STUDIES ON THE 5-AMINOLEVULINATE SYNTHASE GENE AND ITS REGULATION

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SEQUENCE ANALYSIS OF THE CHICKEN ALV-S GENE

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SUMMARY

5-Aminolevulinate synthase (ALV-S) is the first enzyme of the heme biosynthetic pathway. This thesis is concerned with control of hepatic ALV-S synthesis by negative feedback regulation by heme. Briefly, the chicken ALV-S gene has been isolated and characterised, and transcription run-on experiments in isolated rat liver nuclei have demonstrated that control of ALV-S synthesis in liver is exerted primarily at the level of transcription initiation.

1. A full-length cDNA clone (p105B1) for chick embryo liver 5-aminolevulinate synthase (ALV-S) was used as a hybridization probe to isolate 13 clones from a chicken genomic library. These clones were characterized by restriction enzyme analysis and Southern blot hybridization with p105B1. The clones overlapped, spanning a contiguous region of 40 kb of genomic DNA. One clone (designated λ cALA-S 1) which hybridized to DNA from both the 5' and 3' ends of p105B1 was chosen for further analysis.

2. Total chicken genomic DNA and λ cALA-S 1 were each digested with several different restriction enzymes and analysed by Southern blot hybridization with p105B1, at high and low stringency. Identical hybridization patterns were observed, indicating that λ cALA-S 1 contains the entire ALV-S gene. Also, this result provides strong evidence that ALV-S is coded for by a single gene, and that no other related sequences are present in the chicken genome.

3. Sequences hybridizing to p105B1 were contained within two **Bam**HI restriction fragments of 4.3 kb and 5.6 kb. These were cloned from λ cALA-S 1 into pUC19. DNA prepared from each of these subclones was used to generate a library of random overlapping clones in M13, and restriction fragments overlapping the 5' end of the gene and the internal **Bam**HI site were also subcloned into M13 for sequencing. The sequence of a 5.2 kb region of DNA including the 4.3 kb **Bam**HI fragment is presented in this thesis. This sequence extends 995 bp upstream of the mRNA transcription start site.

4. The gene spans 6.9 kb and is divided into 10 exons (156-280 bp), split by 9 introns (91-1100 bp). The exon-intron boundaries all conform to the GT-AG rule and the consensus sequences for eukaryotic splice junctions. Sequences with homology to the branch-point sequences implicated in the splicing mechanism are also present.

5. The ALV-S promoter contains many elements which have been shown to be important in the control of eukaryotic gene transcription. Two TATA boxes are present, at positions -30 and -71 relative to the transcription start site (+1) and CAAT boxes are located approximately 70 bp upstream of each TATA box.

Several features common to many eukaryotic housekeeping genes are also found. Five GC hexanucleotide boxes are present within 110 bp of the transcription start site. The promoter region has a high G+C content, averaging 62% G+C in a 1.5 kb region extending from 995 bp upstream of the transcription start site into the first intron. This high G+C content is also associated with clustered CpG dinucleotides.

An unusual tandemly repeated element is present at position -160 relative to the transcription start site (+1). The 10 bp sequence CCCC(T/C)CATGG is reiterated 3 times in tandem and the sequence CCCCTCA also occurs twice, within a 50 bp segment of DNA. Another tandemly repeated element occurs at position -97. The 6 bp sequence CACGCC is repeated twice with a one nucleotide overlap and sequences similar to this occur in the yeast and bacterial ALV-S genes.

6. The transcriptional regulation of the rat ALV-S gene by heme and porphyrinogenic drugs was examined by transcription run-on experiments using nuclei isolated from the livers of normal rats and from animals treated with heme or its precursor ALV, or with the porphyrinogenic drug 2-allyl-2-isopropylacetamide (AIA), or with both. Treatment of normal animals with heme or ALV reduced ALV-S transcription to undetectable levels. Administration of AIA for 4 h increased ALV-S transcription 10 fold, and heme or ALV treatment completely prevented this induction. An unexpected finding was that transcription of a drug-inducible cytochrome P450 gene was also repressed by heme. Control experiments showed that the effects of heme and drugs were specific, since transcription of the serum albumin gene was unaffected. Heme and ALV had no effect on elongation of the ALV-S or cytochrome P450 transcripts **in vitro**, suggesting that administration of heme or drugs alters the rate of transcription initiation. The relative levels of ALV-S mRNA and enzyme activity correlated closely with the transcription rate of the ALV-S gene, and it is therefore likely that the major control of hepatic ALV-S synthesis is at the level of transcription initiation.

DECLARATION

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

In all of the experiments described in this thesis, the author was involved as the principal worker, however some of the work described in Chapters 4 and 5 was undertaken in collaboration with Adrienne Day and Dr. Gopesh Srivastava.

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.

Deborah Jane Maguire

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ABBREVIATIONS

AIA:	2-allyl-2-isopropylacetamide
ALV:	5-aminolevulinate
ALV-S:	5-aminolevulinate synthase
bp:	basepair
dNTP:	deoxyribonucleotide triphosphate
ddNTP:	dideoxyribonucleotide triphosphate
DTE:	dithiothreitol
DTT:	dithioerythritol
kb:	kilobase
PBG:	porphobilinogen
pfu:	plaque forming units
PMSF:	phenylmethylsulphonyl fluoride
poly(A):	poly adenylic acid
TCA:	trichloroacetic acid

URO decarboxylase: uroporphyrinogen decarboxylase

All other abbreviations were as listed in the Journal of Biological Chemistry "Instructions to Authors" (1986).

CHAPTER ONE

GENERAL INTRODUCTION

1.1 INTRODUCTION

5-Aminolevulinate (ALV) is the first committed intermediate of the tetrapyrrole biosynthetic pathway (Fig. 1.1). The tetrapyrroles are a diverse group of biological compounds, including heme (and the bile pigments produced by heme degradation), chlorophyll, the corrins (e.g. vitamin B12) and the phycobilin pigments of algae.

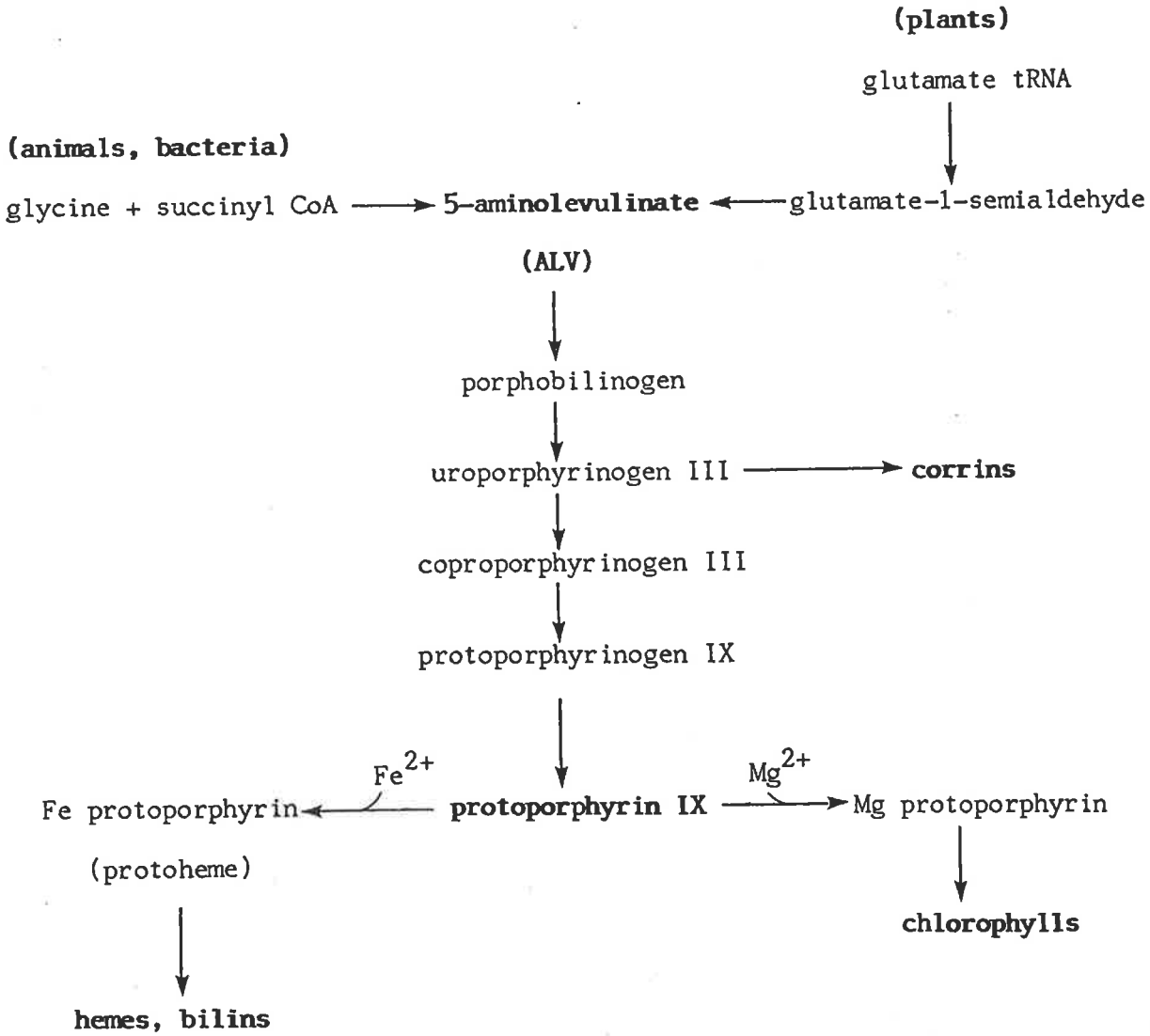
This thesis is concerned with the regulation of ALV and heme synthesis in animals, and a brief introduction to these topics is presented in this chapter. The porphyrias, a group of genetically inherited disorders of heme metabolism, are also discussed, since one of the longer term aims in understanding the control of heme biosynthesis is to aid in the prevention and treatment of these diseases. Recent reviews of these subjects include those by May **et al.** (1986), Maines (1984), Ibrahim **et al.** (1983) and Kappas **et al.** (1983).

1.2 THE HEME BIOSYNTHETIC PATHWAY

Heme is essential for the function of all aerobic cells since it is required as the prosthetic group for many hemoproteins including the cytochromes of both the mitochondrial respiratory chain and the microsomal mono-oxygenase system, the oxygen carriers hemoglobin and myoglobin, and enzymes such as catalase, peroxidase and tryptophan pyrrolase. In humans, 80% of heme is synthesized in the erythropoietic cells of the bone marrow, providing heme largely for the production of hemoglobin. Most of the remaining heme is synthesized in the liver, and the bulk of this is incorporated into the various cytochrome P450s, the terminal oxidases of the microsomal mono-oxygenase system (Tait, 1978).

FIGURE 1.1

THE TETRAPYRROLE BIOSYNTHETIC PATHWAY



In bacteria and animals, the first enzyme of the heme biosynthetic pathway (Fig. 1.2) is 5-aminolevulinate synthase (E.C. 2.3.1.37). 5-Aminolevulinate synthase (ALV-S) catalyzes the condensation of glycine and succinyl CoA to form ALV. The enzyme requires pyridoxal phosphate as a cofactor. In the liver, the levels of ALV-S are very low, and it is the rate-limiting enzyme of heme biosynthesis. The other enzymes of the pathway apparently occur in non-limiting amounts in the liver, apart from porphobilinogen deaminase (see below), which is present at a level similar to that of ALV-S, and may become limiting under conditions of increased ALV production (Kappas **et al.**, 1983). Further evidence for the rate limiting role of hepatic ALV-S ~~is~~ that addition of exogenous ALV, but not ALV precursors, increases the rate of heme synthesis (Granick and Urata, 1963; Granick, 1966; De Matteis and Gibbs, 1972; De Matteis, 1975). In keeping with its regulatory function, ALV-S has a very short half-life (around 35 min in rat liver; Hayashi **et al.**, 1972) compared to most other mitochondrial proteins, which have an average half-life of around 5 days (Kappas **et al.**, 1983).

In animals, ALV-S functions in the mitochondria, where its substrate succinyl CoA is produced. The next four reactions in the pathway take place in the cytosol (Fig. 1.2). ALV dehydratase catalyses the condensation of two molecules of ALV to form the pyrrole, porphobilinogen (PBG). Four molecules of PBG are condensed by PBG deaminase to produce a linear tetrapyrrole, hydroxymethylbilane. This cyclizes in the presence of uroporphyrinogen III cosynthetase to form the type III isomer of uroporphyrinogen (URO), and four sidechain carboxyl groups are removed by URO decarboxylase, producing coproporphyrinogen III. The last three enzymes of the pathway are located in the mitochondria. Oxidative decarboxylation by coproporphyrinogen oxidase produces protoporphyrinogen IX and this is oxidized to protoporphyrin IX by protoporphyrinogen IX oxidase.

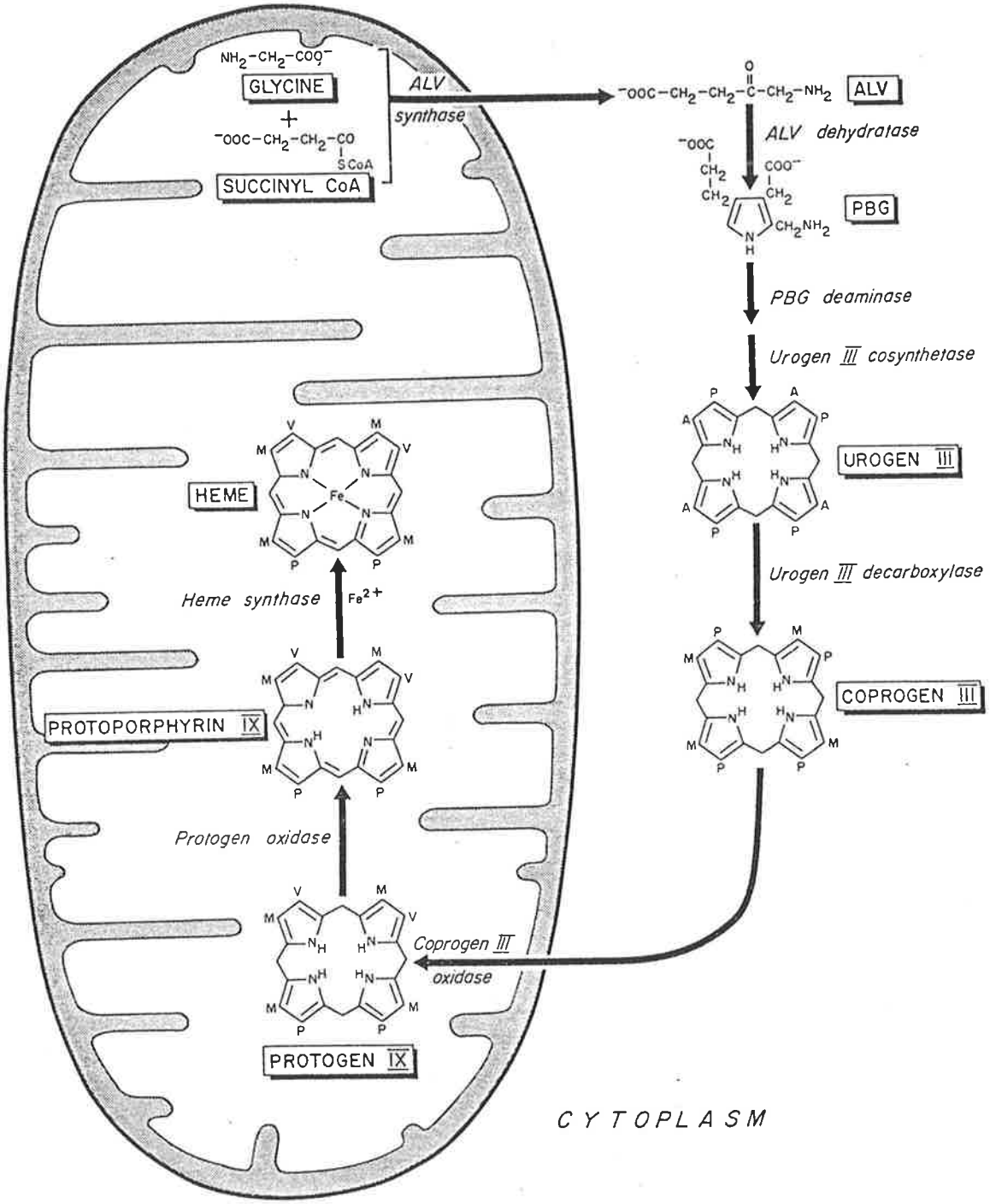


FIGURE 1.2 **THE HEME BIOSYNTHETIC PATHWAY**

ALV, 5-aminolevulinate; **PBG**, porphobilinogen; **UROGEN**, uroporphyrinogen **COPROGEN**, coproporphyrinogen; **PROTOGEN**, protoporphyrinogen; **A**, acetate; **M**, methyl; **P**, propionate; **V**, vinyl. (From May **et al.**, 1986).

Finally, heme is formed by the insertion of Fe^{2+} into protoporphyrin IX, catalyzed by ferrochelatase (Kappas **et al.**, 1983).

ALV-S activity has not been demonstrated in greening plants (Granick and Beale, 1978) and if ALV-S does in fact exist in plants, it plays only a minor role in the synthesis of ALV. Instead, ALV is synthesized in the chloroplast by a novel method in which glutamate is activated by attachment to a chloroplast-encoded tRNA, reduced to glutamate-1-semialdehyde and transaminated to form ALV (Kannangara **et al.**, 1984; Schoen **et al.**, 1986). The ALV is used largely in the production of chlorophyll but is probably also utilized for heme synthesis. All of the heme biosynthetic pathway intermediates are present in the chloroplast, since the steps from ALV to protoporphyrin IX are common to the biosynthetic pathways for both heme and chlorophyll (see Fig. 1.1), and ferrochelatase, required to form heme from protoporphyrin IX, is also found in chloroplasts (Porra and Lascelles, 1968).

ALV may also be synthesized from glutamate in animals, by transamination of the intermediate 4,5-dioxovalerate (DOVA) with alanine. Labelling studies in duck blood have suggested that as much as 25% of ALV may be derived from glutamate (Franck **et al.**, 1984), but further direct evidence is required to confirm this. Alanine:DOVA transaminase has been isolated from liver mitochondria (Varticovski **et al.**, 1980), but the physiological role of this enzyme is unclear since it also catalyzes the formation of glycine, by glyoxylate transamination, and this latter catalytic activity is far greater than transamination of DOVA (Noguchi and Mori, 1981).

cDNA clones have been isolated for many of the heme biosynthetic enzymes, including the following : rat and human spleen and human lymphoblast PBG deaminase (Grandchamp **et al.**, 1984; Raich **et al.**, 1986;

Dr. Y. Nordmann, personal communication); rat and human spleen URO decarboxylase (Romeo **et al.**, 1984; Romeo **et al.**, 1986); chicken, rat and human liver ALV-S (Borthwick **et al.**, 1984 and 1985; Srivastava **et al.**, 1987; Dr. I. Borthwick, personal communication); and rat and human liver ALV dehydratase (Bishop **et al.**, 1986; Wetmur **et al.**, 1986). The isolation of bacterial PBG deaminase and bacterial and yeast ALV-S genes has also been reported (Thomas and Jordan, 1986; Leong **et al.**, 1985; Urban-Grimal **et al.**, 1986). At the time the work in this thesis was commenced, none of the genes for any of the heme biosynthetic enzymes of higher eukaryotes had been isolated. The cDNA clones have been used in the investigation of many aspects of heme biosynthesis, and this is discussed in the relevant sections below.

1.3 STRUCTURE, PROPERTIES AND LOCALIZATION OF ALV-S

Eukaryotic ALV-S has only recently been isolated in its native form (Borthwick **et al.**, 1983). In order to obtain the native enzyme, rapid purification in the presence of protease inhibitors is required, since the enzyme is highly susceptible to proteolytical degradation. The native enzymes from chick embryo or rat liver mitochondria have molecular weights, as judged by SDS polyacrylamide gel electrophoresis, of 68 000 and 70 000, respectively (Borthwick **et al.**, 1983; Srivastava **et al.**, 1982). Prior to the development of the rapid purification method in this laboratory, ALV-S had been isolated only in a form of molecular weight 49 000 (Whiting and Granick, 1976a). This was probably the result of proteolytic degradation, since treatment of the native enzyme with proteases **in vitro** also produces a protein with a molecular weight of around 50 000 (Borthwick **et al.**, 1983). Interestingly, the degraded enzyme retains full catalytic activity.

The catalytic properties and sequential reaction mechanism of the native enzyme have been characterized (Pirola **et al.**, 1984a and 1984b). The enzyme is activated by high ionic strength solutions and by lead, and inhibited by p-hydroxymercuribenzoate, zinc and mercury ions. Native ALV-S is not inhibited by heme or hemin[1] at physiological levels (Pirola **et al.**, 1984a). This contrasts with previous work (Scholnick **et al.**, 1972; Whiting and Granick, 1976a; Paterniti and Beattie, 1979) using partially purified or degraded enzyme, which indicated that hemin inhibited ALV-S activity. Evidence against a physiological role of heme in feedback inhibition of ALV-S catalytic activity was obtained from experiments in isolated mitochondria in which the rate of endogenous heme generation was increased by supplying exogenous protoporphyrin and iron. Ferrochelatase and ALV-S activity were assayed and it was shown that ALV-S activity does not decrease, even when the rate of heme formation is increased to a level estimated to be at least 75 times the rate **in vivo** (Wolfson **et al.**, 1979).

As is the case for many other mitochondrial proteins (Hay **et al.**, 1984), ALV-S is synthesized in the cytosol as a higher molecular weight precursor protein, predominantly on free rather than membrane bound polysomes. This has been demonstrated by experiments in which ALV-S was synthesized from isolated polysomes or mRNA by **in vitro** translation, and analysed after immunoprecipitation (Whiting, 1976; Srivastava **et al.**, 1982 and 1983a). The chick embryo and rat liver precursor proteins have molecular weights of 74 000 and 76 000 respectively, and pulse chase labelling studies **in vivo** support the view that the higher molecular weight cytosolic enzyme is converted to the mature form by proteolytic

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1 Heme is ferroprotoporphyrin and is the form that exists in hemoproteins in the cell. Heme is readily autooxidized **in vitro** to ferriprotporphyrin 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 used experimentally.

cleavage during transport into the mitochondria (Yamauchi **et al.**, 1980; Srivastava **et al.**, 1982 and 1983a). Transport of ALV-S has also been demonstrated **in vitro.** The precursor form of ALV-S was synthesized by **in vitro** translation of isolated chicken liver polysomes, and after incubation with liver mitochondria it was shown that the enzyme had been incorporated into the mitochondria, with a concomitant reduction in size to that of the mature protein (Hayashi **et al.**, 1983).

Transport of ALV-S into the mitochondria is negatively regulated by heme. This is a unique and specific mechanism which is also physiologically very significant, since succinyl CoA, one of the required substrates for ALV-S, is generated only within the mitochondria. The cytosolic precursor protein is consequently presumed to be catalytically inactive **in vivo**, although there is no direct evidence for this. The cytosolic form of the enzyme can be detected by assaying **in vitro** in the presence of exogenous succinyl CoA (Hayashi **et al.**, 1972), although it is not known with certainty that it is the precursor form of the enzyme (rather than the proteolytically degraded form) which is catalytically active.

Evidence for the effect of heme on ALV-S translocation was first provided by Hayashi **et al.** (1972), who showed that in rats in which ALV-S levels were elevated by drug treatment (Section 1.4B), administration of hemin caused accumulation of enzyme in the cytosol which was associated with a drop in the level of intra-mitochondrial enzyme. This has been supported by kinetic studies, utilizing pulse-chase techniques and a specific antibody to detect ALV-S (Yamauchi **et al.**, 1980). Inhibition of ALV-S transport by heme has also been demonstrated in adult and embryo chicken liver (Ohashi and Kikuchi,

1972; Srivastava **et al.**, 1983b), and heme also prevented translocation of chicken ALV-S **in vitro** in the system discussed above (Hayashi **et al.**, 1983).

This novel negative feedback mechanism appears to be specific for ALV-S. The transport of ALV-S and another mitochondrial enzyme, pyruvate carboxylase, were examined by pulse labelling studies in chick embryo liver, and specific antibodies were used to quantitate each of the enzymes in the same mitochondrial preparations. Heme had no effect on the transport of pyruvate carboxylase, under conditions where ALV-S transport into the mitochondria was completely prevented (Srivastava **et al.**, 1983b).

Inhibition of translocation by heme would provide a rapid mechanism to prevent accumulation of catalytically functional ALV-S within the mitochondria. As discussed in Section 1.4A, it seems likely that the primary effect of heme is to repress transcription. Heme repression may occur at a post-transcriptional step, possibly translation. However, this is supported by indirect evidence only, and higher levels of heme are required than for transcriptional repression. If this is the case, it is possible that synthesis of ALV-S by translation of preexisting mRNA could still take place for some time, even after transcription of ALV-S mRNA ceased. The half-life of ALV-S mRNA in drug treated rats after administration of hemin was estimated as 1 hour, by measuring the decrease in the ability of isolated liver polysomes to direct **in vitro** translation of ALV-S (Yamamoto **et al.**, 1982a). Prevention of mitochondrial transport by heme may thus provide a more rapid mechanism for preventing the accumulation of enzymatically active ALV-S than repression of transcription alone.

Electron microscopy has provided further information on the structure and localization of ALV-S. Native chick embryo mitochondrial

ALV-S is a dimer with two identical subunits, which are curved and lie parallel to each other in a 'head to tail' arrangement (Pirola **et al.**, 1984c). Immunocytochemical studies, using monoclonal and polyclonal antibodies specific for ALV-S and a protein-A gold labelling technique, have shown that ALV-S in rat liver mitochondria is associated almost exclusively with the matrix side of the inner mitochondrial membrane (Rohde **et al.**, 1987). In these studies, the cytosolic form of ALV-S was also observed, ruling out the possibility that enzyme activity previously detected in the cytosol (see above) was an artifact due to leakage from the mitochondria during isolation of the cytosolic fraction. In these experiments, it was also shown that administration of the heme precursor ALV, which is rapidly converted to heme **in vivo** (Anderson **et al.**, 1981), reduced the level of protein-A gold labelling in both the mitochondria and cytoplasm in drug-treated rats. In some animals, accumulation of the precursor protein in the cytosol is observed after elevation of the levels of ALV-S by drug treatment (Section 1.4B), even in the absence of heme administration (Kikuchi and Hayashi, 1981). It has been proposed that this is a direct result of the mitochondria being unable to accommodate further enzyme, but the results from the immunocytochemical studies in rat liver do not support this. Accumulation of enzyme in the cytosol is observed even at early stages of drug induction, when the mitochondria still have the capacity to accomodate further enzyme (Rohde **et al.**, 1987). The reason for the cytosolic accumulation is unknown.

The complete amino acid sequences of both chick embryo and rat liver ALV-S have been deduced on the basis of sequence from cDNA clones which were recently isolated in this laboratory (Borthwick **et al.**, 1984 and 1985; Srivastava **et al.**, 1987). Alignment with the N-terminal sequence obtained from the mature protein indicates that both the chick and rat enzymes have a 56 amino acid N-terminal transit peptide which,

as discussed above, is thought to be cleaved during transport into the mitochondria. This transit peptide is predominantly basic, and if it is assumed to be in an α-helical conformation, then the basic residues are positioned mainly on one side. This amphiphilic helical structure may be required for targeting of proteins to the mitochondria since it seems to be the only common characteristic shared between the mitochondrial transit peptides which have so far been characterized; no significant primary sequence homology is apparent (von Heijne, 1986). The first 38 amino acids of the ALV-S presequence are highly conserved (87%) between rat and chicken, with less homology over the remainder (39%). In the mature protein sequence, the first 135 amino acids from the N-terminus are poorly conserved (47%; Pirola, 1986). It has been suggested that this N-terminal region may be required for interaction of the enzyme with protein or membrane components within the mitochondrion (Pirola, 1986). The remaining two thirds of the protein, extending to the C-terminus, is highly conserved (91%) and presumably contains the catalytic site.

The protein sequence of yeast ALV-S has been deduced from the gene sequence (Urban-Grimal **et al.**, 1986). The C-terminal region of the yeast protein is 41% homologous to the conserved C-terminal two thirds of the chicken enzyme. The N-terminal region (approximately 65 amino-acids) is not significantly homologous, but is nevertheless suggested to have a similar function to the chicken N-terminal region, encoding a mitochondrial transit peptide and a region required for structural interactions within the mitochondria (Urban-Grimal **et al.**, 1986).

It was proposed that the N-terminal 35 amino acids, which are rich in basic residues, represented the mitochondrial transit peptide. This is consistent with the size estimated by comparison of the molecular

weights (measured by SDS polyacrylamide gel electrophoresis) of the immunoprecipitated precursor protein, synthesized by **in vitro** translation or pulse-labelled **in vivo**, and the mature form of the protein, observed **in vivo** or after processing of the cytoplasmic form **in vitro** by the addition of isolated mitochondria (Urban-Grimal **et al.**, 1986). The exact site of cleavage needs to be confirmed directly, by obtaining N-terminal protein sequence from the mature protein, however it has been demonstrated that the nine N-terminal amino acids are sufficient to direct transport of a heterologous protein (β-galactosidase) into mitochondria (Keng **et al.**, 1986).

As mentioned above, the yeast N-terminal region, has no apparent homology with those of chicken or rat. This is not surprising, considering the general lack of sequence homology between mitochondrial transit peptides, but could explain why mitochondrial transport of the yeast ALV-S is not inhibited by heme (Urban-Grimal **et al.**, 1986), unlike the rat and chicken enzymes (see above). In view of the apparent association of excess levels of ALV and ALV-S with neurotoxic effects, observed in the porphyrias and other diseases (Section 1.6), one can speculate that the development of heme inhibition of translocation to provide a more rapid control mechanism for preventing accumulation of ALV-S and synthesis of ALV, was an important evolutionary adaptation in higher eukaryotes.

The putative 35 amino-acid transit peptide of yeast ALV-S is followed by a hydrophobic sequence of about 20 uncharged amino acids flanked by positively charged ones, which it was suggested might allow interaction of the protein with the mitochondrial membrane. This sequence shows no apparent homology with the analogous regions of chicken and rat. However, this sequence is in any case only poorly

conserved between rat and chicken, possibly indicating a structural rather than catalytic role, such that more variability can be tolerated.

Bacterial ALV-S is smaller than the rat and chicken enzymes, with a molecular weight of around 50 000, even when purified (from **Rhodopseudomonas spheroides**) under conditions which prevent degradation of the native chicken or rat enzymes (Pirola, 1986). Amino acid sequence data for bacterial ALV-S has been obtained from N-terminal sequencing of the **Rhodopseudomonas spheroides** protein (Pirola, 1986) and also deduced from the complete **Bradyrhizobium japonicum** gene sequence (Dr. B. Chelm, personal communication) and partial sequence from the **Rhizobium meliloti** gene (Leong **et al.**, 1985). There is distinct amino acid sequence homology between tbe bacterial protein and the conserved C-terminal region of the eukaryotic enzymes (Pirola, 1986). Within this region, short sequences with high homology to the chicken and rat (Pirola, 1986) or yeast enzymes (Urban-Grimal **et al.**, 1986) have been noted, and these may be important for the catalytic mechanism. Alignment of the N-terminal bacterial amino acid sequence with the eukaryotic ALV-S sequences reveals that the bacterial enzyme lacks the first 145 amino acids of the rat and chicken proteins (Pirola, 1986) and the corresponding region, comprising the N-terminal 65 amino acids, of the yeast protein (Urban-Grimal **et al.**, 1986). It is interesting that bacterial ALV-S is similar in size to the catalytically active core of the chicken enzyme which is produced by proteolytic degradation (discussed above). It seems likely that these are evolutionarily related, and that the eukaryotic ALV-S evolved from the bacterial enzyme by the addition of N-terminal sequences to encode the transit peptide as well as an additional region required for interaction with the mitochondrion.

