STUDIES ON THE TRANSCRIPTIONAL REGULATION OF THE 5-AMINOLEVULINATE SYNTHASE GENE

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THESIS SUMMARY

5-Aminolevulinate synthase (ALV-S) is the first enzyme of the heme biosynthetic pathway. The rate of heme synthesis is primarily controlled by feedback repression of ALV-S transcription by the end-product heme. This thesis is concerned with understanding the transcriptional control of the ALV-S gene, with particular interest in the mechanism of end-product repression. Briefly, the expression of the chicken ALV-S gene has been examined in *Xenopus laevis* oocytes and the *cis*-acting sequences essential to expression defined. A rat ALV-S genomic clone has been partially characterised and expression of the rat ALV-S gene examined in the rat hepatoma H4-II-E-C3 cell line. The sequence in the rat ALV-S gene responsive to heme has been localised to an area in the 5' flanking region.

1. A variety of recombinants have been constructed containing various amounts of the chicken ALV-S gene and the expression of these constructions in *Xenopus laevis* oocytes examined by primer extension analysis. This study has shown that a minimum of 80bp of ALV-S 5' flanking sequence is required for maximum transcription and that sequences as far downstream as +4014 do not contribute to the expression of the chicken ALV-S gene in Xenopus oocytes.

2. Within the 5' flanking region of the chicken ALV-S gene are a number of sequences with homology to *cis*-acting elements known to contribute to the expression of other genes. The role of these sequences in ALV-S expression was examined by constructing mutants in which these sequences were altered, singly and in combinations, by oligonucleotide site-directed mutagenesis. The resulting mutants were expressed in *Xenopus laevis* oocytes and their relative efficiency quantified by primer extension analysis. It was found that only a single GC box at position -78 and the TATA box at position -20 were required for maximal expression of the chicken

ALV-S gene in Xenopus oocytes. Both these sequences are located within the previously defined minimal sequence requirements as outlined above.

3. The endogenous *Xenopus laevis* oocyte ALV-S was examined and found to be under feedback repression by heme. Conditions for maximal repression of oocyte ALV-S were optimised. A chicken ALV-S construction M13chALV-7 was injected into oocytes known to be heme repressed. Expression of this injected chicken ALV-S construction could not be reduced by heme under any of the conditions tested.

4. A rat ALV-S genomic clone was isolated by Dr I. Borthwick and used for further studies on the transcriptional control of ALV-S. RG-1 was restriction mapped and the translational start site localised by southern blot hybridisation analysis. 2Kb of the rat ALV-S gene was subsequently sequenced. The presence of an intron in the 5' untranslated region was predicted from the sequence analysis and the intron-exon boundaries were defined by mung bean nuclease protection and primer extension analysis. Analysis of rat liver mRNA by primer extension defined two transcriptional start sites at positions +1 and +3. Surprisingly, there appears to be little homology between the 5' flanking regions of a number of ALV-S genes from different species.

5. pIBICAT-ALV1 was transfected into H4-II-E-C3 cells and these were subsequently incubated in growth medium with and without 1μM heme-arginate. Total H4-II-E-C3 RNA was isolated and the endogenous level of H4-II-E-C3 ALV-S mRNA measured by northern blot hybridisation analysis. In the presence of heme the level of H4-II-E-C3 ALV-S mRNA was reduced by approximately 50%, but when pIBICAT-ALV1 was present, the level of ALV-S mRNA was increased both in the presence and absence of heme. This result is consistent with the conclusion that the sequences responsible for heme repression are located in the pIBICAT-ALV1 construction and implies that heme repression of the ALV-S gene is mediated by a *trans*-acting factor.

6. A series of recombinants were constructed containing increasing amounts of the rat ALV-S gene in front of the CAT structural gene, in the recombinant plasmid pIBI-76. Some recombinants were constructed with the intron in the 5' untranslated region of the rat ALV-S gene deleted by oligonucleotide site-directed mutagenesis. These clones were transfected into the highly differentiated rat hepatoma H4-II-E-C3 cell line by electroporation and the transient expression of the CAT gene measured in the presence and absence of 1μM heme-arginate. It was found that all the constructions except, pIBICAT-ALV1 and pIBICAT-ALV1ΔI and pIBICAT-ALV2 expressed at approximately 50% of the positive control pIBISVCAT. pIBICAT-ALV1 and pIBICAT-ALV1ΔI both expressed at a level approximately 35% of the level of pIBISVCAT and pIBICAT-ALV2 expressed at only 16% of the level of pIBISVCAT. The level of expression of pIBICAT-ALV1, pIBICAT-ALV1ΔI and pIBICAT-ALV2 was also further reduced 50% by the addition of 1μM heme-arginate to the growth medium. It was therefore concluded that the sequences responsible for heme repression of the ALV-S gene are common to these three constructions and therefore likely to be located between positions -1200 and -476 in the rat ALV-S gene.

Sequences essential to the efficient expression of the rat ALV-S gene in H4-II-E-C3 cells were also found in two regions of the ALV-S gene. As the expression of pIBICAT-ALV2 was significantly lower than that of either pIBICAT-ALV1 or pIBICAT-ALV1ΔI there are likely to be sequences essential to the maximal expression of the ALV-S gene between position -1200 and -2700. The level of expression of pIBICAT-ALV4, the shortest ALV-S construction, was 50% of the level of pIBISVCAT. Thus it was concluded that the 160bp 5' to the rat ALV-S transcriptional start site must also contain sequences essential to the efficient expression of the ALV-S gene. No difference in the level of expression of constructions with and without the intron was observed, therefore it seems likely that the intron in the 5' non-translated region of the gene plays no role in either the efficient expression of the ALV-S gene or heme repression, at least in this cell type.

DECLARATION

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

In all of the experiments described in this thesis, the author was involved as the principal worker, though some of the work in Chapter's 5 and 6 was undertaken in collaboration with Dr. Iain Borthwick.

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.

Jason Andrew Loveridge

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ABBREVIATIONS

Abbreviations are as described in The Journal of Biological Chemistry "Instructions to authors" (1987). Additional abbreviations are listed below.

ALV-S: 5-aminolevulinate synthetase.

ALV: 5-aminolevulinic acid

AIA: allylisopropylacetimide

EGTA: ethyleneglycol-bis-(B-amino-ethyl ether)N,N'-tetra acetic acid.

PEG: polyethylene glycol.

DTT: dithiothreitol.

TEMED: N,N,N',N'-tetramethyl-ethylene-diamine.

BCIG: 5-bromo-4-chloro-3-indolyl-B-D-galactoside.

ITPG: isopropyl-B-D-thio-galactopyranoside.

ddNTP: dideoxyribonucleotide triphosphate.

dNTP: deoxyribonucleotide triphosphate

PMSF: phenylmethylsulphonylfloride.

bp: base pair

Kb: kilobase

poly(I): poly inosine

poly(C): poly cytosine

poly(A): poly adenylic acid

TMACL: tetramethylammonium chloride.

CHAPTER 1

INTRODUCTION

Chapter 1: Introduction

1-1 The heme biosynthetic pathway

Heme is synthesised in animal cells by the pathway depicted in Figure 1-1. The first enzyme, 5-aminolevulinate synthase (ALV-S) and the last three enzymes, coprogen III oxidase, protogen oxidase and heme synthase (ferrochelatase) are all localised in the mitochondria whilst the intervening enzymes are found in the cytosol. The substrate for ALV-S, succinyl coenzyme A is generated in the mitochondrion but otherwise the significance of this compartmentalisation is unknown.

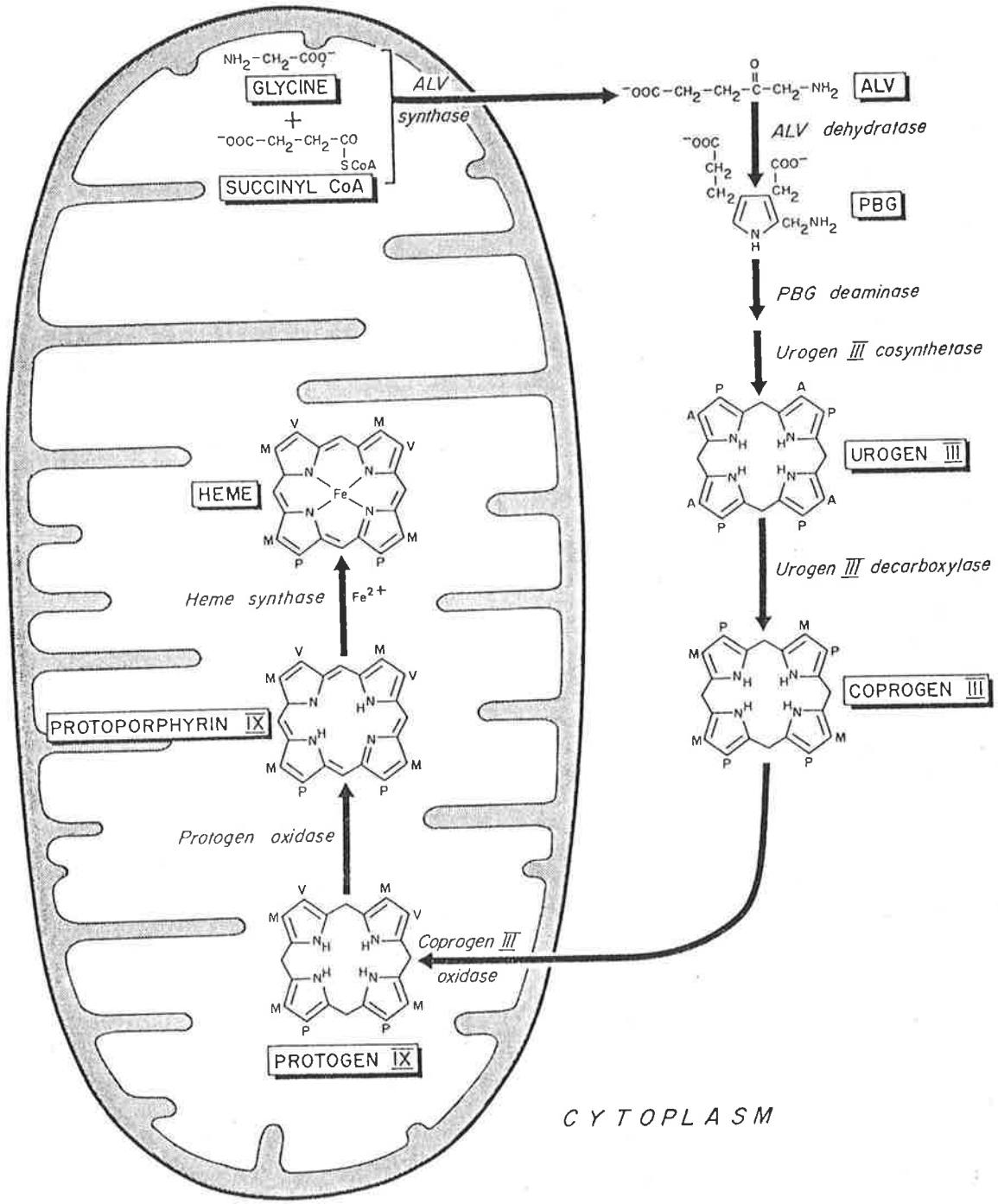
Heme is incorporated as a prosthetic group into hemoproteins such as catalase, peroxidase, hemoglobin, myoglobin, tryptophan pyrrolase and the cytochromes of both the microsomal mono-oxygenase system and the mitochondrial respiratory chain. The most active cells for heme biosynthesis are the erythropoietic cells of the bone marrow where heme is required for hemoglobin production, and in the hepatic cells where heme is mainly incorporated into cytochrome P-450's (Tait, 1978).

ALV-S (EC 2.3.1.37) is the first, and at least in the liver, the rate limiting enzyme of heme biosynthesis (Granick, 1966 Kappas *et al.*, 1983,). It is a pyridoxal phosphate-dependent enzyme which catalyzes the condensation of succinyl coenzyme A and glycine to form ALV (Fig. 1-1). The level of ALV-S in the liver is normally low in comparison with the other heme biosynthetic enzymes, which occur in apparently non-limiting amounts. An exception to this is porphobilinogen deaminase which is also present at low levels and may become limiting under conditions of high ALV-S production (Kappas *et al.*, 1983).

In green plants, heme biosynthesis occurs by a different mechanism in which ALV is synthesised from glutamate (Kannangara *et al.*, 1984, Schoen *et al.*, 1986). It has been claimed that (Franck *et al.*, 1984) ALV can also be synthesised from glutamate in animals. This has been based on labelling studies in duck blood cells which suggested that as much as 25% of cellular heme is derived from glutamate. However further work is required to establish that this pathway contributes significantly to heme biosynthesis in animals.

Figure 1-1.

The heme biosynthetic pathway.


GLYCINE
SUCCINYL CoA
ALV synthase
ALV
ALV dehydratase
PBG
PBG deaminase
Urogen III cosynthetase
UROGEN III
Urogen III decarboxylase
COPROGEN III
Coprogen III oxidase
PROTOGEN IX
Protogen oxidase
PROTOPORPHYRIN IX
Heme synthase
Fe2+
HEME
CYTOPLASM

cDNA clones have been isolated for most of the heme biosynthetic enzymes, from a variety of sources. These include: bacterial, yeast, chicken, rat and human ALV-S (Leong *et al.*, 1985, Urban-Grimal *et al.*, 1986, Borthwick *et al.*, 1984, Borthwick *et al.*, 1985, Srivastava *et al.*, 1988); rat and human ALV dehydratase (Bishop *et al.*, 1986, Wetmur *et al.*, 1986); bacterial, rat and human porphobilinogen deaminase (Thomas and Jordan, 1986, Grandchamp *et al.*, 1984, Raich *et al.*, 1986); rat and human uroporphyrinogen III decarboxylase (Romeo *et al.*, 1984, Romeo *et al.*, 1986).

1-2 Control of heme biosynthesis

The regulation of heme biosynthesis is thought to occur by end-product repression on the rate limiting enzyme, ALV-S. Granick (1966) first showed, using chick embryo liver cells, that ALV-S levels could be increased by drugs and that heme prevented this induction. This led to the first hypothesis that heme acted as a corepressor of ALV-S transcription and that inducing drugs competed with heme for the aporepressor. This hypothesis was subsequently altered to suggest that drugs could act simultaneously at two levels, by increasing ALV-S gene transcription and decreasing heme levels (Granick and Beale, 1978).

Although the initial models suggested that drugs acted directly as positive effectors of gene transcription, the currently held view is that heme repression is the sole control on ALV-S synthesis and that inducing drugs act indirectly by reducing the level of heme, thereby derepressing ALV-S (May *et al.*, 1986). This is supported by the fact that ALV-S can be induced in the complete absence of drugs, by heme depletion alone. Initially it was shown by Srivastava *et al.* (1980) that induction of ALV-S in AIA induced cultured chick embryo liver cells could be maintained after the removal of the inducing drug by adding desferrioxamine, an inhibitor of ferrochelatase. Induction of ALV-S has been achieved in the absence of drugs, in cultured chick embryo liver cells, by the use of succinyl acetone and levulinic acid, inhibitors of ALV-dehydratase (Schoenfeld *et al.*, 1982).

The major factor leading to drug induction of ALV-S in liver is probably the induction by the drug of cytochrome P450 synthesis (reviewed in Bock and Remmer, 1978), since all drugs that induce ALV-S also induce cytochrome P450 synthesis (Meyer, 1982). Further support for a correlation detween ALV-S and cytochrome P450 induction is the fact that drug induction of rat ALV-S mRNA is observed only in tissues in which cytochrome P450 mRNA is induced by drug (Srivastava *et al.*, 1988). Induction of cytochrome P450 constitutes a significant drain on the amount of free heme in the hepatic cell since up to 70% of hepatic heme is utilised in cytochrome P450 synthesis (Marver and Schmid, 1972). Therefore it appears that the induction of ALV-S by drugs is probably due solely to heme depletion (May *et al.*, 1986).

1-2-1 The effect of heme on the ALV-S enzyme

It has been suggested in numerous reports that heme inhibits the activity of the ALV-S enzyme (Scholnick *et al.*, 1972, Whiting and Granick, 1976, Paterniti and Beattie, 1979) In contrast, Pirola *et al.* (1984) have shown that heme or hemin does not inhibit the isolated chick embryo liver ALV-S or rat liver ALV-S, even up to concentrations of 100μM (May *et al.*, 1986). The reasons for the conflicting results are not known. In isolated chick embryo liver cells concentrations as low as 10nM inhibit ALV-S synthesis and concentrations higher than 20μM are toxic, thus it seems unlikely that heme acts physiologically to inhibit the activity of ALV-S.

1-2-2 The effect of heme on the translocation of ALV-S into the mitochondria

Most mitochondrial proteins are synthesised on cytoplasmic polysomes as larger precursors which are subsequently imported into the mitochondria. *In vitro* translation and immunoprecipitation experiments have shown this to be the case for ALV-S (Whiting, 1976, Srivastava *et al.*, 1982, Srivastava *et al.*, 1983). During transport into the mitochondria the higher molecular weight ALV-S precursor is

converted to the mature form by proteolytic cleavage (Yamauchi *et al.*, 1980, Srivastava *et al.*, 1982, Srivastava *et al.*, 1983).

The first evidence that heme negatively regulates the transport of ALV-S into the mitochondria was provided by Hayashi *et al.* (1972), who showed that administration of hemin to induced rats caused the accumulation of ALV-S enzyme in the cytosol and a drop in the level of the intra-mitochondrial enzyme. Kikuchi and Hayashi (1981) subsequently proposed that heme blocked the transport of the ALV-S precursor into the mitochondria.

This novel negative feedback mechanism appears to be specific for ALV-S. Srivastava *et al.* (1983) used specific antibodies and pulse-labelling in chick embryo liver to demonstrate that heme inhibits the transport into the mitochondria of ALV-S, but not that of another mitochondrial enzyme, pyruvate carboxylase. The inhibition therefore appears to be a specific one.

1-2-3 The effect of heme on ALV-S translation

There is conflicting evidence concerning the inhibition of ALV-S translation by heme. Whiting (1976) showed that polysomes isolated from drug induced chick embryo liver were able to complete the synthesis of ALV-S in an *in vitro* system. This was not inhibited by concentrations of heme up to 10 μmol/l. Yamamoto *et al.* (1983) by contrast reported that ALV-S synthesis on rat liver polysomes is inhibited at the elongation step in a rabbit reticulocyte-lysate system by concentrations of heme greater than 20μM. Further work (May *et al.*, 1986) using a wheat germ translation system failed to demonstrate any effect of heme on translation of ALV-S mRNA. Concentrations of heme up to 100μM were used.

1-2-4 The effect of heme on ALV-S gene transcription

Heme repression of ALV-S mRNA synthesis was first indicated by experiments which demonstrated that the level of translatable ALV-S mRNA in chick embryos (Whiting, 1976) or rats (Yamamoto *et al.*, 1982) was increased by treatment with

drugs, and that hemin prevented this. Indirect evidence was also provided by Srivastava *et al.* (1980) who showed that repression of cultured chick embryo liver cell ALV-S by hemin (20-50nM) mimicked the effect of cordycepin, an inhibitor of transcription.

The first direct evidence that heme reduces the level of ALV-S mRNA was obtained by Srivastava *et al.* (1988) who measured the level of ALV-S by a hybridisation assay utilising a rat ALV-S cDNA clone. This showed that hemin prevented any increase in ALV-S mRNA levels in drug treated rats or chick embryos (Beckman, 1984). The basal level of ALV-S mRNA in the liver, brain, kidney, heart and testes was also reduced by administration of hemin to rats (Srivastava *et al.*, 1988). Subsequent *in vitro* nuclear run-on experiments in chick embryo (Maguire, 1987) and rat (Srivastava *et al.*, 1988) liver cell nuclei clearly demonstrated that heme reduces the level of ALV-S mRNA by inhibiting the transcription of the ALV-S gene.

1-3 The regulation of eukaryotic gene transcription

The synthesis of prokaryotic mRNA basically involves the transcription of an appropriate gene by RNA polymerase. In an eukaryotic cell the synthesis of mRNA is much more complex. The primary mRNA transcript with few exceptions must be polyadenylated, spliced, and in some cases capped and methylated before the mRNA is transported into the cytoplasm for RNA translation (for review see Nevins, 1983, Bird, 1986).

Of central importance to the study of eukaryotic gene expression is the regulation of transcription. In eukaryotes this regulation might occur at any of the steps in mRNA production described above. However it is believed that the main control is at the level of transcriptional initiation.

The following discussion will deal specifically with the regulation of transcriptional initiation of protein coding genes transcribed by RNA polymerase II.

1-4 Promoters of higher eukaryotic genes transcribed by RNA polymerase II

A promoter for an eukaryotic gene is a segment of DNA that functions to initiate and modulate transcription. Promoters of higher eukaryotic genes transcribed by RNA polymerase II contain a number of defined elements which have either been found to be common to all RNA polymerase II transcribed genes, or are specific to individual genes or groups of genes.

A higher eukaryotic RNA polymerase II promoter is shown in Figure 1-2. Promoter elements can be divided up into three main groups; the TATA box, immediate upstream elements, and enhancers/silencers.

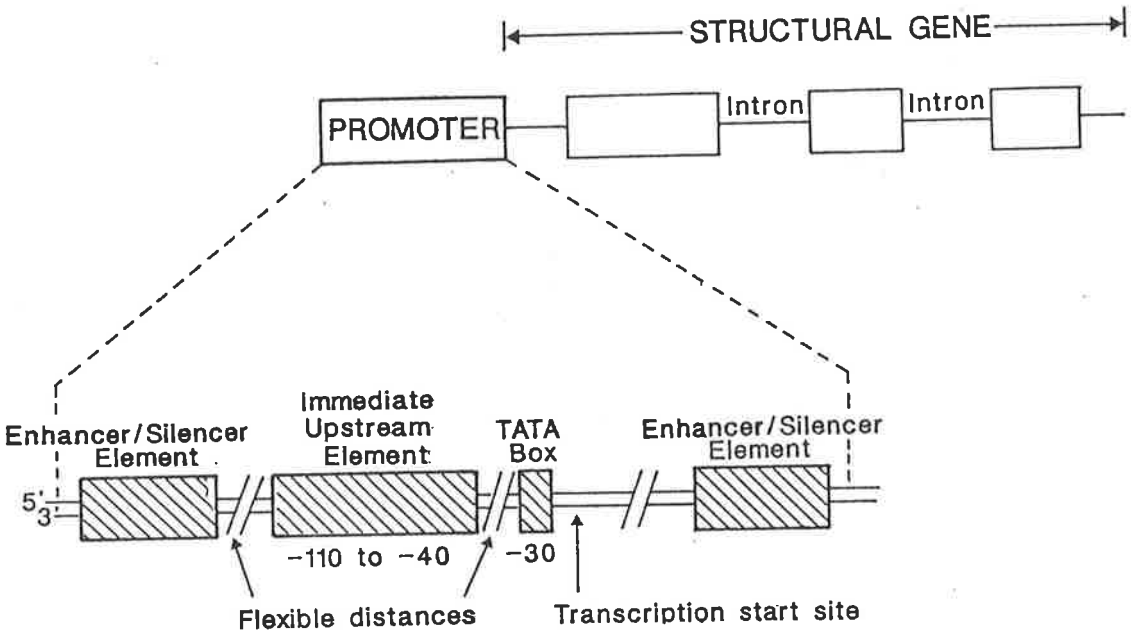
1-4-1 TATA box

Almost all promoters in higher eukaryotes contain a TATA box of consensus sequence $TATA^{A}/_{T}A^{A}/_{T}$ (Corden *et al.*, 1980), which is located between 25 and 30bp upstream of the transcriptional start site (CAP). This *cis*-acting sequence element determines the position of transcriptional initiation (for review see Breathnach and Chambon, 1981). Mutation in this region usually generates 5' heterogeneity in the RNA transcripts, which may or may not be associated with a drop in transcriptional efficiency (Grosschedl and Birnstiel, 1980, Wasylyk *et al.*, 1980, Ghosh *et al.*, 1981, Grosschedl *et al.*, 1981, Hen *et al.*, 1982, Zarucki-Schulz *et al.*, 1982, Concino *et al.*, 1983).

Some eukaryotic housekeeping genes (genes expressed in all tissues) which are transcribed by RNA polymerase II do not possess TATA box elements and produce transcripts which are heterogeneous in the 5' end. At present this includes; the xanthine phosphoribosyl transferase gene (Melton *et al.*, 1986, Patel *et al.*, 1986), human (Chen *et al.*, 1984, Yang *et al.*, 1984) and hamster (Mitchell *et al.*, 1986) dihydrofolate reductase gene, mouse adenine phosphoribosyl transferase gene (Dush *et al.*, 1985), human adenosine deaminase (Valerio *et al.*, 1985), human phophoglycerate kinase (Singer-Sam *et al.*, 1984) and the hamster 3-hydroxy-3-methylglutaryl

Figure 1-2.

Sequence elements commonly found in higher eukaryotic promoters of genes transcribed by RNA polymerase II. The types of elements found and their relative location in the promoter are shown.


STRUCTURAL GENE
PROMOTER
Intron
Intron
Enhancer/Silencer Element
Immediate Upstream Element
TATA Box
Enhancer/Silencer Element
5'
3'
-110 to -40
-30
Flexible distances
Transcription start site

coenzyme A (HMG CoA) reductase gene (Reynolds *et al.*, 1984). The site of transcriptional initiation in some of these genes such as the mouse dihydrofolate reductase gene (Dynan *et al.*, 1986) is thought to be determined by GC rich sequences located upstream of the CAP site (Melton *et al.*, 1984, Reynolds *et al.*, 1985).

In yeast genes, the TATA box is located at a variable distance from the CAP site and is required mainly for efficient transcription. The site of transcriptional initiation is determined by sequences near or at the CAP site (Gaurente, 1984).

1-4-2 Immediate upstream elements

Further upstream from the CAP site is a region containing one or more diverse elements which contribute to the efficiency of transcription. A large number of these immediate upstream elements have been identified. Some, such as the GC box, (McKnight *et al.*, 1981, Kadonaga *et al.*, 1986) or the CCAAT box (Benoist *et al.*, 1980, Efstratiadis *et al.*, 1980), are found in many different promoters. Other genes which are specifically induced by external stimuli contain such elements as the heat shock (Pelham, 1982), or metal (Carter, 1984, Karin *et al.*, 1984, Stuart *et al.*, 1984,) regulatory elements. In general, the functioning of the immediate upstream elements is distance dependant, relative to the TATA box.

1-4-3 Enhancers and silencers

The activity of many promoters is modulated by enhancers or silencers. The 'enhancing' or 'silencing' sequence should be on the same molecule of DNA, function independently of orientation, operate at large and variable distances from the CAP site and be able to operate either upstream or downstream of the CAP site (Banerji *et al.*, 1981, de Villiers and Schaffner, 1981, Moreau *et al.*, 1981, Fromm and Berg, 1983, Schaffner, 1985).

Although there is no consensus sequence for enhancers, the prototype enhancer is found in the SV 40 virus early gene promoter (Fromm and Berg, 1982, Fromm and Berg, 1983). This enhancer contains a core sequence, GTGG$^{A}/_{T}{}^{A}/_{T}{}^{A}/_{T}$G, which is

common to several different viral and cellular enhancers (Laimins *et al.*, 1982, Weiher *et al.*, 1983).

Enhancers can be divided into two basic classes; those that respond to signals (inducible enhancers) and those that are active only at specific times during development, or only in particular tissues (temporal or tissue specific enhancers).

Inducible enhancers include those that mediate transcriptional responses of particular genes to stimuli such as steroid hormones (Chandler *et al.*, 1983, Gaub *et al.*, 1987), poly(I)-poly(C) (Goodbourn *et al.*, 1985) or heavy metals (Serfling *et al.*, 1985). Inducible enhancers can activate heterologous promoters and therefore by definition, act positively. Such activation has been demonstrated to be different for individual enhancers and can be mediated either by the simple binding of a positive *trans*-acting factor, the displacement of a negative *trans*-acting factor, or a combination of both (Maniatis *et al.*, 1987).

Tissue specific enhancers have been identified in the SV 40 early promoter (Gorman *et al.*, 1985), the rat insulin 1 promoter (Nir *et al.*, 1986), the rat α-fetoprotein promoter (Muglia and Rothman-Denes, 1986), and the immunoglobin heavy chain enhancer (Imler *et al.*, 1987 and references therein).

Silencers are essentially enhancers which repress transcription rather than activate it. These sequences have also been shown to function in regulating tissue specific expression (Nir *et al.*, 1986, Cereghini *et al.*, 1987) and in modulating the action of viral enhancers (Borreli *et al.*, 1984, Gorman *et al.*, 1985, Velcich and Ziff, 1985). In yeast, silencers are important sequence elements in mating-type specific expression (Brand *et al.*, 1985).

1-5 Transcriptional regulation by *trans*-acting factors

The *cis*-acting sequences essential to the transcription of protein coding genes by RNA polymerase II have been found to mediate their function through the action of sequence specific DNA binding proteins, or *trans*-acting factors (for review see Dynan and Tjian, 1985). In contrast to prokaryote activators and repressors, little is known

about the transcriptional factors associated with the promoters of RNA polymerase II transcribed genes.

Transcriptional factors which bind to the region of the promoter containing the TATA box have been identified in *Drosophila* (Parker and Topol, 1984, Wu, 1984, Wu, 1985) and HeLa cell extracts (Shi *et al.*, 1986). A factor from mammalian cell extracts has been shown to bind the TATA box and is essential to transcriptional initiation (Davison *et al.*, 1983).

The CCAAT box sequence is a crucial component of many promoters. This sequence appears to modulate basal levels of transcription (Myers *et al.*, 1986), although in some cases it may also modulate transcription levels during differentiation or induction (Collins *et al.*, 1985, Gelinas *et al.*, 1985, Graves *et al.*, 1985, Bienz and Pelham, 1986, Coen *et al.*, 1986). Many different *trans*-acting factors which bind to the canonical CCAAT sequence have been identified and some have been partially purified. These include a factor from murine erythroleukemia cells (Cohen *et al.*, 1986), CTF and NF1 from HeLa cells (Jones *et al.*, 1985, Jones *et al.*, 1987), NF-Y from the B-lymphoma line M12 (Dorn *et al.*, 1987), CCAAT binding factor from sea urchin testes (Barberis *et al.*, 1987) and CBP from rat liver cells (Graves *et al.*, 1986). The relationship between all these factors is not well established. Jones *et al.* (1987) have shown that CTF and NF1 were indistinguishable in polypeptide composition, DNA binding properties, immunological cross-reactivity, and in *in vitro* stimulation of DNA replication and transcriptional initiation. In contrast, NY-1 and CBP appear to be different factors (McKnight and Tjian, 1986, Dorn *et al.*, 1987). There are thus several different CCAAT binding proteins.

By far the best characterised DNA binding protein of RNA polymerase II transcribed gene promoters is the factor Sp1. Sp1 was isolated from HeLa cells (Dynan and Tjian, 1983, Dynan and Tjian, 1983a, Jones *et al.*, 1985). It binds to the well defined GC box element of consensus sequence $G/_{T}GGGCGG^{G}/_{A}{}^{G}/_{A}{}^{C}/_{T}$ (Kadonaga *et al.*, 1986). This is normally located between 50 and 100bp upstream of the TATA box (Gidoni *et al.*, 1984, Jones *et al.*, 1985). Binding of Sp1 enhances transcription by

RNA polymerase II 10-50 fold from a variety of promoters (including the SV 40 early promoter) that contain at least one GC box (Kadonaga *et al.*, 1986). No shared regulatory feature is evident among the diverse promoters which possess Sp1 binding sites. Thus it cannot be explained why some promoters have Sp1 binding sites and others do not. Possibly in the former promoters, Sp1 provides a basal level of transcription which can then be modulated by other positive or negative factors.

Sp1 has been purified 100,000 fold to an estimated 95% homogeneity. It is estimated there are approximately 5000-10,000 Sp1 molecules present in each cell (Kadonaga *et al.*, 1986).

There is a great number of functionally different enhancer and silencer sequences found in both cellular and viral genes. Not surprisingly, the *trans*-acting factors which bind to these sequences and mediate their action are also varied.

Trans-acting factors that bind to viral and cellular enhancers have been identified in mouse 3T6 cells which interact with the B enhancer of polyoma virus (Piette *et al.*, 1985), in A431 cells which interact with the c-*fos* enhancer (Prywes and Roeder, 1986), in B lymphoma cells which bind the immunoglobulin (Ig) heavy chain and kappa light chain enhancers (Senn and Baltimore, 1986) and from MG63 cells which bind to the enhancer present in the β-interferon promoter (Zinn and Maniatis, 1986). In some cases the *trans*-acting factor has been extensively purified, but the biochemical activities of such factors are essentially unknown.

Cloning of the genes that encode *trans*-acting factors has in some cases allowed the functional analysis of the *trans*-acting factor itself. Such studies on the steroid receptors have identified the individual domains of the receptor protein which bind DNA, steroid, and which stimulate transcription (Danielsen *et al.*, 1986, Giguere *et al.*, 1986, Kumar *et al.*, 1986, Miesfield *et al.*, 1986, Godowski *et al.*, 1987). The DNA binding domain of the steroid receptor has a cysteine-rich *finger* region which is thought to interact with the major groove of the DNA helix, a structural feature which has also been found in the DNA binding domain of TFIIIA, a Xenopus 5S gene *trans*-acting factor (Miller *et al.*, 1985).

1-6 Models for the regulation of transcription by *trans*-acting factors

Most *trans*-acting factors interact with *cis*-acting DNA sequences to regulate the transcription of RNA polymerase II transcribed genes. There are several models postulated to explain the mechanism of action of such *trans*-acting factors, most of which are based on models for prokaryotic genes and prokaryotic *trans*-acting factors.

In the simplest case of an element located close to the site of transcriptional initiation, a factor would bind to the appropriate *cis*-acting sequence and by direct protein-protein contact induce a conformational change in the DNA or the initiation complex (Dynan and Tjian, 1985). Such a model could account for the activation of the gene coding for the *Drosophila* heat shock protein, where the most proximal activation sequence is only 15bp upstream of the TATA box. In most promoters however, the *cis*-acting sequences are probably too far upstream for direct protein-protein contact (Dynan and Tjian, 1985).

There have been several mechanisms postulated to account for the ability of upstream factors to initiate and modulate transcription at a distance;

1. After binding to the upstream *cis*-acting sequence the factor may move (slide, track) along the DNA to interact with other proteins which can then initiate transcription. No experimental result directly argues against this as a model for the action of eukaryotic *trans*-acting factors (Ptashne, 1986). However for the strongest presumed case of sliding in prokaryotes (the site specific recombination of the ends of the bacteriophage Mu), sliding has been eliminated as a possible mechanism (Craigie and Mizuuchi, 1986).

2. The bound upstream factor may remain in place but induce a conformational change in the DNA which would allow other proteins to bind and initiate transcription. This seems unlikely as a number of *trans*-acting factors have been isolated and all recognise helical DNA (Ptashne, 1986). For example, it has been shown that the λ repressor does not greatly alter the structure of DNA upon binding (Anderson *et al.*, 1985). One specific suggestion (Nordheim and Rich, 1983), that enhancers are

recognised as Z-DNA (normal DNA is in the B confirmation) has been proved to be incorrect by Zenke *et al.* (1986). These workers showed that alternating purines and pyrimidines, the likeliest Z-forming sequences, are not required for enhancer function.

3. A second factor may bind alongside the first *trans*-acting factor to create a scaffold of protein which can finally interact with the site of transcriptional initiation. Although this model has not been experimentally disproved, Ptashne (1986) believes it is unlikely to occur over thousands of base pairs of DNA.

4. Factors bound upstream allow the DNA to condense into a compact nucleoprotein structure, inducing the formation of an 'active' conformation, or inducing the disruption of an 'inactive' configuration. Although there is no direct experimental evidence for this model, it has been proposed to explain the activation of promoters by enhancers (Dynan and Tjian, 1985).

5. Transcriptional initiation is facilitated by the interaction of *trans*-acting factors bound at widely separated sites, with the intervening DNA looping or bending to allow protein-protein interaction.

The most direct demonstration of interaction between DNA bound proteins with DNA looping comes from work on the λ-phage repressor. Hochschild and Ptashne, (1986), demonstrated by DN'ase footprinting that the λ repressor binds cooperatively to operator sites separated by integral numbers of helical turns, the DNA in between bending to accommodate the interaction. Direct visual proof of DNA looping has been obtained by Griffith *et al.* (1986) utilising electron microscopy. The electron micrographs show two λ repressor molecules bound to the DNA and to each other with the intervening DNA looped out. That repressors might use this mechanism in *E. coli* is also suggested by the requirement for two operator sites in three different operons, arabinose (Dunn *et al.*, 1984), galactose (Irani *et al.*, 1983), and deo (Dandanell and Hammer, 1983).

