Chapter1

INTRODUCTION
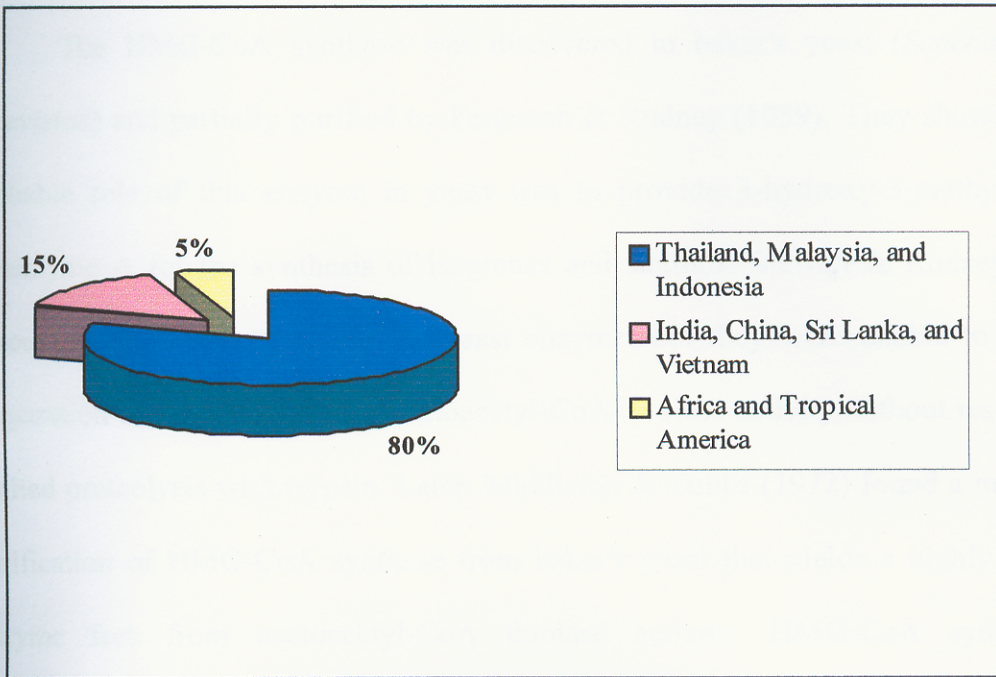
I. Introduction

Rubber tree (*Hevea brasiliensis*) is one of the significant crops for the Thai economy. The number of advantages that the Thai rubber industry has over other its rivals includes high potential to compete in the world market due to its lower production cost, Thailand's favorable climate for rubber planting, and production efficiency, all of which lead Thailand to rank first among the world's rubber producers. The rubber trees are planted in rows on rubber plantations (Figure 1) that cover vast tracts of land. At present, Thailand has become the largest natural rubber producer and exporter in the world and is recognized as the world's leading natural rubber producer and exporter, ahead of Malaysia and Indonesia. Together these three major rubber producers account for 80% in the world's production (Figure 2). Thailand produced 22 million tons of natural rubber, 30 percent of world production and exported 1.9 million tons, generating 57.5 billion baht of foreign currency earnings (Thailand Board of Investment, 1998). Natural rubber produced from *H. brasiliensis* is a valuable commodity in today's economy. A vast number of products are made from it, including washers, gloves, gaskets, tubing, waterproof clothing, toys, erasers, belts, elastics, condoms, bottle stoppers, footwear, and insulation for electrical wiring. The largest single use of rubber is in the manufacture of pneumatic tires, which consumes 60% to 70% of the total world production each year. Therefore, the high yield *H. brasiliensis* clone for rubber latex needs to be further developed,

in order to support industries to add value and maximize the resource's benefit to the country.

Figure 1. A plantation of rubber trees (*Hevea brasiliensis*).

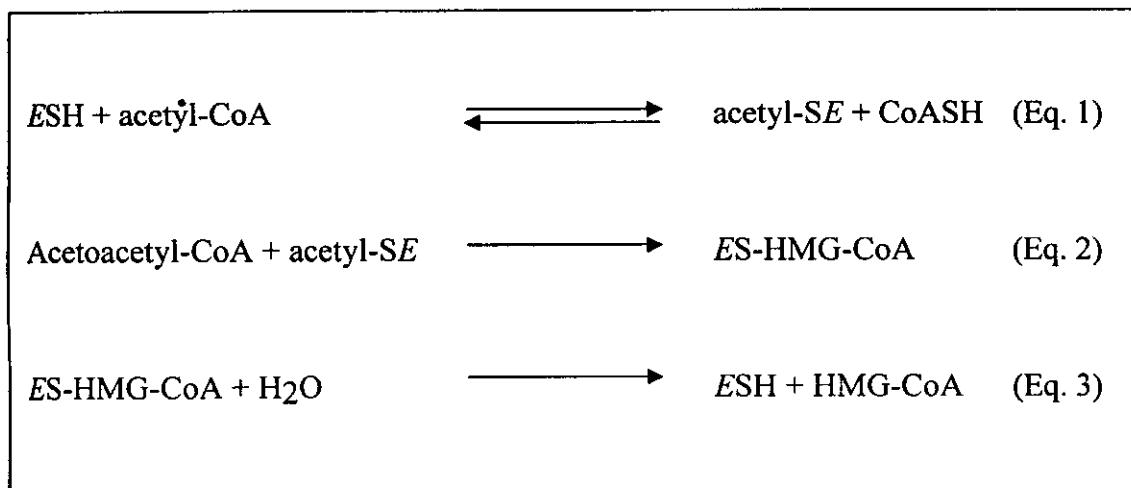**Figure 2. The chart represents the world natural rubber production-2001.**

The data was obtained from Thai Rubber Latex Corporation Public

Company Limited (www.thaitex.com) and The Natural Rubber Industry.

The enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase (EC

4.1.3.5) catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA to form HMG-

CoA plus free CoA. The HMG-CoA synthase is an enzyme in the biosynthesis

pathway of isopentenyl pyrophosphate (IPP), the precursor of rubber latex and other

isoprenoids. Isoprenoids and lipids synthesized from 5-carbon isoform units of IPP,

serve diverse and numerous functions in all living organisms, such as secondary

metabolites in other plants and in bacteria, as well as the synthesis of cholesterol and

ketone bodies in animals.

The HMG-CoA synthase was discovered in baker's yeast (*Saccharomyces cerevisiae*) and partially purified by Ferguson & Rudney (1959). They showed that a probable role of this enzyme in yeast was to provide 3-hydroxy-3-methylglutaryl Coenzyme A for the synthesis of isoprenes and steroids. Stewart & Rudney (1966) succeeded in further purifying the yeast enzyme, but they were unable to free the preparation from contaminating acetoacetyl-CoA thiolase activity without resorting to limited proteolysis with trypsin. Later, Middleton & Tubbs (1972) found a method of purification of HMG-CoA synthase from baker's yeast that yields a highly purified enzyme free from acetoacetyl-CoA thiolase activity. HMG-CoA synthase is susceptible to irreversible inhibition by a wide variety of alkylating and acylating agents. This result suggested that an acetyl-enzyme is a normal intermediate in the reaction mechanism.

Two isoforms of HMG-CoA synthase were reported in chicken (Clinkenbeard et al. 1975a), one cytosolic and the other a mitochondrial enzyme. Cytosolic HMG-CoA synthase is always found in the mevalonate pathway and acts as the control site, co-ordinating with HMG-CoA reductase of isoprenoid and cholesterol biosynthesis whereas mitochondrial HMG-CoA synthase is the control site of the ketogenesis pathway (Clinkenbeard et al. 1975b). Two HMG-CoA synthase isoforms also found in chicken liver were characterized by Reed & Lane (1975). They were different chemical entities, but some uncertainty remained as to whether only one gene produced these two proteins that catalyzed the same reaction.

Miziorko et al. (1975) established that the enzymic formation of HMG-CoA implicated at least three partial reactions in the synthesis of HMG-CoA by the following process in Figure 3. In first step, acetyl group of acetyl-CoA was transferred to the cysteinyl sulfhydryl of enzyme to give an acetyl-S-enzyme intermediate. Subsequently, the thioester intermediate attacks the acetoacetyl-CoA to produce an enzyme-S-HMG-CoA intermediate which was hydrolyzed to be HMG-CoA.

$$ESH + acetyl\text{-}CoA \rightleftharpoons acetyl\text{-}SE + CoASH \quad (Eq. 1)$$

$$Acetoacetyl\text{-}CoA + acetyl\text{-}SE \longrightarrow ES\text{-}HMG\text{-}CoA \quad (Eq. 2)$$

$$ES\text{-}HMG\text{-}CoA + H_2O \longrightarrow ESH + HMG\text{-}CoA \quad (Eq. 3)$$

Figure 3. The three reactions in the synthesis of HMG-CoA.

