1998-05-04

Role of MAP Kinases in the Induction of Heme Oxygenase-1 by Arsenite: Studies in Chicken Hepatoma Cells: A Dissertation

Kimberly Kirstin Elbirt
University of Massachusetts Medical School

A Dissertation Presented

By

Kimberly Kirstin Elbirt

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY

May 4, 1998

BIOCHEMISTRY AND MOLECULAR BIOLOGY

ROLE OF MAP KINASES IN THE INDUCTION OF HEME OXYGENASE-1 BY ARSENITE: STUDIES IN CHICKEN HEPATOMA CELLS

A Dissertaion Presented

by

Kimberly Kirstin Elbirt

Approved as to style and content by:

_____
Kendall Knight, Ph.D., Chair of Committee

_____
Roger Davis, Ph.D., Member of Committee

_____
Allan Jacobson, Ph.D., Member of Committee

_____
Craig Peterson, Ph.D., Member of Committee

_____
Robert Troxier, Ph.D., Member of Committee

_____
Herbert L. Bonkovsky, M.D., Dissertation Mentor

_____
Thomas B. Miller, Ph.D., Dean of the Graduate
School of biomedical sciences

Department of Biochemistry and Molecular Biology

May 4, 1998

ACKNOWLEDGMENTS

First, I would like to thank those people who provided reagents and materials. Thanks to P. Nielsen for a gift of TMH-ferrocene and A. Roy for a supply of perfused rat liver. Thank you to Dr. N. Ahn, (Univ. of Colorado, Boulder, CO) for providing the MEK1 constructs, J. Raingeaud, (Univ. of Mass. Medical School, Worcester, MA) for providing the MKK6 constructs, J. S. Gutkind (NIH, Bethesda, MD) for providing the MLK3 constructs, T. H. Lu (Univ. of Mass. Medical Center, Worcester, MA) for providing the genomic chicken heme oxygenase-1 clone, and D. L. Williams (SUNY, Stony Brook, NY) for providing the GAD-28 plasmid and LMH cells. This research was supported by grants from the National Institutes of Health awarded to HLB (DK-38825), RJD (CA-65861) and the Pittsburgh Supercomputing Center (RR 06009). The contents of this publication are solely the responsibility of the author and do not necessarily reflect the official views of the United States Public Health Service or the National Institutes of Health.

On a more personal note, I would like to thank my advisor, Dr. Herbert Bonkovsky, for being a true mentor, for providing continual support and gentle guidance throughout my graduate education. I would also like to thank the members of the Bonkovsky lab for their patience, concern, and friendship. Many thanks to Sue Donohue for her technical assistance and to Tze Hong Lu for helpful advice and discussions. In particular, thank you to Joyce Pepe and

Otto Gildemeister for not only technical assistance, but also their kindness and concern as to my general well-being, in and out of the lab. Special thanks to Richard Lambrecht for countless revisions, innumerable discussions related to work and recreation, and for teaching me exceptional organizational skills. More special thanks to Tina Tortorelli for being a true friend, a "partner in crime", and for keeping me at least partly sane through the last year.

Special thanks to all of the members of my advisory committee for their fair and intelligent criticism throughout my thesis research. Thank you to the members of the Jacobson lab for helping me to perfect my Northerns. Many thanks also to Roger Davis for his helpful conversations and advice, for providing the MAP kinase components, and for allowing me to perform MAP kinase activity assays in his lab. Thank you to the members of the Davis lab for helping me find my way around and for providing necessary reagents and supplies. I would especially like to thank Alan Whitmarsh for his help with MAP kinase activity assays, and for allowing me to use his reagents, and more importantly, his bench space, whenever I needed.

My greatest appreciation to Dr. Kenneth Hetzler for countless discussions, for his immeasurable patience, his honesty, and his faith, without whom I would not have survived. Finally, thank you to my husband, Adam, who has been a godsend in the final year of my research and preparation, for having constant faith in me and in my ability to complete this journey, especially when I doubted myself, and for being my greatest fan.

ABSTRACT

The chicken hepatoma cell line, LMH, was evaluated with respect to its usefulness for studies of the regulation of heme metabolism. Levels of δ-aminolevulinate synthase mRNA and accumulation of porphyrins were used to evaluate the heme biosynthetic pathway. Regulation of heme oxygenase-1 by known inducers was used as a measure of heme degradation. The induction of heme oxygenase-1 by sodium arsenite was characterized. AP-1 transcription factor elements and MAP kinase signal transduction pathways that modulate expression of endogenous heme oxygenase-1 and transfected heme oxygenase-1 reporter gene constructs in response to arsenite were delineated.

In initial studies, the drug glutethimide was used alone or in combination with ferric nitrilotriacetate to induce δ-aminolevulinate synthase mRNA. Levels of porphyrins, intermediates in the heme biosynthetic pathway, and levels of δ-aminolevulinate synthase mRNA were increased by these treatments in a manner similar to those previously observed in the widely used model system, primary chick embryo liver cells. The iron chelator, deferoxamine, gave a characteristic shift in the glutethimide induced porphyrin accumulation in primary hepatocytes, but was found to have no effect on LMH cells. Heme mediated repression of δ-aminolevulinate synthase mRNA levels was similar among primary hepatocytes and LMH cells. Heme oxygenase-1 was regulated

by heme, metals, heat shock, and oxidative stress-inducing chemicals in LMH cells. Heat shock induction of heme oxygenase-1 mRNA levels was observed for the first time in primary chick embryo liver cells. These data supported the further use of LMH cells to elucidate mechanisms responsible for modulating heme oxygenase-1 gene expression in response to inducers.

The remainder of the studies focused on the role of heme oxygenase-1 as a stress response protein. The oxidative stress inducer, sodium arsenite was used to probe the cellular mechanisms that control the expression of heme oxygenase-1. A series of promoter-reporter constructs were used to search the heme oxygenase-1 promoter for arsenite responsive elements. Several activator protein-1 (AP-1) transcription factor binding elements were identified by computer sequence analysis. Three of these sites, located at -1578, -3656, and -4597 base pairs upstream of the transcription start site, were mutated. The arsenite responsiveness of the reporter constructs containing mutated AP-1 elements was less than that of the same constructs containing wild type AP-1 elements. At least part of the arsenite-mediated induction of heme oxygenase-1 required the activity of AP-1 transcriptional elements.

The MAP kinase signal transduction pathways and heme oxygenase-1 are activated by similar stimuli, including cellular stress. MAP kinases have been shown to exert control over gene expression through effects on the AP-1 family of transcription factors. The MAP kinases ERK, JNK, and p38 were activated by arsenite in LMH cells. Constitutively activated components of the

ERK and p38 pathways increased expression of heme oxygenase-1 promoter-luciferase reporter constructs. Arsenite-mediated induction of heme oxygenase -1 was blocked by dominant negative ERK or p38 pathway components, and by specific inhibitors of MEK (upstream ERK kinase) or p38. In contrast, reporter gene expression was unchanged in the presence of constitutively activated JNK pathway components. Dominant negative JNK pathway components had no effect on arsenite induced heme oxygenase-1 gene activity.

In summary, LMH cells were characterized as a new model system for the study of heme metabolism. This cell line was then used to delineate promoter elements and signaling pathways involved in the arsenite responsiveness of heme oxygenase-1 gene expression. Three AP-1 transcription factor binding sites in the heme oxygenase-1 promoter region were required for responsiveness to arsenite. The MAP kinases ERK and p38 were shown to play an integral role in arsenite-mediated induction of heme oxygenase-1. These studies elucidate one facet of heme oxygenase-1 regulation, and provide tools that will be useful in delineating additional regulatory mechanisms.

TABLE OF CONTENTS

CHAPTER III

CHAPTER IV

CHAPTER V

CLONING AND CHARACTERIZATION OF THE CHICKEN HEME OXYGENASE-1 PROMOTER 118

CHAPTER VI

ROLE OF MAP KINASES IN SODIUM ARSENITE-MEDIATED INDUCTION OF HEME OXYGENASE-1 149

CHAPTER VII

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

AIA	allylisopropylacetamide
AIP	acute intermittent porphyria
ALA	δ-Aminolevulinic acid
ALAD	δ-Aminolevulinic acid dehydratase
ALAS	δ-Aminolevulinic acid synthase
AP-1	Activator protein 1
ARS	Sodium arsenite
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
C/EBP	CCAAT-enhancer binding protein
CAT	Chloramphenicol acetyl transferase
cDNA	Complimentary DNA
CELCs	Chick embryo liver cells
CoA	Coenzyme A
CRE	Cyclic adenosine monophosphate responsive element
DDC	3,5-diethoxycarbonyl-1,4-dihydrocollidine
DFO	Deferroxamine
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
FC	Ferrochelatase
FeNTA	Ferric nitrilotriacetate
GCK	Germinal Centre Kinase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Glu	Glutamate
Glut	Glutethimide
Gly	Glycine
GST	Glutathione-S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO	Heme oxygenase
HPLC	High performance liquid chromatography
HSP	Heat shock protein
JNK	c-Jun N-terminal kinase
LMH	White Leghorn strain M hepatoma
LPS	Lipopolysaccharide
Luc	Luciferase
MAPK	Mitogen activated protein kinase

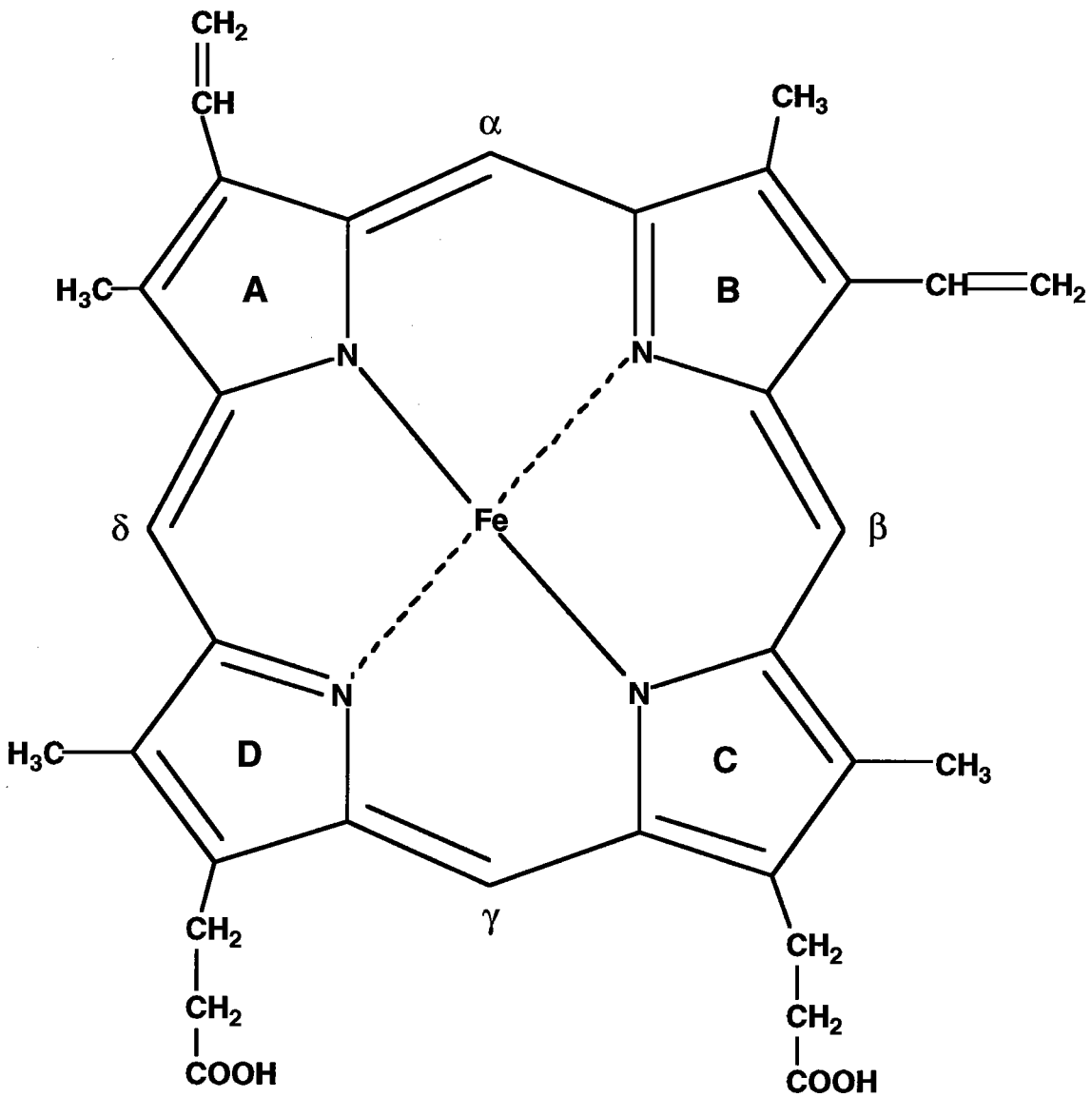
MAPKAP-K2	MAP kinase activated kinase 2
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MEK	MAPK/ERK kinase
MePN	Metalloporphyrin
MKP	MAP kinase phosphatase
MLK	Mixed lineage kinase
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NO	Nitric oxide
PAGE	Polyacrylamide gel electrophoresis
PAK	p21 activated Ser/Thr kinase
PAR	4-(2-pyridylazo)resorcinol
PBGD	Porphobilinogen deaminase
PCA	Perchloric acid
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PGK	Phospho glycerol kinase
Pro	Proline
RLU	Relative light units
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Ser	Serine
SRE	Serum response element
ß-gal	ß-galactosidase
STAT	Signal transducers and activators of transcription
TCF	Ternary complex factor
TGF	Transforming growth factor
Thr	Threonine
TMH-ferrocene	3,5,5-Trimethylhexanoyl ferrocene
TPA	12-O-tetradecanoylphorbol-13-acetate
TRE	TPA-response element
Tyr	Tyrosine
UROD	Uroporphyrinogen decarboxylase
UTR	Untranslated region

CHAPTER I
INTRODUCTION AND OBJECTIVES

Introduction

Significance of heme and heme metabolism. Heme (ferrous protoporphyrin IX) plays a major role in the health and normal functioning of cells and organisms mainly by acting as the prosthetic group for hemoproteins. The tetrapyrrole ring structure of heme (Figure 1.1) enables hemoproteins to perform reduction and oxidation reactions that are necessary for cell and tissue growth and maintenance. Hemoglobin and myoglobin are essential for respiration and gas exchange. The mitochondrial and microsomal cytochromes are the major hemoproteins that carry out steroid biosynthesis, detoxification and activation of xenobiotics, and energy generation. Hemoproteins also contribute to cellular defense mechanisms. Cellular protection is attributed to antioxidant proteins such as catalase, superoxide dismutase, and most recently, heme oxygenase, the rate-limiting enzyme of heme degradation. The balance of heme anabolism and catabolism in cells is essential to proper functioning. Lack of heme may result in the failure of cells to produce enough energy to sustain themselves, whereas excessive "free" or loosely-bound heme may lead to excessive oxidative stress, causing damage to lipids, proteins, and nucleic acids. Therefore, the biosynthesis and degradation of cellular heme are tightly controlled by several mechanisms.

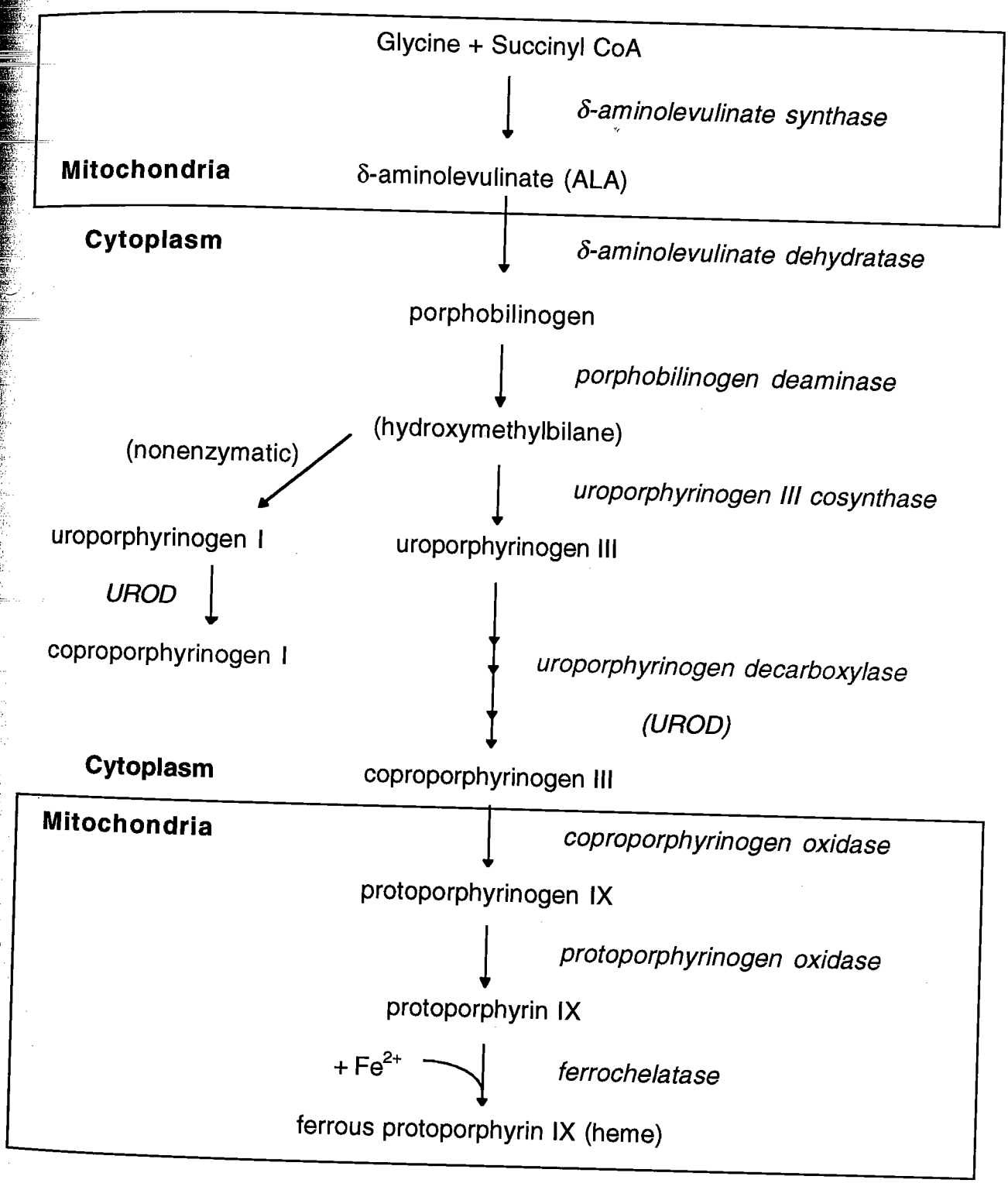
Figure 1.1: The structure of heme, ferrous protoporphyrin IX. According to common reference, the pyrrole rings are labeled A through D, and the methenyl bridges between the rings are labeled α,β,γ, and δ. The substituents on the pyrrole rings are different for uroporphyrins (acetate and propionate), coproporphyrins (methyl and propionate), and protoporphyrins (methyl, propionate, and vinyl, as shown here). Mesoporphyrin derivatives have 1 methyl and 1 ethyl group on each of the A and B rings, and 1 methyl and 1 propionate group on each of the C and D rings. Protoporphyrin and mesoporphyrin may chelate other divalent cations (Mn, Co, Cu, Sn, Zn, etc.) to form metalloporphyrins.



Ferrous Protoporphyrin IX
(HEME)

Biosynthesis of heme. Heme biosynthesis is a multi-step process that has been well characterized [1-5]. The major sites of heme synthesis are hepatocytes and developing erythrocytes, for which the overall steps in heme biosynthesis are similar. Heme synthesis begins and is completed in the mitochondria, with intermediate steps occurring in the cytosol (Figure 1.2). The first committed step of heme biosynthesis is the formation of δ-aminolevulinic acid (ALA), the precursor of porphyrins and chlorophylls, as well as the corrin ring of vitamin B_{12}. In the mitochondria, δ-aminolevulinic acid synthase (ALA synthase) [E.C. 2.3.1.37] catalyzes the condensation of succinyl CoA and glycine to form ALA. In the cytosol, ALA dehydratase (ALAD) [E.C. 4.2.1.24] links two molecules of ALA to form the monopyrrole, porphobilinogen (PBG). Porphobilinogen deaminase (PBGD) [E.C. 4.3.1.8] then converts four molecules of PBG into the linear tetrapyrrole, hydroxymethylbilane, which in the presence of uroporphyrinogen III cosynthase [E.C. 4.2.1.75], is subsequently converted to the asymmetric cyclic tetrapyrrole, uroporphyrinogen III, by inversion of pyrrole ring D (See Figure 1.1). In the absence of the cosynthase, hydroxymethylbilane undergoes spontaneous nonenzymatic cyclization to form uroporphyrinogen I. The formation of heme specifically requires the asymmetric isomer, uroporphyrinogen III. Through a series of four successive decarboxylations, uroporphyrinogen III is converted to coproporphyrinogen III by the next enzyme in the pathway, uroporphyrinogen decarboxylase (UROD) [E.C. 4.1.1.37]. UROD also catalyzes the formation of coproporphyrinogen I from

Figure 1.2: Schematic diagram of the heme biosynthetic pathway. Heme synthesis begins and ends in the mitochondria, with intermediate steps occurring in the cytoplasm of the cell. The substrates and products are indicated for each step. Enzymes are indicated in italics.


Glycine + Succinyl CoA
δ-aminolevulinate synthase
Mitochondria
δ-aminolevulinate (ALA)
Cytoplasm
δ-aminolevulinate dehydratase
porphobilinogen
porphobilinogen deaminase
(hydroxymethylbilane)
(nonenzymatic)
uroporphyrinogen III cosynthase
uroporphyrinogen I
uroporphyrinogen III
UROD
coproporphyrinogen I
uroporphyrinogen decarboxylase
(UROD)
Cytoplasm
coproporphyrinogen III
Mitochondria
coproporphyrinogen oxidase
protoporphyrinogen IX
protoporphyrinogen oxidase
protoporphyrin IX
+ Fe2+
ferrochelatase
ferrous protoporphyrin IX (heme)

uroporphyrinogen I. The next step is catalyzed by the mitochondrial enzyme, coproporphyrinogen oxidase [E.C. 1.3.3.3], which oxidatively decarboxylates the two propionic groups of the A and B rings of coproporphyrinogen III to vinyl groups, resulting in the formation of protoporphyrinogen IX. Protoporphyrinogen oxidase [E.C. 1.3.3.4] then oxidizes the methylene groups which link the pyrrole rings of protoporphyrinogen IX, to methenyl groups to form protoporphyrin IX, the substrate for the final step of heme biosynthesis. Heme synthesis is completed by ferrochelatase (FC) [E.C. 4.99.1.1] through insertion of a ferrous iron atom into protoporphyrin IX to form heme (Ferrous protoporphyrin IX).

Regulation of heme biosynthesis in erythroid cells. During development, erythroid cells must maximize the production of the heme-containing oxygen transport protein, hemoglobin, to last for the lifetime of the erythrocyte. Two enzymes of heme biosynthesis have isoforms that are erythroid specific: ALA synthase, and porphobilinogen deaminase [4]. ALA synthase is encoded as two separate genes: erythroid-specific (located on the X chromosome [6]) and non-specific or housekeeping. The erythroid-specific and non-specific isozymes of porphobilinogen deaminase result from differential transcription and splicing of a single gene product. All of the other enzymes of heme biosynthesis are the same for developing erythrocytes and hepatocytes, and other cells. In developing erythrocytes, heme increases: 1) the transcription and translation of the erythroid form of ALAS; 2) the formation of the erythroid form of

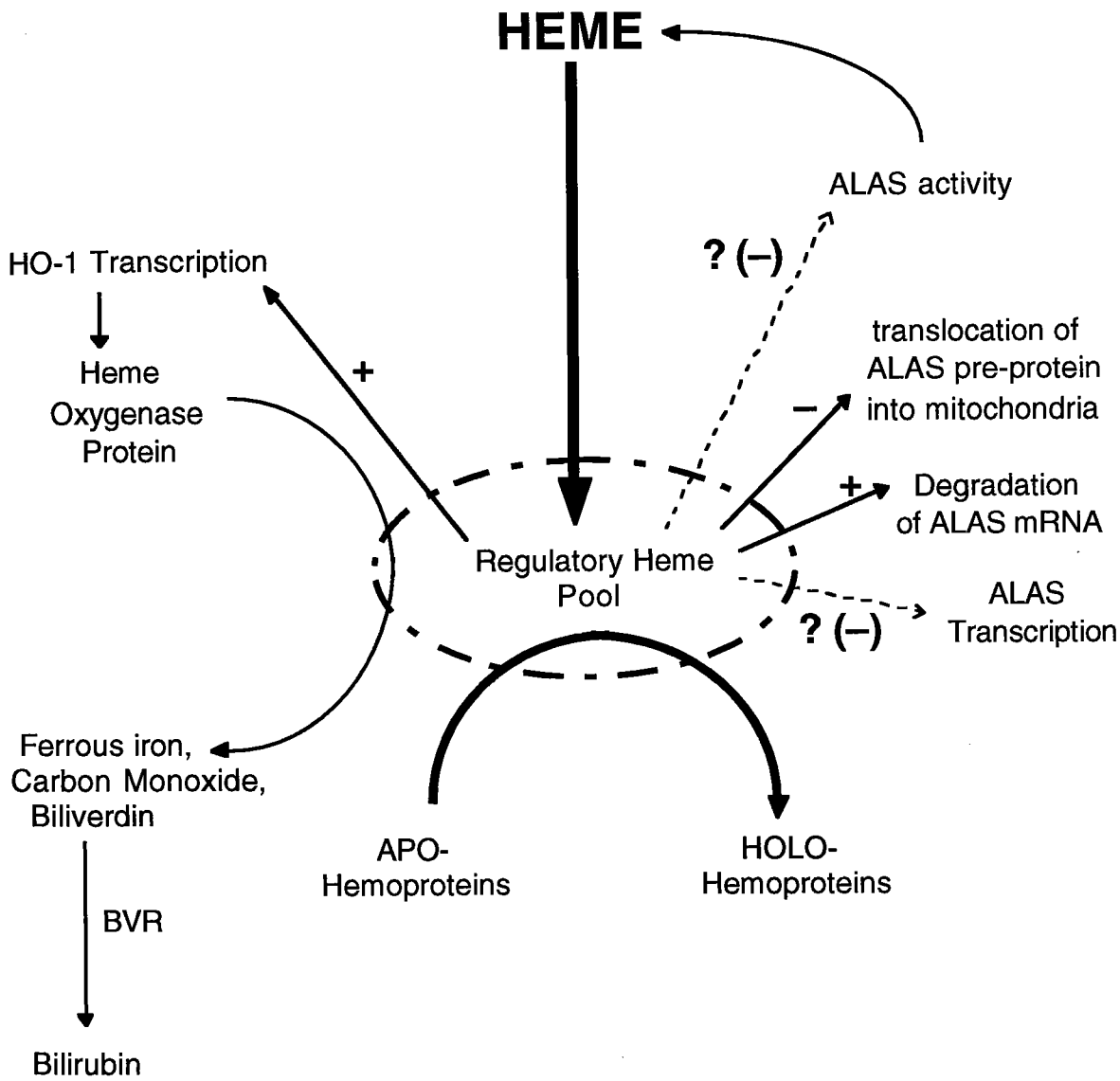
porphobilinogen deaminase; and 3) the synthesis of many other proteins, including the other enzymes of heme biosynthesis and the globins. The overall rate of erythroid heme biosynthesis is dependent on the supply of iron for the final step, catalyzed by ferrochelatase [7-11].

Regulation of hepatic heme biosynthesis. Hepatic heme synthesis accounts for approximately 15% of the total heme produced in humans [2,5]. In hepatocytes, heme is utilized primarily (65%) as the prosthetic group for microsomal cytochrome P450 [2,5]. Hepatic heme synthesis is controlled primarily by the rate of formation of ALA. As the first and rate-limiting enzyme, ALA synthase has been shown to be the major site of regulation of hepatic heme synthesis. Basal ALA synthesis is generally low, but is highly inducible (up to 100-fold) by many hormones (progesterone, estrogens, and testosterone). Chemicals or drugs can induce ALA synthase by several means: 1) by directly up-regulating ALA synthase transcription (e.g. barbiturates and hydantoins), 2) by increasing demand for heme through induction of cytochrome P450, 3) by rapidly destroying cytochrome P450 heme (e.g. chemicals such as allylisopropylacetamide (AIA) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)), and 4) by inhibiting other heme biosynthetic enzymes and causing a depletion of hepatic heme (e.g. succinylacetone, an inhibitor of ALA dehydratase, or iron chelators, which inhibit ferrochelatase). These chemicals and drugs are often used to induce or mimic porphyria in animals or cell culture models.

The regulatory heme pool. Hepatic ALA synthase is subject to negative feedback regulation by heme, the end-product of the pathway. It is generally accepted that a small, rapidly turning-over "regulatory" heme pool exists in hepatocytes [12,13], and that the heme present in this pool is in dynamic equilibrium with the heme incorporated into hemoproteins and other cellular heme pools (Figure 1.3). The concentration of heme in the hepatic heme pool is maintained at concentrations estimated at 10^{-7} to 10^{-8} M by a balance between ALA synthase and heme oxygenase, the enzyme that controls heme degradation [1,2,4,5,12]. Both of these enzymes are regulated by heme. The regulatory heme pool controls heme biosynthesis by several mechanisms: 1) by decreasing the transcription of ALA synthase mRNA (although this has been shown in rodents only); 2) by decreasing the translation of ALA synthase mRNA into protein; and 3) by interfering with the processing of the ALA synthase precursor protein and uptake of the enzyme by mitochondria. Heme may also directly inhibit ALA synthase enzyme activity, but only at a much higher concentration of heme ($K_i = 2 \times 10^{-5}$ M) than that required for regulation of HO or ALA synthase gene expression [2,4,12]. Thus, depletion of regulatory heme generally increases ALA synthesis, whereas excess heme represses ALA synthesis.

Diseases associated with defects in heme synthesis. Deficiencies in heme biosynthetic enzymes may lead to various disease states, the most common being porphyria. Porphyrias were so named due to the characteristic

Figure 1.3: Effects of the regulatory heme pool on hepatic heme metabolism. Heme exerts regulatory effects on both ALA synthase and heme oxygenase-1 to control both its synthesis and its degradation. Heme increases the transcription of HO-1, leading to increased production of HO-1 protein, which catalyzes heme degradation. Several steps in the production of functional mature ALA synthase protein may be inhibited by heme as indicated by minus signs, or by the plus sign indicating increased degradation of ALA synthase mRNA. Regulatory heme is incorporated into apo-hemoproteins as the prosthetic group to form functional holo-hemoproteins. Heme may also increase the transcription of apo-hemoproteins, especially the cytochrome P450 proteins.


HEME
ALAS activity
? (–)
HO-1 Transcription
Heme
Oxygenase
Protein
+
translocation of
ALAS pre-protein
into mitochondria
–
+
Degradation
of ALAS mRNA
Regulatory Heme
Pool
ALAS
Transcription
? (–)
Ferrous iron,
Carbon Monoxide,
Biliverdin
APO-
Hemoproteins
HOLO-
Hemoproteins
BVR
Bilirubin

accumulation and excretion of porphyrins in patients with these disorders. Because only porphyrinogens are substrates for all except the last step of the biosynthetic pathway, very little porphyrin normally accumulates in cells. Porphyrinogens are colorless and have no fluorescent properties. However, the porphyrinogens are very reactive, and are easily oxidized to their corresponding porphyrins. Oxidation can occur spontaneously in the presence of light and oxygen, or it can be catalyzed by cytochrome P450 mono-oxygenase mediated mechanisms upon activation by xenobiotics such as aromatic hydrocarbons [14]. Porphyrins are stable, colored compounds (usually red to purple), that absorb light at about 400 nm (Soret band), and fluoresce when excited. The buildup of porphyrinogens and their oxidation to porphyrins cause the photosensitivity that is observed in most forms of porphyria. The properties of porphyrins have facilitated the detection and diagnosis of the various forms of porphyria. These colored, fluorescent compounds are excreted and can be quantitated by fluorescence spectroscopy or high performance liquid chromatography.

The erythroid form of ALA synthase is critical for erythrocyte development [15]. A deficiency in erythroid ALA synthase leads to X-linked sideroblastic anemia. Deficiencies of the other enzymes in the heme biosynthetic pathway result in porphyrias [2,3]. Porphyrias are categorized as erythroid or hepatic, depending on the site with the highest accumulation of porphyrin precursors. The hepatic porphyrias are characterized mainly by cutaneous photosensitivity,

neurological symptoms, buildup of porphyrin precursors in the blood and tissues, and excretion of porphyrins and/or their precursors in the urine and feces. The most common form of hepatic porphyria is porphyria cutanea tarda, which is characterized by photosensitivity and a high incidence of liver disease, e.g. cirrhosis. The most common form of acute hepatic porphyria is acute intermittent porphyria (AIP), for which the primary symptoms are abdominal pain accompanied by neurological symptoms. Hormones, drugs, and nutritional factors may induce or exacerbate porphyric attacks, which may include muscular paralysis, seizures, respiratory failure, and eventually death [2,3]. Table 1.1 lists the disease states associated with deficiencies of the enzymes involved in heme biosynthesis.

Table 1.1: Disease states related to deficiencies in heme biosynthetic enzymes.

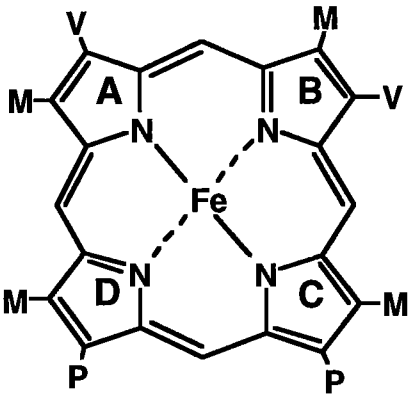
Enzyme Deficiency	Disease and Inheritance Pattern
δ-aminolevulinic acid synthase erythroid	X-linked sideroblastic anemia
δ-aminolevulinic acid dehydratase hepatic	Nearly complete lack of activity, autosomal recessive: ALAD deficiency porphyria
Porphobilinogen deaminase hepatic	Heterozygous, autosomal dominant: acute intermittent porphyria
Uroporphyrinogen III cosynthase erythroid	Homozygous, autosomal recessive: congenital erythropoietic porphyria
Uroporphyrinogen decarboxylase hepatic	Heterozygous, variable: porphyria cutanea tarda; homozygous, autosomal recessive: hepatoerythropoietic porphyria
Coproporphyrinogen oxidase hepatic	Heterozygous, autosomal dominant: hereditary coproporphyria
Protoporphyrinogen oxidase hepatic	Heterozygous, autosomal dominant: variegate porphyria
Ferrochelatase erythroid	Heterozygous, autosomal dominant: erythropoietic protoporphyria

The Degradation of Heme

Catalytic heme degradation. The catalytic breakdown of heme to bilirubin was first described by Tenhunen *et al.* in 1968 [16,17]. Heme degradation is much less complicated than its biosynthesis (Figure 1.4). Heme oxygenase (HO) [E.C. 1.14.99.3] is the first and rate-limiting enzyme of heme catabolism. In association with NADPH ferrihemoprotein reductase (NADPH-cytochrome P450 reductase) [E.C. 1.6.2.4] and in the presence of NADPH or NADH, HO catalyzes the breakdown of heme into equimolar amounts of carbon monoxide, iron, and the linear tetrapyrrole, biliverdin. The reductase-HO complex accepts electrons from NADPH or NADH to maintain the heme iron in the reduced state, which is required for the binding of molecular oxygen, and to oxidatively cleave the α-methenyl bridge between pyrrole rings A and B of the heme molecule [18,19]. In most mammalian, but not avian species, biliverdin is subsequently reduced to bilirubin by biliverdin reductase (BVR) [E.C. 1.3.1.24]. The enzymatic activity of HO can be competitively inhibited by metalloporphyrins with the central iron atom replaced by tin, cobalt, zinc, or manganese [19].

Characterization of heme oxygenase. Two isoforms of HO, HO-1 and HO-2, transcribed from separate genes have been characterized [20-22]. HO-1 has been detected in most vertebrate tissues, with the highest activities in the spleen, liver, and kidneys. Among other functions, HO-1 is thought to play a role in regulating the amount of heme (in the regulatory heme pool) available to

Figure 1.4: The pathway for heme degradation. The first and rate-limiting enzyme of heme degradation is heme oxygenase. The same reaction is carried out by both isoforms, HO-1 and HO-2. The oxidation of heme requires oxygen, NADPH ferrihemoprotein reductase, and NADPH or NADH as a source of reducing equivalents. The reaction requires a total of 3 oxygen molecules and 6 reducing equivalents per molecule of heme that is degraded. Enzymes are indicated in italics.

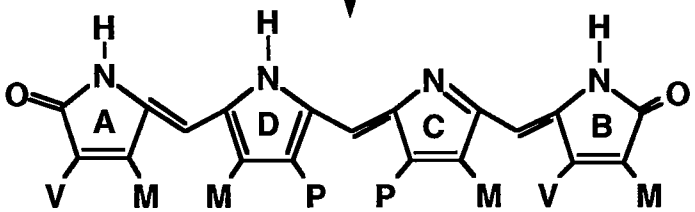


Ferrous Protoporphyrin IX (HEME)

NADPH → NADP

Heme Oxygenase

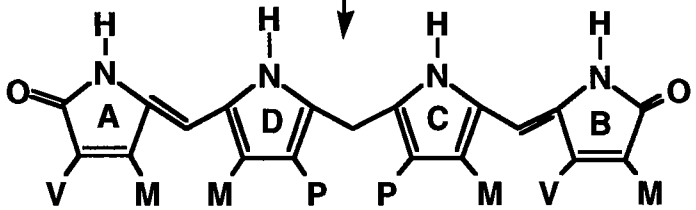
CO, Fe^{3+}



Biliverdin

NADPH → NADP

Biliverdin Reductase



Bilirubin

form hemoproteins, such as the microsomal cytochromes P450, which play key roles in a vast array of reactions, including steroid biogenesis and phase I drug metabolism [23-26]. The HO-1 genes from rodents [27-29], humans [22,30-34], and chicken [35-37] have been cloned and characterized. The chicken HO-1 protein has 296 amino acids with a predicted molecular mass of 32 Kd [35]. The length of the cDNA is 1258 bases, and shares 69% identity with rat HO-1 and 76% identity with human HO-1 cDNAs [35]. The region from amino acids 128 - 136 and histidine-25 correspond to the heme binding and catalytic site, and are conserved among HO-1 proteins from rat, human, and chicken [36,38,39].

A very effective way to study the importance of a gene for cellular metabolism and survival is to obtain a gene knockout animal, or a knockout cell line. Recently, a strain of mice lacking the HO-1 gene has been described. Recent studies by Poss and Tonegawa [40,41] in HO-1 deficient mice have highlighted the important metabolic and cytoprotective roles of this gene. The mice exhibited an incapacity to modulate body iron stores properly and were more sensitive to hepatic injury by iron, indicating that HO-1 plays an important role in iron utilization [40]. After exposure to oxidative damage-causing agents, such as hemin, hydrogen peroxide, or cadmium, the mice were hypersensitive to cytotoxicity when given additional hemin or hydrogen peroxide. When subsequently challenged with endotoxin, HO-1 deficient mice were highly susceptible to hepatic necrosis or death [41].

Model Systems for the Study of Heme Degradation

Current model systems. The inducibility and regulation of HO-1 have been investigated using a number of model systems, including intact rodents, primary cultures of rat hepatocytes and chick embryo liver cells (CELCs) [23,42-47], and various cell lines: Hepa, FEK_4, L929, C6, HepG2, HeLa, etc. [27,33,48-53]. A widely-used model that closely approximates what occurs *in vivo* and in humans is that of primary cultures of chick embryo liver cells (CELCs). This system has been characterized and used successfully by many laboratories for investigations of numerous aspects of heme metabolism. Unlike rat hepatocytes [50,54], CELCs retain normal inducibility of ALA synthase [45,46,55-59]. They also retain normal inducibility and levels of expression of other heme enzymes, such as the cytochromes P-450 [47,50,55-57,60,61] and HO-1 [18,23,45], making this a powerful system for the study of heme metabolism in vitro. However, there are some limitations to this primary culture system: the cells obtained are somewhat heterogeneous; the isolation must be repeated each week to obtain more cultures, resulting in weekly variations and limitations on the number of cultures that can be set up; and such primary cultures may only be used for experiments lasting up to 5-7 days, making stable transfections impossible.

LMH hepatoma cell line. Transient transfections of primary CELCs have provided valuable insights into the regulation of gene expression [62,63]. However, recent work suggested differences in HO-1 expression between

transient and stable transfectants [28]. Since long-term transient and stable transfections were not possible in primary cultures of CELCs, proliferating cell lines were investigated. LMH cells are the first chicken hepatoma cell line to be established. This cell line was derived from an hepatocellular carcinoma induced by feeding diethylnitrosamine to a male White Leghorn strain M chicken [64]. As an immortal cell line, LMH cells provide some potential advantages over primary cultures: continuous long-term culture, cell type homogeneity, and a virtually endless supply. Using this cell line, Oddoux and Grieninger [65,66] have demonstrated normal responses and behavior with respect to plasma protein synthesis and hormonal regulation (with the exception of a defect in fibrinogen secretion), comparable to that observed in primary chicken hepatocytes. LMH cells have also been used successfully in transfection studies exploring regulation of the genes for chicken apolipoproteins AI and II [67-70], vitellogenin II [71], and in studies investigating insulin responsiveness [72,73].

Regulation of Heme Oxygenase-1 Expression

Inducibility of heme oxygenase. Many chemical and environmental stimuli are known to induce HO-1, including its substrate heme [23,74,75], other metalloporphyrins (MePNs) [28,57,74,76], transition metals [23,77,78], inflammatory cytokines [79-82], prostaglandins [83,84], ultraviolet light (UV) [48,85,86], phorbol esters [87], heat shock [29,52,75], and other chemical initiators of cellular stress responses, such as hydrogen peroxide [85], lipopolysaccharide (LPS) [79,88], and sodium arsenite (ARS) [52,85,89,90]. In contrast, HO-2 is found mainly in the brain and testis, is unresponsive to any of the inducers of HO-1, and is essentially uninducible [21,22,28].

HO-1 has been characterized as a heat shock protein (HSP32), and growing evidence supports a role for HO-1 in protecting cells from oxidative stress [48,85,89,91-96]. The protection afforded by HO-1 seems to be due to the production of biliverdin and bilirubin, which have been shown to be potent antioxidants [92,97]. The degradation of heme also leads to production of CO, which is hypothesized to be a signaling molecule that may upregulate cGMP production in a manner similar to nitric oxide (NO) [98,99]. Sodium arsenite causes oxidative stress, is a potent inducer of HO-1 in many cell types and systems, and has been described as a tumor promoting agent [89,90,93,100]. HO-1 most recently has been suggested to play roles in protecting cells from oxidative stress produced during growth and development, and in tumors, with discrete

differences in expression observed between normal and cancerous cell types [101-106].

Previous studies have defined two general mechanisms for induction of HO-1. Heme, the heme precursor δ-aminolevulinate (ALA), and substances in the class of phenobarbital-like drugs (e.g., phenobarbital, glutethimide, or phenytoins) act via a heme-dependent mechanism; whereas transition metals act by a heme-independent mechanism [18,23,31,76,107,108]. Heme and cobalt chloride, acting by two disparate mechanisms, are potent inducers of HO-1, and have been widely used for the characterization of HO-1 regulation [23,32,44,77,109]. Distinctions between the mechanisms utilized by metals, heme, and heat shock to induce HO-1 have also been described [29,52,74,79,110]. Despite these differences, the effects of diverse factors on HO-1 gene expression appear to be exerted chiefly at the transcriptional level, suggesting that multiple signal transduction pathways mediate induction of HO-1 gene transcription in response to a multitude of cellular stimuli.

Regulation of heme oxygenase-1 gene expression. Several *cis*-acting promoter elements involved in mediating HO-1 gene expression have been elucidated [33,111-113]. In one study, stably transfected HO-1 reporter gene constructs were used to locate elements required for induction of the murine HO-1 gene by heme and heavy metals [28]. Putative regulatory elements were identified between 3.5 Kb and 12.5 Kb upstream from the transcription start site. Evidence was presented for a basal level inducer and heme-responsive

element located as far as 10.5 Kb upstream of the transcription start site in the mouse HO-1 gene promoter [87]. Several potential binding sites for known transcriptional regulators were identified in the human HO-1 promoter which may account for induction by a variety of inducers, including heme [114]. Other work has located regions of the HO-1 promoter that mediate cadmium, heat shock, hypoxia, and LPS responsiveness [27,29,87,88,113,115,116].

The role of AP-1 in heme oxygenase-1 gene expression. Several recent studies have presented evidence supporting induction of HO-1 gene expression through the utilization of AP-1 elements present in the promoter regions of human, murine, and avian HO-1. (For a review of the AP-1 family of transcription factors, see Karin *et al.* [117]). Glutathione depletion-mediated induction of rat HO-1 was shown to occur through AP-1 activation [118], and in human fibroblasts, depletion of glutathione induced the expression of HO-1 and c-Fos, an AP-1 transcription factor family member [119]. Inamdar *et al.* identified a composite heme-responsive element that was derived from an extended AP-1 element [111]. Studies by Alam *et al.* [27,28,49,87] in murine hepatoma (Hepa) and fibroblast (L929) cells have identified and characterized enhancer sequences in the mouse HO-1 gene including AP-1 sites that bind c-Fos and c-Jun heterodimers and are responsive to phorbol ester, cadmium, heme, and several other inducers. The heme-, metal-, and arsenate responsive element identified by Alam *et al.* [87] was termed a "core B" element, which includes the recognition sequence for the AP-1 family of transcription factors. Transcriptional activation

of HO-1 by LPS in mouse macrophages has also been attributed to a distal 5' AP-1 binding site located approximately 4 Kb upstream of the transcription start site [88]. Finally, Lu *et al.* have shown that arsenite and cobalt chloride treatments increase binding to an AP-1 consensus element in the chicken HO-1 promoter [37,120]. However, the signal transduction pathways and transcription factor complexes that target these elements have been largely unexplored.

The Mitogen-Activated Protein (MAP) Kinases

The mitogen-activated protein (MAP) kinases are components of signaling cascades which, in response to extracellular stimuli, target transcription factors, resulting in the modulation of gene expression. MAP kinases have two major distinguishing features: 1) they are activated by phosphorylation of threonine and tyrosine residues within protein kinase domain XIII, and 2) they are proline-directed serine/threonine protein kinases that require a minimum recognition sequence of Ser/Thr-Pro. The activation of MAP kinases is performed by upstream dual-specific kinases, called MAP kinase kinases (MAPKKs), which are in turn phosphorylated by MAP kinase kinase kinases (MAPKKKs). In addition, the MAP kinases can be regulated by MAP kinase phosphatases, such that the overall response to a signal is a complex balance between phosphorylation by upstream kinases and dephosphorylation by phosphatases [121,122].

MAP kinases are activated under conditions similar to those that induce HO-1 transcription, *e.g.*, following exposure of cells to phorbol ester, cytokines, ultraviolet light, heat shock, LPS, ceramide, and inducers of oxidative stress, including arsenite [110,123-131]. Three major MAP kinase subfamilies that mediate physiological responses have been described: Extracellular-signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (a homologue of the yeast HOG1 kinase). A comparison of the characteristics of each MAP kinase

family and their major signaling cascade components are represented in Table 1.2. The complexity of these signaling cascades is further enriched by the occurrence of multiple isoforms for each family. In general, growth and proliferation signals are mediated primarily by ERK, and cellular stress signals are mediated mainly by JNK and p38. The specificity of signaling by these kinases, their targets, and the upstream components that mediate activation in response to cellular stimuli have been the subject of several reviews [121,122,126,132-138].

In a recent review, Treistman presented a comparative analysis of MAP kinase pathway targets in *S. cerevisiae*, *D. melanogaster*, *C. elegans*, and mammals [135]. Physiological targets of the MAP kinases range from other cytosolic kinases or membrane-bound tyrosine kinase receptors (MAPKAP-K2, EGFR) to cytoskeletal components and nuclear transcription factors. In particular, the AP-1 family of transcription factors has been shown to be regulated in several ways by all three MAP kinase pathways [117,139,140].

