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Molecular Cloning and Developmental Expression of the Catalytic and 65-kDa Regulatory Subunits of Protein Phosphatase 2A in *Drosophila*

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cDNA clones encoding the catalytic subunit and the 65-kDa regulatory subunit of protein phosphatase 2A (PR65) from *Drosophila melanogaster* have been isolated by homology screening with the corresponding human cDNAs. The *Drosophila* clones were used to analyze the spatial and temporal expression of the transcripts encoding these two proteins. The *Drosophila* PR65 cDNA clones contained an open reading frame of 1773 nucleotides encoding a protein of 65.5 kDa. The predicted amino acid sequence showed 75 and 71% identity to the human PR65 α and β isoforms, respectively. As previously reported for the mammalian PR65 isoforms, *Drosophila* PR65 is composed of 15 imperfect repeating units of ~39 amino acids. The residues contributing to this repeat structure show also the highest sequence conservation between species, indicating a functional importance for these repeats. The gene encoding *Drosophila* PR65 was located at 29B1,2 on the second chromosome. A major transcript of 2.8 kilobase (kb) encoding the PR65 subunit and two transcripts of 1.6 and 2.5 kb encoding the catalytic subunit could be detected throughout *Drosophila* development. All of these mRNAs were most abundant during early embryogenesis and were expressed at lower levels in larvae and adult flies. In situ hybridization of different developmental stages showed a colocalization of the PR65 and catalytic subunit transcripts. The mRNA expression is high in the nurse cells and oocytes, consistent with a high equally distributed expression in early embryos. In later embryonal development, the expression remains high in the nervous system and the gonads but the overall transcript levels decrease. In third instar larvae, high levels of mRNA could be observed in brain, imaginal discs, and in salivary glands. These results indicate that protein phosphatase 2A transcript levels change during development in a tissue and in a time-specific manner.

INTRODUCTION

The importance of protein phosphorylation in *Drosophila* development is evident from the identification of serine/threonine protein kinases involved in cell fate determination or terminal differentiation of specific cell types (reviewed in Siegfried *et al.*, 1990a). The genetic and biochemical analyses possible with *Drosophila* make it an attractive system for the study of the protein kinases and phosphatases involved in specific signaling pathways.

The catalytic subunits of the two major serine/threonine protein phosphatases, type 1 (PP1)[1] and type 2A (PP2A), have been identified in *Drosophila* on the basis of their enzymatic characteristics (Orgad *et al.*, 1987) and by molecular cloning (Dombradi *et al.*, 1989; Orgad *et al.*, 1990). Mutations in the *Drosophila* PP1 87B gene (Axton *et al.*, 1990) lead to a delay in the progression

[1] Abbreviations used: kb, kilobase; PP1, protein phosphatase type 1; PP2A, protein phosphatase type 2A; PR65, 65-kDa regulatory subunit of protein phosphatase 2A.

through mitosis and cause lethality in late larval stages. The involvement of protein phosphatases in mitotic control events is also seen in fungal mutants, in which defects in cell division result from mutations in genes homologous to the mammalian PP1 and PP2A (reviewed in Cyert and Thorner, 1989). Experiments with the protein phosphatase inhibitor okadaic acid in *Xenopus* oocytes have shown the involvement of PP2A in the control of $p34^{cdc2}$ protein kinase at the entry into mitosis (Goris *et al.*, 1989; Felix *et al.*, 1990). This result was supported by the purification of an inhibitor of $p34^{cdc2}$ protein kinase, termed INH, which was identified as a form of PP2A (Lee *et al.*, 1991). Hence, protein phosphatases in a broad spectrum of species appear to be involved in cell division control. *Drosophila* provides a means to analyze the function of these enzymes in a multicellular organism, where early synchronous mitoses in the syncytial stage can be distinguished from asynchronous spatially restricted mitoses in later embryonal development (Glover, 1989).

Several different holoenzymes of PP2A have been purified from a number of species and tissues (reviewed by Ballou and Fischer, 1986; Cohen, 1989; Shenolikar and Nairn, 1991). The "core" structure of the holoenzyme is a heterodimer consisting of a 36-kDa catalytic subunit tightly bound to a regulatory subunit of 65 kDa (PR65). Trimeric forms of PP2A have been purified consisting of the two core subunits associated with either a 54-, 55-, or 72-kDa regulatory subunit. Although the purified holoenzymes show different substrate specificities and regulatory properties in vitro (Imaoka *et al.*, 1983; Takeda *et al.*, 1985; Waelkens *et al.*, 1987; Chen *et al.*, 1989), the in vivo function of the regulatory subunits still is understood poorly.

