THE UNIVERSITY OF CALGARY

The Ecdysone-Induced Regulatory Cascade in the Silkmoth Ovary: The HR3 and E75 Genes

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

CALGARY, ALBERTA

SEPTEMBER, 1998

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ABSTRACT

In *Bombyx mori*, the hormone 20-hydroxyecdysone is required for oogenesis to occur. Therefore it is of interest to isolate ecdysone inducible genes. Four cDNA clones corresponding to two early response genes, previously identified in *Drosophila*, were isolated and characterized in *B. mori*. These are BmHR3A, and three isoforms (A-, C- and D-) of BmE75.

During oogenesis the temporal expression of BmHR3A is reciprocal to that of BmGATA (a transcriptional activator of late chorion genes) and parallels the expression of BmESP (egg specific protein). BmHR3A binds to an 11 bp sequence within the promoter of both the BmESP and BmGATA genes and may be a transcriptional activator of BmESP and/or repressor of BmGATA genes.

BmE75C and BmE75D are new isoforms, not previously identified in *Drosophila* or lepidopteran insects. Due to their temporal expression during oogenesis, BmE75C and BmE75D (which is an ovary specific factor) may regulate early chorion gene expression.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Kostas Iatrou for his guidance and support during the years of my master's program. I would like to thank Hong Qian for her assistance in sequencing the isolated HR3 clones. I also extend my sincere thanks to Dr. Luc Swevers for his intial screening of the Lambda cDNA vitellogenic library for E75 and for his assistance in the Northern hybridizations. I would also like to express my thanks to my committee members, Dr. Lashitew Gedamu and Dr. Jim McGhee for their helpful discussions and suggestions.

On a personal note I would like to thank my parents, Basiliki Eystathioy, Michael Stevens, and Sophia Sarkisian for their support during my master's program.

DEDICATION

To my parents

for their support through the years

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LIST OF ABBREVIATIONS

20E	20-hydroxyecdysone
A	Adenine
Amp	Ampicillin
ATP	Adenosine triphosphate
bp	Base pair
C	Cytidine
°C	degree centigrade
cDNA	Complementary DNA
cpm	Counts per minute
ddNTP	Dideoxyribonucleotide 5-triphosphate where N indicates adenosine, cytidine, guanosine, or thymidine
DNA	Deoxyribonucleic acid
Dnase	Deoxyribonuclease
dNTP	Deoxyribonucleoside 5-triphosphate where N represents adenosine, cytidine, guanosine or thymidine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
EcR	Ecdysone receptor
EcRE	Ecdysone response element

ESP	Egg specific protein
G	Guanine
HRE	Hormone response element
kb	1, 000 bases
L	Liter
M	Molar
ml	Milliliters
ORF	Open reading frame
pfu	Plaque forming units
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpm	Revolutions per minute
RAR	Retinoic acid receptor
ROR	RAR-related orphan receptor
RORE	ROR response element
RXR	retinoic X receptor
SDS	Sodium dodecyl sulfate
Sevag	Chloroform: isoamyl alcohol (24:1)
svp	seven-up
T	Thymine
tll	tailless
Tris	Tris-(hydroxylmethyl)-aminomethane

U	Uracil
USP	Ultraspiracle
vol	Volume
w/v	Weight/volume
%	Percent
µl	Microliters

INTRODUCTION

Information on *Bombyx mori*

Reasons for studying oogenesis in *Bombyx mori*

The lepidopteran silkmoth *Bombyx mori* is an excellent system to investigate the process of oogenesis for three important reasons. First, in the ovary all stages of ovarian development are present, which allows concurrent analysis of changes occuring during oogenesis, using material from only a single female. This provides a certain advantage by allowing one to examine time and tissue specific gene expression in a single ovary. Secondly, the information obtained from insects provides some insights into hormonally triggered processes occuring in higher vertebrates because of the high degree of conservation in the structure of steroid receptors and their regulatory mechanisms. Lastly, the information obtained could be used in the future for insect pest control by genetic means.

Focus of the lab

Our studies focus on the developmental program of follicular cell differentiation and aims at the isolation and characterization of regulatory factors that govern the transition from previtellogenesis to vitellogenesis and from vitellogenesis to choriogenesis.

Structure of the *B. mori* ovary

During pupation and pharate adult development, the ovary consists of four ovarioles, and each ovariole consists of a string of follicles. Each follicle is at a different

developmental time point within oogenesis along this alignment, with two hours of developmental distance separating each follicle from its neighbor (Swevers and Iatrou, 1992). Therefore all different stages of oogenesis are simultaneously present in one female pharate adult seven days after larval-pupal ecdysis. In early pupae (day 1), the first follicles enter vitellogenesis, a process which involves the accumulation of yolk proteins and the generation of the vitellin membrane. During vitellogenesis the female specific protein vitellogenin is produced by the fat body and incorporated in the oocyte as vitellin. Also during vitellogenesis, the follicular cells synthesize an egg specific protein (ESP), as the predominant yolk protein found in the egg (Sato and Yamashita, 1991).

Five days after larval-pupal ecdysis choriogenesis begins (Sato and Yamashita, 1991; Swevers and Iatrou, 1992), whereby chorion proteins are being synthesized to build the chorion or eggshell. The chorion is a complex structure created by over 100 genes (review by Goldsmith and Kafatos, 1984) which is of vital importance for the egg and the developing embryo. The chorion consists of over 100 different proteins which are products of six closely related gene families : A, B, CA, CB, HcA and HcB (Rodakis and Kafatos, 1982; Rodakis et al, 1982, 1984; Tsitilou et al, 1983; Iatrou et al, 1984; Lecanidou et al, 1986; Hibner et al, 1988).

A follicle is composed of three cell types, the oocyte and nurse cells, both derived from the germ line, and the follicular cells which surround the follicles and are of mesodermal origin (Kafatos et al, 1977). The follicular cells which form an epithelium surrounding the oocyte and nurse cells are responsible for transporting yolk protein precursors towards the oocyte and synthesizing the vitellin membrane during

vitellogenesis. During choriogenesis the follicular cells synthesize and secrete the eggshell or the chorion proteins which will encompass the egg.

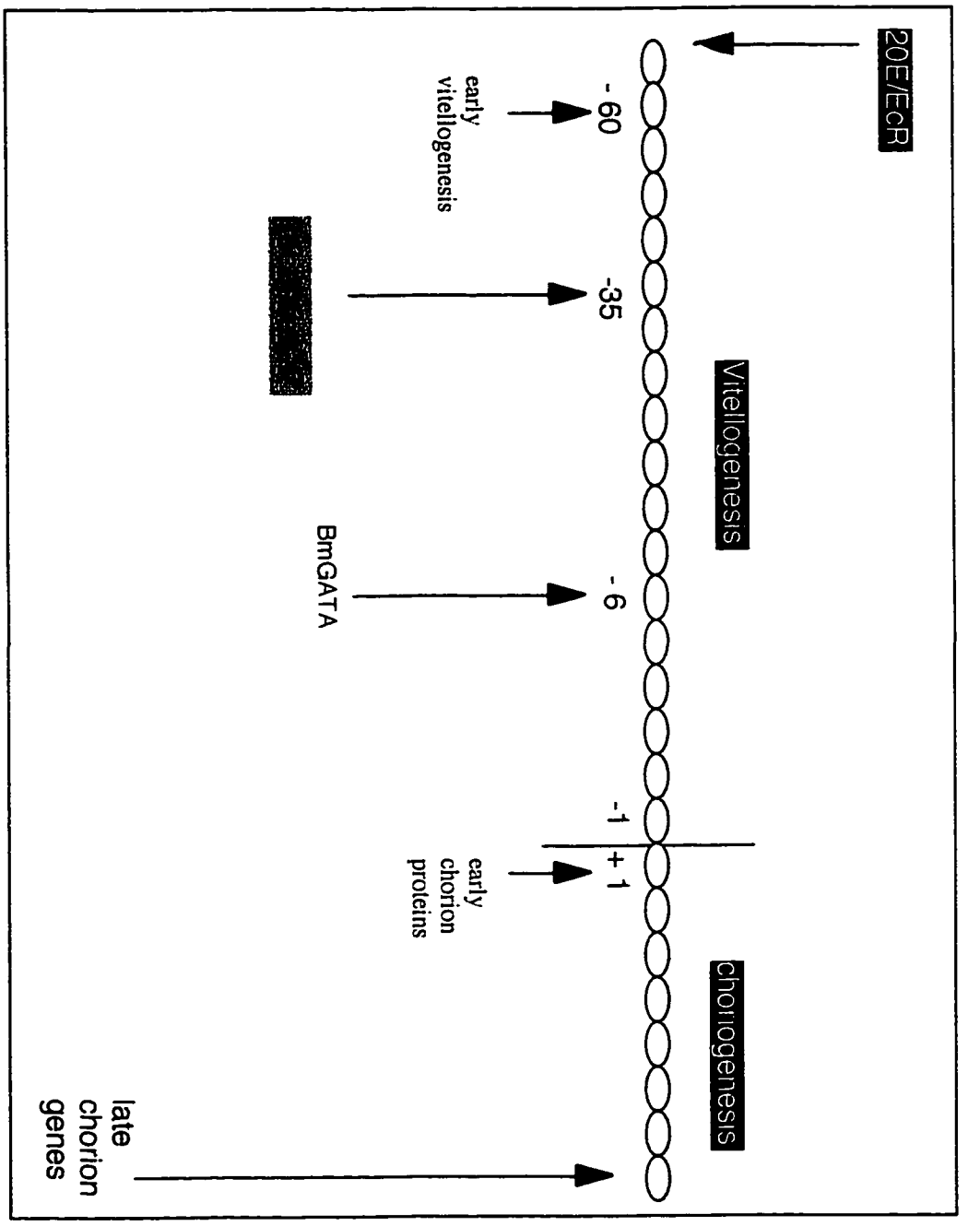
In *B. mori* a system has been developed to number the vitellogenic and choriogenic follicles i.e. the different stages of oogenesis. Negative numbers are assigned to the vitellogenic follicles, with stage -1 representing the follicle in the last stage of vitellogenesis and stage -60 representing a very immature follicle in the beginning of vitellogenesis. Choriogenic follicles are labeled with positive numbers; +1 represents the follicle in the first stage of choriogenesis and the higher the number the more mature the follicle is in the process of choriogenesis.

Some molecular events of oogenesis known in *B. mori*

The goal of our lab is to identify the molecular events occuring during oogenesis, between the initial trigger of ecdysone (ecdysone is required for the initiation of oogenesis; see discussion: The role of ecdysone during oogenesis) and the eventual formation of the chorion membrane.

To date a number of molecular events have been identified during oogenesis and are shown in a summary format in figure A. During oogenesis, approximately stage -35 of vitellogenesis, follicles acquire the competence to enter the program of choriogenesis in later stages. *In vitro* cultures show, that follicles at stage -35 and earlier are unable to synthesize early chorion proteins in later stages, while follicles taken later than stage -35 are able to enter choriogenesis and synthesize early chorion proteins in later stages (I. Dinnetz and K. Iatrou, unpublished).

Figure A. A schematic representation of follicles during the different stages of oogenesis, vitellogenesis and choriogenesis are indicated. Some of the molecular events known to occur during oogenesis (before this study was conducted) are marked underneath the follicles and the stages of these events are shown. 20E/EcR represents the ecdysone receptor complexed with ecdysone, the initial trigger for oogenesis to occur. The last stage of vitellogenesis and the first stage of choriogenesis are indicated by -1 and +1, respectively. The presence of receptor X at stage -35 represents the receptor thought to provide the follicles with competence to later enter choriogenesis, upon interaction with a ligand from the hemolymph.


20E/EcR
Vitellogenesis
Chorilogenesis
- 60
-35
- 6
-1
+ 1
early vitellogenesis
BmGATA
early chorion proteins
late chorion genes

Initially this stage had been placed at -17 (Swevers et al, 1992), however possibly due to a different strain and an aritificial diet this critical stage is now placed to an earlier point at stage -35 (I. Dinnetz and K. Iatrou, unpublished).

A hypothesis raised by these observations is that follicular cells at stages -35 and later express a receptor (receptor X) which interacts with a ligand in the circulating hemolymph. This interaction between receptor X and its ligand at stage -35 results in the autonomous capability of these follicles to later enter choriogenesis. It is thought that follicular cells at stages -36 and earlier do not express this receptor, and therefore follicles at those stages are unable to interact with the ligand in the hemolymph and therefore cannot differentiate autonomously to chorion-producing follicles *in vitro*. An initial hypothesis raised was that the ligand responsible for triggering receptor X was Bombyxin, an insulin-like peptide that is present in pupal hemolymph during mid-vitellogenesis. Receptors for Bombyxin had previously been shown to be present on the ovary (Fullbright et al, 1997). Hence a *Bombyx* insulin-like receptor was isolated. However one possibility is that this insulin-like receptor may not be receptor X, because during oogenesis there is no upregulation of mRNA expression at stage -35. Therefore to date the identity of this receptor X remains unknown. The regulatory cascade triggered by the interaction with the external factor present in the hemolymph and receptor X results in late vitellogenesis in the expression of BmGATA which is an essential transcriptional activator of late chorion genes. BmGATA is a member of the family of "GATA"transcription factors (Drevet et al, 1994) which becomes expressed at the end of vitellogenesis at stage -6. The functional activity of BmGATA is thought to hinge on the phosphorylation/dephosphorylation state

of the protein which affects the distribution of the protein between the nucleus and cytoplasm (Skeiky et al., 1994).

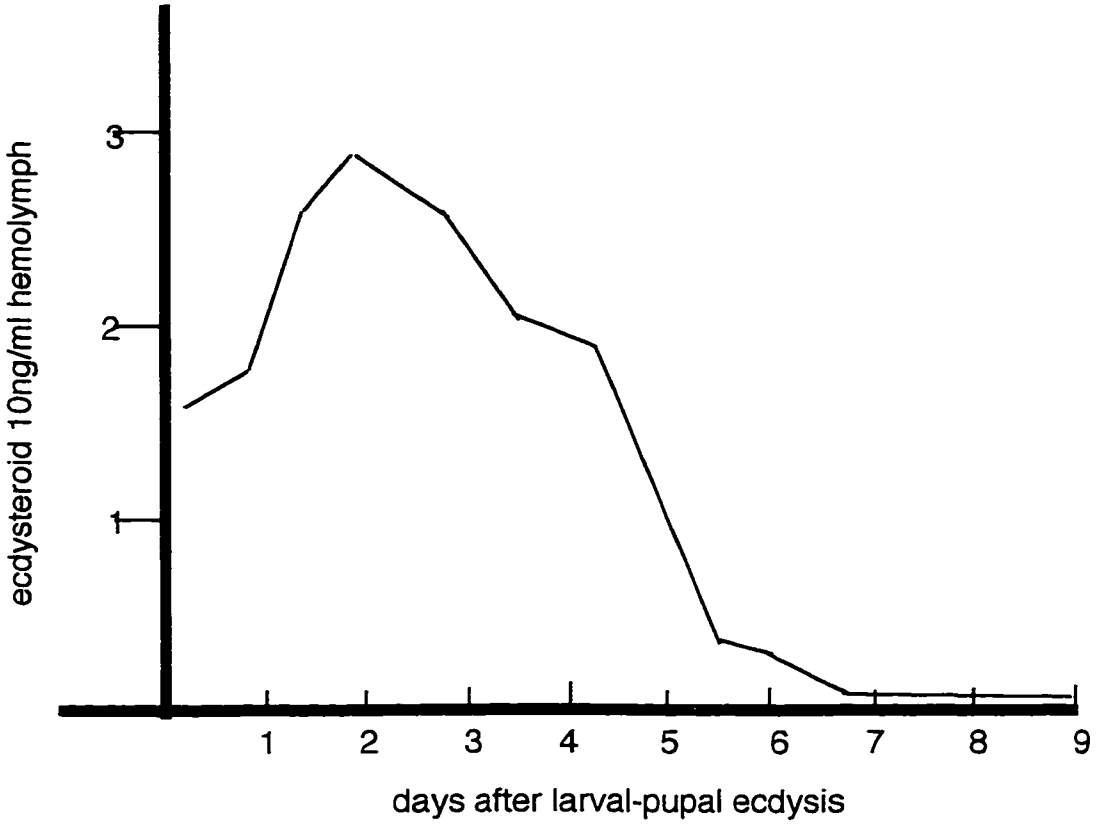
In summary, the hypothesis is that ecdysone induces a developmental program that results in the expression of receptor X at stage -35, which in turn triggers a program resulting in the expression of BmGATA and the initiation of choriogenesis.

The role of ecdysone during oogenesis

Ecdysone is the insect molting hormone important for the metamorphosis. In *Bombyx*, during larval development the ecdysone titer in the hemolymph is high in day 3 spinning larvae. Before pupation the hormone levels decline and then in day 2 pupae (two days after larval-pupal ecdysis) the hormone level is highest and subsequently declines to basal levels six days after the start of pupation (Tsuchida et al, 1987; Calvez et al, 1976) (see figure B for the titer of ecdysone in hemolymph). Ecdysone is then later synthesized by the ovary in day 5 pupae (five days after larval-pupal ecdysis) (Ohnishi and Chatani, 1977; Legay and Hirn, 1976).

A key factor required for the initiation of oogenesis during early pupation, is the converted form of ecdysone circulating in the hemolymph which is 20-hydroxyecdysone (20E). In *Bombyx*, ecdysteroids are synthesized and released from the prothoracic gland after stimulation by the prothoracicotropic hormone (PTTH) that is released from the brain (Nagasawa et al, 1984). Removal of the brain from the female silkworm (at the spinning larval or early pupal stage) results in arrested ovarian development which can be restored following injection of crude PTTH (Tsuchida et al, 1987). Isolated abdomens,

Figure B. The titer of ecdysteroids in the hemolymph of *Bombyx mori*. Days after larval pupal ecdysis are shown. (Taken from Tsuchida et al, 1987).


ecdysteroid 10ng/ml hemolymph
3
2
1
1
2
3
4
5
6
7
8
9
days after larval-pupal ecdysis

obtained by ligation which separates the thorax from the abdomen, prepared within 12 hours after pupation also shows arrested ovarian development which can be restored upon injection with 20E (Tsuchida et al, 1987). Due to the important role of ecdysone during oogenesis the ecdysone receptor (EcR) polypeptide has been cloned and characterized in *B. mori* (Swevers et al, 1995) (see below). Since the hormone 20E is necessary for ovarian development, it follows that 20E also triggers a regulatory cascade event in the follicular cells surrounding the oocyte. This cascade eventually results in the activation of chorion gene batteries whose products form the chorion membrane.

LITERATURE REVIEW

The study of the effect of ecdysone at the molecular level

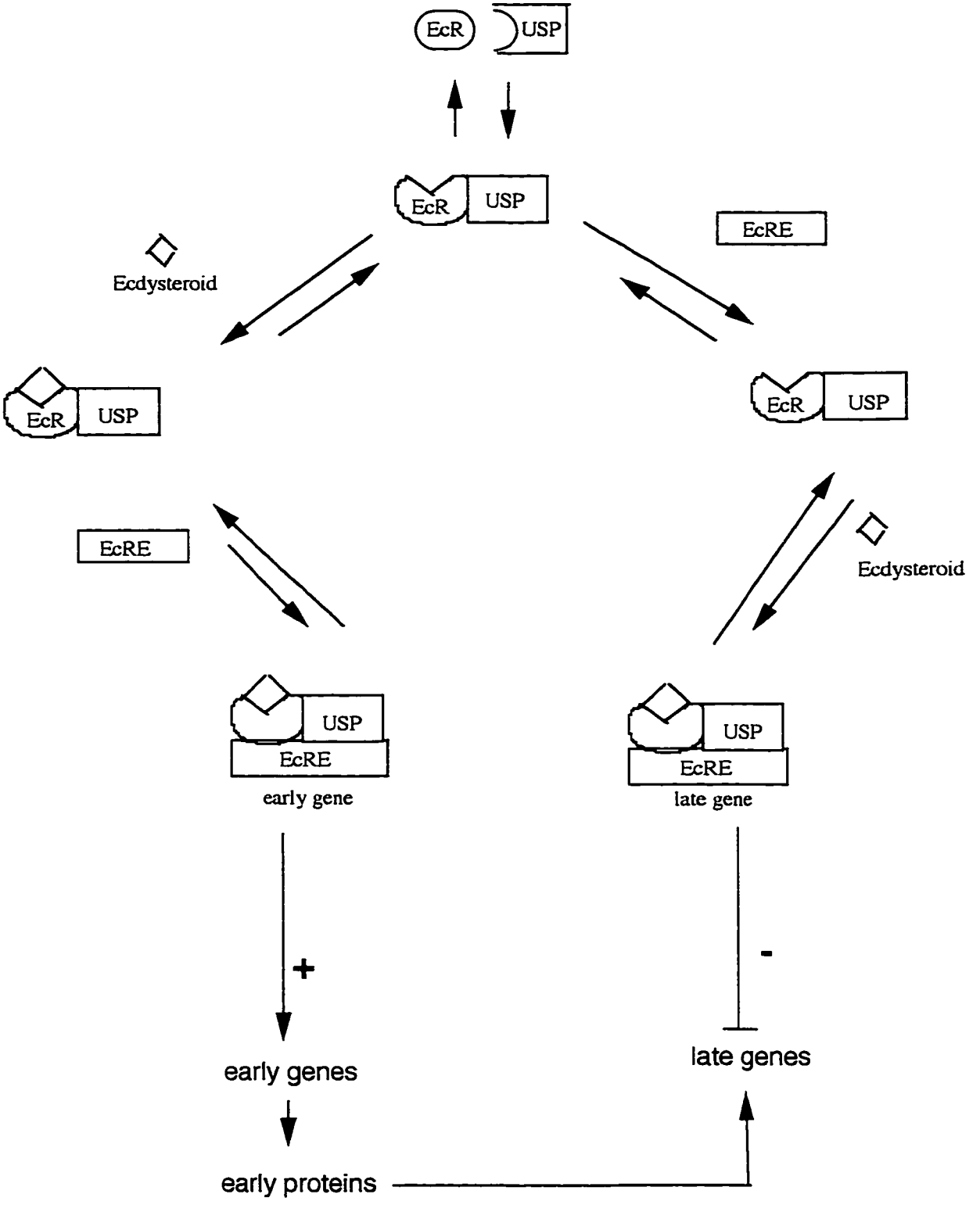
The question then arises as to how 20E triggers a molecular cascade that eventually results in the formation of the chorion by the follicular cells. The effects of ecdysone at the molecular level were initially studied in the larval salivary gland polytene chromosomes. Using the Dipteran *Chironomus tentans*, Clever and Karlson demonstrated that chromosomal puffing responses are triggered following injection of purified ecdysone (Clever and Karlson, 1960). Further experimentation done by Ashburner and his colleagues using cultured larval salivary glands of *Drosophila melanogaster* showed that following the addition of ecdysone to the culture medium, six major puffs were induced within minutes. After approximately four hours early puff formation was highest and then regressed (Ashburner, 1972). This was followed by the appearance of more than 100 late ecdysone-induced puffs (Ashburner, 1972). Interestingly, protein synthesis was required both for the regression of the early puffs and the formation of the late puffs, implying the importance of the early ecdysone-induced proteins in these events (Ashburner, 1974a). In addition a number of other key observations were made. First, when testing the effects of different ecdysone concentrations it was observed that the size of the early puffs varied over a broad range of ecdysone concentration (600 fold), whereas a more narrow response was seen for the late puffs (5-fold concentration range) (Ashburner, 1973). This suggests that ecdysone is not required as a sustained signal for late gene activation but rather functions as a trigger. Second, if ecdysone is removed in less than two hours after its initial administration no late puff formation is observed. Formation of early-late puffs

(62E, 78D, and 93D) is unaffected by ecdysone withdrawal, while late-late puffs are induced prematurely (63E, 71CE, and 82F) indicating late-late puffs are initially repressed by ecdysone (Ashburner, 1976).

All of these findings culminated in the Ashburner model (Fig C) explaining the effect of ecdysone on polytene chromosome puffing (Ashburner, 1974b). This model suggests that ecdysone complexed to its receptor activates "early" genes (residing in early puffs) and represses "late" genes (residing in late puffs). Subsequently, the products of the early genes repress their own expression while they activate late gene expression. It is believed that the products of the late genes are involved in defining the morphological (and physiological) properties of the different target tissues. Several "early response" genes have been cloned and in accordance with the Ashburner model they encode transcription factors.

The Ashburner model has been extended into the tissue coordination model (Burtis et al, 1990; Thummel et al, 1990a) because transcripts of the early response genes have been identified in many other larval and imaginal tissues of *Drosophila* larvae, indicating a broad role of these gene products in the ecdysone response. Different tissues are predicted to have different patterns of late gene expression because the late gene products define the morphological and functional properties of the target tissue. Therefore any target tissue of ecdysone is thought to display the molecular events exhibited by Ashburner's model. The ecdysone receptor was initially cloned in *Drosophila* and shown to form a complex with ecdysone (Koelle et al, 1991) .

Figure C. The data obtained from the *Drosophila* salivary gland chromosomal puffs culminated into Ashburners model which states that the ecdysone receptor complexed to USP, activates early response genes (following the trigger of ecdysone) whose protein products then activate transcription of late response genes. EcR-ecdysone receptor, USP-ultraspiracle, EcRE-ecdysone response element.


EcR
USP
EcR
USP
Ecdysteroid
EcRE
EcR
USP
EcR
USP
EcRE
Ecdysteroid
USP
EcRE
early gene
USP
EcRE
late gene
+
-
early genes
late genes
early proteins

It has since then been cloned in *Choristoneura tentans*, *Manduca sexta* and *Bombyx* (Imhof et al, 1993; Fujiwara et al, 1995; Swevers et al, 1995).

A few years after the initial isolation of EcR in *Drosophila*, the EcR was shown to heterodimerize with ultraspiracle (USP) (Yao et al, 1992; Yao et al, 1993; Thomas et al, 1993). USP, a member of the nuclear hormone receptor superfamily is the *Drosophila* homologue of the mammalian retinoid X receptor (RXR), RXR forms both homodimers and heterodimers with several vertebrate receptors to mediate hormonal response (Yu et al, 1991; Kliewer et al, 1992; Bugge et al, 1992). It has been shown that EcR (Koelle et al, 1991) and USP (Yao et al, 1992) co-localize on the ecdysone inducible puffs of the polytene chromosome (Yao et al, 1993). For high affinity hormone binding both EcR and USP are required (Yao et al, 1993). Moreover for high affinity DNA binding, and transactivation heterodimer formation between EcR and USP are also required (Yao et al, 1993; Thomas et al, 1993). Ultraspiracle, is the *Drosophila* homolog of the vertebrate RXR receptors, (Henrich et al, 1990; Oro et al., 1990) and has been isolated in *Bombyx* and *Choristoneura* (Tzertzinis et al, 1994; Perera et al, 1998).

