

# sericologia

**Journal of Silkworms**

**Revue des Vers à Soie**

Publiée par la Commission Séricicole Internationale  
Published by the International Sericultural Commission  
25, quai Jean-Jacques Rousseau - 69350 La Mulatière - France

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## FOREWORD

Given the success of the first edition of the International Workshop on the Molecular Biology and Molecular Genetics of Lepidoptera, initiated by Marian Goldsmith and Fotis Kafatos, we had all agreed to pursue these meetings every three years. With Karen Sprague we took over the organization of this second workshop.

In spite of the absence of the USSR and Bulgaria, several representatives of which came in 1988, the number of countries participating still amounted to 13, Puerto Rico and Belgium being present this time.

The number of participants, which, at a point, we feared would be too high for the accommodation facilities of the Orthodox Academy of Crete, was in fact lower (66) than during the first workshop (79). This is partly due to withdrawals which occurred in early 1991 owing to the Gulf War and partly to the fact that some registered people were prevented from coming at the last minute (about 10 people altogether).

In fact, 53 % of the first workshop participants did not come back to Kolymbari in 1991 and 53 % of the second workshop participants were attending for the first time. If all come again to the third workshop, what a crowd that will make !

For my part, I appreciated very much the spirit in which the 2nd International Workshop on the Molecular Biology and Molecular Genetics of Lepidoptera was prepared and held. All the participants were very co-operative and understanding vis-à-vis the organizers and hitches were overcome calmly and good-humouredly.

Of course, everybody wished that the boat trip had taken place. Poseidon decided otherwise ! It will have to be next time...

This special issue of *Sericologia*, devoted to our workshop, is above all intended for refreshing memories. In fact, the abstracts of the talks do not reflect the great richness of the oral presentations and, particularly, of the discussions which took place throughout the week, outside the sessions themselves.

These informal and very fruitful contacts are indeed the main feature of the International Workshop on the Molecular Biology and Molecular Genetics of Lepidoptera, as envisaged by the pioneer organizers of 1988.

I am certain that all the participants share this view since they decided to gather together again in the same context and place in 1994.

On behalf of everyone, Karen and I thank Thomas Eickbush and Ingrid Faye for accepting to take over the organization of the third workshop.

See you soon...

G rard Chavancy,  
Editor



**But where is Karen ?...**

Photograph : Madeleine COULON-BUBLEX.

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## HORMONAL REGULATION OF HORMONE RECEPTORS AND TRANSCRIPTION FACTORS IN *MANDUCA* EPIDERMIS

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Regulation of epidermal gene expression by 20-hydroxyecdysone (20HE) and juvenile hormone (JH) requires the presence of intracellular receptors for these hormones which in turn may be regulated by the hormonal milieu. In *Manduca* epidermis JH is specifically bound by a 29 kD nuclear protein [Palli *et al.* (1990), *Proc. Nat. Acad. Sci USA*, 87, 796-800]. This protein is found in the epidermis throughout both the 4th and 5th larval intermolt periods and in the pupa, but decreases during the larval molt and disappears at the time of pupal commitment. Culture experiments showed that the disappearance at commitment was due to the action of 20-HE in the absence of JH. The use of allatectomized larvae showed that reappearance of the 29 kd protein in both the 5th instar and the pupa was dependent on the presence of JH during the ecdysteroid rise causing the respective molts. Thus, both ecdysteroid and JH regulate the presence of the JH receptors.

The 29 kD JH-binding protein was purified and proved to be N-terminally blocked [Prestwich, G. (1991), *Insect Biochem.*, 21, 7-15]. Limited digestion with the endoproteinase Lys-C provided partial amino acid sequences, two of which showed about 35 % and 47 % similarity to the bovine interphotoreceptor retinoid binding protein (Touhara, Atkinson and Prestwich, unpublished). Using oligonucleotides based on these sequences, we have identified by PCR and are characterizing two cDNAs that may encode the JH receptor.

In another approach to isolation of the JH receptor, we used the human retinoic acid receptor (hRAR) cDNA to isolate a gene from *Manduca* ("RAR") which is 66 % and 27 % similar in the DNA and ligand binding regions respectively to that of hRAR. Preliminary experiments indicate that this molecule binds neither iodovinylmethoprenol (a JH analog) nor iodoponasterone. The *Manduca* "RAR" is however 97 % and 67 % similar in the DNA and ligand binding regions respectively to DHR3, a *Drosophila* member of the steroid hormone superfamily (Koelle, Talbot, and Hogness, unpublished). Two "RAR" transcripts (3.8 and 4.5 kb) are expressed in the embryo, larva, and pupa at the time of the molts. Culture experiments showed that expression was induced in intermolt epidermis by 20HE with maximal expression occurring between 6 and 12 hrs followed by a subsequent decline by 18 hrs. Induction was primarily independent of protein synthesis, but the decline required this synthesis. In the absence of 20HE, the mRNA declines with a half line of 2 hrs. *In situ* show that the transcripts are present primarily in the nucleus after 3 hrs of 20HE (only the 4.5 kb transcript), then by 12 hrs are primarily in the cytoplasm (equal amounts of the two transcripts). Earliest expression is found in the bristle cells. Thus, the *Manduca* "RAR" encodes an ecdysone-inducible, DNA binding protein that likely is a transcription factor necessary for the cascade of gene activation and inactivation initiated by ecdysteroids at the time of the molt.

Supported by NSF and NIH.

**cDNA-CLONING, CHARACTERIZATION, AND EXPRESSION  
OF A PUPAL CUTICULAR PROTEIN GENE IN *GALLERIA  
MELLONELLA* L.**

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Transdetermination of epidermal cells, i.e. the switch from pupal to adult expression of cuticular protein genes, occurs in the pupa of *Galleria* during the first 24 h after pupal ecdysis. As a prerequisite for the molecular analysis of cellular events during transdetermination, a cDNA library was constructed utilizing mRNA isolated from epidermal cells of 3 h old pupae. cDNA was cloned in an expression vector (*Lambda gt11*). The library was screened with an antibody specific to cuticular proteins.

An insert of 1.2 kb was subcloned into a plasmid vector (pUC 9) and characterized by restriction analysis, sequencing, and hybrid selected *in vitro* translation. It corresponds to a pupal cuticular protein of 28 kD.

The fragment was used as a probe in Northern blot hybridization to trace the expression of the corresponding cuticular protein gene in the course of the first 48 h after pupal ecdysis. A transcript appears at the onset of transdetermination (pupal age 3 h) and disappears in the postdeterminative period before adult differentiation begins (pupal age 36 h).

## ISOLATION AND CHARACTERIZATION OF THE GENES FOR TWO CUTICULAR PROTEINS THAT ARE REGULATED DURING METAMORPHOSIS

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The cuticular proteins of *Hyalophora cecropia* can be separated into two distinct families, one is found in rigid cuticles, the other in flexible cuticles. Two major cuticular proteins, one from each family, were selected for further analysis. Data from protein micro-sequencing was used to design probes and primers that enabled us to select the corresponding cDNAs. The correct, cloned, cDNA for HCCP 12 was selected with a non-degenerate primer. For HCCP 66 we utilized PCR with highly degenerate primers (768-fold for the N-terminal region and 1536-fold for the internal sequence) to obtain a fragment of the correct cDNA and used this to select a cloned cDNA. The cDNAs were used in turn to isolate clones from a genomic library. Northern, Southern and RACE (rapid amplification of cDNA ends) analyses as well as sequence data were used to determine copy number and promoter usage in different anatomical regions and metamorphic stages. For one cuticular protein, HCCP 12, we found that a single gene and a single promoter are used in all three metamorphic stages. Both genes had introns interrupting the signal peptide, a common feature of cuticular protein genes. The conceptual translation of HCCP 12 is somewhat similar to flexible proteins from diverse species. HCCP 66 has provocative regions of identity to locust and *Drosophila* cuticular proteins. Although the two *Cecropia* proteins have limited sequence similarity, hundreds of bases, surrounding a GTCT repeat, in the upstream region of HCCP 12 are very similar to the 5' end of the intron of HCCP 66. These data have implications for students of metamorphosis and cuticle structure.

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## STORAGE PROTEIN GENE EVOLUTION

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The cDNA sequences for a number of storage proteins have been reported : arylphorin and the methionine-rich protein from *Manduca sexta* [Willot *et al.* (1989), *J. Biol. Chem.*, 264, 10952 ; Corpuz *et al.* (1991), *Insect Biochem.*, 21, 265 ; and Wang and Wells, unpublished] ; arylphorin and the methionine-rich protein from *Bombyx mori* [Fuji *et al.* (1989), *J. Biol. Chem.*, 264, 11020 ; Sakurai *et al.* (1988), *Nucleic Acids. Res.*, 16, 7717] ; a juvenile hormone-suppressible storage protein from *Trichoplusia ni* [Jones *et al.* (1990), *J. Biol. Chem.*, 265:8596] ; and arylphorin from *Calliphora vicina* [Naumann and Scheller (1991), *Biochem. Biophys. Res. Commun.*, 177, 963] ; and a flavin binding storage protein from *Heliothis virescens* [Miller *et al.*, unpublished]. In addition, cDNA sequences for two of the hemocyanin subunits from *Eurypelma californicum* have been published [Voit and Feldmaier-Fuchs (1990), *J. Biol. Chem.*, 265, 19447]. We thought it reasonable to test, as rigorously as possible, the suggestion that the insect storage proteins and the hemocyanins have evolved from a common ancestor, and to determine whether the various insect storage proteins are members of distinct gene families.

We first aligned the deduced amino acid sequences using the procedure of Feng and Doolittle [(1987) *J. Mol. Evol.*, 25, 351]. The aligned amino acid sequence was then used to produce an aligned nucleic acid sequence. The phylogenetic relationships between the proteins can be investigated using distance methods and the amino acid sequences [Feng and Doolittle] or the nucleic acid sequence [Li *et al.* (1985), *Mol. Biol. Evol.*, 2, 150] ; or by parsimony methods using the nucleic acid sequence [Lake (1987), *Mol. Biol. Evol.*, 4, 167]. Distance methods are based on calculations of the similarity of the sequences, whereas parsimony methods involve character analysis ; i.e. how many base changes are required to change one sequence into another ? The conclusions are as follows :

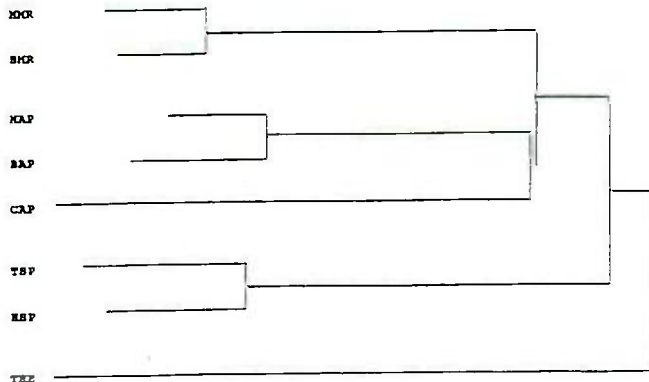
Both parsimony and distance methods yield the same tree.

The tree delineates gene families, not species, i.e. *M. sexta* and *B. mori* arylphorins are more closely related than *M. sexta* arylphorins and methionine-rich proteins.

The lineage of the hemolymph proteins is ancient and/or there are high mutation rates.

The fact that the hemocyanins and storage proteins fit in the same tree supports the suggestion of an ancient lineage.

It should be possible to find related proteins among hemimetabolous insects.



## STRUCTURE AND FUNCTION OF *BOMBYX* LECTIN

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We found proteins with a high hemagglutinating activity in the hemolymph of *Bombyx mori*. The activity was stage specific, peaking at just before the larval ecdysis and prepupal stage. Fluctuation of the activity followed changes of ecdysone-secreting activity of the prothoracic glands during larval development.

To identify which tissue produces hemolymph lectin, ovary, testis, fat body and hemocytes from 5th instar larvae were cultured *in vitro*. Among the tissues tested, hemocytes were expected to be a major source of the lectins. When 20-hydroxyecdysone was added to hemocyte culture medium, production of the lectin was remarkably enhanced, depending on the hormone concentration.

The proteins with hemagglutinating activity in the hemolymph of 5th instar larvae was purified by ammonium sulfate fractionation, gel-filtration on Sephacryl S-300 and affinity chromatography of glucuronic acid, which was a potent inhibitor of the hemagglutinating activity in the hemolymph. SDS-page and Western blotting analysis revealed that the molecular weight of the protein was about 350 kDa and a tetramer composed of two different subunits with molecular weights of ca 88,000 and 90,000. Monoclonal antibodies were prepared against purified 350 kDa lectin. Immunohistological observations revealed the occurrence of lectin in the lumen side of the epidermal cells and cuticular intima of the anterior silk gland of 5th instar larvae. Among the hemocytes, the fluorescence appeared in the granular cells, plasmatocytes and/or prohemocytes. The epidermal cells in the integument and the main tracheae were stained with fluorescent dye after apolysis. The imaginal disc cells were also stained but faintly.

By partial degradation of the hemolymph 350 kDa lectin, a 13 amino acid sequence of the fragmented protein was determined. From a cDNA expression library prepared from hemocyte poly(A) RNA, a clone which possessed a high cross-reactivity to the monoclonal antibody of 350 kDa lectin was isolated. Ongoing analysis of the DNA sequence of the clone may clarify the structure of the lectin gene and, accordingly, the 350 kDa lectin itself.

## IRON TRANSPORT AND STORAGE PROTEINS OF *MANDUCA SEXTA*

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Iron is essential to all organisms. Insects are no exception and, in the case of holometabolous insects, a special problem in storage and transport of iron arises. Some holometabola have access to iron only as larvae, but require it during metamorphosis and adult life. *Manduca sexta*, which is such an organism, uses both a transport protein, transferrin, and a storage protein, ferritin, in its iron economy. The transferrin of this species is a glycoprotein with an apparent molecular mass of 77 kDa, about the same size as mammalian transferrins. It differs, however, in that it binds a single ferric ion, while vertebrate transferrins have two homologous domains, each of which binds a ferric iron. The *M. sexta* protein was isolated and characterized, and polyclonal antibodies were raised in rabbits. Clones were selected from a cDNA library and sequenced. The deduced amino acid sequence indicates a mature protein of 73.4 kDa (excluding the carbohydrate content), which exhibits significant sequence similarity to the vertebrate transferrins. Although the two domains of the insect protein show sequence similarity, only in the N-terminal domain are there conserved amino acids corresponding to the putative iron-binding ligands, suggesting that ferric ion is bound only in the N-terminal domain. Antibodies to the *M. sexta* transferrin cross-react with proteins in the hemolymph of other lepidoptera.

After injection of *M. sexta* with a solution of ferric chloride, ferritin is loaded with iron, and is therefore easily isolated from the hemolymph in a single centrifugation step. This protein is stable to heating at 75 °C, and has a mass greater than 660 kDa. Denaturation of the native form in SDS, followed by gel electrophoresis suggests that the protein is composed of two principle subunits of 34 and 26 kDa, and a minor amount of a third of 32 kDa.

(Supported by an NIH grant, GM 29238)

## MOLECULAR BIOLOGICAL STUDIES OF MOTH OLFACTION

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Moths detect odors via sensilla arrayed along their antennae; somata of sensory neurons are embedded in the epithelium, ensheathed in 3 support cells; dendrites project into the external environment, axons project inwards to make synapses in the brain. Dendrites are incased in hollow cuticular hairs filled with proteinaceous fluid. This fluid contains odorant binding proteins (OBPs) and odorant degrading enzymes (ODEs) which are expressed by the neuronal support cells and secreted into the hair lumen. OBPs are thought to solubilize lipid odors into the fluid and aid in odorant transport through the fluid to receptors. ODEs are thought to rapidly degrade odors to maintain sensitivity to external fluctuations in odorant levels during flight. Sensilla are morphologically and functionally identifiable as pheromone specific or general odorant sensitive.

OBPs are represented by at least 3 classes: pheromone binding proteins (PBP) and the general odorant binding proteins GOBP1 and GOBP2. PBPs associate with pheromone specific sensilla and the GOBPs associate with general odorant sensitive sensilla. Partial N-terminal sequence of 14 OBPs, supported by full length sequence data of 7 OBPs indicates that PBPs are highly variable while GOBPs are highly conserved across species. Variation among PBPs is consistent with these proteins interacting with species specific pheromones while conserved GOBPs indicates that it is advantageous for all species to detect the same general odorant. GOBP1 and GOBP2 are only 50% identical, suggesting that general odors are divided into at least 2 chemical classes. Sensilla derive from epithelial stem cells by 10% of adult development. The stem cells divide and one daughter produces the neurons and one support cell while the other daughter produces the other 2 support cells. Axon and dendrite growth are complete by about 50% development, and final maturation of the antenna occurs at about 90% development with expression of OBPs and ODEs. Staged antennal fragments of male *Manduca sexta* were cultured for 24 hrs in the absence or presence of 20-hydroxyecdysone (20-HE). Removal of tissue from endogenous ecdysteroids led to premature expression of OBP which could be blocked by culture in the presence of 20-HE. This suggests that OBP expression is inhibited, but released when ecdysteroid levels decline to a critical level. This further supports the idea that the events of antennal development are temporally coordinated by circulating and changing ecdysteroid levels.

