# AN ABSTRACT OF THE THESIS OF

Adams Amantana for the degree of Doctor of Philosophy in Toxicology
presented on February 13, 2003.
Title: Characterization of Rodent Selenoprotein W Promoter

Abstract approved: <u>Redacted for privacy</u>

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Rat selenoprotein W (SeW) promoter activity was investigated using different concentrations of cadmium, copper, and zinc. Two fragments (404bp and 1265bp) of the SeW promoter, containing a single metal response element (MRE), were ligated into the multiple cloning site of a pGL3-Basic reporter plasmid. The constructs were transfected into cultured rat C6 (glial) and L8 (myoblast) cells and promoter activity measured by means of luciferase reporter gene fused to the SeW promoter fragments in the reporter plasmid. With post-transfection exposure of these cell lines to these metals, copper and zinc, but not cadmium, significantly increased promoter activity of the unmutated 1265bp (not 404bp) construct ($p < 0.05$) only in the C6 cells. Mutation of the MRE sequence abolished promoter response to metal exposure but did not eliminate promoter activity. The results suggest that SeW expression in glial cells can be increased on exposure to copper and zinc and that this response is dependent on the MRE sequence present in the SeW promoter.

To understand transcriptional regulation of the SeW gene, we used *in vitro* binding assays to identify transcription factors that may be involved in the transcriptional regulation of the SeW gene. Using protein from rat C6 (glial) cell nuclear extracts, oligonucleotides containing putative regulatory elements in the SeW promoter, and antibodies, we were able to show that the specificty protein 1(Sp1) transcription factor binds to the Sp1 consensus sequence in the SeW promoter as well as the MRE. However, the MRE, GRE, AP-1 and LF-A1 did not yield any specific binding. Although, competition analysis showed specific binding at the TFII-1 site, super-shift analysis using anti-TFII-1 antibody did not yield any super-shifted band. Therefore the SeW gene may be a target for Sp1 whose interaction with the SeW promoter may activate or repress the transcription of SeW.

Characterization of Rodent Selenoprotein W Promoter

By

Adams Amantana

A THESIS

Submitted to

Oregon State University

In partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented February 13, 2003
Commencement June 2003

<u>Doctor of Philosophy</u> thesis of <u>Adams Amantana</u> presented on <u>February 13, 2003</u>.

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Adams Amantana, Author

# ACKNOWLEDGEMENTS

To my parents, Dramani and Rose Amantana, I say thank you so much for your love and guidance. I will continue to cherish all you have done for me. Finally, I express my deep appreciation to my parents-in-law, sisters-in-law, my siblings, and all my friends for their love and support.

## CONTRIBUTION OF AUTHORS

Dr. Philip Whanger supervised the entire project and was instrumental in reading and editing the manuscripts. Dr. William Vorachek provided guidance in the design of the experiments, as well as data interpretation, reading and editing manuscripts. Mrs Judy Butler offered technical assistance in cell culture. Dr. Nick Costa and Dr. Walt Ream offered guidance in the design of experiments.

# TABLE OF CONTENTS

# TABLE OF CONTENTS (Continued)

# LIST OF FIGURES

LIST OF FIGURES (Continued)

# LIST OF APPENDICES

# LIST OF APPENDIX FIGURES

# LIST OF APPENDIX TABLES

## DEDICATION

To Vivian Amantana, Rex and Carole Parnell, Ralph and Wilma Hull, Tabas

Amadu, Dramani and Rose Amantana, Cephas and Ernestina Djokotoe.

# Characterization of Rodent Selenoprotein W Promoter

## Chapter 1

### Introduction

During the past sixty years, public perception of selenium evolved from a highly toxic and carcinogenic element to an essential trace element with antioxidant and anticarcinogenic properties. Research findings in the last thirty years provided great insights into biochemical, molecular and genetic aspects of selenium. During this period, data from human and animal nutritional studies defined a vital role for selenium in disease incidence and severity. For example chronic diseases such as cancer, liver dysfunction and cardiovascular disease, smoking, excessive alcohol consumption, may be attributed, in part, to disruption of redox homeostasis, leading to the presence of excess reactive oxygen species and oxidative damage of essential cellular components, including proteins and nucleic acids. Dietary selenium is viewed as a potent regulator of cellular redox homeostasis; therefore it would be an important dietary contributor in reducing the incidence of these abnormalities. For example, Schwarz and Foltz showed that liver necrosis induced in rats by feeding them a purified vitamin E-deficient diet could be prevented by adding selenium [1].

The biological mechanisms of selenium responsible for promoting better health and the extent to which this element is involved remain to be completely understood. Selenium is found in all cells and tissues of the body in concentrations that vary with tissue. Almost all the selenium in animal tissues is associated with protein, and its biological function may be related to unique functions in various selenoproteins [2]. The biological role of selenium in the glutathione peroxidase (GPx) family explained many effects of selenium deficiency. GPx catalyzes the reduction of hydrogen peroxide and organic hydroperoxides to water and relatively less damaging alcohols as a means of detoxification. The indication that there might be additional biologically active forms of selenium has been supported by the finding that about two thirds of selenium present in the organism is not bound to this enzyme alone but is contained in other selenoproteins [3]. These include iodothyronine deiodinases, thioredoxin reductases, and selenophosphate synthetase all of which have known functions. There are several other selenoproteins that have been discovered but their biological functions remain to be determined.

In the last fifteen years, many of the molecular mysteries surrounding selenium have been solved. It is now been recognized that all mammalian enzymes that are selenium-dependent, have selenocysteine at their active sites [4]. One of the unique features in the incorporation of selenocysteine into selenoproteins is the use of the UGA codon, which normally serves as termination signal and

needs an mRNA stem-loop structure, the selenocysteine insertion sequence (SECIS) element located in the 3′ untranslated region and specific translation factors so as to be recognized as the codon for selenocysteine insertion [5]. Unlike any of the other twenty amino acids that are present in proteins, the biosynthesis of selenocysteine occurs on its transfer RNA (tRNA). Selenoproteins have taken advantage of the fact that the pKa of selenocysteine is lower than physiological pH and, therefore this amino acid residue is ideally suited to engage in redox reactions.

## Selenoprotein W

Selenoprotein W (SeW) is an 87-amino acid selenoprotein and contains one selenocysteine residue in its primary structure. This protein is localized predominantly in the cytoplasm, but a small fraction of the total SeW is associated with the cell membrane [6, 7]. SeW is expressed in most tissues including skeletal muscles, heart, testis, spleen, kidney, intestine, brain, liver, and lung [8]. Based on the presence of bound glutathione (GSH) moiety, SeW is thought to function in oxidation-reduction catalysis [9]. SeW expression may be critical to the maintenance of skeletal muscle integrity based on the fact that it is known to contain a motif that strongly resembles the calcium-binding domain present in calmodulin [10].

The possibility that SeW may have an antioxidant function is strengthened by recent findings one of which demonstrated that ectopic expression of SeW renders cells more resistant to hydrogen peroxide and that this resistance is dependent on the availability of GSH [11]. In another study, glial cells with over-expressed levels of SeW showed slightly more resistance to peroxidation than normal cells [12]. These findings suggesting a redox function as one probable biological role of this protein.

**Hypothesis**

We have carried out sequence analysis on the promoter of SeW and this study revealed the presence of several putative regulatory elements. These include a consensus sequence homologous to metal response element (MRE), specificity protein-1 (Sp1) site, glucocorticoid receptor element (GRE), LF-A1, TFII-1, and activator protein-1 (AP-1) sites as well as copper signaling element (CuSE). The presence of these elements suggests that the SeW gene may be regulated by metal response element transcription factor-1 (MTF-1) which binds the MRE, glucocorticoid receptor (GR), AP-1, Sp1, LF-A1, TFII-1 and copper factor 1 (Cuf1) transcription factors through their interaction with the SeW promoter at their respective consensus sequences.

The presence of the MRE, CuSE and other consensus sequences suggest these elements represent specific binding sites for transcription factors. The interactions of the factors with SeW promoter could mediate regulation of the SeW gene necessary for the delicate balance between essential and toxic levels of zinc, copper and related metals and in response to other extracellular signals.

## Objective

Transcription factors are proteins that bind to site-specific response elements near the start site or regulatory region to initiate gene expression. They regulate gene expression by either facilitating or inhibiting the enzyme RNA polymerase in the initiation and maintenance of transcription. They may also activate or inhibit the transcription of target genes by the interaction between themselves or with other proteins. In the short term, transcription factors are involved in mediating responses to growth factors, and a variety of other extracellular signals including hormones and nutrients. For example, GR binds and modulates gene expression in a hormone-dependent fashion with the release of glucocorticoid hormone thereby mediating the biological effect of this hormone. Other transcription factors such as (MTF-1) and the copper factor-1 (Cuf1) bind to the MRE and CuSE, respectively to activate gene expression in response to the presence of metals such zinc, copper and cadmium. In fact transcription factors have been shown to bind to their consensus sequence under the influence molecules such as glucose and micronutrients such as folic acid, vitamins A, D, C, E, and selenocompounds. For instance, Sp1 undergoes a significant fall in binding activity to its consensus sequence in the presence of glucose but this activity is restored by the presence of antioxidants [13].

Also it is shown that purified Sp1 treated with hydrogen peroxide loses its ability to bind its cis-element and the DNA-binding efficiency is fully restored after incubation with dithiothreitol [14]. The findings clearly suggest that Sp1 binding may mediate cellular responses to reactive oxygen species. Also, 1, 4-phenylenebis(methylene)selenocyanate (pXSC) or sodium selenite was found to reduce the consensus site binding of transcription factors Sp1 and Sp3 in a concentration and time-dependent fashion.

On the other hand 1, 4-Phenylenebis(methylene)thiocyanate, the sulfur analog of p-XSC, which is inactive in chemoprevention, had no effect on the oligonucleotide binding of Sp1 and Sp3. These findings could provide further clues as to the mechanisms involved in the chemoprevention of cancer by p-XSC [15]. The binding of some transcription factors are also regulated by reactive oxygen species. For example, an Sp1 expression plasmid, pPacSp1 has been shown to stimulate SOD1-linked CAT expression, suggesting that Sp1 plays a positive role essential for the expression of human MnSOD in response to oxidative stress [16].

We undertook this study to characterize the promoter of the SeW gene by identifying putative regulatory elements and transcription factors that bind to these sequences to give an insight into how the expression of SeW may be regulated. There is no evidence that selenium status has any effect on the rate of transcription initiation for any selenium-dependent gene [17-19].

Presumably due to tissue-specific transcription factors, there are clear developmental changes in selenoprotein expression independent of selenium [20] as well as marked differences in tissue distribution and gender differences in some species [21], hence this study.

In a previous study we examined the effects of different levels of zinc, copper and cadmium on the SeW promoter activity in cultured cells. In this experiment, we developed a plasmid-based transient transfection system in rat C6 (glial) and L8 (myoblast) cell lines. This approach enabled us to determine a sequence-specific effect of these metals on the SeW promoter activity. Mutational analysis was used to further confirm sequence specificity associated with the effect of these metals on promoter activity. To achieve this, fragments of the SeW promoter containing the putative transcription factor binding sites were subcloned into the *Kpn* I and *Hin*d III sites of the Promega pGL3-Basic vector which features the luciferase reporter gene. The resultant plasmid was then amplified in E. coli DH5-alpha and purified by means of cesium chloride-ethidium bromide gradient. Utility of the luciferase reporter gene depends on the absence of a promoter/enhancer sequences and so that expression of this gene will be solely controlled by the cloned SeW promoter fragments. Therefore any luciferase activity that will be registered will be a measure of promoter activity. Promega pGL3-Basic vector which lacks a promoter sequence was used as a negative control and the pGL$_3$-Promoter

which contains the SV40 promoter sequence as positive control. To further determine whether any possible protein interaction with the putative binding sites in the SeW promoter, we employed the electrophoretic mobility shift and supershift assays. Mobility shift analysis is one way to investigate binding of individual transcription factors to specific DNA sequences. The direct or indirect binding of protein factors to nucleic acid sequences can be detected by shifts in electrophoretic mobility of the oligonucleotide. This assay is performed by incubating a purified protein, or a complex mixture of proteins (such as nuclear or cell extract preparations), with $^{32}$P end-labeled DNA fragment containing the putative protein binding site. The reaction products are then analyzed on a non-denaturing polyacrylamide gel. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using unlabeled DNA fragments or oligonucleotides containing a binding site for the protein of interest, or other unrelated DNA sequences. Addition of the non-labeled DNA, the competitor containing the putative binding site of interest, inhibits the shift induced by the protein in the nuclear extract. However, addition of other non-labeled DNA competitors is less effective in inhibiting the shift. Addition of an antibody against the protein in the incubation mixture results in a further retardation, giving rise to a super-shifted band. The super-shift assay exploits the specificity of antibody-antigen reactions and so allows experiments to be done with crude extracts in place of

purified proteins with the aim of determining the protein that is involved in binding with the consensus sequence. The identification of transcription factors that are capable of binding the putative regulatory elements in the SeW promoter will provide an idea as to how the SeW gene may be regulated at the transcriptional level. We showed in this study that Sp1 associates with both the Sp1 site and the MRE in a specific fashion. We demonstrated that an unknown protein interacts with the TFII-1 site suggesting that this site may be a target for some other transcription factor.

# Chapter 2

# EFFECT OF COPPER, ZINC AND CADMIUM ON THE PROMOTER OF SELENOPROTEIN W IN GLIAL AND MYOBLAST CELLS*

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Shortened title: Copper, Zinc, Cadmium and Selenoprotein W.

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**Abstract**

Rat selenoprotein W (SeW) promoter activity was investigated using different concentrations of cadmium, copper, and zinc. Two fragments (404bp and 1265bp) of the SeW promoter, containing a single metal response element (MRE), were ligated into the multiple cloning site of a pGL3-Basic reporter plasmid. The constructs were transfected into cultured C6 (rat glial) and L8 (myoblast) cells and promoter activity measured by means of luciferase reporter gene fused to the SeW promoter fragments in the reporter plasmid. With post-transfection exposure of these cell lines to these metals, copper and zinc, but not cadmium, significantly increased promoter activity of the unmutated 1265bp (not 404bp) construct (p < 0.05) only in the C6 cells. Mutation of the MRE sequence abolished promoter response to metal exposure but did not eliminate promoter activity. The results suggest that SeW expression in glial cells can be increased on exposure to copper and zinc and that this response is dependent on the MRE sequence present in the SeW promoter.

**Key Words:** Selenoprotein W, luciferase, glial cells, transfection, copper, and zinc.

**Introduction**

While investigating the promoter activity of SeW, it was found to stimulate a luciferase reporter gene using copper or zinc. Further investigation revealed that different tissue culture cells (glial or muscle) responded differentially to metals. Mammalian selenoproteins are essential components of several metabolic pathways. For example, glutathione peroxidase (GPx) is an integral component of the antioxidant system [1, 2], and iodothyronine 5'-deiodinase is essential for thyroid hormone metabolism [3-5]. However, several selenoproteins were described that do not have a known function. Two extensively studied selenoproteins without a known function are selenoprotein P (SeP) and selenoprotein W (SeW). SeP is suggested to be important as an antioxidant and involved with transport in the extracellular compartment [6-8]. SeW contains reduced glutathione (GSH) [9], and therefore, is hypothesized to be involved with antioxidant functions.

Although present in a number of tissues, SeW levels are highest in skeletal and cardiac muscles and brain of primates [10] and sheep [11], but highest in skeletal muscle and brain in rodents [12-15]. Since SeW is highest in skeletal muscle, it was suggested to be involved in muscle metabolism [15]. Selenium deficiency resulted in depletion of SeW in all tissues examined except brain in rats [14] and sheep [11]. Studies indicate that dietary selenium supplementation results in increased levels of mRNA for SeW in rat skeletal

muscle [15]. The basis for the effect of selenium on levels of SeW mRNA is now known to be the result of stabilization rather than an effect on transcription of mRNA [16].

It is not known whether selenium is the only trace element that is involved in the regulation of the SeW levels in various tissues. Interestingly, sequence analysis has revealed the existence of several putative regulatory elements in the SeW promoter that based on work with metallothionein suggest that metal ions such as zinc, copper and cadmium may impact SeW promoter activity and thus play a regulatory role in SeW expression. These regulatory elements include a consensus sequence homologous to metal response element (MRE) which is located between −40bp and −20bp, Sp1 site (−90bp and −70bp), glucocorticoid receptor (GR) site (−170bp and −150bp), a site located between −890bp and −870bp that contains the TGGCCC motif which has been shown to bind the liver-specific transcription factor LF-A1 [17], TFII-1 site (−900bp and −890bp), and AP-1 site (−970bp and −960bp) (see in figure 1a). In fact, a number of known transcription factors such as Sp1, AP-1, AP-2, AP-4, and glucocorticoid receptor are shown to be involved in the activation of the transcription of the metallothionein genes in mammalian cells by binding to their specific upstream recognition sites [18]. Therefore, cooperation between the MRE and the other putative regulatory elements may mediate a possible stimulatory effect of metal ions such as zinc and copper and exogenous ones

such as cadmium on promoter activity. Based on this, it is proposed that the sequence of MRE is critical for the response of SeW promoter to these metal ions.

On this basis, mutational analysis was undertaken to examine the role of MRE for directing any possible response of luciferase reporter gene to metal ions. Two fragments (404bp and 1265bp; see figure 1b) of SeW promoter were fused to the luciferase reporter gene and transfected into cultured C6 (rat glial) and L8 (rat myoblast). The cells were exposed to different levels of zinc, copper and cadmium to test promoter response. The resultant SeW promoter construct activities were normalized to the Simian Virus 40 (SV40) promoter.

## Materials

C6 and L8 cells were purchased from American Type Culture Collection (Manassas VA, USA). Dulbecco's Modified Eagle's Medium (DMEM), F-12 nutrient mixture and trypsin were purchased from GIBCO (Grand Island NY, USA). Fetal bovine serum (FBS) was obtained from Summit Biotechnology, Inc (Fort Collins, CO, USA). Penicillin, streptomycin and ampicillin were obtained from SIGMA (St. Louis, MO, USA). HEPES was purchased from Research Organics, Inc (Cleveland, OH, USA). Bovine serum albumen (BSA) and Bio-Rad protein assay reagent were purchased from Bio-Rad (Richmond CA, USA). Luciferase reporter plasmids, containing the ampicillin resistance gene, were purchased from Promega Corporation, Madison WI, USA. Cell culture 6-well plates were obtained from Becton Dickinson Labware (Franklin Lakes, NJ, USA).

**Methods**

**Cell Cultures**

Undifferentiated C6 and L8 cells were cultured in medium that was 90% FD (composed of 50% F-12 nutrient mixture and 50% DMEM) with 10% Fetal Bovine Serum (FBS) supplement.

The FD media contained penicillin G (120µg/ml), streptomycin (200µg/ml), ampicillin (25µg/ml), HEPES (3.6mg/ml) and sodium bicarbonate (1.2mg/ml). The cells were maintained in a humidified atmosphere with 5% $CO_2$ and 95% air at $37^{o}C$. The media was changed on a daily basis until cells reached 80% confluence. Confluence was examined using a light microscope.

**Plasmid construction**

Two fragments of SeW promoter 404bp and 1265bp (with sequences shown in figure 1a) were amplified using the primers shown in table 1. In the case of the unmutated version of 404bp (404N), oligonucleotides IV and I were used and for the unmutated 1265bp (1265N) fragment, oligonucleotides III and I. Oligonucleotides II and III were used to mutate the MRE sequence in each of these fragments. The resultant PCR products were cloned into the *Kpn* I and *Hin*d III sites of the Promega pGL$_3$-Basic luciferase reporter vector. Each plasmid DNA was propagated in the Escherichia coli strain DH5α, purified through two successive ethidium bromide-cesium chloride density gradients and then examined by gel electrophoresis. Plasmid preparation that contained greater than 90% supercoiled DNA was used for transfections. Promega pGL$_3$-Basic and pGL$_3$-Promoter reporter vectors were used as control plasmids.

| Oligonucleotide | Sequence |
| --- | --- |
| ratpro r1 | *Hin*d III<br>5'-GAC*AAGCTT*AGGACTTCAATCTCTGCG-3' |
| ratpro r2 | *Hin*d III<br>5'- GAC*AAGCTT*AGGACTTCAATCTCTGCGCGTGGGTTC<br>GTGCTCACAGCCGAG-3' |
| ratpro f2 | *Kpn*I<br>5'-CGA*GGTACC*TCTGCTGCTGGTGAGCCATTCT-3' |
| ratpro f4 | *Kpn* I<br>5'-CGA*GGTACC*ATTTACTCTGAATCAATTAGGTCTGG-3' |

Table 1. Sequences of oligonucleotides used in the PCR amplification of 404bp and 1265bp of SeW promoter fragments.

