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Ce supplément au volume 28 de SERICOLOGIA est consacré aux résumés des communications présentées à l'Atelier International sur la Biologie moléculaire et la Génétique moléculaire chez les Lépidoptères qui s'est tenu à Kolymbari en Crête, Grèce, du 5 au 10 septembre 1988.

Cet atelier a bénéficié du concours de l'Institut de Biologie moléculaire et de Biochimie d'Héraklion et de la Société Rohm et Hass Co.

Cette réunion était la première du genre. Les difficultés n'étaient donc pas minces. Son succès vient d'abord de la parfaite organisation et de la volonté d'échanges entre les participants. Nous devons cela au travail remarquable fourni par le Dr. Marian R. Goldsmith et le Dr. Fotis Kafatos qui ont su parfaitement préparer et animer les sessions qui regroupaient des équipes venant de treize pays.

Nul doute que cet atelier a été profitable à tous et a permis de faire le point complet sur les travaux en cours et de dégager des perspectives d'avenir très prometteuses.

Gérard CHAVANCY

This supplement to Sericologia Volume 28 is devoted to the abstracts of papers presented at the International Workshop on Molecular Biology and Molecular Genetics of Lepidoptera which was held in Kolymbari, Crete, Greece, from 5 to 10 September 1988.

This workshop was supported by the Institute for Molecular Biology and Biochemistry, Heraklion and by Rohn and Haas Co.

This meeting has been the first one of this kind. Difficulties were thus not slight. Its success is first due to the perfect organization and desire of exchange between participants. We are owing this to the remarkable work of Dr. Marian R. Goldsmith and Dr. Fotis Kafatos who have perfectly prepared and moved the sessions which gathered teams coming from thirteen different countries.

This workshop has undoubtedly been profitable to everyone; it has enabled to take full stock of the work under way and to draw out the prospects of a very promising future.

G. CHAVANCY

Introductory Remarks

Marian R. GOLDSMITH

The Lepidoptera workshop is the offspring of two conference lineages. One is the Silkworm Meeting, which was last held in 1979 under the leadership of Pat Gage at the Roche Institute in Nutley, New Jersey. As you know, silkworm biologists are spread throughout the world, so it has been important for us to come together periodically to exchange information, establish collaborations, and meet new colleagues. Although a couple of attempts were made in the intervening years to reconvene the silkworm meeting, it wasn't until Fotis Kafatos and I set up an ad hoc organizing committee with Bungo Sakaguchi, Yoshi Suzuki, and Jean-Claude Prudhomme about 3 years ago that we found the right catalyst to make things happen. As plans developed, it became obvious that the field was changing rapidly, so that if we restricted the meeting to the silkmoths a lot of important research would be left out. So we decided to organize a workshop around all the Lepidoptera. Our gathering today shows that this is indeed an idea whose time has come.

I think it is fair to say that the Lepidoptera Workshop also arose historically from an event that took place here at the Insect Molecular Biology, the first of its kind, which Fotis organized to precede the much larger International Conference on Molecular and Developmental Biology of Insects in Heraklion. The Kolymbari workshop was so successful that it gave birth to what has become known as the Fly Meeting, now held here every 2 years. The Fly Meeting evolved its own organizational structure: virtually all participants bring abstracts which the organizers arrange into logical sessions just before the meeting starts. In fact, the fourth Fly Meeting ended on Saturday, and nearly 100 people gave presentations.

Since this is the debut of the Lepidoptera Workshop, we decided to provide a framework for the sessions. We have organized it around a series of intermediate length talks given by invited or prearranged speakers. But because we wanted to preserve the immediacy, flexibility, and informality which have been so critical for the success of the Fly Meetings, we have also left time for contributed papers, waiting until our arrival here in Kolymbari to put together the final program. In addition, we decided to leave an open session on the last morning. We'd like to use this time for two purposes: first, to discuss topics that emerge during the course of the Workshop itself; and second, to give ourselves an opportunity to evaluate the status of research in the molecular genetics and molecular biology of Lepidoptera. For this, Adam Wilkins has graciously agreed to provide us with a perspective on the meeting from his point of view as an editor of Bioessays. This will be followed by brief presentations given by the session organizers. I hope that we can emerge from the meeting with a sense of direction, knowing where we have been, and where we'd like to be going in the next few years.

Now, I would like to make a dedication. In response to the Workshop invitation, Ernst Caspari wrote me some time ago that he was ill, but hoped to be well in time to attend the meeting. To my great sadness, I learned recently that Ernst died of cancer on August 11 at the age of 78. The Lepidoptera Workshop continues a scientific tradition that Ernst helped found. I think it is fitting to dedicate the meeting to him.

Ernst had wide-ranging interests, and his work touched on many areas, including biochemical genetics, the nature of the immune system, irradiation and chemical mutagenesis, and DNA transformation in lepidoptera, as well as cytoplasmic inheritance, and developmental and behavioral genetics in mice. He made an impact in these fields not only through his own research, but also by his publication of many review articles and books, and his editorships of the journals Genetics and Advances in Genetics . I would like to give you just a brief summary of some aspects of his work in lepidopteran genetics and biology which are relevant to our conference.

Ernst's earliest published work was in 1933 on the biochemical basis of an eyecolor mutation in, appropriately, the Mediterranean meal moth, Ephestia kuhniella (1). This was his dissertation research, which was carried out in the laboratory of Alfred Kuhn in Gottingen. This work, which included classic experiments on tissue transplantation and analysis of compounds in the ommochrome pathways, established that diffusible substances were involved in pigment formation, and provided part of the background for the emerging 1 gene: 1 enzyme hypothesis. Ernst continued working on and off in this area for over 25 years.

In 1952 Ernst and cobworkers published a brief series of experiments on the nature on insect immunity (2). They showed that repeated imunization of *cecropia and Citheronia regalis* larvae and pupae with phage, *E. coli*, and various other potentially antigenic substances failed to elicit an agglutination reaction characteristic of vertebrate antibodies. This work helped lay to rest the idea that insects acquire specific immunity like vertebrates, lending indirect support to proponents of mechanism of humoral immunity involving the production of nonspecific bacteriocidal factors, which we now know to be true.

Kuhn had used wing scale development in *Ephestia* as a model for pattern formation, and in fact, this was the area in which Ernst first began his doctoral research in an unsuccessful series of experiments to document effects of a brief temperature shock on scale pigmentation. Thirty years later Ernst and coworkers returned to the *Ephestia* wing with its 1000 or so pigmented pattern scales as a highly sensitive system for detecting somatic genetic changes at the level of individual cells.

In 1965, in what is surely one of the earliest studies of DNA-induced transformation in a higher organism, Ernst and Saburo Nawa reported that they had injected mutant, unpigmented larvae with wildtype DNA (3). Indeed, they did detect a low but significant number of pigmented scales on the injected adults, demonstrating directly somatic transformation, but obtained equivocal evidence for inheritance of the induced genetic change. As you know, the problem of obtaining stable transformation remains a critical one in the lepidoptera, and I think it is significant that Ernst recognized its importance over twenty years ago.

In closing, I'd like to tell you about one other aspect of Ernst's life which I feel is an integral part of his role as a scientist. Those who had contact with him found him to be warm, generous, enthusiastic, full of ideas, and above all, encouraging to young people. I feel his loss especially keenly because I took my first college biology course from him as a freshman at the University of Rochester in 1960, which was also his freshman year. I remember well how extraordinary it was for us that Ernst, the Professor, would often drop into the lab and peer over our shoulders, asking with great curiosity and evident interest to know what we were doing or looking at. Years later I was astonished that he remembered me when I began attending Genetics Society meetings. Though he persistently and teasingly, I thought, tried to convince me to switch from *Bombyx* to *Ephestia* or *Galleria* when we would meet from time to time, he always engaged me in animated discussion of my work, and offered insightful and supportive comments. It is as much for Ernst's special qualities as a humanistic and caring scientist and mentor as for his contributions to various fields of genetics that I wish to dedicate the Lepidoptera Workshop to him.

Acknowledgement

These views are echoed in a biographical sketch by Eva Eicher which was published in Advances in Genetics last year (4). Her article provided the main source of historical information on Ernst for this dedication. The issue also includes a complete bibliography of his work.

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INSECT IMMUNITY

INSECT IMMUNITY AND CECROPINS

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Cell-free immunity in the *cecropia* moth and many other insects is based on three families of antibacterial proteins (cecropins, attacins and lysozyme) that are induced by a bacterial infection. The presentation will contain:

1) An introduction to the basic phenomenon of immunity in *cecropia*.

2) A review of the structure and function of cecropins A, B and D.

3) A review of cDNA cloning data that give the structure of the three preprocecropins and the organization of the gene for cecropin B deduced from genomic λ clones.

4) Recent data on the chemical synthesis (together with Andrew and Merrifield) and the enzymic processing of preprocecropins A and B and procecropins A and B as well as some truncated analogs.

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EXPRESSION OF *CECROPIA* IMMUNE PROTEINS IN A BACULOVIRUS VECTOR

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Insects possess an efficient humoral defense system against microorganisms. The final elimination of invading bacteria relies on the induction of three antibacterial proteins; lysozyme, attacin and cecropin, the latter two being unique to insects. Both the small cecropin ($M_r = 4000$) and the larger attacin ($M_r = 20\,000$) molecules do not contain any stable secondary structure in aqueous solution. Upon insertion into a bacterial membrane it was postulated that cecropin folds into an amphipathic, helical structure, thus destabilizing the membrane and causing lysis of the bacterial cell.

In order to study the requirements for an efficient synthesis and export of such potentially membrane disrupting proteins *in vivo*, they were cloned into the *Autographa californica* nuclear polyhedrosis virus system. The correct localization of the foreign gene in the viral genome as well as normal transcriptional levels were confirmed. The virus-mediated expression of cecropin and attacin, proteins originally isolated from *Hyalophora cecropia*, was studied in cells from the related lepidopteran *Spodoptera frugiperda*. Correct processing of preproattacin to proattacin and finally to attacin was inferred to take place based on the pattern obtained from Western blots.

Partially due to proteolysis the protein levels obtained were low, especially for cecropin. To overcome this and facilitate isolation we employed a baculovirus construct containing a fusion of a signal peptide to the ZZ antibody binding domain of protein A from *Staphylococcus aureus* in turn fused to DNA coding for the mature cecropin. A secreted fusion protein was isolated on an IgG-column and cleaved to produce active cecropin. From its electrophoretic mobility it was concluded to contain the C-terminal α-amide group, typical of all natural cecropins.

STRUCTURE AND ORGANISATION OF ATTACIN GENES

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Attacins are a group of antibacterial proteins (20 kD) found in *Hyalophora cecropia.* They are co-induced with a whole set of other proteins which are thought to be of absolute importance for the immune response of these insects. These proteins are synthesized and released into the hemolymph after injection of bacteria but can also be induced by injury. Apart from attacins other antibacterial proteins are lysozyme and cecropins.

Six forms of attacins have been purified. From amino acid and cDNA sequencing data it was deduced that only two types are gene encoded and these could then be enzymatically altered into new forms. One attacin group is basic and one more acidic. Both are active against Gram-negative bacteria.

The analyses of several genomic clones from *H. cecropia* reveal a close linkage between one acidic and two basic attacin genes, which are lying in tandem. The basic and the acidic attacin genes have an opposite direction of transcription and thus could share the controlling region. One of the basic attacin genes and the acidic gene have two introns at homologous positions.

The attacin genes have at least two similar types of octanucleotides, 25-175 bp upstream of the TATA-box. Interestingly enough, these sequences also have been found in the 5'-area of two cecropin genes. These nucleotide sequences are tentative promotor elements and may be the "immune-boxes" of these organisms.

ANTIBACTERIAL PROTEINS SYNTHESIZED BY HEMOCYTES OF *HYALOPHORA CECROPIA*

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Bacterial infections as well as injury induce cellular and humoral defense mechanisms in *Hyalophora cecropia*. Humoral factors are the so-called "immune proteins" P4, attacins, lysozyme, and cecropins (H. Boman & D. Hultmark, 1987). All except protein P4 possess antibacterial activity. One organ of synthesis is the fat body.

During studies with fat body *in vitro* cultures it became obvious that hemocytes are always attached to fat body. In addition some results indicate that hemocytes could influence the synthesis of the "immune proteins" (T. Trenczek and I. Faye, 1988). Therefore it was necessary to find out if the hemocytes themselves are able to synthesize the "immune proteins".

Hemocyte homogenates (western blotting) as well as individual cells (immunohistochemistry) were investigated. After activation *in vivo* with either saline or saline containing *Enterobacter cloacae* β12 and LPS of *E. coli* D21 respectively (a gift from H. Boman), all hemocyte preparations from larvae and pupae contained P4, attacins, lysozyme and cecropins. However, even in samples from untreated animals some hemocytes reacted positively to the appropriate antibody used. Differences in the reaction pattern were found between the different physiological stages. Finally, incorporation studies with ^{3}H-lysine in primary hemocyte culture proved the ability of the hemocytes to synthesize the "immune proteins".

To find out which hemocytes synthesize antibacterial proteins without external activation and why is under present investigation.

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A BACTERIA-INDUCED LECTIN OF *MANDUCA SEXTA* WHICH TRIGGERS COAGULATION

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A feeding-larva specific, bacteria-induced protein has been isolated from the hemolymph of *Manduca sexta*. Two related forms of the enzyme are now known to exist, both dimeric glycoproteins with molecular weights of approximately 72kd. These proteins have been designated M13-1 and M13-2. The addition of a mixture of the purified forms of the two proteins to the hemolymph of non-immune larvae triggers the coagulation response. The protein is a glucose-lectin, and it has been determined that the addition of glucose to the hemolymph blocks the ability of M13 to trigger the coagulation response. The injection of either glucose or anti-M13 rabbit antiserum delay wound-healing of the epidermis suggesting that the M13 participates in some aspect of this process. If M13 is required for wound-healing, and if its action is controlled *in vivo* by glucose concentration, it is obvious that the lowering of glucose would necessarily be localized in order to localize the response. A lowering of the glucose level in the hemolymph at large would of course facilitate a lowering of localized glucose levels. Data show that the level of glucose rapidly drops immediately following the injection of bacteria. It is obvious that this would aid the insect when under, for example, a sustained attack for a gut infection. M13 has thus far been detected only in the epidermis, midgut and hemolymph tissues. When epidermis is damaged, the M13 is rapidly converted to a smaller (66kd) form by partial hydrolysis suggesting a possible activation mechanism.

IMMUNE SUPPRESSION IN INSECT PARASITOID-HOST INTERACTION

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In a hymenopteran parasitoid *Venturia canescens* virus-like particles are found on the egg surface, which are responsible for the protection of the parasitoid against the encapsulation reaction of the host *Ephestia kühniella*. Some of the particle proteins are structurally and probably functionally related to a host protein (p42), which appears to play an important role in the insect immune system. The p42-protein is found in hemolymph and appears to accumulate in the basal lamina of the fat body. The protein is induced to higher levels of synthesis by a number of treatments, including wounding or osmotic disruption of the larva. The most drastic increase of protein synthesis is observed after bacterial infection. The p42-protein in *Ephestia* corresponds to the P4-protein in *Hyalophora cecropia*, a protein induced together with antibacterial proteins.

p42-protein appears to interfere with the encapsulation reaction. Several observations indicate that the protein inhibits coagulation of hemolymph proteins and aggregation of hemocytes to form a capsule. The molecular mechanism of this inhibition is not known. Any property of the p42-protein inhibiting encapsulation would explain the protective function of virus-like particles, provided that the corresponding proteins in the particle actually display a similar effect.

MOLECULAR BIOLOGY OF THE *CAMPOLETIS SONORENSIS* POLYDNAVIRUS

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Campoletis sonorensis virus (CsV, *Polydnaviridae*) is a segmented double stranded DNA virus which has an apparently symbiotic relationship with the parasitic wasp, *Campoletis sonorensis*. CsV replicates in the oviducts of the parasitic wasp and is injected into the wasp's host, *Heliothis virescens*, during oviposition. In the parasitized lepidopteran host, the virus has a dramatic effect on host physiology and viral gene products are believed to play an essential role in the survival of the parasitic wasp's egg and larva. By Northern hybridization analyses two major CsV mRNAs are detected in parasitized *H. virescens* larvae as early as 2 hours post parasitization. cDNA clones representing the two partially homologous CsV mRNAs of 1.6 kb and 1.0 kb show that the two genes are from two separate but closely related CsV genes (Blissard *et al.*, 1987, Virology, 160, 120-134). Nucleotide sequence analysis and comparisons of cloned viral genomic DNA and a cDNA clone demonstrate that the 1.6 kb mRNA is a spliced gene containing introns. By DNA sequence analysis conservation of splice junctions between the 1.0 kb and 1.6 kb mRNAs suggests that the 1.0 kb mRNA is also spliced. To study the proteins encoded by these closely related genes, the open reading frame from each of the related genes was cloned into a baculovirus expression vector. Secretion and glycosylation of these CsV proteins in infected lepidoperan cells (*Spodoptera frugiperda*) were examined. Expression of segment W in the oviducts of the female wasp was also examined. Segment W hybridized to at least 5 CsV mRNAs on Northern blots of poly A mRNA from *C. sonorensis* oviducts. To identify specific CsV mRNAs and map putative viral genes expressed in wasp oviduct tissues, segment W was used to screen a cDNA library of *C. sonorensis* oviduct mRNAs. Three W-positive cDNAs were used to identify CsV mRNAs by Northern blot analyses and to map the locations of three putative CsV genes on segment W.

Theilmann and Summers (1987, J. Virol., 61, 2589) report the presence of a class of repeat elements arrayed either singly or in tandem arrays with an average length of 540 base pairs. To study the role of these in CsV gene expression, clones of the SH DNAs B, H, M, and O^1 were used to probe Northern blots of poly(A)$^+$RNA isolated from *C. sonorensis* reproductive tissue and from parasitized *H. virescens* larvae. All four SH DNAs hybridized to viral transcripts. SH-H, M and O^1 hybridized to messages expressed in both hosts. SH-B and M hybridized to transcripts that were only detected in either *C. sonorensis* reproductive tissue or parasitized *H. virescens* larvae. These results suggest that some CsV genes are expressed in a host specific manner. Hybridization of the 540 bp repeat regions to Northern blots showed that they were all homologous to viral transcripts.

A cDNA clone of a mRNA that is transcribed from the 540 bp repeat region of SH-B was isolated from a λgt10 library and completely sequenced. The sequence data revealed that the 540 bp repeat element was contained within the open reading frame of this gene. These results indicate that transcription units including the 540 repeat elements represent a second gene family to be identified within the CsV genome.

NEUROPEPTIDES AND MOLECULAR NEUROBIOLOGY

STRUCTURE-FUNCTION RELATIONSHIP FOR ADIPOKINETIC HORMONE (AKH) IN *MANDUCA SEXTA*

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Peptides of the AKH family were tested in *M. sexta* for activation of fat body glycogen phosphorylase in larvae and compared with the activity of *Manduca* AKH. It was found that peptides between 8 and 10 amino acids in length (changes are C-terminal) can be highly active, the longer ones having slightly higher activity. The 2 Ser in positions 6 and 7 can be replaced with Pro and Asn, respectively, resulting in practically no change in activity. Replacement of Thr in position 3 with Asn results in only minor reduction of activity. An exchange of Leu by Val in position 2 reduces the activity only slightly. However, replacement of Thr in position 5 with Ser decreases the biological activity of the peptide about 10,000 fold. This could indicate that the receptor for AKH in *M. sexta* is much more specific than the receptor in *Locusta migratoria*, as in that animal only minor differences in the biological activity of different AKHs have been reported. All peptides tested so far had pGlu in position 1, Phe in position 4 and Trp in position 8, so their importance could not be evaluated.

