Three Alternative Promoters of the Rat γ-Glutamyl Transferase Gene Are Active in Developing Lung and Are Differentially Regulated by Oxygen after Birth

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Abstract

The rat γ-glutamyl transferase mRNA transcripts I, II, and III are derived from three alternative promoters, P_I, P_{II}, and P_{III}. In the adult only mRNA III is expressed in the lung. We show that mRNA III gene expression is developmentally regulated in the fetal lung; it is first expressed late in gestation. In contrast to the adult lung, the fetal lung expresses mRNA I, II, and III. The switch from the fetal to the adult pattern of γGT mRNA expression begins within the first 24 h of birth and is complete by 10 d of age. γGT mRNA II disappears within 24 h, mRNA I disappears by 10 d leaving mRNA III as the sole transcript. Alveolar epithelial type 2 cells (AT2) isolated from the adult lung express only mRNA III. When cultured in 21% O_2 mRNA III is maintained, but when cultured in 3% O_2 the fetal pattern of mRNA I, II and III expression is induced. When AT2 cells in hypoxia are exposed to carbon monoxide, mRNA II is suppressed suggesting that a heme-binding protein (responsive to oxygen) may suppress mRNA II expression and may be responsible for the decrease in lung mRNA II seen after birth. A reporter gene under the control of DNA sequences from the γGT P_{III} promoter is activated in transient transfection studies in response to hyperoxia, while a deletion construct retaining an antioxidant responsive element is not. Oxygen appears to regulate each of the alternative promoters of the γGT gene, such that P_{II} is rapidly repressed by a heme-dependent mechanism, P_I is more gradually repressed by a nonheme mechanism and P_{III} is activated by a putative oxygen response element. We hypothesize that similar oxygen-dependent mechanisms regulate other genes in the developing lung at birth. (*J. Clin. Invest.* 1996. 97:1774–1779.) Key words: hypoxia • alveolar epithelium • development • hyperoxia

Introduction

γ-glutamyl transferase (γGT)[1] (EC 2.3.2.2.) is a single copy gene in the rat haploid genome. The regulatory region of this gene is organized into at least four alternative promoters designated P_I, P_{II}, P_{III}, and P_{IV}. In the adult, these alternative promoters are used in a tissue-specific fashion. Alternate promoter utilization and alternative RNA splicing results in the expression of at least five γGT mRNA transcripts, mRNA I, II, III, IV-1, and IV-2. These transcripts differ in their 5′-untranslated regions, but all have the same open reading frame and hence code for a common protein (1, 2). The primary translation product is a single chain propeptide which is endoproteolytically cleaved to form a heterodimer. This protein is expressed as a ectoenzyme in multiple epithelia and as an extracellular protein in several epithelial secretions. γGT functions in the metabolism of glutathione, a redox regulatory molecule (3), and glutathione-substituted molecules such as leukotriene C (4).

In the adult, γGT protein is expressed in two cell types of the distal lung, the alveolar type 2 (T2) cell, and the bronchiolar Clara cell. Amphipathic γGT protein is also present extracellularly in association with surfactant phospholipid (5). The γGT substrate glutathione is abundant in the alveolar lining fluid (6), the Clara cell, and the T2 cell (7). Glutathione appears to be essential for normal T2 cell function as inhibition of its synthesis produces severe structural abnormalities in the cell's mitochondria and lamellar bodies (8). Extracellular glutathione functions as an antioxidant screen to protect the gas exchange surface of the lung (6). Surfactant-associated γGT may function to regulate the size and the turnover of this glutathione pool and produce γ-glutamyl amino acids for cells within the alveolar space (5).

Having characterized the expression of γGT in the adult lung (5), we have studied its expression in developing lung to gain further insight into its regulation and function. We show that the γGT gene is developmentally regulated and that the fetal lung expresses three different γGT mRNA transcripts whereas the adult lung expresses only one. The switch to this adult pattern begins within 24 h of birth and appears to be driven, in part, by the abrupt change in oxygen concentration that takes place at birth. We provide evidence that oxygen is playing a regulatory role for each of the γGT alternative promoters, that the mechanisms of oxygen regulation are different for each promoter, and that one of the γGT promoters may be down-regulated at birth by an oxygen-sensitive heme-binding protein.

