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Molecular Characterization of the *Drosophila melanogaster* Urate Oxidase Gene, an Ecdysone-Repressible Gene Expressed Only in the Malpighian Tubules

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The urate oxidase (UO) gene of *Drosophila melanogaster* is expressed during the third-instar larval and adult stages, exclusively within a subset of cells of the Malpighian tubules. The UO gene contains a 69-base-pair intron and encodes mature mRNAs of 1,224, 1,227, and 1,244 nucleotides, depending on the site of 3′ endonucleolytic cleavage prior to polyadenylation. A direct repeat, 5′-AAGTGAGAGTGAT-3′, is the proposed *cis*-regulatory element involved in 20-hydroxyecdysone repression of the UO gene. The deduced amino acid sequences of UO of *D. melanogaster*, rat, mouse, and pig and uricase II of soybean show 32 to 38% identity, with 22% of amino acid residues identical in all six species. With use of P-element-mediated germ line transformation, 826 base pairs 5′ and approximately 1,200 base pairs 3′ of the *D. melanogaster* UO transcribed region contain all of the *cis* elements allowing for appropriate temporal regulation and Malpighian tubule-specific expression of the UO gene.

In *Drosophila melanogaster*, particular purines serve as precursors of nucleotides and pterins (35, 62). Excess purine is converted to uric acid, which is either stored, excreted, or catabolized, depending on the developmental stage. Uric acid in the third-instar larva and adult is converted by urate oxidase (UO) to allantoin and then excreted by the Malpighian tubules (19, 40). The gene encoding UO is transcribed exclusively within the cells of the Malpighian tubules. UO mRNA is not detected by Northern (RNA) analysis until the beginning of the third-instar larval stage; by the middle of this stage, UO mRNA represents approximately 1% of the total poly(A)$^+$ RNA of the Malpighian tubules (40). 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 (41). 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 after emergence of the adult (40).

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 *Drosophila*, strains *ry*^{2} (3-52.0; 24) and *ma-l* (1-64.8; 9), which have 5- to 10-fold-higher levels of UO mRNA and UO protein in the adult than in the wild-type adult. High levels of UO-inducing factor in the hemolymph of a xanthine dehydrogenase-deficient adult stimulated a 5- to 10-fold increase in UO activity in a wild-type Malpighian tubule transplanted into the abdomen of a xanthine dehydrogenase-deficient adult (19, 21, 41). 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 (21, 22).

Identification of the *cis*-regulatory elements and specific *trans*-acting factors which govern the elaborate pattern of regulation of the *D. melanogaster* UO gene was initiated with the isolation of UO cDNA clones and UO genomic clones. Herein we present a molecular characterization of the UO gene of *D. melanogaster* which includes (i) the sequence of the UO gene and flanking DNA, (ii) the transcription start site of the UO gene, (iii) tissue in situ hybridizations which identify the specific population of cells of the Malpighian tubules that express UO mRNA, (iv) comparison of the deduced amino acid sequence of *D. melanogaster* UO with sequences of plant and animal UO enzymes, and (v) the amount of UO flanking DNA sufficient for appropriate temporal and tissue-specific expression of the UO gene.

MATERIALS AND METHODS

UO cDNA and UO genomic clones. *D. melanogaster* UO cDNAs were isolated from an Ore-R third-instar larval λgt10 library (gift of T. Kornberg; 66) and from our third-instar larval Malpighian tubule library (40) constructed from *ecd*^{1}, a temperature-sensitive 20-hydroxyecdysone-deficient mutant (45), which has a wild-type pattern of UO gene expression at the permissive temperature of 19°C. A UO cDNA probe, cUO2 (designated pcDNAUO2 in reference 40), was used to isolate overlapping genomic clones from a *D. melanogaster* Canton-S λCharon 4A library (51). A UO genomic clone from the *D. melanogaster ecd*^{1} strain was isolated by screening a *Hin*dIII size-limited pBR322 library with cUO2. UO genomic clones were also isolated from a λEMBL4 *Drosophila pseudoobscura* AH133 library (gift of S. Schaffer) and from a λEMBL3 *Drosophila virilis* library (gift of R. Blackman), using cUO2 as a probe.

Southern analysis. High-molecular-weight genomic DNA used for Southern hybridization was isolated according to Bender et al. (3) and purified on an Elutip (Schleicher &

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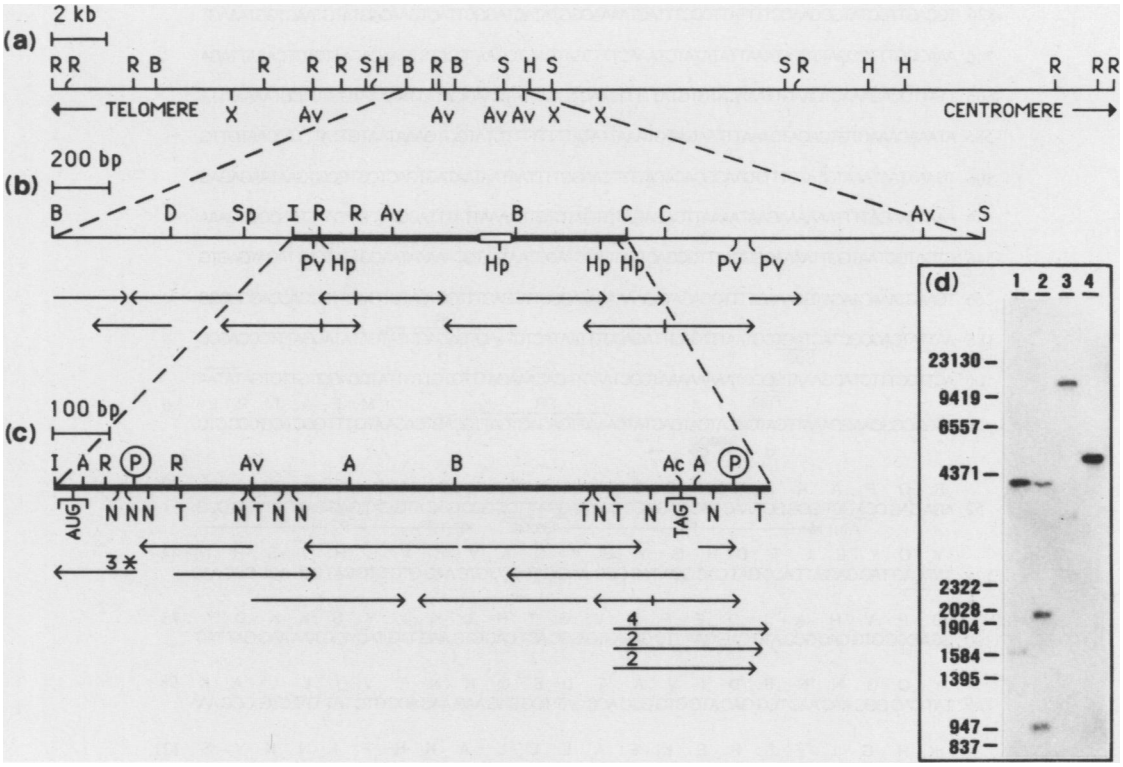
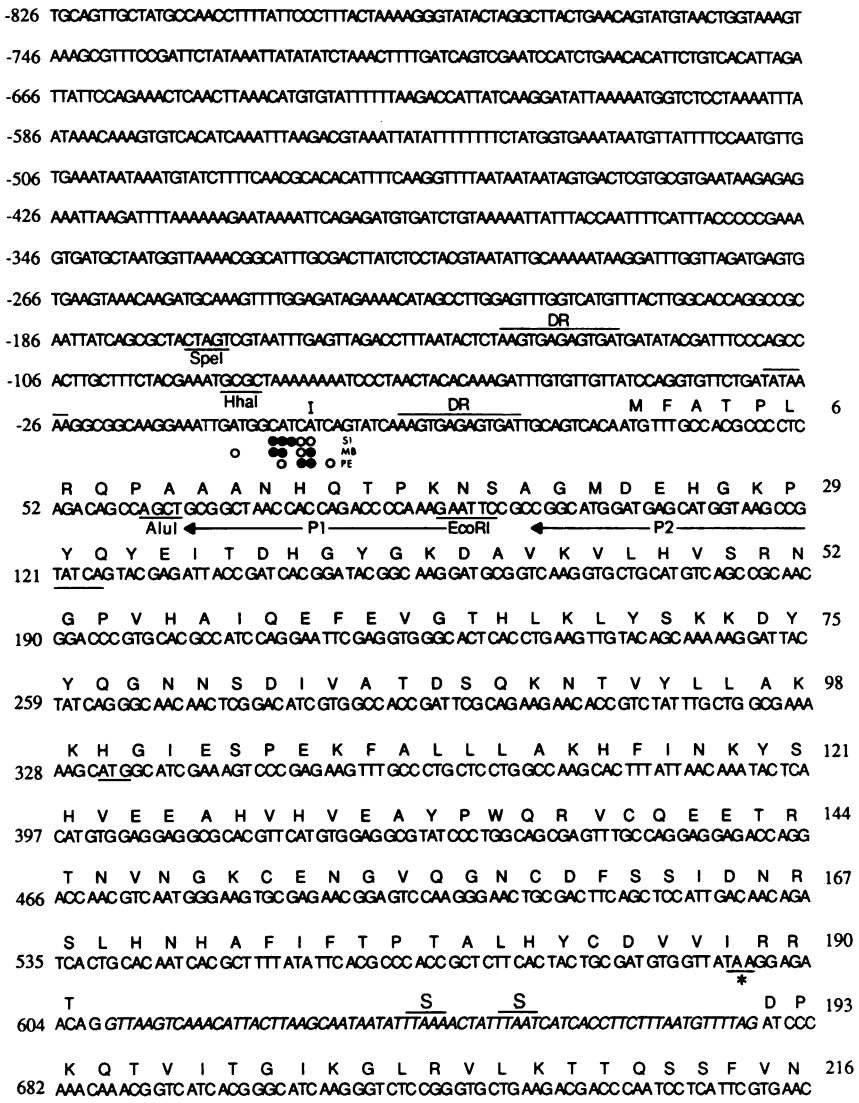


FIG. 1. Restriction map and sequencing strategy for the UO genomic region and 12 independently arising UO cDNAs. (a) Restriction map of 38 kb of genomic DNA including the Canton-S UO gene. (b) Restriction map of the UO gene and flanking DNA, with the directions and regions sequenced designated by arrows. 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 box indicating the 69-base-pair UO intron. (c) Composite restriction map and sequencing strategy of 12 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 positions of the UO translation start and stop sites, respectively. (d) 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 *Hin*dIII (lane 4) was probed with a 5.5-kb *Hin*dIII restriction fragment containing the UO gene. A, *Alu*I; Ac, *Acc*I; Av, *Ava*I; B, *Bgl*II; C, *Cla*I; D, *Dra*I; H, *Hin*dIII; Hp, *Hpa*II; *N*, *Nla*III; Pv, *Pvu*II; R, *Eco*RI; S, *Sal*I; Sp, *Spe*I; T, *Taq*I; X, *Xho*II.