Analogues have been synthesized and will be tested to increase our understanding of the importance of specific amino acids for the biological activity of the peptide.

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EXPRESSION AND REGULATION OF FMRFAMIDE GENE EXPRESSION IN *DROSOPHILA* AND *MANDUCA*

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We are interested in the mechanisms that underlie the precise expression of specific neuropeptides by individual neurons. We have isolated a *Drosophila* gene that encodes multiple and diverse neuropeptides that are related to FMRFamide (Schneider and Taghert, 1988, PNAS 85, 1993). A *Manduca* homolog gene can be detected: it appears to be very similar, although we have not yet cloned it. This talk will (i) summarize current information on the gene in *Drosophila* and *Manduca*, and (ii) introduce developmental studies of embryonic neurons in the *Manduca* gut that express these peptides only if and when they have migrated into a proper gut environment.

(i) The *Drosophila* FMRFamide gene is present in single copy and, in both larval and adult stages, is transcribed into a single mRNA species of ≈1.7 kB. Genomic sequencing and protection assays indicate that the gene is interrupted by at least one intron of ≈2.5 kb. Exon I is 255 bp long; exon II is 1351 and it encodes 15 FMRFamide-containing peptides as well as others. *In situ* hybridization of larvae and of adults reveals a stable pattern of signals in neurons of the brain and segmental ganglia. The strengths of the signals vary according to different neurons. We have made serum antibodies to three synthetic peptides based on the sequence of the cDNA; the three regions selected as epitopes are all distinct from the FMRFamide repeats. Each antiserum stains specific *Drosophila* neurons; with the exception of a small number of brain cells, there is virtually no overlap in the patterns of immunoreactivity. Together the three patterns equal the pattern of ≈100 neurons that are stained by antibodies to synthetic FMRFamide. Each antiserum also stains specific *Manduca* neurons; again, each pattern is different with the exception of a pair of medial neurosecretory neurons in the brain that each specifically stains. All immunoreactive neurons are also stained by anti-FMRFamide antibodies. Northern blot hybridization of *Manduca* mRNA with the *Drosophila* cDNA reveals a single 1.7 kB transcript.

(ii) During embryonic development in *Manduca*, the gut becomes innervated by a large population of neurons that are born and differentiate within it. We have studied one set, the Enteric Plexus cells: these ≈350 neurons emerge from the foregut epithelium as a placode, then undergo complex cell migration from foregut into midgut regions. Only neurons that reach the midgut express FMRFamide-like immunoreactivity. Such expression is only observed in neurons that have completed migration. Surgical interruption of migratory pathways in embryo culture strands many Plexus neurons in the foregut - these stranded neurons do not show any peptide expression. We are currently optimizing *in vitro* methods to culture dispersed neurons and gut cells in order to better understand the potentially inductive influence of the gut environment on neuropeptide expression by Plexus neurons.

THE MOLECULAR BIOLOGY OF ECLOSION HORMONE: FROM SYNTHESIS TO ACTION

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Eclosion hormone (EH) is a neurohormone whose primary actions are behavioral, acting on the insect CNS to trigger behaviors associated with ecdysis. Over the past few years significant advances have been made in studying the molecular biology of both EH synthesis and action on the CNS of *Manduca sexta*.

The complete 62 aa sequence of *Manduca* EH was recently determined (Marti *et al.*, 1987). Based on this sequence an oligonucleotide probe was synthesized and used to screen a *Manduca* genomic library. A genomic clone was isolated and identified as being the EH gene by sequencing a 997 bp fragment of the clone which hybridized with the probe. A portion of the translated sequence matches exactly with the known peptide sequence from residues 5 to 62. Following residue 62 is a termination codon; just upstream from amino acid 5 is a potential 3' splice sequence. This 997 bp probe hybridized to a single band in a genomic Southern indicating a single copy in the genome of *Manduca*. Northern blot analysis showed that the probe hybridizes to a 940 nucleotide RNA from the brain of developing adults. This RNA is present throughout adult development.

Insects are behaviorally responsive to EH only during brief periods at the end of each molt. These response "windows" are regulated by ecdysteroids and are correlated with molecular changes within the CNS. The action of EH is mediated through a biochemical cascade which begins with an elevation in intracellular cGMP which stimulates the phosphorylation of a pair of 54 kDal proteins, the EGPs. (The EGPs are membrane associated proteins but it is not known how their phosphorylation activates the EH target neurons.) For the larval and pupal stages, the response windows are associated with the transient presence of the EGPs (Morton and Truman, 1986). For example, for the pupal molt, the onset of behavioral responsiveness and the appearance of the EGPs both occur 8-10 hr prior to pupal ecdysis. A combination of *in vivo* and *in vitro* treatments showed that both require first the appearance and then the withdrawal of ecdysteroids (Morton and Truman, 1988). By injecting animals with 20-hydroxyecdysone at various times during the normal ecdysteroid decline at the end of the pupal molt, we identified a time after which steroid treatment was ineffective in preventing both of the above. This loss of steroid sensitivity was followed 3-5 hr later by the appearance of the EGPs and the onset of behavioral responsiveness. Studies with actinomycin D and cycloheximide showed that RNA synthesis was required for behavioral responsiveness to EH up to the time of the loss of steroid sensitivity, whereas protein synthesis was required for 5 additional hours, until the insects actually became responsive to EH. The EGPs responded to the latter in a similar way. These data support the hypothesis that the ecdysteroid decline "primes" the CNS to become responsive to EH through the *de novo* synthesis of the EGPs.

Marti T. *et al.* (1987) FEBS Letters, 219, 415-418.
Morton D.B., Truman J.W. (1986) Nature, 323, 264-267.
Morton D.B., Truman J.W. (1988) J. Neurosci., 8, 1338-1345.

ACTIVATION OF NEW GENES DURING THE DEVELOPMENTALLY PROGRAMMED DEATH OF THE INTERSEGMENTAL MUSCLES OF THE TOBACCO HAWKMOTH *MANDUCA SEXTA*.

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Metamorphosis in holometabolous insects provides an excellent model system for examining basic developmental processes such as programmed cell death. The intersegmental muscles (ISM) of *Manduca sexta* begin to atrophy late in adult development and then die quickly following eclosion. These changes in the muscle are caused by the naturally occurring decline in the haemolymph ecdysteroid titer. ISM degeneration is not the result of metabolic shut down, but rather a precisely coordinated event involving the *de novo* transcription and translation of a set of developmentally regulated genes. Muscles were labeled with ^{35}S-methionine, and the newly synthesized protein products separated by 2 dimensional polyacrylamide gel electrophoresis and visualized by film autoradiography. Coincident with the muscles' commitment to degenerate, the expression of several small proteins was greatly diminished, while approximately 8 new proteins appeared. These changes in expression reflect transcriptional rather than translational control, as demonstrated by examining the *in vitro* translation products from muscle mRNAs at different stages of development. Muscle degeneration could be prevented by treating animals with either 20-hydroxyecdysone or low levels of actinomycin D (0.5µg/animal). These treatments prevented the appearance of several of the "new"proteins. We have constructed a cDNA library from the condemned muscles and are in the process of isolating "cell death genes".

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EFFECT OF A BRAIN FACTOR ON SEX PHEROMONE BIOSYNTHESIS IN *PLUSIA CHALCITES* (LEPIDOPTERA: NOCTUIDAE)

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Lepidoptera sex pheromones are mainly C_{12}-C_{16} compounds; their structural diversity is indicated by differences in the number of carbon atoms, position of unsaturation, and functional groups. The proposed reactions involved in the sex pheromone biosynthesis of these insects are related to those involved in the metabolism of fatty acids and include: desaturation, chain shortening (β oxidation), reduction and subsequent oxidation and acetylation. Sex pheromones in Lepidoptera are synthesized by a specialized gland on the ovipositor of the female in a circadian rhythm. Recently it has been reported that sex pheromone biosynthesis in various moths is controlled by a brain factor, suggesting a neuronal or neuroendocrine control of this process. In this study we describe: (1) the presence of a pheromone biosynthesis factor in the head ganglia (brain) of the noctuid *Plusia chalcites*, (2) some of its biological characteristics, and (3) its effect on the sex pheromone biosynthetic pathway in the pheromone gland. Experiments were performed using adult virgin females ligated between the head and the thorax. The ligated females were injected with head ganglia extracts. The pheromone glands were extracted and analyzed for their main pheromone components (Z7-12:Ac, Z9-14:Ac) and putative biosynthetic precursor (Z11-16:Acyl, Z9-14:Acyl, Z7-12:Acyl) content, after base methanolysis, by means of capillary gas chromatography (cGC). An internal standard (10-11:Ac), added to all samples, served for quantitation. Comparison of pheromone components and precursor levels in presence and absence of the brain factor was determined using two controls: (1) untreated females which served for determination of normal gland content, and (2) ligated females which served for determination of basal pheromone content in the absence of the brain factor. The above experiments revealed that a brain factor is involved in the regulation of pheromone biosynthesis in the moth *Plusia chalcites*, and lack of such a factor resulted in a depletion of the moths' main sex pheromone components (Z7-12:Ac, Z9-14:Ac) as well as all of their putative biosynthetic precursors (Z7-12:Acyl, Z9-14:Acyl, Z11-16:Acyl). Injection of head ganglia extracts resulted in a complete recovery of pheromone and precursor content. The Z11 desaturation step is the first one affected by the brain factor. However, the possibility that the other biosynthetic steps (e.g. chain shortening and reduction) are also affected cannot be excluded at this point. Both male and female brains contain a sex pheromone regulatory factor. The stimulatory pattern of the factor from the two sexes was different. In both the level of activity changed with age. Presence of maximal activity in female brain extracts of 4.5-5 day old moths correlates well with the developmental pattern of *Plusia chalcites* sexual maturity, and indicates a close link between the two. The differences in the activity of the male brain factor hint at the possibility that it may be quantitatively and/or qualitatively different from that of the female.

MOLECULAR ONTOGENY OF A SENSORY SYSTEM: THE EXPRESSION OF PHEROMONE BINDING PROTEINS, PHEROMONE DEGRADING ENZYMES AND PHEROMONE RECEPTOR PROTEINS DURING ANTENNAL DEVELOPMENT IN MOTHS.

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Three different proteins have been identified which are associated with the pheromone sensitive sensilla. Pheromone binding proteins (PBPs, 15-16 kDa, soluble, extracellular) have been identified in *Antheraea polyphemus* and *Hyalophora cecropia* (Saturniidae), *Lymantria dispar* (Lymantriidae) and *Manduca sexta* (Sphingidae), on the basis of pheromone binding and amino acid sequence conservation. N-terminal sequences were obtained for the PBPs of each of these species. A full length sequence has recently been derived for the PBP of *M. sexta* via cDNA cloning by T. Gyorgi and M. Lerner. PBPs have been tentatively identified in *A. pernyi* (Saturniidae), *Orgya pseudosugata* (Lymantriidae) and *Heliothis virescens* (Noctuidae) on the basis of common antigenicity and/or tissue specificity.

Pheromone degrading enzymes (soluble, extracellular, varying in size and homology) have been isolated and characterized from both *A. polyphemus* and *M. sexta*, the latter by R. Rybczynski and M. Lerner. Both enzymes degrade their respective substrate pheromones *in situ* at estimated half-life rates of 1-10 msec.

A putative pheromone receptor protein has been identified in *A. polyphemus* (R. Vogt, G. Prestwich & L. Riddiford). This 60kDa protein was shown to bind pheromone in a specific manner, and to be uniquely associated with the membrane of the sensory dendrite. The sensory dendrite membranes of *L. dispar* pheromone-sensitive sensilla contain a limited number of proteins, the major ones being a pair at ca. 60kDa and a trio around 28 kDa on SDS polyacrylamide gels.

Regardless of the length of time of adult development, all of these proteins have been seen to be initially expressed within 2-3 days of adult eclosion. These observations include the PBPs of *L. dispar* and *A. polyphemus* (R. Vogt and G. Prestwich) and *M. sexta* (R. Rybczynski and M. Lerner), and the sensory dendrite-membrane associated proteins of *L. dispar* (R. Vogt and M. Lerner). The PBPs are present at very high concentrations (10-20 mM), and are expressed at a continued high rate well into the adult stage. However, the absolute concentration of PBP plateaus early, suggesting an equally high steady-state of turnover.

The coordinated expression of the pheromone sensilla proteins suggests a final maturation of this sensory system that is hormonally coordinated. We are currently investigating the role ecdysone might have in this coordination.

AMINO ACID SEQUENCE VARIATION IN PHEROMONE BINDING PROTEINS OF MOTHS CORRELATES WITH PHEROMONE STRUCTURE

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N-terminal sequences have been obtained for 5 pheromone binding proteins (PBPs) belonging to 4 moth species. PBPs are 15-16 kDa proteins which are water soluble and extracellular. They are located in the lumen of pheromone sensitive sensilla, and are presumably synthesized and secreted by the tormogen and trichogen accessory cells situated at the base of each sensillum. Studies have suggested that PBPs function to solubilize pheromone molecules within the sensilla, allowing pheromone mobility to interact with membrane associated receptor proteins and inactivating enzymes.

Sequence comparisons will be presented of ca. 20% (30 of ca. 140 amino acids) of the respective PBPs of *Lymantria dispar* (gypsy moth, Lymantriidae), *Antheraea polyphemus* and *Hyalophora cecropia* (Saturniidae) and *Manduca sexta* (Sphingidae). Only *L. dispar* possesses more than one PBP. The *M. sexta* sequence is part of a full length sequence obtained through cDNA cloning by T. Gyorgi and M. Lerner. The other proteins were blotted onto glass fiber filters or immobilon, and subsequently sequenced by a local facility. All PBPs share a high degree of sequence conservation, indicating that they are all homologous. With one exception, the PBPs range between 50% to 67% identity. The exception is one of the *L. dispar* PBPs, PBP_2. PBP_2 and PBP_1 share only 50% of their amino acids, and the identity between PBP_2 and the other sequences ranges downward to 30%. Most of the amino acid replacements would be considered conservative. Nevertheless, there is considerable variation in the sequences of these proteins.

Binding studies suggest that the *L. dispar* pheromone disparlure binds preferentially to PBP_2, not PBP_1. The pheromone of *L. dispar* is an epoxide, 7,8-epoxy-2-methylocatadecane, quite different from the pheromones of *A. polyphemus* ((*E,Z*)-6,11-hexadecadienyl and the corresponding aldehyde) or *M. sexta* ((*E,Z*)-10,12-hexadecadienal). The pheromone of *H. cecropia* is not identified, but presumably is related structurally to the *A. polyphemus* pheromone. Among this group of species, the most different PBP binds the most different pheromone.

These studies suggest two points. First, variation in PBPs of different species is constrained to conservative changes in order to maintain the colligative properties of the protein in the sensillum lymph. Second, the high degree of sequence variation is driven by ligand structure, with similar structured pheromone molecules forcing selection of PBPs of similar sequence. These points focus attention on the role of PBP_1 of *L. dispar*. If it does not bind disparlure, what does it do? Perhaps PBP_1 is present to interact with an *L. dispar* pheromone that has not yet been identified but that is structurally more like the sphingid or saturniid pheromones.

CHARACTERIZATION AND cDNA CLONING OF THE PHEROMONE BINDING PROTEIN FROM THE TOBACCO HORNWORM, *MANDUCA SEXTA*: A TISSUE SPECIFIC, DEVELOPMENTALLY REGULATED PROTEIN

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The pheromone binding protein (PBP), the major soluble protein in olfactory sensilla of male moths, and its cDNA have been characterized from the tobacco hornworm, *Manduca sexta*. Rabbit PBP polyclonal antisera and synthetic oligonucleotides designed from N-terminal amino acid sequence information for purified protein were used as probes to screen a cDNA library constructed in lambda gt11 by using polyA$^+$-RNA from adult *M. sexta* antennae. The isolated overlapping cDNA clones revealed an open reading frame of 504 nucleotides encoding 168 amino acids. Confirmation of the identity of the cDNA clones was obtained by matching the N-terminal amino acid sequence of the purified protein with that derived from the nucleotide code of the cDNA.

All isolated and characterized cDNA clones hybridized to an antenna specific mRNA of approximately 1,400 nucleotide length. Both PBP and its mRNA were detected in female *M. sexta* antennae, although they were less abundant than in male ones. A study of the developmental time course of PBP and the mRNA encoding it reveals that in both sexes they are synthesized just prior to eclosion, and that the percentage of male antennal mRNA encoding PBP shifts from zero to about 20% at that time.

No amino acid sequence homology was observed between the PBP and the members of the binding protein family that, among others, includes the vertebrate odorant binding protein and insecticyanin, a blue biliprotein from *M. sexta*.

GENES, PROTEINS, AND EVOLUTION

DEVELOPMENTAL AND EVOLUTIONARY STUDIES OF MOTH CHORIOGENESIS

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Lamellae constitute the major substructure of the Lepidopteran chorion. We and others have examined lamellogenesis in four moths within the super family Bombycoidea. Lamellogenesis occurs in four temporally distinct modes, the general features of which are identical for the first three modes in all four species. These are lamellar framework formation (an early period event) and lamellar expansion and densification (middle and late period events). The fourth mode, that is species-specific, is the sculpting of the chorion's outer surface. In *polyphemus*, surface structures called aeropyle crowns (AC) form a banded region on the chorion's surface, while in *pernyi*, AC are distributed over the entire surface. AC assemble from a few additional, very late period lamellae that are molded by the "filler" substructure. AC fail to form in *Bombyx* due, at least in part, to insufficient filler. Failure to form AC in *cecropia* is due both to insufficient filler and to the lack of very late lamellae.

We are attempting to understand the molecular basis of these evolutionalry changes in AC formation by comparing very late period events in *polyphemus* and *cecropia*. Filler-forming genes (called E1 and E2) have been isolated and characterized in both species. We find that E genes in *cecropia* are expressed at a level that is only 2-3% of that in *polyphemus*, while timing of expression remains unchanged. There are also spatial differences. In *polyphemus*, high levels of E gene expression occur only in the region producing AC. In the other region, called flat, levels are comparable to that in *cecropia*. These species-specific differences do not result from different numbers of E genes. We also know that E1 and E2 genes are paired in both species, although some features of E gene organization have diverged.

An AC-specific lamellar sequence from *polyphemus*, called "16", has been cloned recently. 16 belongs to the "B" family of lamellar sequences and is closely paired with an AC-specific "A" family member. The 16/A gene pair is flanked by other B:A gene pairs that are not AC specific. Through sequence analysis, we have identified a conserved motif in the immediate 5' flanking region of the 16 gene, the AC-specific A gene, and the E1 and E2 genes; this motif is missing from non-region-specific genes. It may play a role in regionalized expression. In *cecropia*, the gene most similar in sequence to 16 is expressed at a significantly earlier time than in *polyphemus*. Thus, unlike E genes, the AC lamellar genes in *polyphemus* have evolved by a change in timing of expression.

