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Cloning and Characterization of a Bovine Mammary Gland cDNA Encoding Acetyl-CoA Synthetase

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By

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ABSTRACT

Cloning and Characterization of a Bovine Mammary Gland cDNA Encoding Acetyl-CoA Synthetase

By

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Acetate is a major substrate for milk synthesis. The metabolic form of acetate is acetyl coenzyme A (acetyl-CoA) which occupies a central position in the metabolism of the Holstein mammary gland. The mechanism for generating acetyl-CoA is the acetate activation reaction catalyzed by acetyl coenzyme A synthetase (ACS). Acetyl-CoA synthetase is an N-glycosylated protein with a molecular weight of 65 kD. Acetyl-CoA synthetase is not constitutive in the mammary gland. ACS enzyme activity is marginal in a dry gland, reaches peak at maximum milk production and then declines as lactation advances.

A complementary DNA (cDNA) of 4200 base pairs expressing the bovine ACS was isolated by screening a bovine cDNA library with polyclonal antibody produced against the purified protein. Partial sequence analysis revealed an open reading frame of 969 nucleotides corresponding to 323 amino acids. The DNA sequence obtained displayed 42% homology to the *N. crassa* ACS gene. Neither the DNA nor the protein sequence showed significant similarity to other currently published sequences. The

isolated cDNA was expressed in bacteria to yield a catalytically active enzyme. Specific activity of the crude lysate obtained exceeded that of the wild type vector crude lysate by a factor of 2. Using cloned ACS as probe, highest level of ACS mRNA in the mammary gland was found at 120 days postpartum. Southern analysis of bovine genomic DNA suggests that the ACS gene has a single copy per haploid genome. Immunoblot analysis and enzyme assay studies suggest that the rabbit anti-bovine ACS has a high affinity for ACS. The ACS gene is expressed in heart, kidney, and mammary gland but not in liver as judged by northern and western blot analysis. In vitro transcription\translation products of the cloned ACS gene (55 kD) is 8 kD smaller than the native protein. This difference is most probably due to lack of glycosylation in the in vitro translation system. Northern blots probed with the ACS cDNA showed more than one mRNA species, suggesting that alternative splicing and/or multiple promoter(s) may exist for the ACS gene. Alternative regulation mechanisms for ACS gene were proposed to explain ACS mRNA multiple forms and tissue specific expression.

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INTRODUCTION

A characteristic feature of ruminants is the fermentative nature of their digestion, which enables them to survive on high fiber diets. The principal products of fermentation of dietary carbohydrates are short-chain fatty acids, the most important of which are acetate, propionate and butyrate. These fatty acids account for over 70% of the animal's caloric intake. Very little dietary hexose is available for absorption from the gastro-intestinal tract. Even on diets containing high amounts of concentrate, the absorption of glucose from the gut accounts for less than one-third of the whole-body glucose turnover. Therefore, ruminants in both the fed and the fasted state, are critically dependent on gluconeogenesis for provision of glucose. Up to 50% of total glucose requirement of an animal can be met by synthesis from propionate, a process which occurs in the liver. Ruminants have evolved a unique system of metabolism which allows them to spare glucose for essential body functions and to use the ruminally-derived fatty acids as alternate substrates for both energy generation and storage.

Within the ruminant's tissue, absorbed acetate and butyrate are used primarily as energy sources through oxidation via the tricarboxylic acid cycle (TCA cycle). Acetate is also the principal substrate for lipogenesis, while propionate is used largely for gluconeogenesis; with most diets, propionate is the major source of glucose since only small amounts of glucose are absorbed from the intestinal tract. Balance between the supply of glucogenic propionate relative to that of nonglucogenic acetate and butyrate

determines the efficiency with which volatile fatty acids are used for milk production.

Studies with tissue homogenates have demonstrated that different tissues can activate different volatile fatty acids. For example, liver tissue activates propionate, butyrate, and valerate but not acetate. Moreover, heart tissue activates acetate and propionate and lactating mammary gland activates acetate, while kidney tissue activates all these substrates.

The major difference between non-ruminant and ruminant mammary tissue metabolism is that in the latter, acetate can spare the action of glucose by furnishing energy as adenosine triphosphate (ATP) via oxidation in the TCA cycle and carbon skeletons for milk fat synthesis. In contrast, non-ruminant mammary tissue uses glucose as the major metabolic substrate for energy generation and both glucose and acetate for fatty acid synthesis.

Cellular membranes are freely permeable to volatile fatty acids. In order for uptake and subsequent tissue metabolism to occur, volatile fatty acids must first be trapped within a particular cellular compartment by conversion into the coenzyme A derivative. This so-called activation reaction is catalyzed by a series of enzymes termed acyl-CoA synthetases.

The enzyme responsible for trapping acetate is acetyl-CoA synthetase (EC 6.2.1.I). This enzyme occurs in both the mitochondrial and cytosolic fractions of cow and goat mammary tissue. Enzyme activity is negligible in the dry gland, increases just prior to parturition and reaches a peak at maximum milk production and then declines in activity as milk production declines. The role that acetate plays in the mammary

tissue depends on whether acetate is trapped as acetyl-CoA in mitochondria or in the cytosol. Mitochondrial acetate will generate energy in the form of ATP via the TCA cycle, and reducing equivalents for fatty acid synthesis in the cytosol. Mammary gland removes about half of the acetate presented to it. Obviously the absolute amount of acetate removed determines the amount of milk produced.

The development of recombinant DNA techniques and gene cloning systems has opened the prospect of direct manipulation of genes involved in metabolism. The primary purpose of this project is to clone and characterize the bovine acetyl-CoA synthetase gene. Results of this research will help us to understand why different tissues have different patterns of acetyl-CoA synthetase gene expression in relation to the physiological function of each tissue. The overall goal of this research is to identify ways in which tissue uptake of volatile fatty acids could be controlled in an effort to improve feed efficiency and milk production.

LITERATURE REVIEW

In ruminants, very little dietary hexose is available for absorption from the gastro-intestinal tract because of the microbial fermentation of dietary carbohydrate to volatile fatty acids that occurs in the rumen. Volatile fatty acids--principally acetate, propionate and butyrate but also lesser amounts of valerate, caproate, isobutyrate, isovalerate, 2-methylbutyrate and traces of various higher acids--are produced in the rumen as end products of microbial fermentation. During the fermentation process, energy is conserved in the form of ATP and subsequently utilized for maintenance and growth of the microbial population. As far as the microbes are concerned, the volatile fatty acids (VFA) are waste products; however, to the host animal they represent a major source of absorbed energy. With most diets, volatile fatty acids account for approximately 80% of the energy disappearing in the rumen (the remainder being lost as heat and methane) and between 50 and 70% of the digestible energy intake (Sutton, 1972; 1979; Thomas and Clapperton, 1972).

As a result of the fermentation process, ruminants are critically dependent on the process of gluconeogenesis for provision of glucose at all times (Bergman,1973; Young 1977), so that glucose levels can be maintained at adequate levels for critical body functions. Dietary carbohydrates such as cellulose, hemicellulose, pectin, starch and soluble sugars are the main fermentation substrates. They are degraded to their constituent hexose and pentoses before being fermented to VFA via pyruvate. Pentoses

are then converted to hexose and triose phosphate by the transketolase and transaldolase reactions of the pentose cycle. As a result, the majority of dietary carbohydrate metabolism proceeds via hexose, which is metabolized to pyruvate almost exclusively by the Embden-Meyerhof glycolytic pathway.

Acetyl-CoA is an intermediate in the formation of both acetate and butyrate from pyruvate while propionate formation occurs mainly via succinate, although an alternative pathway involving acrylate is also operative. Excess reducing power generated during the conversion of hexose to acetate or butyrate, while utilized mainly by conversion to methane, is also consumed in part during the formation of propionate. Ruminant animals have developed a pattern of intermediary metabolism which allows them, when possible, to utilize volatile fatty acids, primarily acetate, as an alternative substrate to glucose for oxidative and synthetic purposes and to synthesize large quantities of glucose from ruminally-derived propionate. Thus, the volatile fatty acids as well as glucose are major metabolic substrates in these animals.

The manner in which a particular volatile fatty acid can influence carbohydrate and/or lipid metabolism depends on a number of factors. Among them is whether a particular volatile fatty acid can be taken up by a given organ or tissue and incorporated into its metabolic machinery. The use of a given acid depends on the particular organ or tissue involved and the physiological and nutritional status of the whole animal at that time.

Metabolism of Short-Chain Fatty Acids

Propionate

During absorption through the rumen epithelium, 2-5% of propionate is converted to lactic acid with the remainder entering the blood as propionate (Elliot, 1980). Most propionic acid reaching the liver is either oxidized or converted to glucose as shown by the difference between portal and hepatic vein blood propionate concentrations. To enter the TCA cycle, propionyl-CoA must undergo CO_2 fixation by the biotin-containing enzyme, propionyl-CoA carboxylase, to form methylmalonyl-CoA and, subsequently, be converted to succinyl-CoA.

A substantial amount of propionate produced in the rumen is metabolized or converted to other ruminal products before or during absorption. The absorbed propionate, amounts to 40-60% of ruminal production (Steel and Leng, 1973b).

Net hepatic uptake of propionate is more than 90% of the net portal production in cattle (Bairn et al., 1980). Two-thirds of propionate that is taken up by the liver is used for glucose synthesis. The rate of utilization of propionate in glucose synthesis appears to be determined by availability. Hence, propionate utilization is linearly related to its concentration in plasma (Judson and Leng, 1973a). Also, infusion of exogenous propionate into the rumen increases the absolute amount of propionate incorporated into glucose but does not change the fraction of propionate used in glucose synthesis (Veenhuizen et al., 1988). Therefore, it appears that the proportion of propionate converted to glucose is relatively constant under various physiological and nutritional conditions. Similarly, the intravenous infusion of propionate did not change glucose

production and the proportion of glucose derived from propionate increased the proportion of propionate appearing in glucose (Bergman et al., 1966). In addition, intravenous infusion of propionate at double of the normal entry rate of propionate only marginally reduces the hepatic extraction of propionate, from 80-85% to 70-75% while the net hepatic uptake doubles (Bairn et al., 1980). Finally, incorporation of propionate into glucose and the hepatic extraction efficiency do not appear to be influenced by glucoregulatory hormones such as insulin (Brockman, 1990). In addition, glucose infusion sufficient to cause hyperinsulinemia and hyperglycemia does not affect the net hepatic uptake of propionate in cows while the hepatic output of glucose decreases (Bairn et al., 1980). This change in output occurs without change in the amount of propionate converted to glucose (Amaral et al., 1990).

Propionate may influence the utilization of other substrates in glucose synthesis. Since propionate increases the secretion of insulin (Brockman, 1982) and since insulin can decrease the hepatic uptake of lactate, glycerol and other glucose precursors (Brockman, 1985), propionate may decrease hepatic uptake of lactate and other glucogenic substrates through its effects on insulin secretion. Therefore, infusion of exogenous propionate in the absence of changes in plasma insulin concentrations can decrease the hepatic extraction of lactate (Baird et al., 1980). The intravenous infusion of exogenous propionate in fasted sheep was observed to decrease hepatic extraction of lactate and increase hepatic production of lactate, but changes in whole-body lactate production could not be accounted for solely on the basis of the hepatic changes.

Because 85-90% of propionate is removed in a single pass through the liver, only

small amounts of propionate reach other tissues. The hind-limbs of sheep remove about 40% of the propionate in a single pass (Prior et al., 1984). Similarly, the brain removes about a quarter of the propionate available to it (Oyler et al., 1970), but the arterio-venous difference of propionate across the brain is only 3-4% of that of glucose (Oyler et al., 1970). These findings indicate that, quantitatively, uptake of propionate by extrahepatic tissues is of little significance. The main use of propionate is as a substrate for glucose production by the liver, ~~sparing other glucogenic compounds for other uses.~~

Butyrate

Butyric acid is largely converted to ketones during absorption through the rumen epithelium, resulting in very low butyrate levels in the portal blood. β-hydroxybutyric acid (βHBA) accounts for more than 80% of the ketones formed, with the remainder comprised of acetoacetate and acetone. βHBA is oxidized in cardiac and skeletal muscle and is used for fatty acid synthesis in adipose and mammary gland tissue. When butyrate reaches the liver, hepatic tissue rapidly metabolize it, as shown by the difference between the levels of butyrate in the portal and hepatic veins (Bergman, 1975).

Butyrate is the third most important product of carbohydrate fermentation in the rumen (Bergman, 1973), but the amount of butyrate absorbed is low in relation to the amount produced in the rumen (about 20% of butyrate is converted to acetate in the rumen). During absorption, butyrate is largely converted to ketone bodies in the rumen epithelium (Emmanuel, 1980). Consequently, a sheep on a maintenance diet absorbs about 2 $mMol.h^{-1}$ (Katz and Bergman, 1969), compared to ruminal production of 20-40 $mMol.h^{-1}$ (Annison et al., 1967). In cattle the net production of ketone bodies by the

portal-drained viscera is 2-3 times more than by butyrate (Lomax and Bairn, 1983). Ketone body production by the portal-drained viscera decreases during fasting as butyrate production decreases.

More than 80% of absorbed butyrate is removed in a single pass through the liver (Bergman and Wolff, 1971). The liver also produces ketone bodies (Katz and Bergman, 1969) and appears to be able to use butyrate as a substrate (Annison et al., 1963b). Insulin decreases hepatic ketone body production ~~is reduced by insulin~~ (Brokman and Laarveld, 1985). Normally, when dietary butyrate is readily available, insulin concentrations are high. The conversion of butyrate to ketone bodies by ruminal epithelium during absorption allows hepatic ketogenesis to occur at a low rate without impairing conversion of butyrate to ketone bodies.

The most important function of butyrate is as a substrate in the production of ketone bodies. Since butyrate infusion appears to cause hyperglycemia, there is some suggestion that butyrate may be glucogenic. However, butyrate ~~has no~~ do not glucogenic ~~capacity~~ (Annison et al., 1963b). The distribution of radioactivity in glucose indicates that any label from butyrate that appears in glucose is incorporated through the entry of acetyl-CoA into the TCA cycle (Annison, 1963). Thus, the incorporation of butyrate carbons into glucose results in no net synthesis.

Acetate

Although a small amount of acetate absorbed through the rumen wall is converted to ketone bodies, most is carried by portal circulation to the liver unchanged. The initial reaction in acetate metabolism is conversion to acetyl-CoA in the cytoplasm and/or

mitochondria via acetyl-CoA synthetase. Due to absence of the acetate-activating enzyme (acetyl-CoA synthetase) in the ruminant's liver, approximately 80% of acetate reaching the liver escapes oxidation and passes into the peripheral circulation (Bergman, 1975). Once absorbed from the blood, most of acetate is oxidized via the TCA cycle or used for fatty acid synthesis. Acetate is the main precursor for lipogenesis in ruminants because, in the absence of adequate levels of ATP-citrate lyase, glucose can supply only limited quantities of acetyl-CoA for lipid synthesis. Activity of acetyl-CoA synthetase is 2-3 times higher in ruminant adipose tissue than in rat adipose tissue, reflecting the relatively high rate of acetate conversion to fatty acids in ruminant adipose tissue. Production of adequate levels of acetate in the rumen is essential to maintain sufficient quantities of milk fat. Acetate is a precursor of milk fatty acids up to and including palmitic acid.

a. Production

The whole-body production of acetate in a sheep on a maintenance diet is 120-150 mMol. h^{-1} (Bergman and Wolff, 1971). Studies of the arterio-venous differences show that the portal-drained viscera, presumably representing absorption, produces about one-quarter of the acetate difference (Bergman and Wolff, 1971). About 20% of acetate production is due to the liver in fed animals. In fasted animals, the endogenous production of acetate is about the same as in fed animals (Bergman and Wolff, 1971; Pethick et al., 1981).

In lactating ewes, net hepatic production of acetate accounts for about 40% of the whole body acetate turnover (Costa et al., 1976). However, in lactating dairy cows the net hepatic production of acetate is about one-third of that produced by the gut (Lomax

and Bairn, 1983). Increased acetate production in cattle is probably due to increased uptake of free fatty acids by the liver (Costa et al., 1976). While the lactating mammary gland is a net user of acetate, it also produces a small amount of acetate (about 4% of the whole-body production) (King et al., 1985). Mammary production of acetate is about one-quarter of its utilization rate by the organ.

b. Endogenous Acetate

Acetate is produced endogenously from the β-oxidation of fatty acids (Annison and White, 1962). This process occurs primarily in the liver, although other tissues such as heart, brain and skeletal muscle are known to produce endogenous acetate under some conditions.

The observation that acetate is present in the blood of fasted non-herbivores at a concentration of approximately 0.1-0.5 mM (Ballard, 1972) suggests that these animals also produce endogenous acetate, as very little acetate could be provided by their diet. The evidence would therefore suggest a broader role for acetate in the intermediary metabolism of all mammals than has been previously recognized.

The capacity of ruminant liver to produce endogenous acetate is significantly greater than that of its non-ruminant counterpart (Baird et al., 1974; Costa et al., 1976). The livers of lactating cows (Baird et al., 1974) and ewes (Costa et al., 1976) produce substantial quantities of endogenous acetate as do animals made diabetic by alloxan treatment. Knowles et al. (1974) have theorized that this endogenous acetate could be a means of furnishing substrates from adipose tissues which have the metabolic machinery to use large quantities of acetate.

The current state of our knowledge on the source of both exogenous and endogenous volatile fatty acids in ruminants is presented schematically in Figure 1.

c. Utilization

Acetate is rapidly metabolized by ruminants. Estimates of its half-life range from 3-4 minutes (Annison and Lindsay, 1961) to 13 minutes (Jarret et al., 1974). Acetate extraction by the hind-limb is 50-60%, where the net uptake accounts for 20% of the oxygen uptake (Jarret et al., 1976). Acetate extraction is lower during fasting and exercise, when ketone bodies and free fatty acids, respectively, make up the major energy sources and the extraction efficiency of acetate decreases to 15% (Jarret et al., 1976).

The brain also removes acetate from blood. The net uptake by brain may account for about 3% of acetate turnover (Peel and Bergman, 1983). On a molar basis this uptake is equivalent to about 10% of the glucose uptake by the brain, suggesting that the brain is not a major user of acetate. In lactating animals, up to 20% of acetate turnover is accounted for by mammary gland utilization (Pethick and Lindsay, 1982; King et al., 1985). Mammary gland removes about half of the acetate presented to it (Laarveld et al., 1985), and 17-29% of fatty acid synthesis is attributable to acetate (King et al., 1985). Obviously, the absolute amount of acetate removed is a function of milk yield.

Acetate turnover is reduced during insulin deficiency (Jarret et al., 1974) and the uptake by the hind-limb is increased by insulin (Knowles et al., 1974). In untreated diabetic sheep, the extraction of acetate may be as low as 5% (Knowles et al., 1974), compared to 50-60% in normal sheep and treated diabetics (Knowles et al., 1974;

Pethick et al., 1981). However, in untreated diabetic animals the proportion of acetate taken up by the gut, liver and muscle is the same as in treated animals (Pethick et al., 1981). In contrast, mammary gland uptake of acetate is not influenced by insulin (Laarveld et al., 1985). Typically, insulin concentrations are lower in lactating animals than in non-lactating animals, and the difference in the response to insulin allows the body to direct acetate to the mammary gland. When plasma insulin concentrations are low, the uptake of acetate by insulin-responsive tissues is reduced, thereby allowing the mammary gland to use a greater proportion of acetate.

Mechanism of Volatile Fatty Acid Uptake

Uptake of a particular volatile fatty acid depends on whether it can be trapped in a given tissue as the coenzyme A derivative. Cellular membranes are freely permeable to volatile fatty acids; however, membranes are not permeable to the corresponding acyl-CoA derivatives (Spencer and Lowenstein, 1962). This trapping process is analogous to the trapping of glucose in a cell as glucose-6-phosphate by hexokinase. The enzymes responsible for conversion to the acyl-CoA derivatives are termed the acyl-CoA synthetases. These enzymes catalyze the reaction represented by:

(1) Volatile fatty acid + ATP + CoA-SH -----> Acyl-AMP + PP_i

(2) Acyl-AMP + CoA -----> Acyl-CoA + AMP

This biphasic reaction was first proposed by Berg (1956) for acetate activation by yeast acetyl-CoA synthetase.

Acyl-CoA Tissue Distribution and Intracellular Localization

Acyl-CoA synthetases appear to be present in most mammalian tissues.

Furthermore, the short and medium chain acyl-CoA synthetases are members of a small group of enzymes which show a bimodal distribution in the cell, being present both in cytosol and mitochondria (Qureshi and Cook, 1972; Ballard, 1972; Aas and Bremer, 1968; Aas, 1971; Scholte et al., 1971; Barth et al., 1971).

Estimates of the fatty acid activating enzymes in tissue homogenates have been made for rats, guinea pigs, and ruminants (Aas and Bremer, 1968; Aas, 1971; Scholte et al., 1971; Cook et al., 1969; Scholte and Groot, 1975). In all species, each tissue exhibits a very characteristic pattern of volatile fatty acid activation (Figure 2).

Non-ruminants are characterized by significant acetate activating ability in both cytosolic and mitochondrial fractions of liver tissue (Ballard, 1972; Scholte and Groot, 1975). The ruminant animal, however, possesses only marginal acetate activating ability in these compartments. Presence or absence of a cytosolic acetate activating-enzyme may be related to the lipogenic capacity of the liver. Characteristically, lipogenesis occurs primarily in liver in monogastric and in adipose tissue in ruminants (Ingle et al., 1972a; 1972b).

Bauman (1978) has suggested that the site of lipogenesis may be related to the level of volatile fatty acids produced in the gastro-intestinal tract and this in turn would influence the location of the cytosolic acetate activating-enzyme. For example, in animals with cecal fermentation such as rabbits and guinea pigs, some lipogenesis would occur in both liver and adipose sites. In guinea pigs there is some cytosolic acetate activation, although this is lower than that found in a rat (Scholte and Groot, 1975). In ruminants where large quantities of volatile fatty acids are produced in the rumen, lipogenesis has

been shifted to the adipose depots with concurrent loss of the cytosolic acetate activating enzyme in liver tissue.

Biochemical Pathways of Volatile Fatty Acid Metabolism

Mitochondrial Pathway

Mitochondrial activation of acetate or butyrate and subsequent degradation via the TCA cycle yields energy in the form of ATP as well as intermediates required for synthetic purposes. Incorporation at the level of mitochondrion allows the cell to by-pass glycolysis (Bauman, 1976; Ballard et al., 1969; Bauman and Davis, 1974), a process which uses glucose. In ruminants a large portion of energy is derived by peripheral tissues from oxidation of acetate, sparing glucose utilization for other more essential purposes such as brain function, fetal development during pregnancy, and as a precursor for the milk sugar lactose (Young, 1977).

Only volatile fatty acids containing an uneven number of carbon atoms can yield a net synthesis of glucose. Activation of propionate within the mitochondrial matrix and incorporation in the TCA cycle at the level of succinate yields glucose (Figure 3). This process is restricted to organs which possess the necessary gluconeogenic enzymes, namely liver and kidney. Mammary tissue, despite its high requirement for glucose in the lactating state, cannot utilize propionate for glucose synthesis (Scott et al., 1976).

Cytosolic Pathway

Cytosolic activation of acetate and/or butyrate yields substrates for lipogenesis (Bauman and Davis, 1974). In ruminants this occurs primarily in adipose and in mammary tissue when milk fat is being secreted (Ingle et al., 1972a; 1972b; Bauman and

Davis, 1974). In nonruminants species the site of active lipogenesis varies but in most cases occurs primarily in liver (Ingle et al., 1972a; 1972b). Ingle et al., suggest that shifting of lipogenesis from liver to adipose sites in ruminants occurs so that gluconeogenesis, a process on which the ruminant animal is critically dependent for a supply of glucose, can occur at all times.

Lipogenesis and gluconeogenesis are both processes which compete for energy and carbon skeletons. However the metabolic controls exerted on these pathways preclude them from occurring simultaneously (Tepperman and Tepperman, 1970). The primary uses of volatile fatty acids, in the heart, kidney, liver and adipose tissue are shown in Figure 2.

Role of Volatile Fatty Acids in the Intermediary Metabolism of Different Organs and Tissues

Liver Metabolism

The liver is involved in numerous metabolic processes central to the maintenance of homeostasis and productive function. Because of this central role in metabolism, energy expenditures per kilogram of liver tissue are much greater than those of other major tissues in the animal. The primary metabolic activities of the ruminant's liver, as they relate to the whole-animal metabolism and milk production, include gluconeogenesis, ketogenesis and synthesis of lipoproteins rich in triacylglycerol for transport to other tissues. We know a great deal about liver metabolism and its regulation in rats but, unfortunately, our knowledge of ruminant liver metabolism and its regulation is quite limited.

The liver plays a major role in the control of the intermediary metabolism of all mammals by integrating and coordinating the metabolism of the whole body. Most of the incoming dietary nutrients pass via the portal blood system to the liver where they are packaged according to the specific requirements of the animal at that time and then either stored or distributed to the peripheral tissues. In ruminants, the liver can oxidize propionate to carbon dioxide (Hoot et al., 1972) at a rate five times greater than that of acetate oxidation.

Liver is the most important glucose-producing organ, accounting for 85-90% of whole-body glucose turnover in animals either on a roughage diet or fasting (Bergman et al., 1970). The most important substrate for glucose synthesis in fed animals is propionate (Lindsay, 1987), which is the substrate for about half of glucose synthesis.