In *Drosophila* there are three ecdysone receptor isoforms EcR-A, EcR-B1 and EcR-B2 (due to different promoters and alternative splicing), which differ in the N-terminus region but have a common DNA and hormone-binding domain (Talbot et al, 1993). The first EcR identified (Koelle et al, 1991) was the B-isoform. Talbot et al (1993) showed that all three isoforms of the EcR interact with USP. They further showed that different tissues targeted by 20E express different isoforms of the EcR. For example in larval tissues there is more EcR-B1 isoform than the other isoforms whereas in the

imaginal discs there is more EcR-A. This suggests how 20E induction can result in such varied responses.

In order to gain an understanding as to the role of these isoforms a series of experiments were done on EcR-B1 mutants where there was a loss of ecdysone inducible gene expression i.e. E74 and BR-C. The ability of the different isoforms to activate a set of genes was examined in the larval salivary gland of the EcR-B1 mutant. Transgenic expression of EcR-B1 resulted in full activation of primary response genes, whereas for EcR-B2 and EcR-A there was only partial and no activation, respectively (Bender et al, 1997). Some tissues were blocked in their metamorphic responses, i.e. larval midgut cells and midgut imaginal islands, which suggested that the EcR-B1 isoform was the predominant isoform and necessary for normal metamorphosis (Bender et al, 1997). It had previously been observed that E74A and E75A were expressed in imaginal discs (Segraves, 1988; Thummel et al, 1990), where the EcR-A isoform was the predominant isoform in these discs. This suggests EcR-A isoform may be solely responsible in activating the E74A and E75A genes in imaginal discs although EcR-A was unable to activate early response genes in salivary glands (Bender et al, 1997). Therefore different EcR isoforms may be responsible for activating ecdysone inducible genes in different tissues, which adds a level of complexity to the tissue coordination model.

It is interesting that the mechanism of 20E action appears to be conserved among insect species i.e. similar early response genes have been isolated in different species (see below). In *Drosophila*, as well as in the lepidopteran species *Bombyx*, *Manduca*, *Galleria*, and *Choristoneura* ecdysone inducible "early response" genes have been

identified in a variety of tissues such as imaginal discs, fat body, and salivary glands of *Drosophila* or silk glands of *Bombyx* . In *Drosophila*, genes corresponding to the early puffs were isolated based on cytogenetic location (Bender et al, 1983). Three of the six major early puffs have been analyzed at the molecular level, the Broad-Complex (BR-C) gene within the 2B5 locus (Dibello et al, 1991), the E74 gene within the 74EF locus (Burtis et al, 1990), and the E75 gene at position 75B (Feigl et al, 1989; Segraves and Hogness, 1990). All three have been shown to be induced by 20E (Thummel et al, 1990; Segraves and Hogness, 1990; Karim and Thummel, 1992) in addition to the genes HR3 and Ftz-F1 in *Drosophila* (Thummel, 1990; Koelle, 1992; Lavorgna, 1991); the corresponding lepidopteran homologues E75, HR3 and BR-C in *Manduca* (Segraves and Woldin, 1993; Palli et al, 1992; L.M. Riddiford unpublished results); HR3 and E75 in *Galleria* (Jindra, 1994b; Jindra, 1994a); and BmFtz-F1 in *Bombyx* (Sun et al, 1994) and HR3 and E75 in *Choristoneura* (Palli et al, 1996., Palli et al, 1997).

Overview of characteristics of nuclear receptors in the steroid hormone superfamily

Cellular response to steroid and thyroid hormones is mediated by nuclear receptors which bind to their ligands, translocate to the nucleus and regulate transcription. With the isolation of cDNA clones for several receptors in the 1980s came a greater understanding into the characteristics of nuclear receptors (Hollenberg et al, 1985). Analysis of the cloned nuclear receptors through sequence alignments, site-directed mutagenesis, deletion studies and through chimeric receptor molecules, revealed five functional domains (Kumar et al, 1986; 1987). The receptor genes for the steroid, thyroid and retinoid receptors were

cloned in vertebrates (Evans, 1988) and receptors bearing similar domains are grouped into the steroid/thyroid receptor superfamily. The receptors are characterized by the A/B region (N-terminal domain), C-region (DNA binding domain), D-region (hinge domain), E-region (ligand binding domain), and the F-region (C-terminal domain) (Kumar et al, 1986). The function associated with these domains is indicated in Table A. The highest degree of conservation among the receptors exists in the DNA and ligand binding domains. The DNA binding domain or C-region has two C_2C_2 zinc finger motifs. There are two important characteristics of the zinc finger that allow receptors to distinguish among the different hormone response elements. Firstly, the first zinc finger of the DNA binding domain has three amino acids which are responsible for sequence-specific DNA binding known as the P-box (proximal) (Fig D) (Green et al, 1988; Mader et al, 1989; Danielson et al, 1989). Secondly, there are five amino acids between the first two cysteines of the second zinc finger (these amino acids are known as the D-box), which are responsible for identifying the hormone response element (HRE) based on the number of nucleotides separating the HRE half sites (Umesono and Evans, 1989) (Fig D).

The "early response" genes identified in *Drosophila* and lepidopteran species

In Drosophila many members of the steroid hormone receptor superfamily are known (review: Jindra, 1994; Gronemeyer and Laudet, 1995). Some of these genes are listed below.

DHR3 (Koelle et al, 1992)

DHR39, also called Ftz-F1β (Ohno and Petkovich, 1993; Ayer et al, 1993)

Table A. The function for the five domains characteristic of nuclear hormone receptors which belong to the steroid hormone receptor superfamily.

Table A

Function	**Region / References**
Transactivation	A/B (Kumar et al, 1987)
DNA binding	C (Green et al, 1988)
Dimerization	E (Marks et al, 1992; Laudet et al, 1992)
Nuclear localization	D (Picard and Yamamoto, 1987)
Hormone binding	E (Kumar et al, 1986)

Figure D. The P and D-box important for sequence-specific DNA binding and identification of the hormone response element are indicated in the figure. The amino acids of the P-, and D- box are indicated by an asterisk. The amino acids of the P-box are located in the C-terminal region of the first zinc finger. The amino acids of the D-box are located on the N-terminal side of the second zinc finger. BmE75 is used in this figure to show the P and D- boxes.

N-terminal zinc finger
C-terminal zinc finger
P-box
D-box

E75 (Segraves and Hogness, 1990)

E78 (Stone and Thummel, 1993)

EcR (Koelle et al, 1991)

usp (Henrich et al, 1990)

tll (Pignoni et al, 1990)

svp (Mlodzik et al, 1990)

FTZ-F1 (Lavorgna, et al., 1993)

It is interesting that only the ecdysone receptor has a ligand whereas the remaining members are orphan receptors and their ligands remain unknown. It is possible that the missing ligands may be small intracellular regulators. Orphan receptors may be activated by peptides, or through phosphorylation, either activating or deactivating these receptors, or activation may be a secondary effect through signal transduction (O'Malley and Conneely, 1992).

Of key interest is the identification of target genes for the members of the steroid hormone receptor superfamily, because this is how an understanding can be gained into how the hormone signal can regulate the growth and development of the organism as well as the metamorphosis of the larvae into adult insects. A few target genes have been identified such as the BR-C proteins which bind to a few sites on the Sgs-4 glue gene *in vitro*, and some of these binding sites are required for Sgs-4 activity *in vivo* (von Kalm et al, 1994).

Ecdysone response elements

The first ecdysone response element identified was within the hsp27 promoter. (Riddihough and Pelham, 1987; Mestril et al, 1986). Since then, ecdysone response elements have been identified in other genes which are inducible by ecdysone such as Eip28/29 (Cherbas et al, 1991) and Fat body protein 1 (Fbp1) (Antoniewski et al, 1994). Sequence comparison of the identified ecdysone response elements resulted in a proposed consensus EcRE sequence (Cherbas et al, 1991) which is as follows : RG(G/T)TCAnTGA(C/A)CPy.

There are four categories into which nuclear receptors can be grouped depending on the type of HREs the receptors recognize and bind to. The first category consists of steroid hormone receptors, i.e glucocorticoid and estrogen receptors, that recognize HREs as inverted repeats of the consensus AGGAACA or AGGTCA, spaced by 3 bp, and bind to this sequence as homodimers (Klock et al, 1987; Martinez et al, 1987). The second category includes the thyroid hormone, vitamin D3, and retinoic acid receptors (RAR) that bind DNA as a heterodimer partner with the retinoid X receptor (RXR) (Yu et al, 1991; Bugge et al, 1992; Kliewer et al, 1992; Leid et al, 1992; Marks et al, 1992; Zhang et al, 1992). These receptors recognize direct repeats or everted repeats of the HRE half site (Naar et al, 1991; Umesono et al, 1991; Tini et al, 1993). For these receptors heterodimerization is important for DNA binding and transactivation (Glass et al, 1989; Zhang et al, 1992; Kliewer et al, 1992). The third category consists of nuclear receptors, which bind to direct repeats of HREs as homodimers, for example retinoid X receptor (RXR) (Mangelsdorf et al, 1991). The last category consists of orphan nuclear receptors

that bind as monomers to a single HRE half site preceded by a short AT-rich region (Lavorgna et al, 1991; Harding and Lazar et al, 1993).

Section 1 : Review of E75

E75 is an early ecdysone-inducible gene, first isolated in *Drosophila*, and localized to the 75B early puff on the polytene chromosomes in the salivary gland (Feigl et al, 1989; Segraves and Hogness, 1990). In *Drosophila* the E75 gene is approximately 50 kb in length and contains two overlapping transcription units, E75A and E75B, which have common 3'-terminal exons but different 5'-termini (Segraves and Hogness, 1990). The encoded proteins are members of the steroid receptor superfamily, because they contain sequences homologous to the DNA (C-region) and ligand binding domains (E-region) identified in all other members of the steroid receptor superfamily. E75A contains two putative zinc fingers encoded by exon A1 and exon 2 of the gene (characteristic of steroid hormone nuclear receptors; Segraves and Hogness, 1990). In contrast however, E75B contains only the second zinc finger of the DNA binding domain which is encoded by exon 2. Because the DNA binding domain of E75B is incomplete it is thought that E75B is unable to bind DNA. Homologs of E75A and E75B have also been identified in three lepidopteran species, i.e. *Galleria* (Jindra et al, 1994), *Manduca* (Segraves and Woldin, 1993) and *Choristoneura* (Palli et al, 1997). The different isoforms probably arise by differential splicing in addition to usage of two different promoters. In comparison to the vertebrate system the E75A DNA binding domain is highly similar to the vertebrate Rev-erb orphan receptor, which is also a member of the steroid hormone receptor superfamily

(Lazar et al, 1989). Rev-erb has a DNA binding domain with two C_2 C_2 type zinc fingers (Lazar et al, 1989).

Injection of 20E into isolated larval abdomens (last instar larvae) of *Galleria* results in a rapid induction of only the A-isoform of E75. In *Galleria* E75A and E75B both are encoded by mRNAs 2.6 kb long (Jindra et al,1994). Similarly in *Chorisotoneura* E75A mRNA is 2.6 kb long. In *Drosophila* on the other hand, each E75 isoform has two transcripts 4.9 and 5.7 kb (E75A) and 5.2 and 6.0 kb (E75B), that are generated by alternative polyadenylation site usage (Segraves and Hogness, 1990). It is likely that E75 functions in the ecdysteroid regulatory cascade possibly by either activating or repressing specific genes, but to date no target genes for E75 have been identified. However, immunocytological studies using antibodies specific for *Drosophila* E75A and E75B proteins have revealed a number of chromatin binding sites that correlate with those involved in the ecdysone puffing cascade, which includes both early (74EF, 75B) and late (76D, 77E and 78CD) ecdysone inducible puffs (Hill et al, 1993).

Section 2: Review of HR3

Hormone receptor 3 or HR3 has been isolated and cloned in *Drosophila* (Koelle et al, 1992), *Manduca* (Palli et al, 1992) *Galleria* (Jindra et al, 1994a), and *Choristoneura* (Palli et al, 1996, 1997) and belongs to the steroid hormone receptor superfamily. Interestingly, HR3 has also been found in the primitive nematode *Caenorhabditis elegans* (Kostrouch et al, 1995). In *Drosophila*, the HR3 gene maps to the early puff position 46F on the salivary gland polytene chromosomes (Ashburner et al, 1972). Three isoforms of

HR3 are detected in this species with a mRNA sizes of 5.5, 7 kb, and 9 kb during larval and pupal development. The 9 kb transcript is only observed during pupal development. In *Manduca* and *Galleria* two isoforms of HR3 are present, the A- and B- isoforms. In contrast there are four isoforms of HR3 present in *Choristoneura*, the A-, B-, C- and D- isoforms. In the different insects for the A- isoform the mRNA size ranges from 4.5 to 4.6 kb, and for the B- isoform the mRNA size ranges from 3.6 kb to 3.8 kb (Palli et al, 1992; Jindra et al, 1994b; Palli et al, 1996).

In *Manduca* it was clearly demonstrated that culturing fourth instar larval epidermis in the presence of 20E resulted in the induction of MHR3 transcription. The 4.5 kb mRNA was induced by ecdysone within three hours while no early induction of the 3.8 kb mRNA was observed (Palli et al,1992). In *Galleria*, injection of 20E into final instar larval abdomens resulted in the appearance of only the 4.6 kb mRNA within 1.5 hours (Jindra et al, 1994a). Therefore the B-isoform of HR3 (4.5 kb mRNA) is ecdysone inducible whereas the A-isoform is not induced by the hormone. In *Choristoneura* the A-isoform (4.5 kb) is induced within 3 hours (Palli et al, 1996) by the ecdysteroid agonist RH-5992 (Retnakaran et al, 1995), which mimics the action of 20E (Palli et al, 1996). Moreover, the two new isoforms of HR3 which have recently been isolated in *Choristoneura*, the C- and D-isoforms (Palli et al, 1997), are also induced by RH-5992 within 3 hours.

Interestingly, DHR3 has high similarities to the vertebrate RZR/ROR (ROR: RAR-related orphan receptor) family of nuclear orphan receptors (Giguere et al, 1994; Carlberg et al, 1994; Hirose et al, 1994). Members of this family include RZRα (Becker-

Andre et al, 1993), and three splice variants of RORα1, α2, and α3 (Giguere et al, 1994). All four receptors share identical DNA and ligand binding domains and vary in their amino-terminal domain. Unlike DHR3 which is an orphan receptor, however, RZRα and RORα1 have melatonin as a ligand which when complexed to these receptors increases DNA binding (Wiesenberg et al, 1995).

Finally, expression of DHR3 seems to effect the expression of many early response genes and may play an important role in facilitating the ecdysone response. DHR3 protein was observed to bind many ecdysone-induced puffs in the polytene chromosomes such as the Broad-Complex (BR-C) gene within the 2B5 locus, the E74 gene within the 74EF locus, and the E75 gene at position 75B, in addition to the E78 and βFTZ-F1 loci (Lam et al, 1997). DHR3 was shown to be a negative regulator of these genes: ectopic expression of DHR3 was shown to repress BR-C, E74A, E75A, and E78B transcription (Lam et al, 1997). The only exception to this was that DHR3 was shown to be a transcriptional activator of βFTZ-F1 expression (Lam et al, 1997).

Cross-talk among nuclear receptors facilitates the ecdysone response

Pairwise combinations have been carried out with EcR, USP, DHR3, DHR39, βFTZ-F1, E75A and E78A *in vitro* to find out if any of these factors can heterodimerize and bind to an oligonucleotide containing the HRE half site. The only heterodimer pair that formed was the EcR and USP (Horner et al, 1995).

However, the *Drosophila* hormone receptor 38 (DHR38) and its homolog *Bombyx* hormone receptor 38 (BmHR38) have been recently shown to interact with USP

(Sutherland et al, 1995). Interestingly DHR38 competes with USP for EcR *in vitro* therefore DHR38 may act by modulating the ecdysone response.

In *Drosophila* an ecdysone response element (direct element) has also been identified in the coding region of genes ng-1 and ng-2 (nested genes). The ng-1 and ng-2 genes are divergently transcribed in the 3C11 intermoult puff. Interestingly one ecdysone-inducible gene and member of the steroid hormone superfamily, DHR38 (Sutherland et al, 1995) interacts alone or in combination with USP to bind to this element. Competition may exist between the EcR and DHR38 to bind to this response element (Crispi et al, 1998) and this is the first report of a factor competing with the EcR to bind to an ecdysone response element. In addition two factors DHR39 and βFTZ-F1 whose expression is induced and repressed by ecdysone respectively, also bind to this element (Crispi et al, 1998). These observations support the hypothesis of cross talk occurring among the nuclear receptors through development (Crispi et al, 1998). This adds a level of complexity to the Ashburner model; the ecdysone-induced nuclear receptors may be interacting/competing with each other to facilitate the response to the hormone.

In *Drosophila* a functional link has been made between some of the nuclear receptors belonging to the steroid hormone receptor superfamily that exemplifies how these receptors may be working together to control transcriptional activity. This tight control may be imagined to regulate the delicate developmental process of metamorphosis occuring within the insect. Some exquisite results were obtained in 1997 by White et al, who showed that interactions are occuring between HR3 and E75 to control the expression of βFTZ-FI in *Drosophila*.

Although a transcriptional activator of βFTZ-F1 DHR3, acts as a repressor of βFTZ-F1 when dimerized with E75B (White et al, 1997). E75B suppresses activation by interrupting the activation function of DHR3, rather than by inhibiting the binding of DHR3 to DNA (White et al, 1997). The interaction between E75B and HR3 was shown experimentally through the use of a GST-DHR3 column; E75B was able to bind to DHR3 on the column (White et al, 1997). Interestingly the E75B/DHR3 heterodimer can also bind to the E75 gene suggesting a mechanism for early gene repression. Moreover, additional evidence for cross talk among nuclear receptors was demonstrated by the yeast two hybrid system which showed that DHR3 interacts with the EcR through their ligand binding domains (White et al, 1997) and, in addition represses ecdysone-inducibility in the *Drosophila* S2 cell line. Therefore, expression of the early genes may be regulated by DHR3 through the heterodimerization of DHR3 with the EcR.

Besides the three early puffs, 2B5, 74EF, and 75B, that are induced by ecdysone, another early puff 63F, also induced by ecdysone, has been recently characterized (Dibello et al, 1991; Burtis et al, 1990; Feigl et al, 1989; Segraves and Hogness, 1990). This puff contains two ecdysone-inducible genes, E63-1 and E63-2, whose expression seems to be restricted to larval salivary glands (Andres and Thummel, 1995). Interestingly the E63-1 gene encodes for a novel Ca^{2+} binding protein related to calmodulin (Andres and Thummel, 1995). This is interesting because it would bring together a mechanism that integrates signaling by ecdysone through calcium as a second messenger signaling pathway. This is another way in which the ecdysone-induced molecular cascade could be facilitated.

In addition other factors which may influence the ecdysone response are :

- limiting USP; the three isoforms of the EcR have a different affinity for USP.
- there may be other partners for the EcR which would change the function of the EcR.
- the EcR may complex with factors that are not members of the steroid receptor superfamily as has been the case for the glucocorticoid receptor (Jonat et al., 1990;Yang-Yen et al., 1990; Kutoh et al., 1992).

The goal of this Master's thesis

As already mentioned the response to 20E has been studied extensively in *Drosophila* and it has been shown that 20E exerts its effects by binding to a nuclear receptor complex consisting of two nuclear receptors, i.e. the ecdysone receptor EcR and the ultraspiracle gene product (USP) (Yao et al, 1993). In *B. mori*, BmEcR and BmCF1 are expressed in the follicular cells during oogenesis indicating that the ovary is a direct target for the hormone (Swevers et al, 1995). To understand how 20E induces ovarian development the genes that are induced by the 20E-bound EcR/USP complex need to be isolated and identified. Recently one gene in the ecdysone-regulated cascade was isolated and identified as the nuclear receptor BmHNF-4 (Swevers and Iatrou, 1998). However BmHNF-4 behaves as a late gene in this cascade, it is not induced immediately by the EcR complex but only after a delay following the addition of the hormone (Swevers and Iatrou, 1998).

In order to isolate candidate genes that are directly induced in the ovary by 20E at the beginning of pupation, we attempted to isolate silkworm homologs of the ecdysone-response genes identified in other insects. In *Drosophila* and a few Lepidoptera, early and early late genes have been identified which include the nuclear receptors E75 and HR3. My research focused on the isolation of the silkworm homologs of the early response genes HR3 and E75 which have been previously isolated in *Drosophila* as well as the Lepidopteran species *Manduca, Galleria* and *Choristoneura* (HR3: Koelle et al, 1992; Palli et al, 1992; Jindra et al, 1994; Palli et al, 1996 / E75: Segraves and Hogness, 1990; Segraves and Woldin, 1993; Jindra et al, 1994; Palli et al, 1997). It was suspected that these early response genes would be expressed during oogenesis (since the ovary is a target of 20E) and induced by the hormone 20E (as seen in other insects). Isolation of these cDNA clones was important because HR3 and E75 may play an important role in the beginning of oogenesis.

GENERAL MATERIALS AND METHODS

I. DNA PREPARATION

1) Phage preparation of Lambda gt11 in Y1090

An overnight culture of 500 ml of Y1090 grown in NZY medium (1 litre contains 10 g NZ amine, 5 g NaCl, 5 g bacto-yeast extract, 2 g $MgSO_4$-$7H_2O$) was inoculated with 10^5 PFU of Lambda gt11 phage. (In order to ensure efficient lysis, the phage titer was determined by plating phage at different dilutions and by spot titrations). This was placed at 37°C for 30 minutes and then transfered to a 250 ml flask containing 50 ml of NZY media. Once lysis was complete (which occurs in approximately 4-7hrs), 400 μl of chloroform was added to lyse the bacteria. The bacterial debris was spun at 2800xg for 5 minutes, and DNase I was added to the supernatant (10 μg/ml) for 30 minutes at 37°C. Three grams of NaCl and 5 g of polyethylene glycol (PEG) 8000 was added and shaken until it dissolved. This was placed overnight at 4°C. The phage was collected by centrifugation at 11,000xg for 10 minutes. The pellet was resuspended in 5 ml of phage buffer (20 mM Tris-HCl, pH 7.4; 100 mM NaCl; 10 mM $MgSO_4$). This was extracted successively with equal volumes of phenol pH 8.0, phenol/chloroform, and chloroform, and then precipitated with an equal volume of isopropanol and washed with 70% ethanol. The pellet was then dissolved in sterile water (usually in about 100 μl). RNase was then added to this in a final concentration of 25 μg/ml when performing digestion reactions.

2). Quick miniprep

A single transformed colony was used to inoculate 3 ml LB broth (1 litre contains: 10 g peptone, 5 g yeast extract, 10 g NaCl) containing 100 µg/ml of ampicillin and grown overnight at 37°C. One hundred microlitres of this bacterial culture was pelleted at 640xg and resuspended in 30 µl of STE (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and an equal volume of phenol:chloroform (1:1) added. This was then vortexed vigorously and centrifuged at for 2 min at 14, 000xg. The supernatant (approximately 15 µl) was mixed with 2.5 µl of 6X DNA dye (0.25% bromophenol blue, 0.25 % xylene cyanol FF, and 30 % (w/v) glycerol). This was then analyzed on a 1% agarose gel for supercoiled DNA. In such a gel, plasmid DNA with an insert migrates slower than plasmid DNA without an insert. Colonies exhibiting such properties were then analyzed by digesting miniprep DNA (see below) with appropriate enzymes.

3). Alkaline Lysis of Minipreps

From a 3 ml overnight culture, 1.5 ml was removed and centrifuged at 640xg for 5 minutes in order to collect the bacteria. The pellet was redissolved in 100 µl of solution 1 (GET: 50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8.0) and then incubated at room temperature for 5 minutes. To this, 200 µl of freshly prepared solution 2 (0.2 M NaOH, 1% SDS) was added and the mixture was placed on ice for 5 minutes. Next, 150 µl of potassium acetate pH 4.8 was added (potassium acetate was made by adding 60 ml of 5 M potassium acetate, 11.5 ml glacial acetic acid and 28.5 ml water; this solution is 3 M with respect to potassium and 5 M with respect to acetate) and placed on ice for 5 minutes.

This was centrifuged at 14,000xg for 5 minutes, and the supernatant was extracted with an equal volume of phenol/chloroform, and an equal volume of chloroform. The DNA was precipitated by adding two volumes of 95% ethanol, which was left at room temperature for 2 minutes and then centrifuged at 14, 000xg for 5 minutes. The DNA pellet was washed in 70% ethanol and centrifuged as before. The DNA pellet was dried and resuspended in 20 µl water. When digesting this DNA, RNase was added to a final concentration of 20 µg/ml.

4). Large scale Plasmid DNA preparation

Autoclaved TB broth was prepared (12 g peptone, 24 g Bacto-yeast extract, 4.0 ml glycerol, and water to a final volume of 900 ml) and to this 100ml of solution A (0.17M KH_2PO_4 and 0.72 M K_2HPO_4) and 100 µg/µl of ampicillin was added. A 3 ml overnight culture was added to 1 litre of TB broth, and grown overnight at 37°C. A bacterial pellet was collected by spinning at 2800xg for 15 minutes at 37°C. For every 250 ml of TB broth, the pellet was resuspended in 4 ml of ice cold solution 1 (50 mM glucose, 10 mM EDTA pH 8.0, 25 mM TrisCl pH 8.0) and 1ml of 10 µg/ml lysozyme was then added. This was mixed and then incubated on ice for 30 minutes. To this 10 ml of freshly made solution 2 (0.2 M NaOH, 1.0 % SDS), was added and this was then incubated on ice for 10 minutes. To this 7.5 ml of solution 3 (3 M sodium acetate pH 5.2) was added and this was then incubated on ice for 60 minutes. Following centrifugation for 30 minutes at 22, 000xg, the pellet was discarded and the supernatant was extracted in a 50 ml centrifuge tube with an equal volume of isopropanol, and placed at -20°C for 10-

30 minutes. The nucleic acid was collected by centrifugation at 11, 000xg for 15 minutes. The supernatant was discarded and the pellet was redissolved in 8 ml of solution 4 (0.1 M sodium acetate, 50 mM Tris-Cl pH 8.0). Sixteen milliliters of ethanol, was added and then placed at -20° C for 15-30 minutes and then centrifuged once again at 11,000xg for 15 minutes. The pellet obtained was dissolved in 3 ml of autoclaved water to which 3.5 g of cesium chloride, and 240 µl of ethidium bromide (10 mg/ml) was added and dissolved. Centrifugation was then performed at 11,000xg for 10 minutes at 4°C. The supernatant was added to a 3.9 ml poly-allomer "quick seal" tube using a syringe and needle. To collect the plasmid DNA, centrifugation was carried out at 100, 000 rpm from 5 hours to overnight in a TLN100 centrifuge. Once centrifugation was complete, a needle was used to pierce the top of the tube and with another needle and syringe the plasmid DNA band was extracted. To remove the ethidium bromide an equal amount of 1-butanol saturated with 4 M NaCl and 10mM EDTA (in this solution the butanol was the top layer) was added, until all the ethidium bromide was removed. To precipitate the DNA 2.5 volumes of 95% ethanol was added, and then centrifuged at 22, 000xg for 12 minutes to collect the DNA. The DNA was washed with 70% ethanol, and then dissolved in water.

