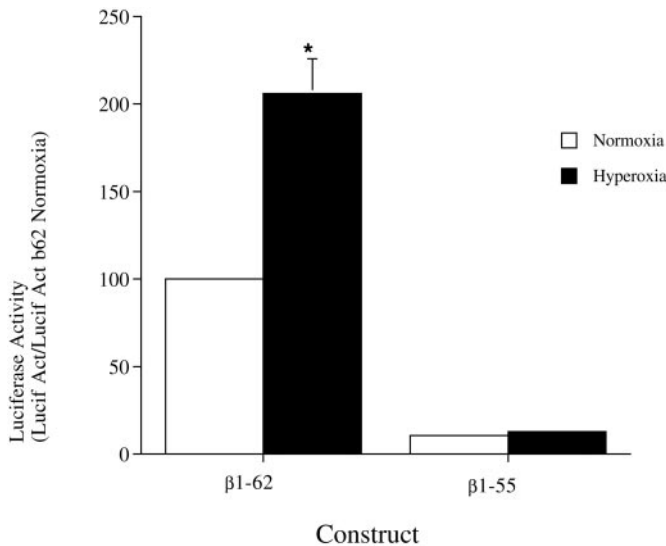


FIG. 3. **Effect of hyperoxia on Na,K-ATPase  $\beta_1$  promoter activity.** MDCK cell transfection: Cells were transfected with either the  $\beta_1$ -62 or  $\beta_1$ -55 construct and exposed to either normoxia or hyperoxia for 48 h. Cells were lysed, and luciferase activity was measured. Data were normalized to protein concentration. Data are reported as -fold increase over control, *i.e.* Na,K-ATPase promoter activity in normoxia. Each data point represents a mean  $\pm$  S.E. of values from four different experiments. \* $p \leq 0.05$  compared with normoxic condition.

ity to 86.2% for the  $\beta_1$ -84 construct and 27.7% for the  $\beta_1$ -62 construct relative to the  $\beta_1$ -102 construct (Table I). Both the  $\beta_1$ -84 and the  $\beta_1$ -62 constructs demonstrated a 2-fold induction in promoter activity in the presence of hyperoxia, thereby localizing a region necessary for hyperoxia induction to a 21-bp region between -62 and -41.

The 21-bp region between -62 and -41, which contained the hyperoxia-inducible element, had an SP1 consensus sequence (GGCGG) spanning from -59 to -55. To eliminate this putative site, a construct was generated via PCR, which spanned from -55 to +151 of the Na,K-ATPase  $\beta_1$  promoter and was subcloned into the pXP1 luciferase expression vector (designated  $\beta_1$ -55). Transfection with the  $\beta_1$ -55 construct in MDCK cells demonstrated a marked decrease in basal promoter activity with only 10.5% promoter activity compared with the  $\beta_1$ -62 construct (Fig. 3). In addition, deletion of this GC element eliminated hyperoxia induction. Therefore, a GC element between -62 to -55 contained a positive regulatory site for induction by hyperoxia as well as basal promoter activity.

To determine whether hyperoxia could up-regulate this element independent of the Na,K-ATPase promoter, we subcloned a dimer of an oligonucleotide that contained the hyperoxia element and spanned from -82 to -44 into a plasmid containing luciferase and a minimal promoter region from the MMTV gene. The empty MMTV plasmid had very low basal promoter activity in MDCK cells and did not up-regulate with hyperoxia (data not shown). In the presence of hyperoxia, the -82/-44-MMTV construct was induced 5.1-fold (Fig. 4). Because this oligonucleotide contained two GC elements (-68/-64 and -59/-55), we eliminated the distal -68 GC site by mutation. A

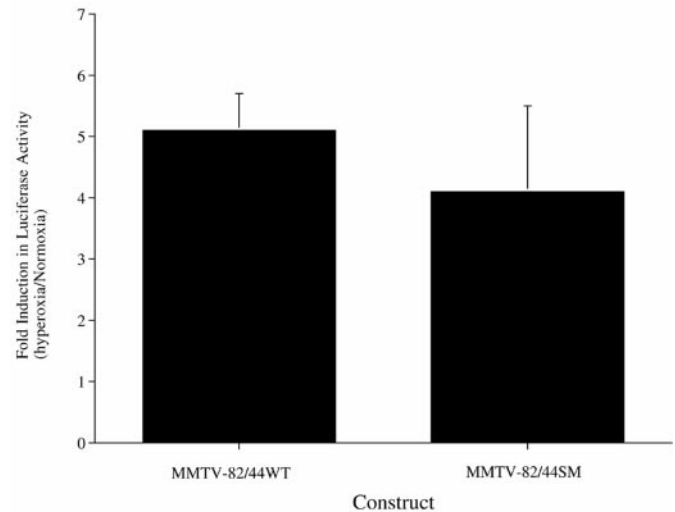


FIG. 4. **Induction by hyperoxia in a minimal promoter construct.** MDCK cells were transfected with either the MMTV-82/44WT or MMTV-82/44SM constructs and exposed to either normoxia or hyperoxia for 48 h. Transfection with the empty MMTV vector showed low promoter activity and no induction by hyperoxia. Cells were lysed, and luciferase activity was measured. Data were normalized to protein concentration. Data are reported as -fold increase over control, *i.e.* Na,K-ATPase promoter activity in normoxia. Each data point represents a mean  $\pm$  S.E. of values from five different experiments.