Lactate accounts for 15% of glucose, with amino acids and other precursors making up the rest. In fasted animals, less propionate is available for use in glucose synthesis. Lactate is also used to a lesser extent and glycerol becomes the most important precursor, its contribution reaching 40% during fasting (Bergman et al., 1968).

The primary difference between liver metabolism of ruminants and non-ruminants is that in the former, gluconeogenesis occurs continuously. Less than 10% of the glucose requirements of ruminants is absorbed from the gastro-intestinal tract (Young, 1977). Therefore, ruminants are dependent on a continual process of gluconeogenesis to provide the remaining 90% of glucose. If the gluconeogenesis process breaks down, severe metabolic disturbances such as ketosis in cattle or pregnancy toxemia and twin lamb disease in sheep can result (Young, 1977; Bergman, 1973).

a. Relationship of Gluconeogenesis to Physiological and Nutritional State

The amount of glucose synthesized in ruminant liver is dependent on the physiological status of the animal. For example, the glucose requirements of a lactating dairy cow producing 89 Lbs of milk/day at peak lactation has been calculated to be 7.4 kg/day (Young, 1977), of which 60% is used for lactose synthesis. Assuming the cow has to synthesize 90% of this, then 6.6 kg of glucose must be synthesized per day. Steers (160-250 kg) fed slightly above the maintenance level still require synthesis of approximately 0.6 kg of glucose per day (Young, 1977). Bergman (1973) has summarized the glucose requirements of the normal non-pregnant, pregnant, and lactating sheep as 100, 180, and 320 g/day respectively. These data emphasize the importance of gluconeogenesis as a continual process in ruminants and highlight the importance of this process during lactation and pregnancy.

The major gluconeogenic precursors in ruminants are propionate and glycogenic amino acids (Young, 1977). Thus maximal rates of gluconeogenesis occurring in the liver of ruminants have been observed 2-4 hours after feeding, when these precursors are available (Bergman, 1973; Trenkle, 1978; Ballard et al., 1969; Katz and Bergman, 1969). In contrast, maximal rates of gluconeogenesis in nonruminants occur during fasting, when glycogenic amino acids and glycerol are made available by the breakdown of the body tissue itself (Young, 1977; Ballard et al., 1969).

b. Volatile Fatty Acids as Carbon Sources for Gluconeogenesis

Propionate is the only volatile fatty acid produced in the rumen that is a major source of glucose (Bergman et al., 1966; Judson et al., 1968). This is because acetate

and butyrate, as well as the even-number long-chain fatty acids derived from dietary fat or adipose tissue are converted to acetyl-CoA. Subsequent metabolism of acetyl-CoA via TCA cycle results in the loss of two carbon atoms as carbon dioxide. Thus,no net gain of oxaloacetate can occur and therefore no net synthesis of glucose (Weinman et al., 1957). At one time, butyrate was thought to be an important gluconeogenic compound (Black et al., 1961; Kronfeld, 1957; Potter, 1952), since butyrate injection resulted in hyperglycemia. However, it is now known that this hyperglycemic action of butyrate is mediated by an increase in glycogenolysis (Phillips et al., 1965; Ash et al., 1964) and possibly also by an increased synthesis of oxaloacetate from pyruvate (Black et al., 1966).

The ability of liver tissue to metabolize propionate increases as lactation progresses (Mathias and Elliot, 1967). Also, mitochondrial propionate activation in liver increases as lactation progresses. This increase is likely a function of increased feed intake, suggesting that the ability to utilize propionate for glucose synthesis might be controlled by suitable dietary manipulations, a fact of some significance to the dairy industry.

Insulin and glucagon are important regulatory hormones of liver carbohydrate metabolism in non-ruminant animals. In ruminants, insulin release is related to feeding (Trenkle, 1978). In view of the low levels of glucose absorbed from the gut, glucose is probably not a major physiological regulator of insulin release in ruminants (Bassett, 1975). The volatile fatty acids propionate and butyrate, but not acetate, have been implicated as physiological regulators of insulin release in ruminants (Carstairs, 1978).

Stimulation of insulin release by volatile fatty acids does not occur in non-ruminants (Horino and Machlin, 1968). Stern et al (1970) report that physiological quantities of volatile fatty acids administered via the gastro-intestinal tract are ineffective in elucidating insulin release.

In the non-ruminant, many of the effects of insulin and glucagon can be directly related to the activity of rate-controlling enzymes of gluconeogenesis, glycolysis and glycogen metabolism. No data are available on the precise hormonal controls of these enzymes in ruminants. The only data available pertain to the level of these enzymes under different physiological and nutritional states. A brief summary of these effects will be discussed here, in view of the importance of these enzymes in controlling flux through the pathways of carbohydrate metabolism (Figure 3).

Phosphoenolpyruvate carboxykinase (PEPCK), a highly-adaptive enzyme in non-ruminants, generally shows little adaptation in cattle (Ballard et al., 1969) or in sheep (Filsell et al., 1960). However, Buttler and Elliot (1970) do find that decreased feed intake is associated with lower levels of PEPCK in dairy cows. This enzyme also exhibits a very characteristic intracellular distribution dependent on the species. In rats, 90% is found in cytosol and 10% within mitochondria (Ballard and Hanson, 1967), while in ruminants the distribution is approximately equally divided between the two compartments (Ballard et al., 1969). These differences in the intracellular distribution must have profound effects on the control of gluconeogenesis, although these effects are not understood at present.

Adaptation of mitochondrial and cytosolic pyruvate carboxylase does occur in

ruminants. In cattle, enzyme activity increases with lactation and increases even further if the lactating animals are starved (Ballard et al., 1969). In sheep, pyruvate carboxylase activity also increases with fasting (Filsell et al., 1969). Fatty acid coenzyme derivatives are known to be allosteric activators of pyruvate carboxylase (Clemants et al., 1978). Such derivatives, particularly propionyl and butyryl-CoA, are produced in large quantities in the ruminant's liver by the action of acyl-CoA synthetases (Cook et al., 1969; Ballard et al., 1969) and could potentially lead to an increase in pyruvate carboxylase activity under certain conditions such as after feeding.

The activities of glucose-6-phosphatase and fructose-1-6- diphosphatase have been shown to increase in liver and kidney of fasted sheep (Filsell et al., 1969). Mackie and Campbell (1972) have found that glucose-6-phosphatase increases in lactating ewes. As already discussed, propionate activation in ruminant liver increases as lactation progresses (Ricks et al., 1978).

These studies suggest that changes in gluconeogenic enzymes do occur in ruminants, albeit of smaller magnitude than those observed in the rat. Based on studies in cattle, Young et al (1969), have interpreted their data to mean that little need exists for changes in gluconeogenic enzymes in ruminants because of a continuous requirement for gluconeogenesis in these animals.

Pyruvate kinase is an important regulatory enzyme of glycolysis in non-ruminants. Pyruvate kinase must be under chronic inhibition while glucose synthesis occurs, to prevent all the phosphoenolpyruvate formed by the action of PEPCK from being converted back to pyruvate. Also, pyruvate kinase activity would be expected to be low

in ruminant liver.

Glycogen stored in non-ruminant liver is a readily available source of glucose for use when dietary glucose levels are inadequate. Ballard et al. (1969) have found that while the activities of glycogen synthetase and phosphorylase decline during development of the mature ruminant, this decrease does not occur in monogastric species such as the rat (Ballard and Olive 1963). Glycogen levels in the livers of lactating cows are extremely low relative to amounts stored in non-ruminant liver. On the basis of this observation Ballard et al. (1969) conclude that animals which do not have large fluctuations in blood glucose in response to feeding do not have the potential to store large amounts of glucose as glycogen.

Ruminants are characterized by their inability to synthesize significant quantities of lipids in liver tissue (Ingle et al., 1972a; 1972b). Acetate, a primary substrate for lipogenesis in ruminants, is not activated by ruminant liver tissue (Quraishi and Cook, 1972).

The biochemical pathways of carbohydrate and lipid metabolism in ruminants and non-ruminants are shown in Figure 3.

Kidney Metabolism

The net renal production of glucose accounts for about 10% of the whole-body glucose turnover (Kaufman and Bergman, 1974). The kidney's contribution to glucose production appears to increase during starvation, lactation and pregnancy (Van der Walt et al., 1983). The amount of propionate reaching the kidney is small when compared to that reaching the liver (Bergman and Wolff, 1971). In vitro studies have shown that

lactate and glycerol can be used by the kidney tissue as effectively as propionate for glucose synthesis (Faulkner, 1980).

In ruminants, kidney tissue does have the capacity to spare utilization of glucose for energy generation, since trapping of acetate can occur in this tissue (Figure 2).

It has been estimated that 8-10% of the total glucose synthesized by the ruminant animal can be made in the kidney (Bergman et al., 1974). This quantity may increase to 15% in fasting animals (Bergman, 1973). Renal gluconeogenesis in humans can increase even further during prolonged fasting (Owen et al., 1969). This increase suggests that under conditions of metabolic stress, such as might occur in a high-producing cow at peak lactation, the kidney might synthesize considerable quantities of glucose. It is not likely, however, that propionate produced in the rumen is a substrate for renal gluconeogenesis. Instead, lactate, pyruvate, glutamine, and glycerol have been shown to be important substrates for renal gluconeogenesis (Kaufman, 1972).

Mammary Gland Metabolism

The major difference between non-ruminant and ruminant mammary tissue metabolism is that in the latter, acetate can spare the action of glucose by furnishing energy as ATP via oxidation in the TCA cycle and by furnishing carbon skeletons for fatty acid synthesis as shown in Figure 4. In contrast, non-ruminant mammary tissue uses glucose as the major metabolic substrate for energy generation and both glucose and acetate for fatty acid synthesis (Bauman and Davis, 1974).

In the lactating state, ruminant mammary tissue takes up tremendous quantities of acetate and glucose from the blood. Ruminant metabolism of these substrates is shown

in Figure 4. Uptake of glucose is essential since a shortage of this metabolite leads to a marked reduction in volume of milk secreted (Davis and Bauman, 1974). Annison and Linzell (1964) have estimated that in the lactating goat, 60-85% of the total glucose available to the animal will be taken up by the mammary gland. This uptake will occur independent of the glucose status (Hove, 1978). Approximately 50-60% of this glucose will be used for lactose synthesis, 23-30% will be metabolized via the pentose phosphate pathway, and less than 10% will be oxidized via the glycolytic pathway (Linzel, 1968). This process is in marked contrast to values obtained with non-ruminant species in which, although about the same amount of glucose is used for lactose synthesis, approximately equal amounts of glucose are metabolized via the glycolytic and pentose phosphate pathways (Davis and Bauman, 1974). Smith (1971) and Chesworth and Smith (1971) observed that there is likely little glucose oxidized to carbon dioxide via the TCA cycle in ruminant mammary tissue. No evidence exists that gluconeogenesis occurs in ruminant mammary tissue (Scott et al., 1976).

Approximately 40% of total acetate available to the body is extracted by the lactating goat udder (Davis and Bauman, 1974). The enzyme responsible for trapping this acetate is acetyl-CoA synthetase (Qureshi and Cook, 1975). The enzyme occurs in both the mitochondrial and cytosolic fractions of cow and goat mammary tissue (Marinez et al., 1976; Ricks et al., 1978). Enzyme activity is negligible in the dry gland, increasing just prior to parturition and reaches peak at maximum milk production and then declines in activity as milk production declines. Enzyme activity can be reinstated by hormone treatment (Marinez et al., 1976).

The role that acetate plays in mammary tissue depends on whether acetate is trapped as acetyl-CoA in mitochondria or in cytosol. Mitochondrial acetate will generate energy in the form of ATP via the TCA cycle, and reducing equivalents for fatty acid synthesis in the cytosol. In lactating goats, acetate oxidation accounts for 23-27% of total mammary carbon dioxide (Annison et al., 1967) and glucose oxidation for 29-49%.

Cytosolic activation of acetate yields a major source of carbon for de novo synthesis of fatty acids in ruminant mammary tissue (Popjak et al., 1951a, 1951b). Approximately 35-45% of total milk fatty acids can be synthesized from acetate (Davis and Bauman, 1974). Numerous studies have shown that glucose cannot serve as a carbon source for fatty acid synthesis in ruminant mammary tissue (Bauman et al., 1970). Most of the remaining fatty acids found in milk fat are taken up preformed from the blood.

A major function of glucose in mammary gland is as a precursor of lactose, which accounts for 50-60% of glucose uptake by bovine mammary gland (Baird et al., 1983). In sheep, glucose uptake by mammary gland may account for 70% of lactose in milk (Oddy et al., 1985). The fractional extraction of glucose by mammary gland (Laarveld et al., 1985) and uterus (Hay et al., 1984) does not change during starvation or insulin administration (Hay et al., 1984; Oddy et al., 1985). Thus, during times of high glucose concentrations in plasma, glucose may be directed toward storage organs, such as adipose tissue for fat synthesis and muscle for glycogen storage. During fasting, lactation or pregnancy, when plasma insulin concentrations are low and less glucose is taken up by adipose tissue and muscle, a greater proportion of glucose is available for the non-insulin-responsive tissues.

Adipose Tissue Metabolism

Adipose tissue plays a central role in metabolism of the lactating animal. In early lactation when cows are in negative energy balance, fatty acids mobilized from adipose tissue provide energy and milk fat fatty acids. During early lactation, changes in adipose tissue functions which are supportive of lactation include increased lipolytic capacity, depressed lipogenic capacity and decreased capacity for blood triacylglycerol uptake. As lactation progresses, lipogenic capacity increases above that of adipose tissue in non-lactating, non-pregnant cows. Lipolytic capacity remains elevated throughout lactation. Newly-synthesized fatty acids released from adipose tissue due to lipolysis can serve as sources of energy and milk fat fatty acids.

In ruminant animals, the major site of fatty acid synthesis is adipose tissue (Ingle et al., 1972a; 1972b). The major site of fatty acid synthesis in pigs is in the adipose tissue (Allen et al., 1976), while in rats fatty acid synthesis occurs about equally in both the adipose tissue and the liver (Leveille, 1967). This shift of fatty acid synthesis to adipose tissue in ruminants allows ruminants to maximize the potential of gluconeogenesis in the liver (Bauman, 1976). Moreover, rates of fatty acid synthesis do vary between the different adipose depots (Ingle et al., 1972). Fatty acid synthesis rates are highest for young growing animals (Allen et al., 1976).

It is well established that in non-ruminants, glucose is the primary carbon source for fatty acid synthesis. In addition, reducing equivalents required for lipid synthesis are generated via the pentose phosphate shunt and the malate transhydrogenation cycle from glucose. These pathways are shown in Figure 5. In ruminants, glucose has been excluded

both as a primary source of carbon for fatty acid synthesis (Hood et al., 1972) and as a primary source of reducing equivalents (Bauman, 1976).

Ruminant tissues that actively synthesize lipids such as adipose and lactating mammary tissues, both contain negligible levels of ATP citrate-lyase and NADP-malate dehydrogenase. The former enzyme is required for shuttling glucose carbon across the mitochondria to the cytosol, the site of fatty acid synthesis. The latter enzyme is involved in the transfer of reducing equivalents. Thus in ruminants, glucose is excluded as a carbon source for fatty acid synthesis.

Acetate in the cytosol is used as the primary substrate for fatty acid synthesis (Hanson and Ballard, 1967). Ingle et al. (1972b) have suggested that reducing equivalents required for fatty acid synthesis in ruminant tissues are generated via action of isocitrate dehydrogenase. Therefore, mitochondrial acetate serves as the substrate for the supply of reducing equivalents in ruminants (Figure 6). Substantial quantities of acetate can be oxidized to carbon dioxide in bovine adipose tissue (Hood et al., 1972). Therefore, acetate can supply energy in the form of ATP for adipose metabolism.

In summary, the ruminant animal, by using acetate as a source of reducing equivalents, as a source of carbon for fatty acid synthesis, and as a source of energy, conserves glucose for more essential functions.

Yeast and fungi cloned acetyl-CoA synthetase genes

Acetyl-CoA Synthetase Gene of *Saccharomyces cerevisiae* (yeast)

Like many other organisms, *Saccharomyces cerevisiae* can use acetate as the sole carbon source. Growth of microorganisms on acetate requires presence of the anaplerotic glyxoylate-cycle enzymes and of essential gluconeogenic enzymes (Kornberg, 1966). The first step of acetate utilization involves activation of free acetate to acetyl-CoA. This step is accomplished by acetyl-CoA synthetase (EC 6.2.1.I).

Acetyl-CoA synthetase is also thought to be involved in ethanol utilization. Converting acetate formed by oxidation of ethanol to acetyl-CoA can occur through action of alcohol dehydrogenase and acetaldehyde dehydrogenase. Acetyl-CoA then enters the glyoxylate-cycle or, alternatively, is used as substrate in the TCA cycle (Wills, 1990).

Virgilio et al. (1992) obtained the *Saccharomyces cerevisiae* acetyl-CoA synthetase gene by selecting for mutants defective in trehalose metabolism. Vectors which carry the disrupted *Saccharomyces cerevisiae* acetyl-CoA synthetase lack the ability to grow in a medium with acetate as sole carbon source. These colonies were purified and sequenced; also, a close homology analysis of the cloned *Saccharomyces cerevisiae* genes with the known sequences of acetyl-CoA synthetase genes of *Neurospora crassa* and *Aspergillus nidulans* indicated homology of 76 and 78%, respectively. *Saccharomyces cerevisiae* acetyl-CoA synthetase gene has an open reading frame (ORF) of 2139 nucleotides, with two potential start codons (ATG). The first ATG of the ORF

is in an unusual context for a translational start site, while the next ATG, 24 codons downstream from the first ATG, is in a more conventional context. This second ATG is in frame with the first and is surrounded by CAAAAATGTCC, a sequence very similar to the consensus for a translational start site. Klein and Jahnke (1968, 1971) suggest that, according to physiological state of the cell, acetyl-CoA synthetase of *Saccharomyces cerevisiae* is either localized in the microsomal or in the mitochondrial fraction. It is possible that different start sites yield enzymes with different subcellular locations (Slusher et al., 1991).

***Aspergillus nidulans* Acetyl-CoA Synthetase Gene**

Sandeman and Hynes (1989) cloned the *A. nidulans* acetyl-CoA synthetase gene. Double stranded cDNA was synthesized from poly $(A)^+$ RNA isolated from wild-type *A.nidulans* grown under conditions of acetate induction. The cDNA was digested with Sau3A, ligated into the BamH1 site of M13mp9 and transfected to generate a small gene bank. Individual plaques were spotted on to agar plates and duplicate nitrocellulose filters were prepared. Differential hybridization to ^{32}P-labeled cDNA probes derived from mRNA from uninduced and acetate-induced cultures were used to screen the filters, and two plaques were identified as hybridizing strongly only to the probe from acetate-induced cDNA.

The plaques were purified and the inserts were used as probes to screen lambda genomic libraries. This screening resulted in two lambda clones. Restriction mapping and southern blot analysis using the M13mp9 cDNA clones as probes allowed approximate localization of the acetate- induced genes and subsequent subcloning into pBR322.

***Methanothrix soehngenii* Acetyl-CoA Synthetase Gene**

It is generally accepted that about 70% of the methane produced in an anaerobic ecosystem is derived from acetate (Gujer and Zehnder, 1983; Zinder et al., 1984). The acetoclastic methanogens play a pivotal role in the anaerobic treatment of organic wastes. Only two genera of methanogenic bacteria, *Methanosarcina* and *Methanothrix*, are capable of metabolizing acetate to methane. To be able to catabolize acetate to methane, acetate must first be activated to acetyl-CoA (Thauer et al., 1989). In the *Methanothrix spp.*, acetate is activated in one step by the enzyme acetyl-CoA synthetase (Jetten et al., 1989; Pelliren et al., 1987).

The Acetyl-CoA synthetase gene (3.4 Kb) was isolated from a genomic library of *Methanothrix soehngenii* by screening with antiserum raised against the purified *Methanothrix* acetyl-CoA synthetase. After introduction into *E.coli*, the acetyl-CoA synthetase gene was expressed, resulting in the production of an immunoreactive protein. However, the fusion protein was 5KD smaller than the known size of purified acetyl-CoA synthetase, but showed a comparable specific activity with the natural protein. Southern blotting of chromosomal DNA from *M.soehngenii* with acetyl-CoA synthetase as a probe revealed that acetyl-CoA synthetase is a single copy gene (Eggen et al., 1991).

The sequence of the acetyl-CoA synthetase gene results in three large open reading frames (ORF). The first ORF, which contains a GTG start codon at position 310 is preceded by a potential ribosomal binding site (AGGA) and stops at TGA stop codon at position 2326. The second open reading frame starts at ATG at position 2609 and the

third ORF starts at the ATG at position 3039. Each ATG site is preceded by a ribosomal binding site but their open reading frames are short. The region upstream of the acetyl-CoA synthetase gene is A-T rich (57%) which indicates that transcription initiation sites will be located in this region. A high percentage of homology is observed when the acetyl-CoA synthetase primary sequence is compared with the amino acid sequence from the *Neurospora crassa* and *Aspergillus nidulans* (42 and 44% respectively) (Eggen et al., 1991).

***Penicillium chrysogenum* Acetyl-CoA Synthetase Gene**

Development of recombinant DNA techniques and gene transfer systems has opened the prospect of direct manipulation of genes involved in penicillin biosynthesis. The filamentous fungus *P. chrysogenum* is of great biotechnological importance for production of β-lactam antibiotic, penicillin. The enzyme acetyl-CoA synthetase is essential for utilization of acetate as a carbon source of the fungus. The enzyme catalyzes the conversion of acetate to acetyl-CoA, which is further metabolized, via the glyoxylate cycle, by the acetate inducible enzymes isocitrate lyase and malate synthetase (Armitt et al., 1976).

The acetyl-CoA synthetase gene was isolated from a *P. chrysogenum* genomic library using defined chemically synthesized oligonucleotide probes. The sequences of the oligonucleotide are derived from conserved regions in the nucleotide sequence of the corresponding genes of *A. nidulans* and *N. crassa* (Connerton et al., 1990). To test whether these oligonucleotide can be used as probes, they were tested in southern blot with chromosomal *P. chrysogenum* DNA, digested with several restriction enzymes. One

of the oligonucleotide probe (5'GATGGCCTC(G,A)GGAATCATGGGAAGGTAGAT3'), showed specific binding. Therefore, this probe is used to screen a *P. chrysogenum* genomic library. A positive colony was identified and the cloned fragment isolated and sequenced. Comparison of the sequence obtained with that from *A. nidulans* acetyl-CoA synthetase gene (Connerton et al., 1990) shows a similarity of 80% in the coding region. In upstream non-coding sequence, a large CT tract and putative TATA boxes are present. These sequences are considered to be involved in initiation of transcription (Punt and Van den Hondel, 1992).

In summary, ruminants have a unique metabolism which relies on acetate, propionate and butyrate as major metabolic substrates. Little glucose is absorbed from the gut. There is constant gluconeogenesis from propionate. Acetate is the main metabolic substrate, sparing glucose for vital functions such as brain metabolism and lactose synthesis. The preferential tissue utilization of acetate, propionate and butyrate has been established. The first step in the metabolism of acetate, propionate and butyrate is the activation reaction, forming the acyl-CoA derivative. Tissue distribution and intracellular localization of acyl-CoA synthetases agrees with results of studies of preferential utilization od acetate, propionate and butyrate. For example, liver does not use acetate because acetyl-CoA synthetase gene is not expressed. To further our knowledge of acetate metabolism it is necessary to study regulation of acetyl-CoA synthetase gene. This requires cloning of acetyl-CoA synthetase. This thesis reports the cloning and characterization of a cDNA encoding acetyl-CoA synthetase.

