ISOLATION OF 5' REGULATORY SEQUENCES FOR RUMINANT ATP CITRATE LYASE

A thesis presented in partial fulfilment of the requirements for the degree of
Master of Science in Biochemistry
at Massey University

Rebecca Jane Sanders
1998

ABSTRACT

ATP citrate lyase is an essential enzyme in the pathway for conversion of glucose to fatty acids in mammalian tissues. The enzyme catalyses the cleavage of cytosolic citrate to acetyl CoA and oxaloacetate in an ATP-dependent reaction. The sequence of the cDNAs for both rat and human ATP citrate lyase have been published and have 96.3% identity at the amino acid level. This high level of identity may also extend to other mammals, including ruminants. The ruminant presents a unique system in which to study the regulation of ATP citrate lyase as levels of expression of the enzyme change during the development of a functional rumen. An analysis of the 5'-regulatory region of ruminant ATP citrate lyase will be important in determining factors that contribute to the developmental regulation of this enzyme in ruminants.

In order to analyse the 5'-regulatory region of ruminant ATP citrate lyase, a probe was constructed with which to screen an amplified bovine genomic library. The probe was produced by cloning a 282 bp PCR product containing rat ATP citrate lyase exon II sequence, amplified from rat genomic DNA. This clone was sequenced to verify that it contained rat ATP citrate lyase exon II sequence.

This probe was then used for northern and Southern blotting, and for screening an amplified bovine genomic library. Northern blotting of rat and lamb total RNA showed that the probe hybridised with rat RNA, but not with lamb RNA. Conditions for hybridisation were not optimised, as hybridisation between the probe and rat RNA was not as specific as expected. The quality of RNA used for preparing the northern blots could have also affected the specificity of hybridisation.

Southern blotting experiments were also inconclusive, as the hybridisation signals seen were not specific. However the probe was shown to hybridise to rat and human genomic DNA.

Screening of the bovine genomic library was unsuccessful, but once conditions for hybridisation are optimised, then the probe could be used to rescreen the amplified bovine genomic library, and isolate a clone containing the 5'-regulatory sequences for bovine ATP citrate lyase.

ACKNOWLEDGEMENTS

I would like to thank Kathryn Stowell and John Tweedie for their supervision, guidance and encouragement during my thesis. I would also like to thank the past and present members of the Twilight Zone for all their help and friendship provided during my time in the lab - it has been a truly memorable experience. Finally, I would like to thank my family and friends for their love and support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

A	adenine
Amp	ampicillin
bp	base pair
BRL	Bethseda Research Laboratories
cDNA	complementary deoxyribonucleic acid
C	cytosine
cpm	counts per minute
Da	dalton
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dCTP	deoxycytidine triphosphate
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetate
EEO	electroendosmosis
G	guanine
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
λ	Bacteriophage lambda
LMP	low melting point
mRNA	messenger ribonucleic acid
µg	microgram
µl	microlitre
mM	millimole
ng	nanogram
nm	nanometres
nt	nucleotide
OD	optical density
PCR	polymerase chain reaction
pfu	plaque forming units
pmol	picomole
RNA	ribonucleic acid

RNAase	ribonuclease
SDS	sodium dodecylsulphate
SSC	standard saline citrate
T	thymine
TAE	Tris acetate EDTA
Taq	*Thermus aquaticus*
TE	Tris (10 mM) EDTA (1 mM) pH 8.0
TEMED	N, N, N', N'-Tetramethylethlyenediamine
U	unit
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

CHAPTER ONE: INTRODUCTION

ATP citrate lyase is a cytosolic enzyme with a role in the cellular distribution of acetyl units. The activity of ATP citrate lyase depends on nutritional status. In conditions of high carbohydrate, acetyl CoA produced from the pyruvate dehydrogenase reaction in the mitochondrion is incorporated into citrate which can be transported into the cytosol. Cytosolic ATP citrate lyase then cleaves citrate and the acetyl CoA produced can be used as a precursor in fatty acid synthesis. Thus carbohydrate is effectively converted into fat. When carbohydrate levels are low, acetyl CoA is channelled into the tricarboxylic acid (TCA) cycle, where the acetyl groups of acetyl CoA are converted to CO_2, NADH, $FADH_2$ and GTP. These products are used to oxidise fuel to produce energy, and to provide intermediates for anabolism.

The ruminant represents a unique opportunity to study regulation in both monogastric and ruminant situations. As part of the glucose-sparing strategy of ruminants, the levels of ATP citrate lyase decrease dramatically when the switch to rumination occurs (Muramatsu *et al.*, 1970). In young ruminants, the pattern of carbohydrate metabolism resembles that of non-ruminants. At this stage of development, the levels and activity of ATP citrate lyase is comparable to that of non-ruminants (Roehrig *et al.*, 1988). However, in adult ruminants, unlike non-ruminants, dietary carbohydrate is converted to short-chain fatty acids in the rumen before absorption. The conversion of glucose to fatty acids in the adult ruminant has been shown to be very low, and is a result of negligible ATP citrate lyase activity (Muramatsu *et al.*, 1970).

A review of lipid biosynthesis will be presented, followed by an introduction to ATP citrate lyase and its potential role in modulating lipid biosynthesis from carbohydrate in the ruminant.

1.1 Lipid Biosynthesis

Fatty acid synthesis in monogastric mammals involves the use of dietary carbohydrate as the primary carbon source, and is a crucial metabolic pathway for the synthesis of triglyceride energy reserves and cellular membrane phospholipids (Kim and Freake, 1996). The pathway is under complex nutritional and hormonal controls, which interact to achieve lipid homeostasis. An imbalance in lipid homeostasis is implicated in the

development of obesity (Cheema and Clandinin, 1996), details of which will be discussed later in this introduction.

A high carbohydrate diet causes a large increase in the activity of key lipogenic enzymes, particularly acetyl CoA carboxylase and fatty acid synthase, and these changes are reflected in increased levels of mRNA for these enzymes. This induction has been shown to be specific, with induction occurring only in liver and adipose tissue (Kim and Freake, 1996).

1.1.1 Acetyl CoA Carboxylase

Acetyl CoA carboxylase catalyses the rate limiting step in the biosynthesis of long chain fatty acids (Kim, 1983). It carries out the ATP-dependent carboxylation of acetyl CoA which results in the formation of malonyl CoA (Figure 1.1)

The fatty acid synthase enzyme system then catalyses the synthesis of long chain fatty acids from malonyl CoA, produced by the action of acetyl CoA carboxylase, acetyl CoA and NADPH. Malonyl CoA and acetyl CoA are then linked to an acyl carrier protein (ACP) via the sulfhydryl termini of the phosphopantetheine groups of ACP and CoA. These ACP derivatives are produced by the action of malonyl and acetyl transacylase enzymes. The malonyl-ACP and acetyl-ACP are then used in the process of long chain fatty acid synthesis (Figure 1.2).

Short term regulation of acetyl CoA carboxylase involves covalent modification, such as phosphorylation, and allosteric control mechanisms affecting enzyme activity (Kim, 1983). Long-term regulation involves changes in the steady-state amount of enzyme present due to changes in the rate of synthesis of the enzyme. Lipogenic tissues in monogastric mammals use glucose as the primary source for the synthesis of long chain fatty acids, and a high carbohydrate diet results in the conversion of excess carbohydrate to triglycerides in the liver (Daniel and Kim, 1996). As this process occurs, there is an increase in activity of many enzymes involved in fatty acid synthesis, including acetyl CoA carboxylase. This increase in activity has been shown to be at least partly at the level of transcription, as shown by an increase in the level of mRNA. Upon refeeding starved chicks with a high carbohydrate diet, increased levels of acetyl CoA carboxylase mRNA were seen (Hillgartner *et al.*, 1996). mRNA for acetyl CoA carboxylase was

Figure 1.1 Reaction catalysed by acetyl CoA carboxylase

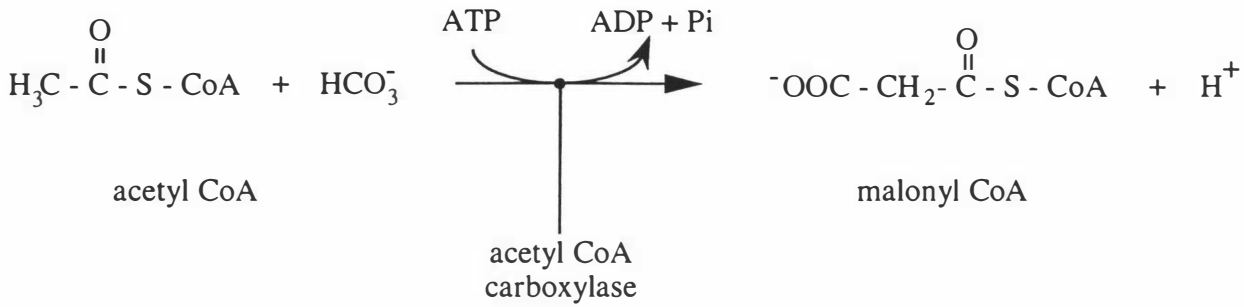
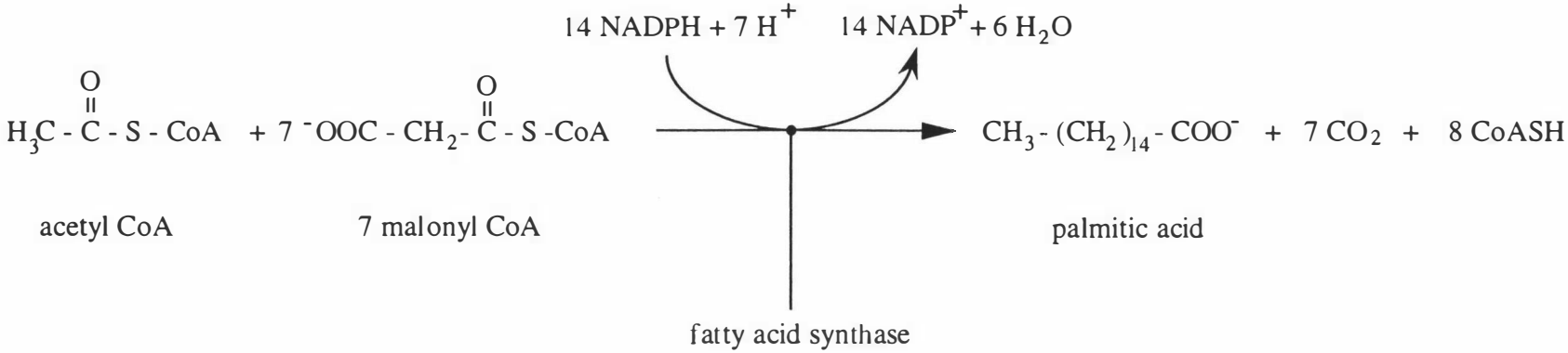


Figure 1.2 Reaction catalysed by fatty acid synthase



initially low in the livers of starved chicks, but an 11-fold increase of acetyl CoA carboxylase mRNA was seen upon refeeding.

1.1.2 SREBPs

Lipid biosynthesis may also be modulated by SREBPs. Sterol regulatory element-binding proteins (SREBPs) are members of the basic-helix-loop-helix-leucine zipper (bHLH-ZIP) family of transcription factors (Wang *et al.*, 1994). These are synthesised as precursors that are attached to the nuclear envelope and endoplasmic reticulum. In sterol-depleted cells, the membrane-bound precursor is cleaved to generate a soluble NH_2-terminal fragment that translocates to the nucleus. This fragment, which includes the bHLH-ZIP domain, activates transcription of genes involved in lipid and cholesterol biosynthesis.

Cleavage of SREBP proteins is enhanced by sterol depletion, and inhibited by the presence of sterols. SREBP proteins are able to stimulate transcription of genes involved in fatty acid synthesis, such as fatty acid synthase, acetyl CoA carboxylase, and lipoprotein lipase, and also those involved in cholesterol metabolism , such as HMG CoA synthase, HMG CoA reductase, and LDL receptor. During differentiation of mouse 3T3-L1 preadipocytes a five-fold increase in LDL receptor mRNA, and a four-fold increase in HMG CoA synthase mRNA were seen, and were quantified by RNase protection assays (Shimomura *et al.*, 1997). At this stage in adipocyte development, the amount of SREBP transcript was nine-fold higher in differentiated adipocytes, as compared to undifferentiated adipocytes.

1.1.3 Obesity

Mis-regulation of lipid biosynthesis can lead to metabolic disorders like obesity. Obesity in humans is a very common problem, and may lead to pathological conditions such as cardiovascular disease, type II diabetes mellitus, and certain forms of cancer. For this reason, obesity has been studied extensively, and interest in this field is beginning to intensify.

Much work looking into obesity has been carried out using genetically obese (*ob/ob*) mice (Clandinin *et al.*, 1996). Results have shown that expression of genes for fatty acid synthase, malic enzyme, acetyl CoA carboxylase and pyruvate kinase (enzymes all

involved in fatty acid synthesis) is higher in the livers of these obese mice. The product of the *ob* gene is leptin, which is expressed and secreted by adipocytes in proportion to their triglyceride stores (Ghilardi *et al.*, 1996; Spiegelman and Flier, 1996). Leptin also circulates in the blood, where its levels correlate with the extent of obesity. The leptin receptor, encoded by the *db* gene, is expressed in the brain, and is involved in transmitting the levels of leptin in the circulation to the brain (Spiegelman and Flier, 1996). Therefore these two proteins are an important signalling system for the regulation of body weight.

It has been shown that *ob* gene expression represses gene expression of acetyl CoA carboxylase, and enzymes involved in fatty acid and lipid synthesis (Bai *et al.*, 1996). In obese mice, there has been a breakdown in the signalling, and these mice are either unable to produce leptin (*ob/ob*), or to respond to it (*db/db*). This results in profound, early onset obesity with persistent excessive food intake, inappropriately decreased energy expenditure, severe insulin resistance, and genetic background-dependent diabetes (Spiegelman and Flier, 1996). However, addition of recombinant leptin to *ob/ob* mice can reverse all symptoms of obesity. Normally, dietary polyunsaturated fats inhibit the expression of lipogenic enzymes such as fatty acid synthase, acetyl CoA carboxylase and malic enzyme. However, not only is the expression of these enzymes higher in obese mice, but these mice also appear to be resistant to the negative control on these enzymes exerted by polyunsaturated fatty acids (Clandinin *et al.*, 1996).

Although extensive research has been carried out into the regulation of fatty acid synthesis and obesity, there is still a lot to discover before the whole process is fully understood.

1.2 Ruminant Lipid Biosynthesis

Lipids are both synthesised and mobilised in adipose tissue (Bauman, 1976). A major difference between ruminant and monogastric lipid synthesis, is that adipose tissue from mature ruminants utilises acetate, a product of rumen metabolism (Hanson and Ballard, 1967), rather than glucose as a carbon source for de novo fatty acid synthesis (Baldwin and Smith, 1971; Ballard *et al.*, 1972). In monogastric mammals given a high-carbohydrate diet, lipids are synthesised from glucose in both adipose and liver tissue. Although the ruminant diet also contains a large amount of carbohydrate, this consists of

mainly cellulose and hemicellulose, which are fermented in the rumen to yield acetate, propionate and butyrate (Ballard *et al.*, 1972).

Adipose and liver tissue of adult ruminants has a limited capacity to use mitochondrial acetyl CoA as a source of carbon for fatty acid synthesis, due to the low levels of ATP citrate lyase (Figure 1.3). This results in the inability to translocate mitochondrial acetyl CoA to the cytosol, consequently ruminant adipose tissue lacks the capacity to convert substrates that generate mitochondrial acetyl CoA into fatty acids.

Ruminants also have a different source of reducing equivalents, due to the absence of the enzyme NADP-malate dehydrogenase (decarboxylation), also known as malic enzyme (Figure 1.3). NADPH required for non-ruminant fatty acid biosynthesis is produced by the action of this enzyme (Cheema and Clandinin, 1996). In ruminant adipose tissue NADPH required for fatty acid synthesis is generated by the pentose phosphate pathway and the isocitrate cycle. These differences in carbon source utilisation acts as an advantage for ruminants in that glucose is spared. There is a substantial requirement for glucose by the central nervous system, and as a nutrient supply for the foetus in late gestation and during lactation (Leng, 1970). Acetyl CoA carboxylase catalyses the regulatory step in lipogenesis in ruminants, thus still enabling fatty synthesis to occur in ruminant adipocytes, although acetate is the carbon source.

This difference in carbon source utilisation between ruminants and non-ruminants seems to be related to nutrient availability. Newborn and foetal ruminants have the ability to use glucose as a carbon source for fatty acid synthesis, and at this stage of development, the activities of ATP citrate lyase and NADP-malate dehydrogenase are similar to that seen in adult rats (Roehrig *et al.*, 1988). Once the rumen is fully developed, the conversion of glucose to fatty acids is restricted by the virtual absence, or negligible activity, of ATP citrate lyase and NADP-malate dehydrogenase (Judson and Leng, 1973). This absence of enzyme activity in ruminants is due to repression of enzyme synthesis, rather than the presence of an inhibitor (Muramatsu *et al.*, 1970). The activities of these two enzymes have been shown to increase upon infusion of glucose, both intravenously and postruminally (Ballard *et al.*, 1972; Bauman, 1976). A 44- and 9-fold increase is seen in the activities of ATP citrate lyase and NADP-malate dehydrogenase, respectively, and this is correlated with an increased rate of glucose incorporation into fatty acids.

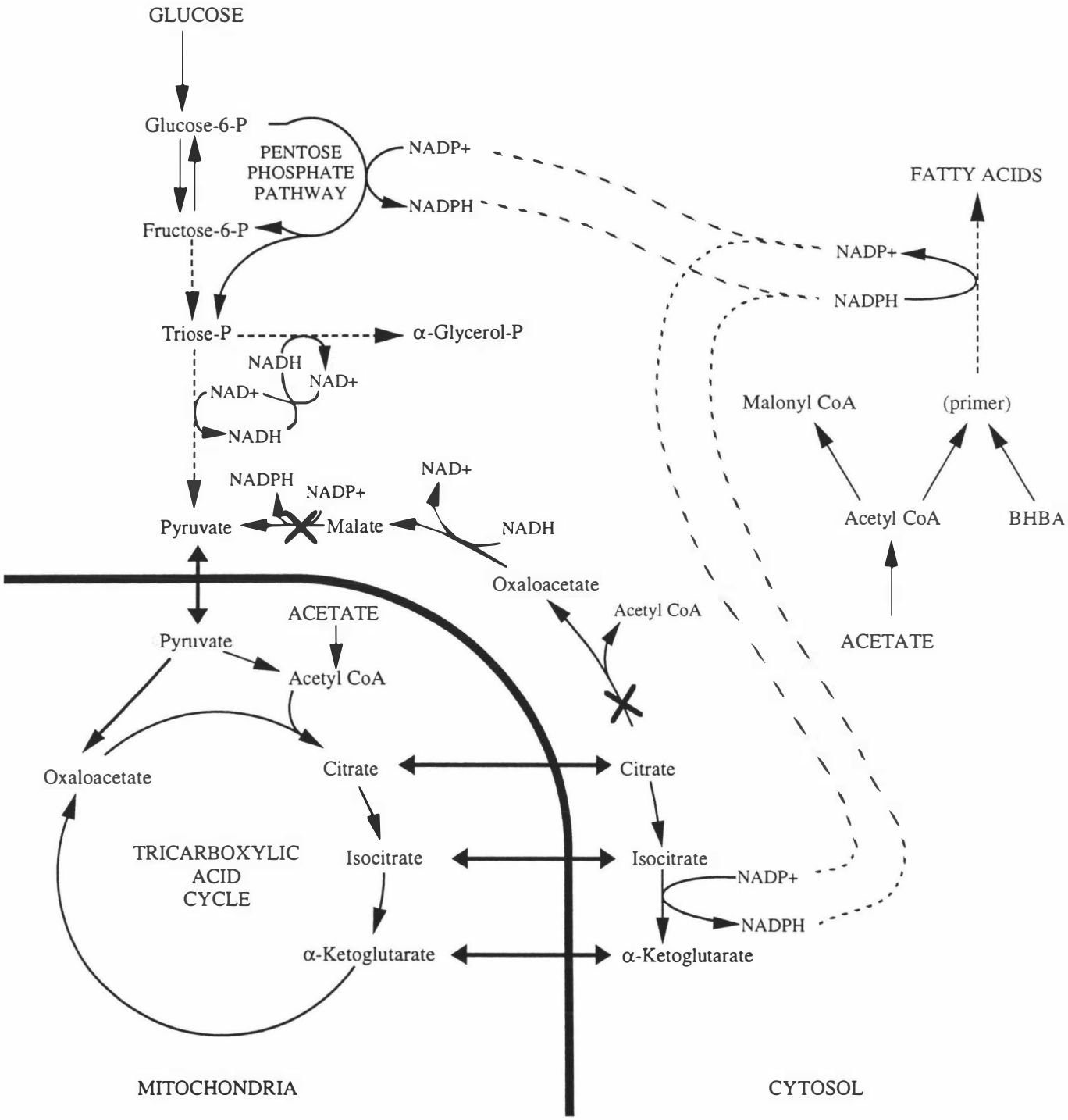


Figure 1.3 Pathways of fatty acid synthesis in ruminant adipose tissue. The negligible activities of ATP citrate lyase and NADP-malate dehydrogenase are denoted by X. BHBA, β-hydroxybutyrate. From Bauman, 1976.

1.2.1 Glucose Sparing in Ruminants

ATP citrate lyase may have an important role in the glucose sparing strategy of animals. Tissues such as the brain have an absolute requirement for glucose. The brain lacks fuel stores, and is unable to use fatty acids from the circulation as an energy source because fatty acids are bound to albumin in plasma, and so do not traverse the blood-brain barrier. If an animal is subjected to a low glucose diet, the available glucose must be used by the brain rather than be converted into fat. Hence, low levels of ATP citrate lyase ensure that fatty acid synthesis from glucose is kept at a very low rate. Conversely, if the diet is high in glucose, there is an adequate energy source for the brain and high levels of ATP citrate lyase cause the surplus glucose to be converted into fat for storage.

Ketone bodies (acetoacetate, D-3-hydroxybutyrate, and acetone), which are formed from acetyl CoA can be used by tissues such as the brain during starvation. Glucose is virtually the sole fuel for the brain, except during starvation, when ketone bodies partly replace glucose as fuel for the brain. Ketone bodies may also therefore play a role in supplying energy to the brain of mature ruminants, at a time when glucose is not supplied by the diet.

Ruminant ATP citrate lyase has not been studied to any great extent as yet, and the regulation of the activity of the enzyme in this group of animals is a new aspect to be investigated. Ruminants present an unique system in which to study the regulation of transcription of ATP citrate lyase, because ATP citrate lyase is expressed at different levels during development. While the animals are milk feeding, and therefore essentially monogastric, they are on a carbohydrate-adequate diet. At this stage, there is sufficient glucose from dietary carbohydrate sources, and it has been shown that there are high levels of ATP citrate lyase (Roehrig *et al.*, 1988), allowing the excess glucose in the diet to be converted into fat. Once the animals switch to a grass diet, and thus are true ruminants, they are on a very low glucose diet, and the cellulose and hemicellulose content of the diet is degraded in the rumen to yield not glucose, but acetate, propionate and butyrate (Ballard *et al.*, 1972). As a functional rumen develops, glucose becomes less important as a source of energy for most of ruminant tissues (Howarth *et al.*, 1968), and any glucose that is obtained from the diet is required for the brain. It has been shown that at this stage of development, there are very low levels of ATP citrate lyase (Roehrig *et al.*, 1988), and thus very little conversion of glucose to fat. This repression can be

overcome in adult sheep, in which the levels of ATP citrate lyase are very low. If adult sheep are infused with glucose, there is an increase in the levels of ATP citrate lyase, as detected by mRNA levels. So some aspect of rumination prevents transcription of ATP citrate lyase. Therefore the promoter region of this gene may provide some clues to explain the molecular mechanisms involved in the regulation of ATP citrate lyase expression.

1.3 ATP Citrate Lyase

The enzyme ATP citrate lyase is found in a wide range of tissues, and functions as a tetramer of 4 identical subunits (Elshourbagy *et al.*, 1990; Elshourbagy *et al.*, 1992), with a total molecular weight of 440kDa (Singh *et al.*, 1976). This enzyme functions in the cytosol to catalyse the cleavage of citrate in an ATP dependent reaction (Figure 1.4), and was first identified in the late 1950's (Srere, 1959).

Glucose is metabolised to pyruvate in the cytosol, and the pyruvate is transported across the mitochondrial membrane, where it is oxidised to acetyl CoA and CO_2 (Spencer and Lowenstein, 1962). Acetyl CoA must be transferred from the mitochondrion into the extramitochondrial space, where the enzymes involved in fatty acid synthesis are situated. The inner mitochondrial membrane is impermeable to acetyl CoA, so the acetyl CoA is converted to citrate, and then transported out of the mitochondrion by the citrate translocator in the mitochondrial membrane (Halperin *et al.*, 1975). The cytosolic citrate is converted to acetyl CoA and oxaloacetate by the action of ATP citrate lyase (Srere, 1959), in an ATP-dependent reaction (Figure 1.4). This reaction supplies acetyl CoA from glucose in the cytosol (Figure 1.5). Thus, acetyl CoA and oxaloacetate are transferred from mitochondrion to the cytosol, in the form of citrate, which is then cleaved by the action of ATP citrate lyase. Acetyl CoA is then available in the cytosol as a precursor for the biosynthesis of fatty acids and cholesterol.

