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STUDIES ON THE REGULATION OF THE HUMAN HEPATIC 5-AMINOLEVULINATE SYNTHASE GENE

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CONTENTS

CHAPTER FIVE

STUDIES ON THE EXPRESSION OF HUMAN HEPATIC ALAS PROMOTER CONSTRUCTIONS IN TISSUE CULTURE CELL LINES

THESIS SUMMARY

5-Aminolevulinate synthase (ALAS) is the first enzyme of the heme biosynthetic pathway in animals. Studies in this group have indicated that the rate of hepatic heme synthesis is controlled by feedback repression of ALAS transcription through heme, the end product of the pathway. This thesis is concerned with characterizing the promoter region of the human hepatic gene for ALAS with the aim of understanding the molecular basis of the negative regulation by heme. Briefly, the base sequence of the human hepatic cDNA was obtained and from this the predicted amino acid sequence of the precursor protein. The human hepatic ALAS gene was isolated from a human genomic cosmid library. The 5' flanking region of the gene was partially characterized and expression of the human ALAS gene promoter was examined in the human hepatoma cell line HepG2 and chinese hampster ovary (CHO) cells. The levels of ALAS and P450IIIA 1 mRNA present in different human liver samples was examined by Northern analysis. Lastly the levels of erythroid and hepatic type ALAS in bone marrow samples from patients with sideroblastic anaemia were investigated.

1. The base sequence of the cDNA for the human hepatic ALAS precursor protein was determined and from this the amino acid sequence was obtained. The amino acid sequence of human hepatic ALAS precursor protein was compared with ALAS sequences from eukaryotic and prokaryotic sources. The N-terminal signal sequences of the human, rat and chicken hepatic ALAS enzymes are highly conserved, particularly the first 42 amino acids. The processed mature mitochondrial ALAS proteins of the human, rat and chicken show strong sequence similarity except for a variable region of approximately 100 amino acids at the N-terminus. The yeast and bacterial ALAS enzymes lack the variable region and are similar to the conserved region of the higher eukaryotic enzyme.

2. The human hepatic ALAS gene was isolated from a genomic cosmid clone that had previously been isolated by Dr. M. J. Bawden. The 5' flanking region of the gene was identified and partially characterized by restriction enzyme mapping and sequence analysis. 1.3kb of the 5' flanking region of the gene was subsequently sequenced. The presence of an intron in the 5' flanking region of the gene was predicted from the sequence of the untranslated region of the cDNA. The presence of the intron was confirmed and the 3' intron-exon boundary was defined by RNAse protection analysis of human hepatic total RNA. The transcriptional start site of the gene was also determined by RNAse protection analysis of human hepatic total RNA. In contrast to the rat hepatic ALAS gene, the human gene was found to have one transcriptional start site. Computer analysis was used to identify putative regulatory consensus elements in both the intron and the 5' flanking region of the gene. The chromosomal localization of the human hepatic ALAS gene was determined by collaboration with Dr. G. Sutherland's group at the Adelaide Children's Hospital.

3. A series of chimeric promoter constructions were prepared containing increasing lengths of the 5' flanking region of the human ALAS gene fused to the human growth hormone reporter gene. Constructions were prepared which contained either all of the intron in the 5' untranslated region of the gene, or due to technical difficulties encountered when attempts were made to generate intronless constructions, the first 140bp of intronic sequence. The expression of the ALAS/hGH promoter constructions were examined by transient expression studies in the human hepatoma cell line HepG2 and CHO cells. In both cell lines the promoter constructions containing the human hepatic gene sequences up to -140bp from the transcription initiation site of the gene were sufficient to generate strong expression of hGH. However, the levels of expression generated by the promoter constructions containing all of the intron in the 5' untranslated region of the gene were considerably lower than the corresponding constructions containing only the first 140bp of the intron. The effect of hemin, its precursor ALA and succinyl acetone, on the expression of the human ALAS promoter constructions in both cell lines was investigated. The human hepatic ALAS promoter constructions were not responsive to treatment with any of these compounds. The expression generated by all six human hepatic ALAS promoter constructions was the same in treated and untreated cells.

The regulation of the endogenous ALAS gene in HepG2 cells was examined by Northern analysis and nuclear transcriptional run-on experiments. Northern analysis established that treatment of the cells with a final concentration of 10μM hemin or 100μM ALA strongly reduced the levels of ALAS mRNA. Treatment of the cells with a final concentration of 1mM succinyl acetone gave a two fold increase in mRNA levels. However, using nuclei isolated from HepG2 cells which had been treated with hemin, ALA, or succinyl acetone for time periods ranging from 2 to 48 hours, it was established that none of these compounds had any detectable effect on the transcriptional rate of the endogenous ALAS gene. Therefore the effects observed by Northern analysis must represent some form of post-transcription regulation perhaps resulting in changes in mRNA stability.

4. The levels of hepatic ALAS mRNA present in nine individual human liver samples were investigated using the human hepatic cDNA probe, and were found to vary up to four fold between individuals. Three of the patients had received dexamethasone therapy prior to organ donation, and the levels of the dexamethasone inducible cytochrome P450IIIA 1 mRNA in the livers of these patients relative to the untreated patients was examined. It was established that the three dexamethasone treated patients all had elevated levels of both ALAS and P450IIIA 1 mRNAs. However, the increases in the two mRNAs were not in parallel and in at least two of the patients it appeared likely that some other factor was contributing to the elevated levels of ALAS mRNA.

5. Recently it has been shown in this laboratory that a second gene for ALAS is expressed exclusively in human erythroid tissue. In collaboration with Prof. S. Bottomley, who kindly supplied the mRNA, the levels of erythroid and hepatic ALAS, α globin, ß globin and glycophorin A mRNAs in the both marrow of sideroblastic patients and healthy individuals, were determined by Northern blot analysis. Bone marrow RNA from a limited number of patients with congenital, acquired and X-linked sideroblastic anaemia was analysed. Decreased levels of erythroid ALAS mRNA were found only in the X-linked sideroblastic patients, and this represented the first indication at the molecular level that ALAS may be involved in the pathogenesis of this disorder. An interesting finding was the associated increased levels of the hepatic form of ALAS in these patients, which may represent de-repression of the gene in response to low cellular heme levels. Reduced levels of α and ß globin mRNAs was also observed in these patients which may also be due to low cellular heme levels. Importantly, the levels of the erythroid specific mRNA for glycophorin A were not reduced in the X-linked patients, and this established that the observed decreases in erythroid ALAS, and α and ß globin mRNA were specific.

The findings in the patients with acquired sideroblastic anaemia were less informative, and the normal levels of erythroid ALAS mRNA found in the bone marrow RNA of these patients, suggest that biochemical heterogeneity may underlie the disorder .

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other University.

In all of the experiments described in this thesis, the author was involved as the principle worker, however some of the work in Chapter 3 was undertaken with assistance from Dr. I. Borthwick, the chromosomal localization of the human hepatic ALAS gene was done in collaboration with Dr G. Sutherland's group at the Adelaide Children's Hospital, and the work described in Chapter 7 was done in collaboration with Prof. Sylvia Bottomley.

To the best of my knowledge, this thesis contains no material previously published by any other person, except where due reference is made in the text.

Helen Moira Healy

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ABBREVIATIONS

Abbreviations are as described in The Journal of Biological Chemistry "Instructions to authors" (1989). Additional abbreviations are listed below.

AIA:	allylisopropylacetimide
ALA:	5-aminolevulinate
ALAS:	5-aminolevulinate synthase
A_n:	Absorbance at wavelength, n.
bp:	base pair
Ci:	Curie
cpm:	counts per minute
dNTP:	deoxyribonucleotide triphosphate
ddNTP:	dideoxyribonucleotide phosphate
DTT:	dithioerythritol
g	gravitational force
HMBA:	hexamethylene bisacetamide
kb:	kilobase
PIA:	propylisopropylacetamide
PMSF:	phenylmethylsulphonyl fluoride
poly (A)	poly adenylic acid
rpm	revolutions per minute
S:	Svedberg unit of sedimentation coefficient
TCA:	trichloroacetic acid
TMACL	tetramethylammonium chloride

CHAPTER ONE

GENERAL INTRODUCTION

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CHAPTER ONE : GENERAL INTRODUCTION

1.1 INTRODUCTION

Heme, a tetrapyrrole ring (porphyrin) complexed with iron, is the essential prosthetic group of a complex family of hemoproteins including the cytochromes of both the mitochondrial respiratory system and microsomal mono-oxygenase system, the oxygen transport and storage proteins hemoglobin and myoglobin, and enzymes such as catalase, peroxidase and tryptophan pyrrolase. The active site of heme is the central iron atom which exists in two stable oxidation states: Fe^{2+} and Fe^{3+}. In the ferrous state the iron can be reversibly oxidised by the transfer of single electrons. It is therefore able to function as a single electron carrier in electron transport chains, and to act as a catalyst in redox reactions involving oxygen or oxygen-containing compounds. In oxygen transport and storage proteins, the ferrous iron atom of the heme group provides for the reversible binding of oxygen. Intracellular factors, such as temperature, pH, and 2-3-diphosphoglycerate concentration, modify the affinity of heme for oxygen, and thereby bring about the dissociation of oxygen to the tissues.

While all aerobic cells synthesize heme to meet their requirement for the various cellular hemoproteins, in humans over 80% of the total heme is synthesized by the erythropoetic cells of the bone marrow, for the production of hemoglobin. Most of the remaining heme is made in the liver, to provide heme primarily for the cytochrome P450 proteins, which are important for the metabolism of endogenous substrates such as steroids and fatty acids, and foreign hydrophobic chemicals such as drugs and environmental contaminents (Tait, 1978; Gonzalez, 1989). As a result, the study of heme biosynthesis in animals has focussed mainly on the hepatic and erythroid cells, and its regulation appears to be quite distinct in the two different cell types, as will be discussed later in this chapter.

5-Aminolevulinate synthase (ALAS; EC 2.3.1.37) is a pyridoxal-phosphate dependent enzyme, which functions in the mitochondria and catalyzes the condensation of succinyl-coenzyme A (succinyl-CoA) and glycine to form 5-aminolevulinic acid (ALA) in the first step of the heme biosynthetic pathway. It has recently been demonstrated that human ALAS exists as two isozymes, the hepatic or housekeeping enzyme which is expressed in all cells, and an

erythroid specific enzyme expressed only in the erythroid cells of the bone marrow. (Bawden *et al.*, 1987; Cox *et al.*, 1990). As yet the properties of the recently described erythroid specific enzyme have not been well characterized. However, it is well established that the hepatic form of ALAS is the rate controlling enzyme of heme biosynthesis in the liver, and probably also in other non-erythroid tissues, with levels of the enzyme fluctuating according to the cells heme requirement.

The regulation of hepatic ALAS expression is of both fundamental and medical interest. The gene is under negative control by the end product of the pathway, heme. While the mechanisms for feedback repression of mRNA production in prokaryotes have been elucidated in detail (Ptashne, 1986), little is known about these mechanisms in higher eukaryotes. In animal cells most attention has focussed on positively regulated systems in which hormones, metabolic inducers and developmental factors increase transcription of genes. One important exception is the *de novo* cholesterol biosynthetic pathway, where two sequential genes in the pathway, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase and HMG-CoA reductase are subject to sterol mediated repression (Gil *et al.*, 1986; Goldstein and Brown, 1984). The information necessary for the transcription of these genes to be repressed by sterols has been localized to their 5' flanking regions (Südhof *et al.*, 1987). However, the mechanism by which repression occurs remains to be elucidated. This thesis is concerned with the regulation of the human hepatic or housekeeping form of ALAS, and of particularly interest is the transcriptional control of ALAS gene expression. An introduction to these topics is presented in this chapter.

One of the longer term aims in studying the regulation of heme biosynthesis, is to aid in the treatment of some of the disorders that result from defective heme biosynthesis. As the porphyrias, and sideroblastic anaemia are particularly relevant to the work in this thesis, they are also discussed briefly in this chapter. More extensive reviews of these topics include those by May *et al.*, (1986), Bottomley and Müller-Eberhard, (1988), and Moore (1990).

Throughout this thesis the term 5' flanking region of the gene has been used in the liberal sense to include the 5' untranslated region of the gene.

1.2 THE HEME BIOSYNTHETIC PATHWAY

The tetrapyrroles are a diverse group of biological compounds, including heme (and the bile pigments produced by heme degradation), chlorophylls, the corrins (e.g. vitamin B12) and the phycobilin pigments of algae. Biosynthesis of the tetrapyrroles follows the same pathway to the intermediate uroporphyrinogen III, which is the universal precursor from which all the other tetrapyrroles are derived. At this branch point uroporphyrinogen III, may either be methylated to be transformed into corrins, or alternatively decarboxylated and converted into heme or chlorophyll. A summary of these pathways is shown in Fig. 1.1.

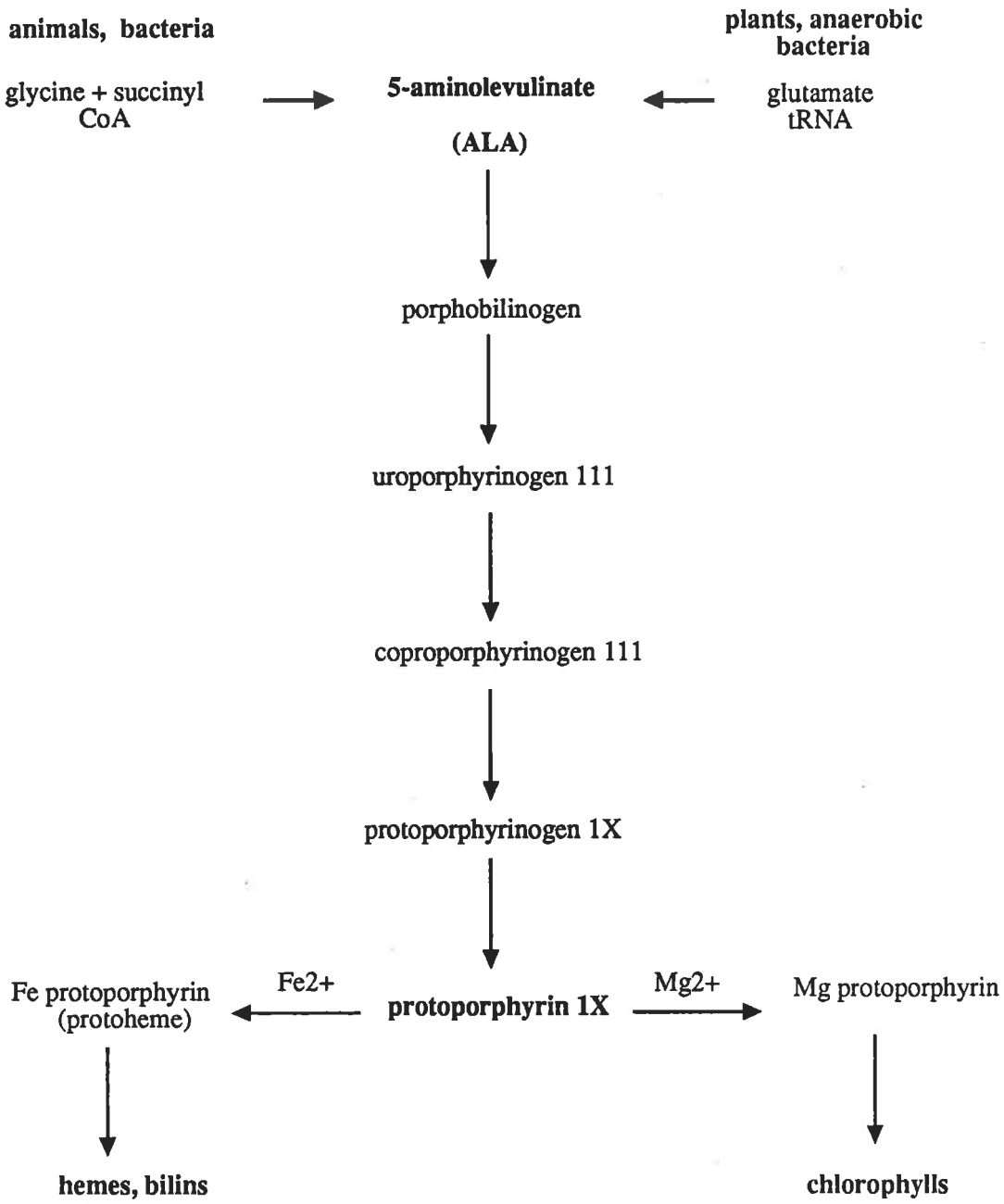
The pathway for the biosynthesis of the tetrapyrrole ring system is broadly similar in all living systems and commences with the biosynthesis of the aminoketone 5-aminolevulinic acid (ALA). There are two distinct routes by which ALA is produced; one in animals and some bacteria which utilizes glycine and succinyl-CoA; and the other more recently shown to operate in plants and some anaerobic bacteria which involves the carbon skeleton of glutamate (Kannangara *et al.*, 1984; Schoen *et al.*, 1986).

In animal cells, ALAS functions in the mitochondria, with the next four reactions in the pathway taking place in the cytosol (Fig.1.2). ALA-dehydratase catalyses the condensation of two molecules of ALA to form the pyrrole, porphobilinogen (PBG). PBG deaminase then polymerizes four molecules of PBG to generate a highly unstable linear tetrapyrrole hydroxymethylbilane called preuroporphyrinogen, which is cyclized in the presence of uroporphyrinogen III cosynthetase to form the key intermediate, the type III isomer of uroporphyrinogen (URO).

In the route to heme, the four side chain carboxyl groups are removed by URO decarboxylase, producing coprophyrinogen III. The last three enzymes of the pathway are located in the mitochondria. Oxidative decarboxylation by coproporphyrinogen oxidase produces protoporphyrinogen IX , which is oxidised to protoporphyrin IX by protophyrinogen IX oxidase. In the final step catalyzed by ferrochetalase (Kappas *et al.*,1983), heme is formed by the insertion of Fe^{2+} into protoporphyrin IX.

FIGURE 1.1

THE TETRAPYRROLE BIOSYNTHETIC PATHWAY



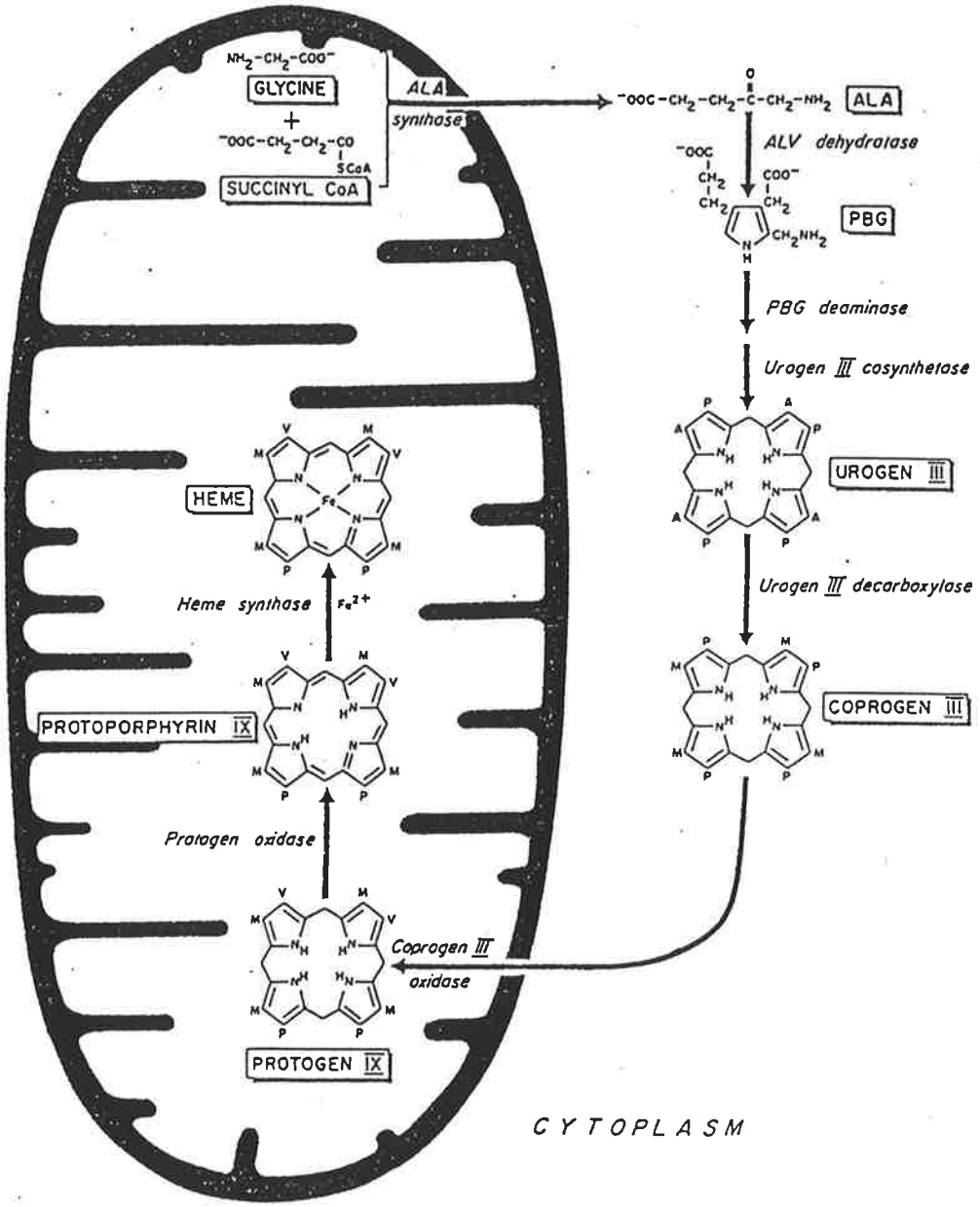


FIGURE 1.2 THE HEME BIOSYNTHETIC PATHWAY

The intermediates and the enzymes involved in the biosynthesis of heme from glycine and succinyl-CoA are illustrated in their subcellular compartments. **ALA**, 5-aminolevulinate; **PBG**, porphobilinogen; **UROGEN**, uroporphyrinogen; **COPROGEN**, coproporphyrinogen; **PROTOGEN**, protoporphyrinogen; **A**, acetate; **M**, methyl; **P**, propionate; **V**, vinyl. (From May *et al.*, 1986).

In plant cells, ALA is synthesized in the chloroplast by a method in which glutamate is activated by attachment to a chloroplast-encoded tRNA, reduced to glutamate-1-semialdehyde and transaminated to form ALA (Kannangara *et al.*,1984; Schoen *et al.*, 1986). The ALA is used largely for the production of chlorophyll but is probably also used for heme synthesis. It has been reported (Franck *et al* ., 1984), that ALA may also be synthesized from glutamate in animals, by transamination of the intermediate 4,5-dioxovalerate (DOVA) with alanine. Labelling studies in duck blood cells have suggested that as much as 25% of cellular ALA may be derived from glutamate (Franck *et al* ., 1984). Further work is required to establish whether this pathway contributes significantly to heme biosynthesis in animals.

Interestingly, some bacteria including *E. coli* and *Salmonella typhimurium*, appear to lack ALAS, and instead synthesize ALA via a pathway similar to the C5 pathway in plants (Avissar and Beale 1989; Elliott, 1989).

1.3 STRUCTURE AND PROPERTIES OF HEPATIC ALAS

ALAS was first described independently by Shemin and co-workers (Kikuchi *et al* ., 1958) in bacterial extracts and by Neuberger and his group in avian preparations (Gibson *et al.*, 1958). The enzyme has since been purified to various extents from many sources including *Rhodobacter spheroides* (Warnick and Burnham, 1971), rat liver (Ohashi and Kikuchi, 1979), *Euglena* (Dzelzkalns *et al.*, 1982), chick embryo liver (Borthwick *et al.*, 1983), and yeast (Volland and Felix, 1984). All ALAS enzymes isolated catalyze the same reaction, the condensation of glycine and succinyl-CoA to yield ALA, CoA, and carbon dioxide. In eukaryotes, the enzyme is normally located in the mitochondria, reflecting the requirement for succinyl-CoA as one of the substrates. If ALAS is strongly induced by drugs in chick embryo liver, activity is also found *in vitro* in cytoplasmic extracts (Srivastava *et al.*, 1983a). Whether this is due to cytoplasmic precursor enzyme being catalytically active, or to its processing *in vitro* or even to mature enzyme being present in the cytoplasm has not been established. Although succinyl-CoA is generated by other enzyme systems such as methyl-malonyl-CoA mutase, succinate thiokinase, and acetacetyl-CoA : succinate transferase, the major source for heme synthesis is from the tricarboxylic acid cycle (Jordan, 1990).

Most mitochondrial proteins are synthesized in the cytosol on free cytoplasmic polysomes as larger precursor proteins which are translocated post translationally into the mitochondria. Targetting to the mitochondria is generally dependent on an N-terminal amino acid signal sequence which interacts with the the mitochondrial translocation apparatus, and is subsequently cleaved as the protein assembles in the mitochondria (von Heijne, 1986; Pfanner *et al.*, 1988; Silver and Goodson, 1989). However, mitochondrial proteins with non-cleavable signal sequences have been described (Hase *et al.*, 1984: Horwich *et al.*, 1985). Furthermore, the carboxyl-terminal region of some mitochondrial precursors have been shown to contain specific import information (Pfanner *et al.*, 1987a and 1988).

It has been shown that both chick embryo and rat hepatic ALAS are initially synthesized as larger precursor proteins each with an amphipathic N-terminal signal sequence of 56 amino acids which is recognized by the mitochondrial transport system (Borthwick *et al.*, 1985). After import across the mitochondrial membrane, the signal sequence is removed to yield the mature enzyme with a molecular weight of 68,000 (Borthwick *et al.*, 1985). A similar situation occurs in yeast where the pre-enzyme, which has a molecular weight of 59,000, is rapidly converted to the mature enzyme with a molecular weight of 55,000 after import into mitochondria (Urban-Grimal *et al.*, 1986). Immunocytochemical studies, using monoclonal and polyclonal antibodies specific for ALAS and a protein-A gold labelling technique, have shown that ALAS in rat liver mitochondria is associated almost exclusively with the matrix side of the inner mitochondrial membrane (Rohde *et al.*, 1990). Crosslinking studies and electron microscopy suggest that the native chicken mitochondrial enzyme exists as a dimer, consisting of two identical subunits each with a relative molecular mass of 70,000 (Borthwick *et al.*, 1985).

Rat and chick embryo liver ALAS proteins have been isolated in their native mitochondrial form (Borthwick *et al.*, 1983), by a rapid purification method developed in this laboratory; the minimum molecular weights of the enzyme subunits are 70,000 and 68,000 respectively. Prior to this the chick embryo mitochondrial enzyme had been isolated to homogeneity, but with a minimum molecular weight of 49,000 (Whiting and Granick, 1976).

This protein was probably a degraded form of the enzyme, since protease treatment of the native enzyme in *vitro*, produces a series of proteins with minimum molecular weights close to 49,000, which interestingly retain full catalytic activity (Borthwick *et al.*, 1983).

While several groups claim to have isolated the cytoplasmic precursor form of ALAS (Nakakuki *et al.*, 1980; Watanabe *et al.*, 1984) the molecular weights of the isolated products suggest the enzyme must have been partially degraded, and it seems unlikely that the native cytoplasmic form of the enzyme has ever been isolated. Therefore, it is difficult to be certain whether the precursor is enzymatically active, but as the preferred substrate, succinyl-CoA, is not present in the cytoplasm whether the enzyme is active or not, is unlikely to have metabolic significance.

All ALAS enzymes require pyridoxal-phosphate for catalytic activity. Studies with a bacterial form of the enzyme showed that pyridoxal-phosphate is bound via a Schiff base to a lysine residue (Nandi, 1978b). Studies in our laboratory with mitochondrial chicken liver ALAS indicate that a lysine residue is also involved in binding the pyridoxal-phosphate moiety (May *et al.*, 1986).

The complete amino acid sequences of the chick embryo, rat and human liver ALAS proteins have been deduced on the basis of sequence from cDNA clones which have been isolated in this laboratory (Borthwick *et al.*, 1984 and 1985; Srivastava *et al.*, 1988; Bawden *et al.*, 1987). Three distinct domains within the eukaryotic enzyme can be defined from the primary sequence; region 1, the mitochondrial targetting domain, region 2, which is part of the mature protein but apparently not required for enzymatic activity, and region 3, the C-terminal two thirds of the enzyme which is highly conserved between all three enzymes (approximately 90%) and presumably contains the catalytic site.

1.4 REGULATION OF HEPATIC ALAS

Heme synthesis is presumably closely regulated in all tissues and coordinated with the expression of numerous apohemoprotein genes. In the last two decades, extensive research has been devoted to understanding how this coordination is achieved. The data available to date

shows there are two distinct regulatory mechanisms, exemplified by the hepatic and erythroid cell types; regulation in hepatic cells will be discussed in this section, while regulation in erythroid cells is discussed later in the chapter.

Heme has been shown to have an autoregulatory role in the liver, being able to regulate the genes responsible for both its own biosynthesis and catabolism. Heme synthesis in this tissue is regulated primarily by feedback repression on the synthesis of ALAS. This appears to take place at a number of levels including repression of ALAS synthesis at the transcriptional and/or translational steps and inhibition of the translocation and/or processing of cytosolic ALAS into the mitochondrial form (May *et al.*, 1986). At higher concentrations, heme also controls the synthesis of the catabolic enzyme heme oxygenase (Kikuchi and Yoshida, 1983), which cleaves heme to form biliverdin (Tenhunen *et al.*, 1969). It has been shown that heme increases heme oxygenase mRNA levels in the liver (Ishizawa *et al.*, 1983), suggesting that induction may be at the level of transcription, and this is supported by the recent description of a putative heme-responsive transcription factor (Shibahara *et al.*, 1987). Therefore, the intracellular level of heme in the liver is autoregulated through both repression of ALAS and induction of heme oxygenase.

The heme which is involved in the regulation of its own biosynthetic pathway is thought to arise from the small "free" cytosolic pool. This heme is probably only "free" in the sense that it is not yet bound as a hemoprotein complex. It most likely represents either newly synthesized heme in transit from the mitochondria (Schmidt, 1973), or a degree of dissociation from hemoproteins (Schmidt, 1973; Bissell and Hammaker, 1976). To date, it has not been possible to directly measure the intracellular heme pool, but the physiological regulatory heme pool has been indirectly estimated to be 10^{-7} to 10^{-9}M (Granick *et al.*, 1975).

The pioneering work of Granick and his colleagues demonstrated that a wide variety of hydrophobic substances, such as foreign chemicals and endogenous steroids, caused an increase in the level of ALAS activity in chick embryo liver cells, while the remaining enzymes in the heme biosynthetic pathway were not significantly affected. It was also shown that hemin added with the inducing agent, repressed the induction of ALAS activity, and provided the first

evidence for end product repression of ALAS by heme (Granick and Urata, 1963; Granick, 1966) Since Granick's initial work, hepatic heme biosynthesis has been studied extensively, and while there is general agreement about the importance of ALAS in regulating the rate of hepatic heme synthesis, the mechanisms by which heme achieves its negative regulation of the level of the enzyme remain obscure.

1.4.1 Inhibition of hepatic ALAS activity by heme.

There has been numerous reports in the literature that heme or hemin[1] directly inhibits the activity of ALAS (Scholnick *et al.*, 1972; Whiting and Granick, 1976; Paterniti and Beattie, 1979). However, ALAS purified to homogeniety from drug induced chick embryo liver or rat liver was not inhibited by heme or hemin concentrations as high as 100μM (Pirola *et al.*, 1984; May *et al.*, 1986). In addition, experiments using intact rat liver mitochondria have established that raising the level of heme has no effect on ALAS activity (Wolfson *et al.*, 1979). However, in a partially purified enzyme extract from rat liver, heme inhibition was observed (Scholnick *et al.*, 1972). While the reasons for these conflicting results are not understood, it is currently thought that feedback inhibition of ALAS activity by heme is not physiologically important. (Pirola *et al.*, 1984; Bloomer and Straka, 1988).

1.4.2 Translational regulation of hepatic ALAS by heme.

There is conflicting evidence concerning the possible role of heme in inhibiting the synthesis of ALAS at the peptide elongation step. Whiting (1976), using an homologous *in vitro* translation system, showed that the synthesis of ALAS by polysomes isolated from drug induced chick embryo liver, was not affected by concentrations of hemin up to 10μM. In contrast, Yamamoto *et al.*, (1983) reported that ALAS synthesis by rat liver polysomes was inhibited at the elongation step when polysomes were allowed to run off in a rabbit reticulocyte-lysate system in the presence of hemin. Similar experiments using a wheat germ cell-free translation system failed to demonstrate any effect of heme on translation of ALAS mRNA, at

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[1] Heme is ferroprotoporphyrin and is the form that exists in hemoproteins in the cell. Heme is readily auto-oxidized *in vitro* to ferrrprotoporphyrin which is called hemin. I have tried to make the distinction in the text between heme, as it exists in the cell and hemin, the form that is used experimentally.

concentrations of hemin up to 100μM. (May *et al.*, 1986). The reason for these conflicting results is not known, but it is possible that heterologous translation systems used in these studies do not reflect the situation found in liver cells. These studies have only addressed the effect of heme on the elongation step of ALAS synthesis, and the important question of whether heme is able to inhibit initiation of translation of ALAS mRNA in hepatic cells, is yet to be to be investigated.

1.4.3 Inhibition of hepatic ALAS translocation into mitochondria.

The first evidence that heme could block translocation of the cytoplasmic precursor ALAS protein into the mitochondria of liver cells was provided by Hayashi *et al.*, (1972), who showed that administration of hemin to drug induced rats caused an accumulation of ALAS activity in the cytosol and a decrease in the level of mitochondrial activity. Kikuchi and Hayashi (1981) subsequently proposed a novel negative feedback mechanism in which heme blocks the transfer of the ALAS precursor into the mitochondria. This proposal has been supported by kinetic studies of the half-lives of the cytosolic and mitochondrial enzymes (Hayashi *et al.*, 1980). Pulse labelling experiments have shown hemin had no significant effect on the net rate of enzyme synthesis 10 to 30 min after administration, but largely suppressed accumulation of the newly synthesized enzyme in the mitochondria (Yamauchi *et al.*, 1980). In these studies it was presumed that hemin treatment prevented the synthesis of new ALAS mRNA, and that the observed cytosolic accumulation of the enzyme resulted from the translation of preexisting message.

Immunological studies in cultured chick embryo liver cells showed that treatment with hemin leads to a cytosolic accumulation of ALAS precursor (Ades *et al.*, 1983; Srivastava *et al.*, 1983b). Inhibition of ALAS transport by hemin has also been demonstrated in adult and chick embryo liver, both in ovo and in culture (Ohashi and Kikuchi, 1972; Srivastava *et al.*, 1983b; Hayashi *et al.*, 1983). This feedback mechanism appears to be specific for ALAS rather than a general effect of heme on the transport of mitochondrial proteins, since it has been shown that heme does not prevent the transfer and processing of pyruvate carboxylase, an unrelated matrix mitochondrial protein (Srivastava *et al.*, 1983b).

The mechanism by which heme inhibits translocation of the precursor protein is unknown. However in recent years there has been mounting evidence that proteins must be partially unfolded in order to pass through the mitochondrial membrane and assemble in the matrix (Eilers and Schatz, 1988; Silver and Goodson, 1989). In addition, there is evidence that an enzyme responsible for keeping proteins unfolded may be located in either the cytoplasm or the mitochondria (Pfanner *et al.*, 1987b). Although direct binding of heme to the precursor has never been reported, it seems reasonable to speculate that when the intracellular concentration of heme is high, heme binds to the precursor protein and inhibits translocation by either preventing unfolding of the precursor, or that heme sterically inhibits the precursor from binding to the mitochondrial transport system. Alternatively, heme may act indirectly through a heme binding factor, which in some way inhibits mitochondrial transport.

1.4.4 Transcriptional regulation of hepatic ALAS

Heme repression of ALAS mRNA synthesis was first indicated by experiments in which treatment of chick embryos (Granick, 1966; Whiting and Granick, 1976) and rats (Yamamoto *et al.*, 1982), with drugs such as 2-allyl-2-isopropylacetamide (AIA) caused an increase in the level of translatable ALAS mRNA. However, administration of hemin with the AIA blocked the induction of ALAS mRNA. These studies provided evidence that inducers of ALAS cause an increase in the level of ALAS mRNA, which can be blocked by the administration of heme.

The isolation in our laboratory of cDNA clones for the rat hepatic ALAS allowed the effect of drugs and heme on the levels of ALAS mRNA to be studied directly. Srivastava *et al.*, (1988), showed that basal levels of hepatic ALAS mRNA are detectable in the rat liver, brain, kidney, heart and testis, in keeping with its housekeeping status. Treatment of the animal with the heme precursor ALA lowered the basal level of ALAS mRNA and prevented the drug induced increase of ALAS mRNA in these tissues. The administration of hemin to the rat, both lowered the basal level of hepatic ALAS mRNA and prevented its drug induced increase, but had no effect on the mRNA level in the extra-hepatic tissues investigated.

Subsequently, *in vitro* nuclear run-on experiments using nuclei isolated from rat liver (Srivastava *et al.*, 1988), demonstrated that administration of hemin or ALA to rats, reduced the basal level of ALAS mRNA and prevented its drug induced increase, by inhibition of transcription of the ALAS gene. The degree of inhibition seen in the nuclear run-on experiments corresponded with the reduction in the levels of ALAS mRNA. Moreover the inhibition was specific as there was no change in the level of transcription of rat serum albumin or ß-actin genes. These results clearly establish that both heme and drugs are able to modulate the levels of ALAS mRNA.

Recent work by Yamamoto *et al.*, (1988), in which rats were treated with the transcriptional inhibitor α-amanitin resulted in estimates of the half-life of mRNA for ALAS of approximately 20 minutes. The molecular basis of this mRNA instability is not known, but it is appropriate for a system which has to be rapidly modulated.

In contrast to the situation in the rat, *in vitro* studies using cultured chick embryonic hepatocytes, have shown that hemin is only partially effective in blocking the drug-mediated induction of ALAS mRNA (Drew and Ades 1986; Hamilton *et al.*, 1988). It seems likely that in this system, heme is controlling ALAS expression predominately at the level of enzyme translocation into the mitochondria. Additional work is needed to understand why the apparent effectiveness of heme repression of ALAS transcription in these two systems is different.

1.4.5 Mechanisms of drug induction of hepatic ALAS.

Granick (1966) originally postulated that heme acted as a corepressor of ALAS transcription and that chemical inducers competed with heme for the aporepressor. However, an alternative model put forward by May *et al.*, 1986, suggested that transcription of ALAS is regulated solely by intracellular heme levels. The chemical inducers of hepatic ALAS are known to also be inducers of apocytochrome P450 genes (Whitlock, 1986). Since these proteins require heme as their prosthetic group, their induction would lower the intracellular heme pool and thereby relieve the feedback repression of ALAS transcription.

Evidence from our laboratory, has shown that ALAS can be induced in the absence of drugs by heme depletion alone. Srivastava *et al.*, (1980b), demonstrated that the induction of ALAS enzyme levels in drug induced cultured chick embryo liver cells could be maintained after removal of the inducing drug, by the addition of desferrioxamine an iron chelator which inhibits the last step of heme synthesis (Strivastava *et al.*, 1980a). Since desferrioxamine by itself did not induce ALAS expression, these results suggested that once induction is established, high levels of ALAS expression in the absence of the inducing drug could be maintained if heme synthesis is blocked. This at any rate is compatible with the idea that inducing drugs exert their effect on ALAS by reduction of heme levels Induction of ALAS activity has also been achieved in the complete absence of drugs, in cultured chick embryo liver cells after induction of heme oxygenase activity with hemin (Strivastava *et al.*, 1980b).

More recently, it has been shown that in rats treated with succinylacetone, a specific inhibitor of ALA dehydratase, the second enzyme in the pathway, the level of hepatic ALAS mRNA increases approximately three fold, but drugs alone cause increases up to 20 fold. This might imply that inhibition of heme synthesis alone is not sufficient for maximal induction of ALAS and May *et al.*, (1990), have proposed that drugs may also increase ALAS transcription through a drug responsive element in the promoter of the ALAS gene. However, this is a speculative model as to date no drug responsive elements in the promoters of either ALAS or the P450 genes have been reported, and it is not easy to envisage how so many chemical inducers with very different structures could act through one common element. Of course the same problem occurs with induction of P450 genes by many different drugs.

While the above experiments indicate that chemical inducers augment ALAS gene transcription indirectly by lowering heme levels, this does not necessarily preclude a direct effect of the inducer on ALAS gene transcription. If induction of ALAS is due to an increased synthesis of apocytochrome P450, then induction of the latter should be necessary and should precede an increase in ALA-S expression. In this regard, Hamilton *et al.*, (1988), have recently shown in embryonic chicken liver cells that treatment with propylisopropylacetamide (PIA) was followed by the rapid and simultaneous accumulation of both ALAS and P450 mRNAs.

Pretreatment of the cells with cycloheximide, an inhibitor of protein synthesis, resulted in the PIA induction of ALAS mRNA being enhanced rather than inhibited. In addition, although cycloheximide treatment is known to increase the stability of labile mRNAs (Shaw and Kamen, 1986), it had no effect on ALAS mRNA in the absence of PIA. These results suggest that the activation of apocytochrome P450 synthesis is not essential for the PIA induced accumulation of ALAS mRNA.

While these results suggest that PIA may be having a direct role on the induction of ALAS gene expression, it should be noted that in this study only the accumulation of mRNA was measured. In our laboratory, we have shown that cycloheximide treatment results in significant ALAS mRNA stabilization in these cells, and therefore it will be necessary to measure newly initiated transcription of these genes to determine whether PIA is directly inducing ALAS expression. In addition, the observed effect may be limited to this class of xenobiotics and not representative of the co-ordinated expression of ALAS and other apocytochromes P450 *in vivo*. The exact mechanism by which drugs mediate ALAS expression remains an important issue that needs to be resolved.

1.5 STRUCTURE AND PROPERTIES OF ERYTHROID ALAS

In recent years there has been considerable debate as to whether ALAS isozymes exist in the liver and erythroid tissue. Initial work was based on studies at the protein level, and the evidence was conflicting. Based on the differential binding to AMP-agarose of guinea pig enzyme, partially purified from erythroid and hepatic cells, Bishop *et al.*, (1981), suggested different forms of ALAS were expressed in these tissues.

Immunological evidence for liver and erythroid ALAS isozymes was found for both the chicken (Watanabe *et al.*, 1983) and rat (Yamamoto *et al.*, 1985). Watanabe *et al.*, (1983) showed that antibody raised against the chicken liver enzyme partially cross-reacted with chicken erythroid ALAS, and that six times more IgG was required to precipitate the erythroid enzyme. Subsequent work demonstrated that the liver enzyme had a minimum molecular weight of 65,000, while the erythroid form had a molecular weight of 55,000. In addition, liver polysomes directed the synthesis of a precursor with a molecular weight of 73,000, while

erythroid polysomes produced a protein with a molecular weight of 55,000 (Watanabe *et al.*, 1984). However, no formal evidence was presented that this protein was in fact an erythroid specific form of ALAS. In addition, since the hepatic form of the enzyme is very susceptible to proteolytic cleavage during isolation, it was suggested the putative erythroid form may represent a degraded form of the hepatic enzyme (May *et al.*, 1986).

Yamamoto *et al.* (1985) used the polyclonal antiserum of Watanabe *et al.* (1983), to identify several putative ALAS cDNA clones in a chicken reticulocyte cDNA expression library. When one of the cDNAs identified was used as a probe in Northern analysis of reticulocyte mRNA, it hybridized selectively to a single species of mRNA with an approximate size of 2kb, that was expressed in chicken reticulocytes but not in the liver. Under low stringency conditions this clone detected a larger transcript in the liver. On the basis of this work, the authors proposed that ALAS is coded for by a minimum of two genes, one erythroid and one liver specific. However, conflicting evidence was presented by Elferink *et al.*, (1987), who demonstrated in the chicken that identical ALAS mRNAs and proteins are present in both liver cells and reticulocytes.

Recently, the issue has been resolved by the isolation and characterization of a cDNA clone for chicken erythroid ALAS mRNA (Riddle *et al.*, 1989). Sequence analysis showed that this clone encodes a preprotein of 518 amino acids with a predicted molecular weight of 53,000, which lacks approximately 100 amino acids of N-terminal sequence present in the hepatic enzyme. The hepatic and erythroid proteins show substantial similarity at the amino acid level, yet at the nucleotide level there are no stretches of similarity greater than 10 nucleotides in length. It has been shown by genomic Southern blotting and RNA protection analysis, that this cDNA is encoded by a distinct ALAS gene which is expressed only in erythroid cells. However, in agreement with Elferink *et al.*, (1987), these investigators showed that the hepatic and erythroid genes are co-expressed in erythroid cells, and while expression of the hepatic enzyme is much lower than that of the erythroid form, this finding explains the apparently conflicting evidence.

A similar conflict existed as to the number of mouse genes encoding ALAS. Schoenhaut and Curtis (1986), used genetic complementation of an *E. coli hemA* mutant, to isolate cDNAs encoding ALAS in mouse. One of the isolated cDNAs was then characterized by partial sequence analysis and used to isolate homologous sequences from an anaemic spleen cDNA library. Using the anaemic spleen cDNA as a hybridization probe, a single band corresponding to a 2.2kb mRNA was detected in samples from liver, anaemic spleen and mouse erythroleukemia (MEL) cells, Additionally, this mRNA was elevated in AIA-treated livers and dimethylsulfoxide (DMSO) induced MEL cells. One discordant feature of this work was the fact that the nucleotide sequence, amino acid sequence and coding capacity of the mouse cDNA, was significantly different from the previously described human, rat and chicken enzymes.

More recently, using a different approach, it has been shown that isozymes for ALAS are expressed in the mouse. Two distinct cross hybridizing cDNAs have been isolated, one from MEL cells and the second from 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) treated mouse liver (Dierks, 1990). Comparison of the sequences of these with those reported for other ALAS cDNAs, shows that the 2.1kb mRNA present in MEL cells is almost identical to the cDNA reported by Schoenhaut and Curtis (1986), while the 2.2kb mRNA expressed in the DDC-treated liver has more similarity with the human, rat and chicken hepatic ALAS cDNAs. In addition, nuclease S1 protection experiments using DNA probes specific for each of these mRNAs, demonstrated the hepatic or housekeeping form of the enzyme is present at a low level in all tissues and cell types that have been tested, including bone marrow, anaemic spleen, and both differentiated and undifferentiated MEL cells. (Dierks, 1990).

The human erythroid ALAS cDNA and the corresponding gene have recently been isolated in our laboratory. While the erythroid ALAS cDNA encodes an enzyme which is similar in size to the human hepatic enzyme, the derived amino acid sequence is quite different. The N-terminal pre-sequences show limited similarity (21%). However, there is considerable similarity (70%) between the carboxyl terminal two thirds of the isozymes, providing further evidence that this region contains the catalytic site of the enzyme.

Southern analysis and chromosome localization of both the liver and erythroid genes has been carried out. There is no doubt that in the human there are two distinct genes for the isozymes of ALAS as the liver gene has been mapped to Chromosome 3, while the erythroid gene is situated on the X Chromosome (Sutherland *et al.*, 1988; Cox *et al*, 1990).

1.6 REGULATION OF ERYTHROID ALAS

In erythroid cells, where the largest amounts of heme are synthesized to provide the prosthetic group for hemoglobin, the regulation of heme biosynthesis is less well defined, as compared with the hepatic system. The erythroid situation differs from that in the liver since it is associated with cell proliferation and differentiation events. Erythroid cells undergo a program of differentiation, or erythropoiesis, during which stem cells which contain no detectable hemoglobin, progress through several defined stages of development to form mature erythrocytes in which more that 95% of the total protein is hemoglobin. (Harris and Kellermeyer, 1970; Marks and Rifkind, 1972). In the terminally differentiated erythrocyte, heme synthesis along with many other cellular processes has ceased, since the mitochondria and protein synthesizing machinery are no longer present and in mammals the nucleus has been extruded.

Erythroid differentiation and proliferation is induced in mammalian bone marrrow cells by the hormone erythropoietin which is synthesized in the kidney and liver in response to hypoxia (Goldberg *et al.*, 1988) Early in this process there is a co-ordinate increase in the activities of the enzymes in the heme biosynthetic pathway (Beaumont *et al.*, 1984). Heme synthesis increases, and is necessary for induction of globin synthesis (Beru *et al.*, 1983). In the maturing erythroid cells, heme is required for initiation of translation of globin mRNA, and is known to inhibit the specific protein kinase responsible for inactivating initiation factor, eIF-2 (London *et al.*, 1987; Edelman *et al.*, 1987; Chen *et al.*, 1989). Following globin synthesis, the mature protein is assembled with two α and two ß globin chains complexing with four heme molecules. The hemoglobin once formed remains stable, enabling the cell to carry out its oxygen carrying function for the life of the cell which is approximately 120 days (Harris and Kellermeyer, 1970)

The study of erythropoiesis and hemoglobin synthesis in normal erythropoietic cells has been limited by several factors, including the difficulty of establishing a homogeneous cell population with respect to differentiation, and maintaining normal erythroid precursors in long term cultures (Marks and Rifkind, 1978). Some of these problems have been overcome by using transformed cell lines such as K562 cells (Hoffman *et al.*, 1980) and MEL cells (Sassa, 1983), in which the response to erythropoietin seen *in vivo,* can be induced *in vitro* by treatment with certain chemicals.

Erythroid differentiation in MEL cells can be induced by chemicals such as DMSO or hexamethylene bisacetamide (HMBA). Hemoglobin accumulation occurs midway through the developmental program and is preceded by an increase in the activities of the enzymes in the heme biosynthetic pathway, including ALAS (Sassa, 1976). Beaumont *et al.*, (1984), demonstrated that treatment of DMSO-treated MEL cells with succinylacetone, which inhibits ALA dehydratase, resulted in a marked increase in ALAS activity compared with DMSO alone and that addition of hemin prevented this. When ALAS mRNA levels were quantitated in DMSO treated MEL cells it was found that while the mRNA level increased during the first 72 hours, succinyl acetone addition either with or without hemin had no effect on this accumulation (Elferink *et al.*, 1988; May and Bawden, 1989). This evidence suggests that in differentiating MEL cells, the negative regulation of ALAS levels by heme is due primarily to a post-transcriptional mechanism.

In normal human bone marrow cultures induced to differentiate by treatment with erythropoietin. ALAS, ALA dehydratase, URO synthase and PBG deaminase are significantly induced by day four and hemoglobin first appears on day seven (Beru and Goldwasser, 1985). Levels of PBG deaminase increase linearly with increasing erythropoietin concentration and are proportional to increasing heme concentration (Sassa, 1980). However, the increase in URO synthase activity is proportional to the increase in ferrochelatase activity (Sassa and Urabe, 1979), suggesting that in normal bone marrow cells PBG deaminase may be the enzyme regulating the amount of heme produced.

The data from these studies suggest that the regulation of heme biosynthesis in normal erythropoietin-induced cells, is different to that seen in transformed erythroleukemic cells lines. The recent development of a MEL cell line that responds to erythropoietin (Klinken *et al.*, 1988), should play an important role in resolving this issue.

Studies of the effect of hemin, co-protoporphyrin and DMSO, on erythroid and hepatic cells, provide further evidence that different regulatory mechanisms of heme biosynthesis operate in these tissues. Heme suppresses ALAS and heme biosynthesis in hepatic cells (Granick and Sassa, 1978), but it stimulates heme biosynthesis in bone marrow cell (Beru *et al.*, 1983), and augments the colony forming capacity of normal murine bone marrow cells (Porter *et al.*, 1979). Co-protoporphyrin, a synthetic metalloporphyrin, also stimulates heme biosynthesis in MEL cells (Chang and Sassa, 1982) while it inhibits ALAS activity in rat liver. DMSO, a potent inducer of heme oxygenase in liver cells, inhibits heme oxygenase in cultured MEL cells and induces erythroid differentiation (Sassa, 1983). This finding suggests that in erythroid cells, not only is the biosynthesis of heme under different regulation than in the liver, but that the mechanism for regulating heme oxygenase is also distinct from that operating in liver cells.

