

# Modulation of glutathione peroxidase expression by selenium: effect on Human MCF-7 breast cancer cell transfectants expressing a cellular glutathione peroxidase cDNA and doxorubicin-resistant MCF-7 cells

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## ABSTRACT

We have studied the effect of selenium on the expression of a cellular glutathione peroxidase, GSHPx-1, in transfected MCF-7 cells and in doxorubicin-resistant (Adr<sup>r</sup>) MCF-7 cells. A GSHPx-1 cDNA with a Rous Sarcoma virus promoter was transfected into a human mammary carcinoma cell line, MCF-7, which has very low endogenous cytosolic glutathione (GSH) peroxidase activity and no detectable message. The transfectant with the highest GSH peroxidase activity among the isolates, MCF-7H6, was characterized. Adr<sup>r</sup> MCF-7 cells, a subline of MCF-7 cells, also has elevated GSH peroxidase activity. GSH peroxidase expressed by MCF-7H6 and Adr<sup>r</sup> MCF-7 cells is similar to the endogenous GSHPx-1 based on molecular weight, immunoreactivity, and metabolic labeling with <sup>75</sup>Se. MCF-7H6 and Adr<sup>r</sup> MCF-7 cells grown in Se-deficient media had  $2.6 \pm 2.4$  (mean  $\pm$  S.D.) and  $4.2 \pm 3.6$  units/mg protein of GSH peroxidase specific activity, respectively. Se supplementation increased GSH peroxidase activity in a concentration- and time-dependent fashion. Enzymatic activity reached a level of  $164 \pm 62$  in MCF-7H6 cells and  $114 \pm 27$  in Adr<sup>r</sup> MCF-7 cells within 5 days of growth in media supplemented with 30 nM Se. Northern analysis revealed that Se-deficient MCF-7H6 cells expressed  $2.1 \pm 0.4$ -fold less GSHPx-1 mRNA than their Se-sufficient counterparts. Similarly, Se-deficient Adr<sup>r</sup> MCF-7 cells expressed  $3.3 \pm 1.8$ -fold less GSHPx-1 mRNA than their Se-supplemented counterparts after the quantity of mRNA was normalized with  $\beta$ -actin. These studies suggest that modulation of GSH peroxidase activity by Se in both MCF-7H6 transfectants expressing pRSV-GSHPx-1 and Adr<sup>r</sup> MCF-7 cells expressing endogenous GSHPx-1 occurs largely at the translational level, and to a lesser degree at the level of mRNA, possibly by stabilizing GSHPx-1 mRNA since the transfected cDNA in MCF-7H6 cells has only 5 nucleotides 5' to the AUG initiation codon.

## INTRODUCTION

Glutathione peroxidases are selenoproteins that catalyze the reduction of various organic hydroperoxides and hydrogen peroxide to lipid alcohols or water, respectively, with glutathione (GSH) as the hydrogen donor. The best characterized GSH peroxidase is a ubiquitous cellular enzyme, GSHPx-1. It contains a selenocysteine at its active site that is encoded by a TGA opal codon (1,2). Recent evidence supports the hypothesis that the selenocysteine moiety is incorporated translationally, and that the expression of GSHPx-1 depends on the availability of selenium (Se) (3–5). Selenium deficiency results in a decrease in GSHPx-1 protein that is detectable by immunoprecipitation (6,7), and by enzyme activity. GSH peroxidase activity in tissues can be increased in individuals with low selenium intake by dietary supplementation with certain Se-containing compounds (8). After the Se level in whole blood reaches 100 ng/ml, GSH peroxidase activity remains constant despite additional Se supplementation (9,10).

It is not clear, however, whether the supply of Se can also affect GSHPx-1 mRNA levels. Conflicting results have been published regarding the effect of Se on GSHPx-1 mRNA levels in rat liver. In these studies, the amount of GSHPx-1 mRNA has been reported as unchanged (11,12), reduced to 7–17% of control level (13), or totally undetectable (14) after 9–19 weeks of selenium starvation. The GSHPx-1 mRNA level in human promyelocytic leukemia HL60 cells was not changed after 7 days of selenium-deprivation (15).

Interpretation of these studies may be obscured by the possibility that multiple alterations may occur in tissues responding to long-term selenium deprivation. In addition to the loss of liver cytoplasmic and plasma glutathione peroxidase activities, other antioxidant molecules and enzymes such as glutathione, glutathione-S-transferase, and catalase may increase in selenium-deficient animals (16–18). Additionally, there are multiple forms of glutathione peroxidase mRNA in human liver cells: the classic cellular GSHPx-1; plasma glutathione peroxidase (manuscript in preparation and 19); and two additional glutathione peroxidase-like mRNAs whose cDNAs have been isolated (20,21). It is possible that when a full-length GSHPx-1 cDNA

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is used as a probe, other related mRNAs from the gene family are also detected, which may obscure the fact that individual family members may be regulated differently by the level of available selenium.

To clarify whether selenium has a direct effect on the level of GSHPx-1 mRNA, we have examined the effect of selenium on MCF-7 human breast carcinoma cells transfected with GSHPx-1 cDNA in an expression construct. The parental MCF-7 cell line expresses very low, if any, endogenous GSH peroxidase activity. Our studies in a transfected clone which expresses high levels of GSHPx-1 activity demonstrate a major effect of selenium on the modulation of GSHPx-1 activity and a minor effect on the level of GSHPx-1 mRNA.

Doxorubicin-resistant (Adr<sup>r</sup>) MCF-7 cells have been shown to express high levels of GSH peroxidase activity (22). It is of interest to determine whether selenium affected the transcription of the endogenous GSHPx-1 gene in a fashion analogous to the regulatory effect of heavy metals on the transcription of the metallothionein gene (23). If this were the case, more dramatic selenium-mediated shifts in Adr<sup>r</sup> MCF-7 mRNA levels and accompanying changes in enzymatic activity might have been expected than those demonstrated in MCF-7H6 cells. Therefore, in parallel experiments, we studied the regulation of GSHPx-1 expression by selenium in Adr<sup>r</sup> MCF-7 and MCF-7H6 cells.

## MATERIALS AND METHODS

### Library Screening, Sequencing, and Transfection

A 21mer of CCGAGAAGGCATACACCGACT which is complementary to nucleotide positions 40–60 of the published human GSHPx-1 cDNA sequence (24) was used as the probe to screen a human liver carcinoma HepG2 cDNA library (generously provided by Dr. Aldon J. Lusis, Univ. of Calif. at Los Angeles) in the lambda gt11 vector. The positive clone was subcloned into the Bluescript<sup>®</sup> plasmid (Stratagene, San Diego, CA) at an *EcoRI* site. DNA sequencing was performed with the Sanger dideoxy sequencing method (25) using a Sequenase kit (United States Biochemical Co., Cleveland, OH). After determining the orientation of the GSHPx-1 gene in the Bluescript plasmid, the insert was cut out with *HindIII* and *BamHI*. To construct pRSV-GSHPx-1, pRSV- $\beta$ -globin (American Type Culture Collection, Rockville, MD) was cut with *HindIII* and *BglIII* to excise the  $\beta$ -globin insert; it was then ligated with the GSHPx-1 fragment.