Oxaloacetate which is formed in the cleavage reaction catalysed by ATP citrate lyase (Figure 1.4) must be returned to the mitochondrion. The inner mitochondrial membrane is also impermeable to oxaloacetate, and so oxaloacetate undergoes a series of reactions to form pyruvate, which readily diffuses into the mitochondrion. These reactions result in the production of cytosolic $NADPH/H^+$, which is then available for the synthesis of fatty acids. Therefore, the action of this enzyme is involved in the recycling of oxaloacetate

Figure 1.4 Reaction catalysed by ATP citrate lyase

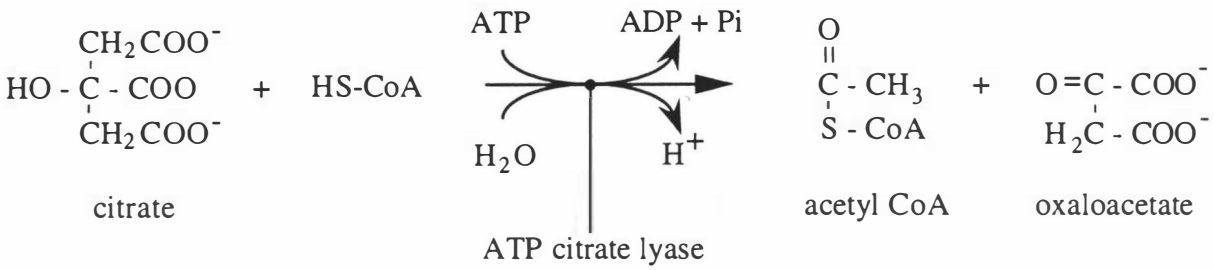
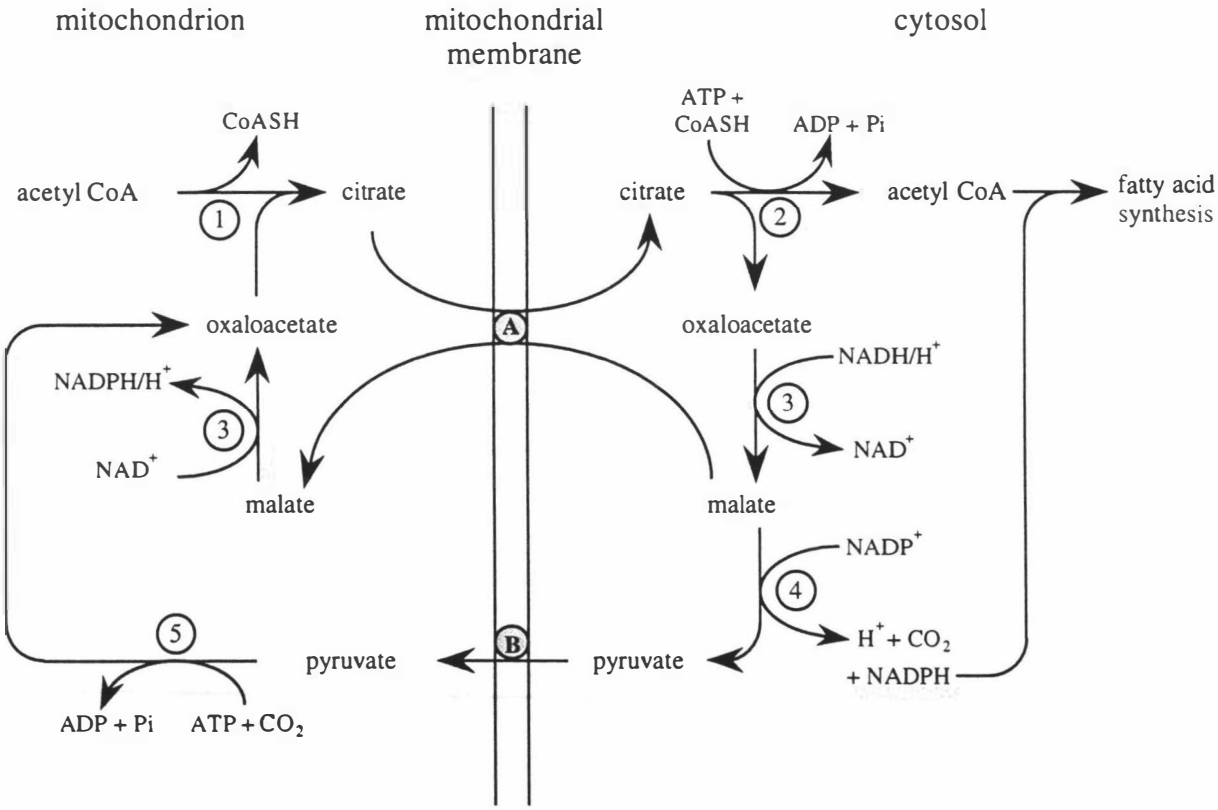


Figure 1.5 Site of action of ATP citrate lyase and its role in providing acetyl CoA for fatty acid synthesis

Enzymes: **1** citrate synthase; **2** ATP citrate lyase; **3** malate dehydrogenase; **4** malic enzyme; **5** pyruvate carboxylase. Transporters: **A** tricarboxylate carrier; **B** pyruvate transporter.



back into the mitochondrion, and the concomitant production of cytosolic $NADPH/H^+$ (Figure 1.5), which is required for the production of fatty acids.

1.3.1 Regulation of ATP Citrate Lyase

ATP citrate lyase is implicated in the regulation of fatty acid synthesis, and an early point of control may be the transport of citrate. Citrate leaves the mitochondrion via the tricarboxylate carrier (Halperin *et al.*, 1975), and this is an important step in the regulation of fatty acid synthesis. The action of this transporter is inhibited specifically by palmitoyl CoA. The amount of acetyl CoA generated in the cytosol is dependent on the amount of citrate which is transported by the tricarboxylate carrier and made available to ATP citrate lyase. Therefore, the amount of citrate is dependent upon the regulation of the mitochondrial citrate transport system. Investigation of this system (Halperin *et al.*, 1975), has shown that the citrate transporter is inhibited under conditions where the rate of fatty acid synthesis is also decreased.

The activity of ATP citrate lyase provides an additional control point in fatty acid synthesis, which varies according to the nutritional state of the animal. A study using rats showed that the changes in activity of ATP citrate lyase correlate with changes in the rate of fatty acid synthesis (Kornacker and Lowenstein, 1965), and so the reaction catalysed by ATP citrate lyase was shown to be involved in fatty acid synthesis. The levels of ATP citrate lyase and fatty acid synthesis were followed in starved rats. In these experiments, the activity of ATP citrate lyase fell to one-third of the normal value after 2 days starvation, and then to one-fifth of the normal value after 6 days of starvation. Fatty acid synthesis also decreased after starvation, so the decrease in activity of ATP citrate lyase as seen during starvation is consistent with the role of ATP citrate lyase in fatty acid synthesis.

Upon refeeding there was a rapid increase of both fatty acid synthesis and ATP citrate lyase activity. This response was dependent on the diet during refeeding. A high glucose diet gave the greatest increase in fatty acid synthesis, ATP citrate lyase activity, and acetate thiokinase activity. A moderate increase was observed when starved rats were refed a balanced diet. However, if a diet high in fat was used for refeeding, levels of ATP citrate lyase and acetate thiokinase returned to normal fed state levels (little change in the rate of fatty acid synthesis), but did not increase past this level during refeeding.

The increase in the level of acetate thiokinase activity was not as high as that of ATP citrate lyase. This suggests that the pathway involving citrate (and thus ATP citrate lyase) is more important for fatty acid synthesis. This increase in activity of ATP citrate lyase was due to an increase in enzyme level via increased protein synthesis, as addition of ethionine (which inhibits protein synthesis) to the refeeding diet suppressed the increase in ATP citrate lyase activity (Kornacker and Lowenstein, 1965).

The enzyme activity and amount of ATP citrate lyase increases during the differentiation of preadipocytes into adipocytes (Benjamin *et al.*, 1994; Wise *et al.*, 1984), which is further evidence for the role of ATP citrate lyase as a primary lipogenic enzyme. This increase has been shown to be due to increased levels of mRNA, so the control of ATP citrate lyase synthesis also appears to be exerted at the transcriptional level.

1.3.2 Phosphorylation of ATP Citrate Lyase

ATP citrate lyase has been identified as a phosphoprotein (Linn and Srere, 1979), and contains 4 phosphorylation sites, 3 of which are structural, and one is a catalytic histidyl site (Benjamin *et al.*, 1994). The catalytic histidyl site is involved in the mechanism of ATP citrate lyase action. A histidine residue in the active site is phosphorylated by Mg-ATP (Elshourbagy *et al.*, 1990), and forms a phosphoenzyme intermediate (Houston and Nimmo, 1984). The formation of this phosphoenzyme intermediate and release is followed by random order binding of CoA and Mg-citrate. Thus, ATP citrate lyase obeys a double displacement mechanism (Houston and Nimmo, 1984). In human ATP citrate lyase, this catalytic histidine had been identified as His765, and mutation of this residue to Ala results in a protein with exactly the same molecular mass, but which is catalytically inactive (Elshourbagy *et al.*, 1992). The catalytic site histidine in rat ATP citrate lyase has also been shown to be phosphorylated by nucleoside diphosphate kinase (NDPK) (Wagner and Vu, 1995). The normal action of this enzyme is to catalyse the phosphorylation of nucleoside 5' diphosphate to triphosphate. The phosphate for the action of NDPK is supplied by ATP, and NDPK is thought to be responsible for maintaining nucleoside triphosphate pools. The physiological significance of the phosphorylation of ATP citrate lyase by NDPK is unclear, as ATP citrate lyase is phosphorylated by Mg-ATP much more rapidly than by NDPK-ATP under normal conditions (Wagner and Vu, 1995). ATP citrate lyase also binds citrate, CoA, ADP, oxaloacetate, and acetyl CoA, and it has been suggested that the binding of one of these

substrates or products may inhibit the phosphorylation of ATP citrate lyase by ATP but not by NDPK. Alternatively, binding of NDPK to the catalytic site of ATP citrate lyase may inhibit the phosphorylation of ATP citrate lyase by ATP and thereby decrease the rate of acetyl CoA formation. However as yet the significance of phosphorylation of ATP citrate lyase by NDPK is unknown.

The three structural phosphorylation sites are spaced 4 amino acids apart, at Thr446, Ser450 and Ser454 (Benjamin *et al.*, 1994; Elshourbagy *et al.*, 1990; Pentyala and Benjamin, 1995). These residues undergo a complex manner of phosphorylation, with different sites being phosphorylated by different kinases. Ser454 is phosphorylated by cAMP dependent kinase, insulin stimulated kinase (Pentyala and Benjamin, 1995), and S6 kinase (Benjamin *et al.*, 1994). Phosphorylation of this site is stimulated by insulin, presumably by increasing the activity of insulin stimulated kinase (Pentyala and Benjamin, 1995). Thr446 and Ser450 are phosphorylated by glycogen synthase kinase-3 (GSK-3), and the sequence around these residues resembles those in glycogen synthase. These sites are also phosphorylated in a hormonally regulated fashion, similar to glycogen synthase (Pentyala and Benjamin, 1995). Phosphorylation of Thr446 and Ser450 is decreased by insulin, which is opposite to the effect of insulin on Ser454 phosphorylation. Insulin exerts this control by decreasing the activity of GSK-3. β-adrenergic agents increase phosphorylation of all of these sites.

As yet, it has not been possible to show that ATP citrate lyase activity is regulated by phosphorylation, and as such, it is not known whether the changes in ATP citrate lyase activity as a function of phosphorylation are physiologically important. Some studies have shown that phosphorylation of ATP citrate lyase by cAMP-dependent protein kinase and/or GSK-3 decreases the apparent V_{max} without changing the apparent K_m (Pentyala and Benjamin, 1995), but this still seems to have no effect on the activity or regulation of ATP citrate lyase.

1.3.3 Transcription and mRNA Levels

The level of protein present at any one time is a result of steady-state levels of protein synthesis and degradation. The change in amount of protein can be caused by a decrease in degradation or an increase in synthesis. This also applies to the levels of mRNA,

where modulation of the rate of transcription, or the rate of mRNA degradation will result in changes in the levels of protein present, and hence enzyme activity.

Changes in ATP citrate lyase enzyme activity occur in response to a change in the rate of transcription, rather than an effect on the enzyme itself. A dramatic drop in the enzyme activity of ATP citrate lyase occurs in ruminants during the switch from monogastric digestion to rumination (Muramatsu *et al.*, 1970; Roehrig *et al.*, 1988). This drop in ATP citrate lyase activity has been shown to be due to a drop in the levels of ATP citrate lyase mRNA, rather than the presence of an enzyme inhibitor (Muramatsu *et al.*, 1970).

The levels of ATP citrate lyase mRNA have been shown to increase in response to glucose, and this increase was shown in cultured hepatocytes, where enhanced mRNA concentrations were seen using total cellular RNA and dot blot hybridisation assays (Fukuda *et al.*, 1996). This mRNA increase is reflected in an increase in enzyme levels and therefore activity (Wise *et al.*, 1984).

1.3.4 Cloning of ATP Citrate Lyase cDNA and Genomic DNA

The entire gene and cDNA for both rat and human ATP citrate lyase have been cloned and sequenced (Elshourbagy *et al.*, 1990; Elshourbagy *et al.*, 1992). The rat ATP citrate lyase gene is present in the genome as a single copy (Kim *et al.*, 1994). A high level of identity has been shown between human and rat ATP citrate lyase (Appendix III). There is 96.3% identity at the amino acid level, and including conservative substitutions, a similarity of 97.7% (Elshourbagy *et al.*, 1992).

The exon-intron regions of rat ATP citrate lyase have also been cloned and identified (Moon *et al.*, 1996), and show that the rat ATP citrate lyase gene spans ~55 kb, and is divided into 29 exons that range in size from 30 to 986 bp (Moon *et al.*, 1996). All the sequences bordering the splice site junctions follow the GT/AG rule. A splicing variant was also identified, which lacks exon 14, a small 30 nucleotide exon. This variant coexists in tissues such as brain, kidney, mammary gland, lung and liver. The complete transcript and spliced variant exist in the same ratio, although the amount of rat ATP citrate lyase mRNA varies among tissues. The physiological significance of the presence of this variant is not yet known.

Variant cDNA sequences of human ATP citrate lyase have recently been identified (Lord *et al.*, 1997). The human ATP citrate lyase cDNA was cloned by RT-PCR, and the sequence produced was analysed. The sequence was found to contain variations such as single nucleotide changes, some of which were silent, while others altered the codon. There were also 4 separate regions of multicodon variance identified. The nucleotide sequences for the two human ATP citrate lyase cDNAs exhibit a high level of similarity (98.7%), and most of the differences are clustered in the regions of multicodon variance. When this variant sequence was cloned and expressed, active recombinant protein was produced. This protein is a tetramer in solution, and characterisation of purified protein showed that its kinetic properties are extremely similar to that of native rat liver ATP citrate lyase and native human ATP citrate lyase (Lord *et al.*, 1997).

Although sequence variability of ATP citrate lyase has been shown, this does not appear to cause any significant difference to the kinetic activity of the enzyme. However, the effect of sequence variability and alternative splicing of ATP citrate lyase mRNA on intrinsic enzyme activity, and the potential regulation by phosphorylation of other posttranslational events is not yet known.

1.3.5 Potential Response Elements

Transcription cannot be initiated by RNA polymerase II alone - it is dependent on other transcription factors in order to bind to the promoter and initiate transcription. RNA polymerase II and these factors constitute the basal transcription apparatus, which is required to transcribe any promoter. The first step in the formation of the pre-initiation complex (PIC) is the binding of the transcription factor TFIID to the TATA box. The TATA box is recognised by the TATA-binding protein (TBP), which is associated with various other protein factors that constitute TFIID. Once TFIID has bound to the DNA, TFIIA joins the complex, followed by TFIIB. RNA polymerase II, with TFIIF associated, is then able to bind to the DNA. Three more factors - TFIIE, TFIIH, and TFIIJ join the complex. Once the PIC has assembled, the polymerase can proceed to transcribe the gene (Lewin, 1994). Formation of the PIC, and therefore the activity of the promoter, can be increased by the presence of transcription factors which bind to enhancer elements. These can act over distances of several kilobases, and can be located either upstream or downstream of a gene. They are thought to stimulate transcription in two ways. Firstly, binding of a transcription factor to an enhancer element could change

the chromatin structure through histone disruption, thus causing a loosening of the DNA, and allowing the basal transcription factors to bind. The second possibility is that protein-protein interactions between transcription factors bound to enhancer elements, and transcription factors associated with the formation of the PIC could either stabilise the PIC, or promote the formation of the PIC, thus enhancing the rate of transcription initiation.

The promoter of rat ATP citrate lyase has been studied, and many different consensus sequences for regulatory elements have been identified (Kim *et al.*, 1994).

Firstly, the rat ATP citrate lyase promoter does not contain a TATA box, which may explain the observation that initiation of transcription of ATP citrate lyase occurs over several bases. There is a CAAT sequence located at positions -95 to -87. A CAAT box is part of a conserved sequence located upstream of the startpoints of eukaryotic transcription units, and is recognised by a large group of transcription factors. The CAAT box plays a role in determining the efficiency of a promoter.

Several sequence elements for the consensus binding site for the transcription factor Sp1 have been identified in the region from -310 to -30 , which is rich in GC content. Sp1 is a ubiquitously expressed transcription factor, and has been implicated in a number of different mechanisms involved in the transcription of genes, including the glucose response (Daniel and Kim, 1996).

Fat specific elements (FSEs) have been identified at -370 to -175, and these may play a role in mediating the induction of the ATP citrate lyase gene in adipocytes. An hepatocyte nuclear factor-I binding site was identified at position -1654, and this site is involved in tissue-specific expression. Sequences between -1288 and -288 match the thyroid hormone element consensus sequence. These elements may be involved in amplifying the effect of thyroid hormone in the induction of ATP citrate lyase during differentiation in adipocytes.

A glucocorticoid responsive element consensus sequence has been identified between -1602 and -842. Glucocorticoid is known to induce lipogenesis in lung and hepatocytes. However, it has been shown that mRNA levels for ATP citrate lyase are not increased by glucocorticoids (Xu *et al.*, 1993). The influence of glucocorticoids on the activity and

mRNA level of ATP citrate lyase, fatty acid synthase and acetyl CoA carboxylase were studied in foetal rat lung. There was an increase in the mRNA for fatty acid synthase, but not for ATP citrate lyase and acetyl CoA carboxylase. Therefore the presence of this glucocorticoid responsive element consensus sequence in the ATP citrate lyase gene is not understood. A cyclic AMP responsive element has been identified at position -1283, and it has been suggested that cAMP decreases the expression of lipogenic enzymes in liver (Kim *et al.*, 1994).

A region from -61 to -49 of the ATP citrate lyase gene has been identified as insulin-responsive (Fukuda *et al.*, 1996). When this region was fused to the chloramphenicol acetyltransferase (CAT) gene, CAT activity was stimulated by insulin. The addition of polyunsaturated fatty acids (PUFAs) resulted in a reduction in the response to insulin. It is not known whether this region contains a PUFA responsive element, or whether the PUFA somehow inhibits the insulin signalling pathway from the insulin receptor to the ATP citrate lyase gene (Fukuda *et al.*, 1996). Binding of Sp1 within this region has also been shown (Fukuda *et al.*, 1997), which suggests that Sp1 may be involved in the regulation of ATP citrate lyase stimulation due to insulin, and suppression due to PUFAs. It has also been shown that dietary PUFAs are potent inhibitors of hepatic fatty acid and triglyceride synthesis, and studies have shown that feeding rats a diet high in PUFAs results in an increase in the levels of mRNA for enzymes involved in fatty acid synthesis (Baldwin *et al.*, 1966; Cheema and Clandinin 1996).

Although many consensus sequences have been identified in the rat ATP citrate lyase gene, the physiological significance of these potential response elements remains unclear.

1.4 Aim of this Project

ATP citrate lyase is expressed in all mammals, and is essential for the conversion of glucose to fat. Ruminant ATP citrate lyase was chosen as the topic of this study because the enzyme in ruminants is subject to transcriptional regulation according to development. For this reason, the study of this enzyme, and aspects of its regulation would help shed light on the complex regulation of enzymes involved in fatty acid synthesis.

A high level of identity between rat and human ATP citrate lyase sequences has been shown, and it is likely that this identity would also extend, at least in part, to ruminants.

By studying the promoter of ruminant ATP citrate lyase, and the regulation of expression of ruminant ATP citrate lyase according to development, new aspects of regulation may be identified.

Ovine ATP citrate lyase was chosen initially because once a suitable probe had been constructed, this could be used to follow the levels of ATP citrate lyase in sheep liver according to development. RNA would be extracted from the livers of lambs at various stages of development and the RNA probed to investigate the levels of ATP citrate lyase mRNA. Sheep livers are a readily available so represent an ideal tissue for this kind of analysis.

Both the cDNA and gene sequence of rat ATP citrate lyase were known, therefore rat as well as sheep RNA was used in the initial approach for preparing a probe. By using the RT-PCR system, RNA was used to synthesise first strand cDNA which was then used as a template for PCR. Primers were designed to amplify sequences containing ATP citrate lyase, using first strand cDNA as a template. The use of sheep RNA from the outset should have provided a PCR product suitable to screen a sheep genomic library. However, unforeseen problems with reproducibility in RT-PCR from sheep mRNA led to the use of rat RNA and rat genomic DNA for the preparation of a probe for ATP citrate lyase.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Materials

2.1.1 Enzymes

Restriction enzymes used were obtained from various companies according to availability and affordability eg. Gibco BRL Life Technologies Ltd, MD, USA; New England Biolabs, MA, USA. T4 DNA ligase and *Thermus aquaticus* (*Taq*) DNA polymerase were purchased from Gibco BRL Life Technologies Ltd, MD, USA. *Pfu* polymerase was purchased from Stratagene, La Jolla, CA, USA. Calf intestinal alkaline phosphatase, proteinase K, and DNase I (bovine pancreas Grade II) were obtained from Boehringer Mannheim, West Germany. Klenow fragment of *E. coli* DNA polymerase I was obtained from New England Biolabs, MA, USA. RNase A and lysozyme from chicken egg white were obtained from Sigma Chemical Company, St Louis, MO, USA. Sequenase version 2.0 was obtained from United States Biochemical Corporation, Cleveland, Ohio, USA.

2.1.2 Chemicals

All general chemicals and reagents used were of analytical grade or better. 1 kb DNA ladder, premixed broth (NZY and LB), agar, LMP agarose, and X-Gal were obtained from Gibco BRL Life Technologies Ltd, MD, USA. IPTG, low electroendosmosis grade agarose, Ampicillin, Tetracycline, and TEMED were obtained from Sigma Chemical Company, St Louis, MO, USA. pBluescript® II SK- was purchased from Stratagene, La Jolla, CA, USA. Acrylamide premix was obtained from BioRad Laboratories, CA, USA. Reaction buffer and $MgCl_2$ for PCR were obtained from Promega, WI, USA; Dynabeads-Streptavidin were purchased from Dynal A.S., Oslo, Norway. NuSieve agarose was obtained from FMC BioProducts, ME, USA.

2.1.3 Miscellaneous Products

Oligonucleotide primers (ATPCL1 and ATPCL2), dNTPs, TRIZOL™ LS reagent, *SUPERSCRIPT*™ Preamplification System, and NACS PREPAC™ columns were purchased from Gibco BRL Life Technologies Ltd, MD, USA. ProbeQuant™ G-50 Micro Columns were obtained from Pharmacia LKB Biotechnology, Uppsala, Sweden.

Oligonucleotide primers (ATPCL5', ATPCL3', and ATPCL3' exon III), and biotinylated oligonucleotide primers (T3 and T7) were purchased from Oligos Etc, CT, USA. 3MM blotting paper was from Whatman, England. Supported nitrocellulose membrane used for Northern and Southern blots were purchased from Sartorius, Germany. The bovine genomic library was obtained from Stratagene, La Jolla, CA, USA. Nitrocellulose filters used for colony lifts were obtained from Sartorius, Germany (82 mm) and Amersham, UK (134 mm). The Rediprime kit, used for labelling DNA was obtained from Amersham, UK. [α^{32}P] dCTP and [α^{35}S] dATP isotopes were purchased from NEN Life Science Products, MA, USA. Xray film was obtained from Fuji Photo Film Company, Japan; fixer and developer were from Eastman Kodak, NY, USA. Several kits were also used: Prepagene from Biorad Laboratories, CA, USA; Wizard™ Maxiprep DNA purification system from Promega, WI, USA; and Genomix Kit for genomic DNA extraction from Talent, Italy.

2.1.4 *Escherichia coli* Genotypes

Strain	Genotype
XL-1 Blue	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi-1 relA*1 *lac*⁻ F' [*proAB* ⁺ *lacI*^{q} *lacZΔM15* Tn*10* (*tet*^{r})]
XL-1 Blue MRA(P2)	*Δ(mcrA)*183 *Δ(mcrCB-hsdSMR-mrr)*173 *endA*1 *supE*44 *thi*-1 *gryA*96 *relA*1 *lac* (P2 lysogen)
DH5-α	*supE44* *Δlac*U169 (ϕ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1
RR-1	*supE*44 *hsdS*20(r_B-m_B-) *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1

Table 2.1 Bacterial strains of *Escherichia coli* used in this study

2.2 Methods

All routine DNA manipulation methods were carried out according to general protocols (Ausubel *et al.*, 1989; Sambrook *et al.*, 1989).

2.2.1 Manipulation of RNA

Total RNA from tissue was prepared using TRIZOL™ LS reagent (Gibco BRL Life Technologies Ltd) according to manufacturers instructions. The TRIZOL™ LS reagent relies on denaturation of protein using a chaotropic salt, followed by extraction of nucleic acid from a phenol phase produced by centrifugation of the tissue/reagent homogenate. Electrophoresis of total RNA was performed according to standard protocols (Sambrook *et al.*, 1989). RNA was quantitated by spectrophotometry at 260 and 280 nm according to standard protocols (Sambrook *et al.*, 1989). RNA species separated by gel electrophoresis were transferred to nitrocellulose membrane by capillary transfer according to standard protocols (Sambrook *et al.*, 1989). Filters were then dried between blotting paper, and baked at 80°C for 2-3 hours in order to immobilise transferred RNA on the nitrocellulose.

2.2.2 Synthesis of cDNA

First strand cDNA was synthesised from RNA prepared from rat or sheep liver tissue, using the *SUPERSCRIPT*™ Preamplification System (Gibco BRL Life Technologies Ltd) with oligo d(T) as a primer according to the manufacturers instructions. First strand cDNA is synthesised by *SUPERSCRIPT*™ reverse transcriptase, and RNA is removed from the resultant RNA/DNA hybrid using RNase H.

2.2.3 DNA Amplification

DNA sequences of rat ATP citrate lyase were amplified by PCR using the protocol recommended by Cetus Corporation Ltd. Reactions were carried out in 0.5 ml tubes overlaid with mineral oil, and typically contained 1x reaction buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 2 mM $MgCl_2$, 0.3 mM deoxynucleotide triphosphates, 250 ng (20 pmol) of each oligonucleotide primer, ~1 ng of template DNA, and 2.5 U *Thermus aquaticus* (*Taq*) DNA polymerase. Plasmid DNA prepared by the rapid boil method (Holmes and Quigley, 1981) was diluted 100 fold to reduce the concentration of any potential inhibitors before the addition of 1 µl to the PCR reaction. Amplification was carried out using a thermal cycler programme for an initial 5 minute denaturation at 95°C

followed by 30 cycles of 95°C for 1 minute, 65-68°C for 1 minute, and 72°C for 1 minute. Reactions were stored at 4°C prior to analysis.

2.2.4 PCR Screening of Colonies

A small amount of *E. coli* cells were added to a PCR reaction containing reaction buffer, deoxynucleotide triphosphates, oligonucleotide primers, and *Taq* DNA polymerase (as described in Section 2.2.3). Amplification was carried out in a thermal cycler, also as previously described.

2.2.5 DNA Digestion and Agarose Gel Electrophoresis

All restriction endonuclease digests were carried out with appropriate buffers using conditions recommended by the supplier. Electrophoresis of DNA fragments was performed in low electroendosmosis grade agarose containing ethidium bromide (0.5 μg/ml) and 1xTAE buffer (0.04 M Tris, 0.02 M acetate, 0.001 M EDTA, pH8.0) according to Sambrook *et al.* (1989). DNA fragment size was confirmed using the 1kb molecular size ladder (Gibco BRL Life Technologies Ltd).

