Isolation and endocrine regulation of an HMG–CoA synthase cDNA from the male Jeffrey pine beetle, *Dendroctonus jeffreyi* (Coleoptera: Scolytidae)

Claus Tittiger *, Chatty O’Keeffe, Cody S. Bongoa, Lana S. Barkawi 1, Steven J. Seybold 1, Gary J. Blomquist

*Department of Biochemistry, University of Nevada, Reno, NV 89557-0014, USA*

Received 31 January 2000; received in revised form 3 May 2000; accepted 8 May 2000

**Abstract**

We have isolated a full length 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-S) cDNA from the male Jeffrey pine beetle, *Dendroctonus jeffreyi* Hopkins, and studied the effects of topical applications of juvenile hormone III (JH III) on its expression. The predicted translation product of this apparently single copy gene has 63% and 58% identity with HMG-S1 and HMG-S2 from *Blattella germanica* (L.), and 61% identity with *Drosophila melanogaster* Hmgs. HMG-S transcript levels remain uniformly low in JH III-treated and control *D. jeffreyi* females, but are induced approximately 2.5- to 5-fold in JH III-treated males. JH III causes a dose- and time-dependent increase in HMG-S transcripts in the male metathoracic–abdominal region. Since monoterpenoid pheromone precursor synthesis and HMG–CoA reductase expression are under the control of JH III in the metathorax of *Ips* bark beetles, the observed HMG-S expression pattern suggests that the isoprenoid pathway is similarly important for semiochemical production in *D. jeffreyi*. © 2000 Elsevier Science Ltd. All rights reserved.

**Keywords:** Coleoptera; *Dendroctonus*; cDNA; Juvenile hormone; HMG–CoA synthase; Isoprenoid; Pheromone

1. Introduction

In vertebrates, the isoprenoid pathway leads to a variety of sterol and nonsterol products and has been extensively studied in the context of controlling cholesterol biosynthesis (Goldstein and Brown, 1990). Since insects lack the ability for de novo cholesterol production (Beenakkers et al., 1985), all products from the pathway are nonsterols in these animals. The sesquiterpenoid juvenile hormone (JH) has long been considered among the most important isoprenoid products in insects (Koyama et al., 1985; Feyereisen and Farnsworth, 1987; Couillaud, 1991), but the importance of the timely production of dolichol, ubiquinone, and protein prenyl groups in development and reproduction is also becoming increasingly clear (Havel et al., 1992; Lai et al., 1998; van Doren et al., 1998; Castillo-Gracia and Couillaud, 1999). Recently, an additional role for the isoprenoid pathway in insects, pheromone synthesis, has been demonstrated with the observation that male *Ips* bark beetles use it to produce monoterpenoid (C10) aggregation pheromone components (Ivarsson et al., 1993; Seybold et al., 1995; Tillman et al., 1998). Since plant monoterpenoids are produced via the DOX/P, or GAP/Pyruvate pathway (Arigoni et al., 1997), and since geranyl diphosphate (C10) generally serves as an intermediate for longer chain isoprenoids in vertebrates (Laskovics and Poulter, 1981), monoterpenoid pheromone production in the Coleoptera is a rare example of mevalonate-based monoterpenoid biosynthesis.

One of the key regulatory enzymes of the isoprenoid pathway is 3-hydroxy-3-methylglutaryl coenzyme A synthase (EC 4.1.3.5, HMG-S), which catalyzes the condensation of acetyl–CoA and acetoacetyl–CoA to form HMG–CoA. The only published molecular studies of
insect HMG-S have been done in the German cockroach, *Blattella germanica* (L.). This insect has two genes encoding apparently cytosolic enzymes (Martinez-Gonzalez et al., 1993; Buesa et al., 1994). Their expression patterns appear coordinated in early embryos, but are complementary during larval stages. Their activity and expression are also modulated differently in different tissues during the 8 day gonadotrophic cycle, suggesting their involvement in regulating the isoprenoid pathway during egg production (Casals et al., 1996). An expressed sequence tag (EST) for *Drosophila melanogaster* Hmgs (GenBank accession number LP4424) was recovered during a P-element mutagenesis screen (Sparadling et al., 1999), but the expression pattern has yet to be reported. The signals governing the isoprenoid pathway in insects remain mostly unknown, though in male *I. paraconfusus* and *I. pini*, JH III plays a major role in regulating HMG–CoA reductase (EC 1.1.1.34, HMG-R) transcript levels and enzyme activity (Tittiger et al., 1999; Tillman, Lu, Dwinell, Tittiger, Hall, Blomquist and Seybold, unpublished results).