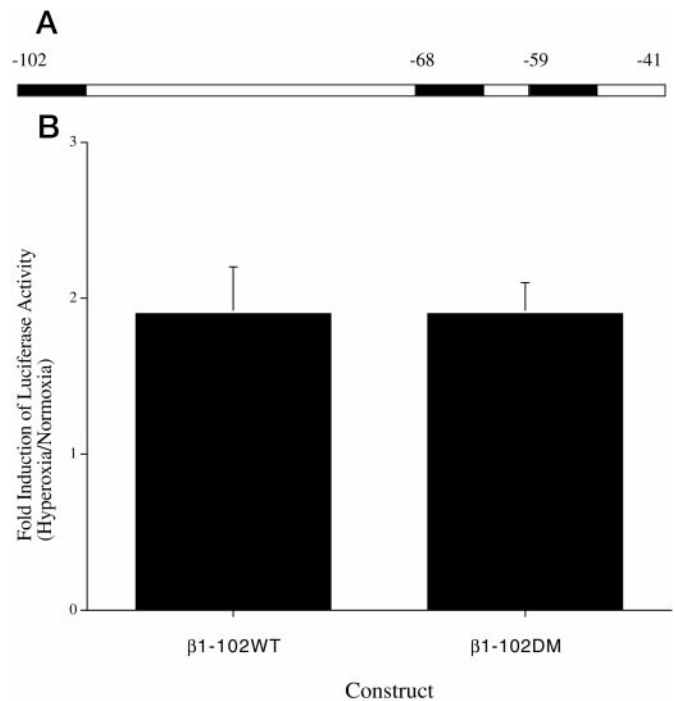
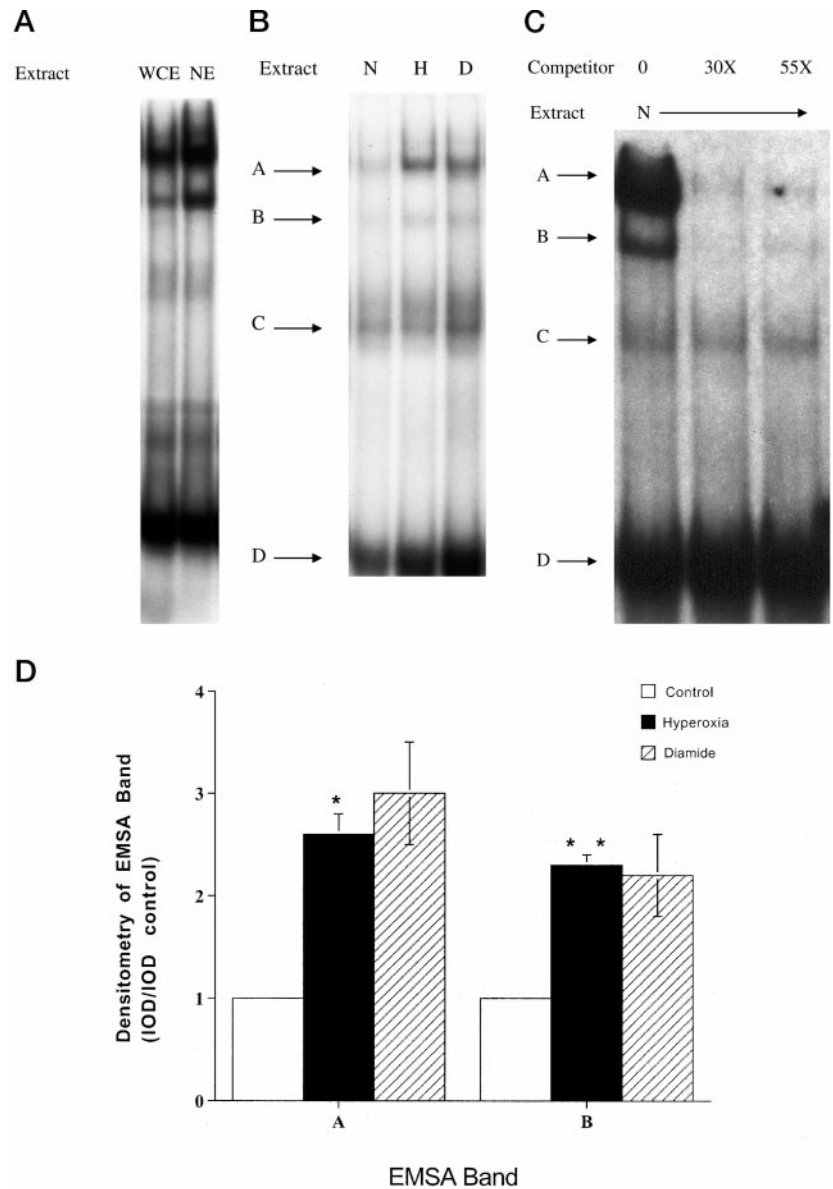


FIG. 5. **Induction by hyperoxia by the GC site at -102.** A, GC sites in the proximal Na,K-ATPase  $\beta_1$  promoter. B, transfection experiments of MDCK cells with either the  $\beta_1$ -102 or  $\beta_1$ -102DM constructs, which were exposed to either normoxia or hyperoxia for 48 h. Cells were lysed, and luciferase activity was measured. Data were normalized to protein concentration. Data are reported as -fold increase over control, *i.e.* Na,K-ATPase promoter activity in normoxia. Each data point represents a mean  $\pm$  S.E. of values from three different experiments.

similar construct containing a multimer of the -82 to -44 oligonucleotide with a mutation of the GC site at -68 to -64 was also inducible by hyperoxia, 4.1-fold (Fig. 4). This confirms that the GC site at -59 is sufficient for hyperoxia induction of transcription.

