A novel putative insect chitinase with multiple catalytic domains: hormonal regulation during metamorphosis

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We have used differential display to identify genes that are regulated by juvenile hormone in the epidermis of the beetle *Tenebrio molitor*. One of the genes encodes *T. molitor* chitinase 5 (TmChit5), a chitinase possessing an unusual structure. Sequence analysis of TmChit5 identified five ‘chitinase units’ of approx. 480 amino acids with similarity to chitinase family 18. These units are separated by less conserved regions containing putative PEST (rich in proline, glutamic acid, serine and threonine) sequences, putative chitin-binding domains and mucin domains. Northern-blot analysis identified a single transcript of approx. 9 kb, whose abundance correlated with that of

20-hydroxyecdysone during metamorphosis. Injection of pupae with 20-hydroxyecdysone alone, or in combination with cycloheximide, indicated that *TmChit5* expression is directly induced by the hormone. Further experiments indicated that methoprene (a juvenile hormone analogue) indirectly induced *TmChit5* mRNA expression. On the basis of the present results and previous studies, we propose a molecular mechanism for cuticle digestion during the moulting process.

Key words: cuticle, ecdysone, juvenile hormone, moult.

INTRODUCTION

Chitin is a homopolymer of β -1,4-*N*-acetyl-D-glucosamine and constitutes an important component of the cell wall of various fungi [1], the exoskeleton of arthropods [2] and the egg shell of nematodes [3]. Endochitinases (EC 3.2.1.14) [4] are found in chitin-containing organisms, as well as in organisms that do not contain chitin, such as plants, bacteria and vertebrates. In these organisms, chitinases may have a defensive role against pathogens or pests [5]. In arthropods, chitin associates with proteins to form the cuticle exoskeleton and the peritrophic matrix in the midgut lumen. During moulting, the old cuticle is digested while a new one is deposited [6]. Insect chitinases belong to family 18 of hydrolytic enzymes [7] and exhibit a high degree of similarity among them. In addition to the integument, a chitinase has been found in the midgut of the mosquito *Anopheles gambiae*, where it is believed to be involved in hydrolysing chitin from the peritrophic matrix [8]. Interest in chitinases has increased in view of their possible uses as selective biopesticides [9].

Chitinase expression plays important roles during post-embryonic development of insects. As the insect grows, the cuticle needs to be degraded and a new one synthesized in its place. Each moult is induced by a pulse of the steroid hormone 20-hydroxyecdysone (20E). The type of moult depends on the titre of juvenile hormone (JH). Larval moults occur in the presence of high titres of JH, whereas the onset of the pupal and adult moults require low levels of JH [10]. Genes that are regulated during the moulting cycles thus appear to be good markers to study how JH and 20E pathways interact to control the post-embryonic development, and especially metamorphosis. The molecular mode of action of ecdysteroids is beginning to be understood as the 20E receptor and numerous target genes have been characterized [11]. However, the mechanisms of action of JH remain largely unknown, because few rapidly regulated genes have been characterized and the nature of the receptor remains controversial. In the beetle *Tenebrio molitor*, the cuticle of the three post-embryonic developmental stages is secreted by the same cell lineage,

but a dramatic switch in gene expression is observed after the pupal–adult reprogramming, and these changes are prevented by application of the JH analogue [12].

We have used the differential display technique [13] to search for new JH target genes expressed in the epidermis of *T. molitor*. In the present study, we report the identification and characterization of a new member of family 18 chitinases, *T. molitor* chitinase 5 (TmChit5), that possesses a novel structure and whose expression is regulated by both developmental hormones. We propose a model to explain how this chitinase may function during the moulting process.

