Isolation and characterization of Drosophila cAMP-dependent protein kinase genes

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We have used mammalian probes to clone genes encoding the catalytic (C) and type I regulatory (RI) components of the cAMP-dependent protein kinase in Drosophila. Both Drosophila gene products are very similar in amino acid sequence (RI, 71%; C, 82%) to their respective mammalian counterparts, implying homologous activity. A single Drosophila type I regulatory subunit gene is the source of at least three distinct transcripts originating from different promoters and spliced to a common body that would encode a full-length analog and two amino-terminally truncated variants of the mammalian RI protein. The RI locus also includes two intronic genes of unknown function. A single highly conserved catalytic subunit gene (DC0) was found that codes for a single polypeptide. It was used to isolate 11 further more distantly related apparent protein kinase genes. Two of these genes (DC1 and DC2) are sufficiently similar in sequence (45% and 49% amino acid identity, respectively) that they could conceivably encode products of overlapping function. Two further genes are very similar in sequence to bovine cGMP-dependent protein kinase. The remaining putative gene products include amino acid sequence motifs characteristic of serine-threonine protein kinases but cannot, from the available data, be defined as homologous to specific protein kinases of other organisms.

[Key Words: Drosophila; cAMP-dependent protein kinase; protein kinase genes]

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Many extracellular stimuli elicit diverse intracellular responses through the action of the second messenger, cAMP (Sutherland 1972). The binding of extracellular ligands to a variety of specific cell-surface receptors activates membrane-associated GTP-binding proteins that either stimulate [Gs] or inhibit [Gi] adenylate cyclase, which catalyzes the synthesis of cAMP at the inner face of the plasma membrane (Gilman 1984). In this way, intracellular cAMP concentration can respond to different hormones and neurotransmitters in different cell types (Nimmo and Cohen 1977; Nestler et al. 1984). A cAMP signal is propagated by binding of cAMP to the regulatory subunits (R) of the inactive cAMP-dependent protein kinase holoenzyme (R,C), causing dissociation of catalytic (C) and regulatory subunits. The activated monomeric catalytic subunits phosphorylate, and thereby alter, the activity of one or more protein substrates appropriate for the execution of a given intracellular response (Krebs and Beavo 1979). Activation of the cAMP-dependent protein kinase is held to be virtually the only direct response to intracellular cAMP signals in eukaryotes (Kuo and Greengard 1969; Coffino et al. 1976; Walter et al. 1977; Nakamura and Gold 1987; Toda et al. 1987b). Therefore, cAMP-dependent protein kinase is a key enzyme in linking many agonists to corresponding intracellular effectors and is expected to participate in cellular communication in several different physiological situations.

The specificity of the response mediated by cAMP-dependent protein kinase in a given cell depends upon the array of functional receptors on the cell surface and the spectrum of kinase substrates and other effector proteins present in the target cell. Specific physiological roles for cAMP-mediated signal transduction have been defined in a number of organisms. Most prominent among these are the control of glycogen and lipid metabolism in mammals by cascades of enzyme phosphorylation (Cohen 1982), modulation of activity of excitable cells, probably by direct phosphorylation of ion channel proteins (Kandel and Schwartz 1982; Osterreider et al. 1982; Siegelbaum et al. 1982), and less well-characterized roles in the control of meiosis (Maller and Krebs 1977; Watanabe et al. 1988) and mitosis (Matsumoto et al. 1982; Shin et al. 1987; Toda et al. 1987; Tanaka et al. 1988), neuronal development (Haydon et al. 1984; Lankford et al. 1988; Rydel and Greene 1988), sensory transduction (Pace et al. 1985; Avenet et al. 1988; Tonosaki and Funakoshi 1988), and selective transcriptional activation of genes (Lamers et al. 1982; Hashimoto et al. 1984; Wyn-
Two roles for cAMP signaling in *Drosophila* already have been inferred from genetic analysis. *Drosophila* carrying mutations that alter cAMP phosphodiesterase \(\textit{dunce}\) or adenylyl cyclase \(\textit{rutabaga}\) activities are deficient in learning and memory tests [Byers et al. 1981; Livingstone et al. 1984]. This has been interpreted to suggest a role for cAMP in the modulation of ion channel activity at the appropriate synapses [Quinn and Greenspan 1984]. Female *Drosophila* carrying the \textit{dunce} mutation are also sterile, further investigation of which suggests that cAMP signals are used in early embryogenesis [Kiger and Salz 1985; Bellen et al. 1987]. The mutations \textit{rutabaga} and \textit{dunce} affect the rates of synthesis and degradation of cAMP, respectively, and alter ambient cAMP levels, but retain the ability to alter cAMP levels and to respond to such alterations. Genetic inactivation of the cAMP-dependent protein kinase should prevent all cAMP signaling. Therefore, it should be possible by genetic manipulation of cAMP-dependent protein kinase to address the entire spectrum of cAMP-mediated signal transduction in a multicellular organism and to pursue the molecular details of specific transduction pathways.