Some properties of mitochondrial HMG-CoA synthase purified from ox liver were reported by Page & Tubbs (1978). The enzyme was obtained essentially free from thiolase, and the kinetic behavior was like that of the HMG-CoA synthase from chicken liver mitochondria and yeast (Miziorko & Behnke, 1985). They found that this enzyme is irreversibly inhibited by the active site-directed inhibitor 3-chloropropionyl-

CoA. Enzyme modification has been postulated to involve alkylation of an active site cysteinyl sulfhydryl group. The enzyme is a dimer with an overall molecular weight of about 100,000 Dalton (Lowe & Tubbs, 1985).

Later, Gil et al. (1986a, b) reported the cloning and sequencing of a full-length 3.3 kb cDNA from hamster. The cytosolic HMG-CoA synthase gene spans 20 kb. After that, they also investigated the conserved sequence and splicing pattern in human and hamster. The existence of two separate genes, mitochondrial and cytoplasmic, for HMG-CoA synthase was firmly established by Ayte et al. (1990). They cloned the cDNA and then in 1993 the gene for rat mitochondrial HMG-CoA synthase waŝ studied.

HMG-CoA synthase is reported in corpora allata of insects presenting in the juvenile hormone (JH) biosynthesis pathway (Couillaund, 1991). HMG-CoA synthase was also assayed during a gonadotrophic cycle of the insect (*Diploptera punctata*) by Couillaud & Feyereisen (1991). Russ et al. (1992) determined the nucleotide sequence of a hepatic cDNA encoding human cytosolic HMG-CoA synthase. After that, Martinez et al. (1993) and Buesa et al. (1994) found two genes encoding apparently cytosolic HMG-CoA synthase in the insect, *Blattella germanica*. The human mitochondrial HMG-CoA synthase was studied by Boukaftane et al. (1994). In the same year Mitchell (1994) also found HMG-CoA synthase in the worm (*Caenorhabditis elegans*).

Montamat et al. (1995) isolated and characterized the 1.7 kb cDNA encoding *Arabidopsis thaliana* HMG-CoA synthase. Morever, in 1995 Katayama et al. (1995) cloned and sequenced the *hcs* gene which encodes HMG-CoA synthase of the fission yeast (*Schizosaccharomyces pombe*). Wegener's group (1997) reported *Pinus*

sylvestris cDNA encodes a globular protein assumed to be similar to mammalian cytosolic HMG-CoA synthase. Tittiger et al. (2000) reported the isolation and endocrine regulation of HMG-CoA synthase cDNA from the male Jeffrey pine beetle (*Dendroctonus jeffreyi*).

Alex et al. (2000) reported that the expression of *Brassica juncea* HMG-CoA synthase is developmentally regulated and stress-responsive. Moreover, in the same year HMG-CoA synthase was sequenced not only in some bacteria [*Staphylococcus*, *Enterococcus*, and *Streptococcus* by Wilding et al. (2000a and b)] but also in the archaebacteria, *Halobacterium sp. NRC-1*, by Ng et al. (2000). As is apparent from all of the above, HMG-CoA synthase is a widely distributed enzyme. In fact, there are nearly no organisms known that lack HMG-CoA synthase.

The HMG-CoA synthase in *Hevea brasiliensis* has been reported in 1995 by Suvachittanont and Wititsuwannakul. Later, the gene for *H. brasiliensis* HMG-CoA synthase (*hmgs*) was cloned and studied its expression by Suwanmanee et al, 2002 and 2004, respectively. It is possible that the rubber biosynthesis pathway was co-ordinately regulated by the activity of both HMG-CoA synthase and HMG-CoA reductase and there are three genes encoding for HMG-CoA reductase in *H. brasiliensis* (Chye et al. 1992). Therefore, the goal of this study was to investigate a new *hmgs* gene in *H. brasiliensis* for understanding the molecular mechanism of rubber biosynthesis is keen scientific and economic interest, which may provide knowledge for improving the high yield *H. brasiliensis* clone for rubber latex production.

II. Literature review

1. HMG-CoA synthase in bacteria and yeasts

HMG-CoA synthase as well as HMG-CoA reductase is present in the mevalonate pathway, and is required for the synthesis of mevalonate, the precursor of isoprenoid molecules, such as dolichol, cholesterol, ubiquinone, heme, and farnesylated proteins. As an example of the last named, in *Saccharomyces cerevisiae*, deletion of the HMG-CoA reductase gene (*hmg1* and *hmg2*) or HMG-CoA synthase (*erg13*) renders the cells auxotrophic for mevalonate (Basson et al. 1986). The mevalonate pathway was found to influence the function of Ras proteins, which are homologue of mammalian oncogenic proteins.

Red Star bakers' yeast (*Saccharomyces cerevisiae*) was the first organism in which HMG-CoA synthase was studied. In early studies, HMG-CoA synthase was identified and partially purified, but it was still contaminated with acetoacetyl CoA thiolase activity. The enzyme was found to have maximum activity at about pH 9 and was inhibited by HMG-CoA (Ferguson & Rudney, 1959). HMG-CoA synthase from bakers'yeast was further purified; trypsin and chymotrypsin partially inactivated HMG-CoA synthase and increased its K_m for acetyl-CoA 100 fold (Stewart & Rudney, 1966).

A method for improved purification of HMG-CoA synthase from bakers'yeast was reported later. These preparations had an average specific activity of 2.1 units μmol/min/mg, with contamination by thiolase less than 0.2 %. The molecular weight is 130 kDa; the enzyme is irreversibly inhibited by alkylating and acylating agents (Middleton & Tubbs, 1972).

Kinetic and chemical experiments were also conducted in yeast; an ordered reaction pathway was observed in which acetyl-CoA reacts to give a covalent acetyl-enzyme intermediate. The effect of independent variation of both acetyl-CoA and acetoacetyl-CoA concentrations on the initial velocity at pH 8.0 and 8.9 gave results compatible with a sequential mechanism, during which a modified enzyme tentatively identified as an acetyl-enzyme. The reaction of HMG-CoA synthase proceeds by a Ping Pong kinetic mechanism as shown in Figure 4. A mechanism was firstly proposed in which HMG-CoA synthase is acetylated by acetyl-CoA with the release of CoA to give a covalent stable acetyl-enzyme intermediate, which then condenses with acetoacetyl-CoA, yielding a covalent derivative between HMG-CoA and enzyme, which is then rapidly hydrolyzed to free enzyme and product (Middleton & Tubbs, 1974 and Miziorko et al. 1975).
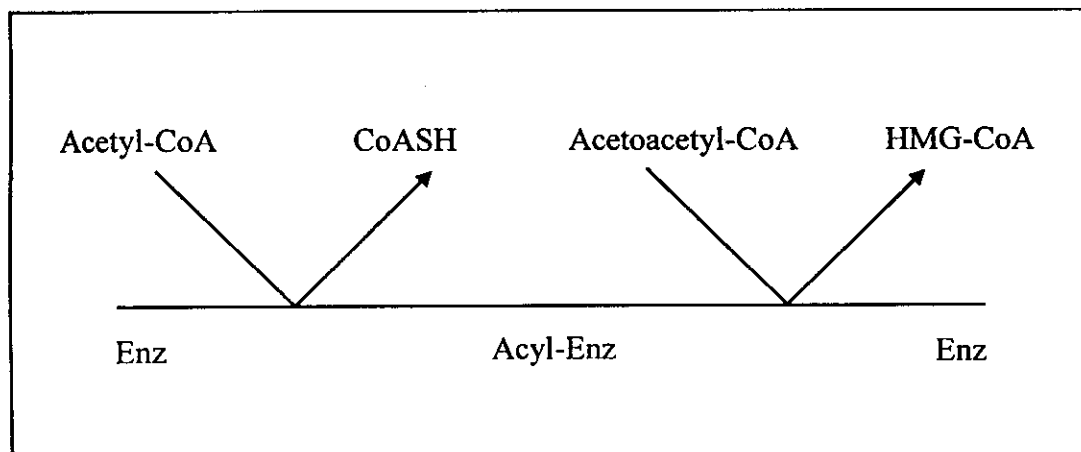
Figure 4. The Ping Pong mechanism of enzyme HMG-CoA synthase.