EXPERIMENTAL

Animals and hormone treatments

Mealworms (*T. molitor*) were mass-reared at 25 °C on chicken food containing 1 % yeast. Dating of larvae and pupae was based on the observation of eye migration [14] or time (in h) after pupation. Treatment with methoprene (a JH analogue) was performed by topical application of 1 μ g of methoprene in acetone on to 16 h-old pupae. Moulting hormone treatments were performed by injecting 2 μ g of 20E dissolved in 2 μ l of physiological serum into 4 h-old pupae. Cycloheximide (2 μ g) was also injected. We found that 8 h after injection, 94 % of [α -³⁵S]methionine incorporated into proteins was inhibited by this dose (result not shown). Cycloheximide toxicity was assessed by following development of injected pupae. We found that these pupae completed metamorphosis, whereas adult ecdysis was delayed by 2 days.

mRNA isolation

Wing epidermal RNAs were extracted from pupae as described previously [15]. For each extraction, RNA integrity was verified by gel electrophoresis. Polyadenylated- [poly(A)⁺] containing

Abbreviations used: 20E, 20-hydroxyecdysone; JH, juvenile hormone; poly(A)⁺, polyadenylated; TmChit5, *Tenebrio molitor* chitinase 5; TmEcR, *T. molitor* ecdysone receptor.

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Figure 1 Deduced protein sequence of TmChit5

The five internal repeats (Units 1–5), typical of family 18 chitinases, are boxed. The chitin-binding domains are in bold letters. The PEST sequences (underlined) and mucin domains (dashed underline) are indicated. The amino acids located in the putative catalytic sites are indicated by an asterisk when they fit the consensus sequence or by a dash when different. The arrow indicates the predicted peptide cleavage site.

RNAs were enriched by oligo(dT) cellulose-affinity chromatography [16]. In order to remove contaminating chromosomal DNA, mRNAs (2 µg) were incubated for 15 min at 37 °C with 10 units of recombinant RNAs in ribonuclease inhibitor (Promega) and 10 units of DNase I (Life Technologies) in a solution containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl and 1.5 mM MgCl₂. The reaction was stopped by heating at 65 °C for 10 min.

Differential display

mRNA differential display was performed essentially as described previously [17]. Reactions were carried out in duplicate for all

samples. PCR was performed with the cycling parameters as follows: 94 °C for 30 s, 42 °C for 2 min, 72 °C for 30 s for 30 cycles, followed by 72 °C for 5 min. The amplified cDNAs were then separated on a 6% (w/v) polyacrylamide sequencing gel. AmpliTaq DNA polymerase was obtained from PerkinElmer and [α -³⁵S]dATP (1250 Ci/mmol) from Amersham Biosciences.

Recovery and reamplification of cDNAs

The DNA sequencing gels were dried, exposed to X-ray film and the bands of interest were cut out. The DNA was allowed to diffuse out by incubating the gel slice in 150 µl of double-distilled

water for 15 min at 95 °C, followed by ethanol precipitation using glycogen as a carrier. The cDNA was dissolved in 10 µl of water and reamplified as described, except that [α - 32 P]dATP was added in the reaction. The amplified fragment was then used as a probe in Northern-blot experiments.

Northern-blot experiments and Northern capture

Since a positive band may contain more than one cDNA, Northern blotting was used for affinity capturing the cDNA of interest [18]. Poly(A)⁺-enriched RNA (4.5 µg) or total RNA (15 µg) were separated by formaldehyde/agarose gel electrophoresis (1.1 % gel), transferred on to a Hybond-N⁺ membrane (Amersham Biosciences) and UV-cross linked (2000 J/cm²). Prehybridization and hybridization were performed at 65 °C in a solution containing 6 × SSC (1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/5 × Denhardt's solution (Denhardt's is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidene/0.002 % BSA)/0.5 % SDS/100 µg/ml salmon sperm DNA/10 % (w/v) dextran sulphate. Hybridization with a radiolabelled cDNA probe was carried out for 16 h. After hybridization, the membrane was washed three times in 2 × SSC/0.1 % SDS at 20 °C for 20 min and twice in 0.2 × SSC/0.1 % SDS at 65 °C for 30 min, prior to exposure to X-ray film. The cDNA hybridized to the differentially expressed mRNA was eluted by incubating the membrane slice in 150 µl of water for 5 min at 95 °C. cDNA was ethanol-precipitated and reamplified as described above. The PCR fragments were purified by agarose-gel electrophoresis using QiaQuick kit (Qiagen) and cloned into the pMOSBlue T-vector kit (Amersham Biosciences).

