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Molecular analysis of cDNA clones and the corresponding genomic coding sequences of the *Drosophila* dunce$^+$ gene, the structural gene for cAMP phosphodiesterase

(learning and memory)

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ABSTRACT **We have isolated and sequenced cDNA clones representing portions of the polyadenylylated transcripts of the dunce$^+$ gene. These define an open reading frame of 1086 bases and some of the 5′- and 3′-untranslated regions of the transcripts. The deduced amino acid sequence is strikingly homologous to the amino acid sequence of a Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase isolated from bovine brain and more weakly related to the predicted amino acid sequence of a yeast cAMP phosphodiesterase. These homologies, together with prior genetic and biochemical studies, provide unambiguous evidence that dunce$^+$ codes for a phosphodiesterase. In addition, the dunce$^+$ gene product shares a seven-amino acid sequence with a regulatory subunit of cAMP-dependent protein kinase that is predicted to be part of the cAMP binding site. We also identify a weak homology between a region of the dunce$^+$ gene product and the egg-laying hormone precursor of *Aplysia californica*. The open reading frame is divided in the genome by four introns.**

Several mutants of *Drosophila melanogaster* defective in learning/memory processes have been isolated. The first and best studied of these are the mutants carrying lesions at the dunce (*dnc*) locus. These mutants show defective conditioned behavior in several different situations in which associative learning is evaluated (1–3). Careful examination of the initial level of learning after training *dnc* flies has revealed that they display appreciable learning, but they have an abbreviated memory of the associations they develop (3–5). Furthermore, *dnc* flies are abnormal in nonassociative learning (6) and in certain courtship behaviors that depend on prior experience (7–9). These findings suggest that the *dnc*$^+$ gene product plays a central role in the biochemical mechanisms underlying conditioned behaviors.

Clues of the biochemistry potentially underlying the memory dysfunction in *dnc* flies came from the studies of Byers *et al.* (10). Specifically, it was shown that *dnc* flies are deficient in the activity of one form of cAMP phosphodiesterase (PDEase), a member of the family of enzymes that hydrolyzes cyclic nucleotides to 5′-nucleotides. *Drosophila* has two major forms of cyclic nucleotide-dependent PDEase, each having a different molecular weight and substrate specificity and responding differently to the Ca^{2+}-dependent activator protein calmodulin (11). The *dnc* mutants exhibit an aberrant cAMP PDEase but have normal levels of the Ca^{2+}/calmodulin (Cam)-dependent PDEase (12–15). This and other indirect evidence suggest that *dnc*$^+$ is the structural gene for cAMP PDEase (11), but conclusive evidence has been lacking. Several different molecules are known to regulate the PDEases post-translationally (16–19), so the hypothesis that *dnc*$^+$ codes for a molecule that interacts with and activates the PDEase catalytic moiety has remained a formal possibility.

Another phenotype conferred by *dnc* mutation is female sterility (20). This phenotype has not been studied in as much detail as have the behavioral and biochemical phenes, but some points are clear. Null mutant females lay few if any eggs because of a requirement for *dnc*$^+$ activity in somatic cells (J. Kiger, personal communication), and the female sterility can be suppressed without removing the cAMP PDEase defect (ref. 20; J. Kiger, personal communication) and the behavioral phenotypes (21) by several different suppressor elements.

Despite some ambiguity in the nature of the *dnc*$^+$ gene product, these observations have been interpreted that cAMP metabolism is intimately involved in female fertility and behavioral plasticity. To further our understanding of the gene and its importance in cyclic nucleotide metabolism and normal physiology, we have continued its study at the molecular level. The gene was isolated by walking along the chromosome (22) and six RNAs, with sizes of 9.6, 7.4, 7.2, 7.0, 5.4, and 4.5 kilobases (kb) were identified as *dnc*$^+$ gene transcripts (23). In this paper, we report the analysis of several cDNA clones originating from these transcripts and the corresponding genome sequences.

