Genetic dissection of the learning/memory gene *dunce* of *Drosophila melanogaster*

Yuhong Qiu and Ronald L. Davis^{1,2}

Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030 USA.

The *dunce* (*dnc*) gene of *Drosophila melanogaster* codes for cAMP phosphodiesterase (PDE) and is required for normal learning/memory and for female fertility. The expression of the gene is elevated in mushroom bodies, brain structures implicated in olfactory learning and memory. In this study several chromosomal deletions and inversions that remove increasingly larger portions of the *dnc* gene from its 5' end and progressively more of the five known transcription start sites (tss) were used to assess the functions of the various transcriptional units. Surprisingly, the *dnc* PDE activity, female fertility, mushroom body expression, learning, and memory were unaffected by the removal of tss1 and tss2. tss3 was required for elevated mushroom body expression but not for female fertility nor initial learning. tss4 contributed to learning and the female fertility function, whereas tss5 contributed to female fertility. The results indicate that the structural complexity of the gene is of biological significance, with individual transcriptional units serving different biological functions.

[Key Words: *dunce* gene; cAMP phosphodiesterase; mushroom bodies; learning/memory]

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Previous studies of the *dunce* (*dnc*) gene of *Drosophila melanogaster* have shown that it is among the most complex of eukaryotic genes characterized to date (Davis and Davidson 1986; Chen et al. 1987; Qiu 1991; Qiu et al. 1991). It extends over >148 kb of the *Drosophila* genome, contains at least 19 exons, and encodes a minimum of 10 RNAs ranging in size from 4.2 to 9.6 kb. Five classes of *dnc* transcripts have been identified from cDNA cloning, with each originating from a distinct transcription start site. Alternative splicing patterns of RNAs from these five transcriptional units predict a minimum of eight protein products, which from sequence homology (Chen et al. 1986), direct expression (Qiu et al. 1991), and biochemical defects exhibited by *dnc* mutants (Byers et al. 1981; Davis and Kiger 1981) are known to be isoforms of the enzyme cAMP phosphodiesterase (PDE). In addition, at least seven genes are nested within two separate and large introns of *dnc* (Chen et al. 1987; Furia et al. 1990, 1993). Six of these intronic genes [Sgs-4, Pig-1, and nested genes (ngs) 1, 2, 3, and 4] have been characterized and appeared to be unrelated to *dnc* function, because they are expressed primarily during larval stages and mainly within salivary glands. This expression pattern is incongruent with the major phenotypes produced by *dnc* mutation (see below).

In addition to the structural complexity, the biological

functions exerted by the gene are diverse. Mutations in the *dnc* gene result in two major phenotypes: One is female sterility. Females carrying *dnc* null alleles lay very few eggs. The egg-laying defect has been attributed to a requirement for *dnc*⁺ activity in somatic cells, because mosaic females with *dnc* PDE activity in somatic tissues but absent in the germ line can produce and deposit eggs (Bellen et al. 1987). The eggs laid, however, fail to develop, owing to defects in DNA replication, mitosis, and nuclear migration (Bellen and Kiger 1988; Whitehouse-Hill et al. 1992). Therefore, the *dnc* PDE is required in somatic cells for egg deposition and in germ cells for proper zygotic development.

A second class of phenotype expressed by *dnc* flies includes defects in conditioned behavior (Dudai et al. 1976; Dauwalder and Davis 1991; for review, see Dudai 1988). The *dnc* mutant flies are defective in acquisition and/or short-term memory when tested in several different olfactory associative learning situations, with negative (Dudai et al. 1976; Dudai 1983; Tully and Quinn 1985) or positive reinforcement (Tempel et al. 1983). In addition, *dnc* flies fail to perform normally in the nonassociative learning situations of habituation and sensitization (Duerr and Quinn 1982). The deficit in more than one form of learning suggests that the *dnc* gene product is involved in fundamental processes underlying learning/memory. Two physiological phenotypes have been observed that are potentially related to the deficits in conditioned behavior. First, Delgado and colleagues discovered a cAMP-activated K⁺ channel in larval muscle

¹Corresponding author.

²Present address: Center for Learning and Memory, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 USA.

that is persistently activated in *dnc* mutants (Delgado et al. 1991). Second, certain types of synaptic plasticity at the neuromuscular junction are compromised in *dnc* mutants (Zhong and Wu 1991).

Immunohistochemical studies (Nighorn et al. 1991) have shown that the *dnc* PDE is concentrated in the mushroom body neuropil and is found at a low level in the general neuropil of the adult brain. It is also found at a homogeneously low level in the neuropil of the thoracic ganglia and the abdominal ganglia. RNA in situ hybridization has shown that *dnc* RNAs are enriched in mushroom body perikarya (Nighorn et al. 1991), suggesting that the elevated mushroom body expression of the *dnc* gene is attributable, at least in part, to transcriptional control and/or RNA stability. These studies are consistent with electrophysiological work and genetic and physical lesioning experiments, which suggest that mushroom bodies play an important role in insect information processing and integration (Erber et al. 1980; Schildberger 1984; Heisenberg et al. 1985). However, the question of whether the elevated mushroom body expression of the *dnc* gene is important for some part of learning/memory processes has not been addressed.