Schuell, Inc.). DNA digested with restriction enzymes was size fractionated in an agarose gel and transferred onto nylon membrane (Hybond-N; Amersham Corp.) with 6× SSC (SSC is 0.1 M NaCl plus 0.015 M sodium citrate). The Southern blot was prehybridized at 42°C overnight in 50% formamide–50 mg of sheared salmon sperm DNA per ml– 0.1 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.04)–0.1 M NaCl–0.1% Sarkosyl–0.1% Ficoll–0.1% polyvinylpyrrolidone 40–0.1% bovine serum albumin. Modified prehybridization solution with 40% formamide and 10% dextran sulfate served as the hybridization mixture. The *Hin*dIII genomic fragment containing the UO gene (Fig. 1a) used as a probe for Southern analysis was random primer labeled with [α-^{32}P]dATP to a specific activity of 4.6×10^8 cpm/μg by the method of Feinberg and Vogelstein (17), and 2×10^7 cpm was incubated with the blot at 42°C overnight in 20 ml of hybridization solution. After hybridization, the blot was washed at 50°C for 2 h in two to four washes of 0.1 × SSC–0.05% *N*-laurylsarcosine–0.02% sodium pyrophosphate and then autoradiographed. For the isolation of UO clones from the *D. pseudoobscura* and *D. virilis* libraries, the stringency of the hybridization conditions was reduced by using 35% formamide and 42°C washes for *D. pseudoobscura* and by using 40°C as the hybridization and wash temperature for *D. virilis*.

Northern analysis. Total RNA used in Northern analysis of transformed files was prepared by grinding 30 animals from various stages of development, 25 hand-dissected Malpighian tubules, or the remaining tissue from the animals in a 1.5-ml Eppendorf microfuge tube, using a ground glass homogenizer and 450 μl of extraction buffer (3 M LiCl, 6 M urea, 10 mM sodium acetate [NaOAc; pH 5.0], 0.2 mg of heparin per ml, 0.1% sodium dodecyl sulfate). The RNA was precipitated in the grinding buffer for 1 to 8 h at 4°C and centrifuged for 15 min. The pellet was washed with 100 μl of wash buffer (4 M LiCl, 8 M urea), centrifuged for 15 min, suspended in 100 μl of 0.1 M NaOAc (pH 5.0)–0.1% sodium dodecyl sulfate, extracted with 100 μl of phenol-chloroform and then with 100 μl of chloroform, and precipitated for 4 to 8 h at −20°C with 2 volumes of ethanol in the presence of 0.2 M NaOAc. This RNA extraction procedure is a variation of a published protocol (71). RNA was recovered by centrifugation for 15 min, washed with 70% ethanol, vacuum dried, dissolved in 74% formamide–26% formaldehyde, denatured for 5 min at 65°C, and loaded onto an agarose-formaldehyde gel. RNA, size fractionated in an agarose-formaldehyde gel, was blotted onto nylon membrane (Hybond-N; Amersham) with 10× SSC. The Northern blot was prehybridized and hybridized in solutions identical to those of Wood et al. (82).

The oligonucleotide (5′-TAATTGCATGCGGCGAAGT



TATATAT GTA-3') 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 1.3×10^8 cpm/μg with [γ-^{32}P]dATP. After 6 hours of prehybridization at 42°C, the Northern blot was hybridized for 3 days at 50°C in 20 ml of hybridization solution with 1.6×10^7 cpm of the end-labeled oligonucleotide. Since the oligonucleotide probe is 66% A+T, the Northern blot was washed at 50°C according to Wood et al. (82) in the presence of tetramethylammonium chloride, which raises the melting temperature of A · T base pairs to that of G · C base pairs. To confirm the presence of RNA in each lane, the Northern blot was stripped and reprobed with 1.5×10^7 cpm of a *Hin*dIII-*Pst*I restriction fragment containing a portion of the *D. melanogaster ras* gene (59), which was random primer labeled (17) to a specific activity of 8.6×10^8 cpm/μg. The Northern blot was hybridized and washed, using the procedure described above for the Southern blot.

Sequencing. Restriction fragments containing the UO transcription unit were cloned into the single-stranded bacteriophage vectors M13mp18 and M13mp19 and sequenced according to Sanger et al. (74), using [α-^{35}S]dATP and DNA polymerase I, Klenow fragment (Bethesda Research Laboratories, Inc.), or avian myeloblastosis virus (AMV) reverse transcriptase (Bio-Rad Laboratories). Figures 1b and c show the sequencing strategy for the UO genomic and cDNA clones. All restriction sites used in constructing M13 subclones were verified by sequencing across that same restriction site present within other subclones. Each subclone was sequenced by using high and low concentrations of dideoxynucleotides, and the DNA was separated on 8 and 6% polyacrylamide sequencing gels, respectively. The majority of the sequencing data was derived from both strands of the UO genomic DNA and UO cDNA. Many subclones were sequenced twice, some by using 7-deaza-2′-deoxyguanosine-5′-triphosphate as a substitute for dGTP (Boehringer Mannheim Biochemicals).

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 T4 polynucleotide kinase and [γ-^{32}P]dATP (50). 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 (Fig. 3c), was

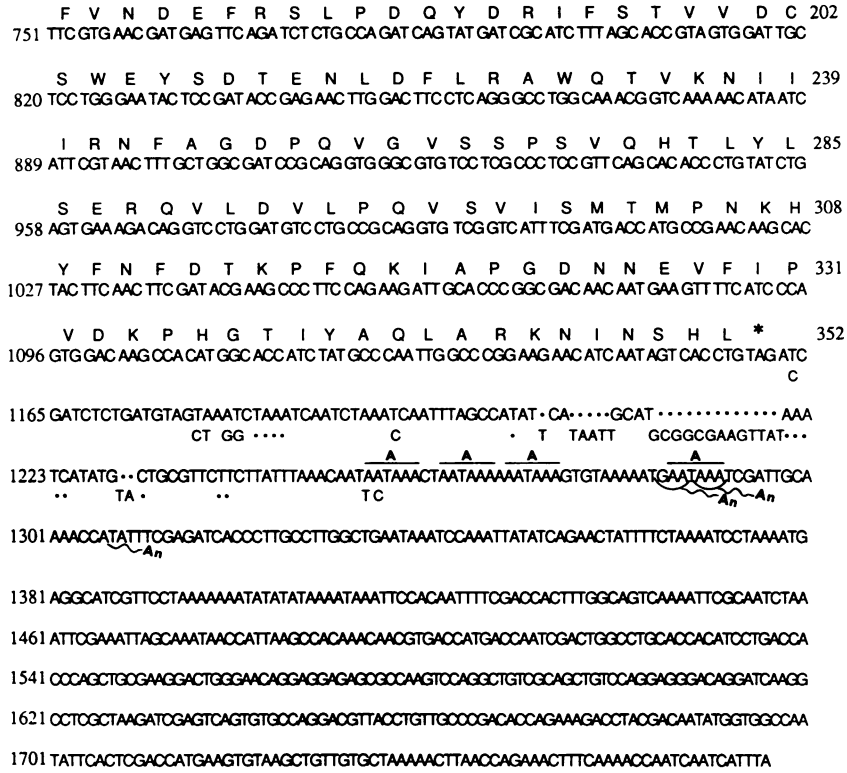


FIG. 2. Sequence of the *D. melanogaster* Canton-S UO gene and flanking DNA and the deduced amino acid sequence of the UO protein. The DNA sequence is numbered in the left margin, with +1 at transcription start (I). The deduced amino acid sequence is numbered in the right margin beginning with the translation 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. 3a), and primer extension experiments (PE) (Fig. 3b and d) are schematically represented by closed circles 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. 3c). 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 GenBank/EMBL protein banks. The sequence of the 69-base-pair UO intron in italics has 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 nucleotides 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.

isolated. Total RNA was extracted with guanidine hydrochloride from whole third-instar larvae and adults (42). Poly(A)$^+$ RNA was isolated on an oligo(dT)-cellulose column (Pharmacia). Approximately 10^5 cpm of probe S and 20 μg of poly(A)$^+$ RNA from Ore-R third-instar larvae, 10 μg of poly(A)$^+$ RNA from Ore-R adults (data not shown), or 10 μg of poly(A)$^+$ RNA from ry^2 12-h adults was dissolved in 40 μl of 80% formamide–40 mM PIPES (pH 6.4)–400 mM NaCl–1 mM EDTA, boiled for 10 min, and allowed to hybridize for approximately 12 h at 42°C. After the annealing step, 350 μl of S1 reaction buffer (11) was added to each sample along with 50 U of S1 nuclease (Boehringer Mannheim) or 150 U of mung bean nuclease (Pharmacia), and the samples were incubated at 37°C for 1 h. 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 (60). 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 min 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 (Boehringer Mannheim) and treated as described above.