Extracellular-signal regulated kinase. The ERK pathway was the first of the MAP kinases to be cloned in mammals. Components of the ERK pathway were found to be homologous to protein kinases of the pheromone response pathway in *S. cerevisiae* [141]. The regulatory motif for ERK activation is Thr-Glu-Tyr. Phosphorylation at both Thr and Tyr is required for full activation [142]. Two isoforms that are similarly regulated are commonly referred to as p42/p44 MAPK or ERK1/2. ERK1/2 is activated mainly in response to mitogenic stimuli,

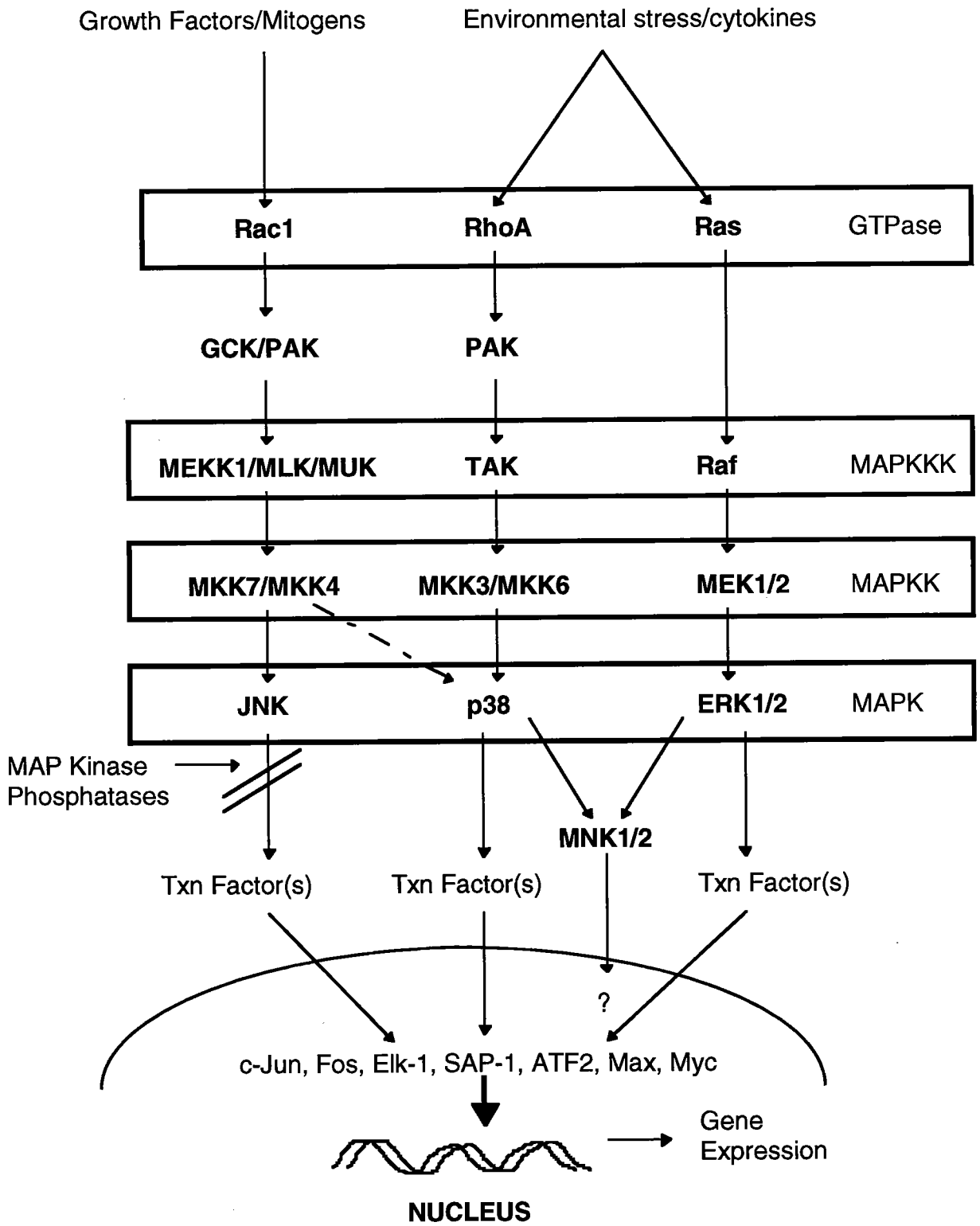
Table 1.2: Characteristics of the three major MAP kinase families. Below each of the families, the upstream cellular stimuli, phosphorylation sequences required for activation, and several downstream targets are indicated. The families are grouped by their characteristic phosphorylation motifs, which differ in the amino acid that is found between the Thr and Tyr that are phosphorylated for activation. Activating stimuli are more similar between JNK and p38, but the downstream targets are more similar between p38 and ERK. Some of the well-characterized targets (substrates) for each kinase are listed. Many other targets (not shown here) have been proposed for each kinase.

MAP Kinase:	JNK/SAPK	p38/CSBP/RK	ERK
Activators:	Inflammatory cytokines UV radiation Environmental stresses Heat Shock Ischemia/reperfusion Ceramide	Inflammatory cytokines UV radiation Environmental & osmotic stresses Heat Shock Ischemia/reperfusion LPS (endotoxin)	Mitogens EGF Phorbol ester
Phosphorylated At:	Thr - Pro - Tyr	Thr - Gly - Tyr	Thr - Glu - Tyr
Substrates:	c-Jun ATF2 Elk-1	ATF2 Elk-1 Max SAP-1 GADD153/CHOP MAPKAP-K2	Elk-1 ATF2 c-Myc STAT EGFR cPLA2

for which the activation pathway has been well-defined [121,141]. Cell surface receptor tyrosine kinases such as the EGF and PDGF receptors activate a signaling cascade involving the G protein Ras, the MAPKKK Raf, the MAPKK MEK1/2, and ERK1/2, which then targets cytoskeletal proteins, other kinases, or transcription factors (Table 1.2 and Figure 1.5) [121,132,134,140,141,143]. A specific inhibitor of MEK1/2, PD98059, has helped to more clearly delineate cellular targets of ERK [144-147]. ERK effects changes in cellular morphology through phosphorylation of cytoskeletal proteins, including microtubule-associated proteins. Phospholipases that control arachidonic acid metabolism, and protein kinases (including $p90^{RSK}$ (RSK), for which c-fos is a substrate) are cytosolic targets for ERK. ERK also regulates transcriptional activity through phosphorylation of several transcription factors, including Elk-1, c-Myc, ATF-2, C/EBPβ, and STAT proteins [121,122,135].

C-jun N-terminal kinase. The cloning of JNK helped to establish the current model of several parallel MAP kinase cascades in mammals that modulate physiological responses to diverse cellular stimuli. Several pathways have been identified in yeast, but there is no known yeast homologue for JNK. Activation of JNK occurs in response to environmental stress and inflammatory cytokines, and is therefore also referred to as stress-activated protein kinase (SAPK) [129,148]. The phosphorylation site of the JNK family is a Thr-Pro-Tyr motif. There are several upstream activation mechanisms for JNK. Characterized mechanisms involve Rho family low molecular weight G-proteins (Cdc42 and

Figure 1.5: Comparison of the mammalian MAP kinase cascade components. Each MAP kinase is activated through a cascade of sequential phosphorylations of protein kinases. The cascades are grouped by level (MAP kinase, MAPK kinase, MAPKK kinase, etc.) to provide comparisons of the components between the MAP kinase families. MKK4 may activate both JNK and p38 (indicated by interrupted arrow), MKK7 is specific for JNK only. Downstream targets shown as transcription factors (Txn Factors) may also be cytosolic components or other kinases (see Table 1.2 and text). For clarity, the possible role of phosphatases in the modulation of MAP kinase activity is indicated for JNK only, but applies to all three MAP kinase pathways. Co-operative interaction or signal integration among the MAP kinase families may occur in the cytosol, via activation of a kinase, or in the nucleus, through dimerization of transcription factors activated by separate MAP kinases.



Rac1), p21 activated kinase (PAK), MEKK1, MUK, or MLK3 (mixed lineage kinase), MKK4 or MKK7, and JNK (Figure 1.5). JNK was discovered by and named for its primary function, regulation of the transcription factor c-Jun. Activation of JNK leads to the induction of apoptosis [127,149]. Other transcription factor targets of JNK include ATF2 and Elk-1.

p38 MAP kinase, homologue of HOG 1. The third MAP kinase family to be described is p38, the homologue of the yeast high osmolarity glycerol regulated MAP kinase, HOG 1. In response to environmental stress or inflammatory cytokines, p38 is activated by phosphorylation at the Thr-Gly-Tyr motif. The pathway of activation involves Rho family G-proteins (Cdc42/Rac1), PAK, TAK (TGFβ-activated kinase) or MEKK1, MKK3 or MKK6, and p38 (Figure 1.5). Activation of p38 inhibits cell growth and promotes apoptosis. Recently, a class of pyridinyl imidazoles (the cytokine-suppressive anti-inflammatory drugs) were identified as specific inhibitors of the p38 family of MAP kinases [150-152]. These inhibitors have facilitated elucidation of the cellular effects of p38. Primary targets for p38 are MAPKAP-K2, the Myc binding protein Max, and the transcription factor components Elk-1, SAP-1, ATF2, and GADD153.

Signal integration by MAP kinases. Recent studies have established mechanisms that allow integration of the ERK and p38 MAP kinase pathways. Novel protein kinases that are activated by both ERK and p38 MAP kinases have been described [153,154]. These kinases, termed MNK1 and MNK2, are proposed to be a convergence point for co-operative action of the growth-factor

regulated ERK pathway and the stress-regulated p38 pathway [153,154]. A second example is provided by Ets transcription factors that are phosphorylated and activated by both ERK and p38 MAP kinases [155,156]. Investigation of MAP kinase involvement in the UV response of HeLa TK- and NIH3T3 cells revealed co-operative serum response element (SRE) and ternary complex factor (TCF) activation by ERK and p38 pathways [156]. Both of these pathways were found to be required for induction of c-fos gene transcription by UV irradiation [156]. Similar mechanisms may contribute to the integration of MAP kinase pathways at the HO-1 promoter.

Activation of MAP kinases by arsenite. Arsenite has been shown to activate MAP kinases [110,123,157,158], however, this arsenite-mediated activation has not been linked to a cellular gene response. Two recent studies have investigated the ability of sodium arsenite to activate MAP kinases. One study found that arsenite activated ERK, JNK, and p38 MAP kinases in PC12 cells [131]. A separate study in HeLa cells found that JNK and p38 MAP kinases were activated by arsenite, however, no activation of ERK was observed [159]. The discrepancy in MAP kinase activation by arsenite may be explained by the fact that these two studies were done in different cell types [131,159]. It is generally acknowledged that MAP kinases can be differentially regulated by the same stimuli in diverse cell types.

It has also been proposed that arsenite affects gene expression by modulating the activities of transcription factor complexes bound to AP-1

elements in the promoter regions of several genes [118,159,160]. Putative AP-1 sites are present in the promoter regions of mammalian [87,88,111,113] and chicken HO-1 [37]. Since the MAP kinase signaling cascades have been shown to target AP-1 elements, they are potential mediators of the arsenite induction of HO-1 [117,139,140].

Research Objectives

Objective I: To establish whether LMH cells are a good model system for the study of heme metabolism. The aim of the work reported here was to study the expression and regulation of heme biosynthesis and degradation [4,8,16,17,26,27,40] in LMH cells, comparing results to those previously found in CELCs. Initially, growth maintenance and culture conditions were established for LMH cells. The comparative dose-responses and time courses of induction of HO-1 mRNA and heme oxygenase activities by heme and cobalt chloride were characterized. In addition, a measurable response to other treatments previously shown to increase HO-1 mRNA levels or heme oxygenase activities in normal CELCs was demonstrated for LMH cells. During these studies a small but significant heat shock induction of HO-1 mRNA in CELCs was detected, which had not been described previously.

Objective II: To establish whether AP-1 transcription factor complex binding sites modulate sodium arsenite induction of HO-1. The ability of arsenite to increase transcription of endogenous HO-1, and the activity of transfected luciferase reporter gene constructs under control of the HO-1 promoter were investigated. Transient transfection assays using reporter constructs containing successive deletions of the cHO-1 promoter sequence were used to identify regions involved in modulating arsenite-mediated HO-1 gene expression. HO-1 reporter constructs containing mutated AP-1 elements

were constructed and tested for responsiveness to arsenite treatment. The results from these experiments suggest that AP-1 elements are involved in the sodium arsenite-mediated induction of HO-1 gene expression.

Objective III: To establish whether the mitogen-activated kinases modulate induction of HO-1 by arsenite. The mitogen activated protein (MAP) kinases were good candidates as signaling molecules for controlling HO-1 expression, since many of the activators of MAP kinases also induce HO-1. In LMH cells, the MAP kinases, ERK, JNK, and p38, were tested for activation by treatments that were known to induce HO-1. Sodium arsenite was a highly efficacious inducer of HO-1 and activator of all three MAP kinases. Arsenite-mediated activation of MAP kinases and induction of endogenous HO-1 mRNA expression were closely correlated. Therefore, arsenite was chosen to further investigate the involvement of MAP kinases in cHO-1 gene expression. Constitutively activated and dominant negative components of the MAP kinase signaling pathways were utilized to delineate the roles of ERK, JNK, and p38 in HO-1 gene expression as measured using HO-1 promoter-driven luciferase reporter gene constructs. Inhibitors of the ERK and p38 signaling pathways were also employed to further analyze the involvement of these kinases in arsenite-mediated induction of HO-1. Results from these experiments demonstrated roles for the ERK and p38 MAP kinase families in the sodium arsenite-mediated induction of cHO-1.

CHAPTER II
MATERIALS AND METHODS

Materials

General materials. Tissue culture dishes and 6 well plates were from Corning, Corning, NY; culture flasks were from Falcon, VWR Scientific, Bridgeport, NJ. Gelatin and fetal bovine serum were from Difco, Detroit, MI. Opti-MEM, Williams' E and Waymouth's MB 752/1 media, and LipoFECTAMINE™ Reagent were from Life Technologies, GIBCO/BRL, Grand Island, NY. Biliverdin and heme were from Porphyrin Products, Logan, UT. Oligo dT (12-18 bases) was from Pharmacia, Piscataway, NJ. Iron chloride for quantitative iron assays was from VWR Scientific, Bridgeport, NJ. Dexamethasone was from Gensia Pharmaceuticals, Irvine, CA. Chloroform, glycerol, isopropanol, potassium phosphate, and sodium vanadate were from Fisher, Pittsburgh, PA. Actinomycin D, adenosine triphosphate (ATP), bovine serum albumin (BSA), cadmium chloride, cobalt chloride, deferoxamine mesylate (DFO), dimethylsulfoxide, EDTA, EGTA, formaldehyde (37% v/v), formamide, glycylglycine, NADPH, O-nitrophenyl ß-galactopyranoside (ONPG), penicillin, phenylmethyl sulfonylfluoride (PMSF), p-hydroxymercuribenzoic acid, piperacillin, sodium arsenite, sodium chloride, sodium citrate, sodium dodecyl sulfate (SDS), sodium hydroxide, streptomycin, 3,5,3'-triiodo-L-thyronine, triton

X-100, Trizma base, and trypsin were from Sigma, St. Louis, MO. Dithiothreitol (DTT) and 4-(2-pyridylazo)resorcinol (PAR), mono sodium salt hydrate were from Aldrich Chemical Co., Inc., Milwaukee, WI. 3,5,5-Trimethylhexanoyl ferrocene (TMH-ferrocene) was provided as an orange powder by P. Nielsen, Universitätskrankenhaus, Eppendorf, Hamburg, Germany. Kinase inhibitors PD98059 and SB203580, were from CalBiochem, La Jolla, CA. Ultraspec RNAzol® was from Biotexc, Houston, TX. Nitrocellulose (0.45 μm) was from Schleicher and Schuell, Keene, NH. All ^{32}P -radionucleotides were from New England Nuclear, Boston, MA. All chemicals were of the highest purity available.

Cells and DNA. Fertilized Barred Rock chicken eggs were from Carousel Farms, Holliston, MA. LMH cells and the pGAD-28 plasmid were generous gifts from D. L. Williams, Dept. of Pharmacological Sciences, SUNY-Stony Brook, Stony Brook, NY. The rat HO-1 cDNA was a gift from S. Shibahara, Tohoku University School of Medicine, JAPAN. The pALX plasmid containing the cDNA of the housekeeping form of chicken ALA synthase was a gift from D. Engel, Northwestern University, Evanston, IL. The lambda FIX II clone containing genomic chicken heme oxygenase-1 promoter sequence, and pCHO3.6-CAT were provided by T. H. Lu, Division of Digestive Disease and Nutrition, University of Massachusetts Medical Center, Worcester, MA. The pGL3 Basic and pGL3 Control plasmids were gifts from G. Gil, Division of Digestive Disease and Nutrition, University of Massachusetts Medical Center, Worcester, MA. The

pPGK-ßgal plasmid was a gift from P. Dobner, Department of Molecular Genetics and Microbiology, University of Massachusetts Medical Center, Worcester, MA. Expression vectors for signaling pathway components and MAP kinases have been described: ERK2 [155]; MEK1 [161]; MEKK1 [162,163]; JNK1 [164]; MLK3 [165]; MKK6 [166]; Ras and Raf [167], as has the expression vector for dominant negative c-Jun (TAM67) [168]. Luciferase Assay Reagent, Wizard™ plasmid DNA preparation kits, and the primers GLprimer2 and RVprimer3 were purchased from Promega, Madison, WI. The QuickChange™ Site-Directed Mutagenesis Kit and Quickhyb® hybridization solution were from Stratagene, La Jolla, CA. DNA sequencing was performed by Dana Farber Cancer Institute, Boston, MA, and by the Nucleic Acids Facility, University of Massachusetts Medical Center, Worcester, MA.

Methods

Cell culture and preparation of chemicals. Cultures of primary chick embryo liver cells (CELCs) were freshly prepared each week by Otto Gildemeister and Joyce Pepe as described [23,44-46,56,57,169]. LMH cells were maintained in Waymouth's MB 752/1 complete medium (Appendix A); and routinely passaged twice a week [64]. Complete and serum-free Williams' E and Waymouth's media contained penicillin/streptomycin. In some experiments, 1μg/mL piperacillin was added to the culture medium. In experiments involving both CELCs and LMH cells, cells were plated on Corning dishes 6 cm in diameter. CELCs were prepared and plated on uncoated dishes in 5 mL Williams' E complete medium (Appendix A). LMH cells were resuspended and plated at 80% confluence in 5 mL Williams' E complete medium on dishes coated with 0.1% (w/v) gelatin. On the day of treatment, the cells were washed once with 5 mL of Williams' E complete medium. Preparation of and treatment with chemicals were exactly as described for primary CELCs [23,44-46,56,57,169]. Sodium arsenite, cadmium chloride, cobalt chloride, and heme were prepared as described [23,44-46,56,57,64,169]. All other chemicals were freshly prepared on the day of treatment and added directly to the culture medium (Appendix B).

Porphyrins assays

Fluorometric porphyrins assays. LMH cells or CELCs were prepared and seeded into 3-cm wells in 6-well plates. On the morning of treatment, medium was changed to Williams' E complete. Cells were typically exposed to treatments for 18 hours. From the time of treatment, all steps were carried out in subdued lighting. The medium was removed to 12 x 75 mm culture tubes and assayed separately. The plates were washed once with 1X PBS, and harvested in 2 mL PBS by scraping with a rubber spatula. Cells and media were disrupted by sonicating for 6 seconds with a sonicator (Bronson model 450, VWR Scientific, Bridgeport, NJ) equipped with a microtip (output: 2, duty cycle: 30). Sonicates were centrifuged at 1,000 x g for 10 minutes and aliquoted into tubes for protein determination (50 μL each) and four-side-clear 4 mL cuvettes (200 μL each) for porphyrin determinations. To extract porphyrins and porphyrinogens and oxidize porphyrinogens to porphyrins, 1.8 mL of perchloric acid (PCA)/methanol/water (5/50/50, v/v/v) was added to the cuvettes. Known amounts (50 μg/L) of uroporphyrin and coproporphyrin were assayed as controls for standardization. A Perkin-Elmer LS 50B spectrophotometer-fluorometer fitted with an R928 (red wavelength sensitive) photomultiplier was used to measure the fluorescence at each of three wavelength excitation/emission pairs (400 nm/595 nm, 405 nm/595 nm, and 410 nm/605 nm); excitations were measured at a slit width of 5 nm, emissions at a slit width of 20 nm. Data were input into a Microsoft Excel worksheet that automatically

calculated the levels of uroporphyrin, coproporphyrin, and protoporphyrin in each sample based on the results of porphyrin standards and Grandchamp equations [170]. The amount of protein in each sample was determined by the BCA method (See Protein assays).

High performance liquid chromatography (HPLC) porphyrins assays. HPLC porphyrin determinations were performed on a Waters Baseline 810 HPLC System, equipped with a Shimadzu RF-551 Fluorometer with a R-928 (red sensitive) photomultiplier tube, essentially as described [171]. Cells were seeded and harvested as described for fluorometric assays. All steps were performed under subdued lighting. Porphyrins and porphyrinogens were extracted and oxidized from 500 µL of each sonicate with 10 µL of 10 N hydrochloric acid. The tubes were centrifuged at 1,000 x g for 10 minutes to remove cellular debris, and 250 µL of supernatant was injected using a WISP autoinjector apparatus. Porphyrins were separated on a C_{18} reverse-phase microbondpak column, with a gradient consisting of two solvents: A, 1:1 (v:v) 0.1 M ammonium phosphate, pH 4.5, and methanol; B, 100% HPLC-grade methanol. A linear gradient from 70% A, 30% B to 100% B was run over 7 minutes with a flow rate of 1.5 mL/minute, followed by 100% B for another 11 minutes at the same flow rate. Porphyrin standards were run every three samples.

Quantitative iron assays. On the day of treatment, cells were washed and the medium changed to Williams' E complete. Treatments were for 18 hours, except for TMH-ferrocene which was for 4 hours. Cells were washed once with 1X PBS and harvested in 1 mL of PBS. A scintillation vial was weighed before and after adding cells to obtain a wet weight for the cellular material. The vials were placed in an 80°C oven overnight to dry. After drying, the cellular material was split into three samples and transferred to 30 mL Kjeldahl tubes, which were weighed before and after addition of cellular material to obtain dry weights for each triplicate. Cells were digested by heating for 3 minutes in 1:1 (v:v) sulfuric acid and nitric acid. Samples were transferred to 16 x 100 mm tubes, and allowed to cool. Standards containing known quantities of iron were prepared and assayed with the samples. Each tube received 2 mL of 0.5 M potassium phosphate buffer, 1.75 mL of 6 M ammonium hydroxide, 100 μL of 10% hydroxylamine, 5.3 mL of Milli-Q water, and 500 μL of 0.1% 4-(2-pyridylazo)resorcinol (PAR), mono sodium salt hydrate. Tubes were inverted gently to mix and allowed to stand at room temperature overnight. The next morning, the absorbance of the iron-PAR adduct was read at 500 nm on a spectrophotometer. Suitable standards were run simultaneously to generate a linear standard curve. The iron concentrations were calculated as μg iron/mg of dry weight using the equation from the best-fit linear regression standard curve and the known dry weights of the samples.

Isolation of RNA. Total RNA was isolated using Ultraspec RNAzol®, following the manufacturer's protocol. The cells from one 6 cm culture dish or from one 3 cm well of a 6 well plate were harvested directly into 0.5 mL of Ultraspec RNAzol® and total RNA isolated as described [57]. Cells were harvested by scraping with a rubber spatula. The lysate was transferred into 1.5 mL microcentrifuge tubes, and 100 μL of chloroform was added. The tubes were vortexed briefly and incubated on ice for at least 15 minutes. Following centrifugation (14,000 x g at 4°C for 15 minutes), the top phase was transferred to a new tube. An equal volume (300 - 400 μL) of isopropanol was added to precipitate the RNA. The tubes were vortexed and incubated at -20°C for at least 1 hour. Following centrifugation (14,000 x g for 15 minutes), the supernatant was removed, and the pellet was washed with 0.5 mL of 75% ethanol/DEPC-treated water. Pellets were dried in a vacuum apparatus, and resuspended in 50 μL of DEPC-treated water for dot blots or 0.5% SDS for Northern blots. Samples were stored at -20°C or incubated at 65°C for 45 minutes to dissolve the RNA. Nucleic acid purity was assessed by determining the absorbance ratio at 260 nm/280 nm (1.80 or higher was required) [172]. RNA concentrations were estimated from the absorbance at 260 nm (1 AU = 40 μg/mL RNA) [173].

Preparation of radiolabeled probes. Radiolabeled probes for detection of mRNA for the housekeeping form of ALA synthase, chicken HO-1, and chicken glyceraldehyde phosphate dehydrogenase (GAPDH) were synthesized by random priming and incorporation of α^{32}P-dCTP using the DECAprime II™ DNA Labeling Kit from Ambion Inc., Austin, TX or the Ready-To-Go™ DNA Labeling Beads from Pharmacia Biotech, Piscataway, NJ. The ALA synthase probe template was a 1.7 Kb Eco RI fragment of the pALX plasmid containing the ALA synthase cDNA. The cHO-1 probe template was a 1.3 Kb polymerase chain reaction (PCR) product obtained using T7 and T3 primers, with the cHO-1 cDNA as the PCR template. The GAPDH probe template was a 530 base pair (bp) PCR product obtained using the linearized pGAD-28 plasmid as template and the primers GAP1: 5'- GAAAGTCGGA GTCAACGGAT TTG -3' and GAP2: 5'- TGGCATGGAC AGTGGTCATA AGAC -3'. The PCR program for HO-1 and GAPDH probes was as follows: delay, 94°C for 3 min.; 30 cycles of: segment 1, denaturation (94°C for 1 min. 30 sec.), segment 2, renaturation (54°C for 2 min.), segment 3, elongation (72°C for 2 min.); delay, 72°C for 10 min.; soak at 4°C. Poly-A and chicken heat shock protein 70 (HSP 70) probes were end-labeled with α^{32}P-dCTP using the DECAprime II™ DNA Labeling Kit from Ambion Inc., Austin, TX. The Poly-A probe template was a 12 - 18 base polythymidylate oligonucleotide from Pharmacia. HSP 70 was detected using an oligonucleotide probe complementary to the plus strand of the cDNA from base pairs 2518 to 2542 (5'— AGAGACAAAC ATCCAGAATA CAAGG —3').

Quantitation of messenger RNA.

Northern blots After isolation, RNA was dissolved in 0.5% SDS, and incubated at 65°C for 45 minutes. The volume of RNA required for 10 μg per sample was calculated from the absorbance measured at 260 nm, and samples were prepared by diluting in DEPC-treated water. HEPES buffer (Appendix A) was added, and the samples were heated to 85°C for 15 minutes. Sample loading dye (2 μL of 0.02% bromophenol blue) was added to each tube. The samples were spun briefly, and 20 μL of sample or marker was loaded into each well of a 1.2% agarose/formaldehyde gel containing ethidium bromide. The gel was run at 80 Volts for 2 hours using 1X HEPES running buffer. The gel was washed in DEPC-treated water 3 times for 20 minutes to remove excess ethidium and formaldehyde, and photographed on a transilluminator. The RNA was transferred to a nitrocellulose filter by capillary action overnight using 20X SSC. Following transfer, the RNA was immobilized by ultraviolet (UV) light crosslinking. The blot was prehybridized in Quickhyb® for 30 minutes at 65°C. The blot was hybridized for 1 hour in Quickhyb® containing a radiolabeled HO-1 probe that specifically recognized the ~1.3 Kb chicken HO-1 mRNA (see Preparation of radiolabeled probes), and washed with 0.1X SSC three or four times for a total of 1 to 2 hours at 65°C to remove nonspecific binding. The amount of specific RNA was visualized by autoradiography, and quantitated using a PhosphorImager and Image Quant software (Molecular Dynamics, Sunnyvale, CA). For normalization, the blot was stripped by

washing in boiling hot DEPC-treated water 3 times, allowing the water to come to room temperature between each cycle. Blots were rehybridized with a radiolabeled GAPDH probe that specifically recognized the ~1.3 Kb chicken GAPDH mRNA (see Preparation of radiolabeled probes), and GAPDH mRNA was quantitated in the same manner as cHO-1 mRNA. The levels of HO-1 message in each lane were normalized to the levels of GAPDH message.

Dot blots. Dot blots were performed essentially as described [57]. After isolation, total RNA was resuspended in 50 µL of DEPC-treated water. Samples were prepared for loading as follows, for three duplicate blots: 20 µL of DEPC-treated water, 36 µL of 20X SSC, and 24 µL of 37% formaldehyde were added to 40 µL of RNA and incubated at 65°C for 15 minutes. For each duplicate blot, 200 µL of 10X SSC was added to the samples. The dot blot apparatus was set up with two pieces of prewet Whatman 3M paper and one sheet of prewet nitrocellulose. The wells were washed once with 10X SSC, 200 µL of each sample was loaded per dot, followed by a second wash with 10X SSC. The nitrocellulose was removed from the apparatus and the RNA was UV-crosslinked to the membrane using a Stratalinker. Blots were prehybridized with Quickhyb® at 65°C for 30 minutes. Each blot was hybridized at 65°C for 1 hour in Quickhyb® containing a radiolabeled probe (see Preparation of radiolabeled probes) previously shown by Northern blot to bind only the mRNA of interest. After hybridization, the blots were washed with 0.1X SSC three or four times for a total of 1 to 2 hours at 65°C to remove nonspecific

binding. Quantitation of specific binding was performed using a PhosphorImager and Image Quant software. The amount of specific mRNA was normalized to total poly-A mRNA, measured on a duplicate blot probed with a radiolabeled poly-T probe as described [57] (see Preparation of radiolabeled probes), or to GAPDH mRNA, measured on a duplicate blot probed with a radiolabeled GAPDH probe, as described [57].

Heme oxygenase activity assays. Heme oxygenase activity was measured by a modification of the method of Tenhunen *et al.* [17], as described previously [23,46,107,169]. This assay measures the conversion of heme to biliverdin by a two-step reaction. In the first step heme is converted to biliverdin by heme oxygenase (HO). In the second step, biliverdin is converted to bilirubin by biliverdin reductase (BVR). All mammals express endogenous BVR; however, chickens do not express this enzyme. Exogenous BVR was obtained as a 105,000 x g supernatant of a 20% homogenate of perfused rat liver as described [174]. Under the assay conditions, the amount of BVR added to reactions was capable of reducing 1.5 nmoles of biliverdin/minute in a total volume of 1 mL, enough to ensure complete conversion of all of the biliverdin formed by HO to bilirubin.

LMH cells or CELCs were plated in 6 cm dishes as described above. The dishes were washed twice with 1X PBS, and harvested in 0.6 mL of Harvest Buffer (Appendix A) by scraping with a rubber spatula. Each cell suspension was sonicated for 3 seconds using a sonicator (Bronson model 450, VWR Scientific, Bridgeport, NJ) equipped with a microtip (output: 2, duty cycle: 30). A 50 μL aliquot of the lysate was used for protein determinations. Two sets of 16 x 100 mm culture tubes per sample were set up, one for control and one as test. Each reaction contained 200 μL of cell sonicate (0.6-0.8 mg of homogenate protein from CELCs or 0.4-0.6 mg of homogenate protein from LMH cells), 70 mM potassium phosphate, pH 7.4, 5 mM deferoxamine mesylate

(Appendix B), BVR (20 μL of rat liver 105,000 x g supernatant), and 4 mM NADPH (3.96 mg/mL in 0.1 potassium phosphate) to make a final volume of 1 mL. The tubes were incubated in a 37°C shaking water bath for 5 min. The remainder of the assay was performed in subdued lighting. Test reactions were started by adding heme-albumin (40 μM final concentration, Appendix B) to the test set of tubes, the control tubes received heme-albumin after the reaction was stopped. After 10 minutes at 37°C, the tubes were placed in an ice bath. To stop the reactions, 20 μL of 50 mM p-hydroxymercuribenzoic acid was added to each tube. The tubes were mixed by vortexing. Using a spectrophotometer (Aminco DW 2000), a split beam wavelength scan from 450 to 550 nm was done for each sample. The rate of bilirubin formation was calculated from the difference in absorbance at 470 nm and 540 nm. The following equation was used to calculate the amount of biliverdin formed, as described [17,23,107]: $\Delta\varepsilon$ 470-540 nm = 66 mM^{-1} cm^{-1}.

$$\frac{OD(470\text{-}540) \times 1.010 \text{ mL final volume} \times 1000 \text{ pmole}}{0.066 \text{ OD/nmole bilirubin/mL} \times 0.2 \text{ mL init. vol.} \times 10 \text{ min.} \times \text{mg prot/mL} \times 1 \text{ nmole}}$$

= pmoles bilirubin/minute x mg protein

Subcloning. The pCHO3.6-Luc reporter plasmid was constructed by subcloning 3728 base pairs of the chicken HO-1 proximal promoter from a CAT construct (pCHO3.6-CAT, provided by T. H. Lu) into the pGL3 Basic plasmid vector, upstream of the luciferase reporter gene. The ligation junctions were verified by sequencing.

Deletion constructs were produced by first cloning a 7.1 Kb fragment of the genomic chicken HO-1 promoter into the pGL3 basic luciferase reporter vector, followed by restriction enzyme digestion and self-ligation of the backbone to excise the deleted region. A lambda FIX® II vector containing approximately 12 Kilobases (Kb) of genomic chicken heme oxygenase-1 (cHO-1) sequence, including some coding sequence and ~10.5 Kb of promoter sequence, was digested with the restriction enzymes Xba I and Xho I. The 7.1 Kb fragment (sequence reported in Appendix E) was ligated into a pGL3 Basic luciferase reporter vector that had been digested with the restriction enzymes Nhe I and Xho I. The ligation mix was transformed into DH5α cells plated on Luria Broth agar plates supplemented with 100 μg/mL of ampicillin, and incubated overnight at 37°C. DNA minipreps for several colonies were tested for insertion of the 7.1 Kb fragment by restriction enzyme digests. Colonies that were positive for pCHO7.1-Luc were verified by sequencing the ligation junctions using the commercial primers GLprimer2 and RVprimer3 (Promega). Deletion constructs were made by digesting pCHO7.1-Luc with Mlu I and the respective enzyme for each construct (see Figure 5.1). Restriction enzyme cut

sites were blunted prior to ligation: for a 3' overhang, the DNA was treated with T4 Polymerase; for a 5' overhang, the DNA was treated with Klenow fragment. Following ethanol precipitation, blunt-end ligation was performed. The Xho I sites were ligated proximal to the start of the luciferase gene. The distal restriction enzyme site corresponding to each deletion was ligated with the Mlu I site at -7085 bp (this numbering is relative to the 7.1 Kb fragment). Positive colonies were identified by DNA minipreps followed by a diagnostic Xba I and Xho I restriction enzyme digest. The ligation junctions of each deletion construct were verified by sequencing.

Site-directed mutagenesis. AP-1 elements located at -1578 bp, -3656 bp, and -4597 bp upstream of the cHO-1 transcription start site were mutated, as shown in Figures 5.7 - 5.9. For each AP-1 site, two of the seven bases that comprise the consensus AP-1 element were mutated. The two changes were made in base pairs that disrupt the wild type palidromic structure, and would therefore be likely to be important for transcription factor binding [117]. The bases chosen for mutagenesis have also been reported to interfere with transcription factor binding [27,37,49,87,111,160]. AP-1 mutants were constructed with the QuickChange™ Site-Directed Mutagenesis Kit using templates and PCR primers as listed in Table 2.1. Table 2.2 lists the primer sequences. The pCHO2.5-Luc mutants have two base-pair mutations in the AP-1 element at -1578 bp. The pCHO4.6-Luc mutants have two base-pair mutations in the AP-1 element at -3656 bp, at -1578 bp, or at both sites. The pCHO5.6-Luc series are single, double, or triple mutants containing two base-pair mutations in the AP-1 elements at -4597 bp, -3656 bp, -1578 bp, at two of these sites, or at all three sites. The PCR reactions were set up with 5 μL of 10X reaction buffer, 30 ng of DNA template, 125 ng of each oligonucleotide primer, and 1μL of dNTP mix in a final volume of 50 μL. Just before starting thermocycling program, 2.5 U of Pfu DNA Polymerase was added to each reaction and 30 μL of mineral oil were used to overlay the liquid in each tube. The PCR thermocycling program was performed as suggested in the manufacturer's protocol: segment 1, soak for 30 seconds at 95°C; segment 2, 14 cycles of: denaturation for 30 seconds at 95°C;

renaturation for 1 minute at 55°C, and synthesis for 2 min./Kb of plasmid length (16 minutes for mutations of pCHO2.5-Luc; 20 minutes for mutations of pCHO4.6-Luc; 23 minutes for mutations of pCHO5.6-Luc) at 68°C; segment 3, soak at 4°C. The wild type DNA was then digested with 10 U of Dpn I for 1 hour at 37°C. 1 - 3 μL of mutant DNA was transformed into 50 μL of supercompetent *E. coli* cells as described.

Table 2.1: Templates and Primers for Site-Directed Mutagenesis of AP-1 Elements. The cHO-1 AP-1 mutants were constructed using the listed templates and primers. Four sets of primers were used in combination with wild type or confirmed mutant constructs, as shown, to obtain all of the listed mutants (see Figures 5.7-5.9). F = forward primer, R = reverse primer. See Table 2.2 for primer sequences.

Mutant construct:	PCR Template:	PCR Primers Used:	
pCHO2.5mut1-Luc	pCHO2.5-Luc	2.5mut F1	2.5mut R1
pCHO2.5mut2-Luc	pCHO2.5-Luc	2.5mut F2	2.5mut R2
pCHO4.6mut1-Luc	pCHO4.6-Luc	2.5mut F1	2.5mut R1
pCHO4.6mut2-Luc	pCHO4.6-Luc	2.5mut F2	2.5mut R2
pCHO4.6mut3-Luc	pCHO4.6-Luc	4.6mut F	4.6mut R
pCHO4.6mut13-Luc	pCHO4.6mut36-Luc	2.5mut F1	2.5mut R1
pCHO4.6mut23-Luc	pCHO4.6mut36-Luc	2.5mut F2	2.5mut R2
pCHO5.6mut1-Luc	pCHO5.6-Luc	2.5mut F1	2.5mut R1
pCHO5.6mut2-Luc	pCHO5.6-Luc	2.5mut F2	2.5mut R2
pCHO5.6mut3-Luc	pCHO5.6-Luc	4.6mut F	4.6mut R
pCHO5.6mut4-Luc	pCHO5.6-Luc	5.6mut F	5.6mut R
pCHO5.6mut13-luc	pCHO5.6mut1-Luc	4.6mut F	4.6mut R
pCHO5.6mut14-Luc	pCHO5.6mut4-Luc	2.5mut F1	2.5mut R1
pCHO5.6mut24-Luc	pCHO5.6mut4-Luc	2.5mut F2	2.5mut R2
pCHO5.6mut34-Luc	pCHO5.6mut4-Luc	4.6mut F	4.6mut R
pCHO5.6mut134-Luc	pCHO5.6mut14-Luc	4.6mut F	4.6mut R

Table 2.2: Primers for Site-Directed Mutagenesis. The PCR primers used for site-directed mutagenesis are grouped in pairs. The AP-1 elements are underlined, with the mutated bases in **bold** lettering. The wild type AP-1 sequence is: 5'–TGAGTCA–3'. The templates used with each primer pair are listed in Table 2.1.

Primer name:	Primer Sequence:
2.5 mut F1	5′– GCAGAGCAAG ACAGGAAAAG CATGGCT**TC**G TCAGGCTGGG AGCGCTGAG –3′
2.5 mut R1	5′– CTCAGCGCTC CCAGCCTGAC **GA**AGCCATGC TTTTCCTGTC TTGCTCTGC –3′
2.5 mut F2	5′– GACAGGAAAA GCATGGC**G**GA GTC**G**GGCTGG GAGCGCTGAG –3′
2.5 mut R2	5′– CTCAGCGCTC CCAGCC**C**GAC TC**C**GCCATGC TTTTCCTGTC –3′
4.6 mut F	5′– GCAAGATCTG GTTGCTGAG**G AA**GCCCCCCT CCCAGCCAGG C –3′
4.6 mut R	5′– GCCTGGCTGG GAGGGGGGCT **TC**CTCAGCAA CCAGATCTTG C –3′
5.6 mut F	5′– GCAAAGGGGC CCTTAGTTGC TGAG**GA**ACAG CAGAGAGGCC ACGTGAGG –3′
5.6 mut R	5′– CCTCACGTGG CCTCTCTGCT GT**TC**CTCAGC AACTAAGGGC CCCTTTGC –3′

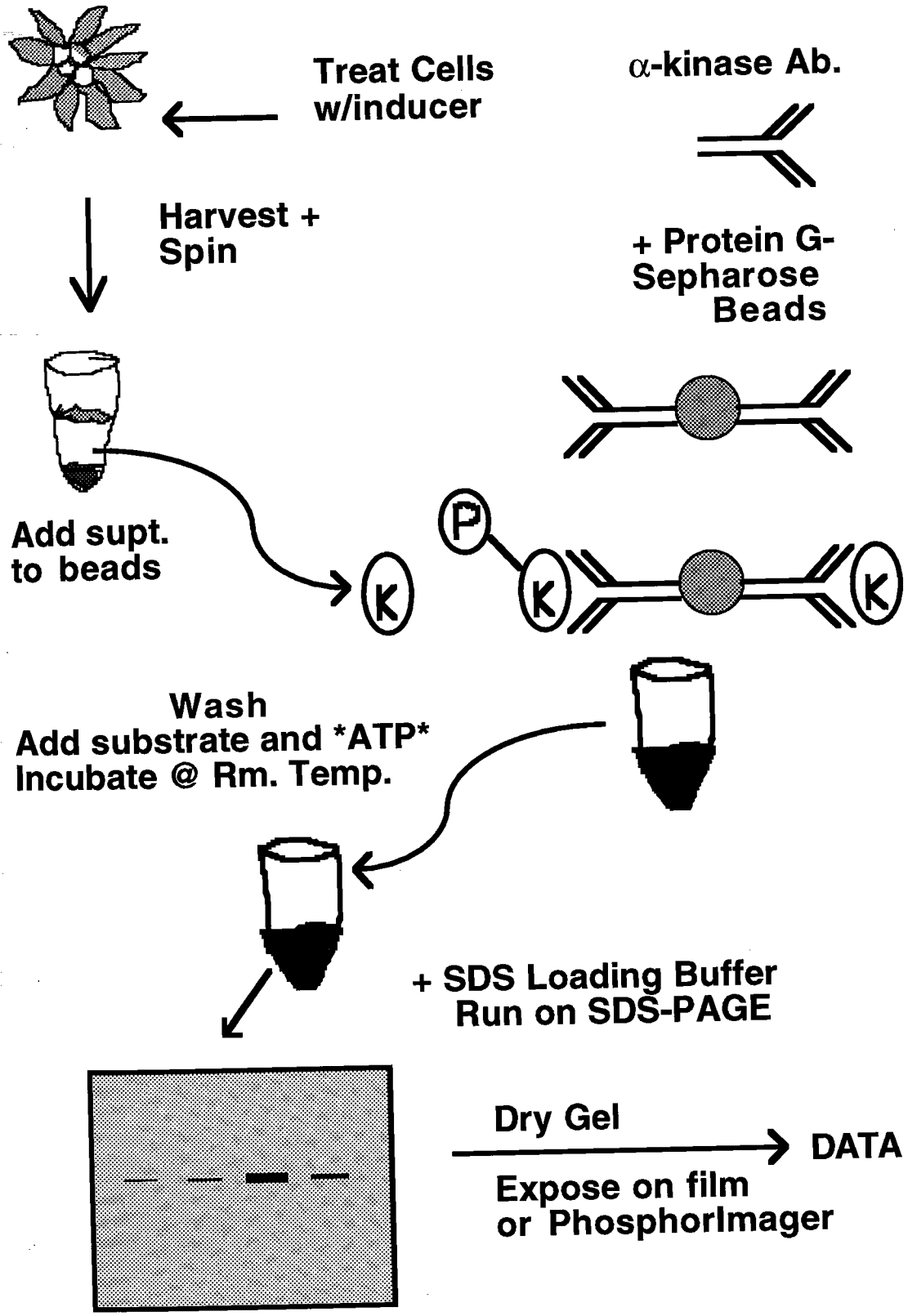
Transfections. LMH cells were plated in gelatin coated 6-well plates at a density of 3.2 x 10^5 cells/well. For transfections, each well received 0.5 µg of pSV-ßgal or pPGK-ßgal, and 0.5 - 1.0 µg of cHO-1 promoter/reporter and/or MAP kinase plasmid DNA using LipoFECTAMINE™ (0.25µg DNA/µL of reagent), according to the manufacturer's protocol. Total DNA transfected was kept constant by adding pBLUESCRIPT KS II+ plasmid DNA. Cells were incubated at 37°C, 3% CO_2 for 5 hours, after which 2 mL of Waymouth's complete medium was added. Incubation was continued overnight at 37°C, 5% CO_2 for a total of 20 - 24 hours. Cells were incubated with serum-free Waymouth's for at least 20 hours prior to treatment with selected chemicals or harvest for assays.

Assessment of reporter gene activity. Reporter gene expression was assessed by quantitation of luciferase activity, normalized to ß-galactosidase activity, and protein content. Some experiments, in which dominant negative ERK kinase components were transfected, were normalized to protein content only, because the ß-galactosidase values were outside the linear range (0.2 to 2.0 absorbance units). For luciferase activities, transfected cells were washed twice with 1X PBS, and harvested by scraping in 250 µL of glycylglycine harvest buffer (Appendix A). Cells were lysed by three cycles of freeze-thaw (3 minutes in liquid nitrogen followed by 3 minutes at 37°C), followed by a 10 minute centrifugation at 14,000 x g at 4°C. The supernatant was retained, and 15 µL aliquots of cell lysate were used for each assay. For luciferase activity measurements, luminometer tubes containing lysates were placed into a Monolight 2010® Luminometer (Analytical Luminescence Labs, Ann Arbor, MI), and 100 µL of Luciferase Assay Reagent was automatically injected. Relative light units produced in 10 seconds were recorded and normalized with ß-galactosidase activities and protein content. For ß-galactosidase activities, 15 µL of cell lysate was added to 200 µL of Z Buffer (Appendix A) and 100 µL of 5 mg/mL O-nitrophenyl galactoside (ONPG) dissolved in 0.1 M potassium phosphate, pH 7.0. The samples were mixed and incubated at 37°C for 1.5 - 2 hours. The absorbance at 420 nm was measured and used for normalization of luciferase activities [173].

Protein assays. Protein concentrations were measured by the bicinchoninic acid method on a Spectronic GENESYS 2 spectrophotometer, using BSA as standard [175]. Protein content of cell lysates was calculated from the BSA standard curve and used for calculation of heme oxygenase activity or for normalization of luciferase activity.

Immune complex kinase assays. Immune complex kinase activity assays were performed as described [176]. Cells were harvested by scraping into 1.0 mL of 1X Triton lysis buffer (TLB, Appendix A). Lysates were centrifuged at 14,000 x g at 4°C for 15 minutes. Anti-ERK2, anti-JNK1 (Santa Cruz Biotechnology, Inc.) or anti-p38 [176] antibodies were bound to Protein G Sepharose beads (10 µL per assay) for at least 30 minutes and washed twice with 1X TLB prior to adding cell lysates. Pre-bound antibodies were incubated with 300 µL of cell extract in a final volume of 500 µL with 1X TLB. After mixing at 4°C for at least 3 hours, the immunoprecipitates were washed three times with 1X TLB, and once with 1X kinase assay buffer (Appendix A). After aspiration, the kinase assay was set up with 10 µL Protein G Sepharose beads/antibody/kinase complex, 26 µL kinase assay buffer, 2 µL of kinase substrate (2 µg of GST-Elk1 for ERK [163], GST-ATF2 for p38 [176], and GST-cJun for JNK [164]), 1 µL 1 mM ATP, 1 µL carrier-free [γ-^{32}P] ATP (approximately 10 µCi/µL) for a final volume of 40 µL. Samples were incubated at room temperature for 30-45 minutes. The reactions were stopped with 3X SDS loading buffer, and 15-20µL were run on SDS-PAGE. This method is schematically represented in Figure 2.1. Results were visualized by autoradiography and quantitated using a PhosphorImager and Image Quant software (Molecular Dynamics, Sunnyvale, CA).

Figure 2.1: Immune-Complex Kinase Activity Assays.