1.4 CONTROL OF HEPATIC ALV-S AND HEME BIOSYNTHESIS

Heme regulates its own synthesis in the liver, by negative feedback repression of the synthesis of ALV-S, the rate limiting enzyme (Granick, 1966; Section 1.2). At higher concentrations, heme also controls its rate of catabolism, by inducing heme oxygenase activity (Tenhunen **et al.**, 1970). Control of ALV-S and heme oxygenase synthesis is thought to be exerted by a small regulatory pool (10-100 nM) of free heme present within the cell (Granick **et al.**, 1975; Kappas **et al.**, 1983).

Evidence for the regulation of ALV-S by heme was first obtained in experiments with cultured chick embryo liver cells. It was demonstrated that ALV-S levels in these cells were dramatically increased by treatment with a number of drugs, and that hemin prevented this increase (Granick, 1966). Alterations in the levels of hepatic ALV-S activity measured after drug or hemin treatment of chick embryos are accompanied by similar changes in the levels of immunoprecipitable ALV-S protein (Whiting and Granick, 1976b) and ALV-S mRNA (discussed below).

Initially, Granick proposed that drugs and heme acted by competing for the same sites on an apo-repressor protein. Drugs would displace heme from the apo-repressor and cause derepression of ALV-S gene transcription. Subsequently, this hypothesis was modified to suggest that drugs acted simultaneously at two levels, by increasing transcription of ALV-S mRNA, and decreasing heme levels, which they suggested acted to repress ALV-S at a post-transcriptional step (Granick and Beale, 1978). The current view is that drug induction of ALV-S transcription is due solely to depletion of the free heme pool, which relieves heme repression of the gene (May **et al.**, 1986). The evidence for this, and the proposed mechanisms for depletion of the free heme pool by various drugs, are discussed in the second half of this section. Firstly however, the control of ALV-S synthesis by heme is discussed, in

particular, the steps at which heme may act to repress ALV-S synthesis and the evidence for each of these.

A) Mechanisms for negative feedback regulation of hepatic ALV-S by heme

Regulation of hepatic ALV-S levels by heme has been proposed to occur at one or more of several steps including (a) mRNA synthesis (Whiting, 1976), (b) translation (Sassa and Granick, 1970; Yamamoto **et al.**, 1983), (c) translocation of the precursor enzyme into the mitochondria (Hayashi **et al.**, 1972) or (d) feedback inhibition of enzyme activity (Scholnick **et al.**, 1972).

As discussed in Section 1.3, direct feedback inhibition of enzyme activity is unlikely at physiological levels of heme. However, there is good evidence for heme inhibition **in vivo** of both ALV-S mRNA synthesis and mitochondrial transport of ALV-S (this was discussed in detail in the previous section). Heme repression of ALV-S mRNA levels was first indicated by experiments which demonstrated that drug treatment of chick embryos (Whiting, 1976) or rats (Yamamoto **et al.**, 1982a) increases the amount of translatable ALV-S mRNA in the liver, and that administration of hemin prevents this. This was established by **in vitro** translation of a post-mitochondrial supernatant fraction followed by immunoprecipitation of the products with an ALV-S specific antibody. Indirect evidence for heme repression of mRNA synthesis by physiological levels of heme was also provided in experiments in cultured chick embryo liver cells, in which it was shown that repression of ALV-S levels by very low concentrations of hemin (20-50 nM) mimicked the effect of cordycepin, an inhibitor of RNA synthesis (Srivastava **et al.**, 1980a). This work also provided evidence for a further control at a post-transcriptional level, and this is discussed below.

Direct evidence that heme regulates hepatic ALV-S synthesis by decreasing ALV-S mRNA levels has been obtained using cDNA clones recently isolated in this laboratory to measure the steady-state ALV-S mRNA levels, by filter hybridization. Administration of hemin was shown to prevent the increase in the levels of hepatic ALV-S mRNA after drug treatment of chick embryos (Beckmann, 1984) or rats (Srivastava **et al.**, 1987). Similarly, Drew and Ades (1986) demonstrated, by solution hybridization using a synthetic oligomer, that heme inhibited elevation of ALV-S mRNA levels by drugs or testosterone (discussed in Section 1.4B) in cultured chick embryo liver cells. The basal level (i.e. not drug induced) of hepatic ALV-S mRNA can also be reduced by hemin treatment of rats (Srivastava **et al.**, 1987) and in addition, heme repression of the basal level of ALV-S mRNA in brain, heart, kidney and testes was demonstrated.

A post-transcriptional effect of heme on ALV-S synthesis has been suggested by many workers. This was initially proposed by Sassa and Granick (1970) and later by Tyrrell and Marks (1972) and Strand **et al.** (1972), on the basis of kinetic studies of the decrease in the activity of ALV-S in drug induced cultured chick embryo liver cells after the addition of hemin. It was shown that the effect of hemin was similar to that of the translational inhibitor cycloheximide. However, in the first two instances, the assay system used would probably have detected only the intramitochondrial enzyme and not cytosolic ALV-S, since a succinyl CoA generating system was not used. Consequently, the drop in ALV-S activity could have been due to the effect of heme on translocation (Section 1.3). Although succinyl CoA was supplied in the experiments of (Strand **et al.**, 1972), a very high level of hemin (150 μM) was used. Since the effect of heme on total protein synthesis was not examined by any of the above workers, the possibility of a general toxic effect cannot be excluded.

Experiments in drug induced rats also suggest a post-transcriptional effect of heme on ALV-S synthesis. Hemin and [^{3}H] leucine were administered and ALV-S was measured by immuno-precipitation (Hayashi **et al.**, 1980). The level of ALV-S in the mitochondria dropped, and ALV-S in the cytosol increased slightly, as expected because of the effect of heme on mitochondrial transport (Section 1.3). In addition however, the **total** level of ALV-S declined rapidly, by approximately 50% after 30 minutes, although heme did not affect general protein synthesis. In a separate experiment, it was shown that the level of ALV-S was not decreased significantly within the first 30 min after administration of the transcriptional inhibitor a-amanitin. These authors concluded that in addition to preventing transport of ALV-S into the mitochondria, heme inhibits synthesis of ALV-S at a post-transcriptional step, probably at the level of translation. However, they also pointed out that their experiments did not necessarily exclude the possibilitity that heme also acts simultaneously at a transcriptional step of ALV-S synthesis.

The work of Srivastava **et al.** (1980a) provided evidence for a dual effect of heme, at both a transcriptional and post-transcriptional level. It was proposed that the initial effect was probably at the level of transcription, since at low concentrations of hemin (up to 50 nM), repression of drug induced ALV-S in cultured chick embryo liver cells followed kinetics similar to those of cordycepin, a transcriptional inhibitor. At higher concentrations (up to 2 μM), the kinetics of repression by heme resembled those seen with cycloheximide. It was also shown that hemin had no toxic effect on general protein synthesis at concentrations up to 5 μM. From this work it was proposed that the primary effect of heme is at the level of transcription, but that an additional post-transcriptional mechanism operates at higher heme concentrations, probably at the level of translation of ALV-S mRNA.

The physiological significance of the proposed post-transcriptional mechanism is still unclear. There are conflicting reports on the effect of heme on translation of ALV-S mRNA. Yamamoto **et al.** (1983) demonstrated that hemin inhibited synthesis of ALV-S from isolated rat liver polysomes in a rabbit reticulocyte lysate system, but the levels used (20-60 μM) were very high and unlikely to occur **in vivo** (Granick **et al.**, 1975). In contrast, Whiting (1976) showed that hemin at concentrations of up to 10 μM had no effect on the synthesis of ALV-S from chick embryo liver polysomes in a postmitochondrial chick embryo supernatant fraction, and experiments in this laboratory have shown that 100 μM hemin has no effect on translation of ALV-S mRNA in a wheat germ cell-free system (May **et al.**, 1986). In all of these experiments, only translation elongation was measured; the effect of heme on the initiation of translation of ALV-S has not yet been investigated.

Since there is no evidence that physiological levels of heme repress translation, other post-transcriptional mechanisms for heme repression of ALV-S synthesis should be considered. One possibility is that heme destabilizes the protein. Although Sassa and Granick (1970) demonstrated that hemin (5 μM) had no effect on the half life of ALV-S in drug induced cultured chick embryo liver cells, their assay probably only detected intra-mitochondrial enzyme, and this possibility should therefore be reexamined. Alternatively, heme may destabilize ALV-S mRNA, or inhibit its processing or transport from the nucleus. Now that ALV-S cDNA probes are available (Section 1.2), these can be tested directly. Until a direct effect of physiological levels of heme on a post-transcriptional step of ALV-S synthesis can be conclusively established, the role of this mechanism in the regulation of ALV-S synthesis remains uncertain.

B) Mechanisms for drug induction of hepatic ALV-S

As discussed above, the level of hepatic ALV-S can be markedly increased in experimental animals by treatment with a variety of drugs, and this reflects corresponding increases in the levels of ALV-S protein and mRNA. Although the initial models suggested that drugs acted directly as positive effectors of gene transcription, the current model proposes that drug action is indirect and that drugs act solely by depleting the regulatory heme pool, with consequent derepression of ALV-S synthesis (May **et al.**, 1986). This is supported by the fact that ALV-S can be induced in the absence of drugs, by heme depletion alone. This was first suggested by experiments in which it was shown that induction of ALV-S in drug induced cultured chick embryo liver cells could be maintained after removal of the inducing drug, by adding desferrioxamine, an inhibitor of heme synthesis (Srivastava **et al.**, 1980a). Induction of ALV-S has been achieved in the complete absence of drugs, in cultured chick embryo liver cells after induction of heme oxygenase activity with hemin (Srivastava **et al.**, 1980b) or by the use of succinyl acetone and levulinic acid (which inhibit ALV dehydratase) to inhibit heme biosynthesis (Schoenfeld **et al.**, 1982).

It has been proposed that drugs can deplete the regulatory heme pool and induce ALV-S synthesis by several mechanisms, including (a) inhibition of heme biosynthesis, (b) destruction of heme, (c) induction of apoproteins which require heme as their prosthetic group, or (d) a combination of these (Meyer, 1982). The major factor leading to drug induction of ALV-S in liver is probably the induction by the drugs of cytochrome P450 apoprotein, since all drugs examined which induce ALV-S have also been found to induce cytochrome P450 synthesis (Meyer, 1982). Cytochrome P450s are a family of enzymes which are induced by a wide variety of lipophilic compounds, including drugs and other foreign

compounds as well as endogenous hormones. The cytochrome P450s catalyze the conversion of these to more polar products which can then be excreted (Bock and Remmer, 1978). Induction of cytochrome P450 apoprotein constitutes a significant drain on the regulatory heme pool since up to 70% of hepatic heme is utilized in the synthesis of this hemoprotein (Marver and Schmid, 1972). Further support for a correlation between ALV-S and cytochrome P450 induction is the fact that drug induction of rat ALV-S mRNA (detected by filter hybridization with ALV-S cDNA clones), is observed only in tissues in which cytochrome P450 mRNA is also induced by the drug (i.e. the kidney and liver; Srivastava **et al.**, 1987). In heart, brain, spleen and testis, neither cytochrome P450 or ALV-S mRNA are increased by drug treatment. It is interesting that ALV-S in testis can be induced by human chorionic gonadotropin (HCG) and that this induction is prevented by administration of the heme precursor, ALV (Srivastava **et al.**, 1987). HCG induces tissue-specific cytochrome P450 proteins (Waterman and Simpson, 1985) which are not drug inducible. Similarly, Drew and Ades (1986) demonstrated that testosterone induces ALV-S mRNA levels in chick embryo liver and that this is inhibited by heme. It was suggested that this was due to induction of hepatic cytochrome P450 by testosterone.

The highest levels of ALV-S induction are seen with drugs which not only induce cytochrome P450 but also deplete the heme pool by additional mechanisms. Examples of such drugs are DDC (3,5-diethoxycarbonyl-1,4-dihydrocollidine), which converts the cytochrome P450 heme moiety to N-methyl protoporphyrin, a powerful inhibitor of ferrochelatase, the terminal enzyme of heme biosynthesis (De Matteis **et al.**, 1973), and AIA (2-allyl-2-isopropylacetamide), which destroys the heme moiety of cytochrome P450 (De Matteis, 1970). Synergistic induction of ALV-S is observed when drugs which inhibit heme synthesis by different mechanisms (e.g. AIA and DDC) are given together, or when drugs which induce

cytochrome P450 are given along with chemicals such as desferrioxamine, levulinate or succinyl acetone, which inhibit heme synthesis (Srivastava **et al.**, 1980a; Schoenfeld **et al.**, 1982). This is further supporting evidence of the role of heme depletion in mediating induction by drugs.

1.5 ERYTHROID HEME BIOSYNTHESIS

The bulk of the body's heme synthesis takes place in the erythroid cells of the bone marrow, where it is used to provide the prosthetic group of hemoglobin. The control of heme biosynthesis in erythroid cells has not been as well characterized as in liver, because of the complex nature of erythroid differentiation and the variety of systems which have been used in its study. The available evidence on the regulation of erythroid heme biosynthesis is presented below, although it will become obvious that much more work is needed to provide a clear picture of the situation. The nature of the rate limiting step in erythroid heme bisynthesis is discussed, and then the question of whether ALV-S synthesis is under feedback regulation by heme in these cells. There is conflicting evidence as to whether heme biosynthesis in erythroid and hepatic tissues differs in these two points. In the last section, the evidence for tissue-specific isozymes of certain of the heme biosynthetic enzymes is presented.

Erythroid cells undergo a program of differentiation (erythropoiesis) in which primitive stem cells, which contain no detectable hemoglobin, develop through several characteristic stages to form mature erythrocytes in which more than 95% of the total protein is hemoglobin (Harris and Kellermeyer, 1970; Marks and Rifkind, 1972). During this process, the levels of the heme biosynthetic enzymes are increased to cope with the extra demand for heme for globin (Sassa,

1976). In the terminally differentiated erythrocyte, heme synthesis has presumably ceased, along with many other cellular processes, since the mitochondria and protein synthesizing machinery are no longer present, and (in mammals) the nucleus has been extruded. Hemoglobin remains stable, enabling the cell to carry out its function as an oxygen carrier until cell death after approximately 120 days (Harris and Kellermeyer, 1970).

Heme has a complex role in erythroid differentiation. As well as providing the prosthetic group in hemoglobin, heme inactivates a translational repressor of protein synthesis (Ochoa **et al.**, 1979). Heme may have an additional function in the differentiation process, although this is not well defined. In transformed erythroid cells (MEL cells, see below), heme triggers the early events of the erythroid differentiation program, including the induction of globin RNA synthesis (Ross and Sautner, 1976) and synthesis of the heme biosynthetic enzymes (Granick and Sassa, 1978). Heme also appears to be required for terminal differentiation (Eisen, **et al.**, 1978; Lo **et al.**, 1981). This has been proposed on the basis of experiments with variant clones of MEL cells in which heme synthesis is limited, although apparently sufficient to enable production of normal levels of globin. These cells fail to undergo terminal differentiation unless exogeneous heme is added. However, heme alone is not sufficient for terminal differentiation and treatment with DMSO, a chemical inducing agent, is also required.

Investigation of the control of the heme biosynthetic enzymes during erythropoiesis has been complicated by difficulties in obtaining homogeneous cell populations at a particular stage of differentiation. Systems which have been used include fetal liver, bone marrow or anemic spleen, which contain a heterogeneous mixture of non-erythroid cells as well as erythroid cells at various stages of differentiation (Stephenson

et al., 1971; Marks and Rifkind, 1972). Cultured chick blastoderm is devoid of myeloid and lymphoid cells and contains predominantly erythroid precursor cells, which develop from colourless cells to erythrocytes over a period of 24-48 hours in culture (Levere and Granick, 1967). MEL cells (Friend virus-transformed murine erythroleukemia cells) are blocked at the proerythroblast stage and can be induced to differentiate semi-synchronously **in vitro** by the addition of DMSO or other chemicals (Friend **et al.**, 1971). Studies of heme biosynthesis have also been carried out in reticulocytes (e.g. Ponka and Schulman, 1985), which can be obtained in a fairly homogeneous preparation from anemic animals. These cells represent a relatively late stage of erythroid differentiation; they contain hemoglobin, and (in mammals) the nucleus has been extruded but the mitochondria and the protein synthesizing machinery are still present.

ALV-S appears to be the rate limiting enzyme for heme biosynthesis in erythroid precuror cells, since addition of ALV to cultured chick blastoderm accelerates the formation of hemoglobin (Levere and Granick, 1967). In the erythroid precursor cells, the heme biosynthetic enzymes are present at relatively low levels, sufficient to provide heme for the formation of essential hemoproteins such as the respiratory cytochromes. However, when the precursor cells have been induced to differentiate, as already stated, the activities of many of the enzymes of the heme biosynthetic pathway are increased, in order to provide heme for the synthesis of hemoglobin (Sassa,1976).

In MEL cells induced to differentiate with DMSO, induction of the heme biosynthetic enzymes apparently occurs by a sequential mechanism, with ALV-S activity being the first to increase, followed by ALV dehydratase and PBG deaminase. Finally, ferrochelatase activity increases, and this is associated with the onset of heme synthesis

(Sassa, 1976; Rutherford **et al.**, 1979). The sequential appearance of heme biosynthetic enzymes has also been observed in fetal mouse liver, in which it was shown that the increase in ALV-S precedes that of ALV dehydratase and ferrochelatase (Freshney and Paul, 1971). The increase in ALV-S and ALV dehydratase activities also precedes the onset of heme synthesis in a human erythroleukemic cell line (Hoffmann **et al.**, 1980) and in mouse bone marrow, cultured **in vitro** and induced to differentiate with the hormone erythropoietin (Ibrahim **et al.**, 1982). It should be noted that in contrast to the above results, Beaumont **et al.** (1984) found no significant differences in the time course of increases in ALV-S, PBG deaminase, ALV dehydratase or ferrochelatase activity in DMSO treated MEL cells; the reason for this discrepancy is not clear.

It has been postulated that induction of the heme biosynthetic enzymes in DMSO treated MEL cells is due to activation of the genes coding for these enzymes, since it is inhibited by actinomycin D (Sassa, 1976). There is clear evidence that the increase in the levels of at least three of the enzymes occurs by **de novo** synthesis. In DMSO treated MEL cells, induction of ALV dehydratase is accompanied by increased levels of immunologically reactive protein (Beaumont **et al.**, 1984; Chang and Sassa, 1984). In the case of PBG deaminase and URO decarboxylase, the increased level of enzyme is accounted for by a coordinate increase in the corresponding mRNA (Grandchamp **et al.**, 1985).

Once induction of the heme biosynthetic enzymes has commenced, ALV-S is apparently no longer rate limiting for heme biosynthesis in erythroid cells. This was initially proposed on the basis that the induction of ALV-S activity during the first 24 hours after DMSO treatment of MEL cells is not accompanied by an increase in heme synthesis. Instead, induction of heme synthesis follows the same time course as that of ferrochelatase. Both increase at about 3 to 4 days

(Sassa, 1976; Rutherford **et al.**, 1979), suggesting that ferrochelatase is the rate limiting enzyme for heme biosynthesis in differentiating erythroid cells. This is supported by the fact that analysis of porphyrins excreted by differentiating MEL cells revealed the presence of protoporphyrin, but not of other porphyrins (Rutherford **et al.**, 1979).

Ferrochelatase may remain the rate limiting step even after the levels of all the heme pathway enzymes have been induced. Evidence for this was obtained by Ponka and Schulman (1985) who demonstrated that ALV does not stimulate ^{59}Fe incorporation into heme in rabbit reticulocytes. Control experiments demonstrated that inhibition of heme synthesis by isoniazid (an inhibitor of ALV-S), is reversed by ALV, establishing that the exogenous ALV is available for heme synthesis. It was concluded that ALV-S is not rate limiting for heme synthesis in reticulocytes. On the other hand when the rate of iron uptake is increased, by use of a compound which bypasses the transferrin pathway, uptake of ^{59}Fe and incorporation of ^{59}Fe or [2-^{14}C] glycine into heme are stimulated. This was interpreted to suggest that some step in the supply of iron for ferrochelatase activity is limiting (Ponka and Schulman, 1985).

This finding is in contrast to work by Ibrahim **et al.** (1978), who provided indirect evidence that ALV-S might be the rate limiting step in rabbit reticulocytes, by demonstrating that incorporation of [^{14}C] ALV into heme is greater than that of [^{14}C] glycine. PBG deaminase has also been proposed as the rate limiting enzyme in differentiating erythroid cells (Beru and Goldwasser, 1985), since it is the only enzyme whose activity is significantly increased after erythropoietin induction of cultured bone marrow cells. Induction of PBG deaminase in this system is temporally associated with an increase in heme synthesis. Further

work is required to clarify whether these apparently conflicting results reflect differences in the experimental systems used.

There are also conflicting views on the control of ALV-S by heme in erythroid cells. It has been proposed by Granick and Sassa (1978) that erythroid ALV-S synthesis is positively controlled by heme, since hemin treatment of MEL cells results in an increase in the level of incorporation of [^{14}C] glycine into heme. However, as discussed above, heme also increases the levels of other enzymes of the pathway, as well as globin mRNA. The increase in ALV-S might simply be a secondary effect due to induction by heme of the early erythroid differentiation program (Eisen **et al.**, 1978; Ibrahim **et al.**, 1983). In any case, incorporation of [^{14}C] glycine is only an indirect assay for ALV-S. When Beaumont **et al.** (1984) assayed ALV-S activity directly, they found no increase after hemin treatment of MEL cells, although it was confirmed that the level of PBG deaminase increased. This work also provided evidence for negative regulation of erythroid ALV-S synthesis by heme although the step at which this occurs was not identified. It was shown that succinyl acetone (an inhibitor of heme synthesis) increases the level of ALV-S in DMSO treated MEL cells. Furthermore, this increase is prevented by heme.

Another apparent difference in the control of heme synthesis in hepatic and erythroid cells is the fact that erythroid ALV-S is not inducible by drugs (Wada **et al.**, 1967) which dramatically increase the level of hepatic ALV-S. This may reflect the absence in erythroid cells of inducible cytochrome P450s (Guengerich and Liebler, 1985), which are thought to mediate drug induction of ALV-S in the liver (Section 1.4B), rather than any more fundamental difference in the regulation of hepatic and erythroid ALV-S synthesis. However, there is no direct evidence to support this proposition as yet.

Because of the apparent differences in heme biosynthesis in erythroid and hepatic tissues, the possibility of tissue specific isozymes or control mechanisms has been investigated for some of the heme biosynthetic enzymes.

Northern hybridization analysis reveals a single size species of mRNA for PBG deaminase in a range of human erythroid and non erythroid tissues, although the amount of mRNA was higher in erythroid tissues (Raich **et al.**, 1986). Analysis of the relative transcription rate of the PBG deaminase gene, by **in vitro** transcription in nuclei isolated from two erythroid cell lines (HEL and K562) and a lymphoid cell line (HL60), demonstrated that the transcription rates were the same, although the K562 cells contained about twice as much PBG deaminase mRNA. This would suggest that the level of PBG deaminase mRNA in these cells is modulated at a post-transcriptional level. However, the difference in the level of mRNA in the erythroid and non-erythroid cell lines (only about two fold) was not as great as that observed between erythroid and non-erythroid tissues **in vivo**, and it was suggested that the regulation of PBG deaminase mRNA levels in the transformed cell lines might not be a true indication of the situation **in vivo.**

Sequence analysis of erythroid and non-erythroid PBG deaminase cDNA clones has revealed a short additional sequence at the 5' end of the non-erythroid mRNA (from a lymphoblastoid cell line) which is not present in the erythroid mRNA (Dr. Y. Nordmann, personal communication). This difference would not have been resolved by Northern analysis and is supported by the existence of erythroid and non-erythroid forms of the PBG deaminase enzyme which vary in molecular weight and electrophoretic mobility. Southern hybridization analysis using a human cDNA clone as probe indicates that the PBG deaminase (Raich **et al.**, 1986) gene occurs in single copy in the human genome. The finding of a single structural

gene for PBG deaminase suggests that the two PBG deaminase mRNAs (discussed above) may arise through differential promoter usage or splicing of the same gene.

Although there is no evidence for tissue specific isozymes of URO decarboxylase, evidence for tissue specific transcriptional control has been presented. Southern and Northern hybridization analysis using the human cDNA clone as probe indicates that the URO decarboxylase gene occurs in single copy in the human genome and that a single size mRNA for URO decarboxylase is present in a range of human erythroid and non-erythroid tissues, including adult liver (Romeo **et al.**, 1986). As observed with PBG deaminase, the basal level of URO decarboxylase mRNA is much higher in erythroid tissues. This is apparently due to tissue-specific activation of the URO decarboxylase gene. It was shown that the 4 fold higher level of mRNA in an erythroid cell line (compared to a lymphoid cell line) is accounted for by a 4 fold increase in the relative level of transcription of the URO decarboxylase gene, measured by **in vitro** transcription in isolated nuclei.

The existence of an erythroid specific ALV-S isozyme has been suggested on the basis of differences in catalytic properties and affinity for CoA-agarose (Woods and Murthy, 1975; Bishop **et al.**, 1981) as well as differences in the size (of both the mature and precursor proteins) and immunological properties of liver and erythroid ALV-S (Watanabe **et al.**, 1983; Yamamoto **et al.**, 1985). Yamamoto **et al.** (1985) reported the isolation of a putative chicken erythroid ALV-S cDNA clone, although no sequence data were presented. Northern hybridization experiments with this clone indicated distinct homologous mRNAs of different sizes in liver, brain and erythroid tissues of chicken. On the basis of this evidence, Yamamoto **et al.** (1985), proposed the existence of a multigene family for ALV-S, suggesting that separate

genes for erythroid and hepatic ALV-S could explain the apparent differences in the control of ALV-S and heme synthesis in these tissues. However, work presented in this thesis (Chapter 3) provides strong evidence against this proposal.

In conclusion, there appear to be many differences between heme biosynthesis in erythroid and hepatic tissues. It is probably justifiable to say that there is no clear picture of how heme biosynthesis is controlled in erythroid cells and a much more systematic approach is required to fully understand this complex problem.

1.6 THE PORPHYRIA DISEASES

The porphyrias are a group of diseases in which the control of heme biosynthesis has been disturbed because of a specific defect, usually genetically determined, in one of the enzymes of the pathway (Table 1.1). Recombinant DNA technology has recently been used to define the molecular defect in a case of hepatoerythropoietic porphyria. It was shown that a single base change in the uroporphyrinogen decarboxylase mRNA caused replacement of a glycine residue in the protein by a glutamic acid. The resulting enzyme is unstable and rapidly degraded **in vivo** (de Verneuil **et al.**, 1986). Recombinant DNA technology should prove useful in the characterization of other types of porphyria as well, since cDNA clones for many of the heme biosynthetic enzymes are now available (Section 1.2). The porphyrias have been reviewed extensively, most recently by Kappas **et al.** (1983), Rimington (1985) and Hindmarsh (1986), and only a summary of the more interesting aspects discussed in these reviews which are relevant to the control of ALV and heme synthesis is presented here.

TABLE 1.1

CHARACTERISTICS OF THE PORPHYRIAS

CONDITION	INHERITANCE	ENZYME DEFICIENCY	EXCRETED INTERMEDIATES		MAJOR SYMPTOMS	
			ALA and PBG[1]	Porphyrins[2]	Photosensitivity	Neurological
NON-ACUTE PORPHYRIAS						
Congenital erythropoietic porphyria	Autosomal recessive	Uroporphyrinogen III synthase	No	URO, COPRO	Yes	No
Porphyria cutanea tarda	Autosomal dominant and sporadic	Uroporphyrinogen decarboxylase	No	URO, ISOCOPRO	Yes	No
Hepatoerythropoietic protoporphyria	Autosomal recessive	Uroporphyrinogen decarboxylase	No	URO, ISOCOPRO	Yes	No
Protoporphyria	Autosomal dominant	Ferrochelatase	No	PROTO	Yes	No
ACUTE PORPHYRIAS						
Acute intermittent porphyria	Autosomal dominant	PBG deaminase	Yes	No	No	Yes
ALV dehydratase deficiency	Autosomal recessive	ALV dehydratase	ALV only	No	No	Yes
Variegate porphyria	Autosomal dominant	Protoporphyrinogen oxidase	Yes	COPRO, PROTO	Yes	Yes
Hereditary coproporphyria	Autosomal dominant	Coproporphyrinogen oxidase	Yes	COPRO	Yes	Yes

1 ALV, 5-aminolevulinate; PBG, porphobilinogen

2 URO, uroporphyrin; COPRO, coproporphyrin; ISOCOPRO, isocoproporphyrin; PROTO, protoporphyrin

The heme biosynthetic pathway is usually closely regulated, so that the intermediate products are normally found only in trace amounts. However, in each of the different types of porphyrias, the specific enzyme defect results in a partial block in the heme pathway and causes the accumulation and excretion of a characteristic pattern of heme biosynthetic intermediates which depends on the site of the blockage (Table 1.1). These intermediates can include ALA, PBG and various porphyrins, which are produced by spontaneous oxidation of the corresponding porphyrinogen. Excess production of these intermediates has been correlated with the two main types of symptoms observed in the porphyrias, (a) cutaneous photosensitivity, which is clearly associated with porphyrin accumulation and (b) neuropsychiatric symptoms, seen in the acute hepatic porphyrias. This latter group of symptoms may be linked to increased ALV-S levels and accumulation of ALV during acute attacks, and this is discussed in more detail below.

Although the underlying enzyme deficiency in each type of porphyria is found in all tissues examined, actual metabolic expression of the defect and overproduction of specific intermediates is often dependent on tissue-specific variables. In erythropoietic protoporphyria for example, in which the genetic defect is a deficiency in ferrochelatase (Table 1.1), erythroid cells appear to be the main source of overproduction of protoporphyrin. In most cases the liver probably does not contribute significantly to the production of excess protoporphyrin in this disease (Kappas **et al.**, 1983). It is possible that the defective enzyme only becomes rate-limiting in erythroid tissus, since in normal liver the levels of ferrochelatase are 20 fold higher than in erythroid cells. Consequently even the deficient level of enzyme in the liver is probably not rate limiting. However, ferrochelatase may be the rate limiting enzyme in normal developing erythroid cells (Section 1.5), so that the deficient levels of ferrochelatase in erythroid cells of a

porphyric patient are probably insufficient, resulting in a partial block in the pathway and accumulation of protoporphyrin.

The acute hepatic porphyrias, acute intermittent porphyria (AIP), hereditary coproporphyria (HCP) and variegate porphyria (VP) (Table 1.1), are particularly interesting in relationship to the topic of this thesis, the regulation of ALV-S synthesis. These diseases can remain latent for many years and are characterized by intermittent attacks of neurological symptoms, which are precipitated by factors such as steroid hormones, drugs, and nutrition. In VP and HCP, but not AIP, cutaneous photosensitivity occurs as a result of the accumulation of porphyrins. In acute attacks of these diseases, excess excretion of ALA and PBG and increased levels of ALV-S are observed (Kappas **et al.**, 1983). This may be explained by the fact that many of the drugs which are known to precipitate acute attacks have been demonstrated to induce the cytochrome P450 drug metabolizing system in the liver (De Matteis, 1978). These same drugs also produce biochemical effects in experimental animals which resemble the symptoms seen in acute porphyric attacks, and this has provided a useful experimental model for the study of these diseases. As discussed in Section 1.4B, induction of the cytochrome P450 drug metabolizing system may lead to depletion of the cellular free heme pool, resulting in the induction of ALV-S synthesis observed in acute attacks. PBG will also accumulate, since the levels of hepatic PBG deaminase are low (Section 1.2) and the enzyme probably becomes limiting under these conditions.