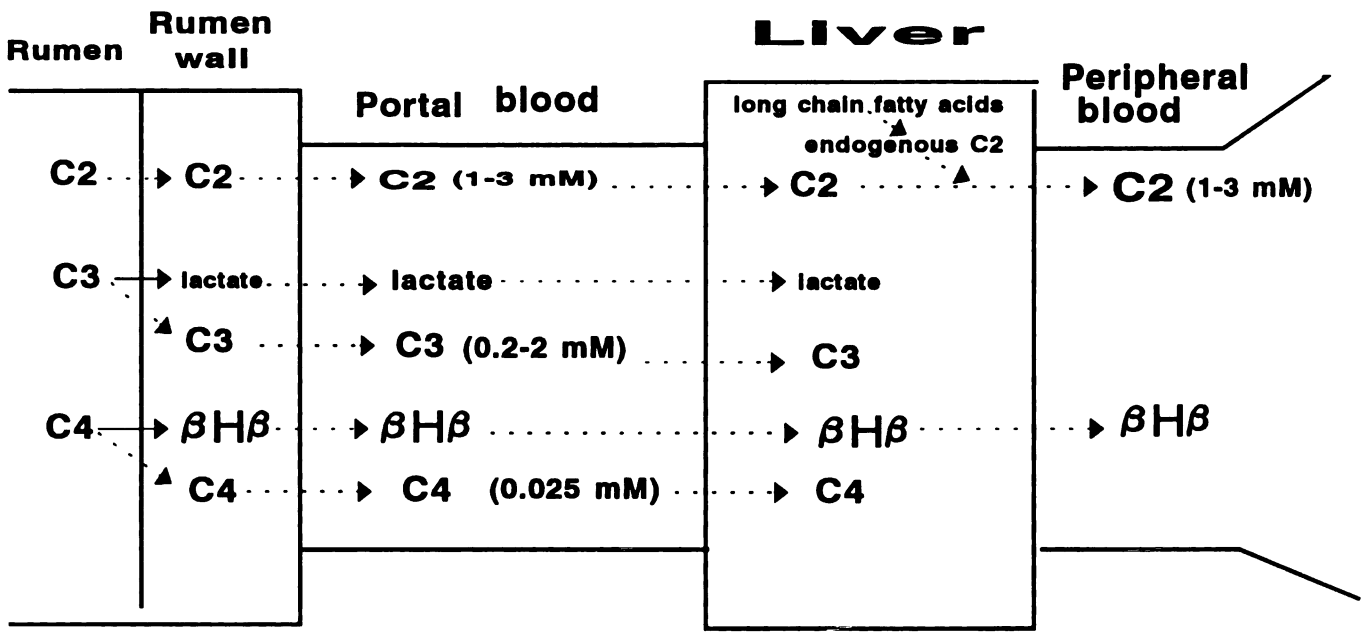


Figure 1. Volatile fatty acids available to the different ruminant tissues

C2 = acetate C3 = propionate C4 = butyrate

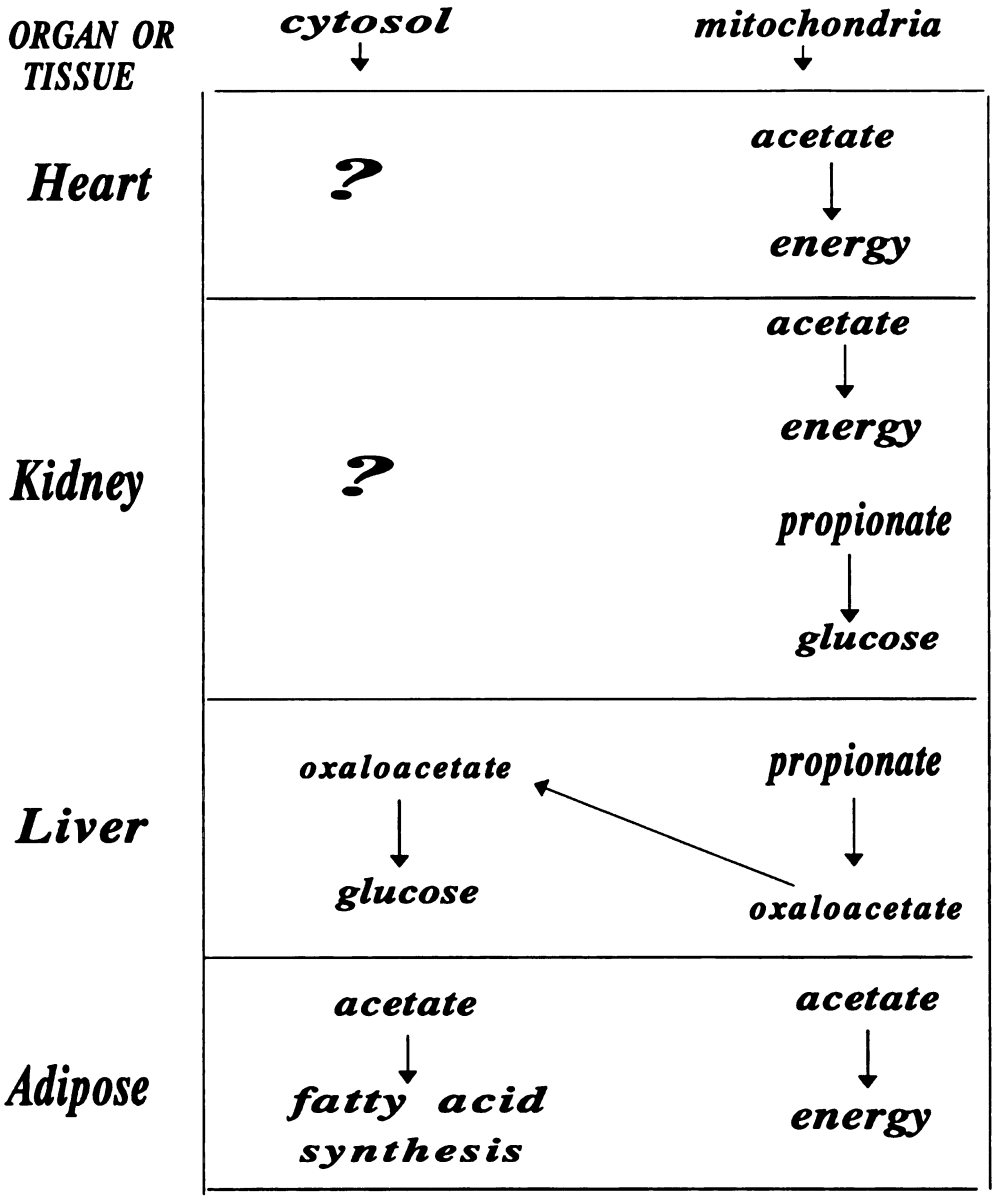


Figure 2. Primary functions of volatile fatty acids in the intermediary metabolism of ruminant heart, kidney, liver and adipose tissue.

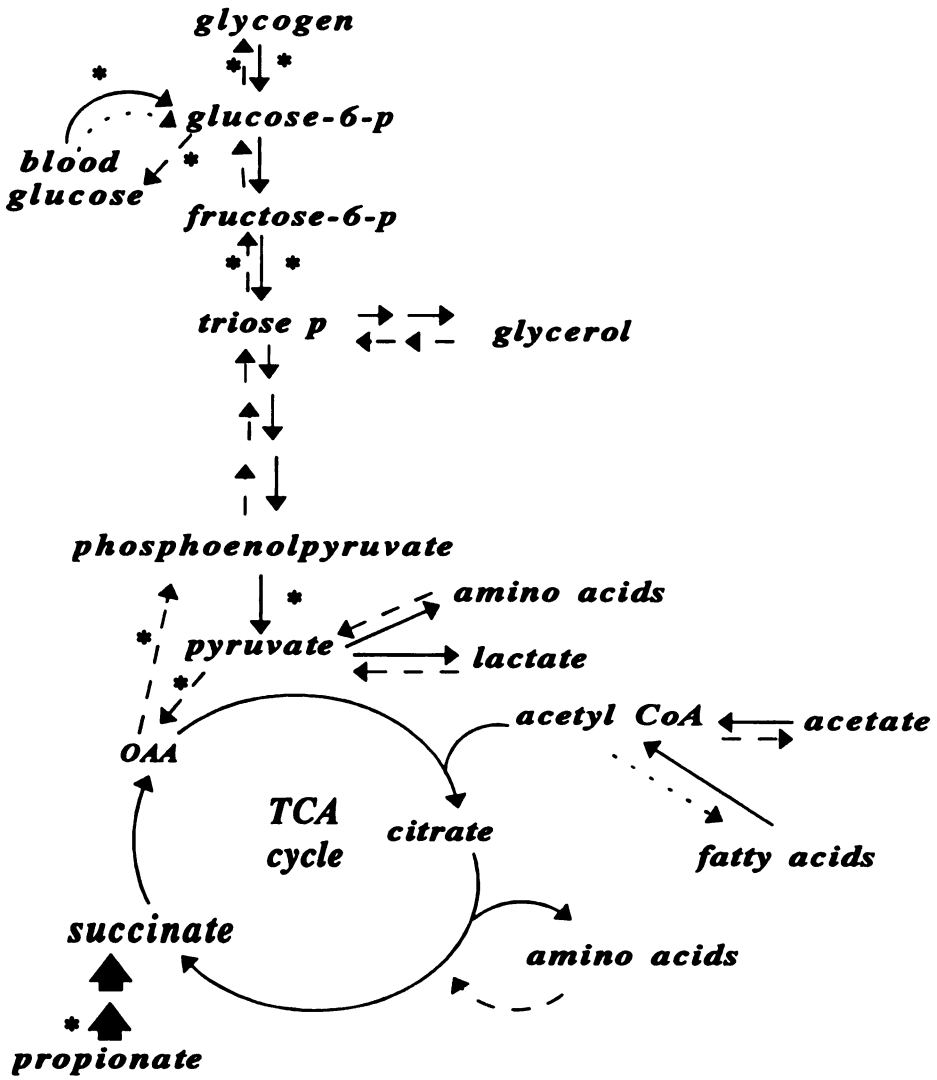


Figure 3. Biochemical pathways of carbohydrate and lipid metabolism operating in the liver of ruminant and non-ruminant animals.

- – – – → ***gluconeogenesis***
- ➡➡ ***ruminants only***
- . . . . . → ***blocked in ruminants***
- * **regulatory enzymes**

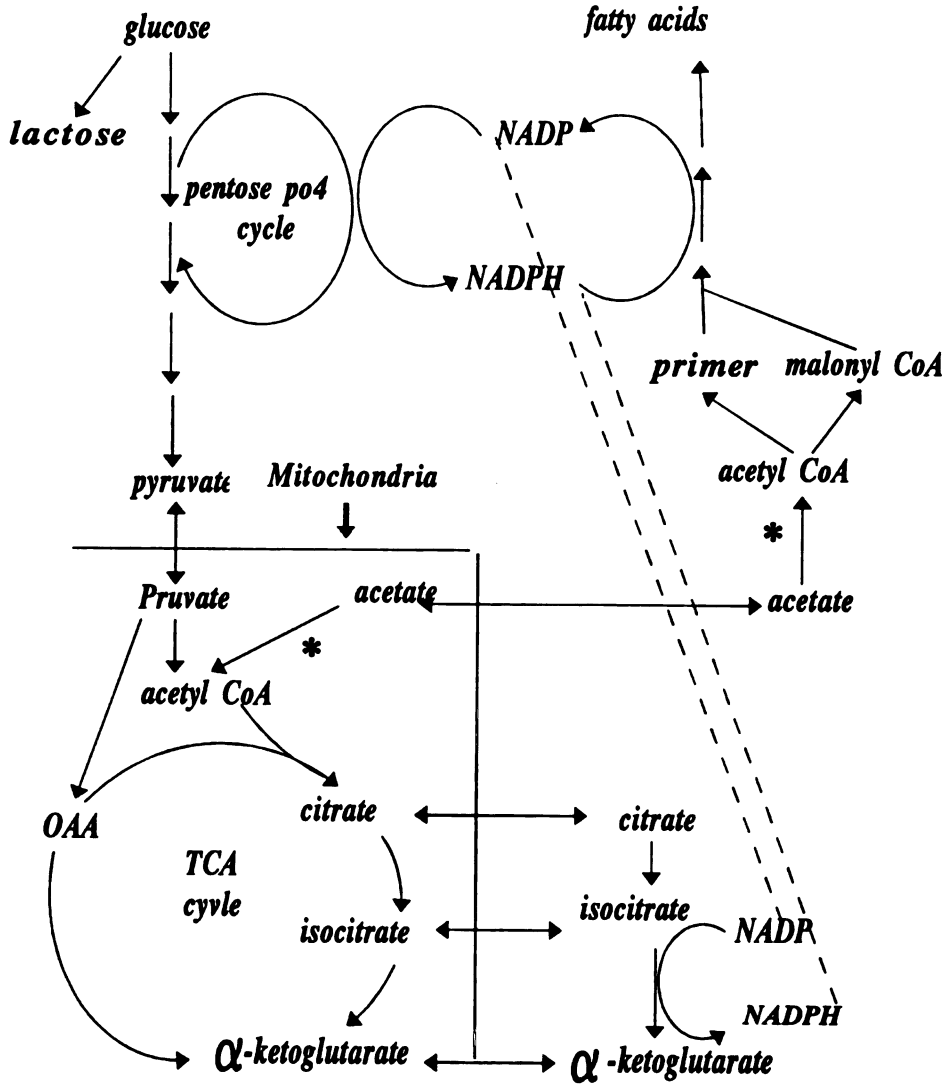


Figure 4. Biochemical pathways of carbohydrate and lipid metabolism in lactating ruminant mammary tissue.

* Acetyl CoA synthetase (absent in dry gland, induced when gland becomes functional).

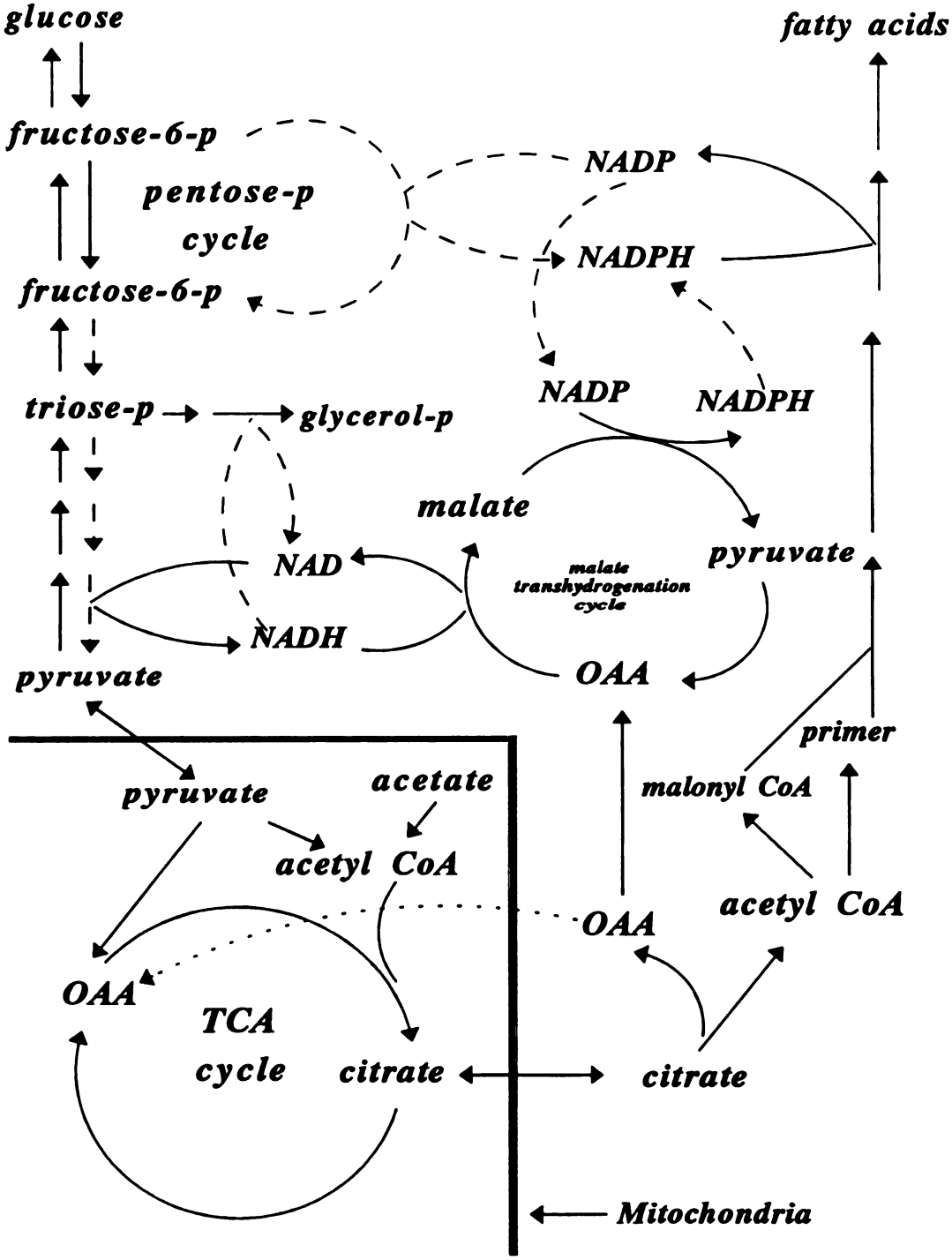


Figure 5. Biochemical pathways of fatty acid synthesis in non-ruminant liver tissue

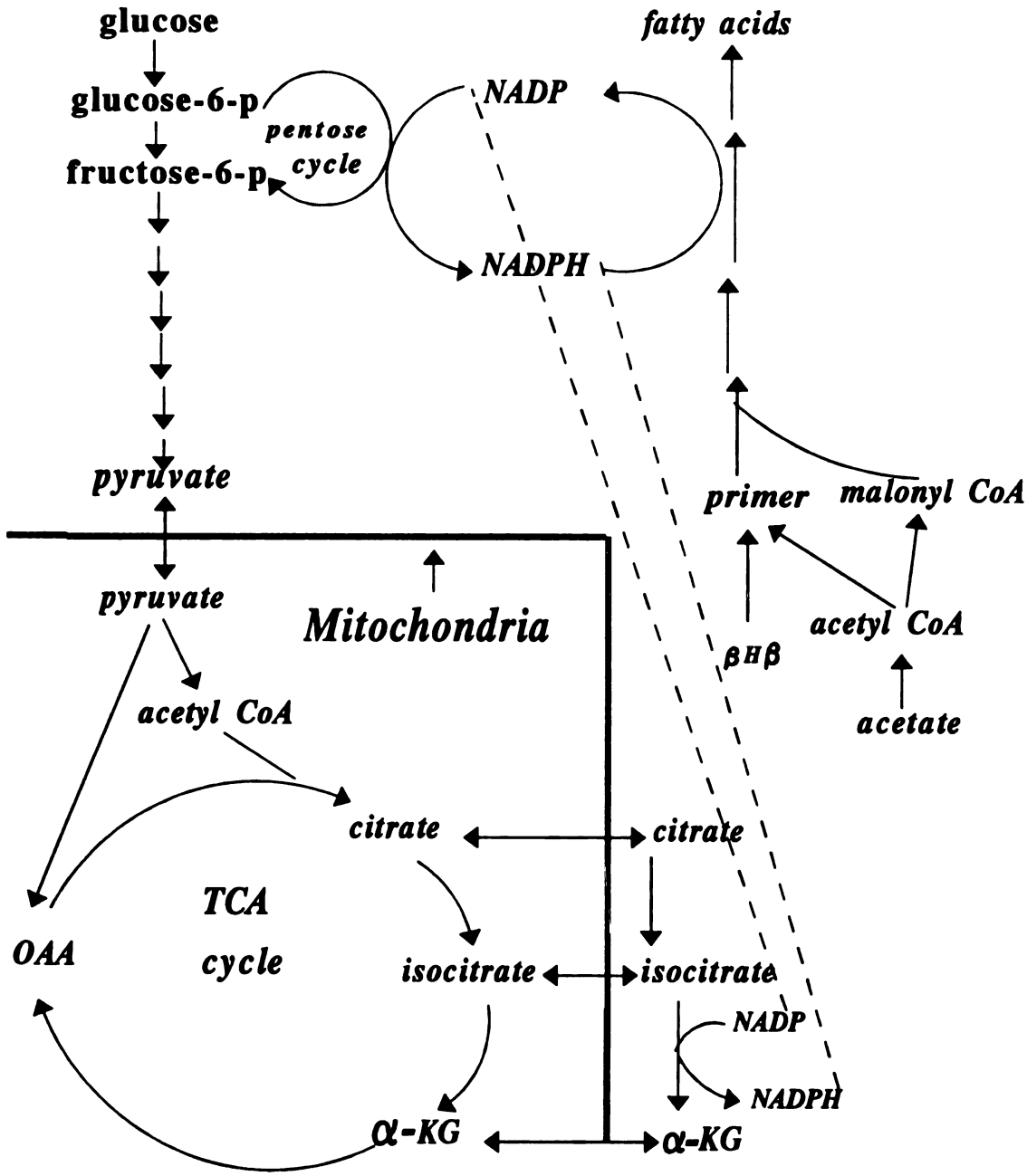


Figure 6. Biochemical pathways of fatty acid synthesis in ruminant adipose tissue

MATERIALS AND METHODS

Extraction of Cow Mammary Gland Total RNA

To avoid the accidental introduction of trace amounts of RNases from potential sources in the laboratory, a number of precautions were used. All glassware was made RNase free by baking at 180°C overnight (at least 12 hr). Sterile, disposable plastic ware, which are RNase free, was used for the preparation and storage of the RNA without pretreatment. The ultra pure water and all reagents (except the ones containing Tris) were treated with 0.1% diethyl pyrocarbonate (DEPC) and sterilized by autoclaving, to insure that they also were RNase free. All solutions and buffers were prepared by using RNase free glassware, and DEPC treated water. Also, a set of chemicals was reserved for work with RNA only (handled with baked spatulas). RNA's were kept on ice at every step except during resuspension of pellets and during column chromatography, both of which were done at room temperature (R.T. = 23-25 °C).

RNA was isolated from frozen (-80 °C) mammary gland tissue by the guanidinium-isothiocyanate cesium chloride procedure described by Glisin et al (1974) and Ullrich et al. (1977). All glassware tubes and reagents were RNase free. Briefly, 4 grams of mammary gland tissue were broken into small pieces under liquid nitrogen and homogenized in 40 ml of homogenization buffer (4 M guanidinium isothio-cyanate, 25 mM Na citrate, pH 7.0, 0.1 M β-mercaptoethanol and 0.5% sarkosyl). To remove the cell debris, the homogenate was centrifuged in sterile centrifuge tubes at 1,500 x g for 5 minutes at room temperature. The supernatant was transferred to a sterile tube and

DNA sheared by passing the supernatant through an 18 gauge needle 12 times. The supernatant was then centrifuged at 5,000 x g at 15°C for 20 minutes. Twelve ml of the supernatant were then layered on 26.5 ml of 5.7 M cesium chloride and 0.1 M EDTA at pH 7.5 and centrifuged at 126 x kg and 20°C for 24 hours using a Beckman SW28 rotor. The supernatant above the CsCl cushion was removed with a sterile pasteur pipette, then all but one ml of CsCl were removed with a second sterile pipette. The centrifuge tube was sectioned just above the remaining CsCl using a razor blade. CsCl was removed and the RNA pellet washed with 70% ethanol and dried in a speedvac. The RNA was dissolved in 0.4 ml of TE buffer, pH 7.6, (10 mM Tris-HCl, pH 7.6, 1 mM EDTA) and transferred to a sterile 2 ml microfuge tube. The solution was extracted once with a 1:1 mixture of chloroform and TE saturated-phenol and once with chloroform. RNA was precipitated by adding 40 μl of RNase-free 3 M sodium acetate, pH 5.2, and 1 ml of ice cold ethanol and held at -70°C for 20 minutes. RNA was collected by centrifuging at 12,000 x g for 15 minutes at 4°C and washed with 70% ethanol and dried for 2 minutes in a speedvac. The RNA pellet was dissolved in 250 μl of TE buffer (pH 7.6) and stored at -70°C. RNA was diluted 100 times with sterile DEPC treated H_2O and the concentration measured in a spectrophotometer at 260 nM. RNA formaldehyde gels were run to ensure that was no significant degradation of the RNA samples before isolation of poladenylated RNA.

Formaldehyde Gel Electrophoresis

This method is adopted from those of Lehrach et al. (1977) and Goldberg (1980). RNA was examined on 1.2% agarose-formaldehyde gels. 1.2 g of agarose were

dissolved in 71.8 ml H_2O and 10 ml of 10 x MOPS (1 x MOPS is 0.04 M MOPS, pH 7.0, 10 mM Na acetate, 1 mM EDTA) by heating in a microwave oven. The solution was cooled to 60°C and 17.8 ml of formaldehyde were added. A 11.5 x 15.5 cm gel was poured and 5 to 25 μg of RNA in 5 μl of 1 x TE buffer were mixed with 15 μl of sample buffer (200 μl 10 x MOPS, 350 μl formaldehyde, and 1 ml of formamide), and heated at 65°C for 15 minutes. Then 4 μl of sterile loading buffer were added (50% glycerol, 1 mM EDTA, 0.4% xylene cyanol and 0.4% bromophenol blue). The electrophoresis buffer was 1 x MOPS. Electrophoresis was at 100 V for 2-2.5 hours. The gel was stained one hour with a solution of 0.1 M ammonium acetate containing 0.5 μg/ml of ethidium bromide, destained for 30 minutes in 0.1 M ammonium acetate and examined under UV light.

Isolation of Polyadenylated RNA

Polyadenylated [Poly(A^+)] RNA was isolated from total RNA by using oligo(dT)-cellulose columns (Pharmacia Cat. # 279258) according to manufacturers instructions. The column was washed once with a solution of 0.1 N NaOH and 0.5 mM EDTA then 2 times with 1 ml of equilibration buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, and 0.5 M NaCl). RNA (600 μg) in 1 ml of TE buffer, pH 7.6, was heated at 65°C for 5 minutes and placed in an ice bath. Then 0.2 ml of high salt buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA and 3 M NaCl) were added. One ml was transferred to the oligo(dT)-cellulose column. The column was centrifuged at 350 g for 2 minutes and then washed two times with 0.25 ml of high salt buffer by centrifuging at 350 g for 2 minutes. The column was then washed 3 times with 0.25 ml of equilibration buffer. To

isolated poly(A^+) RNA the column was eluted 4 times with 250 μl aliquots of TE buffer, pH 7.4, at 65°C. The entire eluate was collected in a RNase-free sterile tube.

Construction of Bovine Mammary gland cDNA Library

First Strand cDNA Synthesis

cDNA was synthesized using a Promega kit (Cat. # C2100). The kit uses the procedure introduced by Okayama and Berg (1982) for the first strand synthesis. Second strand cDNA was synthesized by a modification of Gubler and Hoffman method (Gubler and Hoffman, 1983). Parallel reactions were performed during both first and second strand synthesis reactions so that mass yields could be determined by labeling either the first strand or second strand.