Primer extension analyses. Primer extension was performed by digesting 1 μg of the *Spe*I-*Eco*RI restriction fragment (Fig. 2) with *Alu*I endonuclease and end labeling the resulting restriction fragments with T4 polynucleotide kinase and [γ-^{32}P]dATP (50). The mixture of end-labeled fragments was separated on an 8% sequencing gel, and the 30-nucleotide strand complementary to UO mRNA, designated P1 (Fig. 3c), was purified. Approximately 4×10^4 cpm of the labeled P1 was added to either 5 or 6 μg of poly(A)$^+$ RNA from Ore-R third-instar larvae, from ry^2 12-h adults, or from Ore-R 12-h 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 (30), placed at 65°C for 1 min, incubated at 37°C for 1 h. The hybridization products were ethanol precipitated and dissolved in reaction buffer (30) containing fresh ultrapure deoxynucleoside triphosphates (Pharmacia) and 10 U of AMV reverse transcriptase (Pharmacia) and incubated at

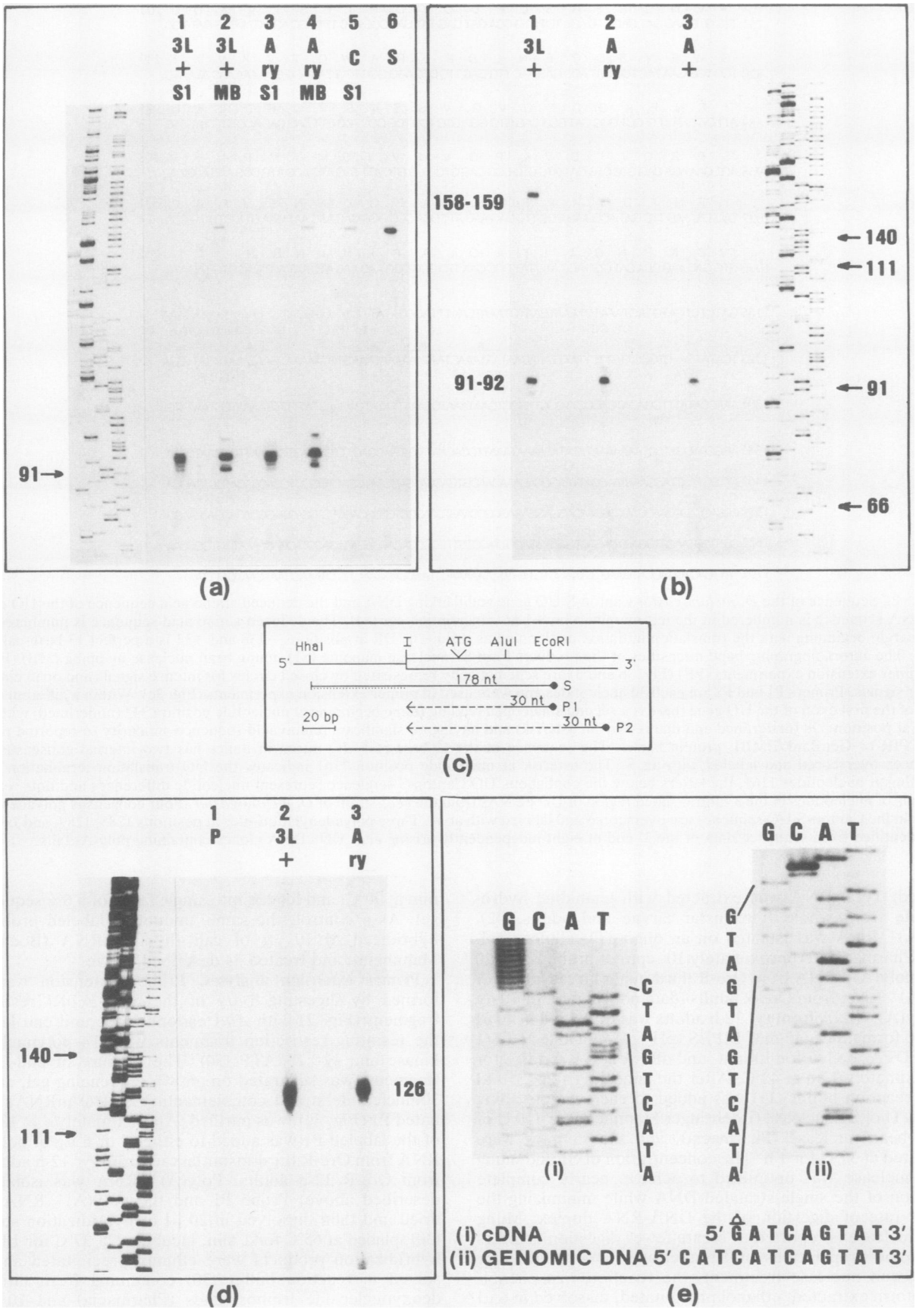

1 3L + S1
2 3L + MB
3 A ry S1
4 A ry MB
5 C S1
6 S
91
(a)
1 3L +
2 A ry
3 A +
158-159
91-92
140
111
91
66
(b)
HhaI
I
ATG
AluI
EcoRI
5'
3'
178 nt
S
30 nt
P1
20 bp
30 nt
P2
(c)
1 P
2 3L +
3 A ry
140
111
126
(d)
G C A T
C T A G T C A T A
(i)
G T A G T A G T C A T A
(ii)
(i) cDNA 5' GATCAGTAT 3'
(ii) GENOMIC DNA 5' CATCATCAGTAT 3'
(e)

FIG. 3. Mapping the transcription start site for the UO gene of *D. melanogaster*. (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-h adults (A, ry; lanes 3 and 4). DNA-RNA hybrids were digested with 50 U of S1 nuclease in lanes 1 and 3 and 400 U 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 U 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 a 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, and 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-h adults (A, ry; lane 2), or 6 μg of poly(A)$^+$ RNA from Ore-R 12-h 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 and mung bean nuclease mapping, placing transcription initiation at position +1 (I; Fig. 2). The origin of the 158-nucleotide product is discussed in 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. 2) was determined to be the transcription initiation site for *D. melanogaster* UO mRNA. (d) P2 (panel c and Fig. 2), 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. 2), 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).

42°C for 1 h. The extension products were phenol-chloroform extracted, ethanol precipitated, vacuum dried, dissolved in 6 μl of standard DNA sequencing loading dye, denatured for 3 min 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 (Fig. 2 and 3c). Hybridization and extension reactions were performed as described above, using 4×10^4 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-h 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.

Tissue in situ hybridizations. Abdomens from ry^2 12-h adults were frozen, sectioned, and hybridized to sense and antisense UO RNA probes according to Raikhel et al. (69). The RNA hybridization probes were generated by transcription in the presence of [α-^{35}S]dUTP from the T7 and T3 promoters of pBluescript KS(+) (Stratagene) containing the *Eco*RI-*Acc*I restriction fragment of cUO2 (Fig. 1c). Full-length transcripts, confirmed by Northern analyses, were hydrolyzed for 30 min in the presence of 0.1 M $NaHCO_3$ 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. (69). There was no significant difference in the total number of cells (480 ± 42) of the Malpighian tubules of Ore-R and ry^2 adults as determined by counting Feulgen-stained nuclei in Malpighian tubule whole-mounts (data not shown).

Malpighian tubules were hand dissected in *Drosophila* Ringer solution (79), permeabilized, and fixed according to a procedure developed for *Drosophila* embryos (16). 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 cover slip treated with Sigmacote (SL-2; Sigma), and flattened by pressing firmly on the cover slip. The cover slip was then removed by rinsing in 95% ethanol. Autoradiography was performed according to Raikhel et al. (69).

Amino acid comparisons. The deduced amino acid sequences of UO from *D. melanogaster*, soybean (61), rat (70), mouse, baboon, and pig (83) were compared by using the program FASTP (48). Each of the six deduced amino acid sequences was divided into approximately three equal parts, and all pairwise comparisons were made. The FASTP algorithm will only detect and optimize an alignment with the insertion of gaps in a single region established by the initial alignment (48).

P-element germ line transformation. A 3.2-kilobase (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 reporter gene (64). The *Pst*I sites are at position -826 and approximately 1.2 kb downstream of the UO transcribed region. The resulting UO P-element construct was coinjected with the helper P-element pπ25.7 according to Rubin and Spradling (72) into white-deficient embryos, Df(1)w,yw67$^{c23(2)}$, which were homozygous for the second chromosome from the wild-type *D. melanogaster* Canton-S strain. The sequence of the UO gene on the Canton-S second chromosome contains several nucleotide differences in the 3′ untranslated region from that of the UO gene isolated from a stock containing the temperature-sensitive 20-hydroxyecdysone mutation, ecd^1 (Fig. 2). This sequence difference allowed 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 above). All transformants were made homozygous for a single P-element insertion.

Nucleotide sequence accession number. The sequence reported has been assigned EMBL accession number X51940.