The proximal promoter of the Na,K-ATPase  $\beta_1$  subunit con-

**FIG. 6. Mobility shift assays of MDCK whole cell extracts demonstrate increased binding in cells treated with hyperoxia or diamide.** A double-stranded DNA probe, which spanned from  $-82$  to  $-44$  (sequence is under "Materials and Methods") of the  $\beta_1$  Na,K-ATPase promoter was radiolabeled with  $^{32}\text{P}$ , incubated with extracts, and electrophoresed on a 4% polyacrylamide gel as described under "Materials and Methods." A, MDCK cells were incubated under normoxic conditions and either whole cell extracts (WCE) or nuclear extracts (NE) were obtained as described under "Materials and Methods." Reactions contained  $6.6\ \mu\text{g}$  of extract and  $0.3\ \text{ng}$  of probe. B, MDCK cells were incubated for 24 h in 1) normoxia (N), 2) hyperoxia (H), or 3) diamide (D), and WCE were obtained as described under "Materials and Methods." Reactions contained  $8.0\ \mu\text{g}$  of extract and  $0.2\ \text{ng}$  of probe. Arrows A and B represent specific DNA-protein binding, arrow C represents nonspecific binding, and arrow D represents excess free probe. In cells exposed to hyperoxia or diamide, there was increased binding of bands A and B. The data are representative of three different experiments. C, competition experiments with excess cold probe, 30X = 30-fold molar excess and 55X = 55-fold molar excess. Reactions contained  $8.0\ \mu\text{g}$  of extract and  $0.2\ \text{ng}$  of probe. Bands A and B were eliminated by competition with excess probe, whereas there was no competition with band C. N, extracts under normoxic conditions. D, quantitative scanning densitometry of bands A and B from gel 6B. Each data point represents the mean  $\pm$  S.E. of values from three separate experiments and is the ratio of the integrated optical density (IOD) of the signal to that of control cells (normoxia). \* $p < 0.05$ , \*\* $p < 0.005$  compared with normoxic control.



tained three separate GC boxes between  $-102$  and  $-41$  (Fig. 5A). We have demonstrated, in our transfection experiments with serial deletions, that the GC element at  $-59$  is sufficient to induce the up-regulation by hyperoxia. To determine whether the distal GC site ( $-102$  to  $-98$ ) could up-regulate Na,K-ATPase  $\beta_1$  promoter activity, we mutated the proximal GC sites at  $-68$  and  $-59$  using site-directed mutagenesis of the  $\beta_1$ -102 construct. Mutation of these two proximal GC sites resulted in a marked decrease in basal promoter activity to  $24.1 \pm 11.6\%$  of the wild type activity. However, induction by hyperoxia (1.9-fold) was maintained in the mutant  $\beta_1$ -102 construct ( $\beta_1$ -102DM), identical to the wild type promoter construct ( $\beta_1$ -102WT; Fig. 5B). Therefore, the GC element at  $-102$  to  $-98$  also was capable of up-regulating transcription in the presence of hyperoxia.

**Electrophoretic Mobility Shift Assays**—The transfection experiments with the deletion constructs revealed a GC box, consistent with a SP family consensus site, that was necessary for hyperoxia induction. We performed EMSA to determine whether there was specific protein-DNA binding correlating to this particular GC box. To determine whether hyperoxia altered protein binding, EMSA of a double-stranded oligonucleotide spanning the putative hyperoxia site ( $-82$  to  $-44$ ) was

performed using nuclear extract (NE) from MDCK cells treated with 12 h of normoxia or hyperoxia. Using NE, we observed only a slight increase in binding (1.3- to 1.5-fold, data not shown). Because oxidation of nuclear binding proteins may be playing a role in the regulation by hyperoxia, but could be masked by redox changes during their extraction, we decreased the DTT concentration to see if this reducing agent altered binding. In the absence of the reducing agent, DTT, we were unable to isolate NE due to significant protein precipitation. We were able to obtain whole cell extracts (WCE) in the presence of very low levels of reducing agents, and the binding pattern was identical for NE and WCE (Fig. 6A). Electrophoretic mobility shift assays of a double-stranded oligonucleotide spanning the putative hyperoxia site ( $-82$  to  $-44$ ) incubated with WCE from MDCK cells in normoxia conditions demonstrated binding of three bands (Fig. 6B). Two of the bands, designated A and B, represented specific binding, because competition with excess cold oligonucleotide resulted in decreased binding (Fig. 6C). The third band, C, represented nonspecific binding, because the binding pattern did not change with excess cold probe.

