sericologia

Revue des Vers à Soie **Journal of Silkworms**

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

Directeur Gérant	H. BOUVIER	**Managing Director**
Rédacteur en Chef	G. CHAVANCY	**Chief Editor**
Rédacteur Adjoint	A. FOURNIER	**Deputy Editor**

COMITE DE REDACTION
EDITORIAL BOARD

AKAPANTHU S. (Thaïlande / *Thailand*)
BEAULATON J. (France)
BECK D. (France)
BOSQUET G. (France)
CAPPELLOZZA L. (Italie / *Italy*)
CROIZIER G. (France)
COUBLE P. (France)
DANDIN S.B. (Inde / *India*)
DAS B.C. (Inde / *India*)
DATTA R.K. (Inde / *India*)
DOIRA H. (Japon / *Japan*)
FONSECA T.C. (Brésil / *Brazil*)
FOURCHE J. (France)
FREDDI G. (Italie / *Italy*)
GOLDSMITH M.R. (USA)
HORIE Y. (Japon / *Japan*)
IATROU K. (Canada)
JOLLY M.S. (Inde / *India*)
KOBAYASHI M. (Japon / *Japan*)
LEA H. Z. (Corée / *Korea*)
LEGAY J.-M. (France)
LIU S.-X. (Chine / *China*)
LU H.-S. (Chine / *China*)
MANO Y. (Japon / *Japan*)
MAUCHAMP B. (France)
PERSOONS C.J. (Pays-Bas / *The Netherlands*)
PRUDHOMME J.-C. (France)
REALI G. (Italie / *Italy*)
SHIMURA K. (Japon / *Japan*)
SHIRATA A. (Japon / *Japan*)
SOMASHEKAR T.H. (Inde / *India*)
SONWALKAR T.N. (Inde / *India*)
TAKAHASHI R. (Brésil / *Brazil*)
TAZIMA Y. (Japon / *Japan*)
TRENCZEK T. (Rép. tchèque / *Czech Republic*)
VAGO C. (France)
VEY A. (France)
XIA J.-G. (Chine / *China*)
YAMASHITA O. (Japon / *Japan*)
YANAGAWA H. (Japon / *Japan*°

FOREWORD

Better late than never. I hope you will agree with this saying because Sericologia supplement dedicated to Kolimbari 4th workshop, which you are receiving today should have been published at the beginning of 1998.

Unfortunately, serious problems did hamper the running of the International Sericultural Commission. On top of financial problems, we had to face the demise of our secretary, Catherine Ferrier.

A lot of time was needed to recover the material and human means necessary to catch on the publishing delays of our journal. This has been attained today and I am pleased to release the abstracts of the contributions which were presented in 1997 in Kolimbari.

In view of the quality of these contributions, it will be probably an additional reason to invite you to participate to the 5th workshop, which will be held in August 2001... It was about time !

I will ask you to please forgive me for this delay and I hope you will be satisfied with the International Sericultural Commission's support to the Workshop on the Molecular Biology and Molecular Genetics of Lepidopera.

May I wish you some good works for the 5th edition.

Gérard Chavancy
Secretary General of the International
Sericultural Commission

Sericologia Editor

INTRODUCTION

These abstracts come from papers presented at the Fourth International Workshop on the Molecular Biology and Genetics of the Lepidoptera held at the Orthodox Academy of Crete, Kolymbari, Crete, August 24-30, 1997.

A perusal of the abstracts should provide a glimpse of the exciting new information that was revealed at the meeting. There has been considerable progress in developing vectors and techniques for transforming cells and tissues, and continued hope for germ line transformation. We had presentations that dealt, at least peripherally, with aspects of rearing and enhanced silk production, a concern for countries involved in sericulture. Participants are aware that lepidopterans are major agricultural pests, and some presentations were directed towards manipulating viruses or understanding parasites that might be useful in control strategies. We had an update on genome mapping projects. All participants recognize lepidopterans as excellent model systems and the vast majority of the papers focused on molecular analyses of interesting aspects of insect lives and metabolism including diapause, growth and metamorphosis, endocrine action, immunity and regulation of the production of silk, cuticle and chorion.

We had 75 participants from 14 countries. The abstracts might, to some, reveal almost infinite diversity of subjects and approaches. Yet, given our shared interest in lepidoptera there was no difficulty in finding common ground. This was evident in the discussions that followed every paper and by the collaborations that were established. Indeed, a special feature of these workshops continues to be the exchanges that occur in the special atmosphere of the Academy that is so conducive to in depth discussion. One has to be there to gain that benefit. So we urge those who are stimulated by these abstracts to contact the next organizing committee.

Kostas IATROU & Judy WILLIS

LIST OF PARTICIPANTS
(number after names indicate presentation)

Bill Baumbach (P-15)
Insecticide Discovery, American Cyanamid Co.
P.O. Box 400, Clarksville Road, Princeton, NJ 08543, U.S.A.
baumbachb@pt.cyanamid.com

D. Benaki (39)
Department of Biology, Division of Cell Biology and Biophysics
University of Athens
Athens 157 01, Greece

Max Bergoin (4, P-5)
Laboratoire de Pathologie Comparee CC101, Unité de Virologie Moleculaire
Universite Montpellier II, Pl. E. Bataillon, 34095 Montpellier Cedex 5, France
bergoin@ensam.inra.fr

Spencer Berry (24)
Department of Biology, Wesleyan University
Middletown, CT 06457, U.S.A.
sberry@wesleyan.edu

David P. Bown (P-16)
Department of Biological Sciences, University of Durham
Science Labs, South Road, Durham, DH1 3LE, England
D.P.Bown@durham.ac.uk

Sharon C. Braunagel (16, 17)
Texas A & M University, Department of Entomology
College Station, TX 77843-2475, U.S.A.
M-Summers@tamu.edu

Pierre Couble (4, 44, 49, P-5, P-20)
Centre de Génétique Moléculaire et Cellulaire, Universite LYON I
43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne cedex, France
pierre.couble@univ-lyon1.fr

R. K. Datta (35, P-17)
Central Sericultural Research and Training Institute
Srirampura, Mysore 570 008, India
rkdatta@csrti.ernet.in

David L. Denlinger (21)
Department of Entomology, Ohio State University
1735 Neil Avenue, Columbus, OH 43210-1220, U.S.A.
denlinger.1@osu.edu

V. Economidou
Department of Biology, Division of Cell Biology and Biophysics
Univesity of Athens, Athens 157 01, Greece

Ingrid Faye (34)
Genetics Department, S-106 91 Stockholm, Sweden
ingrid.faye@genetics.su.se

Elaine C. Fitches (P-14)
Department of Biological Sciences, Science Laboratories
South Road, Durham DH1 3LE, England
E.C.Fitches@durham.ac.uk

Malcolm J. Fraser, Jr. (2)
Department of Biological Sciences, University of Notre Dame,
Notre Dame, IN 46556, U.S.A.
malcolm.j.fraser.1@nd.edu

Michael Friedlander (27)
Department of Life Sciences, Ben Gurion University of the Negev
P.O. Box 653, Beer Sheva 84105, Israel
frdln@bgumail.bgu.ac.il

Annie Garel (44, P-20)
Centre de Génétique Moléculaire et Cellulaire, Université LYON 1
43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne cedex, France
garel@univ-lyon1.fr

Marian R. Goldsmith (11, 12)
Biological Sciences Department, 100 Flagg Road
University of Rhode Island, Kingston, RI 02881-0816, U.S.A.
mki101@uriacc.uri.edu

K. P. Gopinathan (15, 50, P-10)
Microbiology and Cell Biology Department
Indian Institute of Science, Bangalore 560 012, India
kpg@mcbl.iisc.ernet.in

Karl H. J. Gordon (14)
CSIRO Division of Entomology, GPO Box 1700
Canberra, ACT, 2601, Australia
karlg@ento.csiro.au

Tom Grigliatti (1, 7, P-3)
Department of Zoology - UBC, 6270 University Boulevard
Vancouver, BC, Canada V6T 1Z4
grigliat@zoology.ubc.ca

Stavros Hamodrakas (39)
Department of Biology, Division of Cell Biology and Biophysics
University of Athens, Athens 157 01, Greece
shamodr@atlas.uoa.gr

Wajiro Hara (13, 52, P-7)
Ohwashi 1-2, Tsukuba, Ibaraki 305, Japan
whara@.nises.affrc.go.jp

David G. Heckel (11)
Department of Biological Sciences
132 Long Hall, Clemson University, Clemson, SC 29634, U.S.A.
dheckel@clemson.edu

Kostas Iatrou (6, 8, 54)
Department of Medical Biochemistry, The University of Calgary
3330 Hospital Drive, NW, Calgary, AB T2N 4N1, Canada
iatrou@acs.ucalgary.ca

Susumu Izumi (P-11)
Department of Biology, Tokyo Metropolitan University
Minami Ohsawa 1-1, Hachioji-shi, Tokyo 192-03, Japan
izumi-susumu@c.metro-u.ac.jp

Don Jarvis (9)
Department of Molecular Biology
University of Wyoming - Laramie, WY, 82071, U.S.A.
DLJarvis@uwyo.edu

Eric Julien (44, 49, P-20)
Centre de Génétique Moleculaire et Cellulaire
Université LYON 1
43 Boulevard du 11 Novembre 1918, 69622 Villeurbanne cedex, France
julien@biomserv.univ-lyon1.fr

Fotis Kafatos
European Molecular Biology Laboratory
Meyerhofstrasse 1/D-69117, Heidelberg, Germany
lonstrup@embl-heidelberg.de

Atuhiro Kishimoto (P-11)
Department of Biology, Tokyo Metropolitan University
Minami Ohsawa 1-1, Hachioji-shi, Tokyo 192-03, Japan
kishimoto-atuhiro@c.metro-u.ac.jp

Eiichi Kosegawa (P-7)
Ohwashi 1-2
Tsukuba, Ibaraki 305, Japan
kose@affrc.go.jp

Bettina Kraemer (47)
Zoologie I, Theodor-Boveri-Institut fuer Biowissenschaften (Biozentrum)
Universitaet Wuerzburg, Am Hubland, 97074 Wuerzburg, Germany
bkraemer@biozentrum.uni-wuerzburg.de

Eleftheria (Lara) Kravariti (48)
University of Athens, Dept. of Biology, Division of Biochemistry and Molecular Biology, Panepistimiopolis, Kouponia
157 01 Athens, Greece
molbiol@biology.dp.uoa.gr

M. Krishnan (40, 42)
Department of Biotechnology, Bharathidasan University
Tiruchirappalli 620 024, Tamil Nadu, India

Rena Lecanidou (48)
University of Athens, Department of Biology, Division of Biochemistry and Molecular Biology
Panepistimioupolis,157 01 Athens, Greece
rlecanid@atlas.uoa.gr

Bernard Mauchamp (P-6)
Unité Nationale Séricicole, INRA, 25 Quai J.-J. Rousseau
69350 La Mulatiere, France
mauchamp@cismsun.univ-lyon1.fr

Kenichi Mikitani (P-14)
Center for Biotechnology, Department of Biosciences at Novum
Karolinska Institutet, S-141, 57 Huddinge, Sweden
kenichi.mikitani@csb.ki.se

Hajime Mori (5)
Department of Applied Biology, Faculty of Textile Science
Kyoto Institute of Technology, Kyoto 606, Japan
hmori@ipc.kit.ac.jp

Javare Gowda Nagaraju (P-4)
Seribiotech Research Laboratory, Central Silk Board
W.C. Road, Bangalore - 86, India
jnagaraju@oeb.harvard.edu

Yumiko Nakajima (P-2)
Gene Research Center, University of the Ryukyus
1 Senbaru, Nishihara-cho Nakagami-gun, Okinawa 903-01, Japan
nakajima@grc.u-ryukyu.ac.jp

Marc Niere (37)
Free University Berlin, Institute of Zoology, Koenigin-Luise-Str. 1-3
D-14195 Berlin, Germany
awiesner@zedat.fu-berlin.de

Teruyuki Niimi (23)
Lab of Sericulture and Entomoresources
School of Agricultural Sciences, Nagoya University
Furo-cho, Chikusa, Nagoya 464-01, Japan
niimi@agr.nagoya-u.ac.jp

X. Nirmala (42)
Department of Biotechnology
Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India

Nadja Oellers (P-12)
BAYER AG, Central Research, ZF-BTB, Building Q18
D-51368 Laverkusen, Germany
Nadja.Oellers.No@bayer-ag.de

S. R. Palli (20)
Canadian Forest Service
1219 Queen Street E, Sault Ste.Marie, Ontario, Canada P6A 5M7
rpalli@am.glfc.forestry.ca

Tom Pfeifer (1, 7, P-3)
Department of Zoology - UBC
6270 University Boulevard, Vancouver, BC, Canada V6T 1Z4
pfeifer@zoology.ubc.ca

Amornrat Promboon (P-1)
Department of Biochemistry, Faculty of Science
Kasetsart University, Bangkok 10900, Thailand
fsciarp@nontri.kv.ac.th

Jean-Claude Prudhomme (49)
CGMC, Universite Lyon 1, 43 Bd. du 11 Novembre 1918
69622 Villeurbanne Cedex, France

John Eric Rebers (47)
Department of Biology, Northern Michigan University
1401 Presque Isle, Marquette, MI 49855-5341
jrebers@nmu.edu

Ralf K. Reinhardt (P-8)
Universitaet Ulm, Abteilung Allgemeine Zoologie (Bio I.), M24
Albert-Einstein-Alle 11, D89081 Ulm, Germany
ralf.reinhardt@biologie.uni-ulm.de

Steven M. Reppert (18, 19)
Jackson 1226, Mass General Hospital
Boston, MA 02114, U.S.A.
reppert@helix.mgh.harvard.eud

Lynn M. Riddiford (53, 56)
Department of Zoology, University of Washington
Box 351800, Seattle, WA 98195-1800, U.S.A.
lmr@zoology.washington.edu

George C. Rodakis (48)
University of Athens, Department of Biology
Division of Biochemistry and Molecular Biology
Panepistimioupolis,157 01 Athens, Greece
grodakis@atlas.uoa.gr

Corinne Royer (4, P-5)
Unite Nationale Sericicole, INRA, 25 Quai J.-J. Rousseau
69350 La Mulatiere, France
Royer@cismsun.univ-lyon1.fr

Ivo Sauman (18, 19, 24)
Institute of Entomology, Czech Academy of Sciences
Branisovska 31, 370 05 Ceske Budejovice, Czech Republic
Sauman@entu.cas.cz

C. (Babis) Savakis (3)
FDN Research & Technol. Hellas
Institute of Molecular Biology and Biotechnology
POB 1527, Irakilon 71110, Crete, Greece
SAVAKIS@myia.imbb.forth.gr

Klaus Scheller (10, 43)
Cell and Developmental Biology, University Biocentre
Am Hubland, D-97074 Wurzburg, Germany
scheller@biozentrum.uni-wuerzburg.de

Otto Schmidt (28,32)
Department of Crop Protection, University of Adelaide
Waite Campus, Glen Osmond SA 5064, Australia
oschmidt@waite.adelaide.edu.au

Paul D. Shirk (2, 25, P-18, P-19)
USDA ARS CMAVE, P.O. Box 14565
1700 SW 23rd Drive, Gainesville, FL 32604, U.S.A.
pshirk@gainesville.usda.ufl.edu

Amit Singh (P-10)
Microbiology and Cell Biology Department
Indian Institute of Science, Bangalore 560 012, India
amit@mcb1.iisc.ernet.in

Karen Sprague (51)
Institute of Molecular Biology
University of Oregon, Eugene, OR 97403-1229, U.S.A.
ksprague@molbio.uoregon.edu

Sathyanarayan Sriram (15)
Microbiology and Cell Biology Department
Indian Institute of Science, Bangalore 560 012, India
sriram@mcb1.iisc.ernet.in

Max D. Summers (16, 17)
Texas A & M University, Department of Entomology
College Station, TX 77843-2475, U.S.A.
m-summers@tamu.edu

Luc Swevers (54)
Department of Medical Biochemistry, The University of Calgary
3330 Hospital Drive, NW, Calgary, Alberta T2N 4N1, Canada
swevers@acs.ucalgary.ca

J.-Luc Thomas (4, P-6)
Unite Nationale Sericicole, INRA
25 Quai J.-J, Rousseau, 69350 La Mulatiere, France
thomas@cismsun.univ-lyon1.fr

Shuichiro Tomita (P-2, P-9)
National Institute of Sericultural and Entomological Science
1-2 Ohwashi, Tsukuba, Ibaraki, Japan
tomita@nises.affrc.go.jp

Tina Trenczek (33)
Institute of Zoology, Justus-Liebig University Giessen, Stephanstrasse 24
D-35390 Giessen, Germany
tina.e.trenczek@allzool.bio.uni-giessen.de

V. Vanisree (40)
Department of Biotechnology, Bharathidasan University
Tiruchirappalli 620 024, Tamil Nadu, India
viji@cnld.rect.ernet.in

Richard G. Vogt (55)
Department of Biological Sciences, University of South Carolina
Columbia, SC 29208, U.S.A.
vogt@biol.sc.edu

Susanne C. Wache (31)
Graduate Center for Toxicology, 334 HSRB, Chandler Medical Center
University of Kentucky, Lexington, KY 40536, U.S.A.
swache@ukcc.uky.edu

Bruce A. Webb (29)
Department of Entomology, S-225 Agricultural Sciences Center North
University of Kentucky, Lexington, KY 40546-0091, U.S.A.
bawebb@pop.uky.edu

Paul M. M. Weers (38)
Lipid and Lipoprotein Research Group, 327 HMRC
University of Alberta, Edmonton, Alberta, Canada T6G 2S2
pweers@gpu.srv.ualberta.ca

Michael A. Wells (36)
Biochemistry Department, Biological Sciences West
P.O. Box 210088, The University of Arizona
Tucson, AZ 85721-0088, U.S.A.
mawells@u.arizona.edu

Judith H. Willis (45)
Department of Cellular Biology
University of Georgia, Athens, GA 30602, U.S.A.
jhwillis@cb.uga.edu

Peter Wolbert (46)
Theodor-Boveri-Institut fuer Biowissenschaften (Biozentrum)
Lehrstuhl Zoologie I, Am Hubland, D-97074 Wuerzburg, Germany
wolbert@biozentrum.uni-wuerzburg.de

Okitsugu Yamashita (22, 23)
School of Agricultural Sciences
Nagoya University Chikusa, Nagoya, Japan 464-01
ymstokit@nuagr1.agr.nagoya-u.ac.jp

Xiao-Fan Zhao (26)
Department of Biology Life Sciences College
Shandong University
Jinan Shandong 250100, China
wsw@sdu.edu.cn

Michal Zurovec (10, 41)
Institute of Entomology Czech Academy of Sciences
Branisovska 31 Ceske Budejovice 37005, Czech Republic
zurovec@entu.cas.cz

SESSION 1

TRANSFORMATION AND RECOMBINANT PROTEIN EXPRESSION

1. Pfeifer / Ring / Lee / GRIGLIATTI (Canada):
The search for mobile elements in the lepidopterans. 13

2. FRASER / Elick / Shirk (USA):
Analysis of piggyBac transposable element excision in cultured insect cells and embryos. 14

3. Klinakis / SAVAKIS (Greece):
Mobilization of Minos, a *Drosophila* transposon, in lepidopteran cells. 15

4. Bossin / Romane / Royer / Thomas / Couble / BERGOIN (France):
Transformation of lepidopteran cultured cells by *Junonia coenia* densovirus vectors as an approach to develop gene transfer in insects. 16

5. MORI / Yamao / Katayama (Japan):
Targeting of a marker into fibroin light-chain gene of the silkworm. 17

6. Farrell / Shikata / Hashimoto / Behie / IATROU (Canada):
A gene of BmNPV required for progression into the virulent phase: its use in the development of a lepidopteran transducing vector. 18

7. PFEIFER / Hegedus / Theilmann / Grigliatti / Canada:
Expression of proteins in stable insect cell lines. 19

8. Farrell / Behie / IATROU (Canada):
Glycoproteins, cytoplasmic proteins, and nuclear factors secreted at high efficiency from transformed lepidopteran cell lines. 20

9. JARVIS (USA):
Analysis and modification of the N-glycosylation pathway in lepidopteran insect cells 21

1

THE SEARCH FOR MOBILE ELEMENTS IN THE LEPIDOPTERANS

T. A. PFEIFER, M. RING, B. LEE & T. A. GRIGLIATTI

Mobile genetic elements have been found in virtually all organisms for which significant amount of genomic analysis has been done. They are valuable tools for molecular genetics with applications including germ line transformation, enhancer traps, insertional mutagenesis and phylogenetic markers. We have used several techniques for the isolation of transposons and retrotransposons from pest insects; we report our results using PCR technology here. Degenerative primers based on regions of high amino acid similarity in coding regions of retrotransposons and a DNA repeat-like element were used to amplify fragments of these respective mobile elements. The amplified fragments were sequenced and positive clones used as probes to recover full-length elements from genomic DNA libraries. Using the retrotransposon designed primers, we successfully recovered a non-LTR retrotransposon from *Peridroma saucia* and an LTR retrotransposon *Lymantria dispar*. The non LTR element was found to be highly degenerative and has high similarity to *Fw* and *Doc* from *D. melanogaster*. The LTR element from *Lymantria dispar* had high DNA sequence similarity to Ted from *T. ni*, but the recovered element is not mobile due to frameshifts in the protein coding sequence. Analysis of populations of gypsy moths indicated that more than 30 copies of the element are present. In addition, we have also identified and sequenced a mariner-like element from the gypsy moth. Analysis of gene sequence shows a few disruptions in the open reading frame with terminal repeats characteristic of this element. Sequence analysis demonstrates high similarity to the *Cecropia* mariner-like elements.

2

ANALYSIS OF PIGGY BAC TRANSPOSABLE ELEMENT EXCISION IN CULTURED INSECT CELLS AND EMBRYOS

M. J. FRASER, ELICK & P. D. SHIRK

Abstract non available.

3

MOBILIZATION OF *MINOS*, A *DROSOPHILA* TRANSPOSON, IN LEPIDOPTERAN CELLS

C. SAVAKIS, A. KLINAKIS, T. LOUKERIS & T. PAVLOPOULOS

We have recently demonstrated (Loukeris et al., 1995 Science 270: 2002) that the *Minos* transposon from *D. hydei* can be used for germ line transformation of the medfly *Ceratitis capitata*, an agricultural pest in a dipteran family which diverged from that of *Drosophila* about 100 million years ago. This opens the way for developing *Minos*-based tools for transformation of other, more distantly related species.

Minos belongs to the *Tc1-mariner* superfamily of eukaryotic transposons; it is absent from *D. melanogaster* and from the medfly, but it can transpose into chromosomes of these species in the presence of *Minos* transposase. For the medfly, a wild-type version of the medfly *white* gene was used as marker in injections of homozygous *white* medfly host embryos, and transformants were recognised as clusters of phenotypic revertants of the mutation among the progeny of back crosses of the injected flies to homozygous *w* flies. This scheme is quite efficient, allowing detection of transformants at very low frequencies.

However, to assess the function of transformation tools in new target species, a method is required that (a) does not depend on the expression of an (untested) marker and (b) does not require two generations for detection of events. For this purpose, an assay for transposon function was developed that is based on the detection of excision and/ or transposition in plasmids that are introduced into embryos or cultured cells. A mixture of three plasmids is used for this assay; one (the helper plasmid) carrying the transposase gene under heat shock promoter control, a second (the donor) carrying a transposon marked with an antibiotic resistance gene (*Tet*^{R}), and a third (the target) carrying a conditional lethal gene and an antibiotic resistance different from that of the donor (*Cam*^{R}). The target plasmid carries the *Bacillus subtilis* sucrase gene *SacRB*, which is lethal for *E. coli* grown in media containing sucrose. Transpositions of the *Tet*^{R} - marked transposon into the sucrase gene are detected by plasmid rescue in *E. coli* using appropriate selection media.

Minos transposition has been demonstrated by this method in mosquito (*Aedes aegypti*) and in lepidopteran (*Spodoptera frugiperda*) cultured cells, as well as in *Drosophila* embryos and cell lines. The frequency of transposition in the cell lines of the three species is between 12 and 5.1 events per 10^5 target plasmids rescued. Although a transposase gene without the 60-bp intron present in *Minos* is functional in all three species, the native transposase gene is functional only in *Drosophila* and in *Aedes*, suggesting the *Minos* intron may not be spliced correctly in Lepidoptera. We conclude that *Minos* can form the basis of a germ line transformation system for Lepidoptera.

