

ARTICLES

## Enhanced Binding of Sp1/Sp3 Transcription Factors Mediates the Hyperoxia-Induced Increased Expression of the Lung Type I Cell Gene *T1 $\alpha$*

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**Abstract** The transcription factor Sp1 plays an important regulatory role in transactivation of the lung type I cell differentiation gene *T1 $\alpha$* . Like other lung cells, type I cells may encounter changes in oxygen concentration during the lifetime of the organism. We found that exposure of mice to hyperoxia rapidly increases expression of *T1 $\alpha$*  and other type I cell genes, and that returning the mice to normoxia quickly decreases expression. Likewise hyperoxia increases both endogenous *T1 $\alpha$*  expression in lung epithelial cell lines and transcription of luciferase (Luc) from *T1 $\alpha$*  promoter deletion constructs. Using wild-type promoter fragments and gel shift assays, we determined that Sp1/Sp3 and a key Sp *cis*-element in the proximal promoter mediate the hyperoxic response. Mutations of this element and inhibition of Sp–DNA binding by mithramycin block the hyperoxic response. Western analyses of cell homogenates show that the overall abundance of Sp1 and Sp3 proteins is not altered by hyperoxia. However, the abundance of nuclear Sp1 increases after short hyperoxic exposures, suggesting that signaling pathways activated by hyperoxia lead to Sp protein translocation, perhaps as a result of increased Sp phosphorylation. *J. Cell. Biochem.* 89: 887–901, 2003. © 2003 Wiley-Liss, Inc.

**Key words:** alveolar type I cell genes; *T1 $\alpha$* ; aquaporin-5; caveolin-1; hyperoxia; Sp1/Sp3 transcription factors; Sp translocation

Many observations on gene regulation indicate that changes in O<sub>2</sub> tension and redox state have widespread influences on transcription [Zhu and Bunn, 1999; Allen and Tresini, 2000]. In hyperoxia, the molecular responses can be

mediated through direct oxidation of specific molecules [Fridovich, 1998; Leonarduzzi et al., 2000], particularly lipids, by generation of the toxic oxygen intermediates superoxide, hydroxyl radical, and hydrogen peroxide, or by activation of a classical signaling pathway, such as epidermal growth factor receptor (EGF-R) [Takeyama et al., 2000].

However, for most oxygen-responsive mammalian genes the specific signaling pathways, transcription factors, and *cis*-elements that initiate changes in gene transcription have not been identified. Several studies of hyperoxic animals or cells show that the transcription factors NF- $\kappa$ B [Pahl and Baeuerle, 1994; Pepperl et al., 2001; Islam and Mendelson, 2002], AP-1 [Yang et al., 2000; Comhair et al., 2001], and nuclear response factor 2 (Nrf2) [Cho et al., 2002a,b] can mediate transcriptional changes in target genes, and a recent study demonstrates that increased DNA-binding of Sp1/Sp3 [Wendt et al., 2000] transcription factors can also activate oxygen-responsive genes.

Abbreviations used: Transcription factors: Sp1, 2, 3, 4: AP-1, NF- $\kappa$ B, c-jun, C/EBP; EGF-R, epidermal growth factor receptor; SP-A, -B, -C, -D, surfactant proteins A, B, C, D; AQP-5, aquaporin 5; TBST, Tris-buffered saline with Tween-20; BSA, bovine serum albumin; CMV-lacZ, cytomegalovirus promoter-driven  $\beta$ -galactosidase.

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However, details of these molecular regulatory events and signaling pathways are not yet well characterized.

The lung can be directly exposed to dramatic changes in O<sub>2</sub> tension. At birth, the newborn is exposed to a sudden increase in oxygen tension, moving from the relatively hypoxic pre-natal environment to post-natal normoxia. Several lung genes appear to be responsive to this rapid, physiologic change in oxygen concentration [Das et al., 1999; Ibe et al., 2000; Kitley et al., 2000]. In the clinical setting, high oxygen concentrations are used therapeutically in critically ill patients. Although essential for survival of the patient, high O<sub>2</sub> exposure has been shown to damage small pulmonary vessels and alveolar epithelial cells [Crapo et al., 1980; Thet et al., 1983]. These oxygen-induced injuries lead to enhanced expression of protective molecules such as anti-oxidant enzymes [Ho et al., 1996], to an influx of inflammatory cells that may cause additional injury [Ward et al., 2000], and to later repair [Crapo, 1986] and/or fibrosis [Crapo, 1986]. Although the expression of many molecules in the lung increases or decreases during these processes, there is no clear understanding of how the molecular changes relate to one another or how they are regulated at the molecular level.

Hyperoxia-induced alveolar injury in experimental animals is commonly used as a model for exploring the cellular responses to injury and the processes of tissue repair. At the cellular level the response of the peripheral lung to hyperoxia is well known. The earliest morphologically detectable change is endothelial cell damage [Crapo et al., 1980], leading to increased capillary leaks [Nogee et al., 1991], that may eventually produce alveolar edema. With longer exposures electron microscopic studies show that type I alveolar cells, the cells that line 93–95% of the peripheral lung surface, are damaged and detach from the basement membrane [Crapo, 1986]. Type II alveolar epithelial cells, the source of pulmonary surfactant, resist oxygen damage better than type I and endothelial cells and play a key role in tissue repair [Evans et al., 1975]. When type I cells are lost, a yet unidentified signal triggers proliferation of nearby type II cells [Evans et al., 1975]. Some of the mitotic progeny begin to express the type I cell molecular phenotype, and the cells eventually acquire the attenuated morphology of mature type I cells. The general signaling

pathway that directs these complex tissue and molecular events is not known.

This sequence of events suggested the possibility that type I cell mRNAs might decrease per total lung tissue as type I cell number decreases due to injury and cell death. This decrease might be followed by an increase in type I cell mRNAs to normal levels, or above, as the repair process proceeds. We tested these predictions using T1 $\alpha$  [Rishi et al., 1995] and aquaporin 5 (AQP-5) [Nielsen et al., 1997] mRNAs as cell-specific markers for the type I cell phenotype. In certain experiments, we also measured caveolin-1 expression although this gene is expressed by both type I and endothelial cells in the normal lung. The type I cell marker mRNAs do not behave as predicted. Instead of a decrease in mRNAs in O<sub>2</sub>-exposed mice, we observed large increases in expression of these genes by 48–72 h of hyperoxia (total exposure time 72 h). The mRNAs fell rapidly to control levels upon returning the animals to room air.

We then showed that T1 $\alpha$ , AQP-5, and caveolin-1 genes also respond to hyperoxia in a lung epithelial cell line, thus dissociating changes in gene expression from issues of tissue injury, repair, and changes in cell number that confound interpretation of data from whole lungs. Studies using T1 $\alpha$  promoter-luciferase (Luc) constructs, mutational analysis, and molecular inhibitors demonstrate that the hyperoxia-induced increases in transcription are mediated by increased Sp1/Sp3 binding to the proximal promoter.

## MATERIALS AND METHODS

### Mouse Exposures

Normal adult C57BL mice (19–21 g), a strain sensitive to effects of hyperoxia, were obtained from Charles River Laboratories (Wilmington, MA) and housed under standard normoxic conditions for at least 1 week. Food and water were supplied ad libitum. Groups of six mice were placed in plexiglass boxes containing >95% O<sub>2</sub> or room air (controls). Oxygen concentrations were measured daily with an oxygen monitor (J.H. Emerson, Cambridge, MA). Three animals at each time point were sacrificed at 24, 48, and 72 h exposure and thereafter at 1, 5, 14, and 28 days post-exposure recovery. Animals were anesthetized with intraperitoneal sodium methohexital (EliLilly, Indianapolis, IN), following which the abdominal aorta was severed

to exsanguinate the animal. Lungs were removed for biochemical or immunohistochemical analyses as described below.