2.2.6 Purification of Fragments from Agarose Gels

DNA fragments separated by gel electrophoresis were excised under illumination by long wavelength UV light (366nm), and then extracted from the agarose using the Prep-A-Gene Kit from BIO RAD.

2.2.7 Quantitation of DNA

An aliquot of DNA was analysed by agarose gel electrophoresis alongside quantitation standards (10-100 ng), prepared by digesting a known amount of vector with a restriction endonuclease. Fragments were visualised under UV illumination, and the concentration was determined by comparison to the quantitation standards.

2.2.8 Preparation of Vectors for Subcloning

Digested vector to be used for subcloning (1-5 μg) was treated with 1 μl calf alkaline phosphatase for 10-20 minutes at 37°C. This was then treated with 2 μl proteinase K (10 mg/ml) and 2 μl 20% SDS for 60 minutes at 37°C. DNA was extracted with phenol chloroform, and then ethanol precipitated and resuspended in TE (20-100 μl).

2.2.9 Ligation of DNA

Ligations were carried out according to Sambrook *et al.*, (1989), using the T4 DNA ligase enzyme.

2.2.10 Transformation of Competent Cells

Competent *E. coli* XL-1 Blue cells suitable for transformation were prepared using calcium chloride (Sambrook *et al.*, 1989), *E. coli* DH5α cells were purchased from Gibco BRL Life Technologies Ltd, and competent *E. coli* RR1 cells were prepared according to Inoue *et al.* (1990). All competent cells were transformed by heat shock according to Sambrook *et al.* (1989). Agar plates used for plating cells after transformation were supplemented with appropriate antibiotics.

2.2.11 Preparation of Plasmid DNA

Small amounts of plasmid DNA were prepared by extraction of DNA from a bacterial culture using the rapid-boil technique according to Holmes and Quigley (1981). Large amounts of plasmid DNA were obtained by alkaline lysis of the bacterial cells, followed by Wizard Maxiprep DNA Purification Systems™ (Promega) according to the manufacturers instructions. This system is based on purification of DNA by selective binding to a modified DEAE-silica gel anion exchange resin.

2.2.12 Single Stranded DNA Sequencing

Sequences of interest were amplified by PCR using one biotinylated and one non-biotinylated primer in a volume of 50 µl. An aliquot (5 µl) of each reaction was analysed by agarose gel electrophoresis to determine the presence of the appropriate products. 40 µl of the PCR reaction was mixed with streptavidin paramagnetic M-280 dynabeads and the strands were separated by alkali treatment according to the manufacturers instructions. Templates were sequenced by the dideoxy chain termination method originally developed by Sanger *et al.* (1977) using Sequenase version 2.0. Sequencing gels were prepared and electrophoresed as described in Sambrook *et al.* (1989). Dried gels were autoradiographed overnight at room temperature, and autoradiographs were developed and analysed manually.

2.2.13 Double Stranded DNA Sequencing

Double stranded sequences of DNA (3-5 µg DNA template in a volume of 16 µl) were denatured (2 µl of 2 M NaOH and 2 µl of 2 mM EDTA added) for 5 minutes at room temperature, then neutralised (3 µl of 3 M NaOAc pH 4.8). Samples were then ethanol precipitated, and resuspended in sterile water. Templates prepared in this way were sequenced by the dideoxy chain termination method originally developed by Sanger *et al.* (1977) using Sequenase version 2.0. Sequencing gels were prepared and electrophoresed as described in Sambrook *et al.* (1989). Dried gels were autoradiographed overnight at room temperature, and autoradiographs were developed and analysed manually.

2.2.14 Labelling DNA Probes with ^{32}P

DNA probes were labelled with [α^{32}P]-dCTP using either the random primers DNA labelling system (Gibco BRL Life Technologies Ltd) as directed by the manufacturers instructions, or the Rediprime DNA labelling protocol (Amersham Life Sciences). Radioactively labelled probes were purified using ProbeQuant™ G-50 Micro Columns (Pharmacia Biotech) according to manufacturers instructions. The specific activity and degree of incorporation of radioactively labelled DNA was determined by scintillation counting in a Beckman LS8000 scintillation counter.

2.2.15 Hybridisation using DNA Probes

Prehybridisation

All colony lifts and Southern blots were treated in the same manner. DNA was UV cross-linked to membranes by exposure to UV illumination for 2 minutes per side. Filters were prehybridised for 2-3 hours in a rotary oven at 68°C in 100-200 ml of prehybridisation solution (6x SSC containing 5x Denhardts [1% ficoll (Type 400), 1% polyvinylpyrrolidine, 1% bovine serum albumin] and 100 µg/ml herring sperm DNA).

Hybridisation

Hybridisation was performed in a rotary oven at 68°C overnight. Radiolabelled probe was added at a concentration of $5x10^7$cpm/µg DNA per ml of hybridisation solution (1 M NaCl, 50 mM $NaH_2PO_4.2H_2O$, 2 mM EDTA, 0.5% SDS, 5x Denhardts [1% ficoll (Type 400), 1% polyvinylpyrrolidine, 1% bovine serum albumin], and 100 µg/ml herring

sperm DNA). The labelled DNA probe was boiled for 5 minutes and placed on ice before addition to the prehybridised lifts or blots.

Washing

Hybridisation solution was discarded and the filters washed as follows, unless otherwise stated. Filters were washed twice [6x SSC, 1% SDS] for 1 hour at the hybridisation temperature, followed by two washes for 30 minutes in 6x SSC, 0.5% SDS at the same temperature, and finally a high stringency wash for exactly 30 minutes in 1x SSC which had been prewarmed to hybridisation temperature.

2.2.16 Autoradiography

All filters and blots were wrapped in plastic film to prevent drying out during exposure to X-ray film in autoradiography cassettes. Autoradiography was carried out in the presence of intensifying screens at -70°C. Films were developed manually using Kodak developer and fixer.

2.2.17 Isolation of Genomic DNA

Genomic DNA was isolated from both rat spleen and bovine white blood cells using a genomic DNA extraction protocol (GENOMIX Kit) by Talent. This kit uses an initial lysis step followed by extraction with chloroform. The DNA is selectively precipitated using cationic detergents. The detergent is then eliminated using an ion exchanger and finally the DNA is ethanol precipitated. Details of buffers and solutions in the kit were not given. Quantitation of genomic DNA isolated was assessed both spectrophotometrically, and by gel electrophoresis of an aliquot in a 0.7% agarose gel in TAE.

2.2.18 Digestion of Genomic DNA

Digestion of genomic DNA was carried out in sterile 0.5 ml microcentrifuge tubes according to the following protocol.

Genomic DNA	~20 µg
React™ buffer	10 µl
Sterile water	up to 100 µl
Restriction enzyme	2U/µg DNA

Reactions were incubated at 37°C overnight. An aliquot (5 µl) was analysed by agarose gel electrophoresis to determine the efficiency of the digestion. Reactions which required further digestion were incubated with additional restriction enzyme (2U/µg DNA) for 2-4 hours. The correct proportion of React™ buffer was maintained in these incubations.

2.2.19 Electrophoresis of Genomic DNA

Prior to electrophoresis, digested genomic DNA was concentrated by ethanol precipitation according to Sambrook *et al.* (1989). Samples were resuspended in a small volume of sterile TE buffer pH 8.0 and heated to 65°C for 10 minutes with loading dye before electrophoresis in 0.7% agarose gel in TAE. Electrophoresis was carried out for ~24 hours at ~10 V or until the dye front was ~4 cm from the end of the gel tray (11 cm). The DNA fragments were stained with ethidium bromide (0.5 µg/ml), visualised, and photographed under UV illumination.

2.2.20 Southern Transfer

DNA fragments separated by gel electrophoresis were transferred to nitrocellulose membrane by capillary transfer according to Southern (1975) or as described by the supplier of the transfer membrane. Filters were then dried between blotting paper, and either baked at 80°C for 2-3 hours, or exposed to UV light in order to immobilise transferred DNA on the nitrocellulose.

2.2.21 Screening of Bacteriophage Library

The bovine genomic library was screened according to the manufacturers instructions. The first round of screening was carried out with a bacteriophage density of approximately 50,000 plaque forming units (pfu) per plate. This was decreased to approximately 200-300 pfu for second round screening. Dilutions of phage stocks for plating were prepared in SM buffer (0.1 M NaCl, 8 mM $MgSO_4$, 20 mM Tris-HCl pH 7.5, 0.1% gelatin). Replicas of the λ plaques were transferred to nitrocellulose filters which were subsequently hybridised with ^{32}P-labelled DNA probe (Sambrook *et al.*, 1989).

2.2.22 Preparation of Plating Cells

XL-1 Blue MRA(P2) cells (Section 2.1.4) required for plating of the bovine genomic library, were grown in NZY media (5g NaCl, 2g $MgSO_4.7H_2O$, 5g yeast extract, 10g NZ amine (casein hydrolysate), per 1 litre). Plating cells were always prepared freshly before use, by innoculating a 5 ml broth of NZY with a single colony of XL-1 Blue MRA(P2). This was supplemented with sterile maltose and $MgSO_4$ (final concentrations 20% and 1 M, respectively), and grown overnight at 30°C, with shaking. Cells were harvested by centrifugation (2000 rpm, 10 minutes in a bench top centrifuge), and resuspended in 10 mM $MgSO_4$ to OD_{600}=0.5.

2.2.23 Plaque Lifts

Duplicate lifts were taken from each plate (pre-chilled at 4°C), with each filter marked in order to identify plate number and orientation. Filters were left to transfer for 2 minutes (first lift) and 4 minutes (second lift). They were then transferred, plaque side up, to blotting paper and denatured (2 minutes; 1.5 M NaCl, 0.5 M NaOH), neutralised twice (5 minutes; 1.5 M NaCl, 0.5 M Tris HCl pH 8.0), and rinsed (30 seconds; 0.2 M Tris HCl pH 7.5, 2 x SSC). Filters were left to air dry, and exposed to UV illumination in order to cross-link the DNA onto the filter. Agar stock plates of transfers were stored at 4°C, and filters were stored at room temperature until use.

CHAPTER THREE: RESULTS AND DISCUSSION

3.1 Introduction

Isolation of the 5' regulatory sequences of ruminant ATP citrate lyase required a probe which could be used to screen a ruminant genomic library. Initially, RNA was isolated from both rat and lamb liver and used to synthesise first strand cDNA. This first strand cDNA was then used as a template for PCR, using specific primers designed to amplify either the entire coding region (3.3 kb product), or exon II (202 bp) of the ATP citrate lyase gene. The PCR product was then cloned, sequenced, and used as a probe to screen a genomic library.

3.2 Design of PCR Primers

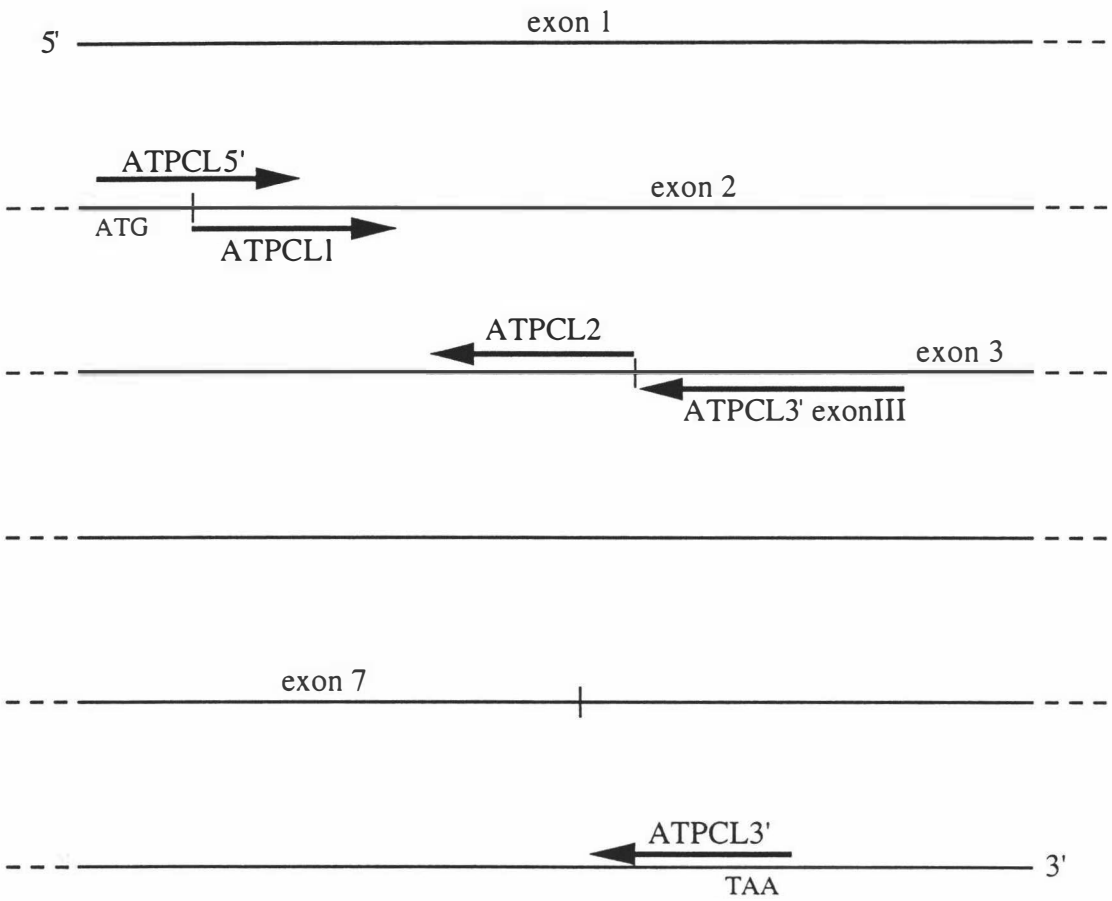
Oligonucleotide primers were designed at two stages during this study, for use with PCR techniques, to amplify regions of ATP citrate lyase. There is significant inter-species divergence of sequence in the 5' and 3' untranslated regions of ATP citrate lyase, so these regions were not used for primer design.

Initially, three oligonucleotide primers were designed : ATPCL5', ATPCL3', and ATPCL3' exon III (Appendix I) (Figure 3.1). The sequences of these primers were based on the homology between rat and human ATP citrate lyase, with the assumption that a similar level of identity (97% at the DNA level between rat and human) might extend to sheep. Degenerate primers were designed for use on both rat and lamb cDNA templates.

ATPCL5' was designed to anneal to the 5' end of the coding region of ATP citrate lyase (Appendix II). This primer has an *Nde* I site incorporating the ATG start codon, a feature that would enable cloning into pT7-7; expression of the cDNA in this vector would allow development of this project to include enzyme studies. An *Xba* I site was also incorporated into this primer to allow cloning into the plasmids pBlueScript™SK(+/-) or pGEM-3Zf(+/-).

ATPCL3' was designed to anneal to the 3' end of the coding region of ATP citrate lyase (Appendix II), and contains a *Cla* I site to enable cloning into pT7-7, and a *Sal* I site for cloning into pBlueScript™SK(+/-) or pGEM-3Zf(+/-). PCR with these two primers was

Figure 3.1 Schematic representation of PCR primers and rat ATP citrate lyase cDNA sequence



expected to amplify the entire coding region of ATP citrate lyase (Appendix II), giving a PCR product of 3.3 kb.

The second 3' primer, ATPCL3' exon III, is an alternative primer which could be used in conjunction with ATPCL5'. ATPCL3' exon III was designed as a perfect match to rat cDNA, and contained a *Sal* I restriction site to enable cloning into pBlueScript™SK(+/-) or pGEM-3Zf(+/-). PCR, with this 3' primer and ATPCL5' using a rat cDNA template, would amplify a 282 bp region containing exon II and exon III sequences of ATP citrate lyase (Appendix II).

3.3 Isolation of Total RNA

RNA was extracted from two types of tissues, rat and neonatal lamb liver, and used for first strand cDNA synthesis (Section 3.4) and northern blotting (Section 3.11). Primers for RT-PCR had been designed (Section 3.2) based on the homology between rat and human ATP citrate lyase sequences. Therefore PCR with these primers on first strand cDNA from rat liver RNA should act as a positive control for the process of cDNA synthesis and DNA amplification. Neonatal lamb liver was used as a source of RNA because this stage of development is thought to correlate with high levels of both ATP citrate lyase enzyme and mRNA (Roehrig *et al.*, 1988). In contrast, tissue from adult ruminants should have negligible amounts of ATP citrate lyase mRNA (Judson and Leng, 1973a). If the RT-PCR approach was successful with lamb mRNA, the resulting probe could be used to screen a sheep genomic library. A sheep liver cDNA probe should be a more suitable probe than one constructed from rat mRNA, as the degree of homology between sheep and rat ATP citrate lyase is not known.

Total cellular RNA was extracted using the TRIZOL™ LS reagent, from rat and neonatal lamb liver tissue (Section 2.2.1) that had been previously collected and stored at -70°C. Isolated RNA was analysed spectrophotometrically to determine the concentration and purity of the samples (Section 2.2.1) (Table 3.1).

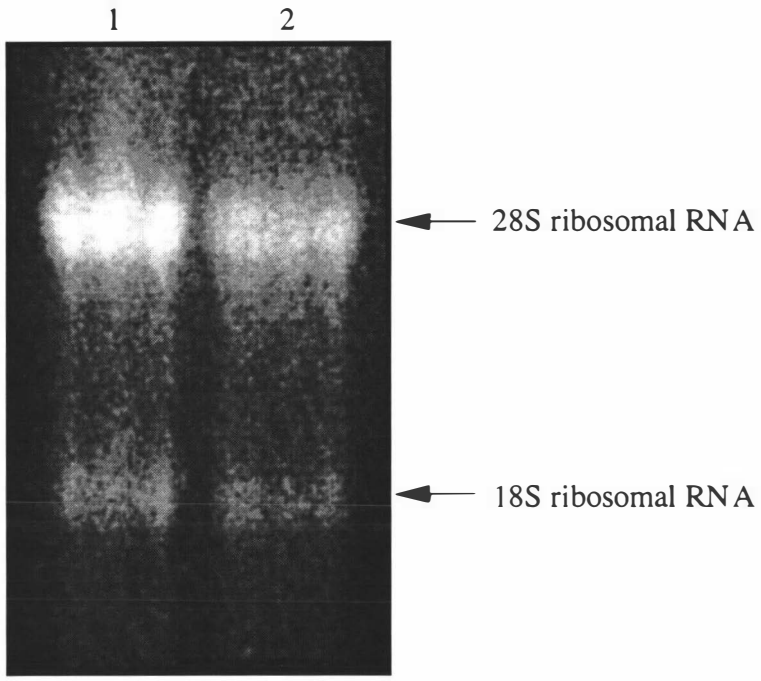
Date	Tissue	Amount	Yield	Purity A_{260}/A_{280}	Use
24/1/95	lamb liver	~200 mg	960 µg	2.08	Northern blotting RT–PCR
30/1/95	rat liver	~200 mg	436 µg	1.69	not used
3/2/96	rat liver	~200 mg	1392 µg	1.86	Northern blotting RT–PCR
9/3/96	rat liver	~200 mg	850 µg	1.84	RT-PCR
9/3/96	lamb liver	~200 mg	544 µg	1.79	RT-PCR
5/12/96	lamb liver	~200 mg	540 µg	1.66	not used
5/12/96	rat liver	~200 mg	1280 µg	1.81	RT–PCR

Table 3.1 Isolation of total RNA from rat and lamb liver tissue. Expected yield was 6-10 µg RNA/mg tissue (TRIZOL™ LS Reagent Product Sheet, Gibco BRL Life Technologies Ltd).

The expected yield of RNA from ~200 mg of tissue using the TRIZOL™ LS reagent was 1200-2000 µg RNA. However, the actual amount of RNA extracted ranged from 436-1392 µg, which was lower than the expected yields. The low yields could have been due to incomplete homogenisation or lysis of tissue samples, or due to incomplete redissolving of the final RNA pellet. Although the yield was not as high as expected, the purity of the RNA was high, with most A_{260}/A_{280} ratios close to a value of 1.8, which indicates pure RNA.

Samples of isolated RNA were separated by electrophoresis (Section 2.2.1), and the presence of two discrete ribosomal RNA bands indicated that intact RNA had been prepared (Figure 3.2). This RNA was subsequently used for cDNA synthesis (Section 3.4) and/or northern analysis.

Figure 3.2 Total RNA isolated from neonatal lamb and rat liver tissue



Total RNA isolated from neonatal lamb and rat liver tissue was analysed on a 1.5% low EEO agarose containing 1x MOPS buffer (1M MOPS, 250 mM sodium acetate, 5 mM EDTA, pH 7.0) and formaldehyde (17.6 ml/100 ml). The gel was submerged in 1x MOPS running gel buffer and electrophoresis was carried out for ~3 hours at 100 mA. Samples were loaded with 1 µl loading buffer (containing bromophenol blue, xylene cyanol and glycerol) and 2 µl ethidium bromide (10 mg/ml) and after electrophoresis the RNA was visualised with a UV transilluminator.

1: Neonatal lamb liver RNA (28.8 µg)

2: Rat liver RNA (27.8 µg)

3.4 Synthesis of cDNA

First strand cDNA was synthesised using oligo d(T) as a primer from total cellular RNA extracted from either rat or lamb liver using the Superscript Preamplification System (Section 2.2.2) (Figure 3.3). Oligo d(T) anneals to the polyA tail of the mRNA, and acts as a primer for first strand cDNA synthesis using the enzyme reverse transcriptase. Once the cDNA had been synthesised, the RNA was removed using RNaseH, leaving first strand cDNA ready for PCR amplification.

3.5 PCR with ATPCL5' and ATPCL3' Primers

PCR (Section 2.2.3) was initially carried out using first strand cDNA as a template with oligonucleotide primers designed to amplify the entire coding sequence of ATP citrate lyase (3.3 kb).

Attempts were made to optimise the following PCR parameters: primer concentration, $MgCl_2$ concentration, and dNTP concentration. Primer concentration was varied, with the first PCR reaction containing 100 pmol of each primer in a total volume of 50 µl. These conditions did not result in the production of a PCR product, so a primer titration was carried out. Primers were added to the PCR reaction in six different amounts, ranging from 50 - 250 ng (4 - 20 pmol) per PCR reaction. This decrease in primer concentration resulted in the production of a very low yield of PCR product from rat liver first strand cDNA.

In an attempt to increase the yield of PCR product from rat liver first strand cDNA, a magnesium titration was then carried out. Six different $MgCl_2$ concentrations were used ranging from 0 - 10 mM Mg^{2+}. A product was obtained (Figure 3.4) with a magnesium concentration of 2 mM. This magnesium concentration was used for further PCR reactions with these primers.

The dNTP concentration was also optimised, by using three different concentrations of dNTP in the PCR reaction: 15 mM, 22.5 mM and 30 mM. The optimal dNTP concentration was 22.5 mM.

PCR reactions with ATPCL5' and ATPCL3' primers and lamb liver cDNA were carried out. Despite varying several PCR parameters, including two magnesium titrations, where

the magnesium concentration ranged from 0 - 10 mM, and another titration with freshly prepared first strand cDNA from lamb liver, a PCR product was not obtained at any stage using RT-PCR with lamb mRNA and these primers.

At this stage, RT-PCR with lamb mRNA was abandoned. A positive control was provided with the RT-PCR kit, which indicated whether the reverse transcriptase reaction had been successful; primers included in the kit could be used to show whether PCR had been successful. This positive control was always included with first strand cDNA synthesis and PCR reactions, and this always produced a 523 bp product (Figure 3.5), indicating that the kit and subsequent PCR reactions were not at fault. There are several possibilities for the failure of PCR with these primers and lamb first strand cDNA.

1 There may have been considerable lack of homology between the PCR primers and lamb cDNA sequences. These primers were designed based on the published DNA sequences for rat and human ATP citrate lyase. The high level of homology between these two species may extend to sheep. However, it could be that the same level of homology does not extend to sheep ATP citrate lyase, and if this is the case, then PCR with these primers may not be successful.

2 There was no definitive method of determining whether first strand cDNA was being produced from sheep liver mRNA. This method does not directly indicate whether first strand cDNA has been produced.

In retrospect, a more direct method could have been used to perform gel analysis of first strand cDNA reaction in the presence of a tracer, but this was not carried out at the time of these experiments. Both control and rat RT-PCR reactions were successful, which suggests that the kit and PCR procedure were both fully functional.

3 It is possible that the RNA extracted from lamb liver did not contain mRNA for ATP citrate lyase. This is a distinct possibility, because the tissue used had been obtained from a stillborn lamb, and at present there is uncertainty about ATP citrate lyase expression prior to, and at birth in ruminants (Roehrig *et al.*, 1988).

At this stage of the project, the best option may have been to obtain fresh lamb livers, from lambs at a stage of development that is known to have high levels of ATP citrate lyase expression. Unfortunately this need did not coincide with the lambing season, so no suitable lamb livers were available.

In an attempt to proceed with the project, the rat PCR product which had been successfully produced, was cloned for use as a probe to screen a genomic library.