Recently, we described the molecular cloning of the PR65 subunit of PP2A from human and porcine cDNA libraries (Hemmings *et al.*, 1990). Analysis of cDNAs revealed that two genes, termed PR65α and β, encode this subunit. Both isoforms consist of 15 imperfect repeating units of ~39 amino acids. Here we report the isolation of cDNA clones encoding the *Drosophila* homolog of the catalytic and PR65 subunits of PP2A, the determination of their chromosomal localizations, and the analysis of the temporal and spatial expression of their transcripts during *Drosophila* development.

METHODS

Isolation and Sequence Analysis of Drosophila Catalytic and PR65 Subunit cDNAs

Two *Drosophila* cDNA libraries, prepared from RNA isolated from 8- to 12-h embryos (λgt11) and adult flies (kindly provided by Dr. Tom Kornberg [University of California at San Francisco, San Francisco, CA] and Dr. Bernd Hovemann [ZMBH, Heidelberg, Germany]), initially were screened at low stringency with a 2.2 kilobase (kb) human PR65α cDNA (Hemmings *et al.*, 1990) and a 1.7 kb human PP2A catalytic subunit α cDNA (Stone *et al.*, 1988) for the embryonal and adult cDNA library, respectively. The *Drosophila* adult cDNA library subsequently was screened with sequenced *Drosophila* PR65 cDNA fragments using standard procedures (Maniatis *et al.*, 1982). Probes were radiolabeled using the random priming method (Feinberg and Vogelstein, 1983). Positive clones were plaque purified and cDNA inserts were prepared from phage DNA (DPC-7 and DPR65-14) or inserts were amplified by the polymerase chain reaction (Saiki *et al.*, 1985) from crude phage preparations (DPR65-16-1 and DPR65-16-2) using phage-specific primers. cDNAs were subsequently subcloned as *Eco*RI or *Sal* I fragments into pBluescript (Stratagene, San Diego, CA). For sequence analysis, nested deletions were created by exonuclease III/mung bean nuclease digestion or by using convenient restriction sites. cDNAs were sequenced on both strands by the dideoxy chain termination method (Sanger *et al.*, 1977) using Sequenase (United States Biochemical, Cleveland, OH) according to the manufacturer's instructions with T3/T7 promoter or gene-specific primers. DNA sequence data were analyzed using the University of Wisconsin Genetics Computer Group software package (Devereux *et al.*, 1984).

Northern Blot Analysis

Total RNA (20 μg) isolated by the method of Chomczynski and Sacchi (1987) from different developmental stages of wild-type *Drosophila melanogaster* (Canton S strain) was fractionated on a 0.8% agarose-formaldehyde gel and transferred onto a nylon membrane (Bio-Rad, Glattbrugg, Switzerland). Hybridization was carried out at 42°C in the presence of 50% formamide as described previously (Khew-Goodall and Hemmings, 1988) using the radiolabeled 1.3 kb DPC-7 cDNA or the 1.2 kb insert of DPR65-14 as probes. Loading and transfer efficiency was monitored by probing the blot with a *Sal* I/*Hin*dIII 0.6 kb *Drosophila ras* genomic fragment (Mozer *et al.*, 1985). Transcript sizes were estimated using mRNA markers (Bethesda Research Laboratories, Bethesda, MD).

In Situ Hybridization

The catalytic subunit locus was determined on chromosome squashes from Canton S strain using biotinylated DPC-7 fragment as probe as described in Langer-Safer *et al.* (1982). The PR65 locus was determined in Oregon R strain chromosomes using biotinylated DPR65-16-1 fragment as probe as described in Saunders *et al.* (1989).

RNA localization by in situ hybridization of tissue sections was essentially as described by Baumgartner *et al.* (1987). Paraformaldehyde-fixed cryostat sections (8 μm) from several developmental stages (Canton S strain) were hybridized with ^{3}H probes (the 1.3 kb fragment from DPC-7 or a 2.2 kb fragment assembled from DPR65-16-1 and DPR65-16-2) labeled by nick translation. Slides were exposed to Kodak (Rochester, NY) NTB-2 emulsion for 4 w at 4°C and developed in Kodak D-19 developer at 15°C. Slides were then stained with 5% Giemsa in phosphate buffer and were mounted in DePeX mounting medium (BDH Chemicals Ltd., Poole, Great Britain).