RESULTS

DNA sequence of the UO gene. The restriction maps and strategies used to sequence *D. melanogaster* UO genomic DNA and cDNA clones are diagrammed in Fig. 1. Genomic DNA from *D. melanogaster* Canton-S was restricted with four different endonucleases and probed with the 5.5-kb *Hin*dIII restriction fragment containing the UO gene (Fig. 1a). The genomic restriction fragments that hybridized to the 5.5-kb *Hin*dIII DNA probe were equivalent in size to the restriction fragments of the cloned UO gene (Fig. 1d),

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 (Fig. 1d) and Ore-R as well as in several mutant strains (data not shown). In contrast to the single-copy UO gene of *D. melanogaster*, there is a tandem duplication of the UO gene in some strains of *D. virilis* (S. Lootens et al., unpublished data). 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 Fig. 2. Several strain-specific nucleotide differences, deletions, and additions in the 3′ transcribed but untranslated region of the UO gene are located between positions +1179 and +1255 (Fig. 2).

The UO 5′-flanking genomic sequence contains a 13-base-pair direct repeat (DR; AAGTGAGAGTGAT, at −138 and +11; Fig. 2). 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 the sequence of UO from a plant and several vertebrates. Within the protein-coding region of the UO gene there is a 69-base-pair intron that has GU-AG consensus splice junctions (58) and two 3′ intronic splice signal sequences, TTAAA and TTAAT (overscored and labeled with an S; Fig. 2) 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 (37).

Within the 3′ untranslated region of the UO gene from Canton-S are four AATAAA elements (overscored and labeled with an A; Fig. 2), identical to the consensus polyadenylation sequence of Proudfoot and Brownlee (67), which are just upstream of the three polyadenylation sites (An in Fig. 2). Comparisons of UO genomic clones from a *D. melanogaster* Canton-S library with UO cDNA clones from a *D. melanogaster* ecd^1 Malpighian tubule library revealed many sequence differences in the 3′ transcribed but untranslated region. Two of these nucleotide changes in the UO gene from the ecd^1 strain at positions 1254 and 1255 (Fig. 2) 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 (Fig. 2). Based on the sequence data from eight ecd^1 UO cDNA clones, the UO gene gives rise to messages, after 3′ endonucleolytic cleavage but before polyadenylation, of 1,224, 1,227, and 1,244 nucleotides, which is consistent with Northern analyses showing UO poly(A)$^+$ RNA to be approximately 1,400 nucleotides (40). Whether endonucleolytic cleavage and polyadenylation occur 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 (4).

Transcription initiation of the UO gene. Two possible overlapping transcription initiation sequences (ATCATCA, −3; Fig. 2) and (ATCAGTA, +1; Fig. 2) were first identified upstream of the UO translation initiation codon by their resemblance to a *D. melanogaster* transcription initiation consensus sequence ATCA(G/T)T(C/T) (31). The transcription initiation site (+1; Fig. 2) for the *D. melanogaster* UO gene was confirmed by three independent experimental procedures: (i) S1 nuclease and mung bean nuclease mapping, (ii) primer extension analyses, and (iii) 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-h adults that were hybridized to single-stranded probe S (Fig. 3c). 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 (Fig. 3a). Protected products of 91, 94, and 95 nucleotides and a minor protected product of 92 nucleotides were observed with mung bean nuclease. Such microheterogeneity in S1 and mung bean nuclease mapping has been reported to be an artifact of incomplete or overdigestion with S1 nuclease or the interference of a cap G (8, 29, 77, 80).

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 (Fig. 2 and 3c) 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 poly(A)$^+$ RNA from Ore-R third-instar larvae and ry^2 12-h adults was used (Fig. 3b). 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 Fig. 2. The lengths of the primer extension products, with 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 the P2, a synthetic primer, just downstream of P1 (Fig. 2 and Fig. 3c). Primer P2 was hybridized to poly(A)$^+$ RNAs from Ore-R third-instar larvae and ry^2 12-h adults, both sources having high levels of UO mRNA. Only a single extension product of 126 nucleotides resulted (Fig. 3d), which also maps the transcription start site of the UO gene to position +1 (Fig. 2).

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 (Fig. 1c). 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 (Fig. 3e). No UO cDNAs were isolated that 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 (Fig. 2), 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 (Fig. 1c). The second class of cDNAs hybridized to the *Eco*RI-*Spe*I probe but not to cUO2 and contained cDNAs of approximately 3,800 base pairs in length with restriction maps unlike that of the UO. This latter class of cDNAs may encode the message

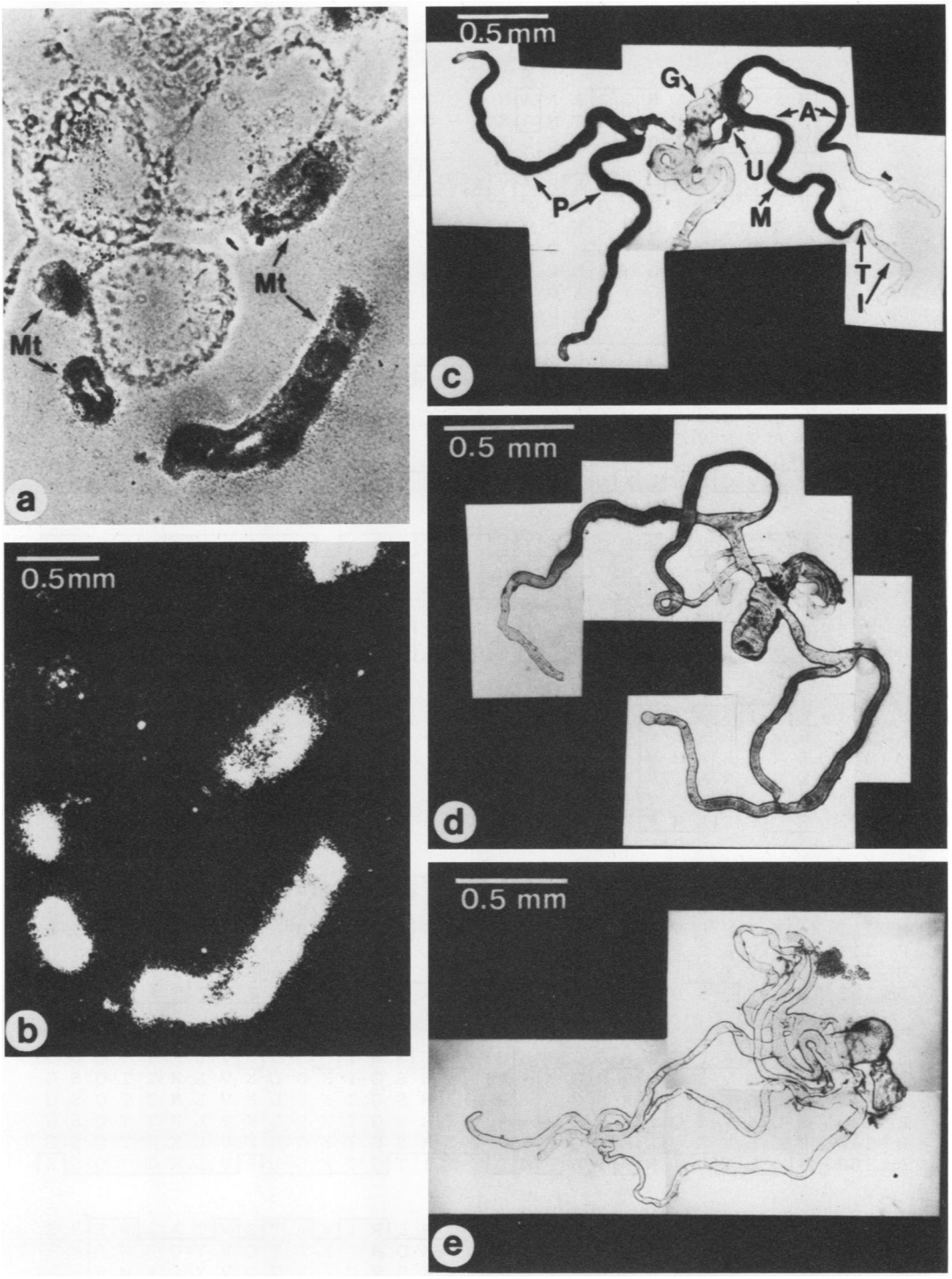
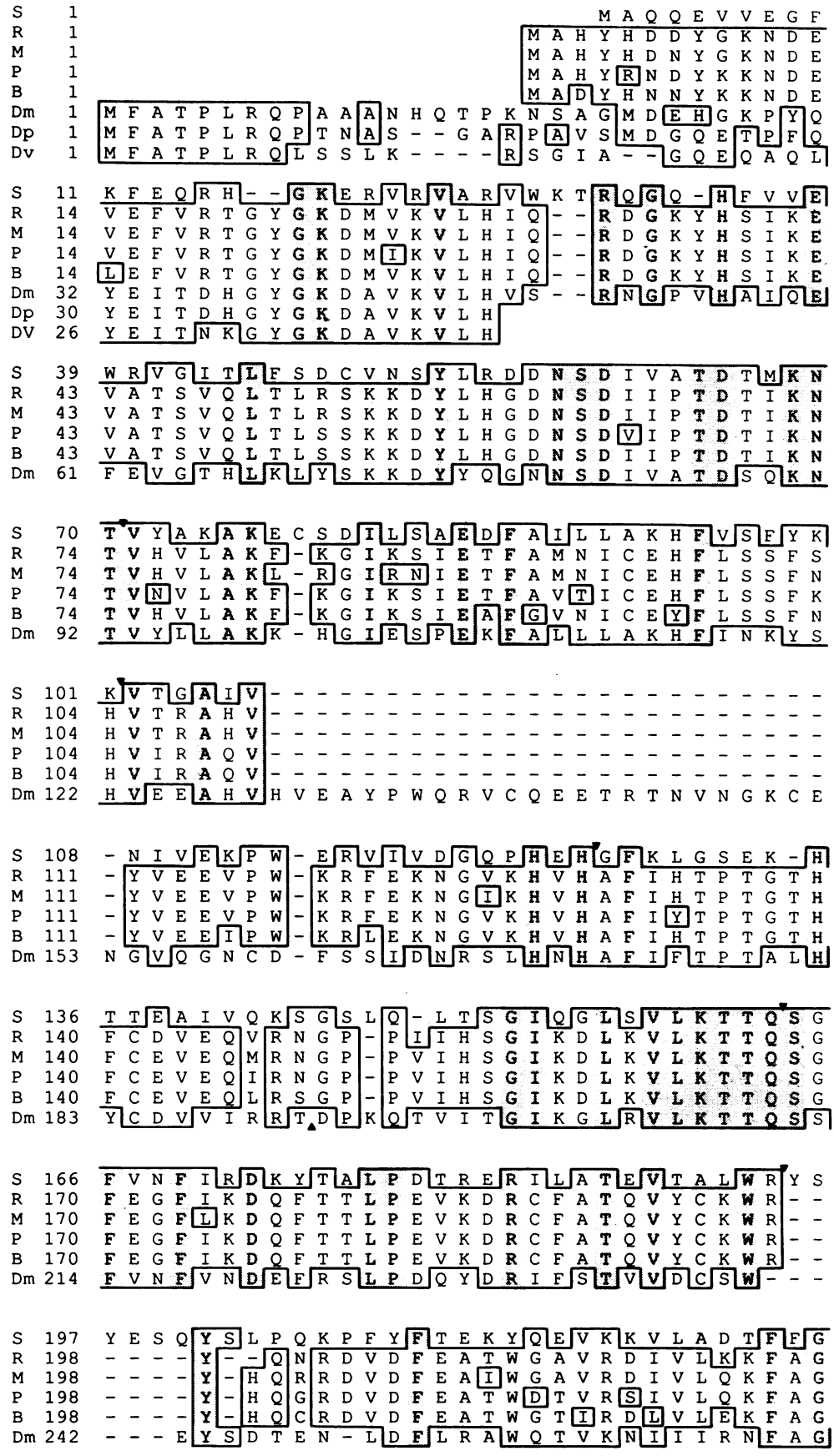