We have recently started examining choriogenesis in species of other superfamilies. Choriogenesis in *Manduca sexta* (superfamily Sphingoidea) differs in two major ways from *cecropia*: 1) framework formation occurs throughout lamellogenesis rather than only during the early period, and 2) lamellar expansion does not occur. A model that accounts for these differences will be tested by comparing nucleic acid sequences from *Manduca* and *polyphemus*. Interesting morphogenetic differences in other superfamilies have been observed and will permit continued molecular developmental and evolutionary analysis of choriogenesis.

ORGANIZATION AND EVOLUTION OF THE CHORION GENE FAMILIES OF *BOMBYX MORI*

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The genes that encode the chorion proteins of *Bombyx mori* are clustered within a small segment of chromosome 2. We have cloned approximately 550 kilobases of DNA from this locus. Genes are clustered in the locus according to their time of expression during choriogenesis. Within a temporal cluster, genes are arranged in divergently transcribed gene pairs; the two members of each pair are members of different gene superfamilies (α and β). Nuclease sensitivity and *in situ* hybridization experiments suggest that virtually all genes are active and are probably expressed in all cells of the follicle monolayer.

In the developmentally "late" chorion region, 15 gene pairs have been identified. DNA:DNA hybridization and extensive sequence analysis show a patchwork distribution of sequence variants indicating that extensive homogenization has resulted from a mechanism similar to gene conversion. The gene conversion-like events are not uniformly distributed along the gene pair but are highest near the 3' ends in regions of simple-sequence DNA encoding the carboxyl-terminal end of the late chorion (High-cysteine, Hc) proteins.

In the developmentally "early" chorion region, 5 gene pairs have been isolated and sequenced. The α member of each pair shows evidence of extensive homogenization by gene conversion. However, the β genes that are paired with this family are not undergoing this process. Conversion events in the three gene families showing homogenization (2 late, 1 early) correlate with the presence of repeats of the DNA sequence GGXGGX which encode Gly-Gly residues in the chorion proteins; This sequence has similarities to recombination "hotspots" detected in other multigene families. GGXGGX sequences are also found in the middle chroion gene families. The chorion genes are an intriguing example of how particular features of a gene's DNA sequence can have important evolutionary consequences independent of its coding capacity.

CUTICULAR PROTEINS

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About half of the protein content of a mature cuticle can be extracted with strong denaturing agents. This soluble fraction has many distinct proteins. Virtually nothing is known about the insoluble fraction. Nor do we know how proteins and chitin interact to produce cuticles with diverse properties.

Individual proteins have been purified in other laboratories by column chromatography and in my laboratory by blotting 2D gels onto glass fiber filters. Computer assisted comparisons of partial and complete sequences obtained from purified proteins and from cDNAs and genes for cuticular proteins have revealed three distinct cuticular protein families.

Class 1. Proteins from flexible cuticles of two orders. *Drosophila* (Fristrom, Snyder), *Sarcophaga* (Lipke), *Manduca* (Riddiford), *Hyalophora* (Willis).

Class 2. Proteins from the hard cuticles of *Locusta* (Andersen). These proteins are high in alanine, and have some similarity to moth chorion and cockroach oothecal proteins.

Class 3. Proteins from rigid cuticles of *Hyalophora* (Willis). These are high in serine and their amino termini resemble those from certain neurofilaments. The neurofilament headpieces are essential for protein alignment.

Our finding (Cox and Willis, 1985. Insect biochem., 15, 349) that different anatomical regions of the integument yield different soluble cuticular proteins might indicate, as we have postulated, that different groups of genes are used to build cuticles with different physical properties. Some critics have speculated that the regional differences in soluble proteins reflect solely differential sclerotization. Translations of RNAs carried out in collaboration with Lynetta Binger were used to learn what messages were present in the epidermis of different anatomical regions and metamorphic stages. We could match translation products run on 2D gels with cold cuticular protein standards when rabbit reticulocyte products were processed with canine microsomes. Products from wheat germ translations underwent spontaneous processing. In all cases, translation products which matched major cuticular proteins came only with RNAs taken from the appropriate anatomical region. Hence differential gene action and not differential sclerotization appears to underly the spatial distribution of cuticular proteins. While we do not understand why the abundant soluble cuticular proteins are minor translation products, some of the translation products may represent the proteins which are rendered unextractable either through sclerotization or bonding to chitin.

CUTICULAR TANNING REACTIONS IN *MANDUCA SEXTA*

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Insect cuticle is tanned in part by highly reactive quinonoid metabolites that cross-link protein and chitin polymers in the presumptive exocuticle. These sclerotizing agents are the products of phenoloxidase-catalyzed oxidations of N-acylated catecholamines. We have focused our investigation on tanning reactions that occur in the pupal cuticle of the tobacco hornworm, *Manduca sexta*. N-β-Alanyldopamine (NBAD), NBAD-3-0-glucoside and N-β-alanylnorepinephrine (NBANE) are the major diphenolic compounds detected in hemolymph or cuticle during sclerotization. Whereas NBAD occurs in both hemolymph and cuticle, NBANE and NBAD glucoside are present in cuticle and hemolymph, respectively. Three phenoloxidases have been partially purified from the integument of pharate pupal *M. sexta*. Two are low and high molecular weight soluble tyrosinases capable of hydroxylating tyrosine at a low rate and oxidising *o*-diphenols to *o*-quinones at a much higher rate. The third enzyme is an insoluble laccase-like oxidase that was solubilized from the cuticle by trypsin treatment. It oxidizes both ortho- and para-diphenols. The formation of *o*-benzoquinones and subsequent products either by chemical oxidation with periodate or enzymatic oxidation with phenoloxidases was monitored by liquid chromatography with electrochemical detection (LCEC) using ascorbic acid as a quinone reducing agent and benzenesulfinic acid as a nucleophilic quinone trap. Similar products were obtained from both the chemical and enzymatic oxidations. The formation of NBANE indicated that tautomerization of the *o*-quinone to a *p*-quinone methide occurs followed by addition of water to electrophilic sites. The data support a tanning mechanism in insect cuticle involving *o*-benzoquinones and *p*-quinone methides that form covalent bonds via ring or side chain carbons, respectively, with nucleophilic groups from cuticular biopolymers.

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IS *LOCUSTA MIGRATORIA* AN HONORARY LEPIDOPTERAN?

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Whereas *Drosophila melanogaster* is now the molecularly best known metazoan, and enough molecular biology has been done with Lepidoptera to support a meeting, our laboratory is almost unique in undertaking to study genes of an orthopteran. The genome of *L. migratoria*, although smaller than those of some other acridiids, is twice the size of the human genome, 12 times those of several Lepidoptera and 40 times that of *Drosophila*. 5-Methylcytosine accounts for 1.5% of the total cytosine. An *Alu*-like 195-bp repeat (Lm-1 element) occurs close to, and in introns of, some structural genes, although the total repetitive DNA content, estimated at 30%, is not exceptional. The mitochondrial genome is 15.2 kb and has a gene order like that of *Drosophila*. rDNA occurs in 4000 copies, which are reduced by one-third in polyploid mature female fat body. Sequences coding for a 10kD hemolymph protein, a probable chorion protein, and a putative microvitellogenin have been cloned but contain long introns and are not yet fully mapped. Extensive intra- and intergenic DNA of no known function may account for the large genome and exempt the locust from the molecular fellowship of the lepidoptera. Two vitellogenin (Vg) genes, and the "microvitellogenin", are expressed coordinately under JH stimulation. The Vg genes belong to a super family with those of other insects (except *Drosophila* and its relatives), invertebrates and vertebrates. In the Vg 5'-flanking DNA, two sequences have been identified which also occur upstream from JH regulated cockroach oothecin genes, and are putative JH-response elements. In the hope of developing a gene expression assay system, DNA has been injected into early locust embryos, and transient expression of phspCAT has been obtained.

THE APOLIPOPHORIN-III GENE FROM *MANDUCA SEXTA*

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The sequence of the gene for an insect apolipoprotein, apolipophorin-III (apoLp-III), from the tobacco hornworm, *Manduca sexta*, has been determined. The gene contains four exons: exon 1 contains a relatively short 5' untranslated region and the first 15 amino acids of the signal sequence; exon 2 contains the remaining 3 amino acids of the signal sequence, the 5 amino acids of the pro sequence and residues 1-64 of the mature protein; exon 3 codes for residues 65-118 of the mature protein; and exon 4 codes for residues 119-165 of the protein plus the 3' untranslated sequences. The apoLp-III gene differs from most vertebrate apolipoprotein genes in lacking an exon in the 5' untranslated region and by the fact that the coding sequences are contained in 3, not 2, exons.

Using the progressive alignment algorithm of Feng and Doolittle (J. Mol. Evol., 25, 817-842, 1987), it was shown that the coding sequences in apoLp-III exon 2 are related to the coding sequences of the first coding exon of all vertebrate apolipoproteins. Based on this alignment an evolutionary tree was constructed which suggested that these exons were derived from a common ancestor. ApoLp-III exons 2, 3 and 4 were shown to be related to the last coding exon of the vertebrate apolipoproteins A-II, C-I, C-II, and C-III and the evolutionary tree suggests that the current genes were derived from an ancestor most related to exon 2 of apoLp-III.

It was shown that the apoLp-III gene is expressed in a cyclic manner during larval development with maximum expression occurring during the feeding period, and that adult fat body contains 2-10 fold higher levels of the apoLp-III mRNA. Restriction mapping, cDNA sequencing, and primer extension analysis all indicated that both larval and adult apoLp-III have the same mRNA transcripts and utilize the same coding sequences of a single apoLp-III gene.

From the genomic library we isolated a clone containing a 571 bp insert which appears to be a pseudogene for apoLp-III. The pseudogene corresponds to the 5' 546 bp of the apoLp-III mRNA (731 bp) and is 85% identical to the mRNA in nucleic acid sequence. The pseudogene contains a polyA tail and extends over two of the three intron/exon splice sites, which have been properly spliced. The pseudogene is not expressed in any life stage of the insect. We estimate the pseudogene entered the *Manduca sexta* genome approximately 20 million years ago.

VITELLOGENIC PROTEINS OF *MANDUCA SEXTA*

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Manduca sexta eggs contain several proteins that are taken up from the hemolymph during oogenesis. These include vitellogenin; a large (500 KDa) phosphoglycolipoprotein; lipophorin, the principal lipoprotein of the hemolymph; insecticyanin, a blue biliprotein; and microvitellogenin, a relatively small (26 KDa) female-specific protein. Microvitellogenin is equimolar with vitellogenin in the egg.

Microvitellogenins have been isolated from hemolymph and eggs and shown to be identical. Microvitellogenin is synthesized in fat body, but not in ovaries; mRNA was isolated from adult female fat body and cDNA was prepared and cloned in λ gt 11. A clone containing a nearly full length transcript was isolated and sequenced. The cDNA probe was used to demonstrate that the microvitellogenin gene is expressed only in females. Expression begins during the last larval instar. The probe was also used to screen a genomic library. A 3.6 Kb fragment containing the microvitellogenin gene was isolated and sequenced. The microvitellogenin gene contains a single 460 bp intron in the 5' non-coding region. Upstream of the intron are sequences with similarity to regions of the *Drosophila* YP genes and the locust vitellogenin genes. One such region, the H-box, has been postulated to be the site of ecdysteroid receptor binding, since it has homology to the mammalian steroid receptor-binding site. Indeed, it has now been shown that ecdysteroids appear to control the synthesis of microvitellogenin.

Microvitellogenin shows a high degree of homology to the non-sex limited 30 KD proteins of *Bombyx mori*.

GENE ORGANIZATION AND DEVELOPMENTAL GENETICS

THE GR^B DELETION OF THE CHORION LOCUS OF *BOMBYX MORI*: LOCALIZATION OF THE LEFT BREAKPOINT AND ISOLATION OF THE DELETION JUNCTION

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In the silkmoth *Bombyx mori*, choriogenesis occurs through the developmentally controlled deposition of several related classes of chorion proteins onto the oocyte by surrounding follicular cells. In the Gr^B mutant strain a distinctive family of proteins (Hc) normally expressed late in choriogenesis, as well as several proteins of middle development specificity, are missing due to the deletion of the corresponding genes from the chorion locus. In addition, a smaller set of proteins normally confined to mid-choriogenesis is found to be prolonged in expression in homozygous mutant but not heterozygous individuals. To elucidate the molecular organization of the chorion locus in the Gr^B genotype, we scanned a part of the wild type locus represented by a chromosomal walk of 270 kb through library screening and genomic DNA hybridizations using a series of probes representing low abundance sequences. A chromosomal clone, GrB4, whose sequences showed the expected characteristics of the deletion junction, was isolated from a partial EcoRI library of mutant genomic DNA. Through comparative hybridizations, mapping and sequencing, the precise location of one of the deletion breakpoints was also identified on one of the clones mapping in the characterized part of the wild type locus. Attempts to locate the other breakpoint in wild type DNA and to extend the structural characterization past the deletion junction through chromosomal walking were unsuccessful, due to the apparent absence of these sequences from libraries of wild type and mutant genomic DNA, respectively. Hybridizations of the deletion region on clone GrB4 to cDNA derived from follicular RNA indicate that no gene sequences are directly interrupted by the deletion and reveal the presence of a gene sequence of unknown function one to five kilobases to the right of the deletion junction.

FUNCTIONAL ASPECTS OF THE CONSERVED 5' FLANKING REGION OF A TRANSCRIBED CHORION PSEUDOGENE

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We have characterized a chorion pseudogene which resides in the late part of the chorion locus of the silkmoth *Bombyx mori*. This pseudogene (pseudo Hcb 12/13) resembles a HcB gene and its prominent feature is the fact that it is transcribed into an apparently non-functional RNA. In the 5' flanking region of the pseudogene, a partial promoter element is retained. It extends for only 2/3 of the length of the consensus sequence for the common flanking region of the Hc gene pair and is 90% homologous to the consensus sequence. This indicates that the 5' flanking region of the pseudogene is sufficient for transcriptional activity of the downstream pseudogene sequence.

The remarkable sequence conservation of the 5' flanking region of the pseudogene relative to the pronounced drift in the body of the pseudogene suggests either a gene conversion event or that this region serves an important function which makes it indispensable for the locus. Such a function was suggested by the results of a homology search through the NHI nucleic acid database which revealed that the 5' flanking region of the pseudogene bears homology to sequence elements present in the nuclear matrix attachment site of the *Drosophila* Sgs-4 gene. In order to ascertain whether or not the pseudogene upstream sequences harbor a nuclear matrix binding site, we prepared nuclear matrices from silkmoth follicular cells which are actively expressing chorion genes and separated the matrix bound DNA fraction from the unbound fraction. We have found that the pseudogene fragment is present in the DNA fraction that binds to the nuclear matrix. To determine the region responsible for binding to the nuclear matrix, we carried out *in vitro* binding assays. Our results demonstrate that the 5' flanking region of the pseudogene has the highest binding potential when compared to the intron or the 2nd exon of the pseudogene.

However, in a parallel experiment we observed that the common 5' flanking region from an actively transcribed Hc gene pair is also capable of binding to the nuclear matrix *in vitro*. This implies that there may be more than one nuclear matrix binding site along the chorion locus. Currently, we are addressing this question through further experimentation.

ANALYSIS OF TWO SEX-LIMITED TRANSLOCATION STRAINS OF *BOMBYX MORI* THAT COVER CHORION STRUCTURAL GENES

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In *Bombyx mori*, chorion genes are organized in tandem arrays in several large DNA fragments that map into two clusters on chromosome 2. Cluster *Ch 1-2* includes a 270 kb walk that covers middle A and B and late Hc genes; *Ch 3* probably corresponds to the early locus of about 120 kb. To clarify further the organization and function of chorion genes, we have examined two sex-limited translocation strains, r06 and r07, in which the proximal end of chromosome 2 is attached to the W chromosome. Females carry dominant alleles of the larval pigmentation gene *p*, which maps to the tip of chromosome 2, on the translocated segment; both sexes carry recessive *p* genes on chromosome 2.

To determine whether the translocations covered any chorion genes, we carried out Southern blot restriction analysis of genomic DNAs isolated separately from males and females, probing them with a set of clones corresponding to several chorion gene families expressed at different developmental stages. These included the early gene families 5H4, 2E6, 6A2, and 6C11, the middle gene families L11 and L12, and the late gene families HcA and HcB. In the presence of wildtype second chromosomes carrying a full complement of chorion genes, r06 and r07 showed sex-specific differences only for the L11 probes, suggesting that the translocated fragments covered only one side of the 270 kb walk in *Ch 1-2*, terminating before the Hc array. To confirm this result in a more favorable genetic background, we examined DNA isolated from r06 and r07 in the presence of the homozygous *Gr-B* deletion on chromosome 2. This deletion lacks all Hc and many middle genes, and is probably confined to *Ch 1-2*. Blots probed with Hc-specific clones at high stringency showed identical hybridization patterns with DNA isolated from the homozygous deletion alone, consistent with the absence of Hc genes from the two translocations. Preliminary analysis of these DNAs with a probe derived from the central domain of B-L12 showed an extra band in females of both r06 and r07, again showing that the translocations include middle chorion genes.

We confirmed these results by examining chorion proteins produced by the translocations over the homozygous deletion. We were unable to detect any Hc proteins in two-dimensional gels, but found an apparently normal set of A and B sized proteins encoded by the translocated segment. r07 produced 5 more proteins than r06, suggesting that the r07 break covers a larger segment of the chorion locus. Moreover, the chorions of both strains were functionally and macroscopically normal, maintaining viable embryos during longterm chilling as well as HCl activation to break diapause. This contrasts with the homozygous deletion alone, which produces a very thin eggshell that rapidly dehydrates and is effectively female sterile.

We examined transverse chorion sections by SEM to see the effects of the various mutations on chorion ultrastructure. In wildtype backgrounds, r06 and r07 produced normal chorions with parallel lamellae and a compact outer crust. Homozygous *Gr-B* chorions lacked a distinct outer crust and showed extreme disorder except for an occasional thin surface region. Neither translocation produced a detectable outer crust in the deletion background, consistent with the results in *Gr-B*, and with the long held idea that this part of the chorion is formed by deposition of Hc proteins. By contrast, r07 produced apparently normal inner lamellae, fully rescuing this aspect of the mutation. Lamellae produced by r06 showed highly irregular stacking, however, suggesting that this translocation lacks or underproduces an element covered by the deletion and required for lamellar orientation.

ANALYSIS OF THE Gr^{16} CHORION MUTATION OF *BOMBYX MORI*

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Mutations at the *Gr* or grey egg locus of *Bombyx mori*, which maps near the proximal end of chromosome 2 (6.9 m.u.), produce chorions with abnormal ultrastructure and protein composition. We have been studying Gr^{16}, a spontaneous, dominant allele which is suspected to have arisen in wildtype strain Old European 16 (OE16), in an effort to determine the underlying basis of this mutation. The two-dimensional (2D) protein patterns of mature homozygous, heterozygous, and wildtype chorions were identical, confirming OE16 as the likely strain in which the Gr^{16} allele arose. These results showed that the mutation does not cause a major primary or secondary loss of chorion proteins, as is the case with Gr^{B} and Gr^{col}, respectively, nor does it carry significantly altered structural genes for the predominant chorion proteins.

