Cloning and characterisation of the cDNA and gene for sheep liver arginase

A thesis presented in partial fulfilment of the requirements

for the degree of Master of Science

in Biochemistry

at

Massey University

Palmerston North

Richelle Kaye Marshall

1997

ABSTRACT

Arginase (arginine amidinohydrolase, EC 3.5.3.1) is a ubiquitous enzyme, notably found in the liver of ureotelic animals. It plays a critical role in the hepatic metabolism of most higher organisms as a cardinal component of the urea cycle (Jenkinson *et al.*, 1996). Arginase has also been identified in numerous organisms and tissues where there is no functioning urea cycle. In animals, many extrahepatic tissues have been shown to contain a second form of arginase, closely related to the hepatic enzyme but encoded by a distinct gene or genes and involved in a host of physiological roles. Recent interest in arginase has been stimulated by it's demonstrated involvement with the metabolism of nitric oxide. Subcloning the sheep hepatic cDNA sequence would allow a ruminant arginase to be compared with other known arginases. Probing a sheep genomic library for the arginase gene could ultimately lead to the characterisation of regulatory elements of the gene.

Partial purification of sheep liver arginase was carried out to develop a DNA probe to screen a sheep liver cDNA library for the cDNA sequence but the protein was N-terminally blocked. An attempt was made to electroelute arginase from an SDS-PAGE gel with a view to cleaving the purified protein and sequencing some of the resulting peptides. But arginase could not be purified sufficiently for successful electoelution.

Total RNA was isolated from both sheep and rat liver. A product of the expected size was produced by RT-PCR on the rat RNA template, but could not be subcloned into a vector. PCR performed on a sheep cDNA library generated a PCR product which was subcloned and sequenced. The sequence had no similarity with known arginase sequences, and showed that the reverse primer sequence was present at both ends of the PCR product.

A region of the human arginase cDNA sequence was PCR amplified from the expression plasmid pTAA12. The PCR product was radiolabelled, and used as a probe to screen a sheep liver cDNA library. No positive clones were identified. Northern blot analysis of RNA isolated from sheep liver was carried out. The blot was probed with a fragment of the human arginase cDNA sequence. Nonspecific binding was observed.

ACKNOWLEDGMENTS

I would first of all like to thank my supervisors Mark Patchett and Kathryn Stowell. Thankyou for your assistance and advice throughout the practical work and writing of this thesis.

I would like to acknowledge Jo Mudford for carrying out the protein sequencing; Trenna Bythe for carrying out the isoelectric focussing step; Shaun Lott and Rachel Page for their technical assistance in the lab.

Thankyou to the past and present members of the Twilight zone, and other members of the Biochemistry department for their friendship and support throughout my masters.

I would finally like to thank my family for all their support and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A_{260}	Absorbance at 260 nm
A_{280}	Absorbance at 280 nm
AMP	Ampicillin
APS	Ammonium persulphate
AR	Analtical reagent
ATP	Adenosine triphosphate
bp	Base pair(s)
BRL	Bethesda research laboratories
BSA	Bovine serum albumin
CAPS	3-(Cyclohexylamino)-1-propanesulfonic acid
C/EBP	CCAAT/enhancer binding protein
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CIP	Calf intestinal phosphatase
CM	Carboxymethyl
CSPD®	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(-5'-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-yl) phenyl phosphate
dATP	Deoxyadenosine triphosphate
dCTP	Deoxypyrocytidine triphosphate
dNTP	Deoxynucleotide triphosphate
DEPC	Diethylpyrocarbonate
Dept	Department
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetate
EEO	Electroendosmosis
h	Hours(s)
HIS	Histidine
HNF-4	Hepatocyte nuclear factor-4
IEF	Isoelectric focussing
IPTG	Isopropyl β-D-thiogalactopyranoside
kb	Kilobase
kDa	Kilodalton
LB	Luria-bertani
min	Minutes(s)
Mn	Manganese
MOPS	3-(N-Morpholino) propanesulfonic acid
mRNA	Messenger RNA
M_r	Relative molecular weight
NA	Not applicable
NADH	Nicotinamide adenine dinucleotide
PCR	Polymerase chain reaction

PEG	Polyethylene glycol
Pfu	Plaque forming units
pKS	pBluescript® KS II
PMSF	Phenylmethylsulfonyl fluoride
PNK	Phosphonucleotide kinase
Q	Ubiquinone
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revs per minute
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
s	Second(s)
S	Subunit
SD	Standard deviation
SDS	Sodium dodecyl suphate
SDS-PAGE	Sodium dodecyl suphate-polyacrylamide gel electrophoresis
Taq	*Thermus aquaticus*
TAE	Tris acetate EDTA
TEMED	N,N,N′,N′-tetramethylethylenediamine
Tris	Tris-(hydroxymethyl) aminomethane
TsAP	Temperature sensitive alkaline phosphotase
T4 PNK	T4 phosphonucleotide kinase
U	Unit
UV	Ultraviolet
(v/v)	Volume: volume ratio
(w/v)	Weight: volume ratio
X-gal	5-bromo-4-chlor-3-indoyl β-D-galactopyranoside

Chapter 1

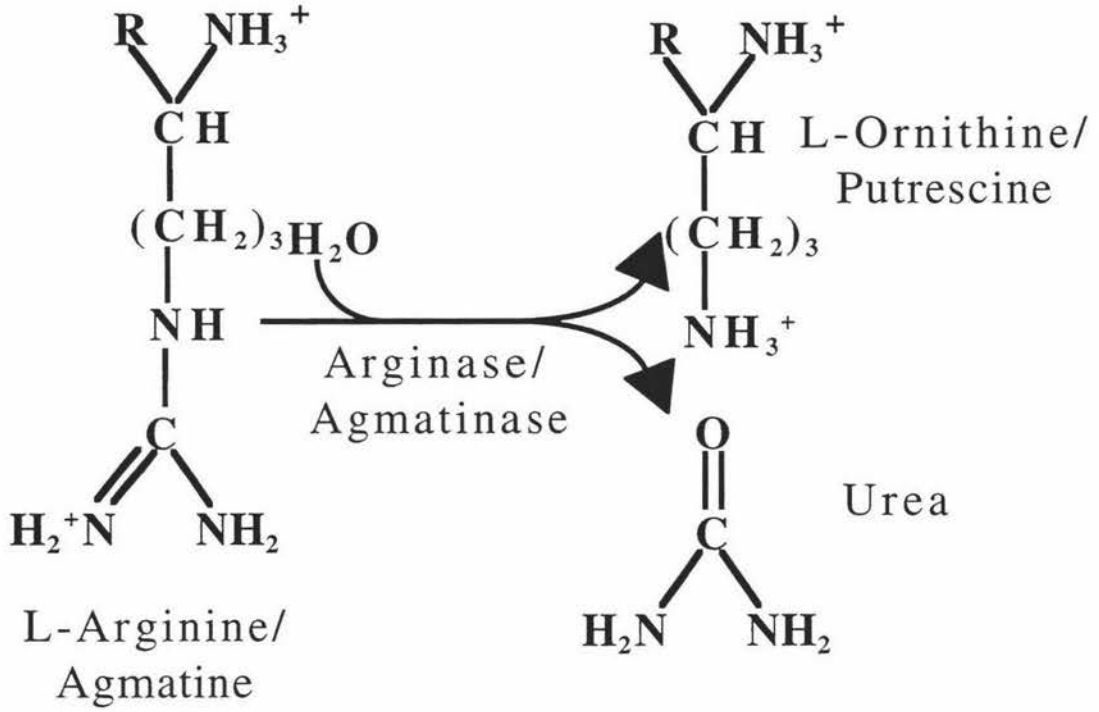
INTRODUCTION

1.1 Diversity of arginase

Arginase has been found in a number of organisms including bacteria, fungi, plants, and animals. The full nucleotide sequence of arginase has been determined for the following species: *Bacillus caldovelox*, *Bacillus subtilis*, *Coccidioides immitis*, *Agrobacterium tumefaciens*, *Synechocystis sp.*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Emericella nidulans*, *Neurospora crassa*, *Arabidopsis thaliana*, *Xenopus laevis*, *Rana catesbiana*, mouse, rat and human. In several of these species especially the higher vertebrates more than one arginase gene has been cloned. Figure 4.1 shows the amino acid sequence alignment of these arginases.

Sequence analysis suggests that members of the arginase family share a common evolutionary origin with the agmatinase (agmatine ureohydrolase, EC 3.5.3.11) family (Ouzounis and Kyrpides, 1994). Both families are involved in arginine catabolism pathways and hydrolyse their substrates (arginine and agmatine respectively) to produce urea (see Figure 1.1). Sequence analysis has also revealed that these families also show similarities with the forminoglutamate hydrolase enzyme from *Klebsiella aerogenes* , and to a protein encoded by an open reading frame in the HMf locus of the archaebacterium *Methanothermus fervidus.*

Figure 1.1 The enzyme reactions catalysed by arginase and agmatinase



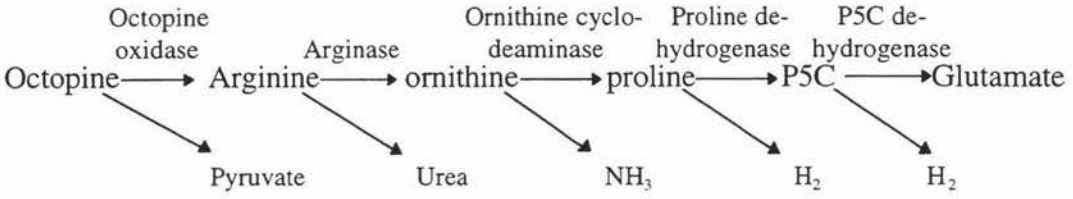
Arginase catalyses the hydrolysis of L-arginine to L-ornithine and urea, whereas agmatinase catalyses the hydroylsis of agmatine to putrescine and urea. In the reaction catalysed by arginase the R-group is a carboxylic acid group, whereas in the reaction catalysed by agmatinase the R-group is a hydrogen atom.

The function of the reaction catalysed by arginase and the regulation of the enzyme differ depending on the organism. Some of these differences are described below.

1.1.1 *Agrobacterium tumefaciens*

Inoculation of plant wound sites with *A. tumefaciens* induces crown gall plant tumour development. The crown gall tumour synthesises opines (an amino acid derivative), the most common of which are octopine and nopaline. Opines are catabolised by the bacterium and used as its sole energy, carbon and/or nitrogen source. Arginase is the second enzyme in this catabolic pathway (Figure 1.2) (Cho *et al.*, 1996).

Figure 1.2 The proposed pathway for catabolism of octopine

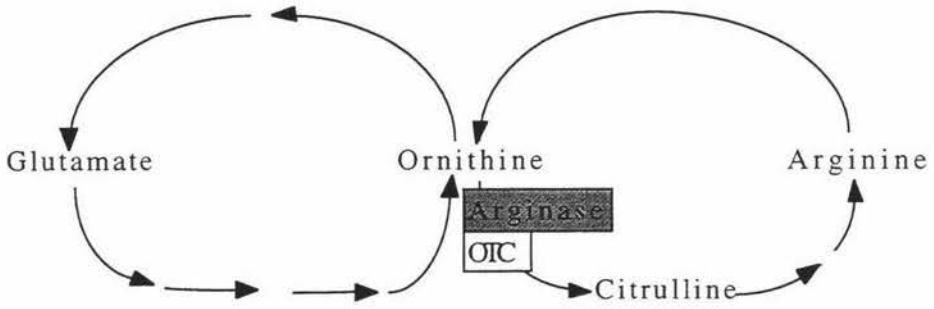


Octopine is catabolised to glutamate in 5 steps. Arginase is the second enzyme in the pathway, converting arginine to ornithine and urea (Cho *et al.*, 1996).

1.1.2 *Saccharomyces cerevisiae*

Arginase acts in *S. cerevisiae* as the first enzyme of the arginine catabolic pathway. Arginase also regulates the activity of ornithine carbamoyltransferase. When the catabolic pathway is functioning, arginase will bind to ornithine carbamoyltransferase in the presence of ornithine and arginine, inhibiting its activity and preventing the wasteful recycling of ornithine back into arginine (Messenguy and Wiame, 1969; Messenguy *et al.*, 1971) (Figure 1.3). This type of regulation is termed 'epienzymatic' and involves a trimer of arginase binding to a trimer of ornithine carbamoyltransferase, to inhibit the latter enzyme (Jenkinson *et al.*, 1996). Epienzymatic regulation has also been observed in *Bacillus subtilis* (Issaly and Issaly, 1974). Palus (1983) speculated that arginase and ornithine carbamoyltransferase are able to interact with a high degree of specificity because they are structurally similar and may have a common evolutionary origin.

Figure 1.3 Regulation of ornithine carbamoyltransferase by arginase when the arginine catabolic pathway is active



Arginase binds to OTC (ornithine carbamoyltransferase) inhibiting citrulline and subsequent arginine synthesis from ornithine. This inhibition allows ornithine to be catabolised into glutamate through the arginine catabolic pathway.

1.1.3 Plants

In *Arabidopsis thaliana* the conversion of seed storage protein to seedling protein involves arginine breakdown, catalysed by arginase. A 10 fold increase in seedling arginase activity during the 0 to 6 day interval after germination has been observed (Zonia *et al.*, 1995). This is consistent with the observation that arginase activity in soybean axes (the region where the leaf meets the stem) increased sharply during germination (Kang and Cho, 1990). Many plant species store seed protein nitrogen as arginine, which is released and catabolised during germination (Polacco and Holland, 1993).

1.1.4 *Xenopus laevis*

Three nonhepatic forms of arginase have been cloned since the gene for the liver-type enzyme was isolated. They were found to have 60% identity with mammalian and *X. laevis* liver arginase proteins. Distinct regulatory patterns of these *X. laevis* arginases have been observed during metamorphosis and their expression has been shown to be developmentally regulated (Patterton and Shi, 1994). Only traces of arginase mRNA are detected in pre-metamorphic tadpoles, but it's accumulation increases very markedly at the onset of natural metamorphosis and is constitutively expressed at high concentrations upon completion of this developmental process. This increase in activity compensates for

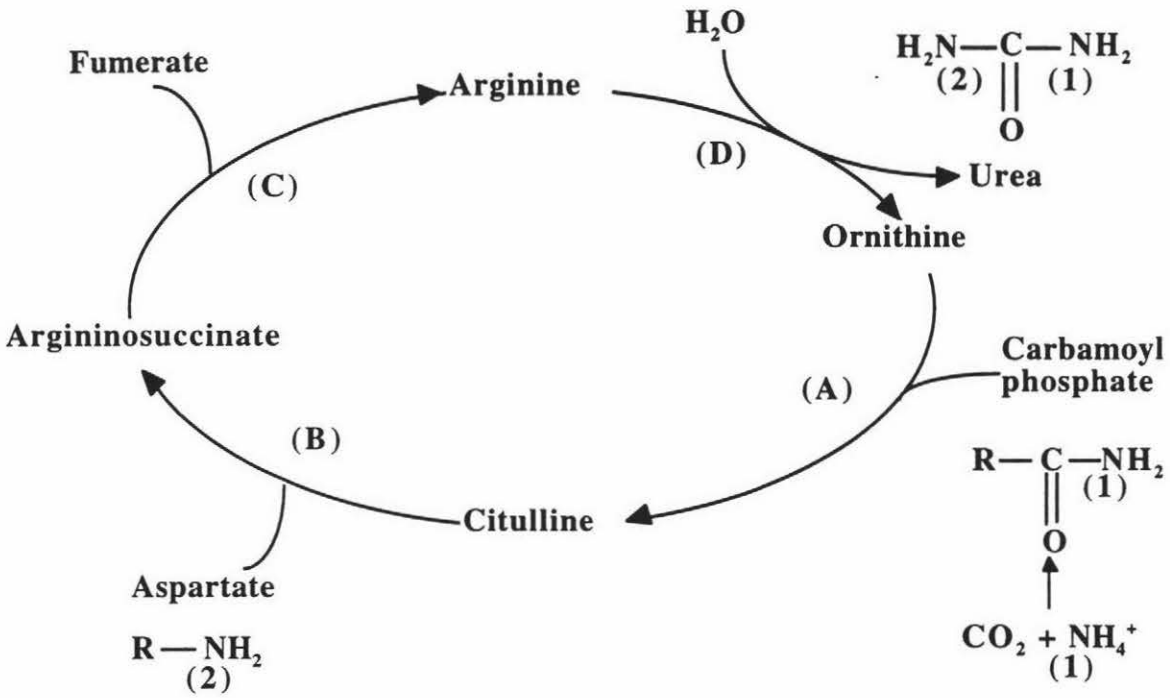
the change from ammonia excretion to urea excretion as the aquatic tadpole is transformed into an amphibious adult (Cohen, 1970; Weber, 1967).

1.1.5 Humans

In humans two isoforms of arginase have been identified, AI and AII for the hepatic and non-hepatic isoforms respectively. Both isoforms of arginase share certain physico-chemical properties but are immunologically distinct (Wang *et al.*, 1995).

The AI isoform is one of the five essential enzymes of the urea cycle. It is the last enzyme in the cycle, catalysing the hydrolysis of L-arginine to L-ornithine and urea (Figure 1.4). The role of the urea cycle is to take ammonia formed during the breakdown of amino acids and convert it into the excretory product urea (Stryer, 1995). The average adult human excretes ~10 kg of urea per year (Kanyo *et al.*, 1996), and this keeps the body in nitrogen balance. The L-arginine structure (see Figure 1.1) incorporates 4 nitrogen atoms, making L-arginine well suited to its role in nitrogen removal.

Figure 1.4 The urea cycle



One of the nitrogen atoms [labelled (2)] of urea synthesised by this pathway is transferred from an amino acid, aspartate. The other nitrogen atom [labelled (1)] and the carbon atom are derived from NH_4^+ and CO_2. Ornithine is the carrier of these carbon and nitrogen atoms. The cycle is as follows: first a carbamoyl group is transferred from carbamoyl phosphate to ornithine to form citrulline, in a reaction catalysed by ornithine carbamoyltransferase (A); argininosuccinate synthetase catalyses the condensation of citrulline and aspartate (B); argininosuccinase cleaves argininosuccinate to arginine and fumarate (C); arginine is then hydrolysed to urea and ornithine by arginase (D) (Stryer, 1995).

1.2 Liver-type arginase deficiency

In humans, deficiency in the AI isoform is an autosomal recessive disorder (Uchino *et al.*, 1995), which is characterised clinically by argininemia, progressive mental impairment, growth retardation, spasticity, and periodic episodes of hyperammonemia which can cause coma. Argininemia is heterogeneous at the molecular level, being produced by a variety of point mutations rather than substantial deletions within the structural gene (Grody *et al.*, 1992). Based on *in vitro* expression tests, these mutations can be considered either moderate or severe. While the quality of life can severely deteriorate in most patients suffering from argininemia, some do show remarkable improvement in

neurological symptoms while on controlled diets. Plasma arginine levels during treatment were found to be correlated to the severity of the various types of molecular defects in the arginase gene (Uchino *et al.*, 1995). Studies carried out by Uchino *et al.* (1995) showed that patients with two severely mutated alleles did not respond to dietary treatment and plasma arginine levels were over 400 μM. Whereas patients with argininemia resulting from one moderately mutated, two moderately mutated or one severely mutated allele(s) have been observed to respond well to dietary treatment, with plasma arginine levels below 300 μM. Normal adult arginine levels range between 100-300 μM (Curtius and Roth, 1974).

Patients suffering from arginase deficiency can not tolerate a protein-rich diet because amino acids ingested in excess of the minimum daily requirements for protein synthesis are deaminated in the liver, producing free ammonia in the blood. NH_3 is toxic in animals with excessive amounts of NH_3 causing alkalisation of cellular fluids, which effects cellular metabolism in a complex manner. In addition, high levels of NH_4^+ lead to an increase in the formation of glutamine through the glutamine synthesis pathway (Figure 1.5). This pathway occurs in the brain and depletes cellular NADH and α-ketoglutarate required for ATP production in the cell. Overall, NH_3 may interfere with the very high levels of ATP production required to maintain brain function (Lehninger, 1993).

Figure 1.5 Glutamine synthesis pathway (Stryer, 1995)

$$NH_4^+ + \textbf{α-Ketoglutarate} + NADH + H^+ \rightarrow \textbf{Glutamate} + NAD^+ + H_2O$$

$$\textbf{Glutamate} + NH_4^+ + ATP \rightarrow \textbf{Glutamine} + ADP + P_i + H^+$$

Arginase deficiency, the rarest of the inborn errors, has been considered the least lifethreatening by virtue of the typical absence of neonatal hyperammonemia and the longer lifespan of affected individuals. The longer lifespan has been attributed to the fact that ureagenesis can still occur in these patients through a second arginase isoform AII, located predominantly in the kidney. In patients studied from infancy, AI activity was absent in the liver, and levels of AII activity were elevated (Grody *et al.*, 1993).

1.3 Nonhepatic arginase

Extrahepatic arginase AII is found in many locations. AII may be located within the mitochondrial matrix, but neither the subcellular location nor the number of extrahepatic arginase isoforms have been determined precisely. AII may have a number of different roles in different tissues and in different organisms including participation in polyamine metabolism, proline and glutamate synthesis, γ-amino-butyric acid (GABA) formation, immune system function and nitric oxide synthesis (Jenkinson *et al.*, 1996).

In general, both AI and AII enzymes are similar physicochemically. They have similar reaction kinetics, share a requirement for the cofactor manganese and have similar sizes and quaternary conformations (Jenkinson *et al.*, 1996). The amino acid sequence of the mitochondrial isoform is 59% identical to AI and has a 22 residue putative N-terminal mitochondrial targeting sequence. It is synthesised as a larger precursor of ~40 kDa and is imported into the mitochondria for processing to the mature form of ~38 kDa (Gotch *et al.*, 1996).

1.4 Structure and function of arginase

Arginase in its active form is a oligomeric enzyme with a subunit molecular weight of 30-42 kDa (Jenkinson *et al.*, 1996) requiring the divalent ion manganese (Mn^{2+}) for both activity and structural stability.

A comparison of the amino acid sequences shows that three histidine residues have been highly conserved across evolutionary divergent species. These residues are His101, His126 and His141 in the rat. His101 and His126 are thought to be ligands to the binuclear Mn^{2+} centre of the enzyme; His141 has been proposed to have a catalytic role (Cavalli *et al.*, 1994).