In extracts from cells treated with hyperoxia (Fig. 6, B and D), we now demonstrated increased binding of bands A and B,

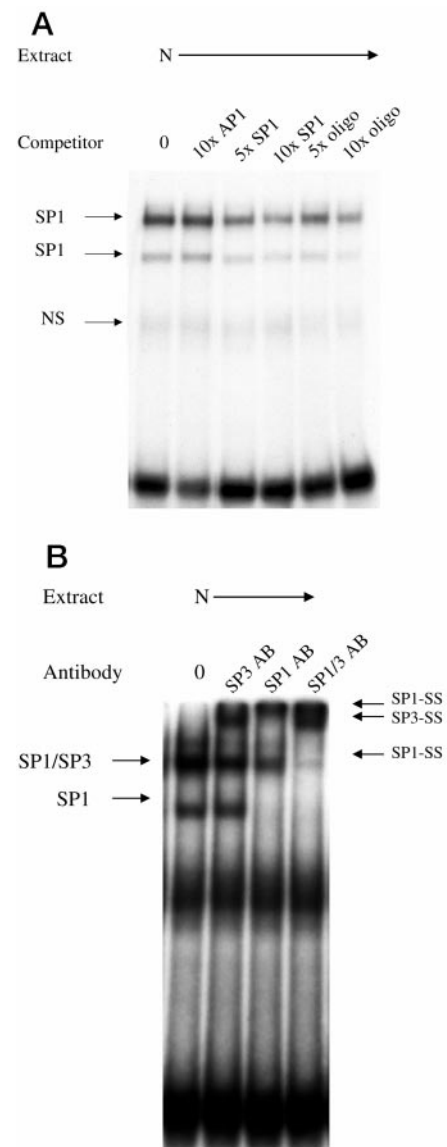
2.6- and 2.3-fold. In addition, we treated cells with 0.2 mM diamide, a thiol oxidizer, for 12 h in normoxic conditions to determine if thiol-disulfide oxidation influenced protein binding. WCE were collected for EMSA, which demonstrated increased binding of both bands, A and B, in extracts from diamide-treated cells. This effect was identical to the increased binding seen with hyperoxia (Fig. 6, B and D). Therefore, treatment with the thiol oxidizer, diamide, resulted in increased protein binding identical to that seen in hyperoxia.

To identify the binding proteins, we performed competition experiments in our EMSA by incubating the radiolabeled oligonucleotides with WCE and excess cold probe containing an SP1 or an AP1 consensus site. The oligonucleotide containing the SP1 consensus site binds members of the SP1 family and competed with our radiolabeled probe which spanned the putative hyperoxia site (Fig. 7A). This is consistent with SP1 binding to our hyperoxia site. This competition was specific, because an oligonucleotide containing an AP1 consensus site did not compete with our DNA probe containing the putative hyperoxia site (Fig. 7A).

To confirm that SP1 binds to the hyperoxia regulatory region, we performed EMSA in the presence of SP family antibodies to demonstrate supershifting (Fig. 7B). Because a number of SP family members can bind to the GC consensus sequence, we performed EMSA with both SP1 and SP3 antibodies. An oligonucleotide containing the hyperoxia-responsive element (-82 to -44) was incubated with WCE from MDCK cells treated with hyperoxia and SP-specific antibodies. Supershift assays demonstrated that SP1 antibodies supershifted both bands A and B, whereas, SP3 antibody supershifted only band A. Therefore, band A contained SP1- and SP3-specific proteins binding to the GC consensus site. When the oligonucleotide was incubated with both SP1 and SP3 antibodies, both bands A and B supershifted. These data, in combination with the competition experiments, demonstrated that SP1 and SP3 bind to the hyperoxia element that contains a GC consensus sequence.

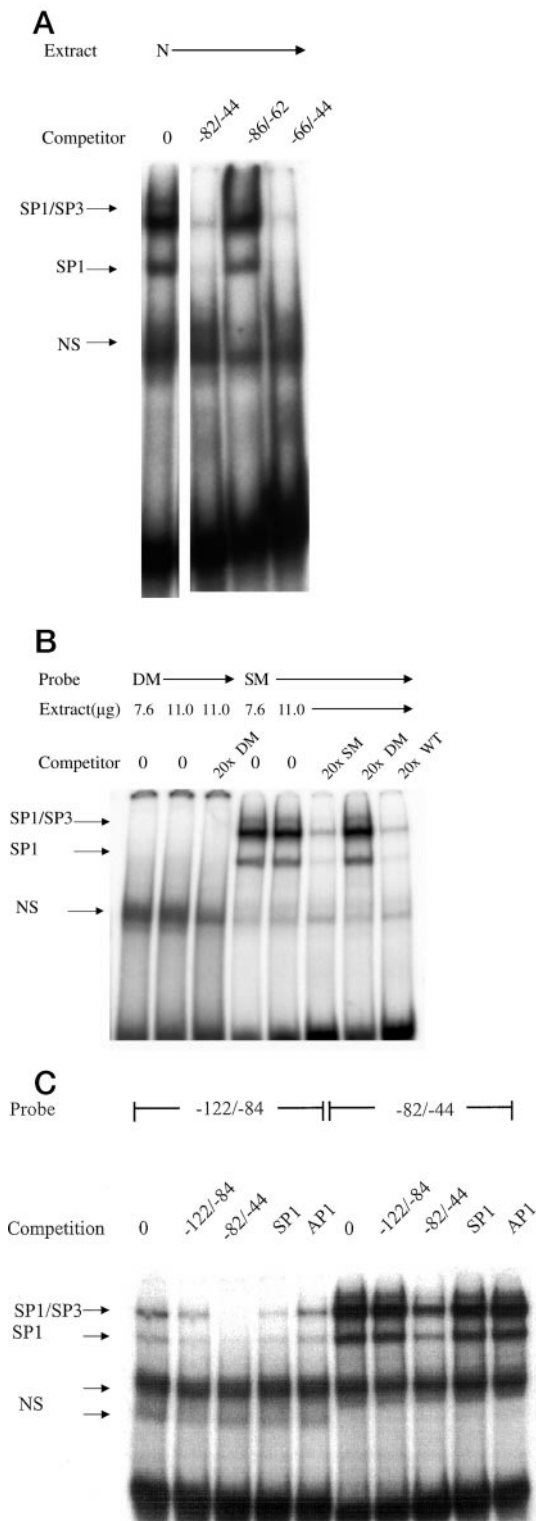
The transfection experiments demonstrated a 7-bp region (-62 to -55) sufficient for hyperoxia induction, which contained a GC element consistent with a SP1 consensus site. Competition and supershift assays demonstrated SP1 and SP3 binding. The sequence between -102 to -55 contained three GC elements consistent with SP family consensus sequences. These occur at -102 to -98, -68 to -64, and -59 to -55. Sequential deletion of the GC sites demonstrated that the GC site at -59 was sufficient for hyperoxia induction. However, the distal site, -102 to -98, was capable of induction by hyperoxia when the proximal sites, -68 and -59, were eliminated by site-directed mutagenesis. To identify which sites had higher affinity for the SP proteins we performed a series of competition EMSA. In EMSA using the -82 to -44 probe, competition assays demonstrated strong competition with cold oligonucleotides containing the GC site at -59 (-66/-44 and -82/-44 oligonucleotides; Fig. 8A). In contrast, competition assays with an oligonucleotide (-86/-62) containing only the GC site at the -68 position demonstrated weak competition (Fig. 8A). Although we have not ruled out some contribution of the GC site at -68, the GC element at -59 was sufficient for hyperoxia induction, and the SP proteins have a higher binding affinity for this site.