The exact cause of the neurological symptoms associated with the acute porphyrias is unclear, although these may be related to increased levels of ALV-S, ALV and PBG which are observed during acute attacks of these diseases. Excess excretion of ALV and PBG is not found in the non-acute porphyrias (Table 1.1). It is interesting that neurological

symptoms remarkably similar to those observed during attacks of the acute porphyrias are also seen in a number of diseases in which the common characteristics are ALV dehydratase deficiency, with a consequent accumulation of ALV, but not PBG. These include lead poisoning, in which ALV dehydratase is inhibited by lead, and some forms of hereditary tyrosinemia, where the abnormal metabolite succinylacetone, is a structural analogue of ALV and hence a competitive inhibitor of ALV dehydratase. A form of porphyria, as yet unnamed, has been described in which ALV dehydratase is the deficient enzyme. This is characterized by excess production and excretion of ALV but not PBG, and neurological symptoms similar to those of the acute porphyrias. This is suggestive, but certainly not conclusive evidence that excess production of ALV, or a metabolite of this, may be responsible for neurotoxic symptoms. One hypothesis is that ALV competitively inhibits binding of the central nervous system neuro-transmitter γ-aminobutyric acid, to synaptic membranes in brain tissue. However, although there is some evidence for such an effect **in vitro**, neurotoxic effects of ALV have not been demonstrated **in vivo** (Kappas **et al.**, 1983).

Therapeutic treatment of the porphyrias has been directed at trying to prevent the induction of ALV-S and consequent accumulation of ALV, by heme infusion, which is assumed to act by feedback repression of ALV-S synthesis, or by glucose administration, since glucose prevents drug-mediated induction of ALV-S **in vivo** (Marver **et al.**, 1966). The biochemical basis of this "glucose effect" is not well understood, but it may be mediated by changes in cAMP levels (Giger and Meyer, 1981). The effectiveness of glucose or heme therapy has been difficult to assess, since porphyric attacks often subside spontaneously and it is not feasible to undertake appropriate control experiments. As mentioned above, drug induction of ALV-S in experimental animals by drugs (Section 1.4B) mimics the biochemical symptoms observed in the acute

porphyrias and this should prove useful for the study of these diseases. A detailed knowledge of the molecular mechanisms for the control of ALV-S synthesis may enable the development of a more effective therapy for this group of potentially fatal diseases.

1.7 AIMS OF THIS THESIS

The work in this thesis was aimed at answering some basic questions about the control of ALV-S synthesis in the liver.

As a first step, isolation of genomic clones for the ALV-S gene was required, in order to determine the sequence of the gene. This is an essential basis for defining the regulatory sequences, and eventually the precise molecular mechanism of ALV-S gene control. It was also necessary in order to investigate whether a multigene family exists for ALV-S, as proposed by Yamamoto **et al.** (1985).

An additional aim was to determine directly whether heme and drugs alter the rate of transcription of the ALV-S gene. To answer this question, the regulation of the ALV-S gene was investigated by **in vitro** transcription run-on experiments in isolated nuclei. Cytochrome P450 transcription was also examined, in view of the proposed role for cytochrome P450 induction in the control of ALV-S synthesis. At the same time, the levels of ALV-S mRNA and protein were determined, by Northern blot hybridization analysis and enzyme assay, to assess the relative contribution of transcriptional and post-transcriptional control of ALV-S levels.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and reagents

The sources of some of the more important reagents were as follows :

agarose, low melting temperature : Bethesda Research Laboratories (BRL)

2-allyl-2-isopropylacetamide (AIA) : Roche

5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (BCIG) : BRL

dextran sulphate : Pharmacia

Ficoll 400 : Pharmacia

hemin : Porphyrin Products, Utah

polyethylene glycol 6 000 : BDH Chemicals

tetracycline : Upjohn Pty. Ltd.

Sarkosyl : Ciba-Geigy

N,N,N',N'-tetramethylethenediamine (TEMED) : Tokyo Kasei

tRNA, **E.coli** : BRL

urea (ultra pure): Merck

The following were obtained from Sigma :

acrylamide, agarose (Type 1), 5-aminolevulinic acid (ALV), ampicillin, bisacrylamide (N',N'-methylene-bis-acrylamide), chloramphenicol, deoxyribonucleotide triphosphates (dNTPs), dideoxyribonucleotide triphosphates (ddNTPs), dithioerythritol (DTE), dithiothreitol (DTT), ethylenediaminetetra-acetic acid (EDTA), ethylene glycol-bis(β-amino-ethyl ether) N,N,N',N'tetra-acetic acid (EGTA), isopropylthiogalactoside (IPTG), heparin, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (Hepes), pyridoxal-5'-phosphate (PLP), salmon sperm DNA, spermidine, spermine.

All other chemicals and reagents were of analytical grade or the highest available purity.

2.1.2 Enzymes

calf intestinal phosphatase : Boehringer Mannheim

E. coli deoxyribonuclease I (DNase) : Sigma

E. coli DNA polymerase I : BRESA (Biotechnology Research Enterprises, South Australia Pty. Ltd., University of Adelaide)

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

lysozyme : Sigma

proteinase K : Boehringer Mannheim

ribonuclease A (RNase A) : Sigma

The stock solution (10 mg/ml) was incubated at 100°C for 15 min to inactivate any DNase activity.

T4 DNA ligase : BRESA

Restriction enzymes : BRL or New England Biolabs

2.1.3 Buffers

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

SM : 10 mM NaCl, 10 mM $MgSO_4$

SSC : 150 mM NaCl, 15 mM sodium citrate

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

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

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

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

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

TS : 25 mM Tris-HCl pH 8.0, 15% sucrose

2.1.4 Radiochemicals

[α-^{32}P]dATP (1800 Ci/mmol) : BRESA

[α-^{32}P]dCTP (1800 Ci/mmol) : BRESA

[α-^{32}P]UTP (1500 Ci/mmol) : BRESA

2.1.5 Cloning vectors

pBR322, M13mp8, M13mp9, M13mp18, M13mp19 : A gift from Dr. M. J. Bawden.

pUC19: A gift from P. Vize.

2.1.6 Bacterial strains

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

(1) **E. coli** MC1061 (Casadaban and Cohen, 1980)
host for recombinant plasmids,

(2) **E. coli** JM101 (Messing, 1979)
host for M13 bacteriophage,

(3) **E. coli** LE392 (Murray **et al.**, 1977)
host for λ Ch4A bacteriophage.

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 -20°C, or -80°C for long term storage. Single colonies of bacteria, obtained by streaking the glycerol stock onto agar plates of a suitable medium (Section 2.2.1.7) were used to innoculate liquid medium, and bacterial cultures were grown at 37°C with continuous shaking to provide aeration

2.1.7 Bacterial growth media

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

1) Growth medium for E. coli MC1061

Luria (L) broth : 1% Bacto-tryptone (Difco), 0.5% yeast extract (Difco), 1% NaCl, adjusted to pH 7.5 with NaOH. Ampicillin (50 μg/ml) or tetracycline (20 μg/ml) were added where appropriate

for growth of transformed bacteria, to maintain selective pressure for the plasmid.

2) Growth media for E. coli LE392

Luria (L) broth plus 0.2% maltose : as above, supplemented with 0.2% maltose.

NZCYM medium : 1% NZ amine A, 0.5% yeast extract, 0.5% NaCl, 0.1% Casaminoacids (Difco), 0.246% $MgSO_4$, adjusted to pH 7.5 with NaOH.

3) Growth media for E. coli JM101

Minimal salts medium plus glucose : 1.05% K_2HPO_4, 0.45% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.05% sodium citrate, supplemented after autoclaving with 0.02% $MgSO_4$, 0.0005% thiamine-HCl and 0.2% glucose.

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

4) Solid media

Agar plates were prepared by supplementing the above media with 1.5% Bacto-agar. Soft overlays were 0.7% agar or agarose in L broth or 2 x YT medium.

2.1.8 Miscellaneous

Bovine serum albumin (Pentax Fraction V) : Sigma

Nitrocellulose (BA 85) : Schleicher and Schuell

M13 universal sequencing primer : BRESA

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

GF/A glass fibre filter discs : Whatman

2.2 METHODS

2.2.1 GENERAL RECOMBINANT DNA METHODS

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

a) Growth, maintenance, and preservation of bacterial strains
b) Plaque purification of bacteriophage lambda
c) Purification of closed circular DNA by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients
d) Digesting DNA with restriction endonucleases
e) End-repair or end-labelling of DNA fragments using the Klenow fragment of **E. coli** DNA polymerase I
f) Gel electrophoresis
g) Isolation of DNA fragments from low melting temperature agarose and acrylamide gels
h) **In situ** hybridization of bacterial colonies or bacteriophage plaques
i) Southern transfer
j) Purification of nucleic acids
k) Quantitation of DNA

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.

2.2.1.1 Preparation of plasmid or M13 replicative form DNA

a) For the preparation of pUC or pBR322-derived plasmid DNA, a 5 ml overnight culture of the transformed bacteria (Sections 2.2.1.4 and 5) was diluted into 500 ml of fresh medium and grown (with the appropriate antibiotic) until the absorbance at 600 nm reached 0.6-0.8. Chloramphenicol (200 µg/ml) was added and the culture grown overnight to amplify the plasmid.

b) For the preparation of double-stranded replicative form DNA from M13 clones, a 1.5 ml phage culture (Section 2.2.1.6A) was grown overnight and added to 100 ml of 2 x YT medium, together with 10 ml of an overnight culture of JM101, and incubated for 5 h at 37°C with shaking.

Cells containing the plasmid or replicative form DNA were pelleted by centrifugation at 5 000 x g for 10 min and one of the following methods was used to purify the DNA.

A) Alkaline lysis procedure

(modified from Birnboim and Doly, 1979)

Cells were resuspended in 3 ml of TES buffer and kept on ice while the following additions were made, with gentle mixing between each addition:

a) 1 ml of lysozyme, 8 mg/ml in TES buffer, for 20 min,

b) 8 ml of 0.2 M NaOH, 1% SDS, for 10 min,

c) 5 ml of 3 M sodium acetate pH 4.6, for 10 min.

Cellular debris and chromosomal DNA were pelleted at 35 000 x g for 30 min. The supernatant was aspirated (avoiding the pellet) and

incubated with 50 µg of RNase A for 30 min at 37°C. After phenol chloroform extraction, the nucleic acids were precipitated by the addition of 2 volumes of ethanol or an equal volume of isopropanol for 30 min at room temperature, followed by centrifugation at 12 000 x g for 15 min. The pellet was resuspended in 4 ml of 0.4 M NaCl, 6.5% PEG 6 000, left on ice for 1 h and centrifuged for 10 min at 12 000 x g at 4°C. The pelleted DNA was washed with 70% ethanol, dried **in vacuo**, resuspended in 500 µl of H_2O and transferred to an Eppendorf microcentrifuge tube. This step was repeated, and the DNA was resuspended in 100 µl of TE buffer.

B) Gentle lysis - caesium chloride gradient procedure

(modified from Godson and Sinsheimer, 1967)

Cells were resuspended in 7.5 ml TES buffer and kept on ice while the following additions were made, with gentle mixing between each addition:

a) 1.5 ml of lysozyme, 5 mg/ml in TES buffer, for 5 min,
b) 3 ml of 0.25 M EDTA pH 8.0, for 15 min,
c) 12 ml of 1% Brij 58, 0.4% sodium deoxycholate, 50 mM Tris-HCl pH 7.0, 25 mM EDTA, for 10 min.

The solution was then centrifuged at 35 000 x g for 30 min and the supernatant carefully decanted. The DNA was purified by caesium chloride gradient centrifugation essentially as described by Maniatis **et al.** (1982).

2.2.1.2 Preparation of pBR322, pUC and M13 cloning vectors

DNA was linearized by digestion with the appropriate restriction enzyme (Maniatis **et al.**, 1982). To prevent self-ligation of the vector, 5' terminal phosphate groups were removed by incubating the restricted

vector (5 µg) in 50 mM Tris-HCl pH 9.0, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ with 0.5 units of calf intestinal phosphatase in a final volume of 200 µl, for 1 h at 37°C. The DNA was extracted with phenol, ethanol precipitated and isolated after electrophoresis in low melting temperature agarose (Maniatis **et al.**, 1982). The DNA was resuspended at a concentration of 20-50 ng/µl, for use in ligation reactions.

2.2.1.3 Ligation reactions

A 10 µl reaction contained 20-50 ng of vector DNA, the DNA restriction fragment, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP and 1 unit of T4 DNA ligase. For cloning in pUC or pBR322, equimolar amounts of DNA fragment and vector were added; for M13, a three-fold molar excess of the DNA insert was used. The reactions were incubated at 4°C for 12-24 h or at 25°C for 3 h. A control ligation with vector DNA only was set up and included in the subsequent transformation to determine background levels of uncut or recircularized vector DNA.

2.2.1.4 Transformation of E.coli MC1061 with pUC or pBR322 recombinants

0.5 ml of an overnight culture of **E.coli** MC1061 was diluted into 50 ml of fresh L-broth and grown at 37°C with shaking, until the absorbance at 600 nm reached 0.6-0.8. The culture was chilled on ice for 10 min and pelleted by centrifugation at 4 000 x g for 5 min at 4°C. The cells were gently resuspended in 25 ml of ice-cold 0.1 M $MgCl_2$, pelleted immediately, resuspended in 2.5 ml of ice-cold 0.1 M $CaCl_2$ and kept on ice for 1 h. 3-10 µl of the DNA ligation reaction mix (Section 2.2.1.3) was diluted with 100 µl of 0.1 M Tris-HCl pH 7.4, added to 0.2 ml of the competent cells and kept on ice for a further 30 min with occasional gentle mixing. The cells were heat-shocked at 42°C for 2 min, placed on ice for 30 min, allowed to equilibrate to room

temperature for 15 min and then incubated with 0.5 ml of L-broth at 37°C for 20 min. The transformed cells were mixed with 3 ml of 0.7% L-agar and poured onto L-agar plates containing the appropriate antibiotic.

2.2.1.5 Transformation of E.coli JM101 with M13 recombinants

0.5 ml of an overnight culture of **E.coli** JM101 was diluted into 50 ml of 2 x YT medium and grown until the absorbance at 600 nm reached 0.4-0.6. A few ml of the cells were saved as plating bacteria (see below) and the remainder were pelleted by centrifugation at 4 000 x g, for 5 min at 4°C. The cells were gently resuspended in 2.5 ml of ice cold 50 mM $CaCl_2$ and kept on ice for 30 min. 3-10 µl of the DNA ligation reaction mix (Section 2.2.1.3) was added to 0.2 ml of the competent cells and kept on ice for 40 min with occasional gentle mixing. The cells were heat-shocked at 42°C for 2 min, mixed with 3 ml of 0.7% YT-agar, 10 µl of BCIG (20 mg/ml), 10 µl of IPTG (20 mg/ml) and 200 µl of plating bacteria, and poured onto minimal medium + glucose agar plates.

2.2.1.6 Preparation of M13 single-stranded DNA

A) Small-scale preparation of DNA for sequencing

Recombinant plaques were toothpicked (or 10 µl of a phage stock was added) into 1.5 ml of a 1:40 dilution of an overnight JM101 culture in 2 x YT broth, and grown with vigorous shaking for 5 h at 37°C. Cells were pelleted by centrifugation in an Eppendorf centrifuge for 10 min, and the supernatant removed carefully into a fresh tube and recentrifuged. 0.3 ml of 2.5 M NaCl, 20% PEG 6 000 was added to 1.2 ml of the supernatant (the remainder was saved as a stock at -20°C). After 15 min at room temperature the phage pellet was collected by centrifugation for 5 min. All traces of the supernatant were removed and the pellet was resuspended in 0.1 ml of 10 mM Tris-HCl pH 8, 0.1 mM

EDTA and extracted with an equal volume of buffer-saturated phenol. The aqueous phase was re-extracted with 0.5 ml of diethyl ether and ethanol precipitated. The phage DNA was collected by centrifugation, washed in 70% ethanol, dried **in vacuo**, resuspended in 25 µl of TE buffer and stored at -20°C.

B) Large-scale preparation of single-stranded DNA from M13 recombinants

A 100 ml phage culture was grown as described in Section 2.2.1.1B. The bacterial cells were pelleted by centrifugation at 5 000 x g for 10 min and phage were precipitated from the supernatant by the addition of 25 ml of 2.5 M NaCl, 20% PEG 6 000, at room temperature for an hour. The phage were pelleted by centrifugation at 5 000 x g for 10 min and resuspended in 1 ml of TE buffer after all traces of the supernatant had been removed. 0.25 ml of the PEG solution was added and the phage were reprecipitated for 15 min at room temperature and collected by centrifugation for 5 min in an Eppendorf microcentrifuge. All traces of the PEG solution were removed and the phage were resuspended in 200 µl of TE buffer and extracted twice with phenol-chloroform, and once with 2 volumes of ether. The DNA was ethanol precipitated and resuspended in 200 µl of TE buffer.

2.2.1.7 Complementarity testing of single-stranded M13 recombinants

To determine which strand of a particular subcloned DNA fragment was present in a single-stranded M13 recombinant, hybridization analysis was carried out using an arbitrarily selected or previously sequenced recombinant as reference. 2 µl of the test DNA was added to 2 µl of the reference DNA with 4 µl of 0.1 M Tris-HCl pH 7.4, 0.1 M $MgCl_2$, 0.5 M NaCl, and 2 µl of 50% glycerol, 1% SDS, 0.2 M EDTA, 0.2% bromophenol blue, and incubated at 65°C for 1 h. The samples were electrophoresed on a 1% agarose minigel, with 2 µl of reference DNA as a

marker. The DNA was visualised after ethidium bromide staining. Single-stranded M13 clones with inserts identical to the reference clone comigrate with the reference, whereas clones containing the complementary strand are retarded, as they have hybridised to the reference DNA, decreasing their mobility.

2.2.1.8 Dideoxy chain termination DNA sequencing procedures

A) Standard method

DNA sequence analysis of M13 single-stranded DNA was carried out essentially as described by Sanger **et al.** (1977).

1) **Hybridization:** 2.5 ng of universal primer (a 17-mer supplied by BRESA) was annealed to 8 µl of the template DNA preparation (about 1 µg) in a 10 µl reaction containing 10 mM Tris-HCl pH 8.5, 5 mM $MgCl_2$, by boiling for 3 min and allowing the reaction mix to cool slowly over 1 h.

2) **Polymerization:** 2 µl of [α-^{32}P]dATP (approximately 10 µCi) was lyophilized, and the hybridization mixture, 1 µl of 10 mM DTT, and finally 1 µl of Klenow fragment (1 unit) were added. The mixture was vortexed to resuspend the label and 2.5 µl was added immediately to each of 4 tubes containing 2 µl of the appropriate N^O-ddNTP work solution (freshly made from stock concentrates, see details below). After mixing, samples were incubated at 37^{O}C for 15 min, then 1 µl of chase solution (250 µM of each dNTP in 5 mM Tris-HCl) was added and the incubation continued for a further 15 min at 37^{O}C. 5 µl of formamide loading buffer (deionized formamide with 0.1% bromophenol blue, 0.1% xylene cyanol and 20 mM EDTA) was added to each tube, the samples boiled for 3 min and then analyzed on a sequencing gel (see below).

The N^O-ddNTP work solutions were as follows :

A-work solution: 0.4 mM ddATP:A^O (200 µM TTP, 200 µM dCTP, 200 µM dGTP, 5 mM Tris-HCl pH 8), 1:1.

C-work solution: 0.2 mM ddCTP:C^O (200 µM TTP, 10 µM dCTP, 200 µM dGTP, 5 mM Tris-HCl pH 8), 1:1.

G-work solution: 0.3 mM ddGTP:G^O (200 µM TTP, 200 µM dCTP, 10 µM dGTP, 5 mM Tris-HCl pH 8), 1:1.

T-work solution: 0.1 mM ddTTP:C^O (200 µM dCTP, 10 µM TTP, 200 µM dGTP, 5 mM Tris-HCl pH 8), 1:1.

3) DNA sequencing gels: 1 µl of each sample was electophoresed on a 6% polyacrylamide, 7 M urea gel (20 cm x 40 cm x 0.2mm) in TBE buffer. The gels were pre-electrophoresed at 20 mA for 30 min prior to loading the samples. Electrophoresis was at 20-25 mA, until the tracker dyes had migrated the desired distance. After electrophoresis the gels were washed with 1 litre of 10% acetic acid, followed by 1 litre of 20 % ethanol, and baked at 100°C. Autoradiography was generally carried out overnight at room temperature.

B) Sequencing with dITP

This was as described above, except that dGTP in the N^O and chase solutions was replaced by dITP (deoxyinosine triphosphate), and a lower concentration of ddGTP was used. The N^O-ddNTP work solutions were as follows :

A-work solution: 0.4 mM ddATP:A^O (200 µM TTP, 200 µM dCTP, 200 µM dITP, 5 mM Tris-HCl pH 8), 1:1.

C-work solution: 0.2 mM ddCTP:C^O (200 µM TTP, 10 µM dCTP, 200 µM dITP, 5 mM Tris-HCl pH 8), 1:1.

G-work solution: 0.1 mM ddGTP:G^{o} (200 µM TTP, 200 µM dCTP, 100 µM dITP, 5 mM Tris-HCl pH 8), 1:1.

T-work solution: 0.1 mM ddTTP:C^{o} (200 µM dCTP, 10 µM TTP, 200 µM dITP, 5 mM Tris-HCl pH 8), 1:1.

2.2.2 METHODS USED FOR ISOLATION AND CHARACTERIZATION OF ALV-S GENOMIC CLONES

2.2.2.1 Screening of a chicken genomic library in λ Charon 4A by plaque hybridization

The chicken genomic DNA recombinant library (Dodgson **et al.**, 1979) was supplied by Dr. J. Welles. The library was constructed by partial **Hae**III/**Alu**I digestion of chicken erythrocyte DNA, followed by ligation of **Eco**RI linkers to 14-22 kb fragments which were then inserted into **Eco**RI digested λ Charon 4A.

Screening of the genomic library by **in situ** plaque hybridization, plaque purification, growth of the λ recombinants and titration of phage stocks were all essentially as described by Maniatis **et al.** (1982). In the initial screening, a total of 1.1 x 10^{6} pfu (plaque forming units) were screened in batches of 50 000 pfu, on 15 cm petri plates. In subsequent rounds of screening for plaque purification, either 100-1000 pfu were grown on 8 cm plates, or plaques were toothpicked in a grid onto a lawn of bacteria and grown overnight. Nitrocellulose filters were pulled from each plate (in duplicate for the initial screen), baked, prehybridized and hybridized for 16 h at 42°C with 35-50 ng/ml of nick translated pl05B1 (Section 2.2.2.2), with 6 ml of hybridization mix per 15 cm filter, or 1.5 ml per 8 cm filter. Hybridization conditions were essentially those described by Maniatis

et al. (1982), except that SDS was not used, and 5 % dextran sulphate and 20 mM potassium phosphate pH 7.4 were included in the hybridization mix. The filters were washed finally in 0.2 x SSC, 0.1% SDS, at 65°C for 60 min and autoradiographed at -80°C overnight or for several hours at room temperature, depending on the strength of the hybridization signal.

2.2.2.2 Preparation of [^{32}P]-labelled DNA probes by nick translation

Nick translation of DNA was carried out essentially as described by Maniatis **et al.** (1982). Entire recombinant plasmids or specific restriction fragments isolated after electrophoresis on low melting temperature agarose gels (Maniatis **et al.**, 1982) were used. A 20 µl reaction containing 50-100 µCi each of [α-^{32}P]dATP and [α-^{32}P]dCTP (2500 Ci/mmol), 50 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT, 50 µg/ml BSA, 0.2-1 µg of DNA, 1 unit of **E. coli** DNA polymerase I and 40 pg of DNase I, was incubated at 16°C for 2-3 h. The reaction was stopped by the addition of 5 µl of 0.5 M EDTA, pH 8.0 and 5 µl of 10% SDS. 5 µl of denatured salmon sperm DNA (10 mg/ml), 65 µl of NET buffer and 100 µl of 4 M ammonium acetate were added and the DNA was precipitated with 400 µl of ethanol at -80°C for 15 min followed by centrifugation at 12 000 x g for 10 min. The DNA was washed with 1 ml of chilled ethanol, dried **in vacuo** and resuspended in 100 µl of TE buffer. 1 µl of the nick translated DNA was spotted directly onto glass fibre discs to measure total radioactivity and the TCA-precipitable radioactivity in a 1 µl aliquot was also determined (Section 2.2.4.1). Over 95% of total radioactivity in the ethanol precipitate was TCA-precipitable, and the specific activity of the probe was 1-2 x 10^8 cpm/µg DNA. In later experiments a nick translation kit supplied by BRESA was used, similar specific activities were obtained.

Immediately before adding to the hybridization mix, the nick translated DNA was denatured by boiling for 10 min, after the addition of 10 M NaOH to a final concentration of 0.3 M. The probe was then snap cooled on ice and neutralized with an equal volume of 4 M ammonium acetate.

2.2.2.3 Preparation of DNA from λ recombinants

DNA was prepared from recombinant λ phage essentially as described by Kao **et al.** (1982). 10^8 pfu were added to 0.25 ml of SM buffer, 0.25 ml of 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 0.25 ml of an overnight culture of **E. coli** LE392 (Section 2.2.1.6), and incubated at 37°C for 10 min with gentle shaking. 50 ml of NZCYM medium was innoculated with the phage culture and incubated at 37°C with vigorous shaking until lysis of the bacterial cells occurred (3-6 hrs). 5 drops of chloroform were added and after further incubation at 37°C for 10 min, bacterial debris was pelleted by centrifugation for 15 min at 30 000 x g at 4°C. 50 µg of DNase I and 100 µg of RNase A were added to the supernatant, which was kept for 1 h (or overnight) on ice. The phage were pelleted by centrifugation at 40 000 x g for 3 h at 4°C, and resuspended in 400 µl of 0.1 M Tris-HCl pH 8, 0.3 M NaCl. The pellet was extracted once with phenol, once with chloroform and twice with ether. The DNA was obtained by centrifugation, after ethanol precipitation at room temperature for 15 min, and resuspended in TE buffer.

2.2.2.4 Southern blot hybridization analysis

DNA was digested with the appropriate restriction enzymes and electrophoresed on 0.7% agarose gels. The DNA fragments were partially hydrolysed by soaking the gels in 0.25 M HCl for 10 min, and transferred to nitrocellulose filters by the method of Southern, as described by Maniatis **et al.** (1982). The filters were baked **in vacuo** for 2 h and

prehybridized for 5 h at 65°C, in 5 x SSPE, 0.1 % each of Ficoll, polyvinylpyrrolidone, bovine serum albumin, SDS, and sodium pyrophosphate, 0.5 mg/ml denatured salmon sperm DNA and 10% w/v dextran sulphate. Hybridization was carried out for 16 h under the same conditions, with 20-50 ng/ml of nick translated DNA probe (Section 2.2.2.1). Filters were washed twice at room temperature for 15 min, in 2 x SSPE, 0.1% SDS, 0.1% sodium pyrophosphate and twice at 65°C for 30 min, in 0.1 x SSPE, 0.1% SDS, 0.1% sodium pyrophosphate. Low stringency hybridization was carried out similarly, except that the temperature for hybridization and the final washes was reduced to 52°C, and 0.5 x SSPE was used in the final washes. Autoradiography was carried out for several hours at room temperature, or overnight or longer at -80°C, depending on the strength of the hybridization signal.

2.2.3 METHODS USED FOR ISOLATION OF NUCLEI AND TRANSCRIPTION RUN-ON ASSAYS

2.2.3.1 Treatment of animals

Male Wistar rats weighing 250-300 g were starved overnight before use. AIA (20 mg/ml in saline, 30 mg/100 g body weight) was injected subcutaneously 4 h prior to sacrifice. Freshly made solutions of ALV (200 mg/ml in H_2O, 100 mg/100 g) or heme (6.5 mg/ml, 5.2 mg/100g) were administered intraperitoneally, 4 or 14 h prior to sacrifice. Heme (65 mg) was dissolved in 400 µl of 1 M NaOH and 9.6 ml of 0.1 M Tris-HCl pH 7.6 was added slowly.

The liver was perfused with ice-cold saline, excised, weighed and divided into separate portions (where appropriate) for the assay of ALV-S activity and protein content (Section 2.2.4.2) and for isolation

of nuclei for transcription assays (Section 2.2.3.2). Total cellular RNA was also prepared for analysis by Northern or slot-blot hybridization. RNA preparation and analysis was carried out by Dr. G. Srivastava, according to the methods described by Elferink **et al.** (1987).

2.2.3.2 Preparation of rat liver nuclei

Nuclei were isolated from rat liver by the method of Hewish and Burgoyne (1973), as modified by Schibler **et al.** (1983). 2 g of liver was homogeneized in 20 ml of homogeneizing buffer (0.3 M sucrose, 15 mM Hepes pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 5 mM DTT, 0.1 mM PMSF), with 10-20 strokes of a glass-teflon Potter-Elvehjem homogeneizer (clearance 0.23 mm), motor-driven at about 500 rpm.

The homogenate was filtered through 4 layers of cheesecloth, layered over 10 ml of 30% sucrose in homogeneizing buffer and centrifuged for 10 min at 2 500 rpm (750 x g) in an HB-4 rotor at 4°C. The pellet was resuspended in 8 ml of Buffer B (2 M sucrose, 15 mM Hepes pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 5 mM DTT, 0.1 mM PMSF), layered over 4 ml of Buffer B and centrifuged for 20 min at 20 000 rpm (75 000 x g) in an SW41 rotor at 4°C.

The supernatant was aspirated and the nuclei resuspended in 1.5 ml of storage buffer (20 mM Tris-HCl pH 7.9, 75 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 50% glycerol) and centrifuged at 12 000 x g for 15 sec at 4°C, in an Eppendorf microcentrifuge . The pellet was resuspended in an equal volume of storage buffer, and a 1 µl aliquot was diluted in 400 µl of storage buffer and counted using a hemocytometer. The

concentration was adjusted to 3 x 10^8 nuclei per ml. The yield of nuclei was 3-5 x 10^7/g liver (wet weight).

2.2.3.3 Transcription run-on assays

Nuclei were used for transcription run-on experiments immediately after isolation. Transcription reactions contained 100 mM Tris-HCl pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 1.5 mM $MnCl_2$, 0.4 mM EDTA, 0.1 mM PMSF, 1.2 mM DTT, 30% glycerol, 1 mM each of ATP, CTP and GTP, 2 µM of unlabelled UTP, 10-100 µCi of [α-^{32}P]UTP and 1.5 x 10^7 nuclei, in a final volume of 150 µl. Reactions were incubated for the required time (usually 15 min) at 26°C. When Sarkosyl (0.5%), heparin (1 mg/ml) or α-amanitin (2 µg/ml) were included, these were preincubated with the reaction mixture for 5 min on ice. To monitor incorporation of [^{32}P]UTP, the TCA-precipitable radioactivity of 1-5 µl aliquots was determined in triplicate by a modification of the method described in Section 2.2.4.1. Carrier RNA was not required, and before adding the TCA, aliquots were added to Eppendorf microcentrifuge tubes containing 100 µl of 10% SDS, 10mM EDTA. This results in much lower background radioactivity (Marzluff, 1978).