MCF-7 cells were cotransfected with calcium phosphate precipitated pRSV-GSHPx-1 and pSV2-*neo* DNA (26). MCF-7 genomic DNA was also added as the carrier DNA. Ten micrograms of each were added to  $2 \times 10^6$  cells plated in 100 mm tissue culture dishes. Stable transfectants were selected by resistance to 400  $\mu$ g/ml G418 (Sigma Chemical Co., St. Louis, MO). To determine the spontaneous rate of mutation of MCF-7 cells to G418-resistance, we also transfected MCF-7 cells with carrier DNA only.

### Cell Culture and Metabolic Labeling with <sup>75</sup>Se

MCF-7 cells and a doxorubicin-resistant (Adr<sup>r</sup>) subline of MCF-7 cells (kindly provided by Dr. Kenneth Cowan, Medicine Branch, NCI, NIH, Bethesda, MD) were maintained in minimal essential medium (MEM) with Earle's salts, L-glutamine, sodium pyruvate and 5% heat-inactivated fetal bovine serum (HI-FBS). HepG2 cells (a gift from Dr. Barbara Knowles, Wistar Institute of Anatomy and Biology, Philadelphia, PA) and A498 cells

(American Type Culture Collection, Rockville, MD) were maintained in the same media supplemented with 10% HI-FBS. To study the Se-deficient state, Adr<sup>r</sup> MCF-7 cells were grown in DMEM/F12 medium containing 0.5% HI-FBS; MCF-7H6 cells were grown in the same medium supplemented with 5  $\mu$ g/ml each of insulin and transferrin, and 200  $\mu$ g/ml of G418. Se content in FBS (GIBCO) assayed in 120 lots averaged 26 ng/ml. Selenium supplementation was performed by growing cells in the medium containing sodium selenite at concentrations from 1 to 300 nM. To label cells with <sup>75</sup>Se, 3  $\mu$ Ci of <sup>75</sup>Se-selenious acid with specific activity of 31 mCi/mg (New England Nuclear, Boston, MA) was added to 10 ml of medium containing 5 or 10% FBS. Cells were plated at a density of  $2 \times 10^5$  cells per 100 mm tissue culture dish to allow 4–6 days for metabolic incorporation of <sup>75</sup>Se. Cells were harvested by either trypsinization or mechanical scraping.

### Enzyme and Protein Assays

Harvested cells were washed once in phosphate buffered saline (PBS); after centrifugation, the volume of the cell pellet was adjusted with a small aliquot of PBS containing a mixture of protease inhibitors described in the immunoprecipitation section below. A protein concentration of 1–5 mg/ml was obtained. Cells were disrupted on ice by sonication with 5–7 one second bursts of a Branson Sonifier Cell Disrupter 250 (Branson Sonic Power Co., Danbury, CT). Supernatants resulting from a 20 min centrifugation of the cell homogenate at 4°C and 16,000  $\times g$  were used for enzyme assays. GSH peroxidase activity was determined by following the oxidation of NADPH at 340 nm as previously described (27). The reaction mixture contained 0.1 M potassium phosphate, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 2 mM reduced glutathione (GSH), 1 unit of glutathione reductase, and 0.2 mM NADPH. When 1.2 mM *t*-butylhydroperoxide was used as the substrate, assays were performed at pH 8.0; and when 0.15 mM H<sub>2</sub>O<sub>2</sub> was used, 1 mM sodium azide was added to the reaction mixture to inhibit endogenous catalase activity, and the assays were completed at pH 7.0 (28). Glutathione-S-transferase (GST) activity was assayed using chlorodinitrobenzene as the substrate according to a published method (29). Protein concentration was determined with either Lowry's method (30) or BCA assay (Pierce, Rockford, IL). Bovine albumin was used as the standard. One unit of enzymatic activity was defined as one nmole NADPH consumed/min. The specific enzymatic activities are presented as the mean  $\pm$  standard deviation units/mg protein.

### Raising Rabbit Anti-RBC-GSHPx Antisera and Immunoprecipitation

Human erythrocyte GSHPx-1 (RBC-GSHPx-1) was purchased from Sigma. It was further purified by fractionation through a  $2 \times 36$  cm G-200 Sephadex column. Fractions collected from the G-200 column were assayed for GSH peroxidase activity; the most active fractions were pooled. This step removed several high molecular weight impurities when analyzed with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). This partially purified GSHPx-1 was used for immunization of New Zealand white rabbits (Hazelton Research Products, Inc., Denver, PA) at a dose of 100  $\mu$ g protein per animal after reconstitution with Ribi adjuvant (RIBI Immunochem. Research, Inc., Hamilton, MT). Rabbits were injected once every two weeks for a total of six weeks (4 injections) to generate a rabbit anti-RBC-GSHPx-1 antiserum.

<sup>75</sup>Se-labeled cells were washed 3× with PBS and harvested with trypsin or by scraping. The cells were resuspended in buffer A containing PBS, 0.5% Triton X-100, 5 mM EDTA, 5 mM EGTA, 5 μM 1,10-phenanthroline. A 1000-fold concentrated protease inhibitor stock consisting of 17 mg/ml phenylmethylsulfonyl fluoride (PMSF), 5 mg/ml leupeptin, 5 mg/ml pepstatin A, and 17 mg/ml benzamide was added to the cells before sonication. The cytosolic fractions were obtained after centrifuging the sonicates at 16,000 ×g for 20 min. Aliquots of <sup>75</sup>Se-labeled cytosols with 1,000–5,000 cpm were added to equal volumes of diluted rabbit antisera, and incubated at 4°C with gentle agitation overnight. A 25–50 μl aliquot of washed protein A-bacterial adsorbent (*Staph. A.*, ICN Immunobiologicals, Lisle, IL) was added to the mixture, and the suspension was agitated for 1 hour at 4°C. The precipitates were washed 6 times with buffer A and 0.5% SDS. The *Staph. A.* step which appeared to be the limiting component was repeated once to ensure quantitative precipitation. <sup>75</sup>Se was counted with the <sup>125</sup>I window of a Gamma counter.

### Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sample preparation and SDS-PAGE were performed as described (31). The gels were composed of 12% acrylamide and 0.32% bis-acrylamide and electrophoresis was performed at a constant current of 5–7 mA per gel overnight. The gels were fixed with a mixture of 10% glacial acetic acid, 40% methanol and 50% water for 30 min and dried for exposure to X-ray film. Prestained molecular weight markers were purchased from Sigma.