5). Genomic DNA extraction

The tissue was homogenized in buffer A (20 mM Tris-HCl pH 7.9, 150 mM NaCl, 50 mM EDTA, 0.5% SDS, and 100 µg/ml proteinase K), and incubated at 37°C from two hours to overnight, and then extracted with an equal volume of phenol/chloroform/isoamyl (25:24:1). This was then centrifuged at 10,000xg for 5 minutes, and the DNA

precipitated with an equal volume of isopropanol. The DNA was then dissolved in TE (TE: 10mM Tris-HCl pH 8.0, 1 mM EDTA p.H 8.0) containing 50 µg of RNase for one hour. To this 0.2% SDS and 50 µg Proteinase K were added and this was then incubated at 37°C for 15-30 minutes. This was extracted twice with phenol/chloroform/isoamyl (25:24:1) and then extracted once with chloroform/isoamyl alcohol (24:1). The DNA was precipitated with 0.25 M ammonium acetate and 2.5 volumes of ethanol, overnight at -20°C or on dry ice. The DNA was centrifuged at 10,000xg for 5 minutes and washed with 70% ethanol. The DNA was then dissolved in water.

2. RADIOACTIVE LABELING OF DNA

1). Random Oligonucleotide labelling

Twenty five ng of the DNA fragment required to make the probe, was dissolved in 30 μl of water. The DNA was denatured by boiling for 5 minutes. Then 10 μl of 5x OLB mix (see below), 2 μl of 10 mg/ml BSA, 5 μl of ^{32}P dCTP, and 10 units of Klenow were added and left overnight in a lead container at room temperature. The DNA was precipitated with 10 μg yeast tRNA, 1/10 vol 3 M sodium acetate pH 5.2, and 2.5 vol 95% ethanol, and placed on dry ice for 10 minutes. This was centrifuged at 14,000xg for 15 minutes, washed with 70% ethanol,and then the pellet was dissolved in 50 μl of water. For counting 1 μl of the probe was spotted on a 3 mm glass filter, counted, and then the filter was washed with 10 ml of 5% TCA (ice cold), 10 ml 95% ethanol, dried and counted again to determine the efficiency of incorporation.

5x OLB mix: contains: solution A: solution B: solution C as 100:250:150

solutions: **Solution O**: 1.25 M Tris-HCl pH 8.0, 0.125 M $MgCl_2$

Solution A: 1ml solution O + 18 µl 2-mercaptoethanol + 5 µl dATP + 5 µl dTTP + 5 µl dGTP (note: each dNTP was previously dissolved in 3 mM Tris-HCl pH 7.0, 0.2 mM EDTA pH 7.0, at a concentration of 0.1 M).

Solution B: 2 M Hepes pH 6.6 adjusted with 4 M NaOH

Solution C: Random hexanucleotides evenly suspended in TE 7.0 (TE: 10mM Tris-HCl pH 7.0, 1 mM EDTA) at 90 OD/ml.

2). PCR probe

To prepare a radioactive probe by PCR, 1ng of template DNA along with 100 µmole of forward and reverse primers were utiiized. To this, 10x PCR buffer (500mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl pH 8.3, 0.02% gelatin, 1 mg/ml BSA), d(AGT)TPs to a final concentration of 25 µM, 50 µCi (a-P^{32})dCTP and 1 unit Taq polymerase was added in a total reaction volume of 10 µl. 20 µl of mineral oil was added on top of the 10 µl volume. (PCR conditions: 94°C/1min, 55°C/1min, 74°C/ 1min). Once the PCR reaction was finished, the mineral oil was removed and the DNA, was precipitated with 10 µg yeast carrier RNA, 1/10 volume of 3 M sodium acetate, 2.5 volumes of 95% ethanol, and placed on dry ice for about 30 minutes. The DNA pellet was collected by centrifugation at 14, 000xg for 12 minutes.

All the radioactive probes, were passed through a Sephadex G-50 column and precipitated twice, once overnight at -20°C and then the following day for 4 hours again

at -20°C.

3. ISOLATION OF DNA FROM AGAROSE GELS

1). Low melting agarose for isolation of DNA

A TAE agarose gel was prepared (1X TAE: 40 mM Tris-acetate, 10 mM EDTA; adjusted to pH 7.9 with glacial acetic acid). The DNA was separated in the gel and a well was cut underneath the DNA band of interest. In this empty well low melting agarose of 0.8% was added and allowed to solidify. The DNA band was run into the low melting agarose and then cut out. An Eppendorf tube was pre-weighed before adding the gel slice, then according to the weight of the agarose gel slice, an equal amount of TE (4 M NaCl, 10 mM EDTA)

(1 ml/1 g of gel) was added. This was incubated at 60-65°C for about 10 minutes. Then a phenol, phenol/chloroform, chloroform extraction was carried out using an equivalent amount to the TE buffer initially added. The DNA was precipitated with 1/10 volume 3 M sodium acetate and 2.5 volumes of 95% ethanol and placed at -20°C for 2 hours or on dry ice for 30 minutes. This was followed by centrifugation at 14,000xg for 12 minutes, and the DNA was further washed with 70% ethanol and dried.

2). Isolation of DNA by DEAE elution

After the DNA was well separated on the agarose gel, a piece of DEAE paper was placed below the DNA band of interest. A piece of DEAE paper was also placed above the DNA band of interest, in order to prevent contamination with other DNA. Once the

DNA was run onto the DEAE paper, the paper was removed from the gel and any remains of agarose were washed away with double distilled water. The DEAE paper was placed in an Eppendorf tube containing about 200 µl of high salt buffer (50 mM Tris-HCl pH 8.0, 1 M NaCl, 10 mM EDTA pH 8.0) and the tube was placed at 55°C for 30 minutes. The DNA was precipitated with 1/10 vol 3 M sodium acetate pH 5.2 and 2.5 volumes of 95% ethanol and precipitated on dry ice for more than 1 hour. The DNA was centrifuged at 14, 000xg for 12 minutes, and washed with 70% ethanol.

4. TRANSFER OF NUCLEIC ACID

1). Northern blot set up

Northern transfer was done using 3 mM NaOH as a buffer. The transfer was set up as shown in Appendix A. The transfer was done overnight, and then the membrane was baked at 80°C for 2 hours.

2). Southern blot transfer

Once the DNA was run on the gel, a picture was taken of the gel with a ruler beside the gel. The gel was then denatured by treating it for 30 minutes with a solution of NaOH and NaCl (87.66 g NaCl, 20 g NaOH in a 1 L solution), and then rinsed in the tray with double distilled water. The gel was placed in a neutralization solution containing NaCl and Tris base (87.66 g NaCl, 60.5 g Tris base in 1 L solution with the pH adjusted to 7.5) for 30 minutes. The transfer was set up as shown in Appendix B.

5. PREPARATION OF RNA

Ten to thirty ovarian follicles were lysed in 400-500 µl of lysis buffer (7 M urea, 2% SDS, 0.135 M NaCl, 0.01 M Tris pH 7.8, and 1 mM EDTA). The follicles were gently mixed and placed on ice for a few minutes. Two hundred µl of phenol/chloroform pH 6.7 (5:1) was added vortexed and centrifuged for 1 minute at 14,000xg. This was repeated until there was no longer an interphase (approximately 2-3 times). Sevag (chloroform 24: 1 isoamyl alcohol) extraction was done and the DNA was precipitated with an equal volume of isopropanol. The RNA was collected by centrifugation at 14,000xg for 10 minutes and was dissolved in freshly autoclaved water. Less than 1 µg of RNA per follicle was obtained.

1). Methylmercury gel

All glass plates, spacers and combs were treated with 2% Absolve (a soap that removes RNase) and then rinsed with autoclaved water or double distilled water. A 1% methylmercury agarose gel was prepared (0.22-0.24 g of agarose, 4 ml of 5x running buffer and 16 ml autoclaved water). The running buffer was composed of 50 mM boric acid, 5 mM disodium tetraborate and 10 mM sodium sulfate anhydrous, with no pH adjustments required. Once the agarose was dissolved, 200 µl of 1 M methylmercury (in the fume hood!), was added quickly and mixed. Once the gel had solidified, the comb was gently removed and the wells were cleaned with a razor blade. The gel was run in 1x running buffer (50 mM boric acid, 5 mM disodium tetraborate, 10 mM sodium sulfate) at 30 mA. All the material which had been in contact with methylmercury was rinsed with

0.5 M ammonium acetate. The gel was then stained with 100 ml of 0.5 M ammonium acetate containing 100 µl of ethidium bromide (1 mg/ml) for about 20 minutes. A photograph was then taken with a ruler next to the gel. To 5 µg of RNA, 4 µl of 6x dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glyerol) was added and water made up the remaining volume to 16 µl total volume. The RNA ladder was also prepared in the same way, and 1 µg of RNA ladder (Gibco BRL) was used. The samples were heated at 60°C for less than one minute, and then quenched on ice.

2). Formaldehyde gel

A 1.5% agarose gel was prepared with DEPC treated water and contained a final concentration of 1X MOPS buffer (0.02 M MOPS pH 8.0, 8 mM sodium acetate, 1 mM EDTA pH 8.0) . Once cooled to 60°C, formaldehyde was added to a concentration of 2.2 M. The formaldehyde gel was poured on a vertical casting gel apparatus. The loading buffer (67% formamide, 2.9 M formaldehyde, 0.7 X MOPS buffer, 0.1 mg/ml ethidium bromide and dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) , was added to the RNA in a volume ratio of 1 to 1. The running buffer for the gel was composed of 1 X MOPS buffer and 1 M formaldehyde. The gel was run at 50 volts which took approximately two hours. Once the gel had finished running it was soaked in 0.5 M ammonium acetate for 30 minutes in order to remove most of the formaldehyde. The RNA was tranfered onto Hybond N+ using 3 mM sodium hydroxide as a buffer.

6. PREPARING COMPETENT CELLS

In a 500 ml flask 100 ml of LB broth (1 litre contains: 10 g peptone, 5 g yeast extract, 10 g NaCl) was inoculated with 1 ml of a 3 ml overnight bacterial culture. This was grown until the OD_{600} read 0.4-0.6. Once this O.D was reached, the culture was placed on ice for 10 minutes and centrifuged at 4000 g for 10 minutes. The pellet was resuspended in a total of 50 ml of 0.1 M $MgCl_2$ and placed on ice for 20 minutes. This was centrifuged at 4000xg for 10 minutes and the pellet was resuspended in 5 ml of 0.1 M $CaCl_2$ and inoculated on ice for 1 hour. Sterile glycerol was added to a final concentration of 15% and placed into a -70°C freezer.

7. TRANSFORMATION OF CELLS

One third of the 10 µl ligation mixture was mixed gently with the competent cells, and incubated on ice for 30 minutes after which the cells were heat shocked at 42°C for 2-3 minutes. This was then left at room temperature for a few minutes and 1 ml of LB media was added and the tube was placed in a 37°C shaker for 1 hour. The cells were centrifuged at 640xg for 5 minutes. The supernatant was removed and the pellet was resuspended in 100 µl of LB broth, and spread onto an LB-AMP plate. For blue white selection the transformed cells treated with 40 µl of X-gal (20 mg/ml stock) and 4 µl of IPTG (200 mg/ml) stock were spread on an LB-AMP plate .

8. SCREENING LIBRARIES

1). Screening of the cDNA library

Eight large plates of NZY agar (1 litre contains 10 g NZ amine, 5 g NaCl, 5 g bacto-yeast extract, 2 g $MgSO_4$-$7H_2O$ with 15 g/l of bacto-agar) were plated with $2x10^4$ PFU of lambda gt11/plate. Specifically $8x10^4$ PFU were added to 2 ml of SM buffer (5.8 g NaCl, 2 g $MgSO_4$-$7H_20$, 50 ml 1M Tris-HCl pH 7.5, 10 ml 1% gelatin solution, for a 1 L solution) and this was added to 2 ml of overnight grown Y1090 bacteria (grown in NZY medium). This was divided into four tubes containing 1 ml each and 9 ml of top agarose was added, mixed and poured onto the NZY plate. For secondary screening plate 100-200 plaques/plate were plated.

2). Screening of the genomic library

Approximately 18,000 PFU of Charon 4A/plate were plated on NZCY-M plates (1 litre contains 10 g NZ amine, 5 g NaCl, 5 g bacto-yeast extract, 1 g casamino acids, 2 g $MgSO_4$-$7H_2O$ with 15 g/l of bacto-agar). Before lifting plaques of plates, the plates were placed at 4°C for at least 30 minutes. On every membrane a label was placed with a pencil. The membrane was placed on the plate for 1 minute for the first lift and 3 minutes for the second lift (two minutes were added for each successive lift). Each membrane was marked for orientation on the plate by making holes in the membrane with a syringe needle asymmetrical. Once the membranes were removed from the plate, the membranes were treated as follows. The membrane was placed (plaque side up) on a 3 MM filter paper soaked in 0.5 M NaOH for 2-3 minutes. The membranes were then soaked with 1M

Tris-HCl, pH 7.5 for 2-3 minutes. Following this the membranes were rinsed in 2X SSC (one liter of 20X SSC contains 175.3 g of sodium chloride and 88.2 g of sodium citrate) with gentle shaking for 30 minutes. The filters were air dried and oven baked for 2 hours at 80°C.

3). Spot titrations

A series of different dilutions of phage were prepared in SM buffer. (SM buffer: 0.1 M NaCl, 5 mM $MgSO_4$, 50 mM Tris-HCl pH 7.5, 0.01% gelatin). A certain dilution of phage was then added to 100 µl of overnight Y1090 culture (grown in NZY), and incubated at 37°C for 15 minutes. After this incubation period 4 ml of liquid top agarose was added, mixed and plated on a NZY plate. The plates were inverted and incubated at 37°C overnight.

9. DNA SEQUENCING

1). Preparing miniprep DNA for sequencing

Miniprep DNA was prepared by the alkaline lysis method, RNase (20 µg/ml final concentration) was added to this and placed at 37°C for 1 hour. A phenol/chloroform followed by a chloroform extraction was carried out and the DNA precipitated with 1/10 vol 3 M sodium acetate and 95% ethanol and dissolved in 20 µl total volume. Five µl of this was used for sequencing with the PE Applied Biosystems sequencing kit. A 10 µl wax pellet was then used to cover the sequencing reaction. Following the PCR reaction the DNA was precipitated using 1/10 volume 3 M sodium acetate and 95% ethanol,

placed on dry ice for 10 minutes followed by -20°C for 2 hours. This was then centrifuged at 14,000xg for 12 minutes and washed with 50 µl of 70% ethanol, dried under vacuum and sent for sequencing.

2). Manual dideoxy sequencing:

Five µg of DNA was denatured by adding 1/10 volume of 2 M NaOH, 2 mM EDTA pH 8.0 in a total final volume of 40 µl. This was incubated at room temperature for 10 minutes. The DNA was precipitated at -70°C for at least 30 minutes (or -20°C overnight) followed by centrifugation at 14,000xg for 15 minutes and then washed with 500 µl of 70% ethanol. The DNA was dissolved in 7 µl of ice cold water, 1 µl of 200 ng primer and 2 µl of annealing buffer (5X sequenase buffer-USB) were added. This was incubated at 65°C for 2 minutes and slowly cooled down to room temperature. The labelling reaction was set up as follows: 2 µl of labeling mix (7.5 µM dCTP, dGTP, dTTP), 1 µl of 100 mM DTT, 0.5 µl dATP-^{35}S and 2 µl of sequenase (diluted enzyme 1:8). This was placed for 5 minutes at room temperature (or for shorter periods of time if sequences were required close to the primer). To terminate the reaction, 3.5 µl of the labeling reaction was added to 2.5 µl of the appropriate ddNTP termination mixture (80 µM of the four dNTPs + 8 µM of one of the ddNTP), and incubated for 15 minutes at 37°C. The reaction was stopped by adding 4 µl of Stop solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue, and 0.05% xylene cyanol). The samples were boiled before loading on a 6% polyacrylamide gel.

3). Other sequencing

A DNA sequencing kit, dRhodamine terminator cycle sequencing ready reaction, was used (PE Applied Biosystems).

10. CLONING OF PCR FRAGMENTS

1). Phosphorylation of primers

Primers were phosphorylated in a 100 µl total reaction volume containing 10 µg of the primer, kinase buffer (NEB) to a final concentration of 1x, ATP to a final concentration of 1 mM and 30 units of T4 polynucleotide kinase. This was incubated at 37°C for 1 hour, which was followed by a phenol/chloroform, chloroform extraction and the DNA was then precipitated with 1/10 volume of 3 M sodium acetate pH 5.2, and 2.5 volumes of ethanol, overnight at -20°C.

Multiple PCR reactions with these phosphorylated primers were set up and in order to ensure that an adequate amount of DNA was obtained, multiple PCR reactions were set up. The PCR product was purified from the agarose gel followed by a phenol/chloroform, chloroform extraction. The DNA was precipitated and dissolved in 20 µl of water, from which 1 µl was used to quantify the amount of DNA.

The PCR product was made blunt ended in a 40 µl reaction volume which contained the following: 2 µg of PCR product, 1/10 of 10 mM dNTP, 1/10 of 10x NEB2 buffer, 1/10 of 10 mg/ml BSA, and 4.8 units of T4 DNA polymerase (USB). This was incubated at 37°C for 10 minutes, followed by a phenol / chloroform extraction and the DNA was precipitated as above. The DNA was dissolved in 10 µl of water and 1 µl was

used for quantification on an agarose gel.

To prepare the vector for blunt end ligation, pBS/SK+ was cut with SmaI (or EcoRV). For 5 µg of cut pBS/SK+, 5 units of calf intestinal phosphatase (CIP; Promega) was added and incubated at 37°C for 15 minutes. Another 5 units of CIP was again added and incubated for a further 45 minutes at 55°C. CIP was heat inactivated at 75°C for 10 minutes. Then a phenol/chloroform extraction and DNA precipitation was performed as described above. The DNA was redissolve in 10-20 µl water and 1 µl was used for quantification on an agarose gel.

A blunt ended ligation between the vector and insert was set up in a 10 µl final volume containing 100 ng PBS, 100 ng PCR fragment (3X molar excess), ligase buffer to a final concentration of 1x, ATP to a final concentration of 0.5 mM and 1 unit of T4 DNA ligase. This was incubated at room temperature for 4-6 hours, and 10 µl of ligation mix without DNA (buffer, ATP, and enzyme) was added. This was followed by an overnight incubation at room temperature.

11. REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION (RT-PCR)

A 2X RT mix was prepared as follows: 2 mM dNTPs, 0.2 mg/ml BSA, 0.02 M DTT, 2X RT-buffer (Gibco/BRL), 100 Units RT enzyme. Five µl of this was added to the following RNA mix : 1 µg RNA, 200 ng oligo dT, 5 units RNA guard. The total reaction was made up to a volume of 30 µl and then placed at 42°C for 60 minutes. Ten percent of this cDNA mix was then used in a PCR reaction (in a total final volume of 50 µl)

containing : 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.1% Triton X-100), 200 µM dNTP, 200 ng forward and reverse primer, 1 unit Tsg polymerase (Sangon). The PCR conditions were as follows 94°C/1 minute denaturation, 50°C/1 minute annealing, 74°C/1 minute extension, at 35 cycles with initial denaturation at 94°C for 4 minutes. Ten to twenty microlitres of the PCR reaction was then loaded on a 1 % agarose gel and analyzed.

12. LIGATION AND TRANSFORMATION

The ligation reaction was set up as follows : 50 ng vector, 3 molar excess insert, 1 mM ATP, 1X ligation buffer (Gibco BRL), 1 unit T4 DNA ligase (Gibco BRL), sterile water up to 10 µl. Conditions for the ligation reaction were 16°C and room temperature overnight, for sticky and blunt end ligations, respectively.

13. *In vitro* TRANSCRIPTION AND TRANSLATION

The plasmid DNA containing the HR3A insert was linearized, an aliquot run on an agarose gel to ensure the plasmid was linearized, and this was then phenol/chloroform extracted and precipitated. The reaction for *in vitro* transcription was set up as follows in a 100 µl reaction: 1X T7 transcription buffer (USB) 3 mM each of ATP, GTP, CTP, UTP, 0.7 units RNasin ribonuclease inhibitor, 1 unit T3 polymerase (sense was read from T3), and 3 µg of linearized DNA. This reaction was placed at 37°C for four hours. Two µl were kept separate in order to run on an agarose gel (see below). This was then extracted with phenol/chloroform (5:1) and chloroform, centrifuged for 2 minutes each

time at 14, 000xg. The DNA was precipitated with 1/10 volume sodium acetate, 2.5 volume of 95% ethanol at 14, 000xg for 10 minutes and then washed with 70% ethanol. The pellet was resuspended in 50 µl of sterile water. Two µl were loaded on a 1% agarose gel in addition to the 2 µl kept before the phenol/chloroform reaction, in order to determine if any DNA loss occurred. Ten µl of the *in vitro* transcription reaction containing an equivalent of 0.6 µg of DNA template was added to the following : 60 µl rabbit reticulocyte (Promega), 1.7 µl each of amino acids without methionine, amino acids without cysteine, and amino acids without leucine, 2.6 µl RNasin and water to a final volume of 86 µl.

CHAPTER 1

Cloning of the hormone nuclear receptor

HR3 and analysis of its expression

in the silkmoth ovary

ABSTRACT

The homolog of the A-isoform of hormone receptor 3 (DHR3) of *Drosophila*, was isolated from a cDNA library of silkmoth vitellogenic ovarian follicles. Three mRNA species encoding HR3 are observed in follicular cells during oogenesis, 3.5 kb, 4.5 kb, and 6 kb, which we denote as the A-, B-, C- isoforms, respectively. The A- and C- isoforms are not induced by ecdysone in developmentally arrested abdomens, however the B-isoform is strongly induced within four hours. During oogenesis, the expression pattern of BmHR3A is reciprocal to that of BmGATA, a transcriptional activator of late chorion genes, and coincides with expression of the BmESP gene which encodes for an egg specific protein. Bandshift experiments with a RORE-like element (RORE-elements; McBroom et al, 1995) isolated from the promoters of BmGATA and BmESP genes, shows binding with BmHR3A indicating that BmHR3A may be a negative and positive regulator of BmGATA and BmESP, respectively.

INTRODUCTION

To isolate candidate genes that are directly induced in the ovary by 20E at the beginning of pupation, silkworm homologs of the ecdysone-inducible genes identified in other insects were targeted. In *Drosophila* and a few lepidopteran insect species, 20E early and early-late response genes have been identified which include the nuclear receptors E75 and HR3. The goal was to isolate silkworm homologs of the early response gene HR3 which has been previously isolated in *Drosophila* as well as the lepidopteran species *Manduca, Galleria* and *Choristoneura* (Koelle et al, 1992; Palli et al, 1992; Jindra

et al, 1994; Palli et al, 1996). HR3 is a member of the steroid hormone receptor superfamily and its expression has been shown to be induced by 20E *in vitro*, and to follow the ecdysteroid titer during development *in vivo*.

In the ovary of *B. mori*, during pupation three isoforms of HR3 are observed. One of these isoforms has been isolated and designated BmHR3A. The isolation and initial characterization of this isoform is described. BmHR3A is expressed in ovarian follicles during early and middle vitellogenesis and behaves as a late gene which is not directly induced by 20E. Putative target genes for BmHR3A during oogenesis include those encoding the transcriptional activator BmGATA, and the egg-specific protein (ESP), a yolk protein produced by the follicular cells during vitellogenesis.

MATERIALS AND METHODS

Animals

The silkmoth strain used was obtained from the Forest Pest Management Institute. Sault Ste-Marie, Canada. Growing and rearing of larvae, pupae and moths were done as previously described (Swevers and Iatrou, 1992).

Ligation procedure

Day 3 spinning larvae were ligated between the thorax and abdomen region using silk thread. The head/thorax region was removed and the isolated abdomen was kept for 3 days at room temperature before any further experimental manipulation. Developmentally

arrested abdomens were injected with 10 µg of 20-hydroxyecdysone (previously dissolved in 5 µl of 50% ethanol), between the 5th and 6th abdominal segment. RNA was extracted from ovaries isolated at different time intervals after injection.

Nucleic acid extraction

Genomic DNA was isolated from silkgland tissue of 5th instar larvae as previously described (Drevet et al., 1994). RNA was prepared from follicular cells and tissues as previously described (Skeiky and Iatrou, 1991; Swevers and Iatrou, 1998).

Reverse transcription coupled polymerase chain reaction (RT-PCR)

Degenerate oligonucleotide primers were designed based on the DNA binding domain and hinge region of *Manduca* HR3 (Palli et al., 1991) and had the following sequences, DF1 (forward primer): 5' AT(A/C/T) CC(GATC) TG(TC) AA(GA) GT(GATC) TG(TC) GG 3', and DR2 (reverse primer): 5' AC (C/T)TC (G/A)TC (C/T)TC (C/T/A/G)AC (C/T)TT (C/T)TC 3' (Fig. 1.1). RT-PCR reactions were carried out as described before (Drevet et al., 1995) using total RNA from vitellogenic follicles as template. PCR amplifications were done for 30 cycles at 94°C/1 min, 55°C/ 1 min, 72°C/1 min. The 270 bp RT-PCR product (double underlined in Fig 1.1) obtained was cloned, sequenced and after conceptual translation, it was shown to have 100% amino acid identity to the corresponding region in *Drosophila*, *Manduca*, and *Galleria* HR3.

cDNA library screening

A Lambda gt11 cDNA library of follicular cells from vitellogenic follicles (Swevers et al., 1995) was screened with the 270 bp RT-PCR product (see above) under stringent conditions; 70°C hybridization and washes were carried out at this temperature. Three hybridizing clones were obtained and their inserts were subcloned, either as EcoRI fragments or as PCR products obtained by amplification using Lambda gt 11 forward and reverse primers (Invitrogen), into the EcoRI or EcoRV site, respectively of pBS/SK$^+$.