Statistical analysis of data. Experiments were repeated two to four times; except for immune complex kinase assays and Northern blots, every experiment included at least triplicate samples for each treatment group. Representative results from single experiments are presented. Statistical analyses were performed with JMP 3.0.2 software (SAS Institute, Cary, NC). Differences in mean values were assessed by ANOVA, with the Tukey-Kramer correction for multiple pair-wise comparisons, or Dunnett's test versus a control. For experiments with non-normally distributed data, the Wilcoxon/Kruskal-Wallis (Rank Sums) test was used. P values <0.05 were considered significant.

CHAPTER III

CHARACTERIZATION OF HEME BIOSYNTHETIC PATHWAYS IN THE CHICKEN HEPATOMA CELL LINE, LMH

Introduction

The purpose of the studies in this chapter was to characterize porphyrin and heme biosynthesis in a potential model cell system, LMH cells. The accumulation of porphyrins (the constituents of the heme biosynthetic pathway) and expression and regulation of ALA synthase (normally the rate-limiting enzyme of heme biosynthesis) were studied.

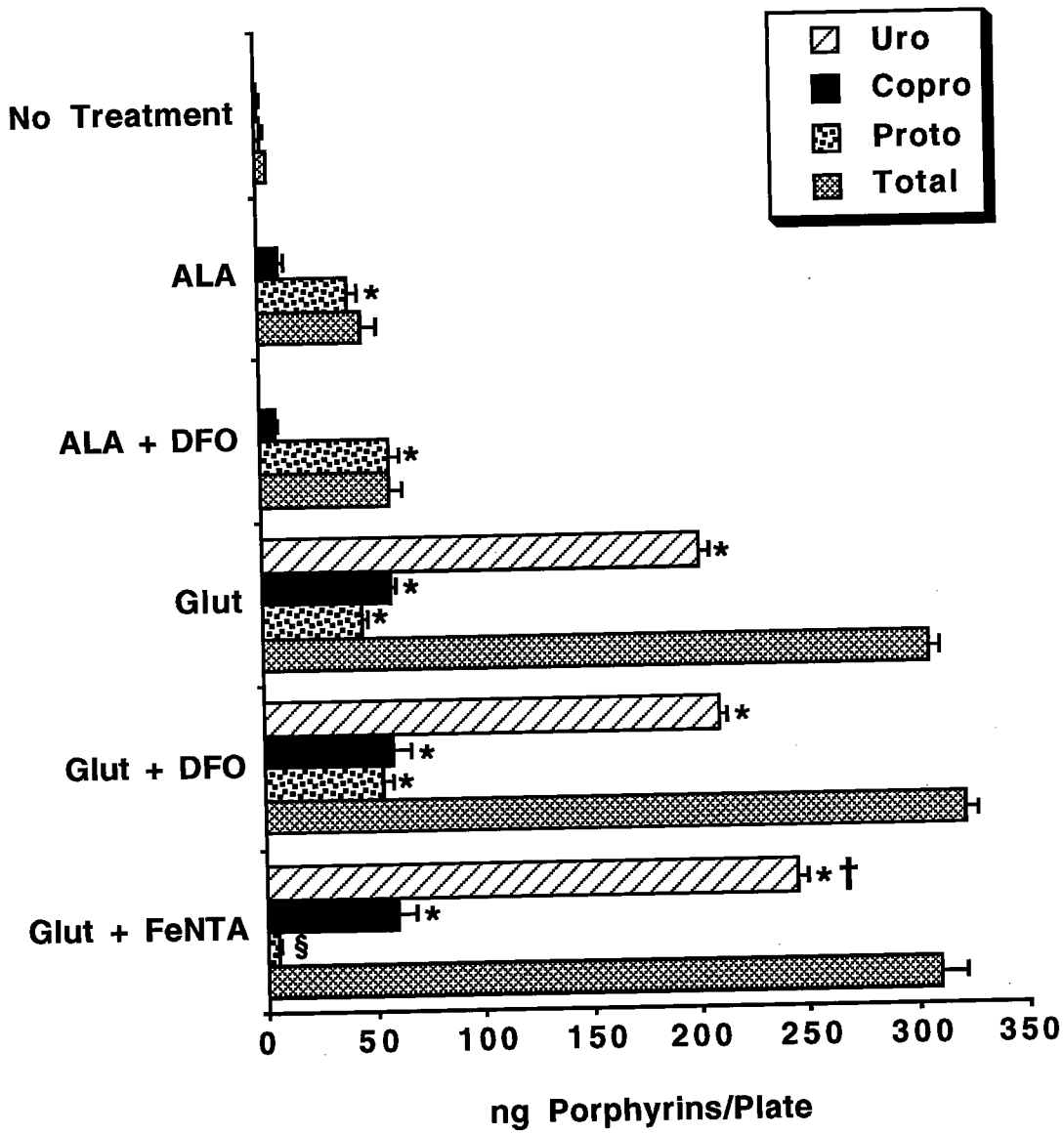
A widely used model system for studies of the regulation of heme metabolism is primary cultures of chick embryo liver cells (CELCs). Primary CELCs retain all of the characteristics of normal hepatic function and mimic human metabolism more closely than mammalian systems, such as rat. LMH cells were derived from a liver tumor dissected from a male White Leghorn chicken that had been treated with intraperitoneal injections of diethylnitrosamine. Preliminary experiments were performed to investigate the heme biosynthetic pathway in these cells, since this is a transformed cell line in which heme metabolism had not been characterized previously. The purpose of these studies was to ensure that the pathways of heme biosynthesis in LMH cells were intact as compared to CELCs, and to determine if LMH cells would be an appropriate model system for the study of heme biosynthesis.

Results

LMH cell culture and growth rate. LMH cells were routinely passaged 1:4 or 1:6 approximately twice a week. Cells were cultured in 25 mL flasks coated with 0.1% gelatin. It was later discovered that the cells attach and grow normally even in the absence of gelatin. After routine culture conditions for LMH cells had been established, their growth rate was measured by trypan blue staining. The number of cells per plate was counted at the time of plating (0 hours), and again at 12, 24, 48, and 72 hours. The average doubling time was calculated to be 21 hours.

Porphyrin accumulation in LMH cells versus primary CELCs. Defects in the biosynthetic enzymes lead to accumulation of certain types of porphyrins. Chemicals that increase the production of porphyrins, either alone or in combination with chemicals that block steps in heme biosynthesis, were used to analyze the response of LMH cells as compared to primary CELCs. Primary CELCs have shown increased production of porphyrins in response to δ-aminolevulinic acid (ALA), glutethimide (Glut), and the combination of glutethimide and ferric nitrilotriacetate (FeNTA). In Figure 3.1, LMH cells were given exogenous ALA to increase porphyrin production. When treated with ALA alone or in combination with the ferrochelatase inhibitor, deferoxamine (DFO), LMH cells accumulated mainly copro- and protoporphyrins. At the concentration tested (250 μM), DFO had little effect on porphyrin accumulation.

Figure 3.1: Porphyrin accumulation in LMH cells. LMH cells were treated with 35 μM δ-aminolevulinate (ALA) alone or in combination with 250 μM deferoxamine (DFO), 50 μM glutethimide (Glut) alone or in combination with 250 μM deferoxamine or 50 μM FeNTA for 18 hours prior to harvest. Harvest and assays were performed as described in Methods. Data represent mean + SEM, n=3. *Significantly greater than No Treatment, $P < 0.001$. †Significantly greater than glutethimide alone, $P < 0.001$. §Significantly less than glutethimide alone, $P < 0.001$.


Uro
Copro
Proto
Total
No Treatment
ALA
ALA + DFO
Glut
Glut + DFO
Glut + FeNTA
*
†
§
0
50
100
150
200
250
300
350
ng Porphyrins/Plate

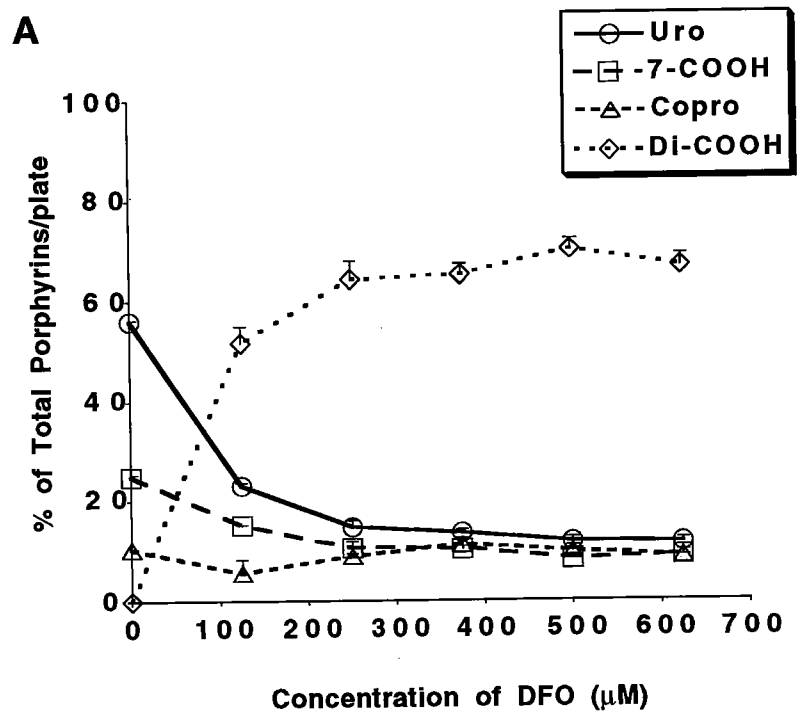
Addition of iron to CELCs by treatment with FeNTA is thought to synergize porphyrin accumulation either by increasing cytochrome P-450 dependent oxidation of porphyrinogen to porphyrin or by inducing HO-1 activity, thereby reducing the size of the regulatory heme pool (See Figure 1.3)[12,13]. When treated with glutethimide alone, LMH cells accumulated large quantities of uroporphyrin, and some copro- and protoporphyrin. The combination of Glut and FeNTA produced a shift in porphyrin levels as compared to glutethimide alone with a decrease in protoporphyrin and an increase in uroporphyrin. FeNTA alone did not cause porphyrin accumulation (data not shown). Surprisingly, when DFO was given in combination with glutethimide, there was no change in porphyrin accumulation. Unlike CELCs, LMH cells did not exhibit a shift from mostly uroporphyrin, with some copro- and protoporphyrin to mostly protoporphyrin in the presence of DFO. When given in combination with either ALA or Glut, 250 μM DFO had no effect on porphyrin accumulation in LMH cells.

Chelation of intracellular iron should remove iron from the heme biosynthetic pathway resulting in inhibition of iron incorporation into protoporphyrin to form heme by the final enzyme of heme biosynthesis, ferrochelatase. This should result in an increase in protoporphyrin, with a corresponding decrease in uro- and coproporphyrins. The lack of effect of DFO in LMH cells was further investigated by inducing porphyrin production with glutethimide, and then treating with increasing doses of DFO. To investigate possible differences in iron metabolism between the two cell types, the more

accurate method of high performance liquid chromatography was used to quantitate porphyrins. Figure 3.2 shows the lack of effect of DFO in LMH cells versus primary CELCs. Changes in porphyrin levels (increased protoporphyrin, decreased uro-, 7-carboxyl-, and coproporphyrins) were observed for CELCs, while up to 625 μM DFO caused no change in LMH cells. This lack of effect may be due to differences in iron transporters, the presence of cellular proteins with higher affinity for iron than DFO, or lack of uptake of DFO in LMH cells, so that it is unable to chelate cellular iron and inhibit ferrochelatase activity.

Cellular iron accumulation. Since LMH cells exhibited a noticeable difference in porphyrin accumulation when treated with DFO, cellular iron accumulation was studied. Cellular iron was quantitated in LMH cells or CELCs that had been treated with DFO, or with exogenous sources of iron, either FeNTA or TMH-ferrocene (Figure 3.3). In untreated cells, iron contents were similar. When treated with DFO, CELCs showed no significant change in iron content, LMH cells had no detectable cellular iron, suggesting that LMH cells may not be permeable to DFO, but that it may remove iron from the cells. DFO probably penetrates into CELCs to chelate intracellular iron and remains within the cells, resulting in iron levels similar to control values. There were slight differences in the type of exogenous iron preferred by the two cell types, LMH cells took up FeNTA to a greater degree than TMH-ferrocene, whereas CELCs absorbed more TMH-ferrocene than FeNTA (Figure 3.3). These results are consistent with the hypothesis of a difference in uptake or metabolism of iron

Figure 3.2: HPLC determination of porphyrin accumulation. Porphyrin accumulation was measured in primary CELCs **(A)** and LMH cells **(B)** in the presence of 50 μM glutethimide and increasing concentrations of deferoxamine. Primary CELCs or LMH cells were treated with 50 μM glutethimide and increasing doses of deferoxamine: 0, 125, 250, 375, 500, or 625 μM for 18 hours. Harvest and assays were as described in Methods. Di-carboxyl porphyrin represents protoporphyrin levels, 7-carboxyl porphyrins represent intermediates between uroporphyrin and coproporphyrin. All sample groups represented were treated with 50 μM glutethimide to induce porphyrin synthesis. The rising concentrations of DFO did not significantly alter the values for total porphyrins/mg protein, data was transformed to show percent of total porphyrins/mg protein. Data represent mean + SEM, n=3. **(A)** CELCs; For uro- and 7-carboxyl porphyrins, all data points are significantly lower than glutethimide only (0 μM), $P < 0.01$; for di-carboxyl (proto) porphyrins, all data points are significantly higher than glutethimide only (0 μM), $P < 0.001$. **(B)** LMH cells; no deferoxamine treated group was significantly different from the glutethimide only (0 μM) group.


A
Uro
-7-COOH
Copro
Di-COOH
% of Total Porphyrins/plate
100
80
60
40
20
0
0
100
200
300
400
500
600
700
Concentration of DFO (μM)

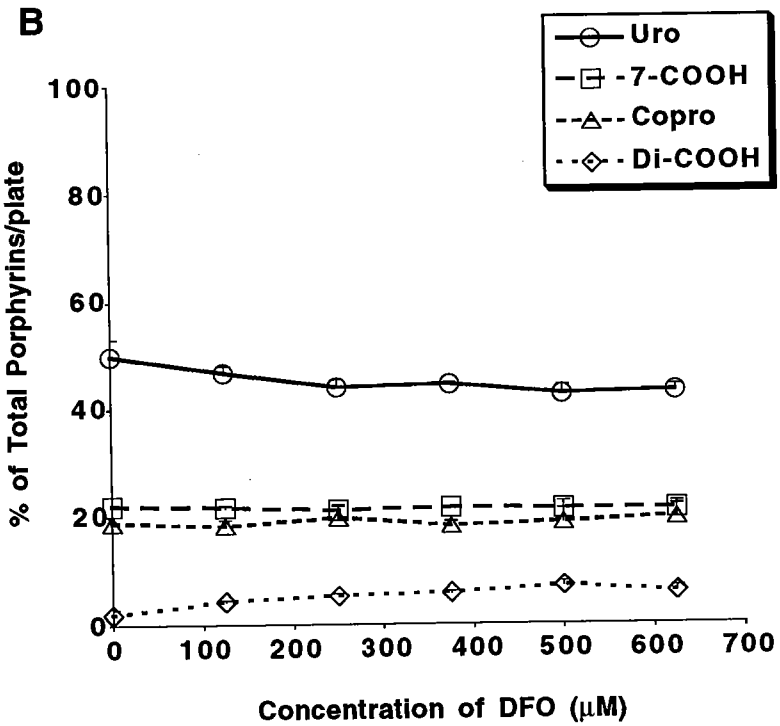
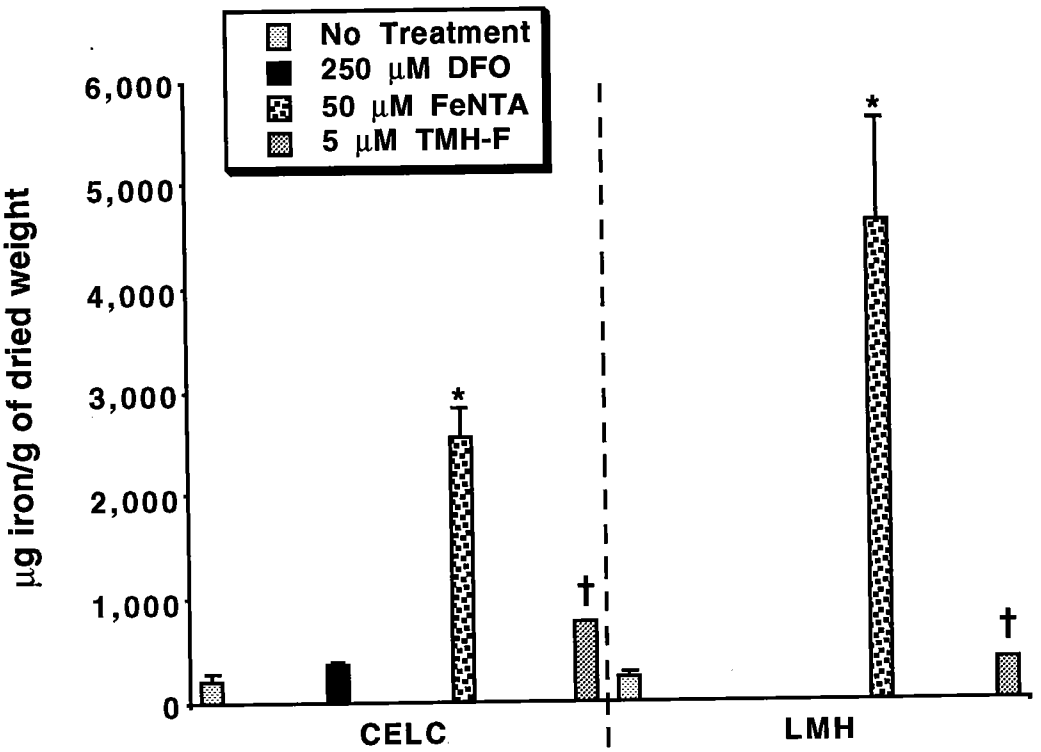

B
Uro
-7-COOH
Copro
Di-COOH
% of Total Porphyrins/plate
100
80
60
40
20
0
0
100
200
300
400
500
600
700
Concentration of DFO (μM)

Figure 3.3: Cellular iron content in CELCs and LMH cells. The effects of an iron chelator and exogenous iron sources on cellular iron content were tested in primary CELCs and LMH cells. Primary CELCs and LMH cells were treated with 250 μM deferoxamine or 50 μM FeNTA for 18 hours, or 5 μM TMH-ferrocene for 4 hours prior to harvest. Harvest and assays were as described in Methods. Data represent mean + SEM, n=3. *Significantly greater than all other treatments, $P < 0.01$. †Significantly different between CELCs and LMH cells by paired t-test, $P < 0.001$.


No Treatment
250 μM DFO
50 μM FeNTA
5 μM TMH-F
μg iron/g of dried weight
6,000
5,000
4,000
3,000
2,000
1,000
0
*
†
*
†
CELC
LMH

between the two cell types. The differences in porphyrin and iron metabolism were not investigated further.

Upregulation of ALA synthase mRNA. ALA synthase is the enzyme that catalyzes the rate-limiting step in heme biosynthesis. In primary CELCs, phenobarbital-like drugs (e.g. glutethimide) caused increased ALA synthase activity and mRNA levels. Synergistic induction of ALA synthase mRNA was observed in response to the combination of glutethimide and FeNTA [45,107].

Since ALA synthase is the key enzyme controlling production of heme, ALA synthase mRNA levels were measured in LMH cells in response to treatments that upregulate or downregulate ALA synthase expression. ALA synthase mRNA was quantitated in LMH cells treated with increasing doses of glutethimide, FeNTA, or both (Figure 3.4). Glutethimide alone produced a modest increase in ALA synthase mRNA, while FeNTA alone had no effect. Synergism was observed in LMH cells given the combined treatment, however higher doses than those in CELCs were required to reach maximal induction.

Downregulation of ALA synthase mRNA. Increased cellular heme leads to decreased ALA synthase expression through several mechanisms: decreased translocation of precursor protein into mitochondria, decreased mRNA translation, and in rat, decreased transcription [1-5]. A time course was performed in LMH cells to test for decreased ALA synthase mRNA in the presence of heme. Figure 3.5 shows that heme decreased the levels of ALA synthase mRNA as early as 2 hours after administration in LMH cells. This

Figure 3.4: Effects of glutethimide, FeNTA, or both, on ALA synthase mRNA levels in LMH cells. LMH cells were exposed to increasing concentrations (0, 25, 50, 100, or 200 μM) of glutethimide, FeNTA, or the combination of glutethimide and FeNTA for 18 hours prior to harvest. Harvest, RNA isolation, and quantitation were performed as described in Methods. Data represent mean + SEM, n=3. *Significantly greater than No treatment and glutethimide alone, $P < 0.001$. **Significantly greater than No treatment or FeNTA alone, $P < 0.05$. †Significantly greater than No treatment and glutethimide alone, $P < 0.01$.

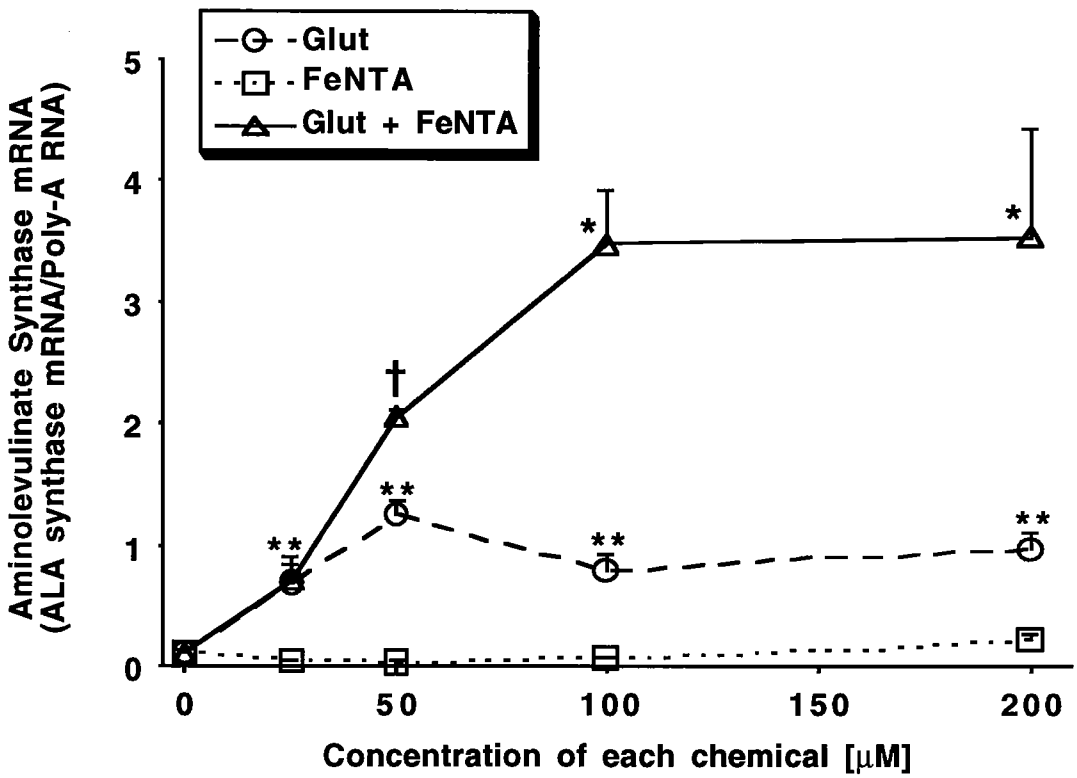
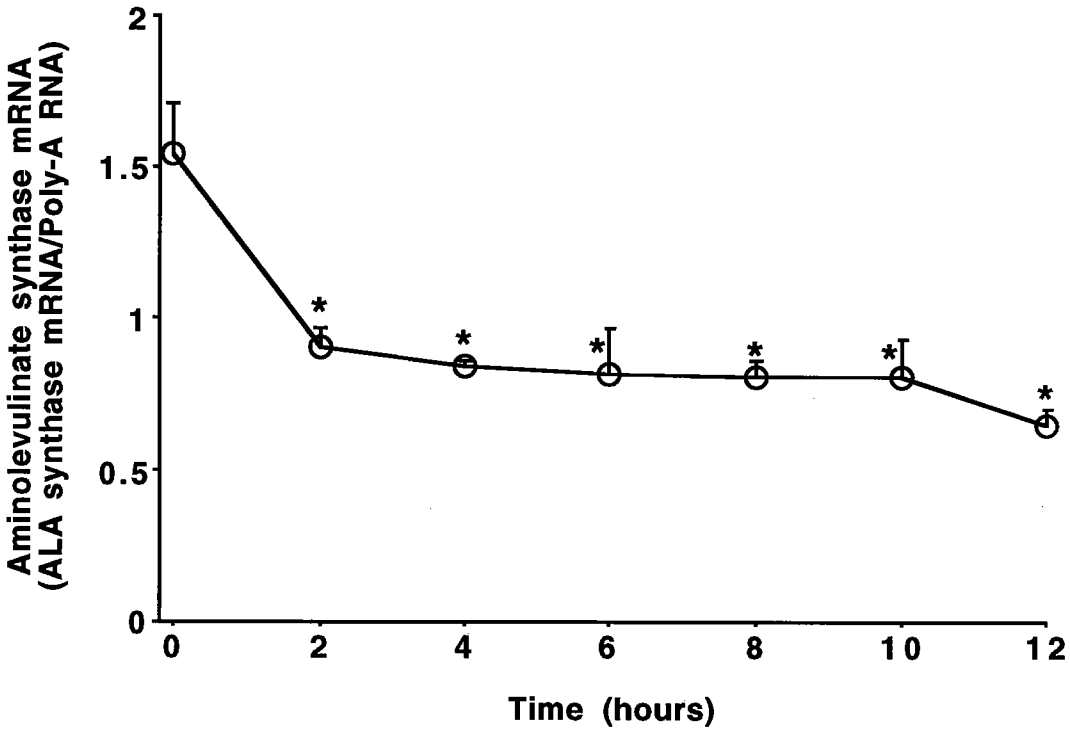


Figure 3.5: Heme-dependent repression of ALA synthase mRNA levels in LMH cells. LMH cells were exposed to 20 μM heme for 2,4,6,8,10, or 12 hours prior to harvest. Harvest, isolation, and quantitation of RNA were performed as described in Methods. Data represent mean + SEM, n=3. *Significantly different from No Treatment (0 hours), $P < 0.001$.



repression was maintained throughout the 12 hour duration of the experiment.

Since the heme-dependent regulation of ALA synthase mRNA is a major component of the normal regulation of heme biosynthesis, a side-by-side comparison of ALA synthase mRNA levels in LMH cells versus CELCs was performed (Figure 3.6). Heme-dependent repression of ALA synthase mRNA was observed for both cell types. In the presence of heme, ALA synthase mRNA levels were 28% of control in CELCs, and 64% of control in LMH cells. The comparison indicated that both the basal and heme-repressed levels of ALA synthase mRNA were higher in LMH cells than in CELCs. This may be due to the differing natures of these two cell types: LMH cells are rapidly metabolizing and have a high growth rate, whereas primary CELCs quickly become dormant, eventually dying within 7 - 10 days.

Regulation of ALA synthase mRNA by metalloporphyrins in LMH cells. Finally, metalloporphyrins other than heme have regulatory effects on ALA synthase activity and mRNA levels [4,44,57,177]. Tin mesoporphyrin and zinc mesoporphyrin repressed ALA synthase activity and mRNA in primary CELCs that had been induced with glutethimide and FeNTA [44,57]. Copper protoporphyrin caused a slight increase in induced activity and manganese protoporphyrin was not tested [44]. Manganese protoporphyrin, copper protoporphyrin, tin mesoporphyrin, zinc mesoporphyrin, and iron protoporphyrin (heme) were tested for the ability to regulate ALA synthase mRNA levels in LMH cells (Figure 3.7). Both concentrations of heme tested reduced ALA synthase

Figure 3.6: Comparison of heme-dependent repression of ALA synthase mRNA in CELCs and LMH cells. Cells were either left untreated or treated with 20 μM heme for 6 hours prior to harvest. Harvest, isolation, and quantitation of RNA were performed as described in Methods. Data represent mean + SEM, n=4. *Significantly different from all others, $P < 0.001$. †Significantly different from untreated LMH cells, and from Heme-treated CELCs, $P < 0.01$. §Significantly different from untreated CELCs, $P < 0.01$.

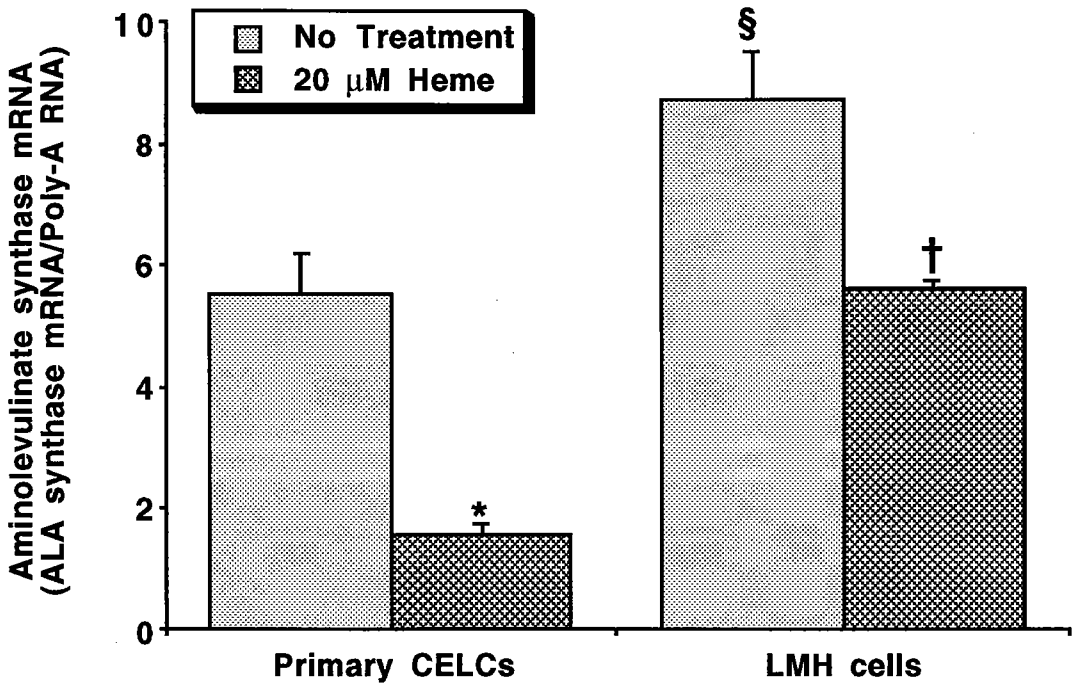
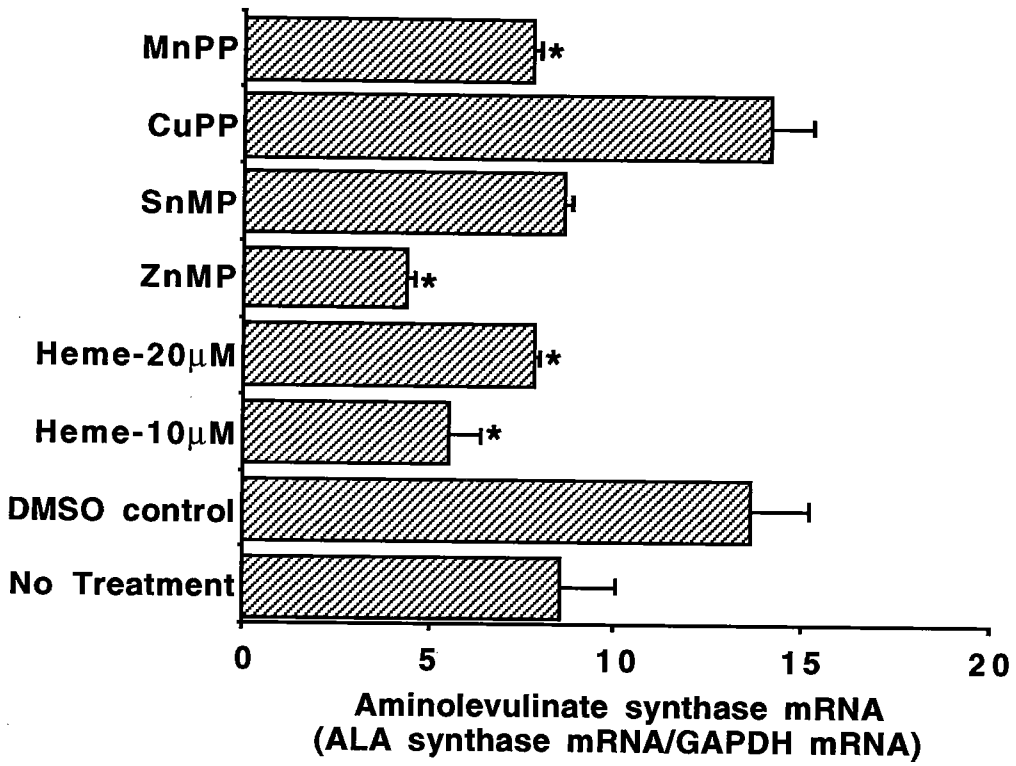

No Treatment
20 μM Heme
Aminolevulinate synthase mRNA
(ALA synthase mRNA/Poly-A RNA)
10
8
6
4
2
0
*
§
†
Primary CELCs
LMH cells

Figure 3.7: Effects of metalloporphyrins on ALA synthase mRNA levels in LMH cells. Cells were treated with 10 μM or 20 μM heme, or 10 μM of the other metalloporphyrins: manganese protoporphyrin, copper protoporphyrin, tin mesoporphyrin, or zinc mesoporphyrin, for 6 hours prior to harvest. The metalloporphyrins were dissolved in DMSO, therefore, a DMSO solvent (1 μL per mL of culture medium) control was also included. Harvest, isolation, and quantitation of RNA were performed as described in Methods. Data represent mean + SEM, n=3. *Significantly less than DMSO control, $P < 0.0055$.



mRNA levels in LMH cells. Manganese protoporphyrin and zinc mesoporphyrin also decreased levels of ALA synthase mRNA. Repression by tin mesoporphyrin was not statistically significant. No repression was observed for copper protoporphyrin and the DMSO solvent control.

Conclusions

LMH cells exhibited porphyrin and heme metabolism similar to that of primary CELCs. Unlike primary rat hepatocyte cultures, LMH cells more closely exhibit characteristics observed in primary CELCs, porphyrin accumulations are inducible and regulation of ALA synthase seems normal.

The major difference between primary CELCs and LMH cells was in the type of porphyrin that accumulated, especially in the presence of deferoxamine. There may be differences in cellular iron metabolism. In all other aspects of heme biosynthesis tested, LMH cells were similar to primary CELCs, supporting the use of this cell line for studies of heme metabolism and its regulation.

CHAPTER IV

CHARACTERIZATION OF HEME OXYGENASE-1: COMPARISON OF LMH CELLS TO PRIMARY CULTURES OF CHICK EMBRYO LIVER CELLS

Introduction

Heme oxygenase catalyzes the degradation of heme into biliverdin, carbon monoxide, and iron. Two forms of this enzyme, heme oxygenase-1 and -2, have been identified; only heme oxygenase-1 (HO-1) is subject to induction by heme, metal ions, and other chemical and physical perturbations (e.g. drugs, oxidants, and heat shock). Primary chick embryo liver cells (CELCs) are widely used for the study of heme metabolism because of their ease of preparation, low cost, and high degree of similarity to human heme metabolism. Nonetheless, this system has some limitations: new cultures must be prepared every week; the resulting cell populations are somewhat heterogeneous; and cells are short-lived, limiting the feasible duration of time course and transfection studies. LMH cells are the first chicken hepatoma cell line to be established.

The aim of the work summarized in this chapter was to characterize the regulation of heme oxygenase-1 in LMH cells, and to compare this regulation to that previously described in primary CELCs [23,44,56,57,107,169,178]. Initially, culture

conditions which provided optimal induction of HO-1 were established. The induction of HO-1 was then assessed by measuring changes in mRNA levels or enzyme activities in response to several treatments, including heme, metals, sodium arsenite, and heat shock, which have been shown to increase the expression of heme oxygenase [23,29,32,51,52,74,85,89-91,100,178]. The comparative dose-responses and time courses of induction of HO-1 mRNA and heme oxygenase activities by heme and cobalt chloride were characterized. In addition, LMH cells exhibited a measurable response to other treatments previously shown to increase HO-1 mRNA levels or heme oxygenase activities in normal CELCs. Similarities were observed with respect to regulation of HO-1 expression in primary hepatocytes and LMH cells. During these studies, a small but significant heat shock induction of HO-1 mRNA in CELCs was detected. This was the first reported measurable heat shock response of HO-1 in CELCs or LMH cells. These experiments support the use of LMH cells as a model for the study of HO-1 regulation.

Results

Initial detection of HO-1 mRNA in LMH cells. For the initial characterization of heme degradation in the LMH cell line, HO-1 mRNA levels were measured in response to treatments previously shown to increase these levels in primary CELCs. To detect a response, LMH cells were treated with heme or cobalt at concentrations and for exposure times formerly shown to produce robust inductions in CELCs, namely, 10 μM heme for 5 hours or 75 μM cobalt chloride for 18 hours [23,46]. Heme caused a 5.8-fold increase and cobalt chloride caused a 3.8-fold increase in HO-1 mRNA levels in LMH cells (Table 4.1).

Gelatin-coated dishes enhance induction in LMH cells. In previous work, others have found that LMH cells required gelatin coating of culture vessels to achieve monolayer growth [69]. In contrast, CELCs are routinely cultured directly on plastic dishes [23,44,45,56,57,59,61,77,109,178]. After initial studies with heme and cobalt chloride had confirmed demonstrable increases in HO-1 mRNA levels in LMH cells, culture conditions were analyzed to optimize the similarities between CELCs and LMH cells and to maximize the inducible response of LMH cells to selected chemicals. Effects of the medium or other factors relating to the conditions of cell cultures were tested. Although gelatin was not necessary for growth of LMH cells, gelatin coating of dishes enhanced the responsiveness of

Table 4.1: Effects of heme or cobalt chloride on levels of HO-1 mRNA in LMH cells. LMH cells were treated with 10 μM heme for 5 hours or 75 μM cobalt chloride for 18 hours prior to harvest. Harvest, isolation, and quantitation of RNA were as described in Methods. For each sample, HO-1 mRNA was quantitated by dot blot and normalized to total poly-A RNA measured on a duplicate blot. Data represent mean + SEM, n=3. *Significantly greater than No Treatment control ($P < 0.0001$).

Treatment	Levels of HO-1 mRNA (Arbitrary Units)	Fold Induction
No Treatment	17.6 ±3.40	-
Heme	102.0 ±12.0*	5.8
Cobalt Chloride	67.5 ±16.2*	3.8

LMH cells to treatments (Figure 4.1). Accordingly, subsequent experiments were conducted with LMH cells grown on gelatin-coated dishes.

Dose response and time course of heme and cobalt induction of HO-1 mRNA. Next, dose-response and time course relationships for heme or cobalt chloride treatment on HO-1 mRNA levels were investigated. LMH cells were treated with increasing doses of heme for 7.5 hours (Figure 4.2A) or cobalt chloride for 18 hours (Figure 4.2B), and HO-1 mRNA levels were measured. The data from both treatments describe typical saturation curves. The dose of cobalt chloride required to reach maximal response in LMH cells was found to be slightly higher than that observed for maximal HO activity, and induction of HO-1 mRNA or HO-1 CAT reporter constructs in CELCs [31,36,37,45,57]. Concentrations of ≥20 μM heme, or ≥150 μM cobalt chloride, led to maximal increases in the amount of HO-1 mRNA in LMH cells. Differences in concentrations required for maximal induction (e.g., for cobalt chloride) cannot be attributed to differences in the medium, which was identical for primary CELCs and LMH cells; the only difference in extracellular environment was the presence of gelatin-coating for LMH cells. It is possible that gelatin may bind some chemicals such as cobalt, making them less available to the cells.

In other systems, regulation of HO-1 expression has been shown to occur principally at the level of transcription, unless heme oxygenase activity has been inhibited [18,28,29,44,57,178]. Most previous studies in CELCs quantitated heme

Figure 4.1: Effects of gelatin coating of culture dishes on induction of HO-1 mRNA in LMH cells. Cells were plated on uncoated culture dishes (shaded bars) or on dishes coated with 0.1% gelatin (hatched bars), and treated with 10 μM heme for 5 hours or with 75 μM cobalt chloride for 18 hours. Harvest, isolation, and quantitation of RNA were performed as described in Methods. Data represent mean + SEM, n=3. *Significantly greater than control, ($P < 0.0005$). †Significantly greater than the same treatment without gelatin coating, ($P < 0.05$).

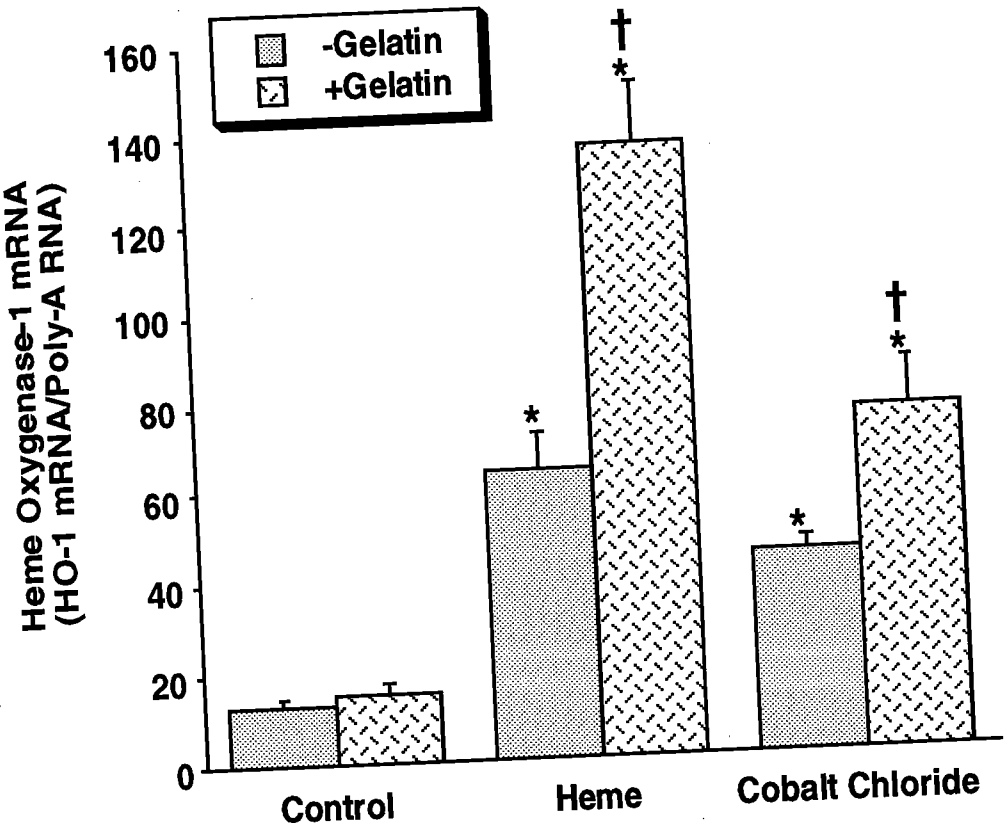
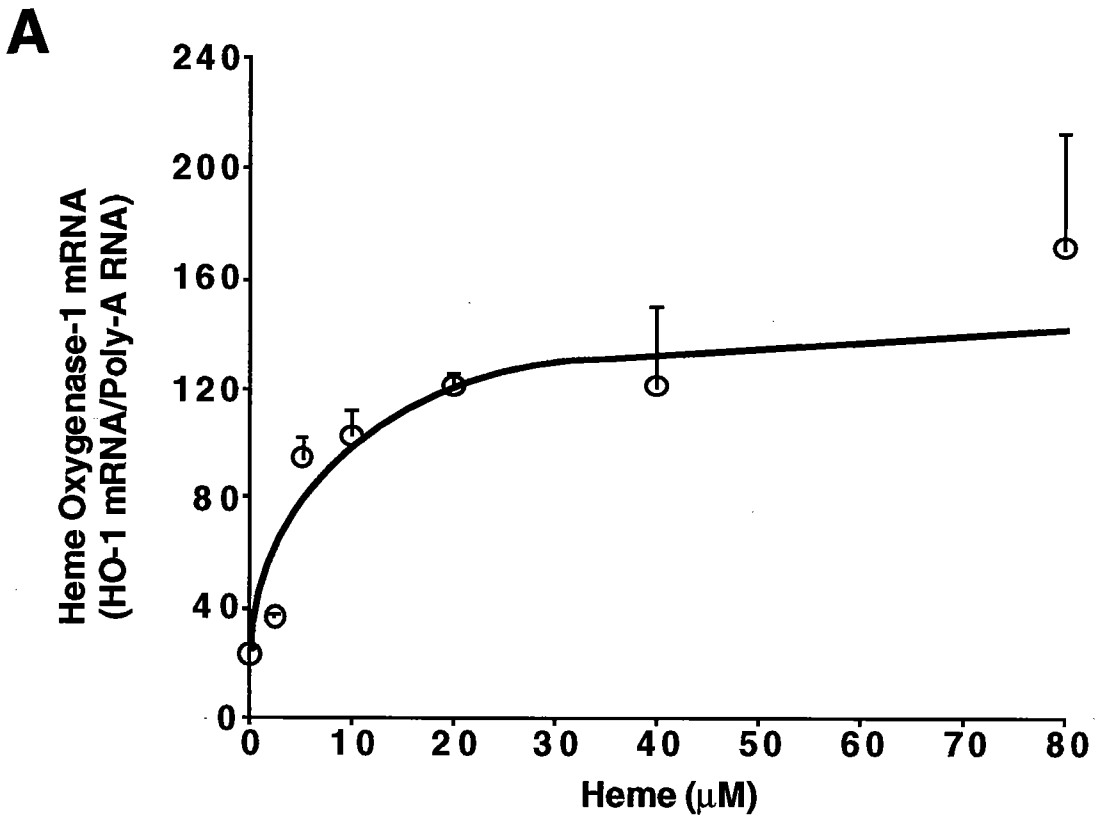
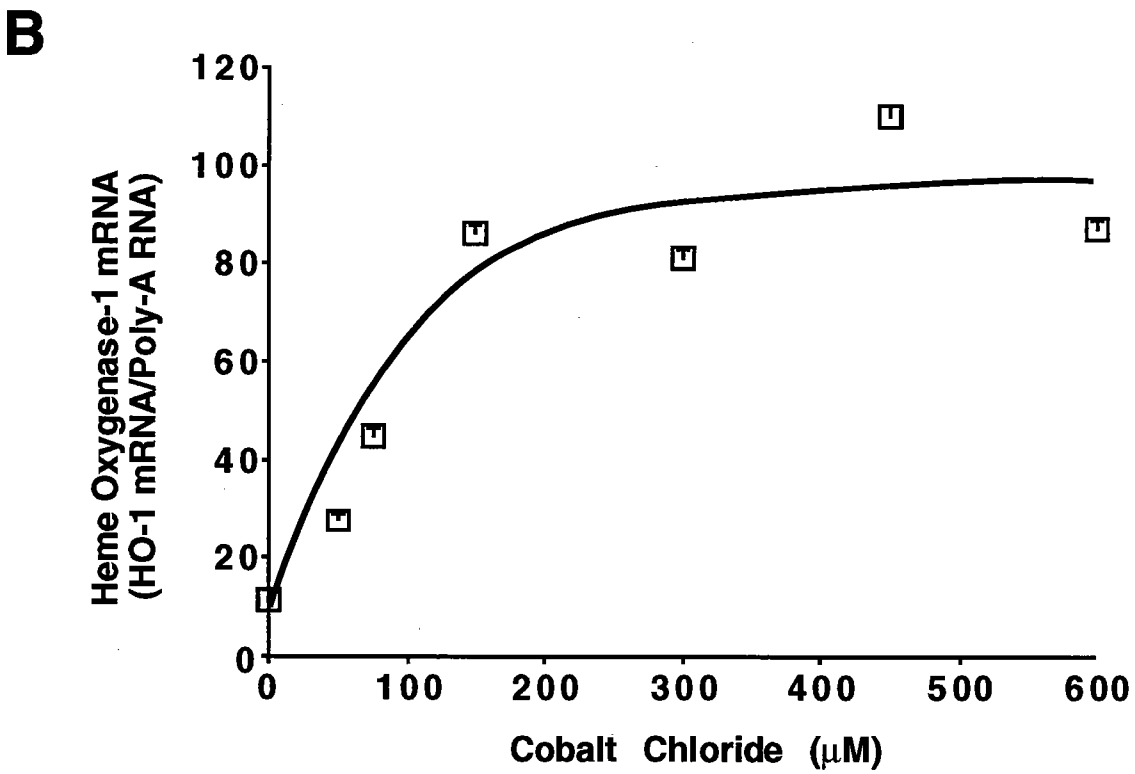


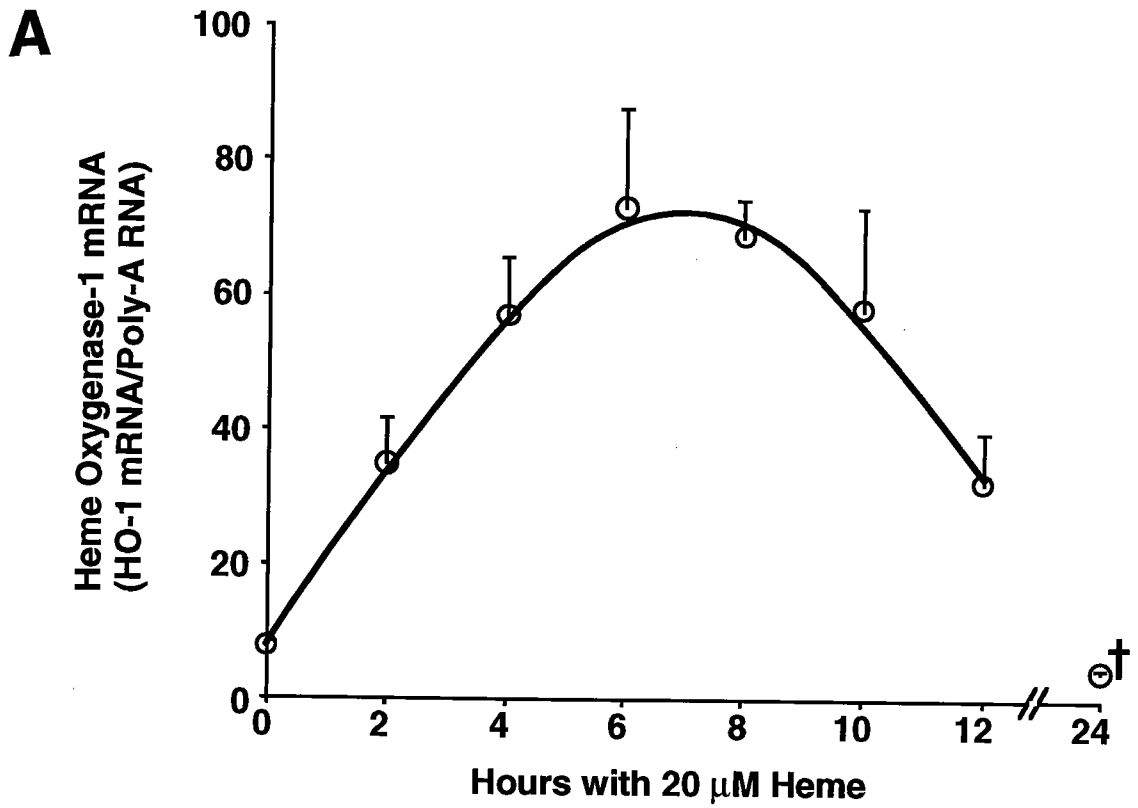
Figure 4.2: Dose-response curves for induction of HO-1 mRNA by heme or cobalt chloride. LMH cells were treated with the indicated concentrations of **(A)** heme for 7.5 hours or **(B)** cobalt chloride for 18 hours. Harvest, isolation, and quantitation of RNA were performed as described in Methods. Data represent means + SEM, n=3.


