

A Dissertation Presented

By

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UPREGULATION OF HEME PATHWAY ENZYME ALA SYNTHASE-1 BY
GLUTETHIMIDE AND 4,6-DIOXOHEPTANOIC ACID AND
DOWNREGULATION BY GLUCOSE AND HEME

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There is a story in the more than 6000 year old Indian scriptures that sums up my thought. A Yogi (one who searches for truth), is questioned by The Almighty Himself as a test, “Who is more important?” “Is it God or Guru (Teacher)?” The Yogi replied unhesitantly, “It is the Guru, my dear God” “For it is the teacher who provides the knowledge and path that helps the disciple appreciate anything including God”.

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ABSTRACT

5-Aminolevulinic acid synthase-1 (ALAS-1) is the first and normally rate-controlling enzyme for hepatic heme biosynthesis. ALAS-1 is highly inducible, especially in liver, in response to changes in nutritional status, and to drugs that induce cytochrome P-450. The critical biochemical abnormality of the acute porphyrias, a group of disorders of heme synthesis, is an uncontrolled up-regulation of ALAS-1. High intakes of glucose or other metabolizable sugars and intravenous heme are the cornerstones of therapy for acute attacks of porphyrias and both repress the over-expression ALAS-1, although their mechanisms of action have not been fully characterized.

In this work, the chick hepatoma cell line, LMH, was characterized with respect to its usefulness in studies of heme biosynthesis and compared with chick embryo liver cells (CELCs), a widely used model for studies of heme metabolism. The inducibility of ALAS-1 mRNA and enzyme activity and accumulation of porphyrins by chemicals were used to evaluate heme biosynthesis in LMH cells. Repression of ALAS-1 mRNA and induced activity by exogenous heme (20 μ M) was shown to occur in LMH cells as in CELCs. In addition, a synergistic induction of ALAS-1 enzyme activity was observed in LMH cells, as shown previously in CELCs, by treatment with a

barbiturate-like chemical, Glutethimide (Glut), in combination with an inhibitor of heme synthesis, 4,6-dioxoheptanoic acid (DHA). This induction of ALAS-1 enzyme activity is analogous to what occurs in patients with acute hepatic porphyrias and LMH cells were used to further characterize effects of Glut, DHA, glucose, and heme on ALAS-1.

A “glucose effect” to decrease Glut and DHA-induced ALAS-1 enzyme activity was obtained in LMH cells and CELCs in the absence of serum or hormones. This “glucose effect” was further characterized in LMH cells using a construct containing approximately 9.1 kb of chick ALAS-1 5'-flanking and 5'-UTR region attached to a luciferase/reporter gene (pGcALAS9.1-Luc). Glut (50 μ M) and DHA (250 μ M) synergistically induced luciferase activity (5-fold) in LMH cells transiently transfected with pGcALAS9.1-Luc. Addition of glucose (11 or 33 mM), in a dose-dependent manner, decreased the Glut+DHA up-regulation of pGcALAS9.1-Luc activity. Gluconeogenic or glycolytic substrates such as fructose, galactose, glycerol and lactate, but not the non-metabolizable sugar sorbitol, also down-regulated pGcALAS9.1-Luc in LMH cells. The cAMP analog 8-CPT-cAMP, augmented Glut induction of ALAS-1, indicating that the glucose effect may be partly mediated by changes in cAMP levels.

The remaining studies focused on delineating the synergistic effect of Glut and DHA, and heme-dependent repression of ALAS-1. The 9.1 kb construct was compared with a construct containing the first 3.5 kb (pGcALAS3.5-Luc). The drug and heme effects were shown to be separate as drug induction was present in -3.4 to +0.082 kb region while the heme responsiveness was present in the -9.1 to -3.4 kb region. Using computer sequence analysis, several consensus activator protein-1 (AP-1) sites were found in the 9.1 kb ALAS-1 sequence but no consensus direct repeat (DR)-4 or DR-5 type recognition sequences for nuclear receptors were identified in the drug-responsive 3.5 kb region. Deletion constructs containing +0.082 to -7.6 kb (pGcALAS7.6-Luc) and +0.082 to -6.2 kb (pGcALAS6.3-Luc) cALAS 5'-flanking and 5'-UTR region were generated and tested and pGcALAS6.3-Luc was shown to have heme-dependent repression of basal and Glut and DHA-induced activity.

A recently identified 167 bp chick ALAS-1 drug responsive enhancer (DRE) was PCR amplified and inserted upstream of the 9.1 kb (pGcALAS9.1+DRE), a 0.399 kb (+0.082 to -0.317) (pGcALAS0.3+DRE), and pGL3SV40 construct (pGL3SV40+DRE). DRE mediated the up-regulation of pGL3SV40+DRE construct by Glut was ~15-30 fold but

interestingly only 3.2 and 3.7-fold for pGcALAS9.1+DRE and pGcALAS0.3+DRE constructs, respectively.

In summary, in LMH cells drugs up-regulate ALAS-1 through non-DRE element(s) in the first 3.5 kb of ALAS-1 5'-flanking and 5'-UTR region and heme down-regulates ALAS-1 and determines the extent of the drug response through element(s) in the -6.3 to -3.5 kb region of ALAS-1 5'-flanking region.

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ABBREVIATIONS

AIP	Acute intermittent porphyria
AIA	Allylisopropylacetamide
ALA	5-Aminolevulinic acid
ALA-pyrrole	2-Methyl-3-acetyl-4-propionic acid pyrrole
ALAS	5-Aminolevulinic acid synthase
ALAD	5-Aminolevulinic acid dehydratase (PBG synthase)
AP-1	Activator protein - 1
ATP	Adenosine triphosphate
bp	base pairs
BSA	Bovine serum albumin
LMH	Leghorn male hepatoma
Luc	Luciferase
kb	kilobases
cALAS	Chick ALAS-1
CBP	CREB-binding protein
cDNA	complimentary DNA
CELC	Chick embryo liver cells
CoA	Coenzyme A
CoreB/AP-1	AP-1 with 4 bp extension
Copro	Coproporphyrin
8-CPTcAMP	8-(4-Chlorophenylthio) adenosine monophosphate
CREB	c-AMP response element binding protein
CXR	Chicken xenobiotic receptor
CYP	Cytochrome(s) P450
Dex	Dexamethasone
DFO	Deferoxamine
DHA	4,6-Dioxoheptanoic acid
DMAB	p-Dimethylaminobenzaldehyde
DMSO	Dimethyl sulfoxide
2-DOG	2-Deoxy α -D-glucopyranoside
DR	Hexamer half-site direct repeat
DRES	Drug-responsive Enhancer Sequences
EDTA	Ethylenediaminetetraacetic acid
FC	Ferrochelatase
FeNTA	Ferric nitrilotriacetate

β -gal	β -Galactosidase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G418	Geneticin
Gluc	Glucose
Glut	Glutethimide
Gly	Glycine
HO	Heme oxygenase
HPLC	High-performance liquid chromatography
Luc	Luciferase
MePN	Metalloporphyrin
Metyrapone	2-Methyl-1,2-di-3-pyridyl propadone
mRNA	messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
3-OMG	3- <i>O</i> -methyl glucose
PB	Phenobarbital, sodium salt
PBG	Porphobilinogen
PBGD	PBG deaminase
PBS	Phosphate buffered saline
PCA	Perchloric acid
PCN	5-Pregnene-3 β -ol-20-one-16 α -carbonitrile
PCR	Polymerase chain reaction
PIA	2-Propyl-2-isopropylacetamide
Proto	Protoporphyrin
RLU	Relative light units
RNA	Ribonucleic acid
RU-486	Mifepristone
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SV-40	Simian virus 40
T ₃	3,5,3'-triiodo-thyronine
TCA	Trichloroacetic acid
TPA	12- <i>O</i> -tetradecanoyl phorbol 13-acetate
TRE	TPA response element
Uro	Uroporphyrin
5'-UTR	5'-Untranslated region
VP	Variegate porphyria

CHAPTER I

INTRODUCTION AND OBJECTIVES

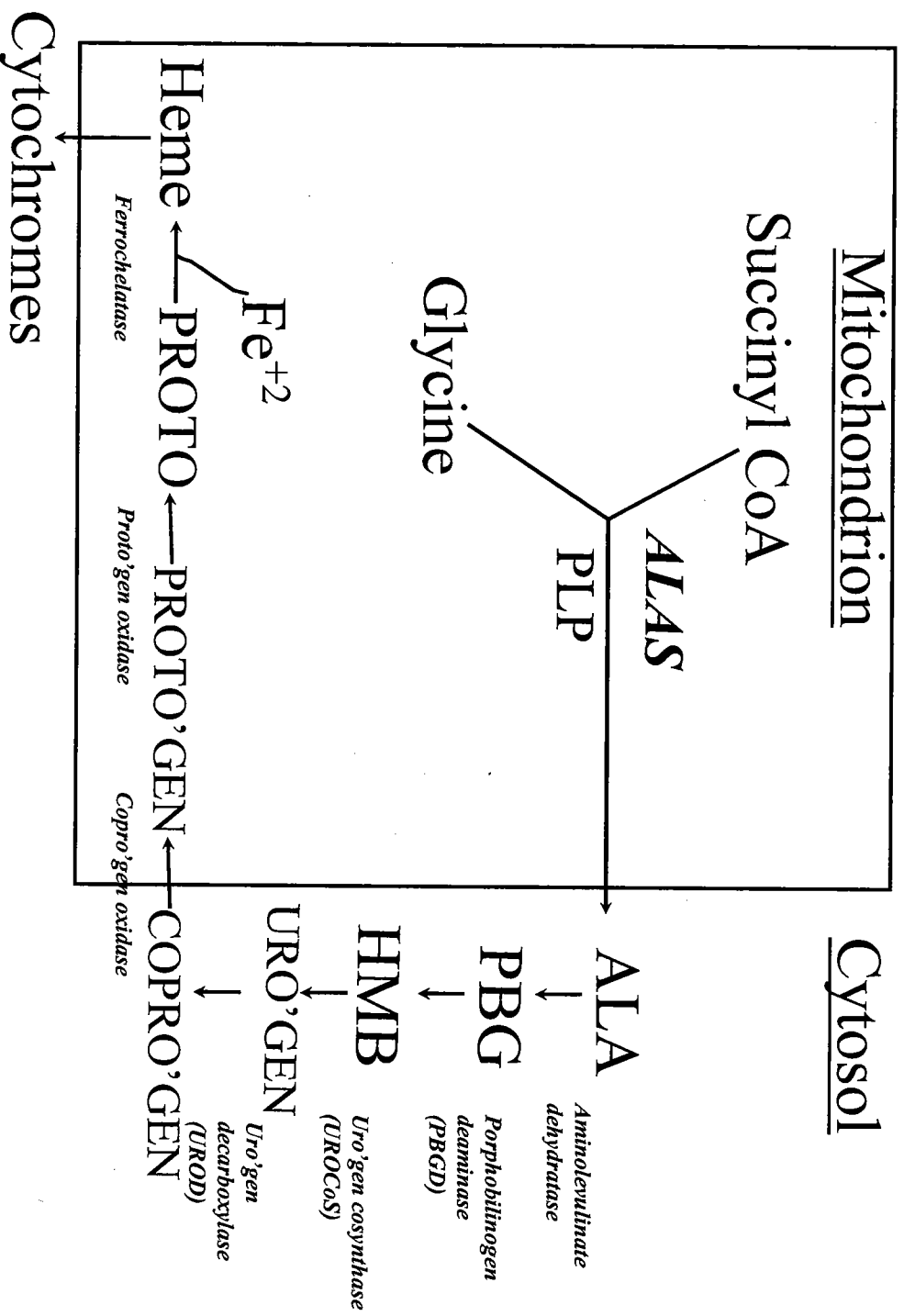
Introduction

Heme is essential for the health and function of nearly all cells, acting predominantly as a prosthetic group for oxygen-carrying proteins and enzymes involved in oxidation/reduction or electron transport reactions.

Heme biosynthesis is a multi-step pathway that is present in virtually all cell types. In eukaryotes, erythroid cells and hepatocytes are the major sites of heme biosynthesis (Ades 1990). 5-Aminolevulinate synthase [Succinyl-CoA:GlycineC-Succinyl transferase, E.C. 2.3.1.37] (ALA synthase or ALAS) is the first and rate-controlling enzyme in the heme biosynthetic pathway in animals (May et al., 1986). ALAS is a pyridoxal 5'-phosphate-dependent enzyme that exists as a homodimer in the mitochondrial matrix where it catalyzes the condensation of glycine and succinyl CoA to form 5-aminolevulinate (ALA) (Kappas et al., 2000). In mammals and birds, ALAS is encoded by 2 genes: ALAS-1, which is expressed ubiquitously, and ALAS-2, which is expressed only in erythrocytes and erythroid precursors (Riddle et al., 1989; Bishop et al., 1990; May et al., 1995) (Figure 1.1).

Figure 1.1. Schematic diagram of the hepatic heme biosynthetic pathway. ALAS-1 catalyses the formation of ALA from glycine and succinyl CoA using pyridoxal 5'-phosphate (PLP) as a cofactor. ALA is converted through a series of steps catalyzed by enzymes (*in italics*) in the mitochondrion or the cytoplasm into heme. Heme synthesis begins and ends in the mitochondrion. Heme is incorporated into apo-hemoproteins (cytochromes shown) to form functional holo-hemoproteins. ALA, 5-aminolevulinic acid; PBG, porphobilinogen; HMB, hydroxymethyl bilane; URO'GEN, uroporphyrinogen III; COPRO'GEN, coproporphyrinogen; PROTO'GEN, protoporphyrinogen; and PROTO, protoporphyrin.

Heme Biosynthetic Pathway



Defects in heme synthesis and metabolism may have far-reaching pathological and biochemical effects. For example, the porphyrias (to be discussed on p. 17) are a group of diseases in which there are defects in normal heme synthesis, with elevated levels of ALAS-1, and overproduction of heme precursors occurring during acute attacks (Kappas et al., 1989; Bonkovsky et al., 1990). Insufficient supply of heme may affect levels and activities of key cellular hemoproteins, including mitochondrial cytochromes, catalase, tryptophan pyrrolase, and cytochrome P-450s (CYP) and b5. These latter cytochromes play a key role in the metabolism of many endogenous and exogenous compounds, including lipophilic drugs, toxins, carcinogens, steroids, prostaglandins and inflammatory mediators (Coon et al., 1992; Guengerich 1992; Nebert and Russell, 2002) and are induced by the lipophilic substances they metabolize (Okey 1990).

Induction of ALAS-1 by chemicals: Chemicals can induce ALAS-1 by more than one means. A general mechanism of induction of ALAS-1 by chemicals is thought to be by the depletion of a small, but critical, pool of heme within hepatocytes (Granick et al., 1975) (See The Regulatory Heme Pool). Recent studies (Fraser et al., 2002 and Fraser et al., 2003), in chick hepatoma (LMH) cells, demonstrate that certain lipophilic drugs can act

directly to increase transcription of the ALAS-1 gene, as summarized below (see Chemicals that induce CYP).

Chemicals that induce ALAS-1 by increasing heme degradation:

Porphyrogenic chemicals (chemicals that lead to an increase in porphyrinogens) such as 2-allyl-3-isopropylacetamide (AIA) increase ALAS-1 activity by depleting the heme pool by increasing the breakdown of heme to non-physiological products (De Matteis et al., 1977), while others, such as the combination of glutethimide (Glut) and ferric nitrilotriacetate (FeNTA), have been shown to do this by inducing the normal pathway for heme degradation via the enzyme heme oxygenase-1 (HO-1) in chick embryo liver cells (CELCs) (Lincoln et al., 1988; Cable et al., 1991).

Chemicals that induce ALAS-1 by inhibiting heme biosynthesis:

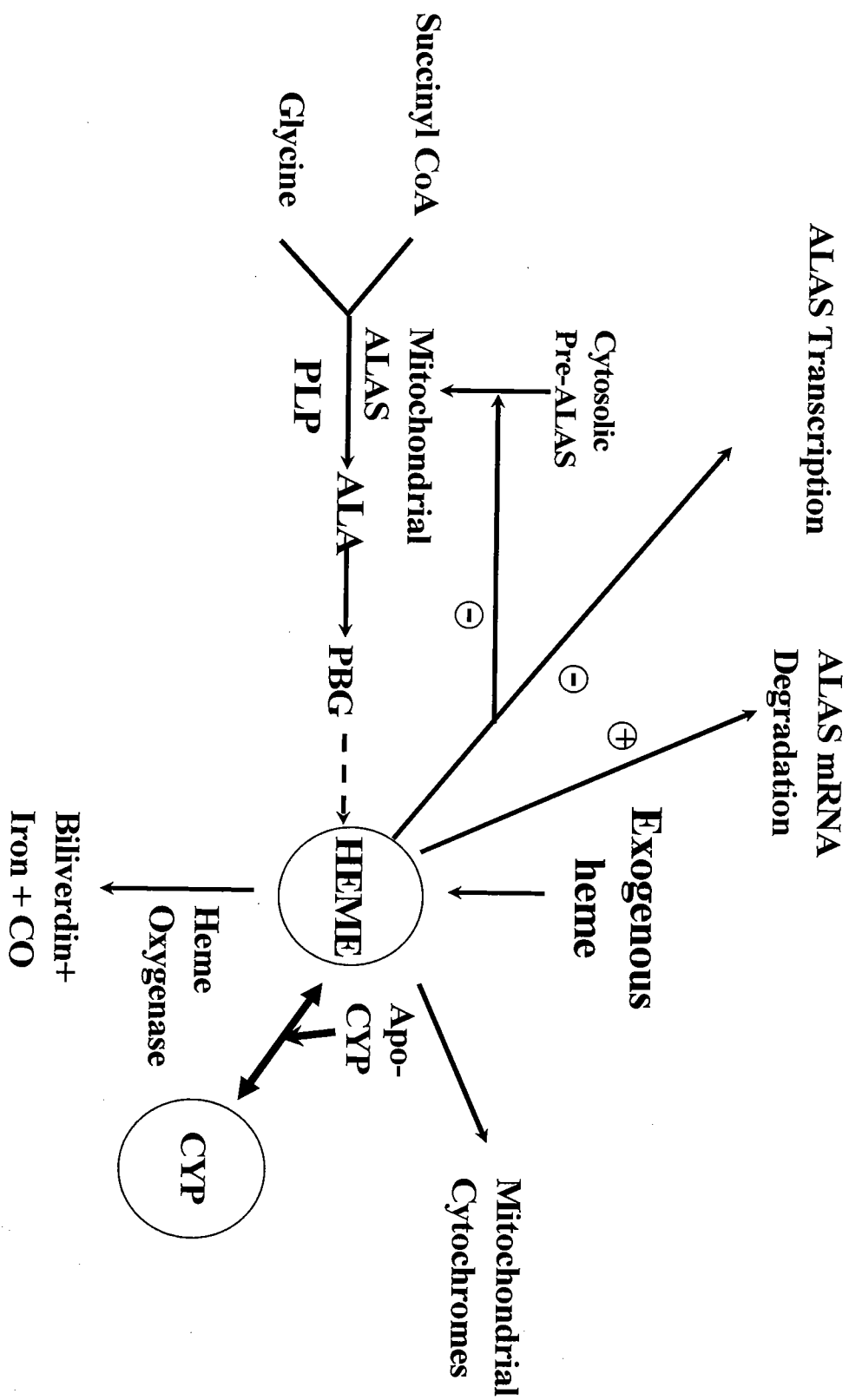
Another mechanism for the depletion of heme is by inhibition of normal heme synthesis (Kappas et al., 2000). For example, 4,6-dioxoheptanoic acid (DHA) and deferoxamine (DFO) are inhibitors of ALA dehydratase (the second enzyme in the heme biosynthetic pathway) and ferrochelatase (the terminal enzyme of the pathway), respectively (Figure 1.1). However, even profound inhibition of the heme biosynthetic pathway *per se* is not able to

produce a marked induction (≥ 5 fold) of ALAS-1 activity in CELCs (Giger and Meyer 1983). Such induction requires the presence of lipophilic chemicals that act directly to increase production of the synthase as discussed below and/or to increase the demand for heme by the synthesis of new molecules of CYP(s) (May et al., 1986; Kappas et al., 2000).

Chemicals that induce CYP: CYP(s) are a super family of proteins that are now believed to include 57 genes (Nebert and Russell 2002). CYPs utilize most (~60%) of the heme that is synthesized in the liver (hereafter termed endogenous heme) for assembly into holocytochrome P450(s) (Kappas et al., 2000) (Figure 1.1). ALAS-1 expression and heme levels are increased by drugs and other chemical inducers of CYP apoproteins (May et al., 1995; Lindberg et al., 1996; Jover et al., 2000).

Chemicals that induce CYPs were thought to also increase ALAS-1 expression indirectly by heme-dependent mechanisms (Srivastava et al., 1980; Kappas et al., 2000) (Figure 1.2). Experimental results with phenobarbital (PB), and other porphyrinogenic chemicals, demonstrated an increase in CYP and ALAS-1 activity (De Matteis and Gibbs, 1972; Giger and Meyer, 1981), and induction of apohemoproteins was envisioned to first cause an increase in demand for heme, thereby leading to a depletion of the

Figure 1.2. Effects of the regulatory heme pool on hepatic heme metabolism. Heme exerts regulatory effects on both ALAS-1 and HO-1 to control both its synthesis and degradation. ALAS-1 catalyses the formation of ALA, from glycine and succinyl CoA, which then through a series of intermediary steps catalyzed by the enzymes of the heme biosynthetic pathway, forms heme (Figure 1.1). Several steps in the production of functional mature ALAS-1 protein are modulated by exogenous or endogenous heme as indicated by the minus signs, or by the plus sign indicating increased degradation of ALAS-1 mRNA. Regulatory heme is incorporated into apo-hemoproteins (such as CYPs and mitochondrial cytochromes) as the prosthetic group to form functional holo-hemoproteins. Heme increases transcription of HO-1, leading to increased production of HO-1 protein, which catalyzes heme degradation.



heme pool followed by an induction of ALAS-1 (Kappas et al., 2000) (Figure 1.2). However, further experiments in CELCs investigating the relationship between induction of CYP2H1 and ALAS-1 have shown that mRNAs for CYP2H1 and ALAS-1 were simultaneously increased after treatment with PB-like drugs, and that inhibitors of heme synthesis had little or no effect on ALAS-1 mRNA expression (Hamilton et al., 1988).

Overexpression of a CYP protein in primary chick cells to levels similar to those achieved following induction by PB (presumably to deplete the heme pool) was shown not to result in ALAS-1 induction (Jover et al., 1996). The recent discovery that orphan nuclear receptors (members of a super family of transcription factors) mediate drug induction of CYP2H1 (Handschin and Meyer, 2000) and ALAS-1 (Fraser et al., 2002) and the isolation of a chick xenobiotic nuclear receptor (CXR) (Handschin et al., 2000) that mediates this induction give further support for the direct effect of drugs on ALAS-1.

These studies indicate that ALAS-1 gene expression can be increased directly by chemicals. However, the effect of the regulatory heme pool on induction of ALAS-1 by drugs has remained unclear from the above studies. This is partly because the regulatory heme pool is known to be rapidly turned over (see The Regulatory Heme Pool), making it hard to determine

the extent of depletion of the heme pool in a particular cell (Kappas et al., 2000).

In spite of these limitations, indirect evidence for the heme pool hypothesis has been obtained from heme-deficient animal models and from cells treated with chemicals that affect heme synthesis (Sassa and Kappas, 1982; Lukton et al., 1988; Brady and Lock., 1992; Kolluri et al., 1999; Jover et al., 2000). A recent study in normal and heme-deficient mice showed that PB treatment increases hepatic ALAS-1 mRNA, supporting the concept that PB directly affects ALAS-1 expression (Jover et al., 2000). In the same study, mice that were deficient in heme synthesis (PBGD-deficient mice) had a synergistic increase in ALAS-1 mRNA levels in response to PB, as compared to normal mice (Jover et al., 2000). The synergistic increase of ALAS-1 mRNA by drugs in the above described heme-deficient animal model involved repetitive doses of PB (that also induces CYP) (Jover et al., 2000) suggesting that heme-deficiency can augment drug-induction of ALAS-1.

Regulation of ALAS-1 by heme: As mentioned before, heme functions not only as a prosthetic group of oxygen-carrying proteins and enzymes involved in oxidation/reduction or electron transport reactions, but

also as an important molecule that regulates metabolic pathways (Sassa and Nagai, 1996). In 1966, the finding by Granick et al., that heme negatively regulates its own biosynthesis by modulating the production of ALAS-1 in the liver, was a seminal demonstration of feedback regulation by the end-product of the first enzyme in the pathway. Since then it has been shown that heme negatively regulates ALAS-1 by a variety of mechanisms, including repression of transcription, increased degradation of mRNA, and inhibition of mitochondrial translocation as described below and as summarized in Figure 1.2.

Transcriptional repression of the ALAS-1 gene: The half-life of the mRNA of hepatic ALAS-1 is reported to be short; from 1-5 h (Drew and Ades, 1989; Hamilton et al., 1991). Because of this, transcriptional repression could be an effective mechanism for regulation of ALAS-1. While heme has been shown to up-regulate gene expression by functioning as a ligand for transcription factors (Creusot et al., 1989; Zhang et al., 1998; Ogawa et al., 2001), the molecular mechanism by which heme represses ALAS-1 gene transcription, have remained elusive. In earlier work in intact rats, it was shown that heme down-regulated transcription of ALAS-1 (Srivastava et al., 1988; Yamamoto et al., 1988; Srivastava et al., 1990) and

had no effect on stability of the mRNA of ALAS-1 (Yamamoto et al., 1988). However, other evidence indicates that heme might be involved in regulation of ALAS-1 mRNA stability in primary rat hepatocytes (Cable et al., 2000). Data from CELCs showed that the effects of heme on ALAS-1 could be accounted for solely by decreasing half-life of the mRNA (Drew and Ades, 1989; Hamilton et al., 1991) without any change in ALAS-1 gene transcription (Hamilton et al., 1991). The differences in transcriptional regulation between chick and other model systems require further investigation.

Increase of ALAS-1 mRNA degradation: As just mentioned, in CELCs the half-life of ALAS-1 mRNA has been shown to decrease from 180-220 minutes to 70-90 minutes in the presence of heme, (1-10 μ M) (Drew and Ades, 1989; Hamilton et al., 1991) without any change in gene transcription (Hamilton et al., 1991). Data from rats, however, showed that the half-life of the ALAS-1 mRNA is unchanged in the presence of heme (Yamamoto et al., 1989; Srivastava et al., 1990), but ALAS-1 gene transcription is decreased as discussed above. Recent evidence, however, indicates that heme is involved in the regulation of ALAS-1 mRNA stability in primary rat hepatocytes (Cable et al., 2000).

Decrease of mitochondrial import of pre-ALAS-1: ALAS-1 is encoded in the nuclear genome but functions as an enzyme in the mitochondrial matrix (May et al., 1995). ALAS-1, synthesized in the cytosol on ribosomes as pre-ALAS-1 (Whiting et al., 1976), is imported into the mitochondria (Ohashi et al., 1978). Heme inhibits the translocation and processing of the cytosolic pre-ALAS through heme regulatory motifs (HRM) in the amino terminal of the pre-ALAS protein (Srivastava et al., 1983; Hayashi et al., 1983; Lathrop and Timko, 1993). Although these studies described above were carried out with the mouse erythroid ALAS-2, the same sequences exist in ALAS-1 (Zhang and Guarente, 1995), rat hemopexin (Nikkila et al., 1991), and the yeast heme activated transcription factor (HAP1) (Zhang et al., 1998). The existence of this motif in several heme-regulated proteins suggests that this may be a general mechanism for regulation by heme.

Summary of regulatory mechanisms: In summary, heme negatively regulates ALAS-1 by a variety of mechanisms including repression of gene transcription in rats (Srivastava et al., 1988), decreasing the half life of ALAS-1 mRNA and import of the pre-ALAS-1 protein from cytoplasm to mitochondria in chick cells (Srivastava et al., 1983; Hamilton et al., 1988).

Chemicals that induce CYPs, directly and simultaneously increase ALAS-1 and CYP mRNAs in CELCs and chick hepatoma (LMH) cells (Hamilton et al., 1988; Hamilton et al., 1991; Fraser et al., 2002). Heme has been shown to repress ALAS-1 gene transcription in rat livers (Yamamoto et al., 1989; Srivastava et al., 1990) but not in chick liver cells (Drew and Ades, 1989; Hamilton et al., 1991). Finally, a mouse with a targeted disruption of PBGD, the third enzyme of the heme synthesis pathway, showed a synergistic increase in ALAS-1 mRNA after PB treatment (Jover et al., 2000).

However, the molecular mechanism(s) such as the signaling pathways, *cis*-acting element(s), or transcription factor complexes by which heme regulates basal and drug-mediated ALAS-1 gene expression have been largely unexplored.

The Regulatory Heme Pool

The observed effects of heme on ALAS-1 led to the formulation of a concept referred to as the 'regulatory' 'free', or 'unassigned' heme pool (Sinclair and Granick, 1975; Granick et al., 1975; Kappas et al., 1989; Bonkovsky et al., 1990). According to this hypothesis, hepatocytes contain a small, but crucial, heme pool that functions as a barometer of the cell's heme requirements (Figure 1.2). Heme is envisioned to exchange between this pool and the various hemoproteins of the cells (Figure 1.2). The free or "regulatory" heme pool is presumed to be very small, and rapidly turn-over (Ketterer et al., 1976; Vincent et al., 1989; Müller-eberrhard and Fraig 1993), making it practically impossible to measure to prove/disprove this hypothesis. There is some indirect evidence for this hypothesis obtained from heme-deficient animals (Jover et al., 2000) and cells in culture, as described below.

In cultured cells, an increase in the size of the regulatory heme pool (eg, after administration of exogenous heme) represses ALAS-1 activity (Hamilton et al., 1988; Cable et al., 1993; Kolluri et al., 1999) and increases transcription of the HO-1 and metallothionein (MT) genes (Alam and Smith,

1992; Smith et al., 1993; Cable et al., 1994). In contrast, conditions that deplete the regulatory heme pool (e.g., drugs in combination with inhibitors of heme synthesis) tend to synergistically increase activity of ALAS (Hamilton et al., 1988; Russo et al., 1994). However, it has been well established that heme pool depletion *per se* will not give rise to high (≥ 5 -fold) activities of ALAS-1 such as those seen in models of acute porphyrias (Giger and Meyer 1983). Such high induction requires the presence of lipophilic chemicals in addition to chemicals that affect heme synthesis (Hamilton et al., 1988; Russo et al., 1994).

Several studies have suggested that drugs can directly increase ALAS-1 gene expression (Hamilton et al., 1988; Fraser et al., 2002 and Fraser et al., 2003), and that simultaneous inhibition of heme synthesis results in synergistic increases in ALAS-1 mRNA levels (Sassa and Kappas 1982; Hamilton et al., 1988; Jover et al., 2000). However, the effect of the “free” or “regulatory” heme pool on drug-mediated induction of ALAS-1 gene expression has not been demonstrated.

The Porphyrrias

Introduction: The porphyrias are a group of inherited or acquired disorders caused by deficiencies in the activities of the enzymes of the heme biosynthetic pathway (Table 1.1). Therefore, in the porphyrias, the fundamental defect is in heme synthesis. While inherited porphyrias are due to a gene defect, their phenotype often remains silent, both biochemically and clinically, and requires another insult, either genetic or environmental. Some of the important endogenous and environmental factors known to precipitate or exacerbate acute attacks of porphyria are endocrine factors, inadequate nutrition or exposure to drugs and other foreign chemicals (Bonkovsky et al., 2000). Eight enzymes are involved in the synthesis of heme and with the exception of ALAS an enzymatic defect at each step of heme synthesis is associated with a form of porphyria (Table 1.1).

Classification of porphyria: Porphyrrias are traditionally grouped into hepatic and erythropoietic porphyrias depending upon the major site of expression of enzymatic defect, either hepatic or erythropoietic cells. Another categorizing scheme is based on the occurrence in some patients of acute symptomatic attacks interspersed by non-symptomatic latent periods

Table 1.1: Enzyme defects, clinical symptoms, and inheritance of human porphyria. Classifications of porphyria, the enzyme defects and the inheritance are listed in the order that the enzymes occur in the heme biosynthetic pathway. Note that photosensitivity is a feature of all of the porphyrias that are associated with porphyrin accumulation as seen in the defects associated with enzymes that are distal to ALAS, whereas neurological features occur in those enzyme defects that are proximal to ALAS. ALAD, ALA dehydratase; PBGD, PBG deaminase; UROD, uroporphyrinogen III decarboxylase; COPRO'GEN, coproporphyrinogen; PROTO'GEN, protoporphyrinogen; AR, autosomal recessive; and AD, autosomal dominant.

<u>Porphyria</u>	<u>Enzyme</u>	<u>Classification</u>	<u>Inheritance</u>	<u>Principal Symptoms</u>
ALAD Deficiency Porphyria	ALAD	Hepatic	AR	Neurovisceral
Acute Intermittent Porphyria	PBGD	Hepatic	AD	Neurovisceral
Congenital Erythropoietic Porphyria	UROCoS	Erythropoietic	AR	Photosensitivity
Porphyria Cutanea Tarda	UROD	Hepatic	AD	Photosensitivity
Hepatoerythropoietic Porphyria	UROD	Erythropoietic	AR	Photosensitivity
Hereditary Coproporphyrria	COPRO'GEN	Hepatic	AD	Neurovisceral,
	OXIDASE			photosensitivity
Variegate Porphyria	PROTO'GEN	Hepatic	AD	Neurovisceral,
	OXIDASE			photosensitivity
Erythropoietic Protoporphyrria	Ferrochelatase	Erythropoietic	AD	Photosensitivity

and therefore, are classified as chronic or acute porphyrias.

Symptoms of porphyria: Elevated ALAS-1 plays a key role in precipitating the acute porphyrias (Kappas et al., 2000). As a result, abnormally elevated levels of porphyrins and/or their precursors, e.g. ALA and PBG are produced in excess, accumulate in tissues, and are excreted in urine and stool (Kappas et al., 1989 and 2000). Two major symptoms of the porphyrias are cutaneous photosensitivity and/or neurological disturbances. Many chemicals that exacerbate porphyria are called porphyrinogenic.

Treatment of Porphyria: Patients suffering from acute hepatic porphyrias should avoid exposure to porphyrinogenic compounds, because of the extreme and debilitating symptoms that occur after such exposure (Kappas et al., 2000). Heme infusion and placing the patient on a high carbohydrate diet, as a method to reduce hepatic ALAS activities, are treatments that utilize the biochemical knowledge about the regulation of hepatic heme biosynthesis. Reversion of ALAS activities to normal is associated with an asymptomatic state (Bonkowsky et al., 1971; Pierach 1982; Kappas et al., 2000).

Research Objectives

Objective 1: To determine if LMH cells, a chick hepatoma cell line, are a good model comparable to CELCs, for the study of heme biosynthesis and regulation of the heme biosynthetic enzyme, ALAS-1. In CELCs and LMH cells, similar patterns of response of ALAS-1 activities and mRNA levels were obtained following treatments known to affect heme biosynthesis. In addition, a synergistic induction of ALAS activity by Glut and DHA, previously shown in CELCs, was demonstrated in LMH cells. Similarly, heme-dependent repression of ALAS-1 mRNA levels for both cell types and of induced-ALAS activities for LMH cells were demonstrated. These results suggest that LMH cells provide a useful model system for further studies of regulation of hepatic heme metabolism.

Objective II: To establish a "glucose effect" on ALAS-1 enzyme activity in LMH cells and CELCs in the absence of serum or hormones, and to determine whether glucose or other metabolizable sugars decrease the activity of transfected reporter gene constructs under control of the ALAS-1 promoter. The optimal glucose conditions for induction by Glut and DHA and dose-responses of glucose repression of Glut and DHA and Glut and

FeNTA-mediated induction of ALAS-1 enzyme activity were characterized in LMH cells and CELCs. Other metabolizable sugars and the non metabolizable sugars, 2-DOG and sorbitol, were tested for their ability to alleviate Glut- and DHA-upregulation of a reporter construct containing approximately 9.1 kb of 5'-flanking region of the chick ALAS-1 gene in LMH cells. These results establish that both glucose and other metabolizable sugars lead to down-regulation of an approximately 9.1 kb construct containing the 5'-flanking and 5'-UTR region of the chick ALAS-1 gene. These effects on enzyme activity and promoter constructs were observed in the absence of insulin, glucagon, other hormones or serum.

Objective III: To characterize effects of drugs, and selected chemicals that inhibit heme synthesis such as, DHA, and heme, on the upstream regulatory region of the chick ALAS-1 gene. Activation of the ALAS-1 promoter by the combination of an inducer of ALAS-1, Glut, and an inhibitor of heme synthesis, DHA, and the effect of exogenous heme on basal and Glut-and DHA-induced reporter activity was tested in transient transfection assays. The effects of drugs (induction) and heme (repression) of ALAS-1 were shown to be regulated by separate and distinct regions of the cALAS-1 promoter. Transient and stable transfection assays using

reporter constructs containing successive deletions of the cALAS-1 promoter sequence were used to further delineate (from 5622 bp to 2819 bp) the 5'-flanking region of the gene involved in mediating heme-dependent repression of ALAS-1. Drug-responsive enhancer (DRE) sequences upstream (~ -13 kb) of the -9.1 kb region of ALAS-1 gene, that mediate 30-40-fold up-regulation of ALAS-1, were PCR amplified and shown to be repressed by the early promoter region of ALAS-1 (which is not repressed by heme). Results from these experiments suggest heme can repress ALAS-1 *cis*-acting element(s) in the -6.3 to -3.5 kb 5'-flanking region of the gene and that although drugs can directly activate the -3.5 to +0.082 kb region, the extent of drug induction is dependent upon heme levels in the cell.

