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MOLECULAR LOCALIZATION, DEVELOPMENTAL EXPRESSION AND NUCLEOTIDE SEQUENCE OF THE ALPHA-METHYLDOPA HYPERSENSITIVE GENE OF DROSOPHILA

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ABSTRACT

The region surrounding the dopa decarboxylase gene of Drosophila contains a cluster of functionally related genes, many of which affect cuticle development and/or catecholamine metabolism. In this report we describe the molecular mapping and sequencing of a full-length cDNA copy of a transcript that maps to the alpha-methyldopa hypersensitive region. Developmental RNA blots show stage-specific patterns of transcription and multiple RNA transcripts. A 2-kb transcript is most abundant at about 12 hr of embryogenesis, and lower levels are detected throughout most of embryogenesis. Lower levels of this transcript are also detected in adults. Smaller stage-specific transcripts are detected in late third-instar larvae. This pattern of transcription is consistent with the known lethal phases and phenotypes of the alpha-methyldopa hypersensitive gene (amd). Based on map position, pattern of transcription, homology with dopa decarboxylase and association with altered DNA in mutants, we conclude that this transcript represents the amd gene.

THE alpha-methyldopa hypersensitive gene of Drosophila, l(2)amd (abbreviated amd) (SPARROW and WRIGHT 1974), maps immediately adjacent to the dopa decarboxylase gene (Ddc) (0.002 cM) (WRIGHT et al. 1982) and participates in catecholamine metabolism in an unknown way (MARSH and WRIGHT 1979, 1986). In insects, catecholamines serve as cross-linking agents for cuticle sclerotization (hardening), as neurotransmitters in the central nervous system, and as precursors for melanin formation (WRIGHT 1977). Mutations of amd exhibit a dual phenotype of recessive lethality and dominant conditional lethality. The lethal phase of the recessive lethal phenotype is embryonic hatching, with dying embryos showing abnormal cuticles as evidenced by thin friable cuticles and necrotic anal organs (SPARROW and WRIGHT 1974). The dominant phenotype is manifested by the lower LD_{50} of amd/+ heterozygotes reared on food containing dopa analogues, (e.g., alpha-methyldopa) (SPARROW and WRIGHT 1974). Resistance to dietary alpha-methyldopa is determined primarily by the level of amd+ gene product (MARSH and WRIGHT 1986). The observa-
tion that larvae fed alpha-methyldopa die at the larval molts and at pupariation exhibiting a thin friable pupal cuticle, whereas adult females fed dopa analogues become sterile and lay eggs that cannot complete embryogenesis, supports the conclusion that the amd locus plays a vital role in cuticle development (Marsh and Wright 1979). Thus, the amd locus is thought to be functionally related to the nearby Ddc locus (Marsh and Wright 1979, 1986).

Analysis of deletion breakpoints indicates that the genes in this region are very densely clustered on a molecular scale (e.g., at least six genes within 17.9–23 kb, Gilbert, Hirsh and Wright 1984). Although this physical organization has potential significance for regulation of these genes, the only gene that has been physically mapped in this region is the Ddc gene. We wished to determine the physical organization of the transcription units surrounding the Ddc gene and to identify the amd gene. In this communication, we report the isolation of a new chromosomal deficiency [Df(2L)NST] that uniquely localizes the Bg locus (Wright et al. 1981) and leaves only the amd and l(2)Bd loci in the interval immediately distal to Ddc. By cDNA cloning and RNA blotting, we have identified a transcription unit mapping in the 1- to 4-kb interval distal to Ddc, and we report the precise physical mapping and nucleotide sequence of a complete cDNA for this transcript and the underlying genomic DNA. The location and pattern of expression of this gene is consistent with the known location, lethal phase and phenotype of amd. The gene maps only 2.5 kb from Ddc, shows extensive structural homology with Ddc (Eveleth and Marsh 1986) and contains DNA alterations associated with recently recovered amd mutations (E. S. Pentz and T. R. F. Wright, personal communication). Based on these observations, we assign this transcription unit to the amd gene.