**Transfections**

Both cell lines were seeded ~ $1.07 \times 10^5$ cells per well (based on flow cytometer) in 3 ml of growth medium supplemented with FBS. The cells were then placed in a $CO_2$ incubator at $37^oC$ for 24 h allowing the cells to reach ~ 80% confluence. For maximum transfection of cells, 2 µg DNA per well contained in 137µl of suspended calcium chloride precipitate was added to the culture medium covering the cells and transfected to ~ 80% confluence. The calcium chloride precipitation of vector DNA was conducted at $37^oC$ for 20 min in HEPES buffered saline at pH 7.4. After a 4-h of incubation at $37^oC$, the media was aspirated. Cells were washed with fresh assay medium, shocked with 2 ml of 15% glycerol for 2 min, and then washed with assay medium again. The quantity of vector DNA, volume of DNA precipitate suspension, and growth medium were optimized to accommodate the use of 6-well culture plates. Cells were fed with 3 ml growth media (10% FBS) per well and a 24-h expression period for the reporter gene was allowed.

**Post-transfection treatments**

This experiment was conducted to evaluate the short-term effect of different levels of metals on the SeW promoter activity in cultured C6 and L8 cells. Promega pGL₃-Basic and pGL₃-Promoter (contains the SV40 promoter) plasmids served as controls and were not incubated with any metals. The plasmids containing the mutated and unmutated versions of 404bp and 1265bp promoter fragments were incubated with physiological and excess levels of cadmium, copper and zinc as the chlorides.

At 24-h post-transfection treatment, plates were removed from the incubator and medium aspirated. Each well was washed twice with 900 µl ice-cold 1x PBS (pH 7.4). Subsequently, a lysate was prepared using 300 µl of 1x reporter lysis buffer (Promega Corporation, Madison WI, USA) with slight modification of the supplier's instructions. In this experiment, the lysate was centrifuged at 4°C for 30 minutes instead of 2 minutes. The supernatant was transferred into fresh tubes and stored at −80°C to await reporter gene (luciferase) activity and total protein assays. Luciferase activity was measured by means of a luminometer (LKB Wallac 1250 Luminometer). Total protein was measured in a 96-well plate by the dye-binding assay of Bradford (1976) using a SpectraMax 250 plate reader with BSA as a standard.

## Statistical analysis

The experimental data was examined for normal distribution prior to statistical analysis. Mean values were compared by a one-way analysis of variance (ANOVA) with Tukey's honest-significant difference (HSD) method for comparing groups.

**Results**

Full length and fragments of the SeW promoter are presented in figure 1a, so the locations of the two fragments can be seen. Sequence analysis showed the presence of several putative regulatory elements within −1265bp and −1bp of the SeW promoter (figure 1b).

. Figure 1. (a) Full length and fragments of SeW promoter.

```
                                                        -1265
                                                        CGTTG
                   -1250
ACATATAAAA   GCCTCTGCTG   CTGGTGAGCC   ATTCTTGGTA   GAAGTGGGGA   GGTGGAGGGA
-1200                                                              -1150
ATGGAGGGAG   TAGGTGGGCG   TGCCATGGAG   TTCATAGGGT   GATAAGAAAC   TAGAGAGAAC
                                        -1100
AATACTTAAA   TTCTTTGTGA   ATGAATATCT   CCTGTTATCG   AATCTGTTCA   GCTTTCAAGG
                                  -1050
AGAAAGTCAG   GATCAGGAAT   CCTAAACCCT   TAAATCATTT   GTCCACTTTT   ACCTTTTTTC
                            -1000
CTTTTTTTTT   TTTTTTTTTT   TTGCGGAGCT   GGGGATCGAA   CCCAGGGCCT   TGCGCTTCCT
                      -950
AGGCAAGCGC   TCTACCACTG   AGCTAAATCC   CCAACCCCCC   CGTTTTCCTT   TCTTGATATG
-900                                                              -850
GTCTCTCTAC   GTAGACCTGG   CCCTGTCTGT   CCTGGAACGC   GCTACATAGG   CTAGATTGGC
                                              -800
CTCGAACCCA   CAGAGATTTC   TTGACTGCTG   GCATTAAAGG   CAACGCCTGG   ATTTTTTGTT
                            -750
TTGAGACAAG   CTGCTCTTGT   AGACCAGACT   GGCTTCAAAC   TCCCTAAGTA   GCAAGGATAA
                      -700
CCTTGAACTC   AATCCTTCAG   TCTCCTCTTC   CCAAATGCTG   GGATTAGAGG   CTTGGACCAC
                -650
AGGCCCCAGA   TAATGGGGTG   CTAGGGACCC   AACCTAGGAC   TTTATAATAT   GCTAGGCAAG
-600                                                              -550
TCCTCTGCGC   CAACTGCGCT   ACAGCCCAAG   CCCCATTCCA   GGACTTTCTA   TAGACCTTTC
                                              -500
TGCTGGAAGG   CAGCGAGAAG   GCCCAAAAAG   AGGGACTGCA   TGATTTGGGA   GTGCGCCGGG
                                        -450
GGGGTCTGTC   TGTGATGAGA   CTGTTGGGCC   TAACGCGCAC   ACTGGGACTC   GCAGTTTCAT
                            -400
TTTTTCAGAC   TTAGGTCCAT   TTACTCTGAA   TCAATTAGGT   CTGGGGAGGA   GAGATCCATC
                -350
CCAATTGTCT   CCCGATGCGT   GCCTGCTCTG   CCCCTCCCAG   ACACGGGTAA   CTGAATTTCA
-300                                                              -250
GTTCCAACCA   ATTATCTGCC   TCGAGCCACC   GCCCTGCGGG   CTGAACCATT   ACGGCACAGA
                                              -200
GAAGGGACAG   CGAGGGGCTC   TCAAGTTACT   GGTCCCAGGG   ATGCCGTCTG   TGGAGACAAC
                                  -150
CCCAGGATAT   AAAGAGAACA   GTAGGGAGTC   TTGGTTTGGG   TCGCGTCGGT   GGCCACAGCC
                      -100
TTCGTCGTGC   TCTGGAACCT   CGGTCTTCGC   ATCACCCTGC   TCCCCCCACG   TACTTGGTTT
                -50
GCTCCGACTC   TTGAGGTACT   CGGCTGTGCG   CACGGCCCCA   CGCGCAGAGA   TTGAAGTCCT

                                        MRE CS 3 (1.15e-05)
 TGCTGCTAGT  +10
  EXON 1                    CTGTGAG    CACGAACC  ◄——— MUTANT MRE
                                                      SEQUENCE

CONSENSUS SEQUENCE   ——►  CTNTGCR    CNCGGCCC
```
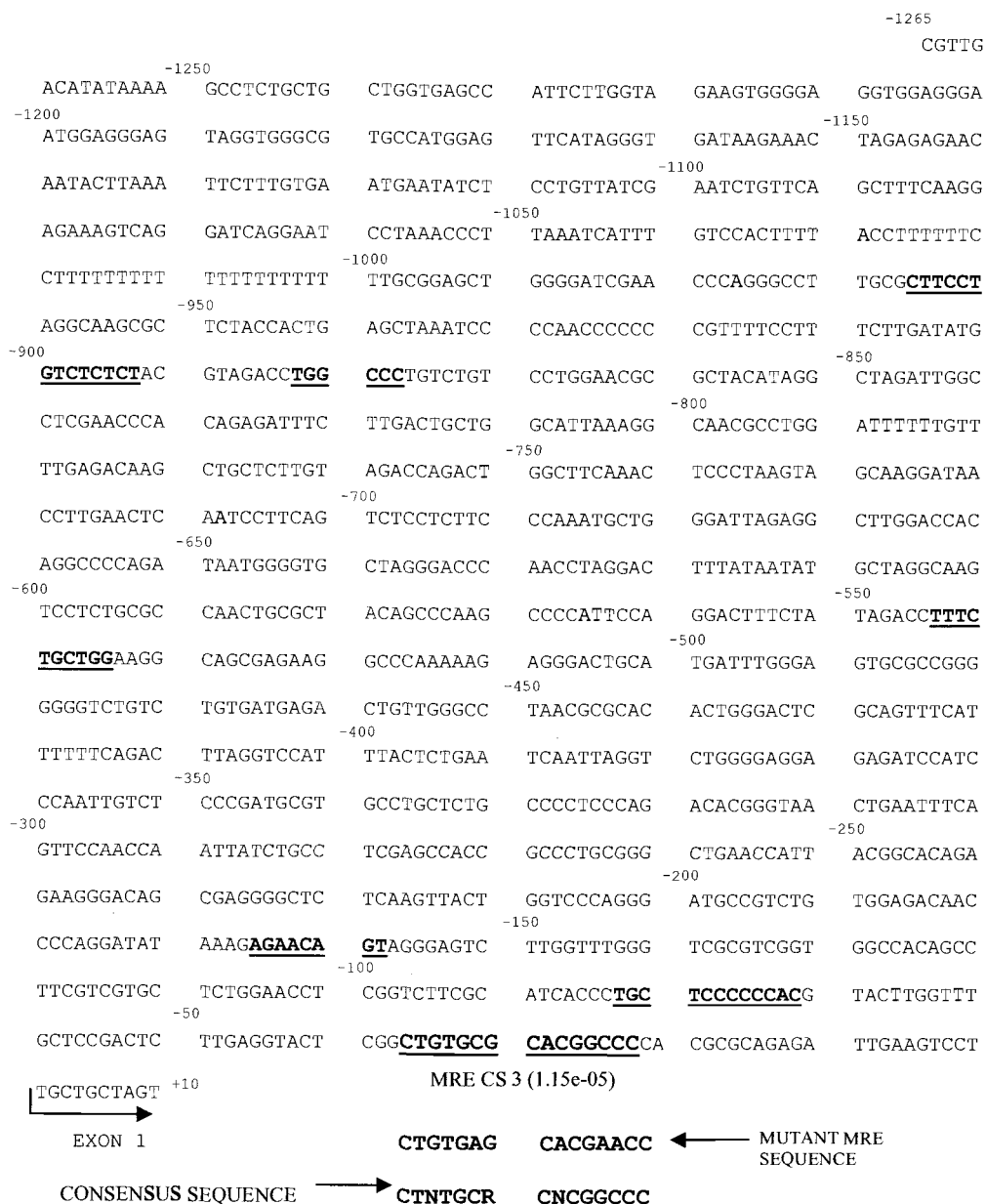
Figure 1. (b) Sequence of SeW promoter fragment between –1265bp and –1bp. This sequence was obtained from the local vector NTI database. Also shown are the modified MRE and the consensus MRE sequences. Underlined sequences represent putative regulatory elements.

Both the 1265 and 404 bp is located adjacent to Exon 1. Luciferase reporter gene analysis showed that activities of the unmutated and mutated versions of 404bp and 1265bp promoter constructs were significantly higher (approximately 4-fold) than that of the SV40 promoter in C6 cells as shown in figures 2-5. To ascertain the functional role of the SeW promoter MRE in metal response, the sequence of the MRE was modified by nucleotide substitution to create mutants of 404bp and 1265bp constructs. After these constructs were transfected into cultured C6 and L8 cells and exposed to various levels of cadmium, copper and zinc the mutational analysis revealed that SeW promoter activity in the metal exposed and control samples were not statistically different (data not shown). Also the mutation of the MRE had very little effect on promoter activity ($p < 0.05$); promoter activity changed but was not completely eliminated as shown in figures 2a and 2b.
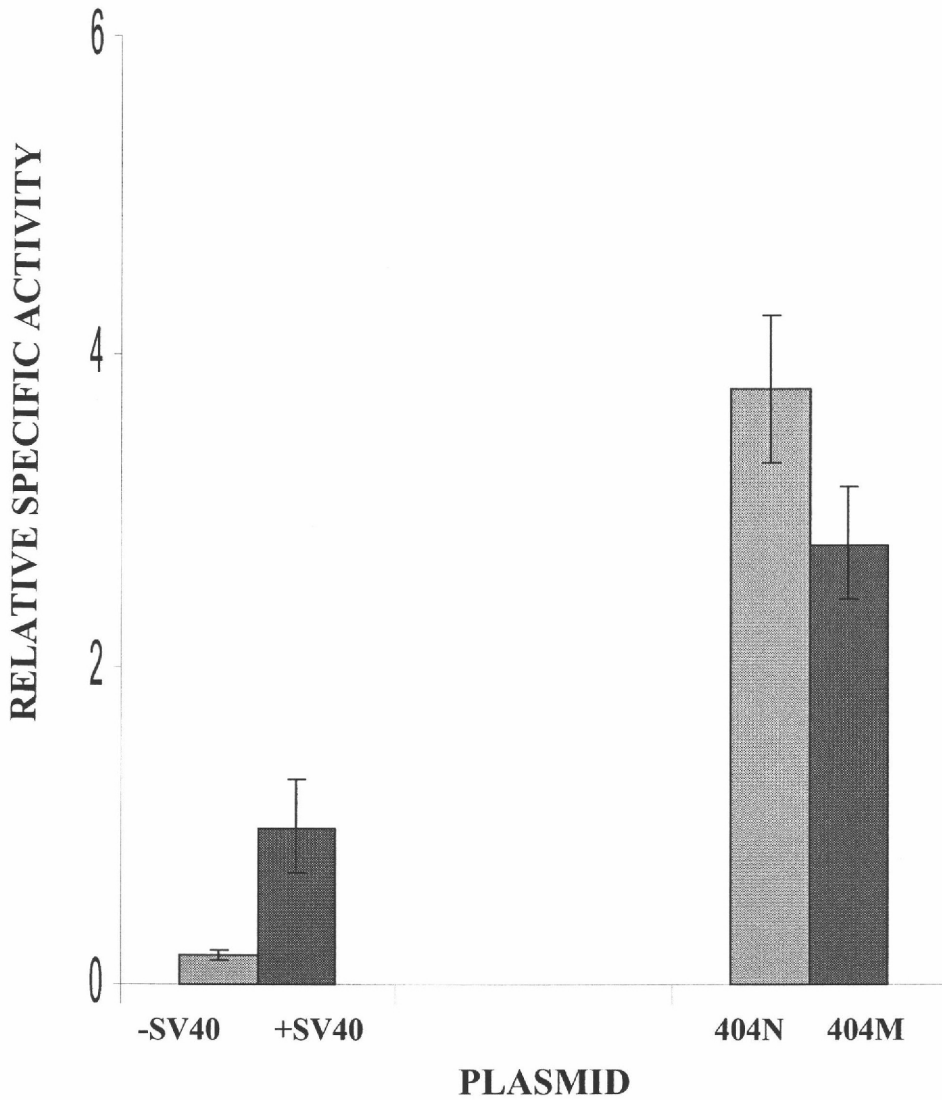
Figure (2a). Activity of unmutated (N) and mutant (M) versions of 404bp promoter constructs in 6-day differentiated C6 (rat glial) cells. Cells were transfected with constructs and incubated at 37oC. A 24 hr period was allowed for luciferase reporter gene expression. Each data point is the mean of 6 transfections. Promoter activity is expressed as specific activity relative to the SV40 promoter. Values are expressed as mean ±SE.
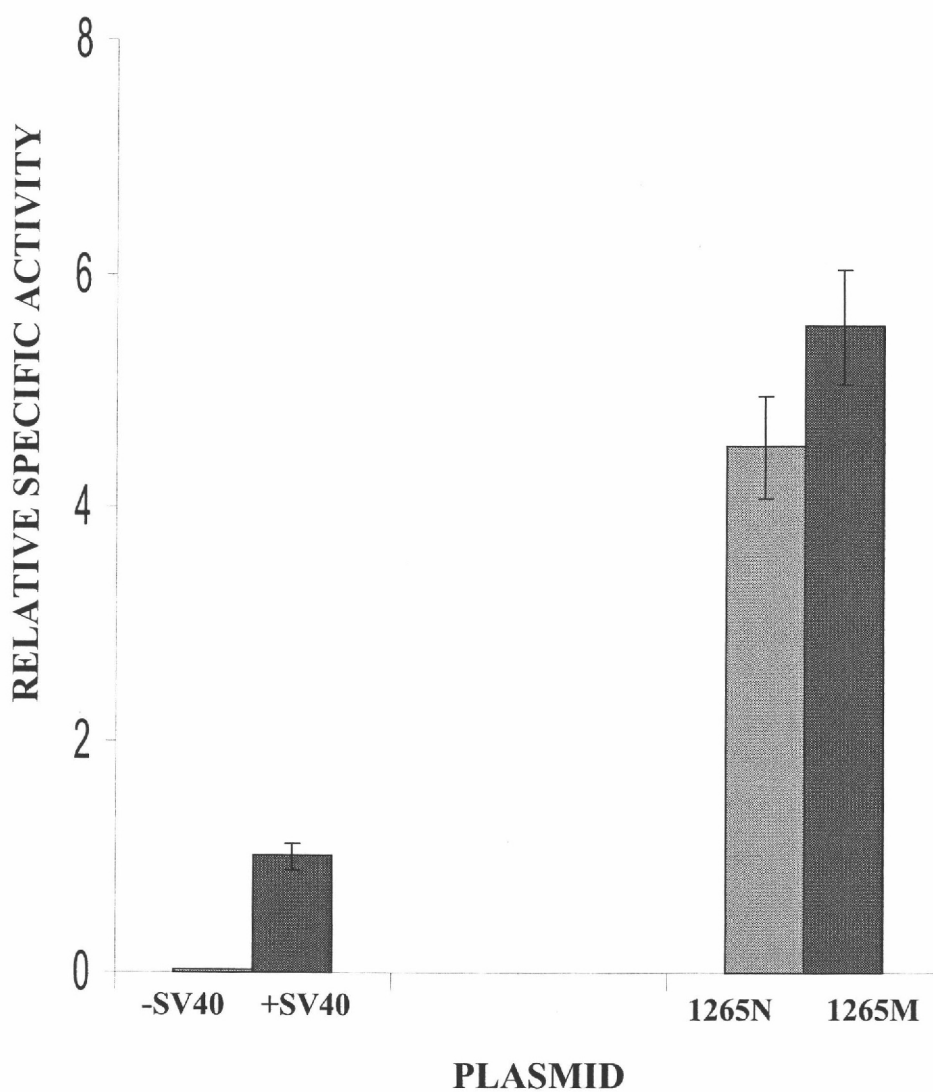
Figure (2b). Activity of unmutated (N) and mutant (M) versions of 1265bp promoter constructs in 6-day differentiated C6 (rat glial) cells. Cells were transfected with constructs and incubated at 37oC. A 24 hr period was allowed for luciferase reporter gene expression. Each data point is the mean of 6 transfections. Promoter activity is expressed as specific activity relative to the SV40 promoter. Values are expressed as mean ±SE.

In the presence of the lowest level of copper (31µM) tested, the activity of the treated unmutated 1265bp plasmid was 2-fold higher than the control in glial cells but there was no effect on the activity of the unmutated 404bp plasmid (figure 3). However, a 5-fold higher amount of copper did not result in greater activity of the unmutated 1265bp plasmid, indicating a plateau was reached.

Figure 3. Effect of copper on activity of SeW promoter in a 6-day differentiated C6 (rat glial) cells. Cells were transfected with promoter constructs and incubated at 37oC with different levels of metal over a 24 hr period. Each data point is the mean of 6 transfections. Promoter activity is expressed as specific activity relative to the SV40 promoter. Values are expressed as mean ±SE. N represents the unmutated fragment.
** $p < 0.05$

A similar pattern was observed when zinc was tested in glial cells. At $31\,\mu M$, the promoter activity of the unmutated 1265bp plasmid activity was approximately two-fold higher and like copper, the unmutated 404bp plasmid did not respond (figure 4).