Based on our studies of the endocrine regulation of de novo aggregation pheromone biosynthesis in *Ips* beetles, we became interested in determining if the isoprenoid pathway is similarly regulated for semiochemical production in the Jeffrey pine beetle, *Dendroctonus jeffreyi*. As a first step to address this question, and to contribute to the general knowledge of HMG–S in insects, we isolated an HMG-S cDNA from *D. jeffreyi* and studied its regulation and tissue distribution following topical applications of JH III.

2. Materials and methods

2.1. Insects and treatments

Sections of *D. jeffreyi*-infested *Pinus jeffreyi* Grev. and Balf. trees from Shasta County, CA (T22N, R4E, S21, VI-19-98), and Carson City County, NV (T15N, R19E, S31, XI-4-98), were kept in darkened rearing chambers in a greenhouse (Browne, 1972). Emerging adult *D. jeffreyi* were collected in glass jars, sexed, and *I. paraconfusus* and *I. pini*, JH III plays a major role in regulating HMG–CoA reductase (EC 1.1.1.34, HMG-R) transcript levels and enzyme activity (Tittiger et al., 1999; Tillman, Lu, Dwinell, Tittiger, Hall, Blomquist and Seybold, unpublished results).

Two degenerate primers were designed based on conserved sequences of other metazoan HMG-Ss. The forward primer corresponded to amino acids 190–199 of *B. germanica* HMG-S, and the reverse to a.a. 328–336 (Martinez-Gonzalez et al., 1993). Their sequences were: GCI TA(G/C) GA(G/C) TT(G/C) TA(G/C) AA(A/G) CC and CA(G/C) GIN GTG TAC AT(A/G) TTN CC, respectively. PCR amplifications were done in 100 μl containing 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 50 pmol each primer, 10 μl template cDNA, and 2.5 U Taq DNA polymerase (Life Technologies). The cycling parameters were: 1 min at 95°C, followed by 35 cycles of 40 s at 94°C, 1 min at 52°C, 3 min at 72°C, and a 15 min extension at 72°C. Inserts were purified with a Microcon 100 spin cup (Amicon, Bedford, MD), ligated into pT7Blue (Novagen, Madison, WI), and sequenced.

2.3. cDNA library construction and screening

Total RNA was isolated using a guanidinium isothiocyanate–CsCl centrifugation procedure (Glisin et al., 1974) from 11 male insects that had been treated with 13.3 μg JH III. Poly(A)+ RNA was further purified using oligo-dT push columns (Stratagene, La Jolla, CA) and used to construct a library in λZAP II (Stratagene). The HMG-S cDNA fragment in pT7Blue was labeled with [³²P]dCTP (NEN Dupont, Boston, MA) by PCR (Mertz and Raschchian, 1994) using the T7 and U19 primers that flanked the insert. Replica nylon filters (Nytran+, Schleicher and Schuell, Keene, NH) containing 1.5 × 10^⁶ plaque forming units (pfu) of the primary library were hybridized with the ³²P-labeled HMG-S cDNA fragment in 50% formamide, 5 × SSC, 50 mM sodium phosphate, pH 7.0, and 7% SDS at 42°C. The filters were washed twice at room temperature, 10 min/wash, with 2 × SSC, 0.1% SDS, and then once at 58°C for 20 min with 0.2 × SSC, 0.1% SDS. Positive plaques were imaged using a BioRad Molecular Imager. Duplicate positive plaques were purified after a second round of screening.