MATERIALS AND METHODS

Preparation of plasmid DNA, restriction analysis and subcloning were as described by Maniatis, Fritsch and Sambrook (1982). Embryonic (1–5.5 hr) and pupal (1-day pupae) cDNA libraries were a kind gift of D. Hogness and M. Goldschmidt-Clermont (Stanford University) and were prepared in the lambda gt10 vector. Screening of the libraries was as described by Huynh, Young and Davis (1985), except that we grew phage on a RecA- host because of early instability problems with some isolates.

DNA sequence was determined by the dideoxy chain termination method of Sanger, Nicklen and Coulson (1977). Growing and manipulating the phage was performed as described by Messing (1983). Reactions were performed using nucleotides from P-L Biochemicals, following the manufacturers protocol with minor modifications. Reactions were run on 0.20 mm X 40 cm urea gels at 50 watts. After running, the gel was dried by bonding directly to one of the glass plates which had been treated with 3(trimethoxysilyl)propyl methacrylate (Aldrich) and exposed to X-ray film.

RNA was prepared by aqueous phenol extraction and oligo-dT chromatography (Geitz and Hodgetts 1985). RNA was separated on 1.3% agarose gels containing formaldehyde and blotted to nitrocellulose. Radiolabeled strand-specific probes (approximately 109 cpm/μg) were prepared by in vitro transcription of the cloned cDNA using T7 RNA polymerase (U.S. Biochemicals) according to the manufacturers directions. Molecular sizes were determined relative to RNA standards synthesized as above. Hybridization was in 50% formamide, 0.8 M NaCl, 0.1 M PIPES, pH8, 0.01% SDS, 5 × Denhardt's and 100 μg/ml salmon sperm DNA at 65° and washing 3X at 70° in 50 mM NaCl, 20 mM sodium phosphate, pH6.5, and 1 mM EDTA. The amount of intact RNA transferred was confirmed and adjusted relative to the signal obtained with actin.
probes (Fyrberg et al. 1983). Quantitation of signal was accomplished using an LKB Ultrascan.

The RNA blots shown in Figure 3A contain approximately 5 µg of poly-A+ RNA, except for the 0- to 4-hr lane, which contains a fivefold excess relative to the adjacent lanes in order to visualize the weak band at 2.0 kb. The 20- to 24-hr adult lanes were exposed 1.7 times longer than the earlier lanes, again in order to better visualize the weak signal. The lanes in Figure 3B contain 10 µg of poly-A+ RNA and were judged to have equivalent amounts of intact RNA based on hybridization with actin. The 5' and 3' specific amd probes used were 5' probe = from −270 (cDNA start) to the PstI site at 666, less the intron sequences and 3' probe = 1313 to cDNA end at 1980.

Analysis of DNA sequences was performed on an IBM-PC or Compaq computer employing a series of programs modified from Schwindinger and Warner (1984) by Alan Goldin (California Institute of Technology).

RESULTS

Isolation of cDNA clones: Genetic mapping predicts that amd mutants lie in the 1- to 4-kb interval distal to Ddc (Marsh and Wright 1979; see Discussion). Initial screening of embryonic RNA blots, using a probe that covered the 1- to 10.5-kb region distal to Ddc, detected transcript(s) of approximately 2 kb from this region. We used this probe to screen two cDNA libraries made from 1- to 5.5-hr embryo and pupariating larval RNA, respectively. Four clones isolated from the embryonic library appear to represent various portions of the same transcription unit based initially on their common restriction patterns. By using these cDNAs to probe blots of cloned genomic DNA, we were able to localize the cDNA transcripts on the genomic map, as well as confirm their overlapping patterns of hybridization. The overlapping cDNA clones all derived from the interval 2.5-4.7 kb distal to the Ddc gene. One clone had an insert of approximately 1.8 kb and was chosen for subsequent analysis. The other clones were judged to represent subfragments of this larger clone by restriction pattern and hybridization. We also analyzed one cDNA, recovered from the late third-instar library, that is homologous to the 3' portion of the larger transcript and that has the same poly-A addition site.