FIG. 4. Spatial distribution of UO mRNA among the cells that comprise the Malpighian tubules from the adult. Panels a and b show phase-contrast (a) and dark-field illumination (b) of the autoradiographic image of a sectioned ry^2 12-h adult abdomen hybridized to a UO antisense ^{35}S-labeled RNA probe synthesized from the *Eco*RI-*Acc*I restriction fragment of cUO2 (Fig. 1c). Hybridization signals were detected exclusively within the Malpighian tubules (Mt). (c) Bright-field images of whole-mount Malpighian tubules from a ry^2 12-h 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 opaque material within the preparations also present in the control sense-strand in situ hybridizations (e). (d) Whole Malpighian tubules from an Ore-R 12-h adult were hybridized to the antisense ^{35}S-labeled UO RNA probe used for panels a and b. (e) No detectable signal was present when ry^2 12-h adult Malpighian tubules were hybridized to a sense ^{35}S-labeled UO RNA probe.


S 1 M A Q Q E V V E G F
R 1 M A H Y H D D Y G K N D E
M 1 M A H Y H D N Y G K N D E
P 1 M A H Y R N D Y K K N D E
B 1 M A D Y H N N Y K K N D E
Dm 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
Dp 1 M F A T P L R Q P T N A S - - G A R P A V S M D G Q E T P F Q
Dv 1 M F A T P L R Q L S S L K - - - - R S G I A - - G Q E Q A Q L
S 11 K F E Q R H - - G K E R V R V A R V W K T R Q G Q - H F V V E
R 14 V E F V R T G Y G K D M V K V L H I Q - - R D G K Y H S I K E
M 14 V E F V R T G Y G K D M V K V L H I Q - - R D G K Y H S I K E
P 14 V E F V R T G Y G K D M I K V L H I Q - - R D G K Y H S I K E
B 14 L E F V R T G Y G K D M V K V L H I Q - - R D G K Y H S I K E
Dm 32 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
Dp 30 Y E I T D H G Y G K D A V K V L H
DV 26 Y E I T N K G Y G K D A V K V L H
S 39 W R V G I T L F S D C V N S Y L R D D N S D I V A T D T M K N
R 43 V A T S V Q L T L R S K K D Y L H G D N S D I I P T D T I K N
M 43 V A T S V Q L T L R S K K D Y L H G D N S D I I P T D T I K N
P 43 V A T S V Q L T L S S K K D Y L H G D N S D V I P T D T I K N
B 43 V A T S V Q L T L S S K K D Y L H G D N S D I I P T D T I K N
Dm 61 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
S 70 T V Y A K A K E C S D I L S A E D F A I L L A K H F V S F Y K
R 74 T V H V L A K F - K G I K S I E T F A M N I C E H F L S S F S
M 74 T V H V L A K L - R G I R N I E T F A M N I C E H F L S S F N
P 74 T V N V L A K F - K G I K S I E T F A V T I C E H F L S S F K
B 74 T V H V L A K F - K G I K S I E A F G V N I C E Y F L S S F N
Dm 92 T V Y L L A K K - H G I E S P E K F A L L L A K H F I N K Y S
S 101 K V T G A I V -
R 104 H V T R A H V -
M 104 H V T R A H V -
P 104 H V I R A Q V -
B 104 H V I R A Q V -
Dm 122 H V E E A H V H V E A Y P W Q R V C Q E E T R T N V N G K C E
S 108 - N I V E K P W - E R V I V D G Q P H E H G F K L G S E K - H
R 111 - Y V E E V P W - K R F E K N G V K H V H A F I H T P T G T H
M 111 - Y V E E V P W - K R F E K N G I K H V H A F I H T P T G T H
P 111 - Y V E E V P W - K R F E K N G V K H V H A F I Y T P T G T H
B 111 - Y V E E I P W - K R L E K N G V K H V H A F I H T P T G T H
Dm 153 N G V Q G N C D - F S S I D N R S L H N H A F I F T P T A L H
S 136 T T E A I V Q K S G S L Q - L T S G I Q G L S V L K T T Q S G
R 140 F C D V E Q V R N G P - P I I H S G I K D L K V L K T T Q S G
M 140 F C E V E Q M R N G P - P V I H S G I K D L K V L K T T Q S G
P 140 F C E V E Q I R N G P - P V I H S G I K D L K V L K T T Q S G
B 140 F C E V E Q L R S G P - P V I H S G I K D L K V L K T T Q S G
Dm 183 Y C D V V I R R T D P K Q T V I T G I K G L R V L K T T Q S S
S 166 F V N F I R D K Y T A L P D T R E R I L A T E V T A L W R Y S
R 170 F E G F I K D Q F T T L P E V K D R C F A T Q V Y C K W R - -
M 170 F E G F L K D Q F T T L P E V K D R C F A T Q V Y C K W R - -
P 170 F E G F I K D Q F T T L P E V K D R C F A T Q V Y C K W R - -
B 170 F E G F I K D Q F T T L P E V K D R C F A T Q V Y C K W R - -
Dm 214 F V N F V N D E F R S L P D Q Y D R I F S T V V D C S W - - -
S 197 Y E S Q Y S L P Q K P F Y F T E K Y Q E V K K V L A D T F F G
R 198 - - - - Y - - Q N R D V D F E A T W G A V R D I V L K K F A G
M 198 - - - - Y - H Q R R D V D F E A I W G A V R D I V L Q K F A G
P 198 - - - - Y - H Q G R D V D F E A T W D T V R S I V L Q K F A G
B 198 - - - - Y - H Q C R D V D F E A T W G T I R D L V L E K F A G
Dm 242 - - - E Y S D T E N - L D F L R A W Q T V K N I I I R N F A G

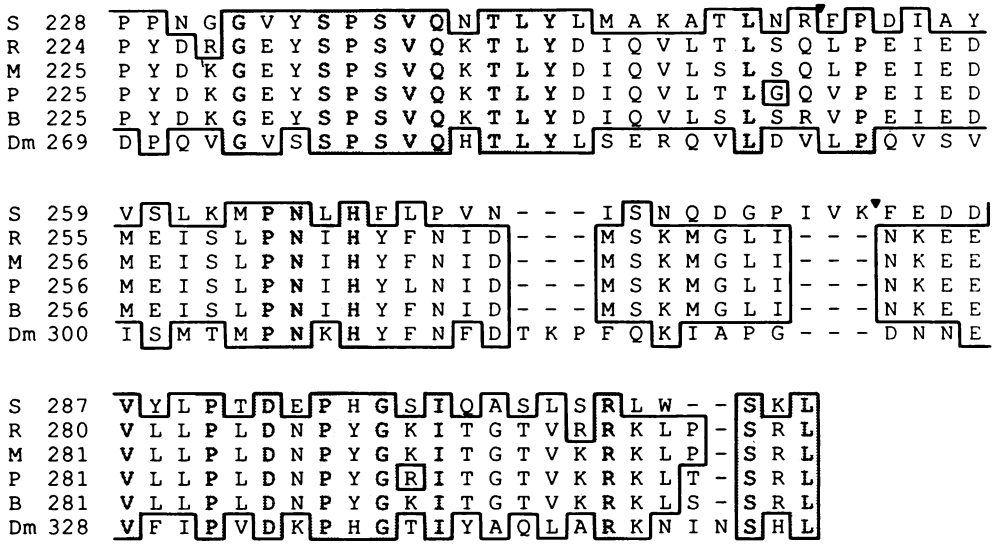


FIG. 5. Alignment of the deduced amino acid sequences of UO from *D. melanogaster* (Dm), soybean (S; 61), rat (R; 70), mouse (M), pig (P), and baboon (B; 83) and the first 39 and 43 amino acids from the amino termini of UO from *D. virilis* (Dv) and *D. pseudoobscura* (Dp), respectively. The single-letter amino acid code is used. Three deduced amino acid sequences for rat UO have been reported which differ from one another at the amino and carboxy termini (33, 57, 70). In this figure, the deduced rat UO sequence from Reddy et al. (70) was used since it appears to be full length. To establish the comparison and accommodate the larger *D. melanogaster* UO protein, a gap was introduced in the middle of all the other UO amino acid sequences at a site that showed no similarity between *D. melanogaster* and other five UO sequences. All other gaps were created by the FASTP program to optimize the alignments (48). 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 UO identical in all six species are shaded. The location of introns for *D. melanogaster* UO and soybean uricase II are indicated by triangles.

that is primed by P1 and gives rise to the 158-nucleotide extension product.