Here we report the isolation and characterization of *Drosophila* genes for the type I regulatory subunit and a catalytic subunit of cAMP-dependent protein kinase. Eleven further apparent serine–threonine kinase genes have been isolated by cross-hybridization between DNAs encoding kinase domains and characterized by limited sequencing. The complete coding sequence of two of these genes has been determined and suggests that they may encode additional catalytic subunit variants of the cAMP-dependent protein kinases.

### Results

**Isolation of Drosophila type I regulatory subunit gene**

A single segment of DNA in the *Drosophila* genome was identified by hybridization of a bovine cDNA probe \(p62C12\), Lee et al. 1983 for the type I regulatory subunit \(\text{RI}\) at low stringency to *Drosophila* genomic DNA blots and a *Drosophila* genomic library [Maniatis et al. 1978]. Several overlapping genomic \(\lambda\) bacteriophage were isolated, from which a short sequence recognized by the bovine probe was identified and used to isolate corresponding cDNAs. The sequence of one of these cDNAs was determined and shown to include an open reading frame \(\text{ORF}\) that could be translated into a protein of similar length and sequence to mammalian regulatory subunits \(\text{class I} \text{ RNA in Figs. 1 and 3}\). The translation product is clearly more similar to the mammalian type I \(71\%\) amino acid identity) than type II \(32\%\) amino acid identity) regulatory subunit sequence [Takio et al. 1982; Titani et al. 1984; G.S. McKnight, pers. comm.; see legend to Fig. 1].

Hybridization of this cDNA to polytene chromosomes showed the *Drosophila* RI gene to lie on the left arm of the third chromosome in polytene division 77F. This gene appears to be the only gene in the *Drosophila* genome capable of encoding a type I regulatory subunit, as no related sequences could be detected by low-stringency nucleic acid hybridization.

**Diverse RNAs are generated from the RI gene by differential splicing and polyadenylation**

Characterization of cDNAs, RNA blots, and S1-nuclease protection experiments have shown that multiple RNA species derive from the RI gene. They result from differential splicing both upstream of and within the coding
sequence of the gene and by the use of different polyadenylation sites distal to the coding region [Fig. 2]. Differential splicing of transcripts leads to the generation of at least three putative RI polypeptide products, two of which are truncated by 57 and 80 residues, respectively, at the amino terminus relative to the largest Drosophila RI protein [Figs. 2 and 3].

Restriction enzyme mapping and partial sequence analysis of 18 independent cDNA clones indicated that any of four different sequences can be spliced to a single site immediately prior to the codon for amino acid 59 of the largest (class I) RI product. The location of these four possible upstream exons has been determined by comparison of genomic and cDNA sequences. The methionine codons indicated in Figures 1 and 2 as likely initiators of translation are the furthest 5′ in the respective long ORFs of class I, II, III, and IV RNAs. The theoretical primary translation product of class I RNA is of roughly the same length as the murine RI protein, the product of class II RNA is missing residues 2–58 but is otherwise identical, and the product of class III and IV RNAs lacks residues 2–81 [Figs. 1–3].

Two minor variants of class I RNA which specify the same polypeptide can be inferred from individual cDNAs [Ib and Ic in Fig. 2] that indicate splicing in the 5′-untranslated sequence between a single donor and two alternative acceptors [see legend to Fig. 1 for exact intron locations]. The major form of class I RNA [Ia in Fig. 2] is not spliced in this region, as shown by S1-nuclease protection of the entire Ia exon using adult head RNA [data not shown] and by the similarity of RNA blot hybridization patterns using probes derived from class Ib intron and exon sequences [Fig. 4].

The 5′ ends of class I, II, and IV RNAs have been mapped by primer extensions [see legend to Fig. 1; data not shown] and also by S1-nuclease protection for class I and II RNAs [data not shown]. Class III RNA is sufficiently rare that it has not been detected by these means or on RNA blots and is exemplified by only a single cDNA. Heterogeneous transcription initiation sites for class I RNAs span a region of 36 nucleotides. The apparently unique 5′ ends of class II and IV RNAs are separated from the 5′ end of type I RNA and each other by more than 4 kb [Fig. 2]. At least three different promoters therefore are used by the Drosophila RI gene, each apparently specifying a distinctive RNA form of unique coding potential. The three major forms of RI transcript, I, II, and IV, appear to be regulated indepen-


parent association between the site of polyadenylation and the use of a given promoter or splicing pathway.