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1. *Abbreviations used in this paper:* F18 cell, fetal epithelial cells isolated from the lung at the 18th day of gestation; γGT, γ-glutamyl transferase; HIF-1, hypoxia-inducible transcription factor; RT, reverse transcriptase; T2 cell, pulmonary alveolar type 2 cell.

Methods

Materials. Adult male Sprague-Dawley rats and timed-pregnant female Sprague-Dawley rats were obtained from Zivic-Miller Laboratories, Zelienople, PA. All materials for cell culture were as described in references 9 and 10. Materials for PCR were as described in reference 5. Tri-Reagent for RNA isolation was from Molecular Research Center, Inc., Cincinnati, OH. Plasmid DNA constructs for CMV-β-galactosidase were kindly supplied by Dr. Alex Mitsialis, Section of Biomolecular Medicine, Boston University School of Medicine (Boston, MA). Those for γGT-CAT were as described in reference 11. Plasmid maxi-preps were prepared and purified with a Wizard Maxi-kit obtained from Promega Corp. (Madison, WI). Purified chloramphenicol acetyltransferase and β-galactosidase enzymes were obtained from Promega Corp. ^{3}H-acetyl coenzyme A (sp act 1–10 Ci/mmol) was obtained from New England Nuclear, Boston, MA. DEAE dextran was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Chloroquine and *o*-nitrophenyl-β-D-galactopyranoside were from Sigma Chemical Co. (St. Louis, MO). DNA electrophoresis standards were from Gibco BRL (Gaithersburg, MD) (1-kb DNA ladder) or New England Biolabs Inc. (Beverly, MA) (pBR322 DNA-Msp1 digest).

Lung tissue and cell source. Fetal and postnatal lung tissue samples were selected at various times based on the developmental profile of enzyme activity as described in reference 12. Lungs studied were 18 d gestation, just before enzyme activity was apparent, 21 d of gestation when fetal activity was near peak, at three time points within the first 24 h after birth (2, 12, and 24 h), 10 d into neonatal life when activity was again rising, and at 6–8 wk of age. Primary lung epithelial cells were isolated from fetal Sprague-Dawley rats at 18 d of gestation (F18) using collagenase dispersion, differential adherence, and density gradient centrifugation as described in reference 9. Primary adult type 2 cells (T2) were isolated by intratracheal elastase digestion and differential adherence on IgG-coated plates as described (10). Cells were cultured with MEM supplemented with 10% bovine fetal serum. A human hepatoma cell line, HepG2, and a subclone of SV40T-T2, a lung alveolar type 2 cell line immortalized by SV40 large T antigen (13), were maintained and passaged in the same media. The SV40T-T2 subclone was selected for high transfection efficiency.

Oxygen exposure conditions. Gas mixtures of 0, 3, 40, or 95% oxygen with 5% CO_2 and balance nitrogen or 10% CO, 5% CO_2 and balance nitrogen were obtained from Wesco Medical Gases, Inc. (Bellerica, MA). Cells were exposed to these gas mixtures in modular incubator chambers which were obtained from Billups-Rothenberg, Inc., Del Mar, CA. Conditions of exposure were as described by Farber et al. (14).

RNA analysis. Total RNA was isolated from lung tissue with guanidinium hydrochloride and cellular RNA with Tri-Reagent according to manufacturer's guidelines. RNA was quantitated by spectrophotometry and analyzed by reverse transcriptase (RT)-PCR as described (5). 1 μg of total lung RNA was reverse transcribed in a 20-μl volume using the γGT-specific oligonucleotide 5′-TCTCTCCTTCC TGAAGCACC (15) and the β-cytosolic actin oligonucleotide 5′-CCA-CATCTGCTGGAAGGTG G (16). A 2-μl aliquot of this cDNA solution was enzymatically amplified in a vol of 100 μl using 2.5 U of AMPLITAQ recombinant Taq DNA polymerase according to Promega Corp. specifications. The thermal cycler unit was programmed for 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. An aliquot of the primary PCR reaction was diluted 1,000-fold and used as a template for a secondary PCR reaction with nested primers. Template DNA or primers were omitted from one PCR reaction as a negative control. The rat cytosolic β-actin gene was amplified as a positive control for RNA integrity in all samples. A 3-μl aliquot of the secondary PCR reaction was analyzed by electrophoresis in a 1.5% agarose gel, and the PCR product was visualized after staining the gel with ethidium bromide. To identify specific mRNAs, an upstream primer from the unique 5′ untranslated region of each mRNA species was used with a downstream primer from the coding domain exactly as described in reference 5. In one experiment, upstream and downstream primers were selected from the coding domain to identify any γGT mRNA nonselectively, and these were 5′-GCT-TTGTGCGAGGTGTTCTG and 5′-CCATCGTCTGGAAGGTAGA, and 5′-CTCAGCGGGCCCGTGCTG and 5′-GGCGGTTGGGTG-AGTGGT in the primary and the secondary PCR reaction, respectively.