Spatial distribution of UO mRNA by in situ hybridization. In situ hybridizations of UO mRNA were performed to identify the population of cells of the Malpighian tubules containing UO mRNA in the wild-type 12-h Ore-R adult and in the xanthine dehydrogenase-deficient (ry^2) 12-h adult, which has a 5- to 10-fold-higher level of UO mRNA (19, 40). At least two mechanisms could account for this higher level of UO mRNA in the Malpighian tubules of ry^2 12-h adults: (i) an increase in the amount of UO mRNA within the same population of cells of Malpighian tubules or (ii) 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 (Fig. 1c) hybridized exclusively to the Malpighian tubules in sections of ry^2 12-h adult abdomens (Fig. 4a and b). No hybridization signal was detected by 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, the hindgut, or the cells that comprise the transitional segment or the initial enlarged segment of the Malpighian tubules as defined by Wessing and Eichelberg (81) (Fig. 4c to e). There was a weak hybridization signal within the ureter and within the cells at the extreme distal end of the posterior tubule (Fig. 4c 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-h adult as compared with the same population of cells of the Ore-R 12-h adult (compare Fig. 4c and d), which is consistent with 5- to 10-fold-higher level of UO mRNA and UO activity in the ry^2 adult than in the Ore-R adult (19, 40, 41).

Deduced UO amino acid sequence and protein sequence comparisons. The *D. melanogaster* UO transcription unit contains two in-frame methionine codons in the amino-terminal region (Met-1 and Met-23; Fig. 2). If Met-1 is utilized as the translation initiation codon, the deduced M_r for the UO peptide would be 39,989, which is not significantly different from the apparent M_r of 40,480 ± 1,340 estimated for the purified UO protein (20). If Met-23 is used to initiate translation, the deduced M_r would be 37,701, which is significantly less than the apparent molecular weight. The scanning model for translation initiation (39) 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 (7). 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 (32, 63, 78). Whether Met-1 or Met-23 is the translation start for the UO protein could not be determined directly, since the amino terminus of the purified UO protein was blocked and could not be sequenced (T. B. Friedman, unpublished results). Consequently, a molecular evolutionary comparison between UO of *D. pseudoobscura* and *D. virilis* (Fig. 5), which diverged from *D. melanogaster* approximately 30 and 40 million years ago, respectively (55, 56), 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 UO from *D. melanogaster*, *D. pseudoobscura*, and *D. virilis* are

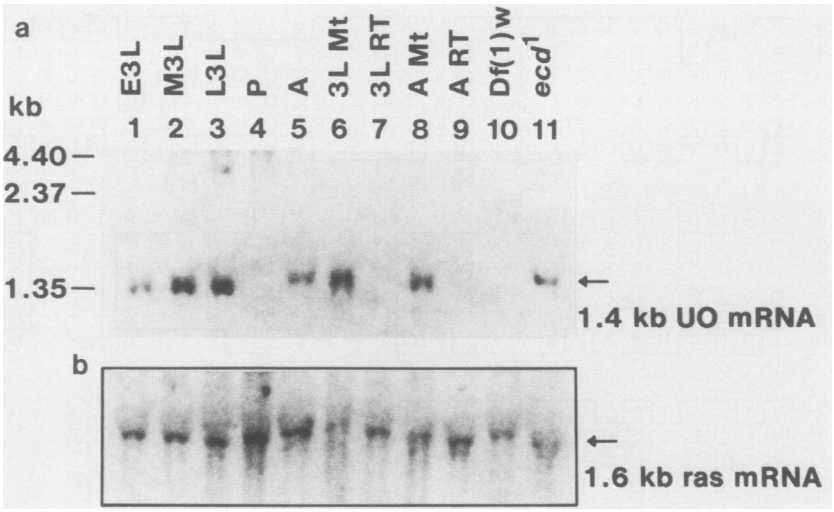


FIG. 6. Northern analysis of UO mRNA in transformants. (a) Total RNA from 60 animals (lanes 1 to 5, 10, and 11), 50 dissected Malpighian tubules (lanes 6 and 8), and the remaining tissue after dissection of the Malpighian tubules (lanes 7 and 9) was probed with an oligonucleotide that hybridized exclusively 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 to 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 did 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. Shown at the left margin are the positions of the size standards present in the 0.24- to 9.5-kb RNA ladder (BRL). (b) To check for the presence of RNA in each lane, the Northern blot was reprobed with the *Hin*dIII-*Pst*I fragment of the *D. melanogaster ras* gene which recognizes a 1.6-kb transcript expressed uniformly during development (59).

identical (Fig. 5). 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 (Fig. 5). The first eight codons of the deduced UO protein in *D. virilis* contain four synonymous substitutions when compared with 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 *Drosophila* species.

The amino acid sequence comparison between UO of *D. melanogaster*, soybean (61), rat (70), mouse, pig, and baboon (83) is shown in Fig. 5. There is 32 to 38% amino acid sequence identity between UO of *D. melanogaster* and UO of the five other species. Though not indicated in Fig. 5, many of the nonidentities represent conservative evolutionary amino acid changes (48). Among the 22% of the amino acid resides identical in the deduced amino acid sequences of UO from soybean, rat, mouse, pig, baboon, and *D. melanogaster*, there are four histidine residues (in *D. melanogaster*, His-170, His-172, His-182, and His-308) which may be involved in copper binding (49, 83). UO is a peroxisomal enzyme (12, 28, 44), and we note that the deduced amino acid sequence at the carboxy terminus of UO is Ser-His-Leu for *D. melanogaster*, Ser-Lys-Leu for soybean, and Ser-Arg-Leu for rat, mouse, pig, and baboon. These tripeptide sequences are also found at the carboxy termini of some, but not all, peroxisomal proteins (26, 27, 47, 53). Any one of these three carboxy-terminal tripeptides is sufficient for targeting a reporter protein to peroxisomes (25). On the basis of the similar carboxy termini of UO proteins compared here (Fig. 5) and from the data on targeting of some peroxisomal proteins (26, 27, 53), we suggest that a serine residue followed by a positively charged amino acid and then a carboxy-terminal leucine is the peroxisomal targeting sequence for UO of *D. melanogaster*, plants, and vertebrates.

DNA sequence sufficient for UO gene expression. A P-element construct containing the 3.2-kb *Pst*I fragment which includes the UO gene from the *ecd*^{1} strain was transformed into *D. melanogaster* by using P-element-mediated transformation (72). Five independent lines were made homozygous for a single P-element insertion and confirmed by Southern analysis (data not shown). Using Northern analysis, an oligonucleotide specific for message transcribed from the UO transgene (see Materials and Methods) detected UO mRNA from transformed third-instar larve and adults but not from pupae (Fig. 6). Shown in Fig. 6 is the Northern analysis of a single transformed line. Four additional independent transformed lines were analyzed in an identical fashion, with the same results as in Fig. 6. In *D. 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 with RNA extracted from the remaining tissues demonstrated that UO mRNA from the UO transgene is expressed only within the Malpighian tubules (Fig. 6).

DISCUSSION

Structural features of the UO gene. The UO gene of *D. melanogaster* is structurally compact, consisting of two exons separated by a 69-base-pair intron (Fig. 2). The *D. melanogaster* UO gene is transcribed from a single promoter, yielding UO mRNA of 1,224, 1,227, and 1,244 nucleotides, depending on which one of three 3′ endonucleolytic cleavage sites is utilized (Fig. 2 and 3). A few genes from

both vertebrates and invertebrates have been shown to have multiple polyadenylation sites (14, 23, 43, 54, 76) and in some cases the polyadenylation sites are closely spaced (34, 68, 75). The biological significance of multiple adjacent polyadenylation signals and sites remains to be determined (13).

The UO gene of *D. melanogaster* has a complex developmentally and tissue-specific pattern of expression. UO mRNA is present within the main segment cells of the Malpighian tubules (see Results and Fig. 4) of third-instar larvae and adults (19, 40). 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 amid a background of dissimilar DNA sequence (5, 6, 18, 36). In both *D. melanogaster* and *D. pseudoobscura*, among 520 base pairs upstream of the UO gene there are seven conserved motifs 8 to 14 nucleotides in length (T. B. Friedman et al., unpublished data). One of the conserved motifs, TATAAAA, identical to the consensus TATA box element (10), is present at position −31 in relation to the *D. melanogaster* UO transcription start site at +1 (Fig. 2). Bracketing the TATA box (−31) and the UO transcription start site at +1 of the *D. melanogaster* UO gene is a perfect 13-base-pair direct repeat (DR, AAGT GAGAGTGAT), 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 *D. melanogaster* (65). In particular, each DR motif has two adjacent copies of the sequence (AGTGA) also found within the region providing 20-hydroxyecdysone induction of the *hsp22* heat shock protein gene of *D. melanogaster* (38, 65). The similarity of DR motifs to proposed steroid hormone receptor-binding sites (2, 65) 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 (41). The UO *Eco*RI-*Hha*I and *Hha*I-*Spe*I restriction fragments (Fig. 2) each contain a single DR element and show binding to purified 20-hydroxyecdysone receptor in a gel retardation assay. In addition, a 22-base-pair double-stranded synthetic oligonucleotide (5′-CTCTAAGTGAGAGTGATGATGG-3′) containing the DR sequence (positions −142 to −115; Fig. 2) showed binding to a purified 20-hydroxyecdysone receptor in a gel retardation assay (R. Voellmy, unpublished data).