A 69 kDa membrane protein of *Antheraea polyphemus* pheromone specific sensory dendrites was previously shown to be a candidate pheromone receptor by labeling with photoaffinity analog of the sex pheromone. A 69 kDa protein is the most abundant protein of these membranes, and the sequence of this protein has been determined through cDNA cloning. The protein was purified and N-terminally sequenced, and this data was used to isolate positive clones. The protein, RP11, is homologous to a human derived protein, CD36. Human CD36 resides in platelet and endothelial cell membranes, has a single transmembrane domain, and binds either an erythrocyte (RBC) membrane protein or an extracellular matrix (ECM) protein to mediate cytoadhesion of infected RBC during malaria infection. By homology, moth RP11 may represent a new class of cell-cell or cell-ECM recognition receptor, and thus may be involved as a developmental regulator of neurite growth.

## ISOLATION, CHARACTERIZATION, AND cDNA SEQUENCE OF TWO FATTY ACID-BINDING PROTEINS FROM THE MIDGUTS OF *MANDUCA SEXTA* LARVAE

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Fatty acid-binding proteins (FABP) are members of a superfamily of hydrophobic ligand binding proteins. This superfamily contains low molecular weight proteins (14-17 kDa) which occur in the cytoplasm and bind ligands such as fatty acids, sterols, and retinol (Veerkamp *et al.*, 1991). Until recently, the only well characterized FABPs have been isolated from vertebrate tissues. Two abundant, low molecular weight proteins (MFB1 and MFB2), resembling the vertebrate FABPs, were purified from the midgut cytosol of larval *Manduca sexta*. Both proteins were found to bind fatty acids in a 1:1 molar stoichiometric ratio. Immunological screening demonstrated that both proteins were restricted to the midgut in a gradient distribution reminiscent of the mammalian liver and intestine FABPs.

The cDNA clones for MFB1 and MFB2 both encode proteins of 131 amino acids that are rich in lysine and acidic residues. Analysis of the progressive amino acid sequence alignment of the MFBs with 6 mammalian FABPs reveals a number of shared features : 9 conserved glycines, presumably important in turns of the  $\beta$ -strands ; a basic amino acid in the position corresponding to the residue reported to participate in binding the carboxyl group of the fatty acid ; and conservation of many of the residues important in binding the aliphatic portion of the fatty acid.

This work was supported by NIH grant HL39116 to M. Wells and NIH Fellowship GM13656 to A. Smith.

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PROTEIN STRUCTURE AND FUNCTION