Work carried out by Kanyo *et al.* (1996) found that rat arginase is a trimer with two Mn^{2+} ions bound to each subunit (see Figure 1.6). Both of these Mn^{2+} ions are needed for full enzymatic activity. The reaction catalysed by arginase is thought to involve a nucleophilic attack whereby Mn^{2+} is substituted for H_2O. It has been suggested that a common protein-derived ligand or water molecule bridges the Mn^{2+} ions (Cavalli *et al.*, 1994). The enzyme

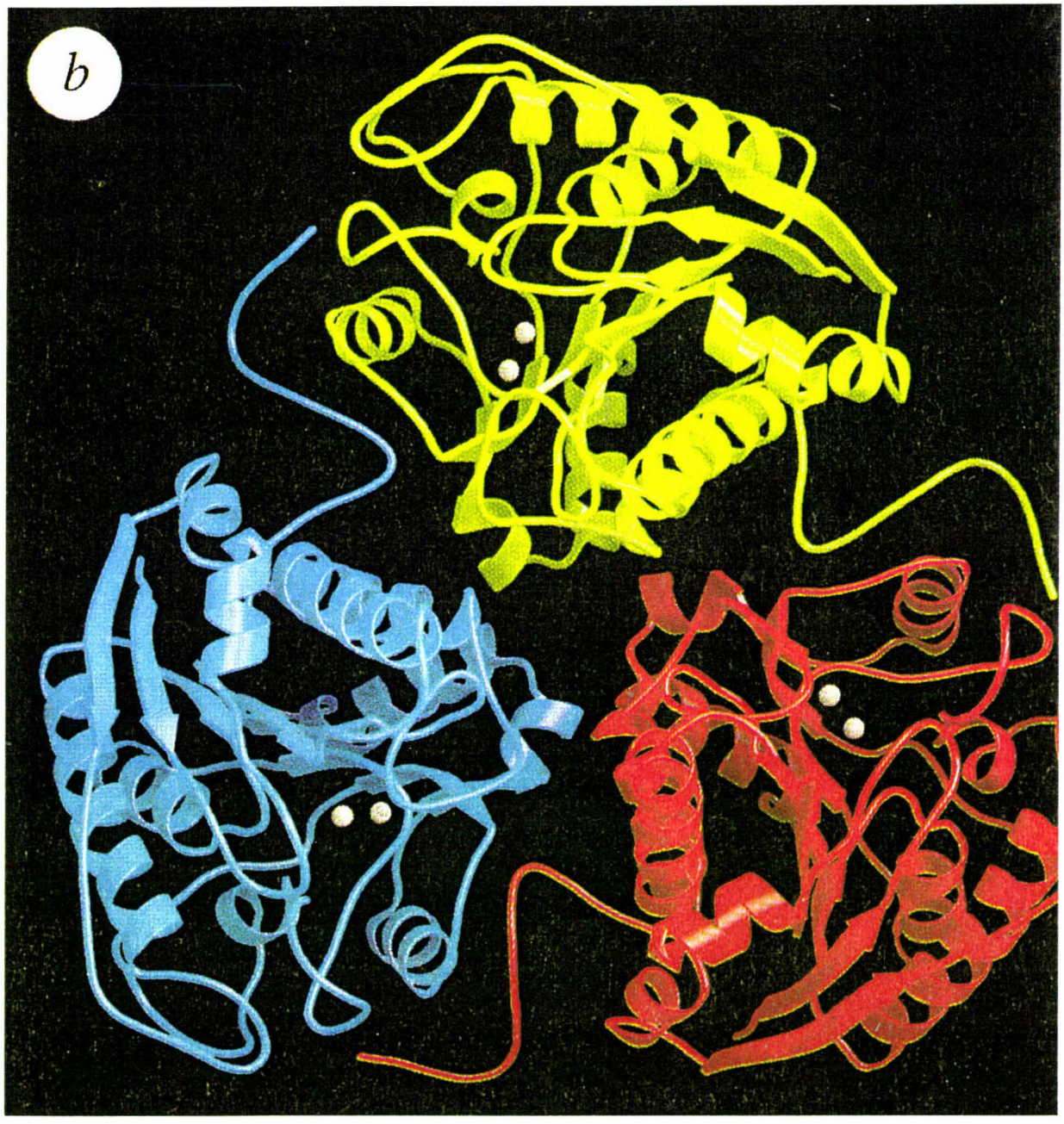
bound metal is thought to be inaccessible to added chelators due to the lack of rapid inhibition of activity in their presence. Mn^{2+} activation is temperature independent in the rat suggesting that the metal site is located in a protein pocket and that conformational flexibility of the protein is an important factor in metal binding (Reczkowski and Ash, 1994). X-ray crystal structures of both the rat and *B. caldovelox* arginase have confirmed that the binuclear Mn^{2+} centre is located in a pocket (Bewley *et al.*, 1996).

Inhibition studies have led to the conclusion that the second metal of the binuclear center could coordinate the substrate through the carboxylate group, or serve to modulate the activity of the water molecule bound to the adjacent metal ion (Reczkowski and Ash, 1994).

Rat arginase is capable of catalysing the hydrolysis of L-argininamide, L-homoarginine, L-argininic acid and agmatine but 15-5000 fold slower than the k_{cat} for L-arginine. The enzyme is competitively inhibited by L-ornithine (L-ornithine binds at the same location as the substrate) indicating a rapid-equilibrium random mechanism for the enzyme (Reczkowski and Ash, 1994).

A decrease in liver arginase activity is seen in rats fed a Mn-deficient diet, therefore Mn is strongly implicated in the regulation of hepatic arginase activity (Visek *et al.*, 1992; Brock *et al.*, 1993).

Figure 1.6 The structure of rat hepatic arginase



Ribbon-plot of the rat arginase trimer; the Mn^{2+}- Mn^{2+} cluster in the active site of each monomer is represented by a pair of spheres (Kanyo *et al.*, 1996).

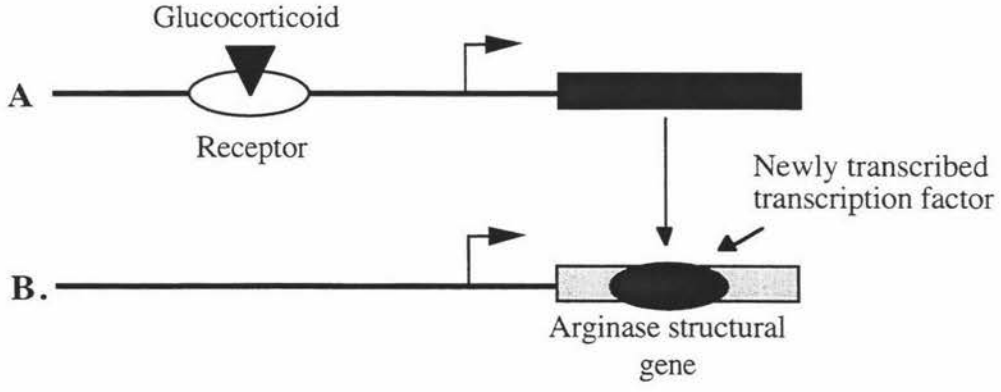
1.5 Transcriptional Regulation of Arginase

Morris *et al.*, (1987) and Schimke *et al.*, (1962) both observed that after birth in humans, the arginase gene is up-regulated by nutritional changes including protein intake. Glucocorticoid hormones and glucagon are responsible for mediating this developmental and nutritional activation of the arginase gene (Morris *et al.*, 1987; Dizikes *et al.*, 1988; Nebe and Morris, 1988; Ulbright *et al.*, 1993).

1.5.1 Arginase response in humans to glucocorticoid hormones

The transcription of the gene for arginase is induced by glucocorticoids in a delayed secondary manner (Figure 1.7). The secondary response seems to be mediated by a transcription factor(s) that is synthesised *de novo* through the primary response. mRNA accumulation in the secondary response follows a delayed time course, typically requiring a time lag of several hours and is sensitive to inhibitors of protein synthesis such as cycloheximide (Gotch *et al.*, 1994). Cycloheximide is an antibiotic which inhibits the peptidyl transferase (peptide bond formation) step of protein synthesis (Stryer, 1995).

Figure 1.7 Proposed model for the delayed secondary response to glucocorticoid



A) The primary response to glucocorticoid is brought about by binding of the glucocorticoid-receptor complex (represented by filled-in triangle and oval) to regulatory sequences of target genes.

B) The secondary response is mediated by one or more transcription factors that are synthesised *de novo* through the primary response. The hooked arrows represent the transcription start sites (Takiguchi and Mori, 1995).

An enhancer region residing in an ~200 bp segment around intron 7, located 11 kb downstream of the rat arginase gene transcription start site, has been identified. Induction of a reporter gene under the control of the arginase enhancer exhibited a delayed time course compared with that under the control of the mouse mammary tumour virus promoter, which shows a typical primary glucocorticoid response. Therefore it has been suggested that the arginase enhancer is responsible for mediating the secondary glucocorticoid response (Takiguchi and Mori, 1995). Four protein binding sites have been detected in this enhancer region, two of which are recognised by CCAAT/enhancer binding protein (C/EBP) family members. The C/EBP family members can be regarded as candidates for transcription factors that mediate the secondary response of the arginase gene (Takiguchi and Mori, 1995).

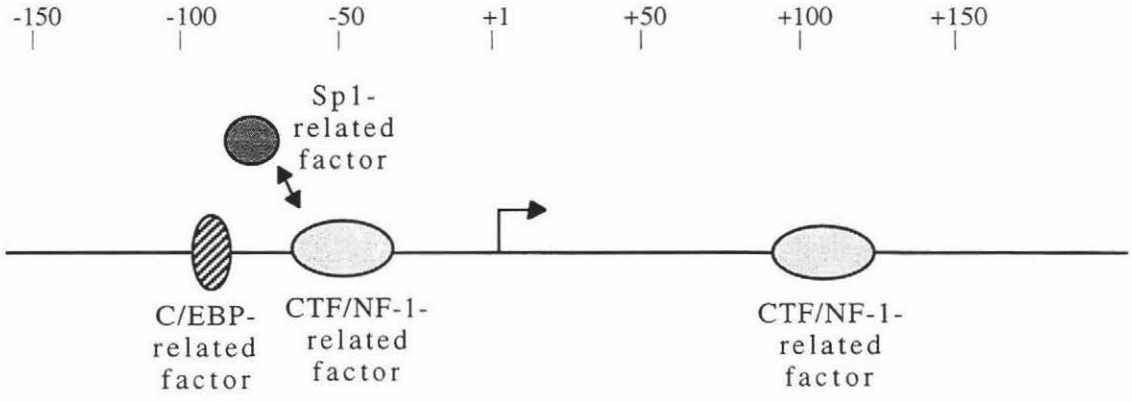
The genes of the four urea cycle enzymes (Figure 1.4) are generally regulated in a coordinated manner both developmentally and nutritionally, therefore one or more shared transcription factors may be involved in this coordinated regulation. At present the most likely candidates are the C/EBP family members which have been found to interact with the genes of three urea cycle enzymes (Takiguchi and Mori, 1995).

1.5.2 Promoter analysis

The rat and human arginase genes are about 12 kb long and are split into eight exons. A comparison of these two genes revealed that the immediate 5′ flanking region of the human gene up to position -105 is 84% identical with the corresponding segment of the rat gene (Takiguchi *et al.*, 1988). The cap site was determined for the human arginase gene by S1 nuclease mapping and primer extension. A TATA box-like sequence was located 28 bases upstream from the cap site, and sequence similar to the binding sites of the transcription factor CTF/NF-1 (a CAAT box-binding protein), was located 72 bases upstream (Takiguchi *et al.*, 1988). 5′ deletion analysis in rat liver nuclear extracts revealed a positive regulatory region spanning the nucleotides -90 to -51 (Figure 1.8). Two protein-binding sites were found to overlap this -90 to -51 bp region by DNase I footprinting (Takiguchi and Mori, 1991). The more upstream site around -90 bp binds C/EBP-related factors, and the -60 bp site is recognised in a mutually exclusive manner, by two factors each related to CTF/NF-1 (Santoro *et al.*, 1988) and Sp1 (Takiguchi and Mori, 1995). Another site, around +100 bp, is recognised by a CTF/NF-1 related factor (Takiguchi and Mori, 1995). Recent work carried out by Chowdhury *et al.* (1996) found

that the human arginase promoter activity was repressed by hepatocyte nuclear factor-4 (HNF-4). They speculated that HNF-4 is involved in fine regulation of the arginase gene in the liver, or shutdown of the gene in nonhepatic tissues without binding directly to the promoter region. It has not yet been determined how this repression by HNF-4 occurs as there is no evidence of any direct binding of HNF-4 to the arginase promoter region (Chowdhury et al., 1996).

Figure 1.8 Schematic representation of the rat liver-type arginase promoter



The hooked arrow indicates the transcription start site. Ovals show binding factors. The dual arrow represents mutually exclusive binding (Takiguchi and Mori, 1991).

To date, most work has been carried out using the rat liver arginase cDNA to investigate the promoter region. Given that the human arginase promoter region has a high identity with the rat, regulation in the human may be similar.

1.5.3 Regulation of pig arginase in response to hormones

It has been observed that urea is produced in enterocytes (within cells) of post-weaning pigs but at a much lower level than in the liver (Meijer *et al.*, 1990). Wu (1995) found there was a 50-100 fold increase in arginase activity in enterocytes of post-weaning pigs compared with newborn and suckling ones. This increase in activity was found to be sufficient to account for the increased rate of urea production from arginine, ammonia and glutamine in enterocytes of post-weaning pigs. It has been suggested that this induction is produced in response to increased plasma concentrations of the circulating steroid hormone cortisol which is the major glucocorticoid secreted by the adrenal cortex in pigs

(Worsaae and Schmidt, 1980). It had been reported previously that administration of cortisol to 5-8 day old rats results in a marked increase in hepatic arginase to adult levels within 24 h (Greengard *et al.*, 1970). Cortisol also increases both the mRNA level and the enzyme activity of arginase in cultured rat hepatocytes (Morris, 1992). Therefore cortisol may mediate the induction of arginase, and consequently urea synthesis, within the cells of post-weaning pigs.

1.6 Arginase activity in ruminants

In ruminants, the degradation of nitrogenous compounds by rumen micro-organisms leads to the production of high levels of ammonia in the rumen. As a consequence high levels of ammonia assimilation occur in the rumen to prevent ammonia toxicity. Enzymes utilising ammonia in the rumen epithelium include glutamate dehydrogenase, glutamate synthetase, therefore arginase and other urea cycle enzymes may not be the main mechanisms for ammonia detoxification in the rumen epithelium (Aminlari and Vaseghi, 1992). Table 1.1 illustrates that arginase activity from rumen tissue is no more than 15% the observed activity from liver extracts of sheep and cattle.

Table 1.1 Mean (± SD) specific activity of arginase in crude extracts from different tissues of sheep and cattle (Aminlari and Vaseghi, 1992)

Tissue	**Specific activity (U / mg)**
Sheep liver	**0.40 ± 0.04**
Sheep rumen	**0.06 ± 0.008**
Cattle liver	**0.42 ± 0.03**
Cattle rumen	**0.05 ± 0.01**

Attempts to sequence sheep liver arginase cDNA were performed during the course of this thesis because 1) little is known about ruminant arginases and 2) access to both sheep liver tissue and a sheep liver cDNA library was available.

1.7 Experimental aims of thesis

The primary aim of this project was to sequence sheep liver-type arginase cDNA. This sequence could then be compared with other known arginases.

Initially it was intended to partially purify arginase to obtain protein sequence information to develop a DNA probe to screen a sheep liver cDNA library. As this proved unsuccessful several PCR-based approaches were attempted. RT-PCR on sheep liver RNA, PCR on cDNA from a sheep liver cDNA library (using degenerate primers designed to conserved regions of known arginase protein sequences), and RT-PCR on rat liver RNA (using primers designed to rat arginase cDNA sequence) were all tried. Another approach involved the use of a fragment of the human arginase cDNA as a probe to screen a λ-ZAP sheep liver cDNA library for a positive clone.

Chapter 2

MATERIALS AND METHODS

The restriction enzymes *Eco*R 1, *Hin*d III and *Nhe* I, dNTPs, all modifying enzymes, TRIZOL™ LS Reagent, NZY powder, 1 Kb DNA molecular size markers and the Superscript preamplification Kit were all supplied by Gibco Bethesda Research Laboratories Life Technologies Inc. (Penrose, Auckland, NZ).

The restriction enzymes *Mun* I, *Fok* I and *Sty* I were supplied by New England Biolabs (MA, USA).

10x PCR buffer and 25 mM $MgCl_2$ were supplied by Promega Corporation (Madison, USA) or Gibco Bethesda Research Laboratories Life Technologies Inc. (Penrose, Auckland, NZ). *Taq* polymerase was supplied by Boehringer Mannheim (Germany) or Gibco.

PCR primers were obtained from Amrad Pharmacia Biotechnology Ltd. (Auckland, NZ) or Oligos etc Inc. (CT, USA).

CM-Sepharose, Reactive Red 120, L-arginine, mineral oil, SDS 6H molecular weight markers, low EEO agarose, lysozyme and ampicillin were all supplied by Sigma Chemical Co. (St. Louis, USA).

Sephadex G-200 gel filtration beads and Phenyl Sepharose were both from Pharmacia LKB Biotechnology (Uppsala, Sweden).

Acrylamide, bis-acrylamide, TEMED, SDS and EDTA were supplied by Serva (Heidelberg, Germany).

Sequenase version 2.0, Coomassie Brilliant Blue R-250 and G-250 were supplied by United States Biochemical Corporation (Cleveland, Ohio, USA).

40% Acrylamide / bis-acrylamide solution, hydroxyapatite powder, analytical grade mixed ion-exchange resin AG 501-X8 (D), (20-50 mesh), and Prep-a-Gene DNA purification system were supplied by Biorad Laboratories (California, USA).

The phagemid pBluescript® KS II was supplied by Strategene Ltd (La Jolla, CA, USA).

The Magic / Wizard DNA purification system™ was supplied by Promega Corporation (Madison, USA).

Absolute ethanol, 75% ethanol, AR methanol, formamide, ethidium bromide dichloromethylsilane and diethyl pyrocarbonate were all supplied by BDH (Poole, England). Formaldehyde, chloroform and glycerol were Univar grade reagents (Auburn, NSW, Austalia).

Microsep™ centrifugal concentrators were supplied by Filtron Technology Corporation (Northborough, MA).

The PVDF (Immobilan™-P) transfer membrane was from Millipore (MA, USA).

RX medical X-ray film was from Fuji Photo Film Company Ltd. (Japan). Photographic developer and fixer was purchased from Eastman Kodak (NZ, USA).

The rediprime random labelling kit and nylon membrane (Hybond™ N^+) were supplied by Amersham, Life Science (England).

Calf intestinal phosphatase and the DIG High Prime DNA labelling and detection starter kit were from Boehringer Mannheim (Germany).

Radioactive [α-^{32}P]-dATP and [α-^{32}S]-dATP was supplied by DU PONT NEN™.

The amplified sheep liver λ-ZAP cDNA library was prepared by C. Stayner in February 1993; kindly provided by John Tweedie (Dept of Biochemistry, Massey University).

The pTAA12 expression plasmid was kindly provided by Toshiaki Kono, Institute for Medical Genetics, University Medical School (Kumamoto, Japan).

pTG3954 was kindly provided by Gareth Morgan, Sir William Dunn School of Pathology (University of Oxford, UK).

2.1 Partial purification of sheep liver arginase

Unless otherwise stated all procedures in this section were carried out at room temperature (20-25°C). Supernatant and column fractions were stored at 4°C between purification steps. After each step a 300 µl sample was stored at -70°C for activity and protein assays. All buffer solutions used for purification procedures were adjusted to the required pH at room temperature.

2.1.1 Step 1 Extraction

50 g of sheep liver (from a sheep which died of Battens disease 9/9/94) was homogenised in 2 volumes of 0.02 M Tris-HCl extraction buffer, pH 7.5, containing 0.1 M KCl and 0.05 M $MnCl_2$ at high speed in a waring blender. 0.5 ml of 0.2 M PMSF in acetone was then added to inhibit serine proteases. The homogenate was centrifuged for 15 min at 20 000 x g in a SS34 rotor (sorval) at 4°C. The supernatant was decanted and stored on ice.

2.1.2 Step 2 Acetone

1.5 volumes of acetone (previously chilled to -10°C) was slowly added to the homogenate in a -10°C salt bath. The temperature was kept below 5°C to prevent denaturation of the arginase enzyme which occurs in the presence of acetone at high temperatures. The solution was allowed to stand for 10 min on ice before centrifuging at 20 000 x g for 5 min carefully sealed in tubes in a GSA rotor (sorval) at -10°C. The supernatant was discarded and the pellet was resuspended in 35 ml (0.7 volumes of original liver weight) of buffer consisting of 0.02 M MOPS-NaOH, 0.1 M aspartic acid, 0.05 M $MnCl_2$, pH 7.5. The resuspension was centrifuged for 10 min at 4°C at 20 000 x g in a SS34 rotor. The supernatant was carefully decanted off and carried forward to the next step.

2.1.3 Step 3 Dialysis

The supernatant was placed in a dialysis bag and dialysed overnight against 2 L of 0.01 M MOPS-NaOH buffer, pH 7.5.

2.1.4 Step 4 Heat treatment

The dialysed supernatant was heated at 60°C for 25 min, cooled on ice and centrifuged for 10 min at 20 000 x g in a SS34 rotor at 4°C. The supernatant was decanted off and stored on ice at 4°C.

2.1.5 CM-Sepharose CL-6B column

A CM-Sepharose CL-6B cation exchange column was used for the first chromatographic step in the purification.

Buffers

Starting buffer: 0.02 M MOPS-NaOH; pH 6.7

Salt gradient buffer: 0.5 M KCl in 0.02 M MOPS-NaOH; pH 6.7

Preparation, pouring and running of the CM-Sepharose CL-6B ion exchange column was carried out at 4°C.

50 ml of preswollen CM-Sepharose CL-6B matrix was washed in starting buffer to remove any traces of the 20% ethanol that the ion exchanger was stored in. The washed matrix was mixed with 17 ml of starting buffer to form a slurry which was then degassed at 4°C for 1 h. The degassed slurry was poured into a 15 x 2.5 cm column and the resin equilibrated at a flow rate of 1 ml / min with 100 ml (2 bed volumes) of starting buffer.

Before loading the column the heat-treated supernatant was diluted to 100 ml with starting buffer. This solution was then loaded onto the gel at 0.4 ml / min. Column was washed with starting buffer at 0.6 ml / min for 90 min before eluting the column using a linear KCl salt gradient at 0.6 ml / min over 800 min. At the end of the gradient, the column was re-equilibrated in starting buffer.

2.1.6 CM-Sepharose batch step

Attempts to bind arginase to a CM-Sepharose column failed, therefore a CM-Sepharose batch step was used to remove those proteins which did bind.
20 g of CM-Sepharose beads were equilibrated in starting buffer (refer section 2.1.5) The starting buffer was then washed off, and the heat treatment step supernatant added to the equilibrated beads. The slurry was kept on ice at 4°C and mixed gently every 5 min for 30 min to keep the beads in suspension. The slurry was then centrifuged at 3000 x g in a SS34 rotor at 4°C for 10 min and the supernatant collected. The matrix was resuspended in starting buffer and centrifuged again as above. The supernatants were combined and centrifuged once more as above to ensure removal of all of the CM-Sepharose matrix.

2.1.7 Gel filtration

Running buffer: 30 mM MOPS-NaOH, 20 mM aspartic acid, pH 7.5.

A gel filtration column was made by suspending 2 g of Sephadex G-200 superfine beads in 500 ml of running buffer. The gel beads were allowed to swell and degas for 5 h at 90°C. After cooling to room temperature the slurry was poured into a 37 x 1 cm column. Degassed running buffer was gravity feed through the column overnight at a flow rate of 2.5 ml / h to pack the gel. The concentrated sample was centrifuged for
30 s before loading 0.36 ml of supernatant on the column. 200 µl of the supernatant was stored at -70°C for later analyses. Once the sample had been loaded, running buffer was gravity feed through the column at 2.5 ml / h. 30 min fractions were initially collected from the column. After 4.5 h, 12.5 min fractions were collected.

2.1.8 Reactive Red 120 column

Due to the unusual nature (see Section 3.1.1) of sheep liver arginase it was found that the hepatic sheep arginase form did not bind to a cation-exchange column. However it did bind to a Reactive Red 120 resin.

Buffers

Starting buffer: 0.02 M MOPS-NaOH, pH 6.7

Salt gradient buffer: 1 M KCl in 0.02 M MOPS-NaOH, pH 6.7

1 L of each buffer were made up and filtered to remove any solid particles. The column was prepared, poured and run at 4°C.