HMG-CoA synthase was also studied in fission yeast (*Schizosacchoromyces pombe*). The 1.7 kb single copy *hcs* gene encoding HMG-CoA synthase from this

organism was cloned and sequenced (Katayama et al. 1995). The open reading frame

encoded 447 amino acids, which corresponds to a protein with a molecular weight of

49 kDa. The predicted amino acid sequence of the *hcs* gene product has homology

with the HMG-CoA synthase of rat (47.8 % identity), chicken (49.2 % identity),

hamster (47.1 % identity) and human cells (46.9 % identity). The genomic sequence

revealed that the fission yeast *hcs* gene has two introns, one located at the 5'

untranslated leader sequence of the *hcs* gene and the other between nucleotide

positions 60-236. Farnesylation is important for the function of a protein such as Ras

protein and the M-factor that have essential roles in sexual differentiation in

Schizosacchoromyces pombe.

In bacteria, isoprenoid, the principal product of IPP (isopentenyl-diphosphate),

includes the lipid carrier undecaprenol, which is involved in cell wall biosynthesis

menaquinones and ubiquinones involved in the electron transport chain (Meganathan,

1996), and carotenoids (Johnson et al. 1996). Two pathways for the biosynthesis of

IPP have been described: the classical mevalonate pathway and the glyceraldehyde 3-

phosphate (GAP)-pyruvate pathway (Rohmer et al, 1993). All bacteria were thought to

use only the nonmevalonate pathway until recently. Genomic analysis revealed that

the gram-positive cocci and the spirochete, *Staphylococci, Streptococci, Enterococci,*

and *Borrelia burgdorferi;* possess genes predicted to encode all of the enzymes in the

mevalonate pathway, unlike *Bacillus subtilis* (gram-positive) and most gram-negative

bacteria, which possess instead components of GAP-pyruvate pathway as shown in

Table 1 (Wilding et al. 2000a).

Table 1. Distribution of mevalonate and GAP-pyruvate pathway for IPP biosynthesis (Wilding et al. 2000a)

Organism	Presence of	
	Mevalonate pathway	GAP-pyruvate
Gram-negative bacteria		
Enterobacteriaceae	-	+
Haemophilus influenzae	-	+
B. burgdorferi, M. fulvus, C. ethylica	+	-
Gram-positive bacteria		
Mycobacteria, B. subtilis	-	+
Streptomyces spp.[a]	+	+
Low-G+C gram-positive cocci	+	-
Archea	+	-
Fungi	+	-
Higher plants		
Cytoplasmic	+	-
Plastid	-	+
Algae	+[b]	+[c]
Animals	+	-

[a] Some species of *Streptomyces* posses both pathways simultaneously

[b] Including species such as *S. obliquus, Chlorella fusca,* and *Chlamydomonas reinhardtii*

[c] Including species such as *E. gracills*

The eubacterial steptomycete strain appears to possess genes that encode both pathways. Genomic disruption experiments have shown that the mevalonate pathway enzymes are essential for the growth of gram positive cocci; *Staphylococcus aureus*, and mutants auxotrophic for mevalonate were severely attenuated for virulence in mice, Wilding et al. (2000b).

The HMG-CoA synthase present in the mevalonate pathway has not been well studied in bacteria with regard to purification and properties. However, the gene encoding HMG-CoA synthase was sequenced for identification, evolution, and for phylogenetic trees of bacteria.

The nucleotide sequence of the HMG-CoA synthase gene was determined, and the amino acid composition inferred for several bacteria, such as *Staphylococcus haemolyticus* (388 residues), *Streptococcus pyogenes* (391 residues), and *Enterococcus faecalis* (383 residues) (Wilding et al. 2000a), as well as *Staphylococcus aureus sub sp. aureus N315* (388 residues) (Kuroda et al. 2001) and *Streptococcus pneumoniae* (398 residues) (Tettelin et al. 2001). HMG-CoA synthase is also readily detectable in several sequenced archeabacteria genomes such as *Halobacterium sp.NRC-1* (445 residues) (Ng et al. 2000).

2. HMG-CoA synthase in animals

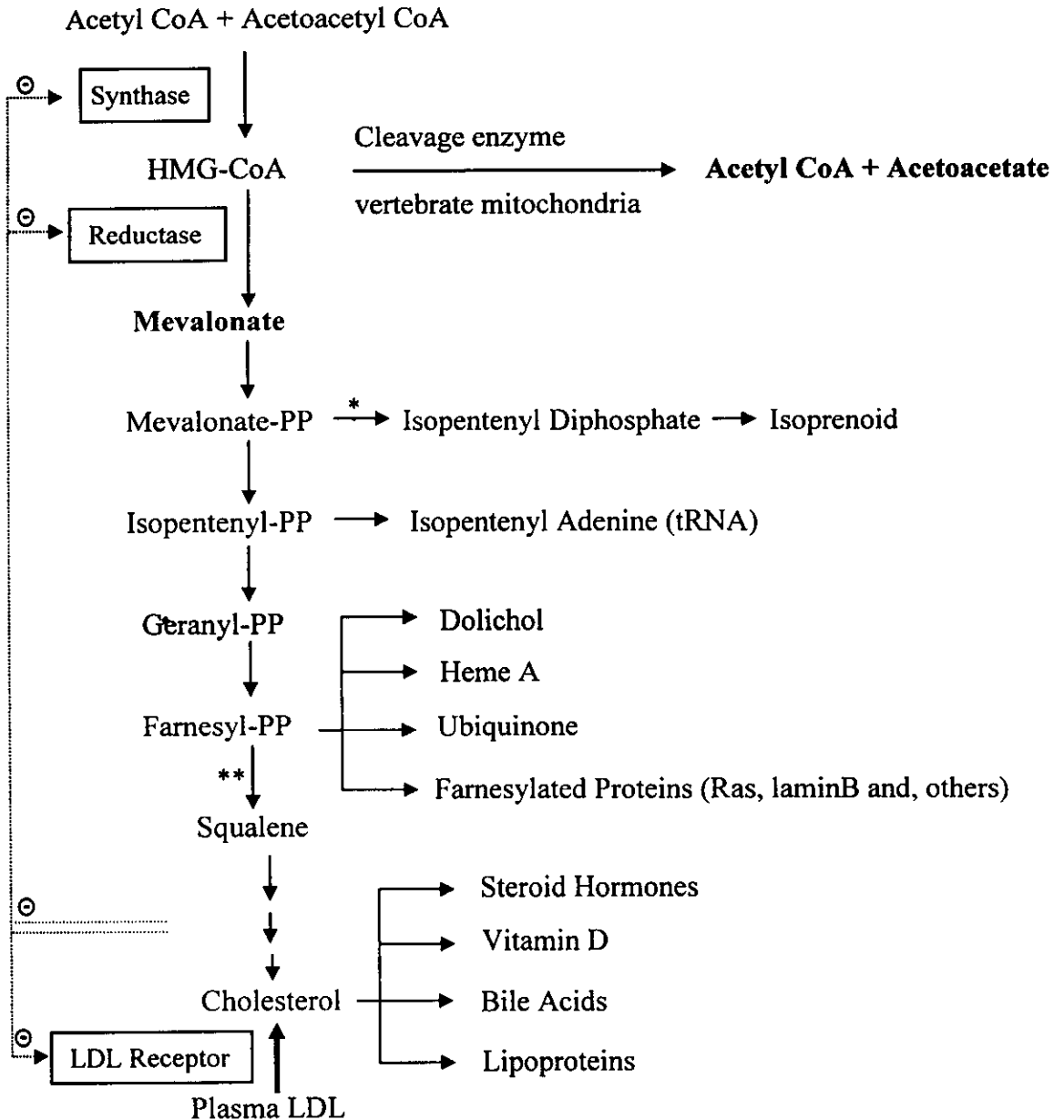Most of the studies on the HMG-CoA synthase have been focused on the mevalonate pathway in mammals because the synthesis of mevalonate is the rate-limiting step in cholesterol synthesis in the cytoplasm, which is clinically important as the target of cholesterol-lowering drugs. In vertebrates, there are two distinct HMG-CoA synthases, a cytosolic and a mitochondrial enzyme. The cytosolic HMG-CoA

synthase catalyzes the second step of cholesterol pathway to produce HMG-CoA transformed into mevalonate by the action of HMG-CoA reductase. This starts the isoprenoid pathway which, in addition to cholesterol as the main end-product, produces several important products, such as ubiquinone, dolichol, isopentenyl adenosine and farnesyl groups, which covalently modify proteins (Figure 5). The mitochondrial HMG-CoA synthase is localized in the mitochondrial matrix and occupies a potentially favorable position for the regulation of hepatic ketogenesis. HMG-CoA produced inside the mitochondria is transformed into acetoacetate by the action of HMG-CoA lyase, acetoacetate is then transformed into β-hydroxybutylrate and acetone. All of these are known as ketone bodies, Reed et al. (1975).