MATERIALS AND METHODS

Four cDNA libraries in phage λgt10 and one in a plasmid vector were screened. The adult cDNA libraries were obtained from T. Bargiello and M. Young (Rockefeller University, New York) and L. Kauvar and T. Kornberg (University of California, San Francisco). Pupal libraries were from S. Falkenthal (Ohio State University, Columbus), N. Davidson (California Institute of Technology, Pasadena), and M. Goldschmidt-Clermont and D. Hogness (Stanford University, Palo Alto, CA). We also screened the embryonic library from Stanford. The most extensive screening procedures were conducted with the Rockefeller library. Approximately nine million phage were screened from this library. The five independent clones obtained were each recovered more than once, suggesting that we have saturated this library. From one to four times the number of independent recombinants present in the other libraries were screened. The inserts of the cDNA clones were digested with various restriction enzymes and small fragments were subcloned into M13 vectors for sequencing. The small cDNA clones were sequenced on both strands. Clones ADC1 and ADC7 were sequenced completely on one strand and partially on the second, but the genome

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Abbreviations: PDEase, phosphodiesterase; Cam, Ca^{2+}/calmodulin; kb, kilobase(s); CAP, catabolite gene activator protein; RI and RII, type I and type II regulatory subunits of cAMP-dependent protein kinase; cGK, cGMP-dependent protein kinase; ELH, egg-laying hormone.

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sequence was obtained for both strands. The IBM-compatible programs (24) were used for analysis of protein sequences. The nucleic acid sequences were analyzed with Staden's programs (25), which we have modified to run on IBM microcomputers (unpublished work). The *Drosophila* codon usage table was compiled from known or suspected protein-coding genes recovered from GenBank† or the primary literature sources and include *DRAS1, DRAS2, Act88F, Act79B, Dash, Dsrc, Adh, Rp49, Cp1, Cp2, Yp1, Yp2, Hsp70*, and *Sgs4*.

RESULTS AND DISCUSSION

Sequence of *dnc*$^+$ cDNA Clones. Genomic sequences that code for *dnc*$^+$ RNAs span at least 25 kb (ref. 23; see also Fig. 6). Restriction fragments that are unique in sequence were nick-translated and used to screen five different cDNA libraries to recover cloned copies of the *dnc*$^+$ poly(A)$^+$ RNA molecules. Two of these cDNA libraries represent the RNA population in adult flies; two, the RNAs found in pupae; and one represents embryonic RNA. Previous developmental RNA blotting experiments had indicated that the complexity and the abundance of *dnc*$^+$ RNAs is greatest during the pupal and adult stages (23). In some screens, the probe was a mixture of genomic fragments comprising *dnc*$^+$ coding regions; in others, only the probe representing coordinates 40–42 (see Fig. 6) was used since this probe contains the greatest sequence homology to *dnc*$^+$ RNAs (23). Mixtures of fragments with some representing more 5′ regions of *dnc*$^+$ were included to help recover clones with truncated 3′ ends.

More than 10^7 cDNA clones were screened from the five different libraries. One positive clone was recovered from the Stanford Oregon R embryonic library. Five other independent positive clones were recovered from the Canton S adult library constructed at Rockefeller University. No positive clones were recovered from the other cDNA libraries. The number of positive clones recovered indicates that *dnc*$^+$ RNAs exist at very low abundance levels. The Rockefeller library contains approximately 10^6 independent recombinants and we recovered five independent clones, suggesting an RNA abundance level of about 5 parts per million. Our estimates from semiquantitative nuclease S1 analysis (unpublished work) also put the abundance of these transcripts at no more than 10^{-5} of the mass of the poly(A)$^+$ RNA fraction.

The two largest cDNA clones, named ADC1 and ADC7, were both recovered from the Rockefeller library and the results of the analysis of these clones are presented here. These clones are 2.0 and 2.2 kb, respectively, and overlap by 1448 residues (Fig. 1). The other cDNA clones recovered are small (<500 base pairs) and the sequence analysis of these clones provided no important information beyond that gathered from ADC1 and ADC7, so they are not detailed here. The cDNA clones probably represent the 5.4- and/or the 7.2-kb RNA transcripts, since these are found at higher abundance levels than other transcripts in the adult RNA population (23). None of the clones contains a poly(dA) terminus representing the poly(A)$^+$ end of the RNAs and they do not contain sequences representing the 5′ end of *dnc*$^+$-encoded transcripts.