Transient transfection assay. 24 h before transfection, one million cells were plated per 60-mm tissue culture dish with MEM supplemented with 10% FCS. Fresh medium was supplied on the day of transfection. The SV40T-T2 cells were transfected with DEAE/dextran and chloroquine. The plates were washed with PBS then exposed for 30 min to 1 ml of a solution of DEAE dextran (500 μg/ml) containing 2 μg of CMV-β-galactosidase DNA and 10 μg of either γGT-P_{III}-CAT DNA, pCAT basic vector DNA as a negative control, or pSV2-CAT as a positive control. 3 ml of serum-supplemented media containing 200 μM chloroquine was then added directly for 2 h. Thereafter the cells were washed with fresh medium and incubated in 21 or 95% oxygen for 48 h. HepG2 cells were transfected by precipitating DNA with calcium phosphate. The calcium phosphate/DNA precipitate was prepared by combining 8 μg of γGT-P_{III}-CAT DNA, 2 μg of CMV-β-galactosidase DNA, and 7 μg of pCAT basic vector DNA and 16 mM $CaCl_2$ per plate in an equal volume of 2× Hepes-buffered saline and allowing the precipitate to form for 10 min. Cells were exposed to this precipitate overnight followed by glycerol shock for 1 min. Then cells were washed extensively and cultured with fresh medium in 3 or 40% oxygen for 48 h. In each case the cells were harvested, and cell lysates were prepared with Promega Corp. lysis buffer diluted in TEN buffer (40 mM Tris, pH 7.4, 1 mM EDTA, 150 mM NaCl). Equal aliquots of this extract were assayed for β-galactosidase activity using *o*-nitrophenyl-β-D-galactopyranoside as substrate and CAT activity using the method of diffusion of reaction products into liquid scintillation fluid, along with standard amounts of enzyme, exactly as described in Sambrook et al. (17). Each transfection experiment was performed in duplicate and was repeated three times (n = 6). CAT activity was normalized by β-galactosidase to control for transfection efficiency. Results for the SV40T-T2 cell line are expressed as a percentage of the positive control with the mean and the SEM. For HepG2 cells, the results were normalized to the lowest value of the given construct in 3% oxygen and presented as the mean and the SEM (n = 3).

Results

γGT mRNA expression in lung development. No PCR product was detected from any γGT mRNA transcript in lung RNA obtained at 18 d gestation, while products from γGT mRNA I, II, and III were present in lung RNA at 21 d of gestation (Fig. 1 *top*). This fetal pattern was also present in RNA samples obtained from the perinatal lung at 2 and 12 h after birth. However, by 24 h after birth, the PCR product from γGT mRNA II was absent while that for γGT mRNA III and I persisted (Fig. 1 *bottom*). The lung RNA samples from 10 d neonatal rat, like that from the adult, contained only the mRNAIII transcript (Fig. 1 *top* and reference 5).

γGT mRNA expression in primary cultures of fetal lung epithelial cells. Epithelial cells isolated at 18 d of gestation (F18 cells) have previously been shown to express the alveolar type 2 cell marker MPA-gp200 protein, SP-C mRNA, and epithelial cytokeratins (9, 18, 19). These cells do not express γGT mRNA transcripts when first isolated, but express both mRNA I and mRNA III when cultured in 21% oxygen for 3–4 d. When cultured in 3% oxygen, only mRNA I is expressed, and in 40% oxygen only mRNA III is expressed (Fig. 2).

