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EVOLUTION OF SEQUENCE AND REGULATION OF THE DROSOPHILA UO GENE: SPECIES-SPECIFIC PATTERNS OF EXPRESSION ATTRIBUTED TO TRANS-ACTING REGULATORY CHANGES

By

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ABSTRACT

EVOLUTION OF SEQUENCE AND REGULATION OF THE DROSOPHILA UO GENE: SPECIES-SPECIFIC PATTERNS OF EXPRESSION ATTRIBUTED TO TRANS-ACTING REGULATORY CHANGES

By

Lori Lyn Wallrath

The urate oxidase (UO) gene of Drosophila is an excellent paradigm for studying the evolution of gene regulation. The *D. melanogaster* UO gene is expressed only in the third instar larva and adult while the *D. pseudoobscura* UO gene is expressed only in the adult. The *D. virilis* UO gene is expressed only in the third instar larva. In these three species, UO activity is present exclusively within the Malpighian tubules. Interspecific sequence comparisons of the 5' flanking DNA of the UO gene from *D. melanogaster, D. pseudoobscura* and *D. virilis* identified possible cis-regulatory elements of the UO gene. Within the 826 bp upstream of the *D. melanogaster* UO transcription start site there are six sequence elements (9-16 bp) conserved between the *D. pseudoobscura* and *D. melanogaster* UO genes and three sequence elements conserved between the *D. virilis* and *D. melanogaster* UO genes. All of the *D. melanogaster* UO cis-regulatory elements required for appropriate UO expression reside between positions -826 and +350 with respect to the UO transcription start site, since this amount of sequence conferred a *D. melanogaster* UO pattern of expression on a *D. melanogaster* UO-lacZ fusion gene.

To investigate the molecular mechanisms accounting for the differences in regulation of the UO gene, the *D. pseudoobscura* and *D. virilis* UO genes were

integrated into the genome of *D. melanogaster* using P-element mediated germ line transformation. The *D. pseudoobscura* and the *D. virilis* UO transgenes were expressed only in the Malpighian tubules, indicating that the mechanism restricting UO expression to the Malpighian tubules has been conserved among these three species. Unexpectedly, the *D. pseudoobscura* and *D. virilis* UO transgenes displayed a *D. melanogaster* UO temporal pattern of regulation. These data indicate that the differences in regulation of the UO gene among these three Drosophila species is dictated by changes in the temporal expression of trans-acting regulators required for UO gene expression, and not by evolutionary changes in the sequence of the cis-acting regulatory elements of the UO gene. This is a novel observation and has important implications for understanding the molecular events responsible for evolution.

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INTRODUCTION

With the application of molecular biology to the study of gene regulation, it is feasible to establish the genetic bases for regulatory differences among species. Regulatory mutations have been proposed to account for much of the diversity among organisms, yet this theory has been based solely upon inference (Wilson, 1975; King and Wilson, 1975). The molecular changes in gene regulation which bring about evolution are largely uncharacterized.

The discovery of overtly different temporal patterns of expression of the urate oxidase (UO) gene among species of Drosophila prompted an investigation to reveal the molecular basis of these regulatory differences. Initially, a detailed analysis of the *D. melanogaster* UO gene was undertaken. The *D. melanogaster* UO gene is expressed exclusively in the main segment cells of the Malpighian tubules of third instar larvae and adults. The sequences of *D. melanogaster* UO genomic and cDNA clones were determined and the transcription start site of the *D. melanogaster* UO gene was identified. Sequence analysis of the 5' region of the *D. melanogaster* UO gene revealed a consensus TATA box at position -34 relative to the transcription start site and a 13 bp direct repeat element (DR) at positions -138 and +11 which have sequence similarity to proposed 20-hydroxyecdysone receptor binding sites (Pongs, 1988). Repression of the UO gene prior to the pupal stage has previously been shown to be in response to the steroid hormone, 20-hydroxyecdysone (Kral et al., 1982). The effects on the expression of a UO-lacZ fusion gene upon removal of one or both of the DR elements are reported herein.

An interspecific sequence comparison of the 5' flanking DNA of the UO gene from *D. melanogaster, D. pseudoobscura* and *D. virilis* was performed to identify conserved motifs that are possible cis-control elements involved in the regulation of the UO gene. An interspecific sequence comparison is one strategy to identify cis-acting regulatory elements. Evolutionary processes obliterate ancient sequence homologies where nucleotide sequence is not under functional constraints and evolutionary processes conserve sequence which has functional properties. Within the 826 bp upstream of the *D. melanogaster* UO transcription start site there are six short sequence elements (9-16 bp) conserved between the *D. pseudoobscura* UO and *D. melanogaster* UO genes and three sequence elements conserved between the *D. virilis* UO and *D. melanogaster* UO genes. All of the *D. melanogaster* UO cis-regulatory elements required for appropriate UO expression reside between positions -826 and +350 with respect to the UO transcription start site, since this amount of sequence conferred a *D. melanogaster* UO pattern of expression on a *D. melanogaster* UO-lacZ fusion gene. To more precisely localize the cis-regulatory elements of the *D. melanogaster* UO gene that are important for temporal regulation and Malpighian tubule-specific expression, a new scheme was formulated for systematically and efficiently deleting large segments of the 5' UO flanking DNA using oligonucleotide-directed *in vitro* mutagenesis.

As part of this molecular evolutionary comparison of Drosophila UO, the coding region of the UO gene from *D. pseudoobscura* and *D. virilis* were also sequenced and compared to that of the *D. melanogaster* UO gene. Both the *D. pseudoobscura* UO and *D. virilis* UO genes are structurally similar to the *D. melanogaster* UO gene. There is 72% amino acid identity among the *D. melanogaster, D. pseudoobscura* and *D. virilis* UO deduced amino acid sequences. In contrast to the sequence similarity of the Drosophila UO genes,

the temporal regulatory pattern of UO has changed dramatically since the divergence of these three species. The *D. melanogaster* UO gene is expressed only within the Malpighian tubules of third instar larvae and adults. The *D. pseudoobscura* UO gene is expressed only within the Malpighian tubules of adults while the *D. virilis* UO gene is expressed only within the Malpighian tubules of the third instar larvae.

To investigate the molecular mechanism accounting for these regulatory changes, the *D. pseudoobscura* and *D. virilis* UO genes were integrated into the genome of *D. melanogaster* using P-element mediated germ line transformation. In several independent transformed lines, the *D. pseudoobscura* UO and the *D. virilis* UO transgenes were expressed only in the Malpighian tubules, indicating that the mechanism restricting UO expression to the Malpighian tubules has been conserved between these three species. Unexpectedly, the *D. pseudoobscura* UO and *D. virilis* UO transgenes displayed a *D. melanogaster* UO temporal pattern of regulation. The most likely interpretation of these data is that the differences in regulation of the UO gene among these three Drosophila species is due to changes in the temporal expression of one or more trans-acting regulators required for UO gene expression, and not due to changes in the cis-elements of the UO gene. In the few cases examined to date, species-specific differences in the temporal and tissue-specific expression of a particular gene have been attributed to cis-acting evolutionary changes (Brady and Richmond, 1990; Bray and Hirsh, 1986; Fisher and Maniatis, 1986; Wu et al., 1990). If the species-specific temporal patterns of UO expression are due to trans-acting regulators, as the data presented here suggest, then this is a novel and important observation and establishes the UO gene in Drosophila as a valuable model system to study the evolution of gene regulation.

I. CHAPTER ONE: Sequence and tissue-specific expression of the *D. melanogaster* UO gene.

A. Introduction

In Drosophila, particular purines serve as precursors of nucleotides and pterins (Johnson and Friedman, 1983; O' Donnell et al., 1985). Excess purine is converted to uric acid which is either stored, excreted or catabolized depending on the developmental stage. In *Drosophila melanogaster*, uric acid in the third instar larva and adult is converted by urate oxidase (UO) to allantoin and then excreted by the Malpighian tubules (Friedman, 1973, Kral et al., 1986). The gene encoding UO is transcribed exclusively within the cells of the Malpighian tubules. UO mRNA is not detected by Northern analysis until the beginning of the third instar larval stage, and by the middle of this stage, UO mRNA represents approximately 1% of the total poly(A)$^+$ RNA of the Malpighian tubules (Kral et al., 1986). By the end of the third instar larval stage, UO mRNA and UO protein abruptly disappear in response to a rising concentration of the steroid hormone 20-hydroxyecdysone (Kral et al., 1982). The UO gene remains transcriptionally inactive from the late third instar larval stage through the pupal stage with UO mRNA reappearing exclusively within the Malpighian tubules following emergence of the adult (Kral et al., 1986).

Though the molecular mechanisms are unknown, two experimentally distinguishable phenomena are involved in this reactivation of transcription of the UO gene in the adult: a UO inducing factor in the hemolymph and an autonomous clock-like mechanism in the Malpighian tubules. The UO inducing

factor was first detected in xanthine dehydrogenase deficient strains of Drosophila, *ry*^{2} (3-52.0; Glassman, 1965) and *ma-l* (1-64.8; Chovnick et al., 1968), which have five- to tenfold higher levels of UO mRNA and UO protein in the adult as compared to the wild type adult. High levels of UO inducing factor in the hemolymph of a xanthine dehydrogenase deficient adult stimulated a five- to tenfold increase in UO activity in a wild type Malpighian tubule transplanted into the abdomen of a xanthine dehydrogenase deficient adult (Friedman, 1973; Friedman and Johnson, 1977; Kral et al., 1982). The autonomous timer was detected when a Malpighian tubule from a pupa was transplanted into a newly emerged adult host. Expression of the UO gene in the transplanted pupal Malpighian tubule was delayed until sufficient time had passed for the transplanted pupal Malpighian tubule to be equivalent in age to a tubule of a newly emerged adult (Friedman and Johnson, 1977; Friedman and Johnson, 1978).

Identification of the cis-regulatory elements which govern the elaborate pattern of regulation of the *Drosophila melanogaster* UO gene was initiated with the isolation of UO cDNA clones and UO genomic clones (Kral et al., 1986). This chapter describes the molecular characterization of the UO gene of *D. melanogaster* which includes: (1) the sequence of the UO gene and flanking DNA, (2) the transcription start site of the UO gene, (3) tissue *in situ* hybridizations which identify the specific population of cells of the Malpighian tubules which express UO mRNA, and (4) comparison of the deduced amino acid sequence of *D. melanogaster* UO to plant and animal urate oxidase enzymes. These observations represent the necessary background information for the interspecific comparisons of the UO sequence and regulation and the identification of UO regulatory regions.

B. Results

1. Sequence of the *D. melanogaster* UO gene.

D. melanogaster UO cDNA clones were previously isolated from a third instar larval Malpighian tubule cDNA library made from the temperature sensitive 20-hydroxyecdysone deficient strain *ecd*^{1}(Kral et al., 1986). A cDNA probe, cUO2 (Figure A1; all figures and tables with an "A" preceding the number are located in Appedix B), was used to isolate additional cDNA clones from an Ore-R third instar library (Poole et al., 1985) as well as overlapping genomic clones from a *D. melanogaster* Canton-S lambda Charon 4A library (Maniatis et al., 1978). A UO genomic clone from the *D. melanogaster ecd*^{1} strain was also isolated by screening a *Hind*III size limited pBR322 library with cUO2. A list of all the Drosophila stocks used in this study is shown in Table A1.

The restriction maps and strategies used to sequence *Drosophila melanogaster* UO genomic and cDNA clones are diagrammed in Figure A1, panels a, b and c. Genomic DNA from *D. melanogaster* Canton-S was restricted with four different endonucleases and probed with the 5.5 kb *Hind*III restriction fragment containing the UO gene (Figure A1, panel d). The genomic restriction fragments which hybridized to the 5.5 kb *Hind*III DNA probe were equivalent in size to the restriction fragments of the cloned UO gene, indicating that no obvious rearrangements of the UO region occurred during cloning of the genomic DNA. Southern analyses also showed that the UO gene is single copy in the wild type strains Canton-S (Figure A1) as well as in several mutant strains (Figure A2). The composite DNA sequence of the UO gene derived from *D. melanogaster* Canton-S genomic clones, Ore-R cDNA clones and *ecd*^{1} cDNA clones is shown in Figure 1. The *D. melanogaster* genomic and cDNA nucleotide sequences are available in the EMBL GenBank and DDBJ Nucleotide Sequence Databases under the accession number X51940.

Figure 1. The sequence of the *D. melanogaster* Canton-S UO gene, flanking DNA and the deduced amino acid sequence of the UO protein. The DNA sequence is numbered in the left margin with +1 at transcription start (I). The deduced amino acid sequence is numbered in the right margin beginning with the translation initiation methionine as residue 1. DR at positions -138 and +11 is a perfect 13 base pair direct repeat. The autoradiographic band intensities of the products from S1 nuclease mapping (S1), mung bean nuclease mapping (MB) (Fig. 2a) and primer extension experiments (PE) (Figure 2b and d) are schematically represented by closed dots for intense signals and open circles for weaker signals. Primers P1 and P2 are each 30 nucleotides and were used in primer extension experiments (Fig. 2c). Within a different reading frame of the first exon of the UO gene there is a second small open reading frame beginning at nucleotide position 332 (underlined) with a stop codon at position 598 (underlined and marked by an asterisk) and having no significant amino acid sequence similarity to reported peptides in the PIR or GenEMBL protein banks. The sequence of the 69 base pair UO intron, in italics, has the two internal consensus splice sequences overscored and labeled with an S. The asterisk at nucleotide position 1161 indicates the UO translation termination codon. Beginning at nucleotide 1163, the letters below the contiguous UO Canton-S sequence represent nucleotide differences and dots represent deletions of nucleotides in the 3' untranslated region of UO cDNAs from the *ecd*^{1} strain of *D. melanogaster*. Four consensus polyadenylation signals in the Canton-S UO sequence are overscored and labeled with an A. Three polyadenylation sites at positions 1285, 1289 and 1306 (An) were identified from sequence data of the 3' end of eight independently arising *ecd*^{1} UO cDNA clones containing poly(A) tails.

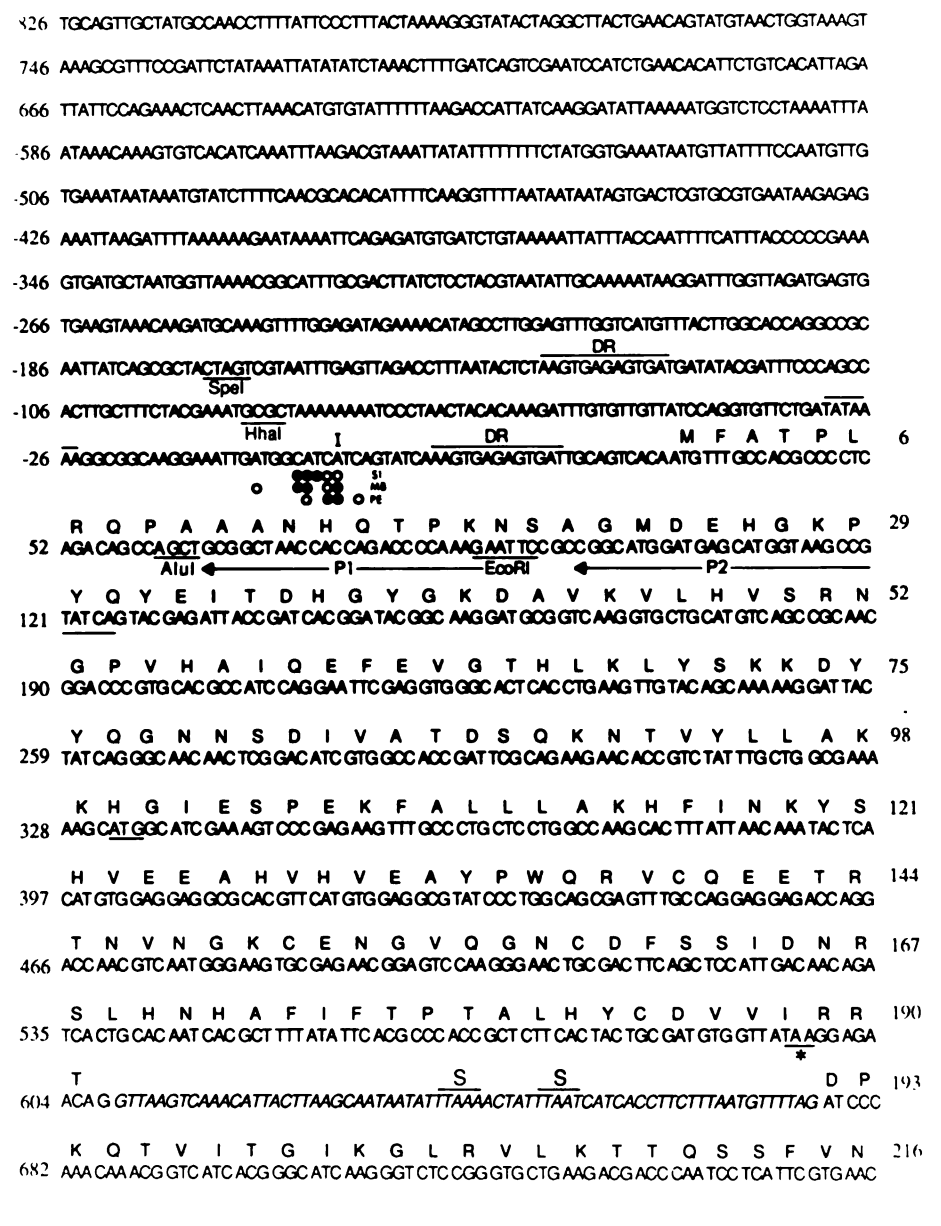

826 TGCAGTTGCTATGCCAACCTTTTATTCCCTTTACTAAAAGGGTATACTAGGCTTACTGAACAGTATGTAACTGGTAAAGT
746 AAAGCGTTTCCGATTCTATAAATTATATATCTAAACTTTTGATCAGTCGAATCCATCTGAACACATTCTGTCACATTAGA
666 TTATTCCAGAAACTCAACTTAAACATGTGTATTTTTTAAGACCATTATCAAGGATATTAAAAATGGTCTCCTAAAATTTA
586 ATAAACAAAGTGTCACATCAAATTTAAGACGTAAATTATATTTTTTTTCTATGGTGAAATAATGTTATTTTCCAATGTTG
506 TGAAATAATAAATGTATCTTTTCAACGCACACATTTTCAAGGTTTTAATAATAATAGTGACTCGTGCGTGAATAAGAGAG
426 AAATTAAGATTTTAAAAAAGAATAAAATTCAGAGATGTGATCTGTAAAAATTATTTACCAATTTTCATTTACCCCCGAAA
346 GTGATGCTAATGGTTAAAACGGCATTTGCGACTTATCTCCTACGTAATATTGCAAAAATAAGGATTTGGTTAGATGAGTG
266 TGAAGTAAACAAGATGCAAAGTTTTGGAGATAGAAAACATAGCCTTGGAGTTTGGTCATGTTTACTTGGCACCAGGCCGC
186 AATTATCAGCGCTACTAGTCGTAATTTGAGTTAGACCTTTAATACTCTAAGTGAGAGTGATGATATACGATTTCCCAGCC
106 ACTTGCTTTCTACGAAATGCGCTAAAAAAAATCCCTAACTACACAAAGATTTGTGTTGTTATCCAGGTGTTCTGATATAA
-26 AAGGCGGCAAGGAAATTGATGGCATCATCAGTATCAAAGTGAGAGTGATTGCAGTCACAATGTTTGCCACGCCCCTC
52 AGACAGCCAGCTGCGGCTAACCACCAGACCCCAAAGAATTCCGCCGGCATGGATGAGCATGGTAAGCCG
121 TATCAGTACGAGATTACCGATCACGGATACGGCAAGGATGCGGTCAAGGTGCTGCATGTCAGCCGCAAC
190 GGACCCGTGCACGCCATCCAGGAATTCGAGGTGGGCACTCACCTGAAGTTGTACAGCAAAAAGGATTAC
259 TATCAGGGCAACAACTCGGACATCGTGGCCACCGATTCGCAGAAGAACACCGTCTATTTGCTGGCGAAA
328 AAGCATGGCATCGAAAGTCCCGAGAAGTTTGCCCTGCTCCTGGCCAAGCACTTTATTAACAAATACTCA
397 CATGTGGAGGAGGCGCACGTTCATGTGGAGGCGTATCCCTGGCAGCGAGTTTGCCAGGAGGAGACCAGG
466 ACCAACGTCAATGGGAAGTGCGAGAACGGAGTCCAAGGGAACTGCGACTTCAGCTCCATTGACAACAGA
535 TCACTGCACAATCACGCTTTATATTCACGCCCACCGCTCTTCACTACTGCGATGTGGTTATAAGGAGA
604 ACAG GTTAAGTCAAACATTACTTAAGCAATAATATTTAAAACTATTTAATCATCACCTTCTTTAATGTTTTAG ATCCC
682 AAACAACGTGTCATCACGGGCATCAAGGGTCTCCGGGTGCTGAAGACGACCCAATCCTCATTCGTGAAC
DR
SpeI
HhaI
I
DR
M F A T P L 6
R Q P A A A N H Q T P K N S A G M D E H G K P 29
AluI
P1
EcoRI
P2
Y Q Y E I T D H G Y G K D A V K V L H V S R N 52
G P V H A I Q E F E V G T H L K L Y S K K D Y 75
Y Q G N N S D I V A T D S Q K N T V Y L L A K 98
K H G I E S P E K F A L L L A K H F I N K Y S 121
H V E E A H V H V E A Y P W Q R V C Q E E T R 144
T N V N G K C E N G V Q G N C D F S S I D N R 167
S L H N H A F I F T P T A L H Y C D V V I R R 190
T S S D P 193
K Q T V I T G I K G L R V L K T T Q S S F V N 216

Figure 1.

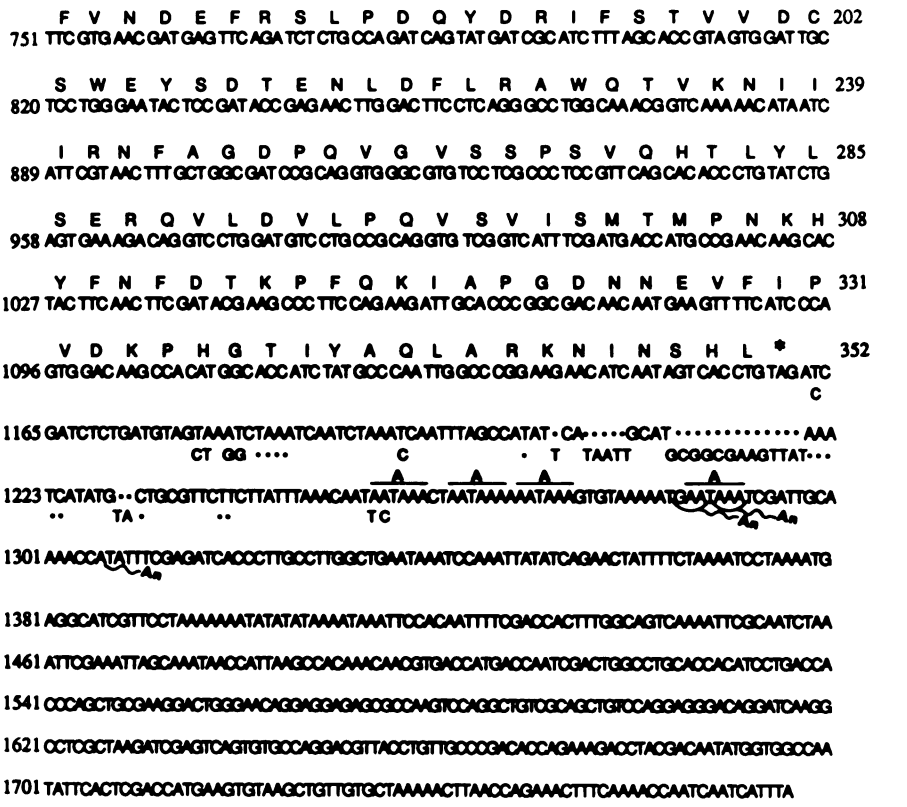


Figure 1 (cont'd).

The UO 5' flanking genomic sequence contains a thirteen base pair direct repeat, (DR; AAGTGAGAGTGAT, at -138 and +11, Figure 1). The DR element at -138 is designated the distal DR (dDR) and the DR element at +11 is designated the proximal DR (pDR). Each DR element includes a perfect direct repeat of the sequence AGTGA with an axis of symmetry centered at the G nucleotide at positions -132 and +17. Downstream of the DR element is a single, long open reading frame, beginning 34 nucleotides 3' of the UO transcription start site, coding for a 352 amino acid sequence similar to urate oxidase from a plant and several vertebrates. Within the protein coding region of the UO gene there is a 69 base pair intron which has GU-AG consensus splice junctions (Mount, 1982) and two 3' intronic splice signal sequences, TTAAA and TTAAT, (overscored and labeled with an S, Figure 1) which are similar and identical, respectively, to the Drosophila consensus splice signal sequence C/T T A/G A T/C proposed by Keller and Noon (1985).

Comparisons of UO genomic clones isolated from *D. melanogaster* Canton-S with UO cDNA clones isolated from *D. melanogaster ecd*^{1} revealed many sequence differences in the 3' transcribed but untranslated region of the UO gene (position +1179 to +1255, Figure 1). These nucleotide differences were confirmed by Southern analysis of genomic DNA isolated from Canton-S and *ecd-1* which was digested with restriction enzymes that recognized the 3' untranslated region of the *ecd-1* UO gene, but not the Canton-S UO gene (Figure A2). The discovery of these nucleotide differences in the 3' untranslated region of the UO gene made it possible to create a strain-specific oligonucleotide probe which would selectively hybridize, on Northern blots, to UO mRNA derived from a P-element encoded *ecd*^{1} UO gene and not to UO mRNA derived from the endogenous Canton-S UO gene (Chapter Three). The ability to detect message from a UO gene introduced into *D. melanogaster*

stock was of extreme importance for studying the regulation of the UO gene since a UO deficient *D. melanogaster* stock was not available.

Within the 3' untranslated region of the UO gene from Canton-S are four AATAAA elements (overscored and labeled with an A, Figure 1), identical to the consensus polyadenylation sequence of Proudfoot and Brownlee (1976), which are just upstream of the three polyadenylation sites (An, Figure 1). Two of the nucleotide differences between the Canton-S and *ecd*^{1} UO genes (positions 1254 and 1255, Figure 1), abolish the first of four polyadenylation signals (AATAAA) so that the UO gene from the *ecd*^{1} strain has only three poly(A) signals and three polyadenylation sites (Figure 1). Based on the sequence data from eight *ecd*^{1} UO cDNA clones, the UO gene gives rise to messages, after 3' endonucleolytic cleavage but prior to polyadenylation, of 1224, 1227 and 1244 nucleotides which are consistent with Northern analyses showing UO poly(A)$^+$ RNA to be approximately 1400 nucleotides (Kral et al., 1986). Whether endonucleolytic cleavage and polyadenylation occurs at the nucleotides G or A and T or A at the first and third polyadenylation sites has not been determined. In general, cleavage and polyadenylation after a C or an A are preferred sites (Birnstiel et al., 1985).

2. Determination of the transcription initiation site of the UO gene.

Two possible overlapping transcription initiation sequences (ATCATCA, -3, and ATCAGTA, +1, Figure 1) were first identified upstream of the UO translation initiation codon by their resemblance to a *Drosophila melanogaster* transcription initiation consensus sequence A T C A G/T T C/T (Hultmark et al., 1986). The transcription initiation site (+1, Figure 1) for the *D. melanogaster* UO gene was confirmed by three independent experimental procedures:

(1) S1 nuclease and mung bean nuclease mapping, (2) primer extension analyses and (3) the sequence of three cDNA clones that extended fully 5' and were capped with a 7-methylguanosine residue. S1 nuclease mapping experiments used poly(A)$^+$ RNAs from Ore-R third instar larvae, Ore-R adults (data not shown) and *ry*^{2} 12 hour adults which were hybridized to single-stranded probe S (Figure 2c). The S1 mapping of the 5' end of the UO transcript revealed protected products of 93 to 95 nucleotides in length and weaker products of 91 and 92 nucleotides for all poly(A)$^+$ RNA preparations examined (Figure 2a). Protected products of 91, 94 and 95 nucleotides and a minor protected product of 92 nucleotides were observed with mung bean nuclease. Such micro-heterogeneity in S1 and mung bean nuclease mapping has been reported to be an artifact of incomplete or over digestion with S1 nuclease or the interference of a cap G (Weaver and Weissmann, 1979; Hentschel et al., 1980; Cherbas et al., 1986; Takeshima et al., 1988).

Primer extension analyses were used as a second method to identify and confirm the site(s) of transcription initiation. Using P1 as a hybridization primer (Figures 1 and 2c) and the poly(A)$^+$ RNA fractions described above for the S1 and mung bean nuclease mapping, products of 91 and 92 nucleotides and weaker products of 89 and 94 nucleotides resulted upon primer elongation with AMV reverse transcriptase. In several independent primer extension experiments, a clearly discernible band at 158 nucleotides also resulted when using poly(A)$^+$ RNA from Ore-R third instar larvae and *ry*^{2} 12 hour adults (Figure 2b). With the exception of the 158 nucleotide primer extension product, a schematized presentation of the data from S1 and mung bean nuclease mapping and the primer extension analyses of the transcription start of the UO gene are shown in Figure 1. The lengths of the primer extension products, with

Figure 2. Mapping the transcription initiation site for the *D. melanogaster* UO gene. (a) S1 and mung bean nuclease mapping of the 5' end of UO mRNA. Genomic fragment S (panel c) was end-labeled with ^{32}P, and the strand complementary to UO mRNA was hybridized with 20 μg of poly(A)$^+$ RNA from wild type Ore-R third instar larvae (3L, +, lanes 1 and 2). Probe S was hybridized to 10 μg of poly(A)$^+$ RNA from *ry*^{2} 12 hour adults (A, ry, lanes 3 and 4). DNA:RNA hybrids were digested with 50 units of S1 nuclease in lanes 1 and 3 and 400 units of mung bean nuclease in lanes 2 and 4. As a control, lane 5 contained 10 mg of calf thymus tRNA (C) hybridized to the probe and digested with 50 units of S1 nuclease. Lane 6 contained only the S probe (S). Protected products were separated on a 6% sequencing gel and sized in relation to an M13 sequence ladder. Both S1 and mung bean nuclease digestion resulted in protected products of 91 to 95 nucleotides for the larval (3L) and the adult (A) mRNA. (b) Identification of the transcription start site of the UO gene by primer extension analyses. DNA fragment P1 (panel c) was end-labeled, the strand complementary to UO mRNA was hybridized to 5 μg of poly(A)$^+$ RNA from Ore-R third instar larvae (3L, +, lane 1), 5 μg of poly(A)$^+$ RNA from *ry*^{2} 12 hour adults (A, ry, lane 2) or 6 μg of poly(A)$^+$ RNA from Ore-R 12 hour adults (A, +, lane 3). After primer extension using AMV reverse transcriptase, the products were separated on an 8% sequencing gel and sized using an M13 sequence ladder. An extension product of 91 nucleotides was detected in all samples tested, consistent with S1/mung bean nuclease mapping, placing transcription initiation at position +1 (I, Fig. 2). The origin of the 158 nucleotide product is discussed within the text. (c) Diagram of the strategy used for the S1 and mung bean nuclease mapping and primer extension experiments. The open box represents a 5' portion of the UO transcription unit. The single stranded DNA probes are indicated by the solid lines with their ^{32}P-labeled terminal nucleotides shown as closed circles. Dots placed under the solid line of probe S indicate the region digested by S1 or mung bean nuclease. Dashed lines show the extension of probes P1 and P2 by AMV reverse transcriptase. On the basis of the 5' mapping data, position I (Fig. 1) was determined to be the transcription initiation site for *D. melanogaster* UO mRNA. (d) P2 (panel (c), and Fig. 1), a synthetic ^{32}P end-labeled 30-mer, complementary to UO mRNA, was hybridized to 10 μg of poly(A)$^+$ RNA from Ore-R third instar larvae (3L, +; lane 2) and to 10 μg of poly(A)$^+$ RNA from *ry*^{2} adults (A, ry; lane 3). Only a single extension product of 126 nucleotides, mapping to +1 (Fig. 1), was observed. Lane 1 is the primer P2 alone (P), which gave a signal at the position of 30 nucleotides (present in the lower portion of the gel not shown here). (e) Sequence comparison of the 5' end of a UO cDNA clone (i) with the corresponding genomic sequence (ii).

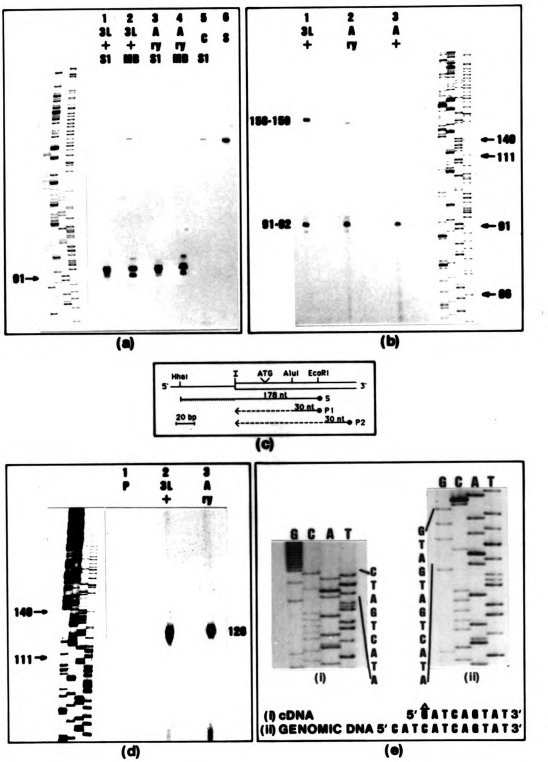

1 2 3 4 5 6
3L 3L A A G T
+ + ry ry C S
S1 MB S1 MB S1
91
(a)
1 2 3
3L A A
+ ry +
159-160
91-92
140
111
91
66
(b)
NheI
I
ATG
AluI
EcoRI
5'
3'
178 nt
S
30 nt
P1
30 nt
P2
20 bp
(c)
1 2 3
P 3L A
+ ry
140
111
129
(d)
G C A T
C T A G T C A T A
G C A T
G T A G T A G T C A T A
(i)
(ii)
(i) cDNA
(ii) GENOMIC DNA 5' GATCATCAGTAT3'
(e)

Figure 2.

the exception of the 158 nucleotide band, were consistent with the transcription start site mapped with S1 and mung bean nuclease.