#### Expression Studies in Cell Lines

Mouse lung epithelial cell lines were exposed to normoxia (95% air/5% CO<sub>2</sub>) or 95% O<sub>2</sub>/5% CO<sub>2</sub> in vitro. E10 cells, kindly provided by Dr. A. Malkinson (University of Colorado) and Dr. R. Ruch (Medical College of Ohio), are a spontaneously immortalized epithelial cell line from adult mouse lung derived originally by Dr. A. Lykke (University of New South Wales) [Smith et al., 1985]. Cells were cultured in CMRL 1066 medium (Gibco-BRL, Gaithersburg, MD), 10% fetal bovine serum (Gibco-BRL), 0.5 mM glutamine (Gibco-BRL), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco-BRL). MLE-15 cells (mouse lung epithelial cells immortalized in vivo with an SP-C driven SV40T antigen [Wikenheiser et al., 1993]), provided by J.A. Whitsett (University of Cincinnati), were cultured and transfected as described previously [Ramirez et al., 1997].

Mithramycin A (stock solution 1 mM in 100% ethanol) (Sigma, St. Louis, MO #M 6891) was added to cultured cells at a final concentration of 100 or 500 nM. Cells were incubated for 30 min in normoxia (95% air/5% CO<sub>2</sub>). Dishes (10 cm) of E10 cells were then divided into six groups: normoxia or hyperoxia alone; normoxia or hyperoxia with 100 or 500 nM mithramycin. Duplicate plates of each condition were harvested at 24 and 48 h.

#### RNA Purification and Northern Analysis

Methods for total RNA purification for Northern analyses have been described previously [Ramirez et al., 1997; Cao et al., 2000]. RNA was extracted using TRIZOL reagent (Gibco-BRL) and denatured with glyoxal/DMSO. Five to ten micrograms of RNA were electrophoresed on 1.5% agarose gels, blotted onto nylon membranes (HyBond N<sup>+</sup>, Amersham, Piscataway, NJ) and cross-linked with UV light. Prehybridization and hybridization were performed as described previously [Ramirez et al., 1997] using [ $\alpha$ -<sup>32</sup>P]dCTP labeled probes prepared with the random-primer method. Probes were as follows: T1 $\alpha$ , 642 bp murine cDNA; AQP-5, 366 bp murine cDNA; caveolin-1, 838 bp human coding region; SP-D, 1,253 bp murine cDNA; SP-B, 550 bp rat cDNA. Blots were washed with high stringency buffers and visualized by auto-

radiography. A probe for the 18S ribosomal subunit (Ambion, Austin, TX) was used to assess equal loading. Relative amounts of RNA were determined by densitometry (ImageQuant, Molecular Dynamics; Amersham, Piscataway, NJ) using 18S as the normalizing value.

#### T1 $\alpha$ Promoter-Deletion Constructs

The preparation of the -170 bp, -1.3 kb, and -10 kb T1 $\alpha$  promoter-Luc constructs has been previously reported [Ramirez et al., 1997, 1999]. Cells were transfected using the DEAE-dextran-chloroquine method described previously with 5 µg total DNA using carrier plasmid DNA to compensate for differences in molecular weights. Cells were seeded into 60 mm plates and used at ~60–70% confluency. Luc activity was measured in cell extracts using the Promega Luciferase Assay kit (Promega, Madison, WI). Data were normalized to  $\beta$ -galactosidase expression from a co-transfected cytomegalovirus promoter-driven  $\beta$ -galactosidase (CMV-lacZ) construct. Data shown are the mean of greater than three experiments and were analyzed by the *t*-test. Differences of  $P < 0.05$  were considered significant. Data are shown as the level of Luc activity from the T1 $\alpha$  promoter construct relative to the promoterless construct 0-luc.

An Sp1 site in the -100 bp promoter is a critical regulator of T1 $\alpha$  expression [Ramirez et al., 1997; Cao et al., 2000]. We, therefore, tested the O<sub>2</sub>-responsiveness of a promoter fragment mutated in 2/6 or 4/6 bases of this element [Ramirez et al., 1997; Cao et al., 2000]. Mutated fragments were generated by PCR, verified by sequence analysis, and cloned into the pGL3 vector as described previously [Ramirez et al., 1997]. Cell transfection methods and assays are as described above.

#### Protein Extraction From Cells and Nuclei

Cultured cells (10 cm plates) were washed 3 $\times$  in PBS containing 0.5 mM sodium orthovanadate, 5 mM sodium fluoride, 2 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM NaF, 10 mM  $\beta$ -glycerophosphate, followed by lysis in 0.5 ml RIPA buffer containing 2% SDS and the above inhibitors. Lysates were boiled for 10 min. After addition of 1.0-ml RIPA buffer without SDS, lysates were aspirated 10 $\times$  through a 22-gauge needle, and centrifuged at 14,000g for 20 min. Supernatants were used for immunoprecipitations. Nuclear

extracts were prepared using a micro-method as previously described [Ramirez et al., 1997].

### EMSA

Gel shift assays were performed using wild type T1 $\alpha$  oligonucleotide probes (–57 to –95 bp) spanning the Sp1/Sp3 binding site (–89 to –95 bp) as described previously [Ramirez et al., 1997]. Purified complementary oligonucleotides with 3–4 bases of protruding 5'-ends were annealed as described previously [Ramirez et al., 1997], and 20 pmol were labeled with [ $\alpha$ -<sup>32</sup>P] dCTP using DNA polymerase large Klenow fragment (New England Biolabs, Beverly, MA). After purification on NucTrap columns (Stratagene, LaJolla, CA), probes were recovered in 100  $\mu$ l final volume at  $2 \times 10^5$  cpm/ $\mu$ l and diluted 1:10. Nuclear protein extracts (5  $\mu$ g) and 1  $\mu$ g poly-[d(I-C)] (Boehringer Mannheim, Indianapolis, IN) were incubated on ice for 10 min in 20  $\mu$ l buffer (12 mM HEPES, pH 7.9, 4 mM Tris pH 7.9, 25 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 10% (v/v) glycerol, 1 mM dithiothreitol, 0.2 mM PMSF). Labeled oligonucleotide (15–20 fmol,  $\sim$ 20,000 cpm) was added and incubated for 20 min. For Sp1 or Sp3 supershifts, mixtures were incubated with 1–2  $\mu$ l antibody. Sp1 supershifts were performed with an antibody recommended for EMSA procedures (Geneka Biotechnology, Inc., Montreal, Canada; rabbit anti-peptide #20012540). Supershifts for Sp3 were performed using goat anti-human peptide antibodies (Santa Cruz #sc-644-G; Santa Cruz, CA).

EMSA assays demonstrating inhibition of Sp1 binding by mithramycin (Sigma) were carried out as above except that the probe was preincubated with 100 or 500 nM (final concentration) mithramycin A for 1 h on ice. Nuclear proteins (5  $\mu$ g) from normoxic or hyperoxic (4 h) cells were used for the binding assays.

### Immunoprecipitations

Cell lysates containing 500  $\mu$ g total protein were precleared with 2  $\mu$ l normal goat serum (for 30 min at 4°C) followed by 25  $\mu$ l pre-washed protein A–G agarose (for 30 min at 4°C) and centrifugation at 14,000g. Supernatants were incubated with 10  $\mu$ l primary antibodies against Sp1 (goat anti-rat, Santa Cruz #sc-59-G) or Sp3 (as before) (for 2 h at 4°C); 30  $\mu$ l pre-washed protein A–G agarose beads were added (for 2 h at 4°C) followed by centrifugation at 14,000g (for 1 min at 4°C). Precipitates were washed 4 $\times$

with RIPA buffer containing inhibitors (above). SDS–polyacrylamide gel (PAGE) sample buffer (1 $\times$ , 30  $\mu$ l) was added followed by 5–10 min boiling. Samples were electrophoresed on 7.5% PAGE under reducing conditions and transferred to PDVF membranes (Millipore Corp., Marlborough, MA).