In eukaryotes, this mechanism has been postulated to occur in the SV 40 promoter. Takahashi *et al.* (1985) demonstrated that insertions of 5bp or 15bp between the enhancer and the 21bp repeats decreased the efficiency of transcription more

drastically than 10 or 21bp insertions. A similar result was found for insertions between the 21bp repeats and the TATA box. A simple interpretation is that the factor bound to the enhancer contacts other proteins bound to the 21bp repeats, which in turn contacts those factors bound at the TATA box, with the intervening DNA looping out (Ptashne, 1986). The results also imply that for this to occur efficiently, all the factors involved must be on the same side of the DNA helix (Takahashi *et al.*, 1985).

1-7 Aims of this thesis

The work in this thesis was aimed at understanding the transcriptional control of the ALV-S gene. ALV-S is an interesting gene to study because it is an end-product repressed gene and this type of regulation is very poorly understood in higher eukaryotes.

A chicken ALV-S genomic clone was available in our laboratory which was already sequenced and found to contain an interesting array of *cis*-acting sequences in the 5' flanking region. It was of some interest to define the role of these sequences in the transcriptional control of the ALV-S gene. Expression studies on the chicken ALV-S gene were conducted in the established Xenopus oocyte expression system to define the role of these regulatory sequences. Because of experimental limitations in further studying the chicken ALV-S gene, our attention was turned to the corresponding rat gene.

Characterisation of part of the rat ALV-S gene and subsequent expression studies were conducted in the homologous rat hepatoma H4-II-E-C3 cell line in a specific attempt to define the mechanism by which heme represses the transcription of the ALV-S gene.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2: Materials and Methods

2-1 Materials

2-1-1 Chemicals and reagents

All chemicals were of analytical grade, or of the highest grade available. Most chemicals and reagents were obtained from a range of suppliers, the major sources of some of the more important chemicals and reagents are listed below;

Acrylamide, ALV, ATP, ddNTP's, dNTP's, DTT, pyridoxal phosphate, levulinic acid and N,N'-methylene-bisacrylamide: Sigma

Agarose and low melting point agarose: B.R.L.

Temed and xylene cyanol: Tokyo Kasei

Bromocresol purple, Nonidet P40, formamide and PEG 6000: B.D.H.

Urea (ultra pure): Merck

Cation exchange resin dowex AG50W-X8 (H+ form, 200-400 mesh): Biorad Laboratories

Heme: Porphorgenic Products

AIA: Roche

2-1-2 Enzymes

Enzymes were obtained from the following sources;

AMV reverse transcriptase: Molecular Genetic Resources.

E. coli DNA polymerase I (Klenow fragment): Boehringer Mannheim and Biotechnology Research Enterprises of South Australia (BRESA)

Chick embryo ALV-S and succinyl CoA synthase: were gifts from B. A. Pirola

Chloramphenicol acetyl transferase: P. L. Biochemicals

Proteinase K: Boehringher Mannheim

Restriction endonucleases: Boehringer Mannheim, New England Biolabs and Pharmacia

T4 polynucleotide kinase: Boehringer Mannheim and BRESA

Calf intestinal phosphatase and ribonuclease A: Sigma

T4 DNA ligase: BRESA

Coenzyme A: Sigma

Mung bean nuclease: Pharmacia

2-1-3 Radiochemicals

(2,3-^{14}C)-succinate (10-60 mCi/mmol), L-(^{35}S)-methionine (1420 Ci/mmol in aqueous solution) and D-threo-(dichloroacetyl-1-^{14}C)-chloramphenicol (53 mCi/mmol in 0.25mol/l Tris-Cl, pH 7.5): Amersham

(α-^{32}P)dATP (1800 Ci/mmol): BRESA

(γ-^{32}P)ATP (1800 Ci/mmol):BRESA

(α-^{32}P)dCTP (1800 Ci/mmol): BRESA

(α-^{32}P)UTP (1800 Ci/mmol): BRESA

2-1-4 Bacterial strains

E. coli MC1061 and *E. coli* ED8799 (Casadaban and Cohen, 1980): Hosts for recombinant plasmids, were gifts from Dr. M. Bawden, this department.

E. coli JM101 (Messing, 1979): A host for M13 bacteriophage, was a gift from Dr. I. Borthwick, this department.

E. coli LE392 (Murray *et al.*, 1977): A host for λ phage, was a gift from Dr. D. Maguire, this department.

E. coli BB4: A host for recombinant plasmids, was purchased from Stratagene.

2-1-5 Bacterial media

All bacteria except *E. coli* JM101 and *E. coli* BB4 were grown in Luria-broth (L-broth) or on Luria-agar plates. *E. coli* JM101 was grown in minimal media, or 2 x YT and on minimal plates. *E. coli* BB4 was grown in L-broth containing 10 μg/ml tetracycline.

L-broth: 1% (w/v) amine A, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0.

L-agar plates: contained L-broth with 1.5% (w/v) bacto-agar.

Minimal medium: 2.1% (w/v) K_2HPO_4, 0.9% (w/v) KH_2PO_4, 0.2% (w/v) $(NH_4)_2SO_4$, 0.1% (w/v) tri-sodium citrate, 0.4% (w/v) glucose, 0.0001% (w/v) thiamine.

Minimal medium plates: contained minimal medium with 1.5% (w/v) bacto-agar.

2 x YT broth: 1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.0.

All media and buffers were prepared with deionised water and sterilised by autoclaving (20psi for 25 minutes at 140°C), except the heat labile reagents which were filter sterilised. All glassware and other utensils were rendered RN'ase and DN'ase free by autoclaving as above.

2-1-6 Tissue culture cell lines

Rat hepatoma H4-II-E-C3 (Pitot *et al.*, 1964) was a gift from Kerry Fowler, The Murdoch Institute, Royal Childrens Hospital, Parkville, Victoria.

2-1-7 Tissue culture media

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

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

Growth Medium: 1 X 1 litre packet DMEM (Gibco), 28 mmol/l $NaHCO_3$, 19 mmol/l glucose, 20 mmol/l Hepes, pH 7.3, 50,000 Units Gentamicin (Gibco). The solution was filter sterilised as above.

Foetal Calf Serum: CSL

2-1-8 Cloning vectors

M13mp19, pSP64 and pUC-19: BRESA

pIBI-76: International Biotechnologies Inc.

2-1-9 Cloned DNA sequences

M13(H2B): A gift from Dr. R. Sturm, this department.

pchALV-2 and pchALV-3: Chicken ALV-S promoter containing constructions, were gifts from Dr. I. Borthwick, this department.

λcALA-S1: A chicken ALV-S genomic clone, was a gift from Dr. D. Maguire, this department.

p101B1: A rat ALV-S cDNA clone, was a gift from Dr. G. Srivastava, this department.

p105B1: A chicken ALV-S cDNA clone, was a gift from Dr. D. Maguire, this department.

pchACTIN: A chicken β-ACTIN cDNA clone, was a gift from Dr. S. Dalton, this Department.

2-1-10 Synthetic oligonucleotides

Synthetic DNA primers were synthesised by BRESA. The primer sequences are listed below;

A. Primer extension and sequencing primers

P1: 5'-dAGGGACTCGGGATAAGAATGGGC-3'

P2: 5'-dGCGGAGGACGCTAAACCG-3'

P3: 5'-dGCCAGCTAGGGAGGACTC-3'

P4: 5'-dGGGATGAACGCTGAGCC-3'

P5: 5'-dGGCTCGGGCATAGTGGCACAACGCGC-3'

P6: 5'-dGACGCGGGCCAGGAACGGGCAGCGCCGCAC-3'

P7: 5'-dGCTGGCTGGAGGCAAAGCGT-3'

P8: 5'-dCACTTCTTTGGCTTTCTTGGC-3'

M13 Universal Sequencing Primer (17mer): 5'-dGTAAAAC-GACGGCCAGT-3'

M13 reverse sequencing primer (25mer): 5'-CACACAGGAA-ACAGCTATGACCATG-3'

B. Oligonucleotide site-directed mutagenesis primers

TATA-I: 5'-dGGGGGCGGAGCAGGAATTACCCTCAG-3'

TATA-II: 5'-dGGAGCCGGCAGGGCTGGGTAAGGGCGGCGGCC-3'

CCAAT-I: 5'-dCACCACTGGGACGGATCACGGCTCGG-3'

CCAAT-II: 5'-dCCCCGCCCACTCGGTCACGCCACGCC-3'

GC-I: 5'-dCAGCACAAGCCCTTCCCACTCCATCA-3'

GC-II: 5'-dGCGTCCTCGGGGTTGGAGCATAAATT-3'

GC-III: 5'-dGCTATATAAGGGTTGCGGCCGCGA-3'

2-1-11 Miscellaneous materials

Nitrocellulose (BA85): Schleicher and Schuell

X-Ray film: Fuji RX Photo Film Company,Tokyo, Japan

Kodak Diagnostic film X-Omat AR, USA

2-2 Methods for the study of *Xenopus laevis* oocyte ALV-S

2-2-1 ALV-S radiochemical assay

The method of Brooker *et al.* (1982) was used.

(i) Cation exchange column

Dowex AG50W-X8 (H+ form, 200-400 mesh) resin was converted to the Na+ form by extensive washing in 1 mol/l sodium acetate, pH 3.9 and packed in small plastic columns (6mm X 50mm). These were sequentially washed with 10ml of 1 mol/l sodium hydroxide (until the eluent was pH 9.0), with 20ml of water (until pH 7.0). It was then eqilibrated with 10ml of 0.1 mol/l sodium acetate buffer, pH 3.9. The resin was regenerated after use by first washing with 10ml 1 mol/l sodium acetate, pH 8.5 and then following the procedure outlined above.

(ii) Assay solutions for ALV-S

5X Assay Cocktail: 250 mmol/l Tris-Cl, pH7.4, 500 mmol/l glycine, 100 mmol/l $MgCl_2$, 75 mmol/l ATP, 5 mmol/l Ca/Mg EDTA (prepared as described by Sinclair and Granick, 1977), 138 mmol/l sodium levulinate, 125 mmol/l NaCl and 5 mmol/l dithioerythritol. This cocktail was stored frozen at -20°C and was stable for several months.

Succinate Solution: 5 mmol/l sodium succinate and 50 mmol/l Tris-Cl, pH 7.4. To this was added (2,3-^{14}C)-succinate to a final specific activity of 6.6 µCi/µmol or 33 µCi/ml.

(iii) Preparation of Xenopus oocyte mitochondria.

Each batch of 100 oocytes was placed in a glass Potter-Elvehjem homogeniser and rinsed in 5ml of cold PEST buffer (0.01 mmol/l pyridoxyl phosphate, 0.1mmol/l Ca/Mg EDTA, 0.25 mol/l sucrose and 5 mmol/l Tris-Cl, pH 7.4) before hand homogenisation in 2.5ml of the same buffer. The homogenate was centrifuged at 2,000 X g and 4°C for 5 min and the supernatant collected. The pellet was resuspended in 2ml of PEST buffer and recentrifuged as above.

Mitochondria were isolated from the combined supernatants by centrifugation at 12,000 X g and 4°C for 5 min. The mitochondrial pellets were then gently resuspended in 172µl of PET buffer (PEST buffer minus sucrose), adjusted to 0.1% Triton X-100, and left on ice for 15 min.

The amount of protein in the mitochondrial suspension was estimated by the Bradford protein assay (Bradford, 1976).

(iv) Assay procedure for ALV-S

To 1ml of the 5X assay cocktail (see section 2-2-1-ii) was added 12µl of 100 mmol/l pyridoxyl phosphate, 3mg of coenzyme A and 66µl of *E. coli* succinyl-CoA synthase (1 U/µl) (see section 2-2-1-ii).

For each assay, 30µl of the above solution, 30µl of ^{14}C-succinate solution and 90µl of oocyte mitochondrial suspension were mixed together and incubated at 37°C for 60 min.

The reaction was then terminated by the addition of 100µl of 10% trichloroacetic acid, 15µl of 10 mmol/l ALV and 15µl of 1 mol/l sodium succinate. After 10 min on ice the tubes were centrifuged at 12,000 X g for 5 min, the supernatant removed and the pellet washed by resuspension in 5% trichloroacetic acid, recentrifuged and the supernatants combined.

To the combined supernatants 1.5ml of 1 mol/l sodium acetate buffer, pH 4.6, was added and the pH adjusted to between 3.7 and 4.1 with glacial acetic acid. This solution was then layered onto a Dowex AG50W-X8 ion-exchange column (see section 2-2-1-(i)) and washed sequentially with 10ml of 0.1 mol/l sodium acetate buffer, pH 3.9, 10ml of methanol, 0.1 mol/l sodium acetate buffer, pH 3.9 (2:1 v/v) and 10ml of 10 mmol/l HCL. The ALV was finally eluted with 5ml of 1.0 mol/l sodium acetate, pH 8.5, and was immediately adjusted to pH 4.5 with HCL.

To convert ALV to the pyrrole, 200ml of acetylacetone was added, the tubes loosely capped and heated at 80°C for 15 min. The solution was cooled on ice for 10 min and the pyrrole extracted into 18ml (3 X 6ml) of ethyl acetate saturated with 1 mol/l sodium acetate, pH 4.6. The ethyl acetate extractions were evaporated to dryness at 60°C in a rotary evaporator and the residue dissolved in 3ml of toluene scintillation fluid containing 30% v/v Triton X-100.

The amount of ^{14}C-ALV was determined by liquid scintillation spectroscopy and the enzyme activity was expressed as pmoles of ALV formed per milligram of mitochondrial protein per hour.

2-2-2 The measurement of total protein synthesis in Xenopus oocytes

Xenopus oocytes were isolated and separated into single oocytes as in section 2-4-1. 10 oocytes per treatment were incubated at 18-21°C for 12 hours in either Barth's saline (see section 2-4-1) containing 15µCi L-(^{35}S) methionine, or in Barth's

saline containing 15μCi L-(^{35}S) methionine plus the appropriate ALV-S inducing and repressing drugs.

At the end of this period the oocytes were washed twice in cold Barth's saline and each group homogenised in a glass Potter Elvehjem homogeniser at 4°C. 10μl of each homogenate was mixed with 10μl 10% bovine serum albumin, 100μl 20 mmol/l D,L-methionine, 100μl 30% trichloroacetic acid and 90μl water. To this, 5ml of 5% trichloroacetic acid was added and the mixtures allowed to stand on ice for 10 min, then heated to 100°C for 10 min and cooled on ice for 5 min.

The suspensions were filtered through Whatman GFA filters, then each filter was washed first with 10ml 5% trichloroacetic acid, then with 10ml ethanol. The filters were dried under a 500W lamp, then each was placed in a scintillation vial containing 3ml of toluene scintillation fluid. The relative amounts of ^{35}S-methionine labelled protein were determined by liquid scintillation spectroscopy on a Beckman LS 7500 liquid scintillation counter.

2-3 General recombinant DNA methods

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

Propagation and maintenance of bacterial and virus strains; quantification of RNA and DNA; autoradiography; agarose and polyacrylamide gel electrophoresis; large scale isolation of plasmid DNA by the alkaline lysis procedure; large scale preparation of λ DNA; rapid small scale isolation of plasmid DNA; DNA and RNA precipitations; phenol/chloroform extractions; transformation of *E. coli* MC1061 or *E. coli* ED8799 using the calcium chloride procedure; Southern blot hybridisation analysis of DNA; Northern blot hybridisation analysis of RNA.

2-3-1 Transformation procedure for M13 recombinants

A single colony of *E. coli* JM101 from a minimal medium plate was inoculated into 10ml of minimal media and the culture incubated overnight at 37°C with shaking.

The culture was then diluted 100 fold into 50ml of 2 X YT and again incubated at 37°C with shaking until it reached an absorbance at A600 of 0.5. The cells were pelleted by centrifugation at 2,000 X g and 4°C for 5 min, resuspended in 10ml of ice cold 50 mmol/l $CaCl_2$ and left on ice for 30 min. These cells were recentrifuged and gently resuspended in 2.5ml of ice cold 50 mmol/l $CaCl_2$. 200µl of this cell suspension was mixed with varying amounts of the transforming DNA (0.1-5µl) and left on ice for 40 min. The transformation mix was then incubated at 42°C for exactly 2 min and to it was added 3ml of 2 X YT broth containing 0.7% bacto-agar, 20µl of 20 mg/ml BCIG in dimethylformamide, 20µl of 24 mg/ml of ITPG in water and 200ml of an overnight JM101 culture diluted 1 in 5 in 2 X YT broth, mixed by inversion and plated directly onto a minimal medium plate, which was then incubated at 37°C overnight.

2-3-2 Transformation procedure for pIBI recombinants

A single colony of *E. coli* strain BB4 from a L-agar plate containing 10 µg/ml of tetracycline was inoculated into 10ml of L-broth containing 10 µg/ml of tetracycline and the culture incubated overnight at 37°C with shaking. The overnight culture was then diluted 100 fold into 50ml of L-broth plus 10 µg/ml of tetracycline and the incubation continued at 37°C with shaking until the culture reached an absorbance at A600 of 0.5. The cells were pelleted by centrifugation at 2,000 X g and 4°C for 5 min, resuspended in 2.5ml of ice cold 25 mmol/l $CaCl_2$, 10 mmol/l $MgCl_2$ and left on ice for 60 min. 200µl of this cell suspension was mixed with varying amounts of the transforming DNA (0.1-5µl) and left on ice for 40 min. The transformation mix was then incubated at 42°C for exactly 2 min, 1ml of L-broth was added and the cells incubated at 37°C for 60 min. At the end of this period 3ml of L-broth containing 0.7% bacto-agar was added. After mixing by inversion it was plated directly onto L-agar containing 100 µg/ml of ampicillin and 10 µg/ml of tetracycline and incubated at 37°C overnight.

2-3-3 Restriction endonuclease digestions

All restriction endonuclease digestions were performed in accordance with the conditions set down by the manufacturer of each enzyme.

Reactions were stopped by the addition of EDTA, pH 8.0 to a concentration of 5 mmol/l and subsequent phenol/chloroform extraction and DNA precipitation. Or secondly, by the addition of a one third volume of urea loading buffer (4 mol/l urea, 50% (w/v) sucrose, 50 mmol/l EDTA, pH8.0 and 0.1% (w/v) bromo-cresol-purple).

2-3-4 Elution of DNA from polyacrylamide gels

The gel slice containing the DNA was placed in an eppendorf tube with 300µl of elution buffer (0.5 mol/l ammonium acetate, 1 mmol/l EDTA and 0.1% SDS) and incubated at 37°C overnight. The buffer was then aspirated away from the gel slice and the DNA ethanol precipitated.

2-3-5 Elution of DNA from low melting point agarose

The gel slice containing the DNA was placed in an eppendorf tube containing 200µl of NET buffer (200 mmol/l NaCl, 10 mmol/l Tris-Cl, pH 7.4 and 1 mmol/l EDTA) and the agarose melted by heating at 65°C. This was extracted twice with hot (65°C) phenol and once with phenol/chloroform. The DNA was finally precipitated by the addition of ethanol.

2-3-6 5' end labelling of DNA primers using T4 polynucleotide kinase

Normally 100-200ng of oligonucleotide primer was 5' end-labelled in a 10µl reaction containing 50 mmol/l Tris-Cl, pH 7.4, 10 mmol/l $MgCl_2$, 1 mmol/l DTT, 8µl of lyophilised γ-^{32}P-ATP (approximately 40µCi) and 1 Unit of T4 polynucleotide kinase. The reaction was incubated at 37°C for 40 min and then 5µl of formamide loading dye (100% formamide, 0.1% (w/v) bromocresol purple, 0.1% (w/v) xylene-cyanol and 20 mmol/l EDTA) was added and the primer purified by electrophoresis on a 10-20% polyacrylamide gel.

2-3-7 Densitometric quantitation of bands on autoradiograms

Quantitation of bands on autoradiograms was performed on a Zeineh soft laser scanning densitometer (SL-504-XL) linked to a Varian CDS-401 computer. Each autoradiogram was scanned at least four times and the values averaged by the computer.

2-3-8 DNA sequencing by the dideoxy-chain termination procedure

(i) Preparation of single strand template

M13 phage plaques were toothpicked into 1ml of a fresh overnight JM101 culture (grown in minimal media) which had been diluted 1:40 in 2 X YT broth. After incubation at 37°C with vigorous shaking for 5 hours, the culture was centrifuged for 5 min in an eppendorf centrifuge. The supernatant was poured into an eppendorf tube containing 200µl of 2.5 mol/l NaCl and 20% PEG 6000, and left at room temperature for 15 min. The single stranded phage particles were pelleted by centrifugation in an eppendorf centrifuge for 5 min. The supernatant was aspirated and the pellet resuspended in 100µl of 10 mmol/l Tris-Cl, pH 8.0, 0.1 mmol/l EDTA. This was phenol/chloroform extracted and the DNA recovered by ethanol precipitation. The DNA pellet was resuspended in 25µl of 10 mmol/l Tris-Cl, pH, 8.0, 0.1mmol/l EDTA and stored frozen at -20°C.

(ii) Sequencing reactions

The method of Sanger *et al.* (1977) was used. Four separate reactions (each specific for one of the bases in DNA) were used in the sequence analysis of the insert of a single stranded M13 template. In each of the reactions, a primer, appropriate to the insert being sequenced, was extended in the presence of a different ddNTP.

The method described below is for the sequencing of one M13 clone, but can be readily used for the concurrent sequencing of up to 24 clones.

(a) Hybridisation

2.5ng of primer was annealed to 6µl of M13 single stranded template in a 10µl volume containing 10 mmol/l Tris-Cl, pH 8.0, 50 mmol/l NaCl, 10 mmol/l $MgCl_2$ by heating the solution to 70°C for 3 min and then allowing it to cool to room temperature over approximately 60 min.

(b) Polymerisation

1µl of α-^{32}P-(dATP) (approximately 7µCi) was lyophilised, the hybridisation mix added, vortexed to dissolve the dry α-(^{32}P)-dATP and then 1µl of 10 mmol/l DTT added. 1.5µl of dNTP mix (200 µmol/l dTTP, dCTP, dGTP, 5 mmol/l Tris-Cl, pH 8.0, 0.1mmol/l EDTA) and 1.5µl of 0.5 mmol/l of each individual ddNTP were added together. 2µl each of these mixtures were added separately to each of four eppendorf reaction tubes.

0.5µl of DNA polymerase I (Klenow fragment) (1 U/µl) was added to the hybridisation mixture α-^{32}P-dATP DTT solution. 2µl of this was then added to each of the four reaction tubes and the solutions were mixed by centrifugation for 10 seconds. After 15 minutes incubation at 37°C, 1µl of chase solution (500 µmol/l dATP, 5 mmol/l Tris-Cl, pH 8.0 and 0.1 mmol/l EDTA) was added to each of the four tubes, mixed by centrifugation for 10 seconds and incubated for a further 10 min at 37°C.

To stop the reactions, 4µl of formamide loading buffer was added and the samples boiled for 5 min. A small volume of each reaction mixture was then loaded onto a sequencing gel.

In certain cases such as the sequencing of the chicken ALV-S site-directed promoter mutants and the rat ALV-S genomic recombinants, polymerisation reactions were performed at 50°C rather than 37°C to reduce DNA secondary structure.

(iii) Sequencing gels

Products of the dideoxy-chain terminator sequencing reactions were separated by electrophoresis on polyacrylamide gels, usually of dimensions 20cm X 40cm X

0.35mm, or 40cm X 40cm X 0.35mm, depending on the number of recombinants to be sequenced. These gels were usually made by the following method. A 100ml mixture of 6.5% (w/v) acrylamide monomer (20:1 acrylamide to bisacrylamide) in TBE buffer (89 mmol/l Tris-borate, 89 mmol/l boric acid and 2 mmol/l EDTA) containing 7 mol/l urea was prepared, 750µl of 10% (w/v) ammonium persulphate and 60µl of TEMED was added and the mixture poured into a gel mould and allowed to polymerise.

Gels were pre-electrophoresed for 40 min in TBE at 20mA prior to loading. Urea or debris was then removed from the sample wells by flushing with TBE. Samples were loaded with a 100µl glass micropipette under low current (5mA) and then the gel was electrophoresed at 25-30mA.

After completion of electrophoresis, the gels were fixed with 500ml of 10% (v/v) acetic acid, washed with 1 litre of 20% (v/v) aqueous ethanol, dried and exposed to X-Ray film for 4-24 hours at room temperature.

2-3-9 Complementarity testing of single stranded M13 recombinants

Single stranded M13mp19 recombinants were complementarity tested to determine the orientation of the subcloned DNA fragment in the M13mp19 vector relative to an arbitrarily selected recombinant as a reference. 2µl of the reference DNA was added to 2µl of the test DNA, 4µl of (100 mmol/l Tris-Cl, pH 7.4, 100 mmol/l $MgCl_2$, 500 mmol/l NaCl) and 2µl of (50% glycerol, 1% SDS, 200 mmol/l EDTA 0.2% bromophenol blue), and incubated at 65°C for 60 min. The samples were electrophoresed on a 1% agarose minigel, with 2µl of the reference DNA as a marker. The DNA was then examined by staining in ethidium bromide and analysis on a short wave UV light box.

Single stranded M13 recombinants with inserts identical to the reference recombinant, co-migrate with the reference recombinant, whereas recombinants containing the complementary strand are retarded in the gel as they hybridise to the reference DNA.

2-3-10 Ligation reactions

Ligation reactions were set up in a 10µl volume containing 20-50ng of vector DNA, varying quantities of the DNA restriction fragment, 50 mmol/l Tris-Cl, pH 7.5, 10 mmol/l $MgCl_2$, 1 mmol/l DTT, 1mmol/l riboATP and 0.2 Units of T4 DNA ligase. For cloning in pUC-19 or pIBI-76 vectors, equimolar amounts of DNA fragment and vector was added. For M13 vectors a 3 fold molar excess of the DNA fragment was used. The reactions were incubated at 4°C for 12-16 hours and subsequently transformed as in sections 2-3, 2-3-1, or 2-3-2 depending on the vector. A control ligation with vector DNA only was included to determine background levels of uncut or recircularised vector DNA.

2-4 Methods for the expression of ALV-S recombinant clones in *Xenopus laevis* oocytes

2-4-1 Injection of Xenopus oocytes

The method of Mertz and Gurdon (1977) was used.

(i) Animals

Xenopus laevis females were obtained from several sources. These were, Dr. Ray Harris (Pharmacology Department, South Australian Institute of Technology, South Australia); Dr. Keith Dixon (Department of Biological Sciences, Flinders University, South Australia); Dr. D. Williamson (Department of Pathology, University of Canterbury, Christchurch, New Zealand); Xenopus Ltd (Holmsedale Nursery, South Nutfield, Redhill, Surrey, England); Xenopus I (Ann Arbor, Michigan, U.S.A.).

(ii) Frog dissection

Frogs were anaesthetised by submersion in 1 litre of 0.1% ethyl-m-aminobenzoate for approximately 15 min, removed, and placed in an ice bath for a further 10 min. The anaesthetised frog was placed on a tray of ice and the skin swabbed with a solution of 0.5% hibitane and 70% aqueous ethanol to remove any slime and to sterilise the area for surgery.

A small incision was then made on either side of the ventral midline. The required number of ovarian lobes were cut off with scissors and placed immediately in cold modified Barth's saline (section 2-4-1-vi). Once the incision had been sutured, the frog was placed in an angled dish with it's nose out of the water until it revived. A single toad could be used up to eight times, depending on the number of oocytes in the ovary.

(iii) Oocytes

The excised ovarian lobes were rinsed in cold modified Barth's saline, teased apart into small clumps and then into single oocytes with grade 5 watchmakers forceps. These single oocytes were stored in small petri dishes containing modified Barth's saline at 18-21°C. Oocytes could be kept for injection in this state for up to 4 days.

(iv) Apparatus for the injection of Xenopus oocytes

Microinjection needles were hand made from 100µl glass micro-capillaries (BLAUBRAND, intraMARK).

The needle was connected to an Agla, screw controlled syringe (Wellcome, Australia) by plastic tubing of 1mm internal diameter and held and manouvered by a micromanipulator (Micro Techniques, Oxford Ltd). The tubing and needle were filled with medicinal paraffin coloured with Fast Red dye enabling discrimination of the paraffin-aqueous interface.

Injections were carried out using a cold light scource for illumination (Volpi, Intralux 150H), under a dissecting, stereozoom microscope (Kyowa) at a magnification of approximately 15X.

(v) Oocyte microinjection technique

(a) Preparation of the DNA for injection

DNA for injection was prepared by CsCl gradient centrifugation (Maniatis *et al.*, 1982). Typically this DNA was dissolved in 88 mmol/l NaCl, 10 mmol/l Tris-Cl,

pH 7.4, and injected into the oocyte at a concentration that varied between 2 and 1000 ng/µl.

(b) Filling the microinjection needle

Usually 1µl samples of the DNA to be injected were brought to the microscope stage on a piece of parafilm. The tip of the paraffin filled needle was introduced below the surface of the droplet and a portion of the sample drawn into the needle by screwing out the syringe.

(c) Nuclear injection

Each oocyte to be injected was transferred from a petri dish to a dry microscope slide (1-8 oocytes/slide) with a wide mouth pasteur pipette. Excess fluid was removed from around the oocyte with a pasteur pipette and the slide transferred to the microscope stage.

Using microscopic observation, the oocytes were manouvered with a pair of forceps such that the animal hemisphere of the oocyte was orientated toward the needle. Although the nucleus is not visible, it occupies a constant position within the oocyte thus facilitating insertion of the needle into it. The oocyte was held with forceps and the needle inserted to about a quarter of the depth of the oocyte, exactly over the apex of the animal pole. Once the needle was positioned, the syringe screw was turned to deliver 25nl of sample per oocyte, by watching the red paraffin meniscus move down the calibrated shaft of the needle. The needle was withdrawn and the injected oocytes washed off the slide into another petri dish containing modified Barth's saline. For each DNA sample 30 oocytes were injected and then incubated for 12 hours at 18-21^{o}C in modified Barth's saline.

(vi) Modified Barth's saline

Modified Barth's saline was made from four stock solutions:

Solution A, 3.52 mol/l NaCl, 40 mmol/l KCL, 96 mmol/l $NaHCO_3$, 600 mmol/l Tris-Cl, pH 7.6; Solution B, (82 mmol/l $MgSO_4.7H_20$); Solution C, 30 mmol/l $Ca(NO_3)_2.4H_2O$, 41 mmol/l $CaCl_2.6H_2O$; Solution D, 10 mg/ml penicillin, 10 mg/ml streptomycin.

To make 1 litre of Modified Barth's saline 25ml of solution A, 10ml of solution B, 10ml of solution C and 1ml of solution D were mixed, and diluted to 1 litre with water.

Solutions A,B,C were stored at 4°C, whilst solution D was stored at -20°C

2-4-2 Isolation of RNA from Xenopus oocytes

The method of Probst *et al.* (1979) was used. Oocytes were homogenised in a loose fitting glass homogeniser in 0.5ml of a solution containing 10 mmol/l Tris-Cl, pH 7.5, 1.5 mmol/l $MgCl_2$, 10 mmol/l NaCl, 1% SDS and 0.5 mg/ml proteinase K. After incubation at 37°C for 30 min, EDTA was added to a final concentration of 10 mmol/l. The mixture was then extracted three times with phenol/chloroform and once with chloroform only. The aqueous phase was made to 200 mmol/l with NaCl and the oocyte RNA precipitated by the addition of three volumes of cold ethanol. After centrifugation for 15 min in an eppendorf centrifuge the pellet was washed in 70% aqueous ethanol, dried *in vacuo* and taken up in 100µl of water. Oocyte RNA could be stored at -80°C indefinitely. Each oocyte usually yielded between 4 and 6µg of total RNA.

2-4-3 Primer extension analysis of oocyte RNA

The method of McKnight *et al.* (1981) was used. 2ng of the appropriate ^{32}P-5' end labelled oligonucleotide primer was added to 10µg of oocyte RNA and ethanol precipitated. The RNA/primer pellet was resuspended in 10µl of 200 mmol/l NaCl, 10 mmol/l Tris-Cl, pH8.3, heated to 80°C for 3 min and allowed to anneal at 42°C for 60 min. Following hybridisation, 24µl of RT buffer (10 mmol/l Tris-Cl, pH 8.3, 10 mmol/l $MgCl_2$, 10 mmol/l DTT, 1 mmol/l dATP, 1 mmol/l dCTP, 1 mmol/l dTTP, 1

mmol/l dGTP and 6 Units of reverse transcriptase) was added and the mixture incubated at 42°C for 60 min.

The extension products were precipitated by the addition of 100µl of cold ethanol and pelleted by centrifugation in an eppendorf centrifuge for 15 min. The pellet was washed in 70% aqueous ethanol, dried *in vacuo* and resuspended in 5µl of water. 5µl of formamide loading buffer was added and the primer extended products electrophoresed on a 7% sequencing gel (section 2-3-8-iii) and visualised by autoradiography.

2-4-4 Oligonucleotide site-directed mutagenesis of M13 recombinants

The method of Zoller and Smith, (1984) was used. 0.5 pmol/l of single stranded M13 recombinant DNA (prepared as in section 2-3-8-i) was mixed with 10 pmol/l 5'-phosphorylated mutagenesis primer, 5.0 pmol/l 5' phosphorylated M13 universal sequencing primer, 50 mmol/l Tris-Cl, pH 7.4, 10 mmol/l $MgCl_2$ and 66 mmol/l NaCl in a total volume of 15µl. This mixture was heated to 65°C for 5 min and then left to cool at room temperature for a further 5 min.

To the above mixture was added 5µl 10 mmol/l riboATP, 5µl 10 mmol/l DTT, 5µl dNTP mix (500 µmol/l dATP, 500 µmol/l dCTP, 500 µmol/l dGTP, 500 µmol/l dTTP), 1µl T4 DNA ligase (1 U/µl), 2µl Klenow fragment of DNA polymerase I (2.5 U/µl) and 17µl of water. This solution was mixed and incubated at room temperature for 12-16 hours.

0.1-2.0µl of the mutagenesis mixture was used to transform *E. coli* JM101 as in section 2-3-1, 1µl usually yielding 150-200 M13 plaques.

(i) Plaque screening

A plate containing approximately 100 plaques was chosen and cooled at 4°C for 30 min. A dry piece of nitrocellulose was placed on the plate, its position marked and left for 2 min. The filter was removed and air dried for 10 min whilst a second identical piece of nitrocellulose was placed on the plate, its position marked and left for

5 min. This second filter was subsequently removed and air dried for 10 min. Both filters were then heated at 80°C *in vacuo* for 2 hours.

Filters were prehybridised in 10ml of 90 mmol/l NaCl, 90 mmol/l Tris-Cl, pH 7.6, 9 mmol/l EDTA, 0.5% NP-40, 5X Denhardts solution (0.4% bovine serum albumin, 0.4% polyvinyl pyrolidone, 0.4% ficoll) and 100 µg/ml sheared salmon sperm DNA (Maniatis *et al.*, 1982) at 42°C for 2 hours.

At the end of this period the filters were removed and 100ng of ^{32}P-5' phosphorylated mutagenesis primer (kinased as in section 2-3-6) was added to the prehybridisation mixture. The filters were then placed back in the hybridisation mixture and incubated at 42°C for 12-15 hours.

After hybridisation, filters were removed and washed twice with 100ml 6xSSC (1 mol/l NaCl, 0.1 mol/l sodium citrate, 3 mmol/l EDTA, pH 7.4) for 5 min at room temperature, then once in 20ml TMACL solution (3 mol/l TMACL, 50 mmol/l Tris-Cl, pH 8.0, 2 mmol/l EDTA, 0.1% SDS) for 5 min at room temperature to remove any unbound mutagenesis primer. Mutagenesis primer bound to wild type plaques was removed by a further 60 min (2 x 30 min periods) washing in TMACL solution at 5°C below the theoretical melting temperature of the mutagenesis primer. The filters were wrapped in Vitafilm and autoradiographed overnight at -80°C.

(ii) Mutant confirmation

Mutant plaques were identified by lining up the developed autoradiogram, nitrocellulose filter and the 2 X YT plate. All M13 mutant recombinant clones were identified by M13 dideoxy chain terminator sequencing, as described in section 2-3-8.