26 ml of resin was washed with starting buffer to remove the NaCl that the resin was stored in. Then 9 ml of starting buffer was added to the resin so that the gel: buffer ratio is Approximately 3: 1. The buffer and resin were mixed to form a slurry which was degassed at 4°C for 1 h. The resin was poured into a column and allowed to settle before being equilibrated overnight by running 100 ml of starting buffer at 0.1 ml / min.

Following the CM-Sepharose batch step the supernatant was loaded onto the Reactive Red 120 column at a flow rate of 1 ml / min. The column was washed by applying MOPS-NaOH buffer to the column at 0.6 ml / min for 90 min. Arginase was then eluted from the resin using a linear KCl gradient (0-1 M, 800 min) at 0.6 ml / min. 7.5 ml fractions were collected in a fraction collector.

2.2 Arginase activity assay

The activity assay carried out by Geyer and Dabich (1971) was modified and performed as follows. 200 µl of a 10 mM L-arginine (free base) substrate solution was equilibrated at 60°C for 2 min in 1.5 ml plastic tubes. 23 µl of appropriately diluted arginase samples were added to the tubes and incubated in a heating block at 60°C for 5 min. The reaction was stopped by adding 0.7 ml of acid reagent and 0.462 ml of colour reagent, then incubated in a heating block at 95°C for 20 min. The tubes were allowed to cool and then urea production was measured at 520 nm in a spectrophotometer (Cary 1, Biolab Ltd, NZ). 23 µl of a 2 mM urea solution was used as a reference.

Colour reagent: 3.6 mM thiosemicarbazide, 61.7 mM diacetylmonoxamine

Acid reagent: 0.05 ml of 0.06 M $Fe_2(SO_4)_3$ in 56.7% phosphoric acid diluted to 100 ml with 10% sulphuric acid

The chromophore formed when urea reacts with diacetylmonoxamine in hot acid is not completely stable, such that colour fades with time at a rate that depends on light intensity. A reference and blank were included with each set of assays so that this instability could be corrected for by converting the absorbance back to µmoles of urea in the sample.

2.3 Protein determination

The Bradford (1976) protein determination method was modified by Spector (1976) and used as follows.

Coomassie Blue dye reagent:
- 100 mg Coomassie Brilliant blue (G-250)
- 50 ml ethanol
- 100 ml 88% orthophosphoric acid
- to 1 L with Milli-Q water

50 µl of appropriately diluted protein sample was mixed with 0.95 ml of dye reagent and left to stand at room temperature for 15-20 min. The absorbance at 595 nm of each sample was then measured. A standard curve was constructed from known amounts of BSA covering the range 0-25 µg BSA .

2.4 Constituting, running and processing the modified Laemmli discontinuous SDS polyacrylamide gels

The Laemmli discontinuous PAGE system (Laemmli, 1970) was modified by Sedmak and Grossberg (1977) and used as follows:

Solutions

Resolving gel acrylamide solution:
- 32 g acrylamide
- 0.2 g bis-acrylamide
- to 100 ml with Milli-Q water

Stacking gel acrylamide solution: 5 g acrylamide
0.105 g bis-acrylamide
to 50 ml with Milli-Q water

0.25% (w/v) of analytical grade mixed ion-exchange resin (AG 501-X8(D), 20-50 mesh) was added to the acrylamide solutions and these solutions were stored in brown bottles at 4°C. The resin settles out to the bottom of the bottle, so liquid was taken from the top of the solution.

Resolving gel buffer solution (4x): 1.5 M Tris / HCl pH 8.7 (20°C), 0.4% SDS

Stacking gel buffer solution (8x): 1 M Tris / HCl pH 6.7 (20°C), 0.8% SDS

Electrode buffer: 72 g glycine, 15 g Tris, 2.5 g SDS, to 2.5 L with Milli-Q water

2x SDS sample buffer: 15% (v/v) glycerol, 2% (w/v) DTT, 0.005% bromophenol blue, 6% (w/v) SDS, 0.125 M Tris / HCl pH 6.7. Stored in aliquots at -20°C.

The information in the "Instruction manual for the mighty small II slab gel electrophoresis unit SE250" (Hoefer) was used to cast and run all SDS-PAGE mini-gels.

To prepare 30 ml of a 15% resolving gel mix, (15% / 32%) x 30 ml = 14.06 ml of resolving gel acrylamide solution and 7.5 ml of resolving gel buffer solution were made up to 30 ml with Milli-Q water. 15 ml of stacking gel mix was prepared by adding 7.5 ml of stacking gel acrylamide solution to 1.875 ml of stacking gel buffer solution and making the volume up to 15 ml with Milli-Q water.

Once the resolving gel mix was at room temperature, 18 µl TEMED and 108 µl 10% APS were added to initiate polymerisation and the mix was poured into gel casting apparatus (Hoefer). Each unpolymerised resolving gel mix was overlayed with 70 µl water-saturated butanol, and allowed to polymerise for 60 min. Polymerisation of the stacking gel mix was initiated by adding 18 µl TEMED and 90 µl 10% APS. The butanol layer

was washed off the polymerised resolving gel, the stacking gel mix poured on top, the comb inserted and the stacking gel allowed to polymerise. Gels were stored up to 1 month at 4°C in glad wrap.

Samples were made up to 15 µl with Milli-Q water. An equal volume of 2x SDS sample buffer was added, samples were vortexed thoroughly and boiled for 3 min. 15 µl was then loaded onto a minigel, typically 0.75 mm thick. An SDS-6H standard was run next to samples. The SDS-6H standard contains 3 mg of a lyophilised mix of 6 proteins: carbonic anhydrase from bovine erythrocytes, M_r 29 000; egg albumin, M_r 45 000; bovine albumin, M_r 66 000; phosphorylase b from rabbit muscle, M_r 97 000; β-galactosidase from *E. coli*, M_r 116 000 and myosin from rabbit muscle, M_r 205 000.

Electrophoresis was initially carried out at 10 mA per 0.75 mm thick gel until the dye front reached the bottom of the stacking gel, the current then was increased to 15 mA. Gels were water-cooled during electrophoresis.

Gels were stained in Coomassie Brilliant Blue (R-250) with gentle shaking for 30 min and destained for 30 min periods in 5: 1: 5 destain with gentle shaking and then left overnight in Welcome destain.

5: 1: 5 destain:	methanol, acetic acid and water in a 5: 1: 5 ratio
Welcome destain:	acetic acid, methanol and water in a 1: 1: 18 ratio
Coomassie Blue stain:	0.1% Coomassie Brilliant Blue R-250 in 30% methanol / 10% acetic acid

2.5 Electroblotting

Solutions

CAPS-MeOH transfer buffer:	Made up 100 ml of a 0.1M CAPS-NaOH buffer, pH 11. Added 100 ml methanol (MeOH) and made up to 1 L with Milli-Q water.

Upper electrode buffer: 2 mM thioglycollic acid in 100 ml of electrode buffer (see Section 2.4).

Samples were made up to 30 µl with Milli-Q water and an equal volume of 2x SDS-PAGE buffer was added. The samples were vortexed, boiled for three min and loaded in the 1 cm wide wells of a 1.5 mm thick gel, next to the SDS 6H molecular weight makers loaded in a 0.5 cm wide well. Electrophoresis was carried out using the thioglycollic acid electrode buffer, at room temperature in the fumehood. Samples were electrophoresed at 25 mA through the stacking gel, and then at 30 mA through the resolving gel. Once the dye front had run to the bottom of the resolving gel electrophoresis was stopped.

For the following procedures gloves were worn at all times:
The SDS 6H molecular weight marker lane and one sample lane were cut from the gel and stained with Coomassie Brilliant Blue (R-250) as described in Section 2.4. A piece of PVDF membrane was cut to the same size as the remaining gel and then immersed in AR MeOH for 10 sec followed by equilibration in transfer buffer for 15 min. The gel was also placed in the transfer buffer for 15 min. Following the 15 min equilibration, a gel / PVDF / blotting paper sandwich was prepared and placed into the electroblotting apparatus with the gel closest to the negative electrode and the membrane closest to the positive electrode.

Electroblotting was carried out for 90 min at 150 mA. Once electroblotting was complete, the PVDF membrane was removed and soaked in Milli-Q water for 10 min while shaking, stained with 0.1% Coomassie Brilliant Blue (R-250) in 50% methanol for 5 min, destained for 2-5 min in 5: 1: 5 destain several times, washed in Milli-Q water for 5 min and air dried. Once the membrane was dry, it was stored in glad wrap at -20°C for protein sequencing. The gel was also stained and destained as described in Section 2.4) to test how much protein had transferred from the gel to the membrane.

2.6 DNA manipulation

Both ethanol precipitation and phenol / chloroform extraction of DNA were performed according to standard protocols (Sambrook *et al.*, 1989 and Ausubel *et al.*, 1987).

2.6.1 Restriction endonuclease digests

Digestion of DNA was carried out following manufacturers instructions. At least 1 U of enzyme was added per 1 µg of DNA digested. The DNA concentration of digests never exceeded 0.1 µg / µl. DNA was digested for 3 h at 37°C unless otherwise stated.

2.6.2 Gel electrophoresis

Electrophoresis of DNA fragments was performed in a low electroendosmosis grade agarose gel containing ethidium bromide (0.2 µg / ml) in a 1x TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) buffer (Sambrook *et al.*, 1989). 1 kb molecular size markers were used to determine the approximate size of DNA fragments. Unless otherwise stated, 1% agarose gels were used to separate DNA fragments. Prior to loading samples into wells, loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% glycerol in water) was added to increase sample density and allow the progress of migration to be monitored.

2.6.3 Purification of fragments from agarose

PCR primers and reactants reduce DNA-modifying enzyme and restriction endonuclease activity, thus markedly reducing the ability of ends to be ligated. Gel purification of DNA was used to eliminate contaminating enzymes and reactants of PCR, and purify DNA fragments of restriction enzyme digests. DNA bands separated by gel electrophoresis were excised under illumination by medium wavelength (302 nm) UV light. To purify 500 bp to 3 kb DNA fragments from agarose gels the Prep-a-Gene purification system was used. A spin elution method (Koenen *et al.*, 1989) was used to purify fragments less than 500 bp in length.

2.6.4 Plasmid preparation

Plasmid DNA was isolated using the 'rapid boil' technique according to Sambrook *et al.* (1989).

2.6.5 Large scale maxipreps

Plasmid DNA was prepared using the Magic / Wizard DNA purification system™.

2.6.6 PCR

DNA sequences were amplified using the polymerase chain reaction (PCR). 5 µl of 10x PCR buffer, 4 µl of 25 mM $MgCl_2$, 6µl of 2.5 mM dNTPs mix (2.5 mM of each dNTP), 5 µl of each primer which had previously been diluted to 5 pmol / µl and 1 µl of template DNA was added to each 0.5 ml sterile PCR tube. The reaction mixes were then made up to 49 µl with Milli-Q water. The reaction mixes were centrifuged in a bench top centrifuge at top speed for 30 sec, and 1.5 U of *Taq* polymerase was then added to each. The reaction mixes were overlayed with mineral oil to eliminate evaporation during cycles and then placed in a thermocycler (Omn-E, Hybaid) preheated to 95°C. The PCR cycles were carried out as follows. Initial melting at 94°C for 5 min, followed by 30 cycles of melting step at 95°C for 1 min, annealing step at 60°C for 1 min and extension step at 72°C for 1 min. As a positive control for the PCR, primers 1917 and 1916 were used to amplify a 300 bp region from template DNA (pTG3954). For the negative control all reactants of the PCR were added to a 0.5 ml PCR tube except template DNA. This is the standard PCR, which was varied in annealing temperature, Mg^{2+}, dNTP and primer concentrations for several of the PCRs.

2.6.7 cDNA synthesis

The superscript preamplification system was used to prepare first strand cDNA from RNA samples following the guidelines supplied with the kit.

2.6.8 T4 DNA polymerase

Taq polymerase adds one or two extra deoxyadenosine 5′ triphosphate nucleotides on the 3′end of the newly synthesised cDNA strands. The 3′ to 5′ exonuclease activity of T4 DNA polymerase was used to remove these overhangs from PCR products creating DNA molecules with blunt ends. T4 DNA polymerase was also used to fill recessed 3′ ends.

2.6.9 Dephosphorylation of vector

To prevent religation of digested vector DNA, 5′ phosphates were removed using TsAP or CIP. The digested vector was treated with TsAP following the manufacturers instructions. CIP was used according to the method stated in Sambrook *et al.* (1989).

2.6.10 Kinasing DNA

5′ phosphates were added to the ends of PCR products using T4 PNK, to enable ligation between blunt ended vector and PCR-amplified DNA. The kinase reaction was carried out according to the manufacturers (Gibco Bethesda Research Laboratories Life Technologies Inc., Penrose, Auckland) guidelines.

2.6.11 Ligations

T4 DNA ligase was used to ligate vector and insert DNA ends together. Ligation reactions were set up with the following molar ratios of insert: vector DNA ends: 3: 1, 2: 1 and 1: 1. Ligation reactions were carried out according to the manufacturers (Gibco Bethesda Research Laboratories Life Technologies Inc., Penrose, Auckland) guidelines. For blunt end ligations an additional 1 U of T4 DNA ligase was added following overnight incubation and then incubated for the remainder of the day. Two controls were set up for the ligation reaction. Control 1 contains vector DNA only and provides an indication of how efficiently the vector was digested. Control 2 contains vector DNA and ligase. This control indicates how efficient dephosphorylation of the vector was.

2.6.12 Transformation into competent cells

Transformation was carried out following the method described by Pope and Kent (1996), using $CaCl_2$-competent *E. coli* XL-1 Blue cells, prepared as described in Sambrook *et al.* (1989). A positive control for the cell viability and contamination was prepared by plating 100 µl of competent cells onto an LB plate and a negative control was prepared by plating 100 µl of competent cells onto an LB-AMP plate. LB and LB-AMP plates were prepared following the method described in Sambrook *et al.* (1989). When α-complementation was used for the selection of recombinant plasmids, cells were plated

out in the presence of IPTG and X-gal. 20 µl of a 200 mg / ml IPTG stock solution and 50 µl of a 20 mg / ml X-gal stock solution were added to the plates with the *E. coli* XL-1 Blue cells. Plates were incubated at 37°C overnight.

2.6.13 T-vector preparation

To prepare T-vector 5 µg of pKS plasmid DNA was digested with *Eco*R V, phenol: chloroform extracted and ethanol precipitated. Resuspended the pellet in 87 µl of sterile water and then incubated with 10 µl PCR buffer, 2 µl 100 mM dNTPs and 1 µl *Taq* polymerase for 2-3 h at 70°C, centrifuging the tube every 30 min in a benchtop centrifuge for 30 sec. The volume was then made up to 200 µl with sterile water before being extracted with phenol: chloroform and ethanol precipitated. The T-tail vector was stored dry at -20°C.

2.6.14 Siliconising Eppendorf tubes

Siliconising Eppendorfs used for Prep-a-Gene isolating and lyophilising DNA fragments was found to increase the amount of low molecular weight DNA isolated. Eppendorfs were siliconised according to the method described in Sambrook *et al.* (1989).

2.7 DNA sequencing

Sequencing gels were prepared and electrophoresed as described in Sambrook *et al.* (1989). Dried gels were autoradiographed overnight onto RX medical X-ray film at room temperature. DNA sequences were read manually. Double stranded templates were sequenced by the dideoxy chain termination method using radioactive [α–^{35}S]-dATP according to the instructions in the Sequenase version 2.0 DNA sequencing kit (8th Ed.).

2.8 Library screening

Library screening was carried out following the recommendations in the Strategene manual. For first round screening, a total of 20 000 pfu of a λ-ZAP sheep cDNA library were screened.

Solutions

SM buffer:	5.8 g NaCl 2.0 g $MgSO_4.7H_2O$ 50 ml 1 M Tris-HCl, pH 7.5 5 ml 2% gelatin made up to 1 L with Milli-Q water and autoclaved
Solution 1:	1.5 M NaCl, 0.5 M NaOH
Solution 2:	1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0
2x SSC:	0.3 M NaCl, 0.03 M Sodium citrate, pH 7.0, autoclaved
Hybridisation solution:	6x SSC, 0.5% SDS, 5x Denharts
Prehybridisation solution:	6x SSC, 1x Denharts
10x Denharts solution:	2% Ficoll, 2% polyvinylpyrolidone, 2% BSA

Probe DNA was labelled with [α-^{32}P]-dCTP using the rediprime random labelling kit (Amersham Life Science, England).

Following hybridisation, nitrocellulose filters (Dassel, Germany) were autoradiographed at -70°C with intensifying screens.

2.9 Northern blot

2.9.1 RNA extraction

RNA was extracted from liver tissue using TRIZOL™ LS Reagent following the instruction manual for using TRIZOL™ LS Reagent in an RNase free environment. RNA pellets were resuspended in 20 µl of DEPC treated water. Isolated RNA concentrations were calculated using a spectrophotometer and the $A_{260/280}$ ratio was determined. Pure

RNA has an $A_{260/280}$ ratio of 2.0 (Sambrook *et al.*, 1989). Isolated RNA was aliquoted into several 0.5 ml sterile Eppendorf tubes and stored at -70°C to keep freeze thawing to a minimum and prevent RNA degradation.

2.9.2 1% agarose-MOPS-formaldehyde gel

Buffers:

Sample buffer: 0.5 ml deionised formamide
0.16 ml formaldehyde solution (in 10-15% methanol)
0.2 ml 5x MOPS running gel buffer
0.14 ml distilled water

5x MOPS buffer: 1 M MOPS, 250 mM Sodium acetate, 5 mM EDTA, pH 7.0 with NaOH

20 µg RNA samples were run on a 1% agarose-MOPS-formaldehyde gel. RNA gels were prepared by melting 1.5 g of low EEO agarose in 62.5 ml of distilled water. 20 ml of 5x MOPS buffer which had previously been heated to 55°C, and 17.6 ml of formaldehyde were mixed with the agarose solution, heated to 55°C and then poured into a gel caster in a fumehood.

The samples were prepared for loading onto the gel by adding 20 µl of sample buffer to 20 µg of RNA and denaturing for 10 min at 60°C. Following denaturation the samples were cooled on ice for 2 mins, and 1 µl of loading dye (0.4% bromophenol blue, 0.4% xylene cyanol, 1 mM EDTA and 50% glycerol) and 2 µl of ethidium bromide (10 mg / ml) was added.

Samples were then electrophoresed for 3 h at 100 mA in 1x MOPS buffer in a fumehood.

2.9.3 Preparation, running and probing

Preparation, running and dismantling of the Northern blot was carried out using the methods recommended by Darling *et al.* (1994). RNA was blotted onto Hybond^{TM}-N^+ nylon transfer membrane.

A nonradiolabelled probe was prepared using the DIG rediprime labelling kit (Boehringer) according to the manufacturers instructions given in the DIG system user guide. The Northern blot was then probed using this nonradiolabelled probe, according to the manufacturers instructions.

Chapter 3

PARTIAL PURIFICATION AND CHARACTERISATION OF SHEEP LIVER ARGINASE

An attempt was made to partially purify sheep liver-type arginase with a view to electroblotting the protein for N-terminal sequencing. The N-terminal amino acid sequence would have been used to design a redundant oligonucleotide. This oligonucleotide could then have been used directly as a probe to screen a sheep liver cDNA library, or used to generate a DNA probe by PCR.

3.1 Sheep liver arginase partial purification procedure

Due to the success of the purification scheme for rat liver-type arginase (Schimke, 1970), this scheme was used as a guide to partially purify sheep liver arginase.

To measure the amount of arginase activity present in samples, an activity assay that detects the urea produced by the arginase-catalysed hydrolysis of substrate (L-arginine) was performed as described in Section 2.2. The activity assay provided a qualitative means of identifying the samples which contained arginase.

3.1.1 Results and discussion

The initial purification steps, extraction, acetone, dialysis and heat treatment were performed as described in Sections 2.1.1 to 2.1.4.

Activity assays and specific activity analysis (Table 3.1) was carried out on fractions collected from the initial steps in the purification. There was a decrease in activity following the dialysis and heat steps which was not observed in the rat arginase purification. The decrease in specific activity following the dialysis step could be a result of Mn^{2+} being dialysed out of the sample. This may have caused a partial depletion of Mn^{2+} cofactor from the active site of the enzyme, inactivating a proportion of the enzyme.

Table 3.1 Specific activity of fractions collected from the initial stages of the sheep and rat liver arginase partial purification procedures

	Sheep liver arginase purification		**Rat liver arginase purification**	
Step	Protein (mg / ml)	Specific activity (U / mg)	Protein (mg / mg)	Specific activity (U / mg)
Homogenate	25.4	28	49.0	6.0
Acetone	23.4	56	34.0	10.5
Dialysis	20.0	31	14.7	62.0
Heat	14.8	27	5.5	209.0

Specific activities of samples collected from the initial steps of the sheep liver arginase purification were compared with results published for the rat liver arginase purification (Schimke, 1970).

The decrease in specific activity following the heat step may be a consequence of not removing all of the acetone prior to heating. If the acetone was not adequately removed, inactivation of arginase would have occurred during the subsequent heat step. This possibility was not investigated. The heat step could have been performed prior to the acetone step as was done for the rabbit arginase purification (Vielle-Breitburd and Orth, 1972) to avoid any chance of inactivation of arginase during the heat step caused by the presence of acetone in the supernatant. The stability of the sheep liver arginase enzyme was also considered as a probable factor as to why there was an observed decrease in arginase activity following the heat step. The sheep arginase protein may not be as thermostable as rat arginase.

The heat treatment supernatant was then applied to a CM-Sepharose column and arginase eluted by a KCl salt gradient (Section 2.1.5). Arginase activity assays (see Table 3.2) performed on fractions eluted from the column showed only low levels of activity. The majority of activity was present in the unbound solution, collected as two fractions, labelled S1 and S2 in Table 3.2, demonstrating that unlike rat liver arginase, sheep liver arginase does not bind efficiently to the CM-Sepharose column.

Table 3.2 Arginase activity analysis of fractions collected from the CM-Sepharose chromatography column

Sample	**Absorbance (520 nm)**
Substrate blank	0.000
Standard 1	0.439
Standard 2	0.468
Wash through (S1)	2.725
Wash through (S2)	2.529
Fraction 16	0.426
Fraction 19	0.270
Fraction 21	0.144
Fraction 23	0.076
Fraction 26	0.041

Arginase activity analysis; 23 µl samples were assayed for arginase activity following the method stated in Section 2.2.

Blank: Water was substituted for enzyme sample.

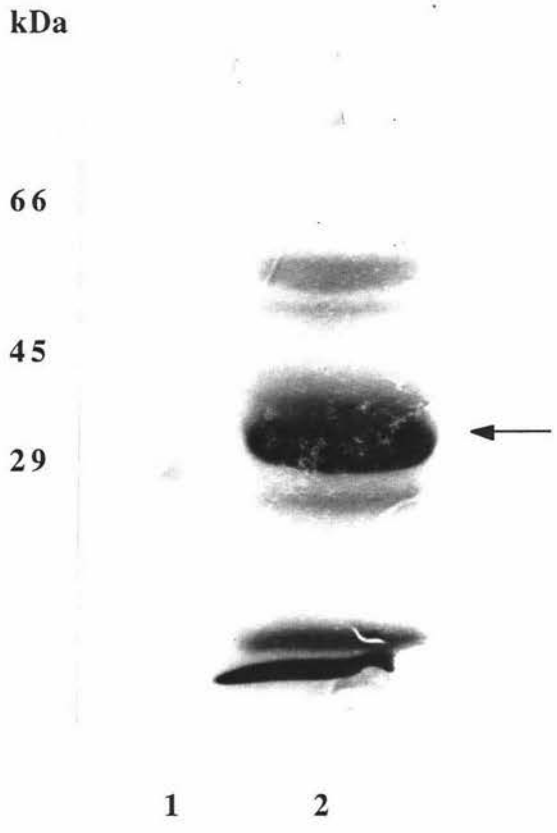
Standards 1 and 2: 2 mM urea standard was substituted for enzyme sample.