In summary, regulation of heme biosynthesis in erythroid cells appears to be different from that seen in hepatic cells. The recent demonstration that two forms of ALAS exist, one a proposed hepatic or housekeeping form and one erythroid specific, has helped explain much of the previously conflicting evidence. Currently, there are still key issues that remain to be resolved, particularly the precise mechanisms by which heme achieves its regulatory control of ALAS in both tissues. Ongoing work in our laboratory involving the characterization of both human ALAS genes and comparison of their expression in hepatic and erythroid cells, is aimed at addressing these fundamental questions.

1.7 DISORDERS OF HEME BIOSYNTHESIS

In addition to being the major heme producing organs, the liver and developing red blood cells are the principal sources of excess porphyrin or porphyrin precursor production, in

a number of inherited and drug induced disorders of porphyrin synthesis in humans. Therefore, understanding the molecular mechanisms responsible for the regulation of heme and porphyrin synthesis in these tissues, is a problem of both fundamental and clinical importance.

Specific defects in heme biosynthesis constitute the basis for several disease states; of these the porphyrias have been studied most extensively. This has given valuable insight into the physiological and biochemical aspects of heme biosynthesis, as well as the porphyrias themselves. Sideroblastic anaemia is a less common disorder and the fundamental defect(s) involved, less defined, but impaired erythroid heme synthesis appears to be an important component of the syndrome.

1.7.1 The Porphyrias.

The porphyrias are a group of diseases in which the control of heme biosynthesis has been disturbed because of a specific defect, usually inherited, in one of the seven enzymes beyond the first step of the heme biosynthetic pathway. The porphyrias have been reviewed extensively, most recently by (Rimington, 1985; Hindmarsh, 1986; Bottomley and Müller-Eberhard 1988, and Moore 1990). Therefore, only those aspects of the three acute hepatic porphyrias, namely, acute intermittent porphyria (AIP), hereditary coproporphyria (HCP), and variegate porphyria (VP), which are most relevant to the regulation of ALAS and heme synthesis are presented here.

The three acute porphyrias are autosomal dominant genetic diseases, which arise as a result of defects at three separate steps in the pathway. Normally, the heme biosynthetic pathway is regulated so that intermediates of the pathway are present only in trace amounts. However, in all three types of acute porphyria, there is a deficiency of one enzyme which causes a partial block in the heme biosynthetic pathway, resulting in accumulation and excretion of excess ALA and PBG, together with a characteristic pattern of other heme biosynthetic intermediates, depending on the actual site of the defect (Kappas *et al.*, 1983).

These diseases may be latent for extended periods, with attacks being precipitated by exposure to one or more of the metabolic or environmental factors, including systemic illness,

certain drugs, steroid hormones, stress, starvation and menstruation (Kappas *et al.*, 1983; Bottomley and Müller-Eberhard 1988).

In all three disorders the activity of the deficient enzyme is reduced by approximately 50% (Mc Coll *et al.*, 1981), and although the enzyme defect peculiar to each type of porphyria is present in all tissues, metabolic expression of the defect resulting in the overproduction of heme precursors appears to occur only in the liver, as far as can be detected. Presumably, in the latent phase of acute porphyria, the level of ALAS is low, and despite the defective biosynthetic pathway, sufficient heme is produced by the liver to repress ALAS in the normal manner. However, many of the drugs known to precipitate acute attacks are also known to induce the cytochrome P450 drug metabolizing system in the liver (De Matteis, 1978). When this is induced, the amount of heme synthesized may be inadequate to cope with the cell's increased requirement. The lowered heme level it can be postulated, would then result in de-repression of ALAS, and in turn in the formation of excess heme precursors. The liver is the major site for metabolizing drugs by the cytochrome P450 system, and it is also the major site for the over-production of heme precursors seen in acute porphyria (May *et al.*, 1986). Further support for this hypothesis comes from studies of patients with acute attacks who have shown increases in hepatic ALAS levels of 10-14 fold (Yamamoto *et al.*, 1985).

The dominant symptoms of acute porphyria are neurological and psychiatric abnormalities. Most common is an autonomic neuropathy with abdominal pain, vomiting, tachycardia, disturbances in hypertension and urinary retention. Less common is motor neuropathy, which in some cases may involve the respiratory muscles and become life threatening. In some cases anxiety may dominate and lead to frank psychosis. In VP and HCP but not AIP, cutaneous photosensitivity occurs as the result of accumulation of porphyrins (Poh-Fitzpatrick, 1982).

The cause of the neurological symptoms associated with the acute porphyrias is unclear. It is possible that the increased circulating levels of ALA, PBG and porphyrins, may have a direct effect on the nervous system. An alternative explanation has been put forward by Corriea and Lunetta (1989), based on their work with porphyric rats. Depletion of heme in the

porphyric patient may result in impaired activity of the hepatic heme enzyme, tryptophan pyrrolase. As a result liver tryptophan levels would increase, which may eventually lead to elevated brain tryptophan and serotonin levels giving rise to neurological symptoms. A lot more work in this area is required before the neurological aspects of these conditions is understood.

Treatment of the porphyrias has been mainly directed towards trying to prevent the induction of ALAS and the subsequent accumulation of the heme precursors. The administration of high carbohydrate intake often results in significant improvement of the clinical symptoms (Bottomley and Müller-Eberhard 1988) and is based on the observation that in animals, the induction of ALAS is blocked by glucose (Bonkowsky *et al.*, 1973). Although the biochemical basis for this treatment is not understood, Yamamoto *et al.*, (1982) and DeLoskey and Beattie (1984) have proposed that glucose prevents the transport of ALAS into mitochondria by somehow promoting an increase in free heme levels. A study of porphyrin content in fasting and fed pigs has led to the proposal that fasting may in some way inhibit the activity of coproporphyrinogen oxidase (Smith and El-Far, 1980), thereby lowering the heme levels and allowing ALAS induction. Clearly more work is needed to establish how glucose exerts its effect on the symptoms of porphyria.

In some cases of porphyria, intravenous administration of hematin is used. This generally reduces the urinary excretion of heme precursors and presumably overcomes the heme deficiency enough to reverse the clinical abnormalities in the majority of, but not all, patients. In some females where attacks are associated with changes in oestrogen levels, the administration of a luteinizing hormone releasing hormone (LHRH) agonistic analogue has been beneficial (Bottomley and Müller-Eberhard 1988).

While the biochemical basis for the clinical symptoms and treatment of the acute porphyrias are not well understood, progress has been made at the molecular level. Immunological studies suggest that AIP has a heterogeneous genetic basis, and studies at the molecular level in which the mutations have been identified in affected individuals, show this to be the case (Grandchamp and Nordmann, 1988; Grandchamp *et al.*, 1989; Grandchamp *et al.*, 1989a). Hopefully, a more detailed knowledge of the molecular mechanisms involved in the

regulation of ALAS will enable the development of therapy able to prevent the acute phase of these conditions from being induced

1.7.2. Sideroblastic Anaemia

Sideroblastic anaemia constitutes a varied group of anaemic states occurring in man. If it arises by inheritance it is irreversible but it also may be associated with or reversibly in association with certain external factors such as ethanol abuse, anti-tuberculous drugs, chloramphenicol or copper deficiency, in which case the condition may be reversible. The clinical recognition of these disorders is based on the presence of hypochromic-microcytic red cells in the peripheral blood and abnormal ring sideroblasts in the bone marrow. The abnormal ring sideroblasts are red cell precursors which contain stainable iron-laden mitochondria, which either partially or completely encircle the nucleus (Bottomley, 1980). The synthesis of globin chains in the defective reticulocytes of sideroblastic patients is abnormal in two respects. Firstly synthesis is asynchronous there is an apparent deficiency of the synthesis of α chains, and secondly a large proportion of both α and ß chains synthesized are not associated with heme but are free in the cell as αß dimers. Addition of heme to these cells *in vitro* stimulates the synthesis of both chains, but particularly the α chains, and eliminates the free dimer pool (White *et al.*, 1971; Bottomley, 1980). *In vitro* studies of iron metabolism have not detected any abnormalities in either iron uptake or transfer to the mitochondria, but reduced incorporation of iron into heme has been demonstrated (Bottomley and Moore, 1985). In addition, the incorporation of glycine and ALA into heme is decreased (Bottomley and Müller-Eberhard, 1988). ALAS activity in the bone marrow is reduced in most types of sideroblastic anaemia, and this reduction is mainly evident in early erythroid development (Fitzsimmons *et al.*, 1988). Therefore, defective heme biosynthesis is thought to be the major disturbance underlying the condition (Bottomley and Müller-Eberhard, 1988).

Some patients respond to pharmacological doses of pyridoxine. ALAS activity in extracts of bone marrow cells from certain patients is enhanced by pyridoxal-phosphate the co-factor of ALAS (Bottomley and Müller-Eberhard,1988). Individual studies have shown that the ALAS from bone marrow cells of pyridoxine-responsive patients has either an altered affinity

for pyridoxal-phosphate (Konopka and Hoffbrand 1979), or an increased susceptibility of the enzyme to a mitochondrial protease which is overcome by pyridoxine administration (Aoki *et al.*, 1979).

Of particular interest among the many forms of sideroblastic anaemia, is the congenital form of the disorder, in which the majority of clinically affected cases are males. Family studies have shown that many related females suffer minor erythroid disorders, although usually no anaemia, and suggest the disorder follows an X-linked form of inheritance (Bottomley, 1980). Among the male cases considerable heterogeneity is observed in the clinical course of the disease, including the severity of symptoms, age of onset, and morbidity, which implies they may in fact represent a range of defects, rather than a single common biochemical disorder (Bottomley, 1980; Bottomley and Müller-Eberhard,1988). Isolation of the human erythroid ALAS gene in our laboratory, and its subsequent localization to the X-chromosome at Xp11.2 (Cox *et al.*, 1990), supports the proposal that defective erythroid ALAS is the principle cause of the condition, and it should now be possible to determine at the molecular level, whether mutations in this enzyme play a role in sideroblastic anaemia.

1.8 REGULATION OF EUKARYOTIC GENE EXPRESSION

Since the work in this thesis was primarily aimed at understanding how the gene for hepatic ALAS is regulated at the transcriptional level, a brief review of eukaryotic gene regulation is presented.

Crucial to the normal functioning of both individual cells and the organism as a whole, is the precise regulation of protein synthesis. In eukaryotic cells, the mRNA template for protein synthesis, has to be transcribed, processed and then transported to the cytoplasm, where it is translated on the ribosomes. While regulation may occur at any of these steps, a major level of control is believed to be at the initiation of transcription.

Early studies of transcriptional regulation in eukaryotes, identified DNA sequences *cis* to the transcription initiation site, which were shown to be the binding sites for *trans* acting factors able to modulate transcription of the gene. The situation is now known to be far more

complex. Control sequences are often able to interact with more than one protein. *Trans*-acting factors are often multimers, whose subunits are able to interact in various combinations each combination with its own specific function. This discussion will focus on some of the more interesting aspects involved in the transcriptional regulation of eukaryotic gene expression.

1.8.1 RNA polymerase II and transcription initiation factors.

Activation and regulation of the promoters of protein coding genes are mediated by the interaction of the general initiation factors, which interact with the common core-promoter elements, and gene-specific transcriptional factors that recognize and bind to particular *cis* DNA control elements (For reviews see Ptashne, 1988; Johnson and McKnight, 1989). The most common core-promoter element located 25-30bp upstream from the RNA start site, is the TATA sequence, which appears to fix the site of transcription initiation. Some housekeeping genes which lack this element, produce transcripts which are heterogeneous at the 5' end (Dynan and Tjian 1985; Melton *et al.*, 1986; Jones *et al.*, 1988).

The first step in the pathway of transcription initiation by RNA polymerase II is the binding of the general transcription factor TFIID to the TATA element (Davidson *et al.*, 1983; Van Dyke *et al.*, 1988; Buratowski *et al.*, 1989). This binding then nucleates the assembly of the other general transcription factors TFIIA, TFIIB, TFIIE and RNA polymerase II into an active transcription complex (Van Dyke *et al.*, 1988; Buratowski *et al.*, 1989).

The rate of transcription initiation is determined by the gene-specific activators and repressors that recognize sequences situated away from the TATA box. In principle, events at the TATA box may be regulated at any one of the steps or factors involved in core promoter assembly (Buratowski *et al.*, 1989; Hahn *et al.*,1989). While the regulation of transcription initiation is poorly understood, the recent cloning of the yeast gene for TFIID (Hahn *et al.*, 1989; Horikoshi *et al.*, 1989) is an important breakthrough. The yeast protein can substitute for human TFIID *in vitro* (Horikoshi *et al.*, 1989), and provides yet another example of the striking conservation of the transcriptional machinery between eukaryotes. Hahn *et al.*, (1989), have demonstrated that the protein is able to promote transcription *in vitro* from nonconsensus TATA

elements, which suggests the existence of multiple forms of the TATA element does not necessarily require multiple TATA binding factors.

1.8.2 Multiple factors recognize upstream control elements.

Upstream from the transcription start site there may be located additional promoter elements which contribute to the efficiency of transcription. A large number of these elements have been identified. Some such as the CCAAT and the GGGCGG homologies have been found in many different promoters (Dynan and Tjian 1985; Kadonga *et al.*, 1986), whereas others such as the heat-shock element (Pelham 1982) or the metallothionein element (Karin, 1984), appear to have a more specialized role.

Initially, factors were defined by the sequence they bound, but it has now become clear that in several cases, multiple factors recognize the same consensus sequence. For example, the octamer transcription factor-1 (OTF-1) sequence has been shown to bind a variety of factors, some which are ubiquitous and some specific to lymphoid cells (Jones *et al.*, 1988). Similarly, the activating transcription element (ATF), also known as the cAMP responsive element (CRE), has been shown to interact with eight distinct proteins (Berk and Schmidt, 1990).

One example which has been extensively studied in the last few years is the consensus CCAAT sequence, which is located 60-80bp from the transcription start site, and is critical for the transcription of numerous cellular and viral genes. A multiplicity of CCAAT binding factors that recognize this sequence have been described (Dorn *et al.*, 1987; Chodish *et al.*, 1988; Raymondjean *et al.*, 1988; Santoro *et al.*, 1988), some of which have been shown to be composed of at least two different subunits (Chodish *et al.*, 1988). In addition, Nuclear factor 1 (NF-I), which was originally defined by its activity in replication, has subsequently been shown to bind to the CCAAT sequence (Jones *et al.*, 1987). The CCAAT binding factor has been purified to apparent homogeneity from HeLa cells using affinity chromatography, and was found to consist of a family of proteins, which are able to function both as transcriptional activators, and initiators of replication (Santoro *et al.*, 1988). The exact relationship of these proteins to each other is not clear but they appear to arise from a single gene by alternative

splicing of mRNA transcripts (Santoro *et al.*, 1988). It remains to be determined whether these factors recognize a particular variant of the consensus sequence, or whether binding is influenced by adjacent flanking sequences.

1.8.3 Enhancers of gene expression

Gene expression in mammalian cells is often controlled by remote enhancer sequences which are able to operate in an orientation independent manner and may be located either upstream or downstream from the transcriptional start site of the gene. They contain multiple binding sites for nuclear transcription factors, and were the first DNA sequences found to confer tissue specificity (Serfling *et al.*, 1985).

The SV40 enhancer which is active in a wide range of cell types, was the first such element to be descibed and remains the best characterized. Mutagenesis studies of the SV40 enhancer have identified short DNA segments or motifs, which bind specific *trans* acting nuclear factors. Interestingly, these motifs reside in functional domains that by themselves have either weak or no enhancer activity, but act synergistically to give high levels of activity (Zenke *et al.*, 1986).

The mechanism by which enhancers exert their remote activation of gene expression is not known but several theories have been put forward. Current evidence favours the looping model, in which the enhancer and promoter associate directly or indirectly with each other to initiate transcription, and as a consequence the intervening DNA is looped out (Schatt *et al.*, 1990). Evidence for this model comes from studies *in vitro* where it has been shown that the effect of an enhancer can be transmitted to a promoter even when the two are not co-valently linked (Müller-Storm *et al.*, 1989). While considerable progress has been made in this area the precise definition of the regulatory roles of enhancer sequences and the factors they interact with remains to be determined.

1.8.4 Control of transcriptional factor activity.

Posttranslational modification of a particular transcription factor provides a rapid and versatile mechanism by which to govern its activity, and may potentially operate by either

activating or repressing its activational functions, or by directing it to a particular subcellular location.

Evidence is accumulating that the activity of several transcription factors is mediated by changes in their phosphorylation state. A recent study has shown that nuclear factor-κB (NF-κB) is activated *in vitro* by phosphorylation of its inhibitor protein-κB (Ghosh and Baltimore, 1990). In contrast, the activity of the enhancer binding activator protein-I (AP-I), a heterodimer of c-Jun and c-Fos, is induced by dephosphorylation (Berk and Schmidt, 1990).

Glycosylation is traditionally regarded as being restricted to proteins situated on cell membranes or within intracellular organelles, although O-linked N-acetylglucosamine proteins have been detected in the nucleus and some other cellular compartments. Jackson and Tjian, (1988), demonstrated a number of different transcription factors, including Sp1, CTF and AP-1, bear multiple O-linked N-acetylglucosamine residues. Furthermore, within families of closely related transcription factors, only a subset of the families were glycosylated. This work suggests differential glycosylation may be important in the functional differences between transcription factors, and may be used by the cell to regulate transcription factor activity. It will be interesting to see whether the glycosylation state of transcription factors is different in different tissues and at different stages of development.

1.8.5 The influence of chromatin structure.

The bulk of DNA in a differentiated eukaryotic cell is packaged by histones and other proteins into compact nucleosome and supranucleosome structures that are not readily available for transcription. These structures are assembled during replication, in a sequence independent manner by pathways that are poorly understood, but once formed are usually stable throughout the life of the cell (Weintraub, 1985). Alterations in chromatin structure occur over regulatory regions of expressed or potentially expressed genes, which renders the DNA hypersensitive to nucleases (Weintraub, 1985; Pederson *et al.*, 1986). These regions are often devoid of nucleosomes, and gene specific and core promoter elements are often occupied by specific binding proteins (Gross and Garrard, 1988).

The relationship between nucleosome assembly, and transcription factor binding with respect to promoter activity, has been investigated *in vitro* using systems that allow nucleosome assembly under physiological conditions. The assembly of DNA into nucleosome structures *in vitro* results in templates that are refractory to transcription factors added subsequently, whereas the formation of a stable complex between TFIID and the promoter prior to nucleosome assembly allows transcription initiation from templates containing nucleosomes (Workman and Roeder, 1987; Knezetic *et al.*, 1988). These results suggest there may be at least two steps at which transcription may be regulated *in vivo*. The first is the establishment of stable active complexes within chromatin structures (Weintraub, 1985), and the second is the utilization of such complexes for transcription initiation by RNA polymerase (Workman *et al.*, 1988).

Pina *et al.*, (1990), using minichromosomes containing the mouse mammary tumour virus promoter, that is controlled by steroid hormones, have shown that hormone receptors bind naked DNA and reconstituted nucleosomes with similar affinities, but that NF-1, a transcription factor essential for efficient transcription of the promoter, only binds the naked DNA. It is conceivable that the hormone binding to the already DNA bound receptor, causes a structural change to the nucleosome which then makes it accessible for NF-1 to bind. This would provide a mechanism by which transcription can be turned off in the absence of the hormone. Although a great deal more work needs to be done before the influence that chromatin structure has on transcriptional regulation *in vivo* is established, these studies do imply that it may have a critical role at least in establishing the inactive or active state of cellular genes.

Understanding the molecular mechanisms, involved in the transcriptional control of gene expression has become one of the major goals of modern biology. While considerable advances have been made both in our understanding of the phenomenon, and in the development of new techniques with which to gain further knowledge, there is still a great deal of work to be done before even the most fundamental mechanisms involved are completely understood.

1.9 THE AIMS OF THIS THESIS

One of the major aims of the work in this laboratory is to understand the control of heme biosynthesis, so that this knowledge can be used to aid in the treatment of the diseases associated with defective heme biosynthesis. As ALAS is the first and rate limiting enzyme in the heme biosynthetic pathway, in the liver and probably most other non-erythroid tissues, attention has focussed on understanding the molecular basis for the control of ALAS. Of particular interest is how hepatic ALAS is negatively regulated by the end product of the pathway, heme, and induced in the liver by the large number of endogenous substrates and foreign hydrophobic chemicals that induce the cytochrome P450 system.

Evidence from this laboratory (and others) suggests that one major level at which heme regulates hepatic ALAS is at the level of transcription of the hepatic ALAS gene, although little is known about how this regulation occurs. The aims of the work presented in this thesis can be divided into two parts. Firstly, in an attempt to understand the transcriptional regulation of the human hepatic ALAS gene, the gene was isolated from a human cosmid library. The 5' flanking region of the gene was identified and characterized. Using this information expression studies of the human hepatic ALAS gene promoter were carried out in the homologous human hepatoma HepG2 cell line, in an attempt to define regions of the promoter important to transcription of the ALAS gene.

The second aim of this study, was to determine whether ALAS is the defective enzyme in sideroblastic anaemia, and more particularly if the decrease in ALAS activity observed in these patients is the result of decreased expression of the ALAS gene. To this end the levels of hepatic and erythroid ALAS mRNA present in the bone marrow of sideroblastic patients were investigated.

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER TWO : MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Drugs, chemicals and reagents

2-allyl-2-isopropylacetamide (AIA) was a generous gift from Roche, Australia. Hemin was purchased from Porphyrin Products Inc., U.S.A.

The following were obtained from Sigma Chemical Co.:

Acrylamide, agarose (Type 1), ampicillin, bisacrylamide (N,N'-methylene-bis-acrylamide), bovine serum albumin (BSA), chloramphenicol, deoxyribonucleotide triphosphates (dNTPs), dithiothreitol (DTT), ethidium bromide, ethylenediaminetetra-acetic acid (EDTA), ethylene glycol-bis (beta-amino-ethyl ether) isopropyl-thiogalactoside (IPTG), N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid (Hepes), ribonucleotide triphosphates (rNTPs), o-nitrophenyl-B-D-galactopyranoside (ONPG), salmon sperm DNA, sodium dodecyl sulphate (SDS), spermidine, spermine, Tris base.

Sources of other important reagents were as follows : 5-bromo-4-chloro-3-indolyl beta-D-galactopyranoside (BCIG): BRL Ficoll 400: Pharmacia phenol: BDH chemicals polyethylene glycol 6000: BDH chemicals N,N,N',N'- tetramethethylethenediamine (TEMED): Tokyo Kasei trichloroacetic acid (TCA): Univar Pty. Ltd tRNA, *E. coli:* BRL

Kits for dideoxy DNA sequencing, oligo-labelling of DNA and *in vitro* synthesis of RNA were obtained from Biotechnology Research Enterprises of South Australia (Bresatec) All other chemicals and reagents were of analytical grade.

2.1.2 Radiochemicals

[alpha-^{32}P] dATP (1800 Ci/mmol), [alpha-^{32}P] dCTP (1800 Ci/mmol), [gamma-^{32}P] ATP (>2000 Ci/mmol) and [alpha-^{32}P]rUTP (1500 Ci/mmol) were purchased from Bresatec.

2.1.3 Enzymes

All restriction enzymes used during the course of this work were purchased from either Pharmacia or Toyobo Inc.

Other enzymes were obtained from the following sources:

Calf intestinal phosphatase : Boeringher Mannheim

E. coli DNA polymerase I (Klenow fragment) : Bresatec.

lysozyme : Sigma

proteinase K : Boeringher Mannheim

ribonuclease A (RNase A) : Sigma The stock solution (10mg/ml) was incubated at 100°C for 10 min to inactivate any DNase activity.

ribonuclease T1 (RNase T1) : Pharmacia

T4 DNA ligase : Bresatec.

T4 DNA polymerase : Integrated Sciences Pty. Ltd.

2.1.4 Buffers

Denhardt's solution : 0.1%(w/v) Ficoll, 0.1%(w/v) polyvinylpyrrolidine, 0.1%(w/v) BSA

NET : 100mM NaCl, 1mM EDTA, 10mM Tris HCl pH 7.5

SSC : 150mM NaCl, 15mM sodium citrate

SSPE : 150mM NaCl, 10mM NaH_2PO_4, 1mM EDTA

TAE : 40mM Tris-acetate, 20mM sodium acetate, 1mM EDTA, pH 8.2

TBE : 90mM Tris, 90mM boric acid, 2.5mM EDTA, pH 8.3

TE : 10mM Tris-HCl pH 7.5, 0.1mM EDTA

TES : 25mM Tris-HCl pH 8.0, 10mM EDTA, 15% sucrose

All buffers were sterilized by autoclaving or where necessary by filtration through a Sartorius ™ Minisart NML 0.2μm filter.

2.1.5 Cloning vectors

M13mp18, M13mp19 were purchased from Bresatec.

pBS KS^- and pBS SK^+ were purchased from Stratagene.

pGEM 3Zf(+) was purchased from Promega.

pIBI 76 was purchased from International Biotechnologies,Inc.

pPTZ 18R, 19R and pCH 110 were purchased from Pharmacia.

pSP 64 and pSP 72 were a generous gift from Dr. P. Krieg.

2.1.6 Cloned DNA sequences

The following cloned DNA sequences used as probes throughout this study were generous gifts from the following :

H2L cDNA for glycophorin A : Dr. Minora Fukuda, La Jolla Cancer Research Foundation, La Jolla, California.

pHF ß-actin : Dr. L. Matchoss, Stanford University, California.

pSV40hGH : Mr. G. Ryan, Institute of Medical and Veterinary Science. Adelaide.

pRHO1 full length cDNA for heme oxygenase : Dr. S. Shibahara, Friedrich Miescher-Institute Switzerland.

pRr18S cDNA for 18S ribosomal RNA : Dr J Mercer, Murdoch Institute, Melbourne.

2.1.7 Synthetic oligonucleotides

Synthetic DNA primers were synthesized by Brestec. The primer sequences are listed below;

SR 145 : 5'-dAGGGACTCGGGATAAGAATGGGC-3'

SR 357 : 5'-dCGAAGGAGGCGGGCACTCAAGTCGA-3'

DS 844 : 5'-dGAAGCTTGCTGGCTCCTGCGCTGAGGACTG-3'

RRS 943: 5'-dCCAACCGTCGACAAGTCCAAACGAAAGCTTCGTTGCCCTTGTCC-3'

M13 Universal sequencing primer (17mer) : 5'-dGTAAAACGACGGCCAGT-3'

M13 Reverse sequencing primer (25mer) : 5'-dCACACAGGAAACAGCTATGACCATG-3'

2.1.8 Bacterial strains

The following *E. coli* K12 strains were used :

(1) *E. coli* BB4 : supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1tonA ΔlacU169 F'[proAB$^+$ lacI^q lacZΔM15 Tn10 (tet^r)] host for recombinant plasmids and M13 bacteriophage.

(2) *E. coli* ED 8799 : hsdSk metB7 supE (glnV)44 supF (tyrT)58 lacZΔM15 host for recombinant plasmids, a gift from Dr. M. J. Bawden (this Department).

(3) *E. coli* DH5α: supE44 ΔlacU169 (p80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 host for recombinant plasmids, obtained from the *E. coli* Genetic Stock Centre, Yale University, New Haven.

(4) *E. coli* XL1-Blue : supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac$^-$ F'[proAB$^+$ lacI^q lacZΔM15 Tn10 (tet^r)] host for recombinant plasmids and M13 bacteriophage, purchased from Stratagene.

(5) *E. coli* DB 1161 : thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 λ^- trp-27 (B or C) rac^- sbcB15 hisG4 rfbD1 recA56 srl-300::Tn10 recB21 recC22 endA5 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1 Λ(hsd)26 (r^- m^-).

(6) *E. coli* DB 1317 : λ^- supF58 trp-89::Tn5 recD1014 hsdR2 Zjj-202::Tn10

E. coli strains DB1161 and DB1317 are both host strains which prevent *rec* A- independent deletions in recombinant DNA propagated in *E. coli* , (Ishiura *et al.*, 1989) and were obtained from the *E. coli* Genetic Stock Centre, Yale University, New Haven.

Stock cultures of these (and plasmid transformed bacteria) were prepared by dilution of an overnight culture with an equal volume of 80% glycerol and stored at either -20°C, or -80°C for long term storage. Single colonies of bacteria, obtained by streaking the glycerol stock onto agar plates of suitable medium (Section 2.1.9) were used to innoculate liquid growth medium, and the bacterial cultures were grown at 37°C with continuous shaking to provide adequate aeration.

2.1.9 Bacterial growth media

Growth media were prepared in double-distilled water and sterilized by autoclaving, antibiotics and other labile chemicals were added after the solution had cooled to 50°C.

(1) Luria (L) broth : contained 1% (w/v) Bacto-tryptone (Difco), 0.5% (w/v) yeast extract (Difco), 1% (w/v) NaCl, adjusted to pH 7.0 with NaOH. Agar plates were prepared by adding 1.5% (w/v) Bacto-agar (Difco) to the L broth. Ampicillin (50 μg/ml) or tetracycline (10 μg/ml) were added where appropriate for growth of transformed bacteria, to maintain selective pressure for the plasmid.

(2) 2 x YT Medium : 1.6% Bacto-tryptone, 1% yeast extract, 0.5% NaCl, adjusted to pH 7.5 with NaOH.

(3) Solid Media : Agar plates were prepared by supplementing the above media with 1.5% Bacto-agar. Soft overlays were 0.7% agar in L broth or 2 x YT medium.

2.1.10 Tissue culture cell lines

Human hepatoma HepG2, and Chinese Hampster Ovary (CHO) cell lines were purchased from the American Type Cell Culture (ATCC) Laboratory.

2.1.11 Tissue culture media

Phosphate buffered saline (PBS) : 136mmol/NaCl, 2.6mmol/KCl, 1.5mmol/KH_2PO_4 and 8mmol/ Na_2HPO_4, pH 7.4, was sterilized by autoclaving (20 psi for 25 min at 140°C).

Trypsin/EDTA solution : 0.1% trypsin (Difco) and 1 x EDTA Versene buffer solution (CSL), was sterilized by filtration through a 0.2μM filter (Whatman).

Growth Medium for HepG2 cells: 1 x 1 litre packet Dulbecco's minimal essential medium, (DMEM) (Gibco), 28mmol/l $NaHCO_3$, 19mmol/l glucose, and 20mmol/l Hepes, pH 7.3, was supplemented with 50,000 Units Gentamycin (Gibco), and filter sterilized as described above.

Growth medium for CHO cells : 1 x 1 litre packet Ham's F12 with L-glutamine (Gibco), and 28mmol $NaHCO_3$, was supplemented with 50,000 Units Gentamycin (Gibco), pH 7.4, and filter sterilized as described above.

Foetal Calf Serum : CSL

Nu Serum™ : Collaborative Research Inc.

2.1.12 Miscellaneous

GF/A glass fibre filter discs and 3MM paper : Whatman Ltd

Kodak Diagnostic film X-Omat AR, USA

Nitrocellulose (BA 85) and Nytran 0.45μm : Schleicher and Schuell

X-ray film : Fuji Photo Film Co. Ltd, Tokyo, Japan

2.2 RECOMBINANT DNA METHODS

2.2.1 General DNA methods

The following methods were performed essentially as described in Maniatis *et al.*, (1982) :

Growth, maintenance and preservation of bacterial and viral strains; quantitation of DNA and RNA; autoradiography; agarose and polyacrylamide gel electrophoresis; DNA and RNA precipitations; phenol/chloroform extractions; end-filling or end labelling of DNA fragments using the Klenow fragment of *E. coli* DNA polymerase I.

All manipulations involving viable organisms which contained recombinant DNA were carried out in accordance with the regulations and approval of the Australian Academy of Science Committee on Recombinant DNA and the University Council of the University of Adelaide.

2.2.2 Plasmid DNA preparation.

The rapid alkaline hydrolysis procedure of Birnboim and Doly (1979) was used for the isolation of plasmid DNA from 2 ml overnight cultures for analytical restriction digests. This method was also employed for the bulk preparation of plasmid cDNAs from 50 ml cultures for use as probes either for radiolabelling in Northern hybridization analysis (Section 2.3.2) or for immobilisation to nitrocellulose filters for nuclear transcription run-on analysis (Section 2.4.2).

DNA used for transfection of tissue culture cell lines, was routinely grown up in 500ml cultures innoculated with 4.5ml from a 5ml overnight culture. The plasmid was extracted using the alkaline lysis procedure described above and further purified either by gel filtration on the HPLC using a Superose-6 preparative grade column matrix (Pharmacia), or by anion-exchange using a Qiagen™ 500 column (Diagen) to remove any contaminating RNA, thereby allowing accurate quantitation of the DNA by spectrophotometry.

2.2.3 Restriction enzyme digestions

In analytical digests, 0.5-1μg of DNA was incubated with 2-5 units each of the appropriate restriction enzyme(s) for a minimum of 2 hours in the buffer conditions specified by the manufacturer. Reactions were terminated by the addition of a 1/3 volume of urea load buffer and electrophoresed on 1% mini-agarose gels in TBE buffer.

In preparative digests, 5 μg of DNA was restricted in a reaction volume of 30μl, and the desired DNA fragments were isolated as detailed below.

2.2.4 Preparation of cloning vectors

Plasmids and the replicative form of M13 were linearized with the appropriate restriction enzyme(s) To prevent self-ligation of the vector, 5' terminal phosphate groups were removed by incubation in 50mM Tris-HCl pH 9.0, 1mM $MgCl_2$, 0.1mM $ZnCl_2$, with 0.5 units of calf intestinal phosphatase (CIP), in a final volume of 50ml for 1 hr at 37°C The vector DNA was isolated after electrophoresis on a 1.0% agarose TAE gel using a Geneclean™ kit according to the manufacturers' instructions. The DNA was resuspended at a concentration of 20-50 ng/μl, for use in ligation reactions.

2.2.5 Preparation of DNA restriction fragments.

The DNA to be digested was incubated with the appropriate restriction enzyme(s) as described above (Section 2.2.3) and all restriction fragments were isolated from either a horizontal 0.8%-2.0% agarose gel or a vertical 8% polyacrylamide gel, depending on the size of the DNA restriction fragment(s). Bands representing restriction fragments were visualised under UV light following staining with ethidium bromide, and the appropriate fragment(s) excised from the gel. Fragments isolated from polyacrylamide gels were eluted from the gel slice by incubation in 400μl of 0.5M ammonium acetate, 0.1% SDS, at 37°C for 16 h. The DNA was precipitated by the addition of 2.5 volumes of 100% ethanol, washed in 70% ethanol, air dried and resuspended in 10-20 μl of 0.1mM EDTA. Alternatively, the DNA fragments were run on 0.8%-2.0% TAE agarose gels and the DNA isolated using the Geneclean™ protocol.

2.2.6 Ligation conditions.

A 10µl reaction contained 20-50ng of vector DNA, the DNA restriction fragment, 50mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 1mM DTT, 1mM ATP, and 1-2 units of T4 DNA ligase. For cloning into plasmid vectors, or M13, a 2-3 molar excess of restriction fragment insert to vector DNA was used. The reactions were incubated for either 4 hours at 26°C, or overnight at 4°C. A control ligation with vector only was set up and included in the subsequent transformation to determine background levels of uncut or recircularized vector DNA.

2.2.7 Transformation procedure of *E. coli* with plasmid recombinants

A single colony of the *E. coli* host strain was innoculated into 5ml of L-broth (where appropriate the L-broth was supplemented with an antibiotic) and the culture incubated overnight at 37°C with continuous shaking. The overnight culture was then diluted 100 fold into 50ml of L-broth (plus antibiotic) and the incubation continued at 37°C, with shaking, until the culture reached an absorbance at A_{600} of 0.6-0.8. The cells were then pelleted by centrifugation at 2,000 x g for 5 min, resuspended in 2.5ml of ice cold 0.2M $MgCl_2$, 0.5M $CaCl_2$ and left on ice for 60 min. 200µl of this cell suspension was mixed with 2-5µl of the DNA ligation reaction mix (Section 2.2.6) and left on ice for 40 min. The cells were then heat shocked at 42°C for 2 min, L-broth plus 20mmol/l glucose was added (1ml if the overlay method of plating was used or 100µl if the cells were spread directly onto the agar plates), and the cells were incubated at 37°C for at 20-30 min. The transformed cells were then plated onto L-agar containing 50µg/ml of ampicillin, either by spreading with a wire spreader, or the cells were mixed with 3ml of 0.7% L-agar overlay, and poured onto the plates. The agar plates were routinely incubated at 37°C overnight.

2.2.8 Transformation procedure for M13 recombinants

A single colony of *E. coli* (strain BB4 or XL1-B) was innoculated into 5ml of 2 x YT broth and grown at 37°C overnight with continuous shaking. The culture was then diluted 100 fold into 50ml of 2 x YT and incubated at 37°C with shaking until it reached an absorbance at A_{600} of 0.4-0.6. The cells were pelleted by centrifugation at 2000 x g for 5 min, and

resuspended in 2.5ml of ice cold 50mM $CaCl_2$. 200µl of this cell suspension was mixed with 2-5µl of the DNA ligation mix and left on ice for 40 min.The cells were heat shocked at 42°C for 2 min, and 3ml of 2 x YT soft agar, containing 20µl of 20 mg/ml BCIG, 20µl of 24 mg/ml IPTG and 200µl of an overnight culture of plating bacteria was added. The transformed cells were poured onto L-agar plates containing 10µg/ml of tetracycline and incubated at 37°C overnight.

2.2.9 Preparation of M13 single-stranded template DNA

M13 phage plaques were innoculated into 1.5 mls of a 1:40 dilution of competent BB4 cells in 2 x YT broth, and grown with vigorous shaking for 5 hours at 37°C. Cells were pelleted by centrifugation in an Eppendorf centrifuge for 5 min. The supernatant was carefully removed into a fresh tube and recentrifuged. 200µl of 2.5M NaCl ,20% PEG 6000, was added to 1.2ml of the supernatant (40% glycerol was added to the rest which was saved as stock at -20°C). After 15 min at room temperature the single stranded phage pellet was collected by centrifugation in an Eppendorf centrifuge for 5 min. All traces of the supernatant were removed, and the pellet was resuspended in 100µl of 10mM Tris-HCl pH 8.0, 0.1mM EDTA and extracted with an equal volume of buffer-saturated phenol. The aqueous phase was re-extracted 3 x with 500µl of diethyl ether and ethanol precipitated. The phage DNA was collected by centrifugation, washed in 70% ethanol, air dried, resuspended in 25µl TE buffer, and stored at -20°C.

2.2.10 Preparation of single-stranded plasmid template DNA

Single colonies of transformed bacteria were innoculated into 1.5ml cultures of L-broth containing 0.001% thiamine and 50µg/ml ampicillin, and grown overnight at 37°C with good aeration. The cultures were then diluted 1:40 grown to an A_{600} of 0.6-0.8, and again diluted 1:40. K107 helper phage at a multiplicity of infection of 10 was added, and the cells were incubated at 37°C for 1 h, kanamycin 70µg/ml was then added and the cells incubated overnight at 37°C with good aeration. The single stranded DNA was isolated as described above.

2.2.11 Complementary testing of single-stranded M13 Clones

To determine which strand of a particular subcloned fragment was present in a single-stranded M13 recombinant, hybridization analysis was carried out using an arbitrarily selected or previously sequenced recombinant as a reference. 2µl of the test DNA was added to 2µl of the reference DNA with 4µl of 0.1M Tris-HCl pH 7.4, 0.1M $MgCl_2$, 0.5M NaCl, and 2µl of 50% glycerol, 1% SDS, 0.2M EDTA, 0.2% bromophenol blue, and incubated at 65°C for 1 h. The samples were electrophoresed on a 1% agarose mini-gel, with 2µl of the reference DNA as a marker. The DNA was visualised after ethidium bromide staining. Single-stranded M13 clones with inserts identical to the reference clone co-migrate with the reference, whereas clones containing the complementary strand are retarded, as they have hybridized to the reference DNA, thereby decreasing their mobility.

2.2.12 Dideoxy-chain sequencing analysis

Single stranded template DNA (8µl) was annealed in 10mM Tris-HCl pH 8.0, 1mM $MgCl_2$ with 5-7 ng of the appropriate primer (1µl) in a final volume of 10µl. The mixture was heated at 95°C for 3 minutes, and incubated at 50°C for at least 15 minutes.

Sequencing was performed using the Sanger dideoxy method (1977), with the sequencing reagents supplied in the Bresatec DSK-A kits. The sequencing reactions were performed in accordance with the protocol accompanying the kits.

Double stranded sequencing was performed using plasmid DNA purified on a Qiagen™ column 3-5µg of plasmid was used per reaction. The DNA was denatured in 0.2M NaOH, 2mM EDTA for 30 min at 37°C. The mixture was then neutralized by the addition of 0.1 volumes of 3M sodium acetate pH 4.6, and the DNA precipitated with 3 volumes of ethanol. The DNA pellet was collected by centrifugation, washed in 70% ethanol, and resuspended in 7µl of 0.1mM EDTA. The sequencing reactions were carried out as for single stranded DNA, using a Sequenase® version 2.0 kit in accordance with the protocol accompanying the kit.

2.2.13 Gel electrophoresis of DNA for sequence analysis.

Sequencing reactions (1µl) were electrophoresed on 6% polyacrylamide gels containing 7M urea in 1 x TBE buffer at 1800 V. After electrophoresis, gels were washed in 10% (v/v) acetic acid to remove the urea, followed by washing with 20% (v/v) ethanol, and baked at 100°C for 40 minutes. The gels were then autoradiographed for 4 to 16 hours at room temperature. All sequencing data was compiled and analysed using a VAX computer (Section 2.6.3).

2.2.14 Preparation of [^{32}P]-labelled DNA probes

(i) Oligo-labelling of DNA

In all experiments, a Bresatec kit was used for the oligolabelling of recombinant plasmids. 0.1-0.5 µg of DNA was [^{32}P]-labelled in a 25µl reaction containing 100mM Tris-HCL pH 7.6, 20mM $MgCl_2$, 100mM NaCl, 200µg/ml BSA, 4µM each of unlabelled dGTP and dTTP, 100µCi each of [α^{32}-P] dATP and [α^{32}-P] dCTP, and 5.0 units of the large Klenow fragment of *E. coli* DNA polymerase I.

The reaction was incubated at 37°C for 30 minutes and then stopped by the addition of 5µl of 0.5 M EDTA, pH 8.0 and 5µl of 10% SDS. 10µl of tRNA (10 mg/ml), 50µl of NET buffer and 125µl of 4M ammonium acetate were then added and following the addition of 400µl of ethanol, the DNA was precipitated at either -80°C for 30 minutes or at -20°C overnight. The DNA was pelleted by centrifugation for 30 minutes at 12,000 x g, washed with 1 ml of ice-cold 70% ethanol, air dried, and resuspended in 388µl of TE buffer.

Determination of TCA-precipitable radioactivity using 1µl aliquots (Section 2.6.1) demonstrated that over 90% of total radioactivity in the ethanol precipitate was TCA-precipitable. The specific activity of probes was generally 1-2 x 10^8cpm/µg. Immediately before adding to the hybridization mix, the the oligo-labelled DNA was denatured by incubation at 100°C for 10 mins, after the addition of 10M NaOH to a final concentration of 0.3M. The probe was then snap cooled on ice and neutralised by the addition of and equal volume of 4M ammonium acetate.

(ii) 5' end-labelling of synthetic DNA oligonucleotides.

The synthetic DNA oligonucleotides used as probes were ^{32}P-labelled at the 5' end using [gamma-^{32}P] -ATP and T4 polynucleotide kinase. The reaction mixture contained 10mM $MgCl_2$, 50mM Tris-HCl pH 7.4, 5mM DTT, 0.1mM spermidine, 0.1mM EDTA, 100 µCi [gamma-^{32}P] ATP and 2 units of T4 polynucleotide kinase in a final volume of 10µl. This was incubated at 37°C for 30 minutes. Following the addition of 10 µl formamide loading buffer, the reaction was run on a 20% polyacrylamide gel at 32 mAmps for 60 minutes to separate the [^{32}P]-labelled oligomer from unincorporated label. The labelled oligomer was localized by autoradiography, excised from the gel and eluted in 0.5 M ammonium acetate, 1mM EDTA, 0.1% SDS at 37°C for 16 h. This solution was then used directly for hybridizations.

2.2.15 Southern transfer and hybridization conditions

DNA was digested with the appropriate restriction enzymes and electrophoresed on a 0.8-1.0% (depending on the size of the DNA fragments being separated) agarose gel in 1 x TBE. Following staining with ethidium bromide, the gel was visualised under UV light, and photographed. The DNA fragments were partially hydrolized by soaking the gel in 0.25M HCl for 10 minutes, and transferred to nitrocellulose according to the method of Southern, as described by Maniatis *et al.*, (1982). Following transfer the filters were baked *in vacuo* for 2 h and prehybridized for 4 hours in 6 x SSC, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidine, 0.1% (w/v) BSA, 0.1% SDS and 200µg/ml of heat denatured sonicated salmon sperm DNA at 65°C. Hybridization was carried out for 16 hours in the same conditions with the addition of 20-50 ng/ml of [^{32}P] labelled DNA probe (Section 2.2.14). If 5'-end-labelled synthetic DNA oligonucleotides were used as the probe (Section 2.2.14), the hybridization temperature was lowered to 37-42°C depending on the length of the oligonucleotides. Filters were washed in 2 x SSC, 0.1% SDS, 0.1% sodium pyrophosphate for 15 minutes at room temperature, followed by two washes in 0.2 x SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 65°C for 30 minutes each. Autoradiography of the filter was performed at either at room temperature for several hours, or overnight or longer at -80°C, depending on the strength of the hybridization signal.

2.2.16 Oligonucleotide site-directed mutagenesis of ALA-S recombinant clones

0.5pmol of single stranded recombinant DNA was mixed with 10pmol of 5' phosphorylated mutagenesis primer, 10pmol of 5' phosphorylated reverse sequencing primer or universal sequencing primer (depending which plasmid vector the recombinant clone had been inserted into), 0.05M Tris-HCl pH 7.4, 0.01M $MgCl_2$, and 66M NaCl, in a total volume of 15µl. The mixture was heated to 65°C for 5 min and left to cool at room temperature a further five min. 5µl of 0.01M rATP, 5µl 0.01M DTT, 5µl dNTP mix (500µM of each dATP,dCTP, dGTP and dTTP), 1µl T4 DNA ligase (1U/µl), 2 µl Klenow fragment of DNA polymerase (2.5U/µl) and 17µl of water were added and the reaction.was incubated at room temperature for 12-16 h. 1-3 µl of the resulting mutagenesis mixture was used to transform *E. coli* BB4 as described in Section 2.2.7 usually yielding 200-500 colonies per plate.

2.2.17 Colony screening

Colonies were picked onto three replica plates containing nitrocellulose stamped with a numbered grid, and grown at 37°C overnight. One plate was used as the master plate and stored at 4°C. The nitrocellulose filters were removed from the other two plates and layered onto a sheet of Whatman 3MM paper saturated in 10% SDS for 5 min after which they were transferred to a second sheet of Whatman 3MM saturated in 0.5M NaOH and 1.5M NaCl for 5 min and finally to a third sheet of Whatman 3MM saturated in 0.5M Tris-HCl pH 8.0, 1.5M NaCl, for 5 min. The filters were air dried and baked at 80°C *in vacuo* for 2 h.

The filters were prehybridized in 10ml of 0.4M NaCl, 0.09M Tris-HCl pH 7.6, 0.009M EDTA, 0.5% NP-40, 5 x Denhardt's solution (Section 2.1.4) and 100µg/ml of sonicated salmon sperm DNA, at 42°C for 2 h. 100ng of ^{32}P-5' phosphorylated primer, kinased as described in Section 2.2.14 (ii), was added to the prehybridization mixture and incubated at 42°C for 16-24 h.

After hybridization the filters were washed twice in 100mls 6 x SSC, 0.003M EDTA, pH 7.4 for 5 min at room temperature, then once in 20ml tetramethylammonium chloride

solution (3M TMACl, 0.05M Tris-HCl pH 8.0, 0.002M EDTA, 0.1% SDS) for 5 min at room temperature to remove any unbound mutagenesis primer. Mutagenesis primer bound to wild type DNA was removed by further washing 2 x 30 min in TMACl solution at 5°C below the theoretical melting temperature of the mutagenesis primer (Maniatis *et al.*, 1982). The filters were autoradiographed overnight at -80°C.

Mutant colonies were identified by aligning the developed autoradiogram and the nitrocellulose filters, corresponding colonies were grown up from the master plate and the mutation verified by analytical restriction enzyme mapping and sequence analysis.

2.3 METHODS FOR ISOLATION AND ANALYSIS OF RNA

2.3.1 Preparation of total RNA from human liver.

Total RNA was extracted from 2.5 g of tissue using the guanidinium isothiocyanate procedure described by Chomczynski and Sacchi (1987), modified in the following way. Firstly, the volumes used in the published extraction procedure were scaled up. Secondly when RNA is prepared from liver, glycogen tends to co-purify with the RNA, this was removed by precipatation of the RNA with 3 volumes of 4M Na acetate at 0°C overnight. The RNA was recovered by centrifugation at 8000 x g for 15 min at 4°C, and resuspended in 0.1mM EDTA.

2.3.2 Isolation of RNA from tissue culture cells

Total RNA was extracted from tissue culture cells, essentially as described by Chomzynski and Sacchi (1987).

Following RNA extraction, the absorbance values at 260 nm and 280 nm of each RNA sample were determined on a Shimadzu UV-160A spectrophotometer. The A_{260}/A_{280} ratios of the RNA samples were consistently in the range 1.6 - 2.0. The relationship of one A_{260} unit equal to 40 µg/ml RNA was used in the calculation of RNA concentrations.

2.3.3 Northern hybridization analysis of RNA

Northern hybridization analysis of total RNA was carried out by denaturation on 1% agarose gels containing 1.1M formaldehyde, and transfer onto either BA85 nitrocellulose or

Transfer was carried out in [redacted]IETS™ medium and verified by visualization of the filter under UV illumination.

Nytran™ (Schliecher and Schuell) filters. Following transfer, the filters were either baked for 2 hours *in vacuo,* or irradiated with 120mjoules of UV radiation in a Stratagene UV Stratalinker™ 1800 which results in the RNA being covalently cross-linked to the filter (manufacturer's instruction manual). Filters were pre-hybridized for 4-16 hours at 42°C in 50% formamide, 5 x SSC, 5 x Denhardt's solution 0.1% SDS, 0.05% sodium pyrophosphate, and 200 µg/ml of sonicated salmon sperm DNA. Hybridizations were carried out for 18-24 h under exactly the same conditions, except for the addition of radiolabelled probe (1-5 x 10^8 counts/µg). Filters were washed in 2 x SSC, 0.1% SDS at room temperature for five and twenty minutes, followed by one wash in 2 x SSC, 0.1% SDS at 60°C for forty minutes.

