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The two duplicated insecticyanin genes, *ins-a* and *ins-b* are differentially expressed in the tobacco hornworm, *Manduca sexta*

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ABSTRACT

Two gene-specific probes were generated from the unique sequences in the 3′ non-coding regions of the two insecticyanin genes, *ins-a* and *ins-b* to study the developmental expression of these genes in *Manduca sexta*. Both genes were initially transcribed in the freshly hatched first instar larvae and then expressed in the epidermis and to a lesser degree in the fat body during every larval feeding stage. In the epidermis of the 4th and 5th instar larvae, both mRNAs appeared shortly before ecdysis and accumulated to maximal levels within a day. As the larval epidermis became pupally committed on day 3 of the 5th (final) instar, INS-a mRNA quickly decreased, whereas INS-b mRNA showed a second peak of accumulation. In the fat body, both genes showed a similar expression pattern within the 4th instar to that of the epidermis except that levels were lower and *ins-b* mRNA dominated. In the final instar, only *ins-b* mRNA was present in significant amount. These findings not only reveal that the two duplicated insecticyanin genes have diverged in their expression pattern but also demonstrate, for the first time, that fat body also expresses insecticyanin genes.

INTRODUCTION

Insecticyanin is a blue pigment found in the larval epidermis and in the hemolymph of the larvae, pupae and adult of the tobacco hornworm, *Manduca sexta* (1). Its main function is to provide camouflage for protecting the larval development (2–3). Biochemical studies have revealed that insecticyanin is a biliverdin IXγ-associated protein (1, 4–5) and belongs to the lipocalin family of proteins recently described by Pervaiz and Brew (6). Two-dimensional gel electrophoresis resolved two different isoelectric forms: INS-a (pI 5.5) and INS-b (pI 5.7) (7–8). The recent cloning of the insecticyanin genes clearly demonstrated that the two major isoelectric forms are encoded by a pair of duplicated genes, *ins-a* and *ins-b* (9).

It has been shown that members of the duplicated gene family often display tissue- and temporal-specific expression during development (10). For example, expression of the different members of the chorion gene families in *Bombx mori* displays clear temporal, yet overlapping patterns which contribute to the normal choriogenesis (11). Whether *ins-a* and *ins-b* are also differentially expressed is explored in the present investigation.

In the lipocalin family, the closely-related lepidopteran member is the bilin binding protein (BBP) of the butterfly, *Pieris brassicae*. Although insecticyanin and BBP have similar three-dimensional structure, the BBP cDNA shows only 52% homology to either INS-a or INS-b cDNA (12). In the whole animal, BBP mRNA is initially detectable in the second instar and shows significant amount in the 4th, 5th instar larvae and apparently peaks in the pupae and adults. BBP mRNA is maily transcribed by fat body and gonads.

In constrast to BBP, insecticyanin, as previously shown, is synthesized by the larval epidermis (7–8, 13). It is not known, however, whether any other tissues also express insecticyanin genes and when these genes are initially transcribed. In the present studies, we have generated two gene-specific probes based on the unique sequences in the 3′ non-coding regions and analysed their tissue- and temporal-specific expression of the two insecticyanin mRNAs at different developmental stages. Our results clearly show that the two insecticyanin genes have diverged in their expression pattern and that fat body is another tissue expressing *ins-a* and *ins-b* with relatively lower levels.

MATERIALS AND METHODS

Chemicals

Radioactive chemicals were purchased from Amersham Corp., Arlington Heights, IL. The molecular biology reagents and enzymes were obtained from Bethesda Research Laboratories, Gaitherberg, MD; New England Biolabs, Beverly, MA; Promega Biotech, Madison, WI; Stratagene, La Jolla, CA and United States Biochemical Corp. The majority of other chemicals and antibiotics were purchased from Sigma.

