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Evolution

Evolutionary changes in the expression pattern of a developmentally essential gene in three *Drosophila* species

(gene regulation/*dopa decarboxylase* gene/*α-methyldopa hypersensitive* gene/*Drosophila melanogaster*/*Drosophila simulans*)

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ABSTRACT **The hypothesis that morphological evolution may largely result from changes in gene regulation rather than in gene structure has been difficult to test. Morphological differences among insects are often apparent in the cuticle structures produced. The *dopa decarboxylase* (*Ddc*) and *α-methyldopa hypersensitive* (*amd*) genes arose from an ancient gene duplication. In *Drosophila*, they have evolved nonoverlapping functions, including the production of distinct types of cuticle, and for *Ddc*, the production of the neurotransmitters, dopamine and serotonin. The *amd* gene is particularly active in the production of specialized flexible cuticles in the developing embryo. We have compared the pattern of *amd* expression in three *Drosophila* species. Several regions of expression are conserved in all three species but, surprisingly, a unique domain of expression is found in *Drosophila simulans* that does not occur in the closely related (2–5 million years) *Drosophila melanogaster* or in the more remote (40–50 million years) *Drosophila virilis*. The "sudden" appearance of a completely new and robust domain of expression provides a glimpse of evolutionary variation resulting from changes in regulation of structural gene expression.**

Zuckerkandl and Pauling (ref. 1; p. 100) conjectured 30 years ago that many phenotypic differences may result from changes in the temporal and spatial regulation of structural gene expression rather than from changes in functional properties due to changes in amino acid sequence. A corollary of this hypothesis would be a disparity between morphological and molecular evolution—i.e., "two organisms may be phenotypically more [or less] different than they are on the basis of the amino acid sequences of their polypeptide chains" (ref. 1, p. 100). That organismal evolution may be largely modulated by changes in gene regulation rather than by changes in the amino acid composition of proteins has indeed been proposed as the explanation for observed discrepancies between rates of phenotypic and molecular evolution (2–5).

Demonstrating the role of gene regulation in the adaptive evolution of multicellular organisms has proven elusive. Pattern formation genes that appear responsible for the dramatic morphological differences between orders and classes have not been implicated in the small morphological differences which often distinguish the different species in a genus. Insight into the more subtle differences between closely related species has often been sought by examining structural or activity variants of enzymes. For example, natural populations of *Drosophila melanogaster* exhibit differences in activity for several enzymes due to variation of both cis and trans-acting regulatory elements—e.g., *alcohol dehydrogenase* (*Adh*; ref. 6), *Cu-Zn superoxide dismutase* (*Sod*; ref. 7), and others (8–12). Experimentally, adaptation of *D. melanogaster* to increased levels of ethanol has occurred without structural changes in the *alcohol dehydrogenase* enzyme, but rather by increasing the amounts of this enzyme present in the flies (13). However, most of the enzymes investigated are not essential for viability and several are not cell autonomous (e.g., *Adh* and *Sod*; refs. 6 and 7). The genes encoding such enzymes may not be subject to stringent control of their pattern of expression. Furthermore, none of the genes studied affects the terminal morphological features that often distinguish closely related species, such as pigmentation patterns or cuticle elements.

The *dopa decarboxylase* (*Ddc*) and *α-methyldopa hypersensitive* (*amd*) genes provide a unique opportunity to examine morphological evolution. They are paralogs that arose by gene duplication and are essential for viability and fertility (14, 15). They are the only terminal differentiation genes that are essential for cuticle development whose regulation has been extensively studied in *Drosophila* (16–26). In addition, *Ddc* activity is required for neurotransmitter production (20, 22, 24, 26–29). Because the two genes encode essential cell autonomous functions, their pattern of expression should be subject to significant selective pressure and thus should provide a unique opportunity to test Zuckerkandl and Pauling's suggestion that changes in temporal and spatial regulation of structural genes may be a frequent source of adaptive variation.