**RI polypeptides**

It is not known whether stable polypeptides that are incorporated into cAMP-dependent protein kinase holoenzyme are produced from each of the RNA species described. RNAs synthesized in vitro from cDNA templates to resemble the splicing variants I–IV can be translated in a rabbit reticulocyte lysate to yield polypeptides of a size consistent with initiation at amino acid 1 [class I], 58 [class II], and 81 [classes III and IV] of a protein of equivalent size to murine type I regulatory subunit [data not shown]. Regulatory subunit protein can be detected specifically in vivo by labeling crude extracts with the radioactive photoaffinity analog, [32P]8-azido-cAMP [Walter et al. 1977]. For all stages of development from embryo to adult, two major bands are seen corresponding to full-length RI and RII proteins by the criteria of gel mobility and a characteristic shift in mobility when 6 M urea is included in the gel [Foster et al. 1984; and data not shown]. Hence, both class I RNA and its translation product are detected throughout the *Drosophila* life cycle. Truncated versions of RI, the theoretical primary translation products of RNAs II–IV, are not clearly evident at any developmental stage but may be obscured by proteolytic products of full-length RI and RII protein from which they could not be definitively distinguished in this assay.

**Genes within the largest RI intron**

Hybridization and sequence studies have defined a separate gene [18c] within the largest intron of the RI gene that is transcribed in opposite orientation to RI at high levels in pupae and adults (Figs. 2 and 4). There is also evidence from RNA blots and the isolation of a cDNA that suggests that a second low-abundance transcript (13a) derives from a distinct location in the same intron (Fig. 2). Because sequences from the region of the RI locus containing the 18c transcript and possibly also the 13a transcript are found in similar form in about six other locations in the genome, it was necessary to compare 13a and 18c cDNAs with genomic sequences form the RI intron to verify that they represent transcripts of the RI gene intron and not of similar cross-hybridizing sequences from another location (Fig. 5). The longest ORF in the 18c cDNA sequence is 215 amino acids long. This deduced protein product contains no stretches of hydrophobic residues sufficient to span a lipid bilayer, is very basic in the carboxy-terminal half, and has not been found to be similar in sequence to any protein in the NCBI-PIR protein sequence database (December 1987). The genomic loci that cross-hybridize to the 18c gene are clustered at polytene chromosome division 77F and some, if not all, are transcribed and translated into polypeptides similar to the 18c product as judged by hybridization to RNA blots and sequence determination of two cDNAs, B4A and H15A (Fig. 5).
cAMP-dependent protein kinase catalytic subunit gene

A cDNA probe for the mouse cAMP-dependent protein kinase catalytic subunit (pMC4; Uhler et al. 1986a) hybridized at low stringency in DNA blots of Drosophila genomic DNA predominantly to one segment of DNA and detectably to at least two others (data not shown). Genomic phage corresponding to the major site of hybridization were isolated and subsequently used to identify corresponding cDNAs. DNA sequences of cDNA and genomic DNA revealed a coding sequence uninterrupted by introns and capable of specifying a protein of the same length and with 82% amino acid identity to the mouse catalytic subunit, Cα (Figs. 6 and 7). A glycine is present as the second residue in the primary translation product of both mammalian and Drosophila genes.
Figure 5. DNA sequence of the RI intronic gene, 18c. The DNA sequence of the RI locus extending from between intronic transcripts high-stringency hybridization of probes from this region were investigated by cloning, limited sequencing, and genomic DNA blots (1452) of 18c is 68 nucleotides from the splice donor junction of class I RI RNA (written here in opposite polarity to transcription).

This residue is myristylated in the bovine protein (Cart et al. 1982). The Drosophila catalytic subunit gene homolog (DC0) has been mapped by in situ hybridization to polytene chromosome region 30C1-6. This gene has also been isolated by Foster et al. (1988). Only a single third-base substitution distinguishes the two reported sequences of DC0.

At least four RNA species of different size derive from
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Figure 6. *Drosophila* catalytic subunit gene, DC0. The genomic DNA sequence that includes the coding region for the *Drosophila* catalytic subunit gene, DC0. The genomic DNA sequence that includes the coding region for the catalytic subunit gene, DC0 (Fig. 7). The genomic DNA sequence that includes the coding region for the catalytic subunit gene, DC0 (Fig. 7). Probes derived from the 3' end of RNA species (Fig. 7) hybridized to only perhaps by the use of different sites of polyadenylation.

The relative abundance of these RNA forms varies during development, the shortest species being particularly prominent in embryos and the longest in adults. Comparison of a representative cDNA, cDCAT, with genomic sequences indicates a single intron in the 5'-untranslated sequence. The first DC0 exon was shown by primer extension to initiate one nucleotide upstream of cDCAT (not data shown) and is shared apparently by all four DC0 RNA species differ principally at their 3' ends, underlining the complex nature of mRNA processing in *Drosophila*. The 5' end of RNA of the DC0-cDCAT form has been mapped to position 391 by primer extension of oligonucleotides complementary to nucleotides 424-456 and 495-524. Underneath is the deduced translation product of the longest ORF, which is 82% identical in sequence to mouse catalytic subunit, Ca (Fig. 9).