From the sequences of ADC1 and ADC7 we have been able to obtain significant information about a *dnc*$^+$-encoded protein. The RNA-like strand of these clones defines an open reading frame of 1086 nucleotides. The sequence of the open reading frame with some flanking sequence and the amino acid sequence of the predicted translation product are presented in Fig. 2. Using the first ATG as the start and translating the open reading frame through to the first in-phase stop codon would produce a protein molecule of 40,000 daltons. The first ATG does not exhibit upstream sequences characteristic of eukaryotic initiator codons (26). The second ATG in the open reading frame resides 30 nucleotides downstream from the first, but this one also does not have characteristic initiator codon sequences. We tentatively assign the first ATG as the initiator codon because of the known preference to utilize the first ATG (26).

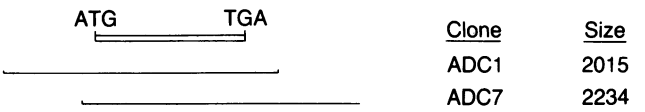

FIG. 1. Alignment of *dnc*$^+$ cDNA clones. Line segments represent extents of *dnc*$^+$ cDNA clones and their overlap determined from sequence comparisons. The location of the long open reading frame defined by ADC1 and ADC7 is depicted.

The size of the open reading frame immediately suggests its occurrence is not fortuitous and that it is probably translated into a protein molecule. The DNA sequence of the long open reading frame was analyzed for codon usage with computer graphics (25) by comparison to a codon usage table compiled from 14 different *Drosophila* protein-encoding genes. Much of the sequence of the long open reading frame conforms to the codon usage bias of other *Drosophila* protein-encoding genes. However, some regions of the long open reading frame score relatively low with respect to codon preference, especially the region from 620 to 730 and that from 1330 to the stop codon. The *dnc*$^+$ open reading frame also displays the base periodicity expected for a protein-encoding sequence (25, 27). These analyses show that the *dnc*$^+$ open reading frame exhibits the properties of other protein-encoding genes, so we conclude that the open reading frame is very likely to be translated *in vivo*.

Two unusual features of the open reading frame are to be noted. First, the region from 620 to 730 has an A+T content of about 70%, which is quite high for protein-encoding regions. This high A+T content is reflected in the unusual codon usage for the region noted above and is confined to a single exon (see Figs. 2 and 6). Second, the carboxyl-terminal sequence of the predicted protein is produced by a series of codon repeats. Thirteen of 20 codons between residues 1261 and 1320 correspond to a GRN (R = puRine) motif. This results in a highly acidic region of the protein, since, of the 20 amino acids, half are glutamic or aspartic acid residues. Region 1321–1350 is composed of mostly ACN codons, coding for eight threonines out of ten. The region 1372–1399 is formed from GGN codons, which translate into a string of nine glycine residues. The significance of the codon repeats is unknown.

The *dnc*$^+$-Encoded Protein Is Homologous to Bovine and Yeast PDEases. Because prior genetic and biochemical analyses suggested that *dnc*$^+$ codes for cAMP-dependent PDEase, we compared the sequence of the putative translation product with the partial protein sequence of the Cam-dependent PDEase from bovine brain (28) and the conceptual translation product of the yeast *PDE2* gene (29). One segment from the Cam-dependent PDEase of 54 residues is strikingly homologous to the *dnc*$^+$ translation product. Within a stretch of 57 amino acids of the *dnc*$^+$ product, there exist 32 amino acids that match the bovine PDEase sequence for an identity value of >50% (Fig. 3). A contiguous stretch of 12 amino acids within this region is completely conserved between bovine PDEase and the *dnc*$^+$ gene product. The *dnc*$^+$ gene product is more weakly homologous to the product of the yeast *PDE2* gene. [These homologies are explored in more

†National Institutes of Health (1983) Genetic Sequence Databank: GenBank (Research Systems Div., Bolt, Beranek, and Newman, Inc., 10 Moulton Street, Cambridge, MA 02238), Tape Release No. 36.0.