In situ hybridizations to whole-mount embryos were performed according to the method described by Tautz and Pfeifle (1989) with the following modifications. Digoxygenin labeling was performed in the presence of 1 mg/ml random hexamer primers for 14 h at 14°C followed by 6 h at 37°C after readdition of 2 U DNA polymerase. Alkaline phosphatase coupled anti-digoxygenin antibody (Boehringer Mannheim, Mannheim, FRG) was diluted 1:2000 and preabsorbed for 4 h with fixed embryos. After the color reaction (30–120 min), embryos were dehydrated and mounted in Canada Balsam for microscopy.

RESULTS

Molecular Cloning of the Catalytic and PR65 Subunits of PP2A from Drosophila

A human PP2A catalytic subunit α cDNA (Stone *et al.*, 1988) was used to isolate the *Drosophila* homolog from a *Drosophila* adult cDNA library. The isolated clone, DPC-7 (Figure 1), contains a 5′ nontranslated region of

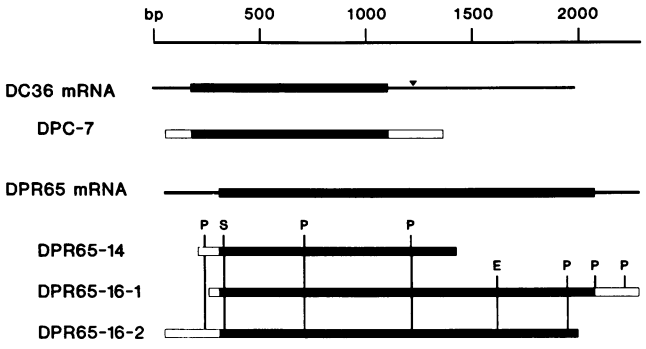

Figure 1. Map of cDNA clones encoding the catalytic and PR65 subunits of *Drosophila* PP2A. The diagram shows the mRNAs encoding the catalytic (DC36) and the PR65 (DPR65) subunits with the corresponding cDNAs below each. The coding region is indicated by a solid box and the 5′ and 3′ untranslated regions by a thin line for the mRNA and as open boxes for the cDNAs. The DC36 mRNA corresponds to the published sequence (Orgad *et al.*, 1990) and the arrowhead indicates the position of the first polyadenylation signal. Restriction sites for *Eco*RI (E), *Pst* I (P), and *Sal* I (S) in the DPR65 clones are indicated.

121 nucleotides, 927 nucleotides coding, and 258 nucleotides 3′ noncoding region ending with 13 A residues. During the course of this work, the sequence of a cDNA encoding the *Drosophila* catalytic subunit was published (Orgad *et al.*, 1990). The sequence of DPC-7 was identical to the reported sequence from nucleotide −121 to 1184, thus lacking some of the noncoding regions.

To clone the *Drosophila* homolog of the PR65 subunit, a human PR65α cDNA (Hemmings *et al.*, 1990) was initially used to screen a *Drosophila* embryo cDNA library at reduced stringency. A single positive clone, DPR65-14 (Figure 1), was isolated and subsequently used to screen the *Drosophila* adult cDNA library for full-length clones. This yielded several additional cDNA clones, of which DPR65-16-1 and DPR65-16-2 were further characterized. Restriction mapping indicated that all three cDNAs overlapped and were sufficient to encompass the complete coding region of the PR65 mRNA (Figure 1). Mapping and sequence analysis of three additional cDNAs indicated that they were identical to the ones described here.

The three overlapping clones were sequenced and found to span an open reading frame of 1773 nucleotides flanked by 5′ and 3′ nontranslated regions of 257 and 170 nucleotides, respectively (Figure 2). The sequence around the predicted initiator AUG was consistent with those found in other *Drosophila* mRNAs (C/AAAC/AAUG) (Cavener, 1987). Comparison of the *Drosophila* PR65 nucleotide sequence with the mammalian PR65α and β cDNAs showed 68 and 61% identity in the coding region, respectively, but with no significant homologies in the noncoding regions. The *Drosophila* PR65 cDNAs contained neither a poly(A) tail nor a polyadenylation signal, indicating that the 3′ noncoding region was incomplete.

