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Characterization of cDNA and genomic clones encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase from *Hevea brasiliensis*

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Abstract

Hevea brasiliensis is the major producer of natural rubber which is *cis*-1,4-polyisoprene. The enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) is involved in the biosynthesis of rubber and other plant products. We have used a hamster HMGR cDNA clone as a heterologous hybridization probe to isolate and characterize cDNA and genomic clones of HMGR from *H. brasiliensis*. Sequence analysis revealed that these clones fall into two different classes, HMGR1 and HMGR2. Comparison of the two classes shows 86% nucleotide sequence homology and 95% amino acid homology. The carboxy-termini of *Hevea* HMGRs are highly homologous to those of hamster, yeast and *Arabidopsis* HMGR. The amino-terminus of *Hevea* HMGR contains two potential membrane-spanning domains as in *Arabidopsis* HMGR while seven such domains are found in the HMGRs of other organisms. The apparent molecular mass of *Hevea* HMGR was estimated in western blot analysis to be 59 kDa. Northern blot analysis indicated that the HMGR1 transcript of 2.4 kb is more highly-expressed in laticifer than in leaf. Genomic Southern analysis using 3′-end cDNA probes indicates the presence of at least two HMGR genes in *Hevea*.

Introduction

The enzyme HMGR that converts HMG-CoA to mevalonate is involved not only in isoprenoid biosynthesis in plants, but also in sterol biosynthesis in yeast and cholesterol biosynthesis in mammals. In plants, some of these isoprenoid compounds include phytoalexins, growth regulators (abscisic acid, gibberellins and cytokinins), chlorophylls, tocopherols, phylloquinones, ubiquinone, plastoquinone, carotenoids and dolichol.

Natural rubber, a unique isoprenoid compound, is obtained commercially from the latex of *Hevea brasiliensis*, a member of the Euphorbiaceae. *H. brasiliensis* is an economically-

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The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X54657 (*H. brasiliensis* HMGR1 gene), X54658 (*H. brasiliensis* mRNA for HMGR2) and X54659 (*H. brasiliensis* mRNA for HMGR1).

important crop in South-East Asia and this has led us to initiate investigations on the biosynthesis of natural rubber in this plant. Mevalonate also serves as precursor in the biosynthesis of natural rubber, which is *cis*-1,4-polyisoprene of high molecular weight (4×10^6) [39]. HMGR has been reported to be present in the pelleted portion of centifuged latex in *H. brasiliensis* and has been implicated to be membrane-bound [16, 34, 35]. This enzyme is known to require NADPH and thiol compounds for its activity [36].

We are interested in studying the regulation and expression of the genes involved in the biosynthesis of natural rubber. Since HMGR has been shown to be the rate-limiting step in cholesterol biosynthesis in mammals [9], we are interested in examining its role in rubber biosynthesis in *H. brasiliensis*. To this end, we have isolated the genes encoding *Hevea* HMGR. In the present paper, we describe the cloning and characterization of *Hevea* HMGR cDNA and genomic clones.

Materials and methods

Plant material

Hevea brasiliensis RRIM600 plants were grown in a growth chamber under a 12 h light/12 h dark cycle at 25–30 °C. Total cell RNA and poly(A) RNA were isolated from various tissues of young plants (1–24 months old) as described [28]. Laticifer RNA was prepared from the latex of field-tapped trees [21]. Genomic DNA was obtained from young leaves and purified by CsCl centrifugation [27].

Construction and screening of cDNA and genomic libraries

Double-stranded cDNA was synthesized from poly(A) RNA extracted from young *Hevea* leaves by the method of Gubler [15]. Following the addition of *Eco* RI linkers the cDNA was ligated to the *Eco* RI site of λgt11 [17] and packaged *in vitro* with Gigapack-Plus (Stratagene) according to the manufacturer's instructions. The resultant library was screened in duplicate by plaque hybridization under conditions of low-stringency in solutions containing 30% formamide. A 0.6 kb *Pst* I fragment containing the 3′-coding region of the hamster HMGR cDNA [12] whose sequence was found to be highly conserved in yeast HMGR [3] was used as a heterologous hybridization probe. Putative positive clones were purified and their DNAs isolated by CsCl gradient centrifugation [27].