In animals, HMG-CoA synthase was first found in chicken liver (Reed & Lane, 1975). Twenty to forty percent of the HMG-CoA synthase from avian and rat liver is localized in the cytoplasmic compartment, the remainder residing in the mitochondria. Chicken liver mitochondrial HMG-CoA synthase activity consists of a single enzymic species with a pI of 7.2, whereas the cytoplasmic activity is composed of at least two species with pI values of 4.8 and 6.7 (Clinkcnbeard et al. 1975a).

The molecular weight of native mitochondrial synthase from avian liver of 105 kDa showed that it is a homodimer of 53-57 kDa monomers. Antibodies against mitochondrial HMG-CoA synthase do not react against the cytosolic HMG-CoA synthase. Mg^{2+} also inhibits mitochondrial HMG-CoA synthase, whereas cytosolic HMG-CoA synthase is activated by this cation (Reed et al. 1975).

Acetyl CoA + Acetoacetyl CoA

⊖ → [Synthase]

HMG-CoA   —— Cleavage enzyme ——→ **Acetyl CoA + Acetoacetate**
          vertebrate mitochondria

⊖ ⇢ [Reductase]

Mevalonate

Mevalonate-PP —*→ Isopentenyl Diphosphate —→ Isoprenoid

Isopentenyl-PP —→ Isopentenyl Adenine (tRNA)

Geranyl-PP ┌→ Dolichol

            ├→ Heme A

Farnesyl-PP ┼→ Ubiquinone

         **↓ └→ Farnesylated Proteins (Ras, laminB and, others)

Squalene

⊖   ┌→ Steroid Hormones

           ├→ Vitamin D

Cholesterol ┼→ Bile Acids

⊖ → [LDL Receptor] ↑ └→ Lipoproteins
Plasma LDL

Figure 5. Organization of the mevalonate pathway

This was adapted from Goldstein and Brown (1990). (⊖ = Negative
feedback inhibition, * = in plants but not animals, ** = in mammals but not plants
and insects)

Acetyl-CoA reacts stoichiometrically with a cysteinyl sulphydryl group on avian liver HMG-CoA synthase to yield acetyl-S-enzyme. The ability of the acetylated enzyme, upon addition of acetoacetyl-CoA, to form HMG-CoA indicates that the acetylated cysteine residue is at the catalytic site and acetylation of synthase is the rate-limiting step in HMG-CoA synthase (Miziorko et al. 1975).

Avian liver mitochondrial HMG-CoA synthase is irreversibly inhibited by the active site-directed inhibitor 3-chloropropionyl-CoA. A cysteinyl residue at the active site is the chloropropionyl-CoA target; this observation partially elucidated the active site structure for the ketogenic mechanism (Miziorko & Behnke, 1985). The acetylated cysteine corresponds to position 129 in the sequence deduced from cDNA data for the hamster cytosolic enzyme (Gil et al. 1986b) and in avian cytosolic HMG-CoA synthase also indicates that Cys^{129} is the principal target (Misra et al. 1995).

The imidazole of histidine-246 in the avian enzyme may play a role in anchoring the second substrate, acetoacetyl-CoA, by interacting with the carbonyl oxygen of the thioester functionality (Misra & Miziorko, 1996). Kinetic studies have also been conducted on the mitochondrial enzyme from ox liver; its behavior is like that from chicken and yeast (Page & Tubbs, 1978). The relative molecular mass of ox liver mitochondrial HMG-CoA synthase is about 100,000 Da and it appears to be a dimer of identical subunits (Mr 47,900 Da) (Lowe & Tubbs, 1985).

In hamsters, the feeding of cholesterol led to a decrease of more than 85 % in the amount of mRNA for both HMG-CoA synthase and HMG-CoA reductase in liver, suggesting that the mRNAs for two cytosolic enzymes are coordinately regulated by cholesterol (Gil et al. 1986a). In hamsters the two negatively regulated genes have extraordinarily complex 5' untranslated regions (Reynolds et al. 1985). In yeast, the

complex 5'-flanking region contains multiple AUG codons and serves as the target site for feedback suppression of translation (Tzamarias et al. 1986).

The gene for hamster cytosolic HMG-CoA synthase is unusual in that it contains a variably spliced intron in the region of the gene corresponding to the 5' untranslated region, thereby producing mRNAs that contain either one or two exons in this region (Gil et al. 1986b). The mRNA for hamster HMG-CoA synthase contains an optional exon in the 5' untranslated region. Approximately 50% of the mRNA transcripts in the UT-1 line of cultured Chinese hamster ovary cells contain a 5'-untranslated region of 68 nucleotides that is interrupted by a single intron located 10 nucleotides upstream of the initiator AUG (nucleotides position-10). The other 50% of the mRNAs have a longer 5' untranslated region of 127 nucleotides, resulting from the insertion of an extra exon of 59 nucleotides at position-10. It is called an "optional" second exon (Gil et al. 1986b).

A similar alternative splicing pattern for cytosolic HMG-CoA synthase was observed in three human tissues: cultured fibroblasts, fetal adrenal gland, and fetal liver. A cDNA for cytosolic human synthase had 90% identity with the hamster sequence in the region corresponding to the optional exon. This sequence contains 20 of 26 nucleotides that are identical immediately upstream of the initiator AUG codon in the mRNA for hamster HMG-CoA reductase, the enzyme that follows the synthase in the isoprenoid biosynthetic pathway. These findings raise the possibility that the optional exon plays an important, conserved functional role in human and hamsters (Gil et al. 1987).

Studies on the HMG-CoA synthase in rat confirm that there are two genes for HMG-CoA synthase. Rat mitochondrial and cytosolic HMG-CoA synthases are

encoded by two different genes (Ayte et al. 1990). The full-length 1,994 bp cDNA encompasses the entire transcription unit of the rat mitochondrial HMG-CoA synthase, which encodes a polypeptide of 508 residues with a 57 kDa molecular mass. The protein is 65% identical with hamster cytosolic HMG-CoA synthase. Percent identity of HMG-CoA synthases among some animals is shown in Table 2.

Table 2. Comparison of percent amino acid identity in HMG-CoA synthase among some vertebrate animals.

		Cytoplasm (%)				Mitochondria (%)			
		rat	hamster	human	chicken	rat	mouse	human	pig
Cytoplasm (%)	rat	-	97.1	94.4	84.1	65.5	66.0	67.0	65.5
	hamster		-	95.2	83.7	65.5	66.0	67.0	65.6
	human			-	84.0	66.0	66.5	67.5	66.2
	chicken				-	65.8	66.4	67.0	67.0
Mitochondria (%)	rat					-	98.5	89.0	83.2
	mouse						-	91.0	84.3
	human							-	84.7
	pig								-

In rat mitochondrial HMG-CoA synthase, a 19 amino acids sequence region, probably corresponding to the catalytic site, is highly similar (90%) with the chicken liver mitochondrial HMG-CoA synthase. The 37 amino acids at the N-terminal are not present in the cytosolic enzyme. The hydrophobic and hydroxylated nature of the residues of this part of the protein suggests that it is a leader peptide to target HMG-CoA synthase inside mitochondria. The rat cytosolic HMG-CoA synthase is 3.4 kb by southern blot analysis (Ayte et al. 1990). Rat liver cytosolic HMG-CoA synthase exhibits a diurnal rhythm of enzyme activities, which coincides with the diurnal rhythm of HMG-CoA synthase protein, this enzyme was repressed by fasting and cholesterol feeding (Royo et al. 1991). In contrast, the mitochondrial HMG-CoA synthase was increased by fasting.

The nucleotide sequence of the coding region of the hepatic human cytosolic HMG-CoA synthase cDNA was determined (Russ et al. 1992). An open reading frame of 1,560 nucleotides constitutes the complete coding region of human HMG-CoA synthase. The nucleotide and deduced amino acid sequence show high resemblance to known mammalian cytosolic sequences (hamster: cDNA 92%, protein 95% identity; rat: cDNA 86%, protein 94% identity); similarity to chicken HMG-CoA synthase was slightly lower (cDNA 74%, protein 84% identity). The putative catalytic region and the three cysteine residues delivering reactive sulfhydryl groups (amino acid positions 129, 224, 268) turned out to be completely conserved.

The full-length cDNA of human liver mitochondria HMG-CoA synthase was reported by Mascaro et al. (1995). A 2058 bp cDNA encodes a polypeptide of 508 amino acid residues and 56,635 Da. The amino acid sequence identity with rat mitochondrial and human cytosolic HMG-CoA synthase is 89% and 67%,

respectively. Its mRNA levels were high in liver and colon, low in testis, heart, skeletal muscle, and kidney, and faint in pancreas.