### Western Blots

PVDF membranes were incubated in 1 $\times$  Tris-buffered saline with Tween-20 (TBST) containing 5% dry milk for 1 h, exposed overnight to Sp1 (above; 1:1,000) or Sp3 (data not shown) antibodies (1:250 dilution in 1 $\times$  TBST containing 1% dry milk), and then to donkey anti-goat secondary antibody (1:10,000, 1 h, RT). Serine-phosphorylated Sp1 protein was detected with rabbit anti-phosphoserine antibody (RDI-PHOSSER abr, Research Diagnostics, Inc., Flanders, NJ). Membranes were blocked with 1 $\times$  TBST containing 2% bovine serum albumin (BSA) (Fisher, Pittsburgh, PA) and incubated overnight with primary antibody (1:1,000, 4°C) in 1 $\times$  TBST with 0.5% BSA. Binding of HRP-labeled secondary antibodies was detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Total protein per lane was used to normalize the data. Histone H1 was detected with a rabbit polyclonal antibody against amino acids 1–219 of the human protein (Santa Cruz, #sc-10806) using a 1:100 dilution in 1 $\times$  TBST containing 1% dry milk followed by incubation in HRP-labeled anti-rabbit secondary antibody. Western blots for T1 $\alpha$  were performed as described previously [Ramirez et al., 2003].

### Immunohistochemistry

Lungs were fixed in 4% paraformaldehyde and embedded in paraffin, and tissue sections were deparaffinized, washed, and blocked for non-specific staining as described previously [Williams et al., 1996]. Sections were exposed overnight (4°C) to 1:50 dilutions of a hamster monoclonal antibody against a 36–38 kDa protein of thymic epithelial cells [Farr et al., 1992] that (hybridoma 8.1.1, Developmental Studies Hybridoma Bank, University of Iowa, [www.uiowa.edu/~dshbwww/](http://www.uiowa.edu/~dshbwww/)). This antibody specifically recognizes only T1 $\alpha$  protein in adult mouse lung [Ramirez et al., 2003]. Slides were washed, and exposed to horseradish peroxidase-conjugated goat anti-hamster secondary antibody (1:50) followed by tyramide signal amplification

as directed by the manufacturer (Perkin-Elmer, Boston, MA) and 3, 3', 4, 4'-diaminobenzidine-HCl as enzymatic substrate. Sections were counterstained with methyl green (Vector Labs, Burlingame, CA).

## RESULTS

### Hyperoxia Increases Type I Cell Gene Expression in Intact Animals

Figure 1A shows representative ( $n = 3$ ) Northern blots of total lung mRNA from mice before (control), during (24, 48, 72 h), and after (1, 5, 14, 28 days) exposure to  $>95\%$   $O_2$  (three animals per time point) probed sequentially for the type I cell genes *T1 $\alpha$* , *AQP-5*, caveolin-1 (also expressed in lung endothelial cells), and the type II/Clara cell gene *SP-D*. Densitometry of these data normalized to the normoxic control value (Fig. 1B,  $n = 3$ ) show that *T1 $\alpha$*  and *AQP-5* mRNAs are increased greater than fivefold over control values by the third day of hyperoxia. In contrast, *SP-D* and caveolin-1 mRNA levels do not increase until day 5 after ending the exposure. The abundance of *T1 $\alpha$*  and *AQP-5* mRNAs declines quickly upon returning the animals to normoxia and within 24 h the levels are about 50% of the peak hyperoxia levels. *T1 $\alpha$*  returns to the normoxic control level by post-exposure day 5 and *AQP-5* by day 14. Thus the time courses of increased expression of *T1 $\alpha$*  and *AQP-5* mRNAs are similar as are the

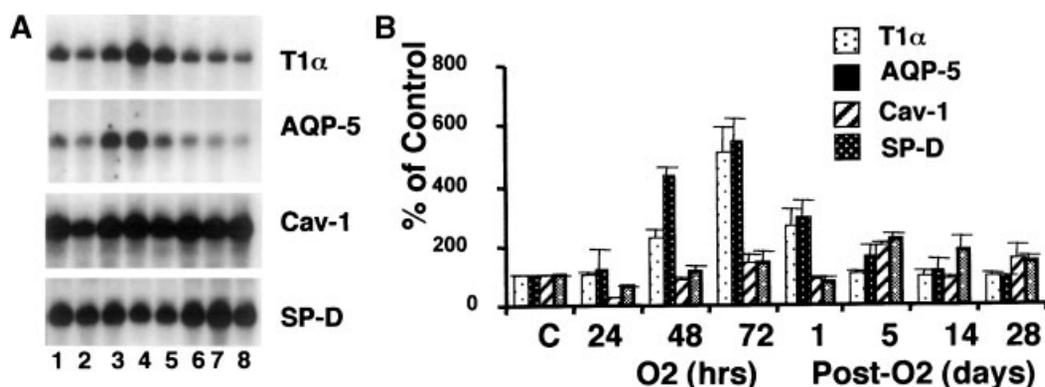
patterns of decrease when the animals are returned to normoxia. In contrast caveolin-1, a gene expressed by both type I and endothelial cells, and *SP-D*, expressed by type II and Clara cells but not type I cells, mRNAs show little change until post-exposure day 5.

Caveolin-1 and *SP-D* mRNAs are increased on post-exposure day 5 and stay elevated throughout the 28 days studied. The peak increases in caveolin-1 and *SP-D* mRNAs are about twofold, thus of a magnitude less than *T1 $\alpha$*  and *AQP-5* mRNAs. With these four peripheral lung genes, there appear to be two waves of increased expression, the first in the exposure period and the second in the post-exposure period.

Immunohistochemical localization of *T1 $\alpha$*  protein in 72 h oxygen-exposed lungs indicates that the increased expression occurs in type I cells (Fig. 2). No *T1 $\alpha$*  protein is detected in alveolar type II cells (Fig. 2B), small bronchiolar cells (Fig. 2A), endothelial (Fig. 2C), or connective tissue cells. Thus increased *T1 $\alpha$*  mRNA expression detected in whole lung homogenates is not the result of induction of ectopic expression in other cell types.

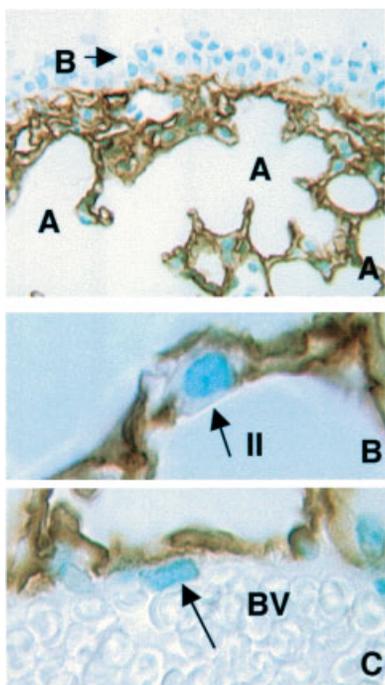
### Hyperoxia Increases Endogenous Type I Cell mRNA but not a Type II Cell mRNA Expression in Lung Epithelial Cell Lines

The E10 epithelial cell line was derived spontaneously from adult mouse lung and has been



**Fig. 1.** **A:** Northern blots of 10  $\mu$ g total RNA from lungs exposed to  $>95\%$  oxygen for 72 h followed by 28 days recovery in normoxia. The lung epithelial genes analyzed are *T1 $\alpha$*  and aquaporin 5 (*AQP-5*) (type I alveolar epithelial cells), caveolin 1 (type I and endothelial cells), and *SP-D* (type II and Clara cells). **Lane 1:** Normoxia control. **Lanes 2–4:** 24 h (lane 2), 48 h (lane 3), and 72 h (lane 4) of hyperoxia. **Lanes 5–8:** Day 1 (lane 5), day 5 (lane 6), day 14 (lane 7), and day 28 (lane 8) post-exposure normoxia. **B:** Densitometry of Northern data from (A) ( $n = 3$ ) with

normoxic control values normalized to 100%. *T1 $\alpha$*  and *AQP-5* mRNAs increase rapidly to fourfold to fivefold the control values within 48–72 h of hyperoxia and decrease to about 50% of peak values within 24 h return to normoxia, followed by a slower decline to control levels between days 5 and 14. Caveolin-1 and *SP-D* mRNAs increase during the post-exposure recovery with peak increases of about twofold on day 5. By day 28 expression levels of all four genes have declined and are close or equal to the normoxic control levels.