2.4. Genomic Southern blotting

Genomic DNA was isolated from 10 individual adult females using a protocol based on Chia et al. (1985), digested with *Eco* RI, and transferred to nylon membrane (Hybond N+, Amersham, Piscataway, NJ) following separation on a 0.8% agarose gel. The membrane was hybridized with the ³²P-labeled HMG-S cDNA fragment in 50% formamide, 0.12 M sodium phosphate, pH
at 42°C, and washed twice for 10 min at room temperature with 2 x SSC, 0.1% SDS, and once for 10 min at 45°C with 0.4 x SSC, 0.1% SDS. The membrane was then hybridized separately with radiolabeled λ phage DNA to visualize the marker.

2.5. Northern blotting

For the tissue distribution study, adult beetles were dissected into approximately male, thoracic, and abdominal body regions. The dissections were approximate because the external morphology obscures the true borders between the body regions. The tissues were frozen at −80°C until total RNA was isolated using TRIzol Reagent (Life Technologies) according to the supplier’s protocol. Two blots were prepared using dissected male and female tissues [Fig. 4(B) and (C)]. For both the time-course and JH III dose–response studies, triplicate samples of five male thoraces per sample were used.

RNA was separated on glyoxal agarose gels (Sambrook et al., 1989), transferred to Hybond N membranes (Amersham), and fixed by UV cross-linking. Membranes were hybridized with the mouse HMG-S cDNA as described above for the Southern blot. Both probes were labeled with [γ-32P]dCTP by PCR using the F1 and R5 primers (Fig. 1). Membranes were washed as described above for the library screens, except that the high stringency washes were for 30 min at 65°C for the HMG-S probe, and for 30 min at 42°C for β-actin. The membranes were imaged with a BioRad molecular imager and densitometry was done with Molecular Analyst software (BioRad, Hercules, CA).

2.6. Sequence analysis

Inserts from all recombinant plasmids and PCR products were sequenced by primer walking using the dRhodamine dye-terminator kit (Applied Biosystems, Foster City, CA) and an ABI Prism 310 sequencer. Both strands of all clones were sequenced at least once. Sequence analysis was done using the PCGene software package (Version 6.85, IntelliGenetics, Mountain View, CA). The conceptual translation of D. melanogaster Hmgs (FlyBase annotation FBan00043116, protein ID number AAF577996) was obtained through FlyBase (1999). Protein sequences were aligned using CLUSTAL W (Thompson et al., 1994). The D. jeffreyi HMG-S cDNA sequence is available from Genbank, accession number AF166002.

3. Results

3.1. HMG-S cDNA

Amplification of first strand cDNA prepared from male D. jeffreyi using degenerate primers corresponding to well conserved portions of metazoan HMG-Ss resulted in a ~0.4 kb fragment whose predicted translation product had over 50% identity with the corresponding portions of HMG-S from humans (Boukaftane and Mitchell, 1997) and B. germanica HMG-S1 and HMG-S2 (Buesa et al., 1994; data not shown). In order to determine the full length sequence, a cDNA library was constructed and hybridized with the 32P-labeled D. jeffreyi HMG-S fragment. A screen of 7 x 10^7 pfu of the primary library yielded six positive plaques. Five were purified after a second round of screening. Preliminary analysis showed that they were of the same length and sequence, and one was chosen for complete sequencing. The 2015 bp cDNA contains a 1371 bp open reading frame flanked by 58 bp and 584 bp 5’ and 3’ untranslated regions (utrs) (Fig. 1). The 3’ utr has two polyadenylation signals, located 18 and 29 bp from the 3’ end. There are several 9 and 10 bp inverted repeats in the 3’ utr which may form stem–loop structures. Alternatively, transcript destabilizing motifs (Wernborg et al., 1995) are also present at positions 1398 and 1542.