A genomic library was constructed by ligating *Sau* 3A partially-digested and size-fractionated (15–25 kb) DNA [18] to the *Bam* HI site of site of λ DASH (Stratagene). HMGR cDNA was used to screen this library for HMGR genomic clones.

DNA sequence analysis

DNA fragments containing the HMGR gene were subcloned into M13mp18 [42] for DNA sequencing [33]. The sequence of both stands were determined using synthetic oligonucleotide primers which were approximately 0.3 kb apart.

Northern blot analysis

For northern blot analysis, 20 μg of total RNAs were denatured at 50 °C in the presence of glyoxal, separated by electrophoresis in 1.5% agarose gel and blotted onto Hybond-N (Amersham) filters. The conditions of blotting, prehybridization and hybridization were as recommended by the manufacturer.

Southern blot analysis

For genomic Southern analysis, high molecular weight DNA (20 μg) was digested with various restriction endonucleases, separated by electrophoresis in 0.7% agarose gels and blotted onto nitrocellulose filters according to standard procedures [27]. Filters were prehybridized in

6 × SSC, 0.5% SDS, 10% dextran sulphate and 100 μg/ml single-stranded salmon sperm DNA, then hybridized with labelled probe at 65 °C for 16 h under the same conditions.

Primer extension analysis

A ^{32}P-end-labelled oligomer (5′-GCATGCTTT-CGGTGGTGGAGCCGGCCGGTGGTGTC-CATGT-3′) complementary to the cDNA sequence from positions + 59 to + 98 (Fig. 1) was hybridized to 50 μg of total laticifer RNA. Extension with reverse transcriptase was carried out following standard protocols [1].

Western blot analysis

A peptide (VSGNYCTDKKPAAINWIEGRG) of twenty-one amino acids corresponding to amino acids 682–702 of hamster HMGR (Fig. 5) was synthesized chemically. 5 mg of this peptide was coupled to keyhole limpet haemocyanin (KLH-Sigma), essentially by the method of Nivison and Hanson [30]. In order to induce anti-peptide antibodies, two rabbits were subcutaneously injected with 100 μg of KLH-coupled peptide mixed with an equal volume of Freund's complete adjuvant. Subsequently four injections at weekly intervals were carried out with the same amount of coupled peptide emulsified in Freund's incomplete adjuvant. Blood was collected after two weeks of last booster dose of coupled peptide and the sera were stored at − 70 °C. Antibodies were further purified by binding them with Protein A-Sepharose CL6B, followed by selective elution using 50 mM glycine pH 3.0, 0.5 mM NaCl. The eluate was stored at − 70 °C.

For western blot analysis, soluble proteins from leaf and latex were extracted as described previously [21]. The fractionation of freshly tapped latex from RRIM600 into bottom fraction and C-serum was done according to Sipat [34]. Proteins from the bottom fraction were solubilized by homogenizing the pellet in 5 mM triethanolamine buffer (pH 7.2) containing 5 mM dithiothreitol and 0.5% Triton X-100. The homogenate was centrifuged at 40000 × ***g*** for 30 minutes and the supernatant was collected. 50 μg of total protein from leaf, latex, C-serum and bottom fraction was separated on 5% polyacrylamide SDS-PAGE according to Laemmli [23]. Proteins were electro-transferred to Hybond-C (Amersham) in 20 mM Tris, 150 mM glycine and 20% methanol. Antibodies were diluted to 1 : 250 and pre-incubated with 10 μg/ml of haemocyanin for two hours at 4 °C. Western blot analysis was done using alkaline phosphate-conjugated antibodies from Proto-Blot system (Promega) following the instructions of the manufacturer.

Results

Isolation and DNA sequence analysis of Hevea *HMGR cDNA and genomic clones*

The observation that the deduced amino acid sequence of yeast HMGR exhibits 64% homology to amino acid residues 620–810 of hamster HMGR [3] suggests that this region may also be conserved in the plant enzyme. We therefore used a 0.6 kb *Pst* I fragment of hamster HMGR cDNA encoding these amino acid residues to screen our leaf cDNA library for *Hevea* HMGR clones. A total of three *Hevea* cDNA clones were isolated and these clones contain inserts of 1.3, 0.9 and 0.8 kb. On sequence analysis the 1.3 kb cDNA was found to differ from the 0.9 kb and 0.8 kb cDNAs. The 1.3 kb cDNA was classified as HMGR1 cDNA while the other two cDNAs as HMGR2 cDNA. Comparison of the two classes shows 86% nucleotide sequence homology and 95% amino acid homology (Fig. 1). Homology was less conserved at the 3′-untranslated region than at the coding region (Fig. 1).