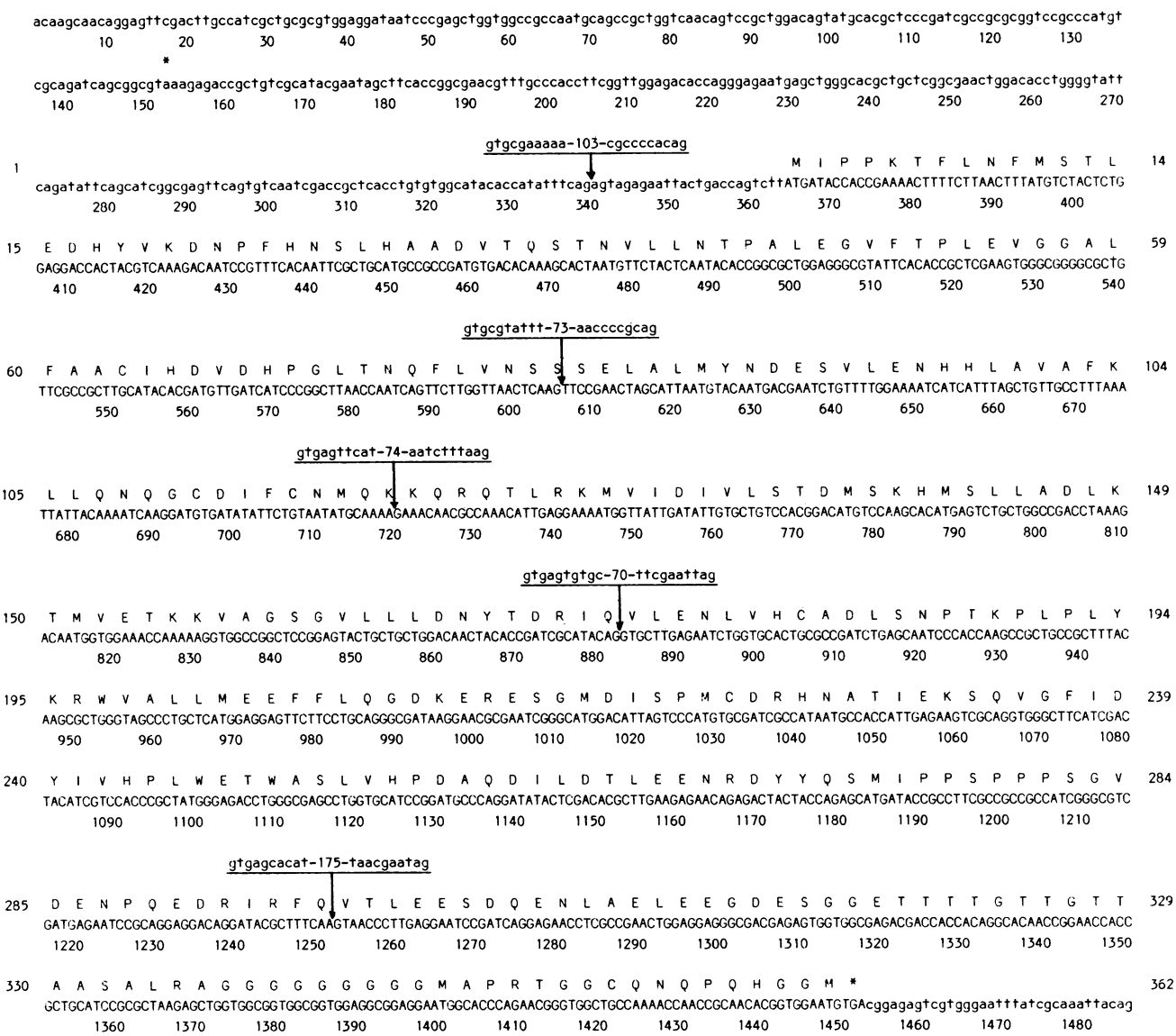

FIG. 2. Sequence of the long open reading frame in *dnc*$^+$ cDNA clones. Residue 1 is the first nucleotide of the first exon located in the 2.5-kb *Hin*dIII/*Eco*RI fragment (see Fig. 6). The sequence flanking the open reading frame is shown in lower case letters. Stop codons are marked with asterisks, including the in-frame stop codon 5′ to the first ATG. The boundary sequences and the sizes of the introns are shown above the position at which the introns interrupt the cDNA sequence.

detail by Charbonneau *et al.* (28).] Most importantly, these homologies, along with the prior genetic and biochemical evidence, conclusively identify *dnc*$^+$ as the structural gene for cAMP PDEase.