33



Figure 4. Effect of zinc on activity of SeW promoter in a 6-day differentiated C6 (rat glial) cells. Cells were transfected with promoter constructs and incubated at 37oC with different levels of zinc for a 24 hr period. Each data point is the mean of 6 transfections. Promoter activity is expressed as specific activity relative to the SV40 promoter. Values are expressed as mean ±SE. N represents the unmutated fragment.
** $p < 0.05$

In contrast to copper, an increase in zinc levels up to 77 $\mu$M resulted in a lower activity compared to 31 $\mu$M. Microscopic examination revealed abnormal morphology and a decrease in confluence and in both cell lines with this highest level of zinc. Overt toxicity was evident when the cells were exposed to 154 $\mu$M zinc. Although there were slight increases in the promoter activity of unmutated 404bp and 1265bp plasmids, cadmium did not result in statistically significant stimulation of promoter activity in the C6 cells (figure 5).
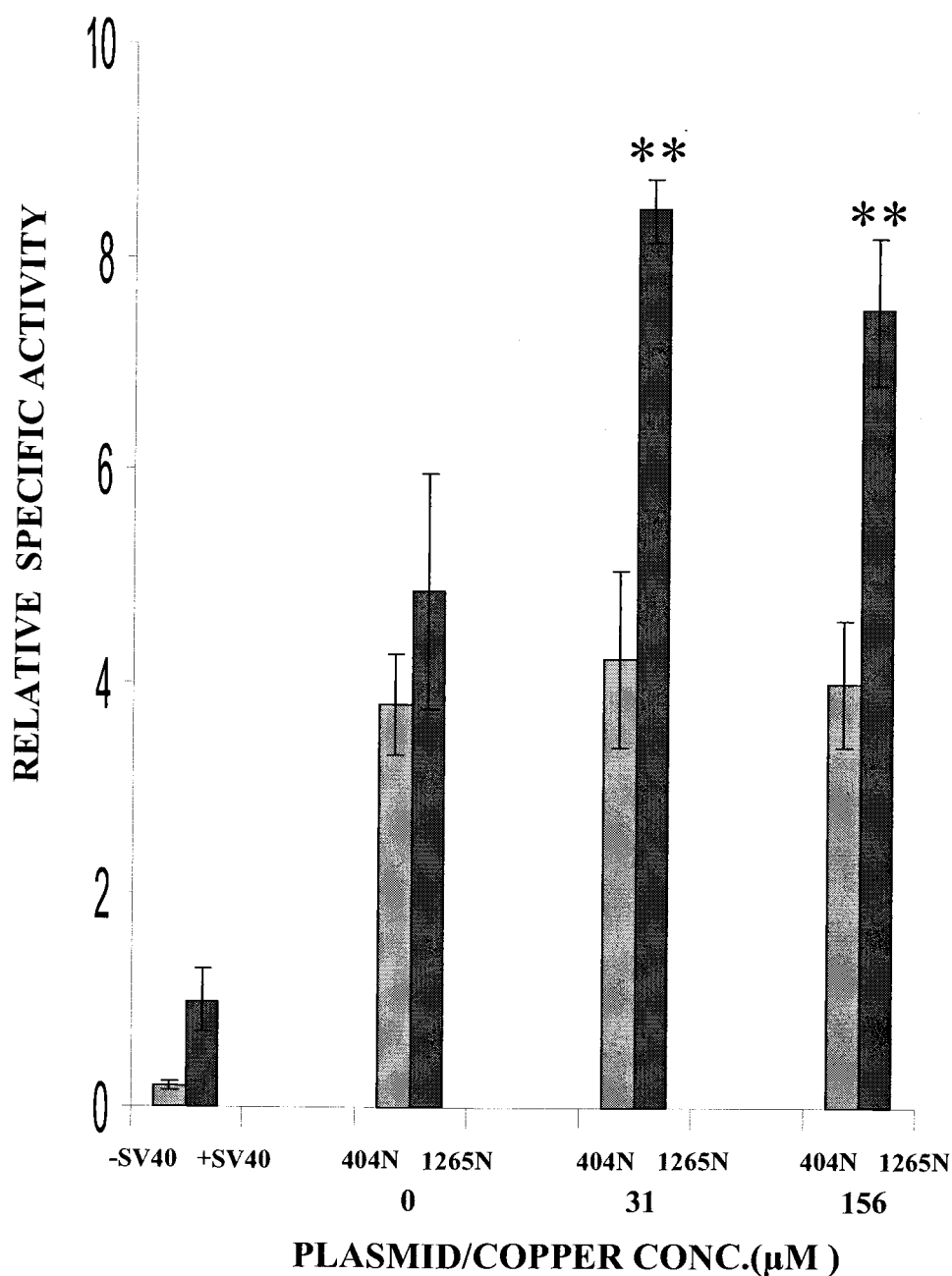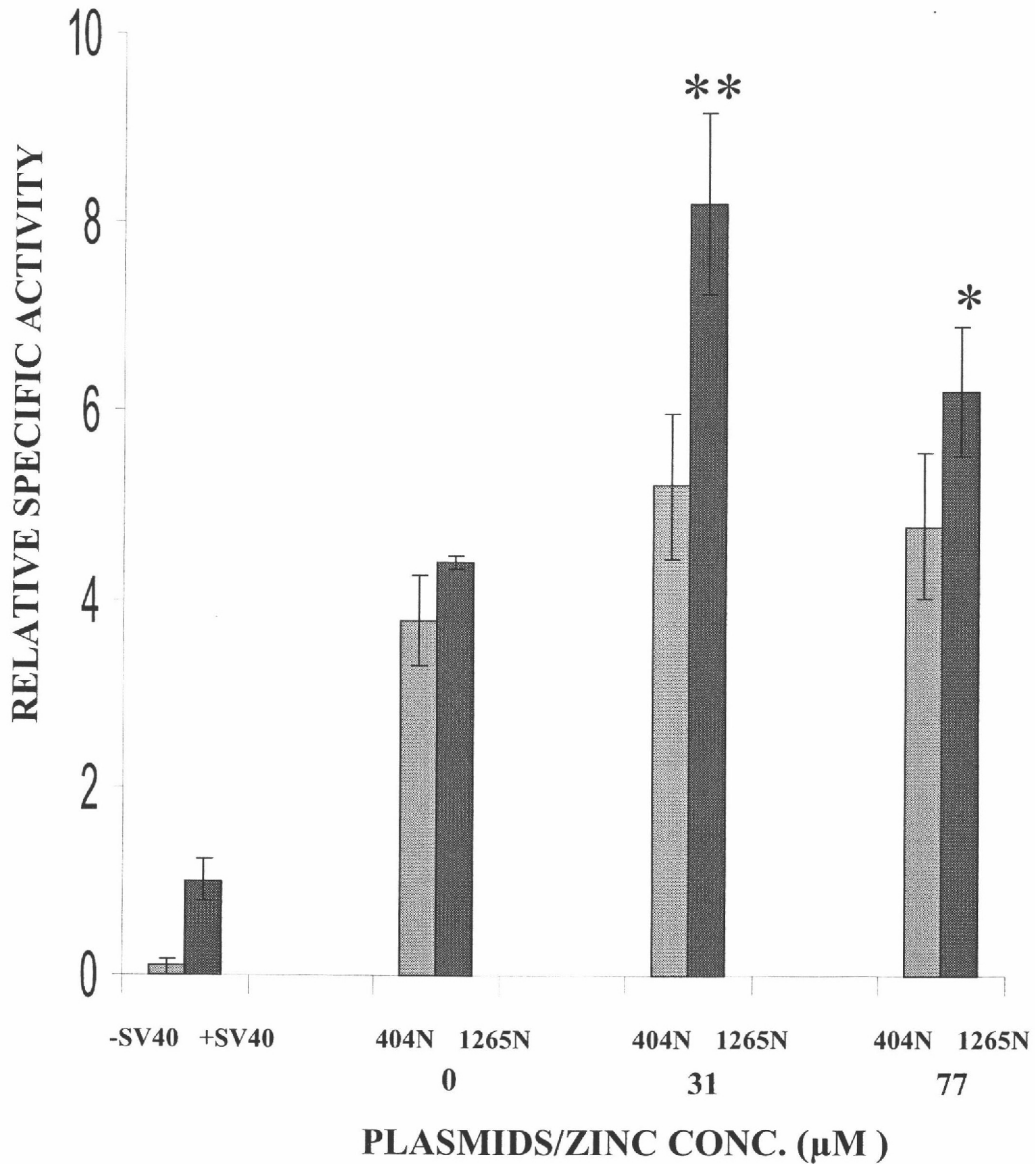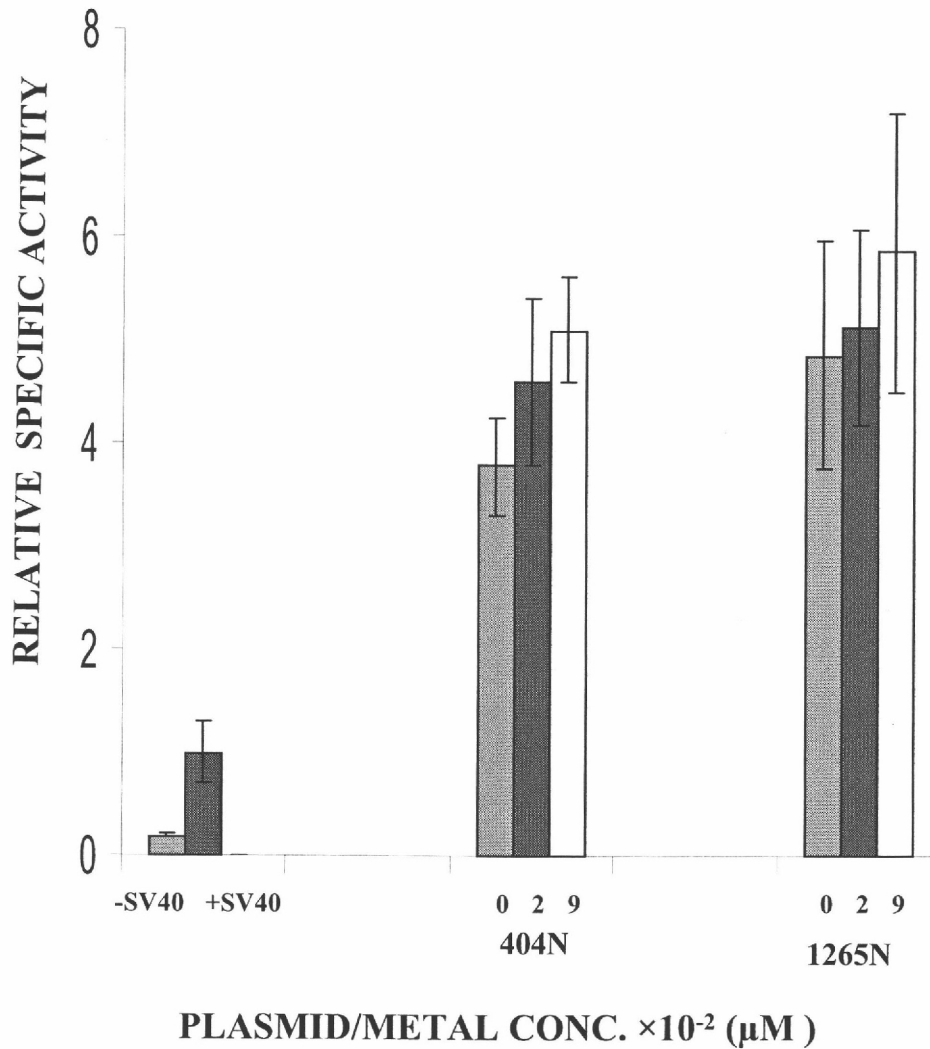
Figure 5. Effect of cadmium on activity of SeW promoter in a 6-day differentiated C6 (rat glial) cells. Cells were transfected with promoter constructs and incubated at 37oC with different levels of metal for a 24 hr period. Each data point is the mean of 6 transfections. Promoter activity is expressed as specific activity relative to the SV40 promoter. Values are expressed as mean ±SE. N represents the unmutated fragment.

With respect to the L8 cells, there was no increase in promoter activity in response to the presence of any of the metals in any of the treatment samples (data not shown). We do not have an explanation for this lack of response to metals in the L8 cells.

**Discussion**

The $pGL_3$-Basic reporter vector lacks a eukaryotic promoter and enhancer sequences, and thus the fused SeW promoter fragments exclusively drove the luciferase gene expression. In this way the SeW promoter activity was monitored throughout these experiments. This made it possible for the two constructs of the SeW promoter to be fused to the luciferase reporter gene so the response to zinc, copper and cadmium could be monitored.

Transfection of C6 cells with 404bp and 1265bp plasmids yielded basal SeW promoter activity approximately 4-fold greater than that of the SV40 promoter (figures 2-5). Based on the stimulatory effect of copper and zinc on SeW promoter activity in the glial cells, it is speculated that metal exposure will stimulate promoter activity with a resultant induction of SeW expression. Figures 3 and 4 showed that a 5-fold increase in copper and a 2.5-fold increase in zinc did not further increase promoter activity, suggesting that SeW promoter responses to metal is not dose-dependent within the range used in this study. There was no evidence of SeW promoter response to the exposure of the metals tested in L8 cells (data not shown).

Mutational analysis of the MRE allowed us to assess the functional role of this response element to metal exposure. The mutation of the MRE sequence (shown in figure 1a) did not abolish basal promoter activity suggesting that this sequence is not critical to basal promoter activity. However, since the

modification of the MRE sequence quenched the SeW promoter response to zinc and copper, it confirms our hypothesis that the substituted nucleotides in the MRE sequence are critical to the metal induction of SeW promoter. The mechanism underlying this observed metal activation of the SeW promoter is unknown. It may be mediated by a precise protein-DNA interaction or some other novel mechanism for metal-regulated process. Shinji et al. [19] showed that the mutant human metallothionein-IIA (hMT-IIA) gene defective in binding MTF-1 is unable to support zinc-induced reporter gene expression. Thus, the failure of the mutant version of the SeW promoter to respond to metal exposure may result from the creation of a sequence defective in protein binding. Therefore, it is proposed that the observed SeW promoter activation by copper and zinc is mediated by a sequence-specific interaction between the MRE and a transcription factor. The modification of this response element therefore decreased or abolished protein-DNA interaction with a consequent loss in activity. Apart from protein-DNA, metal response could also result from unknown mechanism(s) independent of transcription factor-MRE interaction. In fact, it was reported that transition metals, other than zinc, that activate MT gene expression may do so by mechanisms independent of DNA binding activity of MTF-1 [20].

Interestingly, unlike the unmutated 1265bp promoter fragment, the unmutated 404bp fragment did not respond to treatment with metal as shown in figures 3,

4 and 5. This may be correlated to promoter length in that there are putative regulatory elements present in the 1265bp construct such as TGGCCC motif, TFII-1 site and AP-1 site but absent in the 404bp. However, the fact that the disruption of the MRE sequence in 1265bp construct abolished metal response suggests that although these regulatory elements may contribute to metal response they do so only in the presence of the intact MRE sequence.

The results in this study show that copper and zinc exert a stimulatory effect on rat SeW promoter activity in the C6 cells but not the L8 cells and that this response is dependent on the intact MRE sequence. There is no explanation for lack of response in the L8 cells. Nucleotide substitution in the MRE sequence will lead to a complete loss in promoter response to metal ion exposure. Whether this observed promoter response to copper and zinc has any effect on the expression of SeW in the glial cells is yet to be determined.

**Acknowledgements**

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Chapter 3

# IDENTIFICATION OF PUTATIVE TRANSCRIPTION FACTOR-BINDING SITES IN RODENT SELENOPROTEIN W PROMOTER

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# IDENTIFICATION OF PUTATIVE TRANSCRIPTION FACTOR-BINDING SITES IN RODENT SELENOPROTEIN W PROMOTER

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**Abstract**

To understand transcriptional regulation of the SeW gene, we used *in vitro* binding assays to identify transcription factors that may be involved in the transcriptional regulation of the SeW gene. Using protein from rat C6 (glial) cell nuclear extracts, oligonucleotides containing putative regulatory elements in the SeW promoter and antibodies, we observed that specificty protein 1(Sp1) transcription factor is binds to the Sp1 consensus sequence in the SeW promoter as well as to the metal response element (MRE). Although, competition analysis showed specific binding at the TFII-1 site, super-shift analysis using anti-TFII-1 antibody did not yield any super-shifted band. Therefore, the SeW gene may be a target for Sp1 whose binding to various regulatory sequences of the SeW promoter may activate or repress the transcription of SeW. The MRE, GRE, AP-1 and LF-A1 sites were also tested but no evidence was obtained for specific binding as indicated by lack of competition with unlabeled probes.

Keywords: Selenoprotein W promoter; transcriptional regulation; Specificity protein 1 (Sp1); C6 (glial) cells.

**Introduction**

There is increasing evidence that the biological functions of selenium are mediated by selenoproteins and thus it is important to identify, characterize and determine the functions of additional selenoproteins. One of these whose function is yet to be determined is selenoprotein W (SeW). SeW is a small, (87-amino acid) protein and contains one selenocysteine residue. The expression of SeW may be an important component of the cellular defense system against oxidative stress since it is capable of binding reduced glutathione (GSH) [1]. This is demonstrated by the fact that glial cells with over expressed levels of SeW showed more resistance to peroxidation than normal cells [2] suggesting a redox role as one probable function.

Similar to other selenoproteins, selenocysteine incorporation into SeW requires the interpretation of UGA as a codon for selenocysteine incorporation that will otherwise function as a stop codon and a tRNA, which serves as the site for the synthesis of selenocysteine from serine that recognizes the UGA codon [3, 4]. The interpretation of the UGA codon is made possible by the SECIS element, which forms a conserved stem- loop in the 3' UTR of the SeW mRNA [5-7]. Although information is available about the nature of SeW in terms of, amino acid sequence, distribution, and the regulatory effect of selenium on its expression, the mechanisms regulating its gene transcription are unknown. In a previous study we showed the presence of several putative

regulatory elements in the rat SeW promoter [8]. These include a metal response element (MRE), glucocorticoid response element (GRE), Sp1, LF-A1, TFII-1, AP-1 sites as well as a copper signaling element (CuSE). These may serve as binding sites for various transcription factors that are involved in the initiation, enhancement or repression gene transcription.

Using a plasmid-based luciferase reporter gene system, we showed that the 1265bp fragment of the promoter had a strong activity, which was 4-5 times that of the strong SV40 promoter. Also, treatment with different levels of copper and zinc increased promoter activity 2-4 fold in rodent glial cells [8]. An MRE has also been found in the promoter region of a selenoprotein P-like protein gene, but in contrast to the one for SeW neither cadmium nor zinc caused a significant alteration in the mRNA for this selenoprotein (5). Zinc was shown to be more toxic to glial cells than copper (4). In addition to a MRE in the promoter of SeW a copper signaling response element (CuSE) was also found and it is concluded that this is the reason the cells were more sensitive to zinc than to copper. This observation is strengthened by the presence of both MRE and CuSE.

Transcriptional regulation usually involves the recognition and binding to specific promoter elements by transcription factors, leading to the activation or repression of a target gene expression. Transcriptional activation is controlled by a complex series of inhibitory or synergistic reactions that reflect many

intra-and extra-nuclear regulatory or signaling processes. Thus this response may be mediated by the binding of one or more transcription factors to the MRE or some other putative regulatory element in the promoter of SeW leading to the expression of the SeW gene. Presently it is not known whether the regulation of SeW expression is pretranslational or post-translational. It is known that dietary selenium supplementation results in increased levels of SeW mRNA in rat skeletal muscle [9].

To offer an insight into the transcriptional regulation of the SeW gene, we used *in vitro* binding assays to identify transcription factors that are capable of binding the SeW promoter. We showed in this study that Sp1 associates with both the Sp1 site and the MRE in a specific fashion. We also showed that an unknown protein interacts with the TFII-1 site suggesting that this site may be a target for some other transcription factor.

## Materials

C6 cells were purchased from American Type Culture Collection (Manassas VA, USA). Dulbecco's Modified Eagle's Medium (DMEM), F-12 nutrient mixture and trypsin were purchased from GIBCO (Grand Island NY, USA). Fetal bovine serum (FBS) was obtained from Summit Biotechnology, Inc (Fort Collins CO, USA). Penicillin, streptomycin and ampicillin were obtained from SIGMA (St. Louis, MO, USA). HEPES was purchased from Research Organics, Inc (Cleveland, OH, USA). NE-PER $^{TM}$ Nuclear and Cytoplasmic Extraction Reagents were obtained from PIERCE (Rockford IL, USA), [Gamma-$^{32}$P] ATP was obtained from Perkin Elmer Life Sciences Inc. (Boston, MA, USA). Oligonucleotides were purchased from SIGMA (Woodlands TX, USA). Affinity-purified goat polyclonal antibody, raised against human TFII-1, was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA) and rabbit polyclonal antibody, raised against human Sp1, was obtained from Serotec Inc. (Raleigh, NC, USA).