Sequence analysis: To map this transcript more precisely and to determine its structure in detail, we sequenced the largest cDNA and the underlying genomic DNA by the dideoxy chain termination method. We first prepared a detailed restriction map of both the genomic and cDNA clones. Clones for sequencing were constructed using defined start points chosen to lie every 2-300 base pairs (bp) apart. Several isolates of each clone were checked for identity by C-test (Messing 1983) before sequencing. The sequencing strategy is shown in Figure 1. The complete genomic sequence is given in Table 1, with the regions corresponding to the mRNA and the deduced translation indicated. All six base restriction enzyme sites that were predicted by the sequence or known from restriction mapping were confirmed.

The 3' end of the gene was identified by the presence of A residues at the end of the cDNA sequence and the presence of the EcoRI linker sequence at the end of the A's. We sequenced the terminus of the late third-instar cDNA clone and found that it was identical to the embryonic cDNA clone and had the same poly-A addition site as the early embryonic cDNA clone. The se-
Genomic map: Genetic analysis places the B gene series distal and the C gene series proximal to Ddc (WRIGHT et al. 1981). Recovery of additional...
The TATA sequence at -320 is overlined and identified by "tata"; the mRNA start site is indicated by a slash at -284; the ribosome binding sequence of HAGENBUCHEL et al. (1978) is underlined at -110; the protein sequence deduced from the cDNA is indicated; the poly-A addition signal is at 1957 and the site (mature mRNA).

Underlining denotes the 5' and 3' untranslated sequences found in the cDNA (mature mRNA).
chromosomal deficiencies (Brittnacher and Ganetzky 1983; Gilbert, Hirsh and Wright 1984) places three of these genes in the 50-kb region immediately distal to Ddc; namely, amd, Bd and Bg. We have recovered a new deficiency, DF(2L)NST, which fails to complement alleles of l(2)Bd, amd, Ddc and all of the C genes (i.e., l(2)37Ca,Cb,Cc,Cd,Ce,Cf,Cg) (P. D. L. Gibbs and J. L. Marsh, unpublished observations). This deficiency extends to a region approximately 30 kb distal to the Ddc gene (Pentz and Wright 1986) and, in conjunction with others already described (Gilbert, Hirsh and Wright 1984), provides a unique genetic localization of the Bg gene and identifies the Bd and amd genes as the only known complementation groups in the cytogenetic interval immediately distal to Ddc.

Our cDNA clones map to the same cytogenetic interval as amd and Bd. Spencer, Geitz and Hodgetts (1986) have recently mapped an additional transcript to this region that we shall designate Cs, following the suggestion of Wright et al. (1981). The genomic organization of these transcripts is shown in Figure 2. The transcript that we have sequenced lies in the interval 2.5–4.7 kb distal to the 3′ end of the Ddc transcript and is transcribed from the opposite strand as Ddc but from the same strand as the Cs transcript. Pentz and Wright examined seven radiation and EMS-induced amd mutations by DNA blotting (E. S. Pentz and T. R. F. Wright, personal communication).
**TABLE 3**

Codon usage and amino acid content of the deduced *amd* protein

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Codon No.</th>
<th>Codon</th>
<th>No.</th>
<th>Codon No.</th>
<th>Codon</th>
<th>No.</th>
<th>Overall amino acid content</th>
<th>Frequency of occurrence (%)</th>
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<td>(GCA) 19</td>
<td>(GGT) 7</td>
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<tr>
<td>Arg</td>
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<td>(CGA) 6</td>
<td>(CCA) 8</td>
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<tr>
<td>Asp</td>
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<td>(GTC) 4</td>
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<td>Gln</td>
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<tr>
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<td>Ile 20</td>
<td>4.31</td>
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<tr>
<td>Leu</td>
<td>(TTA) 12</td>
<td>(CTG) 22</td>
<td>Leu 48</td>
<td>10.34</td>
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<tr>
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<td>Lys 20</td>
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<tr>
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<td>(TAC) 14</td>
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<td>3.66</td>
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<tr>
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<td>(TGG) 6</td>
<td>(ACG) 3</td>
<td>Trp 6</td>
<td>1.29</td>
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<tr>
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<td>(TAC) 5</td>
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<tr>
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<td>(TAC) 1</td>
<td>END 1</td>
<td>0.22</td>
<td></td>
<td></td>
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</tbody>
</table>

Codons are given in parentheses and are followed by the number of times each codon is used. On the right, the overall amino acid content is given with the percent occurrence listed. There are 464 amino acids with 49 Arg and Lys and 53 Asp and Glu residues, respectively.