Avian myeloblastosis virus (AMV) reverse transcriptase was diluted 10 fold with a solution containing 10 mM potassium phosphate, pH 7.4, 0.2% Triton X-100, 2 mM dithiothreitol (DTT), and 10% glycerol, and kept on ice 30 minutes before initiating the reaction. Five μg of poly(A^+) RNA were annealed to 2.5 μg of oligo dT_{15} primer by heating at 70°C for 5 minutes then cooled to room temperature. Five μl of a solution containing 250 mM Tris-HCl, pH 8.3, 250 mM KCl, 50 mM Mg Cl_2, 2.5 mM spermidine, 50 mM DTT, 5 mM each of dATP, dCTP, dGTP, and dTTP were added followed by 25 units of RNasin (ribonuclease inhibitor), 2.5 μl of 40 mM sodium pyrophosphate (preheated to 70°C), 75 units of AMV reverse transcriptase, and sterile DEPC treated water to a final volume of 25 μl. The reaction was carried out at 42°C for 60 minutes and stopped by placing the tubes on ice. (To follow first strand synthesis, 5 μl of the reaction mixture were incubated with 5 μCi of [α^{32}P] dCTP. Ninety-four μl

of 50 mM EDTA were added to the tube containing $\alpha^{32}P$. This solution was passed through Sephadex G-50 to remove [$\alpha^{32}P$]dCTP and checked for first strand synthetase using alkaline gel electrophoresis and autoradiography).

Second Strand cDNA synthesis

The second strand of cDNA was synthesized by adding to the reaction mixture 10 μl of a solution containing 500 mM Tris-HCl, pH 7.2, 900 mM KCl, 30 mM Mg Cl_2, 30 mM DTT, and 0.5 mg/ml of BSA followed by 23 units of *E. coli* polymerase 1, 0.8 units of *E. coli* RNase H and sterile DEPC treated water to a final volume of 100 μl. The reaction was carried out at 14°C for 4 hours. (To follow second strand synthesis, 10 μl of the reaction mixture were transferred to a tube containing 5 μCi of [$\alpha^{32}P$] dCTP and incubated as above. Ninety μl of 50 mM EDTA were then added to the tube containing ^{32}P and this reaction mixture was examined for second strand synthesis.)

T4 Polymerase Blunting of cDNA

To produce cDNA with blunt ends, the double stranded cDNA was then heated at 70°C for 10 minutes and centrifuged briefly in a microfuge for 4 seconds at 12,000 x g at room temperature, and then incubated at 37°C for 10 minutes in the presence of 10 units of T_4 DNA polymerase. The reaction was stopped by placing the reaction mixture on ice and adding 10 μl of 200 mM EDTA. The reaction mixture was extracted with an equal volume of a 1:1 mixture of chloroform and phenol equilibrated with TE buffer followed by extraction with chloroform. The cDNA was precipitated from the aqueous phase by adding 0.1 volumes of 2.5 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold ethanol and allowed to stand 2 hours at -20°C followed by centrifuging 15 minutes

at 4°C. The cDNA pellet was washed with 70% cold ethanol, dried in a speedvac, dissolved in TE buffer, pH 7.6, and the yield measured at 260 nm using a spectrophotometer.

Alkaline Gel Electrophoresis

Alkaline agarose gels (McDonell et al., 1977) are used to check the size of the first and second strands of cDNA synthesize by reverse transcriptase. 0.9% agarose was dissolved in water and cooled to 60°C. NaOH was added to a final concentration of 50 mM and EDTA to 1 mM and the solution poured in the gel casset (12 x 15 cm). After the solution solidified the comb was removed and the gel covered with 1 x alkaline buffer (50mM NaOH, 1 mM EDTA, pH 8.0). The DNA sample (100 μl) was precipitated and dissolved in a solution containing 20 μl of 50 mM NaOH, and 1 mM EDTA. Then 4 μl of sample loading buffer (60 mM NaOH, 1.2 mM EDTA, 3.6% ficoll 400, 0.03% bromophenol green, 0.05% xylene cyanol FF) were added to the DNA. The DNA was placed in the wells (0.5 cm in width) and electrophoresis (50 V) was carried out until the dye had migrated approximately two thirds of the length of the gel. The gel was removed and placed in 7% trichloroacetic acid (TCA) for 30 minutes at room temperature and then dried overnight under several layers of paper towels. The dried gel was wrapped in Saran Wrap and examined using autoradiography at -70°C.

Ligation of EcoRI Adaptors to Double Stranded cDNA

Small cDNA fragments were removed using Sephacryl S-400. The reaction mixture to ligate EcoRI adaptors to the cDNA was 3 μl of a solution containing 300 mM Tris-HCl, pH 7.8, 100 mM Mg Cl_2, 100 mM DTT, 10 mM ATP, 3 μl BSA (1 mg/ml), 1 μl

of cDNA (234 ng), 1 μl of a solution containing 1 pmol of EcoRI adaptors

($^{5'}$A A T T C C G T T G C T G T CG$^{3'}$

$^{3'}$G G C A A C G A C A GC$_p$$^{5'}$),

7.5 Weiss units (Weiss et al., 1968) of T_4 DNA ligase (1.5 μl), and sterile deionized H_2O to a final volume of 30 μl. The reaction was carried out for 18 hours at 15°C followed by heating at 70°C for 10 minutes. The reaction mixture was cooled to room temperature. To phosphorylate the 5' end of the EcoRI adaptors, the following was added to the reaction mixture: 4 μl of a solution containing 700 mM Tris-HCl, pH 7.6, 100 mM Mg Cl_2, and 50 mM DTT, 2 μl of ATP (0.2 nmol), 10 units of T_4 polynucleotide kinase, and sterile deionized H_2O to a final volume of 40 μl. The reaction was carried out at 37°C for 30 minutes, then 60 μl of TE buffer, pH 7.6, were added and the reaction mixture was extracted one time with a 1:1 mixture of chloroform and phenol equilibrated with TE buffer and one time with chloroform. Unligated EcoRl adaptors were removed by using Sephacryl S-400 column. The cDNA was precipitated with Na acetate cold ethanol, washed with 70% ethanol, dried in speedvac and dissolved in 5 μl of 1 x TE buffer, pH 7.6.

Ligation of cDNA into λgt11

Sephacryl-400 purified cDNA was ligated into the EcoRI site of λgt11 (Young and Davis 1983 a,b) the reaction mixture contained 2 μl of a solution of 300 mM Tris-HCl, pH 7.8, 100 mM Mg Cl_2, 100 mM DTT, 10 mM ATP, 1 μl of cDNA, 1 μl of EcoRI cut, dephosphorylated λgt11 DNA (0.5 μg), 1 Weiss unit of T_4 DNA ligase and sterile deionized water to a final volume of 10 μl. The reaction was carried out at 22°C for 3

hours. Fifty μl of Packgene extract (Promega Cat. # K3154) were added and the solution incubated for 2 hours at 22°C. A solution containing 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 10 mM Mg SO_4 and 25 μl of chloroform were added. The packaged DNA was stored at 4°C.

Titration of cDNA library

The titer of λgt11 phage was determined by standard procedures using *E. coli* Y1090r$^-$ (Promega packgene kit). To demonstrate recombinant phage, 100 μl of diluted (10^2-10^7) λgt11 were incubated with 100 μl (2 X 10^8 cells) of *E. coli* Y1090r$^-$ for 20 minutes at 37°C. The phage-infected *E. coli* were added to 4 ml of sterile top agarose (10 g bacto tryptone, 5 g bacto yeast extract, 10 g NaCl, and 8 g of agarose in 1 liter of deionized H_2O) which contained 4 μl ampicillin (50 μg/ml of H_2O), 40 μl of 100 mM isopropyl β-D-thiogalactopyranoside (IPTG) in H_2O, 40 μl of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in dimethyl-formamide. The top agarose at 50°C was layered onto a 90 mm Petri dish containing 25 ml of low-agar-ampicillin (10 g bacto tryptone, 5 g bacto yeast extract, 15 g bacto agar, and 5 g NaCl per liter of deionized water which was autoclaved and cooled to 50°C before adding ampicillin at 50 μg/ml). The plates were incubated overnight at 37°C. Each agar plate had about the same number of clear and blue-colored plaques indicating that about 50% of the plaque forming units were recombinant, as expected.

cDNA Library Amplification

The library was amplified by the plate lysate method using the restriction minus host Y1090rk$^-$. Recombinant λgt11 phage library in *E. coli* 1090 was plated on 90 mm agar

plates at 5 x 10^4 recombinants per plate, and incubated at 43°C until the plaques appear and merge (9 hours). The plates were cooled to 4°C and each plate overlayed with 4 ml (SM) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 8 mM $MgSO_4$, .01% gelatin) and incubated at 4°C overnight. The SM buffer was removed and each plate was washed with 1 ml of SM buffer. The collected SM buffer was aliquoted in 1.5 ml eppendorf tubes containing 25 μl of chloroform and stored at 4°C (Sambrook et al., 1989). The amplified phage was titered as previously described, and DMSO was added to a final concentration of 7% and the library was stored at -80 C.

Preparation of Rabbit anti-Acetyl CoA Synthetase

Acetyl CoA synthetase was purified from a bovine mammary gland taken 60 days post-partum as previously described (Qureshi and Cook, 1975; Ricks and Cook, 1981). The procedure involved $(NH_4)_2SO_4$ fractionation of a mitochondrial extract followed by column chromatography using DEAE-cellulose and calcium phosphate gel. Fifty μg of acetyl CoA synthetase in 300 μl of potassium phosphate buffer, pH 7.0, and 300 μl of Freund's complete adjuvant (Sigma Chemical Co. F5881) were injected into New Zealand White rabbits. Second and third injections were at 2 week intervals using half the amount in the first injection and 150 μl of Freund's incomplete adjuvant (Sigma Chemical Co. F5506). Blood serum was collected two and four weeks after the third injection. IgG was purified using DEAE Affi-Gel Blue Gel (Bio-Rad 153-7307).

Screening of Mammary Gland λgt11 Library With Rabbit Anti-Holstein ACS IgG

This procedure was adapted from Mierendorf et al (1987). The λgt11 library was screened with rabbit anti-Holstein mammary gland ACS and goat anti-rabbit gamma

globulin containing alkaline phosphatase.

One ml of an overnight culture of *E. coli* (Y1090r) was added to 50 ml of LB media, pH 7.5, (10 g bacto-tryptone, 5 g bacto-yeast, 5 g NaCl, 10 mM $MgCl_2$, 50 μg/ml of ampicillin and 0.2% maltose, in one liter of sterile water), and incubated aerobically at 37°C until the O.D. at 600 nm was 0.5. This requires about 3 hours. Then 0.6 ml (10^8 cells) were incubated with 10^5 λgt11 for 20 minutes at room temperature. Infected *E. coli* Y1090r$^-$ were mixed with 7.5 ml of top agarose, poured on a 150 mm agar plate, and incubated at 42°C for 4 hours. The plate was overlaid with a dry nitrocellulose filter which previously had been saturated with 10 mM IPTG in sterile deionized water. The plates were then incubated at 37°C for 8 hours. The nitrocellulose filter was washed with a Tris-buffered saline plus Tween 20 (TBST) (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) and then blocked with 1% BSA in TBST. The filter was incubated with 7.5 ml of rabbit anti-Holstein ACS for 30 minutes and then washed 3 times with 20 ml aliquots of TBST. Each wash was for 5 minutes at room temperature. The filter was then incubated at room temperature for 30 minutes with 15 ml of TBST containing a 1:7,500 dilution of goat anti-rabbit gamma globulin-alkaline phosphatase conjugate. The filter was washed three times with 20 ml aliquots of TBST. To identify plaques with ACS fusion protein, the filter was incubated with 10 ml of solution containing 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM Mg Cl_2, 66 μl of nitro blue tetrazalium (50 mg/ml in 70% dimethyl formamide), and 33 μl of 5-bromo-4-chloro-3 indolyl phosphate (50 mg/ml in dimethyl formamide) and incubated in the dark at room temperature for 35 minutes. Positive plaques appear as

purple dots on the nitrocellulose filter. Approximately 750,000 plaques were screened.

ACS Enzyme Assay

Acetyl coenzyme A synthetase was assayed using the acetate dependent disappearance of the sulfhydryl group of coenzyme A as described by Qureshi et al. (1975). In a total volume of 0.2 ml the reaction mixture contained 5 μmol of $MgCl_2$, .17 μmol of coenzyme A, 16 μmol of Tris-HCl, pH 8.6, and 5 to 10 μg of enzyme protein. Blank tubes did not contain coenzyme A and standard tubes did not contain acetate. The enzyme assay was carried out at 37°C for 10 minutes. The reaction was stopped by adding 2.8 ml of nitroprusside reagent prepared according to Grunert and Phillips (1951) and the optical density at 520 nm recorded exactly after 30 seconds. The enzyme protein was adjusted to an optical density difference between standard and complete reaction tubes of 0.075 to 0.250. An optical density difference of 0.185 is equivalent to the disappearance of 0.1 μmol of coenzyme A. One unit of enzyme activity is defined as the activation of 1 μmol of acetate per hour. Specific enzyme activity is expressed as units/mg of protein.

ACS activity of fusion proteins AR8 and ATC5 was determined in protein isolated from *E. coli* Y1089r$^-$ grown in the presence of λgt11 carrying AR8 or ATC5 cDNA. Briefly, *E. coli* Y1089r$^-$ was grown in LB medium at 37°C to late log phase. Then, *E. coli* Y1089r$^-$ was infected with λgt11 at a multiplicity of infection (MOI) of 5 for 20 minutes at 32°C, added to top agar and plated as previously described. Colonies were isolated and tested at 32 and 42°C to determine lysogens. Then, 100 μl of liquid broth media were inoculated with a single colony and incubated at 32°C to OD of 0.5 at 600

nm, followed by incubation at 42°C for 20 minutes. The culture was further incubated aerobically at 37°C for 4 hours in the presence of 10 mM IPTG. The culture was centrifuged at 3,000 x g for 5 minutes at 24°C and resuspended in TEP buffer (100 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) and stored at -70°C. The cells were lysed by freezing and thawing 4 times followed by sonication for 10 minutes in a waterbath sonicator (Branson Ultrasonic Corporation Cat. # 26373). The extract was centrifuged at 11,000 x g for 10 minutes and the supernatant taken for enzyme assay.

Isolation of λgt11 AR8 and AT5 DNAs by Using PEG 8000

Ten ml of LB medium were inoculated with *E. coli* Y1090r⁻ and incubated at 37°C overnight under aerobic conditions. Prewarmed LB medium (50 ml) was inoculated with 1 ml of the overnight culture and incubated at 37°C under aerobic conditions to OD of 0.5 at 600 nm (3-4 hours). Five ml of the bacteria were inoculated with 10^{10} pfu of λgT11, incubated at 37°C for 20 minutes, then added to 500 ml of prewarmed LB medium. The flask was incubated at 37°C under aerobic conditions until lysis occurred (4-6 hours). Ten ml of chloroform were added and the flask was incubated at 37°C under aerobic conditions for 15 minutes. The culture was cooled to room temperature and five hundred μg of pancreatic DNase I and 500 μg of RNase were added to 500 ml of lysed culture and incubated 30 minutes at room temperature. Solid NaCl was added to a final concentration of 1 M. The flask was placed on ice for 1 hour and the contents centrifuged at 11,000 x g for 10 minutes at 4°C. To isolate λgt11, solid polyethylene glycol 8,000 (PEG) was added to the supernatant at room temperature to a final

concentration of 10% (w/v) and the material was stored overnight at 4°C. PEG was collected by centrifuging at 11,000 x g for 10 minutes at 4°C (Yamamoto et al., 1970). The PEG pellet was suspended in 5 ml of TM buffer (50 mM Tris-HCl, pH 7.8, and 10 mM Mg SO_4) and λgt11 was removed from the PEG by extracting with an equal volume of chloroform. Fifty μl of chloroform were added to the aqueous phase which contains λgt11 and the material was stored at 4°C. Titers were determined as previously described.

Purifying λgt11 DNA Using Ultracentrifugation in a Glycerol Gradient

λgt11 was purified by centrifuging in a glycerol gradient. Eight ml of 5% glycerol in TM buffer were layered over 6 ml of 40% glycerol in TM buffer. Thirteen ml of λgt11 in TM buffer were layered over the 5% glycerol and the contents were centrifuged at 141,000 x g for 2.5 hours at 4°C. The supernatant was discarded and the λgt11 pellet was suspended in 0.5 ml of TM buffer and transferred to a microcentrifuge tube. To remove any *E. coli* DNA and RNA, pancreatic DNase and RNase were added to a final concentration of 5 μg/ml and 1 μg/ml, respectively. The reaction mixture was incubated at 37°C for 30 minutes. EDTA, pH 8.0, was added to a final concentration of 20 mM. The reaction mixture was then digested 1 hour at 56°C with proteinase K at a concentration of 0.5 μg/ml in the presence of 0.5% SDS. After digestion, the reaction mixture was cooled to room temperature and extracted once with phenol saturated with TE buffer, once with a 1:1 mixture of chloroform and phenol saturated with TE buffer, and finally once with chloroform. The aqueous phase was dialyzed overnight at 4°C against 3 liters of TE buffer, pH 8.0 (Vande Woude et al., 1979). The λgt11 DNA was

then ethanol precipitated as previously described.

DNA Restriction Digest

The reaction mixture used to isolate cDNA from λgt11 DNA contained 1 μl of λgt11 (0.28 μg), 1 μl of a buffer containing 10 mM Tris-HCl, pH 7.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM dithioerythritol, 0.2% (v/v) Triton X-100, and 50% (v/v) glycerol, 1 μl of EcoRI (8-12 units), and 7 μl of sterile deionized water. The reaction was carried out at 37°C for 1 hour. Then 2 μl of gel loading buffer were added that contained 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 15% ficoll 400 in sterile deionized water. The reaction mixture was heated 5 minutes at 65°C and the entire mixture subjected to agarose gel electrophoresis. This procedure was used for all restriction enzyme digests except that a buffer appropriate for the particular restriction enzyme was used.

DNA Electrophoresis

For electrophoresis of DNA, a 0.8% agarose solution was prepared in TBE buffer (0.089 M Tris-base, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) by heating in a microwave oven. The solution was cooled to 50°C and ethidium bromide added to a final concentration of 0.5 μg/ml. Seventy ml of the solution were poured into a 11 x 9.5 cm plate using a 10-well comb to sample wells 0.7 cm wide. After the solution gelled, the comb was removed and TBE electrophoresis buffer was added to the apparatus to a height of about 0.25 ml above the gel. DNA samples were placed in the sample wells and a potential difference of 50 V applied until the tracking dye moved about two thirds of the length of the gel (Sambrook et al.,1989). The gel was examined under UV light.

Gels were photographed with a polaroid camera.

DNA Elution

The section of agarose gel containing the DNA to be examined was placed in the slot of an electro-eluter (IBI UEA Cat. # 46000). TBE buffer was added and air bubbles were removed from the DNA collection channels. Seventy μl of solution containing 10 mM Na acetate and 0.025% bromophenol blue were placed in the DNA collecting channel. A potential difference of 100 V was applied for 30 minutes. The electro-eluted DNA was transferred to a microcentrifuge tube and precipitated with absolute ethanol for 10 minutes at room temperature, washed with 300 μl of 70% ethanol, dried in a speedvac, and dissolved in minimal volume of TE buffer pH 7.6.

Subcloning cDNA into pUC19

One μg of pUC19 was digested with 10 units (1 μl) of EcoRI in a total volume of 10 μl for one hour at 37°C. Then 1 μl (one unit) of calf intestinal alkaline phosphatase and 2 μl of dephosphorylation buffer (500 mM Tris-HCl, pH 8.5, 1 mM EDTA) were added and the total volume adjusted to 20 μl with sterile deionized water. The reaction mixture was incubated 15 minutes at 37°C and then extracted once with a 1:1 mixture of chloroform and phenol equilibrated with TE buffer and once with chloroform. DNA in the aqueous phase was ethanol precipitated as previously described, washed once with 70% ethanol, dried 1 minute using a speedvac, and dissolved in 10 μl of TE buffer, pH 7.6. Two μl (200 ng) of this pUC19 were examined using 0.8% agarose gel to confirm that pUC19 was digested with EcoRI. Then 200 ng were added to 100 ng of cDNA with EcoRI ends, the volume adjusted to 8 μl, and the contents incubated 5 minutes at 45°C.

One μl of 10 x T_4 DNA ligase buffer and 1 Weiss unit (1 μl) of T_4 DNA ligase were added and the tube incubated 4 hours at 16°C. The DNA was then precipitated with ethanol as previously described, washed, dried in a speedvac, and dissolved in 5 μl of sterile deionized water.

Transformation of *E. coli* DH5α with pUC19

Subcloning efficiency DH5α competent cells (BRL cat. # 8265SA) were used for routine subcolning into plasmid vectors according to the manufactuer's procedure. *E. coli* DH5α stored at -70°C was thawed on ice. Five μl of pUC19 containing cDNA were added to 50 μl of *E. coli* DH5α, mixed gently with the pipet tip, and incubated 30 minutes on ice. The cells were heat shocked by incubating 20 seconds at 37°C followed by incubation 2 minutes on ice. Then 950 μl of LB medium were added and the tube incubated 1 hour at 37°C. The cells were isolated by centrifuging 20 seconds at 3,000 x g. Eight hundred μl of supernatant were removed and the cells were suspended in the remaining 200 μl of LB medium. To isolate cells containing recombinant pUC19, 40, 60, and 100 μl of the cell suspension were spread on agar plates containing IPTG, X-Gal, and ampicillin using a sterile glass rod and incubated overnight at 37°C. Positive colonies were selected and cultured in 5 ml of LB medium containing ampicillin (50 μg/ml), and then incubated aerobically overnight at 37°C. The DNA mini prep described above was used to amplify and further purify. The plasmid was digested with EcoRI and the cDNA examined using 0.8% agarose to confirm the presence of cDNA.

Small Scale Plasmid DNA Preparation

This protocol is a modification of the methods of Brinboim and Doly (1979) and

Ish-Horowicz and Burke (1981). Fifteen ml of LB media containing 50 μg/ml ampicillin were inoculated with *E. coli* DH5-α infected with recombinant plasmid (pUC19) and grown overnight at 37°C in an orbital rocker. Then 1.5 ml were centrifuged at 12,000 x g for 2 minutes in a microcentrifuge. The supernatant was discarded and an additional 1.5 ml of culture added followed by centrifuging 2 minutes. The cells were suspended in 100 ml of a solution containing 50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8.0. After standing 5 minutes at room temperature, 200 μl of a freshly prepared solution containing 0.2 M NaOH and 1% sodium dodecyl sulfate (SDS) were added and the tube placed in ice for 5 minutes. Then 150 μl of ice cold 3 M potassium acetate, pH 4.8, were added and the contents mixed by briefly vortexing and then incubated on ice for 5 minutes. The contents were centrifuged at 12,000 x g for 5 minutes and the supernatant transferred to a clean microcentrifuge tube and centrifuged at 12,000 x g for 5 minutes. The supernatant was transferred to a clean tube and RNase was added to a final concentration of 50 μg/ml. The tube was incubated 30 minutes at 37°C. The sample was extracted two times with a 1:1 mixture of chloroform and phenol equilibrated with TE buffer and once with chloroform. DNA was precipitated by adding 0.1 volume of 3 M sodium acetate, pH 5,2, and 1 ml of ice cold ethanol kept at -20°C, and left at -20°C overnight. The tube was then centrifuged at 12,000 x g for 10 minutes. The pellet was washed twice with 70% ethanol and dried for 5-10 minutes in a speedvac. DNA was dissolved in 16.8 μl of water, then 3.2 μl of 5 M NaCl were added. To further purify the DNA, 20 μl of a 13% aqueous solution of PEG 8,000 were added, mixed gently, and the contents incubated on ice in a cold room for 1 hour. The sample

was centrifuged at 12,000 x g for 10 minutes at 4°C. The DNA pellet was washed once with 1 ml of ice cold 70% ethanol, dried in a speedvac, and then dissolved in 20 μl of water. This DNA sample was sequenced using Sequenase (Version 2.0, U.S. Biochemicals).

Random primer labeling of DNA fragments

DNA fragments were routinely labeled to high specific activity by the random hexamer priming method (Feinberg and Vogelstein, 1983). A kit based on this method was obtained from Boehringer Mannheim labeling (kit # 1004760) and used as described by the manufacturers. 50 ng of DNA in 9 μl of sterile deionized water were heated at 95°C for 10 minutes and cooled in ice. The complete reaction mixture contained 50 ng DNA in 9 μl, 2 μl of random primer buffer (2 mM Tris-HCl pH 7.0, 0.2 mM EDTA, 4 mg/ml BSA, 2 M N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) pH 6.6 and random hexanucleotide primers), 3 μl of a mixture of dATP, dGTP, and dTTP each at .5 mM, 5 μl of 3.3 mM [α^{32}P]dCTP containing 50 μCi (3,000 Ci/mmol), and 1 μl (2 units) of Klenow enzyme (exonuclease free). The reaction was carried out at 37°C for 1 hour and stopped by adding 2 μl of 200 mM EDTA. Free [α^{32}P]dCTP was removed from the reaction mixture using Sephadex G-50 spun-column chromatography.