4

TRANSFORMATION OF LEPIDOPTERAN CULTURED CELLS BY *JUNONIA COENIA* DENSOVIRUS VECTORS AS AN APPROACH TO DEVELOP GENE TRANSFER IN INSECTS

H. BOSSIN, C. ROMANE, C. ROYER, J. L. THOMAS, P. COUBLE & M. BERGOIN

We have investigated the capacities of *Junonia coenia* densovirus (*JcDNV*) – derived vectors to stably transform lepidopteran cell lines, with the aim of developing appropriate tools for gene transfer in lepidoptera. Starting from a cloned infectious sequence of *Jc*DNV DNA, we have constructed several vectors expressing reporter genes (lacZ, CAT, Luc, GFP, NeoR) under control of the viral promoter P9 driving expression of capsid polypeptides. By transfecting these constructs to SPC-SL-52 and Sf9 lepidopteran cell lines, we demonstrated that the reporter genes could be expressed either transiently or stably in these cells. The results of analysis of DNA extracted from stably transformed cells strongly suggested that the recombinant *Jc*DNV genomes can integrate into cell DNA when the non structural (NS) genes are functional. Several attempts have been made to increase the rates of cell transformation since this may be a key step for successful transformation of insects via these integrative vectors. Unexpectedly, we observed that the deletion of the viral NS3 gene (pJneoΔNS 3 construct) resulted in a 10-fold increase of SPC-SL-52 cell transformation efficiency (10^4 to 3.10^5). Furthermore, even in the absence of selection pressure (pJlacZΔNS construct) this deletion favoured the persistence of β-gal expression for over 200 cell generations at a significant high level (50 to 90 %) in cloned cells as well as in a mixture of cell clones. In contrast, β-gal activity rapidly declined in cell transfected with the pJLacZ construct.

A second approach to use *Jc*DNV-derived vectors for insect transformation is the large production of transducing viral particles. While progressing on the establishment of a packaging cell line, attempts have been made to produce recombinant viral particles either by transfecting transformed cells with a plasmid providing in *trans* both structural and non structural viral functions or by transfecting a pJGFP construct to cells infected with a recombinant *Ac*NPV expressing the *Jc*DNV structural polypeptides. None of these approaches proved efficient for large scale production of virus particles so far.

We have also assayed *Jc*DNV-derived vectors for gene transfer in insect embryos. By injecting the PjlacZΔNS3 construct to *Drosophila melanogaster* embryos at preblastodermal stage, we monitored β-gal activity at all stages of G0 and G1 generations. We observed an intense activity in various tissues of G0 larvae and adults, showing that the vector was maintained throughout the larval, pupal and adult stage. Southern blot analyses of adult drosophila genomic DNA revealed intense signals using a viral DNA probe, strongly suggesting that the viral DNA had amplified during the course of development. However, attempts to detect viral DNA in the individuals from the G1 generation were unsuccessful. By injecting pJlacZΔNS3 DNA into *Bombyx mori* embryos, β-gal activity could be detected in both somatic and germ line cells.

These results will be discussed with regard to the development of viral vectors efficient for insect transgenesis.

5

TARGETING OF A MARKER GENE INTO FIBROIN LIGHT-CHAIN GENE OF THE SILKWORM

H. MORI, M. YAMAO & N. KATAYAMA

Autographa californica nuclear polyhedrosis virus (AcNPV) can be utilized as a vector to transfer a foreign gene into the genomic DNA of the silkworm, *Bombyx mori* (Mori *et al.*, 1995). Although AcNPV multiplicates in the silkworm larvae, the AcNPV-infected larvae survive without symptoms of nuclear polyhedrosis and larval-pupil ecdysis was observed. Pupal-adult metamorphosis of AcNPV-infected pupae was arrested, however, the metamorphosis was induced by administration of ecdysteroid hormone. Luciferase gene, expressed under control of hsp promoter, was introduced into AcNPV DNA. When the female 5th instar larvae were inoculated with the recombinant AcNPV, luciferase activities were detected in the virus-infected larvae and pupae, and in the newly hatched larvae of the next generation. PCR analysis demonstrated that the luciferase gene was transmitted through generations and AcNPV was a useful vector for the transovarian transmission of foreign genes in the silkworm.

In order to construct a gene targeting vector, upstream (5 kbp) and downstream (0.5 kbp) sequences of exon 7 of fibroin light-chain gene (L-chain gene) of the silkworm were amplified by PCR, respectively. The green fluorescent protein (GFP) gene as a marker gene was cloned between 5 kbp and 0.5 kbp PCR fragments and GFP would be expressed as a fusion protein with L-chain protein. Polyhedrin gene of AcNPV was replaced with the chimeric gene consisted of L-chain gene and GFP gene. The expression of the chimeric gene was considered to be controlled under the L-chain gene promoter. After female 4th or 5th instar larvae were inoculated with the recombinant AcNPV, the female moths were mated with normal male moths. DNA was extracted from the newly hatched larvae of the next generation (F1) and used for detection of GFP gene by PCR amplification. The remaining larvae were reared on the artificial diet. After hemocytes from each larvae were collected, DNA was extracted and used as template for PCR. Animals harboring GFP gene were selected and mated. DNA of larval hemocytes of the F2 generation was extracted and screening of GFP gene-targeting silkworm was performed by PCR.

6

A GENE OF BmNPV REQUIRED FOR PROGRESSION INTO THE VIRULENT PHASE: ITS USE IN THE DEVELOPMENT OF A LEPIDOPTERAN TRANSDUCING VECTOR

P. J. FARRELL, M. SHIKATA, Y. HASHIMOTO, L. A. BEHIE & K. IATROU

The study of gene expression in lepidopteran insects is hampered by the lack of a suitable transformation system. One of our approaches toward a solution to this problem has been to engineer the BmNPV into an infectious, yet non-virulent, self-replicating extrachromosomal entity of BVAC (baculovirus artificial chromosome).

The development of BVACs has been based on the identification of a temperature-sensitive mutation in one of the genes of BmNPV. At the non-permissive temperature, this mutation prevents the virus from progressing into its virulent phase, yet allows its genome to replicate in the infected cells, without affecting the physiological state of the cells. Viral chromosomes replicating in this manner are apparently shared between daughter cells following mitosis and cell division.

A transformed Bm5 cell line over-expressing the wild-type form of the relevant gene product was developed and shown to be capable of rescuing the temperature-sensitive mutation at the non-permissive temperature. Two transfer vectors, TV#1 and TV#2, were also constructed and used in conjunction with the packaging cell line to generate two recombinant baculoviruses, BVAC#1 and BVAC#2, that express the LacZ reporter gene but lack the gene product affected by the temperature-sensitive mutation. Plaque-purified BVAC#1 and BVAC#2 are capable of completing a normal infection cycle in, and kill the rescuing cells. However, although these viruses are capable of infecting normal Bm5 cells and expressing the reporter enzyme in them, they are unable to kill their hosts. The latter appear to remain physiologically normal for prolonged periods of time. Male pupae were also infected with BVAC#1 and BVAC#2 by haemocoelic injections and the resultant adult males were used in crosses with normal females. The analysis of the F1 progeny to determine whether β-galactosidase is expressed in them and, if so, to what extent is in progress.

7

EXPRESSION OF PROTEINS IN STABLE INSECT CELL LINES

T. PFEIFER, D. HEGEDUS, D. THEILMANN & T. GRIGLIATTI

In our studies involved with chromatin-associated proteins and intra cellular vesicle trafficking, we required a system for expressing proteins in stable insect cell lines. We have developed a set of expression vectors for use in the introduction and expression of genes in both dipteran and lepidopteran lines. The vectors created are small, carry the single selection system Zeocin that is usable in both *E. coli* and insect cells. Stable clonal cell lines expressing the protein of interest can be generated in three to four weeks and these cell lines appear to be stable when selection is removed. We have tested this system with several complex proteins. The first is a highly processed human melanotransferrin that is glycosylated and attached to the cell membrane via a glypiated anchor. The second is a small orthopteran insect hormone that is correctly processed, secreted into the medium and is highly bioactive. Other proteins expressed include protein kinases, a transporter and a β-glucosidase. A comparison of the production of these proteins in both lepidopteran and dipteran cell lines will be discussed.

We gratefully acknowledge our working collaborations with the following groups and individuals:

Synapse Technologies Inc., Vancouver, BC
Kinetek Pharmaceuticals Inc., Vancouver BC
Dr John Phillips and Lab – University of British Columbia
Dr Cam Donly – Agriculture Canada, London, Ontario.
Dr Stan Caveney – University of Western Ontario
Dr Francis Choy – University of Victoria

8

GLYCOPROTEINS, CYTOPLASMIC PROTEINS, AND NUCLEAR FACTORS SECRETED FROM STABLY TRANSFORMED LEPIDOPTERAN CELLS AT HIGH EFFICIENCY

P. J. FARREL, L. A. BEHIE & K. IATROU

We have developed a virus-free protein expression package that corrects many of the malevolent characteristics of the baculovirus expression system. We have tested the efficacy of this system for several classes of recombinant proteins including secreted glycoproteins, cytokines, nuclear factors, cytoplasmic proteins, and membrane proteins. In this system Lepidopteran insect cell lines (Bm5, Hi5, etc.) are initially co-transfected with two plasmids, one expression plasmid and the other conferring resistance to antibiotic. This is followed by antibiotic selection, isolation of highly productive clones, and, finally scale-up for continuous protein expression in bioreactors. High protein expression levels result from the exploitation of three genetic elements in the expression cassette: the strong and constitutively active *Bombyx mori* (silkmoth) cytoplasmic actin promoter is used to drive foreign gene expression, and is further stimulated over 1,000-fold by the presence of both an enhancer and a transcriptional activator.

To demonstrate the capabilities of this system, we first expressed a secreted insect glycoprotein, the reporter enzyme juvenile hormone esterase (JHE), in Bm5 (silkmoth) cells. Levels over 100 mg/L were obtained from static cultures of a cloned cell line grown in serum containing medium and 200 mg/L were obtained from a suspension culture. With cells grown on EC400 serum-free medium in a stationary culture, yields of the order of 150 mg/L were obtained. These yields compare very favourably to those obtained from a JHE-expressing recombinant baculovirus (AcNPV) that we evaluated, which could only produce 4 mg/L of JHE in a static culture of Sf21 cells. Assessments of the levels of expression of JHE by the transformed cell lines over a period of 14 months have shown that expression levels remain stable.

The ability to continuously express a normally non-secreted protein into culture supernatant, without lysing the cell, would eliminate many steps in an otherwise complicated purification process. For several cytoplasmic proteins and nuclear factors, we observed that the simple attachment of a signal peptide-encoding sequence to the 5' end of their genes is insufficient for secretion. We have now developed a novel "shuttle" module that directs efficient secretion of such proteins outside the cell and can also facilitate purification of the recombinant proteins. This module has been tested with the bacterial cytoplasmic protein CAT and with BmCF1, the heterodimerization partner of the silkmoth ecdysone receptor subunit BmEcR. The accumulation levels for these intracellular proteins in the cell culture supernatants appear to be comparable to those obtained from the JHE over-expressing cell lines.

9

ANALYSIS AND MODIFICATION OF THE N-GLYCOSYLATION PATHWAY IN LEPIDOPTERAN INSECT CELLS

D. L. JARVIS

For the past several years, our lab has been studying the N-glycosylation pathway in lepidopteran insect cells. The model glycoprotein we have used for most of the studies is gp64, a major component of budded baculovirus particles. We have found that gp64 produced by various lepidopteran insect cell lines has four N-linked glycans, some of which are processed to endoglycosidase H-resistant structures, but none of which contain galactose or sialic acid. By contrast, we found that gp64 produced in mammalian cells contains both galactose and sialic acid. These results suggested that the insect cell lines used for our studies were unable to produce gp64 with extended N-linked side chains containing penultimate galactose and terminal sialic acid, even though this protein clearly had the potential to acquire these modifications.

These results led us to construct a novel baculovirus vector capable of expressing β-1,4 galactosyltransferase early in infection, prior to the peak time of gp64 expression. Sf9 cells infected with this recombinant virus produced gp64 which had at least one N-linked side-chain that had been extended by the addition of galactose. This was the first demonstration that early baculoviral vectors can be used to modify the carbohydrate processing capabilities of lepidopteran insect cells. Subsequently, we isolated a stably-transformed Sf9 cell subclone (SfGalT) that constitutively expresses β-1,4 galactosyltransferase activity. SfGalT cells infected with a wild-type baculovirus produced gp64 with at least one N-linked side-chain that contained galactose. Furthermore, SfGalT cells infected with a conventional recombinant baculovirus encoding human tissue plasminogen activator under polyhedrin control produced tissue plasminogen activator with at least one N-linked glycan containing galactose. These results were the first to demonstrate that stable transformation of lepidopteran insect cells with immediate early expression plasmids can be used to extend the N-glycosylation pathway of lepidopteran insect cells.

Thus, we have demonstrated two different approaches that can be used to produce more authentic mammalian glycoproteins in the baculovirus-insect cell expression system. Recombinant baculovirus expression vectors, themselves, can be engineered to express carbohydrate processing enzymes early in infection, prior to the time at which the gene of interest is expressed. Alternatively, stably-transformed insect cells can be engineered to express carbohydrate processing enzymes constitutively and then used as hosts for conventional recombinant baculoviruses that express a glycoprotein of interest under polyhedrin control.

SESSION 2

PHYLOGENY / GENOME ANALYSIS

10. ZUROVEC / Sehnal / Scheller (Czech Republic):
Evolutionary features of H-fibroin genes in several lepidopteran species 25

11. HECKEL / Gahan / Goldsmith / Shimada / Raijmann / Tabashnik / Trowell (USA):
Linkage mapping in lepidoptera: technical challenges, practical applications, and comparative genomics 26

12. Marino / Mills / Knapp / GOLDSMITH (USA):
Molecular linkage mapping in the silkworm: status and prospects 28

13. HARA (Japan):
Sequence and linkage analysis of cDNA clones in the silkworm, *Bombyx mori* 29

10

GALLERIA MELLONELLA H-FIBROIN AND EVOLUTION

M. ZUROVEC, C. YANG, B. DRDOVA & M. HRADCOVA

Fibroins have been studied for many years because of their commercial importance and availability in relatively pure form. Four major structural types of fibroins were recognized by means of X-ray diffraction. It was possible to find correlation between these types and amino acid composition of fibroins, as well a relationship between fibroin types and taxonomic positions of their producers. We can, however, encounter several interesting exemptions, when a whole taxon exhibits a uniform X-ray type except for one species or a small group of species which secrete fibroins of a different type. For example, *Bombyx* fibroin, which was allocated to class I, seems to be unique among Bombycoidea since almost all other studied species of this superfamily, including *Antheraea yamamai*, produce class III fibroins. Fibroin of class I was found only in species belonging to a distant lepidopteran superfamily Noctuoidea. Interestingly, however, spider silks have a very similar structure and are classified to the same X-ray groups. Our working hypothesis is that structural constraints and concerted evolution of repetitive sequences play important roles in fast evolution of fibroin genes, and that similar fibroin types evolved independently several times. We began gathering data on the changes of fibroin structure in lepidopteran evolution.

First *H-fibroin* genes were cloned and partially sequenced in *Bombyx* and *Antheraea*, which are both members of the superfamily Bombycoidea. We have analysed *Heavy chain fibroin* gene from a more distant lepidopteran species, *Galleria mellonella* (superfamily Pyraloidea). *Galleria* fibroin was classified as type IIIb and contains oligo Ala sequences in a complex hierarchical pattern of repeats. Similarly as *Bombyx*, also *Galleria* contains N-terminal nonrepetitive stretch of more than 100 hydrophilic amino acids. By extending the published N-terminal amino acid fibroin sequence of *Antheraea yamamai*, we were able to align sequences of the three species, find a conserved region and obtain preliminary information about the rate of nucleotide and amino acid substitutions which were extremely high. We also found that the unique sequences of *Galleria* and *Antheraea* fibroins (both of class III) show more mutual homology than *Bombyx* fibroin (class I) to either of them. Since morphological analysis leaves no doubt about the monophyletic origin of Bombycoidea, the evolution of fibroin gene had to be exceptionally fast. Utilization of the stretch of unique N-terminal sequences for a gene tree construction could provide information indispensable for understanding the evolution of fibroins and other genes encoding extensive amino acid repeats.

11

LINKAGE MAPPING IN LEPIDOPTERA: TECHNICAL CHALLENGES, PRACTICAL APPLICATIONS AND COMPARATIVE GENOMICS

D. G. HECKEL, L. G. GAHAN, M. R. GOLDSMITH, L. E. L. RAIJMANN, B. E. TABASHNIK & S. C. TROWELL

Construction of linkage maps in Lepidoptera is complicated by the large number of small chromosomes and the lack of general computational techniques that account for achiasmatic oogenesis in females. But progress has been greatly facilitated by development of molecular markers based on DNA polymorphisms, and the extension of the lod score method to deal with the unique genetic system of Lepidoptera. We describe linkage mapping efforts in some Lepidoptera.

In the tobacco budworm *Heliothis virescens*, a polyphagous noctuid crop pest, all 31 chromosomes are marked with multiple markers, including 20 allozymes, 35 RFLPs, 200 RAPDs and 250 AFLPSs. Interspecific crosses with *H. sublexa* followed by backcrosses generated high degrees of polymorphism and greatly facilitated map construction. An integrated map incorporating different marker classes has been produced for Linkage Group 9, and serves as the basis for a positional cloning strategy to isolate a gene that confers high resistance to the insecticidal toxin produced by the bacterium *Bacillus thuringiensis* (Bt, Heckel *et al.* 1997, J. Econ. Entomol 90: 75-86) that occurs in field populations at a frequency of about 10^{-3} (Gould et al. 1997, PNAS 94: 3519-3523). This resistance threatens the effectiveness of the new transgenic cotton varieties that express Bt toxins, and we want to clone this gene and study its mode of action, to prevent or delay the occurrence of resistance in the field.

In the domesticated silkworm *Bombyx mori*, extension of the likelihood method facilitated analysis of an F2 cross between two inbred strains, and produced a first-generation linkage map base on RFLPs (Shi et al. 1995, Genet Res. 66: 109-126) that is now being extended by the incorporation of RAPDs.

In the small ermine moth *Yponomeuta padellus*, use of the principle of forbidden recombinants permitted a relatively small dataset on allozymes to serve as the first step in construction of a linkage map (Raijmann et al 1997, Heredity in press). Comparative linkage mapping among other *Yponomeuta* is hoped to shed light on their evolutionary relationships and clarify the interesting patterns of hostplant shifts occurring in this genus.

In the diamondback moth *Plutella xylostella*, a pest of tropical vegetable crops worldwide, an AFLP map is being created to map and characterize genes conferring resistance to Bt. Diamondback moth is still the only insect species with Bt resistance occurring in field populations, and different populations appear to have different genetic bases of resistance. The linkage map is expected to sort these out, and to indicate which Bt resistance mechanisms may be homologous to those in *Heliothis*.

In the cotton bollworm *Helicoverpa armigera*, a destructive pest of cotton in Australia, Asia and Africa we are constructing a linkage map based on AFLPs and two linkage groups contributing to cytochrome p450-mediated insecticide resistance have been identified. Analysis of Bt resistant strains is also in progress. Interspecific crosses between *armigera* and other species of *Helicoverpa* have

succeeded in producing polymorphic mapping populations that are being analysed with RFLP probes from *H. virescens*, to compare and integrate the *Heliothis* and *Helicoverpa* maps.

High-throughput multiple marker systems such as RAPDs and AFLPs have greatly reduced the technical barriers to linkage mapping in any species of choice, including the historically difficult Lepidoptera. But comparison of linkage maps of different species poses a different set of problems that will have no easy solution. As a first step in dealing with this situation, we propose a set of anchor loci for comparative genomics, to serve as reference points for integrating genetic maps of different Lepidopteran species. We have synthesized PCR primers for several of these and will distribute them to any group that agrees to collaborate in the development of a comparative genomics for the Lepidoptera.

12

MOLECULAR LINKAGE MAPPING IN THE SILKWORM, *BOMBYX MORI:* STATUS AND PROSPECTS

S. W. MARINO, D. R. MILLS, J. KNAPP & M. R. GOLDSMITH

We are constructing a molecular linkage map in the silkworm using RFLPs (restriction fragment length polymorphisms) derived from cloned cDNAs from an ovarian library, and other isolated sequences. Our standard mapping strains are p50, a south Chinese race, and C108, a Chinese genetically improved race. The same mapping strains are being used by other laboratories involved in molecular map construction. Our initial map (Shi et al., 1995, Genet. Res. 66: 109-126) which was made using an F2 population, accounted for 24 of the 28 chromosomes, and contained 61 molecular markers. Because DNA from this mapping population had become scarce and genotyping a new mapping would be relatively costly given the large number of linkage groups, we changed mapping strategy to meet the interim goal of placing two codominant, highly reliable RFLPs on each linkage group. This would provide a tool for genotyping progeny of crosses for defining QTLs (quantitative trait loci), which is a major goal of this laboratory's work, and for anchoring the molecular maps to physical maps which are in the process of being constructed in other laboratories.

Our new mapping strategy used backcrosses between heterozygous F1 females, which have no crossing over in *Bombyx* (or any Lepidoptera) and homozygous males. This allowed us to characterize relatively small mapping populations (24 individuals per cross) with only a single marker per mapped chromosome, and produce unambiguous evidence for or against synteny (linkage) with 95% confidence. As previously, we screened the cDNAs against Southern blots of parental and F1 DNA to find polymorphisms using a small subset of restriction enzymes, namely Eco RI, Hind III, and Pst I, and discarded any clones that gave poor or weak signals, were monomorphic, or produced patterns too complex to interpret readily. Summary information on screening the library will be presented. This approach produced 35 new follicular cell expressed sequences which we have assigned to the original preliminary linkage groups (PLGs) or to new independent chromosomes using C108 and p50 backcrosses. We also obtained preliminary linkage evidence for 3 known genes (*Bm engrailed, Bm LSP,* and *Bm caudal*), for a subset of bands corresponding to a transcription factor family (GATA-β1), and for a low copy number family of transposable elements in the silkworm genome (K 1.4) which are scored as dominant markers.

The present map contains a total of 96 molecular markers, including 71 anonymous cDNAs, 10 sites for the transposable element Mag, 2 sites for K 1.4 and 13 identified sequences. This encompasses 27 of the 28 linkage groups, with one of them carrying a single P-dominant maker.

We will also describe the application of this synteny strategy to attempt to identify QTLs associated with cocoon shell weight, in which we are genotyping populations with PCR-based makers from a published RAPD map (Promboon *et al.*, 1995, Genet. Res. 66: 1-7).

13

SEQUENCE AND LINKAGE ANALYSIS OF cDNA CLONES IN THE SILKWORM *BOMBYX MORI* L.

W. HARA, E. KOSEGAWA, K. MASE, S. NAGAOKA & K. OKANO

At the beginning of GENOME PROJECT, we try to construct the model systems in the silkworm, *Bombyx mori* L. We have examined probes for RFLP analysis, non-isotope Southern blot by using individual DNAs, and what kind of strains must be used for gene targeting of QTLs. More than 60% of the anonymous cDNA clones showed distinct RFLP in between p50 and J02.

We have analyzed the nucleotide sequences and linkage groups of cDNA clones.

Sequence data for identification of clones gave new information, first of all, clones those gave identical bands and had unique sequences included many unknowns and five ribosomal protein like genes and three heat-shock-protein-like genes, second, most of the clones gave smear bands had repetitious sequences like Bm1 and Bmc1, and the third, clones those gave distinct but multi-bands included AT rich sequences of 3' of the genes and cross-hybridized between them.

Linkage analysis of these unique clones were done by using individual DNAs from the segregants of the cross between p50 × J02 female and J02 male, resulted in 8 linkage groups and 11 independent clones, now.

SESSION 3

BACULOVIRUS FUNCTION

14. GORDON / Brooks / Bawden / Dorrian / Hanzlik (Australia):
Infection of lepidopteran larvae with a small RNA virus induces massive shedding of midgut cells. **33**

15. SRIRAM / Acharya / Gopinathan (India):
Regulation of hyper-transcribed very late gene promoters in *Bombyx mori* nuclear polyhedrosis virus (BmNPV). **34**

16. SUMMERS / Braunagel (USA)
Molecular signals in the integral membrane protein of AcMNPV E66 that direct wild type and reporter fusion proteins to intranuclear membranes. **35**

17. BRAUNAGEL / Summers (USA)
A functional role of FP25K and ODV-E56 in baculovirus ODV envelope protein transport and intranuclear viral maturation. **36**

14

INFECTION OF LEPIDOPTERAN LARVAE WITH A SMALL RNA VIRUS INDUCES MASSIVE SHEDDING OF MIDGUT CELLS

K. H. J. GORDON, E. M. BROOKS, A. L BAWDEN, P. J. LARKIN & T. N. HANZLIK

We are exploring how the midgut of lepidopteran larvae responds to infection by small RNA viruses. *Helicoverpa armigera* stunt virus (HaSV) belongs to the tetravirus family, whose members are small (40 nm) icosahedral viruses which have only been isolated from lepidoptera (see Hanzlik, T. N. and Gordon, K. H. J. (1997) Advances in Virus Research 48: 101-168 for a review).

HaSV is a simple virus with two genomic RNAs. RNA 1 (5312 nucleotides) encodes a 187 kD protein with limited homology to the RNA-replicases of other RNA viruses of plants and animals, whereas RNA 2 (2478 nucleotides) encodes the 71kD capsid precursor and an overlapping gene for a 17 kD protein of unknown function which mutational studies indicate may regulate replication of the two genomic RNAs. A surprising feature of the HaSV RNAs is the presence of an aminoacylatable 3'-tRNA ^{Val}-like structure, which lacks a pseudoknot and is the first such structure found on an animal virus. HaSV is one of only two tetraviruses with a bipartite genome; the other viruses in this family, including the type member *Nudaurelia* βVirus (NβV), have a single genomic RNA of 6 kb in size which carries both the replicase and capsid protein genes.