**Fig. 2.** A: Immunoperoxidase staining for T1 $\alpha$  protein in mouse lung after 72 h hyperoxia shows a pattern characteristic of type I cell proteins outlining the alveolar lumens (A) with no immunoreactivity of bronchiolar epithelium (arrow B). B, C: Type II alveolar epithelial cells (II, arrow) and endothelial cells (arrow) in pulmonary blood vessels (BV) lack immunoreactivity. These staining patterns indicate that hyperoxia does not induce T1 $\alpha$  expression in cells other than type I cells. Methyl green counterstain.

used as an experimental model for studies of alveolar type II cell functions. However, these cells do not express surfactant protein mRNAs including SP-C mRNA, a definitive and commonly used marker for the type II cell phenotype. Northern analyses (Fig. 3A) show that E10 cells express mRNAs for the type I cell proteins T1 $\alpha$ , AQP-5, and caveolin-1 under both normoxic and hyperoxic conditions. Densitometric analyses of three blots (Fig. 3B) shows statistically significant increases in these mRNAs at 48 h hyperoxia. For T1 $\alpha$  and AQP-5 the magnitude of increase in this cell line is similar to those observed in intact animals. In vivo hyperoxia increases T1 $\alpha$  mRNA about  $\sim 2.5\times$  the normoxia levels after 48 h; the in vitro increase is about  $3.8\times$  at the same time period. AQP-5 mRNA is increased by hyperoxia about  $4.2\times$  in vivo and  $3\times$  in vitro at 48 h. In contrast to analyses of whole lung homogenates that reflect caveolin-1 levels in both endothelial and type I cells, hyperoxia also results in a significant increase in expression of caveolin-1

mRNA in E10 cells at 48 h (Fig. 3). Thus when measured directly in a type I cell line, the caveolin gene responds to a hyperoxic stimulus consistent with other type I cell genes. Likewise there is an increase in T1 $\alpha$  protein in the hyperoxia-exposed cells (Fig. 3C).

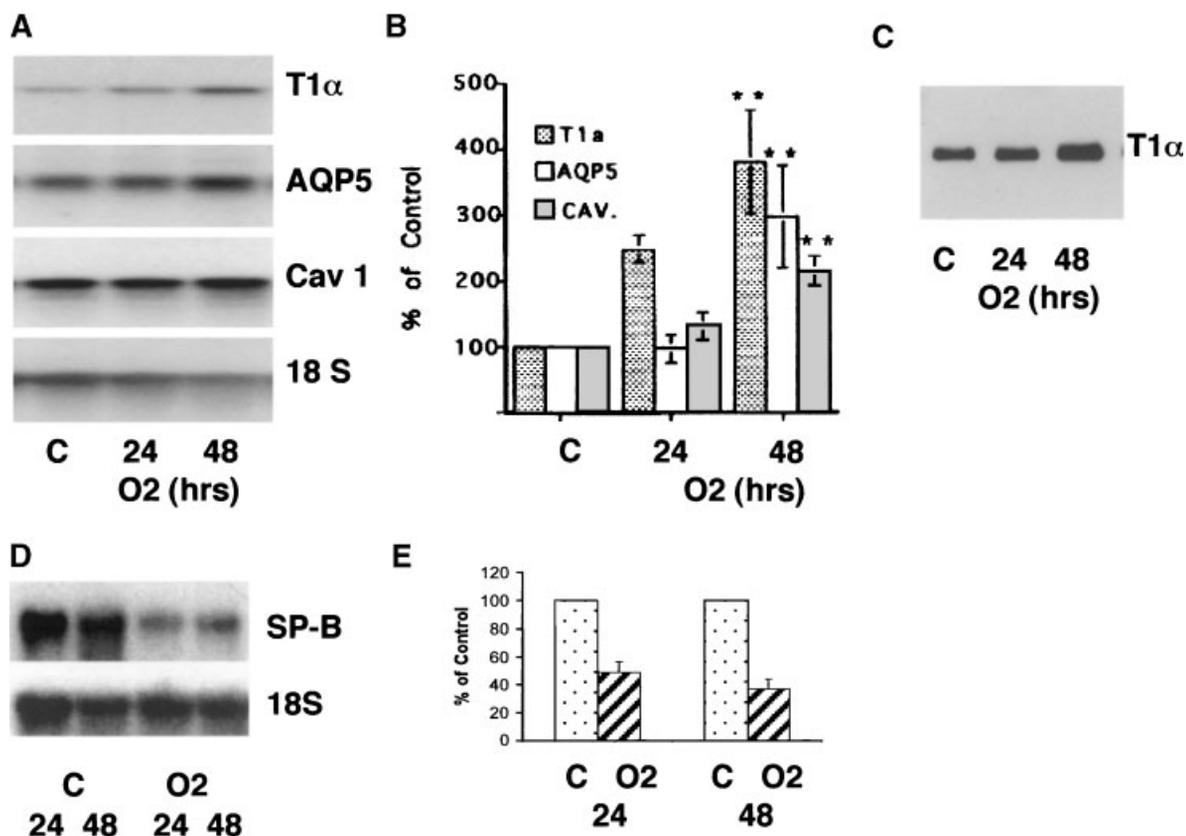
SP-B mRNA (type II cell and Clara cell marker) is not detectable in E10 cells under standard culture conditions (data not shown). To determine if expression levels of other peripheral lung epithelial genes with functional Sp promoter elements [Margana and Boggaram, 1997; Margana et al., 2000], particularly those expressed by type II cells, would increase in response to hyperoxia, MLE cells were exposed to normoxia and hyperoxia and analyzed at 24 and 48 h for changes in endogenous SP-B mRNA. In contrast to the increases observed for type I cell genes, SP-B mRNA abundance was decreased at both 24 and 48 h of hyperoxia (Fig. 3D,E).

#### Critical Sp Element Is Conserved in Rat and Murine Proximal Promoters

Previous studies characterizing T1 $\alpha$  transcriptional regulation used the rat promoter [Ramirez et al., 1997]. We, therefore, compared sequences of the proximal promoter regions of the rat and murine genes (not shown). This analysis indicates an overall  $>90\%$  identity between the two species and shows complete conservation of the functionally important Sp element between  $-89$  and  $-95$  bp. A more proximal Sp element ( $-42$  to  $-56$  bp) has not been shown to be functionally important [Ramirez et al., 1997].

#### Hyperoxia Increases Luc Expression From T1 $\alpha$ Promoter-Reporter Constructs

To localize promoter regions containing hyperoxia-responsive *cis*-elements MLE cells were transduced with T1 $\alpha$  promoter-Luc constructs as in previous studies [Ramirez et al., 1999]. The cells were allowed to recover overnight from transfection procedures before initiating the 24 h hyperoxic exposure. Figure 4A compares Luc activity in control and O<sub>2</sub>-exposed cells using  $-170$  bp,  $-1,251$  bp, and  $-10$  kbp promoter fragments. O<sub>2</sub> exposure significantly increases Luc expression from the  $-170$  to  $-1,251$  bp promoter fragments. The percent increase from these fragments relative to the normoxic value is similar, i.e.,  $\sim 35$ – $40\%$ . As



**Fig. 3.** A: Northern analyses show that hyperoxia increases endogenous expression of type I cell mRNAs in E10 cells. B: Densitometry of three blots from three separate experiments shows that all three mRNAs are induced twofold to fourfold by 48 h hyperoxia with a fold increases similar to those in intact animals (Fig. 1)  $**P < 0.002$  ( $T1\alpha$ ),  $< 0.001$  (AQP-5),  $< 0.05$  (caveolin-1). C: The time course of increases in  $T1\alpha$  protein

shown by Western blot of hyperoxic E10 cells corresponds with changes in mRNA. D: Hyperoxia does not increase endogenous mRNA expression of SP-B, a representative type II cell gene, in MLE cells under similar conditions. E: Densitometry of blots ( $n = 3$ ) shows that SP-B expression is decreased during the 48 h exposure. Expression levels are normalized to 18S mRNA.