2.2.3.4 Preparation of [^{32}P]RNA

[^{32}P]RNA was prepared as described by Vannice **et al.** (1984). Reactions were terminated by the addition of 750 µl of 0.48% SDS, with 100 µg of **E. coli** tRNA as carrier. After gentle mixing, 900 µl of 100 mM sodium acetate pH 5.0, 20 mM EDTA was added and RNA was isolated after extraction with 1.8 ml of H_2O-saturated acidic phenol. The top phase was collected, 3 M Na acetate was added to a final concentration of 0.2 M and the RNA precipitated by the addition of 2.5 volumes of ethanol. After precipitation for 1 h on dry ice, or overnight at -20°C, the RNA was collected by centrifugation for 30 min at 10 000 rpm

(16 000 x g), in an HB-4 rotor at 4°C. The pellet was washed with ethanol, dried **in vacuo** and resuspended in 200 µl H_2O. The TCA-precipitable radioactivity in 1 µl was determined in triplicate (Section 2.2.4.1).

2.2.3.5 Recombinant DNA used in transcription run-on assays

The 1.8 kb internal **Pst**I fragment from pl01Bl, a rat ALV-S cDNA clone containing a 2.2 kb insert (Srivastava **et al.**, 1987), was subcloned into the **Pst**I site of M13 mpl9. The identity and orientation of the clones was confirmed by partial sequence analysis. Restriction digestion with appropriate enzymes was used to determine the size and number of inserted fragments. Clones with full-length (1.8 kb) inserts were obtained in only one orientation, clones in the other orientation repeatedly deleted large portions of the inserted DNA. Fortunately, the orientation which cloned in full-length was that complementary to the ALV-S mRNA. Southern analysis of rat genomic DNA cut with several restriction enzymes, using [^{32}P]-labelled pl01Bl as probe, detects only unique fragments, indicating that no repetitive sequence elements are present in this clone (Dr. I. Borthwick, personal communication).

Other clones used in the transcription experiments were PB-7, a rat cDNA clone for a phenobarbital-inducible cytochrome P450 (provided by Dr. A. Anderson), a chicken β-actin cDNA clone (provided by S. Dalton) and a chicken serum albumin cDNA clone (provided by Dr. A.H. Hobbs). These clones contained inserts of 1.8, 1.7 and 1.9 kb respectively, in the **Pst**I site of pBR322. Actin and albumin genes have been highly conserved throughout evolution and the chicken actin and albumin clones hybridized readily to rat transcripts under the conditions used.

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

5 µg of DNA (double-stranded for pBR322-derived clones, single-stranded for M13 constructs) was applied to a nitrocellulose filter using a slot-blot apparatus (Schleicher and Schuell). For each slot, 5 µg of DNA, in 200 µl of TE buffer, was incubated with 20 µl of 3 M NaOH for 30 min at 65°C, the mixture was then neutralized with 220 µl of 4 M ammonium acetate and kept on ice. The nitrocellulose filter was soaked first in H_2O and then in 2 M ammonium acetate, for 10 min each. The DNA (final volume 440 µl) was applied to the nitrocellulose under gentle suction and the filter was air-dried and baked at 80°C **in vacuo**, for 1 h.

Duplicate filters (15xmm x 50mm) were prehybridized in 1 ml of 50% formamide, 5 x SSC, 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.1% sodium pyrophosphate, 0.1 % SDS, 100 µg/ml **E.coli** tRNA and 0.2 % each of Ficoll, polyvinylpyrrolidone and bovine serum albumin, at 52°C overnight. Hybridizations were carried out in the same solution with 2 x 10^6 cpm of [^{32}P]RNA (unless otherwise stated), for 72 hr at 52°C. Filters were washed twice at room temperature for 30 min in 2 x SSC, 0.1 % SDS, 0.1 % sodium pyrophosphate, then twice in 0.5 x SSC, 0.1 % SDS, 0.1 % sodium pyrophosphate at 65°C for 60 min, and autoradiographed and analysed as described below (Section 2.2.3.7).

2.2.3.7 Autoradiography and densitometric quantitation

Nitrocellulose filters were exposed to Kodak XAR-5 film with an intensifying screen at -80°C and the signal was quantitated by densitometric scanning of the resulting autoradiogram, using a Zeineh Soft Laser Densitometer. Preliminary experiments established that the hybridization signal was within the linear response range of the densitometer and proportional to the time of exposure.

2.2.4 MISCELLANEOUS METHODS

2.2.4.1 Measurement of TCA-precipitable radioactivity

1-5 µl of sample was added to 100 µl H_2O, with 100 µg of carrier (denatured salmon sperm DNA for DNA samples, or **E.coli** tRNA for RNA samples). 1-5 ml 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 25 ml of ice-cold 5% TCA, 1% sodium pyrophosphate followed by 10 ml of ethanol. The discs were dried and counted in scintillation fluid (0.3% 2,5-diphenyloxazole, 0.03% 1,4-bis-2-(4-methyl-phenyl-oxazolyl)-benzene), in toluene, in a Beckmann LS 7500 liquid scintillation counter.

2.2.4.2 Preparation of subcellular fractions and assays for ALV-S activity and protein

1 g of liver was homogeneized in 10 ml of 0.1 M pyridoxal-5'-phosphate, 0.25 M sucrose, 50 mM Tris-HCl, pH 7.6, in a Potter-Elvehjem glass homogeneizer with a loose-fitting motor driven teflon pestle. Cell debris and nuclei were pelleted by centrifugation at 500 x g for 5 min at 4°C. The supernatant was centrifuged at 3 000 x g for 5 min and the centrifugal force was increased to 12 000 x g for a further 5 min. The mitochondrial pellet was resuspended in 1 ml of 0.1 mM pyridoxal-5'-phosphate, 50 mM Tris-HCl, 1 mM DTT, pH 7.6 and Triton X-100 was added to a final concentration of 1%. The 12 000 x g supernatant was saved in some experiments for the determination of cytosolic ALV-S.

ALV-S activity was measured in triplicate using a colorimetric assay (Whiting and Granick, 1976a) which relies on the regeneration of the substrate succinyl CoA from CoA and succinate, catalyzed by

bacterial succinyl CoA-synthase. The assay mix contained, in a final volume of 150 µl: 50 mM Tris-HCl pH 7.6, 10 mM succinate, 100 mM glycine, 50 mM Mg SO_4, 0.25 mM pyridoxal-5'-phosphate, 17 mM ATP, 1 mM DTE, 0.3 mM CoA and 2 units of **E.coli** succinyl CoA synthase (kindly supplied by C. Elferink), and 50 µl of sample. The reaction was initiated by the addition of the sample and incubated at 37°C for 15 min. The reaction was stopped by the addition of 100 µl of cold 10% (w/v) TCA, immediately placed on ice for 10 min and centrifuged for 5 min in an Eppendorf microcentrifuge. 150 µl of the supernatant was mixed with 75 µl of a 10:1 (v/v) mixture of 1 M sodium acetate pH 5.5 and acetylacetone and incubated for 10 min at 80°C.

After cooling on ice for 15 min, the pH was carefully adjusted to 7.5-8.0, with approximately 15 µl of 5 M NaOH, and aminoacetone pyrrole was extracted with ether, as described by Lien and Beattie (1982). 220 µl was taken from the aqueous phase (containing ALV pyrrole) and the pH was immediately readjusted with 16 µl of glacial acetic acid. These steps were carried out as quickly as possible on one reaction at a time, to minimize exposure to alkaline pH. Finally, 236 µl of Ehrlichs reagent was added and the absorbance at 555 nm was measured after 10 min, with a Varian double beam spectrophotometer. The amount of ALV-S was calculated using a molar extinction coefficient of 58 $mM^{-1}cm^{-1}$. All assays were corrected for a blank to which TCA had been added at zero time.

Protein was estimated by the method of Bradford (1976) using bovine serum albumin as the standard.

CHAPTER THREE

ISOLATION AND CHARACTERIZATION OF THE CHICKEN ALV-S GENE

3.1 INTRODUCTION

As a prerequisite for understanding the molecular basis of control of ALV-S gene expression, the structure of the gene and its regulatory regions must be defined. This chapter describes the isolation of a series of chicken ALV-S genomic clones, using a chicken liver ALV-S cDNA clone (pl05Bl; Borthwick **et al.**, 1984; 1985) as a probe. These were characterized by restriction enzyme mapping and Southern blot hybridization, to define the extent of the ALV-S coding sequences for subsequent sequence analysis (Chapter 4).

In addition, the question of whether different genes for hepatic and erythroid ALV-S are present in the chicken genome was examined. As discussed in Section 1.5, it has been proposed that tissue-specific isozymes of ALV-S are present in erythroid and hepatic tissues. This view arose initially because of differences in the properties and sizes of the partially purified enzymes, and because of apparent differences in the control of heme biosynthesis in hepatic and erythroid tissues, in particular the responses of erythroid and hepatic ALV-S to heme and drugs. Recently, it has been proposed that ALV-S in chicken is coded for by a multigene family (Yamamoto **et al.**, 1985). This was concluded from Northern blot hybridization experiments using a single stranded RNA probe derived from a chicken erythroid ALV-S cDNA clone. It was found that the probe detected different sized ALV-S mRNA species in RNA from liver, erythroid and other tissues. When the blots were washed with RNase A, only the signal derived from erythroid (or brain) RNA remained, suggesting the nucleotide sequence of erythroid ALV-S mRNA differs from that in other tissues.

The availability of cloned ALV-S gene sequences enabled direct investigation of this proposal at the gene level, by comparing the ALV-S sequences in the cloned genomic DNA with those in total chicken genomic DNA, using Southern blot hybridization analysis.

3.2 RESULTS

3.2.1 Isolation and characterization of chicken ALV-S genomic clones

A chicken DNA recombinant library in bacteriophage λ Charon 4A (Dodgson **et al.**, 1979) was screened by plaque hybridization with nick-translated pl05Bl (Section 2.2.2.1). pl05Bl is a full-length cDNA clone for chicken liver ALV-S, recently isolated in this laboratory (Borthwick **et al.**, 1984; Borthwick **et al.**, 1985). 1.1×10^6 recombinant phage, representing approximately 12 genomic equivalents (Limbach and Wu, 1983), were screened in duplicate. 13 positively hybridizing plaques were detected and purified by 4 or 5 further rounds of screening (Fig. 3.1). Phage DNA was prepared (Section 2.2.2.3) and digested with **Bam**HI and/or **Eco**RI. The restriction pattern indicated that the clones, designated λ cALA-S (chicken ALA-Synthase) 1-13, contained overlapping genomic DNA inserts of 15-19 kb, which spanned a contiguous 40 kb region of the chicken genome (Fig. 3.2). Six of the clones at one end of this region (λ cALA-S 7-12) were very similar. Subsequent sequence analysis (of λ cALA-S 1; Chapter 4) revealed that these clones terminate in an area of high G+C content which is rich in **Hae**III and **Alu**I sites. These restriction enzymes were used in the construction of the library (Dodgson **et al.**, 1979) and this may explain the high proportion of clones at this end of the gene. A similar finding was reported in the isolation of chicken cytochrome c clones from a similar library (Limbach

FIGURE 3.1

DETECTION OF CHICKEN ALV-S GENOMIC CLONES BY PLAQUE HYBRIDIZATION

Recombinant phage were screened after transfer to nitrocellulose filters, by hybridization to nick-translated p105B1 (Section 2.2.2.1). In the initial screening (**A**), approximately 1.1 x 10^6 pfu were screened, in batches of approximately 50 000 pfu on 15 cm plates. Plaques were transferred to duplicate nitrocellulose filters. The signal from one positively hybridizing plaque is arrowed. Subsequent rounds of screening were at lower density (100-1000 pfu per 8 cm plate). An autoradiograph from the final round of screening of one of the recombinants is shown (**B**).

A.

B.

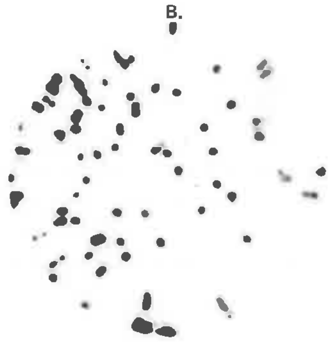


FIGURE 3.2

RESTRICTION MAP OF THE CHICKEN ALV-S GENE LOCUS

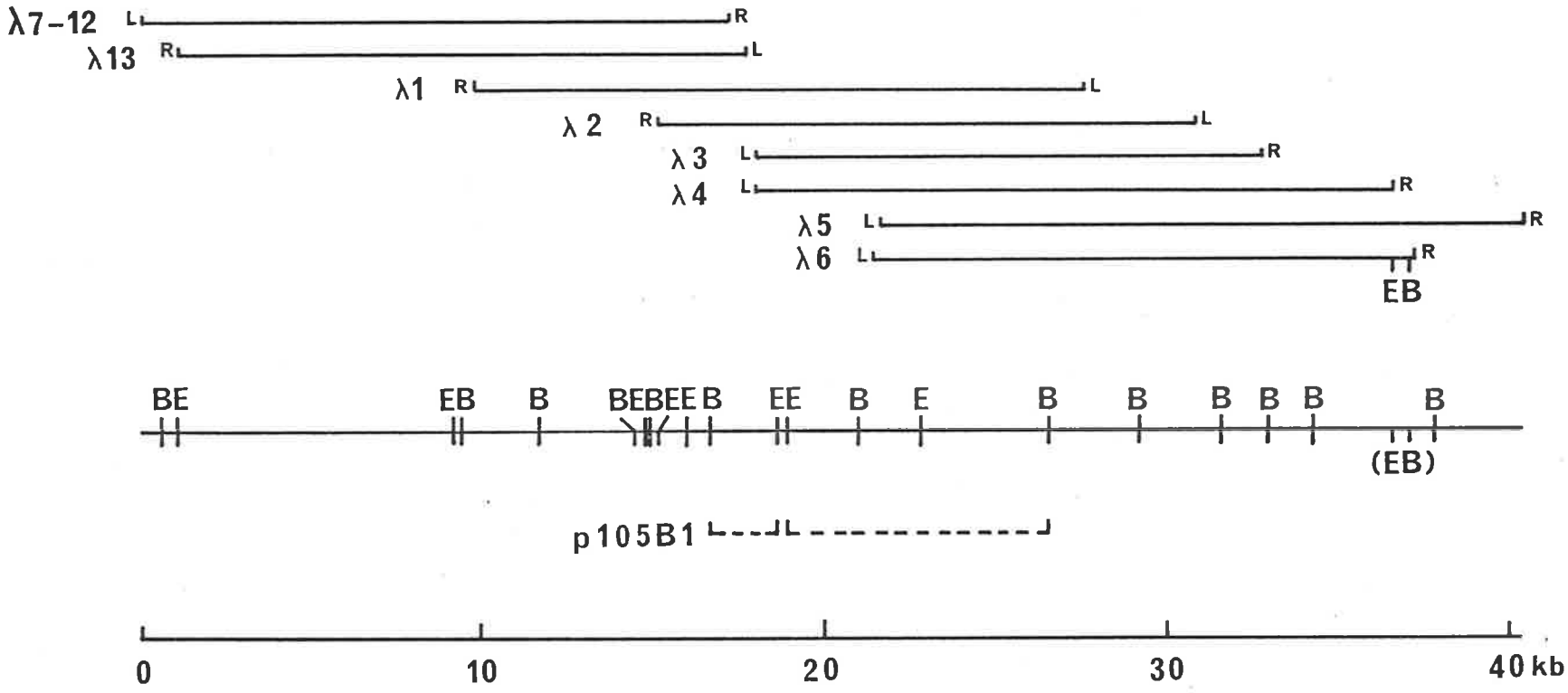
The restriction map of 40 kb of chicken genomic DNA from 13 overlapping clones (λ cALA-S 1-13) is shown. The region spanned by the inserted genomic sequence of each of the clones is indicated above the map. The restriction sites in brackets are present in λ cALA-S 6 but not in λ cALA-S 5, these clones may represent two different alleles. The dashed line indicates restriction fragments which hybridized to p105B1 in Southern blot hybridization experiments (Section 2.2.2.4).

B : BamHI

E : EcoRI

R : right arm of λ Charon 4A vector

L : left arm of λ Charon 4A vector


λ7-12
λ13
λ1
λ2
λ3
λ4
λ5
λ6
L
R
EB
BE
EB
B
BEBEE B
EE
B
E
B
B
B
B
B
B
(EB)
p105B1
0
10
20
30
40 kb

and Wu, 1983). Alternatively, the overrepresentation of these clones may due to amplification of the genomic library.

3.2.2 Southern blot hybridization analysis of the genomic clones

Southern blot hybridization analysis (Section 2.2.2.4) of the 13 ALV-S genomic clones, digested with **Bam**HI and/or **Eco**RI, demonstrated that the sequences hybridizing to pl05Bl were contained within two **Bam**HI genomic fragments of 4.3 and 5.6 kb, or four **Bam**HI/**Eco**RI fragments of 2.0, 2.1, 1.8 and 3.8 kb (from left to right in Fig. 3.2). A small internal **Eco**RI fragment which mapped between the 2.0 and 2.1 kb **Eco**RI fragments did not hybridize to pl05Bl (Fig. 3.2), indicating that the chicken ALV-S gene must contain at least one intron. This is also evident from the fact that the full-length ALV-S cDNA insert of pl05Bl contains no **Eco**RI or **Bam**HI restriction sites and is only 2.2 kb in length (Borthwick **et al.**, 1985), whereas the ALV-S gene contained at least three internal **Eco**RI sites and one internal **Bam**HI site, and spanned a minimum of 4.2 kb (as judged from the restriction map presented in Fig. 3.2).

Further Southern blot hybridization analysis was carried out to determine whether both ends of the gene had been isolated, and to define the transcriptional orientation of the gene within the cloned genomic DNA. Four clones which spanned the entire 40 kb of cloned DNA were digested with **Eco**RI and **Bam**HI and probed with three **Pst**I fragments of pl05Bl, including the 3' and 5' terminal fragments (Sections 2.2.2.2 and 4). λ cALA-S 1, which had a genomic DNA insert of 18 kb, hybridized to both the 3' and 5' terminal **Pst**I fragments (a and d) of pl05Bl (Fig. 3.3, Lane 2), indicating that it was likely to contain the entire ALV-S gene, as well as at least 7 kb of 5' flanking DNA. The transcriptional orientation of the ALV-S gene within the cloned genomic DNA is indicated by an arrow in Fig. 3.4.

FIGURE 3.3

HYBRIDIZATION OF SUBFRAGMENTS OF p105B1 TO CHICKEN ALV-S GENOMIC CLONES

λ cALA-S 1, 4, 5 and 7 were digested with **Bam**HI and **Eco**RI and electrophoresed on a 0.7% agarose gel. The DNA fragments were visualised after staining with ethidium bromide (**B**), and analysed by Southern blot hybridization (Section 2.2.2.4) using nick-translated **Pst**I fragments of p105B1 as probes (Section 2.2.2.2). The autoradiographic signals resulting from hybridization with the indicated **Pst**I fragments of p105B1 (**a**, **c** or **d**) are shown in (**A**).

1 λ cALA-S 7

2 λ cALA-S 1

3 λ cALA-S 4

4 λ cALA-S 5

M1 **Hin**dIII digested λ DNA size markers
(23.1, 9.4, 6.6, 4.3, 2.3, 2.0, 0.5 and 0.1 kb)

M2 **Hin**fI digested pBR322 DNA size markers
(1631, 517, 506, 396, 344, 298, 221, 220, 154 and 75 bp)

A.

B.

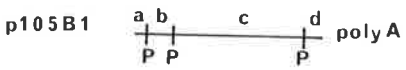


FIGURE 3.4

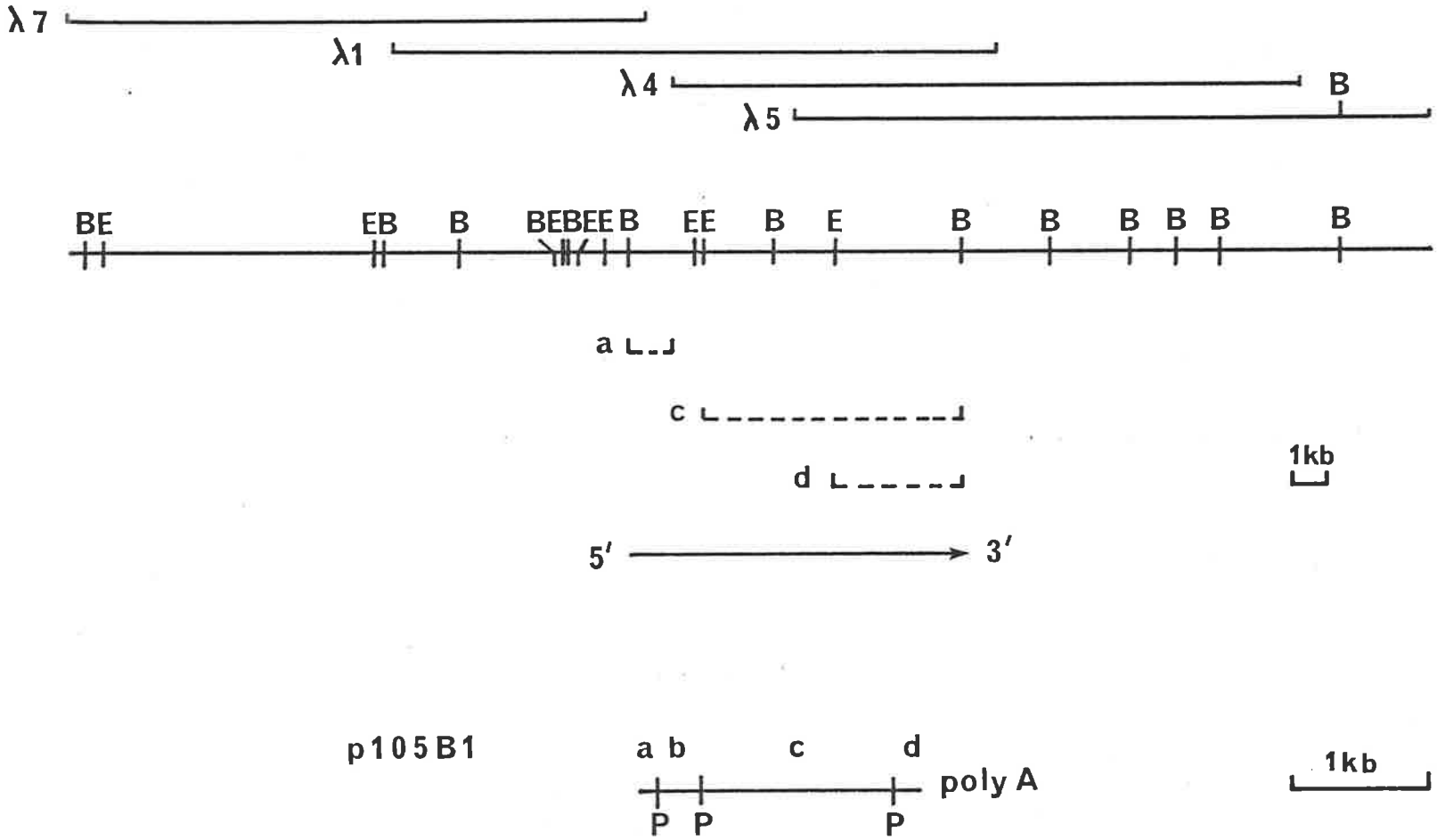
HYBRIDIZATION OF SUBFRAGMENTS OF p105B1 TO CHICKEN ALV-S GENOMIC CLONES

The region spanned by the genomic DNA inserts of λ cALA-S 1, 4, 5 and 7 is indicated. Restriction fragments hybridizing to specific **Pst**I fragments of p105B1 (**a**, **c** or **d**), determined by Southern analysis as described in Figure 3.3, are marked by a dashed line. The transcriptional orientation of the gene is indicated by an arrow.

B : **Bam**HI

E : **Eco**RI

P : **Pst**I


λ7
λ1
λ4
B
λ5
BE
EB
B
BEBEE B
EE
B
E
B
B
B
B
B
B
a
c
d
1kb
5′
3′
p105B1
a b
c
d
poly A
P P
P
1kb

λ cALA-S 1 was chosen for further restriction enzyme analysis in an attempt to delineate the extent of the gene sequences more precisely. λ cALA-S 1 was digested with **Eco**RI, **Bam**HI, **Sac**I or **Hin**dIII, and all possible double combinations of these, and the digested DNA was analysed by Southern blot hybridization using nick-translated p105B1 as probe (Section 2.2.2.4). The region hybridizing to p105B1 spanned 7.7 kb, from a **Bam**HI site at the 5' end to a **Sac**I site at the 3' end (Fig. 3.5). Only restriction sites in the hybridizing region of λ cALA-S 1 were mapped, the non-hybridizing restriction fragments could not all be assigned because of the size of the clone.

3.2.3 Southern blot hybridization analysis of total chicken genomic DNA

Since a total of 13 clones had been isolated from a library of 12 genomic equivalents, and these all mapped to a single genomic locus, it seemed likely that only a single ALV-S gene existed in chicken. In view of the controversy over the existence of separate forms of ALV-S in hepatic and erythroid tissue (Section 1.5) and the proposal of a multigene family for ALV-S by Yamamoto **et al.** (1985), extensive Southern blot hybridization analysis of total chicken genomic DNA was carried out to clarify whether other ALV-S genes were present in the chicken genome.

Total DNA isolated from a single chicken, (kindly provided by L. Mattschoss) and cloned λ cALA-S 1 DNA were digested with several different restriction enzymes (see Fig. 3.6). Southern blot hybridization analysis using nick-translated p105B1 as probe (Section 2.2.2.4), revealed identical hybridization patterns for both total genomic and cloned DNAs (Fig. 3.6). All of the hybridizing bands in the digested chicken total genomic DNA could be accounted for by corresponding bands derived from λ cALA-S 1. For instance, the 4.3 kb and 5.6 kb fragments in the **Bam**HI digested total chicken DNA (Panel B, Lane 2) are matched by identical sized fragments in the cloned DNA

FIGURE 3.5

RESTRICTION MAP OF THE CHICKEN ALV-S GENE IN λ cALA-S 1

The restriction map of the region of λ cALA-S 1 which hybridizes to pl05Bl in Southern blot hybridization experiments (Section 2.2.2.4) was determined as outlined in the text. The relative position of the genomic DNA insert of λ cALA-S 1 is indicated and restriction fragments which hybridized to pl05Bl are marked by a dashed line.

B : BamHI

E : EcoRI

H : HindIII

S : SacI

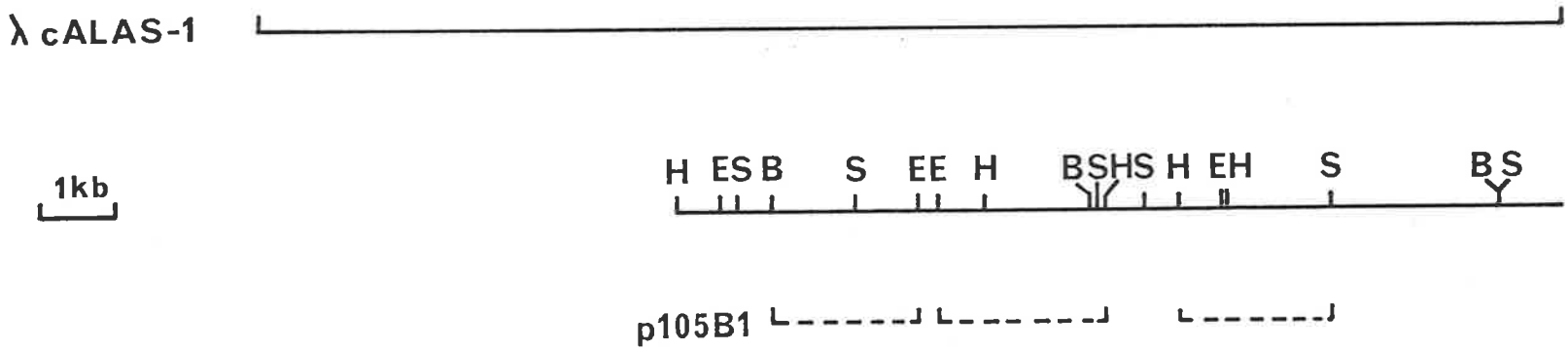
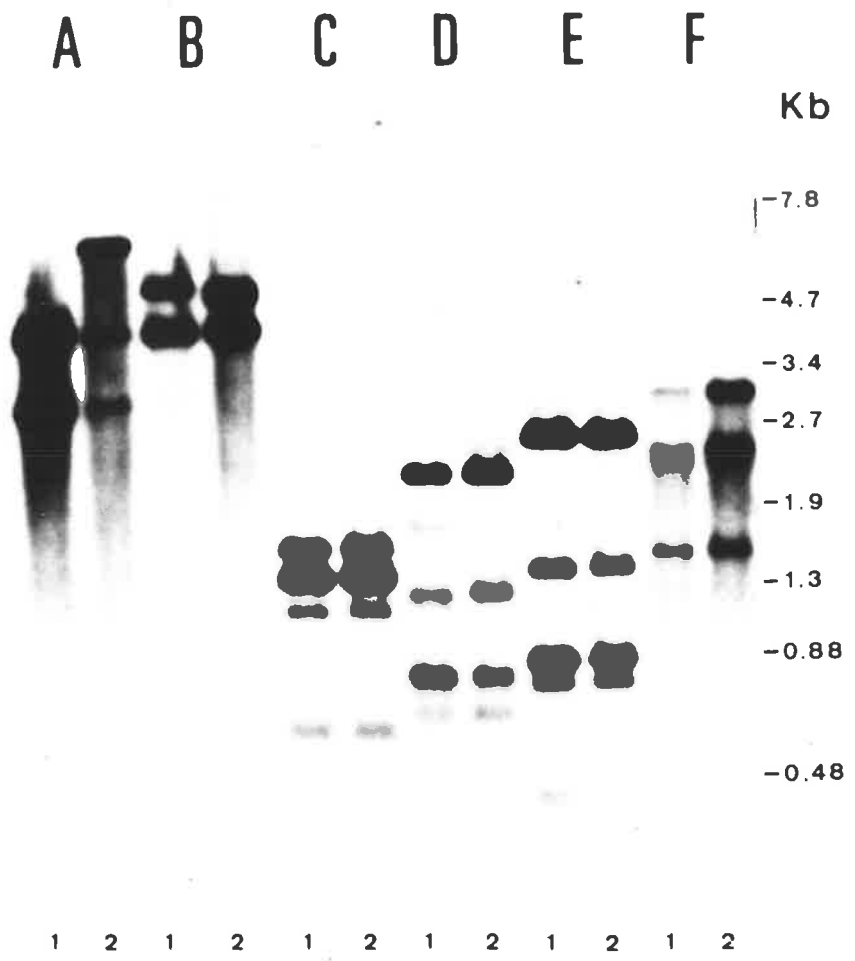

λ cALAS-1
1kb
H ES B S EE H BSHS H EH S BS
p105B1

FIGURE 3.6

SOUTHERN BLOT HYBRIDIZATION ANALYSIS OF TOTAL CHICKEN GENOMIC DNA

5 ng of DNA from the genomic clone λ cALA-S 1 (Lane 1) and 10 μg of total chicken genomic DNA (Lane 2) were digested with : **Ava**II (A); **Bam**HI, (B); **Hgi**AI (C); **Pst**I/**Bam**HI (D); **Pvu**II/**Bam**HI (E); or **Sac**I (F). Southern blot hybridization analysis was carried out using nick translated p105B1 as a probe (Section 2.2.2.4) and the resulting autoradiogram is shown. The size of **Eco**RI digested bacteriophage SPP-1 DNA marker fragments, which were electrophoresed on the same gel, is indicated.


A B C D E F
Kb
7.8
4.7
3.4
2.7
1.9
1.3
0.88
0.48
1 2 1 2 1 2 1 2 1 2 1 2

(Panel B, Lane 1). The additional band in the **Ava**II digested genomic DNA (Panel A, Lane 2) and in the **Pst**I/**Bam**HI digested cloned DNA (Panel D, Lane 1) are partial digestion products. When the hybridization and washing steps were repeated at reduced stringency (Section 2.2.2.4), an increase in non-specific background hybridization was observed but no additional bands were detected in total chicken DNA (not shown). Thus, no sequences homologous to pl05Bl, other than those contained within the genomic clone λ cALA-S 1, are present in the chicken genome.