CHAPTER II

MATERIALS AND METHODS

Materials

General materials. Levulinic acid was from Aldrich, Milwaukee, WI. Tissue culture dishes and 6 well (3 cm) plates were from Corning, Corning, NY; culture flasks were from falcon, VWR Scientific, Bridgeport, NJ. Methanol and 2,4-pentanedione (acetyl acetone) were from EM Science, Gibbstown, NJ. Chloroform, glycerol, potassium phosphate, and isopropanol were from Fisher, Pittsburgh, PA. Fetal bovine serum, geneticin (G418), LipoFECTAMINE™ Plus, opti-MEM, trypsin, Williams' E powder and Waymouth's MB 752/1 liquid were from Invitrogen Corporation, GIBCO/BRL, Grand Island, NY. 2-Allyl-3-isopropylacetamide (AIA) was from Hoffmann-La-Roche, Inc., Nutley, NJ. Random hexamers (pd(N)6) and Oligo-dT (12-18 bases) was from Pharmacia, Piscataway, NJ. Hemin chloride and porphyrin standards (uroporphyrin, coproporphyrin, and protoporphyrin) were from Porphyrin Products, Logan, UT. Luciferase Assay Reagent was from Promega, Madison, WI. Maxi- Midi- and Mini-prep plasmid DNA isolation and agarose gel extraction kits were from

Qiagen Corporation (Valencia, CA). Adenosine triphosphate (ATP), bovine serum albumin (BSA), 8-(4-chlorophenylthio) adenosine monophosphate (8-CPTcAMP), coenzyme A, deferoxamine mesylate (DFO), 2-deoxy α -D-glucopyranoside (2-DOG), dexamethasone (Dex), p-dimethylamino benzaldehyde, dimethylsulfoxide, EDTA, EGTA, ethanol, ferric chloride, formaldehyde (37% v/v), formamide, fructose, galactose, glacial acetic acid, glutethimide, glycine, glycyglycine, lactate, mercuric chloride, 2-methyl-1,2-di-3-pyridyl propadone (metyrapone), mifepristone (RU-486), NADPH, nitrilotriacetic acid (NTA), o-nitrophenyl β -galactopyranoside (ONPG), penicillin, perchloric acid, phenobarbital (disodium salt), potassium monophosphate, 5-pregnene-3 β -ol-20-one-16 α -carbonitrile (PCN), pyridoxal-5'-phosphate, sodium acetate, sodium chloride, sodium dodecyl sulphate (SDS), sodium hydroxide, sorbitol, streptomycin, succinic thiokinase (EC 6.2.1.4), 3,5,3'-triiodo-L-thyronine (T3), and Trizma base were from Sigma (St. Louis, MO). Gelatin was from J. T. Baker, Phillipsburg, NJ. Ultraspec RNAzol® was from Biotecx, Houston, TX. QuickHyb® was from Stratagene, La Jolla, CA. Nitrocellulose (0.45 μ m) was from Schleicher and Schuell, Keene, NH. All 32 P-radionucleotides were from New England Nuclear, Boston, MA. Radiolabeled probes were

generated using the DECAprime II DNA Labeling Kit from Ambion, Austin, TX or Ready-To-Go DNA Labeling Beads from Pharmacia Biotech, Piscataway, NJ. All chemicals were of the highest purity available.

Cells and DNA. Fertilized Barred Rock chicken eggs were from Carousel Farms, Hopkinton, MA. LMH cells and pGAD-28 plasmid were a generous gift from D. L. Williams, Department of Pharmaceutical Sciences, SUNY-Stony Brook, Stony Brook, NY. Chick ALAS-1 cDNA used as a template for generating labeled probe was a gift from D. Engel, Northwestern University, Evanston, IL. The pGcALAS3.5-Luc and pGcALAS9.1-Luc constructs containing chick ALAS-1 promoter sequence were kindly provided by Dr. Timothy J. Sadlon and Dr. Brian May, Department of Molecular Biosciences, University of Adelaide, South Australia, Australia. The pPGK- β gal plasmid was a gift from P. Dobner, Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, MA. PGKneo plasmid was a kind gift from Dr. Eva Lee, University of California, San Diego, CA. PGL3SV40 construct, primer RV primer3 and primerG12 were purchased from Promega, Madison, WI. DNA sequencing was performed by the Nucleic Acids Facility, University of Massachusetts Medical School, Worcester, MA.

Methods

Cell culture and preparation of chemicals. LMH cells were maintained in Waymouth's MB 752/1 complete medium (Appendix A) on 0.1% (w/v) gelatin-coated flasks and passaged routinely 1 to 2 times a week. Cultures of CELCs were obtained every week and plated in Williams' E medium (supplemented with 1.5 μ M T3, and 0.76 μ M dexamethasone) as previously described (Lincoln et al., 1988). Complete (containing 10% v/v fetal bovine serum) and serum-free Williams' E (Appendix A) and Waymouth's media were supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin. CELCs and LMH cells were plated on 6 cm Corning dishes for most experiments, except for transient transfections of LMH cells, when cells were plated on 6 well (3 cm) plates. LMH cells were trypsinized, centrifuged, resuspended, and plated at 50-60% confluence in 3 or 5 mL Waymouth's complete medium. After 24 h, the cells were washed 1X with 2 mL of Waymouth's complete medium to remove any unattached cells and then 3 or 5 mL of the same medium was added. For transfection experiments with LMH cells, 24 h after plating cells were washed as described above and transfected with Luc constructs and pPGK β -gal as described (see

Transfections). For experiments comparing CELCs and LMH cells, on the day of treatment, LMH cells and CELCs were washed 1X with 2 mL Williams' E serum-free medium and 5 mL of the same was added. Preparation and treatment with chemicals was exactly as described for CELCs (Lincoln et al., 1988). All chemicals were freshly prepared on the day of treatment and added directly to culture medium (Appendix B). For experiments testing the glucose effect on ALAS, LMH cells and CELCs were isolated as described above except on the day before treatment the medium on the cells was changed to Williams' E medium without serum, T3, glucose or pyruvate. Twenty-four h later, selected plates received specified amounts of glucose or other selected metabolizable sugars such as fructose, galactose, glycerol and lactate or the non-metabolizable sugars - sorbitol and 2-DOG. After 2 h of exposure to glucose, the cells were treated with Glut (50 μ M) in combination with DHA (250 μ M) or FeNTA (50 μ M). After 16 h of treatment with chemicals, cells were harvested and ALAS-1 (enzyme or Luc) activity and protein content were determined.

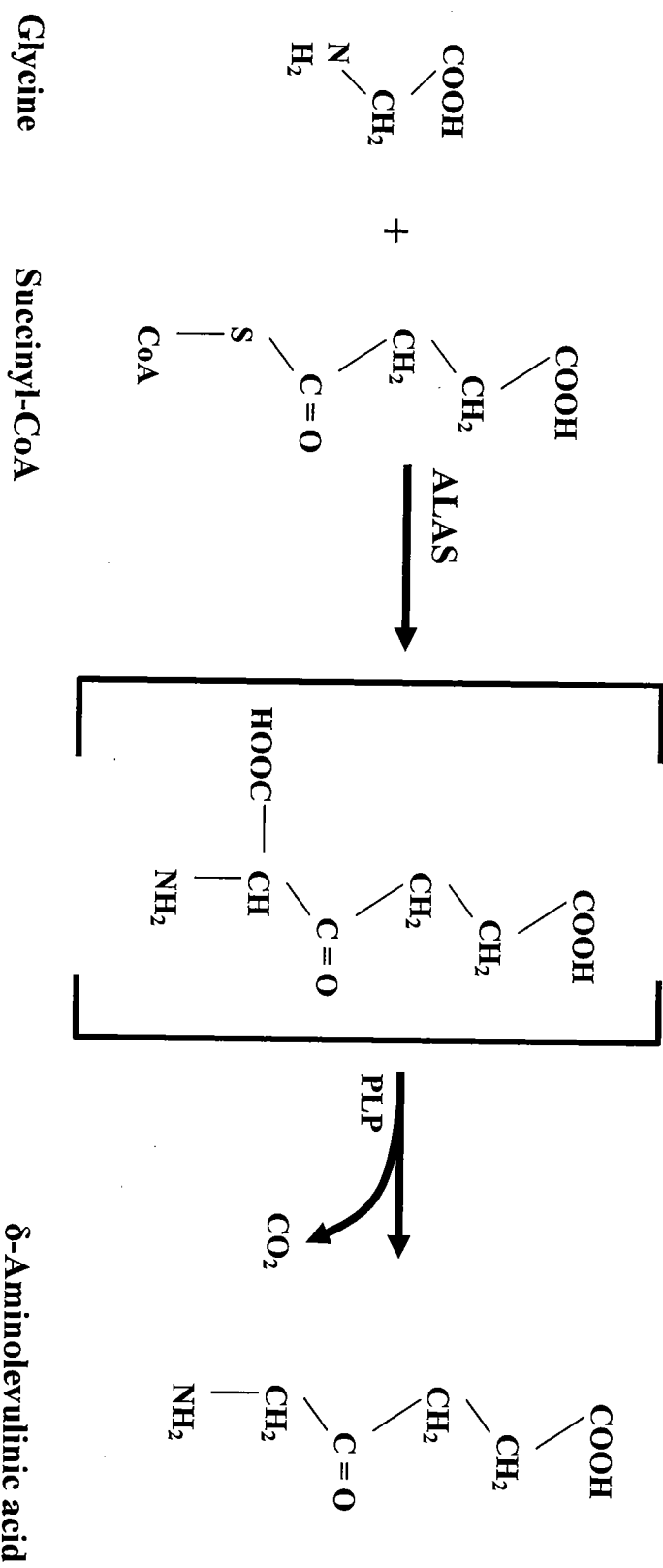
Measurement of ALAS enzyme activity. ALAS activity was measured essentially as described previously (Cable et al., 1991) and as described below except that the samples were snap-frozen in liquid nitrogen as whole

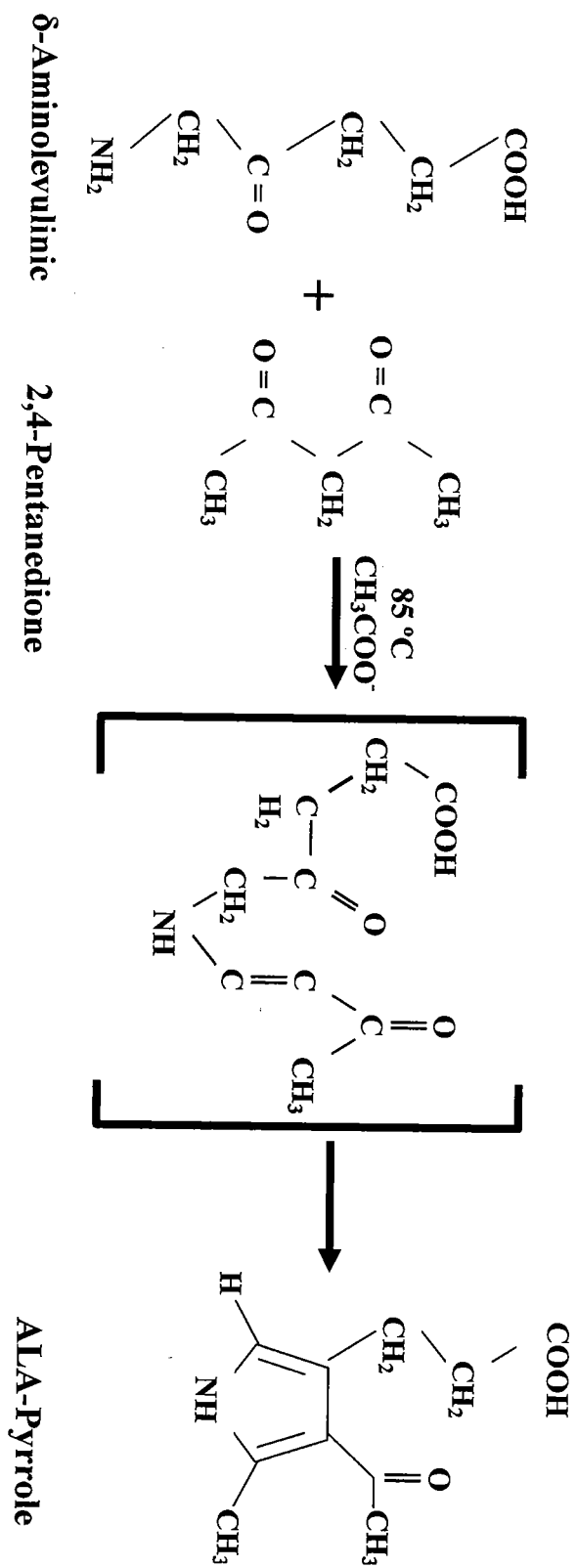
cells and stored at -80°C for 1-2 weeks prior to assay. Initial experiments showed that ALAS activity was stable under these storage conditions. The assay measures the rate of conversion of glycine and succinyl-CoA in the presence of the cofactor PLP to form ALA. The ALA formed is converted into a pyrrole (Figure 2.1) and reacted with Ehrlich's reagent p-dimethyl aminobenzaldehyde (2% DMAB) to form a colored Ehrlich's pyrrole salt (Figure 2.1). The activity of ALAS is determined from the concentration of this product obtained by measuring $\Delta\text{OD}_{555-650\text{ nm}}$ (Mauzerall and Granick, 1956; Sinclair and Granick, 1977; Lien and Beattie, 1982). LMH cells or CELCs were plated on 6 cm dishes as described above. After 24 h the cells were treated with selected chemicals. After 18 h the plates were washed twice with 1X PBS and harvested in 700 μL of assay buffer (Appendix A) designed to maintain pH at 7.4 for at least 30 minutes at 37°C . Each cell suspension was sonicated for 6 seconds using a sonicator (Bronson Model 450, VWR scientific, Bridgeport, NJ) equipped with a microtip (output: 2, duty cycle: 30). Each sonicate (150 μl) was added to each of three 12 x 75 mm culture tubes, one blank and two assay duplicates. To the "blank" tubes, at time $t=0$, trichloroacetic acid (TCA) to a final concentration of 4% (w/v), and 10 μl of 1M glycine (pH 7.0), were added after which they were kept on

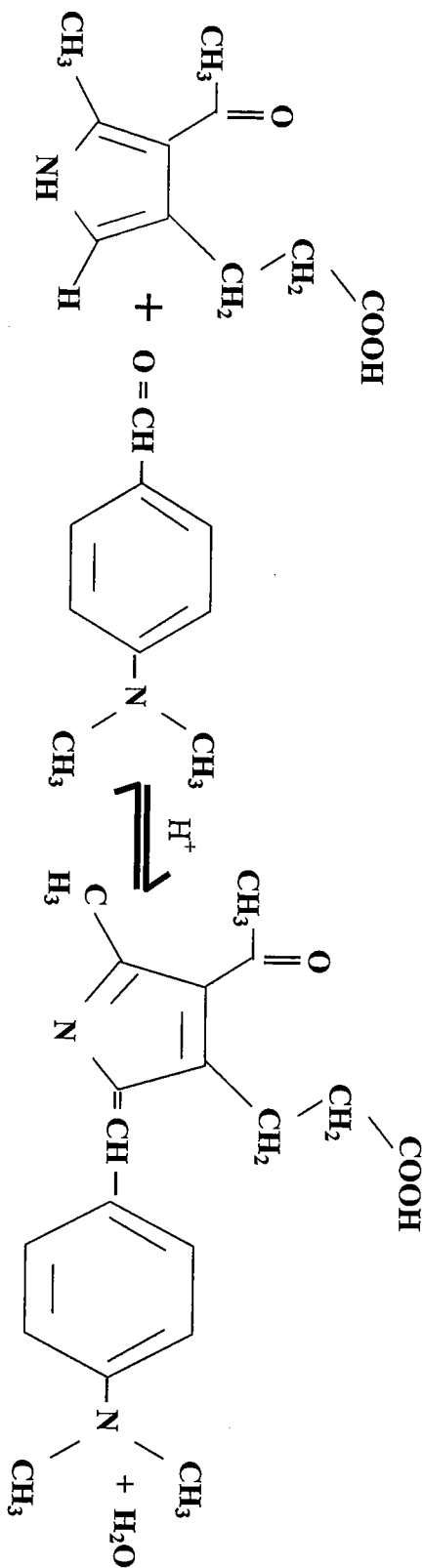
ice throughout the incubation. Each "blank" tube was used to zero the corresponding "assay" tubes during spectrophotometry. Glycine (1M; 10 μ l) pH 7.0, was added to the assay tubes at $t=0$ and incubated at 37°C in a shaking water bath for 30 minutes after which TCA was added to the tubes to a final concentration of 4% (w/v). The blank and assay tubes were treated identically from this point onwards. The samples were centrifuged for 10 minutes at 1000 x g and 150 μ L of the supernatant containing ALA was placed in a separate tube. 100 μ L of 10% (v/v) 2,4-pentanedione in 1.0 M sodium acetate (pH 4.6) was added to the TCA supernatant, and the mixture was incubated at 85° C for 15 minutes to form an ALA-pyrrole (2-methyl-3-acetyl-4-propionic acid pyrrole) in a Knorr-type condensation reaction (Figure 2.1). The ALA pyrrole was converted into a chromophore by the addition of 250 μ L of modified Ehrlich's reagent (DMAB), 0.375% (w/v) mercuric chloride in a 4:21 (v/v) solution of 70% w/v perchloric acid: glacial acetic acid). The chromophore has a $\Delta\epsilon_{555-650\text{ nm}} = 65\text{ mM}^{-1}\text{cm}^{-1}$ (Lien and Beattie 1982; Sinclair and Granick, 1977). The mono Ehrlich's salt, DMAB adduct, a chromophoric Ehrlich's salt forms in solution with a maximum absorbance after 10 minutes. This timing is important as longer incubation (>15 minutes) allows a secondary reaction to form a colorless dipyrrole

Figure 2.1: Mechanisms of the reactions utilized in the assay for ALAS.

Reaction 1: The reaction carried out *in vitro* and catalyzed by ALAS using glycine and succinyl CoA with PLP as a cofactor. Reaction 2: The formation of an ALA pyrrole (2-methyl-3-acetyl-4-propionic acid) (Viale et al., 1987a). This reaction converts the ALA formed by the enzyme into an Ehrlich's reactive species. Reaction 3: Conversion of the ALA pyrrole to a chromophore, the characteristic red Ehrlich's ALA salt (Viale et al., 1987b). Solutions of this compound are quantified and the nanomoles of ALA formed in the *in vitro* enzyme reaction are calculated using the equation described under Measurement of ALAS-1 enzyme activity.

Reaction 1

Reaction 2

Reaction 3

ALA-Pyrrole

p-Dimethylaminobenzaldehyde

Chromophore Ehrlich's ALA Salt

phenyl methane (Lien and Beattie, 1982) which can lead to a decrease in absorbance.

Nanomoles ALA =

$$\frac{\text{OD (555-650)} \times .75 \text{ mL final volume in cuvette} \times .25 \text{ mL final volume}}{.065 \text{ OD/nmole ALA/mL} \times \text{path length in cuvette} \times .15 \text{ mL initial volume}}$$

Measurement of porphyrins. Cells and medium from each plate were harvested together and disrupted by sonicating for 6 seconds with a microtip sonicator (Branson Heat Systems W225 sonicator, Danbury, CT). From the time the cells were treated, all steps were carried out in subdued lighting. Measurement of total porphyrins and of uro-, copro-, and proto-porphyrin were carried out using a spectrofluorometric procedure as previously described (Grandchamp et al., 1980). Briefly, the samples were extracted by adding 1 mL 5% (v/v) perchloric acid in methanol to 1 mL of sonicate, centrifuged at 1000 X g for 10 minutes and the supernatant aliquotted into cuvettes for fluorescence measurements. Fluorescence was measured with a luminescence spectrometer (LS50B, Perkin Elmer, Norwalk, CT) at the following excitation/emission wavelength pairs: 400/595, 405/595, and 410/595 nm. The results were compared with known concentrations (50 µg/mL) of uroporphyrin, coproporphyrin, and protoporphyrin as standards.

In previous studies, direct comparisons of the spectrofluorometric and HPLC methods for detecting porphyrins (Sinclair et al., 1986; Bonkovsky et al., 1987) have shown good agreements (coefficients of variation <10%).

Uroporphyrin and heptacarboxyporphyrin on HPLC are detected spectrofluorometrically as 'uroporphyrin'; hexacarboxy-, pentacarboxy-, and copro-porphyrins are measured as 'coproporphyrin'; and tri- and dicarboxyporphyrins as 'protoporphyrin' (Sinclair et al., 1986; Bonkovsky et al., 1987).

Isolation of RNA. Total RNA was isolated using Ultraspec RNAzol®, according to the manufacturer's protocol. After removing medium, 0.5 mL Ultraspec RNAzol® was added to the plates and cells from each 6 cm dish were harvested by scraping with a rubber spatula. Total RNA was isolated as described (Cable et al., 1994). Briefly, the cellular lysates were transferred into 1.5 mL microfuge tubes, 100 µL of chloroform added, vortexed, and incubated on ice for 15 minutes. After 15 minutes, the mixtures were centrifuged at 14,000 x g and at 4°C for 15 minutes, which causes them to separate into a heavier, lower, cloudy organic layer and an upper, more transparent aqueous phase. The aqueous phase was carefully removed with a

pipette, without touching the interphase, and transferred to a new tube. An equal volume (300-400 μ L) of cold isopropanol was added, the tubes vortexed briefly but vigorously and incubated at -20°C for 1 h. After 1 h, the tubes were centrifuged (14,000 \times g at 4°C for 15 minutes), the supernatants removed, and the pellets washed with 0.5 mL of 75% (v/v) ethanol to remove any residual isopropanol. The pellets, which contain RNA, were dried in a Speed-Vac (Savant), and suspended in 50 μ L of DEPC-treated water for dot blots or 0.5% SDS for northern blots, samples were either stored at -20°C or used directly for northern or dot blots. For blotting, the samples were incubated at 65°C for 45 minutes, and the absorbance ratio at 260 nm / 280 nm was determined. Samples with a 260 nm / 280 nm ratio of 1.8 or higher were considered sufficiently free of contaminating proteins, and the RNA concentrations were estimated from the absorbance at 260 nm (1 AU = 40 μ g / mL RNA) (Sambrook et al., 1989).

Preparation of radiolabeled probes. Radiolabeled probes for detection of mRNA for chicken ALAS-1, and chicken glyceraldehyde phosphate dehydrogenase (GAPDH) were synthesized by random priming and incorporation of $\alpha^{32}\text{P}$ -dCTP using Ready-To-GoTM DNA labeling beads

from Pharmacia Biotech (Piscataway, NJ) or the DECAprime II™ DNA Labeling Kit from Ambion, Inc. (Austin, TX). The chicken ALAS-1 (cALAS-1) probe template was a 1.7 kb EcoR I fragment of the pALX plasmid containing the cALAS-1 cDNA. 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'-GAA AGT CGG AGT CAA CGG ATT TG-3' and GAP2: 5'-TGG CAT GGA CAG TGG TCA TAA GAC-3'. The PCR program for GAPDH was as follows: delay, 94°C for 3 minutes; 30 cycles of segment 1, denaturation, (94°C for 1 minute 30 seconds), segment 2, renaturation (54°C for 2 minutes), segment 3, elongation (72°C for 2 minutes); delay 72°C for 10 minutes; soak at 4°C. Poly-A probe template was a 12-18 base polythymidylate oligonucleotide from Pharmacia Biotech. The Poly-A probe was end-labeled with $\alpha^{32}\text{P}$ -dCTP using T4 polynucleotide kinase and the DECAprime II™ DNA Labeling Kit from Ambion Inc. and used as a control for RNA loading.

Quantitation of messenger RNA.

Dot Blots. The dot blotting protocol used total RNA and a probe that had previously been shown to react with the mRNA of interest (Cable et al.,

1991; Cable et al., 1994). After RNA isolation, as described in Methods, (see Isolation of RNA), total RNA was resuspended in 50 μ L of DEPC treated-water. Samples were prepared for loading onto the dot blot as follows, for three blots: 20 μ L of DEPC-treated water, 36 μ L of 20X SSC, and 24 μ L of 37% formaldehyde were added to 40 μ L of RNA and incubated at 65°C for 15 minutes. For each duplicate blot, 200 μ L of 10X SSC was added to the samples. The pre-wet (DEPC-water for 5 minutes, rinse with 10 X SSC) nitrocellulose membrane was placed between two sheets of pre-wetted Whatman 3M paper on the dot blot apparatus. The wells were washed once with 10X SSC and 200 μ L of RNA was loaded per well in triplicate. The wells were washed twice with 10X SSC. Duplicate nitrocellulose membranes were prepared in the same way. The membranes were removed from the dot-blot apparatus and the RNA cross-linked to the membranes using a UV Stratalinker (Stratagene, La Jolla, CA). The blots were prehybridized with QuickHyb® at 65°C for 30 minutes. After prehybridization, selected radiolabeled probes (see Preparation of radiolabeled probes) were boiled and added to the QuickHyb® solution at 65°C for 1 hour. After hybridization, the blots were washed with 0.1X SSC

at 25°C for 30 minutes with three buffer changes to remove non-specific binding. The amount of specific mRNA was quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and Image Quant software and normalized to total Poly-A mRNA, measured on the duplicate blot probed with the radiolabeled Poly-T probe or to GAPDH mRNA, measured on the duplicate blot probed with the radiolabeled GAPDH probe, as described (see Preparation of radiolabeled probes).

Plasmid construction.

pGcALAS3.5-Luc. The construct containing approximately 9.1 kb of the 5'-flanking region and 5'-UTR of ALAS-1 was a kind gift from Dr. Brian K. May, Department of Molecular Biosciences, University of Adelaide, Adelaide, Australia and constructed as described below. The 5'-flanking region of chick ALAS-1 (cALAS-1) gene was obtained from the λ clone (cALAS-1) (Maguire et al., 1986). The early promoter region and 5'-UTR of (cALAS-1) (-299 to +82 bp) was first amplified with Pfu DNA polymerase (Stratagene, La Jolla, CA) using the primers 5'-AAT GAG AAG CTT TGA GAT TTG TG-3' and 5'-CCG CCT CCA AGC TTC CTC CTG-3'. The PCR product was then digested with Hind III (underlined) and cloned into the Hind III site of a modified pGL3 Basic plasmid vector (Promega,

Madison, WI) in which the Not I site at 4651 bp was destroyed by blunting with T4 DNA polymerase to make pGcALAS0.3-Luc. To construct pGcALAS3.5-Luc, the vector pGcALAS0.3-luc was digested with Sma I and a Sma I fragment (-3447 to -165 bp) isolated from λ clone cALAS-1 was inserted.

pGcALAS9.1-Luc. The construct containing approximately 9.1 kb of the 5'-flanking region and 5'-UTR of ALAS-1 was a kind gift from Dr. Brian K. May, Department of Molecular Biosciences, University of Adelaide, Adelaide, Australia and constructed as described below. To construct pGcALAS9.1-Luc, the vector pGcALAS0.3-Luc, was digested with Not I and Xho I, and an approximately 9.1 kb Xho I to Not I restriction fragment isolated from the λ clone cALAS-1 was inserted. The approximately 9.1 kb (-9067 to +82 bp) fragment represents the parental fragment from which all other deletions were generated. Deletion constructs were produced by restriction enzyme digestion and ligation of the excised region as described below. Two mg of pGcALAS9.1-Luc containing approximately 9.1 kilobases (kb) of genomic chicken 5-aminolevulinate synthase-1 (cALAS-1) sequence was digested with Xho I and subsequently 1 mg was digested with Pml I or Pac I. The ends were blunted with T4 DNA

polymerase and dNTPs and the restriction fragments separated on a 0.8% agarose gel. The fragments of expected sizes were excised from the gel using a sharp razor blade under UV light and placed in microfuge tubes. The DNA in the gel pieces was extracted using the QIAquick[®] gel extraction kit. The DNA was quantitated and ligated using T4 DNA ligase, along with appropriate no ligase and no DNA controls. The ligation mix was transformed into DH5 α competent cells and plated on Luria Broth agar plates supplemented with 100 μ g/mL ampicillin, and incubated overnight at 37°C. Positive colonies were identified by DNA minipreps and screened for the deletions by restriction enzyme digestion with Sma I. Colonies that were positive by restriction digestion were verified by sequencing junctions using the commercial primer RV primer3 (Promega, Madison, WI).

Drug Responsive Element (DRE) Constructs of pGcALAS9.1-Luc,

pGcALAS0.3-Luc and pGL3promoter: The chick ALAS-1 -13793 to -13271 bp 5'-flanking region containing a 167 bp drug responsive enhancer sequence (DRE) (Fraser et al., 2002) was first amplified using ~1 ng of chick genomic DNA and primers 5'- AGT ACA GCT ATG GAT CTG TT-3' and 5'-CCG CCT CCA AGC TTC.CTC CTG-3'. PCR generated a 522 bp

product which was sequenced and found to contain the DRE and to be identical to the selected region. A second PCR was performed using gel extracted PCR1 product as template with the same primers but containing Mlu I sites on the ends 5'-cga cgc gt AGT ACA GCT ATG GAT CTG TT-3' and 5'-acg cgt cg GTC TTG ATA GGG CTA ACT TT-3' (Mlu I sites in lower case). The 538 bp PCR2 product was digested with Mlu I and cloned into the Mlu I site (upstream of the Xho I site) (Appendix E) of cALAS-1 promoter-reporter constructs, pGcALAS9.1- and 0.3-Luc, and into the multiple cloning site of a heterologous promoter construct, pGL3SV40-Luc (Promega Madison, WI). This was done by ligation of Mlu I -digested (and gel-extracted) PCR2 product with 0.5-1 µg of {Mlu I-digested and calf-intestinal phosphatase (CiP)-treated} pGcALAS9.1-Luc and pGL3SV40-Luc and pGcALAS0.3-Luc using T4 DNA ligase, with no ligase and no DNA controls. The ligation mix was transformed into DH5α competent cells and plated on Luria Broth agar plates supplemented with 100 µg/mL ampicillin, and incubated overnight at 37°C. Positive colonies were identified by DNA minipreps and screened for the DRE by restriction enzyme digestion with Mlu I. Single colonies that were positive by restriction digestion were verified by sequencing junctions using the

commercial primer RV primer3 (Promega, Madison, WI) and found to be identical to the selected region.

Transfections.

Transient transfection: LMH cells were plated on 0.1% (w/v) gelatin coated 6-well (3 cm) plates at a density of 1.5×10^5 cells/well. After 24 h each well was transfected with 0.5 μ g of pGK- β gal and 0.5 μ g of cALAS-1 promoter/reporter DNA using LipoFECTAMINE™ Plus (0.33 μ g/ μ L of reagent), according to the manufacturer's instructions. In the DRE experiments, cells were on 24-well (1.4 cm) plates and the transfections, treatments were performed similarly to that described here. Total DNA transfected was kept constant by adding pBLUESCRIPT KS II+ plasmid DNA. Cells were then incubated at 37°C, 5% CO₂ for 3 h, after which 2 mL of Waymouth's complete medium was added and incubated overnight. Twenty-four h after transfection, cells were washed and plated with Waymouth's medium containing penicillin-streptomycin but no serum and treated with selected chemicals.

Stable transfection: LMH cells were stably transfected essentially as described above with 0.3 μ g of pGKNeo and 1.5 μ g of cALAS-1

promoter/reporter DNA. Twenty-four h after transfection, each well of transfected cells was trypsinized and transferred onto 4 to 6, 10 cm plates in Waymouth's complete medium (with serum but without G418). The cells were allowed to recover overnight and in the morning the medium was changed to Waymouth's complete medium supplemented with 250 $\mu\text{g/mL}$ G418. The cells were maintained in G418 medium for 3-4 weeks until most of the cells died. Cells transfected with the neomycin resistance gene survived as single cells, which then grew into single colonies in 3-4 weeks. Such colonies were isolated using trypsin and transferred into gelatin coated 48-well plates and subsequently, after reaching confluence, into 24-well (1.4 cm), 12-well (2.12 cm), and 6-well (3 cm) plates containing Waymouth's complete medium with 250 $\mu\text{g/mL}$ G418. Putative stably-transfected colonies were identified by testing their luciferase activity. Eight-ten independent single colonies were isolated and tested for each construct and only colonies containing basal luciferase activity and inductive/repressive responses after treatment with chemicals similar to transiently transfected cells were used in further studies described.

Assessment of reporter gene activity. Reporter gene expression was assessed by quantitation of luciferase activity, normalized to β -galactosidase (β -gal) activity, and protein content in the case of transient transfection experiments, or just protein content in the case of stable transfection experiments. For luciferase activities, transfected cells were washed 2 times with 1X PBS, and harvested in 250 μ L of glycylglycine harvest buffer (Appendix A). Cells were lysed by freeze-thaw (3 cycles of 3 minutes in liquid nitrogen and 3 minutes at 37°C). The cellular fragments were centrifuged for 10-15 min at 14,000 X g in the cold. The supernatant (15 μ L each) was assayed for luciferase and β -gal activities and protein content. For luciferase activity measurements, luminometer tubes were placed into a Monolight 2010[®] Luminometer (Analytical Luminescence Labs, Ann Arbor, MI) and 100 μ L of Luciferase Assay Reagent (Promega, Madison, WI) was automatically injected. Relative light units (RLU) produced in 10 seconds were recorded and normalized with β -gal activities (for transient transfections) and protein content. β -gal activities were measured by a colorimetric assay (Sambrook et al., 1989). The cell lysate (15 μ L) was added to 200 μ L of Z-buffer (Appendix A) and 100 μ L of 5 mg/mL ONPG dissolved in 0.1 M potassium

phosphate, pH 7.0, and incubated at 37°C for 30-60 minutes. The absorbance at 420 nm was measured as a measure of β -gal activity which was used normalization of luciferase activity.

Measurement of protein concentrations. The amount of protein in the sample was measured by the bicinchoninic acid method (BCA) on a GENESYS 2 (Thermo Spectronic, Rochester, NY) or Synergy HT spectrophotometers (BIO-TEK Instruments INC., Winooski, VT) using BSA as standard (Smith et al., 1985). A protein standard curve was generated in each experiment and used to determine the protein content of each sample.

Statistical analysis of data. In all experiments, each condition included at least triplicate samples. All experiments were repeated two to six times with consistent results. Statistical analyses were performed using JMP 3.0.2 software (SAS Institute, Cary, NC). Initial descriptive statistics showed that the results for continuous variables were distributed normally. Therefore, the differences in mean values were assessed by ANOVA, with the Tukey-Kramer correction for multiple pair-wise comparisons. P values <0.05 were considered significant.

CHAPTER III

CHARACTERIZATION OF HEME BIOSYNTHETIC PATHWAY IN THE CHICKEN HEPATOMA CELL LINE, LMH AND CHICK EMBRYO LIVER CELLS (CELCS)*

Introduction

The purpose of the studies in this Chapter was to characterize the heme biosynthetic pathway and regulation of ALAS-1 (the rate-controlling enzyme for heme biosynthesis) in a continuous cell culture model, LMH, in comparison with chick embryo liver cells (CELCS). The inducibility and regulation of ALAS-1 have been investigated using a variety of models, including intact animals (Bonkowsky et al., 1973; Bonkowsky et al., 1978) and primary liver cell cultures (Cable et al., 1991; Russo et al., 1994). Chick embryo liver cell culture yields data similar to that observed in whole animals such as chick embryos (Ades and Stevens 1988; Hamilton et al., 1988), mice (Smith et al., 1989), rats (Marks et al., 1987) or man (Kappas et al., 1989; Bonkovsky et al., 1990). Thus, CELC are a widely used culture model that closely approximates what occurs *in vivo* and in humans (Healey

et al., 1981; Bonkovsky et al., 1985). Unlike rat hepatocytes (Bissel et al., 1980) CELCs, simply cultured on plastic dishes without special coatings or supplements to standard culture media, retain normal levels and inducibility of ALAS-1 mRNA and activity (Cable et al., 1991; Russo et al., 1994), CYP (Hamilton et al., 1988) and HO-1 (Cable et al., 1991). Therefore, CELCs are a powerful and convenient system for the study of heme metabolism *in vitro*. However, there are some limitations to the primary culture system: the cells obtained are somewhat heterogeneous; the isolation must be repeated every week, resulting in culture-to-culture variations and practical limitations on the number of culture dishes that can be employed in any given experiment. Even then, they are short-lived and eventually become overgrown by fibroblasts (O. Gildemeister, Unpublished observations), limiting the feasibility of time-course and transfection studies.

LMH cells are a chicken hepatoma cell line derived from a liver hepatocellular carcinoma induced by giving intra-peritoneal injections of diethyl nitrosamine to a male White Leghorn chicken (Kawaguchi et al., 1987). LMH cells, an immortal cell line, have several advantages over CELCs; continuous long-term culture, cell-type homogeneity, and a virtually

* Results published Reference # 71

endless supply. In LMH cells, the induction of HO-1 in response to treatments such as heme, transition metals, sodium arsenite, and heat shock was similar to that seen in CELCs (Gabis et al., 1996). It was also shown recently that LMH cells respond to a variety of drugs by induction of CYP2H1, a major drug-metabolizing enzyme in chick liver (Handschin et al., 2000).

The research presented in this Chapter characterizes the heme biosynthetic pathway and regulation of ALAS-1 in LMH cells and compares these results to those in CELCs. The purpose of these studies was to determine if LMH cells are as good a model as CELCs for studies of heme biosynthesis and regulation of ALAS-1.