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Drosophila cAMP-dependent protein kinase genes

A

B

E

L1

P1

H

B

DC0

DC1

DC2

Figure 7. Temporal expression of catalytic subunit, DC0, and related genes, DC1 and DC2. Single-stranded probes specific for the coding regions of (A) DC0 (3180–2951, written 5' to 3'; Fig. 6), (B) DC1 (1067–260, Fig. 10), and (C) DC2 (2017–1770, Fig. 11) were hybridized to equivalent RNA blots of poly(A)+ RNA (5 μg per lane) from the indicated developmental stages (0–24 hr embryos, E; first-larval instar, L1; one-day-old pupae, P1; adult head, H; and adult body, B). As for Fig. 4, all samples contained the same amount of intact actin 5C RNA, and similar patterns of bands were seen in the late larval and pupal stages as for early larval and pupae, respectively (data not shown). DC0 and DC2 transcripts are present at all stages and the largest transcript in each case is particularly abundant in adult head. DC1 transcripts are not detectable in larvae, are more prevalent in adult body than head, and are of apparently altered size in embryos. (D) RNA samples (5 μg poly(A)+ RNA per lane) from 0– to 15-hr embryos (E) and adults (A) have been hybridized to single-stranded probes specific for the 5' exon of the DC0 gene (5'; 14777–1471564–392, Fig. 6), the 3' end of cDNA, DC0-cDH5 (M; spanning a region 3.9 to 3.6 kb downstream of the 5' end of the gene, Fig. 8), and the 3' end of cDNA, DC0-cDD3 (3'; spanning a region 5.1 to 4.8 kb downstream of the 5' end of the gene, Fig. 8). The latter probe (3') also hybridized strongly to a transcript of 0.7 kb (not shown), indicating either an overlapping transcript or that the 3' end of cDNA DC0-cDD3 is composite. (E) The use of a probe biased toward DC2-cDH2-specific sequences of DC2 (DC2-5' in Fig. 8; corresponding to DC2-cDH2 nucleotides that replace nucleotides 952–649 in DC2-cDDS, Fig. 11) indicates that the 2.4-kb RNA species corresponds to DC2-cDH2 and the 3.0-kb species to DC2-cDD5, confirming that the two major DC2 transcripts have different 5'-untranslated sequences spliced to the same coding region.

Genes related to DC0

Both the mouse and Drosophila catalytic subunit genes recognize several other regions of DNA in the Drosophila genome under relaxed hybridization conditions (data not shown). Successive attempts were made to isolate these loci by screening a genomic library with Drosophila catalytic subunit gene probes at low stringency. In the second round, previously isolated loci were used to counterscreen at high stringency so that new loci could be discerned immediately. Eleven new distinct loci were isolated and the region responsible for hybridization determined and sequenced. In all cases, portions of the DNA sequence could be translated into amino acid sequences that are conserved among serine-threonine protein kinases, indicating that each of these loci may represent a distinct protein kinase gene (Fig. 9). The chromosomal location of each of these genes is given in Table 1. Several of the genes have been shown to be expressed by hybridization to RNA blots and cDNAs have been isolated for all but one (5-23), indicating that they are transcribed. Two of the genes [DG1, DG2] appear to encode proteins akin to the kinase domain of mammalian cGMP-dependent protein kinase [Takio et al. 1984]. Two others [DC1, DC2] are particularly similar to the cAMP-dependent protein kinase catalytic subunit and have been analyzed further. There are insufficient sequence data from the remaining putative protein kinase genes to determine whether they are homologs of specific protein kinases characterized in other species. There is substantial similarity between the deduced protein product of 8-6 and the Saccharomyces cerevisiae CDC28 gene product (52 identities in 94 amino acids; Lorincz and Reed 1984), and the 3-2 deduced amino acid sequence is similar to that of protein kinase C species (50 identities in 85 residues with bovine PKC-α; Parker et al. 1986).