Animals

Tobacco hornworm larvae were reared individually on an artificial diet at 25.5°C under a 12 L:12 D photoperiod and 60% relative humidity (14). Lights-off is arbitrarily designated as

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24:00. Fertilized eggs were collected every hour from a tobacco plant, then allowed to develop at 26°C, 17 L:7 D for a given time, then the embryos collected and the RNA extracted. The 1st to 3rd instar larvae were staged from the time of hatching or ecdysis. For the 4th and 5th stages, only larvae that ecdysed within 2 h of lights-on and at lights-off ± 2 h on day 0 respectively were used.

Probe synthesis

The unique sequences present in the 3'-non-coding region of the two insecticyanin cDNAs (9) were purified through sequential restriction digestion and agarose gel separation and then labeled with α-^{32}P-dATP according to Feinberg and Vogelstein (15).

Isolation of RNAs

RNA was extracted from either larval epidermis and fat body (4th and 5th instar larvae, fresh pupae and pharate adult), or the whole organism (embryos, first, second and third instar larvae) using 7.6 M guanidine−HCl in 0.1 M potassium acetate solution (16) as modified by Hiruma *et al.* (17). Further modifications were introduced to increase RNA stability in prolonged storage. After the initial precipitation, the RNA pellet was washed by centrifugation first in 70% ethanol, then in 100% ethanol, and dried at room temperature. This pellet was resuspended in Tris−EDTA (10 mM Tris, 1 mM EDTA, pH 8.0) buffer and precipitated by 2.5 volume of 100% ethanol at −70°C for 20 to 40 minutes. The recovered pellet was then treated the same way as the initial pellet. The final RNA pellet was redissolved in Tris−EDTA buffer and immediately stored at −70°C.

Northern blot analyses

Twenty μg of total RNA from epidermis, fat body, whole embryo or larva at various stages were denatured, electrophoresed on formaldehyde-agarose (1.5%) gels and transferred to Hybond nylon membrane (Amersham) according to established methods (18−20). After transfer, the RNA blot was either baked at 80°C under vacuum for 2 hours or cross-linked by 5 min UV irradiation. Pre-hybridization was conducted at 42°C for 2 to 6 hours in following buffer condition: 50% formamide, 6×SSPE (1×SSPE is 0.15M NaCl, 12mM Na phosphate, pH 7.4, 1.2mM EDTA), 5×Denhardt's solution, 0.1% SDS, 200 μg/ml denatured herring sperm DNA. Hybridization was conducted in the same temperature and buffer condition for 36 to 42 hours with ^{32}P-labeled specific probes (>2×10^6 cpm/ml). After hybridization, the filter was washed once in 2×SSPE, 0.2% SDS for 30 minutes at room temperature, then the wash conditions differed for the two probes as determined experimentally (see Fig. 2). For the INS-a probe, the filters were placed in 0.2×SSPE, 0.1% SDS heated to 52°C, then shaken at the room temperature for 20 minutes; this procedure was repeated once. The same procedure was used for the INS-b probe except that 0.1×SSPE, 0.1% SDS heated to 62°C was used. The washed filter was then exposed to Kodak XAR-5 film.

Dot blot analyses

Five μg of total RNA from epidermis or fat body was denatured by heating at 70°C for 15 min in 100 μl mixture of 20×SSC and formaldehyde, and spotted on either Hybond (Amersham) or nitrocellulose (Gibco BRL) membrane using a Schleicher and Schuell or a Gibco BRL manifold (21). Hybridization, wash and exposure procedures were the same as described above.

Densitometry scanning

Fluorograms of the northern and dot blots were quantitated by densitometric scanning using a computerized scanner, model 300 (ImageQuant). A pooled RNA sample extracted from epidermis of 7 day 1 (22:00 AZT) 5th instar larvae was used as the standard density of 1.0 for all blots.