A Short, but Perfect, Homology Is Found Between the *dnc*$^+$-Encoded PDEase and a Regulatory Subunit of cAMP-Dependent Protein Kinase, Which Localizes Sequences Potentially Involved in Binding cAMP. Since the cAMP PDEase must contain residues that bind the substrate molecule cAMP, we compared the sequence of the *dnc*$^+$-encoded PDEase with the sequences of known cyclic nucleotide-binding proteins. These include the *Escherichia coli* catabolite gene activator protein (CAP), the mammalian regulatory subunits of type I (RI) and type II (RII) cAMP-dependent protein kinase, and cGMP-dependent protein kinase (cGK). Each of the latter three proteins binds two molecules of cyclic nucleotide, probably through two homologous domains.

Although no extended homologies were found, the *dnc*$^+$-encoded PDEase does exhibit a short but interesting homology to RII. The homology is confined to a region of seven contiguous amino acids, which are shown in Fig. 4. Others have demonstrated that unrelated proteins occasionally exhibit octamers of perfect homology (30), but there are two reasons for believing that this identical heptamer is more than a fortuitous match. First, the heptamer contains a tyrosine and a methionine, two amino acids which are relatively rare

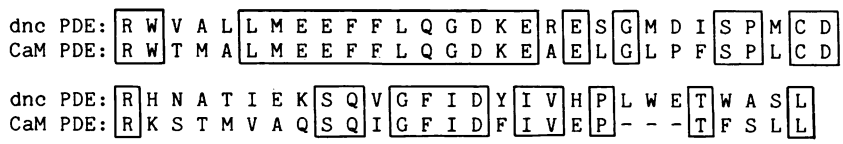

FIG. 3. Highly conserved region between the *dnc*$^+$-encoded protein and bovine PDEase. Residues 196–252 of the *dnc*$^+$ translation sequence (Fig. 2) are aligned with a portion of the sequence of bovine Cam-dependent PDEase (28). Homologous residues are boxed.

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dnc    81 S S E L A L M Y N D E
RIIa  202 F G E L A L M Y N T P
RIIb  332 F G E L A L V T N K P
RIa   198 F G E L A L I Y G T P
RIb   322 F G Q I A L L M N R P
cGKa  165 F G E L A I L Y N C T
cGKb  289 F G E K A L Q G E D V
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FIG. 4. Homology between a portion of the *dnc*$^+$-encoded PDEase and cyclic nucleotide binding proteins. *dnc*$^+$-encoded PDEase residues 81–91 are aligned with the identical sequence in RII. Also shown are similar sequences from other cyclic nucleotide binding proteins. The designations "a" and "b" refer to sequences within the two homologous domains of RI, RII, and cGK. Homologous residues are boxed.

in protein molecules. The occurrence of two infrequently used amino acids in the conserved heptamer makes its fortuitous existence less likely. Second, the conserved heptamer in RII is thought to interact with the bound cAMP molecule because it aligns with sequences in CAP that by crystallographic studies are known to be close to bound cAMP (31). Fig. 4 also illustrates related sequences in the two homologous regions of RI and cGK that have been proposed to be part of their respective cyclic nucleotide binding domains (32, 33). Therefore, we propose that the short but perfect homology is part of the cyclic nucleotide-binding site in cAMP PDEase. As in CAP, the complete cAMP-binding site in PDEase may be comprised of four or five separate subsegments that when folded form the cAMP pocket (31).

A Region of *dnc*$^+$-Encoded Protein Is Weakly Homologous to the Precursor of the *Aplysia californica* Egg-Laying Hormone (ELH). We searched the protein library for other proteins homologous to the *dnc*$^+$-encoded PDEase. One other protein in this library consistently met criteria suggesting a remote, but possible, relationship to a portion of the PDEase molecule. Surprisingly, this homologous protein is the precursor of the *A. californica* ELH (34).

ELH is synthesized as a larger precursor from which the neuropeptide is released by cleavage at two sets of dibasic amino acids. The homology between the *dnc*$^+$-encoded PDEase and the ELH precursor extends across the ELH peptide and into the region that encodes the carboxyl-terminal portion of the precursor (Fig. 5). Fifteen residues are identical between the PDEase and the ELH precursor over a stretch of 47 amino acids, giving an identity value of >30%. Statistical analysis of the homology (24) produced Z values consistently >9 after optimization. This value is believed to indicate a possible relationship.