### Southern and Northern Analysis

High molecular weight DNAs were isolated from the nuclear pellets of MCF-7 cells and its transfectants by RNAase and proteinase K digestion (32). Total RNAs were isolated from postnuclear supernatants as previously described (33). Messenger RNAs were isolated by passing total RNAs through an oligo(dT)-cellulose column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) once. Routinely, restricted DNAs were resolved by electrophoresis in 0.7% agarose gels. RNAs were resolved by electrophoresis in 1.1% agarose gels containing formaldehyde. For Northern analysis, RNAs were transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) or Zeta-Probe (Bio-Rad Lab., Richmond, CA) membranes for hybridization. For DNA analysis, in-gel hybridization was performed (34).

The cDNA clones of GSHPx-1 and chicken β-actin (35) were labeled with <sup>32</sup>P-dCTP using Multiprime DNA Labelling Systems (Amersham, UK). GST-π mRNA was probed with an oligonucleotide of 22 bases: GAAGGAGATCTGGTCTCC-CACA, which is complementary to nucleotides 432–453 of the human GST-π cDNA (36). The oligonucleotide was end-labeled with γ-<sup>32</sup>P-ATP by T4 polynucleotide kinase (United States Biotechnology Co., Cleveland, OH) (37). Unincorporated radioactivity was removed by passage through a Nick® column (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). To probe with cDNA, Northern filters were prehybridized in a mixture of 5× Denhardt's solution, 5× standard saline citrate (SSC), 0.05 M NaPO<sub>4</sub>, 50% formamide, 0.1% SDS and 0.1 mg/ml denatured Herring sperm DNA. The hybridization mixture had the same content except Herring sperm DNA was replaced by the denatured labeled nucleotides. With the oligonucleotide probe, the prehybridization mixture contained 6× Denhardt's solution,

6× SSC, 10 mM EDTA, 0.5% SDS and 0.1 mg/ml denatured Herring sperm DNA. The prehybridization was done at 42°C for 2–4 hours, and the hybridization was done at 52°C overnight. The washing condition for cDNA probes was with 0.1× SSC and 0.1% SDS at room temperature for 15 min and then at 52°C for 20 min twice. The washing condition for the oligonucleotide probe was with 6× SSC and 0.1% SDS for a similar time and temperature (34). When high background was found in gels or blots, further washing in the same wash buffer at 37°C for several hours was performed to eliminate the background. *Hind*III digested lambda DNA and the BRL RNA ladder (Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, MD) were used as molecular weight markers for DNA and RNA, respectively.

To quantitate the amount of specific message on the filters, a GS300 Transmittance/Reflectance Scanning Densitometer with the GS360 Data System for an IBM PC (Hoefer Scientific Instruments, San Francisco, CA) was used to scan the X-ray films. Multiple exposures of filters were made to ensure that the X-ray films to be scanned were not saturated.

## RESULTS

### Cloning of GSHPx-1

An oligonucleotide of 21 bases complementary to nucleotides 40–60 of the human kidney GSHPx-1 cDNA sequence (24) was used to screen a HepG2 library in lambda gt11. This 21mer was complementary to a region 5' to the TGA codon that encodes the selenocysteine. One clone was isolated and sequenced after subcloning into Bluescript® vector. The partial sequence that we have obtained from this clone was in complete agreement to that of the human kidney GSHPx-1 cDNA. It extends 5 nucleotides beyond the ATG initiation codon, has an internal *Eco*RI site, and a polyadenylate tail. However, it is missing at least 300 bp of 5' untranslated region found in a larger cDNA clone isolated from a human liver library (38).

### Transfection of GSHPx-1

To express the GSHPx-1 clone, we subcloned it into a mammalian expression vector, pRSV, which contains a long terminal repeat and promoter regions of the Rous Sarcoma virus as well as the RNA processing signals of SV40 (39–41). The MCF-7 human breast carcinoma cell line was chosen as the recipient of transfection because of its low endogenous level of GSHPx-1 peroxidase activity and its undetectable GSHPx-1 mRNA.

Cells were cotransfected with pRSV-GSHPx-1 and pSV2-*neo* DNA along with MCF-7 DNA as the carrier. The control cells were transfected with the carrier DNA to determine the background level of G418 resistance. Three weeks after selection with geneticin, the number of surviving clones was scored. One clone was found in three control 100 mm dishes plated with 3.6×10<sup>5</sup> cells each, while an average of 15 clones was found in each pSV2-*neo* DNA transfected dish plated with 4.1×10<sup>5</sup> cells each. Thus, although the efficiency of transfection was rather low (3.6×10<sup>-5</sup>), transfectants of interest were readily found.

One hundred and four G418-resistant clones were isolated, mostly as individual clones. Of 32 isolates assayed for GSHPx-1 peroxidase activity, eleven expressed elevated levels of enzyme activity with *t*-butylhydroperoxide as the substrate. Six isolates of single clones were assayed multiple times with both *t*-butylhydroperoxide and hydrogen peroxide as substrates under standard culture conditions (Table 1). MCF-7D1 was resistant

**Table 1.** Glutathione peroxidase activity of MCF-7 transfectants

Cell line <sup>a</sup>	Substrate	
	<i>t</i> -butylhydroperoxide	H <sub>2</sub> O <sub>2</sub>
	units/mg protein	
MCF-7 Wild Type	0.2 ± 0.1 (9) <sup>b</sup>	0.4 ± 0.2 (4)
MCF-7D1	0.2 ± 0.1 (3)	0.8 ± 0.2 (2)
MCF-7G7	4.4 ± 2.0 (2)	2.3 ± 0.5 (2)
MCF-7G1	6.0 ± 2.3 (3)	2.6 ± 0.6 (2)
MCF-7G11	12.5 ± 4.6 (4)	7.5 ± 1.3 (3)
MCF-7E1	17.4 ± 5.8 (2)	4.3 ± 0.2 (2)
MCF-7H6	19.6 ± 4.9 (4)	15.7 ± 1.6 (8)
HepG2	7.4 ± 1.9 (3)	4.4 ± 0.9 (4)
A498 1	8.3 ± 6.8 (2)	10.8 ± 2.6 (4)
Adr <sup>r</sup> MCF-7	10.6 (1)	10.4 ± 4.0 (2)
Subclones of MCF-7 cells <sup>c</sup>		
c1		0.3 ± 0.2 (2)
c2		0.4 ± 0.4 (2)
c3		0.8 ± 0.2 (2)
c5		ND <sup>d</sup> (2)
c10		ND (2)

<sup>a</sup> Cells were grown in MEM media with 5% or 10% FBS.

<sup>b</sup> Enzyme activity is expressed as mean ± S.D. units/mg protein; numbers in parentheses are the number of assays performed.

<sup>c</sup> Clones of untransfected MCF-7 cells derived from single cells.