In EMSA using an oligonucleotide containing a probe (-82 to -44) with a single mutation (SM) of the GC element located at -68 and preserved -59 GC box, protein binding was identical to that seen with the wild type oligonucleotide (Fig. 8B). Whereas, all SP1 and SP3 protein binding was eliminated with an oligonucleotide containing a double mutation (DM) of both

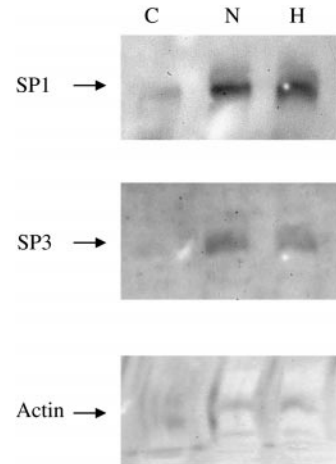


**FIG. 7. Binding of SP proteins to the Na,K-ATPase  $\beta_1$  proximal promoter.** WCE were obtained from MDCK cells maintained in normoxia for 24 h as described under "Materials and Methods." A double-stranded DNA probe, which spanned from -82 to -44 (sequence under "Materials and Methods") was radiolabeled with  $^{32}\text{P}$ , incubated with WCE, and electrophoresed on a 6% polyacrylamide gel. *N* = extracts under normoxic conditions. *A*, competition experiments consisted of the radiolabeled DNA probe incubated with WCE and excess (5-fold molar excess = 5*x* and 10-fold molar excess = 10*x*) cold double-stranded DNA oligonucleotides containing: oligo (-82 to -44 sequence), AP1 (AP1 consensus sequence), or SP1 (SP1 consensus sequence). Reactions contained 6.5  $\mu\text{g}$  of extract and 0.7 ng of probe. *B*, supershift experiments consisted of incubating WCE with antibodies to SP1 and/or SP3. Reactions contained 6.6  $\mu\text{g}$  of extract and 0.3 ng of probe. *Arrows* to the right indicate positions of SP1- and SP3-specific complexes and their respective antibody complexed supershifts. The data are representative of four different experiments. *NS*, nonspecific protein binding.

GC sites (-68 and -59), demonstrating that mutation of the -59 site was necessary to eliminate SP1 and SP3 binding (Fig. 8B). In EMSA with the SM probe, competition assays demonstrated competition with excess cold SM and wild type oligonucleotides, both which contain the intact -59 GC box. There was no competition with excess cold DM oligonucleotides, which contained the mutated -59 GC site. This confirmed that the GC element at -59 is sufficient for hyperoxia-induced promoter activity and SP protein binding, whereas the GC site at -68 is a weak competitor for SP family member binding and



**FIG. 8. Competition of hyperoxia element with other GC elements.** WCE were obtained from MDCK cells maintained in normoxia for 24 h as described under "Materials and Methods." *N*, extracts under normoxic conditions. *A*, a double-stranded DNA probe, which spanned from -82 to -44 (sequence under "Materials and Methods") was radiolabeled with  $^{32}\text{P}$ , incubated with WCE (6.6  $\mu\text{g}$ ), and electrophoresed on a 6% polyacrylamide gel. Competition experiments consisted of the radiolabeled DNA probe incubated with WCE and excess (30-fold molar excess) cold double-stranded DNA oligonucleotides containing: -82 to -44 sequence, -86 to -62 sequence, or a -66 to -44 sequence. Oligonucleotides containing the -59 to -55 GC site demonstrated greater competition. *B*, a double-stranded DNA probe, which spanned from -82 to -44 (sequence under "Materials and Methods") was radiolabeled and electrophoresed as described above. The probe contained either a single mutation (*SM*) at the -68 GC site or a double mutation (*DM*) at the -68