The 158 nucleotide primer extension band could not be due to hybridization of P1 to a second site downstream on the UO mRNA as the sequence of P1 has no similarity to any other site along the UO mRNA, even allowing for 14 mismatches out of 30 nucleotides. The unexpected primer extension product of 158 nucleotides was not due to a second upstream promoter responsible for high levels of UO mRNA. This conclusion was derived from an additional set of primer extension experiments with P2, a synthetic primer, just downstream of P1 (Figures 1, 2c and A3). Primer P2 was hybridized to poly(A)$^+$ RNAs from Ore-R third instar larvae and *ry*^{2} 12 hour adults, both sources having high levels of UO mRNA. Only a single extension product of 126 nucleotides resulted (Figure 2d) which also maps the transcription start site of the UO gene to position +1 (Figure 1).

To confirm the location of the UO transcription start site three different UO cDNA clones from a third instar larval cDNA library were sequenced at the 5' end (Figures 1 and A1). These cDNA clones extend to nucleotide A at +1 followed by a G nucleotide not present at the corresponding position of either the Canton-S genomic sequence or the *ecd*^{1} UO genomic sequence (Figure 2e). No UO cDNAs were isolated which had an additional 67 base pair 5' exon.

A clue concerning the origin of the 158 nucleotide primer extension band was obtained when the *Spe*I-*Eco*RI fragment, containing the 5' transcribed region of the UO gene including the sequence of P1 (Figure 1), was used as a probe to screen a third instar larval cDNA library. Two classes of cDNAs were obtained. One class of cDNAs hybridized to the *Eco*RI-*Spe*I probe and also to cUO2 (Figure A1). The second class of cDNAs hybridized to the *Eco*RI-*Spe*I probe but not to cUO2 and contained cDNAs of approximately 3800 base pairs in

length with restriction maps unlike that of the UO. This latter class of cDNAs may encode the message which is primed by P1 and gives rise to the 158 nucleotide extension product. No further work was done to characterize the 158 nucleotide extension product.

3. Spatial distribution of UO mRNA by *in situ* hybridization.

In situ hybridizations of UO mRNA were performed in order to identify the population of cells of the Malpighian tubules containing UO mRNA in the wild type 12 hour Ore-R adult and in the xanthine dehydrogenase deficient (ry^2) 12 hour adult which has a five- to tenfold higher level of UO mRNA (Friedman, 1973, Kral et al., 1986). At least two mechanisms could account for the five- to tenfold higher level of UO mRNA in the Malpighian tubules of ry^2 12 hour adults: (1) an increase in the amount of UO mRNA within the same population of cells of Malpighian tubules or (2) recruitment of additional cells expressing UO mRNA from those cells which comprise the Malpighian tubules. To examine these two possibilities, UO sense and antisense RNA probes were hybridized *in situ* to sectioned and whole mount Malpighian tubules. An [α-^{35}S]dUTP-labeled antisense RNA probe synthesized from the *Eco*RI-*Acc*I template of cUO2 (Figure 1) hybridized exclusively to the Malpighian tubules in sections of ry^2 12 hour adult abdomens (Figure 3a and b). No hybridization signal was detected using a sense UO RNA probe hybridized to alternate sections (data not shown). Whole-mount Malpighian tubules from Ore-R adults and ry^2 adults were hybridized with a UO antisense RNA probe. There were no detectable UO transcripts in the midgut, hindgut or the cells which comprise the transitional segment or the initial enlarged segment of the Malpighian tubules as defined by Wessing and Eichelberg (1978) (Figure 3c and d). There was a

Figure 3. Spatial distribution of UO mRNA among the cells which comprise the *D. melanogaster* adult Malpighian tubules. Phase contrast (a) and darkfield illumination (b) of the autoradiographic image of a sectioned ry^2 12 hour adult abdomen hybridized to a UO antisense ^{35}S-labeled RNA probe synthesized from the *Eco*RI-*Acc*I restriction fragment of cUO2 (Fig. 1c). Hybridization signals were detected exclusively within the Malpighian tubules (Mt). (c) Brightfield images of whole mount Malpighian tubules from a ry^2 12 hour adult were hybridized to the antisense ^{35}S-labeled RNA probes as described for panels (a) and (b). The autoradiographic image revealed hybridization mainly within the mid-segment (M) of the anterior pair of tubules (A) and along the entire posterior pair of tubules (P) with a small amount of hybridization within the cells of the ureter (U). Hybridization did not occur within the transitional segment (T) or the initial segment (I) of the Malpighian tubules. Dark areas along the gut (G) attached to the Malpighian tubules are not exposed silver grains but are opaque material within the preparations which also present in the control sense-strand *in situ* hybridizations (e). (d) Whole Malpighian tubules from an Ore-R adult 12 hour adult were hybridized to the antisense ^{35}S-labeled UO RNA probe in (a) and (b). (e) No detectable signal was present when ry^2 12 hour adult Malpighian tubules were hybridized to a sense ^{35}S-labeled UO RNA probe.

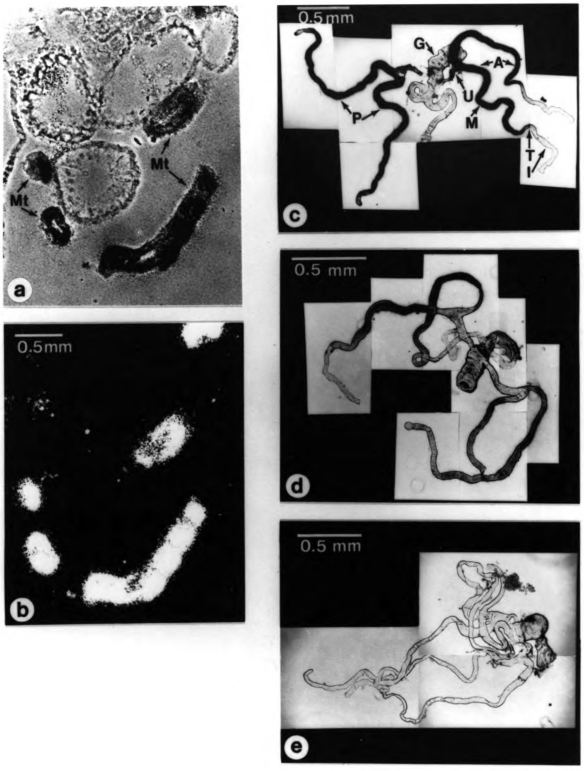


Figure 3.

weak hybridization signal within the ureter and within the cells at the extreme distal end of the posterior tubule (Figure 3c and d). In both ry^2 adults and Ore-R adults, UO transcripts accumulate within the main segment cells of the anterior Malpighian tubules and along the length of the posterior tubules. There was a far greater number of silver grains over the whole-mount Malpighian tubules of the ry^2 12 hour adult as compared to the same population of cells of the Ore-R 12 hour adult (compare Figure 3c and d), which is consistent with five- to tenfold higher level of UO mRNA and UO activity in the ry^2 adult as compared to the Ore-R adult (Friedman, 1973; Kral et al., 1982; Kral et al., 1986).

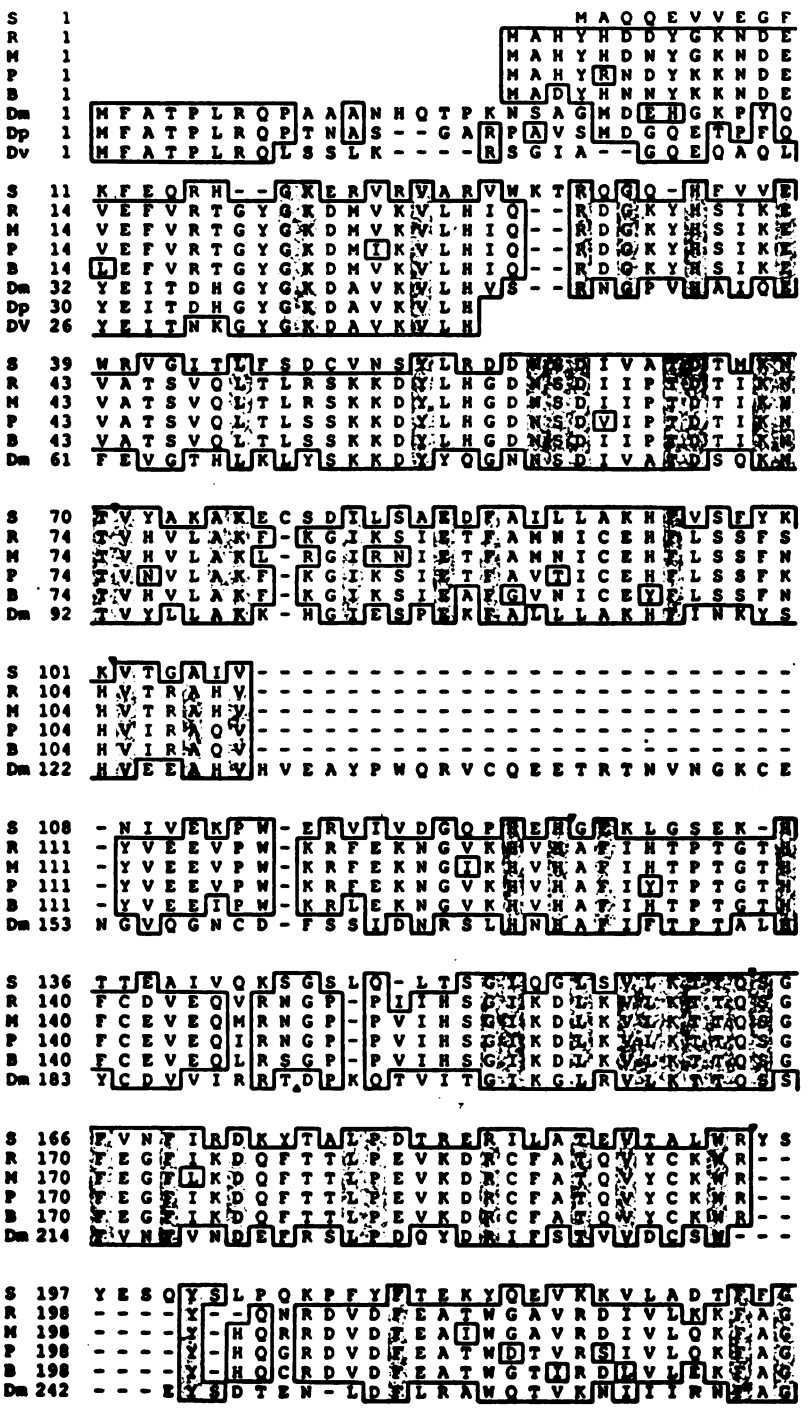
4. Deduced amino acid sequence and protein sequence comparisons of UO.

The *D. melanogaster* UO transcription unit contains two in-frame methionine codons in the amino terminal region (Met-1 and Met-23, Figure 1). If Met-1 is utilized as the translation initiation codon, the deduced M_r for the UO peptide would be 39,989 daltons which is not significantly different from the apparent M_r of 40,480 ±1340 estimated for the purified UO protein (Friedman and Barker, 1982). If Met-23 is used to initiate translation, the deduced M_r would be 37,701 daltons which is significantly less than the apparent molecular weight. The scanning model for translation initiation (Kozak, 1989) would predict that Met-1 is the translation start site for the UO gene in *D. melanogaster*. Met-1 is in good sequence context for a Drosophila translation initiation codon (Cavener, 1987). The four nucleotides upstream of Met-1 of the UO gene are identical to the four nucleotides preceding the start codon of other Drosophila genes (Tőrők and Karch, 1980; O'Tousa et al., 1985; Ito et al., 1988). Whether Met-1 or Met-23 is the translation start for the UO protein could not be examined directly since the amino terminus of the purified UO protein was blocked and could not be

sequenced (Friedman, unpublished results). Consequently, a molecular evolutionary comparison between urate oxidase of *D. pseudoobscura* and *D. virilis* (Figure 4) which diverged from *D. melanogaster* approximately 35 million and 60 million years ago, respectively (Beverley and Wilson, 1984) was used to determine which of the two in-frame methionine codons, Met-1 or Met-23, is the UO translation start site.

The sequences of the first seven amino acids of urate oxidase from *D. melanogaster*, *D. pseudoobscura* and *D. virilis* are identical (Figure 4). The *D. pseudoobscura* deduced UO amino acid sequence has a methionine codon (Met-21) in the corresponding position to Met-23 of the *D. melanogaster* deduced UO protein sequence. However, the *D. virilis* deduced UO amino acid sequence does not have a second methionine residue in the amino terminal region (Figure 4). The first eight codons of the deduced UO protein in *D. virilis* contain four synonymous substitutions when compared to the first eight codons for the deduced amino acid sequence of *D. melanogaster* UO protein, while immediately upstream of Met-1, in both species, the preceding 32 nucleotides show no DNA sequence similarity. Taken together, these data indicate that Met-1 is the UO translation start codon in all three species of Drosophila.

The amino acid sequence comparison between urate oxidase of *D. melanogaster*, soybean (Nguyen et al., 1985), rat (Reddy et al., 1988), mouse, pig and baboon (Wu et al., 1989) is shown in Figure 4. There is 32% to 38% amino acid sequence identity between urate oxidase of *Drosophila melanogaster* and urate oxidase of the five other species. Though not indicated in Figure 4, many of the non-identities represent conservative evolutionary amino acid changes (Lipman and Pearson, 1985). Among the 22% of the amino acid residues identical in the deduced amino acid sequences of urate oxidase from soybean, rat, mouse, pig, baboon and Drosophila, there are four histidine residues (Dm: HIS-170, HIS-172, HIS-182 and HIS-308) which may be



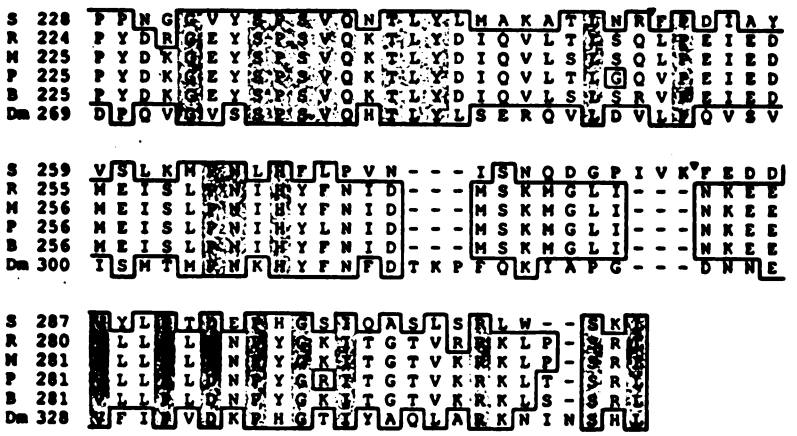


Figure 4. Alignment of the deduced amino acid sequences of UO from *Drosophila melanogaster* (Dm), soybean (S), rat (R), mouse (M), pig (P) and baboon (B) and the first 39 and 43 amino acids from the amino termini of UO from *Drosophila virilis* (Dv) and *Drosophila pseudoobscura* (Dp), respectively. The single-letter amino acid code is used. Three deduced amino acid sequences for rat urate oxidase have been reported which differ from one another at the amino and carboxy termini (Ito et al., 1988; Motojima et al., 1988; Reddy et al., 1988). In this figure the deduced rat urate oxidase sequence from Reddy et al. (1988) was used since it appears to be full length. To establish the comparison and accomodate the larger *Dm* UO protein, a gap was introduced in the middle of all the other UO amino acid sequences at a site which showed no sequence similarity between *Dm* and the other five urate oxidases. All other gaps were created by the FASTP program to optimize the alignments (Lipman and Pearson, 1985). The UO amino acid sequence of *D. melanogaster* shows 32%, 38%, 37%, 36% and 35% identity to the UO amino acid sequence of soybean, rat, mouse, pig and baboon, respectively. Boxed areas indicate identical amino acids found in two or more of the UO proteins. Amino acid residues of urate oxidase identical in all six species are shaded. The location of introns for *D. melanogaster* UO and soybean uricase II are indicated by solid triangles.

involved in copper binding (Mahler, 1958; Wu et al., 1989). Urate oxidase is a peroxisomal enzyme (deDuve and Baudhuin, 1966; Lazarow and Fujiki, 1985; Hayashi et al., 1976) and the deduced amino acid sequence at the carboxy terminus of urate oxidase from Drosophila is Ser-His-Leu, soybean is Ser-Lys-Leu and rat, mouse, pig and baboon are Ser-Arg-Leu. These tripeptide sequences are also found at the carboxy termini of some, but not all, peroxisomal proteins (Miyazawa et al., 1989; Gould et al., 1990; Lewin et al., 1990). Any one of these three carboxy terminal tripeptides is sufficient for targeting a reporter protein to peroxisomes (Gould et al., 1990). On the basis of the similar carboxy termini of urate oxidase proteins compared here (Figure 4) and from the data on targeting of some peroxisomal proteins (Gould et al., 1988; Gould et al., 1989; Miyazawa et al., 1989), a serine residue followed by a positively charged amino acid and then a carboxy terminal leucine is likely to be involved in the peroxisomal targeting of UO of Drosophila, plants and vertebrates.

C. Discussion

The UO gene of *D. melanogaster* is structurally compact, comprised of two exons separated by a 69 base pair intron (Figure 1). The *D. melanogaster* UO gene is transcribed from a single promoter yielding UO mRNA of 1224, 1227 and 1244 nucleotides depending on which one of three 3' endonucleolytic cleavage sites is utilized (Figures 1 and 2). A few genes from both vertebrates and invertebrates have been shown to have multiple polyadenylation sites (Setzer et al., 1980; Mlodzik and Gehring, 1987; Dreesen et al., 1988; Garbe et al., 1989; Laird-Offringa et al., 1989) and in some cases, the polyadenylation sites are closely spaced (Johnson et al., 1987; Seeger et al., 1988; Quan and Forte, 1990). The biological significance of multiple adjacent polyadenylation signals and sites remains to be determined (Denome and Cole, 1988).

The UO gene of *D. melanogaster* has a complex developmental and tissue-specific pattern of expression. UO mRNA is present within the main segment cells of the Malpighian tubules (Figure 3) of third instar larvae and adults (Friedman, 1873; Kral et al., 1986). It is assumed that the UO gene is regulated at the level of transcription. The pattern of expression of a UO-lacZ fusion transgene supports this assumption (Figure 10, Chapter Three). However, nuclear run-on experiments are needed to address whether the dramatic decline in the steady state level of UO mRNA at the end of the third instar stage is due to a decrease in UO gene transcription and/or a decrease in the half-life of UO mRNA.

Putative regulatory sequences involved in tissue-specific expression, developmental timing and quantitative regulation of the UO gene can be identified by several methods. Comparison of the DNA sequence of the flanking region of the homologous gene in different species is one approach for identifying putative cis-regulatory elements which are detected as conserved motifs highlighted amidst a background of dissimilar DNA sequence (Blackman and Meselson, 1986; Bray et al., 1988; Fenerjian et al., 1989; Kassis et al., 1989). The application of this method to examine the 5' flanking DNA of the UO gene is described in Chapter Three. Another method used for identifying putative cis-regulatory elements is to examine the flanking DNA of a particular gene for sequences which are well characterized cis-elements of known function. The sequence at position -31 to -37 of the *D. melanogaster* UO gene was identified as a TATA box using this method. Bracketing the TATA box and the UO transcription start site at +1 of the UO gene is a perfect 13 base pair direct repeat (DR), AAGTGAGAGTGAT, beginning at positions -138 and +11. The sequence of the DR motif is similar to a proposed 20-hydroxyecdysone consensus sequence found upstream of six 20-hydroxyecdysone inducible genes of Drosophila (Pongs, 1988). The possible role of the DR motif in 20-

hydroxyecdysone repression of the UO gene is discussed in Chapter Three.

A third method to identify cis-regulatory elements of a gene is to compare the flanking sequence with the regulatory regions of other genes that share some aspect of temporal and tissue-specific gene control. Urate oxidase is the only gene thus far reported in Drosophila which is expressed exclusively within the Malpighian tubules. The *white* gene (*w*) and the *alcohol dehydrogenase gene* (*Adh*) are expressed in the Malpighian tubules but also in other tissues (Fjose et al., 1984; Lockett and Ashburner, 1990). Nevertheless, there may be similar cis-acting regulatory elements in the flanking DNA of the *w*, *Adh* and UO genes which may be involved in Malpighian tubule expression. An 864 bp region of the *w* gene of *D. melanogaster* was reported to be necessary for expression in the Malpighian tubules (Pirrotta et al., 1985). Tissue-specific expression of the *Adh1* gene of *D. mulleri* requires the presence of two regulatory elements, the "A box" and "B box" (Fischer and Maniatis, 1988).

A DNA sequence similarity search was performed using regions of the *w* and *Adh-1* genes important for Malpighian tubule expression and the flanking DNA of the UO gene. This search revealed a sequence in the 5' flanking DNA of the UO gene, AAAGTAAAGCG (-751, Figure 1), that was similar to the sequence AAAGTACAGTG in the Malpighian tubule-specific region of *w* (+4223, O' Hare et al., 1984; Pirrotta et al., 1985) and to the sequence AAAGTAAAACG in the middle of the *Adh-1* "B box" (-227, Fischer and Maniatis, 1988) which is critical for transcription of *Adh-1*. (This sequence is not found in the upstream flanking DNA of the *D. pseudoobscura* UO and the *D. virilis* UO genes which are described in Chapter Two). The sequence AAAGTAAAGCG of the *D. melanogaster* UO gene resides within the region deleted in the P-element construct P[($w^{\Delta+}$)del(-808, -702)DmUO-lacZ] (Chapter Three) which will be returned to the germ line for functional testing. It will be of interest to examine

the tissue-specific expression of the UO-lacZ transgene in transformants carrying this construct in order to establish whether this sequence has a possible role in the Malpighian tubule-specific expression of the *D. melanogaster* UO gene.

In general, a fine structure map of cis-acting regulatory elements of a gene is the necessary first step to understanding the intriguing questions concerning gene regulation. The molecular analysis of *D. melanogaster* UO determined that this gene is structurally compact and has a complex expression pattern and, therefore, is amendable to a variety of methodologies for studying gene regulation. The determination of the structure, sequence and pattern of expression of the *D. melanogaster* UO gene was essential background information for the investigation of evolutionary changes in sequence and regulation of Drosophila UO.

CHAPTER TWO: Evolutionary changes in the sequence of the UO gene of *D. melanogaster, D. pseudoobscura* and *D. virilis.*

A. Introduction

Sequence comparisons of the homologous gene from different species can be used to identify important structural and regulatory features of a gene or protein (Lipman and Pearson, 1985; Doolittle, 1989). Evolutionary processes conserve sequence which has functional relevance and randomizes and sometimes eliminates sequence which is functionally unimportant. Sequence comparisons have been successful in the identification of important structural and regulatory features of many Drosophila genes (Bray et al., 1989; Treier et al. 1989; Seeger and Kaufman, 1990; Shea et al., 1990). An interspecific sequence comparison of the UO gene was included as part of the molecular analysis reported here.

Since conserved sequence is likely to be important, a molecular evolutionary analysis of the UO gene was made in an attempt to (1) identify conserved regions of the UO protein to establish structure-function relationships and (2) to identify cis-acting regulatory elements in the flanking DNA of the UO gene that are potential candidates for cis-regulatory elements. The UO genes from *D. melanogaster, D. pseudoobscura* and *D.virilis* were cloned (Kral et al., 1986; Friedman et al., 1991; Lootens et al., 1991) and compared. *D. pseudoobscura* and *D. virilis* are estimated to have diverged from *D. melanogaster* 35 million to 60 million years ago (Beverley and Wilson, 1984). These three species were chosen for the comparison since the time of divergence of these species has been shown to be long enough for randomization of nonessential DNA

sequence (Henikoff and Eghtedarzadeh, 1987; Riley, 1989).

B. Results

1. Nucleotide and deduced amino acid comparisons of UO of *D. melanogaster, D. pseudoobscura* and *D. virilis.*

A restriction map of the *Drosophila pseudoobscura* genomic region showing the UO transcription unit and the subclones used for sequencing 2.24 kb of DNA from the *D. pseudoobscura* UO region are shown in Figure A3. Southern analyses demonstrated that the *D. pseudoobscura* UO genomic clones were derived from *D. pseudoobscura* strain AH133 and that no obvious rearrangements had occurred during construction of the genomic library or of subclones used for sequencing. Southern analysis also confirmed that the UO gene was single copy in *D. pseudoobscura* AH133 (Friedman et al., 1991).

A restriction map of the *Drosophila virilis* genomic region and the subclones used for sequencing 1.8 kb of DNA from the *D. virilis* UO region are shown in Figure A4. Extensive restriction mapping and Southern analyses were performed using the UO genomic clones and DNA isolated from several strains of *D. virilis* (Lootens et al., 1991). These data clearly indicated that the UO gene was tandemly duplicated in some strains of *D. virilis* and that the genomic clones were derived from a strain containing this tandem duplication of the UO gene. The tandemly duplicated *D. virilis* UO genes are designated Dv UO1 and Dv UO2 (Chapter Four). The Dv UO1 sequence is presented here. Elucidation of the mechanism of recombination that resulted in this tandem duplication among some strains of the *D. virilis* is part of the Ph.D. thesis research project of Susan Lootens.

The *D. pseudoobscura* and *D. virilis* genomic sequences are available in the EMBL GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X51940 and X57114, respectively. The transcription start

sites of the *D. pseudoobscura* and *D. virilis* UO genes were inferred on the basis of the position and sequence of the experimentally determined *D. melanogaster* UO transcription start site. Both the *D. pseudoobscura* and *D. virilis* inferred transcription start sites, GGCATCAGTCAGTCAT and GGCCTCATCGGAAT, respectively, (Figure A5) match the consensus transcription start site identified for several other Drosophila genes (ATCA(G/T)T(C/T), Hultmark et al., 1986). In the *D. pseudoobscura and D. virilis* UO transcribed region, the first ATG codon following the transcription start site is likely to be the translation initiation codon (Figure A5 and discussed in Chapter One). The sequence contexts of the UO translation initiation codons (ATG) from *D. pseudoobscura* (TAGAATGTTT) and *D. virilis* (CAGTATGTTT) are similar to those reported for other Drosophila genes (Cavener, 1987). Between the *D. melanogaster* and *D. pseudoobscura* deduced amino acid sequence, the first nine amino acid residues are identical with only two silent nucleotide substitutions. Between the *D. melanogaster* and *D. virilis* deduced amino acid sequence, the first eight amino acid residues are identical with eight silent nucleotide substitutions (Figure A5).

The *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO introns are 69 bp, 62 bp and 55 bp, respectively (Figure A5). The position of the *D. pseudoobscura* and *D. virilis* UO introns was inferred from a comparison of the *D. pseudoobscura* and *D. virilis* sequence to the *D. melanogaster* UO cDNA and genomic sequence. The intronic consensus donor (GT) and acceptor (AG) splice sites (Mount, 1982) present in the *D. melanogaster* UO gene are conserved in the *D. pseudoobscura* UO and *D. virilis* Dv UO1 genes with the intronic positions bifurcating an aspartic acid codon between the first and second positions in all three species (Figures 5 and A5).

The *D. pseudoobscura* and *D. virilis* UO nucleotide sequences are 82.2% and 73.2% identical to the *D. melanogaster* UO nucleotide sequence,

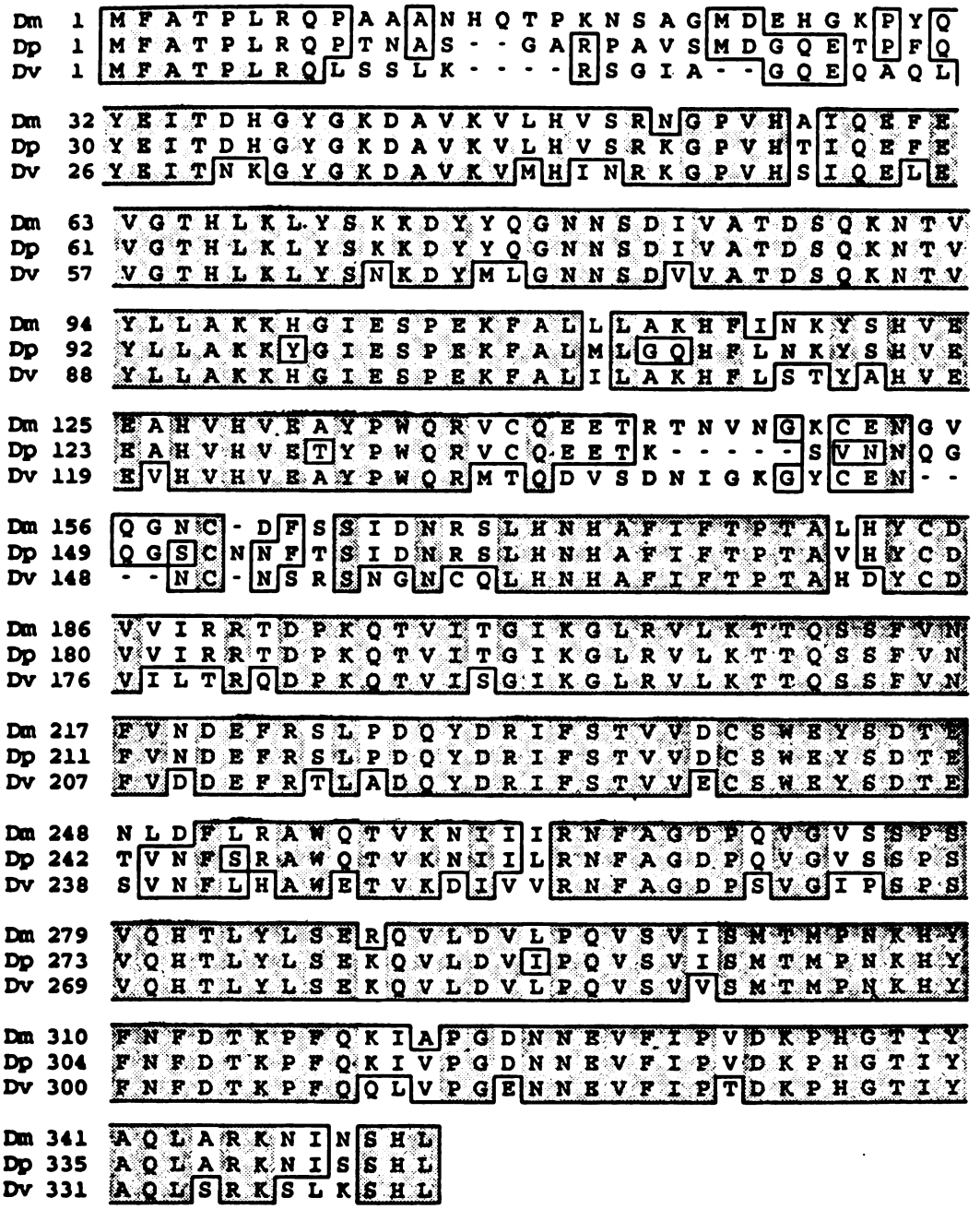


Figure 5. Alignment of the deduced amino acid sequence of UO of *D. melanogaster* (Dm), *D. pseudoobscura* (Dp) and *D. virilis* (Dv). The single-letter amino acid code is used. There is 72% identity among the UO protein from these three Drosophila species. Boxed areas indicate identical amino acid resides in two of the three UO proteins. Shaded amino acid residues are identical in all three Drosophila species. Dashes (-) represent gaps identified by FASTP (Lipman and Pearson, 1985).

respectively (Figure 5 and Table A3). The calculated peptide molecular weights for the *D. pseudoobscura* and *D. virilis* UO protein are 39,266 and 38,680 daltons, respectively, as compared to the *D. melanogaster* value of 39,989 daltons. This difference in size of the UO protein among these three Drosophila species has been confirmed by Western blot analysis (Figure 6). The first exon of the *D. pseudoobscura* and *D. virilis* UO gene encodes 185 and 181 amino acids, respectively, while the first exon of *D. melanogaster* UO encodes 191 amino acids. The second exon of the *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO genes each encodes 161 amino acids (Table A3).

Between *D. pseudoobscura* and *D. melanogaster*, the UO protein-coding region has accumulated 185 nucleotide substitutions from a total of 1038 nucleotides (17.8%) resulting in 42 amino acid replacements, 79% having occurred in exon 1 (Figure A5 and Table A2). Between *D. virilis* and *D. melanogaster*, the UO protein-coding region has accumulated 275 nucleotide substitutions from a total of 1026 nucleotides (26.8%) resulting in 86 amino acid replacements, with 68% occurring in exon 1 (Figure A5 and Table A2). The *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO exon 1 are 607, 590 and 585 nucleotides, respectively. Between *D. pseudoobscura* and *D. melanogaster* there are 106 nucleotide substitutions in UO exon 1 resulting in 48 synonymous changes and 33 amino acid replacements, of which 29 are evolutionarily conserved as defined by Lipman and Pearson (1985). Between *D. virilis* and *D. melanogaster* there are 162 nucleotide substitutions in UO exon 1 resulting in 53 synonymous changes and 59 amino acid replacements, of which 39 are evolutionary conserved changes.