The human mitochondria HMG-CoA synthase is encoded by the *hmgcs2* gene, which spans 20 kb of genomic DNA on chromosome 1p13-p12 (Boukaftane et al. 1994) and contains 10 exons. Comparative analysis of all known mitochondrial and cytosolic HMG-CoA synthases shows a high degree of conservation near the N-terminal that decreases progressively toward the C-terminal; indicating that the two enzymes arose from a common ancestor gene. Comparison of the gene structure of mitochondrial and cytosolic HMG-CoA synthase is also consistent with a duplication event. HMG-CoA synthase gene duplication accounts for the appearance of HMG-CoA synthase within the mitochondria around the time of emergence of early vertebrates. The event linked preexisting pathways of beta oxidation and leucine catabolism and created the HMG-CoA pathway of ketogenesis, thus providing a lipid-derived energy source for the vertebrate brain (Boukaftane et al. 1994).

The mitochondrial activity and mRNA level of HMG-CoA synthase in adult mammals is increased by fasting, fatty acids feeding, cAMP, diabetes, glucagon administration, and the transition from fetal to suckling state (Serra et al. 1993), whereas re-feeding and insulin repress the transcriptional state and enzyme activities (Casals et al. 1992). Cytosolic HMG-CoA synthase is expressed in most tissues and is considered a house-keeping gene, while mitochondrial HMG-CoA synthase is expressed mostly in liver, colon, ceacum and testis of adult mammals, and also in the small intestine of suckling animals (Serra et al. 1996). Pig mitochondrial HMG-CoA synthase showed a tissue-specific expression pattern. As with rat and human, the gene

is expressed in liver and large intestine. However, the pig differs in that mRNA was not detected in testis, kidney or small intestine (Adams et al. 1997)

The cytosolic HMG-CoA synthase and mitochondrial HMG-CoA synthase are clearly revealed that they are the products of two different genes and that they are differently regulated. Structural and functional comparison of the promoter regions of the two synthases showed that the two promoters are very different.

Cytosolic HMG-CoA synthase contains sterol regulatory elements (SREs) in the promoter to modulate transcriptional activity by sterols, mediated by sterol regulatory element binding proteins (SREBPs)-1 and-2 (Yokoyama et al, 1993; and Hua et al. 1993) which are not present in the promoter of mitochondrial HMG-CoA synthase. Cytosolic HMG-CoA synthase is regulated by negative feedback from cholesterol. A low cellular sterol concentration induces the transcription of the gene for cytosolic HMG-CoA synthase (Gil et al. 1986b). This negative feedback is mediated by SREBPs. When the sterol levels decrease, the mature forms of SREBPs are released from the endoplasmic reticulum by a two–step proteolytic process; they then translocate to the nucleus and activate the transcription of genes related to cholesterol and fatty acid metabolism such as the HMG-CoA synthase gene (Brown et al. 1997). Fluvastatin, a hypocholesteremic drug, increased the transcriptional activity of cytosolic HMG-CoA synthase. This indicates that the increase in transcriptional activity in the HMG-CoA synthase gene attributable to fluvastatin is a consequence of the activation of the proteolytic cleavage of SREBPs by reduced levels of intracellular cholesterol (Mascaro et al. 2000).

Conversely, the rat mitochondrial HMG-CoA synthase contains liver-specific enhancer elements in the proximal promoter region and elements that mediate the

enzyme's multihormonal regulation (Gil-Gomez et al. 1993). The gene for the rat mitochondrial HMG-CoA synthase spans at least 24 kb and contains 10 exons and 9 introns. Exon 1 contains the untranslated sequences of the transcript and includes the coding region for the putative mitochondrial-targeting signal (35 amino acids). The 1,148 bp of the 5' flanking region contains typical promoter elements including a TATA box and several putative recognition sequence for transcription factors in controlling both basal-level and hormone-modulated transcription rates. The region is responsible for multi-hormonal regulation of gene transcription (Table 3).

Fatty acids induce the transcription of the gene for mitochondrial HMG-CoA synthase both *in vivo* and *in vitro* (Casals et al. 1992). *Cis*-elements include several known sequences for transcriptional regulation of mitochondrial HMG-CoA synthase gene as shown in Figure 6. Transcriptional activation by fatty acids seems to be mediated by a member of the nuclear hormone receptor superfamily, termed peroxisome proliferator-activated receptor (PPAR), which is a ligand-activated transcription factor. PPAR can bind to a specific DNA sequence, called the PPRE; elements of this kind have been located upstream of several genes (Tugwood et al. 1992). This activation process is dependent on heterodimer formation between PPAR and *cis*-retinoid receptor (RXR). An element, peroxisome proliferator-regulatory element (PPRE) that is responsive to PPAR for activation by fatty acids has not been detected in the promoter of the cytosolic HMG-CoA synthase gene (Rodriguez et al. 1994). The occurrence of the nuclear receptor responsive element (NRRE) which binds to PPAR, chicken ovalbumin upstream promoter transcription factor (COUP-TF) and hepatocyte nuclear factor-4 (HNF-4) and the CRE binding site has been demonstrated.

Table 3. Potential regulatory *cis*-elements in the mitochondrial HMG-CoA synthase gene.

Abbreviations: IRE, insulin regulatory element; CTF, CCAAT-binding transcription factor binding site; NF1, nuclear factor 1 binding site; C-EBP, CCAAT enhancer binding protein; GRE, glucocorticoid regulatory element; NRRE, nuclear receptor responsive element; CRE, cAMP regulatory element.

Cis-element	Sequence	Gene position
TATA	TATAAA	-28
Sp1	GGCGGG	-54
NRRE	AGACCTTTGGCCC	-92
IRE	TGATGTTTTC	-130
CTF-NF1	TGGCA	-520, -836
CRE	GTGCGTCA	-546
C-EBP	AGTCAAAAG	-778
GRE	GCTACAGGTTGTGCT	-995
NF1-like	TGGCA	-520, -836, -1140
c-Jun	TGTGTCA	-249
	TGCGTCA	-554
	TGACTCC	-781
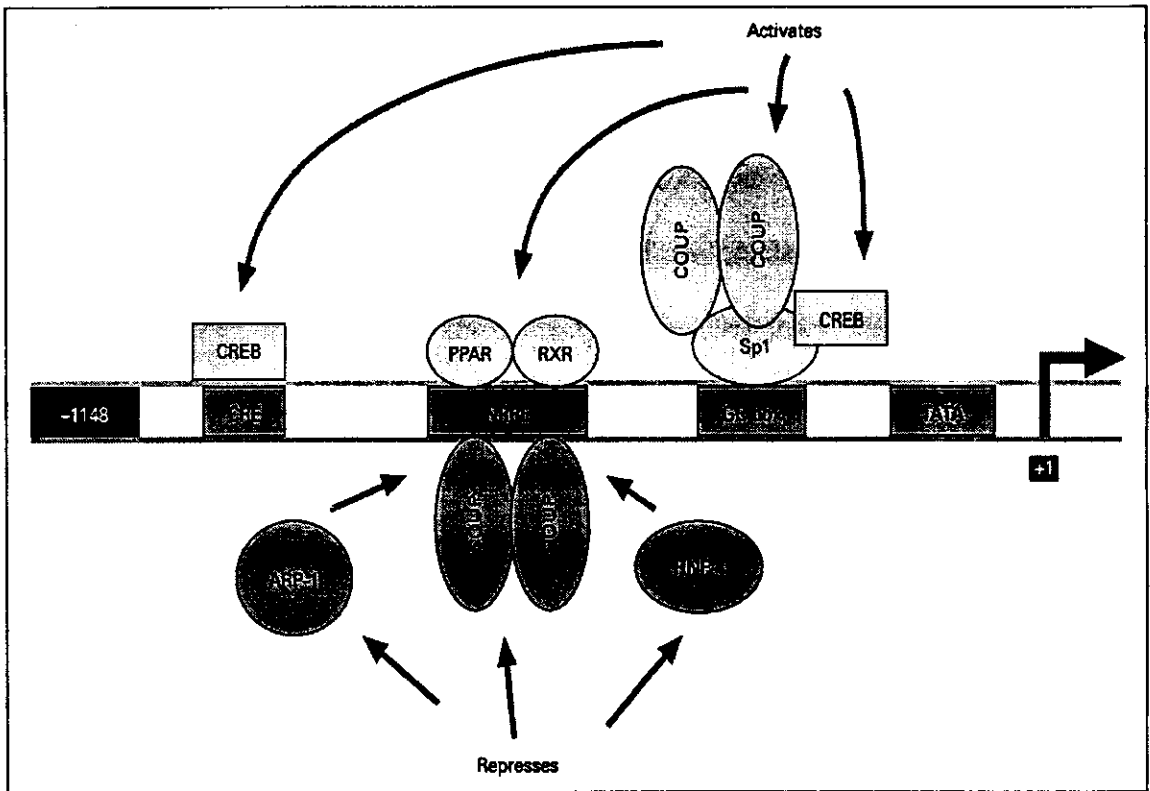
Figure 6. *Cis*-elements of the mitochondrial HMG-CoA synthase promoter.