Wash through (S1) and (S2): The wash through was collected in two fractions, which were labelled S1 and S2.

Fractions 16-26: Fractions collected during elution step of the CM-Sepharose column.

In the Schimke, 1970 rat liver arginase purification, a CM-cellulose matrix was used rather than a CM-Sepharose matrix. Would expect to observe similar binding behaviour whether a CM-Sepharose or CM-cellulose matrix was used, because it is the CM group that provides the negative charge for arginase to bind to the matrix. Therefore this difference does not explain why arginase did not bind to the CM-Sepharose matrix. Using a centriprep-30 k cut off concentrator, the S1 fraction, that contained the highest level of activity was concentrated to 0.7 ml. The concentrated protein sample was analysed by SDS gel electrophoresis as described in Section 2.4.

Figure 3.1 SDS-PAGE of the concentrated sample that was electroblotted following the CM-Sepharose step



SDS-PAGE analysis of the gel that was blotted onto a PVDF membrane. 30 µl of sample was mixed with 30 µl of 2x SDS-PAGE sample buffer, boiled for 3 min and 30 µl loaded into a 1 cm wide well of a 1.5 mm thick, 15% SDS-PAGE gel. Gel electrophoresis and staining were performed as described in Section 2.5.

Lane 1: SDS-PAGE molecular weight markers (Sigma Co.).

Lane 2: Concentrated protein sample; 120 µg of total protein was loaded.

Arrow: Points to the band that was N-terminally sequenced.

SDS-PAGE analysis showed that the concentrated sample was a heterogeneous mix of proteins with a major band migrating at approximately 35 kDa. The concentrated sample was then electroblotted onto a PVDF membrane as described in Section 2.5 and the 35 kDa protein N-terminally sequenced by J. Mudford (Dept of Biochemistry). Figure 3.1 shows the gel half that was not electroblotted onto a PVDF membrane. The N-terminal amino acid sequence of the 35 kDa protein was VHAK. This sequence is not present in any known arginases at the N-terminus. A database search suggested that the sequenced protein may be an hepatic catalase enzyme. VHAK is a conserved region found near the N-terminus of all known catalases. All known catalase sequences are larger than 35 kDa, but the protein may be proteolysed to produce a protein 35 kDa in size, suggested by the presence of a trypsin site immediately upstream of the VHAK region. In the purification of the human liver arginase carried out by Kuhn *et al.* (1995), arginase was observed to migrate as a band with an apparent M_r of 37 000, higher than the deduced M_r of 34 734 from the predicted amino acid sequence (met on) and the M_r value of 35 000 published in Berüter *et al.* (1978) and Ikemoto *et al.* (1990). It is possible that sheep liver arginase would show similar anomalous migration on an SDS-PAGE gel. In Figure 3.1, a number of protein bands migrated on the SDS-PAGE in the region of 37 kDa, therefore further purification was performed to remove some of the other bands in this area.

Sheep hepatic arginase bound poorly to the CM-Sepharose matrix, but as other proteins did, this ion-exchange step was carried out in batch form to remove these proteins. The CM-Sepharose batch step was performed as described in Section 2.1.6.

A gel filtration column (Section 2.1.7) was attempted as a further purification step following the CM-Sepharose batch step. Sheep liver arginase was expected to be in its trimeric (105-115 kDa) form, and the red coloured Haemoglobin protein present in the CM-Sepharose batch step supernatant was a 64 kDa tetramer, as the gel filtration step was carried out under nondenaturing conditions. The Haemoglobin protein which was expected to move through the gel filtration column more slowly than arginase was used to indicate roughly when arginase should elute. Only a small amount of activity was recovered from the column therefore the gel filtration step was not carried out in further sheep liver arginase purifications.

A Polyethylene glycol (PEG) step was also attempted as a further step in the purification, following the CM-Sepharose batch step. PEG has been used successfully to precipitate

and concentrate many enzymes with good recovery of enzyme activity (Scopes, 1994; Fried and Chun, 1971). The PEG step was initially carried out on a small scale. 0-20 µl of 50% PEG solution was added to 20 µl of the CM-Sepharose supernatant in a 1.5 ml Eppendorf tube and the volume made up to 40 µl with 0.02 M MOPS-NaOH, pH 6.7. Each Eppendorf tube was vortexed and left at room temperature for 30 min before centrifuging at maximum speed for 5 min in a bench top centrifuge and assaying the supernatant for arginase activity. No pellets were observed after centrifugation.

Addition of Mn^{2+} at particular PEG concentrations would lower solubilities to produce high levels of arginase activity in *B. caldovelox* (Mark Patchett, Perscomm). It was hoped that this behaviour might be observed and exploited in the purification of sheep liver arginases. To this end the following tests were carried out. 19.2 µl of 50% PEG in 0.02 M MOPS pH 6.7 was placed into a 1.5 ml Eppendorf tubes. 0.8 µl of 0.02 M MOPS-NaOH, pH 6.7 and 20 µl of the CM-Sepharose supernatant was added and the Eppendorf tube incubated at room temperature for 30 mins. Following incubation the Eppendorf tube was centrifuged at maximum speed for 5 min in a benchtop centrifuge. 0.3 µl of 1 M $MnCl_2$ was then added, the Eppendorf tube mixed and then centrifuged in a benchtop microcentrifuge for 5 min at maximum speed. No precipitate was visible following mixing or centrifugation. The supernatant was assayed in case the pellet was too small to be visible (Table 3.3). An increase in the absorbance in the presence of $MnCl_2$ was observed. Precipitation of arginase would be accompanied by a decrease in absorbance, therefore arginase did not precipitate in the presence of both PEG and Mn^{2+}. The increase in absorbance was due to an increase in activity in the presence of $MnCl_2$ which may be due to an increase in the proportion of active sites that contain the essential Mn^{2+} metal ion cofactor.

Table 3.3 Arginase activity analysis of supernatants collected from the PEG step

Sample	Absorbance (520 nm)
Blank	0.000
Standard	0.302
24% PEG + $MnCl_2$	1.582
24% PEG	0.857

Arginase activity analysis; 23 µl of each sample was assayed for arginase activity following the method described in section 2.2.

Blank: Water is substituted for sample.

Standard: 2 mM urea standard substituted for sample.

3.2 The first column chromatography step in the purification

Several chromatographic matrices including Q-Sepharose, hydroxyapatite, and Reactive Red 120, were tested for their ability to bind arginase.

3.2.1 Results and discussion

The binding of arginase to these matrices was tested as described below.

Hydroxyapatite powder was hydrated and washed several times in 0.02 M potassium phosphate buffer, pH 6.7 and then equilibrated in 0.02 M MOPS-NaOH buffer, pH 6.7. For both Reactive Red 120 and Q-Sepharose, 1 g of preswollen matrix was equilibrated in 0.02 M MOPS-NaOH buffer, pH 6.7. For each matrix the following steps were then carried out.

(1) 200 µl of settled resin was transferred to 1.5 ml Eppendorf tube to which 400 µl of heat treated supernatant was added. A control for each matrix was set up by adding 200 µl of 0.02 M MOPS-NaOH buffer to 400 µl of heat treated supernatant.

(2) The tubes were mixed on a slowly rotating wheel for 10 min at 4°C.

(3) The experiment and control tubes were centrifuged at top speed in a benchtop microfuge centrifuge. The supernatants were diluted and then assayed for arginase activity (see Table 3.4).

Table 3.4 Arginase activity analysis of supernatants following incubation of arginase with the chromatography matrices hydroxyapatite, Reactive Red 120 and Q-Sepharose

	Sample	**Absorbance (520 nm)**	**% Activity**
	Blank	0.000	
	Standard 1	0.325	
	Standard 2	0.339	
Hydroxyapatite	Buffer + arginase (control)	0.258	100
	Matrix + arginase	0.010	4
Reactive Red 120	Buffer + arginase (control)	0.252	100
	Matrix + arginase	0.060	24
Q-Sepharose	Buffer + arginase (control)	0.098	100
	Matrix + arginase	0.090	92

Arginase activity analysis; 23 µl of diluted supernatant was assayed following the method described in section 2.2. The % of arginase activity remaining in the supernatant after incubation and centrifugation.

Blank: Water was added substituted for arginase sample.

Standard 1 and 2: 2 mM urea was substituted for arginase sample.

Hydroxyapatite: Supernatants were diluted 100-fold and assayed for arginase activity.

Reactive Red 120: Supernatants were diluted 60-fold and assayed for activity.

Q-Sepharose: Supernatants were diluted 100-fold and assayed for arginase activity.

The absorbance readings could not be compared directly because they were all diluted by different factors. The observed variation in the control absorbance readings was most likely a result of a combination of factors. The colour reaction is time dependant, therefore an error is generated if the samples have not been incubated for exactly the same length of time before being assayed. Other errors include pipetting errors, calibration

errors, experimental errors and limits of accuracy of the assay. It was also observed, that the arginase activity measured using the assay did not appear to be linearly dependant on the amount of arginase enzyme present, essentially making it very difficult to compare directly different experiments or sets of arginase activity assays. Arginase bound strongly to both the Reactive Red 120 and hydroxyapatite matrices: 76% of the total activity bound to the Reactive Red 120 matrix, while 96% bound to the hydroxyapatite matrix. Arginase did not bind to the Q-Sepharose matrix. In hind site these experiments should have been repeated to get more consistent assay results.

No further tests were carried out using the Q-Sepharose matrix, because arginase was previously observed not to bind the Q-Sepharose matrix under the conditions tested. Supernatants were removed from the Reactive Red 120 and hydroxyapatite Eppendorf tubes and arginase eluted from each of the matrices as follows.

1. Hydroxyapatite

500 µl of a 0.8 M potassium phosphate buffer, pH 6.7, was added to the 1.5 ml Eppendorf tubes and mixed on a slowly rotating wheel for 5 min at 4°C.

2. Reactive Red 120

0.2 ml of 2 M KCl was added to the Eppendorf tube and mixed on a slowly rotating wheel for 5 min at 4°C.

Following incubation the Eppendorf tubes from each step were centrifuged and the supernatant's were assayed for arginase activity (see Table 3.5).

Table 3.5 Arginase activity analysis following elution from the hydroxyapatite and Reactive Red 120 chromatography matrices.

Sample	Absorbance (520 nm)	Corrected Absorbance
Blank	0.000	0.000
Standard 1	0.328	0.328
Standard 2	0.347	0.347
Hydroxyapatite eluent	1.250	4.40
Reactive Red 120 eluent	1.850	3.70

Arginase activity analysis; 23 µl of undiluted supernatant was assayed from each tube following the method described in section 2.2. The corrected absorbance accounts for the dilution factor carried out during the elution step.

Blank: Water was substituted for arginase sample.

Standard 1 and 2 : 2 mM urea standard was substituted for arginase sample.

The results of the arginase assay described in Table 3.5 can not be compared directly because each matrix was diluted with a different volume during the elution step. It was calculated that 8% and 16% of activity was recovered on batch elution from the red agarose and hydroxyapatite resins respectively. The low recovery of arginase activity may be due to progressive loss of Mn^{2+} as a result of partial inactivation of arginase by salt. In hind site realise that Mn^{2+} should have been included in the buffers.

Hydroxyapatite is made up of the crystalline calcium phosphate. Unlike matrices based on beaded agarose, the hydroxyapatite crystalline particles adsorb proteins on their surface. Consequently the adsorptive capacity is relatively low and the use of this matrix in enzyme purification is limited to a late-stage procedure (Scopes, 1994). As the hydroxyapatite column would have been the first column in the purification, it was decided that the Reactive Red 120 column would be more suitable to use at this stage of the purification procedure.

3.3 Reactive Red 120 column

3.3.1 Results and discussion

The CM-Sepharose batch step supernatant was loaded onto the Reactive Red 120 column as described in Section 2.1.8. Fractions 35 to 60 were assayed for arginase activity (see Table 3.6). The results of these initial arginase assays showed that arginase had eluted from the column over the large range of fractions, 38 to 57. A second round of arginase assays were carried out on fractions 41 to 45, which contained the highest levels of activity with a 200-fold dilution (Table 3.7). A line diagram representation of the relative arginase activity in each fraction is shown in Figure 3.2. Fractions 39 to 47 were analysed by SDS-PAGE (Figure 3.3). The results of the gel when compared with the arginase assays, showed that the intensity of a band running at approximately 38 kDa corresponded to the relative level of arginase activity. Fractions 43 and 44 which had the highest level of arginase activity and the strongest band intensities on the SDS-PAGE, were combined.

Specific activity analysis (see Table 3.8) showed that the pooled fractions from the Reactive Red 120 column contained 10-fold greater activity than the heat step supernatant, indicating that this chromotography step is effective in purifying arginase from other proteins in the mix. Comparisons made between the rat and sheep arginase purification schemes, suggest that a further purification step may be needed. The protein determination methods used in the two purification procedures were different, which may limit the value of comparison between the final specific activities.

Table 3.6 Arginase activity analysis of fractions collected from the Reactive Red 120 chromatography column

Fraction No.	**Absorbance (520 nm)**
Blank	0.000
Standard	0.402
35	0.05
36	0.12
37	0.25
38	1.12
39	2.60
40	3.01
41	3.05
42	3.06
43	3.08
44	3.02
45	2.97
46	3.04
47	2.96
48	3.08
49	2.61
50	2.37
51	2.24
52	2.22
53	1.94
54	1.39
55	1.64
56	1.55
57	1.21
58	0.79
59	0.61
60	0.44

Arginase activity analysis; 23 µl of each fraction was assayed for arginase activity following the method described in section 2.2.

Blank: Water was substituted for arginase sample.

Standard : 2 mM urea standard was substituted for arginase sample.

Table 3.7 Arginase activity analysis of Reactive Red 120 column fractions 41-45 which contained the highest levels of activity

Fraction no.	Absorbance (520 nm)
Blank	0.000
Standard	0.381
41	0.165
42	0.245
43	0.314
44	0.492
45	0.253

Arginase activity analysis; 23 µl of the 200-fold diluted fractions 41 to 45 were assayed following the method described in section 2.2.

Blank: Water was substituted for arginase sample.

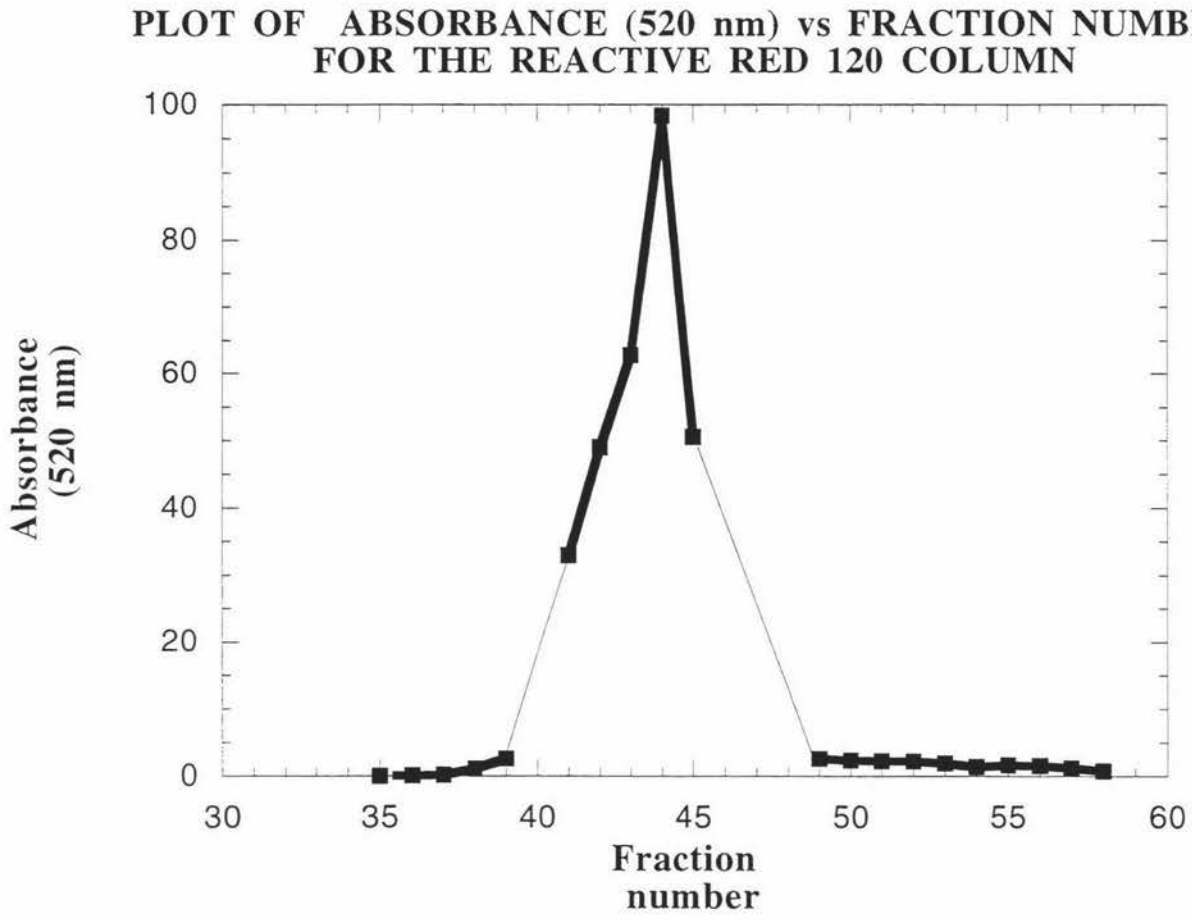
Standard: 2 mM urea was substituted for arginase sample.

Table 3.8 Specific activity comparison between the sheep and rat arginase purification schemes

	Sheep liver arginase purification		**Rat liver arginase purification**	
Step	Protein (mg / ml)	Specific activity (U / mg)	Protein (mg / ml)	Specific activity (U / mg)
Homogenate	23.50	80.0	49.0	6.0
Heat	11.20	9.2	34.0	95.0
Pooled fractions 43 + 44	0.67	1224.0	NA	NA
Ammonium sulphate	NA	NA	8.6	5310.0

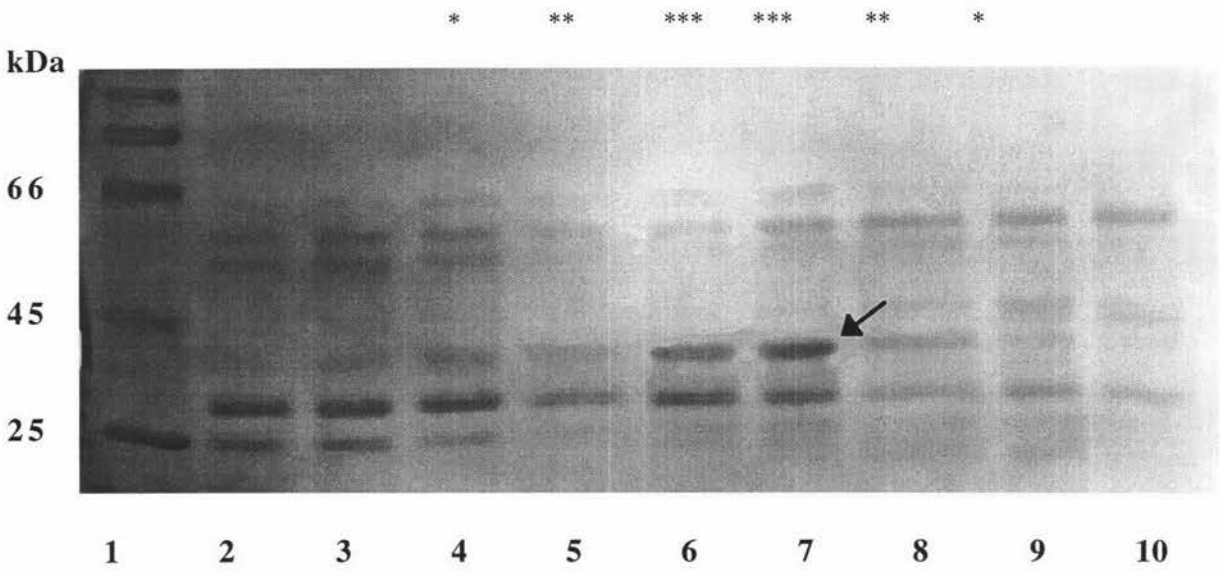
The specific activity of sample collected from the final step of the sheep liver arginase purification was compared with final step of the rat purification (Schimke, 1970).

Figure 3.2 Elution profile of arginase from the Reactive Red 120 fractions.



The absorbance at 520 nm for fractions 41 to 45 was corrected for the 200 fold dilution carried out on these fractions (see Table 3.7) during the arginase activity assay.

Figure 3.3 SDS-PAGE analysis of fractions 39-47 eluted from the Reactive Red 120 chromatography column



15 µl of each fraction were mixed with 15 µl of 2x SDS-PAGE sample buffer, boiled for 3 min and loaded 15 µl onto a 15% SDS-PAGE gel. Gel electrophoresis and staining was performed as described in Section 2.4.

Lane 1: SDS-PAGE molecular weight markers (Sigma Co.)

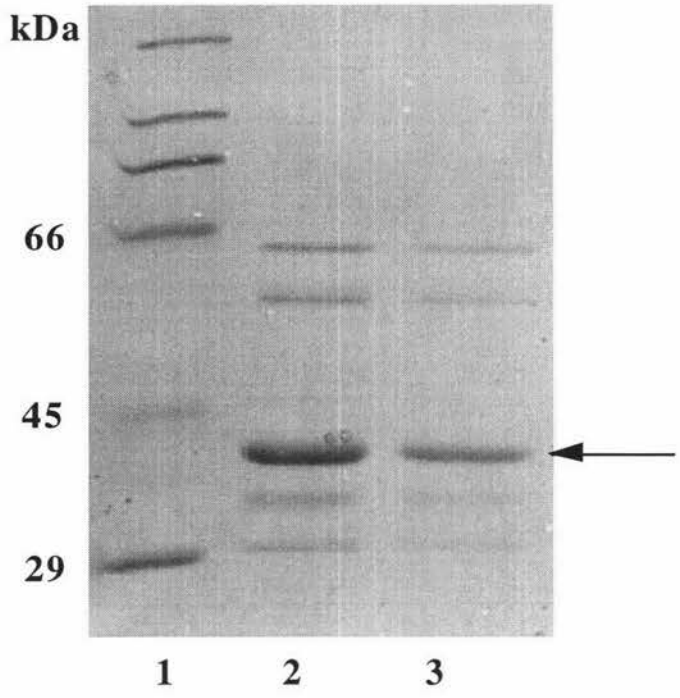
Lanes 2 to 10: Fractions 39 to 47 respectively.

Arrow: Points towards the putative sheep liver arginase band.

* Indicates the relative amount of arginase present in each fraction loaded on the SDS-PAGE. The more stars, the higher the level of activity.