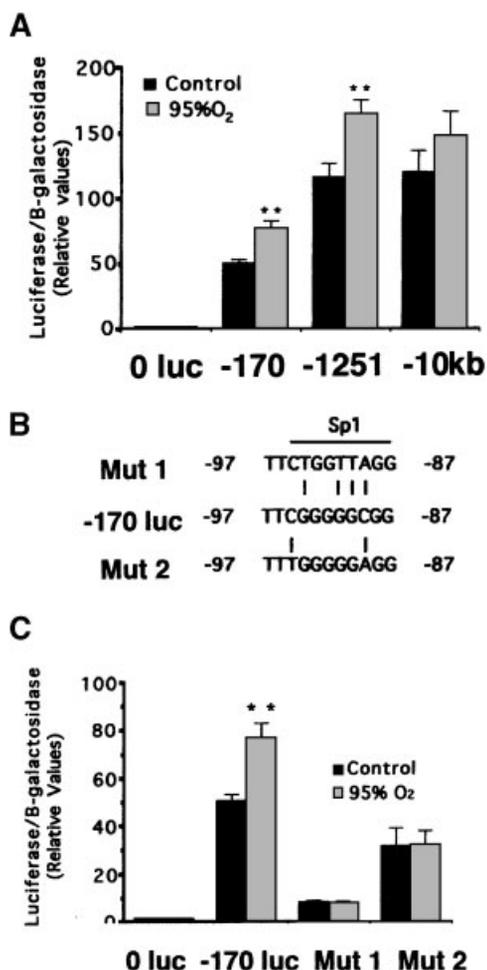
there the  $-1,251$  and  $-10$  kbp fragments do not drive additional increases relative to the proximal promoter, the  $-170$  bp region likely contains most of the elements required for the  $O_2$ -dependent increases in expression. Studies using E10 cells and the  $-170$  and  $-1,251$  bp promoters confirm that hyperoxia increases transcriptional activity with patterns and levels of expression similar to MLE cells (data not shown).

Previous studies using mutated promoters have shown that an Sp *cis*-element at  $-89$  to  $-95$  bp is critical for activation of the  $T1\alpha$  gene [Ramirez et al., 1997; Cao et al., 2000]. We, therefore, prepared promoter-Luc constructs mutated at either 2/6 or 4/6 bases of the  $-89$  to  $-95$  element within the  $-170$  bp promoter (Fig. 4B). Figure 4C shows that these mutations block the hyperoxic-induced increase in  $T1\alpha$ -driven Luc expression. As expected these muta-

tions also markedly decrease expression from this promoter fragment in normoxic conditions.

#### **$O_2$ Increases Sp1/Sp3 Binding to the $-89$ to $-95$ Sp *cis*-Element**

Previous gel shift studies have shown that nuclear proteins from normoxic cells bind to a  $T1\alpha$ -promoter oligonucleotide probe spanning the  $-57$  to  $-95$  bp region. Competition and mutation analyses show, respectively, that binding is specific and limited to the  $-89$  to  $-95$  element [Ramirez et al., 1997]. Two major shifted bands representing Sp1 and Sp3 protein-DNA complexes are present, although the Sp1 (upper) band is composed of two complexes. Figure 5A shows a representative gel shift analysis using the same oligonucleotide probe [Ramirez et al., 1997] and nuclear proteins from E10 cells exposed to normoxia or hyperoxia (1, 2, 4 h). At 4 h of hyperoxia increases in both Sp1



**Fig. 4.** A: Hyperoxia increases transcriptional activity of T1 $\alpha$  promoter-luciferase (Luc) fragments by activation of elements in the -170 bp proximal promoter (n = 3). Although significantly different from 0-Luc, the fold increase driven by the -1,251 bp promoter is not significantly different from the -170 bp promoter, suggesting that critical oxygen-responsive regulatory elements are restricted to the proximal promoter. Values are relative to the 0-Luc construct.  $P < 0.05$ . B: Mutations in the -89 to -95 Sp cis-element are shown relative to the wild-type -170 bp sequence. Mutation 1 (Mut 1) contains four base substitutions while mutation 2 (Mut 2) contains only two substitutions. C: Compared to the wild-type -170-luc, both mutations block hyperoxia-induced increases in Luc expression. Transcriptional activity in normoxia is markedly reduced by these mutations as previously reported.  $P < 0.05$ .

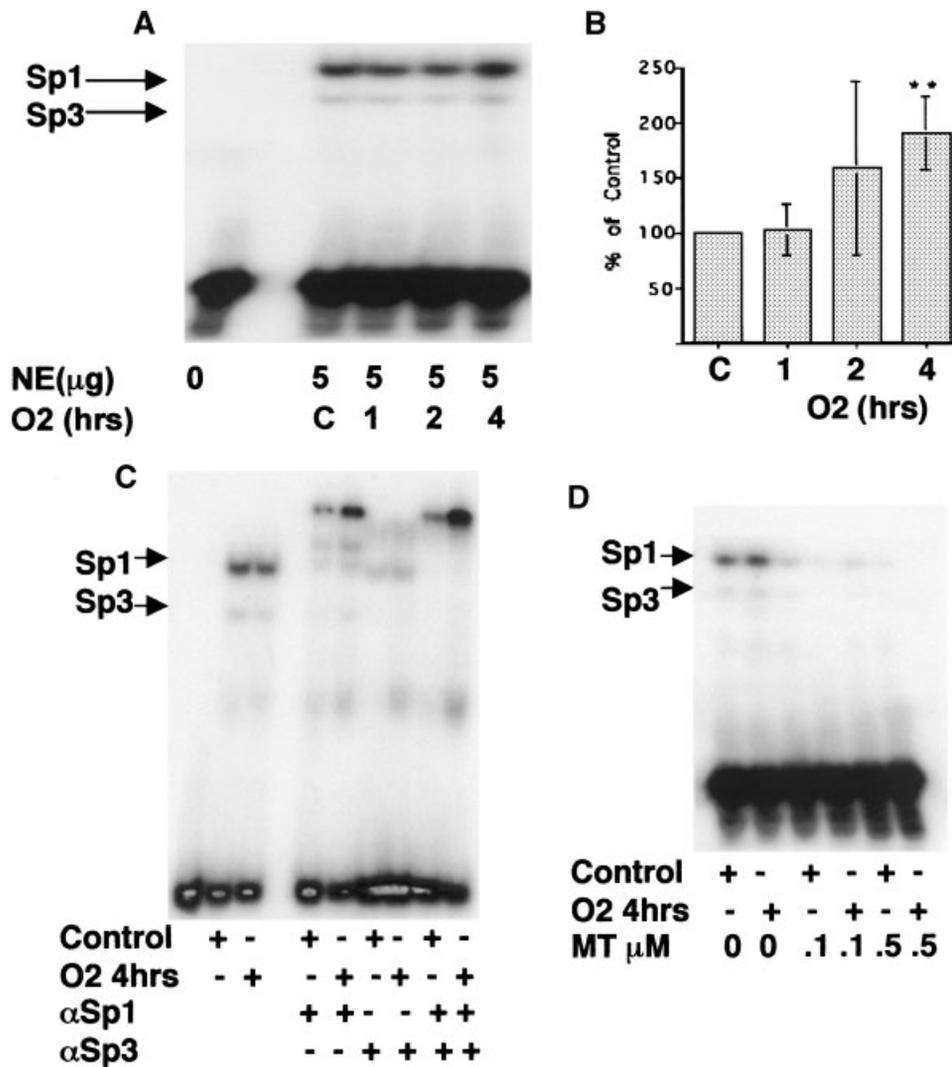
and Sp3 bands are apparent. Figure 5B shows the densitometric analysis of Sp1 binding of three EMSA experiments; hyperoxia values are normalized to normoxic controls. There is a  $\sim 2\times$  increase in binding at 4 h of hyperoxia that is statistically significant. Although the 2 h hyperoxia level is increased, it is not statistically significant compared to either control or 1 h levels.