The structural complexity and the functional diversity of the *dnc* gene prompted us to investigate their relationship. We took advantage of the numerous existing chromosomal deletions and inversions in the region, which alter various portions of the *dnc* gene at its 5' end. Flies carrying the rearranged chromosomes were assayed for *dnc* PDE activity, female fertility, adult brain expression, initial learning, and 90-min memory in a classical conditioning paradigm. We assessed the function(s) of various transcriptional units by correlating the phenotypes with the deletion of specific transcriptional start sites (tss). We found that several transcriptional units contribute a unique increment to the overall expression pattern and are involved in specific biological functions. The deficiency analysis also allowed us to study the relationship between the elevated mushroom body expression of the *dnc* gene and the learning/memory ability of the flies.

Results

Notchchromosomal rearrangements

A total of 10 chromosomal rearrangements were used in this study, all selected from studies on the *Notch* gene, which is adjacent to *dnc* (Fig. 1). The right breakpoints of three of the rearranged chromosomes, *Df(1)N^{68f19}*, *In(1)N^{76b8}* and *Df(1)N^{54l9}*, were mapped previously by other groups (McGinnis et al. 1980; Artavanis-Tsakonas et al. 1983; Kidd et al. 1983). The right breakpoint of *Df(1)N⁶⁴ⁱ¹⁵* was mapped in an early *dnc* study (Davis and Davidson 1984). Because the right breakpoint of *In(1)N^{82k}* is within the *Notch* gene, it was not mapped further. The right breakpoints of the remaining five rearrangements were mapped by genomic blotting experiments by use of probes across the *dnc* locus (data not shown). Their precise locations on a restriction map of

this region are shown in Figure 2. The right breakpoint of *Df(1)N^{75j31}* falls within an uncloned region between exons 0.9 and 1.0.

The rearrangements were classified into five groups (Fig. 1) according to the positions of their right breakpoints relative to the known transcription start sites. The first group includes *Df(1)N^{68f19}* and *In(1)N^{82k}*. The right breakpoints of these chromosomes fall within the *Notch* locus so that the *dnc* locus is most likely left intact. *Df(1)N^{81k}*, *Df(1)N^{75j31}*, *In(1)N^{76b8}*, and *Df(1)N^{54l9}* form a second group and remove tss1 and tss2. *Df(1)N^{79f}* is the sole member of the third group and it removes tss1, tss2, and tss3. The fourth group [*Df(1)N⁶⁴ⁱ¹⁵* and *Df(1)N^{69h9}*] removes tss1–tss4. In *Df(1)N^{81l6}*, all five transcription start sites are removed.

Because each *Notch* chromosome was obtained in an unidentified genetic background, each stock was Cantonized by backcrossing to Canton-S for eight generations, allowing for free recombination of the X chromosomes to minimize the effect of genetic background. The chromosomal translocation *w⁺Y* (McGinnis et al. 1980) was then introduced to cover the *Notch* lethality. *Df(1)N⁻/w⁺Y* flies were established into stocks with an attached-X chromosome. Males from the stocks were used in all subsequent studies except for the immunohistochemical and female fertility experiments. For the immunohistochemical studies, the *Notch* lethality was covered by CosP479BE, a third chromosome transposon containing the *Notch* gene (Ramos et al. 1989).

PDE activity

Because *dnc* is the structural gene for the enzyme cAMP PDE (Chen et al. 1986; Qiu et al. 1991), the *dnc* PDE activity was assayed in whole-fly homogenates of each rearrangement stock to determine the contribution of each transcriptional unit to overall *dnc* PDE activity. No change in *dnc* PDE activity was observed after removing tss1 and tss2 (Fig. 3), suggesting that the RNAs initiated at these sites contribute little to the total *dnc* PDE activity. Removal of tss3 resulted in a 46% decrease in *dnc* PDE activity, suggesting that the RNAs transcribed from tss3 contribute about half of the total *dnc* PDE activity. Elimination of tss4 did not decrease the *dnc* PDE activity further, indicating that it contributes little to the total *dnc* PDE activity. Removal of all the sites including tss5 resulted in a complete lack of *dnc* PDE activity, suggesting that the RNAs transcribed from tss5 are responsible for the other half of overall *dnc* PDE activity. Therefore, the RNAs transcribed from tss3 and tss5 are the major contributors to *dnc* PDE activity.

Female fertility

One of the phenotypes exhibited by *dnc* flies is female sterility (Byers et al. 1981; Salz et al. 1982; Bellen et al. 1987). To determine which tss confers female fertility, each rearranged chromosome was placed in *trans* to *dnc^{M14}*, an amorphic allele (Davis and Kiger 1981; Salz et al. 1982). The resulting females were assayed for fertility.