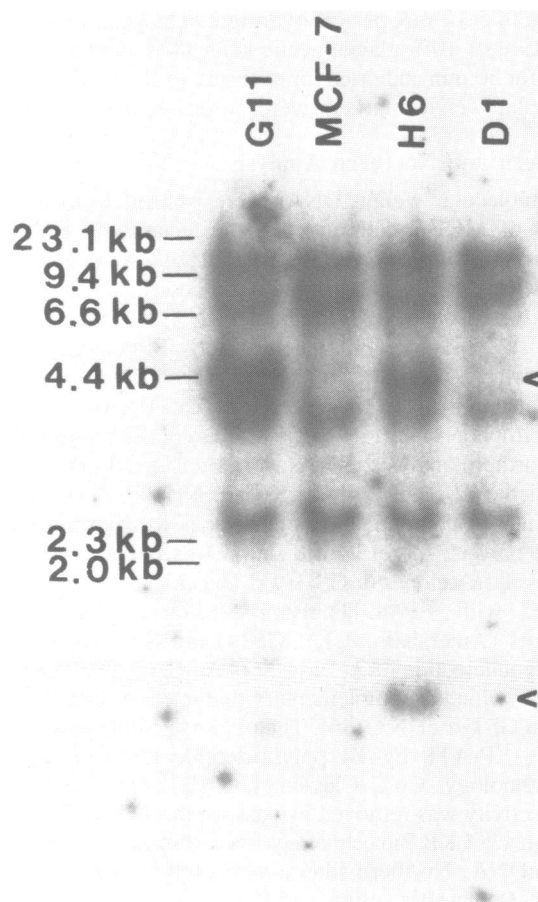
<sup>d</sup> ND: not detectable.

to G418 but expressed a basal level of GSH peroxidase activity. To ensure that the elevated enzymatic activity in the transfectants was due to transfection rather than clonal variation resulting from heterogeneity in the cell population, we isolated five clones of MCF-7 cells started from single cells and analyzed them for GSH peroxidase activity. Similar to the parental cells, they all had low enzymatic activity (Table 1). One of the transfectants, MCF-7H6, which had the highest GSH peroxidase activity, was analyzed further. Under standard tissue culture conditions, i.e. 5% FBS for MCF-7 cells and its derivatives or 10% FBS for HepG2 (liver cells) and A498 (kidney cells), MCF-7H6 cells expressed GSH peroxidase activity which was higher than HepG2 cells and doxorubicin-resistant MCF-7 cells (22), and equivalent to the A498 cell line.

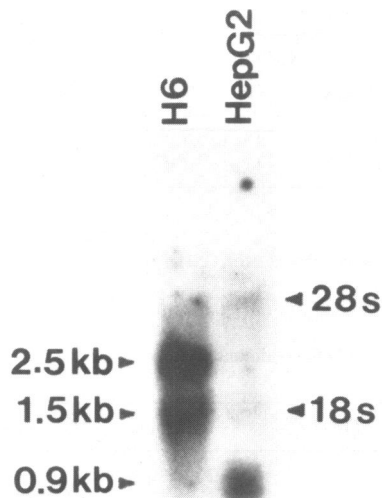
#### Characterization of GSH Peroxidase gene expressed in MCF-7H6 and Adr<sup>r</sup> MCF-7 Cells

To establish MCF-7H6 as a genuine transfectant, its DNA was isolated along with that of two other transfectants, MCF-7G11 and MCF-7D1, and the MCF-7 parental cell line. Southern analysis of these DNAs digested with *Hind*III is shown in Figure 1. Multiple bands were found in all of these cells when probed with the oligonucleotide specific for GSHPx-1, consistent with previous reports (11,42). As expected, MCF-7D1, with only a basal level of GSH peroxidase activity, did not have any additional bands which would represent pRSV-GSHPx-1 DNA. MCF-7H6 and MCF-7G11, which express elevated enzyme activities, both had the same additional DNA of 4.4 kb. The intensity of the 4.4 kb band in the MCF-7G11 DNA was stronger than that in the MCF-7H6 DNA, suggesting that MCF-7G11 had more copies of pRSV-GSHPx-1 integrated. In addition, the MCF-7H6 transfectant had an additional 1.05 kb band not present in MCF-7G11.

RNAs isolated from cells expressing a high level of endogenous GSH peroxidase activity (such as HepG2, A498, and Adr<sup>r</sup>



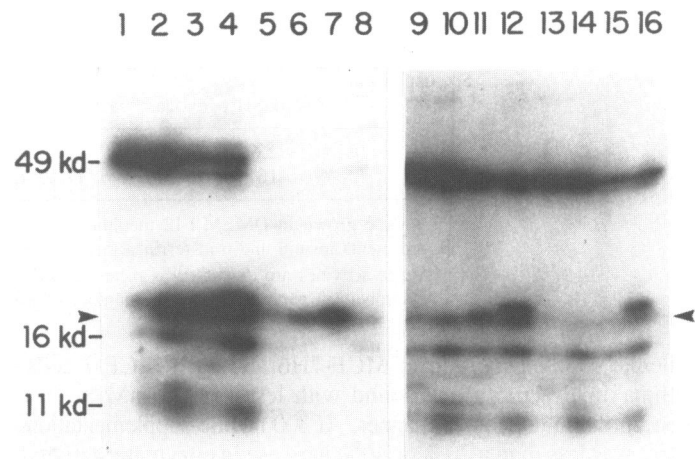
**Figure 1.** Southern analysis of DNA isolated from MCF-7G11, MCF-7, MCF-7H6 and MCF-7D1 cells. Ten  $\mu$ g of DNA were digested with *Hind*III and separated on a 0.7% agarose gel. The end-labeled oligonucleotide of GSHPx-1 was used to probe the DNA. The arrow indicates the presence of the transfected GSHPx-1 DNA.



**Figure 2.** Northern analysis of GSHPx-1 message. Total mRNAs isolated from the transfectant MCF-7H6 and HepG2 cells were separated on a 1.1% agarose gel and transferred to nitrocellulose. Messages of 2.5 kb and 1.5 kb were hybridized to the oligonucleotide probe in MCF-7H6 cells, while the endogenous message was shown to be 0.9 kb in HepG2 cells. The size of the messages was determined by BRL RNA ladder.

MCF-7) expressed an mRNA of 0.9 kb when probed either with GSHPx-1 cDNA or the oligonucleotide. No detectable GSHPx-1 mRNA was found in either total RNA or polyA-containing RNA isolated from MCF-7 cells (data not shown). In contrast to the 0.9 kb mRNA found with endogenous GSHPx-1 mRNA, MCF-7H6 expressed two larger GSHPx-1 mRNAs of 2.5 kb and 1.5 kb (Figure 2).