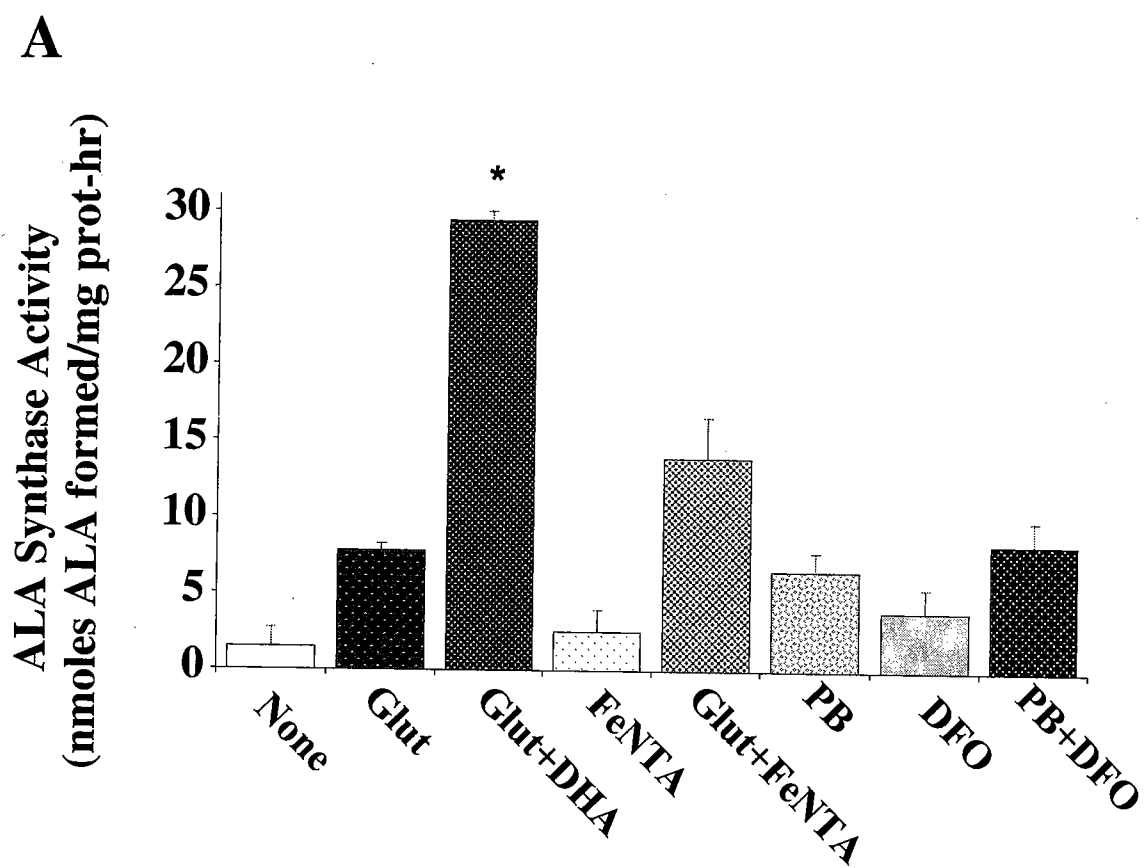
Results

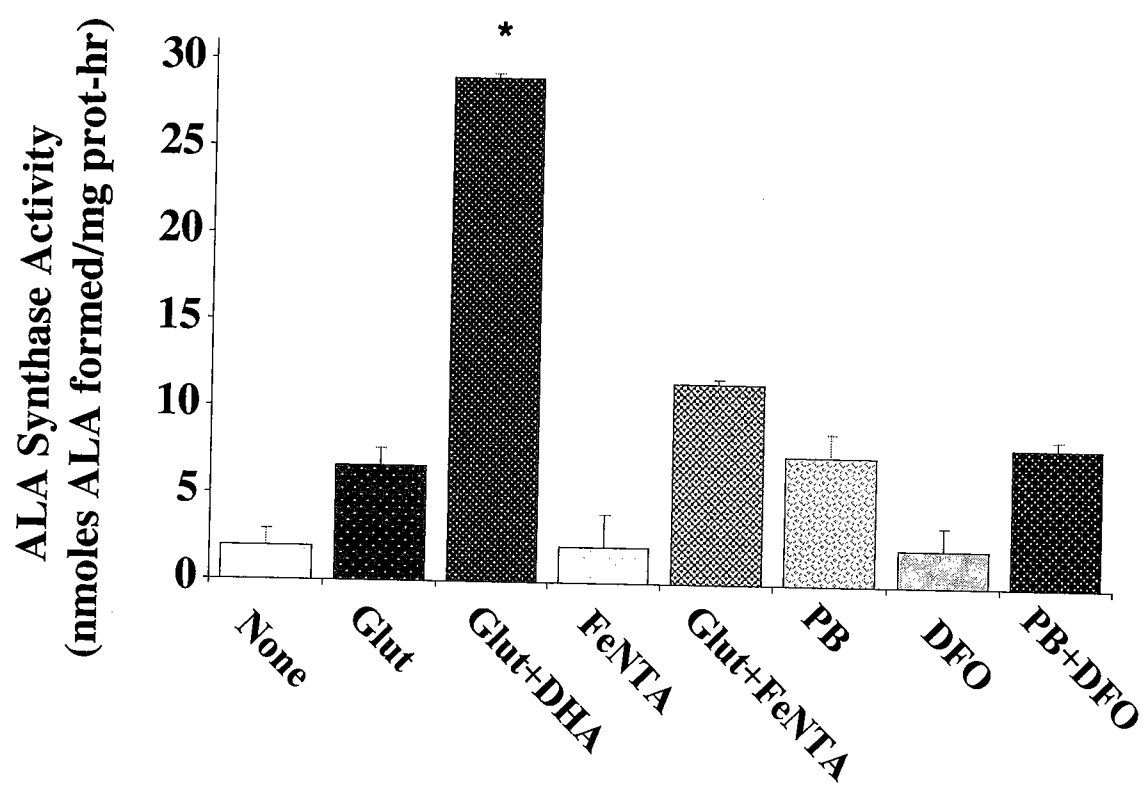
Comparative effects of selected chemicals that affect heme synthesis on ALAS activity in CELCs and LMH cells. It has been shown previously in CELCs that treatment with selected combinations of chemicals increases ALAS-1 activity and that elevated activity is maintained from 12 to 24 h after treatment (Hamilton et al., 1988; Lincoln et al., 1988; Cable et al., 1991; Russo et al., 1994). Hence, in this study, ALAS activity was measured in both CELCs and LMH cells after 18 h of exposure of cells to treatments. All of the selected treatments increased ALAS activity to varying degrees (Figures 3.1 A and B). Phenobarbital (PB) and glutethimide (Glut), either alone or in combination with 4,6-dioxoheptanoic acid (DHA) or deferoxamine (DFO), increased ALAS activity several fold over control. Among the combinations tested, the combination of Glut and DHA was more effective ($P < 0.001$) at increasing ALAS activity than the combinations of Glut and ferric nitrilotriacetate (FeNTA) or PB and DFO in both CELCs and LMH cells (Figures 3.1 A and B).

It was found in CELCs that, by making several modifications in the assay itself (freeze-thaw to facilitate cellular lysis) and by treating them with

Figure 3.1. Comparative effects of selected chemicals that affect heme biosynthesis on ALAS enzyme activity in CELCs (A) and LMH cells (B).

CELCs and LMH cells were treated with chemicals and maintained at 37°C for 18 h, after which cells were harvested, sonicates prepared and used to measure ALAS enzyme activity as described in Chapter II. Concentrations of the selected chemicals were as follows: Glutethimide (Glut) - 50 μ M; ferric nitrilotriacetate (FeNTA) - 50 μ M; 4,6 dioxoheptanoic acid (DHA) - 250 μ M; phenobarbital (PB) - 800 μ M; and deferoxamine (DFO) - 250 μ M in CELCs and 500 μ M in LMH cells, respectively. As shown in Figures 3.2 and 3.7, 250 μ M DHA alone had no significant effect on ALAS activity in CELCs and LMH cells. Data represent means + SE from 3-5 independent experiments in triplicate. *Significant increase vs. none, Glut alone $P < 0.001$.



B

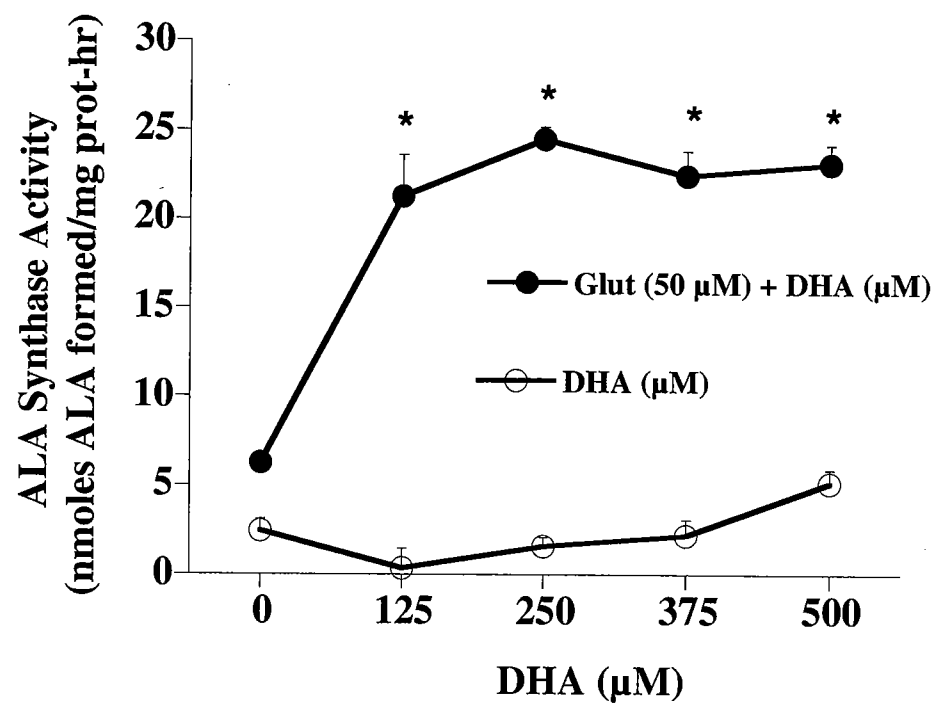
the selected chemicals 24-36 h (rather than 36-48 h) after isolation, we were able to achieve even higher ALAS activity after Glut and DHA treatment (Figure 3.1 A) than those previously achieved (Russo et al., 1994). Hence in further experiments these procedures for treatment and cell lysis were routinely used.

Effect of increasing concentrations of DHA on ALAS activity in CELCs or LMH cells either untreated or treated with Glut: The combination of Glut (50 μ M) and DHA (0-500 μ M) synergistically increased ALAS activity in both CELCs and LMH cells compared with the effect of either Glut or DHA alone (Figure 3.2). The DHA concentration that gave maximal increase in ALAS activity in combination with Glut (50 μ M) was 250 μ M. Hence in further experiments these were the concentrations of Glut (50 μ M) and DHA (250 μ M) used.

Effect of selected chemicals on level of ALAS-1 mRNA in CELCs and LMH cells. With the exception of FeNTA alone in CELCs, each of the selected chemicals increased the levels of ALAS-1 mRNA (Figure 3.3). The fold increases in ALAS-1 mRNA after 6 h of exposure to the combinations tested, were not as large as those obtained for ALAS activities after 18 h

Figure 3.2. Effect of increasing concentrations of DHA on ALAS enzyme activity in CELCs (A) or LMH cells (B) left untreated or treated with glutethimide. CELCs and LMH cells were either left untreated or treated with increasing concentrations of DHA (0, 125, 250, 375 or 500 μM) and/or Glut (0 or 50 μM) for 18 h prior to harvest. Cells were then harvested, sonicates prepared and used to measure ALAS activity as described in Chapter II. Data represent means + SE from 3-5 independent experiments in triplicate. *Significant increase vs. none, Glut alone, or DHA alone $P < 0.001$. The EC_{50} was calculated to be 106.6 μM Glut for A ($R^2 = 0.990$), and 143.512 μM Glut for B ($R^2 = 0.982$) by Non-linear Regression analysis using the Hill, 4 Parameter method.

A



B

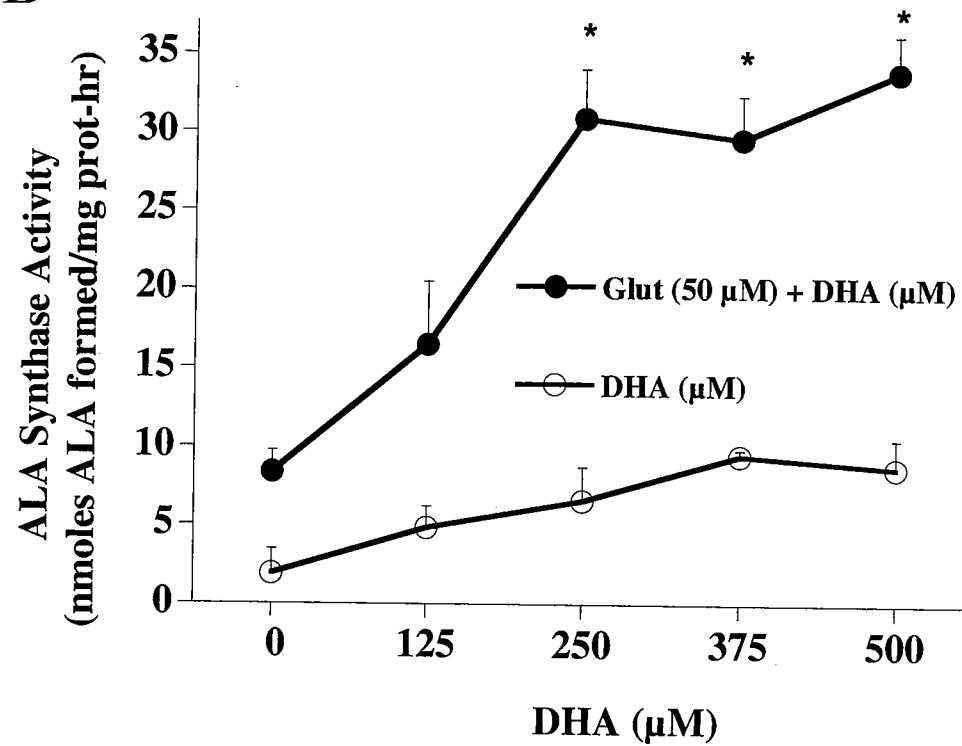
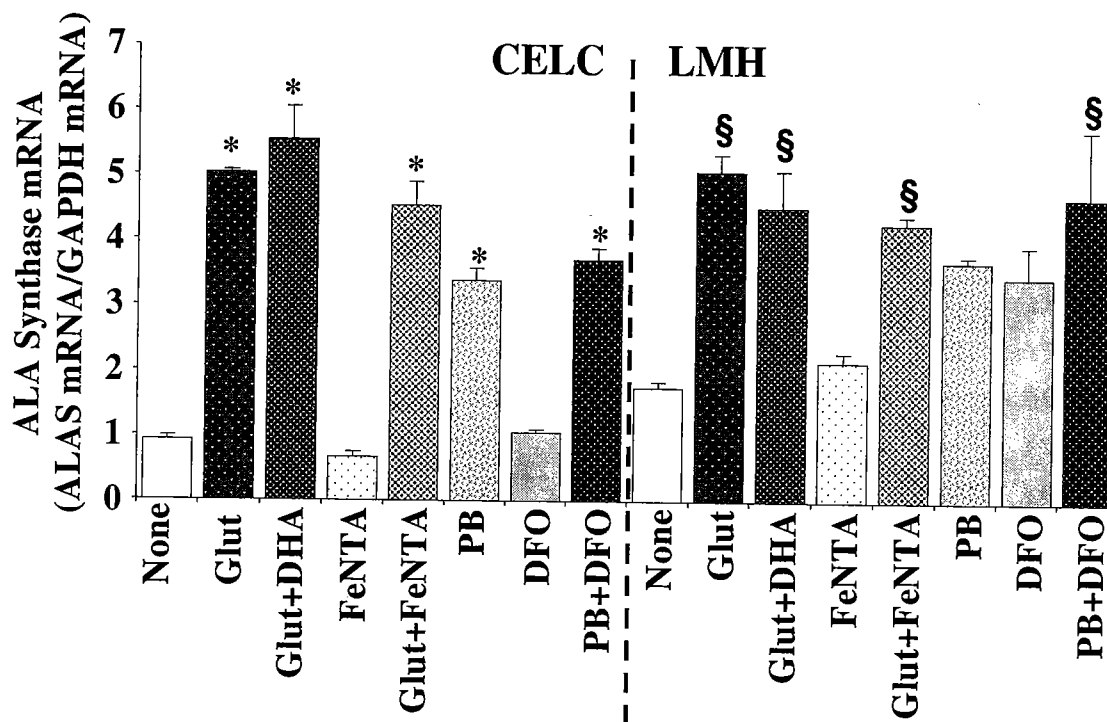


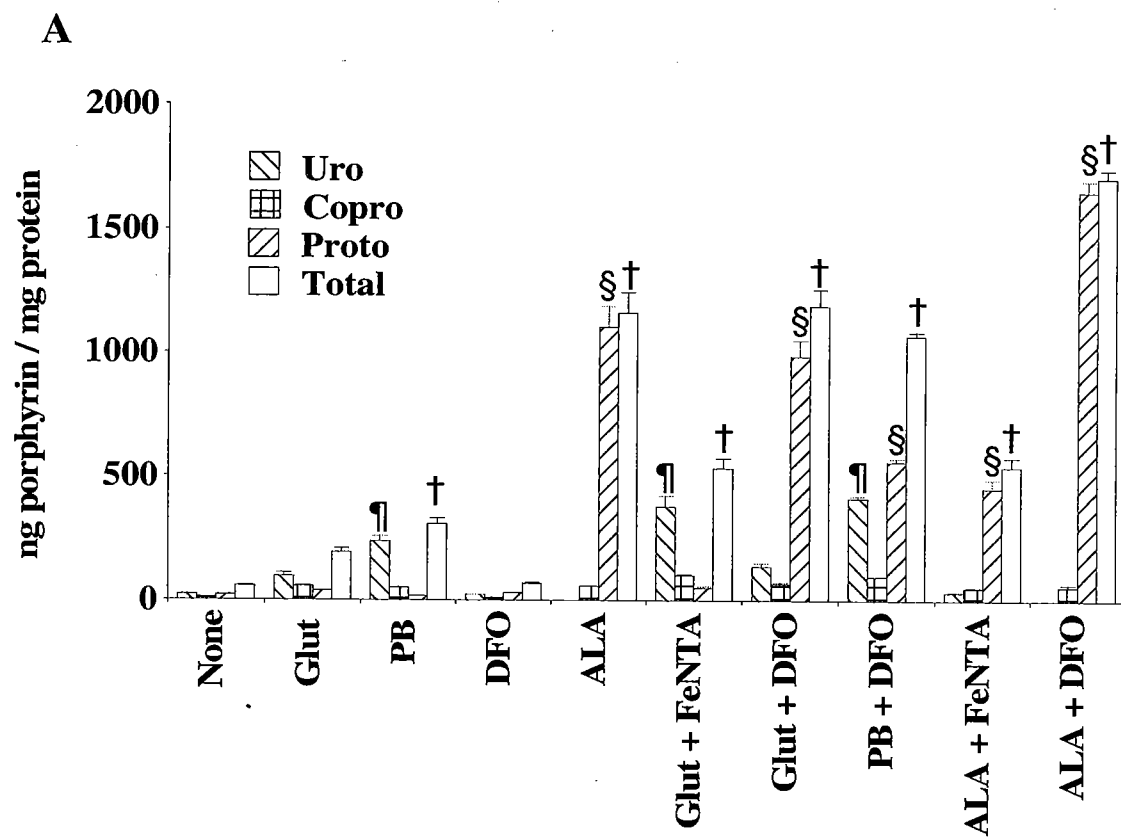
Figure 3.3. Effect of selected chemicals on expression of ALAS-1 mRNA in CELCs and LMH cells. CELCs and LMH cells were treated with chemicals that affect heme biosynthesis for 6 h prior to harvest. Total RNA was isolated and specific mRNAs detected by dot blots as described in Chapter II. Chemical concentrations were as indicated in legend to Figure 3.1. The amount of ALAS-1 mRNA was normalized to GAPDH mRNA, which was shown previously to remain constant under the conditions (Cable et al., 1994). Results are mean + SE from 3-5 independent experiments done in triplicate. * Significant increase vs. none $P < 0.001$. § Significant increase vs. none $P < 0.05$.

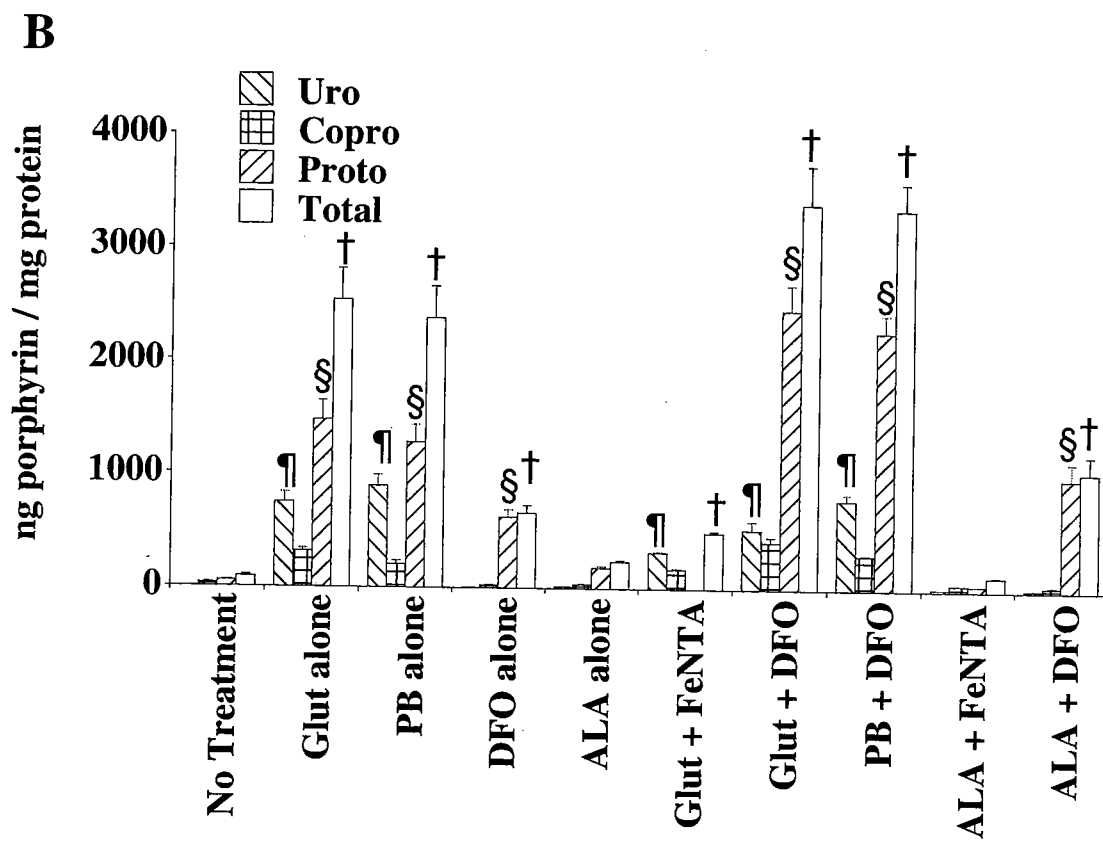


of treatment with the same combinations (Figures 3.1 A and B). In contrast to that seen for enzyme activities, effects of Glut in combination with FeNTA or DHA on ALAS-1 mRNA levels were not significantly different from the effects of Glut alone. This is in keeping with previous studies (Hamilton et al., 1988; Russo et al., 1994) which showed that the synergistic induction of ALAS-1 requires prolonged treatment (12-18 h) with the inducers (Glut and DHA) (Russo et al., 1994).

Porphyrin accumulation in CELCs and LMH cells in response to selected chemicals that affect heme synthesis. Barbiturates like PB and barbiturate-like compounds, such as Glut, increase the levels of ALAS-1 mRNA in CELCs (Hamilton et al., 1988; Jover et al., 1996) (Figure 3.3). The effect of these chemicals alone or in combination with DFO and FeNTA on porphyrin accumulation was tested in LMH cells and CELCs. As previously described (Bonkovsky 1989), Glut or PB alone led to accumulation of porphyrins in CELCs (Figure 3.4 A), indicating that the uninhibited pathway is able to convert most of the intermediates – ALA, PBG, and the porphyrinogens, into heme (Figure 1.1). PB or Glut in combination with DFO which inhibits normal heme biosynthesis by chelating iron (Figure 1.1), resulted in an accumulation of porphyrins, a major proportion ($\sim 1/2$) of which was

Figure 3.4. Porphyrin accumulation in CELCs (A) or LMH cells (B) in response to selected chemicals that affect heme biosynthesis. CELCs and LMH cells were treated with the different chemicals for 18 h prior to harvest. Cells were harvested, sonicates prepared, porphyrins extracted using PCA:MeOH and assayed by the method of Grandchamp et al., as described in Chapter II. The concentrations of the chemicals used were as described in Figure 3.1, except the concentration of ALA was 35 μ M. Data are presented as nanogram (ng) porphyrin / mg protein. Data represent means + SE from 3-5 independent experiments done in triplicate. § Significant increase in protoporphyrin vs. none $P < 0.001$. † Significant increase in total porphyrin vs. none $P < 0.001$. ¶ Significant increase in uroporphyrin vs. none $P < 0.001$.



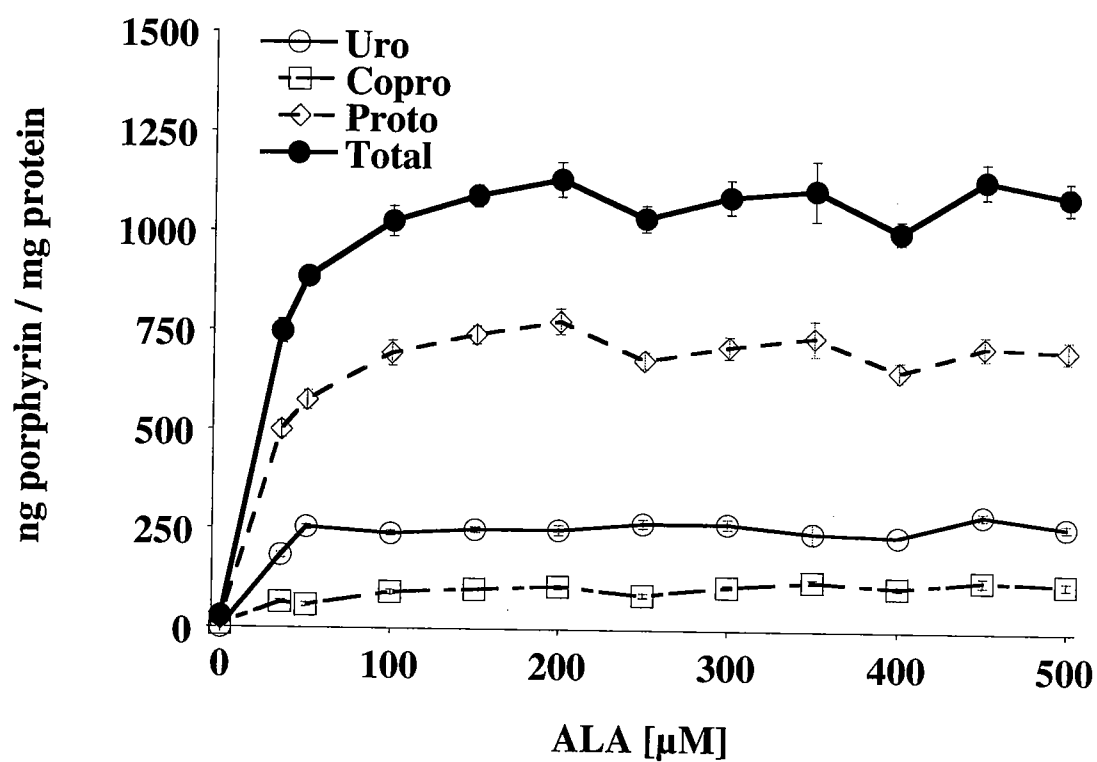


protoporphyrin (Figures 3.4 A and B). ALA alone at 35 μ M, markedly increased porphyrin accumulation in primary CELCs (Figure 3.4 A), but had relatively little effect in LMH cells (Figure 3.4 B). In contrast, DFO alone did not have much effect in CELCs but the combination of ALA or an inducer of ALAS-1 such as, Glut or PB, with DFO, led to significant accumulations of protoporphyrin (Figure 3.4 A). Glut or PB alone produced an increase in porphyrin accumulation that was greater in LMH cells than in CELCs (Figures 3.4 A and B). When DFO was added in combination with Glut or PB, a further increase in total porphyrin, mainly protoporphyrin, was observed in LMH cells as in CELCs (Figures 3.4 A and B). In preliminary dose-response studies, we found that 250 μ M DFO was sufficient to produce near maximal effect in CELCs, while LMH cells required DFO at 500 μ M to produce the same effect. Thus, these concentrations of DFO were used.

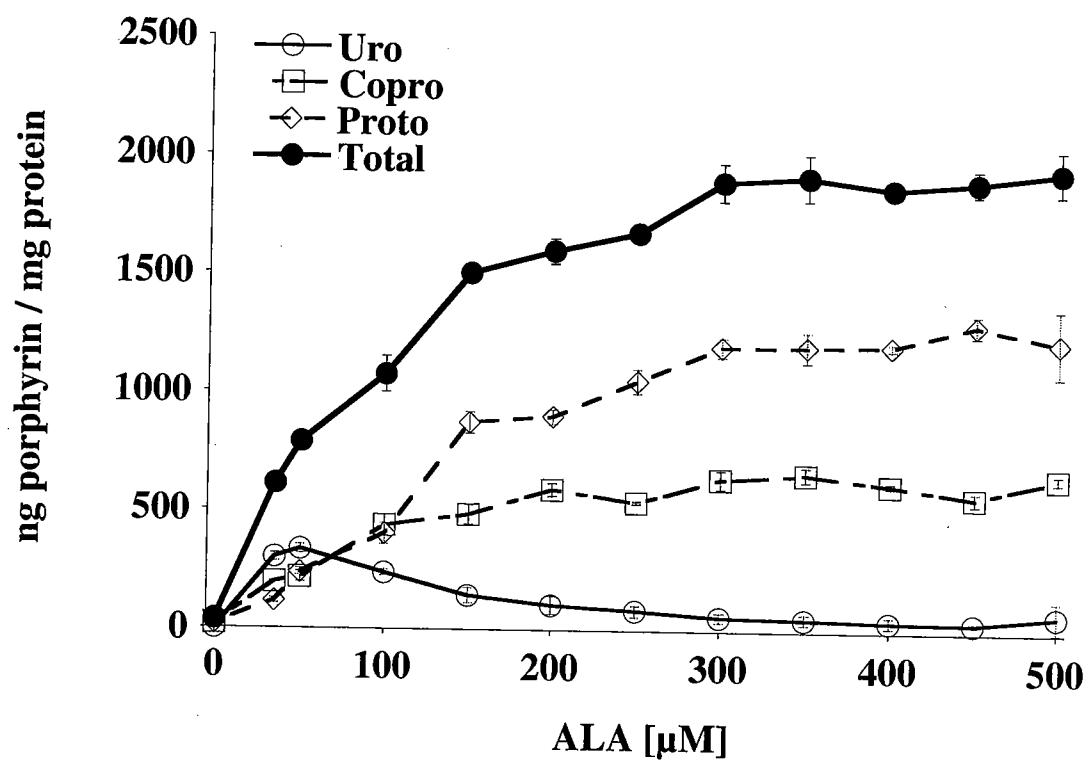
ALA dose-response. In order to determine why 35 μ M ALA did not lead to accumulations of porphyrins in LMH cells to the same extent as did in CELCs, a broader range of concentrations of ALA (0-500 μ M) were studied. We found that porphyrins in LMH cells increased with increasing ALA concentration, reaching a maximal response at 350 μ M (Figure 3.5 B),

Figure 3.5. Effect of increasing concentrations of ALA on porphyrin accumulation in CELCs (A) or LMH cells (B). CELCs and LMH cells were either left untreated or treated with increasing concentrations of δ -aminolevulinic acid (ALA; 0, 35, 50, 100, 150, 200, 250, 300, 350, 400, 450 or 500 μ M) for 18 h prior to harvest. Cells were harvested, sonicates prepared, and porphyrins extracted using PCA:MeOH and assayed by the method of Grandchamp et al., as described in Chapter II. Data are presented as ng porphyrin/mg protein. Data represent means \pm SE from 3-5 experiments in triplicate. The EC_{50} was calculated to be 24.9 μ M ALA for A ($R^2 = 0.985$), and 93.6 μ M ALA for B ($R^2 = 0.992$) by Non-linear Regression analysis using the Hill, 4 Parameter method.

A



B



whereas CELCs showed increased porphyrin accumulation with increasing concentrations of ALA up to a concentration of 100 μ M, after which a plateau was reached (Figure 3.5 A).

Effect of heme on basal ALAS-1 mRNA. Since heme-dependent repression of ALAS-1 is a major component of the normal regulation of heme biosynthesis in CELCs (Cable et al., 1991; Russo et al., 1994), we tested if heme repression of ALAS-1 mRNA occurs in LMH cells as well. In the presence of heme (20 μ M, 6 hour exposure), the uninduced ALAS-1 mRNA was decreased in both CELCs (28% of control, $P < 0.001$) and LMH cells (64% of control, $P < 0.001$) (Figure 3.6).

Effect of heme on induced-ALAS activity. In LMH cells the ALAS activity that had been increased by pretreatment with Glut and DHA or FeNTA for 12 h was decreased (to 68% of control, $P < 0.01$ and to 55% of control $P < 0.05$ respectively) after exposure to 20 μ M heme for 6 h (Figure 3.7).

Figure 3.6. Effect of heme on basal ALAS-1 mRNA. Cultures of CELCs and LMH cells were either left untreated or treated with 20 μ M heme for 6 h and total RNA was harvested. Harvest, isolation, detection and quantitation of RNA were performed as described in Chapter II. Data represent means + SE from 3-5 experiments in triplicate. ¶ Significant decrease from all others $P < 0.01$. ** Significantly different from untreated LMH cells, and from heme-treated CELCs $P < 0.01$.

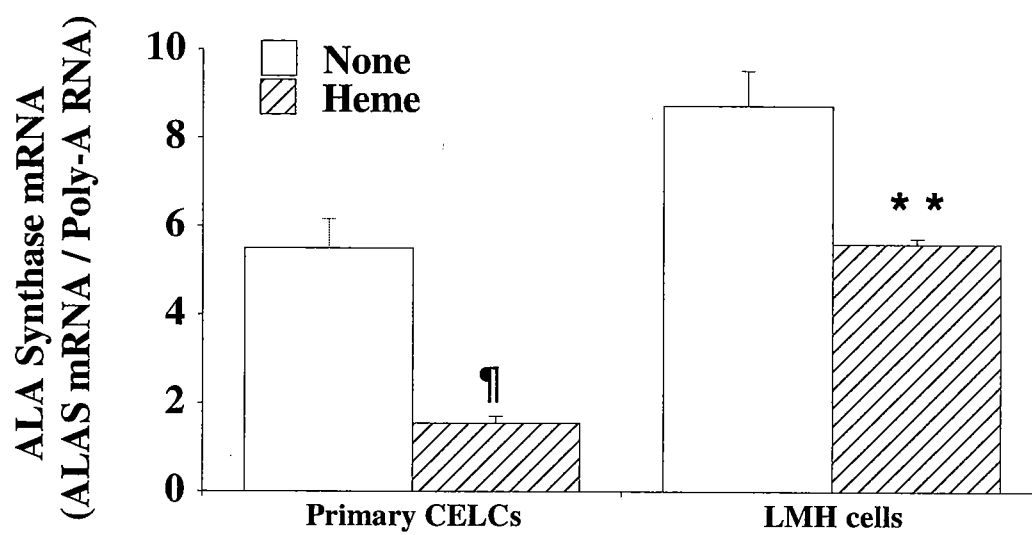
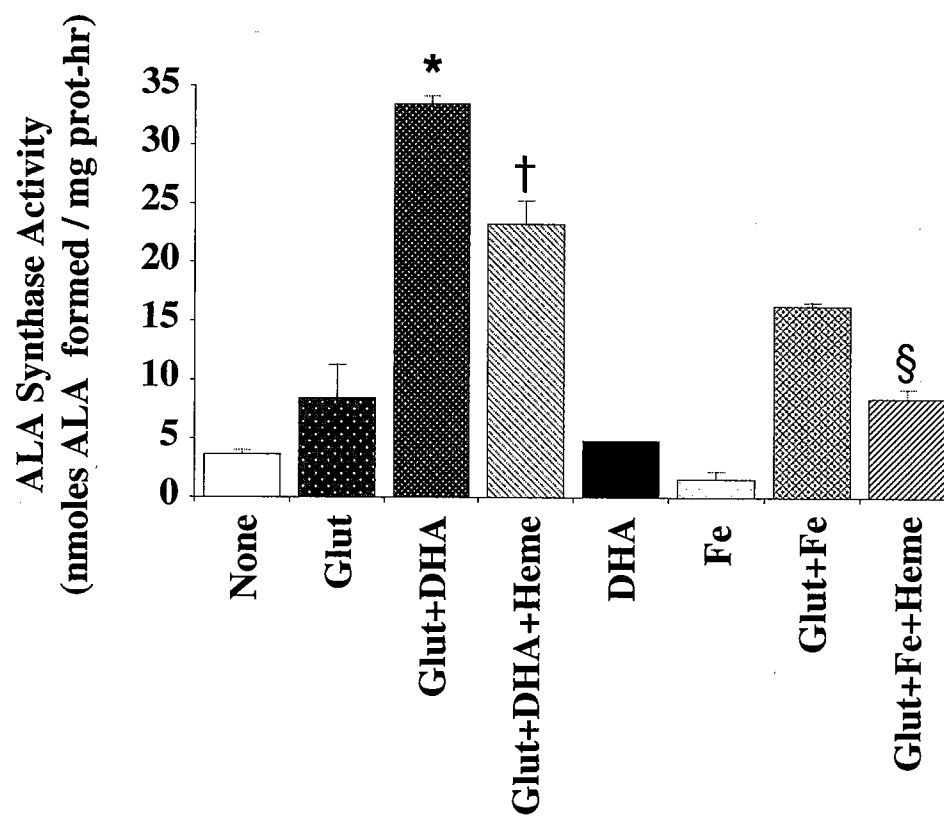


Figure 3.7. Effect of heme on induced-ALAS activity. LMH cells were maintained at 37 °C for 12 h under the following inducing conditions: untreated, glutethimide (Glut, 50 μ M) alone or in combination with either ferric nitrilotriacetic acid (FeNTA, 50 μ M) or 4,6-dioxoheptanoic acid (DHA, 250 μ M). LMH cells were then treated with 20 μ M heme for 6 h. The cells were harvested, sonicated, and assayed as described in Chapter II. Data represent means \pm SE from 3-5 experiments in triplicate. *Significant increase vs. Glut alone, DHA alone $P < 0.001$. † Significantly different from treatment without heme ($P < 0.01$). § Significantly different from treatment without heme ($P < 0.05$).



Conclusions

The utility of LMH cells as a model system for the study of hepatic heme biosynthesis and regulation of ALAS-1 has been demonstrated. LMH cells exhibited similar responses to CELCs with respect to induction of ALAS-1 mRNA and enzyme activity in response to selected chemicals that affect heme biosynthesis. The absolute levels of ALAS-1 mRNA and activity were similar in LMH cells and CELCs. Porphyrin accumulation and overproduction were seen in response to treatment with chemicals that are routinely used in models of porphyria. LMH cells displayed a synergistic induction of ALAS activities in response to Glut and DHA. Synergistic effects of the combination of Glut and DHA to increase ALAS activity might be explained by their effects on heme biosynthesis, by a direct effect of these chemicals on expression of ALAS gene or by a combination of both. Thus, LMH cells provide a useful system for further investigation of the mechanism by which this occurs.