The protein coding region of exon 2 of the UO gene from both *D. pseudoobscura* and *D. melanogaster* has 482 bp with 79 nucleotide substitutions of which 56 (70.9%) are in the third codon position. When compared with exon 2 of *D. melanogaster* UO, exon 2 of *D. pseudoobscura* UO

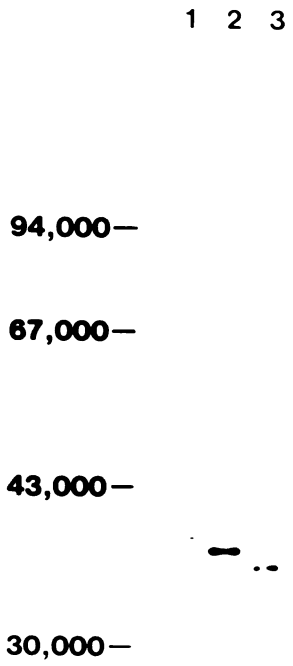


Figure 6. Western analysis of UO protein of *D. melanogaster, D. pseudoobscura* and *D. virilis*. Whole cell homogenate from two Malpighian tubules of *D. melanogaster* third instar larvae (lane 1), one half of a Malpighian tubule from *D. pseudoobscura* (lane 2) and one half of a Malpighian tubule from *D. virilis* (lane 3). UO protein was detected by Western analysis using rabbit polyclonal antibodies made against apparently homogeneous pure *D. melanogaster* UO protein (Friedman and Barker, 1982; Kral et al., 1986). The positions of protein molecular weight standards are given in the left margin. The UO M_r calculated from the deduced amino acid sequence of UO of *D. melanogaster, D. pseudoobscura* and *D. virilis* is 39,989, 39,266 and 38,680 daltions, respectively.

has only 9 amino acid replacements of which 8 are conserved replacements. The protein coding regions of exon 2, for both *D. virilis* and *D. melanogaster* UO genes, have 482 bp with 113 nucleotide substitutions of which 80 (70.8%) are in the third codon position. When compared to exon 2 of the *D. melanogaster* UO gene, the exon 2 of *D. virilis* UO has 27 amino acid replacements with 26 being conserved replacements.

2. *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO codon usage.

Third-codon-positions evolve at a rate approximating selective neutrality and have been used as a measure of the degree of divergence among species (Henikoff and Eghtedarzadeh, 1987; Riley, 1989). In Drosophila, the third-codon-position averages 71.4% G+C (Stramer and Sullivan, 1989). The third position of *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO are 71.0%, 76.3% and 63.7% G+C, respectively. The codon usage for UO genes of *D. melanogaster*, *D. pseudoobscura* and *D virilis* (Table A3) is similar to other Drosophila proteins (Shields et al., 1988).

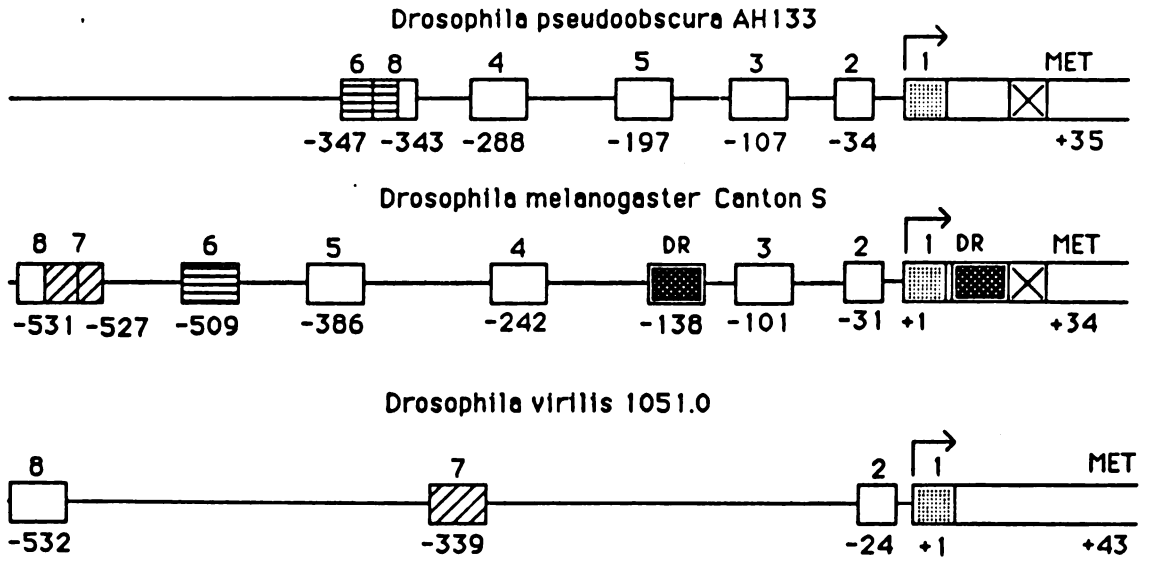
To examine the degree of divergence among the UO genes of *D. melanogaster*, *D. pseudoobscura* and *D. virilis*, third-codon-position differences, adjusted for codon bias, were compared for threonine, proline, alanine, glycine and valine, each having four codons. The deduced amino acid sequences of UO of *D. melanogaster* and *D. pseudoobscura* contain 90 conserved residues for these five amino acids. With random codon usage, 68 (75%) of the third-codon-positions of these amino acids would be expected to have changed to synonymous codons. When corrected for codon usage bias as described in Materials and Methods (Appendix A), 47 different third-codon-positions would be expected to have changed while 37 (79%) were observed to have changed since the divergence of *D. melanogaster* and *D. pseudoobscura* (Table A4).

The deduced *D. melanogaster* and *D. virilis* UO amino acid sequences contain 79 conserved residues for the five amino acids listed above. With random codon usage, 60 of the third-codon-positions of these amino acids would be expected to have changed to synonymous codons. When corrected for codon usage bias, 43 different third-codon-positions would be expected to have changed and in fact, 43 were observed to have changed since the divergence of *D. melanogaster* and *D. virilis* (Table A4). These results indicate that the additional time of divergence between *D. virilis* and *D. melanogaster,* as compared to *D. melanogaster* and *D. pseudoobscura,* has allowed for most of the unconstrained sequence of the UO gene to have changed.

3. DNA sequence comparisons in the 5' flanking DNA of the *D. melanogaster, D. pseudoobscura* and *D. virilis* UO genes.

When comparing the flanking DNA of a homologous gene among sufficiently diverged species, only sequence with important coding and regulatory function is conserved. The *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO flanking sequences were compared using dot matrix analyses (Pustell and Kafatos, 1982; Pustell and Kafatos, 1984). Conserved stretches of nine or more nucleotides, allowing for one nucleotide mismatch, were used as the search parameters.

Eight conserved sequence elements 9 to 16 bp in length (E1-E6 and E8) were identified after a comparison of the 5' flanking DNA of the *D. melanogaster* UO gene to the 5' flanking DNA of the *D. pseudoobscura* UO gene. Four conserved sequence elements (E1, E2, E7 and E8) were identified when comparing the 5' flanking DNA of the *D. melanogaster* UO gene to that of the *D. virilis* UO gene, with three of these elements (E1, E2 and E8) also shared between the 5' flanking DNA of the *D. virilis* and *D. pseudoobscura* 5' UO genes. The position and sequence of each conserved element is shown in



Element	Dm sequence	position	Dp sequence	position	Dv sequence	position
1	GGCATCATCAGTAT	-6	GGCATCAGTCAGTCAT	-6	GGCCTCATCGGAAT	-6
2	TATAAAAGG	-31	TATAAAAAGA	-34	TATAAAATG	-24
3	CTTTCTACGAAAT	-101	CTTTCTACTAAAT	-107	NP	
4	TGGAGATAGAAA	-242	TGGAGATAGAAA	-288	NP	
5	TCTGTAAAAATTA	-386	TCTGTAGAAATTA	-197	NP	
6	TTGTGAAATA	-509	TTGCGAAATA	-347	NP	
7	TAATGTTAT	-527	NP		TAATGTTAT	-339
8	GAAATAATGT	-531	GAAATAGTGT	-343	AAAATAGTGT	-532

Figure 7. The relative positions and the sequences of the conserved elements in the 5' flanking DNA of the UO genes of *D. melanogaster, D. pseudoobscura* and *D. virilis.* E1 matches the consensus transcription initiation site for Drosophila genes (Hultmark et al., 1986). E1 in the *D. melanogaster* UO 5' flanking DNA has been experimentally determined to be the UO transcription start site (Figure 2, Chapter One). E2 corresponds to a consensus TATA box sequence (Corden et al., 1980). The DR element is present only in the *D. melanogaster* UO gene and is similar in sequence to a proposed 20-hydroxyecdysone receptor binding element (Pongs, 1988).

Figures 7 and A5. Two of the elements, E1 and E2, are in similar positions relative to the transcription start sites of the *D. melanogaster, D. pseudoobscura* and *D. virilis* UO genes. E1 matches the Drosophila transcription start consensus sequence (Hultmark et al., 1986) and contains the nucleotide that has been identified for *D. melanogaster* (Figure 1) and inferred for *D. pseudoobscura* and *D. virilis* (Figure A5) to be the UO transcription initiation site. E2 matches the TATA box consensus sequence (Corden et al., 1980) and is at position -31, -34 and -24 with respect to the transcription start site of the *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO genes. Other than E1 and E2, the sequences of the evolutionary conserved elements, do not correspond to any cis-regulatory element reported to date.

C. Discussion

An interspecific DNA sequence comparison of the UO gene from different Drosophila species was chosen as the starting point to identify Drosophila UO protein structure/function relationships and Drosophila UO cis-regulatory elements. Due to the short length of the UO gene, interspecific comparisons of UO were tractable.

A sufficient period of time has transpired since the divergence of *D. melanogaster, D. pseudoobscura* and *D. virilis* that only sequence with functional importance should be conserved. Approximately 78% of the expected third-codon-position changes for alanine, glycine, proline, threonine and valine, when corrected for codon bias, have changed between *D. melanogaster* and *D. pseudoobscura* and 100% between *D. melanogaster* and *D. virilis* UO genes. This amount of third-codon-position change found in the Drosophila UO genes is consistent with observations from comparisons of the *Adh* (Schaeffer and Aquadro, 1987), *Gart* (Henikoff and Eghtedarzadeh, 1987), *hsp82* (Blackman and Meselson, 1986) and *Xdh* (Riley, 1989) genes of

D. melanogaster and *D. pseudoobscura* and the *hsp82* (Henikoff and Eghtedarzadeh, 1987) gene of *D. melanogaster* and *D. virilis*.

1. Deduced amino acid sequence comparisons of *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO.

There is 72% identity among the deduced amino sequences of *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO (Figures 5 and Table A2). The amino terminal extension of *Drosophila melanogaster* UO, not found in vertebrate or plant UO (Figure 4), is also present in *D. pseudoobscura* and *D. virilis* UO. Perhaps this amino extension has a functional role specific for Drosophila UO. The histidine residues conserved among *D. melanogaster*, vertebrate and plant UO, may be involved in copper binding and were discussed in Chapter One. The carboxy terminal tripeptide sequence (SHL) of UO is found in all three species of Drosophila and may be involved in peroxisomal targeting of UO. This issue was discussed in Chapter One. The variable region of Drosophila UO, from *D. melanogaster* UO amino acid positions 138 to 169 (Figure 5), is coincident with a region not present in the vertebrate and plant UO (Figure 4). This variable region may represent evolutionarily unconstrained sequence.

2. Conserved sequence elements in the 5' flanking DNA of the *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO genes.

In addition to tentatively identifying structure/function relationships of UO by molecular evolutionary comparisons, a second goal of the interspecific sequence comparisons was to determine the location and sequence of putative *D. melanogaster* UO cis-acting regulatory elements which may be responsible for the timing, tissue-specific expression and level of UO gene expression during development. Depending on the particular gene, cis-acting regulatory

elements may reside close to (Raghavan et al., 1986; Martin et al., 1989; Cereghini et al., 1987) or far from (Giangrande et al., 1987; Johnson et al., 1989; Bergson and McGinnis, 1990) the coding region as well as within an intron (Bingham et al., 1988; Bruhat et al., 1990; Kassis, 1990) and may be as small as 8 to 14 bp (Berg and von Hippel, 1988; Karim et al., 1990).

Well characterized consensus cis-regulatory elements have been compiled for several viral, mammalian, Drosophila and plant genes (Jones et al., 1988; Wingender, 1988; Biggin and Tjian, 1989). Since there are no obvious sequence landmarks or rules for predicting the locations of uncharacterized cis-regulatory elements, the location and assignment of function to cis-regulatory elements and identification of the transcription factors interacting with a particular gene can be a difficult task. An interspecific sequence comparison is a high resolution method facilitating the location and sequence of cis-acting regulatory elements. Using computer based homology matrix analyses (Pustell and Kafatos, 1984; Martinez-Cruzado et al., 1988), comparisons are made between the DNA sequence flanking the coding region of homologous genes from different species. Naturally occurring mutations will eliminate ancient homologies where nucleotide sequences are not under tight functional constraints, while the nucleotide sequences of some cis-regulatory elements are conserved.

Interspecific sequence comparisons have been made for several Drosophila genes to identify potential functional regions of proteins and putative cis-acting regulatory elements (Table A5). For some of these Drosophila genes, candidate cis-acting regulatory elements were identified as evolutionarily conserved sequence. Subsequently, experimental evidence demonstrated that many of the small evolutionarily conserved sequence motifs in the flanking DNA are functional regulatory cis-elements that bound particular trans-acting factors (Table A5). For example, a 16 bp element conserved within the 5' flanking DNA

of the Drosophila *dopa decarboxylase* (*Ddc*) gene of *D. melanogaster* and *D. virilis* has been shown to be essential for *Ddc* transcription in the central nervous system and binds to a factor present in embryonic nuclear extracts (Bray et al., 1988; Bray et al., 1989).

For several reasons a molecular evolutionary comparison probably does not identify all cis-acting regulatory elements of a particular gene. Cis-regulatory elements smaller than 7 bp are not easily discovered in sequence comparisons. It is also possible that a particular trans-acting factor may bind to cis-elements of strikingly different sequence and still bring about the same regulatory effect. There are examples in which regulatory factors bind dissimilar sequence elements (Pfeifer et al., 1987; Baumruker, 1988). A particular cis-acting sequence and trans-acting factor of a given species could have experienced co-evolution such that their counterparts in the other species no longer share sequence similarity. For these reasons, a molecular evolutionary comparison should be coupled with complementary methods, such as deletion analyses and fine structure mapping with point mutations, when identifying the regulatory elements of a gene.

As for the Drosophila UO gene, molecular evolutionary sequence analyses revealed several potential candidate cis-regulatory elements (Figures 7 and A5). The elements E1 and E2 represent the transcription start site and the TATA box of the UO genes, respectively. The sequence of the conserved elements E3 to E8 do not match the sequence of any characterized cis-regulatory element. The functions of these elements, if any, cannot currently be discerned.

From P-element mediated germ line transformation of a UO-lacZ fusion gene (Chapter 3), the DNA sequence 826 bp upstream and 350 bp downstream of the *D. melanogaster* UO transcription start site has been shown to be sufficient for the appropriate *D. melanogaster* UO temporal and spatial pattern of

expression. The elements in the 5' flanking DNA conserved between *D. melanogaster*, *D. pseudoobscura* and *D. virilis* reside within this DNA sequence and are likely to be cis-acting regulatory elements of the *D. melanogaster* UO gene. The location of these conserved elements has been used as one guide for selecting regions to be deleted from the the 5' flanking DNA of a UO-lacZ fusion gene (Chapter Three).

III. CHAPTER THREE: Identification of the location of regulatory regions required for *D. melanogaster* UO gene expression.

A. Introduction

Appropriate expression of most eukaryotic genes depends on the interaction of trans-acting regulatory factors and cis-acting DNA sequences. With the ability to clone a particular gene from an organism, make alterations in the regulatory region of that gene, introduce the altered gene into the genome of the organism and assay the effects of the alterations on transcription of the gene, many of the mysteries concerning gene regulation have been revealed. This approach has been used to understand the regulation of several genes of mice, Drosophila and plants (Garbadebian et al., 1986; Chung and Keller, 1990; Ohshima et al., 1990; Todo et al., 1990; Vassar et al., 1991). Studies using *in vivo* functional analysis of gene regulation have revealed generalities and continue to uncover novelties with regard to the location and sequence of cis-regulatory elements. With the exception of house-keeping genes, cis-acting regulatory sequences are frequently comprised of general promoter elements, the transcription start site and TATA box, as well as other factor-binding sites that may be relatively close or at great distances from the transcription start site (Reghavan, 1986; Giangrande et al., 1987; Bergson and McGinnis, 1990).

This chapter concentrates on the localization of cis-regulatory elements for the *D. melanogaster* UO gene and includes the application of a newly formulated method of deletion analysis to the 5' region of the UO gene. The method involves the site-specific removal of stretches of DNA as large as 235 bp in length. This technique is expedient, reliable and generally applicable to

the study of any region of DNA for which the sequence is known.

B. Results

Determination of the location of the regulatory region of the *D. melanogaster* UO gene was possible because the *D. melanogaster* UO gene is structurally compact with a transcribed region of approximately 1.4 kb. Moreover, there is an efficient germ line transformation system available for Drosophila (Rubin and Spradling, 1982). When reintroduced into the genome of *D. melanogaster,* most *D. melanogaster* genes with a sufficient amount of flanking DNA, are appropriately regulated (Goldberg et al., 1983; Richards, 1983; Scholnick et al., 1983; Spradling and Rubin, 1983). Transformed stocks are stable for many years and analyses of gene expression can be performed throughout development and on large numbers of isogenic animals. For these reasons, different P-element plasmids containing the UO gene were constructed and transformed into *D. melanogaster* using P-element transformation (Rubin and Spradling, 1982).

1. UO flanking DNA sequence sufficient for appropriate UO gene expression.

P[($w^{+\Delta}$)DmUOPstI], a P-element construct containing the 3.2 kb *Pst*I fragment which includes the UO gene from the *ecd*^{1} strain (Figure A1), was inserted into the P-element vector CaSpeR (Pirrotta, 1988) and integrated into the genome of *D. melanogaster* using P-element mediated transformation described by Rubin and Spradling (1982) with some modifications. A detailed description of the transformation procedure used here is found in Materials and Methods (Appendix A). This 3.2 kb *Pst*I fragment contains the UO gene with 826 bp of 5' flanking DNA and approximately 1200 bp of 3' flanking DNA. By performing the appropriate genetic crosses of the P-element transformants (see

Materials and Methods, Appendix A), five independent lines were made homozygous for a single P-element insertion. An example of a genomic Southern analysis performed to determine the number of P-element integrations is shown in Figure A6.

In vivo transcription of the UO transgene of construct P[($w^{+\Delta}$)DmUOPstI] was examined by Northern analysis. An oligonucleotide specific for message transcribed from the UO transgene of construct P[($w^{+\Delta}$)DmUOPstI] (Chapter One, Materials and Methods and Table A6) detected UO mRNA from transformed third instar larvae and adults but not from pupae on Northern blots. In wild type *Drosophila melanogaster*, UO expression is confined to the Malpighian tubules. Northern analysis of RNA from hand-dissected Malpighian tubules of third instar larvae and adult transformants compared to RNA extracted from the remaining tissues demonstrated that UO mRNA from the UO transgene is only expressed within the Malpighian tubules. Shown in Figure 8 is the Northern analysis of a transformed line, designated 1M11D, possessing the UO transgene of construct P[($w^{+\Delta}$)DmUOPstI]. Four additional independent transformed lines were analyzed in an identical fashion with the same results as in Figure 8. A list of all the UO P-element constructs used in this study, the different transformed lines and the results of functional analysis of the UO transgenes is shown in Table A7.

2. UO flanking DNA insufficient for appropriate UO gene expression.

In order to determine if 174 bp of 5' flanking DNA and approximately 300 bp of 3' flanking DNA would provide appropriate UO transgene expression, a second P-element plasmid designated P[(hspw^{+})DmUOSpeIStuI] was constructed. The *Spe*I-*Stu*I restriction fragment containing the UO gene

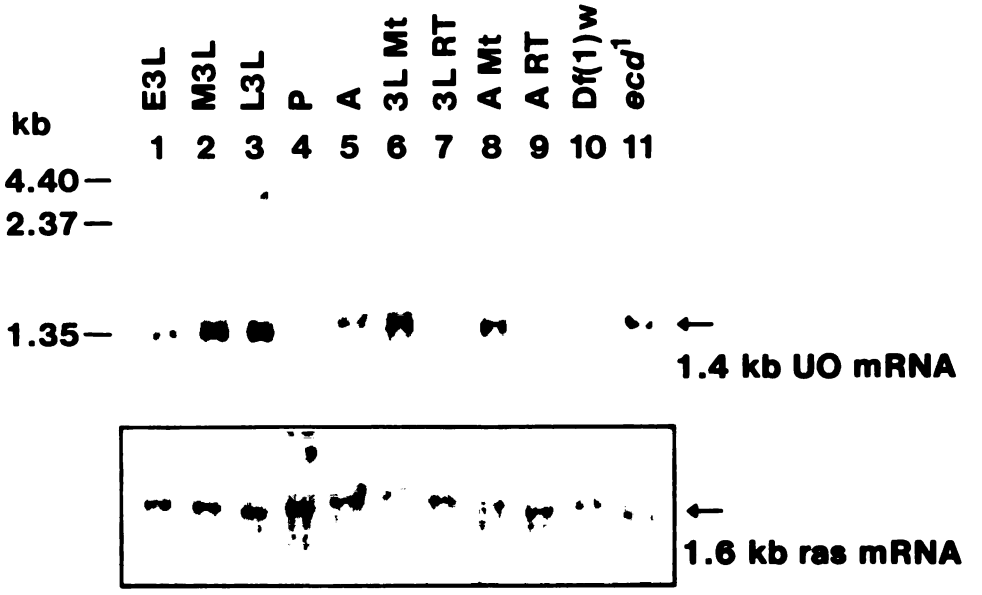


Figure 8. Northern analysis of UO mRNA in a *D. melanogaster* stock, 1M11D, transformed with P[($w^{+\Delta}$)Dm UOPstI]. Total RNA from 60 animals (lanes 1-5, 10 and 11), 50 dissected Malpighian tubules (lanes 6 and 8) and the remaining tissue after dissection of the Malpighian tubules (lanes 7 and 9) was probed with an oligonucleotide which hybridized exclusively to the UO mRNA transcribed from the UO gene introduced via P-element transformation. UO mRNA was detected in transformed early (E3L), mid-(M3L) and late (L3L) third instar larvae (lanes 1, 2 and 3, respectively) as well as the adult (A; lane 5). UO mRNA was not detected in the pupae (P, lane 4). UO expression in the transformants was confined to the Malpighian tubules of the third instar larvae (3L Mt, lane 6) and the adult (A Mt, lane 8) and not present in the remaining tissue after Malpighian tubule dissection (RT, lanes 7 and 9). UO mRNA from the host strain Df(1)w does not hybridize to an oligonucleotide specific for UO RNA transcribed from the UO transgene (lane 10). Lane 11 contains total RNA from adults of the *ecd*^{1} stock from which the UO gene present in the P-element construct was derived. The positions of RNA size standards are shown in the left margin. To verify the presence of RNA in each lane, the Northern blot was reprobed with a *Hind*III-*Pst*I fragment of the *D. melanogaster ras* gene (boxed area) which hybridizes to a 1.6 kb transcript expressed uniformly during development (Mozer et al., 1985).

isolated from *D. melanogaster ecd-1* strain was inserted into the *Xba*I and *Stu*I restriction sites of the P-element vector pW8 (Klemenz et al.,1987) and transformed into *D. melanogaster*. Two transformed lines homozygous for a single copy of the UO transgene were analyzed. Using the 29-mer specific for UO mRNA from the *ecd-1* strain (Chapter One, Materials and Methods and Figure A6), Northern analysis of both transformed lines revealed that expression of the UO transgene was barely detectable. Even with a high specific activity probe a faint band representing UO mRNA from the transgene was only detectable after long exposures of the autoradiograph. The Northern analysis of one transformed line, 3M9B, is shown in Figure 9. Further deletion analysis of the 5' flanking DNA of the UO gene between positions -826 and -174, relative to the UO transcription start site at +1 (Figure 1), is discussed below.

3. Expression of a UO-lacZ fusion gene.

It was important to establish whether UO regulatory elements resided in the 3' flanking DNA of the UO gene and/or within the 69 bp *D. melanogaster* UO intron. A hybrid gene, therfore, was constructed, with 826 bp of 5' UO flanking DNA and the transcribed region encoding the first 104 amino acids of UO fused in-frame with the *E. coli lacZ* gene, and inserted into CaSpeR. The details of the construction of this UO-lacZ fusion gene is diagramed in Figure M1 and discussed in Materials and Methods (Appendix A). This fusion gene lacked the UO intron and all 3' UO flanking DNA.

The *E. coli lacZ* gene was chosen as a reporter gene for many reasons. The bacterial β-galactosidase protein can tolerate large additions to its amino terminus and still remain functional (Casadaban et al., 1980; Shapira et al., 1983). Expression of β-galactosidase from a transgene can be easily and quickly scored in tissues of Drosophila using histochemical staining techniques

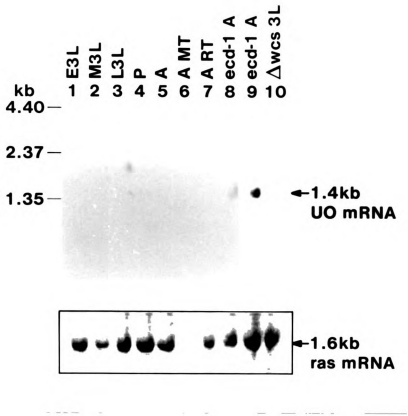


Figure 9. Northern analysis of UO mRNA from a *D. melanogaster* stock, 3M9B, transformed with P[(hspw$^+$)DmUOSpeI-StuI]. Total RNA from 60 animals (lanes 1-5 and 8-10), 60 dissected Malpighian tubules from adults (A MT, lane 6) and the remaining tissue after dissection of the Malpighian tubules (A RT, lane 7) was probed with an oligonucleotide which hybridized exclusively to the UO mRNA transcribed from the UO gene introduced via P-element transformation. UO mRNA was not detected in transformed early (E3L), mid-(M3L) and late (L3L) third instar larvae (lanes 1, 2 and 3, respectively) as well as pupae (P, lane 3) and adults (A, lane 5). UO mRNA from third instar larvae of the *white* deficient host strain (Δwcs) does not hybridize to this oligonucleotide specific for mRNA transcribed from the UO transgene (lane 10). Lanes 8 and 9 contain total RNA from adults of the *ecd*^{1} stock from which the UO gene present in the P-element construct was derived. The positions of RNA size standards are indicated in the left margin. To verify the presence of RNA in each lane, the Northern blot was reprobed with the *Hind*III-*Pst*I fragment of the *D. melanogaster ras* gene (boxed area) Very little hybridization to the *ras* probe occurred in lane 6 due to the reduced amount of total RNA present in the dissected Malpighian tubules compared to all other lanes.

(Raghavan et al., 1986) which eliminates the need for Northern or Western analysis of transformants. In addition, β-galactosidase activity can be quantitatively measured within a single fly (Simon and Lis, 1987). By using the solutions of the appropriate pH (Materials and Methods, Appendix 1) when performing the histochemical staining and quantitative assays, endogenous β-galactosidase (Fuerst et al., 1987) activity is usually not detected. Occasionally, however, histochemical staining of the endogenous β-galactosidase is noted in the alimentary canal of *D. melanogaster* (Bello and Couble, 1990).

The P-element plasmid containing the UO-lacZ fusion gene, designated P[($w^{+\Delta}$)DmUO-lacZ] (Figure M1 and Table A7; all figures with an "M" preceding the number are located in Materials and Methods, Appedix A), was transformed into *D. melanogaster* embryos. Three independently transformed lines, each containing a single insertion of the UO-lacZ transgene (Southern analyses not shown) were analyzed for β-galactosidase activity by histochemically staining of whole transformed flies and dissected tissue in a staining solution containing X-gal (Materials and Methods).

Three transformed lines containing the UO-lacZ transgene showed β-galactosidase expression in a temporal and tissue-specific pattern identical to that of the wild type *D. melanogaster* UO gene (Figure 10). β–galactosidase was detected only in the Malpighian tubules when third instar larvae and adults were stained as whole organisms (data not shown). β–galactosidase staining of Malpighian tubules dissected from a third instar larva and adult transformant are shown in Figure 10, panels a and c. Interestingly, β-galactosidase staining in the adult was confined to the main segment cells of the Malpighian tubules, the same subset of cells which express UO mRNA in wild type *D. melanogaster*. Compare Figures 3c and 10c for temporal expression of the wild type UO gene and the UO-lacZ transgene. Malpighian tubules of first

Figure 10. Histochemical staining for β-galactosidase activity of a *D. melanogaster* stock, 3F6D, transformed with P[($w^{+\Delta}$)DmUO-lacZ]. Dissected Malpighian tubules from a transformed mid-third instar larva (a), late third instar larva (b), mid-pupa (c) and adult (d) after histochemical staining with X-gal as a substrate for β-galactosidase activity.

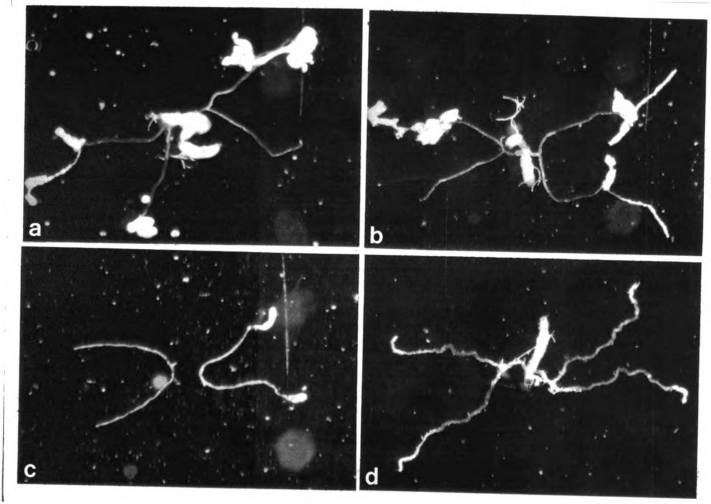


Figure 10.

instar larvae never stained for β-galactosidase, even after extended incubation in the staining solution (6 hours to overnight, data not shown). Malpighian tubules of second instar larvae as well as very early pupal Malpighian tubules rarely exhibited staining for β-galactosidase activity, and if so, the staining was confined to a small number of cells within the Malpighian tubules (data not shown). There did not appear to be a particular pattern as to which cells of the Malpighian tubules expressed β-galactosidase during the late second to early third instar larva transition when the *D. melanogaster* UO gene becomes transcriptionally active or which cells expressed β-galactosidase during the late third instar to early pupal transition when the UO gene is silenced. Clearly, by the mid pupal stage, β-galactosidase activity from the UO-lacZ transgene was not detected in the Malpighian tubules (Figure 10b). Newly emerged adults never showed β-galactosidase staining (data not shown), which is consistent with UO enzyme activity first being detected at two or three hours after emergence (Friedman and Johnson, 1977). Approximately 12 to 18 hours after emergence, β-galactosidase activity is detected histochemically in adult Malpighian tubules as discrete patches of blue cells in the main segment region of the Malpighian tubules (data not shown) and after 24 hours, virtually all of the main segment cells of the Malpighian tubules are stained blue (Figure 10c). This implies that the strict timing of the appearance UO activity in the adult (Friedman and Johnson, 1977) is mimicked by the UO-lacZ transgene.

4. Deletion of the DR elements of the *D. melanogaster* UO gene.

Given that 826 bp of 5' flanking DNA and the first 350 bp immediately downstream of the *D. melanogaster* UO transcription start site provided wild type *D. melanogaster* UO expression pattern on the lacZ gene, this UO-lacZ transgene was used as a reporter system to monitor the effects of site specific mutations made within the 5' flanking DNA of the UO gene. One sequence

element of interest was the direct repeat element (DR, Chapter One) that shares sequence similarity with a proposed 20-hydroxyecdysone receptor binding site (Pongs, 1988). Due to this sequence similarity and the ability of the DR elements to bind purified 20-hydroxyecdysone receptor (R. Voellmy, unpublished data), the DR element was a candidate regulatory element for 20-hydroxyecdysone repression of the *D. melanogaster* UO gene (see discussion and Chapter One).

Using the technique of Vandeyar et al. (1988), site-directed mutagenesis was performed to delete the distal DR element (positions -138 to -126, Figure 1) and the proximal DR element (positions +11 to +23, Figure 1) from the 826 bp of flanking DNA shown to be sufficient for UO gene expression. The resultant P-element constructs were identical to the construct P[($w^{+\Delta}$)DmUO-lacZ] except that one or both of the DR elements were deleted (Figure 11). The P-element construct lacking the distal DR element is designated P[($w^{+\Delta}$)del(-138, -126)DmUO-lacZ], the P-element construct lacking the proximal DR element is designated P[($w^{+\Delta}$)del(+11, +23)DmUO-lacZ] and the the P-element construct with both DR elements deleted is P[($w^{+\Delta}$)de(-138, -126) (+11, +23)DmUO-lacZ] (Figure 11). The site-directed mutagenesis and the details of the construction of these P-element plasmids are described below and in Materials and Methods and diagrammed in Figures 11, M1 and M2. The effects of the DR deletions on the regulation of the UO-lacZ transgene were analyzed for β-galactosidase activity by the histochemical staining procedure described above.