γGT mRNA expression in primary cultures of adult lung epithelial T2 cells. Alveolar type 2 cells (T2 cells) isolated from

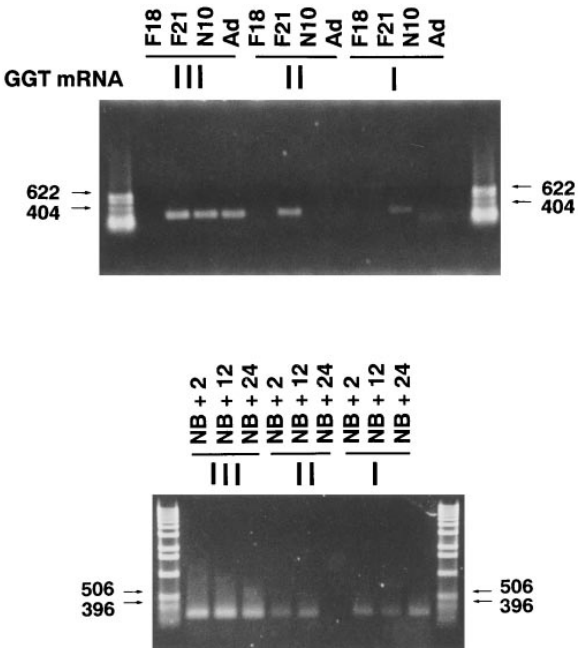

Figure 1. γGT mRNA ontogeny in lung. Total lung RNA was assessed for three γGT mRNA species using RT-PCR as described in Methods. Top panel shows PCR results of RNA obtained from gestational ages 18 (*F18*) and 21 (*F21*), neonatal age 10 days (*N10*), and adult (*Ad*) while bottom panel shows the same technique applied to RNA obtained from perinatal lung 2, 12, and 24 h after birth. DNA standards in top are pBR322 DNA-Msp I digest (404-bp fragment is identified) and, in bottom, 1-kb DNA ladder (396-bp and 512-bp fragments are identified).

the lung of the adult rat express only mRNA III when cultured for 48 h in 21% oxygen as do adult rat lungs. When cultured for 48 h in 40% oxygen, again only mRNA III is expressed (data not shown). However, when cultured in 3% oxygen, all

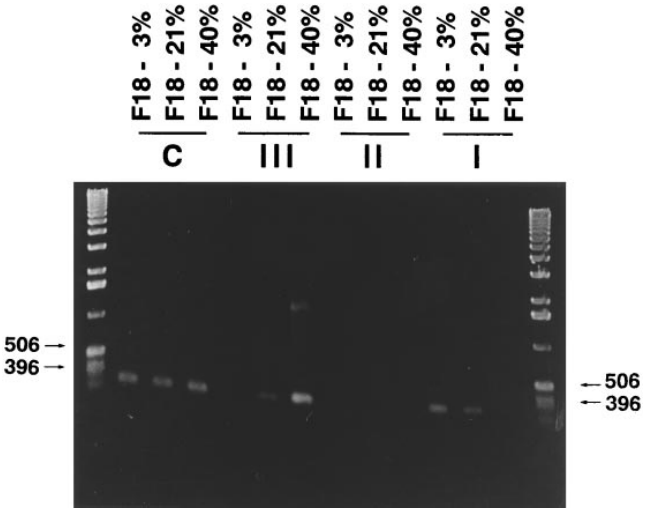

Figure 2. Oxygen switches γGT mRNA expression in F18 cells. Primary fetal lung epithelial cells were cultured in 3, 21, or 40% oxygen and RT-PCR analysis for γGT mRNA were as described in Methods. *C*, nonselective product using primers in coding domain. Standards are 1-kb DNA ladder (396- and 512-bp fragments are identified).