Figure 3.3 RT-PCR scheme used with *SUPERSCRIPT*™ Preamplification System

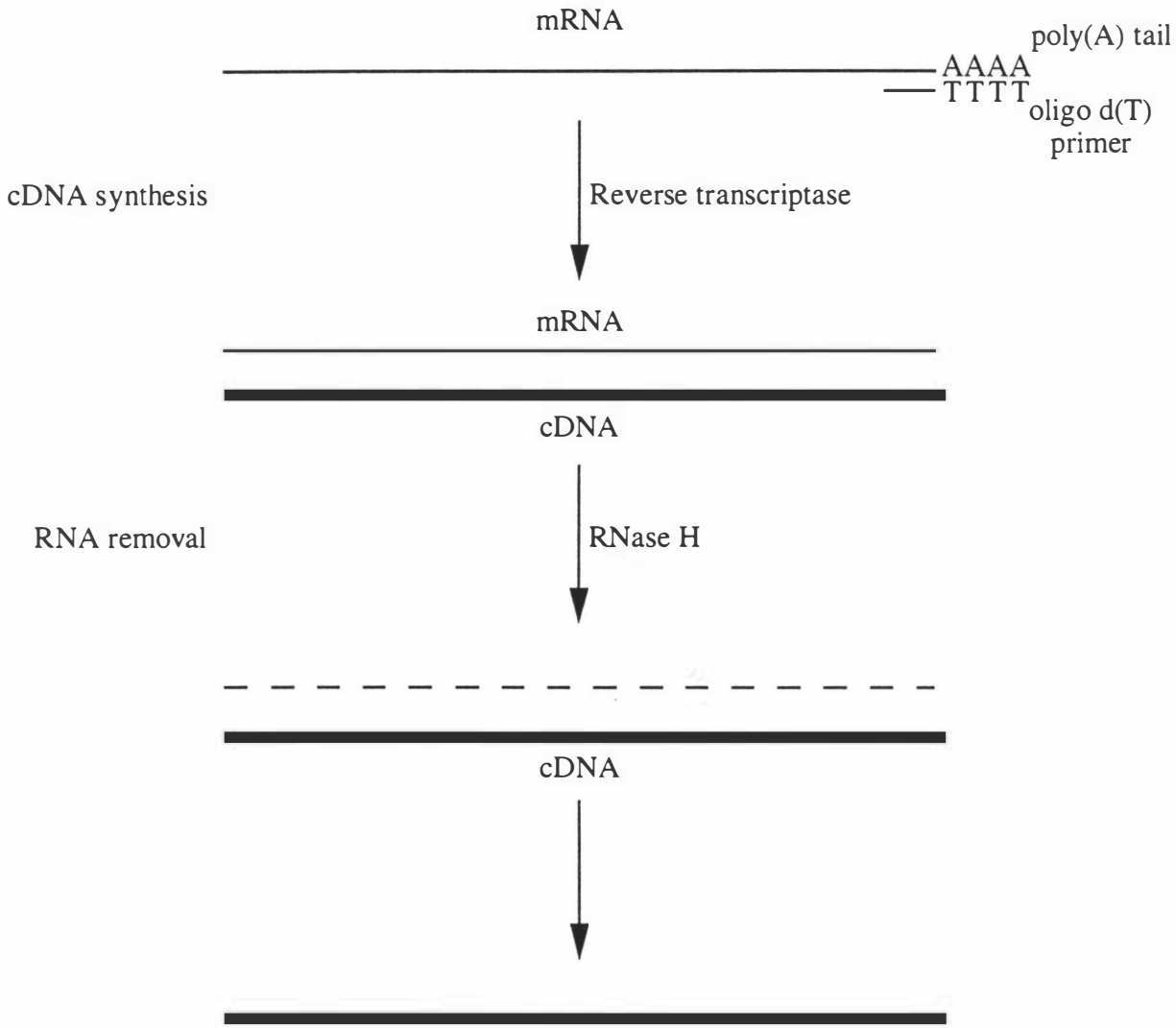
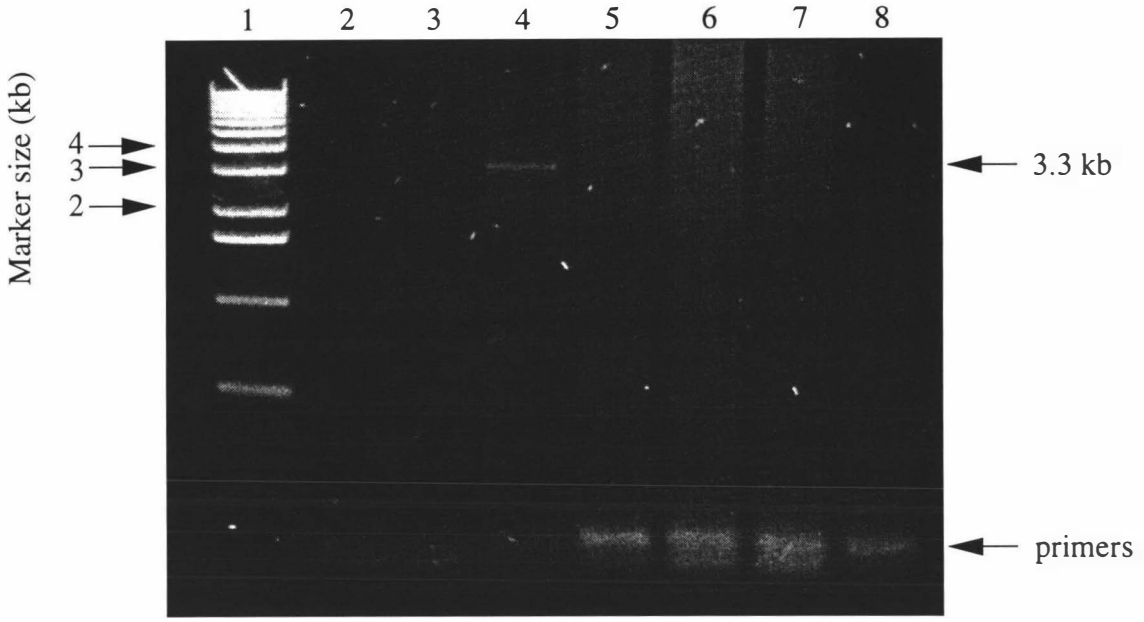


Figure 3.4 3.3 kb PCR product amplified from rat liver first strand cDNA with ATPCL5' and ATPCL3' primers



A 5 µl aliquot of each PCR reaction was analysed by electrophoresis in a 1% agarose gel containing 1x TAE at 88V for 60 minutes. DNA was stained using ethidium bromide and visualised with a UV transilluminator. Components of PCR reactions are described in Section 2.2.3.

1: BRL 1 kb ladder

2: Negative control (All components of PCR reaction except liver first strand cDNA)

3: 0 mM Mg^{2+}

4: 2 mM Mg^{2+}. A 3.3 kb fragment of rat ATP citrate lyase amplified from rat liver first strand cDNA using primers ATPCL5' and ATPCL3' and *Taq* polymerase is indicated.

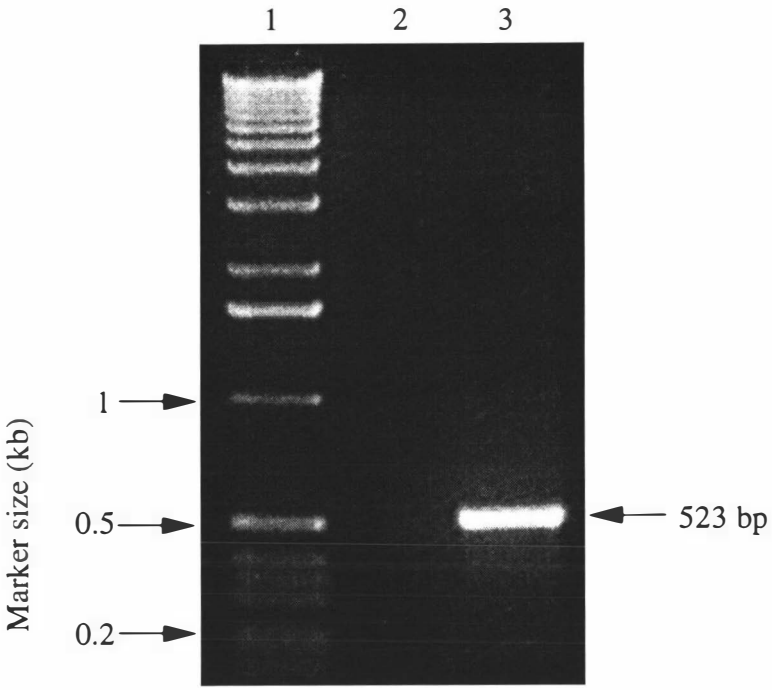
5: 4 mM Mg^{2+}

6: 6 mM Mg^{2+}

7: 8 mM Mg^{2+}

8: 10 mM Mg^{2+}

Figure 3.5 Positive control in RT-PCR reactions



A 5 µl aliquot of each PCR reaction was analysed by electrophoresis in a 1% agarose gel containing 1x TAE at 88V for 60 minutes. DNA was stained using ethidium bromide and visualised with a UV transilluminator.

1: BRL 1 kb ladder

2: Negative control lane, containing all components of the PCR reaction except first strand cDNA.

3: Positive control - 523 bp fragment amplified using primers and template supplied in the kit.

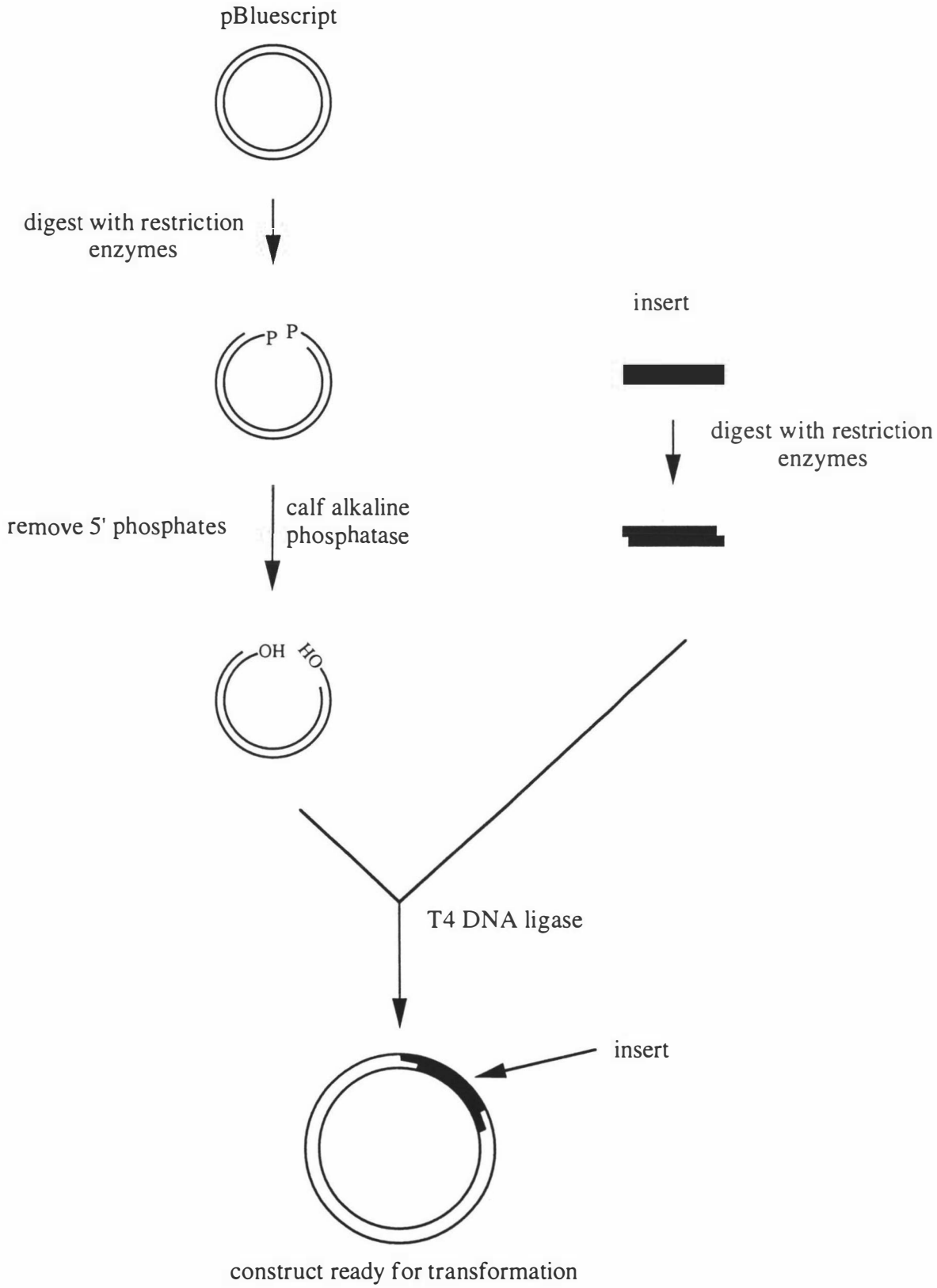
3.5.1 Subcloning of 3.3 kb PCR Product Representing Rat ATP Citrate Lyase cDNA

PCR products were subcloned into the vector pBlueScript™ SK- (Appendix III) as blue/white selection could be used to screen for recombinant clones. Vector was digested with two restriction enzymes *Xba* I and *Cla* I (Section 2.2.5), giving cohesive ends to enable directional cloning. Approximately 3 µg of vector was digested, and an aliquot analysed by agarose gel electrophoresis to check for complete digestion. The vector was then prepared for subcloning (Section 2.2.8) (Figure 3.6).

Several PCR reactions containing the 3.3 kb PCR product were pooled, digested with *Cla* I and *Xba* I (Section 2.2.5), and treated with proteinase K to inactivate enzymes from the digestion. The DNA was separated from proteins using phenol/chloroform extraction, and then concentrated by ethanol precipitation and resuspended in 15 µl of sterile water. Both vector and insert were electrophoresed alongside quantitation standards, and the concentration of each sample determined (Section 2.2.7).
The ratio of free ends available for ligation was calculated in order to successfully ligate the vector and insert. Vector and insert were both ~3 kb in size, so 1 ng of vector would have approximately the same number of free ends as 1 ng of insert. An insert:vector ratio of 3:1 is recommended (Sambrook *et al.*, 1989) for ligation to be successful, with no more than 200 ng DNA in a ligation reaction. Therefore 60 ng of insert was ligated with 20 ng of vector (Section 2.2.9). Competent *E. coli* XL-1 Blue cells were transformed (Section 2.2.10) with the ligation reaction, and left to grow overnight at 37°C.

White colonies were transferred into 5 ml of LB broth (10g tryptone, 5g yeast extract, 5g NaCl, per 1 litre) containing ampicillin (100 µg/ml), and allowed to grow overnight at 37°C. These cells were then harvested by centrifugation, and the plasmid DNA was isolated using the rapid-boil method (Section 2.2.11). The plasmid DNA recovered was then digested with diagnostic enzymes (Section 2.2.5) to identify recombinant plasmids with the 3.3 kb insert. As the size of the insert and plasmid were similar (insert 3.3 kb; vector 3.2 kb), a diagnostic digest that excised the insert would result in two products of similar size, 3.2 and 3.3 kb. These would be difficult to separate and visualise on an agarose gel. Instead, digestion with one enzyme with a unique site would cut a recombinant plasmid with the 3.3 kb insert into a 6.5 kb linear molecule (3.2 kb vector + 3.3 kb insert).

Figure 3.6 Ligation scheme of pBlueScript with PCR product



Recombinant plasmids that did not contain the 3.3 kb insert would be digested to give a 3.2 kb linear molecule. These two bands would be easily identified on an agarose gel.

Plasmid DNA was digested with either *Cla* I or *Xba* I (enzymes used for cloning), but only 3.2 kb products were observed on the gel. Therefore none of the white colonies contained plasmids with the 3.3 kb insert.

Additional PCR reactions to obtain the 3.3 kb ATP citrate lyase cDNA PCR product were carried out with an extra cycle of 72°C for 10 minutes at the end of the PCR cycle, in order to finish any unextended copies. This approach was repeatedly unsuccessful in producing a PCR product. Another enzyme, *Pfu* polymerase, was used in the PCR reaction, but this was also ineffective in producing a PCR product. This was repeated unsuccessfully on first strand cDNA prepared from fresh rat liver RNA.

In another attempt to obtain the 3.3 kb PCR product, a PCR reaction was carried out on some 3.3 kb PCR product that had already been produced and used in the cloning exercise. 1 µl of PCR product was added to a 50 µl PCR reaction, but this was also unsuccessful.

At this stage, this approach was abandoned because of the low level of success. One explanation for the lack of ability to produce the 3.3 kb PCR product could be that the reverse transcriptase enzyme was not always able to produce cDNA the full length of the mRNA message. Also, the population of full length transcripts may not have been large enough to be successfully amplified by PCR, and 3.3 kb is a reasonably large product to amplify by standard PCR conditions.

Another 3' primer was available, ATPCL3' exon III, which in conjunction with ATPCL5', would amplify a 282 bp region representing exon II and exon III of rat ATP citrate lyase. This primer was used, as the likelihood of producing smaller PCR products would be more successful, and a probe comprising exon II and exon III of ATP citrate lyase would be suitable to screen a library for 5' regulatory regions.

3.6 PCR with ATPCL5' and ATPCL3' Exon III Primers

This second 3' primer (ATPCL3' exon III), designed to anneal to the 3' end of exon III (Section 3.2), was used for PCR with ATPCL5'. A different positive control for PCR was introduced at this stage. This comprised a vector, pTG3954 (Appendix VI) which contains a ~19 kb factor IX gene insert. PCR with 1916 and 1917 primers (Appendix I) amplifies a 344 bp PCR product from this vector.

Many attempts at PCR were made until a 282 bp product was successfully produced. The first PCR reaction used rat liver first strand cDNA prepared on 21/6/95 (Table 3.1), with the same magnesium chloride and primer concentrations and PCR programme used previously (Section 3.4.1). A PCR product for the positive control was observed when an aliquot was analysed by agarose gel electrophoresis, but no other PCR products were seen. For the next PCR reaction, the annealing temperature was reduced to 37°C. No PCR products were observed, not even the positive control, so these conditions were repeated, using rat liver first strand cDNA prepared at a different time (9/3/95). This produced a faint band at the expected size of 282 bp, which indicated that the primers were capable of producing a PCR product. In another attempt to produce this PCR product, several dilutions were made of the cDNA template (produced on 8/1/95). These dilutions were prepared in an attempt to dilute out any contaminating species that may be present in the first strand cDNA, as no cleanup step was carried out after the reverse transcriptase incubation and RNaseH treatment. Amounts used were 5 µl cDNA, 1 µl cDNA, 1 µl of 1:5 dilution, 1 µl of 1:10 dilution, 1 µl of 1:50 dilution, and 1 µl of 1:100 dilution. A PCR product was seen for the positive control, but once again no other PCR products were observed.

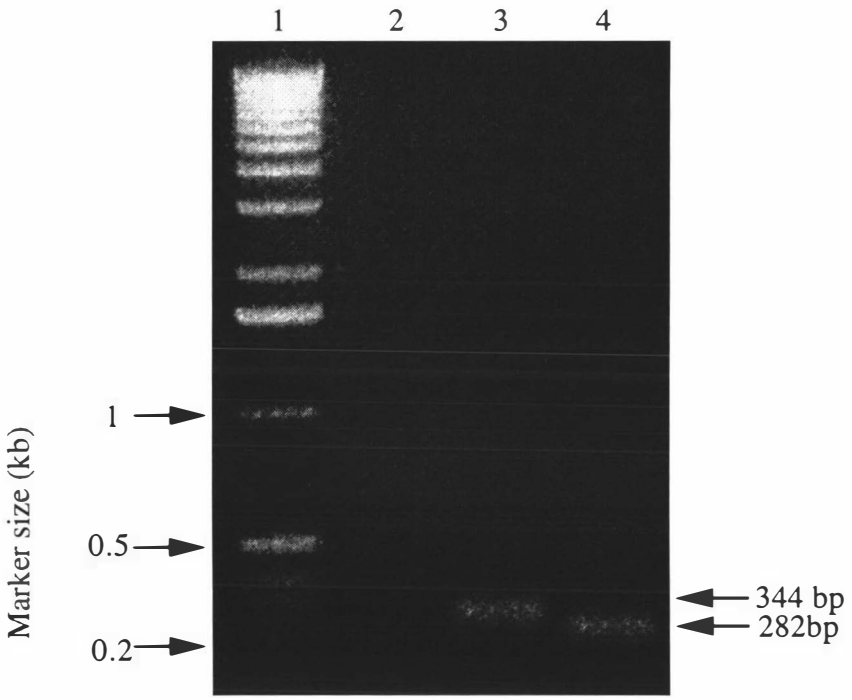
At this stage all new reagents for PCR were prepared, including dilutions of primers, dNTPs, reaction buffer and $MgCl_2$. PCR with these new solutions and freshly prepared cDNA (12/1/96) was unsuccessful.

A different template was then used for PCR as follows: 1 µl of the PCR reaction which gave a faint 282 bp product obtained previously was used in a PCR reaction containing the newly prepared reagents.

This approach successfully produced a PCR product (Figure 3.7). This product was able to be produced reproducibly, and the presence of a single band indicated that only a single product was being produced, despite the unusually low annealing temperature required.

A diagnostic digest was carried out on the 282 bp PCR product to confirm its identity. Two restriction endonucleases, *Apa* I and *Ava* I, were used to digest the PCR product. These enzymes have a single restriction site within the 282 bp PCR product, and the presence of two correct sized fragments after digestion would indicate that the PCR product was the 282 bp sequence of rat ATP citrate lyase. Digestion of the PCR product indicated that it was heterogeneous, as there were also several other bands as well as the expected products. There are two possible explanations for the production of a heterogeneous PCR product. Firstly, a single incorrect product was amplified, which was the same size as the expected product. The second possibility is that two products were amplified by the same primers - the correct product, and another incorrect product of the same size (282 bp). Digestion of this PCR product with several enzymes clearly demonstrates the second option (Figure 3.8A). The expected digest products were observed (Figure 3.8B), indicating that the correct DNA fragment was present, but also several other fragments were present after digestion, indicating heterogeneity. Cloning the correct PCR product should have allowed isolation of the specific band representing ATP citrate lyase cDNA sequence. Therefore, the PCR products were cloned in an attempt to isolate the correct fragment.

Figure 3.7 282 bp PCR product amplified from rat liver first strand cDNA with ATPCL5' and ATPCL3' exon III primers



A 5 µl aliquot of each PCR reaction was analysed by electrophoresis in a 0.7% agarose gel containing 1x TAE at 70V for 90 minutes. DNA was stained using ethidium bromide and visualised with a UV transilluminator.

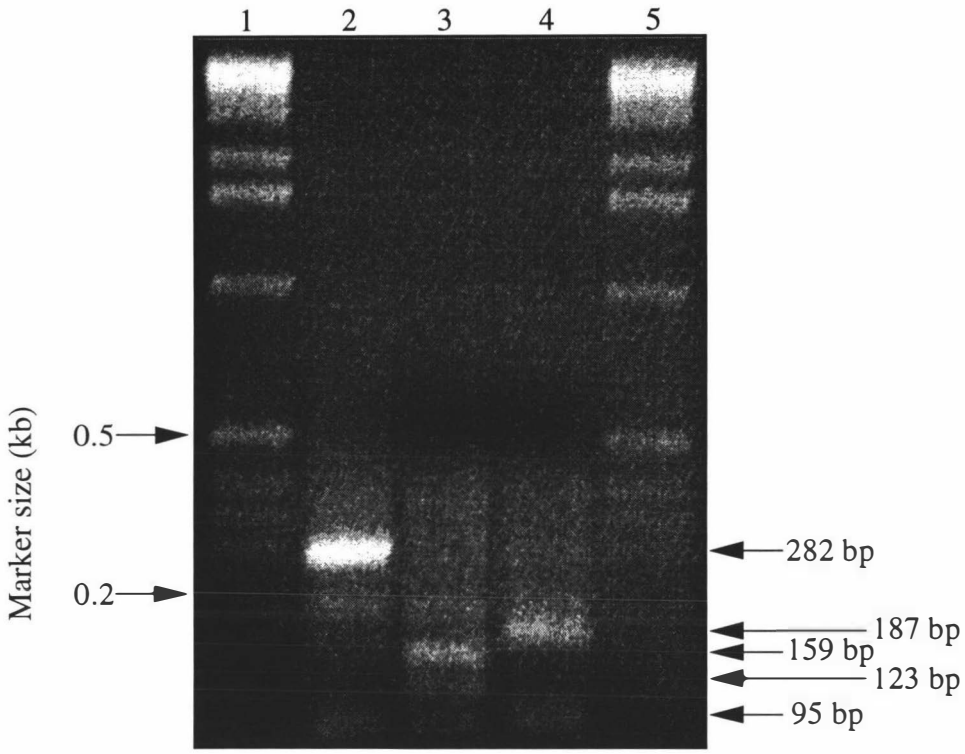
1: BRL 1 kb ladder

2: Negative control

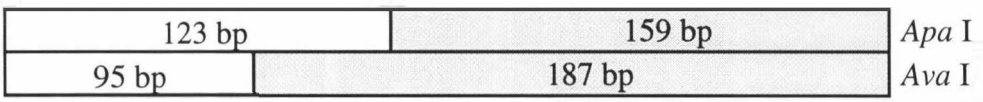
3: Positive control - 344 bp fragment of factor IX gene promoter insert amplified from pTG3954 using primers 1916 and 1917 and *Taq* polymerase.

4: 282 bp product amplified from previously prepared 282 bp product (amplified from rat liver first strand cDNA) and ATPCL5' and ATPCL3'exon III primers and *Taq* polymerase.

Figure 3.8 Diagnostic digest of 282 bp PCR product and schematic representation of expected digest products



B



A: Diagnostic digest of 282 bp PCR product

135 ng of 282 bp PCR product was digested and analysed by electrophoresis in a 2% agarose gel containing 1x TAE at 88V for 60 minutes. DNA was stained using ethidium bromide and visualised with a UV transilluminator.

1: BRL 1 kb ladder

2: Undigested 282 bp PCR product.

3: 282 bp PCR product digested with *Apa* I.

4: " " " " " " *Ava* I.

5: BRL 1 kb ladder

B: Expected digest products of 282 bp PCR product contained a 282 bp insert

3.6.1 Subcloning of 282 bp PCR Product

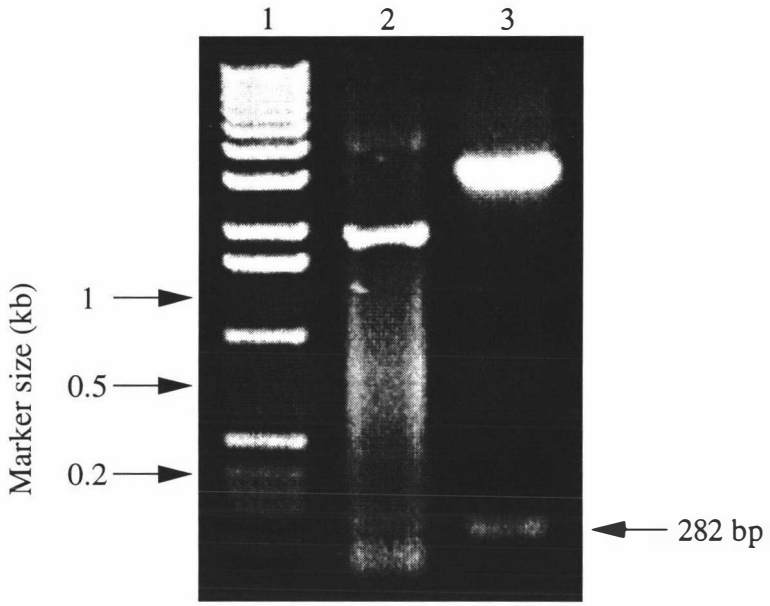
In preparation for directional cloning, the PCR product was digested with restriction endonucleases (Section 2.2.5) *Xba* I and *Sal* I; the vector pBlueScript™ SK- had been digested with the same enzymes. PCR product and vector were ligated as described previously, and competent *E. coli* DH5α cells (Gibco BRL) were transformed with the ligation (Section 2.2.10). Blue/white selection was also carried out with this strain of *E. coli*, in order to identify recombinant transformants. A total of 12 white colonies were from plates of transformed cells, and the plasmid DNA was extracted (Section 2.2.11) and analysed by digestion (Section 2.2.5) with two restriction enzymes, *BstX* I and *Xho* I, which should excise the 282 bp fragment from the plasmid. Of the 12 colonies selected, only one clone contained a 282 bp insert (Figure 3.9), pACL282 (Appendix IV). A large quantity (337.5 μg) of this plasmid DNA was prepared (Section 2.2.11) in preparation for DNA sequencing.