2-5 Methods for the analysis of the rat ALV-S genomic clone RG-1

2-5-1 Dideoxy sequencing of rat ALV-S recombinants

All rat ALV-S recombinants were sequenced by the dideoxy chain termination method described in section 2-3-8, utilising either the universal sequencing primer or

primers synthesised corresponding to the rat ALV-S gene sequence (these primers included P1, P2, P3 and P4, their respective sequences are shown in section 2-1-10).

2-5-2 Mung bean nuclease protection analysis

(i) Construction of pSP(ALVS-A)

10μg of the plasmid vector pSP64 was digested with Pst I. The Pst I cut pSP64 was then heated to 65°C for 10 min, cooled on ice and the 5' phosphates from the free vector ends removed with calf intestinal phosphatase as in Maniatis *et al.*, 1982. The dephosphorylated Pst I cut pSP64 was purified by agarose gel electrophoresis as described in section 2-3-5.

25μg of the rat ALV-S genomic clone RG-1 was digested to completion with Pst I and electrophoresed through 1% low melting point agarose. The 1.3Kb rat genomic fragment was cut out of the gel and the DNA purified (see section 2-3-5).

The 1.3Kb Pst I rat genomic fragment was ligated into the Pst I cut pSP64 vector (section 2-3-10) to produce the recombinant pSP(ALVS-A). The ligation mixture was subsequently transformed into *E. coli* ED8799 as in Maniatis *et al.* (1982).

The orientation of the fragment in the vector was determined by analytical restriction endonuclease digestion with Bam HI. The correct orientation of the insert, relative to the SP6 RNA polymerase initiation site of pSP64 yields a 1.3Kb and a 3.0Kb fragment upon digestion with Bam HI.

(ii) Preparation of ^{32}P-labelled Pst I-Bam HI rat ALV-S genomic fragment

10μg of pSP(ALVS-A) was digested with Bam HI and then electrophoresed on a 1% low melting point agarose gel. The 3.1Kb band was cut out and purified (see section 2-3-5).

1μg of this 3.1Kb Bam HI fragment, containing the SP6 RNA polymerase initiation site of pSP64 and a Pst I-Bam HI rat ALV-S genomic fragment, was added to a reaction mixture containing 10 mmol/l DTT, 40 mmol/l Tris-Cl, ph 7.6, 6 mmol/l $MgCl_2$, 500 μmol/l ATP, 500 μmol/l CTP, 500 μmol/l GTP, 15 μmol/l UTP, 1μg BSA,

100μCi α-(^{32}P)-UTP and 4 Units of SP6 RNA polymerase, in a total volume of 20μl. The above mixture was then incubated at 40°C for 60 min, electrophoresed on a 4% sequencing gel and exposed to X-Ray film for 30 seconds. The band corresponding to 466 nucleotides was cut out, and the ^{32}P-labelled RNA fragment eluted as in section 2-3-4.

(iii) Mung Bean Nuclease digestion

For each digestion 5μg of rat liver poly(A)$^+$ RNA (prepared as in Srivastava *et al.*, 1988) and 15μl of ^{32}P-labelled Pst I-Bam HI rat ALV-S genomic fragment were ethanol precipitated together, washed in 70% ethanol, dried and the pellet dissolved in 10μl of 30 mmol/l sodium acetate, pH 4.6, 50 mmol/l NaCl, 1 mmol/l $ZnCl_2$ and 5% glycerol. This mixture was heated to 65°C for 5 min and allowed to cool slowly to 37°C. When the mixture had cooled, 1μl of mung bean nuclease (containing 0, 10, 75 and 150U, respectively) was added to the different mixtures and the reaction incubated at 37°C for exactly 3 min. At the end of this period 10μl of formamide loading dye was added and the mixture heated to 80°C for 3 min, cooled on ice, and electrophoresed on a 7% sequencing gel (see section 2-3-8-iii). The resulting gel was subsequently autoradiographed.

2-6 Methods for the transient expression of ALV-S recombinants in tissue culture cell lines

2-6-1 General tissue culture techniques

(i) Cell maintenance

All cells were routinely maintained in 75cm^3 flasks (Costar) at 37°C in an atmosphere of 5% CO_2 and were subcultured every 3-4 days. After a maximum of 13 passages, cells were discarded because of a reported decrease in transfection efficiency (Heard *et al.*, 1987).

(ii) Trypsin treatment

Culture medium was removed from the cells and the plate rinsed in PBS before the addition of 2ml of trypsin/EDTA solution. The plate was then incubated at room temperature for 1 min. Following this, the trypsin/EDTA solution was removed. The cells were readily detached by rinsing with 5ml PBS and were then pelleted by centrifugation at 1500 x g for 5 min.

2-6-2 Heme-arginate solution

25mg of hemin and 26mg of L-arginine were mixed in 200µl of water. To this was added 100µl 100% ethanol and the solution mixed again. A further 400µl of water and 300µl of polyethylene glycol was added, and the solution mixed by vortexing. The final concentration of heme was 34 mmol/l, which could then be diluted to the concentration of choice.

2-6-3 Calcium phosphate transfection of rat hepatoma H4-II-E-C3 cells

Transfections were carried out using the calcium phosphate method of Graham and Van Der Eb (1973), as modified by Wigler *et al.* (1979), which is dependant upon the formation of a calcium phosphate-DNA precipitate.

24 hours prior to transfection the cells were seeded at a density of 0.5 x 10^6/60 mm plate. Fresh medium was then added to the cells two hours prior to the addition of the calcium phosphate-DNA precipitate.

The precipitate was prepared from equal volumes of two solutions. Solution A (10µg of DNA to be transfected, 125 mmol/l $CaCl_2$ in 1 mmol/l Tris-Cl, pH 7.9 and 0.1 mmol/l EDTA) was added dropwise to solution B (280 mmol/l NaCl, 50 mmol/l Hepes, pH 7.1, and 1.5 mmol/l NaH_2PO_4) with constant aeration of solution B. Following a 30 min incubation period at room temperature, aliquots of the transfection mix were added to the cell cultures. These cultures were then incubated at 37°C for 18 hours.

The cells were then either osmotically shocked for 1-5 min with 2ml of 25% (v/v) glycerol/ DMEM, or washed twice with 2ml of PBS. Fresh medium was added to both glycerol shocked and PBS treated cells, and the incubation continued at 37°C for a further 48 hours. Cells were harvested by trypsin treatment and assayed for CAT activity as in section 2-6-5.

2-6-4 Transfection of rat hepatoma H4-II-E-C3 cells by electroporation

Transfection of rat hepatoma H4-II-E-C3 cells by electroporation was performed by a modification of the method of Chu *et al.* (1987).

Growing cells were removed with trypsin/EDTA and resuspended in HeBS buffer (20 mmol/l Hepes, pH 7.05, 137 mmol/l NaCl, 5 mmol/l KCL, 0.7 mmol/l Na_2HPO_4, 6 mmol/l dextrose) at a concentration of 5 x 10^6 cells/ml. 500 µg/ml of sonicated salmon sperm DNA was added to the cell suspension and mixed. This solution was then added to 10µg of DNA to be transfected at a concentration of 0.5 µg/ml in the electroporation cuvette Biorad gene pulser.

The cells were incubated on ice for 10 min and then exposed to a single voltage pulse at room temperature on a Biorad Gene Pulser. The cells were placed on ice for 5 min and then gently plated in 60mm dishes containing either DMEM containing 10% FCS, or DMEM containing 10% FCS and 1 µmol/l heme-arginate (see section 2-6-2). Cells were incubated at 37°C for 48 hours and at the end of this period, dead cells were removed by washing in PBS. Attached cells were removed by trypsin/EDTA treatment and assayed for CAT activity as described in section 2-6-5.

2-6-5 Chloramphenicol acetyltransferase (CAT) assay

CAT activity of transfected cell extracts was determined by a modification of the procedure of Gorman *et al.* (1982).

Cell pellets obtained by treatment with trypsin were resuspended in 100µl of 250 mmol/l Tris-Cl, pH 7.6, and lysed by three rounds of freezing at -80°C and

thawing at 37°C. The lysate was centrifuged in a microfuge at high speed for 5 min to remove the cellular debris and the resultant supernatant retained, and made to 5 mmol/l with EDTA. This solution was heated to 60°C for 10 min to reduce deacetylase activity (Andrisani *et al.*, 1987).

The assay mixture contained in an initial total volume of 180µl, 0.4 mmol/l acetyl coenzyme A, 180 mmol/l Tris-Cl, pH 7.6, 0.1µCi ^{14}C-chloramphenicol and 150µg of protein extract, as determined by the method of Bradford (1976). This mixture was incubated for 3h, with 0.4 mmol/l acetyl coenzyme A being added to the reaction mixture every 45 min (Heard *et al.*, 1987). At the end of this period the reaction was stopped by the addition of 1ml of cold ethyl acetate. The phases were mixed by vortexing and the organic phase removed, and subsequently evaporated to dryness.

The resultant residue was dissolved in 10µl of ethyl acetate and the unacetylated ^{14}C-chloramphenicol was resolved from the mono and di-acetylated ^{14}C-chloramphenicol products by thin layer chromatography on silica plates, in a solvent of chloroform/methanol (9:1 v/v). ^{14}C-chloramphenicol products were visualised by autoradiography using Kodak XAR-5 X-ray film. Relative levels of CAT activity were quantified by liquid scintillation spectroscopy of the excised ^{14}C-acetylated chloramphenicol products from the chromatogram.

2-6-6 Oligonucleotide site-directed mutagenesis of pIBI-76 recombinant clones

0.5 pmol/l of single stranded pIBI-76 recombinant DNA was mixed with 10 pmol/l 5'-phosphorylated mutagenesis primer, 5.0 pmol/l 5' phosphorylated reverse sequencing primer, 50 mmol/l Tris-Cl, pH 7.4, 10 mmol/l $MgCl_2$ and 66 mmol/l NaCl in a total volume of 15µl. This was heated to 65°C for 5 min and then left to cool at room temperature for a further 5 min.

To the above mixture was added 5µl 10 mmol/l ribo ATP, 5µl 10 mmol/l DTT, 5µl dNTP mix (500 µmol/l dATP, 500 µmol/l dCTP, 500 µmol/l dGTP, 500 µmol/l

dTTP), 1µl T4 DNA ligase (1 U/µl), 2µl Klenow fragment of DNA polymerase I (2.5 U/µl) and 17µl of water. This solution was mixed and incubated at room temperature for 12-16 hours.

0.1-2.0µl of the mutagenesis mixture was used to transform *E. coli* BB4 as in section 2-3-2, 1µl usually yielding 100-150 colonies.

(i) Colony screening

A plate containing approximately 50 colonies was chosen and cooled at 4°C for 10 min. A dry piece of nitrocellulose was placed very carefully on the plate, the position marked and left for 2 min. The filter was removed and layered onto a sheet of Whatman 3MM paper saturated in 10% SDS for 3 min, removed and transferred to another sheet of Whatman 3MM saturated in 500 mmol/l NaOH and 1.5 mol/l NaCl for 5 min, and finally to another sheet of Whatman 3MM saturated in 500 mmol/l Tris-Cl, pH 8.0, and 1.5 mol/l NaCl for 5 min. At the end of this period the filter was air dried for 30 min and then heated at 80°C *in vacuo* for 2 hours.

The filter was prehybridised in 10ml of 0.4 mmol/l NaCl, 90 mmol/l Tris-Cl, pH 7.6, 9 mmol/l EDTA, 0.5% NP-40, 5X Denhardts solution (0.4% bovine serum albumin, 0.4% polyvinyl pyrolidone, 0.4% ficoll) and 100 µg/ml sheared salmon sperm DNA (Maniatis *et al.*, 1982) at 42°C for 2 hours.

At the end of this period the filter was removed and 100ng of ^{32}P-5' phosphorylated mutagenesis primer (kinased as described in section 2-3-6) was added to the prehybridisation mixture. The filter was placed back in the hybridisation mixture and left at 42°C for 12-15 hours.

After hybridisation the filter was removed and washed twice with 100ml 6xSSC (1 mol/l NaCl, 0.1 mol/l sodium citrate, 3 mmol/l EDTA, pH 7.4) for 5 min at room temperature, then once in 20ml TMACL solution (3 mol/l TMACL, 50 mmol/l Tris-Cl, pH 8.0, 2 mmol/l EDTA, 0.1% SDS) for 5 min at room temperature to remove any unbound mutagenesis primer. Mutagenesis primer bound to wild type plaques was removed by a further 60 min (2 x 30 min periods) washing in TMACL solution at 5°C

below the theoretical melting temperature of the mutagenesis primer. The filter was wrapped in Vitafilm and autoradiographed overnight at -80°C

(ii) Mutant confirmation

Mutant colonies were identified by lining up the developed autoradiogram, nitrocellulose filter and L-agar/60 μg/ml ampicillin plate. All pIBI-76 mutant recombinant clones were identified by analytical restriction mapping.

2-7 Miscellaneous methods

2-7-1 Containment facilities

All manipulations involving 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.

2-7-2 Animal experimentation

All experiments on animals were approved by the Committee for the Ethical use of Animals, University of Adelaide.

CHAPTER 3

DELIMITATION AND CHARACTERISATION OF *CIS*-ACTING DNA SEQUENCES REQUIRED FOR THE EXPRESSION OF THE CHICKEN ALV-S GENE IN *XENOPUS LAEVIS* OOCYTES

Chapter 3: Delimitation and characterisation of *cis*-acting DNA sequences required for the expression of the chicken ALV-S gene in *Xenopus laevis* oocytes

3-1: Introduction

Chick embryo liver ALV-S genomic clones have been isolated in this laboratory and the complete nucleotide sequence of the gene determined by Maguire *et al.* (1986). The chicken gene is 7.6Kb in length and is a single copy gene (Borthwick *et al.*, 1985). Sequence analysis of the 5' flanking region of the chicken ALV-S gene demonstrated the presence of a number of sequences which correspond with *cis*-acting control elements found in the transcriptional regulation of other eukaryotic genes. The sequences identified are as follows: two possible sequences related to the CCAAT box canonical sequence GG$^{C}/_{T}$CAATCT (Benoist *et al.*, 1980, Efstratiadis *et al.*, 1980); multiple copies of the consensus sequence $^{G}/_{T}$GGGCGG$^{G}/_{A}$$^{G}/_{A}$$^{C}/_{T}$ (McKnight *et al.*, 1981, Farnham and Schimike, 1986, Kadonaga *et al.*, 1986, Damante *et al.*, 1987); two potential TATA boxes (consensus sequence TATA$^{A}/_{T}$A$^{A}/_{T}$) (Cordon *et al.*, 1980). It had also been shown that 298bp of the 5' flanking region of the chicken ALV-S gene was sufficient to promote efficient expression of an attached reporter gene in Xenopus oocytes (Maguire *et al.*, 1986).

At the time this work was undertaken, many studies had examined the regulation of eukaryotic transcription, but few had been reported in which a detailed analysis of the *cis*-acting sequence elements had been made. No chicken hepatoma cell lines are available for use in studying chicken ALV-S gene expression. Since the chicken ALV-S gene expressed strongly in Xenopus oocytes, a detailed analysis of the 5' flanking region of the chicken ALV-S gene was made utilising a combination of deletion and site-directed mutational analysis and expression in Xenopus oocytes. The aim of this work was to define those sequences important for ALV-S gene expression in the *Xenopus laevis* oocyte.

3-2 Results

3-2-1 Construction of chicken ALV-S restriction enzyme deletion clones

A series of restriction enzyme deletion clones containing varying lengths of the chicken ALV-S gene was constructed as outlined below. Four of these clones contained the 5' flanking region and a truncated ALV-S gene sequence, while the other three constructions were made with the ALV-S 5' flanking region attached to the chicken histone H2B gene as a reporter gene.

M13chALV-7 was prepared by subcloning into the Hind III site of M13mp19, a 4.1Kb Hind III DNA fragment containing 1.5Kb of the ALV-S 5' flanking region from the chicken ALV-S genomic clone λcALA-S1 (Maguire *et al.*, 1986) (see Fig. 3-1 for illustration of this and the constructions below).

M13chALV-6 was constructed by subcloning a 4.3Kb Bam HI fragment containing 257bp of the ALV-S 5' flanking region, from λcALA-S1 into the Bam HI site of M13mp19.

M13chALV-2 was constructed by cloning a 288bp Bam HI-Pvu II fragment from the ALV-S 5' flanking region into the polylinker Sma I site of M13(H2B) (an M13mp19 construction containing the chicken histone H2B structural gene cloned into the polylinker Hinc II site [Maguire *et al.*, 1986]) in a 5' to 3' orientation. M13chALV-1 was derived from M13chALV-2 by removal of a 94bp Sma I fragment.

M13chALV-4 contained a 532bp HgAI1-Pvu II fragment from λcALA-S1, cloned into the Sma I site of M13(H2B) in a 5' to 3' orientation.

pchALV-5 was constructed by cloning a 397bp Bam HI-Pst I fragment from M13chALV-7 into pUC-19, which had been linearised with Pst I and Bam HI. pchALV-3 was then derived from pchALV-5 by removal of a 115bp fragment, by digestion with Ava I and subsequent re-ligation.

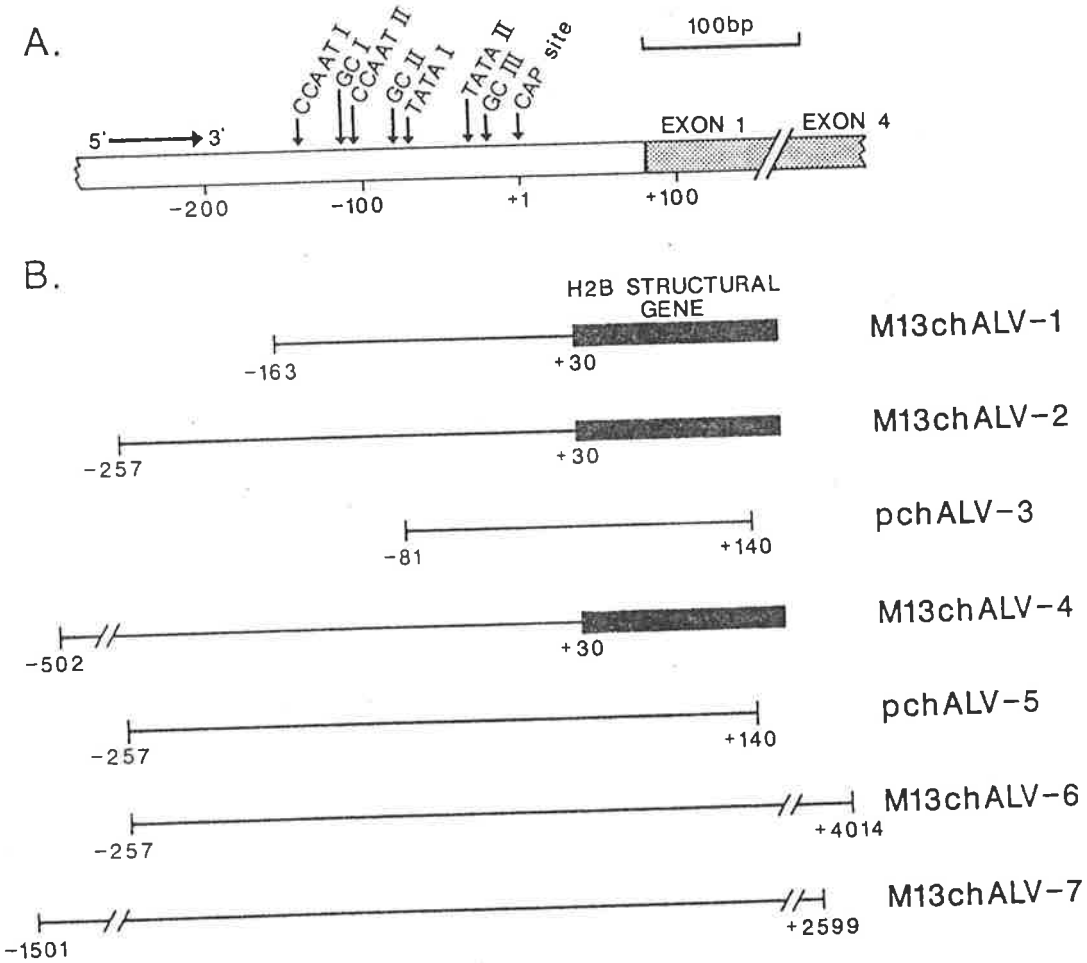
The plasmid p7AT contained the chicken H2B structural gene under the control of its own promoter in the pAT vector (Sturm, 1986) and was co-injected with the ALV-S constructions as an internal control.

Figure 3-1-A.

Schematic map of the putative *cis*-acting sequence elements of the ALV-S gene. Open bar, 5' flanking DNA; shaded bars, exon DNA. Numbers refer to bp of DNA sequence relative to the transcriptional start point (CAP) at +1.

Figure 3-1-B.

Deletion mutations of the 5' flanking region of the ALV-S gene. Details of the construction of the deletion mutations are described in section 3-2-1. The ALV-S gene sequences cloned into either M13mp19, pUC-19 or M13(H2B) are indicated by thin lines. The H2B structural gene is shown as the thick line. The nucleotide positions of the end points of the deletion clones are shown below the thin lines.


A.
CCAAT I
GC I
CCAAT II
GC II
TATA I
TATA II
GC III
CAP site
100bp
5'
3'
EXON 1
EXON 4
-200
-100
+1
+100
B.
H2B STRUCTURAL GENE
M13chALV-1
-163
+30
M13chALV-2
-257
+30
pchALV-3
-81
+140
M13chALV-4
-502
+30
pchALV-5
-257
+140
M13chALV-6
-257
+4014
M13chALV-7
-1501
+2599

The identity of all the restriction enzyme deletion mutants was confirmed by dideoxy sequencing (see section 2-3-8) where possible, or otherwise by analytical restriction mapping. All ALV-S restriction enzyme deletion mutants not containing the H2B structural gene are referred to as ALV-S minigenes for convenience.

3-2-2 Construction of specific deletion-insertion mutants

Specific deletion-insertion mutations of the chicken ALV-S 5' flanking region were introduced into the ALV-S construction M13chALV-7 by site-directed oligonucleotide mutagenesis as outlined in section 2-4-5, utilising the mutagenesis primers listed in section 2-1-10-B. All mutants were confirmed by dideoxy sequencing of the single stranded template (section 2-3-8).

3-2-3 Minimum length of 5' flanking sequence required for expression of the ALV-S gene in Xenopus oocytes

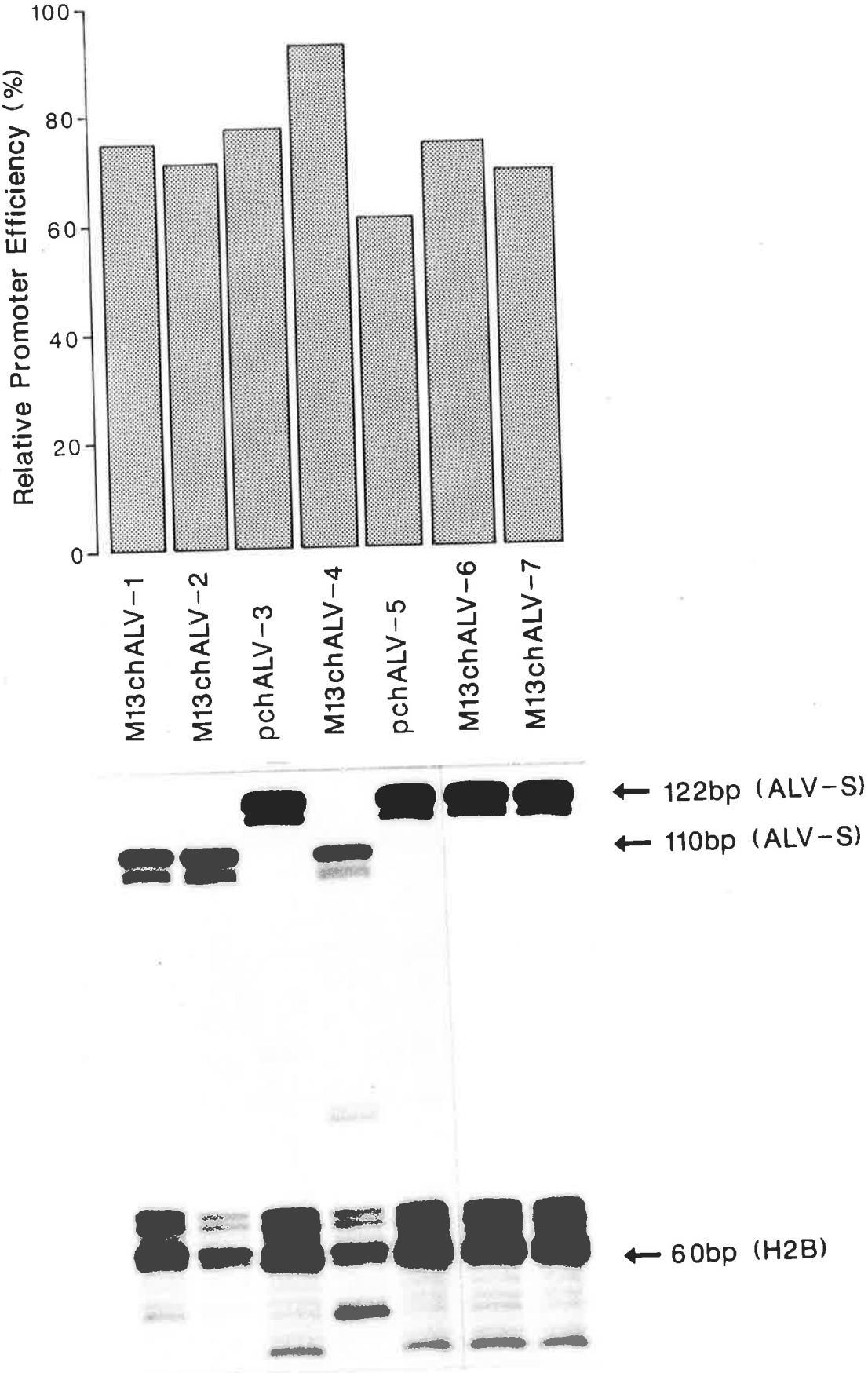
Initial experiments were aimed at defining the minimal length of ALV-S 5' flanking sequence required to direct accurate and efficient transcription. In these experiments various lengths of the 5' flanking region, attached to either an ALV-S minigene or the chicken histone H2B structural gene, were injected into Xenopus oocytes. The construction p7AT was always co-injected as an internal control.

The lengths of the 5' flanking region tested extended 5' from the transcription initiation (CAP) site for 1501, 502, 257, 163 and 81 base pairs respectively (see Fig. 3-1). Each of these constructsions with p7AT, was co-injected into Xenopus oocytes. After 20-24 hours incubation at 18^{o}C, RNA was extracted and isolated as described in section 2-4-2. The amount of RNA transcribed from each construction was analysed by primer extension analysis (see section 2-4-3) and quantified by laser densitometry.

The results of such an experiment are shown in Figure 3-2. Two groups of extension products are seen; extension products from the internal control plasmid p7AT show the characteristic multiple banding pattern of the H2B gene (Wigley, 1987), designated H2B in Figure 3-2. Two extension products are generated by the ALV-S 5'

Figure 3-2.

Primer extension analysis of RNA transcribed in *Xenopus* oocytes from the ALV-S gene deletion mutants. *Xenopus* oocyte nuclei were injected with the deletion mutant indicated, and primer extension analysis performed on isolated RNA as described in section 2-4-3. The lower portion of the figure shows an autoradiogram of the primer extended products from both the deletion mutant (ALV-S) and the internal control plasmid p7AT (H2B). The length and identity of each extension product is indicated. The level of ALV-S expression is shown as a percentage of the level of p7AT expression.


Relative Promoter Efficiency (%)
100
80
60
40
20
0
M13chALV-1
M13chALV-2
pchALV-3
M13chALV-4
pchALV-5
M13chALV-6
M13chALV-7
122bp (ALV-S)
110bp (ALV-S)
60bp (H2B)

flanking constructions (Fig. 3-2, ALV-S). This doublet is probably due to the property of reverse transcriptase to characteristically add an extra base to the genuine extension product, producing a doublet (Forster, 1987).

The results show that constructions containing 80bp or more of 5' flanking sequence expressed at approximately the same high level. Thus sequences contained in the first 80bp can promote efficient transcription of the ALV-S gene in Xenopus oocytes. The results also show that the level of ALV-S gene expression in oocytes was not affected by sequences as far downstream as +4014. Although there is some variation in the level of expression between the various ALV-S constructions (shown in Fig. 3-2), this was not a consistent observation and over many experiments this variation was shown not to be significant.

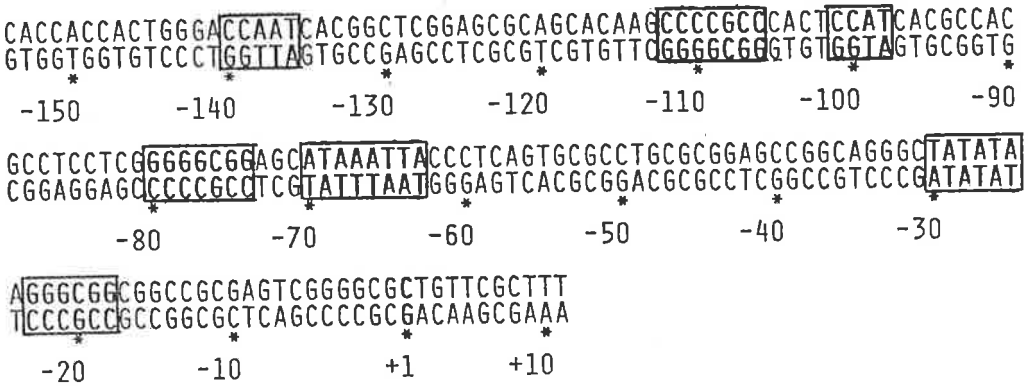
3-2-4: The role of the putative promoter elements in expression of the chicken ALV-S gene

The ALV-S 5' flanking region contains a number of sequences homologous to sequences known to be important for the transcriptional control of other genes (Fig. 3-3). These are, TATA boxes at -28 and -68, CCAAT boxes at -98 and -138 and GC boxes at -20, -78, and the reverse complement at -108. All these sequence elements have been found in the promoter regions of other genes and in many cases contribute to expression both *in vitro* (Hen *et al.*, 1982, Farnham and Schimke, 1986, Sive *et al.*, 1986) and *in vivo* (McKnight *et al.*, 1981, Mellon *et al.*, 1981, Eisenberg *et al.*, 1985, Bergsma *et al.*, 1986). Although some of these elements lie outside the minimum length of 5' flanking region required for ALV-S gene expression, they were examined for effects which may not have been observed in the deletion analysis already described.

Using site-directed mutagenesis a series of mutants were constructed in which these putative elements were altered without changing the natural spatial configuration of the promoter. At the time these mutants were made, all promoter changes were based on the estimations in the literature of what bases are essential to the function of

Figure 3-3.

Nucleotide sequence of the chicken ALV-S gene 5' flanking region. Numbers indicate the nucleotide position of the base pair relative to the transcriptional start point at +1. The putative TATA, CCAAT and GC box sequence homologies are indicated.


CACCACCACTGGGACCAATCACGGCTCGGAGCGCAGCACAAGCCCCGCCCACTCCATCACGCCAC
GTGGTGGTGTCCCTGGTTAGTGCCGAGCCTCGCGTCGTGTTCGGGGCGGGGTGTGGTAGTGCGGTG
-150
-140
-130
-120
-110
-100
-90
GCCTCCTCGGGGGCGGAGCATAAATTACCCTCAGTGCGCCTGCGCGGAGCCGGCAGGGCTATATA
CGGAGGAGCCCCCGCCTCGTATTTAATGGGAGTCACGCGGACGCGCCTCGGCCGTCCCGATATAT
-80
-70
-60
-50
-40
-30
AGGGCGGCGGCCGCGAGTCGGGGCGCTGTTCGCTTT
TCCCGCCGCCGGCGCTCAGCCCCGCGACAAGCGAAA
-20
-10
+1
+10

the element. In addition to individual mutations, combinations of mutations were prepared to test for the possibility of interactive effects. These mutants were all derived from the construction M13chALV-7 and any alterations are shown in Figure 3-4. Each mutant was co-injected into oocytes with the control plasmid p7AT, and the level of transcription from both the mutant ALV-S promoter and the histone H2B promoter measured by primer extension analysis.

Typical results are shown in Figures 3-5 and 3-6. The track labelled M13chALV-7 shows the level of transcription from the unaltered promoter. Alteration of the TATA box at position -68 did not change either the level of transcription of the ALV-S minigene, or the site of transcriptional initiation (Fig. 3-5, TATA-I). In contrast, alteration of the TATA box at position -28 completely abolished transcription of the ALV-S minigene. None was detectable from the TATA-II mutant even when the gel was heavily overloaded (Fig. 3-5, TATA-II). No alternative start sites were observed and no specific transcription was seen from the potential TATA box at -68. When both TATA boxes were mutated in the one construction, as was expected from the above result, no detectable transcription of the ALV-S minigene was observed (Fig. 3-6, TATA-I,II). Thus the TATA box at position -28 is essential for transcription of the chicken ALV-S gene in oocytes, while that at position -68 is non functional.

The ALV-S promoter contains two potential CCAAT boxes located approximately 98 and 138bp upstream from the transcription initiation site. Alteration of either of these CCAAT boxes separately did not alter the level of transcription as compared with M13chALV-7 (Fig. 3-5, CCAAT-I, CCAAT-II). To eliminate the possibility that the CCAAT boxes could functionally compensate for one another when either was mutated, a clone was constructed with both CCAAT box sequences altered (see Fig. 3-4). Expression of this construct in oocytes resulted in a level of transcription equal to that of M13chALV-7 (Fig. 3-6, CCAAT-I,II). This clearly demonstrates that neither of the two potential CCAAT box sequences in the ALV-S promoter region contribute to expression of the ALV-S gene in oocytes.

Figure 3-4.

Schematic representation of the site-directed mutations of the ALV-S gene. All mutations were constructed in the ALV-S minigene, M13chALV-7, as described in section 3-2-2. The unaltered M13chALV-7 containing the putative *cis*-acting sequences is shown on the top line of the figure. All base substitutions in the chicken ALV-S gene are shown in bold print.

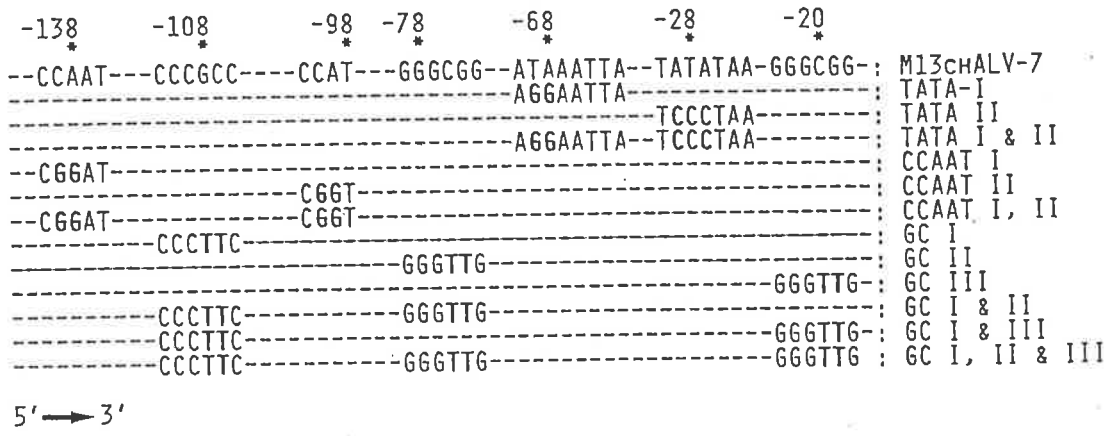

-138
-108
-98
-78
-68
-28
-20
--CCAAT---CCCGCC----CCAT---GGGCGG--ATAAATTA--TATATAA-GGGCGG-: M13cHALV-7
----------AGGAATTA----------: TATA-I
----------TCCCTAA--------: TATA II
----------AGGAATTA--TCCCTAA--------: TATA I & II
--CGGAT----------: CCAAT I
----------CGGT----------: CCAAT II
--CGGAT----------CGGT----------: CCAAT I, II
----------CCCTTC----------: GC I
----------GGGTTG----------: GC II
----------GGGTTG-: GC III
----------CCCTTC----------GGGTTG----------: GC I & II
----------CCCTTC----------GGGTTG-: GC I & III
----------CCCTTC----------GGGTTG----------GGGTTG : GC I, II & III
5'→3'

Figure 3-5.