To evaluate the extent of qualitative changes in patterns of gene expression, we have compared the expression of the *amd* gene in three *Drosophila* species, the two sibling species *D. melanogaster* and *Drosophila simulans* (≈2–5 million years), and the more remotely related (≈40–50 million years) *Drosophila virilis* (30).

MATERIALS AND METHODS

Strains. The strains of *D. melanogaster* (OreR) and *D. simulans* are maintained in our laboratories. *D. virilis* was a kind gift of H. Biessmann (University of California, Irvine). All fly strains were cultured at 25°C on standard corn meal and molasses medium. Eggs were collected for 2–4 hr and aged at 25°C for the appropriate times. Ovaries were dissected from 3- to 7-day-old females, chilled on ice, washed in Ringer's solution, fixed in 10% formaldehyde/phosphate-buffered saline (PBS), and stored at −20°C in ethanol for *in situ* hybridization.

Cloning. A *D. virilis* λ1059 genomic library (kind gift of P. O'Farrell, University of California, San Francisco) was screened by using a probe made from a *D. melanogaster amd* cDNA clone (15). Hybridization was carried out at 50°C in 4× SSPE (20× SSPE = 3.6 M NaCl/0.2 M NaH_2PO_4/20 mM EDTA/0.16 M NaOH, pH 7.0) containing 0.3% SDS, 100 μg of yeast tRNA per ml, and 0.8× Denhardt's solution (50× Denhardt's solution = 1% Ficoll/1% polyvinylpyrroli-

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Abbreviations: *Ddc*, *dopa decarboxylase* gene; *amd*, *α-methyldopa hypersensitive* gene; *Adh*, *alcohol dehydrogenase* gene; *Sod*, *Cu-Zn superoxide dismutase* gene, CNS, central nervous system.

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done/1% bovine serum albumin). Washing conditions were 1× SSPE/0.2% SDS, 50°C.

Detection of mRNA Expression. *In situ* hybridization to embryos or ovaries followed standard protocols (31), with the modification that *D. virilis* embryos were treated with proteinase K for 20 min. Anti-sense RNA probes were labeled with digoxigenin-dUTP by using the Genius kit (Boehringer Mannheim). Extending posthybridization washes for 2 days improved signal clarity. The *D. melanogaster amd* probe was a 1.4-kb fragment derived from a 2-kb *amd* cDNA clone (15). This probe was also used for *D. simulans in situ* hybridization. The *D. virilis amd* probe was transcribed from a 1.6-kb *D. virilis* genomic *amd* clone spanning the majority of the coding sequence and containing a 270-bp intron.

Ddc and *amd* are paralogous genes that share 61% sequence similarity in *D. melanogaster* (ref. 32; GenBank accession nos. X04695 and X04426). The possibility that the *D. virilis amd* probe might hybridize with the *Ddc* mRNA in addition to, or instead of, the *amd* mRNA was tested and excluded by two control experiments as follows. First, *amd* and *Ddc* did not cross hybridize under either standard or reduced stringency conditions (55°C and 42°C for hybridization and posthybridization washes). This was determined by finding that *D. melanogaster* embryos did not exhibit the *amd* pattern when probed with the *D. melanogaster Ddc* probe at either stringency. Second, the *D. melanogaster amd* probe did not cross-react with *D. virilis* embryos. Because the *amd* genes of these two species share 73% of the amino acids in identical positions (unpublished observations), while the *Ddc* gene is more distantly related, we conclude that cross hybridization is not a factor here.

RESULTS

We used *in situ* hybridization to compare the pattern of expression of the *amd* gene in *D. melanogaster*, *D. simulans*, and *D. virilis as* a function of developmental stage (Fig. 1). We found that while all three species shared many common patterns of *amd* expression, the species differed in three ways, including the appearance of a completely unique pattern of expression in the developing *D. simulans* nervous system.