**FIG. 9. Expression of SP1 and SP3 from MDCK cells in normoxia and hyperoxia.** A representative Western blot of WCE collected from cells treated in normoxia (*N*) or hyperoxia (*H*). *C*, protein control lane obtained from the manufacturer of the antibody. Approximately 10  $\mu\text{g}$  of protein/lane was resolved on an 8% polyacrylamide gel and transferred to a nitrocellulose membrane before immunoblotting with polyclonal antibodies to SP1, SP3, or actin according to "Materials and Methods." No differences in protein concentrations were seen in treated or untreated cells.

is not necessary for hyperoxia induction.

Next, we sought to determine the role of the GC box at -102. Transfection experiments with site-directed mutagenesis revealed that the GC site at -102 was capable of induction by hyperoxia. To determine the binding pattern of this GC site, we performed EMSA using an oligonucleotide, which spanned this element (-122/-84). EMSA demonstrated a similar binding pattern to the oligonucleotides that contained the GC site at -59 (Fig. 8C). In addition, competition assays of the -122/-84 probe demonstrated competition with the -82/-44 and the SP1 oligonucleotides, but not with an AP1 oligonucleotide. The -82/-44 oligonucleotide, which contained the -59 GC site, was a very strong competitor of the -122/-84 probe. In contrast, the oligonucleotide containing the GC site at -102 (-122/-84 oligonucleotide) was a weak competitor for the GC binding site on the -82/-44 probe. Thus, the Na,K-ATPase  $\beta_1$  proximal promoter contained three GC sites, and we have demonstrated that either of two sites (-102 or -59) are capable of induction by hyperoxia, however, the GC site at -59 has the highest binding affinity for SP family members and is sufficient for induction by hyperoxia.

**Protein Expression of SP1 and SP3**—The mechanism by which hyperoxia increased SP family member binding to the Na,K-ATPase  $\beta_1$  gene remains unknown. Increased SP binding can be due to increased protein affinity or increased available protein. The latter can be due to either transcriptional or post-transcriptional regulation of SP family members. To determine whether increased available SP1 or SP3 protein was present with hyperoxia, we performed a Western blot on whole cell extract from cells treated with normoxia or hyperoxia (Fig. 9).

and -59 GC sites (see "Materials and Methods"). Competition experiments consisted of the radiolabeled DNA probe incubated with WCE and excess (30-fold molar excess) cold double-stranded DNA oligonucleotides: SM, DM, or wild type (*WT*). Reactions contained 7.5  $\mu\text{g}$  of extract and 0.3 ng of probe. *C*, a double-stranded DNA probe, which spanned from either -122 to -84 or -82 to -44 (sequence under "Materials and Methods") was radiolabeled and electrophoresed as described above. Reactions contained 6.3  $\mu\text{g}$  of extract and 0.3 ng of probe. Competition experiments consisted of the radiolabeled DNA probe incubated with WCE and excess (30-fold molar excess) cold double-stranded DNA oligonucleotide as described previously. *NS*, nonspecific protein binding.

Protein levels of SP1 or SP3 remained constant in the presence of hyperoxia. This suggested that increased SP binding was due to increased affinity and not due to an increase in available protein.

#### DISCUSSION

The Na,K-ATPase is an important protein for maintaining normal cellular function. This is emphasized by its presence in all cells and high levels in cells specialized in sodium transport (1–3). The sodium pump consists of two subunits,  $\alpha$  and  $\beta$ , which are necessary for normal enzyme activity. In some tissues, such as the lung, overexpression or up-regulation of the  $\beta$  subunit results in increased activity, whereas, this is not true for the  $\alpha$  subunit (5, 6). This implies that the  $\beta$  subunit is the rate-limiting subunit.

There are many factors that influence Na,K-ATPase regulation, including oxidant stress. Up-regulation of the Na,K-ATPase gene expression by hyperoxia has been previously described in both lung and kidney epithelia (5, 9, 10–12). In the lung, hyperoxia up-regulates Na,K-ATPase gene expression 2- to 6-fold with a concomitant increase in enzyme activity that is dependent on the duration and type of hyperoxia exposure (5, 9, 10–12). In addition, there is evidence that the up-regulation of pump expression by hyperoxia in MDCK cells increased ion transport as demonstrated by the formation of domes *in vitro* (13). However, the molecular mechanism by which hyperoxia influenced Na,K-ATPase gene expression and enzyme activity remained unknown.

Using MDCK cells exposed to hyperoxia as a model system, we now demonstrate that hyperoxia increased Na,K-ATPase  $\beta_1$  gene expression via an increase in transcription. To define the element necessary for hyperoxia induction of the Na,K-ATPase  $\beta_1$  gene, we performed transfection experiments using sequential deletions of the Na,K-ATPase  $\beta_1$  promoter between –102 and –41. These transfection experiments revealed a GC box between –59 to –55 that was necessary for basal promoter activity and sufficient for induction by hyperoxia. Two other GC sites, which were homologous for SP1 sites, also exist in this region between –102 and –68. Sequential deletions of these elements resulted in partial loss of basal promoter activity, however, the two-fold induction by hyperoxia was preserved. Mutations of the –68 and –59 GC sites within the wild type promoter revealed that the intact –102 GC site, in the absence of the –68 and –59 site, was functional and could be induced by hyperoxia. These data suggested that the –59 GC site is sufficient for hyperoxia induction, however, other GC sites are functional. In addition, there may be additional regulatory sites outside of our cloned promoter, because hyperoxia increased Na,K-ATPase  $\beta_1$  mRNA 5-fold and we identified only a 2-fold induction in promoter activity.