Primer extension analysis of RNA transcribed in *Xenopus* oocytes from the ALV-S site-directed mutants. *Xenopus* oocyte nuclei were injected with the mutant indicated and primer extension analysis performed on isolated RNA as described in section 2-4-3. The lower portion of the figure shows an autoradiogram of the primer extended products from the site-directed mutants (ALV-S) and the internal control plasmid p7AT (H2B). The length and identity of each extension product is indicated. The upper portion of the figure shows the quantitation of the level of expression of the ALV-S site-directed mutants relative to the unaltered M13chALV-7. The level of ALV-S expression is shown as a percentage of M13chALV-7 after calculating expression relative to p7AT.

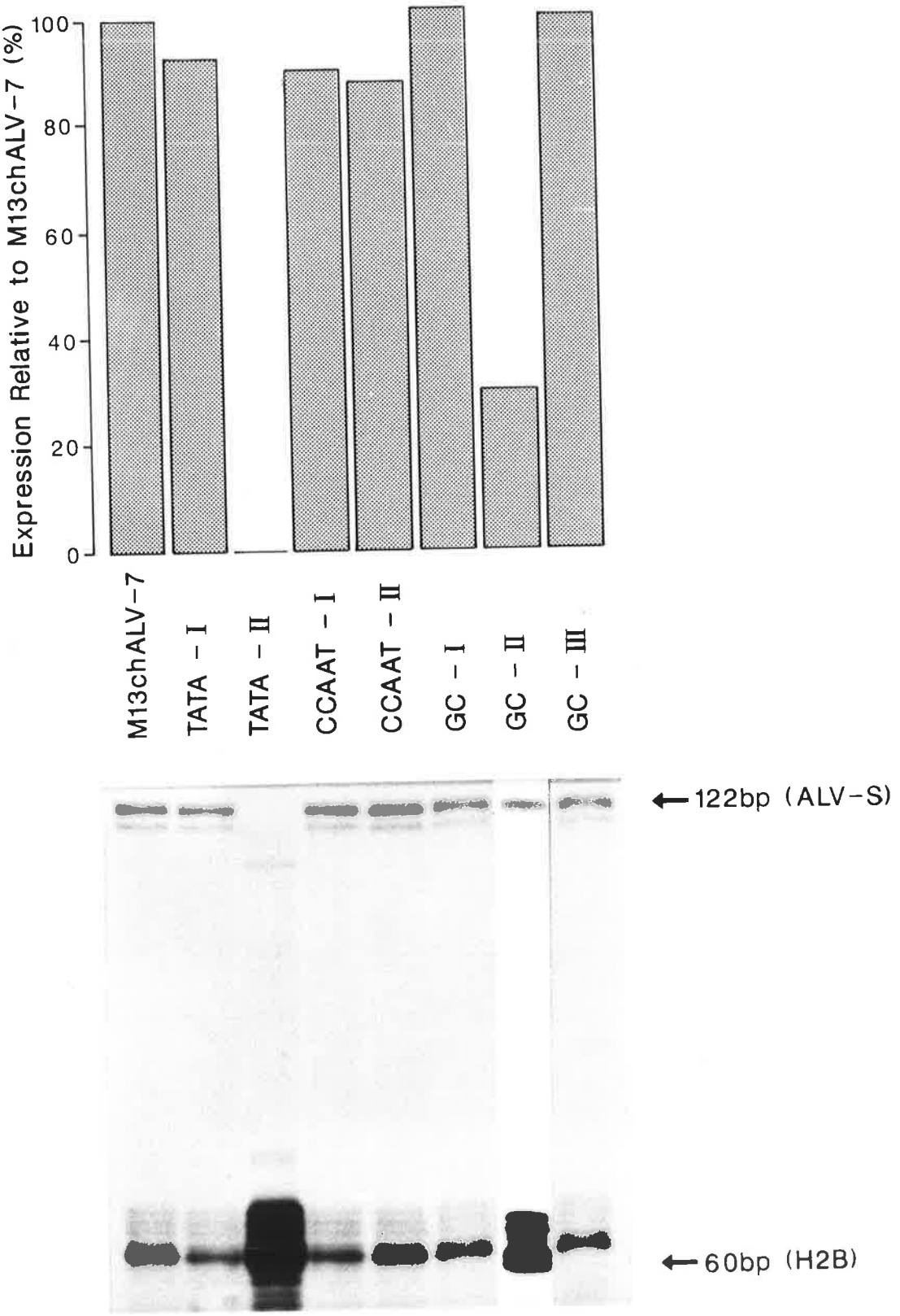
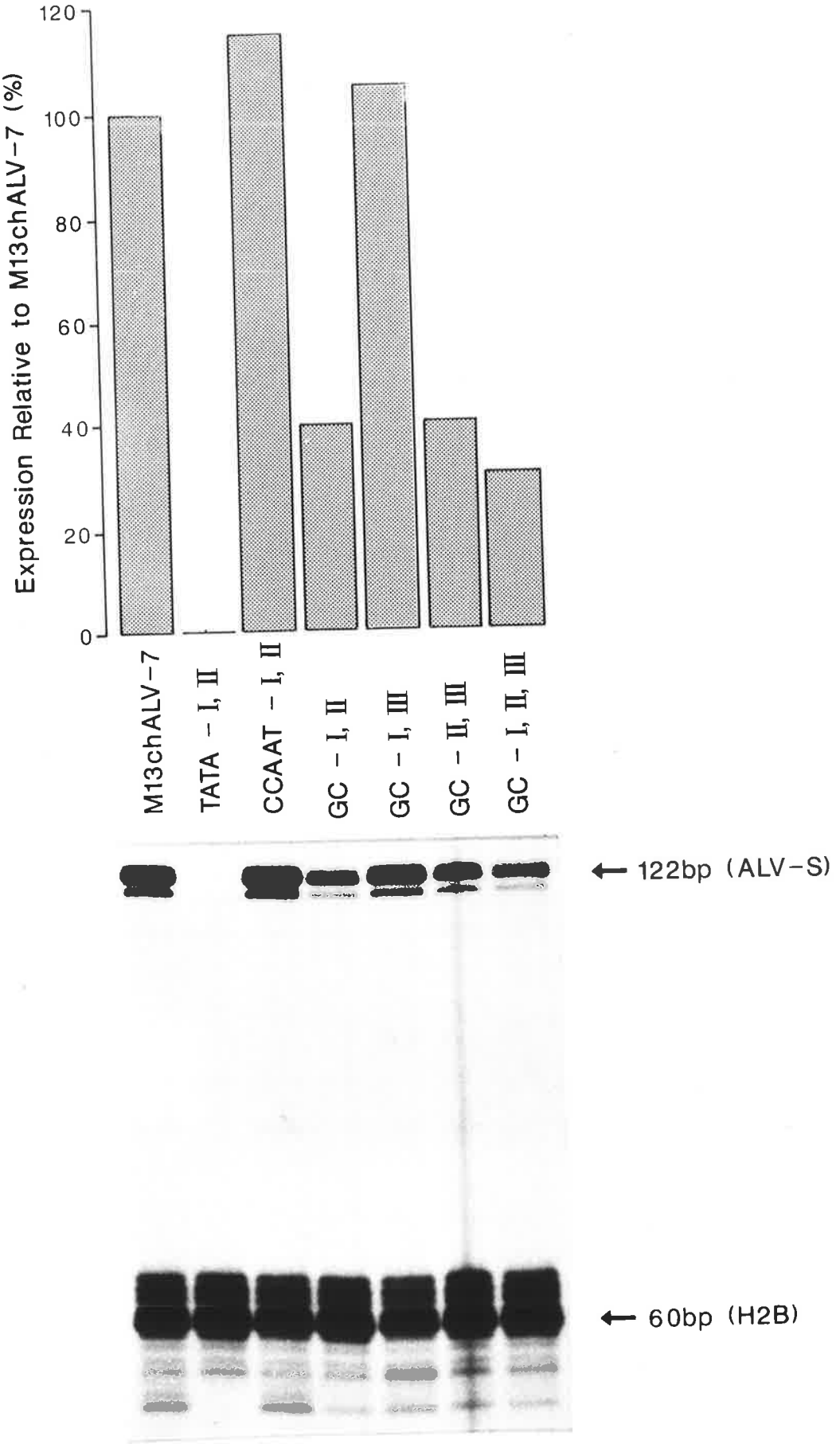

Expression Relative to M13chALV-7 (%)
100
80
60
40
20
0
M13chALV-7
TATA - I
TATA - II
CCAAT - I
CCAAT - II
GC - I
GC - II
GC - III
122bp (ALV-S)
60bp (H2B)

Figure 3-6.

Primer extension analysis of RNA transcribed in *Xenopus* oocytes from the ALV-S site-directed mutants containing more than one mutation. Details as in Figure 3-5.


Expression Relative to M13chALV-7 (%)
120
100
80
60
40
20
0
M13chALV-7
TATA - I, II
CCAAT - I, II
GC - I, II
GC - I, III
GC - II, III
GC - I, II, III
122bp (ALV-S)
60bp (H2B)

The 5' flanking region of the ALV-S promoter contains three potential GC box sequences. Alteration of the GC boxes at either -108 or -20 did not affect the level of transcription of the ALV-S minigene relative to M13chALV-7 (Fig. 3-5, GC-I, GC-III). In contrast, alteration of the GC box at -78 reduced transcription of the ALV-S minigene by approximately 70% (Fig. 3-5, GC-II).

As in the case of the herpes simplex virus thymidine kinase gene (McKnight, 1982), the possibility of cooperation between the GC boxes existed. ALV-S mutants were therefore constructed in which combinations of two of the GC boxes were altered in the one mutant (see Fig. 3-4). A mutant in which all three GC boxes were mutated was also constructed (see Fig. 3-4). The level of transcription of the ALV-S minigene from all promoters in which the GC box at -78 was mutated was reduced by about 70% relative to M13chALV-7 (Fig. 3-6, GC-I,II, GC-II,III, GC-I,II,III). In mutants in which this GC box was intact, the ALV-S minigene was transcribed with the same efficiency as M13chALV-7 (Fig. 3-6, GC-I,III).

This data is consistent with the conclusion that only the GC box at position -78 is involved in transcribing the ALV-S gene in Xenopus oocytes, contributing approximately 70% of the total level of expression. The other two GC boxes do not appear to be utilised for transcription.

3-3: Discussion

The Xenopus oocyte system has been used to study the expression of a number of eukaryotic genes including the chicken skeletal α-actin gene (Bergsma *et al.*, 1986), herpes simplex virus thymidine kinase gene (McKnight and Gravis, 1980, McKnight *et al.*, 1981), sea urchin histone genes (Probst *et al.*, 1979) and the SV 40 late genes (Wickens and Gurdon, 1983). It has proved to be a useful system for gaining information on the sequences involved in gene transcription.

Using this system to study the expression of the chicken ALV-S gene it has been shown that a maximum of 80bp proximal to the transcription initiation site was necessary for efficient transcription of the ALV-S gene in Xenopus oocytes. As all the

constructions expressed at approximately the same level, this would also indicate that sequences 3' from +35 to +4014 of the ALV-S gene were not essential to efficient expression. Therefore sequences in the first three introns of the chicken ALV-S gene (Maguire *et al.*, 1986) play no role in expression. These results indicate that the region of the chicken ALV-S 5' flanking region between -80 and +35 most likely contains all the sequences essential for efficient expression of this gene in oocytes.

The ALV-S 5' flanking region contains a number of potential *cis*-acting regulatory elements (see Fig. 3-3) which were initially identified by their homology to sequences known to play an important role in the expression of other genes. Several of these putative elements, a TATA box and two GC boxes lie within the region between -80 and +35 shown to be essential for maximal ALV-S expression. To define the role of these elements in transcription of the ALV-S gene, mutants were constructed in which these elements were altered singly and in various combinations, and their expression monitored in Xenopus oocytes.

The ALV-S 5' flanking region contains two potential CCAAT boxes at positions -98 (ACTCCATCA) and -138 (GGACCAATC). Mutation of one or both of these elements did not alter the accuracy or efficiency of ALV-S transcription. This is in agreement with the results of the deletion analysis which showed that removal of sequences between -160 and -80 did not alter the level of expression.

The observation that neither of the two CCAAT box sequences of this gene contribute to the expression of the ALV-S gene in oocytes is unusual. Although *in vitro*, the removal of the CCAAT box has little or no effect on transcriptional efficiency (Corden *et al.*, 1980, Grosveld *et al.*, 1981, Hu and Manley, 1981), most studies of genes *in vivo* show that the removal of the CCAAT box causes a reduction in the efficiency of transcription (Benoist and Chambon, 1981, Dierks *et al.*, 1981, McKnight *et al.*, 1981, Mellon *et al.*, 1981, Grosveld *et al.*, 1982, Bergsma *et al.*, 1986). More specifically, removal of the CCAAT box sequence from either the herpes simplex thymidine kinase gene (McKnight *et al.*, 1981), or the chicken skeletal α-actin gene

(Bergsma *et al.*, 1986) resulted in reduced expression when assayed in Xenopus oocytes.

A major problem in interpreting the available data is that in the studies documented in the literature, most rely exclusively on deletion mapping in which it is difficult to determine if the reduction in transcriptional efficiency is due to loss of the CCAAT box, loss of surrounding sequences, or alteration of the spatial arrangement of the remaining DNA sequences. These problems have been avoided by Dierks *et al.* (1983) who utilised single point deletion-substitution mutations of the rabbit β-globin gene CCAAT box. These authors showed that the CCA of the CCAAT consensus sequence was functionally essential. Grosveld *et al.* (1982) previously had shown that the preceding two G's were also essential for this CCAAT box to function efficiently. In view of this, our results would suggest that the ACTCCATCA sequence present at position -68 in the ALV-S gene does not function because it is not a true homolog of the CCAAT canonical sequence. However, the GGACCAATC sequence at position -138 of the ALV-S 5' flanking region, whilst strongly homologous to the consensus sequence may be too far upstream of the transcription initiation site to affect transcription. Most functional CCAAT boxes are found closer to the TATA box.

The ALV-S gene contains three potential GC boxes at positions -20, -78 and -108. Mutation of either of the GC boxes at positions -20 or -108 singly, or in combination with each other failed to alter the level of ALV-S transcription. In contrast, alteration of the GC box at -78, whether individually or in combination with either or both of the other two GC boxes reduced the level of ALV-S transcription by approximately 70%. These results would indicate that only the GC box at position -78 is involved in the transcription of the ALV-S gene in Xenopus oocytes.

The role of the GC box in transcription has been best characterised in the herpes simplex virus thymidine kinase gene (McKnight *et al.*, 1981, McKnight, 1982, McKnight and Kingsbury, 1982, McKnight *et al.*, 1984, Eisenberg *et al.*, 1985, Jones *et al.*, 1985). The GC boxes at -78 and -108 of the ALV-S gene are very similar to those of the herpes simplex virus thymidine kinase gene, both in their sequence and spatial

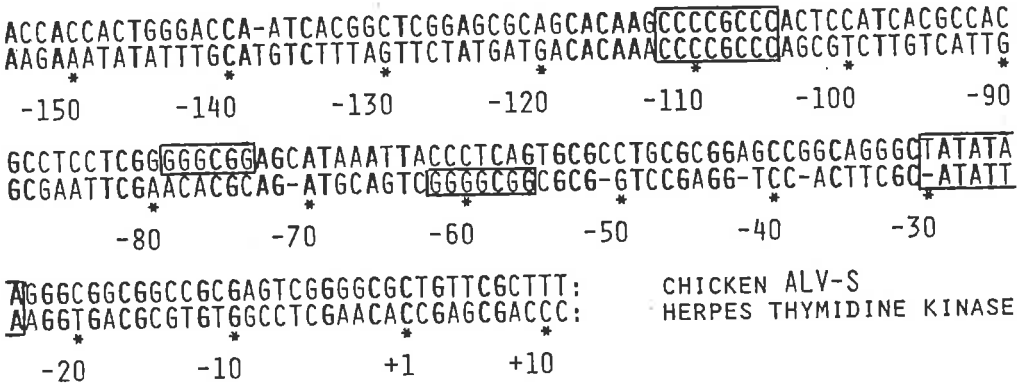
configuration (see Fig. 3-7). In the herpes simplex virus thymidine kinase gene, both GC boxes contribute to expression in oocytes and both function in a mutually dependent manner, although the dependence is not strictly equivalent (McKnight, 1982). This is clearly not the case with the ALV-S gene.

From the data of Kadonaga *et al.* (1986), the GC boxes at -78 and -108 of the ALV-S gene could be considered high affinity binding sites for the transcriptional factor Sp1, whilst the GC boxes of the herpes simplex virus thymidine kinase gene are of medium and low affinity. McKnight *et al.* (1981) has proposed that the strong distal GC box of the herpes simplex virus thymidine kinase gene is dependent on the weaker proximal GC box only because of the formers greater distance from the TATA box. When the herpes simplex virus thymidine kinase distal GC box is interchanged with the proximal GC box, the original proximal GC box now ceases to contribute to expression. It has also been suggested by Kadonaga *et al.* (1986) that the GC box proximal to the 5' side of the TATA box is the most important in genes in which there are multiple Sp1 binding sites present. Here data is presented to support the idea of McKnight *et al.* (1984), that the efficiency of a GC box is dependent on its position in the 5' flanking region of the gene. It is proposed that in the ALV-S gene, the GC box at position -78, because of its proximal position to the TATA box, and because it is most likely a strong GC box (in terms of binding Sp1), is the only GC box that is required for ALV-S expression. The distal GC box at -108 does not function as it may be too far upstream from the TATA box to exert any detectable effect on its own. The GC box at -20 of the ALV-S gene does not function, possibly due to its close proximity to the TATA box and the site of transcriptional initiation.

The ALV-S 5' flanking region contains TATA box like sequences at positions -28 and -68. Alteration of the TATA box sequence at position -68 did not affect either the accuracy or efficiency of ALV-S transcription, whereas alteration of the TATA box at position -28 completely abolished ALV-S transcription. Thus the TATA box at -20 is essential for ALV-S transcription, and cannot be functionally substituted for by other

Figure 3-7.

Nucleotide sequence comparison of the chicken ALV-S and the herpes simplex virus thymidine kinase 5' flanking regions. The optimal alignment of the DNA sequences was performed by computer (NIH molecular biology package, SEQH program) and base matches are indicated in bold print. Sequence elements discussed in the text are boxed. Numbers indicate the nucleotide position of the bp in the ALV-S gene relative to the transcriptional start site (+1).


ACCACCACTGGGACCA-ATCACGGCTCGGAGCGCAGCACAAGCCCCGCCCACTCCATCACGCCAC
AAGAAATATATTTGCATGTCTTTAGTTCTATGATGACACAAACCCCGCCCAGCGTCTTGTCATTG
-150
-140
-130
-120
-110
-100
-90
GCCTCCTCGGGGGCGGAGCATAAATTACCCTCAGTGCGCCTGCGCGGAGCCGGCAGGGCTATATA
GCGAATTCGAACACGCAG-ATGCAGTCGGGGCGGCGCG-GTCCGAGG-TCC-ACTTCGC-ATATT
-80
-70
-60
-50
-40
-30
AGGGCGGCGGCCGCGAGTCGGGGCGCTGTTCGCTTT:
AAGGTGACGCGTGTGGCCTCGAACACCGAGCGACCC:
CHICKEN ALV-S
HERPES THYMIDINE KINASE
-20
-10
+1
+10

sequence elements as shown by Benoist and Chambon (1981) for the SV 40 early promoter.

It is of interest to note that the ALV-S gene is a housekeeping gene which strictly requires a TATA box for transcriptional initiation, whereas most housekeeping genes do not have TATA boxes and initiate transcription from multiple start sites (McGrogan *et al.*, 1985, Reynolds *et al.*, 1985, Singer-Sam *et al.*, 1985). ALV-S is the only housekeeping gene known to have a strict requirement for a TATA box. One other housekeeping gene has been found that has a TATA box and a GC rich 5' flanking region and that is human triose phosphate isomerase (Brown *et al.*, 1985). At this stage the significance of this is unknown.

Many of the sequences examined in this study do not contribute to expression of the ALV-S gene in oocytes, although the work described here does not eliminate the possibility that they could be used in transcription of the ALV-S gene in other expression systems. Our results suggest that caution is advised in assuming that putative *cis*-acting sequences found in the 5' flanking regions of many eukaryotic genes contribute to the expression of those genes.

It is not known why there is variation in the levels of control expression (p7AT).

CHAPTER 4

ATTEMPTED HEME REPRESSION OF CHICKEN ALV-S CONSTRUCTIONS IN *XENOPUS LAEVIS* OOCYTES

Chapter 4: Attempted heme repression of chicken ALV-S constructions in *Xenopus laevis* oocytes

4-1 Introduction

The mechanism of transcriptional control by which heme regulates the synthesis of ALV-S mRNA is of particular interest since most of the well characterised eukaryotic regulatory mechanisms involve positive hormonal or developmental control and very few examples of end-product negative repression have been examined.

The early work of Granick (1966) demonstrated that ALV-S was inducible by drugs and that this induction is prevented by heme. Recent work in our laboratory utilising chicken and rat ALV-S cDNA clones has shown that heme modulates the levels of ALV-S mRNA (Maguire, 1987, Srivastava *et al.*, 1988). At the time the work described in this chapter was started, nuclear run-on experiments had not been started, but subsequent experiments by Maguire (1987) and Srivastava *et al.* (1988) confirmed that the reduction in ALV-S mRNA levels in chick embryo and rat liver was a result of lowered ALV-S mRNA synthesis.

The studies described in the previous chapter defined the roles of potential *cis*-acting sequences in the constitutive expression of the chicken ALV-S gene in the Xenopus oocyte. Transient expression studies in which the chicken ALV-S 5' flanking region was attached to the CAT reporter gene and introduced into mammalian cell lines were pursued by other members of the research group (Day, 1988). However it should be noted that no chicken hepatoma cell lines exist, as far as is known. Therefore it seemed reasonable to examine the expression of the chicken ALV-S gene in Xenopus oocytes in an attempt to understand heme repression.

4-2 Results

Initial experiments were performed to see whether endogenous oocyte ALV-S activity could be detected by the radiochemical assay and then to determine if the oocyte ALV-S was subject to heme repression.

4-2-1 *Xenopus laevis* oocyte ALV-S

Several ovarian lobes were removed from 5 female *Xenopus laevis* toads and the oocytes separated as described in section 2-4-1. 100 oocytes from each toad were homogenised, their mitochondria isolated and assayed for ALV-S activity as outlined in section 2-2-1. As can be seen in Figure 4-1, ALV-S enzyme activity was detected at varying levels in each toad examined.

4-2-2 Succinyl acetone induction of oocyte ALV-S

It is believed that induction of ALV-S is brought about by lowering the level of cellular heme and consequent de-repression of the ALV-S gene. Succinyl acetone, an inhibitor of ALV dehydratase is known to induce ALV-S presumably because it inhibits heme biosynthesis (Schoenfeld *et al.*, 1982). In this set of experiments, ovarian lobes were removed from a single *Xenopus laevis* toad and the oocytes separated. 100 oocytes were then incubated in Barth's saline containing increasing concentrations of succinyl acetone (0-1000 µg/ml), for 12 hours at 18-21°C. Mitochondria were then isolated and assayed for ALV-S activity. It was found that oocyte ALV-S was inducible with succinyl acetone and that the highest levels of induction, a 3 fold increase, occurred at a succinyl acetone concentration of 250 µg/ml (Figure 4-2). This induction was found to be reproducible in oocytes from a number of toads.

Levulinic acid also inhibits ALV-dehydratase and when used in conjunction with succinyl acetone in treating monolayers of chick embryo liver cells has a synergistic effect on the induction of ALV-S (Schoenfeld *et al.*, 1982). Oocytes were isolated from one toad and incubated at 18-21°C for 12 hours in Barth's saline containing 250 µg/ml succinyl acetone plus increasing concentrations of levulinic acid (5-25 mmol/l). At the end of this period, oocyte mitochondria were isolated and assayed for ALV-S activity. The results shown in Figure 4-3 indicate that in oocytes, levulinic acid does not enhance the induction of ALV-S by succinyl acetone; it in fact appears to reduce the level of ALV-S activity. This result was reproducible in oocytes from a number of toads.

Figure 4-1.

ALV-S activity was measured in oocyte mitochondria from 5 different individual *Xenopus laevis* toads. In each case 100 oocytes were homogenised and the mitochondria isolated and assayed for ALV-S activity as in section 2-2-1. Each toad is referred to by a letter, A-E.

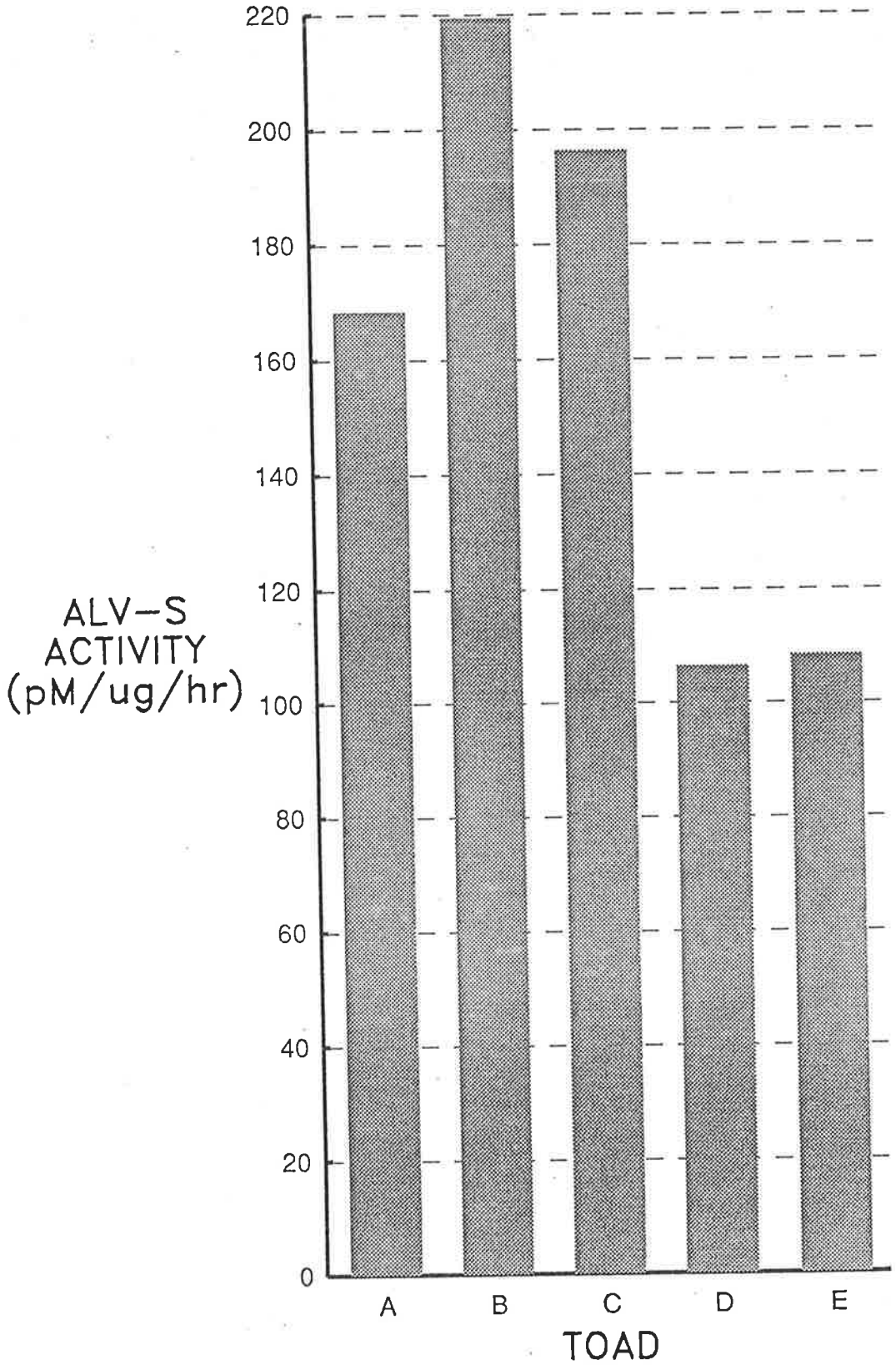

220
200
180
160
140
120
100
80
60
40
20
0
ALV–S
ACTIVITY
(pM/ug/hr)
A
B
C
D
E
TOAD

Figure 4-2.

The response of *Xenopus laevis* oocyte ALV-S activity to increasing concentrations of succinyl acetone. Oocytes were isolated from a single toad and six groups of 100 oocytes incubated in Barth's saline containing increasing concentrations of succinyl acetone for 12 hours at 18°C. At the end of this period mitochondria was isolated and assayed for ALV-S activity as in section 2-2-1. The concentration of succinyl acetone that each group of oocytes were subjected to is shown along the horizontal axis.

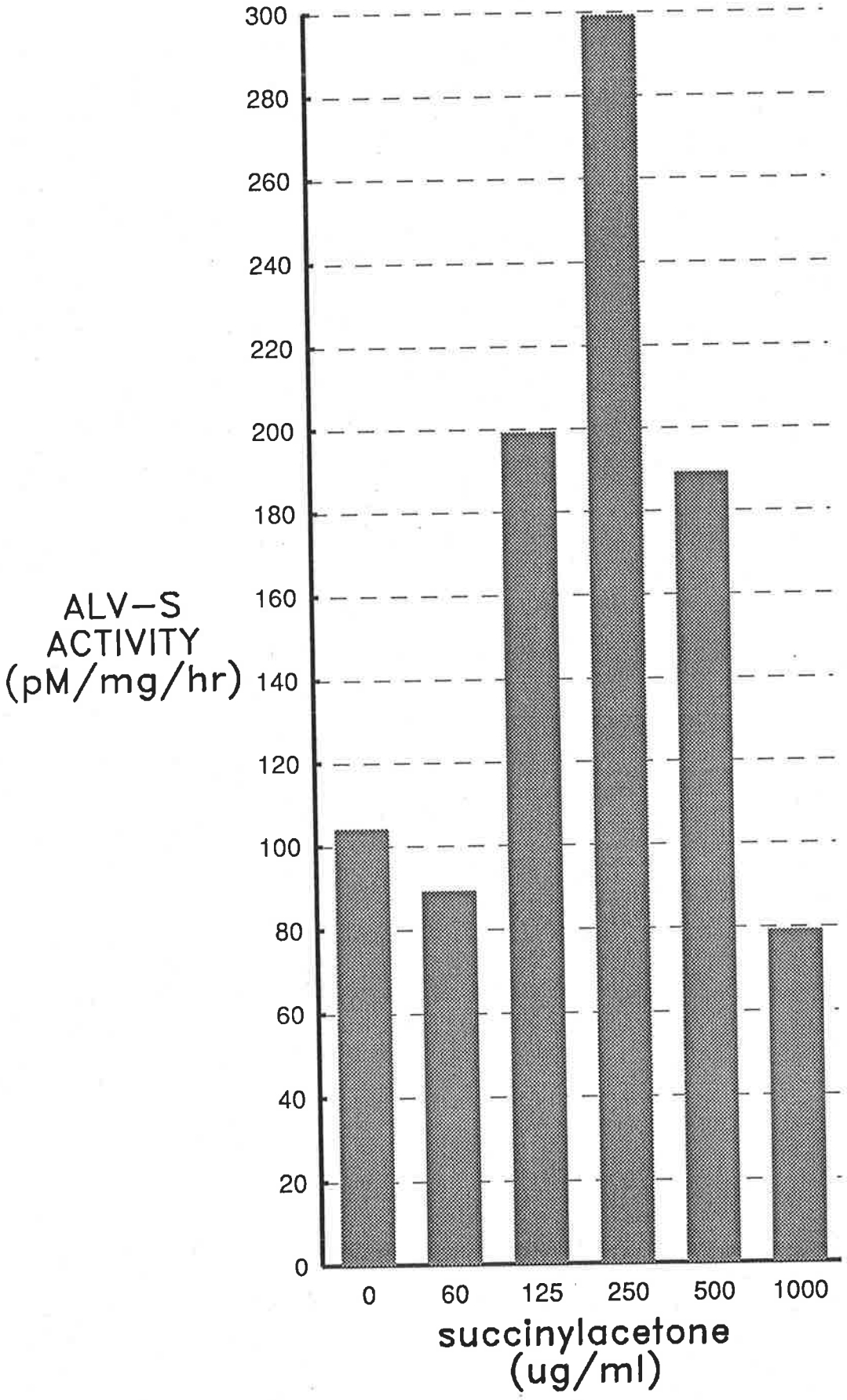
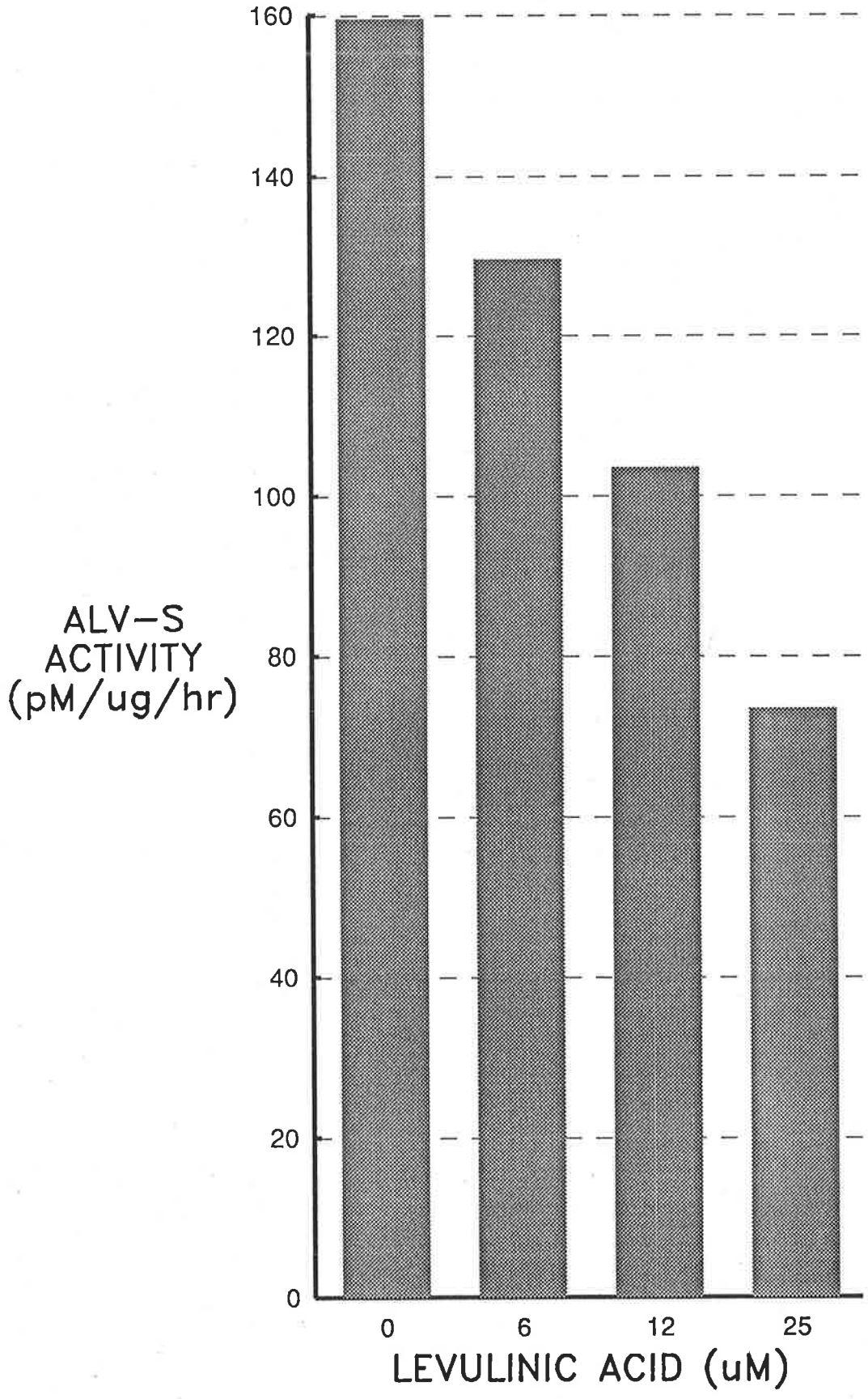

ALV–S
ACTIVITY
(pM/mg/hr)
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
60
125
250
500
1000
succinylacetone
(ug/ml)

Figure 4-3.