The open reading frame encodes a 457 amino acid (a.a.) protein with 63.1 and 58.2% identity with HMG-S1 and HMG-S2 from B. germanica, respectively, and 60.9% identity with D. melanogaster Hmgs (Fig. 2). The overall a.a. identity is 35% when aligned with insect and human cytosolic HMG-Ss, though this value rises to 63% within the putative catalytic domain (Miziorko and Behnke, 1985).

3.2. Gene copy number

Based on the precedent set in all metazoans in which HMG-S has been studied, we expected two HMG-S genes per D. jeffreyi haploid genome. To investigate this, a Southern blot of Eco RI digested genomic DNA was prepared and hybridized with a portion of the HMG-S cDNA. The probe, which contains an internal Eco RI site (Fig. 1), hybridized to ~6.3 and ~1.5 kb fragments in all samples following moderately-low stringency washes (Fig. 3). A very faint signal can also be observed in some samples at ~7.9 kb.

3.3. Tissue distribution and endocrine regulation

In order to determine body regions expressing HMG-S, males and females were dissected approximately into heads, thoraces, and abdomens. The HMG-S probe hybridized with a ~2 kb transcript primarily in the male tissue, while the β-actin probe recognized a ~1.5 kb transcript on Northern blots prepared from both male and female tissues [Fig. 4(A)]. Two metathoracic–abdominal transverse cuts were made that differed dorsally. The first cut was made perpendicular to the anterior–posterior axis, dorsally between abdominal terga 2 and 3, and ended between abdominal sternites 2 and 3 [Fig. 4(B)].
The second cut was made dorsally between the metathoracic postnotum and abdominal tergite 1, was angled posteriorly, and also ended between abdominal sternites 2 and 3 [Fig. 4(C)]. Ventrally, in both cases, the metathorax was separated from the abdomen between the coxal base and abdominal sternite 3 (first apparent ventral sclerite). Terminology follows Lawrence and Britton (1991). When the perpendicular cut was performed, most of the transcript localized in the thoracic section [Fig. 4(B)]. A second blot was prepared from beetles that were dissected with the angled cut. In this case, a slightly higher proportion of message localized in the abdominal section compared to the thoracic section [Fig. 4(C)]. In both blots, control (acetone treated) insects had a uniformly low expression level, while JH III treated males showed an approximately 4-fold induction, most of which was found in the thoracic and abdominal sections, depending on the way the bodies were dissected.

The effects of JH III dose on HMG-S transcript levels were further studied using Northern blots of total RNA prepared at various times following topical application of 13.3 mg JH showed a lag of about 6 h before a modest increase in HMG-S transcript levels [Fig. 5(B)]. Maximal expression (3.6-fold over controls) was reached by 15 h, followed by a gradual decline to near control levels.

4. Discussion

We used a standard combination of PCR and library screening methods to isolate a cDNA for HMG-S from male D. jeffreyi. The fact that the size of all five clones isolated from the library corresponds to the transcript size on Northern blots suggests that the clone we have characterized, designated “DJHMG-S,” is a full length cDNA.
all HMG-Ss reported to date (Misra et al., 1993). Similar to B. germanica HMG-S1, a 6 a.a. gap must be inserted between residues 235–236 of the predicted Dendroctonus protein in order to optimize alignments with B. germanica HMG-S2 and the human enzyme (Fig. 2). As with the other insect sequences, the beetle sequence is shorter than the human sequence at both the N- and C-termini, and there is no recognizable amino terminal targeting sequence, suggesting that the bark beetle enzyme is cytosolic.