Two independent transformed lines homozygous for a single insertion of P[($w^{+\Delta}$) del(-138, -126)DmUO-lacZ] showed no difference in the temporal pattern of β-galactosidase staining when compared to lines transformed with

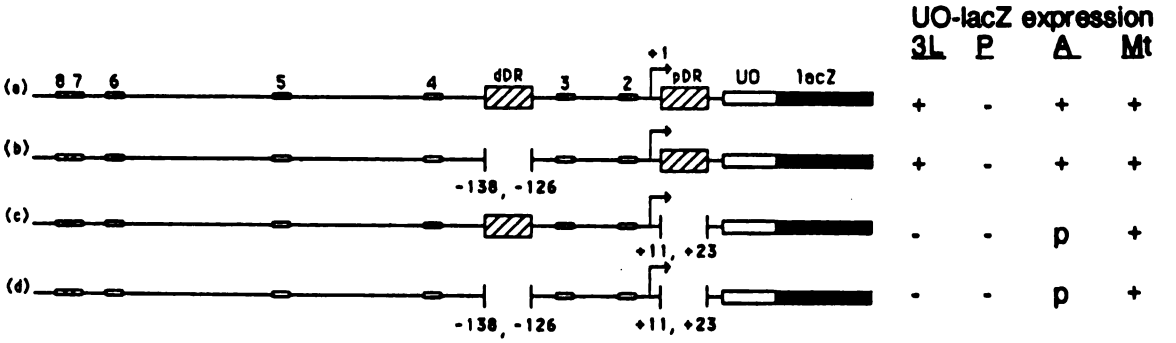


Figure 11. Diagram of the 5' flanking DNA of the *D. melanogaster* UO gene present in the P-element DR deletion constructs and the UO-lacZ expression pattern of transformants. The DR elements are represented by cross-hatched rectangles, with the distal DR element (positions -138 to -126, Figure 1) designated dDR and the proximal DR element (postions +11 to +23, Figure 1) designated pDR. The evolutionarily conserved elements are represented by small open rectangles, the UO coding region is shown as an open large rectangle and lacZ coding sequence is a filled rectangle. Schematic representation of the "wild type" 5' flanking region of the UO gene which is present in P-element construct P[($w^{+\Delta}$)DmUO-lacZ] (a). Schematic representation of the 5' region of the UO gene included in construct P[($w^{+\Delta}$)del(-138, -126)DmUO-lacZ] (b), construct P[($w^{+\Delta}$)del(+11, +23)DmUO-lacZ] (c) and construct P[($w^{+\Delta}$)del(-138, -126)(+11, +23)DmUO-lacZ] (d). Gaps in a line represent the sequence that has been deleted. The numbers below the lines represent the position of the breakpoints of the *in vitro* generated deletions with respect to UO transcription initiation at +1. The numbered nucleotides as well as the sequence between the numbers has been deleted. UO-lacZ expression detected (+) or not detected (-) by histochemical staining for β-galactosidase activity. p=perturbed expression of the UO-lacZ transgene with only some adult flies within a stock showing staining for β-galactosidase activity. 3L=third instar larvae, P= pupae, A=adults and Mt=Malpighian tubules.

P[($w^{+\Delta}$)DmUO-lacZ] (data not shown). β-galactosidase was detected exclusively in the Malpighian tubules of third instar larvae and adults. Four independent transformed lines homozygous for a single copy of P[($w^{+\Delta}$)del(+11, +23)DmUO-lacZ] were analyzed. Interestingly, β-galactosidase could not be detected from the first instar larval through the adult stage (data not shown). Occasionally, a few cells in a third instar larval Malpighian tubule would stain for β-galactosidase activity. In addition, the Malpighian tubules of some adults would stain for β-galactosidase activity. Possible reasons for this variable expression of the UO-lacZ fusion transgene lacking the proximal DR are dealt with in the Discussion section.

Four independently transformed lines containing P[($w^{+\Delta}$)del (-138, -126)(+11, +23)DmUO-lacZ] that were homozygous for a single P-element insertion and one that was heterozygous for a single P-element insertion did not display β-galactosidase staining in any tissue from the first instar larval through the pupal stage. Only a few adults of a given transformed line showed staining for β-galactosidase within the Malpighian tubules (data not shown). However, one line, 2M23, homozygous for a single insertion of P[($w^{+\Delta}$)del(-138, -126)(+11, +23)DmUO-lacZ] showed β-galactosidase staining in all of the adult Malpighian tubules examined, but not in the larval or pupal Malpighian tubules (data not shown). Clearly the deletion of the proximal DR element disrupts the normal pattern of UO gene expression. An interpretation and possible significance of this staining pattern of the UO-lacZ transgene of P[($w^{+\Delta}$)del(-138, -126) (+11, +23)DmUO-lacZ] will be addressed in the Discussion section.

5. Combinative oligonucleotide-directed large deletions in the 5' flanking DNA of the *D. melanogaster* UO gene.

Results from the analysis of UO transgene expression of lines transformed with P[($w^{+\Delta}$)DmUOPstI], P[(hspw^{+})DmUOSpeI-StuI] and P[($w^{+\Delta}$)DmUO-lacZ] (summarized in Table A7) indicated that important cis-regulatory elements of the UO gene reside between position -826 and -174 with respect to the UO transcription start site (Figure 1). A new approach was formulated to efficiently delete large regions (approximately 100 bp) within the 826 bp upstream of the UO gene, which would enable one to analyze single large deletions and large deletions in all possible combinations with one another (Figures 12 and M2). The site-directed mutagenesis technique of Vandeyar et al. (1988) was used to construct deletions within the 5' region of the UO gene which had been subcloned into the bacteriophage M13 (Messing and Vieira, 1982) (Figure 18). This *in vitro* mutagenesis technique involves hybridization of a mutagenic primer and selective methylation of the mutant strand by the incorporation of 5-methyl-dCTP during synthesis of the second M13 strand. The parental M13 strand is degraded with restriction enzymes that nick only the nonmethylated DNA and digestion with exonuclease III. For deletion analysis of the UO cis-regulatory region, 34-mer oligonucleotides (Table A6) were used as deletion primers. The sequence of the two 17-mer "arms" of each primer were complementary to noncontiguous 17 bp sequences at distances of 105 bp to 245 bp from each other along the single stranded UO region subcloned into the M13 vector (Figures 12 and M2). Constructing large deletions by "looping out" the DNA sequence to be deleted are thought to be difficult to obtain (Sambrook et al., 1989). However, for three different 34-mers (Primers #20, #21 and #23, Table A6), large deletions of the UO 5' flanking region were obtained in 38% to 70% of the M13 plaques analyzed.

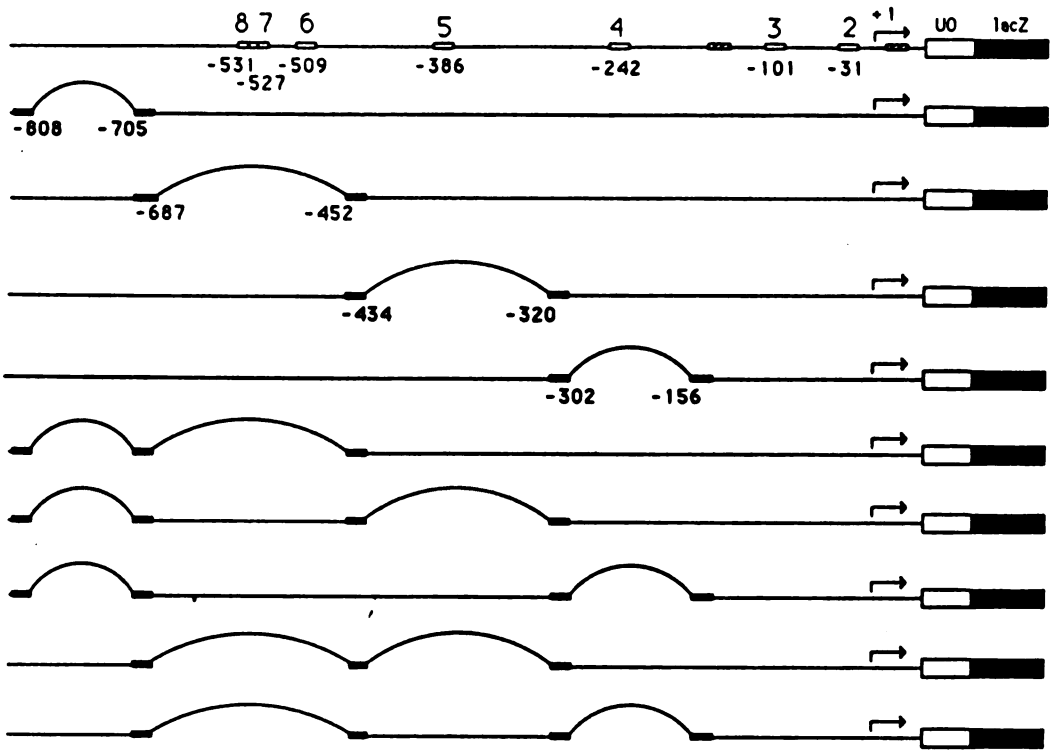


Figure 12. Diagram of the combinative oligonucleotide deletion analysis of the regulatory region of the *D. melanogaster* UO gene. The top line represents the 5' flanking DNA of the UO gene with evolutionarily conserved elements as small open rectangles, the DR elements as cross-hatched rectangles and the UO coding region as a large open rectangle fused in-frame to the *lacZ* coding region, a large filled rectangle. +1 represents the transcription start site of the UO gene with the arrow showing the direction of transcription. Each subsequent line represents the 5' UO flanking DNA included within a deletion construct. Each small, filled rectangle represents 17 nucleotides of a 34-mer primer with the other 17 nucleotides connected by a curved line. The region deleted after mutagenesis is represented by the gap within the line between the two small, filled rectangles. The numbers below the lines indicate the limits of the deletions with the numbered bases deleted. A detailed discussion and diagram of this combinative deletion technique is in the Materials and Methods (Appendix A).

It was confirmed by sequence analysis that a deletion was introduced into the 5' UO flanking region. The single stranded DNA from a clone with a particular deletion was used as a substrate for deletion mutagenesis with another UO 34-mer. Thus, combinative deletions were efficiently and accurately made within the 5' region of the UO gene. The UO 5' flanking DNA, possessing one or more large deletions, was excised from the M13 vector and fused in-frame with the *E. coli lacZ* gene, inserted into CaSpeR and transformed into *D. melanogaster* (Figure M2). The creation of these *D. melanogaster* UO deletion constructs and the analyses of transformants carrying these altered UO genes is a contribution to the ongoing project in Dr. Friedman's laboratory to fine-structure map the location and sequence of cis-acting regulatory elements of the *D. melanogaster* UO gene.

C. Discussion

For appropriate regulation, most eukaryotic genes appear to require multiple discrete cis-acting regulatory sequences and trans-acting regulatory factors which bind them. Cis-acting regulatory elements may act individually or in cooperation with other elements to control the transcriptional activity of a gene (Bouvagnet et al., 1987; Jantzen et al., 1987, Chretien et al., 1988). Regulatory elements include promoters, enhancers, transcription terminators and sequences involved in controlling timing, tissue-specific expression and response to trans-acting molecules (Delaney et. al., 1987; Geyer and Corces, 1987; Hammer et al., 1987; Shermoen et al., 1987; Bray et al., 1988). Depending on the particular gene, cis-acting regulatory elements may reside close to or far from the transcription start site (Steller and Pirrotta, 1985; Giangrande et al., 1987; Reghavan et al., 1986). Cis-acting regulatory elements have been discovered in the 5' and 3' flanking DNA as well as in intronic sequences (Bingham et al., 1988; Bruhat et al., 1990).

1. Delimiting the amount of 5' flanking DNA required for appropriate regulation of the *D. melanogaster* UO gene.

The first step in the identification of the location of the cis-regulatory elements of a particular gene is to delimit the amount of flanking DNA required for appropriate expression of that gene. For the UO gene, P-element plasmids containing either the UO gene with differing amounts of 5' and 3' UO sequence or containing a UO-lacZ fusion gene were transformed into the *D. melanogaster* genome. Data from Northern analyses and histochemical staining for β-galactosidase of the transformants carrying these modified UO genes indicated that more than 174 bp, but no more than 826 bp of 5' UO sequence is required for appropriate temporal and tissue-specific expression of the *D. melanogaster* UO gene (Figures 8 and 9 and Table A7). Regulatory elements that influence the UO gene do not reside in the UO 3' flanking DNA or intronic sequences, since these sequences are not present in the UO-lacZ transgene which exhibits an expression pattern identical to that of the UO gene (Figure 10). It was not surprising that UO cis-regulatory elements were not discovered in the 69 bp intron since all introns harboring regulatory elements to date have been large in size (Bingham et al., 1988). Thus, between -826 and -174 there are critical UO regulatory elements, some which may correspond to the elements identified by the evolutionary sequence comparison described in Chapter Two.

2. The DR element has a role in the expression of the *D. melanogaster* UO gene.

Examination of the 5' flanking DNA of the *D. melanogaster* UO for known cis-regulatory elements identified a direct repeat that shares sequence similarity with a proposed 20-hydroxyecdysone receptor binding site (Pongs, 1988). In particular, each DR motif has two adjacent copies of the sequence (AGTGA)

which is also found within the region providing 20-hydroxyecdysone induction of the *hsp22* heat shock protein gene of *D. melanogaster* (Klemenz and Gehring, 1986; Pongs, 1988). The similarity of DR motifs to proposed steroid hormone receptor binding sites (Pongs, 1988; Beato, 1989) and the position of the two DR motifs relative to transcription start of the UO gene suggest that these elements may be important for the transcriptional repression of the UO gene by 20-hydroxyecdysone (Kral et al., 1982). In collaboration with R. Voellmy (University of Florida, Miami), it was shown that the UO *Eco*RI-*Hha*I and *Hha*I-*Spe*I restriction fragments (Figure 1), each containing a single DR element, bound to purified 20-hydroxyecdysone receptor in a gel retardation assay. In addition, a 22 bp double stranded synthetic oligonucleotide (5' CTCTAAGTGAGAGTGATGATGG 3') containing the DR sequence (position -142 to -115 , Figure 1) showed binding to a purified 20-hydroxyecdysone receptor in a gel retardation assay (unpublished data).

Evidence that 20-hydroxyecdysone is part of the mechanism which represses the UO gene is derived from experiments using *ecd*^{1}, a temperature sensitive 20-hydroxyecdysone deficient strain of *D. melanogaster*. Repression of UO gene transcription in *ecd*^{1} occurs in the late third instar larvae at 19^{o}C as in wild type *D. melanogaster*, but repression of UO gene transcription does not occur in the late third instar *ecd*^{1} larvae at 29^{o}C, the restrictive temperature. In addition, a rapid decline of UO mRNA in *ecd*^{1} at 29^{o}C can be brought about by feeding 20-hydroxyecdysone to late third instar larvae (Kral et al., 1982).

For some genes, different cis-regulatory elements appear to be involved in steroid hormone mediated gene activation and repression (Beato, 1989, Martin et al., 1989; Sakai et al., 1988). It seems possible, however, that a sequence which binds the 20-hydroxyecdysone receptor could function either as an

inducible or repressible element depending on its location. Therefore, the same 20-hydroxyecdysone receptor may function as an activator as well as a repressor. If the 20-hydroxyecdysone receptor interacts with one or both of the DR elements, this complex might interfere with positive trans-acting factors that bind to the TATA box or the ability of RNA polymerase to initiate transcription of the UO gene. There is precedent for interference as a mechanism of gene repression (Drouin et al., 1987; Akerblom et al., 1988; Levine and Manley, 1989). The human chorionic gonadotropin gene is repressed when the glucocorticoid receptor binds to a cis-element and interferes with cAMP binding at an adjacent site (Akerblom et al., 1988).

Given these observations, it seemed plausible that the UO DR element may have a role in 20-hydroxyecdysone mediated repression of the UO gene at the end of the third instar larval stage. As a functional test of this hypothesis, one or both of the DR elements were deleted from the *D. melanogaster* UO-lacZ fusion gene and the DR deletion constructs were transformed into the genome of *D. melanogaster* (Figures 11 and M1). The spatial and temporal developmental regulatory consequences of these deletions were analyzed by histochemical staining for β-galactosidase activity in tissues of transformants. If elimination of the DR element prevented 20-hydroxyecdysone repression of the UO-lacZ transgene, assuming that 20-hydroxyecdysone repression of the UO gene is the only mechanism involved in silencing the *D. melanogaster* UO gene at the end of the third instar larval stage, β-galactosidase staining would be predicted to persist throughout the pupal stage. This result would be in contrast to the absence of β-galactosidase staining of the pupal Malpighian tubules of transformants possessing the "wild type" UO-lacZ transgene which diminishes at the onset of the pupal stage (Figure 10b).

However, deletion of the distal DR element did not change the temporal or tissue-specific pattern of expression of the UO-lacZ transgene (data not shown).

Histochemical staining of β-galactosidase in transformants carrying the UO-lacZ transgene with deletion of the DR element at position -138 occurred in the Malpighian tubules of third instar larvae and adults but not the pupae, identical to the normal pattern of UO regulation. This result raises the possibility that there is more than one mechanism responsible for UO gene repression during the late third instar larval stage.

That a second mechanism may be involved in UO gene repression is derived from an analysis of the expression of the *D. pseudoobscura* UO and *D. virilis* UO1 genes in the genome of *D. melanogaster* (Chapter Four). The *D. pseudoobscura* UO and *D. virilis* UO1 genes do not have the direct repeat element within approximately 800 bp of 5' flanking DNA (Figure 7). Both the *D. pseudoobscura* and *D. virilis* UO genes are expressed during the third instar larval stage and silenced at the beginning of the pupal stage when integrated into the *D. melanogaster* genome. However, it is possible that for these species 20-hydroxyecdysone repression of the UO gene is operating through a cis-acting regulatory element with a sequence unlike the DR elements. There may be many different cis-acting sequences to which the 20-hydroxyecdysone receptor binds. In addition, there may be more than one 20-hydroxyecdysone receptor in Drosophila.

Even though deletion of the distal DR element did not change the UO-like temporal or spatial pattern of expression of the UO-lacZ transgene, the distal DR element may be involved in 20-hydroxyecdysone repression of the *D. melanogaster* UO gene, but the effects of removal of the distal DR may be masked by a second mechanism of UO repression or perhaps the absence of a positive trans-acting factor.

In order to more definitively determine if deletion of the distal elements blocks 20-hydroxyecdysone repression of the *D. melanogaster* UO gene, 20-hydroxyecdysone could be exogenously fed to transformants, possessing the

UO-lacZ transgene with the distal DR deletion, during the late second instar larval stage through the third instar larval stage. This type of 20-hydroxyecdysone feeding experiment has been shown to cause premature repression of the wild type *D. melanogaster* UO gene (Kral et al., 1982). If transformed early third instar larvae carrying the "wild type" UO-lacZ transgene display a premature reduction in β-galactosidase expression after being fed 20-hydroxyecdysone then perhaps transformed larvae carrying the UO-lacZ fusion gene lacking the distal DR element would not exhibit this premature reduction in expression of β-galactosidase. If such results were obtained, these data would suggest that the distal DR element is involved in 20-hydroxyecdysone repression of the *D. melanogaster* UO gene and that a second mechanism is also involved in the silencing of UO at the end of the third instar larval stage. It is possible that the disappearance of a positive UO trans-acting factor could serve as a second mechanism to silence the UO gene prior to pupariation.

Deletion of the proximal DR element (Figures 1 and 11) dramatically altered the level of expression of the UO-lacZ transgene. For the most part, β-galactosidase expression could not be detected at any stage of development from the first instar larval stage through the adult stage by histochemical staining in four different transformed lines possessing P[($w^{+\Delta}$)del(+11, +23) DmUO-lacZ]. Occasionally β-galactosidase staining in the Malpighian tubules of some adults transformed with P[($w^{+\Delta}$)del(+11, +23)DmUO-lacZ] was observed. Infrequent expression of the *lacZ* reporter in the adult suggests that the UO-lacZ fusion transgene, with deletion of the DR element at +11, is capable of being transcribed and translated.

These results raised two questions: (1) Is the DR element at +11 required for induction of UO gene expression? (2) Is 20-hydroxyecdysone required for induction of UO expression? There are Drosophila genes in which 20-

hydroxyecdysone is involved in both induction and repression of transcription (Hanson and Lambertsson, 1983; Ashburner et al., 1984). A test to determine if 20-hydroxyecdysone is required for activation of UO transcription would be to shift the 20-hydroxyecdysone deficient *ecd*^{1} stock from the permissive temperature to the restrictive temperature during the mid-second instar larval stage and then assay for UO enzyme activity or mRNA during the mid-third instar larval stage. If 20-hydroxyecdysone is also a positive regulator of UO expression, the rapid induction of UO mRNA in mid-third larval instar should not occur.

Deletion of the proximal DR element may have also disrupted normal translation of UO mRNA rather than transcription of the UO gene. Since the proximal DR element is between the transcription start site and translation start site of the *D. melanogaster* UO gene, it is possible that the reduction in *lacZ* expression has occurred due to truncation of the UO leader sequence causing a decrease in the translational efficiency of UO mRNA. By deleting the 13 bp DR element at position +11 to +23, the untranslated leader sequence, which is normally 33 nucleotides, is reduced to 20 nucleotides in length. Although the data on the lower limits in size of a leader sequence is not available for Drosophila genes, vertebrate leader sequences are on the average 20-100 nucleotides in length (Kozak, 1987). In several viral genes there are strong translation start codons with only 9-10 nucleotides of leader sequence (Kozak, 1987). However, for some genes, leader sequences of 10 nucleotides or less can reduce the efficiency of translation of an mRNA (Kozak, 1987).

Northern analysis of RNA isolated from third instar larva and adult transformants possessing P[($w^{+\Delta}$)del(+11, +23)DmUO-lacZ] may help to determine if the truncated UO leader is insufficient for proper translation initiation. If Northern analysis of RNA from these transformants shows an

abundance of UO-lacZ mRNA, then deletion of the DR at position +11 has most likely caused a block in translation. One way to determine if it is the the DR sequence itself or the length of the untranslated leader that is essential for β-galactosidase expression from the transgene would be to replace the 13 base pairs that were deleted with a 13 bp sequence, having the same G+C content as the DR element, and then test for restoration of β–galactosidase expression.

Transformants containing the UO-lacZ transgene with both DR elements deleted had essentially the same phenotype as those possessing the deletion of the DR element at +11. Thus, the lack of β-galactosidase expression in the lines transformed with P[($w^{+\Delta}$)del(-138, -126)(+11, +23) DmUO-lacZ] is probably due to the deletion of the DR element at position +11. One line, 2M23, transformed with the UO-lacZ transgene deleted for both DR elements showed frequent β-galactosidase staining exclusively in the adult Malpighian tubules. The reason for UO-lacZ expression in this line, and for infrequent UO-lacZ expression in four other lines transformed with the same construct, is unknown. If the deletion of DR at +11 effects the expression of the UO gene at the level of transcription, perhaps in this transformed line, the site of integration of the UO-lacZ gene has overridden the deleterious effects of the deletion on the proximal DR element. Unique patterns of β-galactosidase have been detected for a variety of different fusion genes when integrated into the *D. melanogaster* genome. The same gene construct can give expression patterns that vary depending on the insertion site (Kassis, 1990; Zink et al., 1991). " Promoterless" genes frequently are transcribed during precise developmental and tissue-specific patterns when integrated at sites near enhancers. These "promoterless" genes have been dubbed "enhancer traps" (Bellin et al., 1989; Wilson et al., 1990). The promoter of the UO-lacZ transgene of line 2M23 may be under the control of a nearby adult enhancer. Examining the expression of

UO-lacZ by histochrmical staining after mobilization of the P-element insert in line 2M23 to a variety of new chromosomal loacations using the Delta 2-3 system (Robertson et al., 1988) would address this issue.

In conclusion, the distal DR element appears to be dispensable with regard to appropriate temporal and tissue-specific regulation of the *D. melanogaster* UO gene. The proximal DR element is essential for appropriate levels of expression of the UO gene. Whether deletion of the proximal DR element has diminished the level of transcription of the UO gene or translation of the UO message is not known. To date, analysis of the DR element has not provided direct evidence that the DR sequence is or is not a binding site *in vivo* for the 20-hydroxyecdysone receptor.

3. Combinative oligonucleotide-directed large deletions as a method of choice for identifying the location of UO cis-regulatory elements.

Determination of the amount of DNA sufficient for appropriate regulation of a particular gene is often the first step in the identification of the cis-regulatory elements. Usually successive deletions are constructed to delimit the amount of DNA necessary for appropriate expression. This can be accomplished by deleting restriction fragments from the target region or by successive digestion with exonucleases such as *Bal*31 or *Exo*III (Edlund et al., 1985; Boulet et al., 1986; Mariani et al., 1988; Chung and Keller, 1990). In the case of the UO gene, along the 826 bp 5' of the transcription start site there were only a few restriction sites which might have been useful for deletion of 5' regions. Therefore, a new scheme for generating site-specific large deletions was formulated (Figures 12 and M2).

There are only a few reported examples of large deletions being created by oligonucleotide-directed *in vitro* mutagenesis. Sambrook et al. (1989) states "the larger the size of the mutation to be constructed, the lower the efficiency of

oligonucleotide-mediated mutagenesis". Although this seems intuitively reasonable, the data presented here suggest a more optimistic outcome for the use of site-directed mutagenesis as an efficient means of systematically deleting large sections of a putative regulatory region of a gene.

Using the site-directed technique of Vandeyar et al. (1988), independent clones with deletions of the 13 bp DR elements of the UO gene were identified in 10% to 25 % of the M13 plaques examined (Chapter Three). However, deletions of three different regions of 5' UO sequence, each approximately 100 bp, were detected in 38% to 70% of the plaques examined. Therefore, it was possible to systematically and efficiently delete approximately 100 bp regions along the 826 bp immediately upstream of the *D. melanogaster* UO gene (Figure 12). This technique should be generally applicable to delete regions of any stretch of DNA for which the sequence is known.

Since the deletions are site-specific there is no need for subsequent mapping of the deletion breakpoints as required for procedures using exonucleases, though in the case of the UO gene the deletion breakpoints were sequenced given the initial concern that this technique might not work. In each case for the 5' UO deletions, the deletion endpoints corresponded precisely to the breakpoints predicted on the bases of the primer sequences. By using site-directed mutagenesis to generate large deletions, there is also no need to have conveniently located restriction enzyme recognition sites. The major advantage of this method for creating particular deletions is that the functional consequences of different combinations of deletions on the regulation of a gene can be analyzed (Figure 12).

For the 5' deletion analysis of the UO gene, the sites of the deletion breakpoints were chosen such that each deletion removed one of the evolutionary conserved elements, with the exception of the deletion from -687 to -452, which removed three closely spaced conserved elements (Figure 12).

The 5' UO regulatory sequences, along with the transcribed portion of the UO gene encoding the first 104 amino acids are being fused in frame with the *lacZ* gene and subsequently inserted into a P-element vector for germ line transformation of *D. melanogaster*. Once certain deleted regions are demonstrated to alter the normal pattern of UO gene regulation, those regions will be characterized in more detail. The subsequent analyses to identify cis-regulatory elements of the *D. melanogaster* UO gene will include smaller oligonucleotide-directed deletions and oligonucleotide directed point mutations.

IV. CHAPTER FOUR: Species differences in the temporal regulation of Drosophila UO gene expression attributed to trans-acting regulatory changes.

A. Introduction

In 1963 Bruce Wallace hypothesized that much of the genetic diversity among species was due to changes in regulatory genes since there was an uncanny conservation of the sequence of structural genes (Wallace, 1963). During the mid 1970's speculation that speciation was driven by changes in regulatory genes or cis-acting regulatory elements flourished. This argument, based on inference, was presented in a more compelling fashion by King and Wilson (1975) after comparing the amino acid sequences of several structural proteins of chimpanzees and humans and finding only a small number of amino acid changes. Wilson and his colleagues had revealed a paradox in which two species with strikingly different anatomical features and modes of behavior had homologous peptides which were on the average 99% identical. Therefore, they hypothesized that evolutionary changes bringing about the species-specific morphology and behavior of species must be due to changes in the mechanisms controlling gene expression, rather than the protein coding sequences of genes.

Naturally occurring regulatory variation is still thought to be an important force in evolution, but supporting evidence is lacking. MacIntyre (1982) points out that regulatory gene variants are abundant in natural populations and molecular techniques will reveal the genetic bases of these regulatory variants. Species differences in the regulation of a particular gene are usually assumed

to be caused either by cis-dominant changes or by mutations elsewhere in the genome affecting trans-acting regulatory factors (MacIntyre, 1982; Dickenson, 1983). In some cases differences in theregulation of homologous genes have been demonstrated to be due to changes in cis-acting regulatory elements (Bray and Hirsh, 1986; Fischer and Maniatis, 1986; Brady and Richmond, 1990; Wu et al., 1990). Evidence for species differences in gene expression due to trans-acting regulatory changes is less well established (King and Wilson, 1975; Csink and McDonald, 1990; Hedrick and McDonald, 1990).

The UO gene of Drosophila is a good biological paradigm to investigate the molecular mechanism underlying the evolutionary changes in gene regulation. There are overt differences in the temporal regulation of UO enzyme activity and UO mRNA abundance during development of *D. melanogaster*, *D. pseudoobscura* and *D. virilis*. The *D. melanogaster* UO protein and enzyme activity are only detected within the Malpighian tubules of third instar larvae and adults (Friedman, 1973, Kral et al., 1982). In contrast, *D. pseudoobscura* UO enzyme activity is present only in the Malpighian tubules of adults while *D. virilis* UO enzyme activity is present only in the Malpighian tubules of third instar larvae.

Differences in temporal regulation of the UO gene among these three species of Drosophila have been investigated using interspecific P-element mediated germ line transformation. The UO genes from *D. pseudoobscura* and *D. virilis* were transformed into the genome of *D. melanogaster* and the regulation of these transgenes was examined. The *D. pseudoobscura* and *D. virilis* UO transgenes exhibited the *D. melanogaster* UO temporal and tissue-specific pattern of expression. The most likely explanation for these data is that the species-specific patterns of expression of the UO gene are due to changes in the temporal regulation of one or more trans-acting factors required for UO gene expression in the third instar larva and adult.

B. Results

1. UO expression in *D. melanogaster* , *D. pseudoobscura* and *D. virilis*.

UO mRNA, protein and enzyme activity were detected in the third instar and adult stages of *D. melanogaster* (Friedman, 1973; Kral et. al., 1982; Figure A7). In contrast UO enzyme activity was detected only in the adult *D. pseudoobscura* strain AH133 (Figure A7). Developmental Northern analysis of RNA isolated from *D. pseudoobscura* AH133 showed UO mRNA present only during the adult stage (Figure 13), consistent with the profile of UO enzyme activity in this species. UO enzyme activity was detected only in the third instar larva of *D. virilis* strains 1051.0 and 1051.48 (Figure A7). Developmental Northern analysis of RNA isolated from *D. virilis* 1051.48 detected UO mRNA only in the third instar larva (Figure 14), consistent with the *D. virilis* UO enzyme profile (Figure A7). Developmental Northern analysis of RNA isolated from *D. virilis* 1051.0, containing a tandem duplication of the UO gene, detected UO mRNA predominantly in the third instar larva and at a lower level in the adult (Figure 14).

2. *D. pseudoobscura* UO expression in a *D. melanogaster* genome.

Given the differences in temporal regulation of the UO genes during development of *D. melanogaster* and *D. pseudoobscura*, we wondered what the pattern of expression of the *D. pseudoobscura* UO gene would be if placed in a *D. melanogaster* genome. The UO gene from *D. pseudoobscura* with approximately 700 bp of 5' flanking DNA and 200 bp 3' flanking DNA (Figure 15A) was inserted into the P-element vector CaSpeR (Pirrotta, 1988) and transformed into *D. melanogaster*. Unique single homozygous insertions of the *D. pseudoobscura* UO transgene in six lines was confirmed by Southern analyses (data not shown). Developmental expression of the *D. pseudoobscura* UO transgene was examined by Northern analysis. An

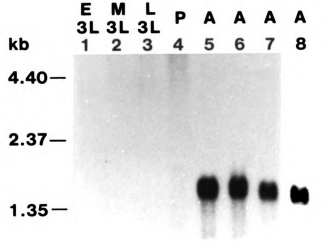


Figure 13. Northern analysis of UOmRNA from *D. pseudoobscura* third instar larvae, pupae and adults. Lanes 1-3 contained total RNA from 60 third instar larvae and lane 4 contained total RNA from 60 pupae. Lanes 5 and 6 contain total RNA from 15 adults and lanes 7 and 8 contain RNA from 7.5 adults. Developmental stages are designated above each lane (E3L, early-third instar larvae; M3L, mid-third instar larvae; L3L, late third instar larvae; P, pupae; A, 36 hour adults). The probe used to detect UO mRNA was a ^{32}P labeled 618 bp AluI restriction fragment derived from the *D. pseudoobscura* UO protein coding region (Figure A3).

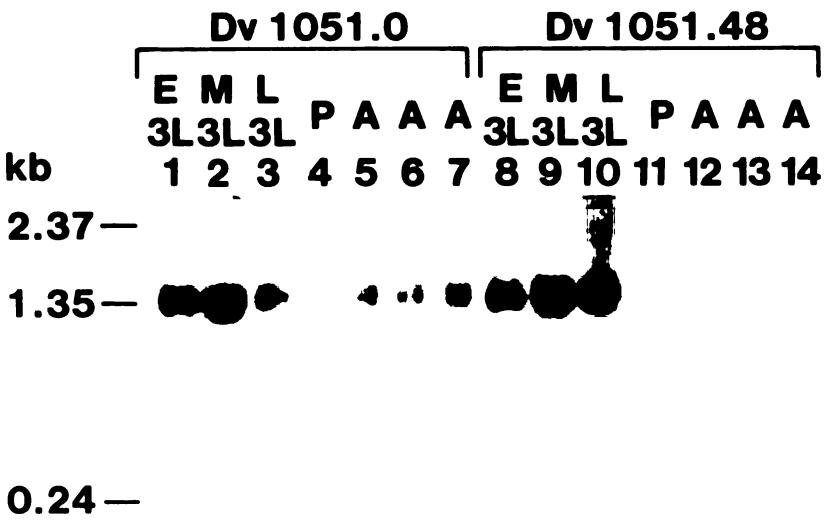


Figure 14. Northern analysis of UO mRNA from third instar larvae, pupae and adults of two wild type strains of *D. virilis*, 1051.0 and 1051.48. *D. virilis* 1051.0 has a tandem duplication of the UO gene. *D. virilis* 1051.48 has a single copy of the UO gene per haploid genome. Total RNA from 30 early, mid- or late third instar larvae (E3L, M3L and L3L, respectively), pupae (P) and adults (A) was isolated, size separated by gel electrophoresis, transferred to a nylon membrane and probed with a ^{32}P-labeled *Hind*III restriction fragment including and extending beyond the entire *D. virilis* UO1 transcribed region (Figure 16). Lanes 1-7 contain RNA isolated from *D. virilis* 1051.0 and lanes 8-14 contain RNA isolated from *D. virilis* 1051.48. The positions of RNA size standards are given in the left margin.