cDNA library screening

A lambda Zap II pupal epidermis cDNA library [19] was screened with radiolabelled cDNA probes. Plasmids containing positive inserts were derived from the phage according to the manufacturer's instructions. Full-length cDNA sequence was obtained by rescreening the cDNA library with PCR probes corresponding to the extremities of the cDNA already cloned.

Slot-blot analysis

Total RNAs were adjusted to 10 × SSC final concentration and applied on to nylon membranes with a suction manifold. Hybridization was performed as described for Northern-blot experiments. To quantify autoradiographic signals, autoradiograms were scanned with a Shimadzu CS-9000 densitometer equipped with an integrated computer. Each membrane was rehybridized with TmL27a cDNA in order to verify that equal amounts of RNA had been loaded. Mann–Whitney U-test was used in statistical treatment of data and only $P < 0.01$ was considered to be significant.

Phylogenetic analysis

The clustal program [20] was used for generating amino acid sequence alignments with chitinases from several arthropods and nematodes as follows: *Penaeus japonicus* Pjchit-1 (GenBank® accession no. D84250), Pjchit-2 (GenBank® accession no. D89751) and Pjchit-3 (GenBank® accession no. AB008027), *Chelonus* sp. (GenBank® accession no. U10422), *Manduca sexta* (GenBank® accession no. L49234), *Bombyx mori* (GenBank® accession no. U86876), *Hymantria cunea* (GenBank® accession no. U86877), *Anopheles gambiae* (GenBank® accession no. AF008575), *Aedes aegypti* 1, 2 and 3 (GenBank® accession nos. AF 26491 and AF26492), *Chironomus tentans* (GenBank® accession no. CAA73686), *Caenorhabditis elegans* (GenBank®

Figure 2 Dendrogram generated from an alignment of chitinase sequences using the Neighbour-Joining method

The clustal program was used to generate amino acid sequence alignments using amino acid sequences of several arthropod and nematode chitinases as described in the Experimental section. *P. japonicus*, Pjchit-1, Pjchit-2 and Pjchit-3; *Chelonus* sp.; *M. sexta*; *B. mori*; *H. cunea*; *A. gambiae*; *A. aegypti* AaChit1, 2, 3 and 4; *Ch. tentans*; *C. elegans*; and *Br. malayi*. For TmChit5, each unit (in bold) was extracted for alignment. The numbers indicate the bootstrap values.

accession no. AAA83586) and *Brugia malayi* (GenBank® accession no. AAA27854). For TmChit5, each unit was extracted for alignment. The dataset was treated by distance analysis with the Neighbour-Joining method [21] as described previously [22]. For the bootstrap analysis, 1000 sets of replications were performed.

RESULTS

Isolation of a new member of the chitinase 18 family of genes

To identify genes potentially regulated by JH, we compared differential mRNA display patterns from alar epidermis. Pupae (16-h old) were treated with either methoprene or acetone (control) and the epidermis was dissected 25 h later. In order to purify the cDNAs and to confirm that the corresponding genes were regulated by methoprene, the differentially amplified bands were eluted, PCR-amplified and subjected to Northern-capture experiments. A 246 bp insert that shared sequence similarity with chitinases from various sources was isolated. This fragment was subcloned and used as a probe to screen a cDNA library.

