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MOLECULAR GENETIC DISSECTION OF JUVENILE HORMONE SYNTHESIS IN *DROSOPHILA MELANOGASTER*

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Abstract

Juvenile hormone (JH) is a major regulator of development and reproduction in insects. We aim at identifying molecular components of JH biosynthesis in the corpora allata (CA) glands and in its allatostatic cerebral regulation, which may serve as preferred sites for intervention for purposes of pest control. *Drosophila* serves here as an ideal model. We are focusing on the *apterous* (*ap*) gene because mutations in it cause female sterility due to JH deficiency.

We have adapted an in vitro bioassay for measuring JH synthesis by isolated glands of larvae and adult *Drosophila*. It has enabled us to identify a novel hormone (JHB₃) unique to the Diptera and to demonstrate its biological activity; to describe developmental parameters of CA activity and to demonstrate negative regulation of CA activity by cerebral neuropeptides that are transmitted to the glands axonally. Transduction of the signal operates via two mechanisms involving cAMP and Ca²⁺. Regulation is likely at the level of the terminal enzymes (epoxidases and terminal transferases) in the JHB₃ biosynthetic pathway. We are in the process of isolating these neuropeptides for sequencing and structure-function studies. We found that glands of *ap* mutants produce as little as 15% of the normal level of JH. Their gross- and ultra- structure is normal except that they exhibit characteristics of inactive glands and we presume that they are deficient in the above enzymes.

We found negative feedback in this system, in agreement with the accepted dogma of endocrine gland regulation, by demonstrating that the production of total JH by isolated CA is inhibited reversibly in the presence of exogenous JHB₃ and JH-III.

We showed that *ap* mutants are defective in certain sexual behaviors which are known in other insects to be JH regulated. Severity of the behavioral abnormality is correlated to the level of JH produced by the mutant.

Novel mutations were generated in the *ap* gene notably by P element insertions. Their phenotypic analyses indicated that the gene is involved in mutationally distinct functions required for pre-adult viability, JH related processes in the larvae and adult, and wing development. The P insertional mutants were used for molecular cloning of the *ap* gene. It was found to encode a protein containing a homeodomain as well as a tandem repeat of the novel cys/his-rich LIM domain. It thus belongs to the family of transcription factors where the LIM region facilitates binding of the homeodomain to the target DNA or serves to bind other proteins that modulate transcription. We presume that *ap* regulates the expression of distinct sets of genes involved e.g. in wing development and in JH-related processes. We are in the process of studying the temporal and spatial expression of the gene in wild type and *ap* mutants and have recently generated anti-*ap* antibodies that will facilitate studies of the cellular processes in which *ap* is involved.

Introduction

The juvenile hormones (JHs), are primary regulators of development and reproduction in all insects, participate in the regulation of the orderly and timely transition (molt) from one developmental stage to the next together with the steroid hormone 20-hydroxyecdysone and are major gonadotropic hormones in the adult. JH regulates the synthesis of yolk proteins in the female fat body and ovary and their uptake into the oocyte; it regulates the synthesis of various proteins in males and females that constitute essential components of the semen and the mature egg respectively; and it mediates the onset of mature sexual behavior in both sexes, presumably via regulation of synthesis and/or release of sex pheromones.

In view of the central role of JH in insect development and reproduction and its uniqueness to arthropods, it has long been recognized that intervention with the JH neuroendocrine system might be a useful approach for biological control of pest populations, replacing the commonly used and environmentally hazardous insecticides. Indeed, some juvenoids, synthetic JH mimics (notably methoprene), are now in use for pest control with some success and anti-juvenoids have been explored as well. However, a more detailed basic understanding of the molecular components of the JH neuroendocrine axis is clearly required for identifying preferred sites for intervention in the biosynthesis of JH itself for purposes of pest management.

Our collaborative research project aims at the molecular dissection of the JH neuroendocrine axis, employing biochemical-physiological, behavioral, genetic and molecular approaches. We chose to utilize *Drosophila melanogaster* as a model organism; although not a pest, it offers unique advantages for molecular-genetic studies over any other insect. Our analysis made use of mutants at the *apterous* (*ap*) gene. This gene is regarded as being implicated in the JH system because the previtellogenic arrest typical of *ap*⁴ homozygous females can be alleviated by external application of JH. This indicates that the mutant ovaries are capable of normal response to JH, but that these flies presumably suffer some deficiency of JH.

Original Objectives

Our original objectives composed four distinct experimental avenues. For convenience these objectives, are quoted here, for each avenue, from the original proposal:

"1) Biochemical-physiological approach: Develop an in vitro assay for measuring JH synthesis in isolated single corpora allata (CA) glands and use it for comparing JH production in wild type and mutant *ap* individuals. Parameters to be examined are: levels of JH production throughout larval development and during reproductive maturation of the adult; capacity of the gland for maximal JH synthesis following exogenous supply of a JH precursor; effect of the brain on CA activity when it is either axonally connected to the gland, disconnected from it or as brain extract; possible accumulation of intermediates in the JH biosynthetic pathway as a result of the cerebral effect or of the defect in *ap*; effect of *ap* mutant brains on JH production by wild type CA and vice versa; pharmacological effects on transduction of the cerebral signal into the CA. These measurements will be conducted also on several *ap* alleles representing classes of different phenotypic severity".

As outlined in the Results section, most of these goals have been achieved and were published in 7 papers which constitute part of this report (see Appendices 1-7). In addition, results regarding histolysis of the larval fat body have been obtained and published (see Results section).