Evidence that 20-hydroxyecdysone is part of the mechanism that 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°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°C, the restrictive temperature. In addition, a rapid decline of UO mRNA in ecd^1 at 29°C can be brought about by feeding 20-hydroxyecdysone to third-instar larvae (41).

For some genes, different *cis*-regulatory elements appear to be involved in steroid hormone-mediated gene activation and repression (2, 52, 73). Nevertheless, it seems possible 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 (1, 15, 46). The human chorionic gonadotropin gene is repressed when the glucocorticoid receptor binds to a *cis* element and interferes with cyclic AMP binding at an adjacent site (1). To clarify the possible negative regulatory function of the DR *cis*-regulatory motifs of the UO gene, the effect on the developmental pattern of transcription of the UO gene by site-specific mutagenesis of one or both DR motifs will be examined in future experiments.

DNA sequence sufficient for UO gene expression. Using P-element-mediated germ line transformation (72), we determined that 826 base pairs of 5′-flanking DNA and approximately 1,200 base pairs of 3′-flanking DNA are sufficient for the complex and tissue-specific pattern of expression of the UO gene (Fig. 6). Further experiments are being performed to more narrowly define the sequences necessary for the developmental timing and Malpighian tubule-specific expression of the UO gene. Identification of potential elements that may be important for UO gene regulation has been accelerated by a sequence comparison of the 5′-flanking DNA of the UO gene from *D. melanogaster* with the 5′-flanking DNA of the UO gene from *D. pseudoobscura* (T. B. Friedman et al., unpublished) and the 5′ sequence of the UO gene from *D. virilis* (L. L. Wallrath et al., unpublished data). During development, both *D. pseudoobscura* and *D. virilis* exhibit a pattern of UO gene expression different from that of the UO gene from *D. melanogaster*. Evolutionarily conserved sequence elements within the flanking DNA of the UO gene may be involved in the common features of UO expression between these three species. Further investigations will determine whether the differences in regulation of the UO gene between these three species are due to changes in *cis* elements or differences in the UO *trans*-acting factors.

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LITERATURE CITED

1. **Akerblom, I. E., E. P. Slater, M. Beato, J. D. Baxter, and P. L. Mellon.** 1988. Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. Science **241:** 350–353.
2. **Beato, M.** 1989. Gene regulation by steroid hormones. Cell **56:**335–344.
3. **Bender, W., P. Spierer, and D. S. Hogness.** 1983. Chromosomal walking and jumping to isolate DNA from the *Ace* and *rosy* loci and the biothorax complex in *Drosophila melanogaster*. J. Mol. Biol. **168:**17–33.
4. **Birnstiel, M. L., M. Busslinger, and K. Strub.** 1985. Transcription termination and 3′ processing: the end is in site! Cell **41:**349–359.
5. **Blackman, R. K., and M. Meselson.** 1986. Interspecific nucleotide sequence comparisons used to identify regulatory and

structural features of the *Drosophila hsp82* gene. J. Mol. Biol. **188**:499–515.
6. **Bray, S. J., W. A. Johnson, J. Hirsh, U. Heberlein, and R. Tjian.** 1988. A *cis*-acting element and associated binding factor required for CNS expression of the *Drosophila melanogaster* dopa decarboxylase gene. EMBO J. **7**:177–188.
7. **Cavener, D. R.** 1987. Comparison of the consensus sequence flanking translation start sites in *Drosophila* and vertebrates. Nucleic Acids Res. **15**:1353–1361.
8. **Cherbas, L., R. A. Schultz, M. M. D. Koehler, C. Savakis, and P. Cherbas.** 1986. Structure of the *Eip28/29* gene, an ecdysone-inducible gene from *Drosophila*. J. Mol. Biol. **189**:617–631.
9. **Chovnick, A., V. Finnerty, A. Schalet, and P. Duck.** 1968. Studies on genetic organization in higher organisms: I. Analysis of a complex gene in *Drosophila melanogaster*. Genetics **62**: 145–160.
10. **Corden, J., B. Wasylyk, A. Buchwalder, P. Sassone-Corsi, C. Kedinger, and P. Chambon.** 1980. Promoter sequences of eukaryotic protein-coding genes. Science **209**:1406–1414.
11. **Davis, L. G., M. D. Dibner, and J. F. Battey.** 1986. Basic methods in molecular biology. Elsevier Science Publishing Co., Inc., New York.
12. **deDuve, C., and P. Baudhuin.** 1966. Peroxisomes (microbodies and related particles). Physiol. Rev. **46**:323–357.
13. **Denome, R. M., and C. N. Cole.** 1988. Patterns of polyadenylation site selection in gene constructs containing multiple polyadenylation signals. Mol. Cell. Biol. **8**:4829–4839.
14. **Dreesen, T. D., D. H. Johnson, and S. Henikoff.** 1988. The brown protein of *Drosophila melanogaster* is similar to the white protein and to components of active transport complexes. Mol. Cell. Biol. **8**:5206–5215.
15. **Drouin, J., J. Charron, J.-P. Gagner, L. Jeannotte, M. Nemer, R. K. Plante, and Ö. Wange.** 1987. Pro-opiomelanocortin gene: a model for negative regulation of transcription by glucocorticoids. J. Cell. Biochem. **35**:293–304.
16. **Edgar, B. A., and P. H. O'Farrell.** 1989. Genetic control of cell division patterns in the Drosophila embryo. Cell **57**:177–187.
17. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:266–267.
18. **Fortini, M. E., and G. M. Rubin.** 1990. Analysis of cis-acting requirements of the *Rh3* and *Rh4* genes reveals a bipartite organization to rhodopsin promoters in Drosophila melanogaster. Genes Dev. **4**:444–463.
19. **Friedman, T. B.** 1973. Observation on the regulation of uricase activity during development of *Drosophila melanogaster*. Biochem. Genet. **8**:37–45.
20. **Friedman, T. B., and A. P. Barker.** 1982. Purification and partial characterization of urate oxidase from *Drosophila melanogaster*. Insect. Biochem. **12**:563–570.
21. **Friedman, T. B., and D. H. Johnson.** 1977. Temporal control of urate oxidase activity in *Drosophila*: evidence of an autonomous timer in Malpighian tubules. Science **197**:477–479.
22. **Friedman, T. B., and D. H. Johnson.** 1978. Autonomous timer in Malpighian tubules. Science **200**:1185–1186.
23. **Garbe, J. C., W. G. Bendena, and M. L. Pardue.** 1989. Sequence evolution of the Drosophila heat shock locus *hsrw*. I. The nonrepeated portion of the gene. Genetics **122**:403–415.
24. **Glassman, E.** 1965. Genetic regulation of xanthine dehydrogenase in *Drosophila melanogaster*. Fed. Proc. **24**:1243–1250.
25. **Gould, S. J., G.-A. Keller, N. Hosken, J. Wilkinson, and S. Subramani.** 1989. A conserved tripeptide sorts proteins to peroxisomes. J. Cell Biol. **108**:1657–1664.
26. **Gould, S. J., G.-A. Keller, M. Schneider, S. H. Howill, L. J. Garrard, J. M. Goodman, B. Distel, H. Tabak, and S. Subramani.** 1990. Peroxisomal protein import is conserved between yeast, plants, insects and mammals. EMBO J. **9**:85–90.
27. **Gould, S. J., G.-A. Keller, and S. Subramani.** 1988. Identification of peroxisomal targeting signals located at the carboxy terminus of four peroxisomal proteins. J. Cell Biol. **107**:897–905.
28. **Hayashi, H., K. Taya, T. Suga, and S. Niinobe.** 1976. Relationship between the peroxisomal core and urate oxidase. J. Biochem. **79**:1029–1034.
29. **Hentschel, C., J.-C. Irminger, P. Bucher, and M. L. Birnstiel.** 1980. Sea urchin histone mRNA termini are located in gene regions downstream from putative regulatory sequences. Nature (London) **285**:147–151.
30. **Hirsh, J., B. A. Morgan, and S. B. Scholnick.** 1986. Delimiting regulatory sequences of the *Drosophila melanogaster Ddc* gene. Mol. Cell. Biol. **6**:4548–4557.
31. **Hultmark, D., R. Klemenz, and W. J. Gehring.** 1986. Translation and transcriptional control elements in the untranslated leader of the heat-shock gene *hsp22*. Cell **44**:429–438.
32. **Hung, M.-C., and P. C. Wensink.** 1983. Sequence and structure conservation in yolk proteins and their genes. J. Mol. Biol. **164**:481–492.
33. **Ito, M., M. Suzuki, and Y. Takagi.** 1988. Nucleotide sequence of cDNA and predicted amino acid sequence of rat liver uricase. Eur. J. Biochem. **173**:459–463.
34. **Johnson, D. H., J.-E. Edström, J. B. Burnett, and T. B. Friedman.** 1987. Cloning of a *Drosophila melanogaster* adenine phosphoribosyltransferase structural gene and deduced amino acid sequence of the enzyme. Gene **59**:77–86.
35. **Johnson, D. H., and T. B. Friedman.** 1983. Purine-resistant *Drosophila melanogaster* results from mutations in the adenine phosphoribosyltransferase structural gene. Proc. Natl. Acad. Sci. USA **80**:2990–2994.
36. **Kassis, J. A., C. Desplan, D. K. Wright, and P. H. O'Farrell.** 1989. Evolutionary conservation of homeodomain-binding sites and other sequences upstream and within the major transcription unit of the *Drosophila* segmentation gene engrailed. Mol. Cell. Biol. **9**:4304–4311.
37. **Keller, E. B., and W. A. Noon.** 1985. Intron splicing: a conserved internal signal in introns of *Drosophila* pre-mRNAs. Nucleic Acids Res. **13**:4971–4981.
38. **Klemenz, R., and W. J. Gehring.** 1986. Sequence requirement for expression of the *Drosophila melanogaster* heat shock protein *hsp22* gene during heat shock and normal development. Mol. Cell. Biol. **6**:2011–2019.
39. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108**:229–241.
40. **Kral, L. G., D. H. Johnson, J. B. Burnett, and T. B. Friedman.** 1986. Cloning a cDNA for *Drosophila melanogaster* urate oxidase. Gene **45**:131–137.
41. **Kral, L. G., D. H. Johnson, M. Wing, and T. B. Friedman.** 1982. Temporal control of urate oxidase activity during development of the third-instar larva of Drosophila: the role of 20-hydroxyecdysone. Dev. Genet. **3**:215–233.
42. **Krawetz, S. A., and R. A. Anwar.** 1984. Optimization of the isolation of biologically active mRNA from chick embryo aorta. BioTechniques **2**:342–347.
43. **Laird-Offringa, I. A., P. Elfferich, J. de Ruiter, and A. J. van der Eb.** 1989. Analysis of polyadenylation site usage of the c-*myc* oncogene. Nucleic Acids Res. **16**:6499–6514.
44. **Lazarow, P. B., and Y. Fujiki.** 1985. Biogenesis of peroxisomes. Annu. Rev. Cell Biol. **1**:489–530.
45. **Lepesant, J.-A., J. Kejzlarova-Lepesant, and A. Garen.** 1978. Ecdysone-inducible functions of larval fat bodies in *Drosophila*. Proc. Natl. Acad. Sci. USA **75**:5570–5574.
46. **Levine, M., and J. L. Manley.** 1989. Transcriptional repression of eukaryotic promoters. Cell **59**:405–408.
47. **Lewin, A. S., V. Hines, and G. M. Small.** 1990. Citrate synthase encoded by the *CIT2* gene of *Saccharomyces cerevisiae* is peroxisomal. Mol. Cell. Biol. **10**:1399–1405.
48. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227**:1435–1441.
49. **Mahler, H. R.** 1958. The role of copper in some enzyme-catalyzed oxidation reactions, p. 311–335. *In* C. A. Lamb, O. G. Bentley, and J. M. Beattie (ed.), Trace elements. Academic Press, Inc., New York.
50. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
51. **Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, and D. Quon.** 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell **15**:687–701.