Conservation of the PR65 Protein Sequence

The open reading frame of the overlapping *Drosophila* PR65 cDNAs predicted a polypeptide of 591 amino acids with a molecular mass of 65 451 Da. The deduced amino acid sequence showed ~75 and 71% identity to the α and β isoforms of both the human and porcine proteins, respectively (Walter *et al.*, 1989; Hemmings *et al.*, 1990; Mayer-Jaekel and Hemmings, unpublished data). Compared with the mammalian proteins, *Drosophila* PR65 has an insertion of two amino acids in the amino terminal region (Figure 3). As with the mammalian proteins, the *Drosophila* PR65 subunit can be arranged in 15 imperfect repeating units of ~39 amino acids where identical or related residues occur at the same positions (Figure 3). The most highly conserved residues (present in ≥11 out of 15 repeats) are proline at position 3, aspartic acid at position 11, and the valine/arginine pair at positions 16/17 of the repeats. Repeat units 1, 10, and 11 of the *Drosophila* protein show the highest sequence conservation with 90–95% identity with the human PR65α protein. The amino acids, which are conserved between the repeats (boxed), are also highly conserved between species (95% similarity compared with 84% over the whole protein), whereas the regions that are most divergent between the *Drosophila* protein and the mammalian PR65 isoforms also show the highest divergence between the mammalian α and β isoforms. The divergence is most pronounced in repeat unit 8 and in the carboxy terminal 40 amino acids.

Chromosomal Localization

In situ hybridization to polytene chromosomes was performed to localize the *Drosophila* PR65 gene using a 1.9 kb DPR65-16-1 cDNA fragment (nucleotides 22–1950). This revealed a single locus at 29B1,2 on the left arm of the second chromosome (Figure 4). Furthermore, genomic Southern analysis confirmed that a single gene encodes the PR65 subunit. Similarly, a single locus encoding the catalytic subunit was found using DPC-7 as probe, which hybridized at 28D on the second chromosome in agreement with the previously reported data (Orgad *et al.*, 1990).

Expression of PP2A Catalytic and PR65 Subunit mRNAs During Drosophila Development

The expression of the PP2A catalytic subunit at different developmental stages was analyzed by probing a Northern blot with the 1.3 kb DPC-7 cDNA (Figure 5). Two major transcripts of ~1.6 and 2.5 kb and a minor one of 5.5 kb were detected. The expression of the 2.5 kb mRNA was high during early embryogenesis and was reduced ~20-fold in late embryos and in larvae. The 1.6 kb transcript decreased only ~60% in later development and was the major form detected in RNA isolated from larvae and adult flies.

Northern blot analysis of *Drosophila* developmental stages using the 1.2 kb DPR65-14 cDNA as a probe

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-263        GAATTCGGAAAAATATTAATTTGTCACCAACGACGTACGTTTTTCGTTCGAAAAGAACAGCAATCAGCGGATAATTGTATGGA   -181

-180  TTCTTAGAAAATTTGCAGCGACGAAATATAGTACCTTGCGAAATTATTTTGTAACCTTGAAGAAGCAAACTGCGAATCGCCAGAGGGCCA   -91

 -90  AATAGAAATCTCGTCGTTTGCCTGCAGAATAGAAGACCCGATTTTCAGTGCGCTTGTGTTTGTGTGTGAGCAGCAGAAACAGCAGAAAAA    -1

   1  ATGGCAGCAAGCGACAAATCGGTCGACGATTCACTATATCCCATTGCGGTTCTAATCGATGAACTGAAAAACGAGGACGTTCAGCTTCGG    90
   1  M  A  A  S  D  K  S  V  D  D  S  L  Y  P  I  A  V  L  I  D  E  L  K  N  E  D  V  Q  L  R     30

  91  TTGAACTCCATCAAGAAACTGTCCACCATTGCACTCGCTTTGGGCGAGGAGCGCACACGGTCCGAGTTGATTCCCTTCCTCACCGAGACC   180
  31  L  N  S  I  K  K  L  S  T  I  A  L  A  L  G  E  E  R  T  R  S  E  L  I  P  F  L  T  E  T     60