**FIGURE 2.**—Organization of transcripts in the Ddc region. Transcripts discussed in this communication are placed on the genomic map. We have used the *HpaI* site near the terminus of the Ddc as a fixed reference point at 0 kb. This is different from the arbitrary zero point that is used by other authors (e.g., Gilbert, Hirsh and Wright 1984) which would lie at a point slightly greater than 860 bp on our map. The position of the *amd* transcript derives from the sequence data reported here. The dashed arrow represents the 2-kb transcript described by Spencer, Geitiz and Hodgetts (1986). Solid bars indicate exons included in the mature RNA or cDNA. Arrows indicate direction of transcription from 5' to 3'. In this figure the centromere is to the right. Restriction enzymes are represented by E( EcoRI, B(BamHI, P(PstI), H(HindIII).
and found two *amd* alleles that showed DNA alterations within the 1-kb *BglII* fragment that is internal to the coding region of the *amd* transcript (662 to 1639 in Table I), thus providing additional and compelling evidence that this transcription unit represents the *amd* gene.

**Developmental expression:** Total cellular and polysomal poly-A+ RNA isolated from several stages of development was subjected to electrophoresis on denaturing agarose gels, transferred to nitrocellulose or nylon membranes and probed with labeled single-stranded RNA complementary to the mRNA. Transfer and quantitation of intact RNA was confirmed by probing with actin probes. The *amd* cDNA strand-specific probes revealed a 2.0-kb *amd* transcript, which was first detectable early in embryogenesis and reached a maximum in mid-embryogenesis (i.e., 12–16 hr, Figure 3A). Very weak signal could be detected even in 0- to 4-hr embryonic RNA when a fivefold excess of this RNA was loaded, as in Figure 3A. Whether this represents carry-over of oocyte transcripts or new synthesis cannot be determined from these data. We did observe the 2.0-kb transcript in 3- to 6-hr embryo polysomal RNA (data not shown). Allowing for a poly-A tail, this transcript size of 2.0 kb agrees well with the size of 1780 nucleotides predicted from the cDNA, and it supports our interpretation of the origin of transcription deduced from the sequence analysis. Low levels of the 2.0-kb transcript were also observed in RNA extracted from adults (Figure 3A). *In situ* hybridization to ovaries shows *amd* transcripts concentrated in approximately stage 8–9 oocytes (K. KONRAD and J. L. MARSH, unpublished observations). Transcripts were also detected on blots of third-instar larval RNA, although the bulk of the hybridization is due to small RNA species present at this stage. RNA blots probed with subcloned portions of the *amd* cDNA indicate that the small RNAs hybridize to the 5' half of the gene and not to the 3' half (Figure 3B). To determine whether the smaller stage-specific transcripts originate from the *amd* gene itself or from a closely related gene, we probed DNA blots at elevated stringency. Even in 1 M Na+, 60% formamide 65° (equivalent to approximately 113° 1 × SSC) the small RNAs hybridize, suggesting that they either share a considerable stretch of strong homology with *amd* or they are bona fide transcripts from the *amd* gene. To determine whether the structural homology between *Ddc* and *amd* (EVELETH and MARSH 1986) is sufficient to cause *amd* probes to react with *Ddc* transcripts, we performed a reconstruction experiment in which *amd* probes were hybridized to *in vitro* synthesized full-length *amd* and *Ddc* transcripts at various stringencies. The hybridization signal with *Ddc* transcripts was less than 10% of that with *amd* and was not detectable at the RNA concentrations found on the Northern blots. Thus, *Ddc* transcripts are not contributing significantly to the signals observed in these studies.