The response of *Xenopus laevis* oocyte ALV-S activity to succinyl acetone plus increasing concentrations of levulinic acid. Oocytes were isolated from a single toad and four groups of 100 oocytes incubated in Barth's saline containing 250 μg/ml succinyl acetone plus increasing concentrations of levulinic acid for 12 hours at 18°C. At the end of this period mitochondria were isolated and assayed for ALV-S activity as in section 2-2-1. The concentration of levulinic acid that each group of oocytes was subjected to is shown along the horizontal axis.


160
140
120
100
80
60
40
20
0
ALV–S
ACTIVITY
(pM/ug/hr)
0
6
12
25
LEVULINIC ACID (uM)

4-2-3 Attempted induction of oocyte ALV-S by AIA

High levels of ALV-S induction can be achieved in chick embryos and rats with AIA, which is believed to reduce the level of the heme pool by inducing hepatic cytochrome P-450 synthesis. It also destroys the heme moiety of cytochrome P-450's (May *et al.*, 1986). Experiments were performed to examine whether this drug induces ALV-S in Xenopus oocytes.

Oocytes were isolated from one toad and incubated in Barth's saline containing increasing concentrations of AIA (0-1000 μg/ml) at 18-21°C for 12 hours. At the end of this period oocyte mitochondria were isolated and ALV-S activity assayed. It was found that AIA did not induce ALV-S enzyme activity at any of the concentrations examined (Figure 4-4). No induction of ALV-S by AIA was found in any of the oocytes examined from different toads.

In isolated embryonic chick liver cells, AIA induction of ALV-S is dependent on the presence of dibutyryl-cAMP (Srivastava *et al.*, 1979). Isolated oocytes were incubated in Barth's saline containing 500 μg/ml AIA and increasing concentrations of dibutyryl-cAMP for 12 hours at 18-21°C. Again oocyte mitochondria were isolated and assayed for ALV-S activity. The data in Figure 4-5 indicates that in oocytes, dibutyryl-cAMP does not enable AIA to induce ALV-S, at least at the concentrations examined. This result was reproducible in oocytes from different toads.

4-2-4 Repression of oocyte ALV-S by ALV

Both basal and induced levels of ALV-S can be repressed in rat, mouse and chick embryo by administration of low concentrations of either heme or the heme precursor ALV (Srivastava *et al.*, 1980, Srivastava *et al.*, 1980a, Anderson *et al.*, 1981). In the following experiments the effect of ALV on Xenopus oocyte ALV-S was investigated.

Oocytes were isolated from one toad and incubated in Barth's saline containing 50μM ALV (Anderson *et al.*, 1981) for 12 hours at 18-21°C. At the end of this period

Figure 4-4.

The response of *Xenopus laevis* oocyte ALV-S activity to treatment with increasing concentrations of AIA. Oocytes were isolated from a single toad and six groups of 100 oocytes incubated in Barth's saline containing increasing concentrations of AIA for 12 hours at 18°C. At the end of this period mitochondria were isolated and assayed for ALV-S activity as in section 2-2-1. The concentration of AIA that each group of oocytes was subjected to is shown along the horizontal axis.

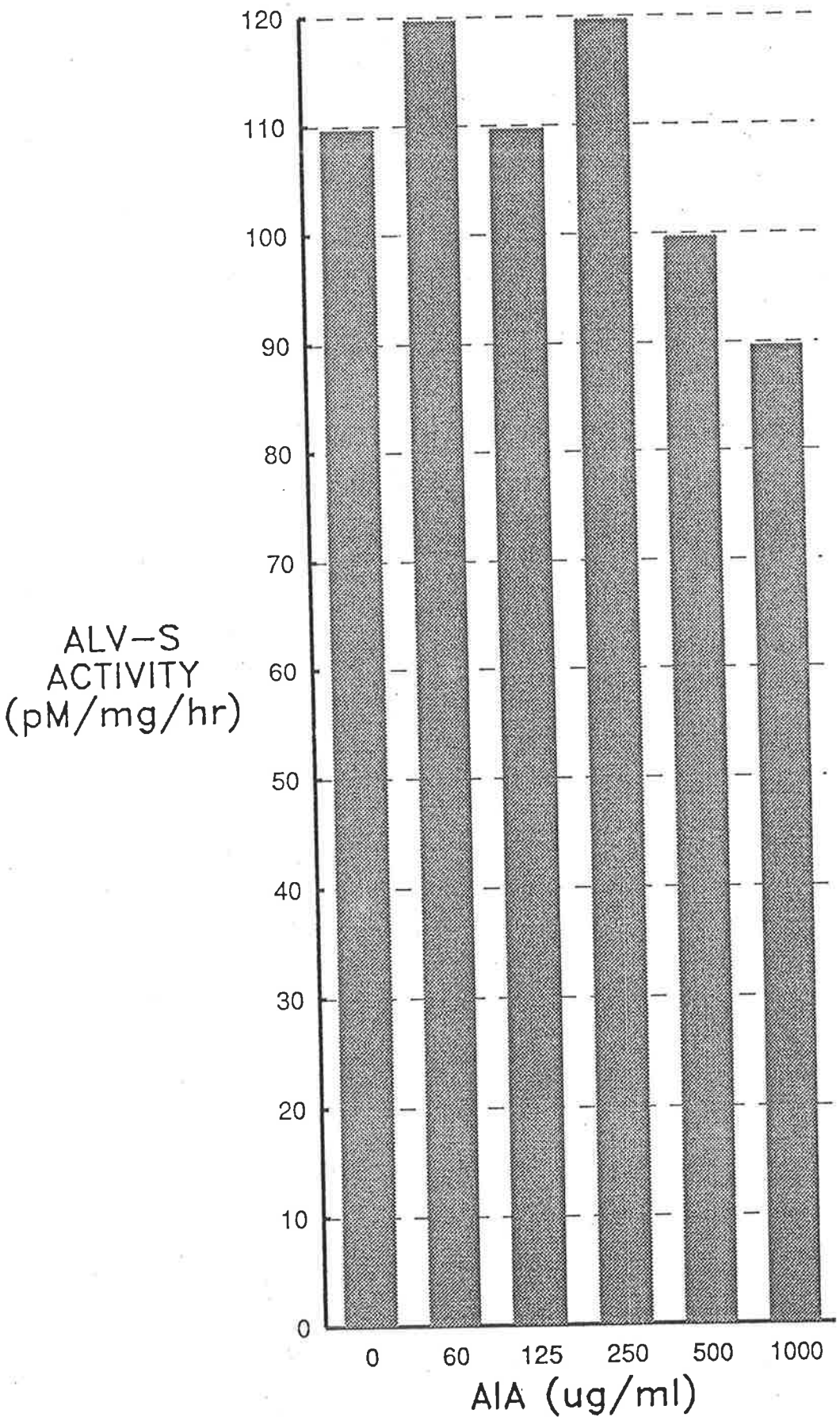
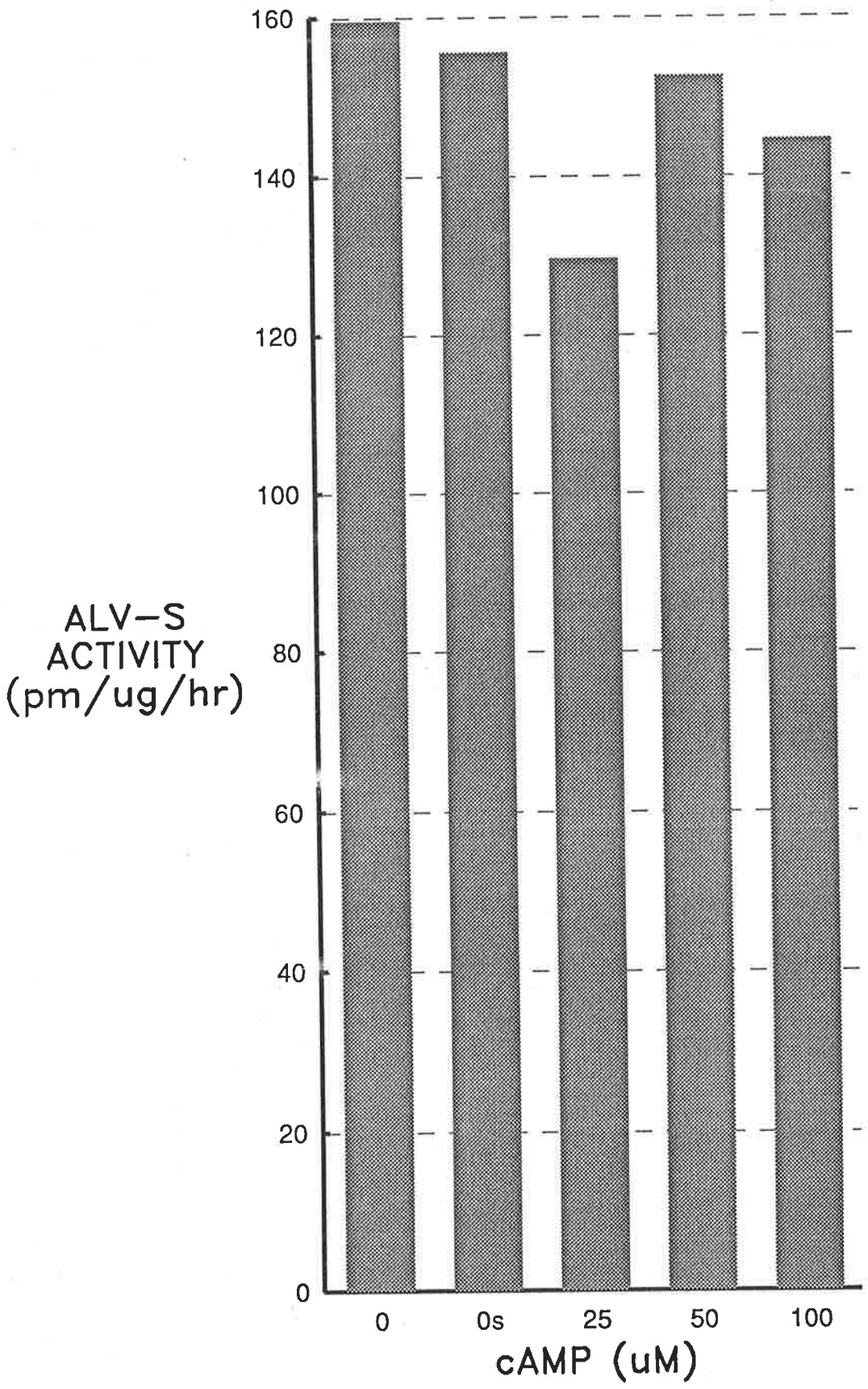

ALV-S
ACTIVITY
(pM/mg/hr)
120
110
100
90
80
70
60
50
40
30
20
10
0
0
60
125
250
500
1000
AIA (ug/ml)

Figure 4-5.

The response of *Xenopus laevis* oocyte ALV-S activity to treatment with AIA plus increasing concentrations of dibutyryl-cAMP. Oocytes were isolated from a single toad and five groups of 100 oocytes incubated in Barth's saline containing 500 μg/ml AIA containing increasing concentrations of dibutyryl-cAMP for 12 hours at 18°C. At the end of this period mitochondria were isolated and assayed for ALV-S activity as in section 2-2-1. The concentration of dibutyryl-cAMP that each group of oocytes was subjected to is shown along the horizontal axis.


160
140
120
100
80
60
40
20
0
ALV–S
ACTIVITY
(pm/ug/hr)
0
0s
25
50
100
cAMP (uM)

oocyte mitochondria were isolated and assayed for ALV-S activity. As shown in Figure 4-6, the basal level of ALV-S enzyme activity was repressed 10 fold in oocytes. This repression was reproducible in all the toads examined.

100 oocytes from one toad were incubated in Barth's saline containing 250 µg/ml succinyl acetone plus 50 M ALV for 12 hours at 18-21°C. At the end of this period, oocyte mitochondria were isolated and assayed for ALV-S activity. Figure 4-6 shows that ALV reduced the level of ALV-S activity in succinyl acetone treated oocytes by 30 fold.

4-2-5 Total protein synthesis in the *Xenopus laevis* oocyte

The possibility existed that the induction of ALV-S by succinyl acetone could be a general response of cellular protein synthesis, or that repression by ALV could be due to toxicity. To investigate this possibility, the level of total protein synthesis in oocytes was measured (see section 2-2-2). Oocytes from an individual toad were incubated under identical conditions of ALV-S induction and repression to those used in sections 4-2-2, 4-2-3 and 4-2-4, except that in each incubation 15µCi L-(^{35}S)-methionine was included. At the end of each incubation the oocytes were homogenised, the protein precipitated with trichloroacetic acid and the amount of protein synthesis quantified by liquid scintillation spectroscopy.

Figure 4-7 shows that the level of total protein synthesis in untreated oocytes and in oocytes treated with ALV, succinyl acetone, or AIA, were approximately the same.

4-2-6 Northern blot hybridisation analysis of *Xenopus laevis* oocyte ALV-S

A northern blot hybridisation analysis of oocyte RNA was undertaken to determine if the effects of succinyl acetone and ALV on oocyte ALV-S were at the transcriptional level.

Figure 4-6.

The response of *Xenopus laevis* oocyte ALV-S activity to treatment with ALV. Oocytes were isolated from a single toad and four groups of 100 oocytes were incubated in Barth's saline (0), or Barth's saline containing 50μM ALV (50), or Barth's saline containing 250 μg/ml succinyl acetone (0+succ), or Barth's saline containing 250 μg/ml succinyl acetone and 50μM ALV (50+succ) for 12 hours at 18°C. At the end of this period mitochondria were isolated and assayed for ALV-S activity as in section 2-2-1.

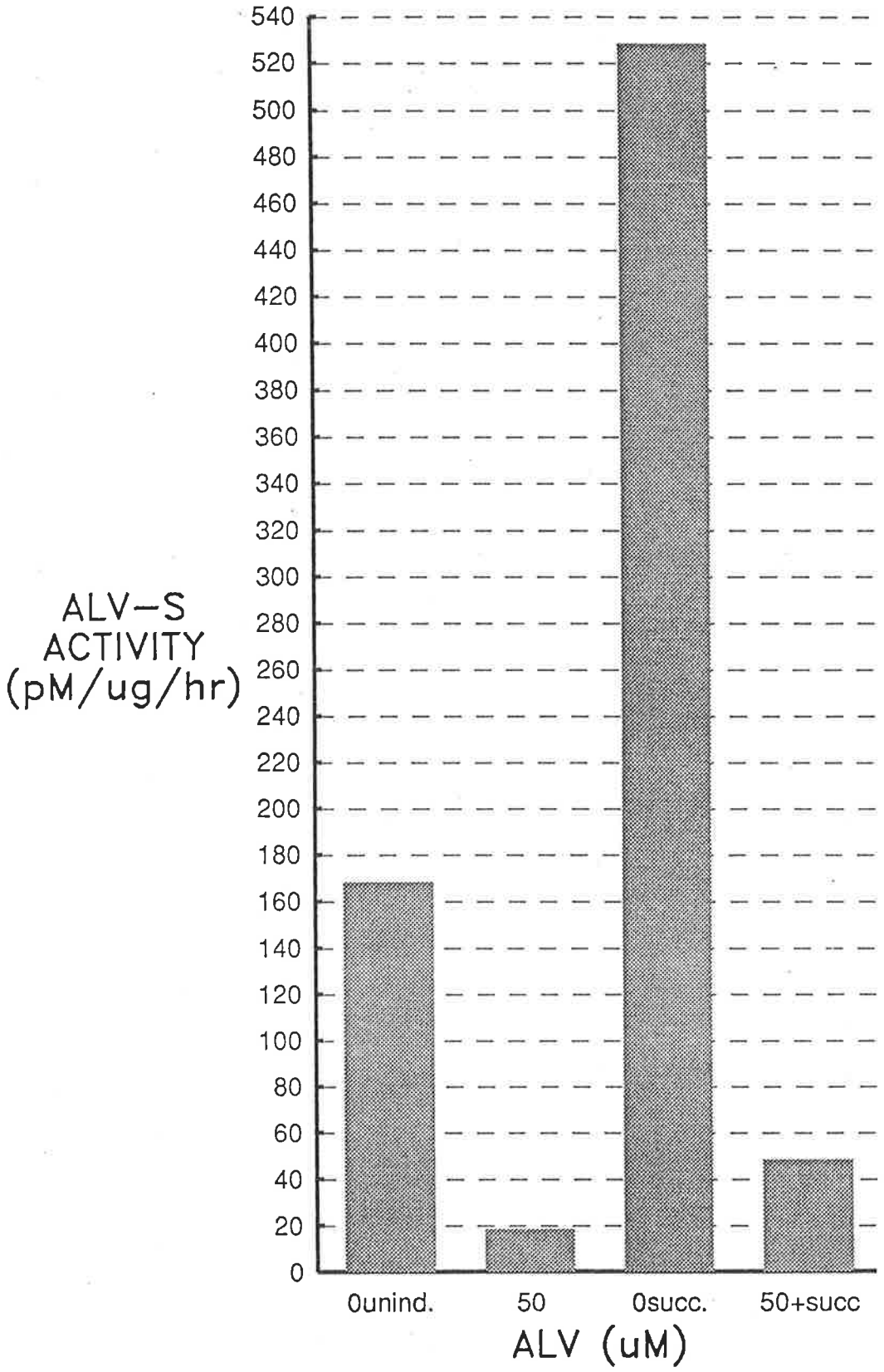

ALV–S
ACTIVITY
(pM/ug/hr)
540
520
500
480
460
440
420
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0unind.
50
0succ.
50+succ
ALV (uM)

Figure 4-7.

A measure of total protein synthesis in *Xenopus laevis* oocytes. Total protein synthesis was measured in groups of 10 oocytes isolated from the one toad as outlined in section 2-2-2. The level of protein synthesis is shown along the vertical axis as cpm of incorporated ^{35}S-methionine/µg total oocyte protein. Along the horizontal axis the various oocyte treatments are listed;

unind: 10 oocytes incubated in Barth's saline.

250 succ: 10 oocytes incubated in Barth's saline containing 250 µg/ml succinylacetone.

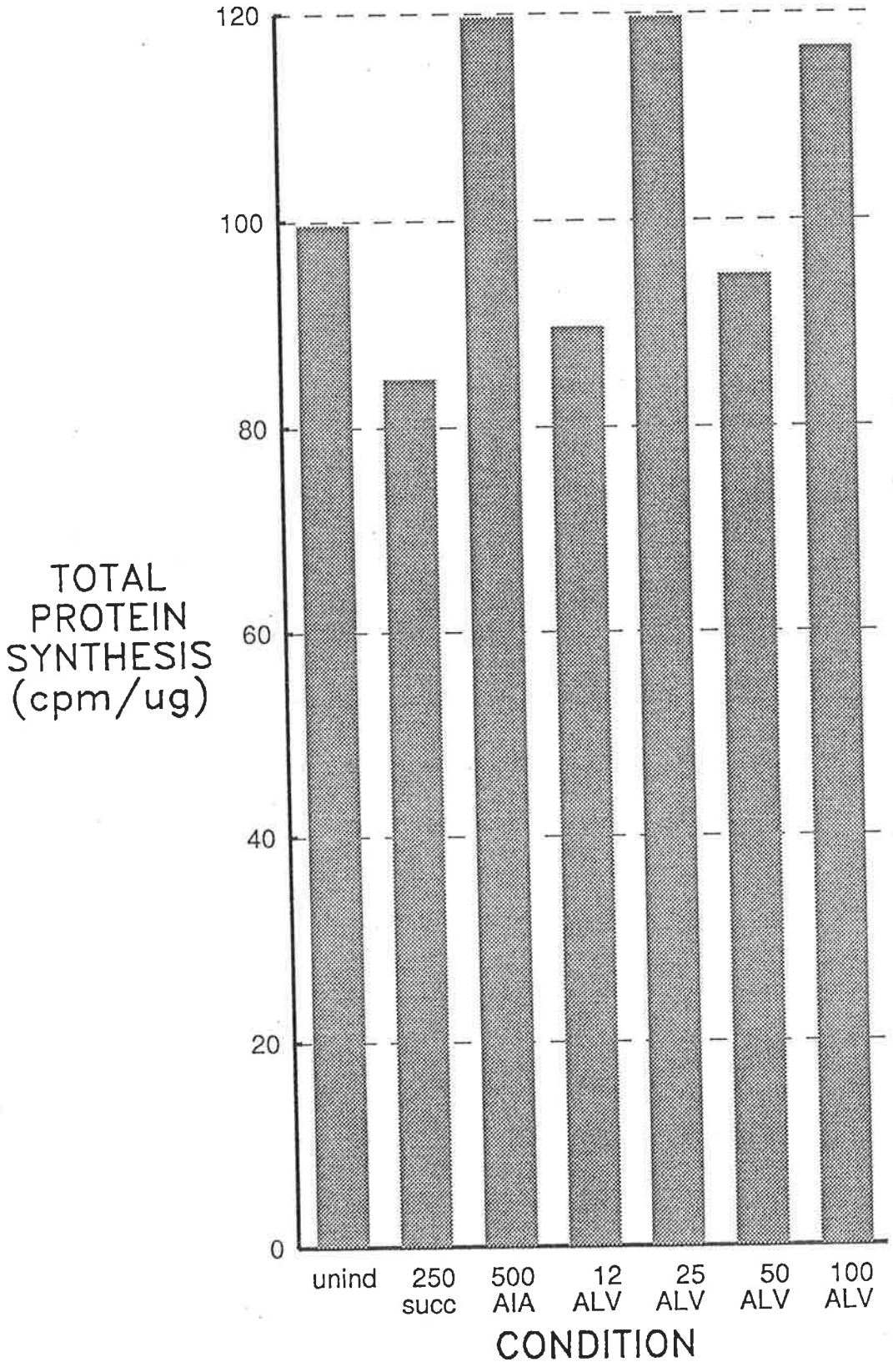
500 AIA: 10 oocytes incubated in Barth's saline containing 500 µg/ml AIA.

12 ALV: 10 oocytes incubated in Barth's saline containing 12µM ALV.

25 ALV: 10 oocytes incubated in Barth's saline containing 25µM ALV.

50 ALV: 10 oocytes incubated in Barth's saline containing 50µM ALV.

100 ALV: 10 oocytes incubated in Barth's saline containing 100µM ALV.


120
100
80
60
40
20
0
TOTAL PROTEIN SYNTHESIS (cpm/ug)
unind
250 succ
500 AIA
12 ALV
25 ALV
50 ALV
100 ALV
CONDITION

100 oocytes from an individual toad were incubated in Barth's saline, or Barth's saline containing either 250 µg/ml succinyl acetone or 50 µmol/l ALV for 24 hours at 18oC. At the end of this period total oocyte RNA was isolated by either the Kressman (see section 2-4-2) or the guanidine hydrochloride method (Srivastava *et al.*, 1988) and from this RNA, poly(A)+ RNA was isolated by chromatography on oligo-dT cellulose (Maniatis *et al.*, 1986).

Northern blot hybridisation analysis of 25µg oocyte total RNA and 25µg of oocyte poly(A)+ RNA from each experiment, was performed essentially as in Maniatis *et al.*, (1982) and probed with a full length chicken ALV-S cDNA clone, p105B1 (Borthwick *et al.*, 1985), or an almost full length rat ALV-S cDNA clone, p101B1 (Srivastava *et al.*, 1988). Unfortunately oocyte ALV-S mRNA could not be detected under any of the conditions used, although the control chicken ALV-S (1µg chick embryo poly (A)+ RNA was always detected (data not shown). Numerous attempts were made to detect oocyte ALV-S mRNA using the two different methods of RNA isolation, listed above, or by utilising either the chicken or rat ALV-S cDNA clones as hybridisation probes, or by reducing both the hybridisation and washing stringencies. Oocyte ALV-S could not be detected under any of the conditions tried.

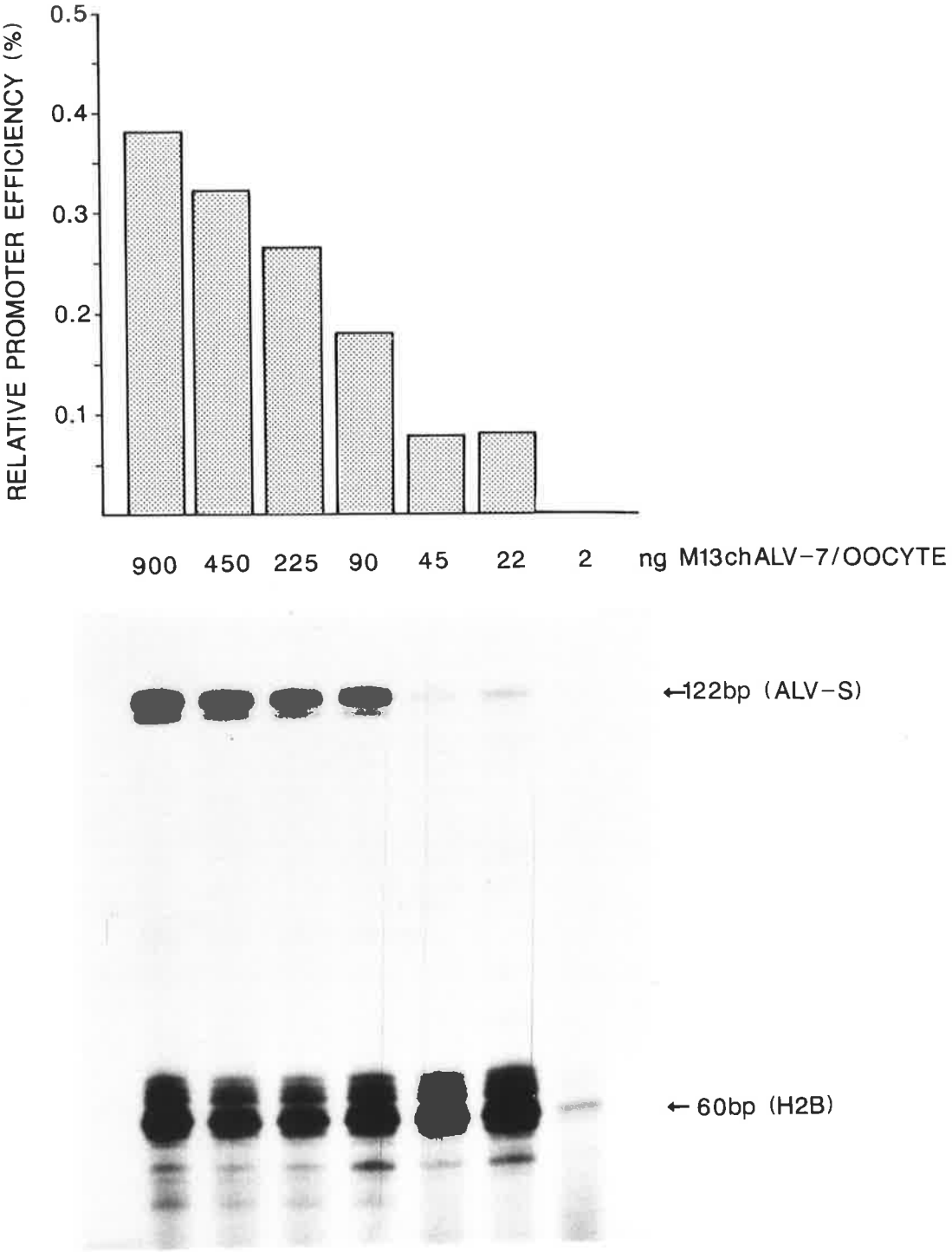
4-2-7 Expression of M13chALV-7 in Xenopus oocytes

M13chALV-7 is an ALV-S minigene containing 1.5Kb of 5' flanking region. The construction of M13chALV-7 is outlined in section 3-2-1. M13chALV-7 was co-injected with the internal control plasmid p7AT (see section 3-2-1) into the nucleus of Xenopus oocytes and the oocytes incubated in Barth's saline for 12 hours. At the end of this period, RNA was isolated as outlined in section 2-4-2 and examined by primer extension analysis (see section 2-4-3) and laser densitometry.

Initially M13chALV-7 was injected at a range of concentrations (2-900ng M13chALV-7/oocyte) to determine the lowest number of M13chALV-7 templates that could be injected and give detectable expression. As shown in Figure 4-8, the amount of RNA synthesised from the M13chALV-7 construction was proportional to the

Figure 4-8.

Primer extension analysis of RNA transcribed in Xenopus oocytes from decreasing amounts of the ALV-S construction M13chALV-7. Xenopus oocyte nuclei were co-injected with 400 ng/oocyte of the internal control p7AT together with decreasing amounts of M13chALV-7 as indicated (ng/oocyte). Primer extension analysis was performed on isolated RNA as in section 2-4-3. 30 oocytes from the one toad were injected for each M13chALV-7 concentration examined. The lower portion of the figure shows an autoradiogram of the primer extended products from both M13chALV-7 (ALV-S) and the internal control plasmid p7AT (H2B). The length and identity of each extension product is indicated. The upper portion of the figure shows the level of ALV-S expression as a percentage of the level of p7AT expression.


RELATIVE PROMOTER EFFICIENCY (%)
0.5
0.4
0.3
0.2
0.1
900 450 225 90 45 22 2 ng M13chALV-7/OOCYTE
←122bp (ALV-S)
← 60bp (H2B)

number of templates injected, and the lowest amount of M13chALV-7 DNA that could be injected and give detectable expression was found to be 22ng M13chALV-7/oocyte.

4-2-8 Expression of M13chALV-7 in heme repressed and succinyl acetone induced Xenopus oocytes

Having established conditions under which the Xenopus oocyte endogenous ALV-S could be induced and repressed, these conditions were then used to examine the expression of M13chALV-7 in induced and repressed oocytes.

M13chALV-7 was co-injected into oocyte nuclei at two concentrations together with p7AT. The two concentrations used were 90ng and 22 ng/oocyte. After injection oocytes were incubated for 12 hours in Barth's saline, or Barth's saline containing 250 μg/ml succinyl acetone, or Barth's saline containing 50μM ALV. At the end of this period, RNA was isolated and analysed by primer extension. As shown in Figure 4-9, the level of M13chALV-7 expression relative to the level of p7AT was approximately the same in uninduced, succinyl acetone induced and ALV repressed oocytes.

As no significant difference was observed in the expression of M13chALV-7 between any of the conditions tried (over numerous repeats of this experiment) oocytes were injected which had been pre-treated for 12 hours in Barth's saline containing either 250 μg/ml succinyl acetone, or 50μM ALV. After injection the oocytes were incubated in fresh media for 12 hours at 37°C before RNA was isolated and analysed by primer extension. This experiment was repeated numerous times and in none of these experiments was any difference in the relative expression of M13chALV-7 observed between the different conditions studied (Fig 4-10).

In a final attempt to obtain heme repression of injected M13chALV-7, increasing concentrations of heme (prepared as in Ross and Sautner, 1976) were co-injected with the M13chALV-7 and p7AT constructions. Heme was co-injected because it is not very soluble in water and may not be absorbed by the oocyte. Oocytes were incubated for 12 hours in either Barth's saline, or Barth's saline containing 50μM ALV. 22 ng/oocyte M13chALV-7 was mixed with increasing concentrations of heme

Figure 4-9.

Primer extension analysis of RNA transcribed in untreated, succinyl acetone induced and ALV repressed Xenopus oocytes from the ALV-S construction M13chALV-7. Xenopus oocyte nuclei were injected with 400 ng/oocyte of the internal control p7AT and either 22 or 90 ng/oocyte of M13chALV-7 as indicated. 30 oocytes from the one toad were injected for each treatment examined. After injection, oocytes were incubated for 12 hours in either Barth's saline, Barth's saline containing 250 µg/ml succinylacetone or Barth's saline containing 50µM ALV. At the end of this period, primer extension analysis was performed on isolated RNA as in section 2-4-3. The lower portion of the figure shows an autoradiogram of the primer extended products from both M13chALV-7 (ALV-S) and the internal control plasmid p7AT (H2B). The length and identity of each extension product is indicated. The upper portion of the figure shows the level of ALV-S expression as a percentage of the level of p7AT expression.

UI90: Oocytes injected with 90 ng/oocyte M13chALV-7 and subsequently incubated in Barth's saline.

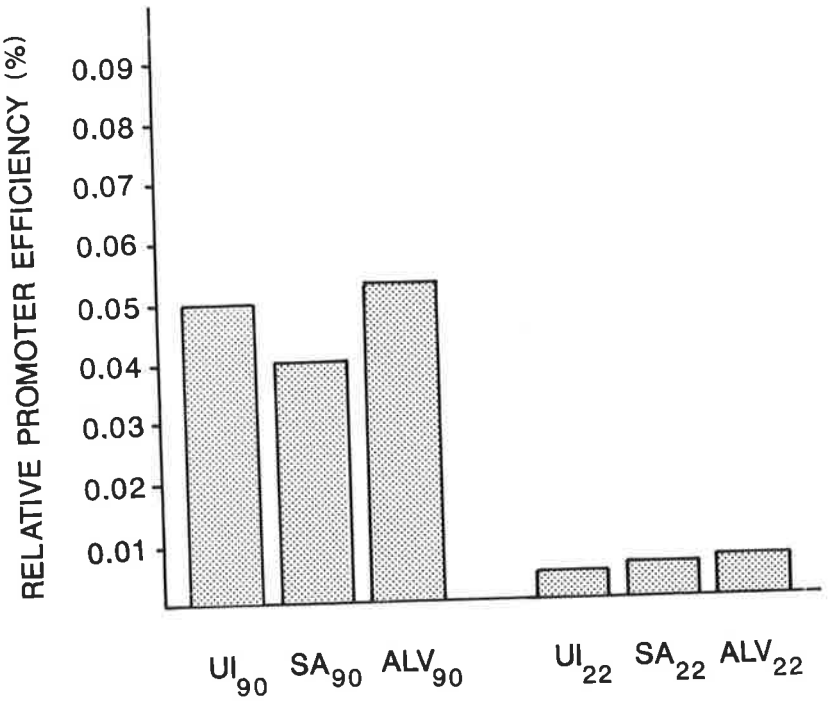
UI22: Oocytes injected with 22 ng/oocyte M13chALV-7 and subsequently incubated in Barth's saline.

SA90: Oocytes injected with 90 ng/oocyte M13chALV-7 and subsequently incubated in Barth's saline containing 250 µg/ml succinyl acetone.

SA22: Oocytes injected with 22 ng/oocyte M13chALV-7 and subsequently incubated in Barth's saline containing 250 µg/ml succinyl acetone.

ALV90: Oocytes injected with 90 ng/oocyte M13chALV-7 and subsequently incubated in Barth's saline containing 50µM ALV.

ALV22: Oocytes injected with 22 ng/oocyte M13chALV-7 and subsequently incubated in Barth's saline containing 50µM ALV.

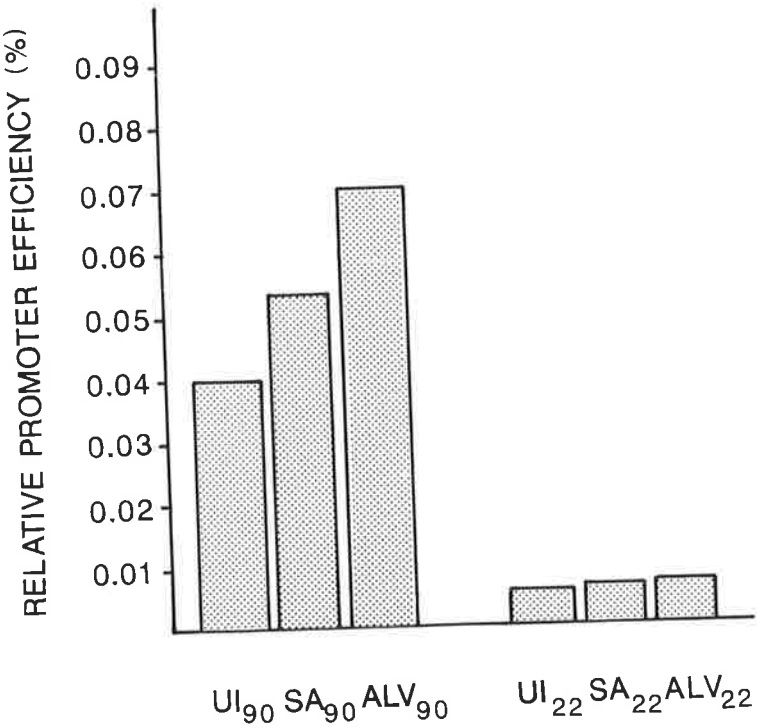

RELATIVE PROMOTER EFFICIENCY (%)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
UI90
SA90
ALV90
UI22
SA22
ALV22


←122bp (ALV-S)
←60bp (H2B)

Figure 4-10.