Since it has been reported that the ultrastructural defect appears at the earliest stages of chorion deposition, we looked for differences in the labelling patterns of early chorion proteins, well before the accumulation of the major components. Although most components were identical, we detected two differentially labeled bands on isoelectric focusing (IF) gels after a 30 min. pulse with radioactive leucine or glycine (the latter abundant in chorion proteins) followed by a 10 min. chase. One band showed relatively high intensity in the homozygous mutant, the other in the wildtype. Both were present at approximately equal intensities in the heterozygote. On 2D gels each of these bands contained a pair of spots with slightly different molecular weights; their pKi's and similar sizes suggested that these sets of proteins might be closely related. To examine the kinetics of synthesis and accumulation of these proteins, we varied amino acid pulse and chase times. In a preliminary analysis of proteins labeled for 5 min. with no chase, the wildtype pattern remained unaltered. However, the mutant accumulated a small amount of material at both the wildtype and mutant-specific pKi's, consistent with the possibility that the mutation affects post-translational modification or stability of these gene products.

We also carried out restriction analysis of genomic DNA from the Gr^{16} homozygote, OE16, and the corresponding heterozygote. Southern blots probed with cloned DNAs corresponding to the early gene families 5H4, 2E6, 6A2, and 6C11 were identical in all three strains. This suggested that the defect in Gr^{16} does not involve a major physical change in early chorion structural genes or in neighboring DNA.

EXPRESSION OF CHORION GENE FAMILIES IN *BOMBYX MORI, BOMBYX MANDARINA* AND THEIR PROGENIES

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Insect eggshell chorions have attracted attention since these have a permeable structure permitting gas-exchange and sperm-entry while funtioning as a protector for the embryos against bacterial and water invasion. These apparently conflicting roles of insect chorions may be supported by their complex architecture resulting from the expression of multigene families, whose structures have been extensively studied by Kafatos' group. It is believed that the complex structures of the chorion genes have made possible the rapid evolution of the eggshells of insects, which have been adapted to the multiple environments on the earth. In this connection it is of interest whether or not any remarkable differences in expression of the chorion genes exist between the domestic silkworm *Bombyx mori* and its supposed ancestor *Bombyx mandarina*, as these are closely related genetically but differ in their behavior and habitation. In the present study, the eggshell architecture, protein constitution and a feature of gene divergence are compared between the two species (as well as their hybrid and progenies) by scanning electron microscopy, gel electrophoresis and Southern blotting analysis, respectively. We found that the morphology of the eggshell of *mandarina* was very specific and seemed to match the outdoor habitation of this species. The *mandarina* chorion had protein components which were scarcely detected in *mori*. Therefore, *mandarina* may have a distinct gene set at the chorion locus in comparison with the domestic silkworm. In fact, the patterns of the Southern blot hybridization analysis of the genomic DNA of *mandarina* differed from those of *Bombyx mori*. The results suggest that the overall structure of the chorion multigene families of *mandarina* is more complex than of *Bombyx mori*. The species-specific features found in the present study were transmitted and segregated clearly in the progenies of their hybrid. Further analysis of the *mandarina* genome is needed to draw conclusive statements in terms of molecular evolution.

GENETIC LINKAGE MAPPING AND INSECTICIDE RESISTANCE IN *HELIOTHIS VIRESCENS*

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The noctuid genus *Heliothis* contains agricultural pests of worldwide importance, in part because of its ability to evolve resistance to chemical insecticides. In addition to its economic importance, insecticide resistance offers the opportunity to study toxicological modes of action and genetic mechanisms for circumventing them. Our studies of resistance in a strain of *Heliothis virescens* (tobacco budworm) to the neurotoxic pyrethroid insecticides showed that variation at a single locus could account for much, but not all, of the resistance. Neurophysiological studies suggested that the major mechanism of resistance is nerve insensitivity to pyrethroid action, perhaps at the site of the sodium channel. Other resistance mechanisms are also present in the strain; however it proved difficult to isolate and characterize them because of the lack of a genetic map. Consequently we are now devoting much of our effort to develop a useful genetic linkage map. Although this approach would have been unthinkable in the past, the potential availability of hundreds of marker loci in the form of DNA restriction fragment length polymorphisms (RFLPs) makes this feasible.

We are using three types of marker loci: 1) enzyme loci, characterized by allozymic variation as revealed by starch gel electrophoresis and enzyme-specific staining: 2) RFLPs in protein-coding regions of the genome, characterized by probing Southern blots with clones from a cDNA library, and 3) RFLPs in randomly chosen single-copy regions of the genome, characterized by probing Southern blots with clones from a pUC8 library of genomic DNA. We have two strategies for mapping these loci using classical genetics: A) Intraspecific variation in marker types 1 and 3 is common, and linkage can be established by analyzing patterns of cosegregation in pedigrees. B) Interspecific variation in 1 and 3 between *H. virescens* and *H. subflexa* is common; with some difficulty the two species can be hybridized, and linkage established by analyzing patterns of cosegregation in backcrosses.

We have "mapped" ten polymorphic enzyme loci in a pyrethroid-susceptible strain by examining all their possible pairwise linkage relationships. Three are sex-linked (AcP, TPI, 6PGD). The seven mapped autosomal loci are mutually unlinked; if female meiosis is achiasmatic in *Heliothis*, then the seven loci mark seven distinct chromosomes (of the 31). Studies on linkage of 4 unmapped autosomal loci and screening for more enzyme polymorphisms are in progress. Crosses to our pyrethroid-resistant strain indicate that the major locus for resistance is linked to none of the ten mapped loci.

Screening of type 3 probes indicates that about 2% of them yield useful single-copy sequences associated with intraspecific RFLP variation. We are currently working out modes of inheritance and linkage to the enzyme markers. Screening of type 2 probes reveals little intraspecific variation, but distinct interspecific variation in about half of them. We continue to screen these in hybrids as we conduct our second round of backcrossing with *H. subflexa*.

Statistical considerations of the efficiency of saturating the linkage map using classical genetical approaches indicate that physical mapping techniques will be essential, especially in mapping the smaller chromosomes. We intend to attempt this by separating the smaller chromosomes by pulsed field gel electrophoresis and probing with marker types 2 and 3.

STRUCTURAL ANALYSIS OF CHROMOSOMAL DNA OF *BOMBYX MORI* WITH PULSED FIELD GEL ELECTROPHORESIS (PFGE)

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Pulsed field gel electrophoresis (PFGE), a technique for the separation of large DNA molecules, has been developed over the last few years. This new method allows restriction maps to be made, and DNA arrangement to be observed, in the size of 50kb to 10Mb. I have used this technique for two studies: (1) analysis of the polymorphism of the *Bombyx* rDNA units in several strains and tissues; (2) separation of chromosomal DNAs of *Bombyx mori*.

(1) The normal unit of *Bombyx* rDNA is about 10kb in length, although two other units interrupted by insertion sequences (IS) are distributed in the rDNA cluster at low frequency (1). The insertion frequencies of these pseudo-units varied in the several strains so far examined. We have analysed the insertion patterns of the sequences in several strains with PFGE. Because SalI sites are found only in the type II IS within the rDNA cluster, we can detect the insertion position by Southern hybridization with rDNA, after the total genome of *Bombyx* is cut with SalI and electrophoresed in PFGE. We found that the insertion patterns were different not only among the strains, but also between the tissues, testis and silkglands of the same larvae. The distributions of the type II insertion sequences in the silkglands were more homogeneous than in the testis. This finding indicates several possibilities, but it may be considered that homologous recombination during the polyploidization of silkglands make the insertion homogeneous.

(2) Gage reported that the haploid genome size of *Bombyx mori* is about 530Mb (2), indicating that the average size of the chromosome is about 19Mb. It is likely that we can separate some chromosomal DNAs of *Bombyx* from the ability of resolution of PFGE. For the separation of chromosomal DNA, it is necessary to change the pulse time to the order of an hour. But at 1-2 min. pulse time we can detect two close bands of about 1-2 Mb. The two bands were hybridized with rDNA and BMC, the repeated sequence specific to *Bombyx*, suggesting that these actually correspond to the chromosomal DNA of *B. mori*. However, they appear to be mitochondrial DNAs because about the same sized bands were also observed in a human sample. When the pulse time was altered to an hour, there seemed to be some smeared bands between 6 to 9 Mb. Most bands, however, were observed to be more than 10Mb, and in several groups. Also, such bands hybridized with BMC. When using the mosaic mutant which is believed to contain chromosome fragments, we observed a smaller band: but it is still unclear whether the band is true or artificial. I have tried to determine the appropriate conditions for good separation of these DNAs.

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GENE ACTION BEFORE EARLY DEVELOPMENT

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Maternal messenger RNAs are synthesized during oogenesis and stored in the oocytes to be activated later to support early development. A general rule seems to be that no new translation of the zygote genome occurs until after the early events of cleavage are complete. In many organisms, the maternal message is stored in an untranslated form until the egg is fertilized. Giant silkmoths fall into this pattern of development, while *Drosophila* deviates from this behavior because translation begins during oogenesis, and thus there is no baseline from which to study the activation of very early syntheses. For this and other structural reasons, we have chosen to approach the question of the synthesis, information content, and activation of maternal message in silkmoths. The experiments which led us to this choice will be summarized.

Our strategy for investigating the role of maternal message in development begins with the premise that messages which specify regional differences in the embryo might be localized by binding to the cytoskeleton of the oocyte. Since the cortical region of the egg is the only portion destined to be subdivided into cells, we have examined this region, and have found a substantial actin-based cytoskeleton. Preliminary hybridization studies using tritiated poly-uridine confirm the presence of polyadenylated message in this region.

We have constructed a cDNA library by reverse transcription of the total maternal mRNA in chorionated but unfertilized eggs. This library was screened against RNAs extracted from detergent-prepared cortical cytoskeleton, and against the remaining ooplasmic RNA. We discovered that many clones hybridized to both cytoskeleton and ooplasmic RNA fractions, but some were confined to only one fraction. We selected clones associated with the cytoskeleton only for further examination. The first clones examined were designated Ec 4b, Ec 20. Ec 4b consists of about 200 nucleotides, while Ec 20 appears to be an almost complete fragment of an allele. Both sequences contain an open reading frame, and Ec 20 contains a polyadenylation signal as well. An anti-sense riboprobe for Ec 4b hybridizes *in situ* to the cortex of the developing oocytes and to the mature, chorionated egg.

The nucleotide sequences of the two probes were similar to chorion proteins from *A. polyphemus*, and we found some hybridization to follicle cells in the developing follicles. To show that this hybridization was not the result of contaminating follicle-cell RNAs, we were able to hybridize to nurse cell cytoplasmic RNA using both *in situ* and dot blot hybridizations. The amino acid sequences deduced from these nucleotides indicated that Ec 4b is similar to mammalian cytokeratin. We speculate from the sequence data and from the global distribution of this clone in the egg cortex, that it may represent a cytoskeletal fibrous element necessary for cellularization of the blastoderm.

CLONING AND EXPRESSION OF A HOMEO-BOX CONTAINING GENE IN *BOMBYX MORI*

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We have cloned a genomic DNA associated with the *E* group mutants, homeotic mutants in *Bombyx mori*. A 180bp homeo-box like sequence having an approximate 82% nucleotide sequence homology with the *Drosophila Antp* homeo-box was found, and its deduced amino acid sequence was identical with that of *Antp*. The transcripts of this gene were detected from before organogenesis in the embryo through the hatching stage. A major transcript was 4.4kb, and a minor one of 6.0kb was also detected.

We also have cloned two kinds of cDNA from the cDNA library prepared from poly(A)$^+$RNA of the embryonic stage. One of the cDNA clones had a 2.2kb insert length, and the other had one of 3.2 kb. It seemed that these were the cDNA clones of the genomic clone from their restriction maps and other results.

The same kind of transcripts were also detected at the larval 4th molting stage. In the epidermis of the thorax 1, 2, 3 and abdominal 1, 2, 3, 4, 5, 6, 7 segments, the major transcript of 4.4kb was detected, but in the head and abdominal 8, 9 segments there were no transcripts detected.

We infer that this might be a homeotic gene in *Bombyx mori* corresponding to *Antp*.

GENE EXPRESSION

REGULATION OF EXPRESSION OF SILK ENCODING GENES IN *BOMBYX MORI*

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The silkglands of *Bombyx mori* consist of modified salivary glands specialized in the production of huge amounts of silk proteins. The posterior part synthetizes the main silk component which consists of two polypeptides - fibroin (370 KD) and P25 (25KD) linked by S-S bonds. This linkage appears to be essential for the correct secretion of this complex as demonstrated by genetic experiments (1).

The two corresponding genes have been cloned and extensively studied (2,3). Their transcription is higly coordinated during development and results in the accumulation of equimolar amounts of the two mRNAs during the animal feeding periods. At molting stages both genes are repressed and the mRNAs accumulated during the previous instar are rapidly destroyed. This defines two main levels of regulation -transcription and mRNA stability-. The detailed analysis of cis acting sequences upstream of the fibroin gene (4) provides a first clue for the understanding of the molecular mechanisms involved in the coordinated activation of these genes. Our current studies in this field will be described in another session of this meeting (see presentation of P. COUBLE.)

The middle part of the silkgland in which these polypeptides are stored until spinning synthesizes a mixture of serine-rich proteins known as sericins. Their function is to glue together the silk fibers of the cocoon. Two genes specifically expressed in the middle silkgland have been isolated (5,6). They code respectively for 4 and 2 sericin polypeptides through differential splicing of their primary transcripts.

The Ser 1 gene synthesizes mRNA of 10.5, 9.0, 4.0 and 2.8 KD which accumulate according to the same kinetics as fibroin/P25 in the posterior silkgland. By means of RNA blots and *in situ* hybridization, we investigated the variations of the distribution of these mRNAs during the last larval instar in different subterritories of the middle silkgland (7). The results show that the gene is only expressed in the 150 posterior cells of this region (250 cells) but that the splicing pathways vary depending on both the topology and the stage of development. The most posterior 42 cells perform successively three distinct maturation processes leading to a development dependant accumulation of successively the 4, the 10.5 and the 9 kb mRNAs. This shows the existence of a regulatory-mechanism at the level of mRNA maturation involving two switches in the splicing capacities of these cells during the fifth instar.

The Ser 2 gene encodes two mRNAs of 5.4 and 3.1 kb, also produced by differential splicing. At the beginning of the instar all the middle silkgland cells express the gene, but, as development proceeds, expression becomes limited to only the anterior cells, those which do not express the Ser 1 gene. Both mRNAs are simultaneously produced during development in the active cells.

The biological consequence of the topological and temporal regulation of these genes is a sequential layering of the different sericins around the silk thread.

Thus the different genes involved directly in silk production show a remarkable diversity in their regulation mechanisms at the cellular level. The nature of the physiological signals responsible, for their overall coordination remains largely unknown, even if a hormonal control can certainly be hypothesized, as will be discussed.

Prospects for future investigations using the new techniques of transgeny will also be discussed.

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HORMONAL CONTROL OF SILKGLAND DEVELOPMENT

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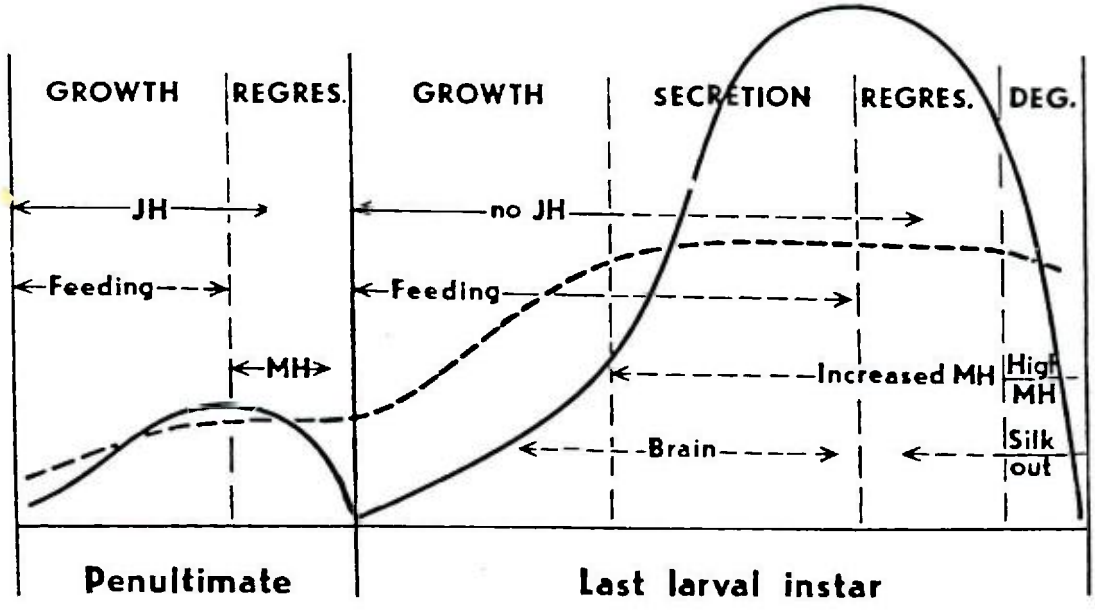
Development of silkglands depends on the supply of nutrients and is controlled by hormones. In early instars, juvenile hormone (JH) causes the glands to develop according to a larval pattern. Each molt-inducing increase of ecdysteroids to 200-400 ng 20-hydroxyecdysone (20E) equivalents per ml hemolymph elicits a profound functional regression of silkglands.

Metamorphic pattern of silkgland development, which is characterized by increased growth rate, higher silk production, and alterations in sericin composition, is established in the last larval instar at a low JH titre. Realization of the new pattern can be delayed or the pattern reverted to the larval one with exogenous JH. Sensivity of silkglands to JH declines with progression of the metamorphic development but some responses can be induced until the termination of DNA synthesis.

The function of silkglands in the last larval instar is stimulated with a brain neurohormone (experiments with *Galleria mellonella*) and with ecdysteroid increase to over 100 ng 20E equiv./ml hemolymph (*Bombyx mori*). This increase subsequently stimulates cocoon spinning and a regression of silkglands. Their degeneration at the end of the last larval instar is caused by the pupation-inducing surge of ecdysteroids (1000-1500 ng 20E equiv./ml). This response to high ecdysteroid titre is encoded in the silkglands within 48 hr of the last instar in *Bombyx* but much later in *Galleria*. Degeneration is accomplished only when silk accumulated in the gland lumen has been discharged or digested.

Control of silkgland growth (broken line, DNA content) and function (solid line, rate of proteosynthesis), is summarized in the chart below. Distinguishable phases in silkgland development are shown by inscriptions Growths, Regression, etc.



STRUCTURES AND EXPRESSION OF STORAGE PROTEIN GENES OF *BOMBYX MORI*

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In the silkworm *Bombyx mori* , storage proteins occur in two forms termed as "SP1" and "SP2", respectively. Both proteins are of molecular weights around 500,000, each composed of six identical subunits, but their amino acid compositions are markedly diffferent. SP1 is characterized by an exceptionally high content of methionine, while SP2 is classified as a lepidopterous arylphorin, being rich in phenylalanine and tyrosine. Several cDNA and genomic clones for the*B. mori* storage proteins were isolated and their structures were analyzed. The exon/intron compositions of two storage protein genes are remarkably similar to each other. Each gene spans over a 4kb region of chromosome and is composed of five exons interspersed with four introns. It is of special significance that the sizes of corresponding exons, except for those of the fifth exons, are identical, within 10 bp in length.

The initiation sites for gene transcription were identified at the nucleotide level. The TATA box-like sequence occurs some 30 bp upstream of the transcription initiation site of each structural gene. The sequence homologous to the SV40 enhancer core structure exists in two adjacent locations in the first intron of the SP1 gene, whereas the same sequence is found in the 5' flanking region of the SP2 gene. The structure highly homologous to the *Drosophila* sequence that confers the fat body specificity of gene expression is found in both storage protein genes. However, the sequence homologous to the putative ecdysteroid-receptor complex binding site found in some of the *Drosophila* genes exists in the SP1 gene but not in the SP2 gene.