RESULTS

Design of the two gene-specific probes to detect the INS-a and INS-b mRNAs

Each of the insecticyanin genes contains a unique sequence in its 3' non-coding region (9). These unique sequences with small boundary regions (less than 23 bp on each side) were cut from the two full length cDNA clones using RsaI and MseI for INS-a (Fig. 1A), or RsaI and DraIII for INS-b (Fig. 1B). The restricted unique INS-a or INS-b cDNA fragments were ^{32}P-labeled and used to hybridize with the two full length cDNAs (9). Wash conditions were then varied systematically for each probe to determine those conditions under which each probe was specific for only one insecticyanin cDNA. As shown in figure 2B, the INS-a probe hybridizes only to INS-a cDNA after a 30-minute wash with 2×SSPE and 0.2% SDS at room temperature followed by two 20-minute washes in 0.2×SSPE and 0.1% SDS heated to 42°C, 52°C or 62°C, then shaken at room temperature (Fig.

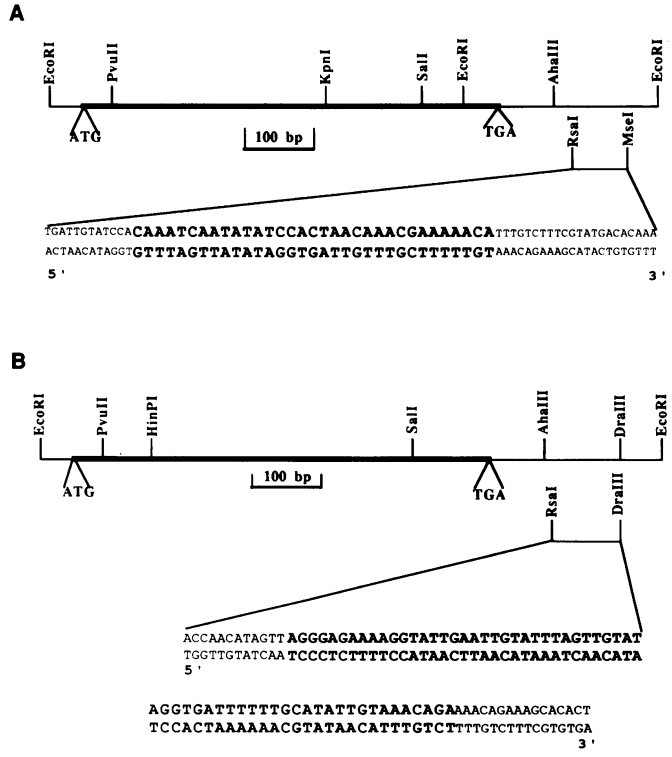

Figure 1. Design of the INS-a and INS-b gene-specific probes. **A.** The DNA fragment containing the 33 bp unique (bold-faced) 3' non-coding sequence of the INS-a gene was cut out from the INS-a cDNA by RsaI and MseI at the two closest restriction endonuclease sites as determined by computer search on a Pustell program. **B.** The DNA fragment containing the 63 bp unique (bold-faced) 3' non-coding sequence of the INS-b gene was similarly cut out from the INS-b cDNA by RsaI and DraIII.

2B). The INS-b probe hybridizes only to INS-b cDNA after the same first wash followed by two 20-minute washes in 0.1×SSPE and 0.1% SDS heated to 62°C and shaken at room temperature (Fig. 2C). These conditions were used for all the experiments described below.

Tissue specificity of *ins-a* and *ins-b* expression

The epidermis has been shown to synthesize, store and secrete insecticyanin (1,7–8, 13, 22). The synthesized insecticyanin is also found in the hemolymph and pericardial cells (22). To determine whether any other tissue makes insecticyanin besides epidermis, we hybridized total RNA from epidermis, fat body, hemocytes, muscle, nervous system (brain and ventral nerve cord) and pericardial cells with the two gene-specific probes. Figure 3A clearly shows that the INS-a gene is expressed mainly in the epidermis with a trace amount of mRNA (about 10% of the average of 2 blots) in the fat body. The INS-b gene is expressed both in the epidermis and in the fat body with the latter containing about 30% of the amount in the epidermis as determined by densitometry on 2 blots (Fig 3B). No insecticyanin mRNAs were detected in 20 μg of total RNA in the other tissues, even after a 10-fold longer exposure (data not shown).