## Methods

### Cell Culture and Preparation of Nuclear Extracts

Undifferentiated C6 (glial) cells were cultured in medium that was 90% FD (composed of 50% F-12 nutrient mixture and 50% DMEM) with 10% Fetal Bovine Serum (FBS) supplement. The FD media contained penicillin G (120μg/ml), streptomycin (200μg/ml), ampicillin (25μg/ml), HEPES (3.6mg/ml) and sodium bicarbonate (1.2mg/ml). The cells were maintained in a humidified atmosphere with 5% $CO_2$ and 95% air at $37^o$C. The media was changed on a daily basis until cells reached 80% confluence. Confluence was examined using a light microscope. Crude nuclear extracts from the cultured C6 cells were prepared using NE-PER$^{TM}$ Nuclear and Cytoplasmic Extraction Reagents kit PIERCE (Rockford IL, USA). Total protein estimates were done on these extracts in a 96-well plate by the Bradford assay using a SpectraMax 250 plate reader with BSA as a standard. These were then stored at $-80^o$C prior to use.

**Electrophoretic mobility shift assay (EMSA)**

This experiment was conducted to determine DNA-binding activity of proteins in the glial cell nuclear extract. Oligonucleotides containing the individual putative transcription factor binding sites were ^{32}P end-labeled using [gamma-^{32}P] ATP and T4 polynucleotide kinase and these were used as probes. The standard reaction mixture (20µl) contained 10 mM Tris/HCL, pH 7.5, 10 mM MgCl$_2$, 50 mM NaCl, 0.5 mM dithiothreitol, 10% glycerol, 0.1µg/µl poly (dI-dC), and 20 fmols ^{32}P-labeled probe. In order to separate free and bound probe, 5µg of nuclear extract protein was incubated in the reaction mixture at 25 °C for 20 min and subsequently electrophoresed on a native 5% polyacrylamide gel in a 0.5X TBE buffer at 200V for 1.5 hours. Gels were dried and subjected to autoradiography. For competition assays, a large excess of corresponding unlabeled double-stranded oligonucleotide was incubated 10 min prior to adding the ^{32}P-labeled probe.

Below are the putative binding-site sequences used as probes:

| Putative binding-sites | Binding proteins |
|---|---|
| 5'-AGGTACTCGG<u>CTGTGCGCACGGCCCC</u>ACGCGCAGA-3' | MTF-1 |
| 5'-CATCACCC<u>TGCTCCCCCCAC</u>GTACTTGGTT-3' | SP1 |
| 5'-CAGGATATAAAG<u>AGAACAGT</u>AGGGAGTCTTGG-3' | GR |
| 5'-GTAGACC<u>TGGCCC</u>TGTCTGTCCTGGAACGC-3' | LF-A1 |
| 5'-TCCTTTCTTGATATG<u>GTCTCTCT</u>ACGTAGA-3' | TFII-1 |

5'-CCAGGGCCTTGCG<u>CTTCCT</u>AGGCAAGCGCTCT-3'          AP-1

For super-shift analysis, 1µl of antibody (in surplus amounts) was incubated with the probe/nuclear extract mixture at 25°C for another 30 min. Reactions were then analyzed by the standard gel-shift procedure as described above.

**Results**

**Gel-shift analysis of SeW Promoter**

To identify possible binding sites for transcription factors within the SeW promoter, *in vitro* binding studies were conducted using the gel-shift technique with nuclear extracts from purified nuclei. Nuclear extracts were incubated with individual $^{32}$P-labeled probes in the presence of poly (dI-dC) as nonspecific competitor. Free and bound probe were separated on 5% native polyacrylamide gels and subjected to autoradiography. Initial experiments using a rat glial cell nuclear extract showed the formation of nucleoprotein-DNA complexes with probes MRE, Sp1, TFII-1, GRE, AP1 and LF-A1.

In the competitive gel-shift analysis, unlabeled double-stranded oligonucleotides containing binding sites for the transcription factors MRE, Spl, TFII-1, GRE, AP1, and LF-A1 were used as competitors in the gel shift assays. The nuclear extract was incubated with either probe in the presence of oligonucleotide competitor. As shown in figures 1 and 2, the retarded bands indicate that the unlabeled Sp1 and TFII-1 binding site oligonucleotides competed specifically with the Sp1 and TFII-1 probes respectively.

Figure 1. Gel-shift assay of Sp1 site oligonucleotide in the presence of competitive oligonucleotide. $^{32}$P-end labeled oligonucleotide was incubated with nuclear extract in the presence or absence of unlabeled competitive oligonucleotide.

Figure 2. Gel-shift assay of TFII-1 site oligonucleotide in the presence of competitive oligonucleotide. $^{32}$P-end labeled oligonucleotide was incubated with nuclear extract in the presence or absence of unlabeled competitive oligonucleotide. The mixture was separated on a 5% native polyacrylamide gel and autoradiographed.

However in the case of the MRE, GRE, AP1, and LF-A1 sites there was no effect when increasing levels of the unlabeled probe was used as shown in figures 3-6.

Figure 3. Gel-shift assay of MRE site oligonucleotide in the presence of competitive oligonucleotide. ^{32}P-end labeled oligonucleotide was incubated with nuclear extract in the presence or absence of unlabeled competitive oligonucleotide. The mixture was separated on a 5% native polyacrylamide gel and autoradiographed.
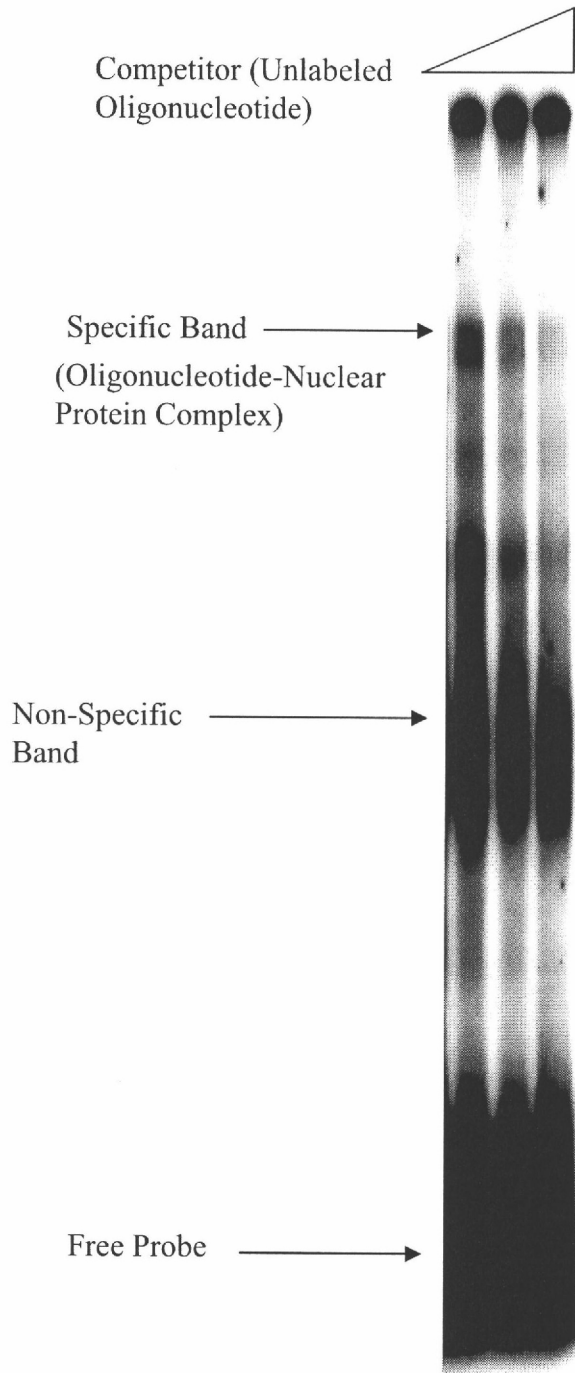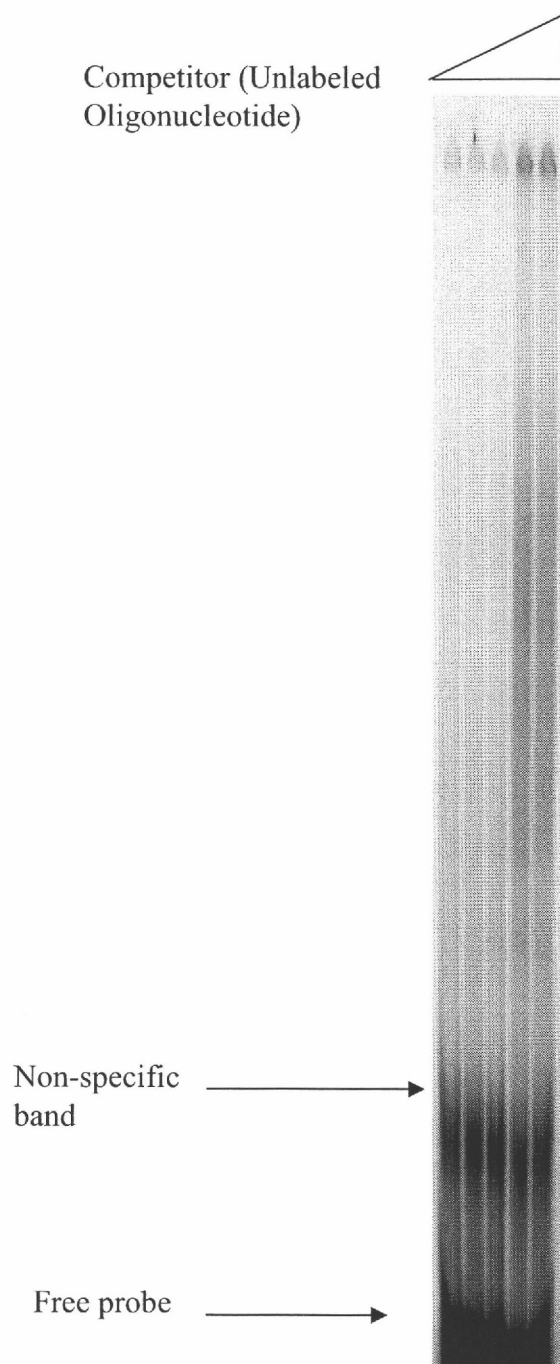
Figure 4. Gel-shift assay of AP-1 site oligonucleotide in the presence of competitive oligonucleotide. $^{32}$P-end labeled oligonucleotide was incubated with nuclear extract in the presence or absence of unlabeled competitive oligonucleotide. The mixture was separated on a 5% native polyacrylamide gel and autoradiographed.

Figure 5. Gel-shift assay of LF-A1 site oligonucleotide in the presence of competitive oligonucleotide. $^{32}$P-end labeled oligonucleotide was incubated with nuclear extract in the presence or absence 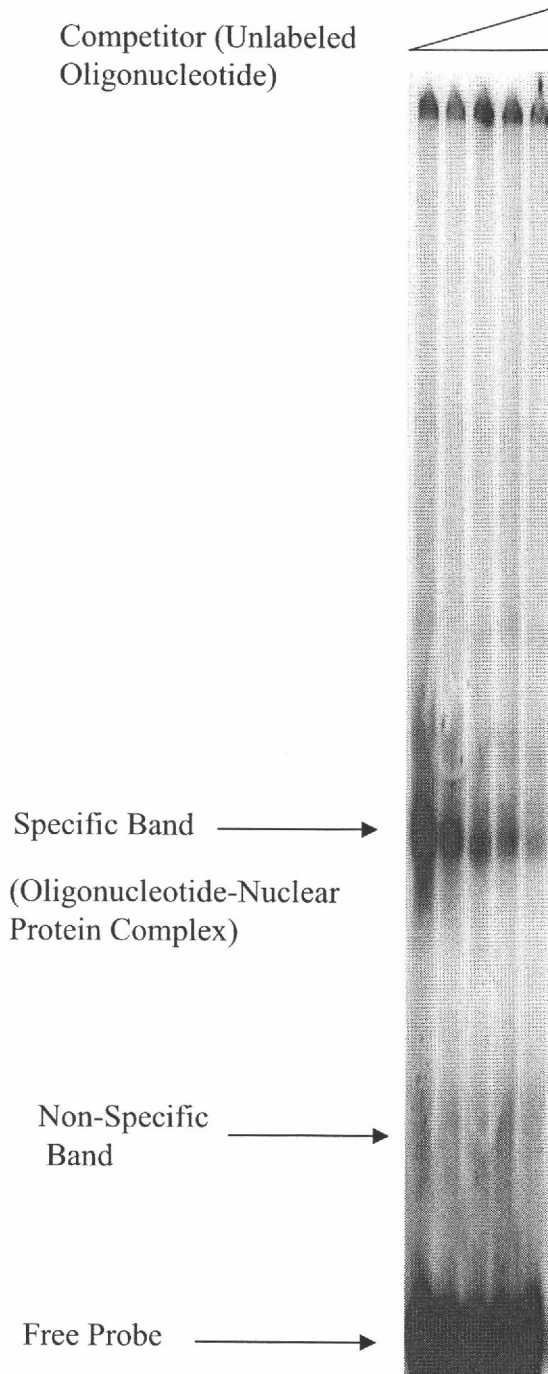of unlabeled competitive oligonucleotide. The mixture was separated on a 5% native polyacrylamide gel and autoradiographed.
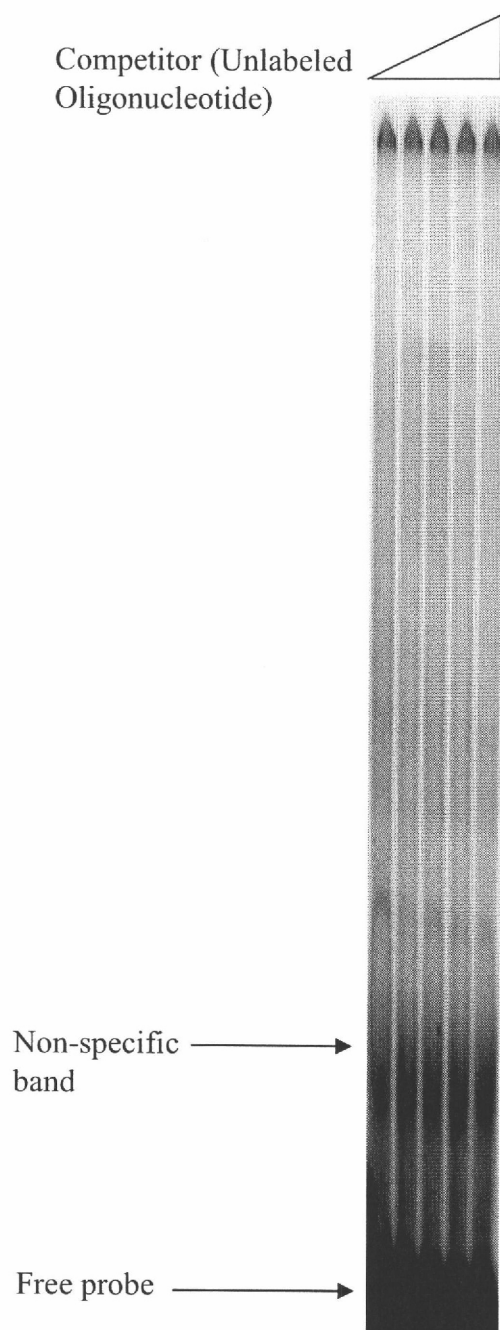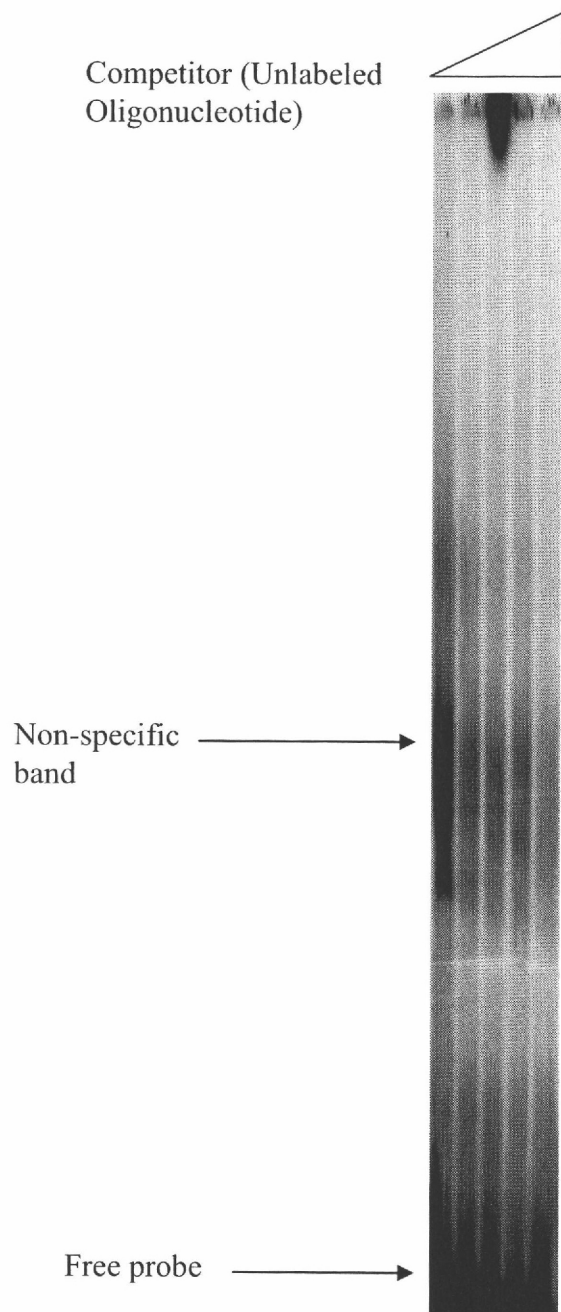
Figure 6. Gel-shift assay of GR site oligonucleotide in the presence of competitive oligonucleotide. $^{32}$P-end labeled oligonucleotide was incubated with nuclear extract in the presence or absence of unlabeled competitive oligonucleotide. The mixture was separated on a 5% native polyacrylamide gel and autoradiographed.

Supershift analysis was conducted on the Sp1 and TFII-1 probes using anti-Sp1 and anti-TFII-1 antibodies. A super-shifted band appeared as indicated in figure 7 (open arrowhead in lane 2). This result shows that the transcription factor Sp1 is a component of the Sp1 oligonucleotide/nuclear protein complex. Although the presence of the unlabeled TFII-1 oligonucleotide competed with the retarded TFII-1 probe-nuclear protein band as shown in figure 2, the super-shift analysis on this probe using anti-TFII-1 antibody did not yield any super-shifted band (figure 8).