"2) Behavioral approach: Use available assays for comparing sexual behavior of wild type and mutant *ap* adults. Parameters to be measured are: female attractiveness, female receptivity, male attractiveness to mature males, male courtship. These aspects will be examined during the first 3-4 days post eclosion during which adult reproductive maturation is attained. They will be studied in several *ap* mutants representing classes of different phenotypic severity".

As outlined in the Results section, all of these goals have been achieved and were published in 2 papers which constitute part of this report (see Appendices 8-9). In addition, preliminary results correlating the hormonal and behavioral defects of *ap* mutants have been obtained (see Results section).

"3) Genetic approach: Generate: a) very severe (lethal) mutations in *ap* that will cause complete loss of its function as well as mutations associated with chromosomal breakpoints in the gene; b) point mutations that will affect specific *ap* phenotypes (functions). Describe the phenotypes engendered by these novel mutation in various heteroallelic combinations for identifying possible roles of *ap* in early development and for elucidation of relations between presumptive functional domains in this gene. Phenotypes to be examined are: anatomy and morphology of embryos, larvae and adults; parameters of their JH biosynthesis and sexual behavior. We will also generate mutations in neighboring genes for characterization of the chromosomal region surrounding *ap* and mutations that will suppress the *ap* phenotypes thereby identifying genes whose products interact with the *ap* protein".

As outlined in the Results section, many of these goals have been achieved and are being prepared for publication. The rest are currently underway (see Results section).

"4) Molecular approach: Generate insertions of the P transposon into *ap* and use them for cloning of the gene. Characterize the structure of the gene; describe its temporal and spatial mode of expression (isolate cDNAs; do developmental RNA blots and in situ hybridization to tissue sections from various

stages of the fly's life cycle). Sequence the gene; identify upstream control elements; manipulate the gene by in vitro mutagenesis and study the resulting phenotypic effects in transgenic flies. Identify the sites of various *ap* mutations on the DNA map and compare their mode of expression to the wild type".

As outlined in the Results section, most of these goals have been achieved and were published in a paper which constitute part of this report (see Appendix 10). The rest are currently underway (see Results section). In addition, progress has been made towards characterization of the *ap* protein (see Results section).

Results

Biochemical-physiological approach:

We have adapted an in vitro radiochemical assay for measuring JH production by isolated individual larval ring-glands and adult corpora allata (CA) from *Drosophila*. This method is based on monitoring the incorporation of [³H]methyl (from labeled methionine) into JH (see Fig. 1) as identified by TLC of hexane extracts of incubation media, using unlabeled JH III and JHB₃ as markers. With this assay we have shown that:

1) The major product of the *Drosophila* ring gland and adult CA is a JH III bisepoxide (JHB₃, Fig. 1). JHB₃ is restricted to the higher Diptera and appears to act as a hormone as evidenced by its ability to rescue vitellogenesis when externally applied, at least as effectively as JH III. The details of the biosynthetic pathway leading to JHB₃ are unknown, but we hypothesize that farnesoic acid is its precursor. Several alternative routes can be envisaged (detailed in Fig. 1). The following observations we have made suggest that the terminal enzymes may be site of regulation of JH biosynthesis:

Production of Total JH (i.e. methyl farnesoate + JH III + JHB₃) is regulated during both the wandering larval stage and reproductive maturation of the adult following eclosion. The absolute increase in the biosynthetic activity (Total JH produced) of the glands in these stages is accompanied by a relative increase in the proportion of JHB₃ and a decrease in JH III and methyl farnesoate. Conversely, there is a decline in the biosynthetic activity of the gland associated with a relative decrease in the amount/proportion of JHB₃ and an increase in JH III and methyl farnesoate. Thus, regulation in both stages apparently operates via control of the levels (or activity) of the putative terminal enzymes as well as the availability of the rate-limiting precursor (farnesoic acid).

JHB₃, which has two epoxide functions, is unique to the higher Diptera. This suggests that the Dipteran terminal epoxidase(s) may have specificities differing from epoxidases found in other insect orders, and that this difference may serve as a preferable target for the control of dipteran pests.

2) The regulatory mechanisms described above are mediated by the brain via allatostatic neuropeptides which down regulate the biosynthetic activity of the gland. Axonal connections to the gland must be intact for this effect to be achieved. The structure of these allatostatic neuropeptides, their mode of action, the site of their production in the brain as well as the regulation of their synthesis and release in response to internal and environmental stimuli remain to be elucidated. They can be extracted from the brain by methanol and we have purified three allatostatic neuropeptides by RP-LC

(see Fig 2). These are being processed now for determination of their amino acid sequence. Extrapolating from published data on, and our own (Applebaum's) experience with other insect neuropeptides, we speculate that these allatostatic neuropeptides are ca. 20 amino acid long.

Once an amino acid sequence is available for one or more of these putative *Drosophila* allatostatins we new avenues will be opened for us for structure-function studies and for molecular analysis of the genes encoding them. Since these are intended for the near future they warrant brief mention here in order to put our achievements so far into a broader perspective and to highlight their contribution therein. Since the putative neuropeptides are short they could be synthesized them in vitro (with normal or modified sequence). Thus, we will utilize these synthetic peptides in order to initiate structure-function physiological and pharmacological studies of them. In parallel, the gene encoding the neuropeptide(s) will be isolated using oligonucleotide probes for screening head cDNA or genomic libraries. By analogy to other neuropeptides it is likely that the corresponding gene(s) encode larger proteins that may be processed post-translationally to give a family of related neuropeptides that can thus be unravelled. The cloned gene/cDNA will allow us to generate in vitro large quantities of the neuropeptide (normal or in vitro mutagenized) and will facilitate the structure-function analysis.