 181  ATATACGATGAGGACGAGGTACTGCTGGCCCTGGCCGACCAACTGGGCAACTTTACTAGTCTCGTTGGTGGGCCAGAGTTTGCCATGTAC   270
  61  I  Y  D  E  D  E  V  L  L  A  L  A  D  Q  L  G  N  F  T  S  L  V  G  G  P  E  F  A  M  Y     90

 271  TTGATTCCGCCCCTCGAGAGTTTGGCCACCGTAGAGGAAACCGTGGTGCGAGACAAGGCTGTGGAATCTCTACGCACCGTGGCCGCTGAG   360
  91  L  I  P  P  L  E  S  L  A  T  V  E  E  T  V  V  R  D  K  A  V  E  S  L  R  T  V  A  A  E    120

 361  CACAGCGCCCAGGATTTGGAGATCCATGTGGTGCCGACACTGCAGCGATTGGTTTCCGGTGACTGGTTCACCTCACGCACCTCTGCCTGC   450
 121  H  S  A  Q  D  L  E  I  H  V  V  P  T  L  Q  R  L  V  S  G  D  W  F  T  S  R  T  S  A  C    150

 451  GGCCTCTTCTCGGTCTGCTATCCACGCGTCACACAGCCAGTGAAGGCCGAGCTGCGCGCCAACTTCCGAAAGCTCTGCCAGGATGAGACA   540
 151  G  L  F  S  V  C  Y  P  R  V  T  Q  P  V  K  A  E  L  R  A  N  F  R  K  L  C  Q  D  E  T    180

 541  CCCATGGTGCGCCGTGCAGCGGCCAACAAGCTGGGCGAGTTTGCCAAGGTCGTTGAGACGGAGTATCTGAAGTCCGATTTGATTCCCAAC   630
 181  P  M  V  R  R  A  A  A  N  K  L  G  E  F  A  K  V  V  E  T  E  Y  L  K  S  D  L  I  P  N    210

 631  TTTGTCCAGCTGGCACAGGATGATCAGGACTCTGTCCGTCTGCTGGCTGTAGAGGCATGCGTAACGATTGCCCAGCTGCTGCCTCAGGAT   720
 211  F  V  Q  L  A  Q  D  D  Q  D  S  V  R  L  L  A  V  E  A  C  V  T  I  A  Q  L  L  P  Q  D    240

 721  GATGTAGAGCACCTGGTTCTGCCCACGCTCCGCCAGTGCGCCAGCGACTCTTCCTGGAGGGTGCGTTACATGGTGGCCGAGAAGTTTGTT   810
 241  D  V  E  H  L  V  L  P  T  L  R  Q  C  A  S  D  S  S  W  R  V  R  Y  M  V  A  E  K  F  V    270

 811  GATCTGCAAAAGGCTGTGGGCCCAGAGATTACTAGGGTGGACTTGGTGCCTGCCTTCCAGTACTTGCTCAAGGATGCCGAGGCCGAGGTT   900
 271  D  L  Q  K  A  V  G  P  E  I  T  R  V  D  L  V  P  A  F  Q  Y  L  L  K  D  A  E  A  E  V    300

 901  CGCGCTGCAGTGGCCACCAAGGTGAAGGACTTCTGCGCCAATCTGGACAAGGTCAACCAGGTGCAAATCATCCTTAGTTCCATTTTGCCC   990
 301  R  A  A  V  A  T  K  V  K  D  F  C  A  N  L  D  K  V  N  Q  V  Q  I  I  L  S  S  I  L  P    330

 991  TATGTCCGCGATCTTGTCTCGGACCCCAATCCTCATGTGAAGTCAGCTCTGGCCTCAGTGATCATGGGCTTGAGTCCCATGCTGGGCGCC  1080
 331  Y  V  R  D  L  V  S  D  P  N  P  H  V  K  S  A  L  A  S  V  I  M  G  L  S  P  M  L  G  A    360

1081  TATCAGACTGTGGAGCAATTGCTCCCCCTGTTCCTTATTCAACTCAAGGATGAGTGCCCAGAAGTGCGCCTAAACATCATCTCAAACCTG  1170
 361  Y  Q  T  V  E  Q  L  L  P  L  F  L  I  Q  L  K  D  E  C  P  E  V  R  L  N  I  I  S  N  L    390