2.3.4 *In vitro* synthesis of [^{32}P]-labelled RNA

10µg of the DNA vector containing the anti-sense DNA template for RNA synthesis was linearized with the appropriate enzyme, separated from any uncut vector by electrophoresis and the template recovered by the gene clean™ protocol. 2µg of this DNA was added to a reaction mix containing 0.01M DTT, 0.04M.Tris-HCl pH 7.6, $MgCl_2$, 500µM of each rATP, rCTP and rGTP, 50µM rUTP,1µg BSA, 100µCi [α–^{32}P] rUTP and 4 units of SP6 RNA polymerase, in a total volume of 20µl. The reaction was incubated at 42°C for 60 minutes, electrophoresed on a 6-8% sequencing gel, and autoradiographed for 30 seconds. The band corresponding to the full length probe was cut out and the labelled probe was eluted overnight in 400µl of elution buffer (0.5M ammonium acetate, 0.001M EDTA,and 0.1% SDS), at 37°C. The buffer containing the probe was then aspirated away from the gel slice and the RNA was stored under ethanol at -20°C

2.3.5 RNA protection analysis

20µg of the test RNA and approximately 50,000 cpm of the single stranded RNA probe were combined in an Eppendorf tube and pelleted by centrifugation. The supernatant was removed and the RNA pellet dissolved in 24µl of deionized formamide, 3µl of water and 3µl of 10 x hybridization buffer (4M NaCl, 0.4M PIPES pH 6.4, and 0.01M EDTA) were

added.The mixture was heated to 85°C for several minutes and then incubated at 45°C overnight.

Following hybridization 350µl of RNase digestion solution was added (0.3M NaCl, 0.01M Tris-HCl pH 7.5, 0.005M EDTA, 4µg/ml RNase A and 2µg/ml RNase T1, and the reaction was incubated at 37°C for 30 min. 5µl of Proteinase K solution (25mg/ml) and 20µl of 10% SDS were added and the incubation continued at 37°C for a further 15 min. The reaction mix was extracted with an equal volume of phenol/chloroform (1:1) and the protected RNA recovered by precipitation after addition of 10µg of carrier tRNA and 1ml of ethanol.

The protected RNA was dissolved in formamide loading buffer (Maniatis *et al.*, 1982), denatured by heating at 85°C for 2 min and analyzed by electrophoresis on a 6-8% sequencing gel. A series of sequencing reactions performed on either single stranded M13 DNA, or single stranded DNA prepared from the clone encoding the protected fragment, was used as a size marker. The gel was autoradiographed at -80°C for approximately one week using Kodak X-Omat sensitive film.

2.4 METHODS FOR ISOLATION OF NUCLEI AND TRANSCRIPTION RUN-ON ASSAYS

2.4.1 Isolation of nuclei from mammalian tissue culture cells

To ensure the cells were in the growth phase they were routinely divided 1:2 the day before the nuclei were to be isolated. The media from four 175cm^2 tissue culture flasks (approx 5 x 10^7 cells) per treatment , was removed and the cells washed with 10mls of PBS. The cells were trypsinized and collected in 10mls of media containing 10% foetal calf serum to inactivate the trypsin. The cells were centrifuged 5 min at 1000 x g, resuspended in PBS and the wash repeated. The supernatant was completely removed and the cell pellet loosened by vortexing gently at half maximal speed for 10 sec prior to the addition of 4mls of NP-40 lysis buffer (10mM Tris-HCl pH 7.4, 10mM NaCl, 3mM $MgCl_2$, and 0.5% NP-40). The vortexing was continued as the buffer was added and for 10 secs afterwards. This allowed the uniform resuspension of the cells and inhibited clumping. (The same method was used to resuspend the

nuclei). The lysed cells were incubated on ice for 5 mins and centrifuged for 5 mins at 1000 x g, at 4°C.

The nuclear pellet was resuspended in 4 mls of NP-40 lysis buffer by vortexing as described above, and centrifuged 5 mins at 1000 x g, at 4°C. The supernatant was discarded, and the nuclei were resuspended in 200µl glycerol storage buffer (20mM Tris-HCl pH 7.9, 75mM NaCl, 0.5mM EDTA, 50% glycerol, 1mM DTT, and 0.1mM PMSF) by gentle vortexing. The nuclei were counted using a hemocytometer and the concentration adjusted to 3 x 10^8 nuclei per ml. The yield of nuclei was approx 3 x 10^7.

2.4.2 Nuclear run-on transcription assay

Nuclei were used in the transcription run-on experiments immediately after isolation. Transcription reactions contained 100mM Tris-HCl pH 7.9, 50mM NaCL, 5mM $MgCl_2$, 1.5mM $MnCl_2$, 0.4mM EDTA, 0.1mM PMSF, 1.2mM DTT, 30% glycerol, 1mM of each ATP, CTP, GTP, 2µM of unlabelled UTP, 10-100µCi of [α-^{32}P]rUTP and 1.5 x 10^7 nuclei, in a final volume of 150µl. Reactions were incubated for the required time (usually 25 min) at 26°C To monitor incorporation of [^{32}P]UTP, the TCA- precipitable radioactivity of 1µl aliquots were determined in triplicate. Before adding the TCA, aliquots were added to Eppendorf microcentrifuge tubes containing 100µl of 10% SDS, 10mM EDTA. This terminates the reaction and lyses the nuclei, resulting in a much lower background radioactivity (Marzluff; 1978).

2.4.3 Preparation of [^{32}P]-labelled RNA

[^{32}P] RNA was prepared as described by Vannice *et al.,* (1984). Reactions were terminated by the addition of 750µl of 0.5% SDS, with 100µg of *E. coli* tRNA as carrier. After gentle mixing, 900µl of 100mM Na acetate pH 5.0, 20mM EDTA was added and RNA was isolated by extraction with 1.8ml of H_2O saturated acidic phenol. The top phase was collected, 3M Na acetate was added to a final concentration of 0.2M and the RNA precipitated by the addition of 2.5 volumes of ethanol. After precipitation for one hour on dry ice, or overnight at -20°C, the RNA was collected by centrifugation for 30 minutes at 10,000 rpm (16,000 x g),

in a HB-4 rotor at 4°C. The pellet was washed with ethanol, and resuspended in 200µl H_2O, and the TCA-precipitable radioactivity in 1µl was determined in triplicate (Section 2.6.1).

2.4.4 Hybridization of [^{32}P] RNA to immobilized DNA

5 µg of DNA was applied to a nitrocellulose filter using a slot-blot apparatus (Schleicher and Schuell). For each slot, 5 µg of DNA in 200µl of TE buffer was incubated with 20 µl of 3M NaOH for 30 minutes at 65°C, the mixture was then neutralized with 220 µl of 4M ammonium acetate and kept on ice. The nitrocellulose filter was soaked first in H_2O and then in 2M ammonium acetate, for 10 min each. The DNA (final volume 440µl) was applied to the nitrocellulose under gentle suction and the filter was then either air-dried and baked at 80°C under vacuum for one hour, or irradiated with 120m joules of UV radiation in a Stratagene UV Stratalinker™1800.

Duplicate filters were prehybridized in 50% formamide, 5 x SSC, 10mM Tris-HCl pH 7.6, 1mM EDTA, 0.1% Na pyrophosphate, 0.1% SDS, 100 10mM Tris-HCl pH 7.6, 1mM EDTA, 0.1% Na pyrophosphate, 0.1% SDS, 100 µg/ml *E. coli* tRNA and 0.2% each of Ficoll, polyvinyl-pyrrolidone and bovine serum albumin, at 52°C overnight. Hybridizations were carried out in the same solution with the [^{32}P] RNA for 72 hours at 52°C. Filters were washed twice at room temperature for 30 minutes in 2 x SSC, 0.1% SDS, 0.1% Na pyrophosphate, then twice in 0.5 x SSC, 0.1% SDS, 0.1% Na pyrophosphate at 65°C for 60 minutes. The filters were then incubated at 37°C in 1 x SSC, 10 µg/ml RNAse A, for 30 minutes, washed for 30 minutes in 1 x SSC, air dried and autoradiographed.

2.5 METHODS FOR THE TRANSIENT EXPRESSION OF ALA-S RECOMBINANTS IN TISSUE CULTURE CELL LINES

2.5.1 Cell maintenance

All cells were routinely maintained in 150 cm^3 flasks (Costar) at 37°C in an atmosphere of 5% CO_2 and were subcultured every 3-4 days. To subculture or harvest the cells the culture media was removed and the cells washed in PBS before the addition of 3mls of trypsin/EDTA solution. The cells were left at room temperature until they began to detach from the flask, when 7mls of culture media was added and the flask washed to remove any

remaining cells. The cells were subcultured by routinely being split 1:4, into fresh media and incubating at 37°C. If the cells were to be harvested they were washed twice in 10mls of PBS, and pelleted by centrifugation at 1200 x g for 5 minutes, before resuspending in the appropriate buffer.

2.5.2 Transfection of human hepatoma HepG2 cells by electroporation

Transfection of human hepatoma HepG2 cells by electroporation was performed by a modification of the method of Chu *et al.*, (1987). To ensure the cells were in the growth phase they were routinely split 1:2 the day before electroporation

The cells were detached using trypsin/EDTA washed 2 x in PBS and resuspended in Hepes buffered saline (HBS) containing 0.02M Hepes pH 7.05, 0.137M NaCl, 0.005M KCl, 700μM $Na_2 HPO_4$, 0.06M dextrose at a concentration of 1x 10^7 cells/ml. 50μg of sonicated salmon sperm, 5-10μg of the DNA construct to be transfected and 500μl of the cell suspension was added to each electroporation cuvette and gently mixed using a pipette.

The cells were incubated at 4°C for 10 min, exposed to a single voltage pulse of 220 volts, capitance 960, and left at room temperature for 10 min before gently plating into 60mm dishes containing 4mls of DMEM plus 2% FCS and 8% Nu serum. Cells were incubated at 37°C for 48-72 hours. In experiments where reagents such as hemin, or ALA were used the cells were routinely left to recover from electroporation and start to express the reporter gene for approximately 24-36 hours. After this time the media was changed the reagents added, and the media assayed for human growth hormone 2-48 h later. Where the cells were co-transfected with the plasmid PCH 110 (which has the reporter gene ß-galactosidase (ß-gal) under the control of the SV40 promoter), as an internal control the cells where harvested and assayed for ß-gal activity. In experiments where reagents were used, hemin was prepared freshly each time as a 1mM dissolved in 0.1% sodium carbonate, once the hemin was dissolved the pH was adjusted to 7.4 with 1M HCl. ALA, the ALA methyl ester, and succinyl acetone were dissolved in PBS at concentrations 100 fold greater than the final concentration in the culture media.

2.5.3 Transfection of chinese hampster ovary cells (CHO) by electroporation

CHO cells were harvested and transfected by electroporation as described above, using the following conditions. The cells were resuspended in PBS at a concentration of 6.25 x 10^6 cells per ml, and 800µl of the cell suspension was used for each transfection. The electrical pulse was 1800 volts and 240 capitance, and the cells were plated into 4mls of F12 media, and treated as described above.

2.5.4 Human growth hormone (hGH) assay

The Allegro™ hGH Immunoassay System which incorporates two monoclonal antibodies with high affinity and specificity for the hormone was used for all hGH assays performed during this study. The two antibodies obtained from mouse Ab (1) and Ab (2), each specific for a different epitome on the hGH molecule, bind without competition or steric interference from each other and form a soluble sandwich complex : Ab (1) - hGH - Ab (2). One of the antibodies is radiolabelled for detection, while the other is coupled to biotin. The subsequent addition to the reaction mixture of an avidin coated bead allows for a specific and efficient means of binding the sandwich complex to a solid phase via the high affinity interaction between biotin and avidin. (manufacturer's information brochure).

In the assay, 100µl of the control standards and samples were incubated with 100µl of a solution containing both the radiolabelled and biotin coupled antibodies, and an avidin coated bead, for four hours at room temperature. At the end of the incubation period, the bead was washed to remove any unbound components and the radioactivity bound to the solid phase measured in an LKB gamma scintillation counter. Since the sandwich complex only forms in the presence of the hGH molecule, the radioactivity of the avidin bound complex is directly proportional to the amount of hGH in the sample

A dose response curve of radioactivity versus concentration was generated using the results obtained from the standard controls. The concentrations of hGH present in the samples was determined directly from this curve.

2.5.5 ß-galactosidase (ß-gal) assay

Cells were harvested as described above washed twice in PBS, and resuspended in 0.25M TrisHCL buffer, pH 7.5 (50µl per 60cm dish of cells) The cells were lysed by freeze thawing three times, cell debris was pelleted by centrifugation for 5 min in an Eppendorf centrifuge and 30-50µl of cell extract was used to assay for ß-galactosidase activity.

1ml of reaction buffer : 0.1M $NaPO_4$ pH7.3, 10mM KCL, 1mM $MgCl_2$, 50mM ß-mercaptoethanol, and 200µl of 2mg/ml ONPG,were added to 20µl of cell extract, and incubated at 37°C until a yellow colour was obvious (40-60 min). The reaction was stopped by the addition of 500µl of 1M Na_2CO_3. The absorbance at A_{420} was measured and the ß-galactose activity expressed as units of ß-gal per mg of protein per hour of reaction.

2.5.6 Bio-Rad Protein Assay

The protein content of the cell extracts was determined using 5µl of cell extract and the Bio-Rad protein microassay procedure according to the manufacturer's instructions. Bovine serum albumin was used as the protein standard.

2.6 MISCELLANEOUS METHODS

2.6.1 Measurement of TCA-precipitable radioactivity

1-5µl of sample was added to 100µl of H_2O, with 100µg of carrier (denatured salmon sperm DNA for DNA samples, or *E. coli* tRNA for RNA samples). 1-5ml of ice cold 5% TCA, 1% sodium pyrophosphate was added and the samples kept on ice for 30 min. The precipitate was collected by filtration through a Whatman GF/A glass fibre disc and washed with 25ml of ice cold 5% TCA, 1% sodium pyrophosphate followed by 10ml of ethanol. The discs were dried and counted in 3ml of Optiscint™ (Pharmacia) scintillation fluid in a LKB Wallac 1214 Rackbeta liquid scintillation counter.

2.6.2 Densitometric quantitation of bands on autoradiograms

Quantitation of bands on autoradiograms was performed on a LKB Bromma ULTASCAN XL enhanced laser densitometer. Exposure times were adjusted so that the signals were within the linear range of the film used.

2.6.3 Computer Programs

DNA sequence overlaps were found using SPCOMP (Dr. A. Sivaprasad, Adelaide), a modified version of DBCOMP (Staden, 1982). ANALYSEQ, a suite of DNA analysis programmes (Staden, 1982) was used to identify restriction enzyme sites, translate coding regions into amino acid sequence and produce sequence data of the complementary strand.

Localised protein sequence similarities were identified using SEQHP (Dayhoff, 1978; Goad and Kanehisa, 1982). Sequence databases GenBank, EMBL, NBRF and VECBase were screened using the suite of programs from Genetics Computing Group of the University of Wisconsin (Devereux *et al.*, 1984) particularly WORDSEARCH, FASTA, TFASA.

CHAPTER THREE

CHARACTERIZATION OF HUMAN HEPATIC ALAS cDNA CLONES.

CHAPTER THREE: CHARACTERIZATION OF HUMAN HEPATIC ALAS cDNA CLONES.

3.1 INTRODUCTION

For a number of years the research interest of our group has focussed on the regulation of heme biosynthesis. Of particular interest is the molecular basis of the negative feedback regulation of ALAS gene transcription observed in rat liver following hemin administration to rats as discussed in chapter one. In early work, the chicken hepatic ALAS gene was isolated, and the complete nucleotide sequence of the gene determined (Maguire *et al.*, 1986). Expression studies using promoter constructs from the chicken gene were carried out in *Xenopus laevis* oocytes and various human transformed cell lines but unfortunately heme repression of the promoter constructs was not observed in either system (Loveridge, 1988; Day 1988). This raised the possibility that a species specific factor may be necessary for heme repression of chicken hepatic ALAS, and further studies are required in cultured chicken hepatocytes to test this.

Other work in our laboratory involved regulation of the rat hepatic ALAS gene. A rat cDNA clone had been isolated, characterized and used to isolate genomic clones containing the promoter region of the rat gene. As mentioned above, it had been established by nuclear run-on transcription experiments, that *in vivo,* the rat ALAS gene is regulated at the transcriptional level by hemin administration (Srivastava *et al.*, 1988). Encouraged by this result, and since the ultimate aim of the work was to understand the regulation of heme biosynthesis in the human liver, it was decided to study the regulation of the human and rat hepatic ALAS genes concurrently. Comparison of the promoter regions could be useful in identifying putative regulatory elements, and the rat could provide a mammalian system in which to test any regulatory model arising from the work. In addition, as there are a number of rat and human hepatoma cell lines, both genes offered the opportunity to study heme regulation of the ALAS gene in homologous systems.

At the time the work for this thesis was undertaken, Dr. M. J. Bawden of this laboratory had isolated seven human ALAS cDNA clones from a λgt 10 liver library, by cross

hybridization with the rat ALAS cDNA clone. The work in this chapter, concerns the base sequencing of the cDNA for the human hepatic ALAS precursor and deduction of the amino acid sequence of the precursor protein. The latter is compared with the amino acid sequences, previously determined, of ALAS enzymes from rat (Srivastava *et al.*, 1988), chicken (Borthwick *et al.*, 1984 and 1985), yeast (Urban-Grimal *et al.*, 1986) and the bacterium *Rhodopseudomonas spheroides* (Pirola, 1986).

3.2 RESULTS

3.2.1 Nucleotide sequence analysis of the human ALAS cDNA clone.

Seven human ALAS cDNA clones, previously isolated as described from a λgt 10 human liver library by Dr. M. J. Bawden, were used to obtain the nucleotide sequence of the coding and untranslated regions of the human ALAS cDNA. The Eco RI linkered inserts of the λ clones had been subcloned, in both orientations into the Eco RI site of the plasmid vector pTZ 18R, and partially characterized by restriction enzyme analysis by Dr. M. J. Bawden. In the work described here, the author was entirely responsible for the sub-cloning of the required restriction enzyme fragments, and the sequence analysis of these clones was shared equally with Dr. I. A. Borthwick.

Initially, the ends of each cDNA clone were sequenced using the dideoxy chain termination method (Section 2.2.12), and this together with the restriction enzyme analysis data facilitated alignment of the clones relative to each other. Appropriate DNA fragments were then subcloned into either M13mp18 or 19 and sequenced. The restriction enzyme sites used for subcloning are indicated in Fig 3.1. The Eco RI restriction enzyme sites shown in Fig 3.1 represent the linkered sites used to clone the λ library inserts into the plasmid vector; the cDNA itself does not have any Eco RI sites. The overall strategy used to obtain 2.15kb of sequence encompassing the complete coding region of the human ALAS precursor protein is shown in the lower part of Fig. 3.1. The arrows indicate the direction and length of sequence obtained from each subclone.

As shown in the upper part of Fig. 3.1, the seven cDNA clones overlapped, and were shown by sequence analysis to extend from 84bp in the 5' untranslated region, to the poly A

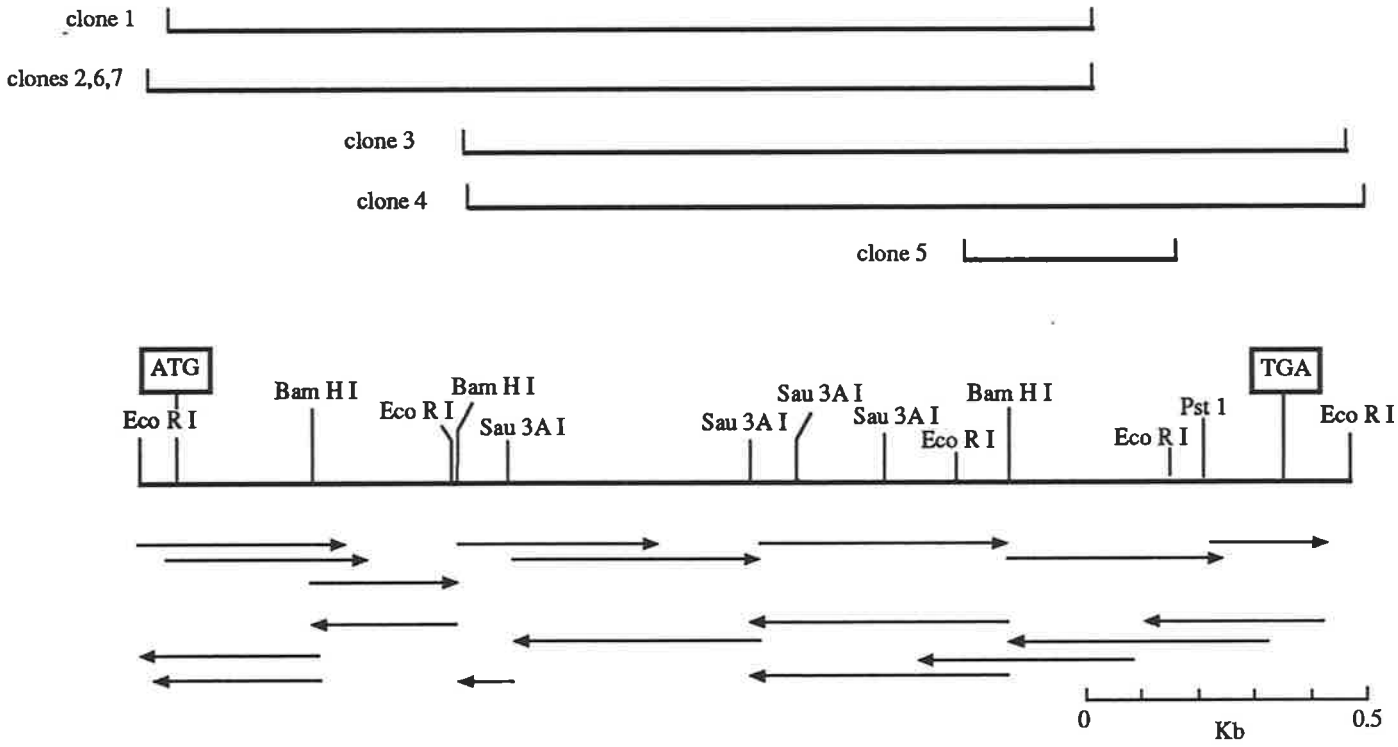


Figure 3.1 Sequencing strategy for the human hepatic ALAS cDNA clones.

The human hepatic ALAS cDNA was sequenced in both directions from seven overlapping clones. The region spanned by each of the clones is indicated above the map. Clones 2, 6 and 7 which proved to be identical are represented by one line. Where necessary, the clones were subcloned into either M13mp 18, 19 or pTZ 18R vectors; only the restriction sites used in sequencing are shown. Note that the Eco RI sites represent the Eco RI linkers used to subclone the lambda cDNA inserts into pTZ 18; they are not present in the cDNA. The arrows indicate the direction and length of sequence obtained from each subclone.

tail. Clones 2, 6 and 7 were found to be identical and extended from 84bp in the 5' untranslated region to approximately 1.7kb into the coding region. Clone 1, was identical to clones 2, 6 and 7 at the 3' end, but at the 5' end it differed in two respects. Firstly, it was approximately 40bp shorter than the other three clones, and at the nucleotide position 21bp 5' to the ATG start codon, contained an additional 11 base pairs not present in the other three clones. The origin of these extra bases is discussed in chapter 4 . Clones 3 and 4, were identical to each other at their 5' ends, and extended from position 0.5kb within the coding region to the 3' untranslated region of the cDNA. However, while clone 4 extended to and included some of the poly A tail, clone 3 stopped 50bp before the poly A tail. Clone 5, the smallest of the group, lay entirely within the coding sequence. It extended from position 1.38-1.85kb of the coding region, and the sequence from this clone matched that obtained from clones 3 and 4 which also span this region.

The nucleotide sequence of the coding region for the human hepatic ALAS precursor protein as determined from the cDNA clones is presented in Fig. 3.2. Computer analysis (Section 2.6.3) was used to predict the amino acid sequence of the encoded protein also shown in Fig. 3.2 above the nucleotide sequence. The translation initiation site and correct reading frame of the human enzyme were deduced by alignment of the human sequence with that previously obtained for the rat (Srivastava *et al.*, 1988), and chicken (Borthwick *et al.*, 1984 and 1985) hepatic ALAS enzymes. The numbers in Fig. 3.2 indicate the nucleotide position of the base pair relative to the translation initiation site.

The sequence of the 5' flanking region and the transcription initiation site of the human hepatic ALAS gene have been determined, and are discussed in Chapter four. From this data it was established that the 5' noncoding sequence of the mRNA is 130bp long, and that the cDNA sequence obtained lacks the first 36bp of the mRNA. As the 5' noncoding sequences of most vertebrate mRNAs fall in the size range of 20-100 nucleotides (Kozak 1987), the noncoding sequence of the human hepatic ALAS mRNA is relatively long. Like many naturally occurring long 5' noncoding sequences the human noncoding sequence is G+C rich (62%) although the significance of this and the effects of leader length on translation and/or mRNA stability are unknown (Kozak 1987).

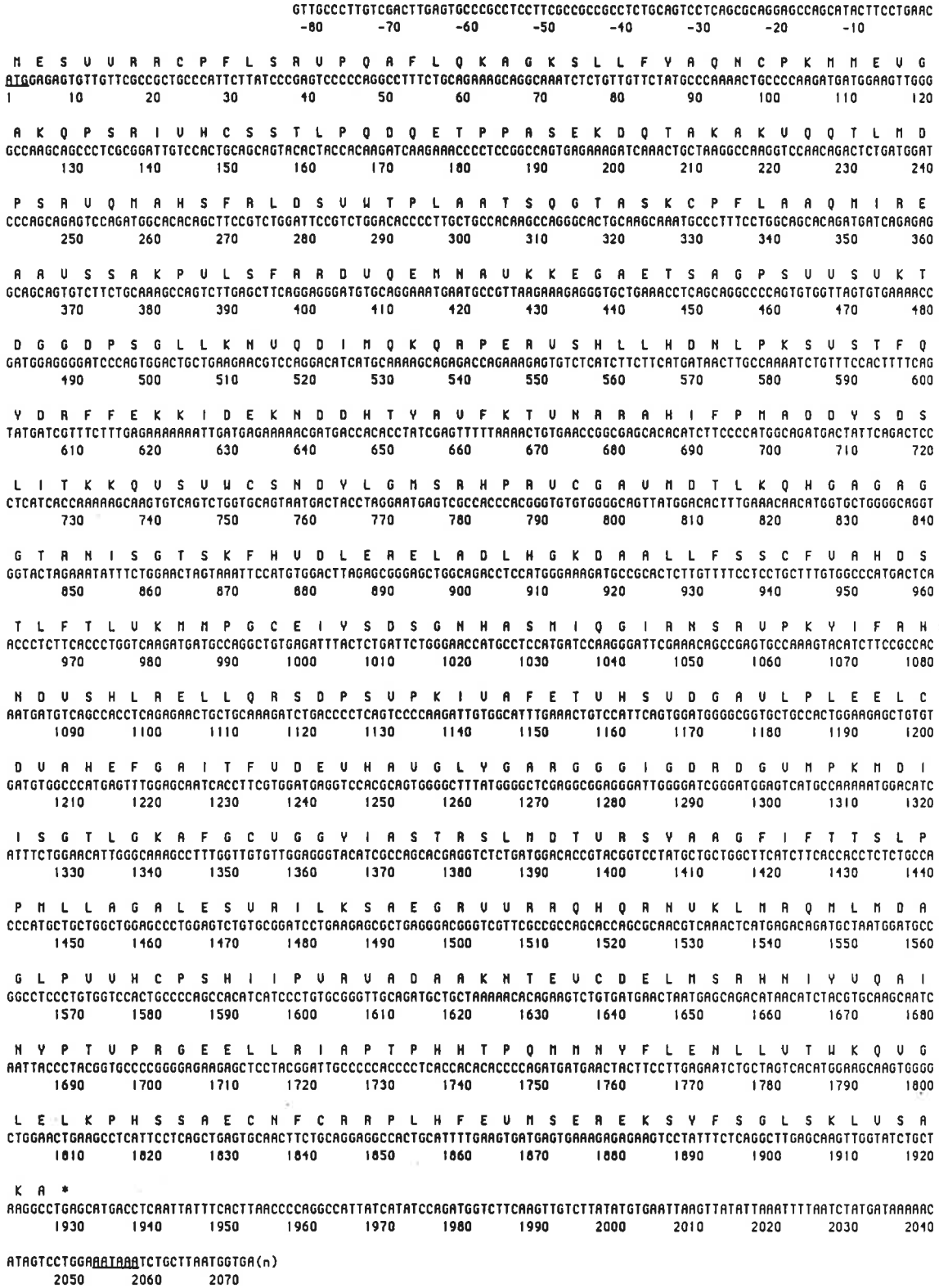


Figure 3.2 Nucleotide sequence of the human hepatic ALAS cDNA clones.

The nucleotide sequence and the predicted amino acid sequence of the human hepatic ALAS precursor protein is shown. Assignment of the correct reading frame was facilitated by alignment with the previously determined cDNA sequences for the rat and chicken hepatic ALAS precursor proteins. The nucleotide sequence extends from 84bp in the 5' untranslated region to the poly A tail. The numbers indicate the nucleotide sequence relative to the putative translational initiation site at +1 (underlined). The predicted mature protein contains 586 amino acids and has an N-terminal transit sequence of 56 amino acids. The translation stop codon TGA is marked with an asterisk. The 3' untranslated region extends 130bp to the AATAAA polyadenylation signal (underlined), situated 14bp from the poly A tail.

Kozak (1987) carried out a comprehensive analysis of the 5' noncoding sequences of vertebrate mRNAs and established a consensus sequence for the initiation of translation (GCCA/GCCATGG). The putative initiation of translation region of the human hepatic ALAS gene complies with the consensus in the most conserved position of the motif, at nucleotide position -3 upstream from the ATG codon where an A residue is found in 97% of vertebrate mRNAs. The human gene also has a C preceding and a G following the ATG, but varies from the consensus sequence at the other 4 positions. There are no other ATG codons upstream from the predicted translational start site. From this ATG codon, the cDNA sequence has an open reading frame of 1926 nucleotides which codes for 642 amino acids. Alignment with the rat and chicken hepatic ALAS amino acid sequences suggests that the first 56 amino acids comprise the N-terminal signal sequence necessary for mitochondrial import of the precursor protein, and the remaining 586 amino acids constitute the mature protein.

The 3' untranslated region of the cDNA extends 138bp from the TGA stop codon to the poly A tail. Within this region situated 15bp upstream from the poly A tail there is an AATAAA sequence. This hexanucleotide, or a closely related sequence, is found 10-30bp upstream of the polyadenylation site in the majority of higher eukaryotic mRNAs (Proudfoot and Brownlee, 1974), and is absolutely required for polyadenylation of the mRNA (Fitzgerald and Shenk, 1981; Wickens and Stephenson, 1984; Zarkower *et al.*, 1986).

3.2.2 Comparison of hepatic ALAS amino acid sequences.

Computer analysis (Section 2.6.3) was used to compare the deduced amino acid sequence of the human hepatic ALAS, with the sequences of the rat and chicken hepatic ALAS, and in addition the yeast and bacterial forms of ALAS. The amino acid sequence comparisons are presented in Fig. 3.3. The numbering of the amino acids refers to the human enzyme. Amino acids which are identical to those found in the human enzyme are shaded grey.

The N-terminal 56 amino acids of the human, rat and chicken hepatic ALAS enzymes, which comprise the signal sequences necessary for mitochondrial import, are highly conserved between these species. As can be seen in Fig. 3.3, conservation of the first 42 amino acids is particularly high, with the percentage of amino acid sequence which is identical being 98%

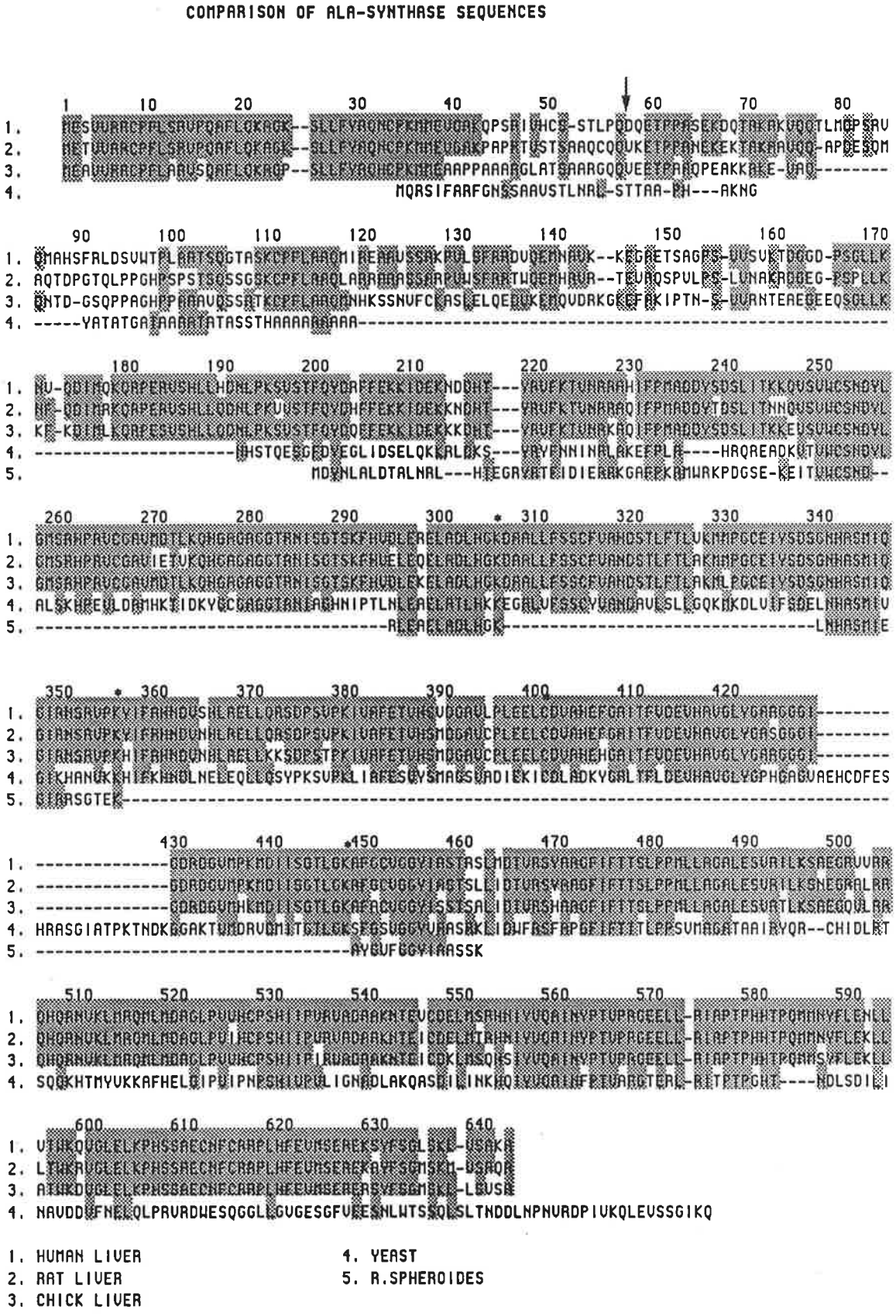


Figure 3.3 Comparison of hepatic ALAS amino acid sequences.

The amino acid sequences of the human, rat, and chicken hepatic ALAS precursor proteins, deduced on the basis of their cDNA nucleotide sequence are shown. The amino acid sequence of the yeast enzyme, deduced from the gene sequence, and the amino acid sequence of the *Rhodopseudomonas spheroides* bacterial ALAS enzyme obtained from amino acid sequencing are also presented. Amino acids identical to the human ALAS enzyme are shaded grey. The numbering of amino acids refers to the human enzyme.

between human and rat, and 79% between human and chicken. Interestingly, the predicted signal sequence of the yeast ALAS enzyme (Urban-Grimal *et al.*, 1986) has no similarity to the signal sequences of the higher eukaryotic enzymes. This high degree of conservation suggests that this part of the signal sequence may play a functional or regulatory role and this is discussed in Section 3.3.

As the signal sequences of most mitochondrial proteins are generally less than 40 amino acids in length (Vassarotti *et al.*, 1987; Pfanner *et al.*, 1988), the ALAS signal sequences are quite large by comparison, although the significance of this, if any, is unknown. The signal sequences of all four eukaryotic ALAS enzymes are positively charged, which is a feature of all mitochondrial signal sequences determined so far (Pfanner *et al.*, 1988; Silver and Goodson, 1989). Interestingly, the putative proteolytic cleavage site between two glutamine residues is conserved between the rat and chicken ALAS enzymes, but not in the human enzyme where it is predicted to occur between a glutamine and aspartic acid residue, and is indicated by an arrow in Fig. 3.3.

The 586 amino acids which comprise the mitochondrial or mature human hepatic ALAS enzyme are highly conserved between the higher eukaryotes, with the overall percentage of amino acid sequence that is identical being 84% between human and rat, and 81% between human and chicken. Most of the sequence variability is found in the first 100 amino acids. In this region the percentage of amino acid sequence which is identical to the human enzyme, is only 51% for rat and 38% for chicken ALAS. The analogous region of the yeast protein is much shorter (29 amino acids) and has no sequence similarity to the other species. Currently it is not known what, if any role this region of the protein performs.

In contrast, the region of the mature protein extending from amino acid 164 of the human enzyme to the COOH-terminus, is highly conserved between the higher eukaryotes with the percentage of amino acid sequence which is identical being 92% between human and rat, and 91% between human and chicken. The corresponding region of the yeast protein has only 40% of its amino acid sequence identical to that of the human enzyme. While the bacterial ALAS enzyme is considerably smaller than the eukaryotic enzymes, when aligned with this conserved region, 47% of the amino acid sequence of the bacterial enzyme is identical to the

human ALAS sequence. This high degree of conservation between species implies that the active site of ALAS is probably contained within this region. As can be seen in Fig. 3.3, located between amino acid 248 and the COOH-terminus of the human enzyme, there are several stretches where the amino acid sequence is highly conserved between all five ALAS enzymes and these are likely to be important for the catalytic activity of the enzyme.

Previous work in our laboratory suggests that both chick embryo and rat liver ALAS exist as dimers of identical subunits (Pirola, 1986; May *et al.*, 1986). The relative molecular weight of each subunit of the human enzyme (assuming it has similar subunits) can be deduced from the amino acid sequence. The 586 amino acids predicted to comprise the mitochondrial enzyme have a deduced minimum molecular weight of 64,863, compared to 64,956 for the predicted rat protein, which has the same number of amino acids. The chicken enzyme is slightly smaller consisting of 579 amino acids with a calculated minimum molecular weight of 63,903. All three enzymes have a predicted NH_2-terminal signal sequence of 56 amino acids, and the calculated minimum molecular weights of the ALAS precursor proteins are; 71,140 for the human, 71,100 for the rat and 70,209 for the chicken.

As binding of the pyridoxal-phosphate moiety is thought to involve a lysine residue (Nandi, 1978b; May *et al.*, 1986), it is interesting to note that there are three lysine residues which are conserved between all five ALAS enzymes shown in Fig. 3.3. These residues, indicated by an asterisk in Fig. 3.3, are at positions 305, 355 and 447. There is also a conserved cysteine residue at position 400. This is of interest since there is evidence that a cysteine residue is important for the catalytic activity of the ALAS enzyme (Pirola, 1986). The amino acid sequence has been deduced for the human (Cox *et al.*, 1990), chicken (Riddle *et al.*, 1989), and mouse (Schoenhaut and Curtis, 1986) erythroid ALAS enzymes, and for the prokaryotic form from *Bradyrhizobium japonicum* (McClung *et al.*, 1987), from their cDNA nucleotide sequences. Interestingly, two of these three lysine residues at positions 355 and 447 and the cysteine residue at position 400 are conserved between all of these ALAS enzymes. The lysine at position 305 is replaced by a glutamine in the chicken erythroid ALAS (Riddle *et al.*, 1989), implying that it is not the lysine involved in binding the pyridoxal phosphate moiety.

A search of the Genebank data base (Section 2.6.3) for other pyridoxal phosphate-dependent enzymes with similar substrates detected an *E. coli* enzyme, 2-amino-3-ketobutyrate CoA ligase (also called aminoacetone synthase) which catalyses the reversible cleavage/condensation reaction between 2-amino-3-ketobutyrate and glycine + acetyl-CoA (Aronson *et al.*, 1988). Alignment of the amino acid sequence of the *E. coli* enzyme with the human hepatic ALAS sequence, shown in Fig.3.4, established that the two enzymes have a highly conserved region (74%) located between amino acids 440 and 460 of the human ALAS sequence, and amino acids 237 and 257 of the bacterial enzyme. The lysine residue located at position 447 of the human enzyme, indicated by an asterisk in Fig.3.4, lies within this conserved region and is present in the corresponding bacterial sequence at position 245. However, the lysine located at amino acid 355 of the human ALAS sequence is replaced by an arginine residue in the corresponding sequence of the bacterial enzyme. From this evidence it seems likely that the lysine residue at position 447 is the one involved in binding the pyridoxal-phosphate co-factor of human hepatic ALAS. Interestingly, the cysteine residue located at position 400 in the human ALAS enzyme sequence is also conserved in the corresponding bacterial 2-amino-3-ketobutyrate CoA ligase sequence at position 197 (Fig 3.4), which strongly suggests that it is the cysteine residue that in some way contributes to the catalytic mechanism of the 5-aminolevulinic acid reaction.

3.2.3 Generation of a human hepatic ALAS cDNA construct containing the entire coding sequence.

Computer analysis (Section 2.6.3) of the nucleotide sequence of the human hepatic ALAS cDNA shown in Fig. 3.2 established that a unique Bgl II site is situated at nucleotide position 1120, and confirmed there were no Hind III sites. As the multiple cloning site (MCS) of the vector pTZ 18R has one Hind III site, this information enabled a cDNA construction to be prepared which contained the entire coding region of the human ALAS protein, by the cloning strategy outlined in Fig. 3.5.

The cDNA clones 1 and 3 shown in the upper region of Fig. 3.1, are overlapping and together contain the entire coding region of the protein. Both contain the unique Bgl II site. A

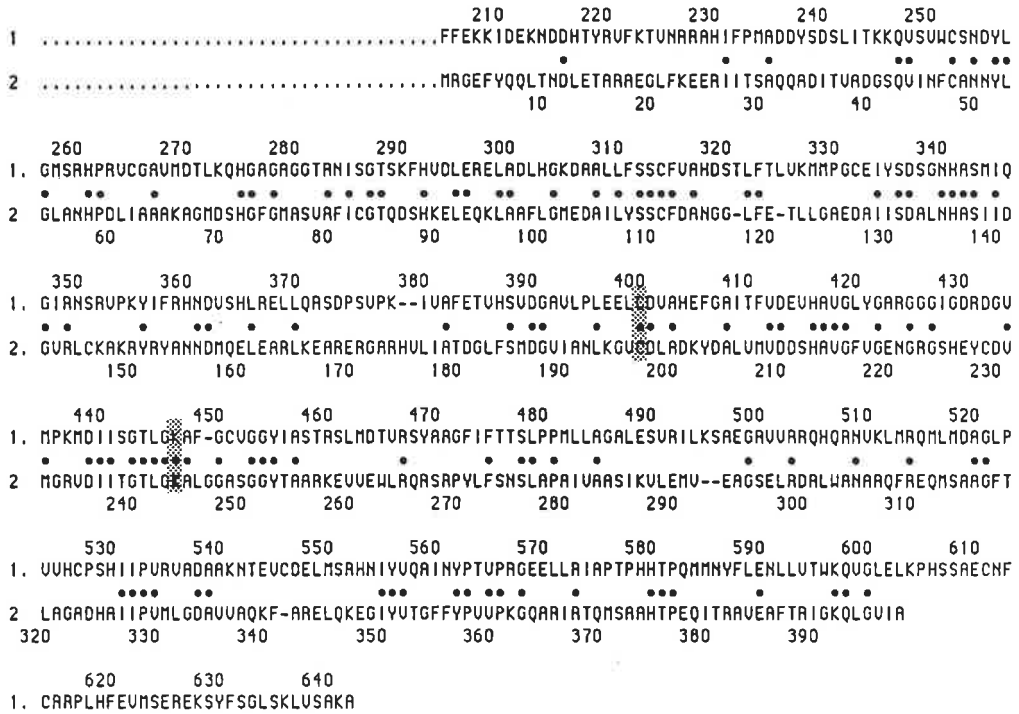


Figure 3.4 Alignment of the amino acid sequences of human hepatic ALAS and *E. coli* 2-amino-3-ketobutyrate CoA ligase

The amino acid sequence of the presumed catalytic domain of the human hepatic ALAS is presented on line one, the numbering above the line refers to the human enzyme. The entire amino acid sequence of *E. coli* 2-amino-3-ketobutyrate CoA ligase is presented on line two, the numbers below line two refer to the *E. coli* enzyme. Both amino acid sequences were deduced on the basis of their cDNA nucleotide sequences. Identical amino acids are indicated by dots. The lysine and cysteine residues, at positions 400 and 447 respectively in the human ALAS sequence, which are conserved between all known ALAS enzymes and the *E. coli* enzyme and thought to be involved in the catalytic mechanism are shaded grey.

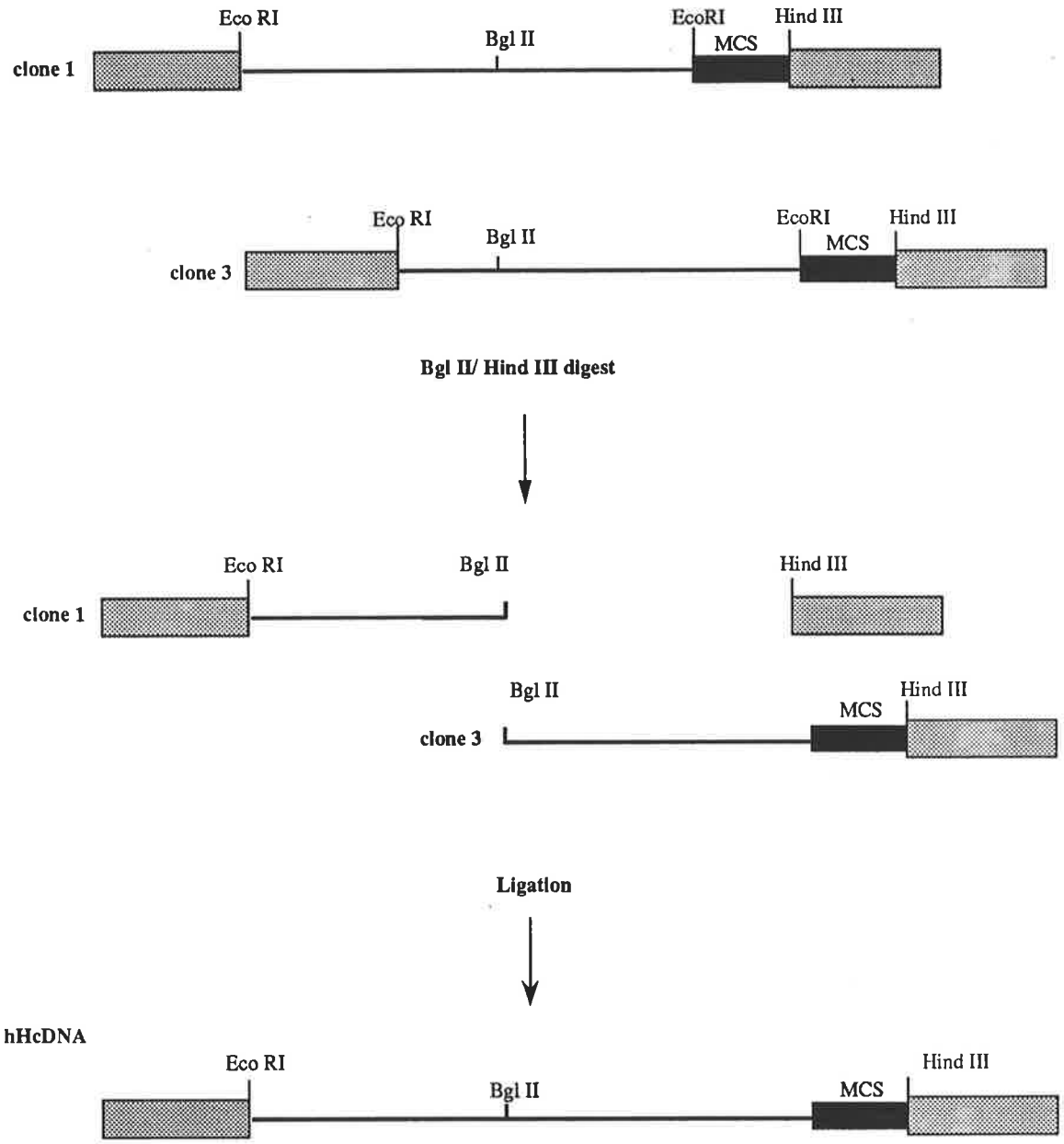


Figure 3.5 The generation of a full length human hepatic ALAS cDNA construct.

The cloning strategy used to prepare a full length human hepatic cDNA construction (hHcDNA) from the overlapping cDNA clones 1 and 3 is shown. The unique Bgl II site present at nucleotide position 1120 in the cDNA is indicated. The Hind III site shown refers to the site present in the multiple cloning site (MCS) of the pTZ 18R vector. The Eco RI sites are the sites used to subclone the inserts from the lambda cDNA library into pTZ 18R.

Bgl II/ Hind III restriction enzyme digest of clone 1, left the 5' first 1120bp of the cDNA attached to the pTZ vector, while the incomplete 3' region.was removed. A Bgl II / Hind III digest of clone 3 generated a Bgl II/ Hind III fragment which contained the 3' region of the cDNA from position 1120bp in the coding region to approximately 50bp past the stop codon. This fragment was inserted into clone 1, in place of the incomplete 3' end, thereby generating a cDNA construct which contained the entire coding region and some of the 5' and 3' untranslated region. This construct was designated hHcDNA, and unless otherwise specified is the hepatic cDNA probe used throughout this thesis.

3.3 DISCUSSION

In this Chapter, several cDNA clones for human hepatic ALAS were analysed and the deduced amino acid sequence for the complete precursor protein was determined. This sequence was compared with that of other ALAS sequences from eukaryotic and prokaryotic sources. The processed mature mitochondrial proteins of human, rat and chicken hepatic ALAS show strong sequence similarity except for a variable region of approximately 100 amino acids at the N-terminus. There is evidence that these 100 amino acids can be cleaved off without affecting enzyme activity (Pirola 1986). Moreover, the yeast and bacterial enzymes lack this variable region, with the remainder of the mature protein being very similar to that of the other ALAS enzymes, and this strongly suggests that the variable region is not required for catalytic activity.

The lysine residue most likely to be involved in binding the pyridoxal-phosphate co-factor of the human hepatic form of ALAS was deduced from comparisons of all known eukaryotic and prokaryotic ALAS amino acid sequences, and from comparison of the human ALAS amino acid sequence with the amino acid sequence of *E coli* 2-amino-3-ketobutyrate ligase. As mentioned previously, this ligase is a pyridoxal-phosphate dependent enzyme and has a catalytic function similar to ALAS, as it catalyses the reversible cleavage/condensation reaction between 2-amino-3-ketobutyrate and glycine + acetyl-CoA (Aronson *et al.*, 1988). Studies conducted in this laboratory in which the catalytic mechanism of native eukaryotic ALAS has been investigated, have shown that the presence of a sulphydryl residue near the lysine residue responsible for binding the pyridoxal phosphate moiety is important for the

catalytic activity of the enzyme (Pirola, 1986). The role of the sulphydryl remains unclear but similar results with the bacterial form of ALAS from *Rhodobacter. spheroides* have led to the proposal that it may participate in proton removal/addition during the course of the ALAS reaction (Nandi, 1978a). Interestingly, the catalytic activity of *E coli* 2-amino-3-ketobutyrate ligase is also thought to involve an active sulphydryl residue (Mukherjee and Dekker, 1987) and a cysteine residue was found to be conserved between the two proteins.