Figure 1. Schematic representation of the right breakpoints of the *Notch* rearrangements in the *dnc* genomic region. An arbitrary coordinate system extending from -90 to $+47$ and measured in kilobases is illustrated (Davis and Davidson 1984) but omits 7.3 kb of sequence contained in a transposable element inserted in the Canton-S strain between coordinates 2 and 5. The position of exon 0.9 at approximately -90 is estimated (see Qiu et al. 1991) because the complete region between exons 0.9 and 1 has not been cloned. Exons of the *dnc* gene are represented as numbered boxes. They are not drawn to scale. A portion of the *Notch* gene is represented by the open box. Long arrows indicate the approximate locations of the right breakpoints of the deficiency or inversion chromosomes. Chromosomal rearrangements are represented by their abbreviated names [i.e., 82k instead of *In1N*^{82k}]. The rearrangements are classified into five different groups indicated by brackets and a group number. Splicing patterns of five classes of RNAs (I–V) and the transcription start sites tss associated with the known transcriptional units are shown (see Qiu et al. 1991; Qiu 1991). The putative open reading frame in each class is defined by ATG and STOP. tss1 has not been mapped and tss5 has been putatively delimited to a 10-kb region by breakpoint mapping of *Df(1)N*^{69h9} and a recently recovered cDNA clone (Qiu 1991).

The results (Fig. 4) showed that the elimination of the first three tss did not affect female fertility. The fertility decreased dramatically, however, in *Df(1)N*^{64j15}/*dnc*^{M14} and *Df(1)N*^{69h9}/*dnc*^{M14} females and was eliminated in *Df(1)N*⁸¹¹⁶/*dnc*^{M14}. This suggests that the RNAs initiated at tss4 and tss5 both are required for normal female fertility. Interestingly, although *Df(1)N*^{64j15}/*dnc*^{M14} and *Df(1)N*^{69h9}/*dnc*^{M14} females generated few progeny, most laid numerous eggs while *Df(1)N*⁸¹¹⁶/*dnc*^{M14} females were deficient in egg laying (not shown).

Immunohistochemistry

Drosophila mushroom bodies are brain structures housing ~2500 perikarya (Technau and Heisenberg 1982; Balling et al. 1987) located in the dorsal and posterior

cortex of each brain hemisphere along with their axonal and dendritic processes. The cells send dendrites ventrally to form the calyx, where extrinsic fibers make contacts with the mushroom body. Their axons pass in a highly ordered and parallel fashion in a ventral and anterior direction to form the peduncle. Each peduncle divides near the anterior margin of the brain. One branch extends dorsally to form the α -lobe; the other extends medially to form the β - and γ -lobes. Many extrinsic fibers link the lobes to other parts of the brain (Schildberger 1983, 1984).

Previous studies have shown that *dnc* PDE immunoreactivity is concentrated in the mushroom bodies of the adult brain but is expressed at a low level throughout most brain neuropil (Nighorn et al. 1991). To determine whether the RNAs initiated at particular transcription

Figure 2. Restriction map of *dnc* genomic region and the right breakpoints of the *Notch* rearrangement chromosomes. The coordinate system numbers shown here correspond to those in Fig. 1, except the 7.3-kb transposable element is included (the broken line between coordinates +2 and +5). The restriction enzymes from which the map was constructed are at the left end of each row. The sizes of the fragments are indicated by the numbers (in kilobases) above the line. The coordinate line is continued from the first row to the fifth row with some overlap at the end of the first row/beginning of the second row and the end of the second row/beginning of the third row. Exons of *dnc* are represented as numbered solid boxes. They are not drawn to scale. The genomic fragments to which the right breakpoints of the rearrangements were mapped are indicated by the double-headed lines with the names of the rearrangements underneath. The right breakpoint of a chromosome translocation, $w^+ Y$, is also shown (McGinnis et al. 1980; Davis and Davidson 1984). The shaded box within the 3.1-kb *Hind* III fragment is the 2.2-kb *Xho*I fragment that contains a putative mushroom body enhancer. The positions and direction of transcription of seven intronic genes are indicated by arrows above the restriction map. The directions of the centromere and the telomere are indicated by arrows. The gene *indnc* has only been defined by RNA blotting experiments (Chen et al. 1987).

start sites are responsible for the elevated *dnc* PDE in the mushroom bodies, immunohistochemistry was used to examine adult brain expression in Canton-S and *Notch* rearrangement flies mounted side by side in fly collars (Heisenberg and Bohl 1978; Nighorn et al. 1991). Although the immunohistochemistry results are not objectively quantifiable, the experiment was performed a minimum of three times for each genotype and each time multiple flies (12–20) of a given genotype were examined to gain a subjective impression of the relative staining level.