HaSV is highly specific for the midgut of heliothis larvae. It accumulates in all three major cell types comprising the midgut epithelium: the differentiated columnar and goblet cells and the regenerative cells located in the surrounding basal membrane, although it binds strongly only to a putative receptor on the surface of the goblet cell cavity. It is pathogenic only to the first three larval instars; in contrast, fourth instar larvae appear to recover from infection with very high viral doses and are able to pupate and emerge.

The restriction of virus infection to the midgut makes this an ideal system to study the response of insect cells to virus infection *in vivo*. Immunohistochemical studies at the electron, light and confocal microscope levels on midgut pathology in larvae infected at the first instar show that HaSV rapidly induces a massive increase in shedding of rejected midgut cells above the level of sloughing normally found in healthy larvae, whose growth is characterized by significant increases in both size and number of the columnar and goblet cells. This increased shedding observed upon infection results in irreversible degeneration of the midgut of young larvae. However, shedding of infected cells in larvae infected at later stages may explain the ability of these larvae to clear the midgut of HaSV infection in a successful defence response.

In order to study this infection in more detail, cDNA clones corresponding to the complete virus genome have been assembled in plasmids allowing transient expression of virus RNA and capsid protein in isolated plant cells. Infectious particles are assembled and infect larvae feeding on the protoplasts. Using this system we have inserted reporter genes into the viral genome in order to follow the course of virus infection. HaSV-infected midgut cells show DNA fragmentation characteristic of apoptosis. Using mutant recombinant forms of HaSV, we are further exploring the links between viral replication and the apoptotic response. One aim is to ask whether expression of apoptosis-modulating genes carried by recombinant HaSV affects the observed pathology.

15

REGULATION OF HYPER TRANSCRIBED VERY LATE GENE PROMOTERS IN *BOMBYX MORI* NUCLEAR POLYHEDROSIS VIRUS

S. SRIRAM, A. ACHARYA & K. P. GOPINATHAN

The hyper transcribed, very late gene promoters, polyhedrin (*polh*) and *p10*, from baculoviruses have been extensively exploited for high level expression but the mechanism underlying the high levels of transcription is not clear. Several late gene expression factors (*lefs*) have been implicated in this α-amanitin resistant transcription. We have cloned and sequenced a *late gene expression factor (lef2)* from *Bombyx mori* Nuclear Polyhedrosis Virus (BmNPV) which shares 96% homology at DNA level to its counter part from the prototype virus, *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV). The Bm*lef2* encodes a 209 aa protein, harbouring a highly Cys rich C-terminal domain and it *trans*-activates very late gene expression in transient expression assays. The deletion of the C-terminal domain inactivates the transcriptional activation. The temporal transcription profiles of *lef2* revealed the presence of a 1.6 kb transcript in both delayed early and late periods following virus infection. Transcription start site mapping by primer extension analysis identified the presence of an aphidicolin sensitive late transcript arising form a TAAG motif located at −352 nt with respect to +1 ATG of *lef2* and an aphidicolin insensitive early transcript originating 35 nt down stream to a TATA box located at −312 nt with respect to +1 ATG. Both early and late transcripts harboured long 5'UTRs with stable stem loop structures. Inactivation of Lef2 synthesis at late time points by antisense *lef2* transcripts resulted in drastic reduction in very late gene transcription whereas the immediate early gene transcription remained unaltered. The expression of antisense *lef2* transcripts at late time points did not significantly affect the viral DNA replication while the presence of early antisense transcripts caused a 20-30% reduction in viral DNA replication. Based on these results, Lef2 could be ascribed a bifunctional role in both very late gene transcription and viral DNA replication.

16

MOLECULAR SIGNALS IN THE INTEGRAL MEMBRANE PROTEIN OF AcMNPV E66 THAT DIRECT WILD TYPE AND REPORTER FUSION PROTEINS TO INTRANUCLEAR MEMBRANES

M. D. SUMMERS & S. C. BRAUNAGEL

Baculovirus occlusion derived virus (ODV) obtains its envelope from an intranuclear source of membranes. The molecular pathways for viral envelope protein trafficking, targeting and maturation as membrane precursors in the nucleoplasm of the cell is not known. To study this, the viral genes for several proteins identified as specific for the occlusion derived virus were cloned, sequenced and localization of the protein determined during the course of infection. Western blot, immunoelectron microscopy and fractionation of purified virus into capsid and envelope confirmed that E66 was specific to the ODV envelope. And, ODV-E66 is present in microvesicles and unit membrane structures on the infected nucleus. These results suggest that virus-induced intranuclear microvesicles and unit membrane structures in the nucleus are ODV envelope precursors.

A series of recombinant viruses containing deletions of ODV-E66 amino acid sequences revealed that a 23AA hydrophobic amino terminal domain fused to a reporter protein (GFP) was sufficient to direct the 23AA-GFP reporter to intranuclear membrane vesicles and the ODV envelope. These studies also show 23AA GFP reporter in cytoplasmic membranes juxtaposed to the nucleus and the inner and outer nuclear membrane. These results were confirmed with a 24AA hydrophobic N-terminal sequence of AcMNPV-E25 similar to that of 23AA-ODV-E66. This suggests that the trafficking of ODVE66 and ODV-E25 is associated with the outer and inner nuclear membranes; and, that this trafficking and localization is dependent on the 23N-terminal amino acid sequence. To identify other proteins that interact with E66 in trafficking and localization, studies were conducted using FP mutant infected cells. The results show that a fully functional FP25K protein is involved in the E66 pathway of trafficking into intranuclear membranes.

17

A FUNCTIONAL ROLE OF FP25K AND ODV-E56 IN BACULOVIRUS ODV ENVELOPE PROTEIN TRANSPORT AND INTRANUCLEAR VIRAL MATURATION

S. C. BRAUNAGEL & M. D. SUMMERS

Baculovirus infection induces membrane structures within the nucleus of an infected cell and these membranes act as precursors for the viral envelope of occluded derived virus (ODV). For successful envelopment of ODV, proteins destined for the viral envelope must be:

i) Properly transported into the nucleus and incorporated into the membrane precursors;

ii) Mature nucleocapsids must recognize the membrane precursors and undergo envelopment. Our studies have identified several proteins that function in these pathways. Current work is directed to identify the mechanism or structural domain of FP25K required for proper transport of BV/ODV-E26 and ODV-E66. We have identified a protein that locates to the viral envelope of both BV and ODV, BV/ODV-E26 and followed transport to the cell surface and into the nucleus of this protein. This work has led to a model of protein and/or nucleocapsid transport that includes FP25K and cellular actin.

The second stage of viral nucleocapsid envelopment is mediated at least in part, by the ODV-E56 protein. When ODV-E56 is fused to the reporter GFP, the fusion protein is transported to the intranuclear microvesicles, however the majority of the nucleocapsids do not acquire their envelope and the nucleus fills with membrane microvesicles and non enveloped nucleocapsids. Thus, ODV-E56GFP is transported normally, however recognition of nucleocapsids and membrane precursors is seriously compromised. Additionally, viral occlusions form, but are mostly devoid of virus. The relationship and role of FP25K, BV/ODV-E26 and ODV-E56 in protein transport and viral maturation and assembly will be discussed.

SESSION 4

MOLECULAR CLOCKS, DIAPAUSE, GERM CELLS

18. REPPERT / Sauman (USA):
Circadian clock neurons in the silkmoth *Antheraea pernyi*: novel mechanisms of period protein regulation. 39

19. SAUMAN / Tsai / Roca / Reppert (Czech Republic):
Period protein controls circadian egg hatching behavior in the silkmoth, *Antheraea pernyi* 40

20. PALLI / Ladd / Gojtan / Retnakaran (Canada):
Molecular analysis of overwintering diapause in spruce budworm *Choristoneura fumiferana*. 41

21. Lee / DENLINGER (USA) :
Diapause regulation in the gypsy moth. 42

22. YAMASHITA (Japan):
A new peptide (VAP peptide) and a new egg system for studies on diapause of the silkworm, *Bombyx mori*. 43

23. NIIMI / Yamashita / Yaginuma (Japan):
Cold inducible genes in diapause eggs of the silkworm, *Bombyx mori*. 44

24. Sauman / BERRY (USA):
Apoptosis in the *Manduca* ovary. 45

25. SHIRK / Broza / Hapner / Perea (USA):
Alpha-crystallins are chaperones in germ cells of moths. 46

26. ZHAO / Wang (China):
Studies of egg proteinases in *Samia*, *Antheraea* and *Helicoverpa*. 47

27. FRIEDLÄNDER (Israel):
Sperm dimorphism in Lepidoptera. 48

18

CIRCADIAN CLOCK NEURONS IN THE SILKMOTH *ANTHERAEA PERNYI*: NOVEL MECHANISMS OF PERIOD PROTEIN REGULATION

S. M. REPPERT & I. SAUMAN

We examined *period* protein (PER) regulation in the brain of the silkmoth *Antheraea pernyi*. PER expression is restricted to the cytoplasm and axons of 8 neurons with no evidence of temporal movement into the nucleus. These neurons appear to be circadian clock cells, because PER and *per* mRNA are colocalized and their levels oscillate in these cells, *timeless* protein immunoreactivity is co-expressed in each PER-positive neuron, and clock protein and mRNA oscillations are all suppressed in these neurons by constant light. A *per* antisense RNA oscillation was detected that is spatially restricted to PER-expressing cells, suggesting a new mechanism of PER regulation. PER-positive neurons and their projections are strategically positioned for regulating prothoracicotropic hormone and eclosion hormone, two neurohormones under circadian control. Differences in the molecular details of PER expression and regulation between the brains of silkmoths and fruitflies provide new insights into the mechanisms of clock gene regulation.

19

PERIOD PROTEIN CONTROLS CIRCADIAN EGG HATCHING BEHAVIOR IN THE SILKMOTH, *ANTHERAEA PERNYI*

I. SAUMAN & S. M. REPPERT

We examined the molecular basis of the circadian control of egg hatching behavior in the silkmoth *Antheraea pernyi*. Egg hatching is rhythmically gated, persist under constant darkness, and can be entrained by light by midembryogenesis. The time of appearance of photic entrainment by the silkmoth embryo coincides with the appearance of Period (PER) and Timeless (TIM) proteins in eight cells in embryonic brain. Although daily rhythms in PER and/or TIM immunoreactivity in embryonic brain were not detected, a robust circadian oscillation of PER immunoreactivity is present in the nuclei of midgut epithelium, *per* antisense oligodeoxynucleotide treatment of pharate larvae on the day before hatching consistently abolishes the circadian gate of egg hatching behavior. *per* antisense treatment also causes a dramatic decrease in PER immunoreactivity in newly hatched larvae. The results provide direct evidence that PER is a necessary element of a circadian system in the silkmoth. More over, our recent brain transplantation studies strongly suggest that the circadian clock which controls the gating of egg hatching resides in the brain.

20

MOLECULAR ANALYSIS OF OVERWINTERING DIAPAUSE IN THE SPRUCE BUDWORM, *CHORISTONEURA FUMIFERANA*

S. R. PALLI, T. R. LADD, K. GOTJAN, J. W. BARRET, F. QILI, M. PRIMAVERA & A. RETNAKARAN

The spruce budworm, *Choristoneura fumiferana*, exhibits an obligatory overwintering diapause as a 2nd instar larva. The role of proteins associated with this overwintering diapause was investigated. *C. fumiferana* larva produces large quantities of two hexameric proteins (diapause associated protein 1 & 2, DAP1 & 2, sub unit sizes 72 and 74 kDa) that are diapause related. These proteins accumulate in the 1st instar larval hemolymph beginning at four days after emergence and reach maximum levels by seven days. High levels of these proteins are maintained throughout diapause. The mRNAs (2.4 kb) coding for these proteins were present in large quantities during all days of the 1st instar larval stage but decreased to lower levels as soon as the larvae molted into 2nd instar larval stage and entered diapause. The mRNAs reappear in the fat body of the last larval instar for 2-3 days after which they disappear once again. The DAP1 promoter region contains the ecdysone response element and the AGGTCA monomer binding site that bind to the ecdysone receptor complex and the *Choristoneura* hormone receptor 3 respectively. We have constructed a recombinant baculovirus expressing the Green fluorescence protein (GFP) under the control of DAP1 promoter. GFP was detected in CF-203 cells within 3 hrs after inoculation with this virus. Upon inoculation of *C. fumiferana* larvae with the recombinant virus, GFP was detected in all the baculovirus infected tissues.

We have used differential display of mRNAs technique to identify genes whose mRNAs either increase or decrease during overwintering diapause. One of these genes was identified as an insect chaperonin. Chaperonins have been shown to be involved in the proper folding of proteins. Another gene was identified as an insect defensin. Defensins are proteins that have antibacterial activity and are induced in response to bacteria and / or stress. In *C. fumiferana* larvae defensin mRNAs are induced during the 2nd instar larval diapause in response to cold. Unlike other insect defensins which are synthesized in the fat body or hemocytes, CfDefensins are synthesized in the midgut.

Supported by the Canadian Forest Service, and the Science and Technology Opportunities Fund.

21

DIAPAUSE REGULATION IN THE GYPSY MOTH

K-Y. LEE & D. L. DENLINGER

With the exception of *Bombyx mori*, very little is known about the mechanisms that regulate embryonic diapause. We have addressed this question in the gypsy moth *Lymantria dispar*, a species that enters an overwintering, obligatory diapause as a pharate first instar larva. Several lines of evidence suggest a novel regulatory mechanism for diapause regulation in this species. We present evidence that this diapause is initiated and maintained by an elevation of ecdysteroids and is terminated by a decline in the ecdysteroid titer. The success of this work was dependent upon our discovery of a 55 kDa gut protein that is uniquely synthesized during diapause. This protein provided a valuable marker for monitoring the diapause status of the gypsy moth. Ligation behind the prothorax halted synthesis of the protein but synthesis could be restored by co-culturing pharate larvae in hanging drop cultures with prothoracic glands. Agents such as KK-42 that block ecdysteroid synthesis can be used to avert the diapause, and diapause termination can be prevented by maintaining an elevated level of ecdysteroids. And, a nondiapausing strain of the gypsy moth can be forced to enter diapause by exposure to ecdysteroids.

Other experiments underway indicate that most midgut enzymes are greatly reduced during diapause, but one enzyme, alkaline phosphatase, increases dramatically in activity at the onset of diapause. An examination of brain proteins indicates a conspicuous shut-down in synthesis of a major brain protein (42 kDa) at the onset of diapause. At diapause termination the 45 kDa protein is again highly expressed. Partial amino acid sequence determination suggests that the 45 kDa protein is actin, and this was confirmed using anti-actin antibodies. RT-PCR using primers designed from the sequence of the gypsy moth actin mRNA indicates that appearance of actin mRNA in the CNS follows the same developmental pattern of expression as observed for the 45 kDa protein. The expression of actin thus emerges as a powerful tool for monitoring the diapause status of the gypsy moth brain.

REFERENCES:

LEE K.-Y. & D. L. DENLINGER (1996) Diapause-regulated proteins in the gut of pharate first instar larvae of the gypsy moth, *Lymantria dispar*, and the effect of KK-42 and neck litigation on expression. J. Insect Physiol. 42: 423-431.

LEE K.- Y. & D. L. DENLINGER (1997) A role for ecdysteroids in the induction and maintenance of the pharate first instar diapause of the gypsy moth, *Lymantria dispar*. J. Insect Physiol. 43: 289-296.

LEE K.-Y., A. P. VALAITIS & D. L. DENLINGER (1997) Further evidence that diapause in the gypsy moth, *Lymantria dispar*, is regulated by ecdysteroids: a comparison of diapause and nondiapause strains. J. Insect Physiol. (in press).

22

A NEW PEPTIDE (VAP PEPTIDE) AND A NEW EGG SYSTEM FOR STUDIES ON DIAPAUSE OF THE SILKWORM, *BOMBYX MORI*

O. YAMASHITA

The diapause hormone (DH) was found in 1951 to be a neurohormone which is secreted from the suboesophageal ganglion (SG) and is responsible for induction of embryonic diapause of the silkworm, *Bombyx mori*. Since 1956, we have made many attempts to purify DH from male adult heads, but failed in purification. In 1991, DH was finally isolated from SGs, and determined to be a 24 amino acid peptide amide. We challenged again to purify some bioactive peptides from male adult heads using organic solvents for extraction. A new peptide (VAP peptide) with a low but significant biological activity was isolated by HPLC with different programmes. VAP peptide was a hydrophobic peptide composed of 68 amino acids, in which Val, Ala, Pro and His accounted for 80%. By producing a lot of the recombinant VAP peptide, we decided that VAP peptide is not a diapause inducing peptide, but a strong synergist enhancing DH activity when administrated exogenously. Thus, VAP peptide or its smaller fragments is useful as a molecular tool for analysis of DH action, and as a potent enhancer of DH in the field of sericultural industry.

The diapause occurs as an alternative developmental program in the life cycle and is accomplished by the dynamic change of biochemical events. The silkworm embryonic diapause is realized by a sequential shift in carbohydrate metabolisms. In this process, DH acts to induce trehalase gene expression in developing ovaries, through which action DH directs a series of diapause associated metabolisms. This central dogma for diapause biochemistry is reconsidered by preparing a new egg system. A new trehalase inhibitor, trehazolin, was systematically applied with various doses to pupae at different stages. Injection of trehazolin reduced glycogen content in ovaries and eggs at various levels, and some eggs completely lost glycogen. The glycogen deficient eggs maintained the ability to undergo embryogenesis. DH expressed its full activity to induce diapause eggs in the trehazolin treated eggs as the controls with injection of DH alone. Consequently, we can produce a new egg system for reconsideration of the diapause metabolism other than the glycogen-sorbitol metabolism.

23

COLD INDUCIBLE GENES IN DIAPAUSE EGGS OF THE SILKWORM, *BOMBYX MORI*

T. NIIMI, O. YAMASHITA & T. YAGINUMA

Environmental signals such as photoperiod and temperature are important for the regulation of insect diapause. How environmental temperature acts as a signal at molecular level is totally unknown. To understand the molecular mechanism of the action of temperature on insect diapause, we use the silkworm, *Bombyx mori*, as an experimental material.

The diapause of *Bombyx* is precisely regulated by temperature at the two phases of diapause. One is the programming of diapause of the next generation by the incubation temperature during the middle stage of embryogenesis of bivoltine races. The other is the termination by cold acclimation. We focus on the latter phenomena in this workshop.

When diapause starts, several physiological changes occur. One of the major changes is the accumulation of sorbitol from glycogen. Sorbitol accumulates at about 150 mM in diapausing eggs. However, sorbitol is converted into glycogen at the termination of diapause by cold acclimation. NAD-sorbitol dehydrogenase (SDH) was found to be a key enzyme for the utilization of sorbitol. The activity of SDH is almost negligible in diapausing eggs, but is increased by acclimation at 5° C.

We have investigated the regulation mechanism of SDH to understand how low temperature induces SDH activity. Immunoblotting and RT-PCR analyses showed that SDH activity was correlated with the amounts of the enzyme protein and its mRNA. SDH mRNA was localized in yolk cells. These results indicate that the yolk nuclei-dependent gene expression of *Bombyx* SDH is induced by acclimation to 5° C in diapause eggs.

To investigate the regulation mechanism of the SDH gene expression, the structure of the SDH gene was analyzed. The 5'-upstream region of the gene contains the sequences similar to cis-elements recognized by members of the steroid receptor superfamily. The regulation of the SDH gene expression by the steroid receptor superfamily gene products is under inverstigation.

To identify other cold inducible genes associated with diapause, we carried out differential display. We compared the difference between high temperature incubation and low temperature incubation. We found a gene which was induced by chilling at 5° C for 10-20 days. We propose that diapause termination may occur by the sequential induction of gene expressions in response to the different chilling period.

24

APOPTOSIS IN THE *MANDUCA* OVARY

I. SAUMAN & S. BERRY

Our initial observations of apoptosis in the moth ovary were made during a study of the behavior of actin in the cortical cytoskeleton of developing oocytes of the tobacco hornworm, *Manduca sexta* (Sauman and Berry, 1993). We noticed that when female moths were injected with an actin cleaving agent, Cytochalasin D (CD), at very modest concentration (10 µg / gm animal), the females were unaffected, except that egg-laying was suspended. Dissection of treated moths revealed that the vitellogenic follicles were destroyed. Previtellogenic follicles appeared unaffected by the treatment and already chorionated eggs were retained and later oviposited. Control males were injected with comparable doses of CD, and died within 24 hours. When the actin-containing tissues of the treated males were examined, structures such as the actin filaments of flight muscles were totally disrupted, but nuclei seemed unaffected. All somatic tissues of the CD treated females appeared normal with the exception of the vitellogenic ovarian follicles. In particular, the flight muscles were still intact and functional, as were all nuclei examined. Our interpretation of these results is that the CD must have been rapidly taken up by the follicles, thus sheltering other tissues from the toxic effects. This theory would be consistent with numerous observations on many different species in which injected compounds are concentrated in the oocytes. A less probable explanation is that females have some detoxifying system not present in males, but we know of no evidence to indicate this alternative. The final conclusion must be the same in either case, CD triggers the premature destruction of vitellogenic follicles, but no other tissues in the female.

The CD initiated death of vitellogenic follicles appears to represent a classic case of apoptosis, while other cell death induced in tissues of the adult males appears to represent necrosis. Under the influence of CD the nurse and follicle cells round up, but organelles such as mitochondria, ER and Golgi Apparatus are not damaged. The nucleus condenses, and as the condensation progresses, blebs break away from the main mass and form extremely dense masses which are Feulgen and Hoechst 33342 positive. DNA extracted from these dying cells produced a typical "nucleosome ladder" when separated on agarose gels. The only deviation from the "classical" picture of apoptosis that we have observed is that we cannot inhibit CD-induced nurse or follicle cell apoptosis with either Actinomycin D, an inhibitor of RNA synthesis nor with Cycloheximide, an inhibitor of protein synthesis.

25

α-CRYSTALLINS ARE CHAPERONES IN GERM CELLS OF MOTHS

P. D. SHIRK, R. BROZA, M. HEMPHILL & O. P. PERERA

α-Crystallyn protein eognates were found in germ cells of the Indianmeal moth, *Plodia interpunctella* (Shirk and Zimowska, Inseet Biochem. Molec. Biol. 27, 149-157). A eDNA elone was isolated for 25,000 kDa moleeular weight polypeptide member of this family, αCP25. Both the DNA sequenee and predieted amino aeid sequenee showed considerable homology with the embryonic lethal gene, *l(2)efl*, in *Drosophila melanogaster*. The predieted amino acid sequenee for αcp25, as well as *l(2)efl*, also showed signifieant sequenee similarity with the α-erystallin A chain polypeptides from the lenses of vertebrate eyes. An N-terminal hydrophobie aggregation site and a C-terminal proteetive binding site eommon to α-erystallin proteins were present in the predieted αep25 amino aeid sequenee. On the other hand, the eDNA sequenee for *αcp25* showed more similarity to small heat shoek proteins in *D. melanogaster*. This evidenee suggests that although the α-erystallin protein eognates in *P. interpunctella* evolved from a gene eommon with small heat shoek protein genes, the amino aeid sequence has converged on a structure similar to that of α-crystallin proteins. An eleetroblot binding assay was used to show that the germ-cell α-crystallins of *P. interpunctella* bind speeifieally with the follieular epithelium yolk protein (FEYP) and that the binding was reversible in the presenee of APT or low pH. Thus, the α-erystallin eognates appear to funetion as ehaperones for the follicular epithelium yolk proteins in the embryos of *P. interpunctella*.

26

STUDIES ON EGG PROTEINASES FROM *SAMIA*, *ANTHERAEA* AND *HELICOVERPA*

X. F. ZHAO & J-X. WANG

Egg proteinases are involved in the degradation of yolk proteins, which are considered being important in the embryonic development. The properties, mechanisms of activation and regulation of egg proteinases are key points of the research. Up to now, the egg proteinases from *Bombyx mori* have been relatively thoroughly studied and four kinds of egg proteinases have been purified and well characterized. One is cystein proteinase, and others are serine proteinases. To compare the proteinases existing in other Lepidopteran insects, egg proteinases in *Samia cynthia ricini*, *Antheraea pernyi* and *Helicoverpa armigera* were investigated in this study.

Results showed that yolk proteins in these insects were degraded at acidic pH when the extracts of oocytes were incubated *in vitro*, which suggested that there were some acidic proteinases existing in oocytes. Proteolytic activities in oocytes were increasing with the development of oocytes, which was similar to that in *Bombyx mori*. The activities in oocytes were effectively inhibited by Chymostatin, Iodoacetate and E-64. Besides, proteolytic activities in oocytes from *Samia cynthia ricini* and *Antheraea pernyi* were also inhibited by Leupeptin and Pepstatin. These results implied that cysteine proteinase and aspartic proteinase existed in oocytes. The proteinases were partially purified from oocytes of these insects. The activities of purified proteinases were inhibited by E-64 as well as other inhibitors, which suggested that they were cystein proteinases.