DNA sequence analysis

Sequence comparisons were performed using the GCG (Genetics Computer. Inc.) sequence analysis software package.

Filter hybridization

Southern blot analysis was carried out with 5 µg of genomic DNA digested with EcoRI, HindIII, and EcoRI/HindIII. The DNA was denatured in 0.5 M NaOH, 0.6 M NaCl and subsequently transferred onto Hybond-N$^+$ membranes (Amersham) using 10 x SSC (Drevet et al, 1994).

Northern blot analysis was carried out with 5 µg of total RNA separated on 1.5% agarose gels containing methyl mercury hydroxide or formaldehyde as a denaturing agent (Bailey and Davidson., 1975; Swevers et al., 1995). Hybridizations and washes were done at 65°C as described (Drevet et al., 1994). The RNA was tranferred onto Hybond N+ using 3mM NaOH.

For Southern hybridization a fragment, corresponding to the C-terminus and the 3' UTR of BmHR3A, generated by digestion of the BmHR3A clone with Bgl II and Hind III (in pBS/SK+), was used as a probe.

For Northern hybridizations, the following DNA fragments were used as probes: (1) a PCR fragment, corresponding to the 5' UTR and N-terminus of BmHR3A, generated by primers FP1: 5'TACGCGCCGAAACTTTCA 3' and RP2: 5' CTCCAGCTTGCTCACAGA 3' (Fig. 1.1), (2) a 1.5 kb PstI-NotI fragment, encompassing the ORF of BmHR3A from the DNA-binding domain until the C-terminus including the 3' UTR; (3) a 1.9 Kb NotI fragment corresponding to the complete egg-specific (ESP) cDNA (Sato and Yamashita, 1991); (4) two PstI fragments corresponding early genes, 6A2 and 6C11 (Eickbush et al, 1985; Lecanidou et al, 1986; Swevers et al, 1995); and (5) a 0.8 Kb XhoI-PstI actin gene fragment (Mounier and Prudhomme, 1986).

Gel retardation assays

The pBS/SK+ subclone containing BmHR3A was linearized with Sal I and *in vitro* transcribed using T3 RNA polymerase (Pharmacia). The transcribed RNA was then translated in rabbit reticulocyte lysate (Promega) using the manufacturer's protocols. Gel mobility shift assays were carried out as previously described (Swevers et al., 1996). Double-stranded oligonucleotide probes used in the bandshift assays were derived from the BmGATA promoter (-588 to -577 nt upstream of the first transcription start site; Lunke and Iatrou, unpublished), and the BmESP promoter (-698 to -687 in Sato and Yamashita, 1991). They contained sequences that were highly similar to monomer nuclear

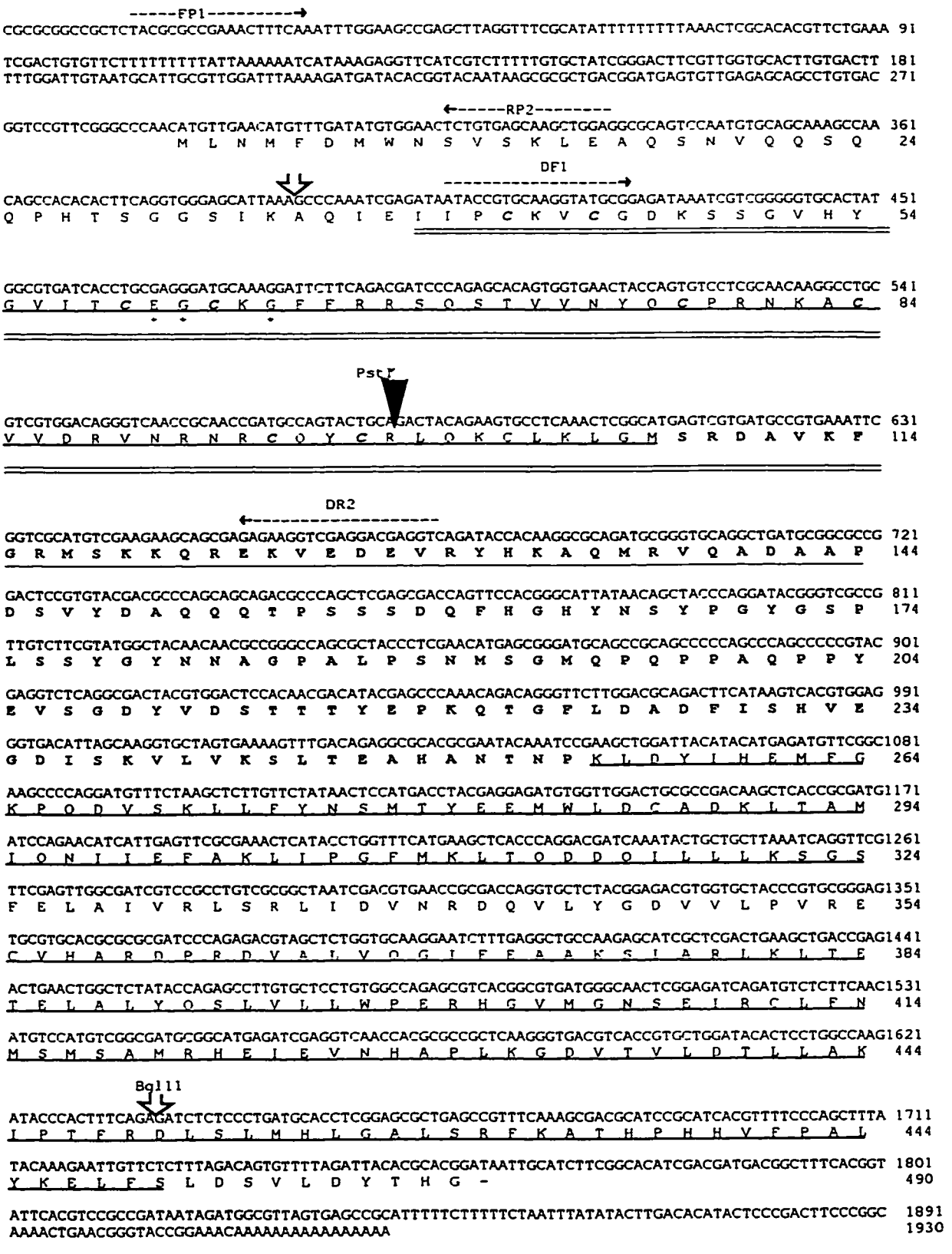
receptor response elements known as ROREs (Giguere et al, 1994; Korner et al , 1995; Fig 1.7).

RESULTS

Characterization of cDNA clones encoding BmHR3A

In order to isolate the homolog of HR3 in *B. mori*, RT reactions were carried out using total RNA from vitellogenic follicles as template. Degenerate primers based on the cDNA sequence of *Manduca* HR3 were used to amplify a 270 bp fragment corresponding to the DNA binding domain and hinge region of *Bombyx* HR3 (Fig 1.1). Cloning and sequencing of this RT-PCR product revealed that there was 100% amino acid identity in the DNA binding and hinge region when compared to corresponding regions of *Manduca*, *Galleria* and *Choristoneura* HR3. This 270 bp RT-PCR product was then used to screen 80,000 PFUs of a vitellogenic cDNA library at high stringency hybridization conditions. Three hybridizing clones were isolated, two contained an identical NotI insert of 1.9 kb and the third one contained a 1.2 kb insert truncated at its 5' end at the DNA binding domain. The 1.9 kb NotI fragment contains a long open reading frame (ORF) that has high amino acid identity with *Manduca*, *Galleria*, *Choristoneura*, and *Drosophila* HR3 (Fig 1.1; Table 1.1). In comparison to the lepidopteran receptors there is a high amino acid sequence identity in the DNA binding domain (100%), the hinge region (71-85%), the ligand binding domain (85-95%) and the C-terminus (F-region) (78-100%) (Table 1.1). In contrast however, the N-terminus of *Bombyx* HR3 shows only low similarity to the lepidopteran receptors (only 16%). *Drosophila* HR3 and the lepidopteran receptors

Figure 1.1: Nucleotide and amino acid sequence of BmHR3A. The five domains characteristic of the nuclear hormone receptor superfamily are highlighted in the figure as follows: the DNA binding domain and ligand binding domain are underlined, and the hinge region is indicated in bold. For the DNA binding domain the cysteine residues of the zinc finger are shown in bold italics. The residues for the DNA half-site recognition P-box are indicated by asterisks. The amino acid residues specific to the A-isoform are separated from the common region with an arrowhead. The primers FP1 and RP2 were used for the generating of a PCR probe that was used in the Northern hybridization reactions. The primers DF1 and DF2 were the initial degenerate primers used in the RT-PCR reaction that resulted in the 270 bp HR3 fragment which was used to screen the library (see Materials and Methods). The PstI site is indicated with a solid black arrow; restriction digestion with PstI and, NotI in pBS/SK+, was used to make the common probe. The BglII site in the BmHR3A cDNA clone is indicated with a white arrow; this restriction digest (BglII and HindIII in pBS/SK+) was used to make the probe for the Southern blot hybridization.


-----FP1---------→
←------RP2--------
DF1
----------→
PstI
DR2
←--------------------
Bgl11

overall, have similar degrees of sequence identity to *Bombyx* HR3 (see Table 1.1). It is interesting however that in the N-terminal region *Drosophila* has a higher amino acid identity (60%) than the lepidopteran receptors (15-16%).

Based on the sequence of the N-terminus and the size of its mRNA (see below) the isoform that was isolated corresponds to the A-isoform of HR3 (BmHR3A; see Palli et al, 1997 for discussion of isoforms). The BmHR3A protein encompasses 490 amino acids predicting a polypeptide of molecular weight of 55.1 kDa. The ORF is preceded by a 5' UTR of 278 bp. The methionine in figure 1.1 corresponds to the translation start site because it is preceded immediately by a Kozak consensus site "CAAC" and, further upstream in the 5' UTR, by translational stop codons in all three reading frames. The ORF is followed by a 3' UTR of 168 bp in which there is no canonical polyadenylation site.

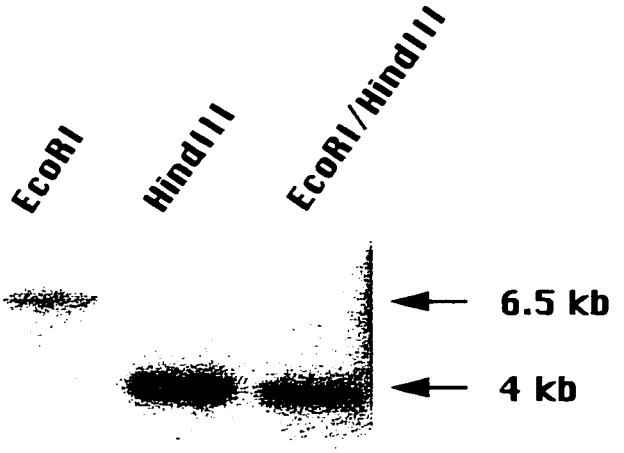
BmHR3 is encoded by a single copy gene

In order to determine the number of genes encoding BmHR3, genomic DNA was isolated from silk glands of 5th instar larvae and subjected to Southern blot analysis. Hybridization was carried out using a short fragment of the BmHR3 cDNA at the 3' end (BglII/HindIII restriction fragment, see Fig 1.1) as a probe. From the restriction digests, a single hybridizing band was obtained (Fig. 1.2) indicating that BmHR3 is encoded by a single copy gene. However this result would have to be verified by using probes specific to other regions of the BmHR3A.

Table 1.1: Comparison of *Bombyx* HR3A with the A-isoform of *Drosophila* (Koelle et al, 1992) *Manduca*, *Galleria*, *Choristoneura* (Palli et al, 1992; Jindra et al, 1994; Palli et al, 1996), in addition to RORα (Giguere et al, 1994) in the 5 different regions which are characteristic to nuclear receptors: N-terminus, DNA binding domain, hinge region, ligand domain and C-terminus. A comparison of total amino acid identity is also provided.

Length / percent amino acid identity						
Insect	N-terminal region	DNA binding domain	Hinge region	Ligand binding domain	C-terminal	total
Choristoneura	101/16%	66 /100%	144 /71%	227 /85%	9 /78%	547/73%
Manduca	103/16%	66 /100%	143 /85%	227 /94%	10/100%	549/78%
Galleria	107/15%	66 /100%	148 /85%	227 /90%	10/100%	558/76%
Drosophila	50 /60%	66 /100%	138 /64%	226 /69%	8 /63%	488/71%
RORα	73 / 12%	66 / 71%	133 / 11%	235 / 35%	17 / 37%	524 / 30%

Figure 1.2: Southern blot hybridization of genomic DNA isolated from the salivary gland of fifth instar larvae and digested with EcoRI, HindIII, and EcoRI/HindIII. Hybridization was carried out using the BglII/HindIII fragment of BmHR3A for a probe (see Fig 1.1). The exposure time was seven days.


EcoRI
HindIII
EcoRI/HindIII
6.5 kb
4 kb

Induction of BmHR3 by 20-hydroxyecdysone (20E)

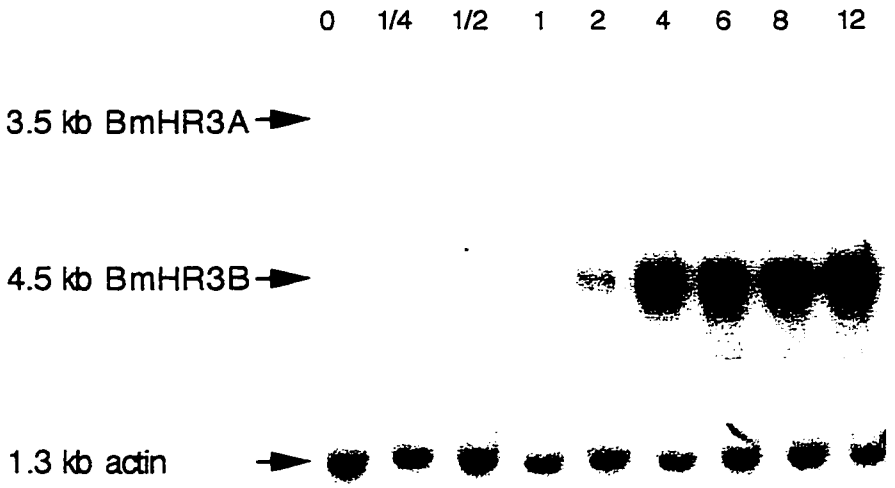
In cultured larval tissues of *Drosophila* and *Manduca*, HR3 mRNA is induced within 1 to 3 hours following exposure to 20E (Palli et al, 1992., Horner et al, 1995., Huet et al, 1995). To examine whether a similar situation occurs in the *Bombyx* ovary, the expression of HR3 was determined in developmentally arrested pupal abdomens. At different time points after injection of 20E, samples were collected and the levels of mRNA expression were analyzed by Northern blot analysis.

Northern blot analysis using a probe specific to the N-terminus of BmHR3A, showed that BmHR3A with a mRNA size of 3.5 kb (Fig. 1.3; see also Fig. 1.4) is not induced by 20E. However, when a probe corresponding to all the regions of HR3, except the N-terminus, was used as a probe, a mRNA species of 4.5 kb was induced between 2 and 4 hours following hormone injection (Fig. 1.3). In *Manduca* and *Galleria* a mRNA of similar size is observed in the epidermal tissue during pupation, which is strongly induced by 20E within 3 and 1.5 hours, respectively (Palli et al, 1992; Jindra et al, 1994). In the spruce budworm *Choristoneura*, of the four isoforms observed, HR3B has been isolated which has a similar size and induction pattern to the unknown isoform observed in *Bombyx*. Therefore it is likely that the 4.5 kb mRNA observed in *Bombyx* ovarian tissue is the B-isoform of HR3 (Palli et al, 1996; 1997).

Expression of BmHR3 in the ovary during metamorphosis

The expression pattern of the BmHR3A isoforms in response to the changing ecdysone titer during pupal and pharate adult development, was examined by Northern blot analysis of ovarian tissue RNA taken from larvae and pupae at different stages of

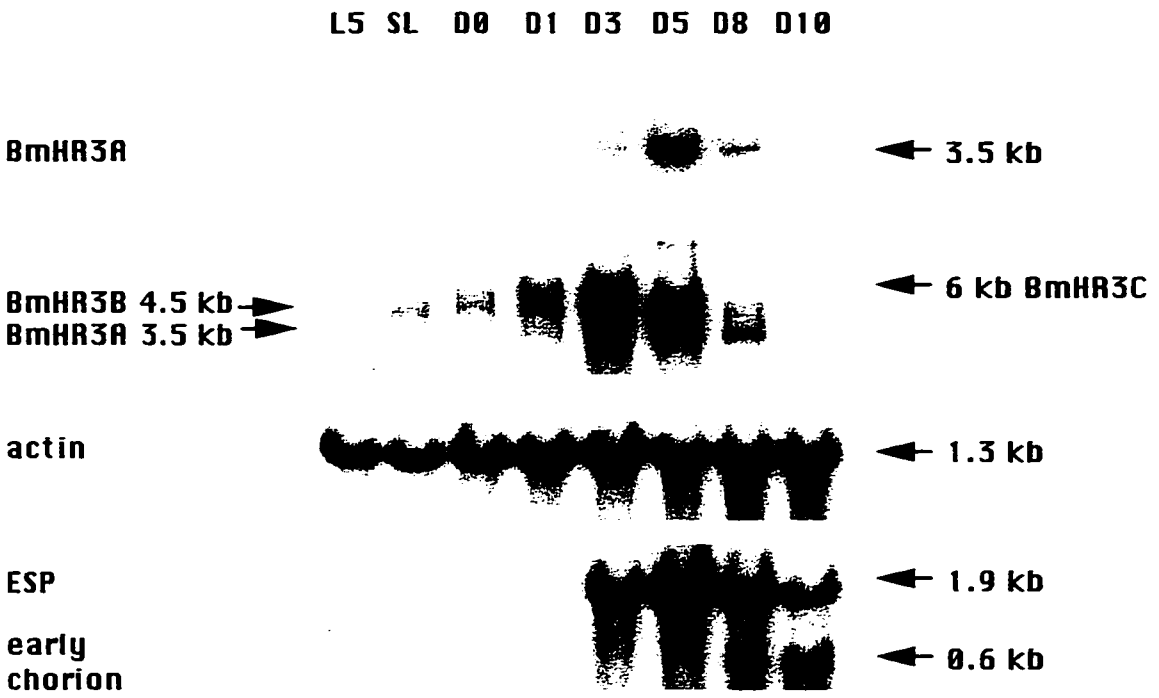
Figure 1.3: Northern blot analysis of RNA isolated at different time points (in hours) from ligated pupae injected with 20E. The different panels were hybridized as follows: upper, with the isoform specific region of BmHR3A (using the PCR amplified product with FP1 and RP1), middle, with the total cDNA clone of BmHR3A minus the isoform specific region (this was obtained by digestion of BmHR3A with PstI and NotI), and, lower with an actin fragment for a loading control. The exposure times were: upper panel, 4 days; middle panel, 1 day; bottom panel, 4.5 hours.


0 1/4 1/2 1 2 4 6 8 12
3.5 kb BmHR3A
4.5 kb BmHR3B
1.3 kb actin

development.

In the hemolymph of *Bombyx*, the ecdysone titer rises in day 3 spinning larvae declines sharply before the pupation, rises again in day 0 pupae, peaks in day 2 pupae and then declines reaching basal levels by day 6 (Calvez et al, 1976). BmHR3 mRNA expression is at its highest when the ecdysone titer is declining in day 3 and day 5 ovaries (Fig 1.4). In day 5 ovaries three mRNA isoforms of HR3 are detected with the BmHR3A total cDNA, minus the isoform specific region, with sizes of 3.5, 4.5 and 6 kb. As already mentioned, hybridization with the isoform specific probe identifies the 3.5 kb mRNA, as the A-isoform of BmHR3. The other two mRNAs likely correspond to the B- and C-isoforms as defined in *Choristoneura* (Palli et al, 1997). Two observations can be made from the expression pattern obtained during metamorphosis (Fig. 1.4). First, the A-isoform of BmHR3 begins to be expressed in day 5 ovaries when the ecdysone titer is low. In contrast the B-isoform is expressed weakly on day 1 and strongly on day 3 concomitant with the rise and decline of the ecdysone titer, respectively. These results, in addition to the results obtained from the hormone injection experiments, indicate the existence of two mechanisms for the up-regulation of BmHR3 levels: first, as an early-late response to 20E (BmHR3B only), and second, as a late response during declining hormone titers (all isoforms).

Figure 1.4: Expression of BmHR3 in the ovary of *B. mori* during metamorphosis. The ovary was isolated during the following stages of development: L5= fifth instar larvae, SL= spinning larvae, D-followed by a number represents the days after larval-pupal ecdysis. Probes used in the hybridization reaction were: 1) the PstI/NotI fragment that encompassed all of BmHR3A minus the isoform specific region and 2) fragments for actin, ESP and early chorion mRNA for loading controls. The exposure times were: first panel, 6 days; second panel, 6 days; third panel, 4.5 hours; bottom panel, 1.5 hours.


L5 SL D0 D1 D3 D5 D8 D10
BmHR3A
3.5 kb
BmHR3B 4.5 kb
BmHR3A 3.5 kb
6 kb BmHR3C
actin
1.3 kb
ESP
1.9 kb
early
chorion
0.6 kb

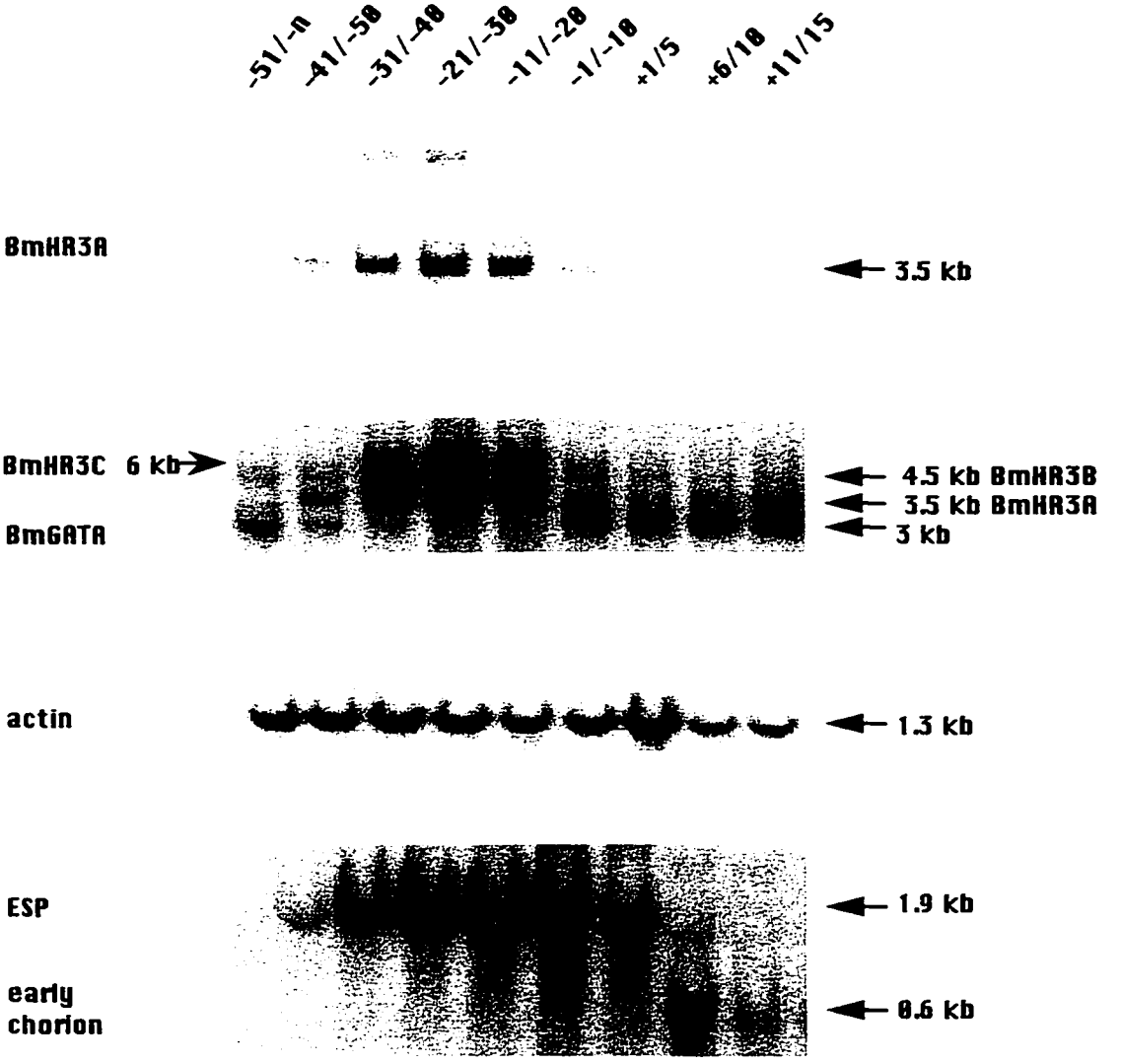
Expression of BmHR3 in the cells of the follicular epithelium during vitellogenesis and choriogenesis

Because high expression of BmHR3 is observed in the ovary of day 5 pupae, the question arises as to which stages of oogenesis BmHR3A is expressed. RNA was extracted from pools of vitellogenic and choriogenic follicles at consecutive stages of development and Northern blot analysis was conducted. All BmHR3 mRNA species showed a similar expression pattern: they are absent in previtellogenic follicles and start to accumulate in the follicles concomitant with the production of the mRNA encoding ESP (Sato and Yamashita, 1991) at stages -41/-50 (Fig 1.5).

Maximal expression of BmHR3 is observed during mid-vitellogenesis (stages -21/-30) and this is followed by a subsequent decline during late vitellogenesis (stages -1/-10). A similar pattern of expression is observed *in vivo*, using polyclonal antibodies raised against *Manduca sexta* HR3 (Lan et al, 1997; K. Iatrou, unpublished).