In an attempt to obtain full-length cDNA clones, the 1.3 kb cDNA was used to screen a random-primed laticifer cDNA library (gift of V. Vanniasingham and S. Sivasubramaniam). We obtained 6 overlapping clones, one of which was used to rescreen the library until a composite full-length cDNA sequence was obtained. A cDNA

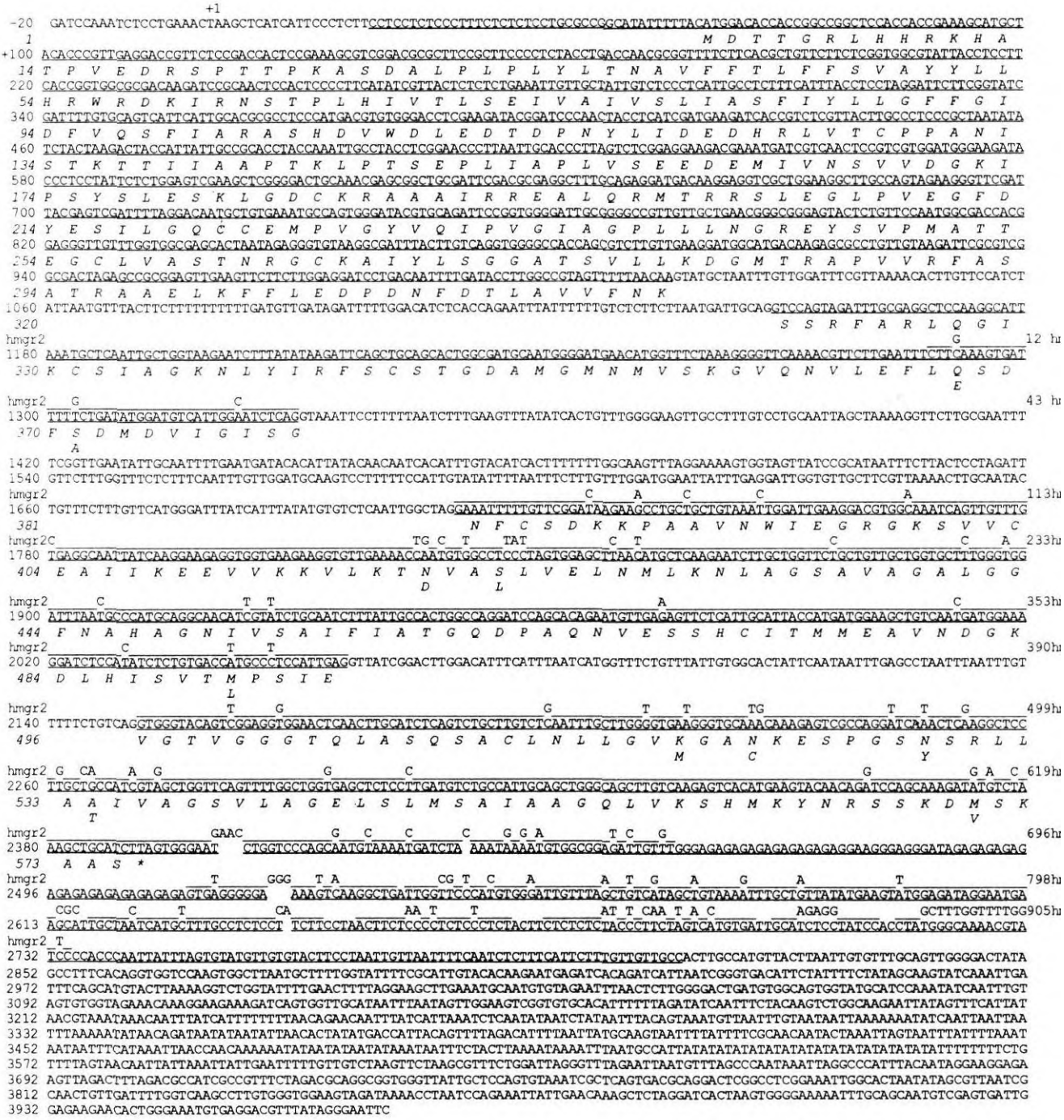

Fig. 1. Nucleotide sequence of 4 kb genomic fragment encoding *Hevea* HMGR1. The composite HMGR1 cDNA sequence is underlined. HMGR1 deduced amino acid sequence is shown below its cDNA. The nucleotides are numbered with respect to the HMGR1 transcription start site as mapped by primer-extension and the amino acid residues are numbered with respect to the ATG start codon. The 5′ border of the 1.3 kb HMGR1 cDNA is located at nucleotide 857. The partial sequence of HMGR2 cDNA is placed above the HMGR1 cDNA sequence and its nucleotide numbers are indicated on the right of the figure. Identical nucleotides in HMGR2 cDNA are denoted by a line and mismatches are shown above HMGR1 cDNA. Changes in the partial HMGR2 amino acid sequence are shown below HMGR1. The 0.8 kb HMGR2 cDNA is located from nucleotide 42 to nucleotide 807 of the HMGR2 cDNA sequence.

library derived from laticifer rather than leaf was used since northern analysis showed that the HMGR1 transcript of 2.4 kb is more abundant in laticifer (Fig. 2A). Sequence analysis of overlapping HMGR cDNA clones revealed that the composite HMGR1 cDNA consists of 41 bp of 5′-untranslated region, 1725 bp of coding region, 416 bp of 3′-untranslated region and a poly (A) tail. As in the case of HMGR from *Xenopus laevis* [11] the consensus sequence for polyadenylation [31] was absent in *Hevea* HMGR cDNAs. Primer extension analysis was used to determine the transcription start site (Fig. 3). We found that this start site is located 19 nucleotides upstream from the 5′ end of the HMGR1 cDNA indicating that our composite cDNA sequence is near full-length (Fig. 1).