3.6.2 Sequencing of pACL282 Clone

Double-stranded DNA sequencing of pACL282 was carried out manually (Section 2.2.13) to confirm its identity. As PCR generates errors during the amplification process, the isolated clones had to be sequenced to check that not only did they contain rat ATP citrate lyase exon II and III sequence, but also that there were no nucleotide substitutions, additions or deletions. Sequencing of both strands revealed that pACL282 did not contain the sequence of rat ATP citrate lyase exons II and III as expected. Alignment of this sequence with a eukaryotic database did not reveal any similarity to known sequences.

This strategy was repeated; two additional positive clones were isolated, containing the 282 bp insert (Figure 3.10). Sequence analysis of these two clones revealed two additional sequences. Therefore 3 clones had been isolated, each containing a 282 bp insert, yet all 3 inserts had a different sequence. DNA sequence comparison using BLAST (Altschul *et al.*, 1990) of these additional clones revealed no homologies with rat ATP citrate lyase.

Figure 3.9 *BstX* I and *Xho* I digest of miniprep plasmid DNA containing the 282 bp insert



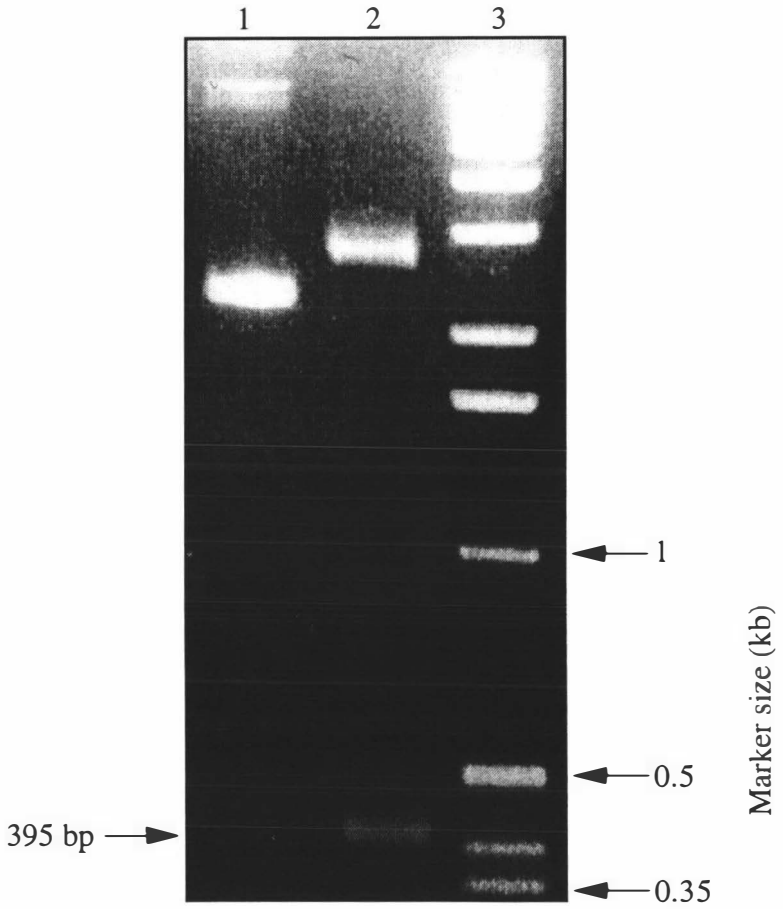
1-2 µg of rapid boil DNA was digested in a total volume of 30 µl. Digest products were analysed by electrophoresis in a 2% agarose gel containing 1x TAE at 80V for 60 minutes. DNA fragments were stained using ethidium bromide and visualised with a UV transilluminator. Only one clone contained the 282 bp fragment, in lane 3.

1: BRL 1 kb ladder.

2: Undigested clone 1 miniprep DNA.

3: Clone 1 miniprep DNA digested with *Bst*X I and *Xho* I. A 282 bp fragment is indicated.

Figure 3.10 *Bss*H II digest of miniprep plasmid DNA containing the 282 bp insert



1-2 µg of rapid boil DNA was digested in a total volume of 20 µl. This total volume was analysed by electrophoresis in a 1.5% agarose gel containing 1x TAE at 90V for 110 minutes. DNA was stained using ethidium bromide and visualised with a UV transilluminator. The 395 bp digest product represents 282 bp of ATP citrate lyase sequence plus 173 bp of multiple cloning site sequence either side of the fragment.

1: Undigested miniprep DNA.

2: Miniprep DNA digested with *Bss*H II. A 395 bp fragment is indicated.

3: BRL 1 kb ladder.

Results of the sequencing of 3 separate clones indicated that this approach was unlikely to be successful in obtaining a clone containing sequence representing rat ATP citrate lyase. Initially, there were many problems encountered with PCR, and this was attempted many times, with different templates until a PCR product was able to be produced. Even then, an unusually low annealing temperature of 37°C was required in order for PCR amplification to be successful.

The main cause for these problems experienced is likely to be due to the primers used for PCR. The length of sequence that had homology with rat ATP citrate lyase sequence was 22 and 21 nucleotides (ATPCL5' and ATPCL3' exon III, respectively). These lengths are considered to be relatively short, and coupled with redundancy, could have caused the primers to anneal at positions other than the target sequence (Erlich, 1991). An annealing temperature of 37°C would have also made incorrect annealing more likely.

For these reasons, another set of PCR primers were designed.

3.7 Design of ATPCL1 and ATPCL2 Primers

ATPCL1 and ATPCL2 primers were designed as 25mer perfect matches to the rat ATP citrate lyase coding sequence. They contained no redundancies, to enable the greatest possibility of specific annealing to the correct sequences. PCR with these two primers would amplify a region of 182 bp, containing the whole of exon II of rat ATP citrate lyase (Appendix II) (Figure 3.1).

ATPCL1 was designed to anneal to the 5' end of exon II. This contained an *Eco*R I restriction site, to enable cloning into pBlueScript™SK (+/-).
ATPCL2 was designed to anneal to the 3' end of exon II, and also contained an *Eco*R I restriction site for cloning.

By this stage in the project, the RT-PCR approach was unable to be used to generate a template for PCR; the kit being used to synthesise first strand cDNA from mRNA had been exhausted. The new primers for PCR, ATPCL1 and ATPCL2, were designed as exonic primers which would amplify a region of rat DNA containing exon sequence only. Therefore genomic DNA would be a suitable template for PCR. As there were no supplies of genomic DNA available, fresh genomic DNA was extracted from rat and bovine sources.

The original aim of this project was to prepare a sheep cDNA probe suitable for screening a sheep genomic library. This work would have been carried out at AgResearch in Dunedin with Professor Diana Hill. However, given the difficulties of preparing a sheep DNA probe and the eventual success with preparing a rat DNA probe, an alternative approach was chosen. Faced with having to use a rat DNA probe for screening a genomic library, but having no knowledge of the sequence homology between rat and sheep, the wisdom of screening the sheep library in Dunedin was questioned. A bovine genomic library was available within the Department of Biochemistry at Massey University; a decision was made to use this library instead.

3.8 Isolation of Genomic DNA

Genomic DNA was isolated from several different tissues (Section 2.2.17) to test for hybridisation of the rat ATP citrate lyase probe using Southern blot analysis. Rat genomic DNA was used as a positive control, because the intended probe contains sequences for exon II of the rat ATP citrate lyase gene. Secondly, bovine genomic DNA was used as a test to determine whether the probe would hybridise to bovine sequences. Successful hybridisation would indicate whether the probe would be useful for screening a bovine genomic library. The homology between exon II of human and rat ATP citrate lyase DNA sequences is 92.3%, therefore the probe would be expected to hybridise to human genomic DNA as well; human genomic DNA was included on the blot as an additional control.

3.8.1 Methods of Genomic DNA Isolation

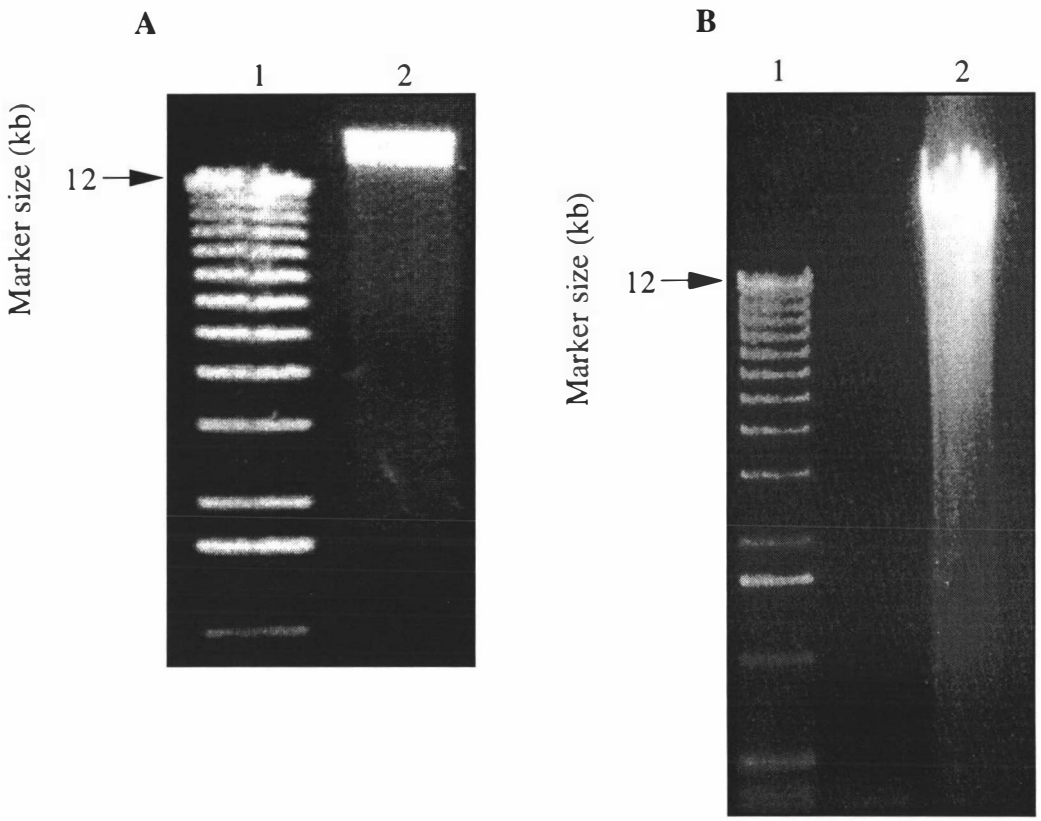
Two different methods were used to extract genomic DNA from rat and bovine tissue, TRIZOL™ LS reagent (Gibco BRL Life Technologies), and Genomix kit (Talent). A comparison of the two methods and the efficiency of extraction are outlined in Table 3.2.

Method	Tissue	Amount Used	Yield	Purity A_{260}/A_{280}
TRIZOL	rat liver	~300 mg	2750 µg	1.70
TRIZOL	rat spleen	~200 mg	1470 µg	1.45
Genomix	bovine WBC	~200 mg	400 µg	1.49
Genomix	rat spleen	~200 mg	135 µg	1.86

Table 3.2 Genomic DNA extracted from rat and bovine tissues.

The yield of genomic DNA was much higher using the TRIZOL™ reagent compared to the Genomix kit, but both methods yielded high molecular weight DNA. Rat genomic DNA extracted using the TRIZOL™ method was used as a template for PCR with ATPCL1 and ATPCL2 primers. Rat (Figure 3.11A) and bovine (Figure 3.11B) genomic DNA prepared using the Genomix kit was used for Southern blot analysis. Human genomic DNA used for Southern blots had been prepared previously using Genomix; this DNA was quantitated, and analysed by gel electrophoresis to check for degradation. The DNA was still of high molecular weight form, and was used for Southern blotting (Figure 3.12).

Figure 3.11 Rat and bovine genomic DNA samples



Samples of genomic DNA were analysed by electrophoresis in a 0.7% agarose gel containing 1x TAE at 88V for 50 minutes. DNA was stained using ethidium bromide and visualised with a UV transilluminator.

A: Genomic DNA extracted from rat spleen

1: BRL 1 kb ladder.

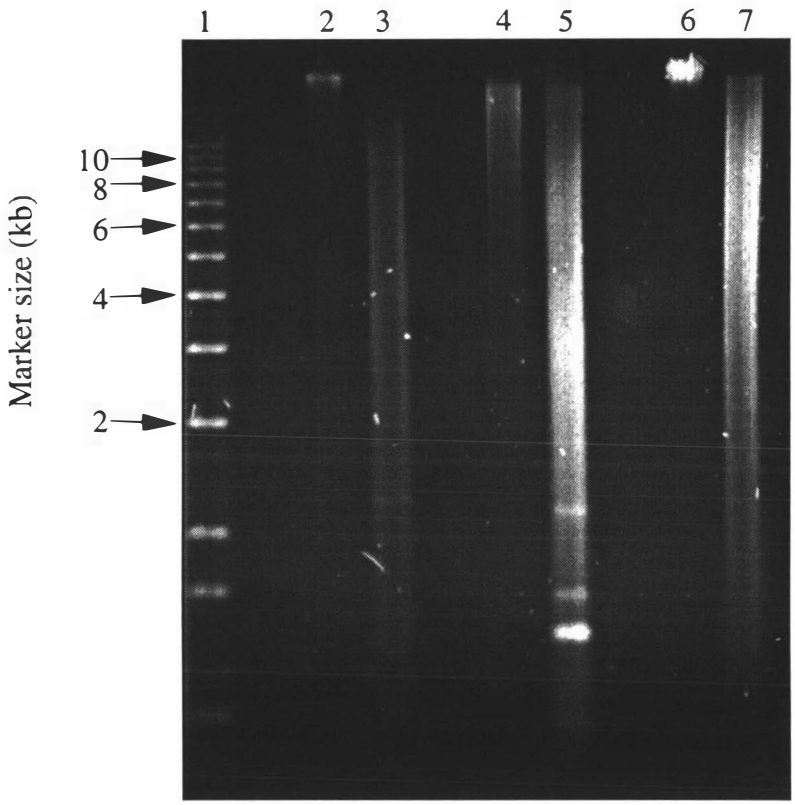
2: ~2 µg rat genomic DNA.

B: Genomic DNA extracted from bovine white blood cells

1: BRL 1 kb ladder.

2: ~5 µg bovine genomic DNA.

Figure 3.12 Genomic DNA samples used for Southern blotting



Rat, bovine and human genomic DNA (~25 ng) were digested with *Eco*R I and analysed by electrophoresis alongside undigested samples (~10 ng) in a 0.7% agarose gel containing 1x TAE at 20V overnight. DNA was stained using ethidium bromide and visualised with a UV transilluminator.

1: BRL 1 kb ladder

2: Undigested rat genomic DNA

3: Digested rat genomic DNA

4: Undigested bovine genomic DNA

5: Digested bovine genomic DNA

6: Undigested human genomic DNA

7: Digested human genomic DNA

3.9 PCR with ATPCL1 and ATPCL2 Primers

ATPCL1 and ATPCL2 were used to amplify exon II of ATP citrate lyase from rat genomic DNA using an annealing temperature of 68°C. A discrete, single product of 202 bp (representing 182 bp of rat sequence and 20 bp of primer sequence, containing restriction sites) was reproducibly generated (Figure 3.13), and produced the expected bands when digested with diagnostic restriction endonucleases (Figure 3.14). This indicated that the correct sequence of DNA had been amplified, so this 202 bp product was cloned into pBlueScript™ SK-.

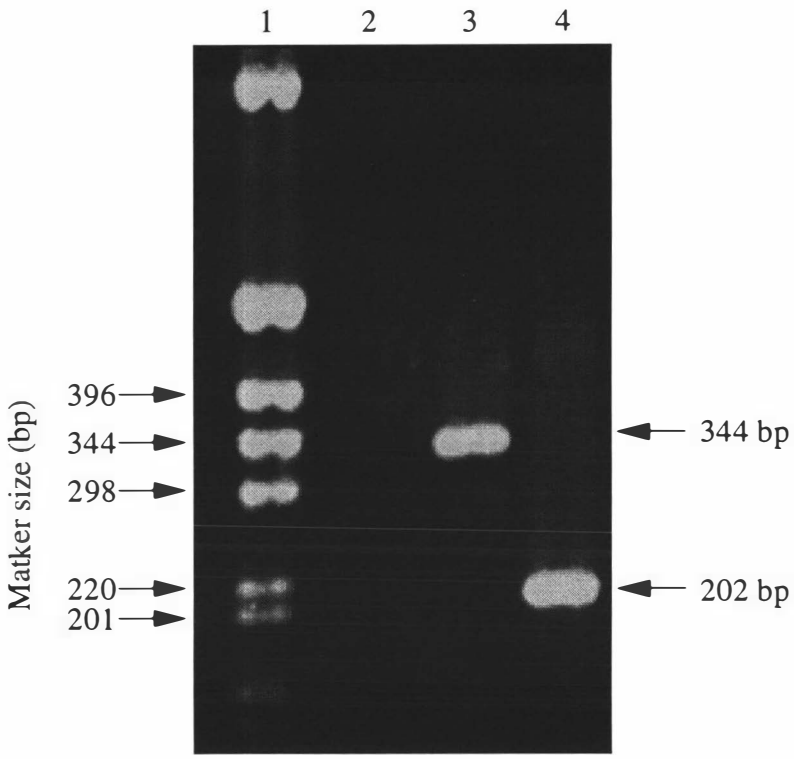
3.9.1 Subcloning of 202 bp Product

The 202 bp PCR product was prepared for ligation by digestion with *Eco*R I (Section 2.2.5), to produce cohesive ends for ligation into the vector, pBlueScript™ SK–, which had also been digested with the same enzyme. The digested PCR product was purified from other digestion products by separation on an agarose gel (Section 2.2.5). The band was then excised, purified from the agarose using Prep-A-Gene (Section 2.2.6), and aliquots of both the purified insert and digested vector were quantitated by agarose gel electrophoresis (Section 2.2.7). The vector was also treated with calf alkaline phosphatase, to remove 5' phosphate groups in order to prevent vector religation (Figure 3.6). Vector and insert were ligated (Section 2.2.9), and competent *E. coli* RR1 cells were transformed (Section 2.2.10) with the ligation reaction.

Previously, *E. coli* XL-1 Blue cells had been used for transformation. These cells were unable to be used at this stage in the work, due to unexplained problems with stock cultures of XL-1 Blue cells. These cells, which do not have ampicillin resistance were able to grow on agar plates containing ampicillin. For some reason, either these cells had been contaminated, or lab stocks of ampicillin were no longer effective.

New stocks of ampicillin were obtained, and a different strain of cells (*E. coli* RR1) were used (Table 2.1). As blue/white selection cannot be used to screen for recombinant clones in these cells, 9 colonies were randomly selected. These were screened using PCR with ATPCL1 and ATPCL2 primers (Section 2.2.4), which would amplify the insert if it was present in the vector. All 9 reactions contained a 202 bp product, indicating that this ligation and transformation had been successful.

Figure 3.13 202 bp PCR product amplified from rat genomic DNA with ATPCL1 and ATPCL2 primers



A 5 µl aliquot of each PCR reaction was analysed by electrophoresis in a 2.5% Nusieve agarose gel containing 1x TAE at 90V for 60 minutes. DNA was stained using ethidium bromide and visualised with a UV transilluminator.

1: BRL 1 kb ladder

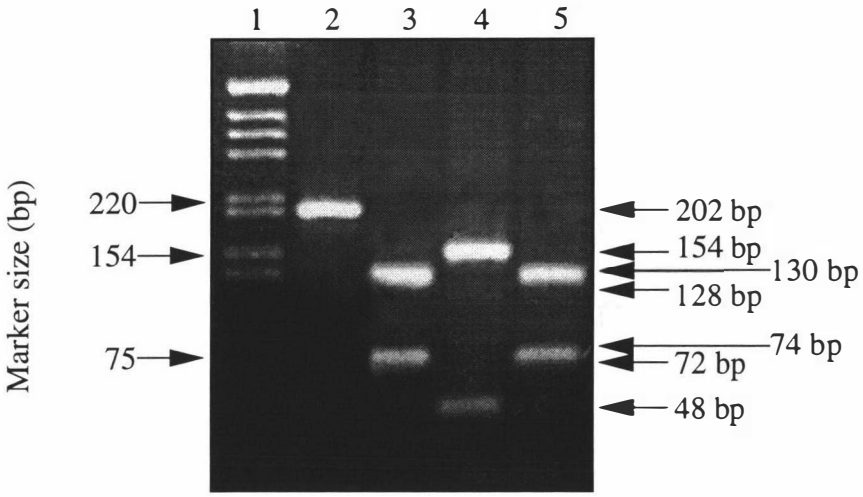
2: Negative control

3: Positive control - 344 bp fragment of factor IX gene promoter insert amplified from pTG3954 using primers 1916 and 1917 and *Taq* polymerase.

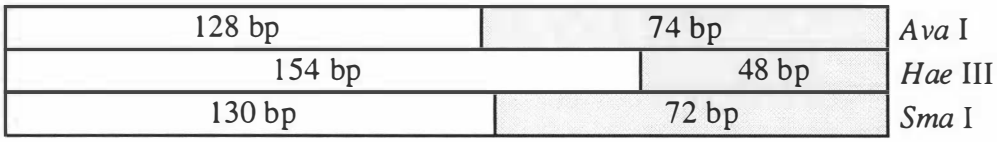
4: 202 bp PCR product amplified from rat genomic DNA using ATPCL1 and ATPCL2 primers and *Taq* polymerase.

Figure 3.14 Diagnostic digest of 202 bp PCR product and schematic representation of expected digest products

A



B



A: Diagnostic digest of 202 bp PCR product

45 ng of 202 bp PCR product was digested and analysed by electrophoresis in a 2.5% Nusieve and 1% agarose gel containing 1x TAE at 100V for 60 minutes. DNA fragments were stained using ethidium bromide and visualised with a UV transilluminator.

1: BRL 1 kb ladder

2: Uncut 202 bp PCR product

3: 202 bp PCR product digested with *Ava* I

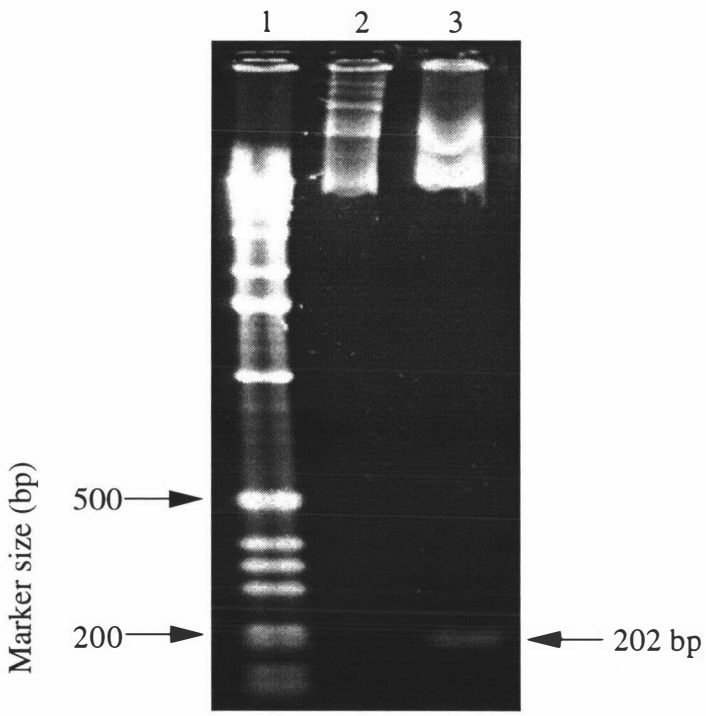
4: " " " " " " *Hae* III

5: " " " " " " *Sma* I

B: Expected digest products of 202 bp PCR product.

Digestion of DNA (Section 2.2.5), prepared from several colonies by the rapid boil method (Section 2.2.11), with the restriction endonuclease *Eco*R I confirmed that two clones contained the 202 bp insert (Figure 3.15). These two clones, named pACL202 (Appendix V), were subjected to DNA sequencing.

Figure 3.15 *Eco*R I digest of miniprep plasmid DNA containing the 202 bp insert



1-2 µg of rapid boil DNA was digested in a total volume of 10 µl, and analysed by electrophoresis in a 2% agarose gel containing 1x TAE at 100V for 60 minutes. DNA was stained using ethidium bromide and visualised with a UV transilluminator.

1: BRL 1 kb ladder

2: Undigested clone 1 miniprep DNA.

3: Clone 1 miniprep DNA digested with *Eco*R I. A 202 bp fragment is indicated.

3.9.2 Sequencing of pACL202 Clones

Two pACL202 clones were sequenced manually on each strand using T3 and T7 primers. Sequencing templates were prepared by PCR using one biotinylated and one non-biotinylated primer (Section 2.2.12). The 202 bp insert was amplified from the clone; the strands of DNA were separated, and then the single strands of DNA were sequenced using Sequenase version 2.0 (Gibco BRL). The resulting sequence confirmed that the two clones contained sequences for rat ATP citrate lyase exon II, both in the same orientation (Appendix V). Confirmation of the sequence of the 202 bp insert enabled the preparation of large amounts of this plasmid, excision of the fragment, and then labelling of this DNA fragment for use in screening of blots and libraries.

E. coli cells containing the pACL202 clone from a 500 ml overnight culture were harvested by centrifugation, and the plasmid DNA extracted using the Wizard Maxiprep DNA Purification System™ (Section 2.2.11). An *Eco*R I digest (Section 2.2.5) on ~14 µg of plasmid DNA was carried out to excise the 202 bp fragment. This fragment was separated from the vector using agarose gel electrophoresis (Section 2.2.5). The insert was purified from the agarose gel using Prep-A-Gene (Section 2.2.6). Once purified in this way, the DNA was resuspended in sterile water, and quantitated by agarose gel electrophoresis (Section 2.2.7). DNA was stored at -20°C until it was required for the subsequent screening of blots and libraries.

3.10 Labelling DNA Probes with ^{32}P

The 202 bp DNA fragment containing sequence for rat ATP citrate lyase exon II was labelled with [α^{32}P]-dCTP (Section 2.2.14) and tested for suitability as a probe in both northern and Southern blots. Two methods were used for labelling DNA, either random primers or Rediprime kit (Table 3.3).