Temporal specificity of *ins-a* and *ins-b* expression in embryos and the early larval stages

Manduca eggs and embryos contain insecticyanin (23). To determine whether any of this insecticyanin is newly synthesized by the embryos, we conducted northern blot analyses with total RNA prepared from the freshly oviposited egg and from embryos of 12, 24, 36, 48 and 72 hours. As shown in the first 6 lanes of Figures 4A and B, no detectable messenger for either INS-a or INS-b was observed in the freshly oviposited egg or developing embryos. Ten-fold longer exposure of the same filter also showed neither INS-a nor INS-b mRNA signals (data not shown). These results clearly demonstrate that insecticyanin in the egg and embryo (23) is accumulated through maternal deposition during oogenesis.

The *Manduca* larva hatches from the egg at about 100 hours at 25°C (24). Northern blot analyses with total RNA from the whole body of various aged larvae showed that INS-b mRNA was detectable in the freshly-hatched first instar larva (Fig. 4B, lane 7 from the left). A trace amount of INS-a mRNA was also present (Fig. 4A, lane 7 from the left). As the larva grows, the two mRNAs increased and attained maximal levels in 24 hours (Fig. 4A & B, lane 9 from the left). Then the two mRNAs gradually disappeared as the larva entered the molting period (lane 10 of Fig. 4A & B). This pattern was also observed during development of the 2nd and the 3rd instar larvae (lanes 11 to 16 of Fig. 4).

Temporal specificity of *ins-a* and *ins-b* expression in the late larval stages and during metamorphosis

In the epidermis, the mRNAs for both genes were present in the freshly-ecdysed 4th instar larvae (Fig. 5). As the larva grew,

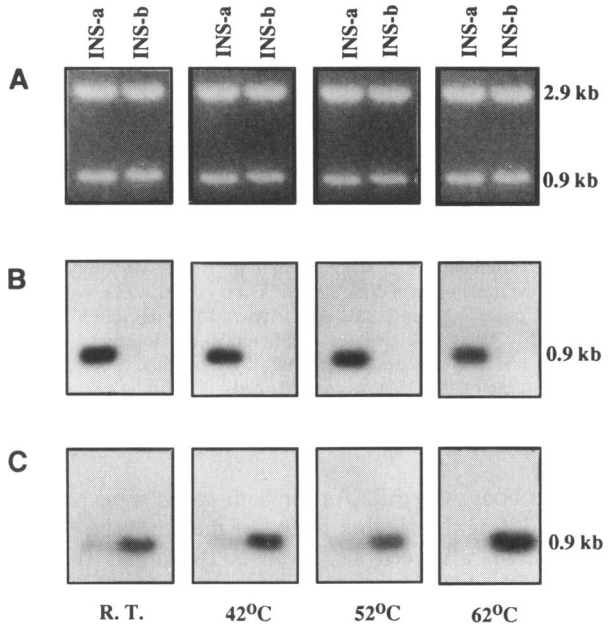

Figure 2. Determination of the hybridization conditions for the two gene-specific probes. (**A**) The two cDNAs (Li and Riddiford, 1992) were digested with BamHI and HindIII (for INS-a cDNA) or EcoRI alone (for INS-b cDNA), separated on an 1.5% agarose-gel, transferred onto Hybond nylon membrane and hybridized with either (**B**) ^{32}P-labeled RsaI–MseI fragment from INS-a cDNA or (**C**) ^{32}P-labeled RsaI–DraIII fragment from INS-b cDNA. After 36-hour hybridization, the filter was washed once in 2×SSPE, 0.2% SDS for 30 minutes at room temperature (R.T.) followed by two washes in either (B) 0.2×SSPE, 0.1% SDS (for the INS-a probe) or (C) 0.1×SSPE, 0.1% SDS (for the INS-b probe) heated to the indicated temperatures and then shaken at room temperature for 20 min. DNA fragment sizes as determined from 1 kb ladder are indicated on the right side.