1171  GATTGCGTTAACGACGTCATCGGTATCCAGCAACTGTCACAGTCGCTTCTGCCCGCCATCGTCGAGCTGGCCGAGGACTCCAAGTGGCGT  1260
 391  D  C  V  N  D  V  I  G  I  Q  Q  L  S  Q  S  L  L  P  A  I  V  E  L  A  E  D  S  K  W  R    420

1261  GTGCGTCTAGCCATCATCGAGTACATGCCTGCTCTGGCCGGTCAGTTGGGTCAGGAATTCTTTGACCAAAAACTGCGCGGTCTCTGCATG  1350
 421  V  R  L  A  I  I  E  Y  M  P  A  L  A  G  Q  L  G  Q  E  F  F  D  Q  K  L  R  G  L  C  M    450

1351  GGATGGCTCAACGATCACGTGTACGCCATTCGTGAGGCAGCCACCCTCAACATGAAGAAGCTCGTCGAGCAGTTCGGAGCTCCCTGGGCC  1440
 451  G  W  L  N  D  H  V  Y  A  I  R  E  A  A  T  L  N  M  K  K  L  V  E  Q  F  G  A  P  W  A    480

1441  GAACAGGCCATAATTCCAATGATTCTGGTTATGTCGCGCAACAAGAACTATTTGCACAGAATGACTTGCTTGTTCTGCCTGAATGTTTTG  1530
 481  E  Q  A  I  I  P  M  I  L  V  M  S  R  N  K  N  Y  L  H  R  M  T  C  L  F  C  L  N  V  L    510

1531  GCAGAGGTCTGCGGCACAGATATCACCACCAAGTTGCTGCTGCCCACAGTTCTCCTGCTTGCCGCTGATCCCGTTGCCAATGTTCGTTTC  1620
 511  A  E  V  C  G  T  D  I  T  T  K  L  L  L  P  T  V  L  L  L  A  A  D  P  V  A  N  V  R  F    540

1621  AACGTGGCAAAGACCCTGCAGAAGATCTCGCCCTTCCTGGAGGCCAGCGTCATTGATGCCCAAGTAAAGCCCACACTCGACAAACTGAAC  1710
 541  N  V  A  K  T  L  Q  K  I  S  P  F  L  E  A  S  V  I  D  A  Q  V  K  P  T  L  D  K  L  N    570

1711  ACAGACACAGATGTCGATGTCAAGCATTTTGCTGCACAGGCCATTGCCGGCATAGCTGCAGCGTAATCAACCTTCTTGCATCTTTTTTTA  1800
 571  T  D  T  D  V  D  V  K  H  F  A  A  Q  A  I  A  G  I  A  A  A                              591

1801  TTCTATTTTTAAAACTGTATGTATTTAACGCCGGCAACAAAACATATTATGTAGCAACAACACTTAAGAACGGAAAATTGTAGTGGATCC  1890

1891  CGGAAGGTGTTAAACGCTGCAGCCGATGCTGGATGAGGGATGAATGGAGCCCCGAATTCC  1950
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Figure 2. Nucleotide and deduced amino acid sequence of the *Drosophila* PR65 cDNAs. Sequences were assembled from the overlapping clones DPR65-16-1 and DPR65-16-2. Nucleotides are numbered starting from the first nucleotide of the proposed initiator codon, and nucleotides extending 5′ of base 1 are designated with negative numbers. The deduced amino acid sequence is given in the single letter code. GenBank accession number M86442.