Canton-S showed intense staining in mushroom body neuropil, including the calyces, the peduncles, and the lobes, and intermediate staining in most other brain neuropil (Fig. 5A–C; Nighorn et al. 1991). The same staining

pattern and intensity was observed in the members of group 1, and also in *Df(1)N^{81k}* and *Df(1)N^{75j31}* of group 2 (not shown), suggesting that the RNAs initiated from *tss1* and *tss2* are unimportant for the proper expression of the *dnc* gene in the adult brain. For *In(1)N^{76b8}* and *Df(1)N^{54l9}* flies, about half the number of flies examined showed slightly lower and less uniform staining in mushroom bodies compared with Canton-S. The remainder appeared similar to Canton-S (Fig. 5D). The conservative interpretation is that the expression might be slightly decreased, although the rearranged chromosomes do not remove any *tss* unaffected by *Df(1)N^{81k}* and *Df(1)N^{75j31}* (Fig. 1). The *Df(1)N^{79f}* rearrangement dramatically decreased staining in the calyces, the peduncles, and the lobes of *Df(1)N^{79f}* flies (Fig. 5E–G) to a

Figure 3. Bar graph of *dnc* PDE activity. The PDE activity of each stock is expressed as picomoles of cAMP hydrolyzed per minute per milligram of fly. The stocks marked with an asterisk (*) are significantly different from Canton-S ($P < 0.05$) as determined by one-way analysis of variance. Error bar, S.E.M. The reason for the elevated levels in *Df(1)N^{75j31}* and *In(1)N^{76b8}* is unknown, although genetic background differences could account for that in *In(1)N^{76b8}* (see text).

level approximating that in most of the brain neuropil. This indicates that *tss3* is of special importance for the elevated *dnc* gene expression in the mushroom bodies. The *Df(1)N^{64j15}* flies showed no staining (Fig. 5H). Thus, the RNAs initiated from *tss4* are likely required for the general neuropil expression found in *Df(1)N^{79f}*, whereas those from *tss5* contribute little, if any, to the expression of the *dnc* gene in the adult brain.

Initial learning/memory

It was important to determine whether the elevated mushroom body expression correlates with learning/memory ability. An olfactory classical conditioning paradigm was used in which olfactory cues were coupled with the negative reinforcement of electric shocks. We define the scores obtained as soon as possible (~3 min.) after training as initial learning, although these scores reflect both acquisition and very short-term memory. Scores obtained at 90 min after training were taken as an indication of memory, although these scores must reflect acquisition, short-term memory, and a longer-term memory, because this time point is past the 0- to 30-min window in which memory is disrupted by anesthesia (Quinn and Dudai 1976).

Three conclusions can be drawn from the initial learning data (Fig. 6). First, the learning indices of *Df(1)N^{81k}*, *Df(1)N^{75j31}*, and *Df(1)N⁵⁴¹⁹* were not significantly different from Canton-S, suggesting that removal of *tss1* and *tss2* has no effect on initial learning. Second, the initial

learning index of *Df(1)N^{79f}* was not significantly different from Canton-S, indicating that the RNAs transcribed from *tss3* are not required for normal initial learning. Thus, the surprising extension of this conclusion is that elevated mushroom body expression is not required for normal levels of initial learning. Third, *Df(1)N^{64j15}* had significantly lower initial learning scores, suggesting that *tss4* is required for normal initial learning. As demonstrated previously, this *tss* contributes to the general neuropil expression of the *dnc* gene. Thus, expression in one or more areas of the general neuropil is apparently required for normal initial learning.

The *In(1)N^{76b8}* flies showed initial learning values significantly lower than flies carrying deficiency chromosomes with breakpoints on the left or right of *In(1)N^{76b8}*. This may be attributable to genetic background, because *In(1)N^{76b8}* is a chromosomal inversion that likely escaped the degree of Cantonization obtained with the chromosomal deletions. This interpretation can also be extended to the anomalously high level of PDE observed in this stock (Fig. 3).

The dispensability of elevated mushroom body expression for initial learning prompted us to study its role in memory. Four conclusions can be drawn from the results of 90-min memory tests (Fig. 7). First, the 90-min retention of *Df(1)N^{81k}* and *Df(1)N^{75j31}* was not significantly different from Canton-S, suggesting that *tss1* and *tss2* are not required for 90-min memory. Second, the score for *Df(1)N^{79f}* was significantly lower than Canton-S, suggesting that the genomic sequence between the break-

Figure 4. Female fertility. The genotypes marked with an asterisk (*) are significantly different from Canton-S ($P < 0.001$). Error bar, S.E.M. (n) The number of females tested.

Figure 5. Immunohistochemistry. Frozen sections of adult brains were stained with an affinity-purified anti-*dnc* PDE antibody (Nighorn et al. 1991) and visualized by peroxidase staining. (A–C) Canton-S; (D) *In(1)N^{76b8}*; (E–G) *Df(1)N^{79f}*; (H) *Df(1)N⁶⁴ⁱ¹⁵*. The lethality of each rearrangement chromosome was rescued using the third chromosomal *Notch*⁺ minigene, *cosP479BE*. [ca] Calyx; [p] peduncle; [β, γ] β-lobe and γ-lobe. The staining intensity shown in D was among the highest observed for this genotype.