Two different forms of cDNA [DC1-cDH1 and DC1-cDD1] have been isolated for the DC1 gene that are distinguished by the use of different splice acceptor sequences located 5 bp apart in the genomic DNA (Figs. 8 and 10). The point of divergence is in coding sequence exactly at the carboxy-terminal border of the kinase domain and therefore leads to the generation of two different polypeptides, each expected to exhibit protein kinase activity [Fig. 3]. One product [DC1a] is of the same
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**Figure 8.** Transcripts of DC0, DC1, and DC2 genes. The DC0 gene is the source of at least four different transcripts (Fig. 7). The coding and 5'-untranslated region of one RNA form, represented by cDNA cDCAT has been determined by comparison of cDNA and genomic sequences. Primer extension showed the 5' end of this RNA to be one nucleotide from the 5' end of DC0-cDCAT. A second cDNA DC0-cDPH5 is contiguous with genomic sequence around the splice acceptor of DC0-cDCAT and may represent an incompletely processed RNA. Both cDNAs DC0-cDPH5 and DC0-cDD3 extend further 3' than DC0-cDCAT, and by restriction site comparison to genomic DNA each lacks an intron of about 11 kb. The structure of these cDNAs relative to genomic DNA further 3' is not known, as indicated by the dashed lines. Both 5' and coding region probes from DC0-cDCAT hybridize to all four DC0 RNAs on RNA blots, whereas probes derived from the middle (M) and the extreme 3' end (3') of the apparent transcription unit only detect the two largest and the single largest DC0 RNA species, respectively (Fig. 7). The transcription pattern of the DC1 gene has been deduced from comparison of genomic sequence to two cDNAs, DC1-cDH1 and DC1-cDD1. The two cDNAs indicate identical splicing patterns except for the different choice of splice acceptors for the third intron that are located 5 bp apart. The reading frames of the polypeptides encoded by the two RNAs therefore differ beyond this point, which corresponds to the carboxy-terminal border of the kinase domain (Fig. 3). The DC2 gene generates two major transcripts (Fig. 7) that are detected by the probe DC2-coding. Two forms of cDNA, represented by DC2-cDD5 and DC2-cDH2, of roughly equivalent size to the two RNA species (3.0 kb and 2.4 kb, respectively) differ 5' of the coding sequence, presumably as a result of alternative splicing. Consistent with this proposal, a probe containing largely sequences specific to DC2-cDH2 (DC2-5') highlights the shorter of the two RNA species on RNA blots (Fig. 7). The two DC2 cDNAs are represented by dashed lines because the corresponding genomic sequences are not known. For all three genes the termination codon and furthest upstream methionine coding in the long ORF are indicated.

Length as the mouse catalytic subunit and of similar sequence throughout (45% amino acid identity), whereas the other (DC1b) is slightly longer and is not related in sequence to catalytic subunit at its unique carboxyl terminus. The relative abundance of RNAs represented by these cDNAs is not known. Collectively, they are transcribed into a species of about 1.6 kb that is detected in embryonic, pupal, and adult RNA samples (Fig. 7). DC1 transcripts, in contrast to DC0 and DC2, are more abundant in adult body than adult head RNA (Fig. 7).

The second catalytic subunit related gene, DC2 is expressed as two major RNA species of 3.0 kb and 2.4 kb, for which corresponding cDNAs have been isolated (Figs. 7 and 8). The complete coding sequence of DC2 has been determined from the longer of these cDNAs (DC2-cDD5, Figs. 8 and 11). Two other independent cDNAs (DC2-cDH2, DC2-cDD6), the longer of which is 2150 bp, appear to derive from the use of an alternative splice donor to pair with an acceptor 81 nucleotides upstream of the initiator codon, although the genomic sequence in this region is not known (Figs. 8 and 11). As for the DC0 gene, the relative abundance of transcripts of identical coding sequence but different untranslated sequence is regulated during development. In this case the shorter DC2 transcript is only detectable post-pupariation (Fig. 7). The inferred amino acid sequence of the DC2 gene product is 49% identical over the entire length of the mouse catalytic subunit but includes an additional 151 amino acids at the amino terminus (Fig. 3). The unique amino-terminal portion of the DC2 gene product is extremely rich in serine, threonine, and acidic residues (Fig. 11) and is not related in sequence in this region to any other known protein sequences (NBRF-PIR protein sequence database December 1987).

**Discussion**

Drosophila genes have been isolated that can encode proteins very similar in primary sequence, and hence presumably of analogical activity, to the mammalian RI and C subunits of the cAMP-dependent protein kinase. The conservation in sequence between these genes (71% amino acid identity for RI, 82% for C) is somewhat greater than for other kinase genes that have been characterized in Drosophila and mammals (abl ~75%, Henkenmeyer et al. 1988; protein kinase C ~64%, Rosenthal et al. 1987; raf ~65%, Mark et al. 1987; src ~40%, Simon et al. 1985). The deduced amino acid sequence of the Drosophila RI protein is particularly similar to its mammalian homolog in the two carboxy-terminal CAMP binding domains (see Fig. 1). Clearly, it is more related to mammalian RI than RII protein (71% versus 32% identity) and in particular, includes a serine (Ser = 96) in a context analogous to that of the bovine RI serine that is phosphorylated by cGMP-dependent protein kinase (Tritani et al. 1984) but does not include a sequence analogous to that around the serine in bovine RII protein (RII Ser = 95; Drosophila RII Gly = 94) that
Drosophila cAMP-dependent protein kinase genes

Mouse C
Drosophila DC0
Drosophila DC1
Drosophila DC2
Ser/Thr kinases
Tyrosine kinases

Drosophila DC0
Drosophila DC1
Drosophila DC2
Ser/Thr kinases
Tyrosine kinases

Drosophila DG1
Drosophila DG2
Drosophila 1C
Ser/Thr kinases
Tyrosine kinases

Drosophila 8-6
Drosophila 7-10
Drosophila 5-23
Drosophila 1J
Drosophila 3-10
Ser/Thr kinases
Tyrosine kinases