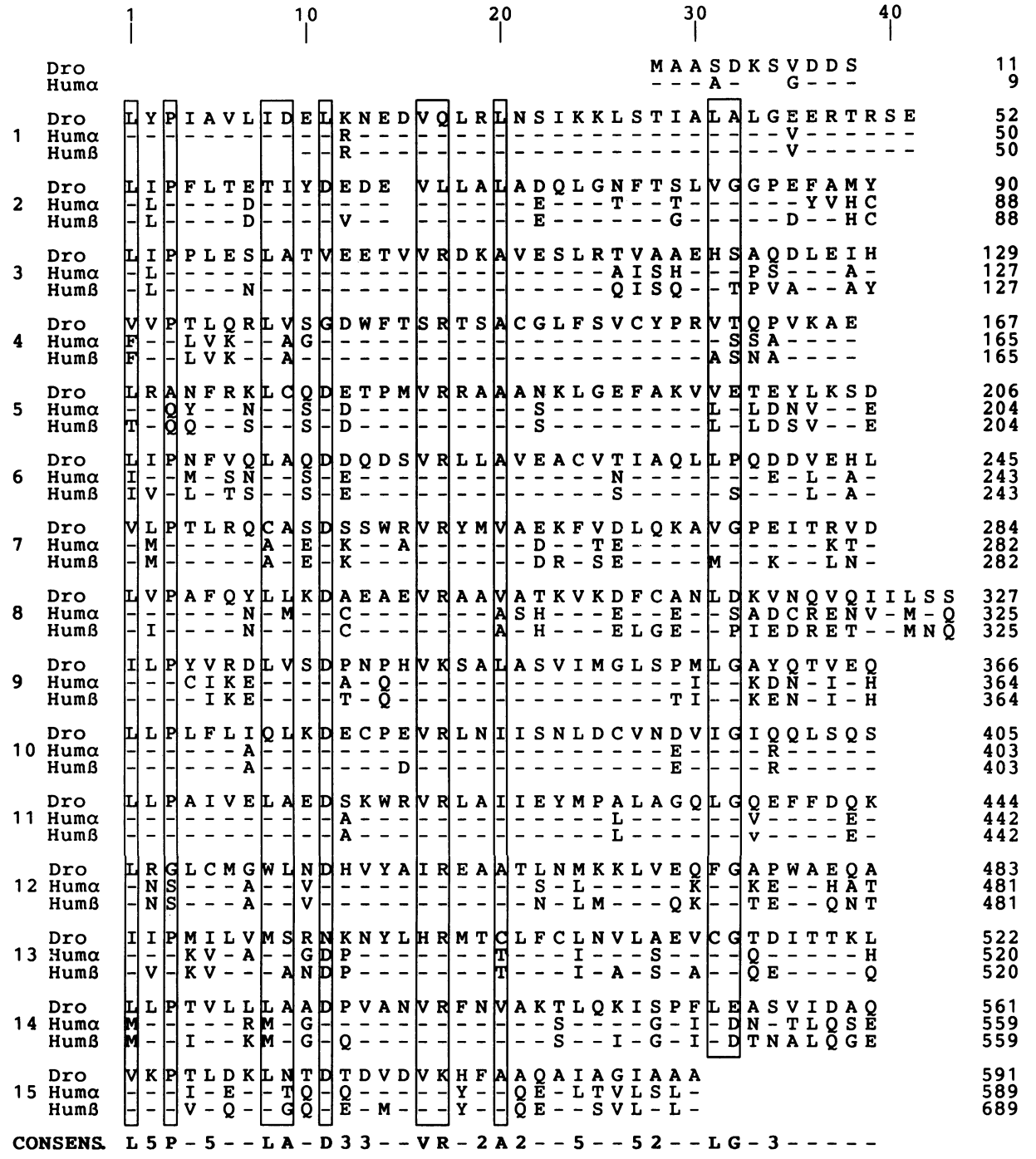

Figure 3. Comparison of the *Drosophila* PR65 protein with the human α and β isoforms. The deduced amino acid sequence of *Drosophila* PR65 was arranged to show the repetitive structure. The deduced amino acid sequence of the human PR65 α and β isoforms (Hemmings *et al.*, 1990; GenBank accession number J02902, M65254) is shown below the *Drosophila* sequence, with identical amino acids indicated as (-). The repeat number is marked on the left of each row and the amino acid position on the right side. The top line gives the position within a repeat and the last line gives the consensus residues for the position. The boxed residues correspond to the positions identified earlier as most highly conserved between the repeats of the mammalian proteins. (Amino acid groups are defined as follows: [1] C; [2] S, T, P, A, G; [3] N, D, E, Q; [4] H, R, K; [5] M, I, L, V; [6] F, Y, W.) Amino acid residues 452–457 of the human PR65β were deduced from the nucleotide sequence determined after reverse transcription of MCF7 poly(A)$^+$ RNA followed by a polymerase chain reaction amplification using primers flanking the two internal *Eco*RI sites (Mayer-Jaekel and Hemmings, unpublished data).