Spun-Column Chromatography

Sterile glass wool was placed in the bottom of a 1 ml plastic syringe and the syringe filled with either Sephadex G-50 (for removing [α^{32}P]dCTP in DNA labeling reactions) or Sephacryl S-400 (for size fractionation of cDNA) in TE buffer pH 8.0. The syringe

was placed in a 15 ml plastic centrifuge tube and centrifuged at 1,600 x g for 3 minutes using a swinging bucket rotor. This process was repeated until the volume of the packed column was 0.9 ml. The column was equilibrated 3 times with the addition of 0.1 ml aliquots of TE buffer pH 8.0. DNA in 0.1 ml was placed on the column and collected in a microcentrifuge tube by centrifuging at 1,600 x g for 3 minutes (Sambrook et al., 1989).

Screening cDNA Library with ^{32}P-Labeled DNA Probes

Oligonucleotide screening was performed as described by Grunstein and Hogness (1975) Agar plates containing *E. coli* (Y1090r$^-$) infected with λgt11 were incubated overnight at 37°C then at 4°C for 2 hours before dry nitrocellulose filters were placed on the agar. After 30 to 60 seconds, the filters were removed using blunt ended forceps. The filters were then placed colony side up on 3 mm Whatman chromatography paper previously saturated with 10% aqueous SDS. After 3 minutes, the filter was placed on 3 mm paper saturated with denaturing solution (0.5 M NaOH and 1.5 M NaCl in H_2O). After 5 minutes the nitrocellulose filter was transferred to 3 mm paper saturated with neutralizing solution (1.5 M NaCl and 0.5 M Tris-HCl pH 7.4). After 5 minutes the filter was transferred to a final 3 mm paper saturated with 2 x SSC (1 x SSC is 150 mM NaCl, 15 mM Na citrate pH 7.0). After 5 minutes the nitrocellulose filter was removed from the 3 mm paper, dried at room temperature, and baked in a vacuum oven for two hours at 80°C (Benton and Davis 1979). The filter was then placed in 2 x SSC for 5 minutes followed by washing in a prewashing solution containing 5 x SSC, 0.5% SDS, and 1 mM EDTA. To reduce background, bacterial debris was gently removed from the

surface of the nitrocellulose filter using lintless tissue premoisturized with prewashing solution.

Each filter was incubated in 15-20 ml prehybridization solution [28.4 ml H_2O, 50 ml formamide, 16.6 ml 6 x SSC, 5 ml of 1 x Blotto (5% non-fat dried milk dissolved in water containing 0.02% sodium azide) and poly(A^+) to a concentration of 1 μg/ml] for at least 4 hours at 42°C. The ^{32}P-labeled double stranded DNA was denatured by heating for 5 minutes at 100°C and then placed on ice. The denatured probe was added to the prehybridization solution containing the filter and incubated overnight at 42°C. The filter was then washed two times with 2 x SSC and 0.1% SDS for 15 minutes at room temperature, then one time with a solution of .1% SDS in .1 x SSC for 30 minutes at 68°C. The filters were dried at room temperature, wrapped in Saran Wrap, and exposed to Kodak XR film for 24 to 48 hours at -70 C with an intensifying screen (Sambrook et al., 1989). Plaques which gave postive signals on the filters were isolated and rescreened until plaque purified.

Isolation of Genomic DNA

Two g of mammary gland tissue were taken from a Holstein cow 60 days post-partum, minced, and placed in 0.7 ml of lysis buffer (0.05 m Tris-HCl, pH 8.0, 0.1 M EDTA, 0.5% SDS, and 350 μg of proteinase K) and incubated 14 hours at 55°C with gentle mixing. Then 0.7 ml of phenol saturated with TE were added and the tube gently mixed for 3 minutes and centrifuged at 12,000 x g for 3 minutes at room temperature. The aqueous phase was extracted with 0.7 ml of a 1:1 mixture of chloroform and phenol saturated with TE buffer, pH 8, by mixing gently for 2 minutes and then centrifuged for

3 minutes. The aqueous phase was transferred to a 1.5 ml centrifuge tube and DNA was precipitated by adding 70 μl of Na acetate (pH 6.0) and 1.4 ml of 100% ethanol. After standing at room temperature for 5 minutes, the tube was centrifuged for 30 seconds at room temperature and the DNA precipitate suspended in 1 ml of 70% ethanol and centrifuged again for 1 minute. The DNA was dried in a speedvac for 2 minutes and then dissolved in 0.1 ml of TE buffer, pH 7.6, by incubating at room temperature overnight and then stored at 4°C (Blin and Stafford, 1976).

Southern Blots

DNA was subjected to agarose gel electrophoresis as previously described. The gel was then gently agitated for 30 minutes in 0.4 M NaOH. A Zeta-Probe membrane (Bio-Rad) having the same dimensions as the gel was placed in 0.4 M NaOH for 15 minutes. An empty pipette tip box was placed in a plastic container containing transfer solution (0.4 m NaOH) then a plexiglass plate was placed on top of the box. A piece of Whatman 3 mm paper was placed on the plexiglass plate such that the side of the paper extended into the transfer solution. The 3 mm paper was equilibrated with transfer solution. The agarose gel was then inverted and placed on the 3 mm paper. Air bubbles were removed using gentle pressure. Next the Zeta-Probe membrane was placed on top of the gel and air bubbles removed. The membrane was covered with 2 layers of 3 mm filter paper previously equilibrated with 0.4 m NaOH. Air bubbles were removed by gently rolling a pasture pipette over the 3 mm filter paper. A stack of paper towels was cut just smaller than the 3 mm paper and placed on the 3 mm paper. The area of the gel was held in place on top of the filter paper with glass plates. Transfer of DNA was

allowed to precede overnight (Southern, 1975). The Zeta-Probe membrane was washed once with a solution containing 0.2 M Tris-HCl, pH 7.5, and 2 x SSC for 15 minutes with gentle shaking. The membrane was blotted dry with Whatman No. 1 filter paper, sandwiched between 3 mm paper, and heated in a vacuum oven for 1 hour at 80°C.

Northern Blots

RNA was subjected to electrophoresis in agarose formaldehyde gel as previously described. The gel was washed two times for 15 minutes each at room temperature in DEPC treated water and then placed in a solution containing 50 mM NaOH and 10 mM NaCl for 15 minutes at room temperature. The gel was then placed in 20 x SSC for 15 minutes. The buffer used to transfer RNA to a Hibond membrane (Amersham # RPN.303N.) previously equilibrated with DEPC water was 20 x SSC. The apparatus and procedure to transfer RNA was as described for southern blots except that the two layers of 3 mm paper placed on the membrane were equilibrated with 20 x SSC (Fourney et al., 1988; Wreschner and Herzberg, 1984). After drying, RNA was fixed to the membrane by exposing to UV light for 10 seconds and then baked in a vacuum oven at 80°C for 2 hours.

Sequencing Gel

The glass plates (38 x 40.5 cm and 38 x 44.5 cm) with spacers were held together by paper clamps. The 8% sequencing gel contained 20 ml of 20% acrylamide (96.5 g acrylamide, 3.35 g methylene-bis-acrylamide, 233.5 g ultra-pure urea, 100 ml 5 x TBE, sterile deionized H_2O to 500 ml), 30 ml of urea mix (233.5 g urea, 100 ml 5 x TBE, sterile deionized H_2O to 500 ml), 0.4 ml of 10% ammonium persulfate, and 20 μl

TEMED. The solution was poured using a 50-ml syringe. A 65 well sequencing comb was inserted and the gel solution was allowed to polymerize at room temperature. After polymerization the gel was mounted in a vertical electrophoresis apparatus (International Biotechnologies Inc. Cat.# 80000) and the comb was removed. Electrophoresis buffer (TBE, pH 8.3) was added to the top and bottom reservoir. Air bubbles at the bottom of the gel were removed using a bent hypodermic needle attached to a syringe (Sambrook et al., 1989). The samples were loaded and electric power applied (55 W).

DNA Sequencing

DNA was sequenced in both directions using Sequanase kit (Version 2.0, U.S. Biochemicals) which implies the chain termination metod (Sanger et al., 1977). DNA in 20 μl was denatured by adding 15.2 μl of a solution containing 2 M NaOH and 2 mM Tris-HCl, and incubating 5 minutes at room temperature. Then the tube was placed in ice and 8 μl of 1 M Tris-HCl, pH 4.5, and 3 μl of 3 M Na acetate were added followed by 75 μl of ice cold 100% ethanol. The sample was stored 20 minutes at -70°C and then centrifuged at 12,000 x g for 5 minutes. The DNA precipitate was washed with 200 μl of ice cold 70% ethanol, centrifuged, and the DNA dried at room temperature using a speedvac. The DNA was dissolved in 7 μl of sterile deionized water. The 5 μg of denatured DNA in 7 μl was annealed to 1 μl of pUC19 forward primer (5'-GTTTTCCCAGTCACGAC-3') in the presence of 2 μl of sequencing buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl). A second tube was prepared using 1 μl of pUC19 reverse primer (5'-CAGGAAACAGCTATGAC-3'). The reaction mixtures were incubated 30 minutes at 37°C. The following was added to the annealed

DNA: 1 μl of 1 M DTT, 2.0 μl of solution containing 1.5 μM each of dGTP, dCTP, and dTTP, 0.5 μl (4 μCi) of [^{35}S]dATP, and 2 μl (3 units) of Sequanase. This labeling reaction tube was incubated 5 minutes at room temperature. For the termination reaction, 2.5 μl of ddGTP solution (dGTP, dATP, dCTP, and dTTP each at 80 μM; 8 μM ddGTP, 50 mM NaCl) were placed in a 1.5 ml microcentrifuge tube labeled G. Similarly, tubes labeled A, T, and C contained 2.5 μl of the ddATP, ddTTP, and ddCTP termination solutions. To each of these 4 tubes prewarmed to 37°C, 3.5 μl of labeling reaction were added. The tubes were briefly centrifuged and incubated at 37°C for exactly 5 minutes. The reaction was terminated by adding 4 μl of stop buffer containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF, thoroughly mixed, and then stored on ice before loading on the sequencing gel. Before loading, the sample was heated 2 minutes at 70°C and then placed on ice. 2.5 μl were loaded on the sequencing gel.

Purification of Rabbit anti-ACS IgG

Nitrocellulose filters containing immobilized proteins were prepared as described previously for immunological screening (*E. coli* Y1090r$^-$ was infected with λgt11 containing ATC5). After blocking the filters with 1% BSA, they were dried and treated 1 hour with a 1:200 dilution of rabbit anti-mammary gland ACS in TBST buffer and then washed 3 times with 7.5 ml of TBST. The IgG bound to ATC5 fusion protein was eluted from the nitrocellulose filter with 3 one minute washes of 7.5 ml of a solution containing 5 mM glycine-HCl, pH 2.3, 150 mM HCl, 0.5% Triton X-100, and BSA (100 μg/ml). The combined washes were made 50 mM with Tris-HCl, pH 7.4 (Weinberger

et al., 1985). This IgG preparation was used in heart-ACS assays and to detect ACS in Western blots of protein from mammary gland, heart, kidney, liver, and spleen.

Immunoprecipitation of Heart ACS Activity

Heart ACS was the $(NH_4)_2SO_4$ precipitate of the mitochondrial extract of fresh tissue (Qureshi and Cook, 1975). The first antibody was rabbit anti-ACS affinity purified. The second antibody was goat anti-rabbit IgG (Sigma). Ten μg of protein in 50 μl of Tris 0.4 M, pH 8.6, were incubated with 250 μl of affinity purified rabbit or with 250 μl buffer (control) for one hour. 50 μl of the second antibody containing 20 μg of IgG were added to the tube containing antibody and 50 μl of buffer were added to the control and the tubes incubated for another hour. In a third tube, 10 μg of protein in 50 μl of Tris 0.4 M, pH 8.6, were incubated with 250 μl of buffer for one hour and then with 50 μl of the second antibody containing 20 μg of IgG for another hour (Weinberger et al., 1985). The tubes were centrifuged at 12,000 x g for 30 minutes, and the supernatant assayed for enzyme activity as above.

SDS-Polyacrylamide Gel Electrophoresis

Proteins were resolved in 8% polyacrylamide. A stock solution of 30% acrylamide/bis was prepared by dissolving 29 g of acrylamide, 1 g of N-N'-methylene-bis-acrylamide in 60 ml of sterile deionized water, heating 10 minutes at 37°C, diluting to a final volume of 100 ml, and filtering through a Nalgene filter having a pore size of 0.45 microns. The 8% polyacrylamide gel mixture consisted of 12.8 ml of 30% acrylamide/bis, 12 ml of 1.5 M Tris-base, pH 8.8, 22.5 ml sterile deionized water, 0.48 ml of 10% SDS (Laemmli, 197), which was mixed and degassed for 10 minutes, and

then 0.36 ml of ammonium persulfate and 25 μl of N-N-N'-N'-tetramethyl-ethylenediamine (TEMED) added. The solution was poured between two glass plates 16 x 19.5 and 20 x 19.5 cm in dimensions leaving a 4 cm space at the top. The gel was overlayed with 0.5 ml of water-saturated isobutanol. After 45 minutes at room temperature, the isobutanol was removed and the top of the gel was washed with sterile deionized water. The stacking gel (4% acrylamide) was prepared by mixing 2 ml of 30% polyacrylamide/bis, 3.75 ml of 0.5 M Tris-base, pH 6.8, 9 ml of sterile deionized water, and 0.15 ml of 10% SDS. The solution was degassed for 10 minutes then 75 μl of 10% ammonium persulfate and 8.5 μl of TEMED added. After pouring this solution on top of the resolving gel, a 15 well comb (0.6 cm wide, 2.8 cm long, and 0.1 cm thick) was inserted in the stacking gel solution (Davis, 1964). The gel was kept in a vertical position for 90 minutes then the comb was removed, the gel was mounted in a vertical electrophoresis apparatus, and electrophoresis buffer (25 mM Tris-base, 25.0 mM glycine, pH 8.3, 0.1% SDS) added to the top and bottom reservoirs. Air bubbles were removed from the bottom of the gel using a bent 20 gauge needle and syringe. The protein samples and molecular weight marker were heated in the loading gel buffer (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) at 100°C for 3 minutes. Then the samples were placed in the wells and 13 mA of current were applied overnight.

Western Blot

The plates from the SDS polyacrylamide gel electrophoresis of proteins were separated using a spatula and the orientation of the gel marked. A nitrocellulose

membrane (Schlucher and Schuell Cat. # 68380) was equilibrated with transfer buffer (1.5 mM Tris-base, pH 8.3, 120 mM glycine, and 20% methanol). The gel was placed in transfer buffer and the stacking gel removed. Four sheets of 3 mm paper were equilibrated with transfer buffer. One-half of the cassette of a Hoeffer electro transferring apparatus (Hoeffer Scientific Instrument Cat. # SE600) was placed in a tray containing transfer buffer. The sponge supplied with the apparatus was placed on top of the cassette. Then the following was placed on the sponge: 2 sheets of Whatman 3 mm paper, 1 nitrocellulose membrane, the polyacrylamide gel, the remaining 2 sheets of 3 mm paper, and a second sponge. The second half of the cassette was assembled to complete the sandwich. The cassette was removed from the tray and placed in the Hoeffer transfer tank. The tank containing transfer buffer and cassette was cooled using circulating ice water. One hundred V were applied for 2 hours in a cold room (Towbin et al., 1979; Burnette 1988). Then the gel was stained with Coomassie blue to determine if protein transfer was complete. The nitrocellulose filter was then examined using rabbit anti-Holstein mammary gland ACS and Promega immunological screening kit as previously described.

Slot Blot

300 μl of a mixture of 500 μl formamide, 162 μl of formaldehyde (37% solution) and 100 μl of 10 x MOPS buffer were added to 100 μl of the RNA sample (1-10 μg) and incubated at 65°C for 5 minutes. The sample was then chilled on ice and 100 μl of cold 20 x SSC were added. Three layers of Bio-Dot SF filter paper (Bio-Rad Cat. # 162-0161) were equilibrated in 10 x SSC and placed on the filter support plate of the slot-blot

apparatus (Bio-Rad Cat. # 170-6542). A nylon membrane (Hybond-N, Amersham RPN.303N.) was also equilibrated with 10 x SSC and placed over the 3 layers of filter paper. The sample well plate was clamped into place and a low vacuum applied. When the buffer had drained from the wells the vacuum was disconnected and the RNA samples were loaded in the wells. Low vacuum was applied again until the solution drained from the wells. Vacuum was disconnected and 500 μl of 10 x SSC were loaded in the wells. Vacuum was applied until the buffer drained. This last procedure was repeated. RNA was fixed to the membrane by exposure to UV light for 30 seconds, dried 30 minutes at room temperature, then under vacuum for 2 hours at 80°C. Prehybridization was 1 hour and hybridization to ^{32}P-labeled probe was overnight at 42°C in 5 x SSC, 5 x Denhardt's solution (1 x Denhardt's solution is 0.02% BSA, 0.02% ficoll, and 0.02% polyvinyl-pyrrolidone), 0.5% SDS, and 10 μg/ml denatured herring sperm DNA (Boehringer Mannheim 223 646). After hybridization the membrane was washed two times for 10 minutes each at room temperature with 2 x SSC and 0.1% SDS and then 30 minutes at 42°C with 0.5 x SSC and 0.1% SDS (Thomas, 1980; White and Bancroft, 1982). Auto-radiography was for 48-72 hours at -80°C. A densitometer (Bio-Rad 165-2040) was used to examine the radiogram. A standard curve was prepared using ATC5 cDNA at concentrations of 1.6-200 pg.

Large Scale Preparation of Plasmid DNA

E. coli (DH5α) transformed with pUC19-ATC5 or pUC19-AR8 was grown in 500 ml LB medium in a shaker overnight. The bacterial cells were harvested by centrifugation at 6,000 x g at 4°C for 10 minutes. The pellet was washed in 100 ml STE

buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and the bacterial cells were collected by centrifugation at 6,000 x g for 10 minutes at 4°C. The pellet was resuspended in 10 ml GET buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA) then 10 ml of freshly prepared solution of 0.2 N NaOH and 1% SDS were added with thorough mixing. The tube was stored at room temperature for 10 minutes then 20 ml of ice cold 3 M potassium acetate, pH 4.8, were added and the contents mixed by inverting the tube several times. Then the tube was stored on ice for 10 minutes. The bacterial lysate was centrifuged at 15,000 x g for 20 minutes at 4°C. The supernatant was transferred to a sterile Corex tube and 6 ml of isopropanol were added, mixed and incubated at room temperature for 15 minutes. The tube was centrifuged at 12,000 x g for 20 minutes at room temperature. The pellet was washed with 5 ml of 70% ethanol and dried in a speedvac for 5 minutes. The dry pellet was dissolved in 11.3 ml of sterile TE buffer, pH 7.5. Then 11.55 g of CsCl were added to the tube and dissolved by inverting the tube several times, and 300 μl of ethidium bromide (10 mg/ml) were added. The solution was placed into a Beckman Ti 65 Quick-Seal tube and the tube sealed and centrifuged at 55,000 x g overnight at 20°C. The supercoiled DNA was collected by inserting an 18-gauge hypodermic needle into the Quick-Seal tube. An equal volume of water saturated isobutanol was added to the DNA, mixed by vortexing and centrifuged at 400 x g for 3 minutes at room temperature to remove the ethidium bromide. This step was repeated 5 times. The DNA was dialyzed against 3 changes of TE buffer, pH 8.0, at room temperature. The DNA was extracted once with a 1:1 mixture of chloroform and phenol saturated with TE buffer, once with chloroform, precipitated with ethanol,

washed with 70% ethanol, dried and dissolved in TE buffer, pH 7.6 (Godson and Vapnek, 1973).

Coupled *in vitro* Transcription/Translation of ATC5

ATC5 was cloned into the expression vector pGEM-3Z which has two promoters, T7 and SP6. pGEM-3Z-ATC5 was transcribed/translated by using the TNT coupled reticulocyte lysate system (Promega cat. # L5020) according to the manufactures instructions.

Computer Facilitated DNA and Amino Acid Sequence Analysis.

The DNA sequence was compiled on an IBM computer using GCG (Genetics Computer Group Inc.) sequence software, the program was run on the unix system at Michigan State University. The composition and the translated amino acid sequence of acetyl CoA synthetase was determined by the same program. Open reading frames of acetyl CoA synthetase sequenced fragments were obtained by using the Map soft ware. Map displays both strands of DNA sequences with a restriction map above the sequence and possible protein translations shown below. Alignments of protein sequence were determined by Bestfet software. Bestfet is a powerful software for identifying the best region of similarity between two sequences whose relationship is unknown. Optimal alignments are found by inserting gaps to maximize the number of matches using the local homology algorithm of Smith and Waterman (1981). Comparison of acetyl CoA synthetase amino acid sequence with the amino acid sequences in the database was done by using the FastA software. FastA is included in the GCG package, it answers the question "What sequences in the database are similar to my sequence?". Also, TfastA

software was used to search for similarity between a query peptide sequence and any group of nucleotide sequences. TFastA translates the nucleotide sequences in all six frames before performing the comparison. It is designed to answer the question, "What implied peptide sequences in a nucleotide sequence database are similar to my peptide sequence". Map, Bestfet, FastA and TFastA softwares are all included in the GCG package.

Materials

Sodium nitroprusside, β-mercaptoethanol, ethidium bromide, dithiothreitol, dimethylformamide, Tween 20, polyethylene glycol 8,000, bromophenol blue, xylene cyanol, MOPS (3-[N-Morpholino]propanesulfonic acid), coenzyme A, goat anti-rabbit IgG, Sarkosyl, diethyl pyrocarbonate, BSA, Freund's adjuvant complete, Freund's adjuvant incomplete, Tris-HCl, and Tris base were from Sigma.

Sodium citrate, sodium acetate, potassium acetate, sodium hydroxide, ammonium acetate, potassium phosphate, EDTA, chloroform, formaldehyde, glycerol were from J.T. Baker. Magnesium sulfate, boric acid, and sodium cyanide were from Mallinckrodt. Sodium chloride, ammonium sulfate, and magnesium chloride were from Columbus Chemical Industries, Inc. Ethanol was from Quantum Chemical Corp. Triton X-100 was from Research Products International.

Cesium chloride, sodium dodecyl sulfate, guanidinium isothyocyanate, formamide, phenol, IPTG (isopropyl-β-D-thiogalactopyranoside), X-Gal (5-bromo-4-chloro-3-endolyl-β-D-galactopyranoside), AMV reverse transcriptase, T_4 DNA polymerase, T_4 DNA ligase, T_4 polynucleotide kinase, pancreatic DNase I, pancreatic RNase, proteinase K, restriction enzymes, pUC19, and Ampicillin were from Boehringer Mannheim.

Ficoll, oligo dT-cellulose, Sephadex G-50, Sephacryl S-400 spin columns, and pALTER plasmid were from Pharmacia. DEAE Affi-Gel Blue Gel was from Bio-Rad. DEA-cellulose, No. 1, and 3 mm Chr filter paper were from Whatman. Nitrocellulose filters were from Schleicher and Schwell.

Agarose was from Gibco. Agar, bacto tryptone, and bacto yeast extract were from

Difco.

E. coli DH5α was from Gifco. Sodium pyrophosphate, nitroblue tetrazolium, 5-bromo-4-chloro-3-endolyl phosphate, RNasin ribonuclease inhibitor , *E. coli* RNase H, oligo dT primers, spermidine, HaeIII, EcoRI adaptors, λgt11, *E. coli* γ1090r$^-$, *E. coli* 1089r$^-$, Packgene, ProtoBlot immunological screening kit, RiboClone, and pCAT-basic were from Promega. Sequanase and deoxy and dideoxy nucleotides were from United States Biochemical Corp.

Results

cDNA Synthesis and Library Construction

RNA Isolation and cDNA Synthesis

Mammary tissue was taken from a Holstein cow 60 days postpartum. Total RNA was isolated by the guanidinium isothiocyanate cesium chloride method. Yield of total RNA ranged from 500-700 μg per gram of frozen tissue. The integrity of total RNA was analyzed by formaldehyde gel electrophoresis (Figure 7). No significant degradation was apparent, as judged by the equal stoichiometry between low (18S) and high (28S) molecular size RNA. By this criterion, RNA was deemed suitable for use in isolating polyadenylated poly(A)$^+$ RNA.

The poly(A)$^+$ RNA fraction represented 0.7 to 1.2% of the total RNA passed over the column, which is in line with the reported percentage of poly(A)$^+$ RNA in higher euocaryotes. Less than 3% was isolated by this method (Aviv et al., 1972).

Once enough poly(A)$^+$ RNA was isolated, single and double stranded cDNA were synthesized. The reaction was carried out with 5 μg of poly(A)$^+$ RNA. Five μci of [α-^{32}P] dCTP added to both the first and second strand synthesis reaction permitted determination of cDNA molecular size using alkaline gel electrophoresis and autoradiography (Figure 8). An increase in density can be seen when the double stranded cDNA (lane 2) is compared to the single stranded cDNA (lane 1). The majority of double stranded cDNA was between 0.3 and 4.5 kb in size. This range was satisfactory since native acetyl-CoA synthetase has a molecular weight of 63 kD; therefore, its predicted

mRNA size should be on the order of 2 kb. The cDNA was blunt-ended and synthetic EcoRI adapters were attached. Separation of free adapters from the cDNA was achieved by sephacryl-S 400 column chromatography. cDNA fragments greater than 400 bases in length were isolated. The purified cDNA was ligated into a unique EcoRI site contained in the phage expression vector λgt11 (Figure 9) (Young and Davis; 1983a). Thus the Holstein mammary acetyl-CoA synthetase cDNA was inserted into the λgt11 lacZ gene, coding for β-galactosidase, at the EcoRI site.