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      *           *           *           *           *
MFB1  AYLGVVYKFDREENFDGFLKSIKGLSEEQVQKYLQYKPSQLVKEGDKYKYISVSSDGTKEVTFESGVE
MFB2  SYLGKVYSLVKQENFDGFLKSAKLSDDKIQALVSDKPTQKMEANGDSYSITSTGIGGERTVSFKSGVE
RFBL  NFSGK  YQVQSQENFEAFMKAMGLPEDLIQKGDIKGVSEIVHEGKVKVLTITYGSKVIHNEFTLGEE
HFBL  SFSGK  YQLQSQENFEAFMKAIIGLPEELIQKGDIKGVSEIVQNGKHFKFTITAGSKVIQNEFTVLEE
RFBI  AFDCT  WKVYRNENYEKFMKMGINVVKRLGAHDNLKLTITQEGNKFTVKESSNFRNIDVVFELGVD
HFBI  AFDST  WKVDRSENYDKFMKMGVNIIVKRLAAHDNLKLTITQEGNKFTVKESSAFRNIEVVFELGVT
HFBI  DAFLGT  WKLVDKSNFDDYMKSLGVGFATROVASMTKPTTIIEKNGDILTTLKTHSTFKNTEISFKLGVE
RFBH  ADAFVGT  WKLVDKSNFDDYMKSLGVGFATROVASMTKPTTIIEKNGDITITKTHSTFKNTEISFQLGVE
      ↑           ↑           ↑           ↑           ↑
      *           *           *           *           *
MFB1  TDDVVQGGPLPIKTTYTVDG  NVTQVVN  SAQGSATFKREYNGDELKVTITSSWDGVAYRYKA
MFB2  FDDVIGAGESVSKMYTVDG  NVVTHVVK  GDAGVATFKKEYNGDDLVTITSSNWDGVARRYKA
RFBL  CELETMTGEKVKAVVMEGDNKMVTTFK  GIKSVTEFNGDTITNTMTLG  DIVYKRVSGRI
HFBL  CELETMTGEKVKVTVQLEGDNKLVTTFK  NIKSVTELNDDIITNTMTLG  DIVFKRISKRI
RFBI  FAYSLADGTELTGTLTMEG  NKLVGKFKRVDNGKELIAVREISGNELIQTYTYE  GVEAKRIFKK
HFBI  FNYNLADGTELRGTWSLEG  NKLIGKFKRTDNGNELNVTREIIGDELVTQTYVYE  GVEAKRIFKDD
HFBI  FDETTADDRKVKSVVTLTG  GKLVLHQQW  DGQETTKVRELIDGKLILTLTHG  TAVSTRTYEKEA
RFBH  FDEVTADDRKVKSVVTLTG  GKLVLHVQKW  DGQETTLTRELSDGKLILTLTHG  NVVSTRTYEKEA
      ↑           ↑           ↑           ↑           ↑

```

\* = identical residues ; ↑ = residues involved in fatty acid binding in RFBI.

Per cent identity (based on aligned regions) :

	MFB1	MFB2	RFBL	HFBL	RFBI	HFBI	HFBH	RFBH	
MFB1 = Manduca FABP 1	MFB1		55.7	29.0	30.7	25.0	25.8	28.4	29.1
MFB2 = Manduca FABP 2	MFB2	55.7		29.8	32.3	20.3	20.3	33.9	34.7
RFBL = Rat FABP-liver	RFBL	29.0	29.8		81.8	28.2	24.8	26.4	25.6
HFBL = Human FABP-liver	HFBL	30.7	32.3	81.8		27.4	24.8	28.8	28.8
RFBI = Rat FABP-intestine	RFBI	25.0	20.3	28.2	27.4		80.8	32.0	32.8
HFBI = Human FABP-intestine	HFBI	25.8	20.3	24.0	24.8	80.8		33.3	32.6
HFBH = Human FABP-heart	HFBH	28.4	33.9	26.4	28.8	32.0	33.3		90.1
RFBH = Rat FABP-heart	RFBH	29.1	34.7	25.6	28.8	32.8	32.6	90.1	

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No comments...

## LINKAGE MAPPING OF INSECTICIDE RESISTANCE GENES IN THE TOBACCO BUDWORM, *HELIOTHIS VIRESCENS*

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The tobacco budworm is a serious agronomic pest in part because of its remarkable ability to evolve resistance to chemical insecticides. Our overall goal is to understand the molecular and population genetics of this rapid response to natural selection. The linkage map we have been developing for tobacco budworm currently has 21 of 31 chromosomes marked with marker genes (defined by allelic variation or RFLPs). We have used this tool to characterize different genetic mechanisms of resistance to various chemical and biological insecticides. *Aceln*, a locus conferring resistance to organophosphorus (OP) insecticides, maps to linkage group 2. This locus controls the sensitivity of acetylcholinesterase to OP inhibition and may be the structural locus for acetylcholinesterase. Analysis of resistance to pyrethroid insecticides shows that loci on at least three chromosomes make a contribution. One of these encodes a sodium channel, the putative target site of this class of insecticides. Analysis of the resistance to the delta-endotoxin of *Bacillus thuringiensis* reveals at least two chromosomes with a major effect on resistance, but the mechanisms involved are still unknown. The linkage mapping approach, since it allows the detection of multiple genetic mechanisms in resistant strains and their subsequent segregation into separate strains for further characterization, will greatly facilitate the molecular analysis of insecticide mode of action and resistance mechanisms in this economically important pest species.

## THE *BOMBYX* GENOME MAPPING PROJECT

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The use of one organism as a reference for basic and applied research is a powerful strategy for rapid acquisition of information and its transfer to a wide range of related species, which are often more difficult to study in the laboratory. With its well-developed genetics and large collections of mutant and inbred wild type strains, the silkworm, *Bombyx mori*, is the species of choice to serve as a model for the lepidoptera. The aim of the *Bombyx* genome mapping project is to construct a molecular map integrated with the classic genetic map at a resolution that will enable cloning of genes, mutations, and chromosome segments of interest for fundamental biological studies, application of genetic engineering techniques, and development of new breeding strategies for silkworm strain improvement.

*Bombyx* has a haploid genome size of 0.5 pg DNA, or about 530 Mb. There are 28 chromosomes; with 205 markers (H. Doira, personal communication), the genetic linkage map totals about 1 000 cM (centiMorgans), or about 530 kb per cM. The initial goals of this project - the first five year plan - are to construct a molecular map at 10 cM resolution, and to begin mapping complex, polygenic traits or QTLs (quantitative trait loci), the targets of practical breeding. Based on the segregation of characters such as heat tolerance, disease resistance, and fecundity in inbred strains, mapping of QTLs promises to uncover new classes of genes and gene complexes of potential interest for basic as well as applied research. Work in a number of plants suggests that we will probably have to map initially about 300 randomly chosen markers to achieve uniform spacing at this resolution.

We plan to use two approaches for detecting molecular markers or RFLPs (restriction fragment length polymorphisms), namely Southern hybridization of genomic blots made from individual segregants of F<sub>1</sub> backcrosses and F<sub>2</sub> crosses, and PCR-based amplification of random arbitrary DNA primers (the so-called Random Amplified Polymorphic DNA or RAPD technique) [Williams J.G.K. *et al.* (1990), *Nucleic Ac. Res.*, 18, 6531]. For Southern probes, we are using characterized *Bombyx* clones for single and low copy number sequences; there is also the possibility of using conserved sequences from other lepidopteran species. In addition, we plan to screen high complexity cDNA libraries for suitable probes. Testing of several inbred strains with chorion, fibroin, rDNA, and Bm Antp clones as probes suggests that RFLPs for expressed sequences are easily found in this species after digesting DNA with 6 base recognition site restriction enzymes. If necessary, we will also screen genomic DNA libraries for additional probes. Finally, we plan to test the feasibility of using the RAPD screening technique, which has major advantages over Southern blots in that the defined oligomers used for detecting DNA level polymorphisms can be made available as published sequences, it can easily accommodate large sample sizes, and the amount of test DNA needed for screening is small.

Although we plan to construct the initial map using about 60 progeny from a single F<sub>2</sub> cross, for the longterm, we are also developing recombinant inbred (RI) lines using the same parental strains (p50, a tropical race, and C108, an improved Chinese race) [Burr B., Burr F.A. (1991), *TIG*, 7, 55]. These homozygous stocks of genetic recombinants generated by successive sib matings from an initial cross between genetically distant parents; theoretically, it takes 8-10 generations to breed homozygous RI lines, or 2-3 years with average silkworm strains. RI lines can then be used as a cumulative database for constructing linkage maps, and, ultimately, for generating homogeneous

populations carrying known genetic variants or gene combination that can be used for testing environmental and other conditional effects on phenotypic expression patterns.

Although I will describe strategies and results from my own laboratory, other groups actively involved in the mapping project include H. Fujii and H. Doira, Kyushu U., M. Kobayashi, T. Shimada and H. Fujiwara, Tokyo U., W. Hara, T. Tamura, and O. Ninaki, National Institute of Sericultural and Entomological Science, Tsukuba, H. Maekawa, National Institute of Health, Tokyo, R.K. Datta, S.N. Chatterjee, J. Nagaraju, and E.G. Abraham, Central Sericultural Research and Training Institute, Mysore, and P. Saksoong, Kasetsart U., Bangkok. I invite the participation of the greater lep community in this project, and hope to engage in discussion about important practical issues, such as obtaining and making clones available, and setting up a readily accessible database.

Supported by the Carolyn and Kenneth D. Brody Foundation.

## HIERARCHICAL CLUSTERING OF 54 RACES AND STRAINS OF MULBERRY SILKWORM, *BOMBYX MORI* L. : SIGNIFICANCE OF BIOCHEMICAL PARAMETERS

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Domestication of mulberry silkworm, *Bombyx mori*, for more than 2500 years led to differentiation into Chinese, Japanese, Asian and European varieties (Gamo, 1983). They are either univoltine, bivoltine or polyvoltine. Looking from the economic viewpoint, different silkworm varieties can be broadly grouped into two classes : (i) one having poor cocoon and yarn character but higher survival potential in tropical areas, (ii) the other group is characterised by higher silk return and better yarn quality but low adaptation to tropical conditions (Chatterjee and Datta, 1989). Recent studies in this institute indicate the presence of differential activity of different enzymes in varieties having different yield potentials. With this background an attempt has been made to test whether correlation between biochemical markers and yield variables can be utilised for classifying the silkworm varieties into different clusters and whether such classification corroborates the geographical variation. Accordingly, an estimation of the activities of amylase, invertase, alkaline phosphatase and protease (pH 7 and pH 10) enzymes in digestive juice and trehalose content in haemolymph of V instar larvae of *B. mori* was carried out. The 54 strains of silkworm used in the study include germplasm collections from India, China, Japan, Korea, Brazil and France. The yield parameters considered are : weight of 10 mature larvae, cocoon shell/single cocoon weight and effective rate of rearing (a measure of survival potential). The estimates of variables were used for hierarchical cluster analysis (Peters and Martinelli, 1989) (UPGMA) on the basis of (i) yield parameters (ii) yield parameters with amylase and invertase activity and (iii) all 10 variables.

Results indicate that with yield parameters alone, 7 clusters can be realised, wherein Pure Mysore and NB1 have maximum distance between them. It is also of interest to note the clustering together of NK4, Nong51 and Boropolu, the coefficient ranging from  $1.9 \times 10^3$  to  $3.1 \times 10^3$ . Inclusion of biochemical parameters for clustering resulted into two broad groups : one having all the breeds with high cocoon weight and shell weight while the other group includes all low yielding silkworm strains from India and also other countries. The application of quick clustering with 10 variables was used to group all the breeds into 5 clusters. The maximum distance observed was between cluster 5 (Moria, NK4, Rong dazao, Dao9 and PCN) and cluster 1 (NB18, NB1, NBHPO, French, French batique, C124, etc.) having different geographic origins.

It is also of interest to note that cluster 1, which includes most of the bivoltines, has the lowest cluster value for amylase (249.9  $\mu\text{g}$ ) and/or highest cluster value for alkaline phosphatase (21.0  $\mu\text{g}$ ). Evolutionary aspects of the genetic channelisation of silkworm races in different countries are discussed against the background of the differences in the biochemical parameters and yield variables of the mulberry silkworm, *B. mori*.

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## CODON USAGE IN HEMOLYMPH PROTEINS AMONG LEPIDOPTERANS

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Codon bias patterns were examined for the 23 known hemolymph proteins from 5 Lepidopteran species. These are *Manduca sexta* : Ala-serpin, microvitellogenin, apolipoprotein III, arylphorin alpha- and beta- subunits, methionine-rich storage protein, transferrin, P4, cecropin B5P ; *Bombyx mori* : 30K proteins 1-5, anti-trypsin, storage proteins 1 and 2 ; *Heliothis zea* : JH esterase ; *Hyalophora cecropia* : cecropins A-D, lysozyme ; *Trichoplusia ni* : acidic JH suppressible protein 1. Comparisons are made between species, within groups of related proteins, and between codons for specific amino acids. In general codon bias is strong but with considerable heterogeneity among genes in a given genome, as among other animals [Li W-H., Graur D. (1991), *Fundamentals of Molecular Evolution*, Sunderland MA : Sinauer Press, 284 pp.].

Codon Frequency Table for 23 Hemolymph Proteins

<u>F</u>	TTT	107	<u>R</u>	CGT	98	<u>T</u>	ACT	125	<u>Q</u>	CAA	141
	TTC	391		CGC	85		ACC	143		CAG	153
<u>L</u>	TTA	67		CGA	34		ACA	83	<u>N</u>	AAT	155
	TTG	146		CGG	17		ACG	55		AAC	350
	CTT	118		AGA	98	<u>A</u>	GCT	219	<u>K</u>	AAA	255
	CTC	158		AGG	62		GCC	182		AAG	378
	CTA	61	<u>S</u>	TCT	94		GCA	93	<u>D</u>	GAT	173
	CTG	178		TCC	91		GCG	104		GAC	365
<u>I</u>	ATT	156		TCA	57	<u>G</u>	GGT	135	<u>E</u>	GAA	267
	ATC	224		TCG	54		GGC	149		GAG	243
	ATA	65		AGT	60		GGA	129	<u>C</u>	TGT	35
<u>M</u>	ATG	294		AGC	146		GGG	23		TGC	68
<u>Y</u>	GTT	172	<u>P</u>	CCT	115	<u>X</u>	TAT	105	<u>W</u>	TGG	114
	GTC	241		CCC	116		TAC	382	<u>X</u>	TGA	5
	GTA	110		CCA	87	<u>H</u>	CAT	54		TAA	15
	GTG	160		CCG	61		CAC	125		TAG	3

## MOLECULAR SYSTEMATICS APPLIED TO LEPIDOPTERA AND THEIR CHORION SEQUENCES

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Alignable coding regions of forty-three chorion sequences from *Bombyx mori* and *Antheraea polyphemus* have been analyzed phylogenetically using the HENNIG86 and PAUP 3.0 software packages. The hypothesis that the root for the bombycoïd chorion gene tree should be placed between the  $\alpha$  and  $\beta$  branch sequences is supported both by outgroup comparison and midpoint rooting. Within each species, sequences expressed over similar timeperiods typically form monophyletic groups; this is most simply explained by rapid rates of gene duplication and loss relative to changes in timing of expression. When all  $\beta$  branch sequences are analyzed together *Bombyx* and *Polyphemus* sequences form nonoverlapping sister groups, with one exception (*m5H4*). A likely interpretation is that most of the observed chorion gene duplication and diversification has occurred since separation of the *Bombyx* and *Polyphemus* lineages. The one exception may result from orthology or close paralogy; that is, *m5H4* is a gene that has not duplicated since separation of the lineages and thus pairs with another *CB* sequence from *Polyphemus* (*pc404-H12*). Inclusion of chorion sequences from *Hyalaphora cecropia*, a member of the same taxonomic family as *Polyphemus*, results in their placement within the *Polyphemus* group. This places historical boundaries on the timing of *Bombyx*- and *Polyphemus*-specific chorion gene duplication and diversification.  $\alpha$  branch sequences yield a less resolved tree that is still consistent with the above conclusions.

In a separate project, we have initiated systematic studies of deep taxonomic splits within the Lepidoptera, using nucleotide sequences as character data. Our initial aim is to identify several previously untested genes that will generate phylogenetic hypotheses concordant with those strongly supported by previous morphological studies. Subsequently, we will initiate studies of the morphologically homogeneous and largely uncharacterized ditrysian superfamilies, whose members constitute 98 % of the modern day species diversity. Towards this goal, we have applied criteria of gene structure and evolution and systematics to search the nucleic acid databases for potentially informative cloned sequences. Of the 23 arthropod genes selected, most were tested in primitive Lepidoptera for cross-hybridization and gene copy number. The most promising candidate genes are now being amplified via PCR in a variety of Lepidoptera as prelude to their sequence analysis.

## A PHYLOGENETIC ANALYSIS OF MOTH CHORION GENE FAMILIES

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The molecular analysis of lepidopteran chorion sequences was extended from silkmoths (superfamily Bombycoidea) to gypsy moth (superfamily Noctuoidea) in order to understand to evolution of the chorion gene superfamily and to place choriogenesis in a broader phylogenetic context. Transmission electron micrographs of eggshells indicate that lamellogenesis in both superfamilies involves early period lamellar framework formation, followed by expansion and densification. SDS-PAGE of chorion proteins synthesized from progressively more mature follicles show generally similar protein sizes, complexities and patterns of synthesis.

A gypsy moth cDNA library was constructed from chorionating follicle cell poly A+ RNA and differentially screened for the abundantly expressed chorion RNAs. Initially, one cDNA was sequenced and compared to the bombycoid chorion sequences. The sequences share 86 % nucleotide similarity over a 124 nt region and similar hexapeptide repeats in a central domain, establishing homology of Ld3 to the bombycoid chorion gene sequences.

Partial sequence analysis of additional cDNAs has identified at least 20 chorion sequences. Of these, 10 have been sequenced in the appropriate regions and used for further analyses. As in the bombycoids, the gypsy moth chorion sequences cluster into two groups (previously called  $\alpha$  and  $\beta$ ) based on coding lengths and the presence of group-specific motifs. Each group possesses a highly conserved region, i.e. the above mentioned central domain.

The alignable portions of all sequences were analyzed phylogenetically using HENNIG86. Putative  $\alpha$  and  $\beta$  branch sequences from both superfamilies clustered separately, providing strong evidence that the origin of the  $\alpha/\beta$  split preceded the noctuoid/bombycoid split. Phylogenetic analysis of  $\alpha$ , and separately of  $\beta$  branch sequences, resulted in species-specific clustering on the tree. This implies that diversification of the gypsy moth genes occurred after the noctuoid/bombycoid split.

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No comments...

## STRUCTURES AND EXPRESSION OF THE MAJOR PLASMA PROTEIN GENES OF *BOMBYX MORI*

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The major plasma proteins, termed "30K proteins", accumulate in the larval hemolymph of *Bombyx mori* in a stage-dependent fashion. To study the mechanism underlying the developmental regulation of gene expression in *B. mori*, we have analyzed the structures and expression of the genes for 30K proteins.

Three genes, each encoding a distinct 30K protein, are clustered on a 46kb region of chromosomal DNA. Sequence analyses revealed that all the 30K protein genes are structurally similar to each other; each gene is composed of a short 1st exon and a protein-coding 2nd exon interspersed by a single intron. Typical TATA box structure and octamer-like sequences occur in the promoter region of each gene. Retrotransposon-like **Bm1** repeats are also present in the 5' upstream region or the intron of each gene. The nucleotide sequences of the 30K protein genes as well as the deduced amino acid sequences of exon are highly homologous to those for microvitellogenin of *Manduca sexta*, suggesting that these plasma protein genes have evolved from a common ancestor.

The transcription initiation site of the 30K protein gene was determined at the nucleotide level. S1 nuclease protection analysis using intron-labeled probe provided evidence that the expression of 30K protein genes is regulated in a stage-dependent manner at the level of gene transcription. Gel retardation analyses indicated the presence of fat body nuclear factors that interact with the 5' upstream region of the 30K protein gene.

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## CHROMOSOMAL FRAGMENT CARRYING THE LARVAL BODY MARKING GENE, STRIPED ( $p^S$ ), OF *BOMBYX MORI*

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Mottling is a particular type of mosaic ; it exhibits an intricate pattern of small patches of two allelic characters in the same individual. Several such genetic mosaics, called "madara", for larval body marking of the silk worm have been induced by X-ray irradiation. Genetic and cytological studies have suggested that they are probably not due to alterations in gene expression ; rather, the somatic loss of chromosomal fragments carrying the genes responsible for larval body marking is presumed to cause the mosaic pattern. At the molecular level, however, the existence of such a chromosomal fragment has not been demonstrated.

Using pulsed field gel electrophoresis (PFGE), I have examined DNA migration patterns in two kinds of mottled mosaic strains (mottled striped strains,  $p^{Sm788}$  and  $p^{Sm872}$ ), and found DNA molecules of about 2.3-2.5 megabases (Mb) only in the mosaic strains. These DNA fragments hybridize strongly with some chorion genes, which are located less than 6.9 cM away from the locus for the larval body marking gene,  $p^S$ , indicating that the fragments contain genes for both striped marking ( $p^S$ ) and the chorion. In the non-mottled  $p^S$  strain, the phenotype before X-ray irradiation, which should not have any chromosomal fragment, no band was detected either on a PFGE gel or after hybridization with the chorion probe. These results suggest that the mottled  $p^S$  strains carry short chromosome fragments which are lost differentially during cell division.

In order to isolate the larval body marking gene  $p^S$ , I have constructed a partial genome library from a chromosomal fragment fractionated by PFGE, using PCR with short adaptors. The library contains a large amount of chorion genes and BmI repeated sequences, but no BmC repeated sequence, suggesting faithful construction specifically for a chromosomal fragment. We are trying to isolate the larval body marking genes from this library.

## THREE EARLY GENES COPIES DISRUPT THE CONTINUITY OF THE LATE LOCUS OF *B. MORI* CHORION GENES

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The morphologically complex eggshell of lepidopteran insects (chorion) is the result of a strictly defined differential synthesis and excretion of more than 150 proteins which are coded by a superfamily of genes. The chorion genes of *Bombyx mori* are clustered in two genetic loci (Ch1-2 and Ch3) of chromosome 2 separated by approximately four map units. Genes which are expressed early in choriogenesis are clustered in chorion locus Ch3, while Ch1-2 contains all the late genes in a continuous region of approximately 140 kb which is flanked by two regions containing most of the middle genes. The typical organization of silkmoth chorion genes is that of  $\alpha/\beta$  divergently transcribed gene pairs which share a short common 5' flanking region (less than 350 bp). The two genes of a gene pair are expressed with the same developmental specificity and share certain sequence characteristics, for example, HcA/B gene pairs are expressed late in choriogenesis and encode high cysteine-rich proteins. Excluding pseudogenes, up to the present only one unpaired silkmoth chorion gene was known, which belongs to the early CB family of *B. mori* genes.

Recently we found that three  $\beta$ -type early chorion gene copies (6F6.1, 6F6.2 and 6F6.3) are dispersed in the late region of chorion locus Ch1-2. Hybridization data indicate that 6F6.2 corresponds to the previously characterized m6F6 cDNA clone and that 6F6.1 and 6F6.3, but not 6F6.2, have next to them  $\alpha$ -type genes. Determination of the complete sequence of 6F6.1 and of the major exon of the A-gene near it shows that these two genes have a convergent rather than a divergent direction of transcription and thus do not constitute a typical gene pair. The sequence data further suggest that 6F6.1 is transcriptionally active. The location of the 6F6 gene copies at points where the polarity of their neighbouring gene pairs is inverted. This could be the result of two tandem duplication and inversion events. Detailed analysis of the 5' flanking region and the intron of 6F6.1 shows that they contain sequences which are homologous to *B. mori* Bm1 repeat elements. Interestingly, the Bm1-type segment of the intron is interrupted by the insertion of a sequence which shows 83 % similarity with part of an intron of a *B. mori* and *B. mandarina* fibroin gene and 75 % similarity with part of the 3' untranslated region of *B. mori* PTTH gene. Moreover, following the Bm1-homologous sequence of the 6F6.1 5' flanking region and preceding the gene promoter region, a short sequence shows sequence identities to nucleotides 40 to 76 of the ErA.1 promoter region. The occurrence of these sequences near one end or within the Bm1 repeat element is suggestive of complex sequence transfer events. Comparative analysis of known *B. mori* chorion  $\alpha$ -gene promoter and of Bm1 repeat elements shows that these two sets of sequences contain common elements. Statistical analysis does not preclude the possibility of chance similarity and therefore a common evolutionary origin of chorion  $\alpha$ -gene promoters and Bm1 elements cannot be inferred. However, the operation of sequence shuffling events seems a likely hypothesis and it should be interesting to examine the possible role of shared and/or unshared very short segments.

**THE ISOLATION OF GENOMIC SEQUENCES FROM  
*MANDUCA SEXTA* RELATED TO *DROSOPHILA*  
*MELANOGASTER* DEVELOPMENTAL GENES**

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The metameric organization of the insect body is specified early in development by a hierarchy of gene interactions. For long germ band insects such as the fruitfly, *Drosophila melanogaster*, the entire embryo is subdivided into segments nearly simultaneously, whereas in short germ band insects such as the tobacco hornworm, *Manduca sexta*, at first only a head region is defined and segments are added progressively as a posterior bud elongates. Several gap genes have been identified in *Drosophila* that divide the embryo into broad domains and provide positional signals for proper segmentation. These genes encode transcription factors that exert their function by forming overlapping gradients along the longitudinal axis. If gap genes exist in *Manduca*, they must have their own distinct patterns of spatio-temporal expression reflecting the different mode of segment formation. Using PCR technology, sequences similar to gap genes and other developmentally relevant genes of *Drosophila* have been isolated from the *Manduca* genome. Sequence analysis suggests that these clones represent *Manduca* homologs of the corresponding *Drosophila* genes and conserved domains can be identified that may be of functional significance. Future experiments are planned to study the regulation and pattern of expression of these genes during *Manduca* embryogenesis and to examine the structural and functional relationship between the *Manduca* and *Drosophila* gene products.

## FUNCTIONAL ANALYSIS OF THE EARLY PROMOTER OF A BACULOVIRUS ENVELOPE GLYCOPROTEIN GENE

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Transcription of the baculovirus (OpMNPV) envelope glycoprotein gene (gp64) in infected *Lymantria dispar* cells is regulated by both early and late promoters. To examine the regulation of the gp64 early promoter, we constructed promoter-reporter (gp64-CAT) fusions and examined transient expression of these constructs in uninfected cells of several insect cell lines. By analysis of 5' deletion mutations, we identified upstream regions containing putative negative and positive regulatory elements. In addition, 5' deletions containing only 34 nt upstream of the transcription start site were transcriptionally active. Transcription was abolished when the TATA box was deleted.