Primer extension analysis of RNA transcribed in Xenopus oocytes (pretreated for 12 hours prior to injection in either Barth's saline, Barth's saline containing 250 μg/ml succinylacetone or Barth's saline containing 50μM ALV) from the ALV-S construction M13chALV-7. Xenopus oocytes were pretreated as above and then their nuclei co-injected with 400 ng/oocyte of the internal control p7AT and either 22 or 90 ng/oocyte of M13chALV-7 as indicated. After injection the oocytes were incubated for a further 12 hours in either Barth's saline, Barth's saline containing 250 μg/ml succinylacetone or Barth's saline containing 50μM ALV. At the end of this period, primer extension analysis was performed on isolated RNA as in section 2-4-3. The lower portion of the figure shows an autoradiogram of the primer extended products from both M13chALV-7 (ALV-S) and the internal control plasmid p7AT (H2B). The length and identity of each extension product is indicated. The upper portion of the figure shows the level of ALV-S expression as a percentage of the level of p7AT expression.

UI90: Oocytes injected with 90 ng/oocyte M13chALV-7 and subsequently incubated in Barth's saline.
UI22: Oocytes injected with 22 ng/oocyte M13chALV-7 and subsequently incubated in Barth's saline.
SA90: Oocytes injected with 90 ng/oocyte M13chALV-7 following 12 hours incubation in 250 μg/ml succinyl acetone and subsequently incubated in Barth's saline containing 250 μg/ml succinyl acetone.
SA22: Oocytes injected with 22 ng/oocyte M13chALV-7 following 12 hours incubation in Barth's saline containing 250 μg/ml succinyl acetone and subsequently incubated in Barth's saline containing 250 μg/ml succinyl acetone.
ALV90: Oocytes injected with 90 ng/oocyte M13chALV-7 following 12 hours incubation in Barth's saline containing 50μM ALV and subsequently incubated in Barth's saline containing 50μM ALV.
ALV22: Oocytes injected with 22 ng/oocyte M13chALV-7 following 12 hours incubation in Barth's saline containing 50μM ALV and subsequently incubated in Barth's saline containing 50μM ALV.


RELATIVE PROMOTER EFFICIENCY (%)
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
UI90 SA90 ALV90
UI22 SA22 ALV22


←122bp (ALV-S)


←60bp (H2B)

(0.5-50μM) and then injected into the nucleus of both untreated and ALV repressed oocytes. Oocytes were then incubated in either Barth's saline or Barth's saline containing 50μM ALV for a further 12 hours before RNA was isolated and analysed by primer extension. As seen in Figure 4-11 no difference in the level of M13chALV-7 expression was observed between the various conditions examined, relative to the internal control.

In all of the experiments described above, duplicate M13chALV-7/p7AT co-injected oocytes were homogenised and assayed by the radiochemical assay (see section 2-2-1) to determine the level of endogenous oocyte ALV-S. In all experiments the level of endogenous oocyte ALV-S was repressed by ALV and induced by succinyl acetone (data not shown).

4-3 Discussion

In the initial studies described in this chapter the effect of a number of agents on *Xenopus laevis* ALV-S was examined to determine if the oocyte ALV-S is regulated by feedback inhibition by heme.

It was first shown that the level of ALV-S activity in the Xenopus oocyte could be measured by the radiochemical assay of Brooker *et al.* (1982). The level of ALV-S activity in the oocyte was variable between oocytes of individual toads. Having established a method for measuring the level of ALV-S in oocytes, a number of experiments were then conducted to determine if the oocyte ALV-S was subject to heme repression.

Succinyl acetone has been shown to induce ALV-S in a wide variety of experimental systems including; malignant murine erythroleukemic cells (Ebert *et al.*, 1981), rabbit reticulocytes (Ponka *et al.*, 1982), human liver and erythrocytes (Lindblad *et al.*, 1977), *Clostridium tetanomorphum* (Brum and Friedmann, 1981) and in monolayers of chick embryo liver cells (Schoefeld *et al.*, 1982). This compound induces ALV-S by inhibiting aminolevulinate dehydratase (Schoenfeld, *et al.*, 1982),

Figure 4-11.

Primer extension analysis of RNA transcribed in untreated, and ALV repressed Xenopus oocytes from the ALV-S construction M13chALV-7. Xenopus oocytes were pretreated in either Barth's saline, or Barth's saline containing 50μM ALV for 12 hours prior to injection. The oocyte nuclei were then injected with 400 ng/oocyte of the internal control p7AT and 22 ng/oocyte of M13chALV-7, or 400 ng/oocyte of the internal control p7AT, 22 ng/oocyte of M13chALV-7 and increasing concentrations of heme (0.5μM, 5μM, or 50μM). These oocytes were incubated for a further 12 hours in either Barth's saline, or Barth's saline containing 50μM ALV and at the end of this period primer extension analysis was performed on isolated RNA as in section 2-4-3. The lower portion of the figure shows an autoradiogram of the primer extended products from both M13chALV-7 (ALV-S) and the internal control plasmid p7AT (H2B). The length and identity of each extension product is indicated. The upper portion of the figure shows the level of ALV-S expression as a percentage of the level of p7AT expression.

UI: Oocytes were co-injected with M13chALV-7 and subsequently incubated in Barth's saline.

0.5: Oocytes were co-injected with M13chALV-7 and 0.5μM heme and subsequently incubated in Barth's saline.

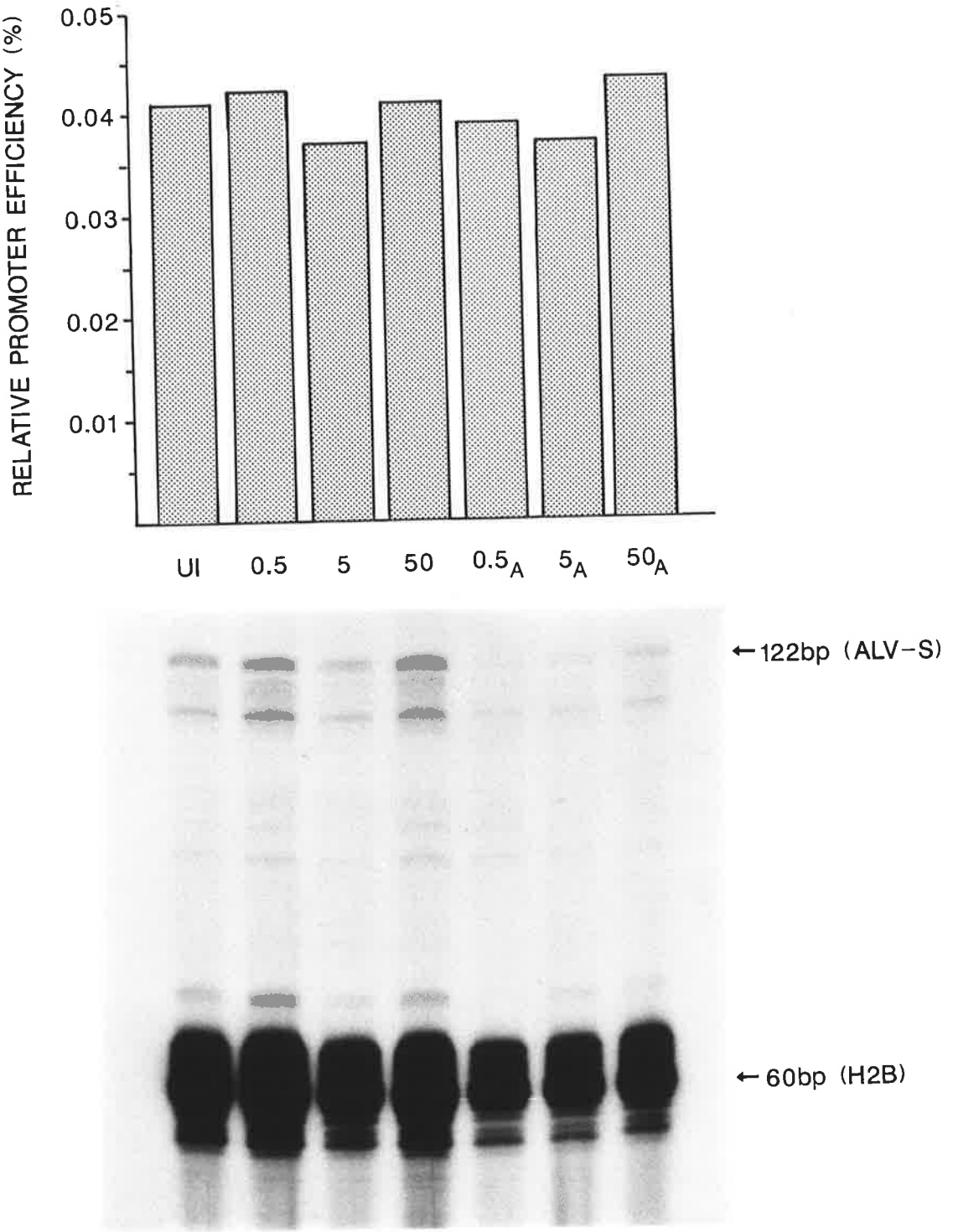
5: Oocytes were co-injected with M13chALV-7 and 5μM heme and subsequently incubated in Barth's saline.

50: Oocytes co-injected with M13chALV-7 and 50μM heme and subsequently incubated in Barth's saline.

0.5_A: Oocytes were co-injected with M13chALV-7 and 0.5μM heme and subsequently incubated in Barth's saline containing 50μM ALV.

5_A: Oocytes were co-injected with M13chALV-7 and 5μM heme and subsequently incubated in Barth's saline containing 50μM ALV.

50_A: Oocytes were co-injected with M13chALV-7 and 50μM heme and subsequently incubated in Barth's saline containing 50μM ALV.


RELATIVE PROMOTER EFFICIENCY (%)
0.05
0.04
0.03
0.02
0.01
UI
0.5
5
50
0.5A
5A
50A
←122bp (ALV-S)
←60bp (H2B)

which leads to a depletion of the regulatory heme pool and consequently to derepression of ALV-S (May *et al.*, 1986).

In Xenopus oocytes it was found that succinyl acetone induced ALV-S optimally at a concentration of 250 μg/ml. This result is consistent with the synthesis of oocyte ALV-S being repressed by heme.

Xenopus oocytes were treated with a combination of levulinic acid and succinyl acetone in an attempt to enhance the ALV-S induction obtained with succinyl acetone alone. Schoenfeld *et al.* (1982) had previously shown that when levulinic acid, which also inhibits aminolevulinate dehydratase, is used in combination with succinyl acetone in treatment of chick embryo liver cells a synergistic induction of ALV-S occurs. In oocytes however, this treatment did not result in enhanced ALV-S induction, but decreased the observed ALV-S enzyme activity.

It is possible that succinyl acetone only partly inhibits the aminolevulinate dehydratase, but sufficiently lowers heme levels to cause derepression of ALV-S. Perhaps complete inhibition of heme biosynthesis obtained in the presence of levulinic acid is damaging to the oocyte, thus reducing ALV-S synthesis. This may explain why concentrations of succinyl acetone higher than 250 μg/ml reduce the observed level of ALV-S activity.

Studies in chicken, rat and mice have shown that the drug induction of ALV-S in liver is probably a secondary consequence of the induction of cytochrome P-450 apoproteins (see section 1-2).

When Xenopus oocytes were treated with increasing concentrations of AIA, no induction of ALV-S was observed even in the presence of dibutyryl-cAMP, which has been shown by Srivastava *et al.* (1979) to be necessary for ALV-S induction in isolated chick embryo liver cells. The failure of AIA to induce ALV-S in the Xenopus oocyte may be due to the fact that the oocyte does not possess a functioning cytochrome P-450 system.

As the endogenous oocyte ALV-S can be induced by depletion of the oocyte heme pool by succinyl acetone treatment, it was then of interest to determine if oocyte

ALV-S could be repressed by increasing the level of heme in the oocyte. The synthesis of ALV-S has been shown to be extremely sensitive to heme. Srivastava *et al.* (1980) have shown that in isolated chick embryo liver cells, heme concentrations of 20-50nM were sufficient to inhibit ALV-S synthesis. ALV, the product of the ALV-S catalysed reaction, can also strongly repress ALV-S synthesis since it is rapidly converted to heme (Srivastava *et al.*, 1979). ALV was chosen in preference to heme for these studies because of its greater water solubility than that of heme. When oocytes were treated with ALV, the observed level of ALV-S activity was dramatically decreased, indicating that the oocyte ALV-S is repressed by heme.

The regulation of hepatic ALV-S levels by heme has been proposed to occur at a number of levels including, feedback inhibition of enzyme activity (Scholnick *et al.*, (1972), translocation of the precursor ALV-S into the mitochondria (Hayashi *et al.*, 1972), translation (Sassa and Granick, 1970, Yamamoto *et al.*, 1983), or at the level transcription (Whiting, 1976). Work in our laboratory using chick embryos (Beckman, 1984) and rats (Srivastava, *et al.*, 1988) has established that heme reduces the level of ALV-S mRNA. Subsequent *in vitro* nuclear run-on experiments in isolated rat and chick embryo liver nuclei have demonstrated that heme specifically inhibits transcription of the ALV-S gene (Maguire, 1987, Srivastava, *et al.*, 1988).

The work presented in this chapter is consistent with the conclusion that oocyte ALV-S is under feedback repression by heme. Confirmation that the repression by heme of oocyte ALV-S is at the transcriptional level could not be achieved as the oocyte ALV-S mRNA could not be detected by northern blot hybridisation analysis, probably due to a very low level of ALV-S mRNA in the oocyte. It is also possible that the chicken and rat cDNA probes did not cross hybridise with the ooctye ALV-S mRNA. However this is somewhat unlikely in view of the high degree of conservation of the amino acid sequence of ALV-S from bacteria to mammals (Pirola, 1987).

With the establishment that heme repression of ALV-S synthesis occurs in Xenopus oocytes it was then investigated whether the expression of the ALV-S construction M13chALV-7 in oocytes was affected by either heme or ALV. This

construction is strongly expressed in Xenopus oocytes as already described in Chapter 3.

Unfortunately the results were disappointing in that expression was unaffected by any of the oocyte treatments that repressed the endogenous ALV-S. Thus oocytes treated with succinyl acetone, ALV or heme, expressed the construction at the same level as untreated oocytes. There are a number of possible reasons for this.

1. There may be too many copies of the M13chALV-7 construction injected for the oocyte to repress. If this is the case, injection of the construction should lead to derepression of the endogenous ALV-S; this was not observed.

2. The repression of M13chALV-7 by heme may take several hours, during which time many transcripts could already have been made from the unrepressed M13chALV-7 templates. This possibility seems unlikely as oocytes pre-treated for 12 hours in either succinyl acetone or ALV prior to injection with M13chALV-7 expressed at the same relative level under all conditions.

3. If heme repression of ALV-S is mediated by one or more *trans*-acting factors, these may be species specific. The Xenopus oocyte transcriptional factor(s) may not bind to the chicken ALV-S gene. It may be valid that Xenopus oocytes have been shown to possess transcriptional factors which interact with other eukaryotic genes (Bergsma *et al.*, 1986, Kadonaga *et al.*, 1986), but this cannot necessarily be extrapolated to the ALV-S gene.

4. Heme repression in the Xenopus oocyte is not at the level of transcription. This explanation is unlikely as heme repression of ALV-S has been shown to be at the transcriptional level in all the organisms in which it has been examined (Maguire, 1987, Srivastava *et al.*, 1988).

5. The construction M13chALV-7 does not contain the *cis*-acting sequences responsive to heme and therefore can never be heme repressed.

Since attempts to obtain heme repression of an injected chicken ALV-S construction in the Xenopus oocyte had proved unsuccessful, it was decided to change

the experimental approach and concentrate this laboratories continued effort on the rat ALV-S gene. This work is described in the following Chapter's.

CHAPTER 5

ANALYSIS OF A RAT ALV-S GENOMIC CLONE

Chapter 5: Analysis of a rat ALV-S genomic clone

5-1 Introduction

In the previous chapter it was shown that although endogenous Xenopus oocyte ALV-S activity was repressed by co-injected heme, this was not exerted on an injected chick embryo ALV-S promoter construction. Subsequently work by others in this research group also failed to obtain heme repression of chicken ALV-S promoter constructions transfected into human cell lines (Day, 1988).

Since attempts to identify heme responsive elements in the chicken ALV-S gene promoter had proven unsuccessful it was decided to reconsider the experimental approach being used. Other work in this laboratory had established that the rat liver ALV-S gene was regulated by heme at the transcriptional level (Srivastava *et al.*, 1988). It was therefore decided to switch studies on the expression of the ALV-S gene from the chicken to the rat. The reasons for this were that it was difficult to see how to usefully extend the chicken ALV-S gene work, whereas the rat gene at least offered the possibility of studying its expression in an homologous rat hepatoma cell line. It was also thought that a comparison of the chicken and rat promoter regions might reveal significant homologies. To this end a rat ALV-S gene was isolated (Loveridge *et al.*, 1988) and characterised as described below.

5-2 Isolation of a rat ALV-S genomic clone

A rat genomic clone was isolated from a lambda Charon 4A rat genomic library by Dr. I. A. Borthwick. Using the methodology of Maniatis *et al.*, (1982) 1.5 X 10^6 plaques were screened using a nick-translated rat ALV-S cDNA clone p101B1 (Srivastava *et al.*, 1988). A rat ALV-S genomic clone was isolated and genomic southern blot hybridisation analysis of this clone utilising p101B1 showed that the rat ALV-S gene was contained in a single Eco R1 fragment of approximately 13Kb in size. This Eco R1 fragment was excised and subcloned into Eco R1 cut pUC-19 and designated RG-1.

5-3 Results

5-3-1 Analytical restriction mapping of a rat ALV-S genomic clone

The rat ALV-S genomic clone RG-1 was digested with a number of restriction endonucleases including Eco R1, Pst I, Sal I, Hind III, Sac I, Stu I, Sma I and Bam HI. The relative locations of these restriction sites are shown in Figure 5-1. Localisation of the translational start site (Fig. 5-1, labelled ATG) was determined by southern blot hybridisation analysis of restriction endonuclease digests of RG-1 using a 5' end labelled oligonucleotide (see section 2-3-6) complementary to the extreme 5' end of the rat cDNA clone (Fig. 5-2) (Srivastava *et al.*, 1988). The sequence of this primer, P1, is shown in section 2-1-10 and the location of the primer in the rat ALV-S gene is shown in Figure 5-1. As shown in Figure 5-2, P1 hybridised to a single DNA fragment (B.) in each restriction endonuclease digest (A.). From this data the position of the translational start point was localised to a region of the rat ALV-S gene.

5-3-2 Sequencing of part of the rat ALV-S gene

Once an overall restriction map of the rat ALV-S genomic clone RG-1 was obtained and the translational start site determined as above, appropriate restriction fragments from RG-1, 5' to the translational start site, were subcloned into M13mp19 for sequencing by the dideoxy chain-termination method (see section 2-3-8). The overall strategy to obtain the sequence of the 5' end of the rat ALV-S gene is shown as a series of arrows in the lower part of Figure 5-1. A total of approximately 2Kb of the rat ALV-S genomic clone RG-1 was sequenced and this is shown in Figure 5-3.

5-3-3 Primer extension analysis of rat liver RNA for ALV-S

Total RNA was isolated from rat liver according to the method of Srivastava *et al.* (1988). Poly(A)$^+$ RNA was then purified by oligo-dT cellulose chromatography (Maniatis *et al.*, 1982). 10μg of rat liver poly(A)$^+$ RNA was analysed by primer extension analysis (see section 2-4-3) utilising two oligonucleotide primers complementary to rat ALV-S mRNA. The location of these primers in the rat ALV-S

Figure 5-1.

A restriction endonuclease map of the rat ALV-S genomic clone RG-1. RG-1 was digested with a limited number of restriction endonucleases and their location mapped as indicated. The location of the rat ALV-S gene CAP sites (CAP), as determined by primer extension analysis (see section 5-3-3), and the translational start site (ATG), determined by southern blot hybridisation analysis (see section 5-3-1), are also marked. The location of the two oligonucleotide primers P1 and P2 are indicated along with the location of the intron in the 5' untranslated region (section 5-3-4).

The arrows in the lower part of this figure indicate the direction and length of sequence obtained from individual M13 clones (see section 5-3-2).

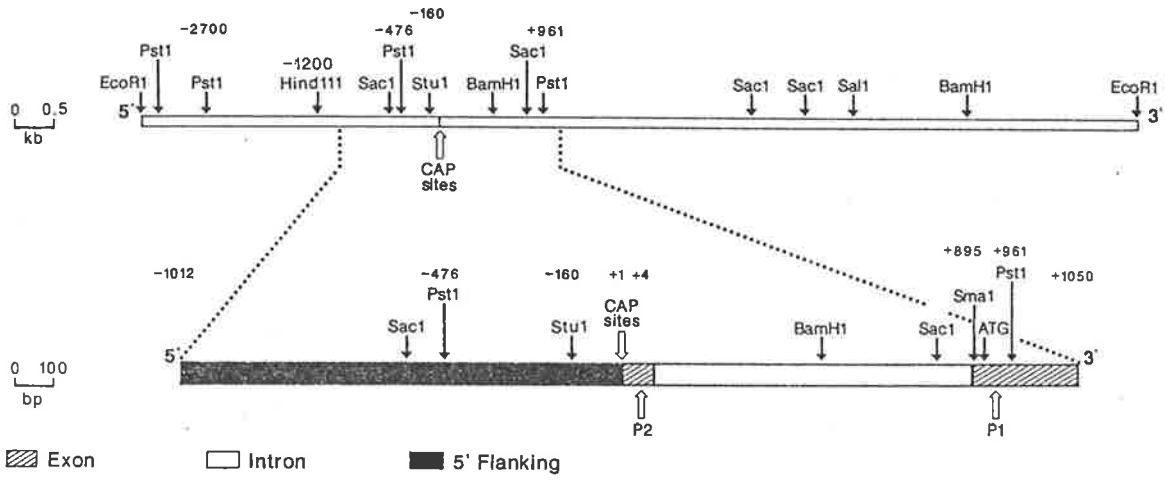
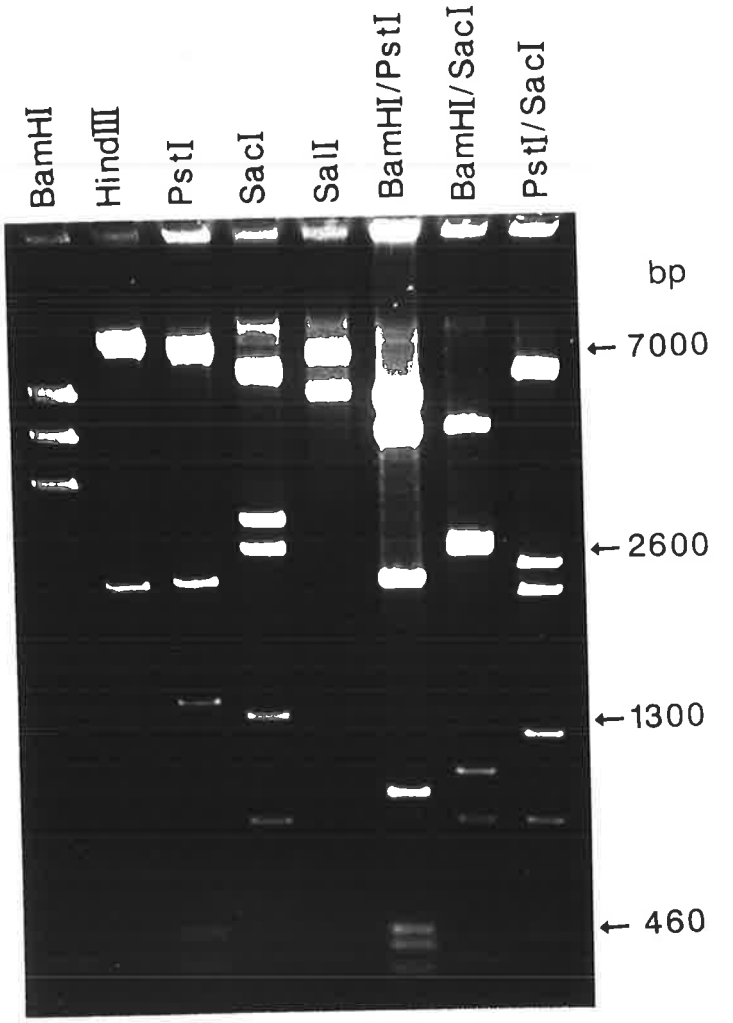

-160
-2700
-476
+961
Pst1
Pst1
Pst1
Sac1
EcoR1
Pst1
-1200
Hind111
Sac1
Stu1
BamH1
Pst1
Sac1
Sac1
Sal1
BamH1
EcoR1
0 0.5
kb
5'
3'
CAP
sites
-1012
-476
-160
+1 +4
+895 +961
Pst1
+1050
Pst1
Sma1
CAP
sites
Sac1
Stu1
BamH1
Sac1
ATG
0 100
bp
5'
3'
P2
P1
Exon
Intron
5' Flanking

Figure 5-2.

Southern blot hybridisation analysis of the rat genomic clone RG-1. 10ng of RG-1 DNA was digested with the restriction endonucleases indicated, electrophoresed on a 1% agarose gel and visualised by staining in ethidium bromide (A). Southern blot hybridisation analysis was carried out using 5' end labelled P1 as a probe and the resulting autoradiogram is shown (B). The size of appropriate DNA fragments is indicated on the left side of the figure.

A.


BamHI
HindIII
PstI
SacI
SalI
BamHI/PstI
BamHI/SacI
PstI/SacI
bp
7000
2600
1300
460

B.

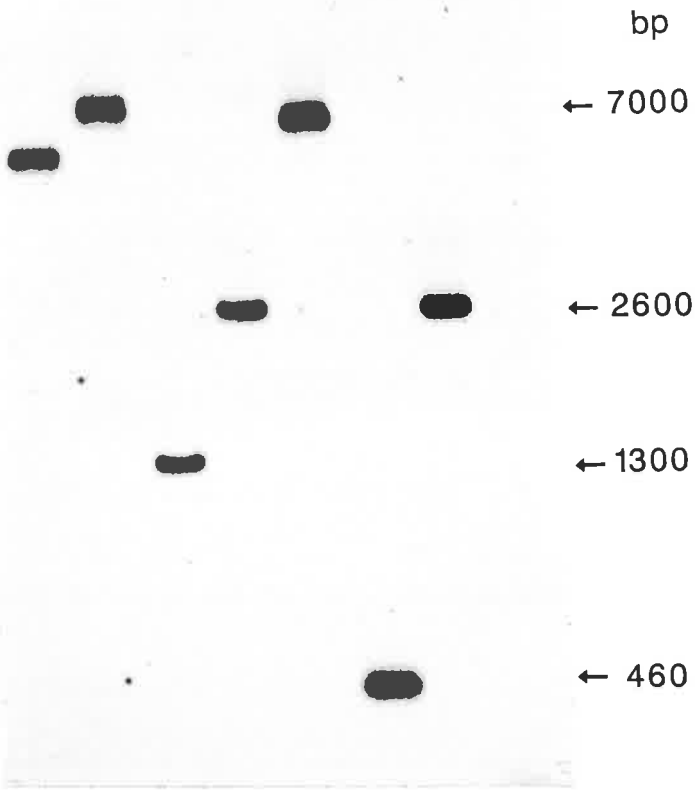

bp
7000
2600
1300
460

Figure 5-3.

Nucleotide sequence of part of the rat ALV-S gene. Numbers indicate the nucleotide position of the base pair relative to the transcriptional start site at +1. Sequences referred to in the text are described below;

Transcriptional start sites

Translational start site

Intron-exon boundaries

TATA box

Oestradiol response element

Core enhancer sequence

NFIII binding site

```
CGGCATTAAGCGGCCGGTGTGGTGGTTAGCCGACGTGACGCATACTTGGCATCGCCCTAGCGCCCGGCTCCGTTTCGCTTTCTTCCCTTC
GCCGTAATTCGCCGGCCACACCACCAATCGGCTGCACTGCGTATGAACCGTAGCGGGATCGCGGGCCGAGGCAAAGCGAAAGAAGGGAAG
-1020       -1010       -1000        -990        -980        -970        -960        -950        -940

CTTTGCTCGGCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGGCACCTCG
GAAACGAGCCGTGCAAGCGGCCGAAAGGGGCAGTTCGAGATTTAGCCCCGAGGGAAATCCCAAGGCTAAATCACGAAATGCCCGTGGAGC
-930        -920        -910        -900        -890        -880        -870        -860        -850

ACCCCAAAAAACTGATTTGGGTGATGGTTCACGTAGTGGGCCATGCGCCGTGATAGACGGTTTTTCGCCCTTGACGTTGGAGTCCACGTT
TGGGGTTTTTTGACTAAACCCACTACCAAGTGCATCACCCGGTACGCGGCACTATCTGCCAAAAAGCGGGAACTGCAACCTCAGGTGCAA
-840        -830        -820        -810        -800        -790        -780        -770        -760

CTTTAATAGTGGACGTCTGTTCCAAACTGGGAACAACAGCTCAACCCTGATCTGACTACATGAGACCTTGTCTCAAATTAAAAAACAAGA
GAAATTATCACCTGCAGACAAGGTTTGACCCTTGTTGTCGAGTTGGGACTAGACTGATGTACTCTGGAACAGAGTTTAATTTTTTGTTCT
-750        -740        -730        -720        -710        -700        -690        -680        -670

TAATTTAGCTGTTCAGTCCTGTGTCAGGCCCTGTGCTTGTCATTGACACCCTCACCATCGGGTGACTAGAACTGTGGAGCTATGAGCTCC
ATTAAATCGACAAGTCAGGACACAGTCCGGGACACGAACAGTAACTGTGGGAGTGGTAGCCCACTGATCTTGACACCTCGATACTCGAGG
-660        -650        -640        -630        -620        -610        -600        -590        -580

ATCTAGCCAGTTCCTCTGTTCCCTGCTCTCTCTTTTCTTCAATTCGGATCAGAACGTATCACCCTTCTGCGGCTGCTTCTTCAATCCCGT
TAGATCGGTCAAGGAGACAAGGGACGAGAGAGAAAAGAAGTTAAGCCTAGTCTTGCATAGTGGGAAGACGCCGACGAAGAAGTTAGGGCA
-570        -560        -550        -540        -530        -520        -510        -500        -490

GTCTGCAGCCCCTTAGCATCTCTTCACCAGGACCATTCTATTCTTGGGCCATTCATCAAGTAAAGAATCCCTGTCATCGATGCAAACAAA
CAGACGTCGGGGAATCGTAGAGAAGTGGTCCTGGTAAGATAAGAACCCGGTAAGTAGTTCATTTCTTAGGGACAGTAGCTACGTTTGTTT
-480        -470        -460        -450        -440        -430        -420        -410        -400

ACCAAGCAAGCCCCTCGTTGATCTGATCTTAAGACCCTGGACTGAAACCCCTTCCTGTGCGCCCAATAAACCTTTTTACCAGGGTGGGGT
TGGTTCGTTCGGGGAGCAACTAGACTAGAATTCTGGGACCTGACTTTGGGGAAGGACACGCGGGTTATTTGGAAAAATGGTCCCACCCCA
-390        -380        -370        -360        -350        -340        -330        -320        -310

TGGTTTACGACCTACGCCGGGGGCCCAGGAGTCTGACGCACAGGGCTGTTCGTGGAAGGTGAAAGAGGGACGAAGGGTAGAGATCCAAAA
ACCAAATGCTGGATGCGGCCCCCGGGTCCTCAGACTGCGTGTCCCGACAAGCACCTTCCACTTTCTCCCTGCTTCCCATCTCTAGGTTTT
-300        -290        -280        -270        -260        -250        -240        -230        -220

AGGGACTAGCCCGTCCACTTGCCCTGCTCTCCTGGCTCAGGCAGCGTAGGCCTAAGACATAGTCACAAGAGTTGGAGCAGAGCCCACACA
TCCCTGATCGGGCAGGTGAACGGGACGAGAGGACCGAGTCCGTCGCATCCGGATTCTGTATCAGTGTTCTCAACCTCGTCTCGGGTGTGT
-210        -200        -190        -180        -170        -160        -150        -140        -130

GAGGGACCTGGCACCCTGCCGGGCCGACTCCGGTAGCGCGATGCGCGCGGCAGGCCGACCCACAGCGCATGCGCAGCGGTCACCCCCGGC
CTCCCTGGACCGTGGGACGGCCCGGCTGAGGCCATCGCGCTACGCGCGCCGTCCGGCTGGGTGTCGCGTACGCGTCGCCAGTGGGGGCCG
-120        -110        -100        -90         -80         -70         -60         -50         -40

TCTATATTAGGCCAACAGCGGCCCTAGCCGAGGCTCGTTCGTGAAGAAGGGCACTGGTCGGTTTAGCGTCCTCCGCTCGAGAGCCACCGCC
AGATATAATCCGGTTGTCGCCGGGATCGGCTCCGAGCAAGCACTTCTTCCCGTGACCAGCCAAATCGCAGGAGGCGAGCTCTCGGTGGCGG
-30         -20         -10          1          10          20          30          40          50

GTCTCGTCGAGAGCCCGCGCAGGTGAGGGCCACGCTCGGTGGTCGCTGGTCGCGGGAACCCGGCGCGCCCCGCCACCGCCGCCACATGCC
CAGAGCAGCTCTCGGGCGCGTCCACTCCCGGTGCGAGCCACCAGCGACCAGCGCCCTTGGGCCGCGCGGGGCGGTGGCGGCGGTGTACGG
 60          70          80          90          100         110          20          130         140

CATCCCCTCCGGGCACCACCTTGGCTCAGCGTTCATCCCCCCTCTTTACCTCTTGGGCTACCTCAGACGCCCCACTACGTGCCCTTCTGA
GTAGGGGAGGCCCGTGGTGGAACCGAGTCGCAAGTAGGGGGGAGAAATGGAGAACCCGATGGAGTCTGCGGGGTGATGCACGGGAAGACT
150         160         170         180         190         200         210         220         230

CATTGGGCGTTTTGACAGGCGCCTTCAGAACTTCTTTCTTACCCTTTCCATTCTGACCCCAGAGAACCTCTGACTCACTGCTGACCCCCT
GTAACCCGCAAAACTGTCCGCGGAAGTCTTGAAGAAAGAATGGGAAAGGTAAGACTGGGGTCTCTTGGAGACTGAGTGACGACTGGGGGA
240         250         260         270         280         290         300         310         320

TCCACCAGTCAGGCTCTGCAATGTCTAGCCCATGCTCCTGACCGAGTCAGCCCATGCCCTTACCCTCTTTCATGCCTCCTTTCCCATGTT
AGGTGGTCAGTCCGAGACGTTACAGATCGGGTACGAGGACTGGCTCAGTCGGGTACGGGAATGGGAGAAAGTACGGAGGAAAGGGTACAA
330         340         350         360         370         380         390         400         410

CTAGCTGTAGGGACATCGACCTTGTCTCGGGGACTCAGGGCTGGTCTAGACTCTTCCCCCTCCAGGGGTTGCTAGGATCCCAGGGAGTCC
GATCGACATCCCTGTAGCTGGAACAGAGCCCCTGAGTCCCGACCAGATCTGAGAAGGGGGAGGTCCCCAACGATCCTAGGGTCCCTCAGG
420         430         440         450         460         470         480         490         500

TCCCTAGCTGGCTGATAGGTGACAGTGACTTTTCCCCTGGCCTTGCTATTGCTCCTGGGTGTGTGCTAAAGTGTCTTAACCTACAGTGTA
AGGGATCGACCGACTATCCACTGTCACTGAAAAAGGGGACCGGAACGATAACGAGGACCCACACACGATTTCACAGAATTGGATGTCACAT
510         520         530         540         550         560         570         580         590

CACCCTTCAAGGACCTCTGGCCTCCGACACCAGTTCTGCCTTGTTCTTCATCTCTACATCCTTAGTTATTGGTCGTCCCTACTTACTCTG
GTGGGAAGTTCCTGGAGACCGGAGGCTGTGGTCAAGACGGAACAAGAAGTAGAGATGTAGGAATCAATAACCAGCAGGGATGAATGAGAC
600         610         620         630         640         650         660         670         680

GTTAAAGCTTGCTTAGGGGTAGGGAGGGTTTCTCTAGAAGTAGAGAATGTAGAAGTTGGTGACCTGGGTAGAGTGTGGCTCCCATGCCTT
CAATTTCGAACGAATCCCCATCCCTCCCAAAGAGATCTTCATCTCTTACATCTTCAACCACTGGACCCATCTCACACCGAGGGTACGGAA
690         700         710         720         730         740         750         760         770

TACTGAGCTCTCTGTTGGTTGCTGTCCTTAGTTTGGTTTGGTTTGGTTTGGTTTTGAGTGGGATGTGTGTCTTGGGCCTCCCTTAACGTC
ATGACTCGAGAGACAACCAACGACAGGAATCAAACCAAACCAAACCAAACCAAAACTCACCCTACACACAGAACCCGGAGGGAATTGCAG
780         790         800         810         820         830         840         850         860

ACTCATCATGGACTCTTGTACAGGACCCGGGACACTTTGCAGACATGGAGACTGTCGTTCGCAGATGCCCATTCTTATCCCTCAGGCCTT
TGAGTAGTACCTGAGAACATGTCCTGGGCCCTGTGAAACGTCTGTACCTCTGACAGCAAGCGTCTACGGGTAAGAATAGGGAGTCCGGAA
870         880         890         900         910         920         930         940         950

TCTGCAG
AGACGTC
960
```

gene is shown in Figure 5-1 (P1 and P2). Two extension products of 143bp and 146bp in length were generated from the P1 oligonucleotide primer and 40bp and 43bp from the P2 oligonucleotide primer (Fig. 5-4).