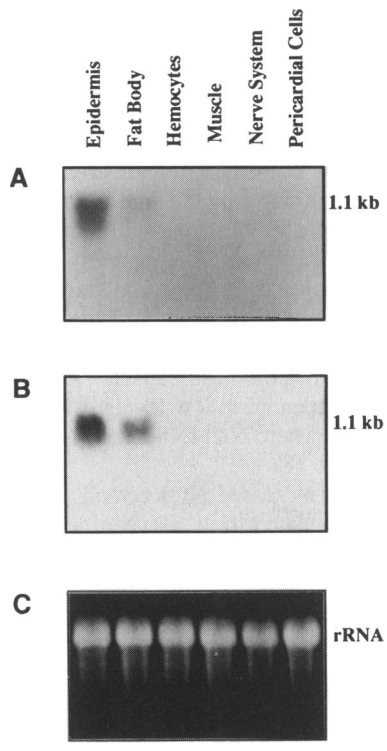

Figure 3. Tissue specificity of expression of the INS-a and INS-b genes. Twenty micrograms of total RNA from various tissues of the day 1 (22:00 AZT) 5th instar larvae were hybridized with either (**A**) the INS-a specific probe or (**B**) the INS-b specific probe and washed in the conditions described in the Materials and Methods. (**C**) Ethidium bromide staining of total RNA. The size is indicated on the right side as determined from the RNA ladder (BRL).

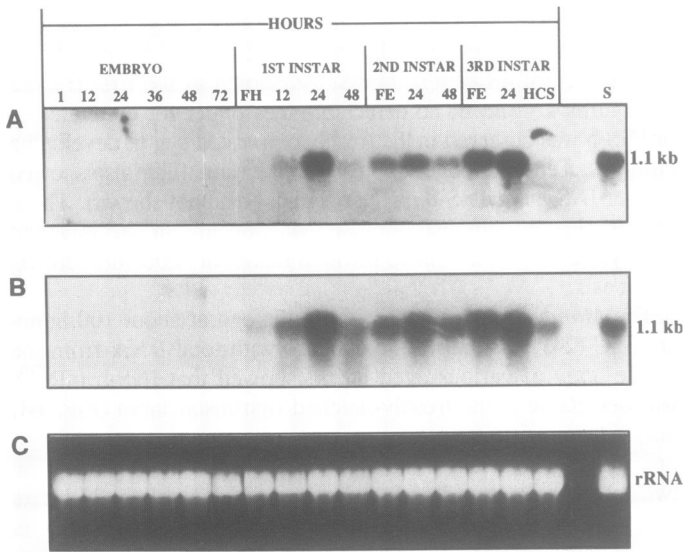

Figure 4. Temporal specificity of expression of the INS-a and INS-b genes in embryos and early larval stages. Twenty micrograms of total RNA from whole eggs (1 h), embryos at indicated hrs after oviposition, and 1st to 3rd instar larvae were hybridized with either (**A**) the INS-a -specific probe or (**B**) the INS-b-specific probe and washed in the conditions described in the Materials and Methods. (**C**) Ethidium bromide stain of total RNA. The size is indicated on the right side as determined from the RNA ladder (BRL). Abbreviations: FH, freshly hatched; FE, freshly ecdysed; HCS, head capsule slippage; S, standard, epidermal RNA extracted from day 1 (22:00 AZT) 5th instar larvae.