Compared with cysteine proteinase from *Bombyx mori*, the activities of cystein proteinases from *Samia cynthia ricini* and *Antheraea pernyi* are obviously lower. The mechanism of activation of the proteinases is kept unknown. The molecular mass of cystein proteinase from *Helicoverpa armigera* is smaller than that from *Bombyx mori*. There are also reports about egg proteinases from other insects. These proteinases appear different characters on molecular mass, optimum pH and inhibitors. The reasons resulted in the differences on the characters of egg proteinases might be because of the different composition of yolk protein, as well as the style of development of oocytes. If they are homology with each other is quite needed to be clarified on molecular level.

27

SPERM DIMORPHISM IN LEPIDOPTERA

M. FRIEDLANDER

The normal lepidopteran male produces two kinds of sperm: nucleate-eupyrene and anucleate-apyrene. But the eggs are fertilized exclusively by the nucleate ones which, however, make up less than 50% of the sperm reaching the spermatheca of the inseminated female. The function of the apyrene-sterile sperm is still obscure. Both kinds of spermatozoa derive from the same bipotential early primary spermatocytes which produce eupyrene spermatozoa in the larvae, and anucleate ones in the pupae and imago. Spermatocytes producing nucleate sperm divide regularly while spermatocytes producing anucleate spermatozoa show asynchronous- asymmetric chromosome segregation and distribution during metaphases – telophases. The nuclei of the prospective anucleate spermatozoa are eventually discarded from the cells during spermatid differentiation. The switch over of spermatocyte commitment from eupyrene to apyrene spermatogenesis is induced by a haemolymph apyrene-spermatogenesis-inducing factor (ASIF), causally related to pupation and becoming active close to the molting to this instar. Thus, (1) testes of penultimate or last larval instar of *Cydia pomonella*, that bear eupyrene spermatogenetic cells only, begin producing apyrene-anucleate spermatids when transplanto into pupae, and (2) in *Actias selene*, apyrene cells appear two days after pupation in precocious pupae produced by allatectomizing penultimate larvae, as in control intact pupae, notwithstanding the precocious pupae are 11-12 days younger than the control ones. Induction of the shift to apyrene differentiation is related to (a) shortening of the meiotic prophase, as determined by radioactive thymidine incorporation, and (b) suppression of synthesis of a meiotic lysine-rich cytoplasmic protein fraction, which is present in the dividing eupyrene spermatocytes. It is still unknown whether the speedy apyrene meiotic prophase is too short to make the synthesis of the lysine-rich protein fraction feasible or whether the lack of this fraction results on the shortening of the apyrene meiotic prophase.

Spermatogenesis is a discontinuous process punctuated by predetermined stations. Progress from one to the next station is under hormonal control. Spermatogenesis continues uninterruptedly and apparently "automatically" until mid meiotic prophase but further progress towards metaphase is induced by a peak of 20-hydroxyecdysone. Thus, experimental removal of this peak in *Manduca sexta* larvae by isolating the abdomen from the thorax which contains the main source of the hormone, causes stoppage of meiosis at diakinesis and, eventually, the primary spermatocytes lyse before reaching metaphase. Implanting active prothoracic glands or injecting 20-hydroxyecdysone into the isolated abdomens, induces meiosis deblocking and renewal of the progress towards metaphase. The deblocking effect is dose-dependent, the higher the dose, the longer the time the spermatocytes arriving diakinesis continue uninterruptedly their development. To investigate the mode of action of the ecdysteroids during meiosis reinitiation, a non-ecdysteroidal ecdysone agonist, that is not metabolized *in vivo*, was applied to isolated abdomens of diapausing *Cydia pomonella* larvae, which display spermatogenesis stoppage at the primary spermatocyte level. Reinitiation of meiosis and the subsequent advance of the spermatocytes from diakinesis towards metaphase is a "all or none" type of phenomenon in which, it appears, the hormone attaches, and saturates, a determined number of receptors as: (1) a minimal concentration of agonist is needed to reinitiate meiosis and (2) extremely

high concentrations of the agonist neither induce a more effective renewal, nor produce any apparent detrimental effect on spermatogenesis.

FRIEDLÄNDER M. & HAUSCHTECK-JUNGEN E. (1986) Regular and irregular divisions of Lepidoptera spermatocytes as related to the speed of meiotic prophase. Chromosome 93: 227-230.

FRIEDLÄNDER M. & REYNOLDS S. E. (1992) Intratesticular ecdysteroid titers and the arrest of sperm production during the pupal diapause in the tobacco hornworm, *Manduca sexta*. J. Insect Physiol. 38: 692-703.

FRIEDLÄNDER M. & BROWN J. J. (1995) Tebufenozide (Mimic®), a non-ecdysteroidal ecdysone agonist, induces spermatogenesis reinitiation in isolated abdomens of diapausing codling moth larvae (*Cydia pomonella*) J. Insect Physiol. 41: 403-411.

SESSION 5

PARASITES AND IMMUNITY

28. SCHMIDT/Beck/Siekmann/Theopold (Australia) :
Molecular genetics of intraspecific host competition 53

29. WEBB/Cui/Deng (USA) :
Real and apparent complexity in polydnavirus genomes : linkages between genome organization and viral gene function. 54

30. JONES (USA) :
Precocious metamorphosis and immune suppression induced in host Lepidoptera by regulatory agents injected by adult female wasps of *Chelonus curvimaculatus*. 56

31. WACHE/Jones (USA) :
Participation of venom and polydnavirus in parasite escape from host immune reactions. 57

32. SCHMIDT/Asgari/Theopold (Australia) :
Maternal wasp secretions protect the egg and larva against the host immune reaction 58

33. TRENCZEK (Germany) :
Studying insect cellular defense mechanisms with the help of monoclonal antibodies 59

34. FAYE/Roxstrom-Lindquist/Tapia/Bettencourt/Redd/Toutain (Sweden) :
Functional aspects of hemolin, an insect immune protein of the immunoglobulin superfamily (IgSF). 60

35. DATTA/Ponnuvel/Jain (India) :
Purification, characterization of antiviral protein in the gut juice of silkworm, *Bombyx mori* L. 61

28

MOLECULAR GENETICS OF INTRASPECIFIC HOST COMPETITION

O. SCHMIDT, BECK, SIEKMANN & THEOPOLD

Abstract non available.

29

REAL AND APPARENT COMPLEXITY IN POLYDNAVIRUS GENOMES' LINKAGES BETWEEN VIRAL GENOME ORGANIZATION AND VIRAL GENE FUNCTION

B. A. WEBB, L. DENG, L. CUI

Polydnaviruses are insect viruses carried by some parasitic hymenoptera that inhibit lepidopteran immunity and development after parasitization. Polydnaviruses are vertically transmitted as proviruses and are integrated in the wasp's chromosomal DNA. Polydnaviruses replicate only in specialized cells of the female reproductive tract, the calyx cells, for transmission to parasitized insects during oviposition. In lepidopteran larvae, polydnaviruses infect several tissues, most notably hemocytes, and express a host-specific subset of viral genes that alter host physiology. Importantly, viral gene expression occurs in the absence of detectable viral DNA replication in parasitized insects. Polydnaviruses are unusual in that they are the only segmented DNA viruses. Moreover, genome segmentation and an obligate association with parasitic hymenoptera are characteristics shared by both polydnavirus genera although the phylogenetic distribution of and virion structure strongly suggest that the two polydnavirus genera arose from evolutionarily independent virus lineages. Therefore, characteristics common among polydnaviruses (i.e. genome segmentation and association with parasitic wasps) may be derived.

Our laboratory has pursued a two-track approach to the study of the *Campoletis sonorensis* poydnavirus under the premise that viral genome organization and virus function in parasitized insects are inter-related. The unique viral genome organization of polydnaviruses may result from their unique associations with parasitic wasps. One line of research targets functional analysis of viral genes expressed in parasitized insects using a molecular approach. We have described lesions in the insect immune response that include inhibition of encapsulation and melanization as well as the failure of the antibacterial immune response. An abundantly expressed viral gene family, the cys-motif genes, have been linked to inhibition of encapsulation but are not associated with the lesions in humoral immunity (i.e. melanization and antibacterial protein synthesis). Inhibition of encapsulation requires abundant expression of at least one and possible all of the cys-motif genes. The second line of research seeks to describe the fundamental organization of polydnavirus genomes and elucidate links to viral gene function. The CsPDV genome has two fundamental types of genome segments. Those that hybridize strongly to other genome segments ; and those that do not hybridize to other segments. Sequence analysis of CsPDV segments that hybridize to segment W demonstrate that the W cross-hybridizing segments are nested (smaller segments are produced from the parental segment by intra-molecular recombination) with all segments produced from a single, integrated proviral segment. Moreover, the copy number of genes encoded on nested segments is increased as a result of segment nesting and appears to be responsible, in part, for the abundant expression of the cys-motif genes. Approximately half of the CsPDV genome segments are not nested and are integrated at distinct genomic loci. These "unique" segments are not well characterized but appear to encode genes that are less abundantly expressed and have not been linked to specific functions in parasitized insects.

In summary, polydnavirus genome organization may be associated, in part, with controlling viral gene expression in parasitized larvae and may reflect the evolution of polydnaviruses within the constraints of their obligate associations with parasitic wasps.

Related references :

DIB-HAJJ S.D., WEBB & B.A & SUMMERS M.D (1993) Structure and evolutionary implications of a "cysteine-rich" *Campoletis sonorensis* polydnavirus gene family. *Proc Natl Acad Sci USA* 90:3765-3769.

XU D. & STOLTZ D.B. 1993. Polydnavirus genome segment families in the ichneumonid parasitoid Hyposoter fugitivis. J. Virol. 67:1340-1349.

LI X. & WEBB B.A (1994) Apparent functional role for a cysteine-rich polydnavirus protein in suppression of the insect cellular immune response. J. Virol. 68:7482-7489.

CUI L. & WEBB B.A (1996) Isolation and characterization of a member of the cysteine-rich gene family from *Campoletis sonorensis* polydnavirus J. gen. Virol.77:797-809.

SOLDEVILA A., HEUSTON S. & WEBB B.A (1997) Purification and analysis of a polydnavirus gene product expressed using a poly-histidine baculovirus vector. Insect Biochem and Mol. Biol.27:201-211.

SHELBY K.S. & WEBB B.A (1997) Polydnavirus infection inhibits translation of specific growth-associated host proteins. *Insect Biochem and Molec. Biol.* 27:263-270.

CUI L. & WEBB B.A (1997) Homologous sequences in the *Campoletis sonorensis* polydnavirus genome. J. Virol.

WEBB B.A & CUI L. (1997) Relationships between polydnavirus genomes and viral gene expression. J. of Insect Physiol.

30

PRECOCIOUS METAMORPHOSIS INDUCED IN HOST LEPIDOPTERA BY REGULATORY AGENTS INJECTED BY ADULT FEMALE WASPS OF *CHELONUS CURVIMACULATUS*

D. JONES & S. WACHE

In normally regulated larval heteromorphosis of *Trichoplusia ni*, a 4th, 5th or other numbered instar is a "preultimate" instar, and will normally continue larval molting, if the larva has not yet surpassed the critical (minimal) size threshold corresponding to attainment of the "ultimate" (metamorphic) instar.

Natural injection of *T. ni* embryos with venom/calyx fluid of female *Chelonus* sp. near *curvimaculatus* caused "penultimate" 4th or 5th instar larvae that would normally molt at least once more, to a 5th/6th instar, to instead precociously metamorphose without another larval molt. These effects were observed in naturally-injected insects that never contained either a parasite larva, a viable parasite embryo, or a parasite egg.

The regulatory basis of expression of heteromorphic developmental programs was assessed by two-dimensional electrophoretic analysis of hemolymph proteins during the normal and experimentally manipulated feeding stages of the 3rd, 4th and 5th (final) instar larvae, and during the prepupal stage, of *Trichoplusia ni*. Following the expression of a normal 3rd instar pattern, such stung, pseudoparasitized host larvae were observed to omit expression of the 4th instar program, including omission of the proteins heteromorphically specific to that instar, and instead then express an essentially normal final instar pattern. Thus, normal expression of the final instar feeding stage pattern, was not invariably coupled to prior expression of the penultimate instar-specific proteins or pattern. Also, expression of the full program of the final instar feeding stage was epistatic to the penultimate instar program, i.e., the protein pattern unique to the penultimate larval instar was not co-expressed with the precociously expressed final instar pattern. Larvae developmentally redirected in this manner failed to fully express the final instar prepupal stage pattern of protein expression, due at least in part to failed expression of prepupal ecdysteroids, but this was shown not to arise from omission of any of the first 4 larval instars *per se*. Additionally, arylphorin was precociously highly expressed in parasitized hosts in a manner independent of a decline in the host JH titer. Therefore, the main target of the venom/calyx fluid activity to induce precocious metamorphosis appears to be an event upstream of the decline in JH production by the corpora allata.

A general mode for mechanisms of action of chelonine venom/calyx fluid, and larvae, to cause precocious host metamorphosis and suppressed prepupal development is presented that is based on the current "size threshold" model of normal lepidopteran development, rather than the older, displaced "instar count" model. By basing the model for chelonine regulation of host development on the current "size threshold" model for normal development, the proposed model for chelonine action both accounts for observations reported on various species of that subfamily and makes useful, testable predictions.

31

PARASITIC WASP (*CHELONUS NEAR CURVIMACULATUS*) VENOM EFFECTS IN SUPPRESSION OF THE HOST (*TRICHOPLUSIA NI*) IMMUNE RESPONSES : BLOOD MELANIZATION ACTIVITY AND CELLULAR ENCAPSULATION

S.C. WACHE, D. JONES

Selective removal of wasp venom immediately after its natural injection into embryos of *Trichoplusia ni* results in a delayed death and cellular encapsulation of the parasite larva in its juvenile host. This removal of venom also results in significantly less suppression of the host blood's production of brown/black melanins, compared to observed when the venom is fully present. However, this change did not arise through altered expression of the major polydnavirus transcript, which was expressed at similar levels in both hosts that were venom-deficient and hosts that received a full complement of persistent venom. The presence of naturally injected venom and polydnavirus, in the selective absence of a parasite larva, results in the hemolymph melanization process being shifted toward pathways that produces yellow/red blood upon melanization. Finally, the presence of a normal complement of all three components (venom, polydnavirus and parasite larva) has no apparent effect to block host embryonic melanization, but does prevent visible production of either brown/black eumelanin-like products or yellow/red pheomelanin-like products by the host larva. These results demonstrate that the fully-present, naturally-injected venom has effects on both parasite escape from encapsulation and host melanization reactions.

32

MATERNAL WASP SECRETIONS PROTECT THE EGG AND LARVA AGAINST THE HOST IMMUNE REACTION

S. ASGARI, U. THEOPOLD & O.SCHMIDT

In *Cotesia rubecula* the developing embryos and larvae are protected against the host defence reactions by a combination of two different mechanisms involving the evasion of the host defence and the suppression of the cellular capacity to mount an encapsulation reaction by a polydnavirus (CrV) encoded protein. Both mechanisms are essential for the completion of wasp growth inside the caterpillar. Since the inactivation of hemocytes by the virus-coded suppressor is not observed until a few hours following parasitization a surface protection of egg depositions by maternal secretions must be effective to protect the egg and possibly the symbiotic viruses against the immediate attack by a healthy host. Assuming that viruses and eggs are protected by a similar surface coat, we raised antibodies against purified polydnaviruses and tested the egg surface for cross-reacting proteins. Subsequent experiments showed that two (32 and 65 kDa) proteins on the egg surface cross-react with the anti-virus antibodies, and that the protective properties on the egg can be masked with specific antibodies leading to an encapsulation of the injected egg inside the caterpillar. To analyze the protective surface properties at the molecular level we cloned the major 32 kDa protein which is produced in the calyx gland of the wasp and attached to the surface of the egg on its passage from the ovary into the oviduct. The recombinant protein confers protection to coated objects in a cellular encapsulation assay suggesting that a layer of p32 either precludes recognition of foreignness or prevents cellular attacks by a local inactivation of the host defence system.

In this system, a transient suppression of the host's defense system is observed four to six hours after parasitisation, and we have isolated the virus gene that codes for the immune suppressor (CrV1). Purified recombinant CrV1, synthesized by baculovirus and plasmid expression systems, were injected into caterpillars and produced the same cytological alterations suggesting that the polydnavirus-related inactivation of hemocytes is due to a single glycoprotein. The protein probably interacts with the hemocyte surface and causes an intracellular breakdown of actin cytoskeleton. Our experiments suggest that the active suppression of the host immune system is caused by an inactivation of cytoskeleton-dependent cellular processes, like hemocyte cell membrane changes and microparticle formation. The transient inactivation of hemocytes provides immune protection for the developing parasitoid larva when it emerges from the egg shell.

33

STUDYING INSECT CELLULAR DEFENSE MECHANISMS WITH THE HELP OF MONOCLONAL ANTIBODIES

T. TRENCZEK

Monoclonal antibodies (mabs) have been generated against *Hyalophora cecropia* and *Manduca sexta* hemocytes. These markers allow for the identification of hemocytes and the study of cellular defense reactions of insects in more detail.

The mabs can be used :

(1) to label/identify insect hemocytes,
(2) to isolate distinct hemocyte populations,
(3) to analyse and purify various hemocyte factors,
(4) to study the function of distinct hemocytes.
(5) to compare hemocytes of various insects, and finally
(6) to analyse hemocyte differentiation.

Some examples will be presented :

(1) The mabs obtained recognize distinct hemocyte populations that are already identified by morphological characterization, i.e. plasmatocytes (PL) or granular cells (GR) or oenocytoids (OE). In addition, some antibodies detect antigens that are present on certain groups of hemocytes, e.g. GRs and OEs, or PLs and SPs, or GRs and PLs. Thus, hemocytes can be detected and distinguished in various conditions when they can not be identified by their morphology, for example during aggregation or encapsulation.

(2) Hemocytes can now be isolated by cell sorter, magnetic beads or panning.

(3) One protein present in granules of GRs of *H. cecropia* could partially be characterized by mabHC1A6.

(4) Certain functions of hemocytes can be visualized like the uptake of LPS in *H. cecropia* and *Galleria mellonella* by granular hemocytes using FITC-labeled LPS of *E.coli* and indirect immunohistochemistry with secondary antibodies coupled to Texas Red. Using mabs to block hemocyte functions, we were able to demonstrate that the antigen corresponding to mab MS#13 (an integral membrane protein of PLs with MWr of 90-100,000) is involved in adhesion processes during aggregation/encapsulation.

(5) Some of the mabs also bind to hemocytes of other insect species. Species tested so far are : *G. mellonella, Pieris brassicae, Spodoptera littoralis, Drosophila melanogaster, Musca domestica, Gromphadorina sp., Tenebrio sp.*. The percentage of crossreactive mabs decrease with the phylogenetic distance between the species. Some antigens are localized on/in the same morphological hemocyte type (mab MS#7 detecting granules in Grs of several species), others show a different distribution (mab MS-X-22C10 binds only to PLs of *G. mellonella* but nearly to all cells of *M. sexta)*.

Major contributions to the investigations were provided from E. WILLOT, M.R. KANOST & E. GATEFF, F. SIEG, F. SCHOLZ & C. WIEGAND.

This research was supported by the Gunnar Hansson Stiftelse & DFG (Tr254/1-2) to TRENCZEK & the NIH (AI3 1084) to M.R. KANOST.

34

FUNCTIONAL ASPECTS OF HEMOLIN, AN INSECT IMMUNE PROTEIN OF THE IMMUNOGLOBULIN SUPERFAMILY (IgSF)

I. FAYE, H.LANZ MENDOZA, K. ROXSTROM LINDQUIST, I. TAPIA, R. BETTENCOURT, D. PEREIRA & Y. ASSAFAW REDDA

Hemolin is an insect molecule related to immunoglobulins and neural cell adhesion molecules. It is present at low levels in the naive pupae *Hyalophora cecropia*, and strongly upregulated by injection of bacteria, lipopolysaccharides (LPS) or phorbolester (PMA).

Hemolin was shown to enhance phagocytocis of yeast by both hemocytes and *Drosophila* mbn-2 cells. The phagocytic activity was further increased by addition of LPS. This activity was correlated with increased protein kinase C phosphorylation, which in turn could be inhibited by PKC inhibitors.

By UV-cross linking experiments, hemolin was shown to bind to *E. coli* LPS. This binding could be competed by Lipid A, indicating its specificity of binding to Gram-negative bacteria. Soluble hemolin inhibits hemocyte aggregation, a finding that may be explained by the recently identified membrane form of hemolin and by the homophilic binding properties of soluble hemolin.

By the use of the yeast two-hybride system a hemolin binding protein, Jippy, was isolated from a *Drosophila melanogaster* disc cDNA library. Alteration of the N-glycosylation site in the third Ig-like domain of hemolin diminished the hemolin-Jippy interaction.

Recently, a PCR generated fragment based on the use of degenerate hemolin primers has been isolated from an *Anopheles gambiae* λZAP library.

35

PURIFICATION AND CHARACTERIZATION OF ANTIVIRAL PROTEIN IN THE GUT JUICE OF SILKWORM, *BOMBYX MORI* L

R.K. DATTA, K.M. PONNUVEL & A.K. JAIN

In insects the initial defence mechanisms against pathogens entering *per os* could be expected in the tissues along the alimentary canal and especially in the digestive juice. Among silkworm genetical stocks maintained in the institute, existence of differential level of tolerance to BmNPV infection was noticed. In order to understand the mechanism of differential tolerance in silkworms, seventeen nondiapausing silkworm breeds were screened for tolerance to BmNPV (1×10^5 PIB/ml) by bioassay and identified Hosa Mysore and Nistari as susceptible and tolerant breeds, respectively. The breeds were further characterised for tolerance and susceptibility to BmNPV infection from the occurrence of total protein and alkaline protease in the gut juice of the larvae. Silkworm larvae were per orally inoculated with BmNPV polyhedra (1×10^5 PIB/ml) and the digestive tract was compartmentalised into foregut, midgut and hindgut, 24h of post inoculation (PI). The gut juice was collected at 24h intervals for 4 days from each compartment and the total protein (Lowry *et al.*, 1951) and alkaline protease activity (Eguchi *et al.*, 1976) were estimated.

The total protein in the foregut and midgut of BmNPV inoculated tolerant silkworms sharply increased upto 4th day of PI, while in the BmNPV inoculated susceptible silkworm the total protein level was comparatively lower than that in tolerant breeds during the same period. The alkaline protease activity in the foregut and midgut of BmNPV inoculated tolerant silkworms also increased sharply upto 3rd day and decline on 4th day. Similar trend in increase in alkaline activity was observed in susceptible silkworm breeds but the increase was comparatively low.

Infectivity of BmNPV in the midgut of identified silkworm breeds were studied through electron microscopy. Second instar silkworms were per orally inoculated with BmNPV polyhedra (10^5 PIB/ml) and ultrathin sections stained in uranyl acetate and lead citrate were observed at 75 × 1000 magnification. The results indicated that infection of the midgut tissue of susceptible silkworm breed within 12h of PI, while in tolerant breeds the midgut tissue was free of virions upto 24h of PI.

In order to sequence the protein with antiviral activity, the gut juices from susceptible and tolerant silkworms were collected and subjected to step wise (10-60 %) ammonium sulphate precipitation. The partially purified proteins (30-60 % fractions) were filtered through sephadex G100 and the fractions were tested for antiviral activity. Fractions A and C were found antiviral and subjected to 10 % SDS PAGE. The protein bands at 28 kD (Fraction C) and 66 kD (Fraction A) were further tested for antiviral activity and sequenced following the method described by Matsudaira (1987). The 28 and 66 kD proteins were antiviral and these reduced the infectivity by 84 and 88 %, respectively. The N-terminal sequence of aminoacids of these proteins is as follows.

28 kD : -Phe-Asp-Leu-Gly-Glu-Arg-Asp-Val-Val-Phe
66kD : -Glu-Gln-Gly-Ala-Tyr-Arg-Val-Pro-Trp-Phe-Lys-Ile-Leu-

These studies reveal the existence of a correlation between alkaline protease activity including occurrence of antiviral proteins (28 and 66 kD) and higher tolerance. At present attempts are being made to clone the genes producing antiviral proteins by using reverse genetics.

SESSION 6

PROTEIN STRUCTURE/FUNCTION

36. WELLS/Sundermeyer/Karnas (USA) :
Molecular biology of lipophorin synthesis in *Manduca sexta*. 65

37. NIERE/Meifllitzer/Ziegler/Dettloff/Weise/Weisner (Germany) :
Molecular cloning of *Galleria mellonella* apolipoprotein III cDNA 66

38. WEERS/Van der Horst/Ryan (Canada) :
Bacterial over-expression of recombinant apoLp-III 67

39. Benaki/HAMODRAKAS (Greece) :
Structural studies of peptide analogues of silkmoth chorion protein segments in solution and in the solid state 68

36

MOLECULAR BIOLOGY OF LIPOPHORIN BIOSYNTHESIS IN *MANDUCA SEXTA*

M. A. WELLS, K. SUNDERMEYER & K. J. KARNAS

Insects have a single high-density hemolymph lipoprotein called lipophorin, which is composed of 60 % protein and 40 % lipid. Lipophorin contains two apolipoproteins, and each particle contains one molecule of apolipophorin-I (apoLp-I ; Mr ≈ 250,000) and one molecule of apolipophorin-II (apoLp-II ; Mr ≈ 80,000). Lipophorin is synthesized in the fat body and secreted into the hemolymph where it serves as a reutilizable shuttle for the transport of lipids between insect tissues.