Primer extension analysis of rat liver RNA utilising the P2 primer indicated that there were two species of rat ALV-S mRNA differing in length at the 5' end by 3bp. The length of these extension products localised the rat ALV-S transcriptional start sites (CAP site) to positions +1 and +3 of the rat ALV-S gene, as shown in Figure 5-3. Primer extension analysis of rat liver RNA utilising the P1 primer showed that the rat ALV-S 5' untranslated region was 107bp in length. Interestingly, as P1 and P2 are separated in the rat ALV-S gene by over 900bp, as determined in section 5-3-2, these results implied the existence of an intron in the 5' untranslated region of the rat ALV-S gene.

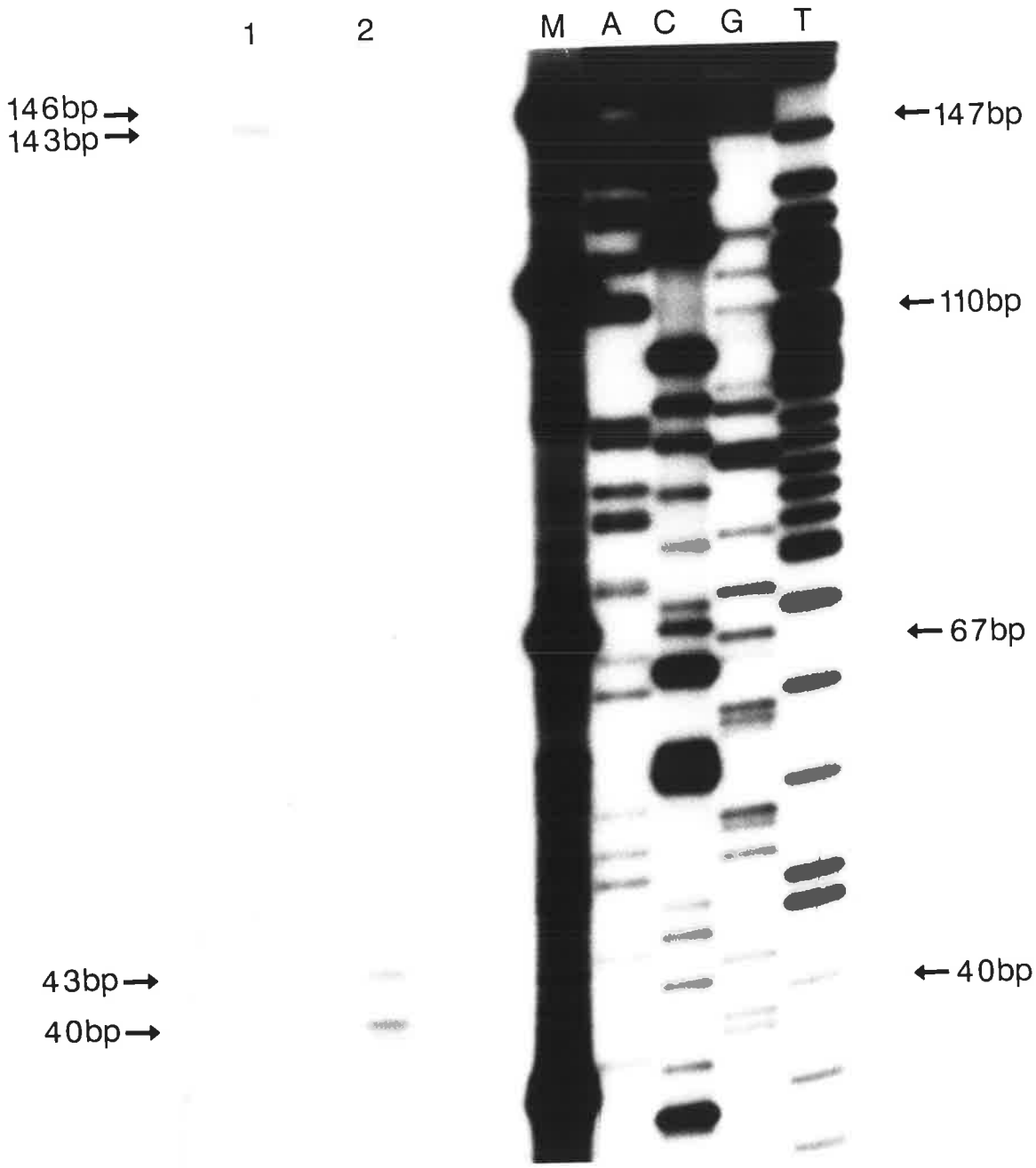
5-3-4 Mung bean nuclease protection analysis

Analysis of the rat ALV-S gene sequence, shown in Figure 5-3 predicted the presence of two RNA splice sites at positions +83 and +890. Also, primer extension analysis of rat liver poly(A)+ RNA strongly implied the presence of an intron in the 5' untranslated region of the rat ALV-S gene. To define the 3' splice site of the predicted intron, the technique of mung bean nuclease protection (outlined in section 2-5-2) was adopted.

A single Pst I rat genomic fragment from positions -476 to +961 in the rat ALV-S genomic clone RG-1, containing the DNA sequence predicted to contain the 3' RNA splice site, was subcloned into the pSP6 vector (which contains the SP64 RNA polymerase initiation site) and designated pSP(ALVS-A). By digestion of pSP(ALVS-A) with Bam HI and subsequent run-off in an *in vitro* transcription system containing the SP6 RNA polymerase, a ^{32}P-labelled RNA fragment was generated which was complementary to the sequence spanning the 3' splice site. This ^{32}P-labelled RNA fragment was annealled to rat liver poly(A)+ RNA and digested with increasing amounts of mung bean nuclease, which digests only single stranded RNA.

Figure 5-4.

Primer extension analysis of rat liver RNA for ALV-S. 10μg of rat liver poly(A)+RNA was analysed by primer extension as outlined in section 2-4-3, utilising the P1 (track 1) and P2 (track 2) oligonucleotide primers. The length of the extension products is indicated on the left hand side of the figure. This was determined by direct comparison of the lengths of the primer extended products with the HpaII cut pUC-19 markers (track M) [the size of these markers are shown on the right hand side of the figure] and a series of dideoxy chain termination sequencing reactions performed as in section 2-3-8 on single stranded M13mp19 (tracks A, C, G, T).


1
2
M A C G T
146bp
143bp
147bp
110bp
67bp
43bp
40bp
40bp

As shown in Figure 5-5, a single fragment of length 71bp was protected from nuclease digestion, indicating that the 3' RNA splice site was located at the predicted position of +890 (as shown in Figure 5-3). This sequence was strongly homologous to the consensus acceptor sequence of Breathnach *et al.* (1978). The 5' splice site could then be deduced from the length of the primer extended product using the P1 oligonucleotide primer. This site was also in agreement with the predicted position of +83 (Figure 5-3) and was strongly homologous to the donor consensus sequence as defined by Mount (1982). The location of the intron in the rat ALV-S genomic clone RG-1 is shown in Figure 5-1.

5-3-5 Analysis of the 5' untranslated region of the rat ALV-S gene

The sequence of part of the rat ALV-S gene is shown in Figure 5-3. The CAP sites are located at positions +1 and +3, as defined by primer extension analysis and the intron is located in the 5' untranslated region, with intron-exon boundaries at positions +83 and +890.

Some well defined regulatory sequence elements commonly observed in eukaryotic genes transcribed by RNA polymerase II are found in the vicinity of the rat ALV-S CAP site (Fig. 5-3 and Table 5-1). At position -28 there is a sequence bearing strong homology to the TATA box consensus sequence (Breathnach and Chambon, 1981), but no putative CCAAT box sequences (Benoist *et al.*, 1980) upstream. An incomplete homology to the GC hexanucleotide box (McKnight *et al.*, 1984) is present at position -300, and a sequence shown to bind NFIII at position -398. A putative oestradiol responsive element (ERE) (Klock *et al.*, 1987) is located in the intron at position +530, as well as a sequence homologous to the core enhancer element (Weiher *et al.*, 1983) upstream of the TATA box at position -995. Although these sequences are present in the rat ALV-S gene it is not known at this stage if they are functional, or play any role in the transcription or regulation of the ALV-S gene.

Figure 5-5.

Mung bean nuclease protection of rat liver RNA. The protection of 5µg rat liver poly(A)+RNA was performed as described in section 2-5-2 utilising 0 Units (track 1), 10 Units (track 2), 75 Units (track 3) and 150 Units (track 4) of mung bean nuclease. The size of the protected RNA/DNA hybrid is shown on the right hand side of the figure. This was determined by direct comparison of the size of the protected hybrid with the size of HpaII cut pUC-19 markers [the size of these markers is shown on the left hand side of the figure] and a series of dideoxy chain termination sequencing reactions performed as in section 2-3-8 on single stranded M13mp19 (not shown).

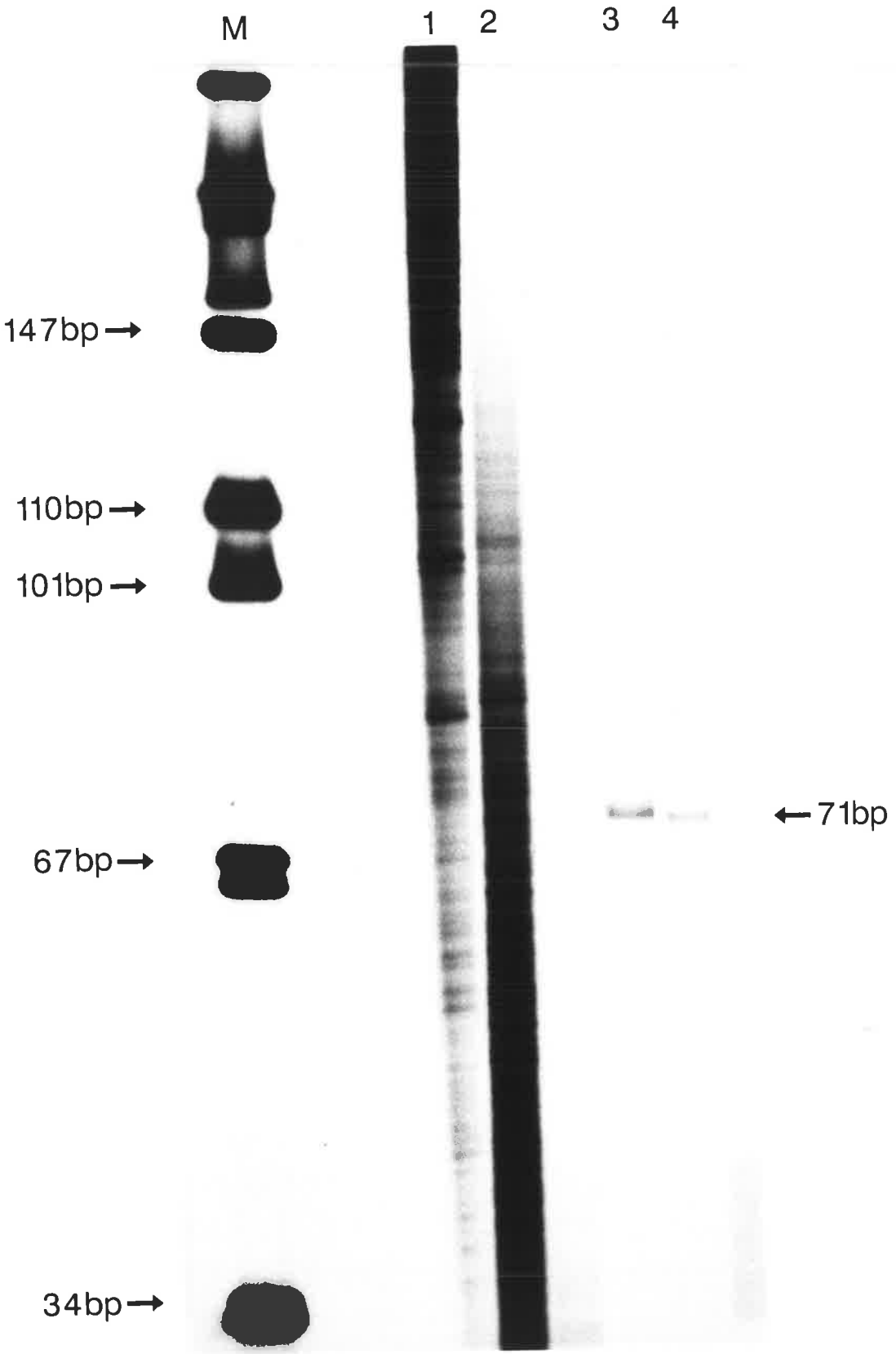

M
1
2
3
4
147bp
110bp
101bp
67bp
34bp
71bp

Table 5-1

Potential regulatory sequences in the rat ALV-S 5' flanking region

CONSENSUS SEQUENCE	FUNCTION	REFERENCE	MATCH IN ALV-S GENE[b]	POSITION[a]
TATAA/TAA/T	transcriptional initiation	Breathnach and Chambon (1981)	**TATATTA**	-28
G/TGGGCGGG/AG/AC/T	Sp1 binding, activation of transcription	Kadonaga *et al.* (1986)	**CCCCGCC**	+130
			GGGTGGGG	-300
GTGGA/TA/TA/TG	core enhancer sequence	Weiher *et al.* (1983)	**GTGGTTAG**	+995
AGGTCACAGTGACCT	oestradiol response element	Klock *et al.* (1987)	**AGGTGACAGTGACTT**	+530

a The position of the sequence is relative to the CAP site at +1

b Nucleotides which match the consensus sequence are in **bold type**

5-4 Discussion

As already described, much work was done on the analysis of the chicken ALV-S gene and its expression in an attempt to elucidate the mechanism by which the transcription of the ALV-S gene is repressed by heme. Expression studies on the chicken ALV-S gene have been conducted in Xenopus oocytes (this thesis) and in human cell lines (Day, 1988), but were unsuccessful in defining the mechanism of heme repression. Because of these problems, attention was turned to the rat ALV-S gene. A genomic clone was isolated with the idea that a comparative sequence and expression analysis should aid in the definition of regulatory sequence elements.

A rat ALV-S genomic clone (RG-1) was isolated by Dr. I. Borthwick. This clone, 13Kb in length, contains approximately 5Kb of sequence 5' to the translational start site and two thirds of the structural gene (Loveridge *et al.*, 1988). RG-1 was restriction mapped utilising a limited number of restriction endonucleases. The translation start point (ATG) was located by southern blot hybridisation analysis of restriction endonuclease digested RG-1 with the 5' end labelled oligonucleotide P1, the latter corresponding to the extreme 5' end of the rat ALV-S cDNA sequence (Srivastava *et al.*, 1988). It should therefore hybridise only to those restriction fragments containing the translational start site. Once the relative location of the restriction endonuclease sites and the position of translation initiation was known, appropriate rat ALV-S restriction endonuclease fragments 5' to this point were cloned into M13mp19 for sequence analysis. Rat ALV-S DNA sequence was obtained for the 2Kb immediately 5' to the translational start point and is shown in Figure 5-3.

Primer extension analysis of rat mRNA indicated the presence of two ALV-S mRNA's, differing in size at the 5' end by 3bp. This is in contrast to the chicken ALV-S gene which only has a single transcriptional start site (Maguire *et al.*, 1986). Although many housekeeping genes generate multiple RNA's with heterogeneity at the 5' end, these genes do not possess TATA boxes (Singer-Sam, *et al.*, 1984, McGrogan *et al.*, 1985) and appear to initiate transcription from GC rich sequences (Melton *et al.*, 1984, Reynolds *et al.*, 1985). In contrast, the chicken ALV-S gene has a strict

requirement for a TATA box to initiate transcription (Chapter 3, this thesis) and the rat ALV-S gene also has a TATA like sequence in the correct position at -28. Whether the rat ALV-S gene has a strict requirement for a TATA box as in the chicken ALV-S gene is yet to be determined.

Analysis of the rat ALV-S gene sequence predicted the presence of an intron in the 5' untranslated region of the gene. The location of the 3' intron-exon boundary was determined by mung bean nuclease protection analysis. Once the 3' boundary had been identified, the location of the 5' intron-exon boundary was calculated from the length of the primer extension product generated with the P1 oligonucleotide. It is not an uncommon feature of eukaryotic genes to have introns in the 5' untranslated region (Reynolds *et al.*, 1984, Reynolds *et al.*, 1985, Gil *et al.*, 1986), but this is again in contrast to the chicken ALV-S gene which does not have such an intron.

The rat ALV-S gene sequence was compared with those of the chicken (Maguire *et al.*, 1986), human (H. Healy, unpublished results), *Rhizobium meliloti* (Leong *et al.*, 1985) and *Saccharomyces cerevisiae* (Urban-Grimal *et al.*, 1986). The aim was to search for conserved sequence elements which may be involved in heme regulation. Surprisingly, little overall homology was found between any of these ALV-S sequences outside the coding regions, except between the human and rat genes. The ALV-S genes from both of these species have introns in their respective 5' untranslated regions and the 3' end of these introns contain areas of strong homology (Table 5-2). Both these introns also contain oestradiol responsive sequence elements.

It is noteworthy that the chicken ALV-S 5' flanking region contains several putative sequence elements most of which were subsequently found to lack any role in expression, at least in Xenopus oocytes (Chapter 3, this thesis). The rat ALV-S 5' flanking region contains none of these sequence elements, with the exception of a TATA box at position -28 and a possible GC box at -300.

Several sequences were found in the rat ALV-S gene which were homologous to transcriptional sequence elements known to play a role in the expression of other genes (Table 5-1). A putative oestradiol response element was found in the intron of

Table 5-2

Comparision of the 5' end of the ALV-S structural gene from different species

SPECIES	SEQUENCE[a b] (5' to 3')	REFERENCE
rat	**ATGGAG**ACT**GT**CGTTCGCAGA**TGCCCATTCTTATCCC**TCAGGC**CTTTCTGC**AG	Borthwick *et al.* (1984)
chicken	**ATGGAG**GCG**GTGGTG**CGGCGC**TGCCCGTTCCTGGCCCGCGTCTC**GCAGGC**CTT**	Bawden *et al.* (1987)
human	**ATGGAG**AGT**GTTGTT**CGCCGC**TGCCCATTCTTATCCC**GAGTCCCCCAGGC**CTT**	

a Homology between all three species shown in **bold type**

b Sequence begins at translational start site

the rat ALV-S gene. This sequence is present in the human ALV-S gene, in a number of oestrogen responsive genes such as the vitellogenin genes (Wahli *et al.*, 1982, Martinez *et al.*, 1987) and has been shown to be responsive to oestrogen (Klock *et al.*, 1987). The ERE is very closely related, but distinctly different from the glucocorticoid response elements (GRE) (Klock *et al.*, 1987) found in the chicken ALV-S gene and in steroid responsive genes such as ovalbumin (Gaub *et al.*, 1987). These elements may be important in relation to the porphyria diseases, a group of diseases in which the control of the heme biosynthetic pathway is disturbed because of a defect in one of the enzymes of the pathway (Kappas *et al.*, 1983). Physiological problems associated with over accumulation of heme precursors occurs in patients only after the onset of puberty (Kappas *et al.*, 1983).

A sequence found to bind the transcription factor NFIII was found at position -398 of the rat ALV-S gene. NFIII is a DNA binding protein isolated from HeLa cells which was found to stimulate the initiation of Adenovirus type 2 DNA replication. This octanucleotide sequence has also been shown to bind NFIII in the histone H2B promoter, the immunoglobin light and heavy chain promoters, a U2 snRNA enhancer, an immunoglobulin heavy chain enhancer and the SV 40 enhancer, suggesting that NFIII could function as a general DNA binding factor (Pruijn *et al.*, 1987).

A sequence containing the core enhancer domain was also found upstream of the rat ALV-S TATA box. This sequence is found in several viral enhancers (Weiher *et al.*, 1983, Garcia *et al.*, 1987) and its deletion from the SV 40 72bp repeat results in a marked decrease in transcriptional efficiency (Wildeman *et al.*, 1986, Zenke *et al.*, 1986). The sequence is known to bind a number of transcriptional factors (Davidson *et al.*, 1986, Sen and Baltimore, 1986, Garcia *et al.*, 1987, Nabel and Baltimore, 1987).

A common eukaryotic immediate upstream control sequence which is found in the rat ALV-S 5' flanking region is the GC box (McKnight *et al.*, 1984). This sequence is known to bind the transcriptional factor Sp1 (Dynan and Tjian, 1985, Kadonaga *et al.*, 1986) and is commonly found in the promoters of housekeeping genes (Reynolds *et al.*, 1984, Osborne *et al.*, 1985) such as ALV-S. The homology of the rat ALV-S

putative GC box at position -300 is incomplete in comparison to the consensus sequence (Kadonaga *et al.*, 1986) and its location possibly too far upsteam of the TATA box sequence but recent work has shown that such incomplete sequences are still capable of binding Sp1 (Garcia *et al.*, 1987). It is possible that such a sequence might have some function in modulating expression of the rat ALV-S gene; a GC box has been shown to be essential to the maximum expression of the chicken ALV-S gene in oocytes (Chapter 3, this thesis).

CHAPTER 6

DELIMITATION OF THE SEQUENCES ESSENTIAL TO HEME REPRESSION OF THE RAT ALV-S GENE

Chapter 6: Delimitation of the sequences essential to heme repression of the rat ALV-S gene

6-1 Introduction

Recent work by Srivastava *et al.* (1988) in this laboratory has shown that the level of ALV-S mRNA is reduced in a number of rat tissues by the injection of heme-arginate and that this regulation is, at least in the liver, at the level of transcription. In the previous chapter a genomic clone for rat ALV-S was obtained and the 5' untranslated region characterised by DNA sequencing, primer extension analysis and mung bean nuclease protection. This therefore permitted examination of the expression of the rat ALV-S gene with the aim of identifying and delimiting those DNA sequences responsible for heme repression of ALV-S gene transcription.

Initially, a series of ALV-S promoter constructions was made containing increasing lengths of the rat ALV-S gene connected to the CAT reporter gene in the plasmid pIBI-76. Expression of these constructions was then examined by transient expression in the rat hepatoma H4-II-E-C3 cell line in the presence and absence of heme-arginate. The level of endogenous ALV-S mRNA was also examined in these cells.

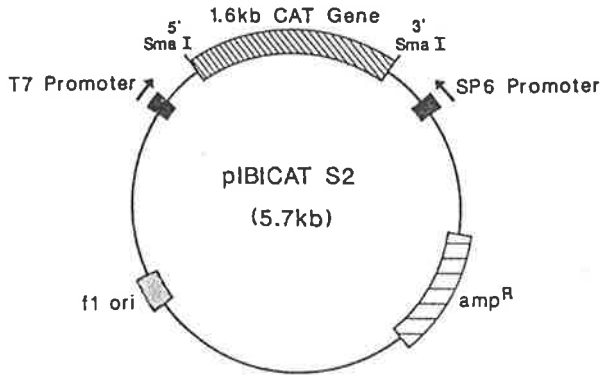
6-2 Results

6-2-1 Construction of rat ALV-S/CAT chimeric plasmids

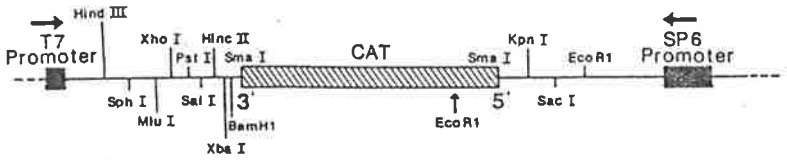
A series of expression vectors containing the chloramphenicol acetyl transferase (CAT) gene was prepared in the plasmid pIBI-76 in order to access a broad range of restriction enzyme sites for the cloning of promoter fragments. As pIBI-76 contains the *E. coli* f1 origin of replication, single stranded DNA can also be made for sequencing and for oligonucleotide site-directed mutagenesis (Fig. 6-1). To this end, a 1.6Kb Hind III-Bam HI DNA fragment containing the CAT structural gene, the SV 40 't' intron and the SV 40 early region polyadenylation signal (obtained from A. Day, this laboratory), was cloned in both orientations into either the Sma I site, or the Hinc II site of pIBI-76 (Fig 6-1). These four constructions, designated pIBICAT-H1, pIBICAT-H2, pIBICAT-

Figure 6-1.

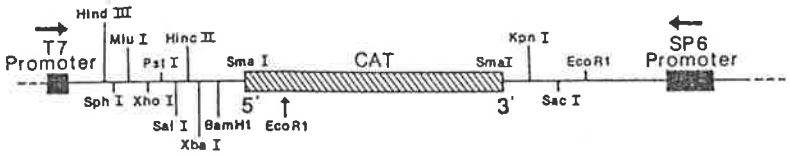
A map of the four starting vectors utilised for the construction of ALV-S/CAT chimeric plasmids; pIBICAT S1, pIBICAT S2, pIBICAT H1 and pIBICAT H2. The orientation of the CAT gene, T7 RNA polymerase initiation site, SP6 RNA polymerase initiation site and restriction endonuclease map of the pIBI-76 polylinker is shown next to the name of each vector. At the top of the figure is a map of the vector pIBICAT S2 indicating the size of the vector and the relative location of the CAT gene, *E. coli* origin of replication, ampicillin resistance gene and T7 and SP6 RNA polymerase initiation sites.



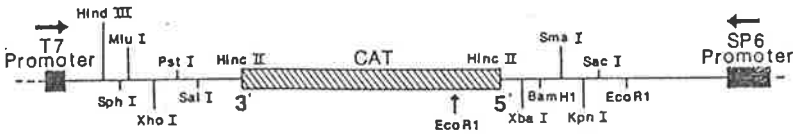
pIBICAT S1



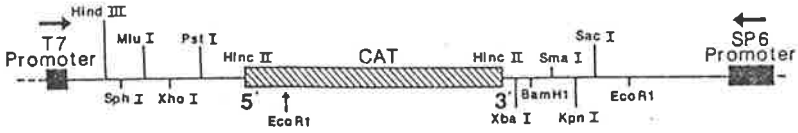
pIBICAT S2



pIBICAT H1



pIBICAT H2



S1 and pIBICAT-S2 were used as starting plasmids for the construction of chimeric ALV-S/CAT constructions (Fig. 6-1).

The positive control vector pIBISVCAT was constructed by cloning a 500bp Acc I-Hind III SV 40 early promoter fragment (obtained from A. Day, this laboratory) into the Sma I site of pIBICAT-H1 (see Fig 6-2).

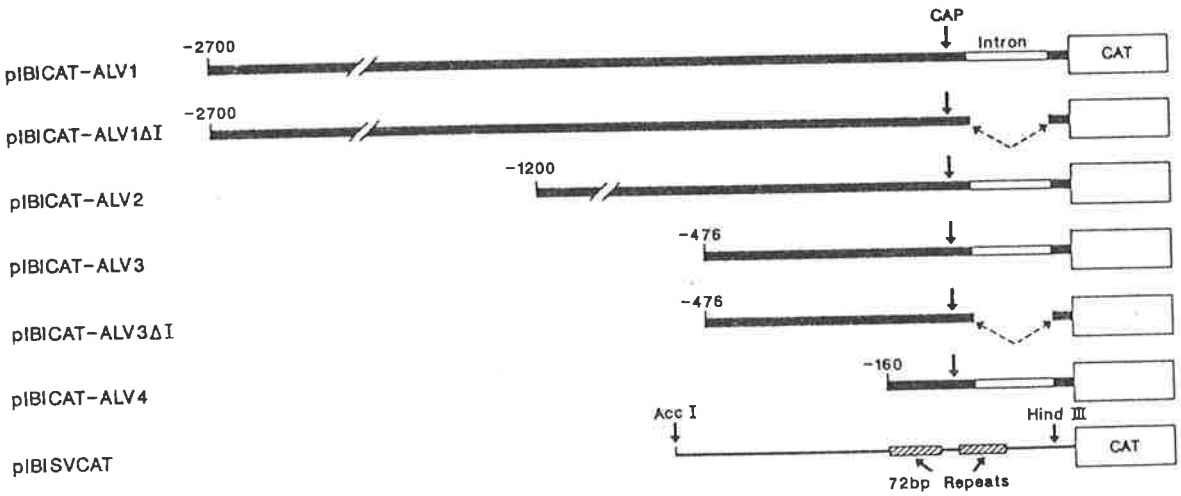
A progenitor plasmid for the ALV-S/CAT constructions was prepared by cloning the 1.4Kb Pst I fragment from positions -476 to +961 of the rat ALV-S gene into the Pst I site of pIBICAT-S2, in the correct orientation relative to the CAT gene. To bring the 5' untranslated sequence of the ALV-S gene adjacent to the CAT gene coding sequences, a portion of the ALV-S coding sequence was removed from the progenitor plasmid by digestion at the unique restriction enzyme sites for Sal I (in the pIBI-76 polylinker) and Sma I (in the ALV-S gene). The remaining vector plus ALV-S insert was then end-filled with *E. coli* DNA polymerase I (Klenow fragment) as described in Maniatis *et al.* (1982) and religated to produce the construction pIBICAT-ALV3. This construction contains rat ALV-S gene sequences from -476 to +896, and includes the intron in the 5'untranslated region of the rat ALV-S gene (see Fig. 6-2). Plasmid pIBICAT-ALV1 was prepared by inserting the 2.3Kb Pst I rat genomic fragment immediately 5' to the 1.4Kb Pst I fragment in Pst I cut pIBICAT-ALV3, in the correct orientation, to produce a plasmid containing rat ALV-S gene sequences from -2700 to +896 (Fig. 6-2). Digestion of pIBICAT-ALV1 with Hind III and subsequent religation of the vector, removed the ALV-S 5' flanking sequences from -1200 to -2700 in pIBICAT-ALV1 to produce the construction pIBICAT-ALV2 (see Fig. 6-2). pIBICAT-ALV4 was prepared by cloning the 1056bp Stu I-Sma I rat ALV-S genomic fragment, spanning bases -160 to +896 of the ALV-S gene, into the Sma I site of pIBICAT-H1 (see Fig. 6-2).

Two intronless mutants were prepared by oligonucleotide site-directed mutagenesis (see section 2-6-6) of pIBICAT-ALV1 and pIBICAT-ALV3 to produce pIBICAT-ALV1ΔI and pIBICAT-ALV3ΔI, respectively. The orientation of all constructions was confirmed by restriction endonuclease mapping.

Figure 6-2.

Rat ALV-S/CAT chimeric plasmids. Details of the construction of the ALV-S/CAT chimeric plasmids is described in section 6-2-1. The ALV-S gene fragment cloned in front of the CAT gene in the vectors pIBICAT S2 and pIBICAT H1 are indicated by thick lines. The nucleotide positions of the 5' end of each deletion mutant, relative to the CAP site at +1, are shown above the thick line. Two constructions, pIBICAT-ALV1ΔI and pIBICAT-ALV3ΔI have had the intron specifically removed by oligonucleotide site-directed mutagenesis as is indicated by the broken arrows.

The positive control vector pIBISVCAT, constructed as in section 6-2-1 is also shown.


CAP
Intron
CAT
pIBICAT-ALV1
-2700
pIBICAT-ALV1ΔI
-2700
pIBICAT-ALV2
-1200
pIBICAT-ALV3
-476
pIBICAT-ALV3ΔI
-476
pIBICAT-ALV4
-160
Acc I
Hind III
CAT
pIBISVCAT
72bp Repeats

6-2-2 Validity of the chloramphenicol acetyltransferase assay

It was of some importance before the transfection experiments were done to determine if the CAT assay, performed as outlined in section 2-6-5, gave linear results and thus was a true measure of the relative efficiencies of the ALV-S/CAT constructions. CAT assays were performed as described in section 2-6-5 except that known, increasing quantities of CAT enzyme was added to 25μg of H4-II-E-C3 cell lysate and assayed under the exact conditions as were the cell lysates from H4-II-E-C3 cells transfected with the ALV-S/CAT constructions. As seen in Figure 6-3, the CAT assay was linear for the range of CAT enzyme concentrations assayed. As the level of CAT activity generated from the ALV-S/CAT constructions was well within the range tested, it can be concluded that the CAT assay used in these experiments does give a true estimate of the relative efficiencies of the ALV-S/CAT constructions.

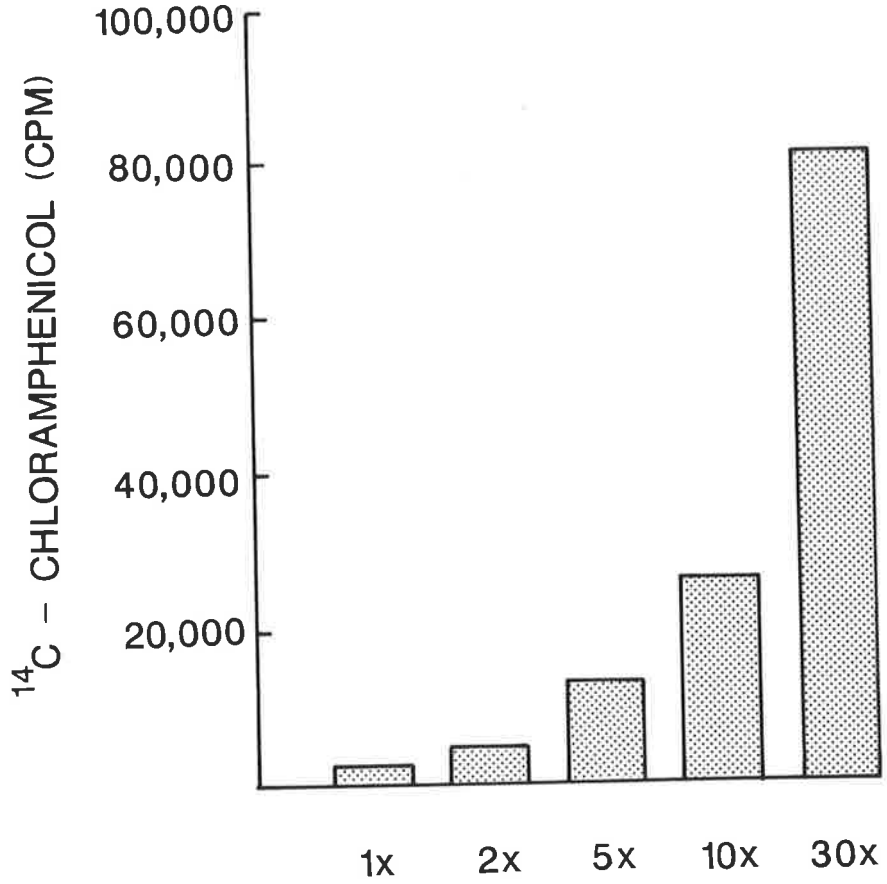
6-2-3 Optimisation of the transfection of H4-II-E-C3 cells

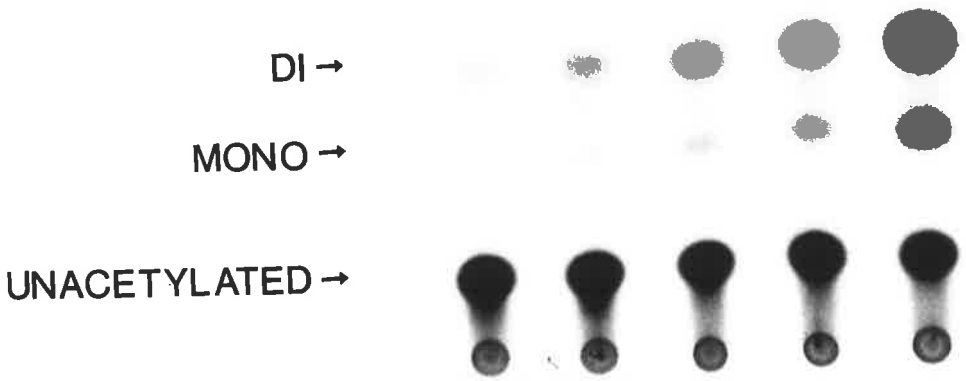
DNA can be introduced into tissue culture cells by a number of means (reviewed in Spandidos and Wilkie, 1984, Chu *et al.*, 1987). The methods of electroporation (Chu *et al.*, 1987) and calcium chloride precipitation (Graham and Van Der Eb, 1973) were examined, to optimise the transfection efficiency of the rat hepatoma cell line H4-II-E-C3. The H4-II-E-C3 cell line was chosen for these studies as it is a highly differentiated rat liver cell line (Pitot *et al.*, 1964). Moreover, heme repression had been demonstrated to occur at the transcriptional level in rat liver (Srivastava *et al.*, 1988) and the H4-II-E-C3 cell line was homologous to the species from which the gene being examined was isolated.