3) Presumptive transduction of the allatostatic signal to the glands has been studied to date in the larval ring-glands. Transduction operates via two mechanisms involving cAMP and Ca^{2+} . Incubation of the glands in the presence of agents which act to increase the intracellular levels of cAMP (e.g. 3-isobutyl-1-methylxanthine) resulted in a reversible reduction in hormone production that could be overcome by the addition of the JH-precursor farnesoic acid to the medium. Direct determination of the levels of endogenous cAMP in CA cells following incubation in the presence of allatostatic factors could not be performed because the larval CA is inseparable from the larval ring gland which contains other endocrine glands as well. The involvement of Ca^{2+} was demonstrated by incubating the ring glands in the presence of a Ca^{2+} channel blocker (lanthanum chloride) and in the presence of Ca^{2+} - free medium containing EGTA. These treatments abolished JHB₃ production completely in a manner reversible by inclusion of farnesoic acid in the medium.

4) Homozygotes for the non-vitellogenic *ap*⁴ mutation produce absolute levels of Total JH as little as 6-fold less than the wild type level in adults, and about half of the wild type level in larvae. This is a chronic effect rather than a delay in the onset of JH production. The absolute low level of Total JH in the mutant is associated with a decrease in the proportion of JHB₃ and an increase in JH III and methyl farnesoate, indicating that in *ap* mutants the activity or availability of the terminal epoxidases in the JH biosynthetic pathway is reduced. Interestingly, preliminary observations suggest that cerebral regulation of CA activity is defective in *ap* mutants.

5) The above mentioned functional studies of the endocrine glands during the larval-pupal-adult transformation were correlated with their ultrastructure. Thus an ultrastructural analysis using light, scanning, and electron microscopy of the larval ring gland (which composes, in addition to the CA, the prothoracic gland, PG, where ecdysone is produced) has been conducted in wild type animals and *ap* mutants. Special attention was paid to the morphological changes that the glands undergo during metamorphosis, namely degeneration of the PG and migration of the CA to a new position in the adult. We found that the normal, active PG, displays several unique ultrastructural features that reflect

increased steroidogenic activity, including extensive infoldings (intracellular channels) of the cell membrane; an increase in both smooth endoplasmic reticulum (SER) and elongated mitochondria. PG degeneration is characterized by: a gradual decrease in its ability to synthesize ecdysteroids; a decreasing quantity of SER and mitochondria; the absence of intercellular channels; cytoplasmic fragmentation; and the separation of the PG from the CA and corpus cardiacum. By contrast, the PG of third instar larvae of the conditional larval lethal *l(3)ecd^{1u}* not only fails to produce ecdysteroids at normal levels at the restrictive temperature (29°C), but also acquire abnormal morphological features, e.g. an accumulation of lipid droplets, a disappearance of SER and a drastic reduction of membrane infoldings.

Similar analysis was conducted on glands of the mutants *ap^{56f}* and *ap⁴*. The mutant CA apparently have normal size and gross morphology. However the amount and size of the lipid droplets, putative depots of the precursors of JH, in the *ap^{56f}* CA are positively correlated with the insect's age but are also present in much lower quantities than in wild type glands. The ultrastructural picture of the adult CA of *ap⁴* reveals features typical of inactive glands, namely low concentration of both smooth endoplasmic reticulum, mitochondria and mitochondrion-scalariform junction complexes indicative of precocious degeneration in agreement with our above mentioned results that this non-vitellogenic mutant is JH-deficient. In contrast to *ap⁴*, adult *ap^{56f}* homozygous females are fertile and the degree of oogenesis is indistinguishable from that of wild type adults, although in vitro production of JH by the mutant gland is much lower than that of wild type glands. The ultrastructural profile of the *ap^{56f}* CA is also similar to that of wild type CA. Some possible explanations for the apparent lack of correlation between CA ultrastructure and function in *ap^{56f}* as compared to *ap⁴* are discussed in the accompanying paper.

6) We have also examined another phenomenon defective in *ap* flies, namely histolysis of the larval fat body (for background see our original grant proposal), in particular its hormonal control by JH and ecdysteroids. We have compared it in wild type animals and in JH-deficient (*ap*) and ecdysone-deficient (*ecd¹*) mutants. We found that fat body cells of wild type (C-S), *ap^{56f}/ap^{56f}*, *ap^{1s78j}/ap^{1s78j}*, *ap⁴/+* and *ecd¹/ecd¹* females all underwent histolysis normally during the 72 hours following adult eclosion. Only *ap⁴/ap⁴* adults failed to histolyse cells normally. Attempts to demonstrate histolysis in vitro were unsuccessful, even in the presence of juvenile hormones, larval ring glands or adult ovaries. In all of the genotypes examined other than *ap⁴/ap⁴* a significant proportion of larval fat body cells were dead at any time. In the *ap⁴/ap⁴* animals almost all cells remained viable. Experiments with homozygotes for the temperature sensitive allele *ap^{1s78j}* have shown that the temperature sensitive period for histolysis is early in pupal development (ca. 72-96 hours before adult eclosion for an upshift from the permissive to the restrictive temperature, 18°C -> 27°C). This is in agreement with the temperature sensitive period we have observed for both JH synthesis by the adult CA as well as for the aberrant female sexual behavior of this mutant.