We previously identified a 4 nt sequence (CAGT) conserved at the transcription start sites of several baculovirus early genes and numerous insect genes. To determine the sequence requirements for transcription initiation, we synthesized and cloned a 43 nt synthetic promoter which consisted of gp64 sequences from the TATA box through the transcription start site. Analysis by transient expression and *in vitro* transcription demonstrated that the 43 nt synthetic promoter was transcriptionally active and initiated accurately.

To investigate the function of sequences within the 43 nt synthetic promoter, we generated a series of Linker Scanning (LS) mutations. In addition, we also generated mutations that altered the spacing between the conserved TATA box and CAGT motif. Mutations within the TAT box or CAGT motif (at the transcription start site) resulted in decreased or undetectable CAT activity in transient expression assays. Mutant promoters were also analyzed for transactivation by IE1. Analysis of LS mutations by *in vitro* transcription showed that only mutations in the TATA box eliminated accurate transcription initiation. Mutations that altered the spacing between the conserved TATA box and start site CAGT were also examined to determine which element was responsible for start site selection. Both LS and spacing mutations indicate that the TATA box is the primary element controlling start site selection in the synthetic promoter.

Preliminary experiments in which the TATA box was deleted from larger promoter constructs indicate that the gp64 early promoter may contain an additional basal promoter element that is TATA independent. Deletion and LS mutagenesis experiments suggest that the second element is not located within sequences downstream of the TATA box.

## RETROTRANSPOSABLE ELEMENTS R1 AND R2

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R1 and R2 are non-LTR retrotransposable elements that exclusively integrate into specific sites in the 28S ribosomal RNA genes. They have been found in a few to over 50 % of the 28S genes from most insects (43 of 47 species tested), as well as in representative species of millipedes, centipedes, and arachnids. Experiments concerning the possible mode of evolution of these elements and the mechanisms they use for sequence-specific integration will be discussed.

Nucleotide sequence analysis of R1 and R2 from a number of insect species suggests that these elements have been horizontally transferred between different taxonomic groups. R1 or R2 from closely related species, or even the same species, were sometimes found to be very divergent in sequence. Conversely, R1 and R2 elements from divergent insects were sometimes found to be similar in sequence. For example, two families of R1 were found in the gypsy moth, *Lymantria dispar*. One of these *L. dispar* R1 families has greater sequence similarity to the R1 element of the silkworm, *Bombyx mori*, than it has to the other R1 family in *L. dispar*.

Our efforts to understand the mechanism by which R1 and R2 are able to integrate specifically into the 28S genes have focused on the enzymatic activities synthesized by these elements. R2 elements encode a single open-reading frame (ORF) approximately 1100 amino acids in length that contains reverse transcriptase, endonuclease and nucleic acid binding (gag-like) domains. This ORF from the R2 element of *B. mori* has been expressed in *E. coli*. An endonuclease is made which recognizes a 30 bp region of the 28S gene and specifically cleaves the DNA at the insertion site. Surprisingly, RNA is required for the enzymatic activity of this enzyme. The properties of the RNA-protein complex formed by this ORF and a possible role of the RNA component will be described. A model will be given for the integration of R2 elements, and non-LTR elements in general, which accounts for our observations that (a) the 5' end of the elements can be any sequence, (b) the presence of highly conserved sequences near the 3' end of the elements, and (c) the requirement of RNA for endonuclease cleavage.

## Section : Transgenic expression

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## TOWARD TRANSGENIC SILKWORMS

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Transgenic *Bombyx mori* would be a very valuable tool for the study of the regulation of gene expression during the development of the animal. For this purpose, we recently made preliminary experiments to assay the feasibility of genetic manipulation of the silkworm genome.

By injecting naked heterologous linear DNA into the egg, we were able to recover somatically transformed animals which have integrated the foreign sequences, but in a modified form. The improvement of the technique and the possibility of transforming the germ line is under study.

Another approach is to make use of the retrotransposon, Mag, to construct a specific vector for inserting foreign DNA. This element has many of the characteristics of the Gypsy family mobile elements found in *Drosophila* and yeast, particularly in the coding sequence, which is organized in two open reading frames encoding a gag-like and a pol-like protein, respectively. The most distinctive feature of Mag is the presence of short direct terminal repeats 77 nt long instead of typical LTRs. However, the existence of a putative internal promoter and of a polyadenylation signal within these terminal repeats, which, moreover, are bordered by a tRNA complementary sequence and a polypurine stretch, suggests that Mag could function in the standard way. In order to use this element as a gene vector we have synthesized its proteins by introducing the coding sequence into a producing baculovirus. Results indicate that the ORF1 encoded peptide is efficiently produced, but that the ORF2 one is poorly synthesized, probably because this necessitates the occurrence of a special frame-shifting event.

In order to construct a helper vector able to produce Mag proteins, we tested the activity of ubiquitous promoters such as those of the actin A3 gene and the immediate early gene of the *Autographa baculovirus*. Both are highly efficient in promoting the activity of a reporter gene in embryonic vitellogenins and in different cultured insect cell lines. Their activity in the embryo itself remains undetectable. Experiments are in progress to use these promoters to direct the synthesis of Mag proteins within the cells of very early embryos.

## DEVELOPMENT OF A PLASMID VECTOR PROMOTING STABLE CHROMOSOMAL INTEGRATION OF TRANSGENES INJECTED INTO PREBLASTODERM STAGE SILKMOTH EMBRYOS

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Preblastoderm stage embryos of the domesticated silkmoth, *Bombyx mori*, were injected with a plasmid vector containing a complete copy of a *Bombyx* dispersed repeat element and a marker sequence derived from the 2  $\mu$ m plasmid of the yeast, *Saccharomyces cerevisiae*. Injected embryos were allowed to develop to maturity, crossed to each other and sacrificed after mating. PCR analyses of residual gonad DNA demonstrated that over 70 % of these adults carried the injected vector DNA in their gonads (spermatogenic cells and follicle) and that all positive individuals contained the injected vector sequences as integral parts of their chromosomes.

Crosses amongst the F1 progeny of injected individuals were subsequently carried out and the gonads of the F1 generation (parents of F2) were similarly analyzed by PCR to determine whether the injected vector persisted in the F1. Of 32 F1 individuals analyzed, all of them progeny of a cross between two injected parents, 28 (14 males and 14 females) were found to be positive while 4 females were found to be negative. This transmission rate suggests that chromosomal integration of the vector sequences occurs prior to or at the time of establishment of the germ cell progenitors. Quantitative PCR analyses revealed that, under the specific injection conditions employed in our experiments, F1 progeny carry between 10 and 100 integrated copies of the vector sequences per haploid genome.

Analyses of genomic DNA of F2 progeny by PCR and Southern hybridization are currently in progress to determine the underlying mechanism of integration (most likely homologous recombination) and the stability of integrated sequences. Additional quantitative parameters for achieving stable integration of fewer transgene copies in the germ line are considered and will be presented together with considerations regarding the applicability of our method for embryo transformation to other lepidopteran and non-lepidopteran insect species.

This work has been supported by the Canadian National Network of Centres of Excellence for "Biotechnology for Insect Pest Management" (Insect Biotech Canada) and the Medical Research Council of Canada.

## Session 4 : Hormonal Control of Physiology and Gene Expression

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## LOCALIZATION OF THE SITE OF STORAGE AND OF SYNTHESIS OF THE ADIPOKINETIC HORMONE IN *MANDUCA SEXTA*

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Adipokinetic hormone (AKH) injected into *Manduca sexta* results in the activation of glycogen phosphorylase in fat body and in the mobilization of lipids. Extracts of the corpora cardiaca, but not of other nervous tissues, mimic the effect of AKH. In the corpora cardiaca there are 7-8 intrinsic neurosecretory cells, localized in the half adjacent to the corpora allata. If the corpora cardiaca are divided into two halves, one containing the intrinsic neurosecretory cells, then nearly all the AKH activity is found in the half with the intrinsic neurosecretory cells. Immunocytochemical staining of the nervous system with an antiserum against AKH shows strong staining of the intrinsic neurosecretory cells, proving that AKH is stored in the corpora cardiaca. However, it does not show that AKH is synthesized in the corpora cardiaca. Some antibodies stain cells in the brain, so AKH could be stored in the corpora cardiaca but synthesized in the brain.

*In situ* hybridization with a DNA probe for the AKH gene indicated that AKH is synthesized in the intrinsic cells of the corpora cardiaca.

(Supported by NIH A1 26905, GM 29238 and A1 28368).

### ADDENDUM

What we thought to be *in situ* hybridization was found to be binding of streptavidin to an unknown substance, possibly to biotin bound by a biotin binding protein. However, we have clearly shown by bioassays and immunocytochemistry that only the intrinsic neurosecretory cells of the corpora cardiaca contain AKH in *M. sexta*. We are convinced that AKH is synthesized in the corpora cardiaca and Northern analysis supports this assumption. We will repeat the *in situ* hybridization with a different detection system.

## CLONING, SEQUENCE AND REGULATION OF JUVENILE HORMONE SUPPRESSIBLE PROTEINS FROM LEPIDOPTERAN HEMOLYMPH

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A cDNA library was prepared from mRNA isolated from the Lepidopteran *Trichoptusia ni* during larval-pupal metamorphosis. Differential probing identified clones for mRNAs which are suppressible by exogenous juvenile hormone treatment. *In vitro* transcribed cDNAs from these clones were translated *in vitro* and challenged with antiserum specific for a known acidic, juvenile hormone-suppressible hemolymph protein (AJSP-1) that is associated with larval metamorphosis. Three clones were found which encoded immunoreactive translation products whose identity was confirmed by comparison with the N-terminal sequence of the mature AJSP-1 protein with the cDNA sequence. As inferred from the cDNA sequence, widely distributed as well as more localized stronger sequence similarities indicate that the protein is distantly to hemocyanins and hemocyanin-like insect proteins. However, on the basis of amino sequence and composition, immunological reactivity, and hormonal sensitivity, the protein is distinct from previously described insect proteins. Its juvenile hormone suppressibility can be ascribed to suppression of the mRNA. RNA blot analysis using the cloned cDNA as a probe demonstrated that the transcript (approximately 2.8 kilobases) is of very low abundance during the penultimate stadium, but becomes very abundant during the last larval stadium, when juvenile hormone rapidly declines. Furthermore, treatment of larvae prior to metamorphic commitment with a juvenile hormone analog strongly suppresses the abundance of the message. However, after metamorphic commitment expression of the transcript becomes refractory to hormone treatment. A number of other JH-suppressible, metamorphosis-associated proteins similar to the above have been identified, and their cDNAs and genomic sequences cloned. Comparison of the 5' flanking regions prior to the structural genes has permitted identification of sequences common to the putative promoter regions of these genes.

## REGULATION OF DOPA DECARBOXYLASE GENE EXPRESSION IN THE *MANDUCA SEXTA* LARVAL EPIDERMIS : 20-HYDROXYECDYSONE SUPPRESSES THIS GENE EXPRESSION VIA PROTEIN SYNTHESIS

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Dopa decarboxylase (DDC), which converts dopa to dopamine is important for cuticular melanization and sclerotization in insects. Using the *Drosophila* DDC gene, we have isolated the *Manduca* DDC gene which hybridizes with an RNA that produces a 49 kDa translation product precipitable by the *Drosophila* DDC antibody. Sequence analysis shows 73 % homology with *Drosophila* DDC at the amino acid level and a conserved 15/19 bp sequence within the promoter region (AAAATATGATAGTGTGCAT). The sequence TGCA is important for the repressive action of ecdysteroid on the *Drosophila* Sgs-3 glue gene.

This gene encodes a 3.1 kb mRNA in the epidermis, which appeared 12 hr after head capsule slippage (HCS) and reached a maximal level 7 hr later. Peak expression was two-fold higher in melanizing allatectomized larvae and could be repressed to normal levels by application of 0.1 µg/ml juvenile hormone I. This increase in DDC mRNA was prevented by infusion of 1 µg/hr 20-hydroxyecdysone (20-HE) for 18 hr beginning 2 hr after HCS or by culturing this epidermis with 1 µg/ml 20-HE for 24 hr. Culture of Day 2 4th instar larval epidermis (before the ecdysteroid rise) showed that an initial exposure to 2µg/ml 20-HE for 17 hr followed by 24 hr in hormone-free medium was necessary for this later increase in DDC mRNA. The continuous presence of 20-HE for 41 hr prevented the appearance of the DDC mRNA. Thus, ecdysteroid during a larval molt is first necessary to determine the later expression of DDC and the subsequent decline of ecdysteroid is required for this expression to occur. To study how 20-HE prevent DDC expression, we cultured epidermis with 2 µg/ml 20-HE for 41 hr, then added either 10 µg/ml cycloheximide or anisomycin for the final 3 hr of culture. Under these conditions, DDC mRNA increased despite the continuous presence of 20-HE. By contrast, in the presence of both cycloheximide and 3 µg/ml α-amanitin, the DDC mRNA level remained low, indicating that the increase was due to new mRNA synthesis. When cycloheximide was injected into larvae at the HCS stage, DDC mRNA precociously appeared within 4 hr. These results indicate that 20-HE suppresses DDC mRNA expression via stimulation of a short-lived inhibitory protein(s), presumably a transcription factor. A model for steroid coordination of the molt will be presented.

Supported by USDA and NIH.

## ECDYSTEROID RECEPTORS AND THE REGULATION OF NEURONAL FATES DURING METAMORPHOSIS IN *MANDUCA SEXTA*

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At metamorphosis the larval nervous system undergoes a complex series of changes as it is transformed into that of the adult. On the cellular level, neurons show a number of different fates including remodeling, programmed cell death, and the maturation of arrested, immature neurons. These diverse fates are played out in response to ecdysteroids acting in the absence of juvenile hormone.

Spatial and temporal aspects of ecdysteroid receptor (EcR) expression in the CNS of *Manduca* have been studied using monoclonal and polyclonal antibodies raised against the EcR of *Drosophila*. Results from blocking experiments and comparison of antibody staining with binding of radiolabeled ecdysteroids (Fahrbach and Truman, 1989 ; Fahrbach, in press) argue that the antibodies recognize the legitimate *Manduca* EcR. During metamorphosis EcR shows a complex spatial and temporal pattern of nuclear expression in the CNS that can be correlated to the different types of neuronal responses. During the molt to the last larval stage, EcR levels are very low in neurons although glia and trachea show moderate levels of expression. EcR levels in neurons first begin to rise between days 2 and 3 of the 5th larval stage, peak at high levels by wandering (W) and W+1 and then fall to below detectability by the time of pupal ecdysis. Unlike the larval neurons, the nests of arrested, adult-specific cells show very low or undetectable levels of receptors through this larval-pupal transition. EcR is initially present at the start of adult development in most neurons, but disappears through most of development, only to reappear at low levels as metamorphosis is nearing completion. A notable exception to this pattern is the neurons that are fated to die at the end of metamorphosis. These cells show extremely high levels of EcR throughout all of adult development. The significance of these diverse patterns of EcR expression will be discussed.

**ISOLATION OF MONOCLONAL ANTIBODIES AGAINST AN ALLATOTROPIC FACTOR RELEASED BY BRAINS OF *GALLERIA MELLONELLA* LARVAE**

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Neuronal signals as well as neurohormonal substances modulate the capability of the corpora allata (CA) to synthesize juvenile hormone (JH). The larval CA of *Galleria mellonella* are stimulated by an allatotrophic factor released by the brain. We report experiments to measure the influence of brains and various brain fractions to induce i. supernumerary moulting of last larval instars and ii. JH synthesis of CA cultured *in vitro*. One 20 kDa polypeptide, partially purified by FPLC and identified by SDS-PAGE, is released by the brain into the culture medium where it accumulates and enhances the capability of the CA to synthesize JH up to 4.2-fold.

Monoclonal antibodies were raised against polypeptides prepared from brains of previously chilled larvae. Working up the hybridoma supernatants we found 4 fractions which inhibited the production of chilling-induced supernumerary moults as well as the activation of *in vitro* JH synthesis of CA by co-incubation with chilled brains. By means of immuno-histochemical methods we identified in the larval brains of prechilled larvae a group of medial cells which were selectively stained and are therefore thought to serve as a source of an allatotrophic factor.

This work was supported by the Deutsche Forschungsgemeinschaft (Sche 195/6) and a travel grant of the Universitäts-bund Würzburg to MIB.

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No comments...

## THE REGULATION OF THE IMMUNE PROTEIN GENES OF *HYALOPHORA CECROPIA*

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The *Cecropia* immune protein genes (Boman *et al.*, 1991) coding for two attacins, lysozyme and two cecropins contain a putative promoter sequence homologous to the binding site of the mammalian transcription factor NK- $\kappa$ B. These genes are strongly induced by bacteria, lipopolysaccharides (LPS) and phorbol esters (PMA) and are also slightly turned on by wounding (Sun *et al.*, 1991a, 1991b). Similarly, the genes governed by NF- $\kappa$ B are involved in immune, inflammatory and acute phase responses and can be transcriptionally activated by LPS and PMA (Lenardo and Baltimore, 1989). Gel retardation experiments have shown that only nuclear extracts from induced insects contain a factor that binds to the  $\kappa$ B-like sequence.

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## ACTIN IN LEPIDOPTERAN OOGENESIS

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The cortex of lepidopteran oocytes contains a substantial cytoskeleton which is rich in actin. Maternal messenger RNAs are attached to this cortical scaffolding, and are translated only after fertilization. Using confocal laser scanning of developing oocytes treated with fluorescent-labeled Phalloidin and anti-actin antibodies, we have been able to determine the organization of the cortical cytoskeleton in great detail, and to speculate on possible models for the establishment and growth of the scaffolding as the size of the oocyte increases.

In addition to its function as an anchoring point for mRNAs, actin may also serve as a storage depot for enzymes. We have investigated one such enzyme which would not be expected to have a role in oogenesis. This is RNA polymerase II which is responsible for the transcription of mRNA. No transcription is detectable in the ooplasm during either oogenesis or early preblastoderm development, but large amounts of RNA pol. II are detected in the ooplasm of unfertilized eggs which will transcribe *in vitro* when supplied with template DNA. This pol. II can be detected by fluorescent-labeled alpha Amanitin, and coincides with the pattern of actin distribution in the cortex. Both the Amanitin and Phalloidin patterns are disrupted to a similar extent by Cytochalasin D, suggesting that the pol. II is associated with actin. The pattern of appearance of pol. II during oogenesis is consistent with its synthesis in the fatbody, release into the blood and endocytotic uptake by the oocyte.

Actin also is a prominent structural element in the nuclei of the ovarian nurse cells, and its distribution parallels that of chromosomal DNA. These apparently anomalous results may be explained by a suggested role of actin in supporting transcription.

## TEMPORAL AND SPATIAL REGULATION OF THE GENE ENCODING THE SILK PROTEIN P25

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The P25 gene in the silk gland of *Bombyx mori* exhibits narrow cell specificity of expression and alternative repressed and derepressed status at molting and intermolting periods, respectively. These properties are shared by the gene encoding fibroin, to which P25 is bound. Moreover, both genes are transcribed at the same high level, strengthening the hypothesis that they are regulated via very similar sets of cis- and trans-activators.

P25 gene function was studied by *in vitro* and *in vivo* approaches. Scanning the -441, +1 upstream sequences by gel retardation assays led to the identification of a series of putative cis-acting elements that bind specifically to nuclear proteins. Among these, two factors, SGF-B and BmF-A, were more particularly investigated because of their affinity to sequences homologous to the enhancer of the fibroin gene.

SgF-B is a silk gland-specific nuclear protein. No differential properties were detected between this SGF-B factor extracted from posterior cells and its version in middle silk gland cells; similarly, developmental analysis did not reveal modification of its structure. In contrast, BmF-A appeared as a ubiquitous protein whose properties vary according to the molting cycle. Namely, a fast-migrating form in the gel retardation assay correlates with the moment at which gene expression is repressed.

*In vivo* functional assays of the P25 gene sequence made use of the capacity of transgenic *Drosophila* to reproduce developmental- and cellular-specificity characteristics of its expression in anterior larval salivary gland cells. Transformed lines with 5' recurrent deletions coupled to a reporter gene showed that the -441 to -165 domain upstream of the fibroin gene-homologous sequence is required for expression, probably by promoting cell-restricted interactions. Applying a similar strategy to the 5' flanking sequence of the fibroin gene failed to reveal any expression in the salivary gland of *Drosophila*, but showed activity in some other specific organs. Interestingly, combining P25 and fibroin gene upstream sequences abolished the expression of the reporter gene in the anterior cells and triggered its expression in cells of the imaginal disc of the salivary gland and, subsequently, in the adult gland. This suggests that the mixed promoter induces specific and positive recognition in a different territory of the *Drosophila* organ. Other gene constructs are currently being analysed to delineate the promoter configuration required for *in vivo* regulation of the P25 gene.

## TRANSCRIPTIONAL REGULATION OF FLY AND MOTH CHORION PROMOTERS

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Chorion promoters from three species of moth (*Bombyx mori*, *Antheraea pernyi* and *A. polyphemus*) are transcriptionally active in transgenic flies with a high degree of tissue and developmental stage specificity (Mitsialis *et al.*, 1989). Although short similar sequence motifs have been identified in chorion promoters from both *Drosophila* and moths much of the sequence is different and the promoters cannot be aligned with certainty. A radical difference is that moth promoters are bidirectional, directing coordinately the divergent transcription of two genes, A and B. Divergent transcription also occurs in transgenic flies transformed with chorion promoters. Several *Drosophila* cDNAs for putative chorion regulatory factors have been isolated on the basis of binding of their products to functionally important regions of a *Drosophila* late chorion promoter, *s15*. We are testing the proteins expressed from these cDNAs for their role in recognizing and regulating moth chorion promoters. As a model system we are using the *B. mori* A/B *L12* gene pair which is expressed in flies with the specificity of the endogenous *s15* gene. The cDNAs for the putative chorion regulatory proteins CF1 and CF2 (Shea *et al.*, 1990) have been expressed in bacteria and the protein produced is being used to compare binding specificities towards *s15* and *L12* sequences.

We are trying to answer the following questions concerning the regulation of moth chorion promoters :

1. Do the same transcription factors regulating endogenous fly promoters act on and regulate moth promoters introduced in transgenic flies ?
2. Do homologues of the fly factors exist in *Bombyx* and how conserved are they in terms of sequence and regulatory properties ?
3. Is molecular recognition between factors and promoters constant in the two species, or do these interacting macromolecules co-evolve ?

Using DnaseI footprinting and methylation interference we found that CF1 and CF2 bind on at least two sites on the *L12.A/B* promoter. CF1, a member of the nuclear hormone receptor superfamily, binds to a region containing the essential hexamer element TCACGT on the *s15* promoter. It recognizes two sites on the *L12* promoter : a site within the B half, also containing the essential TCACGT element, and a site within the A half which contains an "imperfect hexamer", ACACGT. Another feature of these binding sites is their similarity to hormone response elements (HREs). The sites are shown below along with an ecdysone response element which is included for comparison. These sites and what is known about the structure and properties of hormone receptors raise the possibility that CF1 may bind on the promoter as a dimer and mediate the action of some unidentified hormone, possibly a choriogenesis-triggering signal from the oocyte.

## TRANSCRIPTION MECHANISMS