Transcription factors PPAR, RXR, Sp1 and CREB bind to the mitochondrial HMG-CoA synthase promoter at different sites, activating transcription. Other transcription factors, such as HNF-4 and ARP-1, repress transcription by competing with the heterodimer PPAR–RXR at the NRRE site. COUP-TF can either activate or repress transcription, depending on the binding site and the tissue. Abbreviation: ARP-1, apo A1 regulatory protein-1.

The cAMP-induced transcription of the mitochondrial HMG-CoA synthase gene is mediated by the CRE-binding protein (CREB) through both Sp1 and CREB (Hegardt, 1999) binding sites in the promoter of the gene (Figure 4). The COUP-TF is another member of the nuclear hormone receptor superfamily. COUP-TF activates the transcription of mitochondrial *hmgs* gene, irrespective of the NRRE locus, by binding to a fragment comprising nucleotides -62 to +28 (Rodriguez et al. 1997), through an interaction with the transcription factor Sp-1, which binds to a GC box present in the promoter. In addition, COUP-TF competes with PPAR for binding to the same NRRE sequence (positions -104 to -92), and this competition leads to inhibition of the transcriptional activation produced by the heterodimer PPAR–RXR (Hegardt, 1999) as shown in Figure 4. On the other hand, HNF-4 represses the mitochondrial HMG-CoA synthase gene in transiently transfected HepG2 cells (Rodriguez et al. 1998). This repression seems to be caused by competition between HNF-4 and PPAR in the promoter of mitochondrial HMG-CoA synthase by binding to the same locus that binds PPAR and COUP-TF (Figure 4).

Insects do not have squalene synthase (farnesyl-diphosphate farnesyl transferase) or lanosterol synthase (Beenakkers et al. 1985), so they cannot synthesize cholesterol *de novo*. Therefore, from the acetyl-CoA in the mevalonate pathway leads to other specific isoprenoids via farnesyl pyrophosphate, such as the juvenile hormome (JH), which is synthesized by the corpora allata (Schooley and Baker, 1985). JH has a crucial role in the maintenance of the larval form vitellogenesis and also in the maturation of the reproductive system (Feyereisen, 1985).

Since HMG-CoA synthase and HMG-CoA reductase are generally considered the rate-limiting steps in vertebrate cholesterol biosynthesis pathway, it has been

suggested that they could also be the rate-limiting step in JH biosynthesis. HMG-CoA synthase measured during a gonadotrophic cycle in *Diploptera punctata* corpora allate showed that the dramatic changes in the JH III synthetic rate are reflected in the enzymatic rates of HMG-CoA synthase (Couillaud and Feyereisen, 1991).

Blattella germanica is an insect that has two cytoplasmic HMG-CoA synthase genes; both are regulated in the ovary during the gonadotrophic cycle (Buesa et al. 1994). One is a 1658 bp cDNA that encompasses the entire transcription unit of the HMG-CoA synthase gene (*hmgs-1*). The cDNA encodes a polypeptide of 453 amino acids (Mr 50338 Da) that is similar to vertebrate HMG-CoA synthase (74-76% conserved residues). The HMG-CoA synthase transcript was differentially expressed throughout *Blattella germanica* development. Analysis of RNA from different adult female tissues shows high HMG-CoA synthase mRNA levels in the ovary and lower levels in brain and muscle (Martinez-gonzalez et al. 1993). The other HMG-CoA synthase gene in *Blattella germanica* has a 1,716 bp cDNA with a deduced protein of 455 residues with a molecular mass of 51,424 Da. The two synthases have 69% identical amino acid residues, both lack an N-terminal leader peptide to target the protein into mitochondria and neither corresponds to the mitochondrial form found in vertebrate animals. Both HMG-CoA synthase genes are expressed differently throughout development. Analysis of adult tissues shows higher expression in ovary and fat body (Buesa et al. 1994). HMG-CoA synthase and reductase showed coordinated expression and activity in the fat body of *Blattella germanica* during the first gonadotrophic cycle, vitellogenesis (Casals et al. 1996). The activity changes more than 10 fold in the corpora allata and fat body, and peak activities correspond to maximum rate of JH and vitellogenin production.

The catalytic properties of recombinant *hmgs-1* in *Blattella germanica* were observed by Cabano et al, 1997. Morever, *hmgs1* in *Blattella germanica* has structural and functional features of an active retrogene (Casals et al. 2001). This gene shows several feature of processed gene (retroposons); it contains no introns but has a short direct-repeat sequence at both ends. An atypical feature is the presence at both ends of the gene of short inverse repeats flanked by direct repeats. There is neither a TATA box nor a CAAT box in the 5' region and *hmgs1* gene derives from *hmgs2*.

The full-length HMG-CoA synthase from Jeffrey pine beetle, *Dendroctonus jeffreyi* Hopkins was isolated and the effect of topical applications of JH III on enzyme expression studied (Tittiger et al. 2000). A single copy gene has 63% and 58% identity with *hmgs1* and *hmgs2* from *Blattella germanica* and 61% identity with *Drosophila melanogaster hmgs*. *Dendroctonus jeffreyi hmgs* transcript levels remain uniformly low in JH III-treated and control *Dendroctonus jeffreyi* females, but are induced approximately 2.5-5 fold in JH III- treated males. JH III causes a dose-and time-dependent increase in *hmgs* transcripts in the male metathoracic-abdominal region.
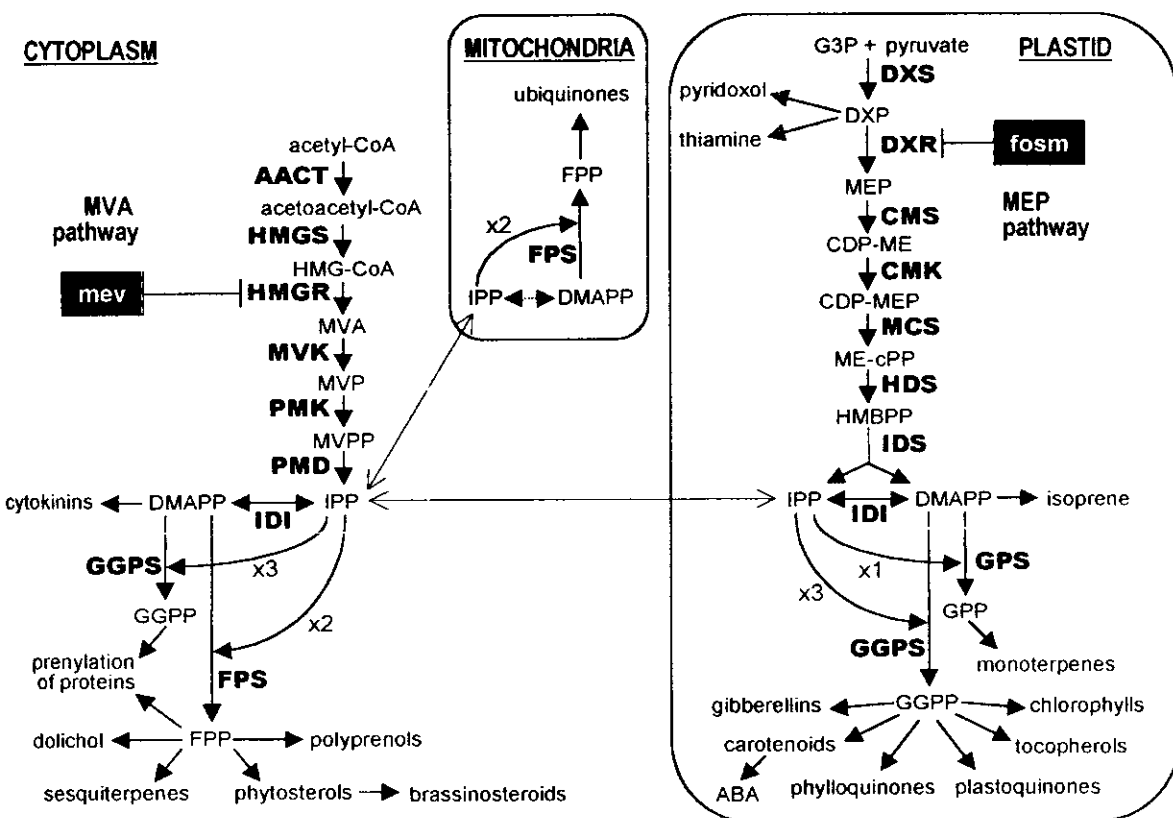
In addition the HMG-CoA synthase cDNA from African clawed frog; *Xenopus laevis* is submitted in GenBank in the accession number AAH42929. It is apparent that HMG-CoA synthase has been well studied in animals because of its medical importance. This thesis, however, will be mainly concerned with the plant genes. Nonetheless, many of the inputs studied in animals may have relevance to plants. In particular, the regulation of enzyme activity, at many levels, has been explored in animals.