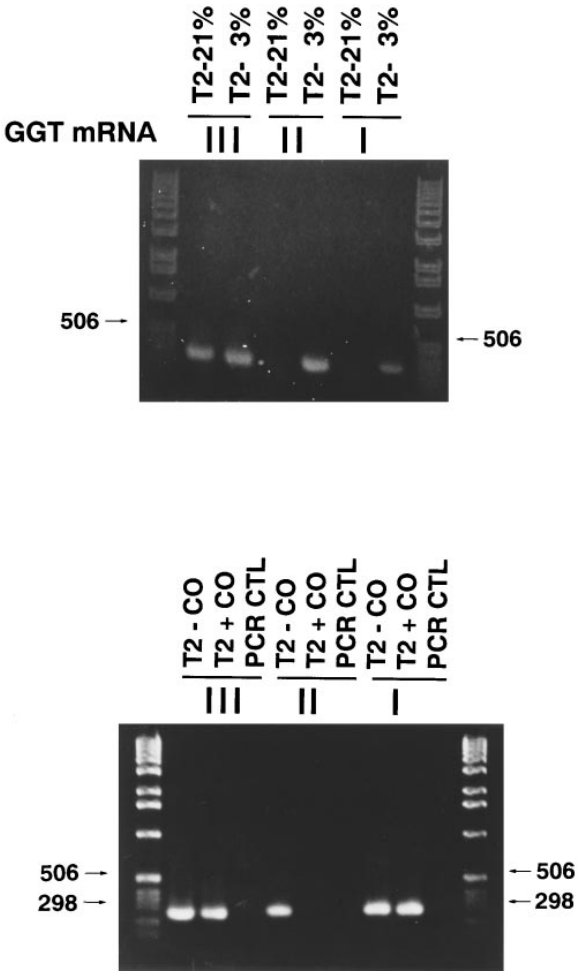

Figure 3. Hypoxia induces and carbon monoxide silences γGT mRNAII expression in T2 cells. Cell culture conditions and RT-PCR analysis of mRNA were as described in Methods. Top panel shows the pattern of γGT mRNA expression in T2 cells cultured in 21% versus 3% oxygen. Bottom panel shows the effect of carbon monoxide on the pattern of mRNA expression in hypoxia. DNA standards in top and bottom panels are 1-kb DNA ladder (298- and 512-bp fragments are identified).

three mRNA transcripts appeared within 48 h, recapitulating the setting in the in vivo fetal lung at 21 d, where mRNA I, II, and III are all expressed (Fig. 3 *top*). T2 cells were then cultured in 3% oxygen for 48 h (expressing mRNA I, II, and III), and switched into 0% O_2 in the presence or absence of 10% CO for 24–36 h. In the CO-exposed cells, mRNA II vanished, but mRNA I and III persisted, suggesting that CO, likely by binding a hemoprotein, suppressed expression of mRNA II (Fig. 3 *bottom*).

γGT-P_{III}-reporter gene expression in lung and liver cell lines. Since mRNA III was the only γGT transcript in the adult lung and in AT2 cells at > 21% oxygen, experiments were done to study the affect of hyperoxia on γGT mRNA III expression. Two cell lines were transiently transfected with a plasmid DNA that encoded a CAT reporter gene under the control of rat γGT-P_{III} sequence from −1578 to +55 (11). In the subclone of the SV40T-T2 cell line, CAT activity was significantly different between 3 and 21% oxygen ($P < 0.01$, Stu-

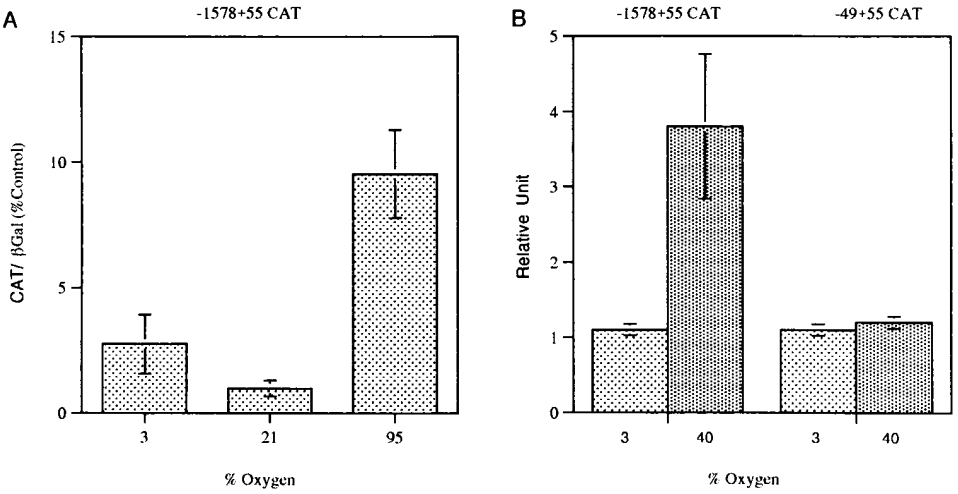

Figure 4. Hyperoxia activates γGT-P_{III}-CAT in SV40T-T2 and HepG2 cells. Cells were transfected with DNA, exposed to various oxygen concentrations, then extracted as described in Methods. CAT activity was normalized by β-galactosidase activity to correct for transfection efficiency. *A* shows γGT (−1578+55)-CAT in SV40T-T2 cells in 3, 21, or 95% oxygen. *B* shows the same construct plus γGT(−49+55)-CAT in HepG2 cells in 40% oxygen relative to that in 3% oxygen.