Method	% incorporation	cpm incorporated	cpm/μg	Use
Random Primers	7	2.07×10^6	8.3×10^7	Northern Blotting
Random Primers	8	1.07×10^6	4.30×10^7	Northern Blotting
Random Primers	10	1.09×10^9	4.34×10^{10}	1° Library Screen
Random Primers	26	1.45×10^6	5.78×10^7	2° Library Screen
Random Primers	35	2.97×10^6	1.19×10^8	2° Library Screen Northern & Southern Blotting
Random Primers	31	2.07×10^8	8.26×10^9	3° Library Screen Northern & Southern Blotting
Random Primers	31	5.48×10^8	2.19×10^{10}	2° Library Screen Northern & Southern Blotting
Random Primers	65	2.70×10^7	1.08×10^9	Southern Blotting
RediPrime	70	1.37×10^7	5.49×10^8	Southern Blotting

Table 3.3 Methods used for labelling DNA, and final use of this labelled DNA.

There were also two methods used for removing unincorporated nucleotides from the DNA labelling reaction, NACS PREPAC™ columns and ProbeQuant™ G-50 micro columns. The first of these methods was used only once, as it was found to be very difficult to get a sufficient amount of probe from the column. The ProbeQuant™ G-50 micro columns were very simple to use as well as effective, and so were used for later labelling reactions.

DNA labelled with the random primers system was used to screen northern blots (Section 3.11.1). DNA was also labelled using the Rediprime system, which enables labelling of

DNA in a gel slice; DNA labelled in this way was used for probing Southern blots (Section 3.13.1).

There was a distinct advantage of using the Rediprime random primer labelling kit (Amersham Life Science). DNA requiring labelling is simply excised from an agarose gel, thus removing the need to extract the DNA band from the agarose. This avoided any possible loss of DNA during a cleanup step, and also saved time. DNA samples were found to be labelled to approximately the same extent, and at nearly the same rate as purified DNA.

3.11 Northern Blotting

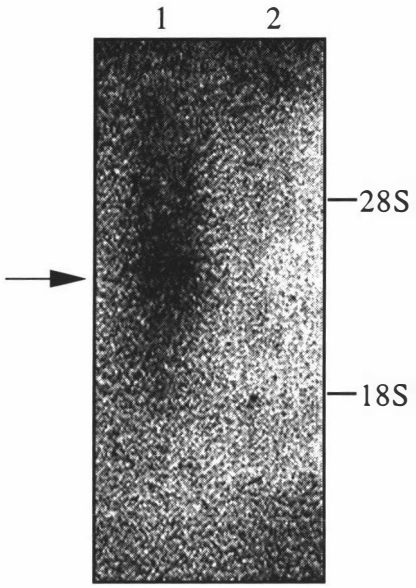
Northern blotting was carried out to test the probe for hybridisation to rat and sheep RNA. The results of this would give some indication as to whether this probe would be useful for screening a library.

It has been shown that ATP citrate lyase enzyme levels in newborn and young ruminants are high, and then drop dramatically upon the development of a fully functional rumen. It would be useful and informative to know whether this drop in enzyme levels is mirrored by a drop in ATP citrate lyase mRNA levels. A probe that could hybridise to ATP citrate lyase mRNA could also be used to analyse the change in levels of ATP citrate lyase mRNA that occur during development in ruminants.

3.11.1 Northern Blot Analysis

Northern blotting was carried out with total RNA from both rat and neonatal lamb liver in order to see if the probe that had been constructed (exon II sequences of rat ATP citrate lyase) would be useful for this type of investigation. These blots were then hybridised with the radioactively labelled probe (Section 2.2.15). Hybridisation occurred between the probe and rat RNA (Figure 3.16), with a smear of radioactivity seen in the lane containing rat RNA. However hybridisation between the probe and lamb RNA was not observed.

Figure 3.16 Results of northern blotting with the 202 bp probe



Nitrocellulose membrane containing samples of rat and lamb total RNA were hybridised to a radiolabelled probe derived from rat ATP citrate lyase exon II sequences, washed at 68°C (conditions for washing are described in Section 2.2.15), and autoradiographed overnight at -70°C.

1: ~10 µg rat RNA.

2: ~10 µg lamb RNA.

This shows that the conditions used enabled hybridisation between the probe and rat RNA, but the hybridisation was not as specific as expected, as a hybridisation signal of a single band should have been seen with rat RNA. In hindsight, an annealing temperature of 68°C may have been too low. Hybridisation of the probe with rat RNA at a higher temperature may have been more specific. However, 68°C may have been too high to detect hybridisation between the probe and lamb RNA, as homology between these two sequences is not known. Alternatively, the quality of the mRNA may not have been high enough to use for this kind of procedure. Problems were also experienced with RT-PCR and lamb mRNA (discussed in Section 3.4.1), so this could explain why hybridisation did not occur between this probe and lamb mRNA. The production of a hybridisation smear with the rat RNA also suggests that some degradation of the sample had occurred, although this was not evident on the agarose gel (Figure 3.2).

There were also problems experienced with producing rat liver cDNA, and RT-PCR with this cDNA was not particularly successful. The same RNA was used for both cDNA synthesis and northern blotting, which could explain why the results seen here were not as expected.

3.12 Screening of Bacteriophage Library

Despite the fact that the probe did not hybridise specifically to total rat RNA, the probe was used to screen a bovine genomic library. Problems experienced with northern blotting could have been attributed to RNA degradation, which may have caused the smear of hybridisation seen. RNA degradation may have also been a factor in the problems experienced with first strand cDNA synthesis and PCR.

The library used for screening (Section 2.2.21) was a Lambda FIX® II Custom Genomic Library (amplified bovine genomic library), supplied by Stratagene. This library had previously been used to isolate clones containing 5' regulatory regions of lactoferrin (Bain, 1995), and so should be useful for obtaining another clone containing ATP citrate lyase 5' regulatory sequences.

3.12.1 Titering Library

Serial dilutions of the phage library were made with SM buffer. The estimated amplified titer was 1.5 x 10^{10} pfu/ml, so several dilutions were made within this range. Plating cells (Section 2.2.22) were added to the different dilutions of phage, and plated onto NZY plates (140 mm). Plates were inverted and left to grow overnight at 37°C. The number of plaques on each plate were counted, and these results were used to calculate the titer of the library (Table 3.4).

Dilution	Volume plated (µl)	Plaques	pfu/ml
10^{-2}	10	confluent	-
10^{-2}	100	confluent	-
10^{-4}	10	confluent	-
10^{-4}	100	confluent	-
10^{-6}	10	334	3.34 x 10^{10}
10^{-6}	100	confluent	-
10^{-8}	10	7	7 x 10^{10}
10^{-8}	100	48	4.8 x 10^{10}
10^{-10}	10	no plaques	-
10^{-10}	100	1	10 x 10^{10}

Table 3.4 Results of titering phage library.

The average titre of this library was ~6 x 10^{10} pfu/ml, slightly higher than estimated by the manufacturer. The difference probably reflects experimental errors involved in dilution and plating; the titre of this library has not decreased during storage at -70°C, and thus is a high quality library.

The number of plaques required for screening was calculated, based on the size of the bovine genome (3 x 10^{9} bp) and the average size of the insert contained in the library (9 kb - 23 kb).

Calculations were as follows:

$$N = \frac{\ln (1 - P)}{\ln (1 - I/G)}$$

where:

N = number of clones needed to be screened

P = probability of having a representative library (99% = 0.99) - probability that target sequence will be screened

I = average size of insert (9 kb - 23 kb)

G = size of the genome (3×10^9 bp)

The number of plaques needed to be screened ranged from 6×10^5 (if the inserts were 23 kb) to 1.5×10^6 plaques (for inserts containing 9 kb). In order to screen a representative number of plaques, 1×10^6 plaques would be required. Therefore the library was plated at a density of ~5×10^4 pfu/plate on 20 plates, 140 mm in diameter.

3.12.2 Plaque Lifts

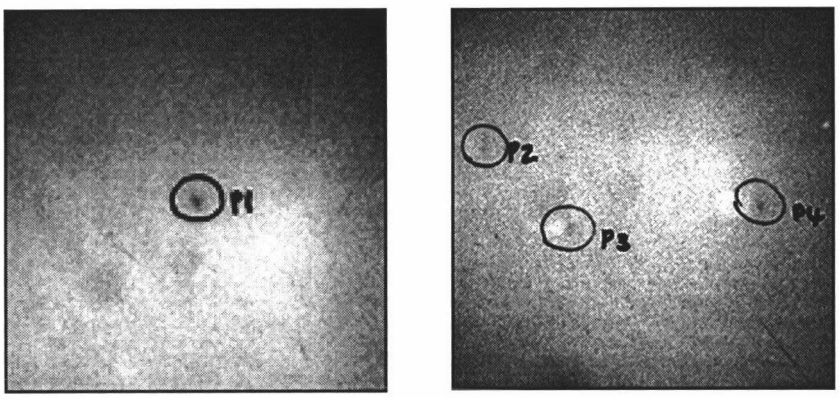
Once the required plaque density had been reached (confluence for first round screening, discrete plaques for subsequent rounds), plates were removed from the 37°C incubator, and left to chill at 4°C. A dry nitrocellulose filter was placed onto the plate and orientation marks were made through the filter and plate while DNA transfer was taking place (Section 2.2.23).

3.12.3 Hybridisation

Filters of plaque lifts were hybridised (Section 2.2.15) with ^{32}P labelled probe (rat exon II sequences), and autoradiographed (Section 2.2.16). After first round screening, four putative plaques were identified (Figure 3.17). These were subjected to a second round of screening; no hybridisation signals were obtained which suggested that none of these four putative plaques contained sequences to which the probe could hybridise. Another attempt at first round screening was also unsuccessful. There are several possibilities which could explain why library screening was not successful.

1 The library being used for screening was an amplified library. The process of amplification of libraries can produce a population of clones that may not necessarily be representative of the genome. This could mean that there were no clones (or not a large enough number) present containing ATP citrate lyase sequence.

Figure 3.17 Four putative plaques identified after first round screening of the bovine genomic library with the 202 bp probe



Autoradiograph of hybridisation filters used in screening the Stratagene bovine genomic library. The library was plated and lifts prepared as described in Section 3.12.2. Filters were hybridised to a radiolabelled probe derived from rat ATP citrate lyase exon II sequences as described in Sections 3.11 and 3.13, washed (60°C, 6x SSC, 1% SDS for 30 minutes; 60°C 2x SSC, 0.1% SDS for 30 minutes), and autoradiographed overnight at -70°C. Plaques are labelled P1 - P4.

Screening of an unamplified library could prove more successful. This library would be more likely to contain a clone with ATP citrate lyase 5' regulatory sequences.

2 The level of homology between rat and bovine ATP citrate lyase exon II sequences is not high enough to allow hybridisation. Therefore, a lower hybridisation temperature could be used.

3 Although the number of plaques required was calculated, perhaps more plaques would have been required. An amplified library was being used, and this may have required a larger number of plaques to be screened.

3.13 Southern Analysis

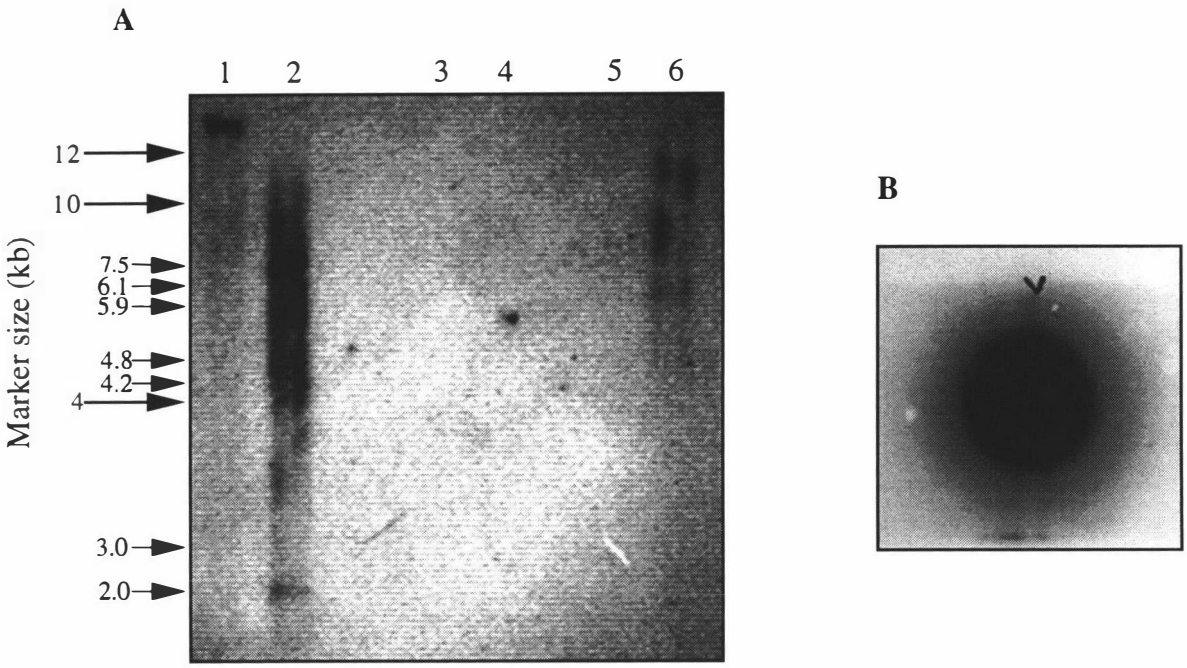
Since the library screening was unsuccessful, the probe was tested for ability to hybridise to genomic DNA using Southern blotting.

Samples of genomic DNA (~20 µg) were digested (Section 2.2.18) with *Eco*R I and checked for completeness of digestion by agarose gel electrophoresis. Samples were concentrated by ethanol precipitation to facilitate loading into the wells in the gel. Electrophoresis was carried out (Section 2.2.19) alongside samples of uncut genomic DNA (Figure 3.12), and then transferred onto nitrocellulose membrane (Section 2.2.20) by the process of Southern transfer (Southern, 1975). This membrane was then hybridised with ^{32}P labelled DNA (202 bp product) (Section 2.2.15).

3.13.1 Probing Southern Blot

Radioactively labelled DNA containing sequences for exon II of rat ATP citrate lyase was used to probe a Southern blot containing undigested and digested samples of rat, bovine and human genomic DNA. Autoradiography revealed several hybridisation signals (Figure 3.18). The positive control was included to show that the probe was able to hybridise to itself (Figure 3.18B); an aliquot of unlabelled probe (~100 ng) was spotted onto a piece of nitrocellulose, exposed to UV light to cross-link it onto the filter, and then treated in the same way as the blot.

Figure 3.18 Hybridisation of Southern blot with 202 bp probe



A: Southern Blot

Digested (~25 ng) and undigested (~10 ng) genomic DNA samples were separated by electrophoresis in a 0.7% agarose gel (Figure 3.12) and transferred to nitrocellulose membrane. The membrane was hybridised to a radiolabelled probe derived from rat ATP citrate lyase exon II sequences, washed (60°C, 6x SSC, 1% SDS for 30 minutes; 60°C 2x SSC, 0.1% SDS for 30 minutes), and autoradiographed overnight at -70°C.

1: Undigested rat genomic DNA

2: Digested rat genomic DNA

3: Undigested bovine genomic DNA

4: Digested bovine genomic DNA

5: Undigested human genomic DNA

6: Digested human genomic DNA

B: Positive control

An aliquot of unlabelled probe (~100 ng) was spotted onto nitrocellulose and treated in the same way as the Southern blot.

A hybridisation signal was detected in both lanes containing rat genomic DNA (Figure 3.18A). In the uncut rat genomic DNA lane, there was a band seen at the level corresponding to high molecular weight DNA (>12 kb). This indicated that the probe was able to hybridise to rat genomic DNA, which was expected. In the lane containing digested genomic DNA, there are several bands that can be seen (7.5 kb, 6.1 kb, 5.9 kb, 4.8 kb, 4.2 kb, 3.0 kb, and 2.2 kb), within a smear between 10 and 4 kb. The presence of these multiple bands suggests that the genomic DNA was incompletely digested.

There was no hybridisation observed between the probe and bovine genomic DNA, which indicates that perhaps the homology in this region (exon II) between bovine and rat ATP citrate lyase sequences is not as high as that between rat and human ATP citrate lyase.

A hybridisation signal could be seen in the lane containing digested human genomic DNA, with a smear between 12 and 6 kb, and a band at 7 kb. This confirms the high level of homology between rat and human ATP citrate lyase sequences.

A lack of discrete bands with digested genomic DNA suggests either incomplete digestion or low stringency. Considering that the hybridisation temperature was 68°C, which may have been too high to allow hybridisation with sequences less than 100% homologous, the reason for the smear of hybridisation seen is more likely to be due to incomplete digestion.

These results show that the conditions used allowed a degree of hybridisation between the rat probe and genomic DNA. Therefore, this probe was able to anneal to complementary sequences of genomic DNA.

Tm is the melting temperature of a perfect hybrid to a target sequence. Hybridisation should be carried out at 25°C lower than the Tm (Sambrook *et al.*, 1989). The Tm was calculated as follows:

$$Tm = 16.6\log[Na^+] + 0.41(\%GC) + 81.5 - 0.65(\%\ \text{formamide}) - \frac{500}{\text{bp in duplex}}$$

For the reaction conditions: $[Na^+] = 0.05M$

$\%GC = 55\%$

bp in duplex = 182 bp

% formamide = 0%

$$Tm = 16.6\log(0.05) + (0.41)(55) + 81.5 - \frac{500}{182}$$

$$= 80$$

Therefore a hybridisation temperature closer to 55°C should have been used. Also, if the homology between the probe and target sequences is less that 70%, then it is unlikely that the probe will detect these sequences. This could well be the case, and while the homology between rat and bovine ATP citrate lyase sequence is unknown, these results suggest that the homology between these two sequences is likely to be low.

The hybridisation signals seen were very weak, which could have been due to the hybridisation temperature of 68°C being too high. There was also a faint smear of hybridisation in the lane containing digested human genomic DNA. The probe was expected to anneal to human genomic DNA, due to the high level of homology between rat and human ATP citrate lyase sequences. More specific hybridisation signals may have been observed if hybridisation conditions were optimised. All hybridisation was carried out at 68°C, except for the last hybridisation reaction, which was carried out at 60°C. This lower temperature was the only time when hybridisation was seen. Also, the temperature for washing of the blot was 60°C, which was also lowered from 68°C. If this problem had been addressed earlier in the project, then perhaps more success would have been obtained with northern and Southern blotting. While these results show that hybridisation does occur between the probe and both rat and human genomic DNA, hybridisation conditions need to be optimised. Time constraints meant that this option could not be explored.

3.14 Summary

This work has produced a probe for rat ATP citrate lyase which has been shown to hybridise to both rat and human genomic DNA. Many difficulties were experienced initially, involving PCR amplification to prepare a probe, such as reproducibility of the reaction. Sequencing of the 282 bp PCR product showed that it was not the expected sequence, which to some extent explains the difficulty experienced in PCR. Once new primers were obtained, a reproducible PCR product was easily produced, and this was sequenced to show that it was authentic. In contrast, few problems were encountered with PCR using the new primers, and only a minimal amount of optimising of PCR conditions was required. This highlighted the need for careful design of PCR primers, of which length seemed to be an important factor in this case. Of the first set of primers, ATPCL5' and ATPCL3' exon III were fairly short in length (22-mer and 21-mer), with both the primers containing one mismatch each, which may have been enough to compromise the ability of these primers to anneal to specific sequences. Also, the length of the product that was being amplified with ATPCL5' and ATPCL3' was 3.3 kb. This is a reasonably large stretch of DNA to try and amplify by standard PCR conditions, which may explain why problems were experienced in producing this product.

The new primers (ATPCL1 and ATPCL2) were designed as 25-mer perfect matches to the rat cDNA sequences, and as such did not contain any redundancies. Digestion of the PCR product obtained with the new primers gave the correct digestion pattern; sequencing confirmed that it represented exon II of the rat ATP citrate lyase gene. This PCR product was successfully cloned and sequenced, and was then used for northern and Southern blotting, and for screening an amplified bovine genomic library.

Results of northern blotting showed that this probe hybridised with rat RNA, but not with lamb RNA. Hybridisation with rat RNA produced a smear, which was not as expected. A single band should have been observed. The hybridisation temperature used was 68°C, which may have been sub-optimal; specific hybridisation may have been inhibited. Also, the quality of mRNA may not have been high enough to use for this type of procedure, and problems were also experienced when this RNA was used for RT-PCR. No hybridisation was observed with lamb mRNA, but this could also have been due to hybridisation temperature and/or degradation of RNA.

The results of Southern blotting showed that no hybridisation signal was seen between the probe and bovine genomic DNA; hybridisation occurred between the probe and both rat genomic DNA and human genomic DNA.. However, the hybridisation signals seen were not specific, and could be due to incomplete digestion of genomic DNA. The hybridisation temperature that may have also been too high.

Screening of the amplified bovine genomic library was unsuccessful, and there were many factors which may have contributed to this. The use of an amplified, as opposed to an unamplified library could mean that there were no clones containing ATP citrate lyase 5' regulatory sequences present. The level of homology between rat and bovine ATP citrate lyase exon II sequences may not be high enough to allow hybridisation. Finally, because an amplified library was used, it is possible that more plaques should have been screened.

Once conditions for hybridisation of the probe and bovine genomic DNA are optimised, then this probe should be useful for isolating a bovine genomic clone containing 5' sequences for ATP citrate lyase.

CHAPTER FOUR: FUTURE DIRECTIONS

The results of this project leave many possibilities for future work. At this stage, a probe containing exon II sequences of rat ATP citrate lyase has been prepared. This has been shown to hybridise to both rat and human genomic DNA, but not to bovine genomic DNA or lamb liver RNA. However, this probe may still be useful, provided that the conditions for hybridisation are optimised to ensure significant binding of the probe to bovine genomic DNA.

4.1 Optimisation of Hybridisation Conditions

The probe that has been constructed is able to hybridise to rat and human genomic DNA, however the hybridisation signal seen was a non-specific smear (Section 3.13.1). Therefore, the conditions for Southern blotting using rat and human genomic DNA should be optimised before investigating binding to sheep or bovine genomic DNA. The problems encountered with northern blotting may have been due to sub-optimal hybridisation conditions, or because neonatal lamb liver may not contain detectable quantities of ATP citrate lyase message. Screening of the Southern blots with the probe would need to be repeated, and conditions for hybridisation should be optimised. The most sensitive parameter for hybridisation of nucleic acids is temperature. At a much lower hybridisation temperature, the stringency is lower, and therefore this would increase the chance of the probe annealing to similar sequences.

If a hybridisation signal is successfully obtained at a low temperature, the annealing temperature could be gradually increased, so as to get a more specific hybridisation signal. If the hybridisation temperature is lowered, and this does not increase the specificity of hybridisation sufficiently, then other factors affecting hybridisation can be altered, such as formamide and salt concentration. Adjusting the concentration of these will affect the Tm, which is the melting temperature of a perfect hybrid to a target sequence. Hybridisation is normally carried out at 25°C lower than the Tm. An increase in the Tm will increase the specificity at the same hybridisation temperature. A decrease in the Tm will decrease the specificity at the same hybridisation temperature.

For example, if the % formamide is increased to 20% and 50%, then the calculation of Tm is as follows (while keeping the other conditions the same as described in Section 3.13.1):

$$Tm = 16.6\log[Na^+] + 0.41(\%GC) + 81.5 - 0.65(\%\ \text{formamide}) - \frac{500}{\text{bp in duplex}}$$

$$Tm = 16.6\log(0.05) + (0.41)(55) + 81.5 - (0.65)(20) - \frac{500}{182}$$

$$Tm = 67$$

If the % formamide is incerased further to 50%, then the Tm is as follows:

$$Tm = 16.6\log(0.05) + (0.41)(55) + 81.5 - (0.65)(50) - \frac{500}{182}$$

$$Tm = 47$$

By increasing the % formamide, the Tm is decreased. Therefore, hybridisation at the same temperature will be more specific under these conditions.

If the salt concentration is increased, the opposite effect is seen. By increasing the salt concentration from 0.05M to 0.5M, the Tm is increased as follows:

$$Tm = 16.6\log(0.5) + (0.41)(55) + 81.5 - \frac{500}{182}$$

$$Tm = 96$$

By increasing the salt concentration, the specificity of hybridisation is decreased, thus allowing mismatches to occur.

Once the conditions for hybridisation have been optimised for rat and human genomic DNA, then screening of a sheep and/or bovine genomic library will be possible. These sequences may have a low level of homology, and so less stringent conditions will need to be used, due to the lower specificity of the probe to the target sequences.

4.2 Screening a Bovine Genomic Library

Once a probe has been identified that specifically hybridises to bovine genomic DNA, the bovine genomic library could be rescreened using the optimised hybridisation conditions.

There are two options concerning the type of library to use for screening. Firstly, an amplified bovine genomic library can be screened. If this proves unsuccessful, then the next option would be to try and screen an unamplified bovine genomic library. Once a bovine genomic clone containing the 5' regulatory sequences for ATP citrate lyase is isolated, it will be sequenced to confirm that it contains sequences corresponding to the 5'-regulatory region. This sequence could be used to compare rat and human ATP citrate lyase sequences, to identify any differences or similarities between species. Also, sequence comparisons with regulatory elements could be carried out, to identify any elements present that could contribute to the unique regulation of ATP citrate lyase seen in ruminants.

Secondly, this clone could be used to screen a sheep genomic library. There should be more success in screening a sheep library with a probe obtained from a bovine clone compared to rat; there should be a greater degree of homology between sheep and bovine sequences than between rat and bovine sequences.

4.3 Obtaining a Sheep cDNA Probe

If lowering the hybridisation temperature, and screening the bovine genomic library with a rat probe is not successful, then another approach would need to be used. The rat probe could be used to screen a sheep liver cDNA library, at low stringency. The probe was constructed from exonic sequences within the rat ATP citrate lyase gene, so this would still be useful for screening a cDNA library. Screening a cDNA library with a probe containing exon II sequence provides the opportunity to isolate sequences in the 5' untranslated region. This would be a more useful probe with which to screen a genomic library; it would be more likely that this approach would detect a clone containing the 5' regulatory region.

As the calculations show below, screening of a cDNA library will require fewer plaques to be screened, because of the absence of intron sequences. The number of plaques needed to be screened, based on the size of the cDNA (4.3 kb), and the average size of the insert contained in the library (~1 kb for an unsized cDNA library) is as follows:

$$N = \frac{\ln (1 - P)}{\ln (1 - I/G)}$$

where: N = number of clones needed to be screened

P = probability of having a representative library (99% = 0.99)

I = average size of insert (1 kb)

G = size of the genome. In this case, the size of the cDNA (4.3 kb)

Based on this relationship 1.98×10^4 plaques would need to be screened. This is a significant decrease to the 1×10^6 plaques that were required when screening the amplified bovine genomic library. The library could be plated at a density of $\sim 2 \times 10^4$ pfu/plate on 140 mm plates, and only 1 plate would need to be used. By screening fewer plaques, there would be less time spent on screening, and it would be more manageable dealing with only 1 filter of plaque lifts as opposed to 20. Also, a cDNA probe could be used to study mRNA expression over time without the need for 5'-regulatory regions.

This clone could then be used to screen the sheep genomic library - and the level of identity (ie. 100%) would mean that obtaining a clone should be fairly straightforward, and that fairly stringent conditions could be used.