EMSA of oligonucleotides containing the –59 GC site demonstrated that hyperoxia increased binding of two bands that contained SP1 and SP3 as demonstrated by supershift and competition assays. Competition assays revealed that the –68 and –102 sites were weak competitors for SP1 and SP3 binding compared with the –59 site, suggesting the –59 site had higher affinity for SP1 and SP3. The EMSA demonstrated increased binding in the band containing SP1 alone and the band containing both SP1 and SP3. Therefore, it is unknown whether SP1 protein binding increased solely, or whether there was a concomitant increase in SP3 binding as well. In many systems, SP1 activates transcription, such as the Na,K-ATPase  $\alpha_3$  subunit gene (19). The role of SP3 in gene regulation varies between different promoters and cell types. SP3 represses SP1 transcriptional activation of the human thrombin receptor and uteroglobin gene (20), whereas, it up-regulates SP1 transcriptional regulation in the hepatic growth factor promoter (21).

There are two possibilities in our system. First, SP1 and SP3 may be acting synergistically to activate Na,K-ATPase  $\beta_1$  transcription and hyperoxia might increase the binding of both factors. Alternatively, SP3 may be playing an inhibitory role and hyperoxia might lead to increased SP1 binding and an increased SP1/SP3 ratio with subsequent increased transcription.

SP1 and SP3 belong to a transcription factor family that contain three zinc finger motifs. Promoter elements containing the core sequence GGG(C/T/A)GG bind several transcription factors, including members of the SP family (22). The SP transcription factor family members bind with different affinities to these DNA sites. This binding specificity is cell-specific, promoter-specific, and varies according to the consensus sequence (23, 24). These transcription factors are ubiquitous in mammalian cells and contain several different isoforms, all which bind to the core sequence and vary in abundance (20, 25, 26). SP1 plays a critical role in promoters without TATA or CAAT consensus sequences, however, SP1 also is functional in genes with TATA boxes (27, 28). The Na,K-ATPase  $\beta_1$  promoter contains a TATA box at –31, however, its function has not been demonstrated. SP1 has been described as a positive regulator of transcription, whereas, SP3 has been shown to either activate or repress transcription in different cell types (22, 29–33). The role of SP3 is promoter and cell type-specific, and the ratio of SP1 to SP3 may be important in transcriptional regulation.

The SP family members are usually associated with basal promoter activity, however, they contain zinc finger sulfhydryl groups that are sensitive to oxidation and direct redox changes alter SP regulated transcription (34, 35). Wu *et al.* (35) demonstrated that oxidizing conditions led to a decrease in SP1 binding and promoter activity in HeLa cells. This occurred in extracts from cells treated with oxidizing agents and extracts treated directly. In our system, hyperoxia and the oxidizing agent diamide led to increased DNA binding. Hyperoxia is felt to be toxic to cells due to oxidant injury, and this may be the mechanism of gene induction. The amount of injury by hyperoxia varies between different cells, and, in MDCK cells, it has been demonstrated that hyperoxia decreases proliferation in subconfluent cells, whereas, cellular death remains unchanged (36). Diamide is an agent that oxidizes sulfhydryl groups; therefore, thiol oxidation may be a mechanism by which hyperoxia induced Na,K-ATPase  $\beta_1$  subunit promoter activity. These redox changes may affect SP3 and/or SP1 binding capacity directly or indirectly. Alternatively, we sought to determine if hyperoxia directly influenced SP1 or SP3 expression. Hyperoxia up-regulated  $\beta$ -enolase and pyruvate kinase-M promoters by down-regulating the expression of the inhibitory factor, SP3, thereby increasing the SP1/SP3 ratio (37). In our system, hyperoxia did not change protein levels of either SP1 or SP3 suggesting altered binding affinity was responsible for increased binding on EMSA.

In summary, we demonstrate that hyperoxia increased Na,K-ATPase  $\beta_1$  transcription via a GC element in its proximal promoter. Multiple GC boxes exist in the Na,K-ATPase  $\beta_1$  proximal promoter, at least two of which are capable of up-regulating transcription in the presence of hyperoxia. These sites bind the SP family members SP1 and SP3, which increase in binding in the presence of hyperoxia. These transcription factors are necessary for both basal and hyperoxia-induced regulation of the Na,K-ATPase  $\beta_1$  promoter, and this represents a novel oxidant stress-dependent regulation by SP1/SP3 transcription factors.