In all three species, *amd* expression was detected at the cellular blastoderm stage in an anterior dorsal domain (Fig. 1 *B*, *H*, and *N*). The first differences were apparent immediately prior to this when *amd* transcripts accumulated in an anterior dorsal patch in *D. melanogaster*, (Fig. 1*A*) while in *D. simulans* transcripts accumulated in an anterior ring (Fig. 1*G*). Expression was not apparent in *D. virilis* at this stage, but it did appear later at the cellularization stage (Fig. 1 *M* and *N*). During formation of the cellular blastoderm, *amd* expression refined to a small anterior dorsal patch in all three species. At gastrulation, these dorsal cells (arrowheads, Fig. 1 *B*, *H*, and *N*) moved and came to line the developing foregut (e.g., the stomadeum and pharynx) at the extended germ band stage. At this stage, *amd* expression became quite robust in the foregut of *D. melanogaster* (fg in Fig. 1*C*) and *D. virilis,* while foregut staining was less intense, although clearly present, in *D. simulans* (fg in Fig. 1*I*). Later, foregut staining was indistinguishable in all three species. A second site of expression was apparent in the anlage of the malphigian tubules and was similar in all three species (mt in Fig. 1 *C*, *I*, and *O*).

Surprisingly, a novel pattern of expression was observed in *D. simulans* embryos. *amd* was expressed in a segmentally repeating pattern of cell clusters in the developing nervous system (arrowheads along the ventral margin in the lateral view in Fig. 1*I* and along the ventral midline in the ventral view in Fig. 1*J*; see also a higher magnification Fig. 2). This expression occurred before the nervous system was fully functional and before production of neurotransmitters; yet, it was quite specific, reproducible, and robust. This expression was never seen in *D. melanogaster* or *D. virilis*, even when overstained (not shown), and it was transient, disappearing at about the time of dorsal closure (Fig. 1*K*), although some staining in the mature larval nervous system appeared later in all three species (data not shown; ref. 26).

As development proceeded, expression in the foregut and hindgut was common to all three species (fg and hg; Fig. 1 *F*, *L*, and *R*). As the embryonic hypoderm began to deposit cuticle, all three species exhibited segmentally repeating stripes of *amd* expression, although there were differences in the patterns. Staining in *D. melanogaster* initially appeared at the anterior boundary of each segment (brackets in Fig. 1*E*), whereas in *D. simulans* and *D. virilis* stripes of expression occurred at both the anterior and posterior boundary of each segment (brackets in Fig. 1 *K* and *Q*). At later stages, the expression pattern in the three species became similar on both the dorsal and ventral sides of the embryo (data not shown). Thus, during embryogenesis, three differences in timing and location of *amd* expression were evident between the three species, including a completely new pattern of expression that appeared only in *D. simulans* embryos.

We also tested for the expression of *Ddc* in embryos. Enzyme assays of embryos at different stages have suggested that DDC activity does not appear until late, after cuticle had been deposited (e.g., ≥16 hr of development) (16, 33). The phenotype of *Ddc* null embryos was consistent with this measurement, as the only apparent defect in mutant embryos was lack of melanization and sclerotization of the mouth hooks and denticles which often prevents hatching (21, 22). *Ddc* mutant embryos that managed to hatch from the egg survive until pupariation (21, 22). Consistent with these observations, no staining of *Ddc* mRNA was detected in embryos until the time of cuticle deposition (data not shown). After that time, we were unable to assess expression in whole mount preparations due to the inability of the probe to penetrate the cuticle.

We also compared *amd* expression during oogenesis in all three species by using whole-mount *in situ* hybridization (Fig. 3). The expression of *amd* in follicle cells as well as in nurse cells, starting with the earliest egg chambers emerging from the germarium, was virtually the same in all three species.

DISCUSSION

Comparing the pattern of expression of *amd* in three related species provides a glimpse of the nature of regulatory variation that is the raw material of adaptive selection. By comparing genes that are orthologous, selective pressures retained between the species for functions essential in all may be revealed, while distinct pressures unique to some but not other species may become apparent.