λgt11 was chosen because libraries constructed in this vector can be screened with nucleic acid hybridization probes or with antibodies directed against the protein of interest. Also, since genes cloned into λgt11 can be expressed as β-galactosidase fusion protein or, in some cases, as non-fusion protein, it is often possible to measure the activity of an expressed protein in recombinant λgt11 lysogenies.

Recombinant phage was packaged into phage heads with one packaging reaction. The packaged phage was infected into *E. coli* Y1090 cells and tittered. Total number of recombinant phage was greater than 2×10^7 plaque forming units (pfu) before library amplification. Average recombinant frequency was between 50-60% (Figure 10). The abundance of acetyl-CoA synthetase mRNA species in the poly(A)$^+$ RNA fraction was unknown at this point. Assuming that the message level was equivalent to the acetyl-CoA synthetase protein level in mammary total protein extract (approximately 0.001%) (Cook e al., 1975), a cDNA library containing 2×10^7 recombinant plaques before amplification would be expected to contain a maximum of 200 plaques of the acetyl-CoA synthetase cDNA. Even if this assumption was in error by two orders of magnitude, one could still

reasonably expect to isolate an acetyl-CoA synthetase cDNA from a library of this size.

Isolation and characterization of a positive plaque from the bovine λgt11 library

Immunological screening

The bovine mammary gland cDNA library was screened using rabbit anti-bovine acetyl-CoA synthetase. Twelve positive colonies were observed out of 6 x 10^5 plaques. Rescreening of the 12 clones with the same antibody at a low pfu density (200 plaques/plate) revealed one positive plaque. This plaque was designated λAR8, the plaque was recombinant (Figure 11). During primary screening, the color of the false positive signals was observed to be weaker than that of the positive one (Figure 12). This phenomena may be caused by the nonspecific binding of the antibody and bacterial proteins. To minimize this the rabbit antiserum was pretreated with *E. coli* protein extract.

Phage DNA mini-preps were performed on each clone and the purified phage DNA was subjected to EcoRI restriction analysis to determine the cDNA insert size. The fragments were separated by agarose gel electrophoresis. Colony λAR8-ACS contained a 200 bp cDNA fragment (Figure 13).

Studies performed on λAR8

To determine if λAR8 cDNA fragment can be used to screen the bovine mammary library, it was amplified and subjected to enzyme assay, Southern blot analysis and DNA sequencing.

Acetyl-CoA synthetase activity in λAR8 fusion protein

When the recombinant λgt11 infects its host cell, the ACS-β-galactosidase fusion protein, the product of the recombinant lacZ gene will be synthesized (Carmichael et al.,1977).

Acetyl-CoA synthetase activity was measured in 5 μg of fusion protein produced from *E. coli* 1089 infected with wild-type λgt11 and with λAR8. Also, these fusion proteins were treated with the rabbit anti-bovine acetyl-CoA synthetase antibody and with anti β-galactosidase monoclonal antibody. Enzyme activity was 29% higher in the λAR8 fusion protein as compared to the λgt11 fusion protein. Acetyl-CoA synthetase-specific activity decreased by 35% and 100% when the recombinant λAR8-ACS fusion protein was treated with rabbit anti-bovine acetyl-CoA synthetase or β-galactosidase monoclonal antibody, respectively (Table 1). On the other hand, neither rabbit anti-bovine acetyl-CoA synthetase or β-galactosidase monoclonal antibody affected the enzyme activity of λgt11 fusion protein.Protein A-positive *S. aureus* cells was used as second antibody.

Southern blot analysis

A. Probing of fungal ACS DNA with λAR8

N. crassa acetyl-CoA synthetase gene was generously provided by Dr. Connerton (Connerton et al 1990). *N. crassa* acetyl-CoA synthetase gene is 6 kb, and is cloned in pEMBL 8 vector in Hind III restriction sites. *N. crassa*-pEMBL DNA was digested with HindIII. The fungal acetyl-CoA synthetase DNA was separated in 0.8% agarose gel and transferred to a nitrocellulose filter. This membrane was probed with λAR8 cDNA fragment (200bp). The wash conditions were 0.3X SSC, 0.1% SDS at 55°C. λAR8

cDNA fragment bound to the fungal acetyl-CoA synthetase gene (Figure 14).

B.Genomic Southern blot analysis

Bovine genomic DNA was digested with EcoRI and separated by agarose gel electrophoresis. A Southern blot of this gel was then probed with the λAR8 cDNA fragment (200 bp). The filter was washed 15 minutes with 0.3X SSC, 0.1% SDS at 55°C. Autoradiography showed one band (Figure 15).

Sequence of the λAR8 cDNA

The positive recombinant clone (λAR8) containing Holstein mammary acetyl-CoA synthetase cDNA, judged by antibody screening, Southern blot and enzyme assay was amplified, and the recombinant λgt11 DNA was purified (see materials and methods). The purified DNA was digested with EcoRI and the DNA fragments were separated in agarose gel (Figure 13). The 200 bp fragment was subcloned into a pUC19 vector (Figure 16) at the EcorI site, and insertion was confirmed by EcoRI digestion of the recombinant pUC19-AR8 DNA and agarose gel analysis (Figure 17). The corresponding double stranded pUC19-AR8 DNA was sequenced in both directions by using the dideoxy chain termination method (Sanger et al., 1977). The universal primer was used to prime the sequencing reaction. Sequencing analysis showed that λAR8 DNA is 228 bp (Figure 18). Homology search in the gene bank revealed that, AR8 has no homology with common proteins, instead AR8 showed homology (14-24%) to the ATP using enzymes.

Screening of the cDNA library with α-^{32}P labeled AR8

The bovine mammary gland library was rescreened by plating 1 x 10^6 recombinant

phage plaques which were transferred to nitrocellulose filters and hybridized to the 228 bp EcoRI restriction fragment of AR8 (Figure 19). Twenty eight positive plaques were isolated. These plaques were plaque purified, by several rounds of screening. Phage DNA mini preps were performed for all 28 of the purified colonies and the isolated DNA was digested with EcoRI. Analysis of the digests by agarose gel electrophoresis, revealed that 9 colonies had an insert of 4.2 kb (Figure 20). This phage was selected for further study and designated λATC5. This 4.2 fragment was subcloned into pUC19 vector (pUC19-ATC5) at the EcoRI site, and insertion was confirmed by EcoRI digestion of the recombinant pUC19-ATC5 DNA and agarose gel analysis (Figure 21).

λATC5 fusion protein enzyme assay

Fusion protein was prepared from λATC5-ACS and wild type λgt11, as described "in materials and methods." Again the fusion protein was treated with either rabbit anti-bovine acetyl-CoA synthetase polyclonal antibody or with β-galactosidase monoclonal antibody. Acetyl-CoA synthetase fusion protein specific activity increased 93% in the λATC5 fusion protein compared to λgt11 fusion protein (control). In addition, acetyl-CoA synthetase-specific activity decreased by 85% and 50% when the fusion protein was treated with anti β-galactosidase and the rabbit anti-bovine acetyl-CoA synthetase (Table 2). However, rabbit anti-bovine ACS and β-galactosidase treated λgt11 fusion protein did not show change in activity.

Immunoblot analysis of ATC5 fusion protein

Positive plaque ATC5 was further analyzed by western blotting of the acetyl CoA synthetase-β-galactosidase fusion protein produced by this plaque (Figure 21). Bands in

panel A were visualized by Coomassie blue staining, while the bands in panel B were visualized by treating with the anti-β-galactosidase antibody. The recombinant λgt11 fusion protein showed two bands, 180 and 116 kD (lane 2 in panel B), and the nonrecombinant λgt11 fusion protein showed one band, 116 kD (lane 3 in panel B).

Southern blot analysis of λATC5 DNA

Bovine genomic DNA was digested with EcoRI and subjected to agarose gel electrophoresis. A Southern blot of this gel was then probed with λATC5 cDNA fragment. The filter was washed for 15 minutes with 0.3X SSC, 0.1% SDS at 55°C. The autoradiography showed one band (figure 22). Band size was the same as that detected using AR8.

Another Southern blot of *N. crassa* ACS DNA was prepared and probed with λATC5 cDNA fragment. λATC5 cDNA bound to the *N. crassa* acetyl-CoA synthetase gene (Figure 23).

Northern blot analysis of mRNA from bovine tissue

To determine the size of acetyl-CoA synthetase mRNA. A northern blot of poly(A^+) RNA from Holstein heart, liver, kidney and mammary gland was hybridized with ATC5 cDNA fragment. To increase the band intensity the probe was labeled with both α-^{32}P dCTP and α-^{32}P dATP. Surprisingly, different species of mRNA were detected in different tissues (Figure 24). The number of acetyl-CoA synthetase mRNA bands found was 3, 1, 2 and 4 for heart, liver, kidney and lactating mammary gland, respectively. The multiple forms of ACS mRNA ranged from 0.8 to 5.2 kb.

Slot blot Analysis of ACS mRNA

To investigate the effect of lactation on acetyl-CoA synthetase mRNA levels, poly(A^{+}) RNA was isolated from a dry, and 60, 120 and 180 days postpartum mammary gland tissue. A slot blot of these mRNA was probed with ATC5 cDNA fragment (Figure 25). The filter was washed for 15 minutes with 0.3X SSC, 0.1% SDS at 55°C. Acetyl-CoA synthetase mRNA increased 3 fold, at 120 days postpartum compared to 60 and 180 days of lactation. However, in the dry mammary gland acetyl-CoA synthetase level was very low. This experiment demonstrates that the gene is not transcribed in the dry mammary gland. On the other hand, mammary acetyl-CoA synthetase gene transcription increases to a maximum at 120 days of lactation and declines as lactation advances. This agrees with the measurement of enzyme activity during lactation. This data suggests that this gene is controlled at the transcription level.

Antibody studies

A. Western blot analysis

To verify that the polyclonal antibody used to screen the library detects acetyl-CoA synthetase, and to investigate the expression of the ACS gene in different tissue, a series of western blot analyses were undertaken. Western blot of mitochondrial protein extracts from heart, liver, kidney, mammary gland and spleen, were treated with polyclonal rabbit anti-bovine acetyl-CoA synthetase and then with goat anti-rabbit alkaline phosphatase-conjugated IgG (Figure 26). The acetyl-CoA synthetase polyclonal antibody used in this experiment has nonspecific reactivity (Figure 26) with other proteins. Therefore, this polyclonal antibody, was purified by incubating with the acetyl-CoA

synthetase fusion protein produced by λATC5. This purified antibody was used again in a western blot analysis of mitochondrial extracts from liver, heart, mammary gland, kidney and spleen (Figure 27). The purified antibody bound to one band in heart, kidney and mammary gland tissue with apparent molecular weight of 65 kD. For spleen tissue, however, the band size was 78 kD, and there was no binding to the liver protein extract. Consequently, this demonstrates that the immunological screening of the λgt11 library detected ACS. In addition, acetyl-CoA synthetase gene is expressed in heart, kidney and mammary tissue, but not in liver. For spleen the band at 78 kD is unexplained.

B. Tissue extract enzyme assay

Rabbit anti-ACS used in the immunological screening of λgt11 library was affinity purified using the fusion protein produced by λATC5-ACS. This antibody was then shown to remove 90% of acetyl-CoA synthetase activity from the preparation of partially purified Holstein heart acetyl-CoA synthetase (Table 3). Accordingly, the purified antibody is highly specific for acetyl-CoA synthetase.

Restriction map

The partial restriction map of pUC19-ATC5 is presented in Figure 29. To determine restriction sites, restriction enzymes that recognize 6 bp sequence, which theoretically occurs every 4096 bp were used. Therefore a 6 bp cutter would cut ATC5 once. To determine the exact site of each restriction enzyme, pUC19-ATC5 was first cut with a single restriction enzyme then with two restriction enzymes at the same time (Figure 28).

In vitro transcription\translation of ATC5

The initial plasmid construct was derived by subcloning the λgt11-ATC5 4.2 kb EcoRI fragment into the EcoRI site of the pGEM-3Z vector (Figure 30). Plasmid DNA mini preps were prepared to select the pGEM-3Z-ATC5 clone. To confirm the presence of ATC5, pGEM-3Z-ATC5 DNA was subjected to EcoRI digestion and the fragments separated by agarose gel electrophoresis. pGEM-3Z is designed to allow transcription by T7 and SP6 polymerase. Activation of both promoters was carried on separately. The activation of T7 resulted in production of a protein (55 kD), while the activation of SP6 did not (Figure 31).

Partial sequencing and analysis of ATC5

In the pUC19-ATC5 construct, the universal primer was used to prime the sequencing reaction. Four oligonucleotide sequencing primers were synthesized to sequence the overlaps. As such, a fragment of 1130 bp starting at the 5' end, was completely sequenced in one direction (5'--> 3') and 216 bp were sequenced at the 3' end (3'---> 5'). The sequencing strategy used to sequence the pUC19-ATC5 is shown in Figure 29.

Analysis of the completed sequence data by the GCG DNA software revealed additional genetic elements, including the location of the restriction enzyme sites, a possible open reading frame (ORF), and a possible transcription start codon (Figure 32). With respect to six base restriction sites, 34 restriction enzyme sites were found: 11 are unique sites, and 23 are duplicated sites. A map of these sites and their relative positions in the cDNA sequence is shown in figure 32.

Discussion

For the last 25 years our laboratory has been involved in the enzymological characterization of proteins and metabolic substrates affecting milk production by ruminants. Acetate is a major substrate for milk synthesis. The metabolic form of acetate is acetyl-CoA. The mechanism for generating acetyl-CoA is the acetate activating reaction catalyzed by acetyl-CoA synthetase (ACS). Our laboratory has determined ACS tissue distribution, intracellular localization, physical and catalytic properties, and changes in activity in mammary gland throughout lactation. Molecular studies were the next logical extension of this work to further our understanding of acetyl-CoA synthetase. Specifically, the objective of this project was to clone bovine ACS and characterize the cDNA encoding ACS.

All of these objectives has been achieved. A secondary objective was to study the effect of lactation on ACS gene expression. These analyses resulted in interesting observations with respect to ACS multiple mRNA forms in different tissues.

As is often in science, the answer to a question leads to many others. Though this discussion will focus primarily on the implication of my results, some emphasis will also be placed on questions which need to be answered in order to complete the ACS project. These are regulation of ACS gene, ACS gene expression between milking periods, evolution of the ACS gene, and production of pure recombinant ACS for research purpose.

The ACS cDNA

The ACS cDNA has a continuous ORF, beginning at the 5' terminus and continuing for 323 amino acid residues. Obviously the cDNA can not be judged to be full length depending on a partial sequence. However, several observations indicate that the λgt11-ATC5 codes for the full-length ACS protein. The first observation revolves around the size of the fusion protein. The fusion protein will have a higher molecular weight than that of wild type λgt11 β-galactosidase and react with the β-galactosidase antibody. Indeed, immunoblotting analysis of the fusion protein gave these results (Figure 21). Thus, it can be seen that the fusion protein which has a molecular weight of 180 kD is 63 kD larger than λgt11 β-galactosidase (116.3 kD) and reacts with anti-β-galactosidase. In contrast, E. coli Y1089 (control) show no bands corresponding to the fusion protein. Therefore, the western blot analysis of the fusion protein presents strong evidence that ATC5 is a full length cDNA.

Another observation which may offer evidence to support the full-length character of ACS cDNA concerns the size of the native and in vitro translated acetyl-CoA synthetase. The molecular weight of the product of transcribed\translated pGEM-3Z-ATC5 gene is slightly smaller (8 kD) than the molecular weight of the native protein. The in vitro translation system is a cell free system. Therefore, glycosylation is not expected to take place in this reaction. The native protein was reported to contain more than 0.7% carbohydrate by weight, such as N-acetylneuraminic acid, fucose and glucose (Stamoudis and Cook 1975). Due to the lack of a glycosylation system the in vitro translated protein would have a lower molecular weight than the native protein. SDS-

PAGE analysis showed this difference, (Figure 27 and Figure 31). In addition a western blot of mitochondrial extracts from several ruminant tissues demonstrated that rabbit anti-bovine ACS bound to 63 kD protein. However, the size of recombinant ACS is 55 kD. If a significant amount of N-linked suger is present on the native protein, a minimum increase of 2.5 kD should be expected and would be detected by these methods (Mayes and Waterfield, 1984). The fact that the two proteins have different molecular weight supports glycosylation of the native enzyme.

Finally, the enzymatic activity of both recombinant and native ACS provides strong evidence that ACS cDNA is a full length transcript. Recombinant ACS behaves identically to the native protein with regard to enzyme activity. The clone that contains the full-length ACS cDNA showed higher enzyme activity (94%) compared to enzymatic activity of the wild type vector (Table 2). In addition, the purified ACS antibody was capable of removing the ACS activity of the recombinant ACS fusion protein and also from a heart mitochondrial extract.

Southern blot analysis and gene structure

ACS appears to be a single copy gene. A single hybridizing band was seen in the bovine EcoRI genomic digests which have been analyzed by southern blot analysis. This result and the fact that full-length ACS cDNA does not contain EcoRI sites rules out the possibility of more than one gene copy. Analysis of the ATC5 5' end, and sequence of the ACS genomic DNA will provide information necessary to determine the organization of ACS, and where introns are located.

Both the full-length ACS cDNA and the 228 bp fragment hybridized with the

fungal ACS gene. This hybridization occurred under high stringency. This indicates high homology between the bovine and fungal ACS genes. Also, this result suggests that the ACS gene is a conserved gene. Hiroyuki (1991) extracted a conservative sequence motif from the alignment of the firefly luciferase family. Acetyl-CoA synthetase derived from *N. crassa* and *A. nidulans* was identified as a member of the firefly luciferase family. The motif sequence shares several characteristics with the phosphoproteins and the nucleotide-binding proteins of the luciferase family.

If there is high homology between bovine ACS and other ACS genes, the evolution of ACS would be a very interesting subject to study, because acetyl-CoA metabolism performs a diversity of functions in the physiology of procaryotic and eucaryotic organisms. The main mechanism for synthesis of acetyl-CoA is the activation reaction catalyzed by ACS. Acetyl-CoA is the common metabolic form of acetate, and it is a key intermediate in pathways such as, the citric acid cycle, the glyoxylate cycle, amino acid metabolism, and the synthesis of polyhydroxybutyrate. Acetyl-CoA metabolism in procaryote provides energy for cell growth. Acetyl-CoA in non-ruminant mammals was viewed as an initial substrate for fatty acids synthesis. While, in ruminant animals acetyl-CoA is looked at as a source of energy, and a source of carbon of fatty acid synthesis in adipose tissue and the mammary gland. In addition, the metabolic fate of acetyl-CoA is different in the cytoplasm and the mitochondria. The fact that ACS in organisms higher up evolutionary ladder is part of very specialized pathways which result in energy production (ATP) or energy storage (fat synthesis) suggests that ACS probably evolved on the basis of selection for energy metabolism. Because the substrates for acyl-

CoA synthetases are similar (fatty acids) and because there appears to be a high homology between acyl-CoA synthetases, it is interesting to understand how they evolved from a common ancestral gene.

Northern blot analysis of ACS mRNA

Another interesting, yet unexpected, result was the detection of multiple forms of ACS mRNA. This result suggests that this gene is differently controlled in different bovine tissue. The level of ACS mRNA is low relative to the level of ACS protein in the cell (0.001% of total mammary protein). This suggests that the ACS mRNA is efficiently translated. The existence, in ruminant tissue, of several forms of ACS mRNA does not necessarily mean that the enzyme coding sequence is different.

ACS mRNA multiple forms within a tissue can be explained on the basis of differential splicing. On the other hand mRNA multiple forms among tissues can be explained on the basis of alternative promoters. Kim et al (1989) reported that rat acetyl-CoA carboxylase (ACC) has multiple forms of mRNA, having identical coding sequences, but heterogenous 5'-untranslated regions. Rat ACC has three forms of mRNA in the mammary gland and two forms in the liver. This suggests a multiple copy gene. However, rat ACC is a single copy gene per haploid chromosomal set (Lopez et al., 1988). Rat ACC is activated by different promoters in liver and in mammary gland. Thus, ACC mRNA class 1 is produced in the mammary gland and ACC class 2 is produced in the liver. The different mRNA forms within each organ are then produced as a result of alternative splicing. The 5' untranslated regions (5'UTR) can affect the translation efficiency of mRNA. Lopez and Kim (1991) reported that the rat mammary

mRNA transcripts had a 6-9 fold better transnational efficiency than the ACC liver mRNA. This increase in translation rate is due to a GC rich region (50 bp) in the 5' UTR of mammary gland ACC mRNA. These results are consistent with the hypothesis that the 5'UTR in the ACC mRNA may be involved in post-transcription control, of ACC gene expression.

Regulation of the ACS gene may be similar to the ACC gene. To determine this possibility an oligonucleotide complementary to ACS cDNA 5' end (100 bp) should be used in a linked primer extension\S1 nuclease experiment. This might be followed by characterization of intron-exon structure of genomic ACS.

Acetyl-CoA synthetase mRNA multiple forms might play a role in ACS gene post-transcription regulation. Factors which bind specific sequences in RNA and alter stability have been found in animals (Koeller et al., 1989; Ross, 1989). These factors might exist in a certain bovine tissue and not in others. There are two fairly well characterized systems involving changes in mRNA stability in animal cells that have been studied. These are *c-myc* mRNA and iron transferrin mRNA. In both systems, specific sequences in the 3' untranslated region of the gene have been shown to be necessary for regulation. In the case of *c-myc* mRNA, the sequences of interest are AUUUA repeats in its 3' UTR. AS shown in figure 32, ATC5 3' UTR is AUUU rich. Repeats of this sequence when placed in the 3' UTR of a reporter gene can dramatically reduce the stability of its RNA (Shaw and Kamen, 1986). Specific proteins have been shown to bind to this AUUUA motif in vivo (Myer et al., 1992) and site specific changes in this sequence that prevents binding of the AUUUA binding factor also result in a more stable

RNA (Malter, 1989). With the iron transferrin mRNA, the 3' UTR was shown to impart an iron dependent decrease in the stability of RNA from a reporter gene. A hypothetical hairpin loop structure within the U rich region of the 3' UTR was shown to bind a protein factor, and specific changes in these sequences that disrupt the hairpin formation also prevented the protein binding and resulted in a more stable RNA (Mullner and Kuhn, 1988). Since, bovine ACS 3' UTR is AUU rich (Figure 32), these sequences might play a role in ACS gene regulation. Also, protein factors which bind U rich regions might exist in one tissue and not in others.

In the above cases, it is generally assumed that binding of proteins to specific sites in the 5' or 3' ends of mRNA protects these sites from endonucleases with specifities to the same or nearby sequences (Malter, 1989; Mullner et al., 1989).

Other mechanisms which may be functioning to bring about changes in mRNA stability are those which specifically alter the affinity of poly $(A)^+$ RNA binding proteins which are involved in protecting the 3' end of specific RNA (Bernstein and Ross, 1989). It has been observed that generally mRNA with longer poly A tails are usually more stable than mRNA with shorter poly A tail (Shapiro et al., 1987). The reason for this is thought to be due to the presence of poly A binding proteins (PABP) that may be involved in protecting the 3' end of mRNA from 3'--> 5' exonucleases (Ross, 1989). There is some evidence to support the proposal that the AU rich regions which have been correlated with reduced mRNA stability (including the AUUUA sequences in the *c-myc* gene) may compete for PABP with the poly (A) tail, and thus leave the 3' end open to degradation by exonuclease (Bernstein et al., 1989)

The different bovine ACS mRNA forms in the different tissues, might help explain tissue specific expression of ACS. Acetyl-CoA synthetase is continuously active in the heart and kidney and not active in the liver. This might be explained by the presence or absence of PABP. It is possible to isolate the protein factors by using the gel mobility shift assay.

Acetyl-CoA synthetase mRNA is very low (Figure 25). Therefore, for further study it may be necessary to amplify ACS mRNA using PCR and ACS specific probes.

Slot blot analysis

Milk production involves the coordinated regulation of many genes. Acetyl-CoA synthetase transcription was higher at 120 days postpartum than in 60, 180 or in the dry gland. ACS activity is marginal in a dry mammary gland, increases from parturition to peak milk production and then declines in activity as lactation advances (Marinez et al., 1976) which agrees with the slot blot results (Figure 25). These results suggest that the expression of this gene is very tightly controlled.

Changes in ACS mRNA level suggest that there is regulation at the transcription level. Therefore, the regulatory regions would be located up stream from the 5' end. The upstream regulatory regions (URE) are short sequences in the promoter that act as binding sites for proteins that can interact either directly or indirectly with transcription factors (TF) or other proteins in the transcription complex in order to facilitate complex formation and/or RNA polymerase II binding (Klein-Hitpass et al., 1990). URE can affect transcription even when they are positioned some distances from the transcription start site (Struhl, 1987). However, it has been found that when such sequences were

placed at varying distances from the start site, full activity was only observed when the distance to the TATA box was altered in multiples of ten to eleven bases (Takahashi et al., 1989). This suggests that the URE binding proteins must be present in a specific orientation for enhanced RNA polymerase activity, and that this requirement may be fulfilled if there is sufficient distance between the URE and the site of complex formation.