3.3 DISCUSSION

In the work described in this chapter, a series of chicken genomic clones were isolated which contain sequences complementary to pl05Bl, a chicken liver ALV-S cDNA clone. Together, these clones represent a 40 kb region of the chicken genome. Southern blot hybridization analysis demonstrated that one of these clones (λ cALA-S 1) was likely to contain the entire ALV-S gene within a 7.7 kb segment of DNA. The hybridizing sequences were contained entirely within two **Bam**HI fragments of 4.3 kb and 5.6 kb, and these fragments were subcloned and used for sequence analysis of the chicken ALV-S gene, as discussed in the next chapter. This confirmed that λ cALA-S 1 contains the entire chicken ALV-S gene, which is 6.9 kb in length.

Extensive Southern hybridization analysis of total chicken genomic DNA (Section 3.2.3) has provided strong evidence that the ALV-S gene is present as a single copy in the haploid chicken genome. It is highly unlikely that homologous genes are present since these would have to be completely identical with respect to the recognition sites for six

different restriction enzymes. If related genes coding for tissue-specific isozymes of ALV-S are present in the chicken genome, as suggested by Yamamoto **et al.** (1985), then they are not significantly homologous to the liver ALV-S cDNA clone pl05Bl.

Evidence for a single species of ALV-S in erythroid and hepatic tissues has subsequently been provided in our laboratory, at both the protein and mRNA levels (Elferink **et al.**, 1987). In Western blotting experiments using an antibody raised against purified hepatic ALV-S, the same sized protein (68 000 Da) was detected in extracts from both liver and reticulocytes. The 68 000 Da form of ALV-S was detected in reticulocytes only when these were rapidly boiled under denaturing conditions, since ALV-S appears to be subject to protease degradation in reticulocytes to an even greater degree than in liver. It seems likely that previously reported differences in size between the erythroid and hepatic ALV-S (Watanabe **et al.**, 1983) can be attributed to proteolytic breakdown during isolation.

It has been further demonstrated by RNase mapping and primer extension studies that ALV-S mRNA is identical in chicken liver and reticulocytes (Elferink **et al.**, 1987). Furthermore, a single sized ALV-S mRNA species was detected in various chicken tissues including liver, reticulocytes, brain and heart, in Northern blot hybridization experiments using the chicken liver cDNA clone pl05Bl as probe. A similar comparison of ALV-S mRNA in rat tissues, using a rat liver ALV-S cDNA clone, has demonstrated that hepatic and erythroid ALV-S mRNA are also identical in the rat (Srivastava **et al.**, 1987). The Northern blot hybridization experiments in chicken were carried out using hybridization and washing stringencies even lower than those used by Yamamoto **et al.** (1985), so it was expected that the multiple mRNA species observed by these workers would be detected, if homologous to

the liver cDNA clone. The reason for the differences between the results of Yamamoto **et al.** (1985) and those of Elferink **et al.** (1987) is not clear.

In conclusion, the results presented in this chapter have provided evidence that only a single ALV-S gene homologous to the chicken liver cDNA clone pl05Bl is present in the chicken genome. Since Elferink **et al.** (1987) have shown that liver and erythroid tissues of chicken almost certainly contain identical ALV-S mRNA and protein species, it seems likely that a single gene functions in all tissues. Any differences in the control of ALV-S gene expression in erythroid and hepatic tissues may reflect control by different regulatory proteins in each tissue, rather than the existence of separate genes.

CHAPTER FOUR

SEQUENCE ANALYSIS OF THE CHICKEN ALV-S GENE

4.1 INTRODUCTION

In the previous chapter, the isolation of several genomic clones for ALV-S was described. One of these clones, λ cALA-S 1, contained the entire ALV-S coding region as well as 7 kb of 5' flanking sequence and was chosen for sequence analysis, with the aim of delineating the exon-intron structure of the ALV-S gene and as a basis for further studies to define any regulatory elements which might control expression of the gene. The negative feedback control by heme of transcription of the ALV-S gene (discussed in Chapter 5) was of particular interest, since most of the well-characterized eukaryotic regulatory systems involve positive hormonal or developmental control, and very few examples of negative control mechanisms in eukaryotes have been studied.

At the time the work described in this thesis was commenced, few housekeeping genes, such as that for ALV-S, had been sequenced. Housekeeping genes are expressed in all tissues, generally at low levels, and encode essential functions required for cell growth and maintenance. Although these genes account for the vast majority of active genes in all cells (Carneiro and Schibler, 1984), their regulation is poorly understood, since most studies in eukaryotes have focused on tissue-specific genes. In order to understand the constitutive regulation of ALV-S gene, it was of interest to analyse any structural features it might have in common with other housekeeping genes.

4.2 RESULTS

4.2.1 Preparation of M13 subclones of λ cALA-S 1 and sequence analysis of a 5.2 kb region of the chicken ALV-S gene

The coding sequences of λ cALA-S 1 were contained entirely within two adjacent **Bam**HI restriction fragments of 4.3 and 5.6 kb (Section 3.2.2, Fig. 3.2). These were subcloned into the **Bam**HI site of pUC19 to facilitate preparation of large quantities of the **Bam**HI inserts (Section 2.2.1). A "shotgun" library of random overlapping clones was constructed from each of the **Bam**HI fragments by A. Day, using the method of Deininger (1983). Briefly, the restriction fragments were self-ligated to form concatemers, sonicated to produce fragments of 200-500 bp, end-repaired using Klenow and subcloned into the **Sma**I site of M13mp19 (Section 2.2.1). Sequencing of the library derived from the 5.6 kb **Bam**HI fragment was carried out by A. Day.

The library generated from the 4.3 kb **Bam**HI fragment (representing the 5' end of the gene) was sequenced by myself. In addition, several restriction fragments derived from the 4.3 kb **Bam**HI pUC clone were subcloned into M13 (Section 2.2.1), to obtain sequence in regions that were under-represented in the library. Restriction fragments were also subcloned directly from λ cALA-S 1 to obtain a further 750 bp of 5' flanking sequence. A small **Hin**dIII-**Bgl**II fragment which overlaps the **Bam**HI site between the 4.3 and 5.6 kb fragments was also subcloned from λ cALA-S 1 and sequenced, to confirm that these two fragments were in fact adjacent. The overall strategy used to obtain the sequence of 5.2 kb of the chicken ALV-S gene is shown in Fig. 4.1.

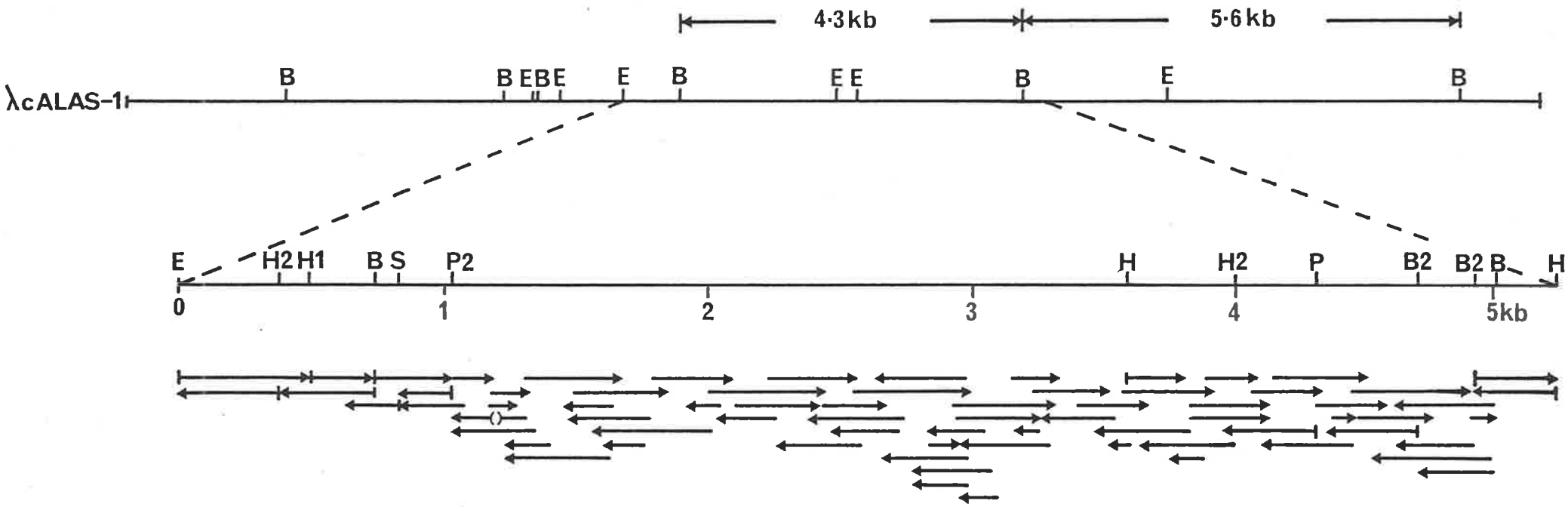
DNA was sequenced by the dideoxy chain-termination method of Sanger **et al.** (1977) (Section 2.2.1.8). A total of 58 clones were sequenced

FIGURE 4.1

SEQUENCING STRATEGY FOR THE 5' END OF THE CHICKEN ALV-S GENE

The arrows indicate the direction and length of sequence obtained from a particular M13 clone. In M13 clones derived by subcloning specific restriction fragments, the restriction site from which sequencing commenced is represented by a vertical line. The remaining clones were from the "shotgun" library derived from the 4.3 kb BamHI fragment (see text). The EcoRI/BamHI restriction map of the genomic insert of λ cALA-S 1, and the 4.3 kb and 5.6 kb BamHI fragments used to generate the "shotgun" libraries, are shown for reference. In the map representing the sequenced portion, only those restriction sites used specifically for M13 cloning are indicated.

B, BamHI; B2, BglII; E, EcoRI; H, HindIII; H1, HgiAI; H2, HincII; P, PstI; P2, PvuII; S, SmaI.

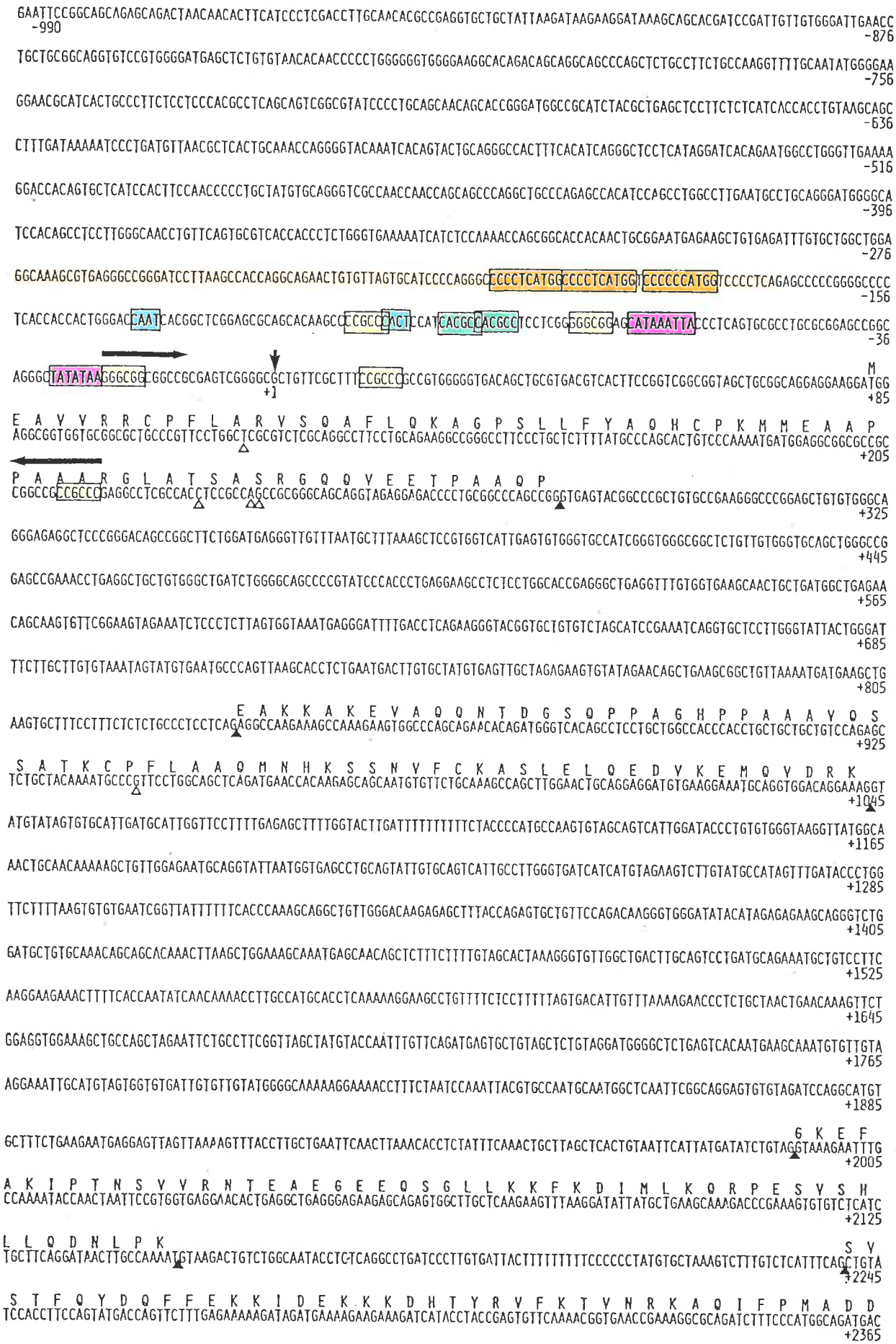

4·3kb
5·6kb
λcALAS-1
B
B E B E
E
B
E E
B
E
B
E H2 H1 B S P2
H H2 P B2 B2 B H
0
1
2
3
4
5kb

from the "shotgun" library, each providing 100-450 bases of sequence. Because of the high G+C content of some areas (Section 4.2.3), much of the sequence was obtained using dITP in the sequencing reactions instead of dGTP (Section 2.2.1.8B). This eliminates secondary structure due to GC base pairing, which otherwise causes anomalous migration of the DNA in the sequencing gels, resulting in band "compression" (Gunn and Symons, 1980). Both strands were sequenced completely and the sequence is presented in Fig. 4.2.

The sequence matched that of the chicken ALV-S cDNA clone pl05Bl (Borthwick **et al.**, 1985), with a few exceptions which are indicated in Fig. 4.2. Most of the differences were silent base substitutions which did not affect the amino acid sequence. Two base substitutions in codon 53 alter the amino acid from alanine encoded by the cDNA sequence to serine in the genomic sequence. These differences may reflect either strain or allelic variations or cloning artifacts. The sequence data were in agreement with the restriction mapping data presented in Chapter 3 (compare Figs. 3.5 and 4.3), confirming that no major rearrangements had occurred during any of the subcloning steps.

4.2.2 The structure of the chicken ALV-S gene

The intron-exon arrangement of the gene was determined by alignment of the genomic sequence with the sequence of the cDNA clone pl05Bl, and is depicted in Fig. 4.3. This figure is compiled from the sequence data presented in Fig. 4.2, together with sequence provided by A. Day for the 3' end of the gene. The gene spans 6.9 kb of DNA and is divided into 10 exons of fairly uniform size (156-280 bp), split by 9 introns of more variable length (91-1100bp) (Table 4.1). The exon-intron boundaries all conform to the GT-AG rule (Breathnach **et al.**, 1978). The splice junctions are in reasonable agreement with the consensus sequence (Table 4.1), and putative lariat branch-point sequences, which have been



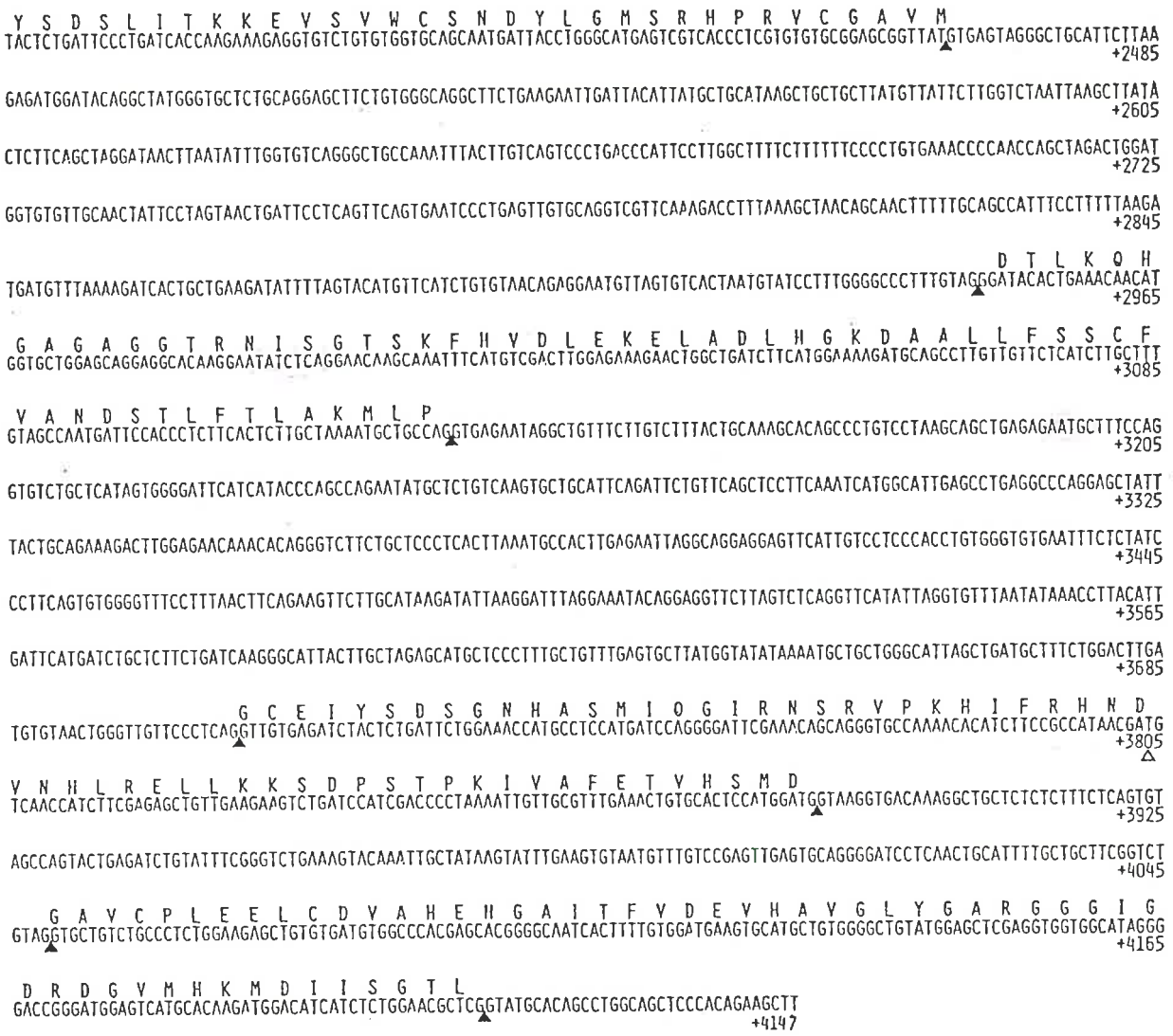


FIGURE 4.2 NUCLEOTIDE SEQUENCE OF THE 5' END OF THE CHICKEN ALV-S GENE

Intron-exon boundaries are indicated (▲) and amino acids encoded by the exons are indicated in one-letter code. The transcription initiation site is marked with a bold arrow. The CAAT, TATA and GC boxes and the tandem repeat elements are boxed. A 12 bp indirect repeat sequence overlapping two of the GC boxes is arrowed. Differences from the cDNA nucleotide sequence (Borthwick et al., 1985) are indicated (△).

TATA box

CAAT box

GC box

10 bp repeat element

6 bp repeat element

FIGURE 4.3

EXON-INTRON STRUCTURE OF THE CHICKEN ALV-S GENE

Exons 1-10 are represented as filled boxes. The ATG start codon and TGA stop codon are indicated, as well as the functional TATA box and the polyadenylation signal (AATAAA; Proudfoot and Brownlee, 1976).
The restriction sites present in the sequenced region are indicated:

B : **BamHI**

E : **EcoRI**

S : **SacI**

H : **HindIII**

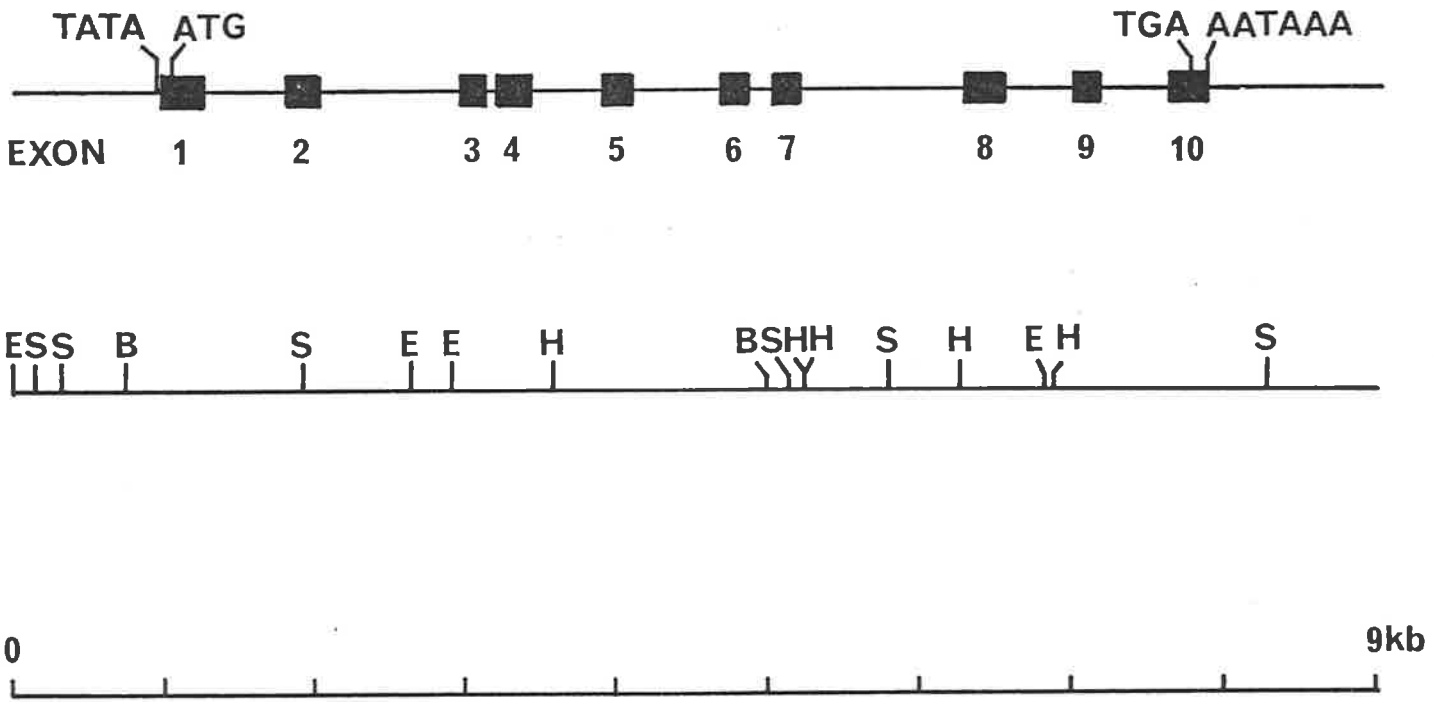

TATA
ATG
TGA
AATAAA
EXON
1
2
3
4
5
6
7
8
9
10
E S S
B
S
E E
H
B S H H
S
H
E H
S
0
9kb

TABLE 4.1

SPLICE SITES IN THE CHICKEN ALV-S GENE

EXON	length (bp)	DONOR[a] exon	DONOR[a] intron	INTRON	length (bp)	ACCEPTOR[a] intron	ACCEPTOR[a] exon
consensus[b]:		$\frac{C}{A}$AG	GT$\frac{A}{G}$AGT			CCCCCCCCCCC$\frac{C}{T}$N$\frac{C}{T}$AG	G
1	280	CCG	GTGAGT	1	556	CTCTGCCCTCCTCAG	A
2	207	AAG	GTATGT	2	950	TTATGATATCTGTAG	G
3	156	AAT	GTAAGA	3	91	TTTGTCTCATTTCAG	C
4	223	TAT	GTGAGT	4	483	TGGGGCCCTTTGTAG	G
5	185	CAG	GTGAGA	5	578	GGGTTGTTCCCTCAG	G
6	180	ATG	GTAAGG	6	160	TGCTTCGGTCTGTAG	G
7	165	TCG	GTATGC	7[c]	1100	AATTGTTTTTTTTAG	G
8	269	AGG	GTAGTA	8	457	AGTTTGGAACTTCAG	G
9	163	TCG	GTGAGT	9	449	CCCTGTTTGTAACAG	A
10	275						

a When ambiguities in the precise splice site existed, exon intron boundaries were determined by applying the GT-AG rule (Breathnach **et al.**, 1978). The GT and AG dinucleotides are in bold type.

b Mount (1982)

c This intron was not fully sequenced, the size was determined from restriction mapping data.

implicated in the splicing mechanism (Ruskin **et al.**, 1984), have been identified at the 3' end of the introns (Table 4.2).

4.2.3 The G+C content of the chicken ALV-S gene.

The G+C content varies considerably along the chicken ALV-S gene (Fig. 4.4A, Table 4.3). A notable feature is the high G+C content of the 5' end of the gene. The region extending from position -995 of the 5' flanking sequence to position +530 in intron 1 has an overall G+C content of 62%. By comparison, the average G+C content for the entire gene and its flanking regions is 48% and that of the chicken genome is 43% (Shapiro, 1976). The G+C content of the 3' end of the gene is closer to the average value for the chicken genome, although that of the exons is generally slightly higher (exons 2-10, average 47% G+C; introns 2-9, average 43%). A slight preference for G+C has been noted for the coding regions of other genes (McClelland and Ivarie, 1982; Smith **et al.**, 1983) and may reflect differential codon usage.

An unusually high frequency of CpG dinucleotides occurs in the 5' flanking region and exon 1 (Figs. 4.4B, 4.4C). This dinucleotide is normally rare in the DNA of higher vertebrates. In the chicken genome for instance, the frequency of CpG dinucleotides is only 1.1%, five-fold less than would be expected if the distribution of nucleotides were completely random (Setlow, 1976). This may be partly due to the high level of cytosine methylation in CpG dinucleotides in higher vertebrates. 5-Methylcytosine can mutate by spontaneous deamination to thymidine, thus CpG dinucleotides would be converted to TpG or CpA, depending on which strand the mutation occurs (Salser, 1977; Bird, 1980). In the ALV-S gene, CpG residues are clustered at the 5' end of the gene and, to a lesser extent, in exons 4 and 6 (Fig. 4.4B). In contrast, GpC dinucleotides are distributed fairly evenly along the gene (Fig. 4.4B). Over most of the gene, the frequency of CpG dinucleotides

TABLE 4.2

PUTATIVE LARIAT BRANCH-POINTS IN THE CHICKEN ALV-S GENE

INTRON	branch point consensus[a]: $CT\frac{G}{A}A\frac{C}{T}$ ..... 10 to 60 bp ..... AG		
1	TTGCTAGAGAAGTGTATAGAACAGCTGAAGCGGCTGTTAAAATGATGAAG	CTGAA	GTGCTTTCCTTTCTCTCTGCCCTCCTCAG
2	AGTTTACCTTGCTGAATTCAACTTAAACACCTCTATTTCAAACTGCTTAG	CTCAC	TGTAATTCATTATGATATCTGTAG
3	ACCTCTCAGGCCTGATCCCTTGTGATTACTTTTTTTTTCCCCCCTATGTG	CTAAA	GTCTTTGTCTCATTTCAG
4	AAGATATTTTAGTACATGTTCATCTGTGTAACAGAGGAATGTTAGTGTCA	CTAAT	GTATCCTTTGGGGCCCTTTGTAG
5	CCCTTTGCTGTTTGAGTGCTTATGGTATATAAAATGCTGCTGGGCATTAG	CTGAT	GCTTTCTGGACTTGATGTGTAACTGGGTTGTTCCCTCAG
6	GCTATAAGTATTTGAAGTGTAATGTTTGTCCGAGTTGAGTGCAGGGGATC	CTCAA	CTGCATTTTGCTGCTTCGGTCTGCAG
7	GGCAACCATTCTGATGGGTGGAAGGAAGAAGCTTGGGTTGCTTACAGCTT	TTAAT	TTCTGAATTGTTTTTTTTAG
8	TTTGACTCCTGTGCTGACGATCCTGGGCTGACAACTTTGCTACCTTGTGG	CTGAT	ACTGAAACAAAGTTTGGAACTTCAG
9	ATTTCTTTCCTGGGATAACTATTGTGTCAGTTCTCTTTTGCTGGAGTAAT	CTGAT	GTTCTGAGATGTGATCTTGGTGCAATTTCCCTGTTTGTAACAG

The most likely branch points have been assigned using the consensus sequence of Keller and Noon (1984)[a] and have been placed in the central column.

Sequences matching the consensus are in underlined, those which are 80% homologous are in bold type.

FIGURE 4.4

THE G+C COMPOSITION OF THE CHICKEN ALV-S GENE

A) The G+C content of the chicken ALV-S gene

The % G+C along the gene was plotted using a window size of 21 bases. The average G+C content of the total chicken genome (43%; Shapiro, 1976) is indicated by an arrow. The broken line represents the unsequenced region of intron 7.

B) Distribution of CpG and GpC dinucleotides

Each vertical line represents one GpC dinucleotide (top) or one CpG dinucleotide (bottom).

C) Frequency of CpG dinucleotides

$(CpG)_{obs}$ = observed frequency of CpG dinucleotides

$(CpG)_{exp}$ = expected frequency of CpG dinucleotides

= (frequency of C) x (frequency of G)

The ratio of CpG_{obs}/CpG_{exp} was determined for the flanking regions of the gene and for each intron and exon. The average ratio for the entire chicken genome (calculated from data in Setlow, 1976) is indicated by an arrow.