Since multiple forms of GSH peroxidases have been reported in liver and its derived cells (20,21,38), it is important to know if the GSH peroxidase expressed in both MCF-7H6 and Adr<sup>r</sup> MCF-7 cells was GSHPx-1. The two GSHPx-like mRNAs were not detectable in Adr<sup>r</sup> MCF-7 or MCF-7H6 cells when a cDNA and an oligonucleotide were used as probes (data not shown). Total cellular proteins were metabolically labeled with <sup>75</sup>Se-selenite in Adr<sup>r</sup> MCF-7, MCF-7H6, HepG2 and A498 cells. The cytosolic GSH peroxidase activity in these cells was chromatographed at  $\approx$ 88 kDa through a Sephadex G-200 column. The enzymatic activity could be removed from cytosol after immunoprecipitation with anti-RBC-GSHPx-1 antibodies but not with antibodies made against human plasma GSH peroxidase (data not shown). The <sup>75</sup>Se-labeled immunoprecipitates were analyzed by SDS-PAGE (Figure 3). The 22 kDa <sup>75</sup>Se-polypeptide from a Adr<sup>r</sup> MCF-7, MCF-7H6, and A498 cells but not HepG2 cells were completely removed from the supernatants after two rounds of *Staph. A.* adsorption, which appeared to be the limiting component.



**Figure 3.** Autoradiograph of a <sup>75</sup>Se-polypeptide immunoprecipitated with anti-RBC-GSHPx-1 antisera resolved by SDS-PAGE. Lanes 1–4 represent total <sup>75</sup>Se-proteins in A498, Adr<sup>r</sup> MCF-7, MCF-7H6 and HepG2 cells, respectively; lanes 5–8 represent immunoprecipitates from cells in the same order; lanes 9–12 represent aliquots of post-immunoprecipitated supernatants of cells in the same order; lanes 13–16 represent post-immunoprecipitated supernatants after incubation with additional *Staph. A.* adsorbent. Arrows indicate a 22 kDa <sup>75</sup>Se-polypeptide.

#### Effect of Selenium on Growth and GSH Peroxidase Activity in MCF-7H6 and Adr<sup>r</sup> MCF-7 Cells

Serum contains nondialyzable selenium which could be a potential source of selenium for cells grown in tissue culture. In order to achieve a Se-deficient state, MCF-7H6 cells were grown in medium containing 0.5% serum supplemented with insulin and transferrin (Table 2). Unlike MCF-7H6 cells, Adr<sup>r</sup> MCF-7 cells do not need additional supplementation with insulin and transferrin (Table 3). DMEM/F12 medium was used instead of MEM because of its enriched nutrients. In the absence of serum, both cell lines became moribund within two weeks. Supplementation of the medium with 10 nM 17 $\beta$ -estradiol in addition to insulin and transferrin was not sufficient to maintain cell viability in the absence of serum. Both cell lines had similar growth rates in the low-serum medium supplemented with 0–300 nM sodium selenite and in 5% serum; a doubling time of  $\approx$ 48 hours was determined for all growth conditions when assayed by protein concentration or cell count (data not shown). Sodium selenite at 1.2  $\mu$ M was cytotoxic to both cell lines, most cells became nonviable within 7 days after treatment. Similar GSH peroxidase activities were obtained in cells grown in dialyzed versus nondialyzed serum. In addition, exogenous estrogen did not have any effect on GSH peroxidase activity (data not shown).

GSH peroxidase activity increased rapidly in Se-deficient MCF-7H6 cells upon the addition of 30 nM Se. We detected 39 units/mg protein of activity 4 hours after the addition of Se compared to a baseline activity of  $2.6 \pm 2.4$  units/mg protein. MCF-7H6 cells reached near maximal levels of GSH peroxidase in 5 days with a plateau at 150–300 units/mg protein (Figure 4a). Adr<sup>r</sup> MCF-7 cells reached near maximal levels of  $\approx$ 100 units/mg protein 3 days after the addition of 30 nM selenium (Figure 4b). The activity of Se-independent glutathione-S-transferase was also assayed as a control; it was unchanged throughout the experiments.

The levels of GSH peroxidase activity varied with the amount of Se supplementation in both MCF-7H6 and Adr<sup>r</sup> MCF-7 cells (Figures 5a and 5b). Near maximal activities of  $164 \pm 62$  and  $114 \pm 27$  units/mg protein were obtained with 30 nM sodium

**Table 2.** Effect of serum and selenium on GSH peroxidase and GST activities in MCF-7H6 cells<sup>a</sup>

Se supplement	0.5% FBS + IT		5% FBS	
	GSH peroxidase <sup>c</sup>	GST <sup>c</sup>	GSH peroxidase	GST
–	2.6 ± 2.4 (6)	13 ± 8 (5)	18 ± 5 (5)	15 ± 3 (3)
30 nM	164 ± 62 (5)	17 ± 13 (4)	59 ± 26 (3)	11 ± 3 (3)

<sup>a</sup> Cells were grown in DMEM/F12 medium.

<sup>b</sup> IT represents insulin and transferrin at 5 µg/ml each.

<sup>c</sup>Enzymatic activities are expressed as mean ± S.D. units/mg protein; H<sub>2</sub>O<sub>2</sub> was substrate for GSH peroxidase assay; numbers in parentheses are the number of assays performed.

selenite supplementation in MCF-7H6 and Adr<sup>r</sup> MCF-7 cells; submaximal activity was found with less than 10 nM Se in a concentration-dependent manner. At 300 nM Se-supplementation, there was less than a 20% or 40% increase in enzymatic activity, respectively, with MCF-7H6 and Adr<sup>r</sup> MCF-7 cells compared to those cells grown in the presence of 30 nM Se (Figures 5a, 5b and unpublished observations).

The rate of GSHPx-1 degradation was also estimated in these cells based on the loss of enzymatic activity after removing Se from the tissue culture medium. Cells were grown in medium containing 0.5% serum supplemented with 30 nM sodium selenite for 7 days to reach maximal enzyme levels. At the beginning of the experiments, the cells were transferred to fresh medium without selenium. The half-lives of GSHPx-1 determined from two experiments were 2.7 days in MCF-7H6 cells and 2.5 days in Adr<sup>r</sup> MCF-7 cells (Figures 6a and 6b). In Adr<sup>r</sup> MCF-7 cells, GSH peroxidase activity decreased in an apparent biphasic fashion, the half-life of 2.5 days was determined from the slower phase. These half-lives were similar to that determined by Knight and Sunde as measured by the loss of GSH peroxidase activity in rat liver (43). However, when these investigators used antibodies to determine GSHPx-1 protein levels, a half-life of 5.2 days was found.

#### Effect of Selenium on GSHPx-1 mRNA Level in MCF-7H6 and Adr<sup>r</sup> MCF-7 Cells

Polyadenylated mRNAs were isolated from MCF-7H6 cells grown in Se-deficient or Se-supplemented media. Three sets of mRNAs were studied in parallel and analyzed for GSHPx-1 mRNA (Figure 7). Only the 2.5 kb band was quantified. The 1.5 kb band overlapped with 18S RNA, which hybridized nonspecifically with the probes in two Northern blots. We found 2.1 ± 0.4 fold less GSHPx-1 mRNA in Se-deficient compared to Se-supplemented cells after normalization with β-actin. The endogenous mRNAs of GSHPx-1 and GST-π were undetectable and could not be analyzed in MCF-7H6 cells.