These data suggest that fat body histolysis in *Drosophila* may not be a strictly JH dependent process as has been thought, rather that it may be another phenotype unique to the *ap⁴* allele. This possibility will be critically examined, now that an apparently true-null *ap* allele (lacking *ap* transcript, see below) is available to us.

7) We have initiated an investigation of the regulatory interplay between the JH and ecdysteroid systems by examining the ability of JHB₃ and its putative precursor JH-III, to modulate the in vitro production of both ecdysteroids and JHs by isolated ring glands, and brain-ring gland complexes, of third instar wild type larvae. We found that both JHB₃ and JH-III inhibited the in vitro production of ecdysteroids by these ring glands and brain-ring glands complexes in a reversible manner, although JHB₃ had greater efficacy. Methyl farnesoate, the precursor of JH-III and JHB₃, did not affect ecdysteroid production. Likewise, the production of total detectable JH (JHB₃ + JH-III + methyl farnesoate) by the corpus allatum portion of the isolated ring gland was also inhibited reversibly in the presence of exogenous JHB₃ and JH-III, but not methyl farnesoate.

Taken together these data are indicative of negative feedback in this system, in agreement with the accepted dogma of endocrine gland regulation.

In view of our finding (see below) that the *ap* gene encodes a transcription factor we hypothesize that the normal role of this gene in the JH neuroendocrine system is to either regulate expression of genes encoding these enzymes in the CA cells or of genes encoding allatostatic neuropeptides in the brain. Molecular genetic tools which we have generated during the past three years (see below) will allow us to examine this hypothesis more directly.

Behavioral approach:

The JH deficiency in *apterous* (*ap*) mutants offered an avenue for studying the role of JH in *Drosophila* reproductive behavior. To that end, using established procedures for assaying sexual behavior, we have assessed female attractiveness (sex appeal), female receptivity, male sex appeal and male courtship in various *ap* alleles during the reproductive maturation through the first three days after eclosion and compared them with the behavior of wild type flies.

Briefly, our results indicate that:

- 1) Mutations in *ap* prolong the period of young male sex appeal to mature males, reduce both male courtship and mating propensity.
- 2) Mutations in *ap* do not affect female sex appeal but reduce their receptivity.
- 3) These behavioral defects are observed also in hemizygotes and in various heteroallelic combinations indicating that they map to the *ap* gene and do not result from genetic background effects.
- 4) These behavioral anomalies do not result from the wing defect typical of *ap* mutants since: a) They are manifested also when the *ap* mutant is examined as a courtship object, a status that does not involve wing vibration; b) They are evident even when the wing vibration component is subtracted from the performance data of the control wild type flies; c) They are not displayed by two other wingless mutants we have examined: *vg* and *nub*.
- 5) The level of JH synthesis in different *ap* mutants during reproductive maturation appears to correlate with the severity of their abnormal female receptivity. The correlation to other *ap*-specific behavioral symptoms currently being examined.
- 6) In preliminary experiments external application of a JH analog to the mutant flies appears to be

capable of restoring at least one aspect of their normal reproductive behavior, namely female receptivity. This indicates that the behavioral symptoms of *ap* and its hormonal defect are causally related.

7) In a further attempt to correlate *ap* hormonal and behavioral defects we have used the temperature-sensitive allele *ap^{ts78j}* and examined the sexual behavior of these adult flies following different regimens of temperature shift-up and shift-down during their larval and pupal development. Our results suggest that the temperature sensitive periods (TSP, i.e. the period during which the *ap*⁺ gene product is required) for both the hormonal and the behavioral effects of *ap* mutations are similar, namely at the first half of the pupal period. These results lend further support to the suspected causal relation between the hormonal deficiency and the aberrant reproductive behavior of *ap*.

Genetic approach:

A. Mapping the chromosomal region surrounding *ap*: An EMS mutagenesis (F2 lethal screen) has been conducted aimed at obtaining point mutations in *ap* and in the surrounding chromosomal region. Point mutants in *ap* induced alleles are likely to affect individual functions if, as suggested in our original grant proposal, discrete functional domains do exist in *ap*, and will allow us to study each presumptive subfunction separately. The additional benefit from this approach is the recovery of lethal mutations in other genes uncovered by the chromosomal deletion (abbreviated hereafter Df-2) used as a tester in this scheme. Thus, a detailed map of the genetic region surrounding the *ap* gene can be derived.

This mutagenesis has yielded 93 independent mutations (out of 5539 chromosomes screened). They were mapped using available deficiencies that delete parts of this chromosomal region (Fig. 3) and have been subsequently crossed to each other in order to establish which belong to the same gene (i.e. complementation group). The result of this mapping and complementation analysis are presented in Fig. 4. In total the mutants generated identify 57 complementation groups, three of which are previously identified genes in the region (*ap*, *nap* and *EcR*, see Fig.4). Considering the size of the deletion used for this screen (Df-2, see Fig. 3) this is a relatively large number. Nevertheless it is evident from Fig. 4 that we have not reached yet a saturation of the region, since many complementation groups are represented by only one mutation. Using a variety of calculations based on Poisson's distribution we extrapolate that at most 26 additional genes exist in this region.