ApoLp-I and -II arise from a common precursor. A 10,138-bp cDNA from the fat body of the tobacco hornworm, *Manduca sexta*, which encodes the precursor protein was cloned and sequenced [the Precursor Protein of the Structural Apolipoproteins of Lipophorin : cDNA and Deduced Amino Acid Sequence. K. Sundermeyer, J. K. Hendricks, S. V. Prasad, and M. A. Wells, *Insect Biochem. Molec. Bio.* 26, 735-738 (1996)]. The cDNA has a single 9,915-bp open reading frame beginning at an initiating ATG at bp 59 and extending to a stop codon at position 9,974. This open reading frame encodes a 3,305 amino acid protein with a molecular mass of 366,812 Da. Signal peptide cleavage is predicted to occur after residue 23, leaving a 3,282 amino acid precursor protein. The precursor protein is arranged with apoLp-II at the amino terminal end and apoLp-I at the carboxyl terminal end. The sequence RGRR, which occurs at the carboxyl terminal end of apoLp-II, is the consensus cleavage site for subtilisin-like convertases.

A number of studies suggest that apoLp-I is found predominantly on the surface of lipophorin and is responsible for directing the metabolism of lipophorin in the hemolymph. ApoLp-II is somehow "sequestered" away from the aqueous environment and may be responsible for maintaining the structure of the surface phospholipid monolayer of lipophorin. The basic hypotheses on which our experiments are based are (1) apoLp-II is responsible for recruiting the phospholipid monolayer of lipophorin from the ER membrane during lipophorin biosynthesis ; (2) apoLp-II plays an important role in the structural stability of the phospholipid monolayer in lipophorin ; and (3) apoLp-I plays a critical role in stabilizing the lipophorin particle after it has been assembled. In order to test these hypotheses a number of experiments are in progress : [1] Several cDNAs, encoding portions of apoLp-II, are being constructed and will be analyzed by *in vitro* transcription and translation in the presence of microsomes with the aim of determining the role of apoLP-II in phospholipid recruitment. [2] A yeast expression vector is being constructed to analyzed apoLp-II expression in yeast with the aim of deducing the pathway for lipophorin assembly through the use of yeast mutants ; and [3] the apoLp-I+II precursor cDNA is being cloned into a baculovirus expression system to enable production of native and site-directed mutants of the nascent lipophorin particle. These particles will be used to analyze receptor-binding domains and the mechanism(s) of lipid delivery from midgut to lipophorin and from lipophorin to fat body. Progress in these goals will be discussed. Supported by NIH grant GM 50008.

37

PARTIAL cDNA-SEQUENCING OF THE HEMOLYMPH-PROTEIN APOLIPOPHORIN III (APOLP-III) of *GALLERIA MELLONELLA*

M. NIERE, C. MEIBLITZER, M. ZIEGLER, M. DETTLOFF, C. WEISE & A. WIESNER

ApoLp-III is well examined in regard to its role in lipid-metabolism of flying insects. Especially from investigations with *Manduca sexta* and *Locusta migratoria* it is concluded that the main function of this protein is the stabilization of diacylglycerol (DAG)-loaded low density lipophorin (LDLp) during the lipid-transport between fat body and wing-muscle cells (Blacklock and Ryan, Insect Biochem. Molec. Biol. 24, 855-873, 1994). The protein is also present in larval hemolymph, however usually not associated with lipophorin (Ziegler et al., Insect Biochem. Molec. 25, 101-108, 1995). Therefore new, still unknown roles can be postulated for larval apoLp-III. Recent immunological studies support this idea. It was shown that the intrahemocoelic injection of isolated apoLp-III provokes a dose-dependent linear increase of antibacterial activity in the hemolymph of *Galleria-mellonella*-larvae (Wiesner et al., J. Insect Physiol. 43, 383-391, 1997).

Further investigations about the mode of action of apoLp-III during the immune-stimulation require high amounts of the pure protein. For this reason we decided to establish an expression system for producing recombinant *Galleria-mellonella*-apoLp-III. Our knowledge about the complete primary structure of the native protein (Weise et al., in preparation) facilitated the amplification of cDNA-sequences by heterologous PCR and 3'-Race-PCR. With these methods 439 bp of the apoLp-III-cDNA could be determined. The missing sequence of the 5'-end of the mRNA was tried to be identified by other PCR-methods with single-sided specificity. However, neither ligation-anchored PCR nor 5'-Race-PCR have so far been performed successfully. Our sequence data reveal high similarities to the known apoLp-III-cDNA-sequences from other lepidopteran species. In contrast, the apoLp-III-cDNA-sequences of other insect orders show only a low degree of correspondence to the partial sequence of *Galleria-mellonella-apoLp*-III.

38

BACTERIAL OVEREXPRESSION OF RECOMBINANT APOLP-III ; STRUCTURE-FUNCTION STUDIES

P. M. M. WEERS, V. NARAYANASWAMI, D.J. VAN DER HORST & R. O. RYAN

Apolipophorin III (apoLp-III) is an exchangeable apolipoprotein found in the hemolymph of several insect species and well characterized in the Sphinx moth *Manduca sexta* and the locust *Locusta migratoria*. It exists in lipid free and lipid bound forms. The lipid free protein exists as a bundle of 5 elongated amphipathic α-helices (1). The protein plays a key role in the enhanced diacylglycerol transport during insect flight by stabilizing lipid enriched lipophorin particles. Both *M. sexta* and *L. migratoria* apoLp-III have been successfully cloned in the bacterial pET expression vector, which bears a pelB leader sequence directly upstream of the apoLp-III gene (2). This enables targeting of the synthesized apoLp-III to the periplasmic space, where the signal sequence is cleaved off. Following cleavage full length apoLp-III escapes the bacteria and appears in the medium accumulating to concentrations up to 100 mg/L. Recombinant apoLp-III has been isolated and purified from the medium by a single step reversed-phase HPLC. In contrast to *M. sexta* apoLp-III which is non-glycosylated, native *L. migratoria* apoLp-III is glycosylated at Asn 16 and Asn 83. However, overexpression of the non-glycosylated *L. migratoria* apoLp-III in *E. coli* results in a stable recombinant protein with a molecular weight of about 17.5 kDa. Circular dichroism spectroscopy revealed that both the recombinant *M. sexta* and *L. migratora* apoLp-III have a high α-helical content. Functionally, both the proteins elicited lipid-binding properties in terms of ability to bind to DAG-enriched LDL particles. Coupled to the availability of the high resolution structure of apoLp-III the ability to express recombinant protein offers powervul possibilities to carry out site directed mutagenesis studies (3). Studies are currently in progress to dissect structure-function relationships in these model exchangeable apolipoproteins.

1. Breiter, D. R., Kanost, M. R., Benning, M. M., Wesenberg, G., Law, J. H., Wells, M. A., Rayment, I., & Holden, H. M. 1991. Molecular structure of an apolipoprotein determined at 2.5-Å resolution. Biochemistry 30: 603-608.

2. Ryan, R. O., Schieve, D., Wientzek, M., Narayanaswami, V., Oikawa, K., Kay, C. M., & Agellon, L. 1995. Bacterial expression and site-directed mutagenesis of a functional recombinant apolipoprotein. J. Lipid Res. 36: 1066-1072.

3. Narayanaswami, V., Wang, J., Kay, C. M., Scraba, D. G., & Ryan, R. O. 1996. Disulfide bond engineering to monitor conformational opening of apolipophorin III during lipid binding. J. Biol. Chem. 271: 26855-26862.

39

STRUCTURAL STUDIES OF PEPTIDE-ANALOGUES OF SILKMOTH CHORION PROTEIN SEGMENTS, IN SOLUTION AND IN THE SOLID STATE

D. BENAKI & S. J. HAMODRAKAS

Silkmoth chorion is a model system to study how structural proteins fold and self-assemble to form complex, physiologically important structures. Data collected utilizing X-ray diffraction, laser-Raman and infrared spectroscopy from intact chorions indicate the existence of a common molecular denominator, the beta-pleated sheet, that apparently dictates, the self-assembly process, in agreement with theoretical predictions (1).

Peptides representative of certain evolutionary conservative parts of chorion proteins were synthesized and their structure is currently being studied, mainly in order to elucidate principles that govern chorion protein folding and assembly. Laser-Raman and infrared spectroscopic conformational studies of these peptides, in a variety of conditions, in solution and in the solid state, indicate the abundance of antiparallel beta-pleated sheet in the structure of these peptides. CD and NMR studies are in progress to determine their 3D-structure. The structure of these peptides and their assembly properties suggest that they might be promising candidates for the design of new biomaterials.

1. Hamodrakas, S. J. (1992) "Molecular Architecture of Helicoidal Proteinaceous Eggshels" in "Results and Problems in Cell Differentiation", Vol. 19, (Case, S. T. ed.), Springer-Verlag, Berling-Heidelberg

SESSION 7

FAT BODY AND SILK GLANDS

40. Krishnan/VANISREE (India) :
Differential synthesis and uptake of storage protein (SP) by various fat body tissues during development of *Bombyx mori*. 71

41. Yang/Kodrik/ZUROVEC/Sehnal (Czech Republic) :
Low molecular weight proteins of Lepidopteran silk 72

42. NIRMALA/Krishnan (India) :
Sensitivity of fibroin in silk glands of *Bombyx mori* to dietary protein levels. 73

43. SCHELLER/Yang/Sehnal (Germany) :
Hormonal control of wax moth sericin genes. 74

44. JULIEN/Horard/Nony/Garel/Couble (France) :
Two factors drive posterior cell specific expression in the silk gland of *Bombyx mori* 75

40

DIFFERENTIAL SYNTHESIS AND UPTAKE OF STORAGE PROTEIN (SP) BY VARIOUS FATBODY TISSUES DURING DEVELOPMENT OF *BOMBYX MORI*

M. KRISHNAN & V. VANISHREE

Insect fatbody is a versatile tissue performing myriad of functions. Depending on their locations, the tissue has been traditionally recognised as peripheral and pervisceral and frequently compared to mammalian liver and adipose tissue. Since structural changes take place rapidly during larval development, it is difficult to distinguish between regional or developmental differences. Recently, in 1992, it was established that in *Heliothis zea* the fat body that turns blue, due to the sequestration of the blue coloured storage protein, is the perivisceral tissue that is assigned the role of storage and the colourless peripheral tissue is the synthetic tissue. The punch line of their report was a fact that such specialised tissues exist in other insect species that lack a colored storage protein. Our search in this direction in *Bombyx mori* revealed that fatbody tissues at different locations differ in their morphology from the last instar stadium through adult life. Our *in vitro* and electrophoretic studies revealed the differential storage protein synthetic capacities of these tissues and the specialised organ of storage during stages in *B. mori*. Studies pertaining to the levels of SPI mRNA transcripts in different fat body tissues are under progress.

41

LOW MOLECULAR WEIGHT PROTEINS OF LEPIDOPTERAN SILK

C. YANG, D. KODRIK, M. ZUROVEC & F. SEHNAL

Lepidopteran silk is a complex of proteins which are classified into two groups - fibroins and sericins. Fibroins, which are produced in the posterior section of silk glands, make up the hydrophobic core of the silk fibre, whereas sericins from the middle section provide the core with a sticky coating, which is soluble in hot water. Several genes encoding major silk proteins, such as *H-chain fibroin*, *L-chain fibroin*, *P-25*, *Sericin 1* and *Sericin 2*, have been cloned and studied in two distantly related lepidopteran species *Bombyx mori* and *Galleria mellonella*.

Electrophoretic analysis of cocoon proteins from these two species indicates that the list of known proteins is incomplete. Several poorly soluble cocoon proteins with molecular weight of 8-17.5 kDa were detected. Using a probe derived from the N-terminal sequence of the 17.5 kDa component of *Galleria* silk, we isolated a silk gland-specific cDNA encoding 167 amino acids, of which 17 were identified as the signal peptide. Notable differences between the encoded sequence and the amino acid composition of other silk proteins included high proline content (34 residues or 20.26 % by weight), lack of cysteines, and presence of two kinds of short amino acid repeats. Northern analysis demonstrated that the gene coding for this protein is expressed in both the posterior and the middle sections of the silk glands, but in no other tissues. To accentuate the fact that both the fibroin and the sericin producing cells are involved in the secretion of the newly identified protein, this protein was given the name seroin. We found that expression of the *seroin* gene fluctuates during development in correlation with nutrient supply and hormonal changes ; the content of *seroin* mRNA is high in the feeding larvae, declines at ecdysis, reaches a maxinum during cocoon spinning, and thereafter rapidly drops to underdetectable level. *In vivo* and *in vitro* experiments showed that the drop is caused by ecdysteroid hormones and can be prevented with juvenile hormone.

N-terminal sequencing of several silk proteins of *Bombyx* revealed that two low molecular components share 5 of 11 identified amino acids of their N-termini with the N-terminus of *Galleria* seroin and obviously represent seroin homologues. These results suggest that serions are a general component of lepidopteran silk but occur in different sizes in different species. The conservation of a serion sequence suggests that these proteins may have an important role in silk secretion or may be crucial for some of the physico-chemical properties of the silk.

42

SENSITIVITY OF FIBROIN IN SILK GLANDS OF *BOMBYX MORI* TO DIETARY PROTEIN LEVELS

M. KRISHNAN & X. NIRMALA

Fibroin is the major silk protein produced by the posterior silk gland and stored in the middle silk gland during the fifth instar development. Fibroin gene expression is known to be under the control of hormones and nutrition. The evidence for the nutritional regulation silk gene expression is the increase in cocoon weight and shell weight when silkworm larvae are supplemented with nutrients like aminoacids, carbohydrates or proteins. The present study is aimed to identify the effect of dietary protein levels on the accumulaion of fibroin in *Bombyx mori*. Feeding the chosen hydrolysed soy protein to silkworm larvae at the rate of 2mg/female larva and 4mg/male larva/day during the fifth instar development increased the total weight of silk glands. Parallel to this an increase in the levels of fibroin heavy (H) and light (L) chains were also observed on a 4-15 % SDS-PAGE and the same was confirmed by densitometric scanning. The homology of the H and L chain polypeptides in control and supplemented groups was confirmed by Western blotting. Studies pertaining to the levels of fibroin (H & L), mRNA silk glands with respect to dietary protein are under progress.

43

HORMONAL CONTROL OF WAX MOTH SERICINE GENES

K. SCHELLER, C. YANG & F. SEHNAL

Juvenile hormones and ecdysteroids are known to regulate many biological processes, usually by controlling gene expression at the transcriptional level. On the example of the sericin genes of *Galleria mellonella* we demonstrate the complexity of hormonal regulation by studying the expression of genes which are probably not the primary targets for the hormones.

1. Two sericin genes (MG1 and MG2) are expressed in the silk glands of caterpillars. The glands consist of a posterior section, where the water-insoluble core of the silk fibre is synthesized, a middle section (MSG) and a narrow outlet. The expression of sericin genes is restricted to particular sections of the MSG, as shown by *in situ* hybridization.

2. The MG1 and MG2 genes generate (probably by alternative RNA splicing) several transcripts whose patterns change during the penultimate and last larval instars, as demonstrated by Northern analyses.

3. The total contents of MG1 and MG2 transcripts decline during moulting to the last larval instar. Since a similar decline of the transcripts occur in cultured silk glands when α-amanitin is added to the medium we assume that moulting is associated with a great reduction of sericin gene transcription. The corresponding mRNAs are short-lived and their contents diminish rapidly when transcription ceases. Transcription is restored after ecdysis. We conclude that the moult-inducing surge of ecdysteroids inhibits the transcription of the sericin genes.

4. The 1.9 kb MG1 -MRNA is detectable as long as JH is measurable in last instar larvae. We have now verified this result by culturing silk glands of VII/3 larvae. When the medium contained JH, the glands produced 1.9 kb mRNA, whereas glands kept without JH switched to the synthesis of 4.2 kb mRNA. MSG of VII/6 larvae have lost the potential to produce 1.9 kb mRNA *in vitro*. The 4.2 kb species was the only mRNA which could be recovered. However, in the presence of JH, the 1.9 kb RNA becomes detectable. We interpret the effect of JH on the MSG of both VII/3 and VII/6 as a hormonal regulation of RNA splicing. This JH action depends on protein synthesis because it is totally inhibited by cycloheximide or anisomycin.

5. The most obvious effect of ecdysteroids on the sericin genes of cultivated silk glands is a rapid decline of mRNAs in response to high 20E concentrations. From our experiments we furthermore conclude that 20E affects both the rate of transcription and the stability of the mRNAs. JH counteracts the ecdysteroid-induced degradation of MG1 and MG2 transcripts.

Conclusions :

- Low ecdysteroid titres stimulate sericin-mRNA transcription in MSGs.
- High ecdysteroid concentrations inhibit the transcription of the sericin genes and enhance the degradation of the corresponding mRNAs.
- JH maintains the "larval type" splicing of MG1 transcripts.
- JH "stabilizes" mRNA against degradation induced by ecdysteroids.

44

TWO FACTORS DRIVE POSTERIOR CELL SPECIFIC EXPRESSION IN THE SILK GLAND OF *BOMBYX MORI*

E. JULIEN, B. HORARD, P. NONY, A. GAREL & P. COUBLE

The mechanisms by which spatial restriction of gene expression operates in the silk gland of the silkworm were studied using the gene encoding the silk/chaperon protein P25, the transcription of which is specific of posterior cells. By combining an *in vivo* chromatin analysis of the *P25* promoter region (using DNase I-aided LMPCR) and an *in vivo* promoter study in silk gland cells (using a biolistic-reimplantation method), we could show that two factors are necessary and sufficient to induce posterior cells specific expression (ie, no activity was ever observed in sericin secreting median cells). They are SGFB, a silk gland restricted factor present in both posterior and median cells, and PSGF, a novel factor that is likely PSG specific. The concerted action of the two factors and of an upstream enhancer complexe provides full transcription of the P25.

In PSG cells, the SGFB/PSGF cis-acting elements define a short MNase hypersensitive region and the transcribed domain displays no nucleosome phasing. In contrast, a repressive nucleosome phasing features MSG cells chromatin : this phasing correlates with the binding of BMFA, an ubiquitous factor that could play repressor activity.

The interaction of SGFB and PSGF to their cognate element was also examined during the IVth larval molt when *P25* is fully repressed in PSG cells. Repression correlates with the absence of interaction of the two factors with their respective target sequence. The stability of the SGFB cellular pool during larval development may suggest that the availability of PSGF itself regulates P25 expression. However, as a molt specific DNase I hypersensitive site occurs at the same time at - 290, other unknown factors may also be involved in turning off the gene.

As a whole, our data show that the PSG temporary inactivation of *P25* at molting and its permanent repression in MSG are driven by distinct mechanisms.

SESSION 8

TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL CONTROL OF CUTICULAR GENE EXPRESSION

45. Gu/WILLIS (USA) :
Characterization of moth wing development *in vivo* and *in vitro* 79

46. KRAEMER/Wolbert (Germany) :
Down regulation of expression of a pupal cuticle protein gene by transcriptional and post-transcriptional control mechanisms during metamorphosis in *Galleria*. 80

47. REBERS/Niu/Knepper (USA) :
Regulation of the *Manduca* MSCP14.6 cuticle gene 81

45

CHARACTERIZATION OF MOTH WING DEVELOPMENT *IN VIVO* AND *IN VITRO*

S. GU & J. H. WILLIS

RT-PCR and *in situ* hybridization were used to examine distribution of messages for two cuticular proteins in the wings of *Hyalophora cecropia*. Unexpected findings resulted ;

(i) Both methods revealed abundant HCCP 66 mNRA in pupal hind-wing epidermis, a tissue that secretes a flexible cuticle with HCCP 12 protein but no trace of HCCP 66 protein.

(ii) HCCP 12 mRNA was detected in the dorsal epidermis of the fore-wing, but no HCCP 12 has ever been extracted from the overlying rigid cuticle.

(iii) The wing imaginal discs from final instar larvae contain abundant message for HCCP 12 but none for HCCP 66. Message is found in both the peripodial membrane and disc proper. Since pupal forewings secrete copious HCCP 66, the HCCP 12 message is not there "in anticipation" of pupal cuticle formation. Rather the presence of HCCP 12 mNRA supports the morphological evidence presented by Svacha (Devel. Biol. 154 : 101) that lepidopteran wing imaginal discs are not "embryonic cells" but actually secrete cuticle.

Our results and those from other laboratories, indicate that the action of JH in regulating metamorphosis involves directing a myriad of processes ranging from quantitative regulation of gene expression to post-transcriptional control. Studies are under way to learn if a suitable model system for studying metamorphosis might be the cell line IAL-PID2 derived from wing imaginal discs of the Indian meal moth *Plodia interpunctella* (Lynn and Oberlander, J. Insect Physiol. 29 : 591). A subline (PID2A) has been selected that responds within 48 hrs. to 20E (30 nM) by detaching from the culture dish. This ecdysteroid-induced detachment is prevented with juvenoids ; JH I is effective at 0.01 nM. Retinoic acid, linoleic acid and linolenic acid are ineffective even at 100 nM, whereas farnesol prevents ECD-induced detachment at 100 nM and JH II acid is effective at 10 nM. Sevala and Davey (Experientia 45 : 355) showed PDBU (100 nM) can mimic the action of JH in inducing follicle cell patency in *Rhodnius* ; the protein kinase C inhibitor, H-7, blocked JH action. PDBU had no effect on PID2A cells even at 5 µM, but H-7 interfered somewhat with JH action at the concentration they used, 100 µM. The relation of 20E-induced detachment of PID2A cells to metamorphic processes is unknown. We could not demonstrate a critical period where 20E exposure rendered cells insensitive to JH. Yet the data on sensitivity to JH analogues indicate that PID2A cells appear to be a promising system for studying juvenoid action.

46

DOWNREGULATION OF EXPRESSION OF A PUPAL CUTICLE PROTEIN GENE BY TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL CONTROL MECHANISMS DURING METAMORPHOSIS IN *GALLERIA*

B. KRÄMER & P. WOLBERT

In holometabolous insects, a metamorphic step in postembryonic development (larva to pupa or pupa to adult, respectively) is accompanied by reprogramming of gene expression in a stage and tissue specific manner. One of the earliest events in this process is to shut off the program of gene expression of the foregoing stage. In order to study the underlying control mechanisms, we have established the developmental profiles of (1) transcription, (2) transcript accumulation and degradation, (3) translatability of a pupal cuticle protein mRNA (GmPCP52) during reprogramming of epidermal cells in the pupa of *Galleria*.

(1) The actual transcription rate was analyzed by nuclear run-on transcription assays utilizing isolated epidermal nuclei of carefully timed pupae. Nuclei were incubated with ^{33}P-UTP. Labelled RNA was hybridized to immobilized GmPCP52 antisense RNA and visualized by autoradiography. (2) The relative amount of the GmPCP52 transcript in epidermal cells was determined by Northern blot analysis of total RNA. The transcript was detected by Digoxigenin-labelled antisense RNA. (3) The loss of *in vitro* translatability was shown to be accompanied by poly(A) tail shortening. Length of the GmPCP52 poly(A) tail was analyzed by RNaseH digestion assays. PAGE followed by blotting and hybridization with Digoxigenin-labelled antisense RNA shows changes of poly(A) tail lengths with high resolution. Experiments were carried out with normally developing pupae and after injection of 20E or JH, respectively, immediately after pupal ecdysis.

Remarkable differences exist between the developmental profiles of transcription, transcript abundance, and poly(A) tail shortening or, respectively, the loss of *in vitro* translatability. This indicates post-trancriptional and/or translational control mechanisms to guarantee precisely timed synthesis of the GmPCP52 protein.

Our results demonstrate, that expression of the GmPCP52 gene is differently and independently regulated at the level of transcription, transcript accumulation and degradation, and translatability of the corresponding mRNA. Putative targets of hormonal action are discussed.

47

REGULATION OF THE *MANDUCA* MSCP14.6 CUTICLE GENE

J. E. REBERS, J. NIU & M. A. KNEPPER

The *Manduca sexta* MSCP14.6 cuticle gene produces RNA that is found throughout the epidermis in larvae and is spatially restricted in pupal and adult stages. Both 20-hydroxyecdysone and juvenile hormone (JH) are important in controlling the amount of RNA present in the epidermis during development. To map potential ecdysone and JH response elements, segments of the MSCP14.6 gene have been inserted into a luciferase reporter vector, followed by transfection into *Manduca* cells, to test their ability to confer either ecdysone or JH responsiveness upon the reporter gene. A 3.5 kb genomic DNA fragment that includes 5' flanking DNA, exon 1, and a portion of intron 1 was first used. A reporter construct including this DNA fragment shows a 50 % decline in luciferase activity when cells transfected with the construct are treated with 20-hydroxyecdysone. No significant change inluciferase activity was seen when the cells were treated with methoprene, a JH analogue. Preliminary results indicate that a reporter construct made using a 577 N genomic fragment, which includes both potential EcREs noted above, shows an ecdysone response similar to the reporter construct that includes the larger genomic DNA fragment. The 5' flanking DNA of MSCP14.6 is approximately 60 % identical to the *H. cecropia* HCCP12 gene, including a segment in which 25/27 nucleotides are identical. To determine if this region is conserved in other Lepidoptera, which would be expected if it is a regulatory DNA segment, PCR primers were designed using the conserved 27 N 5' flanking region and exon I from MSCP14.6 and HCCP12. Amplification of genomic DNA from *Bombyx mori* resulted in a 400 N product, while DNA from *Lymantria dispar* produced a 200 N product. A *Bombyx* genomic library has been screened using the PCR product to isolate a potential homologue ot the MSCP14.6 and HCCP12 genes.