Interestingly we noticed that the temporal expression pattern of BmHR3 in follicular cells was reciprocal to that of BmGATA (Fig. 1.5). BmGATA is a zinc finger protein that binds to the "GATA" sites in late chorion gene promoters in *B. mori*, and is hypothesized to be a transcriptional regulator of late chorion genes. During oogenesis BmGATA shows a bimodal pattern of expression. It is present in previtellogenic follicles but becomes down-regulated during early and middle vitellogenesis when BmHR3B accumulates in the follicles. BmGATA mRNA becomes re-induced in late vitellogenic follicles (stages -1/-10, see also, Drevet et al, 1995), concomitant with the decline in

Figure 1.5: Expression of BmHR3 and BmGATA during oogenesis. The RNA was extracted from follicles at different stages of vitellogenesis (every 10 stages) and choriogenesis (every 5 stages). The top panel was probed with BmHR3A isoform specific. The second panel from the top was probed with BmHR3A total (minus the isoform specific region) and BmGATA probes. The third panel was probed with actin. The bottom panel was probed with ESP and early chorion as loading controls. The exposure times were: first panel, 24 hours; second panel, 24 hours; third panel, 4.5 hours; bottom panel, 1.5 hours. Please note that the level of cytoplasmic actin RNA varies during vitellogenesis, it is not a result of unequal RNA loading. In addition there is an upregulation of cytoplasmic actin RNA in the beginning of choriogenesis (Swevers et al, 1995).


-51/-41
-41/-31
-31/-21
-21/-11
-11/-1
-1/+1
+1/+6
+6/+11
+11/+15
BmHR3A
3.5 kb
BmHR3C 6 kb
BmGATA
4.5 kb BmHR3B
3.5 kb BmHR3A
3 kb
actin
1.3 kb
ESP
early chorion
1.9 kb
0.6 kb

BmHR3 expression (Fig. 1.5).

BmHR3 binds to the BmGATA promoter and the ESP promoter

Insect HR3 nuclear receptors closely resemble the vertebrate ROR receptors (retinoic acid related-orphan receptors) (77% amino acid identity in the DNA binding domain, Giguere et al, 1994); specifically BmE75 shares 71% amino acid identity with RORα (see Table 1.1). The vertebrate ROR receptors have been shown to bind as monomers to nuclear receptor half sites (A/G GGTCA) preceded by an A/T rich sequence (RORE-elements; McBroom et al, 1995).

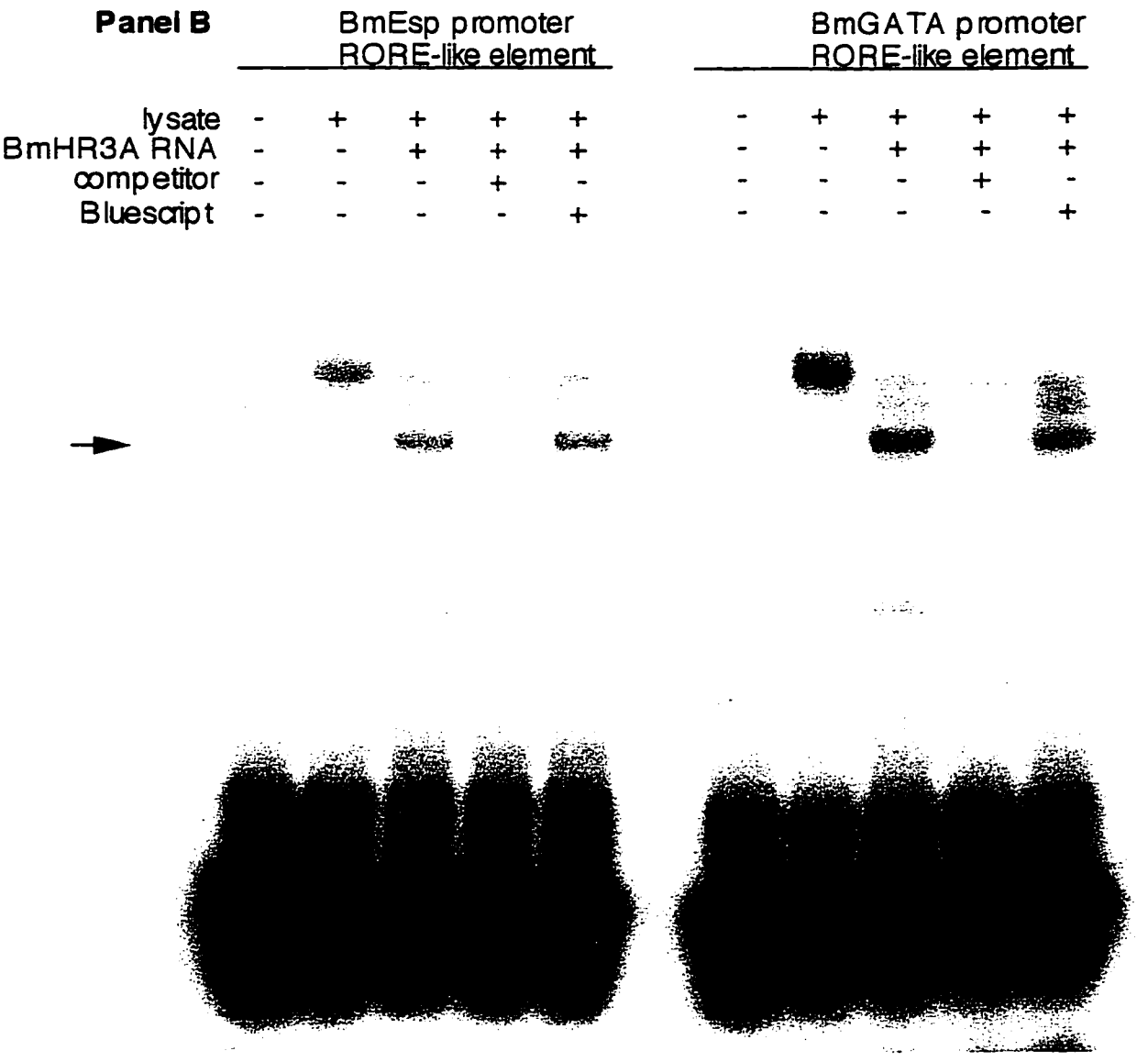
As described earlier the temporal expression pattern for BmHR3 in follicular cells was reciprocal to that of BmGATA (Fig 1.5). Concomitant with the expression of the BmHR3 gene, BmGATA mRNA is absent, and the reciprocal pattern is also observed. Therefore this was worthy of further investigation because of the possible link between BmHR3 and the expression of late chorion genes. Furthermore, the observation that BmHR3 expression parallels the expression pattern of BmESP also warranted investigation because of the possibility that BmHR3 acts as a transcriptional activator of BmESP. A RORE-like element was found in each of the BmGATA and the BmESP promoters. One such sequence was found, approximately 588 bp upstream of the BmGATA transcriptional start site (M. Lunke and K. Iatrou., unpublished results) with a consensus of 9 out of 11 base pairs (Fig 1.6). Also a putative binding site was identified 720 bp upstream of the transcriptional start site in the ESP promoter (Fig 1.6). Two double stranded oligonucleotides were synthesized which contained the RORE-like elements of the ESP and GATA promoters, and these DNA fragments were used in a

Figure 1.6: Panel A: sequence comparison of the RORE element with the consensus sequence found in the BmESP promoter (720 bp upstream of the transcriptional start site) and BmGATA promoter (approximately 588 bp upstream of the transcriptional start site). Panel B: Bandshift assay with *in vitro* transcribed and translated BmHR3A (L.S and K.I., unpublished). The oligonucleotides used in the bandshift experiments are the fragments indicated in panel A (the RORE-like elements of the BmGATA promoter and the BmESP promoter). For a specific competitor unlabeled BmGATA and BmESP RORE-like fragment was used. For a non-specific competitor Bluescript was used. The exposure time was three days.

Panel A

	11 10 9 8 7 6 5 4 3 2 1
Consensus sequence of RORE-element	A A A N T A G G T C A
	T T G
BmGATA RORE-like element	C A A T C A G G T C A
BmESP RORE-like element	T T A A T G G G T C A

Panel B

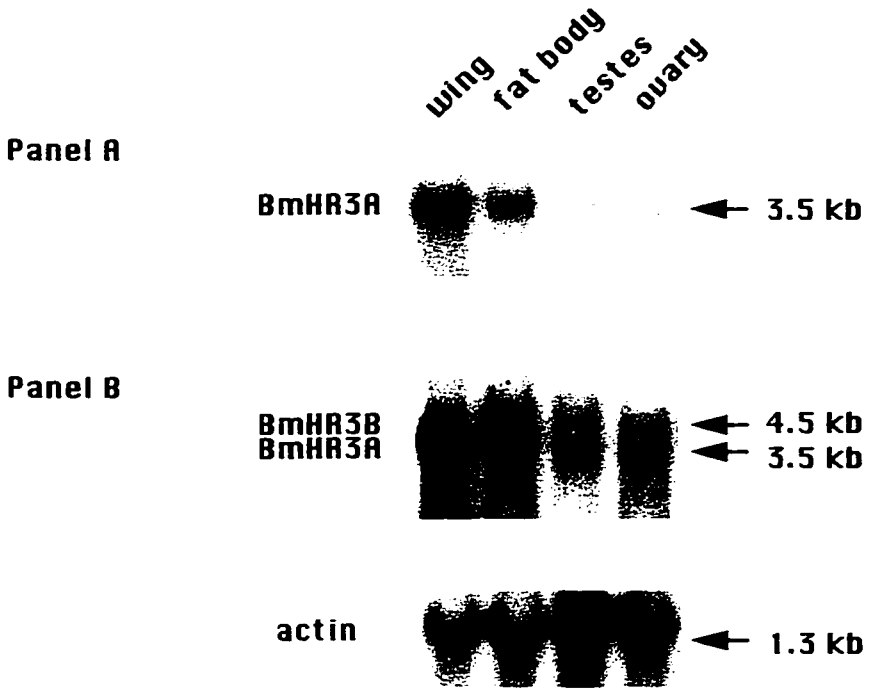


bandshift analysis (Luc Swevers and K. I, unpublished) with *in vitro* transcribed/ translated BmHR3A. BmHR3A binds to the double stranded oligonucleotides identified for both BmGATA and BmESP, to give specific complexes that can be competed out with specific competitors but not with non-specific competitor DNA (L.S and K. I., unpublished) (Fig 1.6). Although the unprogrammed lysate contains factors that bind to the RORE-like elements, this complex has a different molecular mass than that obtained with the lysate programmed with BmHR3A. Therefore BmHR3A is capable of interacting with these RORE-like elements *in vitro*.

Expression of BmHR3 mRNA is not ovary-specific

The expression pattern of BmHR3 during follicular development in the ovary indicates an important function for BmHR3 in the regulation of follicular cell-specific genes such as ESP and BmGATA (Figs 1.5 and 1.6). To examine whether BmHR3 is an ovary-specific regulator, the distribution of BmHR3 mRNA among different pharate adult tissues was examined (Fig 1.7). BmHR3 mRNA is present in all tissues examined and is particularly highly expressed in fat body and wing epidermis. Interestingly wing epidermis RNA extracts only contain the 3.5 kb mRNA corresponding to the A-isoform, while in the testis an additional HR3 mRNA species of a smaller size (2.9 kb) than the A-isoform mRNA is expressed (Fig 1.7). BmHR3 accumulation patterns in the other tissues during pupal and pharate adult development also parallel those observed in the ovary: low levels of the B-isoform (4.5 kb) are present in early pupae while major expression of both A and B isoforms occurs during declining ecdysteroid titers (data not shown).

Figure 1.7: Northern hybridization of different tissues taken from day 6 pupae. Panels A and B were hybridized with: A) the isoform specific region of BmHR3A (amplified by primers FP1 and RP1, Fig 1.1) and B) the PstI and NotI fragment of BmHR3A which contains all of BmHR3A except for the isoform specific region. The bottom panel has been hybridized with actin. The exposure times were: top panel, 10 days; middle panel, 10 days; bottom panel, 4.5 hours.


wing
fat body
testes
ovary
Panel A
BmHR3A
3.5 kb
Panel B
BmHR3B
BmHR3A
4.5 kb
3.5 kb
actin
1.3 kb

DISCUSSION

An isoform of HR3 was isolated which was termed BmHR3A based on the properties of the HR3 mRNA isoforms of *Choristoneura*. In *Choristoneura*, four isoforms of HR3, A, B, C and D have been observed, from which the B and C isoforms have been cloned (Palli et al, 1997). The isoform isolated from *Bombyx* has been designated as BmHR3A, for the following reasons: 1) the similar mRNA size between CHR3A and BmHR3A, (3.8 kb versus 3.5 kb, respectively); 2) the expression of both CHR3A and BmHR3A is not induced by the hormone 20E (Fig 1.3); and 3) in the N-terminus, BmHR3A shares a higher amino acid identity with the N-terminus of the A-isoform of *Drosophila* HR3 (60%) than with the isoforms isolated from *Manduca* and *Galleria* (16 and 15%, respectively), which, by deduction are the B-isoform.

In the developing ovary of *Bombyx*, two other isoforms of HR3 are observed in addition to HR3A, which we named the B- and C- isoforms for reasons explained below. The B -isoform and the C- isoform of HR3 are encoded for by a 4.5 kb and 6 kb mRNA respectively (Fig 1.5). The 4.5 kb mRNA most likely corresponds to the B- isoform isolated in *Manduca* and *Galleria* (Palli et al, 1992., and Jindra et al, 1994) for two reasons: 1) its size is comparable to *Manduca* (4.5 kb) and *Galleria* (4.6 kb) and 2) due to its strong induction by 20E within 4 hours compared to 3 hours in *Manduca*. The 6 kb mRNA of BmHR3 corresponds to an unknown isoform which may in fact correspond to the C-isoform of *Choristoneura*. An additional isoform of HR3 has also been isolated recently in *Bombyx* which is similar to BmHR3A, except for an insertion of 180 amino acids in the hinge region (K. Ito and K.Iatrou, unpublished results). Northern

hybridizations indicate that this isoform is encoded by a mRNA of approximately 6 kb. This isoform of HR3 was identified in the yeast-two hybrid system in an attempt to isolate factors interacting with the nuclear receptor BmE75 (K. Ito and K.Iatrou, unpublished results). Interestingly in *Choristoneura*, a C-isoform has been described (encoded by a 5.4 kb mRNA) that has a unique N-terminus and also a 180 amino acid insertion in the hinge region (Palli et al, 1997). This suggests that the third isoform observed in *Bombyx* may be the C-isoform of HR3. During ovarian development, the expression pattern of the HR3 isoforms is as follows. First, in spinning larvae before larval-pupal ecdysis, the B-isoform is strongly induced by 20E. Then during oogenesis when the ecdysone titer is declining (2-3 days after larval pupal ecdysis) BmHR3A is induced during early vitellogenesis (stage -41/-50), whereas strong expression of the B and C isoforms is detected in later stages (stages -31/-40). The expression of all isoforms of BmHR3 declines sharply and is undetectable during choriogenesis. Therefore HR3 is a late gene in the regulatory cascade induced by 20E during early pupation because it is expressed in the later stages of vitellogenesis. This differs from DHR3 which acts as an early-late 20E response gene (Huet et al, 1995).

A peculiarity with the developing ovary is that during pharate adult development various ecdysteroids, including 20E, start to accumulate in the developing follicles (Legay et al, 1976; Ohnishi et al, 1977). However accumulation of ecdysteroids in the ovary starts 4-5 days after larval- pupal ecdysis i.e. 1-2 days after HR3 isoforms start to accumulate in the follicular epithelium (Fig 1.4). Thus, no correlation can be made between the increase in the ecdysteroid titer in the hemolymph or follicles and the

induction of HR3 isoforms during vitellogenesis. This indicates that HR3 expression in the ovary is mainly regulated in an ecdysteroid-independent fashion.

Southern blot analysis shows that BmHR3 is a single copy gene in the *Bombyx* genome (Fig 1.2) when a fragment specific to the C-terminus and 3' UTR was used (see Fig 1.1). Because HR3B is induced by the hormone while HR3A and C are not, HR3B must be transcribed from a different promoter. However HR3A and C show a similar accumulation pattern in vitellogenic follicles and it is therefore possible that these mRNA isoforms originate from the same promoter by 20E-regulated alternative splicing.

The presence of three isoforms of HR3 mRNA in the silkmoth ovary, all of which share the same DNA binding domain and are simultaneously expressed in follicular cells during vitellogenesis, raises the question as to whether the different isoforms may regulate different target genes. Such is the case with the vertebrate homologs of HR3, the ROR or RZR receptors, whose different isoforms have a preference for different DNA binding sites (Giguere et al, 1994). Both the N-terminus and the hinge region have been implicated in the regulation of DNA binding carried by the zinc-finger region and the carboxy-terminal end of the DNA binding domain (McBroom et al, 1995). With regard to HR3 the situation may be similar because of the presence of isoforms that may differ in their N-terminus. Selective binding of a particular HR3 isoform to a RORE-like element probably also depends on interactions with other factors that bind to adjacent sites in the DNA. Hence because BmHR3 is not an ovary specific factor, other factors may be responsible for tissue specific gene expression, eg. expression of the ESP gene.

The bandshift assays (Fig 1.6) conducted with oligonucleotides containing RORE-

like sites whose sequence was derived from the promoters of the BmESP and BmGATA genes, showed positive binding by BmHR3A (Fig 1. 6) suggesting that BmHR3A may regulate BmGATA and BmESP gene expression. However a future confirmation of this result is necessary by conducting another bandshift using the same RORE-like elements (as above) except for a mutation in the oligonucleotide (i.e. one base pair change) to ensure that binding by BmHR3A is indeed specific. However based on the binding obtained (Fig 1.6) and on the temporal expression pattern of BmHR3A, BmGATA and BmESP during vitellogenesis, one hypothesis is that BmHR3A is a negative and positive regulator of BmGATA and BmESP, respectively. In order to determine whether BmHR3A regulates the aforementioned factors *in vivo* as suggested by the bandshift assays, BmHR3A was expressed in a *B. mori* derived cell line (Bm5 cells). However BmHR3A did not activate or repress the BmESP or BmGATA promoter (L.S and K. I., unpublished), nor was any effect observed on the activity of a basal promoter containing two copies of either RORE-like element, that was used in the bandshift assay (Fig 1.6) (L.S., M.Lunke., and K.I., unpublished results). There are two possible explanations for this result. First, it is possible that the sequences used for the bandshift experiment do not provide sufficient binding sites *in vivo* and, second, BmHR3 may require a co-repressor or a co-activator in order to function as a repressor or an activator. Both possibilities require further investigation in order to understand what functional role BmHR3A has in the ovary during pupation.

In *Drosophila*, DHR3 is induced as an early-late response to 20E at the end of the third larval instar stage (Horner et al., 1995; Huet et al, 1995) and plays an important role

in the larval-prepupal transition by arresting the early response to 20E and inducing the mid-prepupal specific transcription factor βFTZ-F1 (Lam et al, 1997). The activity of DHR3 is modulated by E75B, (which lacks a complete DNA binding domain; White et al, 1995), which prevents DHR3 from activating the transcription of βFTZ-F1. Homologs of the *Drosophila* early response gene E75A have been isolated in *B. mori*. However during oogenesis, the factors BmE75A, HR3 and βFTZ-F1, are not expressed coordinately (T.E., L.S., and K.I., unpublished results; see also Chapter 2). Therefore there is no temporal succession of mRNA accumulation of E75A, HR3, and FTZ-F1 during follicular development in *Bombyx* as is observed during the larval-prepupal transition in *Drosophila*. This suggests that there is a different developmental program with different regulatory factors during follicular cell differentiation in *Bombyx* .

Recently it was found through employment of the yeast two hybrid system that BmE75 (E75 cDNA clone minus the isoform specific region) interacts with BmHR3A (K. Ito; and K. Iatrou, unpublished). Therefore if the *in vitro* interaction between BmHR3A and BmE75 also occurs *in vivo*, by analogy to *Drosophila* BmE75 may regulate the activity of BmHR3A.

During mid-vitellogenesis at approximately stage -35, follicles acquire the competence to enter choriogenesis autonomously whereas follicles earlier than this stage are unable to enter choriogenesis in later stages (I. Dinnetz and K.I., unpublished). Initially this stage had been placed at -17 (Swevers et al, 1992), however studies with a new strain of *B. mori* that is maintained on artificial diet has put the stage for induction of the autonomous part of the choriogenic program at -35 (I. Dinnetz., L. S and K. I,

unpublished). It is hypothesized that a receptor is expressed at this stage and that interaction between this receptor and a ligand from the hemolymph may provide this competence. The abrupt expression of BmHR3A during vitellogenesis at stage -41/-50, leads to the interesting hypothesis that BmHR3 may be the receptor that confers this competence in the follicular cells. The reason for this assumption is as follows. The vertebrate counterpart of *Drosophila* HR3, RORα (Giguere et al, 1994; Carlberg et al, 1994), needs a ligand for receptor transactivation (melatonin; Wiesenberg et al, 1993). Due to the high amino acid identity between RORα and BmHR3A in their DNA binding domain (see Table 1.1) it is possible that BmHR3A also has a ligand possibly one similar in structure to melatonin. Therefore, the finding that ROR or RZR is not an orphan receptor, suggests that its homolog BmHR3 may not be an orphan receptor and that it is capable of responding to a hormone from the hemolymph.

All the observations and assumptions made regarding BmHR3A suggest that this factor may play a pivotal role during oogenesis in *Bombyx*. Is BmHR3A the determining receptor? Could there be a dual function for BmHR3A both as a transcriptional activator of BmESP and a negative regulator of BmGATA? Further studies into the problem of assigning definitive role to BmHR3A should prove very interesting.

CHAPTER 2

Isolation and partial characterization of the hormone nuclear receptor

E75: isoforms BmE75A, BmE75C and BmE75D

in the silkmoth ovary

ABSTRACT

Three cDNA clones corresponding to isoforms of BmE75 were isolated. One isoform shares a high degree of amino acid identity with *Drosophila*, *Manduca* and *Galleria* E75A, and is therefore designated BmE75A. The other two isoforms BmE75C and BmE75D are new isoforms of the E75 gene not previously identified in other insect species. The three isoforms are distinguished from one another by their unique N-termini. BmE75C is characterized by a proline rich N-terminal region while the other two isoforms have no noticeable characteristics.

BmE75C and BmE75D may be involved in regulating the expression of early chorion proteins, because BmE75C and BmE75D are abruptly upregulated during the transition from vitellogenesis to choriogenesis in day 6 pupae. The temporal expression pattern of these factors during oogenesis suggests that these factors may play important roles during oogenesis. This is supported by the observation that BmE75C is expressed only in the ovary in day 6 pupae, and that BmE75D is an ovary specific factor.

INTRODUCTION

The goal was to isolate cDNA clones corresponding to genes that are directly induced by 20E at the beginning of pupation in the *B. mori* ovary. Candidate genes would be homologs of early and early late genes such as the nuclear receptors E75 and HR3, which have been identified in *Drosophila* and lepidopteran insect species. The goal was to isolate silkworm homologs of the early response gene E75 which has been previously isolated in *Drosophila* as well as the lepidopteran species *Manduca*, *Galleria* and

Choristoneura (Segraves and Hogness, 1990; Segraves and Woldin, 1993; Jindra et al, 1994; Palli et al, 1997). E75 is a member of the steroid hormone receptor superfamily and its expression has been shown to be induced by 20E *in vitro*.

Three isoforms of E75 were isolated from the *B. mori* ovary, the A-, C- and D-isoforms. The isolation and initial characterization of these isoforms is described. All three isoforms of BmE75 are expressed in ovarian follicles during vitellogenesis but surprisingly, two of the isoforms, the C- and D- isoforms, are abruptly expressed at the end of vitellogenesis. Moreover these two isoforms are expressed only in the ovary in day 6 pupae when choriogenesis begins. The temporal expression of these isoforms raises a question as to whether these isoforms may play a role in choriogenesis by regulating the expression of early chorion genes.

MATERIALS AND METHODS

Animals

Information on the strain of the silkmoth, and rearing, are provided in Chapter 1.

Ligation Procedure

The ligation protocol is described in Chapter 1.

Nucleic Acid Extraction

The protocol for nucleic acid extraction is provided in Chapter 1.

cDNA library screening

The Lambda gt11 cDNA library made from vitellogenic follicles (Swevers et al, 1995) was screened with the DNA binding domain of *Galleria* E75A (kindly provided by Dr. Marek

Jindra). Specifically, a 550 bp fragment containing the E75A specific region and the DNA binding domain of *Galleria* E75A (Jindra et al, 1994) was used. Thirty nine hybridizing clones were obtained, whose inserts were subcloned as NotI fragments into the NotI site in pBS/SK+.

DNA sequencing analysis

The protocol for DNA sequencing is provided in Chapter 1.

Filter hybridization

The protocol for filter hybridization is described in Chapter 1. The probes utilized in the Southern and Northern hybridizations are specified below.

For the Southern hybridization, a fragment corresponding to the the ligand binding domain of BmE75 was generated by a PCR reaction using primers P4: 5' CTCATCTGTATGTTTGAC 3' and RP3: 5' CCGATCCGGAGTGATGAG 3' (Fig 2.1).

For plaque screenings: primers specific for E75A, P2A: 5' GTGATGTCTCCG GATAGT 3' and RP1: 5' CGTGGTACCGTCAAATT 3' and specific for E75C, P2C: 5' AGCCTCCGGAAGGATTA 3' and RP1: 5' CGTGGTACCGTCAAATT 3' and for internal control, common region primers used P4: 5' CTCATCTGTATGTTTGAC 3' and RP3: 5' CCGATCCGGAGTGATGAG 3' (Fig. 2.1, 2.2, 2.4).