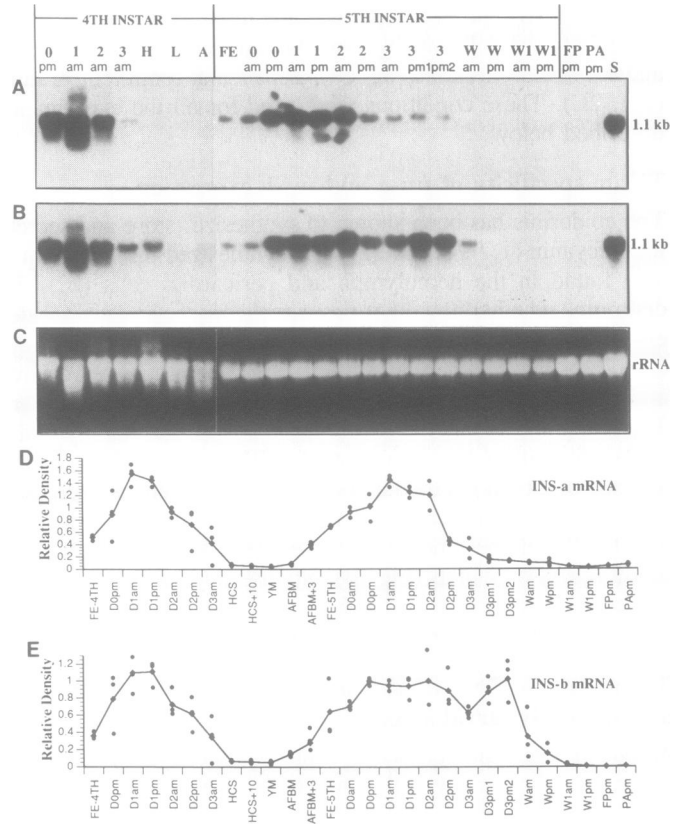

Figure 5. Differential expression of the INS-a and INS-b genes in the epidermis of the 4th and 5th larval stages, fresh pupae and pharate adults. Twenty micrograms of total epidermal RNA were hybridized with either (**A**) the INS-a specific probe or (**B**) the INS-b specific probe and washed in the conditions as described in the Materials and Methods. (**C**) Ethidium bromide stain of total RNA to show loading. The size is indicated on the right side as determined from the RNA ladder (BRL). Numbers refer to days of each stage. Abbreviations: A, onset of air-filled head capsule (6 hours before ecdysis); FE, freshly ecdysed; FP, fresh pupa; H or HCS, head capsule slippage; L, liquid-filled head capsule with brown mandibles showing (20 hours after head capsule slippage); PA, pharate adult; S, standard, epidermal RNA extracted from day 1 (22:00 AZT) 5th instar larvae; W, wandering; W1, the day after the onset of wandering. am: 6:00 am (11:00 AZT); pm, 5:00 pm (22:00 AZT); pm1, 5:00 pm (22:00 AZT); pm2, 10:00 pm (03:00 AZT, day 4). (**D**) and (**E**) Quantitative analyses of differential developmental expression of the INS-a and INS-b genes as determined by dot-blot hybridization and densitometric scanning (see Materials and Methods). Each solid circle represents a mRNA sample from an individual animal and the curve crosses the average value (square) from different animals at the same stage.

both mRNAs increased and were maximal by the morning of day 1. During this period, individual larvae showed variable levels of both INS-a and INS-b mRNA. By the morning of day 2, both mRNAs began to decline. When the larva entered the molting period on day 3, the two mRNAs decreased substantially and became barely detectable by the time of head capsule slippage (Figure 5, the sample of head capsule slippage used for the northern blot in Figure 5 A and B apparently was aberrant since the other RNA samples from the same stage used for the dot blots in Figures 5D and E showed no detectable INS-b). Then 3–6 hrs before ecdysis into 5th instar soon after the molting fluid had been resorbed from the head capsule (AFBM), both INS-a and INS-b mRNA appeared in low levels, which was consistent with previous studies where both INS-a and INS-b syntheses were detected at this stage (8).