The SP1 subunit is composed of 747 amino acid residues in which methionine comprises 11 mole%. These methionine residues are frequently found in consecutive or every other locations along the sequence. The SP2 subunit contains 704 amino-acids, and phenylalanine and tyrosine amount to over 17% of total residues. There are regions in SP2 where four tyrosine residues are clustered or phenylalanine and tyrosine occur in turns for five consecutive residues. Signal sequence for trans-membrane secretion is identified at the amino terminal domain of each storage protein.

Despite little homology in the nucleotide sequence between the two cDNAs, SP1 and SP2 exhibit nearly 30% homology with respect to the deduced amino acid sequences. Homologous residues appear to be rather concentrated at the terminal one-third of the molecules. It is noteworthy that six consecutive residues are identical in three locations of two storage proteins. These facts are highly suggestive that the genes for two storage proteins of *B. mori* might have evolved from a common ancestor.

Developmental changes in the levels of storage protein mRNAs and their precursors in the fat body well reflect those of the hemolymph concentrations of storage proteins, indicating that the biosyntheses of storage proteins in*B. mori* are regulated at the level of transcription. Cloned storage protein genes are efficiently and faithfully transcribed in a cell-free system derived fromcultured BmN cells. Studies are in progress in search for cellular factors responsible for the stage-and sex-specific transcription of storage protein genes.

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REGULATION OF LARVAL HEMOLYMPH PROTEIN GENE EXPRESSION IN *GALLERIA MELLONELLA*.

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Two separate larval fat body specific cDNA clones were isolated by differential screening of a day-5 last instar *Galleria mellonella* larval fat body cDNA library with day-5 and day-0 last instar larval poly(A)+RNA. Based on the synthetic patterns of larval hemolymph proteins and the sizes of the polypeptides synthesized in hybrid selected translation analysis, these cDNAs are tentatively designated as Lhp 76 and Lhp 82. These cDNAs, Lhp 76 and Lhp 82, hybridize in northern blots to 2200 and 2400 nucleotide long transcripts which are produced only in fat body cells. Developmental northern analysis showed that the transcripts complementary to Lhp 76 are present in 3rd, 4th, 5th, 6th and the last instar larvae and early pupae. On the other hand Lhp 82 complementary transcripts are present only in day-2 and older last instar larvae and in early pupae. Neither transcript was detectable in older pupae, pharate adults or adult moths. During the course of a supernumerary larval molt induced by cold shock or application of JH to day-0 last instar larvae Lhp 82 transcripts were not produced while Lhp 76 transcripts were produced only transiently. Based on these and other observations Lhp 82 is regarded as a last instar specific gene while the Lhp 76 is expressed in all larval stages. Howeve,r these transcripts are not detectable at the time of ecdysis.

Studies on the effect of 20-hydoxycdysone on intact or prothorax-ligated larvae as well as on isolated fat body suggest that this hormone causes a rapid decline in Lhp transcripts. Preliminary results on nuclear run off transcript analysis of isolated fat body nuclei from control and ecdysteroid-treated larvae suggest that this hormone represses transcription of the larval hemolymph protein genes. Similarly, studies on the effect of JH on intact and prothorax ligated penultimate and last instar larvae suggest that JH blocks activation of the Lhp 82 gene. Studies involving starvation of newly ecdysed last instar larvae for varying periods followed by feeding and/or ligation suggest that the nutritional state per se has no effect on Lhp gene expression. But starvation delays Lhp transcript production in these larvae by its effect on the neuroendocrine system of the insect.

Using these cDNA clones the corresponding genomic clones were isolated from a *Galleria* genomic library prepared in EMBL 3. Restriction maps of the clones AGm Lhp 76 and A Gm Lhp 82 have been prepared. The coding and the upstream genetic regions of Lhp 76 have been identified by S1 analysis and have been partially sequenced. Similar studies on Lhp 82 are in progress.

EXPRESSION OF JUVENILE HORMONE SENSITIVE GENES FOR LEPIDOPTERAN STORAGE PROTEINS

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During the last larval stadium of lepidopteran insects, the hemolymph concentration of what are believed to be storage proteins increases greatly. The question then arises "what regulates the hemolymph abundance of these proteins?" Any combination of the levels of transcription, translation, secretion and clearance could be responsible.

I have addressed this question using a model system in which the hemolymph abundance of several of these 'storage proteins' is sensitive to juvenile hormone. This model system is particularly useful since few metamorphosis-associated hemolymph proteins have been identified which are suppressible by JH. However, In *Trichoplusia ni* an acidic and two basic hemolymph proteins are each suppressible by JH (1).

The acidic protein (M.W. 76,000) appears on the second day of the final stadium, and appears on coomassie blue stained gels to be at a constant hemolymph concentration for the remainder of the stadium. Topical treatment of larvae with JH or a JH analog at ecdysis to the final stadium strongly suppresses the hemolymph abundance of the protein on Day 2, in a dose-dependent manner (2). The protein was purified, and a polyclonal antibody preparation against it was made (3). Messenger RNA was prepared from normal and JHA treated larvae by oligo d(T) chromotography. Direct visualization of the mRNA following urea/polyacrylamide gel electrophoresis showed that a predominant band was absent in the sample from treated larvae. A cDNA clone bank for mRNA from day 2 of the final larval stadium was prepared in a transcription vector (4). The bank was screened using a differential probe of end-labeled mRNA from normal *vs.* JHA treated larvae. Clones containing cDNA for JHA suppressible mRNA were then transcribed and translated *in vitro* , and translation products for the 76,000 molecular weight translation product were identified by immunoprecipitation. The identity of the clone was confirmed by comparing the protein N-terminal sequence with the sequence encoded in the cDNA. A full length clone (confirmed by primer-extension analysis) was used in northern analysis of abundance of the mRNA for the protein during the 4th and 5th larval stadia. Only a single-sized transcript was detected, and it was detected only in final instar larvae. Within the final stadium, the transcript was absent on day 1, highly abundant on days 2 and 3, and then absent on day 4(4). Incorporation of ^{35}S-methionine into the protein *in vivo* showed incorporation only on days 2 and 3 (4). However, immunoblot analysis showed that the protein was present in the hemolymph on days 2, 3 and 4, and on into the pupal stage (4). Similar analyses on control *vs.* JHA-treated insects showed a strong suppression of the transcript in JHA-treated larvae, and that the 76,000 molecular weight protein was missing among *in vitro* translation products of mRNA from treated larvae. Collectively the data suggest that prior to steroid-induced metamorphic commitment hemolymph abundance of the protein increases due to increased mRNA abundance, which is permitted by the natural decline in JH(5). After metamorphic commitment, the hemolymph abundance is controlled by a posttranslational mechanism such as degradation or clearance.

The two basic, 73-74,000 molecular weight proteins appear on day 2 coordinately with each other and with the 76,000 molecular weight acidic protein (6). Topical treatment of larvae with a JHA at ecdysis to the final larval instar strongly suppresses their hemolymph abundance. Polyclonal antisera specific for the two proteins were prepared, and immunoblot analysis shows that they are not immunologically related to each other (6), or to the acidic protein (6), or to other abundant hemolymph proteins such as the blue chromoprotein (7). Developmental immunoblot analysis showed that the two proteins are present only on days 2 and 3.

The *Trichoplusia ni* system is seen to offer unusually rich opportunities for study of the molecular basis of JH suppression of protein expression and abundance. Three immunologically unrelated proteins coordinately increase in hemolymph concentration, and later, one stays constant in hemolymph concentration while the other two disappear. It is our goal to analyze upstream regulatory regions of the genes for these proteins to determine if there is a consensus region which can be correlated with JH sensitivity. If we are successful, it will be the first identification of regulatory sequences necessary for proper expression of JH sensivity genes.

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HORMONAL REGULATION OF CUTICULAR GENE EXPRESSION IN *MANDUCA* EPIDERMIS

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The larval epidermis produces endocuticle continuously as the larva feeds and grows. During the molt this synthesis ceases, and at metamorphosis, the cells produce morphologically different cuticles. We have isolated cDNAs for 5 of the larval endocuticular genes and shown that 4 of them are expressed during each larval feeding period and transiently shut off during the molt due to the rise of ecdysteroid. Three of these are permanently suppressed by the action of 20-hydroxyecdysone (20 HE) in the absence of JH at the time of pupal commitment, while expression of the 4th becomes temporally and regionally suppressed at this time. In the pharate pupa and adult it is expressed only in flexible cuticular regions. The 5th gene is a member of a multigene family of at least 5 genes which code for 16 and 17kD proteins that first appear coincident with the formation of thin lamellae in the cuticle on the final day of feeding (day3). Expression of these genes is dependent on a small rise of ecdysteroid on day 2 in the absence of JH, but is not a primary action of 20HE. Thus, they are one of the first expressions of metamorphosis. Sequencing of these genes and of one of the larval endocuticular genes coding for a 14 kD protein shows an overall 34% identity in the coding region although some differences exist in the hydrophobicity profiles. Moreover there is a region of high similarity to *Drosophila* larval cuticle proteins in the C-terminal region with a consensus sequence of G--------G------Y-A-E-GY-------P--P. They also show regions of similarity to flexible cuticle proteins from *Sarcophaga* and *H. cecropia*. There is little similarity in the 5' flanking regions except for a sequence of 35 bases that varies in position relative to the initiation site but shows identity of 19-25 bases among various *Manduca* and *Drosophila* genes.

Although these cuticular genes are not regulated directly by 20HE, we hypothesize that they may be regulated directly by JH. Using the photoaffinity analogs of JH I and II and of methoprene, we have found a 30kD protein in 0.5MKCl extracts of the nucleus and a 38kD protein in the cytosol, both of which specifically bind JH. These proteins are not present in pupally committed epidermis but are present in the pupa. Preliminary experiments show that the 30kD protein binds specifically to DNA. In our search for the JH receptor, we screened, the *Manduca* genomic library with the human retinoic acid receptor cDNA clone and found a cross-hybridizing clone. This clone codes for a single copy gene which is being characterized. A stable derivative of retinoic acid is about 0.1 as active as JH II in the black larval assay.

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CONTROL OF EXPRESSION OF DOPA DECARBOXYLASE IN *MANDUCA* BY ECDYSTEROID AND JUVENILE HORMONE

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During a larval molt, the absence of juvenile hormone (JH) around the time of head capsule slippage (HCS) causes the deposition of premelanin granules containing prophenoloxidase into the newly forming cuticle 13 hr. later. When the ecdysteroid titer declines, the enzyme is activated and melanization occurs 3 hr. before ecdysis. Dopa decarboxylase (DDC) is also required for melanization, since *Manduca* melanin is a dopamine melanin. Epidermal DDC activity increases due to *de novo* synthesis during the last 13 hr. before the final larval ecdysis with the final levels 2-fold higher in melanizing larvae.

To study the control of DDC at the molecular level, we have isolated the *Manduca* DDC gene using the *Drosophila* DDC gene. This gene hybridized with a 3.1 kb mRNA from the epidermis which produced a 49 kD translation product that was precipitable by the *Drosophila* DDC antibody. This RNA appeared 1 hr. after HCS and was maximal 7 hr later. The timing of the expression was essentially identical to that of DDC synthesis in the epidermis. Peak expression was 2-fold higher in allatectomized larvae and coud be depressed to normal levels by JH application at HCS. Infusion of 20-hydroxyecdysone (20-HE) or addition of 20-HE to the culture medium reduced the expression. When 4th instar epidermis was explanted before the molting ecdysteroid rise and cultured with 1 μg/ml 20-HE for 17 hr, then for 24 hr in hormone-free medium, DDC expression increased 3-fold. Continuous exposure to 20-HE inhibited this expression. No increase in expression was seen in the absence of 20-HE during the initial culture period. Thus, ecdysteroid during a larval molt is a necessary trigger for the expression of DDC, but the subsequent decline of the ecdysteroid is required for this expression to occur. JH at the time of HCS determines the level of later expression.

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PROTEIN SYNTHESIS DURING METAMORPHOSIS OF THE EPIDERMIS IN THE PUPA OF *GALLERIA*

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Changes in the synthetic profile of cytoplasmic and nuclear proteins of epidermal cells were investigated during metamorphosis of the *Galleria* pupa. Newly synthesized epidermal proteins were labelled *in vivo* using ^{35}S - methionine and analysed by SDS-polyacrylamide-electrophoresis followed by autoradiographic processing of the gels. Distinct alterations of the pattern of cytoplasmic protein synthesis can be observed during the first two days after pupal ecdysis, which can be grouped into 4 categories:

1) The first changes start as early as 3h to 6h after pupation (group I). They concern bands which increase in intensity from this time on up to a pupal age of 18h and decrease afterwards again.

2) Proteins of group II show a more or less steady decline onwards from 3h after pupation and disappear at a pupal age of 24h.

3) Group III proteins appear at day 2.

4) One band (group IV) increases in intensity up to 12 h and remains constant afterwards .

Similar categories can be found in nuclear proteins.

The main effect of an injection of JH (epofenonane) immediately after pupal ecdysis is the delay of decreasing rates of protein synthesis of cytoplasmic proteins, whereas changes of nuclear proteins are accelerated. The main feature of effects of 20-hydroxyecdysone on changes in synthesis of cytoplasmic as well as of nuclear proteins is an even stronger acceleration. Even proteins which are normally synthesized at day 2 appear within the first 12 h. after pupation. The results are discussed relative to the significance of changes in protein synthesis for the change in commitment at day 1 and the regulatory function of juvenile hormones and ecdysteroids in this context.

EXPRESSION OF THE GLUCOSE DEHYDROGENASE ENZYME IN *LEPIDOPTERA* AND *DIPTERA* : REGULATION OF METAMORPHOSIS

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In *Drosophila* , the *Gld* gene is expressed during each metamorphic stage in correlation with ecdysone titers in a region-specific manner by the epidermis or epidermal derivatives, with switches in the cells expressing the gene occuring at the larval/pupal molt and at adult eclosion (Cox-Foster, Schonbaum, and Cavener, submitted). In *Drosophila melanogaster* embryos, *Gld* mRNA is expressed in the cypeolabrum and mandibular-maxillary regions of the head (stages 14-17) and in cells surrounding the posterior spiracles. In wandering third instar larvae, *Gld* mRNA is expressed by the larval epidermis and large polyploid cells at the base of the anterior spiracles; no *Gld* mRNA is detected in the imaginal disc cells. GLD is secreted during pupariation into the molting fluid and the expression of the gene is required for perculum formation. After pupariation, *Gld* mRNA is expressed by various cells derived from the imaginal discs. These regions include portions of the head, thorax and abdomen. The mRNA is detected in the ejaculatory duct and bulb of pharate adult males and in the spermatheca and oviduct of the pharate adult female; expression is shut off at the end of adult development. In *D. melanogaster, Gld* mRNA expression is turned on again in only the male ejaculatory duct, resulting in a switch from non-sexlimited to sex-limited expression in the reproductive tissues. A comparison of both the expression of the enzyme GLD and the *Gld* gene structure between the Diptera *(Drosophila)* and Lepidoptera (*Manduca* and *Lymantria*) may indicate evolutionarily-conserved regulatory mechanisms and reveal information on the evolution of holometabolous metamorphosis since these insects are evolutionarily distant and have different types of imaginal development. In *Manduca* , the enzyme is detected in late pharate adults immediately preceding the adult eclosion. The enzyme is synthesized by epidermal tissue and is secreted into the molting fluid. Like *Drosophila* , the enzyme is secreted in the epidermal region where the ecdysial line occurs. During *Lymantria* development, the enzyme is synthesized during each larval molt and not detectable during the intermolt period. During the pupa/pharate adult transformation, GLD is detected during Day 0 to Day 6, and again synthesized in late *Lymantria* pharate adults (day 10 and Day 11) preceding adult eclosion on Day 12. Unlike *Drosophila melanogaster*, no synthesis of the enzyme is found in either the adult males or in the adult females of *Lymantria*.

CUTICULAR PROTEINS AND THEIR GENES OF THE SILKMOTH *A. POLYPHEMUS*

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The wing tissue of the silkmoth *A. polyphemus* is being employed as a model system to study ecdysterone and juvenile hormone control of cuticular synthesis. One dimensional and two dimensional analysis of the proteins extracted from untanned cuticles are made up of distinct proteins. Immunological analysis with antibodies raised against these proteins demonstrates that antibodies against pupal and adult proteins do react well with both stage proteins, while antibodies against larval cuticular proteins react only minimally with the pupal and adult cuticular proteins. The mRNA populations of the wing epidermis during pupal and adult development have been extensively characterized by *in vitro* translations and cDNA-mRNA hybridizations. These results also confirm that the protein composition of the cuticles at the different stages are distinct and a few hundred new mRNAs belonging to the moderately abundant class appear specifically at the time of cuticular deposition (Sridhara S., Insect Biochem. 15:103, 1985, 15:333, 1985 and in preparation). Based on these results, cDNA probes specific for the mRNAs expressed at the time of pupal and adult cuticular deposition were prepared and employed to screen a genomic library made in the phage λEMBL 4. About a hundred plaques from each stage demonstrating hybridization were subjected to three rounds of selection and purification. Finally, ten adult clones and seven pupal clones were selected for analysis. Restriction mapping demonstrated that each of the clones contain different fragments of genomic DNA and each (except one pupal clone) contained a single gene. Specific fragments from each clone were subcloned into PUC plasmid vectors. These plasmid DNAs when used as probes confirmed the results obtained with the original clones. The combined results indicate that the genes coding for cuticular proteins in the silkworm may not be clustered. Some of these clones have been used both for developmental and for hybrid-selection translation studies. The mRNAs for the adult genes appear beginning day 10 of adult development while those of pupa appear on day 3. Each of these code for a polypeptide ranging in size from 12 to 50 kilodaltons. Sequencing of some of these clones is in progress for the final confirmation of the clones being true cuticular protein genes.