Drosophila DC0
Drosophila DC1
Drosophila DC2
Ser/Thr kinases
Tyrosine kinases

Figure 9. Catalytic subunit-related kinase genes in Drosophila. The complete amino acid sequences, deduced from cDNA sequence, for the mouse catalytic subunit, Cα (Uhler et al. 1986a), Drosophila catalytic subunit, DC0, and related genes DC1 and DC2 [from amino acid 151] are shown. The mature mouse Cα and DC0 proteins probably lack the amino-terminal methionine residue (Carr et al. 1982), altering residue numbering by one. Partial amino acid sequences deduced from genomic DNA sequence for nine other genes isolated by cross-hybridization to DC0 can also be aligned with each other and with amino acid sequence motifs characteristic of protein kinases, shown on the bottom two lines (Hunter and Cooper 1985). Two of these genes, DG1 and DG2, clearly encode products closely related to the cGMP-dependent protein kinase (Takio et al. 1984). The remaining deduced gene products are more similar in sequence to serine-threonine-specific than tyrosine-specific protein kinases. When compared with other known protein kinase amino acid sequences, substantial similarities between 3-2 and bovine protein kinase C-e (50 identities in 85 residues; Parker et al. 1986) and between 8-6 and S. cerevisiae CDC28 (52 identities in 94 residues; Lorincz and Reed 1984) are apparent. The kinase domain of mouse catalytic subunit defined by sequence similarity to other protein kinases is boxed and extends from amino acid 46 to 281.
Table 1. Sequence identities between putative Drosophila kinase gene products and mouse cAMP-dependent protein kinase catalytic subunit

| Overall % identity | Within kinase domain | Outside kinase domain | Chromosomal location |
|-------------------|----------------------|-----------------------|---------------------|
|                   | % identity | no. of AA | % identity | no. of AA |                 |
| DC0               | 82        | 236      | 89        | 236      | 67                | 115        | 30C |
| DC1               | 45        | 236      | 53        | 236      | 28                | 115        | 100A |
| DC2               | 49        | 236      | 56        | 236      | 28                | 115        | 72A |
| DG1               | 44        | 169      | 44        | 169      | 115               | 21D        |     |
| DG2               | 49        | 138      | 49        | 138      | 115               | 24A        |     |
| 1C                | 44        | 52       | 44        | 52       | 115               | 36A        |     |
| 8-6               | 36        | 94       | 36        | 94       | 115               | 53C        |     |
| 3-2               | 54        | 85       | 54        | 85       | 115               | 45C        |     |
| 7-10              | 43        | 51       | 43        | 51       | 115               | 64F        |     |
| 5-23              | 60        | 20       | 60        | 20       | 115               | 72A        |     |
| I1                | 56        | 48       | 56        | 48       | 115               | 91C        |     |
| 3-10              | 50        | 30       | 50        | 30       | 115               | 17E        |     |
| G kinase          | 42        | 236      | 47        | 236      | 31                | 115        |     |
| Phosphorylase b kinase | 23       | 236      | 35        | 236      | 0                 | 115        |     |
| Myosin light-chain kinase | 21  | 236      | 28        | 236      | 4                 | 115        |     |

The percentage amino acid identity to mouse cAMP-dependent protein kinase catalytic subunit [Cα] [Uhler et al. 1986a] of the deduced products of the putative Drosophila protein kinase genes described in this study and of three representative mammalian serine protein kinases [bovine cGMP-dependent protein kinase, designated G kinase [Reimann et al. 1984]; rabbit phosphorylase b kinase [Reimann et al. 1984]; and rabbit myosin light-chain kinase [Takio et al. 1985]] is tabulated for regions within the kinase domain (amino acids 46–281 of mouse Cα) and outside (residues 1–45 and 282–351). Only limited amino acid sequence is available for some gene products. The number of amino acids over which the comparisons are made is indicated. The chromosomal location of the putative Drosophila kinase genes as determined by in situ hybridization to polytene chromosomes is also given. [AA] Amino acids.

is phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase [Takio et al. 1984]. The deduced Drosophila catalytic subunit gene product includes all of the conserved features of serine–threonine kinases and is substantially closer in primary sequence to the cAMP-dependent protein kinase than to the kinase domain of even its closest acknowledged relative, cGMP-dependent protein kinase [82% versus 42% identity].

In contrast to the mouse, where two very similar RI genes have been found [C. Clegg et al., in press], the Drosophila genome appears to contain only a single RI gene. However, differential splicing within the coding region of the Drosophila gene and the use of different promoters generates transcripts capable of programming the synthesis of RI proteins truncated at the amino terminus by 57 and 80 amino acids in addition to the authentic full-length RI homolog. Proteolysis studies on the mammalian RII protein suggest that the amino-terminal 90 residues of regulatory subunit protein are required for dimerization but not for cAMP and catalytic subunit binding activities [Weldon and Taylor 1985]. The two theoretically truncated polypeptide products of the Drosophila RI gene would therefore be expected to act as monomeric cAMP-dependent regulators of catalytic subunit activity like the Dictyostelium regulatory subunit, which is 68 amino acids shorter than mammalian RII protein [Mutzel et al. 1987].