Total RNA was isolated from Adr<sup>r</sup> MCF-7 cells grown in medium containing 0.5% FBS with or without 30 nM Se supplementation for 7 days, and analyzed for GSHPx-1 mRNA level by Northern blots. Total RNA, instead of polyadenylated mRNA, was analyzed since the endogenous GSHPx-1 mRNA can be easily separated from abundant 28S and 18S rRNAs by gel electrophoresis. Thus, when rRNAs hybridize probes nonspecifically, the specific probing of GSHPx-1 was not affected although it did affect the probing of β-actin whose molecular weight is similar to that of 18S rRNA. Three pairs of RNAs were isolated and quantitated. GST-π and β-actin (including 18S rRNA) RNAs were also analyzed as internal standards for RNA quantitation. One set of Northern blots is shown in Figure 8. When the quantity of GSHPx-1 mRNA was normalized with β-actin, we found 3.3 ± 1.8-fold more GSHPx-1 mRNA in cells

**Table 3.** Effect of culture conditions on GSH peroxidase activity in Adr<sup>r</sup> MCF-7 cells

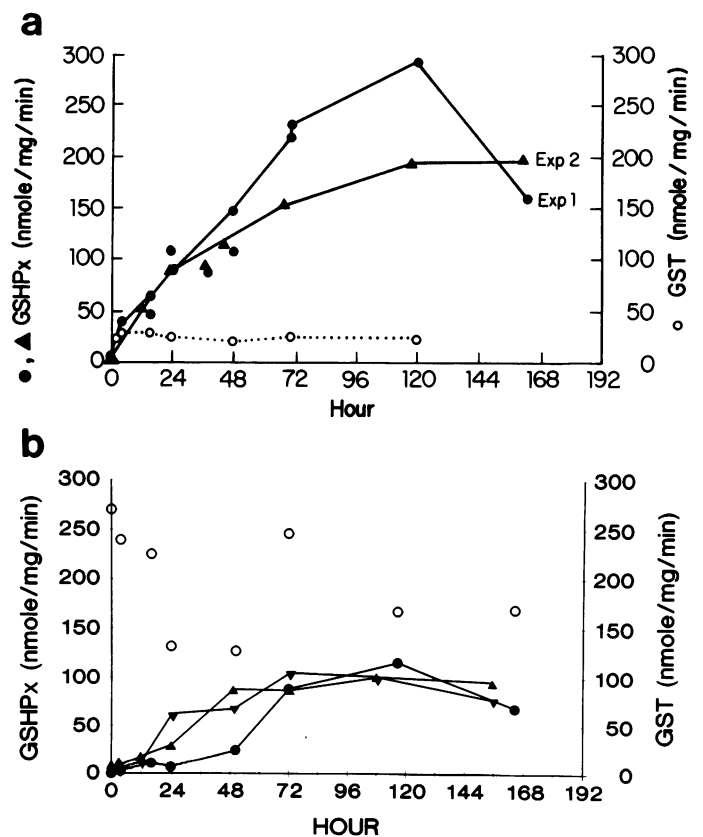
% Serum	IT <sup>a</sup>	– Selenium	+ Selenium <sup>b</sup>
0.5%	–	4.2 ± 3.6 (8) <sup>c</sup>	114 ± 27 (9)
0.5%	+	3.3 ± 3.1 (4)	102 ± 19 (8)
5.0%	–	26 ± 11 (4)	67 ± 17 (2)
10.0%	–	N.D. <sup>d</sup>	100 (1)

<sup>a</sup> IT = 5 µg/ml each of insulin and transferrin in the culture media.

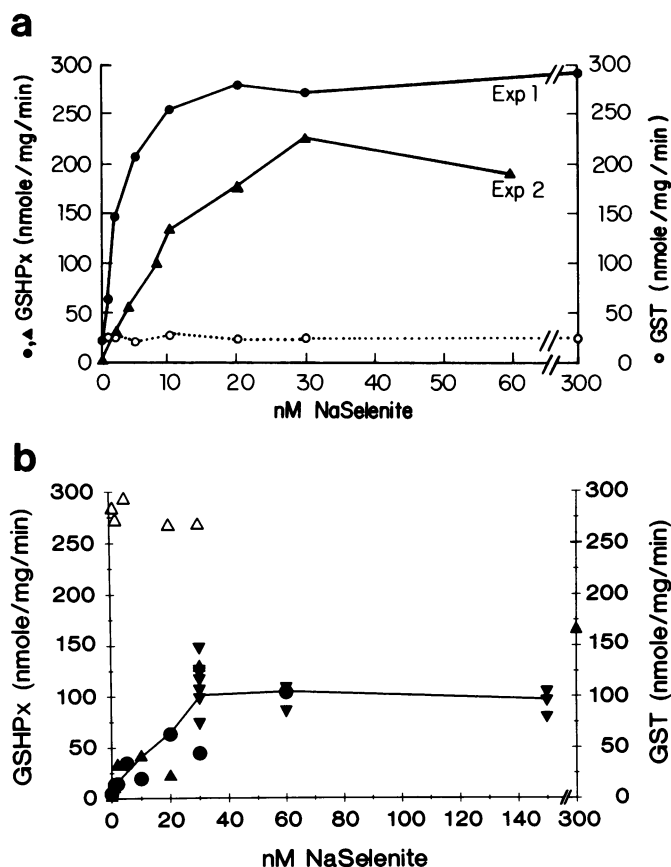
<sup>b</sup> Sodium selenite was used at a concentration of 30 nM.

<sup>c</sup>The activity is expressed as mean ± standard deviation units/mg protein. H<sub>2</sub>O<sub>2</sub> was the substrate. The number in parentheses indicates the number of assays.

<sup>d</sup> N.D.: not determined.



**Figure 4a.** Time course of GSH peroxidase activity changes in MCF-7H6 cells after the addition of 30 nM Se to Se-deficient medium. Two experiments were performed. Cells had been grown in Se-deficient media for at least 7 days before plating at time zero. In experiment 1, glutathione-S-transferase (GST) activity was also assayed as a control. 4b. The effect of Se supplementation over time on GSH peroxidase activity in Adr<sup>r</sup> MCF-7 cells. Sodium selenite at 30 nM was added to medium containing 0.5% fetal bovine serum at 0 time. Three experiments were performed; GSH peroxidase activity was measured over the subsequent 168 hours (filled symbols). GST activity was also measured (open circles).