A
Heme Oxygenase-1 mRNA
(HO-1 mRNA/Poly-A RNA)
240
200
160
120
80
40
0
0
10
20
30
40
50
60
70
80
Heme (μM)


B
Heme Oxygenase-1 mRNA
(HO-1 mRNA/Poly-A RNA)
120
100
80
60
40
20
0
0
100
200
300
400
500
600
Cobalt Chloride (μM)

oxygenase activities, because the increase in activities occurred as a consequence of increased mRNA levels. In CELCs, heme treatment produces a rapid initial increase in levels of heme oxygenase activities and HO-1 mRNA levels followed by a decrease back to control levels by 12 hours [18,23,46,57,178]. In contrast, following addition of cobalt chloride, an initial lag phase of about 12 hours is observed, followed by a steady increase in activities and mRNA levels throughout the duration of the experiment [18,57,77,78,109]. To delineate the temporal response of LMH cells to treatment with heme or cobalt chloride, the induction of HO-1 mRNA levels was studied over 24 hours. As shown in Figure 4.3A, maximal induction was observed 6-8 hours after treatment with 20 μM heme; HO-1 mRNA levels then decreased, reaching control levels by 24 hours. After an initial lag phase of 8-12 hours, 200 μM cobalt chloride caused a steady increase in HO-1 mRNA levels throughout the duration of the experiment (Figure 4.3B). The temporal relationship between treatment and the rise in HO-1 mRNA in LMH cells correlated well with that previously observed in CELCs. For direct comparison, the effects of heme or cobalt chloride on HO-1 mRNA in primary cultures of CELCs versus LMH cells were examined in the same experiment. As shown in Figure 4.4, the fold of maximal induction for each treatment was similar in CELCs and LMH cells; i.e., the efficacy is similar in the two cell types. The potency of heme is also similar among CELCs and LMH cells, but cobalt chloride is more potent in primary CELCs.

Figure 4.3: Time course for induction of HO-1 mRNA by heme or cobalt chloride. Prior to harvest, LMH cells, plated on gelatin-coated dishes, were exposed to either **(A)** 20 μM heme or **(B)** 200 μM cobalt chloride for the indicated times. Harvest, isolation, and quantitation of RNA were performed as described in Methods. Data represent means + SEM, n=3. * No treatment control (24h). †This point is also a 24 hour control.


A
Heme Oxygenase-1 mRNA
(HO-1 mRNA/Poly-A RNA)
100
80
60
40
20
0
0
2
4
6
8
10
12
24
†
Hours with 20 μM Heme

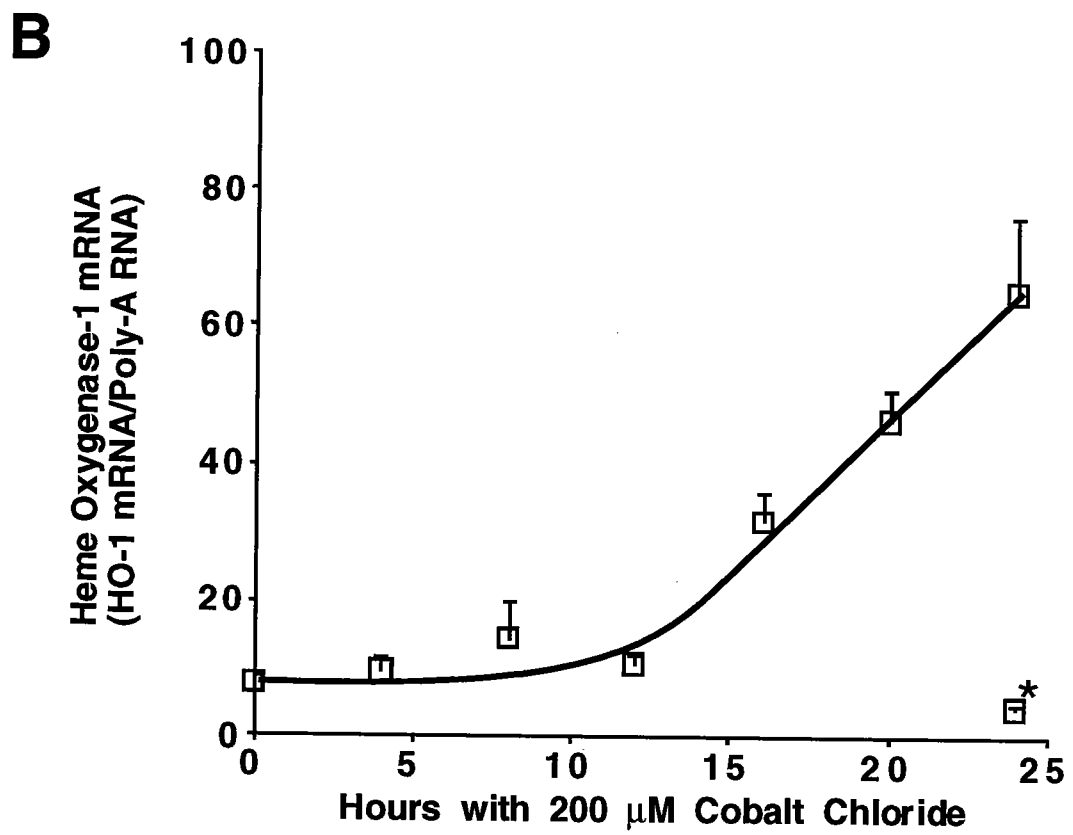
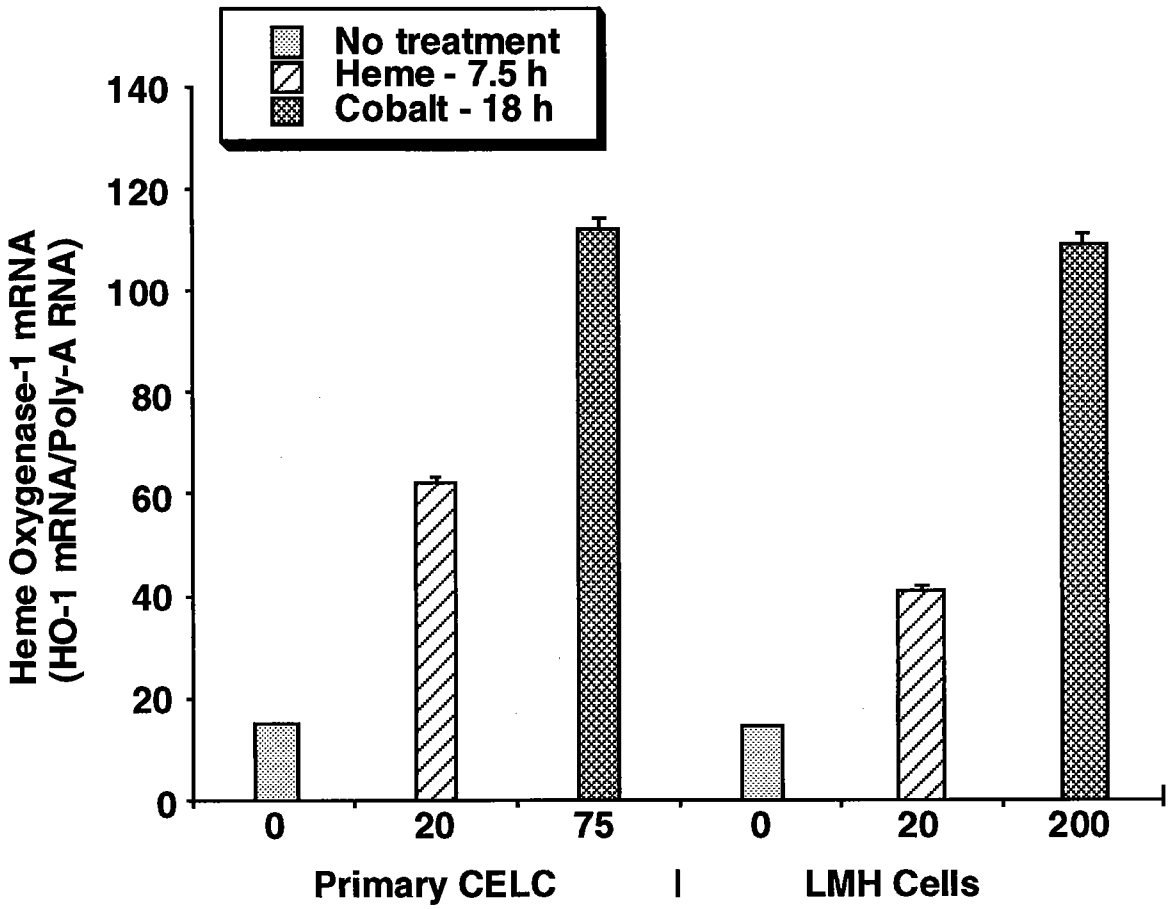

B
Heme Oxygenase-1 mRNA
(HO-1 mRNA/Poly-A RNA)
100
80
60
40
20
0
0
5
10
15
20
25
*
Hours with 200 μM Cobalt Chloride

Figure 4.4: Comparative efficacy of heme or cobalt chloride to increase levels of HO-1 mRNA in CELCs and LMH cells. In a single experiment, both CELCs and LMH cells were simultaneously treated with the appropriate doses of heme or cobalt chloride required for maximal induction for each cell type (heme, 20 μM for both cell types; cobalt chloride, 75 μM for primary CELCs and 200 μM for LMH cells). Cells were treated with heme for 7.5 hours or with cobalt chloride for 18 hours prior to harvest. Harvest, isolation, and quantitation of RNA were performed as described in Methods. Data represent means + SEM, n=4. There were no significant differences in the induction between primary CELCs and LMH cells.


No treatment
Heme - 7.5 h
Cobalt - 18 h
Heme Oxygenase-1 mRNA
(HO-1 mRNA/Poly-A RNA)
140
120
100
80
60
40
20
0
0
20
75
0
20
200
Primary CELC
LMH Cells

Synergistic induction of HO-1 mRNA by glutethimide and ferric nitrilotriacetate (FeNTA). The combination of glutethimide and FeNTA produces a synergistic induction of HO-1 mRNA in CELCs. This induction has been shown to be due to the interdependence of ALA synthase and HO-1 on the levels of cellular heme [45,107]. In Figure 4.5, the ability of glutethimide + FeNTA to produce synergistic increases in HO-1 mRNA was examined in LMH cells. Treatment with glutethimide or the combination of glutethimide + FeNTA increased HO-1 mRNA levels, with the highest induction at 50 μM concentrations of each chemical. FeNTA alone had no significant effect on HO-1 mRNA levels. In addition, synergistic induction of HO-1 mRNA was observed at 50 μM glutethimide + 50 μM FeNTA (Figure 4.5). These data agree with previously observed results for HO activity in CELCs [45,107].

Increased heme oxygenase activity levels correlate with induction of HO-1 mRNA. Similarities in HO-1 regulation for CELCs and LMH cells also were observed when heme oxygenase activities were investigated. To confirm that activities of HO-1 also were increased in LMH cells, both cell types were treated with heme, cobalt chloride, or glutethimide + FeNTA. All treatments increased heme oxygenase activities in a fashion similar to that observed for HO-1 mRNA (Table 4.2). In fact, in this experiment, the heme-mediated induction of activity was greater in LMH cells than CELCs. Glutethimide alone caused no significant increase in heme oxygenase activity in LMH cells (117±6 pmol bilirubin/mg protein). In CELCs, induction of HO-1 mRNA has been correlated

Figure 4.5: Effects of glutethimide, FeNTA, or the combination of glutethimide and FeNTA on levels of HO-1 mRNA in LMH cells. LMH cells were treated with increasing concentrations of glutethimide, ferric nitrilotriacetate, or both, for 18 hours prior to harvest. Harvest, isolation, and quantitation of RNA were performed as described in Methods. Data represent mean + SEM, n=3. *Significantly greater than no treatment control (0 μM), $P<0.05$; †Significantly greater than no treatment control, $P<0.01$; §Significantly greater than glutethimide alone, $P<0.05$.

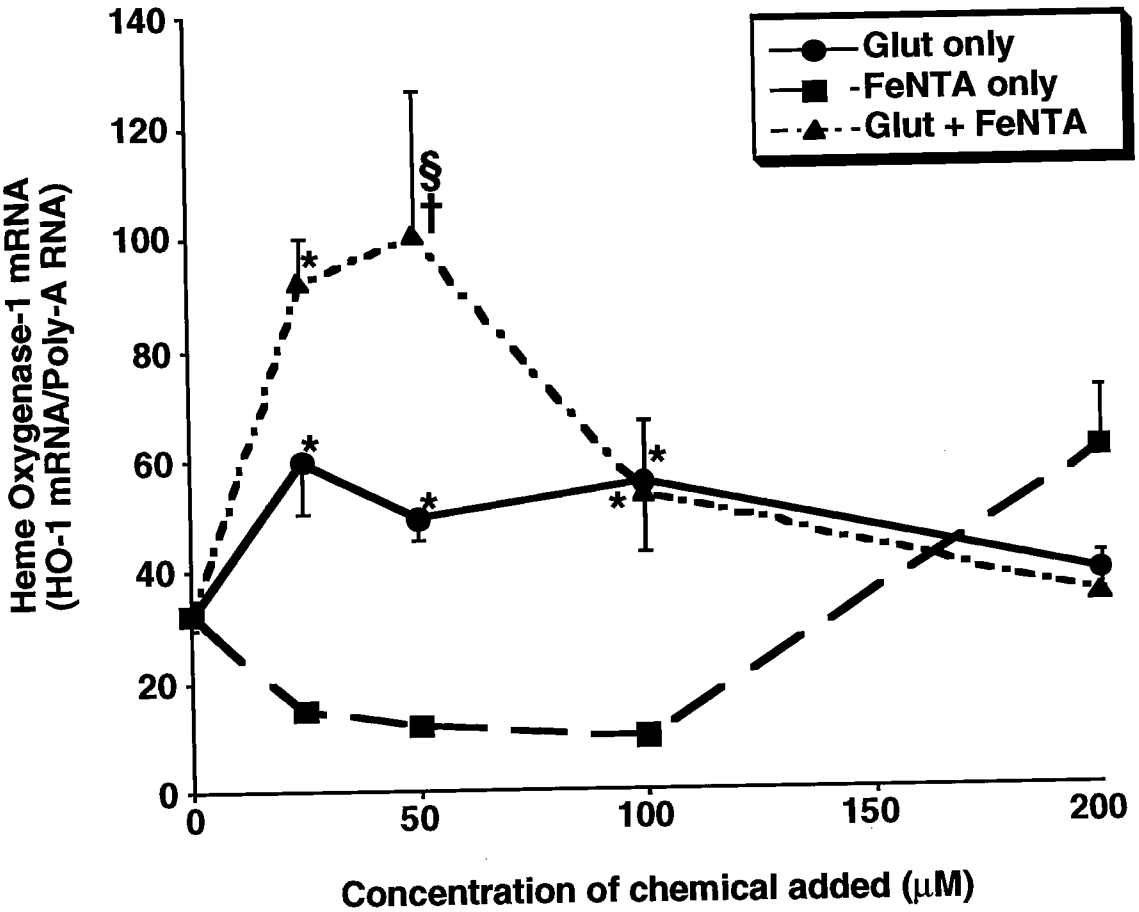

Glut only
-FeNTA only
Glut + FeNTA
Heme Oxygenase-1 mRNA
(HO-1 mRNA/Poly-A RNA)
140
120
100
80
60
40
20
0
0
50
100
150
200
§
*
Concentration of chemical added (μM)

Table 4.2: Comparative effects of selected chemicals on heme oxygenase activities in primary CELCs and LMH cells. Incubation times were: heme - 10 h; cobalt chloride, and the combination of glutethimide and FeNTA - 20 h. Concentrations were: 20 μM heme, 50 μM glutethimide + 50 μM FeNTA for both cell types; 75 μM cobalt chloride for CELCs, and 200 μM cobalt chloride for LMH cells. Cells were harvested and heme oxygenase activity assays were performed as described in Methods. Data represent mean ± SEM, n=4. *Significantly greater than control ($P < 0.0005$). †Significantly greater than control ($P < 0.0001$). §Significantly greater than in CELCs ($P < 0.01$).

Treatment	Heme Oxygenase Activity (pmol bilirubin/mg prot./min)	Fold Induction
Primary CELCs		
No Treatment	55 ±6	1.0
Heme	273 ±14*	5.0
Cobalt Chloride	382 ±14†	7.0
Glut + FeNTA	199 ±9*	3.6
LMH Cells		
No Treatment	77 ±4	1.0
Heme	557 ±14†§	7.2
Cobalt Chloride	429 ±64†	5.6
Glut + FeNTA	292 ±25*	3.8

with subsequent increases in HO-1 protein and enzyme activity [178]. For both cell types, increases in HO-1 mRNA in response to heme or cobalt chloride, and synergy in response to glutethimide + FeNTA also correlated with observed increases in enzyme activity levels. These data are consistent with transcriptional regulation of HO-1 expression in both cell types. Although apolipoprotein II expression was reported to be decreased in LMH cells [69], the expression of several other proteins, including HO-1, was comparable in LMH cells and CELCs [65,66]. The levels of HO-1 protein expression in LMH cells were reported to be similar to those in CELCs, supporting the notion that LMH cells are a useful model not only for assessing regulation of HO-1 mRNA expression, but also HO-1 protein and activity levels.

Effects of non-heme metalloporphyrins on HO-1 mRNA levels. Unlike heme, some other metalloporphyrins have been shown to repress ALA synthase activity and to inhibit heme oxygenase in a competitive manner [44,57,177]. Zinc mesoporphyrin has been shown to inhibit heme oxygenase in several model systems for porphyria, including primary CELCs and in rats and mice [44,46,57,177,179-181]. Tin protoporphyrin has been used in humans with acute porphyria, in combination with intravenous heme, in an attempt to inhibit heme oxygenase and thereby prolong the duration of heme-mediated inhibition of ALA synthase [182-184]. Inhibition of HO-1 mRNA by several metalloporphyrins was tested in LMH cells (Figure 4.6). Both concentrations of heme tested

Figure 4.6: Effects of selected metalloporphyrins on HO-1 mRNA levels in LMH cells. Cells were treated with 10 μM or 20 μM heme; or with 10 μM non-heme metalloporphyrin dissolved in DMSO for 6 hours prior to harvest (MP = mesoporphyrins, PP = protoporphyrins). DMSO was added at not more than 1 μL per mL of medium as a solvent control. Harvest, isolation, and quantitation of RNA was performed as described in Methods. Data represent mean + SEM, n=3. *Significantly less than DMSO solvent control, $P < 0.05$. †Significantly greater than DMSO control, $P < 0.05$.

Heme Oxygenase-1 mRNA
(HO-1 mRNA/GAPDH mRNA)
150
125
100
75
50
25
0
†
†
*
*
No Treatment
DMSO control
Heme-10μM
Heme-20μM
ZnMP
SnMP
CuPP
MnPP

FIG. 6A AND 6B

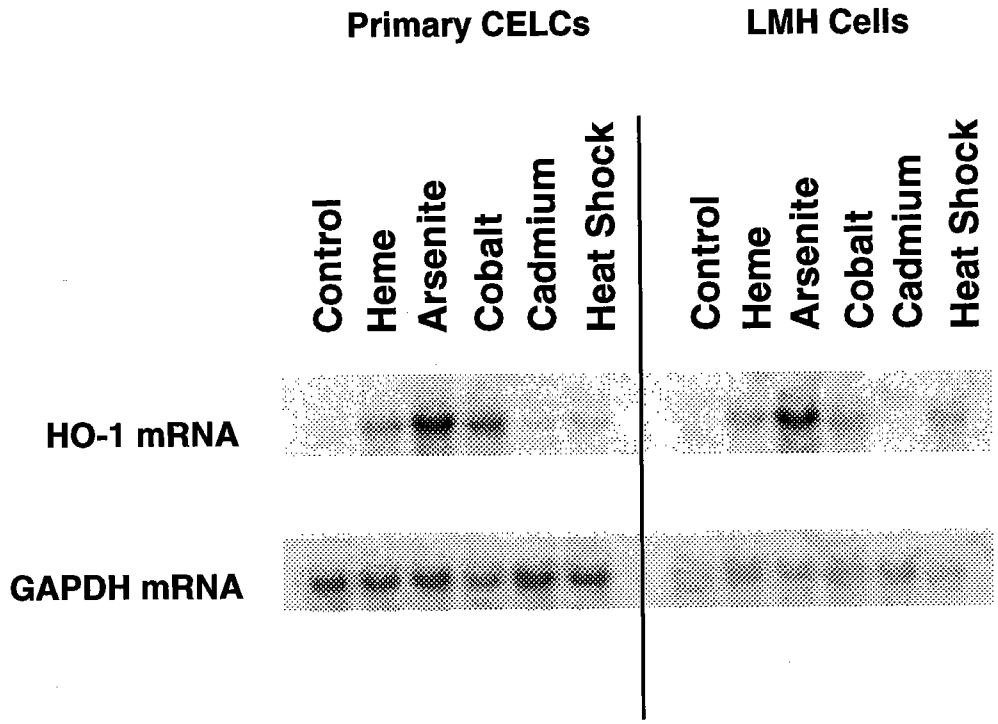
significantly increased HO-1 mRNA levels. No effect on HO-1 mRNA was observed for manganese protoporphyrin (MnPP), while zinc and tin mesoporphyrins (ZnMP and SnMP) resulted in HO-1 mRNA levels lower than DMSO solvent control levels.

HO-1 mRNA induction measured by both Northern blot and dot blot. To further characterize HO-1 regulation in LMH cells, additional chemicals and stimuli were tested, which previously have been shown to elevate HO-1 mRNA levels and enzyme activities in primary CELCs and other cell culture systems [23,29,89,91,169]. Heme, cobalt chloride, cadmium chloride, sodium arsenite, and 3,5,5-trimethylhexanoylferrocene (TMH-ferrocene) were each tested for their abilities to increase the levels of HO-1 mRNA. Dot blot data are presented in Table 4.3. LMH cells were treated with chemicals at concentrations and times used routinely in CELCs. All treatments caused significant increases in HO-1 mRNA levels, with the highest degree of induction by arsenite. Additionally, heme, arsenite, cobalt, cadmium, and heat shock were tested by Northern blot (Figure 4.7) for their ability to induce HO-1 mRNA in CELCs and LMH cells. All treatments gave similar fold inductions for both cell types. Although there have been no previous reports of a heat shock response in primary CELCs, heat shock significantly increased HO-1 mRNA levels in both CELCs and LMH cells. For both cell types, increases in HO-1 mRNA measured by Northern blot were more pronounced than that observed by dot blot for all treatments except for

Table 4.3: Effect of selected chemicals on levels of HO-1 mRNA in LMH cells. Cells were treated with selected chemicals for the indicated times prior to harvest. Concentrations of chemicals and incubation times were: heme, 20 μM for 6 h; cobalt chloride, 200 μM for 18 h; cadmium chloride, 1.5 μM for 18 h; sodium arsenite, 25 μM for 18h; and TMH-ferrocene, 5 μM for 4h. Harvest, isolation, and quantitation of RNA were performed as described in Methods. Relative amounts of mRNA were normalized to total Poly-A RNA. Data represent mean + SEM, n=4. *Significantly greater than control ($P < 0.0001$). †Significantly greater than other treatments ($P < 0.0005$).

TREATMENT	Levels of HO-1 mRNA (Arbitrary Units)	Fold Induction
No Treatment	29.6±2.7	-
Heme	106±16 *	3.6
Cobalt Chloride	135±3.6 *	4.5
Cadmium Chloride	123±13 *	4.2
Sodium Arsenite	177±4.2 *†	6.0
TMH-ferrocene	89.3±4.4 *	3.0

Figure 4.7: Northern blot comparison of HO-1 mRNA induction by selected treatments in CELCs and LMH cells. Prior to harvest, cells were left untreated or exposed to 10 μM heme for 6 h, 75 μM sodium arsenite for 4 h, 200 μM cobalt chloride for 18 h, 1.5 μM cadmium chloride for 18 h, or heat shock at 43°C for 2 h. Harvest, isolation, and quantitation of RNA was performed as described in Methods. A single band located at 1.3 Kb was detected with the probe specific for chicken HO-1. The blot was stripped and rehybridized with a probe specific for chicken GAPDH. HO-1 mRNA was normalized to GAPDH mRNA levels for each lane. Fold inductions in CELCs and LMH cells are presented.



Fold Induction of HO-1/GAPDH mRNA		
Treatment	Primary CELCs	LMH Cells
None	1.0	1.0
10 μM heme	10.8	5.2
75 μM ARS	47.9	35.9
200 μM $CoCl_2$	29.2	5.5
1.5 μM $CdCl_2$	1.1	1.1
Ht Shk (43°C)	3.2	9.8

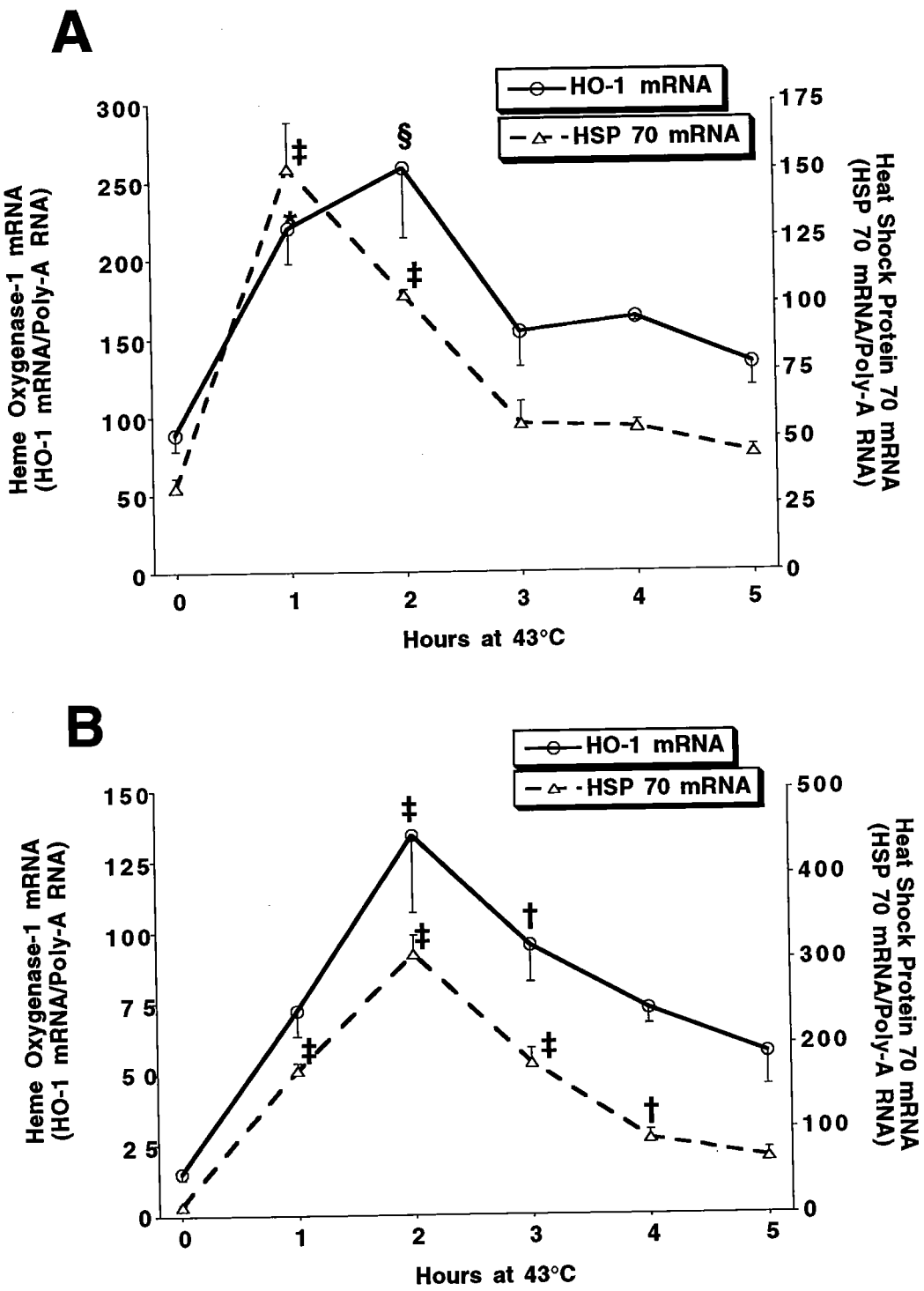
cadmium chloride, which showed no significant induction. For dot blots, less HO-1 mRNA may have remained bound to the membrane after washing, resulting in decreased binding of radiolabelled probe. This may account for the observed differences between Northern and dot blot results.

Studies in intact rats, or with other liver-derived cell lines have indicated variation in the response of HO-1 mRNA to cobalt vs. cadmium. Recently, Alam [27] showed that HO-1 mRNA in the cell lines Hepa and L929 was not induced by cobalt, but was markedly induced by cadmium. In LMH cells, the induction by cobalt is not only measurable, but is at least comparable to that observed for cadmium or heme in other systems [23,77,109]. Northern analysis indicated little to no induction by cadmium, but a significant induction by cobalt was observed. Differences in minimal doses required for maximal induction may be due to cell-specific changes in uptake or metabolism of the chemicals used, or to differences in cell surface-to-volume ratios, as LMH cells are morphologically different from primary CELCs. Despite the requirement for greater concentrations of cobalt chloride in LMH cells, the pattern of induction seems to be generally similar to that observed for CELCs when analyzed by either Northern or dot blots.

Heat shock induction of HO-1 in CELCs and LMH cells. Although heat shock has produced a response in rat, mouse, and some human cell lines (Hep3B) [185], heat shock-mediated induction of HO-1 mRNA has not been detected in other human cell lines (HepG2, HeLa), macrophages, or primary

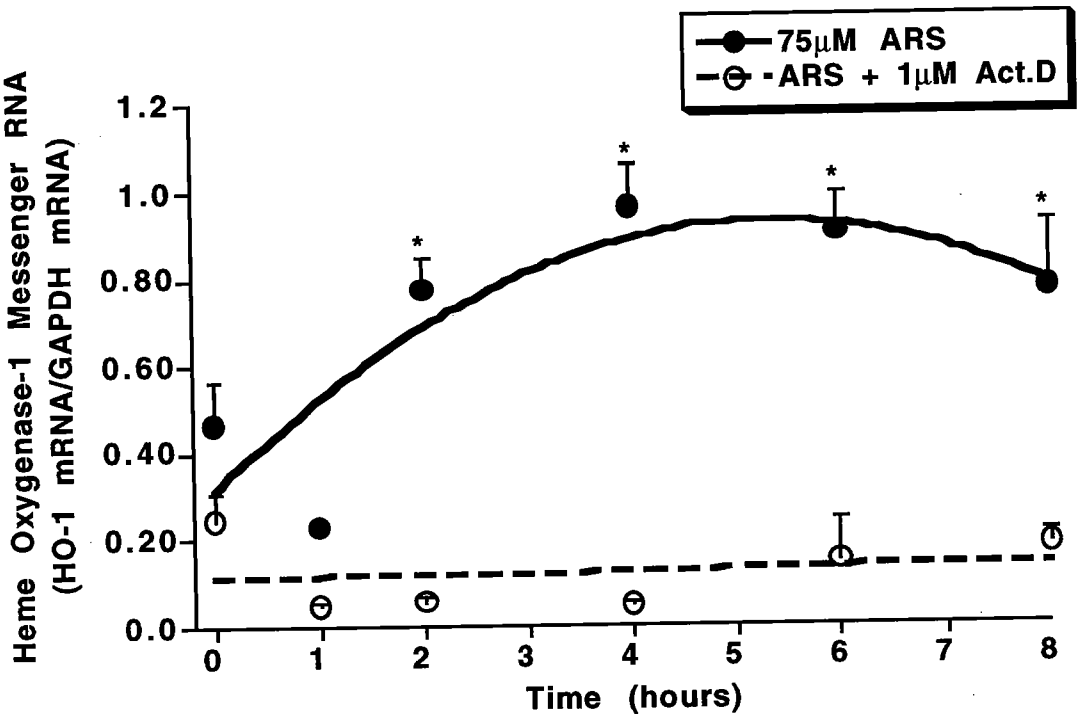
CELCs previous to the work presented here [89,185,186]. Since a heat shock response was observed by Northern blot (Figure 4.7), the ability of CELCs and LMH cells to mount a heat shock response was further investigated by following induction of HO-1 and HSP70 mRNA levels through a 5 hour time course of incubation at 43°C (Figure 4.8). The time courses of the heat shock response in CELCs and LMH cells were similar. Both CELCs and LMH cells were found to respond to heat shock with an increase in HO-1 mRNA, although the fold increase in message levels was considerably greater in LMH cells than in CELCs. As shown in Figure 4.8A, the results for CELCs show a slight but significant increase in HO-1 and HSP70 mRNA levels (2.9-fold and 4.7-fold versus no treatment (0 hour) control, respectively). Peak induction of HO-1 mRNA in CELCs occurred at 2 hours and returned to basal levels by 5 hours. Earlier results in CELCs involved only a 5 hour time point, at which time the slight increase in HO-1 mRNA was already past. The heat shock mediated induction in LMH cells was 9.1-fold for HO-1 mRNA and 26.7-fold for HSP70 mRNA levels (Figure 4.8B). These results with LMH cells indicate that HO-1 mRNA induction follows the same pattern as the HSP70 mRNA, with maximal increase observed after 2 hours of incubation at 43°C. A similar response was seen for HO-1 mRNA in CELCs, but for HSP70, peak mRNA levels were observed slightly earlier, at 1 hour. Heat shock mediated induction was observed in both CELCs and LMH cells despite the notable absence of a putative or functional heat shock element in the chicken HO-1 promoter.

Figure 4.8: Comparison of the effects of heat shock on HO-1 and heat shock protein 70 (HSP70) mRNA levels in CELCs and LMH cells. **(A)** CELCs or **(B)** LMH cells were incubated at 43°C for the indicated times prior to harvest. Harvest, isolation, and quantitation of RNA were performed as described in Methods. Data represent mean + SEM, n=3. *Significantly greater than control ($P<0.05$). †Significantly greater than control ($P<0.01$). §Significantly greater than control ($P<0.005$). ‡Significantly greater than control ($P<0.0005$).


A
HO-1 mRNA
-HSP 70 mRNA
Heme Oxygenase-1 mRNA (HO-1 mRNA/Poly-A RNA)
Heat Shock Protein 70 mRNA (HSP 70 mRNA/Poly-A RNA)
Hours at 43°C
B
HO-1 mRNA
-HSP 70 mRNA
Heme Oxygenase-1 mRNA (HO-1 mRNA/Poly-A RNA)
Heat Shock Protein 70 mRNA (HSP 70 mRNA/Poly-A RNA)
Hours at 43°C

Sodium arsenite induction of HO-1 mRNA in LMH cells. Regulation of HO-1 gene expression has been shown to occur at the transcriptional level for many inducers: heme, the transition metals cobalt and cadmium, and the cellular stressor LPS. A time course was done to examine the level of regulation by the most potent inducer of HO-1 mRNA, sodium arsenite. LMH cells were treated with 75 μM arsenite in the presence or absence of actinomycin D, which was used to inhibit the synthesis of nascent mRNA. HO-1 mRNA levels were quantitated and normalized to GAPDH mRNA, as measured by both Northern and dot blots, with similar results. Data from dot blots are illustrated in Figure 4.9. Peak induction of HO-1 mRNA by arsenite occurred at 4 hours, consistent with previous results. No arsenite-mediated induction of HO-1 mRNA was observed when LMH cells were pretreated for 4 hours with actinomycin D. This data is consistent with transcriptional regulation of HO-1 by arsenite.

Figure 4.9: Sodium Arsenite (ARS) induction of endogenous HO-1 mRNA in LMH cells; and block by actinomycin D. Cells were treated with 75 μM ARS for the indicated times prior to harvest. Some plates received 1 μM Actinomycin D, 4 hours prior to treatment with sodium arsenite. At t = 0, 75 μM ARS was added directly to the medium. Plates were incubated at 37°C until harvest. Total RNA was prepared and levels of HO-1 and GAPDH mRNAs measured as described in Methods. Data represent mean + SEM, n=3. *Significantly greater than control, $P<0.01$.


75μM ARS
-ARS + 1μM Act.D
Heme Oxygenase-1 Messenger RNA
(HO-1 mRNA/GAPDH mRNA)
1.2
1.0
0.80
0.60
0.40
0.20
0.0
0
1
2
3
4
5
6
7
8
Time (hours)
*
*
*
*

Conclusions

The results presented in this chapter support the use of LMH cells as a model system for the study of HO-1 expression. LMH cells have detectable and inducible heme oxygenase-1. Culture conditions that support robust induction were defined. Results from all treatments tested support normal HO-1 regulation and expression in LMH cells, and provide evidence that heme metabolism in LMH cells parallels that previously characterized in primary CELCs. The potencies of some chemical inducers (e.g., cobalt chloride) were lower in LMH cells. However, the efficacies and time courses were very similar to those observed in primary CELCs. Similar increases in heme oxygenase activities were also observed. The effects of non-heme metalloporphyrins on HO-1 mRNA levels in LMH cells also correlated well with previous results in CELCs [44,57,177]. Both zinc and tin mesoporphyrins caused slight decreases in HO-1 mRNA levels in LMH cells. These data lend additional support that control of HO-1 expression in LMH cells is similar to that observed in normal liver cells *in vivo*, and in primary cultures. In addition, induction of HO-1in response to heat shock in primary CELCs was identified for the first time.

In conclusion, LMH cells exhibit levels of heme oxygenase-1, and mechanisms of its regulation similar to those observed in primary chicken hepatocytes. Minor differences were observed in the efficacies of some treatments. Although no heat shock response had previously been reported for

chicken HO-1, induction of HO-1 mRNA was observed in response to heat shock in LMH cells, and for the first time, in CELCs. Actinomycin D blocked arsenite-mediated induction of HO-1 mRNA in LMH cells. This is consistent with a transcriptional regulation mechanism for this chemical. Other facets of regulation of heme metabolism may be elucidated through long-term studies and stable transfections, which are now possible using this cell line.

CHAPTER V

CLONING AND CHARACTERIZATION OF THE CHICKEN HEME OXYGENASE-1 PROMOTER

Introduction

As previously described, studies in which the level of HO-1 induction has been investigated have shown that HO-1 expression is transcriptionally regulated. Several *cis*-acting promoter elements involved in mediating HO-1 gene expression have been elucidated [33,111-113]. In one study [28], stably transfected HO-1 reporter gene constructs were used to locate elements required for induction of the murine HO-1 gene by heme and heavy metals. Putative regulatory elements were identified between 3.5 Kb and 12.5 Kb upstream from the transcription start site. Recently, evidence for a basal level inducer and heme-responsive element located as far as 10.5 Kb upstream of the transcription start site in the mouse HO-1 gene promoter was presented [87]. Other work has located regions of the HO-1 promoter that mediate cadmium, heat shock, hypoxia, and lipopolysaccharide (LPS) responsiveness in rat, mouse, and human HO-1 genes [27,29,87,88,113,115,116]. However, the transcription factor complexes that target these elements have been largely unexplored.

Work summarized in this chapter focused on the induction of chicken HO-1 by the tumor promoter arsenite, which produced the greatest increases in

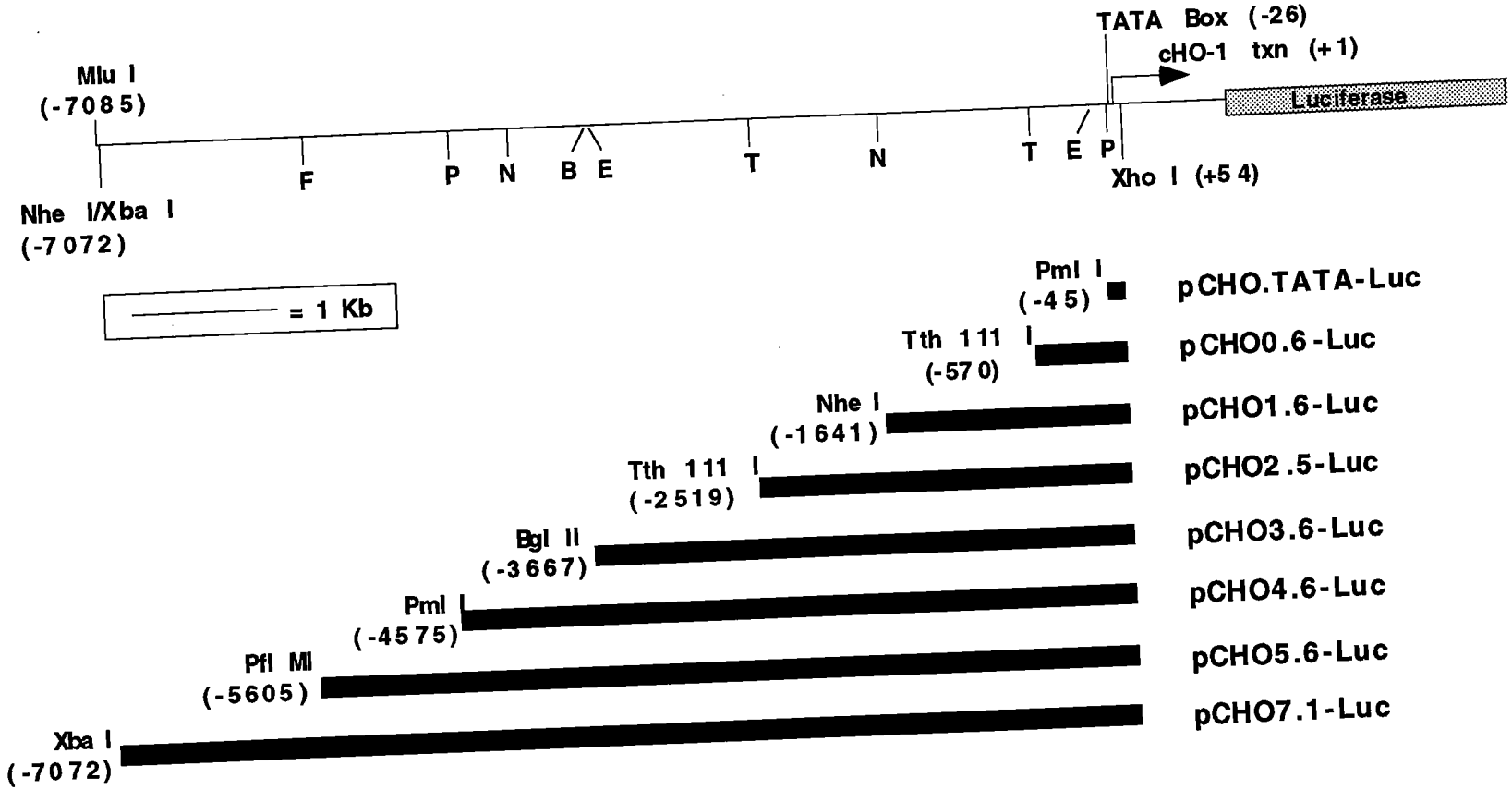
levels of HO-1 mRNA and activities in LMH cells, as shown in Chapter IV. The ability of arsenite to increase transcription of endogenous HO-1, and the activity of transfected luciferase reporter gene constructs under control of the HO-1 promoter, was investigated using LMH cells. Regions of the cHO-1 promoter were subcloned into a luciferase reporter vector, and tested for responsiveness to HO-1 inducers. Transient transfection assays were used to investigate the modulation of HO-1 transcription by arsenite. In other systems, arsenite has been proposed to affect gene expression by modulating the activities of transcription factor complexes bound to AP-1 elements in the promoter regions of several genes [118,159,160]. Putative AP-1 binding sites are present in the promoter regions of mammalian [87,88,111,113] and chicken HO-1 [37]. The roles of three putative AP-1 transcription factor complex binding sites (located at -1578, -3656, and -4597 base pairs, upstream of the transcription start site) in arsenite-mediated induction of HO-1 gene expression were studied.

Results

Construction of cHO-1 promoter-luciferase reporter plasmids. Transcriptional regulation is mediated by the binding of transcription factors to specific sequences in the promoter regions of genes. The recent cloning and characterization of the genomic chicken HO-1 gene [37] has aided the development of tools to elucidate the mechanisms involved in modulating HO-1 gene expression. To facilitate the search for promoter elements involved in arsenite induction of HO-1, a series of cHO-1 promoter-luciferase reporter plasmids were constructed using convenient restriction enzyme recognition sites (Figure 5.1, see Methods for details of cloning). The largest plasmid contained a 7132 bp Xba I - Xho 1 fragment of cHO-1 promoter sequence (including the transcription start site and some 5'-UTR) cloned upstream of the firefly luciferase reporter gene cassette in the commercially available reporter vector, pGL3 basic. Each successive deletion plasmid contained approximately 1 Kb less of the 5' promoter sequence than the corresponding next largest plasmid. Appendix E contains the sequence of the 7132 bp fragment that was cloned into pGL3 basic, and the restriction sites used to create the deletions.