4.4 Sheep Genomic Clone

A sheep genomic DNA library could be screened with a suitable probe, to obtain 5' regulatory sequences of sheep ATP citrate lyase.

4.4.1 ATP Citrate Lyase Expression During Development

The clone obtained from screening a sheep genomic library could be used to construct a probe containing a portion of the coding region of the ATP citrate lyase gene. This probe could then be used to screen blots of total RNA (northern blotting), extracted from sheep livers at different stages of development. As ATP citrate lyase is regulated at the level of transcription, the levels of ATP citrate lyase RNA will reflect the rate of transcription. This type of analysis can be used to determine the developmental pattern of ATP citrate

lyase gene expression. Also, the level of expression will be shown by the intensity of hybridisation. Ribonuclease protection assays could also be used to monitor RNA levels, as this type of analysis is more sensitive than northern blotting.

This information will be useful in understanding the regulation of enzymes involved in lipogenesis in ruminants, and expression of these enzymes during development and the transition from monogastric to ruminant digestion.

4.4.2 Characterisation of Sheep Genomic Clone

Initially it is likely that several clones would be isolated, and these would need to be sequenced in order to identify one which contains the 5' flanking sequences of ATP citrate lyase. This clone will then be characterised further, by restriction analysis and DNA sequencing. These sequences will be compared to both the corresponding regions of rat and human (and possibly bovine) ATP citrate lyase promoter sequences, and also the GenBank database of transcription factor consensus sites. Results of these analyses will shed light on the possible regulatory elements involved in ATP citrate lyase transcription, and also any differences in the regulatory elements present in the promoters of ruminant and monogastric animals.

Further analyses could also be carried out, such as the identification of the transcription start site by primer extension analysis and/or S1 nuclease protection analysis of the poly A^+ RNA isolated from ruminant tissues.

The 5' flanking sequences could also be analysed for promoter activity and potential DNA binding protein sequences. Promoter activity could be measured using reporter gene assays by cloning fragments of the 5' flanking sequences into luciferase reporter gene plasmids, and assaying for transcriptional activity.

Potential DNA binding protein sequences can be identified firstly by sequence analysis, and then confirmed using DNAse I footprinting and electrophoretic mobility shift assays.

In summary, screening a genomic library for the 5' regulatory region of sheep or bovine ATP citrate lyase could be carried out using standard methods once a suitable probe has

been identified. The promoter region can be characterised by a number of conventional methods to define the minimal promoter and putative regulatory elements. This analysis will provide new insights into the molecular mechanisms that are responsible for the regulation of ATP citrate lyase in ruminants, the role this regulation plays in the glucose-sparing strategy of ruminants, and putative transcription factors involved in modulating fatty acid synthesis.

REFERENCES

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215** 403-410

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1989) *Current Protocols in Molecular Biology* Greene Publishing Associates and Wiley Intersciences N.Y.

Bai, Y., Zhang, S., Kim, K. S., Lee, J. K. and Kim, K. H. (1996) Obese gene expression alters the ability of 30A5 preadipocytes to respond to lipogenic hormones. *Journal of Biological Chemistry* **271** (24) 13939-13942

Bain, H. B. (1995) Isolation and characterisation of the 5' region of the bovine lactoferrin gene. *PhD Thesis, Massey University, Plamerston North, New Zealand*

Baldwin, R.L. and Smith, N.E. (1971) Intermediary aspects and tissue interactions of ruminant fat metabolism. *Journal of Dairy Science* **54** (4) 583-595

Baldwin, R.L., Ronning, M., Radanovics, C. and Plange, G. (1966) Effect of carbohydrate and fat intakes upon the activities of several liver enzymes in rats, guinea pigs, piglets and calves. *Journal of Nutrition* **90** (1) 47-55

Ballard, F.J., Filsell, O.H. and Jarrett, I.G. (1972) Effects of carbohydrate availability on lipogenesis in sheep. *Biochemical Journal* **226** 193-200

Bauman, D. E. (1976) Intermediary metabolism of adipose tissue. *Federation Procedings* **35** (11) 2308-2313

Benjamin, W.B., Pentyala, S.N., Woodgett, J.R., Hod, Y. and Marshak, D. (1994) ATP citrate lyase and glycogen synthase kinase-3β in 3T3-L1 cells during differentiation into adipocytes. *Biochemical Journal* **300** 477-482

Cheema, S. K. and Clandinin, M. T. (1996) Diet fat alters expression of genes for enzymes of lipogenesis in lean and obese mice. *Biochimica et Biophysica Acta* **1299** 284-288

Clandinin, M. T., Cheema, S., Pehowich, D. and Field, C. J. (1996) Effect of polyunsaturated fatty acids in obese mice. *Lipids* **31** S13-S22

Daniel, S. and Kim, K.H. (1996) Sp1 mediates glucose activation of the acetyl-CoA carboxylase promoter. *Journal of Biological Chemistry* **271** (3) 1385-1392

Elshourbagy, N.A., Near, J.C., Kmetz, P.J., Sathe, G.M., Southan, C., Strickler, J.E., Gross, M., Young, J.F., Wells, T.N.C. and Groot, P.H.E. (1990) Rat ATP citrate-lyase: molecular cloning and sequence analysis of a full-length cDNA and mRNA abundance as a function of diet, organ,and age. *Journal of Biological Chemistry* **265** (3) 1430-1435

Elshourbagy, N.A., Near, J.C., Kmetz, P.J., Wells, T.N.C., Groot, P.H.E., Saxty, B.A., Hughes, S.A., Franklin, M. and Gloger, I.S. (1992) Cloning and expression of a human ATP-citrate lyase cDNA. *European Journal of Biochemistry* **204** 491-499

Erlich, H. A. (1991) PCR technology: *Principles and applications for DNA amplification.* W. H. Freeman and Company, New York

Fukuda, H., Iritani, N., Katsurada, A. and Noguchi, T. (1996) Insulin- and polyunsaturated fatty acid-responsive region(s) of rat ATP citrate-lyase gene promoter. *FEBS Letters* **380** 204-207

Fukuda, H., Iritani, N. and Noguchi, T. (1997) Transcriptional regulatory region for expression of the rat ATP citrate lyase gene. *European Journal of Biochemistry* **247** 497-502

Ghilardi, N., Ziegler, S., Wiestner, A., Stoffel, R., Heim, M. H. and Skoda, R. C. (1996) Defective STAT signalling by the leptin receptor in diabetic mice. *Proceedings of the National Academy of Sciences (USA)* **93** 6231-6235

Halperin, M. L., Cheema-Dhaldi, S., Taylor, W. M. and Fritz, I. B. (1975) Role of the citrate transporter in the control of fatty acid synthesis. *Advances in Enzyme Regulation* **13** 435-445

Hanson, R. W. and Ballard, F. J. (1967) The relative significance of acetate and glucose as precursors for lipid synthesis in liver and adipose tissue from ruminants. *Biochemical Journal* **105** 529-536

Hillgartner, F. B., Charron, T. and Chestnut, K. A. (1996) Alterations in nutritional status regulate acetyl CoA carboxylase expression in avian liver by a transcriptional mechanism. *Biochemical Journal* **319** 263-268

Holmes, D. S. and Quigley, M. (1981) A rapid boil method for the preparation of bacterial plasmids. *Analytical Biochemistry* **114** (1) 193-197

Houston, B. and Nimmo, H.G. (1984) Purification and some kinetic properties of rat liver ATP citrate lyase. *Biochemical Journal* **224** 437-443

Howarth, R.E., Baldwin, R.L. and Ronning, M. (1968) Enzyme activities in liver, muscle, and adipose tissue of calves and steers. *Journal of Dairy Science* **51** (8) 1270-1274

Inoue, H., Nojima, H. and Okayama, H. (1990) High efficiency transformation of *Escherischia coli* with plasmids. *Gene* **96** (1) 23-28

Judson, G.J. and Leng, R.A. (1973) Studies on the control of gluconeogenesis in sheep: effect of glucose infusion. *British Journal of Nutrition* **29** 159-174

Kim, K. H. (1983) Regulation of acetyl-CoA carboxylase. *Current Topics in Cellular Regulation* **22** 143-176

Kim, K.S., Park, S.W., Moon, Y.A. and Kim, Y.S. (1994) Organisation of the 5' region of the rat ATP citrate lyase gene. *Biochemical Journal* **302** 759-764

Kim, T. S. and Freake, H. C. (1996) High carbohydrate diet and starvation regulate lipogenic mRNA in rats in a tissue-specific manner. *Journal of Nutrition* **126** 611-617

Kornacker, M. S. and Lowenstein, J. M. (1965) Citrate and the conversion of carbohydrate into fat: the activities of citrate-cleavage enzyme and acetate thiokinase in livers of starved and refed rats. *Biochemical Journal* **94** 209-215

Leng, R. A. (1970) Glucose synthesis in ruminants. *Advances in Veterinary Science and Comparative Medicine* **14** 209-260

Lewin, B. (1994) ed. *Genes V* Chapter 29 Oxford University Press

Linn, T. C. and Srere P. A . (1979) Identification of ATP citrate lyase as a phosphoprotein. *Journal of Biological Chemistry* **254** (5) 1691-1698

Lord, K.A., Wang, X. M., Simmons, S. J., Bruckner, R. C., Loscig, J., O'Connor, B., Bentley, R., Smallwood, A., Chadwick, C. C., Stevis, P. E. and Ciccarelli, R. B. (1997) Variant cDNA sequences of human ATP citrate lyase: cloning, expression, and purification from baculovirus-infected insect cells. *Protein Expression and Purification* **9** 133-141

Moon, Y.A., Kim, K.S., Park, S.W. and Kim, Y.S. (1996) Cloning and identification of exon-intron organization of the rat ATP-citrate lyase gene. *Biochimica et Biophysica Acta* **1307** 280-284

Muramatsu, M., Ambo, K. and Tsuda, T. (1970) ATP citrate lyase activity in the liver of newborn lambs. *Journal of Biochemistry* **67** (5) 727-729

Pentyala, S. N. and Benjamin, W. B. (1995) Effect of oxaloacetate and phosphorylation on ATP citrate lyase activity. *Biochemistry* **34** (35) 10961-10969

Roehrig, K., Nestor Jr, K.E. and Palmquist, D.L. (1988) ATP citrate lyase activity in liver and adipose tissue of veal or ruminating calves (*Bos taurus*). *Comparitive Biochemistry and Physiology* **90**B (1) 147-149

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Second Edition, Cold Spring Harbour Laboratory Press.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences (USA)* **87** 4509-4513

Shimomura, I., Shimano, H., Horton, J. D., Goldstein, J. L. and Brown, M. S. (1997) Differential expression of exons 1a and 1c in mRNAs for sterol regulatory element binding protein-1 in human and mouse organs and cultured cells. *Journal of Clinical Investigation* **99** (5) 838-845

Singh, M., Richards, E. G., Mukherjee, A. and Srere, P. A. (1976) Structure of ATP citrate lyase from rat liver. Physicochemical studies and proteolytic modification. *Journal of Biological Chemistry* **251** (17) 5242-5250 1976

Southern, E. M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98** (3) 503-517

Spencer, A. F. and Lowenstein, J. M. (1962) The supply of precursors for the synthesis of fatty acids. *Journal of Biological Chemistry* **237** (12) 3640-3648

Spiegelman, B. M. and Flier, J. S. (1996) Adipogenesis and obesity: rounding out the big picture. *Cell* **87** 377-389

Srere, P. A. (1959) The citrate cleavage enzyme: distribution and purification. *Journal of Biological Chemistry* **234** 2544-2547

Wagner, P. D. and Vu, N. D. (1995) Phosphorylation of ATP citrate lyase by nucleotide diphosphate kinase. *Journal of Biological Chemistry* **270** (37) 21758-21764

Wang, X., Sato, R., Brown, M. S., Hua, X. and Goldstein, J. L. (1994) SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell* **77** (1) 53-62

Wise, L.S., Sul, H. S. and Rubin, C. S. (1984) Coordinate regulation of the biosynthesis of ATP citrate lyase and malic enzyme during adipocyte diferentiation: studies on 3T3-L1 cells. *Journal of Biological Chemistry* **259** (8) 4827-4832

Xu, Z.X., Stenzel, W., Sasic, S.M., Smart, D.A. and Rooney, S.A. (1993) Glucocorticoid regulation of fatty acid synthase gene expression in fetal rat lung. *American Journal of Physiology* **265** L140-L147

Appendix I

Oligonucleotide Sequences

DNA sequence 5' → 3'

ATPCL5' : ATC TCT AGA TCR GCC AAG GCA ATT TAG

ATPCL3' : CAG TCG ACA TCG ATG CTC KGT TAC ATG CTC ATG TGT TCC GG

ATPCL3'

exon III : GCG TCG ACG GTG GCC TCA TGT CCC AGT CG

ATPCL1 : GCG CGA ATT CGT AAA CCA GGT CCC TCT GCA GCC AT

ATPCL2 : GCG CGA ATT CCT GGC TAA GCA GCC AGG GGT GGT CC

1916 : GTG CTG CCA CAG TAA ATG TA

1917 : TGA TGA GGC CTG GTG ATT CT

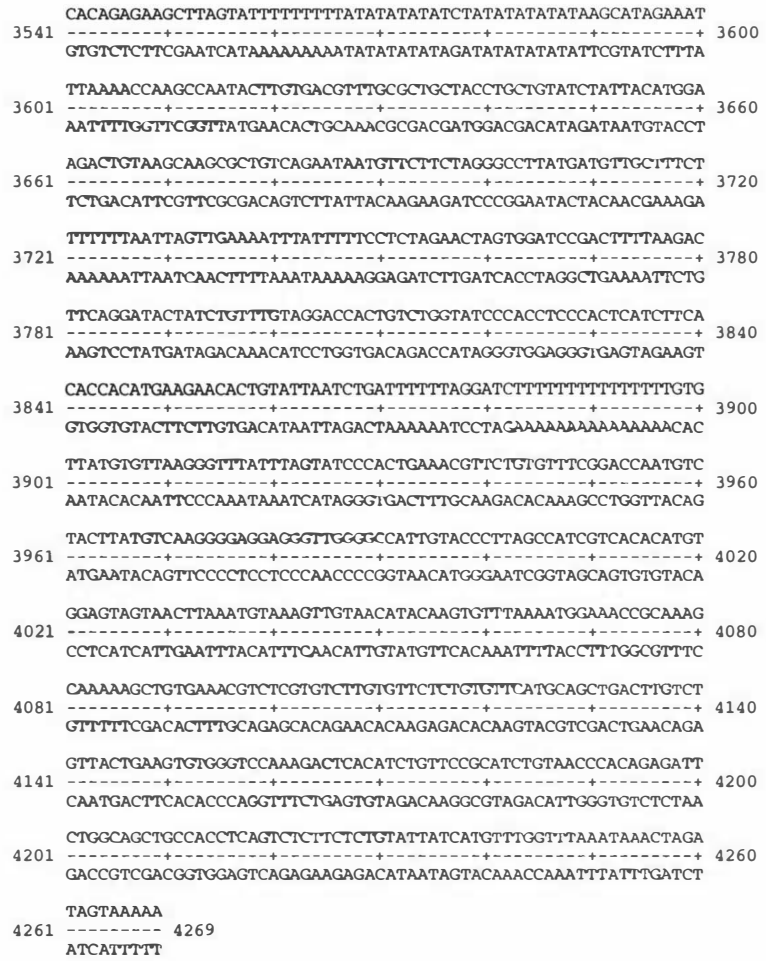
Appendix II

Rat ATP Citrate Lyase mRNA Sequence

Primer locations are indicated by arrows


(Linear) MAP of: j05210.gb_ro check: 6216 from: 1 to: 4269
LOCUS RATCLATP 4269 bp mRNA ROD 18-AUG-1995
DEFINITION Rat ATP citrate-lyase mRNA, complete cds.
ACCESSION J05210
NID g949989
KEYWORDS .
SOURCE rat. . . .
ATPCL1
ATPCL5'
ATPCL2
ATPCL3' exon III

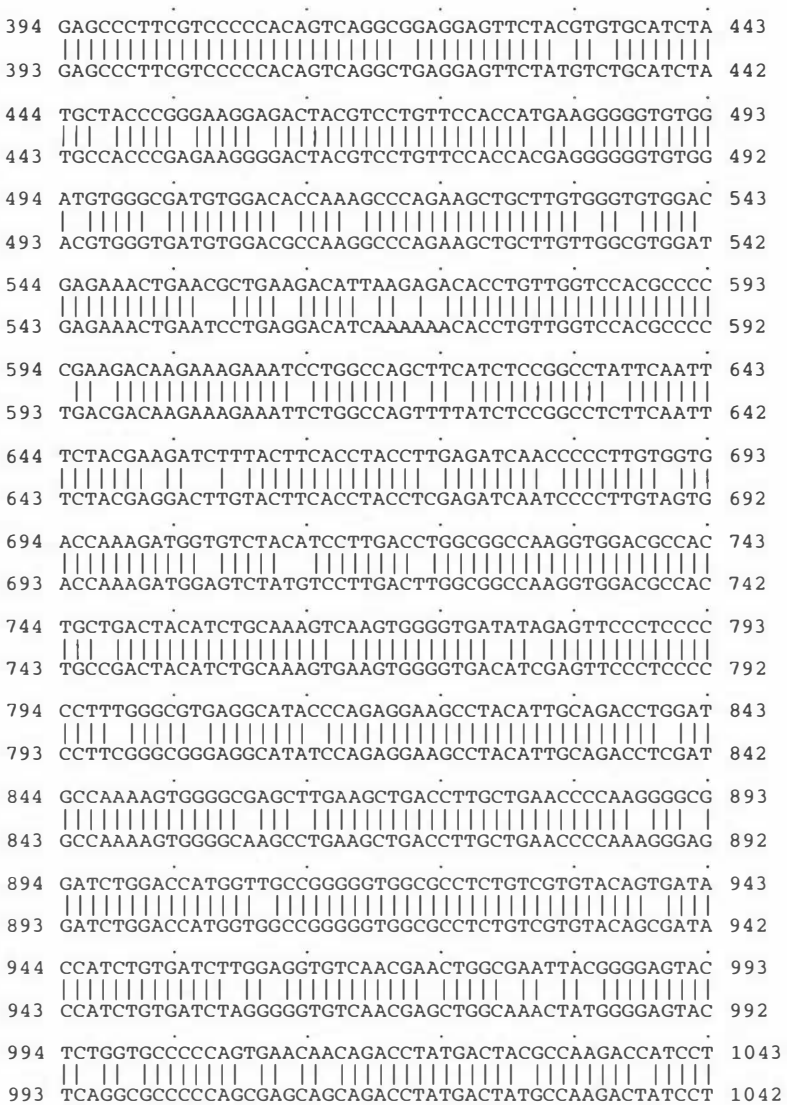

ATPCL3'

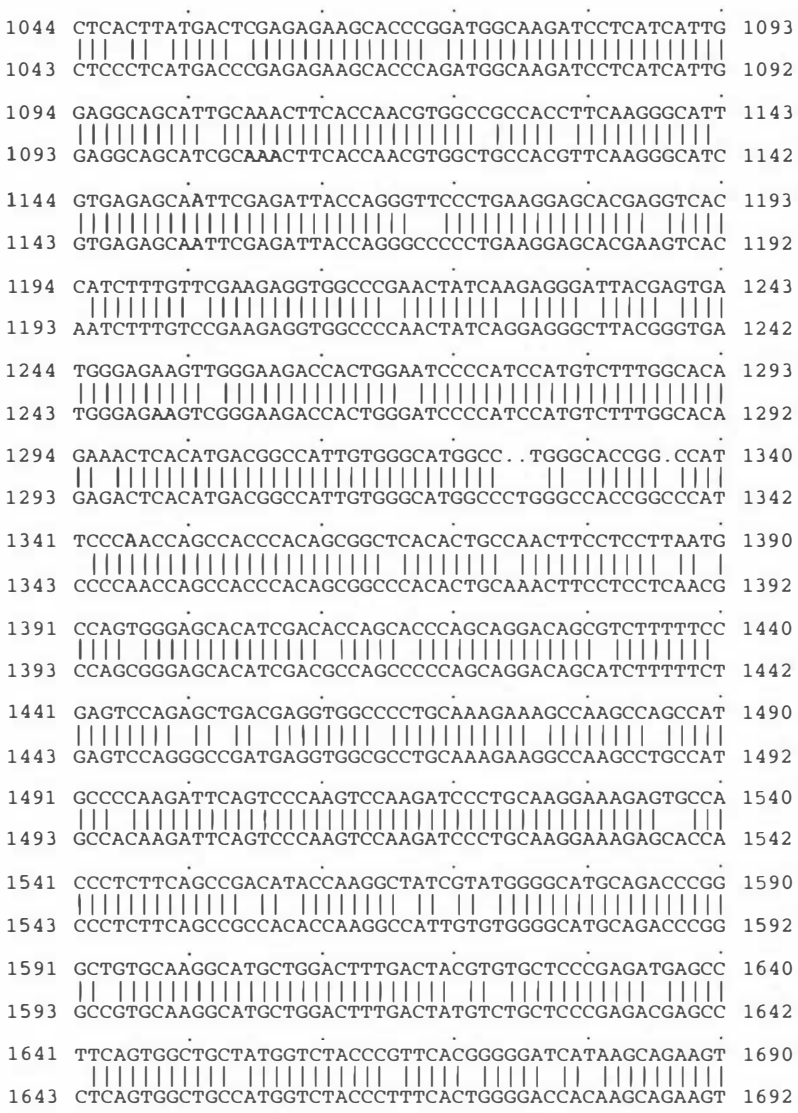

CACAGAGAAGCTTAGTATTTTTTTTTATATATATATCTATATATATATAAGCATAGAAAT
3541 ---------+---------+---------+---------+---------+---------+ 3600
GTGTCTCTTCGAATCATAAAAAAAAATATATATATAGATATATATATATTCGTATCTTTA
TTAAAACCAAGCCAATACTTGTGACGTTTGCGCTGCTACCTGCTGTATCTATTACATGGA
3601 ---------+---------+---------+---------+---------+---------+ 3660
AATTTTGGTTCGGTTATGAACACTGCAAACGCGACGATGGACGACATAGATAATGTACCT
AGACTGTAAGCAAGCGCTGTCAGAATAATGTTCTTCTAGGGCCTTATGATGTTGCTTTCT
3661 ---------+---------+---------+---------+---------+---------+ 3720
TCTGACATTCGTTCGCGACAGTCTTATTACAAGAAGATCCCGGAATACTACAACGAAAGA
TTTTTTAATTAGTTGAAAATTTATTTTTCCTCTAGAACTAGTGGATCCGACTTTTAAGAC
3721 ---------+---------+---------+---------+---------+---------+ 3780
AAAAAATTAATCAACTTTTAAATAAAAAGGAGATCTTGATCACCTAGGCTGAAAATTCTG
TTCAGGATACTATCTGTTTGTAGGACCACTGTCTGGTATCCCACCTCCCACTCATCTTCA
3781 ---------+---------+---------+---------+---------+---------+ 3840
AAGTCCTATGATAGACAAACATCCTGGTGACAGACCATAGGGTGGAGGGTGAGTAGAAGT
CACCACATGAAGAACACTGTATTAATCTGATTTTTTAGGATCTTTTTTTTTTTTTTTGTG
3841 ---------+---------+---------+---------+---------+---------+ 3900
GTGGTGTACTTCTTGTGACATAATTAGACTAAAAAATCCTAGAAAAAAAAAAAAAAACAC
TTATGTGTTAAGGGTTTATTTAGTATCCCACTGAAACGTTCTGTGTTTCGGACCAATGTC
3901 ---------+---------+---------+---------+---------+---------+ 3960
AATACACAATTCCCAAATAAATCATAGGGTGACTTTGCAAGACACAAAGCCTGGTTACAG
TACTTATGTCAAGGGGAGGAGGGTTGGGCCATTGTACCCTTAGCCATCGTCACACATGT
3961 ---------+---------+---------+---------+---------+---------+ 4020
ATGAATACAGTTCCCCTCCTCCCAACCCCGGTAACATGGGAATCGGTAGCAGTGTGTACA
GGAGTAGTAACTTAAATGTAAAGTTGTAACATACAAGTGTTTAAAATGGAAACCGCAAAG
4021 ---------+---------+---------+---------+---------+---------+ 4080
CCTCATCATTGAATTTACATTTCAACATTGTATGTTCACAAATTTTACCTTTGGCGTTTC
CAAAAAGCTGTGAAACGTCTCGTGTCTTGTGTTCTCTGTGTTCATGCAGCTGACTTGTCT
4081 ---------+---------+---------+---------+---------+---------+ 4140
GTTTTTCGACACTTTGCAGAGCACAGAACACAAGAGACACAAGTACGTCGACTGAACAGA
GTTACTGAAGTGTGGGTCCAAAGACTCACATCTGTTCCGCATCTGTAACCCACAGAGATT
4141 ---------+---------+---------+---------+---------+---------+ 4200
CAATGACTTCACACCCAGGTTTCTGAGTGTAGACAAGGCGTAGACATTGGGTGTCTCTAA
CTGGCAGCTGCCACCTCAGTCTCTTCTCTGTATTATCATGTTTGGTTTAAATAAACTAGA
4201 ---------+---------+---------+---------+---------+---------+ 4260
GACCGTCGACGGTGGAGTCAGAGAAGAGACATAATAGTACAAACCAAATTTATTTGATCT
TAGTAAAAA
4261 --------- 4269
ATCATTTTT