Comparing the early pattern of *amd* expression in the sibling species, *D. melanogaster* and *D. simulans*, and the less related *D. virilis* permits three general conclusions. First, some functions are conserved in all three species. Expression in the epidermis, the foregut, the hindgut, the proventriculus, and tracheal system is essential for colorless sclerotization of flexible cuticle during early stages of development. Because this function is conserved in all three *Drosophila* species, it is likely to have arisen shortly after the *Ddc–amd* duplication—i.e., at least before the divergence of the *D. melanogaster/D. simulans* and *D. virilis* lineages. The gene duplication made it possible for *Ddc* and *amd* to become specialized in different aspects of sclerotization of pigmented cuticle and colorless cuticle, respectively. *amd* expression in the female germ line and somatic follicle cells is also conserved in all three species. Insect eggs are surrounded by the outer chorion and the inner vitelline membrane (see ref. 34). The vitelline membrane is flexible, yet highly impermeable. It is composed of proteins that are cross-linked by tyrosine bridges into an insoluble matrix and it is covered by a waxy layer that prevents desic-

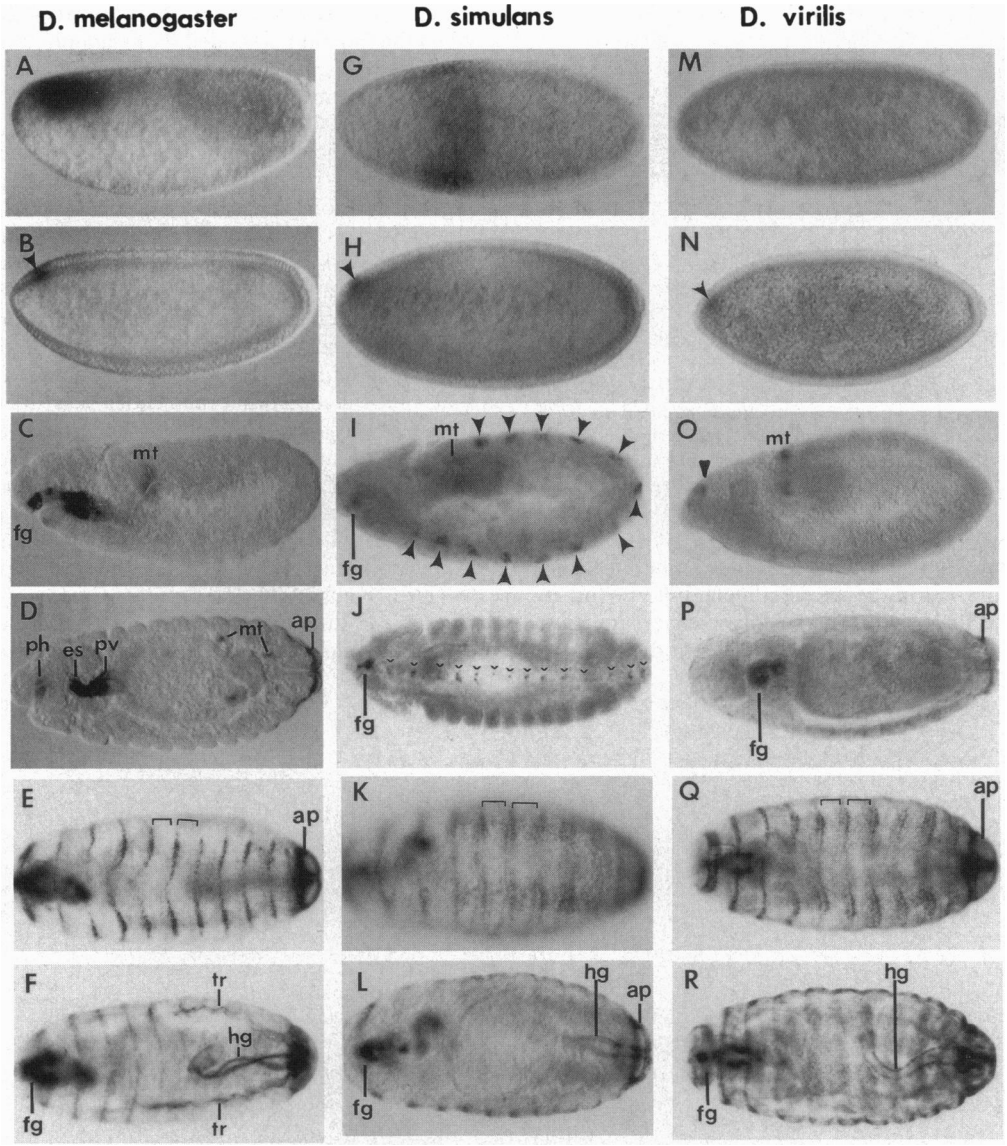

FIG. 1. Embryonic expression of *amd* in *D. melanogaster*, *D. simulans*, and *D. virilis*. Before cellularization (*A*, *G*, and *M*), *amd* is expressed in a dorsal anterior patch in *melanogaster* (*A*) and in an anterior ring in *simulans* (*G*), but is not detectable in *virilis* (*M*). By cellular blastoderm (*B*, *H*, and *N*), all three species exhibit a dorsal anterior patch of *amd* expression (arrowhead). As gastrulation proceeds, the cells of that patch move down and into the invaginating foregut, and in *D. melanogaster*, expression of the *amd* increases in several regions of the foregut (fg in *C* and *I*). Foregut staining in the *D. virilis* embryo shown in *O* is at an earlier stage than the adjacent *D. melanogaster* and *D. simulans* embryos, so that the dorsal anterior patch (arrowhead) has not yet moved into the emerging foregut although, as the foregut develops further (not shown), *amd* expression is indistinguishable from *D. simulans* and *D. melanogaster*. Expression in the foregut is dynamically emerging at this stage in all three species, and the differences in foregut staining seen in *C*, *I*, and *O* are primarily due to slight differences in developmental stage and plane of focus of the embryos selected. During this germ band extension stage (*C*, *I*, and *O*), all three species show staining in the anlage of the malphigian tubules (mt). Surprisingly, a segmentally repeating pattern of expression appears in the developing nervous system of *D. simulans* (arrowheads), which is not seen in *D. melanogaster* or *D. virilis*. As the germ band retracts and segmentation begins, all three species show *amd* expression in the foregut and the emerging malphigian tubules and around the anal plates (ap). In *D*, elements of the foregut are labeled ph (pharynx), es (esophagus), and pv (proventriculus). In *J*, a compromise focus is presented which shows the foregut and nervous system staining (small arrowheads), but the anal plates and malphigian tubule anlage are not visible in this plane of focus. In *P*, the anal plates (ap) and foregut (fg) staining is in focus but not the malphigian tubules (mt) stain. Expression in the *D. simulans* nervous system continues (small arrowheads in *J*) but is transient, disappearing at about the time of dorsal closure. As the external cuticle begins to be laid down (≈16 hr), staining becomes quite pronounced in the foregut (fg) and the hindgut (hg) of all three species (*F*, *L*, and *R*). The ring of *amd* expression around the anal plates (ap) in all three species (*D*, *J*, and *P*) is the first expression in the epidermis. As cuticle is laid down, *amd* is expressed in a narrow band of cells in the anterior of each segment in *D. melanogaster* (brackets in *E*) while in *D. simulans* and *D. virilis* a band on both sides of the segmental groove expresses *amd* (brackets in *K* and *Q*). In late embryos, cells of the tracheal system (tr) begin to stain in all three species (shown only in *F*). Generally, tissues expressing *amd* will produce cuticle—e.g., the external hypodermal cells, the internal hypodermal cells lining the foregut and hindgut, the proventriculus, and the trachea. Exceptions are the anlage of malphigian tubules and the nervous system.