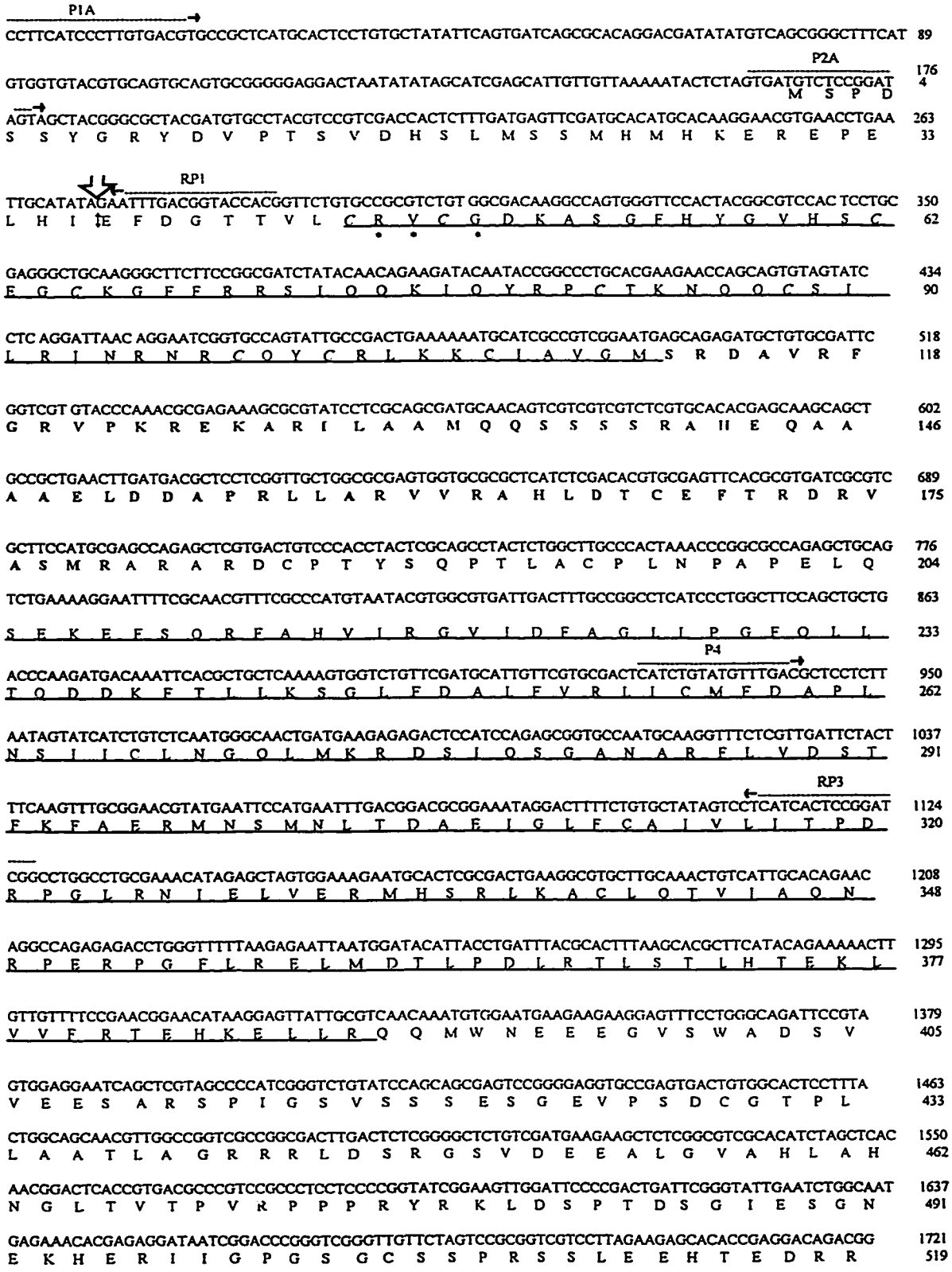
For the Northern hybridizations, the following DNA fragments were used for generating hybridization probes : (1) a PCR fragment corresponding to the 5' UTR and N-terminus of BmE75A, generated by primers P1A: 5' CCTTCATCCCTTGTGACG 3' and RP1: 5' CGTGGTACCGTCAAATT 3' (Fig 2.1), (2) PCR fragment corresponding to the 5' UTR and N-terminus of BmE75C, using primers P1C (Fig 2.2): 5'

CACGATACGCGCTACGA 3' and primer RP1: 5' CGTGGTACCGTCAAATT 3', and (3) another fragment corresponding to the 5' UTR and N-terminus of BmE75D, generated by primers P1D (fig 2.4): 5' GACAGACATGACGGTCAC 3' and primers RP1: 5' CGTGGTACCGTCAAATT 3'. For internal controls the following probes were used (1) a 1.9 kb NotI fragment corresponding to the complete egg-specific (ESP) cDNA (Sato and Yamashita, 1991); (2) two Pst I fragments corresponding to early genes, 6A2 and 6C11 (Eickbush et al, 1985; Lecanidou et al, 1986; Swevers et al, 1995); and (3) a 0.8 Kb XhoI-PstI actin gene fragment (Mounier and Prudhomme, 1986).

RESULTS

The vitellogenic cDNA library was screened in order to identify homologs of *Drosophila* E75. The library was screened using the DNA binding domain from the *Galleria* E75A cDNA clone (kindly provided by Dr. Marek Jindra). A total of 80,000 plaques were screened from which 17 plaque purified clones were obtained. Digestion of these 17 clones with NotI yielded insert sizes from 2.5 to 3 kb. Two clones were chosen based on their size, one of which contained a 2.6 kb fragment and the other a 3 kb fragment. These two clones were sequenced and one was named BmE75A (Fig. 2.1) due to the high amino acid similarity in the N-terminus with *Choristoneura*, *Galleria*, and *Drosophila* E75A (70%, 78% and 49% respectively; Table 2.1; Segraves and Hogness, 1990; Jindra et al, 1994).

Figure 2.1: Nucleotide and amino acid sequence of BmE75A. The five domains characteristic of the nuclear hormone receptor superfamily are highlighted in the figure as follows: the DNA binding domain, ligand binding domain, and polyadenylation signal site are underlined, and the hinge region is indicated in bold. For the DNA binding domain the cysteine residues of the zinc finger are shown in bold italic. The residues for the DNA half -site recognition P-box are indicated by asterisks. The amino acid residues specific to the A-isoform are separated from the common region with an arrow. The primers specific for E75A are P1A and P2A.


P1A
P2A
RP1
P4
RP3

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CCCACCGCGCCCGCCGATGACATGCCCGTGCTCAAACGTGTGCTTCAGGCTCCACCTTTGTACGGCGGAACTTCTACATTGATGGAT  1808
P  T  A  P  A  D  D  M  P  V  L  K  R  V  L  Q  A  P  P  L  Y  G  G  T  S  T  L  M  D    548

GAAACCTACAAACCACATAAGAAATTCCGCGCTATGAGGCGCGACACTGGAGAAGCAGAGGCTCGTCCAGTGCAGCCGACGCCG  1892
E  T  Y  K  P  H  K  K  F  R  A  M  R  R  D  T  G  E  A  E  A  R  P  V  Q  P  T  P      576

TCGCCACAGCCGCTGCACCCGCACCCGGCCAGTCCGGCTCATCCGGCTCATTCGCCGCGACCACCGCGCATTTCTCTGTCATCCACG  1979
V  P  Q  P  L  H  P  H  P  A  S  P  A  H  P  A  H  S  P  R  P  P  R  I  S  L  S  S  T    605

CATTCGGTGCTCGCTAAAAGTTTAATGGAGGGGCCGAGAATGACTCCTGAACAATTAAAACGTACCGATATGATCCAGCAGTAC  2063
H  S  V  L  A  K  S  L  M  E  G  P  R  M  T  P  E  Q  L  K  R  T  D  M  I  Q  Q  Y      633

ATGCGGCGAAATGAAGCTGGTTCTAGTGTGGAAGGATGTACGCTGCGAACTGGAGGGCTGCTGACCTGCTACCGCGGTGCGTCT  2147
M  R  R  N  E  A  G  S  S  V  E  G  C  T  L  R  T  G  G  L  L  T  C  Y  R  G  A  S      661

CCGGCTCCGCCGCCAGTGCTGGCGCTGCAGGTGGACGTGACGGACGCGCCGCTGAACCTCTCCAAGAAGTCGCCGTCGCCGCCT  2231
P  A  P  P  P  V  L  A  L  Q  V  D  V  T  D  A  P  L  N  L  S  K  K  S  P  S  P  P      689

CGTTCGTACATGCCGCAGATGTTAGAGGCGTGAGGCCGGCCCGCGGAGCCGCGAGTCTCCGCCACCCCACCCCGACCTACTTTAAA  2317
R  S  Y  M  P  Q  M  L  E  A  -                                                            699

TTAAATAAACTAGATAATAGTAATCGTTCGCAGATCGTTTTAATATATTATATGTGATAATTCGTGTAGGACGTGTGCGTGCGTGGCC  2405

GGCGCCGAACGAGGAGAGCGCAATAGACTGAAAATTAACATAATTTAAGACTATATAAGTGTAATTTATAATACAATGCAGCGCC  2490

GGTCGTGGTGTGTTTGTGTGTATTGTATGTGCGTATGTGAGCGTGCTCAGTCATTTGTTTGT TAATATAATGACAGGTTGTCGAATTAA  2579

ATAAAACAAATTGTAAGAATTAAAAAAAAAAAAAAAAAAAAAA  2622
```

Table 2.1: Comparison of *Bombyx* E75A with the A-isoforms of *Choristoneura*, *Galleria* (Palli et al, 1997a; Jindra et al, 1994a), *Drosophila* (Segraves and Hogness, 1990) and Rev-erb (Lazar et al, 1989) in the 5 different regions which are characteristic to nuclear receptors: N-terminus, DNA binding domain, hinge region, ligand binding domain and C-terminus. A comparison of total homology is also provided.

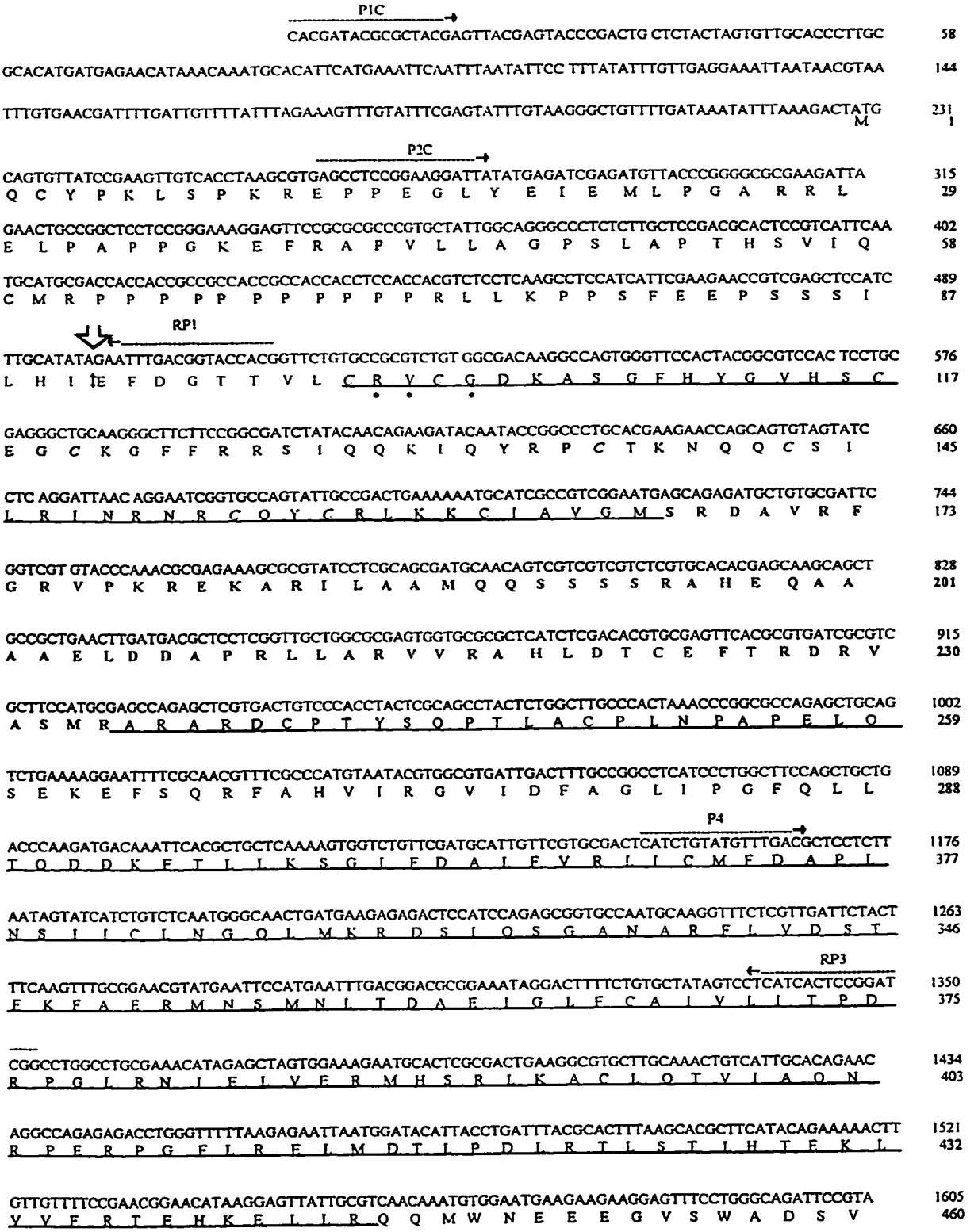
Length / percent amino acid identity						
Insect	N-terminal region	DNA binding domain	Hinge region	Ligand binding domain	C-terminal	total
Choristoneura	46/70%	67/100%	68/97%	210 /94%	308/80%	699/79%
Galleria	46/78%	67 /100%	67 /91%	210/97%	320/85%	710/90%
Drosophila	244/49%	67/98%	68/74%	210/84%	647/81%	1236/82%
Rev-erbα	25/16%	67/73%	230/10%	184/30%	—	508/26%

The other clone encodes a new isoform of E75, designated BmE75C due to its unique N-terminus (see Fig 2.2 and 2.5) . It is characterized by a region of 30 % proline amino acids in the N-terminus (see Fig 2.3).

Interestingly, there is a small percentage of amino acid identity (23%) between BmE75C and DE75C, mostly in the proline rich region stretch of the N-terminus (Fig 2.3). However the similarity is relatively low and therefore BmE75C is considered to be a different isoform than DE75C. Using primers specific to the A- and C- isoforms of E75 (primers P2A / RP1, and P2C / RP1 Fig 2.1 and 2.2), the remaining of the 17 plaque purified plasmids were examined in order to determine whether other isoforms were present. As an internal control primers specific to the DNA binding and hinge region (primers P3A / RP2; see Fig 2.1) were utilized. From this an amplified product should be obtained from all 17 clones because these clones were isolated using the DNA binding domain of *Galleria* E75A. In this manner the third isoform designated BmE75D (Fig. 2.4) was isolated. The proportion of the three isoforms among the 17 clones were 12%, 41% and 47% for the A-, C- and D-isoforms, respectively.

BmE75A has a cDNA sequence length of 2.6 kb and an ORF of 697 amino acids (Fig 2.1). The number of amino acids specific to the isoform-specific region is 34 and the 5' UTR contains 175 bp. In the A/B region, BmE75A is most similar to *Choristoneura* (70%) and *Galleria* (78%). There is high amino acid identity in the common regions among *Drosophila*, *Galleria*, and *Choristoneura*, the DNA binding domain (98-100%), hinge region (74-97%), ligand binding domain (84-97%) and C-terminus (80-85%) (Table 2.1).

Figure 2.2: Nucleotide and amino acid sequence of BmE75C. The five domains characteristic for the nuclear hormone receptor superfamily are highlighted in the figure as follows: the DNA binding domain, ligand binding domain and polyadenylation signal site are underlined, and the hinge region is indicated in bold. For the DNA binding domain the cysteine residues of the zinc finger are shown in bold italic. The residues for the DNA half -site recognition P-box are indicated by asterisks. The amino acid residues specific to the A-isoform are separated from the common region with an arrow. The primers specific for E75C are P1C and P2C.


P1C
P2C
RP1
P4
RP3

```
GTGGAGGAATCAGCTCGTAGCCCCATCGGGTCTGTATCCAGCAGCGAGTCCGGGGAGGTGCCGAGTGACTGTGGCACTCCTTTA   1689
V  E  E  S  A  R  S  P  I  G  S  V  S  S  S  E  S  G  E  V  P  S  D  C  G  T  P  L      488

CTGGCAGCAACGTTGGCCGGTCGCCGGCGACTTGACTCTCGGGGCTCTGTCGATGAAGAAGCTCTCGGCGTCGCACATCTAGCTCAC   1776
L  A  A  T  L  A  G  R  R  R  L  D  S  R  G  S  V  D  E  E  A  L  G  V  A  H  L  A  H      517

AACGGACTCACCGTGACGCCCGTCCGCCCTCCTCCCCGGTATCGGAAGTTGGATTCCCCGACTGATTCGGGTATTGAATCTGGCAAT   1863
N  G  L  T  V  T  P  V  R  P  P  P  R  Y  R  K  L  D  S  P  T  D  S  G  I  E  S  G  N      546

GAGAAACACGAGAGGATAATCGGACCCGGGTCGGGTTGTTCTAGTCCGCGGTCGTCCTTAGAAGAGCACACCGAGGACAGACGG   1947
E  K  H  E  R  I  I  G  P  G  S  G  C  S  S  P  R  S  S  L  E  E  H  T  E  D  R  R      574

CCCACCGCGCCCGCCGATGACATGCCCGTGCTCAAACGTGTGCTTCAGGCTCCACCTTTGTACGGCGGAACTTCTACATTGATGGAT   2034
P  T  A  P  A  D  D  M  P  V  L  K  R  V  L  Q  A  P  P  L  Y  G  G  T  S  T  L  M  D      603

GAAACCTACAAACCACATAAGAAATTCCGCGCTATGAGGCGCGACACTGGAGAAGCAGAGGCTCGTCCAGTGCAGCCGACGCCG   2118
E  T  Y  K  P  H  K  K  F  R  A  M  R  R  D  T  G  E  A  E  A  R  P  V  Q  P  T  P      631

TCGCCACAGCCGCTGCACCCGCACCCGGCCAGTCCGGCTCATCCGGCTCATTCGCCGCGACCACCGCGCATTTCTCTGTCATCCACG   2205
V  P  Q  P  L  H  P  H  P  A  S  P  A  H  P  A  H  S  P  R  P  P  R  I  S  L  S  S  T      660

CATTCGGTGCTCGCTAAAAGTTTAATGGAGGGGCCGAGAATGACTCCTGAACAATTAAAACGTACCGATATGATCCAGCAGTAC   2289
H  S  V  L  A  K  S  L  M  E  G  P  R  M  T  P  E  Q  L  K  R  T  D  M  I  Q  Q  Y      688

ATGCGGCGAAATGAAGCTGGTTCTAGTGTGGAAGGATGTACGCTGCGAACTGGAGGGCTGCTGACCTGCTACCGCGGTGCGTCT   2373
M  R  R  N  E  A  G  S  S  V  E  G  C  T  L  R  T  G  G  L  L  T  C  Y  R  G  A  S      716

CCGGCTCCGCCGCCAGTGCTGGCGCTGCAGGTGGACGTGACGGACGCGCCGCTGAACCTCTCCAAGAAGTCGCCGTCGCCGCCT   2457
P  A  P  P  P  V  L  A  L  Q  V  D  V  T  D  A  P  L  N  L  S  K  K  S  P  S  P  P      744

CGTTCGTACATGCCGCAGATGTTAGAGGCGTGAGGCCGGCCCGCGGAGCCGCGAGTCTCCGCCACCCCACCCCGACCTACTTTAAA   2543
R  S  Y  M  P  Q  M  L  E  A  -                                                           754

TTAAATAAACTAGATAATAGTAATCGTTCGCAGATCGTTTTAATATATTATATGTGATAATTCGTGTAGGACGTGTGCGTGCGTGGCC   2631

GGCGCCGAACGAGGAGAGCGCAATAGACTGAAAATTAACATAATTTAAGACTATATAAGTGTAATTTATAATACAATGCAGCGCC   2716

GGTCGTGGTGTGTTTGTGTGTATTGTATGTGCGTATGTGAGCGTGCTCAGTCATTTGTTTGT TAATATAATGACAGGTTGTCGAATTAA   2805

ATAAAACAAATTGTAAGAATTAAAAAAAAAAAAAAAAAAAAAA                                                2848
```

Figure 2.3. Nucleotide sequence comparison between the C-isoform of *Drosophila* (sequence data provided by Carl Thummel) and the C-isoform of *Bombyx*, in the N-terminus. Sequence similarities are highlighted in bold. The isoform specific region of these two isoforms ends at position 445.

```
        1                                                            50
BmE75C ..... ..... ..... ..... .....
DE75C  MHGGGPGSSG SNIIRRSSGS FPGSGSGSAS KLIKTEPIDF EMLHLEENER

       51                                                           100
BmE75C ..... ..... ..... ..... .....
DE75C  QQDIEREPSS SNSNSNSNSL TPQRYTHVQV QTVPPRQPTG LTTPGGTQKV

      101                                                           150
BmE75C ..... ..... ..... ..... .....
DE75C  ILTPRVEYVQ QRATSSTGGG MKHVYSQQQG TAASRSAPPE TTALLTTTSG

      151                                                           200
BmE75C ..... ..... ..... ..... .....
DE75C  TPQIIITRTL PSNQHLSRRH SASPSALHHY QQQQQPQRQQ SPPPLHHQQQ

      201                                                           250
BmE75C ..... ..... ..... ..... .....
DE75C  QQQQHVRVIR DGRLYDEATV VVAARRHSVS PPPLHHHSRS APVSPVIARR

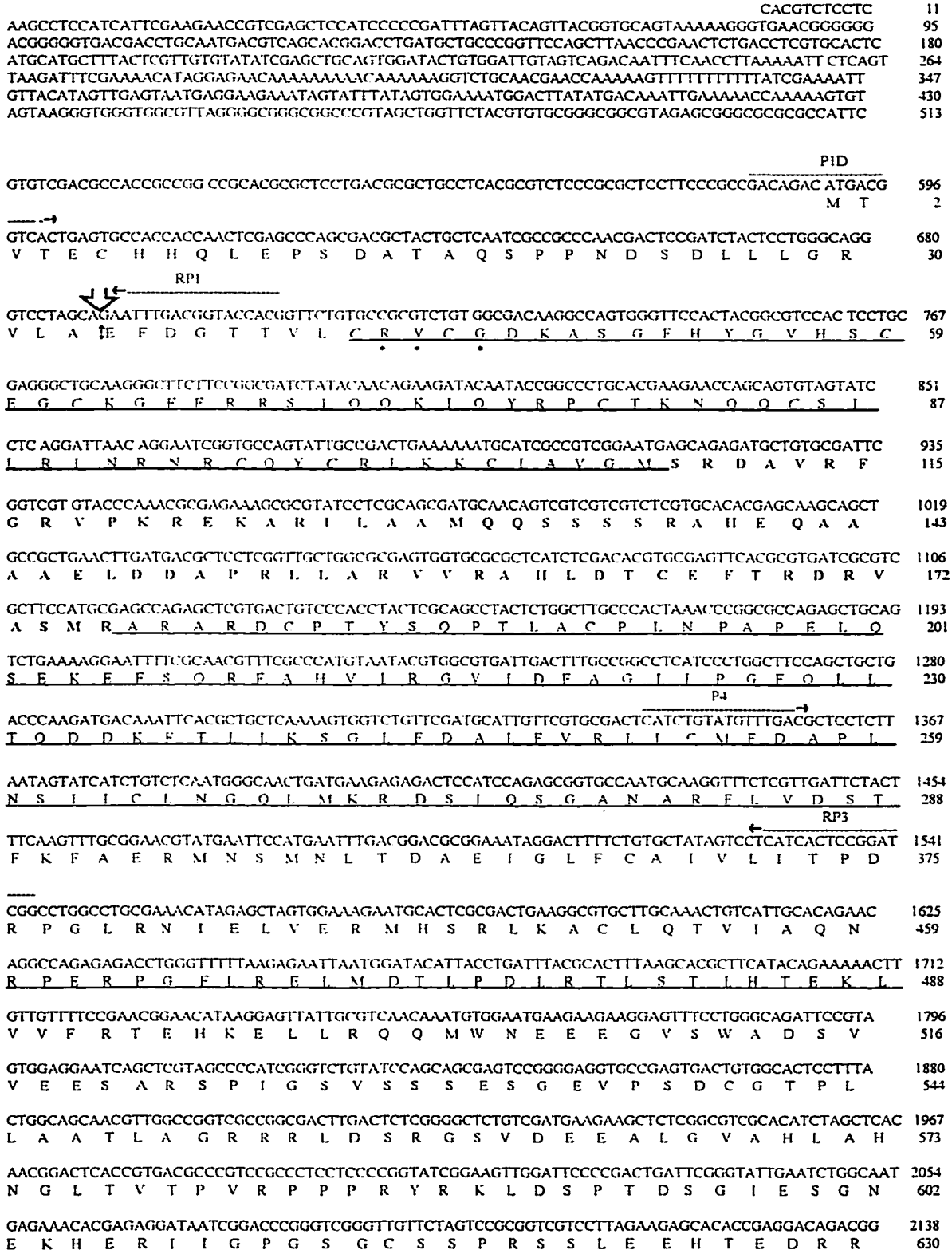
      251                                                           300
BmE75C ..... ..... ..... ..... .....
DE75C  GGAAAYMDQQ YQQRQTPPLA PPPPPPPPPP PPPPPQQQQQ QYISTGVPPP

      301                                                           350
BmE75C ..... ..... .....MQ CYPKLSPKRE PPEGLYEIEM
DE75C  TAAARKFVVS TSTRHVNVIA SNHFQQQQQQ HQAQQHQQQH QQHVIASVSS

      351                                                           400
BmE75C LPGARRLELP APPGKE.FRA PVLLAGPSLA PTH..... ..S.VIQCMR
DE75C  SSSSSAIGSG GSSSSHIFRT PVVSSSSSSN MHHQQQQQQQ QSSLGNSVMR