To obtain HMGR genomic clones, the HMGR1 cDNA was used to screen the genomic library. We obtained several putative clones, one of which, on restriction analysis, produced a 4 kb *Bam* HI-*Eco* RI fragment which hybridized strongly to this probe. Sequence analysis revealed that this genomic clone corresponds to the HMGR1 cDNA (Fig. 1). Three introns were found to interrupt an open reading frame of 575 amino acids which would encode a protein of M_r

Fig. 2. Northern blot analysis. Total RNA from laticifer (Lx) and leaf (Lf) were hybridized to the (A) 1.3 kb HMGR1 cDNA, (B) β subunit of mitochondrial ATP synthase [5] control probe.

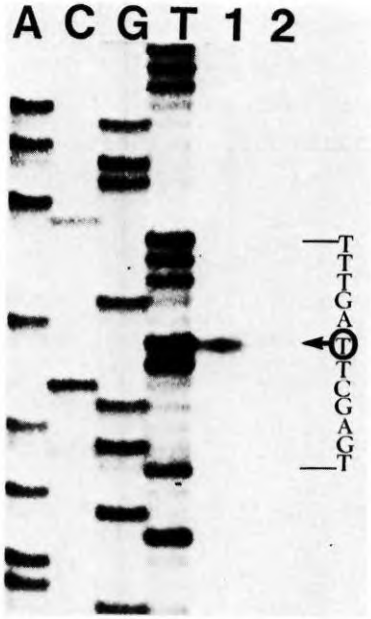

Fig. 3. Primer extension analysis to map the 5′ end of the HMGR1 transcript. A ^{32}P-labelled primer was hybridized to 50 μg of laticifer total RNA (1) and 10 μg of *Escherichia coli* tRNA as control (2). This primer was also used to generate a dideoxy sequencing ladder (ACGT) which was electrophoresed next to the extended product. The template used in this sequencing reaction was derived from the genomic clone for which the sequence is given in Fig. 1.

61 702. The 4 kb genomic fragment contains 1.6 kb of downstream 3′-untranslated region but only 81 bp of 5′-untranslated region. The 5′-upstream region was not present in other restriction fragments of the same λ genomic clone as the *Bam* HI-end of the 4 kb fragment was actually the site where *Sau* 3A was ligated to the *Bam* HI site of the vector λ DASH.