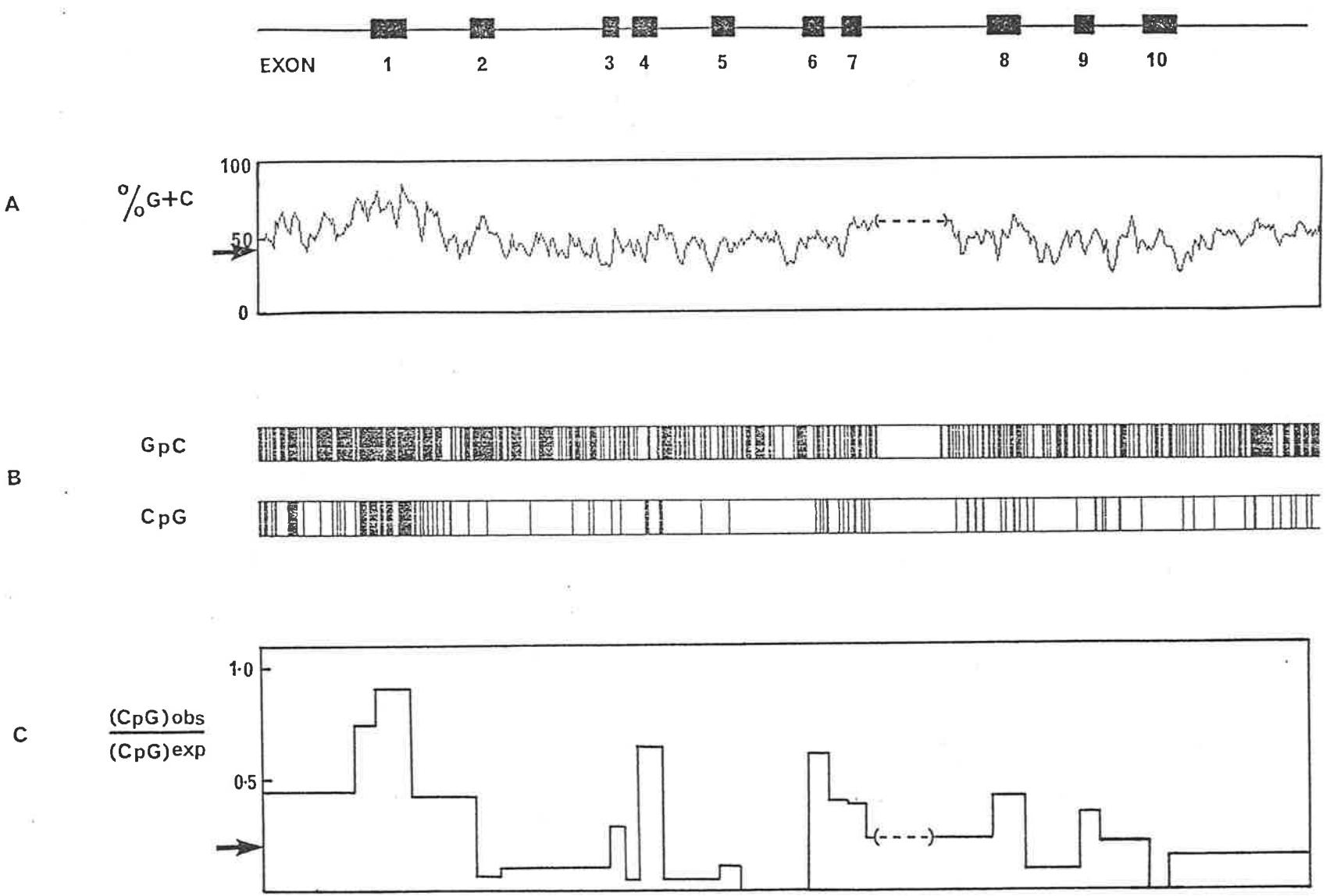

EXON
1
2
3
4
5
6
7
8
9
10
A
%G+C
100
50
0
B
GpC
CpG
C
(CpG)obs / (CpG)exp
1·0
0·5

TABLE 4.3

THE G+C COMPOSITION OF THE CHICKEN ALV-S GENE

REGION	% G+C
5' FLANKING REGION	
-995 to -201	57
-200 to -1	71
EXON 1	72
INTRON 1	54
EXON 2	55
INTRON 2	42
EXON 3	43
INTRON 3	43
EXON 4	47
INTRON 4	42
EXON 5	45
INTRON 5	44
EXON 6	47
INTRON 6	44
EXON 7	58
INTRON 7	46
EXON 8	54
INTRON 8	42
EXON 9	47
INTRON 9	42
EXON 10	40
3' FLANKING REGION	48
AVERAGE :	48

is close to the average frequency for the chicken genome, i.e. much lower than the expected random frequency (Fig. 4.4C). However, in the CpG clusters the frequency approaches that expected for a random sequence of the same base composition (Fig. 4.4C). There is a negative correlation between the levels of CpG and the TpG + CpA content (not shown), supporting the idea that suppression of CpG dinucleotides is a consequence of conversion of methylated cytosine to thymidine.

4.2.4 The 5' flanking region of the chicken ALV-S gene

In primer extension studies of mRNA from chick embryo liver or **Xenopus laevis** oocytes injected with ALV-S expression vectors (undertaken by other workers in this laboratory; Borthwick **et al.**, 1985; Maguire **et al.**, 1986), the length of the extension product is consistent with a transcription start site at the guanosine nucleotide at position +1 (Fig. 4.2). Some well-defined regulatory sequence elements commonly observed in eukaryotic genes transcribed by RNA polymerase II are found in the vicinity of the ALV-S transcription start site (Fig 4.2 and Table 4.4). Two TATA boxes (Breathnach and Chambon, 1981) are located at positions -30 and -71. Two putative CAAT boxes (Benoist **et al.**, 1980) are also present, located about 70 bp upstream of each TATA box, at positions -105 and -139. Five GC hexanucleotide boxes (CCGCCC, or its complement GGGCGG; McKnight **et al.**, 1984) occur at positions -110, -79, -23, +13 and +212.

A number of other features of interest are present at the 5' end of the gene. A unique feature of the 5' flanking region is a tandemly repeated 10 bp sequence, CCCC$^{T}_{C}$CATGG, which is reiterated three times between positions -211 and -180 (Fig. 4.2). Part of this sequence, CCCCTCA, is further repeated, at positions -178 and -159. Another tandemly repeated element occurs at position -97 and contains a 6 bp sequence repeated twice with a one nucleotide overlap, CACGCCACGCC,

TABLE 4.4

POTENTIAL REGULATORY SEQUENCES IN THE CHICKEN ALV-S GENE

CONSENSUS REGULATORY SEQUENCE	POSITION[a]	FUNCTION	REFERENCE	MATCH IN ALV-S GENE	POSITION[a]
TATA(A/T)A(A/T)	-25 to -30	transcription initiation	Breathnach and Chambon (1981)	TATATAA	-30
				CATAAAT	-71
GG(C/T)CAATCT	-70 to -80	transcription efficiency	Benoist et al. (1980)	GCCCACTCC	-105
				GACCAATCA	-139
(G/T)GGGCGG(GGC/AAT)		Sp1 binding, transcription activation	Kadonaga et al. (1986)	TGGGCGGGGC	-113[b]
				GGGGCGGAGC	-80
				AGGGCGCCGG	-23
				CGGGCGGAAA	+10[b]
				CGGGCGGCGG	+209[b]
TGACGTCA	-149 to -179	cAMP induction	Montminy et al. (1986)	TGACTGCA	+29
GTGG(AAA/TTT)G		core enhancer sequence	Weiher et al. (1983)	GTGGAAAG	+1650
				GTGGATAG	+7725
(CA/TG)n		Z-DNA	Rich et al. (1984)	GTATGTATAGTGTGCATTGATGCAT	+1044

a The position of the sequence is relative to the transcription start site (+1).

b Nucleotides which match the consensus sequence are underlined.

c The sequence occurs on the non-coding strand.

(Fig. 4.2). This is of particular interest because a similar sequence, CACGACACGCG, is found at position -120 of the proximal **Rhizobium meliloti** ALV-S promoter (Leong **et al.**, 1985), and the sequence CACGCC occurs between 84 and 98 nucleotides upstream from the multiple transcription start sites in the yeast ALV-S gene (Urban-Grimal **et al.**, 1986).

Many short inverted repeat sequences, with the potential to form stem-loop secondary structures, are present throughout the gene sequence but occur at significantly higher frequency in the 5' flanking region, compared to the remainder of the gene (not shown). Many of these inverted repeats overlap elements already described. In particular, a 12 bp sequence which includes the GC box immediately downstream of the TATA box at position -30, is complementary to a region overlapping the GC box at position +200 (Fig. 4.2). This sequence is made up entirely of G and C nucleotides and has the potential to form a stable secondary structure (ΔG = -41 kcal, Tinoco **et al.**, 1973).

Other possible regulatory sequences in the chicken ALV-S gene (Table 4.4) include an 8 bp palindrome, TGACGTCA, which is proposed to have a role in cAMP induction of gene expression (Montminy **et al.**, 1986) and a stretch of alternating purine-pyrimidine sequence. The latter sequence has potential to form Z-DNA (DNA with a left-handed helical conformation), which has been implicated in both positive and negative gene control mechanisms and in the formation of altered chromatin structure (reviewed by Rich **et al.**, 1984).

4.3 DISCUSSION

The sequence of a 5.2 kb region of the chicken ALV-S gene has been presented here. Together with sequence from the 3' end of the gene provided by A. Day from this laboratory, this represents the first complete gene sequence for any of the heme biosynthetic pathway enzymes (Maguire **et al.**, 1986). The work described in this chapter provides the basis for further studies to define the molecular mechanisms for control of expression of the ALV-S gene by heme or other regulatory factors.

A) The structure of the chicken ALV-S gene

The chicken ALV-S gene spans 6.9 kb and contains 10 exons and 9 introns. The first exon encodes a mitochondrial transit peptide of 56 amino acids and 10 amino acids of the mature enzyme. Together with the next two exons, this segment of the gene encodes an N-terminal region of 145 amino acids which is lacking in the bacterial ALV-S sequence, and may be required for interaction with protein or membrane components within the mitochondrion (discussed in Section 1.3). N-terminal extensions which are thought to have a similar function are also present in yeast and rat. Interestingly, while the rat N-terminal region is strongly homologous to that of chicken, the yeast N-terminal region has no apparent sequence homology. Since neither the bacterial **Rhizobium meliloti** (Leong **et al.**, 1985) or yeast (Urban-Grimal **et al.**, 1986) ALV-S genes contain sequence homologous to the first three exons of the chicken gene, it is unlikely that these exons encode the active site of the enzyme.

Interestingly, the yeast gene contains no introns. By analogy with evolutionary schemes proposed for other proteins (Lonberg and Gilbert, 1985; Gilbert **et al.**, 1986), it is possible that the ancestral ALV-S

gene contained introns but that these were eliminated in yeast and bacteria in the process of streamlining their genomes. In yeast, loss of introns may have occurred after the ancestral eukaryote ALV-S gene evolved from the prokaryote form, by acquiring additional N-terminal sequences needed for interaction with the mitochondrion.

An unusual feature of the gene is a G+C rich region at the 5' end, associated with a high frequency of CpG dinucleotides (Section 4.2.3). Clustered CpG dinucleotides have been observed at the 5' end of many housekeeping genes (Tykocinski and Max, 1984; Max, 1984; Wolf and Migeon, 1985; Lewis **et al.**, 1985) and are often undermethylated (Stein **et al.**, 1983; Chen **et al.**, 1984; Shimada and Nienhuis, 1985). This may be necessary for the constitutive expression of these genes (Stein **et al.**, 1983; Bird, 1986), since methylation has been correlated with inactivation of gene expression. Undermethylation of germ-line DNA would also explain why the dinucleotide is not supressed by mutation, if this is methylation-dependent (Tykocinski and Max, 1984). Nuclease hypersensitivity has been demonstrated in CpG clusters in housekeeping genes (Wolf and Migeon, 1985; Shimada and Nienhuis, 1985) and may also be an indication of a role for these in the maintenance of transcriptional activity. Further studies are required to determine whether the CpG clusters associated with the ALV-S gene are undermethylated or nuclease hypersensitive, and if either of these factors is related to the control of ALV-S gene expression.

B) Potential regulatory elements in the 5' end of the ALV-S gene

Studies of ALV-S gene expression in oocytes and cultured cell lines, undertaken by other workers in this laboratory, have shown that the 5' flanking sequence of the chicken ALV-S gene can promote gene transcription. A 291 bp **BamHI-PvuII** restriction fragment with 257 bp of 5' flanking sequence, extending 34 bp into the first exon of the chicken

ALV-S gene, is able to promote the transcription of a histone H2B reporter gene when introduced into the nucleus of **Xenopus laevis** oocytes by microinjection (Maguire **et al.**, 1986). Similarly, this fragment will support transcription of the chloramphenicol acetyltransferase gene when transfected into human liver or hamster kidney cultured cell lines (A. Day, personal communication). Shorter fragments, containing only 166 bp of 5' flanking sequence, also promote efficient transcription in oocytes and cultured cells (J. Loveridge and A. Day, personal communication).

Several regulatory sequence elements commonly found in RNA polymerase II promoters are present in the promoter region of the ALV-S gene (Fig. 4.2, Table 4.4). These include two pairs of TATA and CAAT boxes and five GC hexanucleotide boxes. The TATA box is found in almost all eukaryotic genes transcribed by RNA polymerase II, usually 25-30 bp upstream of the transcription start site. It functions primarily to define the point of initiation of transcription (Grosschedl and Birnstiel, 1980; Breathnach and Chambon, 1981). The CAAT box (Benoist **et al.**, 1980) is found in many genes, usually 40-100 nucleotides upstream from the transcription start site (Kadonaga **et al.**, 1986). This sequence element is not as highly conserved as the TATA box and its function is less well defined, but in some genes it is required for efficient expression (e.g. Dierks **et al.**, 1983). Transcription factors which bind to TATA or CAAT boxes have been identified (Sawadogo and Roeder, 1985; Jones **et al.**, 1985) and presumably mediate regulation of transcription by these sequences.

Although two consensus TATA and CAAT boxes are present in the chicken ALV-S promoter, primer extension studies (by others in this laboratory) suggest that only the proximal TATA box, at position -30, is functional. mRNA from normal or drug-induced chick embryo liver

* Primer extension analysis of mRNA from chicken liver, kidney, heart, bone marrow and reticulocytes, established that the same TATA box in the 5' flanking region functions in all tissues (Elferink **et al.** 1987).

(Beckmann, 1984; Borthwick **et al.**, 1985), adult chicken liver or reticulocytes, (Elferink **et al.**, 1987) or from **Xenopus laevis** oocytes injected with the ALV-S expression vectors, (Maguire **et al.**, 1986) gives only one major extension product, with a length consistent with direction of initiation of transcription from the -30 TATA box.*

Another common eukaryotic control sequence found in the ALV-S promoter is the GC box (McKnight **et al.**, 1984). In a number of genes, including two housekeeping genes, transcription is activated by the binding of the Hela cell transcription factor Spl to this sequence (Dynan and Tjian, 1985; Kadonaga **et al.**, 1986; Dynan **et al.**, 1986). There are five GC boxes in the ALV-S promoter and the sequence around two of these (at positions -110 and -79 in Fig. 4.2) is a perfect match to the consensus sequence derived by Kadonaga **et al.** (1986) for high affinity binding of Spl (Table 4.4). Multiple copies of the GC box have been found in the control regions of most housekeeping genes which have been sequenced (see, for example, the references cited in Osborne **et al.**, 1985) and may provide for the constitutive basal level of expression of these genes. In housekeeping genes which lack conventional TATA boxes, GC boxes also appear to direct the position of transcription initiation (e.g. Melton **et al.**, 1984; Reynolds **et al.**, 1984), although at heterologous start sites.

A conserved G+C rich sequence of 25 bp, located immediately downstream of the TATA box, was noted in a comparative analysis of eight housekeeping genes in which a TATA box is present (Martini **et al.**, 1986). The chicken ALV-S gene was included in this analysis and has 80% homology with the proposed consensus sequence. It was suggested that this sequence might be of importance in preventing the genes from ever being completely switched off.

An 8 bp sequence, TGACGTCA, is present in the 5' flanking region of eleven eukaryotic cAMP controlled genes, between 49 and 179 bp upstream from the transcription start site (Montminy **et al.**, 1986). This sequence occurs in the chicken ALV-S gene, but downstream of the the transcription start site, at position +30 (Table 4.4). A similar sequence is also present in the in the 5' noncoding region of the rat ALV-S cDNA sequence (I. Borthwick personal communication).

The role of cAMP in the control of ALV-S synthesis gene expression is not clear. Kim and Kikuchi (1974) showed that the AIA-induced increase in levels of ALV-S in rat liver is significantly reduced when cAMP is injected at the same time as AIA, and that cAMP also appears to prevent transport of ALV-S into the mitochondria. Later, it was suggested that this is an indirect effect due to an increase in cellular heme levels after administration of cAMP (Yamamoto **et al.**, 1982b), although the mechanism for this was not established. In contrast, ALV-S induction in isolated hepatocytes is dependent on the presence of cAMP, although this too may be an indirect effect (Srivastava, 1982). However, Friedland and Ades (1985) found that cAMP had no effect on ALV-S synthesis or translocation in isolated hepatocytes. Further experiments are obviously required to directly determine the effect, if any, of cAMP on the control of ALV-S gene expression, and the role of the putative regulatory sequence (see below).

C) Potential heme control elements in the 5' flanking regions of the chicken ALV-S gene.

As well as the more general transcriptional controls discussed above, we are particularly interested in gene-specific regulatory sequences, including those which presumably mediate negative control of ALV-S transcription by heme (see Chapter 5). Few such negative control mechanisms have been described in higher eukaryotes. In bacteria and

yeast, the inhibition of transcription by binding of sequence-specific repressor proteins to the promoter region is a well characterized control mechanism for many genes (Ptashne **et al.**, 1980; Guarente, 1984; Johnson and Herskowitz, 1985) and some evidence for similar mechanisms in higher eukaryotes is available. The best characterized eukaryotic negative control mechanism is the autoregulation of SV40 T antigen gene transcription (Rio **et al.**, 1980). T antigen binds cooperatively to three tandem sites overlapping the transcription start-site and extending 50-70 nucleotides on either side (Myers **et al.**, 1981), inhibiting transcription from the SV40 early promoter, probably by preventing transcription initiation.

Negative feedback mechanisms for two other eukaryotic housekeeping genes have been partly characterized. The hamster gene for 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), the rate-limiting enzyme of cholesterol biosynthesis, is repressed by cholesterol (Osborne **et al.**, 1985) and the human gene for arginosuccinate synthase, a key enzyme in arginine biosynthesis, is repressed by arginine in (Boyce **et al.**, 1986). In both these instances, it has been demonstrated that sequences capable of repressing transcription when fused to a heterologous gene are present in a region extending 300 bp (for the HMG CoA reductase gene) or 149 bp (for the arginosuccinate synthase gene) upstream of the transcription start site. The fusion constructs also contained some downstream sequences, but it was suggested that the region responsible for mediating repression was likely to be upstream of the transcription start site. This is certainly the case for the majority of transcription control mechanisms which have so far been investigated at the molecular level.

Control of ALV-S transcription by heme is also probably mediated by regulatory sequences in the 5' flanking region. This is supported by a

preliminary report that heme repressibility can be conferred on a heterologous gene by sequences upstream from the yeast ALV-S gene (Keng and Guarente, 1985). In the yeast iso-1-cytochrome c and catalase T genes, which are positively regulated by heme, heme regulatory sites have also been defined in a region upstream from the transcription start-site, approximately 400-500 bp upstream for the catalase T gene and 200-300 bp upstream for the iso-1-cytochrome c gene (Guarente and Mason, 1983; Guarente **et al.**, 1984; Spevak **et al.**, 1986).

Candidates for a heme control sequence in the chicken ALV-S gene include the tandemly repeated elements and the inverted repeat sequences discussed in Section 4.2.4. Direct and inverted repeat sequence elements have been implicated in many eukaryotic control mechanisms in which transcription is regulated by the binding of sequence-specific regulatory proteins (e.g. Myers **et al.**, 1981; Brinster **et al.**, 1982; Pelham, 1982; Dierks **et al.**, 1983; Davidson **et al.**, 1983; Guarente **et al.**, 1984; Johnson and Herskowitz, 1985). In the ALV-S gene, a 6 bp element (Fig. 4.2), located between 80 and 120 bp upstream of the transcription start site in the yeast, bacterial and chicken ALV-S genes is of particular interest. A single copy occurs in the yeast gene and two copies of this element are present in an overlapping tandem repeat in the bacterial and chicken genes.

The sequence of the ALV-S promoter was compared with the 5' flanking regions of the **Rhizobium meliloti** and yeast ALV-S genes and with the promoters of the yeast iso-1-cytochrome c and catalase T genes, in order to define further conserved sequence elements which might have a role in heme regulation. Short regions of homology were found, but the significance of these or any of the other potential regulatory sequences described above is difficult to assess fully without a direct functional analysis of the chicken ALV-S promoter. Experiments are

already in progress in this laboratory, using **in vitro** mutagenesis of the promoter region and transient expression systems such as the **Xenopus** oocyte and cultured cell systems described in Section 4.3B, to define the functional elements of the ALV-S promoter, including those reponsible for the basal level of transcription as well as those which mediate control by heme and, possibly, cAMP. The rat and human ALV-S genes have also been isolated in this laboratory and are currently being characterized (Drs. I. Borthwick and M. Bawden personal communication). Comparative sequence analysis of these genes should further aid in the definition of potential regulatory sequence elements.

CHAPTER FIVE

TRANSCRIPTIONAL REGULATION OF THE ALV-S GENE IN RAT LIVER

5.1 INTRODUCTION

The last section of this thesis concerns the control of ALV-S gene expression by heme and porphyrinogenic drugs. Granick (1966) was the first to propose that heme exerts its effect on ALV-S synthesis by repressing ALV-S mRNA transcription (see Section 1.4). Indirect evidence for control of ALV-S synthesis at the level of transcription has been provided by experiments which used transcriptional inhibitors (Srivastava **et al.**, 1980a) or measured the level of translatable mRNA (Whiting, 1976; Yamamoto **et al.**, 1982a). Recently, the isolation of cDNA clones for ALV-S has permitted the direct detection of ALV-S mRNA. It has been demonstrated that alterations in the amount of ALV-S enzyme after treatment of rats and chick embryos with hemin or porphyrinogenic drugs correlate with changes in the steady state levels of ALV-S mRNA (Beckmann, 1984; Srivastava **et al.**, 1987; Drew and Ades, 1986).

Accumulation of ALV-S mRNA could potentially be controlled by altering the rate of gene transcription or by regulation of a post-transcriptional step such as processing, transport or stability of the mRNA (Darnell, 1982), or by a combination of one or more of these. The aim of the work described here was to determine directly whether heme and drugs alter the rate of transcription of the ALV-S gene. To answer this question, the relative rates of transcription were measured by transcription run-on experiments (McKnight and Palmiter, 1979) with nuclei isolated from the livers of rats which had been treated with the porphyrinogenic drug AIA (Section 1.4B), or with either heme or its precursor ALV. At the same time, the levels of ALV-S mRNA and protein were determined by Northern blot hybridization analysis and enzyme assay, to assess the relative contribution of transcriptional and post-transcriptional control of ALV-S levels. As discussed in Section

1.4A, it has been proposed that heme acts primarily at a post-transcriptional level (Sassa and Granick, 1970; Tyrrell and Marks, 1972; Strand **et al.**, 1972).

mRNA synthesis measured in isolated nuclei accurately reflects the transcription rate determined in intact cells (McKnight and Palmiter, 1979; Hofer and Darnell, 1981; Derman **et al.**, 1981; Darnell, 1982). The use of isolated nuclei has the advantage that direct analysis of the transcriptional activity of a gene can be made without the possible complications of post-transcriptional processing or reinitiation of transcription, which occur at very low levels in isolated nuclei (Blanchard **et al.**, 1978; Marzluff, 1978; Marzluff and Huang, 1984).

Transcription in isolated nuclei relies on elongation by RNA polymerase molecules immobilized on their respective genes during isolation of the nuclei. Nascent mRNA chains, initiated **in vivo**, are elongated **in vitro** for a few hundred nucleotides in the presence of a [^{32}P]-labelled nucleotide triphosphate. A complex mixture of transcripts is produced, but the relative amount of a specific gene transcript can be determined by hybridization of the total [^{32}P]-labelled RNA mixture to excess DNA complementary to the transcript of interest, immobilized on nitrocellulose. The specific [^{32}P]-labelled transcript binds to the complementary DNA, and the remaining non-homologous transcripts are washed off. The amount of bound radioactivity is quantitated by autoradiography or scintillation counting. Transcripts from a number of genes can be analysed at the same time, by hybridizing the total RNA to several complementary DNAs immobilized separately on the same filter.

The amount of hybridized RNA, assayed as bound radioactivity, is proportional to the number of RNA polymerase molecules which had initiated transcription **in vivo**, and were engaged in transcription at

the time of isolation of the nuclei (assuming a constant rate of elongation **in vitro**). Therefore, this assay provides a measure of the relative rate of initiation of transcription **in vivo**. If equal amounts of [^{32}P]RNA from different samples of nuclei are used in the analysis, relative transcriptional rates can be compared, both between different samples of nuclei or to a reference gene which is transcribed at a relatively constant rate.

In the experiments described in this chapter, the relative rates of transcription of the ALV-S gene were measured in nuclei isolated from rat liver. Rats were chosen as the experimental animal because conditions for isolation of nuclei and transcription run-on assays are well established for rat and mouse liver (e.g. Derman **et al.**, 1981; Schibler **et al.**, 1983). A rat ALV-S cDNA, recently isolated in this laboratory (Srivastava **et al.**, 1987), was available for the work.

The transcription rate of a drug-inducible cytochrome P450 gene was also examined, in light of the postulated relationship between expression of the ALV-S and cytochrome P450 genes (Section 1.4B). As a control, the transcription rates of the β-actin and serum albumin genes were also determined. These are, respectively, a housekeeping and a liver-expressed gene which were not expected to be transcriptionally regulated by heme or drugs and thus could act as controls for comparing the effects of these agents on transcription of the ALV-S gene.

5.2 RESULTS

5.2.1 The time course and α-amanitin sensitivity of [^{32}P]RNA synthesis in isolated nuclei

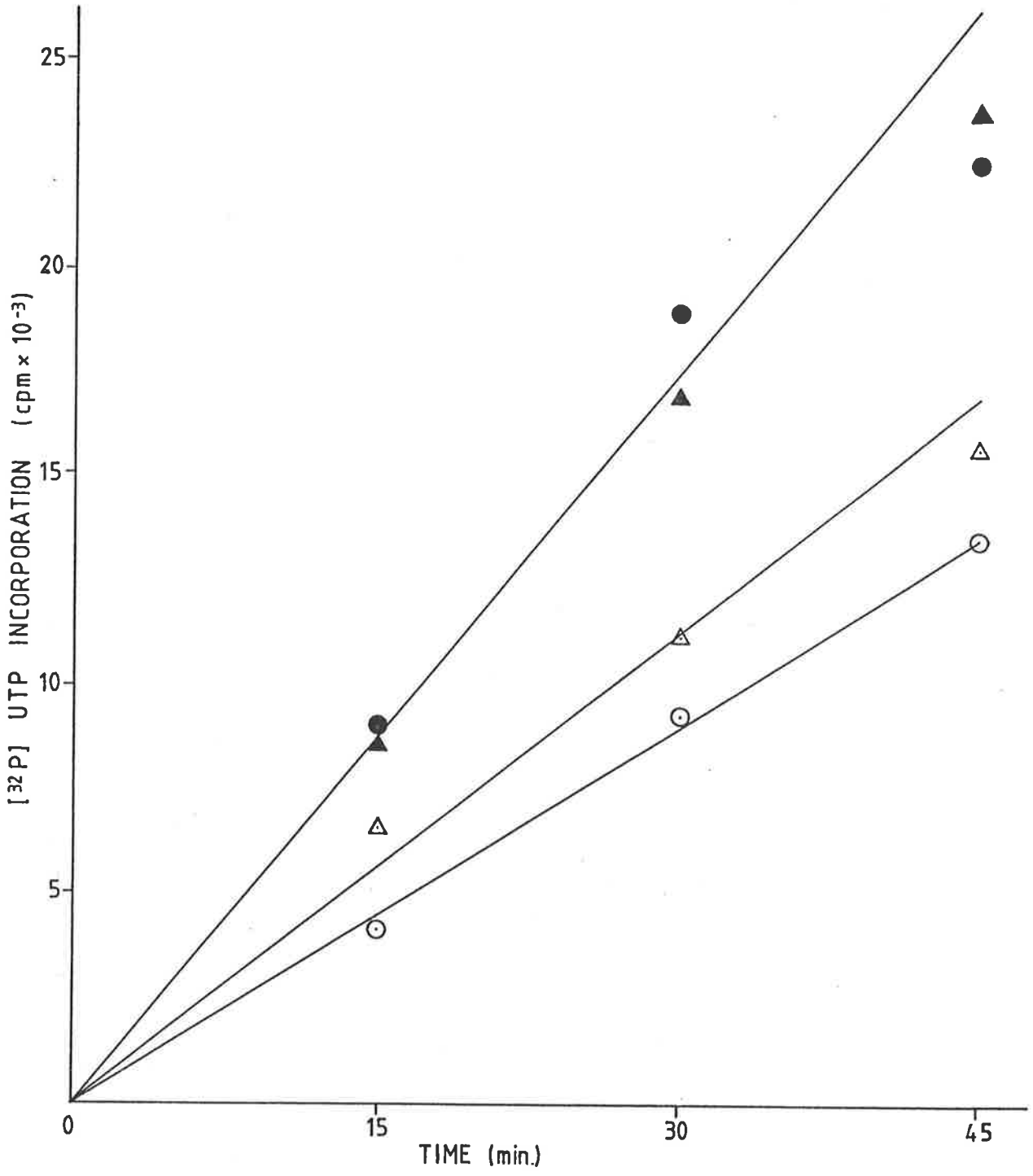
The time course of [^{32}P]UTP incorporation by the isolated nuclei was determined to ensure that this was linear over the time used for the synthesis of [^{32}P]RNA. The α-amanitin sensitivity of [^{32}P]UTP incorporation was also analysed, to determine the proportion of total transcriptional activity due to RNA polymerase II (which synthesizes mRNA), and to ascertain whether this reflected the pattern of RNA synthesis observed **in vivo.**

In initial attempts to isolate nuclei by a rapid procedure (Alterman **et al.**, 1983), incorporation of [^{32}P]UTP into TCA-precipitable radioactivity (Section 2.2.4.1) ceased after 5 min and the amount of incorporated radioactivity then decreased, suggesting ribonuclease contamination (data not shown). Subsequently, the procedure of Schibler **et al.** (1983) was adopted (Section 2.2.3.2). This yields highly purified nuclei with minimal contaminating cellular debris and avoids the use of divalent cations which can activate cellular nucleases (Hewish and Burgoyne, 1973). Nuclei were prepared by this procedure from the livers of control rats or rats treated with AIA for 4 h, and incubated with [^{32}P]UTP (Sections 2.2.3.1-3). Incorporation of [^{32}P]UTP was similar in nuclei from both control and AIA-treated rats and was linear for at least 30 min (Fig. 5.1). For subsequent experiments an incubation time of 15 min was chosen; this permitted sufficient [^{32}P]RNA synthesis, while minimizing opportunity for breakdown or processing of the RNA.

FIGURE 5.1

TIME COURSE OF [^{32}P]RNA SYNTHESIS IN ISOLATED RAT LIVER NUCLEI.

Nuclei from the livers of a control rat (●,○) or a rat treated with AIA (▲,△) were used in transcription run-on assays with 10 μCi of [^{32}P]UTP, with (○,△) or without (●,▲) α-amanitin (2 μg/ml). Aliquots were removed at the indicated times and TCA-precipitable radioactivity was determined.


25
20
15
10
5
0
15
30
45
[32P] UTP INCORPORATION (cpm × 10-3)
TIME (min.)

α-amanitin (2 μg/ml) inhibited [^{32}P]RNA synthesis by about 40% (Fig. 5.1). Since at this concentration α-amanitin specifically inhibits RNA polymerase II (Lindell **et al.**, 1970), it can be concluded that 40% of the total RNA synthesis in this system is carried out by RNA polymerase II, in agreement with other reported values (e.g. Marzluff and Huang, 1984). The synthesis of specific transcripts from the ALV-S, actin and albumin genes (quantitated by hybridization to complementary DNA, as in the next section) was completely inhibited by α-amanitin at 2 μg/ml, confirming that these genes are transcribed by RNA polymerase II (data not shown).

5.2.2 Quantitative hybridization of specific [^{32}P]RNA transcripts

In order to determine the relative transcription rates of specific genes it was necessary to establish conditions for quantitative hybridization of RNA transcripts to filter-bound complementary DNA, and for quantitative analysis of the hybridization signal by densitometry.