The pooled fractions were analysed by SDS-PAGE (Figure 3.4) in preparation for protein blotting and N-terminal amino acid sequencing. The M_r of sheep liver arginase was determined from this SDS-PAGE gel to be 36 500 (Figure 3.5). Several lanes of the pooled protein sample were electroblotted onto a PVDF membrane as described in Section 2.5. No amino acid sequence was generated from the blot presumably because it was N-terminally blocked.

Figure 3.4 SDS-PAGE analysis of the pooled fractions 43 and 44 eluted from the Reactive Red 120 chromatography column



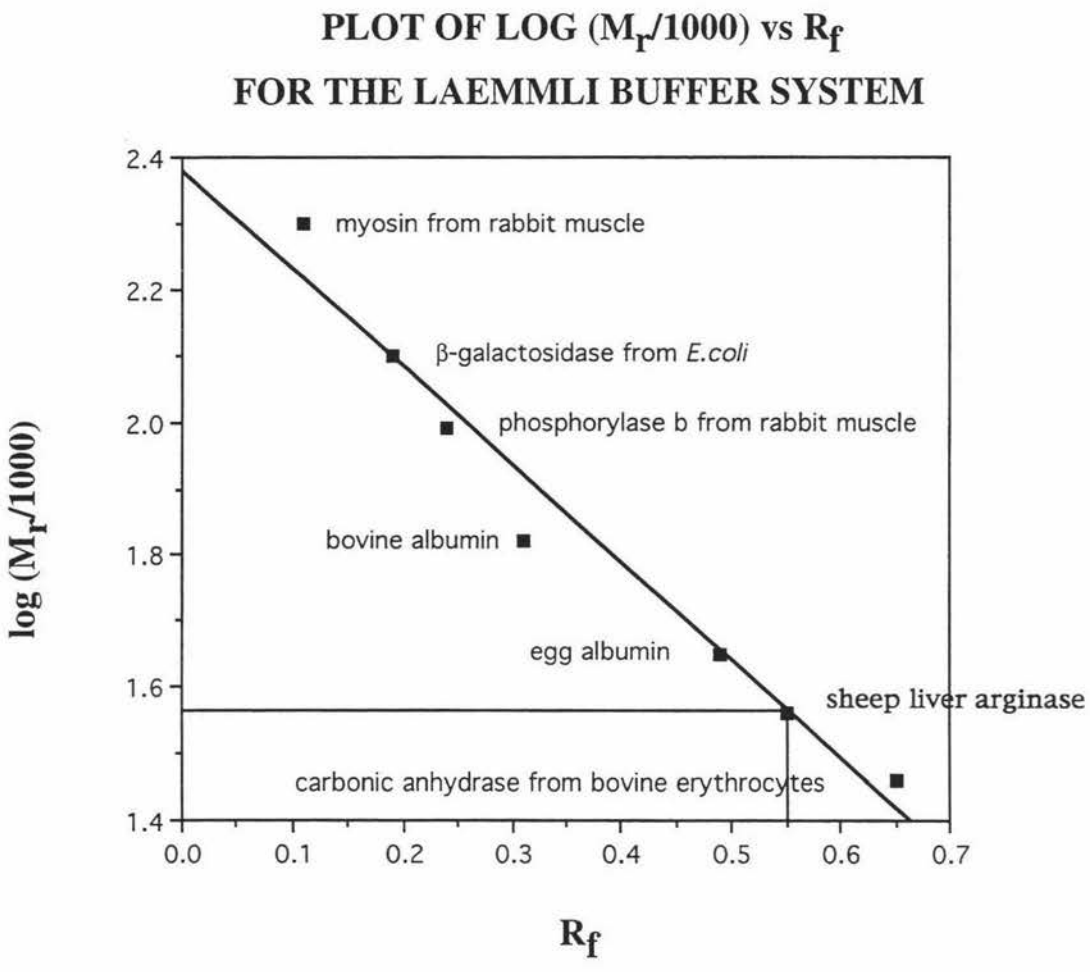
15 µl of each fraction was mixed with 15 µl of 2x SDS-PAGE buffer, boiled for 3 min and loaded 15 µl onto a 15% SDS-PAGE gel. Gel electrophoresis and staining were then performed as described in Section 2.4.

Lane 1: SDS-PAGE molecular weight markers (Sigma Co.).

Lanes 2 and 3: Pooled fraction 43 and 44; 8 and 4 µg loaded in lanes 1 and 2 respectively.

Arrow: Points to the band that was N-terminally sequenced.

Figure 3.5 Determination of the molecular weight of sheep liver arginase by SDS-PAGE



Plot of log (M_r / 1000) versus R_f for the 6 protein molecular weight markers used in the modified Laemmli Tris-glycine buffer system. A line of best fit was drawn between the plotted points.

R_f = retention factor

$$= \frac{\text{distance taken between the middle of the protein band and top of the resolving gel}}{\text{distance between dye front and top of the resolving gel}}$$

N-terminal acetylation of proteins is a common modification especially among eukaryotic proteins (Persson *et al.*, 1985), with the most common acetylated residues being serine, alanine and methionine. The first amino acid residue of all the known arginase sequences is a methionine and is followed in a number of the arginase sequences by a serine residue. If sheep liver arginase has been N-terminally acetylated, the Edman degradation reaction, which requires a free amino terminus would have been prevented.

Acetylated amino acids can be removed by acylamino acid peptidase. The chain length of the peptide influences the activity of acylamino acid peptidase, having the highest activity with peptides up to 10 amino acids in length (Boehringer Mannheim information sheet). If sheep liver arginase was isolated from an SDS-PAGE, it could be chemically cleaved into peptides, treated with acylamino acid peptidase to remove the acetylated residue and the cleaved fragments N-terminally sequenced. A probe could be designed to any one of these fragments for screening a sheep cDNA library, as only a short sequence is needed.

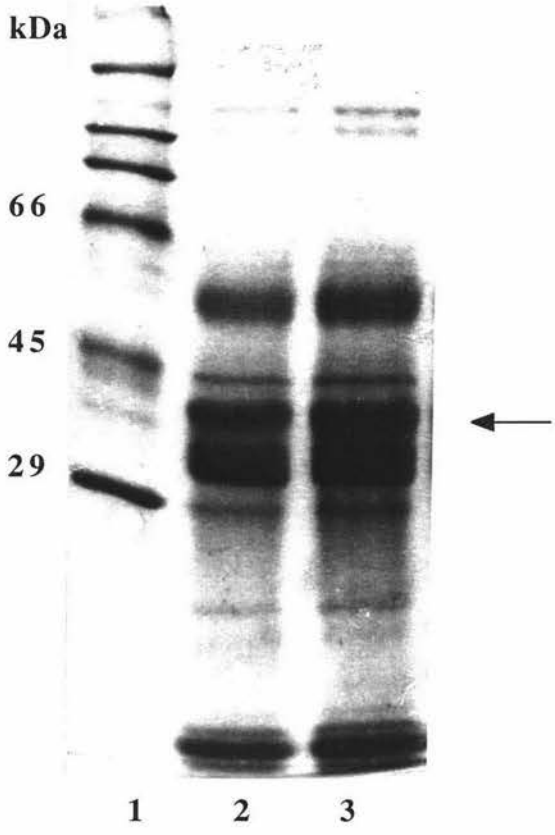
3.4 Electroelution

3.4.1 Results and discussion

Electroelution from preparative SDS-PAGE gels has been used to isolate proteins after partial purification has been carried out (Hunkapiller *et al.*, 1983). The putative arginase band would be cut from the SDS-PAGE gel and eletroeluted from the gel as a means of isolating the protein. The electroeluted protein would then be chemically cleaved to produce peptides that could be sequenced to obtain amino acid sequences of liver arginase. These sequences would then be used to design suitable DNA probes to screen a sheep liver cDNA library.

The purification was carried out a number of times with slight variations. In one such purification the most active fractions from the Reactive Red 120 chromatography step were pooled and concentrated. The concentrated fractions were analysed by SDS-PAGE gel electrophoresis (Figure 3.6).

Figure 3.6 SDS-PAGE analysis of the pooled concentrated active fractions eluted from the Reactive Red 120 chromatography column



SDS-PAGE analysis; 15 µl of sample was mixed with 15 µl of 2x SDS-PAGE sample buffer, boiled for 3 min and loaded 15 µl onto a 15% SDS-PAGE gel. Gel electrophoresis and staining were then performed as described in Section 2.4.

Lane 1: SDS-PAGE molecular weight markers (Sigma Co.).

Lane 2 and 3: Contains similar loadings of the concentrated protein sample.

Arrow: Points to the putative arginase band.

A large number of the bands seen in the SDS-PAGE analysis of concentrated sample had not previously been visible. The possible reasons for the increase in number of bands visible on the SDS-PAGE include: (1) the protein sample was so concentrated that the amount of SDS was no longer sufficient to saturate all the proteins in the sample which

may lead to partial bands being observed on the SDS-PAGE, (2) during the concentration step other proteins may have formed covalent bonds with other proteins, and (3) the occurrence of proteolysis may have lead to new bands on the SDS-PAGE.

The % acrylamide of the resolving gel was varied in an attempt to separate arginase sufficiently from the other bands on the SDS-PAGE, but these efforts were unsuccessful.

3.5 Isoelectric focussing

An isoelectric focussing (IEF) step was carried out as a means of separating the protein on the basis of charge.

3.5.1 Results and discussion

An IEF gel was attempted to further purify the sheep liver arginase on the basis of it's charge properties. This step was kindly performed by T. Blythe (Dept of Biochemistry, Massey University). The pH range of the IEF gel was 3.5 to 9.0.

The IEF step showed a set of poorly resolved bands, with all the bands migrating at about the same place, therefore the degree of separation seen on the IEF gel was not sufficient for it to be an effective purification step.

Chapter 4

PCR-BASED APPROACH

As the sheep liver arginase protein was unable to be purified sufficiently for N-terminal sequencing, a PCR-based approach was attempted to generate a suitable probe for screening a sheep liver λ-ZAP cDNA library.

PCR is a powerful technique for specifically amplifying target DNA sequence. It involves two oligonucleotide primers hybridising to opposite strands of a target DNA sequence, oriented such that DNA synthesis by a thermostable DNA polymerase proceeds across the region between the primers. Since the extension products are also complementary to the primers, the cycle can be repeated. By repeated cycles of denaturation, primer annealing and extension there is a rapid exponential accumulation of the specific target fragment (Old and Primrose, 1989).

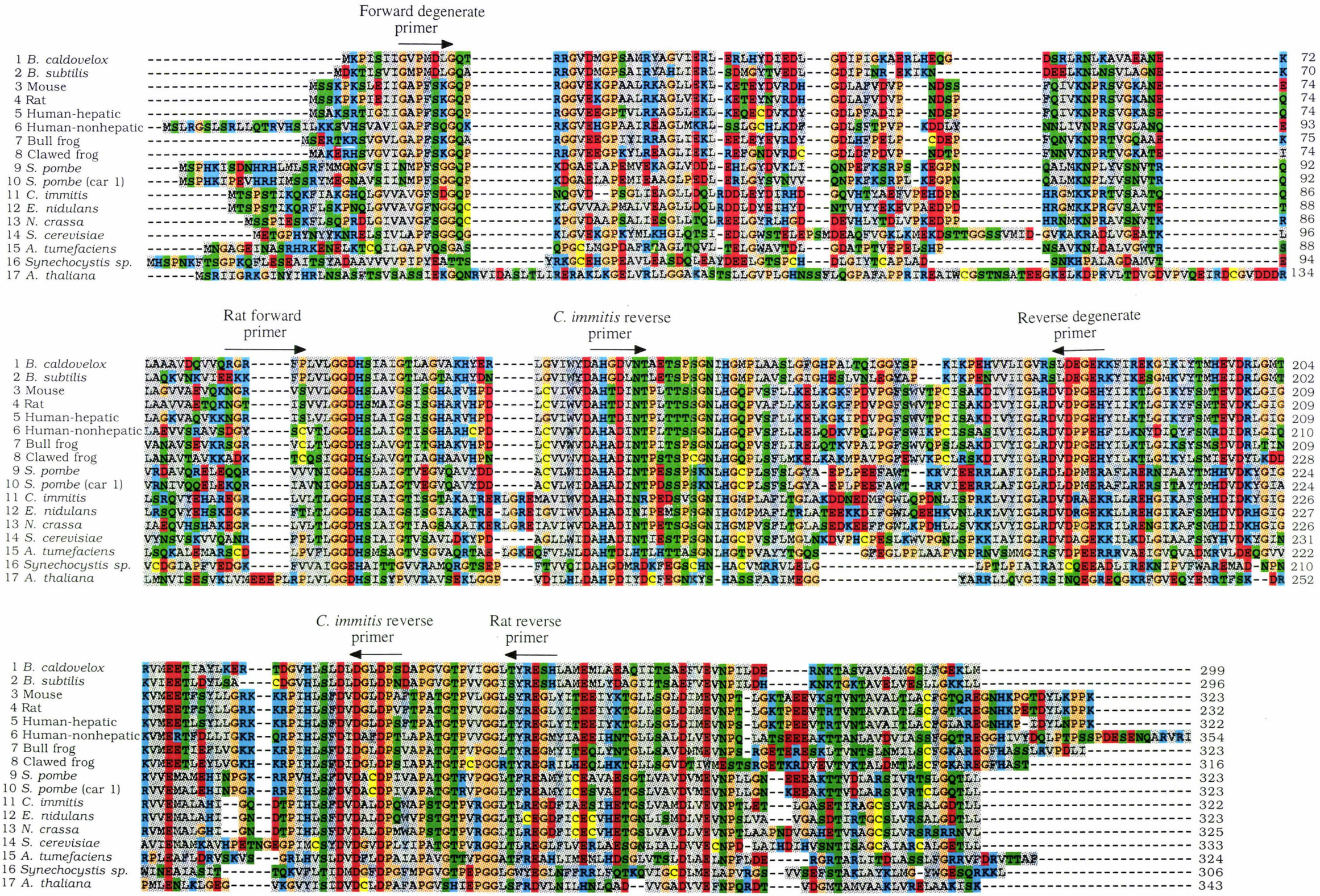
4.1 Primer design

Degenerate primers for an internal fragment of the sheep liver arginase cDNA (see appendix 1) were designed from regions of arginase protein sequences conserved between the known *Coccidioides immitis*, *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae, Neurospora crassa*, *Xenopus laevis,* rat and human arginase sequences. Perfectly matched primers were synthesised to the known rat DNA sequence (Accession no. JO2720) obtained from GENBANK DNA sequence database. The amino acid sequences the perfectly matched and degenerate primers were designed to, are shown in Figure 4.1. The degenerate primers were not designed to all of the sequences shown in Figure 4.1 because at the time they had not been entered into the GENBANK DNA sequence database.

Tried to minimise synthesising primers which would selfcomplement or had secondary structure. Primer pairs were designed to have melting temperatures which were within 10°C of each other, typically between 50 and 60°C. The rat specific primers were designed such that they would amplify a 495 bp conserved middle region of the arginase cDNA, a good size for use as a probe.

Alignment was made using the Clustal W multiple sequence alignment program version 1.6, March 1996. The 18 amino acid sequences that were aligned are from (1) *Bacillus caldovelox* (Bewley *et al.*, 1996); (2) *Bacillus subtilis* (Gardan *et al.*, 1995); (3) mouse (Chieko., 1996); (4) rat (Ohtake *et al.*, 1989); (5) human AI (liver-type) (Haraguchi *et al.*, 1987); (6) human AII (nonhepatic) (Vockey., 1996); (7) *Rana catesbiana* (bull frog) (Iwase *et al.*, 1995); (8) *Xenopus laevis* (clawed frog) (Xu *et al.*, 1993); (9) *Schizosaccharomyces pombe* (Ocampas, 1996); (10) *Schizosaccharomyces pombe* carl gene (Van Huffel *et al.*, 1994); (11) *Coccidioides immitis* (Pan *et al.*, 1995); (12) *Emericella nidulans* (Borsuk *et al.*, 1996); (13) *Neurospora crassa* (Marathe *et al.*, 1993); (14) *Saccharomyces cerevisiae* (Sumrada and Cooper, 1984); (15) *Agrobacterium tumerfacians* (Schrell *et al.*, 1987); (16) *Synechocystis sp.* (Kaneko *et al.*, 1996); and (17) *Arabidopsis thaliana* (Krumpelman *et al.*, 1995). Colour coding, used to show conserved amino acids and constitutive substitutions was generated using the X-align program (David Perkins, University of Leeds, 1993). The amino acids were colour coded as follows: yellow is cysteine (C); brown is proline (P) and glycine (G); grey is valine (V), leucine (L), isoleucine (I), alanine (A) and methionine (M); purple is phenylalanine (F), tryptophan (W) and tyrosine (Y); blue is lysine (K), arginine(R) and histidine (H); red is glutamate (E) and aspartate (D); green is threonine (T), glutamine (Q), serine (S) and asparagine (N). The arrows show which amino acids the primers were synthesised to.

Figure 4.1 Alignment of arginase amino acid sequences

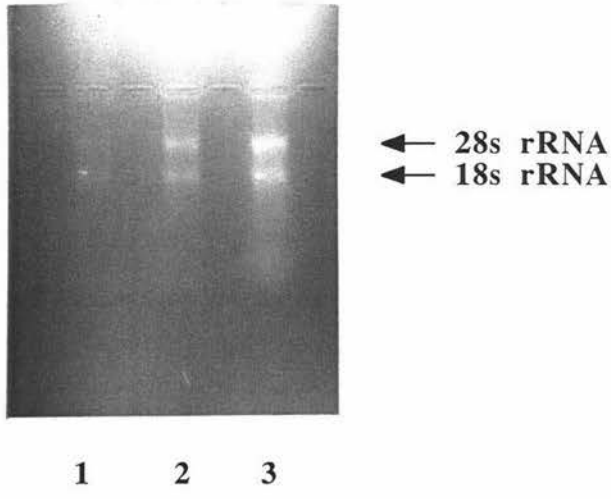


4.2 mRNA isolation

4.2.1 Results and discussion

Prior to RT-PCR being carried out, RNA was isolated from both sheep and rat liver following the method described in Section 2.9.1. A fraction from each of the RNA samples was then run on a 1% agarose-MOPS-formaldehyde gel (Section 2.9.2) to check that the RNA had not degraded. Two bands representing the 28S rRNA and 18S rRNA subunits were visualised on the agarose gel (Figure 4.2) suggesting that the RNA was essentially free of RNA degration. The gel photo presented in Figure 4.2 shows the electrophoresis migration is of RNA isolated from sheep liver. RNA isolated from rat liver has not been shown but was observed to migrate in a similar manner.

Figure 4.2 Agarose gel electrophoresis analysis of RNA isolated from sheep liver



20 µl of sample buffer was added to each 20 µl RNA sample and the RNA was denatured by incubating at 60°C for 10 min. The RNA sample was cooled on ice for 2 min before 1 µl of loading dye and 2 µl of ethidium bromide (0.2 µg / ml) were added. Samples were electrophoresed in a 1% agarose-MOPS-formaldehyde gel and visualised with a UV transilluminator (302 nm). 10, 20 µg and 30 µg of rat RNA was loaded into lanes 1, 2 and 3 respectively.

4.3 PCR amplification

4.3.1 RT-PCR using sheep RNA as the template

RT-PCR involves the synthesis of first strand cDNA, which is then used as the template for PCR amplification. The first strand cDNA reaction was primed by hybridising oligo (dT) to the 3′ poly (A) tails of RNA extracted from sheep liver. The Superscript Preamplification System uses Superscript RNase H-RT to eliminate the RNase H activity (found in other RTs) that degrades mRNA during first strand synthesis.

4.3.1.1 Results and discussion

Using the primer oligo (dT), first strand cDNA was synthesised (Section 2.6.7) from the isolated sheep liver RNA. PCR was then carried out (Section 2.6.6) on the first strand cDNA using the degenerate primers to amplify a region of the sheep liver arginase cDNA. The dNTP and $MgCl_2$ concentrations were varied, and the annealing temperature of the PCR cycles was lowered to 40°C, but no PCR product was produced from sheep liver first strand cDNA.

The successful amplification of a region of the sheep liver arginase cDNA sequence relies on a number of factors, including the isolation of high-quality RNA, successful cDNA synthesis, the primers annealing to the cDNA template and optimal PCR conditions for the generation of a PCR product. Not all of these conditions may have been met because:
(1) The isolation of high-quality mRNA requires a RNase-free environment.
(2) The positive control RNA for the cDNA synthesis reaction was not available therefore it was not possible to verify the performance of the first strand cDNA synthesis reaction.
(3) The degenerate primers are both highly redundant (see Appendix 1). Codon usage may also decrease the probability of hybridisation, if don't take into account that the species looking at does not use certain codons in its amino acid sequences.
(4) On average, only 20 -30 % of the original RNA molecules are reverse transcribed into cDNA (Qiagen PCR handbook, Oct. 1996). The relatively low efficiency of RT reaction combined with high residual content of RNA molecules in the sample after reverse transcription has to be taken into consideration when calculating the appropriate amount of starting template for the PCR.

4.3.2 RT-PCR using rat liver RNA as the template

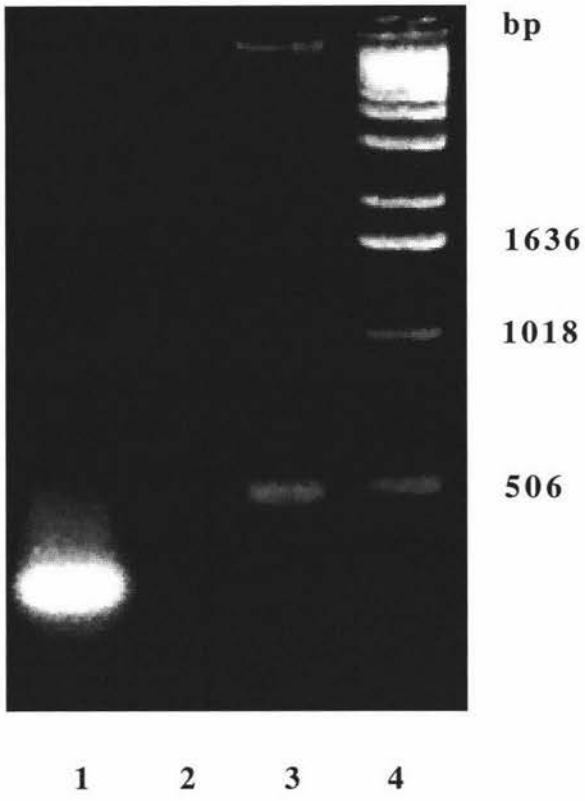
The failure of the RT-PCR on sheep liver mRNA prompted a new approach. Arginase sequences between species show a high degree of conservation, e.g. there is 87% identity between the human and rat arginase amino acid sequences. If this conservation is extended to the sheep, it may be possible to use a fragment of the rat arginase sequence as a probe to screen a sheep liver cDNA library.

4.3.2.1 Results and discussion

A 495 bp cDNA region from rat RNA was PCR amplified (Section 2.6.6) using the specific primers which were designed to the known rat arginase cDNA sequence. The PCR product was analysed by agarose gel electrophoresis (Figure 4.3) as described in Section 2.6.2. The absence of any bands in the negative control lane demonstrated that the observed PCR products were not artefacts of any contaminating DNA acting as a template. The 495 bp product was gel isolated using Prep-a-Gene (Biorad) (Section 2.6.3), and a diagnostic digest on the PCR product was performed.

The 495 bp PCR product was digested with the enzymes *Fok* I and *Mun* I (Section 2.6) and the fragments generated analysed by agarose gel electrophoresis. Fragments of the expected size (Figure 4.4) were obtained indicating that the PCR product was a fragment of the rat arginase cDNA sequence.