Two genes encode 3-hydroxy-3-methylglutaryl-coenzyme A reductase in Hevea brasiliensis

Genomic Southern analysis showed that several bands hybridized to the HMGR2 cDNA probe (Fig. 4A) indicating that more than one HMGR gene is encoded in *Hevea*. To verify that HMGR1 cDNA and HMGR2 cDNA are derived from two different genes we hybridized the same genomic blot to 3′-end probes from these cDNAs.

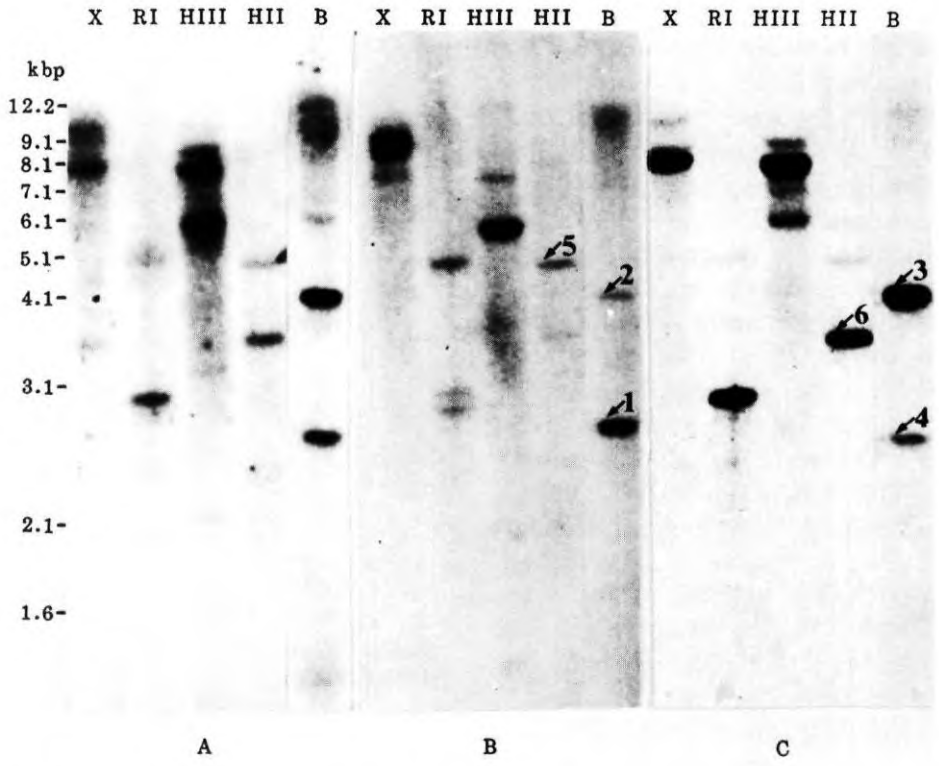

Fig. 4. Genomic Southern analysis *Hevea* genomic DNA (20 μg) was digested with *Xba* I (X), *Eco* RI (RI), *Hin*d III (HIII) *Hin*d II (HII) and *Bam* HI (B), separated by gel electrophoresis and blotted onto nitrocellulose filters. The same genomic blot was hybridized to the following probes: A, HMGR2 cDNA (0.6 kb *Bam* HI-*Eco* RI fragment, positions 289 to 928 in Fig. 1); B, 3′ end of HMGR1 cDNA (0.4 kb *Ava* II-*Eco* RI fragment, positions 2404 to 2809 in Fig. 1); C, 3′ end of HMGR2 cDNA (0.4 kb *Pvu* II-*Eco* RI fragment, positions 563 to 928 in Fig. 1). The bands denoted by arrows 1–6 are discussed in the text.

Sequence analysis had earlier shown that the two classes of cDNAs are least homologous at their 3′ ends. When the genomic blot was hybridized to the 3′-end probe of the HMGR1 cDNA, it was observed that in the *Bam* HI digest, a 2.6 kb band (denoted by arrow 1 in Fig. 4B) hybridized better than a 4.1 kb band (denoted by arrow 2 in Fig. 4B). However, when the 3′-end probe of the HMGR2 cDNA was used, the 4.1 kb band (denoted by arrow 3 in Fig. 4C) gave a stronger signal than the 2.6 kb band (denoted by arrow 4 in Fig. 4C) indicating that the 4.1 kb band corresponds to the HMGR2 cDNA while the 2.6 kb band corresponds to the HMGR1 cDNA.