Detection of basal and induced reporter plasmid luciferase activity. A test transfection was performed using the smallest, pCHO.TATA-Luc, and largest, pCHO7.1-Luc, constructs to detect basal level reporter gene expression, and heme-induced or arsenite-induced reporter gene expression. Controls were:

Figure 5.1: HO-1 promoter-luciferase reporter constructs. Restriction enzyme sites used for creating reporter constructs are shown in relation to the chick heme oxygenase-1 TATA box (3' end = -26 base pairs), cHO-1 txn = transcription start site (+1), and the Xho I cloning site (+54). Abbreviated restriction enzyme recognition sites are designated as follows: Pfl MI (F), Pml I (P), Nhe I (N), Bgl II (B), Eco RI (E), Tth 111 I (T). The restriction enzyme and sequence location used to create each construct are identified beside each representation. Sequence of the proximal promoter region has been deposited with GenBank (Accession number U95209). For the entire 7132 bp chicken HO-1 promoter sequence, see Appendix E. The pCHO3.6-Luc construct was subcloned into pGL3 by ligation of a fragment taken from a chloramphenicol acetyltransferase (CAT) reporter construct. See Methods for details of creation of constructs. See Appendix C for a comprehensive list of plasmid constructs.


TATA Box (-26)
cHO-1 txn (+1)
Mlu I
(-7085)
Luciferase
F
P
N
B
E
T
N
T
E
P
Xho I (+54)
Nhe I/Xba I
(-7072)
= 1 Kb
Pml I
(-45)
pCHO.TATA-Luc
Tth 111 I
(-570)
pCHO0.6-Luc
Nhe I
(-1641)
pCHO1.6-Luc
Tth 111 I
(-2519)
pCHO2.5-Luc
Bgl II
(-3667)
pCHO3.6-Luc
Pml I
(-4575)
pCHO4.6-Luc
Pfl MI
(-5605)
pCHO5.6-Luc
Xba I
(-7072)
pCHO7.1-Luc

nontransfected cells; cells that were subjected to transfection conditions, including the transfection reagent, but received no plasmid DNA; cells that received the pGL3 control plasmid, which contains the luciferase gene under control of an SV40 enhancer and promoter; and cells that received the promoterless and enhancerless vector control plasmid, pGL3 basic. Cells transfected with pGL3 basic were left untreated or treated with heme or arsenite. As shown in Figure 5.2, the highest normalized luciferase reporter gene activity was observed for the positive control vector plasmid, pGL3 control. The negative control vector, pGL3 basic, had a low basal expression which was unaffected by heme or arsenite treatment. Activity of the minimal promoter construct, pCHO.TATA-Luc, was not increased by treatment with heme or arsenite. Basal expression of pCHO7.1-Luc was much greater than pCHO.TATA-Luc or pGL3 basic. No induction over basal was observed by heme for pCHO7.1-Luc, however, arsenite produced a robust (8.3-fold) increase in reporter gene activity from pCHO7.1-Luc.

Responsiveness of pCHO7.1-Luc to cellular stressors. To characterize further the usefulness of this construct as a tool for studying HO-1 gene expression under conditions of cellular stress, the effects of several chemicals implicated in stimulating cellular stress responses were tested. The minimal promoter construct, pCHO.TATA-Luc served as a negative control. The effects of arsenite, cadmium, cobalt, hydrogen peroxide, and LPS were tested on both pCHO.TATA-Luc and the largest construct, pCHO7.1-Luc (Table 5.1). There

Figure 5.2: Initial test for expression and induction of chicken HO-1 promoter luciferase reporter constructs in LMH cells. Cells were transfected with no DNA, or pGL3 control, pGL3 basic, pCHO.TATA-Luc, or pCHO7.1-Luc plasmid DNA approximately 48 hours prior to treatment. Treatments were: 20 μM heme for 8 h or 25 μM sodium arsenite for 8 h. All transfections that received DNA also received pPGK-βgal plasmid DNA for normalization of luciferase activity. Harvest and assays were performed as described in Methods. Data represent mean + SEM, n=3. * Significantly greater than pCHO7.1-Luc, no treatment, $P < 0.001$.

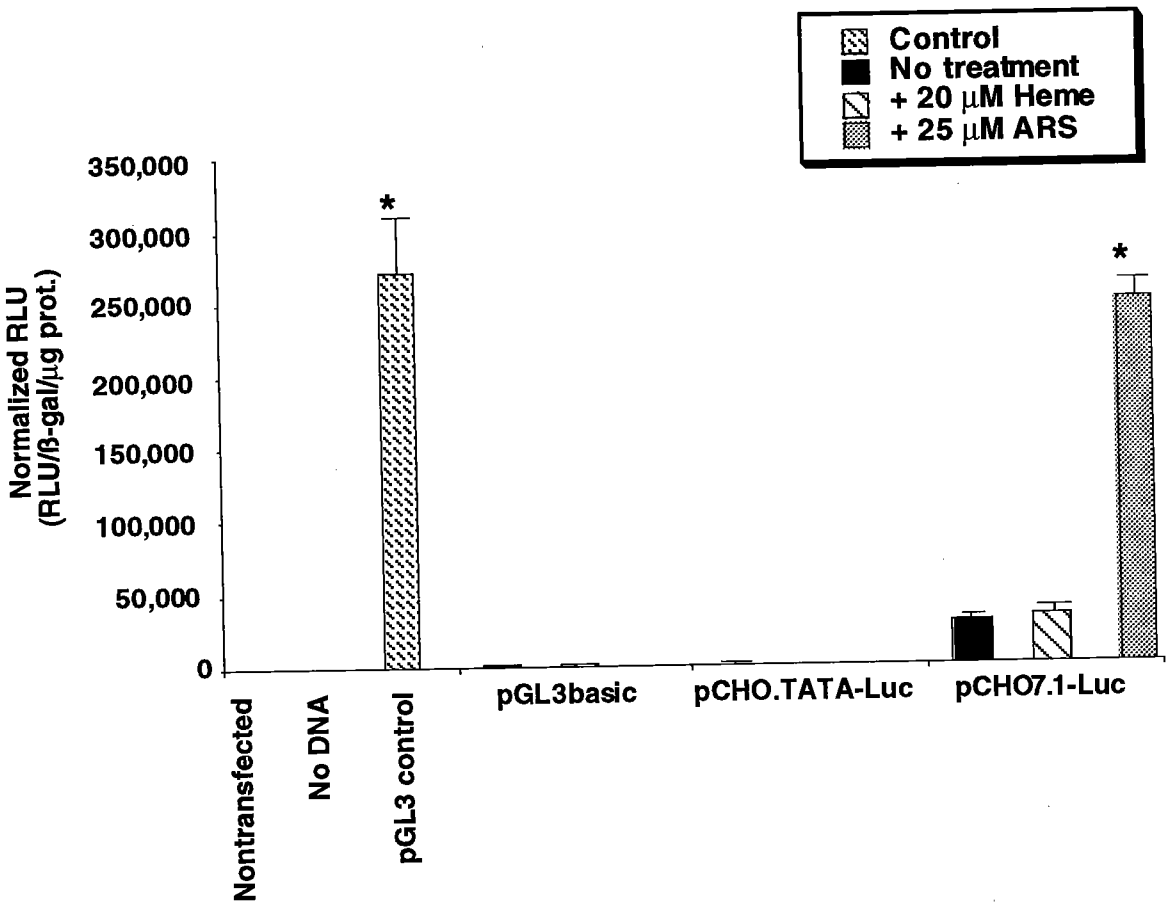

Control
No treatment
+ 20 μM Heme
+ 25 μM ARS
Normalized RLU
(RLU/ß-gal/μg prot.)
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
*
*
Nontransfected
No DNA
pGL3 control
pGL3basic
pCHO.TATA-Luc
pCHO7.1-Luc

Table 5.1: Induction of pCHO7.1-Luc, but not pCHO.TATA-Luc by inducers of endogenous HO-1. LMH cells were transfected with either pCHO.TATA-Luc or pCHO7.1-Luc approximately 48 hours prior to treatments. Cells were treated as follows: 75 μM sodium arsenite for 6 h; 1.5 μM cadmium chloride for 20 h; 200 μM cobalt chloride for 20 h; 0.5 mM hydrogen peroxide for 6 h; and 1.5 μg/mL LPS for 20 h. Harvest and assays were performed as described in Methods. Data represent mean ± SEM. * Significantly greater than no treatment control, $P < 0.001$. †Significantly greater than all other treatments, $P < 0.01$.

Treatment	Normalized RLU ± SEM	Fold Induction
pCHO.TATA-Luc		
None	2410 ± 88	1.0 ± 0.1
Sodium arsenite	1260 ± 166	0.5 ± 0.1
Cadmium chloride	2550 ± 99.4	1.1 ± 0.1
Cobalt chloride	1930 ± 380	0.8 ± 0.2
Hydrogen Peroxide	2540 ± 251	1.1 ± 0.1
Lipopolysaccharide	1640 ± 180	0.7 ± 0.1
pCHO7.1-Luc		
None	3350 ± 246	1.0 ± 0.1
Sodium arsenite	14400 ± 2810	4.3 ± 0.8 *†
Cadmium chloride	7360 ± 696	2.2 ± 0.2 *
Cobalt chloride	4080 ± 353	1.2 ± 0.1
Hydrogen Peroxide	5200 ± 323	1.6 ± 0.1 *
Lipopolysaccharide	3550 ± 264	1.1 ± 0.1

was no significant increase in activity of pCHO.TATA-Luc in response to any treatment tested. Sodium arsenite had the greatest effect on pCHO7.1-Luc reporter gene expression, with a 4.3-fold increase in normalized luciferase activity (Table 5.1). Lesser degrees of induction were observed with this construct after exposure to hydrogen peroxide (1.6-fold) and cadmium chloride (2.3-fold). LPS and cobalt chloride have been shown to induce HO-1 in some experimental systems; however, no induction was observed for LPS or cobalt chloride with pCHO7.1-Luc in LMH cells under these conditions.

The lack of a response to LPS may be explained by a cell-type specific difference; e.g., it is not known whether LMH cells have LPS receptors, which would confer responsiveness to this chemical. In liver, the major effects of LPS are thought to be on Kupffer cells which release cytokines that exert effects on the other cell types [187,188]. Since LMH cells are derived from hepatocytes, they may indeed lack the pathway that directly responds to LPS. Alternatively, the promoter elements required for responsiveness to cobalt chloride and LPS may not be present in the pCHO7.1-Luc construct, or a silencer element may be preventing reporter gene expression.

Optimization of arsenite-mediated induction of pCHO7.1-Luc. Arsenite potently induced both endogenous HO-1 and transiently transfected HO-1 reporter constructs in LMH cells, making this a good system for studying the arsenite-mediated cellular stress response. Further analysis was performed on the arsenite-induced increase in reporter gene expression in cells transfected

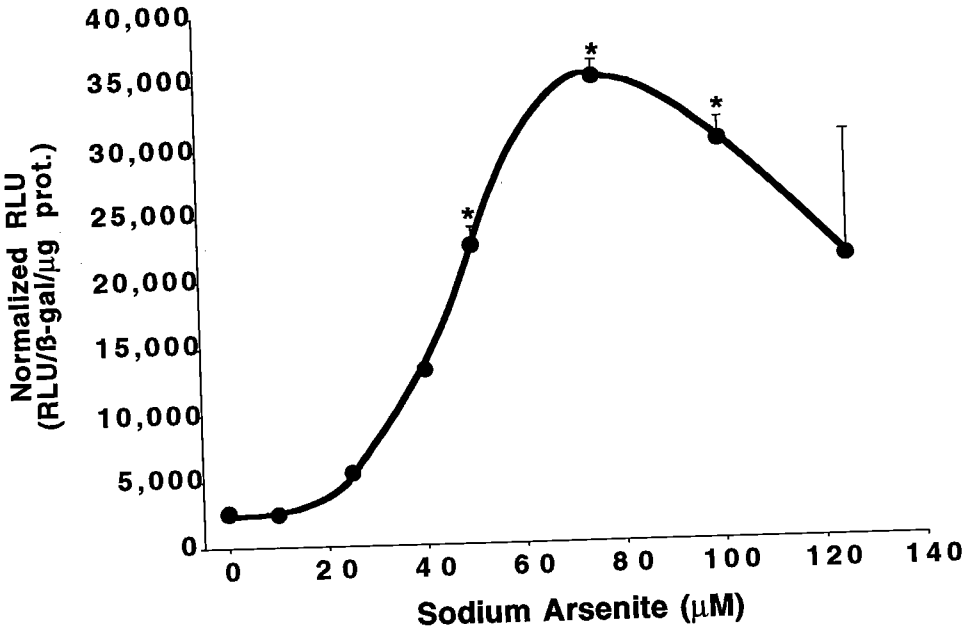
with pCHO7.1-Luc. Dose response and time course experiments established that the most effective dose for arsenite induction of reporter constructs was 75 μM and peak induction occurred at 6 hours (Figure 5.3). Therefore, these conditions were used to further investigate the mechanism of sodium arsenite induction of HO-1 gene transcription.

Induction of transfected heme oxygenase-1 promoter luciferase reporter constructs by sodium arsenite. Sodium arsenite is a potent inducer of HO-1 mRNA in CELCs [30,31,85,89]. In LMH cells, 75 μM sodium arsenite gave a peak induction of HO-1 mRNA (3.9-fold) at 4 hours (Figure 4.9). The highly reproducible, robust induction of HO-1 mRNA expression by arsenite provides a reliable system to study the promoter elements and signaling mechanisms involved. The HO-1 promoter-luciferase reporter constructs were tested for responsiveness to sodium arsenite to define regions of the promoter that may be responsible for the induction effects. Although arsenite induced several constructs, the highest folds of induction were observed for pCHO5.6-Luc and pCHO7.1-Luc (Figure 5.4).

Identification of putative transcriptional elements potentially involved in modulating HO-1 gene expression. The MacVector® analysis program was used to identify putative transcription factor binding site sequences within the cHO-1 promoter region. Many putative transcriptional elements are present in the proximal 7.1 Kb promoter region of the chicken HO-1 gene that was subcloned into pGL3 basic. Figure 5.5 diagrams several potential candidates

Figure 5.3: Dose- and time-dependence of induction of pCHO7.1-Luc by sodium arsenite. LMH cells were transfected with pCHO7.1-Luc plasmid DNA approximately 48 h prior to treatment. Cells were treated with increasing concentrations of arsenite for 8 hours **(A)**, or with 75 μM arsenite for increasing lengths of time **(B)**. Harvest and assays were performed as described in Methods. Luciferase activity (relative light units, RLU) was normalized to ß-galactosidase activity and protein content. Data represent mean + SEM, n=3. *Significantly greater than control (No treatment), $P<0.001$.

A


40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
Normalized RLU
(RLU/ß-gal/µg prot.)
0
20
40
60
80
100
120
140
Sodium Arsenite (µM)

B

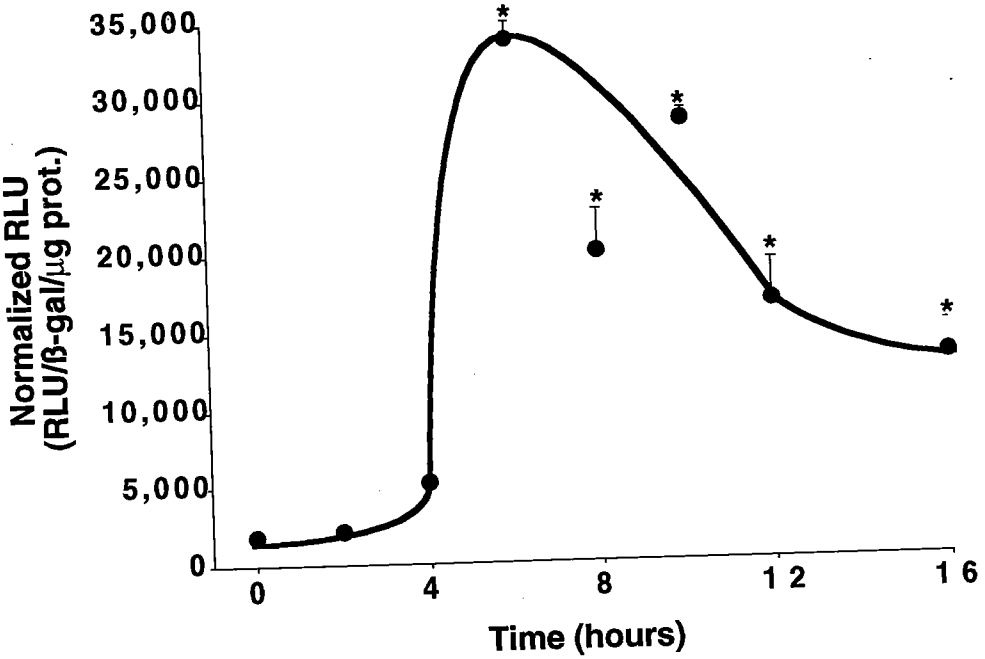

35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
Normalized RLU
(RLU/ß-gal/µg prot.)
0
4
8
12
16
Time (hours)

Figure 5.4: Sodium arsenite induces cHO-1 promoter reporter constructs. LMH cells were transfected with deletion construct and pPGK-ßgal plasmids approximately 48 hours prior to treatment. Cells that received treatment were exposed to 75 μM sodium arsenite for 6 hours prior to harvest. Harvest and assays were performed as described in Methods. Data represent mean + SEM, n=3. *Significantly greater than no treatment and other constructs containing less than 3.6 Kb of HO-1 promoter, $P < 0.001$. †Significantly less induction than 5.6 and 7.1 Kb constructs.

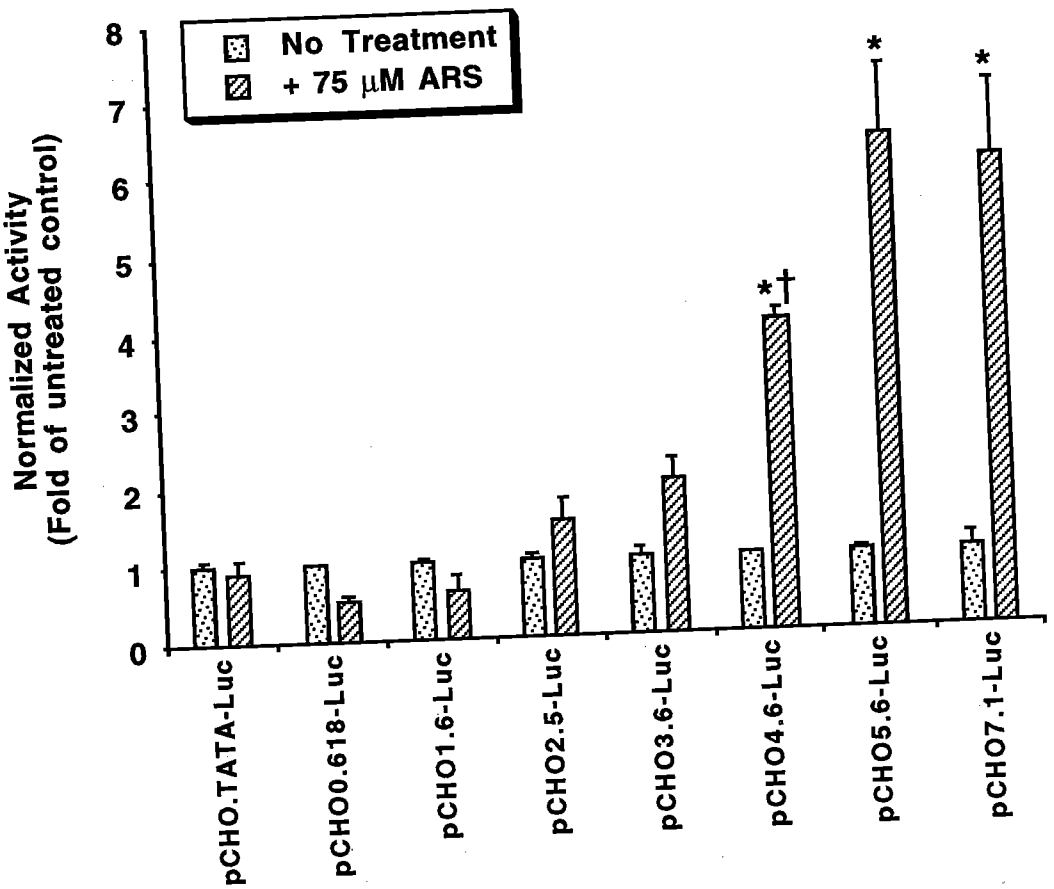
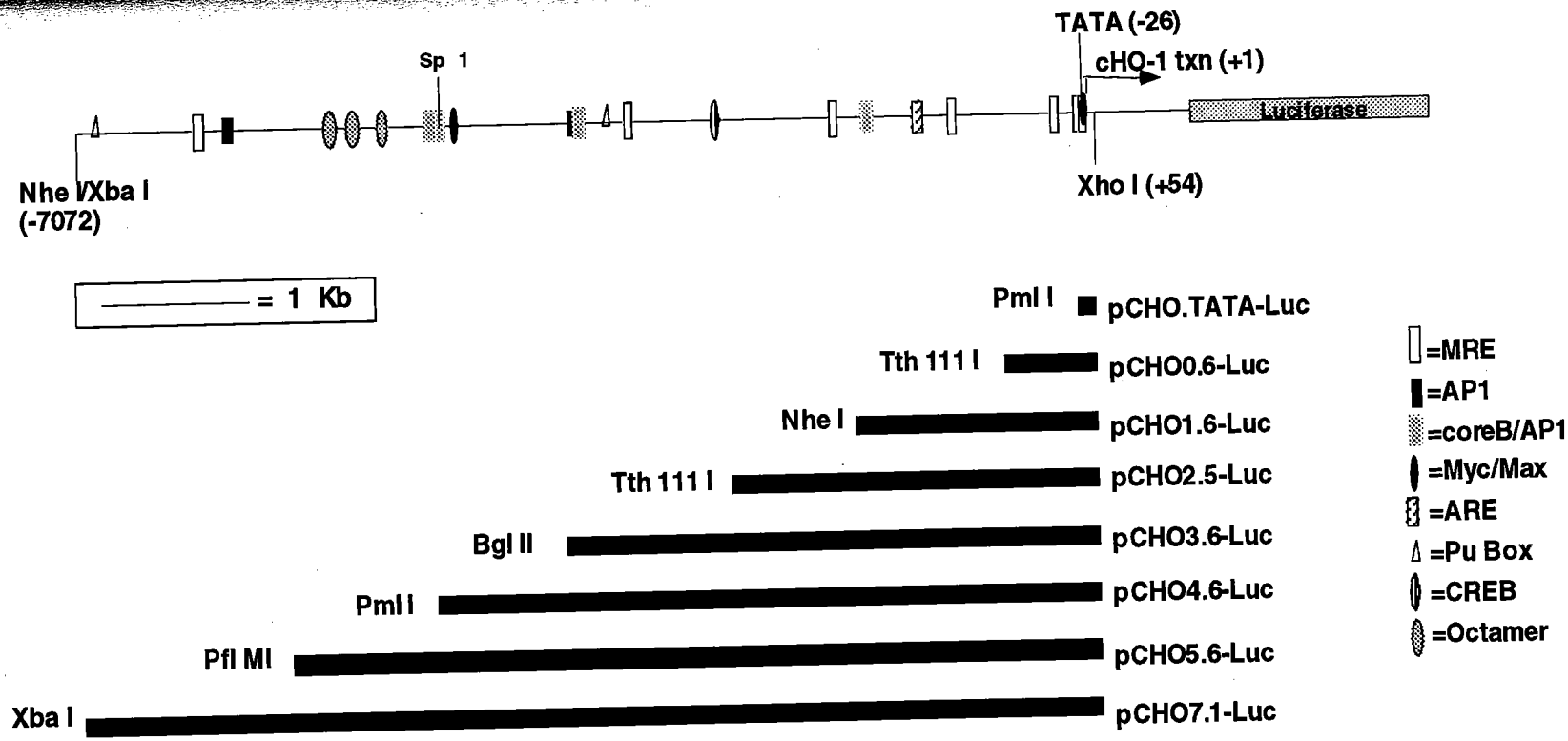

No Treatment
+ 75 μM ARS
Normalized Activity
(Fold of untreated control)
0
1
2
3
4
5
6
7
8
*
*†
*
pCHO.TATA-Luc
pCHO0.618-Luc
pCHO1.6-Luc
pCHO2.5-Luc
pCHO3.6-Luc
pCHO4.6-Luc
pCHO5.6-Luc
pCHO7.1-Luc

Figure 5.5: Computer generated map of putative consensus transcription elements in the proximal 7.1 Kb of the chicken HO-1 promoter. The MacVector® software package was utilized to search for several putative transcription factor binding sites in the 7132 bp cHO-1 promoter sequence. The program was set up to search previously identified nucleic acid subsequences that were likely to be utilized by stimuli known to modulate HO-1 gene expression. Among the elements identified were: metal responsive element (MRE), activator protein 1 (AP-1), core B heme responsive/AP-1 element (coreB/AP-1) [87], oncogene c-myc/max heterodimer binding site (Myc/Max), antioxidant response element (ARE), purine rich binding site (Pu box), cyclic AMP responsive element binding site (CREB), an octamer site (Octamer), and a GC box binding site (Sp1). Each element and its corresponding symbol, except for Sp1, are represented on the right side of the figure. The Sp1 site is labeled above the promoter representation. The deletion constructs are aligned with the element map to allow comparison of the elements present in each construct.


TATA (-26)
Sp 1
cHO-1 txn (+1)
Luciferase
Nhe I/Xba I
(-7072)
Xho I (+54)
= 1 Kb
Pml I
pCHO.TATA-Luc
Tth 111 I
pCHO0.6-Luc
Nhe I
pCHO1.6-Luc
Tth 111 I
pCHO2.5-Luc
Bgl II
pCHO3.6-Luc
Pml I
pCHO4.6-Luc
Pfl MI
pCHO5.6-Luc
Xba I
pCHO7.1-Luc
=MRE
=AP1
=coreB/AP1
=Myc/Max
=ARE
=Pu Box
=CREB
=Octamer

for elements that may modulate cHO-1 gene expression in response to treatments that lead to increased transcription of HO-1 mRNA.

Some evidence has suggested that gene induction by sodium arsenite is mediated through an AP-1 element [37,87]. Guyton *et al.* [160] found that arsenite treatment increased nuclear extract binding to an AP-1 element in the GADD153 (CHOP) gene promoter. In LMH cells transfected with cHO-1 deletion constructs, increased induction by arsenite correlated with the presence of putative AP-1 consensus sites in the distal promoter (Figure 5.4). Several putative consensus AP-1 elements were identified in the 7135 bp sequence of cHO-1 promoter region by the subsequence search function of the MacVector® software program. The role of the three most proximal AP-1 sites (located at -1578, -3656, and - 4597 bp) as transcriptional elements that modulate HO-1 gene expression in response to arsenite was investigated by: 1) testing the effects of arsenite on both negative and positive control plasmids, and 2) by introducing specific base pair mutations in these sites in the context of selected reporter gene constructs.

Effects of arsenite on AP-1 control plasmid expression. Two control plasmids were obtained to investigate transcriptional modulation by AP-1 elements in LMH cells. The negative control plasmid was GAL4-Luc, a construct containing the luciferase gene cassette under control of the yeast GAL4 promoter. The positive control plasmid was 4xTRE-Luc, a construct containing the luciferase gene cassette under control of 4 consecutive TPA-

responsive AP-1 elements. Each construct was transfected into LMH cells that were left untreated or treated with arsenite or the phorbol ester, TPA, and luciferase activity was measured (Figure 5.6). Since these constructs were tested for activation in a chicken hepatoma cell line, the yeast GAL4 promoter was unable to promote treatment-induced expression of the luciferase gene (Figure 5.6). In contrast, the 4xTRE-Luc construct was activated by both treatments, with a 6.2 fold induction by arsenite and an 8.4 fold induction by TPA (Figure 5.6).

Role of AP-1 sites in arsenite induction of cHO-1 reporter plasmids. The role of an AP-1 site as a transcriptional element that may modulate HO-1 gene expression in response to arsenite was investigated. For initial studies, the reporter construct pCHO2.5-Luc, which contains a single consensus AP-1 element located at -1578 base pairs, upstream of the transcription start site was used. The role of this AP-1 site in transcriptional activation was studied by making site-directed mutations in 2 out of 7 base pairs, as illustrated in Figure 5.7A. Two separate mutants were made and tested for their ability to be induced by treatment with arsenite (Figure 5.7B). Both mutant 1 and mutant 2 were incapable of being induced by arsenite treatment. Common transcription factor dimer pairs that bind to AP-1 elements are Jun/Jun, Fos/Jun, or ATF/Jun. The role of these dimer pairs in arsenite induction of HO-1 was studied by co-transfecting a dominant negative c-Jun with the wild type pCHO2.5-Luc. The

Figure 5.6: Arsenite induction of a luciferase reporter construct driven by exogenous AP-1 elements. LMH cells were transfected with GAL4-Luc or 4xTRE-Luc plasmid DNA for approximately 48 hours prior to treatment. Cells were left untreated or treated with 0.1 μM TPA for 6 hours or 75 μM sodium arsenite for 6 hours prior to harvest. Harvest and assays were performed as described in Methods. Data represent mean + SEM, n=3. *Significantly greater than no treatment control, $P < 0.01$.

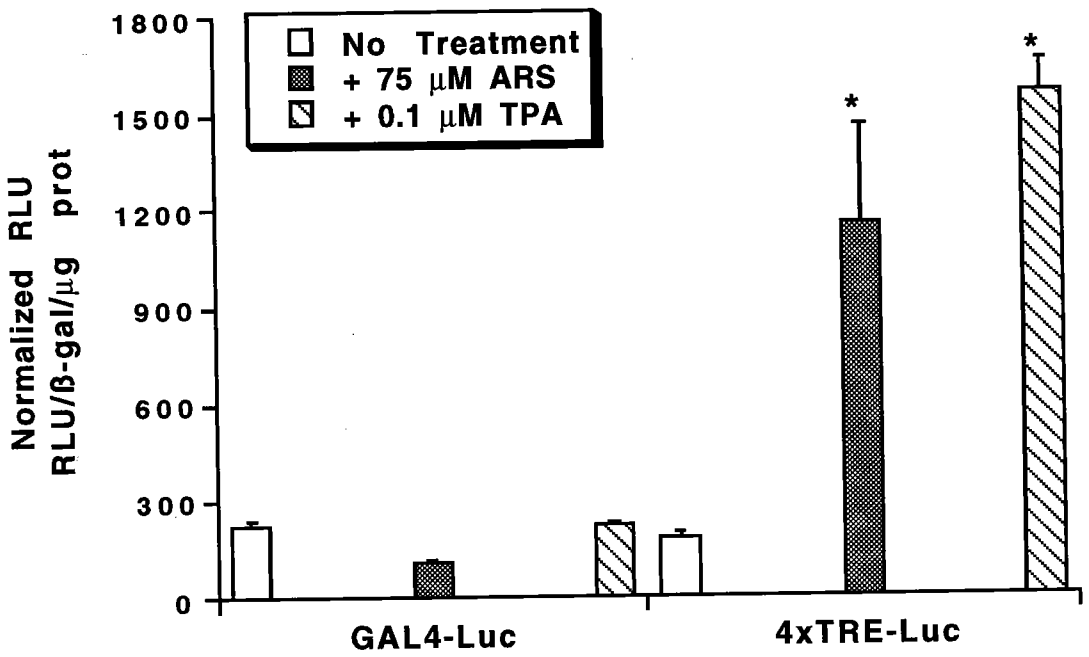
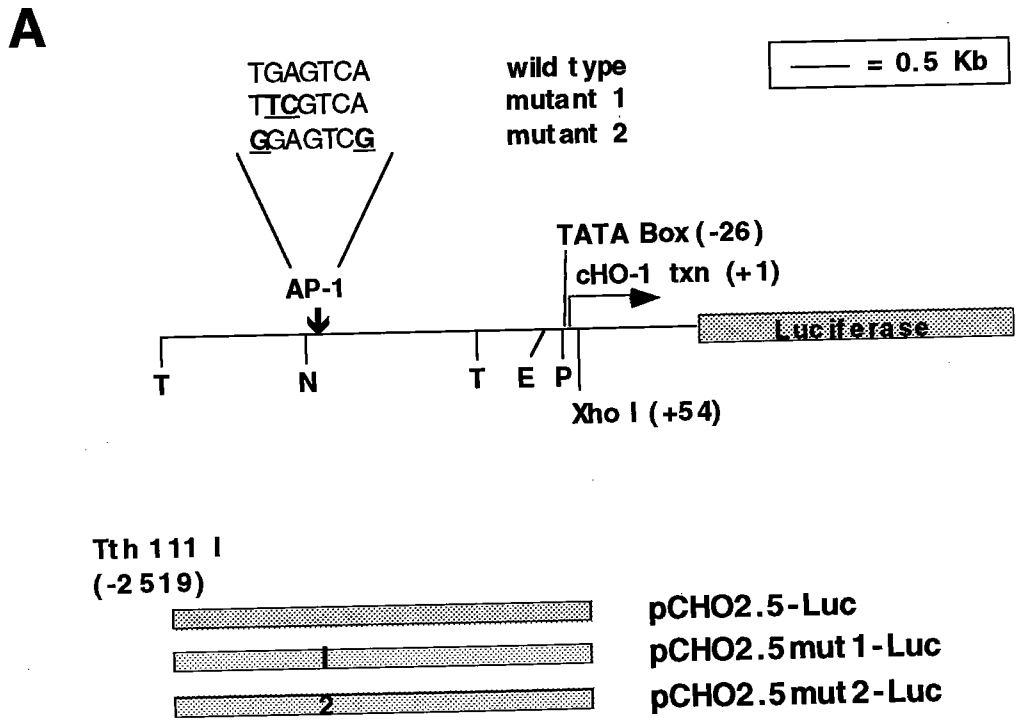
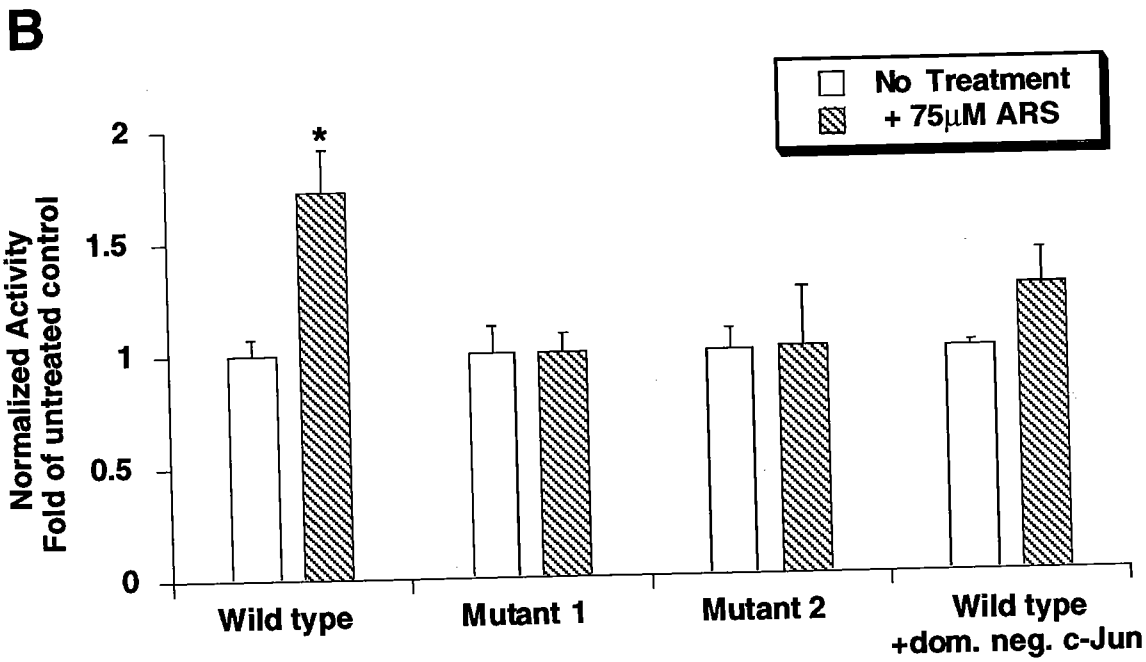

No Treatment
+ 75 μM ARS
+ 0.1 μM TPA
Normalized RLU
RLU/ß-gal/μg prot
1800
1500
1200
900
600
300
0
*
*
GAL4-Luc
4xTRE-Luc

Figure 5.7: Involvement of an AP-1 element in sodium arsenite-mediated HO-1 expression. **(A)**, The pCHO2.5-Luc HO-1 promoter-luciferase reporter construct (created with the Tth 111 I site at -2519 bp of the HO-1 promoter) contains an AP-1 site at -1578 base pairs. Site directed mutants 1 and 2 contain different two-base changes, indicated in **bold** and underlining. Abbreviated recognition sites are designated as follows: Tth 111 I (T), Nhe I (N), Pml I (P), Eco RI (E). The diagram at the bottom of the figure represents the wild type or mutant AP-1 constructs by: solid bar for wild type, or for mutants, a 1 or 2 on the bar indicating the mutation made at that location. **(B)**, LMH cells were transfected with wild type pCHO2.5-Luc, either alone, or in combination with TAM67 (dominant negative c-Jun), or with mutant 1 or mutant 2, and pPGK-ßgal. Following transfection, cells were left untreated or treated with 75 µM ARS for 6 hours. Luciferase activities were normalized to ß-galactosidase activities and protein content. Data are presented as mean fold induction versus untreated control + SEM, n=3. *Significantly greater than wild type, $P < 0.05$.


A
TGAGTCA
TTCGTCA
GGAGTCG
wild type
mutant 1
mutant 2
= 0.5 Kb
TATA Box (-26)
cHO-1 txn (+1)
AP-1
Luciferase
T
N
T
E
P
Xho I (+54)
Tth 111 I
(-2519)
pCHO2.5-Luc
pCHO2.5mut1-Luc
pCHO2.5mut2-Luc

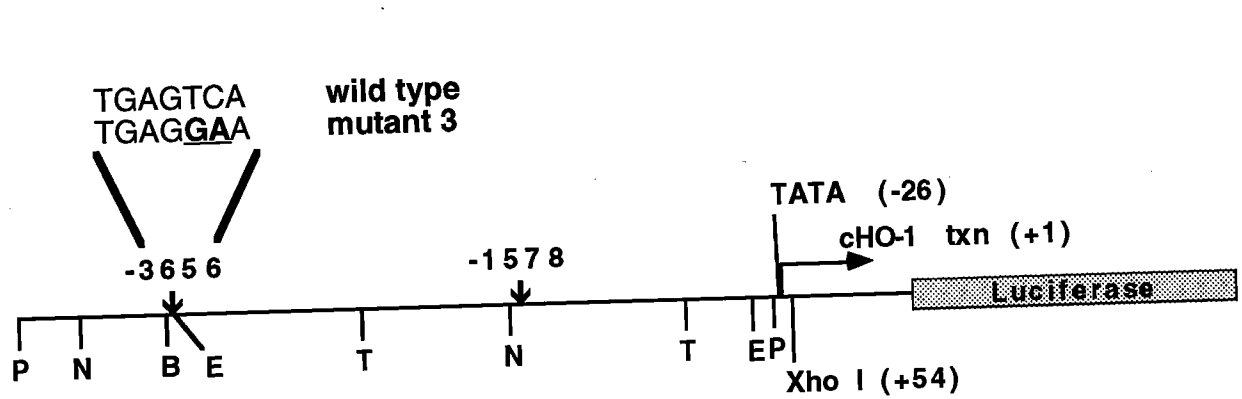

B
No Treatment
+ 75μM ARS
Normalized Activity
Fold of untreated control
2
1.5
1
0.5
0
*
Wild type
Mutant 1
Mutant 2
Wild type
+dom. neg. c-Jun

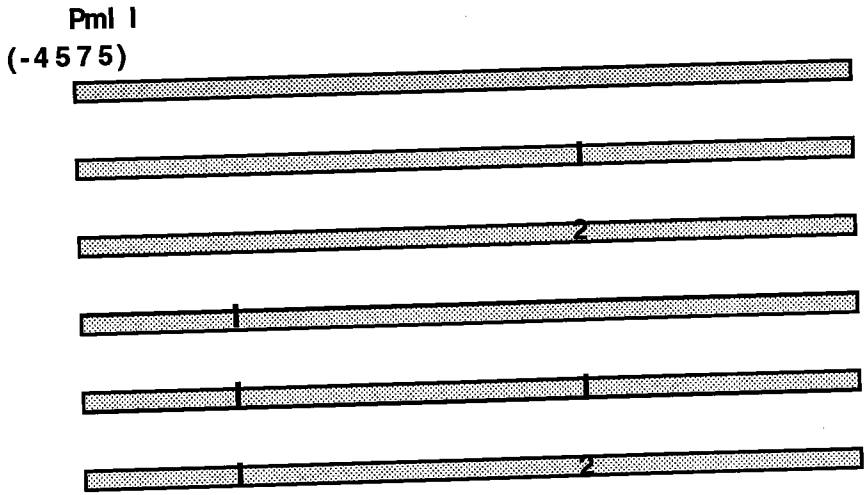
dominant negative c-Jun blocked the arsenite induction of luciferase reporter gene activity.

To further investigate possible cooperativity among the three putative consensus AP-1 elements, two additional reporter plasmids were mutated. The deletion constructs, pCHO4.6-Luc , which encompasses two of the AP-1 sites (Figure 5.8), and pCHO5.6-Luc, containing all three proximal AP-1 sites (Figure 5.9), were used as templates for single, double, or triple AP-1 mutants. The mutations made at -1578 bp are the same as those represented in Figure 5.7A for the pCHO2.5-Luc mutants. The two bases that were mutated in the AP-1 elements at -3656 and at -4597 bp were the same and are shown for the -3656 bp site at the top of Figure 5.8.

The pCHO4.6-Luc series of AP-1 mutants, and the resulting fold induction of luciferase activity of each mutant in response to arsenite treatment are shown in Figure 5.8. Additionally, the pCHO4.6-Luc wild type construct was also co-transfected with a dominant negative c-Jun. The pCHO5.6-Luc series of mutants, and resulting fold inductions by arsenite are shown in Figure 5.9. When LMH cells were transfected with the mutant constructs and tested for arsenite induction of luciferase activity, all mutants except for pCHO5.6mut13-Luc had lowered activities as compared to the wild type construct (Figures 5.8 and 5.9). Some single mutations decreased arsenite reporter gene activity to a similar extent as double mutations or the triple mutation. All three AP-1 elements were necessary for the full response to arsenite. No single AP-1

Figure 5.8: Mutation of AP-1 sites decreases responsiveness of pCHO4.6-Luc to arsenite. The pCHO4.6-Luc promoter reporter plasmid (created with the Pml I site at -4575 bp of the HO-1 promoter) was used as a template for site-directed mutagenesis of AP-1 sites located at -3656, or -1578 bp upstream of the transcription start site. Mutation of the AP-1 site was done by mutating two base pairs out of seven, as indicated at the top of the figure. The mutated bases are shown in **bold** and underlining. Double mutants were made by using pCHO4.6mut3-Luc as template for mutations at -1578, which were the same as for mutations of pCHO2.5-Luc, shown in Figure 5.7A. The bars represent the wild type or mutant AP-1 constructs by: solid bar for wild type, or for mutants, a 1 or 2 on the bar at -1578, indicating which mutation was made at that location, and a 1 at the -3656 site for constructs containing the mutation listed at the top of the figure. The primers and template for each mutant plasmid are listed in Table 2.1 in Methods. LMH cells were transfected with wild type or mutant plasmids approximately 48 hours prior to treatment. Additionally, one set of transfections included wild type pCHO4.6-Luc and a dominant negative c-Jun plasmid, TAM67. Cells were left untreated, or treated with 75 μM sodium arsenite for 6 h. Harvest and assays were performed as described in Methods. Fold inductions (arsenite treated versus untreated) are presented beside the bar representing each construct. Data represent mean ± SEM, n=3. *Significantly less than wild type, $P < 0.01$. †Significantly less than wild type, $P < 0.05$.



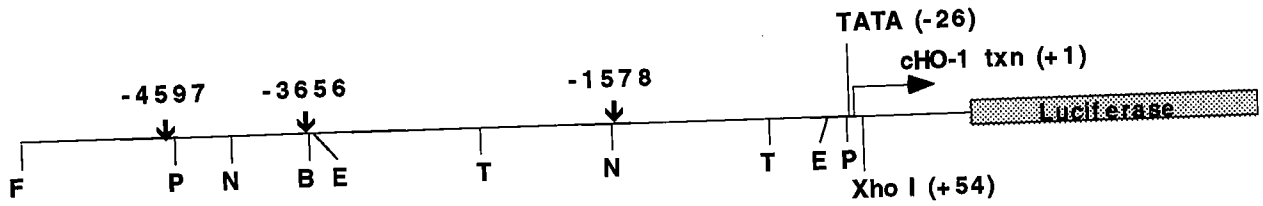


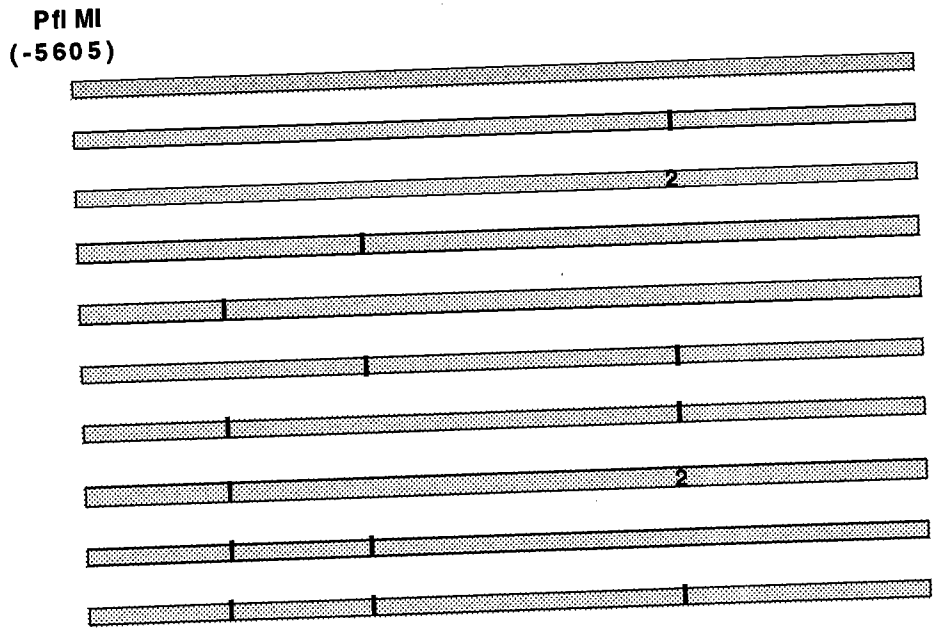
	Fold Induction by 75 μM Arsenite
pCHO4.6-Luc	3.34 ± 0.43
pCHO4.6mut1-Luc	1.78 ± 0.12 †
pCHO4.6mut2-Luc	2.04 ± 0.16 †
pCHO4.6mut3-Luc	1.09 ± 0.10 *
pCHO4.6mut13-Luc	1.15 ± 0.09 *
pCHO4.6mut23-Luc	1.39 ± 0.15 *
pCHO4.6-Luc + dom. neg. c-Jun	1.29 ± 0.22 *

Figure 5.9: Mutation of AP-1 sites decreases responsiveness of pCHO5.6-Luc to arsenite. The pCHO5.6-Luc promoter reporter plasmid (created with the Pfl MI site at -5605 bp of the HO-1 promoter) was used as a template for site-directed mutagenesis of AP-1 sites located at -1578 (mutants 1 and 2), -3656 (mutant 3), or -4597 (mutant 4) bp upstream of the transcription start site. Mutation of each AP-1 site was done by mutating two base pairs out of the seven consensus bases, as indicated at the top of Figure 5.9 for the site at -3656 bp. Mutations at -1578 bp were the same as those of pCHO2.5-Luc, as shown in Figure 5.7A. The bars represent the wild type or mutant AP-1 constructs by: solid bar for wild type, or for mutants, a 1 or 2 on the bar at -1578, indicating which mutation was made at that location, a 1 at the -3656 site in constructs containing the mutation listed at the top of Figure 5.8, and a 1 at the -4597 site in constructs containing a mutated AP-1 at this site. The primers and template for each single, double, or triple mutant plasmid are listed in Table 2.1 in Methods. LMH cells were transfected with wild type or mutant plasmids approximately 48 hours prior to treatment. After transfection, cells were left untreated, or treated with 75 μM sodium arsenite for 6 h. Harvest and assays were performed as described in Methods. Fold inductions (arsenite treated versus untreated) are presented beside the representation for each plasmid. Data represent mean ± SEM, n=3. *Significantly less than wild type, $P < 0.05$. †Significantly less than wild type, $P < 0.01$. §Significantly less than wild type, $P < 0.001$.