More work needs to be done to be certain that the ACS gene is transcriptionlly regulated. This could be done by screening a bovine genomic library with an oligo made from the 5' ATC5 cDNA nontranslated region. Isolated genomic fragments containing the regulatory regions would be able to express a reporter gene.

The northern blot was probed with β-actin to check for mRNA integrity and quantity (Figure 24). However, it is recognized that β-actin is not a good indicator for mammary gland mRNA. Binding of β-actin to mRNA from mammary gland 180 days postpartum was quite low. This low binding might be due to the involution of mammary gland at late lactation.

Western blot analysis

To confirm that the rabbit anti-bovine acetyl-CoA synthetase binds to a biologically active ACS, and to demonstrate ACS tissue-specific expression, western blots were made of mitochondrial tissue extracts from heart, liver, mammary gland, kidney and spleen. Spleen mitochondrial extract was used in this experiment as a negative control. Spleen does not have ACS activity, however, it has a high level of acetyl-CoA hydrolase. Acetyl-CoA synthetase purified antibody bound to a single band in the tissues

that express ACS (heart, kidney, and mammary gland). The band size was 65 kD. This molecular weight agrees with the known ACS molecular weight. These results, in addition to those of the enzyme assay show that rabbit anti-bovine ACS has a high affinity for ACS. In addition the purified antibody did not bind to protein from the liver mitochondrial extract. On the other hand the purified antibody did bind to a 78 kD band in the spleen mitochondrial extract. We do not have an explanation for the spleen band but we think it represents nonspecific binding.

Acetyl-CoA synthetase tissue specific-expression could be studied by finding and characterizing the regulatory regions and the proteins which bind them. Then, the factors which influence the presence, the activation, and the binding of these proteins to the regulatory regions could be determined. Such factors include the spatial and temporal regulation of the expression of the genes which encode these DNA binding proteins, and also the proteins involved in the phosphorylation or the post-transcriptional modifications of these protein factors which may alter there ability to bind to their specific sequences or alter their ability to interact appropriately with the RNA. For example, the chromatin structure in and around the promoter has been demonstrated to alter the binding pattern of the essential components of the mRNA transcription complex (Laybourn and Kadonaga, 1991). Finally, the spatial orientation and packing of the chromatin within the nucleus and the channels through it may even play a role by determining which mRNA is allowed to leave the nucleus and which are physically blocked by the proteins and DNA (Blobel, 1985; Chang and Sharp 1990)

ATC5 Sequence

The ATC5 cDNA was subcloned into pUC19 at the EcoRI site, and the insertion was confirmed by EcoRI digestion of the recombinant pUC19-ATC5 DNA and analysis in agarose gel (Figure 17). The corresponding double stranded pUC19-ATC5 DNA, as the template, was sequenced in one direction using the dideoxy chain termination method. The sequencing strategy is shown in Figure 29.

ATC5 sequence is shown in figure 34. Only 1130 bp were sequenced at the 5' end and 261 bp at the 3' end. The EcoRI recognition sequence, 5'-GAATTC-3', at the 5' and the 3' end was found. Also a potential poly adenylation site (ATAAA) is detected at the 3'end. The GC content of ATC5 is 58%. The cloned ACS cDNA is 4.2 kb. However the expected size of bovine ACS is about 2 kb. Many cDNA are much larger than the coding region. For example, the reported size of bovine retinal pigment epithelium cDNA is 3115 bp, with a coding region of 1549 bp (Hamel et al., 1993). Out of the 1566 nontranslated bp, 1514 bases were located at the 3' end and 52 bases at the 5' end. Also, the mouse liver murine S-adenosyl methionine synthetase was cloned and sequenced (Shigeko et al, 1993). The cDNA was 3232 bp with a coding region of 1188 bp. Out of the 2044 nontranslated bp 1820 bp were located at the 3' end and 224 bp were located at the 5' end. Therefore,it is not uncommon for a cDNA to be much larger than the coding region. Most of the untranslated region is at the 3' end.

A comparison of the ATC5 restriction map with the restriction maps of all the vectors (λgt11, pUC19, pGEM-3Z, and pEMBL8) and fungal DNA used in this project showed that ATC5 is not related to any of these.

ATC5 has 42% homology with the *N. crassa* ACS gene, and the amino acid homology was 9%. The 42% homology is significant; however the 9% homology is very low. Since ATC5 is sequenced in only one direction, a missing bp would not affect the nucleic acid homology, but would result in a major change in the predicted ACS amino acid sequence.

In vitro translation

Earlier studies of acetyl-CoA synthetase indicated that the protein is 63 kD. Therefore, a full length cDNA should encode a protein of 572 amino acids. The native ACS is N-glycosylated. However, the in vitro translation product is not. The lack of glycosylation in the in vitro transcription\translation system explains molecular weight difference between the native and the expressed ACS (about 10 kD).

The main objective of the in vitro translation experiment was to identify the ATC5 5' end. ATC5 was cloned into pGEM-3Z plasmid, and two reporter reactions were performed. The first reaction contained T7 RNA polymerase, and the other contained SP6 RNA polymerase. Activation of T7 promoter produced a protein; however, SP6 did not. These results suggest that the 5' end of ATC5 fragment is under the control of the T7 promoter. This conclusion was further supported by sequencing. To orient ATC5, pGEM-3Z-ATC5 was cut with ScaI and BamHI. Comparing the size of resulted fragments with ATC5 and pGEM-3Z restriction map indicated that the EcoRI-BamHI end is the 5' end of ATC5.

ACS fusion protein enzyme assay

E.coli infected with wild type λgt11 would produce β-galactosidase as a fusion

protein. While, E.coli infected with λgt11-ATC5 would produce β-galactosidase-acetyl-CoA synthetase as a fusion protein. The expression vector λgt11 makes a temperature sensitive repressor (c1857) which is inactive at 42°C and has an amber mutation (S100) which only lyses the hosts containing the amber suppressor supF. On the other hand, E. coli Y1089 has a lac repressor which inhibits LacZ gene expression until derepressed by IPTG, also it is deficient in protease which decreases the degradation of the recombinant fusion protein, and supF which suppresses the phage mutation (S100) (Young and Davis, 1983b). Thus, λgt11 will not lyse Y1089 at 30°C because of its C1857 mutation, and lacZ won't be expressed until the addition of IPTG. Taking advantage of these characteristics, we successfully induced the E. coli 1089 lysogen strains by infecting the cell with recombinant λgt11 and obtained sufficient acetyl-CoA synthetase-β-galactosidase fusion protein for enzyme assay.

We expressed the bovine ACS cDNA in E. coli to verify cloning and functional integrity of recombinant acetyl-CoA synthase. The ACS enzyme activity in infected E. coli was inhibited by rabbit anti-bovine ACS and β-galactosidase monoclonal antibody. In addition recombinant ACS enzyme activity was 2 fold greater than the wild type λgt11 protein. These results suggest that recombinant ACS is a functionable protein, and ATC5 is coded for the whole ACS gene. E. coli expressed a low but detectable level of ACS activity. The rabbit anti-bovine ACS was not capable of removing the bacterial ACS activity. This data shows that the rabbit anti-bovine ACS is highly specific to the bovine protein.

The fusion protein produced from E. coli infected with λgt11-ATC5 showed 94%

increase in enzyme activity. Also, the fusion protein produced by the E. coli infected with λgt11-AR8 reacted with ACS and β-galactosidase antibody.

Here we report the first construction of mammalian ACS expression system in E. coli.

Summary and Conclusions

The results from this body of work have advanced the field of ruminant metabolism. Also, they have provided the tools (namely the bovine ACS gene and the bovine mammary cDNA library) for future studies of how ruminant animals alter gene expression in response to lactation. The bovine ACS gene has been cloned and characterized. The ACS gene has a single copy per haploid chromosome. Acetyl-CoA synthetase enzyme assay results provide evidence that the recombinant ACS is biologically active like the native protein. In addition, the ACS purified antibody is highly specific to ACS. To our knowledge this is the first report of recombinant mammalian ACS.

The fact that lactation can have an effect on ACS gene expression has been directly demonstrated by the slot blot study. Further, data have been presented which provides strong evidence of alternative promoters and\or alternative splicing involved in ACS gene regulation. Finally, the data suggests that ACS is controlled at both the transcriptional and post transcriptional level.

From a biological aspect, the cloning of the bovine ACS makes it possible to study the biochemical events that occur in order to bring about altered gene expression in response to lactation, hormones and different metabolic conditions. From a more physiological aspect, the cloning of bovine ACS makes it possible to test the role of the ACS gene in milk production by utilizing antisense technology to reduce the function of ACS in lactating animal at peak lactation. These studies of the ACS gene will shed light

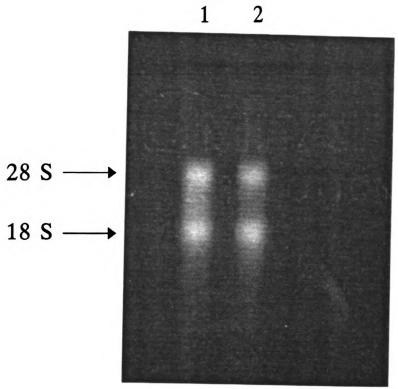
on the biological control of other acyl-CoA synthetases (e.g. propionyl-CoA synthetase).

In addition, the isolation of the bovine ACS promoter(s) is now possible and this may well prove quite valuable in the field of transgenics. The fact that there is tissue specific expression of ACS makes its promoter a good candidate for gene regulation studies.

Finally, there is recent evidence for cross-talk between separate signal transduction pathways (Nishizuka, 1992). Also, there may be several signal transduction pathways that play a role in the regulation of the genes involved in milk production. Thus, these initial studies of the molecular biology of lactation-regulated genes, may provide a starting point for studies of the interaction and the communication between the various signal transduction pathways involved in milk production. The experiments and fields of study discussed in this work should add greatly to our understanding of ruminant gene regulation, biochemistry, and physiology.

Figure 7. Formaldehyde gel electrophoresis of total mammary gland RNA isolated from a Holstein cow 60 days postpartum. Each Lane contains 20 μg of total RNA.

Formaldehyde gel electrophoresis



Gel analysis of RNA

Figure 8. Gel analysis of cDNA. The inset shows the successful first and second strand synthesis (lanes 2 and 4 respectively). Total RNA was used as a control (lane 1 and lane 3).

Alkaline gel electrophoresis

1 2 3 4

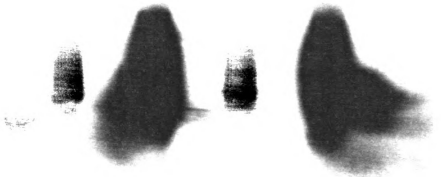


Figure 9. Genetic and restriction enzyme map of the phage cloning vector, λgt11.

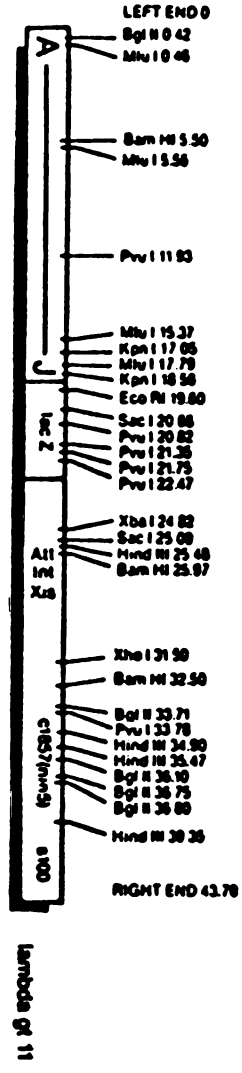

LEFT END 0
Bgl II 0 42
Mlu I 0 46
Bam HI 5 50
Mlu I 5.56
Pvu I 11 93
Mlu I 15.37
Kpn I 17 05
Mlu I 17.70
Kpn I 18 56
Eco RI 19.80
Sac I 20 88
Pvu I 20 82
Pvu I 21.36
Pvu I 21.75
Pvu I 22.47
Xba I 24 82
Sac I 25 09
Hind III 25 48
Bam HI 25.97
Xho I 31 59
Bam HI 32.50
Bgl II 33.71
Pvu I 33 78
Hind III 34.90
Hind III 35.47
Bgl II 36.10
Bgl II 36 75
Bgl II 36 89
Hind III 39 36
RIGHT END 43.78
A
J
lac Z
Alt
Int
Xis
cI857/nin5
s100
lambda gt 11

Figure 10. *E. coli* (Y1090) infected with Holstein mammary gland cDNA library plated on agar plate, containing, ampicillin, IPTG, and x-gal. The plates showed 50 to 60% recombinant colonies (clear).

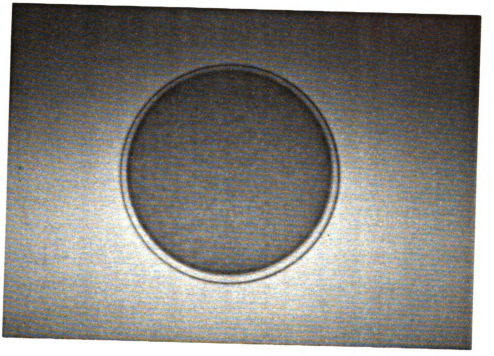


Figure 11. Purification of λgt11-AR8. *E. coli* (Y1090) was infected with λgt11-AR8 and plated on agar plates containing, ampicillin, IPTG, and x-gal.All plaques were clear indicating 100% recombinant λgt11.

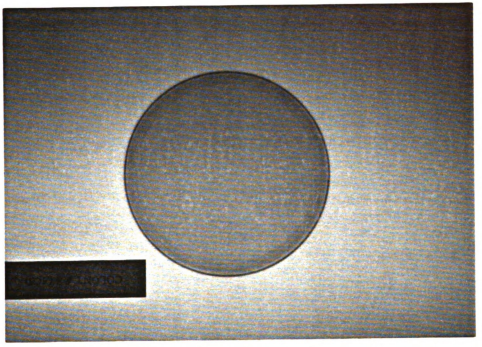


Figure 12. A representative primary screen of Holstein mammary gland cDNA library. Filters were lifted from plates containing 200 plaques and were screened with rabbit anti-bovine acetyl-CoA synthetase. Positive signals (λgt11-AR8) are shown on filter A, and false positive signals (λgt11) are shown in filter B.

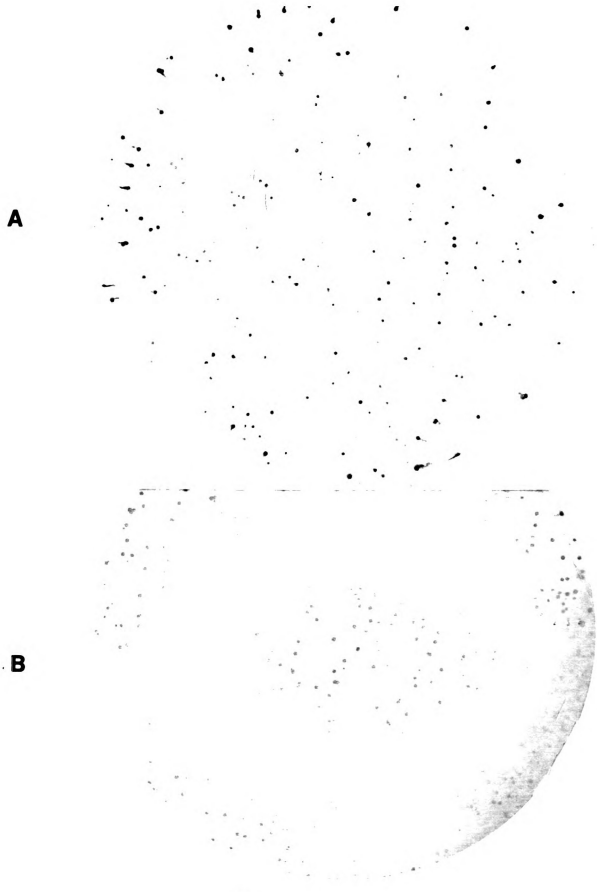

A
B

Figure 13. Isolation of λgt11-AR8 cDNA fragment. λgt11-AR8 DNA was purified with PEG and centrifuged using a glycerol gradient. The DNA was digested with EcoRI and separated in agarose gel. The inset shows DNA marker (lane 1) and digested λgt11-AR8 (lanes 2, 3 and 4).

Isolation of (λgt11-AR8) cDNA fragment

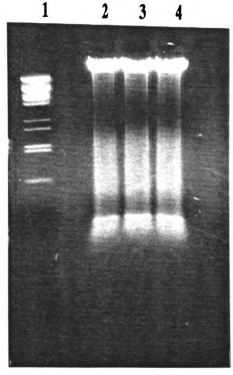


Figure 14. Southern blot of *N. crassa* acetyl-CoA synthetase gene probed with α^{32}P-AR8. The fungal clone was digested with HindIII. DNA was separated in agarose gel. The results indicates that AR8 is homologous to the fungal acetyl-CoA synthetase gene.

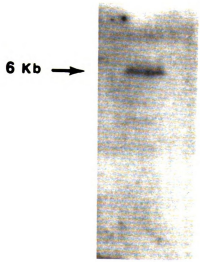

6 Kb

Figure 15. Southern blot of bovine genomic DNA probed with α^{32}P-AR8. Bovine genomic DNA was digested with EcoRI. DNA was separated in agarose gel. The results indicate one gene copy of acetyl-CoA synthetase.

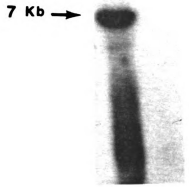

7 Kb

Figure 16. Genetic and restriction enzyme map of the phage cloning vector, pUC19.

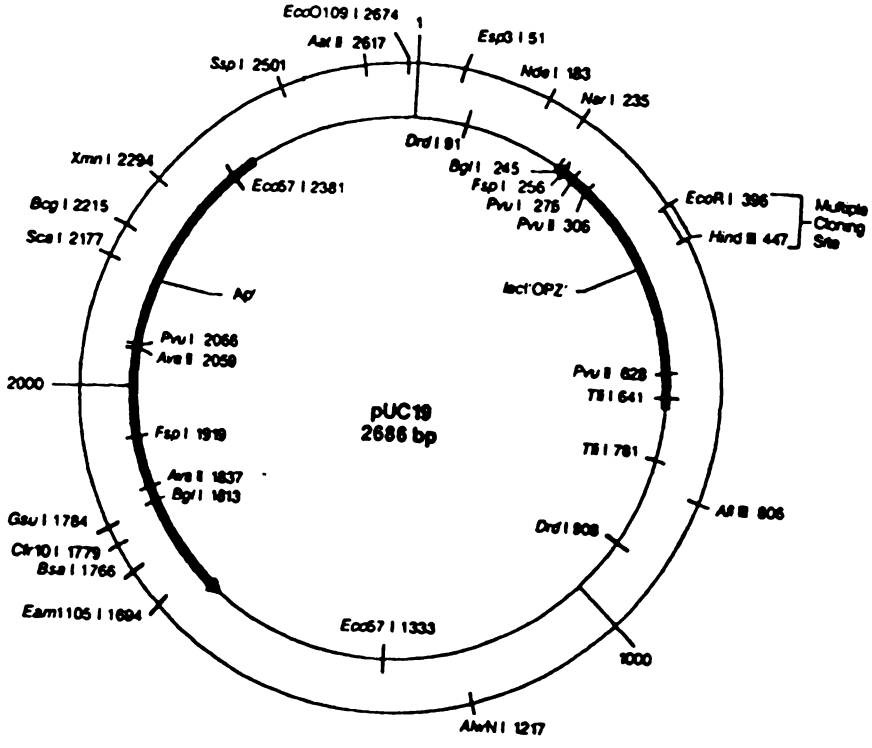

pUC19
2686 bp
1
Esp3 I 51
Nde I 183
Nar I 235
EcoR I 396
Hind III 447
Multiple Cloning Site
lacI'OPZ'
Pvu II 628
Tli I 641
Tli I 781
Afl III 806
Drd I 908
1000
Alw NI 1217
Eco57 I 1333
Eam1105 I 1694
Bsa I 1766
Ctr10 I 1779
Gsu I 1784
Bgl I 1813
Ava II 1837
Fsp I 1919
Pvu I 2066
Ava II 2059
2000
Apr
Sca I 2177
Bcg I 2215
Xmn I 2294
Eco57 I 2381
Ssp I 2501
Aat II 2617
Eco0109 I 2674
Drd I 81
Bgl I 245
Fsp I 256
Pvu I 275
Pvu II 306

Figure 17. Examination of AR8 and ATC5 cloned in pUC19. AR8 and ATC5 were cut from λgt11, separated in agarose gel, eluted and cloned into pUC19. A DNA mini prep was prepared using positive colonies and DNA was cut using EcoRI. The inset shows:lane 1, DNA standard; Lane 2 pUC19-ATC5 digest; and lane 3, pUC19-AR8 digest.

AR8 and ATC5 cloned in pUC19

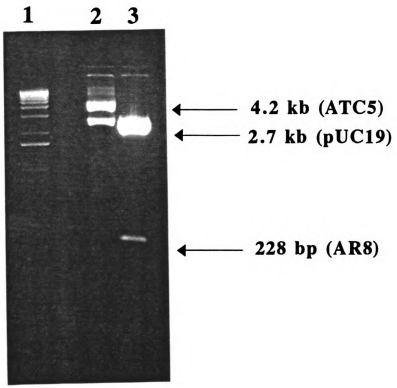


Figure 18. Nucleotide and deduced amino acid sequences of AR8. AR8 was sequenced in both directions; forward primer (panel A); and reverse primer (panel B).

Panel A

```
CCG TTG CTG TCG CGC ACC ACG CAC GCA GCA CAC GGC CCC GCA CAC        45
P   L   L   S   R   T   T   H   A   A   H   G   P   A   H
 R   C   C   R   A   P   R   T   Q   H   T   A   P   H   T
  V   A   V   A   H   H   A   R   S   T   R   P   R   T   Q

AGC CCC ATC TTA CCC ACC CTG CAA CCA ACC CCC GTC GAC TGC CTT        90
S   P   I   L   P   T   L   Q   P   T   P   V   D   C   L
 A   P   S   Y   P   P   C   N   Q   P   P   S   T   A   L
  P   H   L   T   H   P   A   T   N   P   R   R   L   P   Y

ACA CAC CCG CCC CCG CCG TCC GCC AGC ACG GGG GTG AGA ACA AGG        135
T   H   P   P   P   P   S   A   S   T   G   V   R   T   R
 H   T   R   P   R   R   P   P   A   R   G   *   E   Q   G
  T   P   A   P   A   V   R   Q   H   G   G   E   N   K   A

CAC TAG GTC GGC CGG CCA GCG CGA CA                                 161
H   *   V   G   R   P   A   R
 T   R   S   A   G   Q   R   D
  L   G   R   P   A   S   A   T
```

Panel B

```
TGC AGG TCG ACT CTA GAG GAT CCC GGG TAC CGA GCT CGA ATT CCG        45
C   R   S   T   L   E   D   P   G   Y   R   A   R   I   P
 A   G   R   L   *   R   I   P   G   T   E   L   E   F   R
  Q   V   D   S   R   G   S   R   V   P   S   S   N   S   V

TTG CTG TCG CCG TTG CTG TCG CGC TGG CCG GCC GAC CTA GTG CCT        90
L   L   S   P   L   L   S   R   W   P   A   D   L   V   P
 C   C   R   R   C   C   R   A   G   R   P   T   *   C   L
  A   V   A   V   A   V   A   L   A   G   R   P   S   A   L

TGT TCT CAC CCC CGT GCT GGC GGA CGG ACG CCG CGC GGG GGC GGG        135
C   S   H   P   R   A   G   G   R   T   P   R   G   G   G
 V   L   T   P   V   L   A   D   G   R   R   A   G   A   G
  F   S   P   P   C   W   R   T   D   A   A   R   G   R   V

TGT GTA AGG CAG TCG ACG CGG GGG TTG GTT GCA GGG TGG GTA AGA        180
C   V   R   Q   S   T   R   G   L   V   A   G   W   V   R
 V   *   G   S   R   R   G   G   W   L   Q   G   G   *   D
  C   K   A   V   D   A   G   V   G   C   R   V   G   K   M

TGG GGC TGT GTG CGG GGC CGT GTG CGT GCG TGC GTG CGT GCC GGA        225
W   G   C   V   R   G   R   V   R   A   C   V   R   A   G
 G   A   V   C   G   A   V   C   V   R   A   C   V   P   D
  G   L   C   A   G   P   C   A   C   V   R   A   C   R   T

CAC                                                                228
H
```

Figure 19. Screening of λgt11 library with α^{32}p AR8. Filters were lifted from plates containing 200 plaques. Positive plaques are shown on each filter.

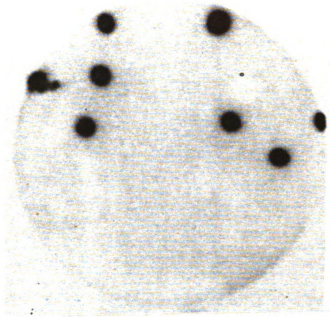


Figure 20. Isolation of ACS recombinant λgt11. λgt11 containing ATC5 was purified with PEG and centrifuged using a glycerol gradient. The DNA was digested with EcoRI and separated using 0.8% agarose gel electrophoresis. The inset shows marker, lane 1; and λgt11-ATC5 lane 2 to 10.