**DISCUSSION**

**Genomic map:** Sixteen lethal genes and the visible gene *hk* have been identified in the *Ddc* region as defined by *Df(2L)TW130* (WRIGHT et al. 1981). The chromosomal deficiencies described here in conjunction with previously described deficiencies establish the following chromosomal order in this region:
STRUCTURE AND EXPRESSION OF \( l(2) \text{amd} \)

**FIGURE 3.**—Developmental RNA blots. Poly-A+ RNA was extracted and run on denaturing agarose gels and was probed, as described in MATERIALS and METHODS. Approximate developmental stages are given above each lane. A, Each lane contains an equivalent amount of poly-A+ RNA. The 0- to 4-hr lane contains fivefold excess of RNA to permit visualization of the weak signal. The 20- to 24-hr and adult lanes are exposed 1.7 times longer than are the others. Molecular weights are given in kilobases. B, Each lane contains 10 \( \mu \)g of poly-A+ RNA probed with 5' and 3' specific probes, as described in MATERIALS and METHODS. Transfer of intact RNA was confirmed by probing with actin.

The \( \text{Bd} \) and \( \text{amd} \) genes are the only two genetic units identified in the approximately 30-kb cytogenetic interval immediately distal to \( \text{Ddc} \). The \( \text{amd} \) gene maps 0.002 cM distal to the \( \text{Ddc} \) gene (WRIGHT et al. 1981), which would place it approximately 1.2 (GELBART, MCCARRON and CHOVNICK 1976) to 4 kb (BOSSY, HALL and SPIERER 1984) distal to the \( \text{Ddc} \) gene. The 3' end of the cDNA transcript we have sequenced lies approximately 2.5 kb from the 3' terminus of the \( \text{Ddc} \) gene (SPENCER, GEITZ and HODGETTS 1986; D. D. EVELETH, J. L. MARSH and R. B. HODGETTS, unpublished observations) on the opposite strand. SPENCER, GEITZ and HODGETTS (1986) describe another 2.0-kb RNA (herein designated as \( \text{Cs} \)) that maps between \( \text{Ddc} \) and \( \text{amd} \) but its pattern of transcription is inconsistent with the \( \text{amd} \) phenotype.

**Dense clustering:** Placing a 2-kb transcript between \( \text{Ddc} \) and the \( \text{amd} \) cDNA would place the 5' start of the \( \text{Cs} \) transcript only 450–650 bp from the 3' terminus and the \( \text{amd} \) gene, without including any introns but allowing for a
poly-A tail. As seen in Figure 2, three transcripts are contained within about 8.5 kb, and the unspliced transcripts from these genes account for almost 8 kb of this region. Thus, the genes in this interval are very densely packed. This dense clustering of genes has complicated the analysis of transcripts in this region, because probes used in other studies have later been found to overlap more than one transcription unit (e.g., SPENCER, GEITZ and HODGETTS 1986). The use of strand-specific probes derived from cDNA clones and the detailed sequence analysis and mapping described here ensure that we are analyzing only a single transcription unit. If transcription termination in Droso-
phila (PRICE and PARKER 1984) occurs as much as 1 kb downstream of the poly-A addition processing site, as has been observed in a number of cases (CITRON et al. 1984; HAGENBUCHLE et al. 1984; BIRNSTEIL, BUSSLINGER and STRUB 1985), the gene arrangement seen here implies that the primary trans-
scripts from these genes overlap one another. The regulatory consequences of this fact (if any) await functional studies.

Developmental expression: The major 2.0-kb transcript is weakly detectable during early embryogenesis, reaches a maximum in mid-embryogenesis at approximately 12–16 hr and is relatively weak again at embryonic hatching. Developmentally, mid-embryogenesis is a period of active cuticle deposition (POODRY 1980), which correlates well with the observation that embryos homo-
zygous for amd mutations show cuticular defects and die at the embryonic/ larval boundary, exhibiting a necrotic anal organ and thin friable cuticles (SPARROW and WRIGHT 1974). In the absence of conditional mutations, there is no direct evidence that amd function is required in adults. However, the effects of dietary alpha-methylldopa on adults are specific to females who, after 2–3 days exposure, lay eggs which do not complete embryogenesis (MARSH and WRIGHT 1979). The appearance of low levels of the 2.0-kb mRNA in adults, and the appearance of amd transcripts in nurse cells of stage 8–9 oocytes, suggests a possible maternal requirement for amd function. The pattern of transcription in third instar larvae is more complex. Smaller, stage-specific transcripts are observed that suggest developmentally specific modes of transcription or processing. Whether the smaller transcripts originate from a closely related gene or from alternate promoters or alternative RNA pro-
cessing events of the amd gene is presently under study.