Supershift assays using Sp1 and Sp3 antibodies show that both proteins contribute to DNA-protein complexes (Fig. 5C) under normoxic and hyperoxic conditions although Sp1 binding is by far the more significant. The Sp1 antibody results in two prominent shifted bands, likely due to differences in Sp1 phosphorylation [Jackson et al., 1990]. Used alone the Sp3 antibody blocks protein-DNA binding resulting in loss of the shifted band but no supershifted complex (Fig. 5C). When used together, Sp1 and Sp3 antibodies supershift or inhibit all Sp-DNA complexes. A minor lower molecular weight DNA-protein complex can be detected in gel shift studies (Fig. 5C); the identity of the protein is unknown. This band appears inconsistently and is not reproducibly shifted by either antibody.

Addition of mithramycin (100 or 500 nM), a antibiotic that binds to GC-rich sequences and acts as a competitive inhibitor of Sp-DNA interactions [Chatterjee et al., 2001; Liu et al., 2002], markedly reduces Sp1/Sp3 interactions with the T1 $\alpha$  promoter probe when present during the DNA-protein binding reactions; mithramycin (500 nM) almost completely blocks interaction of either normoxic or hyperoxic nuclear proteins with this oligonucleotide (Fig. 5D).

#### Mithramycin Blocks Hyperoxia-Induced Increases in T1 $\alpha$ Expression in E10 Cells

E10 cells were treated with mithramycin (100 or 500 nM) to test whether blocking Sp-DNA interactions in intact cells would interfere with induction of T1 $\alpha$  gene expression by hyperoxia. A representative Northern blot of cells harvested after 48 h exposure to hyperoxia shows the expected increase in T1 $\alpha$  mRNA in the absence of mithramycin (Fig. 6A). In hyperoxic cells, mithramycin substantially reduces T1 $\alpha$  mRNA levels by  $\sim 50\%$  (100 nM) compared to hyperoxia without the drug. At 500 nM concentrations the hyperoxic response is completely blocked as shown by densitometry and statistical analysis (n = 3) (Fig. 6B). Thus both concentrations of mithramycin substantially decrease the hyperoxic effects. Mithramycin does not affect basal T1 $\alpha$  mRNA levels in normoxic cells within the duration of the exposure time tested, consistent with the behavior of other genes where it blocks induced, but not basal levels, of expression [Yoo et al., 2002].



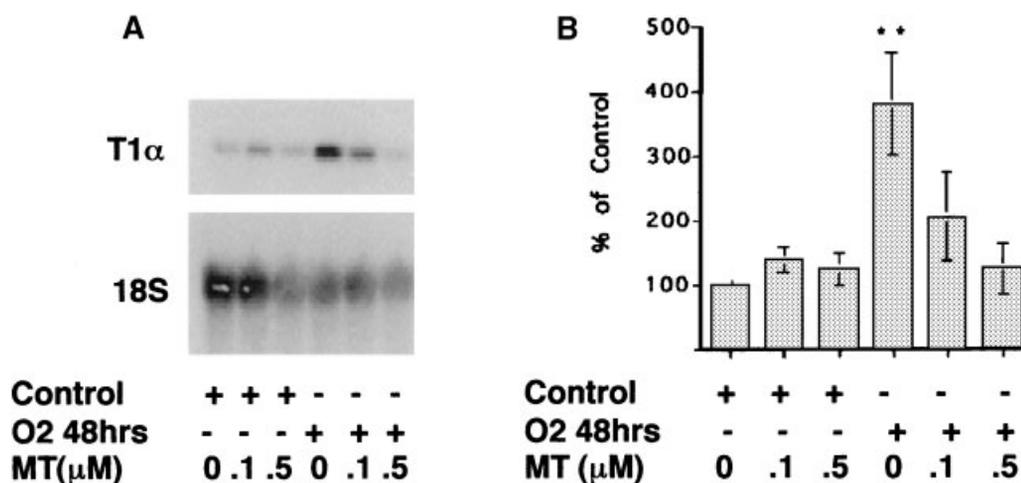
**Fig. 5.** **A:** EMSA analysis of nuclear proteins from hyperoxia-exposed cells shows increased binding of Sp1 protein to an oligonucleotide probe containing the -89 to -95 Sp *cis*-element of the T1 $\alpha$  promoter at 4 h with a smaller increase in Sp3 binding. **B:** Densitometry of the shifted Sp1 band shows statistical significance only at 4 h of hyperoxia ( $n = 3$ ). **C:** Anti-Sp1 antibody alone supershifts two molecular species of which the larger molecular weight is believed to be highly phosphorylated

[Jackson et al., 1990]. Anti-Sp3 antibody supershifts and/or inhibits formation of protein-DNA complexes, and both antibodies used together supershift or inhibit formation of all Sp1 and Sp3 shifted bands. **D:** Mithramycin, a competitive inhibitor of Sp binding, inhibits formation of DNA-protein complexes with this probe in both hyperoxic and normoxic extracts.

### Hyperoxia Increases Nuclear Abundance of Sp1 Protein

Western analysis of nuclear proteins from normoxic or hyperoxic E10 cells used for EMSA assays shows a marked increase in nuclear Sp1 protein content within 2–4 h of initiating the hyperoxic exposure (Fig. 7A); hyperoxia does not increase concentrations of histone H1 in E10 nuclei (Fig. 7A). Therefore, the increase in nuclear Sp1 is not likely to be the result of non-specific changes in nuclear protein content

due to hyperoxia. Densitometry ( $n = 3$ ) normalized to normoxic values shows that hyperoxia results in about a sixfold to sevenfold increase in nuclear Sp1 at 4 h (Fig. 7B). However, hyperoxia does not increase total cellular content of Sp1 in either E10 (Fig. 7C,D) or MLE cells (data not shown). Western blots of Sp1 immunoprecipitates probed for phosphorylated serine residues (Fig. 7E) consistently show increases in Sp1 phosphorylation in E10 cells exposed to hyperoxia (Fig. 7F). Such an increase is also suggested by data in Figure 5C showing prominence of the



**Fig. 6.** A: Mithramycin (0.1 or 0.5  $\mu\text{M}$ ) blocks the hyperoxia-induced increase in T1 $\alpha$  mRNA in E10 cells as shown by Northern analysis. B: Densitometry ( $n=3$ ) indicates that this response is dose dependent with  $\sim 50\%$  inhibition at 0.1  $\mu\text{M}$  and complete inhibition at 0.5  $\mu\text{M}$ . Neither concentration affects baseline expression levels in normoxic cells.

highest molecular weight, phosphorylated species supershifted by Sp1 antibodies in hyperoxic E10 cells.