Figure 6. Initial learning. Males from each stock were trained as populations and the initial learning indices were graphed. The stocks marked with an asterisk (*) have initial learning values significantly different from Canton-S ($P < 0.05$). (n) The number of times each stock was trained. Error bar, S.E.M.

points of *Df(1)N^{75j31}* and *Df(1)N^{79f}*, potentially tss3, is required for normal 90-min memory. Third, the memory scores for *Df(1)N⁵⁴¹⁹* and *In(1)N^{76b8}* were intermediate between Canton-S and *Df(1)N^{79f}*. These rearrangements may disrupt regulatory sequences required for efficient expression from tss3 (see Discussion). Fourth, *Df(1)N^{64j15}* flies had a very low memory score consistent with the poor initial learning.

RNA blots

If a tss is removed in a given rearranged chromosome, its corresponding transcripts should be eliminated. Conversely, if a tss is retained along with its regulatory elements, the corresponding transcripts should be present as in the wild type. To confirm this, RNA blot analysis was performed on *In(1)N^{76b8}*, *Df(1)N^{79f}*, and *Df(1)N^{64j15}* flies, representing groups 2, 3, and 4 respectively. Because several of the *dnc* RNAs transcribed from different tss have similar sizes, exon-specific probes were utilized to determine the presence or absence of specific transcripts. The blot was reprobed with different exon-specific probes and, finally, with an actin 5C probe to normalize the amount of RNA in each lane.

The *dnc* gene encodes an extremely complex set of rare RNAs, ranging in size from 4.2 to 9.6 kb (Davis and

Davidson 1986; Qiu et al. 1991). Classes I and II RNAs have been measured previously with a major transcript of 7.2 kb and a minor one of 9.6 kb (Qiu et al. 1991). When hybridized with single-stranded probe representing exon 1, the two expected transcripts were detected in Canton-S but were absent in the three stocks carrying rearranged chromosomes (Fig. 8A). This was predicted because tss1 and tss2 are removed in all three chromosomal rearrangements.

Class III transcripts include a relatively abundant 7.0-kb transcript and a rarer transcript of 9.5 kb as shown by probing Canton-S RNA with an exon 2.3-specific probe (Fig. 8B, lanes 1 and 2). The RNAs are conserved in *In(1)N^{76b8}* flies (Fig. 8B, lane 3), suggesting that tss3 is functional in the inversion chromosome, at least when considering whole-body RNA expression. In contrast, *Df(1)N^{79f}* and *Df(1)N^{64j15}* flies lack these transcripts as predicted from the absence of tss3 in these two deficiencies (Fig. 8B, lanes 4, and 5).

A single-stranded probe specific to exon 2.8 was used to display class IV transcripts. Exon 2.8 was used as a probe because identical RNAs are detected with exon 2.7-specific probes, although the background is considerably higher with the latter (Qiu et al. 1991). The RNA blot was very complex (Fig. 8C). Two major RNAs, 9.6 kb and 7.4 kb, and two minor RNAs, 7.0 kb and 6.7 kb,

Figure 7. Memory at 90 min. Males of each stock were trained as populations, and the memory indices obtained at 90 min after training were plotted. Bars marked with an asterisk (*) are significantly different from Canton-S ($P < 0.05$). (n) Number of times trained. Error bar, S.E.M.

Figure 8. RNA blots. Exon-specific probes were used to hybridize to adult poly(A⁺) RNA blots prepared from several rearrangement stocks and Canton-S flies. (A) Exon 1-specific probe; (B) exon 2.3-specific probe; (C) exon 2.8-specific probe; (D) probe for the protein coding region (exon 3 to the 5' half of exon 13); (E) actin 5C probe as internal control. (Lanes 1,2) Canton-S; (lane 3), *In(1)N^{76b8}*; (lane 4) *Df(1)N^{79f}*; (lane 5) *Df(1)N^{64j15}*. Although the 6.7-kb RNA in *In(1)N^{76b8}* and *Df(1)N^{79f}* was faint (C, lanes 3,4), its existence was confirmed on a separate RNA blot (not shown). The RNAs observed in C are not observed in D as a result of the different exposure times for the two panels: 5 days for C and 1 day for D. *In(1)N^{76b8}* showed a pattern similar to Canton-S (D, lanes 1–3), although they are missing class I and class II transcripts because these transcripts are low in abundance and similar in size to the transcripts in the other classes. The slightly faster mobility of the 9.5-kb RNA in *In(1)N^{76b8}* (D, lane 3) was specific to this blot and was not observed on a separate blot (not shown). The 9.6-kb RNA in *Df(1)N^{79f}* (D, lane 4) was clearly visible on a separate blot (not shown).

are transcribed from *tss4*. They exist in Canton-S, *In(1)N^{76b8}*, and *Df(1)N^{79f}*, but not in *Df(1)N^{64j15}*, as predicted. Two faint RNAs at ~4.2 kb cannot be *dnc* RNAs because they were detected in *Df(1)N^{64j15}*, which lacks exon 2.8 (Fig. 8C, lane 5). The origin of the 5.0-kb RNA in Canton-S is not clear.