      401                                                           450
BmE75C PPPPPPPPPP PRLLKPPSF. ..... .....EEPSS SIPDLEFDGT
DE75C  PPPPPPPPKV KHASSSSSGN SSSSNTNNSS SSSNGEEPSS SIPDLEFDGT
```

Figure 2.4: Nucleotide and amino acid sequence of BmE75D. The five domains characteristic for the nuclear hormone receptor superfamily are highlighted in the figure as follows: the DNA binding domain, ligand binding domain and polyadenylation signal site are underlined, and the hinge region is indicated in bold. For the DNA binding domain the cysteine residues of the zinc finger are shown in bold italic. The residues for the DNA half -site recognition P-box are indicated by asterisks. The amino acid residues specific to the A-isoform are separated from the common region with an arrow. The primer specific to E75D is P1D.


CACGTCTCCTC 11
AAGCCTCCATCATTCGAAGAACCGTCGAGCTCCATCCCCCGATTTAGTTACAGTTACGGTGCAGTAAAAAGGGTGAACGGGGGG 95
ACGGGGGTGACGACCTGCAATGACGTCAGCACGGACCTGATGCTGCCCGGTTCCAGCTTAACCCGAACTCTGACCTCGTGCACTC 180
ATGCATGCTTTACTCGTTGTGTATATCGAGCTGCAGTGGATACTGTGGATTGTAGTCAGACAATTTCAACCTTAAAAATT CTCAGT 264
TAAGATTTCGAAAACATAGGAGAACAAAAAAAAACAAAAAAGGTCTGCAACGAACCAAAAAGTTTTTTTTTTTATCGAAAATT 347
GTTACATAGTTGAGTAATGAGGAAGAAATAGTATTTATAGTGGAAAATGGACTTATATGACAAATTGAAAAACCAAAAAGTGT 430
AGTAAGGGTGGGTGGCGTTAGGGGCGGGCGGCCCGTAGCTGGTTCTACGTGTGCGGGCGGCGTAGAGCGGGCGCGCGCCATTC 513
P1D
GTGTCGACGCCACCGCCGG CCGCACGCGCTCCTGACGCGCTGCCTCACGCGTCTCCCGCGCTCCTTCCCGCCGACAGAC ATGACG 596
M T 2
GTCACTGAGTGCCACCACCAACTCGAGCCCAGCGACGCTACTGCTCAATCGCCGCCCAACGACTCCGATCTACTCCTGGGCAGG 680
V T E C H H Q L E P S D A T A Q S P P N D S D L L L G R 30
RP1
GTCCTAGCAGAATTTGACGGTACCACGGTTCTGTGCCGCGTCTGT GGCGACAAGGCCAGTGGGTTCCACTACGGCGTCCAC TCCTGC 767
V L A E F D G T T V L C R V C G D K A S G F H Y G V H S C 59
GAGGGCTGCAAGGGCTTCTTCCGGCGATCTATACAACAGAAGATACAATACCGGCCCTGCACGAAGAACCAGCAGTGTAGTATC 851
E G C K G F F R R S I Q Q K I Q Y R P C T K N Q Q C S I 87
CTC AGGATTAAC AGGAATCGGTGCCAGTATTGCCGACTGAAAAAATGCATCGCCGTCGGAATGAGCAGAGATGCTGTGCGATTC 935
L R I N R N R C Q Y C R L K K C L A V G M S R D A V R F 115
GGTCGT GTACCCAAACGCGAGAAAGCGCGTATCCTCGCAGCGATGCAACAGTCGTCGTCGTCTCGTGCACACGAGCAAGCAGCT 1019
G R V P K R E K A R I L A A M Q Q S S S S R A H E Q A A 143
GCCGCTGAACTTGATGACGCTCCTCGGTTGCTGGCGCGAGTGGTGCGCGCTCATCTCGACACGTGCGAGTTCACGCGTGATCGCGTC 1106
A A E L D D A P R L L A R V V R A H L D T C E F T R D R V 172
GCTTCCATGCGAGCCAGAGCTCGTGACTGTCCCACCTACTCGCAGCCTACTCTGGCTTGCCCACTAAACCCGGCGCCAGAGCTGCAG 1193
A S M R A R A R D C P T Y S Q P T L A C P L N P A P E L Q 201
TCTGAAAAGGAATTTTCGCAACGTTTCGCCCATGTAATACGTGGCGTGATTGACTTTGCCGGCCTCATCCCTGGCTTCCAGCTGCTG 1280
S E K E F S Q R F A H V I R G V I D F A G L I P G F Q L L 230
P4
ACCCAAGATGACAAATTCACGCTGCTCAAAAGTGGTCTGTTCGATGCATTGTTCGTGCGACTCATCTGTATGTTTGACGCTCCTCTT 1367
T Q D D K F T L L K S G L F D A L F V R L I C M F D A P L 259
AATAGTATCATCTGTCTCAATGGGCAACTGATGAAGAGAGACTCCATCCAGAGCGGTGCCAATGCAAGGTTTCTCGTTGATTCTACT 1454
N S I I C L N G Q L M K R D S I Q S G A N A R F L V D S T 288
RP3
TTCAAGTTTGCGGAACGTATGAATTCCATGAATTTGACGGACGCGGAAATAGGACTTTTCTGTGCTATAGTCCTCATCACTCCGGAT 1541
F K F A E R M N S M N L T D A E I G L F C A I V L I T P D 375
CGGCCTGGCCTGCGAAACATAGAGCTAGTGGAAAGAATGCACTCGCGACTGAAGGCGTGCTTGCAAACTGTCATTGCACAGAAC 1625
R P G L R N I E L V E R M H S R L K A C L Q T V I A Q N 459
AGGCCAGAGAGACCTGGGTTTTTAAGAGAATTAATGGATACATTACCTGATTTACGCACTTTAAGCACGCTTCATACAGAAAAACTT 1712
R P E R P G F I R E L M D T L P D L R T L S T L H T E K L 488
GTTGTTTTCCGAACGGAACATAAGGAGTTATTGCGTCAACAAATGTGGAATGAAGAAGAAGGAGTTTCCTGGGCAGATTCCGTA 1796
V V F R T E H K E L L R Q Q M W N E E E G V S W A D S V 516
GTGGAGGAATCAGCTCGTAGCCCCATCGGGTCTGTATCCAGCAGCGAGTCCGGGGAGGTGCCGAGTGACTGTGGCACTCCTTTA 1880
V E E S A R S P I G S V S S S E S G E V P S D C G T P L 544
CTGGCAGCAACGTTGGCCGGTCGCCGGCGACTTGACTCTCGGGGCTCTGTCGATGAAGAAGCTCTCGGCGTCGCACATCTAGCTCAC 1967
L A A T L A G R R R L D S R G S V D E E A L G V A H L A H 573
AACGGACTCACCGTGACGCCCGTCCGCCCTCCTCCCCGGTATCGGAAGTTGGATTCCCCGACTGATTCGGGTATTGAATCTGGCAAT 2054
N G L T V T P V R P P P R Y R K L D S P T D S G I E S G N 602
GAGAAACACGAGAGGATAATCGGACCCGGGTCGGGTTGTTCTAGTCCGCGGTCGTCCTTAGAAGAGCACACCGAGGACAGACGG 2138
E K H E R I I G P G S G C S S P R S S L E E H T E D R R 630

```
CCCACCGCGCCCGCCGATGACATGCCCGTGCTCAAACGTGTGCTTCAGGCTCCACCTTTGTACGGCGGAACTTCTACATTGATGGA  2225
P  T  A  P  A  D  D  M  P  V  L  K  R  V  L  Q  A  P  P  L  Y  G  G  T  S  T  L  M  D    659

GAAACCTACAAACCACATAAGAAATTCCGCGCTATGAGGCGCGACACTGGAGAAGCAGAGGCTCGTCCAGTGCAGCCGACGCCG  2309
E  T  Y  K  P  H  K  K  F  R  A  M  R  R  D  T  G  E  A  E  A  R  P  V  Q  P  T  P      687

TCGCCACAGCCGCTGCACCCGCACCCGGCCAGTCCGGCTCATCCGGCTCATTCGCCGCGACCACCGCGCATTTCTCTGTCATCCACG  2396
V  P  Q  P  L  H  P  H  P  A  S  P  A  H  P  A  H  S  P  R  P  P  R  I  S  L  S  S  T    716

CATTCGGTGCTCGCTAAAAGTTTAATGGAGGGGCCGAGAATGACTCCTGAACAATTAAAACGTACCGATATGATCCAGCAGTAC  2480
H  S  V  L  A  K  S  L  M  E  G  P  R  M  T  P  E  Q  L  K  R  T  D  M  I  Q  Q  Y      744

ATGCGGCGAAATGAAGCTGGTTCTAGTGTGGAAGGATGTACGCTGCGAACTGGAGGGCTGCTGACCTGCTACCGCGGTGCGTCT  2564
M  R  R  N  E  A  G  S  S  V  E  G  C  T  L  R  T  G  G  L  L  T  C  Y  R  G  A  S      772

CCGGCTCCGCCGCCAGTGCTGGCGCTGCAGGTGGACGTGACGGACGCGCCGCTGAACCTCTCCAAGAAGTCGCCGTCGCCGCCT  2648
P  A  P  P  P  V  L  A  L  Q  V  D  V  T  D  A  P  L  N  L  S  K  K  S  P  S  P  P      800

CGTTCGTACATGCCGCAGATGTTAGAGGCGTGAGGCCGGCCCGCGGAGCCGCGAGTCTCCGCCACCCCACCCCGACCTACTTTAAA  2734
R  S  Y  M  P  Q  M  L  E  A  -                                                            810

TTAAATAAACTAGATAATAGTAATCGTTCGCAGATCGTTTTAATATATTATATGTGATAATTCGTGTAGGACGTGTGCGTGCGTGGCC  2822

GGCGCCGAACGAGGAGAGCGCAATAGACTGAAAATTAACATAATTTAAGACTATATAAGTGTAATTTATAATACAATGCAGCGCC  2907

GGTCGTGGTGTGTTTGTGTGTATTGTATGTGCGTATGTGAGCGTGCTCAGTCATTTGTTTGT TAATATAATGACAGGTTGTCGAATTAA 2995

ATAAAACAAATTGTAAGAATT                                                                      3016
```

Figure 2.5. Comparison of the amino terminus or isoform-specific regions among the three isoforms of E75. The nucleotide sequence for the amino terminus of each isoform is shown. The amino acid sequences highlighted in bold indicate the common regions between these isoforms.

```
BmE75D
BmE75A
BmE75C                  M  Q  C  Y  P  K  L  S  P  K  R  E  P  P  E  G  L

BmE75D
BmE75A
BmE75C     Y  E  I  E  M  L  P  G  A  R  R  L  E  L  P  A  P  P  G  K

BmE75D
BmE75A                                                              M
BmE75C     E  F  R  A  P  V  L  L  A  G  P  S  L  A  P  T  H  S  V  I


BmE75D     M  T  V  T  E  C  H  H  Q  L  E  P  S  D  A  T  A  Q  S  P
BmE75A     S  P  D  S  S  Y  G  R  Y  D  V  P  T  S  V  D  H  S  L  M
BmE75C     Q  C  M  R  P  P  P  P  P  P  P  P  P  P  P  R  L  L  K  P


BmE75D     P  N  D  S  D  L  L  L  G  R  V  L  A  E  F  D  G  T  T  V
BmE75A     S  S  M  H  K  E  R  E  P  E  L  H  I  E  F  D  G  T  T  V
BmE75C     P  S  F  E  E  P  S  S  S  I  L  H  I  E  F  D  G  T  T  V


BmE75D     L  C  R  V  C  G  D  K  A  S  G  F  H  Y  G  V  H  S  C  E
BmE75A     L  C  R  V  C  G  D  K  A  S  G  F  H  Y  G  V  H  S  C  E
BmE75C     L  C  R  V  C  G  D  K  A  S  G  F  H  Y  G  V  H  S  C  E