Nuclei were isolated from the livers of AIA-treated rats and [^{32}P]RNA was synthesized **in vitro** (Sections 2.2.3.1-4). The [^{32}P]RNA was hybridized to filter-bound DNA (Sections 2.2.3.5 and 6) as indicated (Fig. 5.2) and the hybridized RNA was quantitated by autoradiography and densitometric scanning (Section 2.2.3.7).

For quantitative hybridization of the RNA, the complementary DNA must be present in excess. This was demonstrated by hybridizing increasing amounts of [^{32}P]RNA to filters containing 5 or 10 μg of DNA (Fig. 5.2). The hybridization signal was not increased when the amount of DNA on the filter was doubled (Fig 5.2B) and was directly proportional to the RNA input over a range of 1-4 x 10^6 cpm of RNA (Fig. 5.3). To confirm that the hybridization reaction was driven to completion, "used" hybridization mixtures were rehybridized for a

FIGURE 5.2

QUANTITATIVE HYBRIDIZATION OF [^{32}P]RNA SYNTHESIZED IN RAT LIVER NUCLEI.

[^{32}P]RNA was synthesized in nuclei from AIA-treated rats, and the indicated amounts were hybridized to nitrocellulose filters loaded with: **(A)** 5 µg or **(B)** 10 µg of the following vector or recombinant DNAs (described fully in Section 2.2.3.5):

ALV: ALV-synthase

M13: M13mp19

ACT: actin

pBR: pBR322

ALB: serum albumin

The filters were exposed to X-ray film and the resulting autoradiogram is shown.

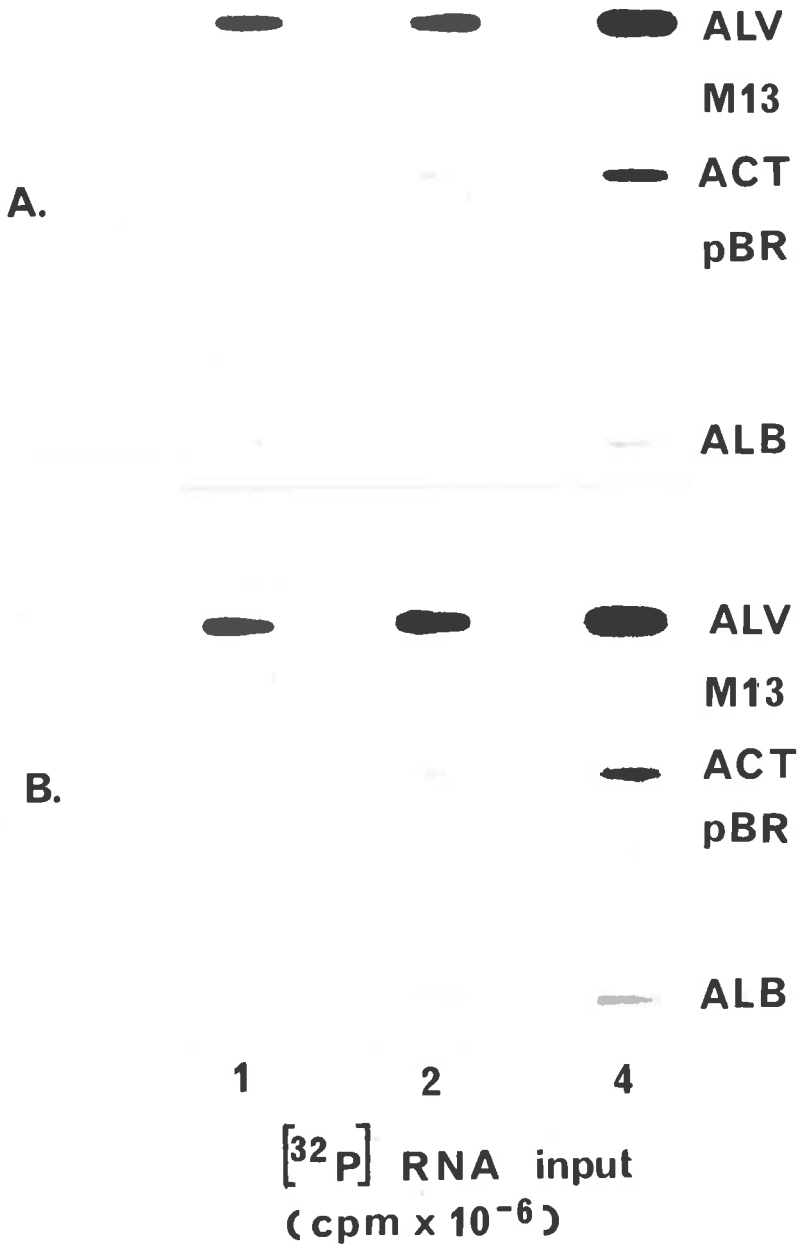

A.
ALV
M13
ACT
pBR
ALB
B.
ALV
M13
ACT
pBR
ALB
1
2
4
[32P] RNA input
(cpm x 10-6)

FIGURE 5.3

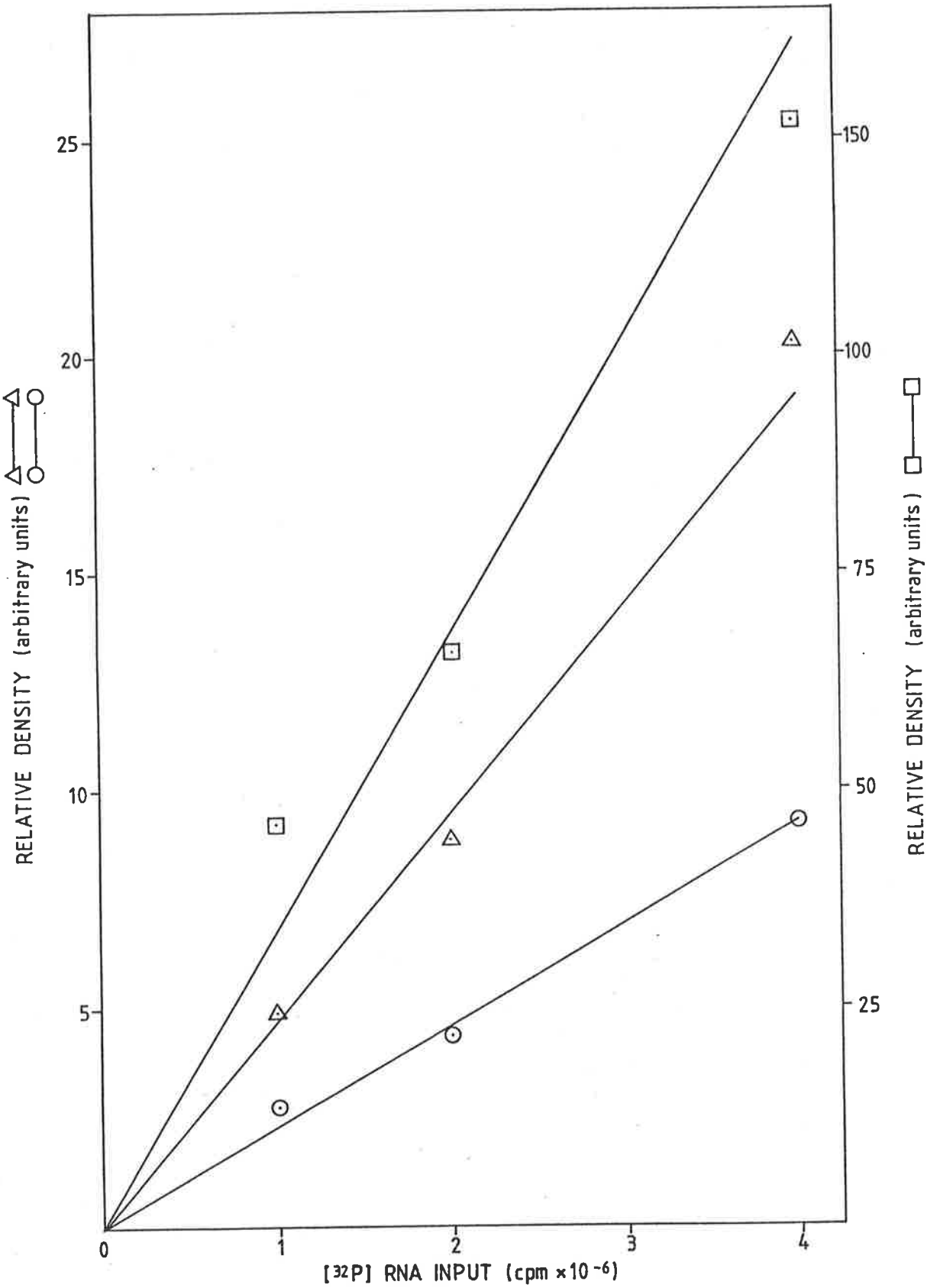
QUANTITATION OF SPECIFIC [^{32}P]RNA TRANSCRIPTS SYNTHESIZED IN RAT LIVER NUCLEI

The signal generated by the following specific [^{32}P]RNA transcripts (hybridized to complementary filter-bound DNA) was quantitated by densitometric scanning of the autoradiogram shown in Figure 5.2. The signals from filters carrying 5 or 10 μg of DNA did not differ significantly and were averaged.

□——□ ALV-synthase

△——△ actin

○——○ serum albumin


RELATIVE DENSITY (arbitrary units)
RELATIVE DENSITY (arbitrary units)
25
20
15
10
5
150
100
75
50
25
0
1
2
3
4
[32P] RNA INPUT (cpm x10-6)

further 72 h to a fresh set of filters, to determine if any unbound [^{32}P]RNA remained. The hybridization efficiency was estimated by this method to be greater than 80 % (data not shown).

Non-specific hybridization to pBR322 or M13 was minimal (Fig. 5.2). For densitometric quantitation of hybridized [^{32}P]RNA, any detectable background due to the vector sequences was subtracted from the hybridization signal. The linearity of the densitometer response was investigated using autoradiograms of a filter to which known quantities of [^{32}P]RNA had been bound directly. The densitometer response was not linear in the lower range and tended to underestimate weak signals by up to 30 % (data not shown). Therefore, hybridization signals were quantitated using autoradiograms exposed for 4-8 days to obtain a reliable signal. Overall, the error in the hybridization signal determined by this method was 10-15 % for strong signals and up to 20-30 % for weaker signals.

5.2.3 Induction of ALV-S and cytochrome P450 transcription by AIA

The relative transcription rates of the ALV-S, cytochrome P450, albumin and actin genes were determined in nuclei isolated from the livers of control or AIA-treated rats (Section 5.2.2). The results of a typical experiment are presented in Figs. 5.4A and 5.5A.

Treatment with AIA for 4 h increased the rate of ALV-S transcription 10-fold, and transcription of the drug-inducible cytochrome P450 gene was also substantially increased (about 6-fold). This is only a minimum estimate of the level of induction of cytochrome P450 transcription, since the cytochrome P450 cDNA clone used in this experiment (PB-7, Affolter **et al.**, 1986) cross reacts with a constitutively expressed cytochrome P450 which is not drug-inducible.

FIGURE 5.4

REGULATION OF ALV-S AND CYTOCHROME P450 TRANSCRIPTION BY AIA AND ALV

A) Induction of ALV-S and cytochrome P450 trancription by AIA

B) Repression of ALV-S and cytochrome P450 transcription by ALV

[^{32}P]RNA was synthesized in nuclei from **(A)** control or AIA-treated rats or **(B)** control or AIA-treated rats administered ALV for 14 h, and hybridized to nitrocellulose filters loaded with 5 µg of the following vector or recombinant DNAs (described fully in Section 2.2.3.5):

ALV: ALV-synthase

M13: M13mp19

ACT: actin

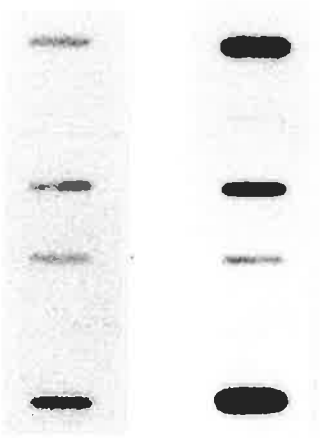
ALB: serum albumin

pBR: pBR322

P450: cytochrome P450

The filters were exposed to X-ray film and the resulting autoradiogram is shown.

A.



CONTROL AIA

B.

CONTROL AIA

FIGURE 5.5

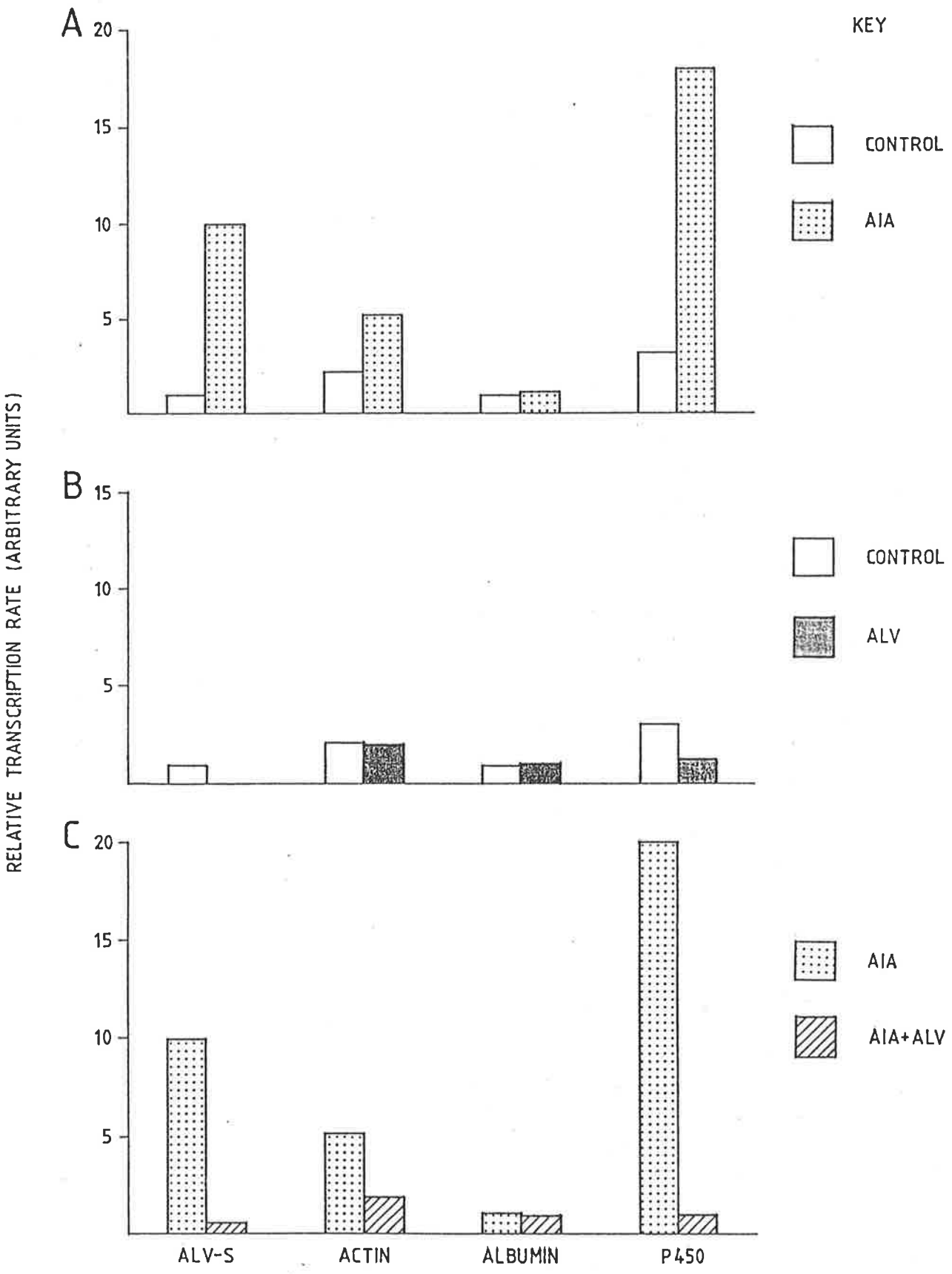
REGULATION OF ALV-S AND CYTOCHROME P450 TRANSCRIPTION BY ALV AND AIA

(A) Induction of ALV-S and cytochrome P450 transcription by AIA

(B) Repression of ALV-S and cytochrome P450 transcription by ALV in control rats

(C) Repression of ALV-S and cytochrome P450 transcription by ALV in AIA-treated rats

The relative transcription rates of the indicated genes were quantitated by densitometric scanning of the autoradiogram shown in Figure 5.4. The values from duplicate filters were averaged.


A
B
C
20
15
10
5
RELATIVE TRANSCRIPTION RATE (ARBITRARY UNITS)
KEY
CONTROL
AIA
CONTROL
ALV
AIA
AIA+ALV
ALV-S
ACTIN
ALBUMIN
P450

Actin transcription was increased slightly by AIA in some experiments (about 2-fold in the experiment shown). AIA and other drugs which induce P450 also induce cell growth and proliferation (Moses **et al.**, 1970; Bock and Remmer, 1978) and it may be speculated that this leads to an increased requirement for actin synthesis. Importantly, however, albumin transcription was not affected by AIA treatment, so induction of gene transcription by AIA is not a general effect.

5.2.4 Repression of ALV-S and cytochrome P450 transcription by heme or its precursor ALV

In the initial experiments, the heme precursor ALV was used to study heme repression. Exogenous ALV is rapidly taken up by the liver and converted to heme **in vivo** (Anderson **et al.**, 1981) and is more conveniently administered than heme, which can present solubility problems.

The relative transcription rates of the ALV-S, cytochrome P450, albumin and actin genes were determined (Section 5.2.2) in nuclei isolated from the livers of control or AIA-treated rats and from control or AIA-treated rats which had been administered the heme precursor ALV for 14 h prior to sacrifice (Section 2.2.3.1). The results from one of two experiments, which gave similar results, are presented in Figs. 5.4 and 5.5.

Unexpectedly, total incorporation of [^{32}P]UTP in nuclei isolated from ALV-treated rats was double that in control nuclei (data not shown). The reason for this is unknown, but it is apparently a general effect. The level of α-amanitin sensitive incorporation, representing total mRNA synthesis, was also doubled. However, the relative transcription rate of the albumin gene remained constant (Figs. 5.5B and 5.5C), i.e. the ratio of albumin mRNA synthesis to total mRNA synthesis

was unchanged, implying that ALV treatment had doubled the absolute level of albumin transcription.

In contrast, ALV treatment of control animals reduced ALV-S transcription to undetectable levels (Fig. 5.5B). In the drug-treated rats, prior administration of ALV completely prevented induction of ALV-S transcription, reducing the level of transcription to below that of normal animals (Fig. 5.5C). A most unexpected result was that ALV treatment completely prevented induction of cytochrome P450 transcription by AIA (Fig. 5.5C) and reduced transcription of the cytochrome P450 gene by about 2-fold in normal animals (Fig. 5.5B). Thus, it appears that transcription of the cytochrome P450 gene, as well as that of ALV-S, is negatively regulated by ALV, a wholly unanticipated result.

Actin transcription was not affected by ALV in the control rats (Fig. 5.5B). ALV administration prevented the 2-fold induction of actin transcription by AIA (Fig. 5.5C), possibly because the drug-induced cellular proliferation discussed in the previous section has also been prevented by ALV. As stated above, the total level of mRNA transcription including that for albumin, was doubled by ALV treatment, although the relative rate of albumin transcription was unaffected. Thus, the repression of ALV-S and cytochrome P450 transcription after ALV treatment is in marked contrast to the effect of ALV on total transcription or, more specifically, that of the albumin gene.

To confirm that the repression of ALV-S and cytochrome P450 transcription by ALV was due to its conversion to heme **in vivo**, the effect of hemin administration was examined in two preliminary experiments. The relative transcription rates of the ALV-S, cytochrome P450, albumin and actin genes were determined (Section 5.2.2) in nuclei isolated from the livers of control or AIA-treated rats and from control

or AIA-treated rats which had been administered hemin for 4 h or 14 h prior to sacrifice (Section 2.2.3.1). In one of the experiments, the yield of [^{32}P]RNA was unexpectedly low and the results obtained were not fully conclusive. Unfortunately, time did not permit further experiments, nevertheless some interesting conclusions can be drawn from this work.

In the first experiment, hemin was administered at the same time as AIA, 4 h prior to killing the animal (Fig. 5.6A). Heme had no significant effect on total incorporation of [^{32}P]UTP or on the relative transcription rates of the ALV-S, actin or albumin genes, in either control or drug-treated rats. However, hemin administration reduced the level of drug induction of cytochrome P450 transcription by 50 % in the AIA-treated rats. This result suggests that the repression of cytochrome P450 transcription by ALV (discussed above) is a consequence of the conversion of ALV to heme.

Since cytochrome P450 transcription, but not ALV-S transcription was repressed by heme in this experiment, it is possible that the level of heme required to repress cytochrome P450 transcription is lower than that required to inhibit ALV-S transcription. This is supported by similar results obtained in an earlier experiment which was undertaken to establish conditions for repression of ALV-S transcription by ALV. ALV was administered for only 4 h, rather than 14 h as in the experiments discussed above. In this experiment, ALV had little effect on ALV-S transcription but reduced cytochrome P450 transcription by 75% in drug-induced animals (Fig. 5.6B). Cytochrome P450 transcription in normal animals was also reduced by approximately 50%, although this is not evident from the photographic reproduction in Fig. 5.6B.

Hemin was administered for 14 h in the next experiment, in the hope that increasing the length of treatment would increase the level of heme

FIGURE 5.6

REPRESSION OF ALV-S AND CYTOCHROME P450 TRANSCRIPTION BY HEME

[^{32}P]RNA was synthesized in nuclei from control or AIA-treated rats. Where indicated, the rats had been administered **(A)** hemin for 4 h, **(B)** ALV for 4 h or **(C)** hemin for 14 h prior to sacrifice. [^{32}P]RNA was hybridized to filters loaded with 5 μg of the following vector or recombinant DNAs (described fully in Section 2.2.3.5):

ALV: ALV-synthase

M13: M13mp19

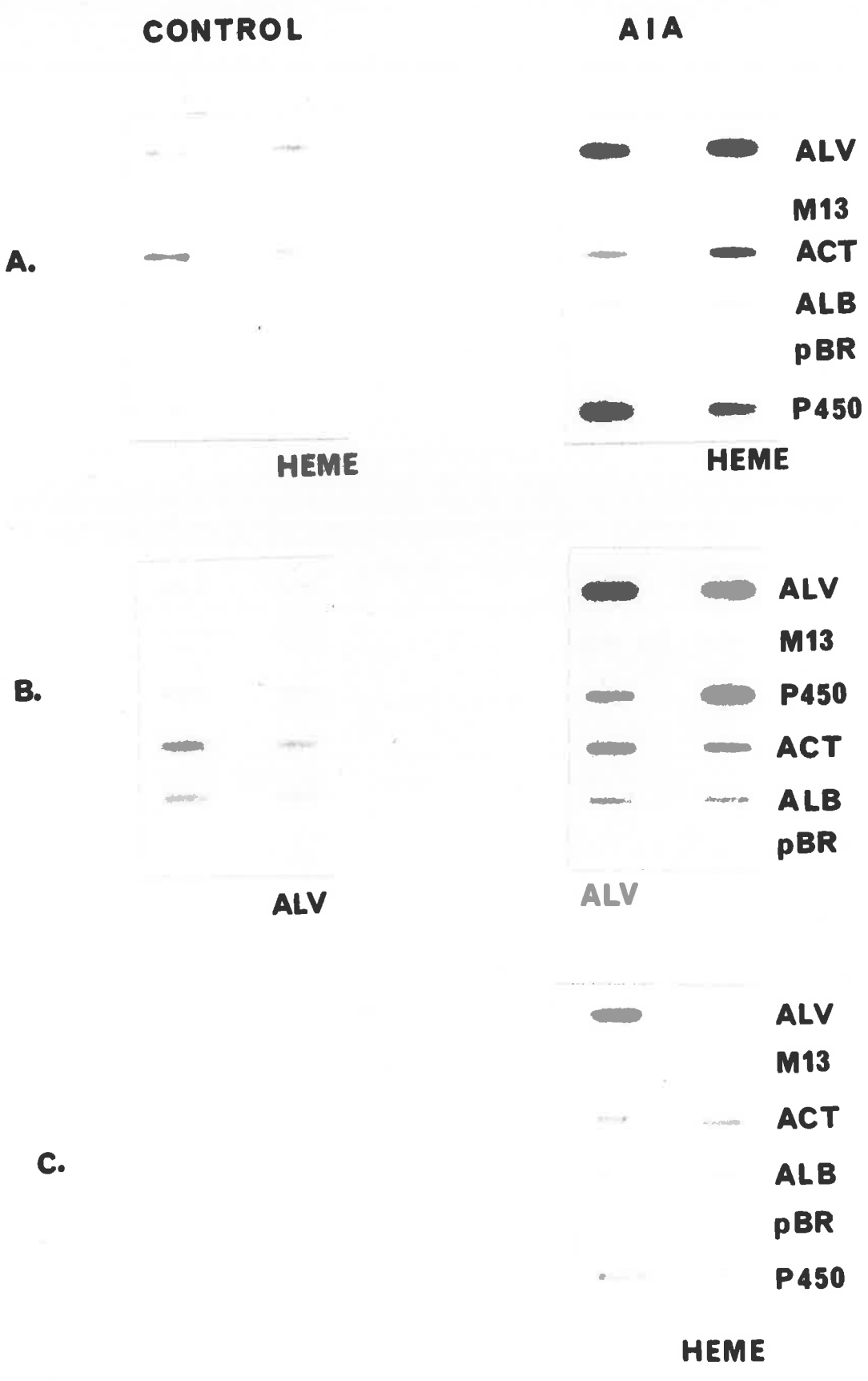
ACT: actin

ALB: serum albumin

pBR: pBR322

P450: cytochrome P450

The filters were exposed to X-ray film and the resulting autoradiogram is shown.


CONTROL
AIA
A.
ALV
M13
ACT
ALB
pBR
P450
HEME
HEME
B.
ALV
M13
P450
ACT
ALB
pBR
ALV
ALV
C.
ALV
M13
ACT
ALB
pBR
P450
HEME

sufficiently to repress ALV-S transcription (as had been found for ALV administration). Unfortunately, the recovery of [^{32}P]RNA in this experiment was very low, so that the hybridizations contained only a sixth of the usual amount of RNA, resulting in very low hybridization signals (Fig. 5.6C). Nevertheless, it is clear that heme has significantly reduced the level of induction of ALV-S transcription by AIA. This strengthens the assumption that repression of ALV-S transcription by ALV is due to conversion of ALV to heme. No conclusions can be made about cytochrome P450 transcription from this experiment since this was much lower than expected in the control drug-treated animal. The reason for this is not known.

5.2.5 The effect of heme on translocation of ALV-S

The failure to obtain repression of ALV-S transcription by heme in the experiment described above, in which heme was administered for 4 h only (Fig. 5.6A), was unexpected. In preliminary experiments to establish conditions for this work, induction of mitochondrial ALV-S activity by AIA was considerably diminished by 4 h heme treatment under the same conditions. One possibility was that heme had prevented the translocation of the enzyme into the mitochondria. In this unique control mechanism, heme prevents transport of ALV-S into the mitochondria, and this results in the accumulation of newly synthesized enzyme in the cytosol. The cytosolic form of ALV-S is a higher molecular weight precursor which is converted to the mature enzyme by proteolytic cleavage of transit peptide during transport into the mitochondria (Section 1.3).

The possibility that heme had prevented mitochondrial transport was investigated by determining ALV-S enzyme activity (Section 2.2.4.2) in mitochondria and post-mitochondrial fractions from the livers of rats treated for 4 h with AIA, either alone or with hemin for 4 h or 14 h,

exactly as in the experiments in Section 5.2.4. The results are presented in Table 5.1.

When hemin was administered for 14 h, ALV-S activity was reduced to negligible levels in both the mitochondrial and post-mitochondrial fractions, i.e. ALV-S synthesis had been completely inhibited. This is consistent with the result of the experiment described above, in which 14 h hemin treatment repressed ALV-S transcription (Fig. 5.6C).

In rats treated with hemin for 4 h only, the mitochondrial level of ALV-S was also diminished, but the amount of ALV-S in the post-mitochondrial fraction had increased, so that the total level of ALV-S was similar to that of animals which had not been administered heme. In fact, total ALV-S activity was actually higher in the hemin-treated rats, this may reflect underestimation of mitochondrial ALV-S activity (relative to cytosolic ALV-S) because of reduced substrate accessibility. This result suggests that treatment with hemin for 4 h has prevented translocation of ALV-S from the cytoplasm. At the same time, the level of ALV-S synthesis is unchanged. This explains the result of the previous section, where treatment with heme for 4 h had no effect on ALV-S transcription (Fig. 5.6A). It appears the level of heme required to inhibit translocation of ALV-S into the mitochondria is lower than that required to inhibit transcription of the ALV-S gene.

5.2.6 The effects of Sarkosyl, heparin, hemin and ALV on transcriptional elongation in vitro

The results presented above clearly demonstrate transcriptional control of the ALV-S and cytochrome P450 genes by heme or its precursor ALV. This was assumed to reflect repression of transcription initiation **in vivo**. However, it was possible that the reduced levels of ALV-S and cytochrome P450 transcripts obtained with nuclei from ALV- or

TABLE 5.1

THE EFFECT OF HEME ON TRANSLOCATION OF ALV-S

TREATMENT	ALV-S ACTIVITY[1] (nmol ALV/h/g liver, wet weight) MITOCHONDRIA	CYTOSOL[2]	TOTAL
AIA	129	ND[3]	129
	97	ND	97
	105	ND	105
AIA+HEME (4 h)	11	186	197
	2	252	254
	8	88	96
AIA+HEME (14 h)	4	ND	4
	16	ND	16
	7	ND	7

AIA was administered 4 h before sacrifice.

Heme was given with the AIA (for a total of 4 h) or 10 h prior to AIA treatment (for a total of 14 h).

1 Each value represents the average of triplicate determinations of ALV-S activity in a separate animal. For each treatment, ALV-S activity was measured in 3 animals.

2 'Cytosol' refers to the 12 000 x g post-mitochondrial supernatant fraction (see Section 2.2.4.2).

3 ND - no detectable activity

In these studies, the molecular form of ALV-synthase which accumulates in the cytosol is not known; it may be the precursor or a proteolytically modified form.

hemin-treated rats might be due to inhibition of the **in vitro** elongation reactions, or to rapid degradation of the newly synthesized transcripts by a specific ribonuclease. These possibilities were tested in the following experiments.

A) The effects of ALV and hemin on transcriptional elongation in vitro

If heme or ALV were retained within the nuclei during the isolation procedure, this might inhibit RNA polymerase II elongation **in vitro.** To test this, ALV and hemin were included in transcription run-on assays.

The relative transcription rates of the ALV-S, cytochrome P450, albumin and actin genes were determined (Section 5.2.2) in nuclei isolated from the livers of control or AIA-treated rats. The transcription run-on assays were carried out in the presence of 50 μM ALV or 0.01-10 μM hemin (Section 2.2.3.3). The results are presented in Fig. 5.7.

Neither ALV (Fig. 5.7A) or hemin (Fig. 5.7B) had any effect on the relative rate of transcription of any of the genes tested, or on total [^{32}P]RNA synthesis (not shown), either in nuclei from control or drug-treated rats. The concentration of ALV used (50 μM) is sufficient to completely repress induction of ALV-S synthesis in cultured cells (Dr. G. Srivastava, personal communication) and heme was tested at concentrations up to 10 μM, much greater than the expected physiological level (10-100 nM; Granick **et al.**, 1975). Therefore, it seems unlikely that the observed decrease in transcription of the ALV-S and cytochrome P450 genes in ALV or hemin treated rats is due to a direct effect of ALV or heme on elongation.