The amino acid sequences of many mitochondrial signal sequences have been determined, and analysis of these has revealed they generally have no primary amino acid sequence similarity, although they do exhibit common properties apparently necessary for their function in membrane transport (von Heijne 1986). In this regard, mitochondrial signal sequences are essentially basic hydrophilic sequences, which carry a small net positive charge, and can potentially form an amphipathic α-helical structure (Vassarotti *et al.*, 1987; Pfanner *et al.*, 1988). The ALAS signal sequences exhibit these properties. They are basic in nature with a predominence of basic over acidic residues, and in the postulated α-helical structure would have charged residues arranged on one side only of the helix (May *et al.*,1986).

A striking feature is the high degree of conservation observed between the N-terminal mitochondrial signal sequences of the human, rat and chicken ALAS enzymes. As discussed in Chapter one, translocation of the hepatic form of ALAS into the mitochondria is inhibited by heme (Hayashi *et al.*, 1980; Srivastava *et al.*, 1983b; Hayashi *et al.*, 1983). Moreover, this inhibition is specific for ALAS, as it has been shown that heme does not prevent the transfer and processing of pyruvate carboxylase, an unrelated matrix mitochondrial protein (Srivastava *et al.*, 1983b). It is possible that in the hepatic cells of higher eukaryotes, when the intracellular levels of heme rise, heme may interact either directly or indirectly with the conserved N-terminal region of the signal sequence. Since precursor proteins have to unfold prior to or during translocation into the mitochondria, and also interact with specific receptors on the mitochondrial surface (Pfanner *et al.*, 1988; Silver and Goodson, 1989) heme binding to the signal sequence may prevent one or both of these processes from occurring, thereby inhibiting the mitochondrial translocation of the ALAS precursor. However, more work is necessary

before the mechanism of heme inhibition of translocation is fully understood, and the region of the ALAS precursor involved is identified.

Interestingly, the transport of yeast ALAS into the mitochondria is not inhibited by heme (Urban-Grimal *et al.*, 1986) and this may explain why the predicted signal sequence of the yeast enzyme has no similarity to the signal sequences of the higher eukaryotes (Urban-Grimal *et al.*, 1986). The development of such a mechanism to inhibit mitochondrial translocation of the ALAS precursor protein would provide the cell with a rapid control mechanism by which to regulate heme biosynthesis in response to changing intracellular heme levels and may have been an important evolutionary adaptation in higher eukaryotes.

The bacterial form of ALAS as expected does not contain the signal sequence. It can be speculated therefore, that the ALAS proteins of the higher eukaryotes have evolved by the addition of the sequences necessary to specify mitochondrial import of the protein. This proposal is supported by the structure of the chicken hepatic ALAS gene, which has been shown to consist of 10 exons. Of these, exon one encodes the N-terminal mitochondrial signal sequence, exons 2 and 3 encode the variable N-terminal region of the mature protein while the remaining exons (4-10) encode the catalytically active protein (Maguire *et al.*, 1986). It is plausible therefore, that the eukaryotic hepatic ALAS proteins have evolved by the addition of the N-terminal mitochondrial signal sequence and the variable N-terminal region to a primitive form of ALAS. As mentioned previously it will be of interest to determine what role, if any, the variable N-terminal region of the mature protein plays in the transport or function of the eukaryotic ALAS enzymes.

Also discussed in this chapter was the generation of a cDNA construction, designated hHcDNA, which contained all of the coding and some of the untranslated region of the human hepatic ALAS precursor protein. As ALAS mRNA is expressed at low levels in the liver (Chapter six this thesis) this construct allowed greater sensitivity when used as the probe for the work described later in this thesis.

CHAPTER FOUR

CHARACTERIZATION OF THE 5' FLANKING REGION OF THE HUMAN HEPATIC ALAS GENE AND ITS CHROMOSOMAL LOCALIZATION.

CHAPTER FOUR: CHARACTERIZATION OF THE 5' FLANKING REGION OF THE HUMAN HEPATIC ALAS GENE AND ITS CHROMOSOMAL LOCALIZATION.

4.1 INTRODUCTION

Previous work carried out in our laboratory investigated the role of the hepatic form of ALAS in various rat tissues. Using cDNA clones for rat hepatic ALAS, Northern analysis of total RNA from various rat tissues, including liver, kidney, heart, testis and brain, was carried out, and demonstrated that the corresponding mRNA was detectable at very low levels in all tissues examined (Srivastava et al., 1988). Furthermore, it was established by primer extension and RNAse mapping studies that the mRNA was identical in all tissues, and this work provided evidence for the proposed housekeeping role of the hepatic form of ALAS. The highest basal levels of the hepatic form of ALAS were seen in the heart and the liver. Administration of hemin or its precursor ALA to rats reduced the basal level of this mRNA in all tissues. Administration of the drug 2-allyl-2-isopropylacetamide (AIA) increased the mRNA level in the liver and kidney, and nuclear transcription run-on experiments demonstrated that both hemin and AIA exerted their effect by altering the rate of transcription of the ALAS gene (Maguire, 1987; Srivastava et al., 1988).

At the time the work described in this chapter was undertaken, the housekeeping function of the hepatic form of ALAS was well established, as described above, but nothing was known at the molecular level about the regulation of the basal expression of the gene or the mechanism(s) by which expression of the gene was repressed by heme and induced by drugs such as AIA. As a first step towards understanding the molecular basis for the transcriptional regulation of the human hepatic or housekeeping ALAS gene, it was important to isolate and characterize the 5' flanking region of the gene. Dr. M. J. Bawden had previously isolated twenty-four genomic clones from a human genomic cosmid library by cross hybridization with the human ALAS cDNA clone, designated clone 1, (Chapter three), and established by Southern analysis of human genomic DNA, that clone 1 hybridized to an Eco RI DNA fragment of approximately 9kb. Of the twenty-four genomic clones that had been isolated, only ten

contained an Eco RI DNA fragment of this size and these clones were selected for further characterization.

In this chapter, isolation of the human hepatic ALAS gene from the genomic cosmid clones is described. Southern analysis using synthetic oligonucleotides complementary to the nucleotide sequence of the 5' region of the human hepatic ALAS cDNA clone (Chapter three), were used to identify the 5' flanking region of the gene. The transcriptional start site of the gene was defined by RNAse protection analysis. The 5' flanking region of the gene was characterized by restriction enzyme mapping and sequence analysis, and computer analysis of the obtained sequence was used to identify putative regulatory elements commonly associated with eukaryotic genes transcribed by RNA polymerase II.

The chromosomal localization of the human hepatic ALAS gene is also described in this chapter. Defective ALAS had been implicated in inherited sideroblastic anaemia, a disorder of heme biosynthesis which follows an X-linked pattern of inheritance (Bottomley, 1980). Therefore, it was of interest to see whether the human ALAS gene was on the X-chromosome, and this work was carried out in collaboration with Dr. G. R. Sutherland's group at the Adelaide Children's Hospital.

4.2 RESULTS

4.2.1 Southern blot hybridization analysis of the human hepatic ALAS gene.

The main purpose of the work undertaken in this section was to isolate the 5' flanking region of the human hepatic ALAS gene. As mentioned above, previous work had established that the human hepatic ALAS cDNA clone, designated clone 1, cross-hybridized with a 9kb genomic Eco RI DNA fragment and ten genomic cosmid clones were selected for further characterization on the basis that they contained an Eco RI DNA fragment of similar size. To determine which, if any, of these cosmid clones contained the 5' flanking region of the gene, the clones were digested with the restriction endonucleases Bam HI, Eco RI, and Hind III and Southern blot hybridization analysis was carried out as described in Section 2.2.15. The resulting filter was probed with a ^{32}P-labelled synthetic oligomer (Section 2.2.14), designated SR 145, which is complementary to the 5' coding region of the human hepatic ALAS cDNA

base sequence, immediately 3' of the ATG translation initiation codon from nucleotide position +22 to +45 (Fig 3.2). This preliminary work established that three of the ten cosmid clones, designated clones 2, 14 and 21, cross-hybridized with this probe (data not shown) and therefore contained 5' coding sequence from the human hepatic ALAS gene.

The cosmid clones 2, 14 and 21, were digested with the restriction endonucleases Bam HI, Eco RI and Hind III, and electrophoresed on a 1.0% vertical agarose gel. The DNA bands were photographed after staining with ethidium bromide (Fig. 4.1A) and bi-directional Southern transfer to nitrocellulose was carried out. The ^{32}P-labelled oligomer SR 145, described above, was used to probe one of the filters and the autoradiograph from this experiment is shown in Fig. 4.1B. SR 145 hybridized to a 4kb Bam HI fragment, a 9kb Eco RI fragment, and an 11kb Hind III fragment all of which were present in the three cosmid clones. It can be seen from Fig 4.1A, that the 9kb Eco RI fragment present in clones 2 and 21 runs as a doublet with another fragment of a similar size, while in clone 14 there is a single band (Fig. 4.1A). As it had previously been established by Southern analysis of human genomic DNA, that the human hepatic ALAS cDNA clone hybridized to an Eco RI DNA fragment of approximately 9kb, this fragment was isolated from clone 14 and subcloned into pTZ 18R, for further characterization.

Restriction enzyme mapping and Southern analysis established that the 4kb Bam HI fragment, which cross-hybridized to the oligomer SR 145, was contained entirely within the 9kb Eco RI fragment. Since this smaller fragment was a more convenient size to analyse, it was isolated from clone 14 and subcloned in both orientations into pTZ 18R. Limited sequence analysis (Section 2.2.12) was obtained from this clone, which established that it extended into the 5' flanking region of the human hepatic ALAS gene to a Bam HI site located only 450bp upstream from the ATG translation initiation start codon (Fig. 4.2), and the remaining 3.55kb extended into the coding region of the gene. In addition, when the 450bp of 5' flanking sequence obtained from this Bam HI clone was compared with that previously obtained for the 5' untranslated region of the human hepatic ALAS cDNA clone (Chapter three), it was found that only 30bp of the obtained sequence was present in the nucleotide sequence of the 5' untranslated region. From this information it was deduced that an intron, greater than 420bp in

Figure 4.1 Southern blot hybridization analysis of the human hepatic ALAS gene.

Human genomic cosmid clones 2, 14, and 21, isolated by cross hybridization with the human hepatic ALAS cDNA clone 1, were digested with Bam HI, Eco RI and Hind III and electrophoresed on a 1.0% vertical agarose gel. The DNA fragments were visualized after staining with ethidium bromide (A), and were transferred to nitrocellulose by bi-directional Southern Blot transfer (Section 2.2.15). The resulting filters were probed using 5' end labelled (Section 2.2.14) synthetic oligomers complementary to (B) the 5' coding region, oligomer SR 145, and (C) the 5' untranslated region, oligomer SR 357, of the human hepatic ALAS cDNA nucleotide sequence.

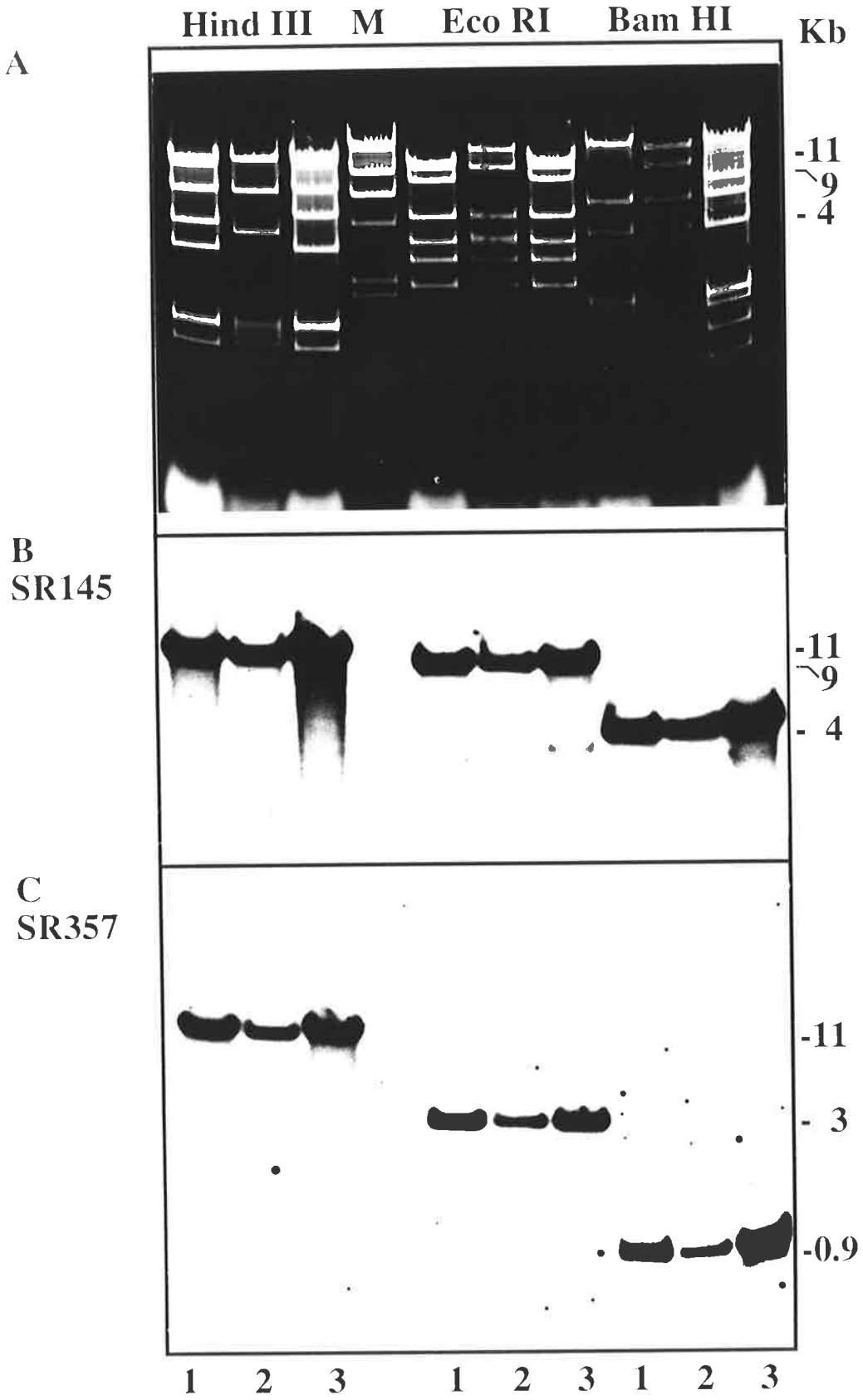
1. Cosmid 21

2. Cosmid 14

3. Cosmid 2

M. Hind III digested λ DNA size markers

(23.1, 9.4, 6.6, 4.3, 2.3, 2.0. 0.5 and 0.1 kb)


A
Hind III
M
Eco RI
Bam HI
Kb
-11
9
- 4
B
SR145
-11
9
- 4
C
SR357
-11
- 3
-0.9
1 2 3 1 2 3 1 2 3

length, was present in the 5' untranslated region of the human hepatic ALAS gene, and in order to identify the promoter region of the gene it was necessary to isolate a further DNA fragment that encompassed the intron.

A second synthetic oligomer, designated SR 357, complementary to the 5' untranslated region of the human hepatic ALAS cDNA clone from nucleotide positions -48 to -73 (Fig 3.2), was synthesized. This oligomer was ^{32}P end-labelled and used to probe the second filter resulting from the bi- directional Southern transfer of clones 2, 14 and 21 described above. From the resulting autoradiograph shown in Fig. 4.1C, it can be seen that the oligomer SR 357 hybridized to an 0.9kb Bam HI fragment, a 3kb Eco RI fragment and an 11kb Hind III fragment. Comparison of the autoradiographs shown in Figs 4.1B and 4.1C established that both oligomers hybridized to the same 11kb Hind III fragment, and implied that this restriction fragment encompassed the intron in the 5' untranslated region of the human hepatic ALAS gene. This 11kb Hind III DNA fragment was isolated from clone 14, subcloned into the plasmid vector pIBI 76, and further characterized as described in Section 4.2.2.

It was deduced from Southern analysis and restriction enzyme mapping that the 9kb Eco RI fragment subcloned from clone 14, extended 8.2kb into the coding region of the human hepatic ALAS gene. It was of interest to determine whether the entire human hepatic ALAS gene was contained within clones 2, 14 and 21, and if so, to determine the approximate size of the gene. In order to do this, one of the filters used for Southern analysis of the cosmid clones described above was stripped and reprobed, using a ^{32}P-oligolabelled DNA fragment (Section 2.2.14) isolated from the 3' region of the human hepatic ALAS cDNA clone, which extended from the Pst I site located at nucleotide position +1840 in the 3' coding region to nucleotide position +1980 in the 3' untranslated region of the cDNA sequence (Fig 3.2). This fragment hybridized to a 3.5kb Eco RI DNA fragment present in clone 14, and to a 9kb Eco RI fragment present in clones 2 and 21, which runs as a doublet with the previously described 9kb Eco RI fragment containing the coding region of the gene (data not shown). The 3' region of the gene has not been further characterized, nor has the precise length of the human hepatic ALAS gene been determined, but from this work it is estimated that the gene spans 13-19kb.

4.2.2 Isolation of the promoter region of the human hepatic ALAS gene.

The 11kb Hind III fragment described above and shown in Fig 4.2, which was subcloned from clone 14 into the plasmid vector pIBI 76, was further characterized using Southern analysis and the two ^{32}P-labelled oligomers SR 145, and SR 357 as probes. This work established that the oligomer SR 145 hybridized to a 2.0kb Bgl II-Hind III DNA fragment, while the more 5' probe, SR 357 hybridized to an adjacent 2.5kb Bgl II fragment (data not shown). These two DNA fragments, indicated in Fig 4.2, were isolated from the 11kb Hind III fragment and subcloned in both orientations into M13 mp18 for sequence analysis. The ends of these two DNA fragments were characterized by sequence analysis (Section 2.2.12) and additional nucleotide sequence was obtained by using the synthetic oligomer SR 357 as a sequencing primer on the 2.5kb Bgl II DNA fragment. This strategy generated approximately 700bp of continuous genomic nucleotide sequence which extended 550bp upstream and 150bp downstream from the Bgl II restriction enzyme site which joined the two fragments (Fig. 4.2). Comparison of the genomic sequence obtained from these clones with the previously determined nucleotide sequence of the 5' untranslated region of the cDNA (Chapter three), established that the putative 5' donor splice site of the intron in the 5' untranslated region of the human hepatic ALAS gene, was located 140bp upstream from the Bgl II restriction enzyme site (Fig 4.2). In addition, the genomic nucleotide sequence upstream from the putative 5' splice site of the intron, corresponded exactly with the 50bp of nucleotide sequence obtained from the cDNA clones that spanned this region. A putative TATA box consensus element was located a further 70 bp upstream and the transcriptional intiation site of the gene was predicted to be approximately 100bp upstream from the putative 5' splice site of the intron.

Computer analysis of the 700bp genomic sequence established that an Eco RI restriction enzyme site was located 80bp 3' of the Bgl II restriction enzyme site. As described in Section 4.2.1, the sequence of the first 450bp of the 5' flanking region of the human hepatic ALAS gene extending to a Bam HI site located within the intron of the 5' untranslated region had already been obtained from the 4.0kb Bam HI clone (Section 4.2.1). Restriction enzyme mapping established that the Eco RI- Bam HI DNA fragment (Fig 4.2) was approximately 300bp in length. This DNA fragment was subcloned and sequenced, thus completing the first

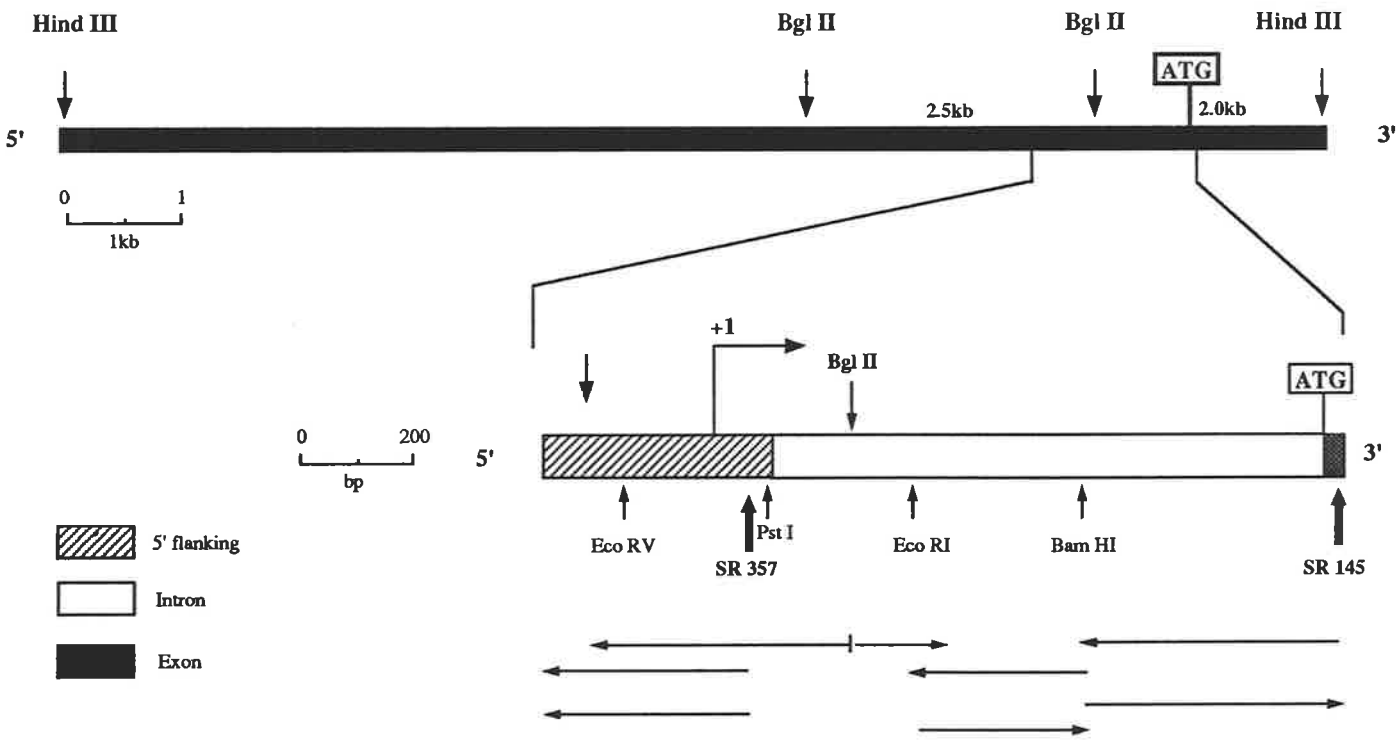


Figure 4.2 Restriction map of the promoter region of the human hepatic ALAS gene.

A restriction map of the Hind III fragment isolated from genomic cosmid clone 14, representing approximately 10kb of the promoter region of the human hepatic ALAS gene, is shown. Restriction enzyme sites important to the work in this thesis are indicated. The location of the transcriptional start site as determined by RNA protection analysis (Section 4.2.3), and the translational start site (ATG) are shown. The hybridization sites of the two oligomers, SR 145, and SR 357 used to isolate this fragment are indicated. The intron in the 5' untranslated region (Section 4.2.1) is represented by an open box.

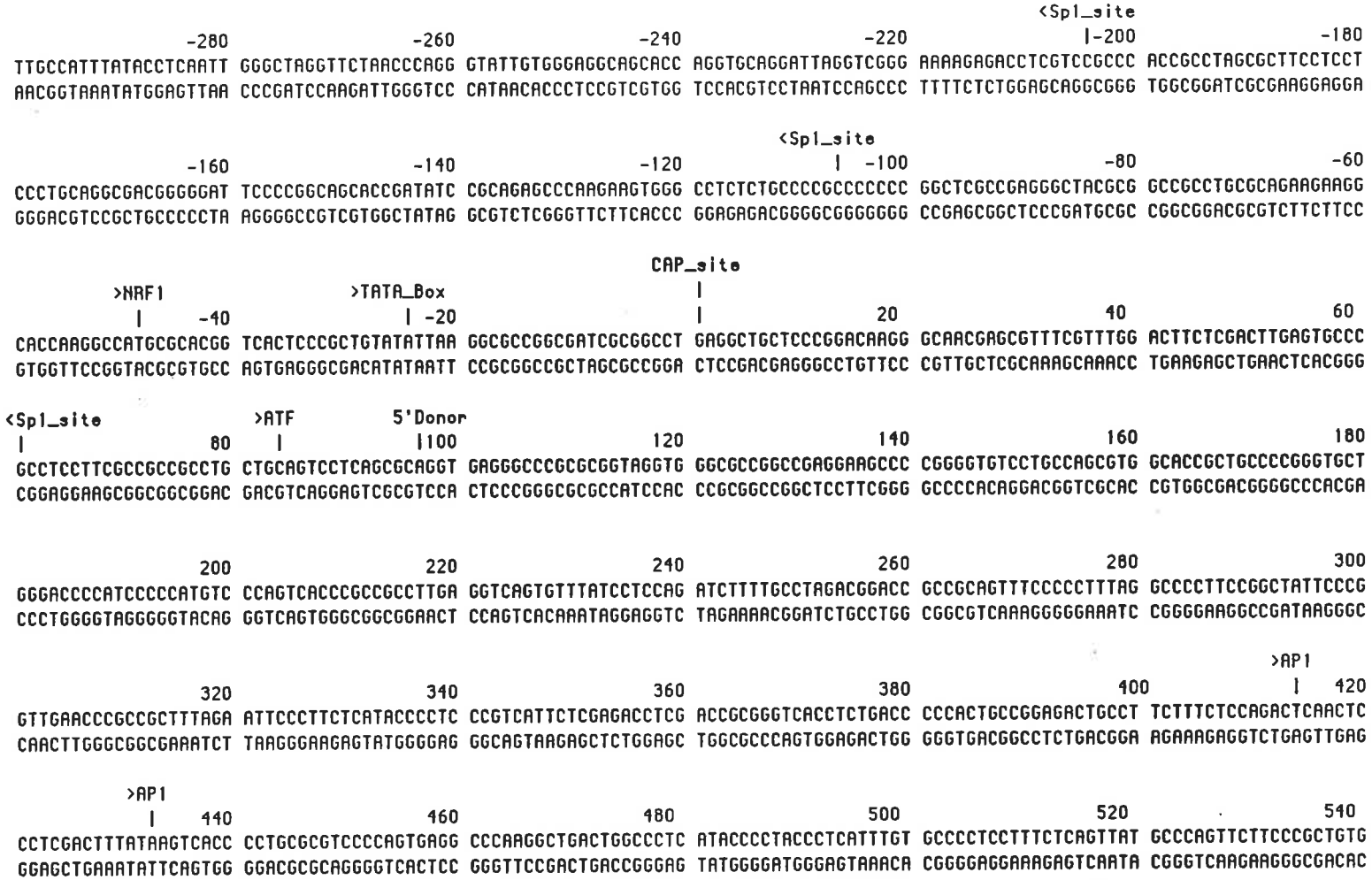
Appropriate restriction fragments were subcloned, into either M13mp18,19 or pTZ 18R vectors, and used to generate the first 1387bps of nucleotide sequence of the 5' flanking region of the human ALAS gene. The strategy used to obtain this sequence is shown in the lower part of the figure. The arrows indicate the direction and length of sequence obtained from each subclone.

1387bp of sequence of the 5' flanking region of the gene. This sequence which extended from the ATG translation initiation codon, included the sequence of the intron in the 5' untranslated region, and approximately 400bp of sequence upstream from the putative 5' splice site of the intron, is shown in Fig 4.3. The overall strategy used to obtain the 1387bp of nucleotide sequence is shown in the lower part of Fig. 4.2, the arrows indicating the direction and the length of sequence obtained from each clone.

From restriction enzyme mapping and sequence analysis it was established that the 11kb Hind III fragment contained approximately 1kb of the coding region of the human hepatic ALAS gene; it encompassed the intron in the 5' untranslated region of the gene, and contained approximately 10kb of the 5' flanking region of the ALAS gene. The human hepatic ALAS gene promoter constructions used in the expression studies described in Chapter five, were prepared from this DNA fragment.

4.2.3 Determination of the transcription initiation site of the human hepatic ALAS gene.

RNAse protection analysis was used to determine the transcription initiation site of the human hepatic ALAS gene. As mentioned above, a putative TATA box consensus sequence was located approximately 120bp upstream from the putative 5' splice site of the intron in the 5' untranslated region of the gene. If this TATA box was functional, then the transcriptional initiation site of the gene would be located 25-30bp downstream from this element. A DNA fragment which spanned this region of the gene, extended from a Pst I site 10bp upstream from the putative 5' splice site of the intron to an Eco RV site located 226bp further upstream (Fig 4.4). This DNA fragment was isolated and subcloned into the *in vitro* transcription vector pSP 72 (which contains the SP6 RNA polymerase phage promoter). Preliminary experiments were carried out in which the conditions for RNAse protection analysis were optimized by varying the concentrations of the enzymes RNAse A and RNAse T1, and the incubation temperature used for the RNAse digestion step. Following overnight hybridization of the RNA probe with total RNA (20μg) isolated from human liver, the RNAse digestion step was carried out at room temperature, 37°C and 42°C respectively with either 40μg/ml RNAse A and 20μg/ml of RNAse T1, or 4μg/ml RNAse A and 2μg/ml of RNAse T1. All other steps were carried out as


Sp1_site
-280
-260
-240
-220
-200
-180
TTGCCATTTATACCTCAATT GGGCTAGGTTCTAACCCAGG GTATTGTGGGAGGCAGCACC AGGTGCAGGATTAGGTCGGG AAAAGAGACCTCGTCCGCCC ACCGCCTAGCGCTTCCTCCT
AACGGTAAATATGGAGTTAA CCCGATCCAAGATTGGGTCC CATAACACCCTCCGTCGTGG TCCACGTCCTAATCCAGCCC TTTTCTCTGGAGCAGGCGGG TGGCGGATCGCGAAGGAGGA
Sp1_site
-160
-140
-120
-100
-80
-60
CCCTGCAGGCGACGGGGGAT TCCCCGGCAGCACCGATATC CGCAGAGCCCAAGAAGTGGG CCTCTCTGCCCCGCCCCCCC GGCTCGCCGAGGGCTACGCG GCCGCCTGCGCAGAAGAAGG
GGGACGTCCGCTGCCCCCTA AGGGGCCGTCGTGGCTATAG GCGTCTCGGGTTCTTCACCC GGAGAGACGGGGCGGGGGGG CCGAGCGGCTCCCGATGCGC CGGCGGACGCGTCTTCTTCC
CAP_site
>NRF1
>TATA_Box
-40
-20
20
40
60
CACCAAGGCCATGCGCACGG TCACTCCCGCTGTATATTAA GGCGCCGGCGATCGCGGCCT GAGGCTGCTCCCGGACAAGG GCAACGAGCGTTTCGTTTGG ACTTCTCGACTTGAGTGCCC
GTGGTTCCGGTACGCGTGCC AGTGAGGGCGACATATAATT CCGCGGCCGCTAGCGCCGGA CTCCGACGAGGGCCTGTTCC CGTTGCTCGCAAAGCAAACC TGAAGAGCTGAACTCACGGG
Sp1_site
>ATF
5'Donor
80
100
120
140
160
180
GCCTCCTTCGCCGCCGCCTG CTGCAGTCCTCAGCGCAGGT GAGGGCCCGCGCGGTAGGTG GGCGCCGGCCGAGGAAGCCC CGGGGTGTCCTGCCAGCGTG GCACCGCTGCCCCGGGTGCT
CGGAGGAAGCGGCGGCGGAC GACGTCAGGAGTCGCGTCCA CTCCCGGGCGCGCCATCCAC CCGCGGCCGGCTCCTTCGGG GCCCCACAGGACGGTCGCAC CGTGGCGACGGGGCCCACGA
200
220
240
260
280
300
GGGACCCCATCCCCCATGTC CCAGTCACCCGCCGCCTTGA GGTCAGTGTTTATCCTCCAG ATCTTTTGCCTAGACGGACC GCCGCAGTTTCCCCCTTTAG GCCCCTTCCGGCTATTCCCG
CCCTGGGGTAGGGGGTACAG GGTCAGTGGGCGGCGGAACT CCAGTCACAAATAGGAGGTC TAGAAAACGGATCTGCCTGG CGGCGTCAAAGGGGGAAATC CGGGGAAGGCCGATAAGGGC
>AP1
320
340
360
380
400
420
GTTGAACCCGCCGCTTTAGA ATTCCCTTCTCATACCCCTC CCGTCATTCTCGAGACCTCG ACCGCGGGTCACCTCTGACC CCCACTGCCGGAGACTGCCT TCTTTCTCCAGACTCAACTC
CAACTTGGGCGGCGAAATCT TAAGGGAAGAGTATGGGGAG GGCAGTAAGAGCTCTGGAGC TGGCGCCCAGTGGAGACTGG GGGTGACGGCCTCTGACGGA AGAAAGAGGTCTGAGTTGAG
>AP1
440
460
480
500
520
540
CCTCGACTTTATAAGTCACC CCTGCGCGTCCCCAGTGAGG CCCAAGGCTGACTGGCCCTC ATACCCCTACCCTCATTTGT GCCCCTCCTTTCTCAGTTAT GCCCAGTTCTTCCCGCTGTG
GGAGCTGAAATATTCAGTGG GGACGCGCAGGGGTCACTCC GGGTTCCGACTGACCGGGAG TATGGGGATGGGAGTAAACA CGGGGAGGAAAGAGTCAATA CGGGTCAAGAAGGGCGACAC

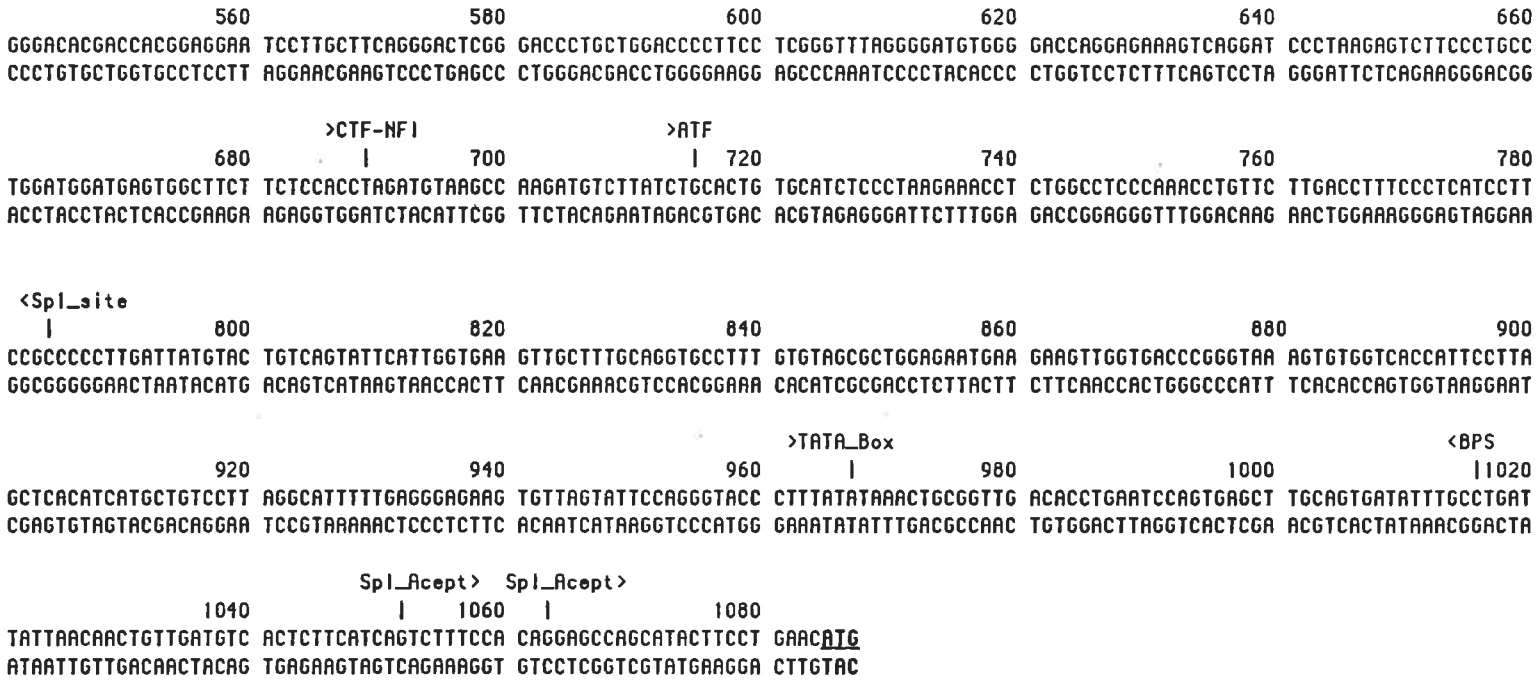


Figure 4.3 Nucleotide sequence of the 5' flanking region of the human hepatic ALAS gene.

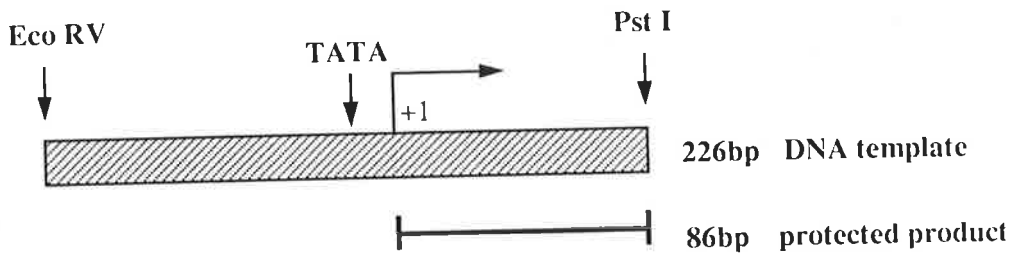
The sequence of the first 1387bp of the 5' flanking region of the human ALAS promoter extending upstream from the ATG translational initiation codon (underlined) is shown. Numbers indicate the nucleotide position of the base pair relative to the transcription initiation (CAP) site at +1, as defined by RNA protection analysis Section 4.2.3. Computer analysis was used to compare the promoter sequence with the consensus sequences of regulatory sequence elements commonly associated with the promoters of eukaryotic genes transcribed by RNA polymerase II. Where the human sequence had one or less mismatches with the consensus sequence of a particular regulatory element, the site is indicated. The intron-exon boundaries and putative branch point site (BPS) of the intron are indicated.

Figure 4.4 Determination of the transcription initiation site of the human hepatic ALAS gene.

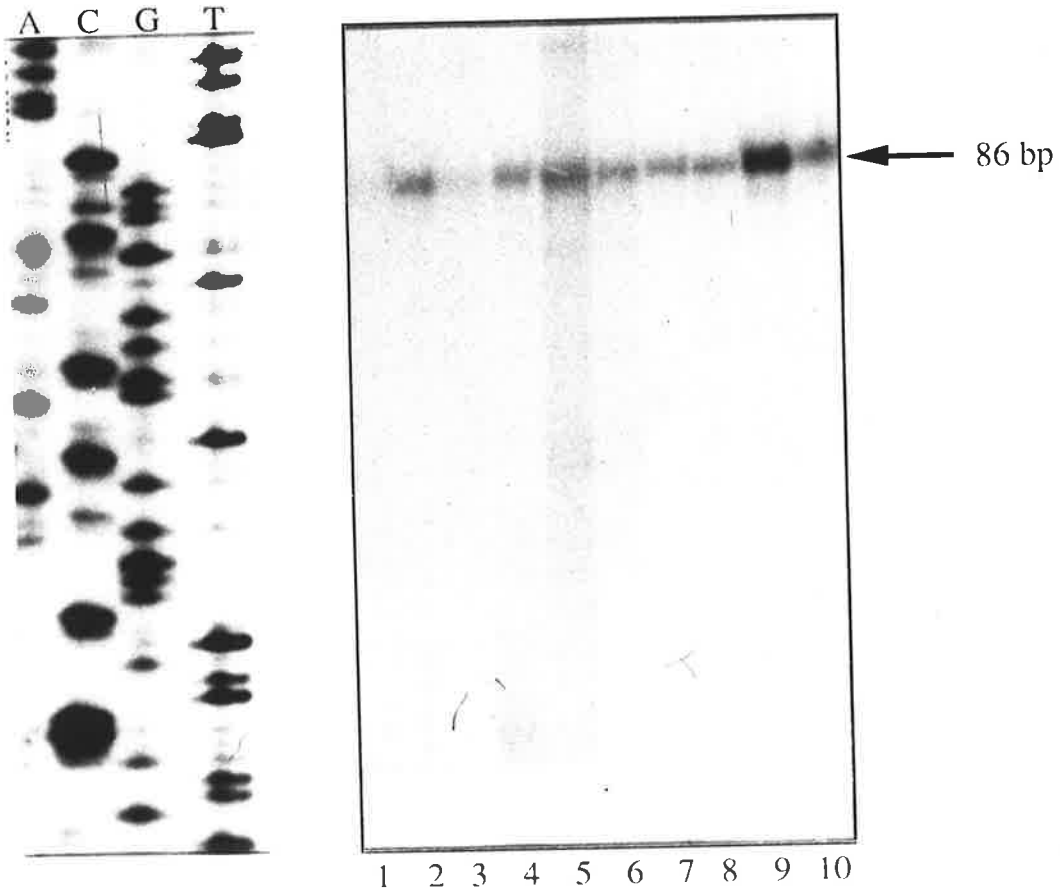
(A) A diagrammatic representation of the Eco RV-Pst I DNA fragment used as the template for *in vitro* synthesis of the ^{32}P-labelled RNA probe used in the RNAse protection assay is presented. The relative positions of the putative TATA consensus sequence and the transcriptional start site of the gene are shown. The mRNA product protected by this probe is shown below the DNA template.

(B) Samples of total RNA (20μg) isolated from 9 individual human liver samples were analysed by RNAse protection as outlined in Section 2.3.5, and the autoradiograph from this experiment is shown. In lanes 1-9 the products resulting from the protection of human hepatic total RNA isolated from nine different adult individuals are shown. In lane 10, the protected product from a human foetal liver RNA sample is shown. The size of the protected RNA/DNA hybrid is indicated on the right hand side of the figure. This was determined by comparison of the protected hybrid with a series of dideoxy chain termination reactions performed as described in Section 2.2.12, on single stranded DNA prepared from a clone which encoded the corresponding region of the ALAS gene. The autoradiographic signals from the protected products were much weaker than from the sequencing reactions, and a shorter exposure of the sequencing reaction is presented.

A


Eco RV
TATA
Pst I
+1
226bp DNA template
86bp protected product

B


A C G T
86 bp
1 2 3 4 5 6 7 8 9 10

described in Section 2.3.5. A single protected band was seen when the RNAse digestion step was carried out at 42°C with 4μg/ml RNAse A and 2μg/ml of RNAse T1(data not shown) and these conditions were used for all subsequent experiments.

RNAse protection analysis of 20μg samples of total RNA was carried out using RNA isolated from nine individual human liver samples and one sample obtained from human foetal liver. A dideoxy sequencing ladder performed on single stranded DNA which encodes this region of the human ALAS gene was included as a size marker, and the autoradiograph from this experiment is shown in Fig. 4.4. While the level of ALAS mRNA varies between adult individuals, (lanes 1-9) and is barely detectable in the foetal liver sample (lane 10) in all 10 of the liver samples investigated, there is a single protected product 86bp in size. From this result it can be concluded that the transcription initiation site of the gene is located 28bp 3' from the putative TATA consensus sequence.

4.2.4 Analysis of the 5' flanking region of the human hepatic ALAS gene.

The sequence of the first 1387bp of the 5' flanking region of the human hepatic ALAS gene is shown in Fig. 4.3; the numbering is relative to the transcription initiation site of the gene (CAP site) which was defined by RNAse protection analysis (Section 4.2.3), and is located at nucleotide position +1. The intron in the 5' untranslated region of the gene extends 954bp with the predicted intron-exon boundaries at nucleotide positions +98 and +1052. The translation initiation ATG codon located at position +1087 is underlined.

Computer analysis (Section 2.6.3) was used to compare the nucleotide sequence of the 5' flanking region of the gene with the consensus sequences of regulatory sequence elements commonly associated with the promoters of eukaryotic genes transcribed by RNA polymerase II. Putative regulatory elements present in the human sequence, which have either one or no mismatches with the consensus sequence are indicated in Fig 4.3. In the vicinity of the transcription initiation site, there is the TATA box consensus element (TATATT) mentioned previously, located between nucleotide positions -22 to -28. Many eukaryotic promoters possess a CCAAT box sequence located between 60 to 80 bp upstream from the transcriptional initiation site, but no consensus CCAAT box sequences (Benoist *et al.*, 1980) were detected

within the first 300bp of the 5' flanking region of human ALAS gene. However, there are three sequences identical to the GC hexanucleotide box consensus sequence (GGCGGG), which binds the ubiquitous transcription factor, SpI (McKnight *et al.*, 1984), located at nucleotide positions -200, -105 and +57. It is interesting to note that there is a putative nuclear respiratory factor I (NRF-I) recognition site (GGATGGCC) at position -46. This sequence is also found in the promoter regions of the nuclear genes for rat and human cytochrome *c*, human cytochrome c_1, rat cytochrome *c* oxidase subunit VIc and mouse mitochondrial RNA processing RNA gene. The protein products of all these nuclear genes function in the mitochondria (Evans and Scarpulla 1989), as does ALAS.

The intron in the 5' untranslated region of the gene also has an interesting array of putative regulatory elements indicated in Fig. 4.3. There are two activator protein-1 (AP-1) sites, T(T/G)AGTCA, clustered between nucleotide positions +414 and +440. AP-1 was first described as a transcription factor required for optimal activity of the human metallothionein promoter II_A (Lee *et al.*, 1987a), but has since been shown to be composed of several polypeptides (Angel *et al.*, 1987; Lee *et al.*, 1987b) which can mediate a transcriptional response to phorbol ester tumour promoters such as 12-O-tetradecanoylphorbol 13-acetate (Angel *et al.*, 1987; Lee *et al.*, 1987b). At nucleotide position +715 there is a consensus sequence, (T/G)(A/T)CGTCA, for the activating transcription factor (ATF). The ATF binding site is identical to a sequence found in promoters that are induced by raising cAMP levels, and there is evidence to suggest that ATF and the nuclear factor which binds the cyclic AMP response element (CREB) are the same factor (Jones *et al.*,1988). A consensus sequence for nuclear factor I, TGGCT(N_3)AGCCAA, is located at nucleotide position +689. As discussed Section 1.8.2, nuclear factor I was originally defined by its activity in replication, but was subsequently shown to also bind to the CCAAT consensus sequence, and is now referred to as CTF-NFI (Jones *et al.*, 1987). In addition, at the 3' end of the intron there is a second putative TATA box consensus sequence located at nucleotide position +963, and a GC element at +783. The possibility that this region functions as an alternative promoter of transcription was investigated using RNAse protection analysis and is discussed in the following Section.

4.2.5 RNAse protection analysis of the 3' region of intron one, of the human hepatic ALAS gene

When the nucleotide sequence of the 5' untranslated region of the human hepatic cDNA clone was obtained (Chapter three) one of the four cDNA clones that spanned the region, designated clone 1, contained an additional 11bp located 21bp upstream from the ATG translation initiation codon and this 11bp sequence was not present in the other three clones. Comparison of the sequence from these cDNA clones with the corresponding genomic sequence obtained from the Bam HI clone (Section 4.2.1) established that the 11bp sequence was present in the gene sequence and was located at the position corresponding to the putative 3' acceptor splice site of the intron in the 5' untranslated region of the gene. This suggested that the intron in the 5' untranslated region may have alternate 3' acceptor splice sites located immediately upstream from the ATG translational initiation codon, and these would generate mRNAs differing by 11bp in length. RNAse protection analysis was used to investigate this possibility.

A 268bp genomic DNA fragment which spanned this region, was cloned into the *in vitro* transcription vector pSP 64. This DNA fragment, shown in the upper part of Fig. 4.5, extends from a Sma I site 208bp 5' of the ATG translation initiation codon to a Pst I site situated 60bp 3' of the ATG. If both putative splice sites were used then the RNA probe generated from this fragment by *in vitro* transcription would protect two products 81 and 92bp in length. This DNA fragment also encompassed the 3' region of the intron which contained the second putative TATA consensus sequence located at nucleotide position +963 and the consensus GC box located at nucleotide position +783 (Fig 4.3). If these elements were functional in promotion of transcription then the RNA probe generated from this DNA fragment would protect an RNA product of size 152bp.

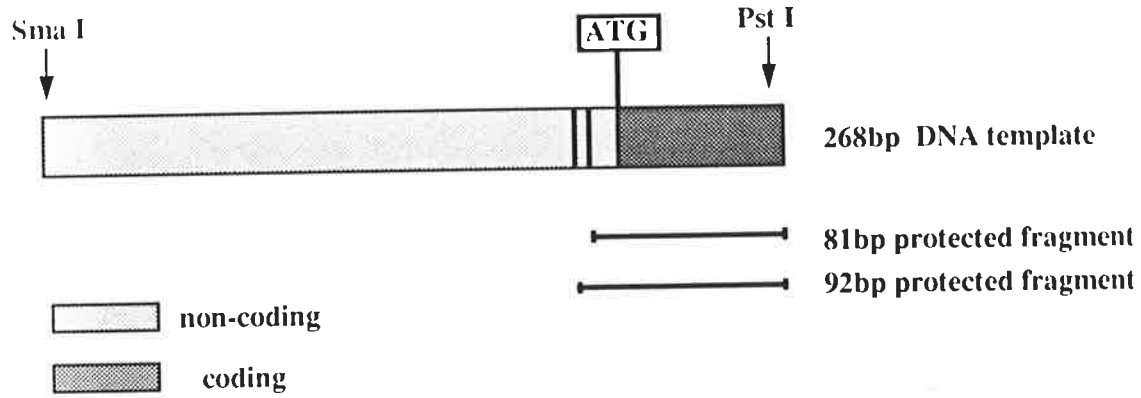
RNAse protection analysis of total RNA (20µgs) isolated from human adult liver and human foetal liver was carried out as described in Section 2.3.5, and the autoradiograph from this experiment is shown in Fig. 4.5. The human foetal liver sample is shown in lane 1; in lanes 2 and 3 different preparations of RNA from the same liver sample were used and in lanes 4-12

Figure 4.5 RNAse protection analysis of the 3' acceptor splice site of intron one in the 5' untranslated region of the human hepatic ALAS gene.