BmE75D     G  C  K  G  F  F  R  R
BmE75A     G  C  K  G  F  F  R  R
BmE75C     G  C  K  G  F  F  R  R
```

BmE75C is 2.8 kb in length with an ORF of 753 amino acids (Fig 2.2). The N-terminus specific to the C-isoform is 90 amino acids in length and the 5' UTR has 228 bp.

Finally BmE75D is also 2.8 kb in length (Fig 2.4) with an ORF of 810 amino acids. The isoform specific region contains 33 amino acids, and the 5' UTR has 593 bp.

As already mentioned this isoform differs from E75A and E75C in the N-terminus (A/B region) of the open reading frame (Fig 2.5). Comparison of the three different isoforms in the N-terminus is shown in Fig 2.5. BmE75A and BmE75C have an identical 3'UTR of 358 bp which contains a canonical polyadenylation site however for BmE75D there is no poly-A tail and the 3' UTR consists of 346 bp (Fig 2.4).

Genomic organization of BmE75

Southern blot analysis was done on genomic DNA isolated from silk glands of the 5th instar larvae. Hybridization was carried out using the 204 bp region of the ligand binding domain amplified by primers P4 and RP3 (Fig 2.1). Although two bands are obtained with the EcoRI digestion, the same two bands are obtained with the EcoRI/HindIII double digest indicating a single gene which is supported by obtaining a single band in the HindIII lane. Therefore the three isoforms of E75 appear to be products of a single gene (Fig 2.6). The intensity of the 20 kb fragment is greater than the 2 and 7 kb genomic fragments because the entire probe is probably binding to the 20 kb fragment whereas only a protion of the probe is hybridizing to the 7 and 2 kb fragments.

Induction of BmE75A and BmE75C by 20-hydroxyecdysone

In *Manduca* and Galleria, E75A is induced by 20E within one hour in isolated abdomens and in 3 hours in fifth instar epidermis, respectively (Zhou et al, 1998; Jindra et

Figure 2.6. Southern blot analysis of genomic DNA extracted from the silk glands of 5th instar larvae. The DNA was digested with EcoRI, HindIII, and EcoRI/Hind III, hybridization was carried out using a PCR fragment generated with primers P4 and RP3 (Fig 2.1) at the 3' end of the cDNA clone. The exposure time was seven days.


EcoRI
HindIII
EcoRI/HindIII
20 kb
7 kb
2 kb

al, 1994). To determine whether the E75 isoforms isolated in *Bombyx* are also hormone-inducible, developmentally arrested pupal abdomens were injected with 20E and, following different time points the mRNA expression levels were examined by Northern hybridization.

BmE75A and BmE75C are both induced by 20-hydroxyecdysone albeit at different times, and are encoded by mRNA of similar sizes (3 kb; Fig 2.7). A similar mRNA size was observed in *Manduca* and *Galleria* upon induction, a 2.5 and 2.6 kb mRNA species, respectively. In *B. mori* the timing of 20E-induction for the A and C- isoforms of BmE75 differs, BmE75A is induced within 30 minutes and reaches maximal induction in 1 hour, whereas BmE75C is induced within 1 hour and reaches maximal induction in 2 hours (Fig 2.7). It remains unknown, whether BmE75D is induced by ecdysone, because the Northern hybridization for this fragment has not been done.

Expression of BmE75A, BmE75C, and BmE75D in the follicular epithelium of the ovary during vitellogenesis and choriogenesis

In order to examine the expression patterns of the different isoforms of E75 during oogenesis, RNA was extracted from pools of vitellogenic and choriogenic follicles and analyzed by Northern blot analysis.

During vitellogenesis BmE75A is expressed continuously but its expression level declines sharply at the beginning of choriogenesis (Fig 2.8).

Figure 2.7. Induction of E75 mRNA isoforms by 20E in ligated abdomens. The probes used in hybridization were isoform-specific probes generated by PCR with the following primers: for E75A: P2A; RP1, for E75C: P2C; RP1, and for E75D: P1D; RP1 (see Figs 2.1, 2.2, 2.3). Time is indicated in hours. The exposure times were: first panel, 7 days; second panel, 1 week; third panel, 1 day; last panel, 4.5 hours.

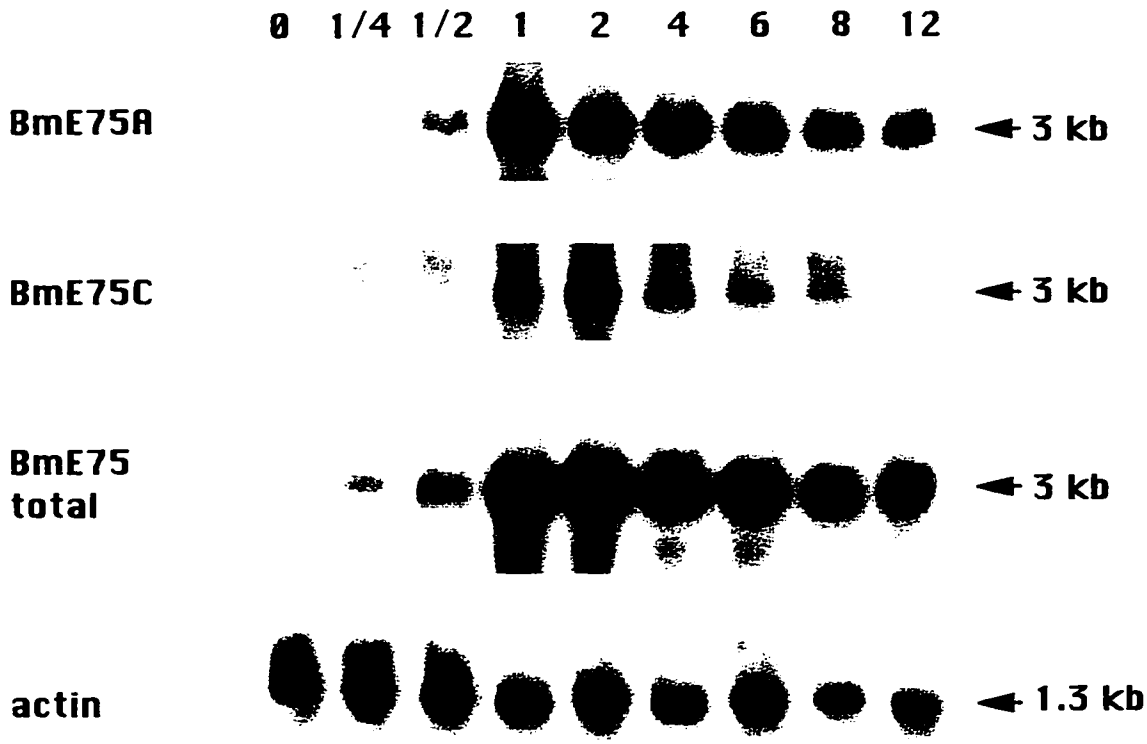
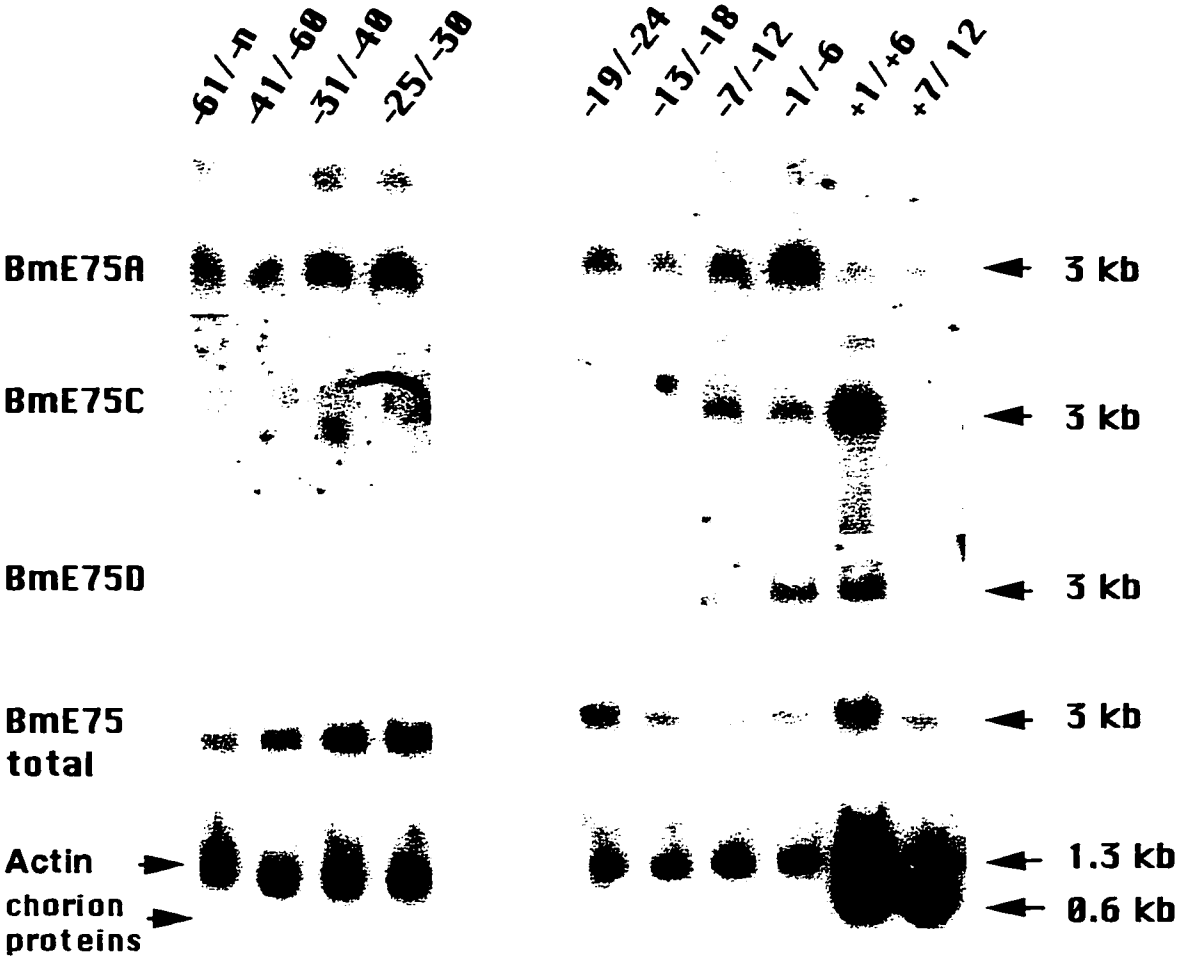

0 1/4 1/2 1 2 4 6 8 12
BmE75A
3 kb
BmE75C
3 kb
BmE75
total
3 kb
actin
1.3 kb

Figure 2.8. Expression of the A-, C- and D- isoforms during oogenesis. The probes used in this hybridization were isoform specific regions generated by PCR with the following primers: for E75A: P2A;RP1, for E75C: P2C;RP1, and for E75D: P1D;RP1 (see Figs 2.1, 2.2, 2.4). The exposure times were: first panel, 4 days; second panel, 4 days; third panel, 7 days; fourth panel, 1 day; bottom panel, 4.5 hours. Please note that the level of cytoplasmic actin RNA varies during vitellogenesis, it is not a result of unequal RNA loading. In addition there is an upregulation of cytoplasmic actin RNA in the beginning of choriogenesis (Swevers et al, 1995).

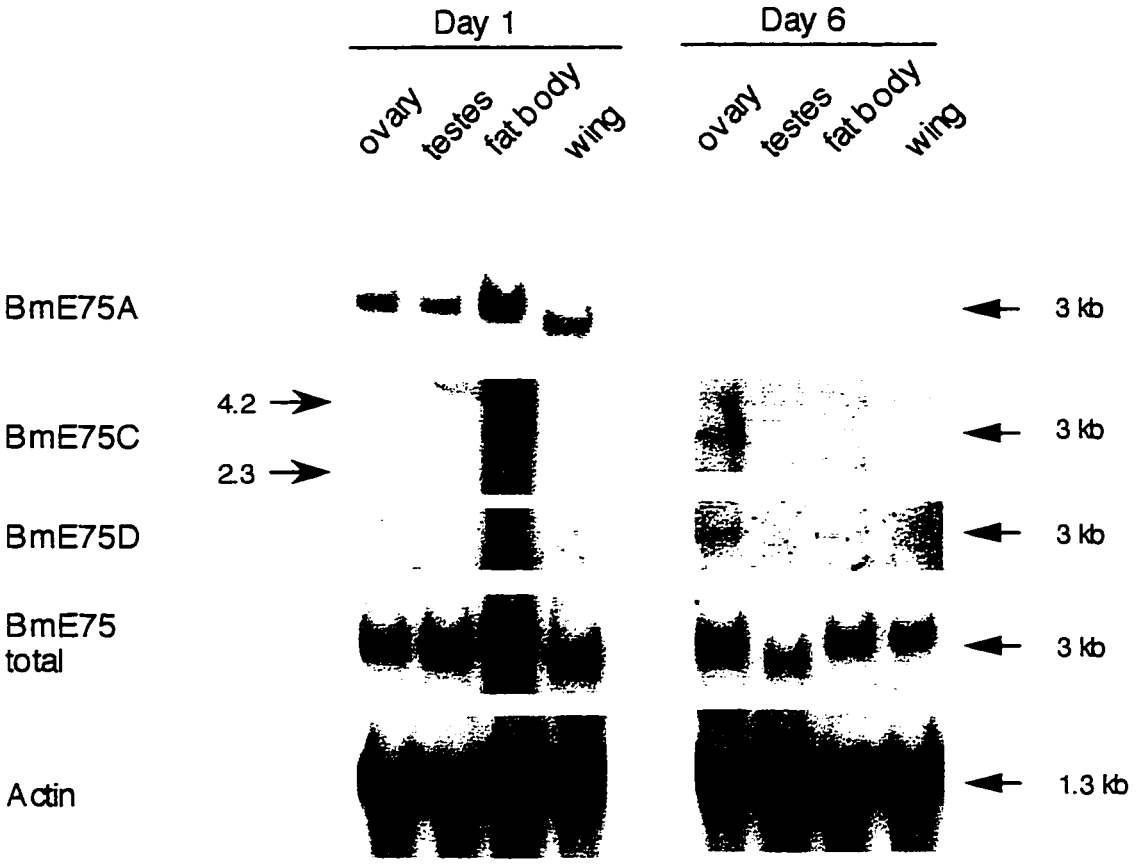

-61/-41
-41/-60
-31/-40
-25/-30
-19/-24
-13/-18
-7/-12
-1/-6
+1/+6
+7/ 12
BmE75A
BmE75C
BmE75D
BmE75 total
Actin
chorion proteins
3 kb
3 kb
3 kb
3 kb
1.3 kb
0.6 kb

In contrast, BmE75C is expressed only in late vitellogenic follicles (-12 to -1) and this is followed by an increased expression level at the beginning of choriogenesis. Moreover as quickly as BmE75C expression is induced, it is no longer present in choriogenic follicles at stage +7. This is coincident with the concomitant expression of early chorion genes at stage +1 of choriogenesis (Fig 2.8). A similar expression pattern is observed with BmE75D which is expressed abruptly during stages -6/-1 and is present in the beginning of choriogenesis, stage +1/+6, but disappears later on. Therefore BmE75D is expressed a few stages later than BmE75C. Also BmE75D mRNA does not appear to be as abundant as are the A- and C- mRNA isoforms.

Tissue Expression of the E75 isoforms

The expression of BmE75 was also examined in different tissues isolated from day 1 (when the ecdysone titer is high) and day 6 pupae (when there is a basal level of ecdysone). BmE75A is expressed in all tissues examined and has higher expression in day 1 pupae than in day 6 pupae, indicative that expression of this isoform follows the ecdysone titer (Fig 2.9). The C-isoform of E75 which corresponds to the 3 kb mRNA species (as observed during oogenesis, fig 2.8), is observed in the fat body of day 1 pupae. In day 6 pupae however BmE75C is present only in ovarian tissue. The D- isoform of E75 is not present in any of the tissues of day 1 pupae, and similar to BmE75C, it is present exclusively in the ovary of day 6 pupae. This suggests that the isoform BmE75D may in fact be ovary specific. Although BmE75C is induced by ecdysone (Fig 2.7), its temporal expression pattern does not follow the ecdysone titer, suggesting that other factors may play a role in regulating this isoform.

Figure 2.9. Expression of BmE75, the A-, C-, and D- isoforms in pupal tissues of day 1 and day 6 pupae (one day and six days after larval-pupal ecdysis respectively). The probes used were isoform specific probes generated by PCR with the following primers: for E75A: P2A;RP1, for E75C: P2C;RP1, and for E75D: P1D ;RP1 (see Figs 2.1, 2.2, 2.3). The exposure times were: first, second, third and fourth panels, 7 days; bottom panel 4.5 hours.


Day 1
Day 6
ovary
testes
fat body
wing
ovary
testes
fat body
wing
BmE75A
BmE75C
BmE75D
BmE75 total
Actin
4.2
2.3
3 kb
3 kb
3 kb
3 kb
1.3 kb

In day 1 pupae two other mRNA species, of 4.2 and 2.3 kb in size are hybridizing to the BmE75C isoform specific probe, which are present only in the ovary at low levels. The origin of these isoforms are unknown. The lower sized mRNAs obtained in the wing of day 1 pupae and testes of day 6 pupae are an artifact due to the uneven migration of the RNA in the gel.

DISCUSSION

Three isoforms of BmE75, A-, C-, and D, have been isolated and characterized. BmE75C and BmE75D are new isoforms of E75 that have not been previously isolated in other insect species. BmE75C is characterized by a proline rich region (28%) in the N-terminal domain. Considering the stage specific expression pattern of BmE75C during oogenesis (abrupt expression during late vitellogenesis and strong expression at the beginning of choriogenesis), this isoform may be an important transcriptional regulator of early chorion genes. Moreover, the overlapping expression pattern of BmE75D during stages -1 to +6 of oogenesis suggests that BmE75D may also have an important role in regulating early chorion gene expression, because it is expressed during the same short temporal period. Regarding BmE75D, this is supported by the ovary specific expression of BmE75D in day 6 pupae (Fig 2.9). Therefore, the fact that BmE75D appears to be an ovary specific factor raises the question that BmE75D may play an important role in initiating and/or regulating choriogenesis.

The expression of BmE75C and BmE75D is probably under the control of other protein factors in addition to 20E. Although BmE75C is an ecdysone inducible gene (Fig 2.7), when the titer of ecdysone is low in day 6 pupae, BmE75C is suddenly expressed

during the transitionary phase between vitellogenesis and choriogenesis. Similarily, BmE75D is only expressed in the ovary of day 6 pupae when the ecdysone titer is low and, during oogenesis, this factor is present only during late vitellogenesis. Therefore, because of their appearance during late vitellogenesis when the ecdysone titer is low in the hemolymph these isoforms cannot be regulated exclusively by 20E.

The Rev-erb family of proteins are orphan receptors (O'Malley and Conneely, 1992) and members of the steroid hormone receptor superfamily. These receptors are closely related to the *Drosophila* E75A, with the high amino acid identity in the DNA binding and ligand binding domains. They are also related to the ROR/RZR gene family (retinoic acid receptor-related orphan receptor) (Retnakaran et al, 1994; Forman et al, 1994; Dumas et al, 1994; Giguere et al, 1994). Rev-erb is encoded by the non-coding strand of the α-thyroid hormone receptor (TR) gene (Lazar et al, 1989; Miyajima et al, 1989) and its DNA binding domain contains two zinc fingers of the C_2C_2 type which is characteristic of the steroid and thyroid hormone receptors (Evans, 1988; Green and Chambon, 1988). Rev-erb does not seem to form heterodimers with the thyroid hormone receptor or the retinoid X receptor, but binds to the RevRE site as a monomer (Harding and Lazar, 1993). Rev-erb, as a homodimer, acts as a repressor when direct repeats of AGGTCA motifs separated by 2 bp (Rev-DR2) are placed upstream of various basal promoters (Harding and Lazar, 1995). Although Rev-erbα and Rev-erbβ can bind as a monomers to DNA and are inactive, RORα induces transcription when bound to the same response element as Rev-erb. Interestingly, when co-expressed, Rev-erb suppresses the transcriptional activity of RORα1 (Forman et al, 1994). Based on this information for

Rev-erb, and due to the high amino acid identity between BmE75 and Rev-erb (73%, see Table 2.1), the possibility exists that BmE75C and/or BmE75D may act as repressors to regulate other genes at the end of vitellogenesis and beginning of choriogenesis. Future experiments into the exact function of these factors will prove very interesting.

Recently the yeast two hybrid system was employed to find potential dimerization partners for BmE75 (Ito and Iatrou, unpublished). Using BmE75 (the E75 cDNA clone minus the isoform specific region), BmHR3A and BmHR3C were isolated indicating that interactions are occuring between these proteins *in vitro*. Although the question remains as to whether these interactions are true *in vivo*, these results suggests that cross talk is occurring among the nuclear receptors to facilitate the ecdysone mediated response.

The finding that BmE75 may interact with BmHR3 (K. Ito and K. Iatrou, unpublished), is also exciting because a link between ecdysone inducible genes and, the possible activation of early chorion genes is established.

In *Drosophila*, E75B (which lacks the first zinc finger of the DNA binding domain) interacts with HR3 and thereby prevents HR3 from activating the βFTZ-F1 gene (White et al, 1998). Although the B-isoform of E75 has been isolated in *Manduca*, *Galleria* (Jindra et al, 1994a; Segraves and Woldin, 1993) the B-isoform for E75 was not isolated from *Bombyx*. Thirty-nine clones were examined from the secondary screening of the vitellogenic follicular cell cDNA library, and only the A-, C- and D- isoforms were present. If BmE75B is expressed in the ovary at low abundance this may suggest that E75B does not play an important role in the ovary. However BmE75B may be an abundant transcript and have an important role in the other tissues targeted by 20E.

Southern blot hybridization (Fig 2.6) has shown that BmE75 is a single gene. Because the expression pattern of the three different isoforms during oogenesis is quite different (see Fig 2.8) these isoforms may be regulated by different promoters, in addition to alternative splicing.

Three genomic fragments of 1.5, 2.5, and 8 kb, of the BmE75 gene have been cloned. The 8 kb fragment contains the E75A exon however the other exons are not present in any of these isolated genomic clones (see Appendix C). Therefore, using these genomic clones as a basis the C- and D- exons and their promoters could be searched for.

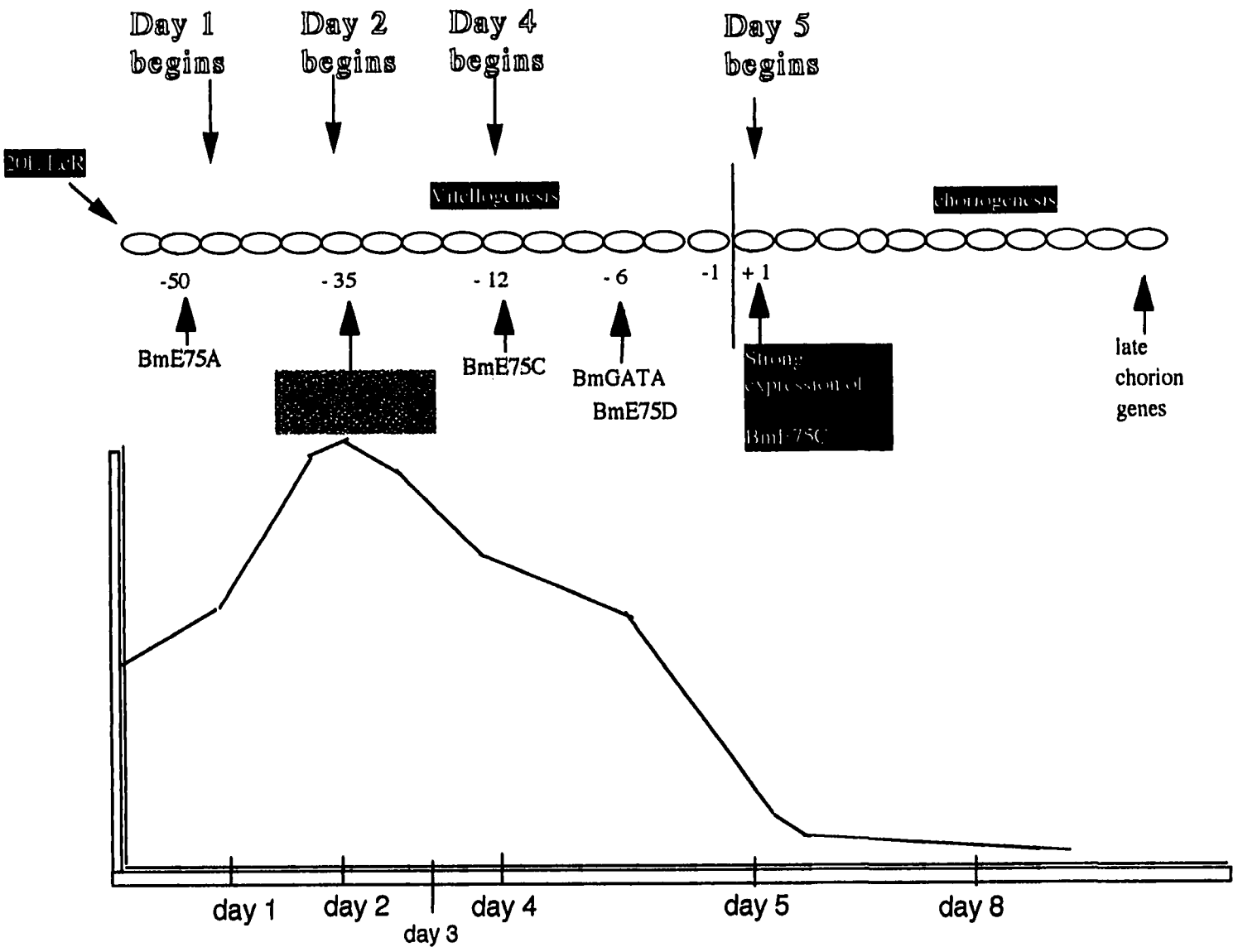
One important observation that has been made concerns a synthetic chemical analog of ecdysone (RH-5992; Retnakaran et al, 1995) which, when injected into ligated pupae binds to the EcR and arrests oogenesis during late vitellogenesis, at approximately stage -6 (L. Swevers and K. Iatrou, unpublished). The choriogenic program in these pupae is not initiated. In these follicles BmE75C is not expressed (L. Swevers and K. Iatrou). Therefore this system could be utilized for factors i.e. BmE75, suspected of being important for initiating choriogenesis. This could be examined by ectopically expressing BmE75C for example, in an *in vitro* culture system to see whether the arrested follicles can enter into choriogenesis. Hypothetically, this would occur on the contigence that the factor expressed ectopically would be sufficient to act alone to initiate the program of choriogenesis. Using this information as a tool, BmE75C and BmE75D could be examined with regard to identifying a function during late vitellogenesis.

Another way in which one could determine if BmE75C and BmE75D regulate early chorion gene expression is to search the promoter elements of early chorion genes for an RORE-like element. An RORE-like element has been found in the promoter of one early chorion gene (see Perspectives of Future Work). It has been previously shown that *Choristoneura* E75A, *Drosophila* E75 and the closest vertebrate homolog, Rev-erb bind to the same RORE -element (Palli et al, 1997; Horner et al, 1995; Harding and Lazar, 1993). Therefore, a bandshift assay using a RORE-element alone first to see whether binding occurs (as a control) and then with an RORE-like element from the early chorion gene could be used to address this question. In this manner more information would be accumulated in order to understand the possible function of the C- and D- isoforms of E75; whether they are involved in choriogenesis, specifically in regulating early chorion genes.

SUMMARY OF RESULTS

The goal of this Master's project was to isolate, clone and determine the temporal expression patterns of ecdysone-inducible genes during oogenesis. A summary of the results obtained are indicated diagrammatically in figure S, along with the ecdysone titer in the hemolymph during pupal development. Four cDNA clones containing high overall amino acid identities to ecdysone inducible genes from *Drosophila* were isolated. These include BmHR3A, and three isoforms of BmE75, A, C, and D. New findings from this study are: (1) we are the first to isolate the A-isoform of HR3 from a lepidopteran insect which is not inducible by 20E; (2) there is an upregulation of BmHR3A expression at stage -35 of vitellogenesis. A question raised is whether BmHR3A is receptor X (the receptor thought to be expressed in follicles at stage -35 and later, that provides the competence to enter choriogenesis); (3) BmHR3A expression during oogenesis parallels and is reciprocal to BmESP and BmGATA, respectively; (4) *in vitro* BmHR3A binds to a RORE-like element in the promoters of BmGATA, a transcriptional regulator of late chorion genes, and BmESP, an egg specific protein, suggesting that BmHR3A may be a negative and positive regulator of these genes respectively; (5) two new isoforms of E75, E75C and E75D, not previously found in any of the other insect species were isolated; (6) the abrupt expression of BmE75C and BmE75D before the beginning of choriogenesis raises the question of whether these factors regulate the expression of early chorion genes; and (7) BmE75D appears to be an ovary specific factor and, therefore, must play an important role in choriogenesis.

Figure S. Summary of results. The stages of oogenesis are indicated, the different isoforms are listed during oogenesis according to when expression occurs. The ecdysone titer is also provided for comparison to the expression of the different isoforms.


Day 1 begins
Day 2 begins
Day 4 begins
Day 5 begins
20E/EcR
Vitellogenesis
chorionogenesis
-50
-35
-12
-6
-1
+1
BmE75A
BmE75C
BmGATA
BmE75D
Strong expression of BmE75C
late chorion genes
day 1
day 2
day 3
day 4
day 5
day 8

Perspectives of Future Work

The isolation of four cDNA clones corresponding to ecdysone inducible genes is an important step to understanding the molecular events of oogenesis in *B. mori*. However the next step is to isolate the target genes directly regulated by these ecdysone inducible factors. A number of future experiments can be undertaken on the basis of the results obtained in this study. First, the chemically synthesized ecdysone analog RH-5992 which arrests oogenesis, during late vitellogenesis could be used in an *in vitro* culture system with ectopically expressed BmHR3, E75A, E75C and E75D, either alone or in different combinations in order to determine if the program of oogenesis can be restored in the arrested follicles. A second experiment would involve bandshift assays. with various regions of the early chorion gene promoters (in addition to searching for RORE-like elements), and the isoforms BmE75C and BmE75D, in order to determine whether these factors can bind; this would suggest regulation of the early chorion genes by BmE75C and BmE75D. One RORE-like element has been found in the intron of an early chorion gene ErB.5 (nucleotide position 263 to 273) which could be utilized in the immediate future in a bandshift assay with BmE75C and/or BmE75D proteins (Hibner et al, 1991).

Regarding BmHR3A the hypothesis was raised from this study that this factor may be the receptor X which triggers the developmental program for the follicles at stage -35 and later to enter choriogenesis. An interesting experiment would be to ectopically express BmHR3A in *in vitro* cultured follicles from stage -35 and earlier, and treat these follicles with hemolymph from more mature pupae. These follicles would then be analyzed by Northern blot analysis and if early chorion protein mRNAs are synthesized in

them but not the control (*in vitro* cultured follicles earlier that stage -35, treated only with hemolymph from more mature follicles), then this would suggest that BmHR3A may be receptor X. Future experimentation in determining the role of these ecdysone inducible factors in the sillkmoth ovary will prove very exciting.

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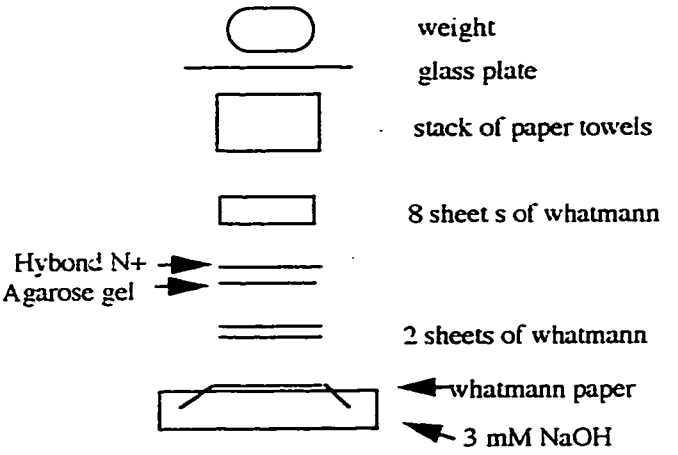
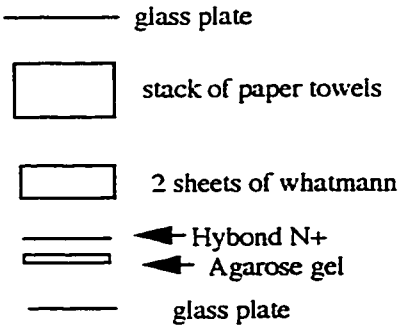
APPENDICES

APPENDIX A The transfer set up for RNA



APPENDIX B The transfer set up for DNA



Appendix C. Subcloned genomic fragments of the BmE75 gene. Three genomic fragments that were subcloned from the initial EcoRI fragment of 18 kb are shown, 8 kb EcoRI/HindIII, 2.5 kb HindIII, and 1.5 kb EcoRI/HindIII fragments. A fragment from the original 18 kb fragment remains to subcloned, as indicated in the figure. Different sets of primers were used in PCR reactions, in addition to sequencing, to deduce the presence of relevant gene sequences on the subcloned genomic fragments. Using primers P1A (5' CCTTCATCCCTTGTGACG 3', nt 1-18 of the BmE75A cDNA clone) and RP1 (5' CGTGGTACCGTCAAATT 3', nt 500-516 of the BmE75A cDNA clone), the E75A isoform-specific region was shown to be present on the 8 kb fragment. Moreover, the first zinc finger of the BmE75 DNA binding domain was found to be present on the 8 kb fragment, and to be separated from the E75A isoform-specific region by a 2 kb intron. Downstream of the first zinc finger is another intron, therefore the entire DNA binding domain is not present on this 8 kb fragment. The above information was obtained by using primers P3 (5' TTCCACTACGGCGTCCAC 3', nt 553-570 of the BmE75A cDNA clone) and T3 of pBS/SK+ in a PCR reaction, and by DNA sequencing using the P3 primer. No PCR products were obtained when using different combinations of primers (based on the BmE75A cDNA clone) with the 2.5 kb genomic fragment. Therefore it remains unknown as to what sequences this fragment contains. The 1.5 kb fragment contains the 3' end of the BmE75A ORF in addition to part of the 3' UTR as determined by PCR reactions with primers P4 (5' AGTCCGGCTCATCCGGCT 3', nt 1929-1946 of the BmE75A cDNA clone) and RP4 (5' ACGCACGCACACGTCCTA 3', nt 2390-2407). The question mark under

the 2.5 kb HindIII fragment indicates our uncertainty with regard to the sequences this fragment contains. The sequences of the portion of the 8 kb EcoRI/HindIII fragment labeled "remaining 4 kb" have yet to be determined as well.

Appendix C Cloned BmE75 Genomic Fragments

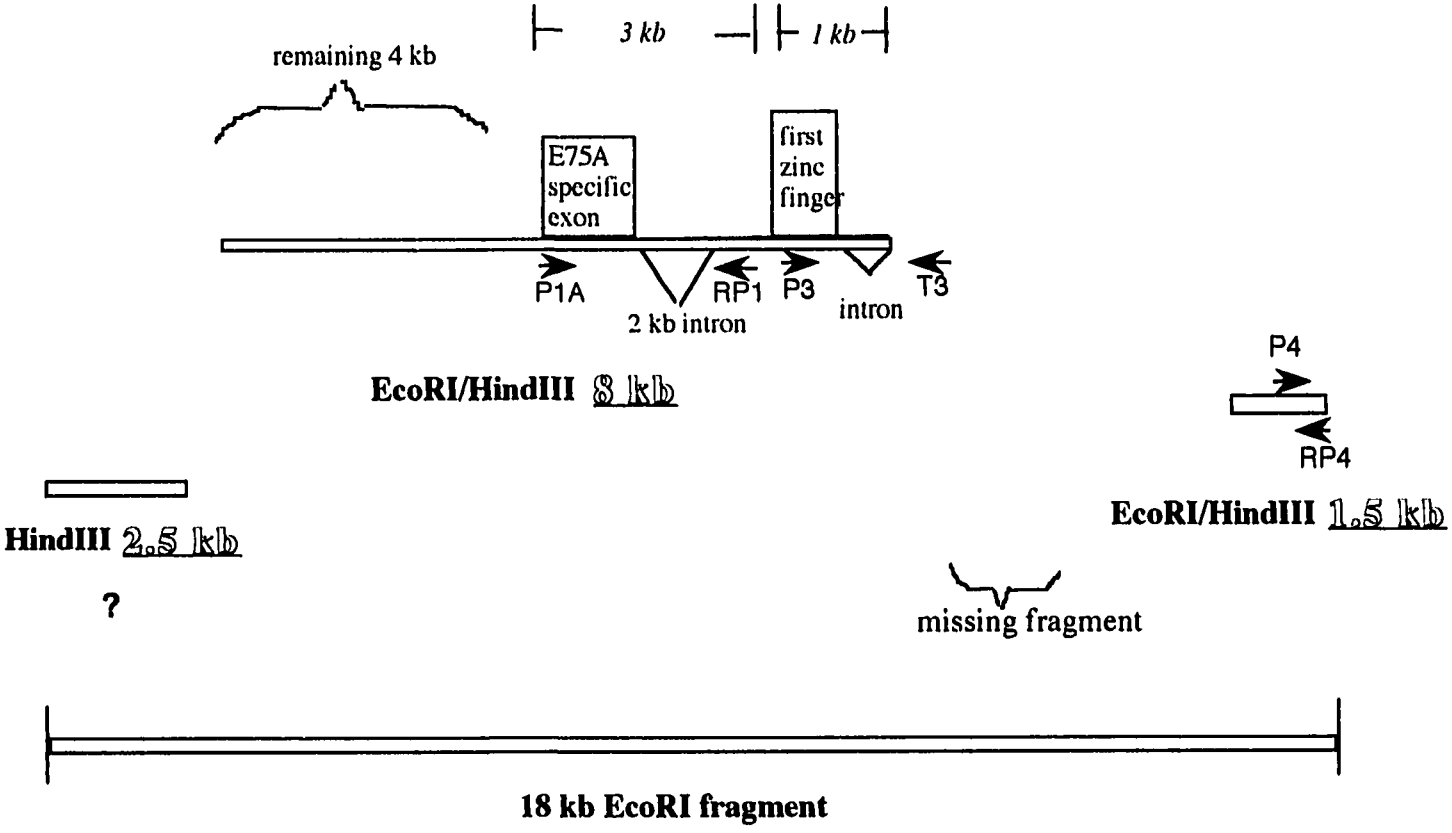
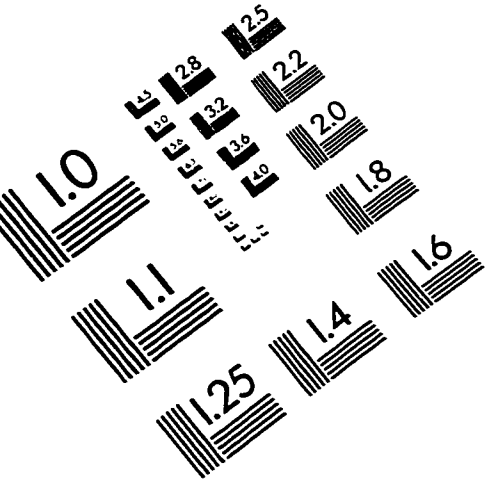
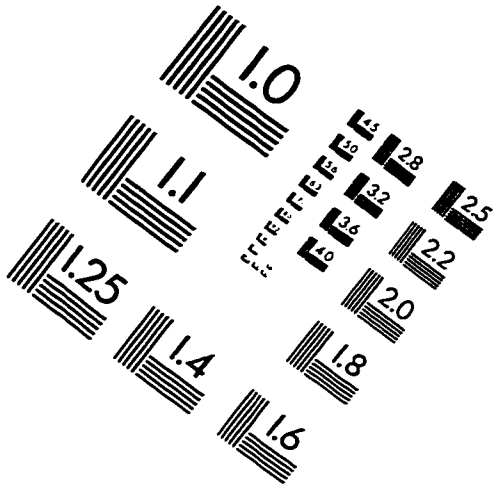
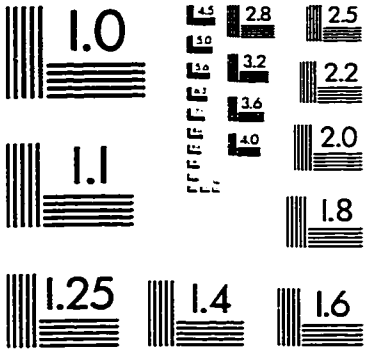
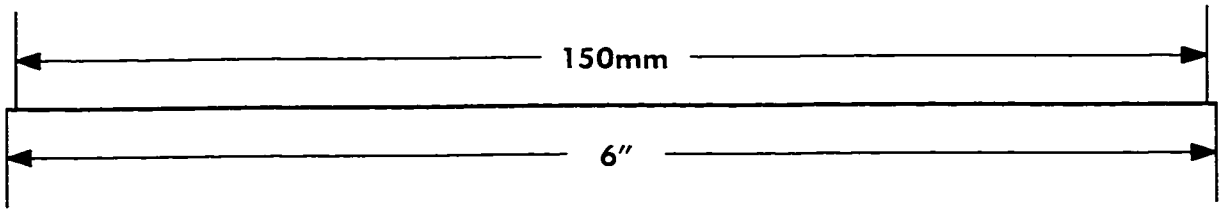


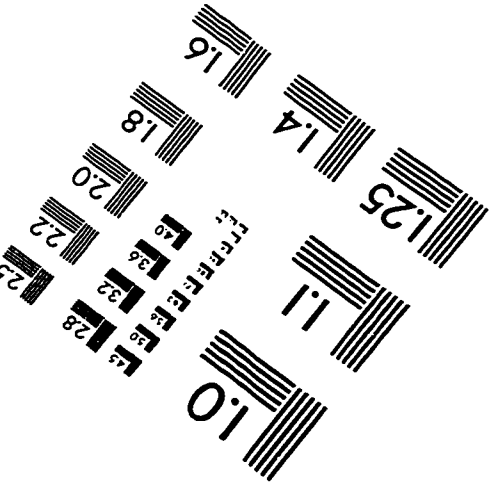
IMAGE EVALUATION
TEST TARGET (QA-3)

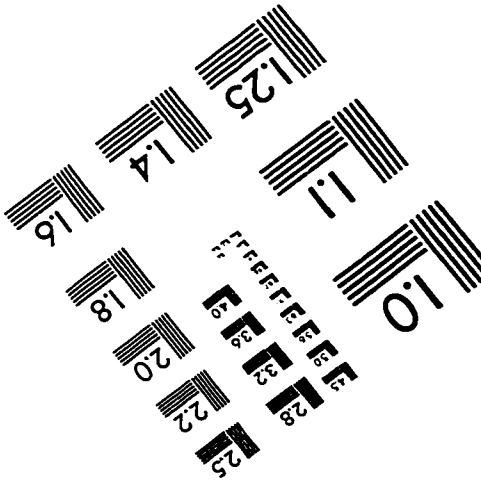












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