↓ = AP-1 Element

——— = 1 Kb





	Fold Induction by 75 μM Arsenite
pCHO5.6-Luc	5.23 ± 0.40
pCHO5.6 mut 1-Luc	2.23 ± 0.10 §
pCHO5.6mut2-Luc	3.60 ± 0.18 †
pCHO5.6 mut 3-Luc	2.17 ± 0.44 §
pCHO5.6 mut 4-Luc	3.73 ± 0.12 *
pCHO5.6 mut 13-Luc	5.63 ± 0.22
pCHO5.6 mut 14-Luc	3.52 ± 0.32 †
pCHO5.6 mut 24-Luc	2.51 ± 0.65 §
pCHO5.6 mut 34-Luc	2.08 ± 0.26 §
pCHO5.6 mut 134-Luc	2.17 ± 0.12 §

mutation completely blocked induction by arsenite, and different combinations of double mutations displayed similar abilities to block arsenite induction. These data are consistent with some level of cooperativity in the response to arsenite among the AP-1 elements that were tested. When one or two AP-1 elements were mutated, the remaining wild type AP-1 element(s) continued to modulate at least a partial response to arsenite. However, since some activation was still observed even when all three AP-1 sites were mutated, either there was still some binding causing activation at these sites, or other transcriptional elements may also be involved in producing the full response to arsenite. Arsenite induction of both pCHO2.5-Luc and pCHO4.6-Luc was significantly lowered when co-transfected with dominant negative c-Jun. These experiments implicate a role for at least one AP-1 element in the sodium arsenite-mediated induction of HO-1 gene expression.

Conclusions

In summary, arsenite induces both endogenous HO-1 mRNA and transfected HO-1 promoter-driven luciferase gene expression. A series of promoter reporter plasmids with selected deletions in the promoter region was used to explore the region of control. The results of studies using control AP-1 plasmids supported the hypothesis that AP-1 elements were involved in arsenite-mediated regulation of HO-1 expression. Experiments in which cHO-1 reporter constructs containing mutated AP-1 elements were shown to be less responsive to arsenite treatment provided further evidence that arsenite modulates HO-1 gene expression by activating AP-1 transcription factor complexes. Together, these data implicate AP-1 as one of the transcription factors that contribute to the majority of the arsenite-mediated induction of HO-1 gene expression.

The data presented in this chapter present a reliable model system that was used to investigate the transcriptional control of cHO-1 gene expression by the cellular oxidant and tumor promoter, sodium arsenite. The major findings of this study are: sodium arsenite is a potent inducer of both endogenous HO-1 and transfected HO-1 promoter-reporter constructs, and AP-1 elements are involved in transcriptional activation of HO-1 by arsenite. However, given the complexity of the HO-1 promoter, and the residual activity of AP-1 mutant constructs, it is likely that other transcription factor elements are also involved in

the modulation of HO-1 gene transcription. Two more AP-1 elements located further upstream were not tested by mutational analysis. These elements, as well as other transcription factor binding sites located further upstream (beyond the sequence of the reporter constructs tested here), may account for the lower induction of reporter constructs as compared to endogenous HO-1 mRNA in LMH cells. The deletion reporter constructs and subsequent mutations described here provide useful tools for further investigations into the transcriptional regulation of cHO-1 by many chemicals or other perturbations.

CHAPTER VI

ROLE OF MAP KINASES IN SODIUM ARSENITE-MEDIATED INDUCTION OF HEME OXYGENASE-1

Introduction

As previously described, HO-1 is an inducible enzyme that catalyzes heme degradation and has been proposed to play a role in protecting cells against oxidative stress-related injury. The induction of HO-1 by the tumor promoter sodium arsenite was investigated in the chicken hepatoma cell line, LMH. In the previous chapter, a cHO-1 promoter driven luciferase reporter construct that was reproducibly induced to high levels (at least 4-fold) in response to sodium arsenite treatment was identified. This construct, pCHO7.1-Luc was used to investigate the role of mitogen-activated protein (MAP) kinases in arsenite-mediated HO-1 gene expression.

The effects of diverse factors on HO-1 gene expression appear to be regulated at the transcriptional level, suggesting that multiple signal transduction pathways mediate induction of HO-1 gene transcription in response to a multitude of cellular stimuli. The MAP kinases are serine/threonine protein kinases that have been shown to be activated under conditions similar to those that induce HO-1 transcription, e.g., following exposure of cells to phorbol ester, cytokines, ultraviolet light, heat shock, LPS,

ceramide, and inducers of oxidative stress [110,123-130]. MAP kinases are components of signaling cascades which, in response to extracellular stimuli, target transcription factors, resulting in the modulation of gene expression. Three major MAP kinase subfamilies that mediate physiological responses were investigated: Extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 (a homologue of the yeast HOG1 kinase).

Arsenite-mediated activation of MAP kinases [110,123,131,157-159] has been demonstrated, however, a cellular gene response to this activation has not been described. Both arsenite [37,118,159,160] and the MAP kinase signaling cascades [139,140] have been shown to target AP-1 elements. Data presented in Chapter V (Figures 5.7 - 5.9) support the involvement of AP-1 in the arsenite-mediated induction of HO-1.

In this study, transient co-transfection assays were used to investigate the mechanism of arsenite-mediated HO-1 gene expression. In LMH cells, sodium arsenite, cadmium and heat shock, but not heme, induced activity of the MAP kinases extracellular-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. To examine whether these MAP kinases were involved in mediating HO-1 gene expression, constitutively activated and dominant negative components of the ERK, JNK, and p38 MAP kinase signaling pathways were utilized. Activated components of the ERK and p38 MAP kinase signaling pathways increased gene expression from an HO-1 promoter-driven luciferase reporter gene construct. A p38 inhibitor, a MEK inhibitor, and dominant

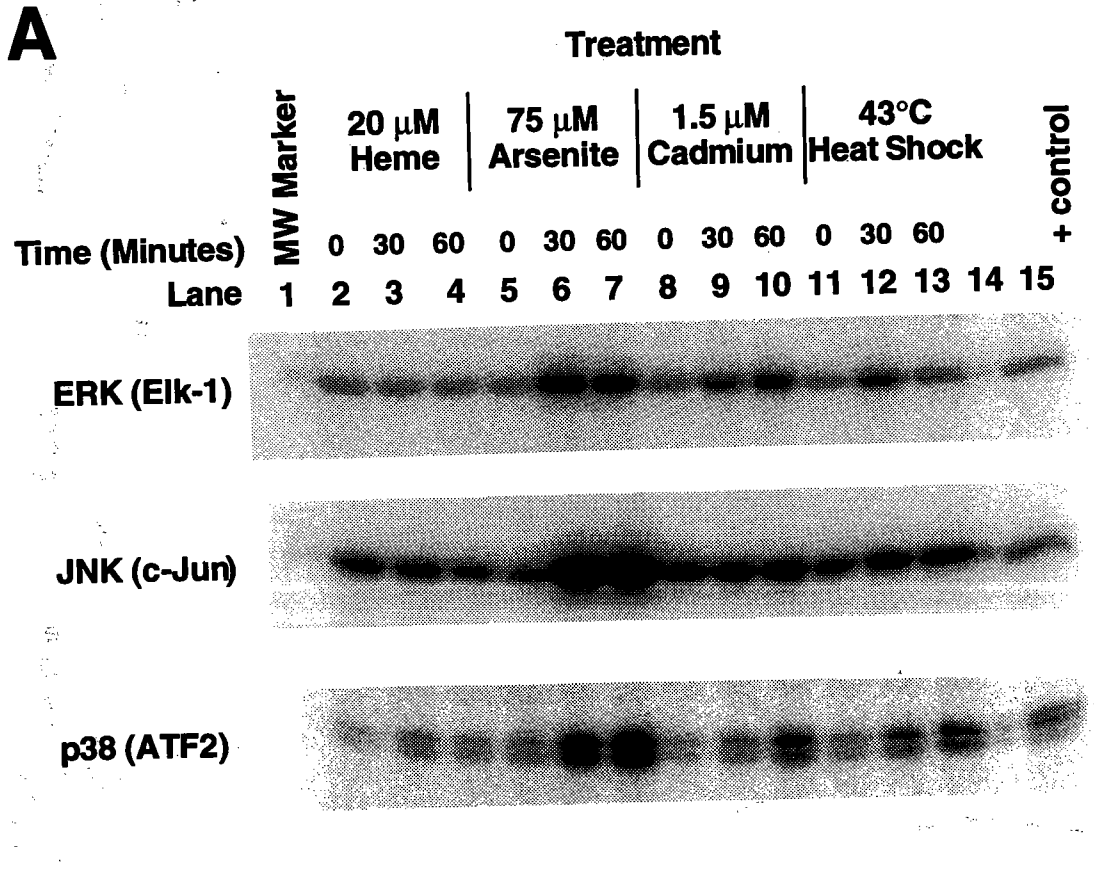
negative components of the ERK and p38 MAP kinase pathways were able to block nearly all of the arsenite-mediated induction of HO-1. In contrast, for the JNK pathway, activated components were unable to induce HO-1 gene expression, and dominant negative components were unable to block arsenite induction. These experiments demonstrate a role for the ERK and p38 MAP kinase families in the sodium arsenite-mediated induction of HO-1 gene expression.

Results

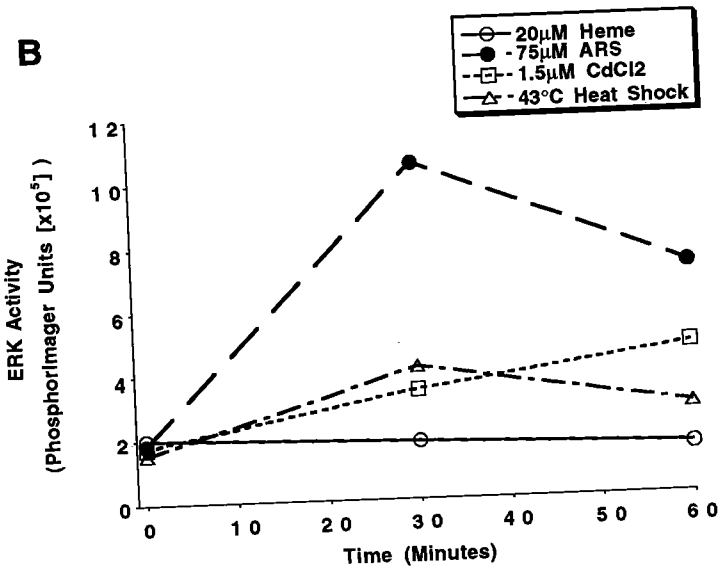
Arsenite activates MAP kinases in LMH cells. The ability of heme, sodium arsenite, cadmium chloride, and heat shock to activate ERK, JNK, and p38 was assessed in LMH cells. Cells were treated with 20 μM heme, a known inducer of endogenous HO-1; 75 μM sodium arsenite, a potent inducer of both endogenous HO-1 and HO-1 reporter gene constructs; 1.5 μM cadmium chloride, a known metal inducer of HO-1; or exposed to heat shock (at 43°C) for 0, 30, or 60 minutes. Immune complex kinase assays were performed to detect changes in MAP kinase activity (Figure 6.1). Treatment with sodium arsenite increased the activity of all three MAP kinases. In contrast, cadmium and heat shock caused only slight increases in ERK and p38 activities, and heme had no effect on any of the MAP kinases in LMH cells. To further characterize the arsenite-mediated activation of the MAP kinases, a more detailed time course was performed (Figure 6.2). The peak activation times observed for arsenite exposure were 10 minutes for ERK, 20 minutes for p38, and 45 minutes for JNK.

Induction of pCHO7.1-Luc by components of MAP kinase signaling cascades. To link activation of MAP kinases with the induction of HO-1 gene expression, several expression constructs encoding constitutively activated or dominant negative components of the MAP kinase signaling pathways were co-transfected with the pCHO7.1-Luc reporter construct (Figure 6.3). The constitutively activated MAP kinase components would be expected to increase

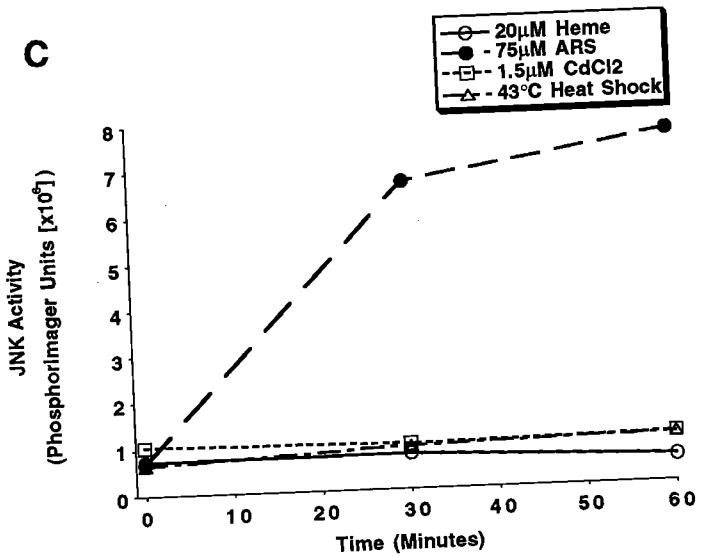
Figure 6.1: Effects of heme, sodium arsenite, cadmium chloride, or heat shock on MAP kinase activity in LMH cells. Cells were treated with 20 μM heme, 75 μM sodium arsenite, 1.5 μM cadmium chloride, or exposed at 43°C (heat shock) for 0, 30, or 60 minutes prior to harvest. **(A)**, Immune complex kinase assays were performed for ERK (top), JNK (middle), and p38 (bottom). Bands indicate phosphorylated substrates: GST-Elk1 for ERK, GST-cJun for JNK, and GST-ATF2 for p38. Lane 14 was left empty, Lane 15 was a positive control (EGF treated LMH cell lysate for ERK, and UV-treated LMH cell lysates for JNK and p38). **(B-D)**, Data from immune complex kinase assays were quantitated with the aid of a PhosphorImager and plotted as time courses of ERK, JNK, or p38 MAP kinase activation.


A
Treatment
MW Marker
20 μM Heme
75 μM Arsenite
1.5 μM Cadmium
43°C Heat Shock
+ control
Time (Minutes)
0 30 60 0 30 60 0 30 60 0 30 60
Lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
ERK (Elk-1)
JNK (c-Jun)
p38 (ATF2)

B


20μM Heme
75μM ARS
1.5μM CdCl2
43°C Heat Shock
ERK Activity
(Phosphorimager Units [x10⁵])
0
2
4
6
8
10
12
0
10
20
30
40
50
60
Time (Minutes)

C


20μM Heme
75μM ARS
1.5μM CdCl2
43°C Heat Shock
JNK Activity
(Phosphorimager Units [x10⁶])
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60
Time (Minutes)

D

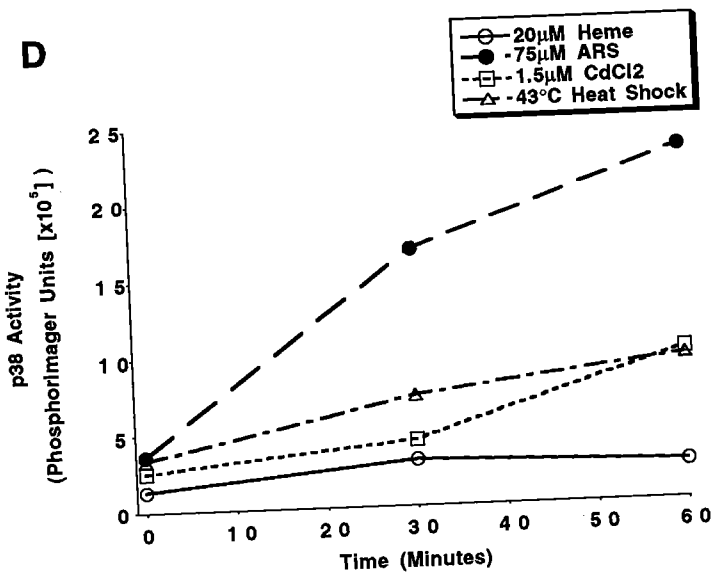

20μM Heme
75μM ARS
1.5μM CdCl2
43°C Heat Shock
p38 Activity
(Phosphorimager Units [x10⁵])
0
5
10
15
20
25
0
10
20
30
40
50
60
Time (Minutes)

Figure 6.2: Time course of MAP kinase activation by sodium arsenite. LMH cells were treated with 75μM ARS for the indicated times. Cell lysates were harvested and immune complex kinase assays specific for ERK, JNK, and p38 MAP kinases were performed. Data are plotted as fold induction relative to untreated control values.

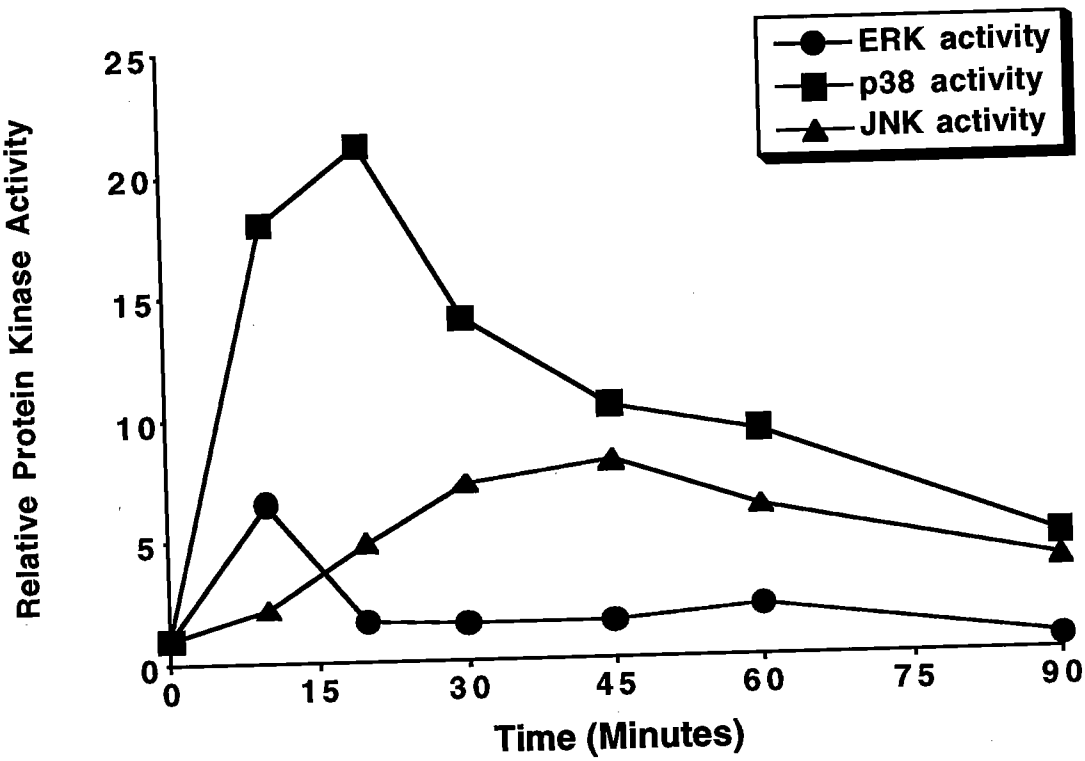
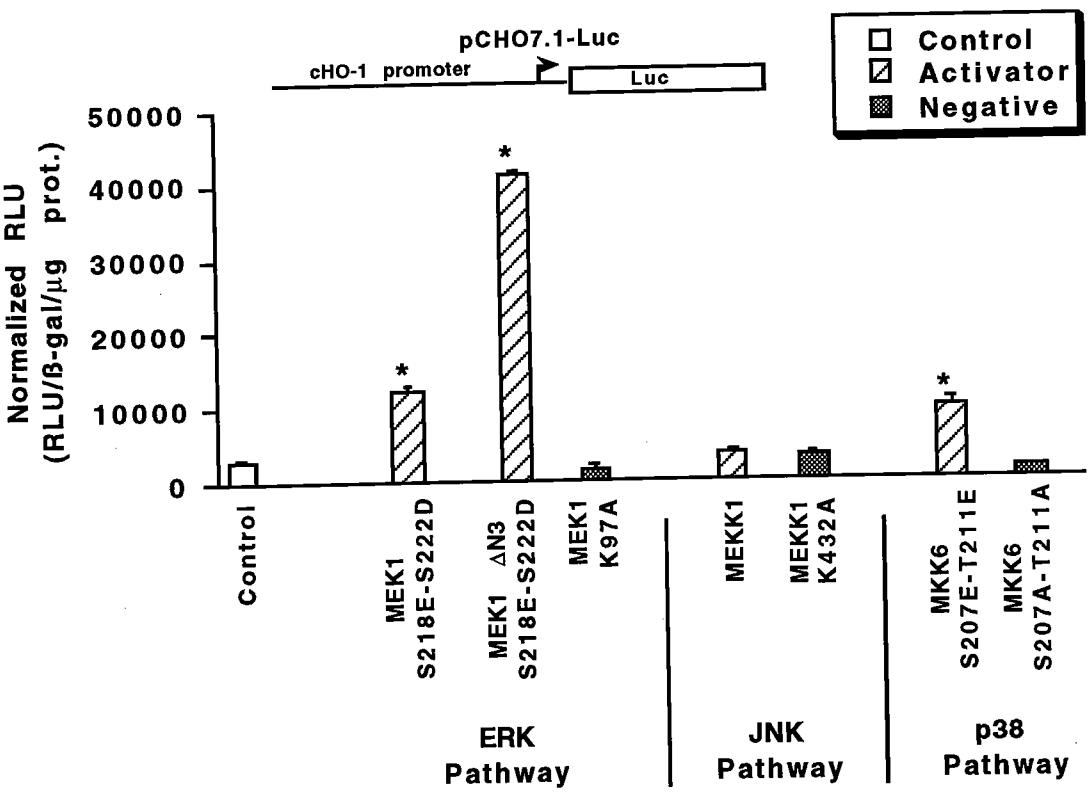

ERK activity
p38 activity
JNK activity
Relative Protein Kinase Activity
25
20
15
10
5
0
0
15
30
45
60
75
90
Time (Minutes)

Figure 6.3: Effect of selected MAP kinase pathway components on luciferase reporter gene activity in LMH cells co-transfected with pCHO7.1-Luc. Constitutive activators of ERK MAP kinase (MEK1S218E-S222D and MEK1ΔN3-S218E-S222D), JNK MAP kinase (MEKK1), and p38 MAP kinase (MKK6S207E-T211E) were tested for their ability to induce reporter gene activity. Dominant negatives of the ERK pathway (MEK1K97A), JNK pathway (MEKK1K432A), and p38 pathway (MKK6S207A-T211A) were also tested. LMH cells were co-transfected with pCHO7.1-Luc, pPGK-βgal, and the indicated MAP kinase construct using LipoFECTAMINE®. Luciferase activities were normalized to β-galactosidase activities and protein content. Data represent mean + SEM, n=3. *Significantly greater than control (pCHO7.1-Luc only), $P < 0.005$.

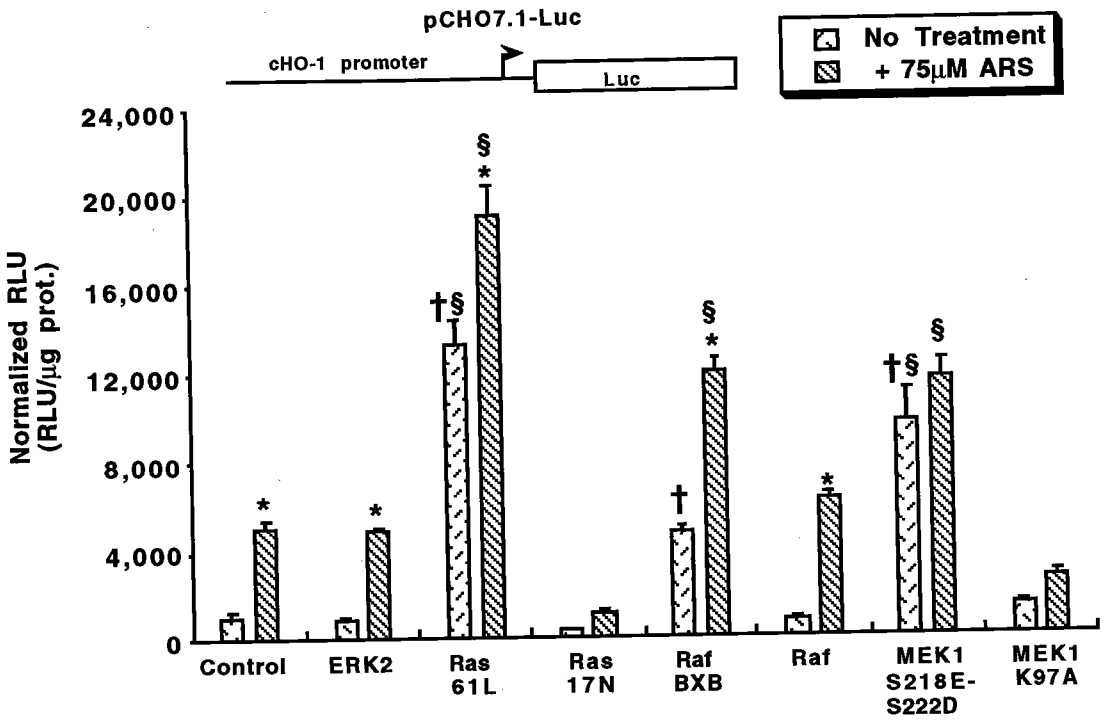

pCHO7.1-Luc
cHO-1 promoter
Luc
Control
Activator
Negative
Normalized RLU
(RLU/ß-gal/µg prot.)
50000
40000
30000
20000
10000
0
Control
MEK1 S218E-S222D
MEK1 ΔN3 S218E-S222D
MEK1 K97A
MEKK1
MEKK1 K432A
MKK6 S207E-T211E
MKK6 S207A-T211A
ERK Pathway
JNK Pathway
p38 Pathway

reporter gene activity if the downstream MAP kinases are involved in transducing cellular signals that control HO-1 gene expression. Constitutively activated forms of MEK1 (the kinase immediately upstream of ERK), MEKK1 (a kinase proposed to act upstream of JNK), and MKK6 (a kinase upstream of p38) were tested for their abilities to induce luciferase gene expression from pCHO7.1-Luc. Activated MEK1 and MKK6 were able to induce reporter gene expression, while no induction was observed for MEKK1 (Figure 6.3). As expected, all dominant negative components failed to affect basal levels of luciferase expression from pCHO7.1-Luc.

Involvement of the ERK signaling cascade in arsenite induction of HO-1.

To investigate the role of ERK in arsenite induction of HO-1, several ERK pathway components (Ras activated and dominant negative, Raf wild type and activated, MEK1 activated and dominant negative, and wild type ERK2) were co-transfected with pCHO7.1-Luc, then left untreated or treated with arsenite. If the ERK pathway was important for arsenite signaling, an activated component of the pathway would be expected to increase luciferase gene activity in the absence of arsenite, whereas the dominant negative construct would be expected to block the ability of arsenite to induce luciferase activity from the reporter gene. As shown in Figure 6.4, the activated Ras, Raf, and MEK1 components all increased luciferase gene activity, indicating each component led to induction of HO-1 gene expression. Dominant negative Ras and MEK1

Figure 6.4: Effects of ERK signaling pathway components on reporter gene activity in LMH cells co-transfected with pCHO7.1-Luc. Wild type Raf, ERK2, or constitutive activators of Ras (Ras 61L), Raf (Raf-BXB), and the ERK pathway (MEK1S218E-S222D) were tested for their ability to induce reporter gene activity. Dominant negatives of Ras (Ras 17N), and the ERK pathway (MEK1K97A) were tested for their ability to block arsenite induction of the reporter gene. LMH cells were co-transfected with pCHO7.1-Luc, pPGK-ßgal, and the indicated MAP kinase construct using LipoFECTAMINE®. Luciferase activities were normalized to protein content. Data represent mean + SEM, n=3. *Significantly greater than untreated, $P < 0.01$. †Significantly greater than untreated control (pCHO7.1-Luc only), $P < 0.01$. §Significantly greater than arsenite treated control, $P < 0.005$.

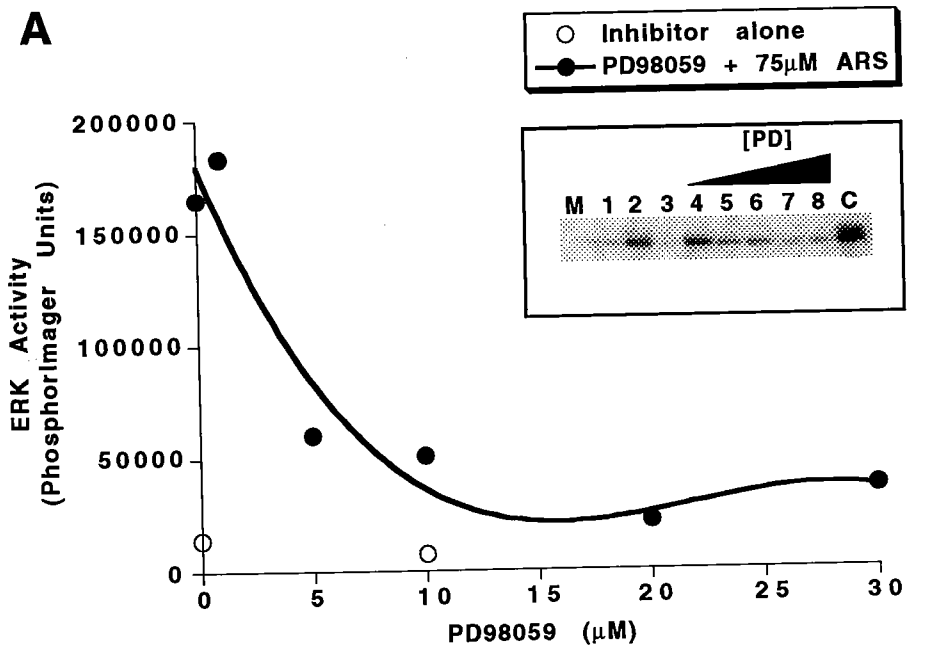

pCHO7.1-Luc
cHO-1 promoter
Luc
No Treatment
+ 75μM ARS
Normalized RLU
(RLU/μg prot.)
24,000
20,000
16,000
12,000
8,000
4,000
0
Control
ERK2
Ras
61L
Ras
17N
Raf
BXB
Raf
MEK1
S218E-
S222D
MEK1
K97A

also decreased induction by arsenite treatment. The wild type ERK2 and wild type Raf gave results similar to the pCHO7.1-Luc only control.

A role for ERK in arsenite-mediated HO-1 expression was supported by another experiment in which ERK activity was blocked using an inhibitor (PD98059) specific for MEK1, the kinase immediately upstream of ERK. This inhibitor (See Appendix D for structure) has been shown to be highly specific (IC_{50} of 2-7 μM) and noncompetitive with respect to the ATP binding site of MEK1 [144-147]. Immunoprecipitated ERK2 from cells treated with PD98059 for 30 minutes prior to receiving arsenite was incapable of phosphorylating the substrate Elk-1. Dose response analysis of the effects of the MEK inhibitor on ERK activity demonstrated inhibition of arsenite activation of ERK (Figure 6.5A). The role of ERK in arsenite induction of pCHO7.1-Luc was then probed using PD98059. As shown in Figure 6.5B, at 30 μM the inhibitor blocked approximately 60% of the arsenite induction of the HO-1 reporter gene.

JNK MAP kinase is not implicated in arsenite induction of cHO-1. Possible involvement of JNK in HO-1 induction by arsenite was studied by co-transfecting components of the JNK pathway with pCHO7.1-Luc. If JNK was an important intermediate in the pathway to HO-1 induction, wild type constructs would be expected either to increase luciferase reporter activity or to give results similar to control, while dominant negative components would block the arsenite increase in reporter gene activity. Figure 6.6 shows that wild type JNK, MEKK1, or MLK3 resulted in luciferase gene activity levels similar to control.

Figure 6.5: Effects of the MEK inhibitor, PD98059, on ERK activity and arsenite-mediated induction of HO-1. LMH cells were left untreated or treated with the indicated concentrations of MEK inhibitor. Some samples received 75μM ARS 30 minutes after addition of inhibitor. **(A)**, Cells were harvested 10 minutes after addition of ARS (40 minutes after inhibitor) and ERK activity was measured by immune complex kinase assays. Data was quantitated with a PhosphorImager and plotted as a dose response curve. [Insert: PhosphorImager scan of the kinase assay gel; M - SDS-PAGE low molecular weight protein standard, Lane 1: No treatment, Lane 2: 75 μM ARS only, Lane 3: 10 μM PD only, Lanes 4 - 8: 75 μM ARS plus increasing concentrations of PD (1, 5, 10, 20, 30 μM), Lane C: EGF treated LMH cell lysates as positive control]. **(B)**, Prior to treatment, cells were transfected with pCHO7.1-Luc, and pPGK-ßgal. Cells were harvested 6 hours after addition of ARS (6.5 hours after inhibitor). Luciferase activities were normalized to ß-galactosidase activities and protein content. Data represent mean + SEM, n=3. **Significantly less than ARS only control, $P < 0.005$. *Significantly less than ARS only control, $P < 0.001$.


A
Inhibitor alone
PD98059 + 75μM ARS
[PD]
M 1 2 3 4 5 6 7 8 C
ERK Activity
(PhosphorImager Units)
200000
150000
100000
50000
0
0 5 10 15 20 25 30
PD98059 (μM)

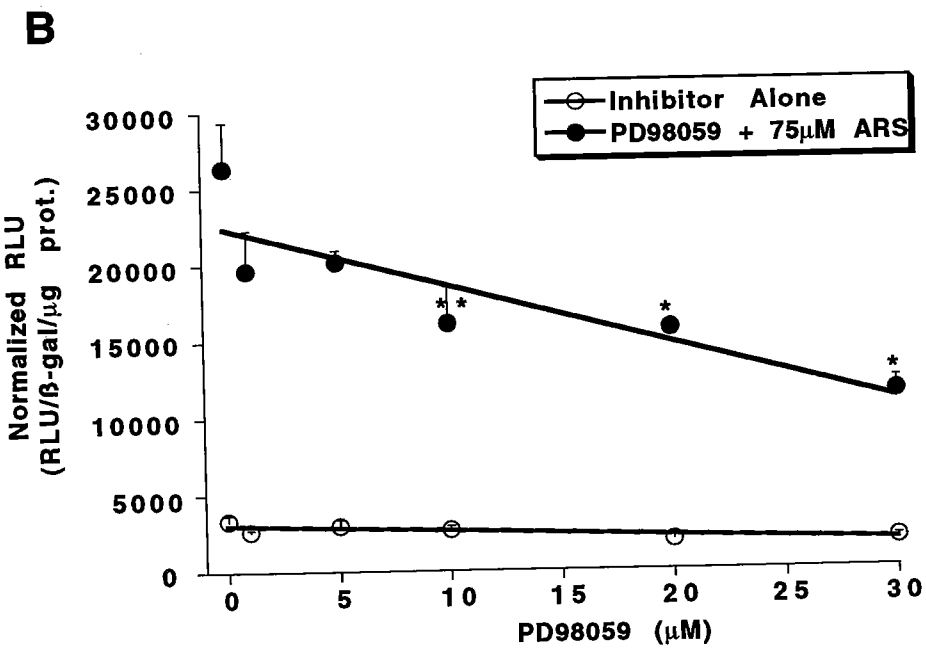
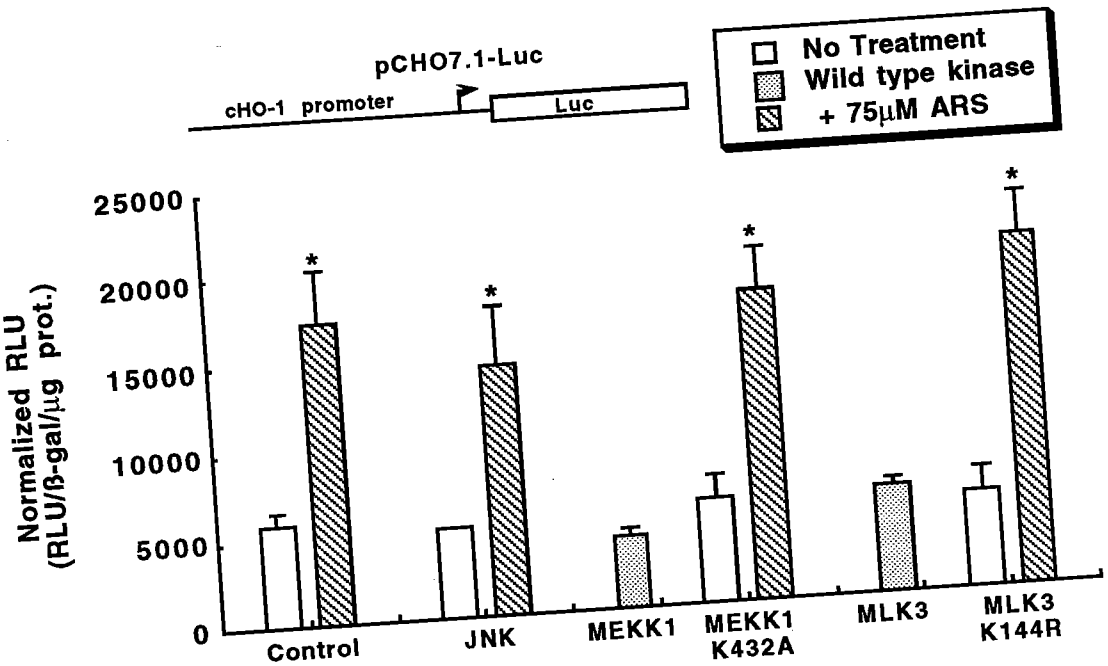

B
Inhibitor Alone
PD98059 + 75μM ARS
Normalized RLU
(RLU/ß-gal/μg prot.)
30000
25000
20000
15000
10000
5000
0
0 5 10 15 20 25 30
PD98059 (μM)

Figure 6.6: Effects of JNK signaling pathway components on reporter gene activity in LMH cells co-transfected with pCHO7.1-Luc. Wild type JNK, and activators of the JNK kinase pathway (MEKK1 and MLK3) were tested for their ability to induce reporter gene expression. Dominant negative JNK MAP kinase pathway components (MEKK1 K432A and MLK3 K144R) were tested for their ability to block arsenite induction of the reporter gene. LMH cells were co-transfected with pCHO7.1-Luc, pPGK-ßgal, and the indicated MAP kinase construct using LipoFECTAMINE®. Luciferase activities were normalized to ß-galactosidase activities and protein content. Data represent mean + SEM, n=3.

* Significantly greater than untreated control, $P < 0.05$.

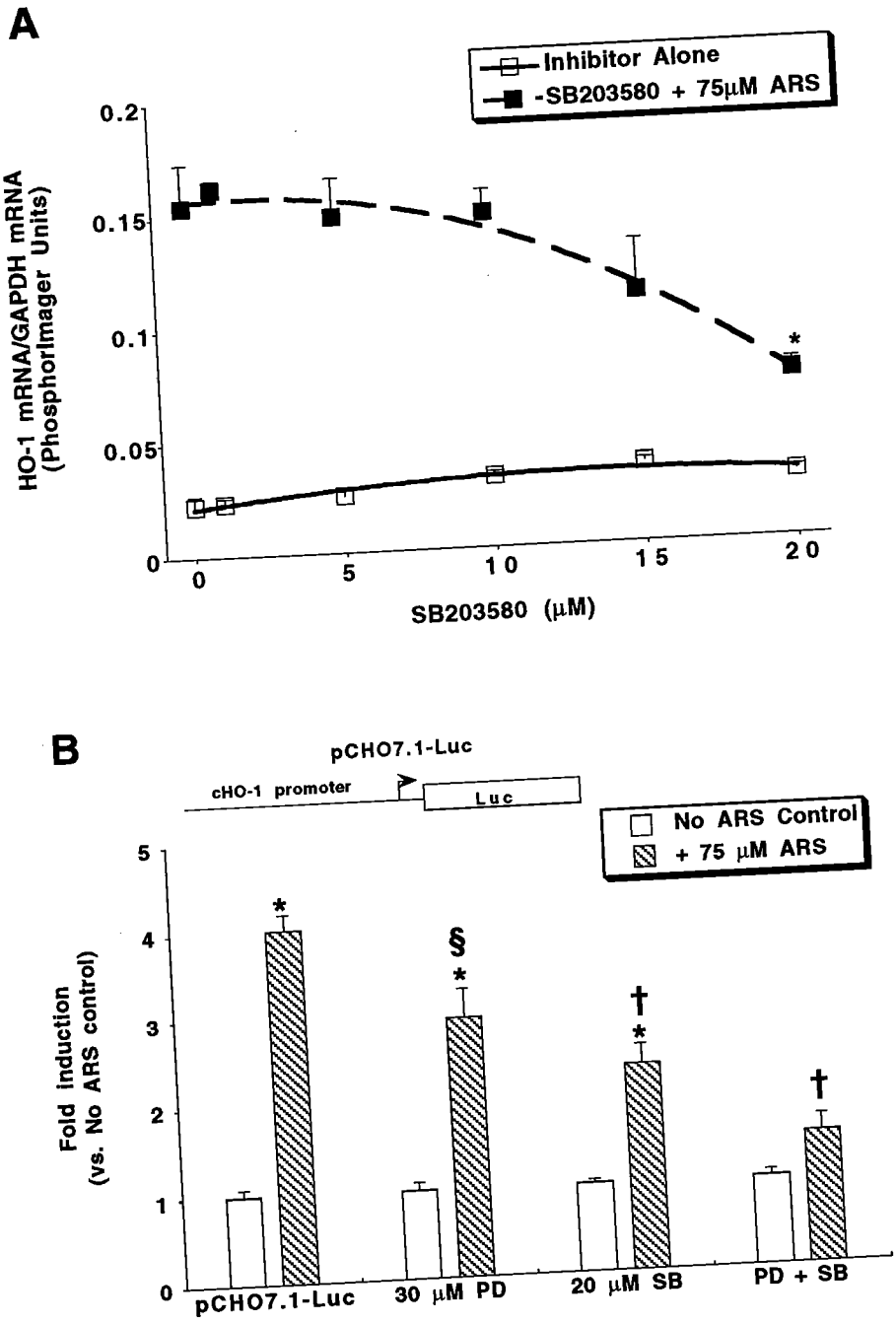

pCHO7.1-Luc
cHO-1 promoter
Luc
No Treatment
Wild type kinase
+ 75μM ARS
Normalized RLU
(RLU/β-gal/μg prot.)
25000
20000
15000
10000
5000
0
Control
JNK
MEKK1
MEKK1
K432A
MLK3
MLK3
K144R

However, in contrast to the results for ERK components, arsenite treatment continued to increase luciferase gene activity in the presence of co-transfected dominant negative MEKK1 or MLK3.

Involvement of p38 MAP kinase in arsenite induction of the cHO-1 gene. In Figure 6.3, an ERK component (MEK), or a p38 component (MKK6), demonstrated the ability to induce luciferase reporter activity. To further delineate a role for p38 in arsenite signaling, a p38 kinase specific inhibitor (SB203580) was tested for the ability to block arsenite induction of HO-1 gene expression. SB203580 is a pyridinyl imidazole (See Appendix D for structure) that specifically binds to p38 kinase with a 1:1 stoichiometry, and competitively inhibits the binding of ATP with a K_i of 21 nM and an IC_{50} of 42 nM [150-152]. Figure 6.7A shows the effects of SB203580 on arsenite induced HO-1 mRNA levels in LMH cells. When cells were pretreated with inhibitor at 20 μM for 30 minutes prior to arsenite treatment, the ability of arsenite to increase HO-1 mRNA levels was decreased to only 47% of the control (no inhibitor). The inhibitor alone caused no significant change in HO-1 mRNA levels.

Since both kinase inhibitors (PD98059 for MEK in Figure 6.5B and SB203580 for p38 in Figure 6.7A), blunted arsenite-mediated induction of HO-1, the combined action of both the MEK and p38 inhibitors on expression of the pCHO7.1-Luc reporter gene was further investigated (Figure 6.7B). Cells were treated with MEK inhibitor, p38 inhibitor, or both, either alone or 30 minutes prior to arsenite treatment. The MEK inhibitor blocked 48%, and the p38

Figure 6.7: Effects of inhibitors of MAP kinase pathways on HO-1 gene expression in LMH cells. **(A)**, LMH cells were treated with the indicated concentrations of p38 kinase inhibitor (SB203580) alone, or 30 minutes prior to addition of 75 μM ARS. Cells were harvested 4 hours after addition of ARS (4.5 hours after inhibitor). HO-1 mRNA was quantitated on dot blots and normalized to GAPDH. Data represent mean + SEM, n=3. *Significantly less than control (ARS only), $P < 0.01$. **(B)**, Prior to treatment, cells were transfected with pCHO7.1-Luc, and pPGK-ßgal. After transfection, cells were treated with either 30 μM MEK inhibitor (PD98059), or 20 μM p38 inhibitor (SB203580), or both, 30 minutes prior to addition of 75 μM ARS. Cell lysates were harvested 6 hours after addition of ARS (6.5 hours after inhibitor(s)). Luciferase activities were normalized to ß-galactosidase activities and protein content. Fold inductions versus no ARS control are presented. Data represent mean + SEM, n=3. *Significantly greater than no ARS control, $P < 0.005$. †Significantly less than ARS treated pCHO7.1-Luc, $P < 0.001$. §Significantly less than ARS treated pCHO7.1-Luc, $P < 0.01$.


A
Inhibitor Alone
-SB203580 + 75μM ARS
HO-1 mRNA/GAPDH mRNA
(PhosphorImager Units)
0.2
0.15
0.1
0.05
0
0
5
10
15
20
SB203580 (μM)
*
B
pCHO7.1-Luc
cHO-1 promoter
Luc
No ARS Control
+ 75 μM ARS
Fold Induction
(vs. No ARS control)
5
4
3
2
1
0
*
§
*
†
*
†
pCHO7.1-Luc
30 μM PD
20 μM SB
PD + SB

inhibitor blocked 68% of the arsenite-mediated increase in luciferase gene activity. This pattern of inhibition is consistent with the patterns of p38 and ERK activation by arsenite (Figure 6.2). Because p38 is activated to a greater extent than ERK (Figure 6.2), it is not surprising that inhibition of arsenite induction by the p38 inhibitor was greater than by the MEK inhibitor. When added in combination, both inhibitors also strongly inhibited (84% inhibition) the increase in luciferase gene activity.

Conclusions

The major findings described in this chapter are: 1) sodium arsenite activates the MAP kinases ERK, JNK, and p38 in LMH cells; 2) activated components of the ERK and p38 MAP kinase pathways are capable of inducing HO-1 gene expression, while dominant negative components block arsenite induction of HO-1; and 3) inhibitors of MEK and p38 differentially inhibit the arsenite induction of HO-1.

Arsenite potently induced both endogenous HO-1 and transiently transfected HO-1 reporter constructs in LMH cells, making this a good system for studying the arsenite-mediated cellular stress response. The results described in this chapter show that there are clear differences between the activation pathways of arsenite and the other treatments tested (heat shock, cadmium, and heme), since all of these conditions elicited different patterns of MAP kinase activation (Figure 6.1).

Some researchers have proposed that heme induces HO-1 by a stress-mediated mechanism [28,48,74,94,111,189]. However, in kinase assays, heme, at concentrations that strongly induce endogenous HO-1, failed to activate any of the MAP kinases (Figure 6.2). Therefore, since heme induces endogenous HO-1 in LMH cells [75], it is likely to do so via a non-MAP kinase-mediated pathway. This is consistent with the results of Cable et. al. [76], who proposed that heme induces HO-1 through a pathway not involving a stress-mediated mechanism.

In LMH cells arsenite activates ERK, JNK, and p38 (Figures 6.1 and 6.2), in agreement with the studies done by Liu *et al.* in Rat-1 and PC-12 cells [131].

Activated components of the ERK pathway, (Ras, Raf, MEK), and an activated component of the p38 pathway (MKK6) were able to induce luciferase gene expression from the pCHO7.1-Luc reporter construct (Figure 6.3). In contrast, MEKK1 and MLK3, components upstream of JNK, were not capable of inducing expression of this HO-1 promoter-reporter construct (Figures 6.3 and 6.6). MEKK1 has been reported to activate a number of kinases, including JNK, IκB kinase, and ERK in some experimental systems (but only when overexpressed) [162,190,191]. Since MEKK1 was unable to induce luciferase gene expression, these pathways of activation are not likely to be involved in mediating the arsenite induction of HO-1. Experiments involving dominant negative components of the ERK and p38 pathways, and the inhibitors of MEK (PD98059) and p38 (SB203580), also provided strong evidence that these two pathways play essential roles in HO-1 gene expression in the presence of arsenite. The combination of MEK and p38 inhibitors was unable to completely block the arsenite induction of pCHO7.1-Luc reporter gene expression. Due to amplification in kinase signaling pathways, a small amount of active kinase may account for the induction observed in the presence of both inhibitors (Figure 6.7). However, arsenite signaling is complex, and may also utilize a pathway that does not involve a phosphorylation event. These results suggest that ERK

and p38 mediate the MAP kinase induction of HO-1, and that JNK is unlikely to be involved.