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MOLECULAR APPROACHES TO ISOLATE INSECT HORMONE RECEPTORS

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Steroid hormones affect development and differentiation in eukaryotes by regulating the transcription of specific genes in target cells. These can bring about a wide range of physiological effects after binding to specific receptors. The hormone receptor complexes bind to specific DNA sequences and/ or acceptor sites within the nucleus and increase the efficiency of transcription initiation from nearby promoters. During the last few years, the genes for the various mammalian steroid hormone receptors have been isolated and the structure-function relationships in these receptors are being actively pursued. These studies have led to the suggestion that all the receptors may be a part of a superfamily of regulatory proteins that have arisen over evolutionary time to match the increasing developmental and physiological demands of more complex eukaryotes (Weinberger *et al*., Cold Spring Harbor Symp. Quant. Biol., 51, 759, 1986. Gehring U. Trends Biochem. Sci., 12, 399, 1987). It has also been hypothesized that a steroid (or steroids) was adapted early in evolution as an important physiological signaling molecule (Karlson, Hoppe-Seyler's Z. Physiol. Chem., 363, 1067, 1983). Ecdysterone, the steroid hormone found in all arthropods, regulating the multitude of physiological functions of insects encompassing growth, ecdysis, metamorphosis, and reproduction, fits this role very well. Based on the structural data of the mammalian steroid hormone receptors and the above mentioned hypothesis, it was predicted that there might be some homology between the receptor(s) for ecdysterone and that of mammalian steroid hormones. Initial screening of dot blots of insect DNAs with labeled human estrogen (HER) and chicken progesterone receptor (CPR) probes (kindly provided by Dr. Bert O'Malley) confirmed the presence of sequences with similarity to the probes employed. The same DNAs on digestion with restriction enzymes, electrophoresis and transfer to membranes have demonstrated the presence of hybridizing fragments of similar sizes in all the DNAs tested. More detailed experiments, including human and rat liver DNAs as controls, have demonstrated that when complete digestion has occurred, only one fragment of about 2.5 kb size of lepidopteran DNAs and two fragments of Dipteran DNAs hybridize strongly to the probes. DNAs of insects belonging to other orders also demonstrate one or two fragments hybridizing with less intensity. Other conclusions that can be drawn from the results are: 1) Fragments that show cross activity with HER always do so with CPR. 2) CPR probe cross reacts with extra fragments not seen with HER. Experiments involving the HER insert and the DNA binding region as probes for various Southern and Northern blots permit the following conclusions: 1) Silk moths represented by *A. polyphemus* and flies by *D. melanogaster* have in their genomes sequences of DNA that hybridize to the mammalian and avian steroid receptor DNAs. 2) The similarity is not restricted to the DNA binding domain of the probe receptors. 3) Insects belonging to other orders contain either similar or related sequences. 4) These sequences are not highly expressed since RNAs from different tissues and different developmental stages tested demonstrate only minimal specific hybridization.

Based on these results, two cDNA libraries have been constructed in the expression vector λgt11 and screened with HER probe, and one clone with an insert of about 6Kb size has been isolated. Characterization of this clone is in progress.

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STUDIES ON SILKMOTH RNA POLYMERASES

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In the past ten years we have witnessed an explosion of information on the structural and functional aspects of eukaryotic genes which are grouped into three classes based on the type of RNA polymerase that transcribes them. Transcription of all three classes of genes show several common features: interaction or recognition of specific DNA regions by specific DNA binding factors (species independent or specific) followed by binding of the respective RNA polymerase and initiation of transcription. Other factors also referred to as transcription factors, that regulate enzyme activity and level of transcription, interact with either DNA and/or DNA-factor-RNA Pol complexes. Hence, whether one is considering RNA transcription *per se* or its regulation during growth and differentiation, the three principal components involved are the genes, the transcription factors and the corresponding RNA polymerase. Compared to the vast amount of data available on the first two components, the information on the enzymes themselves is limited, especially for enzymes I and III. This is due to their occurrence in eukaryotic cells in minute quantities and the difficulty of obtaining them in a pure form. A four step procedure consisting of Heparin Sepharose chromatography, followed by DEAE Cellulose chromatography, Glycerol gradient centrifugation and finally DEAE Sephadex chromatography has been developed in this laboratory to purify the three enzymes from the silk glands, leading to yields of about 20% active enzymes. In order to provide an easier approach to obtain small amounts of rather pure enzymes rapidly, an HPLC technique has been attempted. In keeping with the size estimation of about 600 kilodaltons, the enzymes eluted at 8 min. after Thyroglobulin (670 kilodaltons) and before β galactosidase (570 kilodaltons). The results indicate that gel filtration and ion exchange columns for HPLC can be effectively utilized for RNA polymerase purification.

Purified enzymes I and III have been employed to obtain monoclonal antibodies by the hybridoma technique. After several rounds of selection and cloning by limiting dilution, hybridomas producing antibodies specific for two subunits of polymerase I, two subunits of polymerase III and for a common subunit of all the three polymerases have been selected for further studies. ELISA titrations confirm the specificity of the individual monoclonal antibodies which are only partially inhibitory for enzymatic activity. A radioimmunoassay in which radioactivity of iodinated antimouse antibody bound to the microtiter plates that is directly proportional to the antigen present has been developed. The response was linear with up to 50 ng enzyme. With a view to measure the three enzymes simultaneously in tissue extracts, a sandwich ELISA procedure employing individual monoclonal antibodies and enzymeslinked polyclonal antibody is being developed.

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THE EXPERIMENTAL INSECTICIDE RH 5849 IS A NONSTEROIDAL ECDYSONE AGONIST

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RH 5849 (1,2-dibenzoyl-1-tert-butylhydrazine) is a representative member of a new class of insect growth regulators discovered at the Rohm and Haas Research Labs. It has been found to have ecdysone-like effects in the *Drosophila* K_c cell line and in larvae of the tobacco hornworm *Manduca sexta*.

When ecdysone-susceptible whole K_c cells are exposed to RH 5769 they undergo process elaboration, inhibition of proliferation, and induction of acetylcholinesterase in a manner qualitatively identical to that observed with 20-hydroxyecdysone (20-0HE) treatment. When cytosolic extracts of the cells are prepared which contain high affinity saturable ^{3}H-ponasterone A binding activity, RH 5849 is found to inhibit ponasterone binding in a competitive manner (K_d is increased with increasing RH 5849 concentration but B_{max} remains the same). RH 5849 lowers the kinetic on-rate k_a of ^{3}H-ponasterone A binding to the receptor but does not affect the off-rate K_d . In lines of 20-OHE- and RH 5849-resistant cells, treatment with either compound does not lead to distinguishable morphological differentiation or inhibition of proliferation; these cell lines have lowered levels of specific ^{3}H-ponasterone binding activity. In side-by-side experiments, RH 5849 is consistently less potent than 20-OHE (140-fold for whole cell assays, 30-fold for direct displacement of ^{3}H-ponasterone A from receptor). Taken together, these data indicate that RH 5849 is a weak nonsteroidal agonist of 20-0HE.

However, RH 5849's effect on *M. sexta* larvae is more dramatic. When either fed or injected into whole larvae RH 5849 causes a rapid (about 24 hrs) molting response first manifested as head capsule apolysis, immediately halting feeding and leading to eventual death without ecdysis. This occurs without a rise in the endogenous ecdysone titers (in fact ecdysone titers are actually decreased, implying a negative feedback effect on ecdysone biosynthesis). When injected into either L4D1 or L5D3 isolated abdomens, RH 5849 is 28-fold more potent than 20-OHE at inducing molting or ecdysone-dependent development. When injected or fed to intact L5DO larvae, RH 5849 is 53- and >670-fold as potent, respectively, as 20-OHE. This remarkable potency for a relatively weak agonist is apparently due to its superior metabolic stability and/or transport properties relative to 20-OHE; an EC_{95} (for head cap slippage) oral dosage of RH 5849 leads to a peak hemolymph RH 5849 concentration of 16 µM, exceeding concentrations needed for K_c whole cell differentiation by 3 to 5-fold. Oral administration of much higher concentrations of 20-OHE almost certainly lead to catabolism and rapid excretion.

RH 5849 initiates premature molting at every larval instar of *M. sexta* observed, and at all days during the last larval instar. In addition its effects are evident in a wide variety of lepidopteran families. There is a significant relation between relative ability of several RH 5849 analogs to displace ^{3}H-ponasterone A from its K_c cell receptor and ability to initiate premature head cap apolysis in L3 *M. sexta*. Thus these compounds hold great promise as both physiological tools in research involving ecdysone-utilising invertebrates and as a major new class of "third generation" insecticides.

BIOLOGY AND MOLECULAR BIOLOGY OF JUVENILE HORMONE ESTERASE

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In the species of lepidoptera examined, hydrolysis of juvenile hormone by a group of esterases with a high k_{cat}/K_M ratio is very rapid at certain stages of development. At key stages, these JH esterases appear to work in conjunction with a reduction in the rate of JH biosynthesis to reduce the juvenile hormone titer. In addition to the correlation between the reduction in JH and the appearance of JHE in the hemolymph noted by Weirich, in several species a cause effect relationship between the appearance of the esterase and the reduction of JH titers has been shown by using several classes of selective esterase inhibitors. There are a variety of reasons to study these unique enzymes. First, a regulatory system where degradation plays a major role appears unique in the field of endocrinology. In addition the large increase in hemolymph JHE protein is one of the early markers of the developmental sequence leading to pupation. The regulation of JHE itself is interesting in that its production and release by tissues is very precise and appears to be under different regulatory systems at different times in development. Biochemically the enzyme is interesting in that few detailed studies have been carried out on the catalytic mechanism of esterases in general and even fewer where an endogenous substrate is known. Finally the JHE message may offer a useful tool in developing genetically engineered pest control agents.

A variety of powerful transition state mimics have been synthesized for the enzyme and many have been found to be slow tight binding inhibitors. Using these inhibitors for affinity chromatography, the esterases from several lepidopterous species have been purified to apparent homogeneity. In some cases there clearly were multiple forms based on molecular weight, isoelectric point, presence and extent of glycosylation and interaction with inhibitors. However, all forms hydrolyzed JH efficiently. Because of ease of obtaining biomass, the JHE from *Manduca sexta* was selected for most biological work and that from *Heliothis virescens* for molecular work.

Injection of picomole amounts of affinity purified JHE into mid second stadium larvae of *M. sexta* resulted in a dose dependent blackening of the cuticle in the third stadium. This effect could be reversed in a dose dependent manner with application of pico to nanogram amounts of JH mimics. These data show that JHE has a clear anti-JH effect even at a developmental stage when high secretion of JH is anticipated and when JHE is turned over rapidly.

Hemolymph of prewandering *H. virescens* appears to contain two isoforms of JHE which differ only by a SER-ALA extension at the N-terminus of the minor form. The affinity purified material was used to generate antibodies and an oligonucleotide probe. Screening 400,000 clones of a lambda GT 11 library size selected for transcripts >1350 bp in length yielded 25 immmunoreactive clones five of which hybridize to the probe. Three of these clones were subcloned and found to contain a 3000 bp fragment which hybridized to a 3kb message on a Northern blot and which had additional restriction maps. Sequencing of this insert indicated a 1714 bp ORF with an untranslated 1256 bp 3' tail. Translation of this sequence matches closely the N-terminal sequence from Edman degradation and predicts a molecular weight of 61,012 plus a 19 residue N-terminal signal sequence. Partial sequencing of the other two clones indicated a small level of heterogeneity not surprising from an outbred culture of *H. virescens*.

Using a Bam HI fragment corresponding to about 60% of the N-terminal coding region, RNA's from several tissues were probed at several times during development. Levels of poly A RNA from the fatbody were found to correlate with levels of catalytic activity in the hemolymph at early stages of development.

PARASITE REGULATORY MEDIATORS AND REDIRECTION OF LEPIDOPTERAN HOST PHYSIOLOGY

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Parasitic wasps in the genus *Chelonus*, which oviposit in host eggs, redirect host physiology for 1) precocious initiation of the developmental program leading toward metamorphic commitment (1-3), 2) suppressed development of the precocious prepupa (4-5) and 3) suppression of the host immune response (6). The regulatory mediators responsible for these effects are injected by the ovipositing female (7).

Precocious initiation of metamorphosis is induced by an apparent alteration in, or perception of, the size threshold which signals attainment of the final larval instar. Thus, the larvae prematurely express all programs leading toward metamorphic commitment, not just those programs whose expression is released by JH decline. As part of this redirection of developmental trajectory, JH esterase is prematurely expressed, making the parasite regulatory mediator the only reported capable of inducing premature expression of this enzyme in a preultimate instar larva. Inasmuch as the biochemical basis for this threshold in normal insects is not known, the responsible parasite regulatory mediator may finally give us a tool with which to crack this heretofore intractable problem in insect developmental biology. The 'reprogramming' effect in parasitized larvae is permanent, in that even if larvae are induced by JH treatment to undergo a 'supernumerary' larval molt, precocious initiation of metamorphosis will still take place.

Suppression of precocious prepupal development is due to a suppression in ecdysteroids. Interestingly, none of the pulses of ecdysteroids which cause each of the preceding larval-larval molts is affected. The latent signal for suppression of prepupal ecdysteroids is permanent, in that even after two JH-induced 'supernumerary' molts the larva will still stop development when it reaches the prepupal stage.

Host permissiveness for parasite survival is induced by several regulatory mediators injected by the female. Associated with this effect is a reduced rate of melanization activity in the host hemolymph.

Candidates for regulatory mediators include venom from a venom gland, polydnavirus, and unique proteins in the calyx fluid. Antiserum against the venom proteins has been prepared and used to provide the first report on the time course of entry of parasite venom into its host (6). A number of venom proteins in this wasp show immunological conservation with venom proteins of a wide spectrum of Hymenoptera (8). The venom proteins have been biochemically characterized (9), and N-terminus sequence data for them is now being generated. Combination of biochemical and biological experiments have shown that the venom is necessary for survival of the endoparasite.

The ultrastructure and genome segmentation of the polydnavirus which replicates in the female ovaries of this braconid wasp have been described (10). Molecular studies suggest that virus expression in host Lepidoptera is associated with survival of the endoparasite, and clones containing such expressed genes are currently being sequenced. Use of immunological and molecular approaches to track the entry of the polydnavirus during oviposition have shown that it appears to enter after the venom.

Unique calyx fluid proteins have been identified, and these are currently being biochemically characterized. Antisera for these proteins has also been prepared.

In contrast to the situation reported for model parasites which sting lepidopteran larvae, this egg-larval parasite does not rapidly induce in the host highly abundant, low-intermediate molecular weight proteins. However, a low abundance, high molecular weight protein does appear late in host development, and this protein is associated with the presence of the endoparasite (11). The protein

has been purified and biochemically characterized and is currently being sequenced. Its regulation and function are also being addressed.

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BOMBYX MORI $tRNA_1^{Gly}$ GENES: STRUCTURE AND TRANSCRIPTION

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We report here our preliminary results on the structure and transcription of $tRNA_1^{Gly}$genes in *Bombyx mori*.

From a library of genomic *B. mori* DNA, twenty two $tRNA_1^{Gly}$ genes have been cloned in plasmid recombinant vectors. This set forms the major part of this tRNA multigene family.

Transcription experiments with cell free systems show that the forty base pair 5' flanking region is required for efficient transcription in homologous systems.

The sequence of three of these genes have been determined and their 5' flanking regions compared. Even though some homologies are found in the flanking sequences of these genes and of the other *B. mori* tRNA genes already studied, no consensus sequence can be defined.

From our results, we also demonstrate that the number of $tRNA_1^{Gly}$ genes is between 22 and 35 and that they are clustered to some extent.

TRANSCRIPTION AND TRANSCRIPTIONAL CONTROL

ANALYSIS OF CIS- AND TRANS-REGULATORY ELEMENTS IN *BOMBYX MORI* CHORION GENES.

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Choriogenesis (eggshell formation) serves as an attractive model system for studying *cis*-acting elements and *trans*-acting factors that are important for the regulation of tissue and temporally-specific gene expression. During choriogenesis in the silkmoth, *Bombyx mori* , families of evolutionarily-related chorion genes are coordinately- and sequentially-expressed, culminating in the formation of a well-organized chorion. We have focused our *in vitro* analyses on two prototypical A/B gene pairs (A/B. L12 and A/B. L11), each of which exhibits a distinct developmental expression profile within the middle period of choriogenesis. In parallel, we have conducted *in vivo* studies, using P element-mediated transformation, to characterize the regulated expression of the A/B.L12 gene pair in *D. melanogaster*. *In vitro* binding studies of the 5' flanking regions show that a limited number of protein binding sites are detectable with nuclear extracts from *Bombyx* follicles and silk glands, as well as with extracts from the *Bombyx* cell line, Bm5. More importantly, there are several distinct follicle-specific protein-binding sites within these regions. The *in vivo* analyses, using clustered point mutations, have provided evidence that at least one of the follicle-specific binding sites is important for the temporal regulation during choriogenesis. These *in vivo* studies have also uncovered another region that is involved in regulating the level of expression as well as an additional region, spanning the chorion-specific hexanucleotide TCACGT, that is essential for transcriptional competence of the A/B.L12 promoter.

MULTIPLE NUCLEAR FACTORS INTERACT WITH CONSERVED SEQUENCES IN THE PROMOTER REGION OF SILKMOTH CHORION GENES.

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We have initiated the characterization of macromolecules involved in the determination of tissue specific expression of chorion genes in the silkmoth *Bombyx mori* by studying the interactions of factors present in follicular tissue and other silkmoth cell types with the promoter elements of selected chorion genes.

Initial characterization utilized the intact promoter element of a gene pair of late developmental specificity, HcA/B.12, which was subjected to gel retardation analysis using nuclear extracts prepared from expressing (follicular) and non-expressing (silkgland and Bm5 tissue culture cells) tissues. Binding activities were detected only in extracts of choriogenic follicles and Bm5 cells but not in those of the silkgland. Comparative analysis of the band shifts with the follicular and tissue culture extracts revealed different patterns, suggesting that distinct promoter-interacting factors exist in the two types of tissues. However, with both extracts binding occurs primarily in the HcA 'half' of the promoter.

The inclusion of bivalent actions (Mg^{2+} and Ca 2+) during the incubation with the follicular extracts, led to the stimulation of one of the binding activities at the expense of another, suggesting the presence of multiple distinct trans-acting factors specific for chorion promoter recognition. The binding activities in both follicular and Bm5 extracts were inhibited in the presence of 2mM $ZnCl_2$, implying that they are unlikely to represent zinc finger proteins and that their DNA-binding domains may contain cysteine residues.

Footprinting and methylation interference analysis of the promoter region with follicular extracts have led to the identification of two DNA binding regions (I and II) which interact independently of each other with follicular factors FFI and FFII, respectively. The two binding sites are separated from each other by two-turns of the helix. Footprinting analysis with the Bm5 extracts revealed the presence of a tissue culture factor (BFI) that binds to region I. The results from methylation interference studies indicate that BFI and FFI do not interact to the same residues within region I where binding of both takes place.

Regions I and II are present in all Hc genes of *Bombyx*. Sequence comparisons to the promoter regions of other characterized chorion genes of the A and B classes revealed that regions I and II are conserved in some but not all of the genes of middle developmental specificity. Interestingly, regions I and II are absent from the chorion promoters of *Drosophila melanogaster* as well as from the silkmoth chorion genes which were expressed in *Drosophila* via P- element mediated embryo transformation. The implications of these findings with respect to the developmental program of choriogenesis will be discussed.

DEVELOPMENTALLY REGULATED CHORION ANTISENSE RNA IN THE SILKMOTH *BOMBYX MORI*

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Follicular cells of the silkmoth *Bombyx mori* contain certain considerable quantities of chorion antisense RNA which is transcribed from a high cysteine gene pair, HcA/HcB.12, whose properties we have been investigating. The antisense transcripts of gene HcB.12 have been characterized in detail. Antisene RNA was found to be considerably less abundant than the corresponding mRNA and to have a size similar to that of its sense counterpart.

Primer extension, S1 and RNase protection analyses revealed that antisense transcription initiation occurs 100 NT downstream from the AAUAAA polyadenylation signal of gene HcB.12. The antisense transcripts span the entire second exon and terminate at a stretch of T residues within the intron. Sequence analysis revealed the presence of a sequence matching the consensus "box A" promoter element of RNA polymerase III within the transcribed region near the proximal end of the transcript and multiple SP1-like sequences in the immediate 5' flanking region. These observations, in addition to the finding that the antisense transcripts are not polyadenylated, suggest that antisense RNA may be transcribed by Pol III.

Fractionation of follicular cells into nuclear and cytoplasmic compartments revealed that, like the mRNA, the antisense transcripts are largely localized in the cytoplasm. Only a small fraction of the antisense RNA is in a duplex form with its complementary RNA. The proportion of the duplexed species was increased by as much as 100 fold following reannealing of total RNA *in vitro*.