Figure 3 Developmental profiles of TmChit5 and TmEcR epidermal mRNAs during *T. molitor* metamorphosis as a function of 20E titres (μM) in the haemolymph

Blots were hybridized with cDNA encoding TmChit 5 (A), *T. molitor* ribosomal protein 27a, as loading control, (B) and TmEcR (C). The time is in days. A–M, periods of prepupal extractions; A'–N', periods of pupal extractions; A. Apo, adult apolysis; A. Ecd, adult ecdysis; P. Apo, pupal apolysis; P. Ecd, pupal ecdysis. The concentrations of 20E in the haemolymph during the time periods considered here are given in Figure 5 of [19].

Five overlapping clones were isolated and the cDNA sequence was established. This sequence was 8938 bp long and contained an open reading frame of 2839 amino acids followed by a stretch of 167 untranslated nucleotides containing a putative poly(A)⁺ signal. The deduced amino acid sequence is shown in Figure 1. The calculated molecular mass of the deduced protein, named TmChit5, was 321.6 kDa and the calculated pI was 6.49. The first 20 residues, which define a hydrophobic region, probably corresponded to a signal peptide. A putative cleavage site was predicted to be located between position 20 and position 21 [23]. Sequence alignment revealed that TmChit5 contained five repeats of approx. 480 amino acids, and that the repeats possessed more than 70% similarity between them. The repeats have the consensus sequence (Leu-Ile-Val-Met-Phe-Tyr)-(Asp-Asp)-Glu-(Leu-Ile-Val-Phe-Met)-(Asp-Gln)-(Leu-Ile-Val-Phe-Met)-(Asp-Glu)-Xaa-Glu, where Glu is the catalytic amino acid [24] characteristic of chitinase family 18 [7] and Xaa is any amino acid. This family comprises proteins from organisms as diverse as arthropods, bacteria, fungi, plants, nematodes and vertebrates. For the first and second units of TmChit5, there was one mismatch between the sequence and the proposed consensus pattern of chitinase family 18 active site (histidine at position 7 in unit 1 and asparagine instead of the catalytic glutamic acid in unit 2), whereas the three other units fit the consensus. The units are separated by intervening regions of variable length containing putative PEST (proline-, glutamic acid-, serine- and threonine-rich) sequences. These motifs are postulated to increase susceptibility to proteolysis [25]. The intervening regions also contain four putative chitin-binding domains, each approx. 50 amino acids long, which conform to the consensus CX₁₁CX₅CX₉CX₁₂CX₇C (one-letter code) [26]. Two serine/threonine-rich mucin domains have been also identified between units 2 and 3 [27]. Lysine and arginine residues, potentially cleaved by trypsin, are present at the beginning of each unit.

Phylogenetic analysis

Clustal analysis (neighbour-joining method) was performed using the catalytic domain to compare the deduced amino acid sequences of the five TmChit5 units between themselves and the other family 18 chitinases. Phylogenetic analysis was performed using both distance and parsimony methods, with the bootstrap value as a test of the robustness of each branch. Two nematode chitinases were used as an external group. The overall topology of the deduced phylogenetic tree (Figure 2) was consistent with phylogeny. Units 3, 4 and 5 clustered with chitinases 2, 3 and 4 respectively, from the mosquito *A. aegypti*. The grouping of these sequences was supported well by the bootstrap values and indicated that units 3–5 of Tmchit5 were orthologous to these mosquito chitinases. It should be noted that the chitinase Pjchit-2 from the shrimp *Penaeus japonicus* clusters with the group formed by unit 4 of TmChit5 and AaCHIT3 chitinase from *A. aegypti*. Units 1 and 2 of TmChit5 do not cluster with the other units of TmChit5. Moreover, the group in which unit 1 was attached did not respect the phylogeny, indicating an artefactual position of this unit in the present tree. Indeed, if TmChit5 unit 1 was orthologous to the other chitinases included in this group, it would be attached to the other insect chitinases and not to Pjchit-1.