The locations of the dibasic amino acids at which the ELH precursor is cleaved are shown in Fig. 5. Inspection of the homologous portion of the PDEase shows that the basic amino acid pairs, Lys-Lys, are found at about the same positions as the dibasic pairs in the ELH precursor when the two sequences are aligned. One additional dibasic pair (Arg-Lys) is found in the PDEase at the beginning of the homology.

We regard the potential evolutionary and functional relationship between the *dnc*$^+$ gene product and the *A. californica* ELH precursor as speculative, because it requires invoking a previously unknown organization to the *dnc*$^+$ gene as discussed below. However, certain biological considerations discussed below open the possibility that the structural homology is meaningful.

The *dnc*$^+$ Protein-Encoding Sequence Is Interrupted by Four Introns. As part of our structural studies of the *dnc*$^+$ gene, we have sequenced the 25-kb coding region shown in Fig. 6. The complete sequence of the gene and the intron/exon organization of its 5′ region will be presented elsewhere, but here we present the genomic organization of the sequences that encode the long open reading frame.

Comparison of the genome sequence with that of the cDNA clones reveals that the coding sequences for the PDEase open reading frame are interrupted by four intervening sequences. The locations of the introns and their boundary sequences are shown in Fig. 2 and are illustrated schematically in Fig. 6. All of the introns display boundary sequences conforming to consensus splice sites. The proposed initiator methionine codon is located on an exon of 264 base pairs, which we designate exon 1 of the protein-encoding region. The second exon contains the RII homology. The ELH homology resides on the third exon with the exception of the amino-terminal dibasic residues illustrated in Fig. 5, which are split by an intron. The major PDEase homology (Fig. 3) is found on exon 4 but lesser homologous regions are encoded by each of the other exons, with the exception of exon 5 (28). This exon contains the codon repeats as well as the stop codon.

FURTHER DISCUSSION

Molecular studies of *Drosophila* behavioral mutants have produced some important information regarding the biochemical processes potentially underlying behavioral plasticity. The *dnc*$^+$ gene, which was the first gene identified to play a role in learning/memory processes, encodes a component of the cAMP metabolic system, namely the enzyme cAMP PDEase. The genetic and biochemical data heretofore have suggested this relationship but alternative explanations have also been considered. For example, previous evidence was compatible with the possibility that *dnc*$^+$ codes for a molecule that regulates the PDEase post-translationally and yet potentially plays some other role in neuronal physiology important for normal learning and memory. We present data in this paper that demonstrate sequence homology between the predicted translation product of *dnc*$^+$ and the amino acid sequences of other PDEases. These data assign *dnc*$^+$ as the structural gene for cAMP PDEase with certainty.

The size of the open reading frame is large enough to code for a molecule of about 40 kDa. Previous estimates of the molecular mass of cAMP PDEase have been ambiguous, ranging between 35 and 70 kDa (11, 35). The information presented here indicates that those estimates of about 40 kDa are correct and that larger estimates are due to abnormal behavior of the enzyme, association of the PDEase with other components, or other causes.

The homology between the bovine Cam-dependent PDEase and the *dnc*$^+$-encoded PDEase is substantial and includes a subsequence of 12 amino acids that is identical between the two PDEases. This is extraordinary considering that the two PDEases are representatives of the PDEase

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dnc: M Q K K Q R Q T L R K M V I D I V L S T D M S K H M S L
ELH: S K R I S I N Q D L K A I T D M L L T E Q I R E R Q R Y

dnc: L A D L K T M V E T K K V A G S G V L L L D N Y T D R I Q
ELH: L A D L R Q R L L E K G K R S S G V S L L T S N K D E E Q
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FIG. 5. Homology between a portion of the *dnc*$^+$-encoded PDEase and the ELH precursor of *A. californica*. Residues 117–173 of the *dnc*$^+$-encoded PDEase are aligned with a weakly homologous segment of the precursor to the *A. californica* ELH. The dibasic cleavage sites in the ELH precursor and the potential cleavage sites in the PDEase are underlined. Homologous residues are boxed.