It is interesting to note that the distribution of the complementation groups in the region is not uniform, e.g. one sub-region (B, see Fig.4), which appears to constitute ca. 10% of the entire region studied, contains as many as 21 complementation groups. Another interesting observation is that 8 of the mutants display a complex pattern of complementation since they do not complement two (or more) complementation groups that by other criteria appear to be distinct. The nature of this phenomenon is unclear and is reminiscent of complex genes in *Drosophila*. Simple calculation rules out the possibility that these are all double mutants. These results are being prepared for publication.

B. Induction of novel mutations in *ap*: Three mutageneses have been carried out using a) EMS, b) X-rays or c) P element insertions. a) The above mentioned EMS mutagenesis has yielded also 5 new mutation in the *ap* gene. b) X-rays have been used to generate *ap* mutations associated with gross chromosomal rearrangements that are likely to disrupt the *ap* gene more severely than the preexisting

recessive *ap* alleles and cause complete loss of its function. In addition, breakpoint-associated alleles can subsequently serve as helpful physical landmarks for the molecular cloning of the gene. Of the five breakpoint-associated *ap* alleles we have generated one involves a translocation to the Y chromosome and one is a small deletion surrounding the *ap* chromosomal region. All but the latter appear to be clean breaks in the *ap* gene and are apparently not associated with any minute rearrangements involving nearby genes, as determined by the fact that they do not elicit any mutant phenotype when crossed to mutants in genes flanking *ap*: c) A hybrid dysgenesis protocol has been used for generating in vivo insertional mutations of the P transposable element into the *ap* gene. The aim of this mutagenesis was two-fold: First, P insertional mutations can serve as tool for cloning the gene (see below). Second, insertions of a transposable element into the gene will probably cause a major disruption of its structure and function, and is thus likely to be a highly desired severe or even null mutation. In the initial scheme we have used the $\pi 2$ strain as a source of P elements and obtained five independent insertional *ap* mutations by screening, following a test cross, for the reduced wing phenotype. These strains have been outcrossed and processed for stabilizing the transposon, for eliminating irrelevant P insertions into other chromosomal sites. Several spontaneous revertants have been recovered from each of the insertional alleles. They are traditionally presumed to be partial or complete excisions of the original insert. A second, more extensive scheme involved a combination of the Birm-2 and the $\Delta 2-3$ strains which became available at that time and were shown to be more potent in mobilizing P elements. This mutagenesis has yielded 19 independent insertional *ap* mutations, following a similar screen for aberrant wing morphology in a test-cross. A few of them were recovered in F2 as lethals in combination with the tester *ap* allele. Several revertants have been recovered by now from two insertional alleles from the second set by subjecting them to another round of hybrid dysgenesis and screening for restoration of normal wing morphology.

The new *ap* alleles have been characterized in terms of their phenotypes (development, wing morphology, fertility, longevity) as homozygotes, hemizygotes and in heteroallelic combinations with other, preexisting as well as novel, *ap* alleles. The conclusions are common for mutants generated by the three mutageneses and have been presented in great detail in our previous progress reports. They will therefore be presented herein briefly. The fact that we observe similar phenomena in *ap* with EMS-induced (presumed point) mutations as in transposon and X-rays induced mutations indicates that these phenomena are most likely not the result of unrelated major disruptions in the *ap* chromosomal region but represent genuine aspects of the structure and function of the *ap* gene.

The most salient observations and conclusions are: a) A new *ap*-specific phenotype was observed namely developmental lethality. Embryonic development is not affected in these lethals (as evidenced by the normal hatching of the mutant larvae from the egg), but the larvae become gradually lethargic, their development is prolonged and they eventually die without discernible external defects. These observations raise the possibility that function of the *ap*⁺ gene product is not essential during embryogenesis but is indispensable for vital, conceivably endocrine, functions during larval and possibly pupal development, in addition to the role of *ap* in the adult endocrine system. Hormone levels in the lethal larvae remain to be examined. Since we can not be certain at present that these new lethal alleles represent true null mutations in the *ap* gene, we are examining the possibility that maternal contribution of normal *ap*⁺ product allows the mutant embryos to survive through the embryonic stage,

by eliminating it and looking for prelarval death that would indicate requirement for *ap*⁺ activity in the embryo

b) The phenotypes of the new mutations have revealed interesting complexity in the function of the *ap* gene: we found that the gene can apparently be mutable independently with respect to the three major phenotypes elicited by its mutants, namely lethality, wing defects and hormonal abnormalities (exemplified by non-vitellogenesis). For example, *ap* mutants could be obtained that in various heteroallelic combinations display only lethality while the wings and fertility of the few escapers appear unaffected (e.g. *ap*^{31m}/*ap*^{8b} and *ap*^{3oj}/*ap*^{8a}, Fig.5). Likewise, in many heteroallelic combinations we find apparent interallelic complementation, e.g. the lethal alleles *ap*^{11d} and *ap*^{9e} or *ap*^{31m} and *ap*^{13c}, which are lethal by themselves (as hemizygotes), are completely normal in every aspect in combination with each other (Fig.5). In yet other combinations the only defect of the adults appears to be female sterility associated with a JH-rescuable previtellogenic arrest. Thus, the new mutations have uncoupled the different functions of the *ap* gene. In accordance, the temperature sensitive period for the wing defects in the temperature-sensitive *ap* allele (*ap*^{ts78j}) was shown to be distinct from the temperature sensitive period for the JH-related abnormalities (2nd - 3rd larval instar and early pupal stage, respectively). Furthermore, no apparent correlation was found among many *ap* alleles between the severity of their wing defects and of their JH-related symptoms mentioned above.