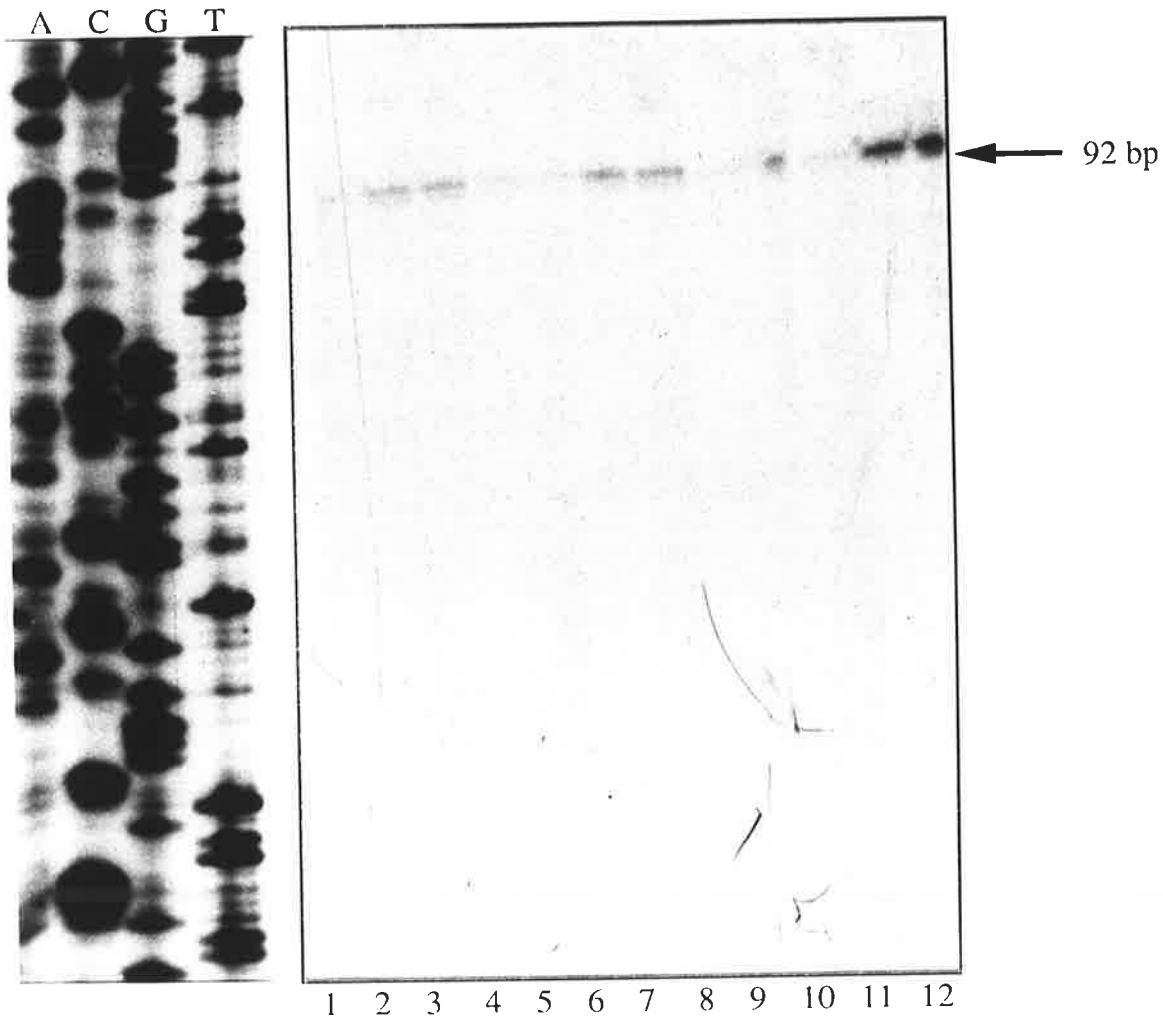
(A) A diagrammatic representation of the Sma I-Pst I DNA fragment used as the template for *in vitro* synthesis of the ^{32}P-labelled RNA probe used in the RNAse protection assay is presented. The translational start site (ATG) of the gene is shown. The two putative 3' acceptor splice sites of the intron are indicate by black lines, and the two possible mRNA products that may be protected by this probe are indicated below the DNA template.

(B) Samples of total RNA (20μg) isolated from nine individual human liver samples were analysed by RNAse protection as outlined in Section 2.3.5, and the autoradiograph from this experiment is shown. The protected product from a human foetal liver sample is shown in lane 1. In lanes 2 and 3, two different RNA preparations from the same adult human liver sample were used for the protection assay. In the remaining lanes (4-12), the protected products from nine individual human liver samples are shown. The size of the protected RNA/DNA hybrid was determined by comparison of the protected hybrid with a series of dideoxy chain termination reactions performed as described in Section 2.2.12.and is indicated on the right hand side of the Figure. The autoradiographic signals from the protected products were much weaker than from the sequencing reactions and a shorter exposure of the sequencing reaction is presented.

A


Sma I
ATG
Pst I
268bp DNA template
81bp protected fragment
92bp protected fragment
non-coding
coding

B


A C G T
92 bp
1 2 3 4 5 6 7 8 9 10 11 12

samples from individual livers are shown. While again the levels of ALAS mRNA varies considerably between individuals, there is only one protected band in all 11 of the liver samples investigated. The size of the protected product calculated from the DNA sequencing ladder, corresponds to the 3' splice site of the intron located at nucleotide position +1052, and includes the additional 11bp found in both cDNA clone 1, and the genomic sequence. This work established that at least in the liver, the preferred 3' acceptor site of the intron is the one located at nucleotide position +1052, and that the consensus TATA and GC boxes located in the 3' region of the intron are not functional in the liver.

4.2.7 Comparison of human and rat intronic sequences.

The sequence of the 5' flanking region of the human hepatic ALAS gene was compared with that of the chicken (Maguire *et al.*, 1986), and rat (Loveridge, 1988). The aim was to search for conserved sequence elements which may be involved in heme regulation. Surprisingly, there is no significant similarities between the chicken gene and the other two species, with the exception of the immediate promoter region where all three genes have a TATA box sequence and a putative SpI consensus site. However, the rat and human genes both contain an intron in the 5' untranslated region of the gene. While the rat intron is slightly smaller (807bp) than the human intron (954bp), both extend to within 30bp of the translation initiation codon. Computer analysis of the introns was carried out and is presented as a matrix plot in Fig. 4.6, where the human promoter sequence is on the horizontal axis and the rat promoter sequence the vertical axis. Regions of similarity greater than 70% between the two sequences are indicated by a line. While there is little similarity between species in the 5' region of the intron, the 3' region has several areas of striking similarity, which do not contain any known regulatory sequences. The strongest regions of similarity, are the most 3', where 20 of 23bp (87%) and 30 of 33bp (91%) are identical.

4.2.8 Chromosome localization of the human hepatic ALAS gene.

The isolation of the human hepatic cDNA clones provided the opportunity to determine the chromosomal localization of the corresponding gene. This work was done in the laboratory of Dr. G. R. Sutherland at the Adelaide Children's Hospital, and while the author did not have

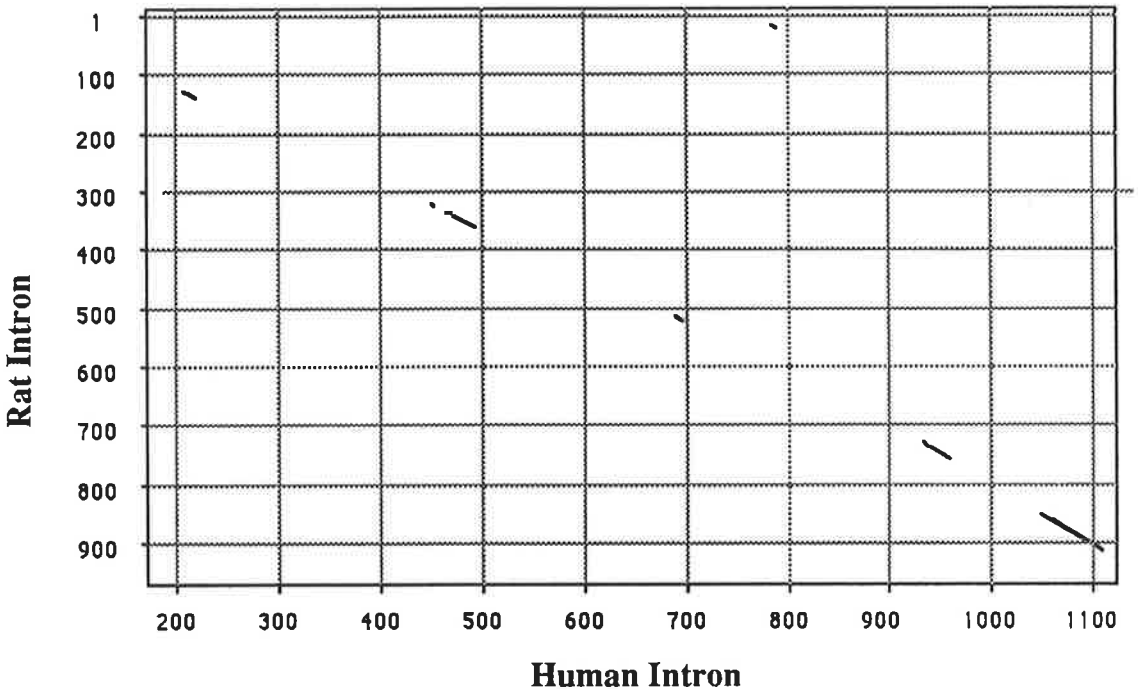


Figure 4.6 Matrix plot comparison of the nucleotide sequence of the introns in the 5' flanking regions of the human and rat hepatic ALAS genes.

The human and rat intronic nucleotide sequences were compared by computer analysis (Section 2.6.3). A matrix plot generated from this comparison is shown. The human intron sequence is shown on the horizontal axis against the rat intron sequence on the vertical axis. Regions of similarity between the two sequences where the nucleotide identity is greater than 70% are indicated.

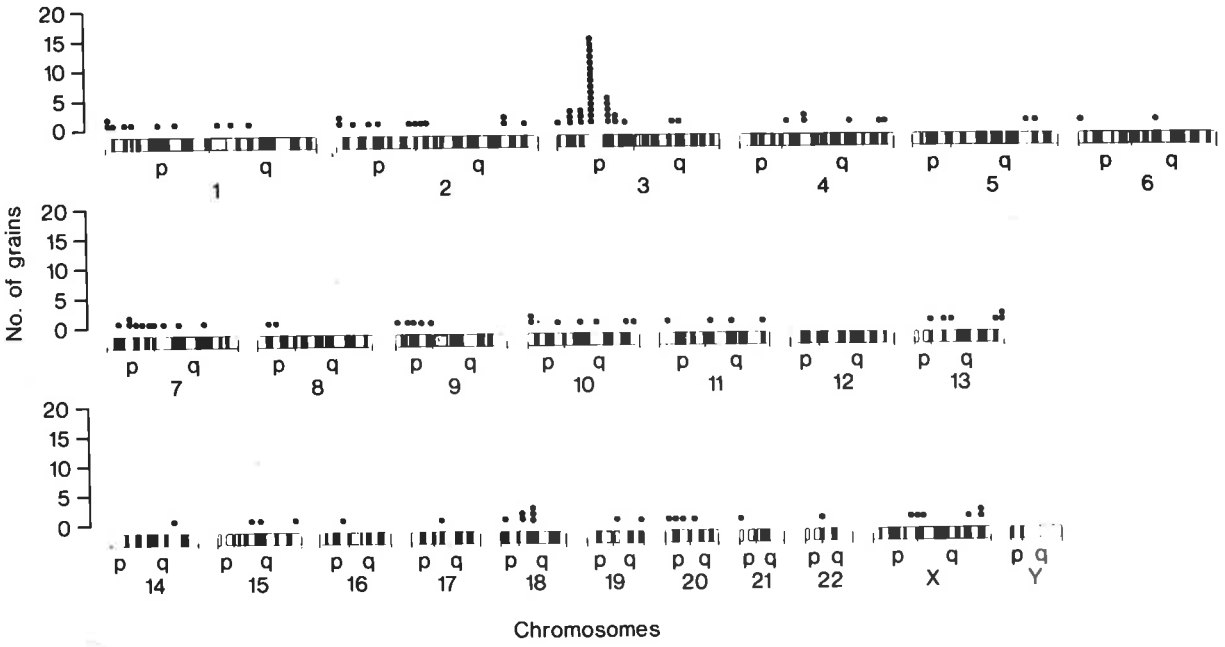
Figure 4.7 Chromosome localization of the human hepatic ALAS gene.

The human ALAS cDNA was labelled with three tritiated nucleotides and used to localize the corresponding human hepatic ALAS gene by *in situ* hybridization.

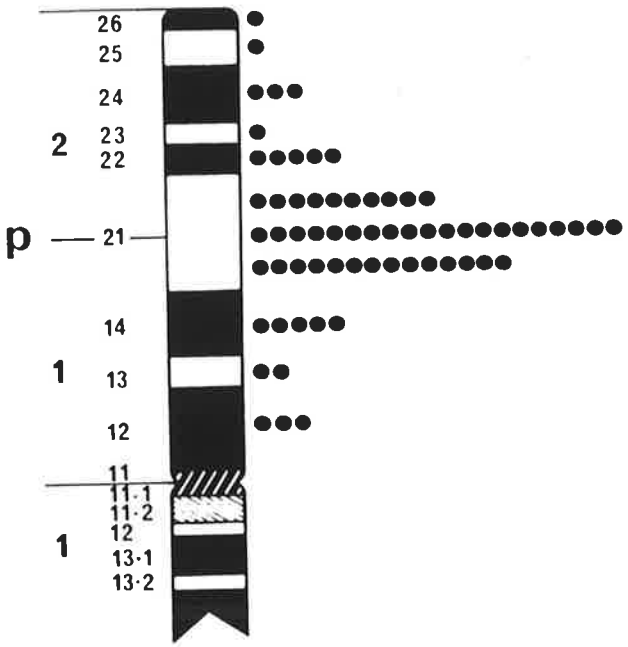
(A) The distribution of silver grains over all chromosomes in 30 metaphases after *in situ* hybridization is shown.

(B) The distribution of silver grains on the short arm of chromosome three from 39 metaphases is presented.

A


No. of grains
20
15
10
5
0
p q
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y
Chromosomes

B


26
25
24
2
23
22
p
21
14
1
13
12
11
11·1
11·2
12
1
13·1
13·2

an active role in this part of the work, apart from supplying the DNA used as the probe, a brief description of the work that was undertaken is presented.

Southern blot hybridization analysis of mouse/human hybrid cells, established that two of the cell lines used showed the presence of human ALAS. The only chromosome region present in these two cell lines and absent from the others is 3pter->3q13.2. Hence the gene was localized by Southern analysis to this region.

Chromosome preparations from two normal males were prepared from synchronized lymphocyte cultures. G-band staining and *in situ* hybridization were carried out using standard methods described by Sutherland *et al.*, (1988). The *in situ* hybridization results (Fig. 4.7A) showed that from 30 metaphases on which there were 122 silver grains, 30 were on 3p (24.6% of all silver grains). The distribution of silver grains from 19 of these 30 metaphases and an additional 20 metaphases having grains over the short arm of chromosome three is shown in Fig. 4.7B. Of 64 grains on 3p, 44 were on band 3p21. Similar results were obtained from the chromosomes of the second normal male (data not shown). To increase the resolution of this result, metaphases expressing the common fragile site at 3p14.2 were examined. From 14 metaphases showing signal on 3p and expressing the fragile site there were 18 silver grains distal to the fragile site, 2 proximal to it and 4 centrally located.

These results indicate that the gene for hepatic ALAS is most likely in band 3p21 and definitely distal to *FRA3B* at 3p14.2.

4.3 DISCUSSION

The work presented in this chapter describes for the first time, the characterization of the 5' flanking region of the human hepatic ALAS gene. Base sequence analysis established that the 5' untranslated region of the human hepatic ALAS gene contains an intron predicted to be 954bp in size. An 11kb Hind III DNA fragment was isolated from a genomic cosmid clone which encompassed the intron and extended approximately 10kb into the 5' flanking region of the gene. RNAse protection analysis of human liver RNA was used to define the transcriptional initiation site of the gene. This work provided the basis necessary for expression studies of

human hepatic ALAS gene promoter constructions to be undertaken, and these are discussed in Chapter five.

The genes for the chicken (Maguire *et al.*, 1986) and rat (unpublished) hepatic ALAS have previously been isolated in our laboratory. The human and rat genes are of similar size, approximately 13kb in length, compared with 6.9kb for the chicken gene. As discussed in Chapter three, the coding regions of the three genes are remarkably conserved, in both size and amino acid sequence. Therefore, the mammalian genes must contain considerably more intronic sequence than the chicken gene.

RNAse protection analysis of human liver RNA established that the human hepatic ALAS gene has a single transcriptional start site located 28bp downstream from a TATA box consensus element. It is interesting to note that although the hepatic ALAS gene is considered to be a housekeeping gene, the human, rat and chicken hepatic ALAS genes all have a TATA box consensus element upstream from the transcriptional initiation site, whereas most housekeeping genes lack this element, and initiate transcription from multiple start sites (Dynan and Tjian 1985; Melton *et al.*, 1986; Jones *et al.*, 1988). The chicken hepatic ALAS gene has been shown, at least in *Xenopus laevis* oocytes, to have a strict requirement for the TATA box element to initiate transcription (Loveridge, 1988), and primer extension analysis of mRNA from chick embryo liver defined a single transcriptional start site (Maguire *et al.*, 1986). In contrast, primer extension analysis of rat liver mRNA indicates that the rat hepatic ALAS gene uses two transcriptional start sites located 3bp apart, despite the fact that a TATA like consensus element is located within the the immediate promoter region of the rat gene at nucleotide position -28bp (Srivastava *et al.*, 1988). It remains to be determined whether the human and rat hepatic ALAS genes have a strict requirement for the TATA box sequence to initiate transcription.

Nucleotide sequence analysis of the 5' flanking region of the human hepatic ALAS gene established that the 5' untranslated region of the gene contained a 954bp intron. The possibility that the intron may have alternative 3' acceptor splice sites was investigated by RNAse protection analysis. Both of the putative splice sites located at nucleotide positions +1052 and +1063 (Fig 4.3) have the consensus sequence CAG which is usually found at the

intronic side of the 3' acceptor splice site (Mount, 1982). In 55% of vertebrate mRNAs this sequence is followed by a G residue, and such a residue is present in this position at the more 3' of the two sites. The other site has a T residue in that position, which is the nucleotide least preferred occurring in only 8% of vertebrate mRNAs (Padgett *et al.*, 1986; Shapiro and Senapathy 1987). RNAse protection analysis established that in all 11 of the human hepatic RNA samples examined, including one human foetal liver sample, the more 5' of the two putative splice sites, located at nucleotide position +1052 was used (Fig.4.5).

The sequences required for intron splicing in higher eukaryotes, are the conserved elements of the 5' and 3' splice junction sites and a somewhat less conserved element the branch point sequence (BPS) at the site of lariat formation (Green 1986; Maniatis and Reed, 1987). The BPS consensus sequence CTGAC, is usually located 18-40 nucleotides upstream from the 3' splice junction (Reed, 1989). A study by Reed and Maniatis (1985), showed that where CAG nucleotides are clustered, splicing usually takes place at the first CAG downstream from the branch point site. However, both the distance and the sequence between the branch point site and the 3' acceptor splice site, play roles in the efficiency of this reaction. As indicated in Fig. 4.3, the first intron of the human ALAS gene has a putative BPS sequence, CTGAT located at nucleotide position +1020, which is 32bp upstream from the more 5' of the two possible 3' acceptor splice sites. Therefore, although the more 5' of the two splice sites is followed by a less favourable T residue, the functional organization of this splice site is more likely to be favoured by the intron splicing machinery, which is consistent with the results found in this study.

Why only one of the four human ALAS cDNA clones isolated that encompassed this region, contained the 11 bp of nucleotide sequence located between the two putative splice sites is not clear. It is possible that under the conditions used to generate the cDNA library, the 5' end of the ALAS mRNA formed a secondary structure which in some way interfered with the reverse transcriptase transcribing the 5' end of the mRNA, and as a result the 11bp were omitted from the transcripts. Alternatively, splicing may occur at the 2nd splice site, but at a very low frequency and below the sensitivity of the RNAse protection technique employed in this study. It may be possible to investigate this alternative by using the polymerase chain

reaction technique to amplify the levels of ALAS mRNA in the samples examined. However, the results of this study show quite clearly that splicing predominately occurs at the more 5' of the two possible splice sites and it seems unlikely that alternative splicing of the ALAS mRNA transcript plays a significant biological role.

The consensus sequences of several transcriptional elements known to play a role in the expression of other eukaryotic genes, were found in the promoter region of the human hepatic ALAS gene (Fig. 4.3). Located in the human ALAS promoter region at nucleotide position -100, is a consensus GC box sequence (McKnight *et al.*, 1984) for the ubiquitous Sp1 transcription factor (Dynan and Tjian, 1985; Kadonga *et al.*, 1986). This GC box sequence is commonly found in the promoter regions of housekeeping genes particularly those lacking a TATA box (Osborne *et al.*, 1985). The element may have some function in modulating the expression of the human hepatic ALAS gene, as interestingly, a GC box located at nucleotide position -78 has been shown to be essential for maximum expression of the chicken hepatic ALAS gene promoter when it is expressed in *Xenopus* oocytes (Loveridge, 1988). A second GC box consensus sequence, is located in the 5' untranslated region of the human ALAS gene at nucleotide position +60. Although the transcription factor Sp1 functions preferentially in an immediate upstream position (Serfling *et al.*, 1985; Schatt *et al.*, 1990), it is possible that this second element modulates expression of the human ALAS gene indirectly by competing with the upstream site for the binding of Sp1. However, further work is required to determine whether the expression of the human hepatic ALAS gene is modulated by either of these elements.

Interestingly, the human hepatic ALAS promoter has a consensus sequence for the recently described nuclear respiratory factor (NRF-1) located at nucleotide position -45. This factor appears to be specific for nuclear genes, the products of which are expressed in the mitochondria, and it has been suggested that this factor is responsible for the co-ordinated expression of respiratory proteins (Evans and Scarpulla, 1989). Since many mitochondrial respiratory proteins use heme as a co-factor it seems logical to suggest that NRF-1 may co-ordinate heme biosynthesis through the transcriptional regulation of ALAS with expression of

the cytochrome respiratory genes. Current work in our laboratory is investigating this possibility.

Functional promoter elements within an intron have only been defined for a few genes (Rossi and De Crombrugghe, 1987, Coulombe *et al.*, 1988; Reisman *et al.*, 1988), including the cytochrome *c* gene (Evans and Scarpulla, 1989), which like ALAS is a nuclear-encoded gene whose product functions in the mitochondria. It has recently been shown that expression of the cytochrome *c* gene is modulated by two adjacent Sp1 sites located within the first intron of the gene (Evans and Scarpulla, 1989). As indicated in Fig. 4.3, the intron in the 5' flanking region of the human hepatic ALAS gene contains many putative regulatory elements. From expression studies of the human hepatic ALAS gene promoter constructions in tissue culture cell lines, described in Chapter five, this intron appears to have a role in the regulation of transcription of the ALAS gene. Therefore, it will be of interest to determine which, if any, of these putative elements are involved.

Comparison of the human and rat intronic sequences show that the 3' region of the intron has several regions of striking nucleotide similarity, which are not present in the chicken ALAS promoter (Fig 4.6). As yet it is not known whether these regions have a functional role. However, it seems unlikely they would be so highly conserved in the intronic region of the gene if they did not either have a specific function or confer some advantage. Since, these sequences are not present in the chicken promoter, it seems unlikely that they are the sequences involved in heme repression of the promoter, unless they interact in some way with other regulatory elements located several kilobases away.

The gene for human hepatic ALAS has been mapped to part of chromosome 3 by Southern blot hybridization analysis of somatic cell hybrids and more precisely to 3p21 by *in situ* hybridization (Fig. 4.7). There was no evidence of other sites of hybridization of the probe to metaphase chromosomes which suggests that the human hepatic ALAS gene is present in the genome as a single copy and there are no pseudogenes for ALAS. It is interesting to note that the genes for the other members of the heme biosynthetic pathway which have been examined to date, are located on different chromosomes; 5-aminolevulinate dehydratase has been localized to 9q34 (Potluri *et al.*, 1987), porphobilinogen deaminase to 11q23 (Wang *et al.*, 1981),

uroporphyrinogen decarboxylase to 1p34 (Povey *et al.*,1985), and coproporphyrinogen oxidase to chromosome 9 (Grandchamp *et al.*, 1983). Therefore, the expression of these genes must be co-ordinated, presumably by some trans-acting mechanism(s), for heme biosynthesis to occur.

Localization of ALAS to chromosome 3p21 was of interest, because at the time this work was undertaken it was thought that there was only one gene, the hepatic or housekeeping form of ALAS, which had been implicated as the deficient enzyme in X-linked sideroblastic anaemia. Since the disease clearly follows X-linked inheritance (Bottomley, 1982), this work showed it could not be the result of mutation of the hepatic ALAS gene. However, when subsequent work established the existence of isoenzymes for human ALAS, and the erythroid specific form was localized to the X chromosome, the involvement of erythroid ALAS in the aetiology of the disease became a distinct possibility and is investigated in Chapter seven.

CHAPTER FIVE

STUDIES ON THE EXPRESSION OF HUMAN HEPATIC ALAS PROMOTER CONSTRUCTIONS IN TISSUE CULTURE CELL LINES

CHAPTER FIVE : STUDIES ON THE EXPRESSION OF HUMAN HEPATIC ALAS PROMOTER CONSTRUCTIONS IN TISSUE CULTURE CELL LINES

5.1 INTRODUCTION

In the previous chapter, the isolation and characterization of the 5' flanking region of the human hepatic ALAS gene was described. This provided the basis for the work presented in this chapter in which expression of the human hepatic ALAS gene promoter in tissue culture cell lines was examined. A series of promoter constructions were prepared containing increasing lengths of the 5' flanking region of the human hepatic ALAS gene fused to the human growth hormone (hGH) reporter gene. Expression of these constructions was then examined by transient expression studies in the human hepatoma cell line HepG2, and the non-hepatic chinese hampster ovary (CHO) cell line.

The human hepatoma cell line HepG2, was chosen for these studies for the following reasons. Firstly, it is an homologous cell line which has retained differentiated liver cell function. The cells are not virally transformed; the presence of a viral genome could potentially interfere with both cellular gene expression and the expression of transfected DNA. Moreover, HepG2 cells have the ability to respond to a limited number of chemicals, including succinyl-acetone and DMSO, which induce the levels of ALAS activity, and both the non-induced and induced ALAS levels of activity are subject to repression by added hemin (Iwasa *et al.*, 1989). Unfortunately however, porphyrogenic chemicals such as phenobarbitone do not induce ALAS levels in this cell line (Iwasa *et al.*, 1989).

The hepatic form of ALAS is considered to be a housekeeping gene and it was of interest to compare the expression generated by the chimeric ALAS/hGH promoter constructions in HepG2 cells with a non-hepatic cell line. CHO cells have been used extensively in many studies of gene expression and have the advantage that following transfection they can survive in growth media for a limited period of time without any added serum. As both foetal calf and Nu™ serum contain some heme, this attribute allowed the responsiveness of the ALAS promoter constructions to added hemin to be studied without contribution from any heme present in the growth media.

5.2 RESULTS

5.2.1 Attempted generation of intronless ALAS/CAT and ALAS/luciferase chimeric plasmid gene promoter constructions.

At the time when the work to generate human ALAS promoter constructions for expression studies commenced, the luciferase gene had recently been isolated from the firefly *Photinus pyralis*, and used as a reporter gene for gene expression studies (de Wet *et al.*, 1987). Depending on the instrumentation used to detect the luciferase activity, this system was reported to be from 30-1000 fold more sensitive than assaying for chloramphenicol acetyltransferase (CAT) expression (de Wet *et al.*, 1987). Other work in this laboratory had shown that the levels of expression that were obtained from rat and chicken hepatic ALAS promoter constructions were low when assayed using the CAT reporter gene system (Day, 1988; Loveridge, 1988). Therefore, it was decided initially to compare the expression of the human ALAS promoter constructions using both the luciferase and CAT reporter gene systems, with the intention of using the luciferase system for further work should it prove to be satisfactory.

Analysis of the sequence of the 5' flanking region of the ALAS gene established that there are convenient restriction enzyme sites which could be utilized to prepare promoter constructions without the intron in the 5' untranslated region of the gene. However, to generate constructions containing the intron it was first necessary to engineer a restriction enzyme site between the 3' splice site of the intron and the ATG translation initiation codon. Therefore, in the first instance it was convenient to generate promoter constructions without the intron.

All the human hepatic ALAS promoter constructions used in this study were generated from the 11kb Hind III restriction fragment, described in Chapter four, which contains approximately 10kb of the 5' flanking region of the ALAS gene. Sequence analysis established that a Pst I site was located at nucleotide position +80bp, which is 12bp upstream from the putative 5' splice site of the intron in the 5' untranslated region of the gene (see Fig.5.1). Restriction enzyme analysis established that the Eco RV site situated at nucleotide position -140bp is a unique site in the 11kb Hind III restriction fragment (see Fig 5.1). Simultaneous restriction enzyme digestion of the 11kb Hind III fragment with the endonucleases Eco RV and

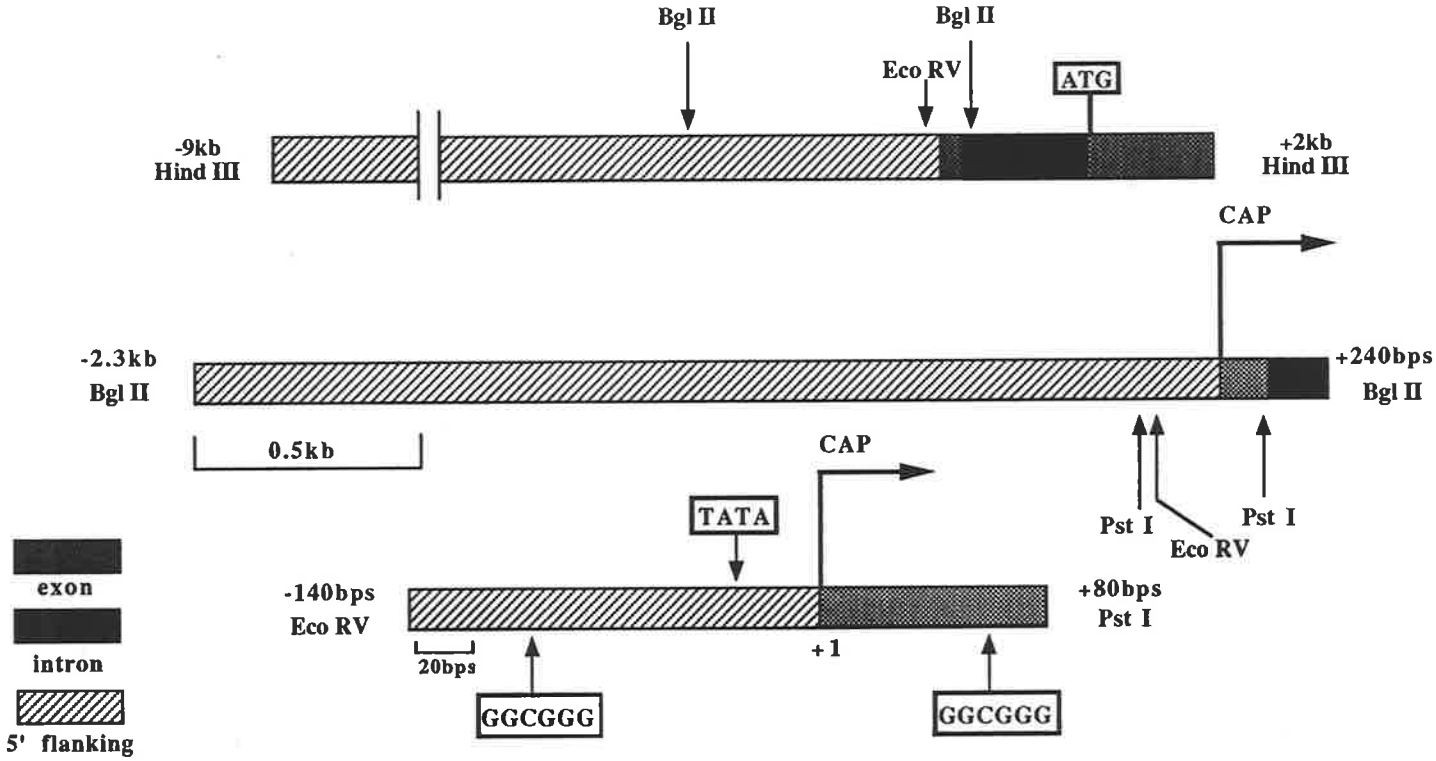


Figure 5.1 Restriction enzyme map of the 5' flanking region of the human hepatic ALAS gene.

The 11kb Hind III DNA fragment (partially characterized in Chapter 4) encompassing the translational start site (ATG) and approximately 10kb of 5' flanking sequence of the hepatic ALAS gene is shown. The Bgl II and Eco RV-Pst I DNA fragments, subcloned from the 11kb Hind III fragment, and used to the generate intronless chimeric ALAS/hGH promoter constructions are also shown. Restriction enzyme sites important to the cloning strategy are indicated. The location of the transcriptional start (CAP) site of the gene is indicated by an arrow. The location of the putative TATA and GC box consensus elements are also shown.

Pst I, generates an Eco RV-Pst I restriction fragment 220bp in size. This fragment had previously been cloned into the plasmid vector pSP 72 and used to define the transcription initiation site of the human hepatic ALAS gene by RNAse protection analysis (Section 4.2.3). The putative TATA box sequence located at nucleotide position -28bp, and two putative GC boxes located at nucleotide positions -105 and +57 bp are also contained within this fragment (see Fig 5.1). As it was of interest to compare the level of expression obtained from this immediate region of the human ALAS promoter with the longer promoter constructions, this fragment was isolated for cloning into CAT and luciferase expression vectors.

The human ALAS promoter has a second Pst I site at nucleotide position -172 (Fig.5.1) therefore, a longer promoter construction could not be generated by using the Pst I site at +80bp described above. To overcome this problem, a Bgl II-Eco RV restriction fragment which contains human ALAS gene sequence from -140bp to -2.3kb (and is continuous with the 220bp Eco RV-Pst I fragment described above) was isolated from the 11kb Hind III fragment. This fragment was then cloned next to the Eco RV-Pst I fragment in the vector pSP 72, using the Eco RV and Bgl II sites in the multiple cloning site of the vector. This allowed the human hepatic ALAS gene promoter sequences from +80bp to -2.3kb to be isolated as a Bgl II-Hind III restriction fragment by using the Hind III site adjacent to the Pst I site in the multiple cloning site of the pSP 72 vector, for cloning purposes.

The expression vectors used in this study were derived from the mammalian expression vector pSV series, which are hybrid plasmid vectors which contain pBR 322 and SV40 sequences (Subramani and Southern, 1983). It had been established that cryptic promoters are present in the pBR 322 region of the pSV vector. Although these promoters require the presence of an enhancer for activity, as much as 14% of transcription from pSV2 (which has the SV40 small-t-antigen splice site and an SV40 polyadenylation signal located downstream from the site into which foreign sequences are inserted) had been found to originate from the amp^r region of the pBR 322 portion of the vector (de Wet *et al.*, 1987). To overcome this problem a vector, designated pSVOA, was constructed which has two copies of the SV40 polyadenylation signal located between the pBR 322 portion of the vector and the 5' end of the inserted gene (de Wet *et al.*, 1987). Both the CAT and luciferase genes had been

cloned into this vector generating the expression vectors pSVOtCAT and pSVOAL respectively. The CAT expression vector pSVOtCAT, was a gift from Dr. J. P. Whitlock, Jr., Stanford University, Stanford California. The luciferase expression vector pSVOAL was obtained from Dr. D. Helinski, University of California, San Diego; La Jolla, California.

Attempts were made to fuse the two human hepatic ALAS gene promoter DNA fragments described above, to the luciferase and CAT reporter genes in the expression vectors pSVOAL and pSVOtCAT. However, when the transformed colonies resulting from these four cloning events were screened, it was found that in all the isolated plasmids examined, up to four kilobases of DNA had been deleted. In most cases (approximately 70%) the plasmids were about 2kb in size, 3kb smaller than the size of the CAT and luciferase expression vectors alone. Initially, a *rec*A$^+$ strain of *E. coli* was used as the host strain for cloning. Two *rec*A$^-$ host strains, DH 5 α and XL-1-B, were then used but the outcome was the same, and of the 500 colonies screened all had undergone large deletions of DNA.

As described previously, the expression vectors pSVOAL and pSVOtCAT, had two copies of the SV40 polyadenylation signal inserted 5' to the reporter gene in addition to a single copy fused to the 3' region of the CAT and luciferase genes. it was possible that some recombination event was occurring between these sequences to bring about the deletions. To investigate this possibility, the CAT and luciferase genes were cloned into the bluescript plasmid vector pBSKS$^-$, orientated in such a way that any cryptic promotion that might arise from the amp^r gene of the vector would read into the 3' region of the reporter gene. Therefore, cryptic promotion would not contribute to any expression generated by inserted promoter sequences. These expression vectors were designated pBSLuc and pBSCAT. However, attempts to clone the two human ALAS promoter sequences described previously, into these expression vectors again resulted in large deletions of DNA.

As the ALAS gene promoter sequences were already cloned into the vector pSP 72 and appeared to be quite stable attempts were made to blunt-end ligate the CAT and luciferase genes behind the promoter sequences in this vector. However, this strategy was also unsuccessful. In all recombinants screened up to 4kb of DNA had been deleted.

To determine whether cloning the human hepatic ALAS promoter sequences in front of a eukaryotic reporter gene would eliminate the deletion problem, the gene for human growth hormone, described by Selden *et al.*, 1986, was obtained from Dr. P. J. McKinnon of this Department. The growth hormone gene was isolated as a 2.1kb Bam HI-Eco RI fragment from pØGH (Selden *et al.*, 1986) and blunt-end ligated behind the promoter sequences in the pSP 72 vector. Unfortunately, this also proved to be unsuccessful and all recombinants screened had deletions of several kb of DNA. Dr. P. J. McKinnon, from another research group in this department, very kindly also tried to clone the human growth hormone gene behind the two human promoter sequences in pSP 72. However, although this cloning attempt was carried out independently, of the two hundred transformants screened all had several kb deletions of DNA.

Reports in the literature had suggested that some *E. coli* strains with *rec*A$^-$, *rec*B$^-$, *rec*C$^-$ and *rec*D$^-$ genotypes or various combinations of these prevented some *rec*A-independent deletion events (Ishiura *et al.*, 1989). Two strains,*E.coli* DB 1317 and DB 116 (see Section 2.1.8 for genotypes), were obtained from the *E. coli* stock centre New York. However, in both of these strains the deletion events still occurred, the only difference being that in all the transformants screened the deletions were the same size (approximately 2kb). The significance of this, if any, is unknown.

As the cloning strategies used up until this stage of the work had relied on directional cloning of the ALAS promoter sequences, it was decided to attempt to clone the entire Bgl II fragment (Fig.5.1) in front of the human growth hormone reporter gene. If this fragment cloned in one orientation only it would establish that the cloning problems were due to an orientation effect. The Bgl II fragment contains the same ALAS gene sequences as the Bgl II-Pst I DNA fragment described above, and in addition the first 140bp of the intron in the 5' untranslated region of the gene. Of the twelve transformants screened from this cloning event, seven had cloned in one orientation and the remaining five in the opposite orientation, which is what might be expected statistically from any cloning event. Extensive restriction enzyme analysis of all 12 isolated plasmids established that there were no deletions of DNA. As the only difference between the Bgl II DNA fragment and the Bgl II-Pst I promoter sequences used previously is the inclusion of the first 140bp of intronic sequence, this result implied that the

140bp of intron had somehow conferred stability during the cloning event. Subsequently, it was shown that the inclusion of these 140bp with the smaller Eco RV-Pst I DNA fragment descibed above, also resulted in that fragment being cloned in a stable fashion next to the human growth hormone reporter gene.

In an attempt to generate an intronless promoter construction by another strategy, the polymerase chain reaction (PCR), was used to generate a fragment which extended from nucleotide position +12bp in the 5' untranslated region of human hepatic ALAS gene to the Eco RV site at -140bp in the 5' flanking region of the gene (Fig. 5.1). The template DNA (100ngs) used was a 2.5kb Eco RV-Hind III fragment (Fig.5.2), which contained the human ALAS gene sequences from -140bp in the promoter region to +1kb in the coding region of the gene. This DNA fragment was isolated from the 11kb Hind III fragment, described in Chapter four, and cloned into the plasmid vector, pBSSK+. A synthetic oligomer (100ngs) designated RRS-943, complementary to the 5' untranslated region of the human ALAS gene, extending from nucleotide position +12 to +50 was used as one PCR primer. This oligomer had a Hind III site incorporated into it by changing the nucleotides, GCG at nucleotide positions +27 to +30 in the human hepatic ALAS gene sequence, to AGC. The reverse sequencing primer (100ngs) which hybridizes to the betagalactosidase gene located upstream from the multiple cloning site of the pBSSK+ vector was the second primer used for the PCR reaction. The polymerase chain reaction was carried out using the following conditions; annealing 55°C, elongation 72°C, and melting 94°C for 30 cycles. The resulting product was digested with the restriction enzymes Hind III and Eco RV, and cloned into the corresponding sites of pBSSK+. However, when an attempt was made to clone this fragment in front of the human growth hormone reporter gene, all of the 12 transformants screened had deleted several kb of DNA.

From the work described above it appears that the human hepatic ALAS gene contains some undefined sequence(s) located between nucleotide positions +50bp and -140bp which bring about the deletion of large amounts of DNA, when attempts are made to clone this sequence next to a heterologous gene. It should be emphasized that the same DNA sequences are quite stable until a heterologous gene sequence is introduced. The stabilizing effect seen when 140bp of intronic sequence were included in the human ALAS promoter constructions

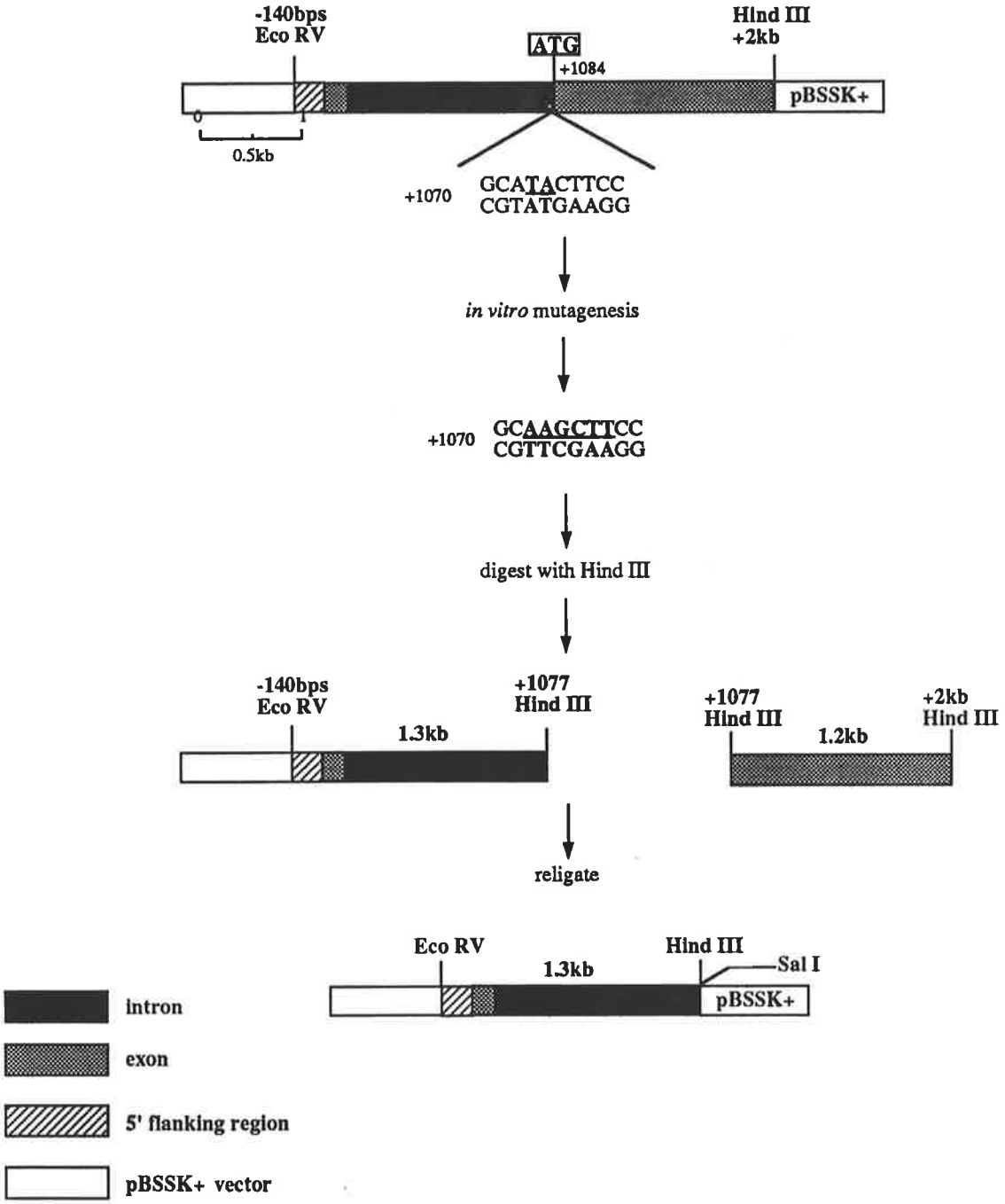


Figure 5.2 The generation of a Hind III restriction enzyme site in the 5' untranslated region of the human hepatic ALAS gene.

The 2.5kb Eco RV-Hind III genomic DNA fragment, subcloned from the 11kb Hind III fragment (Fig.5.1) was used as the template DNA for *in vitro* mutagenesis, A Hind III restriction enzyme site was inserted between the 3' acceptor splice site of the intron in the 5' untranslated region of the gene and the ATG translation initiation codon. The location of the base sequence, and the nucleotides that were altered are indicated. In the lower part of the figure the strategy used to subclone the 1.3kb Eco RV-Hind III that was the outcome of this procedure into the pBSSK+ vector is shown.

cloned in front of the human growth hormone reporter gene, was unexpected. Computer analysis of the nucleotide sequences involved has failed to provide any insight into what might be causing this phenomenon. None of the sequences usually associated with destabilization of propagation of recombinant DNA in *E. coli* hosts, such as palindromic structures (Collins, 1980), inverted repeat sequences (Lilley, 1981) or tandemly repeated sequences (Brutlug *et al.*, 1977) are present in the human ALAS sequence. In addition, a search of the Gene Bank data base failed to detect any sequences similar to the region involved.

Although it would have been preferable to examine expression of the human hepatic ALAS promoter without any of the intron in the 5' untranslated region present, due to the technical difficulties that generating such a construction presented, it was decided to include the first 140bp of the intronic sequence in the human ALAS promoter constructions. The human growth hormone reporter gene system is reliable, quantifiable and at least 10 fold more sensitive than the CAT system (Selden, *et al.*, 1986). As it had been possible to clone this gene next to the human hepatic ALAS promoter, providing the 140bp of intronic sequence were included, it was decided to use the human growth hormone system as the reporter gene for the rest of this study.

5.2.2 Generation of ALAS/hGH chimeric plasmid gene promoter constructions.

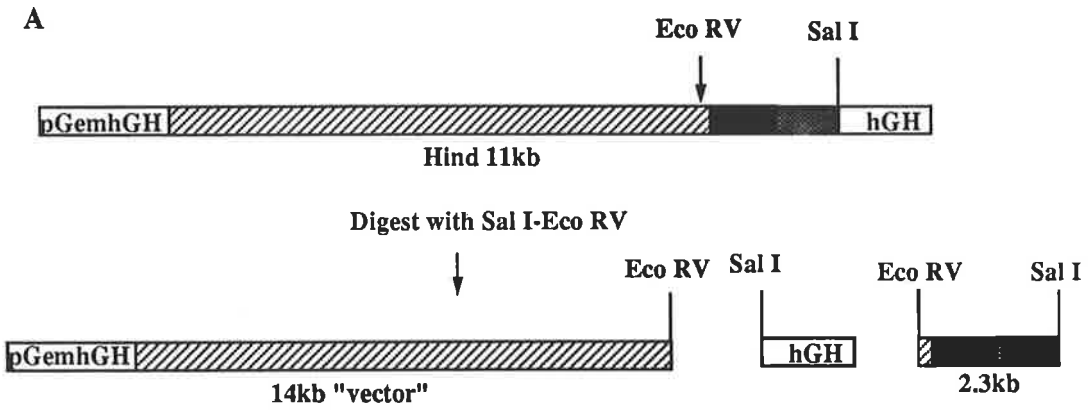
A human growth hormone expression vector was prepared by cloning the gene for human growth hormone isolated as a 2.1kb Bam HI-Eco RI fragment from pØGH (Selden *et al.*, 1986; obtained from Dr. P. J. McKinnon, this Department) into the Bam HI-Eco RI sites of the plasmid pGEM 3Zf+. This particular pGEM vector was chosen because it generated an expression vector with a Sal I site 5' to the growth hormone gene and 3' to the Hind III site of the multiple cloning site. Sal I is the only known restriction endonuclease that does not have a recognition site within the 11kb Hind III restriction fragment. Therefore, this site was important in the cloning strategy used to generate the promoter constructions. The resulting expression vector was designated pGEMhGH, and is shown in the upper part of Fig.5.4.

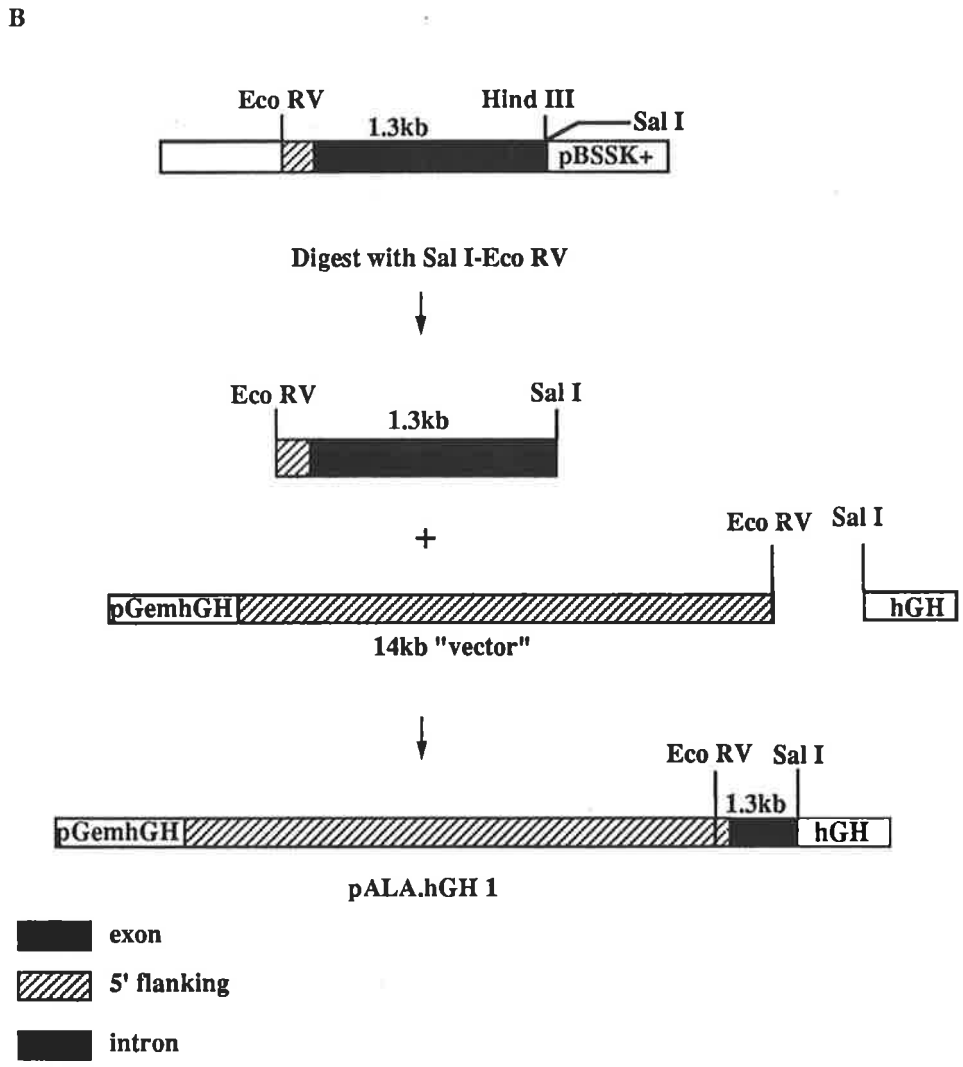
In order to examine expression of the human hepatic ALAS gene promoter and to determine whether the intron has a role in regulation of expression of the ALAS gene, three

pairs of human hepatic ALAS/hGH chimeric promoter constructions were prepared. The promoter constructions contained increasing lengths of ALAS gene promoter sequence, and included either all of the intron in the 5' flanking region of the gene, or because of the technical difficulties described in the preceding section the first 140bp of intronic sequence.