References :

Riddiford, L. M., Baeckman, A., Hice, R. H. & Rebers, J. (1986). Developmental expression of three genes for larval cuticular proteins of the tobacco hornworm, *Manduca sexta*. Dev. Biol. 118, 82-94.

Rebers, J., Horodyski, F., Hice, R. & Riddiford, L. M. (1987). Genomic cloning of developmentally regulated larval cuticle genes from the tobacco hornworm *Manduca sexta*. In UCLA Symposia on Molecular and Cellular Biology, New Series, 49 : Molecular Entomology, J. Law, ed. (New York : Alan R. Liss), pp. 201-210.

Rebers, J. E., Niu, J. & Riddiford, L. M. (1997). Structure and spatial expression of the *Manduca sexta* MSCP14.6 cuticle gene. Insect Biochem. and Mol. Biol. 27, 229-240.

SESSION 9

TRANSCRIPTIONAL CONTROL OF GENE EXPRESSION ; tRNA GENES

48. KRAVARITI/Rodakis/Lecanidou (Greece) :
A protein factor produced in the early stage of *Bombyx mori* choriogenesis can interact with early and late chorion gene elements. 85

49. Mange/Julien/Couble/PRUDHOMME (France) :
Structure and expression of cytoplasmic actin genes in *Bombyx mori*. 86

50. GOPINATHAN/Sharma (India) :
Regulation of glycyl tRNA gene expression in *Bombyx mori*. 87

51. Brown/Mangor/Martinez/Ouyang/Young/SPRAGUE (USA) :
Differential transcription of silkworm tRNA Ala genes. 88

48

A PROTEIN FACTOR PRODUCED IN THE EARLY STAGES OF *BOMBYX MORI* CHORIOGENESIS CAN INTERACT WITH EARLY AND LATE CHORION GENE ELEMENTS

L. KRAVARITI, G. C. RODAKIS & R. LECANIDOU

Three early β-type chorion genes, 6F6.1, 6FF.2 and 6F6.3, are interspersed in chorion locus Ch1-2 instead of early locus Ch3. In attempt to identify *cis*-elements that are essential for the early expression of the 6F6 genes, the -102 to -37 promoter region of the 6F6.2 gene was isolated and its ability to interact with specific proteins was tested. DNA-protein interaction assays revealed a region, -56 to -43, that seems to interact specifically with nuclear extracts from early follicular cells. The main complex formed with this region at the early stages can be competed out by a sequence containing the binding site for a late specific factor, BCFII. It is argued that BCFII is not involved in any complex formation at the early stages of choriogenesis. However, the early binding protein(s) that interact(s) with the -56 to -43 region of the 6F6.2 promoter can also recognise the BCFII binding site. This can probably be attributed to sequence similarity of the involved promoter regions. Interestingly, the -56 to -43 region exhibits high similarity with a sequence found in the bidirectional promoter of a *Drosophila* gene pair. Isolation and characterization of this early protein factor is in progress.

49

STRUCTURE AND EXPRESSION OF CYTOPLASMIC GENES IN *BOMBYX MORI*

A. MANGE, E. JULIEN, P. COUBLE & J. C. PRUDHOMME

The two cytoplasmic actin genes of *B. mori*, *BmA3* and *BmA4*, have been isolated and compared. These linked genes encode very similar actins which differ by only two amino acid residues. *BmA4* exhibits two alternative promoters whereas *BmA3* has a single promoter. The comparison of nucleotide sequences showed that the coding region and the single intron which interrupts it are highly similar and most probably have been recently homogenized by gene conversion. A similar organisation has been described in the other lepidopteran *Helicoverpa armigera*. On the contrary *Drosophila melanogaster* has two different and unlinked cytoplasmic actin genes, which suggests that the observed gene structure was inherited during the evolution of *Lepidoptera*.

The *BmA3* promoter is regulated by two cis-activating sequences, SRE and Act1, located in a short proximal region next to the TATA box. The deletion or mutation of both these sequences results in suppressing the promoter activity in insect cultured cells as well as in biolisticated silk glands. A third element, RA3, located just upstream, has a very strong negative action and its deletion enhances the promoter strength by twenty folds.

The nucleotide sequence of SRE, the strongest positive element, is very similar to that of the Serum Response Element of Vertebrates and we showed, by gel retardation assay, that it interacts with a silk gland protein similar to SRF. *In vivo* footprinting demonstrated that this factor does bind to its target DNA in silk gland nuclei. In Vertebrates, SRF is known to regulate the transcription of the cytoplasmic actin genes by interacting with the transcription factor TFIIF. Our data encourage to test the existence of a similar protein-protein interaction in insects.

The knowledge of the A3 promoter that functions in a large spectrum of insect cells will also be helpful for devising efficient vectors for expressing foreign genes in cultured cells and transgenic animals.

50

REGULATION OF GLYCYL tRNA EXPRESSION IN *BOMBYX MORI*

K. P. GOPINATHAN & S. SHARMA

The $tRNA_1^{Gly}$ genes form a multigene family comprising of 20 copies, in the mulberry silkworm, *Bombyx mori*. They have identical coding sequences but differ substantially in their 5' and 3' flanking regions. Based on *in vitro* transcription in posterior silkgland nuclear extracts, these genes could be classified into 3 groups of high, medium or low levels of expression. We have identified *cis* acting elements modulating transcription, located in the flanking regions of the coding sequences, much farther than believed earlier for Pol III transcribed genes. One characteristic regulatory element has been identified as a TATAA box sequence, which acts either as a positive or negative element depending on its location from the transcription start site. Since the *in vivo* transcription status of these genes is not known, two sets of $tRNA_1^{Gly}$ belonging to the extreme groups of highly transcribed and barely transcribed copies have been examined here for their expression patterns in *B. mori*-derived cell lines following tranfection. We have developed a sensitive and reliable method for directly quantifying the transcription levels of transfecting tRNA genes without relying on the biological activity of the transcript. The strategy involved the insertion of synthetic oligodeoxyribonucleotide sequences into the coding region of the transfecting gene and monitoring the transcripts in an RNase protection assay using an antisense probe that clearly distinguished them from the endogenous tRNAs. The oligonucleotide insertion did not significantly affect the transcriptional status of the genes even though the distance between the A and B boxes was enhanced by 10-15 nt. *In vivo* also the transcription of $tNRA_1^{Gly}$-1 reached very high levels whereas the transcripts arising from $tNRA_1^{Gly}$-6:7 accounted for only 2-5 % of the former, closely resembling the transcription patterns *in vitro*. These individual gene copies having identical coding sequences and consequently the same internal conserved regions, differed only in their flanking sequences which modulate their transcription levels. A combinatorial effect of multiple *cis* regulatory elements and the availability of the corresponding *trans* acting factors ultimately decide the expression status of the individual members of this multigene family.

51

DIFFERENTIAL TRANSCRIPTION OF SILKWORM tRNA^{Ala} GENES

B. BROWN, J. MANGOR, C. OUYANG, L. S. YOUNG & K. U. SPRAGUE

Background

The genes encoding alanine tRNA in the silkworm, *Bombyx mori*, provide a striking example of regulated polymerase III activity. One class of these genes (c) is transcribed constitutively ; the other (SG) is transcribed only in cells of the silk gland, where the resulting increase in alanine tRNA allows efficient silk production. We are investigating the mechanism of regulation by comparing the transcriptional properties of wild type and mutant versions of these genes both *in vitro*, using homologous transcription machinery derived from silk glands or oocytes, and *in vivo*, using cultured *Drosophila* or *B. mori* cells. Our previous work had identified the upstream segment of the tRNA ^{Ala} promoter as the key determinant of C vs SG *in vitro* transcriptional properties[1], and had established that, *in vitro*, C promoter activity is conferred largely by two AT-rich elements within this upstream segment (the "TAT" element at -29, and the "AT" element at -20)[2].

Results

***In vivo* analysis** : We have now investigated the promoter activity of a set of upstream elements *in vivo*, and find that upstream sequences, generally, are active in both *Drosophila* (S2) and *Bombyx* (BmN) cells. The relative activities of specific elements vary between the two systems. In *Drosophila* nearly all of the promoter activity resides in the TAT element[3], whereas in *Bombyx*, both TAT and AT confer activity. In the homologous system, the patterns of sequences with promoter activity *in vivo* or *in vitro* are much alike - a result that supports the physiological relevance of functional analyses performed *in vitro*.

***In vitro* analysis** : To pinpoint the protein-DNA interactions characteristic of C and SG promoters, we have examined direct binding of isolated transcription factors and of partially purified transcription factor complexes[4]. We have also used site-specific cross-linking of polypeptides within intact transcription complexes[5]. We find that isolated silkworm TBP is capable of binding both C and SG promoters, but that it does so with slightly different affinities and at very different locations. Current experiments are aimed at determining whether TBP actually binds these sites when transcription complexes form on the two genes.

The introduction of protein-DNA crosslinks at known positions within upstream promoter elements has allowed us to map the positions of particular polypeptides within active transcription complexes. We find a simple and reproducible pattern of 3 polypeptides (52, 95 and 171 kD) that are preferentially cross-linked from different positions along the C upstream promoter. Our goal is to use this approach to compare the architecture of protein-DNA complexes formed on the C and SG promoters. We will look for qualitative differences (proteins that can be crosslinked to one promoter, but not the other) as well as quantitative differences in the crosslinking efficiency of particular proteins.

1. Young, L. S., N. Takahashi, & K. U. Sprague, 1986, Upstream sequences confer distinctive transcriptional properties on genes encoding silkgland-specific $tRNA^{Ala}$, *Proc.* Natl. Acad. Sci. USA 83 : 374-378.

2. Palida, F. A., C. Hale, & K. U. Sprague, 1993, Transcription of a silkworm $tRNA^{Ala}C$ gene is directed by two AT-rich upstream sequence elements, *Nucl. Acids Res.* 21 : 5875-5881.

3. Experiments performed in collaboration with Alpa Tivedi and Deborah Johnson (University of Southern California).

4. Young, L. S., N. Ahnert, and K. U. Sprague, 1996, Silkworm TFIIIB binds both constitutive and silk gland-specific $tRNA^{Ala}$ promoters but protects only the constitutive promoter from Dnase I cleavage, Mol. Cell. Biol. 16 *: 1256-1266.*

5. Bartholomew, B., G. A. Kassavetis, B. R. Braun, and E. P. Geiduschek, 1990, The subunit structure of transcription factor IIIC probed with a novel photo-crosslinking reagent. EMBO J. 9 : 2197-2205.

SESSION 10

HOMEOTIC GENES ; ECDYSTEROID HORMONE-CONTROLLED DIFFERENTIATION PROGRAMS

52. HARA (Japan) :
BmScr transformed thoracic leg to antenna in BmAntp deletion mutant in the silkworm, *Bombyx mori*. 93

53. Hiruma / Zhou / Shinoda / Sun / Malone / RIDDIFORD:
Orchestration of insect molting and metamorphosis: hormonal regulation and molecular switches 94

54. SWEVERS/Eystathioy/Iatrou (Canada) :
The ecdysone response in the follicular cells of the silkmoth ovary during pupal and pharate adult development. 95

55. VOGT/Franco/Rogers (USA) :
Steroid regulation of olfactory development : ecdysteroid action in olfactory epithelia of larval and adult *Manduca sexta*. 97

56. Lan/Jindra/RIDDIFORD/Hu/Cherbas (USA) :
Activation of MHR3 by different ecdysone receptor-ultraspiracle isoform combinations. 99

52

BmScr TRANSFORMED THORACIC LEG TO ANTENNA IN BmAntp DELETION MUTANT IN THE SILKWORM, *BOMBYX MORI*

W. HARA & K. MASE

cDNA clone showing very strong cross-hybridizing signal with BmAntp genomic clone pBm3.0 was isolated from the embryonic cDNA library in the silkworm, *Bombyx mori*. Sequence analysis was done and showed that this was the Scr homologue in the silkworm. Homeoboxes was very similar between BmAntennapedia and Sex comb reduced homologue in amino acid and nucleotide level. Strikingly this BmScr homeobox was 59/60 similar to *Drosophila* Scr homeobox in amino acid level.

In *Drosophila melanogaster*, most of the homeotic genes including ftz, M repeat was found in nucleotide sequences. In *Bombyx mori*, M repeat sequences were not found in BmAntp And BmScr genes. And these genes had long 3 prime region from the stop codon.

Linkage analysis was done by using RFLP revealed that these genes are closely linked to BmAntp on the same sixth chromosome.

Northern blot analysis was performed at larval and embryonic stages. Transcript was strongly detected from before organogenesis through hatching stages like BmAntp expression and the size of the transcript was 3.2 kb. The transcript was detected at from posterior head to anterior thorax in wild type embryos, but in BmAntp deletion mutant embryo, the transcript was detected at more posterior segment even at abdominal segments.

In Nc mutant proto-thoracic leg (T1) was transformed to antenna, from our results, this transformation was resulted by the Scr expression at thoracic leg without suppression of wildtype BmAntp expression.

53

ORCHESTRATION OF INSECT MOLTING AND METAMORPHOSIS : HORMONAL REGULATION AND MOLECULAR SWITCHES

K. HIRUMA, B. ZHOU, T. SHINODA, G-C. SUN, F. MALONE & L. M. RIDDIFORD

Insect growing and metamorphosis are regulated primarily by two hormones ecdysone and juvenile hormone (JH). Ecdysone and its active metabolite 20-hydroxyecdysone (20E) cause molting that is necessary for continued growth during larval life and also the switching of gene expression that must occur for metamorphosis to proceed. Juvenile hormone is present during larval life and prevents this switching but allows the molting responses to ecdysone ; JH then disappears and ecdysone initiates metamorphosis. In the tobacco hornworm, Manduca sexta, the epidermis which makes the overlying cuticle or exoskeleton plays out three different genetic programs as it makes first a series of larval cuticles during the growth phase, then during metamorphosis first a pupal, then an adult cuticle. When larval epidermis is cultured *in vitro* with high 20E in the presence of JH to mimic the molt, it makes a new larval cuticle. When cultured with low levels of 20E in the absence of JH, it becomes committed to undergo pupal differentiation in response to high 20E. Under these latter conditions we show that the RNAs for the two ecdysone receptor (EcR) isoforms, the Ultraspiracle (USP)-2 isoform, and the transcription factors E75A, MHR3 and Broad-Complex (BC-C) were up-regulated, each in its own time-dependent manner. The initial increases of the two EcR and USP-2 RNAs were unaffected by the presence of JH which prevents the 20E-induced pupal commitment, although the continued up-regulation of the EcRs between 12 and 24 hr was inhibited. By contrast, the presence of JH increased both the sensitivity and the amount of induction of E75A RNA by 20E, increased the accumulation of MHR3 RNA, and completly inhibited the expression of BR-C. It also prevented the delayed induction of USP-1 by 20E. Therefore, the 20E-induced switch from larval to pupal commitment is accompanied by an earlier appearance of the EcR-A isoform, decreased levels of E75A and MHR3, and the first appearance of the metamorphic-specific BR-C, leading to both quantitative and qualitative changes in both the EcR/USP complex and the combination of transcription factors present. At the end of the molt when the ecdysteroid titer declines, βFTZ-F1 RNA increases and may be important in regulation of the onset of cuticle gene transcription. This increase was found to occur precociously in the absence of JH, irrespective of whether or not the epidermis had seen a molting level of ecdysteroid, indicating that JH is the main hormone involved in the regulation of βFTZ-F1. Therefore, JH can modulate both the level and timing of transcription regulated by 20E as well as prevent the turning on of new genes at metamorphosis. Supported by grants from NIH, NSF, and USDA.

54

THE ECDYSONE RESPONSE IN THE FOLLICULAR CELLS OF THE SILKMOTH OVARY DURING PUPAL AND PHARATE DEVELOPMENT

L. SWEVERS, T. EYSTATHIOY, I. LINDSTRÖM-DINNETZ & K. IATROU

The molting hormone, 20-hydroxy-ecdysone (20E) is the master regulator of ovarian development in the early pupa of the silkmoth, *Bombyx mori*. Follicular cells respond to the presence of 20E by engaging in a long term differentiation program that results in the transport of yolk proteins into the oocyte during vitellogenesis and the synthesis and secretion of the eggshell or chorion proteins during choriogenesis. In addition to the well-documented role of 20E at the beginning of pupation, studies involving *in vitro* culturing of ovarioles have suggested that an additional hormonal factor must exist in the hemolymph that induces the developmental program of chorion gene expresssion at a specific stage during mid-vitellogenesis. This stage occurs 3 days after larval-pupal ecdysis (1.5 days prior to the onset of choriogenesis). A major goal of our research is to identify regulatory factors that are induced by 20E at the begininning of pupation and by the unknown hemolymph factor whose presence is required during mid-vitellogenesis. We are also interested in the definition of the interactions that occur among such regulatory factors with target genes that provide the specific phenotypic characteristics of follicular cells at different stages of oogenesis.

To analyze the ecdysone response in follicular cells, we investigate, first, the patterns of expression of several candidate elements of the 20E-induced regulatory cascade during follicular development. The analysis included elements of the ecdysone receptor complex (BmEcR) and BmCF1), a putative modulator of the ecdysone response (BHR38), nuclear receptors that are directly-induced (BmHR3 and BmE75), indirectly-induced (BmHNF-4) or negatively regulated (BmFTZ-F1) by 20E, transcription factors characterized by a "GATA"-type zinc finger containing DNA-binding domain (BmGATAβ) and three homologues of the *Drosophila* early ecdysone-responsive gene Broad-Complex (BmBTBs). Our analysis included an examination of the temporal patterns of 20E-inducibility of expression of specific mRNA (and protein) isoforms, encoded by these genes, in the silkmoth ovary following injection of 20E into ligated pupal abdomens, as well as a determination of their expression profiles in follicular cells of pharate adults 5 days after larval-pupal ecdysis, when the first choriogenic follicles appear in the ovarioles.

Our results have identified two ecdysone-responsive genes, BmHR3 and BmE75, and several other genes which are not directly regulated by 20E, that display potentially important, concomitant or reciprocal, expression patterns. The observed temporal patterns of expression indicate that a specific isoform of BmHR3 may function as a repressor of the gene BmGATAβ and as an activator of the gene encoding the egg-specific protein ESP, and that BmE75C may be a regulatory factor mediating the transition from vitellogenesis to choriogenesis.

Finally, to investigate whether bombyxins have a role in the establishment of the program of choriogenesis during mid-vitellogenesis, we cloned the cDNA for an insulin-like tyrosine kinase membrane receptor and studied its expression during follicle development. Studies to deduce whether

bombyxin-binding activity occurs in membrane extracts of ovarian follicular cells and whether the cloned receptor is capable of transducing bombyxin signals are in progress.

To test the validity of our model for follicular cell differentiation, we intend to examine the effects of follicular cell-specific over-expression or antisense RNA-mediated inhibition of relevant wild-type or mutant regulatory factors in follicular cells on ovarian development.

55

ECDYSTEROID REGULATION OF ODORANT BINDING PROTEIN EXPRESSION AND SENSORY NEUROGENESIS IN *M. SEXTA*

R. G. VOGT, M. D. FRANCO & M. ROGERS,

The olfactory antenna of adult *Manduca sexta* is highly organized into distinct sensory and non-sensory domains. Sensory domains are further spatially subdivided in a highly repeated fashion into distinct regions of differing functional modalities; these regions can be defined by the phenotypes of differing sensilla types and the specific behaviour these support by the different sensilla. We are taking a molecular / histological approach to characterize mechanisms underlying antennal organization and its establishment. For example, we are characterizing a pair of antennal specific and homologous genes which show temporally and spatially specific yet differing patterns of expression throughout larval, pupal and adult life. These genes, encoding the odorant binding proteins PBP and GOBP2, reside as a neighbour, coding regions are separated by only 2500 bp of DNA. In adult male antennae, PBP expresses in sensilla arrayed along the peripheral boundaries of the sensory epithelium of each annulus; GOBP2 expresses in a middle annular region, surrounded by but not overlapping PBP expression. The physical proximity of these genes and their interrelated patterns of expression strongly suggests these genes are regulated in a highly co-ordinated manner. Previous studies demonstrated an ecdysteroid involvement in the expression of PBP and GOBP2 late during adult development; culturing tissue in prematurely low levels of 20-HE induced expression as much as 48 hrs prior to the time of normal *in vivo* expression (1). Recent studies show that larval expression of GOBP2 is regulated through a molt cycle in an ecdysteroid consistent manner.

The adult spatial patterns are established early during the 18 days development of *M. sexta*, at the time of sensory neurogenesis. At pupation, the adult antennal immaginal disk everts, forming the tubular monolayer of cells that will morphogenize into the 80 annular segments of the mature antenna. Initially this tissue secretes pupal cuticle as it lays down upon the pupal surface. Antennal apolysis, occurring 3 days following pupation, marks a major event in antennal development, separating DNA replicative events that associate with mitotic events (neurogenesis during days 1-3) from DNA endoreplicative events that occur in certain support cells following apolysis (days 4-6). DNA replication is histologically visualized in whole-mount following exposure to and incorporation of BrdU.

Neurogenesis follows the selection of Sensory Organ Precursor (SOP) cells from the background epithelium. Beginning late during Day1, SOP cells begin dividing, each producing progeny which differentiate into the neurons and support cells comprising an individual olfactory sensillum. This neurogenesis initiates on Day 1 along the borders of each annulus; neurogenesis continues as a spatial wave progressing to the middle region of each annulus and completing by apolysis. BrdU incorporation is identified as mitotic based on the consistent appearance of clusters of 2 or 4 cells and previous reports (2) of mitotic figures during this phase. Following apolysis, 2 sequential rounds of DNA replication are observed in the region occupied by the pheromone specific sensilla trichoidea and the later site of PBP expression: one round of DNA replication is observed in the region occupied by plant volatile sensitive sensilla basiconica, and the latter site of GOBP2 expression.

Ecdysteroids regulate the temporal / spatial patterns of neurogenesis within the pre-apolysis antenna. Immunocytochemical analysis of EcR expression reveals a temporal / spatial pattern which overlaps the temporal / spatial pattern of BrdU incorporation (neurogenesis). In culture, exposure to either 20-HE (100ng / ml) or ecdysone (500ng / ml) stimulates a significant spatial advancement of neurogenesis in a concentration dependent manner. We are developing this system to explore the interplay between EcR isoforms and the hormones 20-HE and ecdysone in the orchestration of temporal / spatial specification of the olfactory system, as well as the roll apolysis has in isolating neurogenesis (i.e. cell proliferation) from morphogenesis and differentiation in the developing antenna.

1. Vogt RG, Rybczynski R., Cruz M., lerner MR (1993) Ecdysteroid regulation of olfactory protein expression in the developing antenna of the tobacco hawk moth, *Manduca sexta*. J. Neurobiol. 24, 581-597.

2. Sanes JR, Hildebrand JG (1976) Origin and morphogenesis of sensory neurons in an insect antenna. Dev. Biol. 51, 300-319.

56

ACTIVATION OF MHR3 BY DIFFERENT ECDYSONE RECEPTOR-ULTRASPIRACLE ISOFORM COMBINATIONS

Q. LAN, M. JINDRA, X. HU, P. T. CHERBAS & L. M. RIDDIFORD

MHR3 is an ecdysteroid-induced transcription factor of the delayed-early class and is a member of the nuclear hormone receptor family. In *Manduca* 4th instar epidermis maximal expression of two isoforms (3.8 and 4.5 kb) is induced by a molting concentration (2 µg/ml) of 20E in 6 hrs but only about 60 % of that expression occurs in the presence of a protein synthesis inhibitor (Palli *et al.*, Dev. Biol. 150, 306-318, 1992). In *Manduca* GV1 cells, only the 4.5 kb isoform is induced but with the same kinetics and concentration-response as in the epidermis. In both epidermis (Zhou *et al.*, submitted) and the cells (Lan *et al.*, *Insect Mol Biol.* 6, 3-10, 1997), E75A, another member of this family, is more rapidly induced by 20E.

We have now sequenced the promoter region for the 4.5 kb transcript and found 4 ecdysone receptor response elements (EcRE-1,-2, -3 and -4 according to their distance from the transcription start site) and one E75A response element (E75A) within 2.5 kb upstream of the transcription start site. Three of the EcREs are located within the first 1 kb, and the E75ARE is 8 bp downstream from EcRE-3. A bacterial CAT reporter gene was ligated to the 3' end of the MHR3 promoter, and transient transfection assays in the GV1 cells were performed to test for 20E-regulated promoter activity. Activation of the transfected promoter by 20E was similar to the endogenous MHR3 (Lan *et al.*, 1997) ; CAT was detectable only after 3 hrs exposure to 2 µg/ml 20E. The 1 kb promoter was sufficient for full 20E-inducible activity. Deletion analysis showed that this 20E-induced promoter activity was reduced over 2.5-fold when only EcRE-1 and -2 were present and nearly undetectable if only EcRE-1 was present.