Isolation of ATC5 from λgt11-ATC5

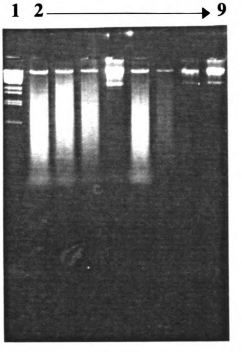


Figure 21. Western blott of ATC5 fusion protein. Panel A and B were detected with Coomaise Brilliant Blue, and antibody against β-galactosidase, respectively. Protein standard is in Panel A lane 1. The second band from the top is β-galactosidase. In Panel A, and B lane 2, E. coli Y1089 extract; lane 3, E. coli Y1089 lysogen extract (ATC5 fusion protein); lane 4 E. coli Y1089 lysogen (λgt11 fusion protein).

SDS-PAGE gel and western blot of ATC5 fusion protein

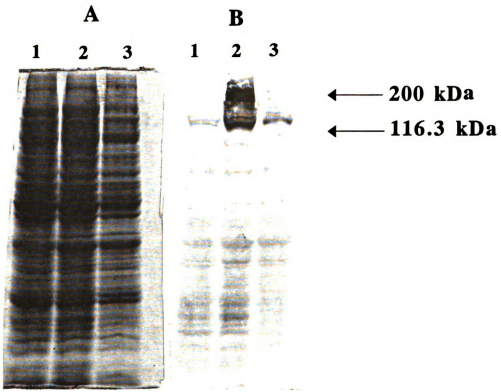


Figure 22. Southern blot analysis of bovine genomic DNA probed with α^{32}P-ATC5. Genomic DNA was digested with HindIII and EcoRI. DNA was separated in agarose gel. The results indicate one gene copy of acetyl-CoA synthetase.

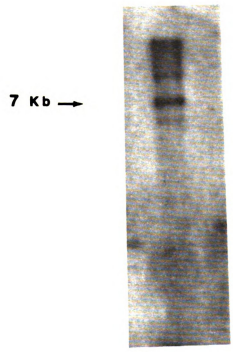

7 Kb

Figure 23. Southern blot of *N.crassa* acetyl-CoA synthetase probed with α^{32}P-ATC5. The fungal clone was digested with HindIII. DNA was separated in agarose gel. The results indicates that AR8 is homologous to the fungal acetyl-CoA synthetase gene.

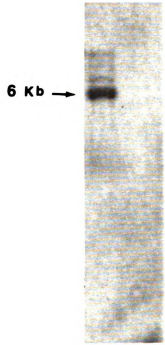

6 Kb

Figure 24. Acetyl-CoA synthetase mRNA multiple forms in different Holstein tissues. poly(A^+)RNA from different Holstein tissues was isolated, separated in formaldehyde gel, transferred to nylon membrane and probed with α^{32}P ATC5. Five μg of poly(A^+)RNA were used in each lane. Lanes: 1, heart; 2, liver; 3, mammary gland and 4, kidney. Heart has 3, mammary gland 3, kidney 2, and liver 1 form of acetyl-CoA synthetase mRNA. The mRNA population ranged from 5.2 to 0.8 kb.

Northern blot of ACS mRNA

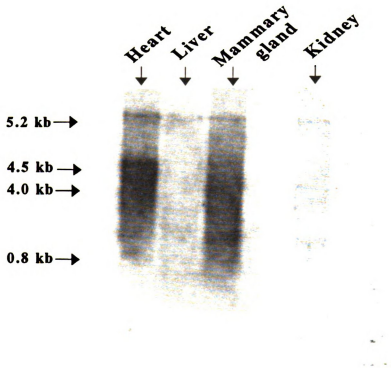


Figure 25. Effect of stage of lactation on relative levels of acetyl-CoA synthetase poly(A^+) RNA in Holstein mammary gland. Mammary gland poly(A^+) RNA was isolated from dry, 60, 120 and 180 day lactating Holstein. Five μg of the mammary poly (A)+ RNA were spotted on nylon filter and probed with α^{32}p-AT5. Acetyl-CoA synthetase mRNA level was highest at 120 days of lactation. In panel A, columns 1,2,3,and 4 represent samples from cows 60, 120, 180 days postpartum and dry mammary gland respectively. β-actin hybridization is shown in panel B.

Effect of lactation on ACS mRNA

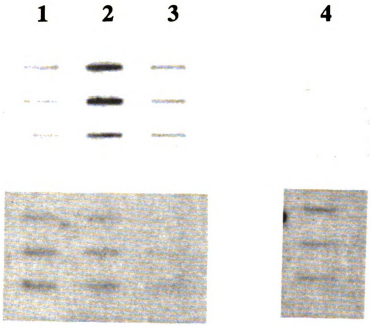


Figure 26. Western blot analysis of mitochondrial extract, examined with rabbit anti-bovine acetyl-CoA synthetase gamma globulin. Mitochondrial proteins were separated in 12.5% SDS-polyacrylamide gel. Alkaline phosphatase-conjugated goat anti-rabbit IgG was used as second antibody. 20 μg of protein was used for each extract. lanes: 1, heart; 2, liver; 3, mammary gland; 4, kidney; and 5, spleen. The acetyl-CoA synthetase antibody used in this experiment appears to have a great deal of nonspecific reactivity to other proteins.

Western blot of mitochondrial protein extracts

1 2 3 4 5

Figure 27.Western blot analysis of mitochondrial extract, examined with rabbit anti-bovine acetyl-CoA synthetase antibody purified using the ATC5 fusion protein. Mitochondrial proteins were separated in 12.5% SDS-polyacrylamide gel. Also, rabbit anti acetyl-CoA synthetase was purified as described in "Materials and Methods." Alkaline phosphatase-conjugated goat anti-rabbit IgG was used as second antibody. Twenty μg of protein was used for each extract. lanes: 1, spleen; 2, kidney; 3, mammary gland; 4, liver; and 5, heart. Accordingly, the antibody against Holstein mammary acetyl-CoA synthetase used in the present study has a reasonably high specificity.

Western blot of mitochondrial protein extracts

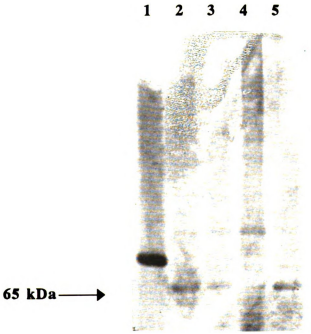
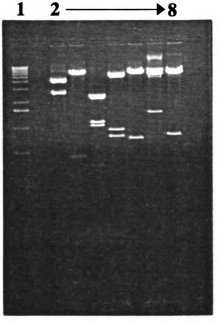


Figure 28. Single and double restriction digests of pUC19-ATC5. pUC19-ATC5 DNA was prepared and digested with several 6 bp cutters. Panel A shows DNA standard; EcoRI; BamHI; BglI, PvuI, SalI, ScaI and SspI digests in lanes 1 to 8 respectively. Panel B shows DNA standard in lane 1 and double digests of pUC19-ATC5 by BglI-EcoRI, BglI-ScaI, BglI-SspI and BglI-SalI are, in lanes 2, 3, 4, and 5, respectively.

Single and double restriction digests of pUC19-ATC5



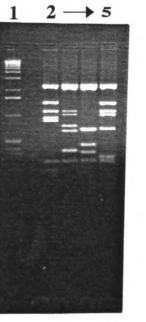


Figure 29. Restriction map and sequencing strategy of ATC5. Restriction sites were determined by single and double endonucleolytic digestions. pUC19-ATC5 was digested with BamHI and SalI. Subclones of the cDNA and the direction in which they were sequenced are given by arrows below the map. The doted arrows represent the location and the direction of the oligos used to sequence the overlap.

ATC5 sequencing strategy

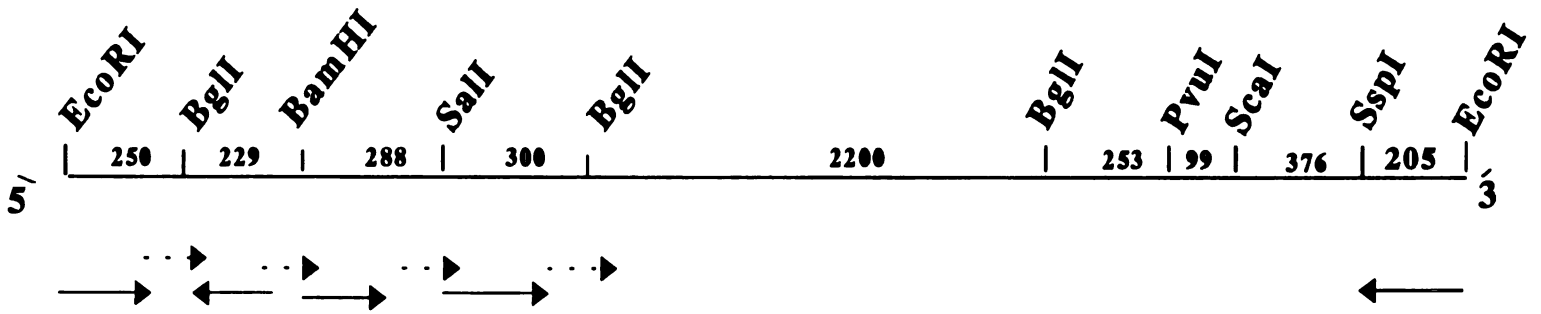


Figure 30. Genetic and restriction enzyme map of the plasmid cloning vector pGEM-3Z.

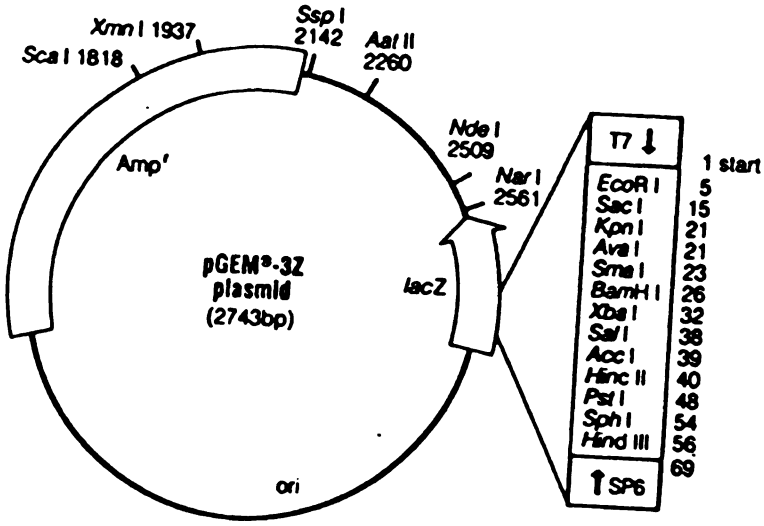

Xmn I 1937
Ssp I 2142
Aat II 2260
Sca I 1818
Nde I 2509
Nar I 2561
Amp'
pGEM®-3Z plasmid (2743bp)
lacZ
ori
T7
EcoR I
Sac I
Kpn I
Ava I
Sma I
BamH I
Xba I
Sal I
Acc I
Hinc II
Pst I
Sph I
Hind III
SP6
1 start
5
15
21
21
23
26
32
38
39
40
48
54
56
69

Figure 31. in vitro translation of ATC5. T7 and SP6 promoters were activated individually. Lane 1, pGEM-3Z (control); lane 2, T7; lane 3 SP6 activation products. Activation of T7 resulted in protein synthesis while activation of Sp6 did not.

Coupled in vitro transcription\translation of ATC5

Figure 32. Nucleotide and predicted amino acid sequence of ATC5. ATC5 was sequenced in one direction. The nucleotide sequence (Panel A) was derived from sequencing one strand. The initiation codon is in bold, and the EcoRI site is underlined. Panel B shows the nucleic acid sequence of ATC5 3' end. Panel C shows the ATC5 homology with *N. carassa*.

A

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CTC TCC CCA TCC TCC CCC CAA GAT TGC ATG CCT GCA GGT CGA CTC        45
L   S   P   S   S   P   Q   D   C   M   P   A   G   R   L

TAG AGG ATC CCC GGG TAC CGA GCT CGA ATT CTC ATG TTT GAC AGC        90
*   R   I   P   G   Y   R   A   R   I   L   M   F   D   S

TTA TCA CTG ATA AGC TTT AAT GCG GTA GTT TAT CAC AGT TAA ATT       135
L   S   L   I   S   F   N   A   V   V   Y   H   S   *   I

GCT AAC GCA GGT CAG GCA CCG TGT ATG AAA TCA TAC AAT GCG CTC       180
A   N   A   G   Q   A   P   C   M   K   S   Y   N   A   L

ATC GTC ATC CTA GGC ACC GTC ACC CTG GAT GCT GAT GGC ATG GCT       225
I   V   I   L   G   T   V   T   L   D   A   D   G   M   A

TGG TAC GAC TGC GGC CCT TGC GGT TAT GCG TCG CGG CCT CTT GCG       270
W   Y   D   C   G   P   C   G   Y   A   S   R   P   L   A

GAT ATC GTC CAT TCC GAC AGC ATG CCA GTC ACT ATG GCC GTG CCG       315
D   I   V   H   S   D   S   M   P   V   T   M   A   V   P

CAG CGC TAT ATG CGT CGA TGC AAT TTC TAT GCG NAC CCG TTC ACG       360
Q   R   Y   M   R   R   C   N   F   Y   A   X   P   F   T

GAG CAC TGT CCG ACC GCC TTT GCC GCC GCC CAG TCC TGC CGT CGC       405
E   H   C   P   T   A   F   A   A   A   Q   S   C   R   R

TAC TTC CAG CCA CTA TCG ACT ACG CGC TCA TGG CGA CCA CAC CCG       450
Y   F   Q   P   L   S   T   T   R   S   W   R   P   H   P

TCC TGT CGA TCC CCG GGC CCT NGC CTC TAC AGG ATC CTC TAC CCC       495
S   C   R   S   P   G   P   X   L   Y   R   I   L   Y   P

GGA CGC ATC GTC CCC GGC ATC ACC GCC NCC ACA GGT GCG GTT GCT       540
G   R   I   V   P   G   I   T   A   X   T   G   A   V   A

GGC GCC TAT ACG CCG ACA TCA CCG ATG GGG AAG ATC GGG CTC GCC       585
G   A   Y   T   P   T   S   P   M   G   K   I   G   L   A

ACT TCG GGC TCA TCA GCG CTT GTT TCG GCG TGG GTA TGG TGT GCA       630
T   S   G   S   S   A   L   V   S   A   W   V   W   C   A

GTC AGT GAT AAG TGG CGG GGG ACT GTT GGG GGC GCC ACT CCT TGC       675
V   S   D   K   W   R   G   T   V   G   G   A   T   P   C

ATG CAC CAT TCC TTG CGG CGG CGT GCT CAA CGG CTC AAC CTA CAC       720
M   H   H   S   L   R   R   R   A   Q   R   L   N   L   H

GGG TGC TTC GAA TGC AGG AGT GCA TGG GAG AGC TCG ACC GAT GCC       765
G   C   F   E   C   R   S   A   W   E   S   S   T   D   A
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TGG AGC TCA CAG AAA GCT TNC ATC CCT GCA GGC CGA CCG ATN CCC        810
W   S   S   Q   K   A   X   I   P   A   G   R   P   X   P

TTG AGA GCC TTC AAC CCA GTC AGC TCC TTC CGG TGG GCN CGG GGC        855
L   R   A   F   N   P   V   S   S   F   R   W   X   R   G

ATG ACT ATC GTC NNC GCA CTT ACA CTG TCT TCT TTA TCA TGC AAC        900
M   T   I   V   X   A   L   T   L   S   S   L   S   C   N

TCG TGG GAC AGG TGC CGG CAG CGA TCT GGG TCA TTT TCG GCG AGG        945
S   W   D   R   C   R   Q   R   S   G   S   F   S   A   R

ACC GCT TTC GCT GGA GCG CGA CGA GGA TCG GCC TGT CGC TTG CGT        990
T   A   F   A   G   A   R   R   G   S   A   C   R   L   R

ATT CGG AAT CTT GCA CGC CCT CGT CGG AGT GTG ATG ACA CTG GTT       1035
I   R   N   L   A   R   P   R   R   S   V   M   T   L   V

CGC TGT CCG TGC ACC TGG AAC TCC GTA TGT TCT GTC CAA GTT CCT       1080
R   C   P   C   T   W   N   S   V   C   S   V   Q   V   P

GCT ATC GGC TTG TTC TTA AAT GCC TGT GAG AGT AGT ACA CTG TAC       1125
A · I   G   L   F   L   N   A   C   E   S   S   T   L   Y

TGT GA                                                           1130
C
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B

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TTTCTTAGAC GTCAGGTGGC ACTTTTTGGG GAAATGTGCG CGGAACCCCT ATTTGTTTAT    60

TTTTCTAAAT ACATTCAAAT ATGTATCCGC TCATGAGACA ATAACCCTGA TAAATGCTTC   120

AATAATATTG AAAAAGCAAG AGTATGAGTA TTCAACATTT CCGTGTCGCC TTATTCCTTT   180

TTTGCGGCAT TTTGCTTCCT GTTTTTTGTA CGCCTATTTT TATAGGTTAA TNTCATGATA   240

ATAATGGTTT CTTAAGACGT CA                                            262
```

C

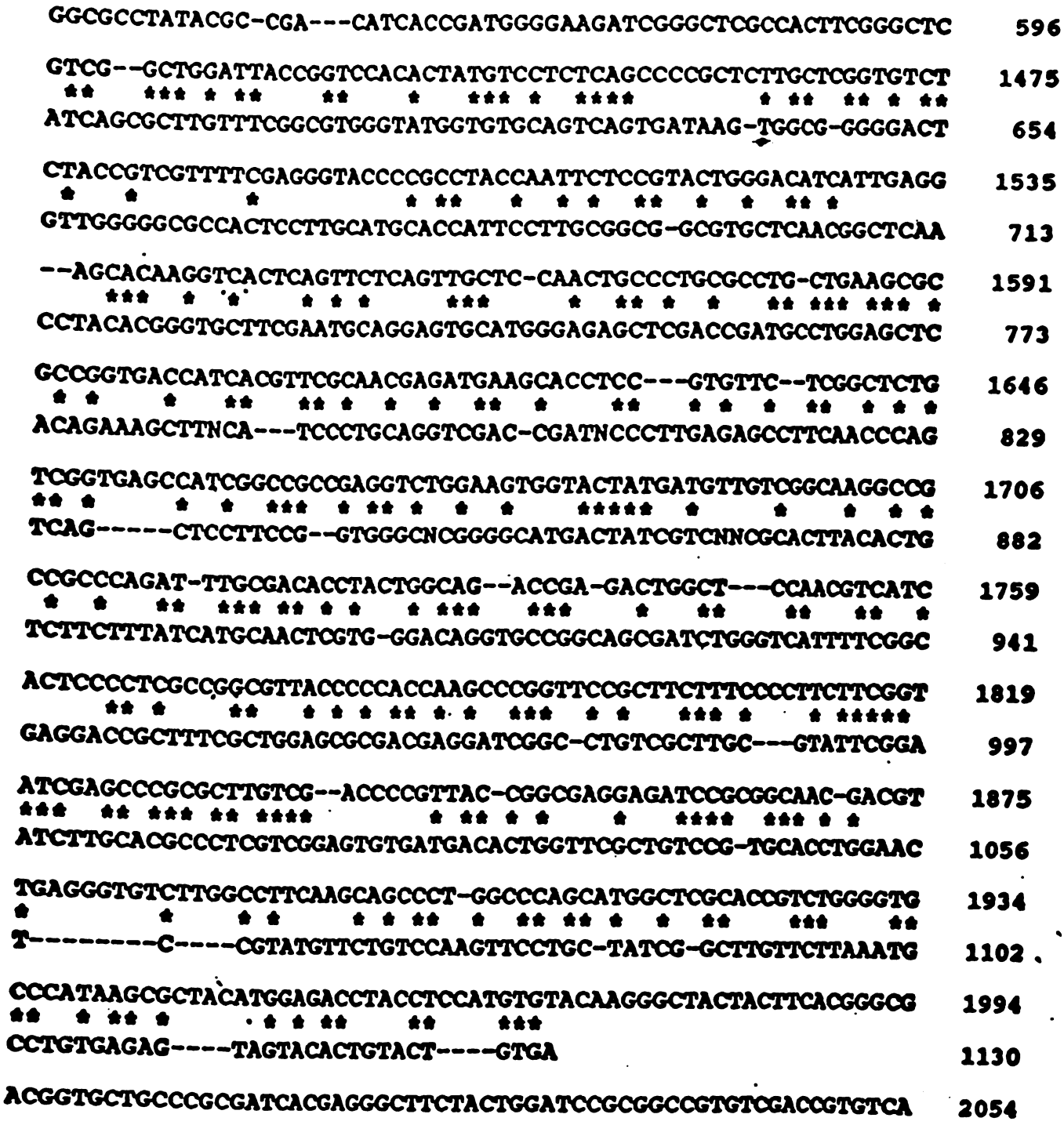
GGCGCCTATACGC-CGA---CATCACCGATGGGGAAGATCGGGCTCGCCACTTCGGGCTC 596
GTCG--GCTGGATTACCGGTCCACACTATGTCCTCTCAGCCCCGCTCTTGCTCGGTGTCT 1475
ATCAGCGCTTGTTTCGGCGTGGGTATGGTGTGCAGTCAGTGATAAG-TGGCG-GGGGACT 654
CTACCGTCGTTTTCGAGGGTACCCCGCCTACCAATTCTCCGTACTGGGACATCATTGAGG 1535
GTTGGGGGCGCCACTCCTTGCATGCACCATTCCTTGCGGCG-GCGTGCTCAACGGCTCAA 713
--AGCACAAGGTCACTCAGTTCTCAGTTGCTC-CAACTGCCCTGCGCCTG-CTGAAGCGC 1591
CCTACACGGGTGCTTCGAATGCAGGAGTGCATGGGAGAGCTCGACCGATGCCTGGAGCTC 773
GCCGGTGACCATCACGTTCGCAACGAGATGAAGCACCTCC---GTGTTC--TCGGCTCTG 1646
ACAGAAAGCTTNCA---TCCCTGCAGGTCGAC-CGATNCCCTTGAGAGCCTTCAACCCAG 829
TCGGTGAGCCATCGGCCGCCGAGGTCTGGAAGTGGTACTATGATGTTGTCGGCAAGGCCG 1706
TCAG-----CTCCTTCCG--GTGGGCNCGGGGCATGACTATCGTCNNCGCACTTACACTG 882
CCGCCCAGAT-TTGCGACACCTACTGGCAG--ACCGA-GACTGGCT---CCAACGTCATC 1759
TCTTCTTTATCATGCAACTCGTG-GGACAGGTGCCGGCAGCGATCTGGGTCATTTTCGGC 941
ACTCCCCTCGCCGGCGTTACCCCCACCAAGCCCGGTTCCGCTTCTTTCCCCTTCTTCGGT 1819
GAGGACCGCTTTCGCTGGAGCGCGACGAGGATCGGC-CTGTCGCTTGC---GTATTCGGA 997
ATCGAGCCCGCGCTTGTCG--ACCCCGTTAC-CGGCGAGGAGATCCGCGGCAAC-GACGT 1875
ATCTTGCACGCCCTCGTCGGAGTGTGATGACACTGGTTCGCTGTCCG-TGCACCTGGAAC 1056
TGAGGGTGTCTTGGCCTTCAAGCAGCCCT-GGCCCAGCATGGCTCGCACCGTCTGGGGTG 1934
T--------C-----CGTATGTTCTGTCCAAGTTCCTGC-TATCG-GCTTGTTCTTAAATG 1102
CCCATAAGCGCTACATGGAGACCTACCTCCATGTGTACAAGGGCTACTACTTCACGGGCG 1994
CCTGTGAGAG----TAGTACACTGTACT----GTGA 1130
ACGGTGCTGCCCGCGATCACGAGGGCTTCTACTGGATCCGCGGCCGTGTCGACCGTGTCA 2054

Table 1. Acetyl-CoA synthetase activity in the fusion protein of λgt11-AR8

Extract	Standard	Test	Δ O.D.	ACS specific activity*
	Without antibody			
E. coli (1089)	0.361	0.331	0.030	19.4
λgt11	0.354	0.316	0.038	24.6
λAgt11-AR8	0.355	0.307	0.048	31.1
	With rabbit anti-bovine ACS			
E. coli (1089)	0.357	0.326	0.031	20.0
λgt11	0.354	0.315	0.039	25.3
λgt11-AR8	0.341	0.341	0.017	11.0
	With monoclonal β-galactosidase antibody			
E. coli (1089)	0.352	0.321	0.031	20.0
λgt11	0.348	0.310	0.038	24.6
λgt11-AR8	0.335	0.336	0.00	0.00

* E.coli is fusion protein extract of E. coli; λgt11, is fusion protein extract of E.coli infected with wild type λgt11; λgt11-ATC5 is fusion protein extract of E. coli infected with λATC5-ACS.

* Specific activity in U\mg protein. One unit is defined as 1 μmol of substrate reacting per hour.

* The results are averages of duplicate determinations for each of the three independent experiments.

Table 3. Effect of purified rabbit anti-bovine ACS antibody on heart ACS activity

Antibody	Standard	Test	Δ O.D.	ACS specific activity*
None	310	286	24	31
Second	310	287	22	29
First and second	310	301	8	10

* The first antibody was rabbit anti-ACS affinity purified using the fusion protein produced by λgt11-ATC5. The second antibody was goat anti-rabbit IgG.

* Specific activity in U\mg protein. One unit is defined as 1 μmol of substrate reacting per hour.

* The results are averages of duplicate determinations.

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