The human hepatic ALAS gene does not have any known restriction enzyme sites between the 3' acceptor splice site of the intron in the 5' untranslated region of the gene and the ATG translation initiation codon. Therefore, in order to prepare gene promoter constructions which included the intron, it was first necessary to engineer a restriction enzyme site in this region of the ALAS gene. Single stranded DNA was prepared from the previously described 2.5kb Eco RV-Hind III fragment in the plasmid vector, pBSSK+ (Fig.5.2) which contained the human ALAS gene sequences from -140bp in the 5' flanking region to +2kb into the coding region of the gene. The single stranded DNA was used as the template for *in vitro* mutagenesis (Section 2.2.16) to change the T and A nucleotides, at positions 1073 and 1074 of the human hepatic ALAS gene sequence (Fig. 5.2) to an A and G respectively. This procedure engineered a Hind III site (AAGCTT) at nucleotide positions +1072 to +1077. Simultaneous restriction enzyme digestion of the mutated clone with the endonucleases Eco RV and Hind III generated a 1.3kb Eco RV-Hind III fragment which contained the ALAS gene sequences from nucleotide position +1077, which is 10bp upstream from the ATG translation initiation codon, to -140bp in the 5' flanking region of the gene (Fig. 5.2). This newly generated 1.3kb Eco RV-Hind III fragment was then cloned into the corresponding restriction enzyme sites of the plasmid vector pBSSK+. This particular Bluescript™ vector was chosen because it has a Sal I site adjacent to the Hind III site in the multiple cloning site of the vector, which allowed this DNA fragment to be isolated as a Eco RV-Sal I fragment for cloning purposes.

The longest pair of human hepatic ALAS promoter constructions used in this study contained human hepatic ALAS gene sequences upstream from the transcription initiation site to approximately -9kb. These constructions were prepared by first cloning the 11kb Hind III fragment (Chapter four) which contained the ALAS gene sequences from approximately +2kb in the coding region to -9kb in the 5' flanking region of the ALAS gene, into the Hind III site in the multiple cloning site of the pGEMhGH expression vector (Fig. 5.4). The resulting clones


A
Eco RV
Sal I
pGemhGH
hGH
Hind 11kb
Digest with Sal I-Eco RV
Eco RV
Sal I
Eco RV
Sal I
pGemhGH
hGH
2.3kb
14kb "vector"


B
Eco RV
Hind III
1.3kb
Sal I
pBSSK+
Digest with Sal I-Eco RV
Eco RV
Sal I
1.3kb
+
Eco RV
Sal I
pGemhGH
hGH
14kb "vector"
Eco RV
Sal I
1.3kb
pGemhGH
hGH
pALA.hGH 1
exon
5' flanking
intron

C

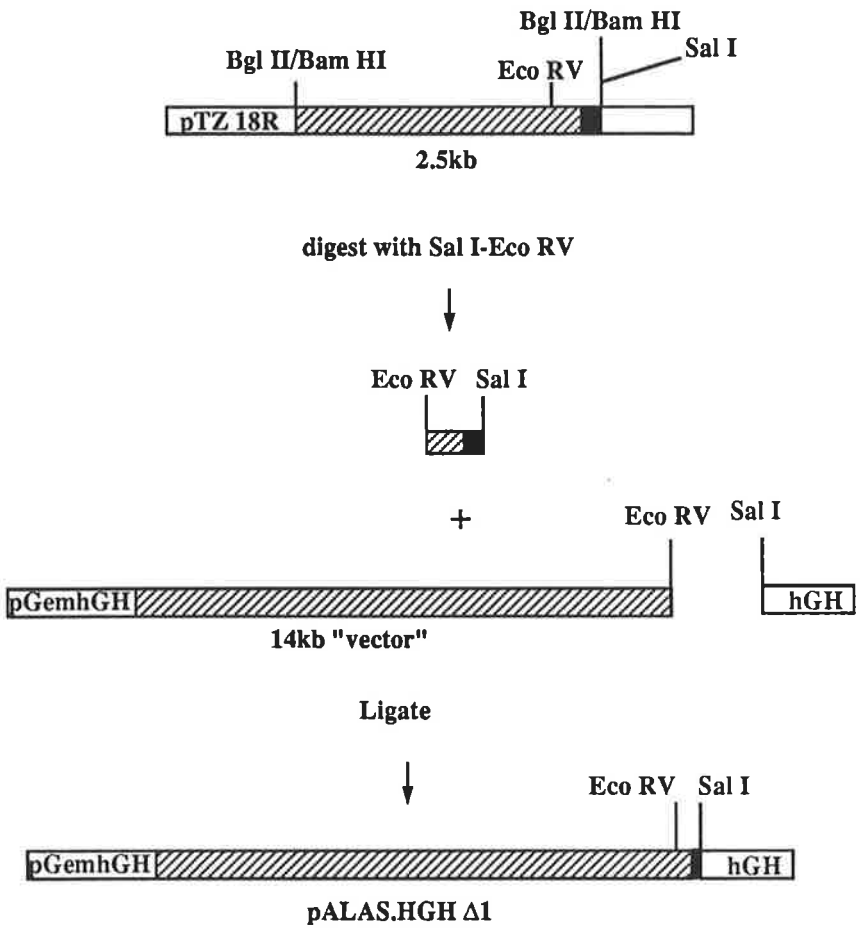


Figure 5.3 The cloning strategy used to generate the chimeric ALAS/hGH promoter constructions, pALA.hGH 1 and pALA.hGH Δ1.

(A) The 11kb Hind III fragment (Fig 5.1) cloned into the expression vector pGEMhGH and relative locations of the unique Eco RV restriction enzyme site and the Sal I site in the multiple cloning site of the pGEM vector are shown. The DNA fragments generated by a simultaneous Sa I-Eco RV restriction enzyme digest are indicated. The larger 14kb fragment was isolated and used as the "vector" to generate the promoter constructions pALA.hGH 1 and pALA.hGH Δ1

(B) The cloning strategy used to generate pALA.hGH 1 from the 1.3kb Eco RV-Hind III DNA fragment generated by *in vitro* mutagenesis (Fig. 5.2) and the 14kb Sal I-Eco RV fragment described above is shown. This strategy generated a promoter construction which contained all of the intron in the 5' flanking region of the human hepatic ALAS gene and approximately 9kb of 5' flanking region.

(C) The cloning strategy used to generate pALA.hGH Δ1 from the Bgl II DNA fragment (Fig 5.1) and the 14kb Sal I-Eco RV fragment described above is shown. This strategy generated a promoter construction which contained the first 140bp of the intron in the 5' flanking region of the human hepatic ALAS gene and approximately 9kb of 5' flanking region.

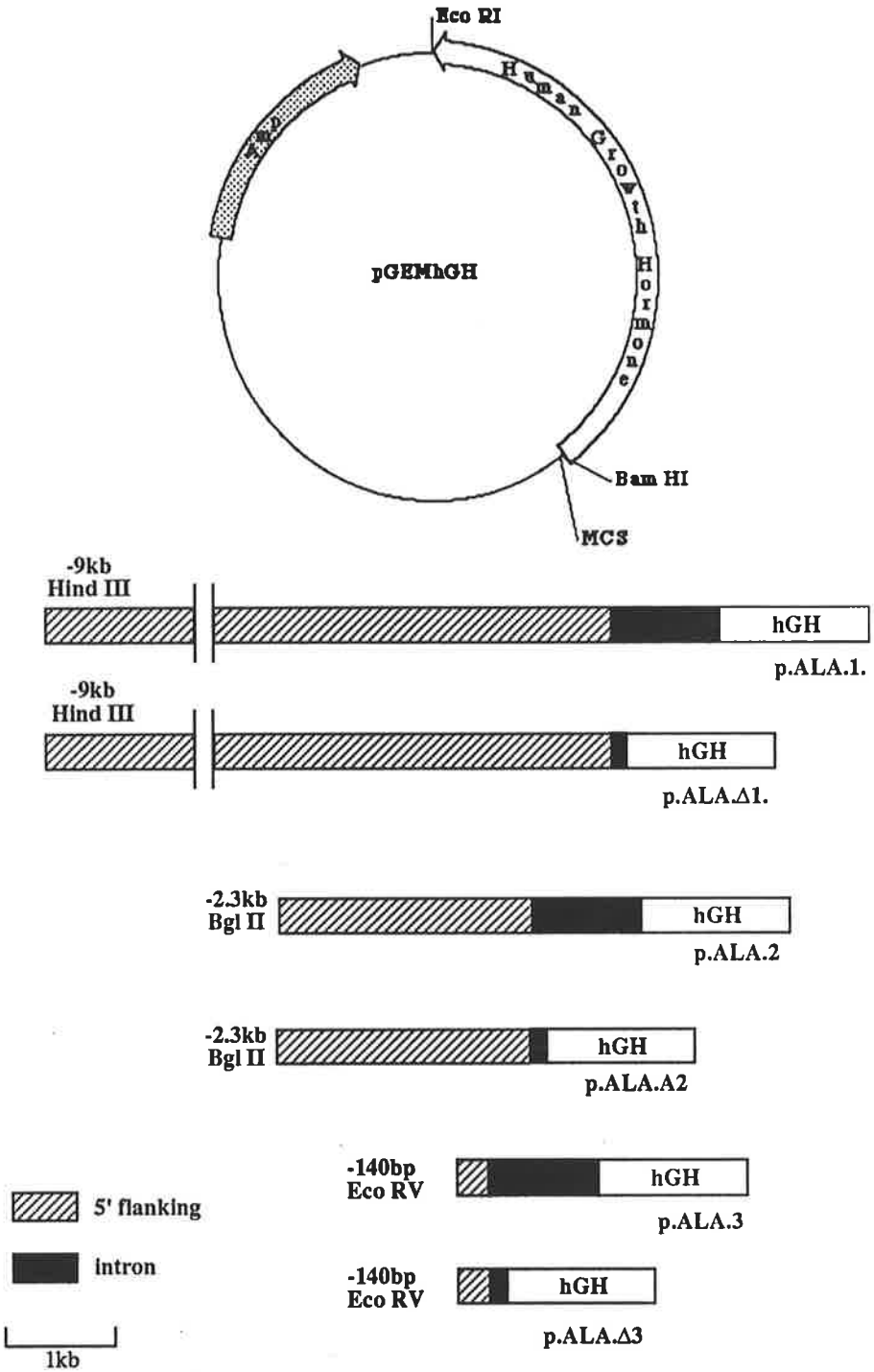


Figure 5.4 The pGEM hGH expression vector and the 6 chimeric human hepatic ALAS/hGH promoter constructions.

The pGEM hGH expression vector used as the progenitor for the chimeric human hepatic ALAS/hGH promoter constructions is shown at the top of the figure.

The six human ALAS/hGH promoter constructions are shown. Details of the construction of the ALAS/hGH chimeric plasmids is described in Section 5.2.3. The 5' flanking region of the ALAS gene is indicated by striped shading. The intron in the 5' flanking region of the gene is indicated by solid black shading. The length of 5' flanking region of the gene relative to the CAP site at +1 are shown at the 5' end of each construction.

were characterized by restriction enzyme analysis to determine the orientation of the ALAS gene sequences relative to the human growth hormone gene. Simultaneous Sal I and Eco RV restriction enzyme digestion of those clones in the orientation with the 3' end of the Hind III fragment adjacent to the 5' end of the coding sequences of the human growth hormone gene, generated the two fragments shown in Fig 5.3. The smaller 2.3kb fragment contained the ALAS gene sequences from +2kb in the coding region to -140bp in the 5' flanking region of the human hepatic ALAS gene. The larger fragment, approximately 14kb in size, contained the human hepatic ALAS gene sequences from -140bp to -9kb in the 5' flanking region of the gene, and the pGEMhGH expression vector. The larger 14kb Sal I-Eco RV fragment was isolated and used as the vector in the cloning strategies described below.

The 1.3kb Eco RV-Hind III fragment generated by the *in vitro* mutagenesis procedure described previously, which contained the ALAS gene sequences from 10bp upstream from the ATG translation initiation codon to -140bp in the 5' flanking region of the gene, was isolated as an Eco RV-Sal I fragment from the pBSSK+ plasmid vector. This fragment was then cloned into the 14kb Sal I-Eco RV vector fragment described above, which contained the 5' flanking sequences of the human hepatic ALAS gene from -140bp to -9kb and the pGEMhGH expression vector. (The cloning strategy used to generate this promoter construction is shown in the first part of Fig. 5.3). This generated an ALAS/hGH chimeric plasmid gene promoter construction, designated pALAS.hGH 1, which contained the human hepatic ALAS gene sequences extending from 10bp upstream from the translation initiation codon to the Hind III site at -9kb, and included all of the intron in the 5' untranslated region of the gene.

The corresponding ALAS/hGH chimeric plasmid gene promoter construction which contained the ALAS gene promoter sequences up to -9kb and included the first 140bp of the intron in the 5' untranslated region of the human hepatic ALAS gene, was prepared by the cloning strategy shown in the second part of Fig. 5.3. The 2.5kb Bgl II fragment which contains the human hepatic ALAS gene sequences from +240bp to -2.3kb (Fig. 5.3) had previously been cloned into the Bam HI site of PTZ 18R (which resulted in both the Bam HI and the Bgl II sites being destroyed) for sequencing and restriction mapping purposes. The 380bp Eco RV-Bgl II fragment, which contained the first 140bp of the intron and extended to

-140bp in the promoter region of the gene, was isolated as an Eco RV-Sal I DNA fragment from the PTZ 18R clone, by utilizing the Sal I site adjacent to the Bam HI site in the polylinker of the PTZ 18R vector. This fragment was then cloned into the 14kb Sal I- Eco RV vector fragment described previously, which contained the human hepatic ALAS gene sequences from -140bp to -9kb in the 5' flanking region of the gene, and the pGEMhGH expression vector. This generated an ALAS/hGH chimeric plasmid gene promoter construction, designated pALAS.hGH Δ1, which contained the human hepatic ALAS gene sequences extending from the Bgl II site at nucleotide position +240bp in the 5' untranslated region of the gene to the Hind III site at -9kb, and included the first 140bp of the intron in the 5' untranslated region of the ALAS gene.

A second pair of ALAS/hGH chimeric plasmid gene promoter constructions were prepared which contained the human hepatic ALAS gene sequences up to the Bgl II site located at -2.3kb from the transcription initiation site of the gene (Fig.5.1). The construction pALAS.hGH Δ1 described above, was digested with Bgl II. (As the Bgl II site in the intron of the ALAS gene at nucleotide position +240bp of the ALAS gene was destroyed when the fragment was cloned into the Bam HI site of PTZ 18R the only remaining Bgl II site present in the promoter construction pALAS.hGH Δ1, is the one located at -2.3kb). The Bgl II ends were end filled using *E. coli* DNA polymerase I (Klenow fragment) as described in Maniatis *et al.*, (1982), before digestion with the endonuclease Sal I, and the resulting 2.5kb Bgl II (blunted)-Sal I DNA fragment was isolated. The pGEMhGH expression vector was digested with Hind III, end filled with *E. coli* DNA polymerase I (Klenow fragment) and then digested with Sal I, to generate a Sal I-blunt ended vector, into which the 2.5kb Bgl II (blunt ended)- Sal I DNA fragment was cloned. This generated an ALAS/hGH chimeric plasmid gene promoter construction which contained the human hepatic ALAS gene sequences from the Bgl II site located at +240bp in the intron in the 5' untranslated region of the gene to the Bgl II site located at -2.3kb in the 5' flanking region of the ALAS gene sequence and included the first 140bp of intronic sequence. This chimeric plasmid gene promoter construction was designated pALAS.hGH Δ2.

The corresponding chimeric plasmid gene promoter construction, which contained the human hepatic gene promoter sequences up to -2.3kb and included all of the intron in the 5' untranslated region of the ALAS gene was generated by the following strategy. The construction pALAS.hGHΔ2 was digested simultaneously with the restriction endonucleases Sal I and Eco RV, and the vector fragment containing the 5' ALAS gene sequences from -140bp to -2.3kb and the pGEMhGH expression vector was isolated. The 1.3kb Eco RV- Hind III DNA fragment (Fig 5.2), which as described previously was generated by *in vitro* mutagenesis and contained the ALAS gene sequences from 10bp upstream from the ATG translation initiation codon, to -140 bp in the 5' flanking region of the gene, was isolated as an Eco RV-Sal I DNA fragment from the pBSSK+ vector. This fragment was then cloned into the pALAS.hGH Δ2 Sal I-Eco RV vector, to generate a promoter construction, designated pALAS.hGH 2, which included all of the intron in the 5' untranslated region of the gene and the human hepatic ALAS gene sequences up to -2.3kb. Restriction endonuclease mapping was carried out to verify the orientations of the ALAS/hGH chimeric plasmid gene promoter constructions, and to ensure that there had been no deletions of DNA, and interestingly, none of these promoter constructions had undergone deletion events.

It was of interest to compare the level of expression obtained from the immediate promoter region of the human ALAS promoter with that of the longer promoter constructions. In order to do this a pair of promoter constructions were prepared which contained the ALAS gene sequences up to the Eco RV site located at nucleotide position -140bp (Fig. 5.1). The putative TATA box sequence located at nucleotide position -28bp and the three putative GC boxes at nucleotide positions -200, -105 and +57 bp are located within this region of the gene (see Fig 5.1). The previously described Eco RV-Hind III fragment which was used to generate the promoter construction designated,pALAS.hGH 1, and contained the ALAS gene sequences from 10bp upstream from the ATG translation initiation codon to -140bp in the 5' flanking region of the gene, was isolated as an Eco RV-Sal I fragment from the pBSSK+ vector (Fig 5.3). Similarly, the 380bp Eco RV-Bgl II fragment used to generate the construction pALAS.hGH Δ1, which contained the first 140bp of the intron and extended to -140bp in the 5' flanking region of the gene, was isolated as an Eco RV-Sal I fragment from the PTZ 18R

clone (Fig. 5.3). These fragments were then cloned into the Sal I-Hinc II sites of the pGEMhGH expression vector. The chimeric promoter construction containing all of the intron in the 5' untranslated region of the gene and the ALAS gene promoter sequences up to -140bp, was designated pALAS.hGH 3,.and the corresponding construction containing only the first 140bp of intronic sequence, was designated pALAS.hGH Δ3. The orientations of these promoter constructions were confirmed by restriction endonuclease mapping and sequence analysis.

In summary, three pairs of human hepatic ALAS/hGH chimeric promoter constructions were prepared for transfection into tissue culture cell lines, and the six constructions resulting from this part of the work are shown in the lower part of Fig 5.4.

5.2.3 Northern analysis of endogenous ALAS mRNA levels in HepG2 cells.

In preliminary experiments, the basal level of endogenous ALA-S mRNA in untreated HepG2 cells cultured in different conditions was investigated. The levels of ALA-S mRNA in cells grown in standard conditions, of DMEM supplemented with 10% foetal calf serum, was found to be very low and since heme present in the foetal calf serum, may contribute to repression of ALA-S mRNA, several other serum substitutes were tried including Opti-MEM®I and Nu serum. The highest level of ALA-S mRNA was seen in cells grown in 8% Nu serum plus 2% foetal calf serum, where the levels of ALAS mRNA were four times higher than in cells grown under standard conditions (compare Fig. 5.5, lanes 1 and 5). Attempts to grow HepG2 cells without any foetal calf serum present were unsuccessful and 2% foetal calf serum was the minimum requirement to allow cell attachment.

The concentration of hemin required to achieve maximal repression of ALA-S mRNA levels was determined by treating the cells with a final concentration of 1, 5 and 10μM hemin respectively for 24 and 48 hours. As can be seen in Fig 5.6, there was no difference in the levels of hepatic ALAS mRNA when the cells were treated with 1, or 5μM hemin for 24 hours. Following treatment of the cells with 10μM hemin for 24 hours, there was a decrease in the level of ALAS mRNA present in the cells, and maximal repression was achieved when the cells were treated with 10μM hemin for 48 hr (see Fig. 5.6, lane 6).

Figure 5.5 Northern blot analysis of hepatic ALAS mRNA levels in HepG2 cells.

Total RNA was prepared from HepG2 cells grown under standard conditions in 10% foetal calf serum or in 8% Nu™ serum 2% foetal calf serum. Cells were either untreated or treated for 48hr with a final concentration of 10μm hemin, 100μm ALA, or 1mM succinyl acetone. 50μg of each total RNA sample was electrophoresed on a 1% agarose gel, containing 1.1M formaldehyde. Following Northern transfer to nitrocellulose (Section 2.3.3) the filter was probed with the ^{32}P-labelled human hepatic ALAS cDNA probe, hHcDNA (Section 2.2.14). The filter was exposed to autoradiography and the autoradiograph from this experiment is shown.

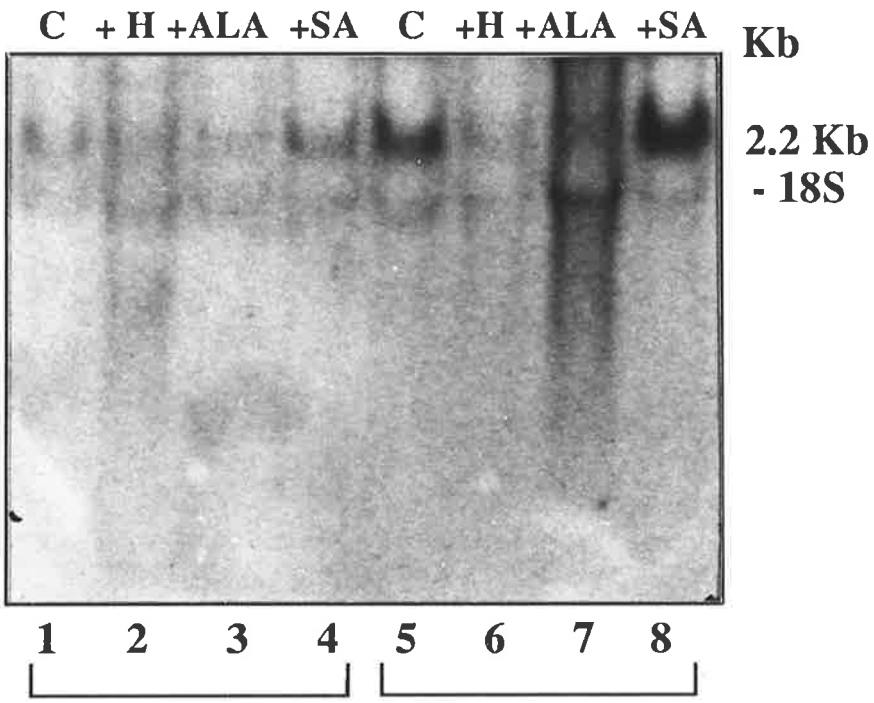
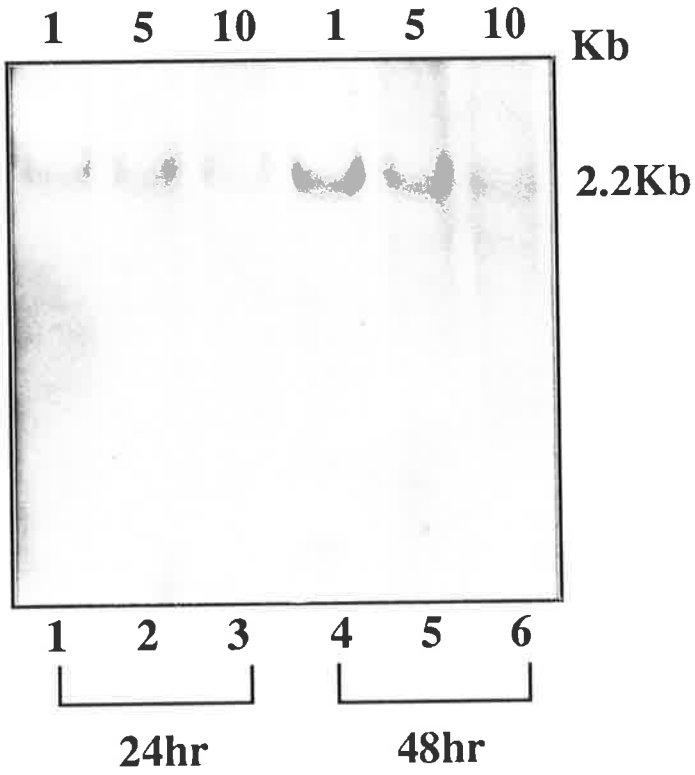

C
+H
+ALA
+SA
C
+H
+ALA
+SA
Kb
2.2 Kb
- 18S
1
2
3
4
5
6
7
8
10% Foetal Calf Serum.
8% Nu Serum
2% Foetal Calf Serum

Figure 5.6 The effect of hemin concentration and time on ALAS mRNA levels in HepG2 cells.

HepG2 cells were grown in 8% Nu™ serum 2% foetal calf serum and were treated with a final concentration of either 1, 5, or 10mM hemin for 24 hr lanes 1-3, or 48 hr lanes 4-6. RNA was then prepared from the cells and 50μg of total RNA was analysed per track.

Heme Treatment (uM)



Cells grown under standard conditions for 48 hr were treated with a final concentration of 10μM hemin, the heme precursor ALA at a final concentration of 100μM, or succinyl acetone at 1mM final concentration; at these concentrations it is known that ALA-S enzyme activity levels are repressed by hemin and ALA and increased by succinyl acetone (Iwasa *et al.*, 1989). Northern analysis of mRNA from treated and untreated cells indicated that the levels of ALAS mRNA were lowered by treatment of the cells with hemin or ALA (compare Fig. 5.5, lane 1 with lanes 2 and 3) and increased by treatment with succinyl acetone (compare Fig.5.5, lanes 1 and 4) although accurate quantitation of the ALAS mRNA levels was difficult because of the low levels present.

When cells were cultured in 8% Nu™ serum and 2% foetal calf serum the effects of hemin, ALA and succinyl acetone on ALAS mRNA levels were clearly seen. Hemin at a final concentration of 10μM strongly reduced levels of ALAS mRNA (compare Fig.5.5, lanes 5 and 6) and a similar result was seen with ALA (Fig. 5.5, lane 7). As can be seen in Fig. 5.5, lane 8, treatment of the cells with succinyl acetone (1mM) gave approximately a two fold increase in ALA-S mRNA levels. The levels of ß-actin mRNA were not affected by these treatments (data not shown). From this preliminary work, it was concluded that the HepG2 cell line was suitable for examining expression of the ALAS promoter constructions. As the levels of ALAS mRNA in cells grown in 8% Nu™ serum and 2% foetal calf serum were significantly higher than in cells grown in 10% foetal calf serum (compare Fig 5.5 lanes 1 and 5) the cells used throughout the rest of this study were routinely grown in DMEM supplemented with 8% Nu™ serum and 2% foetal calf serum.

5.2.4 Optimization of conditions for transfection of the human hepatoma HepG2 cell line.

A large number of methods are available for the introduction of recombinant DNA molecules into mammalian cells *in vitro*, including protoplast fusion, co-precipitation of DNA with either DEAE dextran or calcium phosphate, and various refinements to these basic techniques. However the efficiency of transfection by these methods varies widely between different cell lines and individual experiments, and in many cases can be unacceptably low

(Novak and Rothenberg, 1986; Tur-Kaspa *et al.*, 1986; Maxwell and Maxwell, 1988). The technique of electroporation involves the exposure of cultured cells to a pulsed electric field, which presumably creates transient pore formation in the cell membranes and facilitates the uptake of DNA molecules. Electroporation has been used successfully to introduce DNA into a wide variety of both plant and animal cells in an extremely reproducible fashion (Chu *et al.*, 1987; Maxwell and Maxwell, 1988). Moreover, it had previously been shown in this laboratory that the electroporation method was the most efficient transfection method for introducing rat hepatic ALAS/CAT chimeric plasmids into a rat hepatoma cell line (Loveridge, 1988) Therefore, electroporation as a transfection method for the introduction of the ALAS/hGH chimeric plasmids into HepG2 cells was investigated.

In preliminary experiments, the positive control vector pSVhGH with the human growth hormone gene under the control of the SV40 early promoter was employed to investigate the parameters for transfection of HepG2 cells by electroporation. Voltages ranging from 150-400, at capacitances of 500 and 960 were tested. It was found that electroporation was most efficient at 220V at a capacitance of 960C, and these conditions were used throughout this study. A time course of expression of the positive control vector pSVhGH in HepG2 cells, established that hGH was detectable in the media 18 hours after electroporation and the accumulation of secreted hGH increased over 96 hours.

While optimizing the conditions for electroporation of HepG2 cells it was found that HepG2 cells require the presence of carrier DNA during electroporation. This effect of DNA concentration on transfection efficiency has been reported for a number of other cell lines (Chu *et al.*, 1987; Potter *et al.*, 1984). Using voltages ranging from 150-400, at capacitances of 500 and 960 it was found that when carrier DNA was omitted, cell viability decreased to less than 10% and expression of hGH from the positive control vector pSVhGH was not detectable. Optimal expression from pSVhGH was obtained when electroporation was carried out in the presence of 500μgs/ml of sonicated salmon sperm DNA. The nature of the carrier nucleic acid was found to be important, since the addition of *E. coli* DNA or tRNA led to a decline in hGH activity of 25% and 75% respectively.

Comparison of the levels of hGH expression HepG2 cells obtained from the positive control vector pSVhGH and the ALAS/hGH promoter constructions established that when the cells were grown in DMEM supplemented with 10% foetal calf serum after electroporation, the hGH signal was barely detectable above background. However, when the cells were grown in 8% Nu™ serum and 2% foetal calf serum, the levels of expression were at least four fold higher. Therefore, cells were routinely grown and plated in DMEM supplemented with 8% Nu™ serum and 2% foetal calf serum.

As it was intended to use transient expression studies to compare expression of the ALAS constructions it was important to use an internal control plasmid to normalize for variations in transfection efficiency between experiments. The pCH 110 plasmid which has the gene coding for ß-galactosidase (ß-gal) activity under the control of the SV40 early promoter was obtained from Pharmacia for this purpose. It was important to establish that co-transfection of the plasmid pCH 110 with the ALAS/hGH promoter constructions did not result in competition between the experimental and normalization plasmids for the factors required for gene expression. Competition experiments were carried out in which increasing amounts of the pSVhGH positive control vector, or the ALAS/hGH promoter constructions were co-transfected with a constant amount (5µgs) of the plasmid pCH 110. Up to a total of 30µgs of total DNA was co-transfected with a linear increase in human growth hormone expression (data not shown) and it was concluded that at these concentrations of transfected DNA there was no competition between the plasmids for the factors required for gene expression.

5.2.5 Expression of the ALAS/hGH chimeric plasmids in the human hepatoma HepG2 cell line.

The six human ALAS/hGH constructions, shown in the lower part of Fig. 5.4, were transfected by electroporation into the human hepatoma cell line HepG2 cells (Section 2.5.2). The cells were plated and left to recover for 24 hours before the media was changed. The levels of hGH secreted into the media after a further 24 hours were determined (Section 2.5.4), and the cells were harvested and assayed for ß-gal activity (Section 2.5.5). The levels of human growth hormone expression generated by the expression vector pGEMhGH were determined

and were found to be less than 0.05% of pSVhGH expression. In addition, the levels of expression due to cross-reactivity with any bovine growth hormone present in the growth media were determined and deducted from each experimental value. The levels of hGH generated by each of the ALAS promoter constructions were normalized to ß-gal activity, and the levels of expression generated by each of the ALAS/hGH promoter constructions in four separate experiments, and the standard deviation between experiments, are summarized in Fig 5.7. In Fig. 5.7, the ALAS promoter constructions containing all of the intron in the 5' untranslated region of the gene are represented by dark shading, and those with only the first 140bp of intronic sequence are lightly shaded.

The results shown in Fig 5.7 demonstrate that the minimum sequence requirement for transcription of the ALAS gene in HepG2 cells resides in the promoter construction pALAS.hGH Δ3, which contains the first 140 bp of the intron in the 5' untranslated region of the gene and extends upstream to -140bp from the transcription initiation site. In addition, this construct generated the highest level of expression seen for any of the promoter constructions. As can be seen in Fig 5.7, the levels of expression generated by the ALAS/hGH promoter constructions vary considerably. Although the highest level of expression was clearly generated by the construct pALAS.hGH Δ3, the construction pALAS.hGH Δ2, which contains the ALAS gene sequences up to -2.3kb, also expressed quite strongly with approximately 80% the efficiency of pALAS.hGH Δ3. In contrast, the two promoter constructs pALAS.hGH 1 and pALAS.hGH Δ1, which contained the ALAS gene sequences up to -9kb both generated a much lower level of expression.

Interestingly, in all three pairs of promoter constructions the deletion of the intron (except for the first 140bp) in the 5' untranslated region of the human hepatic ALAS gene resulted in a significant increase in the levels of expression generated by the ALAS gene sequences (Fig 5.7). The effect of the intron is most evident when the levels of expression generated by shortest pair of promoter constructions are compared (Fig 5.7). The expression generated by the promoter construction pALAS.hGH Δ3, which has only the first 140bp of intronic sequence is 150% higher than the corresponding construction pALAS.hGH 3, which contains all of the intron. While the effect is not as dramatic, the influence of the intron is

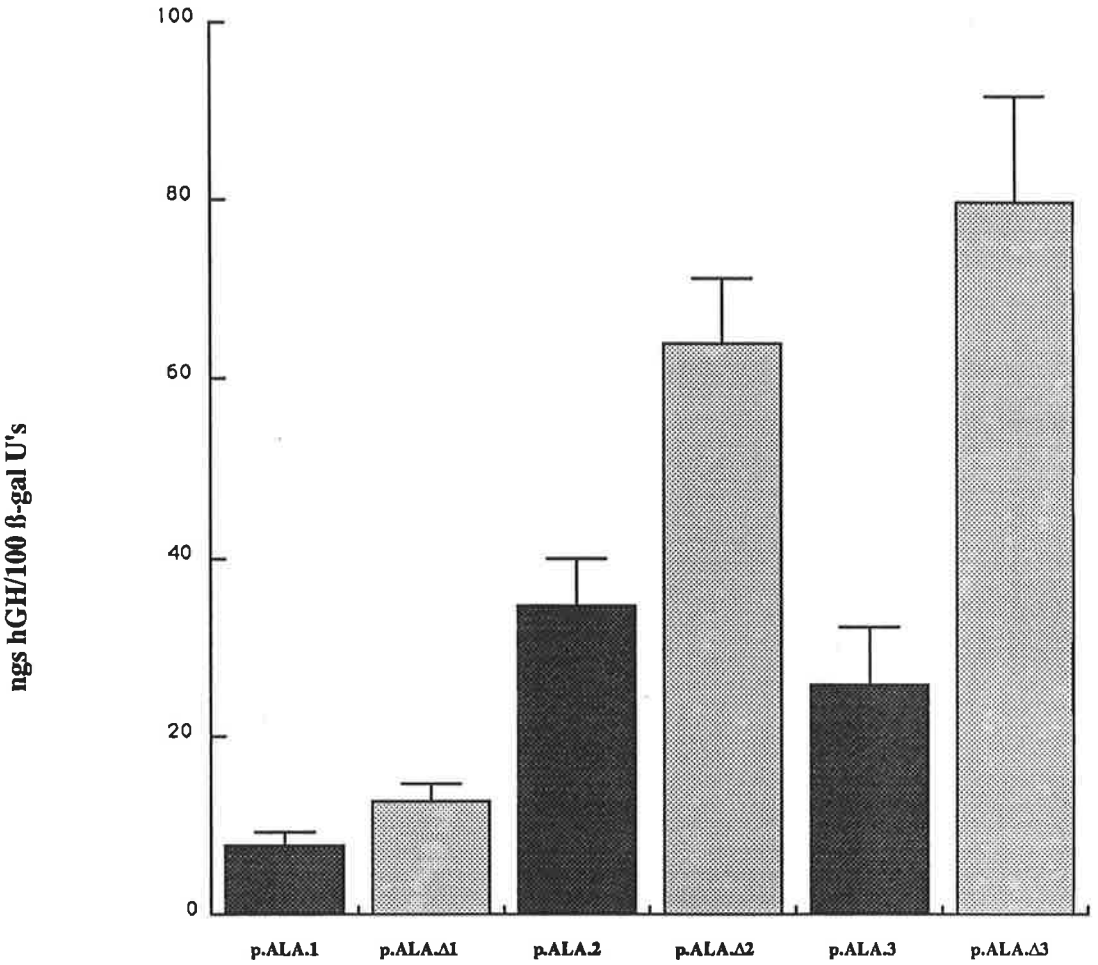


Figure 5.7 Expression of chimeric human hepatic ALAS/hGH promoter constructions in the human hepatoma cell line HepG2.

The human hepatic ALAS promoter constructions described in Section 5.2.3 and shown in Fig. 5.4, were co-transfected with pPCH 110, which has the gene for ß-gal activity under the control of the SV40 promoter, by electroporation (Section 2.5.2) into HepG2 cells. The growth media was assayed for human growth hormone (Section 2.5.4) and 50µl of the cell lysate was assayed for ß-gal activity (Section 2.5.5). The levels of human growth hormone generated by each of the ALAS/hGH constructions, in four separate experiments and the standard deviation between these experiments is expressed as ngs of human growth hormone/100 ß-gal units. The ALAS/hGH promoter constructions containing all of the intron in the 5' untranslated region of the gene are represented by the dark shading, and the constructions containing only the first 140bp of intronic sequence are lightly shaded.

clearly seen when the levels of expression generated by the other two pairs of promoter constructions are compared. As can be seen in Fig.5.7, the level of expression generated by pALAS.hGH Δ2 is 50% higher than pALAS.hGH 2, while that generated by pALAS.hGH Δ1 is 30% higher than pALAS.hGH 1. These results demonstrate that at least in the human hepatoma HepG2 cell line the intron in the 5' untranslated region of the human hepatic ALAS gene is able to modulate the expression generated by the ALAS gene sequences. In addition, since the ALAS promoter constructions which contained the first 140bp of intronic sequence gave the highest levels of expression, the presence of the 5' splice site of the intron in these constructions does not appear to be interfering with the production or secretion of active human growth hormone protein.

5.2.6 The effect of hemin, ALA and succinyl acetone on expression of the ALAS/hGH chimeric constructions in the human hepatoma HepG2 cell line.

As discussed previously, nuclear transcriptional run-on experiments in rats had demonstrated that the rat hepatic ALAS gene is regulated at the transcriptional level by hemin administration (Srivastava *et al.*, 1988). The experiments described in Section 5.2.3, demonstrated that the endogenous levels of hepatic ALAS mRNA in HepG2 cells were modulated by treatment of the cells with hemin, ALA and succinyl acetone (Fig. 5.5). Now that the basal levels of expression generated by the chimeric human hepatic ALAS/hGH promoter constructions had been determined (Fig 5.7), it was of interest to determine whether the human hepatic ALAS/hGH promoter constructions were responsive to the addition of added hemin, ALA and succinyl acetone.

The six human ALAS/hGH constructions shown in the lower part of Fig. 5.4, were transfected by electroporation into the human hepatoma cell line (Section 2.5.2). The cells were divided into duplicate plates and left 24 hours to recover before the media was changed. Cells were then grown in the presence or absence of 10μM hemin for 24 hours, and the media assayed for hGH. The levels of expression of hGH generated by all six ALAS promoter constructions were the same either in the presence of absence of hemin.

To determine whether an effect of hemin was detected at either an earlier or later time point, the experiments were repeated. Cells were transfected and divided into duplicate plates and left 24 hours to recover, after which time the cells were grown in the presence or absence of 10μM hemin. The media were assayed for hGH at time points 2, 4, 6, 8, 12, and 48 hours following the addition of hemin. The presence of hemin did not affect the levels of expression of the ALAS/hGH promoter constructions measured at any of these time points. Using the protocol described above, in which the cells were left for 24 hours after transfection to recover before changing the media and adding reagents, it was found that the levels of expression of the promoter constructions were unaffected by growing the cells in the presence of 100μM of either ALA or the methyl ester of ALA for 4 or 24 hours. Similarly, the presence of 1mM succinyl acetone in the media for 4 or 24 hours did not alter the levels of expression of the ALAS/hGH constructions (data not shown).

5.2.7 Expression of the ALAS/hGH chimeric plasmids in the chinese hampster ovary (CHO) cell line.

CHO cells have two advantages over HepG2 cells. Firstly, they can survive in growth media for a limited period of time (2-3 days) without any foetal calf serum present, and secondly they can be electroporated without using carrier DNA. The six human hepatic ALAS/hGH constructions, shown in the lower part of Fig. 5.4, were transfected by electroporation into CHO cells (Section 2.5.3). The cells were plated and left to recover for 24 hours before the media was changed. After a further 24 hours, the levels of hGH secreted into the media were determined (Section 2.5.4), and the cells were harvested and assayed for ß-gal activity (Section 2.5.5). The levels of hGH generated by each of the ALAS promoter constructions were normalized to ß-gal activity. The results of three separate transfection experiments and the standard deviation between experiments are summarized in Fig 5.8. In Fig. 5.8, the ALAS promoter constructions containing all of the intron in the 5' untranslated region of the gene are represented by dark shading, and those with only the first 140bp of intronic sequence are lightly shaded.

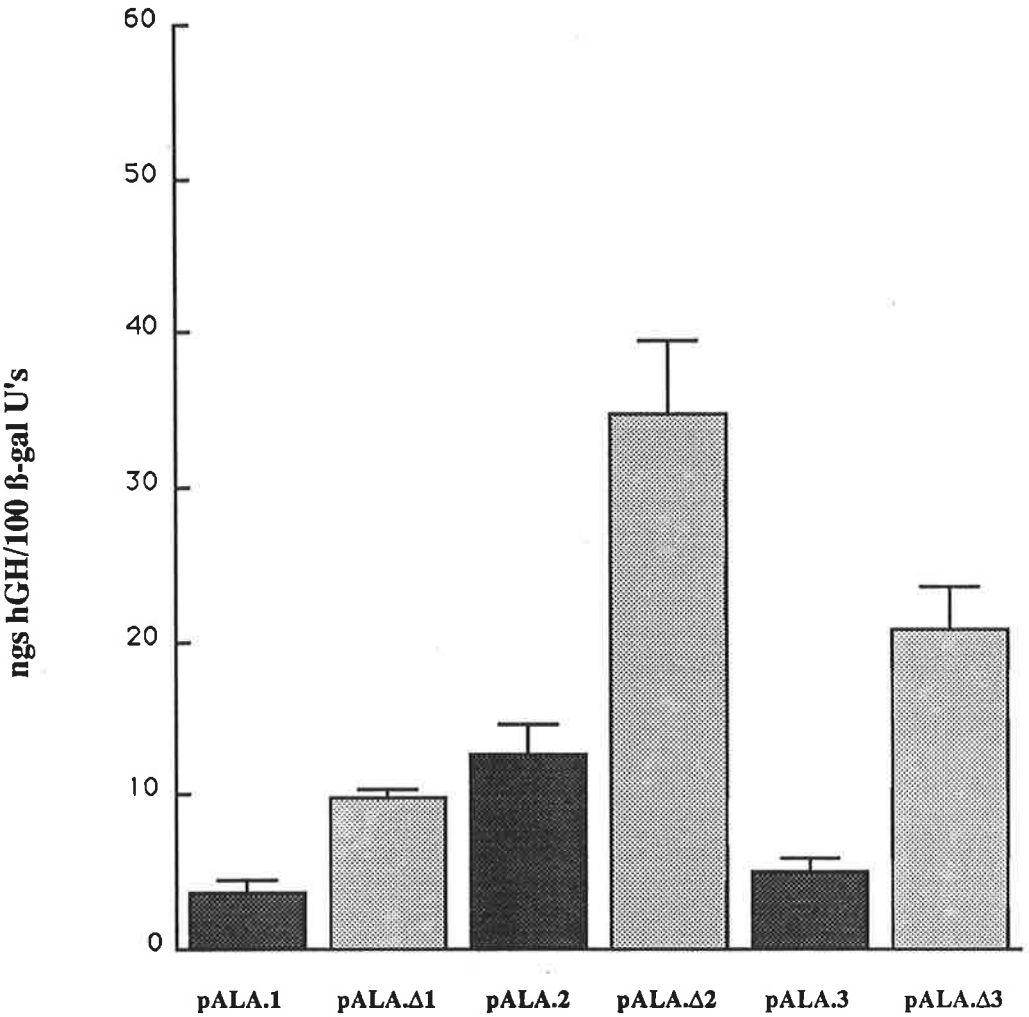


Figure 5.8 Expression of chimeric human hepatic ALAS/hGH promoter constructions in the chinese hampster (CHO) cell line.

The human hepatic ALAS promoter constructions described in Section 5.2.3 and shown in Fig. 5.4, were co-transfected with pPCH 110, which has the gene for ß-gal activity under the control of the SV40 promoter, by electroporation (Section 2.5.3) into CHO cells. The growth media was assayed for human growth hormone (Section 2.5.4) and 50µl of the cell lysate was assayed for ß-gal activity (Section 2.5.5). The levels of human growth hormone generated by each of the ALAS/hGH constructions, in three separate experiments and the standard deviation between these experiments is expressed as ngs of human growth hormone/100 ß-gal units. The ALAS/hGH promoter constructions containing all of the intron in the 5' untranslated region of the gene are represented by the dark shading, and the constructions containing only the first 140bp of intronic sequence are lightly shaded.

The levels of expression of the human hepatic ALAS promoter constructions in CHO cells differs from that seen in the HepG2 cells in that in these cells the highest level of expression was generated by the construction pALAS.hGH Δ2. The construction pALAS.hGH Δ3 in CHO cells was 60% as efficient as pALAS.hGH Δ2. The lowest levels of expression were again generated by the constructions containing the ALAS gene sequences up to -9kb. Interestingly, in the CHO cell line the deletion of the intron (except for the first 140bp) from the promoter constructions had a similar effect as seen in the HepG2 cell line; again the levels of expression generated by the promoter constructions with the first 140 bp of intron sequence was significantly higher than the corresponding constructions containing all of the intron. The effect of the intron was most evident when the levels of expression of the shortest pair of promoter constructions were compared (Fig. 5.8). The levels of expression of the construction pALAS.hGH Δ3, which has only the first 140bp of the intronic sequence present, was 75% higher than the corresponding construction pALAS.hGH 3, which contains the entire intron in the 5' untranslated region of the human ALAS gene. The influence of the intron was also evident when the levels of expression generated by the other two pairs of promoter constructions were compared. The level of expression generated by pALAS.hGH Δ2 was 65% higher than that generated by pALAS.hGH 2, and pALAS.hGH Δ1 was 62% higher than pALAS.hGH Δ1. These results confirm those obtained in the HepG2 cell line and support the proposal that the intron in the 5' untranslated region of the gene is important in the regulation of expression of the ALAS promoter sequences at least *in vitro*, in these two transformed cell lines

The effects of hemin, ALA, and succinyl acetone on the expression of the ALAS promoter constructions in this cell line were also examined. The levels of expression of hGH driven by the ALAS promoter sequences were apparently unaltered when cells were grown in the presence or absence of these compounds for time periods ranging from 4 to 48 hours. Similarly, the addition of 10^{-6}M dexamethasone did not affects the levels of expression of the ALAS promoter sequences (data not shown). These experiments were also carried out using serum free media but again the addition of hemin, ALA or succinyl acetone did not affect the levels of expression generated by the human hepatic ALAS promoter constructions.

Two conclusions can be drawn from these expression studies. Firstly, the intron in the 5' untranslated region of the human hepatic ALAS gene is able to modulate expression of the ALAS promoter constructions, at least in the two cell lines used in this work. Secondly, in these cell lines heme is not having an effect on the levels of expression generated by the human hepatic ALAS promoter constructions. One discordant feature of this work is the apparent regulation of the levels of endogenous ALAS in the human hepatoma cell line by hemin, ALA and succinyl acetone as determined by Northern analysis (Section 5.2.1) and this question is addressed in the following Section

5.2.8 Isolation of nuclei from HepG2 cells and synthesis of [^{32}P] transcripts *in vitro*.

As mentioned previously, the accumulation of mRNA may be regulated by altering the rate of transcription of the gene, and/or by regulation at one or more post-transcriptional steps (Ross, 1989). To determine whether the changes detected by Northern analysis, in the levels of endogenous hepatic ALAS mRNA in HepG2 cells, following treatment of the cells with hemin, ALA and succinyl acetone (Section 5.2.) were due to changes in the rate of transcription of the ALAS gene, nuclear transcription run-on experiments were carried out. As a control, the transcription rate of the 18S ribosomal gene which was not expected to be transcriptionally regulated by hemin or the other reagents used was also determined. The 18S gene was used in preference to ß-actin because preliminary work established that the levels of ß-actin mRNA in HepG2 cells was very low.

A relatively pure preparation of nuclei is an essential prerequisite for nuclear run-on transcription assays. Initially, the procedure used to isolate nuclei from HepG2 cells was a modification of the procedure described by Schibler *et al.*, (1983) which had previously been employed in this laboratory for the isolation of chick embryo and rat liver nuclei. However, the nuclei isolated by this procedure tended to clump particularly at the stage when they were resuspended for the elongation part of the procedure. This problem was largely overcome by using the method described by Ausubel *et al.*, (1989) detailed in Section 2.4.1, which replaces Triton with NP-40 in the cell lysis buffer.

In order to determine whether nuclei isolated by the above procedure were able to support *in vitro* transcription a time course of [^{32}P] rUTP incorporation by the isolated nuclei was carried out according to the procedures detailed in sections 2.4.2 and 2.6.1. The time course also established the period of time over which the incorporation of [^{32}P] rUTP was linear. Since the purpose of carrying out the transcription run-ons was to monitor any changes in transcription of the ALA-S gene in hemin treated cells, it was important to compare the time courses of [^{32}P] rUTP incorporation in nuclei isolated from both treated and untreated cells and these are shown in Fig. 5.9. As can be seen in Fig 5.9, the incorporation of [^{32}P] rUTP was similar in nuclei from both treated and untreated cells, and was linear for 30 minutes after which time incorporation started to decline. In subsequent experiments nuclei were incubated for 25 minutes, which allowed sufficient [^{32}P] mRNA synthesis, while at the same time minimized the opportunity for degradation of the mRNA.

The fungal peptide a-amanitin present at low concentrations (0.5-2μg/ml) in the *in vitro* transcription reactions specifically inhibits RNA polymerase II activity (Marzluff and Huang, 1984). To determine the contribution of RNA polymerase II to the total transcriptional activity *in vitro* α-amanitin (2μg/ml) was added to the nuclei from untreated cells and a time course of [^{32}P] rUTP incorporation assayed. As shown in Fig.5.9 the α-amanitin inhibited total [^{32}P] rUTP incorporation by approximately 71%, as measured by the TCA precipitable radioactivity. This figure is in agreement with other reports of the RNA polymerase II directed component of total transcription *in vitro* which has been found to occur in the range of 50-90%, depending on the cell type (Marzluff and Huang, 1984). These results indicate that the observed increase in [^{32}P] rUTP incorporation following incubation of the HepG2 nuclei is largely due to RNA polymerase II dependent gene transcription.

5.2.9 Quantitative hybridization of specific [^{32}P] labelled RNA transcripts.

In order to determine the relative transcription rates of the ALAS and 18S ribosomal genes it was first necessary to demonstrate that hybridization of [^{32}P] labelled RNA to filter-bound complementary DNA probes was quantitative.