Based on similar observations with other complex genes in *Drosophila* we speculate that the distinctness of the different *ap* functions reflects complexity of its structure or expression. One possibility is that *ap* has a single promoter but a complex pattern of alternative splicing giving rise to several different transcripts each encoding a protein with a different function. An alternative possibility is that the gene is rather small, encodes an essentially single type of transcript but has multiple, differentially utilized promoters, each being specific for a different tissue/stage during the life cycle. The present state of knowledge on the molecular biology of the gene (see below) is insufficient for clear-cut distinction between these options. Our future molecular analysis of the gene aims at these issues.

C. Search for new genes interacting with *ap*: An EMS mutagenesis is underway for generating second-site suppressors of *ap* mutations. These are likely to identify new genes that participate in processes common to the *ap* gene, some of which may well be novel endocrine genes. Two slightly different schemes are being conducted, both utilizing the wing phenotype (i.e. screening for its improvement). Five putatives have been isolated in the first scheme and 7 in the second (out of ca. 15,000 chromosomes screened in each). They are being grown out for verification of their ability to suppress the *ap* wing defect. We are also planning a scheme to isolate second site suppressors that will suppress the *ap*-associated sterility. The two different approaches may yield distinct classes of genes interacting with *ap*. Once verified, the suppressors will be mapped and examined for their own phenotype when homozygous. Their phenotypic effects on *ap* will be compared to their effect on the timing and tissue distribution of *ap* expression using probes available for detection of *ap* transcripts (see below).

Molecular approach:

We have initiated cloning of the *ap* gene using a genomic library obtained from one of our P element-induced *ap* alleles and probing it with a P-probe. While this was underway we learned that

Dr. S. Cohen (Baylor College, Houston), who is interested in genes regulating the development of imaginal discs, has inadvertently obtained, in an "enhancer trap" experiment, a P-lacZ insert with interesting β -galactosidase staining in the larval imaginal discs (see below) which turned out to be an insert in *ap* (based on its wingless phenotype when homozygous and lack of complementation to other *ap* alleles). Subsequently, another P-lacZ insert in *ap* has been obtained by us. Part of the initial characterization of the gene, described below, has been conducted in collaboration with him and recently published (relevant figures are found therein). Further work on the molecular biology of *ap* is being conducted in our lab.

1) The genomic *ap*-specific sequences were used to isolate cDNA clones from a larval library. Two classes of cDNAs were obtained, 3.2 and 4.3 Kb long. Restriction mapping and sequencing provided the following picture: The transcription unit spans at least 21 Kb and its 5' control region has not been identified yet. It contains 4 exons and the two cDNA classes appear to differ only in their 3' untranslated region. Northern blot reveals a 6 Kb *ap* transcript in pupae and adults, suggesting that additional exons (presumably 5' to those shown in Fig. 1 of Appendix 10) remain to be identified (Shtorch and Segal, unpublished). The predicted amino acid sequence of the protein includes a homeodomain which is a motif typical of many DNA binding proteins that serve as regulators of transcription. In addition *ap* has two novel Cys/His-rich regions known as LIM domains which were initially defined in the genes *lin-11*, *isl-1* and *mec 3* all of which contain also a homeodomain and whose protein products were shown to bind DNA in a sequence specific manner. It is believed that the LIM domain facilitates interaction of the transcription factor with the DNA or with other proteins that may modulate its activity. The *C. elegans* genes *lin-11*, and *mec 3* function in the specification or maintenance of cell fate during development of the nematode. Thus, it is tempting to speculate that the *ap* gene product(s) regulates the expression of distinct sets of genes, one involved in wing development and the other in endocrine functions.

2) We have started to map the site of mutations within the cloned *ap* gene, focusing first on *ap* alleles associated with P insertions or with X-ray induced chromosomal breakpoints that should be identifiable by Southern blot. To date, the site of one insert (*ap^{rK568}*) has been mapped to 42 bp upstream of the 5' end of the longest cDNA. This is in accord with the preference of P elements to insert in control regions. None of the 22 other mutants examined so far fall within the cloned 21Kb of *ap* DNA and we presume that they will be found further upstream.

3) The P-lacZ inserts in *ap* have allowed us to examine the normal pattern of *ap* expression as reflected in β -galactosidase staining. Similar results have been obtained by in situ hybridization to embryos and imaginal discs using an *ap* probe. Expression of *ap* in the embryo has a complex and dynamic pattern starting from the stage of germ band extension. It is expressed in the peripheral as well as the central nervous system of the embryo and larva. Its transcripts can be found in most of the imaginal discs, usually occupying regions destined to give rise to the distal portions of the corresponding appendages. This pattern of *ap* expression in imaginal discs is in accord with the prediction that in normal development *ap⁺* is required in those cells which in *ap* mutants are defective or missing as deduced from the wing and haltere phenotype of the mutants.

4) Similar analysis of *ap* expression in the wild type adult has just started with special emphasis on

the brain and neuroendocrine structures. Our preliminary observations indicate that in the head, most β -galactosidase staining occurs throughout the cellular cortex of the brain and adjacent neural tissues (cell-body layer of the lamina, first and second optic chiasmata, antennal glomerulus and antennal nerve, accessory pharyngeal nerve).