The mRNAs then increased after ecdysis as the larva grew. INS-b mRNA accumulated to its maximal level by 24 hrs after ecdysis, while INS-a mRNA attained its peak level slightly later, on the morning of day 1 (Fig. 5). The levels of both INS-a and INS-b mRNAs remained relatively steady until morning of day 2, then began to decline with INS-a mRNA disappearing more rapidly (Fig. 5). As the larval epidermis became pupally committed in the afternoon of day 3 (25), INS-b mRNA transiently increased, whereas INS-a mRNA remained low. Both were undetectable by the day after wandering. Hence, the differential accumulation of the transcripts of the two insecticyanin genes during this transition period correlated well with the previously observed differential syntheses of the two forms of insecticyanin (7–8). These mRNAs were not found thereafter in the epidermis of either fresh pupae or pharate adult (1 day before ecdysis) (Fig. 5).

In the fat body, the mRNAs for both genes were present in low levels during the feeding period of the 4th instar (Fig. 6). By about 12 hrs after ecdysis, levels of INS-a and -b mRNAs were about 40 and 60% respectively of that found in the epidermis of day 1 5th instar larvae based on the dot blot analysis. These RNAs declined during the molt to the 5th instar and INS-a mRNA levels remained low thereafter. By contrast, the INS-b mRNA accumulated to 28% of that found in the epidermis of day 1 5th instar larvae and remained there until day 3 when it declined to undetectable by wandering. None was found in the freshly ecdysed pupae or in the pharate adult.

DISCUSSION

Using the unique sequences in the 3′ non-coding regions of the two insecticyanin genes as gene-specific probes, we have shown

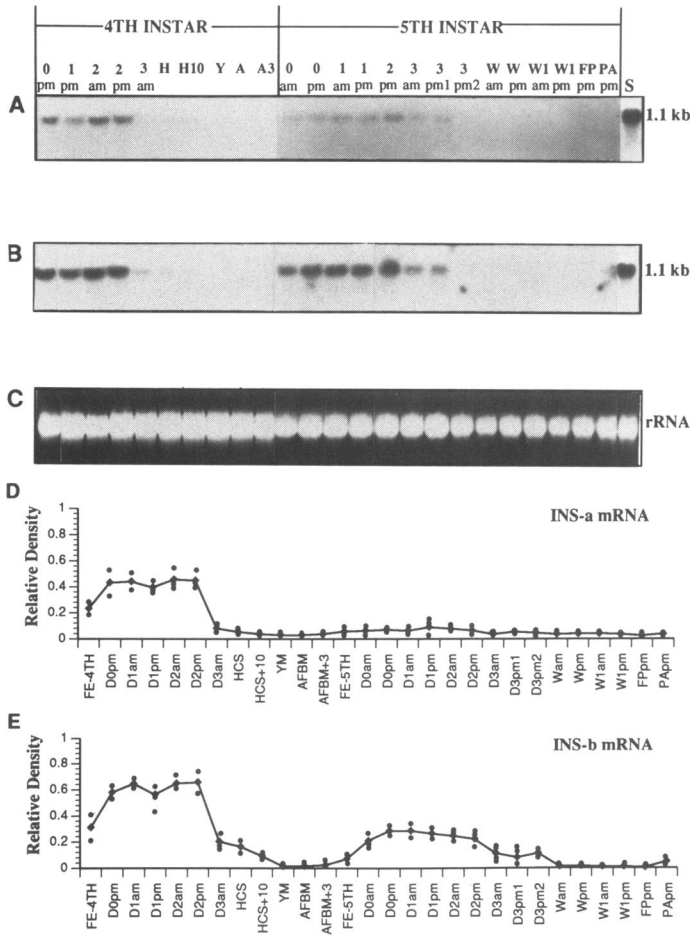

Figure 6. Differential expression of the INS-a and INS-b genes in the fat body of both 4th and 5th instar larvae, fresh pupae and pharate adults. Twenty micrograms of total fat body RNA were hybridized with either (**A**) INS-a-specific probe or (**B**) INS-b-specific probe and washed in the conditions as described in the Materials and Methods. (**C**) Ethidium bromide stain of total RNA. The size is indicated on the right side as determined from the RNA ladder (BRL). Abbreviations are as in Figure 5 plus Y, yellow mandible stage; A3, 3 hours after onset of air-filled head capsule (3 hours before ecdysis). (**D**) and (**E**) Quantitative analyses of differential developmental expression of the INS-a and INS-b genes as determined by dot-blot hybridization and densitometric scanning (see Materials and Methods). Each solid circle represents a mRNA sample from an individual animal and the curve crosses the average value (square) from different animals at the same stage.