Class V RNAs are best visualized in *Df(1)N^{64j15}*, in which all of the RNAs transcribed from other known start sites are eliminated. When a probe specific to the common region of *dnc* RNAs was used (Fig. 8D), three RNAs were observed in *Df(1)N^{64j15}*: a major RNA of 5.0 kb and two very rare RNAs of 7.4 and 4.2 kb (Fig. 8D, lane 5). The same set of RNAs were observed in *In(1)N^{76b8}* and *Df(1)N^{79f}*, along with additional RNAs. This identifies the *dnc* RNAs of 5.0 and 4.2 kb as arising from a *tss* residing to the right of the *Df(1)N^{64j15}* breakpoint (*tss5*). The 7.4-kb RNA, however, was not present in Canton-S. It is possible that the deficiencies remove a distal inhibitory element allowing for some novel transcription after other upstream transcription start sites are removed.

In summary, *tss1* and *tss2* produce RNAs of 9.6 and 7.2 kb, *tss3* produces RNAs of 9.5 and 7.0 kb, *tss4* produces RNAs of 9.6, 7.4, 7.0, and 6.7 kb, and *tss5* produces RNAs of 5.0 and 4.2 kb. And, although the RNA blots are complex as a result of the numerous and rare RNAs, in general, those RNAs expected to be present in the various rearrangements on the basis of breakpoint location are present, and those expected to be absent are absent.

Discussion

Because the *dnc* gene has multiple transcription start sites and confers multiple functions, it was conceivable that different biological functions might be fulfilled by different transcriptional units. This is indeed the case.

Several *Notch* alleles that remove increasingly larger portions of the *dnc* gene from its 5' end were assayed for *dnc* PDE activity, female fertility, adult brain expression, initial learning, and 90-min retention to dissect the function of different transcriptional units. These results are summarized in Figure 9. The results showed that *tss3* and *tss5* each contribute to about half of the total PDE activity. The elevated mushroom body expression is attributable to *tss3*. The *tss4* is responsible for general neuropil expression, initial learning, and female fertility, although the PDE assays indicate that *tss4*-directed expression contributes a negligible portion of the total *dnc* PDE. Complete female fertility also requires *tss5*. Thus, several of the transcriptional units contribute a unique increment of the overall expression pattern and are involved in specific biological functions, demonstrating that the structural complexity of the *dnc* gene is biologically significant.

The removal of *tss1* and *tss2* did not result in any observable phenotypes. There are several possible explanations for this unexpected result. First, the RNAs from *tss1* and *tss2* could be expressed in many cell types and confer a redundant function with downstream transcription start sites. Hence, the function of *tss1* and *tss2* might only become obvious if downstream transcription start sites are inactivated. Second, *tss1* and *tss2* could be important for subtle functions that have not yet been detected. Third, they could be functionless, perhaps having been fortuitously recruited during evolution. Studying the *dnc* gene structure in other *Drosophila* species might provide insights into the last possibility.

The *dnc* PDE activity is required in somatic cells for egg deposition and in the germ line for proper development of the zygotes (Bellen et al. 1987). It is possible that the somatic requirement and the germ-line requirement are controlled separately. The observation that many

Figure 9. Summary of the functions conferred by each transcriptional unit. Exons are represented by solid boxes on the top line. The positions and sizes of the exons are not drawn to scale. The potential open reading frame predicted from each class is marked by ATG and STOP.

eggs were laid by *Df(1)N^{64j15}/dnc^{M14}* and *Df(1)N^{69h9}/dnc^{M14}* females but few of these hatched raises the possibility that RNAs initiated from tss5 are primarily required for egg deposition, whereas those from tss4 might be primarily required for zygotic development.

Although class IV transcripts are involved in initial learning and female fertility, these functions might be dissociable among the several transcripts initiated at tss4. There are three subclasses of *dnc* transcripts (one in IVA and two in IVB) initiated at tss4; each of them could potentially generate a unique *dnc* PDE isoform (see Fig. 1). Different transcripts could have different post-transcriptional or -translational control, or generate translation products with different modifications or stabilities. This would enable a specific isoform to be produced preferentially over others in certain types of cells to fulfill its unique function.