Initially H4-II-E-C3 cells were transfected with 10μg of the positive control vector pIBISVCAT by the calcium chloride procedure outlined in section 2-6-3, with and without a glycerol shock. 48 hours after transfection the H4-II-E-C3 cells were isolated and lysed by freeze-thawing. Cell lysate containing 25μg of protein was assayed for CAT activity as outlined in section 2-6-5. As seen in Figure 6-4 (A.), the

Figure 6-3.

Linearity of the CAT assay. CAT assays were performed as outlined in section 2-6-5 except that known increasing amounts of CAT enzyme (1X, 2X, 5X, 10X, 30X) were assayed in the presence of 25μg of H4-II-E-C3 cell lysate. An autoradiograph of this CAT assay is shown in the bottom of this figure. Indicated are the positions of the substrate, ^{14}C-chloramphenicol (UNACETYLATED) and the two products of the CAT enzyme, monoacetylated ^{14}C-chloramphenicol (MONO) and diacetylated ^{14}C-chloramphenicol (DI). The activity of each concentration of CAT is expressed as the total number of counts/min (cpm) in the mono and diacetylated ^{14}C-chloramphenicol products.


100,000
80,000
60,000
40,000
20,000
^{14}C – CHLORAMPHENICOL (CPM)
1x
2x
5x
10x
30x


DI →
MONO →
UNACETYLATED →

level of CAT expression was low in general, but higher in the cells which had been glycerol shocked. The length of the glycerol shock was optimal at 2 min.

H4-II-E-C3 cells were also transfected with 10μg of pIBISVCAT by electroporation (see section 2-6-4) at a range of voltages. 48 hours after transfection the H4-II-E-C3 cells were isolated and lysed by freeze-thawing. H4-II-E-C3 cell lysate containing 25μg of protein was assayed for CAT activity as described in section 2-6-6. As seen in Figure 6-4 (B.) electroporation was most efficient at around 220V and at a capacitance of 960C. These experiments show that the electroporation method was a more efficient transfection method for introducing DNA into H4-II-E-C3 cells than the calcium phosphate technique and was therefore used for all H4-II-E-C3 transfections.

6-2-4 Effect of heme on H4-II-E-C3 cell ALV-S levels

Before attempting to modulate any expression of the chimeric plasmids by heme administration, it was of importance to determine whether endogenous ALV-S levels in H4-II-E-C3 cells were subject to heme repression and if so whether such a control still operated in the presence of transfected ALV-S gene sequences.

Preliminary experiments demonstrated that the addition of 1μM heme-arginate to the H4-II-E-C3 hepatoma cell culture medium was sufficient to lower the level of activity of the endogenous ALV-S enzyme (assayed as described in section 2-2-1) (Fig. 6-5). The levels of H4-II-E-C3 ALV-S mRNA were then measured to determine if heme affected the transcription of the endogenous ALV-S gene. In these experiments the effect of 1μM heme-arginate was examined on endogenous ALV-S mRNA in H4-II-E-C3 cells which were either, mock transfected (transfected in the absence of pIBICAT-ALVS1), or transfected with pIBICAT-ALV1. The transfection was by electroporation (section 2-6-4). The cells were incubated for 48 hrs after transfection, and total RNA was then isolated by the guanidinium isothiocyanate method of Chomczynsky and Sacchi (1987). RNA samples (20μg) were denatured in formamide and electrophoresed on a 1.1M formaldehyde-1% agarose gel (Maniatis *et al.*, 1982). Following transfer to nitrocellulose, the filter was probed with ^{32}P nick-translated

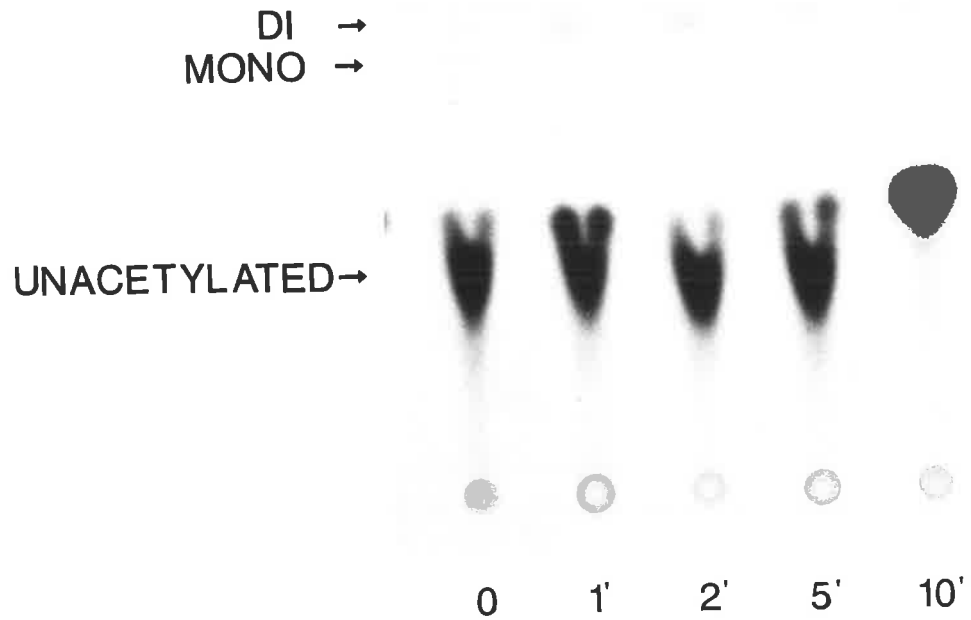
Figure 6-4.

Optimisation of the transfection of H4-II-E-C3 cells.

A. H4-II-E-C3 cells were transfected with the construction pIBISVCAT by the calcium chloride procedure outlined in section 2-6-3, without a glycerol shock (0) and with a glycerol shock of length 1 min (1'), 2 min (2'), 5 min (5') and 10 min (10'). 25μg of H4-II-E-C3 lysate was then assayed for CAT activity as in section 2-6-5 and the components of the assay chromatographed on silica gel plates. An autoradiograph of such a silica plate is shown. The positions of the substrate, ^{14}C-chloramphenicol (UNACETYLATED) and the two products of the CAT enzyme, monoacetylated ^{14}C-chloramphenicol (MONO) and diacetylated ^{14}C-chloramphenicol (DI) are indicated.

B. H4-II-E-C3 cells were transfected with the construction pIBISVCAT by electroporation as outlined in section 2-6-4 at voltages of 180V, 200V, 210V, 220V and 230V as indicated. 25μg of H4-II-E-C3 lysate was then assayed for CAT activity as in section 2-6-5 and the components of the assay chromatographed on silica gel plates. An autoradiograph of such a silica plate is shown. The positions of the substrate, ^{14}C-chloramphenicol (UNACETYLATED) and the two products of the CAT enzyme, monoacetylated ^{14}C-chloramphenicol (MONO) and diacetylated ^{14}C-chloramphenicol (DI) are indicated.

A.


DI →
MONO →
UNACETYLATED→
0
1'
2'
5'
10'

B.

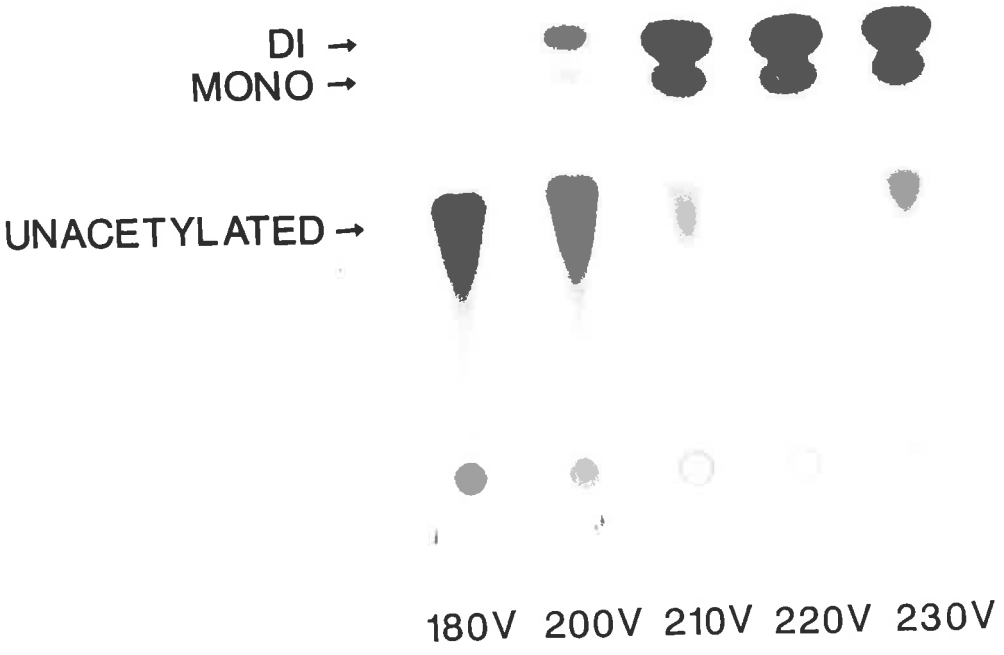
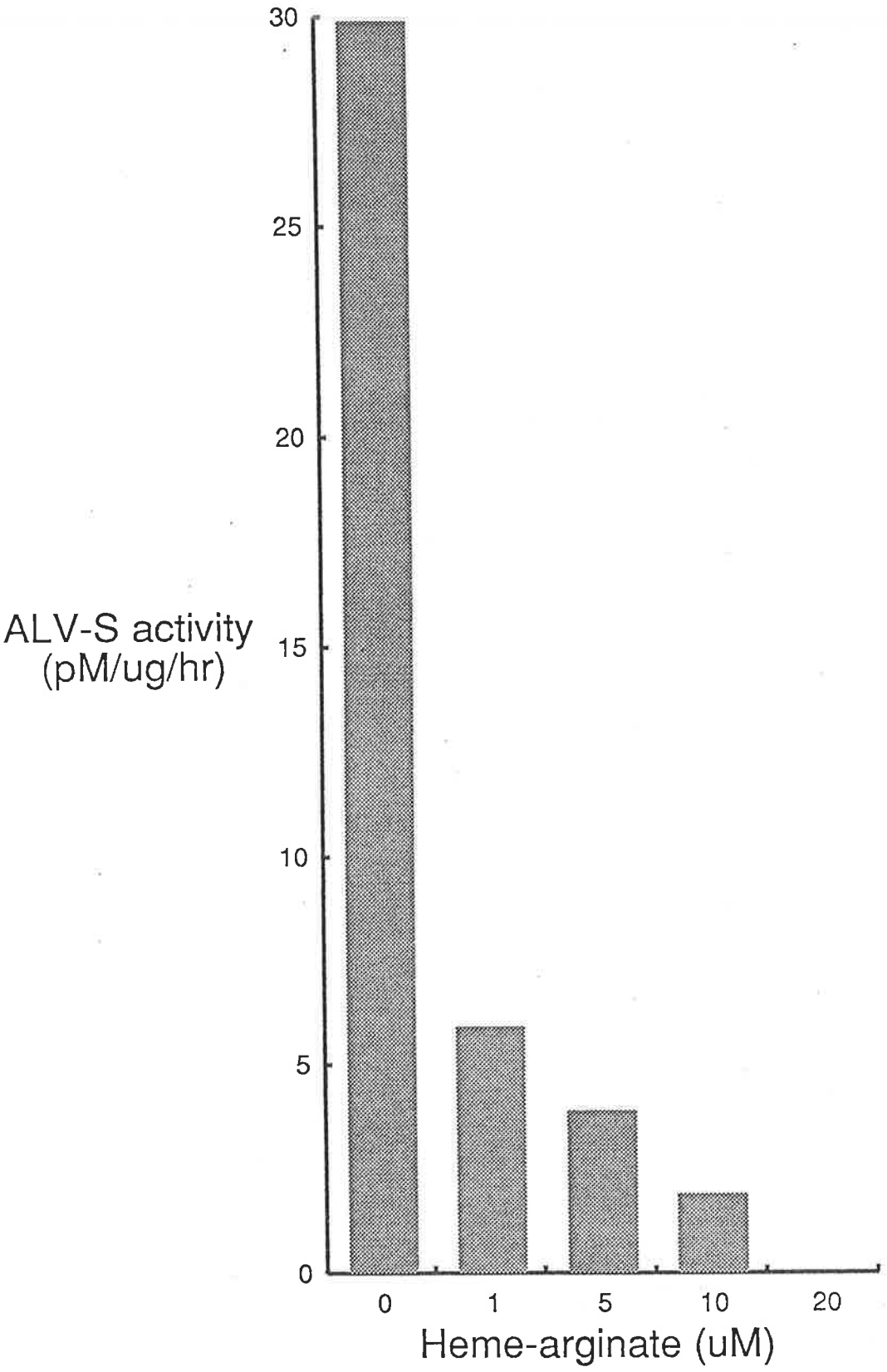

DI →
MONO →
UNACETYLATED →
180V
200V
210V
220V
230V

Figure 6-5.

The response of H4-II-E-C3 ALV-S activity to treatment with increasing concentrations of heme-arginate. ALV-S activity was measured in 100μg of H4-II-E-C3 mitochondrial protein essentially as described in section 2-2-1 and is shown along the vertical axis as pM ALV-pyrole produced/μg of H4-II-E-C3 mitochondrial protein/hr. The concentration of heme-arginate in the cell media is shown along the horizontal axis.


ALV-S activity
(pM/ug/hr)
30
25
20
15
10
5
0
0
1
5
10
20
Heme-arginate (uM)

p101B1; a rat cDNA clone (Srivastava *et al.*, 1988). The results showed that the addition of 1μM exogenous heme-arginate was sufficient to lower the endogenous level of H4-II-E-C3 ALV-S mRNA by approximately 50% (Figure 6-6, A., lanes A and B). Interestingly, when ALV-S mRNA levels were measured in pIBICAT-ALV1 transfected cells it is found that the endogenous ALV-S levels were raised over those of the mock transfected controls and further, that heme-arginate also decreased this level of ALV-S mRNA (Figure 6-6, **A.**, lanes C and D). As a control, the nitrocellulose filter was stripped (Maniatis *et al.*, 1982) and probed for β-actin mRNA (Srivastava *et al.*, 1988). β-actin mRNA levels were found to be approximately the same in all samples (Figure 6-6, **B.**). The heme effect on ALV-S would therefore not appear to be a general non-specific effect. This experiment was repeated several times and was found to be reproducible. These results suggest that rat hepatoma H4-II-E-C3 cells are suitable for the further study of heme repression of the rat ALV-S gene.

6-2-5 Effect of heme on expression of the ALVS/CAT chimeric constructions in the H4-II-E-C3 cell line.

The rat ALV-S/CAT constructions, shown in Figure 6-2, were transfected by electroporation into rat hepatoma H4-II-E-C3 cells (section 2-6-4) and the level of CAT expression measured in the lysates of cells grown in the presence and absence of 1μM exogenous heme-arginate. The level of CAT activity (measured as described in section 2-6-5) generated by each construction was expressed relative to the level generated by the positive control plasmid pIBISVCAT. The results, shown in Figure 6-7, demonstrate that in the absence of added heme-arginate, the pIBICAT-ALV3, pIBICAT-ALV3ΔI and pIBICAT-ALV4 constructions all expressed at approximately 47% of the pIBISVCAT control. In contrast, pIBICAT-ALV1 and pIBICAT-ALVΔI expressed at approximately 36% of the level of pIBISVCAT, whilst pIBICAT-ALV2 expressed at only 15% of the level of pIBISVCAT. These results indicate that the minimum sequence requirement for transcription of the rat ALV-S gene in H4-II-E-C3 cells reside in the first 160bp of ALV-S gene sequence proximal to the CAP site.

Figure 6-6.

The effect of heme-arginate and the construction pIBICAT-ALVS1 on the level of H4-II-E-C3 ALV-S mRNA.

A. H4-II-E-C3 cells were transfected by electroporation (see section 2-6-4). Tracks A and B: mock transfected. Tracks C and D: transfected with the ALV-S/CAT chimeric construction pIBICAT-ALVS1. After transfection the cells were grown in either the absence (tracks A and C) or presence of exogenous 1μM heme-arginate (tracks B and D). RNA was isolated and examined by northern blot hybridisation analysis using nick-translated p101B1 (a rat ALV-S cDNA clone) which specifically hybridises to the 2.3Kb rat ALV-S mRNA (as indicated).

B. The same filter which is shown in Figure 6-6-A. (above) was stripped of all hybridised p101B1 (Maniatis *et al.*, 1982) and reprobed utilising a nick-translated β-actin clone which specifically hybridises to the 1.9Kb rat β-actin mRNA.

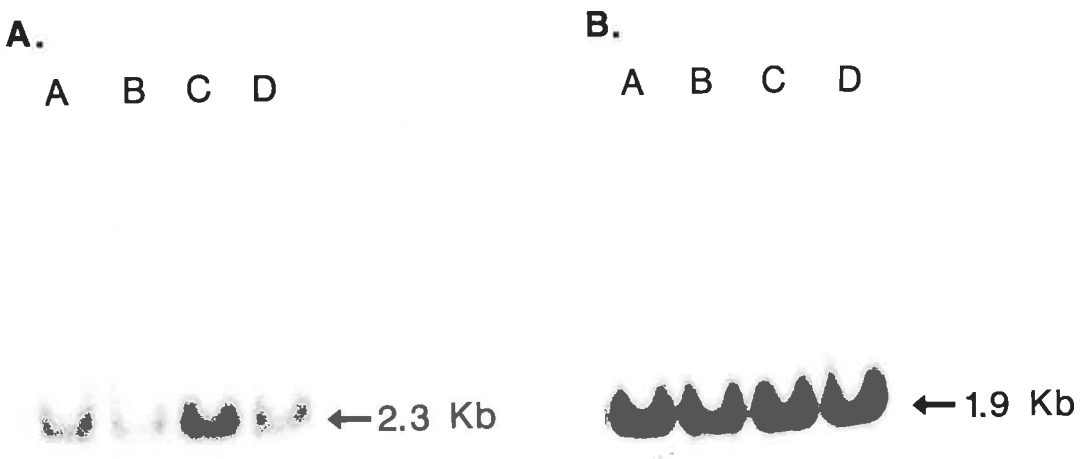

A.
A B C D
←2.3 Kb
B.
A B C D
←1.9 Kb

Figure 6-7.

Expression of rat ALV-S-CAT constructions in the rat hepatoma H4-II-E-C3 cell line in the presence and absence of heme-arginate. The ALV-S constructions described in section 6-2-1 were transfected by electroporation (section 2-6-4) into H4-II-E-C3 cells, which were then grown in either the presence or absence of 1μM heme-arginate. 25μg of H4-II-E-C3 cell lysate was assayed for CAT activity as outlined in section 2-6-5 and the products chromatographed on silica gel plates. An autoradiograph of such a silica plate is shown at the bottom of the figure. The positions of the substrate, ^{14}C-chloramphenicol (UNACETYLATED) and the two products of the CAT enzyme, monoacetylated ^{14}C-chloramphenicol (MONO) and diacetylated ^{14}C-chloramphenicol (DI) are indicated. The level of expression of each ALV-S construction is expressed as a percentage of the positive control vector pIBISVCAT.

EXPRESSION RELATIVE TO pIBISVCAT (%)
100
80
60
40
20
pIBISVCAT
pIBISVCAT (+H)
pIBICAT
pIBICAT (+H)
pIBICAT-ALV1
pIBICAT-ALV1 (+H)
pIBICAT-ALV ΔI
pIBICAT-ALV ΔI (+H)
pIBICAT-ALV2
pIBICAT-ALV2 (+H)
pIBICAT-ALV3
pIBICAT-ALV3 (+H)
pIBICAT-ALV3 ΔI
pIBICAT-ALV3 ΔI (+H)
pIBICAT-ALV4
pIBICAT-ALV4 (+H)
← DI
← MONO
← UNACETYLATED

The addition of 1μM heme-arginate to the cell growth media reduced the level of CAT expression from pIBICAT-ALV1, pIBICAT-ALVΔI and pIBICAT-ALV2 by approximately 50%, but did not alter the level of expression from any of the other constructions. (Fig. 6-7). The deletion of the intron from pIBICAT-ALV1 and pIBICAT-ALV3 did not appear to affect the level of expression of the CAT gene driven by the ALV-S promoter either in the presence, or absence of heme-arginate (Fig. 6-7). The experiments described above were repeated numerous times and the results found to be reproducible. Heme repression of the constructions pIBICATALV-1, pIBICAT-ALVΔI and pIBICAT-ALV2 was not an artifact of the preparation of the heme (see section 2-6-2) as the level of pIBICATALV-1, pIBICAT-ALVΔI and pIBICAT-ALV2 expression was not reduced when transfected H4-II-E-C3 cells were incubated in the presence of the arginate preparation. Heme-arginate was used in these experiments in preference to heme alone due to its increased solubility in water.

6-3 Discussion

Since the early work of Granick (1966), which showed that ALV-S levels in chick embryo liver cells could be increased by drugs and that heme could prevent this increase, it has been thought that the synthesis of heme is controlled by end-product repression of ALV-S. Utilising nuclear run-on experiments in rat liver cell nuclei (Srivastava *et al.*, 1988), work in this laboratory has shown that heme control is exerted at the level of transcription. The present work is aimed at elucidating the transcriptional mechanism by which heme represses the transcription of the ALV-S gene. A rat genomic ALV-S clone was isolated by Dr. I. Borthwick in this laboratory. In the previous chapter, part of the rat ALV-S gene was sequenced and analysed by primer extension and mung bean nuclease protection experiments. This analysis permitted expression studies on the rat ALV-S gene in the rat hepatoma cell line, H4-II-E-C3.

The first question to be answered was whether heme control was exerted in H4-II-E-C3 cells into which the ALV-S/CAT constructions had been transfected, as

well as in cells which had been mock transfected. The results show that the presence of exogenous heme-arginate at a concentration of 1μM reduced the level of endogenous ALV-S mRNA. Heme repression of the ALV-S gene was therefore occurring under the experimental conditions used.

It was also observed that the transfection of pIBICAT-ALV1 into cells caused an increase in endogenous ALV-S mRNA, which was evident even in the presence of heme-arginate, implying that the construction was capable of relieving heme repression. The results infer that heme repression is mediated by a *trans*-acting factor(s). Based on this interpretation, the transcriptional factor(s) would bind to a region of the pIBICAT-ALV1 construction, thus making these factors unavailable for the repression of the endogenous ALV-S gene. Such an interpretation is not unprecedented; comparable *in vivo* competition experiments on the rat insulin 1 gene (Nir *et al.*, 1986), the immunoglobin heavy chain enhancer (Mercola *et al.*, 1985), the metallothionein gene (Seguin *et al.*, 1984), and gene transfer experiments on the immunoglobulin heavy chain (Imler *et al.*, 1987) and early genes of SV 40 (Gorman *et al.*, 1985) have been interpreted in a similar way. Both positive and negative *trans*-acting factors involved in cell type specific expression and induction of transcription have been identified in these experiments.

To confirm this interpretation and to localise the region of the ALV-S gene mediating heme repression, a series of expression studies were undertaken using the rat ALV-S gene. Varying lengths of the rat ALV-S gene were fused to the CAT structural gene and the expression of these constructions examined in the rat hepatoma cell line H4-II-E-C3. It was found that the constructions pIBICAT-ALV3, pIBICAT-ALV3ΔI and pIBICAT-ALV4 all expressed at 45-50% of the positive control, pIBISVCAT. pIBICAT-ALV1 and pIBICAT-ALV1ΔI expressed at only 36-39% of the level of pIBISVCAT, whilst pIBICAT-ALV2 expressed at an even lower, 15% of pIBISVCAT. This result indicates that a maximum of 160bp of rat ALV-S 5' flanking sequence is required for efficient expression of the ALV-S gene in H4-II-E-C3 cells. Within this region there are no known *cis*-acting sequences present, except the TATA box at -28.

In view of the fact that sequences within this region can promote transcription of the CAT gene at a level 50% below that of the strong SV 40 early gene promoter, it would be of some interest to define the positive sequence elements in this 160bp of ALV-S 5' flanking sequence that promote such a transcription rate.

The expression of a series of ALV-S/CAT constructions was examined in H4-II-E-C3 cells in the presence of 1μM exogenous heme-arginate. All constructions expressed at an equivalent level in the presence and absence of additional heme-arginate, except pIBIALVS-1, pIBICAT-ALVΔI and pIBICAT-ALV2. The level of expression of these constructions was reduced approximately 50% by exogenous heme-arginate. These results are consistent with the interpretation that all three constructions contain sequences necessary and sufficient for heme repression.

In the absence of exogenous heme-arginate the level of expression of pIBICAT-ALV1, pIBICAT-ALVΔ1 and pIBICAT-ALV2 was significantly lower than the other constructions. It seems likely that this reduction in expression resulted from repression by endogenous H4-II-E-C3 cellular heme, and/or from heme present in the foetal calf serum component of the media. The construction pIBICAT-ALV2 was repressed by heme-arginate to the same extent as pIBICAT-ALV1 and pIBICAT-ALVΔ1, but expressed at a lower basal level. This result infers the presence of positive *cis*-acting sequence(s) between positions -1200 and -2700 of the rat ALV-S gene. As the only region common to all three constructions is between positions -476 and -1200, this region should contain all the sequences necessary to mediate heme repression.

The removal of the intron from the 5' untranslated region of the rat ALV-S gene did not significantly alter the expression of the rat ALV-S gene in H4-II-E-C3 cells in either the presence, or absence of heme-arginate. This result would indicate that this intron probably plays no role in either promoting efficient transcription, or heme repression of the rat ALV-S gene, at least in these cells. This is in contrast to the HMG-CoA reductase gene, another end-product negatively regulated gene, where removal of the intron from the 5' untranslated region of this gene reduces expression by approximately 80% (Osborne *et al.*, 1985). However, like the ALV-S gene, the intron

in the 5' untranslated region of the HMG-CoA reductase gene plays no role in end-product repression of transcription (Osborne *et al.*, 1985).

Cis-acting sequences involved in negative repression of transcription have been found in a number of genes and most have been shown to behave like enhancers. Consequently, some have been termed *dehancers* (Remmers *et al.*, 1986), or *silencers* (Brand *et al.*, 1985). One better understood function of these sequences is to mediate cell specific expression by *trans*-acting repressors. Enhancers have been shown to be involved in the tissue specific expression of the albumin gene (Cereghini *et al.*, 1987), in modulating the activity of viral enhancers in embryonic cells (Gorman *et al.*, 1985) and in cells expressing the adenovirus E1A gene (Borreli *et al.*, 1984, Velcich and Ziff, 1985), and in the suppression of transcription of the rat insulin gene in nonpancreatic cells (Nir *et al.*, 1986).

End-product repression of transcription as occurs with the ALV-S gene is poorly understood in higher eukaryotes, although it has been elucidated in elegant detail in prokaryotes (reviewed by Ptashne, 1986) and yeast (reviewed by Guarente, 1984, Brent, 1985, Nasmyth *et al.*, 1987, Sternberg *et al.*, 1987). The best studied example of a eukaryotic repressor is SV 40 T-antigen, which stimulates the onset of viral DNA replication and represses the transcription of its own gene during the shift from the early to the late phase of the lytic cycle (Tooze, 1981). Although T-antigen binds to a *cis*-acting sequence in the SV 40 early gene promoter, this binding does not mediate end-product repression. Instead, T-antigen also binds another positive transcriptional factor, AP-2 and prevents it from binding to the SV 40 enhancer (Mitchell *et al.*, 1987).

End-product repression by cholesterol of the HMG-CoA synthase, HMG-CoA reductase and the light density lipoprotein (LDL) receptor genes has been studied in mouse L cells (Osborne *et al.*, 1985) and CHO cells (Sudhoff *et al.*, 1987). In the HMG CoA reductase gene, the sequences conferring repression of transcription have been, as with ALV-S, localised to an area of the 5' flanking region (Osborne *et al.*, 1985). In the LDL receptor gene, a 42bp element has been identified which can confer

end-product repression by sterols on a heterologous promoter. This 42bp element, located in the 5' flanking region, contains two 16bp repeats which exhibit both positive and negative transcriptional activities. Sterols are thought to repress transcription by opposing the action of a positive transcriptional factor that binds to a discrete promoter sequence (Sudhoff *et al.*, 1987).

To postulate a precise model for heme repression of the ALV-S gene, more information is required on the exact location, nature and number of sequences involved. In general, most genes are regulated through the interaction of multiple *cis*-acting sequences and *trans*-acting factors. It seems reasonable, based on our current knowledge of the regulation of other genes and on the data presented in this chapter, that this will be the case with the ALV-S gene.

Repression of gene expression through the action of *cis*-acting sequences and/or *trans*-acting factors is postulated to function by a number of mechanisms which differ with the gene and the function of the repression. Repressors have been shown to bind positive *trans*-acting factors, preventing their association with a sequence element. Such a repressor (T-antigen), prevents the binding of the AP-2 transcriptional factor to the SV 40 enhancer (Mitchell *et al.*, 1987). This mechanism may also mediate the repression of the LDL receptor gene by sterols (Sudhof *et al.*, 1987). The binding of a dominant repressor to a negative *cis*-acting sequence could displace bound positive factors, or prevent (possibly by steric hindrance) the binding of other unbound positive factors as is proposed to mediate the tissue specificity of the immunoglobin heavy chain enhancer (Imler *et al.*, 1987), and to account for the extinction of a differentiated phenotype after cell fusion (Killary *et al.*, 1984, Petit *et al.*, 1986). Negative regulatory elements could also modulate transcription by stabilising the binding of a *trans*-acting factor to its enhancer sequence, inhibiting subsequent interaction of the enhancer with factors bound downstream (Garcia *et al.*, 1987). Any of the above mechanisms could control repression of ALV-S gene transcription by heme, but at this stage there is insufficient experimental data to favor any particular mechanism. It is likely that the efficient transcription of the ALV-S gene requires the action of *cis*-acting sequences in

the regions of the ALV-S gene between positions -160 and +1 and -1200 and -2700. Heme repression appears to be the dominant factor in ALV-S transcription and requires the combined interaction of *cis*-acting sequence(s) located between positions -476 and -1200 of the ALV-S promoter and *trans*-acting factor(s).

The next phase of the work will utilise DN'ase footprinting and gel retardation studies to accurately define the number and nature of the *cis*-acting sequences involved in heme repression and efficient expression of the ALV-S gene. An additional important experiment that time prevented me from doing, but will be performed, is to transfect H4-II-E-C3 cells with constructions not containing sequences responsible for heme represssion, such as pIBICAT-ALV3 and pIBICAT-ALV4, and measure the response of endogenous ALV-S mRNA as in section 6-2-4. It would be expected that these constructions should not be capable of de-repressing the endogenous ALV-S. ALV-S mRNA levels should be similar to the level of ALV-S mRNA in the mock transfected control.

It should be noted that while ALV-S enzyme activity in hepatoma cells is substantially reduced by heme (at least 10 fold, see Fig. 6.5), the level of specific mRNA (Fig. 6.6) and CAT assay analysis of promoter constricts (Fig. 6.7) shows a consistent 50% reduction compared with control.

The explanation for this is that heme acts at two different levels. It inhibits both the transcription of the ALV-S gene and also substantially prevents the uptake of precursor ALV-S protein into the mitochondria where it is catalytically active.

As a final comment, it should be pointed out that the possibility has not been excluded that upstream cryptic promoters contribute to CAT activity measurements. This does seem unlikely, however, since no CAT activity is detected with pSVOCAT. Future experiments must determine whether correct initiation of the CAT transcripts is occurring, using either RNase protection or primer extension analysis.

CHAPTER 7

FINAL DISCUSSION AND SUMMARY

Chapter 7: Final discussion and summary

The transcriptional control of the ALV-S gene is of particular interest since most of the well characterised eukaryotic control mechanisms involve regulation of induction or development, and very few examples of end-product regulated housekeeping genes have been studied.

The work presented in this thesis has provided an initial understanding of the transcriptional mechanism by which the ALV-S gene is regulated and also provides a solid basis for further work aimed at elucidating the molecular mechanism of control of the ALV-S gene by heme. Initially the expression of the chicken ALV-S gene was examined in *Xenopus laevis* oocytes (Chapter 3). It was demonstrated that of the multiple *cis*-acting sequences known to play a role in the expression of a range of genes, few were functional in the chicken ALV-S gene. Only a single TATA box and GC box were found to contribute to the expression of the ALV-S gene in oocytes. The GC box at position -78 contributed approximately 70% of the level of expression of the ALV-S gene. The TATA box at position -28 was found to be strictly required for initiation of transcription of the ALV-S gene. Based on our current knowledge of the function of such *cis*-acting sequences this result was unusual and further work is currently underway in this laboratory examining the function of these *cis*-acting sequences in primary cultured chicken hepatocytes.

The endogenous Xenopus oocyte ALV-S was examined and found to be regulated by heme. Although endogenous ALV-S activity was controlled by heme in oocytes, heme repression of an injected chicken ALV-S construction could not be demonstrated under any of the conditions examined (Chapter 4). Either the construction examined did not contain those sequences responsive to heme or the Xenopus oocyte control proteins did not recognise the chicken sequence(s). If the former explanation is correct, this result could indicate that the heme responsive sequences are probably greater than 1.5Kb upstream of the transcriptional start site in the chicken ALV-S gene.

Because the chicken ALV-S gene had proved to be unsuitable for examining the mechanism by which heme represses the synthesis of ALV-S mRNA, a rat genomic clone was isolated in this laboratory with the aim of making a comparative sequence and expression analysis. Initially the rat ALV-S genomic clone was restriction mapped and the start of translation localised by southern blot hybridisation analysis. The rat ALV-S gene was then partially characterised by DNA sequencing, primer extension analysis and mung bean nuclease protection (Chapter 5). This work demonstrated that the rat ALV-S gene, unlike the chicken, had an intron in the 5' untranslated region and initiated transcription from multiple start sites. Although the rat ALV-S gene contained a number of sequences known to regulate transcriptional initiation in other genes, little overall homology was found with the chicken ALV-S gene.

The final project described in this thesis (Chapter 6) involved an examination of the expression of the rat ALV-S gene in a rat hepatoma cell line. The endogenous level of ALV-S mRNA in H4-II-E-C3 cells was examined and found to be repressible by heme. Interestingly, transfection of an ALV-S/CAT construction was found to increase the level of H4-II-E-C3 ALV-S mRNA. A series of constructions were made containing varying lengths of the ALV-S 5' untranslated region attached to the CAT reporter gene. The relative expression of these constructions was examined in H4-II-E-C3 cells in the presence and absence of exogenous heme. This analysis delimited the sequences responsible for heme repression to an 724bp area of the 5' flanking region of the ALV-S gene, as well as separate sequences essential to the efficient transcription of the ALV-S gene in this cell line.

DN'ase footprinting experiments are currently underway in this laboratory to further delimit the sequences responsible for both the heme repression and efficient transcription of the ALV-S gene, and to identify the *trans*-acting factors mediating these effects.

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