Nuclear Extract      +   +

Anti Sp1 Antibody     −   +

◀ Supershifted Band

◁ Oligonucleotide-Nuclear
   Protein Complex

Free probe ⎯⎯⎯→

Figure 7. Gel-shift assay of Sp1 site oligonucleotide in the presence of anti-Sp1 antibody. 32P-end labeled oligonucleotide was incubated with nuclear extract in the presence or absence of antibodies. The mixture was separated on a 5% native polyacrylamide gel and autoradiographed. The oligonucleotide/nuclear protein complex is indicated by the open arrow head and the supershifted band is indicated by the closed arrow.
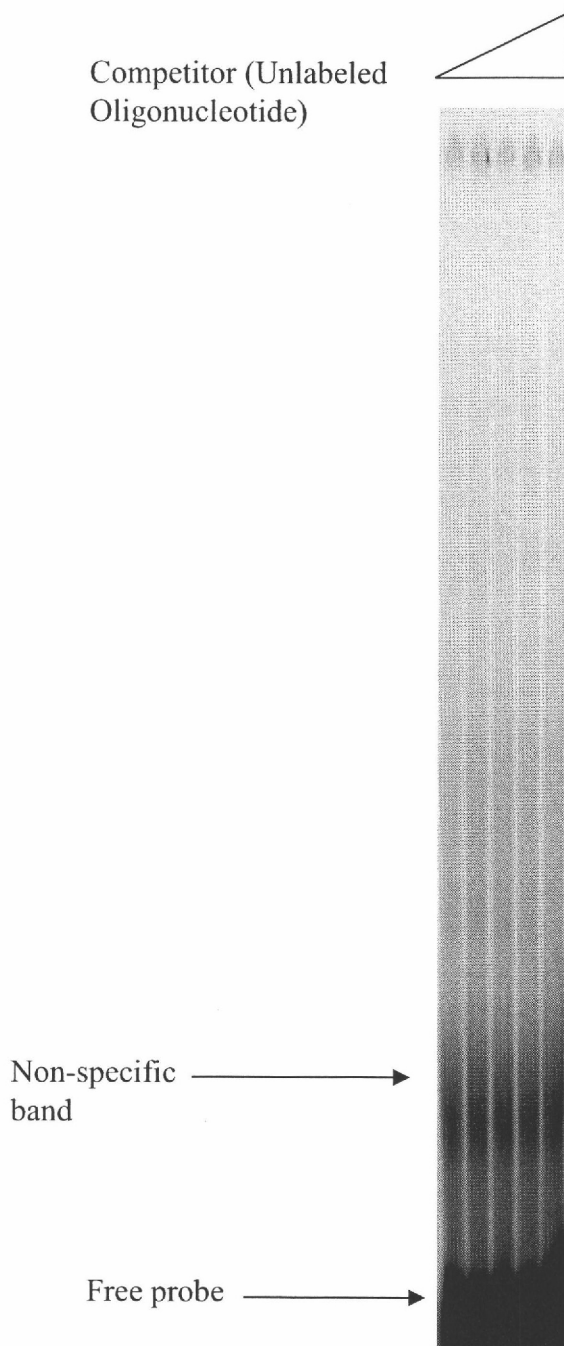
Nuclear Extract    +    +

Anti TFII-1 Antibody    −    +

Specific Band
(Oligonucleotide-Nuclear
Protein Complex)

Free probe
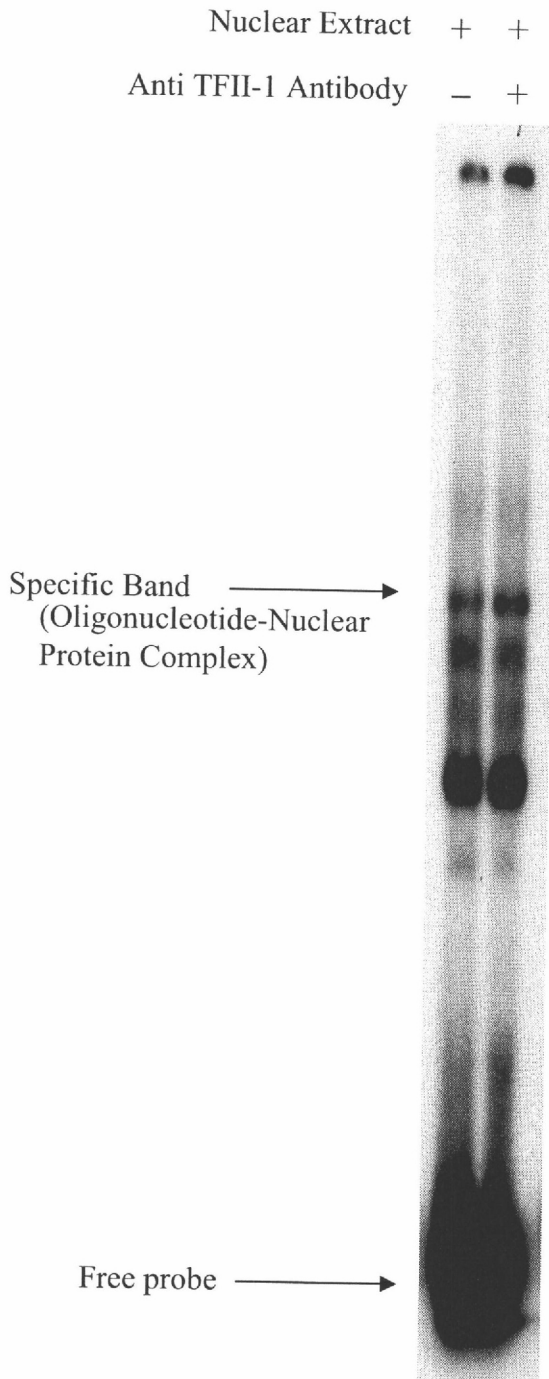
Figure 8. Gel-shift assay of TFII-1 site oligonucleotide in the presence of anti-TFII-1 antibody. 32P-end labeled oligonucleotide was incubated with nuclear extract in the presence or absence of antibodies. The mixture was separated on a 5% native polyacrylamide gel and autoradiographed.

This suggests that, the nuclear protein in the TFII-1 oligonucleotide/nuclear protein complex is not this transcription factor. As shown in figure 9 (lane 2), super-shift analysis using antibodies against Sp1 indicated that this transcription factor binds the oligonucleotide containing the MRE. The reason the retarded band in figure 9 (lane 2) disappeared in the presence of the antibody remains unclear.
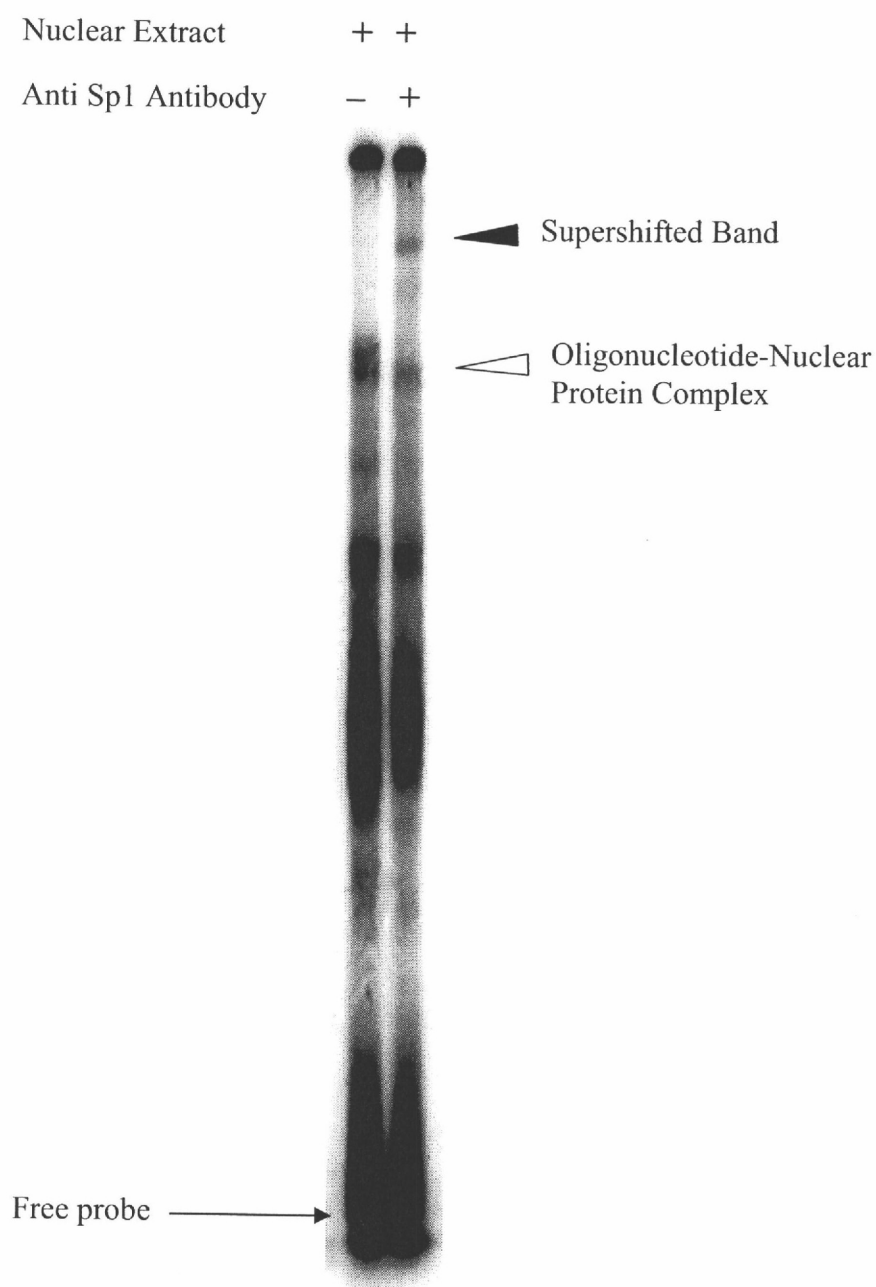
Figure 9. Gel-shift assay of MRE site oligonucleotide in the presence of anti-Sp1 antibody. $^{32}$P-end labeled oligonucleotide was incubated with nuclear extract in the presence or absence of antibodies. The mixture was separated on a 5% native polyacrylamide gel and autoradiographed. The oligonucleotide/nuclear protein complex is indicated by the open arrow head and the supershifted band is indicated by the closed arrow.
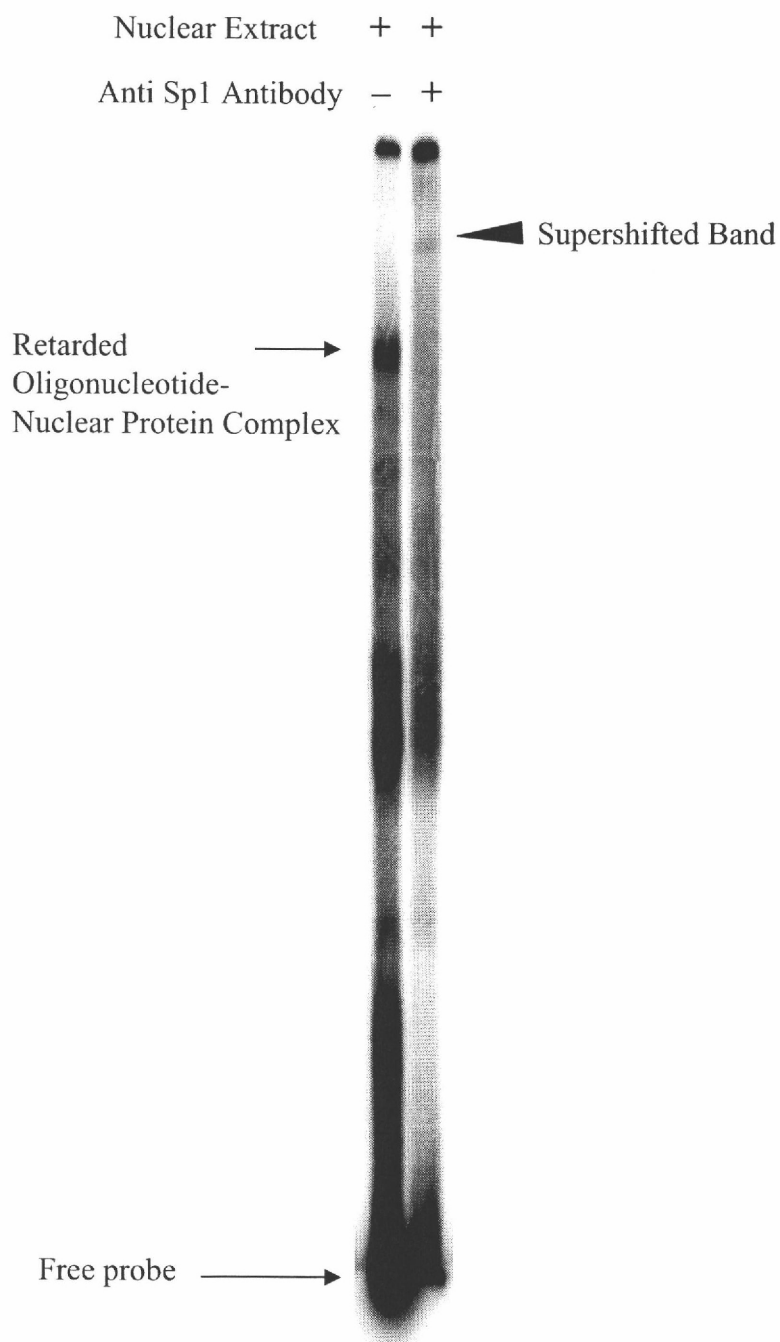
**Discussion**

The initiation of transcription is accomplished via interactions of many different proteins with common and gene-specific regulatory motifs. Clearly, sequence-specific transcription factors play a crucial role in the specificity of transcription initiation. Presently, it is not known whether the regulation of SelW gene expression is pretranslational or post-translational. However, it is known that dietary selenium supplementation results in increased levels of SeW mRNA in rat skeletal muscle [9]. The basis for the effect of selenium on levels of SeW mRNA is now known to be the result of stabilization of SeW mRNA rather than an effect on transcription of mRNA [10].

We have identified putative binding sites in the SeW promoter which include metal response element (MRE), glucocorticoid response element (GRE), Sp1, LF-A1, TFII-1, AP-1 sites and a copper signaling element (CuSE). SP1 was also shown to be present in the promoter of selenoprotein P [11]. By fusing the 5' flanking sequence to a luciferase reporter gene, a TATA box at a putative SP1 site was identified to be necessary for selenoprotein P transcription.

SP1 binding site has been found in the promoter of another selenoprotein, type 1 iodothyronine deiodinase, gene and SP1 binding site immediately 5' to thyroid hormone response element increases basal expression of a 430 bp diol promoter-chloramphenicol acetyltransferase construct in the presence of

unliganded thyroid hormone receptor, decreasing the T3 responsiveness [12]. Interestingly, this does not do so when the complex is placed in its more 5' wild-type location. In another system, fluid shear stress-induced transcriptional activation of the vascular endothelial growth factor receptor-2 gene requires SP1 dependent DNA binding [13]. This suggests that SP1 transcription factor may be wide spread in systems associated with oxidative stress. Sp1 has been shown to bind and regulate the promoter of manganese superoxide dismutases (an antioxidant system) [14]. Since the GPXs are responsible for degrading peroxides, it would appear fruitful to determine the presence of SP1 binding factors in the promoters of these peroxidases.

In another system, zinc finger protein (ZFP) transcription factors are essential for regulation of gene expression in the developing brain [15]. Lead induced development perturbations in hippocampal SP1 DNA-binding was prevented by zinc supplementation and the data suggested that lead and zinc can compete both in vivo and in vitro at the ZFP domain of SP1 with a consequential effect on SP1 DNA-binding. This subsequently affects gene expression and brain development.

We could not find any information on LF-A1 and TFII-1 binding sites in the promoter region of selenoproteins in addition to SeW and thus more work is needed to determine how widespread it is in these selenium requiring proteins.

We conducted electrophoretic mobility shift, competitive and super-shift assays to identify transcription factors that are capable of binding to the SeW promoter to modulate transcription of the SeW gene. In vitro binding assays were conducted where oligonucleotides containing putative binding sites that occur in the SeW promoter and nuclear extracts from cultured rat glial cells were incubated at room temperature. The resultant DNA/protein complexes were then separated on a native 5% polyacrylamide gel. We observed retarded bands in all the oligonucleotides that were tested. To determine whether these bands were specific, we conducted competition assays using increasing levels of unlabeled oligonucleotides. Addition of the non-labeled DNA, the competitor containing the putative binding site of interest, inhibits the shift induced by the protein in the nuclear extract. However, addition of other non-labeled DNA competitors is less effective in inhibiting the shift. Except for the Sp1 and TFII-1 site oligonucleotides the competition assay data did not show any correlation between band intensity and levels of the unlabeled oligonucleotide competitor in the case of GR, LF-A1, AP1, and MRE.

Even though AP-1 did not binding competitively to SeW, selenocompounds have been shown to affect its binding to DNA recognition sites. The binding of AP-1 to its DNA recognition site was shown to be inhibited by selenodiglutathione and selenite in nuclear extracts of 3B6 lymphocytes, and selenite also inhibited AP-1 activation in vivo [16]. Selenodiglutathione was

about ten times more effective than selenite in inhibition of AP-1 DNA binding in nuclear extracts from 3B6 lymphocytes. The inhibition of AP-1 DNA binding by selenite in vitro was prevented by the presence of the reducing agent, dithiothreitol, indicating that reducing conditions will affect the binding of this transcription factor [17]. In another study, selenite was also reported to inhibit AP-1 binding in vitro, as well as repress the expression of a reporter gene in MCF-7 cells [18]. Differences in the effects of various selenocompounds have also been observed. As an example, an inhibition of AP-1-oligonucleotide binding by selenite was observed but no inhibition were observed in the binding by p-XSC in vitro using HCT-116 cells [19]. This difference between the effects of selenite and p-XSC suggests the occurrence of interesting qualitative or quantitative differences in the binding of the two selenium compounds to the subunit components of AP-1.

To identify the proteins that bind to the Sp1 and the TFII-1 sites, we conducted supershift assays with anti-Sp1 and anti-TFII-1 antibodies. The super-shift assay exploits the specificity of antibody-antigen reactions and so allows experiments to be done with crude extracts in place of purified proteins to identify the protein that is involved in binding with the consensus sequence even in the presence of other proteins. The appearance of a super-shifted band in the assay in which the anti-Sp1 antibody was used demonstrated the

presence of Sp1 transcription factor in the rat glial cell nuclear extract which binds the Sp1 site.

It is therefore not surprising that in this study we found Sp1 to bind the SeW promoter since iodothyronine deiodinase and SeW are both selenoproteins and also SeW is proposed to be an antioxidant system. Also because Sp1 is a ubiquitously expressed transcriptional factor and involved in many aspects of eukaryotic gene regulation [20], the binding of Sp1 to the proximal upstream region of the SeW is speculated to play a role in the expression of SeW, suggesting the presence of SeW in a wide variety of cells.

We established that Sp1 binds to the MRE in the rat SeW promoter which is consistent with work done by Ogra et al [21] where they demonstrated that Sp1 recognizes and binds the GC-rich sequence of some MREs and negatively regulates gene expression. Whether the binding of Sp1 to the SeW promoter may be required in the transcriptional regulation of SeW gene remains to be determined. We conclude that Sp1 binds to the MRE in the promoter of SeW and may compete with MTF-1 for the MRE. The absence of a supershifted band in the assay with anti-TFII-1 antibody and TFII-1 site oligonucleotide suggests that this site may be a target for multiple transcription factors.

## Acknowledgements

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# Chapter 4

## Conclusion

The results in this study show that copper and zinc exert a stimulatory effect on rat SeW promoter activity in the C6 cells but not the L8 cells and that this response is dependent on the intact MRE sequence. Lack of response in the L8 cells to metal exposure cannot be explained by the data in this study. Also we conclude that nucleotide substitution in the MRE sequence will lead to a complete loss in promoter response to metal ion exposure and thus the intact MRE is necessary for activation of the SeW promoter by metals. Whether this observed promoter response to copper and zinc has any effect on the expression of SeW in the glial cells remains to be determined. In addition to a MRE in the promoter of SeW, a copper signaling response element (CuSE) was found. Therefore, we propose that the presence of both elements in the SeW promoter is the reason the C6 cells were more resistant to copper than zinc.

The appearance of a super-shifted band in the assay in which the anti-Sp1 antibody demonstrated that the rat glial cell nuclear extract contains the Sp1 transcription factor and that this protein is capable of binding the Sp1 site oligonucleotide in a specific manner. We established that Sp1 is capable of binding to the MRE in the rat SeW promoter. Whether this binding will

influence SeW gene expression in response to metals such as zinc is not known. There was no supershifted band in the assay with anti-TFII-1 antibody and TFII-1 site oligonucleotide suggesting that this site may be a target for multiple transcription factors. Based on our data, the MRE, GRE, AP-1 and LF-A1 sites do not engage in any specific interaction. Whether the non-specific binding of proteins to these sites has any effect on the SeW cannot be determined from the present data. Even though the MRE did not engage in specific DNA/protein interaction, it can be concluded that this site may play a role in SeW promoter activity since a disruption of this sequence led to a complete loss of the observed metal response in the presence of zinc and copper.