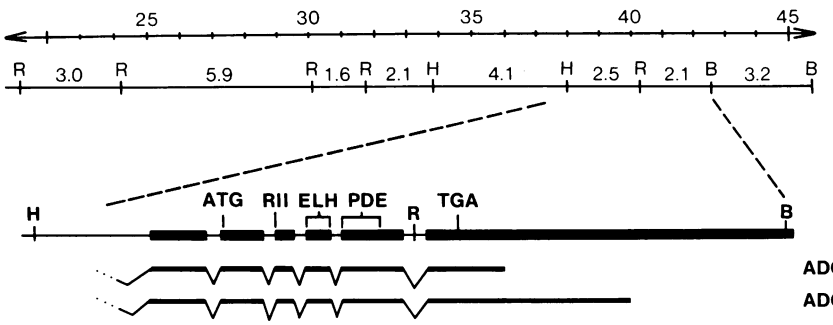

FIG. 6. Intron/exon organization of the genomic region that codes for cAMP PDEase. The coordinate system and restriction fragments that contain *dnc*$^+$ coding sequences are illustrated (R = *Eco*RI, H = *Hin*dIII, B = *Bam*HI). Exons defined by the cDNA clones within the region of the gene analyzed here are depicted in the expanded view of the 3′ portion of the gene. The locations of various landmarks including the RII homology, the ELH homology, and the highly conserved segment to bovine PDEase are shown.

enzyme family from different phyla as well as being different isoforms of the enzyme. The bovine PDEase hydrolyzes both cAMP and cGMP with some preference for cGMP as substrate and is regulated by Ca^{2+} and calmodulin. The *Drosophila* enzyme is specific for cAMP as substrate and is not sensitive to the modulator calmodulin. Interestingly, the *dnc*$^+$-encoded PDEase is more homologous to the bovine Cam-dependent PDEase than to the yeast PDEase (28), even though the yeast PDEase is like the *dnc*$^+$-encoded PDEase in being specific for cAMP and insensitive to calmodulin.

The search for sequences conserved between the *dnc*$^+$ gene product and cyclic nucleotide binding proteins did reveal a short but perfect homology with the RII subunit of cAMP-dependent protein kinase. The sequence Glu-Leu-Ala-Leu-Met-Tyr-Asn is found in the *dnc*$^+$-encoded PDEase, which is also found in RII. This sequence in the RII protein aligns with the corresponding sequence in CAP, which has been found by crystallographic analysis to reside close to cAMP. Thus, it corresponds to one of the four or five subsegments dispersed throughout CAP that fold to form the cAMP binding site, so we have concluded that this heptamer is probably part of the cAMP binding site in the *dnc*$^+$-encoded PDEase molecule. These residues apparently do not interact with cAMP directly but rather the corresponding glutamic acid residue in CAP, which resides in a loop structure, is thought to form an internal salt bridge with the guanidinium group of an arginine located in a long α-helix (36). Interestingly, we did not detect homologies with subsegments that might interact with a bound cyclic nucleotide directly.

A search of the Protein Database‡ (supplied on diskettes by W. R. Pearson, University of Virginia, Charlottesville) identified a weak homology between the *dnc*$^+$-encoded PDEase and the *A. californica* ELH precursor. We should like to stress that some proteins with no obvious biological relationship can exhibit much greater homology (24) than the *A. californica* ELH precursor has to the *Drosophila* PDEase, but several considerations are compatible with the possibility that this remote homology is more than coincidental. In addition to the structural features noted above, an intriguing point consistent with a functional role of the homologous segment is that *dnc* females are sterile, and this sterility is due in part to their failure to lay eggs. Additionally, the female sterility is suppressible by other genetic elements independently of other *dnc* phenotypes, consistent with the possibility that *dnc*$^+$ has at least two different functions. It is also interesting that the ELH homology is nested within the PDEase molecule; but it is confined to its own exon, so that by alternative splicing one of the *dnc*$^+$ transcripts might code for ELH separate from the PDEase molecule.

We have previously described the complexity of the *dnc*$^+$ locus with respect to its transcripts (23). The six transcripts with sizes ranging from 4.5 to 9.6 kb are more and larger than that necessary to code for the enzyme cAMP PDEase. The possibility that *dnc*$^+$ encodes more than one function cannot be eliminated with our current understanding of the locus.

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‡National Biomedical Research Foundation (1985) Protein Sequence Database, Protein Identification Resource (Natl. Biomed. Res. Found., Washington, DC 20007), Release No. 6.0.

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