Heme-dependent regulation of ALAS-1 mRNA levels was seen for both CELCs and LMH cells in the absence of any inducers. In addition, as previously shown by Russo et al., 1994, in CELCs, heme decreased the Glut and DHA-induced ALAS-1 activity in LMH cells.

The major difference between primary CELCs and LMH cells was in the amount (ng) of porphyrins accumulated in response to the inducers of ALAS-1 and ALA. LMH cells needed higher concentrations of ALA, than CELCs, to accumulate the same amount of porphyrin. Different sensitivity to ALA may be due to less avid and/or less rapid uptake of ALA into LMH cells than into CELCs. Requirements for higher concentrations of ALA have been well described in a human hepatoma cell line (HepG2) (Iwasa et al., 1989), in rat hepatocytes (Sinclair et al., 1990) and in embryonic stem cells (Harigae et al., 1998). Whether these differences may also be due to differences in growth rate and metabolism of the given cell types is not known. LMH cells are a rapidly growing tumor-derived cell line with a doubling time of approximately 21 h, whereas CELCs under our conditions of culture are essentially non-proliferating and eventually are overgrown by fibroblasts (O Gildemeister, unpublished observations).

Drugs such as PB and Glut increased ALAS-1 mRNA levels and activities particularly Glut in combination with the inhibitor of heme synthesis, DHA, synergistically increased ALAS activity in LMH cells as in CELCs. Heme (20 μ M) decreased the Glut and DHA-induced ALAS

enzyme activity and mRNA levels in LMH cells as shown previously in CELCs (Hamilton et al., 1988; Russo et al., 1994).

Previous studies in intact rats and CELCs examining the drug induction of ALAS-1 gene expression have been conflicting about the effect of "the regulatory heme pool" on ALAS-1 gene expression vs. mRNA stability (Hamilton et al., 1988; Srivastava et al., 1988; Srivastava et al., 1990). Since LMH cells show characteristics that are very similar to primary CELCs, they provide a useful model system for further studies of the regulation of hepatic ALAS-1 expression by drugs, heme and glucose.

CHAPTER IV

EFFECT OF GLUCOSE AND OTHER METABOLIZABLE SUGARS ON ALAS-1 IN LMH CELLS

Introduction

The chick hepatoma cell line, LMH, and CELCs were used to characterize the effects of glucose and/or its metabolites on the drug induction of ALAS-1. In Chapter III it was demonstrated that LMH cells display normal induction of ALAS-1, accumulation of porphyrins in response to chemicals, and show heme-dependent repression of ALAS-1 mRNA and activity (Kolluri et al., 1999). In addition, treatment of LMH cells with DHA in combination with the drug, Glut, resulted in a synergistic increase in ALAS-1 activity. Therefore, LMH cell cultures yield data (Gabis et al., 1996; Kolluri et al., 1999; Fraser et al., 2002; Fraser et al., 2003) similar to that observed in CELCs (Bonkovsky et al., 1992; Russo et al., 1994; Jover et al., 1996; Hahn et al., 1997) and are therefore a simple, reproducible system for exploring hepatic heme and porphyrin metabolism.

The 'inducible' hepatic porphyrias - acute intermittent porphyria, hereditary coproporphyria and variegate porphyria- are disorders of hepatic

heme biosynthesis caused by inherited partial deficiencies of three specific enzymes of this pathway. They are collectively called “inducible” hepatic porphyrias because “acute” attacks are often precipitated by drugs and are associated with considerable increase in activity of ALAS-1 (Granick and Urata, 1963; Tschudy et al., 1965; Perlroth et al., 1968; Hahn and Bonkovsky, 1998) with enhanced hepatic synthesis and excretion of heme precursors such as ALA, PBG and porphyrins.

It has been known for a long time that administration of large amounts of glucose to patients with hepatic porphyrias induced by drugs can produce significant clinical improvement of human acute intermittent porphyria (Welland et al., 1964) and variegate porphyria (Perlroth et al., 1968), and that dietary and hormonal factors play an important role in the drug precipitation of porphyric attacks (For recent reviews see Bonkovsky 2000; Sassa et al., 2002).

Most of the earlier studies investigating the “glucose effect” on heme synthesis were done on intact animals that were administered porphyrinogenic compounds (Bonkovsky et al., 1973; DeLoskey and Beattie, 1984). One difficulty with defining the events responsible for the glucose effect in intact animals is separating those events attributable

directly to glucose and those events resulting from changes in hormonal balance within the animal. Cells in culture therefore, are useful for deciphering the contributions that specific nutrients vs. hormones make to overall regulation of gene expression.

In 1981, Giger and Meyer showed in CELCs, as had been previously demonstrated in intact animals (Bonkovsky et al., 1973), that fructose, glycerol, and lactate, mimicked the repressive effect of glucose on ALAS activity (Giger and Meyer, 1981). However, CELCs in this study were isolated in Williams' E medium containing 1.5 μ M T3 and this hormone was present in the medium throughout the experiments on the glucose effect. Hence, a possible effect of T3 could not be excluded in the work of Giger and Meyer.

Given that these earlier studies assessed the effects of glucose on ALAS enzymatic activities it is also not known whether the effects of glucose and/or its metabolite(s) on ALAS-1 are mediated at the level of gene transcription. To begin to address some of these unanswered questions, I took advantage of the fact that LMH cells proved to be a good model for the study of chemically induced experimental porphyria (Chapter III). I performed preliminary experiments firstly to establish a "glucose effect" on

ALAS-1 *in vitro* in LMH cells, in the absence of any factors such as serum and hormones, and secondly to determine whether the effects of glucose and/or its metabolite (s) on ALAS-1 are mediated at the level of gene transcription.

Summary of the "glucose effect": Despite extensive studies of chemically-induced experimental porphyria in animals and in cell cultures, it is not known whether the effect(s) of glucose and/or its metabolite(s) on ALAS-1 occur in the absence of hormones and at the level of gene transcription. In addition, the molecular mechanism(s) by which glucose and/or its metabolites regulate ALAS-1 have not been elucidated. Such data may be relevant to understanding of ALAS-1 gene regulation by glucose in patients with 'inducible' hepatic porphyrias.

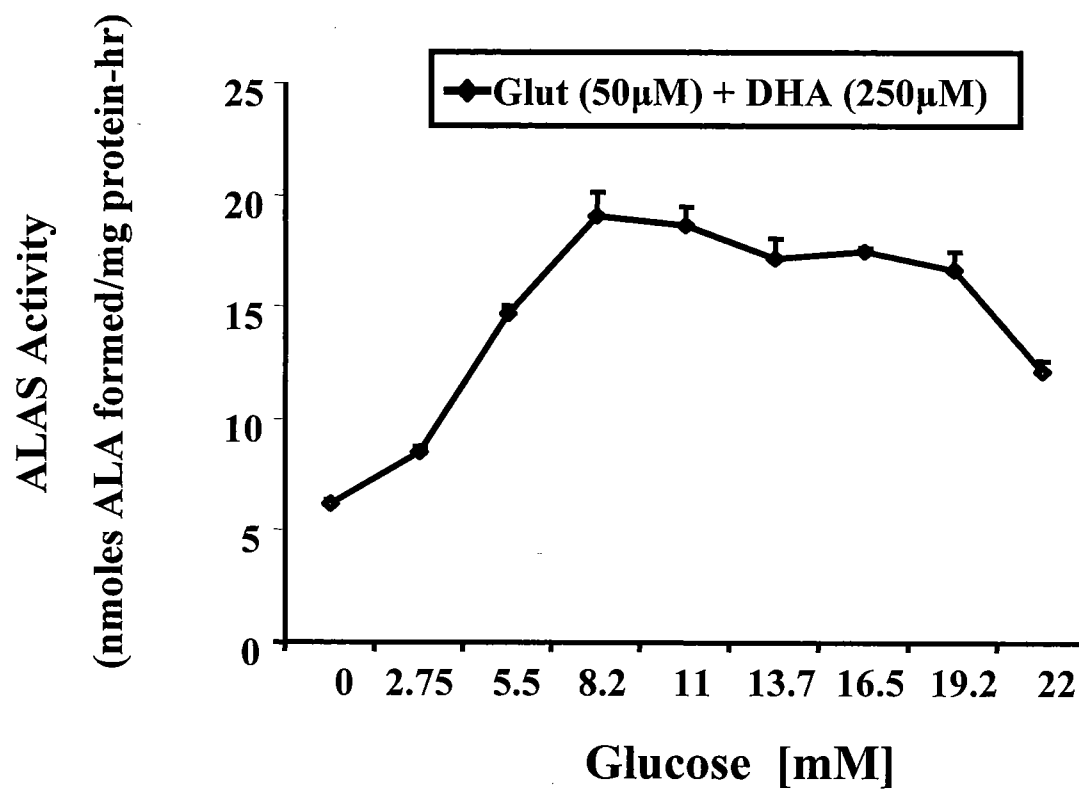
Results

Induction of ALAS activity by selected chemicals under different glucose concentrations in LMH cells. We first wanted to determine the optimum glucose concentration for induction by Glut and DHA. For this purpose, cells grown in glucose- and serum-free Williams' E for 24 h were incubated with Williams' E medium containing glucose (mM) as indicated, for 2 h, and then treated with 50 μ M Glut and 250 μ M DHA. After 16 h of treatment with Glut and DHA, cells were harvested and ALAS activity measured as described in Chapter II. Cells in Williams' E medium containing 2.75 mM glucose did not induce ALAS activity and cells in 5.5 mM glucose induced ALAS activity to a lesser extent in response to Glut and DHA, than those in Williams' E containing 8.25 – 19.25 mM glucose which gave optimal fold induction over control (4.5 fold) (Figure 4.1). Cells in 22 mM glucose had lower Glut and DHA-induced of ALAS-1 activity as compared to cells in 8-19 mM glucose (Figure 4.1).

Effect of increasing concentration of glucose on Glut- and DHA-induction of ALAS activity in LMH cells. Since cells in 5.5-19.25 mM glucose increased ALAS activity several fold over control, 5-11 mM

Figure 4.1. Dose response curve of the amount of glucose required for the optimal induction of ALAS activity by Glut and DHA in LMH cells.

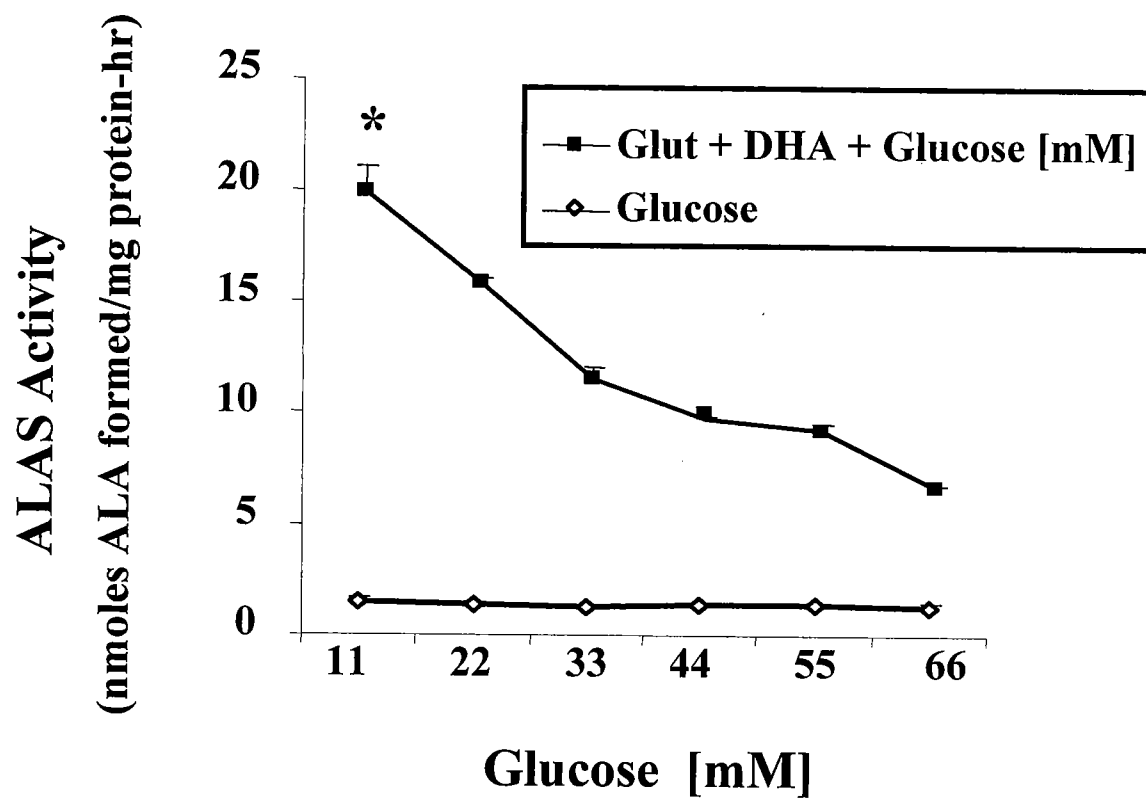
LMH cells grown in glucose and serum-free Williams' E medium for 24 h were changed to Williams' E medium containing 0, 2.75, 5.5, 8.25, 11, 13.75, 16.5, 19.25 or 22 mM glucose for 2 h, and then treated with 50 μ M Glut and 250 μ M DHA. Cells were treated with selected chemicals for 16 h, after which cells were harvested, sonicates prepared and used to measure ALAS activity as described in Chapter II. Concentrations of the chemicals were as follows: Glut, 50 μ M, and DHA, 250 μ M. Data are presented as nanomoles ALA formed/mg protein-hr. Data represent means + SE from 3-5 independent experiments in triplicate. Basal ALAS activity in William's E medium without glucose for untreated samples was 6.32 ± 0.07 nanomoles ALA formed/mg protein-hr.



glucose was used as a standard glucose concentration for induction of ALAS activity in LMH cells. Since regular Waymouth's medium contains 11 mM glucose and regular Williams' E medium 5.5 mM glucose (Appendix A), further experiments testing the "glucose effect" on ALAS-1 were performed on LMH cells in regular Waymouth's medium. The ability of higher concentrations of glucose (> 11 mM) to decrease Glut- and DHA-induced ALAS activity was tested by addition of 11-55 mM glucose to Waymouth's medium to give effective glucose concentrations of 22, 33, 44, 55 and 66 mM, respectively. Glucose concentrations of 22-66 mM produced a dose-dependent decrease in the induction obtained by Glut and DHA, compared to that obtained by Glut and DHA in the presence of 11 mM glucose (Figure 4.2). The basal ALAS activity in regular Waymouth's medium for untreated samples was 1.2 ± 0.017 nmoles ALA formed/mg prot-hr (Figure 4.2) and was 19% lower than that obtained in Williams' E special medium (6.32 ± 0.07 nmoles ALA formed/mg prot-hr) without glucose or sodium pyruvate (Figure 4.1).

Effect of increasing concentrations of sorbitol on Glut- and DHA-induction of ALAS activity. Sorbitol is a non-metabolizable monosaccharide whose molecular weight is very close to that of glucose (182.2 vs. 180.2 for

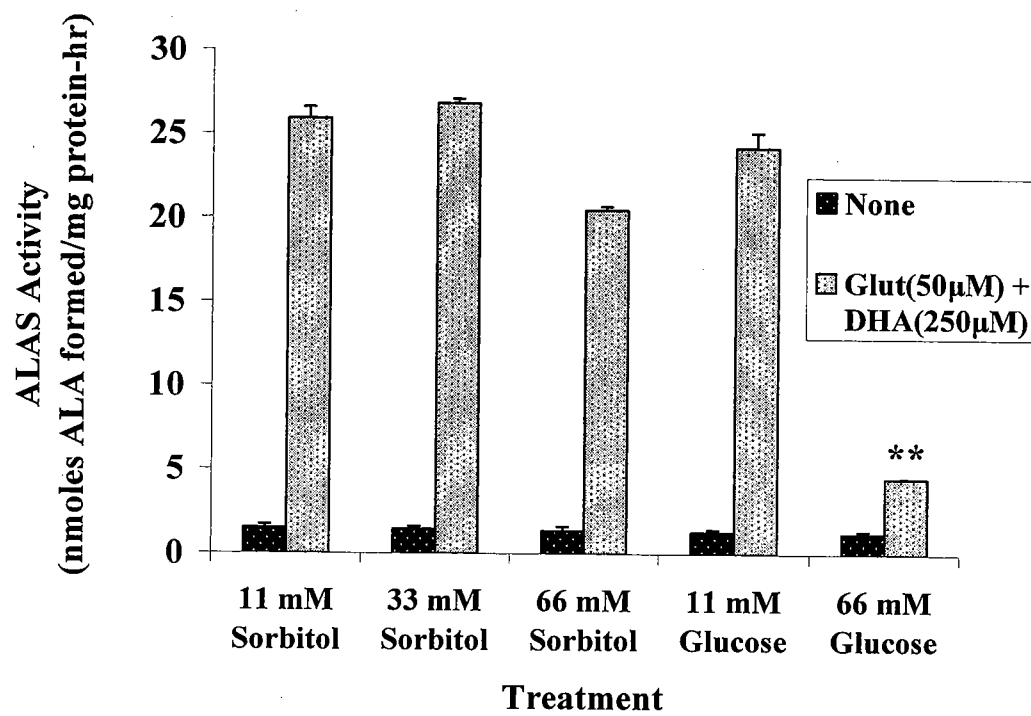
Figure 4.2. Dose response curve of the effect of glucose on the Glut- and DHA-mediated induction of ALAS activity in LMH cells. Effect of increasing concentrations of glucose on ALAS activity in LMH cells treated with 50 μ M Glut and 250 μ M DHA in regular Waymouth's medium containing 11 mM glucose. LMH cells were then treated with increasing concentrations of glucose (11, 22, 33, 44, or 55 mM) to give effective glucose concentrations of 22, 33, 44, 55 and 66 mM respectively for 2 h before treatment with 50 μ M Glut and 250 μ M DHA for 16 h prior to harvest. Cells were then harvested, sonicates prepared and used to measure ALAS activity as described in Chapter II. Data are presented as nanomoles ALA formed/mg protein-hr. Data are expressed as means + SE from 3-5 independent experiments in triplicate. Basal ALAS activity in Waymouth's medium for untreated samples was 1.252 ± 0.017 nanomoles ALA formed/mg protein-hr. *Significantly increase from treatment with Glucose alone or Glucose (33-66 mM) in the presence of Glut and DHA, ($P < 0.01$). The IC_{50} was calculated to be 29 μ M Glucose by Non-linear Regression analysis ($R^2 = 0.986$) using the Hill, 4 Parameter method.



D-glucose). Sorbitol is taken up easily by liver cells though to a lesser extent than glucose. LMH cells in Waymouth's medium without serum, were either left untreated or treated with 50 μ M Glut and 250 μ M DHA in the presence of selected amounts of glucose or sorbitol. Sorbitol (11, 33, or 66 mM) did not decrease the induction of ALAS obtained by Glut and DHA, which was similar to that obtained in 11 mM Glucose (regular Waymouth's medium) (Figure 4.3). Glucose (66 mM), however, did decrease this induction and served as a positive control for the glucose effect (Figure 4.3). From this experiment, it was concluded that the effect of glucose on ALAS activity is specific for glucose and is not due to an increase in tonicity (solute content) of the medium due to addition of large amounts of sugar.

Effect of glucose on Glut- and FeNTA-induced ALAS activity. The next experiment tested whether the effect of glucose to alleviate the Glut and DHA-induction was also true for induction by other chemical combinations. Glut and FeNTA (Fe) have been shown to induce ALAS-1 by inducing the normal mechanism for heme degradation via HO-1 (see Chapter I, Chemicals that induce ALAS-1 by heme degradation) (Cable et al., 1990; Cable et al., 1991). Treatment with Glut and Fe did not induce ALAS in special Williams' E without glucose as observed for by Glut and DHA

Figure 4.3. Effect of increasing concentrations of sorbitol on Glut- and DHA-mediated induction of ALAS activity in LMH cells. LMH cells were grown in regular Waymouth's medium, treated with selected carbohydrates and chemicals that affect heme biosynthesis, and assayed for ALAS activity as described in Chapter II. Glut and DHA concentrations were as indicated in legend to Figure 4.1. Data are presented as nanomoles ALA formed/mg protein-hr. Results are means + SE from 3-5 experiments in triplicate. **Significant decrease from treatment with Glut and DHA in the presence of Sorbitol (11, 33, or 66 mM) or Glucose (11 mM) ($P < 0.001$).



(Figure 4.4 vs. Figure 4.1). However, Glut and Fe in the presence of 11 mM glucose induced ALAS 6-fold and higher glucose concentrations (22 and 33 mM) gave inductions of 3 and 1-fold respectively thereby decreasing the Glut- and Fe-induction of ALAS in a dose-dependent manner (Figure 4.4).

Effect of glucose on Glut- and DHA- induced ALAS activity in CELCs.

CELCs were isolated and maintained in regular Williams' E medium and changed to Williams' E medium without glucose or pyruvate or T3, 24 h before treatment. Cells were then incubated for 2 h with 0, 11 or 33 mM glucose before treating with 50 μ M Glut and 250 μ M DHA or vehicle alone for 16 h. Treatment with Glut and DHA induced ALAS activity several fold over controls at 0 and 11 mM glucose, but glucose at 33 mM decreased this induction in CELCs (Figure 4.5) as seen before for LMH cells (Figure 4.2).

Effect of glucose on Glut- and DHA-mediated induction of pGcALAS9.1-

Luc in LMH cells. To determine whether the effect of glucose on ALAS-1 is mediated at the level of transcription, Glut and DHA induction and the effect of glucose were tested on, pGcALAS9.1-Luc, a construct containing ~ 9.1 kb of 5'-flanking region of chick ALAS-1 gene attached to a luciferase (reporter) gene. PGcALAS9.1-Luc was transiently transfected into

Figure 4.4. Effect of glucose on Glut and Fe-mediated induction of ALAS in LMH cells. LMH cells in glucose- and pyruvate-free Williams' E for 24 h were changed to Williams' E medium containing 0, 11, 22, or 33 mM glucose for 2 h, and then treated with Glut and FeNTA (Fe) for 16 h. Cells were harvested, sonicates prepared, and assayed for ALAS activity as described in Chapter II. The concentrations of the chemicals used were Glut, 50 μ M, and Fe, 50 μ M. Data are presented as nanomoles ALA formed/mg protein-hr. Data are expressed as means + SE of 3-5 independent experiments in triplicate. * Significant increase from all others ($P < 0.01$). **Significant decrease from treatment with Glut and Fe, in the presence of 11 mM Glucose ($P < 0.01$).

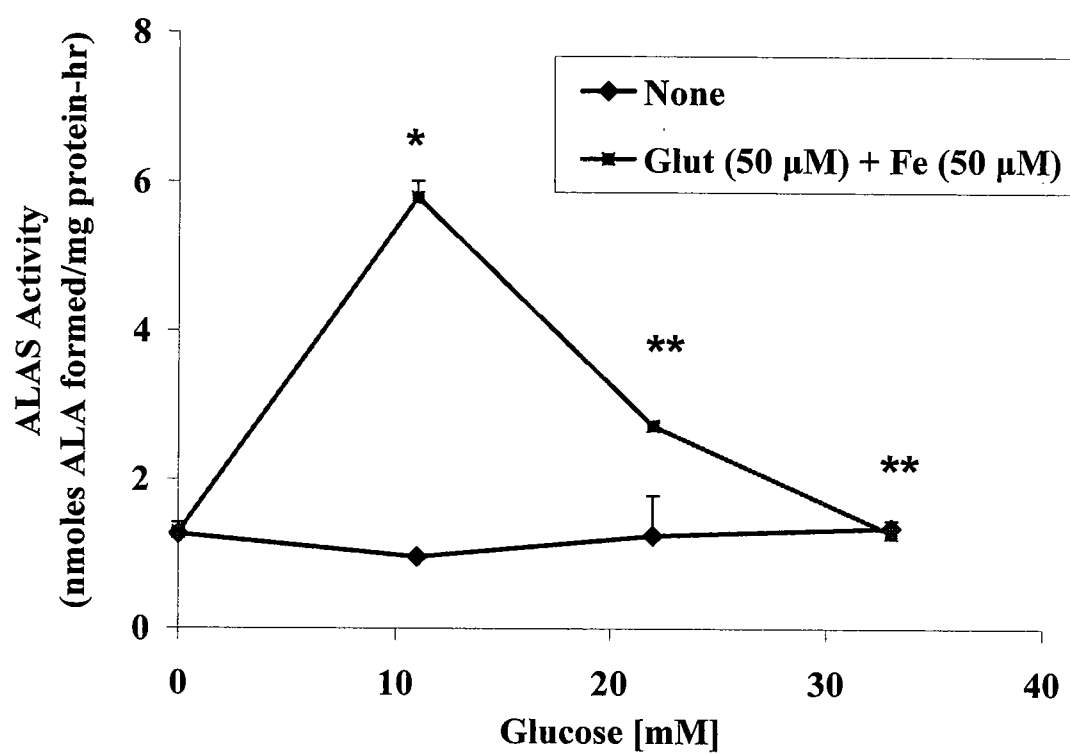
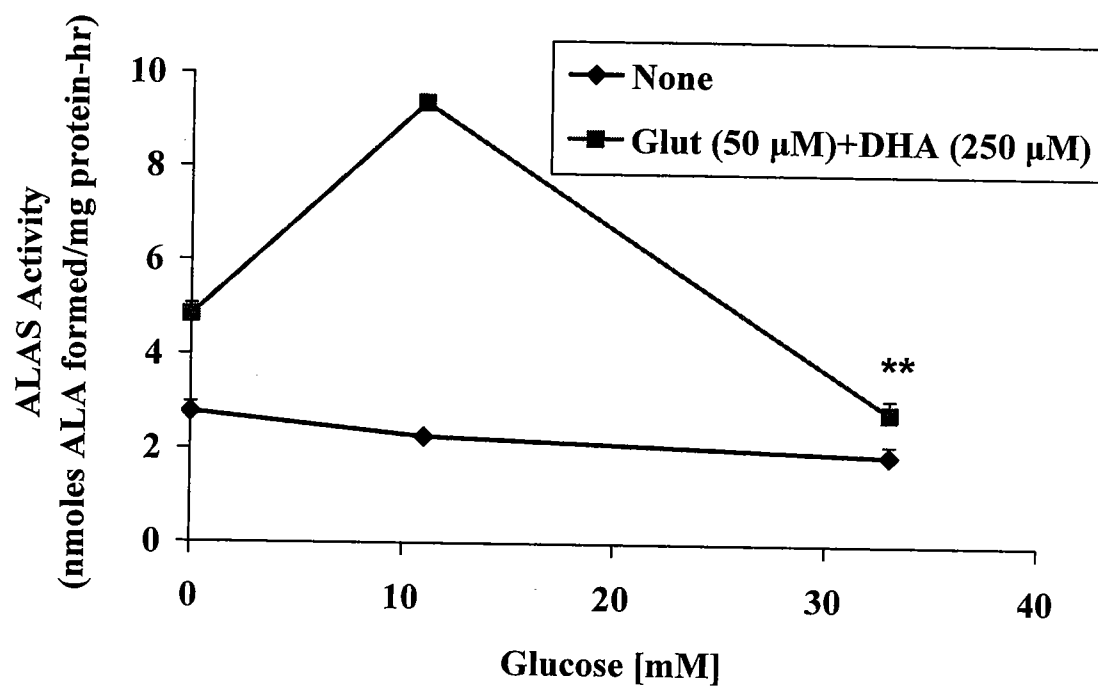


Figure 4.5. Effect of glucose on Glut- and DHA-mediated induction of ALAS activity in CELCs. CELCs were isolated and maintained in regular Williams' E medium and changed to Williams' E medium without glucose or pyruvate or T3, 24 h before treatment. Cells were then incubated for 2 h with 0, 11 or 33 mM glucose before treating with 50 μ M Glut and 250 μ M DHA or DMSO alone for 16 h. Data are presented as nanomoles ALA formed/mg protein-hr. Data represent means \pm SE from 3-5 independent experiments in triplicate. **Significant decrease from treatment with Glut and DHA, in the presence of Glucose (11 mM) ($P < 0.01$).



LMH cells and 24 h after transfection LMH cells were treated with Glut (50 μ M) and DHA (250 μ M) either alone or in combination for 16 h in the presence or absence of Glucose (11 or 33 mM). Glucose (33 mM) reduced the basal luciferase activity of uninduced LMH cells. Glut alone increased reporter activity about 1.5- fold over control while DHA alone did not have any significant effect (Figure 4.6 B). Glut and DHA in combination significantly increased the expression from pGcALAS9.1-Luc in a synergistic manner that was approximately 5-fold over no treatment control ($P < 0.001$) (Figure 4.6 B). The induction by Glut and DHA was reduced by glucose at 11 mM and further reduced by glucose at 33 mM ($P < 0.01$) (Figures 4.6 A and B). Glucose (33 mM) had no effect on basal luciferase activity of a pGL3SV40 construct transiently transfected in LMH cells and treated with increasing concentrations of glucose (11-66 mM) (Figure 4.7).

Effect of glucose analog 2-DOG on reporter activity in LMH cells untreated or treated with Glut and DHA in the presence or absence of glucose.

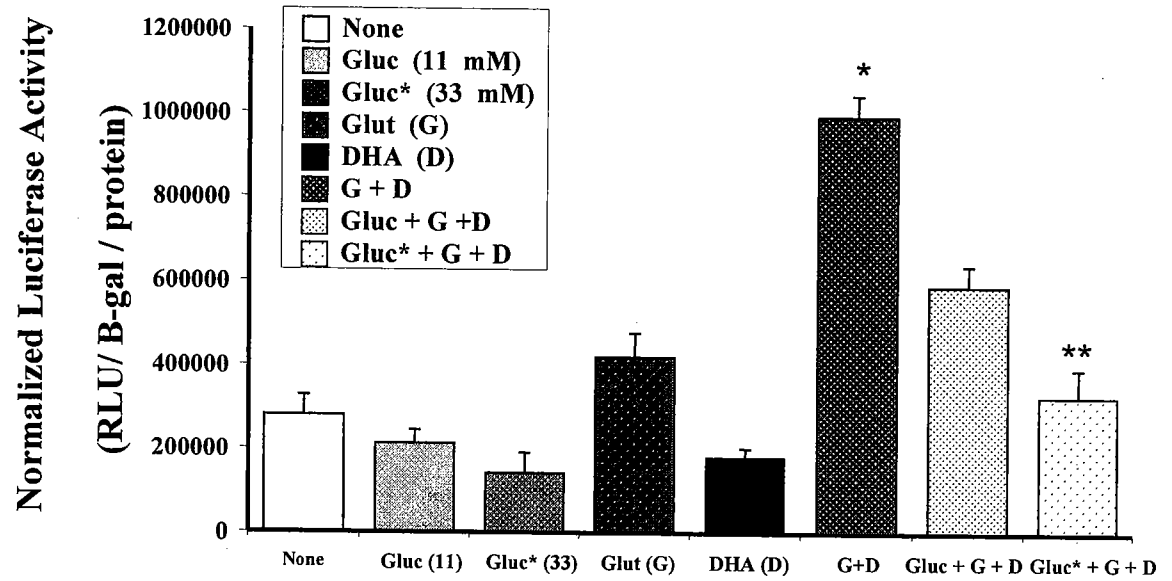
Analogues of glucose such as 3-*O*-methyl glucose (3-OMG) and 2-deoxy glucose (2-DOG) have been shown to block the inhibitory effect of glucose on ALAS enzyme activities (Giger and Meyer, 1981). In order to further characterize the glucose effect on ALAS-1 expression I studied the effects

Figure 4.6. Comparative effects of selected chemicals on luciferase activities in LMH cells transiently transfected with pGcALAS9.1-Luc.

Following transient transfections, LMH cells were either untreated or treated with glucose (11 mM or 33 mM) and/or treated simultaneously with Glut, 50 μ M, or DHA, 250 μ M, alone or in combination for 16 h prior to harvest.

Luciferase, β -gal, and protein concentrations were assayed as described in Chapter II. Luciferase activities are expressed either as normalized relative luciferase units (RLU) (A) or as normalized fold induction over untreated control (B) normalized to β -gal activities and protein concentrations. Data represent means + SE from 3-5 independent experiments in triplicate.

*Significant increase from all others ($P < 0.001$). **Significant decrease from treatment with Glut and DHA ($P < 0.01$).

A

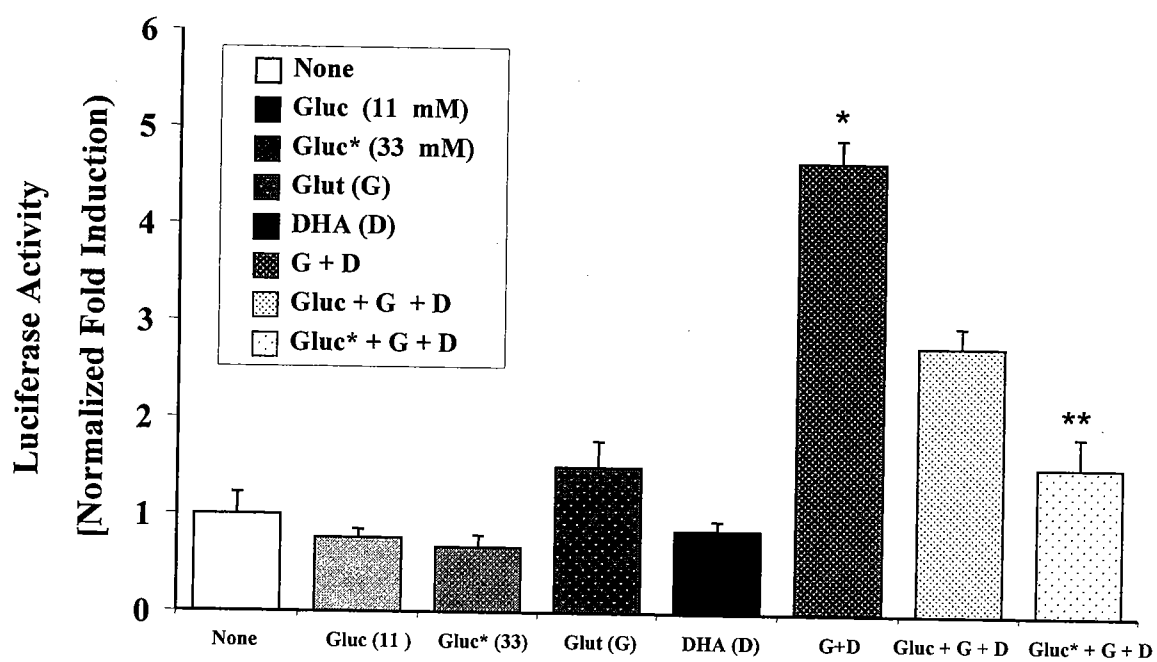
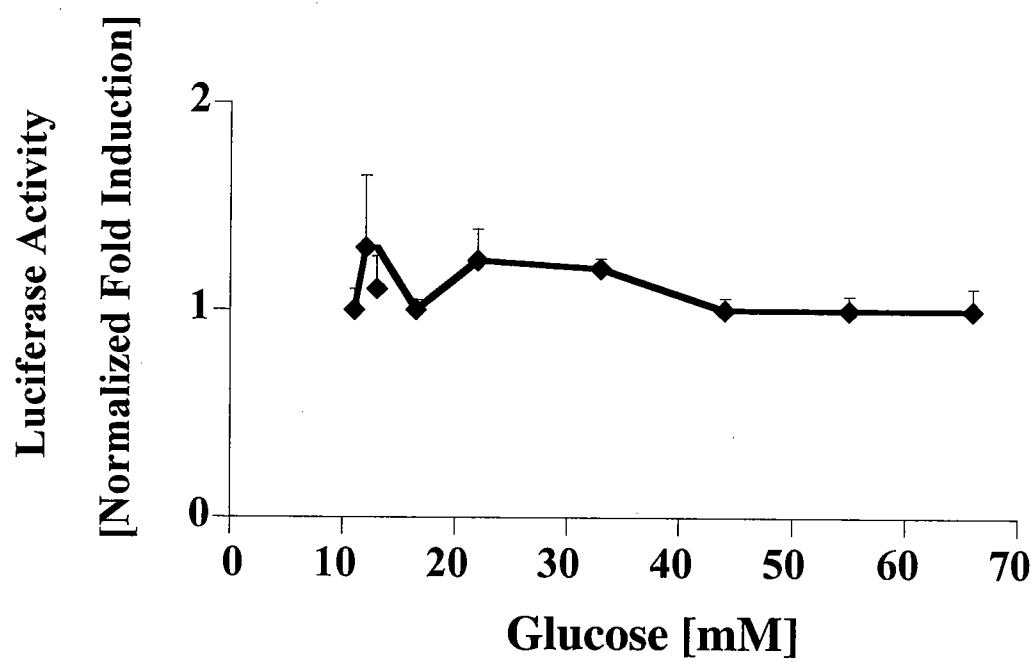
B

Figure 4.7. Effect of glucose on the pGL3SV40 luciferase activity in LMH cells. LMH cells were transfected with pGL3SV40-Luc as described in Chapter II and the effect of increasing concentrations of glucose on luciferase activity was tested in LMH cells maintained in serum-free Waymouth's medium. Briefly, 24 h after transfection, the medium on the plates was changed to serum-free Waymouth's medium (containing 11 mM glucose). LMH cells were then treated with increasing concentrations of glucose (1, 2, 5.5, 11, 22, 33, 44 or 55 mM) to give effective glucose concentrations of 12, 13, 16.5, 22, 33, 44, 55 and 66 mM respectively and then maintained at 37 °C for 16 h prior to harvest. Luciferase, β -gal, and protein concentrations were assayed as described in Chapter II. Luciferase activities are expressed either as fold induction over untreated control normalized to β -gal activities and protein concentrations. Data are expressed as means + SE from 2 independent experiments done in triplicate.



of the glucose analog, 2-DOG, on Glut and DHA induction and on the ability of glucose to alleviate this induction. The compound, 2-DOG, is taken up by liver cells in the same way as glucose and can be phosphorylated into 2-DOG-6-phosphate but cannot be further metabolized. Hence in its presence we would expect an induction in reporter gene activity by Glut and DHA even in the presence of inhibitory concentrations of glucose. LMH cells were transiently transfected with pGcALAS9.1-Luc as described in Chapter II. In the presence of 2-DOG, induction by Glut and DHA was higher than induction by Glut and DHA alone (Figure 4.8).