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APPENDICES

# APPENDIX 1

## Antimutagenic Activity of Selenium-Enriched Green Tea Towards the Heterocyclic Amine 2-Amino-3-methylimidazo[4,5-f]quinoline

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## Abstract

Both selenium and green tea have been reported to exhibit antigenotoxic and cancer chemopreventive properties. We compared the antimutagenic activities of regular green tea and selenium-enriched green tea obtained from Hubei Province, China, towards the heterocyclic amine, 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) in the Salmonella assay. Selenium-enriched green tea obtained by foliar application of selenite exhibited concentration-dependent inhibition of IQ-induced mutagenesis in the presence of S9, and was significantly more effective than regular green tea tested under the same conditions. Analytical studies revealed no major differences in the polyphenol or caffeine content between regular green tea and selenium-enriched green tea, but the latter tea contained approximately 60-fold higher concentrations of selenium compared with regular green tea. The only soluble form of selenium was identified as selenite. The antimutagenic effects of certain individual tea constituents, such as epicatechin gallate and catechin, were enhanced by the addition of selenite to the Salmonella assay. Sodium selenite, sodium selenate, seleno-DL-cysteine, seleno-L-methionine, and L-Se-methylselenocysteine-HCL were not antimutagenic towards IQ when tested alone, but augmented significantly the inhibitory potency of green tea.

The results suggested an enhancing ("co-antimutagenic") effect of selenium in combination with green tea in vitro, but in vivo studies are needed to assess whether there is a synergistic effect of tea and selenium to protect against heterocyclic amine-induced mutagenesis and carcinogenesis.

**Index Entries:** tea polyphenols; heterocyclic amines; selenium; caffeine; Salmonella assay.

**Introduction**

There is growing interest in the possible health benefits of tea consumption, based on evidence from epidemiological and animal studies indicating that tea or constituents of tea might afford protection against the development of various chronic diseases, including cardiovascular disease and cancer (1-3). Some reports show that tea inhibits against carcinogens and mutagens from the human diet, including the heterocyclic amines produced in meat and fish during normal cooking procedures (4-7). Heterocyclic amines such as 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) produce tumors in various target organs of the rat, including the colon (8). Recent investigations showed that white tea, green tea, and black tea reduced the formation of colonic aberrant crypt foci (ACF) and DNA adducts in rats given heterocyclic amines, via changes in carcinogen bioactivation and detoxification pathways (4,9). The bioactivation of IQ, as with other heterocyclic amines, occurs primarily via hepatic cytochrome P4501A2, initially yielding 2-hydroxyamino-3-methylimidazo[4,5-*f*]quinoline (N-hydroxy-IQ). Sulfotransferases and acetyltransferases play an important role in subsequent steps that generate the aryl nitrenium ultimate carcinogen (10). Detoxification pathways involving UDP-glucuronosyltransferase and sulfotransferase isozymes have been identified, and glutathione S-transferases may protect by converting reactive N-acetoxy-intermediates back to the parent compound (11, 12). Tea is shown to induce these conjugation pathways, leading to enhanced glucuronidation

and greater excretion of detoxified metabolites in the urine (4, 9). In addition, the mutagenic activities of N-hydroxylated heterocyclic amines are inhibited directly by tea constituents under aqueous conditions in the absence of exogenous enzyme systems (13-15).

In the course of investigating the antimutagenic properties of different varieties of tea against IQ and other mutagens, we became interested in a selenium-enriched green tea produced in the Hubei province of China. Several studies have linked selenium with cancer chemoprevention. The incidence rates for lung, breast, peritoneum and GI tract cancers, and deaths from lymphoma, were lower in people residing in areas of the US with forage crops rich in selenium compared with those living in areas of low-forage selenium (16). In a prospective study of selenium status and cancer, it was observed that initial plasma selenium concentrations were inversely related to subsequent risk of colonic adenomatous polyps and non-melanoma skin cancer; patients with plasma selenium levels less than the population median (128 ng/ml) were four times more likely to develop one or more polyps and had more than three times the number of polyps per patient (17). A 10-year clinical trial by Clark et al. (18) showed the relative risk for cancer of colon, lung, and prostate to be reduced by 50% in patients taking an oral supplement of selenized brewer's yeast (200µg Se/day).

In animal studies, Se-methylselenocysteine is shown to be the most effective selenocompound against carcinogen-induced mammary tumors in the rat (19)

whereas selenite and selenate, but not selenomethionine, inhibited 3, 2'-dimethyl-4-aminobiphenyl-induced colonic ACF in the rat (20). These findings suggest that the chemical form of selenium may be an important consideration for chemoprevention. In related studies, selenomethionine inhibited azoxymethane (AOM)-induced ACF (21) but not AOM-induced colon tumors in the rat (22), and the synthetic compound 1, 4-phenylene bis(methylene)selenocyanate inhibited the development of tumors in the small intestine and colon of the APC[min] mouse (23). A recent study also described the antigenotoxic properties of selenium in the Drosophila Wing Spot test (24). These results suggest that various selenocompounds have varied effects in different tumor models. Since regular green tea and selenium have been shown to independently reduce tumors incidence, it was of interest to determine whether there is a possible synergistic effect of these against mutagens. This approach was used and selenium-enriched green tea was found to be more effective than regular green tea against IQ-induced mutagenesis in the Salmonella assay. A preliminary report of this work has been presented previously (25).

## Materials and Methods

## Chemicals

The tea standards (+)-catechin, epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechin-3-gallate (ECG), epigallocatechin-3-gallate (EGCG), gallic acid, theobromine, and caffeine, as well as the selenium compounds sodium selenite, sodium selenate, seleno-DL-cysteine, seleno-L-methionine, and L-Se-methylselenocysteine-HCL were purchased from Sigma Chemical Co. (St. Louis, MO). IQ was procured from the National Cancer Institute Carcinogen Repository (Kansas City, MO). For the chemical structures of IQ, N-hydroxy-IQ, and of several major tea constituents the reader is referred to Hernaez et al. (14). Reagents and media for the mutagenicity assays were obtained from sources previously described (7, 14, 15).

## Teas

The two green teas were grown in Jianshi County, Enshi area of Hubei province, China. Tea was sprayed with selenite at concentration of 0.05 μg selenium per ml two weeks before harvest to obtain selenium enriched green tea. The tea was processed with a 10 second pass at 240-280°C, then kept at 100°C for 30-60 minutes, baked in a wok at 100°C for 30-60 minutes, and dried at 60-80°C for one hour to reduce the moisture content to below 5%. Unless stated otherwise, both teas were brewed fresh before each experiment at a concentration of 1 g leaves per 100 ml hot water (1% w/v). The tea was brewed for 5 minutes and passed through a 0.20μm filter prior to subsequent antimutagenesis and analytical studies.

**Analytical studies (polyphenol content)**

Tea components were separated by high-performance liquid chromatography (HPLC) using a Shimadzu VP series instrument equipped with a 25cm X 4.6mm, 5μm particle size, reverse phase column (Supelcosil™ LC-18). The mobile phase was composed of methanol (buffer A) and water (buffer B). Chloroacetic acid was added to both solvents at a final concentration of 0.3% and adjusted to pH 4.5. The gradient program used for the separation of the tea compounds was as follows: 10% buffer A at 0 min, increased linearly to 40% buffer A at 50 min and returned to 10% buffer A at 60 min. The flow rate was 1 ml/min and detection was at 273 nm. The major tea compounds were identified by LC-MS and by co-elution with authentic standards, as reported elsewhere (7, 9).

**Selenium Analysis and Speciation**

After digestion with perchloric and nitric acids, aqueous extracts of tea were subjected to selenium analysis using a fluorimetric method for total selenium (26). In addition, sodium selenite, sodium selenate, seleno-L-methionine, seleno-DL-cysteine, and L-Se-methylselenocysteine standards were used to assist in the identification of chemical species. Separation was performed by reverse phase HPLC using a 15 cm 5 μm-particle Symmetry Shield RP8 Waters column. The chromatogram was obtained using Se-82 specific element detection by interfaced ICPMS (27). The total amount of selenium in the aqueous extracts, starting with one gram of tea in 100 ml hot water (1% w/v), was 1.1 μg and 60.3 μg respectively for regular green tea and selenium-enriched green tea. In addition to direct testing of the teas in the Salmonella assay, some experiments used tea preparations or solutions of individual polyphenols "spiked" with selenium.

**Salmonella assays**

All of the experiments were performed under subdued lighting with IQ in the presence of an exogenous metabolizing system (aroclor-induced rat liver S9), and used Salmonella typhimurium strain TA98, kindly provided by Dr. B. N. Ames (University of California, Berkley, CA). Strain TA98 and IQ were chosen because they are the most used in routine studies of this type. Also IQ is one of the heterocyclic amines produced during the normal cooking process of meat and fish (4-7). Each assay involved a 20-minute pre-incubation of bacteria (0.2 ml), mutagen (6-10 ng IQ in 0.01 ml DMSO), and inhibitor (typically 0.01-0.2 ml) with aroclor-induced rat liver S9 (0.2 ml of 10% v/v S9 mix) at 37°C, followed by the addition of 2 ml soft agar and transfer onto minimal glucose plates. His$^+$ revertant colonies were scored after 2 days of incubation at 37°C in the dark. Plates were carefully examined for signs of possible toxicity, such as thinning of the background lawn, or reduction of spontaneous counts. In some cases, smaller colonies were picked from the plate and confirmed to be His$^+$ revertants by streaking on plates lacking added histidine. None of the treatments reported here were toxic to the bacteria. Experiments were repeated at least twice, using triplicate plates for each dose, and included appropriate vehicle controls (DMSO but no mutagen, plus and minus tea and/or selenium).

## Statistical analysis

In some experiments with selenium and tea constituents, the data were analyzed

for statistical significance by one-way analysis of variance (ANOVA) using SAS

v.8.

**Results**

**Analytical studies**

Several chemical forms of selenium were spiked into tea and the retention times were determined by HPLC (Figure 1). The order of elution and retention times (Rt) were as follows: 1, selenate (1.1 min); 2, selenite (1.2 min); 3, seleno-DL-cysteine (1.6 min); 4, Se-methylseleno-DL-cysteine (2.2 min); 5, seleno-DL-methionine (5.9 min); 6, Se-allyl-seleno-DL-cysteine (8.1 min); 7a, cis-Se-1-propenyl-seleno-DL-cysteine (11.1 min); 7b, trans-Se-1-propenyl-seleno-DL-cysteine (12.2 min); 8, Se-propyl-seleno-DL-cysteine (14.8 min); 9, DL-Selenoethionine (15.7 min). By selenium analysis (Se-82 counts/sec), a single peak was observed in selenium-enriched tea from China (inset, Figure 1); this peak, which corresponded to selenite, was not detected at appreciable levels in regular green tea (not shown).
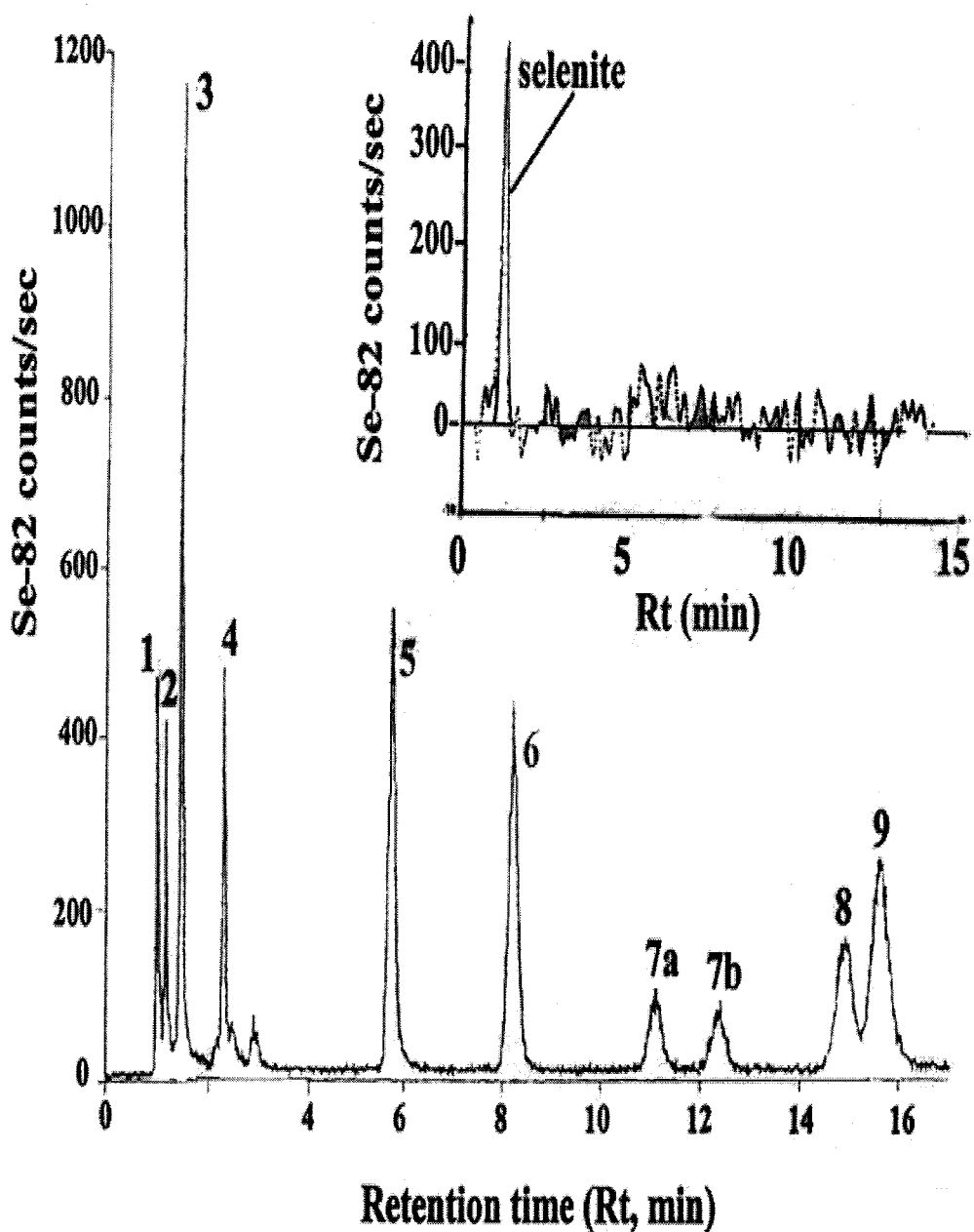
Figure 1. Chromatography of selenium standards after spiking into green tea, and identification of selenite as the major selenium species in a high-selenium tea (inset). Chemicals forms of selenium were separated using heptafluorobutyric acid as an ion-pairing agent in a reverse phase HPLC mobile phase. For identification of peaks 1-9, see text.

The HPLC profiles of the two teas were compared at 273 nm in order to identify possible differences in the major polyphenols, caffeine, and other constituents (Figure 2). Selenium-enriched green tea and regular green tea had essentially identical profiles; the two major peaks were identified as caffeine and EGCG, with additional peaks corresponding to gallic acid, theobromine, theophylline, catechin, EC, and ECG.
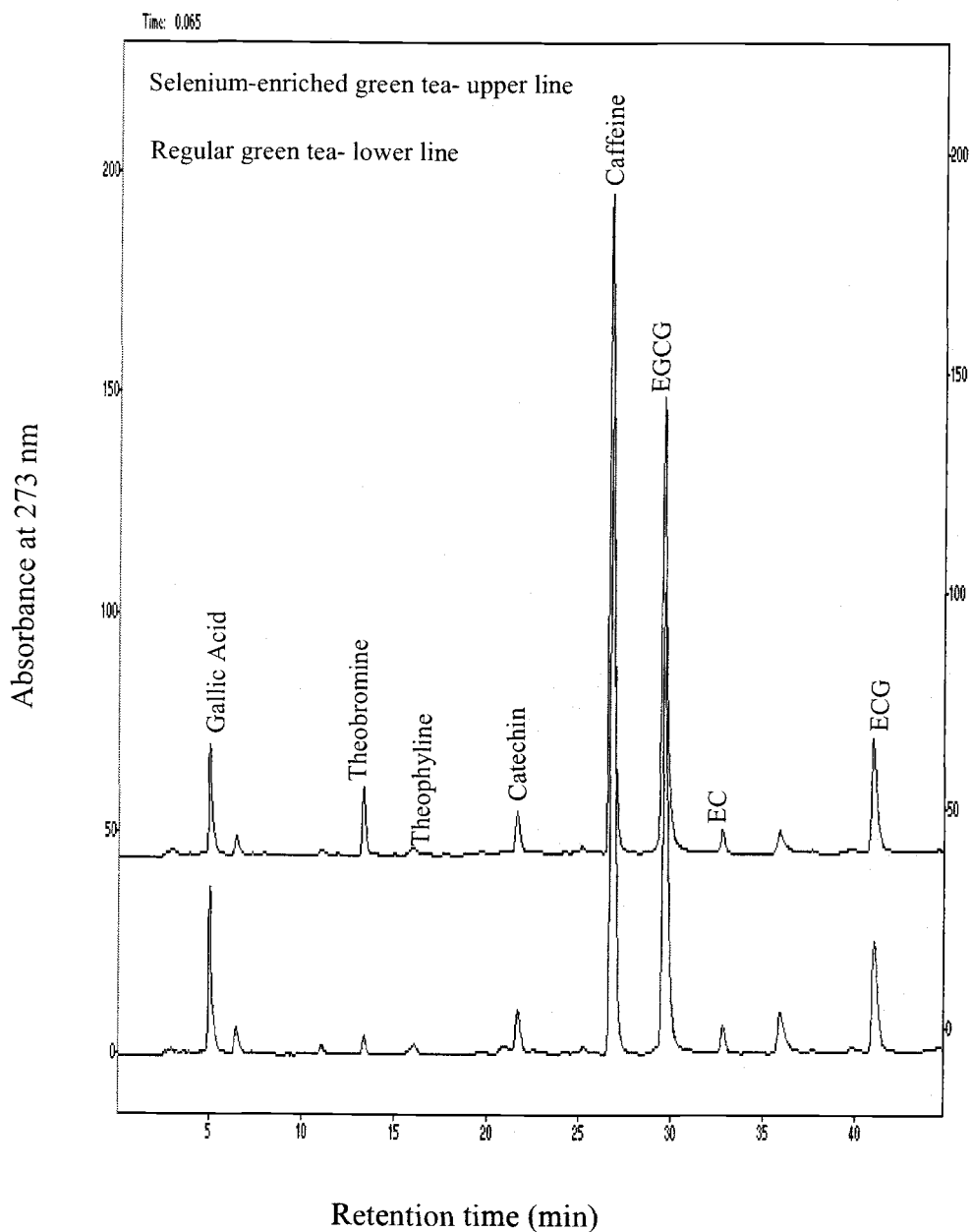
Figure 2. Comparison of selenium-enriched green tea and regular green tea, showing similar levels of the major constituents detected at 273 nm. For HPLC conditions, see text.