Figure 4.3 Agarose gel electrophoresis analysis of the rat PCR product



20 µl of each PCR mix was loaded onto a 1% agarose gel (1x TAE). After electrophoresis the gel was stained with ethidium bromide (0.2 µg / ml) and visualised with a UV transilluminater (302 nm). The PCR product was analysed by agarose gel electrophoresis as described in Section 2.6.2.

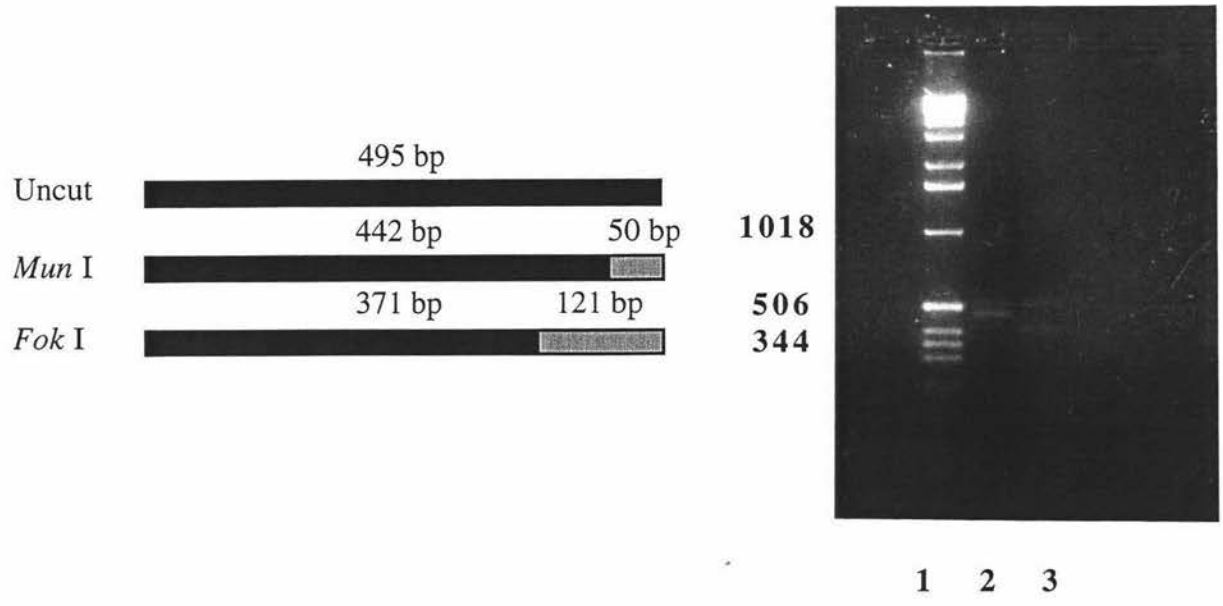
Lane 1: Primers 1917 and 1916 (Franklin, 1995) were used to amplify a 300 bp region from the template DNA (pTG3954) (Franklin, 1995). This was used as a positive control for the PCR. It contained the same concentrations of $MgCl_2$, dNTPs, primers and 10x buffer as reaction tubes.

Lane 2: Negative control. Contains all the reactants of the PCR (including primers 1917, 1916 and the rat forward and reverse primers) except for template DNA.

Lane 3: A 500 bp product was amplified using the rat forward and reverse primers (see Appendix 1 for primer sequences) from template DNA (cDNA synthesised from rat liver).

Lane 4: DNA molecular size markers (1 kb ladder, BRL)

Figure 4.4 Restriction enzyme digest of the rat PCR product



5 µl of each restriction digest was loaded onto a 1% agarose gel (1x TAE). After electrophoresis the gel was stained with ethidium bromide (0.2 µg / ml) and visualised with a UV transilluminator (302 nm). The PCR product was analysed by agarose gel electrophoresis as described in Section 2.6.2.

Mun I digested to produce 50 bp and 442 bp fragments (lane 2). *Fok* I digested to produce 121 bp and 371 bp fragments (lane 3). The 121 bp and 50 bp fragments are not visible on the gel because they are too small. Presence of a band migrating at ~500 bp suggests partial digestion of the PCR product. DNA molecular size markers (1 kb ladder, BRL) were added to lane 1.

4.3.3 PCR on a sheep cDNA library

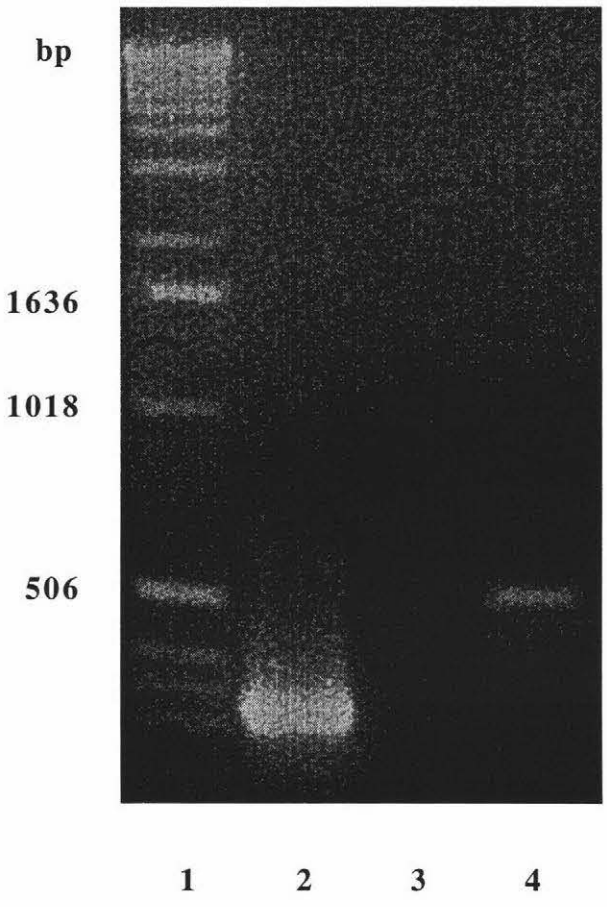
PCR performed using a cDNA λ-ZAP library as template DNA is generally not carried out for the following reasons: (1) the cDNA is packaged into phage particles, therefore the virus coat must be melted to expose the cDNA for PCR amplification, and (2) if arginase is present as a low copy number mRNA in the liver, it may not be represented in the amplified cDNA library that was used as template DNA. Due to the difficulty experienced in carrying out RT-PCR on sheep liver, PCR was attempted using a sheep cDNA library as template DNA. If a sheep arginase PCR product could be produced from the sheep

cDNA library this would mean that (1) arginase cDNA is represented in the cDNA library and (2) the PCR product would be a perfect probe for screening the cDNA library.

4.3.3.1 Results and discussion

PCR carried out on a sheep liver cDNA library using the degenerate primers described in Section 4.1.1 amplified a 500 bp product (Figure 4.9) from a λ-ZAP sheep cDNA library. PCR was carried out as described in Section 2.6.6. The PCR reaction was optimised. The optimised reactant concentrations were 50 µM dNTP mix and 0.2 pmol / µl of each primer being added to each PCR tube (final concentrations in the PCR tube is given). The optimised annealing temperature used was 50°C. Agarose gel electrophoretic analysis of the PCR mix is shown in Figure 4.5.

Figure 4.5 Agarose gel electrophoresis analysis of the PCR product generated from a sheep cDNA library



20 µl of each PCR mix was loaded onto a 1% agarose gel (1x TAE). After electrophoresis the gel was stained with ethidium bromide (0.2 µg / ml) and visualised with a UV transilluminator (302 nm). The PCR product was analysed by agarose gel electrophoresis as described in Section 2.6.2.

Lane 1: DNA molecular size markers (1 kb ladder, BRL).

lane 2: Primers 1917 and 1916 (Franklin, 1995) were used to amplify a 300 bp region from template DNA (pTG3954) (Franklin, 1995). This PCR was used as positive control for the PCR. It contained the same concentrations of $MgCl_2$, dNTPs, primers and 10x buffer as reaction tubes.

Lane 3: Negative control. Contains all PCR reactants (including primers 1917, 1916 and the sheep degenerate forward (1) and reverse primers) except template DNA.

Lane 4: A 500 bp product was amplified from a sheep cDNA library using the sheep degenerate forward (1) primer and the sheep degenerate reverse primer (see Appendix 1 for primer sequences).

4.4 Subcloning of the PCR products

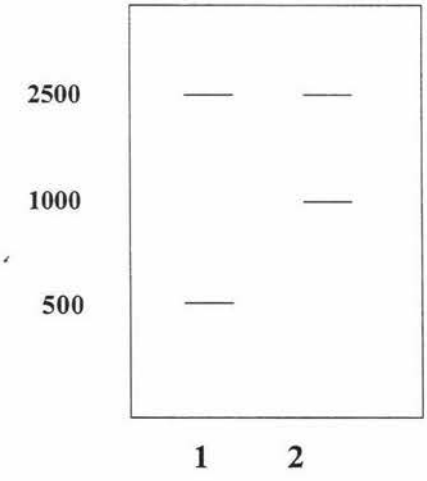
4.4.1 Results and discussion

Both the rat and sheep PCR products from several identical reactions were gel purified as described in Section 2.6.3 using Prep-a-Gene (Biorad), combined, and quantitated by agarose gel electrophoresis (Section 2.6.2), ready for use in subsequent cloning and sequencing steps.

Four different cloning schemes were attempted to ligate the rat and sheep liver cDNA PCR products into pKS. These schemes are described in Figures 4.7 to 4.10.

Vector and insert were ligated as described in Section 2.6.11 and then transformed into $CaCl_2$-competent *E. coli* XL-1 Blue cells following the method outlined in Section 2.6.12. Single colonies were seeded into 5 ml of LB-AMP medium and incubated overnight at 37°C at 200 rpm. The plasmid DNA was isolated and screened for insert DNA, by restriction analysis with *Pvu* II. *Pvu* II should cleave on either side of the multiple cloning site to release a 1 kb fragment (Figure 4.6) if an insert is present.

Figure 4.6 *Pvu* II digest analysis of mini-prep isolated plasmid DNA

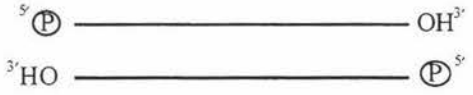


Lane 1 represents what is expected if no insert ligates with the vector DNA, whereas lane 2 represents what is expected if insert DNA does ligate with the vector DNA following digestion with *Pvu* II.

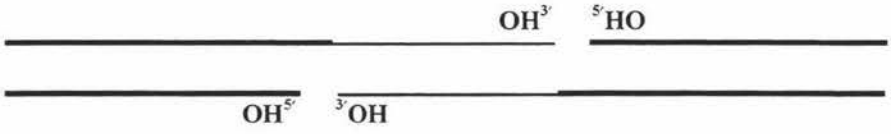
Figure 4.7 Line diagram representation of subcloning scheme 1

(1) pKS was digested with *Eco*R V to produce a blunt-ended vector. Once digestion has gone to completion, the plasmid was gel purified (Section 2.6.3). The plasmid was then dephosphorylated with TsAP to prevent religation of the vector (Section 2.6).

(2) The 495 bp rat liver arginase cDNA PCR product was gel purified from an agarose gel, then phosphorylated with T4 PNK (Section 2.6.10) and blunt-ended with T4 DNA polymerase (Section 2.6.8).



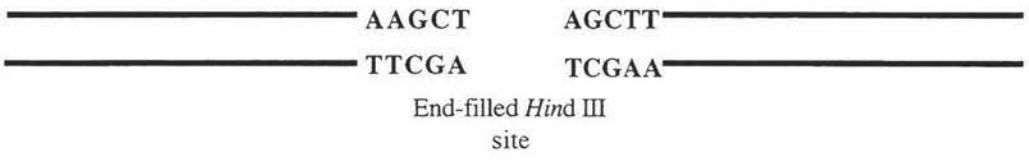
(3) Both vector and insert DNA were quantitated and then blunt-end ligated using T4 DNA ligase.



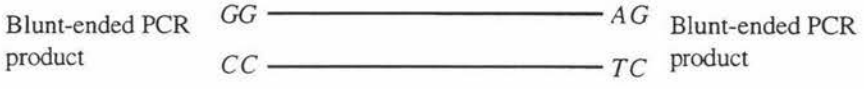
Ligation of vector and insert occurs through bond formation between the 5′ phosphate groups on the insert and the 3′ hydroxyl groups on the vector.

Figure 4.8 Line diagram representation of subcloning scheme 2

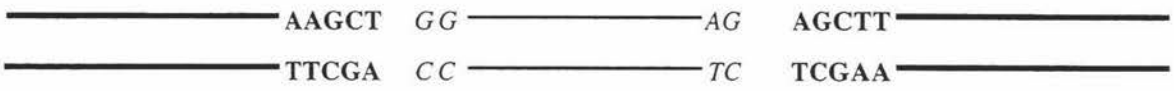
(1) pKS was digested with *Hind* III (Section 2.6.1) to produce a vector with sticky ends. The restriction enzyme was removed from the digest by phenol: chloroform extraction and ethanol precipitation. 5′ overhangs were then filled using the polymerase activity of T4 DNA polymerase (Section 2.6.8), to produce a blunt-ended vector.



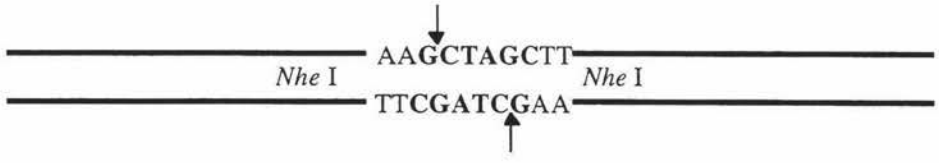
(2) Following the gel purification of the PCR product, the DNA fragment was blunt-ended using the 3′ to 5′ exonuclease activity of T4 DNA polymerase (Section 2.6.8).



(3) Both vector and insert DNA were gel purified, quantitated and then blunt-end ligated together using T4 DNA ligase.



(4) Digested ligation reactions with *Nhe* I (Section 2.6.1) to linearise religated vector. The bases in bold indicate the *Nhe I* recognition site, and the arrows indicate Nhe I digest sites.

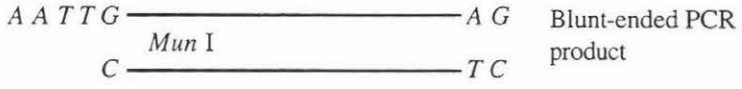


None of the plasmid mini-prep DNA isolated from colonies were found to contain insert DNA.

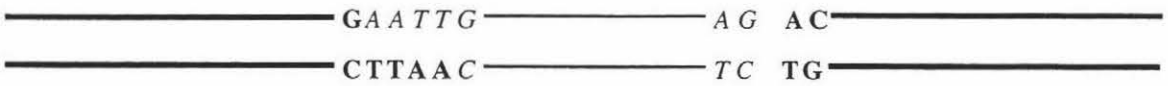
Figure 4.9 Line diagram representation of subcloning scheme 3

(1) pKS DNA was digested with *Eco*R I (Section 2.6.1) and then the reaction products were analysed using agarose gel electrophoresis (Section 2.6.2). Once digestion had gone to completion the vector was ethanol precipitated (Section 2.6) to remove the restriction enzyme. The linear plasmid was then digested with *Hinc* II. The vector was then ethanol precipitated to remove the 22 bp fragment produced by the double digest.

(2) The gel purified PCR product was treated with T4 DNA polymerase to produce blunt ends (Section 2.6.8). The fragment was then *Mun* I digested to produce a sticky end. Phenol: chloroform extraction and ethanol precipitation steps were carried out to remove the restriction endonclease, added dNTPs and the 50 bp fragment produced by the *Mun* I digest (Section 2.6).



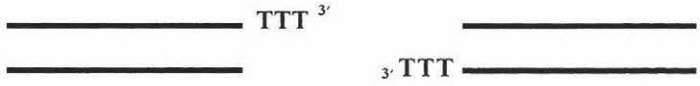
(3) Both the PCR product and vector were quantitated and then ligated with the vector using T4 DNA ligase.



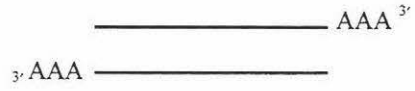
Mun I digesting the PCR product generated a fragment with the same compatible termini as the *Eco*R I digested pKS vector.

Figure 4.10 Line diagram representation of subcloning scheme 4

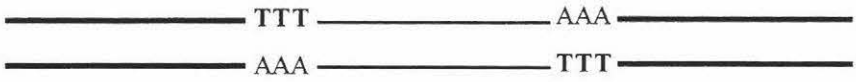
(1) pKS was T-tailed as described in Section 2.6.13. The T-vector was dissolved in 20 µl of sterile water and quantitated by agarose gel electrophoresis (Section 2.6.2).



(2) The PCR product which had been produced by amplification of template DNA using *Taq* polymerase, was gel purified using Prep-a-Gene (Biorad) (Section 2.6.3), and quantitated.



(3) The PCR product was ligated with the T-tailed vector using T4 DNA ligase.



4.5 Analysis of subcloning

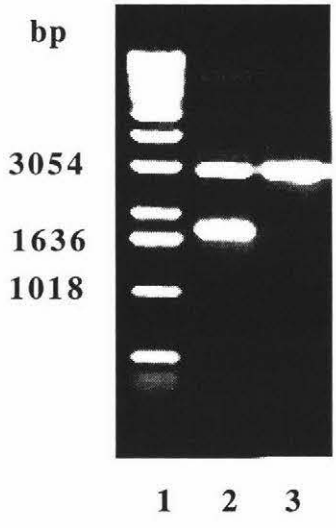
4.5.1 Subcloning the rat PCR product

Attempted ligating the rat PCR product into pKS using ligation schemes 1, 2 and 3.

Cloning scheme I

Following digestion (Section 2.6.1) of the vector with *Eco*R V, the reaction product was analysed on a 0.7% agarose gel (Figure 4.11) as described in Section 2.6.2.

Figure 4.11 Agarose gel electrophoresis analysis of pKS vector digested with the restriction enzyme *Eco*R V



100 ng of digested pKS vector was run next to 100 ng of uncut vector on a 0.7% agarose gel. After electrophoresis the gel was stained with ethidium bromide (0.2 µg / ml) and visualised with a UV transilluminater (302 nm). The PCR product was analysed by agarose gel electrophoresis as described in Section 2.6.2.

1: DNA molecular size markers (1 kb ladder, BRL).

2: Uncut pKS vector which runs as two bands on an agarose gel. The two bands represent supercoiled and covalently closed circular DNA.

3: pKS vector digested with *Eco*R V.

*Eco*R V digested the 3 kb vector once, and the linear product produced by the digest migrated on an agarose gel as a single band (Figure 4.11). The disappearance of the lower band representing the covalently closed circular subset of the uncut vector implies that digestion has gone to completion, but because the linear 3 kb size vector comigrated with the supercoiled subset of the uncut vector the extent of cannot be determined.

Greater than 100 colonies grew on the vector plus ligase control plate and 30 colonies grew on the vector only plate indicating that the vector had not been efficiently dephosphorylated or digested. Both CIP and excess amounts of TsAP were used as methods to remove end phosphates but for unknown reasons the plasmid was unable to be completely dephosphorylated. The inability to completely dephosphorylate the vector or be able to visualise complete digestion on an agarose gel due to the comigration of uncut supercoiled DNA and linear 3 kb vector caused a high background on plates due to religated vector to be observed, making screening for recombinants difficult.

Cloning scheme 2

Due to the difficulty experienced in dephosphorylating the vector, a new ligation experiment was designed which did not require dephosphorylation of the vector (Cloning scheme 2). The ligation experiment was fundamentally the same except any vector that religates during ligation will form the new restriction site *Nhe* I. Digesting the ligation mix with the restriction enzyme *Nhe* I will eliminate background on plates produced as a result of religated vector.

None of the miniprep isolated plasmid DNA that was extracted from colonies contained the 495 bp insert.

Cloning scheme 3

At this stage, a positive clone containing the insert had not been identified. Blunt-end ligations are more difficult than cohesive-end ligations which led to the third cloning scheme being attempted. Assuming that the PCR product is a fragment of the known rat hepatic arginase cDNA, it should be possible to digest both the vector and PCR product to generate DNA molecules with compatible ends. This method avoided having to both phosphorylate the PCR product or dephosphorylate the vector, as religation of the vector was made unlikely by giving it incompatible ends.

Digestion trials suggested that double digestion of pKS with *Eco*R I and *Hin*c II in react buffer 4 was inefficient therefore pKS was serially digested in the optimal buffer for each enzyme.

The ligation reactions were transformed into $CaCl_2$-competent *E. coli* XL-1 Blue cells and plated onto LB-AMP plates in the presence of X-gal and IPTG, allowing the selection of recombinant clones which appeared as white colonies (Section 2.6.12). Non-recombinants appeared as blue colonies. The plasmid was efficiently double digested as no more than 10 colonies were present on either of the ligation control plates. Miniprep plasmid DNA isolated from white colonies was screened for insert DNA following the method described previously in Figure 4.6 but no plasmids containing insert DNA were identified.

4.5.2 Subcloning the sheep PCR product

Cloning schemes 4 and 2 were the methods used to subclone the sheep PCR product into pKS.

Cloning scheme 4

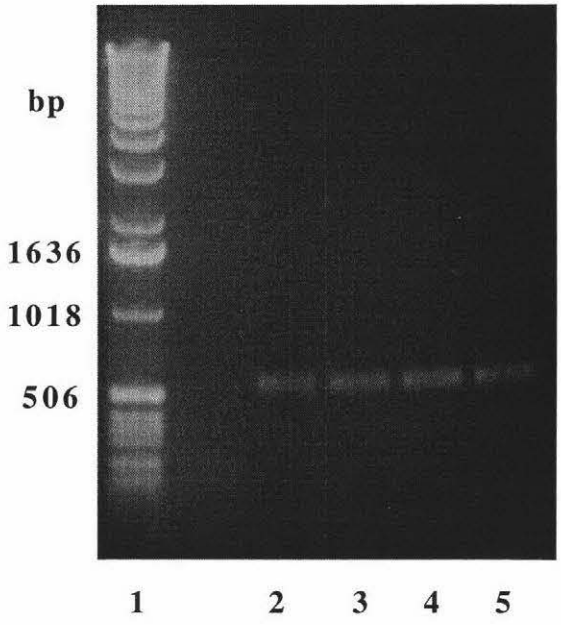
Taq polymerase has a terminal transferase activity that preferentially adds an adenine to the 3′ end of PCR products. Therefore by adding a thymidine to the 3′end of the vector this complements the ends of the PCR product and allows ligation to occur. The ligation reactions were transformed into $CaCl_2$-competent *E. coli* XL-1 blue cells and plated onto LB-AMP plates in the presence of IPTG and X-gal allowing selection of recombinant white colonies. A high number of colonies were present on the control plates. This background may be due to a loss of the 3′ adenine through degradation (Zhou *et al.*, 1995). To help prevent this degradation, newly amplified PCR products were used for cloning. Miniprep plasmid DNA isolated from white colonies were screened for insert DNA but no plasmids contained insert DNA.

Cloning scheme 2

Before attempting to subclone the sheep PCR product into pKS using cloning scheme 2, the PCR product was digested with *Pvu* II, *Nhe* I and *Hin*d III to establish that the PCR product does not contain these restriction enzyme sites (Figure 4.12). A 500 bp fragment

was observed in lanes 2 to 5, indicating that there is neither a *Nhe* I, *Pvu* II or *Hin*d III site in the cDNA sequence.