Effect of selected gluconeogenic or glycolytic substrates on reporter gene activity in LMH cells. The effect of selected carbohydrates and metabolizable sugars on pGcALAS9.1-Luc reporter activity was tested in LMH cells. Glucose by itself (33 mM) reduced the basal luciferase activity of untreated cells. Glut and DHA in combination induced the normalized luciferase activities approximately 4.7-fold over control (Figure 4.9). This induction was reduced significantly by glucose (33 mM), fructose (33 mM), galactose (33 mM), or the gluconeogenic or glycolytic substrates, glycerol (33 mM) and lactate (11 mM) ($P < 0.001$), but not by the non-metabolizable sugar sorbitol (33 mM) (Figure 4.9).

Figure 4.8. Effect of glucose and/or 2-DOG on Glut and DHA-mediated induction of luciferase activities in LMH cells transiently transfected with pGcALAS9.1-Luc. Following transient transfection, LMH cells were either left untreated or treated with glucose (Gluc, 33 mM), 2-deoxyglucose (2-DOG, 11 mM), and/or treated simultaneously with Glut, 50 μ M, and DHA, 250 μ M for 16 h prior to harvest. Luciferase, β -gal activities, and protein concentrations were assayed as described in Chapter II. Luciferase activities are expressed as fold induction over untreated control normalized to β -gal activities and protein concentrations. Data represent means \pm SE from 3-5 independent experiments in triplicate. Δ Significant decrease from cells treated with Glut + DHA and Glut+DHA+2-DOG+Glucose, $P < 0.05$ (Tukey Kramer).

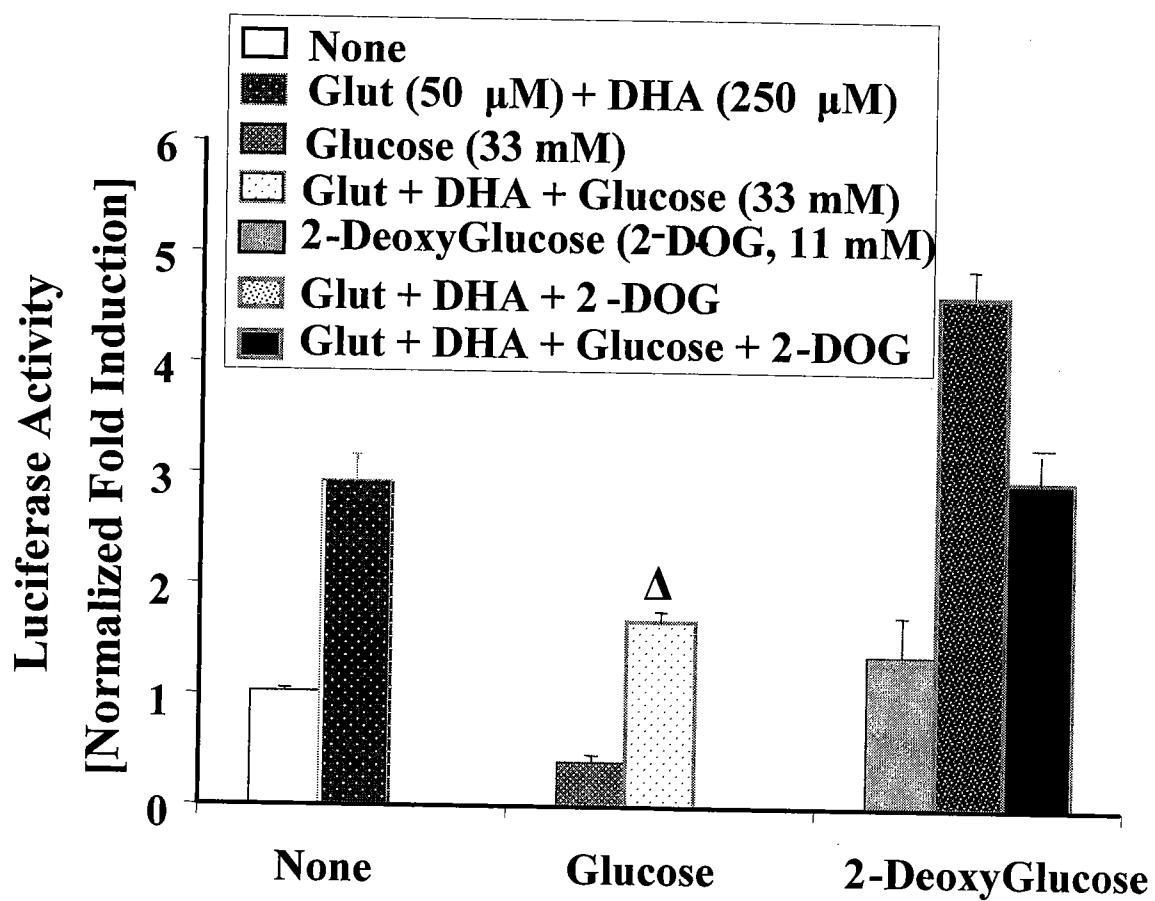
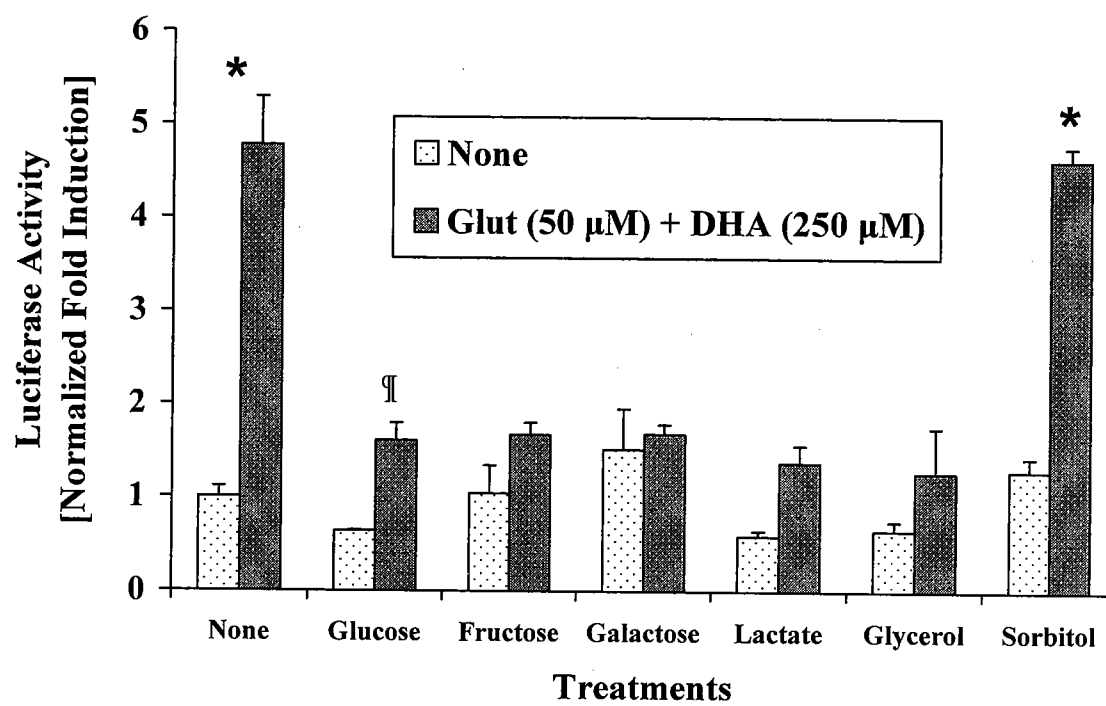
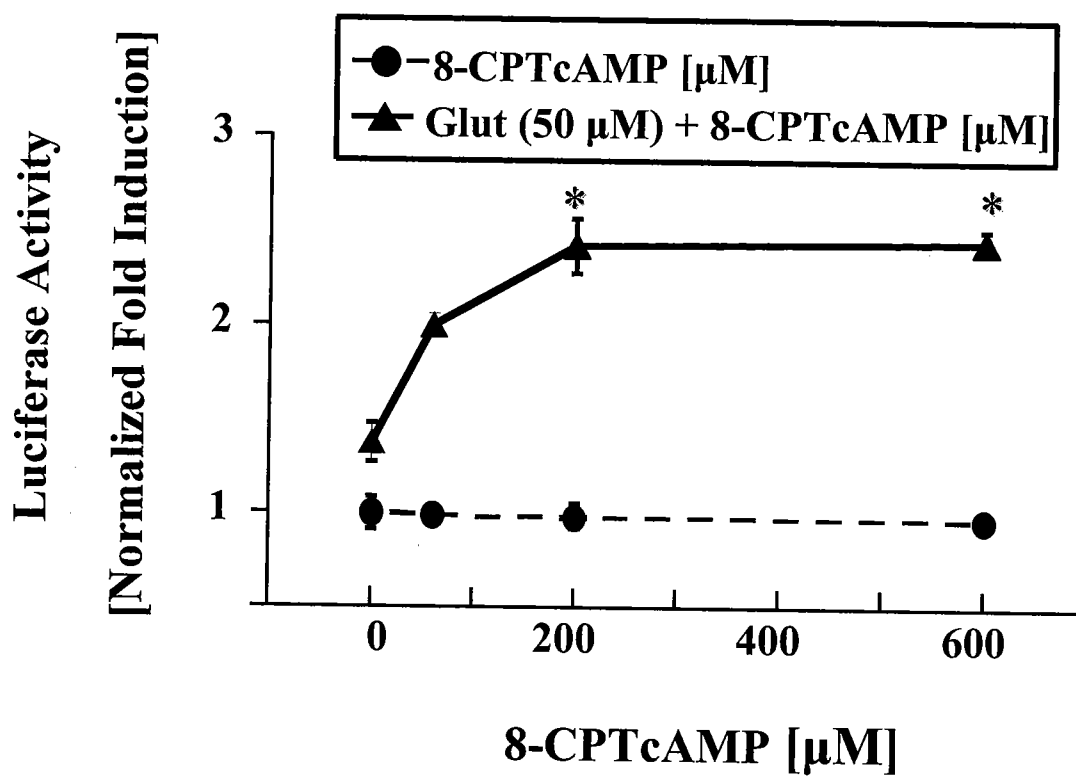


Figure 4.9. Effect of selected carbohydrates on Glut and DHA-mediated induction of luciferase activities in LMH cells transiently transfected with pGcALAS9.1-Luc. Following transient transfection, LMH cells were either left untreated or treated with glucose (33 mM), fructose (33 mM), galactose (33 mM), glycerol (33 mM), lactate (11 mM) or sorbitol (33 mM) and/or treated simultaneously with Glut, 50 μ M, and DHA, 250 μ M for 16 h prior to harvest. Luciferase, β -gal activities and protein concentrations were assayed as described in Chapter II. Luciferase activities are expressed as fold induction over untreated control normalized to β -gal activities and protein concentrations. Data represent means + SE from 3-5 independent experiments in triplicate. *Significant increase from others, $P < 0.001$. ¶Significant decrease from Glut+DHA and, Glut+DHA + Sorbitol, $P < 0.001$.



Effect of 8-CPTcAMP on Glut-induced pGcALAS9.1 reporter gene activity in LMH cells. Changes in intracellular c-AMP levels have been shown previously to increase drug-induced porphyrin biosynthesis in CELCs maintained in serum-free medium (Stephens et al., 1978). To determine if changes in c-AMP levels mediate the glucose effect on ALAS-1, the effect of increasing concentrations of the cAMP analog 8-(4-chlorophenylthio) cyclic adenosine monophosphate (8-CPTcAMP), a long lasting analog of cAMP, was tested on pGcALAS9.1-Luc activity in LMH cells. Glut alone (50 μ M) induced pGcALAS9.1-Luc activity 1.4-fold as seen before (Figure 4.6 and 4.9). Increasing concentrations (60, 200 and 600 μ M) of 8-CPTcAMP alone had no effect on pGcALAS9.1-Luc activity but increased in a dose-dependent manner the Glut-induction of pGcALAS9.1-Luc activity to about 2.5-fold (Figure 4.10).

Figure 4.10. Effect of 8-CPTcAMP on Glut -mediated induction of luciferase activities in LMH cells transiently transfected with pGcALAS9.1-Luc. Following transient transfection, LMH cells were either left untreated or treated with Glut (50 μ M) or 8-CPTcAMP (60, 200, or 600 μ M) or with a fixed concentration of Glut (50 μ M) and increasing concentrations of 8-CPTcAMP (60, 200, or 600 μ M) for 16 h prior to harvest. Luciferase, β -galactosidase activities (β -gal) and protein concentrations were assayed as described in Chapter II. Luciferase activities are expressed as fold induction over untreated control normalized to β -gal activities and protein concentrations. Data are expressed as means \pm SE from 3-5 independent experiments in triplicate. *Significant increase from treatment with 50 μ M Glut alone ($P < 0.001$). The EC_{50} was calculated to be 53.1 μ M 8-CPTcAMP by Non-linear Regression analysis ($R^2 = 1.00$) using the Hill, 4 Parameter method.



Conclusions

The optimal glucose conditions for the induction of ALAS-1 activity in LMH cells by Glut and DHA were established. Treatment with Glut and DHA induced ALAS activity many fold over no treatment control in LMH cells maintained in regular Waymouth's medium (Figure 4.2). Glucose, in the absence of serum or hormones, decreased the Glut- and DHA-, and Glut- and FeNTA- mediated induction of ALAS, in a dose-dependent manner, while the same concentrations of the monosaccharide, sorbitol, had no effect on the Glut- and DHA- induction of ALAS. From this lack of effect of sorbitol we conclude that the decrease in ALAS activity observed with glucose could not be attributed to changes in cell volume, such as that occurring due to addition of large amounts of solute, but due to a specific effect of glucose.

I tested whether the effect of glucose occurs on ALAS-1 gene expression. This was addressed by determining the effects of glucose and other metabolizable sugars on LMH cells transiently transfected with an ALAS-1 promoter-luciferase (Luc) reporter construct. Treatment for 16 h with Glut (50 μ M) and DHA (250 μ M), synergistically induced,

approximately 5-fold over control, the ALAS-1 promoter-reporter activity of a construct containing approximately 9.1 kb of chick ALAS-1 5' flanking region and 5'-UTR attached to a Luc reporter gene (pGcALAS9.1-Luc) (Figures 4.6 A and B). Glucose (11 or 33 mM), in a dose-dependent manner, decreased Glut and DHA-mediated up-regulation of pGcALAS9.1-Luc (Figure 4.6). Gluconeogenic sugars such as fructose, galactose or glycolytic compounds, such as glycerol and lactate, but not the non-metabolizable sugar, sorbitol, mimicked the "glucose effect" on ALAS-1. Sorbitol served as a negative control as in its presence at concentrations equal to those of the other carbohydrates, promoter activity was induced to similar extents as that obtained with Glut and DHA. 8-CPTcAMP, a long-lasting analog of cAMP, potentiated the induction of pGcALAS9.1-Luc by 50 μ M Glut suggesting that the "glucose effect" could be mediated by decreases in cAMP levels.

These results establish that glucose or gluconeogenic or glycolytic substrates can down-regulate hepatic ALAS-1 through a mechanism that requires the first 9.1 kb of the 5'-flanking and 5'-UTR region of the ALAS-1 gene. These effects were observed in the absence of insulin, glucagon, other hormones or serum. I conclude that LMH cells provide an excellent model for delineating the mechanism of the "glucose effect" on ALAS-1.

CHAPTER V

**CHARACTERIZATION OF THE UPSTREAM REGULATORY
REGION OF CHICK ALAS-1 GENE IN LMH CELLS: STUDIES ON
EFFECTS OF HEME, GLUT AND DHA**

Introduction

Under normal physiological conditions, “free” heme levels are low and tightly regulated, as toxicity can occur with increased cellular concentrations of unincorporated heme (Balla et al., 2003; Maya-Monteiro et al., 2004; Gonzalez-Michaca et al., 2004). However, coordinate increases in heme and cytochrome P-450(s) are required for induction by drugs and other chemicals of cytochrome P-450(s) in liver and other tissues (Kihara et al., 1999). This is achieved by induction of ALAS-1 which provides an adequate supply of heme for the generation of functional cytochrome holoproteins (Granick et al., 1975; May et al., 1986). Free heme is envisioned to repress hepatic ALAS-1 by a number of negative feedback mechanisms, including reduction of transcription (Srivastava et al., 1990; Sassa and Nagai, 1996), reduction of mRNA stability (Hamilton et al., 1988; Cable et al., 2002) and inhibition of the transport of the pre-ALAS-1 into the mitochondria (Lathrop

and Timko, 1993) (p. 7). Direct inhibition of ALAS activity by heme has not been found at reasonable heme concentrations (Wolfson et al., 1979).

Results of studies on the effect of heme on ALAS-1 gene transcription have been conflicting in part due to coinciding effects of heme on ALAS-1 mRNA stability (Srivastava et al., 1988; Srivastava et al., 1990; Hamilton et al., 1991). In Chapter III, Glut and DHA in combination were shown to synergistically up-regulate ALAS-1 enzyme activity in LMH cells as detected by spectrophotometry. This up-regulation was repressed by exogenous heme (20 μ M). Due to uncertainties implicit in the several potential mechanisms of the heme repressive effect described above, the transcriptional regulation of ALAS-1 by heme and chemicals that affect heme synthesis were studied using promoter-reporter constructs. In this Chapter, the *cis*-acting elements in the 5'-flanking region and 5'-UTR region of ALAS-1 that mediate heme-dependent repression of ALAS-1 gene transcription were studied with the long-term goal of identifying the heme repressive element(s) that regulate ALAS-1 gene transcription.

In contrast to heme, there is much experimental evidence that ALAS-1 gene expression is increased directly by drugs (Hamilton et al., 1988; Hamilton et al., 1991; Louis et al., 1998; Jover et al., 2000). A recent study

in LMH cells has demonstrated that induction of ALAS-1 by drugs involves the chick xenobiotic-sensing receptor (CXR), a chick homolog of the constitutive androstane receptor, (CAR) (Fraser et al., 2002). The transcriptional activation of cALAS-1 gene by CXR has been shown to occur via two 175 bp and 167 bp drug responsive enhancer sequences (DRES) located at -14,312 to -14,137 bp and -13,592 to -13,426 bp upstream of the transcriptional start site. These DRES's work independently of each other and contain functional direct repeat (DR) 4 and DR5 type recognition sequences for nuclear receptors (Fraser et al., 2002). Characterization of these DRE's does not exclude a role for a regulatory heme pool in modulating the extent of induction by drugs (p. 7). This is partly due to the fact that the "free" or "regulatory" heme pool is presumed to be small and to turn-over rapidly (Ketterer et al., 1976; Vincent et al., 1989; Müller-eberhard and Fraig 1993), making it practically impossible to measure. Indeed, no direct measure of such a pool has been accepted in the field. The level of activity of ALAS-1 is taken as perhaps the most sensitive indirect estimate of the size of the regulatory heme pool (Sassa and Nagai, 1996). Another proposed measure is the degree of saturation of tryptophan pyrrolase by heme (Bonkovsky et al., 1990).

Indirect evidence for the effect of heme pool depletion on induction of ALAS-1 is obtained from heme-deficient mice where heme-deficiency augmented drug-mediated induction of ALAS-1 mRNA (Jover et al., 2000). In this study, treatment of mice deficient in heme synthesis, with drugs that induce CYP and increase the demand for heme, resulted in a synergistic up-regulation of ALAS-1 mRNA levels (Jover et al., 2000). Another line of evidence for the effect of regulatory heme pool comes from studies of cells in culture, assessing effects on enzyme activity of chemicals that inhibit heme synthesis (Sassa 1982; Kolluri et al., 1999; Cable et al., 2000). In these studies, treatment of cells with inhibitors of heme synthesis, in combination with drugs that induce CYP and increase the demand for heme, resulted in synergistic increases in ALAS-1 enzyme activity (Sassa 1982; Kolluri et al., 1999; Cable et al., 2000). Synergistic effects of the combination of chemicals as described above might be explained by their effects on the regulatory heme pool (p. 7), by a direct effect of these chemicals on the expression of ALAS-1 gene, or by a combination of both.

The question of heme regulation of basal and drug-induced ALAS-1 gene transcription was approached by investigating, in LMH cells, the effects of Glut in combination with the inhibitor of heme synthesis, DHA,

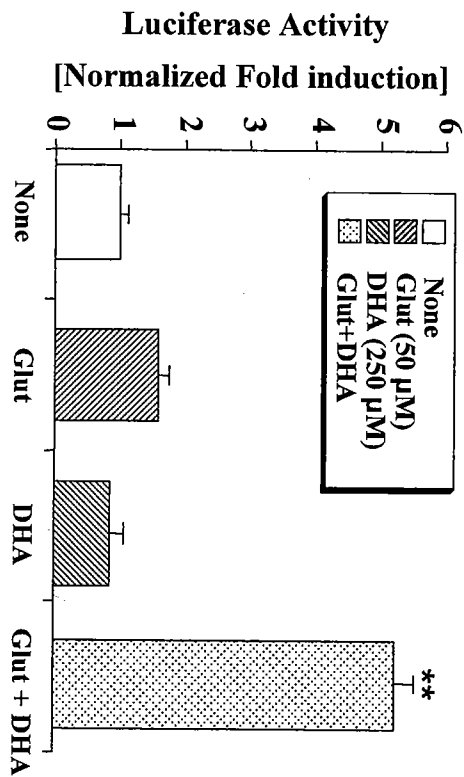
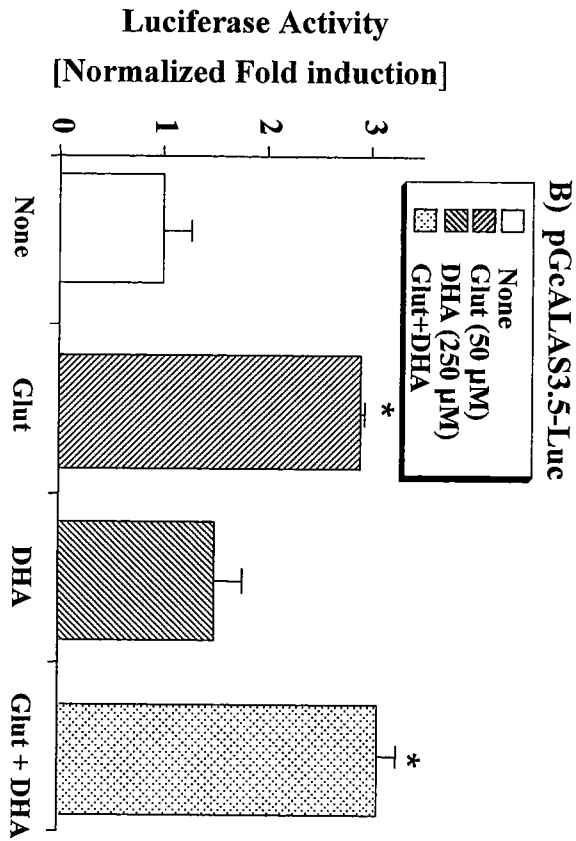
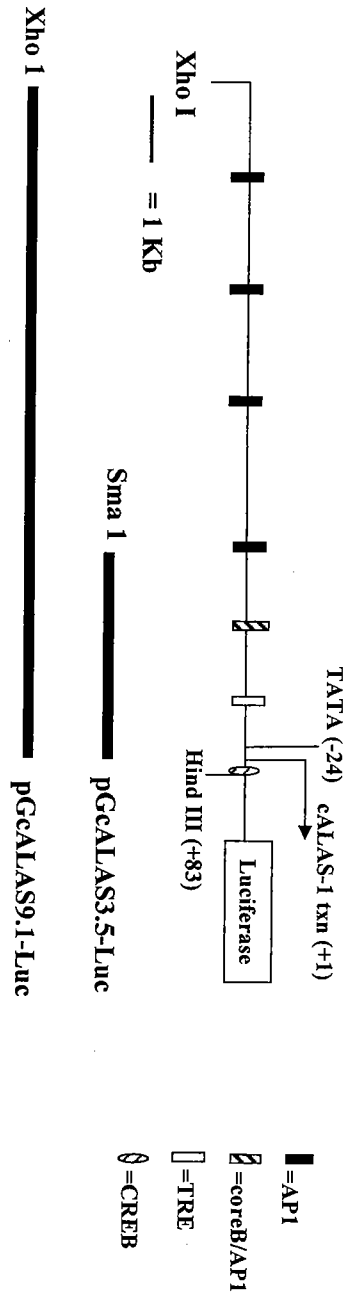
and/or heme on ALAS-1 promoter-reporter (Luc) constructs transiently/stably transfected and treated with the above mentioned chemicals as described in detail next.

Results

Effects of Glut or DHA alone or in combination on ALAS/Luc constructs. For characterization and delineation of the effects of heme and drugs on ALAS-1 promoter, the 9.1- and a 3.5-kb of the 5'-flanking and 5'-untranslated (UTR) region of chick ALAS-1 fused upstream of the reporter (luciferase) gene of a pGL3basic vector (Promega, Madison, WI), as described in Chapter II, were used. These ALAS-1 constructs are termed pGcALAS9.1-Luc and pGcALAS3.5-Luc, respectively. In Chapter IV, Glut in combination with DHA, an effective inhibitor of ALA dehydratase (Cable et al., 2000), the second enzyme of heme biosynthesis, synergistically induced pGcALAS9.1-Luc activity 5-fold over control (Figure 4.6 B). In the experiments described in this Chapter, the question of drug and heme regulation of ALAS-1 was approached by studying transfections of these ALAS-1 constructs in LMH cells, which have been shown to be a good model for studies of heme biosynthesis and regulation of ALAS-1 (Chapter III). The effects of Glut or DHA alone or in combination were tested in LMH cells transiently transfected with pGcALAS9.1-Luc, and compared with the effect of the selected chemicals on the shorter construct - pGcALAS3.5-Luc (Figure 5.1). Glut alone (50 μ M) for 16 h led to a 3-fold

Figure 5.1. CALAS-Luc constructs and effects of Glut and DHA on Luc expression (A) The 5'-flanking and 5'-UTR region of chick ALAS-1 gene showing candidate regulatory elements and structures of cALAS-1 constructs used. Numbering starts at the transcription start point (+1). Comparative effects of selected chemicals on luciferase activities in LMH cells transiently transfected with pGcALAS3.5-Luc (B) or pGcALAS9.1-Luc (C). Following transfections, LMH cells were either left untreated or treated with Glut, 50 μ M, or DHA, 250 μ M, or the combination for 16 h. Cells were harvested and luciferase, β -galactosidase activities (β -gal) and protein concentrations were assayed as described in Chapter II. Luciferase activities are expressed as fold induction over untreated control normalized to β -gal activities and protein concentrations. Results are means + SE from 3-5 independent experiments in triplicate. This experiment was repeated six times with essentially the same results. *Differs from None or DHA alone, $P < 0.01$. In B, the two groups treated with Glut did not differ significantly from one another. **Differs from others, $P < 0.001$. AP, activator protein; CoreB/AP-1, activator protein with 4 bp extension; TRE, TPA-response element; CREB, cAMP response element. Recognition sites for enzymes Xho I, Sma I, and Hind III, used for plasmid construction(s) are also shown.

A) The 5'-UTR and 5'-Flanking region of the Chick ALAS-1 Gene with Candidate Regulatory Elements and Structures of Promoter/Luc-Reporter Constructs Studied



increase in luciferase activity in cells transfected with pGcALAS3.5-Luc (Figure 5.1 B). A lesser increase (1.5-fold) was obtained with 50 μ M Glut alone in cells transfected with pGcALAS9.1-Luc (Figure 5.1 C). In cells transfected with pGcALAS3.5-Luc, the combination of Glut (50 μ M) and DHA (250 μ M) did not significantly increase luciferase activity compared with treatment with 50 μ M Glut alone (Figure 5.1 B). In contrast, in cells transfected with pGcALAS9.1-Luc, the combination of 50 μ M Glut and 250 μ M DHA produced a synergistic induction in luciferase activity compared with 50 μ M Glut or 250 μ M DHA alone ($P < 0.001$) (Figure 5.1 C).

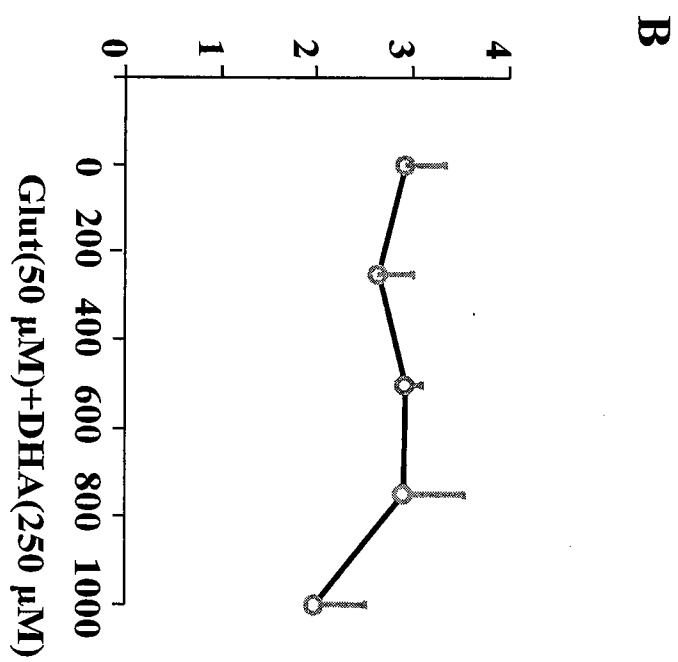
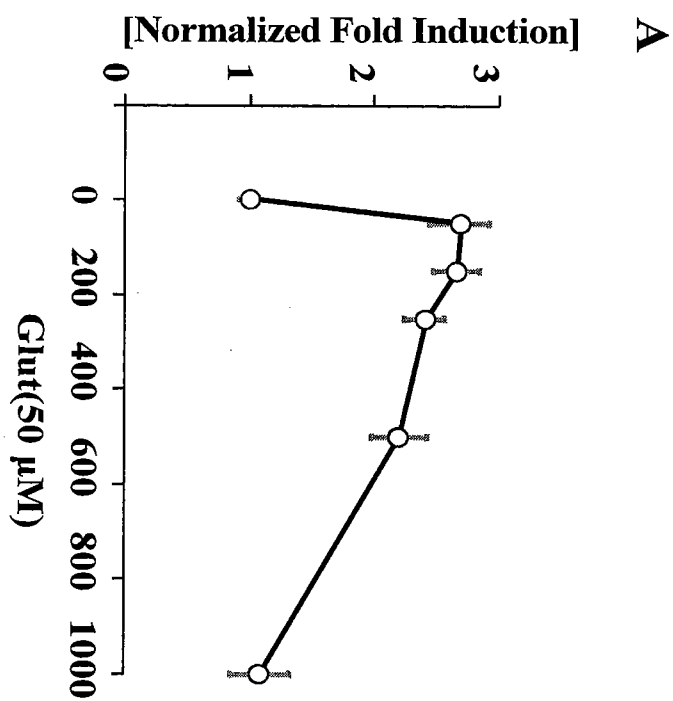
The synergistic induction by Glut and DHA of pGcALAS9.1-Luc, but not of pGcALAS3.5-Luc, suggest that there are negative element(s) in the -3.5 to -9.1 kb region of ALAS-1 5'-flanking region that repress Glut induction in the absence of DHA. Interestingly, DHA alone (250 μ M) did not have any effect on pGcALAS9.1-Luc activity (Figure 5.1 C). This is probably because, it has been shown that even though DHA inhibits *de novo* heme biosynthesis, it does not affect the regulatory heme pool *per se* (Giger and Meyer, 1983; Russo et al., 1994; Kappas et al., 2000), which may exert a regulatory effect on the -3.5 to -9.1 kb region. But Glut and DHA in combination increase the demand for heme because barbiturate-like drugs,

such as Glut induce CYP mRNA and apoprotein synthesis (Jover et al., 1996; Handschin and Meyer, 2000; Kappas et al., 2000) the latter by binding heme decreases the heme pool further (p. 7), leading to the synergistic activation of pGcALAS9.1-Luc, as obtained in the presence of Glut and DHA (Figure 5.1 C).

Glut and DHA dose-response. Because there was no synergistic effect of Glut (50 μ M) and DHA (250 μ M) on pGcALAS3.5-Luc, I investigated whether Glut and DHA at other concentrations can affect pGcALAS3.5-Luc. I first assessed the effect of increasing concentrations of Glut alone (0-1000 μ M). 50 μ M Glut gave maximal up-regulation of pGcALAS3.5-Luc (Figure 5.2 A). The decrease in luciferase activity observed with 1000 μ M Glut was probably due to toxicity at the higher concentration, as reflected by decreased β -gal activity and protein levels. Increasing concentrations of DHA (0-1000 μ M), had no effect on Glut (50 μ M)-induced reporter gene expression from pGcALAS3.5-Luc (Figure 5.2 B), indicating that pGcALAS3.5-Luc was unable to be synergistically induced by DHA.

Because DHA, an effective inhibitor of heme synthesis (Sassa and Kappas, 1982; Giger and Meyer, 1983; Kappas et al., 2000), produced a synergistic increase with Glut of pGcALAS9.1-Luc, but not of

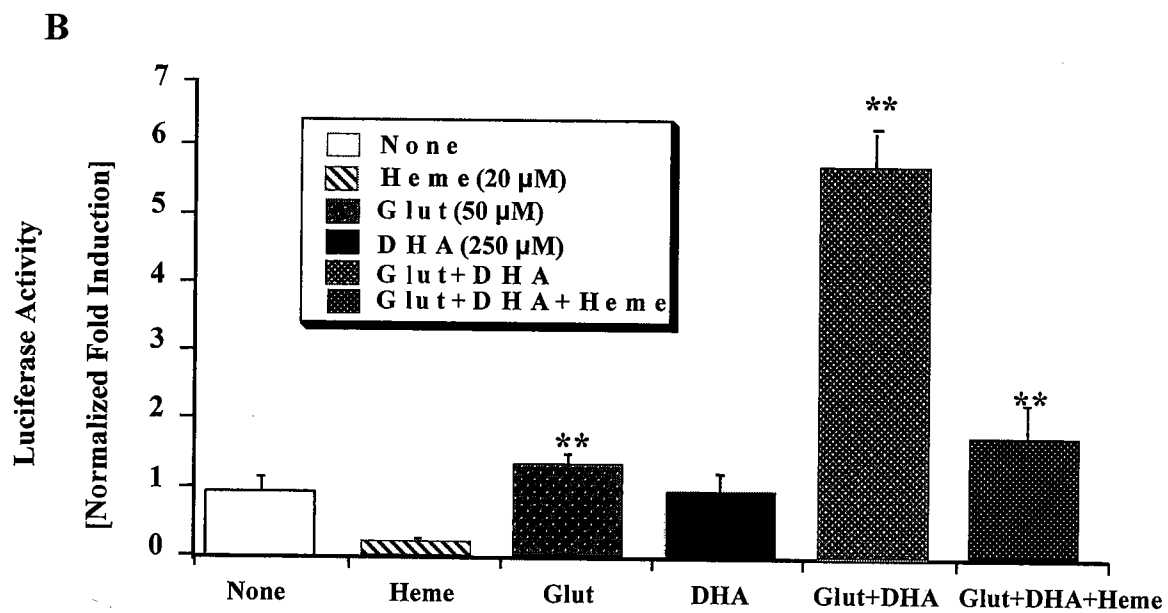
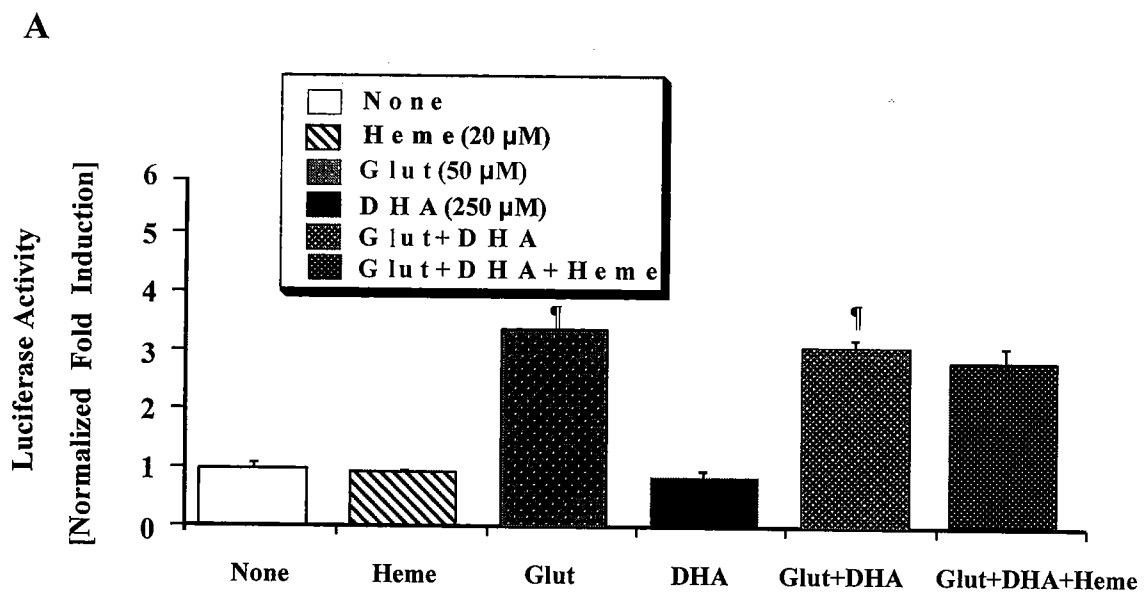
Figure 5.2. Effect of increasing concentrations of Glut alone (A), or DHA in combination with a fixed concentration (50 μ M) of Glut (B) on luciferase activities in LMH cells transiently transfected with pGcALAS3.5-Luc. Following transfection, LMH cells were either left untreated or treated with increasing concentrations of Glut (0, 50, 150, 250, 500 or 1000 μ M) or Glut (50 μ M) and DHA (0, 250, 500, 750 or 1000 μ M) and harvested after 16 h. Luciferase-, β -gal activities and protein concentrations were assayed as described in Chapter II. Results are means \pm SE from 3-5 independent experiments in triplicate. Luciferase activities are expressed as fold induction over untreated control normalized to β -gal and proteins. Values for all concentrations of Glut > 0 μ M except 1000, differ from 0, $p < 0.001$.



pGcALAS3.5-Luc (Figures 5.1 B and C), it seemed likely that element(s) involved in the heme-mediated repression of chick ALAS-1 gene occur in the -3.5 to -9.1 kb region. Synergistic activation of pGcALAS9.1-Luc by Glut and DHA (Figure 5.1 C) might be due to a decrease in the regulatory heme pool that normally mediates heme repression (p. 7), through a mechanism that requires the -3.5 and -9.1 kb region of the chick ALAS-1 gene.