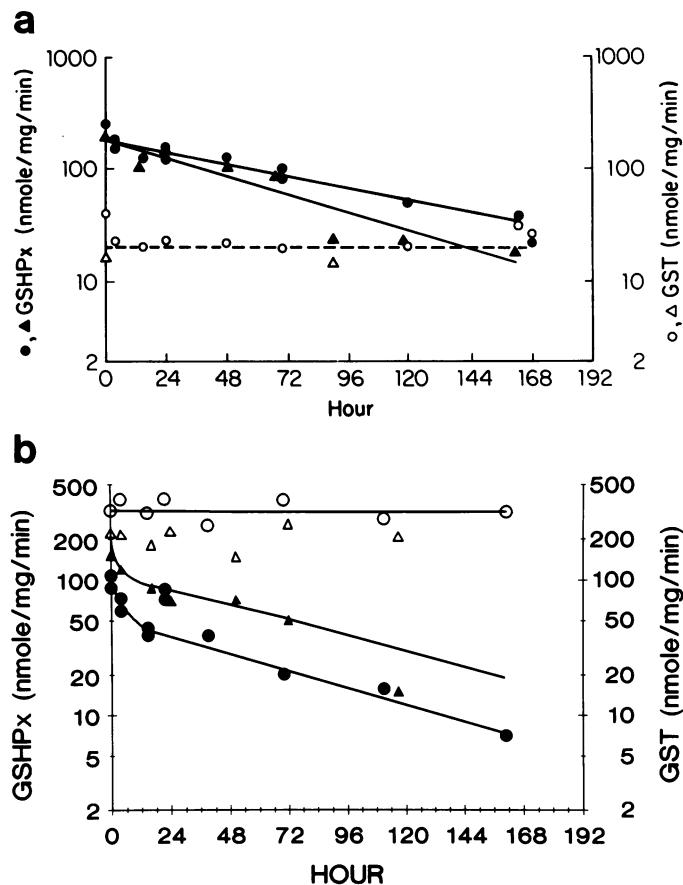


**Figure 5a.** Effect of Se concentration on GSH peroxidase activity in MCF-7H6 cells. Cells had been adapted to Se-deficient media for at least 7 days before being plated for these two experiments. After supplementation with various amounts of Se for 8 days, cells were harvested for enzyme assays. In experiment 1, GST was assayed as a control. 5b. The effect of Se concentration on GSH peroxidase activity in Adr<sup>r</sup> MCF-7 cells. Sodium selenite in the concentration range from 2 to 300 nM was added to tissue culture medium containing 0.5% fetal bovine serum. Three experiments were performed; additional measurements were done at some selenite concentrations. Filled symbols represent GSH peroxidase activity; open triangles represent GST activity.

grown in medium supplemented with 30 nM Se compared to those cells grown in Se-deficient medium. When GSHPx-1 mRNA was standardized with GST- $\pi$ , we found a  $3.1 \pm 1.7$ -fold difference. No difference was found in GSHPx-1 mRNA levels in cells grown in 30 versus 150 nM Se. This is consistent with the similar enzymatic activities found in cells cultured under these conditions.

## DISCUSSION

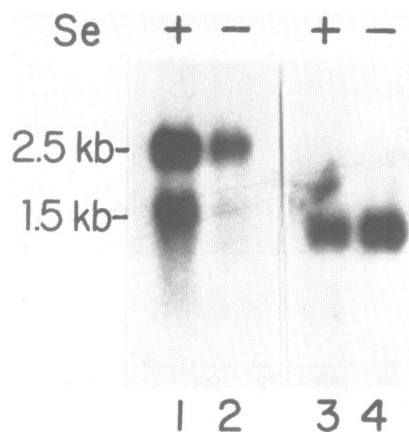
We have transfected MCF-7 cells which have little endogenous GSH peroxidase activity and have isolated a clone expressing high levels of enzymatic activity. After Se supplementation, the transfectant MCF-7H6, expressed  $164 \pm 62$  units/mg protein of enzymatic activity, i.e.  $\approx 50$ -fold increase over that found in Se-deficient cells. Adr<sup>r</sup> MCF-7, a drug-resistant variant of MCF-7 cells, also exhibits a marked degree of responsiveness to the level of Se in the local environment. Its activity was increased 27-fold at  $102 \pm 19$  units/mg protein in cells grown in 0.5% FBS-containing medium supplemented with 30 nM Se. Near maximal enzymatic activity was reached with 30 nM Se supplementation in MCF-7H6 and Adr<sup>r</sup> MCF-7 cells; we found less than a 20% or 40% further increase in enzymatic activity



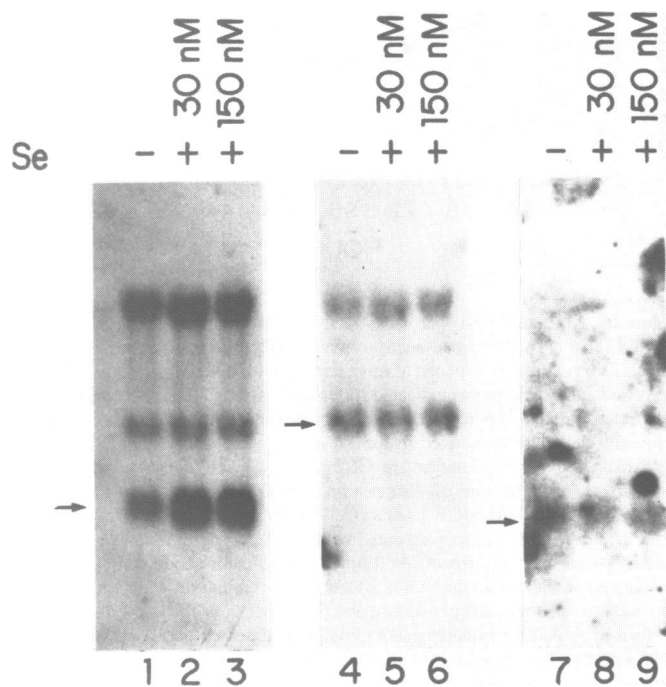
**Figure 6a.** Kinetics of GSH peroxidase activity disappearance during Se depletion in MCF-7H6 cells. Cells had been grown in medium containing 30 nM Se for at least 7 days; they were plated at time zero in Se-deficient medium. Cells were harvested at the time points indicated for enzyme assays. Two experiments were performed: filled circles represent GSH peroxidase activity level and open circles represent GST activity studied in the 1st experiment; filled triangles are GSH peroxidase and open triangles are GST activity levels examined in the 2nd experiment. 6b. The rate of disappearance of GSH peroxidase activity upon removal of Se in Adr<sup>r</sup> MCF-7 cells. Cells were grown in medium containing 0.5% fetal bovine serum supplemented with 30 nM of sodium selenite for 7 days before starting the experiment. At 0 time, fresh medium containing 0.5% fetal bovine serum without selenium was added; cells were harvested at various times and assayed for both GSH peroxidase and GST activities. Two experiments were performed. Filled symbols represent GSH peroxidase activity; open symbols are GST activity. The open and filled circles or the open and filled triangles were measured from the same cells.

by increasing the concentration of Se to 300 nM. Se supplementation at a concentration of  $1.2 \mu\text{M}$  was highly cytotoxic. The biochemical properties of the GSH peroxidase expressed in both MCF-7H6 and Adr<sup>r</sup> MCF-7 cells are similar to those of cellular GSHPx-1 isolated from human erythrocytes: including molecular weights of native molecule and subunit component, immunoreactivity with antibodies raised against human RBC-GSHPx-1, enzymatic activity toward  $\text{H}_2\text{O}_2$  and *t*-butylhydroperoxide, and metabolic labeling with  $^{75}\text{Se}$ .