In summary, arsenite induced MAP kinases that are involved in cellular responses to mitogens and cellular stressors. Disruption of the ERK or p38 MAP kinase pathways by dominant negative components or inhibitors blocked nearly all of the arsenite-mediated induction of HO-1. In contrast, JNK did not seem to play a role in mediating the induction of the HO-1 gene by arsenite.

CHAPTER VII

CONCLUSIONS

Summary and Discussion

Heme metabolism and its regulation were studied in the chicken hepatoma cell line, LMH. LMH cells exhibited regulation of ALA synthase mRNA levels and heme biosynthesis, analogous to that observed in normal chicken hepatocytes. Porphyrin overproduction and accumulation were observed in response to treatment with chemicals that are routinely used for induction in models of porphyria. HO-1 mRNA and activity levels were characterized and correlated with those observed in primary CELCs. Some minor differences were observed in the efficacies of cobalt and arsenite treatments and in the effects of iron chelation on LMH cell metabolism. For the first time, an induction of HO-1 mRNA in CELCs was observed in response to heat shock. Regulation of heme degradation by prototypic inducers and by treatments that cause oxidative stress was studied. These data support the use of LMH cells for further investigation into the regulation of heme metabolism.

HO-1 is induced by a wide array of chemical and physical agents. However, very few studies have been done previously to elucidate the signaling mechanisms utilized by these stimuli. Some evidence has suggested that at least two separate mechanisms of induction exist, one that is heme-dependent,

and another (mediated by transition metals) that is heme-independent [23], but the exact mechanism of signal transduction from the cell surface through second messengers to transcription factors and promoter elements was not delineated.

In Chapter V, HO-1 gene expression in response to sodium arsenite was examined in detail. Studies using transfected HO-1 deletion constructs showed that robust induction by arsenite correlated with the presence of putative AP-1 consensus sites in the distal promoter. Site-directed mutagenesis of these elements revealed an important role for AP-1 transcription factor elements (located at -1578, -3656, and -4597 base pairs upstream of the cHO-1 transcription start site) in the arsenite-mediated induction of HO-1.

The role of MAP kinases as mediators of the arsenite induction of HO-1 was examined. Several investigators have presented evidence that arsenite activates the MAP kinases in several cell lines [131,192]. All three MAP kinase families activate transcription factors that target AP-1 sites [117,139,140]. Transfection of LMH cells with plasmids containing constitutively activated and dominant negative components of each MAP kinase pathway suggested that ERK and p38, but not JNK, modulate most of the arsenite-mediated induction of HO-1. From studies using stably transfected PC12 and transiently transfected Rat-1 cells, Liu et. al. [131] suggested that the cellular response to arsenite is partially regulated by a Ras-dependent mechanism and partially by a Ras-independent mechanism. A response involving both the Ras-dependent ERK, and the Ras-independent p38 pathways (as observed here) is consistent with this result.

Additionally, the recently described MAP kinase, ERK5, is proposed to be activated via a Ras-dependent but Raf independent pathway [193].

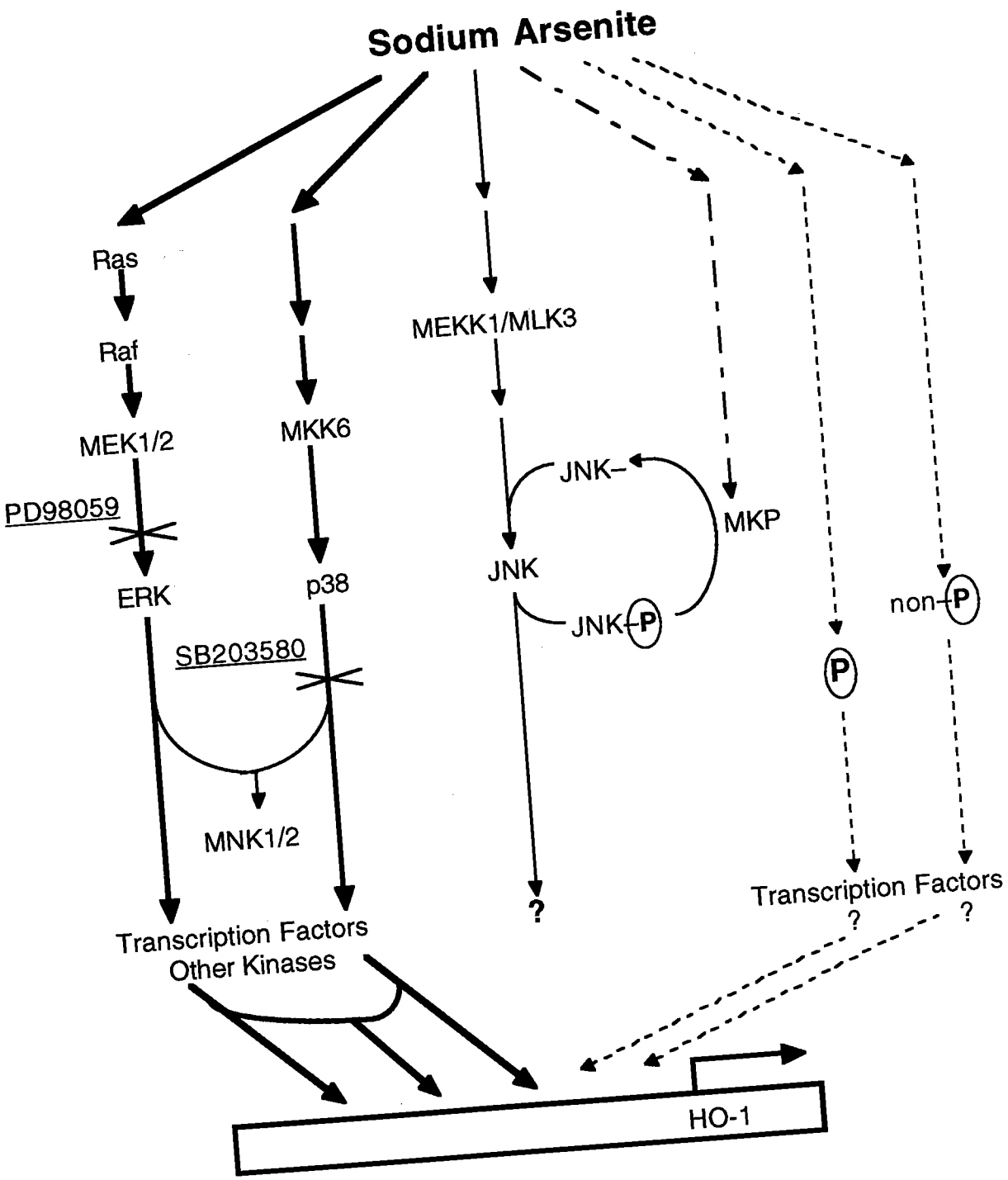
Several possible mechanisms through which arsenite activates both ERK and p38 provide an explanation for cooperative activation of HO-1 gene induction: 1) ERK and p38 may target transcription factors that bind to separate promoter elements required for the tightly controlled expression observed for HO-1; 2) ERK and p38 may activate transcription factors that bind to a single promoter element; or 3) ERK and p38 may target a shared downstream kinase. AP-1 elements are potential targets for the combined action of ERK and p38. ERK can increase c-Fos expression *in vitro*, while p38 activates ATF2, and also increases c-Fos expression. Each kinase activating one of its substrates would lead to formation or activation of AP-1 transcription factor complexes [122,132,135,140,194,195]. In a recent study by Numazawa *et al.*, c-Fos protein expression and ERK activation were shown to be involved in glutathione depletion-mediated oxidative stress induction of HO-1 in human fibroblasts [119]. There are also several other putative promoter elements (Myc/Max, CREB, and STAT3 sites) present in the cHO-1 distal promoter region that may serve as targets for transcription factors activated by MAP kinases (See Figure 5.5).

Another explanation, requiring multiple mechanisms of MAP kinase activation for the full response to arsenite, may involve effects of this chemical on phosphatase activities. Although evidence presented here supports a role for ERK and p38 in HO-1 expression, these studies do not exclude the

possibility that the activity of one or both of these MAP kinases may be increased through phosphatase inhibition, in addition to or instead of activation of their upstream kinase cascades. Phosphatases have critical sulfhydryl residues that are susceptible to the type of oxidation mediated by arsenite. Inhibition of phosphatases may also lead to an increase in MAP kinase activity. In a study of MAP kinase activation by arsenite, Cavigelli *et al.* [159] suggested that arsenite may stimulate AP-1 transcriptional activity by inhibiting a JNK phosphatase. A model representing several possible mechanisms for induction of HO-1 by arsenite is presented in Figure 7.1.

Figure 7.1: Possible mechanisms for induction of HO-1 by sodium arsenite. Most of the arsenite-mediated induction of HO-1 gene expression is transduced by the ERK and p38 MAP kinase pathways (indicated by heavy arrows). Inhibitors that were used to elucidate ERK and p38 involvement are represented by X's through arrows (PD98059, specific inhibitor of MEK; SB203580, specific inhibitor of p38). MNK1/2 is a possible site for cooperation between the ERK and p38 pathways. Also shown at the bottom of the figure are possible mechanisms for cooperation by transcription factors activated by ERK and/or p38.

The remainder of the arsenite signal may be transduced by several other mechanisms as shown. Arsenite activated JNK in LMH cells, but effects on HO-1 gene expression were not observed (light arrows). By inhibiting a MAP kinase phosphatase (MKP) as suggested by Liu et al [159], arsenite may increase MAP kinase activity (indicated by interrupted arrows, — - —). This possibility is illustrated for the JNK pathway, but is applicable to other MAP kinases as well. Alternatively, a signaling mechanism that does not involve MAP kinases may be utilized (indicated by dashed arrows, -----). This mechanism may be phosphorylation dependent (other protein kinases) or it may be a pathway that is independent of phosphorylation events. The question marks represent the unknown effects of JNK activation by arsenite (**?**), and possible signaling through pathways that do not involve MAP kinases (?). (P) = Phosphorylation event.


Sodium Arsenite
Ras
Raf
MEK1/2
PD98059
ERK
MKK6
p38
SB203580
MNK1/2
MEKK1/MLK3
JNK
JNK-
JNK-P
MKP
?
P
non-P
Transcription Factors
?
Transcription Factors
Other Kinases
HO-1

Future Directions

The data presented here support the use of the LMH chicken hepatoma cell line as a good model system for studies involving the regulation of the major enzymes involved in heme biosynthesis and degradation. Other facets of the regulation of heme metabolism may be elucidated through long-term studies and stable transfections, which are now possible using LMH cells.

The sequence reported in Appendix E contains 7.1 Kb of the HO-1 promoter. Several HO-1 promoter luciferase reporter gene deletion constructs were prepared, and their responsiveness to arsenite was characterized. In addition, three putative AP-1 elements were mutated and tested for induction by arsenite. The plasmids and assay system developed throughout the research presented in Chapters V and VI provide useful tools that will facilitate the elucidation of promoter elements and signaling mechanisms utilized by the multitude of stimuli that increase HO-1 gene expression.

Additionally, these studies link two major research fields. Research directed at elucidating the physiological consequences of MAP kinase activation by stress-causing stimuli has emerged concurrently with research directed at delineating the larger role of HO-1 as a cellular stress response protein. In particular, HO-1 has been shown to play a role in protecting cells and tissues from oxidative stress brought about by ischemia/reperfusion [196,197], hypoxia [116], tissue damage [198], hypertension [199], and physical stress [200].

Arachidonic acid and cyclooxygenase metabolites have also been implicated as inducers of HO-1 [201]. The effects of these treatments on MAP kinase activation and phosphorylation of MAP kinase substrates also have been elucidated recently [130,202,203]. When considered together, these studies conceptually link the activation of MAP kinases with induction of HO-1 as integral players in an elaborate cellular stress responsive mechanism.

Although the data presented in Chapter VI indicate that both ERK and p38 MAP kinases are modulators of HO-1 gene expression in response to arsenite, the entire signaling pathway has yet to be delineated. Activity of MAP kinases is maintained by a balance between phosphorylation by upstream kinases and dephosphorylation by dual-specificity and tyrosine phosphatases [143,204-207]. Therefore, MAP kinase activity may be increased either by activation of upstream kinases or by inhibition of regulatory phosphatases. One study by Cavigelli *et al.* suggested that arsenite induced JNK activity in HeLa cells by inhibition of a JNK phosphatase [159]. Further investigation into a phosphatase inhibition mechanism for HO-1 induction is necessary to delineate the contribution of phosphatases to arsenite induction of HO-1.

This work provides a solid foundation for further research, which may investigate: 1) upstream components of the pathway leading from arsenite through MAP kinases to HO-1 gene expression, 2) other non-MAP kinase signal transduction pathways (phosphorylation dependent or independent) that mediate arsenite induction of HO-1, 3) other HO-1 promoter elements or targets

of MAP kinases after activation by arsenite, and 4) a possible indirect role for JNK in HO-1 gene expression.

BIBLIOGRAPHY

1. Ibraham, N. G., Friedland,M. L., and Levere, R. D. (1983) Heme Metabolism in Erythroid and Hepatic Cells. *Progress in Hemotology, Volume XIII* (Brown, E. B., ed) pp. 75-130, Grune & Stratton, Inc.,

2. Bonkovsky, H. L. (1990) Porphyrin and heme metabolism and the porphyrias. *Hepatology: A Textbook of Liver Disease. Volume 1* (Zakim, D. and Boyer, T. D., eds) pp. 378-424, W. B. Saunders Company, Philadelphia, PA

3. Kappas, A., Sassa, S., Galbraith, R. A., and Nordmann, Y. (1989) The Porphyrias. *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beadet, A. L., Sly, W. S., and Valle, D., eds) pp. 1305-1365, McGraw Hill Inc., New York, NY

4. Sassa, S. (1996) Novel effects of heme and heme-related compounds in biological systems. *Curr. Medicinal Chem.* **3**, 273-290.

5. Sassa, S.and Nagai, T. (1996) The role of heme in gene expression. *Intl. J. Hematol.* **63**, 167-178.

6. Cotter, P. D.; Rucknagel, D. L.and Bishop, D. F. (1994) X-linked sideroblastic anemia: identification of the mutation in the erythroid-specific δ-aminolevulinate synthase gene (ALAS2) in the original family described by Cooley. *Blood* **84**, 3915-3924.

7. Ponka, P.and Schulman, H. M. (1985) Acquisition of iron from transferrin regulates reticulocyte heme synthesis. *J. Biol. Chem.* **260**, 14717-14721.

8. Ponka, P., Schulman, H. M., and Cox, T. M. (1990) Iron metabolism in relation to heme synthesis. *Biosynthesis of Heme and Chlorophyll* (Dailey, H. A., ed) pp. 393-434, McGraw-Hill, New York, NY

9. Dandekar, R.; Stripecke, R.; Gray, N. K.; Boossen, B.; Constable, A.; Johansson, H. E.and Hentze, M. W. (1991) Identification of a novel iron-responsive element in murine and human erythroid δ-aminolevulinic acid synthase mRNA. *EMBO J.* **10**, 1903-1909.

10. Ponka, P.and Schulman, H. M. (1993) Regulation of heme biosynthesis: distinct regulatory features in erythroid cells. *Stem Cells* **11 (suppl. 1)**, 24-35.

11. Lash, A.and Saleem, A. (1995) Iron metabolism and its regulation. *Ann. Clin. & Lab. Sci.* **25**, 20-30.

12. Granick, S.; Sinclair, P.; Sassa, S.and Grieninger, G. (1975) Effects by heme, insulin and serum albumin on heme and protein synthesis in chick embryo liver cells cultured in a chemically defined medium, and a spectrofluorometric assay for porphyrin composition. *J. Biol. Chem.* **250**, 9215-9225.

13. Bonkovsky, H. L.; Healey, J. F.; Lourie, A. N.and Gerron, G. G. (1991) Intravenous heme-albumin in acute intermittent prophyria: evidence for repletion of hepatic hemoproteins and regulatory heme pools. *Amer. J. Gastroent.* **86**, 1050-1056.

14. Sinclair, P. R.; Gorman, N.; Walton, H. S.; Sinclair, J. F.; Lee, C. A.and Rifkind, A. B. (1997) Identification of CYP1A5 as the CYP1A enzyme mainly

responsible for uroporphyrinogen oxidation induced by AH receptor ligands in chicken liver and kidney. *Drug Metab. and Disposition* **25**, 779-783.

15. Harigae, H.; Suwabe, N.; Weinstock, P. H.; Nagai, M.; Fujita, H.; Yamamoto, M.and Sassa, S. (1998) Deficient heme and globin synthesis in embryonic stem cells lacking the erythroid-specific δ-aminolevulinate synthase gene. *Blood* **91**, 798-805.

16. Tenhunen, R.; Marver, H. S.and Schmid, R. (1968) The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc. Natl. Acac. Sci. USA* **61**, 748-755.

17. Tenhunen, R.; Marver, H. S.and Schmid, R. (1969) Microsomal heme oxygenase. Characterization of the enzyme. *J. Biol. Chem.* **244**, 6388-6394.

18. Kikuchi, G.and Yoshida, T. (1983) Function and induction of the microsomal heme oxygenase. *Mol. Cell. Biochem.* **53/54**, 163-183.

19. Abraham, N. G.; Lin, J. H. -C.; Schwartzman, M. L.; Levere, R. D.and Shibahara, S. (1988) The physiological significance of heme oxygenase. *Intl. J. Biochem.* **20**, 543-558.

20. Ewing, J. F.; Weber, C. M.and Maines, M. D. (1993) Biliverdin reductase is heat resistant and coexpressed with constitutive and heat shock forms of heme oxygenase in brain. *J. Neurochem.* **61**, 1015-1023.

21. McCoubrey, Jr.,W.K.and Maines, M. D. (1994) The structure, organization and differential expression of the gene encoding rat heme oxygenase-2. *Gene* **139**, 155-161.

22. Shibahara, S.; Yoshizawa, M.; Suzuki, H.; Takeda, K.; Meguro, K.and Endo, K. (1993) Functional analysis of cDNAs for two types of human heme oxygenase and evidence for their separate regulation. *J. Biochem.* **113**, 214-218.

23. Lincoln, B. C.; Healey, J. F.and Bonkovsky, H. L. (1988) Regulation of hepatic haem metabolism. Disparate mechanisms of induction of haem oxygenase by drugs and metals. *Biochem. J.* **250**, 189-196.

24. Bhat, G. J.and Padmanaban, G. (1988) Heme is a positive regulator of cytochrome P-450 gene transcription. *Arch. Biochem. Biophys.* **264**, 584-590.

25. Sardana, M. K.; Sassa, S.and Kappas, A. (1980) Adrenalectomy enhances the induction of heme oxygenase and the degradation of cytochrome P-450 in liver. *J. Biol. Chem.* **255**, 11320-11323.

26. Paine, A. J.and Legg, R. F. (1978) Apparent lack of correlation between the loss of cytochrome P-450 in hepatic parenchymal cell culture and the stimulation of haem oxygenase activity. *Biochem. Biophys. Res. Commun.* **81**, 672-679.

27. Alam, J. (1994) Multiple elements within the 5' distal enhancer of the mouse heme oxygenase-1 gene mediate induction by heavy metals. *J. Biol. Chem.* **269**, 25049-25056.

28. Alam, J.; Cai, J.and Smith, A. (1994) Isolation and characterization of the mouse heme oxygenase-1 gene. Distal 5' sequences are required for induction by heme or heavy metals. *J. Biol. Chem.* **269**, 1001-1009.

29. Shibahara, S.; Müller, R. M.and Taguchi, H. (1987) Transcriptional control of rat heme oxygenase by heat shock. *J. Biol. Chem.* **262**, 12889-12892.

30. Tyrrell, R. M.; Applegate, L. A.and Tromvoukis, Y. (1993) The proximal promoter region of the human heme oxygenase gene contains elements involved in stimulation of transcriptional activity by a variety of agents including oxidants. *Carcinogenesis* **14**, 761-765.

31. Lu, T. H.; Pepe, J.; Gildemeister, O.; Tyrrell, R. M.and Bonkovsky, H. L. (1997) Regulation of expression of the human heme oxygenase-1 gene in transfected chick embryo liver cell cultures. *Biochim. Biophys. Acta* **1352**, 293-302.

32. Yoshida, T.; Biro, P.; Cohen, T.; Müller, R. M.and Shibahara, S. (1988) Human heme oxygenase cDNA and induction of its mRNA by hemin. *Eur. J. Biochem.* **171**, 457-461.

33. Sato, M.; Ishizawa, S.; Yoshida, T.and Shibahara, S. (1990) Interaction of upstream stimulatory factor with the human heme oxygenase gene promoter. *Eur. J. Biochem.* **188**, 231-237.

34. Lavrovsky, Y.; Schwartzman, M. L.and Abraham, N. G. (1993) Novel regulatory sites of the human heme oxygenase-1 promoter region. *Biochem. Biophys. Res. Commun.* **196**, 336-341.

35. Evans, C. -O.; Healey, J. F.; Greene, Y.and Bonkovsky, H. L. (1991) Cloning, sequencing, and expression of cDNA for chick liver haem oxygenase.

Comparison of avian and mammalian cDNAs and deduced proteins. *Biochem. J.* **273**, 659-666.

36. Bonkovsky, H. L.; Healey, J. F.and Pohl, J. (1990) Purification and characterization of heme oxygenase from chick liver. Comparison of the avian and mammalian enzymes. *Eur. J. Biochem.* **189**, 155-166.

37. Lu, T. H.; Lambrecht, R. W.; Pepe, J.; Shan, Y.; Kim, T.and Bonkovsky, H. L. (1998) Molecular cloning, characterization, and expression of the chicken heme oxygenase-1 gene in transfected primary cultures of chick embryo liver cells. *Gene* **207**, 177-186.

38. Sun, J.; Loehr, T. M.; Wilks, A.and Ortiz de Montellano, P. R. (1994) Identification of histidine 25 as the heme ligand in human liver heme oxygenase. *Biochemistry* **33**, 13734-13740.

39. Ito-Maki, M.; Ishikawa, K.; Matera, K. M.; Sato, M.; Ikeda-Saito, M.and Yoshida, T. (1995) Demonstration that histidine 25, but not 132, is the axial heme ligand in rat heme oxygenase-1. *Arch. Biochem. Biophys.* **317**, 253-258.

40. Poss, K. D.and Tonegawa, S. (1997) Heme oxygenase 1 is required for mammalian iron reutilization. *Proc. Natl. Acac. Sci. USA* **94**, 10919-10924.

41. Poss, K. D.and Tonegawa, S. (1997) Reduced stress defense in heme oxygenase 1-deficient cells. *Proc. Natl. Acac. Sci. USA* **94**, 10925-10930.

42. Padmanaban, G.; Rao, M. R. S.and Malathi, K. (1973) A model for the regulation of δ-aminolaevulinate synthetase induction in rat liver. *Biochem. J.* **134**, 847-857.

43. Sinclair, P. R.; Bement, W. J.; Bonkovsky, H. L.; Lambrecht, R. W.; Frezza, J. E.; Sinclair, J. F.; Urquhart, A. J.and Elder, G. H. (1986) Uroporphyrin accumulation produced by halogenated biphenyls in chick-embryo hepatocytes. Reversal of the accumulation by piperonyl butoxides. *Biochem. J.* **237**, 63-71.

44. Cable, E. E.; Cable, J. W.and Bonkovsky, H. L. (1993) Repression of hepatic ∂-aminolevulinate synthase by heme and metalloporphyrins: relationship to inhibition of heme oxygenase. *Hepatology* **18**, 119-127.

45. Cable, E. E.; Healey, J. F.; Greene, Y.; Evans, C. -O.and Bonkovsky, H. L. (1991) Synergistic induction of ∂-aminolevulinate synthase by glutethimide and iron: relationship to the synergistic induction of heme oxygenase. *Biochim. Biophys. Acta* **1080**, 245-251.

46. Russo, S. M.; Pepe, J. A.; Cable, E. E.; Lambrecht, R. W.and Bonkovsky, H. L. (1994) Repression of ALA synthase by heme and zinc-mesoporphyrin in a chick embryo liver cell culture model of acute porphyria. *Eur. J. Clin. Invest.* **24**, 406-415.

47. Lincoln, B. C.and Bonkovsky, H. L. (1987) Hepatic heme catabolism in cultured hepatocytes. *Fed. Proc.* **46**, 2264. (Abstract)

48. Vile, G. F; Basu-Modak, S.; Waltner, C.and Tyrrell, R. M. (1994) Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts. *Proc. Natl. Acad. Sci. USA* **91**, 2607-2610.

49. Alam, J.and Zhining, D. (1992) Distal AP-1 binding sites mediate basal level enhancement and TPA induction of the mouse heme oxygenase-1 gene. *J. Biol. Chem.* **267**, 21894-21900.

50. Bissell, D. M.and Bearn, A. G. (1980) Liver cell culture: Current status and prospects for the study of inherited diseases. *Birth Defects: Orig. Art. Series* **16**, 283-291.

51. Mitani, K.; Fujita, H.; Sassa, S.and Kappas, A. (1989) Heat shock induction of heme oxygenase mRNA in human HEP 3B hepatoma cells. *Biochem. Biophys. Res. Commun.* **165**, 437-441.

52. Mitani, K.; Fujita, H.; Sassa, S.and Kappas, A. (1990) Activation of heme oxygenase and heat shock protein 70 genes by stress in human hepatoma cells. *Biochem. Biophys. Res. Commun.* **166**, 1429-1434.

53. Koningsberger, J. C.; Rademakers, L. H. P. M.; van Hattum, J.; de la Faille, H. B.; Wiegman, L. J. J. M.; Italiaander, E.; van Berge Henegouwen, G. P.and Marx, J. J. M. (1995) Exogenous protoporphyrin inhibits Hep G2 cell proliferation, increases the intracellular hydrogen peroxide concentration and causes ultrastructural alterations. *J. Hepatology* **22**, 57-65.

54. Van Remmen, H.; Williams, M. D.; Heydari, A. R.; Takahashi, R.; Chung, H. Y.; Yu, B. P.and Richardson, A. (1996) Expression of genes coding for antioxidant enzymes and heat shock proteins is altered in primary cultures of rat hepatocytes. *J. Cell. Physiol.* **166**, 453-460.

55. Hamilton, J. W.; Bement, W. J.; Sinclair, P. R.; Sinclair, J. F.and Wetterhahn, K. E. (1988) Expression of 5-aminolaevulinate synthase and cytochrome *P*-450 mRNAs in chicken embryo hepatocytes. *Biochem. J.* **255**, 267-275.

56. Cable, E. E.; Pepe, J. A.; Donohue, S. E.; Lambrecht, R. W.and Bonkovsky, H. L. (1994) Effects of mifepristone (RU-486) on heme metabolism and cytochromes P-450 in cultured chick embryo liver cells. Possible implications for acute porphyria. *Eur. J. Biochem.* **225**, 651-657.

57. Cable, E. E.; Pepe, J. A.; Karamitsios, N. C.; Lambrecht, R. W.and Bonkovsky, H. L. (1994) Differential effects of metalloporphyrins on messenger RNA levels of δ-aminolevulinate synthase and heme oxygenase. Studies in cultured chick embryo liver cells. *J. Clin. Invest.* **94**, 649-654.

58. Rifkind, A. B.; Gillette, P. N.; Song, C. S.and Kappas, A. (1973) Drug stimulation of ∂-aminolevulinic acid synthetase and cytochrome P-450 *in vivo* in chick embryo liver. *J. Pharmacol. & Exptl. Therap.* **185**, 214-225.

59. Sassa, S.and Kappas, A. (1977) Induction of ∂-aminolevulinate synthase and porphyrins in cultured liver cells maintained in chemically defined medium. Permissive effects of hormones on induction process. *J. Biol. Chem.* **252**, 2428-2436.

60. Bonkovsky, H. L.; Cable, E. E.; Cable, J. W.; Donohue, S. E.; White, E. C.; Greene, Y. J.; Lambrecht, R. W.; Srivastava, K. K.and Arnold, W. N. (1992) Porphyrogenic properties of the terpenes camphor, pinene, and thujone. (With a note on historic implications for absinthe and the illness of Vincent Van Gogh). *Biochem. Pharm.* **43**, 2359-2368.

61. Sinclair, J. F.; Sinclair, P. R.and Bonkowsky, H. L. (1979) Hormonal requirements for the induction of cytochrome P450 in hepatocytes cultured in a serum-free medium. *Biochem. Biophys. Res. Commun.* **86**, 710-717.

62. Lu, T. H.; Cable, E. E.; Pepe, J.; Lambrecht, R. W.; Dalton, T.; Andrews, G.and Bonkovsky, H. L. (1994) Regulation of hepatic metallothionein (MT) gene expression: Studies in transfected primary liver cell cultures. *Hepatology* **20**, 227A. (Abstract)

63. Hahn, C. N.; Hansen, A. J.and May, B. K. (1991) Transcriptional regulation of the chicken CYP2H1 gene. Localization of a phenobarbital-responsive enhancer domain. *J. Biol. Chem.* **266**, 17031-17039.

64. Kawaguchi, T.; Nomura, K.; Hirayama, Y.and Kitagawa, T. (1987) Establishment and characterization of a chicken hepatocellular carcinoma cell line, LMH. *Cancer Res.* **47**, 4460-4464.

65. Oddoux, C.and Grieninger, G. (1994) Characterization of a chicken hepatoma cell line with a specific defect in fibrinogen secretion. *Hepatology* **19**, 682-687.

66. Oddoux, C.and Grieninger, G. (1994) Fibrinogen assembly: Insights from chicken hepatocytes. *Hepatology* **19**, 688-693.

67. Bhattacharyya, N.and Banerjee, D. (1993) Transcriptional regulation of the gene encoding apolipoprotein AI in chicken LMH cells. *Gene* **137**, 315-320.

68. Bhattacharyya, N.; Chattapadhyay, R.; Oddoux, C.and Banerjee, D. (1993) Characterization of the chicken apolipoprotein A-I gene 5'-flanking region. *DNA Cell Biol.* **12**, 597-604.

69. Sensel, M. G.; Binder, R.; Lazier, C. B.and Williams, D. L. (1994) Reactivation of apolipoprotein II gene transcription by cycloheximide reveals two steps in the deactivation of estrogen receptor-mediated transcription. *Mol. Cell. Biol.* **14**, 1733-1742.

70. Berkowitz, E. A.and Evans, M. I. (1992) Estrogen-dependent expression of the chicken very low density apolipoprotein II gene in serum-free cultures of LMH cells. *In Vitro Cell. Dev. Biol.* **28A**, 391-396.

71. Seal, S. N.; Davis, D. L.and Burch, J. B. E. (1991) Mutational studies reveal a complex set of positive and negative control elements within the chicken vitellogenin II promoter. *Mol. Cell. Biol.* **11**, 2704-2717.

72. Taouis, M.; Derouet, M.; Caffin, J. P.; Chavanieu, A.and Simon, J. (1993) Insulin receptor and insulin sensitivity in a chicken hepatoma cell line. *Molec. Cell. Endocrinol.* **96**, 113-123.

73. Taouis, M.; Derouet, M.; Caffin, J. P.and Simon, J. (1994) Increased insulin receptor number and insulin responsiveness in a chicken hepatoma cell line. *J. Endocrinol.* **140**, 119-124.

74. Alam, J.; Shibahara, S.and Smith, A. (1989) Transcriptional activation of the heme oxygenase gene by heme and cadmium in mouse hepatoma cells. *J. Biol. Chem.* **264**, 6371-6375.

75. Gabis, K. K.; Gildemeister, O. S.; Pepe, J. A.; Lambrecht, R. W.and Bonkovsky, H. L. (1996) Induction of heme oxygenase-1 in LMH cells. Comparison of LMH cells to primary cultures of chick embryo liver cells. *Biochim. Biophys. Acta* **1290**, 113-120.

76. Cable, E. E.; Pepe, J. A.; Gildemeister, O. S.; Lambrecht, R. W.and Bonkovsky, H. L. (1994) Induction of heme oxygenase by metalloporphyrins in primary chick embryo liver cells: Evidence for a protein- but not a stress-dependent response. *Hepatology* **20**, 179A. (Abstract)

77. Maines, M. D.and Kappas, A. (1977) Metals as regulators of heme metabolism. *Science* **198**, 1215-1221.

78. Sardana, M. K.; Sassa, S.and Kappas, A. (1982) Metal ion-mediated regulation of heme oxygenase induction in cultured avian liver cells. *J. Biol. Chem.* **257**, 4806-4811.

79. Rizzardini, M.; Terao, M.; Falciani, F.and Cantoni, L. (1993) Cytokine induction of haem oxygenase mRNA in mouse liver. Interleukin 1 transcriptionally activates the haem oxygenase gene. *Biochem. J.* **290**, 343-347.

80. Fukuda, Y.and Sassa, S. (1994) Suppression of cytochrome P450IA1 by interleukin-6 in human HepG2 hepatoma cells. *Biochem. Pharm.* **47**, 1187-1195.

81. Fukuda, Y.and Sassa, S. (1993) Effect of interleukin-11 on the levels of mRNAs encoding heme oxygenase and haptoglobin in human HepG2 hepatoma cells. *Biochem. Biophys. Res. Commun.* **193**, 297-302.

82. Strandell, E.; Buschard, K.; Saldeen, J.and Welsh, N. (1995) Interleukin-1 beta induces the expression of hsp70, heme oxygenase and Mn-SOD in FACS-purified rat islet beta-cells, but not in alpha-cells. *Immunol. Lett.* **48**, 145-148.

83. Rossi, A.and Santoro, M. G. (1995) Induction by prostaglandin A1 of haem oxygenase in myoblastic cells: an effect independent of expression of the 70 kDa heat shock protein. *Biochem. J.* **308**, 455-463.

84. Koizumi, T.; Odani, N.; Okuyama, T.; Ichikawa, A.and Negishi, M. (1995) Identification of a cis-regulatory element for D^{12}-prostaglandin J_2-induced expression of the rat heme oxygenase gene. *J. Biol. Chem.* **270**, 21779-21784.

85. Keyse, S. M.and Tyrrell, R. M. (1989) Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc. Natl. Acad. Sci. USA* **86**, 99-103.

86. Basu-Modak, S.and Tyrrell, R. M. (1993) Singlet oxygen: a primary effector in the ultraviolet A/near-visible light induction of the human heme oxygenase gene. *Cancer Res.* **53**, 4505-4510.

87. Alam, J.; Camhi, S.and Choi, A. M. K. (1995) Identification of a second region upstream of the mouse heme oxygenase-1 gene that functions as a basal level and inducer-dependent transcription enhancer. *J. Biol. Chem.* **270**, 11977-11984.

88. Camhi, S. L.; Alam, J.; Otterbein, L.; Sylvester, S. L.and Choi, A. M. K. (1995) Induction of heme oxygenase-1 gene expression by lipopolysaccharide is mediated by AP-1 activation. *Am. J. Resp. Cell Mol. Biol.* **13**, 387-398.

89. Taketani, S.; Kohno, H.; Yoshinaga, T.and Tokunaga, R. (1989) The human 32-kDa stress protein induced by exposure to arsenite and cadmium ions is heme oxygenase. *FEBS Lett.* **245**, 173-176.

90. Lee, T. -C.and Ho, I-C. (1995) Modulation of cellular antioxidant defense activities by sodium arsenite in human fibroblasts. *Arch. Toxicol.* **69**, 498-504.

91. Applegate, L. A.; Luscher, P.and Tyrrell, R. M. (1991) Induction of heme oxygenase: A general response to oxidant stress in cultured mammalian cells. *Cancer Res.* **51**, 974-978.

92. Llesuy, S. F.and Tomaro, M. L. (1994) Heme oxygenase and oxidative stress. Evidence of involvement of bilirubin as physiological protector against oxidative damage. *Biochim. Biophys. Acta* **1223**, 9-14.

93. Lee, T. -C.and Ho, I-C. (1994) Expression of heme oxygenase in arsenic-resistant human lung adenocarcinoma cells. *Cancer Res.* **54**, 1660-1664.

94. Tyrrell, R. M.and Basu-Modak, S. (1994) Transient enhancement of heme oxygenase 1 mRNA accumulation: a marker of oxidative stress to eukaryotic cells. *Meth. Enz.* **234**, 224-235.

95. Choi, A. M.and Alam, J. (1996) Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am. J. Resp. Cell Mol. Biol.* **15**, 9-19.

96. Tyrrell, R. M. (1997) Approaches to define pathways of redox regulation of a eukaryotic gene: the heme oxygenase-1 example. *Methods* **11**, 313-318.

97. Yamaguchi, T.; Terakado, M.; Horio, F.; Aoki, K.; Tanaka, M.and Nakajima, H. (1996) Role of bilirubin as an antioxidant in an ischemia-reperfusion of rat liver and induction of heme oxygenase. *Biochem. Biophys. Res. Commun.* **223**, 129-135.

98. Raju, V. S.and Maines, M. D. (1996) Renal ischemia/reperfusion up-regulates heme oxygenase-1 (HSP32) expression and increases cGMP in rat heart. *J. Pharmacol. & Exptl. Therap.* **277**, 1814-1822.

99. Maines, M. D. (1997) The heme oxygenase system: a regulator of second messenger gases. *Annu. Rev. Pharm. Toxicol.* **37**, 517-554.

100. Brown, J. L.and Kitchin, K. T. (1996) Arsenite, but not cadmium, induces ornithine decarboxylase and heme oxygenase activity in rat liver: relevance to arsenic carcinogenesis. *Cancer Lett.* **98**, 227-231.

101. Kurata, S.; Matsumoto, M.and Nakajima, H. (1996) Transcriptional control of the heme oxygenase gene in mouse M1 cells during their TPA-induced differentiation into macrophages. *J. Cell. Biochem.* **62**, 314-324.

102. Primiano, T.; Kensler, T. W.; Kuppusamy, P.; Zweier, J. L.and Sutter, T. R. (1996) Induction of hepatic heme oxygenase-1 and ferritin in rats by cancer chemopreventive dithiolethiones. *Carcinogenesis* **17**, 2291-2296.

103. Dennery, P. A.and Rodgers, P. A. (1996) Ontogeny and developmental regulation of heme oxygenase. *J. Perinatology* **16**, S79-S83.

104. Lee, P. J.; Alam, J.; Wiegand, G. W.and Choi, A. M. (1996) Overexpression of heme oxygenase-1 in human pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia. *Proc. Natl. Acac. Sci. USA* **93**, 10393-10398.

105. Maines, M. D.and Abrahamsson, P. A. (1996) Expression of heme oxygenase-1 (HSP32) in human prostate: normal, hyperplastic, and tumor tissue distribution. *Urology* **47**, 727-733.

106. Goodman, A. I.; Choudhury, M.; da Silva, J. L.; Schwartzman, M. L.and Abraham, N. G. (1997) Overexpression of the heme oxygenase gene in renal cell carcinoma. *Proc. Soc. Exptl. Biol. Med.* **214**, 54-61.

107. Cable, E.; Greene, Y.; Healey, J.; Evans, C. -O.and Bonkovsky, H. (1990) Mechanism of synergistic induction of hepatic heme oxygenase by glutethimide and iron: studies in cultured chick embryo liver cells. *Biochem. Biophys. Res. Commun.* **168**, 176-181.

108. Lincoln, B. C.; Aw, T. Y.and Bonkovsky, H. L. (1989) Heme catabolism in cultured hepatocytes: evidence that heme oxygenase is the predominant pathway and that a proportion of synthesized heme is converted rapidly to biliverdin. *Biochim. Biophys. Acta* **992**, 49-58.

109. Maines, M. D.and Sinclair, P. (1977) Cobalt regulation of heme synthesis and degradation in avian embryo liver cell culture. *J. Biol. Chem.* **252**, 219-223.

110. Adler, V.; Schaffer, A.; Kim, J.; Dolan, L.and Ronai, Z. (1995) UV irradiation and heat shock mediate JNK activation via alternate pathways. *J. Biol. Chem.* **270**, 26071-26077.

111. Inamdar, N. M.; Ahn, Y. I.and Alam, J. (1996) The heme-responsive element of the mouse heme oxygenase-1 gene is an extended AP-1 binding site that resembles the recognition sequences for MAF and NF-E2 transcription factors. *Biochem. Biophys. Res. Commun.* **221**, 570-576.

112. Raju, V. S.; McCoubrey, Jr.,W.K.and Maines, M. D. (1997) Regulation of heme oxygenase-2 by glucocorticoids in neonatal rat brain: characterization of a functional glucocorticoid response element. *Biochim. Biophys. Acta* **1351**, 89-104.

113. Takeda, K.; Ishizawa, S.; Sato, M.; Yoshida, T.and Shibahara, S. (1994) Identification of a *cis*-acting element that is responsible for cadmium-mediated induction of the human heme oxygenase gene. *J. Biol. Chem.* **269**, 22858-22867.

114. Lavrovsky, Y.; Schwartzman, M. L.; Levere, R. D.; Kappas, A.and Abraham, N. G. (1994) Identification of binding sites for transcription factors NF-kB and AP-2 in the promoter region of the human heme oxygenase 1 gene. *Proc. Natl. Acad. Sci. USA* **91**, 5987-5991.

115. Maeshima, H.; Sato, M.; Ishikawa, K.; Katagata, Y.and Yoshida, T. (1996) Participation of altered upstream stimulatory factor in the induction of rat heme oxygenase-1 by cadmium. *Nuc. Acids Res.* **24**, 2959-2965.

116. Lee, P. J.; Jiang, B. -H.; Chin, B. Y.; Iyer, N. V.; Alam, J.; Semenza, G. L.and Choi, A. M. K. (1997) Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J. Biol. Chem.* **272**, 5375-5381.

117. Karin, M.; Liu, Z.and Zandi, E. (1997) AP-1 function and regulation. *Curr. Opin. Cell Biol.* **9**, 240-246.

118. Oguro, T.; Hayashi, M.; Numazawa, S.; Asakawa, K.and Yoshida, T. (1996) Heme oxygenase-1 gene expression by a glutathione depletor, phorone, mediated through AP-1 activation in rats. *Biochem. Biophys. Res. Commun.* **221**, 259-265.

119. Numazawa, S.; Yamada, H.; Furusho, A.; Nakahara, T.; Oguro, T.and Yoshida, T. (1997) Cooperative induction of c-*fos* and heme oxygenase gene products under oxidative stress in human fibroblastic cells. *Exp. Cell Res.* **237**, 434-444.

120. Lu, T. H., Shan, Y., Pepe,J., Lambrecht, R. W., and Bonkovsky, H. L. (1998) Sodium arsenite and cobalt chloride induce chick heme oxygenase-1 gene expression through oxidative stress and AP-1 activation. (In Preparation).

121. Graves, J. D.; Campbell, J. S.and Krebs, E. G. (1995) Protein serine/threonine kinases of the MAPK cascade. *Ann. New York Acad. Sci.* **766**, 320-343.

122. Bokemeyer, D.; Sorokin, A.and Dunn, M. J. (1996) Multiple intracellular MAP kinase signaling cascades. *Kidney Intl.* **49**, 1187-1198.

123. Meier, R.; Rouse, J.; Cuenda, A.; Nebreda, A. R.and Cohen, P. (1996) Cellular stresses and cytokines activate multiple mitogen-activated-protein kinase kinase homologues in PC12 and KB cells. *Eur. J. Biochem.* **256**, 796-805.

124. van Delft, S.; Coffer, P.; Kruijer, W.and van Wijk, R. (1993) C-fos induction by stress can be mediated by the SRE. *Biochem. Biophys. Res. Commun.* **197**, 542-548.

125. Sachsenmaier, C.; Radler-Pohl, A.; Zinck, R.; Nordheim, A.; Herrlich, P.and Rahmsdorf, H. J. (1994) Involvement of growth factor receptors in the mammalian UVC response. *Cell* **78**, 963-972.

126. Kyriakis, J. M.and Avruch, J. (1996) Protein kinase cascades activated by stress and inflammatory cytokines. *BioEssays* **18**, 567-577.

127. Zanke, B. W.; Boudreau, K.; Rubie, E.; Winnett, E.; Tibbles, L. A.; Zon, L.; Kyriakis, J.; Liu, F. -F.and Woodgett, J. R. (1996) The stress-activated protein kinase pathway mediates cell death following injury induced by *cis*-platinum, UV irradiation or heat. *Curr. Biol.* **6**, 606-613.

128. Lee, J. C.and Young, P. R. (1996) Role of CSBP/p38/RK stress response kinase in LPS and cytokine signaling mechanisms. *J. Leuk. Biol.* **59**, 152-157.

129. Sluss, H. K.; Barrett, T.; Dérijard, B.and Davis, R. J. (1994) Signal transduction by tumor necrosis factor mediated by JNK protein kinases. *Mol. Cell. Biol.* **14**, 8376-8384.

130. Coroneos, E.; Wang, Y.; Panuska, J. R.; Templeton, D. J.and Kester, M. (1996) Sphingolipid metabolites differentially regulate extracellular signal-regulated kinase and stress-activated protein kinase cascades. *Biochem. J.* **316**, 13-17.

131. Liu, Y.; Guyton, K. Z.; Gorospe, M.; Xu, Q.; Lee, J. C.and Holbrook, N. J. (1996) Differential activation of ERK, JNK/SAPK and p38/CSBP/RK MAP kinase family members during the cellular response to arsenite. *Free Rad. Biol. Med.* **21**, 771-781.

132. Davis, R. (1993) The mitogen-activated protein kinase signal transduction pathway (minireview). *J. Biol. Chem.* **268**, 14553-14556.

133. Cobb, M. H.and Goldsmith, E. J. (1995) How MAP kinases are regulated. *J. Biol. Chem.* **270**, 14843-14846.

134. Seger, R.and Krebs, E. G. (1995) The MAPK signaling cascade. *FASEB* **9**, 726-735.

135. Treisman, R. (1996) Regulation of transcription by MAP kinase cascades. *Curr. Opin. Cell Biol.* **8**, 205-215.

136. Kyriakis, J. M.and Avruch, J. (1996) Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.* **271**, 24313-24316.

137. Marshall, M. S. (1995) Ras target proteins in eukaryotic cells. *FASEB* **9**, 1311-1318.

138. Paul, A.; Wilson, S.; Belham, C. M.; Robinson, C. J. M.; Scott, P. H.; Gould, G. W.and Plevin, R. (1997) Stress-activated protein kinases: activation, regulation and function. *Cell. Signal.* **9**, 403-410.

139. Karin, M. (1995) The regulation of AP-1 activity by mitogen-activated protein kinases. *J. Biol. Chem.* **270**, 16483-16486.

140. Whitmarsh, A. J.and Davis, R. J. (1996) Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J. Mol. Med.* **74**, 589-607.

141. Robbins, D. J.; Zhen, E.; Cheng, M.; Xu, S.; Ebert, D.and Cobb, M. H. (1994) MAP kinases ERK1 and ERK2: pleiotropic enzymes in a ubiquitous signaling network. *Adv. Cancer Res.* **63**, 93-116.

142. Her, J. -H.; Lakhani, S.; Zu, K.; Vila, J.; Dent, P.; Sturgill, T. W.and Weber, M. J. (1993) Dual phosphorylation and autophosphorylation in mitogen-activated protein (MAP) kinase activation. *Biochem. J.* **296**, 25-31.

143. Sun, H.and Tonks, N. K. (1996) The coordinated action of protein tyrosine phosphatases and kinases in cell signaling. *Trends Biochem. Sci.* **19**, 480-484.

144. Dudley, D. T.; Pang, L.; Decker, S. J.; Bridges, A. J.and Saltiel, A. R. (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acac. Sci. USA* **92**, 7686-7689.

145. Alessi, D. R.; Cuenda, A.; Cohen, P.; Dudley, D. T.and Saltiel, A. R. (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.* **270**, 27489-27494.

146. Pang, L.; Sawada, T.; Decker, S. J.and Saltiel, A. R. (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *J. Biol. Chem.* **270**, 13585-13588.

147. Servant, M. J.; Giasson, E.and Meloche, S. (1996) Inhibition of growth factor-induced protein synthesis by a selective MEK inhibitor in aortic smooth muscle cells. *J. Biol. Chem.* **271**, 16047-16052.