FIGURE 5.7

THE EFFECTS OF HEMIN AND ALV ON IN VITRO TRANSCRIPTION.

[^{32}P]RNA was synthesized in nuclei from control or AIA-treated rats, in the presence of **(A)** 0 or 50 μM ALV or **(B)** 0-10 μM hemin, as indicated. The RNA was hybridized to nitrocellulose filters loaded with 5 μg of the following vector or recombinant DNAs (described fully in Section 2.2.3.5):

ALV: ALV-synthase

M13: M13mp19

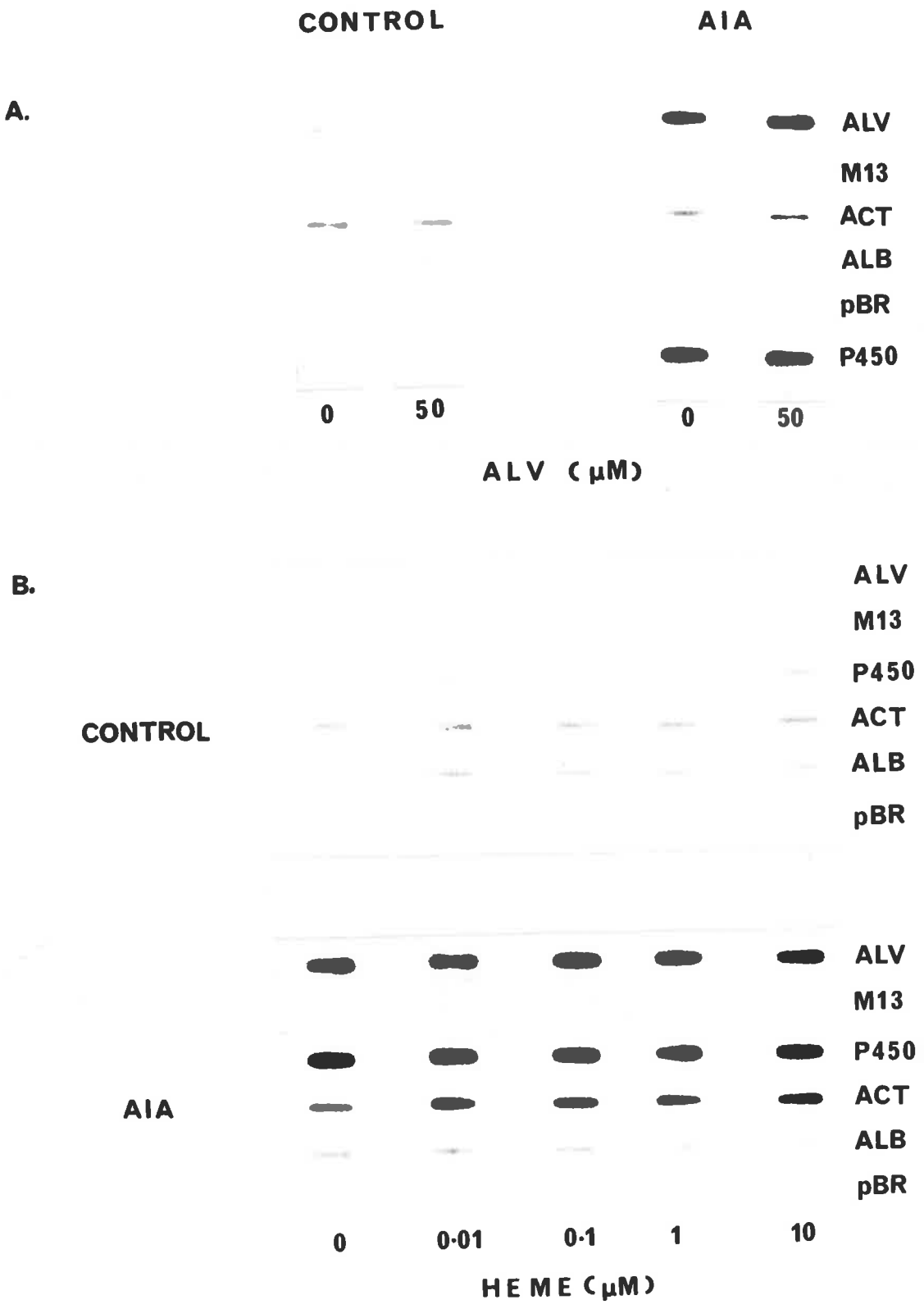
ACT: actin

ALB: serum albumin

pBR: pBR322

P450: cytochrome P450

The filters were exposed to X-ray film and the resulting autoradiogram is shown.


CONTROL
AIA
A.
ALV
M13
ACT
ALB
pBR
P450
0
50
0
50
ALV (μM)
B.
CONTROL
AIA
ALV
M13
P450
ACT
ALB
pBR
0
0·01
0·1
1
10
HEME (μM)

B) The effects of Sarkosyl and heparin on in vitro transcription

The repression of ALV-S and cytochrome P450 transcription in hemin or ALV treated rats could be due to prevention of RNA polymerase movement along the gene by a regulatory protein bound to the DNA. In addition, the newly synthesized ALV-S and P450 transcripts might be rapidly degraded during the transcription assay by a sequence-specific RNase.

These possibilities were tested by including Sarkosyl or heparin in transcription assays of nuclei from ALV-treated rats. Sarkosyl and heparin remove most chromatin proteins but have no effect on RNA polymerase II elongation (Ferencz and Seifart, 1975; Gariglio, 1976). Heparin is also a known RNase inhibitor (Cox, 1976) and both Sarkosyl and heparin release RNA-associated proteins, decreasing the specificity of sequence-specific RNases (Groudine **et al.**, 1981). If transcription elongation along the ALV-S and cytochrome P450 genes has been blocked by a regulatory DNA-binding protein, or if sequence specific RNases are degrading the nascent RNA transcripts of these genes in nuclei from ALV-treated animals, then Sarkosyl or heparin might stimulate transcription of these genes **in vitro.** However, there is no certainty that all DNA binding proteins will be removed, or that a specific ribonuclease will be inhibited, and consequently these experiments are only indicative.

The relative transcription rates of the ALV-S, cytochrome P450, albumin and actin genes were determined (Section 5.2.2) in nuclei isolated from the livers of control or AIA-treated rats which had been administered ALV for 14 h prior to sacrifice (Section 2.2.3.1). In the control rats, ALV treatment reduced transcription of the ALV-S gene to undetectable levels, and that of the cytochrome P450 gene by about 50%. In drug-treated animals, induction of ALV-S and cytochrome P450

transcription, was completely prevented by ALV treatment (data not shown).

Neither Sarkosyl or heparin had any effect on total [^{32}P]RNA synthesis (data not shown) or on transcription of any of the genes tested, apart from a slight reduction in the level of ALV-S and actin transcription (Fig. 5.8). Since neither ALV-S or cytochrome P450 transcription in nuclei from ALV-treated animals was stimulated by Sarkosyl or heparin, it seems likely that repression of ALV-S and cytochrome P450 RNA synthesis is due to prevention of transcription initiation rather than blockage of the RNA polymerase II elongation reaction by a protein bound to the gene or rapid degradation of the newly synthesized transcripts. However, it was recently reported that repression of c-myc transcription in differentiating granulocytes was due to a block in elongation which was not removed by 0.5 % Sarkosyl (Eick and Bornkamm, 1986). This is discussed further in Section 5.3.

5.2.7 The relationship between ALV-S enzyme and mRNA levels and the relative transcription rate of the ALV-S gene

The relative contribution of transcriptional control to the overall level of ALV-S synthesis was investigated by examining the relationship between the relative transcription rate of the ALV-S gene and the steady-state levels of ALV-S mRNA and protein.

Rats were treated with AIA or ALV, or both (Section 2.2.3.1). Portions of liver from the same animal were used for the preparation of nuclei for analysis of ALV-S transcription (Section 2.2.3.1-7) and mitochondria and post-mitochondrial fractions for the assay of ALV-S enzyme activity (Section 2.2.4.2). Total cellular RNA was also prepared and ALV-S mRNA levels were analysed by Northern and slot-blot

FIGURE 5.8

THE EFFECTS OF SARKOSYL AND HEPARIN ON IN VITRO TRANSCRIPION.

[^{32}P]RNA was synthesized in nuclei from control or AIA-treated rats administered ALV for 14 h, in the presence of 0.05 % Sarkosyl **(S)** or heparin (1mg/ml) **(H)**, as indicated. The RNA was hybridized to nitrocellulose filters loaded with 5 µg of the following vector or recombinant DNAs (described fully in Section 2.2.3.5):

ALV: ALV-synthase

M13: M13mp19

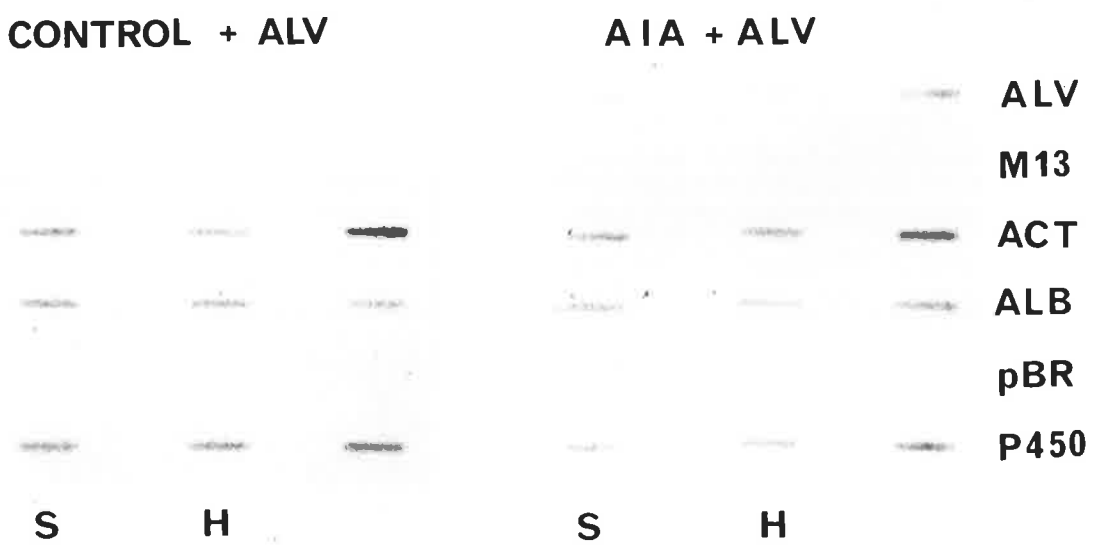
ACT: actin

ALB: serum albumin

pBR: pBR322

P450: cytochrome P450

The filters were exposed to X-ray film and the resulting autoradiogram is shown.


CONTROL + ALV
AIA + ALV
ALV
M13
ACT
ALB
pBR
P450
S
H
S
H

hybridization, essentially as described by Elferink **et al.** (1987). RNA isolation and analysis were performed by Dr. G. Srivastava. The results from one of two experiments (which gave similar results) are presented in Fig. 5.9.

The changes in the transcription rate of the ALV-S gene brought about by treatment with ALV or AIA (see Section 5.2.4) are accompanied by changes of similar magnitude in the steady-state ALV-S mRNA and protein levels (Fig. 5.9). Thus it is likely that modulation of the transcription rate is the major control mechanism regulating ALV-S synthesis in liver.

5.2.3 DISCUSSION

A) Transcriptional control of ALV-S synthesis

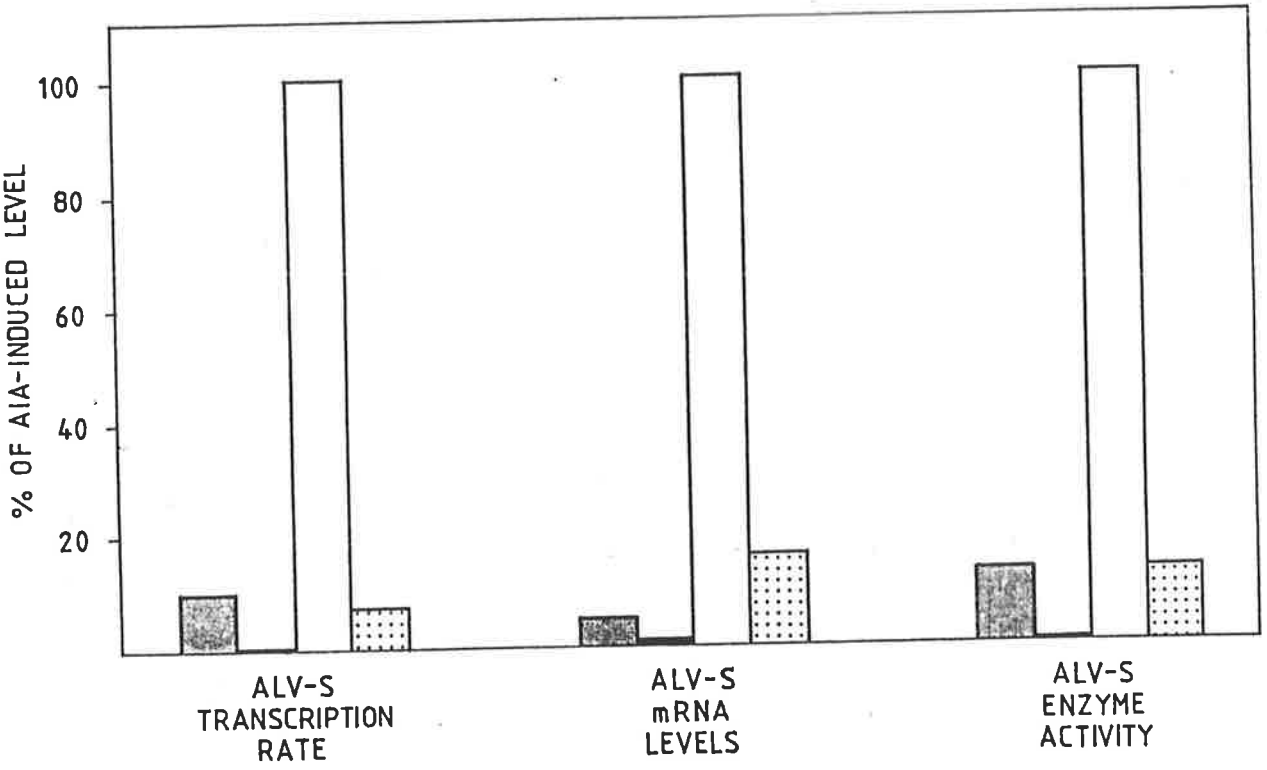
The work presented here demonstrates unequivocally for the first time, that the effects of heme or drug administration on ALV-S levels in experimental animals are due to changes in the rate of transcription of the ALV-S gene. Comparison of the relative levels of ALV-S enzyme activity and mRNA with the relative transcription rate of the gene suggests a high degree of transcriptional control of ALV-S synthesis in liver. This is in contrast to results of earlier workers (Sassa and Granick, 1970; Tyrrell and Marks, 1972; Strand **et al.**, 1972) who concluded, on the basis of indirect evidence from experiments with inhibitors of translation or transcription, that control of ALV-S synthesis by heme is at a post-transcriptional level (Section 1.4A).

The conclusion that ALV-S is controlled primarily at the level of transcription is based on results from a single time point, and further

FIGURE 5.9

COMPARISON OF THE STEADY-STATE ALV-S ENZYME AND mRNA LEVELS WITH THE RELATIVE TRANSCRIPTION RATE OF THE ALV-S GENE.

ALV-S enzyme activity, mRNA levels, and relative transcription rates in the livers of control or AIA-treated rats, or control or AIA-treated rats which had been administered ALV for 14 h, were determined as described in the text (Section 5.2.7). These are expressed as a percentage of the value for the AIA-treated rat.



KEY

CONTROL

CONTROL + ALV

AIA

AIA + ALV

experiments are required to validate this. The time course of ALV-S protein and mRNA accumulation after drug or hemin administration should be investigated and compared with the time course of ALV-S transcription, to determine when the maximum rates of protein and mRNA synthesis occur and how these correlate with the maximum transcription rate of the gene. Unfortunately, time did not permit the author to carry out this work.

Heme repression of the basal level of ALV-S mRNA has been demonstrated in brain, heart, kidney and testis, as well as in the liver, (Srviastava **et al.**, 1987) and it seems likely that a similar transcriptional control operates in these tissues, although this has not been tested directly. The basal levels of ALV-S mRNA vary markedly, the largest amounts occurring in liver, testis and heart, with considerably less in kidney, brain and anemic spleen. This raises the possibility of tissue-specific transcriptional control, which could be tested by examining the transcription rate of the gene in different tissues. Alternatively, tissue-specific controls might operate at a post-transcriptional step, and this is discussed below.

The exact molecular basis for regulation of ALV-S transcription by drugs and heme is unknown although there is much evidence that drugs act only indirectly, by altering heme levels (Section 1.4). The simplest hypothesis for heme regulation of ALV-S transcription, by analogy with previously characterized negative control mechanisms (discussed in Section 4.3C) is that increased levels of heme result in binding of a regulatory protein(s) to the promoter region of the gene, preventing initiation of transcription. Experiments are currently in progress in this laboratory to identify potential **trans** acting regulatory proteins by DNA footprinting and gel retardation assays. It is possible that heme is associated with the putative regulatory protein, or that heme

might even act directly by binding to the gene. In differentiating MEL cells (Section 1.5), heme becomes covalently linked to both proteins and DNA in the nucleus (Lo **et al.**, 1981) and it has been suggested that heme directly effects changes in gene expression associated with erythroid differentiation, by nicking of the DNA (Aft and Mueller, 1983).

An alternative transcriptional control mechanism which cannot be excluded is attenuation, or premature termination of transcription. Strong evidence for such a mechanism has been reported for the control of c-myc and dihydrofolate reductase gene expression (Barsoum and Varshavsky, 1985; Bentley and Groudine, 1986). These genes, like that for ALV-S, are both concerned with house-keeping functions. Sequences within the first intron of the c-myc and dihydrofolate reductase genes are postulated to generate alternative stem-and-loop conformations in the mRNA which cause premature termination of transcription (Farnham and Schimke, 1985; Eick and Bornkamm, 1986). Alternatively, it has been speculated that an antisense mRNA might be involved in this novel control mechanism (Mechti **et al.**, 1986). If the transcription rate of the repressed c-myc gene is determined using probes containing sequences from the first exon only, it is found to be much greater than that determined using probes derived from the 3' end of the gene (Bentley and Groudine, 1986; Eick and Bornkamm, 1986). Similar experiments to determine the transcription rate of specific regions of the ALV-S gene would show whether such a mechanism operates for this gene.

B) Post-transcriptional control of ALV-S synthesis

Although transcriptional control of ALV-S synthesis by heme and drugs has been clearly demonstrated and appears to be the major control mechanism, additional regulation of ALV-S synthesis by post-transcriptional mechanisms (Section 1.4) cannot be excluded by the work presented here, and should be investigated. Now that ALV-S cDNA probes

are available (Section 1.2), mRNA stability during drug induction and heme repression can be measured directly; previously only indirect methods have been available (Marver **et al.**, 1966; Yamamoto **et al.**, 1982a). Another possible post-transcriptional control site is at the level of processing and transport of the mRNA from the nucleus. Analysis of RNA isolated from nuclei, using ALV-S cDNA probes, should clarify whether heme inhibits processing of ALV-S mRNA or causes accumulation of ALV-S mRNA within the nucleus.

It has been suggested that the constitutive expression of most housekeeping genes is regulated by post-transcriptional mechanisms (Carneiro and Schibler; 1984, Derman **et al.**, 1981) and there are many specific examples which support this (e.g. Leys and Kellems, 1981; Groudine and Casimir, 1984; Piechaczyk **et al.**, 1984). As discussed above, the basal level of ALV-S mRNA varies in different tissues, so the possibility of tissue specific control of ALV-S by a post-transcriptional mechanism exists. Some support for this is provided by the finding that the proportion of polyadenylated ALV-S mRNA varies in a tissue-specific manner (Dr. G. Srivastava, personal communication).

C) Heme control of ALV-S translocation

Interestingly, although transcription appears to be the most important mechanism for regulation of ALV-S synthesis, it seems that the primary effect of heme is to block translocation of the enzyme into the mitochondria (Section 1.3). As discussed in Section 5.2.5, administration of hemin together with AIA for 4 hours, inhibited translocation of the enzyme but had no effect on ALV-S transcription. When hemin was administered for 14 hours (10 hours prior to injection of AIA) induction of ALV-S transcription was completely prevented. The simplest hypothesis to explain this result is that repression of ALV-S transcription requires higher levels of heme than repression of

translocation. A similar finding was noted by Yamamoto **et al.** (1981), who reported that the level of heme required to inhibit ALV-S synthesis (measured as enzyme activity) in AIA-treated rats, was greater than that required to inhibit translocation of the enzyme. An alternative explanation is that the mechanism for heme repression of transcription is less direct than that for translocation, so that a longer period of time is required in order for heme to repress transcription.

The differential effect of heme on translocation and transcription should be further investigated, by undertaking time course experiments at varied heme concentrations. These experiments would be more easily carried out in a simpler system such as a cultured hepatocytes, in which the dosage of heme or drugs can be accurately regulated.

As discussed in Section 1.3, inhibition by heme of ALV-S transport into the mitochondria would enable control of the level of catalytically functional ALV-S, since the substrate succinyl CoA is not available in the cytoplasm. The apparent sensitivity of the translocation mechanism to heme makes biological sense, since even if transcription were shut down immediately, synthesis of ALV-S from pre-existing mRNA could still take place. Prevention of ALV-S translocation would therefore provide a more rapid mechanism for controlling the level of enzymatically active ALV-S than repression of transcription alone. However, additional repression of ALV-S synthesis at the level of transcription will ensure tighter control, which is probably very important physiologically, judging from the toxic effects which appear to be mediated by overproduction of ALV-S and ALV in the porphyrias (Section 1.6).

D) Transcriptional regulation of the cytochrome P450 gene

The cytochrome P450 cDNA clone used in these experiments was initially isolated and characterized as being derived from a

phenobarbital-inducible mRNA (Affolter **et al.**, 1986). Induction of the corresponding gene by AIA has not been previously reported but is not unexpected, since phenobarbital and AIA induce the same cytochrome P450 mRNA species in chicken (Brooker **et al.**, 1983). Induction of rat cytochrome P450 transcription by phenobarbital has been demonstrated previously, using isolated nuclei (Hardwick **et al.**, 1983), in agreement with the result obtained here.

The conclusion that cytochrome P450 transcription is negatively regulated by heme is surprising, since others have proposed that heme has a positive effect on cytochrome P450 transcription (Ravishankar and Padmanaban, 1985). These workers have shown that inhibitors of heme synthesis prevent phenobarbital induction of rat cytochrome P450 apoprotein synthesis, mRNA accumulation and gene transcription (Ravishankar and Padmanaban, 1983; 1985). It is difficult to reconcile their results with those reported here. However, since the effect of the inhibitors used by these workers could not be counteracted by adding heme, it is possible that prevention of drug-induction of cytochrome P450 by the inhibitors is due to an indirect effect on some unknown factor other than heme synthesis. Alternatively, since phenobarbital was used by these workers to induce cytochrome P450, whereas the experiments described here used AIA, the effect of heme on induction of cytochrome P450 may be drug-specific. It is not easy to postulate a rationale for this but the possibility cannot be excluded on the available evidence.

Some supporting evidence for heme repression of cytochrome P450 synthesis is available. Many workers have reported a small but reproducible decrease in the level of cytochrome P450 apoprotein after administration of heme or ALV (Marver, 1969; Song **et al.**, 1971; Bock

et al., 1971; Dehlinger and Schimke, 1972; Lagnado, 1980; Anderson **et al.**, 1981).

It might be informative to repeat the experiments of Ravishankar and Padmanaban, using more effective inhibitors of heme synthesis such as desferrioxamine, levulinic acid or succinylacetone, possibly in combination (Srivastava **et al.**, 1980a; Schoenfeld **et al.**, 1982). The effect on ALV-S transcription could be measured at the same time, as a control to indicate the status of the intracellular free heme pool. The work presented in this chapter should also be extended by investigating the effect of heme on cytochrome P450 protein and mRNA levels as well as the transcription rate and mRNA prepared in some of the experiments described is currently being analysed. Once the heme control mechanism for the phenobarbital inducible P450 has been established, it will also be of interest to investigate whether other proteins involved in drug metabolism, or other hemoproteins, are similarly regulated as suggested by Marver (1969).

A negative control region in the promoter of the TCDD-inducible mouse cytochrome $P450_1$ has been defined by deletion mutagenesis experiments (Whitlock, 1986). It has been proposed that constitutive expression of the gene is negatively regulated by interaction of this region with a labile **trans**-acting repressor protein. Activation of gene transcription by the TCDD-receptor complex overrides this negative control. A similar type of dual control mechanism may operate for the drug-induction and heme repression of the AIA-inducible cytochrome P450 described in this work, although much more work is required to establish this.

E) Coordinate control of ALV-S and cytochrome P450 synthesis by heme

The results presented in Section 5.2.4 indicate that induction of transcription of cytochrome P450 may be repressed by lower levels of heme than are required to repress ALV-S transcription. Alternatively, different control mechanisms for the two genes may result in different kinetics for repression of transcription by heme. Investigation of the time-course of induction and repression of these two genes at different levels of heme should clarify this point. Examination of the time course of drug induction will be of particular interest, in view of the postulated relationship between the expression of the two genes. It has been proposed that induction of ALV-S by drugs is in a large part due to lowering of the intracellular heme pool by increased levels of cytochrome P450 apoprotein (Section 1.4B). If this is the case, then induction of cytochrome P450 transcription should precede that of ALV-S. These experiments would be more conveniently undertaken using cultured hepatocytes, so that the levels of heme and drugs can be accurately controlled. Transformed cell lines in which the cytochrome P450 genes retain phenobarbital-inducibility have not yet been obtained, so it would probably be necessary to work with primary cultures.

The physiological significance of the dual control of cytochrome P450 by drugs and heme is not clear, since the established positive regulation of cytochrome P450 by drugs should be sufficient to account for the observed response. Nevertheless, repression of transcription of both cytochrome P450 and ALV-S by heme has been clearly established in the work described in this chapter. Regulation of cytochrome P450 and ALV-S synthesis by heme could provide for coordination of the hepatic response to drugs, in particular the synthesis of cytochrome P450 apoprotein and its prosthetic group, heme. A possible model for the coordinate regulation of cytochrome P450 and ALV-S which is consistent

with the evidence described in this chapter is presented here. This model assumes that the drug induction and heme repression mechanisms for control of cytochrome P450 transcription can operate independently and that the transcriptional rate is a result of the balance between these factors. It is also assumed that drug induction will override repression of transcription at normal physiological levels of heme, in a similar fashion in which TCDD overrides the negative control of the TCDD inducible cytochrome P450 discussed above. Since there is no evidence for either of these assumptions, this model must be regarded as purely speculative.

The inducing drug would activate transcription of the cytochrome P450 gene and newly synthesized apoprotein will combine with heme, depleting the regulatory heme pool. In the case of AIA, the heme moiety of cytochrome P450 is destroyed, increasing the rate of heme depletion. As the level of heme in the regulatory pool drops, ALV-S transcription will increase, and then cytochrome P450 transcription will be further boosted as the heme repression is completely lifted. Thus, synthesis of both cytochrome P450 apoprotein and its prosthetic group, heme, will be at maximum levels for metabolism of the inducing drug. As the levels of drug fall, cytochrome P450 apoprotein synthesis would decrease, so that there is less of a drain on the regulatory heme pool. The level of heme would therefore start to increase again, further repressing cytochrome P450 transcription and then ALV-S transcription so that the normal levels of expression are restored. It is possible that negative regulation of cytochrome P450 transcription by heme is required as a direct mechanism for the efficient shut-down of cytochrome P450 synthesis. When the inducing drug is encountered again, the drug induction mechanism overrides the heme repression mechanism, and the cycle recommences. This model is almost certainly an oversimplification of the true situation, and further work is required to elucidate the

details of the complex interactions which regulate cytochrome P450 and ALV-S synthesis.

It will be of particular interest to determine whether heme represses the ALV-S and cytochrome P450 genes by the same molecular mechanism, although the apparent differential sensitivity to heme (Section 5.2.4) suggests that there are at least some differences. Comparison of the promoter sequence of the chicken ALV-S gene (Chapter 4) with that of a chicken phenobarbital inducible P450 gene (Lisa Mattschoss, personal communication) and the phenobarbital inducible rat cytochrome P450 b and e genes (Suwa **et al.**, 1985) reveals short stretches of homology, although none of these appear to be conserved in all four of the genes. Their significance can be further assessed once the heme control region of the ALV-S gene has been defined, as discussed in Chapter 4.

CHAPTER SIX

CONCLUDING DISCUSSION

The control of ALV-S gene expression is of general interest, since the regulation of negatively controlled housekeeping genes, such as that for ALV-S, is poorly understood. The negative feedback control of ALV-S by heme at the level of translocation is unique, and the coordinate regulation of ALV-S levels by inhibition of both transcription and translocation is a complex and fascinating problem. Ultimately, it is hoped that investigation of the regulation of ALV-S synthesis will lead to the development of a more effective therapy for the porphyrias (Section 1.5).

The work presented in this thesis has provided a thorough basis for further work aimed at elucidating the molecular mechanism of control of ALV-S gene expression by heme. The isolation of the chicken ALV-S gene and the analysis of ALV-S gene sequences in the chicken genome were described in Chapter 3. It was demonstrated, using Southern blot hybridization analysis, that a single gene homologous to the liver cDNA clone pl05Bl was present in the chicken genome. Others in our laboratory have shown that a single species of ALV-S mRNA and enzyme is present in all tissues examined. It therefore seems highly likely that the same ALV-S gene functions in all tissues, in direct contrast to the proposal of Yamamoto **et al.** (1985) that a multigene family for ALV-S exists, with separate genes coding for liver and erythroid-specific isozymes of ALV-S. The sequence of a 5.2 kb region of DNA spanning the 5' end of the chicken ALV-S gene has been obtained and several putative regulatory elements have been identified. Together with sequence for the 3' end of the gene, provided by A. Day of this laboratory, this represented the first complete gene sequence for any of the heme biosynthetic pathway enzymes.

In the final project described in this thesis it was established directly, by transcription run-on experiments in isolated nuclei, that ALV-S synthesis in rat liver is controlled by modulating the rate of transcription. Alterations in the levels of ALV-S mRNA and enzyme after administration of drugs, or heme or its precursor ALV, were accompanied by similar changes in the transcription rate of the ALV-S gene, suggesting that this is the main regulatory mechanism for ALV-S synthesis in rat liver. An unexpected result was that transcription of a drug inducible cytochrome P450 was repressed by heme.

Experiments to extend several aspects of this work are currently underway. A functional analysis of the components of the chicken ALV-S promoter is being undertaken, using deletion mutagenesis to study the role of the putative regulatory sequences in expression from the ALV-S promoter in tissue cultured hepatocytes and **Xenopus laevis** oocytes. Potential regulatory factors are being identified by DNase footprinting and gel retardation assays. Eventually, it may be possible to study transcription of the ALV-S gene completely **in vitro**, using cloned gene sequences and purified protein factors. The transcriptional regulation of the cytochrome P450 and ALV-S genes **in vivo** is also being further investigated. Much work will be required to fully understand the complex role of heme in the regulation of ALV-S, both at the level of transcription and translocation. In addition, the mechanisms responsible for coordination of the induction of hepatic cytochrome P450 and ALV-S in response to drugs, and the apparent dual control of cytochrome P450 transcription by drugs and heme, remain to be elucidated.

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PUBLICATIONS

Nucleotide sequence of the chicken 5-aminolevulinate gene.

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