Correlation with amd: The transcript described by SPENCER, GEITZ and HODGETTS (1986) (Cs) is most abundant in adult males and early embryos and decreases in amount during the first 8 hr of embryogenesis. This pattern of expression is inconsistent with the known phenotype and lethal phases of amd. The location of our transcript to the region 1.2–4 kb distal to Ddc and the appearance of the 2.0-kb transcript, which correlates well with the embryonic lethal phase of amd, are consistent with the genetic observations of the amd gene. The observation that two of seven EMS- and gamma-ray-induced amd mutations show altered restriction patterns that map to the 1-kb BglII fragment contained within the cDNA sequence (E. S. PENTZ and T. R. F. WRIGHT, personal communication) further extends this correlation. We interpret these observations to indicate that this transcript defines the amd gene.
Structure of the gene: The eukaryotic promoter sequence TATAAAA occurs 30 bp upstream of the proposed 5' end of the RNA transcript. This conforms nicely to the consensus sequence (5'-TATA[A/T]A[A/T]-3') and position (3' terminal A or T at -24 ± 4 bp) derived from 60 examples of TATA boxes (Breathnach and Chambon 1981). We have placed the origin of transcription at the last base sequenced from the cDNA (Table 2), although it is quite likely that the mature RNA would extend another few bases, thus placing the TATA box closer to the canonical interval for this structure. It is interesting to note that the TATA homology used in the amd gene is identical to the corresponding sequences in three of four cuticle protein genes analyzed by Snyder et al. (1982). The consensus sequence (5'-GG[C/T]CAATCT-3') is often found approximately 50 bp upstream of the TATA box homology (Breathnach and Chambon 1981). The sequence AGATTGGC is found 45 bp upstream of the TATA homology in our sequence and reads as an inverted “CAAT box,” which matches the consensus in eight of nine positions and, as such, may serve as a “CAAT box.” No strong homology was noted to the GGGCGG sequence implicated in some promoter regions (Dynan and Tijan 1985).

We also note the occurrence of sequences of noteworthy structure because of their symmetry or repetition. One copy of the sequence AATAAACAAA is found at position -414 that is similar to the decamer AATAAACAAAAA found repeated three times in regions flanking the hormonally regulated SGS4 gene sequence (Muskavitch and Hogness 1982). In addition, the sequence GGCTGAAccGAC beginning at position -363 is repeated in inverse at position -335 as CAGagAAGTCGG (lower case letters indicate mismatch), and the sequence GGCTG at -363 is repeated at -342, while the sequence TTGGCTG (-365) is repeated as the inverse complement with AACCGAC (-358). The significance of these sequences is unclear, but they are sufficiently uncommon to stand out in scans for regions of symmetry or repetition and are reminiscent of similar sequence motifs found in the regions flanking other genes, especially ecdysone-induced genes (Muskavitch and Hogness 1982).

The sequences immediately surrounding the AUG initiation codon site do not match the generalized eukaryotic initiation consensus proposed by Kozak (1984); namely, preference for C's and an A at -3 bp (ccAccAUG). The use of this initiation codon indicates that the amd gene has an unusually long 5' untranslated leader (284 bp). In a compilation of 211 leader sequences from eukaryotic genes, Kozak (1984) finds over 70% of the leaders clustered in the 20- to 80-nucleotide range and speculates that the structure of the longer leaders may participate in unusual modes of regulation. This long leader contains four premature AUG codons. Hagenbuchle et al. (1978) propose that ribosomal binding involves base-pairing of sequences in the untranslated leader sequences with sequences near the 3' end of the 18 S rRNA. The sequence at -110, 5'-UUCCGAAG-3' can base pair at seven of its eight positions with the conserved 3'-AAGCCGUC-5' sequence located 7 bp from the 3' end of the 18 S rRNA and, thus, might serve as a ribosome binding site. This proposition would still leave two "nonfunctional" AUG codons before the functional
Figure 4.—Hydropathy plot of *amd* protein. The regional hydropathy values for the *amd* protein were calculated by the method of Kyte and Doolittle (1982) using a window size of 11 amino acids. The plot begins with the amino terminus on the left.