3. HMG-CoA synthase in plants

Plants are capable of synthesizing a myriad of isoprenoid and prenyl lipids. Much less is known in plants about the role of isopentenyl pyrophosphate (IPP) in isoprenoid biosynthesis pathway. Higher plants were shown to possess two distinct biosynthetic routes for IPP biosynthesis; while cytoplasmic sterols form via the acetate/mevalonate pathway. Another biosynthetic pathway of IPP formation using pyruvate and glyceraldehyde-3-phosphate as substrate is called the DXR or GAP-pyruvate pathway; it is present in plastids as shown in Figures 7 and 8 (Lange et al. 2000) and (Rodriguez and Boronat, 2002), respectively. This pathway was first found in eubacteria and green algae. The chloroplast-bound isoprenoids (β-carotene, lutein, prenylchains of chlorophylls and plastoquinone-9) are synthesized via this pathway (Lichtenthaler et al.1996).

In the first isoprenoid biosynthesis pathway, HMG-CoA synthase converts acetyl-CoA and acetoacetyl-CoA to HMG-CoA, and HMG-CoA reductase reduces HMG-CoA to mevalonate, which is subsequently converted to isopentenyl pyrophosphate, the universal precursor for isoprenoids. In plants, isoprenoid compounds such as sterols and ubiquinones, growth regulators (gibberellins, abscisic acid, brassinosteroid, and cytokinin) phytoalexins, and other specialized terpenes including natural rubber are essential for normal growth, development and defense against pathogens (Bach et al. 1995).

B

Pyruvate +D-Glyceraldehyde 3-phosphate

$\downarrow$ DXPS

A

1-Deoxy-D-rylulose-5-phosphate

Acetyl-CoA

$\downarrow$**DXR**

$\downarrow$AACT

2-C-Methyl-D-erythritol 4-phosphate

Acetoacetyl-CoA

$\downarrow$ CMT

$\downarrow$**HMGS**

4-(Cytidine 5'-diphospoh)-2-C-methyl-D-erythritol

HMG-CoA

$\downarrow$ HMGR

CMK

Mevalonate

$\downarrow$ MK

Mevalonate-5-phosphate

2-Phospho-4-4-(Cytidine5'-diphospoh)-2-C-methyl

D-erythritol

$\downarrow$PMK

$\downarrow$ MECPS

Mevalonate-5-diphosphate

2-C-Methyl-D-erythritol-

2,4-cyclodiphosphate

MDC

IPP

IPPI

Isopentenyldiphosphate $\xleftrightarrow{\quad*\quad}$ Dimethylallyldiphosphate

**

Cholesterol

Isoprenoid end products

Figure 7. Biosynthesis of IPP via the mevalonate pathway (A) and the DXP

pathway or GAP-pyruvate pathway (B).

*Plant, ** Animal. The large open arrow indicates the as yet unidentified

step (Lange et al. 2000).

Figure 8. Isoprenoid biosynthesis pathways in the plant cells

HMG-CoA, Hydroxymethyl-glutaryl CoA; MVP, 5-phosphomevalonate; MVPP, 5-diphosphomevalonate; HBMPP, hydroxymethylbutenyl 4-diphosphate; FPP, farnesyl diphosphate; ABA, abscisic acid. The first intermediate specific to each pathway is boxed. Enzymes are indicated in bold: **AACT**, acetoacetyl CoA thiolase (EC 2.3.1.9); **HMGS**, HMG-CoA synthase (EC 4.1.3.5); **HMGR**, HMG-CoA reductase (EC 1.1.1.88); **MVK**, MVA kinase (EC 2.7.1.36); **PMK**, MVP kinase (EC 2.7.4.2); **PMD**, MVPP decarboxylase (EC 4.1.1.33); **IDI**, IPP isomerase (EC 5.3.3.2); **GPS**, GPP synthase (EC 2.5.1.1); **FPS**, FPP synthase (EC 2.5.1.10); **GGPS**, GGPP synthase (EC 2.5.1.29); **DXS** (EC 4.1.3.37); **DXR**, DXP reductoisomerase (EC 1.1.1.267); **CMS** (EC 2.7.7.60); **CMK** (EC 2.7.1.148); **MCS** (EC 4.6.1.12); **HDS**; **IDS**, IPP/DMAPP synthase. The steps specifically inhibited by mevinolin (mev) and fosmidomycin (fosm) are indicated (Rodriguez and Boronat, 2002).

Much that is known about mevalonate biosynthesis is derived from studies on HMG-CoA reductase. By contrast, HMG-CoA synthase in the synthesis of sterols and other isoprenoid compounds has been less studied in plants.

In an attempt to isolate the cDNA encoding HMG-CoA synthase in *Arabidopsis thaliana*, a cDNA was isolated that was shown to complement a yeast mutant defective in HMG-CoA synthase. The nucleotide sequence contained an open reading frame of 1,383 bp which encodes a protein of 461 amino acids with a predicted molecular mass of 51,038 Da for a monomeric unit (Montamat et al. 1995). Wegener et al (1997) isolated an ozone-inducible cDNA from *Pinus sylvestris*, and it was identified as encoding HMG-CoA synthase. It was a globular protein of 474 amino acids with a predicted molecular mass of 52,995 Da. The deduced amino acid sequences showed 41.9% identity to mammalian HMG-CoA synthases. Apparently this protein has a role in plant defense, since ozone exposure elicits defense responses.

There are four HMG-CoA synthase genes present in *Brassica juncea*, a common plant in Asia. *Bjhmgs1*, a HMG-CoA synthase gene in *Brassica juncea,* was isolated and used as an example for study; it is expressed in all plant organs and shows developmental regulation in flower, seed and seedling, with highest expression in early development. In seedlings, expression is highest in young hypocotyls and is induced during the greening of etiolated cotyledons. *Bjhmgs1* is down regulated by abscisic acid, osmotic stress and dehydration, the effects of which arrest seedling growth. Thus, *Bjhmgs1* expression shows correlation with rapid cell division and growth, like *hmgr*. Wounding, or treatment with methyl jasmonate or salicylic acid, induces *Bjhmgs* expression and also *hmgr,* indicating that *hmgr* and *hmgs* expression are coordinately regulated. This suggests that HMG-CoA synthase in *Brassica juncea*

is also involved in defense (Alex et al. 2000). Moreover, the putative *hmgs* mRNA of 1,392 and 1,790 bp nucleotides from *Oryza sativa* and *Zea mays* were deposited in the GenBank under the accession number NM187557 and AY104370, respectively.

4. HMG-CoA synthase in *Hevea brasiliensis*

Hevea brasiliensis Mull. Arg. was discovered by Dr. Jean Mueller, a botanist from Switzerland. Taxonomic classification is as followings.