The three major transcripts of the Drosophila RI gene all derive from different promoters and therefore might be expected to be regulated independently. Class I RNA, which codes for the largest RI protein, can be detected at roughly constant level throughout development as can full-length RI protein by labeling with a radioactive cAMP photoaffinity analog. In contrast, class II and IV RNAs have been detected only in adults where class IV RNA, which codes for the shortest RI polypeptide, is the most abundant RI species. The inferred truncated proteins have only been visualized directly as products of in vitro translation and, indeed, will be hard to distinguish in vivo from proteolytic products of the full-length protein.

The Drosophila RI gene includes two transcription units of polarity opposite to RI within the first and largest intron. The better-characterized intronic gene (18c) appears to be expressed at high levels [10- to 20-fold higher than RI] only in pupae and adults and to be one of at least six members of a clustered gene family that encode related polypeptides of unknown function. The polyadenylation site of this intronic transcript is 68 nucleotides distant from the first exon of the RI gene. Hence, fully processed transcripts of the 18c and RI genes do not overlap.

In mammals there are two [Showers and Maurer 1986; Uhler et al. 1986a,b] and in yeast three [Toda et al. 1987b] very similar genes that encode catalytic subunits of the cAMP-dependent protein kinase. In Drosophila, only one locus that clearly encodes a homologous protein has been found and the coding region of this gene lies on a single exon, making differential RNA pro-
cessing an unlikely source of polypeptide diversity. However, nucleic acid hybridization has revealed 11 additional *Drosophila* genes with substantial sequence similarity to the cAMP-dependent protein kinase catalytic subunit. From the limited sequence determined for most of these genes, it seems likely that they encode serine-threonine-specific protein kinases (Fig. 9). cDNAs have been recovered for all but one of these genes, indicating that they are transcribed. Should all of these genes be functional, this observation argues for the existence of many more kinases than have, as yet, been characterized biochemically (Hunter 1987; Hanks et al. 1988).

Two of the kinase genes isolated, DC1 and DC2, are sufficiently similar to the catalytic subunit of cAMP-dependent protein kinase to raise the question of whether their activity is controlled by cAMP by virtue of association with regulatory subunits. The similarity of mouse catalytic subunit sequence to DC1 and DC2 is substantially lower than to DCO (45% and 49% versus 82% amino acid identity), but is marginally higher than to mammalian cGMP-dependent protein kinase (42% amino acid identity; Takio et al. 1984), its nearest known relative. The sequences of the gene products are only 50-53% identical to bovine catalytic subunit sequence to DC1 and DC2 is substantially lower than to DCO (45% and 49% versus 82% amino acid identity), but is marginally higher than to mammalian cGMP-dependent protein kinase (42% amino acid identity; Takio et al. 1984), its nearest known relative. The sequences of the gene products are only 50-53% identical to bovine catalytic subunit (Toda et al. 1987b). A specific determinant for regulatory subunit binding has not been defined in the catalytic subunit of cAMP-dependent protein kinase, although it is known that alteration of a single residue in the yeast catalytic subunit TPK1-3 gene products are only 50-53% identical to bovine catalytic subunit, but still serve as catalytic subunits, of cAMP-dependent protein kinase genes. The catalytic subunit TPK1-3 gene products are only 50-53% identical to bovine catalytic subunit, but still serve as catalytic subunits, of cAMP-dependent protein kinase genes. The catalytic subunit TPK1-3 gene products are only 50-53% identical to bovine catalytic subunit, but still serve as catalytic subunits, of cAMP-dependent protein kinase genes.
Figure 11. Catalytic subunit-related gene, DC2. The nucleotide sequence of a cDNA, DC2-cDD5, to the DC2 gene is shown. A second cDNA, DC2-cDH2, diverges from this sequence 5' of nucleotide 814, presumably at a splice junction, and the unique sequence of this cDNA is shown below. The first 150 amino acids are not significantly related to sequences of this cDNA are shown above. Neither the location of introns nor the 5' and 3' boundaries of this gene have been determined.
peptide product is of the same length as cAMP-dependent protein kinase catalytic subunit and of similar sequence throughout, the other is slightly longer and unrelated at the carboxyl terminus. Both would be expected to exhibit kinase activity, but it is not clear whether the proteins would differ principally in their mode of activation or substrate specificity.