AUG. We do not know the effect of these codons on translation, but it is possible that the long leader containing “nonfunctional” AUG codons may serve to regulate translation using false starts, as has been observed in some genes (Hunt 1985).

Examination of the predicted amino acid sequence does not reveal a leader peptide resembling a signal sequence (Kreil 1981). The predicted *amd* protein does show one broad lipophilic (hydrophobic) domain (Figure 4) that by attaining average hydropathy value of $>+1.6$ over about 19 residues, has a high probability of participating in membrane binding (Kyte and Doolittle 1982). Four other lipophilic spikes with hydropathy values of $>+1.6$ occur, but they do not meet the length requirements described by Kyte and Doolittle (1982) for membrane binding. A potential glycosylation site Asn-X-Thr (McReynolds et al. 1978) is located eight amino acids from the carboxy terminus of the protein. It is possible that these sequences play a role in the intracellular sorting of the *amd* protein. Immunocytochemical localization of the *amd* protein will provide insight into this possibility.

Sequence comparison of the cDNA clone and the genomic sequence indicated the presence of a 483-bp intervening sequence conforming to the GT–AG rule of 5′ donor 3′ acceptor splice sites. We compared the splice sequences of this gene with the tabulated frequencies of splice junction sequences from approximately 130 genes in a variety of species (Mount 1982). The overall agreement with these tabulated frequencies is remarkably good, with the Drosophila sequence showing a preference for C’s near the splice acceptor where vertebrates show a slight preference for runs of T’s. We also note the occurrence of the sequence 5′-ATAAT-3′ approximately 10, 20 and 30 bp 5′ of the splice acceptor site. This sequence matches in four of five positions with the proposed consensus 3′ splice signal thought to be necessary for lariat formation during splicing in Drosophila (Keller and Noon 1985).

The sequence of the poly-A addition site conforms well to the consensus
sequence characteristic of vertebrate poly-A addition sites (Berget 1984; Birnstiel, Busslinger and Strub 1985). For example, the consensus hexanucleotide sequence AATAAA is found 14 bp upstream of the poly-A addition site that occurs at the T of a TA pair. In addition, the genomic sequences downstream of this region are GT rich, with long runs of blocks of contiguous T residues similar to the sequences seen in a number of vertebrate genes (Birnstiel, Busslinger and Strub 1985).

The functional nature of the reading frame predicted by the sequence analysis was confirmed by fusing a major portion of the predicted frame to the lacZ region of pUC19 (Messing 1983) and showing the production of a new polypeptide. Antiserum raised against this polypeptide cross reacts with a 52-kD protein in extracts of Drosophila embryos (K. D. Konrad and J. L. Marsh, unpublished results), thus corroborating the conclusion based on the polysomal location of the mRNA that the functional reading frame described here is actually expressed as an mRNA and is translated to protein.

Although the metabolic function of the amd gene product is unknown, we know that it does not encode phenol oxidase or dopamine acetyl transferase, the prior and next enzymes in the insect sclerotization pathway (Sparrow and Wright 1974; Pentz, Black and Wright 1986). Nevertheless the rescue of amd mutants with catecholamine metabolites (P. D. L. Gibbs and J. L. Marsh, unpublished observations), the ability of amd to confer resistance to dopa analogues, the effect of amd on cuticle development and its structural similarity to Ddc (Eveleth and Marsh 1986) all suggest a role in catecholamine metabolism. The catalytic activity is currently under investigation.

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LITERATURE CITED


MUSKAVITCH, M. A. T. and D. S. HOGNESS, 1982 An expandable gene that encodes a Drosophila...
glue protein is not expressed in variants lacking remote upstream sequences. Cell 29: 1041–1051.


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