**Antimutagenic activities of regular and selenium-enriched green teas**

In preliminary experiments, teas were brewed at concentrations in the range 0.001% to 0.5% (w/v) for 5 minutes, and equivalent volumes (0.2 ml) were tested in the Salmonella assay (Figure 3). Slight enhancement of IQ-induced mutagenicity was detected at the lowest tea concentrations, but at higher concentrations the selenium-enriched green tea inhibited significantly, without evidence of toxicity to the bacteria. When tested under the same conditions, regular green tea was much less effective at all concentrations in the assay; at the highest concentration in this experiment there was 70% inhibition by selenium-enriched tea versus 35% inhibition by regular tea (P<0.05).
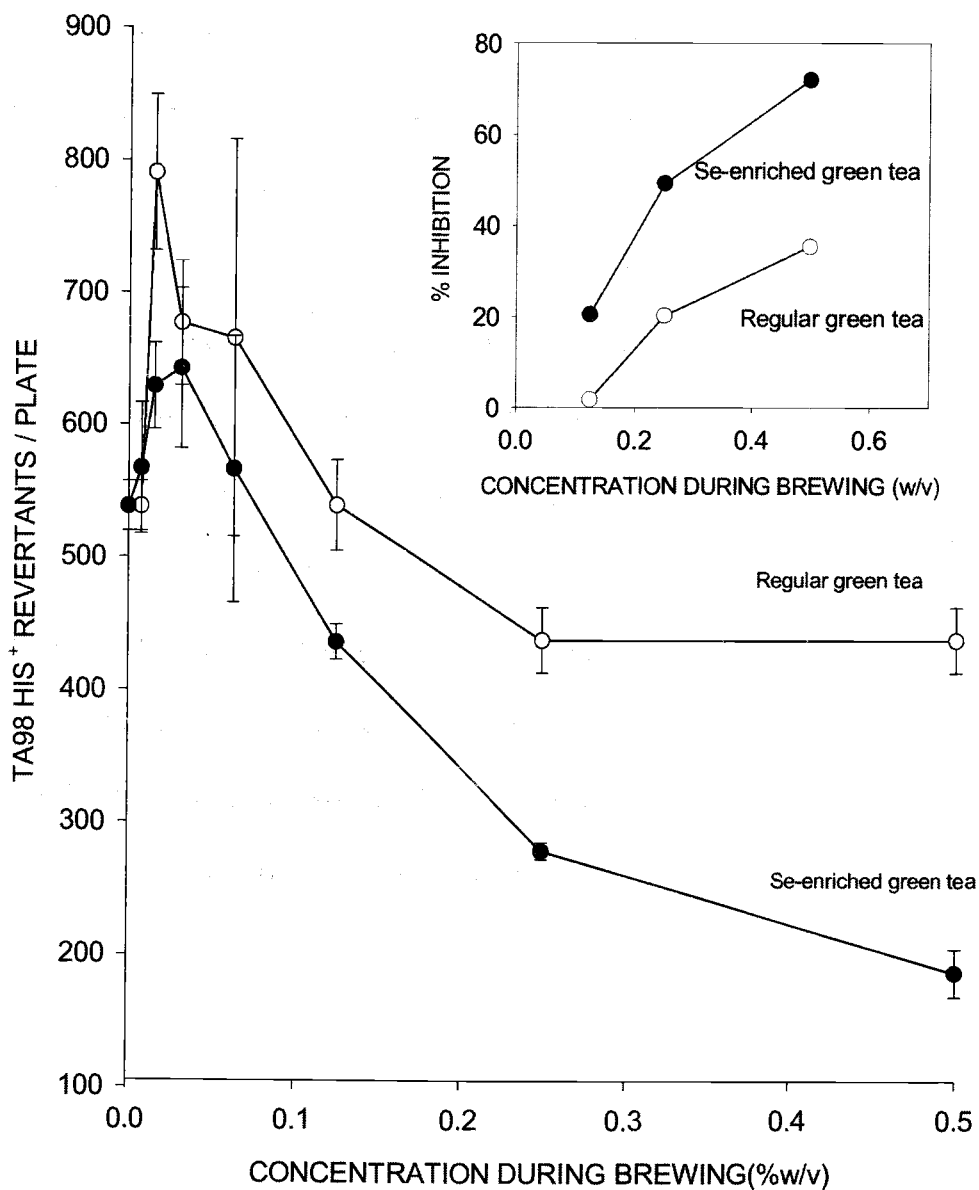
Figure 3. Antimutagenic activities of regular green tea (open symbols) and selenium-enriched green tea (closed symbols) towards IQ in the Salmonella mutagenicity assay. Data are actual counts (mean±SD) for triplicate plates at each dose tested. Concurrent tests with solvent alone (DMSO but no mutagen) provided no evidence of toxicity, based on the criteria stated in the results.

Because selenite was identified in the selenium-enriched green tea (Figure 1), further tests were conducted with regular green tea with additions of sodium selenite. Thus, tea was first brewed at concentrations in the range 0.0625%-1.00% (w/v) for 5 minutes and selenite added to the aqueous extracts to obtain the equivalent amount of selenium as detected in the selenium-enriched green tea. The inhibitory activities of the addition of selenium to tea extracts toward IQ were compared with those of regular green tea extracts, selenium-enriched green tea, and selenite alone. Selenite alone exhibited some degree of antimutagenic activity (Figure 4, open triangles); however, at concentrations in the assay in which selenite was minimally inhibitory, selenium-enriched green tea was highly effective against IQ, and more potent than either the addition of selenite to regular tea or regular green tea alone. At higher concentrations, regular tea, selenium-enriched green tea, and tea plus selenite were equally effective against IQ.
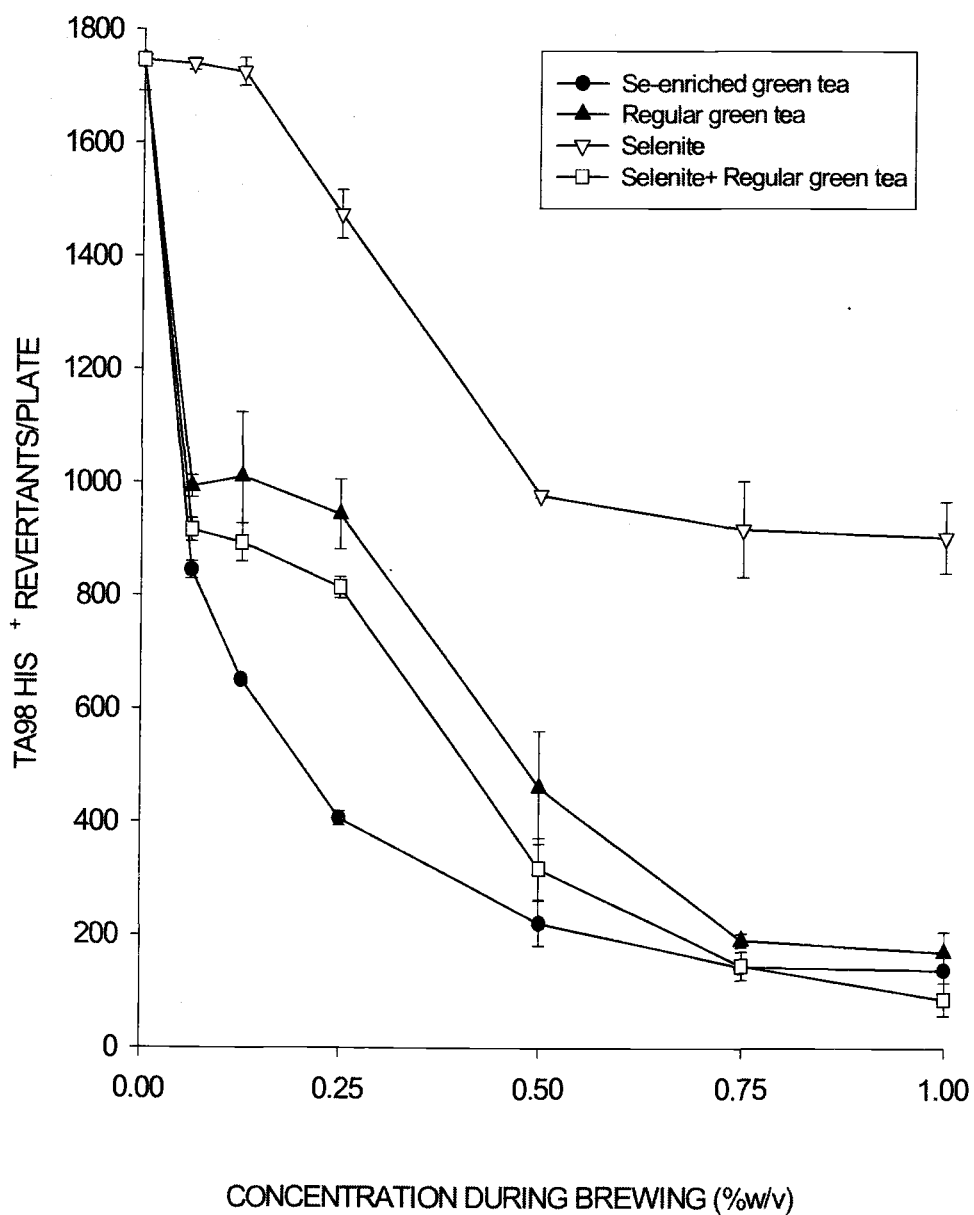
Figure 4. Antimutagenic activity of sodium selenite, green tea, green tea plus selenite, and selenium-enriched green tea against IQ in the Salmonella assay. Tests were performed as described in Figure 3 legend, but with 8 ng IQ/plate, and brew concentrations covered a broader range, up to 1% (w/v). Each data point represents mean±SD, $n$=3.

## Individual tea constituents combined with selenite

To determine whether selenite might act synergistically with individual tea constituents, catechin, EGC, ECG, EGCG, gallic acid, theobromine and caffeine were mixed with selenite and added to the Salmonella assays containing IQ plus rat liver S9 (Table I).

| Tea component | Selenium | % Inhibition | Potency |
|---|---|---|---|
| EGCG | - | 96.9 ± 0.5 | |
| | + | 96.8 ± 0.5 | 1 |
| ECG | - | 70.1 ± 1.5* | |
| | + | 93.7 ± 1.7* | 2 |
| Catechin | - | 58.4 ± 0.7* | |
| | + | 89.9 ± 0.8* | 3 |
| EGC | - | 41.6 ± 4.0 | |
| | + | 42.9 ± 1.3 | 4 |
| Theobromine | - | 15.0 ± 8.2* | |
| | + | 23.4 ± 1.1* | 5 |
| Gallic acid | - | 13.2 ± 4.0* | |
| | + | 39.2 ± 3.0* | 6 |

* $P < 0.05$

Table1. Antimutagenic activity of selenite supplemented individual tea components towards IQ in the Salmonella mutagenicity assay. The tea components (pure standards) and selenite were tested at concentrations in selenium-enriched green tea, as calculated from the HPLC analysis and selenium analysis. EGCG, ECG and EGC represent respectively epigallocatechin-3-gallate, epicatechin-3-gallate and epigallocatechin. Order of potency as shown in table is due to inhibition by tea component without selenium supplementation.

The dose of each tea standard added to the assay was calculated to be equivalent to that present in 0.2 ml tea extract (1% brew concentration, 5 min brew time), based on peak area integration of the data in Figure 3. When tested against IQ in the presence of S9, certain tea constituents were highly effective as antimutagens (EGCG, ECG), some were intermediate (EGC, catechin, caffeine), and others were weak inhibitors (theobromine, gallic acid). From the quantification of selenite in selenium-enriched green tea (Fig. 1) and the data in Fig. 4, the dose of selenite was chosen to give ~25% inhibition of IQ-induced mutagenicity (Table I); significantly greater antimutagenic activities were detected for ECG, catechin, theobromine, and gallic acid after the addition of selenite, and in each case the result was apparently additive (Table I). For example, the data for selenite, ECG, and selenite plus ECG were calculated to be 25.8%, 70.1% and 93.7% inhibition, respectively, the latter value being close to the predicted 95.9% inhibition for additive effects. It is noteworthy that EGCG was highly effective against IQ in the presence or absence of added selenium (96.8% and 96.9% inhibition, respectively).

## Antimutagenic activity of various selenium compounds

To determine whether greater than additive effects might be obtained against IQ, the antimutagenic potencies of several chemical forms of selenium, alone and in combination with regular green tea, were tested in the Salmonella assay (Figure 5).
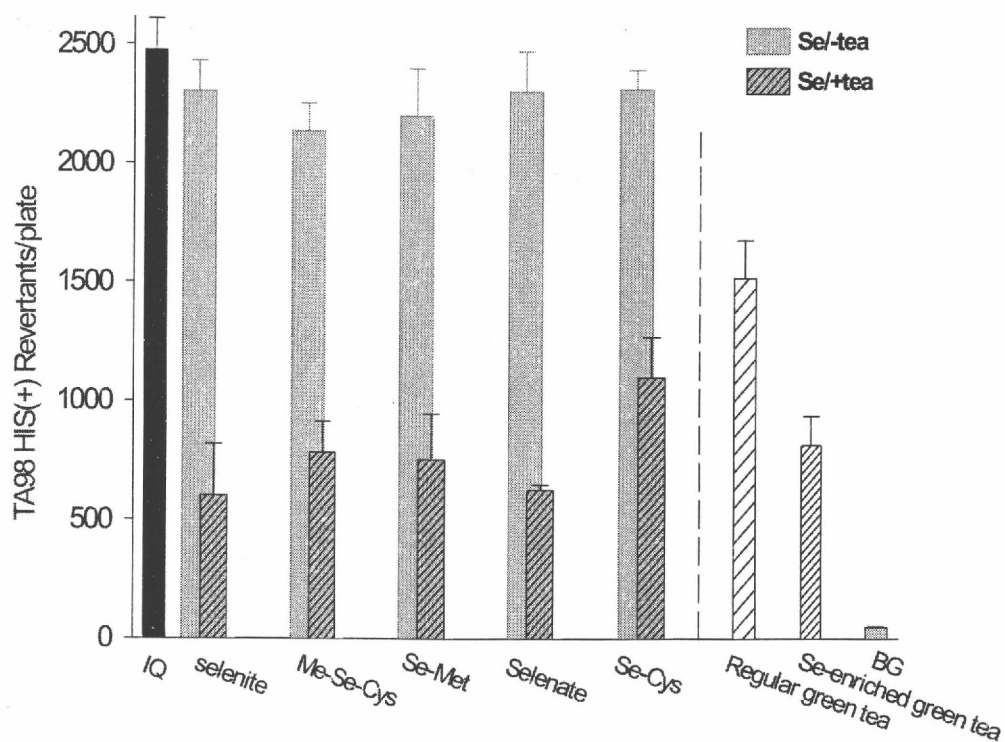
Figure 5. Antimutagenic activities of different forms of selenium in the presence and absence of selenium-enriched green tea. Preincubation tests were performed as described in Figure 4 legend, using 0.2 ml of tea brewed at a concentration of 1% (w/v). Each bar indicates the mean±SD of triplicate plates. Statistical comparisons were made by ANOVA (SAS v.8.0); bars sharing the same superscript are not significantly different, whereas those with different superscripts differ significantly (*P*<0.05). seleno-L-methionine (Se-Met), seleno-DL-cysteine (Se-Cys), L-Se-ethylselenocysteine (Me-Se-Cys), Background (BG) represents DMSO controls with no inhibitor.

By design, the dose of each chemical form of selenium (0.03 µg/plate) was chosen to provide ~10% inhibition against IQ (compare gray bars in Figure 5), thus giving a wide range in which to detect additive versus synergistic effects after the addition of tea to the assay. In this experiment, regular green tea (0.2 ml of 0.25% w/v) inhibited by 40% and selenium enriched green tea from China inhibited by 63%, corresponding favorably with data shown previously (Figure 1). When each of the selenium compounds was added to regular tea, there was a significant enhancement of the antimutagenic potency, comparable to that detected with the selenium-enriched green tea. By statistical analysis, none of the selenium compounds was significantly more effective than another in augmenting the inhibitory potency of regular tea. The results in Figure 5 suggested that equivalent activity was obtained with organic and inorganic selenium compounds (compare gray-hatched bars), and brought the inhibitory potency of regular tea (white bar) to that of the selenium-enriched green tea from China (sliced bar). With the exception of seleno-DL-cysteine (Se-Cys), all other selenium compounds provided greater than additive inhibition against IQ. However, a thorough analysis of the full dose-response curves will be necessary in order to determine whether the effects were truly synergistic. Tests conducted by identical conditions, but using DMSO solvent without mutagen, gave revertant counts that were within the range of background (BG, 50.3±4.9, n=3), with normal appearance of the background lawn of growth (data not shown).

**Discussion**

The results of this study show that green tea from Hubei Province, China, marketed as a selenium-enriched variety inhibited the mutagenic activity of IQ more effectively than regular green tea in the Salmonella assay (Figure 3). The greater inhibitory potency was not associated with higher levels of polyphenols, or other constituents found typically in green tea, since the HPLC analyses revealed no marked differences in polyphenol content between the teas when monitored at 273 nm; the theobromine peak of the selenium-enriched green tea appeared to be elevated as shown in figure 2. Tea constituents were tested individually, without selenium, at concentrations equivalent to those in assays containing the complete tea, and EGCG was most effective, inhibiting the mutagenic activity of IQ by >95%. These results extend earlier work (13, 14), using tea constituents against N-hydroxylated heterocyclic amines in the absence of rat liver S9. In the present study, addition of selenite to the assay augmented the inhibitory activities of certain tea constituents, namely ECG, catechin, theobromine, and gallic acid (Table I).

We obtained the green tea sample expecting it to have been grown on soils containing high levels of this element and became suspicious when selenite was the only detectable form of selenium and when only 10-15% of the total selenium could be extracted from the tea under typical brewing conditions using hot water. After presenting this data to the Chinese workers, they admitted that selenite was

sprayed on the tea. We subsequently added either selenite or selenate to regular green tea and found that only 10-15% of the selenium as selenite could be extracted with hot water whereas 85-90% of the selenium could be extracted when selenate was added. Therefore, it is suggested that selenate instead of selenite should be used for foliar application of selenium to green tea. Plans are to determine the resultant chemical form of selenium when selenate is applied to green tea.

We suggest that selenite is reduced to an insoluble form, such as elemental selenium, by some tea components. It is known that selenite can be reduced by such mild reducing agents as ascorbic acid (28), and although some of the selenium may have been rendered insoluble, the selenium-enriched green tea was highly effective against IQ in the presence of rat liver S9 (Figures 3-5). Additional experiments will be necessary to evaluate the fate of intact selenite versus insoluble form(s), and assess the effect of tea components on selenite reduction.

Our experiments show that all forms of selenium are effective in augmenting the inhibitory activity of regular green tea but by themselves fail to inhibit the mutagenic activtity of IQ as shown in figure 5. A direct interaction is hypothesized (complex formation) between the mutagen and certain tea polyphenols (24) could be facilitated via 'selenium bridges' with some forms of selenium and not with others. However, results in Figure 5 did not support a more

potent action by one form of selenium over another, and we were unable to demonstrate enhanced complex formation between EGCG and IQ (or N-hydroxy-IQ) in the presence and absence of added selenite (data not presented). It would be desirable to be able to screen selenocompounds for their effectiveness as antitumorigenic agents by an in vitro method, but the present data indicate that this cannot be done by the Salmonella assay. No changes were detected upon addition of selenite to suspensions of rat liver microsomes, as might occur in the case of interaction with heme or with cytochrome P450 (data not shown). Additional mechanisms of antimutagenesis remain to be investigated, including enhanced degradation of activated mutagens, and, possibly, bioantimutagenic properties associated with augmented DNA repair.

Enrichment of plants such as broccoli, garlic, onions, and wild leeks by growth on high-selenium soils results in the production of Se-methylselenocysteine as the major selenocompound (29, 30). Se-methylselenocysteine is the most effective selenocompound identified thus far against mammary gland tumor development (19). Other forms of selenium including selenite, selenate, and the synthetic compound 1,4-phenylene bis(methylene)selenocyanate are also shown to be inhibitory against intestinal and colorectal carcinogenesis in the rodent (20, 23). The latter findings are noteworthy in light of the fact that IQ and other heterocyclic amines produce tumors in the small intestine and colon (8). Interestingly, a recent study found a reduction in carcinogen-induced colonic ACF

in the F344 rat that was a function of selenium in high selenium broccoli and not a result of broccoli alone or selenium alone (31, 32). These results along with others (19-22) point to the importance of the chemical form of selenium for chemoprevention in vivo, as well as the dietary context in which the selenium is ingested. Based on the results from the present investigation and others (4, 7, 9), it would be of interest to test various selenocompounds separately and together with green tea against IQ-induced ACF and intestinal tumorigenesis in vivo.

In conclusion, this study has shown that selenium-enriched green tea was more effective than regular green tea against IQ-induced mutagenicity in the Salmonella assay. Organic and inorganic forms of selenium were found to augment the antimutagenic properties of green tea to a similar extent, and selenite increased the inhibitory potencies of individual constituents of tea, such as ECG and (+)-catechin. These findings suggest that selenium compounds combine with tea constituents to protect against heterocyclic amine-induced mutagenesis, and additional studies appear to be warranted into their possible synergistic effects in vivo against ACF and tumor formation in the GI tract.

**Acknowledgements**

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# APPENDIX 2

## Treatment of Tea with Selenium

**Introduction**

Since the previous work indicated that selenium enriched tea would reduce the defects in the Ames test (Amantana et al, 2002), it was of interest to investigate the uptake of selenium by tea. Selenate was used because the prior work indicated that selenite was not the optimal from to use. The tea on Mt Jefferson farms located on Brown's Island Road, which is south of Salem, OR was used for this study.

**Methods**

The first study was conducted during July, 1999, and the second one in July, August and September, 2000. Four different clones, 9-1, 1-1, 2-2, and 1-2, were sprayed with selenium as sodium selenate (2.5grams/gallon water) which contained 15ml/gallon of the surfactant (Ti-700, Loveland Industries, Inc. Box 1289, Greeley, CO 80632) three times on 13, 16, 19, August, 1999. The reason selenate was used is because tea was shown to reduce selenite to insoluble forms (Amantana et al, 2002). During this time there was no rain fall. Samples of old and young leaves were collected initially and each time before the subsequent spraying. The final collection was done on August 23, 1999. The leaves were washed with distilled water (put water in plastic bags with the leaves and let set for 5 minutes) and the water decanted. This was repeated three times. The leaves were processed at 70°C for 12 hours and ground in a small Wiley mill for selenium analysis. Some of the leaves collected on the final collection period were not washed to determine how much selenium would be lost upon washing.