In the case of the *Hin*d II digest, 5.0 kb band (denoted by arrow 5 in Fig. 4B) hybridized strongly to the 3′-end probe of the HMGR1 cDNA while a 3.5 kb band (denoted by arrow 6 in Fig. 4C) hybridized strongly to the 3′-end probe of the HMGR2 cDNA, demonstrating that each band corresponds specifically to their respective cDNA.

Similarly, in the *Xba* I, *Eco* RI and *Hin*d III digests, specific bands were seen to hybridize to these probes (Fig. 4). We concluded that there are at least two different HMGR genes in *Hevea brasiliensis*.

Two hydrophobic domains are conserved in plant 3-hydroxy-3-methylglutaryl coenzyme A reductases.

Comparison of the deduced amino acid sequence of *Hevea* HMGR1, yeast, hamster and *Arabidopsis* reveals that the carboxy-termini are highly-conserved (Fig. 5). It has been reported that the

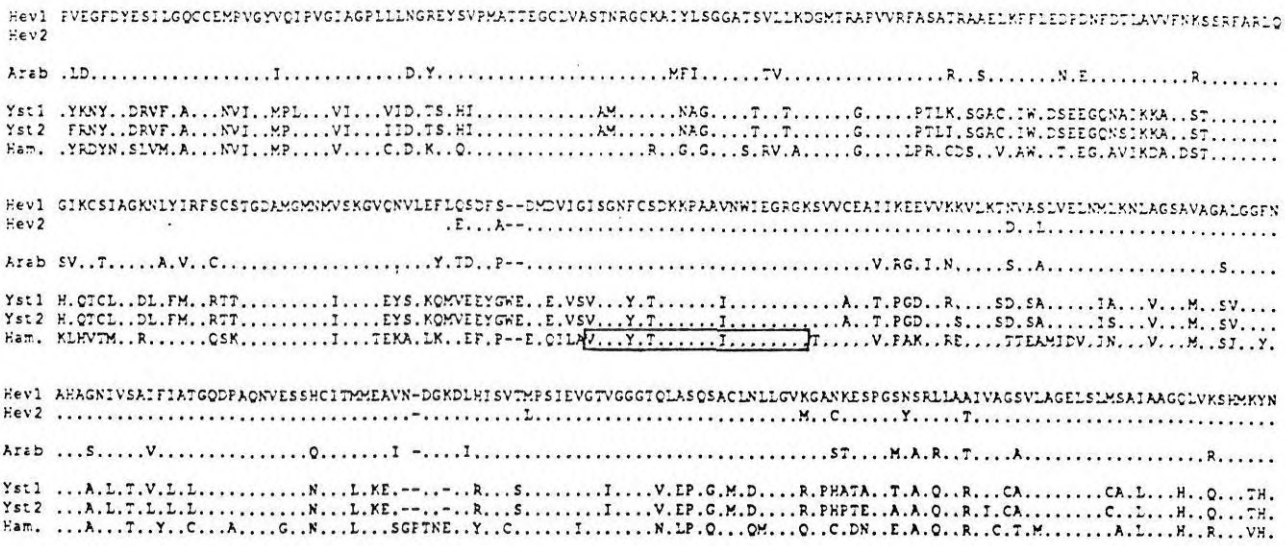

Fig. 5. Comparison at the carboxy-termini of HMGRs from *Hevea*, *Arabidopsis*, yeast and hamster. Hev 1, *Hevea* HMGR1 amino acids 208–564; Hev 2, *Hevea* HMGR2; Arab, *Arabidopsis* amino acids 219–575 [10, 24]; Yst1, yeast HMGR1 amino acids 668–1024 [4], Yst 2, yeast HMGR2 amino acids 664–1020 [4]; ham, hamster amino acids 512–869 [12]. Positions of identity are denoted by dotted lines. The synthetic peptide used for raising antibodies corresponds to amino acids 682–702 of hamster HMGR [12] and is boxed.

catalytic site of the enzyme is located within this region [25]. The predicted amino acid sequence of *Hevea* HMGR1 has 79.9% identity to that of *Arabidopsis thaliana* [10, 24], 52.0% to yeast HMGR2 [4], 56.6% to yeast HMGR1 [4] and 57.1% to hamster HMGR [12].