Figure 4.12 *Nhe* I and *Pvu* II digest of the sheep PCR product



50 ng of PCR product amplified from a sheep liver cDNA λ-ZAP library was digested with *Pvu* II and *Nhe* I as described in Section 2.6, and electrophoresed on a 1% agarose gel next to 50 ng of undigested PCR product. The gel was stained with ethidium bromide (0.2 μg / ml) and visualised with a UV transilluminator (302 nm). The PCR product was analysed by agarose gel electrophoresis as described in Section 2.6.2.

Lane 1: DNA molecular size markers (1 kb ladder, BRL).

Lane 2: PCR product digested with *Pvu* II.

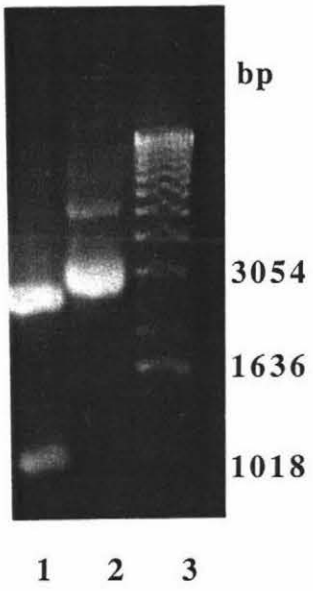
Lane 3: PCR product digested with *Nhe* I.

Lane 4: PCR product digested with *Hin*d III

Lane 5: PCR product undigested

Using this ligation scheme the PCR product was successfully subcloned into pKS. Miniprep plasmid DNA isolated from one colony, digested with *Pvu* II to produce two fragments which when analysed by agarose gel electrophoresis ran at 2500 and 1000 bp (Figure 4.13). Maxiprep DNA was then prepared from the miniprep culture (2.6.5) and quantitated on an agarose gel.

Figure 4.13 Agarose gel electrophoresis analysis of isolated miniprep plasmid DNA



100 ng of digested pKS plasmid was electrophoresed next to 100 ng of undigested plasmid on a 1 % agarose gel. The gel was stained with ethidium bromide (0.2 µg / ml) and visualised with a UV transillumninator (302 nm). The PCR product was analysed by agarose gel electrophoresis as described in Section 2.6.2.

Lane 1: Plasmid DNA digested with *Pvu* II.

Lane 2: Undigested plasmid DNA. The two bands on the gel represent supercoiled and covalently closed circular DNA.

Lane 3: DNA molecular size markers (1 kb ladder, BRL).

4.6 DNA sequencing

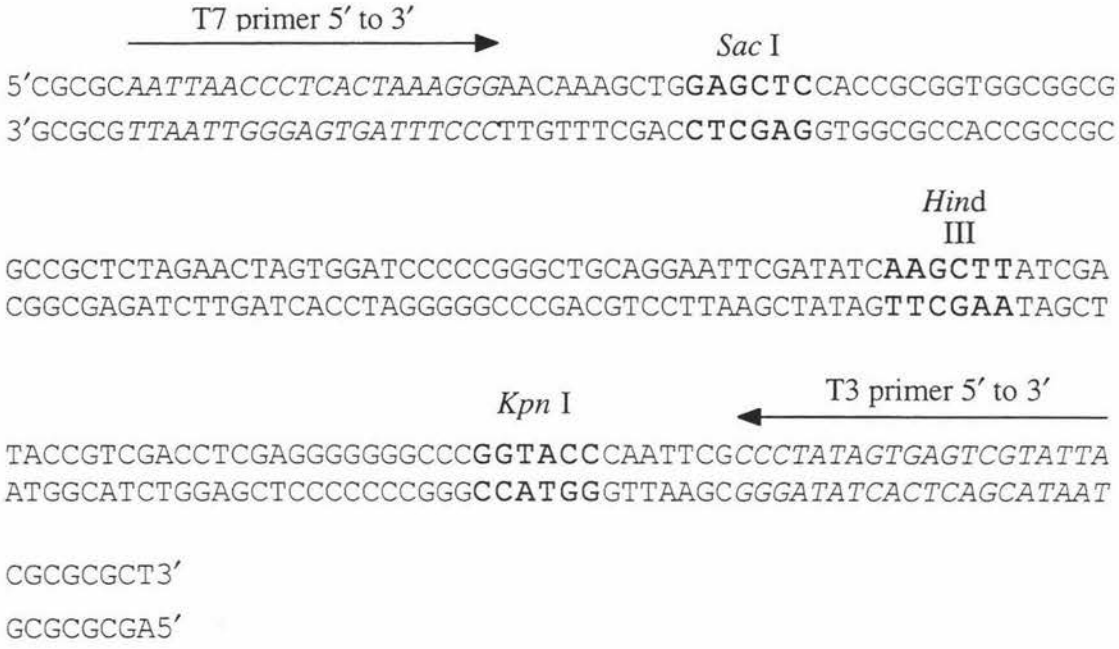
DNA sequencing was used to determine if the cloned PCR product nucleotide sequence was consistent with conserved arginase amino acid sequences.

4.6.1 Results and discussion

Both the isolated miniprep and maxiprep DNA was sequenced (Section 2.7) using the oligonucleotide primers T7 and T3 (refer Appendix 1). T7 and T3 bind to the T3 and T7

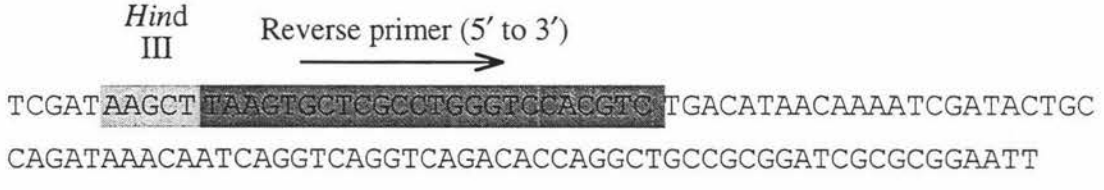
RNA promoters which flank the multiple cloning site of pKS (refer Appendix 2). T3 anneals 50 bp upstream and T7 80 bp downstream from the *Hin*d III site, employed for the subcloning. The nucleotide sequence of the PCR product showed that it was produced by annealing and extension of the reverse primer alone and was not a fragment of the sheep arginase cDNA sequence.

The multiple cloning site region of pKS where T3 and T7 primers anneal is as follows:

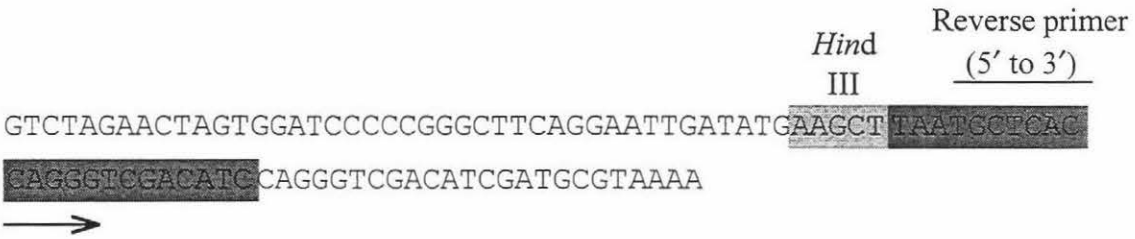


The insert DNA was ligated into a blunt ended *Hin*d III site, therefore the *Hin*d III site was used as a marker of where the insert DNA sequence started from.

The sequence that was produced from the T3 primer was as follows:

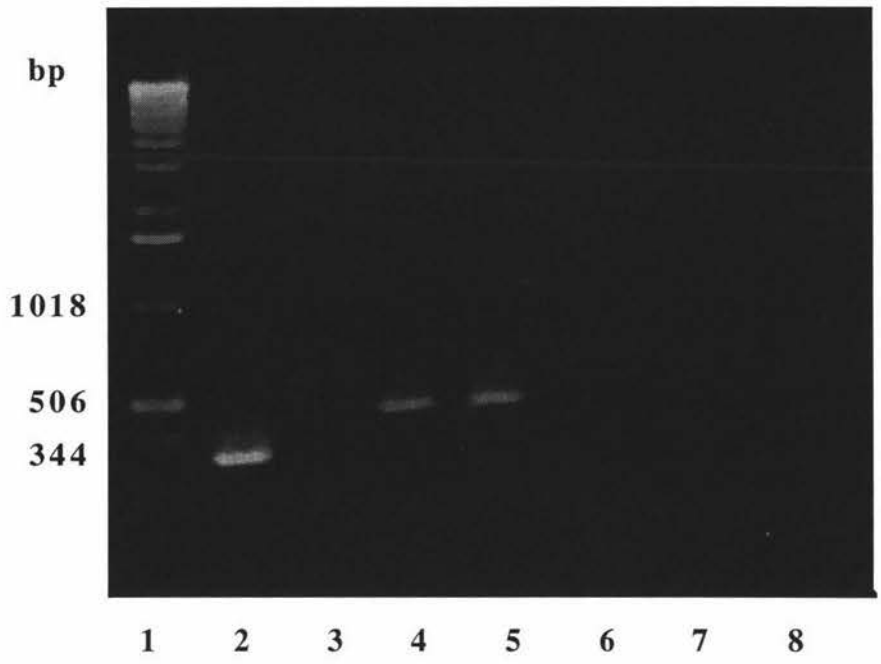


The sequence that was produced from the T7 primer was as follows:



PCR analysis was then carried out (Figure 4.14) to verify these results. A 500 bp product was observed in both, the lane contining reverse and forward primers and the lane containing the reverse primer alone.

Figure 4.14 Agarose gel electrophoresis analysis of PCR products generated from the sheep liver λ-ZAP cDNA library using different primer combinations



20µl of each PCR reaction mix was analysed on a 1% agarose gel (1x TAE), stained with ethidium bromide (0.2 µg / ml) and visualised with a UV transilluminator (302 nm).

Lane 1: DNA molecular size markers (1 kb ladder, BRL).

Lane 2: Primers 1917 and 1916 (Franklin, 1995) PCR amplified a 300 bp region from template DNA (pTG3954) (Franklin, 1995). This was used as a positive control for the PCR. It contained the same concentrations of $MgCl_2$, dNTPs, primers and 10x buffer as reaction tubes.

Lane 3: Negative control. Contains all reactants of the PCR (including primers 1917, 1916 and the sheep generate forward (1), (2) and reverse primers) except for template DNA.

Lane 4: Sheep forward primer (1) and reverse primer (see Appendix 1 for primer sequences) produced a 500bp product from the DNA template (sheep liver cDNA library).

Lane 5: The sheep reverse primer alone produced a 500 bp product from the template DNA (sheep liver cDNA library).

Lane 6: Sheep foward primer (2) and sheep degenerate reverse primer did not produce a PCR product.

Lane 7: Sheep degenerate forward (1) primer alone did not produce a PCR product.

Lane 8: Sheep degenerate forward (2) primer alone did not produce a PCR product.

Chapter 5

SCREENING A λ-ZAP cDNA LIBRARY FOR SHEEP HEPATIC ARGINASE SEQUENCES

An expression plasmid for the full-length cDNA of human liver-type arginase, pTAA12 (Ikemoto *et al.*, 1990) was obtained from Toshiaki Kono (Japan). The percentage identity and similarity between the rat and human arginase amino acid sequences are 92% and 87% respectively. Assuming that the sheep arginase sequence also has a high percentage similarity with the human sequence, a fragment of the human arginase cDNA sequence, derived from the pTAA12 expression plasmid, would make a suitable probe to screen a sheep liver cDNA library.

5.1 Northern blot analysis using the nonradioactive DIG system

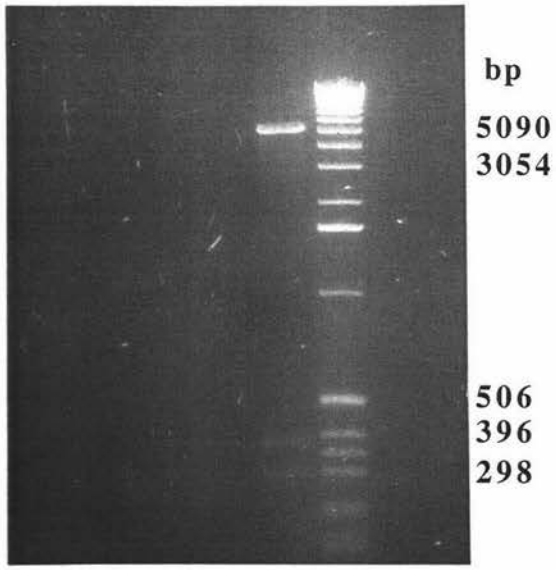
The nonradioactive DIG system uses digoxigenin-11-dUTP, a steroid hapten, to label the cDNA fragment. This alkali-labile probe is easy to strip from blots allowing for repeated probing of the blot. The probe can be stored at -20°C for at least one year without loss of activity. The DIG-labelled DNA probe was generated using DIG-High prime which is a specially developed reaction mixture containing all reagents necessary for random primed labelling, premixed in a 5x concentrated reaction buffer. DIG-High prime generates large quantities of labelled DNA from small amounts of starting material over 20 h of incubation. The DIG-dUTP labelled probe can be detected using anti-DIG-alkaline phosphatase which specifically binds to DIG-dUTP, and a chemiluminescent alkaline phosphatase substrate (CSPD®) which binds to the anti-DIG-AP. CSPD® produces a light signal that can be detected by exposing the membrane to X-ray film (Boehringer Mannheim DIG system user's guide, 1995).

There are three restriction sites for the *Sty* I restriction endonuclease in the pTAA12 expression plasmid, all within the arginase cDNA. Digestion of pTAA12 with *Sty* I produces two fragments, of arginase cDNA 304 bp and 387 bp in length. Either of these fragments should have made a suitable probe for screening a sheep liver cDNA library.

5.1.1 Results and discussion

Approximately 10 µg of the pTAA12 expression plasmid was digested with 10 U of *Sty* I enzyme for 2 h at 37°C. The enzyme *Sty* I is active for only 1 h therefore the plasmid was initially digested with 5 U of *Sty* I for 1 h before adding another 5 U of enzyme and digesting for a further 1 h. The three reaction products were analysed by agarose gel electrophoresis as described in Section 2.6.2. The sizes of the three fragments were consistant with the expected 304 bp, 387 bp and 5.31 kb fragments predicted from a theroretical digest of the pTAA12 plasmid (Figure 5.1). The 387 bp and 304 bp *Sty* I fragments were then purified from the agarose gel (Section 2.6.3). The 387 bp fragment was nonradioactively labelled with DIG-11-dUTP using the DIG high Prime DNA labelling kit (Section 2.9.3).

Figure 5.1 Agarose gel electrophoresis analysis of the pTAA12 human liver arginase expression plasmid following digestion with *Sty* I



20 µl of the digest was electrophoresed on a 1% agarose gel (1x TAE). After electrophoresis the gel was stained with ethidium bromide (0.2 µg / ml) and visualised with a UV transilluminator (302 nm). The digest was analysed by agarose gel electrophoresis as described in Section 2.6.2.

Lane 1: pTAA12 expression plasmid digested with *Sty* I

Lane 2: DNA molecular size markers (1 kb ladder, BRL)

Isolation of total RNA from sheep and rat liver was performed as described in Section 2.9.1. Fractions of the RNA samples were then analysed by agarose gel electrophoresis (Section 2.9.2). 16 ng of the pTAA12 expression plasmid was electrophoresed alongside the RNA samples as a positive control for the Northern blot analysis step. The presence of high molecular weight ribosomal RNA species in the samples and the appearance of the discrete 28S and 18S rRNA bands suggested that the samples were essentially free of ribosomal degradation.

The total cellular RNA, separated by gel electrophoresis (Section 2.9.2) was transferred to a nylon membrane by Northern transfer (Section 2.9.3). A charged nylon membrane (Hybond™ N^+, Amersham, Life Science) was used because it has optimal charge density, allowing the probe to bind tightly without producing high background. The membrane was probed with the DIG-labelled 387 bp fragment of human arginase cDNA and the signal was detected using chemiluminescense (Section 2.9.3). Chemiluminescent detection produced a signal which was visualised as a smear representing nonspecific binding of the probe to both the blotted sheep and rat RNA samples.

DIG-labelled probes tend to produce more background than ^{32}P-labelled probes. With chemiluminescent detection, too high a probe concentration often leads to high background, therefore mock hybridisations need to be carried out in order to optimise the probe concentration. Probe filtration may also need to be carried out in an attempt to reduce nonspecific binding. A [α-^{32}P]-dCTP labelled probe may be a more efficient probe than the nonradioactively labelled probe used.
From the nonspecific binding observed it was decided that a ^{32}P-labelled probe should be used for at least the first round of screening of a sheep liver cDNA library and that the Northern blot should be repeated using a ^{32}P-labelled probe. Unfortunately the Northern blot could not be repeated due to time constraints.

5.2 Library screening

Difficulty in isolating sufficient quantities of the human arginase cDNA *Sty* I fragments due to incomplete digestion by the enzyme and pour recovery during fragment isolation from agarose gels, led to the use of a PCR-based strategy to generate probe DNA. The PCR product was ^{32}P-radiolabelled and the resulting probe was used to screen a sheep liver cDNA library for a clone containing the sheep arginase cDNA sequence.

5.2.1 Results and discussion

A 532 bp region of DNA was PCR amplified (Section 2.6.6) from the pTAA12 expression plasmid using the degenerate primers described in Section 4.1. The PCR cycle annealing temperature was optimised to 55°C.

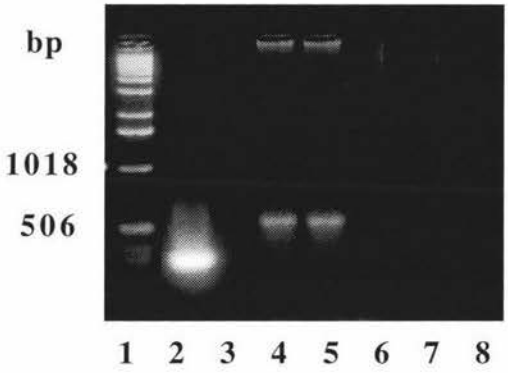
The PCR product was analysed by agarose gel electrophoresis (Figure 5.2) and then purified using Prep-a-Gene (Biorad) (Section 2.6.3). Figure 5.2 shows that no bands were present in the negative control lane, indicating the absence of any contaminants in the PCR which could act as a template. The absence of any extraneous bands in lanes 6, 7 and 8 suggests that the primers were specifically binding to the template cDNA (pTAA12) to produce the 532 bp product. Approximately 20 ng of the PCR product was labelled with [α-^{32}P]-dCTP using the Rediprime random labelling kit (Section 2.8). The PCR product was neither diagnostically digested nor sequenced to demonstrate that the PCR product was amplified from a region of the human arginase cDNA sequence due to time constraints. If the experiment was to be repeated in the future these would need to be carried out.

The amplified sheep liver cDNA λ-ZAP library (titre; 1x 10^6 pfu / ml) was screened with the labelled probe prepared as described above, to identify clones containing the sheep arginase sequence. Approximately 2x 10^4 clones were screened at a density of around 6660 pfu per agar plate. Duplicate nitrocellulose phage lifts were taken from each plate (Section 2.8). The duplicate filters were then probed with the radiolabelled human liver arginase cDNA fragment and autoradiographed as described in Section 2.9.

First round screening of the sheep liver cDNA library produced no positive plaques. Unfortunately, time constraints prevented the repetition of the first round of screening. It was not surprising that no positive clones were identified, as a lower than recommended

number of pfu for an amplified library were screened (2x 10^4 pfu cf 10^6 pfu) (Sambrook *et al.*, 1989). But as it was the first attempt at screening a cDNA library, a lower number of pfu were screened in an attempt to become more familiar with the techniques involved in library screening.

Figure 5.2 Agarose gel electrophoresis of the PCR product generated from the pTAA12 expression plasmid



20 µl of each PCR reaction was loaded onto a 1% agarose gel (1x TAE). After electrophoresis the gel was stained with ethidium bromide (0.2 µg / ml) and visualised with a UV transilluminater (302 nm). The PCR product was analysed by agarose gel electrophoresis as described in Section 2.6.2.

Lane 1: DNA molecular size DNA markers (1 kb ladder, BRL).

Lane 2: Primers 1917 and 1916 (Franklin, 1995) were used to amplify a 300 bp region from the template DNA (pTG3954) (Franklin, 1995). This was used as a positive control for the PCR. It contained the same concentrations of $MgCl_2$, dNTPs, 10x buffer and primers as reaction tubes.

Lane 3: Negative control. Contains all the reactants of the PCR (including primers 1916, 1917 and the sheep degenerate forward (1), (2) and reverse primers) except for template DNA.

Lane 4: A 500 bp product was amplified from the template DNA (pTAA12) using the forward primer (1) and the reverse primer (see appendix 1 for primer sequences).

Lane 5: A 500 bp product was amplified from the template DNA (pTAA12) using the forward primer (2) and the reverse primer.

Lane 6: No PCR product was amplified when using the forward primer (1) alone from the template DNA (pTAA12).

Lane 7: No PCR product was amplified when using the forward primer (2) alone.

Lane 8: No PCR product was amplified when using the reverse primer alone.

Chapter 6

DISCUSSION AND CONCLUSIONS

6.1 Partial purification procedure

Partial purification was attempted first to obtain some amino acid sequence for sheep liver arginase. The purification scheme used by Schimke (1970) to purify rat liver arginase was used as an initial guide for the purification of sheep liver arginase. This scheme was modified after discovering that the sheep enzyme did not behaved differently from the predicted behaviour of the rat enzyme during purification. Several new matrices were tested in these modifications. A negatively charged Q-Sepharose matrix was initially tested because sheep liver arginase did not bind very efficiently to the positively charged CM-Sepharose matrix. In the original purification a CM-cellulose matrix was used therefore it was expected that arginase would behave as for the CM-cellulose matrix. But sheep liver arginase did not bind to the Q-Sepharose matrix. After a number of attempts to purify sheep liver arginase, the N-terminus of the putative arginase was found to be blocked, and the enzyme could not be purified sufficiently for successful electroelution from an SDS-PAGE gel. Had this been possible, the pure protein could have been proteolytically cleaved or cleaved with cyanogen bromide.
C-terminal sequencing of the N-terminally blocked protein could have been carried out, but probably would not have been very helpful because only three or four amino acids can be sequenced using this method. C-terminal sequencing is generally only used to verify the integrity of proteins where the amino acid sequence is known, not proteins of unknown amino acid sequence.

A possible future approach would be to elute arginase from a whole preparative PAGE gel using a Whole Gel Eluter (Biorad). Whole Gel Eluting rapidly extracts all proteins from an entire preparative gel (SDS or nondenaturaing PAGE). Individual bands, or groups of closely spaced bands are collected in liquid fractions, ready for subsequent arginase activity analysis (in the case of elution from an SDS gel, the enzyme would first have to refolded to an active form). This allows a quick and efficient way of identifying the band of interest. The distance between proteins resolved on a PAGE gel must be greater than 5 mm to ensure that the protein is eluted in its pure form without contamination from neighbouring bands. Due to the difficulty experienced in separating arginase sufficiently

from the other bands on an SDS-PAGE mini gel (refer Figure 3.6), the Whole Gel Eluter may not be successful in separating arginase from nearby contaminants. But, the Whole Gel Eluter would be useful in determining with greater certainty which band represents arginase.