Several previous observations have suggested that mushroom bodies are loci for information processing and integration in insects. First, mushroom bodies receive multiple types of sensory information (Erber 1978; Schildberger 1984). For example, electrophysiological studies have shown that mushroom body cells in the cricket depolarize in response to olfactory, mechanical, gustatory, and visual cues (Schildberger 1984). Thus,

mushroom bodies are electrophysiologically capable of information processing and integration. Second, local cooling of the mushroom bodies after olfactory conditioning impairs memory in the honeybee, *Apis mellifera* (Erber et al. 1980). Third, two *Drosophila* mushroom body structure mutants, *mushroom bodies deranged* (*mbd*) and *mushroom bodies miniature* (*mbm*), are defective in olfactory conditioning (Heisenberg et al. 1985). Fourth, the products of the two best characterized learning/memory genes in *Drosophila*, *dnc*, and *rutabaga* (structural gene of Ca^{2+} -calmodulin-sensitive adenylate cyclase; Livingston et al. 1984; Feany 1990; Levin et al. 1992), are concentrated in the mushroom bodies (Nighorn et al. 1991; Han et al. 1992). Together, these observations strongly suggest that mushroom bodies are major anatomical loci for learning/memory.

One objective of this study was to attempt to correlate learning and memory ability with *dnc* PDE expression in the mushroom bodies. The results were unexpected. The normal initial learning displayed by *Df(1)N^{79f}* flies showed clearly that enhanced mushroom body expression is not required for this behavior. This is in contrast to the low initial learning of *Df(1)N^{64j15}* flies in which the *dnc* PDE expression in the brain is completely eliminated and to the low initial learning of other *dnc* alleles

(Tully and Quinn 1985). We offer two possible explanations. Initial learning (acquisition and short-term memory) may not require mushroom body participation. For example, acquisition and short-term memory might be mediated completely by the antennal lobes, the central complex (Heisenberg et al. 1985), or other structures, with mushroom bodies serving later stages of memory. Therefore, the initial learning scores would be unaffected by a reduction of *dnc* PDE in the mushroom bodies but would be affected by a reduction of *dnc* PDE in other brain structures, such as in *Df(1)N⁶⁴¹⁵* flies and in flies carrying point mutation at the gene. Alternatively, physiological processes leading to initial learning might occur in the mushroom bodies but without a requirement for high levels of *dnc* PDE. That is, the low level of *dnc* PDE in the mushroom bodies of *Df(1)N^{79f}* flies might be sufficient for normal initial learning.

Is enhanced mushroom body expression required for 90-min retention? The poor memory and reduction of mushroom body expression in *Df(1)N^{79f}* flies is consistent with this possibility. The *Df(1)N⁵⁴¹⁹* and *In(1)N^{76b8}* flies, however, showed poor retention but clearly had elevated mushroom body expression, although the expression was less uniform than normal flies. Although we cannot eliminate the possibility that some element, unrelated to mushroom body expression, has been eliminated in these flies, which leads to a memory deficit, we favor the hypothesis that enhanced mushroom body expression is important for memory for the following reason: The right breakpoints of both *Df(1)N⁵⁴¹⁹* and *In(1)N^{76b8}* are within a 2.2-kb *XhoI* fragment (Fig. 2) that has been demonstrated to have mushroom body enhancer activity on a minimal promoter/reporter gene construct in transgenic flies (Qiu 1991; Y. Qiu and R. Davis, unpubl.). It is conceivable that the function of the enhancer in these two stocks is crippled by the proximity of the breakpoints so that the expression of the *dnc* gene in mushroom body cells is variably reduced.

Material and methods

Fly genetics

The original non-Cantonized *Notch* alleles listed in Table 1 were generously provided by B. Welshons (Iowa State Univer-

sity, Ames) except *y w^a Df(1)N⁵⁴¹⁹*; CosP479BE/TM6B was kindly provided by S. Artavanis-Tsakonas (Yale University, New Haven, CT) [CosP479BE is a P-element transposon containing the *Notch* gene (Ramos et al. 1989)].

The *w⁺* Y chromosome was placed into a Cantonized background by backcrossing *X^{CANTON-S}/w⁺* Y males to Canton-S females for several generations. The *y dnc^{M14} cv v f* was described previously (Byers et al. 1981; Mohler 1977) and is abbreviated as *dnc^{M14}* in the text. In female fertility assays, individual *N⁻/y dnc^{M14} cv v f* virgin females were placed in a vial with three Canton-S males for a 5-day period and the number of progeny were counted at day 20.

Breakpoint mapping

Because *Notch* lethality in almost all of the stocks was initially covered by a chromosomal duplication [*Dp(1;2)w⁺+51b7*], which also contains a complete copy of the *dnc* gene, the transposon, CosP479BE was introduced instead to cover the lethality to avoid the complications during breakpoint mapping. Genomic DNA was isolated from male flies of each chromosomal rearrangement stock (*N⁻*; CosP479BE x *y w f*: =) according to the procedure by Bingham et al. (1981). The DNA was digested and fractionated on an agarose gel side by side with Canton-S genomic DNA as a control. The DNA was then blotted onto GeneScreen Plus membrane and hybridized with phage genomic clones covering the *dnc* region.