At the amino-terminus, *Hevea* HMGR1 was found to resemble that of *Arabidopsis* in having only two potential transmembrane domains (amino acid residues 32–48 and 65–97 in *Hevea*, amino acid residues 53–69 and 86–118 in *Arabidopsis* [10]) as predicted following the method of Klein *et al.*[19] (Fig. 6). The hydrophobicity plots [22] of the *Hevea* and *Arabidopsis* proteins demonstrate the presence of two hydrophobic regions corresponding to these potential transmembrane domains (Fig. 7). This observation suggests that some degree of conservation occurs at the amino-termini of plant HMGRs. At the first hydrophobic domain all 23 amino acid residues in both *Hevea* and *Arabidopsis* are conserved (Fig. 6). There was 78% homology at the second hydrophobic domain consisting of 33 amino acid residues (Fig. 6). Previous reports have suggested that HMGR in plants are membrane-bound [2, 14]. The occurrence of two such potential transmembrane domains in both the *Hevea brasiliensis* and *Arabidopsis thaliana* [10] enzymes further supports this hypothesis.

By contrast, the HMGR from hamster [25, 37] and yeast [4] have seven hydrophobic regions (Fig. 7) corresponding to seven potential trans-

Fig. 6. Comparison at the amino-termini of *Hevea brasiliensis* HMGR1 (amino acids 24–156) and *Arabidopsis* HMGR (amino acids 45–177). The potential transmembrane domains are underlined and PEST sequences are denoted by dotted lines. Hev1, *H. brasiliensis*; Arab, *A. thaliana*.

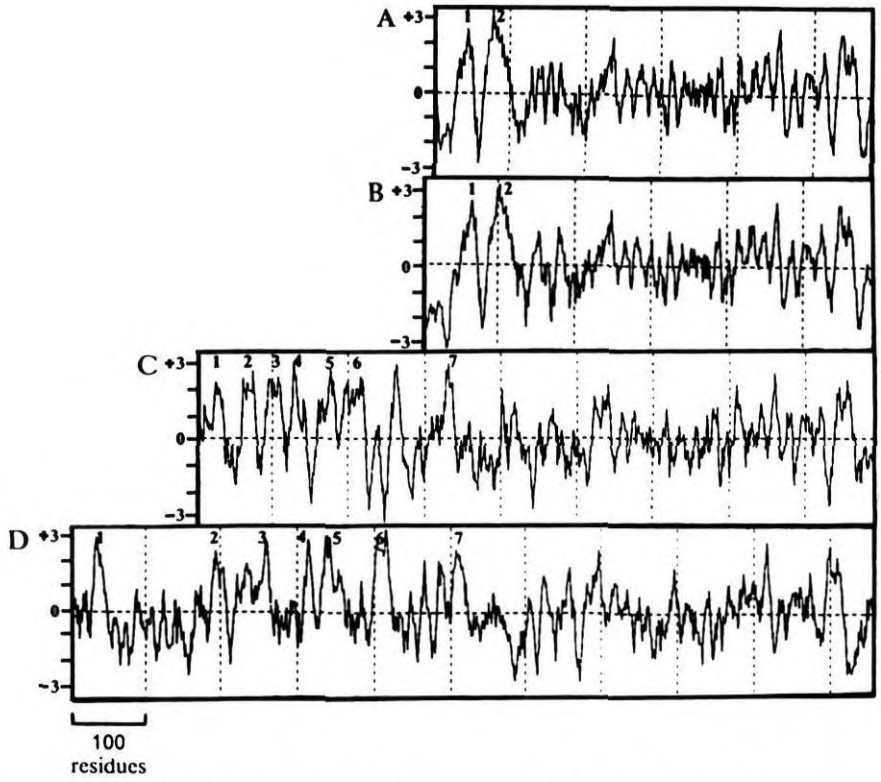

Fig. 7. Hydropathy plots of HMGRs from (A) *Hevea brasiliensis*, (B) *Arabidopsis thaliana* [10], (C) hamster [25] and (D) yeast HMGR1 [4]. The potential transmembrane domains are numerically labelled.

membrane domains. It has been shown in hamster [8, 13, 25] and in yeast [41] that these regions are involved in anchoring the enzyme to the endoplasmic reticulum membranes.