```

Bm L12A      TTGACACGT ATCATTAG
              | |||||. || |
Bm L12B      AAGTCACGT . TTTTGAGAT
              | ||||| | | |
Dm s15       AGGTCACGT . AAATGTCCA
              ||||| | | |
Ecd RE       GGTCA . N . TGACCT
    
```

We are looking for homologues of CF1 and CF2 in *B. mori*. Using PCR with degenerate primers a CF1-homologous cDNA from *B. mori* was isolated, which shows greater sequence similarity to *D. melanogaster* CF1 than any other member of the steroid hormone receptor superfamily and is termed BmCF1. The DNA binding (C<sub>2</sub>C<sub>2</sub> zinc finger) domains show 96 % sequence identity between the species (see below) and the ligand/dimerization domains 51 % identity (71 % similarity). We are comparing in detail the binding properties of the *Bombyx* and *Drosophila* proteins as well as their function in transformed *Drosophila* and in transfected *Drosophila* tissue culture cells.

```

Dm  CSICGDRASGKHYGVYSCEGCKGFFKRTVRKDLTYACRENRNCCIIDKRQRNRCQYCRYQKCLTCGM
    |||||:|||||:|||||:|||||:|||||:|||||:|||||:|||||:|||||:|||||:|||||:|||||:|||||:|||||:|||||
Bm  CSICGDRASGKHYGVYSCEGCKGFFKRTVRKDLTYACREDKNCIIDKRQRNRCQYCRYQKCLACGM
           Pbox                             Dbox
    