Northern blot analysis

RNA isolation was as described previously using guanidinium-thiocyanate homogenization followed by banding in CsCl gradients (Davis and Davidson 1986). Poly(A)⁺ RNA was isolated by passing once over an oligo-(dT)-cellulose column. Ten micrograms of poly(A)⁺ RNA was loaded in each lane and fractionated on 1% formaldehyde-agarose gels. The RNA was then transferred onto Zeta-probe membrane and hybridized to random-primed or single-stranded probes according to the procedure by Davis and Davidson (1984). The single-stranded probes were prepared as described (Burke 1984).

PDE assay

The *dnc* PDE activity was assayed in crude fly homogenates as described previously by measuring the hydrolysis of ³H-labeled cAMP (Davis 1988). Excess cold cGMP was added to the reaction mixture to saturate the PDE, which can hydrolyze both cAMP and cGMP so that only the cAMP-specific *dnc* PDE activity was measured (Davis 1988). Adult flies in each stock were homogenized in groups of five. Each sample was assayed in triplicate. All of the stocks were assayed in each experiment.

Immunohistochemistry

Canton-S flies and flies carrying various *Notch* chromosomal rearrangements were mounted side by side in modified fly collars (Nighorn et al. 1991) to ensure equal treatment throughout the experiments. The heads were processed, sectioned, and stained with an affinity-purified anti-*dnc* PDE antibody as described previously (Nighorn et al. 1991). Streptavidin-peroxidase was used to visualize the spatial distribution of the first antibody.

Learning and memory tests

The classical conditioning apparatus used was a modification of the one described by Tully and Quinn (1985). Flies [5–10 days

Table 1. Chromosomal rearrangements and cytology

Genotype	Chromosomal breakpoints
<i>y w Df(1)N^{68f19}; Dp(1;2)w⁺+51b7</i>	3C1/2–3C6/7
<i>y w In(1)N^{82k}; Dp(1;2)w⁺+51b7</i>	3A2/3–3C7
<i>Df(1)N^{81k}; SM1, CyDp(1;2)w⁺+51b7</i>	3C5/6–3C9/10
<i>Df(1)N^{75j31} sn³; Dp(1;2)w⁺+51b7</i>	3C7/9–3C10/D1
<i>w^a In(1)N^{76b8}; Dp(1;2)w⁺+51b7</i>	3C7/8–3C9/10
<i>yw^a Df(1)N⁵⁴¹⁹; CosP479BE/TM6B</i>	3C5/6–3C10/11
<i>w^a Df(1)N^{79f}; Dp(1;2)w⁺+51b7</i>	3C7–3D1/2
<i>Df(1)N⁶⁴¹⁵; Dp(1;2)w⁺+51b7</i>	3C3/4–3D2
<i>Df(1)N^{69h9} rb; Dp(1;2)w⁺+51b7</i>	3C6–3D1/2
<i>w^a Df(1)N⁸¹¹⁶; Dp(1;2)w⁺+51b7</i>	3C7/9–3D2/3

old) were transferred into fresh food vials 12–24 hr before training. One-half hour before training, the flies were placed in the dark. Training and testing were carried out in the dark under red light at 23°C. A group of 100–120 flies were sequestered in a plexiglas training tube (7.6 cm long with a 1.2-cm internal diam.) lined with a 42 × 76-mm electrifiable grid. Fresh air was drawn through the training tube for 1 min, followed by air drawn over the surface of one odorant. The two odor cues, 3-octanol (OCT) and 4-methylcyclohexanol (MCH) (ICN K&K Laboratory), were delivered to the training tubes or testing apparatuses via air currents drawn by a vacuum source over the surface of the chemicals housed in bubbling flasks (Fisherbrand no. 11-184). Concurrent with the first odor, twelve 1.25-sec, 90 V square-wave pulses were delivered to the grid at 5-sec intervals. After flushing the tube with fresh air for 30 sec, the second odor was presented to the flies for 1 min without shocks. This was followed by 30 sec of fresh air. Air flow during training was 550 ml/min. For testing initial learning, the flies were transferred immediately into the sliding central compartment of a choice chamber and retained for 1 min. The circular central compartment is 2.1 cm in diameter and 0.5 cm in width. The sliding compartment is pushed to a point between two arms, each 2.1 cm in diameter and 10.5 cm in length and connected via silicon rubber tubing to a bubbling flask containing either OCT or MCH. The flies were allowed to disperse for 2 min from the central compartment into the two collection arms. For testing 90-min retention, the flies were transferred back into food vials, kept in the dark for 90 min, and placed into the choice chamber for testing. The learning score of each group of flies was calculated as the fraction of the flies avoiding the shock-associated odor minus the fraction of flies avoiding the control odor (Quinn et al. 1974). A learning index is the average of the learning scores of the two groups of flies: one shocked in the presence of MCH and the second shocked in the presence of OCT. The learning scores obtained were somewhat lower than others reported (Tully and Quinn 1985), perhaps because of the lack of humidity control or differences in the configuration of the apparatuses.

Statistical analysis

The data were analyzed by one-way ANOVA, followed by Tukey's multiple comparisons.

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Y Qiu and R L Davis

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