HcB.12 antisense transcripts are present in follicular cells but absent from non-expressing cells that were examined, including Bm5 tissue culture cells and silkgland. The developmental expression of antisense RNA relative to the sense mRNA was analyzed by dot hybridizationsz of RNA isolated from individual follicles representing all choriogenic stages to single-stranded probes specific for the two types of transcripts. Although subtle differences are evident, the synthesis of antisense RNA appears to be regulated in a fashion similar to that of its complementary chorion mRNA, with both types of RNA being restricted to follicles of late developmental specificity.

Using nuclear extracts prepared from chorion expressing (follicular) and non expressing (Bm5 tissue culture) cells, gel retardation analysis of DNA fragments encompassing regions spanning internal and 3' flanking sequences of gene HcB.12 was performed. Two DNA binding activities were detected in follicular but not Bm5 cells, suggesting the presence of tissue-specific factors that may regulate the expression of the antisense RNA. These trans-acting factors are, therefore, unlikely to represent general Pol III transcription factors.

As of yet, we have not investigated for the presence of antisense transcripts in other chorion gene pairs. The functional role (if any) of the antisense transcripts during late choriogenesis remains undetermined. The possible involvement of such transcripts in some aspects of chorion gene regulation, including the nuclear-cytoplasmic transport, cytoplasmic stabilization or even the translatability of chorion mRNA will be discussed.

TEMPORAL AND SPATIAL REGULATION OF SILK GENE TRANSCRIPTION

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Our major concern has been understanding how cells are specialized and express a set of genes during development. Along this line our target has been a dissection of silk gene regulation in *Bombyx mori*. Transcription of the fibroin H gene begins at around stage 25 of the embryos when the development of the silk gland, originated at stage 19 in the labial segment, is morphologically complete (Ohta *et al.*, 1988. Develop. Growth &Differ. 30, 293). After this first activation the fibroin gene is repeatedly switched on and off in the posterior silk gland (psg) cells during the larval development. Fibroin gene transcription is restricted to the anterior region of the psg in the beginning of the Vth larval instar, and spreads toward the posterior region as the stage proceeds (Obara & Suzuki 1988. Develop. Biol. 127,384).

To analyze the underlying mechanisms controlling the transcription of the fibroin gene in psg and the sericin-1 gene in the middle silk gland (msg) a major strategy we have taken is to develop cellfree transcription systems and to characterize factors involved in the regulation (Suzuki *et al.* 1986. Proc. Natl. Acad. Sci. USA 83, 9522). From embryos and several tissues of various developmental stages and cultured cell lines we have established 18 cell-free systems. Using these systems and deletion templates several cis-acting elements have been identified; the distal upstream element (-238 ~ -73, Enhancer I), the proximal upstream element (72 ~ -32), core promoter (-31 ~ +10), and the second enhancer (+155 ~ +454 . Of these, Enhancer I governs the major activity in enhancing transcription and functions very strongly in psg extracts, weakly in msg extracts, but not significantly in many other extracts. A weak enhancing activity was detectable in stage 25 whole embryo extract but not in stage 17 extract. Differential transcription of the fibroin gene and the sericin-1 gene has been attained in these systems; in the V-2 psg extracts the fibroin gene was preferentially transcribed; while in V-5 msg extracts the sericin-1 gene was a stronger template than the fibroin gene. At least 4 regions controlling ser-1 gene transcription have been mapped; -241 ~ -176, -134 ~ -92, -91 ~ -52, and core promoter region.

Gel shift assays (Suzuki & Suzuki 1988. J. Biol. Chem. 263, 5979) and protection experiments revealed several important regions. For the fibroin gene, Region FE (-220 ~ -180), FD (-170 ~ -135), FC (-135 ~ −100), FB (-90 ~ -60), FA (-60 ~ -35), FTATA (-35 ~ -18), FI (-9 ~ +25), and so on; for sericin-1 gene, SC (-206 ~ -191), SB (-150 ~ -138), SA (-98 ~ -83), and so on. Characterization of factors interacting with these regions is being carried out. Protein(s) interacting with -167 to -155 (ACAATTTAATTAA) in FD and giving a psg-specific complex upon gel shift assay has been purified almost to homogeneity through DEAE-Sepharose, calf thymus DNA-cellulose, and 2 rounds of Enhancer I-Sepharose column chromatography. Repeats of TCAATTAAAT or its variants are detected in FE,FD, and FC including the one at -167 to -158. Interestingly, this consensus sequence is identical with that bound with *Drosophila* homeo box proteins. Binding and protection of *eve* and *zen* homeo domain proteins on these consensus sequences of the fibroin H gene as well as those of the fibroin L and sericin-1 genes have been demonstrated. Significance of these findings will be discussed.

PROTEIN-BINDING PROPERTIES OF THE DNA FLANKING THE P25 GENE OF BOMBYX MORI

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Transcription of genes by RNA polymerase II is regulated by distinct DNA motifs in the vicinity of the transcription initiation site and promoters appear as patchworks of *cis* -acting elements that are recognized by a combination of *trans*-acting factors. In spite of the identification of a variety of *cis* -acting elements and *trans*-acting factors, the molecular mechanisms governing temporally and territorially programmed patterns of transcription are not well understood.

In this regards, the *Bombyx mori* silkgland offers a very attractivmeo del of concerted development - and cell - specific gene regulation. Posterior silkgland cells are highly specialized in the production of the two subunits of the major silk protein. These are fibroin and P25, the products of single copy genes. Fibroin and P25 are produced from a pool of mRNAs whose cell molar concentrations are equal during the feeding period. The expression of these two genes shows the same cell specificities and the same sharp developmental controls of the accumulation of mRNA since their transcription occurs only during intermolts and is abolished during molting stages (1,2). The comparative analysis of the P25 and fibroin gene sequences did not reveal any relationsip between their respective coding seqences. However, the comparison of their upstream DNA has led us to localize highly homologous sequences in the DNA domain close to the transcription start site (3). Functional assays performed by Y. Suzuki's group showed that *in vitro* transcription of fibroin gene templates in a homologous system, is stimulated by the presence of the region -234 to -66 which acts as a strong cis-acting enhancer of the activity of this gene (4). Interestingly, this region coincides with the homologous sequence delimited in the flanking DNA of the P25 gene. Together with the identical space and time regulation of transcription of the P25 gene and fibroin gene, the equal accumulation of both mRNAs argues for the interaction of common *trans*-acting factors with the common sequences.

We used the sensitive *in vitro* DNA band shift assay to examine binding factors to the P25 5' flanking region in crude and fractionated whole cell extracts of posterrior silkglands. We analysed the binding capacity of probes that span the -407 to -18 domain of the 5'-flanking region of the P25 gene and, revealed multiple sites of DNA-protein interactions. In particular, at least three distinct protein-factors bind to the fibroin enhancer-like sequence of the P25 gene. Two of them show independent interaction on two different sequences identified by DNase I footprintings; the third one probably exhibits protein-protein interactions. The recognition sequence of one of these factors is closely homologous to a binding motif of fibroin gene enhancer reported recently by Suzuki and Suzuki (1988). Competition experiments between the two sequences argue for the identity of the corresponding factor.

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EXPRESSION OF GENES ENCODING SILK PROTEIN AND CYTOPLASMIC ACTIN OF *BOMBYX MORI* IN TRANSGENIC DROSOPHILA

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In *Bombyx mori* silkgland, the genes encoding the silk proteins - P25, fibroin and sericins - exhibit high levels of transcription with restriction of developmental and territorial specificities in the posterior and middle silkgland regions. Conversely, the cytoplasmic actin gene A3, whose coding region is highly conserved between species, is expressed at a low level in all the silkgland cells, as well as in other tissues.

As a strategy to unravel the regulatory mechanisms of silkgland expressed genes, we analysed the activity of the P25 and of the A3 genes in *Drosophila* after chromosomal insertion by P element-mediated transformation.

A P25-lacZ hybrid gene was constructed with 1,4 kb of upstream sequence and the first exon of the P25 gene fused with the E.coli β-galactosidase gene-encoding sequence. After injection in *Drosophila* eggs, four lines were isolated, each carrying one structurally intact copy of the hybrid gene. The chromosomal sites of integration were determined by both linkage analysis and *in situ* hybridization of polytene chromosomes. Expression of the hybrid was analysed by direct *in situ* detection of β-galactosidase activity exclusively in third instar larvae tissues, using X-gal histochemical staining. As compared to the recipient strain, all four transformed lines show exclusive β–galactosidase activity in the anterior portion of the salivary glands. This activity was identified to correspond to the bacterial enzyme by in-gel detection after electrophoresis of salivary gland homogenates. Also, quantitative β-galactosidase assays showed variations of enzyme production among the four lines, suggesting the existence of a position effect. Three different double-insert lines were obtained by crossing single -insert lines. In these lines, the resulting β-galactosidase activity reflects the additivity of the expression of the two inserted genes.

These experiments show that the 1.4 kb upstream sequence of the P25 gene carries cis-acting elements responsible for tissue specificity of expression and that these sequences are recognized by Drosophila trans-acting factors. The capacity of *Bombyx* promotors to function in Drosophila, already demonstrated for a chorion gene (Mitsialis and Kafatos, 1985), can thus be extended to a silkprotein gene, whose function has no equivalent in *Drosophila*.

From that it could be supposed that the cytoplasmic actin A3 gene would be expressed in a whole spread manner in *Drosophila* tissues. Four transgenic lines were obtained following germline transformation with a genomic DNA fragment carrying the A3 gene coding region with around 2kb of upstream and of downstream sequences. These lines were characterized by linkage analysis and southern blotting experiments, each showing the presence of one unmodified copy of the actin gene. Preliminary analyses of two transformed lines show the presence of *Bombyx* actin mRNAs in third instar larvae; their tissue distribution is under study.

Mitsialis S.A. and F.C. Kafatos, 1985, Nature 317, 453-456.

CONSTITUTIVE AND REGULATED TRANSCRIPTION BY RNA POLYMERASE III IN *BOMBYX*

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We are determining the requirements for polymerase III transcription in silkworms with the goal of understanding how tissue-specific expression of certain tRNA genes is achieved in this organism. Specifically, there are two classes of alanine tRNAs produced in silkworms. One (the constitutive type) is found in all cell types ; the other is restricted to the silkgland. To discover the mechanisms of this selective gene expression we are examining the transcriptional properties of both kinds of $tRNA^{Ala}$ genes *in vitro*.

In the case of genes that encode the constitutive type of alanine tRNA, we have delineated the elements that act in *cis* , as well as those that act in *trans*, to permit transcription of these templates. We find that the region required for full transcriptional activity of these genes is much larger than the canonical A and B boxes described by other laboratories. Moreover, we have recently discovered a new transcription factor (TFIIID) that is required for transcription of these genes, and that is probably a general component of the polymerase III transcription machinery in other organisms, as well. This factor is required for the formation of stable transcription complexes on tRNA genes, and current work is aimed at determining exactly how it interacts with other transcription components and with tRNA genes. We are using template exclusion, DNA fragment mobility shift, and electron microscopy as assays for the interaction between tRNA genes and the transcription apparatus.

The transcriptional properties of the two classes of silkworm $tRNA^{Ala}$ genes are very different *in vitro* , and we have begun to identify the functionally distinctive features of the genes. We know that the 5' flanking regions of the two genes contain functionally different *cis* -acting elements. We also know that the silkgland-specific type of gene requires positive activation that is not required by the constitutive-type $tRNA^{Ala}$ gene, and we are now isolating the component(s) reponsible for this activation.

STRUCTURE AND EXPRESSION OF TWO MUSCULAR AND ONE CYTOPLASMIC ACTIN GENES IN *BOMBYX MORI.*

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Three actin genes have been isolated from a genomic library with a Drosophila actin cDNA clone as a probe. Their coding sequences and flanking regions have been determined: only one gene, the gene A3, is interrupted by a short intron at position 116, the location of which has not yet been described in other actin genes. Comparison of the coding sequences shows that 2 genes, gene A1 and gene A2, are very closed to each other, while the third one resembles actin cytoplasmic genes.

The number of actin genes in the genome has been determined by hybridization of digested genomic DNA to the 3' end gene regions, which are specific for each gene. 4 actin genes are detected, 2 of them exhibiting 2 allelic forms.

The expression of these 3 genes during development has been analysed using specific 3' end regions either to probe Northern blots or to be hybridized to cDNAs made from different origins. It appears that the genes A1 and A2 are muscular actin encoding genes: gene A1 is very active during the whole ontogeny of *Bombyx* muscles and gene A2 participates only in the elaboration of late pupa thoracic muscles. Gene A3 encodes a cytoplasmic actin since it is expressed in non muscular tissues. It is the only one to be expressed in the silk gland.

The expression of gene A3 has been analysed in more detail during the development of the silk gland. Its transcripts are present in the 2 parts of the gland during molt, rapidly increase after ecdysis, and decrease on and after day 5 of the Vth instar. The middle silk glands can be divided into 5 regions according to the position of 2 natural turns. At the beginning of the Vth instar, the transcripts are almost uniformely distributed and are particularly accumulated in the posterior region at the end of this stage. When Vth instar larvae are treated by a juvenile hormone analog, methoprene, for 6 days, the pattern of transcript accumulation resembles the one observed in younger silk glands.

MOLECULAR BIOLOGY OF VIRUSES AND VIRAL EXPRESSION SYSTEMS

BACULOVIRUS DIRECTED FOREIGN GENE EXPRESSION

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The baculovirus expression vector is a helper-independent recombinant baculovirus which during infection can express abundant quantities of recombinant mRNA and proteins of foreign genes under the control of the virus polyhedrin gene promoter (Smith et *al., 1983* Mol. Cell. Biol. 3:2156). The two major characteristics of this expression vector when compared to other animal virus expression systems are (a) the very abundant expression of foreign genes under the transcriptional regulation of the very strong polyhedrin gene promoter and (b) the production of recombinant proteins in established cultures of lepidopteran insect cells. The vector is widely distributed in the scientific community and is used for the cloning, expression and study of several genes of medical and agricultural importance (Luckow and Summers, 1988, Bio/Technology, 6:47).

The baculovirus vector is the only eucaryotic DNA viral vector for the cloning and expression of genes in insect cells (Summers and Smith, 1987, A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experiment Station Bulletin B-1555) and insects (Marumoto *et al* ., 1987, J.gen. Virol, 68, 2599). It is now a major tool for the studies of the molecular biology of insect systems. Although differences in glycosylation appear to exist, in no case has this been shown to be detrimental to the "authenticity" or function of the recombinant protein. Other major advantages of the baculovirus system include: it is a safe system because it does not utilize a virus which is pathogenic or oncogenic for vertebrates and the insect cells are not transformed or immortalized by an undesirable virus; functionally authentic proteins are abundantly produced; proteins that are normally "toxic" to vertebrate and mammalian cells are also abundantly produced; it is a quick, consistent and reliable system for gene cloning and expression; and the rod-shaped baculovirus nucleocapsid suggests no major limitations in foreign DNA cloning capacity (this has not been tested to any significant degree).

Preliminary results from the use of the new viral vectors such as Ac YM1 (Matsuura *et al.*, 1987) and Ac VL941 (Luckow and Summers, 1988) and new multiple cloning site vectors derived from pAcVL941 suggest that steady state levels of transcription and translation can be improved 2-10 fold for certain genes. There are some indications that certain recombinant proteins may be expressed at nearly the same levels as polyhedrin, which is 1 mg/ml/1-2 x 10^6 cells. It is clear that some membrane-associated or secretory proteins are not as abundantly expressed as expected. At this time, it is not known if insect specific factors (codon usage, glycosylation, signal sequence, efficiency of translation of recombinant mRNA, etc.) are affecting this or if this may be an inherent limitation in the capacity of any eucaryotic cell for "unlimited" production of recombinant products. The major limitation of the system is the lack of knowledge of the molecular biology of polyhedrin gene regulation and the molecular biology of lepidopteran insect cells. It is very likely that as the molecular biology of both of these is better described, significant improvements for the levels of expression of recombinant proteins will be forthcoming by knowledge of how to manipulate the molecular biology and genetics of those cells and genes.

TEMPORAL REGULATION OF IMMEDIATE EARLY AND DELAYED EARLY GENES OF *AUTOGRAPHA CALIFORNICA* NUCLEAR POLYHEDROSIS VIRUS

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Expression of baculovirus genes in infected cells is temporally regulated. The immediate early genes are expressed in the absence of other viral gene products. Expression of the delayed early genes is dependent upon prior synthesis of several immediate early proteins. Late gene expression is concomitant with DNA synthesis and is controlled by the protein products of early genes. Polyhedrin and other very late genes are maximally expressed after the formation of extracellular viral products. We have mapped and identified the *cis* and *trans* -acting factors which control delayed early genes expression.

In order to monitor expression of the delayed early test gene (39K), the bacterial gene chloramphenicol transferase was cloned under the control of the 39K promoter. The major *trans* -activating protein was identified by cotransfection mapping of 39CAT and purified restriction fragments of viral DNA. The active fragment was cloned and sequenced and found to encode a protein of 70,000 mol wt. This gene (IE1) was also shown to *trans* -activate several other delayed early genes.

Accessory factors which augment IE1 *trans* -activation of 39CAT have also been identified. One of these factors is a viral enhancer element. The viral genome contains five regions of homologous DNA which act as enhancer elements when cloned in *cis relative to the delayed early promoter. The five enhancers are similar in nucleotide sequence, and contain several copies of a 26 bp palindrome. Factors which bind to the palindrome were identified using gel retardation and DNase footprint analysis.*

DNA POLYMERASE OF SILKWORM NUCLEAR POLYHEDROSIS VIRUS

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A marked increase in the activity of host-cell nuclear DNA polymerases α and β and the induction of a virus-specific DNA polymerase were observed in pupae of the silkworm *Bombyx mori* after infection with nuclear polyhedrosis virus (BmNPV). The virus infection did not affect the activity of host cell DNA polymerase γ (mitochondrial) in the pupae. The virus-specific DNA polymerase (BmNPV Pol) was purified about 20,000-fold from the infected pupae to near homogeneity. The enzyme consists of one polypeptide 105 kDA and does not possess a reverse transcriptase activity. BmNPV Pol belongs to the "prokaryotic type" of DNA polymerases with strong intrinsic 3' to 5' exonuclease activity specific for single-stranded DNA (proofreading activity). The mode of hydrolysis of ssDNA by BmNPV Pol was strictly distributive: the enzyme dissociated from ssDNA by BmNPV Pol was strictly distributive: the enzyme dissociated from ssDNA after the release of a single dNMP from 3'-end of the polynucleotide and then reassociated with the next polynucleotide being degraded. The processivity of hydrolysis of dsDNA was determined by the primary and the secondary structure of the duplex. In the course of elongation on ssDNA the enzyme was highly processive. Secondary structures in DNA templates arrested driving of BmNPV Pol along the template and caused pauses in polymerization, indicating the purified BmNPV Pol possesses a low ability for synthesis with strand displacement. This result suggests that special DNA-binding proteins are needed for virus replication in the infected cells. Several 3'-modified nucleotide analogues (5'-triphosphates of 2', 3'-dideoxythymidine, 3'-amino-2', 3'-dideoxythymidine and 3'-fluoro-2',3'-dideoxyadenosine) inhibited BmNPV Pol but had no effect on the activity of cellular DNA polymerase α (the enzyme of nuclear DNA replication). These nucleotide analogues efficiently suppressed BmNPV replication in nuclei from the infected silkworms.