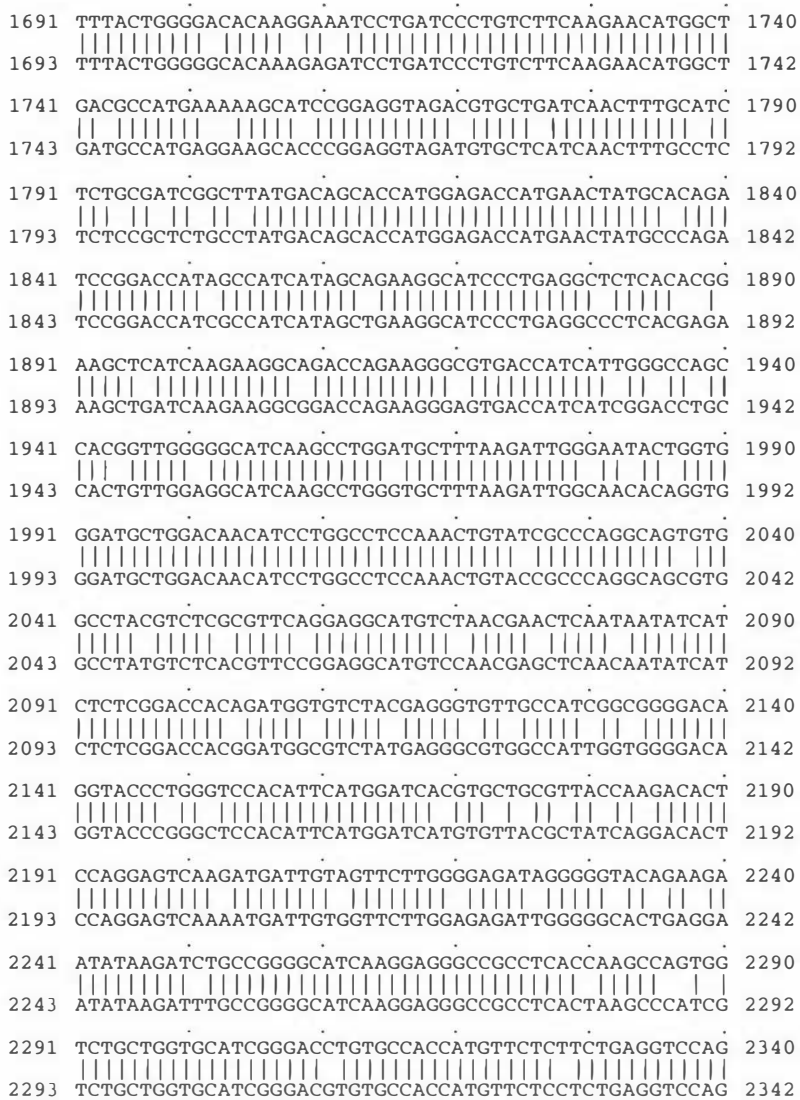
Appendix III

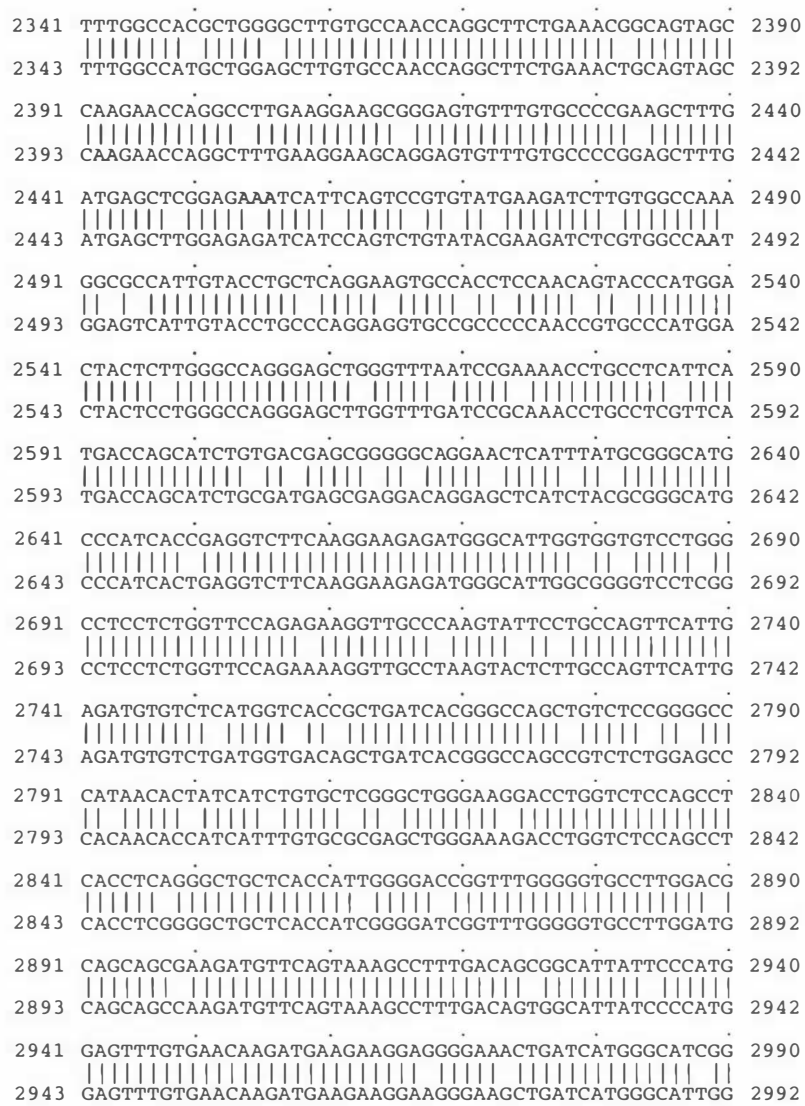
Alignment of Rat and Human ATP Citrate Lyase mRNA Sequences

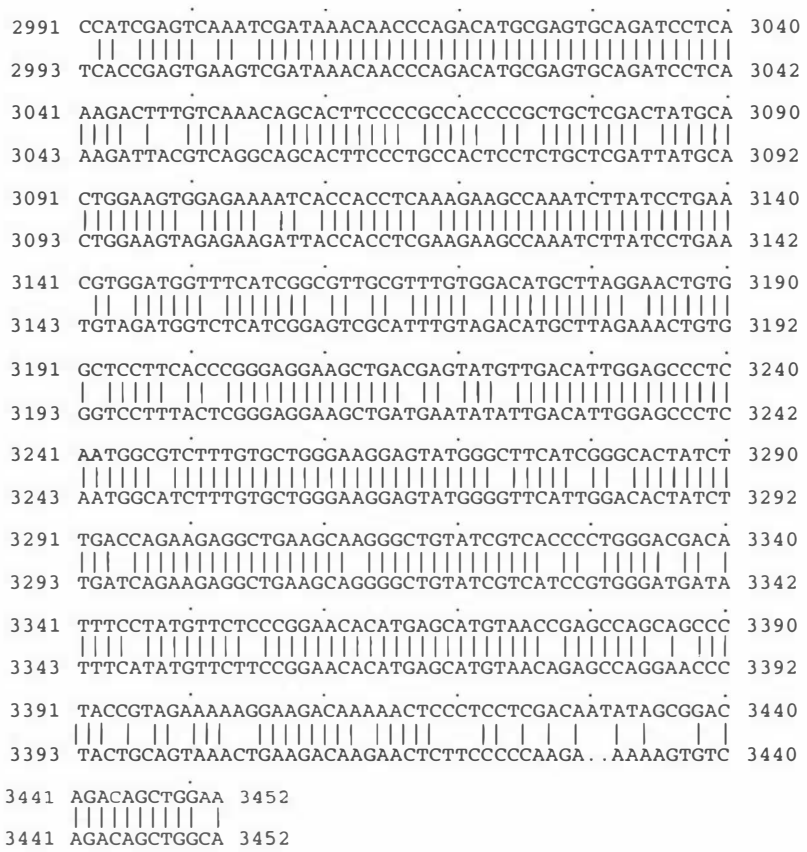
```
BESTFIT of:

DEFINITION  Rat ATP citrate-lyase mRNA, complete cds.
ACCESSION   J05210

to:

DEFINITION  Human ATP:citrate lyase mRNA, complete cds.
ACCESSION   U18197

          Gap Weight:     50      Average Match: 10.000
       Length Weight:      3   Average Mismatch: -9.000

             Quality:  27198             Length:   3412
               Ratio:  7.978               Gaps:      3
 Percent Similarity: 89.639   Percent Identity: 89.639

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   5
                    . =   1

 ratclatp.gb_ro x hsu18197.gb_pr
```




1044 CTCACTTATGACTCGAGAGAAGCACCCGGATGGCAAGATCCTCATCATTG 1093
1043 CTCCCTCATGACCCGAGAGAAGCACCCAGATGGCAAGATCCTCATCATTG 1092
1094 GAGGCAGCATTGCAAACTTCACCAACGTGGCCGCCACCTTCAAGGGCATT 1143
1093 GAGGCAGCATCGCAAACTTCACCAACGTGGCTGCCACGTTCAAGGGCATC 1142
1144 GTGAGAGCAATTCGAGATTACCAGGGTTCCCTGAAGGAGCACGAGGTCAC 1193
1143 GTGAGAGCAATTCGAGATTACCAGGGCCCCCTGAAGGAGCACGAAGTCAC 1192
1194 CATCTTTGTTCGAAGAGGTGGCCCGAACTATCAAGAGGGATTACGAGTGA 1243
1193 AATCTTTGTCCGAAGAGGTGGCCCCAACTATCAGGAGGGCTTACGGGTGA 1242
1244 TGGGAGAAGTTGGGAAGACCACTGGAATCCCCATCCATGTCTTTGGCACA 1293
1243 TGGGAGAAGTCGGGAAGACCACTGGGATCCCCATCCATGTCTTTGGCACA 1292
1294 GAAACTCACATGACGGCCATTGTGGGCATGGCC..TGGGCACCGG.CCAT 1340
1293 GAGACTCACATGACGGCCATTGTGGGCATGGCCCTGGGCCACCGGCCCAT 1342
1341 TCCCAACCAGCCACCCACAGCGGCTCACACTGCCAACTTCCTCCTTAATG 1390
1343 CCCCAACCAGCCACCCACAGCGGCCCACACTGCAAACTTCCTCCTCAACG 1392
1391 CCAGTGGGAGCACATCGACACCAGCACCCAGCAGGACAGCGTCTTTTTCC 1440
1393 CCAGCGGGAGCACATCGACGCCAGCCCCCAGCAGGACAGCATCTTTTTCT 1442
1441 GAGTCCAGAGCTGACGAGGTGGCCCCTGCAAAGAAAGCCAAGCCAGCCAT 1490
1443 GAGTCCAGGGCCGATGAGGTGGCGCCTGCAAAGAAGGCCAAGCCTGCCAT 1492
1491 GCCCCAAGATTCAGTCCCAAGTCCAAGATCCCTGCAAGGAAAGAGTGCCA 1540
1493 GCCACAAGATTCAGTCCCAAGTCCAAGATCCCTGCAAGGAAAGAGCACCA 1542
1541 CCCTCTTCAGCCGACATACCAAGGCTATCGTATGGGGCATGCAGACCCGG 1590
1543 CCCTCTTCAGCCGCCACACCAAGGCCATTGTGTGGGGCATGCAGACCCGG 1592
1591 GCTGTGCAAGGCATGCTGGACTTTGACTACGTGTGCTCCCGAGATGAGCC 1640
1593 GCCGTGCAAGGCATGCTGGACTTTGACTATGTCTGCTCCCGAGACGAGCC 1642
1641 TTCAGTGGCTGCTATGGTCTACCCGTTCACGGGGGATCATAAGCAGAAGT 1690
1643 CTCAGTGGCTGCCATGGTCTACCCTTTCACTGGGGACCACAAGCAGAAGT 1692

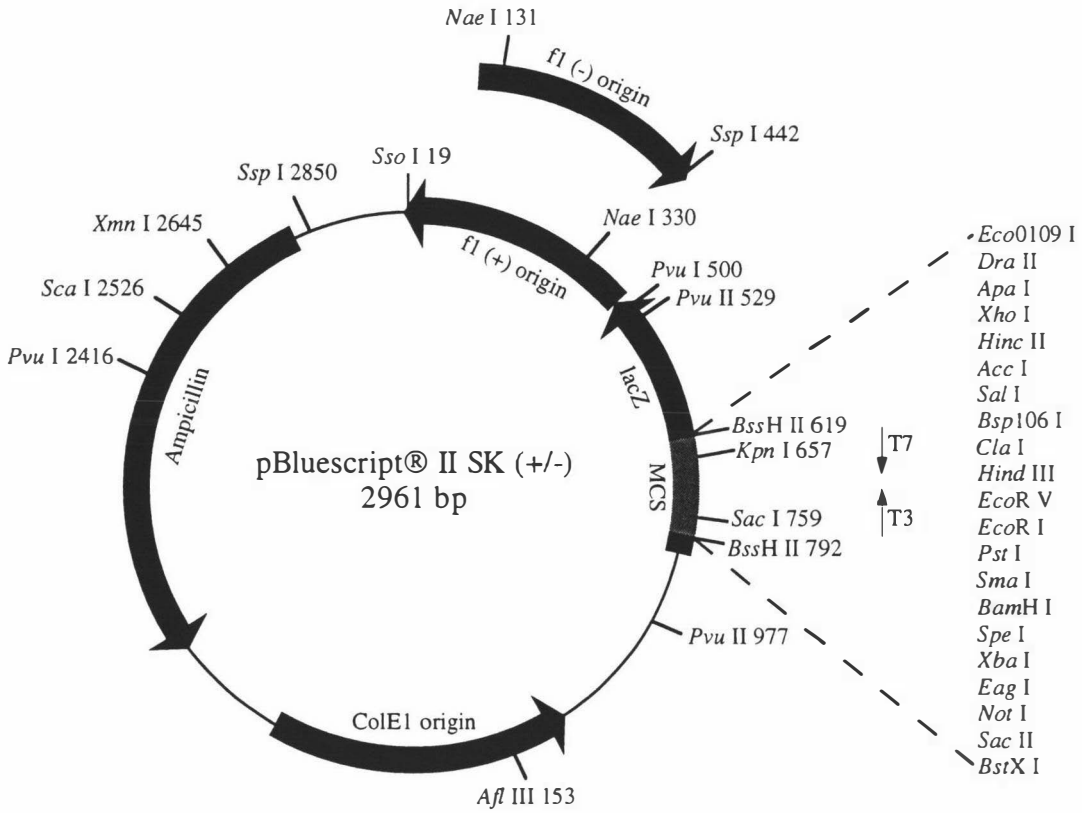

1691 TTTACTGGGGACACAAGGAAATCCTGATCCCTGTCTTCAAGAACATGGCT 1740
1693 TTTACTGGGGGCACAAAGAGATCCTGATCCCTGTCTTCAAGAACATGGCT 1742
1741 GACGCCATGAAAAAGCATCCGGAGGTAGACGTGCTGATCAACTTTGCATC 1790
1743 GATGCCATGAGGAAGCACCCGGAGGTAGATGTGCTCATCAACTTTGCCTC 1792
1791 TCTGCGATCGGCTTATGACAGCACCATGGAGACCATGAACTATGCACAGA 1840
1793 TCTCCGCTCTGCCTATGACAGCACCATGGAGACCATGAACTATGCCCAGA 1842
1841 TCCGGACCATAGCCATCATAGCAGAAGGCATCCCTGAGGCTCTCACACGG 1890
1843 TCCGGACCATCGCCATCATAGCTGAAGGCATCCCTGAGGCCCTCACGAGA 1892
1891 AAGCTCATCAAGAAGGCAGACCAGAAGGGCGTGACCATCATTGGGCCAGC 1940
1893 AAGCTGATCAAGAAGGCGGACCAGAAGGGAGTGACCATCATCGGACCTGC 1942
1941 CACGGTTGGGGGCATCAAGCCTGGATGCTTTAAGATTGGGAATACTGGTG 1990
1943 CACTGTTGGAGGCATCAAGCCTGGGTGCTTTAAGATTGGCAACACAGGTG 1992
1991 GGATGCTGGACAACATCCTGGCCTCCAAACTGTATCGCCCAGGCAGTGTG 2040
1993 GGATGCTGGACAACATCCTGGCCTCCAAACTGTACCGCCCAGGCAGCGTG 2042
2041 GCCTACGTCTCGCGTTCAGGAGGCATGTCTAACGAACTCAATAATATCAT 2090
2043 GCCTATGTCTCACGTTCCGGAGGCATGTCCAACGAGCTCAACAATATCAT 2092
2091 CTCTCGGACCACAGATGGTGTCTACGAGGGTGTTGCCATCGGCGGGGACA 2140
2093 CTCTCGGACCACGGATGGCGTCTATGAGGGCGTGGCCATTGGTGGGGACA 2142
2141 GGTACCCTGGGTCCACATTCATGGATCACGTGCTGCGTTACCAAGACACT 2190
2143 GGTACCCGGGCTCCACATTCATGGATCATGTGTTACGCTATCAGGACACT 2192
2191 CCAGGAGTCAAGATGATTGTAGTTCTTGGGGAGATAGGGGGTACAGAAGA 2240
2193 CCAGGAGTCAAAATGATTGTGGTTCTTGGAGAGATTGGGGGCACTGAGGA 2242
2241 ATATAAGATCTGCCGGGGCATCAAGGAGGGCCGCCTCACCAAGCCAGTGG 2290
2243 ATATAAGATTTGCCGGGGCATCAAGGAGGGCCGCCTCACTAAGCCCATCG 2292
2291 TCTGCTGGTGCATCGGGACCTGTGCCACCATGTTCTCTTCTGAGGTCCAG 2340
2293 TCTGCTGGTGCATCGGGACGTGTGCCACCATGTTCTCCTCTGAGGTCCAG 2342


2341 TTTGGCCACGCTGGGGCTTGTGCCAACCAGGCTTCTGAAACGGCAGTAGC 2390
2343 TTTGGCCATGCTGGAGCTTGTGCCAACCAGGCTTCTGAAACTGCAGTAGC 2392
2391 CAAGAACCAGGCCTTGAAGGAAGCGGGAGTGTTTGTGCCCCGAAGCTTTG 2440
2393 CAAGAACCAGGCTTTGAAGGAAGCAGGAGTGTTTGTGCCCCGGAGCTTTG 2442
2441 ATGAGCTCGGAGAAATCATTCAGTCCGTGTATGAAGATCTTGTGGCCAAA 2490
2443 ATGAGCTTGGAGAGATCATCCAGTCTGTATACGAAGATCTCGTGGCCAAT 2492
2491 GGCGCCATTGTACCTGCTCAGGAAGTGCCACCTCCAACAGTACCCATGGA 2540
2493 GGAGTCATTGTACCTGCCCAGGAGGTGCCGCCCCCAACCGTGCCCATGGA 2542
2541 CTACTCTTGGGCCAGGGAGCTGGGTTTAATCCGAAAACCTGCCTCATTCA 2590
2543 CTACTCCTGGGCCAGGGAGCTTGGTTTGATCCGCAAACCTGCCTCGTTCA 2592
2591 TGACCAGCATCTGTGACGAGCGGGGGCAGGAACTCATTTATGCGGGCATG 2640
2593 TGACCAGCATCTGCGATGAGCGAGGACAGGAGCTCATCTACGCGGGCATG 2642
2641 CCCATCACCGAGGTCTTCAAGGAAGAGATGGGCATTGGTGGTGTCCTGGG 2690
2643 CCCATCACTGAGGTCTTCAAGGAAGAGATGGGCATTGGCGGGGTCCTCGG 2692
2691 CCTCCTCTGGTTCCAGAGAAGGTTGCCCAAGTATTCCTGCCAGTTCATTG 2740
2693 CCTCCTCTGGTTCCAGAAAAGGTTGCCTAAGTACTCTTGCCAGTTCATTG 2742
2741 AGATGTGTCTCATGGTCACCGCTGATCACGGGCCAGCTGTCTCCGGGGCC 2790
2743 AGATGTGTCTGATGGTGACAGCTGATCACGGGCCAGCCGTCTCTGGAGCC 2792
2791 CATAACACTATCATCTGTGCTCGGGCTGGGAAGGACCTGGTCTCCAGCCT 2840
2793 CACAACACCATCATTTGTGCGCGAGCTGGGAAAGACCTGGTCTCCAGCCT 2842
2841 CACCTCAGGGCTGCTCACCATTGGGGACCGGTTTGGGGGTGCCTTGGACG 2890
2843 CACCTCGGGGCTGCTCACCATCGGGGATCGGTTTGGGGGTGCCTTGGATG 2892
2891 CAGCAGCGAAGATGTTCAGTAAAGCCTTTGACAGCGGCATTATTCCCATG 2940
2893 CAGCAGCCAAGATGTTCAGTAAAGCCTTTGACAGTGGCATTATCCCCATG 2942
2941 GAGTTTGTGAACAAGATGAAGAAGGAGGGGAAACTGATCATGGGCATCGG 2990
2943 GAGTTTGTGAACAAGATGAAGAAGGAAGGGAAGCTGATCATGGGCATTGG 2992


2991 CCATCGAGTCAAATCGATAAACAACCCAGACATGCGAGTGCAGATCCTCA 3040
2993 TCACCGAGTGAAGTCGATAAACAACCCAGACATGCGAGTGCAGATCCTCA 3042
3041 AAGACTTTGTCAAACAGCACTTCCCCGCCACCCCGCTGCTCGACTATGCA 3090
3043 AAGATTACGTCAGGCAGCACTTCCCTGCCACTCCTCTGCTCGATTATGCA 3092
3091 CTGGAAGTGGAGAAAATCACCACCTCAAAGAAGCCAAATCTTATCCTGAA 3140
3093 CTGGAAGTAGAGAAGATTACCACCTCGAAGAAGCCAAATCTTATCCTGAA 3142
3141 CGTGGATGGTTTCATCGGCGTTGCGTTTGTGGACATGCTTAGGAACTGTG 3190
3143 TGTAGATGGTCTCATCGGAGTCGCATTTGTAGACATGCTTAGAAACTGTG 3192
3191 GCTCCTTCACCCGGGAGGAAGCTGACGAGTATGTTGACATTGGAGCCCTC 3240
3193 GGTCCTTTACTCGGGAGGAAGCTGATGAATATATTGACATTGGAGCCCTC 3242
3241 AATGGCGTCTTTGTGCTGGGAAGGAGTATGGGCTTCATCGGGCACTATCT 3290
3243 AATGGCATCTTTGTGCTGGGAAGGAGTATGGGGTTCATTGGACACTATCT 3292
3291 TGACCAGAAGAGGCTGAAGCAAGGGCTGTATCGTCACCCCTGGGACGACA 3340
3293 TGATCAGAAGAGGCTGAAGCAGGGGCTGTATCGTCATCCGTGGGATGATA 3342
3341 TTTCCTATGTTCTCCCGGAACACATGAGCATGTAACCGAGCCAGCAGCCC 3390
3343 TTTCATATGTTCTTCCGGAACACATGAGCATGTAACAGAGCCAGGAACCC 3392
3391 TACCGTAGAAAAAGGAAGACAAAAACTCCCTCCTCGACAATATAGCGGAC 3440
3393 TACTGCAGTAAACTGAAGACAAGAACTCTTCCCCCAAGA..AAAAGTGTC 3440
3441 AGACAGCTGGAA 3452
3441 AGACAGCTGGCA 3452

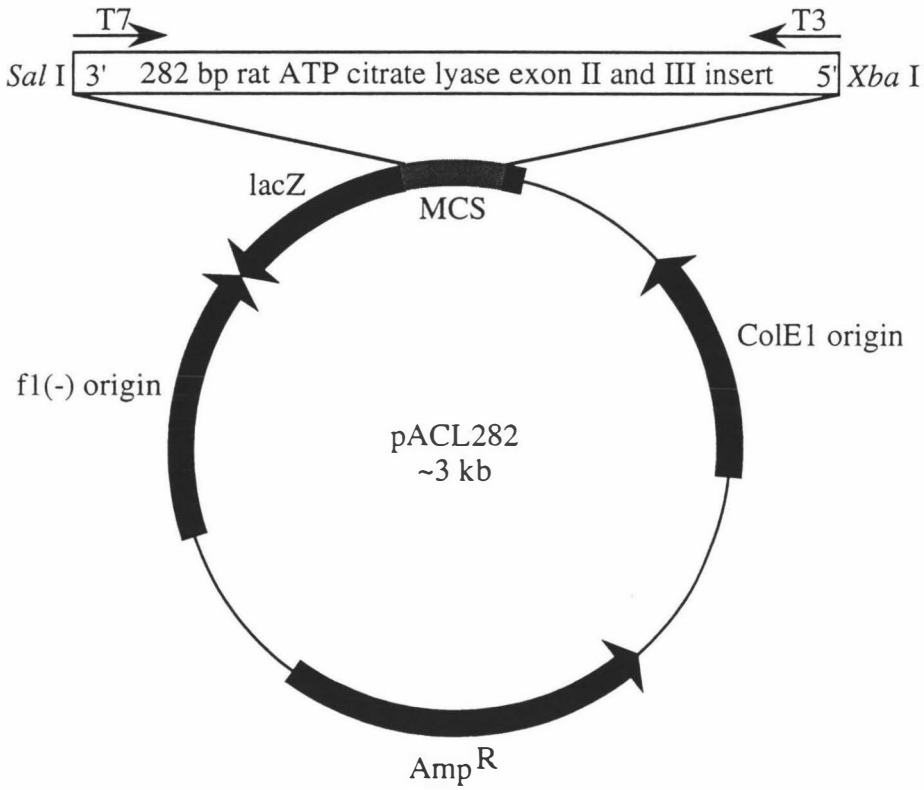
Appendix IV

Map of pBluescript® II SK-



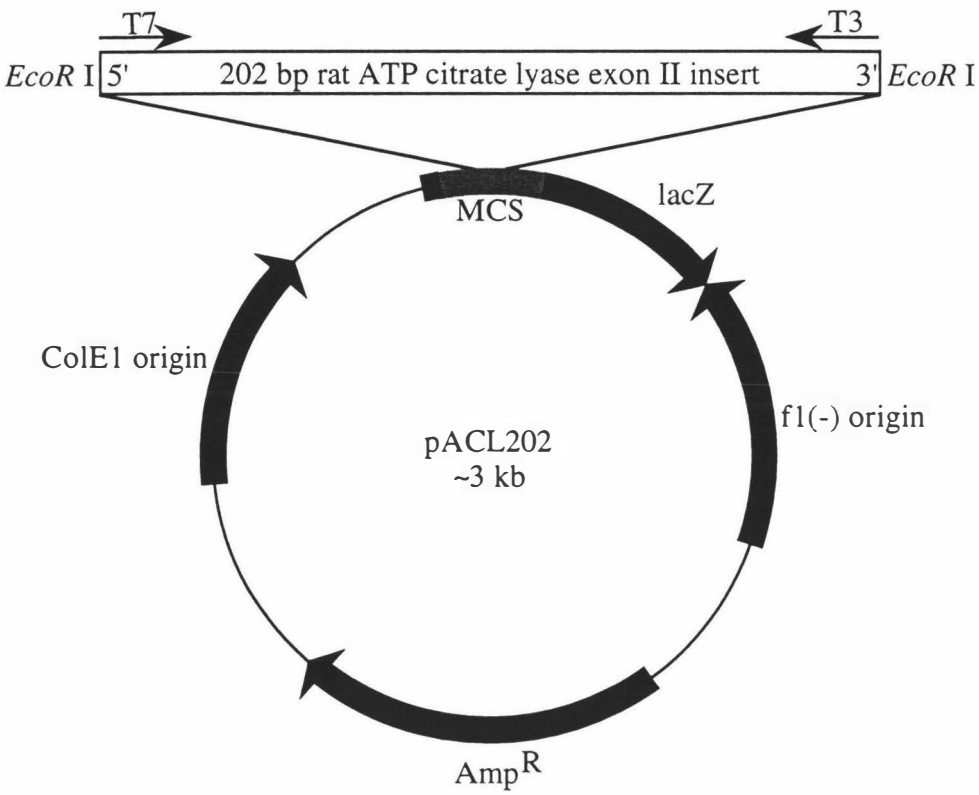
Appendix V

Map of pACL282



Appendix VI

Map of pACL202



Appendix VII

Map of pTG3954