Future attempts to purify sheep liver arginase could benefit from an additional purification step, such as a hydroxyapatite column as the specific activity of the eluent following the Reactive Red 120 step was ~45 fold lower than at the end of the rat purification (Schimke, 1970). This relatively low specific activity may be due to partail inactivation of arginase during the purification procedure. In addition, the resolution of the protein bands on an SDS-PAGE gel could be improved by loading less sample protein in each well. If enough lanes were loaded, it should be possible to recover enough protein for N-terminal sequencing.

6.1.1 Urea activity assay

Large variations in absorbance at 520 nm between duplicate samples were observed. These variations were due to a number of factors including (1) instability of the chromophore generated to light, and (2) the colour reagent aging. These factors meant it was difficult to directly compare values obtained from different activity assays which was why the standard was included with each assay set, to correct for these variations.
Generally Mn^{2+} was not added to the enzyme samples prior to the arginase activity assay. It is therefore possible that some of the observed variations in assay results observed are due to loss of Mn^{2+} ions from a fraction of the active sites of the enzyme. In future assays, samples could be preincubated with Mn^{2+} prior to the assay to gain more accurate measurment of the total arginase protein present in the samples.

6.2 PCR-based approach

6.2.1 Primer design

When designing the sheep degenerate primers Sharg-F1, Sharg-F2 and Sharg-R, it was difficult to predict what regions and specific sequences of the cDNA arginase consensus would make good primers, as what looks good on paper does not necessarily mean the

PCR reaction will be successful. The chances of a successful PCR depend on many factors. For example, it is beneficial to have melting temperatures that differ by less than 10°C of each other. This increases the probability that the PCR product is produced from both the reverse and forward primers, and is not produced as a result of one primer annealing at either both ends of a region of DNA. It is preferable that the primers have high annealing temperatures, as this decreases the probability of nonspecific binding. Two degenerate forward primers were designed so that the very high degeneracy of the forward primer could be at least halved. When the PCR was performed two reactions were carried out, using a different forward primer in each reaction, therefore degeneracy is decreased but all the primer combinations are covered.

Pan *et al.* (1995) designed degenerate primers to conserved regions between the known arginase sequences of *Saccharomyces cerevisiae*, *Neurospora crassa*, *Schizosaccharomyces pombe*, *Agrobacterium tumefaciens*, *Xenopus laevis*, *Ratus norvegicus* and *Homo sapiens* (refer Figure 4.1). Using these primers in the PCR, Pan *et al.* (1995) were able to amplify a region of the *Coccidioides immitis* cDNA arginase gene. The degenerate primers have high melting temperatures which are within one degree of each other (66°C and 65°C) and have a much lower level of degeneracy, 192-fold and 256-fold compared with 576-fold, 2034-fold and 1536-fold of the sheep degenerate primers, which increases the probability of a PCR product being generated. These primers could be used in an attempt to PCR amplify a region of the sheep arginase cDNA.

If a positive clone is identified from screening a λ-ZAP sheep liver cDNA library, a restriction map of the positive clone could be made. The sheep liver arginase cDNA would then be subcloned and sequenced. A sheep liver genomic library could then be probed for the arginase gene with a view to characterising the regulatory elements in the gene. The regulation of the ruminant arginase gene could then be compared with that of monogastric arginase genes.

6.2.2 Subcloning of the PCR products

A number of subcloning procedures to ligate PCR products into the plasmid pKS were tried. The PCR product that was amplified from the sheep liver λ-ZAP cDNA library, and subcloned into pKS, produced only one colony containing a plasmid with an insert.

The PCR process could have produced a mixture of products of the same size. Therefore this cloning procedure could be repeated and several positive clones analysed.

Blunt-end ligations are less efficient than cohesive end ligations (Greene and Guarente, 1987). If more and/or different primers were synthesised in the future, restriction enzyme sites could be added to the ends of the primers to make subcloning easier.

6.3 Screening a sheep liver cDNA library

It would have been preferable to screen a sheep liver cDNA library using a probe designed from a region of the sheep liver arginase sequence, but this could not be obtained. An attempt was made to use a region of the human cDNA as a probe. First round screening of a sheep liver cDNA library was attempted but no positive clones were identified. Due to time constraints, a repeat of this screening using a higher number of plaque forming units was not possible. If this screening is repeated in the future, the region of the human liver arginase cDNA that is amplified should first be checked by digestion and/or sequencing before being used as a probe.

The sheep cDNA library was an amplified library, therefore it is possible that arginase is not well represented in the library (Vogeli and Kaytes, 1987). The process of amplification may have resulted in a library, in which the sheep liver arginase cDNA sequence is under represented. Arginase is a critical enzyme for function in the liver, therefore it was hypothesised that its message would be fairly abundant in the liver and consequently represented in the amplified library.

REFERENCES

Aminlari M, Vaseghi T. Arginase distribution in tissues of domestic animals. *Comp Biochem Physiol* **103**B (2): 385-389, 1992

Ausubel F, Brent R, Kingston R, Moore D, Seidman J, Smith J, Struhl K. in *Current protocols in Molecular biology*. New York: Greene Publishing Associates and Wiley Intersciences, 1987

Berüter J, Colombo J, Bachmann C. Purification and properties of arginase from human liver and erythrocytes. *Biochem J* **175**: 449-454, 1978

Bewley M, Lott J, Baker E, Patchett M. The cloning, expresison and crystallisation of a thermostable arginase. *FEBS Lett* **386** (2-3), 215-218, 1996

Borsuk P, Grzelak A, Weglenski P. Direct submission of *Emericella nidulans* amino acid sequence to *GENBANK database,* 1996

Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248-254, 1976

Brock A, Chapman S, Ulman E, Wu G. Dietary manganese deficiency decreases rat hepatic arginase activity. *Biochem Mol Roles Nutr* 340-344, 1993

Cavalli R, Burke C, Kawamoto S, Soprano D, Ash D. Mutagenesis of rat liver arginase expressed in *Escherichia coli*: role of conserved histidines. *Biochem* **33**: 10652-10657, 1994

Chieko H. Direct submission of the mouse amino acid sequence to *GENBANK database*, 1996

Cho K, Fuqua C, Martin B, Winans S. Identification of *Agrobacterium tumefaians* genes that direct the complete catabolism of octopine. *J Bacteriol* **178** (7): 1872-1880, 1996

Chowdhury S, Gotoh T, Mastaka M, Takiguchi M. CCAAT/enhancer-binding protein β (C/EBPβ) binds and activates while hepatocyte nuclear factor-4 (HNF-4) does not bind but represses the liver-type arginase promoter. *Eur J Biochem* **236**: 500-509, 1996

Curtius H, Roth M. in *Clinical biochemistry*: Principles and Methods. vol **II**, pp 1092. New York: Walter de Gruyter Co., 1974

Darling D, Brickell P. in *Nucleic blotting:* The basics. Oxford: IRL press at Oxford University press, 1994

Dizikes G, Spector E, Cederbaum S. Cloning of rat liver arginase cDNA and elucidation of regulation of arginase gene expression in H4 rat hepatoma cells. *Somat Cell Mol Endocrinol* **2**: 444-451, 1988

Fried M, Chun P. Water-soluble nonionic polymers in protein purification. *Methods Enzymol* **XXII**: 238-248, 1971

Gardan R, Rapoport G, Débarbouillé M. Expression of the *roc*DEF operon involved in arginine catabolism in *Bacillus subtilis*. *J Mol Biol* **249** (5): 843-856, 1995

Geyer J, Dabich D. Rapid method for determination of arginase activity in tissue homogenates. *Anal Biochem* **39**: 412-417, 1971

Greene J, Guarente L. Subcloning. *Methods Enzymol* **152**: 512-522, 1987

Gotoh T, Haraguchi Y, Takiguchi M, Mori M. The delayed glucocorticoid-responsive and hepatoma cell-selective enhancer of the rat arginase gene is located around intron 7. *J Biochem* **115**: 778-788, 1994

Gotoh T, Sonoki T, Nagasaki A, Terada K, Takiguchi M, Mori M. Molecular cloning of cDNA for nonhepatic mitochondrial arginase (arginase II) and comparison of its induction with nitric oxide synthase in a murine macrophage-like cell line. *FEBS Lett* **395**: 119-122, 1996

Greengard O, Sahib M, Knox E. Developmental formation and distribution of arginase in rat tissues. *Arch Biochem Biophys* **137**: 447-482, 1970

Grody W, Kern R, Klein D, Dodson A, Wissman P, Barskey S, Cederbaum S. Arginase deficiency manifestering delayed clinical sequelae and induction of a kidney arginase isozyme. *Hum Genet* **91**: 1-5, 1993

Grody W, Klein D, Dodson A, Kern R, Wissmann P, Goodman B, Bassand P, Marescau B, Kang S, Leonard J. Molecular genetic study of human arginase deficiency. *Amer J Hum Genet* **50** (6): 1281-1290, 1992

Hariguchi Y, Takiguchi M, Amaya Y, Kawamoto S, Matsuda I, Mori M. Molecular cloning and nucleotide sequence of cDNA for human liver arginase. *Pro Natl Acad Sci USA* **84** (2): 412-415, 1987

Hunkapiller M, Lujan E, Ostrander F, Hood L. Isolation of microgram quantities of proteins and polyacylamide gels for amino acid sequence analysis. *Methods Enzymol* **91**: 227-236, 1983

Ikemoto M, Tabata M, Miyake T, Kono T, Mori M, Totani M, Murachi T. Expresson of human liver arginase in *Escherichia coli*: purification and properties o fthe product. *Biochem J* **270**: 697-703, 1990

Issaly I, Issaly A. Control of ornthine carbamoyltransferase activity by arginase in *Bacillus subtilis*. *Eur J Biochem* **49**: 485-495, 1974

Iwas K, Yamauchi K, Ishikawa K. Cloning of cDNAs encoding argininosuccinate lyase and arginase from *Rana catesbeiana* liver and regulation of their mRNAs during spontaneous and thyroid hormone-induced metamorphosis. *Biochim et Biophys Acta* **1260** (2): 139-146, 1995

Jenkinson C, Grody W, Cederbaum S. Comparative properties of arginases. *Comp Biochem Physiol* **114**B (1): 107-132, 1996

Kang J, Cho Y. Purification and properties of arginase from soyabean, *Glycine max*, axes. *Plant Physiol* **93**: 1230-1234, 1990

Kanyo Z, Scolnick L, Ash D, Christianson D. Structure of a unique binuclear manganese cluster in arginase. *Nature* **383**: 554-557, 1996

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S. Sequence analysis of the genome of the unicellular cyanobacterium Synechocystis sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res* **3** (3): 109-136, 1996

Koenen M. Recovery of DNA from agarose gels using liquid nitrogen. *TIG* **5** (5): 137, 1989

Krumpelman P, Freyermuth S, Cannon J, Fink G, Polacco J. Nucleotide sequence of *Arabidopsis thaliana* arginase expressed in yeast. *Plant Physiol* **107** (4): 1479-1480, 1995

Kuhn N, Ward S, Piponski M, Young T. Purification of human hepatic arginase and it's manganese (II)-dependent and pH-dependent interconversion between active and

inactive forms: a possible pH-sensing function of the enzyme on the ornithine cycle. *Arch Biochem Biophys* **320** (1): 24-34, 1995

Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* **227**: 680-685, 1970

Lehninger A, Nelson D, Cox M. in *Principles of Biochemistry*. 2nd Ed. New York: Worth publishers Inc., 1993

Marathe S, Becker E, Yu Y, Weiss R. Direct submission of the *Neurospora crassa* arginase protein sequence to *EMBL/GENBANK/DDBJ data banks*, 1993

Meijer A, Lamers W, Chamuleau R. Nitrogen metabolism and ornithine cycle function. *Physiol Rev* **70**: 701-748, 1990

Messenguy F, Penninckx M, Wiame J. Interaction between arginase and ornithine carbomyltransferase in *S. cerevisiae*. *Eur J Biochem* **22**: 277-286, 1971

Messenguy F, Wiame J. The control of ornithine transcarbomylase activity by arginase in *S. cerevisiae*. *FEBS Lett* **3**: 47-49, 1969

Morris S. Regulation of enzymes of urea and arginine synthesis. *Annu Rev Nutr* **12**: 81-101, 1992

Morris S, Moncman C, Rand K, Dizikes G, Cederbaum S, O'Brien W. Regulation of mRNA levels for five urea cycle enzymes in rat liver by diet, cyclic AMP and glucocorticoids. *Arch Biochem Biophys* **256** (1): 343-353, 1987

Nebes V, Morris S. Regulation of messenger ribonucleic acid levels for five urea cycle enzymes in cultured rat hepatocytes: requirements for cyclic adenosine monophosphate, glucocorticoids, and ongoing protein synthesis. *Mol Endocrinol* **2**: 444-451, 1988

Ocampas M, Direct submission of the Schizosaccharomyces pombe arginase sequence to *EMBL/GENBANK/DDBJ data banks*, 1996

Ohtake A, Takiguchi M, Shigeto Y, Amaya Y, Kawamoto S, Mori M. Structural organization of the gene for rat liver-type arginase. *J Biol Chem* **263** (5): 2245-2249, 1988

Old R, Primrose, S. in *Principles of gene manipulation*: an introduction to genetic engineering. 4th Ed. Oxford: Blackwell Scientific Publications, 1989

Ouzounis C, Kyrpides N. On the evolution of arginase and related enzymes. *J Mol Evol* **39**: 101-104, 1994

Paulus H. The evolutionary history of the ornithine cycle as a determinant of its structure and regulation. *Curr Top Cell Reg.* **22**: 117-200, 1983

Pan S, Zhang M, Cole G. Isolation and characterization of the arginase-encoding gene (arg) from *Coccidioides immitis*. *Gene* **154** (1): 115-118, 1995

Patterton D, Shi Y. Thyroid hormone-dependent differential regulation of multiple arginase genes during amphibian metamorphosis. *J Biol Chem* **269** (41): 25328-25334, 1994

Persson B, Flinta C, Heijne G, Jörnvall H. Structures of N-terminally acetylated proteins. *Eur J Biochem* **152**: 532-527, 1985

Polacco J, Holland M. Roles of ureases in plant cells. *Int Rev Cytol* **145**: 65-103, 1993

Pope B, Kent H. High efficiency 5 min transformation of *Escherichia coli*. *Nucl Acids Res* **24**: 536-537, 1996

Reczkowski R, Ash D. Rat liver arginase: kinetic mechanism, alternate substrates and inhibitors. *Arch Bichem Biophys* **312** (1): 31-37, 1994

Sambrook J, Fritsch E, Maniatis T. in *Molecular Cloning*: A Laboratory Manual. 2nd Ed. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989

Santaro C, Mermod N, Andrews P, Tijan R. A family of human CCAAT-box-binding proteins, active transcription and DNA replication: cloning and expression of multiple cDNAs. *Nature* **334**: 218-224, 1988

Schimke R. Adaptive characteristics of urea cycle enzymes in the rat. *J Biol Chem* **237** (2): 459-468, 1962

Schimke R. Arginase (rat liver). *Methods Enzymol* **XVII** (A): 313-317, 1970

Schrell A, Alt-Moerbe J, Lanz T, Schroeder J. Arginase of Agrobacterium Ti plasmid C58. DNA sequence, properties, and comparison with eucaryotic enzymes. *Eur J Biochem* **184** (3): 635-641, 1989

Scopes R. in *Protein purification*: principles and practice. 3rd Ed. Harrisonburg: R.R.Donnelly and Sons Inc., 1994

Sedmak JJ, Grossberg SE. A rapid, sensitive and versitile assay for protein using coomassie brilliant blue G250. *Anal Biochem* **79**: 544-552, 1977

Spector T. Refinement of the coomassie blue method of protein quantitation. *Anal Biochem* **72**: 248-254, 1976

Stryer L. in *Biochemistry*. 4th Ed. New York: W H Freeman and Co., 1995

Sumrada R, Cooper T. Nucleotide sequence of *Sacchaomyces cerevisiae* arginase gene (CAR1) and its transcription under various physiological conditions. *J Bacteriol* **160** (3): 1078-1087, 1984

Takguchi M, Haraguchi Y, Mori M. Human liver-type arginase gene: structure of the gene and analysis of the promoter region. *Nucl Acids Res* **16** (18): 8789-8802, 1988

Takiguchi M, Mori M. In vitro analysis of the rat liver-type arginase promoter. *J Biol chem* **266** (14): 9186-9193, 1991

Takiguchi M, Mori M. Transcriptional regulation of genes for ornithine cycle enzymes. *Biochem J* **312**: 649-659, 1995

Uchino T, Snyderman S, Lambert M, Qureshi I, Shapira S, Sansaricq C, Smit L, Jakobs C, Matsuda I. Molecular basis of phenotypic variation in patients with argininemia. *Hum Genet* **96**: 255-260, 1995

Ulbright C, Snodgrass P. Coordinate induction of the urea cycle enzymes by glucogon and dexamethasone is accomplished by three different mechanisms. *Arch Biochem Biophys* **301**: 237-243, 1993

Van Huffel C, Dubois E, Messenguy F. Cloning and sequencing of *Schizosaccharomyces pombe* car1 gene encoding arginase. Expression of the arginine anabolic and catabolic genes in response to arginine and related metabolites. *Yeast* **10** (7): 923-933, 1994

Vielle-Breitburd F, Orth G. Rabbit liver L-arginase: purification, properties and subunit structure. *J Biol Chem* **247** (4): 1227-1235, 1972

Visek W, Prior R, Ulman E, Mangian H. Additive depression of liver arginase by dietary deficiencies of arginine and manganese. *FASEB J* **6** (A): 1951, 1992

Vockley J. Submitted the human type II arginase protein sequence to *GENBANK database*, 1996

Vogeli G, Kartes S. Amplification, storage and replication of libraries. *Methods Enzymol* **152**: 407-415, 1987

Wang W, Jenkinson C, Griscavage J, Kern R, Arabolos N, Byrns R, Cederbaum S, Ignarro L. Co-induction of arginase and nitric oxide synthase in murine macrophages activated by lipopolysaccharide. *Biochem Biophys Res Commun* **210** (3): 1009-1016, 1995

Weber R. in *The biochemistry of animal development* 227-301. New York, London: Academic Press Inc., 1967

Worsaae H, Schmidt M. Plasma cortisol and behaviour in early weaned piglets. *Acta Vet Scand* **21**: 640-657, 1980

Wu G. Urea synthesis in enterocytes of developing pigs. *Biochem J* **312**: 717-723, 1995

Xu Q, Baker B, Tata J. Developmental and hormonal regulation of the *Xenopus* liver-type arginase gene. *Eur J Biochem* **211** (3): 891-898, 1993

Zhou M, Clark S, Gomez-Sanchez C. Universal cloning method by TA strategy. *Biotech* **19** (1): 34-35, 1995

Zonia L, Stebbins N, Polacco J. Essential role of urease in germination of nitrogen-limited *Arabidopsis thaliana* seeds. *Plant Pysiol* **107**: 1097-1103, 1995

APPENDIX 1

Details of degenerate primers

Oligo	Length	Tm	Sequence (5' to 3')	Degeneracy
Sheep reverse primer	23 mer	57.9	TARTGYTCNCCDGGRTCVACRTC	576
Sheep forward primer 1	25 mer	68.1	TTGGNGCNCCHTTYTCHAARGGNCA	2304
Sheep forward primer 2	25 mer	66.2	TTGGNGCNCCHTTYAGYAARGGNCA	1536

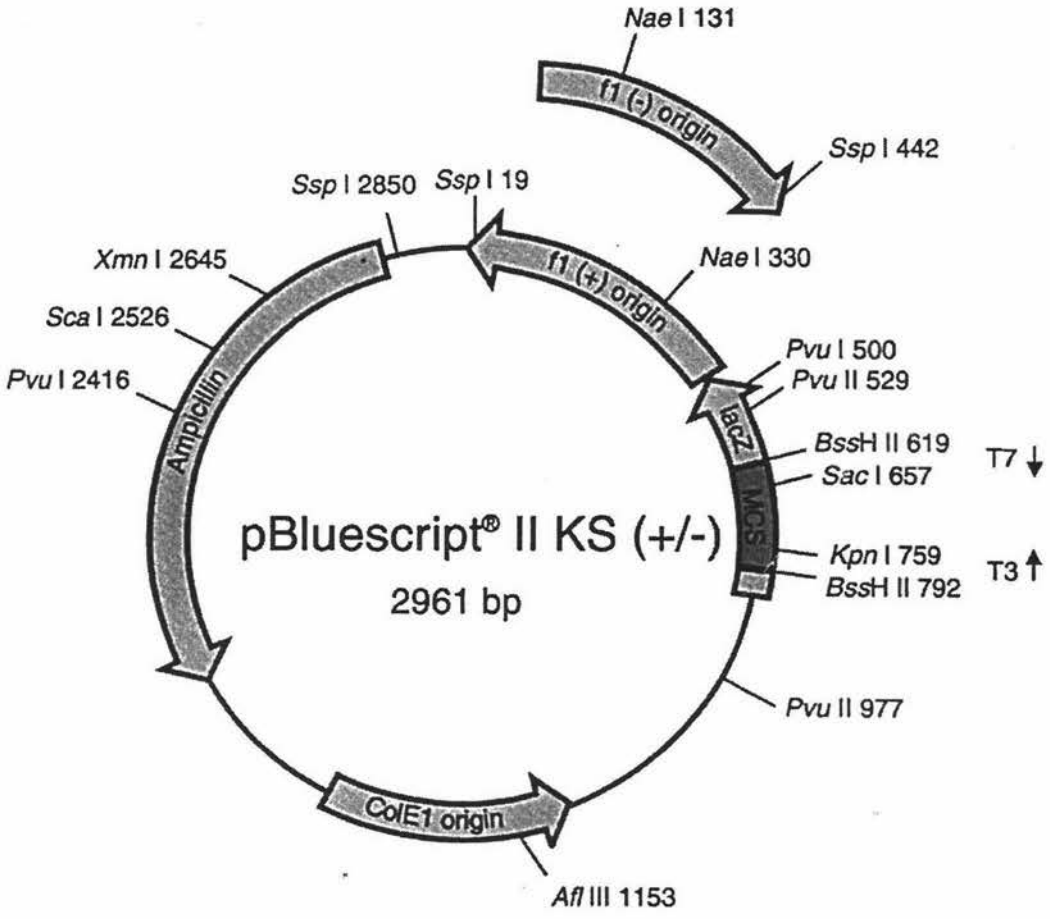
Details of specific rat primers

Oligo	Length	Tm (°C)	Sequence (5' to 3')	Degeneracy
Rat reverse primer	25 mer	57.4	CCAGAAGAATGGAACAATCAGTGTG	NA
Rat forward primer	25 mer	67.5	GTAGAGACCTTCTCTGTAAGATAGG	NA

Details of sequencing primers

Oligo	Length	Tm (°C)	Sequence (5' to 3')	Degeneracy
T3 primer	20 mer	56	AATTAACCCTCACTAAAGGG	NA
T7 primer	22 mer	64	GTAATACGACTCACTATAGGGC	NA

APPENDIX 2


Nae I 131
f1 (-) origin
Ssp I 442
Ssp I 2850
Ssp I 19
Xmn I 2645
f1 (+) origin
Nae I 330
Sca I 2526
Pvu I 500
Pvu I 2416
Pvu II 529
Ampicillin
lacZ
BssH II 619
T7
Sac I 657
MCS
pBluescript® II KS (+/-)
2961 bp
Kpn I 759
T3
BssH II 792
Pvu II 977
ColE1 origin
Afl III 1153