148. Kyriakis, J. M.; Banerjee, P.; Nikolakaki, E.; Dai, T.; Rubie, E. A.; Ahmad, M. F.; Avruch, J.and Woodgett, J. R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* **369**, 156-160.

149. Chen, Y. -R.; Wang, X.; Templeton, D.; Davis, R.and Tan, T. -H. (1996) The role of c-Jun N-terminal kinase (JNK) in apoptosis induced by ultraviolet C and g radiation. Duration of JNK activation may determine cell death and proliferation. *J. Biol. Chem.* **271**, 31929-31936.

150. Cuenda, A.; Rouse, J.; Doza, Y. N.; Meier, R.; Cohen, P.; Gallagher, T. F.; Young, P. R.and Lee, J. C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.* **364**, 229-233.

151. Young, P. R.; McLaughlin, M. M.; Kumar, S.; Kassis, S.; Doyle, M. L.; McNulty, D.; Gallagher, T. F.; Fisher, S.; McDonnell, P. C.; Carr, S. A.; Huddleston, M. J.; Seibel, G.; Porter, T. G.; Livi, G. P.; Adams, J. L.and Lee, J. C. (1997) Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. *J. Biol. Chem.* **272**, 12116-12121.

152. Tong, L.; Pav, S.; White, D. M.; Rogers, S.; Crane, K. M.; Cywin, C. L.; Brown, M. L.and Pargellis, C. A. (1997) A highly specific inhibitor of human p38 MAP kinase binds in the ATP pocket. *Nat. Struc. Biol.* **4**, 311-316.

153. Waskiewicz, A. J.; Flynn, A.; Proud, C. G.and Cooper, J. A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J.* **16**, 1909-1920.

154. Fukunaga, R.and Hunter, T. (1997) MNK1, a new MAP kinase-activated protein kinase isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.* **16**, 1921-1933.

155. Whitmarsh, A. J.; Yang, S. -H.; Su, M. S. -S.; Sharrocks, A. D.and Davis, R. J. (1997) Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. *Mol. Cell. Biol.* **17**, 2360-2371.

156. Price, M. A.; Cruzalegui, F. H.and Treisman, R. (1996) The p38 and ERK MAP kinase pathways cooperate to activate ternary complex factors and c-*fos* transcription in response to UV light. *EMBO J.* **15**, 6552-6563.

157. Trigon, S.and Morange, M. (1995) Different carboxyl-terminal domain kinase activities are induced by heat-shock and arsenite. *J. Biol. Chem.* **270**, 13091-13098.

158. Rouse, J.; Cohen, P.; Trigon, S.; Morange, M.; Alonso-Llamazares, A.; Zamanillo, D.; Hunt, T.and Nebreda, A. R. (1994) A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell* **78**, 1027-1037.

159. Cavigelli, M.; Li, W. W.; Lin, A.; Su, B.; Yoshioka, K.and Karin, M. (1997) The tumor promoter arsenite stimulates AP-1 activity by inhibiting a JNK phosphatase. *EMBO J.* **15**, 6269-6279.

160. Guyton, K. Z.; Xu, Q.and Holbrook, N. J. (1996) Induction of the mammalian stress response gene GADD153 by oxidative stress: role of AP-1 element. *Biochem. J.* **314**, 547-554.

161. Mansour, S. J.; Matten, W. T.; Hermann, A. S.; Candia, J. M.; Rong, S.; Fukasawa, K.; Vande Woude, G. F.and Ahn, N. G. (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. *Science* **265**, 966-970.

162. Minden, A.; Lin, A.; McMahon, M.; Lange-Carter, C.; Dérijard, B.; Davis, R. J.; Johnson, G. L.and Karin, M. (1994) Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. *Science* **266**, 1719-1723.

163. Whitmarsh, A. J.; Shore, P.; Sharrocks, A. D.and Davis, R. J. (1995) Integration of MAP kinase signal transduction pathways at the serum response element. *Science* **269**, 403-407.

164. Dérijard, B.; Hibi, M.; Wu, I-H.; Barrett, T.; Su, B.; Deng, T.; Karin, M.and Davis, R. J. (1994) JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell* **76**, 1025-1037.

165. Teramoto, H.; Coso, O. A.; Miyata, H.; Igishi, T.; Miki, T.and Gutkind, J. S. (1996) Signaling from the small GTP-binding proteins Rac1 and Cdc42 to the c-Jun N-terminal kinase/stress-activated protein kinase pathway. *J. Biol. Chem.* **271**, 27225-27228.

166. Raingeaud, J.; Whitmarsh, A. J.; Barrett, R.; Dérijard, B.and Davis, R. J. (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38

mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.* **16**, 1247-1255.

167. Wartmann, M.and Davis, R. J. (1994) The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. *J. Biol. Chem.* **269**, 6695-6701.

168. Rapp, U. R.; Troppmair, J.; Beck, T.and Birrer, M. J. (1994) Transformation by Raf and other oncogenes renders cells differentially sensitive to growth inhibition by a dominant negative c-jun mutant. *Oncogene* **9**, 3493-3498.

169. Lambrecht, R. W.; Cable, J. W.; Pepe, J. A.and Bonkovsky, H. L. (1994) 3,5,5-Trimethylhexanoylferrocene induction of heme oxygenase activity in normal hepatocytes. *Biochem. Pharm.* **47**, 1669-1676.

170. Grandchamp, B.; Deybach, J. C.; Grelier, M.; De Verneuil, H.and Nordmann, Y. (1980) Studies of prophyrin synthesis in fibroblasts of patients with congenital erythropoietic coproporphyria and one patient with homozygous coproporphyria. *Biochim. Biophys. Acta* **629**, 577-586.

171. Bonkovsky, H. L.; Wood, S. G.; Howell, S. K.; Sinclair, P. R.; Lincoln, B. C.; Healey, J. F.and Sinclair, J. F. (1986) High performance chromatographic separation and quantitation of tetrapyrroles from biological materials. *Anal. Biochem.* **155**, 54-64.

172. Glasel, J. A. 1 (1995) Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios. *BioTechniques* **18**, 62-63.

173. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory Press, Plainview,NY

174. Kutty, R. K.and Maines, M. D. (1981) Purification and characterization of biliverdin reductase from rat liver. *J. Biol. Chem.* **256**, 3956-3962.

175. Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76-85.

176. Raingeaud, J.; Gupta, S.; Rogers, J. S.; Dickens, M.; Han, J.; Ulevitch, R. J.and Davis, R. J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.* **270**, 7420-7426.

177. Cable, E. E.; Gildemeister, O. S.; Pepe, J. A.; Donohue, S. E.; Lambrecht, R. W.and Bonkovsky, H. L. (1996) Hepatic 5-aminolevulinic acid synthase mRNA stability is modulated by inhibitors of heme biosynthesis and by metalloporphyrins. *FEBS Lett.* **240**, 112-117.

178. Srivastava, K. K.; Cable, E. E.; Donahue, S. E.and Bonkovsky, H. L. (1993) Molecular basis for heme-dependent induction of heme oxygenase in primary cultures of chick embryo hepatocytes. Demonstration of acquired refractoriness to heme. *Eur. J. Biochem.* **213**, 909-917.

179. Morgan, W. T.; Alam, J.; Deaciuc, V.; Muster, P.; Tatum, F. M.and Smith, A. (1988) Interaction of hemopexin with Sn-protoporphyrin IX, an inhibitor of heme oxygenase. *J. Biol. Chem.* **263**, 8226-8231.

180. Keino, H.; Banno, T.; Mimura, S.and Kashiwamata, S. (1993) Cobalt-mesoporphyrin inhibits heme oxygenase activity but it does not induce lipid peroxidation in rat brain membranes during photoirradiation. *Biol. Neonate* **63**, 285-289.

181. Smith, A.; Alam, J.; Escriba, P. V.and Morgan, W. T. (1993) Regulation of heme oxygenase and metallothionein gene expression by the heme analogs, cobalt-, and tin-protoporphyrin. *J. Biol. Chem.* **268**, 7365-7371.

182. Dover, S. B.; Graham, A.; Fitzsimmons, E.; Moore, M. R.and McColl, K. E. L. (1991) Haem-arginate plus tin protoporphyrin for acute hepatic porphyria. *Lancet* **338**, 263.

183. Dover, S. B.; Moore, M. R.; Fitzsimmons, E. J.; Graham, A.and McColl, K. E. L. (1993) Tin protoporphyrin prolongs the biochemical remission produced by heme arginate in acute hepatic porphyria. *Gastroent.* **105**, 500-506.

184. Bonkovsky, H. L. (1993) Advances in understanding and treating 'The Little Imitator', acute porphyria. *Gastroent.* **105**, 590-594.

185. Mitani, K.; Fujita, H.; Sassa, S.and Kappas, A. (1991) A heat-inducible nuclear factor that binds to the heat-shock element of the human haem oxygenase gene. *Biochem. J.* **277**, 895-897.

186. Bonkovsky, H. L.; Healey, J. F.; Evans, C. -O.; Brown, K.and Wilkinson, K. (1989) The hepatic heat shock response: Activation of ubiquitin-mediated pathways for cell protein breakdown without induction of heme oxygenase in cultured liver cells. *Gastroent.* **95**, A578.(Abstract)

187. Knolle, P.; Schlaak, J.; Uhrig, A.; Kempf, P.; Meyer zum Buschenfelde, K. H.and Gerken, G. (1995) Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. *J. Hepatology* **22**, 226-229.

188. Cross, A.; Asher, L.; Seguin, M.; Yuan, L.; Kelly, N.; Hammack, C.; Sadoff, J.and Gemski, P.,Jr. (1995) The importance of a lipopolysaccharide-initiated, cytokine-mediated host defense mechanism in mice against extraintestinally invasive *Escherichia coli*. *J. Clin. Invest.* **96**, 676-686.

189. Eyssen-Hernandez, R.; Ladoux, A.and Frelin, C. (1996) Differential regulation of cardiac heme oxygenase-1 and vascular endothelial growth factor mRNA expressions by hemin, heavy metals, heat shock and anoxia. *FEBS Lett.* **382**, 229-233.

190. Lee, F. S.; Hagler, J.; Chen, Z. J.and Maniatis, T. (1997) Activation of the IkBa kinase complex by MEKK1, a kinase of the JNK pathway. *Cell* **88**, 213-222.

191. Yan, M.; Dai, T.; Deak, J. C.; Kyriakis, J. M.; Zon, L. I.; Woodgett, J. R.and Templeton, D. J. (1994) Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. *Nature* **372**, 798-800.

192. Ludwig, S.; Hoffmeyer, A.; Goebeler, M.; Kilian, K.; Häfner, H.; Neufeld, B.; Han, J.and Rapp, U. R. (1998) The stress inducer arsenite activates mitogen-activated protein kinases extracellular signal-regulated kinases 1 and 2 via a MAPK kinase 6/p38-dependent pathway. *J. Biol. Chem.* **273**, 1917-1922.

193. English, J. M.; Pearson, G.; Baer, R.and Cobb, M. H. (1998) Identification of substrates and regulators of the mitogen-activated protein kinase ERK5 using chimeric protein kinases. *J. Biol. Chem.* **273**, 3854-3860.

194. Lee, J. C.; Laydon, J. T.; McDonnell, P. C.; Gallagher, T. F.; Kumar, S.; Green, D.; McNulty, D.; Blumenthal, M. J.; Heys, J. R.; Landvatter, S. W.; Strickler, J. E.; McLaughlin, M. M.; Siemens, I. R.; Fisher, S. M.; Livi, G. P.; White, J. R.; Adams, J. L.and Young, P. R. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. *Nature* **372**, 739-746.

195. Han, J.; Jiang, Y.; Li, Z.; Kravchenko, V. U.and Ulevitch, R. J. (1997) Activation of the transcription factor MEF2C by the MAP kinase p38 in inflammation. *Nature* **386**, 296-299.

196. Takeda, A.; Onodera, H.; Sugimoto, A.; Itoyama, Y.; Kogure, K.and Shibahara, S. (1994) Increased expression of heme oxygenase mRNA in rat brain following transient forebrain ischemia. *Brain Res.* **666**, 120-124.

197. Takeda, A.; Kimpara, T.; Onodera, H.; Itoyama, Y.; Shibahara, S.and Kogure, K. (1996) Regional difference in induction of heme oxygenase-1 protein following rat transient forebrain ischemia. *Neurosci. Lett.* **205**, 169-172.

198. Fukuda, K.; Richmon, J. D.; Sato, M.; Sharp, F. R.; Panter, S. S.and Noble, L. J. (1996) Induction of heme oxygenase-1 (HO-1) in glia after traumatic brain injury. *Brain Res.* **736**, 68-75.

199. Ishizaka, N.; De León, H.; Laursen, J. B.; Fukui, T.; Wilcox, J. N.; De Keulenaer, G.; Griendling, K. K.and Alexander, R. W. (1997) Angiotensin II-

induced hypertension increases heme oxygenase-1 expression in rat aorta. *Circulation* **96**, 1923-1929.

200. Essig, D. A.; Borger, D. R.and Jackson, D. A. (1997) Induction of heme oxygenase-1 (HSP32) mRNA in skeletal muscle following contractions. *Am. J. Physiol. (Cell Physiol.)* **272**, C59-C67.

201. Basu-Modak, S.; Lüscher, P.and Tyrrell, R. M. (1996) Lipid metabolite involvement in the activation of the human heme oxygenase-1 gene. *Free Rad. Biol. Med.* **20**, 887-897.

202. Spiegel, S.and Merrill, A. H.,Jr. (1996) Sphingolipid metabolism and cell growth regulation. *FASEB* **10**, 1388-1397.

203. Clerk, A.; Fuller, S. J.; Michael, A.and Sugden, P. H. (1998) Stimulation of "stress-regulated" mitogen-activated protein kinases (stress-activated protein kinases/c-Jun N-terminal kinases and p38-mitogen-activated protein kinases) in perfused rat hearts by oxidative and other stresses. *J. Biol. Chem.* **273**, 7228-7234.

204. Chu, Y.; Solski, P. A.; Khosravi-Far, R.; Der, C. J.and Kelly, K. (1996) The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity *in vivo* toward the ERK2 *sevenmaker* mutation. *J. Biol. Chem.* **271**, 6497-6501.

205. Groom, L. A.; Sneddon, A. A.; Alessi, D. R.; Dowd, S.and Keyse, S. M. (1996) Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. *EMBO J.* **15**, 3621-3632.

206. Knebel, A.; Rahmsdorf, H. J.; Ullrich, A.and Herrlich, P. (1996) Dephosphorylation of receptor tyrosine kinases as target of regulation by radiation, oxidants or alkylating agents. *EMBO J.* **15**, 5314-5325.

207. Hirsch, D. D.and Stork, P. J. S. (1997) Mitogen-activated protein kinase phosphatases inactivate stress-activated protein kinase pathways *in vivo*. *J. Biol. Chem.* **272**, 4568-4575.

APPENDIX A: MEDIA AND BUFFERS

Media

Comparison of Waymouth's and Williams' E Media:

Component	Waymouth's MB 752/1 Medium, powder (mg/L)	Williams' Medium E (1), powder (mg/L)
Amino Acids:		
L-alanine	-	90
L-arginine	-	50
L-arginine•HCl	75	-
L-asparagine•H2O	-	20
L-aspartic acid	60	30
L-cysteine	-	40
L-cysteine•2HCl	-	40
L-cysteine•HCl•H2O	100.26	-
L-cystine•2HCl	20	26.07
L-glutamic acid	150	50
L-glutamine	350	292
Glycine	50	50
L-histidine	-	15
L-histidine•HCl•H2O	164.1	-
L-isoleucine	25	50
L-leucine	50	75
L-lysine•HCl	240	87.46
L-methionine	50	15
L-phenylalanine	50	25
L-proline	50	30
L-serine	-	10
L-threonine	75	40
L-tryptophan	40	10
L-tyrosine•2Na•2H2O	57.66	50.65
L-valine	65	50
Inorganic Salts:		
CaCl2 (anhyd.)	90.61	200

CuSO4•5H2O	-	0.0001
Fe(NO3)3•9H2O	-	0.0001
KCl	150	400
KH2PO4	80	-
MgCl2 (anhyd.)	112.56	-
MgSO4 (anhyd.)	97.67	97.67
MnCl2•4H2O	-	0.0001
NaCl	6000	6800
Na2HPO4 (anhyd.)	300	-
Na2HPO4•H2O	-	140
ZnSO4•7H2O	-	0.0002
Other Components:		
D-glucose	5000	2000
glutathione (reduced)	15	0.05
hypoxanthine•Na	29	-
methyl linoleate	-	0.03
Phenol red	10	10
sodium pyruvate	-	25
Vitamins:		
ascorbic acid	17.5	2
biotin	0.02	0.5
D-Ca pantothenate	1	1
choline chloride	250	1.5
ergocalciferol	-	0.1
folic acid	0.4	1
i-inositol	1	2
menadione sodium bisulfate	-	0.01
niacinamide	1	1
pyridoxine•HCl	1	1
riboflavin	1	0.1
a-tocopherol phosphate, sodium salt	-	0.01
thiamine•HCl	10	1
vitamin A acetate	-	0.1
vitamin B12	0.2	0.2

Williams' E Medium Complete was supplemented with:
1.5 μM 3,5,3'-triiodo-L-thyronine
0.76 μM dexamethasone
200 μM glutamine

Waymouth's MB 752/1 Complete Medium was supplemented with:
100 U/mL penicillin
100 μg/mL streptomycin
10% (v/v) fetal bovine serum (GIBCO)

Opti-MEM Reduced Serum Media was purchased as a liquid from Life Technologies, Inc. No modification or supplementation was made.

Buffers

Phosphate Buffered Saline (PBS):
10 mM KPi
0.15 M KCl
Adjust to pH 7.4

10X HEPES buffer:
200 mM HEPES
50 mM sodium acetate
10 mM EDTA
pH to 7.2 with NaOH
Filter to sterilize

20X SSC:
3.0 M NaCl
0.3 M sodium citrate
Adjust to pH 7.0 with 10 N NaOH
Autoclave to sterilize

Harvest Buffer (HO Activity assays):
0.1 KPi, pH 7.4
20% glycerol
1.0 mM EDTA
Adjust to pH 7.4

TE Buffer:
10 mM Tris•Cl, pH 7.4, 7.5, or 8.0
1 mM EDTA, pH 8.0

Z Buffer (ß-galactosidase assays):
60 mM $Na_2HPO_4 \cdot 7\ H_2O$
40 mM $NaH_2PO_4 \cdot H_2O$
10 mM potassium chloride
1 mM magnesium sulfate
50 mM ß-mercaptoethanol
Adjust to pH 7.0

Glycylglycine Harvest Buffer (Immune complex kinase assays):

25 mM glycylglycine, pH 7.8
15 mM magnesium sulfate
4 mM EGTA
1 mM DTT *(added just prior to use)

Triton Lysis Buffer (Immune complex kinase assays):

20 mM Tris, pH 7.4
137 mM NaCl
25 mM ß-glycerophosphate
2 mM sodium pyrophosphate
2 mM EDTA
1 mM sodium vanadate *
1% Triton X-100
10% glycerol
1 mM PMSF *
5 µg/mL leupeptin *
5 µg/mL aprotinin *
2 mM benzamidine
0.5 mM DTT *
*(added just prior to use)

Kinase Assay Buffer (Immune complex kinase assays):

25 mM HEPES pH 7.4
25 mM ß-glycerophosphate-Na^+
25 mM magnesium chloride
0.1 mM sodium vanadate *
0.5 mM DTT *
*(added just prior to use)

3X SDS Loading Buffer:

150 mM Tris•Cl (pH 6.8)
300 mM DTT
6 % SDS (electrophoresis grade)
0.3 % bromophenol blue
30% glycerol

Bicinchoninic Acid Protein Assay Stock Reagents:

Solution A:

161 mM sodium carbonate • H_2O
5.67 mM sodium potassium tartrate
113 mM sodium bicarbonate
75 mM sodium hydroxide
3.63 mM bicinchoninic acid
pH adjusted to 11.25 with NaOH

Solution B:

160 mM cupric sulfate

Working reagent:

50 mL Solution A
1 mL Solution B
Mixed well, used within 1 week

APPENDIX B: PREPARATION OF CHEMICALS FOR CELL CULTURE

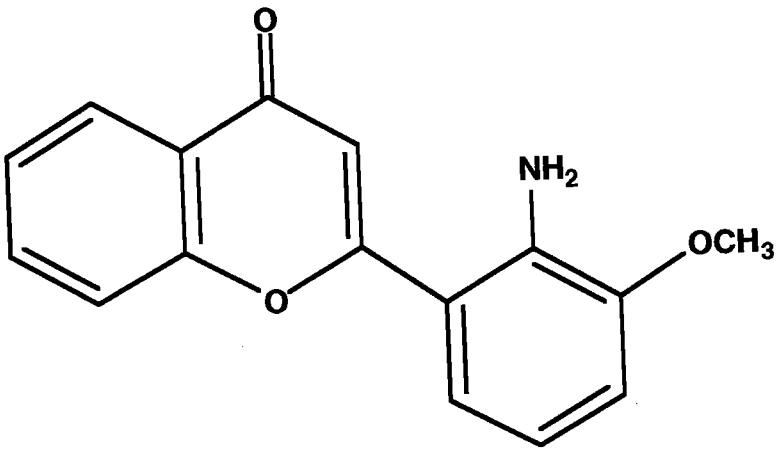
Treatment:	Abbreviation:	Dose in culture:	Preparation:
∂-aminolevulinate	ALA	10 µL/ mL medium, 18 µM final	0.587 mg/ mL ALA in water
Cadmium Chloride	$CdCl_2$		0.22 mg/ mL $CdCl_2 \cdot 2H_2O$ in water
Cobalt Chloride	$CoCl_2$	10 µL/ mL medium, 200 µM final	1.79 mg/ mL $CoCl_2 \cdot 6H_2O$ in water
Deferoxamine	DFO	25 µL/ mL medium, 250 µM final	6.5 mg/ ml DFO in water
Ferric nitrilo-triacetic acid	FeNTA	10 µL/m L medium 50 µM final	1 mL of 4.05 mg/m L $FeCl_3$ in water 1 mL of 14.2 mg/ mL NTA in water 3 mLs of water
Glutethimide	Glut	1 µL/ mL medium, 50 µM final	10.8 mg/m L glutethimide in DMSO
Heme	methemealbumin	260 µL/3 mL medium, 20 µM final [250x=(3000+x)*µM]	100 µL of 6.5 mg/m L hemin in DMSO dilute into 3.9 mLs 1.1% BSA in 40 mM Tris pH 7.4
Hydrogen Peroxide	H_2O_2	1 µL/m L medium, 0.5 mM final	5 mM stock = dilute 8.22 M: 10 µL in 17.64 mL MilliQ
Lipopolysaccharide	LPS;E.coli 0127:B8	1 µL/ mL medium, 1.5 µg/m L final	1.5 mg/m L stock = 1.5 mg LPS in 1.0 mg MilliQ
Sodium Arsenite	NaO2As, ARS	45 µL/m L medium, 75 µM final	5 mM stock = 6.495 mg in10 mL MilliQ sterile filter
TMH-ferrocene	TMH-F	1 µL/m L medium, 5 µM final	1.63 mg/m L in DMSO

APPENDIX C: LIST OF PLASMIDS

NAME OF PLASMID	Contains:	Reference or Source:
MAP Kinase, Vectors, and Control Plasmids		
pCMV/HA-ERK2	wild type ERK	Whitmarsh *et al.*[155]
pSRα/HA-JNK1	wild type JNK	Dérijard *et al.*[164]
pCMV5/MEK1 S218E-S222D	constitutively activated MEK1	Mansour *et al.*[161]
pCMV5/MEK1ΔN3, S218E-S222D	constitutively activated MEK1	Mansour *et al.*[161]
pCMV5/MEK1 K97A	dominant negative MEK1	Mansour *et al.*[161]
pCDNA3/MKK6 S207E-T211E	constitutively activated MKK6	Raingeaud *et al.*[166]
pCDNA3/MKK6 S207A-T211A	dominant negative MKK6	Raingeaud *et al.*[166]
pCMV5/MEKK1	wild type MEKK1	Whitmarsh *et al.*[163]
pCMV5/MEKK1 K432A	dominant negative MEKK1	Whitmarsh *et al.*[163]
pCDNA3/HA-MLK3	wild type MLK3	Teramoto *et al.*[165]
pCEF2/MLK3 K144R	dominant negative MLK3	Teramoto *et al.*[165]
pCDNA1/TAM67	dominant negative c-Jun	Rapp *et al.*[168]
pCMV5-Ras61L	constitutively activated Ras	Wartmann and Davis[167]
pCMV5-Ras17N	dominant negative Ras	Wartmann and Davis[167]
pCMV5-Raf-Flag	epitope tagged wild type Raf	Wartmann and Davis[167]
pCMV5-Raf-BXB	constitutively activated Raf	Wartmann and Davis[167]
GAL4-Luc	luciferase gene driven by yeast GAL4 elements	Roger Davis
4xTRE-Luc	luciferase gene driven by 4 TPA-responsive elements	Roger Davis
pBLUESCRIPT KS II+	DNA filler in transfections	Commercial

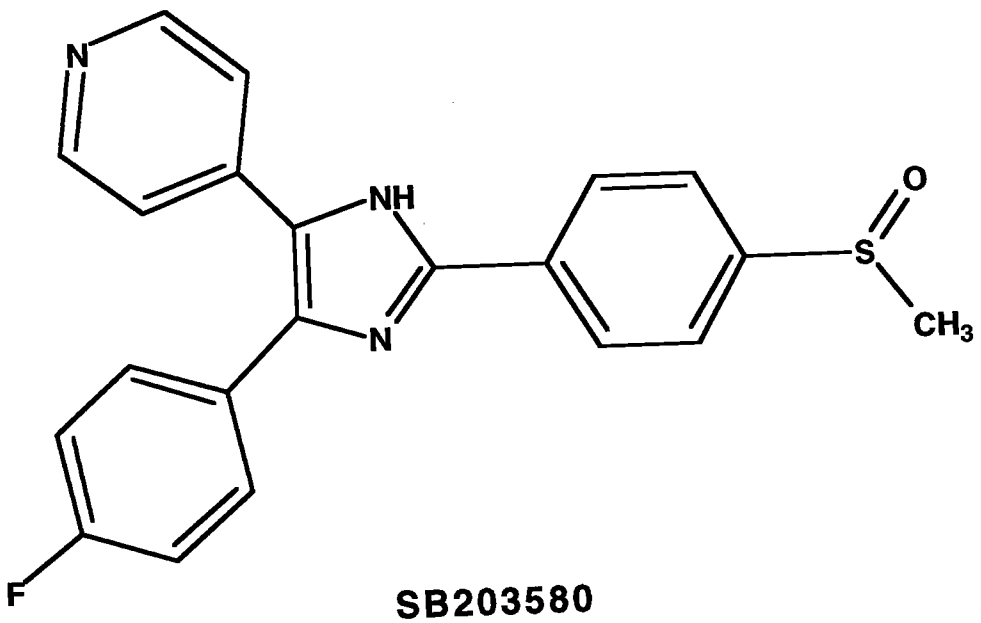
pSV-ßgal	ß-galactosidase w/SV40 promoter	Commercial
pPGK/ß-gal	ß-galactosidase w/PGK promoter	Paul Dobner
pGL3-Control	Luciferase vector w/SV40 promoter and enhancer	Gregorio Gil Promega
pGL3-Basic	Luciferase vector w/no promoter or enhancer	Gregorio Gil Promega
Chick Heme Oxygenase-1 Promoter Reporter Constructs		
pCHO.TATA-Luc	HO-1/luciferase reporter gene	Kim Elbirt
pCHO0.6-Luc	HO-1/luciferase reporter gene	Kim Elbirt
pCHO1.6-Luc	HO-1/luciferase reporter gene	Kim Elbirt
pCHO2.5-Luc	HO-1/luciferase reporter gene	Kim Elbirt
pCHO3.6-Luc	HO-1/luciferase reporter gene	Kim Elbirt
pCHO4.6-Luc	HO-1/luciferase reporter gene	Kim Elbirt
pCHO5.6-Luc	HO-1/luciferase reporter gene	Kim Elbirt
pCHO7.1-Luc	HO-1/luciferase reporter gene	Kim Elbirt
AP-1 Mutants of pCHO-Luc Deletion Constructs		
pCHO2.5mut1-Luc	single AP-1 mutation	Kim Elbirt
pCHO2.5mut2-Luc	single AP-1 mutation	Kim Elbirt
pCHO4.6mut1-Luc	single AP-1 mutation	Kim Elbirt
pCHO4.6mut2-Luc	single AP-1 mutation	Kim Elbirt
pCHO4.6mut3-Luc	single AP-1 mutation	Kim Elbirt
pCHO4.6mut13-Luc	double AP-1 mutation	Kim Elbirt
pCHO4.6mut23-Luc	double AP-1 mutation	Kim Elbirt
pCHO5.6mut1-Luc	single AP-1 mutation	Kim Elbirt
pCHO5.6mut2-Luc	single AP-1 mutation	Kim Elbirt
pCHO5.6mut3-Luc	single AP-1 mutation	Kim Elbirt
pCHO5.6mut4-Luc	single AP-1 mutation	Kim Elbirt
pCHO5.6mut13-Luc	double AP-1 mutation	Kim Elbirt
pCHO5.6mut14-Luc	double AP-1 mutation	Kim Elbirt
pCHO5.6mut24-Luc	double AP-1 mutation	Kim Elbirt
pCHO5.6mut34-Luc	double AP-1 mutation	Kim Elbirt
pCHO5.6mut134-Luc	triple AP-1 mutation	Kim Elbirt

APPENDIX D: STRUCTURES OF MAP KINASE INHIBITORS



PD 98059

2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one, Inhibitor of MAPK/ERK Kinase (MEK)



SB203580

Pyridinyl Imidazole inhibitor of p38 MAP Kinase

APPENDIX E: SEQUENCE OF HO-1 PROMOTER

In the following sequence, restriction enzyme cut sites are underlined. Sites that define the end of a reporter construct (shown in Figure 5.1) are indicated in **bold** type, followed by construct length in Kb in parentheses. All putative transcription factor binding sites are *italicized* and underlined. Activator protein-1 (AP-1) sites that were mutated in functional studies of arsenite responsiveness are labeled AP-1*mut, are underlined and shown in **bold** type. The TATA box is underlined and shown in **bold** type. The transcription start site is shown in ***underlined and italicized bold*** type, and is labeled (+1). Numbering of bases is in reference to the transcription start site = +1.

5′ —
ACGCGCTAGA GTGGGCTTCC AGCCCTCTGC CTGCTGCTGT TAGCCTGGAA -7023
Mlu I-Nhe I/Xba I(7.1)
AGCAAAGGGG GTACGACACA TTACAGGCAG *CAGAGGAAAA* ACACTAATGG -6973
Pu Box
CAATGGAACC CTATTGCTCT AGCTTTTCTT GCACAAGTGC TTGTGGTATG -6923

CAGCACCTCT CACACAACCC CTTCTCTTCC TTTCCTACCC ATCAGTTTAT -6873

CTGGAATCCT ACCCACAGCC CTTGTTTCCG TTGCATGCCA GCCAGTTACC -6823

AAAGCCCTTG TAAGCACACA GGCTTTGAGC TGCGAGTGGT ACGCAGCCTG -6773

CAGGCTGAAG GGGCTGTCTG CTGTGGGTTA GGGTGTGCCT TCAGCAAACT -6723

CTTTGCAGTT AATTTAATTC CCCTATTACT ATCCTTTCCT TCCTTCTTTC -6673

CTCAATACGA CCCTGCCTCC ATCTCGCTTC CACTGATGCC TTCTACCACA -6623

TCCCGCTGTG GTGCAGGGAG GATCTCTTAG CCTCAGATCC AGCCAACAGC -6573

TGTTGACTCC CTGTGTGCCG TAGCAGAGAG CTGTGTTCCC TAAAGCAGGA -6523

GGCCCTCATA GGCACCGTTA CACTGGTGGG AGCATGCTGT TGCTGGGAGA -6473

GCTCGTTCAA GCTGGTGAGC GCAAGGGGGG AAGTGAGACA TCAGTCATCT -6423

CGCAACAGCA AAGCAGCCCT ACAGCAGCCA GGAGTGACTT GCACATGGGG -6373

GAGAAACCCC TTCTGAGCAA ACCACACACA GTGCCTGATC CTGTACGGCA -6323

GCTGGGGAGA TGGGTGCCGA GCCTGGGGCT CC*TGCTCA*TG GGATTTCCTG -6273
MRE
TTGAACGTGG GAGAGTTGCC TTTTTTATTG CACATTAAAG GAGCAAACTC -6223

TTGAACCTCT GCTGAAGGAA CGTGTGTGTT CTGTGCCAGA GTGGGGAAGG -6173

GAGAAGAGGA ATGCAGCCAT GTGGCCACAT TTGCTCTCCA AGTGAGAGCA -6123

CAGTGGTGCT GAGAGGAGGC ATGGCATGGG GCTGAGGGCG CTG*TGAGTCA* -6073
AP-1

AGACAGGTGC TGGCTCCTGG GGAGGTGGCT CCAACCCATC CCAAAATAGC -6023

AAGAGAGTGA GCTACCTCCT CCCCATCCCC TCCCCTCTGC CTTGTGACCA -5973

GACCCAAATG CACATCTCAA TCCATAAATC AGCCAAAGAC ATGCAAGAAA -5923

CAGTTGCTCT GAAAGGGCTC TGTGGTATCC ACAGCATGTC CTCAGAGTCC -5873

TCAGCTGTCC CTGTGCATCT CCTGGTGTCA GACCGGGTGG GAGCTTGGAA -5823

AGCCCTCCCG AGTGGTGGAG TCGCATCCCT GGAGGTGTTC AAGAACCGTG -5773

TGGATGTGGC ACTGAGGGGC ATGGTCAGTG AGCGCGGTGG GGATGGGCTG -5723

ATGGTTGGAC CGGC<u>AGATCT</u> TAGAGGTCTT TTCCAACCTT AATTCTATGG -5673
Bgl II

TCCAATGATT CCGTTGATTA AAAAAAAAAA AAAACACCGA CCCCCTAAGG -5623

CTGTTTGCTG ATTTCTC<u>CCA</u> <u>GCAGATGG</u>CT GTAGTGCCTC GCACGTTGGC -5573
Pf1 MI(5.6)

AGCCTGAGCT CTGCAAGCGG CAGAGGAAGC CCTCTTGCTT CGCAGTGACC -5523

CGCTTAGAGC AGCCCCTGCT ACACAGGACA CCGAACCAAA CAAAGTTGTG -5473

CTTTGTTCTA GGAATTACAC TGCAACCTTT AGCCTGACAC AGACTCACAC -5423

GGTGACCGCT CCGGAGCGTG CACATGGGAG CTGTGTGCAC ACGCGGGCTG -5373

AAGCATCCAT CAAAAACCAC CCTGGGAGCA GGAGCATGAC CAACATAGAC -5323

C*TTTTGCAT*G TTCCGGAGTG CTTGTTTTTA TGTAAGCTGA AAAAAGCCAG -5273
Octamer

AACGTCCTTG CGCCGGGTCA GATTTGCTCC TTATCTCTAA AAATTAAACG -5223

CATATTAAAA GCTCATGAGA *ATTTCCAT*GA TGTTTGGGCT GTGCAGTTCT -5173
Octamer

TGGCATATCA GCACCACATT CCTCAAGGCA AAAAATCCCA ACTCAAATAG -5123

CACATGAAAT TTATTTATAA AGAACAGTTA ATGAACCAGT CTTCTGATTT -5073

CCCACATCAT CTCATGCATC AGCACAGAAG TACCCCTTGC TGGTACACTG -5023

GGAAGGCTGT TCTCTACCCC ACAGG*ATTTT* *CAT*GTTGCTC TACCGTGCCA -4973
Octamer

AATCTTCCTC AAAGCTTTGC CCTGGTAAGC TCCATAAGTT CAGCATGCCC -4923
Hind III
TGTGTGCCAG CTCTGAAATA AAAAGCTCCT TTGCACTCAC TGTGTCTCTG -4873

TGTATTCAGA CAGTGAAGCA GGCACTCCCA CACCCCATCT GTGCCCCAGT -4823

TTCCCCATGA GGAGCAGGAT CTGTCCCAGT CCTGGGGCCA GGGCTCCAGC -4773

ATCACCCGCA TCCTGGGTCA GACCCTCAAA CCCCACAGCC TTCAGGGTGG -4723

GGGTTCCCAC AGATGCTGAC TCATCCTTCC CCAAAGAGGC CATTCTGCTG -4673

AGTCAGGGTC ACGCCAGGCC ACGGGGTTTT GCTGAGCAAC GCTTCTGGGC -4623
coreB/AP-1
TGCGGCAAAG GGGCCCTTAG TTGCTGAGTC ACAGCAGAGA GGCCACGTGA -4573
coreB/**AP-1*mut** MycMax/**Pml I(4.6)**

GGCTGTTTAT CAGGAGGGCG GCTTCCTGCC TGCCGTGCTG AGGTTGCAGC -4523
Sp I
TGTTGTTCCT GGCCCTGCTC CCGTTTCTCA GGACGACCGG AGAAATCTCC -4473

AGAAATAAAC CCCAGCCCCG CAGCACTGCA TGGGACGCGC ATCTCCTCTA -4423

CTCCAGCTGG GAGCCACACA GCGGGGGGTT GGAAGCAGCG GGGTACAGTG -4373

GGGTTTCGCT ACCTGCAGAA CTTGGTTTCC TGTTCTGTCT CCTCCCGTGC -4323

CCTGAGCTGC GGTACAAAGT CCTGTTCGGT GCCTACGTGC GAGTCCTCGA -4273

ATGCAGCCAC TCGCAGCGTG CGCCTGTCTC AGTCAGGGTG CACTTTGCTT -4223

CATGCAGGGC TACGTGGGGT TTGTATTCGC TAGCTTGCCT TGCTCCCTTC -4173
Nhe I
CACACCTCCC TGTGGCTCAC AGACCCCAGT CCCAGCAGGT CGCAGGGTCT -4123

GTCCCCACCG GCACCCGGCC CCAGCCGAAG CCCTTATCCA TACATCTGGA -4073

ACAATGCGTA TTGTAATCAC CGCTTCCTGC TTCCATCACC CTGAGAACCC -4023

GGCACGCACG TCCATCCCCC ACCACATCCG TCTCTGGGGC CACGGCACGG -3973

GCTCTGTGCG GAGCAGCAGC ATCCGTCCCC CAGAGGTGCC ACCGCCACAG -3923

CCCCACGGGG AGGACCCCTT GGTTCTCAGT GCTCCCACCC ACTGTGCTCC -3873

ATCTGTATCT TTGATCTGGG TTCGGGTTGT TCTGCGGCGA GGGCAATAAG -3823

CACAATCCGT TCTGCCAGGG CAGCCTCCTT TTGGTAAACA CGAATTAGCA -3773

CACAGCACGT CCTGTGGACT CCAGCAGTGA CTCTCTCCTT TCACAAGTAG -3723

CTGTACGTGG GTATTGCAGC TGTGTGGGGT TTTTCCCTCT CCATTACACA -3673

GCAAGATCTG GTTGCTGAGT CAGCCCCCCT CCCAGCCAGG CTGAATTCTG -3623
Bgl II(3.6) AP-1*mut Eco RI
CTGTGTCATG GTTTCAGTCC TCCATCAGTT CCCTCTTTGC TGGCCAAGAC -3573
coreB
TGGCTGTGAC CAGGCGGTGC CTCCTTGCAC TGGCCAAGCC GCTGGGAAGC -3523
AATGCATCAG AGCCAAATGA GGAGCGGTGT TCCAGAAGGC AGGAGATCTT -3473
Bgl II
TCTTCTCTGG GGCAGATGGT GTAACCAGAG GGTTCTGGCT GTGAAGCCTA -3423
CAGTCAGGAC AAGCTGTTAG GTCCATAAAG AGGAAAATCT TAGAATCATC -3373
Pu Box
GAATCATTTA AGTTGGAAGG GACTTTTAAA AGGTCATCTG GTCCAACTCC -3323
CCTGCAATGA ACAGGGACAC CTACAGCTAC ATCAGGGTGC TCAGAGCCCC -3273
MRE
TCCAGCCTGA CCCTGGGTGT CTCCAGGGAT AGAGCATCCA CCACCTCTCT -3223
GGATGACCCG CTCCAGTGTT TCACTACCTA TATTATAAAA AACTTCTTTA -3173
TTATATCCAA CCTAAAACTC CCCTCATTTA GTTTGAAATC ATTTCCCCTT -3123
GTCCTATTCT AACAGATCCT GCTAAAGAGC CTGTCCCTTT CGATCTCCCA -3073
CATTTCCCTT CCCCTGATGG CAACACTGAC TCCTCAGTGA CTTCTGTTGC -3023
TGAAGTCTAT TCCGTTCCTC TAACCCATCC CATGACTTAA ACATTTTCTC -2973
AAAGACTCAG ACAGGCAGAT GGAGGTATTT GAGCCCATCA CTATCACAAA -2923
TATCAGCCTC AGGCTGCTTC ATTTATCTTC CTACAAATCC CCCAGGCAAA -2873
CTCAGCAGTA GTCCCACACG ACACCATCCC CTCCGGCACC GTGCAGCCAC -2823
TGATCAGTGA AAGCACAGCA AAAGCAGAGA GGAAGCAATG CAAGTCCTCA -2773
CTGCAGAGCA CTGGATCAAG CCAGAAAAGC TTCAGCCCAG CAATTGCAAA -2723
Hind III
ACTGATGACA GCCTGCTTTC AGTTCTCCCT CCATCTCGCT GCAAGGCCTC -2673
TGGGCTTTTC TGGAGTGAGG AACCTGACCT CACAGCCTCT CCACCTCCAG -2623
CREB
TAACCCCAAC CAAATCAGGC ACAAAACAGT CCTGGGGCAT GGTATGCTTC -2573
CCTGCTCCTA TGGAGGAGCT CACAGAGGAG CGAGCTGTGC CTTGGCTCTT -2523
GTGACCTTGT CTCCTGTGAC CATCCCAGGA GAAGGCAAAG GCCCAGATGC -2473
Tth 111 I(2.5)
AGAAGATGGA ACACTGGAGG ACACCTCAGA GCAAAAATAG GTGATAGGAT -2423

GAGAGGAAAG GGCCTCAAGT TGCACAAGGG GAGGTTCAGG TTGGATGTTA -2373

GGAAAAATGT CTCCTCAGGA AGAGCGGTGC TGCAGTGGCA CAGGCTGCAC -2323

AGGGAGGTGG TGAAGTCACT GTGACTCTAT GAGATTCTGT GACTATGAGA -2273

AAGTTGGAGT TCTTCCTAGG AAGAAGTGTC CTATCTACGT TGGCAGTAAC -2223

ACAATGGGAT ACCCACTCCT GAGTCTCACC TCCCCATCTA CCTCTGCCCT -2173

GCAGGAAAAG TATGGAGGAG GACATTTGGG GCACACCTTT AAGTGCATCC -2123

AAGCATGCAG CTTTTGGGGA GCAGGCAGAC GGGACAAAAG CATTGGCCTA -2073

AATGCAGCTC GAAAGTCCTT AAGAAGAAAT GGAGCTGCAG AACATGGATG -2023

AAAGGAGGAA CATGGTTTAG GGAGGGTAGG TTTTCATATT ATTTCACACT -1973

CATGTAATGA ATAATATCAT TCAGTTTCGT ACCTTTCCTT CTTCCCCCCA -1923

GCAGAACTAT AGGCTCATTG TTTTCCAGCT TCCCATGTCC AAATATGGCT -1873

CACTGAAAGA AGTGAGAACT GTGCTATAAG CACACGATGC CAGTGGAAAA -1823

*TGCTCA*TTTC AGAGCCAGAC CAGTGGCAAA AATGGCACTA ATATCTTCAG -1773

MRE

GCAGGACTGG AACCTCTCTA GGGGCCAAGG GATGTTAAGG GCTTTCTCAC -1723

GCTTGCTTCA AAGACTCGGA CATCCCAATA AATATATTTT ACCCTGTAGC -1673

AAAATAAGCA GTAAGTGGCC TAGGGGTAAG AGCTAGCTTT TGCTAGAATG -1623

Nhe I(1.6)

CTCTGTAGCT TTAAAGAGCA GAGCAAGACA GGAAAAGCAT G*GCTGAGTCA* -1573

coreB/**AP-1*mut**

*GGC*TGGGAGC GCTGAGTAAT CCTTCCTGAG TGTGCACAGT CGCCAGATGT -1523

CAGCCCAGCA CAGAGCTGTG TGCGTGCACA ATCCCTCCAG GCCACTCCTA -1473

TCCATAGCAC GGAACCCAGC TGTGCAGAGG ACACACGAGG CCCACCCAAA -1423

GCCAGGGGAC AGATAGACAG AAGACTTCCC TTCCAGCCCA CAGCAGCAGG -1373

GCAGTGATGG CTTTAAGTGC CCCACACTTT TGGCTCCAAG GCAGAATTTG -1323

TGC*TTTCCCA* AAGCACAGAT GAATGATGCA TCTCTCATTC TGGTTTGCTG -1273

STAT3

AGCTTCCAGC GTAGCTCCAG ATGGAAGAGG GGCCCCACTA CATCTGCGGG -1223

CTGCCTCCTT G*GCTGACGCA* *GC*AGACAAAA CAGCAGAGCA GCCAGCAAGT -1173

ARE

AGCAGGAGCA CCTTGTCCGG CTCTCCTGTC CTAAATCAGA GAAACTATTC -1123

GCTGTTGCAA ATATCTGCAG CCTGCCTCAG TTTCCCCAAA GCCCTGCATC -1073

ACACCTTGGC AGTCTCCTGC ACACAGCAGG TGTGACAACC CCCAGGGTAT -1023

CGGGGGGATG GGCTGCTGTC CTCCTCTATC ACCTCACACA CAGTGC*TGCT* -973
MRE

*CA*CAGCAGGG CCCACATGCC ACGCCAATTC AGTACTTCTG TTGTGTAAGG -923
C/EBP

GAGAAAGGAA AGCATATCCC TGTGCATCAG TGCAGACGCA CCTGTGCAGC -873

ACATGGAAAG AATCATAGAG TCATGGAATC ATAGAATCAT TTGAGTTGGA -823

AGGGACCTTT AAAGGTCATC TGATCCAAAA CCCCTGCAAT GAACAGGAAT -773

GCCTACAGCT CGATCAGACA TTGAAAGGAC CTTTAGATAT TGAAGTATCC -723

CAGGAGCCTT CTCTTCTCCA GGCTGAACAG CCCCAGCTCT CTCAGCCTGT -673

CCTTGTAAGG AGCTGTTCCA TCCCTTGGAC CATTTCTTTG GCCCTCCTCT -623
Pfl MI

GGACGCACTC CAGCAGATCC ATGTCTCTCC TGTACTGAGG GCTCCACATT -573

TGGACACAGT ACTCCAGGTG AGGTCTCACA GCACACAGCA GAGGGGCAGG -523
Tth 111 I(0.6)

GTCACCCCCC TCTCCCTGCT GGCCACACTC CTTTGGATGC TGCCCAGGAC -473

ACAGGTGGCT TTCTGGGCCG TGAGGGCACA CTGCTGGCTC ATGTACAGCT -423

GCCATCCATC AGTACCCCCA AGTTCTTTTG GGCAGGGCTG AGCTTTGAGG -373

GTCCCTGCTG CGCTCCCCTC CGCGAAGCAG GGATGTTCCT ACCCCACAGT -323
NF-kB

TCAGAGCTCT TCGTCCCGTG GGCAGCGAAG CTCCCCGTGC CTTTAGAGGC -273

TCTGAAGA*TG* *CTCA*CAGCTG TGGGTGATAA AACACTGACA CCGGTCTCTT -223
MRE

TCTAGGACAG ATGCTGCAGA AGCTGCATCC CCTCGTTGGT GCTGAGCTGC -173

AGCAGGGGTG AATTCCTCCT GCCCTCCCAG CCGTCCCTGG GGGCCGGGGG -123

GCACAGCAGT GGGAAGGGAA GTGCAGCGTG TTGCAAGAAG CGTG*TGCTCA* -73
MRE

CATGAGGTCT GCGCCTGTGC TGCTCACGTG GCCCGCCTGT ATAAAGCTGG -23
MRE/MycMax/**Pml I(TATA)** **TATA Box**

GAGCCGGAGC CGGGTACAAA G**C**GCAGCGCT TCACGTCCCG GGAGCGGTGG +28
(+1)

GAGAGCTCAA GTAAGGAAAC AACAGGGAAG *CTCGAG* – 3′
Xho I