In order to determine if the effect of DHA on pGcALAS9.1-Luc was mediated through its ability to decrease heme levels in the cells, we assessed the induction by Glut and DHA in the presence of exogenous heme (Figures 5.3 A and B). Heme (20 μ M, 16 h) repressed the basal luciferase activity of pGcALAS9.1-Luc which was reduced by more than 75% (Figure 5.3 B), indicating heme-mediated repression of pGcALAS9.1-Luc. The luciferase activity of pGcALAS9.1-Luc that had been increased by treatment with Glut and DHA, was also repressed, by the addition of heme (20 μ M), to a level similar to that obtained by Glut alone (Figure 5.3 B). In contrast, there was no effect of heme either on the basal or Glut- and DHA-induced reporter gene expression of pGcALAS3.5-Luc (Figure 5.3 A), suggesting that the -3.5 to -9.1 kb region of the chick ALAS-1 promoter contains one or more

Figure 5.3. Comparative effect of exogenous heme on luciferase activities in LMH cells transiently transfected with pGcALAS3.5-Luc (A) or pGcALAS9.1-Luc (B). Transfections, preparation of and treatment with Glut and DHA, and heme were performed as described in Chapter II. Cells were harvested after 16 h of exposure to selected chemicals and assayed as described in Chapter II. Results are means + SE from 8 independent experiments in triplicate. **Differs from Glut + DHA, or Heme alone, $P < 0.001$. *Differs from others, $P < 0.001$. In A, the three groups treated with Glut did not differ significantly from one another. ¶ Differs from None, Heme, or DHA alone, $P < 0.01$.

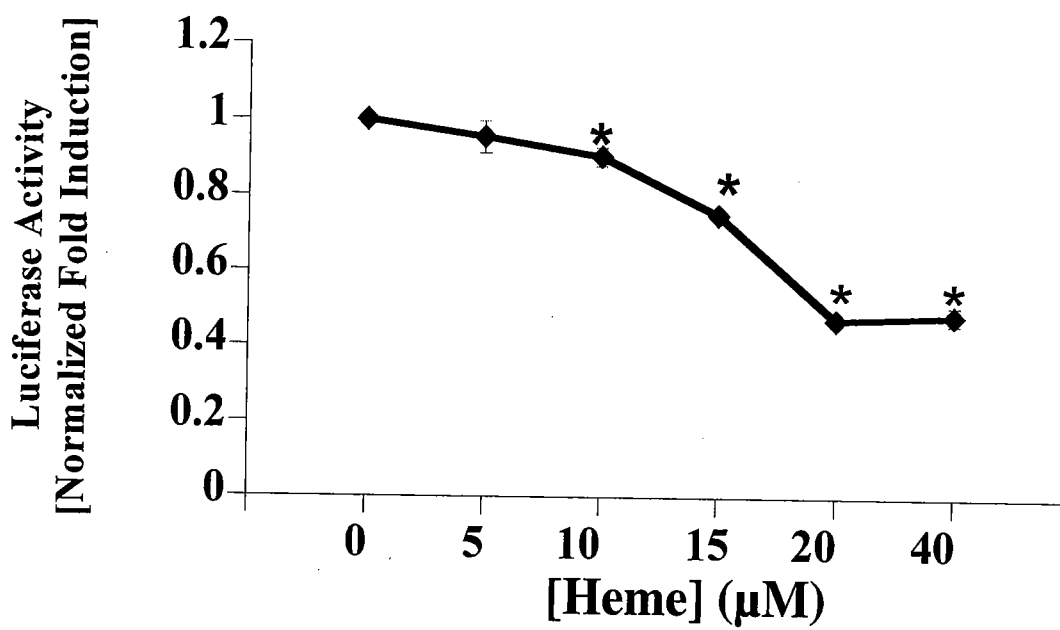


cis-acting elements that mediate heme-dependent repression of ALAS-1. The fact that reporter gene activities were unaffected by heme in Figure 5.3 A, suggests that heme at the concentration used (20 μ M) was not toxic to LMH cells, a finding consistent with other results (Gabis et al., 1996; Kolluri et al., 1999). The induction of pGcALAS9.1-Luc by Glut alone (Figures 5.1 C and 5.3 B), was less than the induction of the 3.5 kb cALAS-1 promoter construct by 50 μ M Glut alone (Figures 5.1 B and 5.3 A) suggesting that there are silencer elements that repress drug induction in the -3.5 to -9.1 kb heme-responsive region.

Effect of increasing concentrations of heme on pGcALAS9.1-Luc activity:

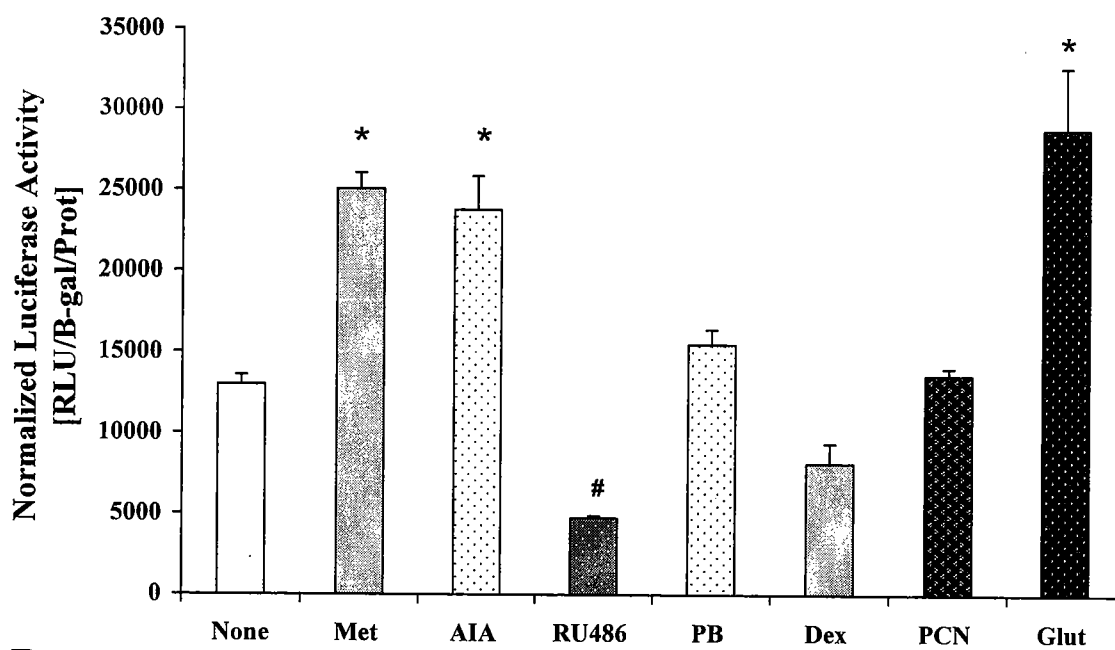
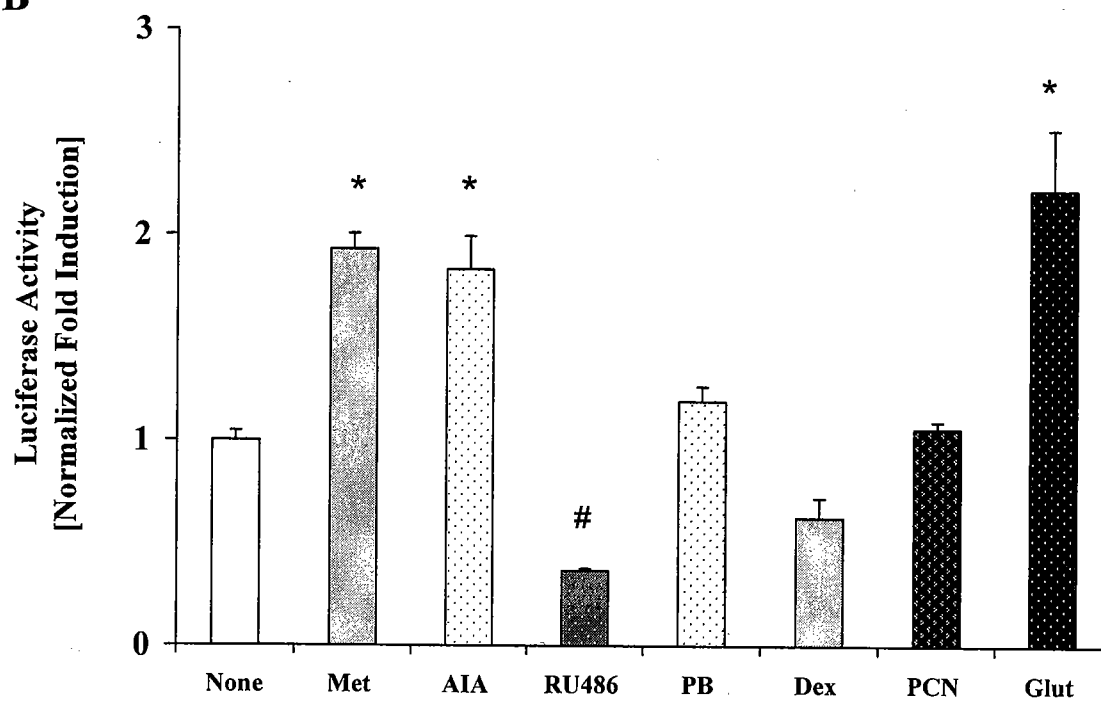
LMH cells transiently transfected with pGcALAS9.1-Luc were treated with heme (0, 5, 10, 15, 20, 40) μ M heme for 16 h, cells harvested, and luciferase, β -gal activities and proteins were measured as described in Chapter II. Increasing concentrations of heme from 0 to 15 μ M reduced luciferase activity in a dose-dependent manner (Figure 5.4). Significant down-regulation by heme was noted at 10 μ M and 15 μ M ($P < 0.001$), and the effect (54% repression of luciferase activity) was maximal at 20 μ M (Figure 5.4). Hence, in further experiments 20 μ M heme was the concentration used.

Figure 5.4. Effect of increasing concentrations of heme on pGcALAS9.1-Luc in transiently transfected LMH cells. LMH cells transiently transfected with pcALAS9.1-Luc were treated with increasing concentrations of heme (0, 5, 10, 15, 20 and 40 μ M) in DMSO and maintained at 37°C for 16 h, after which cells were harvested and used to measure luciferase β -gal activities and proteins. Luciferase activities are expressed as fold induction over DMSO-treated control normalized to proteins. Results are means \pm SE from 3-5 independent experiments in triplicate. *Differs from cells transfected with pGcALAS9.1-Luc and treated with 0 μ M heme, $P < 0.001$.



Effect of selected chemicals that induce CYP on pGcALAS9.1 in stably transfected LMH cells: Recently, it has been shown in LMH cells that treatment with selected drugs increases ALAS-1 mRNA levels and luciferase activity and the effects are mediated, at least in part, by drug-responsive enhancer sequences (DREs) in the 5'-flanking of ALAS-1 (Fraser et al., 2002). In my next studies, pGcALAS9.1-Luc activity was measured in stably-transfected LMH cells after exposure of cells to drugs (Fraser et al., 2002). Stable transfections were used since in preliminary studies, as in other studies, stable incorporation of the constructs was found to provide more consistent and larger chemical-induced changes in Luc activities (Alam et al., 1994; Alam et al., 1995; Shan et al., 2002; Alam 2002). Seven chemicals were screened for their ability to induce the pGcALAS9.1-Luc (Figures 5.5 A and B). Metyrapone (Met; 400 μ M), AIA (250 μ M), and Glut (50 μ M) induced pGcALAS9.1-Luc activity (~2-fold) as compared to control (Figure 5.5 B). There was no effect of 20 μ M PCN and a decrease with 10 μ M RU-486 and 50 μ M Dex, as compared to control (Figure 5.5 B). Subsequent work was done with 50 μ M Glut as it gave consistent and measurable up-regulation of pGcALAS9.1-Luc (Figures 5.5 A and B) at a lower concentration than other phenobarbital-like chemicals.

Figure 5.5. Comparative effects of selected chemicals that induce CYP on pGcALAS9.1-Luc activity in stably transfected LMH cells shown as normalized RLU (A) and normalized fold induction (B). LMH cells stably transfected with pGcALAS9.1-Luc as described in Chapter II were treated with chemicals and maintained at 37°C for 16 h, after which cells were harvested and used to measure luciferase, β -gal activity and proteins as described in Chapter II. Concentrations of the selected chemicals were as follows: Glut - 50 μ M; metyrapone (Met) - 400 μ M; allylisopropyl acetamide (AIA) - 250 μ M; phenobarbital (PB-Na) - 600 μ M; RU486 - 10 μ M; and dexamethasone (Dex) - 50 μ M, respectively. Results are means + SE from 3-5 independent experiments in triplicate. Luciferase activities are expressed as relative luciferase activity (RLU) or fold induction over untreated control, normalized to proteins. *Differs from none, $P < 0.01$. # Differs from none, $P < 0.01$.

A**B**

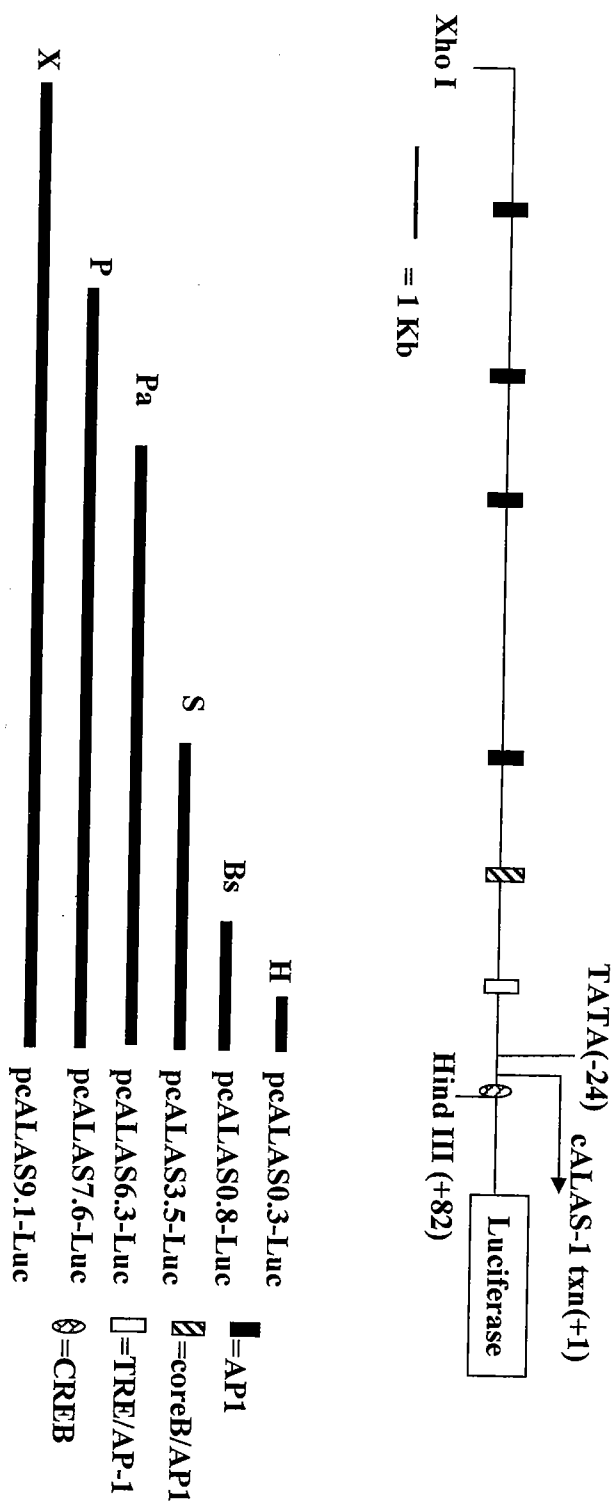
Delineation of the -9.1 to -3.5 kb cALAS-1 region: Since heme-repression of drug-induced reporter activity can be used as a tool to delineate the heme-repressive region of ALAS-1, deletion constructs in the heme-responsive region were made starting from the Xho I site (-9067 bp) (Figure 5.6).

Detailed sequence information or restriction map of the -9.1 to -3.5 kb region was not known when these studies were begun. Therefore, I screened and chose a number of restriction enzymes that were non-cutters in the proximal -3.5 kb or in the vector (pGL3 Basic). PGcALAS9.1-Luc was completely digested with Xho I and subsequently with different enzymes and we looked for two bands that can be easily separated on agarose gels.

Xho I and Pml I or Pac I restriction endonucleases were chosen based on the above mentioned criterion and deletion constructs were generated as described in Chapter II and as shown in Figure 5.6. The resulting deletion constructs and the -9.1 to -3.5 kb of chick ALAS-1 5'-flanking region were sequenced (single strand), collated with the +0.082 to -3.5 kb sequence, and analyzed for putative regulatory elements by computer sequence analysis tools. The approximately 9.1 kb sequence (Appendix E) was also confirmed with a 15 kb sequence of chick ALAS-1 promoter that was recently published (Fraser et al., 2002).

Figure 5.6. The 5'-flanking and 5'-UTR region of chick ALAS-1 gene showing candidate regulatory elements and structures of cALAS-1 constructs. Numbering starts at the transcription start point (+1). Only sites with complete identity to known consensus regulatory elements are shown. Abbreviations used: AP, activator protein (Guberman et al., 2003); CoreB/AP-1 (Alam 2002), activator protein with 4 bp extension; TRE, TPA-response element (Alam and Denz 1992); CREB, cAMP response element (Giono et al., 2001). A partial restriction endonuclease map of the chick ALAS-1 promoter region is also presented. Recognition sites for restriction endonucleases Xho I (X), Pml I (P), Pac I (Pa), Sma I (S), BspM I (Bs) and Hind III (H) are shown.

The 5'-UTR and 5'-Flanking region of the Chick ALAS-1 Gene with Candidate Regulatory Elements and Structures of Promoter/Luc-Reporter Constructs



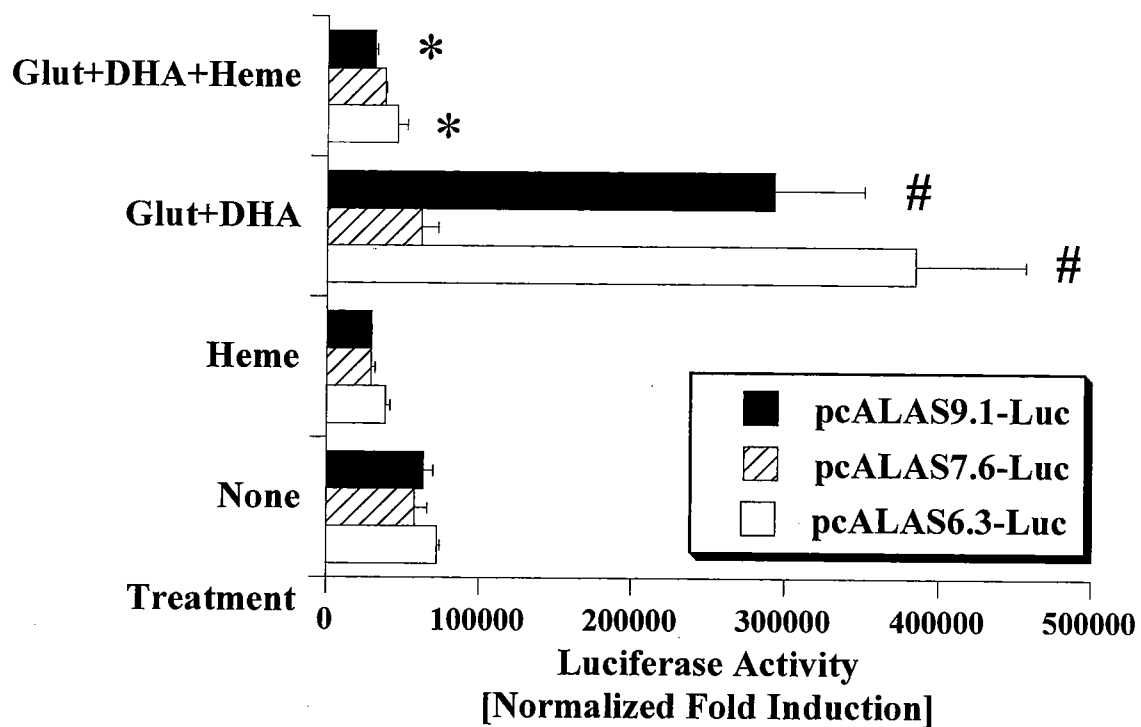
Effects of Glut and DHA, and/or heme on ALAS-Luc constructs in transiently-transfected LMH cells: The effects of Glut (50 μ M) and DHA (250 μ M) and/or heme on ALAS-Luc constructs were tested in LMH cells transiently transfected with pGcALAS9.1-, 7.6-, and 6.3-Luc, as described in Chapter II. Heme (20 μ M) repressed basal luciferase of pGcALAS9.1-, 7.6- and 6.3-Luc to 45%, 50%, and 53% of control, respectively (Figure 5.7 A and B). This effect of heme to repress could also be seen on Glut and DHA-induced luciferase activity of pGcALAS9.1- and pGcALAS6.3-Luc, which were reduced to 11%, and 10% of Glut- and DHA-induced levels in the presence of heme (Figures 5.7 A and B).

The 7.6 kb construct showed similar heme-dependent repression as the 9.1 and 6.3 kb constructs of basal luciferase activity (50 %) but it could not be induced by Glut and DHA (1.07 + 0.18-fold). Therefore, repression by heme in the presence of Glut+DHA showed repression (61%) that was similar to basal repression by heme (50% of basal activity) (Figure 5.7 B).

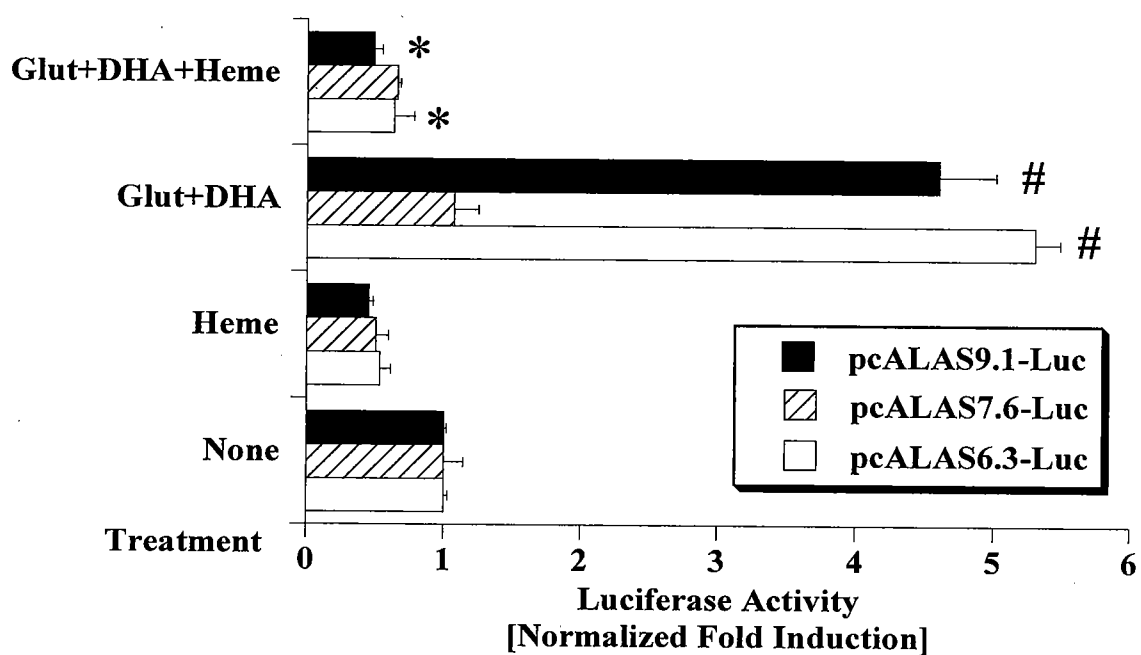
Comparative effects of Glut and DHA, and heme on ALAS/Luc constructs stably-transfected LMH cells: Effects of Glut (50 μ M) + DHA (250 μ M), and/or heme (20 μ M) or DMSO (Me₂SO) alone on ALAS-Luc constructs

Figure 5.7. Comparative effects of Glut and DHA, and heme on ALAS/Luc constructs in transiently transfected LMH cells shown as normalized RLU (A) or normalized fold induction (B). LMH cells transiently transfected with selected ALAS-Luc constructs were treated with chemicals and maintained at 37°C for 16 h, after which cells were harvested and used to measure luciferase activity and proteins. Concentrations of the selected chemicals were as follows: Glut, 50 μ M; DHA, 250 μ M; and heme, 20 μ M. Luciferase activities are expressed as RLU or fold induction over untreated control normalized to β -gal and proteins. Results are means + SE of 6 independent experiments in triplicate. *Differs from cells transfected with pGcALAS9.1- or 6.3-Luc and treated with Glut and DHA, $P < 0.001$. # Differs from all cells transfected with pGcALAS9.1- or 6.3-Luc, $P < 0.001$.

A



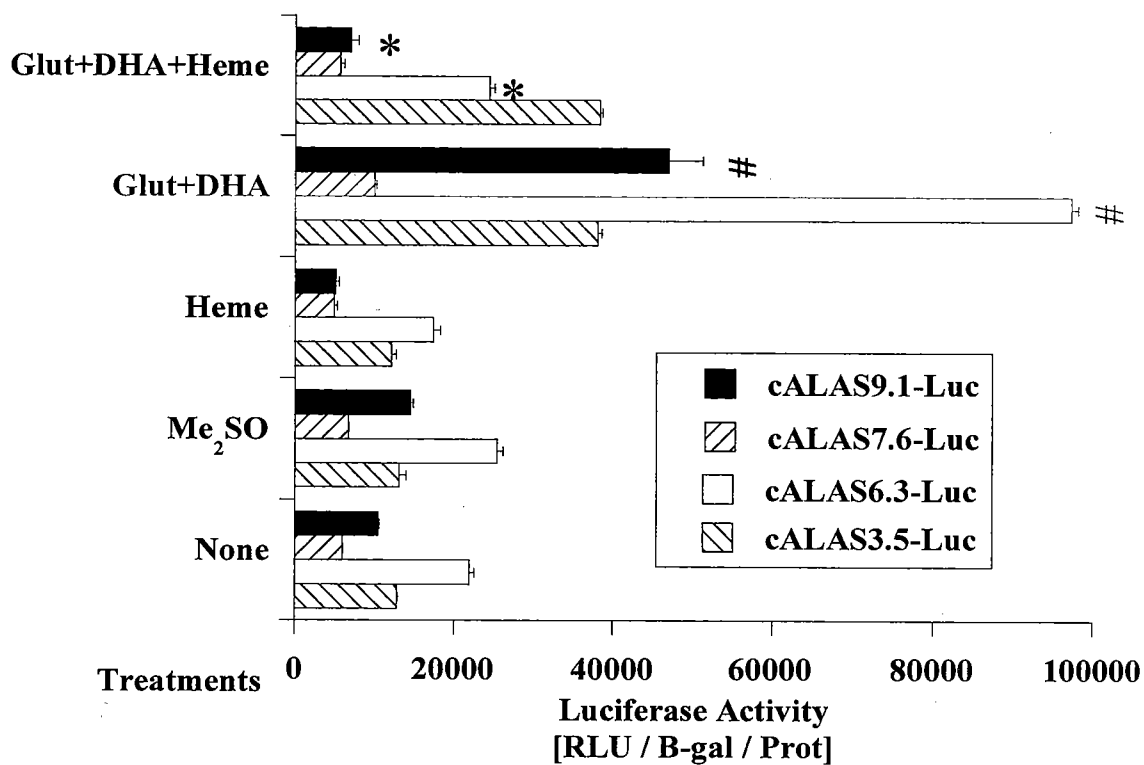
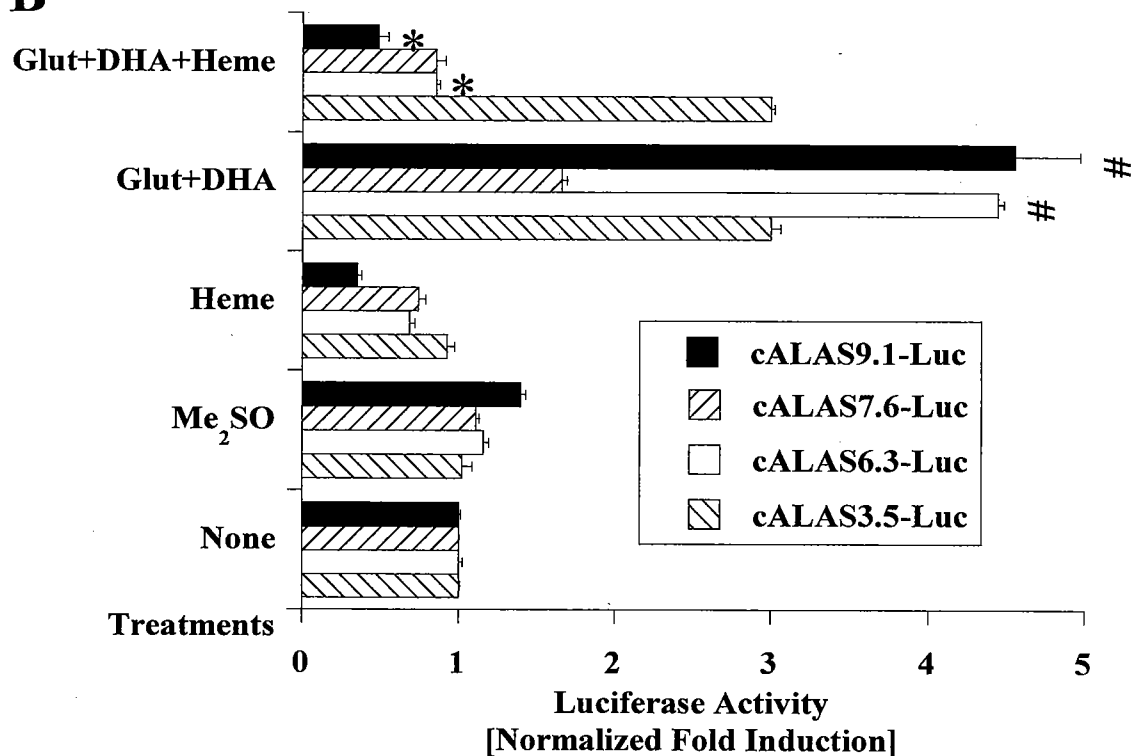
B



were also tested in LMH cells stably-transfected with pGcALAS9.1-, 7.6-, 6.3- and 3.5-Luc, as described in Chapter II. Several independent single colonies were tested and only those found to give similar results to that shown previously for transient transfections (Figures 5.7 A and B) were used (Figures 5.8 A and B). DMSO (Me₂SO) alone had no significant repressive effect on luciferase activity of the different clones, but 20 μ M heme dissolved in DMSO repressed luciferase of pGcALAS9.1-, 7.6- and 6.3-Luc to 35%, 70%, and 68% of control, respectively, whereas the luciferase activity of pGcLAS3.5-Luc was unaffected (Figure 5.8 A). This effect of heme to repress was seen more strikingly on Glut and DHA-induced luciferase activity of pGcALAS6.3-Luc, which was substantially reduced to 19% in the presence of heme (Figures 5.8 A and B). However, as shown before (Figure 5.3 A) the Glut induced activity of pGcALAS3.5-Luc is not affected by the addition of 20 μ M heme (Figure 5.8 A and B). This suggests that the -3.5 to -6.3 kb 5'-flanking region of ALAS-1 contains one or more *cis*-acting element(s) that mediate heme-repression of basal and drug-induced luciferase activity.

Effect of increasing concentrations of Glut on pGcALAS6.3-Luc: Since the 7.6 kb construct could not be induced by Glut and DHA (Figures 5.7 B and

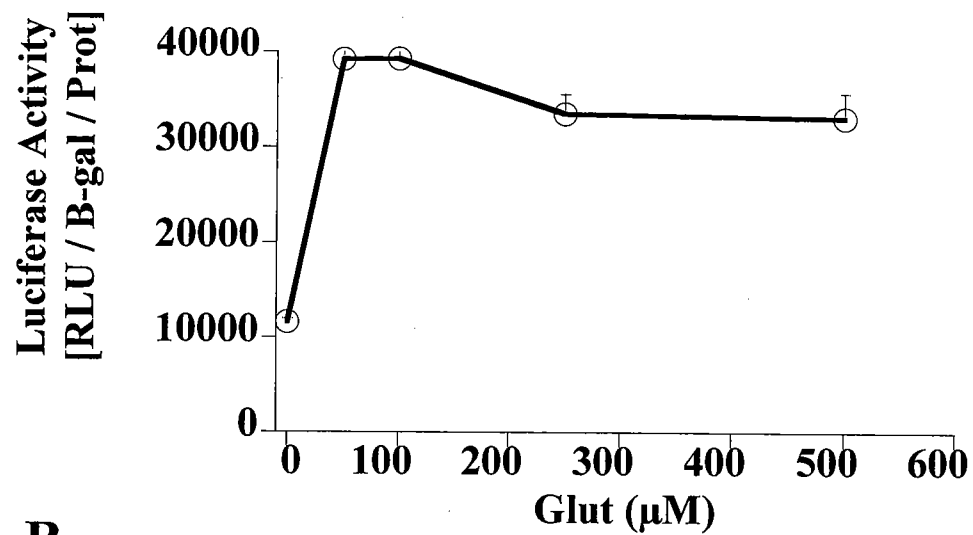
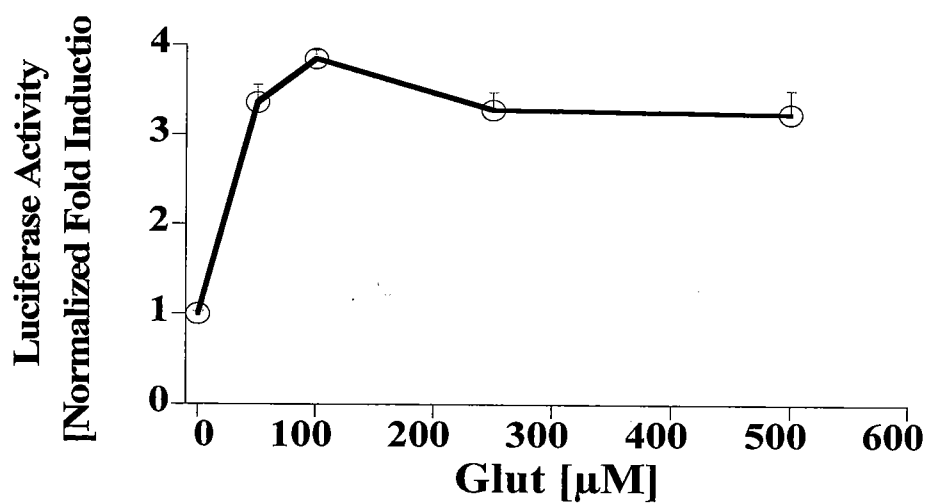
Figure 5.8. Comparative effects of Glut and DHA, and heme on ALAS/Luc constructs shown as normalized RLU (A) or normalized fold induction (B). LMH cells stably-transfected with ALAS-Luc constructs as in Chapter II were treated with chemicals and maintained at 37°C for 16 h, after which cells were harvested and used to measure luciferase activity and proteins as described in Chapter II. Concentrations of the selected chemicals were as follows: Glut, 50 μ M, or DHA, 250 μ M, and heme, 20 μ M. Results are means + SE of 3-5 independent experiments in triplicate. Luciferase activities are expressed as RLU or fold induction over untreated control normalized to proteins. *Significant decrease from cells transfected with pGcALAS9.1- or 6.3-Luc and treated Glut and DHA, $P < 0.001$. # Significant increase from all cells transfected with pGcALAS9.1- or 6.3-Luc, $P < 0.001$.

A**B**

5.8 B, the effect of increasing concentrations of Glut alone (50-500 μ M) on pGcALAS6.3-Luc was determined (Figures 5.9 A and B). Glut (50 μ M and 100 μ M) increased pGcALAS6.3-Luc activity \sim 3.3- and 3.8-fold, respectively, which was then maintained at \sim 3.3-fold at 250 and 500 μ M Glut (Figures 5.9 A and B). The pattern of induction of pGcALAS6.3-Luc by increasing concentrations of Glut (50-500 μ M) (Figure 5.9 B) was similar to that seen before for pGcALAS3.5-Luc (Figure 5.2 A) suggesting that the 6.3 kb construct retains drug-induction as well as heme-mediated repression of basal and Glut and DHA-mediated induction similar to the -9.1 kb construct (Figures 5.7 A and B and 5.8 A and B).

In summary, the data presented till now provide supporting evidence that the +0.082 to -3447 bp region in the 5'-flanking and 5'-UTR of ALAS-1 plays a role in drug-mediated induction of chick ALAS-1 as shown previously (Fraser et al., 2002) and that the -3448 to -6266 bp 5'-flanking region mediates heme-responsiveness. In addition, the observation that the -7676 bp to -6267 bp region (as shown in the 7.6 kb construct) showed heme repression of basal activity but could not be induced by Glut and DHA suggested non-heme-dependent repression of drug induction (Saatcioglu et al., 1994).

Figure 5.9. Effect of increasing concentrations of Glut on pGcALAS6.3-Luc shown as normalized relative luciferase activity (A) and normalized fold induction (B). LMH cells stably transfected with pGcALAS6.3-Luc were either left untreated or treated with increasing concentrations of Glut (0, 50, 250, or 500 μ M) and maintained at 37°C for 16 h, after which cells were harvested and assayed for luciferase activity and proteins as described in Chapter II. Luciferase activities are expressed as relative luciferase activity or as fold induction over untreated control normalized to proteins. Results are means + SE of 3-5 independent experiments in triplicate. Values for all concentrations of Glut > 0 μ M differ from 0, $P < 0.01$. The EC_{50} was calculated to be 40.92 μ M Glut by Non-linear Regression analysis ($R^2 = 0.953$) using the Hill, 4 Parameter method.