The presence of various elements in the 3′ utr invites speculation on their possible roles in regulating HMG-S transcript levels. In their discussion of B. germanica HMG-S, Buesa et al. (1994) noted the presence of putative cytoplasmic polyadenylation signals (Fox et al., 1989) close (|180 nt) to the 3′ terminus to address the observed delay between HMG-S transcript levels and enzyme activity. Two such “UUUUUAU” signals are also present in the DJHMG-S 3′ utr, but at positions 1439 and 1695 — hundreds of bases upstream of the polyadenylation signals. Secondly, the 9 and 10 bp inverted repeats may affect transcript stability by forming stem–loop structures, as has been hypothesized for mitochondrial HMG-S transcripts in rats (Ayte et al., 1993). Alternatively, transcript destabilizing motifs that may be recognized by specific RNases (Wennborg et al., 1995) are also present at positions 1488 and 1542. The presence of these motifs in the D. jeffreyi HMG-S 3′ utr suggests that transcript stability may be modulated, as observed for HMG-S in B. germanica; however, the possible role of any of these sequences in D. jeffreyi must await assays of HMG-S transcript stability, protein levels, and activity.

Under our conditions, we were unable to definitively detect the presence of a second HMG-S gene. Because the cDNA fragment that we used to hybridize with the Southern blot spans an EcoRI site, the presence of two strongly hybridizing fragments (6.3 and 1.5 kb) is consistent with a single copy gene (Fig. 3). The faint, 7.9 kb signal either represents a second gene or a partially digested fragment (6.3 kb + 1.5 kb). We believe the latter is more likely, since a similar pattern was obtained following high stringency washes (not shown). Thus, there appears to be a single HMG-S locus in D. jeffreyi, though we do not yet have enough information to firmly establish HMG-S gene number in this insect. A single
HMG-S locus in metazoans is unusual, but not unprecedented. A survey of the recently completed D. melanogaster genome database revealed a single HMG-S locus at 53C1 on chromosome 2 (FlyBase, 1999). Here, two alternatively spliced transcripts are predicted to encode HMG-S; alternate first exons encoding the 5' utrs splice just upstream of a common second exon, which contains the coding region. One of these, CG4311, has been confirmed during a P-element search (Spradling et al., 1999). A single copy HMG-S gene raises questions about the enzyme’s role in insect metabolism. In vertebrates, one gene encodes a mitochondrial HMG-S for ketone body production, while a second gene encodes a cytosolic form for isoprenoid biosynthesis. It is interesting that all insect HMG-S sequences identified thus far are predicted to be cytosolic, even though insects have ketone body synthetic enzymes such as HMG-CoA lyase (CG10399; FlyBase, 1999), and can metabolize ketone bodies (Bailey et al., 1972; Beis et al., 1980). This suggests that a mitochondrial HMG-S should be present in insects, and calls for studies of the sub-cellular localization of HMG-S in various insect tissues.

HMG-S showed a modest response to topically applied JH III in a dose- and time-dependent manner in male D. jeffreyi. It is surprising that no increase in HMG-S transcript levels was observed in JH III-treated female insects since JH stimulates oogenesis in insects (Riddiford, 1994), and choriogenesis is thought to require synthesis of prenylated proteins. In cockroaches, the two HMG-S genes are coordinately regulated in the ovary during the gonadotrophic cycle, but not in the fat body (Casals et al., 1996). The absence of detectable amounts of HMG-S transcript in female D. jeffreyi may reflect the time point and/or JH III dose chosen for analysis. Alternatively, if the precedent set in cockroaches and other Metazoa holds true in bark beetles, a second, undetected HMG-S gene may be activated in female D. jeffreyi.