```

Alignment of the DNA binding (zinc finger) domains of *Drosophila* (Dm) and *Bombyx* (Bm) CF1 proteins. The residues participating in the recognition helix (Dbox) and protein-protein interactions (Dbox) are underlined.

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## ZINC FINGER MOTIFS AND SILKMOTH CHORION GENE TRANSCRIPTION FACTORS

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Band shift and DNA footprinting assays have been used to identify proteins that bind specifically to the shared promoters of high cysteine (Hc) chorion gene pairs, which are expressed during late choriogenesis. These assays resulted in the identification of two DNA-binding proteins, BCFI and BCFII, whose promoter recognition/binding sequences are conserved in all Hc genes and whose appearance in follicular nuclei coincides with the activation of the Hc genes *in vivo*.

The sequence of the binding site of the major chorion factor, BCFI, contains a perfect match to the consensus sequence of the erythroid-specific transcription factors known collectively as the "GATA-1"-family factors. We have used degenerate oligonucleotides to PCR-amplify from *Bombyx mori* genomic DNA a sequence (ZF1) which, upon conceptual translation, shows extensive homology with the DNA-binding domain of the GATA-1 family of zinc-finger proteins. Using this amplified product as a probe, we have isolated and characterized a 4.3 kb genomic clone (ZF2) which contains GATA-1-like zinc finger motifs separated by an intron. Sequence comparisons as well as Southern analysis suggest that a multigene family encoding this class of zinc-finger proteins exists in *B. mori*.

RT-PCR amplifications of follicular RNAs using primers that encompass the zinc-finger domain of ZF2 resulted in the isolation of two cDNA sequences, ZF2S and ZF2L (for short and long ZF2 cDNAs), which are present in follicular but not in silkgland cell nuclear extracts, and which differ from each other by the presence (absence) of a stretch of 14 amino acids. Considering that only one ZF2 gene was found in *B. mori* genomic DNA under stringent hybridization conditions, these results imply that ZF2 expression may be regulated *via* differential splicing. Moreover, the RT-PCR experiments suggest that ZF2S cDNA is developmentally regulated since, although it can be detected during early choriogenesis, it accumulates maximally in late choriogenic follicles, the stage at which Hc genes expression takes place. ZF2S and ZF2L were subcloned into expression vectors and over-expressed in *E. coli* cells. Band shift assays using bacterial protein extracts have shown that both over-expressed fusion proteins are able to bind specifically to the BCFI recognition sequences of Hc genes. Antibody production is now in progress to find out whether ZF2S and ZF2L represent two variant forms of the chorion transcription factor BCFI.

This work has been supported by the Medical Research Council of Canada.

## TEMPORAL REGULATION OF A SILKMOTH CHORION GENE

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We have identified a short region of the A/B.L12 chorion promoter that is essential for correct temporal regulation during choriogenesis. Mutations in this region (site I, -192, to -218) show a remarkable amount of premature activation at the onset of choriogenesis (stage 11) followed by constitutive expression throughout choriogenesis. Mutations in the center of site I also result in substantially reduced levels of activation. This result can be interpreted as the presence of a negative regulatory element, i.e. a repressor binding site, that normally prevents early expression, in addition to positive elements that control activation during the late phase of choriogenesis as well as overall levels of activity. Site I is also the binding site for follicle-specific protein(s) and immediately adjacent to the binding site (site II, -173 to -192 of B.L12) of a DNA binding protein that is present in follicles and also in a fibroblastic cell line of ovarian origin.

We have used screening of a cDNA expression library ( $\lambda$ gt11) to isolate clones that encode DNA binding proteins. Five different classes of cDNA clones that bind specifically to site I or to site II were isolated. None of these is a homolog of the two cDNA clones that bind to the *Drosophila* s15 chorion promoter.

The features of the cDNA clones include the following details :

cDNA clone  $\lambda$ L15 seems to encode a silkmoth homolog of mammalian HMG-I and HMG-Y high mobility group non-histone chromatin proteins. L15 protein (114 aa) and HMG-I protein (107 aa) are similar in length and contain several oligopeptide repeats of alternating arginines with the core consensus sequence K-(R)-P/G-R-G-R-P. HMG-I proteins have three such repeats, which are thought to mediate binding to AT-rich sequences via the minor groove of DNA, while the L15 protein possesses four such repeats. The L15 mRNA (1500 bases) is developmentally regulated and absent in male adult silkmoths, but present in embryos and second instar larvae in addition to ovaries. Specific binding of L15 protein is abolished by a linker substitution (-184 to -192) adjacent to region A1. Interestingly, even moderate concentrations of bacterially produced L15 protein can form higher order complexes on A/B.L12 promoter fragment.

Three unrelated cDNA clones ( $\lambda$  L5,  $\lambda$  L26,  $\lambda$  L57) encode proteins that seem to bind to sites overlapping most of region A1. The L57 cDNA (4130 bp) include neither 5'- or 3' ends of the corresponding mRNA and contains a continuous open reading frame. The 5'-portion of this open

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reading frame is highly homologous to the yeast transcriptional activator SNF2 (34 % identity, 87 % similarity in a 650 aa overlap). The extensive homology may suggest a highly conserved function, possibly involving protein-protein interaction. In contrast, the DNA binding region in the 3' half of the L57 cDNA has no significant homologies to other entries in the Genbank database.

The DNA binding region of the L5 protein could be delineated to 217 aa by two overlapping cDNA clones that were isolated by functional screening for DNA-binding activity. This DNA binding domain contains two putative nuclear localization signals that also are similar to the K-(R)-P/G-R-G-R-P DNA binding motifs of HMG-I proteins and the L15 protein. Interestingly, L5 cDNAs exist in two alternate forms, one form having an insertion of three codons just in the middle of one of these DNA-binding motifs. This insertion changes the regular spacing of alternating arginines and thereby substantially decreases the potential of about 20 adjacent aa to form  $\beta$ -sheet. Differential expression of the two proteins could have interesting implications for chorion gene regulation. Outside of the DNA binding region L5 protein has a cyteine rich region that shows repeated motifs of cys and has residues similar in arrangement to a Zn-binding domain in the *Drosophila trithorax* gene product but distinct from consensus Zn-finger DNA binding domains. This region could either function as a second DNA binding domain or be important in protein-protein interactions. The remaining cDNAs, L26 and L55, encode proteins with no significant homology to other entries in the Genbank.

## EXPRESSION OF tRNA<sup>GLY</sup><sub>1</sub> GENES IN *BOMBYX MORI* IS REGULATED BY POSITIVE AND NEGATIVE REGULATORY ELEMENTS

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Tissue-specific expression of the tRNA<sup>GLY</sup><sub>1</sub> genes is regulated by cis-acting DNA elements 5' to the transcription start site. Primer extension and S1 nuclease analysis of the transcripts reveal that the initiation of transcription occurs upstream at -4 in one tRNA<sup>GLY</sup><sub>1</sub> clone, pR8, and at -3 and -5 in pBmP1. Deletion of the entire 5' region of pR8 abolishes transcription, as does the deletion of the B promoter. In contrast, deletions between nucleotides -300 to -150 or -300 to -40 cause a rise in transcriptional activity. The presence of specific protein factors capable of interacting with these regions could be demonstrated.

*In vitro* analysis of the protein binding in the 5' regulatory region revealed : (1) a positive regulatory region (-40 to +1) as determined by transcription and gel retardation assays, (2) a negative regulatory region (-300 to -150) as determined by transcription and footprinting experiments.

Transcription competition experiments using the negative element from pR8 indicated the presence of similar regions in four of the six tRNA<sup>GLY</sup><sub>1</sub> clones examined, whereas the positive regulatory element was found to be present in all six tRNA<sup>GLY</sup><sub>1</sub> genes.

## A FACTOR REQUIRED FOR *IN VITRO* TRANSCRIPTION BY RNA POLYMERASE III IS COMPOSED OF RNA

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It is generally assumed that the machinery that transcribes genes is composed entirely of polypeptides. We have discovered that *in vitro* transcription by silkworm (*Bombyx mori*) RNA polymerase III requires a transcription factor that is not a polypeptide (Young *et al.*, 1991a). This component, TFIIR, is distinct from the transcription components previously identified by our laboratory and others: RNA polymerase III, and the accessory factors TFIIIA, TFIIIB, TFIIIC, and TFIID (Ottonello *et al.*, 1987; Young *et al.*, 1991b). TFIIR is required for transcription of both tRNA and 5S RNA genes and its active principle appears to be RNA. It is a macromolecule that is resistant to heat, detergent, phenol, protease and DNase, but is sensitive to alkali and RNase.

Two pieces of evidence argue that the action of TFIIR is specific - that is, that it does not merely titrate a nuclease capable of destroying either the product of transcription or the template. First, the kind of RNA with TFIIR activity is highly specific. Second, the presence or absence of TFIIR does not affect the stability of the template, the transcript, or any other component of the transcription machinery.

Recent work on the purification of TFIIR and on the possibility that a TFIIR analog exists in other organisms will be discussed.

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## Session 6 : Insect Immunity

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**MODE OF ACTION, SITE OF SYNTHESIS, AND CELLULAR INTERACTIONS OF AN INDUCED LECTIN, TERMED SCOLEXIN, IN *MANDUCA SEXTA***

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An inducible insect lectin, termed scolexin (previously termed M13), triggers the coagulation and certain accompanying cellular aggregation responses in the hemolymph during bacterial attack or wounding. Scolexin is a glycoprotein with an approximate native Mr=72 k, and is composed of two subunits of approximately 36 K.

Two forms of scolexin, termed -1 and -2, are regularly observed during induction. Both scolexin -1 and -2 are modified to larger forms of the protein in response to high levels of dietary glucose.

Purified scolexin was radiolabelled with  $^{14}\text{C}$  and used in tissue section autoradiography and cytospin procedures in conjunction with light microscopy. The protein was also subjected to amino-terminal sequencing leading to the determination of a 23 amino acid sequence based on which a mixture of oligonucleotides was deduced and employed as a probe in *in situ* hybridization procedures.

The results suggest the involvement of scolexin in processes such as coagulation and nodulation. The synthesis and activity of the protein may be associated with the glucose levels in the hemolymph.

Preliminary data indicate that the tissues and/or cells responsible for coagulum formation may be the source of scolexin.

## THE APPLICATION OF MONOCLONAL ANTIBODIES TO DISTINGUISH INSECT HEMOCYTE TYPES

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Despite a considerable knowledge on hemocyte morphology very little data is available concerning hemocyte physiology. Only in recent years effort has been undertaken to study hemocytes *in vitro*, to separate them alive into subpopulations for further investigations or to characterize them by labeling with lectins. However, these approaches are limited when a tool is needed to trace hemocytes clearly in an experiment despite their morphological variance and to separate defined subpopulations for biochemical analysis or physiological experiments. The studies undertaken in my laboratory are intended to serve as a basis for investigations of the hemocyte physiology with special respect concerning the defense mechanism of insects against microbial invaders or parasites. Therefore the characterization of hemocytes was carried out by monoclonal antibodies (mAbs) in order to analyze and define the hemocytes of *Hyalophora cecropia* by biochemical-immunological means.

The characterization of four mAbs with distinct staining patterns in the hemocytes of *Hyalophora cecropia* will be demonstrated :

One antibody (HC-1A6) recognizes inclusions in granular hemocytes from diapausing pupae. The corresponding antigen is also present in the hemolymph, however in minute amount. A first approach of purification revealed a protein with high molecular mass ( $> 600,000$  to  $10^6$ ) and probably with multimeric forms (Trenczek and Sieg, 1991).

The second antibody (HC-10B10) recognizes only plasmatocytes, especially those that are extremely adhesive and form pseudopodia rather quickly.

The third and fourth antibody (HC-12F6 and HC-10A10) react to membrane bound antigens of medium sized round cells without pseudopodia. The percentual distribution of cells labeled with these two antibodies indicates the presence of subpopulations within morphologically similar cells. Double labeling experiments proved this hypothesis as well as that morphological different hemocytes have antigens in common.

The results of this investigation clearly demonstrate that the hybridoma methodology can be successfully applied to study insect hemocytes. This approach will enable us to clarify some of the functions of these cells by studying the corresponding antigens, and we will be able to separate hemocyte subpopulations under physiological conditions or to follow some of them in the course of the ontogeny to clarify the cell lineage of the different hemocyte populations.

This investigation is supported by the Gunnar Hanssons Stiftelse and the DFG.

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## STUDIES OF THE PROPART IN PREPROATTACIN USING A BACULOVIRUS VECTOR

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Different types of stress such as bacterial infections induce cellular and humoral defense mechanisms in insects. *Hyalophora cecropia* produce, as a humoral defense, different antibacterial proteins. One group is the attacins. Attacins are synthesized as prepro-proteins and during the transport they are processed into the mature forms. The attacin signal sequence is cleaved off after entry into the endoplasmatic reticulum and the propart is presumably cleaved at two arginine residues. The mature protein is exported into the hemolymph where it exerts its biological function. A cDNA cloning for a basic attacin was cloned into the *Autographa californica* nuclear polyhedrosis virus expression vector. The recombinant virus was used to infect a *Spodoptera frugiperda* cell line and *Trichoplusia ni* larvae. Correct processing of preproattacin into attacin was inferred to have taken place in both these systems, based on the pattern obtained from Western blots.

To understand the function of the attacin propart, we made structural mutants. The transport was followed by pulse-chase labelling of both cell line and fat body tissue. In one construct, one of the arginine residues at the processing site was changed to a glycine residue, thus blocking the cleavage of the propart. In the other construct the complete propart was deleted. Both mutations affected the transport of attacin. For the mutant with an uncleavable propart the extracellular level was approximately 10 % of that of wild type attacin after 180 min of chase. Monensin or Brefeldin A blocked the export from fat body tissues infected with viruses containing the attacin gene. Monensin completely inhibited the processing of the proattacin to mature attacin showing that the processing takes place in the distal subcompartment of the Golgi complex. Tunicamycin had no effect on the processing or transport, showing that glycosylation was not involved.

Larvae infected with virus containing the attacin gene blackened due to melanization 50-65 hours post infection. All larvae infected with virus containing uncleavable proattacin started to melanize in a segmented pattern 24-25 hours post infection. None of the larvae infected with the virus containing the attacin gene without propart turned black. The rather drastic change in appearance caused by these mutations will be further investigated using histochemical techniques.

## INDUCED ANTIBACTERIAL PROTEINS IN SILKWORM, *BOMBYX MORI* L.

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Bacterial infections are known to trigger the synthesis of immune proteins in various insects. Such induced proteins are classified as attacins, cecropins, lysozymes, dipterocins and apidaecins (Boman, 1986; Dunn, 1986; Trenczek, 1989; Casteels *et al.*, 1989). The mulberry silkworm, *Bombyx mori*, an economic insect, is a host for many bacterial and viral diseases (Chitra *et al.*, 1975). Hence an attempt was made to understand the molecular basis of immune response in the present study.

Injection of live bacteria (*E. coli*) into the larvae of tropical silkworm, *Bombyx mori*, induced antibacterial activity in the haemolymph. Antibacterial activity was detected by petridish zonal inhibition assay (Xylander and Nevermann, 1990) and bacteriolytic assay (Hultmark *et al.*, 1980). The antibacterial substance was separated into discrete bands on native PAGE overlaid by bacterial bed. The antibacterial substance was purified to homogeneity by CM-Sephadex C-50 and Sephadex G-100 column chromatography. The purified substance showed antibacterial activity as a single discrete band on gel-overlay as well as coomassie brilliant blue staining. SDS-PAGE of the purified protein revealed a single subunit of low molecular weight (< 7 kDa). The protein was found to have no lysozyme activity. The activity of the substance disappeared on trypsin treatment showing the proteinaceous nature. The protein was found to be heat stable. The results obtained in the present study show that the induced protein is similar in properties to the bacterial induced protein, cecropin of *Hyalophora cecropia*. Further studies on the occurrence and characterisation of the protein in different polyvoltine and bivoltine breeds are in progress.

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## IMMUNE RECOGNITION AND SUPPRESSION IN PARASITOID-HOST INTERACTIONS

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Several parasitoid wasp species are known to successfully circumvent encapsulation in their lepidopteran host. In the hymenopteran wasp, *Venturia canescens*, the egg surface is coated by virus-like particles (VLPs), which efficiently protect the egg from encapsulation by the host caterpillar, *Ephestia kühniella*.

Molecular analysis of VLP-proteins revealed structural similarities to host components. From this observation we conclude that VLPs of the parasitoid evade immune recognition by molecular mimicry of components that are used by the host to distinguish "self" and "non-self".

Using antigenic similarity to VLP-proteins, two different host components have been characterized so far that are involved in immune recognition. One is "hemolin", an insect hemolymph protein containing immunoglobulin-like domain structures. Hemolin is involved in the recognition of bacteria. Its similarities to certain neural cell adhesion molecules suggest that recognition of foreignness might have evolved from components involved in cell-cell recognition.

The other component is called "lining-protein", a hemolymph protein which is found in the hemolymph and on basal membranes of tissues that are exposed to the open circulatory system of invertebrates. The protein is able to form threads or net-like structures due to short collagen-like regions that are interrupted by noncollagen regions. A crosshybridizing protein is found in silk glands and Malpighian tubules of some lepidopteran species that are known to secrete collagen-containing silk proteins.

## HEMOLIN, AN INSECT IMMUNE PROTEIN BELONGING TO THE IMMUNOGLOBULIN SUPERFAMILY

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Insects have an efficient defense system against infections. Especially the inducible antibacterial immune proteins are well characterized. However, the molecular mechanisms by which insects recognize foreignness are not yet known.

Hemolin (previously called P4) has recently appeared as a candidate for a molecule taking part in the recognition of bacteria. It is found in low amount in normal pupae of *Hyalophora cecropia*, and is strongly induced by a bacterial injection. Analysis of cDNA sequence of the protein has shown that it belongs to the immunoglobulin superfamily. It has high sequence similarity to the Ig-part of the *Drosophila* neural cell adhesion protein, neuroglian. Functional *in vitro* analysis has indicated that hemolin is the first hemolymph protein to bind to the bacterial surface where it subsequently forms a complex together with another hemolymph protein (125 kDa). The complex formation seems to be dependent on the sugar-residues of the bacterial LPS-core and on the presence of divalent cations.

## MOLECULAR CLONING OF WASP VENOM PROTEINS AND THEIR EXPRESSION AND PROCESSING IN HETEROLOGOUS SYSTEMS

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In some insect host-parasite interactions the venom of the parasitic wasp has been shown to participate in the immunosuppression of the lepidopteran host, but the identity and molecular parameters of the venom components were unknown. In our studies, a venom protein from an endoparasitic wasp that is necessary for parasite survival has been cloned and expression of deletion mutant constructs for it analyzed for biological activity in a heterologous system. A 32.5 kDa protein from the venom of the parasitic wasp, *Chelonus* sp., is necessary for survival of the wasp in its lepidopteran host. The protein was purified, N-terminal sequenced, peptide-mapped and antiserum for it prepared. It is immunologically conserved with the venom proteins of higher, nonparasitic Hymenoptera whose venom serves a different purpose (e.g. paralysis of lepidopteran prey). A full length cDNA was isolated following immunological screening of an expression cDNA library prepared from venom gland mRNA. The encoded protein did not match reported protein sequences in the GenBank database. Northern analysis showed that the venom glands contained a single transcript corresponding to the protein. The protein appears to possess a signal peptide with unusual charge properties. Biosynthesis of the protein is rapid soon after emergence of the young adult female. The rate of biosynthesis cannot keep pace with the rate of depletion during *ad lib* stinging. Following titration of the venom reservoir, transport of remaining material from the synthesis region occurs faster than synthesis. However, within several hours a massive production of newly synthesized venom restores the reservoir titer to an even higher level than occurred prior to titration. Within the target host, the 32.5 kDa venom protein is processed to two specific lower molecular weight forms. Deletion mutant constructs were prepared and used in transient expression assays in host embryos injected with mutant sequences containing these deleted sequences. These data are the first report of cloning of a nonparalyzing wasp venom protein and of the processing of a wasp venom protein in both the wasp and the heterologous lepidopteran target tissue.