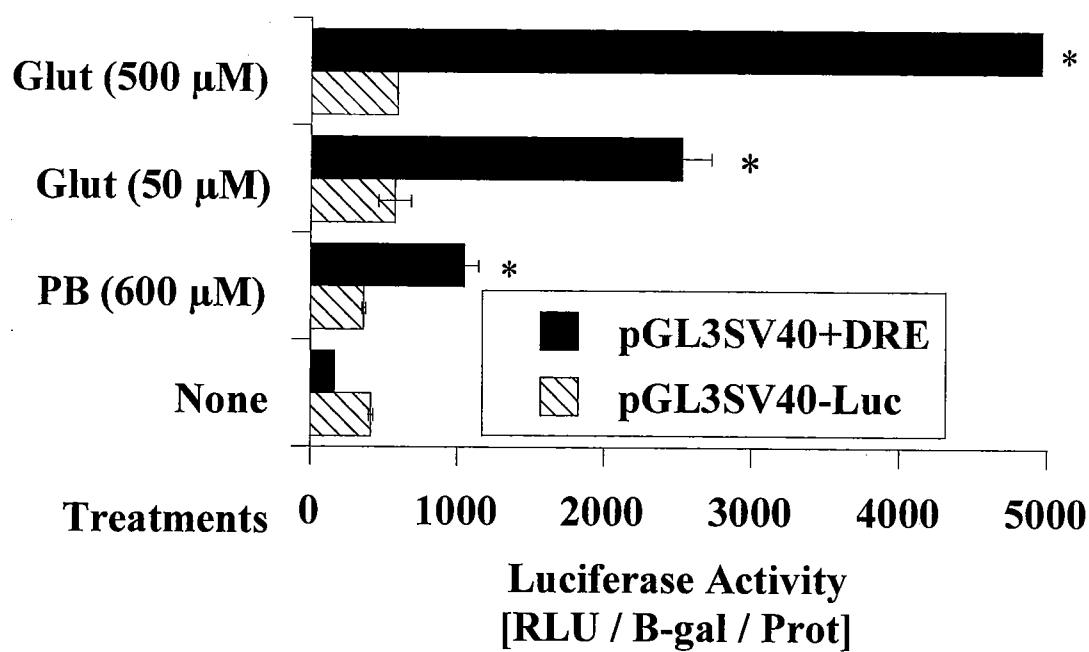
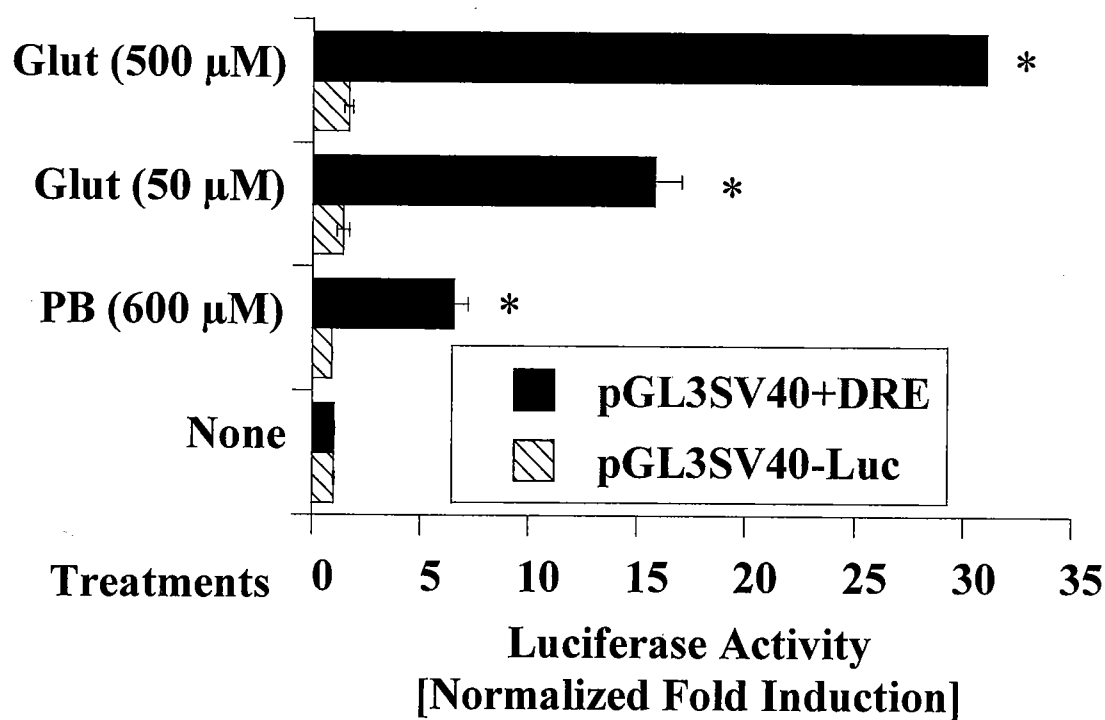
A**B**

Effect of PB, Glut, and heme on Drug-Responsive Element (DRE)

constructs of chick ALAS-1 gene: As mentioned before, two separate (176 bp and 167 bp) drug-responsive enhancer sequences (DRE's) upstream from the -9.1 kb region have been shown recently to play a key role in drug-induction of chick ALAS-1 (Fraser et al., 2002). In an effort to insert the drug-responsive elements (DRE) upstream of the heme responsive region of ALAS-1, a 522 bp region containing the 167 bp enhancer sequence with primers containing Mlu I sites was PCR amplified (Appendix E). This 522 bp PCR product was inserted into the Mlu I site of the multiple cloning sites of pGL3SV40 promoter, pGcALAS 9.1-Luc, and pGcALAS0.3-Luc constructs upstream of the XhoI site in its natural orientation in the cALAS-1 sequence (Chapter II).

The effects of drugs on the above constructs were tested and compared with their effects on constructs without the DRE in transiently transfected LMH cells (Figures 5.10 A and B). As described previously (Fraser et al., 2002), 500 μ M Glut increased pGL3-SV40+DRE activity 31-fold (Figure 5.10 B), as compared with vehicle-treated control. PB-Na (600 μ M) and Glut (50 μ M) induced to a lesser extent i.e. 6.5- and 16-fold respectively over controls (Figure 5.10 B).

Figure 5.10. Comparative effect of drugs on pGL3-SV40 promoter constructs without and with 522 bp PCR product containing cALAS-1 167 bp DRE in transiently-transfected LMH cells. Data are shown as normalized relative luciferase activity (A) and normalized fold induction (B). LMH cells were plated on 24-well (1.4 cm) plates at a density of 0.75×10^6 . Transfections, preparation of and treatment with Glut and PB-Na were performed as described in Chapter II. LMH cells were harvested after 16 h of exposure to selected chemicals and assayed as described in Chapter II. Concentrations of the selected chemicals were as follows: Glut, 50 or 500 μM , phenobarbital (PB), 600 μM . Results are means \pm SE of 7 independent experiments in triplicate. *Differs from Glut, or PB-Na treated LMH cells transfected with pGL3-SV40 promoter without DRE or untreated cells transfected with pGL3-SV40+DRE, $P < 0.001$.

A**B**

In contrast, there was no significant effect of the chemicals on pGL3-SV40 promoter construct (Figures 5.10 A and B) without the DRE.

The effect of Glut (50 μ M) and PB-Na (600 μ M) alone or Glut (50 μ M) in combination with heme was tested on pGcALAS9.1-Luc +DRE and compared with their effects on pGcALAS9.1-Luc. There was no major difference in the basal activity of the two 9.1 constructs with and without DRE (Figure 5.11 A). Addition of the DRE increased the induction by 50 μ M Glut (1.6 to 4.8-fold) and by 600 μ M PB-Na (from none to 3.2-fold) (Figure 5.11 B). 20 μ M Heme repressed Glut induction of pGcALAS9.1-Luc by 53%.

Next the effect of DRE addition on the shorter ALAS-1 construct pGcALAS0.3-Luc, a construct that is sufficient for ALAS-1 basal promoter activity (TJ Sadlon unpublished communication), was tested and compared with the construct without the DRE (Figures 5.12 A and B). Neither heme (20 μ M) nor Glut (50 μ M) had any effect on pGcALAS0.3-Luc construct without the DRE (Figures 5.12 A and B). Interestingly, Glut (50 μ M) increased pGcALAS0.3-Luc+DRE activity only 3.7-fold (Figure 5.12. B).

Figure 5.11. Comparative effect of drugs on pGcALAS9.1-Luc constructs without and with 522 bp PCR product containing cALAS-1 167 bp DRE in transiently-transfected LMH cells. Data are shown as normalized relative luciferase activity (A) and normalized fold induction (B). Transfections, preparation of and treatment with selected chemicals were performed as described in Chapter II. LMH cells were harvested after 16 h of exposure to selected chemicals and assayed as described in Chapter II. Concentrations of the selected chemicals were as follows: Glut, 50 μ M or PB-Na, 600 μ M, and heme, 20 μ M. Results are means \pm SE of 7 independent experiments in triplicate. *Differs from Glut treated LMH cells transfected with pGcALAS9.1-Luc+DRE, $P < 0.01$.

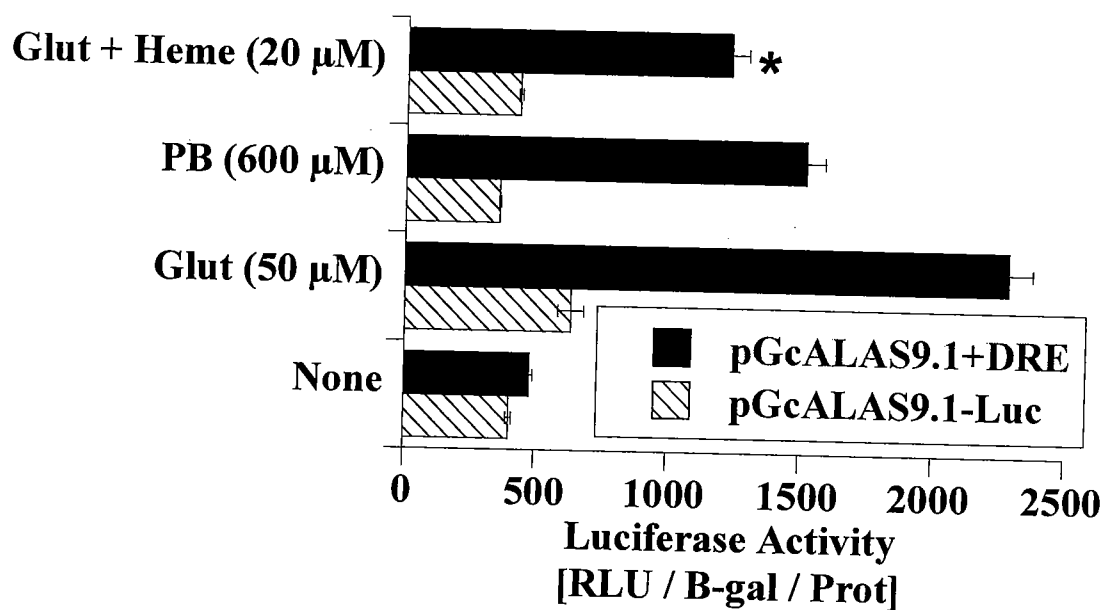
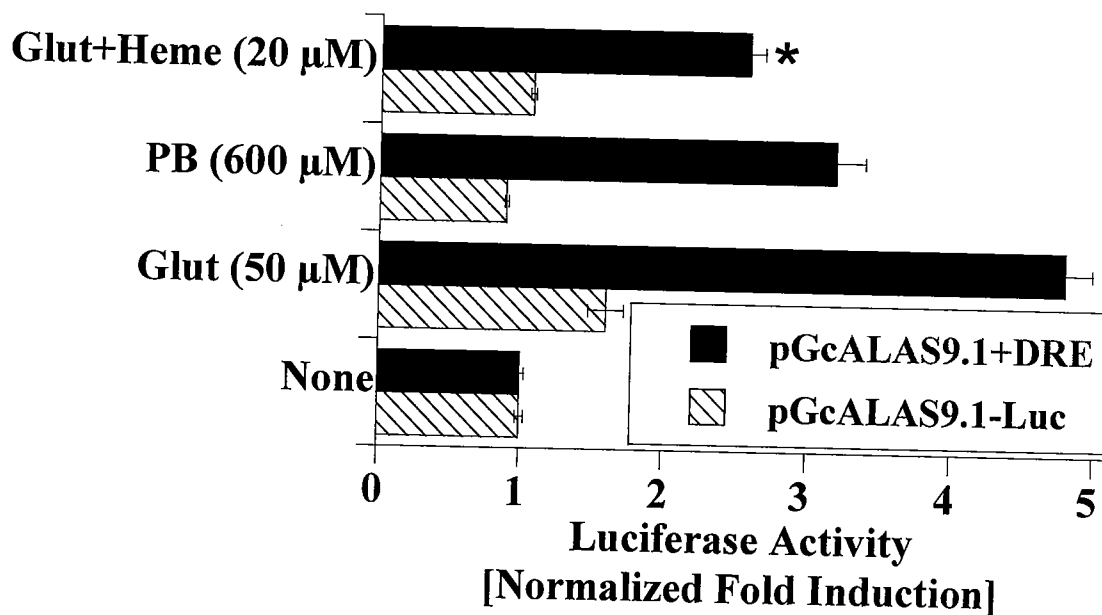
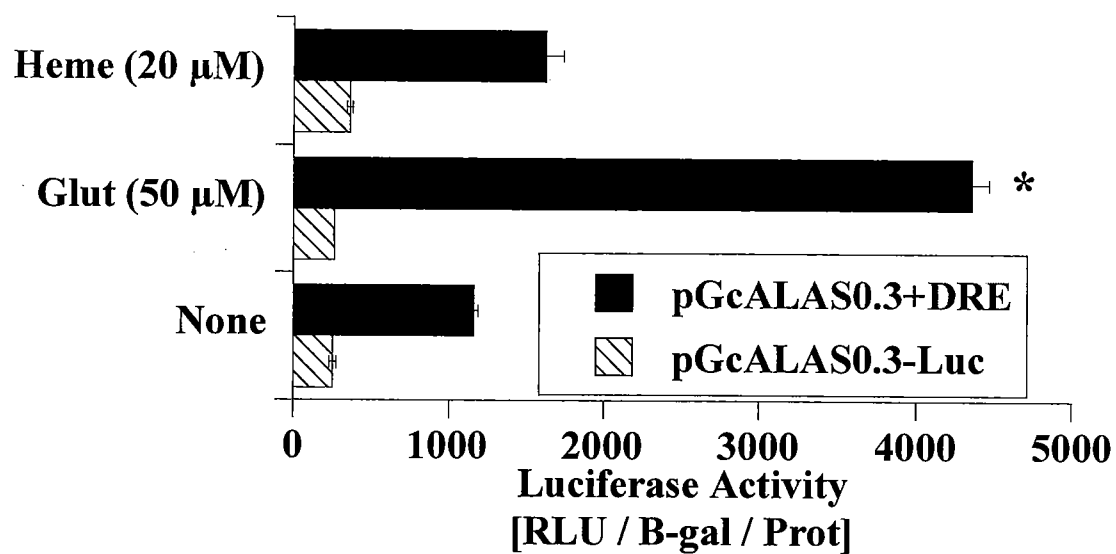
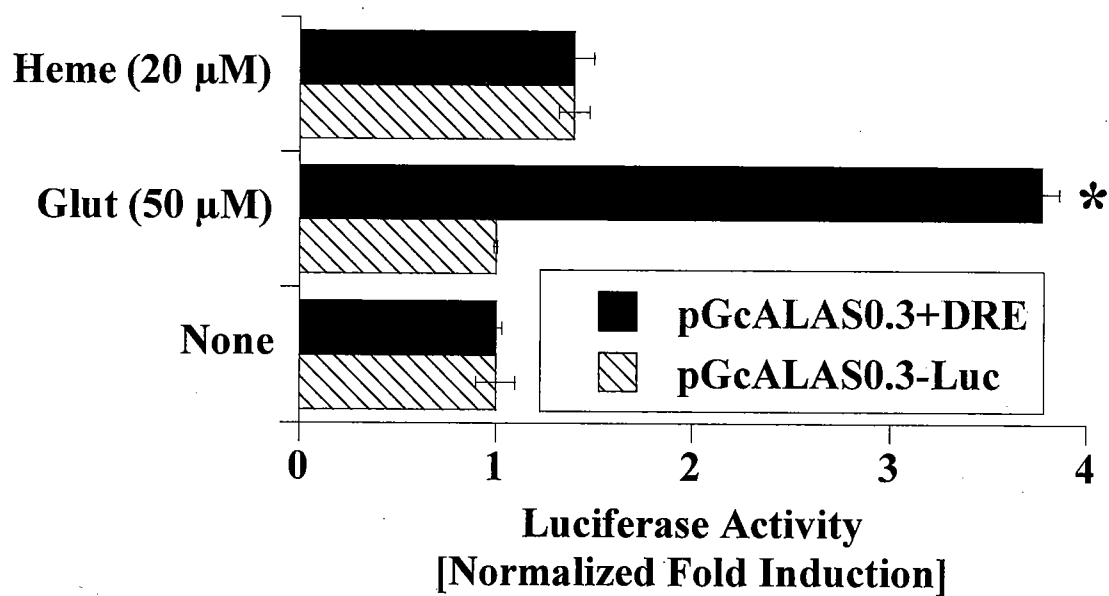
A**B**

Figure 5.12. Comparative effect of drugs on pGcALAS0.3-Luc constructs without and with 522 bp PCR product containing cALAS-1 167 bp DRE in transiently-transfected LMH cells. Data are shown as normalized relative luciferase activity (A) and normalized fold induction (B). Transfections, preparation of and treatment with selected chemicals were performed as described in Chapter II. LMH cells were harvested after 16 h of exposure to selected chemicals and assayed as described in Chapter II. Concentrations of the selected chemicals were as follows: Glut, 50 μ M and heme, 20 μ M. Results are means \pm SE of 7 independent experiments in triplicate. *Differs from Glut treated LMH cells transfected with pGcALAS0.3-promoter construct without DRE, $P < 0.01$.

A**B**

Conclusions

The effects of drugs and heme on several constructs containing chick ALAS-1 5'-flanking and 5'-UTR region attached to a reporter (Luc) gene were tested by transient and stable transfections in LMH cells. Since the combination of 50 μ M Glut and 250 μ M DHA produced a synergistic upregulation of ALAS enzyme activity in LMH cells in Chapter III, Glut alone, DHA alone or in combination, were tested in cells transiently transfected with pGcALAS9.1-Luc or pGcALAS3.5-Luc (Figure 5.1 B and C). 50 μ M Glut increased pGcALAS3.5-Luc (3-fold) but there was no further effect of DHA (0, 250, 500, 750 μ M) or heme (20 μ M) on pGcALAS3.5-Luc (Figure 5.2 A and B and Figure 5.3 A). Searches of the entire 9.1 kb sequence (Appendix E) revealed no consensus DR4 or DR5 type recognition sequences for nuclear receptors that have been shown to be important for drug induction of CYP2H1 and ALAS-1 (Dogra et al., 1998; Handschin et al., 2000; Fraser et al., 2002). This suggests that the first 3.5 kb of cALAS-1 5'-flanking and 5'-UTR region of chick ALAS-1 contains non-DRE elements that contribute to drug-mediated induction of the gene. The Glut-mediated induction of the 3.5 kb and 6.3 kb constructs (~3-fold) was found to be remarkably consistent in this study in both stable and transiently

transfected LMH cells (Figures 5.2 and 5.9). A similar induction by Glut mediated by the non-DRE-containing upstream region of chick ALAS-1 gene was also reported by another group (Fraser et al., 2002). The observation that increasing concentrations of DHA (0, 250, 500, 750 μ M) or 20 μ M heme had no effect on 50 μ M Glut-induced reporter pGcALAS3.5-Luc activity (Figures 5.2 and 5.3) leads to the conclusion that drug induction is separate from the heme responsive region and is mediated through non-DRE element(s) in the first 3.5 kb promoter region of ALAS-1.

PB and other potent porphyrinogenic chemicals such as AIA, or Glut increased pGcALAS9.1-Luc activity approximately 1.5-2-fold over control (Figures 5.3 and 5.5 B). As seen before for ALAS enzyme activity (Figures 3.2 and 3.6), 250 μ M DHA alone had no effect pGcALAS9.1-Luc activity but the combination of 50 μ M Glut and 250 μ M DHA for 16 h produced a synergistic up-regulation, which was repressed by the addition of 20 μ M heme (Figure 5.3 B).

Dose-response studies showed that the heme effect on pGcALAS9.1-Luc was dose-dependent and reached a maximum at 20 μ M heme, which was maintained at 40 μ M heme (Figures 5.4 A and B). The ability of DHA, an inhibitor of heme biosynthesis, to synergistically up-regulate ALAS-1

gene expression and heme to down-regulate basal and Glut-and DHA-induced luciferase activity suggested that the amount of heme in the cell can regulate basal ALAS-1 and also determine the extent of drug induction. Deletion constructs of the heme responsive -9.1 to -3.5 kb region were generated and tested. Addition of 250 μ M DHA in combination with 50 μ M Glut induced pGcALAS6.3Luc 5.3-fold, similar to that of pGcALAS9.1-Luc (4.4-fold) (Figure 5.7 B). Heme (20 μ M) also repressed basal and Glut- and DHA-induced pGcALAS6.3-Luc activity, as in pGcALAS9.1-Luc, suggesting that the 2.8 kb region in the -6.3 to -3.5 kb 5'-flanking region of ALAS-1 contains element(s) that determine heme responsiveness (Figures 5.7 and 5.8 A and B). The pattern of induction of pGcALAS6.3-Luc by increasing concentrations of Glut is similar (~3-fold) to that observed for pGcALAS3.5-Luc (Figure 5.9 A and B vs. 5.2 A). For unknown reasons, the 7.6 kb ALAS-1 construct could not be induced significantly by Glut and DHA, even though it showed heme-dependent repression (50% of basal activity) (Figure 5.7). Further work is needed to establish if the non heme-dependent repression of drug induction in pGcALAS7.6-Luc is mediated by the AP-1 element(s) that are present in the -7.6 to -6.3 kb region of ALAS-1 (Appendix E) (Saatcioglu et al., 1994).

Discussion of heme effects on -6.3 to -3.5 kb region of ALAS-1: The early work of Granick in 1966 established that in chick embryo hepatocyte cultures drug inducibility of hepatic ALAS activity is prevented by the simultaneous administration of heme. The mechanism of this has remained a central question ever since (Sassa and Nagai., 1996). It was originally postulated that heme and drugs compete for a site on a gene-controlling protein. This was challenged by the work of Srivastava et al., (1980) who showed that heme repression appeared to be the sole control and suggested that drug induction was probably a secondary consequence of heme removal. However, it was reported that drugs can directly increase murine and chick ALAS-1 mRNA levels and gene expression (Hamilton et al., 1988), and DR4 and DR5 sites for nuclear receptors, that mediate this induction, have been identified far upstream of the transcriptional start point (Fraser et al., 2002; Fraser et al., 2003).

At the translational and post-translational levels, heme has been shown to reduce ALAS-1 mRNA stability (Drew and Ades 1989; Hamilton et al., 1991; Cable et al., 1996; Cable et al., 2000) and prevent the entry of newly synthesized ALAS-1 into the mitochondrion (Kikuchi et al., 1983). However, with respect to the molecular mechanisms(s) for heme repression

of ALAS-1 mRNA (p. 7), controversy has persisted (Reviewed in May et al., 1995). It has been variously shown that heme represses ALAS-1 at the translational level (decrease of mRNA stability) but not on the transcriptional level (decrease of gene expression) (Hamilton et al., 1991) and vice versa (Srivastava et al., 1988; Srivastava et al, 1990; Ades 1990).

DeMena et al., 1999, reported that in Schneider cells 30 μ M heme (for 24 h) repressed the luciferase activity of a 121 bp region in the 5'-flanking region of the *Drosophila melanogaster* housekeeping ALAS gene. In contrast, effects of heme on ALAS-1 mRNA stability in CELCs and rat hepatoma cells have been observed at a much lower concentration of heme (1 μ M) (Hamilton et al., 1988; Cable et al., 2000).

Due to the complications associated with other mechanism(s) of heme regulation, the studies reported here used ALAS-1 promoter constructs and showed that Glut in combination with DHA, an inhibitor of heme synthesis, synergistically induced ALAS-1. The studies here also show that heme (20 μ M) repressed both basal and expression Glut- and DHA-mediated induction of ALAS-1 by one or more *cis*-acting elements in the -6.3 to -3.5 kb 5'-flanking region.

These differences in results of this and other labs showing transcriptional effects of heme on ALAS-1 (DeMena et al., 1999) compared to the studies showing heme effects on ALAS-1 mRNA stability and no effects of heme on ALAS-1 gene transcription in CELCs (Hamilton et al., 1991) and rat hepatoma cells (Cable et al., 2000), require further elaboration. Hamilton et al, 1991, measured the effects of 1 μ M heme and 0.15 mM DFO on ALAS-1 gene transcription rate in chick embryo hepatocytes after treatment for 1 h with 0.14 mM 2-propyl-2-isopropylacetamide (PIA). They observed no effects of heme or the inhibitor of ferrochelatase, DFO, on the PIA-induced increase in ALAS-1 transcription rate. Cable et al., found effects of 1.25 μ M heme on ALAS-1 mRNA stability starting at 1h and reaching a maximum at 6 h.

In this study, the drug, Glut, in combination with the inhibitor of heme synthesis, DHA, synergistically upregulated ALAS-promoter-luciferase activity and 20 μ M heme downregulated basal and Glut and DHA-induced luciferase activity suggesting that heme (10-20 μ M) regulates ALAS-1 gene transcription. The major differences between the transcriptional effects observed in LMH cells and fruit flies, and the studies showing effects on

mRNA stability is the time of treatment with inhibitors and heme (1-5h) and/or the concentrations of heme (1 μ M vs. 10-20 μ M).

Previous studies on the drug and DHA effects on ALAS-1 enzyme activity have suggested that prolonged (>12 h) incubation with drugs and DHA are required to obtain synergistic induction of ALAS-1 enzyme activity in CELCs (Russo et al., 1994), LMH cells (Kolluri et al., 1999) and rat hepatoma cells (Cable et al., 2000). Another possibility is that the effects of heme on ALAS-1 mRNA stability are seen at heme concentrations of 1 μ M in CELCs (Hamilton et al., 1991) or 1.25 μ M in rat hepatoma cells (Cable et al., 2000) while transcriptional effects of heme in intact rats (Srivastava et al., 1988), LMH cell cultures (this study) and fruit flies (Demena et al., 1999) are obtained at higher heme concentrations (10-30 μ M).

In humans, intravenous infusion of about 4 mg/kg body weight of heme (approximate initial plasma concentration \sim 10 μ M) causes clinical improvement and decreased excretion of heme pathway precursors, ALA and PBG, in patients with acute intermittent porphyria (Bonkowsky et al., 1971; Bonkovsky and Barnard 2000). After intravenous heme infusions the initial plasma heme concentration that results in decreased ALAS-1 activity

in patients with porphyria is ~92-108 μM (Karl Anderson personal communication). In this study, repressive effects on ALAS-1 Luc-reporter constructs are seen starting at 10 μM with a maximal effect at 20 μM heme (Figure 5.4).

Inhibition by heme (40%) of the translocation of an *in vitro* translated pre-ALAS-1 protein into the mitochondrion was observed *in vitro* starting at concentrations of heme from 3 to 30 μM (Hayashi et al., 1983; Yamamoto et al., 1983). These observations about the effects of time of incubation with heme, and the concentrations of heme needed to produce effects on mRNA stability (Hamilton et al., 1991) vs. gene expression [(Srivastava et al., 1988) and the results presented in this Chapter], taken together, suggest different regulatory mechanisms of heme on ALAS-1 mRNA and gene expression based upon the “free heme” concentration (or based on the extent of heme pool depletion) in the liver cell. At low concentrations of heme effects on ALAS-1 mRNA stability (1 μM) and on ALAS-1 translocation (3 μM) into the mitochondrion were observed in chicks (Hamilton et al., 1988; Hayashi et al., 1983) while the effects on ALAS-1 gene expression in this study in LMH cells were observed at higher concentrations of heme (10 and 20 μM).

A 520 bp PCR product containing a 167 bp chick ALAS-1 drug-responsive element is capable of activating pGL3-SV40 promoter construct 15-30-fold in response to Glut (Figure 5.10 B) but activates pGcALAS9.1-Luc and pGcALAS0.3-Luc only 3.2- and 3.7-fold respectively (Figures 5.11 B and 5.12 B). One interpretation for these results is that the +0.082 to 0.3 kb region contains element(s) that contribute to non-heme mediated repression of drug-induction (Figures 5.7 and 5.8) (Kamei et al., 1996; Dilworth and Chambion 2001; Akiyama et al., 2003).

Conclusions: Thus the data presented in this Chapter demonstrate that drugs increase ALAS-1 gene expression (through non-DRE elements in the +0.082 to -3.5 kb region) and that high concentrations of heme down-regulates basal and drug-induction of chick ALAS-1 through element(s) in the -6.3 to -3.5 kb 5'-flanking region.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Studies of the heme biosynthetic pathway: In Chapter III, studies of the heme biosynthetic pathway and its regulation by chemicals and heme are described in LMH cells and compared with primary chick embryo liver cells (CELCs). LMH cells exhibited analogous responses to CELCs in terms of inducibility of ALAS-1 mRNA and activity, in response to chemicals that affect heme biosynthesis. LMH cells and primary cultures exhibited similar increases in levels of ALAS-1 mRNA and activity after treatment with chemicals that affect heme synthesis. Porphyrin accumulation and overproduction were observed in response to treatment with chemicals that are used in models of porphyria. A couple of minor differences were observed in LMH cells in the amount of ALA needed to accumulate porphyrins and the amount of protoporphyrin accumulated by treatment with Glut and PB. Firstly, LMH cells required higher concentrations of ALA (300 μ M) than CELCs (100 μ M) to accumulate the same amount of porphyrin (Figure 3.5). This may be related to differences in uptake of ALA into LMH

cells, as previously shown for human hepatoma cells (Iwasa et al., 1999), rat hepatocytes (Sinclair et al., 1990) and ES cells (Harigae et al., 1998).

Secondly, LMH cells accumulated porphyrins ($\sim 1/2$ was protoporphyrin) to higher levels than CELCs in response to phenobarbital or Glut alone and this accumulation was reduced by addition of $50 \mu\text{M}$ FeNTA (Figures 3.4 A and B). This is probably due the lower levels of iron being available for conversion of protoporphyrinogen to heme in LMH cells vs. CELCs. It is well known that actively replicating, growing cells require more iron than non-replicating cells and LMH cells are consistently more active in this regard (for recent review see Weinberg 2003).

LMH cells and CELCs displayed a marked up-regulation of ALAS activity in response to Glut ($50 \mu\text{M}$) in combination with the inhibitor of heme synthesis, DHA ($250 \mu\text{M}$) (Figures 3.1 B and 3.2 B). LMH cells also upregulated ALAS-1 mRNA after 6h of treatment with Glut alone or in combination with DHA or FeNTA. Since LMH cells were treated with the inhibitors of heme synthesis for only 6 h they did not significantly increase ALAS-1 mRNA levels compared with Glut alone (Figure 3.3). This may suggest that treatment for the given period of time (6 h) and/or the

concentrations of chemicals used is not sufficient to see the effects of the selected chemicals on the heme pool (Russo et al., 1994; Jover et al., 2000).

Because heme-dependent regulation of hepatic ALAS-1 mRNA is a major component of the normal regulation of heme biosynthesis in many species, a side-by-side comparison of ALAS-1 mRNA levels in LMH cells vs. CELCs was performed. Heme-dependent repression of ALAS-1 mRNA was observed for both cell types in the absence of any inducers (Figure 3.6). In addition, as shown previously in CELCs (Russo et al., 1994), heme decreased ALAS activity that has been increased by pretreatment with Glut and DHA and Glut and FeNTA in LMH cells (Figure 3.7). These studies supported the relevance of LMH cells as a useful model for further studying hepatic heme biosynthesis and its regulation.

Studies on the Glucose effect: The critical biochemical abnormality of the acute porphyrias is an uncontrolled up-regulation of ALAS-1 in the liver. In porphyric patients, ALAS-1 is highly inducible, especially in liver, in response to changes in nutritional status, and to many drugs that induce cytochrome P-450 and/or affect heme synthesis. High intakes of glucose or other metabolizable sugars and intravenous heme are the cornerstones of therapy of acute attacks of these types of hepatic porphyrias (Bonkovsky

1990; Sassa 2002). Both glucose and heme are known to repress the uncontrolled over-expression of hepatic ALAS-1 enzyme activity in the acute porphyrias, although their molecular mechanisms of action have not been fully characterized.

In Chapter IV, the glucose effect on ALAS-1 was studied in LMH cells using the combination of Glut and DHA as a model of acute porphyria. In preliminary studies, glucose, in a dose-dependent manner, reduced the Glut and DHA-induced ALAS activity in LMH cells (Figures 4.2 and 4.3) and CELCs (Figure 4.5). Glucose was also shown in LMH cells to abrogate ALAS activity induced by treatment with chemicals previously shown to up regulate ALAS-1 enzyme activity such as Glut and FeNTA (Figure 4.4). Further experiments investigating the dose-response with glucose were completed in LMH cells.

To begin to define the molecular mechanism(s) of the glucose effect, further studies were done by transient transfections of LMH cells with a promoter-reporter construct containing approximately 9.1 kb of chick ALAS-1 5'-flanking region and 5'- untranslated region (UTR) attached to a luciferase reporter (pGcALAS9.1-Luc). Treatment for 16 h with Glut and DHA produced a synergistic (5-fold) up-regulation of ALAS-1 promoter-

reporter activity in LMH cells transiently transfected with pGcALAS9.1-Luc (Figure 4.6). Addition of glucose, in a dose-dependent manner, decreased the Glut and DHA up-regulation of pGcALAS9.1-Luc activity (Figure 4.6). Other metabolizable sugars such as fructose, galactose, and intermediates of glucose metabolism such as glycerol and lactate, but not the non-metabolizable sugar sorbitol, also down-regulated the 9.1 kb construct (Figure 4.8). A long lasting analog of cAMP, namely, 8-CPTcAMP, augmented Glut-induction of pGcALAS9.1-Luc activity in a dose-dependent manner (Figure 4.9). Thus, the “glucose effect” on chick ALAS-1 is dependent upon element(s) in the first 9.1 kb of 5'-flanking region and 5'-UTR (-9.067 to +0.082 kb). Some of this effect may be mediated by the cAMP / CRE located at +0.039 kb (Figure 5.5). These effects were observed in the absence of insulin, glucagon, other hormones or serum. Thus, the “glucose effect” on ALAS-1 is not dependent upon effects of glucose administration on pancreatic islet cells.

Drug and heme studies: ALAS-1 is up regulated by chemicals that also induce CYP (Jover et al., 1996). Some evidence has suggested that at least two mechanisms of drug induction of ALAS-1 exist, one that is by the direct effect of the drug on ALAS-1 gene expression (Hamilton et al., 1988;

Fraser et al., 2002), and another that is mediated by its effect on the heme-pool by induction of cytochrome P-450 apoproteins (heme-dependent) (Sassa and Nagai, 1996; Kappas et al., 2000). However, there has been no direct evidence for an effect of depletion of a regulatory heme-pool on ALAS-1 induction, and *cis*-acting promoter elements that mediate heme-dependent repression of basal or drug-induced ALAS-1 heretofore have not been characterized, in mammals or birds.

In Chapter V, drug and heme studies using transfected (transient / stable) parental ALAS 9.1 kb and 3.5 kb deletion constructs showed that while the +82 to -3447 bp region mediates induction by drugs, the -3448 to -9067 bp region was responsible for heme-mediated repression of drug induction in the absence of DHA (Figures 5.1 B and C). Neither heme nor an inhibitor of *de novo* heme synthesis, DHA, had any effect on Glut induction of the 3.5 kb construct (Figures 5.2 A and B). In contrast, DHA resulted in derepression of drug induction and a synergistic induction of the 9.1 kb construct, which was decreased by the addition of exogenous heme (Figures 5.3 A and B). Taken together these data suggest that distinct and separate regions mediate regulation of ALAS-1 by drugs and heme (Figures 5.3 A and B). In addition, these results indicate that element(s) in the -3448

to -9067 bp region mediate(s) heme-dependent repression of ALAS-1 and are supportive of the hypothesis that a regulatory heme pool (See Chapter I, p. 7) can influence the extent of drug induction of ALAS-1. A search of the entire 9149 cALAS-1 sequence (+82 to -9067) bp, including the heme responsive region (-3448 to -9067 bp), using the MacVector® analysis software program and TRANSFAC Database (<http://www.cbil.upenn.edu>), for putative drug- and heme- responsive elements showed 5 consensus AP-1 sites including an “expanded” AP-1 (Core B/AP-1) located at -1967 bp (Figure 5.5), shown to be important for heme induction of HO-1 (Alam et al., 1992; Alam et al., 1994; Shan et al., 2000; Shan et al., 2002). However, because the +82 to -3447 bp region (pGcALAS3.5-Luc construct) did not show heme-dependent repression (Figure 5.3 A), it suggests that the CoreB/AP-1 at -1967 is not involved in heme-repression of ALAS-1.

Deletions of the -9067 to -3448 region were generated and tested. Transient and stable transfections of LMH cells with further deletions of the heme responsive region, starting from the Xho I site (at -9067) to Pac I (-7676) or Pml 1 (-6266) (Appendix E), retained heme-dependent repression suggesting that the -3448 to -6266 bp cALAS-1 5'-flanking region is involved in mediating heme repression. In addition, the promoter and

proximal 5'-flanking (+82 to -3448) drug-responsive region did not contain recently defined DRE sequences that lie far upstream of the transcription start site and have been shown to participate in the drug-mediated induction of chick and murine ALAS-1 (Fraser et al., 2002; Fraser et al., 2003). Therefore, the +82 to -3447 bp region of ALAS-1 is involved in non-DRE mediated up regulation in response to drugs.

Studies on DRE: Basal expression of the ALAS-1 gene is driven by a number of specific transcription binding sites in the first 300 bp of promoter sequence (Appendix E) such as CREB (Giono et al, 2001) and NRF-1 (Li et al., 1999). The pGcALAS0.3-Luc (+82 to -317 bp) construct was tested in LMH cells and basal activity of luciferase was expressed. This construct was not upregulated by Glut nor repressed by heme and hence was used to help delineate the heme-responsive region. To aid in this effort, DRE sequences recently defined in the chick ALAS-1 gene (Fraser et al., 2002) were inserted upstream of the 0.3- and 9.1-kb constructs and of the enhancerless pGL3-SV40 promoter construct to generate the fusion constructs pGcALAS0.3-Luc+DRE and pGcALAS9.1-Luc+DRE and pGL3-SV40+DRE, respectively. These were tested for drug inducibility and heme repression. As shown previously (Fraser et al., 2002), the pGL3-SV40+DRE

construct was induced many fold [~ 30 -fold by Glut (500 μM) and 16-fold by Glut (50 μM)], but interestingly, pGcALAS9.1-Luc and pGcALAS0.3-Luc were induced by 50 μM Glut only 3.2-fold (1.6 to 4.8-fold) and 3.7-fold, respectively (Figures 5.10, 5.11 and 5.12 B).