A highly efficient expression vector containing the promoter of the immediate early gene *i.e*-1 from AcMNPV was used to overexpress *Ms*EcR-B1, *Ms*USP-1, *Ms*USP-2 and *Ms*E75A proteins in co-transfection assays in the GV1 cells to examine their functions in activation of MHR3 promoter. At least a 25-fold increase in the level of the specific protein was found after these co-transfections. The combination of EcR-B1 and USP-1 caused about a 5-fold increase in MHR3 promoter activity after 3 hr exposure to 2 µg/ml 20E. By contrast, EcR-B1 and USP-2 failed to increase promoter activity above the control. This selective activation of EcR-B1/USP-1 was abolished when only EcRE-1 and -2 remained in the promoter. This result showed showed that the isoforms of EcR and USP genes are functionally different and that the EcRE-3 may be responsive for the selective activation. The *in vitro* transcription-translation product of EcR-B1 formed heterodimes with either USP-1 or USP-2 that both bound Ponasterone A with a k_d=7x10^{-10}M, so this selective activation must be with interaction with the promoter.

When E75A was present along with EcR-B1 and USP-1, the MHR3 promoter activity was over 2-fold higher than with only EcR-B1 and USP-1. This synergistic effect of the E75A protein was decreased when the E75ARE was deleted from the promoter. Thus, we have shown for the first time that full activation of a delayed-early gene (MHR3) by 20E requires the product of an early gene (E75a).

The E75A protein was also found to have a second function. When the EcR-B1, USP-2, and E75A were co-transfected, there was a complete suppression of the 20E induction of promoter activity. Therefore, USP2 is not only insufficient for the activation of the MHR3 promoter but also prevents any synergistic effect of E75A. Possibly USP-2 is involved in the down-regulation of MHR3 at the peak of the ecdysteroid titer.

Supported by grants from NIH.

POSTER SESSION

Mapping / Transposable Elements / Phylogeny

P-1 PROMBOON / Yano / Suzuki / Shimada / Kobayashi (Thaïland) :
Nucleotide sequences of RAPDs in *Bombyx mori*. 103

P-2 NAKAJIMA / Hashido / Tomita / Tsuchida / Takada / Maekawa (Japan):
Transposable elements (BMC1 and BM2) might be derived from the same ancestor . 104

P-3 PFEIFER / Humble / Grigliatti (Canada):
Comparison of the genus *Lymantria* by 5S and ITS sequence analysis 105

P-4 Sethuranaman / NAGARAJU (India):
Cloning and characterization of middle repetitive elements in the silkworm, *Bombyx mori*, and their utility in genetic analysis 106

Transfection / Transformation

P-5 ROYER / Guerin / Romane / Bergoin / Couble (France):
Organization of *Junonia coenia* densovirus DNA in transfected *Spodoptera frugiperda* (Sf9) cells 107

P-6 THOMAS / Bardou / MAUCHAMP / Chavancy (France)
Lac Z gene expression in gonads of *Bombyx mori* larvae and in embryonic tissues following gene gun bombardment 108

Regulation

P-7 KOSEGAWA / Nagaoka / Okano / Hara / Shimizu (Japan):
Structure and expression of HSC70 gene of silkworm, *Bombyx mori* 109

P-8 REINHARDT / Koch (Germany):
Cloning of an ecdysone receptor (EcR) homolog from *Bicyclus anynana*. 110

P-9 TOMITA / Shirai / Kamimura / Fujiwara (Japan):
Binding properties between EcR of *Bombyx mori* and its ligand. 112

P-10 SINGH / Kango-Singh / Pillai / Gopinathan (India):
Developmental characterization of patterning genes in mulberry silkworm species . 113

P-11 KISHIMOTO / Nakato / ISUMI / Tomino (Japan):
Biosynthesis of major plasma proteins in the primary culture of fat body cells from *Bombyx mori* 114

Gene Isolation / Insect Control

P-12 OELLERS / Schulte / Adamczewski (Germany):
Molecular cloning of nicotinic acetylcholine receptor genes from *Heliothis virescens* . 115

P-13 MIKITANI / Gustafsson / Kobayashi (Japan / Sweden):
The cDNA cloning and the characterization of a chitinase gene of the silkworm, *Bombyx mori* 117

P-14 FITCHES / Gatehouse (England):
Mechanisms of action of the insecticidal mannose binding plant lectin from snowdrop (GNA) on the tomato moth, *Lacanobia oleracea* 118

P-15 BAUMBACH (USA):
Insecticide target genes from *H. virescens* cDNA libraries; differential expression between embryo, larva and adult 119

P-16 BOWN / Gatehouse (England)
Differentially regulated inhibitor-sensitive and insensitive protease genes from the phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families 120

P-17 Sivaprasad / Selvakumar / Nataraju / DATTA (India)
Synergistic effect of BmIFV and *Streptococcus*, *Staphylococcus* and *Serratia marescens* in silkworms 122

P-18 Perera / SHIRK (USA)
Two new DEAD box genes expressed in ovaries of the moth, *Plodia interpunctella* . . 123

P-19 SHIRK / Perera / Hemphill / Broza / Zimowska (USA)
cDNA clones of follicular epithelium yolk polypeptides of *Plodia interpunctella* 124

P-20 Horard / JULIEN / Nony / GAREL / COUBLE (France)
Transient expression of modified fusion genes in *Bombyx* silkgland cells after biolistic transformation. 125

P1

NUCLEOTIDE SEQUENCES OF RAPDs IN *BOMBYX MORI*

A. PROMBOON, S. YANO, M. SUZUKI, T. SHIMADA & M. KOBAYASHI

We cloned 36 RAPD bands on the linkage map in *Bombyx mori*. All of them were partially sequenced. Nine (25%) of the 36 RAPDs did not show any homology to other loci on the database whereas 27 (75%) of them were partially homologous to other loci of *B. mori*, indicating that they carried repetitive DNA elements. Eighteen of the multicopy RAPDs contained one or two of the most popular repetitive elements, *Bm1*, *Bm2* or BMC1, in *B. mori*. One multicopy RAPD carried the "mariner" element. Southern hybridization of the cloned RAPD and the genomic DNA confirmed the estimated copy number on the genome. The nucleotide sequences of RAPDs can be used to convert the RAPDs into STS, SCAR or more convenient codominant PCR markers for further analysis of the genome.

P2

TRANSPOSABLE ELEMENTS (BMC1 AND L1Bm : Bm2) MIGHT DERIVE FROM THE SAME ANCESTOR

Y. NAKAJIMA, K. HASHIDO, S. TOMITA, K. TSUCHIDA, N. TAKADA & H. MAEKAWA

Six to nine fragments from 0.6 to 4.2kb, were amplified to genomic DNA of *Bombyx mori* by PCR using inverted repeat of transposon, mariner of *Hyalophora cecropia* as a primer. The longest fragment of 4.2 kb was observed in eight strains tested of *B. mori* and cloned from products to the N4 strain as a plasmid, pBmTNML1, sequenced. This clone contained mariner (1.2kb), L1Bm (SINE-like element of 1? copies) (0.2kb), and retrotransposon, BMC1 (2.8 kb).

The sequence data revealed that L1Bm had been integrated into mariner, and no BMC1 had been done into L1Bm with target sequence duplication at each flanking region. Retrotransposon, BMC1 (full length unit of 5.1 kb), was a member of LI without LTR and about 3500 copies per haploid genome. One of three types of L1Bm was identical 3' end of BMC1 and hence both elements might be derived from the same ancestor. The same member (3.7kb) containing the complete ORF for reverse transcriptase was reported in intron of amylasegene. This suggests that an active reverse transcriptase and integrase complex recognises and integrates the defective R intermediates into the genome. The conserved sequence at the 3' end region in both elements was observed and that sequence might be functioned as recognition sequence the integration of the BMC1 and L1Bm elements by the active reverse transcriptase coding in the same family.

P 3

COMPARISON OF THE GENUS *LYMANTRIA* BY 5S AND ITS SEQUENCE ANALYSIS

T. A. PFEIFER, L. H. HUMBLE & T. A. GRIGLIATTI

In light of the recent dispersal of the Asian gypsy moth *Lymantria dispar* throughout the world, we have undertaken the analysis of rDNA regions for the purpose of providing specific markers that will identify Asian, North American and hybrid varieties of *L. dispar*. In the process we devised markers that could be used to identify other species of the *Lymantria*'s as well as closely related tussuck moths. Two markers, based on restriction enzyme analysis of PCR products, differentiate the geographically separated Asian and North American moths as well as the "hybrid" F1 offspring. Defined crosses were made under quarentine conditions and theses populations, including hybrids, *inter se*, and backcrosses, were raised to the F2 generation. The marker BC-2 correctly identified hybrid moths between these populations and analysis of F2 and backcross data demonstrated that this marker followed normal mendelian genetic predictions. BC-2 can also distinguish between the different species of tussuck moths tested. Finally, we provide phylogenetic comparisons for the tussuck moths based on DNA sequence of the ITS spacer regions and the 5S rRNA genes.

P 4

CLONING AND CHARACTERISATION OF MIDDLE REPETITIVE ELEMENTS IN THE SILKWORM, *BOMBYX MORI* AND THEIR UTILITY IN GENETIC ANALYSIS

B. N. SETHURAMAN & J. NAGARAJU

The silkworm *Bombyx mori* is an organism of an economic and experimental importance. The detection and analysis of DNA polymorphisms in the silkworm is an essential component of molecular mapping, genotype characterisation and marker assisted selection. In order to achieve these goals, we are applying methods that involve the genomic analysis of micro and minisatellites. In the present study, we have identified a number of middle repetitive elements from a silkworm sub-genomic library and tested for their utility as probes to reveal DNA polymorphisms. DNA profiles of individual silkworms of thirteen strains revealed 6 to 7 discrete, easily scorable bands, which were inherited in a Mendelian fashion. The utility of these probes in the genetic analysis of silkworm is discussed.

P 5

EXPRESSION AND ORGANIZATION OF *JUNONIA COENIA* DENSOVIRUS DNA IN *SPODOPTERA FRUGIPERDA* (Sf9) CELLS AND *DROSOPHILA* LARVAL AND ADULT TISSUES

C. ROYER, M. A. GUERIN, C. ROMANE, M. BERGOIN & P. COUBLE

The aim of developing vectors for gene transfer in insects led us to explore the ability of plasmids derived from *Junonia coenia* wild type densovirus to be used as efficient expression and integration systems.

The fate and arrangement of vector DNA were respectively investigated by the detection of a β-galactosidase activity (the selection gene being lacZ gene) in Sf9 cells transfected with densovirus derived plasmids and by southern blot analysis of genomic DNA of several independents LacZ clones. Cloning of the cellular-viral DNA junctions are currently under investigation.

The capacity of such vectors to express, persist and integrate the host genome was first investigated in *Drosophila*.

More recently, the expression JcDNV-derived plasmids was examined in *Bombyx mori* eggs by microinjection and biolistic techniques (see the abstract of J-L Thomas in the same session).

In the light of the results the potentiality to use densovirus derived vectors in insect transgenesis is evaluated.

P 6

LacZ GENE EXPRESSION IN GONADS OF *BOMBYX MORI* LARVAE AND IN EMBRYONIC TISSUES FOLLOWING GENE GUN BOMBARDMENT

J-L. THOMAS, J. BARDOU, B. MAUCHAMP & G. CHAVANCY

Among our attempts to obtain transgenesis in *Bombyx mori*, we have studied the potentiality of gene gun bombardment on different biological samples, i.e. larval gonads (testis and ovaries) and late embryos. Different gene constructs have been used as pA3LacZ comprising the promoter of the actin3 gene of *Bombyx mori* which controls LacZ gene expression (1) or as pBRJZ which contains LacZ gene inserted in the densonuclear virus of *Junonia coenia* (2).

After testis and ovaries ex vivo bombardment with pA3lacZ construct, we can obtain LacZ gene expression in somatic gonad sheet but, especially for the testis, expression in gonocystes and in some spermatocytes. We have not developed this aspect because of the low frequency of spermatocytes staining, probably not enough to obtain germinal transgenesis, and because of the difficulty to succeed in fecondation after reimplantation into the larvae.

The same procedure applied to six day old embryos maintained two days alive in Grace's medium permits us to obtain expression in embryonic tissues with both constructs. The main tests have been made with pBRJZ whose viral genome has integrative potentiality. Although gene bombardment is a capricious technique, we can regularly observe Xgal stainings in epidermal cells and more irregularly in internal organs as cerebrum, muscle, silk gland, intestine and gonad. The main problem is to get the larval development of these embryos. If embryos have a good survival for several days, we do not have the mastership on the following larval development. One time, we have reared a pA3LacZ-bombarded embryo until the fifth larval instar.

At this stage of our biolistic work on embryos, we can say that this method allows us to verify the validity of transient expression of our plasmidic constructs among a set of differentiated tissues. Currently, we are working to overcome the difficulty for obtaining larval development, and to improve the targeting of the gonad with the drop of general tissue damages by the restriction of the shoot area to the gonad carrying segment.

COULON-BUBLEX M., MOUNIER N., COUBLE P. & J-C. PRUDHOMME Cytoplasmic actin A3 gene promoter injected as supercoiled plasmid is transiently active in *Bombyx mori* embryonic vitellophages. Roux's Arch. Dev. Biol. (1993) 202: 123-127.

GIRAUD C., DEVAUCHELLE G. & M. BERGOIN The densovirus of *Junonia coenia* (JcDNV) as an insect cell expression vector. Virology (1992) 186: 207-218.

P 7

STRUCTURE AND EXPRESSION OF HSC70 LIKE GENE OF *BOMBYX MORI* L.

KOSEGAWA, S. NAGAOKA, K. OKANO, W. HARA & K. SHIMIZU

By hot HCl treatment, the diapause of silkworm, *Bombyx mori* is terminated and post-diapause development is enabled to start. Just after the treatment, RNAs that is resembled to HSP70, are induced in hot HCl treated eggs. Heat shock protein, for example HSP70, is suggested the relation to the recovering of cells damaged by physical or chemical stress. Additionally, the molecules have a role of molecular chaperon. We believe that the induction of HSP70 molecule is necessary to terminate diapause by hot HCl. Recently, a HSP like cDNA clone (p49) was isolated from cDNA library constructed by mRNAs of silkworm eggs day 3 in embryogenesis. We will report the construction of the cDNA and gene expression of the gene.

Partial sequence in 5' terminal of the p49 insert fragment was provided to BLAST search of GenBank and revealed high similarity to Drosophila HSC4 gene. HSC4 gene is the typical Heat shock cognate gene (HSC70). The sequence data have also indicated that the 5' terminal of p49 was truncated in comparison to genomic DNA sequences of HSC4 gene.

Since typical HSC70 gene shows constitutive transcription, it is able to distinguish from HSP70 gene. So, northern hybridization analysis was applied to template of total RNAs of both hot HCl treated eggs and non-treated control, by probing with p49 riboprobe. Subsequently, the constitutive signal was observed. It was concerned that p49 gene is a type of HSC70 gene and not of HSP70. Whereas the hybridization signal was slightly stronger in hot HCl treated eggs, the results of northern analysis may be contributed from the hybridization to both HSP70 and HSC70. We are trying to make more specific probe without cross-hybridization.

P 8

PARTIAL CLONING OF AN ECDYSONE RECEPTOR HOMOLOG FROM *BICYCLUS ANYNANA* (SATYRIDAE)

R. K. REINHARDT & P. B. KOCH

The African butterfly *Bicyclus anynana* (Butler, 1879), like some other butterflies can change the pattern of spots on its wings in response to environmental cues. During the rainy season, the butterfly exhibits prominent marginal eyespot patterns which function principally in the deflection of attacks by vertebrate predators. During the dry season, when there is little food and the butterflies show less activity, new generations emerge which show very small or no eyespots and are wholly cryptic (Brakefield, 1984).

Previous studies (Koch et al., 1996) have shown, that in *B. anynana* ecdysteroids regulate developmental speed in the pupa as well as they are involved in pattern determination and pigment synthesis. In the early pupa of *B. anynana* injection of 20-hydroxyecdysone (20E) causes development of increased eyespots.

During late wing differentiation decreasing ecdysteroid titers trigger melanin synthesis. A key enzyme in the pathway from tyrosine to melanin is the dopa decarboxylase (DDC). As we already know, the DDC shows pattern-specific expression in the pupal wing and is expressed only in the future black and grey-brown regions (Bergander *et al.*, 1996). Hiruma (1995) showed that 20E induces a protein which suppresses transcription of the DDC gene. By this mechanism a decreasing ecdysteroid titer allows a time and pattern specific expression of the DDC gene (Koch, 1995).

In order to investigate the role of ecdysone receptor proteins in the complex mechanisms of colour pattern development we cloned a 1000bp fragment of a putative ecdysone receptor of *Bicyclus anynana* to use it as a probe in Northern- and *in situ* hybridizations. This fragment comprises half of the DNA-binding domain and the complete hinge-region and ligand-binding domain. Compared with the ecdysone receptor of *Manduca sexta* (Fujiwara *et al.*, 1995) the gene fragment of the putative ecdysone receptor of *Bicyclus anynana* shows an overall homology of 82% (amino acid level). Moreover, the *Bicyclus* clones show two striking details: one detail is an amino acid exchange in the DNA-binding domain which may influence DNA-binding activity, the other detail is an additional amino acid sequence in the hinge-region, which is unique among the known ecdysone receptors.

Northern Blot hybridization lead to a total length of the *Bicyclus* ecdysone receptor homolog of approximately 2000bp. Using the 3' and 5' RACE method we are on the way to clone the complete gene of the *Bicyclus* ecdysone receptor homolog.

REFERENCES

BRAKEFIELD P. M. The evolutionary significance of dry and wet season forms in some tropical butterflies. Biological Journal of the Linnean Society 22, 1-12 (1984).

FUJIWARA H., JINDRA M., NEWITT R., PALLI S. R., HIRUMA K. & RIDDIFORD L. M. Cloning of an ecdysone receptor homolog from *Manduca sexta* and the developmental profile of its mRNA in wings. Insect. Biochem. Mol. Biol. 25(7),845-856 (1995).

HIRUMA K., CARTER M. S. & RIDDIFORD L. M.

Characterization of the Dopa Decarboxylase gene of *Manduca sexta* and 1st suppression by 20-Hydroxyecdysone. Developmental Biology 169, 195-209 (1995).

KOCH P. B. Colour pattern specific melanin synthesis is controlled by ecdysteroids via dopa decarboxylase in wings of *Precis coenia* (Lepidoptera: Nymphalidae). Eur J. Entomol. 92: 161-167 (1995).

KOCH P. B. , BRAKEFIELD P. M., & KESBEKE F. Ecdysteroids Control Eyespot Size and Wing Color Pattern in the Polyphenic Butterfly *Bicyclus anynana* (Lepidoptera: Satyridae) J. Insect Physiol. Vol 42, No 3, pp 223-230 (1996).

P 9

BINDING PROPERTIES BETWEEN EcR OF *BOMBYX MORI* AND ITS LIGAND

S. TOMITA, K. SHIRAI, M. KAMIMURA & H. FUJIWARA

Ecdysteroids acts through multiple isoforms of the ecdysone receptor (EcR) during metamorphosis and molting of insects. In *Drosophila*, the differential expression of A and B1 isoforms are related to the lineage of the cells on the course of metamorphosis (1)

We have successfully cloned A isoform (BmEcR-A) in addition to B1 (BmEcR-B1) isoform of EcR from *Bombyx mori* and investigated the mRNA expressions of two BmEcR isoforms during molt and metamorphosis. Predicted amino acid sequence of BmEcR-A isoform specific region showed 52% homology to the *Drosophila* EcR A isoform. At the larval-pupal transformation, expression of BmEcR-B1 has relatively predominant in most tissues examined including the wing imaginal disc and three larval tissues (midgut, epidermis and fat body). The anterior silk gland was the only tissue where BmEcR-A was predominantly expressed. In the anterior silk gland, both EcR isoforms were expressed synchronously during the 5th larval instar while expression of the A isoform preceded that of B1 isoform by two days in the 4th instar. Thus, transcription of the BmEcR in this gland is regulated differently between these two instars (2).

These isoforms may directly dictate the expression of the down stream genes by selecting the response elements differently or unknown molecules which may interact differently with EcR isoforms may mediate the ecdysteroid signal. To examine this, we firstly investigated the sequence specificities of DNA-binding properties of BmEcR-A and BmEcr-B. We synthesized the BmEcR-A, BmEcR-B1 and *Bombyx mori* USP (BmUSP) by rabbit reticulocyte *in vitro* translation system. Labeling with ^{35}S-methionine revealed the proteins of proper molecular weight were synthesized. We tried to express B1 isoform of BmEcR fused with thioredoxin at the N terminus in *E. coli*, but the induction of the protein of proper molecular weight was not observed. Western blot analyses suggested the degradation of the product. For BmUSP, we used the GST fusion system for the expression in *E. coli*. The expressed protein showed the proper molecular weight, could be partially by glutathion sepharose column followed by anion exchange column, and released GST portion from USP portion when treated by thrombin.

Using these expressed proteins, we investigated the binding properties between these proteins and ecdysone response element (EcRE) by electrophoresis mobility shift assay (EMSA). We used *Drosophila* hsp27, Fbp1, Lsp2, Sgs4-1 and Eip28/29p EcREs, Pal1 and Direct repeats (DR0 to 5G) as the probe. The experiments using *in vitro* translated BmEcR and BmUSP showed that they could not bind with EcRE alone and only when they formed complex (maybe heterodimer) could bind with EcRE. We could not detect significant effect of 20-hydroxyecdysone, the functional ligand of EcR, on the binding between receptor and probe DNAs. A and B1 isoforms of EcR showed almost the same preferences for the probes. When replacing the USP with bacterially expressed protein, the result was the same as above.

(1) TALBOT W. S. *et al.*, (1993) *Drosophila* tissues with different metamorphic responses to ecdysone express different receptor isoforms. Cell 73, 1323-1337.

(2) KAMIMURA M. *et al.*, (1997) Tissue and stage specific expression of two ecdysone receptor isoforms of the silkworm and their ecdysteroid dependent transcription in cultured anterior silk glands. Eur. J. Biochem. In press.

P 10

DEVELOPMENTAL CHARACTERIZATION OF PATTERNING GENES IN MULBERRY SILKWORM SPECIES

A. SINGH, M. KANGO-SINGH, B. PILLAI & K. P. GOPINATHAN

Pattern formation, an important aspect of developmental biology is in the nascent stage in silkworm and other Lepidopteran insects. Despite the evolutionary conservation of the genes involved in pattern formation, insects exhibit great diversity of pattern. There are striking differences in patterning of *Bombyx mori* as compared to *Drosophila*, the most exploited insect system. Unlike flies, the embryonic development spans to 10 days in silkworm which permits the analysis of patterning events on discrete time scale without overlaps. *B. mori* larvae possess abdominal limbs and two pairs of wing imaginal discs but no imaginal discs for leg and antenna. This projects silkworm as a model system harbouring two different patterning mechanisms, both primitive and advanced, working in parallel for adult appendage development. Therefore, silkworm represents the time window marking the transition of primitive to advanced insects along the evolutionary scale. We have used heterologous antibodies, as well as PCR amplified DNA probes to study the spatio temporal expression of *wingless (wg)* (a gene encoding the signalling molecule involved in segmental patterning during embryogenesis and in wing development) and *Distal-less* (*Dll*) (a gene involved in limb development) *wingless* is expressed globally during development whereas the expression of *Dll* is confined to the appendages. The evolutionary significance of the genetic regulatory mechanism that underlies the development of all insects, in terms of these genes will be discussed.

P 11

BIOSYNTHESIS OF MAJOR PLASMA PROTEINS IN THE PRIMARY CULTURE OF FAT BODY CELLS FROM *BOMBYX MORI*

A. KISHIMOTO, H. NAKATO, S. IZUMI & S. TOMINO

The major plasma proteins in the larval hemolymph of the silkworm, *Bombyx mori* comprise two forms of storage proteins termed respectively as "SP1" and "SP2", and a group of structurally related proteins collectively referred to as "30K proteins". These proteins are synthesized by the fat body cells in a sex-, stage-and tissue-specific manner during the larval development. SP1 exhibits female-specific expression in the fifth (final) instar larvae, and massive accumulation of 30K proteins in hemolymph takes place at late-larval to early-pupal stages. To investigate cellular mechanisms underlying the regulated expression of SP1 and 30K proteins in *B. mori*, we developed a system for the *in vitro* culture of fat body cells, and studied biosyntheses of the proteins in the cultured cells.

The larval fat bodies were treated with dispase, and the cells liberated were cultured under defined condition. Microscopic examination revealed that major fraction of the cells was small and oval, though a fraction of cells was large and spherical, exhibiting an ability to proliferate. By the fourth day of cultivation, the fat body cells adhered on the surface of culture dishes, migrated, clustered and were partly spread to form a thin layer.