dent's *t* test) and was activated ninefold in the presence of 95% oxygen versus 21% oxygen ($P < 0.001$, Fig. 4 *A*). In HepG2 cells, CAT activity from this construct was not different between 3 and 21% oxygen (data not shown) but was activated 3.8-fold when the cells were cultured in 40% oxygen versus 3% oxygen ($P < 0.05$, Fig. 4 *B*). To define a potential role for the antioxidant responsive element in this hyperoxic induction, a deletion construct which retained this element (−49 to +55) was transfected into HepG2 cells under the same conditions. However, activation at 40% oxygen did not occur (Fig. 4 *B*).

Discussion

The γGT gene is developmentally regulated in the lung; γGT mRNA, as well as enzyme activity, are expressed late in gestation. Enzyme activity in the fetus peaks at the time of birth and is only 15% of the adult lung level (12). This pattern of ontogeny parallels that of several other antioxidant enzymes. These activities function to counter oxidative stress during the transition from liquid to air breathing at birth (20). In addition, the type of γGT mRNA expressed differs between the fetal and the adult lung. γGT is a single copy gene in the rat haploid genome that is regulated by multiple alternative promoters (1). In the adult, the various γGT mRNA are expressed in a tissue-specific fashion, and only γGT mRNA III is expressed in the adult lung (5). The data presented here, however, show that the fetal lung expresses at least two other γGT transcripts, mRNA I and II, in addition to mRNA III. The switch from this fetal pattern to the adult pattern occurs after birth and appears to be regulated by the change in ambient oxygen. The fetal pattern of expression can be recapitulated when T2 cells from the adult lung are isolated and cultured in an environment of 3% oxygen. γGT expression in these cells suggests that each γGT mRNA is regulated by a different oxygen-sensitive mechanism (summarized in Table I).

Even though oxygen affects γGT gene expression, it is not likely to be the initial inducer of this gene in the fetal lung for at least two reasons. First, γGT induction occurs at a time in gestation when oxygen content is low and unlikely to have changed. Second, when poorly differentiated fetal lung epithelial cells, F18 cells, are isolated and placed in culture, altered environmental oxygen alone fails to induce all three γGT mRNAs. Some factor other than oxygen must be the inducer of at least γGT mRNA II in the fetal lung. Since γGT is a TATA-less gene, initiators may be needed to recognize the alternative γGT promoters (11), and these factors can be developmentally regulated (21, 22). In fact, the inability of the F18 cell system to express all three γGT mRNA in vitro may reflect their incomplete state of differentiation.

Oxygen can regulate gene expression at multiple levels, including gene transcription where at least two general mechanisms have been defined (23). Reactive oxygen intermediates can produce oxidative stress that is sensed via altered cellular redox state. Redox-regulated gene expression has been well characterized in the eukaryotic NF-κB model (24), and additional redox-sensitive DNA-binding elements such as the antioxidant responsive element (25) have recently been described. In addition to reactive oxygen intermediates, oxygen tension can also affect gene transcription through its ability to alter the conformation of heme-binding proteins. Heme-dependent regulation of gene expression has been well described in the mammalian erythropoietin gene which is activated by hypoxia (26). The ability of carbon monoxide to block this induction has led to the hypothesis that a hemoprotein (not yet identified), acting as an oxygen sensor, functions in the signal transduction pathway leading to enhanced erythropoietin gene transcription (26). At least one hypoxia-inducible transcription factor (HIF-1) and its cognate DNA binding motif have been characterized in this regulatory pathway (27). Two additional DNA elements that respond to changes in oxygen tension have been identified in the human glutathione peroxidase gene (28). Taken together, these observations suggest that

Table I. Summary of γGT mRNA Expression

RNA	Lung					F18 Cells			T2 Cells				Mech
$\%O_2$	F18	F21	Birth	1 d	10 d	3%	21%	40%	0–3%	0%	21%	40%	
%CO										10%			
I	−	+	+	+	−	+	+	−	+	+	−	−	−heme
II	−	+	+	−	−	−	−	−	+	−	−	−	+heme
III	−	+	+	+	+	−	+	+	+	+	+	+	?ORE

ORE, oxygen responsive element.

gene expression can be regulated over a wide spectrum of oxygen concentrations (23). Some of these defined elements or perhaps novel elements may be involved in the regulation of the alternative γGT promoters by oxygen in the lung (Table I).