GENETIC ENGINEERING OF BACULOVIRUSES

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Insect pathogenic viruses, which cause disease in insects, are classified into seven families. The most common group is the family Baculoviridae, which is characterized by their circular double-stranded DNA genome and rod-shaped enveloped virion. Baculoviruses contain 90-160 kb closed circular double stranded DNA as a genome (5). These viruses are believed to contain more than 100 different genes in the genome. Sequencing and hybridization experiments reveal that the genome consists largely of unique sequences which may encode structural or nonstructural polypeptides. Coding sequences are localized tightly in the genome and spaced with short sequences of a junction containing a proposed promoter and poly(A) signal(s). Arrangement of the genes in baculovirus genomes is conserved throughout the various NPVs even between NPVs having relatively low DNA sequence homology.

I would like to discuss the expression of foreign genes in insect cells, especially in the silkworm larva using BmNPV vectors. Applications of baculoviruses to production of pharmaceuticals and genetically engineered viral insecticides are also discussed.

1. BACULOVIRUS AS A GENE TRANSFER VECTOR

As previously discussed by others (8,10), baculoviruses possess several unique characteristics that are extremely advantageous features for a useful expression vector. The virus has 1) a circular double-stranded genome, which is easily modified by a commonly used biotechnological techniques such as specific cleavage and ligation; 2) a rod-shaped capsid which allows it to contain extra DNA fragments; 3) a cell line which is susceptible to viral infection; and 4) the polyhedrin gene. This gene is suitable for the insertion of foreign genes, because it is non-essential for viral reproduction, is a late gene with a strong promoter, and is a marker, detected easily by light microscopy.

2. EXPRESSION OF FOREIGN GENES IN ESTABLISHED CELL LINES AND IN LARVAE OF THE SILKWORM

Many recombinant baculoviruses have been contructed and have successfully expressed different kinds of inserted foreign genes derived from a variety of sources from bacteria to human beings. With the BmNPV vector, the genes encoding the following proteins have been expressed: chloramphenical acetyltransferase, human interferon alpha (1,2,3), Insulin-like growth factor-II (IGF-II) (4), human interleukin 3, mouse interleukin 3 (6), human and mouse interleukin 4, HTLV-1pX(7), HTLV-1 *env* (Nyunoya, personal communication), human immunodeficiency virus (HIV-1) *gag*, *pol*, *sor*, gp41, and gp120, adenovirus E1A (Handa, personal communication), Japanese encephalitis virus *env* , bovine papillomavirus 1 (BPV1)E2(11),HPV6bE2(9,11), and human apolipoprotein A and E (Iwano, personal communication).

2.1 Efficiency of Production. The original viral products, i.e., the polyhedral proteins of BmNPV, generally comprise almost 20% of the total protein in infected cells, and subsequently increase to more than 50% after the degradation of other proteins at a very late stage of infection. About 20 mg of polyhedrin is produced in one fifth instar larva of the silkworm. In contrast, the

quantities of foreign gene products are relatively low, ususally less than a few percent of the original polyhedrin. The reasons for the reduced expression of foreign genes are complex and appear to be affected by several different factors.

One factor affecting the efficiency of the production of foreign gene products is the sequence between the promoter and the translation start. A 71 base sequence is highly concerned in AcNPV and BmNPV (3). Its importance is indicated by deletion experiments in which the removal of more than 10 bases upstream from the translational start in this conserved sequence results in the dramatic reduction of the expression of the foreign gene human interferon a, both in the established cell line and in the silkworm larvae(1).

The efficiency of expression of foreign gene products seems to depend primarily on the characteristics of the foreing protein. On average, levels of expression of nuclear localized or nonstructural proteins are highest, secreted proteins are intermediate, and enveloped proteins are least.

2.2 Cleavage of Signal Peptide and Secretion of Polypeptides. Proteins secreted from cells are first synthesized as precursor molecules containing hydrophobic, N-terminal signal peptides. The signal peptides are cleaved by a peptidase on the membrane of the endoplasmic reticulum when the proteins pass through the membrane.

To examine whether signal peptides of mammals are recognized and cleaved at the correct sites, the mammalian genes encoding secreted proteins have been introduced into BmNPV. The N- terminal amino acid sequences of the secreted polypeptides human interferon alpha (3) and mouse interleukin 3(6) synthesized by cells of *B. mori* are identical with those produced in the original mammalian cells. When silkworms are infected with the recombinant viruses, all foreign gene products are secreted into the hemolymph and the N- termini of the molecules are completely identical with those of mature polypeptides cleaved in mammalian cell lines. The results show that the signal sequences for the secretion of mammalian genes are recognized in the silkworm and are cleaved at the correct sites. This also indicates that the mechanisms involved in the recognition and cleavage signal sequences are conserved throughout many different species in the animal kingdom.

To confirm the importance of the signal peptides for secretion, a recombinant virus carrying human interferon alpha gene without the signal sequence has been constructed. The ATG for the translational start was inserted before the mature interferon sequence and the sequence corresponding to the signal peptide was completely removed by the mutation technique. When the virus infects cells of *B. mori,* most of the interferon activity is found within the cell and little is secreted. Furthermore, the total activity is quite low. Much of the interferon produced in the cytoplasm is probably degraded by endogenous proteinases (2).

2.3 Glycosylation. Glycosylation is a post-transitional modification found abundantly in membrane-bound and secreted proteins in eucaryotes. Some evidence suggests that some of the mechanisms of glycosylation in insects differ from those found in mammals. Glycosylation in mosquito cells is limited; that is, conversion to complex N-linked oligosaccharides does not occur and the structure of oligosaccharides are simple. Experiments aimed at producing glycosylated proteins in heterologous host cells may help to clarify mechanisms and functions of glycosylation.

We have expressed several genes for mammalian glycoproteins using the BmNPV expression vector, both in cultured insect cells and in silkworm larvae. Mouse interleukin 3 was expressed in silkworm larvae. In silkworm larvae, three distinct species of interleukin3 were produced with molecular weights of 22,20, and 18k daltons. The silkworm-produced polypeptides of interleukin 3 were all much smaller than those produced in mammals. Since N-terminal sequencing indicated that the three glycoproteins were identical in polypeptide sequence, the variation in molecular weights appeared to result from variation in glycosylation. This was confirmed in N-glycanase digestion experiments which showed that all species were converted to the sarne, approximately 15 kilodalton, core peptide. Thus, interleukin 3 produced in the silkworm is variably glycosylated and glycosylated to a much lesser extent than in mammals. The recombinant and mammalian interleukin 3 exhibited equal binding affinity, and equal potency in cell proliferation and colony formation assays. The same heterogeneity of produced interleukin 3 was observed when the established cell line was used.

3. USE IN PHARMACEUTICAL PRODUCTION

Many organizations have already started to produce pharmaceutical products using recombinant DNA technology. The pharmaceuticals produced by different procedures have to be tested carefully

and may require considerable time before they are ready for commercial use. Since the silkworm has been used as food by many animals including human beings, it would be a safe "factory" for the production of pharmaceuticals. The purification of the expressed products is important. The foreign gene products secreted into insect hemolymph, e.g., human interferon alpha (3) and mouse interleukin 3 (6), are easily purified by methods commonly used in *in vitro* culture. In the case of interleukin 3, the purification from the hemolymph was much easier than from transfected mammalian cultures containing fetal calf serum.

4. USE IN INSECT PEST CONTROL

Problems withthe use of chemical pesticides, e.g. mammalian toxicity, the appearance and spread of resistant insect pest species, are widely known. This has resulted in increased interest in the use of baculoviruses, some of which have been registered, and are commercially available as viral insecticides. The baculoviruses generally are of low virulence and cause larval death after relatively long periods of infection. Some workers have suggested that by introducing appropriate foreign genes into baculovirus genomes, pathogenicity and insecticidal effectiveness may be increased.

The first approach in the formation of a baculovirus insecticide is to make a recombinant baculovirus of increased toxicity by introducing appropriate foreign genes into the highly expressed polyhedrin gene site. Peptide toxins that block neuronal function and the important peptide hormones are currently being studied. Most of these toxins are toxic only to insects. Infection with such recombinant viruses may cause direct toxicity, alter the behavior, or arrest the development in insects. The onset of these effects may be much faster than the pathology caused by the original parent virus.

Stability of baculoviruses under field conditions is dependent on the occlusion bodies made up of polyhedrin. A recombinant virus still retaining its ability to produce polyhedrin is possible by introducing the foreign gene in the polyhedrin gene site without removing the polyhedrin gene itself. For example, the polyhedrin gene of *S. litura* NPV was introduced into the recombinant BmNPV at a site quite distant from the original polyhedrin gene location. The heterologous polyhedrin gene was expressed with high efficiency together with the human interferon gene.

Furthermore, if the promoter of the polyhedrin gene, used for the gene for insect toxin, is replaced with one of the early gene promoters of NPV or with a wild pest insect promoter which is highly activated in the target organs, such a virus may express the gene for insect toxin at an early stage of infection. If the right gene for insect toxin and the promoter can be isolated and introduced into a baculovirus, the resulting recombinant virus is likely to be an efficient viral insecticide.

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BmNPV-MEDIATED EXPRESSION OF SILKWMOTH CHORION GENES *IN VITRO* AND *IN VIVO*

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We have explored the possibility of utilizing the Nuclear Polyhedrosis Virus of *Bombyx mori* (BmNPV) as a vector for expressing silkmoth chorion chromosomal gene sequences *in vitro* and *in vivo*. To this end, we subjected the cloned polyhedrin gene of BmNPV to deletion mutagenesis and developed two types of vectors which can be used for transferring foreign genetic material into the genome of the virus by homologous recombination in silkmoth tissue culture cells. The first type of vector permits the insertion of foreign gene sequences adjacent to a transcriptionally active polyhedrin promoter which can direct the abundant transcription of passenger genes in cells infected with the corresponding recombinant viruses. We have used it for expressing complete chorion transcription units into Bm5 tissue culture cells. We will demonstrate that the resultant chorion primary transcripts are correctly spliced and polyadenylated in the tissue culture cells to produce, upon translation, chorion polypeptides which can be further characterized by immunoprecipitation with chorion antisera and one- or two- dimensional electrophoretic analysis. The same vector could be similarly used for the identification of other complex transcription units, particularly of insect origin, with the corresponding polypeptides.

The second type of vector was developed for allowing the *in vivo* expression of silkmoth genes under the control of their own promoter. It contains a mutagenized polyhedrin promoter element which is completely devoid of transcriptional activity. We have used this vector for inserting a 3.8 kb fragment of *B. mori* DNA into the polyhedrin gene deletion junction. The inserted DNA comprised a complete chorion gene pair, HcA/HcB.12, which included a common promoter element and considerable lengths of 3'-flanking sequences. Co-transfection of Bm5 cells with DNA from wild type BmNPV and the recombinant vector resulted in the creation, *via* homologous recombination, of recombinant viruses bearing the *Bombyx* chorion gene sequences. Silkmoth pupae of Gr^B strain which lack Hc genes were injected with BmNPV2.HcA/HcB12. Injected animals were dissected at different intervals and their ovaries analyzed for the presence of Hc mRNA sequences transcribed from the chorion genes transduced into the cells of the follicular epitelium by the recombinant virus. The results of our studies indicate that the transduced chorion genes are correctly expressed only in the ovaries of the infected animals but not in other tissues, which appear to accumulate randomly initiated chorion RNA sequences.

COMPARISON OF SEVERAL PROMOTER ACTIVITIES IN CULTURED CELLS AND SILK GLANDS OF *BOMBYX MORI*

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We intend to establish a transformation system at the level of individual cells in *Bombyx mori*. Before doing it, we need an assay system for promoter activity. We tested two systems, cultured cell and silkglands as candidates for the assay of the transient expression. A convenient cell line for transformation must first be established and then we tried to select three cell lines from one already established. Three sublines of SES-Bm-1 that we selected have characteristics of a short doubling time (48hrs), an appropriate absorption to the culture dish and the ability to be effectively transformed by the calcium phophate method. We considered these best to use for transformation.

Firstly we used a combination of hsp70 promoter of *Drosophila melanogaster* , the CAT gene and SV40 terminator as the control gene for the transient expression in cultured cells and silkgland cells. The hsp promoter activity in *Bombyx mori* cells was the same as that in *Drosophila hydei* and rabbit kidney cells. The promoters of fibroin and sericin genes are also active in the three cell lines described above except that they show lower activity than the hsp promoter. However, Moloney leukemia virus promoter is not active in *Bombyx mori* cells. We next established one more useful assay system by the injection of DNA into posterior and middle silkglands cells. We obtained results in the two parts of the silkgland similar to that in the cultured cells. Consequently, we consider that fibroin and sericin promoters seem to be active in our two transient expression systems regardless of cells types and parts of the silkgland.

A NEW *BOMBYX* TRANSPOSON INSERTED IN THE SERICIN II GENE

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An insertion interrupts the intronic region of one of the alleles of the highly polymorphic Sericin II gene. Six to twelve copies of this element are present within the *Bombyx mori* genome in all 30 of the European and Japanese strains screened. This 4561 base pair long element has direct terminal repeats of moderate length (78 nucleotides) and its insertion into the sericin gene has induced the duplication of a 5 nucleotide target sequence, characteristic of a transposition event.

The nucleotide sequence analysis reveals the existence of two open reading frames in the opposite orientation of that of the sericin gene. They occupy together more than 85% of the total sequence . ORF_1 is 257 a.a. in length and contains 2 cysteine motifs characteristic of nucleic acid binding proteins. The ORF_2, 1032 a.a. overlapping ORF_1 exhibits successively similarities with a protease like encoding sequence, a high level of homoloies to Reverse transcriptase-like enzymes.and characteristics of the retroviral endonucleases. Altogether the sequencing data suggest that we are dealing with a new insect retrotransposon which is more related to the copia-like family of *Drosophila* than to any other type on retrotransposa. However, up to now, no detectable transcript has been observed in the tissues analyzed. The presence of the long reading frames makes this transposon a good candidate for a potentially mobile element.

MOLECULAR AND EVOLUTIONARY BIOLOGY OF TWO SEQUENCE SPECIFIC RETROTRANSPOSABLE ELEMENTS FROM *BOMBYX MORI.*

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A fraction of the 28S ribosomal genes in some insect species are interrupted by two insertion elements, which we have called R1 and R2 (formerly called types I and II). Sequence analysis of these two elements from *B. mori* (R1Bm and R2Bm) and *Drosophila melanogaster* (R1Dm and R2Dm) indicates that they are retrotransposons. Sequence comparisons of the same element in these two distant species at the DNA and amino acid levels reveal conserved regions which may be important in the propagation of the elements.

Distribution: Because R1 and R2 occur at single insertion sites within the 28S gene it is possible to assay their presence in widely different organisms even though the level of sequence homology does not permit cross-hybridization. R1 and/or R2 elements are present in every Dipteran species tested, a species of wasp (order : Hymenoptera) and in a species of mealy bug (order: Homoptera), suggesting that they are widely distributed in most modern insects.

Phylogenetic relationships: We have conducted a comparison of the amino acid sequences of R1 and R2 with all other reverse transcriptase containing sequences. All elements can be divided into two major groups. The first group is composed of retroviruses, certain DNA viruses and transposable elements containing long terminal repeats (LTRs). R1 and R2 are grouped with a distinct class of transposons, including I and mammalian L1 sequences, which lack LTRs. These non-LTR elements contain a higher degree of similarity to fungal mitochondrial class II introns than to LTR containing retrotransposons.

Functional Expression: We have functionally expressed in *E. coli* the 1150 amino acid open-reading-frame encoded by the R2 element of *B. mori* . This reading-frame appears to be equivalent to retroviral pol genes which encode protease, RNaseH, reverse transcriptase and integrase activities. We show that the open-reading-frame from R2Bm encodes a double-stranded endonuclease (integrase) which can specifically cleave the 28S ribosomal gene sequence at the insertion site for R2. We have determined the nature of the double-stranded cleavage and the sequences important for recognition by the endonuclease.

STUDY ON THE BIOCHEMISTRY AND MOLECULAR BIOLOGY OF *ATTACUS RICINI* NUCLEAR POLYHEDROSIS VIRUS

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Attacus ricini nuclear polyhedrosis virus (ArNPV) is one of the members of the family Baculoviridae. This paper reports what we have studied about the biochemistry and molecular biology of the virus.

Structural proteins: three components of polyhedra of ArNPV were purified, the virions, nucleocapsids, and polyhedrin, and their polypeptide constitutions were determined. 23 structural polypeptides were revealed for the virion with molecular weights ranging from 10Kd to 105Kd. The nucleocapsids from ArNPV virion treated with NP-contains two predominant polypeptides with MW 36Kd and 32Kd, respectively. A single structural polypeptide was resolved for polyhedrin with MW 27Kd.

Alkaline protease: the alkaline protease present in the ArNPV and the characteristics of the protease were confirmed. The enzyme was purified by affinity chromatograhy with ConA-sepharose. The activity was inhibited by ions such as Hg^{++} and Cu^{++}.

Envelope of polyhedra (PE) : by electron microscopy of ultrathin sections of PE of ArNPV it was observed that lattice matrix protein and multiple capsules were arranged as a dots and rows pattern, and an outer layer. Chemical and biochemical analysis of the purified envelope gave: hexose 103.8 μg PE, pentose 0.343 μg PE, and comparison of the % of Si, P, S, Ca, Mg, and N, C, H, with ArNPV was done.

Viral DNA: Electron microscopy revealed that the genome of ArNPV is double stranded. The mean contour length of the circular molecules is 33 μm. The sedimentation coefficient ($S°_{20,W}$) is 53 S. The % of G+C is 69.6%. Its melting temperature (Tm) is 83.5°C and its cleavage thermal hyperchromic effect is 33.3%. ArNPV-DNA was digested with restricted endonuclease, and cleavage patterns obtained single and double digestion with endonuclease. By using λ-DNA +HindIII and Sppl-DNA+EcoRI fragments as standards, the MW of ArNPV obtained by summation of the MW of fragments ranged from $76X10^6$d to 81 X 10^6d.

Physical map: The physical map of the genome of ArNPV was constructed with restriction endonucleases BgIII, EcoRI, and Kpnl. We also contructed the physical map through triple enzyme digestion and with the help of computer analysis.

Location of polyhedrin gene: By using pMV-VI as a probe and labeling with 35s-dATP by nick translation. The Southern blot and hybridization results demonstrated that the nucleotide sequence of Sal-B, Hind-C and EcoRI-D restriction fragments of ArNPV-DNA were homologous to the cDNA of mRNA of the polyhedrin gene of AcNVPV. And so we suggest that these fragments might contain the coding sequence of the polyhedrin gene of the genome of ArNPV. We also used AcNPV-DNA HindIII-V fragments as a probe to locate the polyhedrin gene on the genome of ArNPV. By using M13 cloning/sequencing system, we have cloned the fragment of ArNPV-pStI into vector M13 p 18, and determined the sequence of the inserted DNA fragment.

Establishment of cell lines from pupal ovary of *Attacus ricini*: We compared the growth of *Attacus ricini* ovarian cells in different media. The data indicated that Medium BLM-TC100 is suitable for culturing pupal ovaries of *Attacus ricini*. Until now, we have gained 17th passage cells within 7 months and found that these cells were susceptible to *Attacus ricini* nuclear polyhedrosis virus.

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