NIH GM 33995.

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## EXPRESSION AND MODIFICATION OF CECROPIN FROM A BACULOVIRUS VECTOR IN DIFFERENT HOSTS

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The cecropins constitute the most effective part of the insect humoral defense system against microorganisms. The final elimination of invading bacteria relies on this small polypeptide ( $M_r = 4000$ ). The molecule does not contain a stable secondary structure in aqueous solution but upon contact with a bacterial membrane, cecropin folds into an amphipathic, helical structure, thus making the membrane permeable and causing lysis of the bacterial cell. A cecropin gene typically contains coding capacity for a signal peptide, a pro-part consisting of two dipeptides, a mature cecropin, and an additional glycine residue that serves as a precursor for the C-terminal  $\alpha$ -amide.

As the expression of the non-globular cecropin is quite demanding we have practiced the baculovirus expression technique using the strong polyhedrin promoter. In the first construct we used cDNA for cecropin B from *Hyalophora cecropia* with the insert taken out just in front of the start ATG. This construct gave very little product. We then switched to cecropin A with 19 bp in front of its ATG start codon cloned into the pVL941 vector 35 bp downstream the original polyhedrin start. High levels of cecropin mRNA was produced in infected *Spodoptera frugiperda* cells. In cell culture supernatants it was possible to detect cecropin cross-reacting material on Western blots but we found that breakdown of cecropin took place both intra- and extra-cellularly. This is not surprising considering its random-coil structure.

To overcome the problem with proteolysis we employed a baculovirus construct containing a fusion of a dimer (ZZ) of the antibody binding domain of protein A from *Staphylococcus aureus* and mature cecropin. This together with an efficient signal sequence facilitated the purification of cecropin A from the medium of *S. frugiperda* cells by affinity chromatography.

In order to further increase the levels of cecropin, *Trichoplusia ni* larvae were used as host. Cecropin A could be obtained after cleavage of the fusion protein with cyanogen bromide. Biological activity as well as correct structure including the C-terminal  $\alpha$ -amide group was shown using electrophoresis with detection of antibacterial activity in conjunction with mass spectroscopy. When expressing cecropin A from its natural cDNA in *T. ni* larvae it was observed that the amidation of the C-terminal was incomplete. We then looked for a better host and found that *Autographa californica* nuclear polyhedrosis virus could propagate in *H. cecropia*. The yield of recombinant cecropin A in *H. cecropia* reached a level of 600  $\mu\text{g/ml}$  hemolymph and about 70 % of the material was amidated.

To find out if the observed difference in expression level was general, four recombinant viruses were used to infect pupae of *H. cecropia*, larvae of *T. ni* and *S. frugiperda* cells in culture. The results showed that *H. cecropia* often is superior to *T. ni* as an expression host. The expression of recombinant proteins in diapausing pupae of *H. cecropia* also has several practical advantages.

## GENE EXPRESSION USING *BOMBYX MORI* BACULOVIRUS

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Expression of the *Bacillus thuringiensis* Aizawa toxin gene was studied in the silkworm using a baculovirus vector. The toxin gene was transferred into the BmNPV transfer vector pBM030, after excision of the coding region from a plasmid containing the toxin gene, removal of upstream nonstructural sequences, and addition upstream of the gene of a BglII site. The recombinant transfer vector was co-transfected with wild-type BmNPV T3 DNA and a recombinant virus was isolated and purified by plaque assay. The recombinant virus was injected into 5th instar *Bombyx mori* larvae, and expression of the toxin gene was analyzed by SDS-PAGE fluorography and Western blot analysis of the haemolymph proteins, and Northern blot analysis of mRNA from fat body. These analyses confirmed expression of the toxin gene in *B. mori* larvae by the recombinant virus.

## CLONING AND COMPLETE SEQUENCING OF A *BOMBYX MORI* BACULOVIRUS DERIVED IMMEDIATE EARLY GENE

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A 3.8 kb *Cla*I fragment of BmNPV, hybridizing to the coding sequence of AcNPV IE-1, has been cloned and sequenced. An open reading frame of 1749 bp, showing 94 % homology to AcNPV IE-1 was observed and indicated BmNPV IEG. Also the 631 bp leader sequence contains identical consensus sequences when compared to AcNPV IE-1 : TATA and CAAT boxes being replaced by TATAAATT and GGCATAAT, respectively.

This leader sequence, minus 38 bp preceding translational start, was inserted in front of the functional *lacZ* gene of plasmid pCHI10 which already contains SV 40 polyadenylation signals. Following transient transformation with the resulting plasmid vector  $\beta$ -galactosidase was positively expressed in as well *Bombyx* (Bm-5), *Spodoptera* (Sf-9) and *Drosophila* (S-2) cells.

From these experiments it can be concluded that the 5' region of BmNPV IEG shows potentials in replacing either *Xenopus* oocyte or mammalian cell based expression systems for the study of insect gene derived expression products.

## RECOMBINANT BACULOVIRUSES FOR LEPIDOPTERAN INSECT PEST CONTROL

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Our activities with respect to genetic manipulation of baculoviruses have focused on three main objectives : (1) testing of specific gene products whose expression may cause incapacitation or death of target insect species ; (2) generation of recombinant viruses expressing such proteins immediately upon infection of the host ; and (3) identification of viral functions responsible for host range restriction.

The testing of proteins with insecticidal activity has been mainly undertaken on deletion mutant forms of the diphtheria toxin which, although capable of arresting protein synthesis in insect cells that express it, should not affect cells that do not actively synthesize it because it cannot be uptaken by them due to lack of the toxin internalization domain. In parallel work we examined the ability of specific recombinant constructs to express the enzyme juvenile hormone esterase and the precursor for the opioid neuropeptide metenkephalin whose expression has been detected in silkmoths and other insect species by immunohistochemical means.

Our work on the generation of "rapid destruction baculoviruses" has focused on the utilization of cellular rather than viral promoters for driving the expression of "insecticidal" genes in recombinant baculoviruses. We have developed an expression cassette which offers a double advantage : immediate expression in infected cells and stability of recombinant baculoviruses as opposed to those whose expression of toxic products is based on early viral promoters that are prone to homologous recombination within the viral genome and consequent genomic instability.

Finally, our efforts on the identification of baculovirus functions that determine host range have focused on the generation of intertypic recombinant viruses through homologous recombination in cell cultures. Although we have failed to produce the anticipated recombinants thus far, information has been derived that should help focus future studies on viral functions occurring at specific times post-infection.

Our work has been supported by the Canadian Network of Centres of Excellence for "Biotechnology for insect pest management" (Insect Biotech Canada).

## STUDIES ON THE IMMUNITY OF CYTOPLASMIC POLYHEDROSIS VIRUS IN SILKWORM, *BOMBYX MORI*

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In order to study the probability of the immunity of cytoplasmic polyhedrosis virus inside the mulberry silkworm (*Bombyx mori* L.) body and its practical effect, we have carried out some laboratory experiments in our institute for three years and undertaken the experiments in production in the countryside for also three years. The large number of experimental data indicated that the immunity effect of CPV was present in the silkworm, and it had a definite practical effect in immunity.

In the solution of 0.114 M sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) of pH value 10.36, the cytoplasmic polyhedra was dissolved, the solution was then put under 27 °C for 24 hrs, so that free virus was utterly set free. Later, 0.02 % formalin (HCHO) was used under 27 °C for inactive treatment for 28-36 hrs so as to make the inactivation of CPV and to produce the vaccine, the concentration of which was equivalent to 100,000,000 per ml.

The above-mentioned vaccine was used to stain on the mulberry leaves which were used to feed the silkworms for 4-6 hrs. After 24 hrs, some active CPV solution was used to feed the vaccinated larvae. After rearing for 10 days, the rate of CPV infection was investigated and comparison was made between it and that of the control group (not vaccinated), from the difference of resulting indices in both cases, the immunity effect was thus known.

After the tests for 20 batches within three years, under similar conditions of attack by the virus, the CPV infection ratio was significantly lower in the vaccinated group than that of the control group. When the CPV infection ratio in the control group was 35-70 %, the indices of the infection ratio of the vaccinated group generally could be decreased by 40-70 %, i.e. the immunity effect could reach 54.4 % on average (Table I).

By investigation of the result of immunity effect, it was shown that there was no reaction of immunity for the newly hatched larvae 12-20 hrs after the vaccine had been taken by them per Os. Only after 24 hrs, the immunity effect began to be produced, this effect could remain relatively stable until the third instar or onward (Tables II and III).

Through the comparison tests of CPV strains from different districts, there were differences in immunity power for the vaccine from various strains. Some of them were stronger in immunity power and more stable.

The experimental result showed that the speed of dissolution of CPV in  $\text{Na}_2\text{CO}_3$  and the temperature of the solution had significant function on the setting free of virion. If the speed of dissolution of it was too fast, the virion was injured, the vaccine produced became ineffective. Similarly, the temperature of  $\text{Na}_2\text{CO}_3$  solution could also affect the extent of CPV dissolution, hence affect the immunity power. For the vaccine of CPV produced by using the gastric juice of the 5th instar larva of mulberry silkworm as solvent, its immunity effect was quite good and relatively stable (Tables IV and V).

Table I. Experimental results of immune effect of cytoplasmic polyhedrosis virus in silkworm.

Batch no.	Group of test	Tested larvae	Incidence of CPV %	Index left %	Immune index %
1st	Vaccine	30 × 6	15.2	41	-59
	Control	30 × 3	37.2	100	
2nd	Vaccine	30 × 6	26.8	38	-62
	Control	30 × 12	70.6	100	
3rd	Vaccine	50 × 12	12.5	30	-70
	Control	50 × 3	42.2	100	
4th	Vaccine	100 × 2	42.9	61	-39
	Control	100 × 3	72.8	100	
5th	Vaccine	100 × 14	28.9	39	-61
	Control	100 × 6	70.1	100	
6th	Vaccine	25 × 6	22.4	57	-43
	Control	25 × 3	39.2	100	

Table II. Effective duration of immune ability of cytoplasmic polyhedrosis virus in silkworm.

Interval time*	Tested larvae	Incidence of CPV %	Index of left %	Immune index %
12 hrs	80	25.6	103	+ 3
Control	80	24.8	100	
20 hrs	80	40.0	91	- 9
Control	80	43.7	100	
24 hrs	80	16.7	40	- 60
Control	80	41.7	100	
28 hrs	80	9.4	35	- 65
Control	80	27.1	100	
32 hrs	80	9.2	27	- 73
Control	80	34.8	100	
36 hrs	80	13.8	25	- 75
Control	80	53.5	100	
44 hrs	80	28.1	37	- 63
Control	80	75.8	100	

\* Interval time means the interval hours from the vaccinated time to attacked time by active CPV.

Table III. Effective duration of immune ability of CPV in silkworm of early stages.

Vaccinated instar larva	Time of attacked CPV	Tested larvae	Incidence of CPV %	Index of left %	Immune index %
I	I - 24 hrs	60	14.5	23	- 77
Control	"	60	63.6	100	
I	II - 24 hrs	60	20.4	52	- 48
I + II	"	60	8.5	22	- 78
II	"	60	2.5	9	- 91
Control	"	60	39.6	100	
I	III - 24 hrs	60	14.3	47	- 53
I + II	"	60	13.6	45	- 55
I + II + III	"	60	4.1	14	- 86
III	"	60	4.7	16	- 84
Control	"	60	30.4	100	

I : 1st instar ; II : 2nd instar ; III : 3rd instar.

Table IV. Immune effect of the different dissolution speeds of cytoplasmic polyhedra.

pH value of Na <sub>2</sub> CO <sub>3</sub> solution	Dissolved time of polyhedra	Treatment time of polyhedra	Tested larvae	Incidence of CPV %	Index of left %	Immune index %
10.5	6-8 hrs	12 hrs	227	11.9	79	- 21
"	"	24 hrs	250	6.4	43	- 57
11.5	0-2 hrs	12 hrs	232	23.4	156	+ 56
"	"	24 hrs	270	25.6	170	+ 70
Control (non-vaccinated)			810	15.0	100	

The study of inactivation by formalin indicated that the inactivation treatment of CPV virion in 0.02 % formalin at 27 °C for 32-36 hrs would give vaccine of better immunity effect.

After the completion of laboratory work, experiment in production was also undertaken in the countryside in sericultural districts for three years, for the identification of its immunity effect. In the first year, 276 egg cards (about 20,000 silkworms/card) were treated with vaccine (newly hatched larvae, per Os), under natural infectious conditions, the index of CPV infection ratio of the vaccinated

area in comparison to the the control (non-vaccinated area) decreased relatively by 43-63 % ; in the second year, vaccination was continued for 5,404 egg cards in the sericultural district, a mean value of 46 % of immunity effect was obtained ; in the third year, further treatment was used for 12,728 egg cards, the immunity effect was about 40 %.

**Table V. Immune effect of the different dissolution temperatures of cytoplasmic polyhedra.**

Batch no.	Temperature of dissolution °C	Tested larvae	Incidence of CPV %	Index of left %	Immune index %
1st	30	109 × 5	61.3	77	- 23
	27	118 × 5	22.5	28	- 72
	24	97 × 5	50.8	64	- 36
	Control	115 × 3	79.6	100	
2nd	30	109 × 5	21.5	62	- 38
	27	103 × 5	10.6	31	- 69
	24	101 × 5	17.9	52	- 48
	Control	141 × 3	34.7	100	

## NUCLEOTIDE SEQUENCE AND TRANSCRIPTION ANALYSIS OF THE *BOMBYX MORI* NUCLEAR POLYHEDROSIS VIRUS P74 GENE

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Control of virulence of baculoviruses is an important subject for the production of efficient biopesticides. The p74 gene of *Autographa californica* multiple polyhedrosis virus (AcMNPV) was found as one of the genes related to virulence of occlusion body. In this presentation we describe the nucleotide sequence and a Northern blot hybridization analysis of the p74 gene of BmNPV.

An isolate of BmNPV D1 has genome size of 126.4 kbp and shows a physical map closely related to those of the BmNPV T3 isolate and AcMNPV E2 isolate. To obtain the DNA sequence of the p74 gene, dideoxy sequencing of cloned DNA fragments in pBluescript was done from 83.6 m.u. to 85.2 m.u. of the genome. Results showed that the BmNPV p74 gene revealed an open reading frame of 1935 bp which showed 94.7 % homology with the p74 gene of AcMNPV. The nucleotide sequences upstream from the open reading frame up to -990 also revealed strong homology to each other. The nucleotide sequence between the open reading frames of p10 and P74 genes of BmNPV is also p10-aaaaacgatttat-p74, which is 2 bps longer than that of AcMNPV. Putative amino acid sequence of the BmNPV p74 gene shows 90.9 % homology against that of the AcMNPV p74 gene. Northern blot hybridization analysis of total RNA prepared from BmN4 cells infected with BmNPV D1 was done at different time points of infection using a part of the open reading frame of the p74 gene. Results showed that this gene expressed as a single transcript in the late phase of the infection cycle and expression level of the gene was very low in comparison of the level of the p10 gene of BmNPV. We are currently constructing recombinant viruses containing deletion of different parts of the p74 gene to see if there is any virulence activity of the p74 gene product in the case of BmNPV.

## Poster Session

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## ANALYSIS OF HAEMOLYMPH AND DIGESTIVE $\alpha$ -AMYLASE ENZYMES IN DIAPAUSING AND NON-DIAPAUSING STRAINS OF SILKWORM, *BOMBYX MORI* L.

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The  $\alpha$ -amylase (EC 3.2.1.1,  $\alpha$ -1,4 glucon-4-gluconohydrolase) hydrolyses-1,4 glycosidic bonds in starch (Bernfeld, 1955). In silkworm, strong amylase activities in digestive juice and haemolymph have been reported and found to be different in their enzymatic properties (Kanakatsu, 1972, 1978 ; Tanaka and Kusano, 1980). The genes responsible for these enzymes have been mapped on autosomes (Doira, 1989).

Quantitative analysis of amylase enzyme in digestive juice and haemolymph has indicated that this enzyme titre in polyvoltine and bivoltine breeds varies (Chatterjee *et al.*, 1987). Thus, the diapausing and non-diapausing strains of the silkworm offer an interesting material for detailed analysis of digestive and haemolymph amylase enzymes.

The digestive amylase of the non-diapausing strain showed maximum activity at pH 9.2 while that in the haemolymph registered the highest activity at pH 6.8. The diapausing strain, NB<sub>1</sub>, revealed no amylase activity at pH range of 4-11 as also on PAGE and hence considered "null" for digestive amylase. On the other hand, the non-diapausing strain, Nistari, registered a strikingly higher digestive amylase activity. Interestingly, the haemolymph amylase in the diapausing strain showed pronounced activity while the non-diapausing strain showed less activity.

The digestive amylase of the non-diapausing strain revealed a distinct developmental (4th and 5th stadia) pattern. It showed higher activity during intermolt and reduced activity during moults (3rd and 4th moults) and towards spinning. The haemolymph amylase, on the other hand, showed systematic increase as the larval development progressed in both strains. The activity reached maximum level on 4th day of fifth stadium while it showed slightly depressed activity during moult and towards spinning. The digestive amylase enzyme of the non-diapausing strain was found to have pI in the alkaline range and hence migrated towards the cathode in both vertical and horizontal PAGE, while the diapausing strain did not reveal any cathodal amylase enzyme. However, a faint anodal band was detected in the digestive juice of both strains. The haemolymph amylase was anodal with pI in the neutral range. The digestive amylase in the non-diapausing strain migrated as three prominent cathodal bands, while the haemolymph amylase migrated as one major and two faint anodal bands.

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## CONCERTED GENE EXPRESSION ACCOMPANIES ELICITED FIBROIN SYNTHESIS

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In order to dissect the molecular mechanisms involved in the production of spider silk, we have developed what has proven to be a fruitful model system. Stimulation of the process evokes a series of gland-specific expressions whose detection has been resolved through time sequence studies. These studies reveal a series of macromolecular syntheses which precede that of the dramatic transient production of the full-size fibroin product. These are expressed as discrete transient events, reproducible both in time and in their relative magnitudes. We have, thus far, detected four waves of transient activities at the subsequently indicated times of incubation of the stimulated glands. Fibroin synthesis peaks after 90 minutes and is preceded by the synthesis of its RNA template by a 60 minute interval. Two waves of small RNA synthesis have been detected, one after 15 minutes, consistently of higher magnitude than the subsequent one, peaking after 45 minutes. The latter serves to optimize the gland's translational milieu through a differential accumulation of the tRNAs cognate to the preponderant amino acids of the tissue-specific product. Of special interest is the bout peaking after 15 minutes in which we have, so far, detected the upgrading of UlsnRNA and 5SRNA. Outstanding and worthy of further inquiry within this peak, was the impressive magnitude of accumulation of alanine tRNA, the only tRNA species upgraded within this particular event. Our studies have shown that the alanine tRNA produced at this time, can be resolved into two isoforms, one constitutive and the other gland-specific. The generation of the latter species correlates with the gland's fibroin synthetic activity. Of particular interest are the parallelisms displayed between the fibroin synthesis strategies of two silk producers, *Bombyx* and *Nephila*, organisms which diverged over 400 million years ago.

Supported by NSF grants # M2586B90, M32891391 and NIHRR088102, RRO364102 to GCC. CC held an NIH Faculty Fellowship during her training.

## NEUROENDOCRINE CONTROL OF SEX PHEROMONE BIOSYNTHESIS IN *HELIOTHIS PELTIGERA*

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Sex pheromone production in female moths is controlled by a cerebral neuropeptide termed pheromone biosynthesis activating neurohormone (PBAN). At present our understanding of the processes involving PBAN, such as : its biosynthesis, secretion, transport, and activation of the target organ, is very limited. In order to widen the understanding of these processes our study concentrated on : a) the characterization of the biological activity of PBAN ; b) analysis of its content as a function of development ; and c) studies of its structure-function activity relationship in the moth, *Heliothis peltigera*.

Our studies demonstrated that sex pheromone biosynthesis in *H. peltigera* females is dependent on the presence of a cerebral factor. Analysis of the biological activity of PBAN, using the ligation bioassay, revealed the presence of the neuropeptide in both male and female head ganglia, as well as its ability to activate sex pheromone biosynthesis during scotophase and photophase and among heterologous moth species. Immunochemical analysis of endogenous PBAN, using specific antiserum for synthetic *Helicoverpa zea* (Hez)-PBAN (PBAN 1-33), in ELISA, revealed the presence of PBAN-like immunoreactivity (IR) as early as the 4th larval instar and an increase in its content as a function of development, reaching a level of 5.0 and 4.6 pmol in 3-day-old adult male and female moths, respectively. The inability of *H. peltigera* females to produce sex pheromone in the absence of the cerebral factor emphasizes the major regulatory role of PBAN in this process, and the presence of PBAN-like IR in larvae suggests an involvement of the neuropeptide in functions other than sex pheromone biosynthesis at early developmental stages.

Studies of the structure-function relationship of PBAN, using synthetic Hez-PBAN (PBAN 1-33) and four shorter peptides derived from its sequence, PBAN 9-33, PBAN 19-33, PBAN 26-33 and PBAN 28-33, revealed that PBAN 1-33 is very potent, stimulating sex pheromone biosynthesis in *H. peltigera* at the picolomolar range. Removal of eight amino acids from the N-terminal region of PBAN 1-33 had no effect on the biological activity, and further truncation of ten amino acids from the N-terminus resulted in partial loss of activity. An octa- and a hexapeptide, derived from the C-terminus of the PBAN, were 100 times less active than PBAN 1-33. The high potency of PBAN 9-33 and the lower potency of PBAN 19-33 and the C-terminally derived shorter peptides suggests that the N-terminal region is not necessary for the onset of the biological activity of PBAN, and that the region between the 9th and 19th amino acids plays an important role in the activity of the neuropeptide.