Fat body cells immediately after dispersion from the female of the fifth instar day-1 larvae synthesized mainly SP1 and SP2. The rate of total protein synthesis in the cells markedly elevated after cultivation for 24 hr, then progressively declined during cultivation for 7 days. By contrast, SP1 was synthesized in nearly a constant rate throughout cultivation. Profile of protein synthesis in the cultivated fat body cells from male larvae was similar to that observed with the female cells, except that SP1 synthesis was severely repressed in the male cells just after dispersion, well reflecting the *in vivo* situation of female specificity of SP1 synthesis. However, SP1 synthesis resumed, though at very low level, after cultivation for 24 hr and the rate of its synthesis significantly elevated on the seventh day of cultivation. The synthesis of 30K proteins was only barely seen in the fat body cells cultured for 24hr, whereas the rate of their synthesis markedly increased as seen in the cells from both sexes on the seventh day of cultivation.

Synthesis of plasma proteins in the cultured fat body cells appears to be related with the change of cellular morphology; the cells in culture dishes start to adhere to the surface of dish and form aggregates after 24hr or so of cultivation, and 30K protein synthesis becomes apparent in the cells few days after the onset of cell aggregation. High level of SP1 synthesis was noted in the fat body cells immediately after dissection from the fifth instar day-1 females, which can be accounted for by assuming that the fat body cells at this stage are already committed to synthesize SP1 before dissection.

Taking together these results it can be concluded that the fat body cells in primary culture faithfully reproduce sex and stage – dependency of plasma protein synthesis, though some delay in gene expression occurs when the cells from molting larvae are cultured. Long-term survival of functional cells in culture will certainly provide a useful system for gene transfection studies. An attempt was made to introduce gene constructs by the method of electroporation into fat body cells. The transfected cells exhibited the specific expression of the SP1 gene construct.

P 12

MOLECULAR CLONING OF NICOTINIC ACETYLCHOLINE RECEPTOR GENES FROM *HELIOTHIS VIRESCENS*

N. OELLERS, T. SCHULTE, S. JAFARI-GORZINI, A. MAELICKE & M. ADAMCZEWSKI

In vertebrates, acetylcholine (ACh) acts as a transmitter at neuromuscular synapses, binding to muscle nACh receptors (nAChRs) of the pentameric structure $\alpha_2\beta(\gamma$ or $\varepsilon)\delta$. At neuronal synapses nAChRs are only assembled from two different types of subunits, α and β (for review see Bertrand and Changeux, 1995).

In insects, Ach is the major excitatory transmitter at neuronal synapses in the nervous system while in contrast to vertebrates, neuromuscular transmission is mediated by glutamate. Neuronal nAChRs are oligomeric transmembrane receptor complexes consisting of ligand binding α and structural β subunits that are distinguished by the presence or absence of two adjacent cystein residues, respectively (Deneris et al., 1991).

Insect neuronal nAChRs have been studied extensively in *Drosophila melanogaster* where, to date, five neuronal nAChR subunits have been cloned (three α and two β). In contrast to vertebrates, no functional receptor could be assembled from these subunits in Xenopus oocytes (for review see Gundelfinger, 1992). Less is known about nAChRs in lepidopteran species.

Our aim is to clone and functionally express the genes encoding the different subunits of the nAChR from a lepidoptera, the cotton bollworm, *Heliothis virescens*.

By Reverse Transcription (RT)-PCR and subsequent screening of cDNA libraries synthesized from polyA$^+$RNA of embryonic and larval stages of *Heliothis*, we have obtained four full length and three partial clones encoding different nAChR subunits, so far. One full length clone (Heli α1) has been sequenced in its entirety, partial sequences have been obtained from the others.

Based on the data presently available, four different α subunits, two different β subunits and one not yet classified subunit exist in *Heliothis virescens*. This is a higher number of nAChR subunits than has been found in any other insect species up to date.

The following clones we have obtained:

Clone	α/βsubunit	length (kb)	most homologous to
Heliα1	α	full length (2.2 kb)	manducaα1; Drosophila ALS
clone 1	α	full length (2.6 kb)	manducaα1; Drosophila ALS
clone 4	α	full length (3.85 kb)	Schistocerca αL1; Drosophila SAD
Clone 10	α	Partial (1.1 kb)	Drosophila SAD

Clone 20	?	full length (2.9 kb)	Drosophila SBD
Clone 22	β	Partial (1.3kb)	Manducaα1
clone 81-6	β	partial (0.83 kb)	Drosophila ARD

The cDNa libraries will be probed for additional nAChR cDNAs and for the purpose of completing already identified partial clones. We are presently trying to functionally express the *H. virescens* subunits in Xenopus oocytes and will start to establish cell lines suitable for application in drug screening assays.

REFERENCES

BERTRAND D. & CHANGEUX J. P. (1995) Nicotinic receptors: An allosteric protein specialized for intercellular communication. Seminar in Neuroscience, 7, 75-90.

GUNDELFINGER E. D. (1992) How complex is the nicotinic receptor system in insects? Trends in Neurosci., 15, 206-211.

DENERIS E. S., CONNOLLY, J. ROGERS S. W. & DUVOISIN R. (1991) Trends Pharmacol. Sci., 12, 34-40.

P 13

THE cDNA CLONING AND THE CHARACTERIZATION OF A CHITINASE GENE OF THE SILKWORM, *BOMBYX MORI*

K. MIKITANI, J.-A GUSTAFSSON & M. KOBAYASHI

The endo-type chitinolytic enzyme chitinase is suggested to be induced by the ecdysteroid and play important roles in the insect moulting and metamorphosis process (1). So far, chitinase genes have been cloned from various organisms including prokaryotes, yeast, plants, and one insect species. Using the degenerated primers based on the reported chitinase amino acid sequence of *Manduca sexta* (2), and the cDNA template prepared from the pre-pupal integument of *Bombyx mori*, we obtained a partial cDNA of the homologue gene of *B. mori*. The 5'-upstream and 3'-downstream sequences of the cDNA were determined by 5'-RACE and 3'-RACE methods, respectively. The deduced amino acid sequence from the *B. mori* chitinase homologue gene showed 83% identity to the sequence of the *M. sexta* chitinase, and it was suggested to be composed of 545 amino acids. The hydropathy plots of the two proteins were highly similar. At the DNA level, the two genes showed 72% identity. In addition to this *M. sexta* chitinase cDNA homologue, the existence of mRNA encoding the second form of C-terminal truncated smaller enzyme of 426 amino acids was also indicated. Southern blot analysis of the *B. mori* genomic DNA with the *B. mori* chitinase gene cDNA probe showed several hybridizing bands. Furthermore, the partial cDNA sequences of the chitinase gene homologues from the three other species of the Lepidopteran insects were determined by the same RT-PCR approach. The deduced amino acid sequences showed high homology between *Agrius convolvuli* and *Manduca sexta* comparing to *Bombyx mori* or *Spodoptera litura*, and the relatively less homology in *Plutella xylostella* from other species.

KRAMER K. J. & KOGA D. Insect chitin: physical state, synthesis, degradation and metabolic regulation. Insect Biochem., 16, 851-877, 1986.

KRAMER K. J., CORPUZ L., CHOI H. K., MUTHUKURISHNAN S. Sequence of a cDNA and expression of the gene encoding epidermal and gut chitinases of *Manduca sexta*. Insect Biochem. Molec. Biol., 23, 691-701, 1993.

P 14

MECHANISMS OF ACTION OF THE INSECTICIDAL MANNOSE BINDING PLANT-LECTIN FROM SNOWDROP (GNA) ON THE TOMATO MOTH *LACANOBIA OLERACEA*

J. GATEHOUSE, J. A. G. GATEHOUSE, E. FITCHES

The mannose-binding lectin from snowdrop, GNA (*Galanthus nivalis* agglutinin), has toxic effects on a wide range of insects, and has been suggested as a protein which could be expressed in transgenic plants for protection against insect pests. The effects of GNA on a model lepidopteran pest *Lacanobia oleracea* (tomato moth) have been studied, both when fed in artificial diet and when expressed in transgenic potato plants (Fitches et al in press). Artificial diet bioassays have shown that GNA, when incorporated at 2% of dietary protein causes significant reduction in larval growth and development. Similar results have been obtained with bioassays on transgenic plants and plant material, although in the latter case low levels of expression of the protein led to the deleterious effects of GNA being reduced. Significant levels of protection of transgenic plants (measured by plant damage) were observed both in growth cabinet assays, and in the glasshouse. Analysis of diet consumption suggests that GNA inhibits nutrient uptake in *L. orelacea*, although the mechanism for this remains to be established. Several authors have suggested that, as is the case for mammals, effects are dependent on lectin binding to suitably glycosylated targets in the insect gut. Proteins that bind GNA *in vitro* have been identified in both whole gut tissue, and in brush border membrane vesicle preparations of *L. oleracea*. The lectin shows strong binding to only a small number of polypeptides. The major GNA binding protein, of approximately 100KDa has been purified using Hitrap Q Seppharose and Microsep ultrafilration. Blockage of the N-terminus of this protein has necessitated the use of enzymatic digests subjected to protein microsequencing. It is hoped that enough sequence information will be obtained to allow the design of oligonucleotides of sufficient length and limited redundancy to allow a *L. oleracea* gut tissue cDNA library to be screened.

Binding of dietary GNA to the gut cells in phytophagous lepidopteran insects would depend on the protein being resistant to proteolysis in the central gut compartment; analysis of faeces from insects fed GNA has shown that the protein can readily be detected in faeces, and no evidence of proteolytic degradation is apparent. In higher animals lectins are able to cross the gut and enter the circulatory system via endocytosis by epithelial cells. This transport mechanism facilitates the action of lectins as growth factors, and induces systemic effects. Similar transport of lectins into the haemolymph of insects has been suggested to occur. In this study, GNA has been identified by Western blotting in extracted haemolymph of *L. oleracea* larvae subsequent to feeding artificial diet containing the lectin (2% dietary protein). This provides evidence that the binding of GNA to gut surface proteins observed *in vitro* also occurs *in vivo*.

GNA can thus exert systemic effects in *L. oleracea*, which may affect development more directly than any effects on nutrient uptake.

P 15

INSECTICIDE TARGET GENES FROM *H. VIRESCENS* cDNA LIBRARIES: CLONING, SEQUENCING AND EXPRESSION STUDIES

W. R. BAUMBACH

Messenger RNAs from *Heliothis virescens* 3 day embryos and 4th instar larva head were used to construct cDNA libraries with about 10^7 members each and average insert size of about 2.0 kb. Insecticide target genes are being cloned from these libraries in preparation for functional expression in insect cells, in mammalian cells and in *Xenopus* oocytes. The first target gene is the insect voltage gated sodium channel (para). Polymerase chain reaction (PCR) was used to isolate the cDNA version of a previously cloned genomic fragment of the *H. virescens* para gene (Taylor *et al.*,). Although para cDNA fragments have been cloned by PCR from RNA representing each stage of development, cDNA clones have thus far not been isolated from the embryonic library. The region of this cDNA fragments, positions 4650 to 5540, displays about 80% homology to the drosophila para gene.

The second target gene is the tyramine (octopamine) receptor (Saudou et al.,). CDna encoding this receptor was found in high abundance in the embryonic cDNA library. The *H. virescens* version of this receptor is about 50% homologous with the drosophola version, with selected regions of higher (e. g. 66%) homology.

Additional work has been done with adult *H. virescens* head RNA. This RNA has proven difficult to isolate due to the high concentration of eye pigments, which in large part copurify with the RNA.

MFJ Taylor, DG Heckel, BM Brown, ME Kreitman and B. Black (1993) Linkage of pyrethroid insecticide resistance to a sodium channel locus in the tobacco budworm. Insect Biochem. Molec. Biol. 23: 763-775.

F. Saudou, N. Amlaiky, J-L. Plassat, E. Borrelli and R. Hen (1990) Cloning and characterization of a Drosophila tyramine receptor. EMBO J 9: 3611-3617.

P 16

DIFFERENTIALLY REGULATED INHIBITOR-SENSITIVE AND INSENSITIVE PROTEASE GENES FROM THE PHYTOPHAGOUS INSECT PEST, *HELICOVERPA ARMIGERA*, ARE MEMBERS OF COMPLEX MULTIGENE FAMILIES

D. P. BOWN, H. S. WILKINSON & J. A. GATEHOUSE

The boll worm or corn ear worm, *Helicoverpa armigera*, (Lepidoptera: Noctuidae) is a serious pest of many crop plants in tropical, subtropical and warm temperate regions of the world. Larvae utilise serine proteases (trypsin and chymotrypsin) to digest plant proteins in their alkaline midguts. As a defence mechanism, many plant species have developed proteinaceous protease inhibitors. *In vitro* assays confirm the reduction of proteolytic activity in larval gut extracts by such inhibitors –notably SKTI (soybean Kunitz trypsin inhibitor). However, when incorporated into artificial diet, effects on growth or mortality of *H. armigera* larvae fed this diet have proved variable and no reports of the effective use of SKTI in transgenic plants have appeared.

Recently, it has been shown that inhibitor-insensitive proteases are induced in various lepidopteran and coleopteran insect species exposed to inhibitors in their diets. The aim of this project is to establish the occurrence of inhibitor-insensitive proteases in *H. armigera*, to study the biochemistry and molecular biology of these proteases, and to investigate the mechanism(s) of their induction.

H. armigera larvae fed artificial diet containing SKTI for 7 days exhibit trypsin-like in activity *in vitro* assays which is not substantially reduced by the addition of SKTI to these assays. This inhibitor-insensitive activity is not induced by starvation or ingestion of a low protein diet. Therefore, lack of amino acid (the product of protein degradation) alone is insufficient to cause the production of these novel enzymes.

When visualised on polyacrylamide gels, activity of larval gut extracts can be seen to be due to multiple enzyme species with differential sensitivity to the inhibitors SKTI (trypsin inhibitor), chymostatin (chymotrypsin inhibitor) and TLCK (trypsin inhibitor). Distinct bands of activity can be seen in the extract from SKTI fed larvae when compared to that from control insects. The sizes of the novel species do not differ greatly from that of the enzymes present in control larval guts.

The inhibition profiles of the trypsin-like activities present in control fed and inhibitor fed insects are similar and confirm that both are due to serine proteases. The exceptions are the effects of the plant derived proteinaceous inhibitors SKTI, LBTI and CpTI (from soybean, lima bean and cowpea respectively). These all inhibit control gut extract to a greater extent than extract from larvae fed SKTI.

cDNA libraries were prepared from mRNA isolated from larval midguts taken from insects fed SKTI-containing or control diet. Many recombinants in both libraries were hybridised by a trypsin-encoding cDNA amplified from *Manduca sexta*. A sequencing screen was performed on 100 cDNAs picked at random from the library prepared from SKTI fed insects.

20% of the clones encoded amino acid sequences homologous to digestive proteases, demonstrating the high level of dedication of the midgut to protease production. Lipases represent the second largest homology group. No amylase encoding cDNAs were isolated, possibly due to an adaptation to the low starch/high sugar content of the diet. The third most abundant species were cDNAs encoding sequences with homology to EPV20, a 20kDa glycoprotein found in bovine milk. The function of the product of these cDNAs (designated HaGut20) within the larval gut is unknown.

The presence of a signal peptide suggests secretion and the product is likely to be an abundant component of total gut protein.

Of the 20cDNAs from the random screen (designated SR#) which encode digestive proteases, 19 encode sequences homologous to serine proteases and one encodes carboxypeptidase. This confirms the results of biochemical studies – that serine protease activity predominates in the larval gut. After complete sequencing, the cDNAs encoding serine proteases can be further subdivided into three classes. From the sequence of the predicted substrate binding site of the encoded enzyme, nine of these appear to encode chymotrypsins and seven encode trypsin-like proteases. The remaining three sequences with homology to serine proteases are diverged from the consensus for this family and although clearly still homologous, it is not possible to say whether they encode active enzymes.

All the digestive protease encoding cDNAs derive from multigene families. There are at least 28 genes encoding members of the serine protease superfamily in *H. armigera* and at least four encoding carboxypeptidases.

The effect of feeding SKTI (for 7 days) to *H. armigera* larvae on digestive protease mRNA levels is not straightforward. Although levels of all the mRNA species corresponding to the chymotrypsin encoding cDNAs tested increase as a result of SKTI ingestion compared to insects fed control diet, the level of increase is not uniform, ranging from two to twelve fold. The levels of trypsin encoding mRNAs detected show a differing response, from an increase up to five fold to a decrease of six fold in SKTI fed larvae depending on which cDNA is used as a probe.

Levels of carboxypeptidase encoding mRNAs tested increase as a result of SKTI ingestion whereas the divergent serine protease mRNAs both increase (SR65) and decrease (SR21) in abundance.

The adaptation of the insect digestive system can be seen to have already started by 24 hours after ingestion of SKTI. Levels of those protease encoding mRNAs in larval midguts which are affected, generally begin to vary between control and SKTI fed larvae by this time.

It is not yet possible to determine which of the individual cDNAs isolated encode SKTI insensitive activity, although up regulation of the corresponding mRNA as a result of SKTI ingestion is a likely indicator. Comparison of the encoded sequences around the region of contact between enzyme and inhibitor (the substrate binding pocket) shows that although the binding pocket itself remains constant, residues flanking this and forming the adjacent surface loops of the enzyme do show variability. This is to be expected as the enzyme has to retain its specific catalytic function (determined by the binding pocket) although the strength of binding to substrates / inhibitors is governed by the surrounding residues.

Expression of the individual cDNAs and biochemical and structural characterisation of their products should allow the nature of the inhibitor-insensitive enzyme to be elucidated.

Key references:

Bown D. P., Wilkinson H. S. and Gatehouse J. A. (1997) Differentially regulated inhibitor-sensitive and insensitive protease genes from phytophagous insect pest, *Helicoverpa armigera*, are members of complex multigene families. Insect Biochem. Mol. Biol., in press.

Broadway R. M. (1995) Are insects resistant to plant proteinase inhibitors? J. Insect Physiol. 41, 107-116.

Johnston K. A., Gatehouse J. A. & Anstee J. H. (1993) Effects of soybean protease inhibitors on the growth and development of larval *Helicoverpa armigera*. J. Insect Physiol. 39, 657-664.

Jongsma M. A. & Bolter C. (1997) Adaptation of insects to plant protease inhibitors. J. Insect Physiol., in press.

Laskowski M. Jr (1985) Protein inhibitors of serine proteases – mechanism and classification. In Nutritional and toxicological significance of enzyme inhibitors in foods (Edited by Friedman M.) ppl-17, Plenum Press, New York.

P 17

SYNERGISTIC EFFECT OF BmIFV AND *STREPTOCOCCUS*, *STAPHYLOCOCCUS* AND *SERRATIA MARCESCENS* IN SILKWORMS

V. SIVAPRASAD, T. SELVAKUMAR, B. NATARAJU & R. K. DATTA

"Flacherie" is a descriptive term used to describe a syndrome where the diseased larva appears flabby, feeble, weak and withered with dysenteric condition. This is caused by a number of bacterial and viral pathogens. A survey on the prevalence of flacherie in silkworm rearings in India indicated that *Bombyx mori* infectious flacherie virus (BmIFV) is usually associated with bacteria, *Streptococcus* sp., *Staphylococcus* sp. and *Serratia marcescens* infecting silkworms. In the present study, the synergistic effect of BmIFV and *Streptococcus*, *Staphylococcus* and *S. marcescens* in the causation of flacherie in silkworms was investigated. NB_{18} silkworms (out of II moult) were per orally inoculated with BmIFV and bacterial isolates (1×10^7 cells /ml) at different concentrations of virus ($IC_{50} \times 10$, IC_{50}, $IC_{50} \times 10^{-1}$ and $IC_{50} \times 10^{-2}$). The silkworms were reared at an alternate temperature cycle of 25° C and 30° C for 12 hours each upto 15 days of post-infection. The mortality due to flacherie was recorded daily after the confirmatory tests (light microscopy and DAC-ELISA). The synergistic relation was observed between BmIFV and low and medium pathogenic bacteria i.e., *Streptococcus*, *Staphylococcus* and *S. marcescens*. Silkworms inoculated with medium pathogenic *Streptococcus*, *Staphylococcus* and *S. marcescens* alone caused a mortality of 44.67, 9.83 and 30.66%, respectively. The percentage of mortality in silkworms inoculated with BmIFV and medium pathogenic *Streptococcus*, *Staphylococcus* and *S. marcescens* ranged from 50.00 – 84.67, 44.66 – 65.66 and 31.66 – 55.66, respectively. The results indicate that bacterial infection followed by virus leads to mortality as early as 5th day of post-infection. The order of synergism with BmIFV was *Streptococcus* > *Staphylococcus* > *S. marcescens*.

P 18

TWO NEW D-E-A-D BOX GENES EXPRESSED IN OVARIES OF THE MOTH, *PLODIA INTERPUNCTELLA*

O. P. PERERA & P. D. SHIRK

Degenerate PCR primers to the A- and B-ATP binding motifs of D-E-A-D box proteins were used to screen a library of *Plodia interpunctella* ovarian cDNA. Clones Piv2-6 and Piv2-17 of the PCR products contained the conserved ATP-A and ATP-B sequences that are common to RNA helicases. Full length cDNA clones isolated from the cDNa library showed these two RNA helicases have considerable regions of similarity with members of the D-E-A-D box protein family and contained most of the consensus motifs present in these proteins. The predicted amino acid sequence of Piv2-6 had 65% similarity with the maternally expressed DEAD5 locus of mouse. The predicted amino acid sequence of Piv2-17 had 42% similarity with the Scl9328.3 locus from *Sacromyces cervisiae* and 33% similarity with the ME31 locus in *Drosophila*. However, the sequences between the conserved motifs of both RNA helicases were highly variable. Northern analysis of RNA from ovaries, tesies and body walls (fat body, epidermis, muscle etc.) showed that the two helicases were highly expressed in the ovaries. Transcript localization and molecular characterization of genomic DNA clones are in progress.

P 19

cDNA CLONES OF FOLLICULAR EPITHELIUM YOLK POLYPEPTIDES OF *PLODIA INTERPUNCTELLA*

P. D. SHIRK, O. P. PERERA, M. HEMPHILL, R. BROZA & G. ZIMOWSKA

The follicular epithelium yolk protein (FEYP), in the Indianmeal moth, *Plodia interpunctella*, is composed of two subunits, YP2 and YP4 and is the major product of the follicular epithelial cells during vitellogenesis. A pharate adult female ovarian cDNA library was constructed in the Lambda ZapII expression vector. Polyclonal antiserum to YP2 and YP4 were used to screen the library. Five partial cDNA clones for YP2 were isolated from a screen of 5000 plaques. The DNA sequence for YP2 showed 100% identity for the 30 amino acid residues derived from the sequencing of the amino terminal of the mature YP2 polypeptide. The 5' region of the mRNA was then determined using 5' RACE. The predicted amino acid sequence from the YP2 cDNA had considerable similarity with Egg Specific Protein (ESP) from *Bombyx mori* and the partial sequence for YP2 from *Galleria mellonella*. These three polypeptides also showed similarity with vertebrate lipases and had a highly conserved lipid binding region. The 5' region of YP2 from Plodia showed considerable divergence from the sequences from both *Bombyx* and *Galleria* and suggested the insertion of a large fragment in this region of the YP2 gene.

No positive clones for YP4 were found in a screen of 10^5 plaques using the YP4 polyclonal antiserum. In order to obtain a cDNA clone, a PCR product was amplified from the Lambda ZAPII ovarian cDNA library. A degenerate forward PCR primer was designed based on six amino acids from the 30 amino acid residues identified in the amino terminal sequencing of the mature YP4. The reverse PCR primer was the T7 primer located in the Bluescript vector. The PCR product was cloned into pCRII and then sequenced. The 5' region of the YP4 gene was determined by 5' RACE. The predicted amino acid sequence from the YP4 cDNA showed considerable similarity with YP4 from *G. mellonella* and only limited similarity with spherulin 2a from the slime mold.

P 20

TRANSIENT EXPRESSION OF MODIFIED FUSION GENES IN *BOMBYX* SILK GLAND CELLS AFTER BIOLISTIC TRANSFORMATION

B. HORARD, E. JULIEN, P. NONY, A. GAREL & P. COUBLE

We have provided an *in vivo* assay using *Bombyx mori* silkglands by which functionning of cloned gene promoters can be efficiently and rapidly measured in an homologous system. This consists in introducing DNA coated tungsten particles into isolated silkglands by biolistic according to a modified protocol in which the DNA is maintained in a hydrate state. The treated organ is then grafted into the haemolymph cavity of a host larva before analysis of transient expression by *in situ* staining or dosage. In these reconstituted physiological conditions the expression is both promoter dependant and cell type specific. We describe here the expression of native Act3 and P25 promotors as well as mutated P25 promotors used to identify the target of regulatory elements such as SGFB and PSGF.

References:

The biolistic, P. COUBLE, 1997. Transgenic animals: Generation and use, L. M. Houdebine Edt, Harwood Academic, Chur 1997, part II section G, pp 209-213.

Bombyx gene promoter analysis in transplanted silk gland transformed by particle delivery system, B. Horard, A. Mangé, B. Pélissier and P. Couble, Insect Molecular Biology, (1994) 3, 261-265.

Differential binding of the *Bombyx* silk gland-specific factor SGFB to its target DNA sequence drives posterior-cell restricted expression, B. Horard, E. Julien, P. Nony, A. Garel and P. Couble, 1997, Molecular and Cellular Biology, 1752-1579.

Directeur Gérant : Dr H. BOUVIER **Dépôt légal : 1er trimestre 2001**