5) Preliminary observations with P-induced *ap* alleles suggest that the pattern of *ap* expression in them is normal at the embryonic and larval stages (except *ap^{ka}*). This may imply either that the lesions in the mutants studied is in the *ap* protein or that *ap* function may be dispensable during the embryonic stage (in accord with the above mentioned observation on the apparently normal embryogenesis of the null and lethal *ap* alleles). It is difficult to extend this second possibility, namely of the distensibility of *ap*, to the imaginal disc since it is obvious from the wing defects that *ap* is required in them. One way for distinguishing between these possibilities is to examine the pattern of distribution of the *ap* protein in these tissues. This requires raising antibodies to the *ap* protein (see #7).

6) We have initiated a functional and molecular dissection of the 5' control region of the gene by generating excisions of the P element in those *ap* mutants where the transposon has inserted upstream of the coding region. A significant portion of such excisions are imprecise and delete also some flanking genomic sequences and these are easily identified by PCR. By correlating the location and size of the deleted 5' sequences in these excisions with their phenotypic effects on the organism we hope to arrive a better understanding of the regulation of *ap* expression.

7) Generation of anti-*ap* antibodies: The entire coding region of the *ap*-cDNA as well as three fragments of it corresponding to the protein's N-terminus, center and C-terminus, were subcloned into the pATH-2 expression vector. Upon induction of the bacteria with β -indol acrylic acid the *ap*-protein or its portions are produced as proteins fused to the N-terminus of the trp-E protein. We have semipurified the fusion protein by separation on SDS denaturing acrylamide gel, excised the corresponding band and injected it into rabbits. Antibodies were obtained directed towards the entire *ap* protein. They recognize the denatured form of the protein as determined by Western blot analysis. The antibodies are currently being tested in immunoprecipitation assays using extracts from *Drosophila* cultured S-2 cells and enriched nuclear fractions from embryos and adult flies. They will soon be used in Western blot and immunohistochemistry to monitor the quantity of the *ap* protein and its spatial distribution, respectively in wild type and *ap* mutant animals. They will also be useful tools for identifying novel proteins that complex/interact with the *ap* protein as well as for identifying possible qualitative changes in the *ap* protein in the various *ap* mutants.

Evaluation of research achievements

Most of the goals set have been achieved and were published: The *in vitro* assay for measuring JH synthesis in isolated single corpora allata has allowed us to study parameters of hormone synthesis during development and reproductive maturation in wild type and *ap* mutant *Drosophila* and demonstrate that the fruit-fly is a useful model for molecular genetic analysis of this system in other insects not amenable to these approaches. Our work has set the stage for detailed examination of the steps in JH biosynthesis which are likely to be targets responsive to intervention for purposes of pest control. To that end we plan to synthesize specific inhibitor of different components in this pathway. We have also been successful in demonstrating cerebral regulation of CA activity and are now working on characterization and molecular analysis of the corresponding neuropeptides. This work should lead to identification of the cerebral sites of synthesis of these neuropeptides and hence will open the way to the analysis of the response of these cells to environmental stimuli that in the future may be manipulated for purposes of pest control. In this context, our demonstration of a correlation between the JH system and reproductive behavior is important as it implies that intervention in this system can regulate the rate of reproduction of a pest population without total eradication which often affects the ecological equilibrium too drastically. unwanted for ecological

Our molecular genetic approach has been especially successful: We have generated a variety of mutants in the *ap* gene and its chromosomal vicinity and their phenotypic characterization will soon be published. The P-transposon-induced mutants were especially instrumental in cloning and the initial molecular characterization of the gene. Information and molecular tools developed along the molecular genetic avenue (DNA probes, antibodies and *ap* mutants to be used in the screen for new endocrine genes) now serve us as valuable probes for studying the JH neuroendocrine system.

In conclusion this research constitutes a pioneering work towards a molecular genetic dissection of the JH neuroendocrine system. Comparable work has been initiated a few years ago on the neuroendocrine system of the molting hormone ecdysone. Since both are the primary insect hormones this work should shed light on an important aspect of insect biology which to date has not been amenable to molecular analysis, primarily because of lack of molecular-genetic tools in insects other than *Drosophila*. Thus this work should serve as a model for the JH system in other insects, notably in pests. We have started with the *ap* gene but our proposed search for its suppressors should open the way for furthering the dissection of the JH system by the identification and comparable study of other such endocrine genes.

Finally, we note that the JH system is unique to insects and as such has long been considered as an attractive target for pest control because interfering in its function may disrupt insect development and reproduction [26]. Indeed, JH analogs have been used with some success as biocontrol agents safer than the commonly used chemical pesticides. However, it soon became evident that while this is a promising avenue for pest control, its improvement requires a more detailed understanding of the molecular basis of the JH system which may suggest which of its components will be a better target for interference for purposes of pest control. Thus, our research is expected to provide also important practical information.

Description of Cooperation

The *in vitro* bioassay for JH biosynthesis has been developed by Segal's and Applebaum's labs. It has been applied by them to CA wild type and *ap* mutant adults and by Gilbert's lab to wild type and mutant larvae and this has allowed the three groups to identify the novel JHB₃. All further physiological studies on JH biosynthesis and its cerebral regulation have been conducted in parallel on larvae in Gilbert's lab and in the adult stage in the two Israeli labs. Technology and information has been exchanged by the three groups and the Israeli investigators, as well as a student from Segal's lab have spent some time in Gilbert's lab refining the bioassay and applying it.

Isolation and purification of the allatostatins have been conducted by Applebaum's lab with some help of Segal's lab in the bioassays.

Analysis of the regulatory interplay between the JH and ecdysteroid systems, of the histolysis of the larval fat body and its hormonal control by JH and ecdysteroids, and of the ultrastructure of the normal and *ap* mutant larval ring gland and pupal-adult CA have all been conducted by Gilbert's lab.