γGT mRNA III is expressed in the fetal lung, and it is the only transcript that is expressed in the lung after postnatal day 10. γGT mRNA III was off in F18 cells in 3% oxygen and was induced in these cells by 21 and 40% oxygen. In T2 cells, mRNA III was not suppressed in 3% oxygen and was expressed in both 21 and 40% oxygen. The activity of the reporter gene under the control of the P_{III} construct (−1578 to +55-CAT) increased in high oxygen concentrations. Although the exact mechanism of oxygen activation is unclear, the deletion experiment presented suggests that any putative oxygen response element is not located in the first 49 bp of the 5′-flanking region, which contains a single copy of the antioxidant responsive element. There is no HIF-1 or oxygen responsive element in the P_{III} sequence described, but further analysis of constructs containing additional upstream DNA sequence are in progress.

γGT mRNA II is expressed in fetal lung which exists at the equivalent of 3% oxygen, but not adult lung which is at 21% oxygen. This mRNA transcript decreases rapidly after birth, and it is undetectable within 24 h. Expression can be induced in T2 cells by culture in 3% oxygen, and this hypoxic induction is reversed by exposure to carbon monoxide. This suggests that a heme-binding protein is involved in the suppression of mRNA II expression by oxygen. As noted above, a similar paradigm has been implicated for heme-binding protein induction of the erythropoietin gene. Only a fraction of the γGT sequence in the P_{II} promoter region has been published, and there is no HIF-1 site present in that portion of the P_{II} promoter. Further work is in progress to determine the remaining sequence and to characterize the location of a possible heme-binding oxygen-sensor which functions to repress this promoter in the lung.

γGT mRNA I is expressed in the fetal but not the adult lung. Its expression is reduced between 1 and 10 d after birth, more gradually than the rapid postnatal decrease in mRNA II. γGT mRNA I was the only transcript induced by hypoxia in the F18 cell system, and it was also induced in T2 cells exposed to hypoxia. The mechanism of oxygen repression of mRNA I is not certain but it differs from that of mRNA II in that it was not affected by carbon monoxide. Hence it is likely that a heme-binding protein is not involved. In addition, there is no HIF-1 binding site within the P_I promoter to account for its hypoxic induction. The regulatory region of this promoter also contains a single copy of the antioxidant responsive element, and this element may play a role in the more gradual repression of mRNA I versus mRNA II after birth.

It has been proposed that genes with multiple alternative promoters provide an organism with greater flexibility in the control of gene expression (29). However, the molecular role for the expression of γGT mRNA III as opposed to mRNA I or II is not yet clear given that the protein product is identical. The mRNA transcripts generated from alternative promoters can have different translational efficiencies and stabilities (29). Further study will be required to establish any such differences for the various γGT mRNAs in lung epithelium. However, the 5′-untranslated region of γGT mRNA III is unique in that it can form a potential stem loop structure. This could provide a level of translational regulation for mRNA III as opposed to mRNA I or II (11). A similar structure has been described in the 5′ untranslated region of a human γGT mRNA species that is expressed in the hepatoma cell line HepG2, and it functions as a tissue-specific active translational enhancer (30).

In summary, our data suggest that oxygen may be regulating the expression of the γGT gene by at least three mechanisms. Whether similar mechanisms are involved in γGT gene regulation in other tissues remains to be determined. Our data concerning the ability of oxygen to modulate γGT mRNA I, II, and III expression also provides a new insight into lung development. If gene induction in the fetal lung is largely determined by an endogenous developmental program, then oxygen, whose concentration suddenly increases at birth, may serve to select for those genes that are essential for survival in the postnatal environment. Nonessential genes will be silenced; essential genes will be maintained or upregulated. A delicate balance likely exists between such a regulatory role and the well described role of oxygen as a redox toxin (31).

Acknowledgments

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