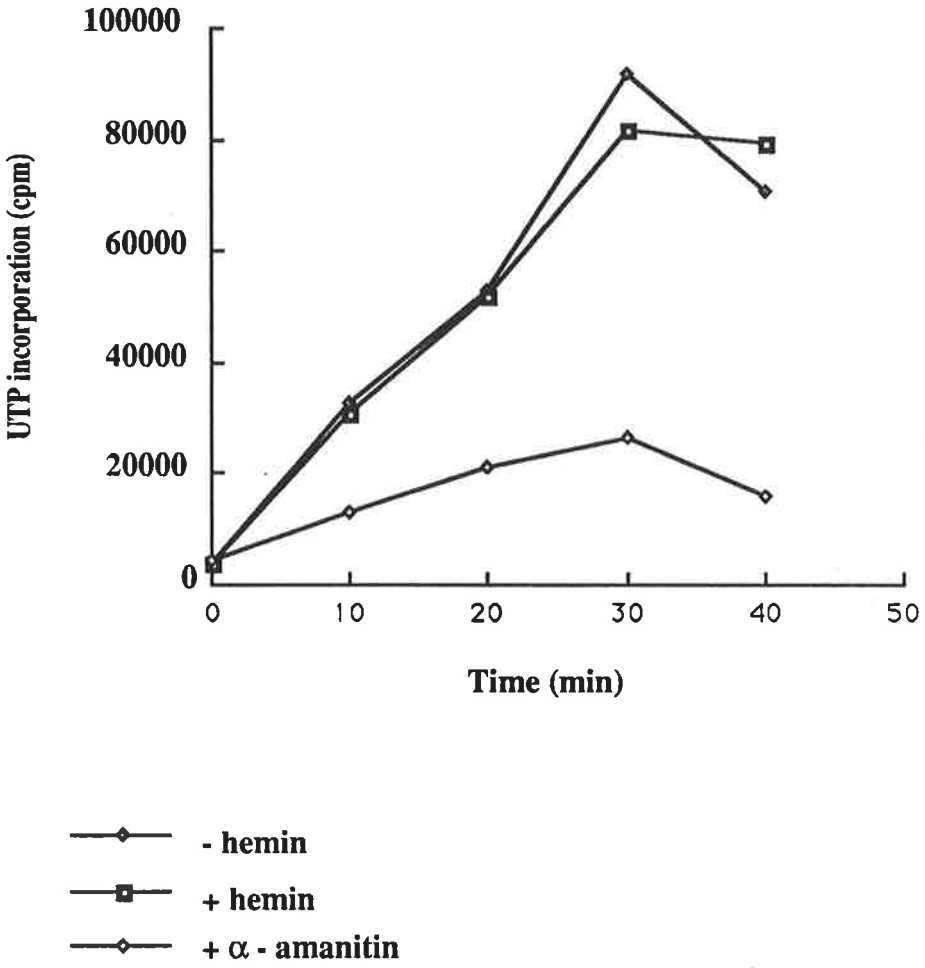


Figure 5.9 Time course of [^{32}P] RNA synthesis in nuclei isolated from HepG2 cells.

The time course of [^{32}P] labelled RNA synthesis using nuclei isolated from HepG2 cells which were either untreated or had been treated with 10μm hemin for 24 hours is shown. A time course of RNA synthesis was also measured in nuclei from HepG2 cells with a-amanitin (2μg/ml) added to the transcription reaction. Aliquots were removed in triplicate at 0, 10, 20, 30 and 40 minutes and TCA precipitable radioactivity was determined as described in Section 2.6.1.

To determine the linearity of the hybridization assay, nuclei were isolated from HepG2 cells and [^{32}P] RNA was synthesized *in vitro* (Section 2.4.1-3). The autoradiographs of the signals obtained by the addition of increasing amounts of [^{32}P] RNA to hybridization reactions with a fixed amount (5µgs) of filter bound DNA (Section 2.4.4) are shown in Fig 5.10A. Based on these results, between 5 and 6 x 10^6 cpm of [^{32}P] RNA were routinely used in further transcription assays.

For quantitative hybridization of the RNA, the complementary DNA employed in hybridization reactions with the [^{32}P] RNA must be present in excess. This was demonstrated by blotting 2, 5, and 10µgs respectively of plasmid DNA onto different filters which were then hybridized to 5 x 10^6 cpm of [^{32}P] RNA derived from nuclei isolated from HepG2 cells. As can be seen in Fig.5.10B, there was no increase in signal intensity between filters containing 5 and 10 µgs of plasmid DNA. 5µgs of plasmid DNA was routinely used in the transcription assay results presented in this chapter.

An additional experiment was performed to confirm that the hybridization reaction was driven to completion. Following 72 hours of hybridization with filter-bound DNA, the hybridization mix was then re-hybridized for a further 72 hours to a fresh filter, to determine whether any unbound [^{32}P] RNA remained. The residual signals were estimated to be less than 10% of those from the first round of hybridization, indicating that approximately 90% of the available transcripts of interest hybridize under the conditions used in these experiments (results not shown).

In these experiments there was no detectable non-specific hybridization to the plasmid vector pBSKS$^-$, which was the vector containing the ALAS probe used in these experiments.

5.2.10 The effect of hemin, ALA and succinyl acetone on the transcription rate of the hepatic ALAS gene in HepG2 cells.

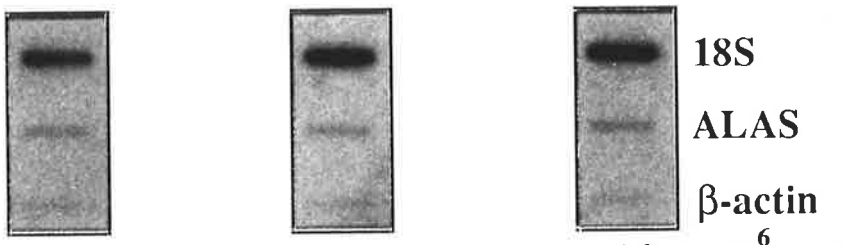
The relative transcription rates of the hepatic ALAS and 18S ribosomal genes were determined, using the conditions outlined in Section 5.2.6, in nuclei isolated from HepG2 cells which were either untreated or grown in the presence of 10µM hemin for 4, 24 or 48 hours. As

Figure 5.10 Quantitative hybridization of [^{32}P] RNA synthesis in nuclei isolated from HepG2 cells.

(A) [^{32}P] labelled RNA synthesized in nuclei from untreated HepG2 cells was isolated as described in Section 2.4.3, and the indicated amounts of TCA-precipitable radioactivity (Section 2.6.1) hybridized with filters loaded with 5μg of each of the indicated plasmid cDNAs. The autoradiographs from this experiments are shown.

(B) Filters were loaded with either 2, 5, or 10 μg of the indicated plasmid cDNAs according to the procedure described in Section 2.4.4. The filters were hybridized with [^{32}P] labelled RNA (5 x 10^6 cpm) synthesized in nuclei isolated from untreated HepG2 cells. The autoradiographs from this experiment are shown.

A



3.0 5.0 6.0 (x 10^6 cpm)

B

2 5 10

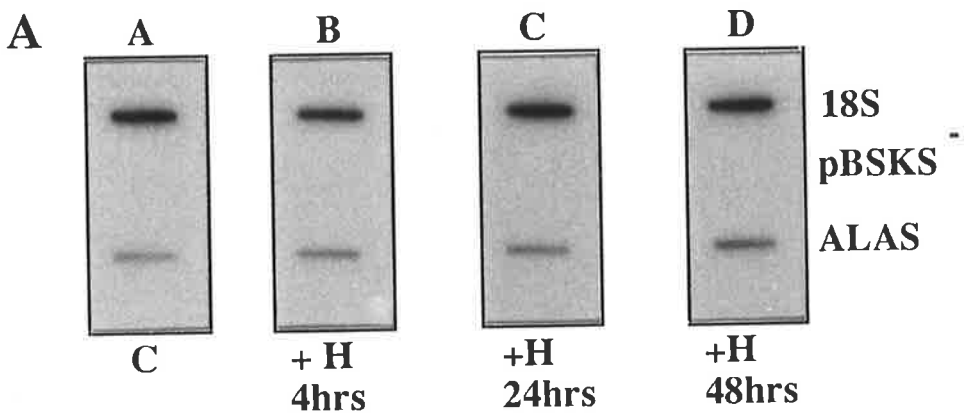
Amount of filter-bound plasmid DNA (μgs)

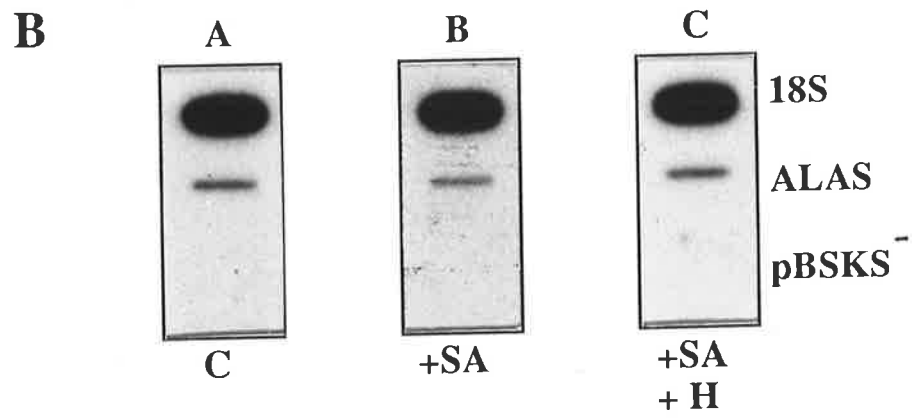
Figure 5.11 The effect of added hemin, ALA and succinyl acetone on the transcription rate of the endogenous hepatic ALAS gene in HepG2 cells.

(A) Nuclei were isolated from cells which were either untreated (panel A) or had been treated with a final concentration of 10μm hemin for 2, 4 and 24 hr (panels B-D) and allowed to transcribe *in vitro*. [^{32}P] labelled RNA was isolated (Section 2.4.3) and hybridized to filters loaded with 5μg of the 2.5kb Eco RV-Hind III genomic ALAS fragment (Fig.5.2), 5μg of vector DNA (pBS) and 0.5μg of 18S ribosomal cDNA.

(B) Nuclei were isolated from cells which were untreated (panel A) or had been treated with a final concentration of 1mM succinyl acetone (panel B) or 1mM succinyl acetone and 10μm hemin (panel C) for 4 hours. The nuclei were allowed to transcribe *in vitro*, and the [^{32}P] labelled RNA was isolated (Section 2.4.3) and hybridized to filters loaded with the same amounts of plasmid DNA described above.

The autoradiographs from these experiment are shown.


A
A
B
C
D
18S
pBSKS
ALAS
C
+ H
4hrs
+H
24hrs
+H
48hrs


B
A
B
C
18S
ALAS
pBSKS
C
+SA
+SA
+ H

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can be seen in Fig 5.11A treatment of the cells with hemin for these time periods did not produce the expected decrease in the transcription rate of the hepatic ALAS gene. A similar result was obtained when the cells were treated with the ALA at 100μM for 2, 4 or 24 hours (results not shown).

It had previously been shown that treatment of HepG2 cells with 0.5mm succinyl acetone for 4 hours resulted in an increase of ALAS activity of 85% (Iwasa *et al.*, 1989). To determine whether succinyl acetone could increase the transcription rate of the hepatic ALAS gene in HepG2 cells, nuclei were isolated from untreated cells, or from cells treated with either succinyl acetone, or succinyl acetone plus 10μM hemin for 4 hours. The results of these experiments presented in Fig 5.11B, show that the transcription rate of the hepatic ALAS gene was unaffected by either of these treatments.

The results of the nuclear run-on experiments demonstrate quite clearly that the transcription rate of the hepatic ALAS gene in HepG2 cells is not influenced by hemin, ALA or succinyl acetone. Therefore, the changes in the levels of ALAS mRNA in HepG2 cells following treatment with these reagents, which were readily detected by Northern analysis, must be the result of a post-transcriptional mechanism(s).

5.3 DISCUSSION

5.3.1 Transcriptional control of ALAS mRNA synthesis in HepG2 cells.

Nuclear run-on experiments with isolated hepatic cell nuclei had previously established that the effects of hemin administration on hepatic ALAS mRNA levels in rats are predominately due to changes in the rate of transcription of the ALAS gene (Maguire, 1987; Srivastava *et al.*, 1988). From this work it was concluded that ALAS is controlled *in vivo* primarily at the level of transcription. However, the authors noted that additional regulation of ALAS synthesis by a post transcriptional mechanism(s) could not be excluded.

The work presented in this Chapter demonstrates unequivocally that the effects of hemin, ALA and succinyl acetone on ALA mRNA levels in HepG2 cells are not due to changes in the rate of transcription of the ALAS gene. Therefore, the observed changes in the steady

state ALAS mRNA levels, detected by Northern analysis, following treatment of the cells with these reagents must be due to a post transcriptional mechanism(s). Therefore, the results obtained in this study, suggest that the regulation of the levels of ALAS mRNA in HepG2 cells is quite different from that seen *in vivo*. While studies conducted in transformed cell lines have provided much insight into the control mechanisms that regulate growth and differentiation in normal cells, transformed cell lines by definition have lost the ability to respond to some of the normal cellular control mechanisms. Therefore, it is possible that HepG2 cells have lost the ability to regulate transcription of the ALAS gene directly in response to changes in cellular heme levels, and as a result may rely on post-transcriptional mechanisms which may be present in hepatic cells *in vivo* but play more of an ancillary role.

It is interesting however, that in other work carried out in this laboratory, using isolated chick embryo primary hepatocytes, similar results to those observed in the HepG2 cell line were obtained. Northern analysis of total RNA from chick embryo primary hepatocytes determined that the levels of endogenous ALAS mRNA are decreased by treatment with 10μM hemin and increased by treatment with succinyl acetone. However, nuclear run-on experiments established that these changes were not due to alterations in the transcription rate of the ALAS gene (Mr C. Hahn personal communication). This raises the possibility that isolated hepatocytes in some way lose the capacity to respond to changes in cellular heme levels by altering transcription of the ALAS gene, and instead rely on post-transcriptional mechanisms to regulate ALAS mRNA levels. An alternative explanation for the results obtained in the chick embryo system is that these cells do not have the same regulatory mechanisms operating in the hepatocytes of mature animals, and it is possible that the HepG2 cells have also reverted to regulatory mechanisms normally present in cells that are not fully differentiated. Ongoing work in this laboratory using primary rat hepatocytes isolated from mature animals to study the transcriptional response of the endogenous rat hepatic ALAS gene should help resolve this question. The advantage that the rat system offers is that the results obtained *in vitro* can be directly related to studies conducted *in vivo*, whereas the human system is limited in that regard. However, hopefully the information obtained from the rat system will assist in elucidating the mechanisms by which heme represses transcription of the human ALAS gene *in vivo*.

5.3.2 Expression of the ALAS/hGH chimeric plasmid promoter constructions in HepG2 and CHO cell lines.

The expression of a series of human hepatic ALAS/hGH promoter constructions was examined in both the human hepatoma HepG2 cell line and CHO cells. To determine whether the intron in the 5' untranslated region of the ALAS gene has a role in the transcriptional regulation of the gene, promoter constructions were prepared that contained either all of the intron or the first 140bp of intronic sequence. In both cell lines (Figs 5.7 and 5.8), the promoter constructions containing the human hepatic ALAS gene promoter sequences up to -140bp from the transcription initiation site of the ALAS gene were sufficient to generate strong expression of hGH, indicating that the minimum sequence required for expression of the ALAS gene, at least in these two cell lines, resides within these sequences.

In both cell lines, the highest levels of hGH expression were generated by the two shorter pairs of ALAS promoter constructions, containing the gene sequences up to -140bp and -2.3kb respectively. The levels of expression generated by these promoter constructions were several fold higher than that generated by the longest pair of promoter constructions which contained the gene sequences up to -9kb. In addition, the expression of hGH generated by the two shorter pairs of ALAS promoter constructions was between 60-75% of that generated by the positive control pSVhGH, which has the human growth hormone reporter gene under the control of the SV40 promoter (data not shown). Therefore, the levels of expression generated by the two shorter pairs of ALAS promoter constructions are somewhat higher than might be expected for a housekeeping gene, while the expression generated by the longest two promoter constructions is perhaps more in keeping with what might be expected. From these results one might speculate that in the 5' flanking region of the ALAS gene, located between -2.3 and -9kb there are sequence(s) which are able to interact with the immediate promoter region in some way to modulate expression of the ALAS gene.

Interestingly, in both cell lines the levels of expression generated by the promoter constructions containing all of the intron in the 5' untranslated region of the ALAS gene were considerably lower than the corresponding constructions containing only the first 140bp of

intronic sequence. This might indicate that the intron contains regulatory sequences which at least in these cell lines decrease the level of expression of the ALAS gene.

Although many genes of higher organisms contain large introns, it is still not clear whether introns are a general site for the placement of critical *cis*-regulatory sequences. Enhancer and modulatory elements have been located within the introns of a growing list of genes including those coding for mouse immunoglobulin chains (Gillies *et al.*, 1983), the *Drosophila yellow* locus (Geyer and Corces, 1987), collagen genes (Rossi and de Crombrugghe, 1987; Bornstein *et al.*, 1987), the chicken δ1-crystalline gene (Hayashi *et al.*, 1987) and the mouse and human 4F2HC genes (Karpinski *et al.*, 1989). In addition, is has recently been shown that sequences present in the first intron of the Drosophila ß3-tubulin gene mediate enhancement of transcription of the gene in a tissue specific manner (Gasch *et al.*, 1989). An interesting proposal has recently been put forward by Brinster *et al.*, (1988) who have suggested that introns contain *cis*-regulatory elements of an unknown nature, other than specific enhancer like elements, that are important for the phasing of nucleosomes relative to important promoter elements. According to this hypothesis, the position and orientation of introns would be critical to ensure correct phasing of the nucleosomes, a property that distinguishes this idea from those invoking enhancer like properties to introns. As the human ALAS intron appears to bring about a decrease in expression of the ALAS gene, it is possible that such a mechanism plays a negative role in the regulation of ALAS expression.

Previous work in this laboratory had failed to obtain repression of chicken hepatic ALAS promoter constructions by the addition of hemin, in both Xenopus oocytes and transformed human cell lines (Loveridge, 1988; Day, 1988). Subsequently work by others in this research group has also failed to obtain hemin repression of rat hepatic ALAS promoter constructions in an homologous rat hepatoma H4-II-E-C3 cell line (G. Bradiotti personal communication). However, both of the chicken and the rat genes had been isolated from lambda genomic libraries with relatively short lengths of 5' flanking sequences, and it was possible that the sequences required for heme repression of the ALAS promoter where not present in the promoter constructions examined. The advantage of the human system was that the gene had been isolated from a cosmid genomic library and consequently it was possible to

prepare promoter constructions containing ALAS promoter sequences up to -9kb. Unfortunately, the results obtained from the expression studies in which the effects of hemin, ALA or succinyl acetone on transcription of the human ALAS promoter constructions were examined, were disappointing in that the cells expressed the promoter constructions at the same level as untreated cells. There are a number of possible reasons for this.

1. The ALAS promoter constructions do not contain the *cis*-acting sequences responsive to heme and therefore can never be heme repressed. Although the longest promoter construction used in this study contained gene sequences up to -9kb this possibility can not be excluded.

2. Heme repression may be mediated by sequences located 3' of the translational start site of the gene. As none of the promoter constructions used in this study contained sequences 3' of the translational start site of the gene this possibility can not be excluded.

3. The repression of transcription may occur over either shorter or longer time periods than those examined in this study. This possibility seems unlikely as changes in the activity of ALAS are readily detectable at the times used in this study (Iwasa *et al.*, 1989) and the constructions expressed at the same relative level under all conditions investigated.

4. HepG2 cells may not have the ability to respond to changes in intracellular heme levels by the same mechanisms seen *in vivo*. The results of the nuclear run-on experiments support this possibility since treatment with hemin, ALA or succinyl acetone do not bring about changes in the transcription rate of the endogenous ALAS gene.

Although it has been shown *in vivo* that hemin or ALA administered to rats reduces the level of hepatic ALAS mRNA predominantly by inhibiting ALAS gene transcription (Maguire, 1987; Srivastava *et al.*, 1988), it is not known whether heme also acts by a previously undetected post transcriptional mechanism to lower ALAS mRNA levels. This possibility is currently under investigation. From the results obtained in this study it seems unlikely that the HepG2 hepatoma cell line reflects the true situation for ALAS regulation seen *in vivo*, and it will be necessary to employ an alternative experimental approach in future work aimed at understanding the transcriptional regulation of human hepatic ALAS.

When attempts were made to generate a completely intronless chimeric ALAS promoter construction an interesting phenomena was encountered. The ALAS gene promoter sequences were quite stable until attempts were made to fuse them to a heterologous reporter gene. This resulted in the deletion of large amounts of DNA, regardless of whether the heterologous gene was from prokaryotic or eukaryotic sources. Several different *E. coli* host strains were tried, including two strains reported to minimize deletions during cloning events. Although initially the deletion events were extremely frustrating, the surprising finding that they could be prevented by the inclusion of the first 140bp of the intron in the 5' untranslated region of the ALAS gene is intriguing. Unfortunately, computer analyses of the sequences involved, and a search of the data base did not provide any insight into the problem. It was beyond the scope of this thesis to pursue the matter further, but it would be interesting in the future to delineate the sequences involved to determine a) what sequences causes the instability and b) whether the stabilizing effect can be transferred to other "problem" sequences. One possibility, yet to be examined is that the cloning of a heterologous gene next to the ALAS promoter sequence causes the overproduction of the protein product of the gene, and this in some way leads to the deletion of the cloned DNA sequences.

CHAPTER SIX

STUDIES ON THE LEVEL OF HEPATIC ALAS mRNA IN HUMAN LIVERS.

CHAPTER SIX : STUDIES ON THE LEVEL OF HEPATIC ALAS mRNA IN HUMAN LIVERS.

6.1 INTRODUCTION

In this chapter, studies are described in which for the first time the levels of hepatic ALAS mRNA present in individual human liver samples were investigated, using the human hepatic cDNA probe, hHcDNA (described in Chapter three). The human liver samples used in this study were obtained from renal or hepatic transplant donors, through the liver bank at the Flinders Medical Centre, and the use of such tissue in this study had local Research Ethics Committee approval. The disclosure of each donor's personal details is carefully monitored by the Ethics Committee, and while a record of the drug therapy administered immediately prior to organ donation was made available, it was not possible to have a more complete medical history.

Three of the patients had received dexamethasone therapy immediately prior to organ donation. It had been shown in the rat that the synthetic steroid, pregnenolone 16 α-carbonitrile (PCN) and dexamethasone induce a unique form of cytochrome P450 designated P450IIIA 1 (Gonzalez *et al.*, 1986). This induction is due to an increase in P450IIIA I mRNA levels (Hardwick *et al.*, 1983), and in the case of dexamethasone has been shown to represent transcriptional activation of the gene (Simmons *et al.*, 1987). More recently, using cultured rat primary hepatocytes, Schuetz *et al.*, (1990) have demonstrated that transcription of the rat P450IIIA 1 gene is also increased by the addition of phenobarbital (PB). A human cDNA orthologous to rat P450IIIA 1, which is induced in human liver by dexamethasone, has been isolated and sequenced (Molowa *et al.*, 1986), and Gonzalez *et al.*, (1988) have shown that the gene encodes nifedipine oxidase. At the time this work was undertaken little was known about the levels of ALAS mRNA present in human liver, and it was of interest to determine if in the dexamethasone treated patients there was an elevated level of ALAS mRNA paralleling the increase in the cytochrome P450IIIA 1 mRNA.

6.2 RESULTS

6.2.1 Northern analysis of human hepatic ALAS mRNA levels in human liver.

To investigate the levels of hepatic ALAS mRNA present in human liver, nine individual liver samples were obtained from the human liver bank and total RNA was extracted separately from each sample using a modification of the guanidinium isothiocyanate procedure, described in Section 2.3.1.

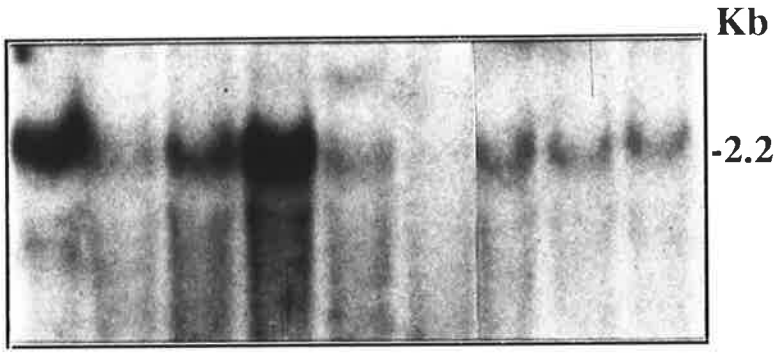
Total RNA (50μg) from each of the nine human liver samples was electrophoresed on a 1% agarose gel containing 1.1M formaldehyde. Following Northern blot transfer to nitrocellulose (Section 2.3.3) the filter was hybridized with the ^{32}P-labelled human hepatic ALAS cDNA probe (Section 2.2.14); the autoradiograph from this experiment is shown in Fig 6.1. The levels of ALAS mRNA were measured by laser densitometry and are represented by bar diagrams below the autoradiograph. The autoradiograph shown in Fig. 6.1, required sensitive X-ray film, two intensifying screens and exposure for 7 days at -80°C to allow detection of the lowest level of ALAS mRNA (lane 6) indicating that the levels of ALAS mRNA present in human liver are low.

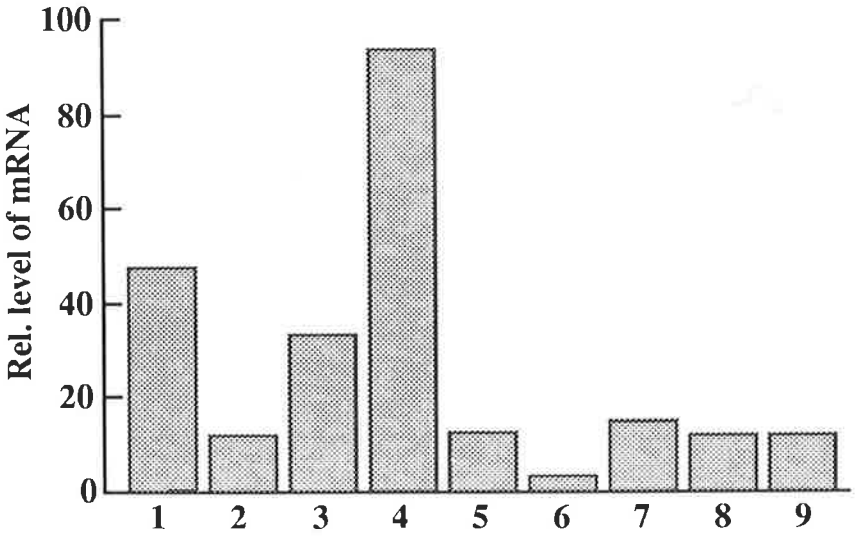
As can be seen in Fig. 6.1, the levels of hepatic ALAS mRNA present in human liver, vary considerably between individuals. Comparison of the levels of ALA mRNA present in the nine liver samples used in this study shows there is a 32 fold difference between the sample with the highest level of ALAS mRNA (lane 4) and the sample with the lowest level (lane 6). The three patients shown in lanes 1, 3 and 4 had received dexamethasone therapy prior to organ donation, and it is interesting to note that the levels of ALAS mRNA present in the liver of these three patients are at least two fold higher (compare lanes 3 and 7) compared with those from the untreated patients. In the six patients who were not known to be receiving drug therapy prior to organ donation, the amount of ALAS mRNA present in the liver varies by up to four fold (compare lanes 6 and 7), and presumably represents variations in the basal levels of ALAS mRNA. However, in view of the incomplete medical history of the donors, one can not be certain whether these fluctuations represent naturally occurring changes in ALAS mRNA

Figure 6.1 Northern blot analysis of hepatic ALAS mRNA levels in human liver.

Total RNA (50μg) prepared from nine human liver samples was electrophoresed on a 1% agarose gel, containing 1.1M formaldehyde. Following Northern transfer to nitrocellulose (Section 2.3.3) the filter was probed with the ^{32}P-labelled human hepatic ALAS cDNA probe, hHcDNA (Section 2.2.14). The filter was exposed to autoradiography and the autoradiograph from this experiment is shown. The levels of ALAS mRNA present in each sample were quantitated by densitometry, and are represented as bar graphs below the autoradiograph.

Hepatic ALA-S





levels or are the result of the patient having received some undisclosed agent(s) which induces ALAS.

6.2.2 Slot blot analysis of human hepatic ALAS, P450IIIA 1 and ß-actin mRNA levels in human liver.

Since the levels of ALAS mRNA in the patients who had received dexamethasone therapy are considerably higher than in the untreated controls, it was of interest to determine whether this increase was associated with an increase in expression of mRNA for the dexamethasone inducible human P450IIIA 1 enzyme.

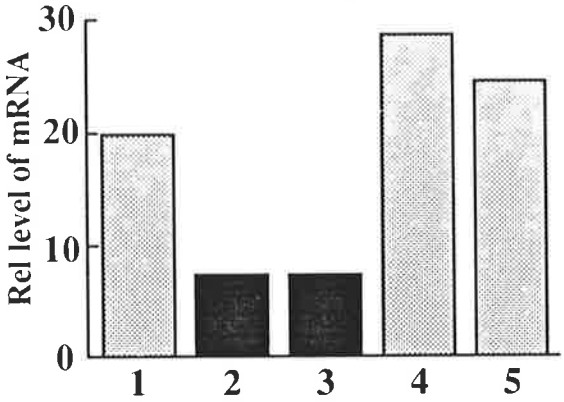
RNA slot blot analysis was used to compare the the levels of ALAS, P450IIIA 1, and ß-actin mRNAs in the dexamethasone treated patients (shown in Fig. 6.1, lanes 1,3 and 4) with two untreated controls (shown in Fig. 6.1, lanes 5 and 7). Total RNA (5µgs per slot), was immobilized on nitrocellulose and the filters probed with the ^{32}P-labelled human cDNAs for hepatic ALAS, P450IIIA 1, and ß-actin, and the autoradiographs from these experiments are shown in Fig. 6.2. Samples from the dexamethasone treated patients are shown in slots 1, 4 and 5; slot 1 corresponds to lane 1 in Fig. 6.1, but the order in which the other two samples were loaded is reversed in Fig. 6.2, and slots 4 and 5 correspond to lanes 4 and 3 respectively in Fig. 6.1. The untreated controls were loaded in the same order as in Fig. 6.1, and are shown in Fig. 6.2 slots 2 and 3. The resulting autoradiographic signals were quantitated by laser densitometry, and are expressed as arbitrary units in bar graphs alongside the corresponding autoradiographs. The bar graphs representing the untreated patients are shown as solid black bars and those representing the dexamethasone treated patients are shaded grey.

As can be seen in Fig. 6.2 A, and as expected from the Northern analysis described above, the dexamethasone treated patients (Fig. 6.2 A, slots 1, 4 and 5), show an increase in the level of hepatic ALAS mRNA relative to the untreated controls (Fig. 6.2 A, slots 2 and 3). The levels of P450IIIA 1 mRNA present in these patients are shown in Fig.6.2B. As this mRNA is detectable in all five liver samples it appears to be constitutively expressed at a relatively low basal level. The levels of P450IIIA 1 mRNA are elevated in the dexamethasone treated patients relative to the untreated patients, and the magnitude of the increase varies from

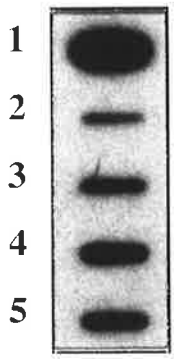
Figure 6.2 Slot blot analysis of human hepatic ALAS, dexamethasone inducible P450IIIA 1, and ß-actin mRNA levels in human liver

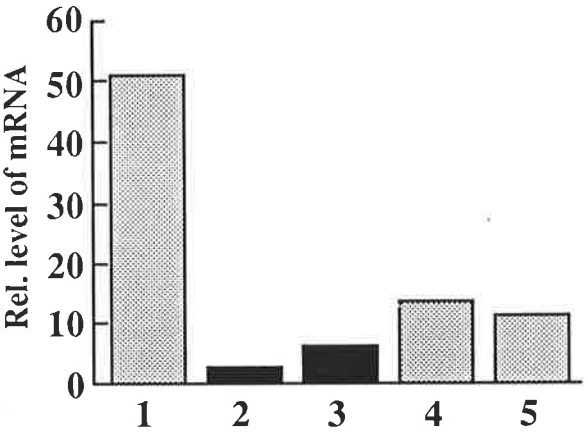
Total RNA (5µg) was analysed from five human liver samples, three of whom had received dexamethasone therapy (slots 1,4 and 5) and two were untreated controls (slots 2 and 3). These samples were immobilized on nitrocellulose, and probed with the oligolabelled cDNAs for (A) hepatic ALAS, (B) the dexamethasone inducible P450IIIA 1 and (C) human ß-actin. The filters were exposed to autoradiography and the autoradiographs from these experiments are shown. The levels of each mRNA were quantitated by densitometry, and expressed as arbitrary units are presented as bar graphs next to the corresponding autoradiograph. The bar graphs representing the untreated patients are shown as solid black bars and those representing the dexamethasone treated patients are shaded grey. Note the arbitrary densitometry units refer to each particular mRNA they are not comparable between autoradiographs.

Hepatic ALA-S

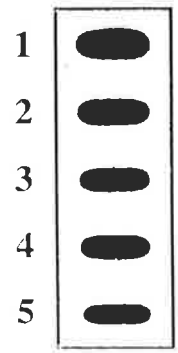


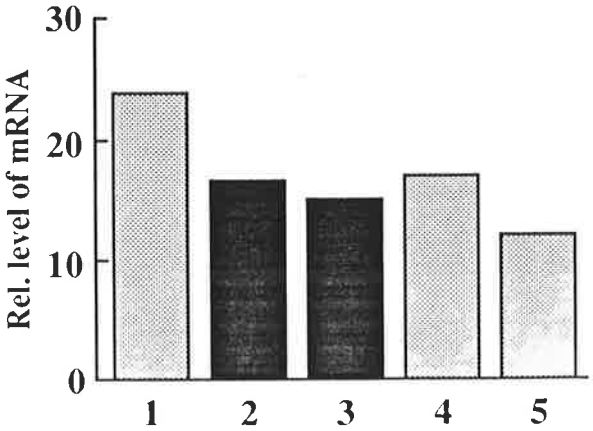
P 450 III A





Beta actin





approximately two fold between slots 3 and 5, to 18 fold between slots 1 and 2. In addition, there is considerable variability in P450IIIA 1 mRNA levels between the dexamethasone treated patients. Although similar levels are seen in the two patients shown in slots 4 and 5, there is approximately a 4-5 fold difference between the patients shown in slots 1 and 5. These differences in P450IIIA 1 mRNA levels may reflect differences in either dosage and/or the length of time the patients were receiving the therapy, but unfortunately this data is not available.

While the levels of hepatic ALAS mRNA are increased in all three dexamethasone treated patients relative to the untreated controls, the increases do not parallel those seen with the P450IIIA 1 mRNA. For example, the patient shown in slot 1 has 77.5% more P450IIIA 1 mRNA than the patient shown in lane 5, but the increase in ALAS mRNA in the patient shown in slot 1 is 20% less than that seen in the patient shown in lane 5. Therefore, while the increase in the levels of hepatic ALAS mRNA seen in the patient shown in slot 1, may be linked to the increase in the level of the P450IIIA 1 mRNA also seen in this patient, it seems likely that in the other two patients there is some other factor(s) contributing to the increased ALAS mRNA levels.

As ß-actin is a housekeeping gene which is unlikely to be affected by dexamethasone therapy, the levels of ß-actin mRNA present in the livers of the five patients were also determined. While the levels of ß-actin mRNA vary between individuals (Fig. 6.2C) importantly, the levels of ß-actin mRNA present in the livers of the dexamethasone treated patients do not exhibit the increases seen in the levels of either hepatic ALAS or P450IIIA 1 mRNA. Therefore, the increases observed in the levels of hepatic ALAS and P450IIIA 1 mRNA in the dexamethasone treated patients probably represent a specific response to the treatment rather than a generalized increase in mRNA synthesis.

DISCUSSION

In this Chapter, the levels of hepatic ALAS mRNA in different human livers was investigated, and were found to vary considerably between individuals. In individuals thought not to be receiving any drug therapy, the level of ALAS mRNA varied by up to four fold, but

as mentioned previously these variations could be due to some undisclosed inducing agent(s) of ALAS. As might be expected from the studies using experimental animals, the three patients receiving dexamethasone therapy prior to organ donation all had elevated levels of ALAS mRNA. However, as the increases seen in the levels of hepatic ALAS and P450IIIA 1 mRNAs are quite different between the dexamethasone treated patients, it seems likely at least in the patients shown in Fig. 6.2A, slots 4 and 5, that some other factor(s) is contributing to the increases in the levels of ALAS mRNA.

The results obtained in this study indicates that the induction of the dexamethasone inducible P450IIIA 1 mRNA in humans, is accompanied by an increase in ALAS mRNA expression. However, the Northern and slot blot analysis of RNA used in this study only measure changes in the level of steady state mRNA. Therefore, from this work it can not be presumed that the observed increases in the levels of ALAS and P450IIIA 1 mRNA are the result of increased transcription of the corresponding genes. It is possible that through some post transcriptional effect dexamethasone may influence the stability of the ALAS and P450IIIA 1 mRNAs, and thereby contribute to the observed increases in the mRNA levels. Support for mRNA stabilization contributing to the observed increases in ALAS mRNA levels, comes from a recent study into the effect of dexamethasone on phenobarbitone-activated transcription of the rat cytochrome P450IIB 1 and 2 genes. Rao *et al.*, (1990) demonstrated in adult rat liver, that dexamethasone has the unusual effect of increasing the levels of P450IIB 1 and 2 mRNA, while inhibiting transcription of the gene, and it has been proposed that mRNA stabilization is the likely reason for this effect. It is possible that in human liver dexamethasone acts by a similar mechanism to positively modulate the level of the P450IIIA 1 mRNA.

At present the induction mechanism of the P450IIIA I proteins is not known. Although induction of the rat IIIA 1 gene, by dexamethasone, has been shown to be due to activation of gene transcription (Simmons *et al.*, 1987). The mechanism by which dexamethasone induces P450IIIA I does not exhibit characteristics typical of other glucocorticoid-induced responses, in which classical glucocorticoid receptors mediate the induction response (Ringold, 1985). For example, in cultured hepatocytes, maximal induction of the cytochrome P450IIIA I protein, requires a dexamethasone concentration that is at least two orders of magnitude higher than that

required to maximally induce tyrosine aminotransferase (TAT) and is far greater than that required to activate the glucocorticoid receptor (Schuetz and Guzelian, 1984). However, whether the response is mediated through the glucocorticoid receptor or through the involvement of some other dexamethasone-modulated protein remains to be determined. If as current evidence suggests, transcription of the hepatic ALAS gene is also induced by dexamethasone, the elucidation of this mechanism may provide valuable insight into how expression of ALAS and the cytochromes P450 are co-ordinated.

CHAPTER SEVEN

STUDIES ON ALAS mRNA LEVELS IN BONE MARROW SAMPLES FROM PATIENTS WITH SIDEROBLASTIC ANAEMIA.

CHAPTER SEVEN: STUDIES ON ALAS mRNA LEVELS IN BONE MARROW SAMPLES FROM PATIENTS WITH SIDEROBLASTIC ANAEMIA.

7.1 INTRODUCTION

The sideroblastic anaemias include a broad range of clinical states, which may be either inherited or acquired, and are recognized by the presence of characteristic ring sideroblasts in the bone marrow, and hypochromic-microcytic red cells in the peripheral blood. As discussed in chapter one, impaired heme biosynthesis in the developing erythroid cell and a consequent ineffective erythropoiesis is the principal disturbance in the pathogenesis of both the congenital and acquired forms of the disorder. Multiple causes appear to underlie the defective heme synthesis as the ring sideroblast abnormality occurs under a variety of circumstances (Bottomley, 1982; Bottomley and Müller-Eberhard, 1988), but in most cases the fundamental derangement of the heme synthesis pathway remains undefined.

Sideroblastic anaemia can be produced in experimental animals by inducing a deficiency of vitamin B_6, which provides pyridoxal-phosphate the essential co-factor for ALAS (Bottomley, 1980; Tanka and Bottomley, 1974). These experiments together with the occasional striking erythropoietic response observed in patients with hereditary and less often with acquired sideroblastic anaemia to pharmacologic doses of pyridoxine, have implicated the involvement of ALAS in the pathogenesis of some types of sideroblastic anaemia (Bottomley and Müller-Eberhard, 1988).

Professor S. Bottomley, a hemotologist at the University of Oklahoma Health Sciences, has studied sideroblastic anaemia (both as a clinician and researcher) for over two decades. Isolation of the human ALAS cDNA in our laboratory, prompted her to seek a collaboration with our group, hoping that together we might gain some insight into the molecular and genetic basis of the disorder. Prof. Bottomley undertook to extract RNA from the bone marrow cells of sideroblastic patients, and to send it to this laboratory for analysis.

It should be emphasized that sideroblastic anaemia, particularly the inherited X-linked form of the disorder is rare. Therefore, the number of samples available to us for analysis was

limited. In addition, as the amount of bone marrow that can be taken from humans without causing unpleasant side effects is also limited, we had to be conservative with the RNA that was extracted from it. Due to these limitations the technical aspects necessary for the collaboration, including extraction of the RNA, transport to Australia, and ensuring that the probe could detect ALAS mRNA in the samples, were established by firstly using RNA from rabbits and then subsequently from healthy human controls.

At the time this study was undertaken it was thought that there was only one gene for ALAS in humans, and so the original work was done using the housekeeping or hepatic type ALAS cDNA, described in Chapter three, as the probe. Initially, RNA samples from two sideroblastic patients were prepared, one with a congenital and the other with an acquired form of the disease. Following the isolation of the human erythroid specific ALAS cDNA in our laboratory, and the subsequent localization of the erythroid ALAS gene to the X chromosome (Cox *et al.*, 1990), RNA was obtained from the bone marrow of two patients with the inherited X-linked form of the disorder and the work was repeated using this erythroid probe.

All the patients and healthy controls involved in this study, were informed as to the nature of the study, and participated voluntarily. I am particularly grateful to the two patients with the X-linked form of the disorder, who had to travel to Oklahoma from quite distant parts of the U.S.A. to undergo the marrow sample collection.

7.2 RESULTS

7.2.1 Northern blot analysis of bone marrow mRNAs for hepatic type ALAS, α, and ß globin, from patients with congenital and acquired sideroblastic anaemia.

As mentioned above, all the bone marrow RNA used throughout this study was extracted from bone marrow aspirate collected in the U.S.A. by Prof. Bottomley. Preliminary experiments established that the RNA had to be extracted from the tissue immediately following collection; storage of the marrow aspirate for even relatively short periods of time resulted in degradation of the RNA. Briefly, the procedure employed by Professor Bottomley is as follows. The bone marrow aspirate was centrifuged to exclude mature erythrocytes and

unneeded cellular and protein material from the nucleated bone marrow cells. (To date there is not an effective way to separate out the erythroblasts from the rest of the nucleated cell population). The percentage of erythroblasts present in the heterogeneous nucleated cell population was determined, before total RNA was extracted, essentially by the guanidinium isothiocyanate procedure, described by Chomczynski and Sacchi (1987). The RNA was then transported as a freeze dried pellet and reconstituted on arrival in Australia.

Northern blot analysis of total RNA from nucleated bone marrow cells was carried out to compare the levels of mRNA for ALAS and α and ß globin, in the bone marrow of sideroblastic patients and healthy individuals. In the initial study, total RNA was prepared from two sideroblastic patients. One sample was from a female with a congenital pyridoxine responsive form of the disorder, and one sample was from a male with an acquired form, which is not pyridoxine responsive. In addition, samples were prepared from two healthy individuals to use as controls. Total RNA (40µg) from the four bone marrow samples was electrophoresed on 1% agarose gels containing 1.1M formaldehyde. Following transfer to nitrocellulose, the filters were hybridized with ^{32}P-labelled probes (Section 2.2.14) for either human hepatic type ALAS, α- globin or ß-globin. In addition to the four bone marrow samples, total RNA (40µg) from two human liver samples, one induced for ALAS and one non-induced sample, were included on the filter probed for hepatic type ALAS mRNA.

The human hepatic type ALAS cDNA construct designated hHcDNA, described in Section 3.2.3, was used as the probe in this initial study, because as discussed above at the time this work was done it had not been established that human ALAS exists as two isozymes. The globin probes used throughout this study were obtained from Dr. B. Rudski, at the Institute of Medical and Veterinary Science, Adelaide. Both probes were human genomic DNA fragments cloned into the Pst I site of the plasmid vector pBR322. The α globin probe was a 1.6kb genomic fragment and the ß-globin probe a 4.4kb fragment.

The autoradiographs of the Northern blot filters obtained from these experiments are shown in Fig 7.1, together with bar graphs representing densitometric quantitation of the autoradiographs. In Fig. 7.1A, the samples of hepatic total RNA, induced and non induced for ALAS are shown in lanes 1 and 2 respectively, and are represented by open bar graphs. The

Figure 7.1 Northern blot analysis of bone marrow mRNAs for hepatic ALAS, α and ß globin in patients with congenital and acquired sideroblastic anaemia.

Total RNA was prepared from the nucleated bone marrow cells of two sideroblastic patients, one from a female with a congenital form of the disorder (Cong.) one from a male with an acquired form (Acq.) and two samples from healthy individuals (C). 40µg of each total RNA sample was electrophoresed on 1% agarose gels containing 1.1M formaldehyde. Following Northern blot transfer to nitrocellulose (Section 2.3.3), the filters were hybridized with ^{32}P-labelled probes (Section 2.2.14) for either (A) human hepatic type ALAS, (B) α- globin or (C) ß-globin. In addition to the four bone marrow samples, total RNA (40µg) from two human liver samples, one induced for ALAS (I) and one non-induced sample (NI), were included on the filter probed for hepatic type ALAS mRNA (A). The autoradiographs from each of these experiments are shown. The autoradiographs were quantitated by densitometry, and the relative amounts of each mRNA expressed as arbitrary units, are presented as bar graphs below each autoradiograph. The arbitrary densitometry units used refer to the relative amounts of each particular mRNA and are not comparable between autoradiographs.

(A)

lane 1 liver RNA induced for ALAS

lane 2.liver RNA not induced for ALAS

lanes 3 and 6 bone marrow RNA samples from healthy individuals

lane 4 bone marrow RNA from patient with congenital sideroblastic anaemia

lane 5 bone marrow RNA from patients with acquired sideroblastic anaemia

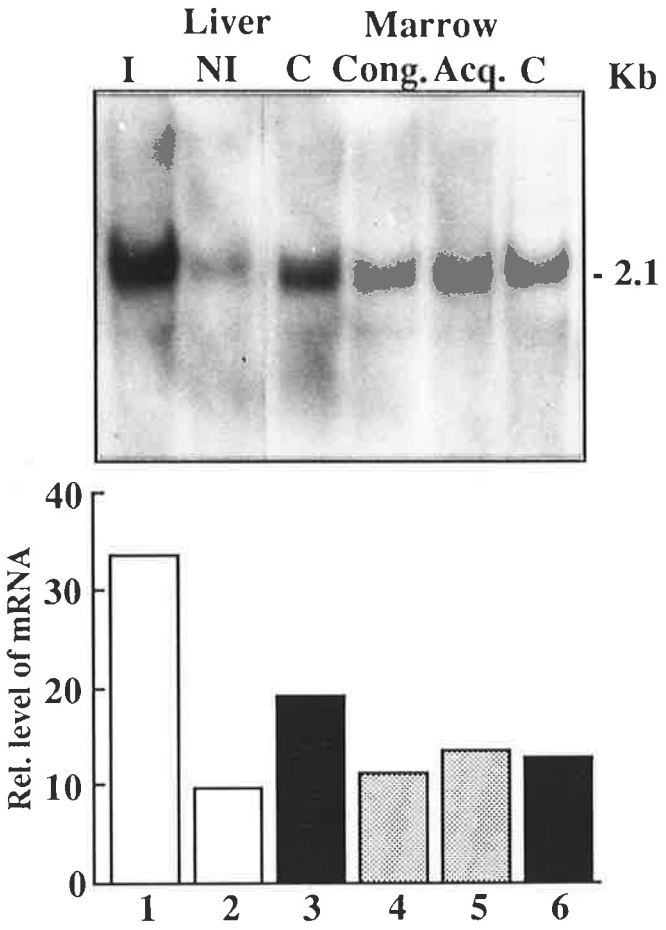
(B) and (C)

lanes 1 and 4 bone marrow RNA from healthy individuals

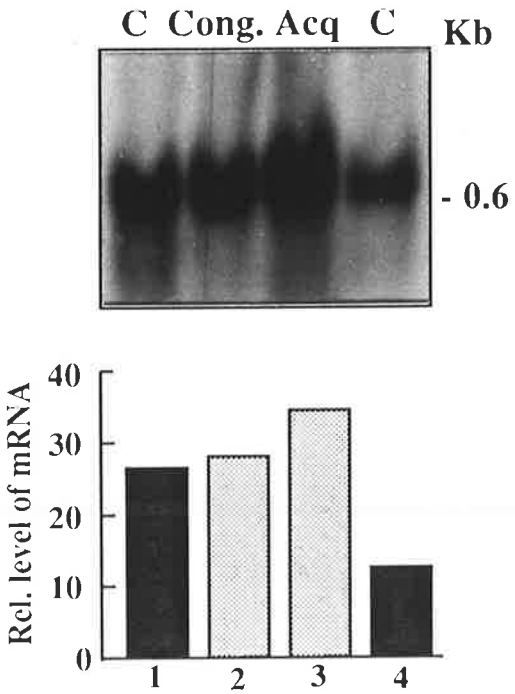
lane 2 bone marrow RNA from patient with congenital sideroblastic anaemia

lane 3 bone marrow RNA from patients with acquired sideroblastic anaemia

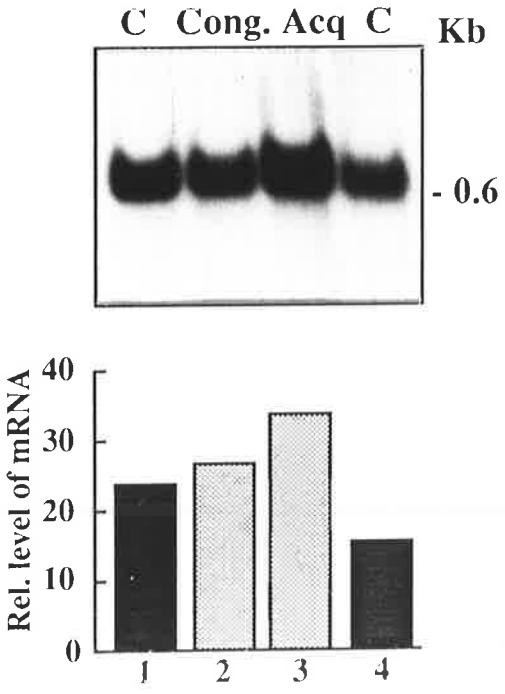
A Hepatic ALA-S



B α– Globin



C β – Globin.



four bone marrow samples are shown in lanes 3-6. In the bar graphs, the sideroblastic patients are represented by grey shaded bars and the healthy controls as solid black bars. As can be seen in Fig 7.1A, the hepatic form of ALAS mRNA is readily detectable in the total RNA isolated from the nucleated bone marrow cells, and similar levels of hepatic type ALAS mRNA are seen in the samples from the sideroblastic patients (lanes 4 and 5) and the healthy controls (lanes 3 and 6).