Figure 15. Northern analysis of the *D. pseudoobscura* UO transgene integrated into the *D. melanogaster* genome. (A). Restriction map of the *D. pseudoobscura* UO region with a schematic diagram of the UO gene and flanking DNA included in the P-element construct P[($w^{+\Delta}$)DpUORI]. The transcribed *D. pseudoobscura* UO region is indicated by a double thick line. Restriction enzyme sites: A, *Alu*I; Ac, *Acc*I; C, *Cla*I; H, *Hind*III; R, *Eco*RI; Sp, *Sph*I; X, *Xho*I; Xm, *Xmn*I. (B). Northern analysis of *D. pseudoobscura* UO mRNA in *D. melanogaster* transformants containing the *D. pseudoobscura* UO transgene. Total RNA was isolated from 15 animals (lanes 1-3, 5 and 11), 30 animals (lanes 4, 5, 11), 60 animals (lane10) and 15 hand-dissected Malpighian tubules (MT, lanes 7 and 9) and the remaining tissue after Malpighian tubule dissection (RT, lanes 6 and 8), size separated by gel electrophoresis, transferred to a nylon membrane and probed with a ^{32}P-labeled *Alu*I restriction fragment of the *D. pseudoobscura* UO gene internal to the UO protein coding region. Early, mid- and late third instar larvae, pupae and adults are designated E3L, M3L, L3L, P and A, respectively. The wash condition used for this Northern analysis was sufficiently stringent that the *D. pseudoobscura* UO probe did not remain hybridized to endogenous UO mRNA from *D. melanogaster white* deficient third instar larvae (Df(1)w, lane 10) but did remain hybridized to UO mRNA from *D. pseudoobscura* AH133 adults (Dp, lane 11). The positions of RNA size standards are given in the left margin. To verify the presence of RNA in the lanes not hybridizing to the *D. pseudoobscura* UO probe, the Northern blot was reprobed with a portion of the *D. melanogaster ras* gene (boxed area) (Mozer et al., 1985). The lack of hybridization of the *ras* probe to RNA in lanes 7 and 9, in comparison to lanes 2-6, 8 and 10, was due to small amounts of total RNA isolated from dissected Malpighian tubules. Nevertheless, lanes 7 and 9 showed strong hybridization to the *D. pseudoobscura* UO probe. Lane 11 containing *D. pseudoobscura* adult RNA showed no hybridization to the *D. melanogaster ras* probe while hybridization with the *D. pseudoobscura* UO probe gave an intense autoradiographic signal.

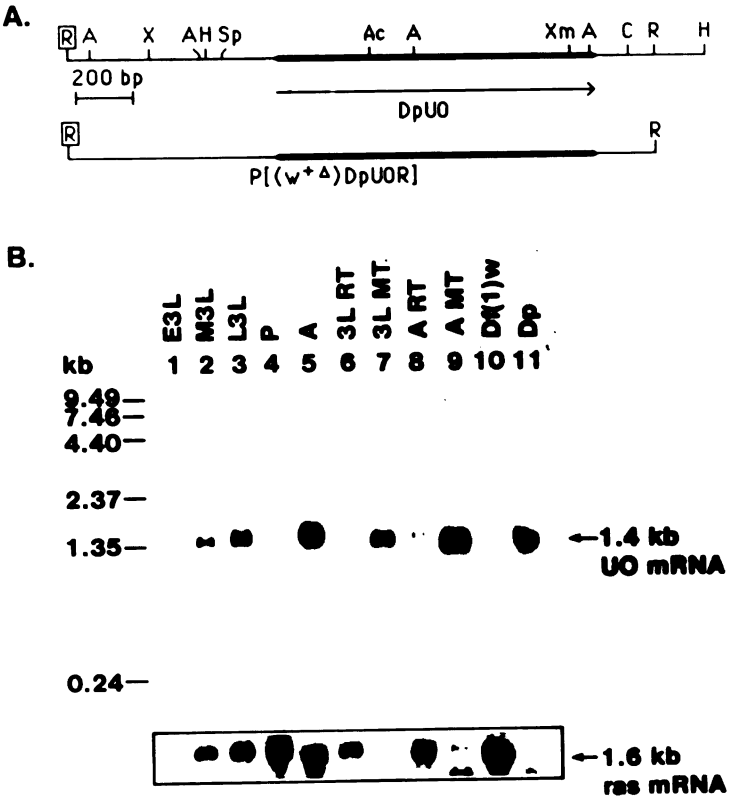

A.
R A X AH Sp Ac A Xm A C R H
200 bp
DpUO
R R
P[(w+Δ)DpUOR]
B.
E3L M3L L3L P A 3L RT 3L MT A RT A MT Df(1)w Dp
kb 1 2 3 4 5 6 7 8 9 10 11
9.49
7.46
4.40
2.37
1.35
←1.4 kb
UO mRNA
0.24
←1.6 kb
rps mRNA

Figure 15.

example of expression of the *D. pseudoobscura* UO transgene in one transformed line, 4F53A, is shown in Figure 15B. In all six transformed lines (Table A7) the *D. pseudoobscura* UO transgene was expressed exclusively in the Malpighian tubules of the third instar larva and adult, a temporal pattern of expression which is unlike the pattern of UO expression of *D. pseudoobscura* AH133 and identical to that of the *D. melanogaster* UO gene.

3. *D. virilis* UO expression in a *D. melanogaster* genome.

The *D. virilis* UO genes were isolated from a library of genomic DNA from a strain containing tandemly duplicated UO genes. The duplicated UO genes are designated Dv UO1 and Dv UO2. Interspecific P-element mediated germ line transformation was used to examine the pattern of expression of the *D.virilis* UO genes in a *D. melanogaster* genome. The *D. virilis* UO1 gene with 3200 bp of 5' flanking DNA and 400 bp of 3' flanking DNA (Figure 16A) was cloned into the P-element vector CaSpeR and transformed into *D. melanogaster*. By Southern analysis (data not shown), five homozygous lines (Table A7) were identified with a single insertion of the *D. virilis* UO1 transgene. Northern analyses of all five transformed lines revealed that the *D. virilis* UO1 transgene was expressed exclusively in the Malpighian tubules of third instar larva and the adult. An example of the expression of the *D. virilis* UO1 transgene in one transformed line, 3M1A, is shown in Figure 16B. The temporal and tissue-specific pattern of expression of the *D. virilis* UO1 transgene was identical to that of the *D. melanogaster* UO gene.

Using the same procedure as described above for the *D. virilis* UO1 gene, the *D. virilis* UO2 gene was integrated into the *D. melanogaster* genome with approximately 4000 bp of 5' flanking DNA and 300 bp of 3' flanking DNA (Figure 16A). Three transformed lines (Table A7) homozygous for a single insertion of the *D. virilis* UO2 transgene (Southern analysis not shown) were examined for

Figure 16. Northern analysis of the *D. virilis* Dv UO1 and Dv UO2 genes separately transformed into the *D. melanogaster* genome. (A). Restriction map of the *D. virilis* UO region showing the Dv UO1 and Dv UO2 genes included in the P-element constructs P[($w^{+\Delta}$)DvUO1PstI)] and P[($w^{+\Delta}$)DvUO2PstI], respectively. Restriction enzyme sites: B, *Bgl*II; H, *Hind*III; P, *Pst*I; R, *Eco*RI; S, *Sal*I; Sp, *Spe*I; X, *Xho*I. (B) Northern analysis of *D. virilis* UO mRNA in *D. melanogaster* transformants containing the *D. virilis* UO1 transgene. Total RNA isolated from 60 animals (lanes 1-5 and 10), 30 animals (lane 11), 60 dissected Malpighian tubules (MT, lanes 6 and 8) and the remaining tissue after Malpighian tubule dissection (RT, lanes 7 and 9) was size separated by gel electrophoresis, transferred to a nylon membrane and probed with a ^{32}P-labeled *Hind*III restriction fragment spanning the entire *D. virilis* UO1 gene. Early, mid- and late third instar larvae, pupae and adults are designated E3L, M3L, L3L, P and A, respectively. (C). Northern analysis of *D. virilis* UO mRNA in *D. melanogaster* transformants containing the *D. virilis* UO2 transgene. Total RNA isolated from 60 animals (lanes 1-5 and 10), 30 animals (lane 11), 60 dissected Malpighian tubules (lanes 6 and 8) and the remaining tissue after Malpighian tubule dissection (lanes 7 and 9) was size separated by gel electrophoresis, transferred to a nylon membrane and probed with the ^{32}P-labeled *Hind*III restriction fragment spanning the entire *D. virilis* UO1 gene. This probe hybridizes equally well to the *D. virilis* UO1 and *Dv* UO2 genomic regions. The wash condition used for the Northern blots did not allow the *D. virilis* UO probe to remain hybridized to the endogenous UO mRNA from the *D. melanogaster white* deficient third instar larvae (Df(1)w, lane 10) but did allow hybridization to UO mRNA from *D. virilis* third instar larvae (Dv, lane 11). The positions of size standards are given in the left margin. The presence of RNA in lanes without a hybridization signal using the *D. virilis* UO probe was confirmed by hybridizing the blot with the *D. melanogaster ras* probe (boxed area) as described in the legend of Figure 3. Lane 11 with RNA isolated from *D. virilis* third instar larvae did not show hybridization to the *D. melanogaster ras* probe while hybridization with the *D. virilis* UO probe gave an intense autoradiographic signal.

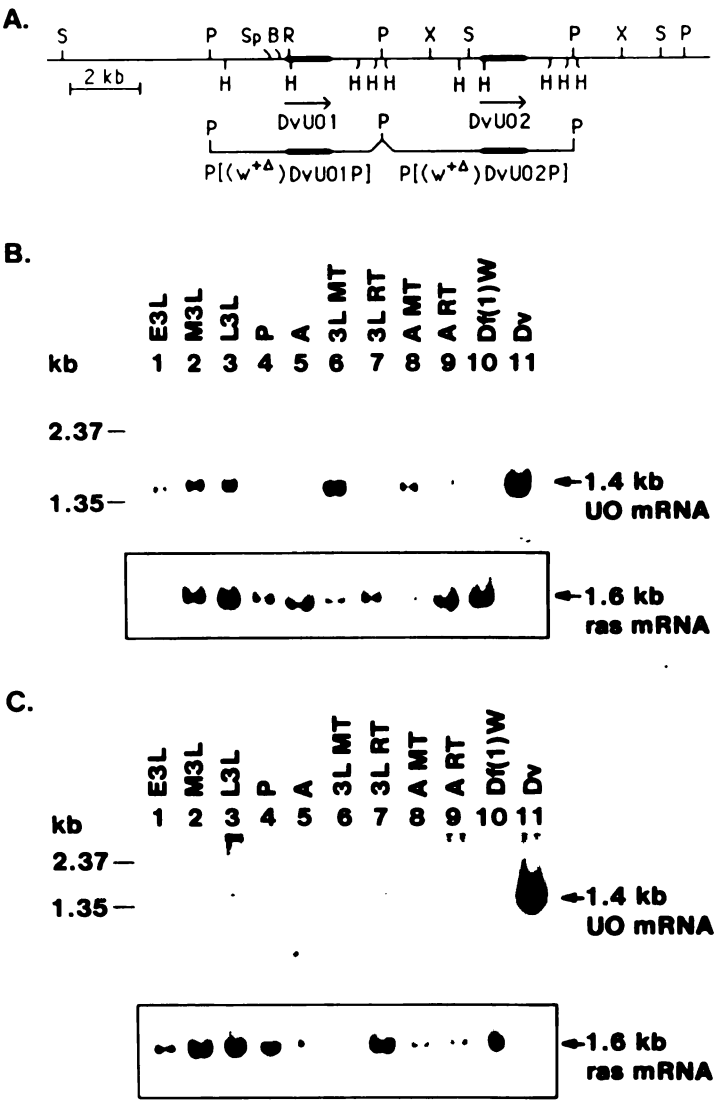

A.
S
P Sp B R
P X S
P X S P
2 kb
H
H
H H H
H H
H H H
DvU01
DvU02
P
P
P
P[(w+Δ)DvU01P]
P[(w+Δ)DvU02P]
B.
E3L
M3L
L3L
P
A
3L MT
3L RT
A MT
A RT
Df(1)W
Dv
kb
1 2 3 4 5 6 7 8 9 10 11
2.37—
1.35—
1.4 kb
UO mRNA
1.6 kb
ras mRNA
C.
E3L
M3L
L3L
P
A
3L MT
3L RT
A MT
A RT
Df(1)W
Dv
kb
1 2 3 4 5 6 7 8 9 10 11
2.37—
1.35—
1.4 kb
UO mRNA
1.6 kb
ras mRNA

Figure 16.

the developmental expression of the *D. virilis* UO2 transgene. A low level of *D. virilis* UO2 transgene expression was detected in the pupae, third instar larva and adult. Furthermore, the majority of *D. virilis* UO2 mRNA was present in the tissue remaining after dissection and removal of the Malpighian tubules. A representative Northern analysis of one transformed line, 4F13D, is shown in Figure 16C. Detection of UO mRNA from the *D. virilis* UO2 transgene by Northern analysis required an eight-fold longer autoradiographic exposure time than for detection of UO mRNA from the *D. virilis* UO1 transgene. Data presented elsewhere indicates that as a result of the site of unequal recombination giving rise to the UO duplication event, the *D. virilis* UO2 gene lacks upstream sequences containing UO cis-acting regulatory elements. As a consequence, the *D. virilis* UO2 gene has an altered temporal and tissue-specific pattern of expression. We attribute the low level of UO mRNA detected in the adult of *D. virilis* strain 1051.0 (Figure 14) to transcription from the *D. virilis* UO2 gene.

C. Discussion

The genetic bases for interspecific differences in the regulation of several Drosophila genes have been investigated by creating interspecific hybrids or P-element transformants. Bray and Hirsh (1986) have identified differences in the expression of the *D. melanogaster* and *D. virilis dopa decarboxylase* (*Ddc*) genes. The *D. virilis Ddc* enzyme activity profile shows a 3-4 fold increase between the early third instar larval and late third instar larval stages while the *D. melanogaster Ddc* enzyme activity profile has a 15-30 fold increase during this same developmental period. In addition, the *D. virilis Ddc* gene is expressed at higher levels than the *D. melanogaster* enzyme in tissues other than the cuticle and central nervous system. This tissue-specific pattern and

quantitative level of expression of the *D. virilis Ddc* gene persisted when integrated into the *D. melanogaster* genome.

There are other examples of species differences in gene expression attributed to cis-acting changes. The *alcohol dehydrogenase* (*Adh*) gene of *D. melanogaster* is expressed in the fat body, hindgut, rectum, Malpighian tubules and male genital disc derivatives while the *Adh-2* gene of *D. mulleri* is only expressed in the adult fat body, hindgut and rectum. Expression of the *D. mulleri Adh-2* transgene in the *D. melanogaster* genome coincides with the tissue-specific pattern of the *D. mulleri Adh-2* gene (Fischer and Maniatis, 1986). Brennan and Dickenson (1988), Brennan et al. (1988) and Wu et al. (1990) have shown that there are tissue-specific differences in the expression of the *Adh* gene of *D. hawaiiensis*, *D. affinidisjuncta* and *D. grimshawi* compared to expression of the *Adh* gene of *D. melanogaster*. The majority of these differences also behave in a cis-dominant fashion when the *Adh* genes of *D. hawaiiensis*, *D. affinidisjuncta* and *D. grimshawi* are separately introduced into the *D. melanogaster* genome.

The *esterase-5* gene of *D. pseudoobscura* is expressed in the eyes and the hemolymph, whereas, the *D. melanogaster* homologue, *esterase-6*, is expressed in the ejaculatory duct. The *D. pseudoobscura esterase-5* gene retains its sex- and tissue-specific expression when transformed into a *D. melanogaster* genome (Brady and Richmond, 1990). For the examples summarized here, interspecific regulatory variation is clearly cis-dominant since the species-specific patterns of expression of the transgenes are maintained in the presence of *D. melanogaster* trans-acting factors.

There are reports of species-specific quantitative differences in gene products that are due to evolutionary changes of trans-acting regulators. Csink and McDonald (1990) discovered differences in the amount of copia mRNA among 37 populations world wide of *D. melanogaster, D. simulans* and

D. mauritiana. There were also intraspecific differences in the level of copia mRNA within *D. melanogaster* populations that showed no correlation with the copy number of copia elements. Using interpopulation hybrids, variation in the amount of copia mRNA among natural populations of *D. melanogaster*, *D. simulans* and *D. mauritiana* was attributed to differences in trans-acting controlling factors. In addition, quantitative differences in the amount of *Adh* activity and cross-reacting material in *D. melanogaster* and *D. simulans* have been suggested to be a consequence of trans-acting modifiers that alter the rate of translation of *Adh* mRNA or the stability of *Adh* protein (Laurie et al., 1990).

There are also a few examples in which species-specific expression of a gene may be the result of both cis-acting and trans-acting genetic differences (Dickenson, 1980; Brennan and Dickenson, 1988; Cavener et al., 1989; Krasney et al., 1990). One of the most clearcut examples is that concerning the regulation of the *aldehyde oxidase* gene of *D. grimshawi* and *D. formella*, two Hawaiian picture-winged Drosophila (Dickenson, 1980). *Aldehyde oxidase* (*AO-1*) is present in *D. grimshawi* third instar larvae at moderate levels in the fat body and barely detectable levels in the salivary gland and carcass. In contrast, *AO-1* is present at high levels in *D. formella* third instar larval salivary glands but at a barely detectable levels in the fat body and carcass. When hybrids were made between *D. grimshawi* and *D. formella*, the hybrids had barely detectable levels of *AO-1* in the salivary gland and fat body, implying negative regulation due to trans-acting controlling factors supplied by *D. grimshawi*. However, the hybrids had high levels of *AO-1* in the carcass. This *AO-1* found in the carcass of the hybrids migrated electrophoretically as the form distinctive of *D. formella*. This implies that the expression of *AO-1* in the carcass is a cis-acting property of the *D. formella aldehyde oxidase* gene.

As I have shown, the *D. melanogaster, D. pseudoobscura* and *D. virilis* UO genes have different temporal expression patterns (Figures 8, 13, 14 and A7).

Two non-mutually exclusive hypotheses to account for the different temporal patterns of UO gene expression among these three species are: (1) the differences in the temporal pattern of expression of the UO gene are due to changes in the cis-acting elements of the UO gene and (2) the differences in the pattern of expression of the UO gene are due to changes in the temporal profile of one or more trans-regulators required for UO gene transcription in the third instar larval and adult stages. The first hypothesis would predict that the species-specific expression of *D. pseudoobscura* or *D. virilis* UO transgenes would persist when integrated into a *D. melanogaster* genome. The second hypothesis would predict that the *D. pseudoobscura* and the *D. virilis* UO transgenes would be expressed in a temporal pattern identical to the *D. melanogaster* UO gene, provided that the *D. melanogaster* UO trans-acting factors recognize the cis-regulatory sequences of the *D. pseudoobscura* and *D. melanogaster* UO genes. When the *D. pseudoobscura* UO gene and the *D. virilis* UO1 gene were transformed into a *D. melanogaster* genome, a temporal pattern identical to that of the *D. melanogaster* UO gene was observed (Figures 15B and 16B), consistent with the second hypothesis.

There is an alternative, but more complex explanation for the *D. melanogaster* UO-like temporal expression pattern displayed by the *D. pseudoobscura* UO transgene and the *D. virilis* UO1 transgene in a *D. melanogaster* genome. This alternative explanation requires the presence of distant negative regulatory elements involved in UO gene repression. It is possible that a cis-element required for repression of the *D. pseudoobscura* UO gene during the third instar larval stage resides outside the boundaries of the *D. pseudoobscura* UO flanking DNA included in the P-element construct P[($w^{+\Delta}$)DpUORI] (Figure 15A). Similarly, an adult cis-acting regulatory element involved in repression of the *D. virilis* UO1 gene would have to reside at a

distance greater than 3200 bp, the 5' limit of the DNA present in the P-element construct P[($w^{+\Delta}$)DvUO1PstI] (Figure 16A). There are examples of Drosophila genes with distant cis-acting regulatory elements (Giangrande et al., 1987; Johnson et al., 1989; Bergson and McGinnis, 1990). However, on the basis of expression of a UO-lacZ fusion gene, 826 bp of 5' UO flanking DNA and the first 350 bp of the *D. melanogaster* UO transcribed region are sufficient for appropriate regulation of the *D. melanogaster* UO gene when reintegrated into the *D. melanogaster* genome (Figure 10). If the *D. pseudoobscura* and the *D. virilis* UO cis-regulatory elements are also within approximately 800 bp of 5' flanking DNA they would have been included in the P-element constructs employed here. Additional P-elements could be constructed that contain the *D. pseudoobscura* and *D. virilis* UO genes with additional 5' and 3' flanking DNA, transformed into *D. melanogaster* and analyzed for UO transgene expression. The problem with such an approach is that there are limitations to the amount of DNA capable of being efficiently transformed into *D. melanogaster* via P-element transposition, with an upper limit of approximately 50 kb (Spradling, 1986).

Additional evidence is needed to unequivocally demonstrate that the species-specific temporal patterns of UO expression are due to changes in trans-acting regulatory genes and not to the exclusion of UO cis-regulatory elements form the P-element constructs. A test, when feasible, would be to perform reciprocal P-element transformations of the UO genes with *D. melanogaster, D. pseudoobscura* and *D. virilis*. The *D. melanogaster* UO, *D. pseudoobscura* UO *and D. virilis* UO1 transgenes in the *D. virilis* genome would be predicted to be expressed only during the third instar larval stage, identical to the developmental pattern of the endogenous *D. virilis* UO1 gene. Similarly, the *D. melanogaster* UO, *D. pseudoobscura* UO and *D. virilis* UO1

transgenes in the *D. pseudoobscura* genome would be expected to be expressed only in the adult.

Reciprocal P-element transformations of *D. pseudoobscura* and *D. virilis* are currently not feasible for several reasons. Although successful transformations of a few Drosophila species other than *D. melanogaster* have been reported, none have involved *D. pseudoobscura* and *D. virilis* (Brennan et al., 1984; Scavarda and Hartl, 1984; Daniels et al., 1985; Daniels et al., 1989; Laurie et al., 1990). It is not known whether the promoters of the *D. melanogaster* reporter genes used for screening transformants are recognized by *D. virilis* transcription factors and whether any of the *D. melanogaster* reporter genes would be expressed in *D. virilis* at levels sufficient for screening.

Once the genes for the UO transcription factors are cloned, evolutionary changes in their expression patterns can be examined in *D. melanogaster, D. pseudoobscura and D. virilis.* A change in the temporal pattern of expression of a trans-acting regulatory gene might be expected to influence the expression of more than one gene since many transcription factors regulate more than one target gene (Costa et al., 1988; Hardon et al., 1988; Hoey et al., 1988; Ruppert et al., 1990). If the species differences in the developmental pattern of the UO gene are a consequence of evolutionary changes in the temporal expression or availability of particular trans-acting factors essential for UO gene expression, it is possible that, in addition to UO, other genes are targets of the same transcription factor(s) and have also experienced a change in regulation since the divergence of *D. virilis, D. pseudoobscura* and *D. melanogaster.*

Although the temporal expression pattern of the *D. melanogaster* UO, *D. pseudoobscura* UO and *D. virilis* UO1 genes have diverged, the Malpighian tubule-specific expression has been conserved. *D. pseudoobscura* and *D. virilis* diverged from *D. melanogaster* approximately 35 million and 60 million years ago, respectively (Beverley and Wilson, 1984). The *D. pseudoobscura*

UO and the *D. virilis* UO1 genes transformed into the genome of *D. melanogaster* are expressed only in the Malpighian tubules. This result implies that the cis-acting elements and the transcription factor(s) required for restricting UO gene expression to the Malpighian tubules have been conserved among these three Drosophila species. It is interesting to note that although the *D. pseudoobscura* and *D. virilis* UO1 transgenes were expressed in the third instar larva and adult Malpighian tubules of *D. melanogaster*, there is very little sequence similarity among the 5' flanking DNA of the *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO1 genes (Figures 7 and A5). Given these results, it seems likely that the UO gene is under the control of cis-acting elements that have small core binding sites or that are poorly conserved at the nucleotide level. Only by performing a thorough mutagenesis analysis of the regulatory region of the UO gene as described in Chapter Three, will the sequences of such elements be revealed.

CONCLUSION

There is evidence that evolutionary changes in cis-acting regulatory elements of a particular gene and evolutionary changes in the trans-acting factors have brought about species differences in gene regulation (Bray and Hirsh, 1986; Fischer and Maniatis, 1986; Brady and Richmond, 1990; Csink and McDonald, 1990; Laurie et al., 1990). However, such differences are often subtle variations of spatial patterns or quantity of mRNA or protein product (Bray and Hirsh, 1986; Fischer and Maniatis, 1986; Laurie et al., 1990). Minor variations in gene regulation make data concerning gene expression of interspecific hybrids and P-element transformants difficult to decipher. In contrast, the differences in the temporal pattern of UO expression are overt with easily discernable results of interspecific P-element transformations.

The Drosophila UO gene has proven to be an excellent and unique model system for studying evolutionary changes in gene regulation. The data collected thus far support the idea that the pattern of UO regulation among *D. melanogaster*, *D. pseudoobscura* and *D. virilis* is different as a consequence of a change in the temporal pattern of one or more trans-acting regulatory factors. Additional tests are needed to unequivocally demonstrate that an evolutionary change in a UO trans-acting regulator has occurred. An obvious first step to understand the differences in UO regulation of these three Drosophila species at the molecular level would be, for example, to identify UO trans-acting factors which are expressed in *D. melanogaster* third instar larvae and adults but only in *D. pseudoobscura* adults and only in *D. virilis* third instar larvae. The identification of the UO trans-acting factors could be accomplished once a thorough analysis of the UO cis-acting regulatory elements has been

completed. This analysis would include the identification of the base pairs within UO cis-acting regulatory elements which are essential for the binding of UO transcription factors.

The small size of the UO gene makes mutation analysis coupled with *in vivo* functional testing possible and expedient. The *D. melanogaster* UO gene has a transcribed region of approximately 1.2 kb and a regulatory region which extends no more than 826 bp 5' of the transcription start site. The *D. pseudoobscura* UO and *D. virilis* UO1 genes are also structurally compact and could be subjected to a similar type of analysis to identify their UO cis-acting regulatory sequences.

In an attempt to obtain clues to the location and sequence of the UO cis-acting regulatory elements, an interspecific sequence comparison of the flanking DNA of the UO gene from *D. melanogaster*, *D. pseudoobscura* and *D. virilis* was performed. Several elements were conserved between *D. melanogaster* UO and *D. pseudoobscura* UO and between *D. melanogaster* UO and *D. virilis* UO, but only one conserved element, discounting basal promoter elements (TATA box and transcription start site), was conserved among all three species. It was surprising to discover that the *D. melanogaster* UO, *D. pseudoobscura* UO and *D. virilis* UO1 genes did not have several conserved elements in 5' regulatory regions of all three genes since the UO genes from these three Drosophila species are all expressed exclusively in the Malpighian tubules. Moreover, the *D. pseudoobscura* UO and *D. virilis* UO1 transgenes were expressed in a pattern identical to the *D. melanogaster* UO gene.

There are several explanations that could account for so few conserved putative UO cis-acting regulatory elements among these three species: (1) the cis-acting elements of the UO gene may consist of small core of binding sites which were missed in the similarity search, (2) the important sites for binding

transcription factors within a UO cis-acting element may be noncontiguous, (3) a particular UO transcription factor may bind a functionally equivalent UO cis-acting element with a dramatically different sequence among these three species, and (4) only a single cis-acting control region of the UO gene, in addition to basal promoter elements, is required for the complex temporal and tissue-specific pattern of expression of the UO gene.

The analysis of the UO gene presented in this thesis is the most clearcut case demonstrated to date of species differences in regulation attributable to changes in trans-acting regulatory factors. The UO gene of Drosophila will continue to be a valuable paradigm for studying the evolution of gene regulation.

APPENDIX A

Materials and Methods

APPENDIX A: Materials and Methods

1. Drosophila stocks.

The Drosophila stocks and their phenotypes used in this study are listed in Table A1. *Drosophila virilis* strains 1051.48 and 1051.1 were obtained from J.S. Yoon of the the National Drosophila Species Resource Center, Bowling Green, Ohio. The *Drosophila pseudoobscura* strain AH133 and the Drosophila *melanogaster white* deficient strain Df(1)w,$y^{67c23(2)}$ were gifts of W. Anderson (University of Georgia, Athens) and V. Pirrotta (Baylor University, Houston) respectively. All stocks were kept at 25°C or at room temperature with the exception of the temperature sensitive mutant *ecd*^{1} (Lepesant, 1978) which was reared at 19°C. Drosophila stocks were maintained on standard media (188.64 g sucrose, 29.1 g brewers yeast, 185.6 g cornmeal, 24 g carageenan per 2230 ml water and 15.2 ml of propionic acid as a preservative) supplemented with active yeast .

2. DNA isolation.

a. Large scale plasmid DNA preparation.

This protocol is a modification of the plasmid purification procedure described by Birnboim and Doly (1979).

1. Grow 10 ml overnight culture of the bacterial strain harboring the plasmid of interest in LB media (10 g bactotryptone, 5 g yeast extract and 10 g NaCl

per liter) supplemented with the appropriate antibiotic.

2. Add 10 ml overnight culture to one liter of M9CA media and periodically monitor O.D.
3. When A_{600} = 0.4 to 0.5 add 5 ml of chloramphenicol (34mg/ml in EtOH).
4. Amplify plasmid overnight (12-16 hours) at 37°C with constant shaking.
5. Chill cells 5 minutes on ice and centrifuge in an IEC Centra-7R at 2,800 rpm for 40 minutes at 4°C. Collect pellet.
6. Pool cells in 40 ml of wash buffer.
7. Centrifuge at 8,000 RPM for 10 minutes in JA21 at 4°C.
8. Resuspend in 3 ml of fresh lysozyme buffer, then add 1 ml of fresh lysozyme buffer containing 8 mg of lysozyme.
9. Incubate 0°C for 30 minutes.
10. Add 8 ml of alkaline SDS. Incubate 0°C for 10 minutes.
11. Add 6 ml of 3 M NaOAc, pH 4.8. Mix by inversion. Incubate 0°C 1 hour.
12. Centrifuge at 17,000 RPM in JA21 for 20 minutes at 4°C.
13. Remove supernatant and place in 40 ml centrifuge tube. Add 1 volume of isopropanol to supernatant and precipitate at -20°C overnight or at -70°C 1 hour.
14. Centrifuge at 10,000 RPM for 15 minutes at 4°C.
15. Pour off supernatant, dry pellet in dissector, resuspend in 4 ml of TE and add 1 ml of 1 M NaCl.
16. Add 10 ml of EtOH. Precipitate overnight at -20°C or 1 hour at -70°C.
17. Centrifuge at 10,000 RPM for 15 minutes at 4°C, pour off supernatant and dry pellet under vacuum.

18. Resuspend pellet in 15 ml of TE (10 mM Tris, 1mM EDTA, pH 7.5).
19. Add 15.9 g CsCl and 0.75 ml of ethidium bromide (10mg/ml).
20. Transfer to 25 ml Oakridge tube. Centrifuge using Beckman TI50.2 at 33K for 36 to 48 hours at room temperature.
21. Collect plasmid band.
22. Extract with H_20 saturated sec-butanol until pink color is lost.
23. Dialyze against one liter of TE for 3 days with several changes of buffer.

Solutions for Plasmid Prep:

M9 Salts

6 grams Na_2HPO_4

3 grams KH_2PO_4

0.5 grams NaCl

1 gram NH_4Cl

dH_20 to 1 liter pH to 7.4

M9CA Media

1000 ml M9 Salts

25 ml 20% glucose

25 ml 20% Casamino acids

2 ml 1% thiamine

1 ml 1 M $MgCl_2$

0.1 ml 1 M $CaCl_2$

4 ml 25 mg/ml stock Ampicillin (or appropriate amount of a different antibiotic)

Wash Buffer (10mM Tris, pH 8, 1 mM EDTA)

10 ml 0.1 M Tris pH 8

0.4 ml 0.25 M EDTA pH 7

18 grams sucrose

dH_20 to 100 ml

Lysozyme Buffer (prepare fresh)

225 ml 20% glucose

200 ml 0.25 M EDTA

125 ml 1 M Tris pH 8

dH_20 to 5 ml

Alkaline SDS (prepare fresh)

2 ml 1.0 N NaOH

1.0 ml 10% SDS

dH_20 to 10 ml

b. Small scale plasmid DNA isolation.

This procedure is used to rapidly obtain approximately one to two micrograms of plasmid DNA starting from a 1.5 ml saturated bacterial culture.

1. For M13 plasmid isolation: pick a single plaque on a plate with a sterile toothpick and place in 1.5 ml of 2XTY (2XTY is 16 g bactotryptone, 10 g yeast extract and 5g NaCl per liter of water) media containing a 1:100 dilution of a saturated culture of an *E. coli* strain capable of being infected by M13 and grow for 12 to 16 hours at 37^{o}C with shaking.

E. *coli* MV1193 was used as the M13 host.

For plasmid isolation other than M13: inoculate 1.5 ml of LB media (10 g bactotryptone, 5 g yeast extract and 10 g NaCl per liter) supplemented with the appropriate antibiotic with a single colony of bacteria harboring a plasmid and grow for 12 to 16 hours at 37°C with shaking.

2. Transfer culture to a 1.5 ml microtube and centrifuge for 10 seconds.
3. Discard supernatant (retain supernatant for M13 phage DNA isolation, see below) and wash pellet with 200 µl of TE pH 8.5.
4. Centrifuge for 10 seconds and discard the supernatant.
5. Resuspend the pellet in 25 µl of Solution I (8% sucrose, 50 mM Tris, 75 mM EDTA, pH 8.5).
6. Add 5 µl of 10 mg/ml lysozyme in 10 mM TE, pH 8.5.
7. Incubate 3 minutes at room temperature.
8. Add 25 µl of Solution II (8% sucrose, 50 mM Tris-HCl, 75 mMEDTA, 1% triton, pH 8.5) and vortex.
9. Place the tube in a boiling water bath for exactly 45 seconds and quickly chill on wet ice.
10. Add 250 µl of Solution III (0.5 M NaCl, 10 mM Tris, pH 8.0) and mix by inversion.
11. Centrifuge for 10 minutes.
12. Remove pellet with toothpick and discard pellet.
13. Add 250 µl of cold isopropyl alcohol and precipitate at -20°C for 1 hour.
14. Centrifuge for 10 minutes at 4°C.
15. Wash pellet with 200 µl of 70% EtOH.
16. Centrifuge for 10 minutes at 4°C.
17. Vacuum dry pellet and resuspend in 20 µl of TE.

c. Small scale M13 phage DNA isolation.