Western blot analysis

Previous studies on *Hevea* HMGR [16, 34, 35] and the presence of two potential membrane-spanning domains in the HMGR1 protein as indicated by our cDNA sequencing data have indicated that the enzyme is membrane-bound. Therefore, we extracted proteins from the bottom fraction of centrifuged latex for western blot analysis and detected a band of 59 kDa (Fig. 8) which is close to the predicted molecular mass (61 702 Da). In both latex and leaf-soluble protein fraction, and also in C-serum, a band of a smaller molecular mass (50 kDa) was observed (Fig. 8). This difference in size corresponds to the presence of two membrane-spanning domains which are presumably cleaved off when HMGR is solubilized during isolation. Peptide cleavage could have occurred at a unique PEST [32] region which lies adjacent to the second transmembrane

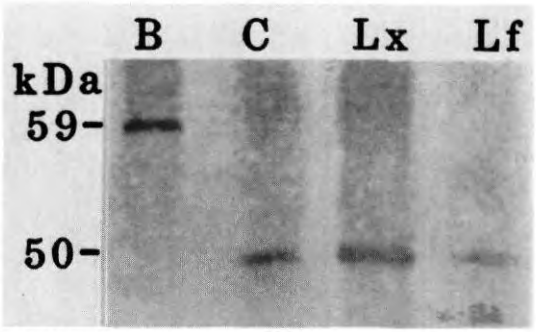

Fig. 8. Western blot analysis using antipeptide antibodies. The size of cross-reacting bands in the bottom fraction (B), C-serum (C), leaf (Lf) and latex (Lx) was calculated using bovine serum albumin (69 kDa), ovalbumin (43 kDa) and carbonic anhydrase (29 kDa) as molecular weight markers.

domain (Fig. 6). A 'PEST' sequence has also been found in *Arabidopsis* HMGR (Fig. 6). The significance of this sequence is unknown as the *Arabidopsis* protein has not been well-characterized.

Discussion

We have identified two classes of HMGR cDNAs in *Hevea brasiliensis* and have shown that they correspond to two different genes in the *Hevea* genome. In yeast [4] and in *Arabidopsis thaliana* [10] two species of HMGR have also been reported while only one form is known to be present in mammals [12, 26]. *Hevea* HMGR1 cDNA was found to be highly homologous (70% nucleotide sequence identity) to *Arabidopsis* HMGR cDNA. This similarity is extended to their genomic clones where in each case, all three introns interrupt the coding sequence at the same positions. A 2.4 kb HMGR mRNA transcript was identified in each of these two plants while a 3.0 kb transcript has been reported in tomato [29]. The occurrence of more than one form of HMGR in plants is not unexpected as it has been previously suggested that subcellular compartmentation of different forms of the enzyme occurs [7]. Plastid and microsomal HMGRs have been identified in pea seedlings [6, 40] and mitochondrial HMGR in sweet potato [38]. A comparison of HMGRs from plants, yeast and mammals has shown that their carboxyl-termini are conserved. *Hevea* and *Arabidopsis* [10, 24] HMGRs show greater homology to each other than to organisms outside the plant kingdom. Comparison of the amino-termini of *Hevea* and *Arabidopsis* HMGRs has shown amino acid conservation at the two hydrophobic regions which correspond to the potential transmembrane domains. We have noted that plant HMGRs have only two such domains while seven are present in HMGRs of other organisms. A 'PEST' sequence has been observed to occur downstream from the hydrophobic regions in *Hevea* (amino acids 106–122), *Arabidopsis* (amino acids 157–176), hamster (amino acids 430–441) and yeast (amino acids 568–588 of HMGR1) reductases. The apparent molecular mass of *Hevea* HMGR was estimated by western blot analysis to be 59 kDa. This compares quite well to the estimated subunit molecular mass of 55 kDa in potato [20].

HMGR functions as an important enzyme in plants since HMG-CoA is a precursor to many vital isoprenoid compounds. The cloning of HMGR genes from plants will be a first step in understanding its various roles. Studies with tomato HMGR cDNA have shown that HMGR is required in the early stages of fruit development but is not required for carotenoid biosynthesis during fruit ripening [29]. The work reported in this paper has provided us with the tools required to elucidate the role of this enzyme in rubber biosynthesis.

Acknowledgments

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