Developmental profile

Variations in TmChit5 mRNA abundance were examined during metamorphosis (Figure 3) and correlated with 20E haemolymph titres. Epidermal RNAs isolated at different times during metamorphosis were submitted to Northern-blot analysis. The TmChit5 probe hybridizes to a single transcript with an estimated size larger than 9 kb, in good agreement with the length of the cDNA sequence. The transcript was undetectable during the prepupal stages until day 5, then appears before apolysis,

Figure 4 Effect of 20E on TmChit5 mRNA transcripts

mRNAs were extracted at 0.5, 2 or 4 h after the treatment. (A) Slot-blot analysis; C, saline injection; 20E, 20E injection. (B) Densitometric analysis of autoradiograms. Each bar represents the mean $\pm$ S.D. of 6 independent densitometric intensities. N.S., not significant; **, highly significant ($P < 0.01$).

increased at day 10 and remained present at a high level until day 15. The decrease in mRNA abundance coincided with the fall of 20E titre. During the pupal stage, the transcript appeared at day 1, thus 12 h before apolysis. As during prepupal development, transcript abundance increased rapidly, peaked at day 3, and decreased sharply when the 20E titre declined. The transcript reappeared at a low level just before adult ecdysis.

Hormonal regulation by 20E

To investigate whether TmChit5 mRNA abundance correlated with 20E titre, 4-h-old pupae were injected with 20E and TmChit5 mRNA abundance was estimated by slot-blot and Northern-blot analyses (Figure 4). Although TmChit5 mRNA abundance was

Figure 5 Effect of cycloheximide on 20E-induced TmChit5 mRNA up-regulation

Northern-blot analysis of mRNA extracted 4 h after treatment with saline alone (C), 20E, cycloheximide (Chx) or by 20E and cycloheximide (Chx + 20E). Densitometric analysis of the autoradiograms is shown. Each bar represents the mean $\pm$ S.D. of densitometric intensities. N.S., not significant; **, highly significant ($P < 0.01$).

Figure 6 Effect of a JHA on TmChit5 epidermal transcripts

JHA was applied topically on to 16-h-old pupae and mRNAs were extracted 4 and 8 h after treatment. Slot-blot analysis was performed and the level of TmChit5 mRNA was quantified. Each bar represents the mean $\pm$ S.D. of 6 independent densitometric intensities. C, mRNAs from control pupae; N.S., not significant; **, highly significant ($P < 0.01$).

the same as controls 30 min after treatment, it was 3.6-fold higher than in controls after 2 h. Peak induction (6-fold) was observed 4 h after 20E injection. The same membranes were hybridized with a probe encoding the *T. molitor* ribosomal protein 27a ('TmL27a') and no significant differences were observed between the different samples (results not shown). These results are consistent with the hypothesis that TmChit5 mRNA expression is directly regulated by 20E. To verify this hypothesis, we assessed the effect of cycloheximide on the

stimulation of TmChit5 mRNA expression by 20E. Cycloheximide had no effect on TmChit5 mRNA induction by 20E (Figure 5), strongly suggesting direct 20E action.

Hormonal regulation by methoprene

In order to determine the time course of methoprene induction, 16-h-old pupae were treated with either the hormone or acetone (as a control), and the mRNAs were extracted at different times after treatment. TmChit5 expression increased 3-fold 8 h after methoprene treatment (Figure 6).