M13 phage DNA for sequencing and site-directed mutagenesis was prepared according to the following protocol.

1. Obtain supernatant form step 3 of small scale plasmid DNA isolation procedure (above).
2. Centrifuge for 10 minutes and transfer supernatant to a new tube.
3. Centrifuge for 10 minutes and transfer supernatant to a new tube.
4. Add 300 µl of 20% PEG, 2.5 M NaCl to supernatant and incubate at room temperature for 15 to 30 minutes.
5. Centrifuge for 10 minutes and discard supernatant.
6. Centrifuge pellet again for 10 minutes and discard supernatant.
7. Resuspend the pellet in 160 µl of TES (20 mM TrisHCl, 10 mM NaCl, 0.1 M EDTA, pH 7.5).
8. Add 160 µl of phenol/chloroform (1:1) and vortex for 5 minutes.
9. Centrifuge for 5 minutes and transfer 140 µl of the top layer to a new tube.
10. Add 140 µl of chloroform and vortex for 5 minutes.
11. Centrifuge for 5 minutes and transfer 120 µl of the top layer to a new tube.
12. Add 15 ul of 2.5 M sodium acetate and 250 µl of EtOH.
13. Precipitate at -20^{o}C for at least 1 hour.
14. Centrifuge 10 minutes at 4^{o}C.
15. Wash pellet with 200 µl of 70% EtOH.
16. Centrifuge for 10 minutes at 4^{o}C.
17. Vacuum dry pellet and resuspend in 10 µl of TES.

3. Genomic DNA extraction from a small number of flies.

This protocol is a modification of the DNA extraction procedure of Bender et al. (1983).

1. Add 187 µl of grinding buffer (0.1 M NaCL, 0.2 M sucrose, 0.1 M Tris-HCL, 0.05M EDTA, pH 9.1), 3 µl DEPC (diethylpyrocarbonate) and 10 µl of 10% SDS to a 1.5 ml microcentrifuge tube.
2. Add 1 to 12 flies and grind at room temperature with a glass pestle that fits snugly inside the tube.
3. Incubate at 65^{o}C for 30 minutes.
4. Add 30 µl of 8 M potassium acetate and incubate at 0^{o}C for 30 minutes.
5. Centrifuge for 10 minutes at 4^{o}C. Transfer the supernatant to a new centrifuge tube and repeat the centrifugation.
6. Mix the supernatant with 250 µl of ethanol. Allow to stand at room temperature for 5 minutes.
7. Centrifuge for 10 minutes at 4^{o}C.
8. Wash pellet 3 times with 70% ethanol, dry pellet under vacuum and resuspend DNA in 10 µl of TE **OR** wash pellet once with 70% ethanol, dry under vacuum, resuspend in 10 µl of TE and perform dot dialysis for 30 minutes.

   Dot Dialysis: Float a Millipore dot dialysis membrane (#VMWP 01300) with the shiny side facing up in a petri dish or small container filled with TE. Carefully load the DNA sample onto the center of the membrane. Allow dialysis to occur for 30 minutes. Remove the sample from the membrane using a pipet. There may be a small loss or gain of sample volume during the dialysis.

9. Digest the entire DNA sample with 10 units of restriction enzyme (less may also work) for 3 hours and load the entire sample into a single lane of an agarose gel.

4. Southern analysis.

DNA samples were electophoresed and blotted onto Hybond-N (Amersham) or Immobilon (Millipore) membrane according to Maniatis et al. (1982). Transferred DNA was crosslinked to the nylon membrane by ultravilolet irradiation. Southern blots was prehybridized at 42^{o}C overnight in 50% formamide, 50 mg/ml sheared salmon sperm DNA, 0.1 M PIPES, pH 7.04, 0.1 M NaCl, 0.1% Sarkosyl, 0.1% Ficoll, 0.1% PVP-40 and 0.1% BSA. Modified prehybridization solution with 40% formamide and 10% dextran sulfate served as the hybridization mixture. Restriction fragments used as probes for Southern blots were size separated on agarose gels, isolated by electroelution (Maniatis et al., 1982) and further purified using an Elutip-D column (Schleicher and Schuell). DNA fragments were labeled with [α-^{32}P]dATP to a specific activity of 1.3 x10^{9} to 8.2 X10^{8} cpm/μg by the method of Feinberg and Vogelstein (1984). Approximately 1X10^{7} to 2 X10^{7} cpm were added to 30 ml of hybridization solution and incubated with the blot at 42^{o}C overnight. After hybridization, the blots were rinsed in 2XSSC, 0.05 % N-laurylsarkosine, 0.02% sodium pyrophosphate and washed at 50^{o}C for two hours in two to four washes of 0.1XSSC, 0.05% N-laurylsarkosine, 0.02% sodium pyrophosphate. Blots were autoradiographed at -70^{o}C with one screen.

5. Small scale total RNA isolation.

This RNA isolation procedure was taken form Richards et al. (1983) with modifications of the volumes used.

1. 30 larvae, pupae or adults or 30 hand dissected Malpighian tubules and the remaining tissue are placed into 450 µl of extraction buffer prepared with DEPC treated water (3 M LiCl, 6 M urea, 10 mM NaOAc pH 5.0, 0.2 mg/ml heparin, 0.1% SDS) in a microcentrifuge and ground with a glass pestle.
2. Incubate on ice for one hour to overnight.
3. Centrifuge for 15 minutes at 4°C.
4. Wash the pellet with 100µl of wash buffer (4 M LiCl, 8 M urea).
5. Centrifuge for 15 minutes at 4°C.
6. Resuspend pellet in 100 µl of 0.1 M NaOAc, 0.1% SDS, pH 5.0 prepared with DEPC treated water.
7. Add 100 µl of phenol/chloroform (prepared according to Maniatis et al., 1982) and vortex for 20 minutes and centrifuge for 10 minutes.
8. Remove aqueous layer transfer to a new tube, add 100 µl of chloroform, vortex and centrifuge for 10 minutes.
9. Remove aqueous layer and transfer to a new tube. Adjust to 0.2 M NaOAc, pH 5.0 and add 2 volumes of EtOH.
10. Place at -20°C for 4 hours or longer, centrifuge for 15 minutes at 4°C, wash pellet with 80% EtOH and vacuum dry pellet.
11. Bring up in 10 µl of denaturing solution (250 µl formamide, 89 µl 37% formaldehyde solution) and load onto Northern gel as described below.

6. Northern analysis.

RNA was recovered from ethanol precipitation (see above) by centrifugation for 15 minutes, washed with 70% ethanol, vacuum dried and dissolved in 74% formamide and 9.7% formaldehyde, denatured for 5 minutes at 65^{o}C and loaded onto 1.2 % agarose, 6.66% formaldehyde gel in 1X MOPS (10X MOPS is 0.5 M MOPS, 0.01 M EDTA, pH 7.0). RNA was size-fractionated in agarose-formaldehyde gels by electrophoresis in 1X MOPS. After electrophoresis, the portion of a gel containing samples to be transferred to a nylon membrane was incubated for 30 minutes at room temperature in 0.15 M NaCl, 0.05 M NaOH with shaking and then in 0.15 M NaCl, 0.1 M Tris pH 8.0 for 30 minutes with shaking. RNA was transferred onto nylon membrane (Hybond-N, Amersham or Immobilon, Millipore) with 10XSSC and crosslinked to the membrane using ultravilolet irradiation. The portion of the gel containing RNA standards was incubated in DEPC treated water for 15 minutes at room temperature and then stained for 15 minutes in 200 ml of DEPC water with 10 µl of EtBr stock solution (10mg/ml). The gel was destained by soaking in DEPC treated water for 5 hours to overnight. DEPC treated water is prepared by adding 250 µl of DEPC to one liter of distilled water, incubating at room temperature overnight and then autoclaving.

The Northern blots in Figures 8 and 9 were prehybridized and hybridized in solutions identical to those of Wood et al. (1985). The oligonucleotide 4 (Table A6), which detects UO mRNA transcribed from the UO gene of the *D. melanogaster ecd*^{1} strain, but not from the UO gene of the *D. melanogaster* Canton-S strain, was end-labeled to a specific activity of 1.3x10^{8} cpm/mg with [γ-^{32}P] dATP using T_4 polynucleotide kinase (Maniatis et al., 1982). After six hours of prehybridization at 42^{o}C, the Northern blots were hybridized for 3 days

at 50°C in 20 ml of hybridization solution with $1.6x10^7$ cpm of the end-labeled oligonucleotide. Since the oligonucleotide probe is 66% A+T, the Northern blots were washed at 50°C according to Wood et al. (1985) in the presence of tetramethylammonium chloride which raises the melting temperature of A·T base pairs to that of G·C base pairs.

DNA restriction fragments, when used as probes for Northern analyses, were prepared according to those described for Southern analysis above and labeled to a specific activity of $3.7x10^8$ to $9.3x10^8$ cpm/μg using [α-^{32}P]dATP (Feinberg and Vogelstein, 1984). Northern blots were prehybridized, hybridized and washed according to procedures for Southern analyses described above. For the *D. pseudoobscura* and *D. virilis* UO probes, Northern blots shown in Figures 15 and 16 were washed at 60°C rather than 50°C. By increasing the temperature of the wash solutions, *D. pseudoobscura* UO and *D. virilis* UO probes only hybridized to mRNA from the *D. pseudoobscura* and *D. virilis* UO transgenes, respectively, and not to the UO mRNA transcribed from the endogenous *D. melanogaster* UO gene.

To verify the presence of RNA in lanes showing no autoradiographic signal after hybridization with a UO probe, all Northern blots were stripped of the UO probe by boiling for ten minutes in 0.1% SDS and reprobed with a *Hind*III-*Pst*I fragment of the *D. melanogaster ras* gene (Mozer et al., 1985). The *ras* gene is expressed uniformly throughout development of *D. melanogaster*. Northern blots were autoradiographed at -70°C with one screen.

7. Sequencing.

Restriction fragments containing the UO transcription unit of *D. melanogaster, D. pseudoobscura* and *D. virilis* were cloned into the single stranded bacteriophage vectors M13mp18 and M13mp19 (Messing and Vieira, 1982) phagemid vectors puc119 (Vieira and Messing, 1987) or pBluescript (Stratagene). The sequencing reactions were performed according to Sanger et al. (1977) using [α-^{35}S]dATP and DNA polymerase I, Klenow (Bethesda Research Laboratories, BRL) and the universal primer (BRL; United State Biochemical, USB) or a synthetic oligonucleotide primer complementary to the sequence internal to a cloned UO sequence (Table A6). After performing sequencing reactions with Klenow purchased from various sources, Klenow obtained from Bethesda Research Laboratories was found to be superior to the other sources under the conditions used here.

Sequence ambiguities due to secondary structure of the cloned DNA were resolved by sequencing with AMV reverse transcriptase (BioRad), Taq polymerase (Strategene) or Sequenase (USB) or substituting 7-deaza-2'-deoxyguanosine-5'-triphosphate for deoxyguanosine-5'-triphosphate (Boehringer Mannheim, BM). When sequencing with Taq polymerase, Sequenase and reverse transcriptase the manufacturers' protocols were used. The majority of the sequencing was performed with Klenow using the protocol found in BRL's M13 Cloning/Dideoxy Sequence Instruction Manual with modifications of the concentrations of the dideoxynucleotides. Each M13 subclone containing a UO sequence greater than 100 bp was sequenced using high concentrations (final concentrations: ddATP, 1.5 X 10^{-5}M; ddGTP, 1.5 X 10^{-4} M; ddCTP, 4.5 X 10^{-5}; ddTTP, 4 X 10^{-4} M) and low concentrations (final concentrations: ddATP, 6.6 X 10^{-6} M; ddGTP, 2 X 10^{-5} M; ddCTP,

3 X 10^{-5} M; ddTTP, 6.6 X 10^{-5}) of dideoxynucleotides and the DNA was separated on 8% and 6% polyacrylamide sequencing gels, respectively. Many subclones were sequenced twice.

Figures 1A, 3A and 4A show the sequencing strategies for the *D. melanogaster*, *D. pseudoobscura* and *D. virilis* subclones, respectively. All restriction sites used in constructing the subclones were verified by sequencing across the same restriction site present within other subclones. I thank Tom Friedman, Jean Burnett, Janice Moskowitz and Susan Lootens for making some of the M13 subclones used for sequencing.

8. S1 and mung bean nuclease mapping.

To determine the transcription start site for the UO gene, S1 and mung bean nuclease mapping were performed by end-labeling 1 μg of the genomic restriction fragment, *Hha*I-*Eco*RI (Fig. 2) with T_4 polynucleotide kinase and [γ-^{32}P]dATP (3000Ci/mmol) (Maniatis, 1982). The two strands of the 178 nucleotide end-labeled fragment were separated on a 6% sequencing gel and the strand complementary to UO mRNA, designated probe S (Figure 3c), was isolated. Total RNA was extracted with guanidine HCl from whole third instar larvae and adults (Krawetz and Anwar, 1985). Poly(A)$^+$ RNA was isolated on an oligo(dT)-cellulose column (Pharmacia). Approximately 1X10^5 cpm of probe S and 20 μg of poly(A)$^+$ RNA from Ore-R third instar larvae, 10 μg poly(A)$^+$ RNA from Ore-R adults (data not shown) or 10 μg of poly(A)$^+$ RNA from ry^2 12 hour adults were dissolved in 40 ml of 80% formamide, 40 mM PIPES pH 6.4, 400 mM-NaCl and 1 mM EDTA, boiled for 10 minutes and allowed to hybridize for approximately 12 hours at 42^{0}C. Following the annealing step, 350 μl of S1

reaction buffer (Davis et al., 1986) were added to each sample along with 50 units of S1 nuclease (Boehringer Mannheim) or 150 units of mung bean nuclease (Pharmacia) and the samples were incubated at 37°C for one hour. The concentration of S1 and mung bean nuclease was optimized to achieve nearly complete digestion of the single stranded DNA while minimizing the slower rate of digestion of the DNA:RNA duplex. Mung bean nuclease is reported to create fewer digestion artifacts than some preparations of S1 nuclease (Murray, 1986). After S1 or mung bean nuclease treatment, the reaction was phenol/chloroform extracted, ethanol precipitated, dissolved in 6 μl of standard DNA sequencing loading dye, denatured for 3 minutes at 90°C and loaded into single lanes of a 6% sequencing gel. As a control, the same amount of labeled probe was hybridized to 10 μg of calf thymus tRNA (BM) and treated in an identical fashion as described above.

9. Primer extension analyses.

Primer extension was performed by digesting 1 μg of the *Spe*I-*Eco*RI restriction fragment (Figure 2) with *Alu*I endonuclease and end-labeling the resulting restriction fragments with T_4 polynucleotide kinase and [γ-^{32}P]dATP (Maniatis et al., 1982). The mixture of end-labeled fragments was separated on an 8% sequencing gel and the 30 nucleotide strand complementary to UO mRNA, designated P1 (Figure 3c), was purified. Approximately $4x10^4$ cpm of the labeled P1 were added to either 5 μg or 6 μg of poly(A)$^+$ RNA from Ore-R third instar larvae, from *ry* ^{2} 12 hour adults or from Ore-R 12 hour adults. Poly(A)$^+$ RNA was isolated as described above. Probe P1 and the poly(A)$^+$ RNA were dried and then dissolved in 20 μl of hybridization solution (Hirsh et

al., 1986), placed at 65°C for 1 minute and incubated at 37°C for 1 hour. The hybridization products were ethanol precipitated and dissolved in reaction buffer (Hirsh et al., 1986) containing fresh ultrapure dNTPs (Pharmacia) and 10 units of AMV reverse transcriptase (Pharmacia) and incubated at 42°C for 1 hour. The extension products were phenol/chloroform extracted, ethanol precipitated, vacuum dried, dissolved in 6 µl of standard DNA sequencing loading dye, denatured for 3 minutes at 90°C and then loaded into single lanes of an 8% sequencing gel. Additional primer extension reactions were performed by end-labeling 400 ng of a synthetic 30 mer, P2, just downstream of P1 (Figures 2 and 3c). Hybridization and extension reactions were performed as described above using $4x10^4$ cpm of the labeled P2 and 10 µg of poly(A)$^+$ RNA from Ore-R third instar larvae or 10 µg of poly(A)$^+$ RNA from *ry*^{2} 12 hour adults. The same results for the primer extension reactions were obtained from several experiments using RNA from two independent isolations of each developmental stage and from each Drosophila strain.

10. Tissue *in situ* hybridizations.

Abdomens from *ry*^{2} 12 hour adults were frozen, sectioned and hybridized to sense and antisense UO RNA probes according to Raikhel et al. (1988). The RNA hybridization probes were generated by transcription in the presence of [α-^{35}S]dUTP from the T_7 and T_3 promoters of pBluescript KS(+) (Stratagene) containing the *Eco*RI-*Acc*I restriction fragment of cUO2 (Figure A1c). Full length transcripts, confirmed by Northern analyses, were hydrolyzed for 30 minutes in the presence of 0.1M $NaHCO_3$ at 60°C yielding an array of RNA fragments of approximately 200 nucleotides capable of permeating sectioned and whole-

mount tissue. Hybridization of the partially hydrolyzed UO sense and antisense RNA probes to the sectioned abdomens and autoradiography were performed according to Raikhel et al. (1988).

Malpighian tubules were hand dissected in Drosophila Ringer solution (Ursprung, 1967), permeablized and fixed according to a procedure developed for Drosophila embryos (Edgar and O'Farrell, 1989). I am greatful to B. Edgar for making this procedure available to me prior to publication.

The UO sense and antisense RNA probes were prepared as described for the sectioned tissue *in situ* hybridizations. After hybridization and final washes, the Malpighian tubules were placed between a poly-D-lysine coated slide and a glass coverslip treated with Sigmacote (Sigma, SL-2) and flattened by pressing firmly on the coverslip. The coverslip was then removed by rinsing in 95% ethanol. Autoradiography was performed according to Raikhel et al. (1988). The preparations were photographed under phase contrast and dark-field illumination using an Olympus Vanox-S phase contrast microscope and a 10X objective.

11. Sequence comparisons.

a. Deduced amino acid sequence comparisons.

The deduced amino acid sequences comparisons in Figures 4 and 5 were made by dividing each deduced amino acid sequence into approximately three equal parts and making all pairwise comparisons. This was done to determine the similarity throughout the entire deduced amino acid sequence since the FASTP algorithm will only detect and optimize an alignment with the insertion of gaps in a single region established by the initial alignment (Lipman and Pearson, 1985, footnote 15).

b. Flanking DNA sequence comparisons.

Comparisons of the flanking DNA of the UO gene of *D. melanogaster, D. pseudoobscura* and *D. virilis* were performed using dot matrix homology analyses (Pustell and Kafatos, 1982; Pustell and Kafatos, 1984) using DNA Inspector IIe (Textco). All pair-wise combinations of *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO flanking DNA were made using search parameters which identified nucleotide stretches of nine or greater matching nucleotides, allowing for one nucleotide mismatch.

c. Calculating the expected number of silent site substitutions.

The number of the expected silent third-position-codon differences between *D. melanogaster* and *D. pseudoobscura* and *D. melanogaster* and *D. virilis* were calculated according to Henikoff and Eghtedarzadeh (1987). The codon bias percentages for Drosophila for the amino acids threonine, proline, alanine, glycine and valine were taken from Shields et al. (1988) and are listed in Table A4. The expected number of third-codon-position differences for each of the five animo acids was calculated using the formula: $(n) \times \{1-\sum[0.01 \times \text{bias } \%)^2\}]$, where n is the number of matching residues between *D. melanogaster* and *D. pseudoobscura* UO or *D. melanogaster* and *D. virilis* UO for one of the five amino acids.

12. P-element Plasmid Construction.

a. P[($w^{+\Delta}$)DmUOPstI].

The 3.2 kb genomic *Pst*I restriction fragment encoding the UO gene from the *ecd*^{1} strain was cloned into the *Pst*I site of the P-element vector CaSpeR containing a modified *D. melanogaster white* (*w*) reporter gene (Pirrotta, 1988). The *Pst*I sites are at position 826 bp upstream and approximately 1.2 kb

downstream of the UO transcribed region.

b. P[(hspw^{+})DmUOSpeI-StuI].

The *Spe*I-*Stu*I restriction fragment encoding the UO gene from the *ecd*^{1} strain was cloned into the *Xha*I and *Stu*I sites of the P-element vector pW8 which has the promoter of the *hsp70* gene linked to the body of the *white* gene as a reporter (Klemenz et al., 1988). The *Spe*I site is 171 bp from the transcription start site of the *D. melanogaster* UO gene and the *Stu*I site is approximately 300 bp downstream of the UO transcribed region. Heat shock is not required for screening transformants since the *hsp70* promoter is transcribed at low levels in the absence of heat shock, providing enough *white* product to produce pigmentation within the eyes of transformed adults.

c. P[(w$^{+\Delta}$)DpUORI].

The *Eco*RI restriction fragment containing the *D. pseudoobscura* UO gene (Figure 15A) was cloned into the *Eco*RI site of the P-element vector CaSpeR. This construct contained the *D. pseudoobscura* UO gene with approximately 700 bp of 5' flanking DNA and approximately 200 bp of 3' flanking DNA .

d. P[(w$^{+\Delta}$)DvUO1PstI].

The 4900 bp *Pst*I fragment containing the *D. virilis* UO1 gene (Figure 16A) with approximately 3200 bp of 5' flanking DNA and 400 bp of 3' flanking DNA was cloned into the *Pst*I site of the P-element vector CaSpeR.

e. P[(w$^{+\Delta}$)DvUO2PstI].

The 5500 bp *Pst*I restriction fragment that includes the *D. virilis* UO2 gene (Figure 16A) with approximately 4000 bp of 5' flanking DNA and 300 bp of 3' flanking DNA was cloned into the *Pst*I site of CaSpeR.

f. P[(w$^{+\Delta}$)DmUO-lacZ].

The *Bgl*II fragment containing the Canton-S UO gene (Figure A1) was isolated from a lambda genomic clone possessing the UO gene. The *Bgl*II fragment was subcloned into the *Bgl*II site of pKC7 (Maniatis et al., 1982) and the resulting plasmid is designated pKC7/B (Figure M1). The subsequent steps performed to make the P-element construct P[(w+Δ)DmUO-lacZ] are diagramed in Figure M1. The *Bgl*II fragment was isolated from pKC7/B and then digested with *Ava*I. The *Bgl*II-*Ava*I fragment containing 826 bp of UO 5' flanking DNA and the first 346 bp of transcribed UO sequence was isolated and the 3' recessed ends were filled in by Klenow in the presence of dNTPs (Maniatis et al., 1982). *Bam*HI linkers (NEB) were ligated to the filled in ends. The products of the ligation were digested with *Bam*HI. Excess *Bam*HI linkers and their cleavage products were removed by fractionating the mixture through a 25 cm x 1.7 cm BioGel P-60 column with TE (BioRad). The fraction containing the *Bam*HI linkered fragment containing the UO gene was digested a second time with *Bam*HI and the digest was loaded onto a 1% agarose gel. The band containing the *Bam*HI linkered fragment was excised from the gel, electroeluted and ethanol precipitated. This *Bgl*II-*Ava*I restriction fragment, now possessing terminal *Bam*HI sites was cloned into the *Bam*HI site of puc119 (Vieira and Messing, 1987). I would like to thank Robin Steinman for ligating the *Bam*HI linkers onto the *Bgl*II-*Ava*I UO fragment.

This UO-containing *Bam*HI fragment was isolated from puc119 and cloned

Figure M1. Construction of the UO-lacZ fusion gene. pKC7/B is a subclone of the Canton-S *Bgl*II restriction fragment containing the UO gene (Figure A1). A portion of the UO coding region is represented by the open rectangle, the *lacZ* coding sequences are represented by a cross-hatched rectangle, the *white* gene coding sequences are represented by a stippled rectangle and *Bam*HI linkers are shown as small filled rectangles. Arrows designate the direction of transcription of the UO and *white* genes. The boxed P's indicate the P-element inverted repeats. Restriction enzyme sites: Av, *Ava*I; Bg, *Bgl*II; Bm, *Bam*HI; P, *Pst*I. The Materials and Methods give a detailed description of the steps diagrammed here.

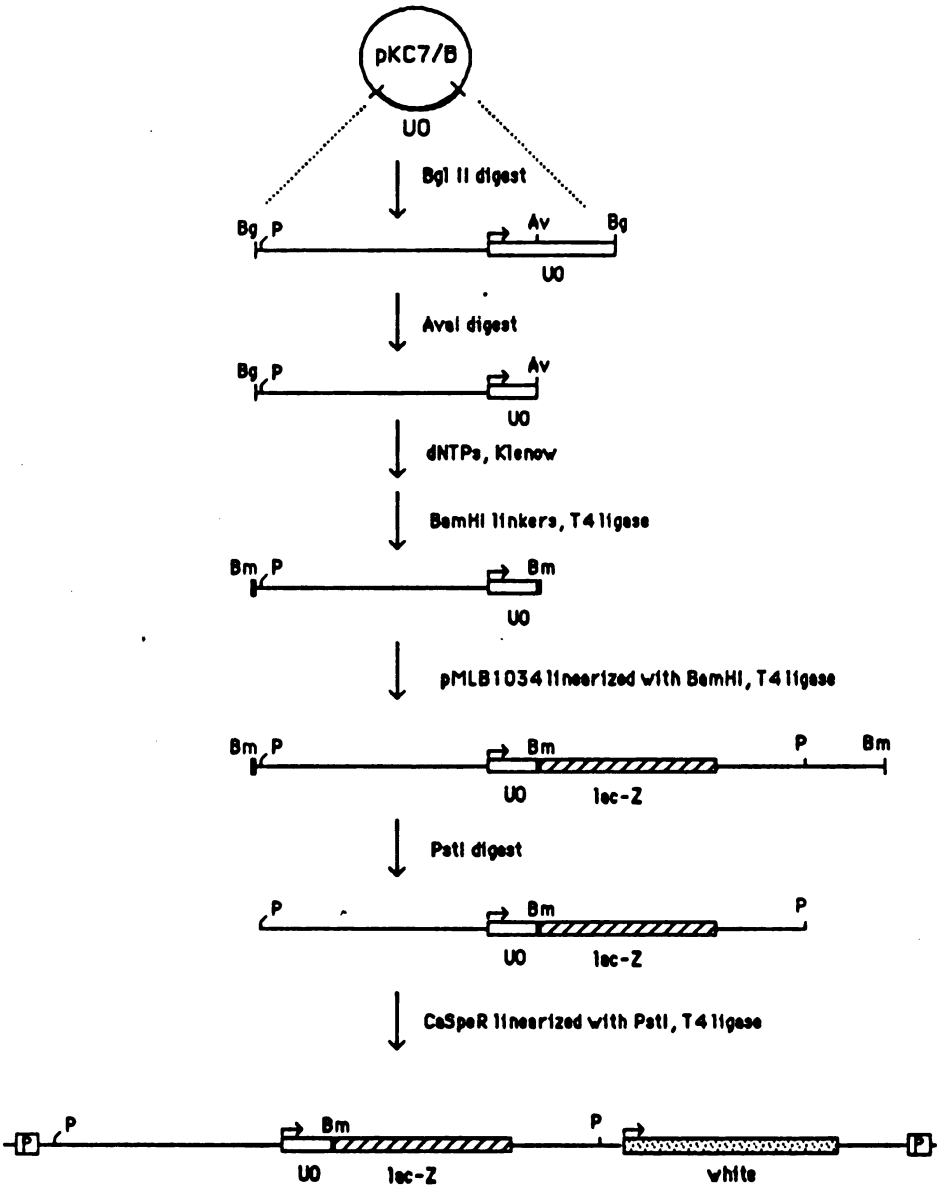

pKC7/B
UO
Bgl II digest
Bg P
Av
Bg
UO
AvaI digest
Bg P
Av
UO
dNTPs, Klenow
BamHI linkers, T4 ligase
Bm P
Bm
UO
pMLB1034 linearized with BamHI, T4 ligase
Bm P
Bm
P
Bm
UO
lac-Z
PstI digest
P
Bm
P
UO
lac-Z
CaSpeR linearized with PstI, T4 ligase
P
Bm
P
P
UO
lac-Z
white

Figure M1.

into M13mp18 and sequenced to verify that no base deletions or changes had occurred during the linkering process since the UO reading frame must be maintained for subsequent steps. The *Bam*HI fragment was isolated from puc119 was also cloned into the *Bam*HI site of the vector pMLB1034 which contains the *E. coli lacZ* gene (Shapira et al., 1983). Appropriate restriction digests were made to select clones in which the *Bam*HI fragment was oriented such that the UO coding sequences were in-frame with the *lac-Z* coding sequences. The UO-lacZ fusion was excised from pMLB1034 by digestion with *Pst*I (Figure M1) and cloned into the *Pst*I site of CaSpeR (Pirrotta, 1988).

g. P[($w^{+\Delta}$)del(-138,-126)DmUO-lacZ].

The M13mp18 subclone of the *Bam*HI fragment containing the UO gene (described above) was subjected to site-directed mutagenesis using the method of Vandeyar et al. (1988). The site-directed mutagenesis method is described in further detail below. In this case, synthetic oligonucleotide #18 (Table A) was used to delete 13 bp of the distal DR element of the *D. melanogaster* UO gene (positions -138 to -126, Figure 1). Possible deletion mutants were screened by performing the T-sequencing reaction of single stranded DNA isolated from the M13 plaques and comparing the sequence ladder to the "T reaction" from the M13 *Bam*HI subclone containing the "wild type " UO gene. Out of 20 plaques screened, two contained the deletion. Plasmid DNA was prepared from an M13 subclone containing the deletion and the *Bam*HI fragment containing the UO gene was isolated. This fragment was fused in-frame with the *lacZ* gene and the fusion gene was excised with *Pst*I and cloned into CaSpeR.

h. P[($w^{+\Delta}$)del(+11,+23)DmUO-lacZ].

The steps to make this construct were identical to those for the construct P[(w+Δ)del(-138,-126)DmUO-lacZ] except that synthetic oligonucleotide #19 (Table A) was used to delete the proximal DR element of the *D. melanogaster* UO gene (positions +11 to +23, Figure 1). Three out of 20 plaques were deleted for this region.

i. P[(w+Δ)del(-138,126)(+11,+23)DmUO-lacZ].

The steps to make this construct were identical to those used to make P[(w+Δ)del(-138,-126) with one exception. The parental single stranded template DNA was an M13 subclone containing a deletion of the DR element from positions +11 to +23. By creating a deletion using primer 19, subclones with both of the DR elements deleted were obtained. Four out of 16 plaques screened had deletions for both the proximal and distal DR elements.

13. Site directed mutagenesis to create large 5' UO deletions.

Large deletions within the 5' region of the *D. melanogaster* UO gene were made using the site-directed mutagenesis method of Vandeyar et al. (1988). Single stranded M13mp18 DNA containing the UO *Bam*HI fragment (section above) was used as the parental DNA for mutagenesis. The steps taken to produce deletions within the 5' flanking DNA of the UO gene are diagramed in Figure M2. Mutagenesis reactions were as described in United States Biochemical T7-GEN *In Vitro* Mutagenesis Instruction Manual. Phosphorylated synthetic primers of 34 nucleotides were independently annealed to the template DNA. Each 17-mer "arm" of a primer hybridized to positions approximately 100 bp apart along the template DNA (Figure 12). After hybridization, the primer was extended in the presence of dATP, dGTP, dTTP,

5-methyl-dCTP and T_7 DNA polymerase. Products which extended along the M13 template to the 3' end of the primer were ligated using T_4 DNA ligase. The final double stranded product containing a "looped out" region to be deleted (Figure M2) was digested with *Msp*I and *Hha*I. These restriction enzymes nick only the nonmethylated parental strand which is subsequently removed by digestion with *Exo*III. The remaining methylated strand which has a deletion for the material between the two primer "arms" was transformed into the non-restrictive host *E. coli* SDM (United States Biochemical), replicated and then infected into an *E. coli* host susceptible to M13 infection. *E. coli* MV1193 was used here.

Deletion mutants were screened by sequencing the "T-reaction" from single stranded DNA prepared from M13 plaques. Examples of deletions spanning approximately 100 bp are rare and considered to be difficult to obtain due to the increased distance between the sites in which the two "arms" of the primer bind. However, in the experiments reported here, large deletions occurred at high frequencies. Mutagenesis with primer #20 (Table A6) yielded three out of eight M13 plaques with a 103 bp deletion, mutagenesis with primer #21 yielded three out of eight M13 plaques with a 235 bp deletion and finally, mutagenesis with primer #23 yielded eleven out of eighteen M13 plaques with a 114 bp deletion.

Combinative deletions were made to examine the effects of simultaneous multiple deletion on the regulation of the UO gene. To construct combinative deletions, M13 template DNA, having a deletion of 5' UO DNA from a previous mutagenesis, was subjected to a second round of mutagenesis with a different primer than the one used to make the original deletion. The primers were designed such that the right "arm" of one primer hybridized to the same seuqence that the left "arm" of another primer hybridized to, in order to make contiguous deletions which would only have the primer binding sites remaining between the deleted regions (Figure 12).

Figure M2. Diagram of the steps to prepare P-element constructs containing combinative oligonucleotide-directed large deletions of the 5' flanking DNA of the *D. melanogaster* UO gene. The M13mp18 sublone contains the Canton-S *Bgl*II-*Ava*I *Bam*HI-linkered restriction fragment of the UO gene. UO, lacZ and *white* coding sequences are represented by an open rectangle, a cross-hatched rectangle and a stippled rectangle, respectively. Arrows indicate the direction of transcription of the UO and *white* genes. BamHI linkers are represented by small filled rectangles. The primer used for *in vito* mutagenesis is shown as a short curved line. M's decorating the double stranded M13mp18 symbolize the presence of 5-methyl-dCTP residues in the strand containing the deletion mutation. A deletion in the 5' flanking DNA of the UO gene after the mutagenesis steps is indicated by a gap. Restriction enzyme sites: Av, *Ava*I; Bg, *Bgl*II; Bm, *Bam*HI; P, *Pst*I. A detailed description of the steps diagrammed here is within the text of Materials and Methods.