In its simplest form the role of cAMP-dependent protein kinase in signal transduction demands only a single constitutively expressed form of the enzyme. The specificity of its action in a given cell can be generated by restricted expression of ligands, receptors, and intracellular effectors subject to regulation by phosphorylation. However, it is clear that several forms of cAMP-dependent protein kinase are expressed in yeast, mammals, Aplysia, and now Drosophila, and that the levels of these isoforms differ among cell types and during development (Eppler et al. 1982, Uhler et al. 1986a,b; Toda et al. 1987a,b). It now appears also that several RNA forms encode each of the components of cAMP-dependent protein kinase in Drosophila and that the relative abundance of some of these forms varies during development. The same Drosophila RI subunit homolog may be synthesized from RNAs that differ at their extreme 5' ends as a result of multiple clustered transcription initiation sites, in the 5'-untranslated sequence due to differential splicing and in the 3'-untranslated sequence due to differential polyadenylation. The Drosophila catalytic subunit homolog is transcribed into four distinguishable RNA species, even though the coding sequence lies on a single exon. These four catalytic subunit RNAs are found in different proportions in embryos and adults (Fig. 7). It is not known if the observed heterogeneity in RNA forms is functionally important and, if so, whether it contributes to regulation of RNA localization, stability, or translation. An effect of upstream methionine codons on translational efficiency has been demonstrated in yeast (Mueller and Hinnebusch 1986) and in mammals (Marth et al. 1988) and may be of relevance to the Drosophila RI gene. Type I RI RNA species include between zero and three methionine codons prior to the presumed translation initiation codon, depending on the exact 5' end and which of three splicing patterns (Ia, Ib, or Ic) is adopted (Fig. 1). Neither the upstream nor the putative initiator is in a context commonly found for initiation codons (Kozak 1984a; Cavener 1987) and considered to be efficiently recognized as such by ribosomes (Kozak 1984b). This is true also for the DC0, DC1, and DC2 genes, all of which include upstream methionine codons in at least some transcripts and one of which (DC2) undergoes differential splicing in the 5'-untranslated sequence. Thus, study of the genes for cAMP-dependent protein kinase in Drosophila has uncovered a number of potentially regulated RNA and protein isoforms that point to unanticipated sophistication in the cAMP signal transduction system.

The identification of clear Drosophila homologs of RI and catalytic components of the cAMP-dependent protein kinase and knowledge of their chromosomal location should allow the isolation of null mutations in these genes and hence insight into the biological role of cAMP-dependent protein kinase in a whole organism. It should also be possible to perturb cAMP-dependent protein kinase activity in Drosophila by germ-line transformation of constructs designed to overexpress wild-type or specifically altered versions of those genes that lead to constitutive activity of catalytic subunit or constitutive inhibitory activity of the regulatory subunit (Clegg et al. 1987, Levin et al. 1988).

Materials and methods

Isolation of genomic DNA and cDNA clones

Genomic clones for Drosophila RI, DC0, and 11 other kinase genes were isolated from a library of sheared Drosophila Canton S genomic DNA in Charon 4A (Maniatis et al. 1978) by low-stringency hybridization (22% formamide, 5 x SSC, 10 x Denhardt's, 42°C, wash 2 x SSC, 42°C) to bovine RI, mouse C, and Drosophila DC0 probes, respectively. Corresponding cDNA clones in λgt10 were isolated from a variety of libraries constructed from RNA of adult heads, third-instar larvae, eye imaginal discs, or total imaginal discs (A.C. Cowman and G.M. Rubin, unpubl.; H. Steller and G.M. Rubin, unpubl.) by high-stringency hybridization (10 x Denhardt's, 5 x SSC, 60°C; wash 0.5 x SSC, 65°C).

Sequence determination of genes

Complete cDNAs or coding regions of genomic DNA determined by low-stringency hybridization to appropriate probes were sequenced by the dideoxy termination method (Sanger et al. 1977), largely using sonicated subfragments cloned into M13mp10 but also by directed cloning into M13mp18 and mp19 vectors (Messing 1983). The DNA sequence presented here has been determined on both strands of either cDNA or genomic DNA for all transcribed regions of the RI, DC0, DC1, and DC2 genes.

RNA analysis

Poly[A] + RNA was purified by one cycle of binding to oligo(dT)-cellulose of RNA prepared from a variety of developmental stages, essentially by tissue disruption and phenol extraction (O'Hare et al. 1983). RNA blots of RNA fractionated on RNA formaldehyde gels and S1-nuclease protection were performed using standard techniques (Maniatis et al. 1982) and uniformly labeled single-stranded probes derived from recombinant M13 single-stranded templates. Primer extension was in each case performed with at least two different oligonucleotides of about 30 nucleotides labeled at the 5' end by [%32P]ATP and T4 polynucleotide kinase as described previously (Zuker et al. 1985).

Chromosomal location of genes

In situ hybridization to polytene chromosomes used nick-translated biotinylated probes from cDNAs or genomic DNAs including sequenced coding regions, as described previously (Zuker et al. 1985).

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Note

Sequence data described in this paper have been submitted to the EMBL/GenBank Data Libraries under accession number Y00220.

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