Class: Angiospermae

Subclass: Dicotyledoneae

Order: Euphorbiales

Family: Euphorbiaceae

Genus: *Hevea*

Species: *brasiliensis*

Scientific name: *Hevea brasiliensis* (rubber tree), a perennial tropical tree originating from South America, is widely cultivated in South America, Africa, and Asia for the production of natural rubber. The unique natural rubber is isoprenoid, cis-1,4 polyisoprene, which is present in latex from the tree. Natural rubber biosynthesis is a side-branch of the ubiquitous isoprenoid pathway as shown in Figure 9 and 10 (Backhaus, 1985). Natural latex oozes from a tap located on the tree, as a milky sap. Special cells of the rubber plant called laticifers produce latex. In general, the latex has a biological function in herbivore defense.
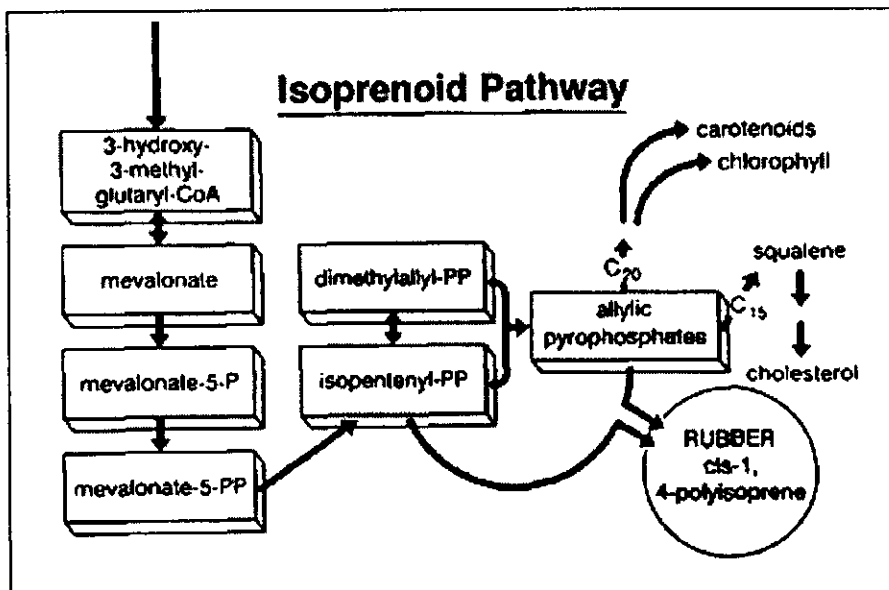
Figure 9. A section of the isoprenoid pathway illustrating the position of natural rubber biosynthesis (Backhaus, 1985).
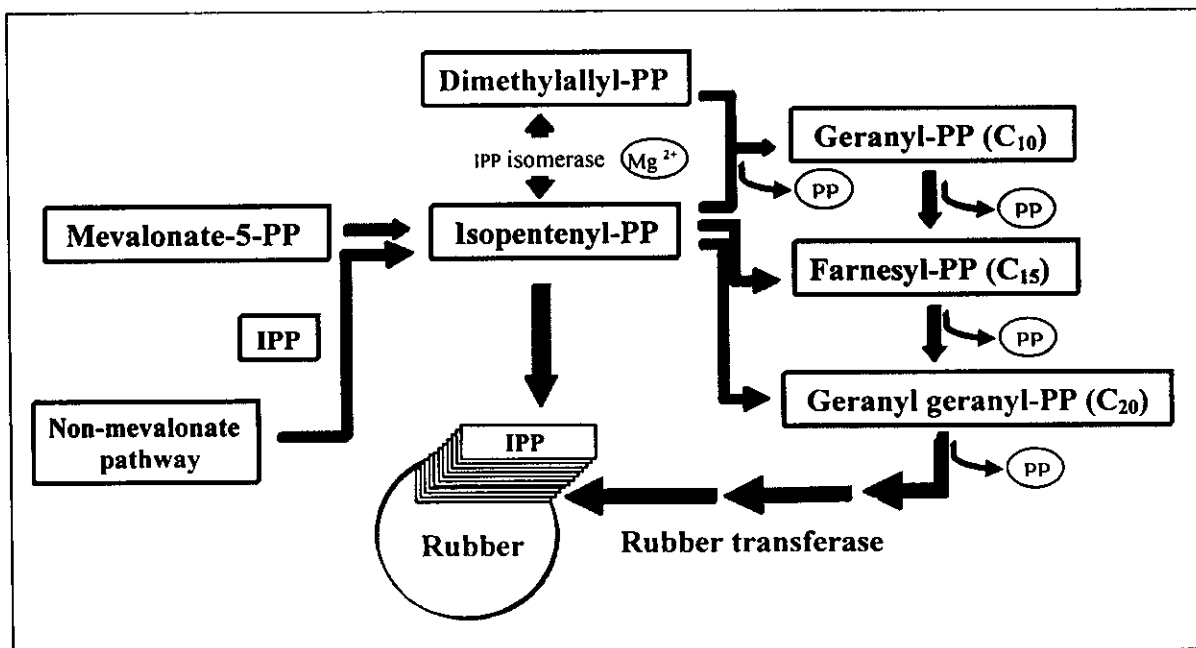

Figure 10. The biosynthesis of natural rubber from iso-pentenylpyrophosphate.

Each new molecule of *cis*-1,4 polyisoprene requires an allylic pyro-phosphate initiator before the isoprene units from IPP can be polymerized.

Specialized plant cells that secrete latex; typically in outer tissues of plant [May be single cell (non-articulated) or connected cells (articulated) forming tubes, canals, or networks]. These laticifers act as a reservoir for biosynthetic materials and metabolic by-products. The laticiferous cells possess the genetic information (DNA) and the machinery needed for protein biosynthesis (RNA, ribosomes) which is indispensable to allow the restoration of the latex lost by the previous tapping (Gomez,1976).

Hevea brasiliensis latex is a cytoplasmic system that contains, besides rubber and the usual cell organelles, two characteristic bodies, the lutoid particle (Homans & Van Gils, 1948) and the Frey-Wyssling complex (Dickenson, 1964). Four fractions can be obtained from ultracentrifugation: the top rubber layer; the zone next to it containing the Frey-Wyssling particles; the clear C-serum or cytosolic fraction; the pellet (bottom fraction) containing the lutoids and other particles (Moir, 1959).

The composition of the fresh latex is rather complex due to its origin and the relative proportions of certain constituents (e.g. proteins and minerals) show important variations depending on many factors (clone, season, and tapping system). All latex's are emulsions, aqueous suspensions of insoluble materials, which include alkaloids, terpenes, resins, phenolics, proteins, sugars, and long-chain hydrocarbons (Table 4). The enzyme HMG-CoA synthase is found in rubber latex, in both C-serum and bottom fractions, but most is in the C-serum (Lynen, 1969). *H. brasiliensis* leaves show very low enzyme activity. The enzyme in C-serum was inhibited by several divalent cations, p-chloromercuribenzoate and dithiothreitol. A positive correlation exists between enzyme activity and the dried rubber content of the latex from each tapping (Suvachittanont & Wititsuwannakul, 1995), which is similar to what is observed for

the HMG-CoA reductase (Wititsuwannakul, 1991). This finding suggests that the rubber biosynthesis pathway is co-ordinately regulated by the activity of both HMG-CoA synthase and HMG-CoA reductase.

Table 4. A typical fresh latex composition from natural rubber products (www.siu.edu)

Components	Percent
Total Solids Content	41.5
Dry Rubber Content	36.0
Amino Acids and N-Bases	0.3
Neutral lipids	1.0
Proteins	1.6
Phospholipids	0.6
Inositols-Carbohydrates	1.5
Salts (mainly K,P and Mg)	0.5
Water	58.5

There are three genes known to code for HMG-CoA reductase in *H. brasiliensis*, called *hmg1, 2*, and *3*. The *hmg1* gene is likely to be involved in rubber biosynthesis, where as *hmg3* is possibly involved in the manufacture of other isoprenoids (Chye et al. 1992).

For HMG-CoA synthase, a 1.8 kb cDNA was isolated from a cDNA library prepared from the C-serum of the rubber latex from *Hevea brasiliensis* (Suwanmanee et al. 2002). Nucleotide sequence analysis revealed an open reading frame of 1,392 base pairs, which encodes a protein of 464 amino acids, with a predicted molecular mass of 51.28 kDa for the monomeric unit. The mRNA level in stem and petiole is higher than in leaves of the seedling whereas in mature rubber trees there is higher mRNA level in latex and petiole than in leaves. The expression of the HMG-CoA synthase gene is higher in laticiferous cells than leaves. Genomic southern blot analysis indicated the presence of three HMG-CoA synthase genes in *Hevea brasiliensis* (Suwanmanee et al. 2002). Higher expression of *hmgs1* mRNA is observed in high yield rubber clone. The expression of *hmgs1* gene in rubber tree is under the influence of various factors, for example, the tapping time and an application of ethephon; an agent that stimulates, latex yield (Suwannamee et al. 2004).

III. Aims of study

Rubber latex is a widely refined natural material for several products in the world and *H. brasiliensis* is an important plant for agriculture in tropical producer countries. It is of great interest to know about the regulation and the expression of the genes involved in the natural rubber biosynthesis pathway. The rubber biosynthesis pathway appears to be coordinately regulated by the activity of both HMG-CoA synthase and HMG-CoA reductase. Previous studies indicated the presence of more than one HMG-CoA synthase gene in *H. brasiliensis* by Southern blotting. Accordingly, studies were undertaken as follows:

1. To investigate a new gene for HMG-CoA synthase from *H. brasiliensis*,

2. To determine the new HMG-CoA synthase mRNA level in various tissues,

3. To compare amino acid sequences of the *H. brasiliensis* HMG-CoA synthase with other species and relative enzyme,

4. To predict the possible secondary structure of *H. brasiliensis* HMG-CoA synthase,

5. To express the *H. brasiliensis* HMG-CoA synthase1 in *E. coli* and assay the activity of recombinant enzymes,

6. To mutate the *hmgs1* gene at the conserved Cys^{117} and Asn^{326} by site-directed mutagenesis.