52. **Martin, M., A. Giangrande, C. Ruiz, and G. Richards.** 1989. Induction and repression of the *Drosophila Sgs-3* glue gene are mediated by distinct sequences in the proximal promoter. EMBO J. **8**:561–568.
53. **Miyazawa, S., T. Osumi, T. Hashimoto, K. Ohno, S. Miura, and Y. Fujiki.** 1989. Peroxisome targeting signal of rat liver acyl-coenzyme A oxidase resides at the carboxy terminus. Mol. Cell. Biol. **9**:83–91.
54. **Mlodzik, M., and W. J. Gehring.** 1987. Expression of the *caudal* gene in the germ line of Drosophila: formation of an RNA and protein gradient during early embryogenesis. Cell **48**:465–478.
55. **Moriyama, E. N.** 1987. Higher rates of nucleotide substitution in Drosophila than in mammals. Jpn. J. Genet. **62**:139–147.
56. **Moriyama, E. N., and T. Gojobori.** 1989. Evolution of nested genes with special reference to cuticle proteins in *Drosophila melanogaster*. J. Mol. Evol. **28**:391–397.
57. **Motojima, K., S. Danaya, and S. Goto.** 1988. Cloning and sequence analysis of cDNA for rat liver uricase. J. Biol. Chem. **263**:16677–16681.
58. **Mount, S. M.** 1982. A catalogue of splice junction sequences. Nucleic Acids Res. **10**:459–471.
59. **Mozer, B., R. Marlor, S. Parkhurst, and V. Corces.** 1985. Characterization and developmental expression of a *Drosophila ras* oncogene. Mol. Cell. Biol. **5**:885–889.
60. **Murray, M. G.** 1986. Use of sodium trichloroacetate and mung bean nuclease to increase sensitivity and precision during transcript mapping. Anal. Biochem. **158**:165–170.
61. **Nguyen, T., M. Zelechowska, V. Foster, H. Bergmann, and D. P. S. Verma.** 1985. Primary structure of the soybean nodulin-35 gene encoding uricase II localized in the peroxisomes of uninfected cells of nodules. Proc. Natl. Acad. Sci. USA **82**: 5040–5044.
62. **O'Donnell, J. M., J. R. McLean, and E. R. Reynolds.** 1989. Molecular and developmental genetics of the *Punch* locus, a pterine biosynthesis gene in *Drosophila melanogaster*. Dev. Genet. **10**:273–286.
63. **O'Tousa, J. E., W. Baehr, R. L. Martin, J. Hirsh, W. L. Pak, and M. L. Applebury.** 1985. The Drosophila nina E gene encodes opsin. Cell **40**:839–850.
64. **Pirrotta, V., H. Steller, and M. P. Bozzetti.** 1985. Multiple upstream regulatory elements control the expression of the Drosophila white gene. EMBO J. **4**:3501–3508.
65. **Pongs, O.** 1988. Ecdysteroid-regulated gene expression in *Drosophila melanogaster*. Eur. J. Biochem. **175**:199–204.
66. **Poole, S. J., L. M. Kauvar, B. Drees, and T. Kornberg.** 1985. The *engrailed* locus of Drosophila: structural analysis of an embryonic transcript. Cell **40**:37–43.
67. **Proudfoot, N. J., and G. G. Brownlee.** 1976. 3′ non-coding region sequences in eukaryotic messenger RNA. Nature (London) **263**:211–214.
68. **Quan, F., and M. A. Forte.** 1990. Two forms of *Drosophila melanogaster* Gsα are produced by alternate splicing involving an unusual splice site. Mol. Cell. Biol. **10**:910–917.
69. **Raikhel, N. V., S. Y. Bednarek, and D. Lerner.** 1988. *In situ* RNA hybridization in plant tissue, vol. B9, p. 1–32. *In* S. B. Gelvin, R. A. Schilperoort, and D. P. S. Verma (ed.), Plant molecular biology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
70. **Reddy, P. G., M. R. Nemali, M. K. Reddy, M. N. Reddy, P. M. Yuan, S. Yuen, T. G. Laffler, T. Shiroza, H. K. Kuramitsu, N. Usuda, R. L. Chisholm, M. S. Rao, and J. K. Reddy.** 1988. Isolation and sequence determination of a cDNA clone for rat peroxisomal urate oxidase: liver-specific expression in the rat. Proc. Natl. Acad. Sci. USA **85**:9081–9085.
71. **Richards, G., A. Cassab, M. Bourouis, B. Jarry, and C. Dissous.** 1983. The normal developmental regulation of a cloned sgs3 'glue' gene chromosomally integrated in *Drosophila melanogaster* by P element transformation. EMBO J. **2**:2137–2143.
72. **Rubin, G. M., and A. C. Spradling.** 1982. Genetic transformation of Drosophila with transposable element vectors. Science **218**:348–353.
73. **Sakai, D. D., S. Helms, J. Carlstedt-Duke, J.-A. Gustafsson, F. M. Rottman, and K. R, Yamamoto.** 1988. Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. Genes Dev. **2**:1144–1154.
74. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
75. **Seeger, M. A., L. Haffley, and T. C. Kaufman.** 1988. Characterization of amalgam: a member of the immunoglobulin superfamily from Drosophila. Cell **55**:589–600.
76. **Setzer, D. R., M. McGrogan, J. H. Nunberg, and R. T. Schimke.** 1980. Size heterogeneity in the 3′ end of dihydrofolate reductase messenger RNAs in mouse cells. Cell **22**:361–370.
77. **Takeshima, H., T. Joh, T. Tsuzuki, K. Shimanda, and Y. Matsukado.** 1988. Structural organization of the mouse mitochondrial malate dehydrogenase gene. J. Mol. Biol. **200**:1–11.
78. **Török, L., and F. Karch.** 1980. Nucleotide sequences of heat shock activated genes in Drosophila melanogaster. I. Sequences in the regions of the 5′ and 3′ ends of the hsp70 gene in the hybrid plasmid 56H8. Nucleic Acids Res. **14**:3105–3123.
79. **Ursprung, H.** 1967. *In vivo* culture of *Drosophila* imaginal discs, p. 485–492. *In* F. H. Wilt and N. K. Wessells (ed.), Methods in developmental biology. Thomas Y. Crowell Co., New York.
80. **Weaver, R. F., and C. Weissmann.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5′ termini of 15 S β-globin mRNA precursor and mature 10 S β-globin mRNA have identical map coordinates. Nucleic Acids Res. **5**:1175–1193.
81. **Wessing, A., and D. Eichelberg.** 1978. Malpighian tubules, rectal papillae and excretion, vol. 2c, p. 1–41. *In* M. Ashburner and T. R. F. Wright (ed.), The genetics and biology of Drosophila. Academic Press, Inc. (London), Ltd., London.
82. **Wood, W. I., J. Gitschier, L. A. Lasky, and R. M. L. Lawn.** 1985. Base composition-independent hybridization in tetramethylammonium chloride: a method for oligonucleotide screening of highly complex gene libraries. Proc. Natl. Acad. Sci. USA **82**:1585–1588.
83. **Wu, X., C. L. Lee, D. M. Muzny, and C. T. Caskey.** 1989. Urate oxidase: primary structure and evolutionary implications. Proc. Natl. Acad. Sci. USA **86**:9412–9416.