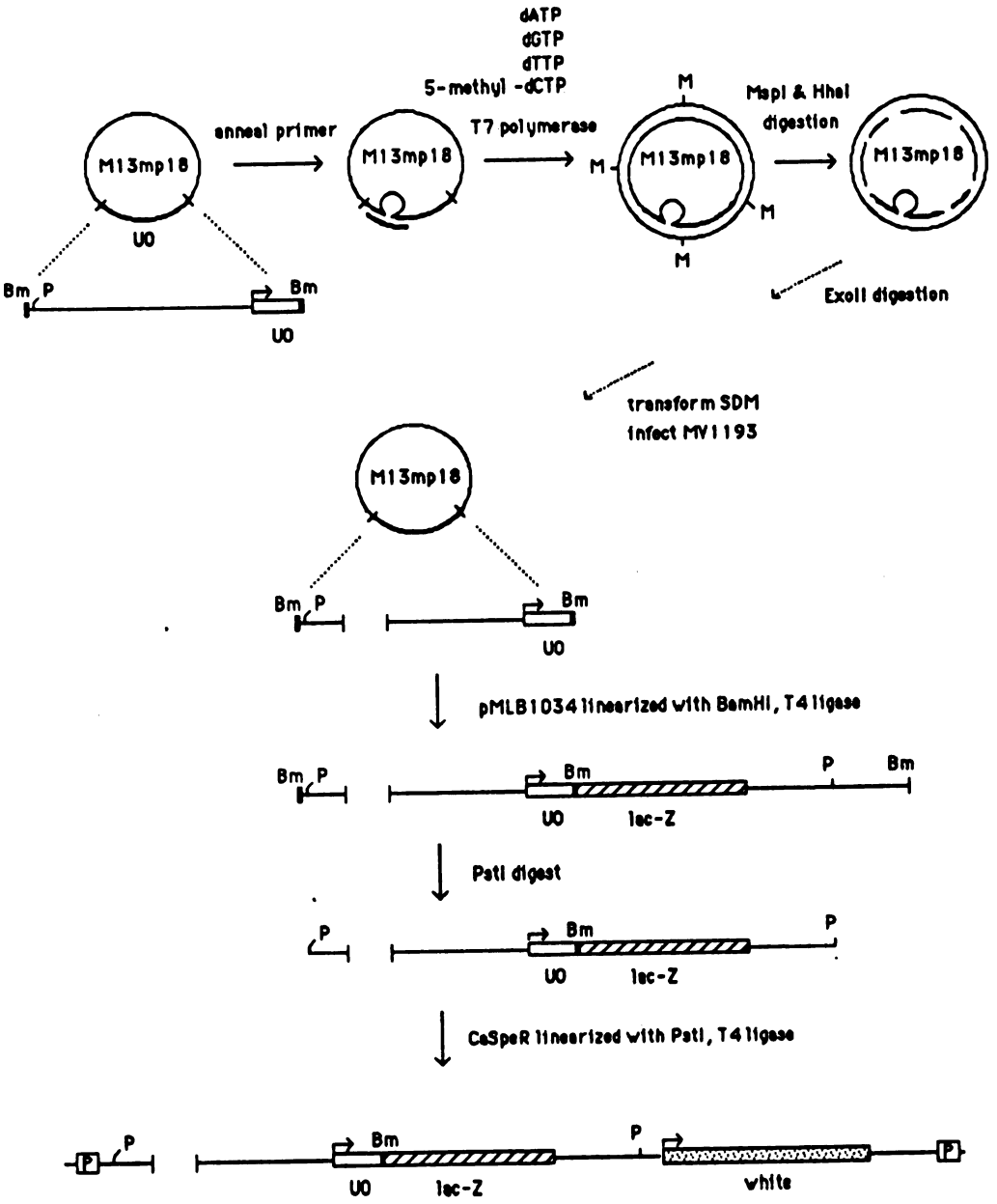

dATP
dGTP
dTTP
5-methyl -dCTP
anneal primer
T7 polymerase
MspI & HhaI
digestion
M13mp18
UO
Bm
P
M
ExoII digestion
transform SDM
infect MV1193
pMLB1034 linearized with BamHI, T4 ligase
lac-Z
PstI digest
CaSpeR linearized with PstI, T4 ligase
white

Figure M2.

14. P-element transformation.

a. Preparation of DNA for micro-injection into *D. melanogaster* embryos.

Having "clean" DNA, free of all chemicals used in the purification procedure, is essential for obtaining high transformation frequencies. DNA should be purified in a cesium chloride gradient and subsequently dialyzed for at least three days (Rubin and Spradling, 1982) as described for a large scale plasmid DNA preparation (above). After dialysis, the P-element construct DNAs were precipitated in the presence of 0.2 M NaCl with 2.5 volumes of ethanol at -20°C. The DNA was recovered by centrifugation at 4°C for 15 minutes and the pellet was washed once with 0.2 M NaCl in 70% ethanol and once with 70% ethanol. The pellet was vacuum dried and resuspended in 50 ml of injection buffer (5 mM KCl/ 0.1 mM NaH_2P0_4, pH 6.8).

A solution of P-element construct DNA at 300 ng/µl of injection buffer and P-element helper plasmid pπ25.7 "wings clipped" at 50 ng/µl of injection buffer was used for micro-injection into *D. melanogaster* embryos. Just prior to injection, the mixture of P-element construct and P-element helper was centrifuged for 10 minutes at room temperature and the solution was transferred to a new tube. This last centrifugation was used to remove debris and precipitation which was not detectable by eye, but capable of clogging embryo injection needles.

b. Embryo injection needle preparation.

Needles used for injections were made from glass capillary tubing with an outside diameter of 1.0 mm and an inside diameter of 0.78 mm (Sutter Instruments Co., #BF100-78-10). Capillary tubes were made into needles using a Flaming Brown micropipette Pluller (Sutter Instruments Co., Model P-80/PC)

P-80/PC) using Program 9. Program 9 was designed by Sutter Instruments given our needle specifications and consisted of the following settings: heat, 32,000 milliamps of current to the filament; pull, 400 milliamps to the pull solenoid; velocity, 230 millivolts of transducer output; time, 5 milliseconds. A boxed heating filament (2 mm x 1.5 mm) and a setting of 50 psi for the nitrogen was used. Needles pulled in this fashion were closed at the ends. In order to break the tip of the needle to make a 1-2 micrometer jagged opening, the needles were gently stroked across the curvilinear surface of a freshly broken microscope slide using a micromanipulator. The microscope edge of the microscope slide was broken using needle nose pliers.

c. Collection of appropriately staged embryos.

The host strain for all P-element transformations was the *white* deficient stock Df(1)w,yw67$^{c23(2)}$, which was made homozygous for the second chromosome from the wild type *D. melanogaster* Canton-S strain. This stock is referred to as Df(1)w,yw67$^{c23(2)}$, Df(1)w and Δwcs within the text. The sequence of the UO gene on the Canton-S second chromosome contains several nucleotides in the 3' untranslated region that are different from those of the UO gene isolated from a stock containing the temperature sensitive 20-hyroxyecdysone mutation, *ecd*^{1} (Figure 1). This difference in UO sequence allowed for discrimination between the message from the UO gene introduced via the P-element and the endogenous Canton-S UO message of the host strain (see Northern analyses in Figures 8 and 9).

Newly emerged *white* deficient adults were collected, fed a rich diet of yeast-honey paste and aged four to five days prior to egg collections. Approximately 300 aged adults were placed in small collection chambers (Santamaria et al.,

1986) and allowed to lay eggs on a lucite tray with a small amount of grape juice-agarose medium supplemented with yeast-honey paste. Collections of eggs were made every half hour by replacing the collection tray with a fresh one. Embryos from the first two half hour collections were discarded due to the lack of synchrony in egg laying. The goal was to obtain stage 2 embryos (Wieschaus and Nüsslein-Volhard, 1986) which had not yet cellularized. Each half hour thereafter, embryos were collected and chemically dechorionated in a 1:1 dilution of bleach in water for 15 seconds with swirling of the solution and the eggs. Dechorinated embryos were aligned on double sticky tape affixed to a coverslip with their posterior end hanging over the edge of the tape. After alignment, embryos were desiccated in a petri dish of Drierite for one to two minutes and then covered with Halocarbon oil (Series 700, Halocarbon Products Corporation).

d. Embryo injections.

Properly prepared embryos covered in Halocarbon oil were viewed for injection through a Wild compound microscope with a 10X objective. Needles were backfilled with the mixture of the P-element construct and the P-element helper plasmid (see above) using a Hamilton syringe and placed into a holder attached to a Leitz micromanipulator. The DNA solution was injected into the pole plasm of the posterior portion of the embryo which gives rise to the germ cells. The solution was driven out of the needle and into the embryos by a pulse of nitrogen (20-25 psi) for 20-40 milliseconds delivered by a Picospritzer II (General Valve Corporation). Only embryos which were at a developmental stage prior to pole cell formation were injected. Appropriately staged embryos have an easily discernable clear area at their posterior end (Wieschaus and Nüsslein-Volhard, 1986). Embryos which were too advanced in development for injection or those which "bled" after injection were discarded. The remainder

of the embryos, all successfully injected, were kept under Halocarbon oil and incubated in humidified petri dishes at 18°C or room temperature. After approximately 24 to 32 hours, first instar larvae hatched and were removed from the Halocarbon oil and placed in groups of 30 per vial of Drosophila media and allowed to develop at 25°C.

The G_0 adults were backcrossed to the *white* deficient host strain in single pair matings. The G_1 progeny from these crosses were screened for pale yellow to wild type brick red eye color. Transformed G_1 adults were backcrossed to the *white* deficient host as single pair matings. The G_2 progeny from a single vial which were heterozygous for a P-element insertion were crossed in single pair matings to each other. G_3 progeny which were homozygous for the P-element insertion usually had a darker eye color than that of the heterozygous G_3 progeny and thus, homozygous G_3 adults from a single vial were mated in pairs to create a homozygous stock. Some stocks possessing P-element integrations that were lethal when homozygous were analyzed for UO transgene expression as heterozygotes.

15. Histochemical staining for β-galactosidase activity.

Whole larvae pupae and adults as well as hand-dissected Malpighian tubules were stained for β-galactosidase activity according to Raghavan et al. (1986). Whole animals were splayed open in 10 mM phosphate buffer, pH 8 and transferred with forceps to the staining solution (0.066 ml 5% X-gal; 0.020 ml 100 mM potassium ferrocyanide; 0.020 ml 100 mM potassium ferricyanide; 0.050 ml 1.0 M sodium phosphate, pH 8, 0.850 ml 35% Ficoll-400). Malpighian tubules were dissected in 10 mM phosphate buffer, pH 8 and transferred in 10-20 µl of dissecting buffer to the staining solution. The tissue was stained for one to six hours and then transferred to a drop of 35% Ficoll on a microscope slide.

A coverslip was carefully overlayed and the preparations were photographed with an Olympus Vanox-S phase contrast microscope using a 10X objective.

16. Synthetic Oligonucleotides.

Synthetic oligonucleotides were synthesized either by Dr. Chris Somerville on an Applied Biosystems DNA Synthesizer or by the Macromolecular Sequencing Facility at Michigan State University. Some oligonucleotides were purified using an OPC column (Applied Biosystems) according to the manufacturers protocol or by HPLC. Some oligonucleotides used for sequencing and site directed mutagenesis were not further purified after synthesis. The sequence of each primer and method of purification is listed in Table A6.

17. Western Analysis.

The Western blotting procedure was similar to that of Kral et al. (1986) and is summarized here. Malpighian tubules were hand-dissected in 0.25 M sucrose, 1.7 mM EDTA, pH 6.9 and transferred in 7.5 ul of dissecting solution to a 1.5 ml microtube and immediately frozen at -70^{o}C. To each frozen sample, 7.5 ul of a two-fold concentrated Laemmli gel loading buffer was added and the samples were boiled for 3 minutes, cooled and loaded onto a 0.1% SDS/ 10% polyacrylamide gel (Laemmli, 1970). Following electrophoresis, the proteins were electroblotted onto an Immobilon-P membrane (Millipore). The portion of the membrane containing the size separated protein molecular weight standards (phosphorylase b, M_r=94,000; albumin, M_r=67,000; ovalbumin, M_r=43,000; carbonic anhydrase, M_r=30,000) was stained with Coomasie Blue. The remainder of the membrane was incubated for one hour in blocking solution and then overnight with the primary antibody. The source of primary

antibody used to detect *D. melanogaster*, *D. pseudoobscura* and *D. virilis* UO was a polyclonal antiserum raised against purified *D. melanogaster* UO protein (Friedman and Barker, 1982). Visualization of the UO proteins occurred by incubating the blot with the secondary antibody, horseradish peroxidase coupled to goat anti-rabbit IgG (BioRad 172-1013), and then 4-chloro-1-napthol (Sigma C-8890) as a substrate for horseradish peroxidase.

APPENDIX B

Figures and Tables

Figure A1. Restriction map and sequencing strategy for the UO genomic region and twelve independently arising urate oxidase cDNAs. (a) Restriction map of 38 kb of genomic DNA including the Canton-S UO gene. (b) Restriction map of the UO gene and flanking DNA with the directions and regions sequenced designated by arrows. The transcription initiation site of the UO gene is at +1 designated by I. The heavy black line represents the coding region of the UO gene with the open rectangle indicating the 69 base pair UO intron.
(c) Composite restriction map and sequencing strategy of twelve UO cDNAs. A UO cDNA (cUO2), spans the region between two *Pst*I sites, Ⓟ, which were introduced during cDNA library construction. The numeral above the arrows designates the number of independently arising cDNAs sequenced for that region. The asterisk indicates the location of a synthetic primer used to sequence the extreme 5' end of the UO cDNAs. AUG and TGA shown on the restriction map indicate the position of the UO translation start and stop sites, respectively. (d) An autoradiograph of a genomic Southern blot of *D. melanogaster* Canton-S high molecular weight DNA restricted with *Sal* I (lane 1), *Ava*I (lane 2), *Eco*RI (lane 3) and *Hind*III (lane 4) was probed with a 5.5 kb *Hind*III restriction fragment containing the UO gene. Restriction sites: A, *Alu*I; Ac, *Acc*I; Av, *Ava*I; B, *Bgl*II; C, *Cla*I; D, *Dra*I; H, *Hind*III; Hp,*Hpa*II; N, *Nla*III; Pv, *Pvu*II; R, *Eco*RI; S, *Sal*I; Sp, *Spe*I; T, *Taq*I; X, *Xho*II. The Southern analysis was performed by T. Friedman.

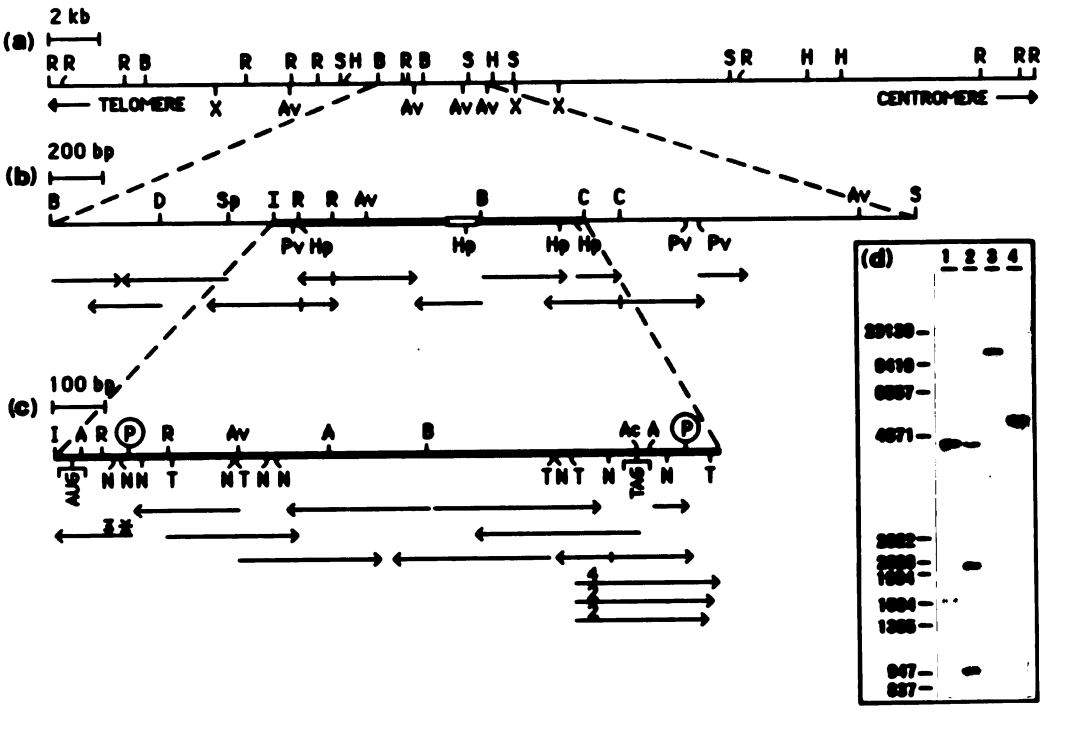


Figure A1.

Figure A2. Southern analysis revealing restriction enzyme recognition site differences in the 3' transcribed but untranslated region of the UO gene from different strains of *D. melanogaster.* Lanes 1, 5 and 9 contain DNA isolated from *ecd*^{1} and lanes 2, 6 and 10 contain DNA isolated from Df(1)w,yw$^{67c23(2)}$. Lanes 3, 7 and 11 contain DNA isolated from Canton-S and lanes 4, 8 and 12 contain DNA isolated for the stock Df(1)w,yw$^{67c23(2)}$ with the Canton-S second chromosome (see Materials and Methods). The DNA samples in lanes 1-4 were digested with *Alu*I. The DNA samples in lanes 5-8 were digested with *Sph*I and the samples in lanes 9-12 were simultaneously digested with *Sph*I and *Pvu*II. The restricted DNA was size separated on an agarose gel, blotted onto nylon and probed with cUO2 (Figure A1). Based on sequence data, *Alu*I and *Sph*I should cut the UO gene of the *ecd*^{1} strain, but not the Canton-S strain, in the 3' transcribed but untranslated region. The approximate 420 bp hybridizing fragment in the DNA samples after digestion with *Alu*I (lanes 1-4) represents an internal fragment within the coding region of UO. The restriction fragment of 610 bp in lane 1 containing *ecd*^{1} DNA which hybridized to the UO probe is the result of cleavage of the 960 from the 1090 bp *Alu*I fragment (lanes 2-4) in the 3' untranslated region of the UO gene. Digestion of *ecd*^{1} DNA within the 3' untranslated region of the UO gene by *Sph*I (lane 5) truncated the 6600 bp UO restriction fragment in the Canton-S DNA (lane 7). Similarly, simultaneous digestion of the *ecd*^{1} DNA by *Sph*I and *Pvu*II (lane 9) truncated the 1,400 bp UO restriction fragment in the Canton-S DNA (lane 11).

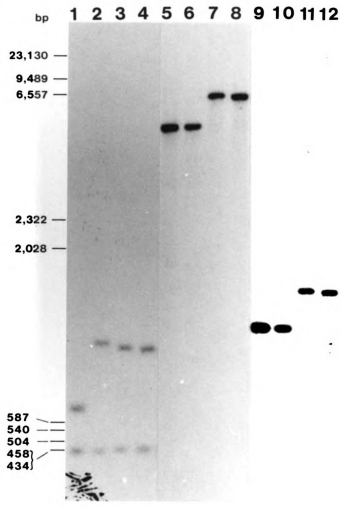

bp
1 2 3 4 5 6 7 8 9 10 11 12
23,130
9,489
6,557
2,322
2,028
587
540
504
458
434

Figure A2.

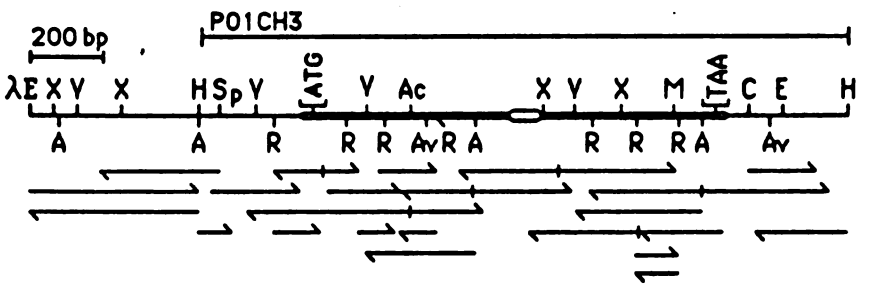


Figure A3. Restriction map and sequencing strategy for a 2.2 kb *D. pseudoobscura* genomic region containing the UO gene. The heavy black line represents the transcribed region of the UO gene with the translation start site (ATG) and translation stop codon (TAA) indicated on the restriction map. The 62 base pair *D. pseudoobscura* UO intron is represented by an open rectangular area. PO1CH3 is a 1.8 kb *Hind*III subclone containing the UO gene. M13 subclones used for sequencing this region are indicated below the restriction map with arrows indicating the direction of sequencing. Restriction sites: A, *Alu*I; Av, *Ava*I; Ac, *Acc*I; C, *Cla*I; E, *Eco*RI; λE,*Eco*RI in λEMBL3 arm; H,*Hind*III; M, *Xmn*I; R, *Rsa*I; Sp, *Sph*I; V, *Ava*II; X, *Xho*II.

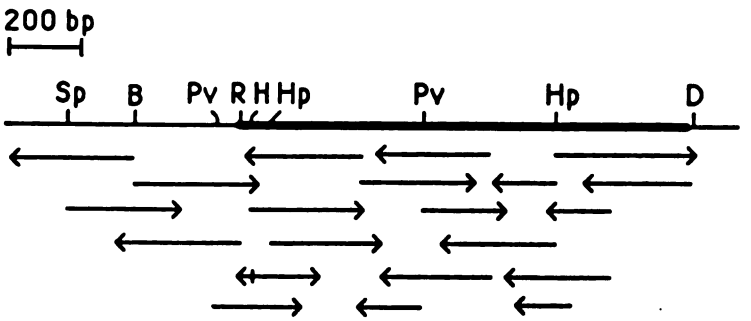
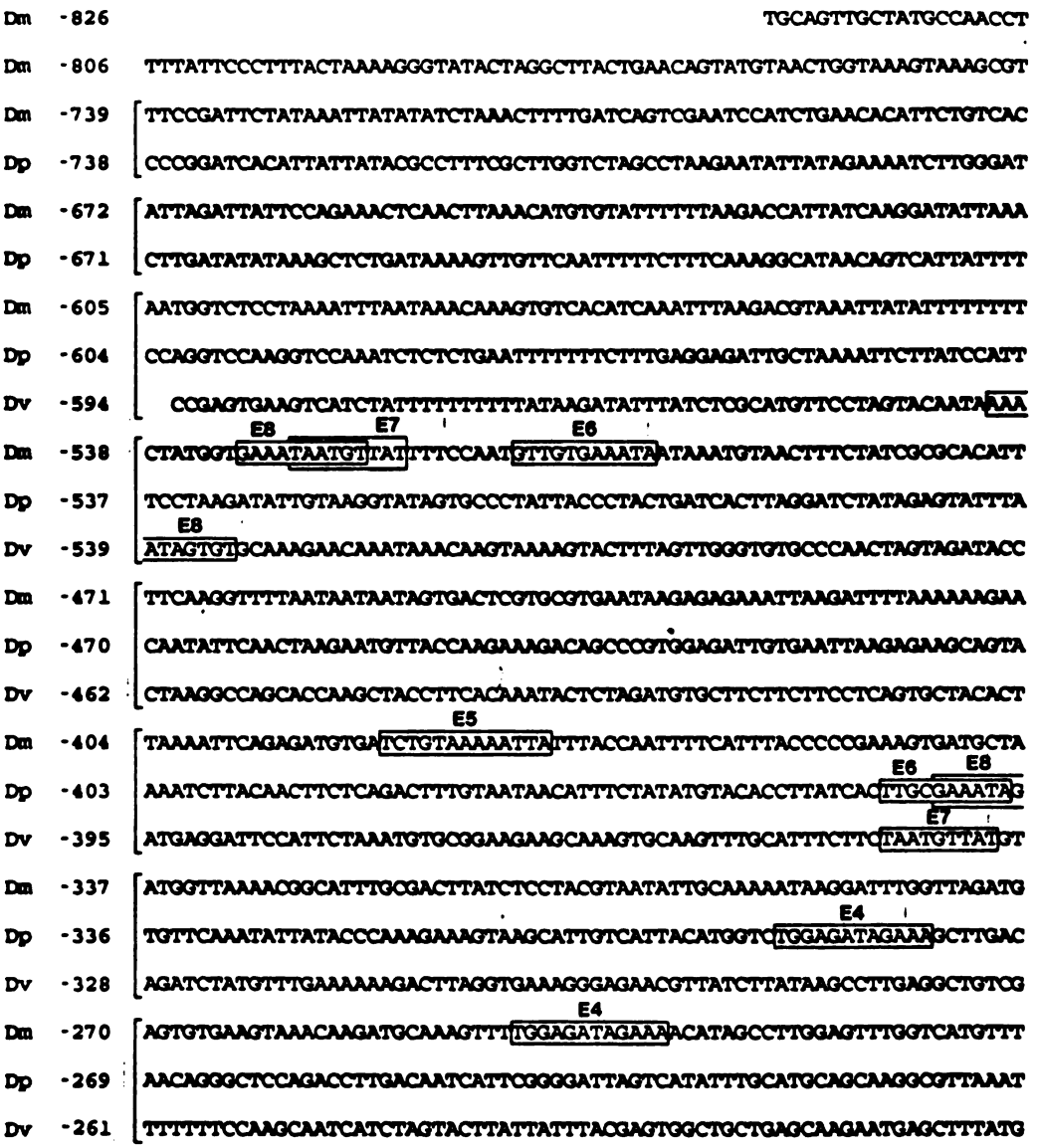


Figure A4. Restriction map and sequencing strategy for a 1.8 kb genomic region containing the *D. virilis* UO1 gene. The heavy black line represents the transcribed region of the UO gene. M13 subclones used for sequencing this region are indicated below the restriction map with arrows indicating the direction of sequencing. Restriction enzyme sites: B, *Bgl*II; D, *Dra*I; H, *Hind*III; Hp, *Hpa*II; Pv, *Pvu*II; R, *Eco*RI; Sp, *Spe*I.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Dm -806 TTTATTCCCTTTACTAAAAGGGTATACTAGGCTTACTGAACAGTATGTAACTGGTAAAGTAAAGCGT
Dm -739 TTCCGATTCTATAAATTATATATCTAAACTTTTGATCAGTCGAATCCATCTGAACACATTCTGTCAC
Dp -738 CCCGGATCACATTATTATACGCCTTTCGCTTGGTCTAGCCTAAGAATATTATAGAAAATCTTGGGAT
Dm -672 ATTAGATTATTCCAGAAACTCAACTTAAACATGTGTATTTTTTAAGACCATTATCAAGGATATTAAA
Dp -671 CTTGATATATAAAGCTCTGATAAAAGTTGTTCAATTTTTCTTTCAAAGGCATAACAGTCATTATTTT
Dm -605 AATGGTCTCCTAAAATTTAATAAACAAAGTGTCACATCAAATTTAAGACGTAAATTATATTTTTTTT
Dp -604 CCAGGTCCAAGGTCCAAATCTCTCTGAATTTTTTTCTTTGAGGAGATTGCTAAAATTCTTATCCATT
Dv -594 CCGAGTGAAGTCATCTATTTTTTTTTTTATAAGATATTTATCTCGCATGTTCCTAGTACAATAAAA
E8 E7 E6
Dm -538 CTATGGTGAAATAATGTTATTTTCCAATGTTGTGAAATAATAAATGTAACTTTCTATCGCGCACATT
Dp -537 TCCTAAGATATTGTAAGGTATAGTGCCCTATTACCCTACTGATCACTTAGGATCTATAGAGTATTTA
E8
Dv -539 ATAGTGTGCAAAGAACAAATAAACAAGTAAAAGTACTTTAGTTGGGTGTGCCCAACTAGTAGATACC
Dm -471 TTCAAGGTTTTAATAATAATAGTGACTCGTGCGTGAATAAGAGAGAAATTAAGATTTTAAAAAAGAA
Dp -470 CAATATTCAACTAAGAATGTTACCAAGAAAGACAGCCCGTGGAGATTGTGAATTAAGAGAAGCAGTA
Dv -462 CTAAGGCCAGCACCAAGCTACCTTCACAAATACTCTAGATGTGCTTCTTCTTCCTCAGTGCTACACT
E5
Dm -404 TAAAATTCAGAGATGTGATCTGTAAAAATTATTTACCAATTTTCATTTACCCCCGAAAGTGATGCTA
E6 E8
Dp -403 AAATCTTACAACTTCTCAGACTTTGTAATAACATTTCTATATGTACACCTTATCACTTGCGAAATAG
E7
Dv -395 ATGAGGATTCCATTCTAAATGTGCGGAAGAAGCAAAGTGCAAGTTTGCATTTCTTCTAATGTTATGT
Dm -337 ATGGTTAAAACGGCATTTGCGACTTATCTCCTACGTAATATTGCAAAAATAAGGATTTGGTTAGATG
E4
Dp -336 TGTTCAAATATTATACCCAAAGAAAGTAAGCATTGTCATTACATGGTCTGGAGATAGAAAGCTTGAC
Dv -328 AGATCTATGTTTGAAAAAAGACTTAGGTGAAAGGGAGAACGTTATCTTATAAGCCTTGAGGCTGTCG
E4
Dm -270 AGTGTGAAGTAAACAAGATGCAAAGTTTTGGAGATAGAAAACATAGCCTTGGAGTTTGGTCATGTTT
Dp -269 AACAGGGCTCCAGACCTTGACAATCATTCGGGGATTAGTCATATTTGCATGCAGCAAGGCGTTAAAT
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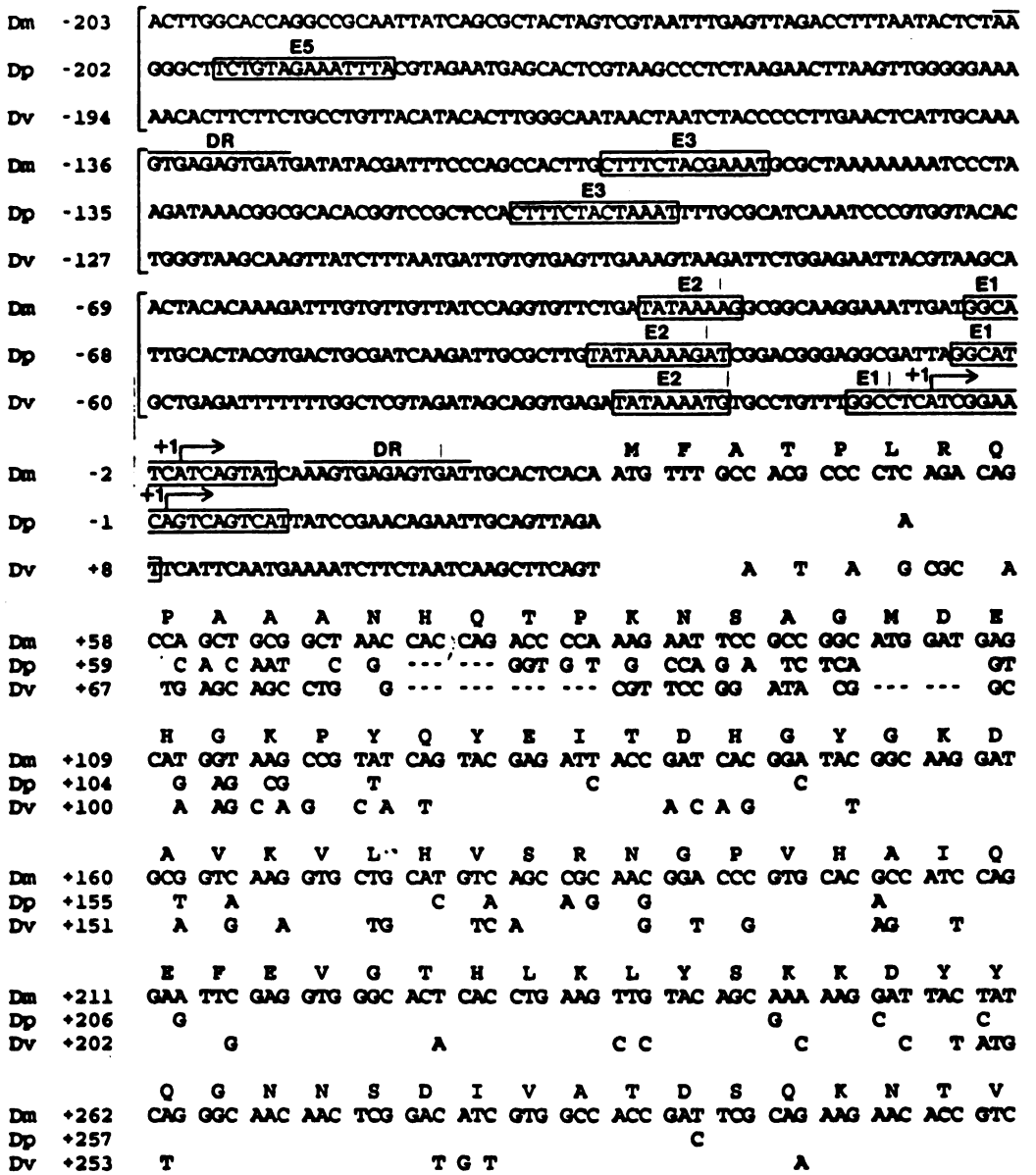
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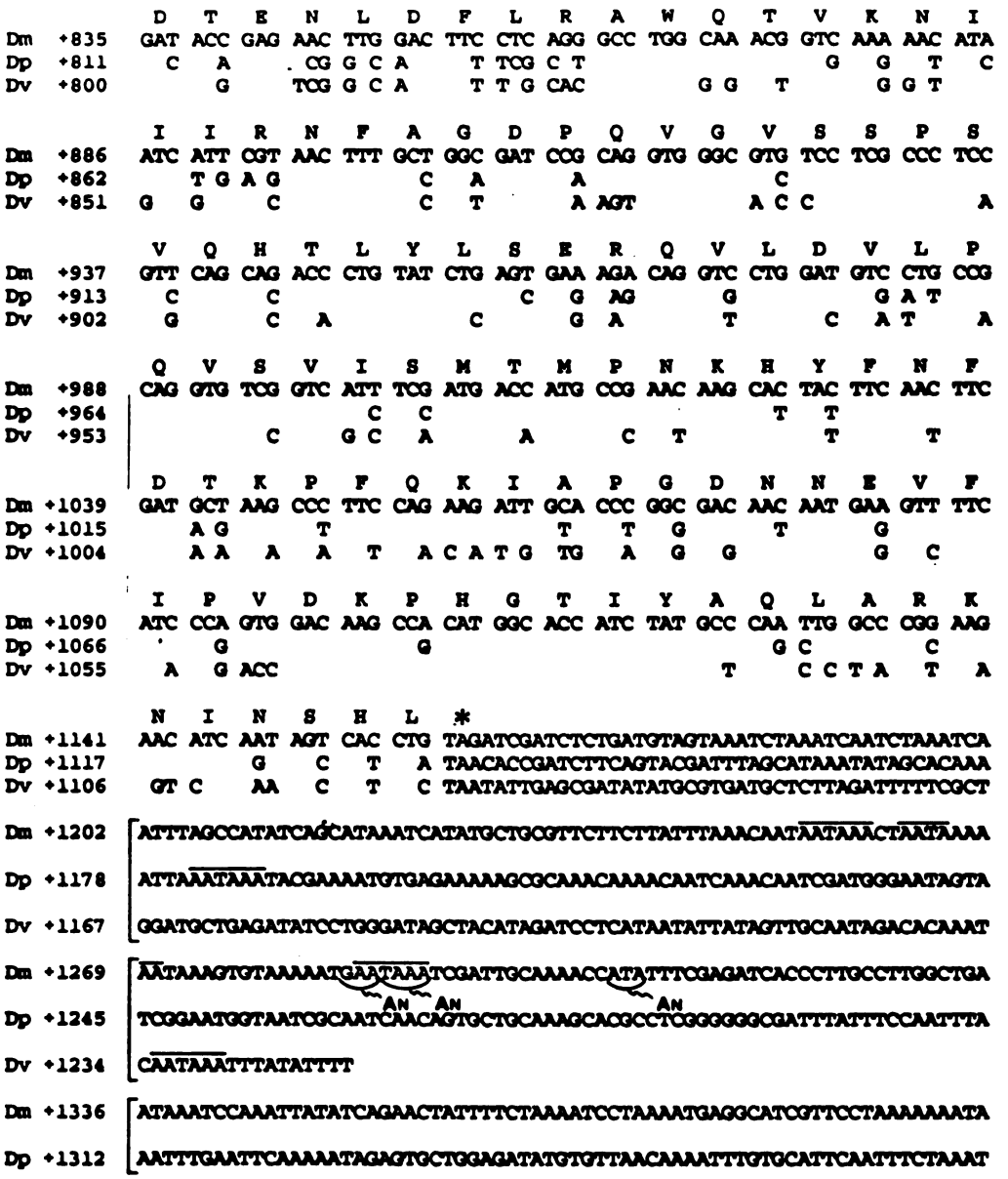
Dp -1
Dv +8
Dm +58
Dp +59
Dv +67
Dm +109
Dp +104
Dv +100
Dm +160
Dp +155
Dv +151
Dm +211
Dp +206
Dv +202
Dm +262
Dp +257
Dv +253
E5
DR
E3
E2
E1
+1
M F A T P L R Q
P A A A N H Q T P K N S A G M D E
H G K P Y Q Y E I T D H G Y G K D
A V K V L H V S R N G P V H A I Q
E F E V G T H L K L Y S K K D Y Y
Q G N N S D I V A T D S Q K N T V

```
          Y  L  L  A  K  K  H  G  I  E  S  P  E  K  F  A  L
Dm  +313  TAT TTG CTG GCG AAA AAG CAT GGC ATC GAA AGT CCC GAG AAG TTT GCC CTG
Dp  +308    C C       C  G     T        G                    T
Dv  +304      C    A      G  A  C        G           A        A

          L  L  A  K  H  F  I  N  K  Y  S  H  V  E  E  A  H
Dm  +364  CTC CTG GCC AAG CAC TTT ATT AAC AAA TAC TCA CAT GTG GAG GAG GCG CAC
Dp  +359  A G     GG C       C C C     G     G                 C
Dv  +355  A A T A   T       T    C G  G   C   T G G  C       A     TA   T

          V  H  V  E  A  Y  P  W  Q  R  V  C  Q  E  E  T  R
Dm  +415  GTT CAT GTG GAG GCG TAT CCC TGG CAG CGA GTT TGC CAG GAG GAG ACC AGG
Dp  +410    C           A C                    T   C                 A  A
Dv  +406    C      A   A         T          C A C AC       T  TT T   GAT

          T  N  V  N  G  K  C  E  N  G  V  Q  G  N  C     D
Dm  +463  ACC AAC GTC AAT GGG AAG TGC GAG AAC GGA GTC CAA GGG AAC TGC --- GAC
Dp  +446  --- --- --- --- --- TCT GT  A T   T CAG  GT   G   C G      AAC A
Dv  +442   AT  T   G    A     T C       C     --- --- --- --- AAC TGT --- A T

          F  S  S  I  D  N  R  S  L  H  N  H  A  F  I  F  T
Dm  +514  TTC AGC TCC ATT GAC AAC AGA TCA CTG CAC AAT CAC GCT TTT ATA TTC ACG
Dp  +497     CT              T C C  G        C       C  C  C  T
Dv  +493  C   A   G      G    T T T CAG        C       C       C       A

          P  T  A  L  H  Y  C  D  V  V  I  R  R  T
Dm  +565  CCC ACC GCT CTT CAC TAC TGC GAT GTG GTT ATA AGG AGA ACA G GTTAAGTCAAA
Dp  +548       G     G G                       A   C C T   G       GTGGGTGCCAG
Dv  +544       G   G  AC G T          C   C A C C G   C   C T CA    GTAGGCACATA

                                                                    D  P
Dm  +619  CATTACTTAAGCAATAATATTTAAAACTATTTAATCATCACCTTCTTTAATGTTTTAG  AT CCC
Dp  +602  GACAATACAAAACGAGTATTACTTAACGCCACTTCCACTTGTGACTTG-------TAG
Dv  +598  TATGGTATGACAGATGCACTTCCTAACAAGTCGTCTGTTTCC--------------AG

          K  Q  T  V  I  T  G  I  K  G  L  R  V  L  K  T  T
Dm  +682  AAA CAA ACG GTC ATC ACG GGC ATC AAG GGT CTC CGG GTG CTG AAG ACG ACC
Dp  +658   G      T           T                 C  T  C  C           C  T
Dv  +647   G   G  T           GT          A   A  G  C  T              A

          Q  S  S  F  V  N  F  V  N  D  E  F  R  S  L  P  D
Dm  +733  CAA TCC TCA TTC GTG AAC TTC GTG AAC GAT GAG TTC AGA TCT CTG CCA GAT
Dp  +709        G      C       T  C              T C C  G      G  C
Dv  +698     A      T          T  T G              C C A G    G G

          Q  Y  D  R  I  F  S  T  V  V  D  C  S  W  E  Y  S
Dm  +784  CAG TAT GAT CGC ATC TTT AGC ACC GTA GTG GAT TGC TCC TGG GAA TAC TCC
Dp  +760   A                    C       T           C              G       G
Dv  +749                                    T       G              G       G
```