#### REFERENCES

- Horisberger, J., Lemas, V., Kraehenbuhl, J. P., and Rossier, B. C. (1991) *Annu. Rev. Physiol.* **53**, 565–584
- Skou, J. C. (1988) *Methods Enzymol.* **156**, 1–25
- Geering, K. (1991) *FEBS Lett.* **285**, 189–193

4. Factor, F., Saldias, F., Ridge, K., dumasius, V., Zabner, J., Jaffe, H. A., Blanco, G., Barnard, M., Mercer, R., Perrin, R., and Sznajder, J. I., (1998) *J. Clin. Invest.* **102**, 1421–1430
5. Carter, E. P., Wangenstein, O. D., O'Grady, S. M., and Ingbar, D. H. (1997) *Am. J. Physiol.* **272**, L542–L551
6. Chalaka, S., Ingbar, D. H., Sharma, R., Zhou, Z., and Wendt, C. H. (1999) *Am. J. Physiol.* **277**, L197–L203
7. Lingrel, J. B., Orlowski, J., Shull, M. M., and Price, E. M. (1990) *Prog. Nucleic Acids Res.* **38**, 37–89
8. Liu, B., Huang, F., and Gick, G. (1993) *Cell. Mol. Biol. Res.* **39**, 221–229
9. Wendt, C., Towle, T., Sharma, R., Duvick, S., Kawakami, K., Gick, G., and Ingbar, D. H. (1998) *Am. J. Physiol.* **274**, C356–C364
10. Harris, Z. L., Ridge, K. M., Gonzalez-Flecha, B., Gottlieb, L., Zucker, A., and Sznajder, J. I., (1996) *Eur. Respir. J.* **9**, 472–477
11. Olivera, W., Ridge, K., Wood, L. D. H., and Sznajder, J. I. (1994) *Am. J. Physiol.* **266**, L577–L584
12. Nici, L., Dowin, R., Gilmore-Hebert, M., Jamieson, J. D., and Ingbar, D. H. (1994) *Am. J. Physiol.* **261**, L307–L314
13. Dreher, D., and Tochat, T. (1992) *Am. J. Physiol.* **262**, C358–C364
14. Rushmore, T. H., Morton, M. R., and Pickett, C. B. (1991) *J. Biol. Chem.* **266**, 11632–11639
15. Pinkus, R., Weiner, L. M., and Daniel, V. (1996) *J. Biol. Chem.* **271**, 13422–13429
16. Meyer, M., Pahl, H. L., and Baeuerle, P. A. (1994) *Chem.-Biol. Interact.* **91**, 91–100
17. Liu, B., and Gick, G. (1992) *Biochim. Biophys. Acta* **1130**, 336–338
18. Wendt, C., Sharma, R., Towle, T., Gick, G., and Ingbar, D. H. (1999) *Chest* **116**, 87S–88S
19. Murakami, Y., Ikeda, U., Shimada, K., and Kawakami, K. (1997) *Biochim. Biophys. Acta* **1352**, 311–324
20. Wu, Y., Ruef, J., Rao, G. N., Patterson, C., and Runge, M. S. (1998) *Biochem. J.* **330**, 1469–1474
21. Jiang, J.-G., Chen, Q., Bell, A., and Zarnegar, R. (1997) *Oncogene* **14**, 3039–3049
22. Marin, M., Karis, A., Visser, P., Grosveld, F., and Philipsen, S. (1997) *Cell* **89**, 619–628
23. Hata, Y., Duh, E., Zhang, K., Robinson, G. S., and Aiello, L. P. (1998) *J. Biol. Chem.* **273**, 19294–19303
24. Netzker, R., Weigert, C., and Brand, K. (1997) *Eur. J. Biochem.* **245**, 174–181
25. Dennig, J., Beato, M., and Suske, G. (1996) *EMBO J.* **15**, 5659–5667
26. Hagen, G., Dennig, J., Preiss, A., Beato, M., and Suske, G. (1995) *J. Biol. Chem.* **270**, 24989–24994
27. Chu, S., Blaisdell, C. J., Lui, M.-Z. M., and Zeitlin, P. L. (1999) *Am. J. Physiol.* **276**, L614–L624
28. Toonen, R. F. G., Gowan, S., and Bingle, C. D. (1996) *Biochem. J.* **316**, 467–473
29. Hagen, G., Müller, S., Beato, M., and Suske, G. (1994) *EMBO J.* **13**, 3843–3851
30. Birnbaum, M. J., van Wijnen, A. J., Odgren, P. R., Last, T. J., Suske, G., Stein, G. S., and Stein, J. L. (1995) *Biochem. J.* **34**, 16503–16508
31. Kumar, A. P., and Butler, A. P. (1997) *Nucleic Acids Res.* **25**, 2012–2019
32. Rajakumar, R. A., Thamocharan, S., Menon, R. K., and Devaskar, S. U. (1998) *J. Biol. Chem.* **273**, 27474–27483
33. Schanke, J. T., Durning, M., Johnson, K. J., Bennett, L. K., and Golos, T. G. (1998) *Mol. Endocrinol.* **12**, 405–417
34. Wu, X., Bishopric, N. H., Discher, D. J., Murphy, B. J., and Webster, K. A. (1996) *Mol. Cell. Biol.* **16**, 1035–1046
35. Hamm-Künsele, B., Schäfer, D., Weigert, C., and Brand, K. (1997) *FEBS Lett.* **403**, 87–90
36. Jyonouchi, H., Sining, S., Mizokami, M., and Ingbar, D. H. (1997) *Nutr. Cancer* **28**, 115–124
37. Discher, D. J., Bishopric, N. H., Wu, S., Peterson, C. A., and Webster, K. A. (1998) *J. Biol. Chem.* **273**, 26087–26093