The JH III-mediated regulation of HMG-S, likely controlling the isoprenoid pathway, suggests that semiochemical production in D. jeffreyi involves the isoprenoid pathway and is regulated in a manner similar to that in Ips bark beetles. In male Ips spp., the isoprenoid pathway is important for the production of monoterpenoid pheromone components (Ivarsson et al., 1993; Seybold et al., 1995; Tillman et al., 1998). The effect of JH III on HMG-R transcript levels in male I. paraconfusus and I. pini (Tittiger et al., 1999; Tillman, Lu, Dwinell, Tittiger, Hall, Blomquist and Seybold, unpublished results) is analogous to that observed for HMG-S in D. jeffreyi, and HMG-R transcript levels in male D. jeffreyi also follow a very similar JH III-regulated expression pattern (Tittiger, Bengoa, Barkawi, Blomquist and Seybold, unpublished results). Furthermore, almost all of the detectable HMG-S transcript localizes in the metathorax and abdomen (Fig. 4), with the slight differences in tissue isolation suggesting that expression localizes near the metathoracic–abdominal border. This body region also has relatively high HMG-R mRNA levels (Hall, Mastick, Tittiger, Seybold and Blomquist, unpublished results), and is the same general area where pheromone precursors are synthesized in I. paraconfusus (Ivarsson et al., 1998). Together, these data suggest that the isoprenoid pathway is involved in semiochemical biosynthesis in male D. jeffreyi.

The complete aggregation pheromone in D. jeffreyi remains uncharacterized (Renwick and Pitman, 1979; Paine et al., 1999), though the bicyclic acetal, frontalin, is a known semiochemical of many Dendroctonus species (Kinzer et al., 1969; Paine et al., 1999). A hypotheti-
Fig. 4. Tissue distribution of HMG-S mRNA in male and female *D. jeffreyi*. (A) Representative Northern blot of RNA isolated from male and female insects that had been treated with acetone (Control) or JH III and hybridized with HMG-S and β-actin. The approximate sizes of transcripts in kb are given on the left. (B) Quantitative presentation of signal strengths for male insects from panel (A). (C) Quantitative representation of data from a second Northern blot (not shown). Grey bars represent control males; black bars represent JH III-treated males. Insects were dissected as diagrammed below the graphs. Expression levels are shown relative to control "thoraces", which were arbitrarily set at one.

cal precursor to frontalin is 6-methyl-6-hepten-2-one (6-MHO), which may arise via the isoprenoid pathway or via the fatty acid pathway from carbon salvaged from leucine, or a combination of both isoprenoid and fatty acid biosynthetic pathways (Barkawi, Bengoa, Blomquist, Francke and Seybold, unpublished results). Preliminary biochemical studies suggest at least some isoprenoid steps are involved in frontalin synthesis, and JH III induces frontalin synthesis in a dose- and time-dependent manner in male *D. jeffreyi* (Barkawi, Blomquist, and Seybold, unpublished results) similar to the pulse of HMG-S transcript (Fig. 5). We are continuing our studies on the regulation of Jeffrey pine beetle pheromone biosynthesis by isolating and investigating cDNAs for other mevalonate pathway enzymes and localizing their expression by in situ hybridizations, and through additional biochemical characterizations of pheromone component synthesis.

Acknowledgements

We thank F. Lu, G. Hall, A.J. Blomquist and B. Strom for assistance in collecting beetles, Dr. W. Francke (Universität Hamburg), and Dr. S.J. Weller (University of Minnesota) for helpful discussions. CO and CSB were supported by the Howard Hughes Undergraduate Scholar Program, University of Nevada. This work was funded by USDA grant 98-35302-6792 to GJB, SJS, and CT, NSF grant IBN-9906530 to SJS and GJB, and a joint Nevada Agricultural Experiment Station and Cooperative Extension Grant to GJB and SJS, and is a contribution of the Nevada Agricultural Experiment Station.

References

Arigoni, D., Sagner, S., Latzel, C., Eisenreich, W., Bacher, A., Zenk, M.H., 1997. Terpenoid biosynthesis from 1-deoxy-d-xylulose in
Fig. 5. Endocrine regulation of HMG-S transcript levels in male D. jeffreyi. Triplicate samples containing thoracic tissues isolated using transverse cuts [Fig. 4(A)] from five beetles per sample were used for each point. (A) HMG-S mRNA levels vs JH III dose at 20 h following JH III application. (B) HMG-S mRNA levels vs. time following topical application of 13.3 μg JH III. In both graphs, transcript levels are shown relative to acetone treated (control) male tissue, which was arbitrarily set at one.