The levels of α and ß-globin mRNA in the four bone marrow samples are shown in Figs 7.1B and C respectively. The greatest variability in the levels of both globin mRNAs are seen between the two healthy controls, where the control shown in lane 1 has approximately two fold more globin mRNA than the control shown in lane 4. The levels of both globin mRNAs in the patient with the congenital form of sideroblastic anaemia are similar to the control shown in lane 1, and are approximately two fold higher than the control shown in lane 4. However, the patient with the acquired form of the disorder has an apparent marginal increase in the levels of both globin mRNAs. This may reflect the severe anaemia seen in this patient (hemoglobin of 6gm/ 100ml) whose bone marrow differential indicated many more early erythroblasts than normal, reflecting a marked erythropoietin drive in response to the low hemoglobin. (Prof. Bottomley, pers. comm.).

7.2.2 Northern Analysis of total RNA prepared from the bone marrow of patients with congenital, acquired and X-linked sideroblastic anaemia.

When the erythroid ALAS cDNA probe became available, Professor Bottomley was able to arrange for two patients with the X-linked form of sideroblastic anaemia to attend her laboratory for bone marrow collection. Total RNA was again prepared from the bone marrow aspirate of these two patients, as described in Section 7.2.1. Samples of total RNA (40µg) prepared from the bone marrow of the X-linked sideroblastic patients, the two patients with the congenital and acquired forms described above, and one sample from a healthy control, were electrophoresed on 1% agarose gels containing 1.1M formaldehyde. Following transfer to Nytran™, the filters were hybridized to ^{32}P-labelled cDNA probes for either human erythroid ALAS, α- globin or ß-globin. The human erythroid probe used throughout this study was a 1.9kb Eco RI DNA fragment, which is located entirely within the coding region of the erythroid

ALAS cDNA. The autoradiographs from these experiments, together with bar graphs representing densitometric quantitation of the autoradiographs are shown in Fig.7.2. In Fig. 7.2, the X-linked sideroblastic patients are represented by grey shaded bars; the other forms of sideroblastic anaemia (lanes 2 and 3) and the healthy control (lane 1) are represented by solid black bars.

It is interesting to note that in Fig 7.2A, both patients with the X-linked form of the disorder (lanes 4 and 5), exhibit a decrease in the level of erythroid ALAS mRNA, relative to both the healthy control and the other two sideroblastic patients. In contrast, the level of erythroid ALAS mRNA seen in the patient with the congenital form of the disorder is marginally higher than that of the control sample (compare lanes 1 and 3). While the patient with the acquired form of sideroblastic anaemia (lane 2) has the highest level of erythroid ALAS mRNA, which as discussed above, is probably the result of a higher and more immature erythroid cell content in the marrow aspirate.

The levels of α and ß globin mRNAs present in these individuals are shown in Fig 7.2 panels B and C respectively. The level of α globin mRNA in the sample from the patient with the congenital form of the disorder is similar to that of the control (compare lanes 1 and 3). While the patient with the acquired form of the disorder (lane 2) has a two fold increase in the level of α globin mRNA relative to the control sample (lane 1). In the patient with congenital sideroblastic anaemia the level of ß globin mRNA is approximately 66% of that seen in the control sample (compare lanes 1 and 3), and the patient with the acquired form (lane 2) has a marginal increase in the level of ß globin mRNA relative to the control (lane 1). However, the levels of α and ß globin mRNA in the two X-linked sideroblastic patients (lanes 4 and 5), are decreased relative to the control sample and the other sideroblastic patients (lanes 1-3). The results of this experiment indicated that the levels of erythroid ALAS, α and ß globin mRNAs in the bone marrow RNA of the X-linked patients were decreased relative to the healthy control. Unfortunately, at the time this work was undertaken there was only one sample of bone marrow RNA from a healthy control available. It was therefore important to compare the levels of these three mRNAs in the bone marrow of the X-linked patients with those of other healthy

Figure 7.2 Northern blot analysis of bone marrow mRNAs for erythroid ALAS, α and ß globin in patients with sideroblastic anaemia.

Total RNA (40μg) prepared from the nucleated bone marrow cells of a healthy individual (C) and four sideroblastic anaemia patients, one sample from a patient with an acquired form (Acq.) one sample from a patient with a congenital form (Cong.) and two samples from patients with X-linked sideroblastic anaemia (X-L) was electrophoresed on 1% agarose gels, containing 1.1M formaldehyde. Following Northern blot transfer to Nytran™(Section 2.3.3), the filters were probed with oligolabelled cDNAs for (A) erythroid ALAS, (B) α- globin or (C) ß-globin (Section 2.2.14). The autoradiographs resulting from these experiments are shown. The autoradiographs were quantitated by densitometry, and the relative amounts of each mRNA expressed as arbitrary units, are presented as bar graphs below each autoradiograph. The arbitrary densitometry units used refer to the relative amounts of each particular mRNA and are not comparable between autoradiographs.

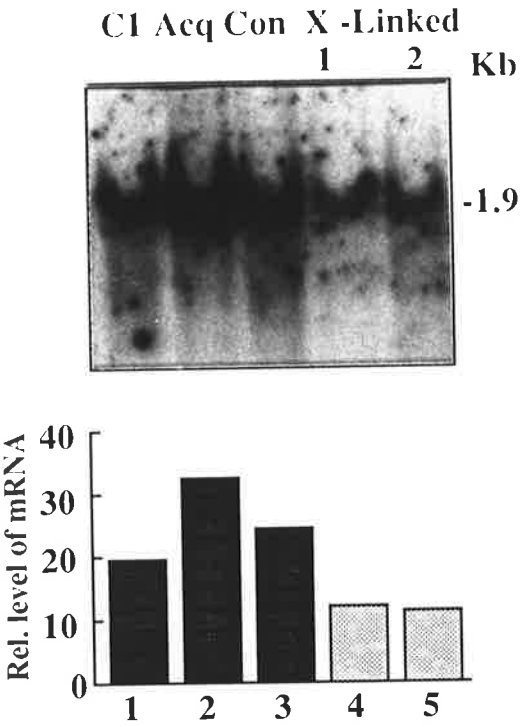
lane 1 bone marrow RNA prepared from a healthy individual.

lane 2 bone marrow RNA from a patient with acquired sideroblastic anaemia.

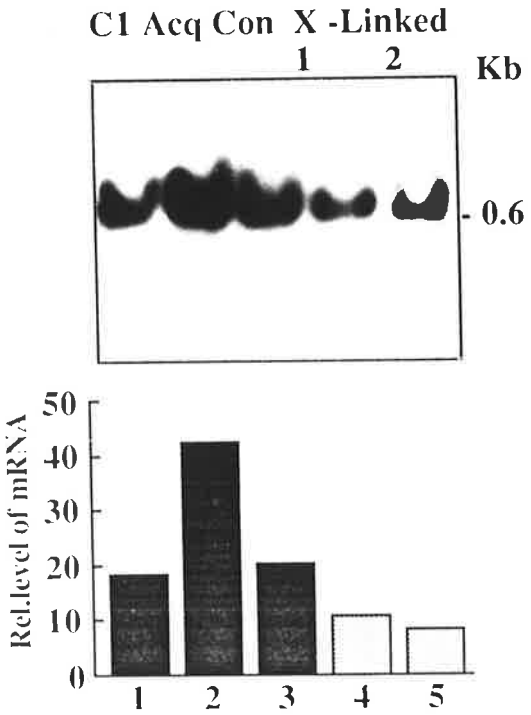
lane 3 bone marrow RNA from a patient with congenital sideroblastic anaemia.

lanes 4 and 5 bone marrow RNA from the X-linked sideroblastic patients.

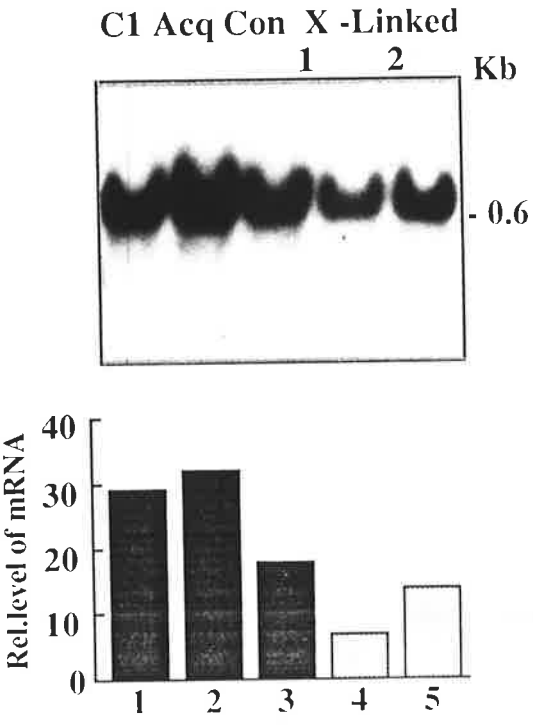
A Erythroid ALA-S



B α – Globin



C β - Globin



individuals. In order to do this, Professor Bottomley prepared total RNA from the bone marrow of a further three healthy controls.

Although the elevated levels of erythroid ALAS, α, and ß globin mRNA observed in the bone marrow RNA of the patient with the acquired form of sideroblastic anaemia, were thought to be a response to the severe anaemia present in that patient, the possibility that they are characteristic of acquired sideroblastic anaemia could not be excluded. To investigate this possibility, Prof. Bottomley prepared RNA from a second patient with an acquired form of the disorder who was not anaemic.

To investigate the levels of hepatic and erythroid ALAS mRNA in the bone marrow of the two X-linked patients the two patients with the acquired form of sideroblastic anaemia and the four healthy individuals, Northern analysis of total RNA (40μg) was carried out as described previously. In addition, 10μg of total RNA from each of these individuals was analysed on filters which were probed for α and ß globin mRNA. It should be noted that in the conditions employed in these experiments the hepatic type and erythroid specific cDNA probes are specific for these mRNAs and detect bands of 2.1 and 2.2kb respectively. The autoradiographs obtained from these experiments were quantitated by densitometry, and bar graphs representing the amounts of mRNA present in each sample are presented below the relevant autoradiograph. In these experiments, the sideroblastic patients are represented by grey shaded bars and the healthy controls are represented by solid black bars. The healthy control sample shown in lane 1 was the same sample used in the previous experiment (Fig. 7.2) and the controls shown in lanes 2-4 were the three more recently prepared samples.

The levels of erythroid and hepatic type ALAS mRNA present in the bone marrow RNA of the sideroblastic patients and the four healthy controls are shown in Fig. 7.3 panels A and B respectively. As can be seen in Fig 7.3A, between the healthy control samples the levels of erythroid ALAS mRNA present in the bone marrow RNA vary up to four fold (compare lanes 2 and 4). However, despite this variation the levels of erythroid ALAS mRNA present in the samples from the two patients with the X-linked form of the disorder are lower than all four of the healthy controls (compare lanes 6 and 7 with lanes 1-4). The sample number used in this experiment is too small to determine whether the decrease in the level of erythroid ALAS seen

Figure 7.3 Northern blot analysis of bone marrow mRNAs for erythroid and hepatic type ALAS in Sideroblastic patients.

Total RNA (40μg) prepared from the nucleated bone marrow cells from four healthy individuals (C) and four sideroblastic patients, two samples from patients with an acquired form (Acq.) and two samples from patients with the X-linked form (X-L) was electrophoresed on 1% agarose gels containing 1.1M formaldehyde. Following Northern blot transfer Nytran™(Section 2.3.3) and probed with oligolabelled cDNAs for (A) erythroid ALAS, (B) hepatic ALAS (Section 2.2.14). On the filter probed for hepatic ALAS 40μg of total liver RNA was substituted for the bone marrow RNA sample from the healthy individual shown in lane 1 (A). The autoradiographs resulting from these experiments are shown. The levels of each mRNA were quantitated by densitometry, and expressed as arbitrary units, are presented as bar graphs below the autoradiographs. The arbitrary units refer to each particular mRNA and are not comparable between autoradiographs.

(A)

lanes 1-4 bone marrrow RNA from healthy individuals

lanes 5 and 8 bone marrow RNA from patients with acquired sideroblastic anaemia

lanes 6 and 7 bone marrrow RNA from patients with X-linked sideroblastic anaemia.

(B)

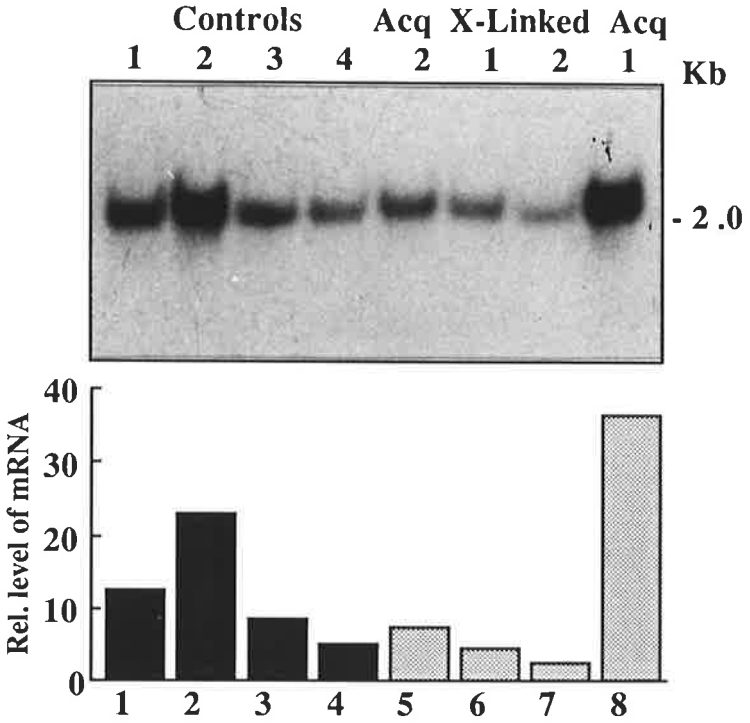
lane 1 hepatic RNA

lanes 2-4 bone marrrow RNA from healthy individuals

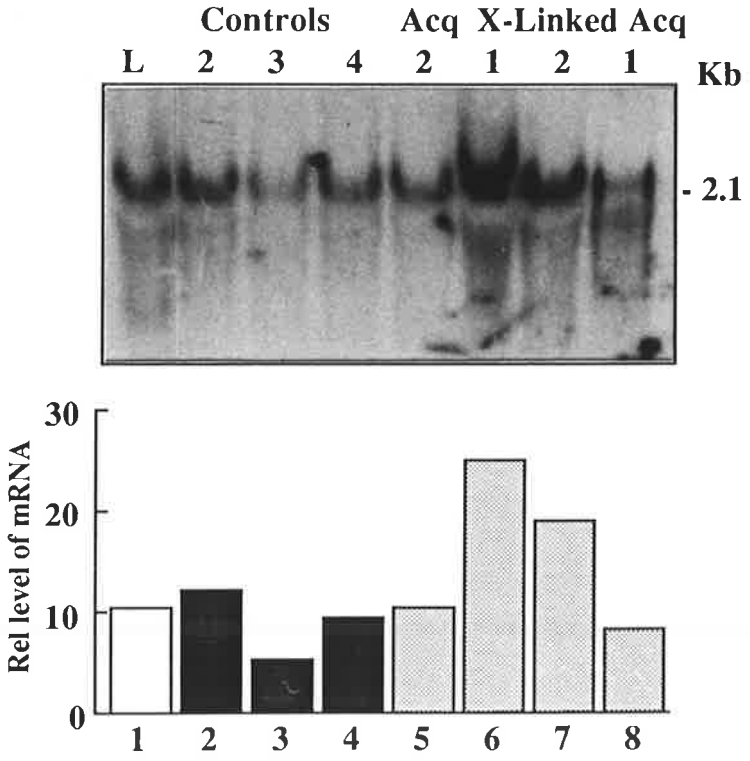
lanes 5 and 8 bone marrow RNA from patients with acquired sideroblastic anaemia

lanes 6 and 7 bone marrrow RNA from patients with X-linked sideroblastic anaemia

A Erythroid ALA-S



B Hepatic ALA-S



in the X-linked sideroblastic patients is statistically significant. However, the mean of the densitometry units for the healthy controls is 12.2, and the range is 5 to 23. The densitometry units for the X-linked sideroblastic patients are 4.5 and 2.5 respectively, and fall below the mean and the lowest range value for the healthy controls.

The levels of erythroid ALAS mRNA present in the bone marrow RNA from the patients with the acquired form of sideroblastic anaemia are shown also in Fig. 7.3A. The sample from the patient with the severe anaemia which was analysed in the previous experiments, is shown in lane 8, and the sample prepared from the second patient who was not anaemic is shown in lane 5. In the non-anaemic patient (lane 5) the level of erythroid ALAS mRNA is lower than that seen in the control samples shown in lanes 1 and 2, and is similar to that seen in the control samples shown in lanes 3 and 4. Comparison between the two patients with the acquired form of the disease shows that the level of erythroid ALAS mRNA seen in the non-anaemic patient is approximately eight fold less than that seen in the patient with the severe anaemia. This result suggests that the high levels of ALAS mRNA observed in the patient with the acquired form of the disorder and the accompanying severe anaemia, are not characteristic of the acquired form of the disease, but are as suspected were brought about by the anaemic state of that patient .

The autoradiograph and bar graphs resulting from Northern analysis of hepatic type ALAS mRNA present in the bone marrow RNA prepared from the four sideroblastic patients and three healthy controls are shown in Fig 7.3B. In this experiment, 40μg of total liver RNA was analysed in lane 1, and the densitometric quantitation of this sample is represented by open bar in Fig 7.3B. 40μg of total RNA prepared from the bone marrow aspirate of three healthy controls are shown in lanes 2-4, and these samples correspond with those analysed in Fig 7.3A, lanes 2-4. As can be seen in Fig 7.3B, hepatic type ALAS mRNA is detectable in all of the RNA samples prepared from the bone marrow, consistent with the housekeeping function of the gene, discussed in Chapter one. The levels of hepatic type ALAS mRNA seen in the bone marrow RNA of the patients with acquired sideroblastic patients (lanes 5 and 8) are higher than the control sample shown in lane 3, and similar to those seen in the control samples shown in lanes 2 and 4. It is interesting to note that while the patient with the acquired form of

sideroblastic anaemia and the accompanying severe anaemia (lane 8), has elevated levels of all three erythroid mRNAs, of the four sideroblastic patients this patient has the lowest the level of hepatic type ALAS mRNA. A surprising result in this experiment is the high levels of hepatic type ALAS mRNA observed in the bone marrow RNA of the patients with the X-linked form of the disease (lanes 6 and 7). The control sample with the highest level of hepatic type ALAS mRNA is the one shown in lane 2, and both of the patients with the X-linked form of the disorder have at least two fold more hepatic type ALAS mRNA than that healthy control sample.

The levels of α and ß globin mRNAs present in 10μg samples of bone marrow RNA prepared from the four sideroblastic patients and the four healthy controls are shown in Fig. 7.4A and B. The control samples are shown in lanes 1-4, the samples from the X-linked sideroblastic are in lanes 6 and 7, the two patients with acquired form of sideroblastic anaemia are in lanes 5 and 8. The patient who was not anaemic is in lane 5, and the sample from the severely anaemic patient is in lane 8. Interestingly, a decrease in the levels of both α and ß globin mRNAs are seen in the patients with the X-linked sideroblastic anaemia, relative to the control samples. In addition, the decrease in the level of α globin mRNA in these patients appears to be more pronounced than the corresponding decrease in the level of ß globin mRNA, which is consistent with the asynchrony of α chain synthesis thought to be associated with the condition (White *et al.*, 1971; Bottomley, 1980).

The levels of both α and ß globin mRNAs in the patient with the acquired form of the disorder, shown in lane 5, are similar to those present in the healthy control samples. However, comparison between the two acquired patients again shows an increase of approximately five fold in the levels of these mRNAs in the patient with acquired sideroblastic anaemia and the accompanying severe anaemia. As discussed previously this result suggests that the elevated mRNA levels seen in this patient are the result of the anaemia, and are not characteristic of the acquired form of the disorder.

The previous experiments showed that the levels of erythroid ALAS, and α and ß globin mRNAs present in the bone marrow RNA of the two X-linked sideroblastic patients were decreased when compared to the levels present in four healthy control samples. It was

Figure 7.4 Northern blot analysis of bone marrow mRNAs for α and ß globin in Sideroblastic patients.

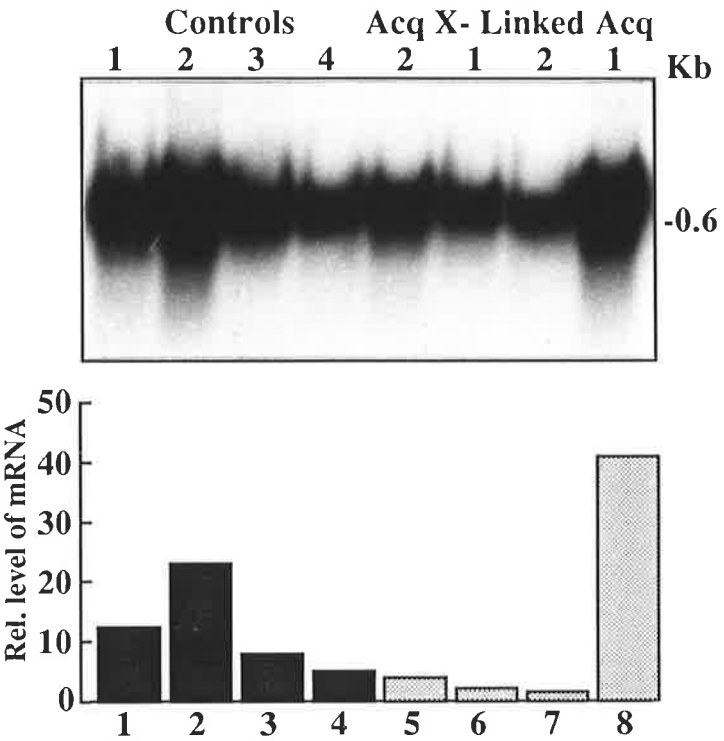
Total RNA (40μg) prepared from the nucleated bone marrow cells from four healthy individuals (C) and four sideroblastic patients, two samples from patients with an acquired form (Acq.) and two samples from patients with the X-linked form (X-L) was electrophoresed on 1% agarose gels containing 1.1M formaldehyde. Following Northern blot transfer Nytran™ (Section 2.3.3) and probed with oligolabelled genomic probes for (A) α- globin or (B) ß-globin (Section 2.2.14). The autoradiographs from these experiments are shown. The levels of each globin mRNA were quantitated by densitometry, and expressed as arbitrary units are presented as bar graphs below the autoradiographs. The arbitrary units refer to each particular mRNA and are not comparable between autoradiographs.

lanes 1-4 bone marrow RNA prepared from healthy individuals

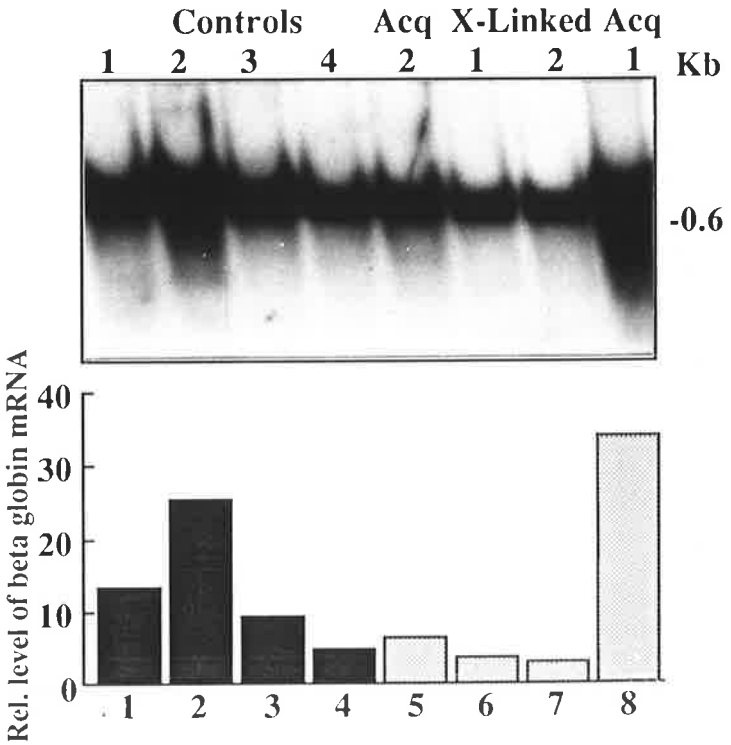
lanes 5 and 8 bone marrow RNA from patients with acquired sideroblastic anaemia

lanes 6 and 7 bone marrrow RNA from patients with X-linked sideroblastic anaemia

A α - Globin



B β - Globin



important to determine whether the observed decreases were specific for these three mRNAs, or represented a general decrease in erythroid mRNA in the bone marrow of these patients. In order to do this, it was necessary to compare the levels of another erythroid specific mRNA, present in the bone marrow RNA of the sideroblastic patients with the healthy control samples.

The major sialoglycoprotein of the human erythrocyte membrane, glycophorin A, is specifically expressed in cells of the erythroid lineage (Siebert and Fukuda, 1986 a and b). As this glycoprotein is a cell surface protein the levels of glycophorin A mRNA present in the erythroid cells of the sideroblastic patients are not likely to be lower than in the healthy controls. Therefore, any differences detected in the levels of glycophorin mRNA would reflect differences in the total amount of erythroid mRNA present in the bone marrow RNA samples. The human glycophorin A cDNA clone, designated H2L, was obtained from Dr. M. Fukuda, La Jolla Cancer Research Foundation, La Jolla. This 900bp clone includes the translation initiation site of the protein and about 350bp of the 3' untranslated sequence. Glycophorin A has at least three differently sized mRNAs and currently it is not clear whether this results from the use of several transcriptional start sites or several polyadenylation sites. Alternatively, they may be due to incomplete or differential splicing of the primary transcript (Siebert and Fukuba, 1986a). The H2L cDNA clone detects two glycophorinA-specific bands approximately 1.7 and 1.0kb in size. The 1.7kb band is the major band detected (Dr. M. Fukuda personal communication) and was used to quantitate the levels of glycophorin mRNA present in the bone marrow RNA samples.

Northern blot analysis of 40μg of total bone marrow RNA from the four sideroblastic patients and the four healthy controls was carried out as described previously. The filter was hybridized to the ^{32}P-labelled human glycophorin A cDNA probe H2L, and the autoradiograph from this experiment is shown in Fig. 7.5A. Two mRNAs were detected a major band of 1.7kb and a minor band of 1.0kb. The 1.7kb band was quantitated by densitometry and bar graphs representing this quantitation are presented below the autoradiograph in Fig 7.5A. The samples from the healthy controls are represented by solid black bars, and the samples from the sideroblastic patients by shaded grey bars.

Figure 7.5 Northern and slot blot analysis of bone marrow mRNAs for glycophorin A and ß-actin in Sideroblastic patients.

(A) Total RNA (40µg) prepared from the nucleated bone marrow cells from four healthy individuals (C) and four sideroblastic patients, two samples from patients with an acquired form (Acq.) and two samples from patients with the X-linked form (X-L) was electrophoresed on 1% agarose gels containing 1.1M formaldehyde. Following Northern blot transfer Nytran™(Section 2.3.3) and probed with the oligolabelled cDNA for human glycophorin A. The autoradiograph resulting from this experiment is shown. The autoradiograph was quantitated by densitometry and the levels of glycophorin A mRNA present in each sample, expressed as arbitrary units is presented as bar graphs below the autoradiograph.

lanes 1-4 bone marrow RNA prepared from healthy individuals

lanes 5 and 8 bone marrow RNA from patients with acquired sideroblastic anaemia

lanes 6 and 7 bone marrrow RNA from patients with X-linked sideroblastic anaemia

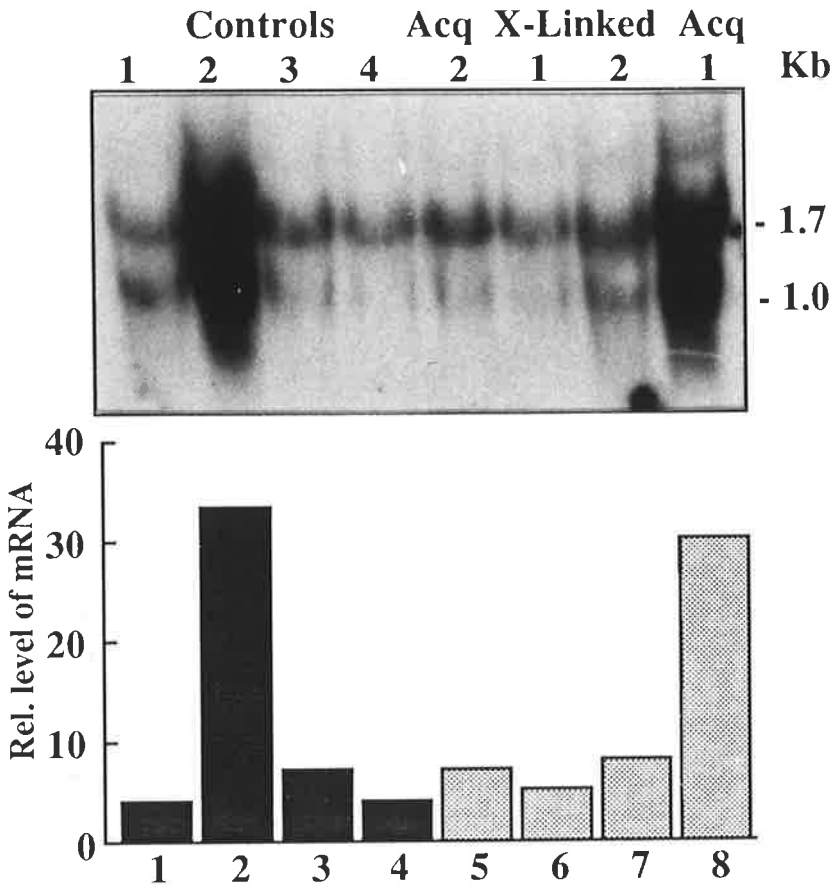
(B) 5µg of total RNA prepared from the bone marrow of a healthy individual (C) a patient with an acquired form of sideroblastic anaemia (Acq.) and samples prepared from two patients with X-linked sideroblastic anaemia (X-L) was immobilized on nitrocellulose, and probed with the oligolabelled cDNA for human ß-actin (Section 2.2.14). The autoradiograph from this experiment is shown. The levels of ß-actin mRNA present in each sample was quantitated by densitometry, and expressed as arbitrary units are presented as bar graphs next to the autoradiograph.

slot 1 bone marrow RNA prepared from a healthy individual

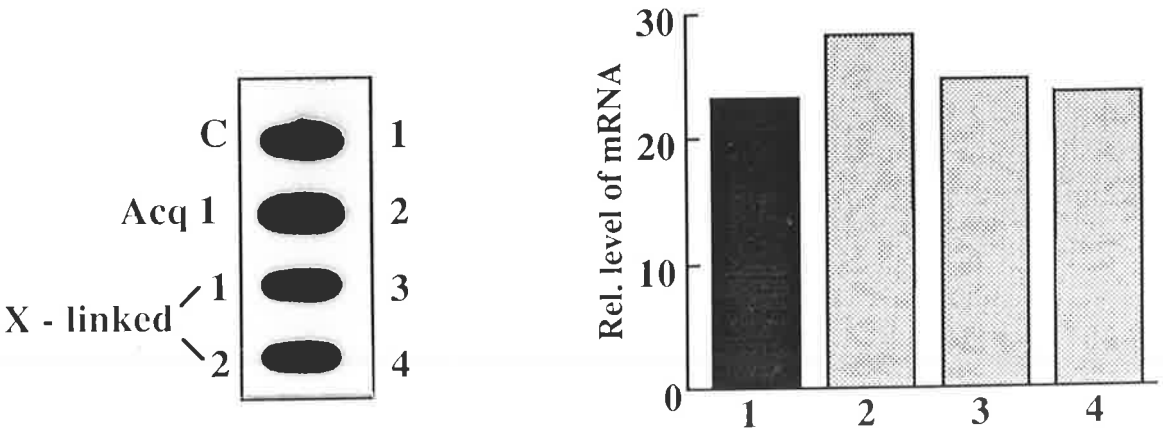
slot 2 bone marrow RNA prepared from a patient with acquired sideroblastic anaemia

slots 3 and 4 bone marrow RNA prepared from two patients with X-linked sideroblastic anaemia

A Glycophorin A



B β -Actin



As can be seen in Fig 7.5A, the levels of glycophorin A mRNA present in the samples from the healthy controls (lanes 1-4) vary between individuals. However, the control sample shown in lane 2 has an exceptionally high level of glycophorin A mRNA, which is at least four times higher than the levels present in the other three samples prepared from healthy controls. As discussed above, this result strongly suggests that the bone marrow aspirate used to prepare the RNA sample shown in lane 2 must have contained a higher percentage of developing erythroid cells. As the high levels of the other three erythroid specific mRNAs support this finding, it is probably not appropriate to compare the levels of erythroid mRNAs present in the sideroblastic patients with this sample. Similarly, the high level of glycophorin A mRNA present in the patient with acquired sideroblastic anaemia and the accompanying severe anaemia (lane 8), which is approximately four fold more than the level present in the other acquired sideroblastic patient who was not anaemic, is consistent with the increased numbers of immature early erythroblasts found to be present in the bone marrow aspirate of this patient from which the RNA sample was prepared.

Importantly, despite the variation in the levels of glycophorin A mRNA present in the control samples, the X-linked sideroblastic patients (lanes 6 and 7) do not have decreased levels of glycophorin A mRNA relative to the controls shown in lanes 1, 3 and 4. Again the sample number used in this study is too small to determine whether the observed differences between the levels of mRNA present in the samples from the healthy controls and the X-linked sideroblastic patients are statistically significant. However, the mean of the densitometry units for the control samples shown in lanes 1, 3 and 4 is 5.2, and the densitometry units for the X-linked sideroblastic patients are 5.2 and 8.2 respectively. This result suggests that the decrease in the levels of erythroid ALAS, and α and ß globin mRNAs observed in the bone marrow RNA samples prepared from the X-linked sideroblastic patients, are not due to an under representation of erythroid cells in the bone marrow aspirate from which the RNA was prepared.

As a further control, the levels of ß-actin mRNA present in the bone marrow RNA samples of the two X-linked patients sideroblastic patients and a healthy control were determined. As ß-actin is a housekeeping gene which is expressed in all cells the levels of

ß-actin mRNA would be expected to remain constant if the amount of mRNA present in the total RNA samples was constant. Total RNA (5µgs per slot) from the two X-linked sideroblastic patients (slots 3 and 4), the patient with the acquired form of the disease (slot 2) and the severe anaemia, and one healthy control (slot 1) was immobilized on nitrocellulose and the filter was probed with the ^{32}P-labelled cDNA for human ß-actin. The autoradiograph from this experiment and bar graphs representing densitometric quantitation of the autoradiograph, are shown in Fig.7.5B. As can be seen in Fig. 7.5B, the levels of ß-actin mRNA present in all three the sideroblastic patients (slots 2-4) are similar to that present in the control sample (slot 1).

7.3 DISCUSSION

As discussed in Section 7.1, since the sideroblastic anaemia syndromes were first recognized defective erythroid heme biosynthesis has been considered the principal underlying disturbance. The reduced ALAS activity associated with the disorder has led to a defective ALAS being implicated, at least as a contributing factor, to the pathogenesis of some types of sideroblastic anaemia (Bottomley and Müller-Eberhard, 1988). The aim of the work described in this Chapter, was to examine the possibility that the reduced levels of ALAS activity observed in sideroblastic anaemia are associated with reduced levels of ALAS mRNA in the erythroid cells of the bone marrow of patients with sideroblastic anaemia.

Initial experiments, were carried out using the human hepatic ALAS cDNA clone to investigate the levels of hepatic type ALAS mRNA in the bone marrow of two sideroblastic patients one with a congenital and one with an acquired form of the disorder. The results of these initial experiments were disappointing. The levels of hepatic type ALAS, α, and ß globin mRNAs present in the RNA prepared from the bone marrow of the two sideroblastic patients were found to be similar to those present in the samples prepared from two healthy controls. However, when the cDNA for the human erythroid specific ALAS isozyme was isolated in this laboratory, and the erythroid ALAS gene subsequently localized to the X chromosome (Cox *et al.*, 1990), the possible involvement of erythroid ALAS in sideroblastic anaemia, particularly in the X-linked form of the disorder, became a strong possibility and the situation was re-examined.

The most important finding in this study was seen when Northern analysis was used to compare the levels of erythroid ALAS mRNA present in RNA prepared from the bone marrow cells of two X-linked sideroblastic patients and from four healthy individuals. While the levels of erythroid ALAS mRNA present in bone marrow RNA were found to vary between individuals, the levels present in the samples from the two X-linked sideroblastic patients were lower than in any of the four samples from healthy individuals (Fig 7.3). This finding represents the first clue at the molecular level that ALAS may be involved in the pathogenesis of this disorder.

Interestingly, a similar result was obtained when the levels of α and ß globin mRNA present in the bone marrow RNA of the two X-linked sideroblastic patients were compared to those present in the samples from the healthy controls. While again the levels of α and ß globin mRNA varied between the four control samples (Fig 7.4) the levels observed in RNA samples prepared from the bone marrow cells of the X-linked sideroblastic patients were lower than those present in any of the four samples prepared from healthy individuals. It is also interesting to note that in the bone marrow RNA of the X-linked sideroblastic patients the decreases observed in the levels of α globin mRNA are more pronounced than the decreases in ß globin mRNA levels (Fig 7.4). As mentioned in Section 7.2.2, this is consistent with the asynchrony of α chain synthesis reported to be associated with the condition (White *et al.*, 1971; Bottomley, 1980).

Importantly, the levels of glycophorin A mRNA present in the bone marrow RNA of the X-linked sideroblastic patients were not lower than those present in three of the healthy control samples (Fig.7.5). This finding established that the decreases seen in the levels of erythroid ALAS and α and ß globin mRNA (Figs 7.3 and 7.4) are specific, and do not simply reflect an under representation of erythroid mRNA in the RNA prepared from the bone marrow aspirate of the X-linked patients. It is also interesting to note that the levels of erythroid ALAS, and α and ß globin mRNAs follow the same pattern in all samples prepared from the healthy controls and the sideroblastic patients (Figs 7.3 and 7.4). For example, the levels of all three of these mRNAs are relatively high in the control sample shown in lane 1, and relatively low in control sample shown in lane four. However, when the levels of glycophorin mRNA are

compared in the corresponding samples, the control sample shown in lane one has marginally less glycophorin A mRNA than the control sample shown in lane four (Fig 7.5).

In the developing erythroid cell, heme is known to regulate the translation of globin mRNA. Heme is required for the initiation of translation of globin mRNA, and an increase in heme biosynthesis precedes the induction of globin synthesis (Beru *et al.*, 1983; Charnay and Maniatis, 1983). In addition, several studies indicate that heme also affects globin production at the transcriptional level. In MEL cells hemin induces accumulation of globin mRNA as well as globin protein and this occurs more rapidly in response to DMSO, the inducer of terminal erythroid differentiation in these cells (Ross and Sautner, 1976). Similarly, inhibition of heme synthesis by isoniazid in MEL cells and mouse foetal liver cells is associated with inhibition of globin mRNA levels, and this is overcome by addition of hemin (Hoffman and Ross, 1980). Finally, in K562 cells, which express low levels of several globin mRNAs constitutively, hemin increases both the rate of transcription and the level of accumulation of the various globin mRNAs (Charney and Maniatis, 1983). The results obtained in this study support the concept that the levels of erythroid ALAS and globin mRNAs present in the developing erythroid cell are in some way linked. However, whether this is by regulation at the transcriptional level, or by some post-transcriptional mechanism(s) or a combination of these remains to be determined.

As mentioned above, the levels of erythroid ALAS, and α and ß globin mRNAs present in the bone marrow RNA analysed from the healthy individuals during this study, were found be quite variable between individuals. It is possible that the levels of these mRNAs in each individual fluctuate with the physiological state of that individual, particularly in circumstances where the hemoglobin level is lowered. Thus it may be questioned as to whether the decreases observed in the levels of these mRNAs in the bone marrow RNA of the X-linked sideroblastic patients are significant, particularly since the sample number used in this study was small. However, as the glycophorin A mRNA levels in the X-linked sideroblastic patients are not lower than the levels present in the three healthy controls, the results of this study do suggest that erythroid ALAS is involved in the pathogenesis of the condition. As further support for this proposal, the inherited form of the sideroblastic anaemia appears to follow an X-linked pattern of inheritance (Bottomley, 1980; Bottomley and Müller-Eberhard,1988), and

defects of erythroid ALAS would be most plausible in the X-linked forms of the disorder since the gene for the erythroid enzyme has been localized to the X chromosome (Cox *et al.*, 1990).

Further work is required to confirm and extend the results obtained in this study before it can be established whether a defective erythroid ALAS is the underlying defect in the condition. If the expression of the erythroid ALAS gene is decreased then the mechanism by which this down regulation of erythroid ALAS expression occurs remains to be elucidated. It is possible that a mutation in the erythroid ALAS gene, or the promoter of the gene, may result in either a decrease in transcription of the gene, or a decrease in the efficiency of the processing and/or transport of the mRNA from the nucleus. Alternatively, a mutated form of erythroid ALAS mRNA may be less stable when it reaches the cytoplasm.

An unexpected result in this study was the apparent increase observed in the levels of hepatic type ALAS mRNA in the bone marrow RNA of the X-linked sideroblastic patients. One might speculate that in the developing erythroblasts of the X-linked sideroblastic patients, the lower levels of erythroid ALAS mRNA cause a corresponding decrease in the intracellular levels of heme. This lowering of the intracellular heme level may then induce transcription of the hepatic form of ALAS, which is known to be responsive to heme levels in other tissues (Srivastava *et al.*, 1988). Induction of the hepatic form of ALAS would then provide the cells with a mechanism by which to partially compensate for the lower levels of erythroid ALAS. However, the RNA used in this study was prepared from a heterogeneous population of nucleated bone marrow cells and it is not known how much of the hepatic type ALAS mRNA is of erythroid origin. Therefore, before this result can be considered significant, it needs to be established that the levels of hepatic type ALAS mRNA are elevated in the developing erythroid cells of the X-linked sideroblastic patients.

Interestingly, the levels of erythroid mRNAs present in the bone marrow RNA of the female patient with the congenital form of sideroblastic anaemia did not show the decreases observed in the X-linked form of the disorder. However, family studies have shown that affected females usually suffer only minor erythroid disorders, and are rarely anaemic (Bottomley 1980). As inactivation of the X chromosome in the cells of human females is a random process, not all of the erythroid cells in the bone marrow of females with the congenital

form of sideroblastic anaemia would be affected. Unfortunately, only one sample was available for analysis from a patient with the congenital form of the disorder. However, the findings in this study suggest that in females with the congenital form of sideroblastic anaemia the affected cells present in the bone marrow of these patients are adequately compensated for by normal cells and the level of erythroid ALAS mRNA appears to be within the normal range. As might be expected from the apparently normal levels of erythroid ALAS mRNA, the levels of α and ß globin mRNAs present in the bone marrow RNA from this patient were similar to those seen in the healthy controls.

In initial experiments, high levels of erythroid ALAS and α and ß globin mRNAs were observed in the bone marrow RNA prepared from the patient with an acquired form of sideroblastic anaemia (Figs. 7.2 and 7.3). As discussed Section 7.2, analysis of the bone marrow aspirate from this patient established that this patient had a very low hemoglobin level, which would cause increased erythropoietin production with consequent erythroid hyperplasia, and this was confirmed by the bone marrow differential. Analysis of bone marrow RNA from a second patient with an acquired form of the disorder, who was not severely anaemic, was then carried out. In the bone marrow RNA from this second patient, the levels of erythroid ALAS and α and ß globin mRNAs, were similar to those present in the samples prepared from the healthy controls (Fig 7.3). The levels of glycophorin A mRNA present in bone marrow RNA of the two acquired sideroblastic patients (Fig 7.5) confirmed that the bone marrow aspirate used to prepare the RNA from the severely anaemic patient contained either more erythroid cells, or a higher percentage of early erythroblasts. From these experiments, it appears that in the acquired form of sideroblastic anaemia, the levels of mRNA for erythroid ALAS and α and ß globin present in bone marrow RNA are not lower than in samples prepared from healthy individuals.

The results in this study do not provide any insight into what might be happening in the acquired form of sideroblastic anaemia, except to suggest that the aetiology may be quite distinct from that of the inherited form. Even among the X-linked cases considerable heterogeneity is observed in the clinical course of the disease, (Bottomley, 1980; Bottomley and Müller-Eberhard,1988), and the two patients with the acquired form of the disorder involved in

this study may represent just one of a whole range of abnormalities which produce the symptoms characteristic of the disease.

Although the results from this work cannot be considered conclusive, they are sufficiently encouraging for the possible involvement of erythroid ALAS in sideroblastic anaemia to be pursued further. Work directed towards examining the levels of erythroid ALAS and α and ß globin mRNAs present in reticulocytes is currently being undertaken. Recent advances in the use of polymerase chain reaction technology for such work indicate that the small amount of erythroid ALAS message present in the reticuloctyes circulating in the blood can be amplified to look for mutations in the mRNA of affected individuals, and Prof. Bottomley is currently pursuing this approach. Ongoing work directed towards finding a restriction fragment polymorphism associated with the disorder, which would allow pre-natal diagnosis and individuals at risk to be identified is also being undertaken in collaboration with our research group. Currently, the work being undertaken in our laboratory is directed primarily towards understanding the transcriptional regulation of the erythroid ALAS gene, which we hope may allow provide some insight into the underlying molecular defect(s) in sideroblastic anaemia.

CHAPTER EIGHT

FINAL DISCUSSION AND SUMMARY

CHAPTER EIGHT: FINAL DISCUSSION AND SUMMARY

Since the early work of Granick (1966) established that ALAS levels in chick embryo hepatocytes could be increased by drugs and that heme could prevent this increase, it has been thought that heme biosynthesis in the liver is controlled by end product repression of the first enzyme in the pathway, ALAS. As discussed in Chapter one, heme is thought to exert this control at a number of levels. It is well established that an important regulatory level is at the import of the ALAS precursor protein into the mitochondria, and recent work carried out in this laboratory provided evidence that *in vivo* heme is able to regulate the transcriptional rate of the rat hepatic ALAS gene. However, the molecular mechanism for this control remains to be elucidated.

The transcriptional regulation of the gene for the hepatic form of ALAS is of particular interest, since most of the well characterized mechanisms of eukaryotic gene expression involve regulation of induction or development, and to date few studies of negatively regulated housekeeping genes have been conducted, with the notable exception of two genes in the *de novo* cholesterol biosynthetic pathway (Gil *et al.*, 1986; Goldstein and Brown, 1984).

The work presented in this thesis was aimed at providing an initial understanding of the transcriptional regulation of the human hepatic ALAS gene, and to provide a solid basis for further work aimed at elucidating the molecular mechanism(s) by which the hepatic ALAS gene is regulated by heme and the drugs which induce the cytochrome P450 proteins.

Initially, the base sequence of the human hepatic ALAS precursor was determined from several different cDNA clones which together encompass the entire coding region of the protein (Chapter three). Comparison of the predicted amino acid sequence of human hepatic ALAS with ALAS sequences from eukaryotic and prokaryotic sources established that the region of the protein thought to contain the catalytic site is highly conserved throughout evolution.

The human hepatic ALAS gene was isolated from a genomic cosmid library by cross hybridization with the cDNA clone (Chapter four). The 5' flanking region of the gene was

isolated and partially characterized by restriction enzyme mapping and DNA sequence analysis. The transcriptional start site of the gene was determined by RNAse protection analysis of total RNA prepared from human liver samples. This work established that unlike the chicken hepatic ALAS gene, but like the rat hepatic ALAS gene, the human ALAS gene contained an intron in the 5' untranslated region. Computer analysis of the rat and human intronic sequences detected several regions of quite high nucleotide sequence similarity, particularly towards the 3' end of the intron. Computer analysis also established that both the immediate promoter region of the gene and the intron contained an interesting array of putative regulatory elements. One particularly interesting element is the NRF-1 consensus sequence. Gel retardation and DNAse footprinting experiments are currently underway in this laboratory, to investigate whether this element is involved in the regulation of the human hepatic ALAS gene.

Chimeric ALAS/hGH promoter constructions were prepared, and their transient expression was examined in HepG2 and CHO cultured cell lines (Chapter five). It was found in both cell lines that the promoter constructions that contained the entire intron in the 5' untranslated region of the gene expressed at a much lower level than the corresponding promoter constructions containing only the first 140bp of intronic sequence. Ongoing work in this laboratory is aimed at determining the intronic sequences responsible for the observed decrease in expression of the promoter constructions. Unfortunately, attempts to achieve repression of the promoter constructions by the addition of hemin or ALA to the transfected cells was unsuccessful in both cell lines, as were attempts to increase expression by the addition of succinyl acetone. Subsequent investigation of transcription of the endogenous hepatic ALAS gene in HepG2 cells, by nuclear transcription run-on experiments established that addition of heme, ALA or succinyl acetone did not affect the rate of transcription of the endogenous ALAS gene. Therefore, it appears likely that the regulation of the ALAS gene in these cell lines involves some other mechanism(s) than that operating in the whole animal.

The levels of hepatic ALAS mRNA present in nine individual human liver samples were investigated by Northern analysis of total RNA, using cross-hybridization with the human hepatic cDNA (Chapter six). Three of the patients had received dexamethasone therapy prior to organ donation and the levels of the dexamethasone inducible P450IIIA 1, were also examined.

Although the levels of both ALAS and P450IIIA 1 mRNAs were found to be elevated in the livers of the patients who had received dexamethasone therapy, the magnitude of the increases were not in parallel, and it seems likely that at least in two of the samples some other agent(s) may have contributed to the high levels of ALAS mRNA observed.

The final project described in this thesis (Chapter seven), involved Northern blot analysis of mRNA prepared from the nucleated bone marrow cells of patients with congenital and acquired sideroblastic anaemia. At the time this work was undertaken, it was well established that the principle characteristic of the disorder is impaired heme biosynthesis but little was known at the molecular level about the biochemical defects underlying the condition. Initially, the human hepatic cDNA was used as the probe to investigate the levels of ALAS mRNA in the bone marrow of two sideroblastic patients, and these were found to be similar to those seen in normal healthy individuals. However, when the human erythroid specific ALAS cDNA was isolated in our laboratory, and the erythroid ALAS gene was subsequently localized to the X chromosome, the involvement of erythroid ALAS in sideroblastic anaemia became a strong possibility and the situation was re-examined.

The steady state levels of erythroid ALAS, hepatic type ALAS, α globin, ß globin and glycophorin A mRNAs in the both marrow of sideroblastic patients and healthy individuals were determined by Northern blot analysis. Despite a limited sample number of patients with various types of sideroblastic anaemia this analysis revealed a spectrum of findings. Decreased levels of erythroid ALAS mRNA were found only in the X-linked sideroblastic patients, and this represents the first indication at the molecular level that ALAS may be involved in the pathogenesis of this disorder. An interesting finding was the associated increased levels of the hepatic form of ALAS in these patients, which may represent de-repression of the gene in response to low cellular heme levels. Low heme levels may also account for the reduced levels of α and ß globin mRNAs in these patients, since as discussed in Chapter seven, there is some evidence that heme affects globin production at the transcriptional level. Importantly, the levels of glycophorin A mRNA were not reduced in these two patients, which established that the observed decreases in erythroid ALAS, α and ß globin mRNA were specific.

The findings in the patients with acquired sideroblastic anaemia were less informative. The normal levels of erythroid ALAS mRNA found in the bone marrow RNA of these patients, suggest that there is biochemical heterogeneity underlying the disorder.

Ongoing work in this area aimed at further analyzing the possible involvement of erythroid ALAS in sideroblastic anaemia, both at the protein and mRNA levels is currently being undertaken both in our laboratory and by Prof. Bottomley.

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