## DISCUSSION

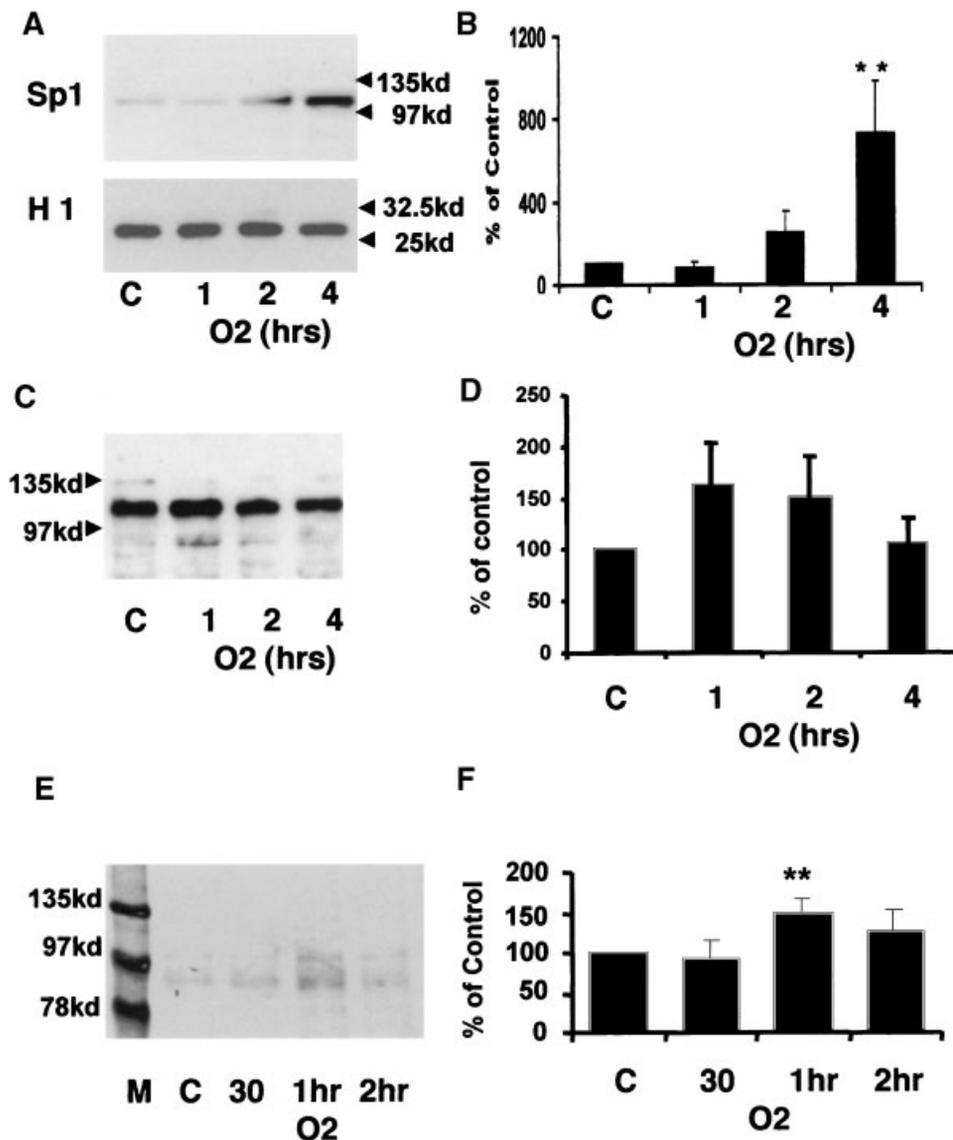
We have shown previously that T1 $\alpha$  basal promoter activity is regulated by Sp1/Sp3, and our studies now implicate Sp1/Sp3 in induced T1 $\alpha$  expression in response to hyperoxia. Several lines of evidence support this conclusion including data from promoter deletion-reporter constructs, mutational analyses, EMSAs and supershifts, and mithramycin inhibition to block binding of Sp proteins and transactivation in intact cells. Furthermore mutations in the T1 $\alpha$  promoter suggest that the -89 to -95 Sp element in the T1 $\alpha$  promoter that we identified as essential for T1 $\alpha$  expression in non-hyperoxic conditions is activated by increased Sp1/Sp3 binding during hyperoxia-induced T1 $\alpha$  expression.

There is considerable information about the Sp family of transcription factors and the widespread roles they play in gene regulation [Black et al., 2001]. Sp1 and Sp3 are members of a large family of transcription factors that have three zinc finger motifs and bind DNA elements rich in GC nucleotides. Of the four Sp family members Sp1, 3, and 4 are widely expressed including in the lung. Little is known about molecular functions and sites of expression of Sp2. Early studies on the general functions of Sp *cis*-elements and transcription factors sug-

gested that this system was primarily involved in transcription of constitutively expressed genes, so-called house-keeping genes, that lacked TATA or CAAT box sequences. However, there are now many examples of Sp transcription factors participating in transactivation of genes that are regulated and induced by different types of stimuli. Thus there is a precedent for our findings that the Sp system mediates the increased expression of T1 $\alpha$  in response to hyperoxia.

It has also been shown that Sp proteins can mediate gene induction in a gene selective manner, with specific target genes being activated while other genes for which Sp-dependent transactivation is important are not affected within the same cellular milieu [Black et al., 2001]. We show that three type I cell genes are induced by hyperoxia in cell lines whereas SP-B, a type II cell gene with a functional Sp element in the proximal promoter [Margana and Bogaram, 1997; Margana et al., 2000] is not. Sequence data show that both AQP-5 [Borok et al., 2000] and caveolin-1 promoters have Sp *cis*-sequences and, in the case of caveolin-1, this element is known to be functional [Bist et al., 2000]. Likewise in intact animals, we show that SP-D, another alveolar epithelial gene with a functional Sp *cis*-element [He et al., 2000], is not induced by hyperoxia.

It is not clear that all inducers activate the Sp system in the same way. A number of molecular changes such as phosphorylation or glycosylation are known to alter Sp protein



**Fig. 7.** A: Western blots of E10 nuclear proteins (10  $\mu$ g protein) show that the concentration of Sp1 protein increases after 2–4 h hyperoxia while concentrations of histone H1 do not change. B: Densitometry ( $n=3$ ) indicates about a sixfold to sevenfold increase in nuclear Sp1 4 h after initiating the exposure. C, D: However, hyperoxia does not increase total cellular Sp1 protein

abundance by Western blot. E: Immunoprecipitates of Sp1 blotted for phosphoserine residues indicate that Sp1 phosphorylation is increased by hyperoxia. M, molecular weight markers. F: Densitometry and statistical analysis ( $n=3$ ) show that there is about a 50% increase in phosphorylated Sp1 in E10 cells after 1 h of hyperoxia.  $P < 0.02$ .

activity [Black et al., 2001] but there is little general understanding of how these are controlled or what cellular events preferentially result in one modification and not in others. In addition the abundance of Sp protein can change in response to an inductive stimulus. Furthermore there is evidence that sulfhydryl groups of Sp1 protein can be oxidized in response to changes in redox state or hyperoxia [Wu et al., 1996]; in some systems, this leads to decreased DNA binding. If Sp1 oxidation occurs in the lung

or cell lines we studied, it does not interfere with transactivation of the T1 $\alpha$  promoter.

In E10 cells, hyperoxia appears to influence translocation of Sp proteins, based on our data showing that Sp1 protein levels in nuclear homogenates increase about  $\sim 6$ – $7\times$  after 4 h hyperoxia. This increase is evident at 2 h exposure but is not statistically significant. The increase in nuclear Sp1 occurs without detectable increases in total cellular Sp1 or Sp3 protein as shown by IP-Western blotting. This

raises the possibility that translocation of Sp protein from cytosol to nuclei results in nuclear accumulation of Sp1; alternatively, there could be a decreased efflux in the opposite direction. The time course of increased nuclear Sp1 coincides with the increased Sp binding shown by EMSA; both are increased at 2 h and statistically significantly increased after 4 h hyperoxia. At first glance, this time course seems prolonged in comparison to the rapidity of many signaling events that occur within a few minutes after addition of soluble stimuli such as growth factors or activation of a signaling pathway. However, this delay is likely explained by recent data from Allen et al. [2001] who demonstrate in an experimental system very similar to ours that equilibration of oxygen tension in culture media with that of the gas phase (100% O<sub>2</sub>) takes about 3 h. When this is taken into account, the E10 cells appear to respond very rapidly, and likely directly, to hyperoxia since we observe increases in nuclear Sp1 protein and enhanced Sp–DNA binding at 2 h hyperoxia.

Increases in nuclear Sp1 protein have not been observed in all Sp responsive systems. Marinovic et al. [2002] report that neither nuclear or cytosolic Sp1 protein levels changes upon induction of ubiquitin expression in muscle cells. MEK1 kinase in the MAPK pathway is involved in this system, suggesting that Sp1 activity is increased by ERK-dependent phosphorylation.