The elevated level of GSH peroxidase activity in our MCF-7H6 transfectant is due to the expression of the transfected DNA rather than the activation of an endogenous gene. It contains the transfected plasmid DNA as shown by Southern blot. In addition, the sizes of GSHPx-1 mRNAs expressed in MCF-7H6 cells are different from that of the endogenous mRNA. Endogenous GSHPx-1 mRNA has a size of 0.9 kb as demonstrated in several



**Figure 7.** Northern blots of polyadenylated mRNAs isolated from Se-supplemented (lanes 1,3) and Se-deficient (lanes 2,4) MCF-7H6 cells. Lanes 1 and 2 were RNAs probed with the GSHPx-1 oligonucleotide. The blot was stripped and reprobed with  $\beta$ -actin in lanes 3 and 4.



**Figure 8.** Northern analysis of GSHPx-1 mRNA levels of Adr<sup>r</sup> MCF-7 cells grown at three Se concentrations. Total RNAs were isolated from cells grown in medium containing 0.5% fetal bovine serum supplemented with 0, 30, or 150 nM sodium selenite. The Northern blots of these RNAs were probed sequentially with GSHPx-1 (lanes 1-3),  $\beta$ -actin (lanes 4-6) and GST- $\pi$  (lanes 7-9); the arrows point to the specific mRNAs. The additional bands were nonspecific binding of 28S and 18S rRNA with GSHPx-1 and  $\beta$ -actin probes.

human cell lines including HepG2 and Adr<sup>r</sup> MCF-7 cells, but is undetectable in MCF-7 cells. Transfectants with increased GSH peroxidase activities have messages of 1.5 kb and 2.5 kb. The larger size is most likely due to the SV40 RNA in the plasmid construct. The 2.5 kb mRNA probably contains the unspliced total transcript of pRSV-GSHPx-1 including the SV40 splicing signal and the SV40 polyadenylation site. The 1.5 kb mRNA may contain a spliced transcript lacking the SV40 small *t* intron.

We have used either a 21-base synthetic probe or the cDNA probe of GSHPx-1 to analyze the human genome. Multiple bands were found with DNA preparations digested with *Hind*III, *Eco*RI, *Bgl*II or *Pst*I after probing. Similar observations have been made with rat genomic DNA (11). In addition, we have isolated a glutathione peroxidase-like cDNA clone, Px2 from both human liver and HepG2 libraries (20). Another glutathione peroxidase-like cDNA which is different from Px2 has been isolated by other investigators from a human liver library (21). Recent work in our laboratory has also determined a sufficient amount of the amino acid sequence of human plasma glutathione peroxidase that we can exclude the possibility that this enzyme is encoded by either of the glutathione peroxidase-like mRNAs (manuscript in preparation). Thus, there are probably at least 4 genes in the glutathione peroxidase family. Perhaps only one of the GSH peroxidase genes that were mapped at human chromosomes 3, 21 and X was for GSHPx-1, the other two genes could be for other members of GSH peroxidase family (42). The cytosolic GSH peroxidase expressed in Adr<sup>r</sup> MCF-7 cells appeared to be GSHPx-1, based on an mRNA which was detected only by the GSHPx-1-specific probe, but not by an oligonucleotide and a cDNA probes for the two GSH peroxidase-like sequences, and the immunoprecipitability of a 22 kDa selenopolypeptide with anti-RBC-GSHPx-1 antibodies but not anti-plasma-GSH peroxidase antibodies.

GSHPx-1 contains a selenocysteine at its active site, which is encoded by a UGA opal codon. An opal suppressor seryl-tRNA has been found in mammalian cells which functions both as a carrier molecule upon which selenocysteine is synthesized and as a direct selenocysteine donor to growing polypeptide chains in response to a specific UGA codon. The biosynthesis of selenocysteine in mammalian cells occurs by conversion of seryl-tRNA<sup>ser</sup> to phosphoseryl-tRNA<sup>ser</sup> and then to selenocysteyl-tRNA<sup>ser</sup> (44). Thus, the Se-dependence of endogenous GSHPx-1 expression, at least at the translational level, has been well established.

We did observe a modest ( $2.1 \pm 0.4$  fold and  $3.3 \pm 1.8$  fold) difference in the GSHPx-1 mRNA levels of MCF-7H6 and Adr<sup>r</sup> MCF-7 cells grown under reduced serum concentrations with or without Se supplementation. The effect of changes in Se concentration occurred both in GSHPx-1 mRNA which had been transcribed from a cDNA containing a heterologous Rous sarcoma virus promoter and from the gene. The effect of Se on GSHPx-1 mRNA levels could have resulted from either transcriptional or post-transcriptional control. Although unlikely, selenium might activate the Rous sarcoma promoter in a fashion similar to the GSHPx-1 promoter to enhance the rate of transcription. However, it seems more likely that selenium may function to stabilize the GSHPx-1 mRNA, resulting in higher levels of this mRNA.

Since the Se concentration present in fetal bovine serum is 26 ng/ml or  $\approx 330$  nM (provided by GIBCO), the Se level in 0.5% FBS is  $\approx 1.7$  nM. It is possible that the low level of Se present in the deficient media was sufficient to stabilize GSHPx-1 mRNA but not to provide enzyme synthesis. However, not all of the Se present in serum is accessible to the cell. Similar enzymatic activity was found in cells grown in 5% and 10% FBS which was  $< 25\%$  of that in cells grown in media supplemented with 0.5% FBS and 30 nM Se. The Se content determined in rat blood was 5.8 ng/ml (73 nM) when animals were fed a normal diet, and 0.3 ng/ml (3.8 nM) when fed with a Se-deficient diet (45). Therefore, the 1.7 nM Se in 0.5% FBS present in cell growth



media is within the range that can be achieved by selenium deprivation *in vivo*. In conclusion, based on our results with the MCF-7H6 transfectant which expresses the cDNA of GSHPx-1, and the Adr<sup>r</sup> MCF-7 cells which expresses the endogenous GSHPx-1 gene, the regulation on GSHPx-1 expression by Se appears to occur mainly at the translational level.

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