Discussion of results with DRE constructs: Although negative binding elements have been described, repression is mainly conducted by interference with other transcription factors (Saatcioglu et al., 1994). Transcriptional coactivators, including the cAMP response element-binding protein (CBP) and its structural homolog p300 (Bannister and Kouzarides 1996) not only link transcriptional activators to the transcription machinery but also have been found to possess intrinsic histone acetyltransferase activity that is required for transcription factor function (Kamei et al., 1996; Perissi et al., 1999; Dilworth and Chambion 2001). Recent evidence indicates that the coactivator CBP and its structural homolog p300 are important for nuclear receptor function (Dilworth and Chambon, 2001; Chakravarti et al., 1996) including CXR-mediated induction of the CYP2H1 gene (Dogra et al., 2003). CBP is also essential for CREB regulation of basal and cAMP-stimulated expression of ALAS-1 (Guberman et al., 2003).

The present finding that drug induction from the DRE construct (pGcALAS0.3Luc+DRE) is repressed by element(s) in the +82 to -317 bp region of ALAS-1 (Figure 5.12 A and B) is in keeping with the notion that competition for limiting amounts of the coactivator CBP/p300 in cells can result in inhibition of gene activation by transcription activators (Kamei et al., 1996; Dogra et al., 2003; Guberman et al., 2003).

These DRE studies suggest that, as seen before for the 7.6 kb construct of ALAS-1, the -0.3 to +0.082 kb 5'-flanking and 5'-UTR region contains element(s) that negatively regulate ALAS-1 induction by drugs as seen previously (Saatcioglu et al., 1994; Dilworth and Chambion, 2001; Dogra et al., 2003).

Conclusions: Drugs up-regulate ALAS-1 mRNA and activity in LMH cells as shown previously in CELCs. This up-regulation is mediated through transcriptional effects of the drugs on non-DRE and DRE-elements in the 5'-flanking region of ALAS-1. Heme and glucose down-regulate ALAS-1 transcription through element(s) in the 5'-flanking region and therefore in their presence induction of ALAS-1 is decreased as seen before in patients with porphyria.

Future Directions

Further characterization of the repressive effect of heme on ALAS-1 constructs: One promising line of experiments is to identify heme-responsive element(s) in the -3445 to -6266 bp region and factor(s) that regulate ALAS-1 promoter expression and that are influenced by heme. Because conveniently located restriction sites are limited in this region, PCR amplification of this region using a high fidelity polymerase, such as Pfu DNA polymerase, and specific primers should be used to generate several smaller deletions of this region. The PCR products can then be amplified using a second set of primers containing Xho1 sites as described in Chapter II for generating the DRE constructs. Following the methods outlined in Chapter II these products can be digested with Xho1 and inserted downstream (as described in Chapter III) of the DRE (which has been inserted in the Mlu1 site of the multiple-cloning site) of the pGL3SV40+DRE-construct, shown in these studies and by Fraser et al., 2002, to increase luciferase activity ~30-40-fold in response to Glut (500 μ M) (Figure 5.10 B).

LMH cells transfected with these constructs can be then treated with heme or DHA alone or in the presence of Glut to delineate the heme responsive region. In light of the studies presented in Chapter V that heme can repress drug-induction of ALAS-1, using the pGL3SV40+DRE construct containing the putative heme responsive region(s) should increase the sensitivity and facilitate easy identification of the key region(s). Once an ~100-50 bp region is identified, it can be analyzed using electrophoretic mobility shift assays and site-directed mutagenesis of candidate regulatory elements in this region with the goal of functional characterization of the heme responsive element(s). Such studies will lead to the ultimate goal of identifying proteins and molecular mechanism(s) that play key roles in heme-dependent regulation of ALAS-1.

Determination of the *cis*-acting element(s) that determine mRNA stability (identification of 3'-UTR elements in the ALAS-1 mRNA) and those that mediate heme responsiveness in the 5'-flanking region of the ALAS-1 gene will shed more light into the question of how heme regulates ALAS-1. The data from this dissertation, that heme can regulate *cis*-acting element(s) in the 5'-flanking region of ALAS-1 and from studies of others demonstrating that heme decreases ALAS-1 mRNA stability and gene

transcription, as discussed in Chapter V, suggest different regulatory mechanisms at different heme levels in the cells. Of particular interest will be studies to determine the heme concentrations and time of treatment with heme or inhibitors of heme synthesis required for heme effects on transcription vs. stability.

The data presented in this dissertation also suggest that glucose and heme can regulate *cis*-acting element(s) in the 5'-flanking region of ALAS-1 as has been seen with ALAS-1 enzyme activity in patients with acute porphyric attacks. Further studies looking into the signaling pathway(s) and component(s) of the pathways that mediate the effects of "glucose" and heme will be of significance in acute porphyria. Of particular interest will be potential cross-talk between the signaling pathways.

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APPENDIX A: MEDIA AND BUFFERS

Media Contents

Waymouth's MB 752/1 Medium, powder		Williams' E Medium, powder
Component		
Amino Acids:	(mg/L)	(mg/L)
L-alanine	-	90
L-arginine	-	50
L-arginine.HCl	75	-
L-asparagine.H ₂ O	-	20
L-aspartic acid	60	30
L-cysteine	-	40
L-cysteine.2HCl	-	40
L-cysteine.HCl.H ₂ O	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.H ₂ O	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-trptophan	40	10
L-tyrosine.2Na.2H ₂ O	57.66	50.65
L-valine	65	50

Inorganic salts:

CaCl ₂	90.61	200
CuSO ₄ .5H ₂ O	-	0.0001
Fe(NO ₃) ₃ .9 H ₂ O	-	0.0001
KCl	150	400
KH ₂ PO ₄	80	-
MgCl ₂	112.56	-
MgSO ₄	97.67	97.67
MnCl ₂ .4H ₂ O	-	0.0001
NaCl	6000	6800
Na ₂ HPO ₄	300	-
Na ₂ HPO ₄ .H ₂ O	-	140
ZnSO ₄ .7H ₂ O	-	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	.05
D-Ca pantothenate	1	1
Choline chloride	250	1.5
Ergocalciferol	-	0.1
Folic acid	0.4	1
I-inositol	1	2
Menadione sodium	-	0.01
Niacinamide bisulphate	1	1
Pyridoxine.HCl	1	1
Riboflavin	1	0.1
α-Tocopherol phosphate, - sodium salt	-	0.01
Thiamine-HCl	10	1
Vitamin A acetate	-	0.1
Vitamin B ₁₂	0.2	0.2

Williams' E Special Medium without glucose or sodium pyruvate contained all of the above **except** 2000 mg/L glucose and 25 mg/L sodium pyruvate.

Williams' E Regular medium was supplemented with:

1.5 μ M 3,5,3'-triiodo-L-thyronine

0.76 μ M dexamethasone

200 μ M glutamine

Waymouth's MB 752/1 Complete Medium was supplemented with:

100 U/mL penicillin

100 μ g/mL streptomycin

10% (v/v) fetal bovine serum (GIBCO)

OptiMEM® Reduced Serum Media was purchased as a liquid from Life Technologies, Inc. No modification or supplementation was made.

Buffers

Phosphate Buffered Saline (PBS):

100 mM KPi
0.15 M KCl
Adjust to pH 7.4

10X HEPES Buffer:

200 mM HEPES
50 mM sodium acetate
10 mM EDTA
Adjust pH to 7.2 with NaOH
Filter to sterilize

20X SSC:

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

Harvest and Assay Stock Buffer (ALAS Activity assays):

35 mM Tris
30 mM NaH_2PO_4
8 mM MgCl_2
1 mM EDTA
15 mM succinate (disodium salt, 6 H_2O)
10 mM sodium laevulinate
Adjust pH to 7.4 with HCl and add
0.5 mM pyridoxal-5'-phosphate
Stable for 1 month at 4°C

Working Reagent: To 25 mL add just before using:

10 mM ATP
0.1 mM CoEnzyme A
5 mM β -mercaptoethanol
100 mM KF.2 H_2O
12.5U succinic thiokinase

Modified Ehrlich's Solution:

Glacial acetic acid 168 mL
 70 % Perchloric acid 32 mL
 Mercuric chloride 0.75 g
 Add 200 mg of p-dimethylaminobenzaldehyde (DMAB)/10 mL
 Ehrlich's reagent just before using.

TE Buffer:

10 mM Tris.Cl, pH 7.5 or 8.0
 1 mM EDTA, pH 8.0

Z Buffer (β -galactosidase assays):

60 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$
 40 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$
 10 mM KCl
 1 mM MgSO_4
 Adjust pH to 7.0; store at room temperature

ONPG (*O*-nitrophenylgalactoside):

4 or 5 mg/mL in 0.1 M potassium phosphate, pH 7.0
 Filter and freeze.

Glycylglycine Harvest Buffer (Luciferase assays):

25 mM glycylglycine, pH 7.8
 15 mM potassium phosphate, pH 7.8
 15 mM MgSO_4
 4 mM EGTA
 1 mM DTT (added just prior to use)

Bicinchoninic Acid Protein Assay Stock Reagents:

Reagent A (0.125% BCA): To 950 mL MQ add

161 mM $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$
 5.67 mM sodium potassium tartrate
 113 mM NaHCO_3
 75 mM NaOH
 3.63 mM bicinchoninic acid
 Adjust pH to 11.25 with NaOH, QC to 1000 mL
 store at room temperature.

Reagent B:

160 mM cupric sulphate. Store at room temperature.

Working Reagent:

Mix 100 volumes of stock Reagent A with 2 volumes of stock Reagent B. Stable for 1 week.

APPENDIX B: PREPARATION OF CHEMICALS FOR CELL CULTURE

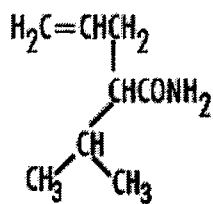
TREATMENT:	ABBREVIATION:	DOSE IN CULTURE:	PREPARATION:
δ -aminolevulinate	ALA	10 μ L/mL media, 18 μ M final	0.587 mg/mL H ₂ O
Allyl isopropyl acetamide	AIA	1 μ L/mL media, 250 μ M final	35 mg/mL DMSO
8-Chlorophenylthio cyclic adenosine monophosphate	8-CPT cAMP	3 μ L/mL media, 60 μ M final	3 mg/0.3 mL H ₂ O
Deferoxamine	DFO	10 μ L/mL media, 250 μ M final	16.25 mg/mL H ₂ O
2-Deoxyglucose	2-DOG	50 μ L/mL media, 12 mM final	37.93 mg/mL H ₂ O
Dexamethasone	DEX	1 μ L/mL media, 50 μ M final	295 mg/mL H ₂ O
4,6-dioxoheptanoic acid (Succinyl acetone)	DHA	.5 μ L/mL media, 250 μ M final	39.5 mg/.5 mL H ₂ O
Ferric nitrilotriacetic acid	FeNTA	10 μ L/mL media, 50 μ M final	1 mL of 4.05 mg/mL FeCl ₃ in H ₂ O 1 mL of 14.2 mg/mL NTA in H ₂ O 3 mLs of H ₂ O

Fructose	Fructose	50 µl/ml media, 11 mM final	41.63 mg/ml H ₂ O
Galactose	Galactose	50 µL/mL media, 11 mM final	41.63 mg/mL H ₂ O
Glucose	Gluc	50 µL/mL media, 11 mM final	41.63 mg/mL H ₂ O
Glutethimide	Glut	.5 µL/mL media, 50 µM final	10.4 mg/.5 mL DMSO
Glycerol	Glycerol	24 µL/mL media, 33 mM final	100 µL of 13.693 moles/lit of glycerol stock 900 µL H ₂ O
Heme		1 µL/mL media, 20 µM final	13.0481 mg/mL DMSO
Lactate	Lactate	50 µL/mL media, 11 mM final	20.80 mg/mL H ₂ O
Met	Met	1 µL/mL media, 400 µM final	90.52 mg/mL DMSO
Mifepristone	RU486	1 µL/mL media, 10 µM final	4.296 mg/mL DMSO
5-pregnene-3β-ol-20-one-16α-carbonitrile	PCN	1 µL/mL media, 20 µM final	6.830 mg/ mL DMSO
Phenobarbital-Na	PB-Na	10 µL/mL media, 600 µM final	15.24 mg/mL H ₂ O
Sorbitol	Sorbitol	50 µL/mL media, 11 mM final	42.1 mg/mL H ₂ O

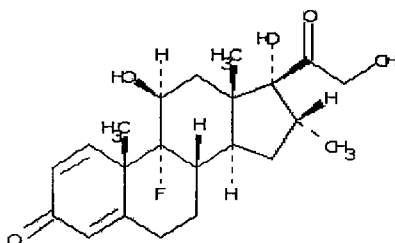
APPENDIX C: LIST OF PLASMIDS

NAME OF PLASMID	CONTAINS:	REFERENCE or SOURCE:
pBLUESCRIPT KS II+	DNA filler in transfections	Commercial
pPGK/ β -gal	β -galactosidase w/PGK promoter	Paul Dobner
pGL3-Basic	Luciferase vector w/no promoter or enhancer	Promega
pGL3-Control	Luciferase vector w/SV40 promoter and enhancer	Promega
pGL3-Promoter	Luciferase vector w/SV40 promoter	Promega
pGcALAS0.3-Luc	ALAS-1/luciferase reporter gene	Sridevi Kolluri
pGcALAS0.3-Luc+DRE (native orientation)	ALAS-1/luciferase reporter gene	Sridevi Kolluri
pGcALAS0.3-Luc+DRE (reverse orientation)	ALAS-1/luciferase reporter gene	Sridevi Kolluri
pGcALAS3.5-Luc	ALAS-1/luciferase reporter gene	Timothy J. Sadlon
pGcALAS4.6-Luc	ALAS-1/luciferase reporter gene	Sridevi Kolluri
pGcALAS6.3-Luc	ALAS-1/luciferase reporter gene	Sridevi Kolluri
pGcALAS8.0-Luc	ALAS-1/luciferase reporter gene	Sridevi Kolluri
pGcALAS9.1-Luc	ALAS-1/luciferase reporter gene	Timothy J. Sadlon
pGcALAS9.1mCREB	ALAS-1/luciferase reporter gene	Timothy J. Sadlon
pGcALAS9.1-Luc+DRE (native orientation)	ALAS-1/luciferase reporter gene	Sridevi Kolluri
pGcALAS9.1-Luc+DRE (reverse orientation)	ALAS-1/luciferase reporter gene	Sridevi Kolluri

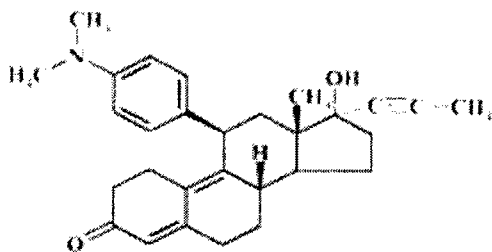
APPENDIX D: STRUCTURES OF CYP INDUCING CHEMICALS



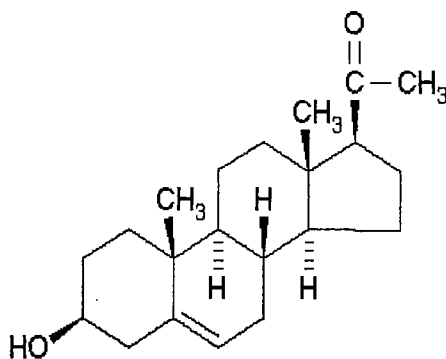
Allyl isopropyl acetamide



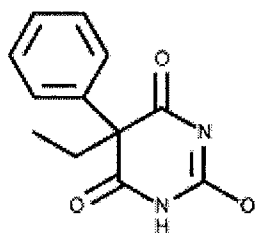
Dexamethasone



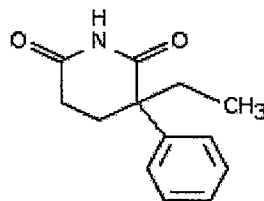
RU486



PCN



Phenobarbital



Glutethimide

APPENDIX E: **SEQUENCE OF ALAS-1 PROMOTER AND 5'-FLANKING** **REGION**

In the following sequence the restriction cut sites are shown by ~ ~ ~ swiggly lines. Sites that define the end of a reporter construct (shown in Figure 5.6) are indicated in **bold** type followed by construct length in kb in parenthesis. The ends of forward and reverse primers used during amplification with Pfu DNA polymerase are underlined. All putative transcription factor sites (eg. Core B/ AP-1 and CREB sites) are **bold underlined**. The TATA box is shown in **bold** type and *italicized*. **Bold** nucleotide indicates **G subs by T** to *create pGcALAS0.3 Hind III site. Numbering of bases is in reference to the transcription start site = +1

XhoI

(9.1)

```

-9107                                     -9067   ~~~~~
                                     CTCGAGATGA
-9057  GCTAACCAAA GCTGAGCAGA CAAGCAATGT GCTCACGCAA CAGAGCTGCA
-9007  CAGAGCCCCA GACCGTGCCA AGTGCTGCCA GCTAGCAGCC CAAGGGCCAC
-8957  CCAGCCTTGT CTCTCATCCA GTCCAACACC AGATGCTTCC CATGTAGGTG
-8907  CAAGAGCCCC ATCCTGCCTG CTGGGGTTTA AGAAGCAGGG TTAAGGCACC
-8857  CCATCTCATG AAAGTATGAT CCTCTCCAAC TGCTGGTGGT GATGAGCTGG
-8807  ACATAAGTAA GCTCAGCTTT GCGGTGGTTA AACTGATTTG TTTCACAGCC
-8757  AGCCATCCTT TTGTTAGGTT CTCTCCTATT GTGGAAACAA GCCCCAGAGA
-8707  GGCTGGAGAT GCTGGGAACA GGAGGTTCTC CCCAGCTGCC ATACAGTGGG
-8657  CAGCTCTCTG TGGCCACCAA TTGTGCCAGA AGCACCGCCG TGCCCTGTGG
-8607  CACAGTGCAT TTGAAGCCTG CTTTGAAATA AAACCAAAGA AGAGTTAAAA
-8557  ACAGGGCAGC CTTGCTAAGG TCTGCTTGAT TCCCCCCTGC TGTCGTATTG

                                     AP-1
-8507  AATGCTGCCG GAGGATTGCT CCATGCAGGG AACAATGCAG AAAAGCCTGG
-8457  ATGAGGAAAG CCTCGTGACG AGGAAGGCTC ATCTGCTTCC CTTAGAGCA
-8407  GCTGCACCCT GCCAAGGACA GATGCGCTGG GCACGTTTGT GGGGATGGCT
-8357  CATAATCAT AAATGAGCAA CTCATTCAAT AACCACACGT TGGTGAGGCA
-8307  GTTTCTAATC GAAGCCTTCC TCCGAGCAA GATAACGGCG GCCCTCCCTG
-8257  TTCTACCAT GGGTACGTGA CAGTGCCCGG CCATCCACAC GGTGCTGAGC
-8207  CGACGTGACA GCCCCAGGGT GCCCTGCCCC TCCCCTGTCT GACTGCACAG
-8157  ATCTTGACAG AGTGAGACTT CTCAGCTGTG TGGGAAGCAC ACTGCATGGC

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-8107 GGTGCCCACG CTTCCCCAAG CAAGAAGCAG CCGGGGCACA GGGCATCTCC
 -8057 AGGCCTGCTG ATATGTAGGG GGTTTGGGAA AGAAGAGAGG ATAGTGAGGG
 -8007 GTATTAAGGG TTGTTGCAAC AGAGGCAAAC ATGAGTGAAG CTGGACTTCA
 -7957 CTCTCTCAAG CCCCACAGCC ATCTGCCCTG GCTGTAGGTG GCAGCTTACA
 -7907 CCAAGGGCCT CCTCTCCTCT CTGTGGGTAT CACACAGCGT CAGCTCTGCT
 -7857 GTGTGCGAAG CAGAGCAGCC CCAGACTAAC TGCCCCAGCA CGGTGCTGGG
 -7807 ATCAGCTCTG GTTTGGGTCC CATTGGCACC CGAGGCTTTT TCTTGTTCGG
 -7757 CATCCCCTTT TCACCCTGGT GGGACCATCC CATCTCCCAG CACGGGCTGC

PmlI (7.6)

~~~~~

-7707 TCCCCAGCTC AGGACTCTGC CACGCAGGCA CGTGGCTGGC AGGACAGACC  
 -7657 TAATTACCAA AACCAAGCTC ATTAAAAGAC AATAAGCCCA GACCGCTAGT  
 -7607 AGACTTGAGA CCTGCTGTCT GCCTCCATGC ACCATTCCCC CACCCAGGG  
 -7557 TACACCAAAC CCTGGGCTGT CTGCCCCTC GCAGGGCAGC ACACGTCCCT  
 -7507 CCTCCATCCT CCTGGGCAGA GCAGTGGAGA AGGGAAAAGC CAGGCTGCCT  
 -7457 GCCACAGCTC TCCATGCTTT CCAGAAAGAC CTGCTCAGCA GAGGAGCCCT  
 -7407 GCTAGAATTC TCCTACTCCT GAAACTGCTT TCTTCCTTCC AGCTGGTGAT  
 -7357 GACCTACATA ATAAGCAGGT CACTGGAACA TGAGAGGGTT TTTTGTGAG  
 -7307 GCAGAGAAGA AAGGCCAAGC AATTCCCCCA CTGTGCTTGC TTCCGAGGAC  
 -7257 AAAGCGAAGT GCTGCTTCCC AGGGGTCTCA TTCAGCCTGA CAGTCAGCTC  
 -7207 TTCCCATCTG AAAGCAGTGG GAGCTGCTGG GATCCCACCG GGGAGGCCAT  
 -7157 TGGACCCTGC ATGGAGCATC AGGCTGCTTG AGGACCGGCT CCATAGGGGG  
 -7107 AGGATGAGGA GAGCCTCGGG GAGCACGAGC CCTCCTCGCA TGAGCAGCAA  
 -7057 ATGTGCCATG AGCAGCCCTA TGCACACGGC TGGGAACCCA CACTGCATGT

**AP-1**

-7007 GATTCAATCT GCTGCACATG GCGAGCTCTC ATCAGTCTGA AGGGAGGGAA  
 -6957 GGAGAGAGCA GGAGAATGTG GAGGAGACGA GGCCCCACCA TGGGGCAACC  
 -6907 CATGCCCAGC TGAAGGAAGG GATTGTGCTTT AGCAAACCTC TGAGTTATTC  
 -6857 CCATCTGGGA CAGCACTCTG CCTGAAAATC TGGCAGGTAA CAAAGGGGTT  
 -6807 AGCAGAGCCT GACTTTCTCA GTCTCTAGGC AGTTCTGCTC CTTTGCTCCT  
 -6757 AAAGGCTTGC TGCAGTGATG CACAAAGCCA GAAATGAGAA GGAAACCGAG  
 -6707 TAGCCCCGAG AGATGGAAAA CCTTGGGAGC CCCCTTCAGC AACAGCATGG  
 -6657 TTTAGGATCT GGTAGCAAAG AACTGGATGG TCCTGAGCCA TTTTTCACAC  
 -6607 CCCTTCCCCC TATCTTGCAG CTGATCTATG CCCACCCTCA GCTCCCTGCT  
 -6557 CCCACATGAA GACAACACTA GGAAAGATGT AGGCCAGCAG CTCTGATCCC

-6507 CATAGCTGGT GCACCACAGG CAAGAAGCTT GCAGTTACCC ACCCGAGGGT  
 -6457 AAAGTGAGGA TCTATTAGGG AAATGGATGT AATGGGAGCC CAGCTGGGGC  
 -6407 TCAGCACAGT GCTGTCAGGG TGCACAGAGC CAGTAACCAG AGCAGAGCAG  
 -6357 GGGAAGAAGC TCTATCAAGC AGGCTGGCAG CTTGGGGAGC TCCAGTACCA

**PacI (6.3)**

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-6307 CTAGCACCAG CTCGGTAAGT AGGTCACAGT TATGATCCTT AATTAATCCT
 -6257 GAAGAGACCC CCATCTGCCA TGGGGAAGAT GACAGATAAG GCAGCTCATT
 -6207 AAGCCAACGG GCAGGCAGAG GGAATGCCAC AGCCCAGCCT AACCCCTACA
 -6157 GATAGCAGGA CAAATCCTGC TCGGTGGCAG TGGCTGCTCT GCCCAGCCTG

BalI

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-6107 CTCCTGGCCA ACAGGTGCTC CAGGCTGTCC CTGTCCCCTG CGAGGAAGCC  
 -6057 CCACCTAAAC CATAGCAGTG TAAGCCTGTC ACAAGGGAGA ATCTGAAGCA  
 -6007 AAGCCAGCCC AGGGGCAGGC AGAGCTGGGC TCCAACAGGG GAGCCATACA  
 -5957 GAGTCTCGAA CCCCAACCAC CTGTGCAGTG CTTACCCTGA GCCCAGCCCT  
 -5907 CCCCACGCCT CCTGGTGCTG CAGGCAGCCA ACATCAGCAG GGACAGCAAA  
 -5857 ACCGGGCAGG GCTTCAGAGG ACTCACGCGG AGCAGGAACA TTAAAAATAG  
 -5807 TGCTCTGAAG GGAGGGATGT GTGACTAACA AGCCCCACGC TGGCAATGGT

**AP-1**

-5757 GGTAGATGGG GGCAGCTTAG TACCAAAGCC ACATCTGCAG AAGCCCTTAT  
 -5707 TAAGGTGTCA CTTGTGCTTG CTGCCACCCC CAGAGGCATT GTGTTTCAGCA  
 -5657 TTGTGCACAA CTGACAGCGT GCCAGAGCTC AGATGGAGCT GCATATGATT  
 -5607 CAACCCGAAC AAGGAGCAAG CAGAAAAGCT GCCAAATAGA CATCAAACAG  
 -5557 TATTTCTACC CAAAGAACT CCGACTAGCA GAGGAACTGA GCCCCAGCCC  
 -5507 TATGCATCAC CACTCTGCTG TGC GTTGTTC CCCTGACCCA AGTGCAGCTG  
 -5457 GGTGAGGGGC TGCAGTGCCA CAAGGTTGGT CCATGAAAAC CAGGTCAGTG  
 -5407 GCTAACAAGC CCAGGAGATA GTGCTAGGGC TTTGGGAAGT ATGCACTGAA  
 -5357 ATCACTTCTT CCAATTAAGA CACAAGCTTA GAAAGCAGTT CTGGCTTATT  
 -5307 TCCTCTTGTA ATCGGAGCTT TAAATCTCAA TTA CTTGCTC AAAAGCTCAT  
 -5257 CATCCTTCGT GGTAAATACA TCTTGGCAGA GAGGTTGCTG TGAAGGAGAT  
 -5207 GCGGTTTTGG GAACCAAGCT GAAGGAAACC CGAAAGGTCA TCACTTTTTG  
 -5157 GAGGCAGCAG TGCAGACAGC ACAGGCAGTA TGGGGCGAGC TACAATCCCA  
 -5107 AGGGCTCACG GGGGAACCCA GAGCTCATCC CCTCTCCCTC CCAGCAGTTC  
 -5057 AGCAGCACGC TCCTTTGCTG TGTCTTCTCC TTGTCCCACC AGCCAAAGTG

-5007 CAATTCCAGG TGCTGTGCCA GCTCTGAAGA CCTTCTGGAC ACCAACGCAA  
 -4957 GACAGCATAG GGATCCAGAG GGTGGCTCC ACTTTGCGTG TTAGAGCTGG  
 -4907 TGGCTCCAGC TTCATGGCTG ATGCCAGGGC TGATTGGCGA TCACCTCTCC  
 -4857 ACTTGTTAAG GAATGGAATG GAATAGAATG GGTTTCCGTT GGAATAAAAC  
 -4807 TATAAAGACC ATCGATTCCA ACTACCAGAC TCCTGCCCAA AGCTGAAACA  
 -4757 TACCATTAAG AGCATTATCC AAATTATCCT CTTAAACATG GACAGGCGTG  
 -4707 AAGCATCAAC CGCCTCCCTA TGAAGCCTTT TCCAGTGACT GATTACCTTC  
 -4657 TCAATAAAGA ATTTTTCCTA GTATCCACTC ATCACGATCC TAATATCCAC  
 -4607 TAACAGGCAC CACTTCAGCC CTAGCATCCC AGCAATAGCA ATGTGCCTTT

**BstEII (4.6)**

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-4557 TGTTAGTGCT GCTGTTGTCT GGAGAGGGTC GGTTACCTGT CACCCCAAAT
 -4507 ATCCCCCAT GTAGAGGCAT CACCCTCCC CACTATATTC TCCCATGGCT
 -4457 CGGTCCCTTC TCCCTGTTTG AGCCAGGAGA GAAGCTTAAA CCAAGGGGTC
 -4407 AGCCTTTATC AGTGCTGAGC GGGGCGTCTC AGGAGGGATA TTAAATATAC
 -4357 AGATATATAA CCAAGCACGT CGTTAAATTT CACACTAGGG ACAGGTTGCA
 -4307 ACATTTTAAG AACATGAACT CCAAATGGTG GGGTTCTTTA GGTTCAACTT
 -4257 TGAAGCACAG ACTTGCCAAG ATCAGAAGTC TTAAAGTCA CACCACATCA
 -4207 CAGCCATTGC TCTACGAGGA GCCTGTGGGA TGGCAGCATG AATCCTTGGG
 -4157 CAAAAATGAC TGCAAGTCCA AAGAGAATAG ATTTTCTGAT GTATGTAAAA
 -4107 GCTCCCTGGG ATAAACAGGG TTCTATGTGA AACCACAGCA CAAATGTTGA
 -4057 TTAAATGATC CGTAGCAAAG CCAGCAGCAC ACATAACTCC CCAGCCTCCC
 -4007 TTGGTTTTCC CCTACAATCA TTTCAGCATG GGAAATTGGA CGTGAAAGTA
 -3957 AAGAGAATGA ATTAAGTGTG TTTTGATTTT TGCCATTTCA TAGCTGGTGC
 -3907 CTTTCCGCTT CTTTGTTGCA CAACGTTTGC ACTTCCAGCT CTTTCGAGGA
 -3857 GGTTTCCTAG AATCAGCTTT TCTCTTCCCT CGCCCAGGGC TGGAGCAATG
 -3807 GATAGTTACA AGAGAAACCA CATGCATCTC ACCCAAGAA GTGGAGCAGT
 -3757 TCCTGAAAGA AGACATTCAA GACTCCAACC GTCACCACCA CCACCAAGCC
 -3707 AACACCCAAC AGGGCTTGGT TTGCTCAGCA CAGCAAGCCA GAGACTTCAA
 -3657 AAGCTTGCTC CTCCAAGCCC TTTAACGCCA CAGATCCCAG CTGGAATGGA
 -3607 GCTCGAACCT CCCAAAAGCC ATCAGGTCAA ACAAGCTGCA CGCAGAAAAG
 -3557 GTTGGCTGGG TCACGGTACC CCATGAAACC TCCCATCCCC AGCTCTCCTG
 -3507 GCTGCTGCTC ACTGGTACTG CTCCCAGTCT GAACGCTGCC ACCTGGCAGG

SmaI (3.5)

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-1857 AGGTCTCCGC ATCAAATGTT GCTTATGGGA ATTCTGGGGA AACTTGCTCA  
 -1807 ACGAATGAGA GATAGGAGGG AAGTACCGAA GCTAACTTCC ACCTGAGCCT  
 -1757 GTTTGGTAAA GCAGCAGCAC GGAAGGGAAC GTCTTCCACA GCAGCTCTTC  
 -1707 ATGGACAGCT GCAGAAATCA GTCTCCCATC TCCAGCCACC CCCCAGGGCA  
 -1657 GGCTATGGAA GCTCTCTGTT CTCCATCCAG CCCAAAAGTG CTGTCCCAGA  
 -1607 CTCCTCTTTC TGGAGCACGG CAAAGGGAGG TTGTGTGGCA ATCCAAGCAA  
 -1557 GCTTTTGCTT AGCCCTGTAG GGTGATAGTA ATGCACTGCC ACAATTAAAC  
 -1507 ACCACGTAAC TAATCCCTTG AAGCCCTAAG AAGTCTTAGT TAATGGCAGC  
 -1457 TTTATTAGTA TTCGCTTCAC TTTCGACACG TAAGGTCTGG CAATGAGGGC  
 -1407 AGTGCTGCAA AGACACCCTT TCCCTTTTCC ACTGCATTTA CCCCCGTTTT  
 -1357 GTTGGCCCTG GAGCTCAAAA TAATCTCCGT GCACTGACAA GGACACATTC  
 -1307 CTTCAGCAGC TCTCCAAGAC AGGGGTCAAG TTCATGTCAT TCCATTGCCG  
 -1257 TTTAGCTCTG CGCAACTCCT GCCTCGTACA AGGTAAAGA AATACGCCAA  
 -1207 AACCAAGCTC TAAAGAAGGG CTACAATCAC ATCTGATTAA CAGCACAGAT  
 -1157 TACAAGGGAG CCTGACAGCA AAGCTGAGAG CTCTGCTTCA CTGGGAGACC  
 -1107 TCTGTGCCAT AGCCAAGGTT GCTGCCTCAG CTTCCAACCT TGAATCAAGT

## AP - 1

-1057 TCGAGTAGAA CATGGATGCT CTCCAGGCTT CAAACTCTTG AATACAAACC  
 -1007 CAAGAGCTTC TGAATTCCGG CAGCAGAGCA GACTAACAAC ACTTCATCCC  
 -957 TCGACCTTGC AACACACCGA GGTGCTGCTA TTAAGATAAG AAGGATAAAG  
 -907 CAGCATGATC CGATTGTTGT GGGATTGAAC CTGCTGCGGC AGGTGTCCGT  
 -857 GGGGATGAGC TCTGTGTAAC ACAACCCCTT GGGGGGTGGG GAAGGCACAG  
 -807 ACAGCAGGCA GCCCAGCTCT GCCTTCTGCC AAGGTTTTGC AATATGGGGA  
 -757 AGGAACGCAT CACTGCCCTT CTCCTCCCAC GCCTCAGCAG TCGGCGTATC  
 -707 CCCTGCAGCA ACAGCACCGG GATGGCCGCA TCTACGCTGA GCTCCTTCTC  
 -657 TCATCACCAC CTGTAAGCAG CCTTTGATAA AAATCCCTGA TGTTAACGCT  
 -607 CACTGCAAAC CAGGGGTACA AATCACAGTA CTGCAGGGCC ACTTTACAT  
 -557 CAGGGCTCCT CATAGGATCA CAGAATGGCC TGGGTTGAAA AGGACCACAG  
 -507 TGCTCATCCA CTTCCAACCC CCTGCTATGT GCAGGGTCGC CAACCAACCA  
 -457 GCAGCCCAGG CTGCCCAGAG CCACATCCAG CCTGGCCTTG AATGCCTGCA  
 -407 GGGATGGGGC ATCCACAGCC TCCTTGGGCA ACCTGTTTCA TGCGTACCA

## TRE

-357 CCCTCTGGGT GAAAAATCAT CTCCAAAACC AGCGGCACCA CAACTGCGGA  
 -307 ATGAGAAGCT GTGAGATTTG TGCTGGCTGG AGGCAAAGCG TGAGGGCCGG

\*Hind III (0.3)



## AflIII

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-257 GATCCTTAAG CCACCAGGCA GAACTGTGTT AGTGCATCCC CAGGGCCCCC

SmaI

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-207 TCATGGCCCC TCATGGTCCC CCCATGGTCC CCTCAGAGCC CCCGGGGCCC

-157 CTCACCACCA CTGGGACCAA TCACGGCTCG GAGCGCAGCA CAAGCCCCGC

-107 CCACTCCATC ACGCCACGCC TCCTCGGGGG CGGAGCATAA ATTACCCTCA

NotI

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-57 GTGCGCCTGC GCGGAGCCGG CAGGGCTATA TAAGGGCGGC GGCCGCGAGT

TATA BOX

-07 CGGGGCGCTG TTCGCTTTCC GCCCGCCGTG GGGGTGACAG CTGCGTGACG

+1

HindIII

CREB

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+44 TCACTTCCGG TCGGCGGTAG CTGCGGCAGG AGGAAGCTT +82

# SEQUENCE OF DRE (522 bp) IN ALAS-1 5'-FLANKING REGION

(13792 to 13270)

In the following sequence the restriction cut sites and forward and reverse primers are shown by ~ ~ ~ swiggly lines. MluI sites added to the ALAS-1 sequence (522+**16 bp**=538bp) are shown in ***bold italics***. Forward and reverse primers used during amplification with Pfu DNA polymerase are shown by ~ ~ ~ swiggly lines and designated PRM. Transcription factor sites (eg. **DR4-1** and **DR4-2** sites for nuclear receptors) shown to mediate drug-induction of the chick ALAS-1 gene (Fraser et al., 2002) are **bold underlined**. Numbering of bases is in reference to the transcription start site = +1

SENSE\_\_PRM

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MluI

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1  CGACGCGTAG TACAGCTATG GATCTGTTCC TCTGGGGCAC ATGCACCTCT
51 TTGGCCCTGA GGTTTTCTCT CTTGCAGCAC GAAGACCTCC ATTCCCCACA
101 TTACAGGCTA GCCCCAAAGT CCTTG GTTCC AGGGGCACAG TTTTATCTAA
151 ATACAGGTTC TTGCTAAGCA AGCAGAGACT GCTGATCTCC CCGGCTGAGT
201 CTGTGCAGCT GCCTCCAGTC CTGAACTTTC TCTGCTGGGA TGAGCAGAGT
251 TCACGCTCGG CTGAACTCGT GACTGTGTCA ACAGGGGGCA TGAAGATCAG
301 CACCAGGCAA AGGTGAGCGG AGTGCACAAG AATGAGGCAG AGACCTTTGG
351 GACAAAGAGT TCCCCACCCG TGGGGCCATC ACTGCATGGA CGCTGATGCC
401 AGGATAGGGC TGGATGGAAT ATGCTGCACC ATCTCCCTGT TGGAGAGCAG
451 ACACATTCCT TTGCCTCTGC CTCTCCGCTC AGTGCCAGCT CTGCAACGTG

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MluI

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501 CCCGCTAAAT AAAGTTAGCC CTATCAAGAC CGACGCGT

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ANTISENSE\_\_PRM