cation (35). *amd* activity is required in both the germ line and the follicle cells for the production of a normal impermeable vitelline membrane (24). Given this essential function for fecundity, it is not surprising that *amd* expression in ovaries has been conserved during evolution. It seems likely, therefore, that the essential function of *amd* in the formation of the vitelline membrane predates the radiation of the *Drosophila* genus (i.e., the divergence of the two subgenera, *Sophophora*, to which *D. melanogaster* belongs, and *Drosophila*, which is the subgenus of *D. virilis*). Because *Ddc* expression is not required

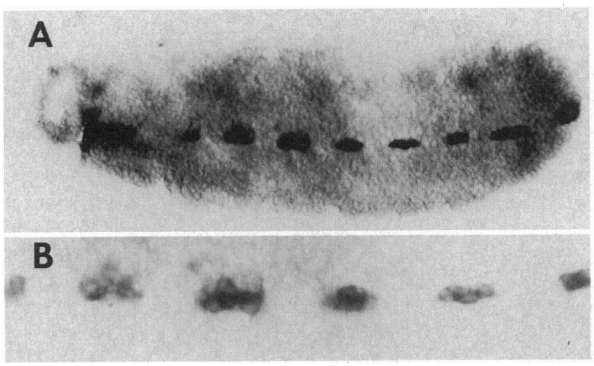

FIG. 2. Expression of *amd* in the central nervous system (CNS) of *D. simulans*. (*A*) Ventral view of a germ band-extended embryo showing a segmentally repeated pattern of *amd* transcription in the ventral mid-line glia. (*B*) Higher magnification showing that the transcription is made up of clusters of nerve cells along the ventral midline.

to cross link the vitelline membrane, it follows that either (*i*) this function was present in the ancestral gene but was lost in *Ddc* after the gene duplication or (*ii*) this is a new function acquired by *amd* that evolved after the *Ddc–amd* duplication.

Second, *amd* expression often precedes the apparent time when the function is needed. For example, *amd* is expressed in the primordia of the foregut, hindgut, proventriculus, and tracheal system much earlier than the onset of cuticle deposition in these tissues. It seems surprising that natural selection would allow the wasted energy required for expressing the *amd* gene when it is not needed. A possible explanation is that regulatory evolution likely capitalizes on transcriptional machinery that is already in place, so that the adaptive advantage of using an existing mechanism for getting *amd* expressed may outweigh the apparent waste of energy in early expression. All the cuticle-secreting tissues are of ectodermal origin. Thus, it is possible that early expression of *amd* utilizes part of the regulatory circuitry of ectodermal tissues. It is also possible that some function other than cuticle sclerotization specifically requires early *amd* expression in the primordia of these tissues, and we are simply unaware of this function. A rigorous experimental test of such a putative function is not possible since maternal contribution may carry early embryos past a phenocritical period, and elimination of *amd* in the germ line leads to the production of eggs that cannot initiate development (24).

Third, completely novel patterns of *amd* expression can appear (or disappear) rapidly in these species even in the absence of an apparent function. *D. simulans* exhibits *amd*