DISCUSSION

We have cloned a *T. molitor* cDNA that shows high amino acid sequence identity with members of the chitinase 18 family. Circumstantial evidence that this protein has enzymic activity is provided by sequence analysis and by the developmental pattern of gene expression. TmChit5 contains five units sharing similarity with members of the chitinase 18 family. All the chitinases possessing the consensus sequence characteristic of this family have chitinase activity, except the human glycoprotein GP39 [28], the lectin concanavalin B isolated from the plant *Canavalia ensiformis* [29] and the insulin-dependent growth factors (IDGFs) from *Drosophila melanogaster* [30]. In the lectin and IDGFs, the essential amino acid for chitinase activity [glutamic acid at position 8 of the consensus sequence] is replaced by glutamine and aspartic acid respectively. Site-directed mutagenesis of this glutamic acid in a tobacco class I chitinase completely destroys enzymic activity and converts the catalytic domain into a chitin-binding domain [31]. Interestingly, this residue was replaced in the second unit of TmChit5, indicating that this unit is probably devoid of enzymic activity. Another essential and invariant amino acid (aspartic acid at position 2) is replaced in the second unit. These observations lead to the speculation that the first two units of TmChit5 have lost their catalytic activity; it is possible that they acquired a different function, such as that of a lectin or growth factor.

The developmental expression profile of TmChit5 mRNA is also in agreement with the hypothesis that this protein has a chitinolytic activity [32–34]. During the last larval instar or during the pupal development, transcript abundance peaks before the deposition of the fibrous cuticle. This time course of expression is in good agreement with studies showing that the transcript appears several days before the digestion of the old cuticle [32–34].

In common with other chitinases, TmChit5 has a typical multi-domain architecture, including a proline/threonine-rich domain, catalytic domains and putative chitin-binding domains, potentially involved in the anchoring of the enzyme to the cuticle (for review see [9]). The unusual structure of TmChit5 with its five repeated units, raises the possibility that a proteolytic-activation mechanism exists. In *B. mori*, an antigen of at least 215 kDa, recognized by an antibody directed against chitinases, does not have chitinase activity and is present in the integument 2 days before the appearance of chitinolytic activity [34]. Based on these data, it was proposed that this protein may be secreted as a zymogen [34]. In *M. sexta*, chitinases are present in the moulting fluid well before the degradation of the old cuticle, and the beginning of this degradation coincides with the appearance of proteolytic enzymes in the moulting fluid [35]. Additional studies [8] have shown that, in the mosquito *A. gambiae*, whole-gut extracts need to be treated with trypsin to detect chitinase activity and putative cleavage sites (Lys-31 or Lys-32) were proposed. Interestingly, lysine or arginine residues are present in a conserved position in the five TmChit5 units (Figure 1). We

speculate that TmChit5 is secreted as a zymogen and is incorporated into the old cuticle through its four chitin-binding domains. Trypsin-like enzymes, which are known to be present in the moulting fluid [32], may cleave the zymogen to generate active units 3, 4 and 5. This proteolysis could be enhanced by the presence of PEST sequences. Confirmation of such a pathway could be obtained by purification and microsequencing of *Tenebrio* epidermal chitinases.

Phylogenetic analysis shows that Tmchit5 units 3, 4 and 5 are orthologous to the mosquito *A. aegypti* chitinases 1 and 2 [36]. These three sequences are located at the same locus and the deduction of the open reading frames led de la Vega et al. [36] to propose two possible versions of the protein sequences. The first results in a protein sequence with three catalytic domains, whereas the second generates three chitinases. Our results strengthen the first hypothesis. Although chitinases have been extensively searched by PCR in *Aedes*, it is possible that the first two units failed to be cloned because the primers were based on the catalytic region, which is not well conserved in the first two units of TmChit5. Moreover, sequence analysis did not reveal the existence of a clear signal peptide. These data seem to indicate that this *Aedes* chitinase may also possess five units. On the other hand, it is possible that the first two units have been lost during evolution.

The phylogenetic tree clearly shows that units 1 and 2 do not cluster with the others and that their position in the tree does not fit the systematics. Since their catalytic site is likely to be inactive, it is possible that these units have rapidly diverged from other chitinases due to loss of functional constraints. Indeed, this phenomenon, whereby genes that have higher mutation rates in comparison with the other genes studied will tend to be displaced at a wrong position (because they appear as being very distant from the other ones), is now well documented [37,38].