D T E N L D F L R A W Q T V K N I
Dm +835 GAT ACC GAG AAC TTG GAC TTC CTC AGG GCC TGG CAA ACG GTC AAA AAC ATA
Dp +811 C A . CG G C A T TCG C T G G T G G T C
Dv +800 G TCG G C A T T G CAC G G T G G T
I I R N F A G D P Q V G V S S P S
Dm +886 ATC ATT CGT AAC TTT GCT GGC GAT CCG CAG GTG GGC GTG TCC TCG CCC TCC
Dp +862 T G A G C A A C
Dv +851 G G C C T A AGT A C C A
V Q H T L Y L S E R Q V L D V L P
Dm +937 GTT CAG CAG ACC CTG TAT CTG AGT GAA AGA CAG GTC CTG GAT GTC CTG CCG
Dp +913 C C C G AG G G A T
Dv +902 G C A C G A T C A T A
Q V S V I S M T M P N K H Y F N F
Dm +988 CAG GTG TCG GTC ATT TCG ATG ACC ATG CCG AAC AAG CAC TAC TTC AAC TTC
Dp +964 C C T T
Dv +953 C G C A A C T T T
D T K P F Q K I A P G D N N E V F
Dm +1039 GAT GCT AAG CCC TTC CAG AAG ATT GCA CCC GGC GAC AAC AAT GAA GTT TTC
Dp +1015 A G T T T G T G G
Dv +1004 A A A A T A C A T G TG A G G G C
I P V D K P H G T I Y A Q L A R K
Dm +1090 ATC CCA GTG GAC AAG CCA CAT GGC ACC ATC TAT GCC CAA TTG GCC CGG AAG
Dp +1066 G G C C
Dv +1055 A G ACC T C C T A T A
N I N S H L *
Dm +1141 AAC ATC AAT AGT CAC CTG TAGATCGATCTCTGATGTAGTAAATCTAAATCAATCTAAATCA
Dp +1117 G C T A TAACACCGATCTTCAGTACGATTTAGCATAAATATAGCACAAA
Dv +1106 GT C AA C T C TAATATTGAGCGATATATGCGTGATGCTCTTAGATTTTTCGCT
Dm +1202 ATTTAGCCATATCAGCATAAATCATATGCTGCGTTCTTCTTATTTAAACAATAATAAACTAATAAAA
Dp +1178 ATTAAATAAATACGAAAATGTGAGAAAAAGCGCAAACAAAACAATCAAACAATCGATGGGAATAGTA
Dv +1167 GGATGCTGAGATATCCTGGGATAGCTACATAGATCCTCATAATATTATAGTTGCAATAGACACAAAT
Dm +1269 AATAAAGTGTAAAAATGAATAAATCGATTGCAAAACCATATTTCGAGATCACCCTTGCCTTGGCTGA
AN AN AN
Dp +1245 TCGGAATGGTAATCGCAATCAACAGTGCTGCAAAGCACGCCTCGGGGGGCGATTTATTTCCAATTTA
Dv +1234 CAATAAATTTATATTTT
Dm +1336 ATAAATCCAAATTATATCAGAACTATTTTCTAAAATCCTAAAATGAGGCATCGTTCCTAAAAAAATA
Dp +1312 AATTTGAATTCAAAAATAGAGTGCTGGAGATATGTGTTAACAAAATTTGTGCATTCAATTTCTAAAT

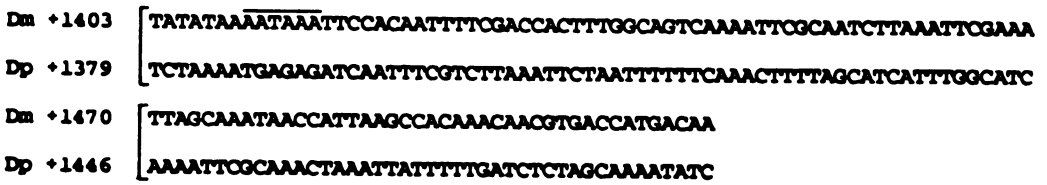


Figure A5. Nucleotide comparisons of the *D. melanogaster* (Dm), *D. pseudoobscura* (Dp) and *D. virilis* (Dv) UO genes. The complete nucleotide and deduced amino acid sequences of the *D. melanogaster* UO gene are shown. For the *D. pseudoobscura* and *D. virilis* UO genes, only the nucleotides within the coding region that differ from the *D. melanogaster* UO gene are shown. Dashes (-) represent nucleotides not present based on the alignment of the deduced UO amino acid sequence of these three species (Figure 5). The transcription start site of the UO gene of each species is designated +1. No attempt was made to align the intron or flanking DNA sequence. Evolutionarily conserved elements (E1 to E8) of the UO 5' flanking DNA (Figure 7) are boxed and shaded. The translation stop codon is indicated by *. The *D. melanogaster* UO sequence has three polyadenylation sites indicated by A_N and four potential polyadenylation signal sequences which are overscored. The *D. pseudoobscura* and *D. virilis* UO genes each have a single polyadenylation consensus signal (overscored).

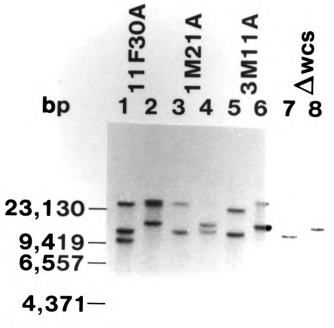


Figure A6. Example of a Southern blot used to determine the copy number of a P-element insertion. Genomic DNA isolated from three different lines (11F30A, 1M21A and 3M11A) transformed with P[($w^{+\Delta}$)DmPstI] and genomic DNA isolated from the *D. melanogaster white* deficient host strain (Δwcs) was digested with *Bam*HI (lanes 1, 3, 5 and 7) and *Xho*I (lanes 2, 4, 6 and 8). *Bam*HI and *Xho*I were used since they restrict the DNA once within the P-element construct. Therefore, a restriction fragment containing the UO transgene would be derived from a second restriction site within the D. melanogaster genome and would be unique to each P-element integration site. The restricted DNA was size separated on an agarose gel, blotted onto nylon and probed with cUO2 (Figure A1). The probe hybridized to an approximate 10,700 bp *Bam*HI DNA fragment and an approximate 11,700 bp *Xho*I DNA fragment of the Δwcs host (lanes 7 and 8). The DNA isolated from 11F30A showed two bands in each lane, in addition to the band corresponding to the endogenous UO gene (the larger band in lane 2 is a doublet), which hybridized to the UO probe (lanes 1 and 2). Two unique bands in this transformed line indicate that there have been two P-element integration events. DNA from strains 1M21A and 3M11A both show one hybridizing band in each lane, in addition to the band corresponding to the endogenous UO gene (lanes 3-6), and therefore, have a single insertion of the UO P-element construct.

Figure A7. UO enzyme activity during development of *D. melanogaster, D. pseudoobscura* and *D. virilis*. UO enzyme activity is present exclusively within the Malpighian tubules of Drosophila and is quantified by the amount of ^{14}C allantoin synthesized from ^{14}C uric acid per minute of reaction time per set of Malpighian tubules from a single animal (vertical axis). The first, second and third instar larval stages and white pupal stage are designated 1, 2, 3 and P, respectively. The sex of the larvae (Δ) assayed for UO activity was not determined. Adult stages are designated in hours after eclosion with ● as males and O as females. The UO enzyme profile for *D. virilis* represents activity in strains 1051.0 and 1051.48. and the UO enzyme activity profile for *D. pseudoobscura* represents activity in strain AH133. The UO enzyme assays were performed by T. Friedman. The UO enzyme activity profile for *D. melanogaster* Ore-R was taken, in part, from Kral et al. (1982). This figure is taken from Wallrath and Friedman (1991).

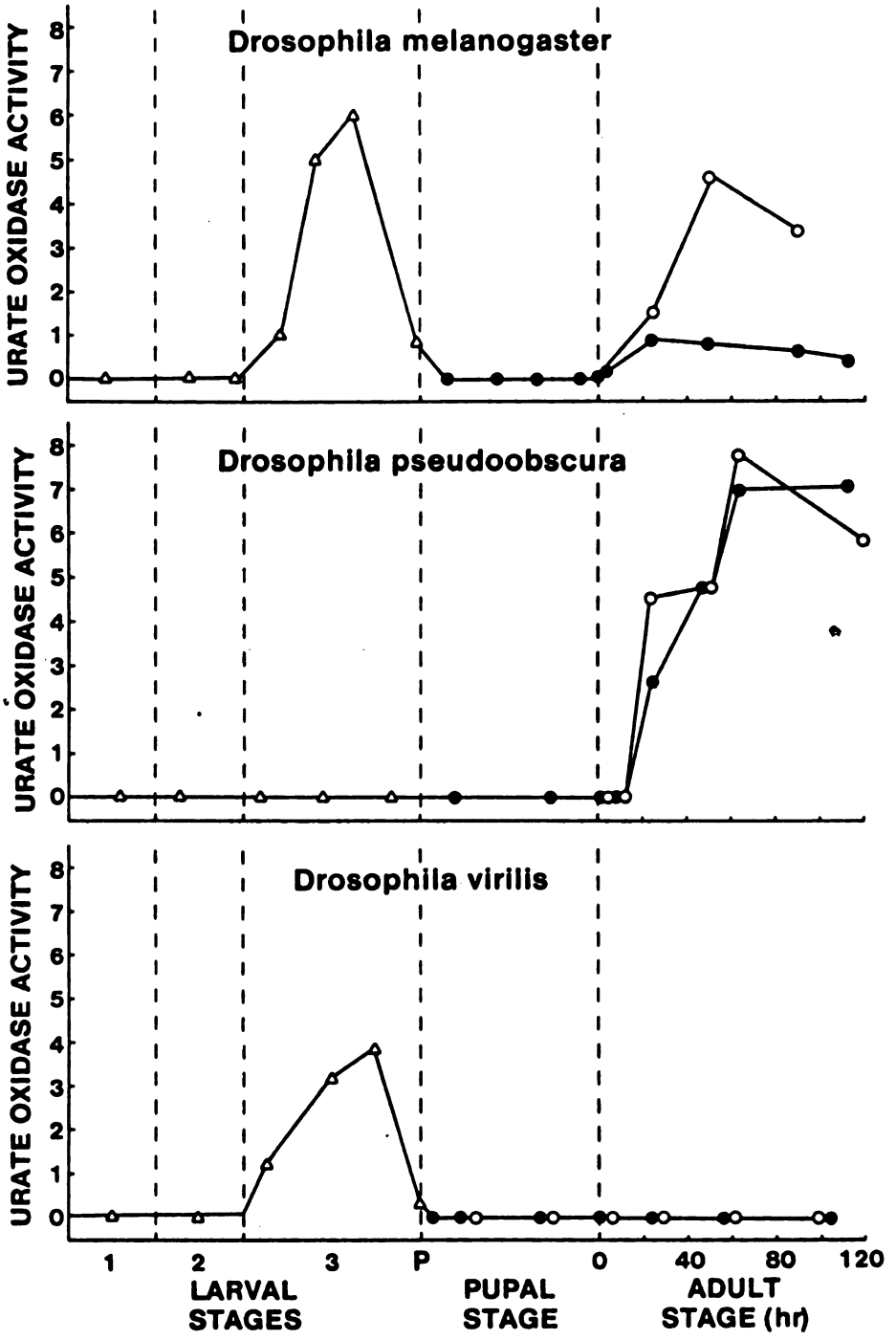

Drosophila melanogaster
URATE OXIDASE ACTIVITY
Drosophila pseudoobscura
URATE OXIDASE ACTIVITY
Drosophila virilis
URATE OXIDASE ACTIVITY
0
1
2
3
4
5
6
7
8
1
2
3
P
0
40
80
120
LARVAL STAGES
PUPAL STAGE
ADULT STAGE (hr)

Table A1. Drosophila stocks.

Stock	Phenotype	UO activity 3rd instar larva	UO activity adult	Reference
D. melanogaster				
Oregon-R	wild type	high	low	Lindsey and Grell (1967)
Canton-S	wild type	high	low	Lindsey and Grell (1967)
ecd^{1}	ts ecdysone deficient	high (19^{0}C)	low (19^{0}C)	Garen et al. (1977)
ry^{2} *xdh* deficient,	high rosy eye color		high	Glassman (1965)
Df(1)w,yw$^{67c23(2)}$	white eyes, yellow body	high	low	Pirrotta et al. (1983)
D. pseudoobscura				
AH133	wild type	absent	high	Schaeffer et al. (1987)
D. virilis				
1051.48	wild type	high	absent	Species Stock Center
1051.0*	wild type	high	abscent	Species Stock Center

**D. virilis* 1051.0 contains a tandem duplication of the UO gene; all other stocks have a single dopy of the UO gene per haploid genome.
ts = temperature sensitive mutation
xdh = *xanthine dehydrogenase*
y = mutant allele of the *yellow* locus
w = mutant allele of the *white* locus
Species Stock Center = National Drosophila Species Resource Center, Bowling Green, Ohio

Table A2. UO sequence changes among *D. melanogaster* *D. pseudoobscrua* and *D. virilis*.

	D. pseudoobscura		*D. melanogaster*	*D. virilis*	
Exon 1, base pairs	590		607	585	
leader sequence, base pairs[a]	34		33	42	
nucleotide substitutions	14				
protein coding region, base pairs	556		574	544	
nucleotide substitutions	106	(19.1%)		162	(29.8%)
codon position 1	23	(21.7%)		37	(22.8%)
codon position 2	18	(17.0%)		32	(19.8%)
codon position 3	65	(61.3%)		93	(57.4%)
transversions	56			86	
transitions	50			76	
number of amino acids	185		191	181	
amino acid identities	151			122	
synonymous (silent) changes	48			53	
replacements	33			59	
conserved replacements[b]	29			39	
amino acid deletions	7			10	
amino acid additions	1			0	
Intron, base pairs	62		69	55	
Exon 2, base pairs	d		608, 612 & 629[c]	d	
protein coding region, base pairs	482		482	482	
nucleotide substitutions	79	(16.3%)		113	(23.4%)
codon position 1	18	(22.8%)		25	(22.1%)
codon position 2	5	(6.3%)		8	(7.1%)
codon position 3	56	(70.9%)		80	(70.8%)
transversions	34			57	
transitions	45			56	
number of amino acids	161		161	161	
amino acid identities	152			134	
synonymous (silent) differences	63			61	
replacements	9			27	
conserved replacements[b]	8			26	
amino acid deletions	0			0	
amino acid additions	0			0	
Exon 1 + Exon 2, protein coding region only					
base pairs	1038		1056	1026	
amino acids	346		352	342	
amino acid identities	303	(87.6%)		256	(74.9%)
deduce M_r of urate oxidase	39,266		39,989	38,680	
nucleotide differences	185	(17.8%)		275	(26.8%)

a The transcription start site of the *D. melanogaster* urate oxidase gene was determined experimentally.
b Conserved amino acid changes were defined by the FASTP program (Lipman and Pearson 1985).
c There are three polyadenylation sites for the *D. melanogaster* UO gene.
d The polyadenylation site(s) has not been determined for the *D. pseudoobscura* and *D. virilis* UO gene and, therefore, the length of the UO exon 2 of these species is not known.

Table A3. Codon Usage of the *D. melanogster*, *D. pseudoobscura and D. virilis UO genes.*

UUU	F	8/6/10	UCU	S	1/1/0	UAU	Y	5/7/9	UGU	C	0/0/2
UUC	F	10/11/5	UCC	S	6/7/5	UAC	Y	10/8/5	UGC	C	4/5/3
UUA	L	0/0/1	UCA	S	1/3/5	UAA	*	1/0/1	UGA	*	0/0/0
UUG	L	2/4/4	UCG	S	10/5/6	UAG	*	0/1/0	UGG	W	3/3/3
CUU	L	1/1/0	CCU	P	2/0/1	CAU	H	5/6/6	CGU	R	3/1/3
CUC	L	1/4/5	CCC	P	8/9/6	CAC	H	10/12/10	CGC	R	5/2/7
CUA	L	2/0/1	CCA	P	2/5/6	CAA	Q	4/5/10	CGA	R	0/1/0
CUG	L	13/13/16	CCG	P	6/4/2	CAG	Q	17/14/9	CGG	R	0/2/0
AUU	I	3/6/2	ACU	T	1/6/3	AAU	N	7/5/8	AGU	S	2/3/5
AUC	I	15/10/10	ACC	T	10/13/7	AAC	N	19/14/12	AGC	S	7/4/7
AUA	I	0/3/4	ACA	T	3/1/7	AAA	K	0/5/8	AGA	R	1/5/1
AUG	M	5/4/6	ACG	T	7/7/5	AAG	K	22/18/15	AGG	R	4/3/0
GUU	V	1/5/7	GCU	A	4/5/2	GAU	D	10/15/14	GGU	G	3/2/2
GUC	V	13/10/8	GCC	A	10/9/4	GAC	D	9/6/5	GGC	G	10/10/11
GUA	V	1/5/4	GCA	A	1/1/3	GAA	E	0/5/4	GGA	G	2/3/2
GUG	V	15/15/13	GCG	A	0/5/7	GAG	E	16/12/14	GGG	G	2/2/2

One-letter symbol is used for amino acids. Codon usage for the *D. pseudoobscura, D. melanogaster* and *D. virilis* UO genes are designated left to right, respectively. * designates translation termination codons.

Table A4. Silent site substitutions of the UO genes for threonine, proline, alanine, glycine and valine.

Amino Acid	Codon	Bias (%)	Number of matching residues[a]		Expected number of differences[b]		Observed number of differences	
			Dp	Dv	Dp	Dv	Dp	Dv
THR	ACU	12.7						
	ACC	80.9	19	17	6.21	5.55	7	11
	ACA	1.9						
	ACG	4.5						
PRO	CCU	10.2						
	CCC	67.7	17	14	8.56	7.05	7	8
	CCA	15.2						
	CCG	6.9						
ALA	GCU	22.0						
	GCC	69.4	12	12	5.60	7.34	6	9
	GCA	4.2						
	GCG	4.4						
GLY	GGU	34.9						
	GGG	0	13	12	8.40	7.75	4	4
	GGA	22.3						
	GGC	42.9						
VAL	GUU	15.6						
	GUC	36.5	29	24	18.40	15.22	13	11
	GUA	2.5						
	GUG	45.4						
	TOTALS		90	79	47.17 (78%)	42.91 (100%)	37	43

Codon usages for Drosophila are from Shields et al. 1988 (high bias group). [a]Difference expected for complete random codon usage. [b]The expected number of differences corrected for codon bias was calculated according to Henikoff and Eghtedarzadeh (1987); expected number of differences = No. matching residues x $\{1-\Sigma[(0.01 \times \text{bias } \%)^2]\}$. Values obtained from a comparison of *D. melanogaster* UO and *D. pseudoobscura* UO are listed under Dp and values obtained from a comparison of *D. melanogaster* UO and *D. virilis* UO are listed under Dv.

Table A5. Interspecific sequence comparisons of Drosophila genes.

Gene	Species	Reference
alcohol dehydrogenase	*D. melanogaster, D. simulans, D. mauritiana, D. orena,*	Bodmer and Ashburner (1984)
	D. melanogaster, D. affinidisjuncta	Rowan and Dickenson (1988)
	D. mojavensis, D. mulleri	Atkinson et al. (1988)
	D. melanogaster, D. mojavensis, D. affinidisjuncta	Ayer and Benyajati (1990)*
	D. melanogaster, D. orena	Moses et al. (1990)
bicoid	*D. melanogaster, D. pseudobscura*	Seeger and Kaufman (1990)
chorion genes	*D. melanogaster, D. subobscura D. virilis, D. grimshawi*	Martinez-Cruzado et al. (1988)
	D. melanogaster, D. subobscura D. virilis, D. grimshawi	Fenerjian et al. (1989)
	D. melanogaster, D. subobscura, D. virilis, D. grimshawi, Bombyx mori, Antheraea pernyi, Antheraea polyphemus	Mitsialis et al. (1989)
	D. melanogaster, D. silvestris, D. heteroneura, D. planitibia, D. subobscura , D. virilis, . D. grimshawi	Martinez-Cruzado (1990)
	D. melanogaster, D. subobscura, D. virilis, D. grimshawi	Swimmer et al. (1990)
	D. melanogaster, D. subobscura D. virilis, D. grimshawi, Bombyx mori, Antheraea pernyi, Antheraea polyphemus	Shea et al. (1990)*
	D. melanogaster, D. virilis, Ceratitis capitata	Konsolaki et al., (1990)
dopa decarboxylase	*D. melanogaster, D. virilis*	Bray and Hirsh (1986)
	D. melanogaster, D. virilis	Bray et al. (1988)*
	D. melanogaster, D. virilis,	Johnson et al. (1989)*
engrailed	*D. melanogaster, D. virilis*	Kassis et al. (1986)
	D. melanogster, D. virilis	Kassis et al. (1989)*
esterase	*D. melanogaster, D. pseudoobscura*	Brady et al. (1990)
fushi tarazu	*D. melanogaster, D. hydei*	Maier et al. (1990)
gart	*D. melanogaster, D. pseudoobscura*	Henikoff and Eghtedarzadeh (1987)
Glucose dehydrogenase	*D. melanogaster, D. pseudoobscura D. virilis*	Krasney et al. (1990)
hsp82	*D. melanogster , D. simulans, D. pseudoobscura, D. virilis*	Blackman and Meselson (1986)
heat shock locus hsrw	*D. melanogaster, D. pseudoobscura, D. hydei*	Garbe et al. (1989)

Table A5 (cont'd).

hunchback	*D. melanogaster, D. virilis*	Treier et al. (1989)
period locus	*D. melanogaster, D. pseudoobscura, D. virilis*	Colot et al. (1988)
	D. melanogaster, D. yakuba	Thackeray and Kyriacou (1990)
ribosomal protein rp49	*D. melanogaster, D. subobscura*	Aguadé (1988)
glue protein sgs3	*D. melanogaster, D. simulans, D. erecta, D. yakuba*	Martin et al. (1988)
β2 tubulin	*D. melanogaster, D. hydei*	Michiels et al. (1987)
	D. melanogaster, D. hydei	Michiels et al. (1989)*
ultrabithorax	*D. melanogaster, D. pseudoobscura, D. funebris, Musca domestica*	Wilde and Akam (1987)
xanthine dehydrogenase	*D. melanogaster, D. pseudoobscura*	Riley (1989)

* Conserved sequences appear to be cis-acting regulatory elements by one of several functional *in vivo* functional tests and/or *in vitro* protein binding studies.

Table A6. Synthetic Oligonucleotide Primers.

Number	5' to 3' Sequence	Application
1^	TCCTTGCCGTATCCG	sequence Dm UO
2^	TAGCACCGTAGTGGA	sequence Dm UO
3^	TCAACTTCGATACGA	sequence Dm UO
4^	TACATATATAACTTCGCCGCATGCAATTA	hybridize to *ecd*[1] UO mRNA
5^	GTTGAGTTTCTGGAATAATC	sequence Dm UO
9^*	TGATACGGCTTACCATGCTCATCCATGCCG	primer extension experiment
13*	CGGAGGGCGAGGAGACG	sequence Dp UO
14*	GGGCTGTCTTTCTTG	sequence Dp UO
15*	TCGTAGATAGCAGGTGAGAT	sequence Dv UO
16	AGAGGCGGTTTGCGTAT	sequence Dv UO
17*	GGTATAGTGCCCTATTA	sequence Dp UO
18	AAACATTGTGACTGCATGATACTGATGATGCC	deletion of DR element at +11
19	TGGGAAATCGTATATCAGAGTATTAAAGGTCTA	deletion of DR element at -138
20	CAGATGGATTCGACTGAGTTGGCATAGCAACTGC	deletion of Dm UO -808 to -703
21	TTCACGCACGAGTCACTCAGATGGATTCGACTGA	deletion of Dm UO -697 to -452
22	TACAGATCACATCTCTG	sequence Dm UO
23	CGTAGGAGATAAGTCGCTTCACGCACGAGTCACT	deletion of Dm UO -434 to -302
24	TTCACACTCATCTAACC	sequence Dm UO
25	AGAGTATTAAAGGTCTACGTAGGAGATAAGTCGC	deletion of Dm UO -302 to -156

* Designated P2 in Chapter One
^ purified by HPLC
* purified by OPC column
The *D. melanogaster* UO, *D. pseudoobscura* UO and *D. virilis* UO1 genes are abbreviated Dm, Dp and Dv, respectively.

Table A7. P-element transformed lines and expression patterns of the UO transgenes

Transformant line	Construct	UO expression 3L	P	A	Mt
1F13B		+	-	+	+
5F15C		+	-	+	+
1F30A	P[($w^{+\Delta}$)DmUOPstI]	+	-	+	+
1M11D		+	-	+	+
3M11		+	-	+	+
1M21A		+	-	+	ND
3M9	P[(hspw^{+})DmUOSpeI-StuI]	-	-	-	-
1M43		-	-	-	-
1F22A		+	-	+	+
3F47A		+	-	+	+
4F53A	P[($w^{+\Delta}$)DpUORI]	+	-	+	+
4M15A		+	-	+	+
3M26A		+	-	+	+
3F6A		+	-	+	+
1F9A		+	-	+	+
2F15A	P[($w^{+\Delta}$)DvUO1PstI]	+	-	+	+
1F19A		+	-	+	+
3M1A		+	-	+	+
2F5B		+	+	+	-
1F10A	P[($w^{+\Delta}$)DvUO2PstI]	+	+	+	-
4F13D		+	+	+	-
3F6D		+	-	+	+
4F14	P[($w^{+\Delta}$)DmUO-lacZ]	+	-	+	+
1F17		+	-	+	+
2F5	P[(w+Δ)del(-138,-126)DmUO-lacZ]	+	-	+	+
2F26		+	-	+	+
9M23		-	-	p	+
1M101	P[($w^{+\Delta}$)del(+11,+23)DmUO-lacZ]	-	-	p	+
1M118		-	-	p	+
1M161		-	-	p	+
4F10		-	-	p	+
2F28		-	-	p	+
2F34	P[($w^{+\Delta}$)del(-138,-126)(+11,+23)DmUO-lacZ]	-	-	p	+
1M1		-	-	p	+
2M23		-	-	+	+

UO mRNA or UO-lacZ expression detected (+) or not detected (-) by Northern analyses or histochemical staining for β-galactosidase activity. p=perturbed expression of the UO-lacZ transgene with only some adult flies within the stock showing β-galactosidase activity. ND=not determined,3L=third instar larvae, P=pupae, A=adults and Mt=Malpighian tubules.

Table A8. List of publications from this work.

Wallrath, L.L., J.B. Burnett and T.B. Friedman (1990). Molecular characterization of the *Drosophila melanogaster* urate oxidase gene: An ecdysone-repressible gene expressed only in the Malpighian tubules. Mol. Cell. Biol. **10**: 5114-5127.

Wallrath, L.L. and T.B. Friedman (1991). Species differences in the temporal pattern of Drosophila UO gene expression are attributed to trans-acting regulatory differences. Proc. Natl. Acad. Sci. USA (in press).

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