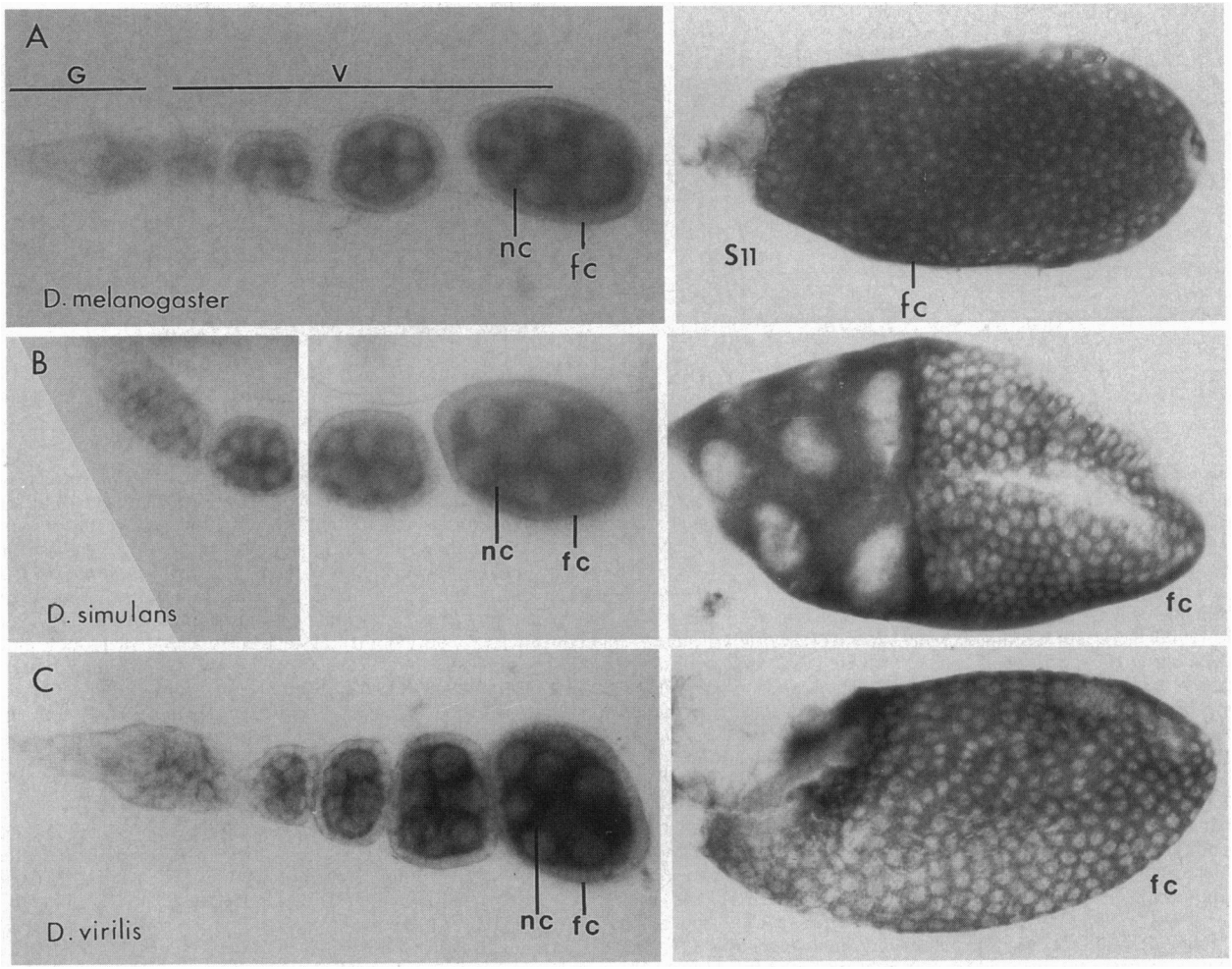

FIG. 3. Comparison of *amd* expression during oogenesis in *D. melanogaster*, *D. simulans*, and *D. virilis*. The expression pattern of *amd* in ovaries was revealed by whole-mount *in situ* hybridization. (*A*) *amd* mRNA is expressed in germ line nurse cells (nc) and somatic follicle cells (fc) of *D. melanogaster*. Expression is detected in developing egg chambers in both the germarium (G) and vitellarium (V). Note that the nuclei of the follicle cells occupy almost the entire cell and, therefore, the stain is evident only as a narrow ring of cytoplasm around the nuclei when viewed laterally. The surface view of the egg chambers [e.g., the stage 11 (S11) egg in *A*] illustrates this as a ring of stain around each follicle cell nucleus. (*B*) *amd* expression in ovaries of *D. simulans*. Again, both nurse cells and follicle cells are stained. (*C*) *amd* expression in ovaries of *D. virilis*. In all three species, expression is already detected in both follicle cells and nurse cells of the earliest egg chambers emerging from the germarium.