that 1) both *ins-a* and *ins-b* are initially transcribed in the freshly hatched first instar larva; 2) fat body is another tissue expressing insecticyanin messengers with INS-b mRNA dominating; 3) *ins-a* and *ins-b* are differentially expressed in both epidermis and fat body during development of *Manduca sexta.*

Manduca eggs contain insecticyanin (23) which must come from maternal deposition during oogenesis since no mRNA was detected at any time during embryogenesis. Instead both insecticyanin mRNAs were first detectable at the time of hatching and increased during the first 24 hrs of feeding which is in contrast to the initial expression of the BBP gene in *Pieris brassicae* (12). The newly hatched first instar larva is nearly colorless and only becomes greenish-blue by 24 hrs (Li and Riddiford, unpublished). The latency between the re-appearance of the blue color and the initial accumulation of insecticyanin mRNAs indicates that either the newly-transcribed insecticyanin mRNAs are not translated or the newly-synthesized insecticyanin does not assemble into the holoprotein, possibly due to a lack of the chromophore, biliverdin IXg, as Kayser (26) demonstrated in the butterfly, *Pieris brassicae.*

The epidermis synthesizes and stores both INS-a and INS-b (1, 7–8, 13, 22) which are secreted into the larval cuticle (8). INS-b is also secreted into hemolymph (8). The present analyses of mRNAs extracted from this and other tissues not only confirmed that the larval epidermis is the major site for synthesis of both forms of insecticyanin but also revealed that the fat body of the 4th and 5th instar larvae express a low level of insecticyanin messengers with INS-b mRNA dominating. Insecticyanin has also been found in the pericardial cells (22), but they must obtain it from the hemolymph since they contained no insecticyanin mRNA. The fact that INS-b mRNA is predominant in the fat body is consistent with predominance of this form in the hemolymph (9). The lack of a blue color and the insecticyanin protein in the fat body (22) is probably due to its immediate secretion after synthesis as Chinzei *et al.* (27–28) recently reported for the bean bug, *Riptortus clavatus.*

Analyses of epidermal RNAs revealed that both the INS-a and INS-b genes were expressed during the feeding period, but the two mRNAs virtually disappeared during the molt period. This developmental pattern of changes of the levels of INS-a and INS-b mRNAs closely parallels that of synthesis of the two forms of this pigment protein (7–8) and likely accounts for the changes in the total epidermal insecticyanin over this time observed by Goodman *et al.* (23). Together, these analyses indicate that the differential expression of the two insecticyanin genes occur at the mRNA level. Such a differential expression pattern is necessary to maintain the normal physiological level of this camouflage pigment and is apparently regulated by two major insect hormones, juvenile hormone and ecdysteroids (Li *et al.*, in preparation).

Although BBP and insecticyanin are two closely-related lepidopteran members of the lipocalin family as mentioned above, their expression show distinct temporal and tissue specificity. During development, BBP gene is expressed in larvae, pupae and adults (12), while *ins-a* and *ins-b* are expressed only in the larvae. BBP are synthesized mainly by fat body and gonads (12), while insecticyanin genes are expressed mainly in larval epidermis and to a lesser degree in the fat body. These difference indicate that the BBP and insecticyanin genes have evolved independently. Moreover, BBP seems to have no camouflage function. The presence of both BBP and insecticyanin at high levels in the hemolymph suggest that the two proteins may have some common function which needs to be further elucidated.

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