Taking into account that unit 4 has a crustacean orthologue, two hypotheses could explain how the unusual structure of TmChit5 was generated during evolution. The first hypothesis implies the existence of a common ancestor in crustaceans and insects of five clustered chitinase genes, which fused during evolution to give rise to a single transcription unit encoding TmChit5. The second hypothesis implies the internal duplication of a chitinase unit module. The analysis of the chitinase-encoding genomic locus of *P. japonicus* may allow us to determine whether these genes are clustered or not, thus confirming or disproving the second hypothesis.

During the prepupal and the pupal stages, TmChit5 mRNA becomes detectable at the same time as many other transcripts which are known to be induced directly by 20E, such as *EcR* [11]. Indeed, two *EcR* isoforms have been cloned in *T. molitor* [19] and present the same developmental profile as TmChit5 (Figure 3). In order to determine whether TmChit5 is a direct target of the 20E receptor, 20E injections were performed in the presence or in the absence of cycloheximide. The results clearly showed that the induction of TmChit5 gene expression by 20E does not require protein synthesis. Thus TmChit5 can be considered as an early responsive gene.

TmChit5 transcript appears in phase with a slight increase in the 20E titre at prepupal apolysis, which could be correlated with the pupal commitment [39]. However, its pupal expression begins 2 days before the increase in haemolymphatic 20E titre. However, the increase in mRNA levels of early responsive genes, such as *EcR* or *E75A* [40], occurs in the absence of detectable 20E variation, whereas these genes are clearly induced *in vitro* by very low levels of 20E. Since we have clearly shown that TmChit5 is a direct target of 20E, it is thus possible that a small difference in the 20E level at this time might be undetectable.

Unexpectedly, TmChit5 mRNA was induced shortly before adult ecdysis in the absence of a major ecdysteroid titre fluctuation. Although similar patterns of gene expression have also been reported for *EcR-B1* in *T. molitor* [19] and *E75A* in *Galleria mellonella* [41], the physiological relevance of TmChit5 increase just before ecdysis remains unknown.

Despite intensive searches for JH-target genes, few have been identified [42–45]. Moreover, to our knowledge, only one study [46] reported the isolation of two genes (named *JH1* and *JH2*), whose transcripts accumulate rapidly in response to methoprene. Although the 5' regulating sequence of *JH1* can confer JH inducibility to a reporter gene, there is no clear evidence that these genes are direct JH targets *in vivo*.

The screen in the present study led to the identification of a new methoprene target, whose expression is rapidly modulated by the analogue in the absence, but not in the presence, of cycloheximide (results not shown). Considering that this up-regulation needs protein synthesis and that JH analogues have an ecdysiotropic effect, it is possible that methoprene acts via a modulation of the 20E titre. To test this hypothesis, 20E titres were determined using an enzyme-linked immunoassay [47] and no significant differences were observed between treated and control animals even 24 hours after JHA application. Northern-blot analysis of early-responsive gene expression could provide a more pronounced and easily detected measurement of brief low-titre pulses than is possible by immunoassay [39]. The response of *T. molitor* ecdysone receptor (*TmEcR*), another early responsive gene induced by 20E, to 20E was studied in methoprene-treated animals. Northern-blot analysis with a cDNA fragment that hybridizes with both TmEcR isoforms revealed no significant effect of methoprene on TmEcR mRNA abundance (result not shown). These results suggest that ecdysone biosynthesis is not enhanced by methoprene treatment and that ecdysone is not involved in the methoprene up-regulation of TmChit5 mRNA expression. Although our data clearly showed that methoprene treatment significantly increases TmChit5 mRNA abundance, the level at which this regulation takes place remains to be determined.

At present, there is no clear evidence on how JH is responsible for such pleiotropic actions and its molecular mode of action remains obscure. The characterization of JH-inducible genes is thus still a prerequisite to elucidate the mode of action of this hormone.

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