Behavioral studies on courtship of *ap* mutants and its correlation with the level of JH biosynthesis by the CA of the mutants have been conducted in Segal's lab (in collaboration with Dr. J. Ringo from Maine University, Orono, ME while he spent a sabbatical leave in Segal's lab).

The entire genetic analysis of the *ap* gene and its chromosomal region have been conducted in Segal's lab. Mutant strains generated in his lab were made available to the other two collaborators for the various avenues of research pursued in their labs.

The molecular work on the *ap* gene has been initiated (in collaboration with Dr. S. Cohen from Baylor College, Houston, TX, as mentioned above) in Segal's lab and is being continued there. Molecular tools for further analyses of *ap* function in the context of the JH neuroendocrine system are continuously being made available by him to Applebaum's and Gilbert's labs.

In conclusion, the combination of expertise of the three investigators and the close contacts and tight interaction between them in person and between their labs have been a major factor in making this collaborative project so productive and successful.

List of publications

- ✓ 1. Richard DS, Applebaum SW, Gilbert LI (1990). Allatostatic regulation of juvenile hormone production in vitro by the ring gland of *Drosophila melanogaster*. *Mol Cell Endocrinol* 68, 153-161.
- ✓ 2. Altaratz M, Applebaum SW, Richard DS, Gilbert LI, Segal D (1991). Regulation of juvenile hormone synthesis in wild-type and *apterous* mutant *Drosophila melanogaster*. *Mol Cell Endocrinol* 81, 205-216.
- ✓ 3. Richard DS, Gilbert LI (1991). Reversible juvenile hormone inhibition of ecdysteroid and juvenile hormone synthesis by the ring gland of *Drosophila melanogaster*. *Experientia* 47, 1063-1066.
- ✓ 4. Dai J-D, Gilbert LI (1991). Metamorphosis of the corpus allatum and degeneration of the prothoracic glands during the larval-pupal-adult transformation of *Drosophila melanogaster*: a cytophysiological analysis of the ring gland. *Dev Biol* 144, 309- 326.
- ✓ 5. Ringo J, Werczberger R, Altaratz M, Segal D (1991). Female sexual receptivity and juvenile hormone synthesis are defective in mutants of the *apterous* gene of *Drosophila melanogaster*. *Behav Genet* 21, 453-470.
- ✓ 6. Ringo J, Werczberger R, Segal D (1992). Male sexual signalling is defective in mutants of the *apterous* gene of *Drosophila melanogaster*. *Behav Genet* 22, 469-487.
- ✓ 7. Cohen B, McGuffin E, Pfeifle C, Segal D, Cohen SM (1992). *apterous*: a gene required for imaginal disc development on *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. *Gene Dev* 6, 715-729.
- ✓ 8. Segal D (1993). Prospects of using *Drosophila* for insect neuroendocrine research. *Arch Insect Biochem Physiol* 22, 199-231.
- ✓ 9. Richard DS, Arnim AE, Gilbert LI (1993). A reappraisal of the hormonal regulation of larval fat body histolysis in female *Drosophila melanogaster*. *Experientia* 49, 150-156.
- ✓ 10. Dai J-D, Gilbert LI (1993). An ultrastructural and developmental analysis of the corpus allatum of juvenile hormone deficient mutants of *Drosophila melanogaster*. *Roux's Arch Dev Biol* 202, 85-94.

Fig. 1. Diagrammatic representation of the possible pathways for the final steps of JHB₃ biosynthesis (after Ref. 10). White arrows indicate known enzymatic steps. The enzymes involved (methyl transferase, MT, and epoxidase, ED) are outlined.

Allele	24b	11d	39a	9e	31m	13c	8b	41c	40c	8a	29k	20b	14b	30j
Df-2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24b		+	+	+	+	+	+	+	+	+	+	+	+	+
11d														
39a														
9e														
31m														
13c														
8b														
41c														
40c														
8a														
29k														
20b														
14b														
30j														

viability

0 >75%
 1 50%-74%
 2 25%-49%
 3 <25%
 4 cannot be tested
 7 viability not tested

wing morphology

0 wild type
 1 mitted wing
 2 bifurc
 3 strap
 4 nub
 5 wing less
 6 dead

Fig. 5 A matrix showing phenotypes of heteroallelic combinations between various novel *ap* mutations. The complete matrix of ca. 40X40 mutants is too large to be shown here. Therefore a small portion of it, involving 14 of the newly induced P-insertional *ap* mutants, is illustrated. The phenotype of each of the mutants in a hemizygous condition (over *Df(ap)*) is shown at the top for reference. Note the extensive and complex pattern of complementation for viability and wing morphology (data for fertility are not shown).

Fig 2. First step purification of *Drosophila* allatostatic factors. Head extract were run on HPLC RP-4 column using linear gradient of CH_3CN / 0.1% TFA (0-60 %). Fractions (1 ml each) were collected and bioassayed for inhibition of JH biosynthesis. Active fractions were rerun for further purification. (a) HPLC profile; (b) activity of 4 most active fractions (A-D correspond to the peaks in (a)).

Fig. 4. Genetic map of the 41-42 chromosomal region. Each number represents one independently isolated lethal mutation. Mutations are grouped vertically according to deficiency mapping (see Fig. 2). Physical order within each sub-division is unknown. Boxes surround mutants belonging to the same complementation group. Overlapping boxes represent cases of complex complementation (see text).