expression in the developing embryonic CNS, whereas such expression is not apparent in *D. melanogaster* or *D. virilis*. This is unexpected given that the *D. melanogaster* and *D. simulans* lineages have diverged from each other only recently compared with the *D. virilis* lineage. A role for catecholamine metabolizing genes to produce neurotransmitters in the CNS is well established. The *dopa decarboxylase* gene is conserved even between flies and man and is expressed in the CNS and peripheral nervous system of insects and mammals. It would appear likely that the CNS function of the *Ddc* gene is ancient, since even the simplest animals have neural networks utilizing catecholamines; in fact, even plants have an enzyme structurally related to *Ddc* (36). However, a potential function for the transient expression of *amd* in the developing nervous system of *D. simulans* is elusive. The expression occurs before functional organization of the nervous system. Interestingly, there are genes which appear to be expressed in this subset of developing CNS cells in *D. melanogaster*, suggesting that *amd* in *D. simulans* may have simply acquired the regulatory sequences to respond to an existing constellation of transcription factors that are present in those cells. It should be noted that *amd* is expressed later in the nervous system of the mature larva. There may be no selectable function for the expression of *amd* in the developing *D. simulans* nervous system as yet, but the target for loss of this expression is likely to be physically extremely small (e.g. on the order of 10 bp or small multiples thereof) and perhaps has not yet been acted upon by selective forces. If *amd* expression in the embryonic CNS of *D. simulans* has a functional role, it would be instructive to discover it, since evolutionary acquisition of a new function for a preexisting gene is thought to be much rarer than the evolutionary loss of function in a duplicated gene.

In *D. melanogaster*, *amd* expression starts at the anterior border of each segment and then spreads to the posterior border, but in *D. simulans* and *D. virilis* it appears simultaneously at both the anterior and posterior borders (Fig. 1 *E*, *K*, and *Q*). Because *D. simulans* and *D. melanogaster* are much more closely related to each other than they are to *D. virilis*, evolutionary parsimony makes it more likely that the pattern of expression observed in *D. virilis* and *D. simulans* be the ancestral one. But why the new pattern of expression would have evolved in *D. melanogaster* in the short interval (2–5 million years) since its divergence from *D. simulans* remains unknown. Is this an adaptive change of unknown significance, or is it an adaptively neutral consequence of an accidental change in the regulatory circuitry of the gene?

Gene duplication followed by acquisition of distinct patterns of expression in the duplicated genes is a common event in evolution. The *Ddc* and *amd* genes are paralogous genes resulting from a gene duplication event that share 61% sequence similarity at the protein level (refs. 15 and 32; GenBank accession nos. X04426 and X04695). Both are essential for viability, but they have evolved completely nonoverlapping functions. *amd* is required for production of soft flexible cuticle (21, 24, 26–29) and for maturation of the vitelline membrane (24). It is also expressed in the CNS of the larva (26), although its catalytic role is not known (37). *Ddc* activity is required to produce the neurotransmitters, dopamine and serotonin, and for sclerotization of pigmented cuticle (21, 29). Other examples of duplication followed by divergence of expression include several globin genes that have evolved nonoverlapping patterns of expression. Notably, some duplicates, such as the δ-globin gene in humans, are hardly expressed and contribute only minimally to the globin pool even when the β-globin gene is lost; yet, the δ locus has been retained. These and other examples of genes that appear to be dispensable but are retained and highly regulated suggest that the pressure to eliminate a nonessential function may be small. If this is true, the conjecture that phenotypic differences may result from changes in the regulation of structural genes rather than in changes in the protein sequences themselves may be supported by the sudden appearance of a completely new and specific pattern of *amd* expression in *Drosophila simulans*.

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