## GENE MAPPING OF THE *BOMBYX ANTENNAPEDIA* BY CLASSICAL AND MOLECULAR GENETICS

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In many organisms homeo-box genes or homeotic genes were identified and cloned. In *Drosophila melanogaster*, two homeotic gene complexes, *Antennapedia* complex (ANT-C) and *Bithorax* complex (BX-C) are identified and are loosely located on the third chromosome.

Whereas RFLP (restriction fragment length polymorphism) are known to be a good candidate to map the genes in many animals and plants.

In *Bombyx mori*, there is a famous metameric mutant, *E* (extra legs) pseudo allele. On this *E* allele, more than 30 mutants have been located and from the characters of the mutants, *E* must be the allele of the homeotic genes.

From the genetic analysis, including gene compensation of lethality, there must be more than three domains of mutations, corresponding to ANT-C and BX-C. From the results of the Southern blots by using the cloned homeotic genes, some *E* are identified to be homeotic mutants those are deleted some parts of the gene structure, mainly in the homeo-box region.

We show here the homeotic gene mapping approach by classical and molecular genetics.

We have already cloned the homeo-box containing gene from the genomic DNA of *Bombyx mori*. This gene was expressed from before the organogenesis through the hatching stage in the embryo, and also expressed at around the larval molting stage. And the homeo-box of this gene was completely homologous to *Drosophila Antennapedia* in amino acid level. So we deduced that this homeo-box containing gene must be an analogue (homologue) of *Antennapedia*.

In *Bombyx*, *Antennapedia* type mutants have never been identified, but there are many homeotic mutants corresponding to BX-C.

So that we used the homeotic mutants as marker genes, and have done linkage analysis of this *Antennapedia* analogue by using the RFLP.

$E^{Ca}/+$  has an additional crescent mark on the third abdominal segment and defect the star mark on the fifth abdominal segment, and homozygote is embryonic lethal and defect all of the abdominal legs.  $E^{Kp}/+$  has an additional abdominal leg at the second abdominal segment and homozygote has also additional crescent marks at the third abdominal segment and is not embryonic lethal.

In Southern blot hybridization with genomic *Bm Antennapedia* clone,  $E^{Ca}/+$  showed 3.0 and 3.6 kb EcoRI fragments whereas  $+/+$  segregant from cross of  $E^{Ca}/+$  showed only 3.6 kb fragment, and  $E^{Kp}$  homozygote showed 2.5 kb fragment only.

After crossing between  $E^{Ca}/+$  and  $E^{Kp}$  homozygote,  $E^{Ca}/E^{Kp}$  males having additional crescent mark and additional abdominal legs were picked up and identified that each individual has 2.5 and 3.0 kb fragment. Segregants from the cross between this  $E^{Ca}/E^{Kp}$  male and  $+/+$  female showing only 3.0 kb fragment were dissected and posterior silk glands were collected. Individual DNAs were prepared and restricted by EcoRI and followed by Southern blots.

Ten individual DNAs from  $E^{Kp}/+$  showed 2.5 and 3.0 kb EcoRI fragment, whereas nine of ten from  $E^{Ca}/+$  showed 3.0 kb and one of them showed 2.5 and 3.0 kb fragments.

These results indicate that this *Bm Antennapedia* located on the sixth chromosome and closely linked to BX-C homologues ( $E^{Kp}$  and  $E^{Ca}$ ).

## LIPOPROTEIN LIPASE FROM *MANDUCA SEXTA* FLIGHT MUSCLE

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In the resting adult sphinxmoth, *Manduca sexta*, lipids are transported in the hemolymph by a high density lipophorin, HDLp. During flight, transport of diacylglycerol (DG) is increased manifold by the conversion of HDLp to a highly lipid loaded low density lipophorin, LDLp. LDLp transports DG from the fat body (site of storage) to the active flight muscle, where DG is hydrolyzed by a lipoprotein-specific lipase (LpL). This flight muscle LpL was partially purified and characterized. The LpL hydrolyzes LDLp-bound DG at a rate twice that of HDLp-bound DG. It is an extrinsic membrane protein that can be extracted with urea. The LpL has a pH-optimum of 7.5, and is only slightly inhibited by salt. The LpL is inhibited by DFP (a serine protease inhibitor), indicating that the LpL has a serine in the active site similar to the vertebrate LpL. Using <sup>3</sup>H-DFP, the DFP-binding protein was identified as a 39 kD band on SDS-PAGE. Since vertebrate lipases are highly conserved, an attempt was started to select a cDNA clone for LpL by the MOPAC-PCR technique. A degenerate oligonucleotide was synthesized coding for a highly conserved sequence of 6 amino acids in vertebrate lipases. The PCR-reaction was primed with this degenerate oligonucleotide and a vector sequence primer, and using a cDNA-library as template. Two PCR-products were obtained, which were sequenced. With the exception of the primer, no sequence similarity with vertebrate lipases could be detected.

**US DEPARTMENT OF AGRICULTURE REGULATIONS AND  
BIOSAFETY ISSUES OF FIELD TESTING TRANSGENIC  
PLANTS RESISTANT TO LEPIDOPTERAN PESTS**

**Q.B. KUBICEK**

USDA APHIS BBEP Rm 841, 6505 Belcrest Road, Hyattsville, Maryland 20782, USA.

The review process for field tests of transgenic organisms of the United States Department Agriculture's (USDA) Animal and Plant Health Inspection Service (APHIS) certifies that there is no significant plant pest risk, even if the organism is derived from a plant pest. The process also assures that there is no significant risk to the environment. APHIS is committed to ensuring the protection of public health and the environment from any potentially harmful effects of biotechnology. To date 176 small-scale field tests have been conducted in the US and 44 of these have been for transgenic plants resistant to Lepidopteran pests. Genes conferring resistance to Lepidopteran pests have been cloned into canola, corn, cotton, potato, rice, tobacco, and walnut. In addition, a corn endophytic bacterium has been genetically engineered to provide its host plant resistance to Lepidopteran pests. An Environmental Assessment (EA) has been prepared for each field test. Components of an EA include an analysis of the molecular biology of the cloned genetic material, biosafety protocols, knowledge of the recipient host plant and donor organism(s), and other parameters. Little is known of the insect pest-transgenic plant interactions and the possible evolution of insect strains that have overcome host resistance. Models to predict development of such strains would be useful to develop strategies to extend the life-span of genes conferring host plant resistance. In addition, detection methods, sampling methodologies, monitoring protocols, and modeling techniques will be needed to assist regulatory agencies evaluate biosafety risk assessment. The development of transgenic plants that are resistant to insect pests may be commercialized in the US by the mid-1990's. A regulatory framework for the review of these products is being formulated.

## CHARACTERIZATION OF DNA POLYMERASE $\alpha$ -PRIMASE COMPLEX FROM *BOMBYX MORI* AND ITS POSSIBLE INVOLVEMENT IN CHROMOSOMAL ENDODUPLICATION

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The silkgland of *Bombyx mori* is a terminally differentiated tissue which undergoes chromosomal endoduplication during the entire larval development. Although the nuclei or cells of the silkgland do not divide, the DNA replication continues throughout larval development period. Concomitant with the increase in DNA contents, the DNA polymerase activity also increases as the development progresses. The enzyme activity is predominantly DNA polymerase- $\alpha$  with no detectable levels of polymerase- $\beta$ . DNA polymerase- $\alpha$  from silkgland extracts has been purified to homogeneity by using ion exchange, gel filtration and affinity chromatography resulting in 4000 fold increase in specific activity. The purified enzyme satisfied the criteria to be designated as polymerase- $\alpha$  based on its sensitivity to inhibition by aphidicolin, sulfhydryl group blocking agents, high concentration of salt and by its lack of sensitivity to ddTTP. The DNA polymerase activity is resident in the 180 kDa subunit of the enzyme. The polymerase is tightly associated with primase activity and initiates primer synthesis in the presence of ribonucleoside triphosphates on a single stranded DNA template. The abundance of DNA polymerase- $\alpha$  in silkglands and its strong association with the nuclear matrix suggest its role in DNA endoduplication process.



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TMAN+ IVGGSTTTIQYPTIVALLFSRNGNTFFQAcCGGIILNRRNVLTAAHcCP HGDVAVNRWRVRSG STYANSGGGAVHNLNRVRIHPNFNRRTLDND
TMOS VVNGQTATIGQFPFQVLLKVELSQRAL cGGSLSDQV SRHeT DGAKSFEVTLGAAVD FEDTTNDGRVVLTATEYHRHEKYNPLFATND
TDRO IVGGSATTISSEFPQISLQRS G SHScGGSIYSANIIVTAAHcL QSVSASVMQVRAGSTWU SSGGVVAKVSSSFKNHEGYNANTMVND
TCRF IVGGTDAVLGEFPYQLSFQETFLGFS FHFcCGASIYENYAITAGHcVYDDYENPSGLQIVAGELTCRFFDMSVNEGSEQTITVSKIILHENFDYDLLDND
TSDF IVGGYECPKHAAPWTVSL N VG YHFcGGSLIAPGWVSAAHc YQRRIQVRLLGEH DISANEGDETYIDSSMVIRHPNYSGYDLDND
TRAT IVGGYTCPEHSVPYQVSL N SG YHFcGGSLINDQVVVSAHc YKSRIQVRLLGEH NINVLEGDEQFINAAKILKHPNYSSWTLNND
TBOV IVGGYTCGANTVPYQVSL N SG YHFcGGSLINSQVVVSAHc YKSGIQVRLLGED NINVVEEGNEQFISAKSIVHPNYSSNTLNND
KERH IVGGTDAPRGKYPYQVSL RA P KHFcGGSISKRYVLTAAHcL VGKSKHQVTVHAG SVLLNKEEAVYNAEELIVNKNYNSIRLIND
KORH IVGGTNPARGKYPYQVSL RA P KHFcGGSISKRYVLTAAHcL VGKSEHQVTV G SVLLNKEEAVYNAKELIVNKNYNSIRLIND
KBOV IVNGEDAVPGSWPQVSLQDS TG FHFcGGSLISEDWVVTAAHc GVTTSDVVVVAGEF DQGLETEDTQVLKIGKVFNKFSILTVVRND
KRAT IVNGEDAVPGSWPQVSLQDK TG FHFcGGSLISEDWVVTAAHc GVTKSDVVVVAGEF DQGSDEENIQVLKIQVFNKFNMFTVRND
CSFC IVGVEAVPNSWPHQAALFID D MYFcGGSLISPEWILTAAHcM DGAGFSDVVVLGAH NIREDEATQVTIQSTDFTVHENYNSFVISND
CHYL IINGYEAYTGLFPYQAGLDITLQDQR RVWcGGSLIDNKWILTAAHcV HDAVSVVVYLGSAVQ YE GEAVVNSERISHSMFNPDTYLND

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TMAN+ IAIMRTTSNIAFNAAQPARIAGAN YNLGDNQVVWAAAGWAIRSGG PSSEQLRHVQVVTVNOATcR SRYASIGRSVTDNMLcSGWLDVGGRDQcQ
TMOS VAVVKLPTPEFENDRVQVVKLPTG SDTFTDREVVVSGWGLQKNGG NVADKLQYAPLTVISNNEc SKAYSPLVIKKSTLcAK GEHKESPcQ
TDRO IAVIRLSSSLSFSSSIKAISLATYN P ANGASAAVSGWGTQSSGSSSIPSQLQYVNVNIVSQSQcA SSTYGYGSQIRNTMICA AASGKDAcQ
TCRF ISLLKLSGLTFNNVAPIALPAQG H TATGNVIVTGWGTTSEGGNT PDVLQKVTVPLVSDATcRFECRDDYGADEIFDSMIcAG VPEGGKDScQ
TSDF IMLIKLSKPAALNRNVDLISLPTGC A YAGEMCLISGWGNTMDGAVS GDQLQCLDAPVLSDAEc KGAYPGMITNNMMcVG YMEGGKDScQ
TRAT IMLIKLSSPVKLNARVAVLPSACA A YAGTQCLISGWGNTLSGNVNNPDLLQCVDAPVLSQADc EAAYPG EITSSMICVG FLEGGKDScQ
TBOV IMLIKLKAASLNSRVASISLPTSCA A SAGTQCLISGWGNTKSSGTSYPDVLKCLKAPILSDSSc KSAYPG QITSNMFcAG YLEGGKDScQ
KERH IGLIRVSKDISITQLVQVVKLPVSN T IKAGDPVVLTGWGRIYVNG PIPNNLQQITLSIVNQQTc KEKHWGLTDSQIcT FTKLGEGAcD
KORH IGLIRVSKDISFTQLVQVVKLPVSN T IKAGDPVVLTGWGRIYVNG PIPNNLQQITLSIVNQQTc KSKHWGLTDSQIcT FTKRGEGAcH
KBOV ITLLKLATPAQFSETVSAVCLPSADD EDFPAGMLCATTGWGKTKYNALKTPKLQQAALPIVSEADc RKYWGS RVTDMVICAG ASGVSScM
KRAT ITLLKLATPAQFSETVSAVCLPNVD DDFPPGTVCATTGWGKTKYNALKTPKLQQAALPIVSEADc KKSWGS KITDVMTcAG ASGVSScM
CSFC IAVVRLPVPVTLTAAIATVGLPST D VGVGTVVTPTGWGLPSSDSALGISDVLRQVDVPIMNSADc DAVYGI VTDGNIICID DSTGGKGTcD
CHYL VALIKIPHVEYTDNIQPIRLPSGEELNNKFENIWATVSGWG QSNTDTVL IQYTVNLVIDNDRc AQEYPPGIIVESTIcD TSTGKSPcF

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***** * * * * *
TMAN+ GDSGGP LYHNGV VVGVCSWGEE cALARFPGVNARVSRSLTGLETTLKFYVRSVIKYRQL * = Conserved in all sequences.
TMOS GDSGGPLVLVLEGEN VQVGVVSFGHAVGcE QGYPGAFARLT # = Active-site residues.
TDRO GDSGGPLVLVSGGV LVGVVSWGYG cAYSNYPGVYADVA VLRSWVVSTANSI T = Trypsin-specific residues.
TCRF GDSGGPLALAASDTGSTYLAGIVSWGYG cARPGYPGVYTEVS YHVDWI KANAV
TSDF GDSGGPVVVVCNGM LQGIVSWGYG cAERDHPGVYTTRVC HYVSWIHETIASV
TRAT GDSGGPVVVVCNGQ LQGIVSWGIG cALPDNPGVYTKVC NFGWIQDTIAAN
TBOV GDSGGPVVVVCSGK LQGIVSWGSG cAQKNKPGVYTKVC NYVSWIKQDIASN
KERH GDSGGPLVLVANGV QIGIVSYGHP cA VGSPNVFTRVY SFLDWIQKNQL
KORH GDSGGPLVLVANGV QIGIVSYGHP cA IGSPNVFTRVY SFLDWIQKNQL
KBOV GDSGGPLVLVCQKNGAWTLAGIVSWGSS TcS TSTPAVYARVT ALMPWVQETLAN
KRAT GDSGGPLVLVCQKDGVWTLAGIVSWGSG VcS TSTPAVYSRVT ALMPWVQQILEAN
CSFC GDSGGPLNLNYDGL TYGITSFGAAAGcEA GYPDAFTRVT YFLDWIQTQTGITP
CHYL GDSGGPFVFVLSDKN LLIGVVSFVSGAGcES GKPVGFSRVT SYMDWIQQNTGIKF

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+ Preliminary sequence from the sequencing of one strand of the cDNA.

POSTER SESSION

## PERFLUORO-CHEMICALS IMPROVE INSECT FAT BODY CULTURE OXYGENATION

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One of the main problems with insect tissue culture is to maintain appropriate oxygenation of the tissue. We addressed this problem by testing the effect of a perfluorochemical solution (perfluorotri-n-butylamine) (PF) on the oxygenation of *Manduca sexta* fat body culture. Two different parameters, protein synthesis and ATP levels, were measured to evaluate the effect of PF.

After 21 h of incubation there was a 5-fold increase in the amount of protein secreted into the culture medium in the presence of PF. The synthesis of two of the major proteins produced by the fat body, arylphorin and high density lipophorin, was, as part of the increase, confirmed by immunoprecipitation. The rate of protein synthesis increased with the concentration of PF in the medium, up to a concentration of 20 %. After 21 h of incubation the ATP levels in fat body incubated in the presence of PF were similar to those found *in vivo*, and significantly higher than without PF.

The results show that PF improves the oxygenation of cultured fat body tissue.

## STUDIES ON THE MODE OF ACTION OF ECLOSION HORMONE ON SILKWORM PHARATE ADULT USING RECOMBINANT HORMONE

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Eclosion hormone (EH) is a neuropeptide transmitter which plays a crucial role to trigger insect eclosion behavior (Truman, 1984). In 1987, three groups independently isolated EHs from *Manduca sexta* (Marti *et al.* and Kataoka *et al.*) or *Bombyx mori* (Kono *et al.*), and their sequencing analyses showed highly conserved sequence between the two species (80 % identical). The primary structures were confirmed in 62 amino acids residues by the sequence analyses of cDNA isolated from the *Manduca* and *Bombyx* brain cDNA libraries.

On the other hand, the signal transduction of *Manduca* EH appears to be mediated via the second messenger, cyclic GMP and it was demonstrated that two ganglionic 54 kDa proteins were specially phosphorylated by the protein kinase activated by cGMP (Morton *et al.*, 1988). However, the extremely low abundance on the natural EH in lepidopteran brain renders it difficult to study the biochemical aspects of the hormone and receptor interaction. To overcome this difficulty, functional expression systems of the EH gene were developed using *E. coli* (Nagasawa *et al.*, 1990), yeast (Hayashi, 1990) and insect cells.

We present the aspect of cGMP induced eclosion behaviors which is similar to the recombinant EH induced one in the silkworm pharate adult developmental stage and moreover, EH is effective to trigger the ecdysis behavior and cGMP plays a crucial role as the second messenger in the EH mediated signal transduction throughout the silkworm life cycle.

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## GENETIC RELATIONSHIPS BETWEEN BACULOVIRUSES

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Baculovirus infections have been reported in more than 500 arthropod species, predominantly of Lepidoptera. The interspecies host range of particular baculovirus isolates is generally narrow, and efforts to understand and broaden host range, and to increase viral pathogenicity, are relevant to the applicability of baculoviruses in pest control strategies.

The two major morphotypic baculovirus subgroups (nuclear polyhedrosis viruses, NPV, and granulosis viruses, GV) have in common a closed circular dsDNA genome of, generally, 100-150 kpb. Morphotypic divisions are mirrored by genomic hybridization studies which indicate low levels of sequence conservation between subgroups; there is, however, a considerable degree of conservation among isolates within each subgroup.

We are investigating the genome organization of an NPV (S1MNPV-B) which is pathogenic for *Spodoptera littoralis* larvae. Homologues to nine genes of the 'prototype' baculovirus AcMNPV have been identified by Southern hybridization. Further characterization of these genes may provide a set of probes for monitoring S1MNPV-B gene expression in host species which do not support complete productive replication of the virus.

Our presentation is intended to provide an 'impressionistic' review of baculovirus molecular genetics, with particular emphasis on comparative aspects of genome organization.

## THE PROGRAM OF CHORIOGENESIS IS ESTABLISHED 32 HOURS BEFORE THE ONSET OF CHORION PROTEIN SYNTHESIS IN *BOMBYX MORI* FOLLICULAR CELLS AND CAN BE IMPLEMENTED IN ORGAN CULTURE

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The chorion of *B. mori* is produced as a result of the sequential activation of chorion gene batteries during the terminal differentiation of follicular cells. Although significant progress has been made recently in identifying factors involved in the activation of silkworm chorion genes expressed at specific stages of choriogenesis, nothing is known about the regulatory factors that control the transition of follicular cells from vitellogenesis to choriogenesis and the transcriptional potentiation of the chorion locus. We, therefore, addressed the following questions : when does the program of choriogenesis become independent of factors extrinsic to the follicle ? ; is the establishment of the program controlled by ecdysone or its receptor at the time of pupation ? ; are there additional non-ovarian factors that participate in its establishment ?

Ovarioles at the onset of choriogenesis (day 5 to day 6 after pupation) were cultured *in vitro* and pulse-labelled either immediately or 24-72 hours later with radioactive aminoacids to examine chorion protein synthesis. It was consistently observed that the last 16 ( $\pm 2$ ) prechoriogenic follicles were capable of undergoing choriogenesis in organ culture. The protein synthetic profiles of cultured follicles were also correlated with corresponding profiles of chorion mRNA accumulation. Twelve prechoriogenic follicles entered choriogenesis after 24 hours of culture, yielding a rate of one follicle entering choriogenesis per 2 hours of culture (NDC strain). After entering choriogenesis, follicles appeared to progress at a slower rate, each proceeding to the next choriogenic stage after 3 hours of culture. While no follicles earlier than "-16" ( $\pm 2$ ) were able to enter choriogenesis after 32 ( $\pm 4$ ) hours, follicles that had already started choriogenesis continued to proceed towards high cysteine chorion protein synthesis at a constant rate for at least 72 hours. Because significantly higher numbers of follicles undergo choriogenesis *in vivo*, it was concluded that the 16th prechoriogenic follicle (stage "-16") represents the stage at which hemolymph factors act to initiate choriogenesis.

Further experiments are currently in progress to correlate the establishment of the choriogenic program at stage "-16" with the appearance of the ecdysone receptor in follicular cells. Finally, we are initiating the construction of a stage "-16" follicular cDNA library to isolate stage-specific clones encoding sequences involved in the establishment of the choriogenic potential in follicular cells.

Luc Swevers is a research assistant of the Belgian NFWO. This work has been supported by the Medical Research Council of Canada.

## INSTRUCTIONS AUX AUTEURS

1) Les articles présentés doivent concerner soit des travaux originaux soit des synthèses. Ils doivent être rédigés en français ou en anglais. Ils doivent avoir trait à la sériciculture qui inclut les vers à soie, les plantes nourricières, la filature de la soie quelle que soit la nature des recherches (fondamentales ou appliquées) et des sujets (biochimie, écologie, économie, virologie, génétique, etc.).

2) Les articles doivent être soumis à : **Dr G. CHAVANCY**. Les articles soumis au Rédacteur doivent être inédits et ne doivent pas être présentés pour publication chez un autre éditeur. Les articles acceptés pour publication dans *SERICOLOGIA* ne pourront être publiés ailleurs sous la même forme sans le consentement écrit de l'éditeur.

3) Les manuscrits et illustrations doivent être soumis en double exemplaire, l'auteur conservant une copie. Dans le cas d'une co-rédaction, les auteurs doivent indiquer le nom de celui qui recevra les épreuves.

4) Les manuscrits doivent être dactylographiés avec un double interligne et des marges importantes. Les auteurs sont priés de rédiger des communications aussi concises que possible. Les renvois de bas de page sont à éviter et les caractères en italique ne doivent pas être utilisés pour la mise en relief.

5) L'article doit être présenté de la façon suivante:

- Le titre suivi du nom de l'auteur et de son adresse complète.
- Le résumé qui doit indiquer le contenu et les conclusions de l'article et doit faire référence à des informations nouvelles. Le résumé ne doit pas excéder 200 mots et peut être divisé en paragraphes numérotés.

- Les mots clés. Une petite liste de mots clés permettant une rapide analyse du contenu de l'article et utilisable pour la compilation de l'index.

- Les articles doivent être divisés en : Introduction, Matériel et Méthodes, Résultats et Discussion. Pour les articles de synthèse, les auteurs peuvent choisir la présentation qui leur convient.

- Remerciements.

- Références. Les auteurs doivent suivre le "Harvard system". Les références doivent être données dans l'ordre suivant : nom de l'auteur et ses initiales, date (entre parenthèses), titre de l'article, nom du journal tel qu'il est abrégé dans la liste mondiale des périodiques scientifiques (4ème édit. 1963), le volume et les première et dernières pages de l'article.

Pour les livres, il est nécessaire d'indiquer le nom de l'auteur et ses initiales, la date de publication, le titre, l'édition, le nombre de pages, le nom de l'éditeur et le lieu d'édition.

Dans le texte, les références doivent être indiquées sous la forme suivante : Dupont (1964) ou (Dupont, 1964). Lorsque la référence comprend plus de deux auteurs, par exemple Dupont, Durand et Martin, l'article doit être cité comme Dupont et coll. sauf si cela peut prêter à confusion. Si l'on cite des articles d'un même auteur publiés la même année, ils doivent être distingués par les lettres a, b, etc.

La référence à un article sous presse doit signifier que l'article a été accepté pour publication et elle doit s'écrire de la façon suivante :

SETOYAMA K. (1962) Effect of water on molecular motion of silk fibroin. *J. Seric. Sci. Jpn.* Sous presse.

6) Illustrations. Le manuscrit sera accompagné de toutes les illustrations nécessaires mais celles-ci ne doivent pas être insérées dans le texte. Toutes les photographies, graphiques et diagrammes doivent être numérotés consécutivement en chiffres arabes, dans l'ordre de citation du texte.

Les photographies sur papier brillant, ou les épreuves positives (pas de négatifs ou de diapositives) doivent être envoyées si possible sans montage et en nombre limité.

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