**Results**

The selenium content of the old and new tea leaves from the four clones is as shown in table II. There were no differences in the uptake of selenium between the four clones of tea and there was 1.1 to 1.8 fold uptake of selenium by the young leaves versus the old ones for an average of 46% greater uptake by the young leaves. There was an average of 28% loss of selenium upon washing the tea leaves with distilled water. The average uptake of selenium by leaves from the four combined clones of tea is shown in figure 1. This figure clearly shows that young leaves took up significantly ($p<0.05$) selenium than the old leaves. The final selenium content of young and old leaves was respectively 101.4±25.6 and 56± 5.4 micrograms per gram leaves.

| | Clone9-1 | | Clone 1-1 | | Clone 2-2 | | Clone 1-2 | |
|---|---|---|---|---|---|---|---|---|
| | Old | New | Old | New | Old | New | Old | New |
| Initial | 0.085 | 0.085 | 0.107 | 0.107 | 0.239 | 0.239 | 0.349 | 0.349 |
| Before 2nd | 8.7 | 33.2 | 10.6 | 28.0 | 8.5 | 19.0 | 12.0 | 20.9 |
| Before 3rd | 35.7 | 82.5 | 61.2 | 90.0 | 51.3 | 104.1 | 40.5 | 80.3 |
| Final | 54.2 | 77.4 | 66.5 | 129.3 | 37.4 | 102.7 | 46.3 | 96.1 |
| Not Washed | 72.9 | 121.5 | 66.1 | 135.6 | 81.7 | 128.7 | 93.3 | 173.6 |

Table II. Selenium content of old and new tea leaves from four clones of tea.
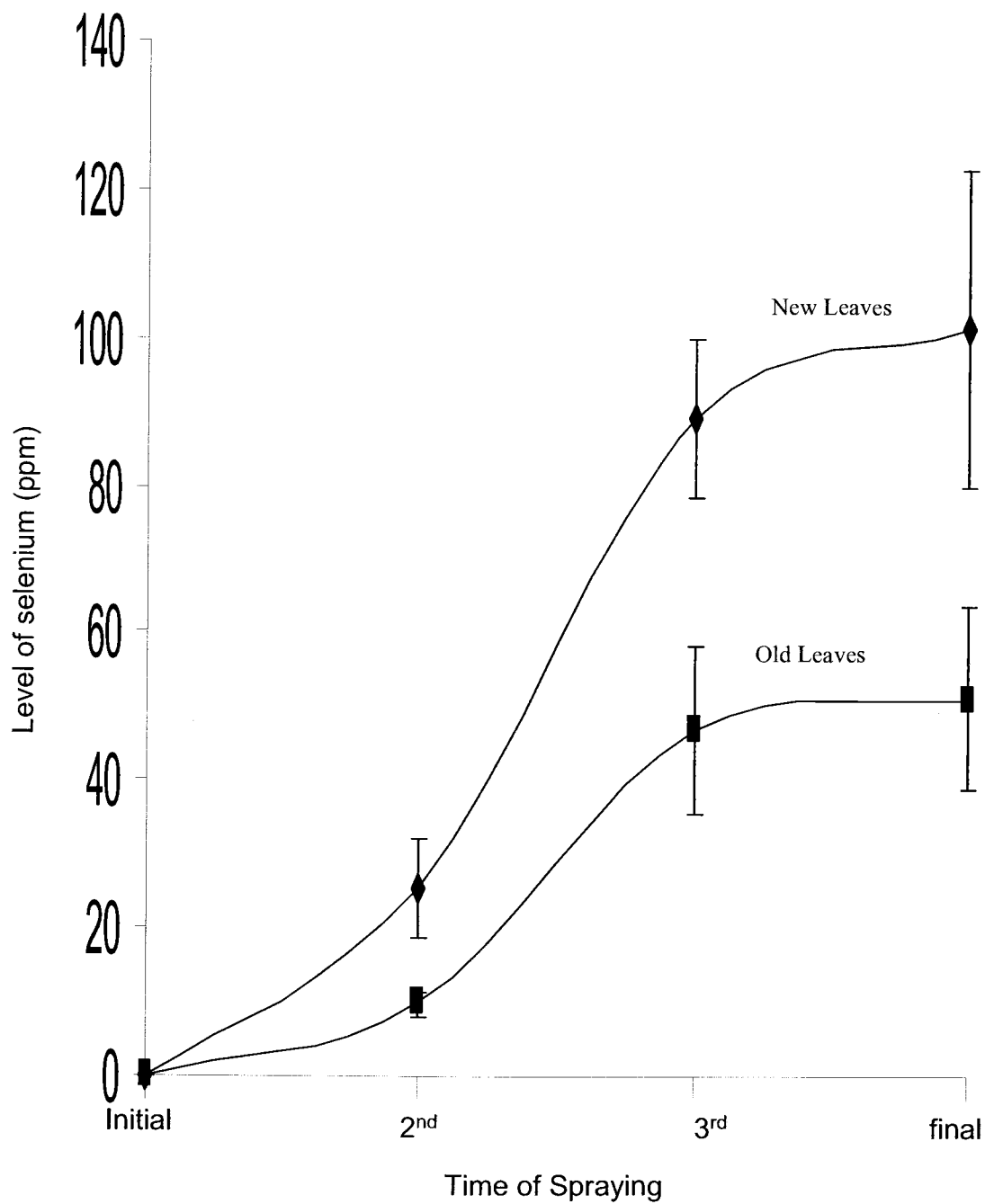
Figure 1. The average uptake of selenium of the four clones of tea. Each value represents the average and mean ± standard error of selenium uptake by the four clones of tea.

The selenium was extracted with hot distilled water (2.2 grams tea in 150 ml water) for 10 minutes. There was no significant difference in the extraction of selenium between the four clones. The percentage of selenium extracted was 85.0, 85.7 86.0 and 83.3 respectively for clones 2-2, 1-2, 9-1 and 1-1. Samples of the old and young leaves were taken from all four clones of tea that were treated the previous year to assess the carry over of selenium. The percentage of selenium remaining one year later (as compared to the final concentration after the third spraying) was respectively for old and young leaves for clones 2-2, 9-1, 1-1, and 1-2 was 12, 16; 42, 22; 49, 24 and 5, 8. It appears clones 9-1 and 1-1 retained more selenium than the other two clones, but no form conclusion can be drawn because only one sample was analyzed for each clone.

Since there was no significant differences in the uptake of selenium between the clones, clone 1-1 was used in the second experiment because there were more of this clone than the others. It was hoped that enough tea would be collected to conduct a rat experiment but this was not accomplished. This is because selenium enriched broccoli has been shown to reduce mammary and colon tumors in rats (Finley at al, 2001) and selenium enriched garlic was shown to reduce mammary tumors in rats (Ip et al, 1992). The tea was sprayed with selenium at the same concentration and surfactant as perfomed in the first experiment. The tea was sprayed on July 10, July 31 and August 20. The final

collection of tea was done on September 11, 2000. Old and young leaves were collected initially and before the subsequent sprayings. The leaves were also collected from untreated tea to serve as control in the rat experiment which was not conducted. The tea leaves were withered for 42 hours at room temperature ($25^{\circ}C$) and the leaves ground in a food grinder. This ground tea was placed as a thin layer on a screen and fermented for three hours at room temperature while covered with plastic wrap. This was stirred a few times during this time, and then dried at $68^{\circ}C$ for about 3 hours in force air oven. Consistent with the first experiment, the young leaves took up significantly more selenium than the old ones (figure 2). There was a gradual increase of selenium content with each spraying with this element. The final selenium content for old and young leaves was respectively 29.4 and 77.9 micrograms selenium per gram tea.
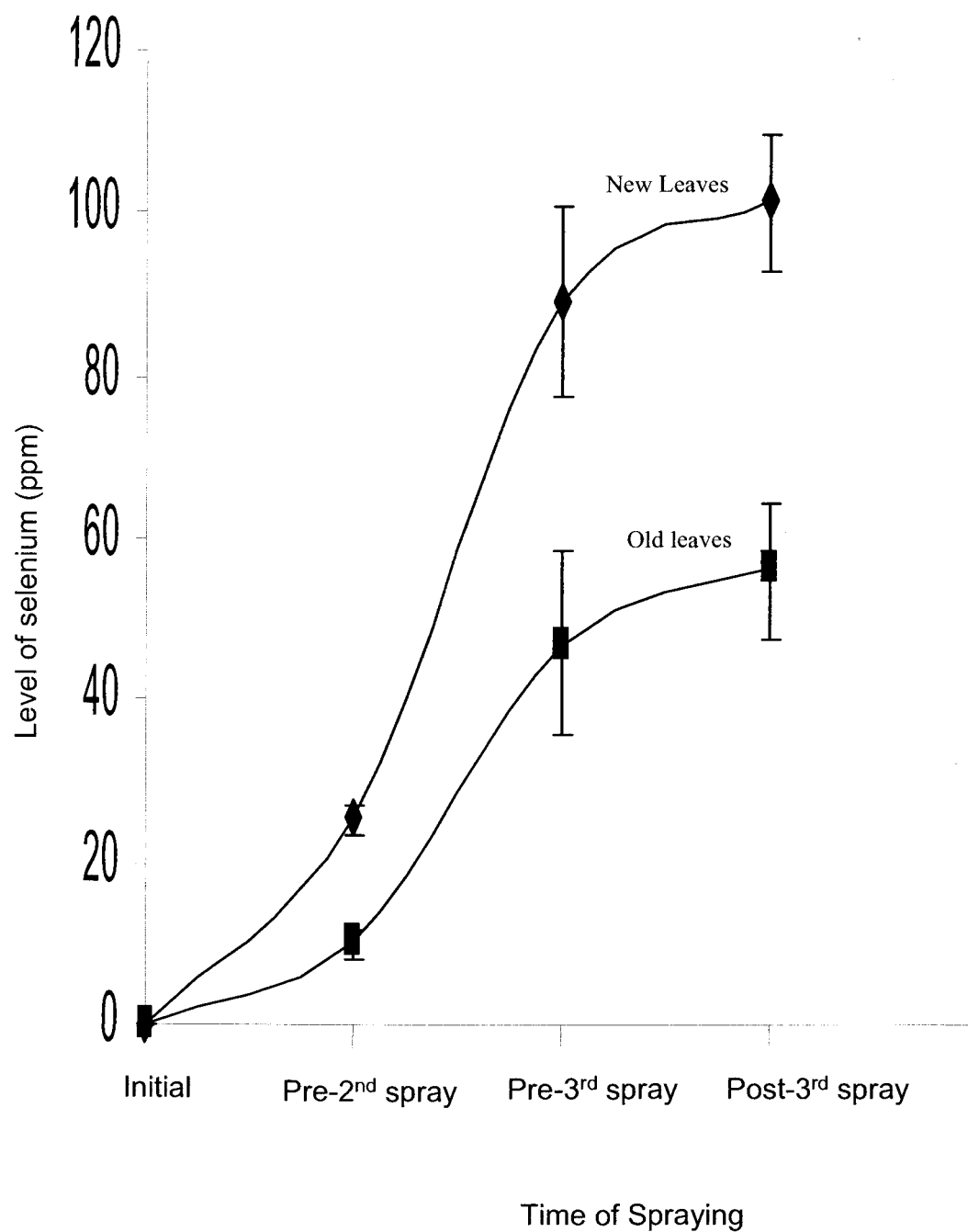
Figure 2. Uptake of selenium by tea clone 1-1 in the second experiment. Each value represents mean ± standard error of three determination of one sample at each time.

After all the samples were dried at 70°C and following digestion with nitric and perchloric acids, the selenium content was determined by a semi-automatic method (Beilstein and Whanger, 1986). Selenium speciation was conducted on hot water extract of tea by HPLC ICP-MS (Casiot et al, 1999). Tea preparation, from the second study was placed in boiling water (2.2grams/150ml water) for 10 minutes. The selenocompounds in the tea extract were separated by HPLC and the selenium peaks analyzed by ICP-MS. The elution patterns for Se-methyl selenocysteine (SeMCys), selenomethionine (Semet), selenite and selenate are shown in figure 3 and the chromatogram for the tea extract is shown in figure 4. Selenate is the predominant peak with a smaller peak for selenite. There are up to four unidentified peaks, but not detectable peaks were found for either SeMCys or Semet. This is in marked contrast to selenium enriched broccoli, garlic, onions, and wild leeks where SeMCys is the predominant selenocompound (Whanger, 2002). This preliminary analysis indicates that additional investigations on the selenocompounds in selenium enriched tea are needed.
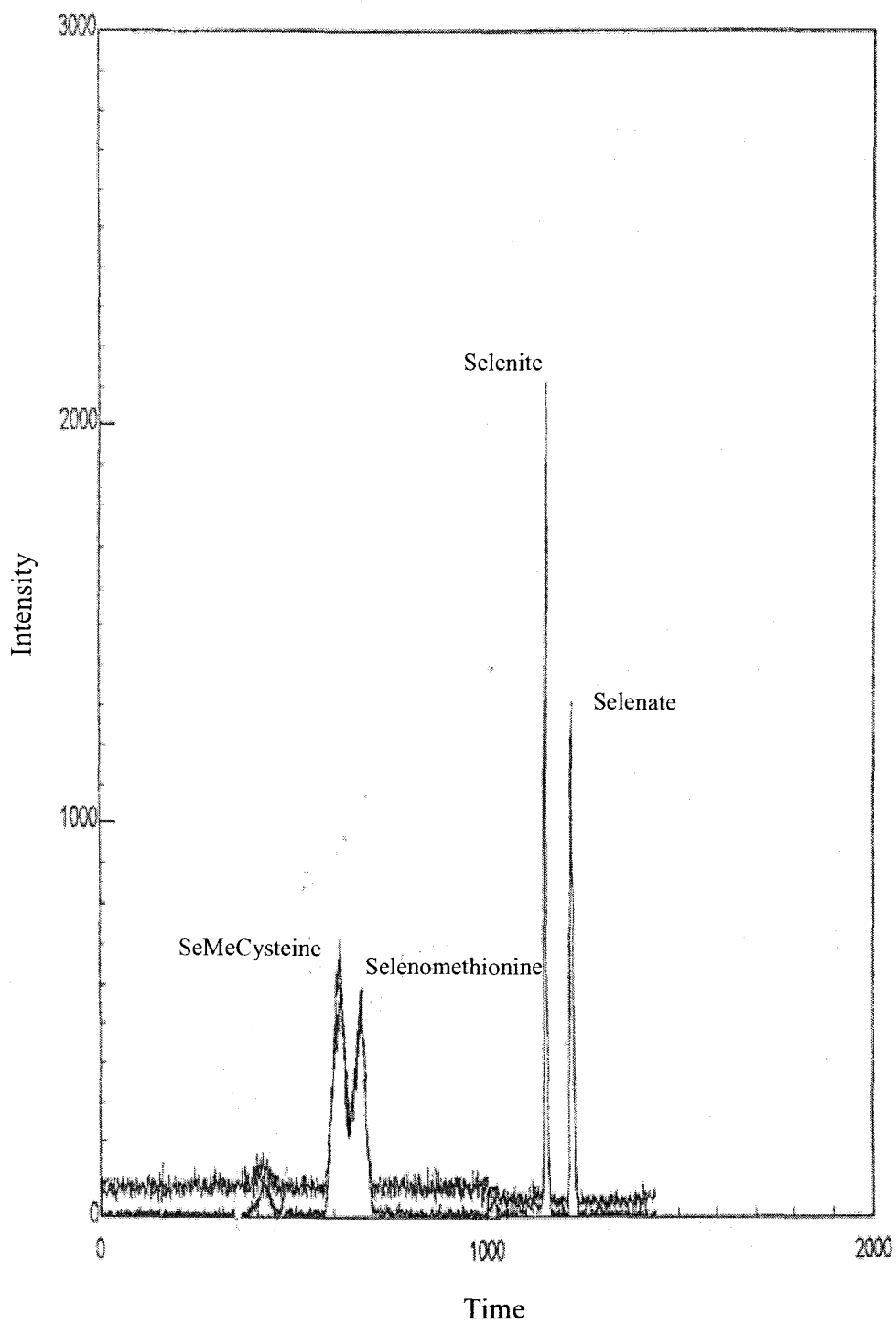
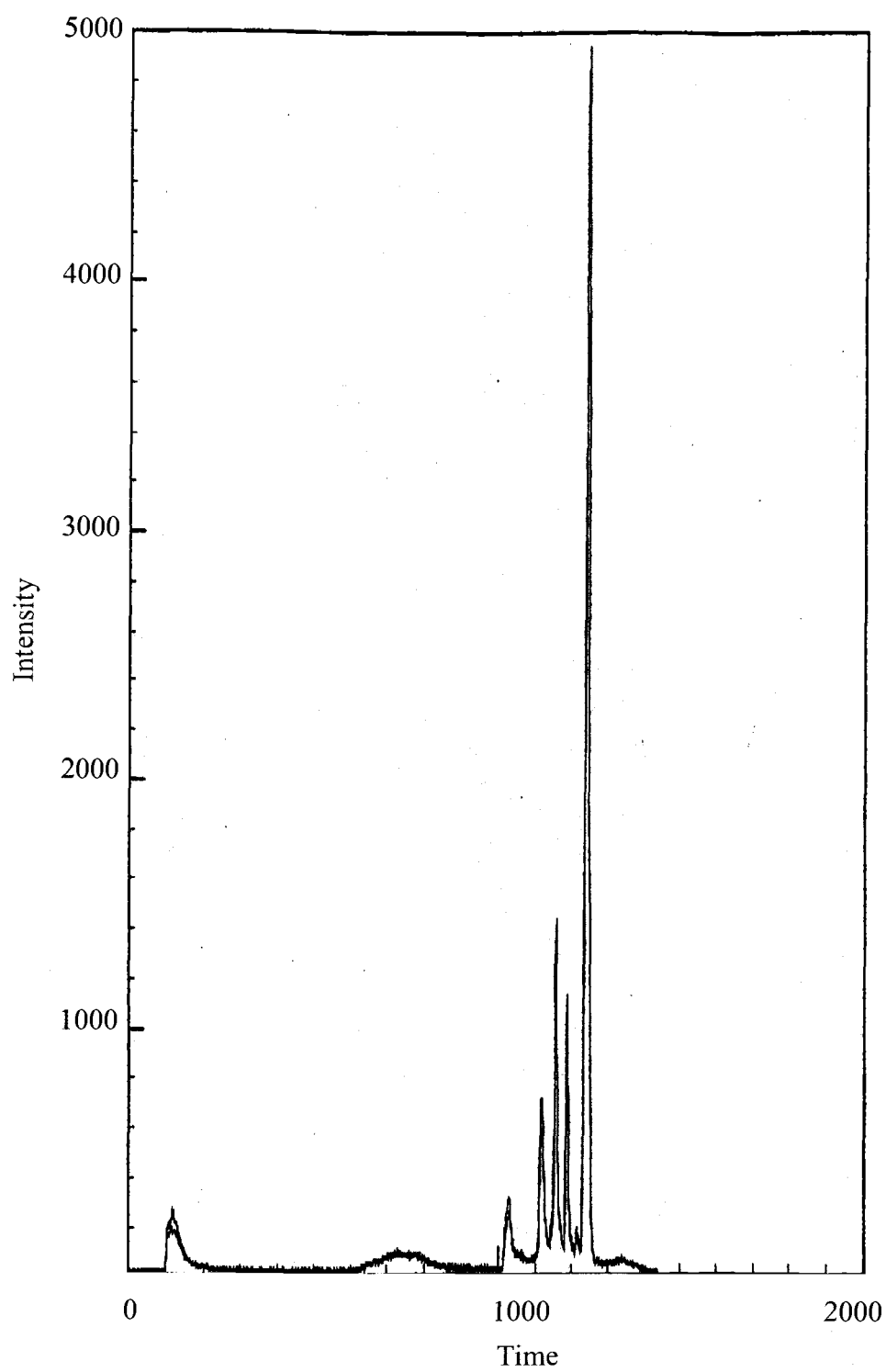Figure 3. Elution patterns for selenium compounds.

Figure 4. Chromatogram for tea extract.

## Acknowledgements

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