We also noted increases in Sp1 phosphorylation, although the increase in the phosphorylated species in hyperoxia was only 40–50% over normoxic levels. This magnitude of increased phosphorylation is similar to that observed by Alroy et al. [1999] in studies of neuregulin activation of Erb receptors using P-19 teratoma cells. However, both P-19 and the lung cell lines normally contain high levels of phosphorylated Sp1 that may make increases difficult to detect on blots. In lung epithelial cell lines, Sp1 is phosphorylated in cells whether in normoxia or hyperoxia, and the T1 $\alpha$  promoter is efficiently transcribed in both. It is possible that there is a small pool of non-phosphorylated Sp1, which is activated upon exposure to hyperoxia. Alternatively hyperoxia could induce phosphorylation of additional serine residues on already partially phosphorylated molecules.

Predicting how Sp phosphorylation will affect transcription is not straightforward. In some

systems, dephosphorylation reduces but does not eliminate DNA binding [Merchant et al., 1999]; in other systems dephosphorylation increases DNA binding and transcription [Zhu and Liao, 2000]. Sp phosphorylation can lead to enhanced molecular interaction with the multi-protein Sp co-activator complex (CRSP) among others [Ryu et al., 1999].

The effects of hyperoxia on E10 cell T1 $\alpha$  gene expression are reduced about 50% by 100 nM mithramycin while at 500 nM hyperoxic induction is completely blocked; mithramycin concentrations in this range have been shown not to suppress  $\beta$ -actin expression [Zhu et al., 2002] or other genes commonly used to normalize mRNA levels including, in our system, levels of 18S. Thus, we believe mithramycin acts by competing for Sp protein binding to DNA in lung cells as expected. Mithramycin does not appear to interfere with T1 $\alpha$  expression in E10 cells maintained in normoxia, consistent with EMSA data that show a lesser effect of MT on Sp1/Sp3–DNA binding in normoxic conditions. We are not certain as to the reason for this difference but suppose that the more complex issues of phosphorylation state and protein–protein interactions are likely to be involved.

Some gene-selective, Sp-mediated transcriptional changes involve interactions with other transcription factors such as NF- $\kappa$ B and Rb, and this also seems likely in our system. We have shown previously that thyroid transcription factor-1 (TTF-1) and HNF3 $\beta$ , a forkhead transcription factor, interact with the T1 $\alpha$  promoter [Ramirez et al., 1997], and there is recent evidence suggesting that TTF-1 and forkhead proteins can be modified as a result of an oxidative environment [Tell et al., 2002]. Oxygen and reactive oxygen species appear to increase DNA binding of TTF-1/NF- $\kappa$ B complexes [Islam and Mendelson, 2002] whereas oxidation of key TTF-1 sulfhydryl groups decreases DNA binding [Tell et al., 2002]. Likewise hydrogen peroxide treatment of PC12 cells increases phosphorylation of the forkhead protein FKHRL1 resulting in cytoplasmic retention, decreased nuclear concentrations, and decreased gene activation [Nemoto and Finkel, 2002].

In addition to possible interactive effects of TTF-1 and HNF3 $\beta$ , a likely candidate for a cooperative role in T1 $\alpha$  gene induction is Nrf2 that binds to the antioxidant response element (ARE) in many genes that encode indirect

antioxidants. Nrf2 is abundantly expressed in lung and is markedly up-regulated by hyperoxia [Cho et al., 2002a]. Nrf2 mRNA is expressed by E10 cells as assessed by RT-PCR (data not shown), and hyperoxia appears to increase cellular concentrations. Sequence analysis identifies an Nrf2 response element (ARE; 5'-ATGAcTAAGC-3') at -1,004 to -1,014 in the upstream regulatory region of the T1 $\alpha$  promoter, and there are two additional sequences with 90% identity to the consensus sequence at more proximal sites. These elements may be involved in responses to hyperoxia but, if so, they must act through the Sp *cis*-element that we have shown to be essential for increased transcription. We are initiating studies to assess the importance of Nrf2-AREs in hyperoxic responses of lung cell lines.

Taken together, our observations on the effects of hyperoxia on type I cell gene expression in intact mice and murine epithelial cell lines show the following: Hyperoxia induces a fourfold to fivefold increase in T1 $\alpha$  mRNA and protein and a similar increase in AQP-5 mRNA. The increases are not the result of induction of expression in cells that normally do not express these genes as shown by immunostaining for T1 $\alpha$ . Increased expression of peripheral lung genes is selective and limited because the mRNAs for genes such as SP-B and SP-D (expressed by type II and Clara cells) are not increased in the models, we have studied.

These conclusions also appear to hold true for caveolin-1. When tested in E10 cells where there are no confounding issues related to the presence of endothelial cells that are early targets of hyperoxic injury, caveolin-1 mRNA is increased ( $\sim 2.5\times$ ) by hyperoxia. The magnitude of increased T1 $\alpha$  and AQP-5 mRNAs in E10 cells approaches that in the intact lung (T1 $\alpha$ ,  $\sim 4\times$ ; AQP-5,  $\sim 3\times$ ), suggesting that these cells likely contain many of the requisite signaling molecules and co-factors that mediate transcriptional responses in the lung. Studies of E10 cells also strongly support the conclusion that increases in the type I cell mRNAs are the result of direct effects on hyperoxia on type I cells and are not likely to be mediated by secondary stimuli arising from the vasculature, other lung cells, or other organs.

There appears to be discrepancy in the time course of T1 $\alpha$  expression between whole lung and lung cell lines. By EMSA there is increased Sp1 binding of E10 cell proteins to the

T1 $\alpha$  promoter at 4 h, but there is not a significant increase in T1 $\alpha$  mRNA in whole lung for about 48 h. However, analysis of whole lung mRNA would not be expected to be sensitive enough to detect early changes in mRNA levels in a cell population that comprises only about 10% of the total cell population. A more likely explanation is that, in animals, there is a complex molecular environment designed to thwart oxidative attacks consisting antioxidant enzymes, cytoplasmic buffers, high levels of glutathione, and surfactant proteins -A and -D that prevents molecular signaling for some time until overpowered. Most of these protective molecules would not be present in E10 cells, perhaps leading to more immediate cellular responses.

The specific molecular functions of T1 $\alpha$ , AQP-5, and caveolin-1, are not well understood in terms of alveolar biology. T1 $\alpha$  knock-out mice fail to form many type I cells during pre-natal lung development and die at birth (term) of respiratory failure [Ramirez et al., 2003]. These animals show evidence of increased peripheral lung cell proliferation, suggesting that T1 $\alpha$  and/or type I cells contribute to a signaling pathway that regulates peripheral lung cell division in the perinatal period. AQP-5 null mice are viable, able to resorb alveolar fluid at birth and alveolar edema fluid in adults [Ma et al., 2000], and show little evidence of defective lung structure or function postnatally, although in other organs AQP-5 protein regulates fluid fluxes [Ma et al., 1999]. Loss of caveolin-1 in mutant mice results in lung fibrosis and increased proliferation of alveolar capillary endothelial cells [Drab et al., 2001; Razani et al., 2001]. Given that the functions of these proteins in normal type I cells are uncertain, the cellular consequences of increased expression in hyperoxia are entirely speculative.

These molecular responses appear to be generally dissociated in their timing from the hyperoxia-induced changes in lung cell populations reported by others. Although details of the hyperoxic exposures differ considerably between studies, some studies show no change or a modest decrease in type I cell number by 40-60 h of hyperoxia with an increase in type II cells at about 7 days. We would expect to see a change in expression of type I cell genes as the post-proliferative type II cells differentiate towards the type I cell phenotype. That these genes respond relatively early in the hyperoxic

exposure leads us to wonder if the T1 $\alpha$  and AQP-5 proteins provide molecular functions that help protect the type I cell against an on-going injury.

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