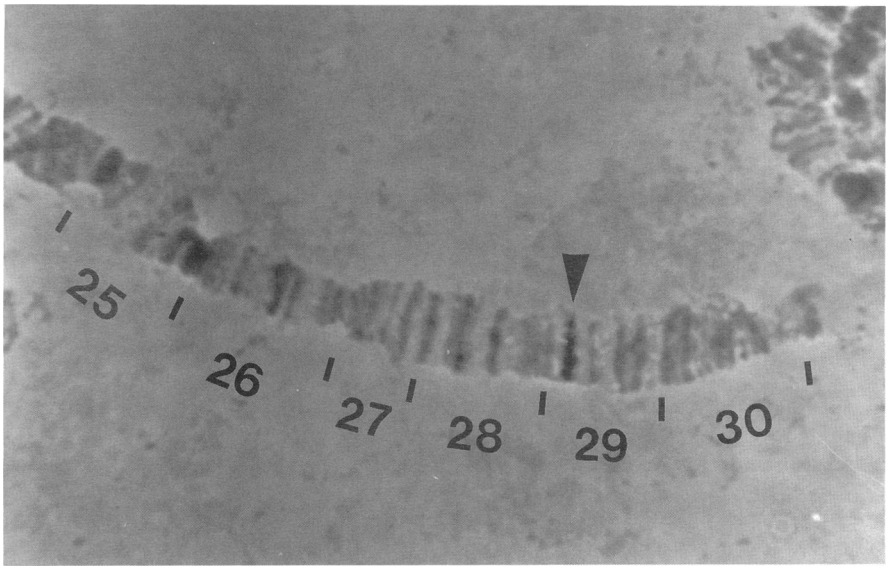

Figure 4. Chromosomal localization of *Drosophila* PR65. In situ hybridization of the DPR65-16-1 cDNA to polytene chromosome salivary gland squashes. The localization of the gene at 29B1,2 on the second chromosome is indicated by an arrow.

showed a major transcript of 2.8 kb and minor ones of 5.5 and 7.5 kb that were expressed at all developmental stages (Figure 5). The major PR65 transcript was expressed at very high levels in embryos from 0 to 12 h, decreased ~10-fold in late embryo, and remained low in larvae and adult flies. The minor transcripts showed less variation during development, and in larvae the 5.5 kb transcript was almost as abundant as the major 2.8 kb mRNA.

Localization of PP2A Catalytic and PR65 Subunit mRNA Expression

Drosophila cryostat sections from different developmental stages were hybridized with either the DPC-7 cDNA (Figure 6) or a probe assembled from the DPR65-16-1 and DPR65-16-2 cDNAs (nucleotides −263–1950) (Figure 7). The transcripts encoding the catalytic and PR65 subunits appear to colocalize during development.

In adult female flies (Figures 6 and 7, A and B), basal levels of the catalytic and PR65 subunit transcripts were detected in all tissues. High expression levels were observed only in the ovaries, restricted to the oocytes and the nurse cells, but only basal levels were detected in the follicle cells. The transcript levels in the egg chamber appear to change according to the stage of oocyte development. Slightly elevated levels were also observed in the brain and in the gut.

During early embryonic development (Figures 6 and 7, C and D), both the catalytic and PR65 subunit transcripts were distributed evenly at high levels. Only in late embryos (Figure 6D, stage 16 according to Campos-Ortega and Hartenstein [1985]) could a slightly higher accumulation of the catalytic subunit transcripts in the developing nervous system be observed (but see below). The overall transcript levels were clearly reduced in later embryonal stages, consistent with the reduction observed by Northern blot analysis (Figure 5).

At the third instar larval stage (Figures 6 and 7, E and F), the catalytic and PR65 subunit transcripts were enriched in the brain lobes (supraoesophangial ganglion), whereas the neuropile showed very low levels of mRNA. Furthermore, increased expression levels were observed in the imaginal discs and surprisingly high levels in the salivary glands.

Whole-mount in situ hybridization was performed to obtain higher resolution of the expression of the mRNAs encoding the catalytic and PR65 subunits during embryonic development. As seen with the cryostat sections, both transcripts were distributed evenly in early embryos. Only when the overall levels of transcripts decreased was a more localized distribution detected. The earliest time that an enrichment of the transcripts in the developing nervous system could be observed was at full germ band extension (Figure 8, Panels A and B), where the expression in the neuroblasts was clearly elevated compared to the epidermal cell layer. At later developmental stages (Figure 8, C and D), the basal expression level was further reduced, and a clear enrichment of the transcripts in the ventral cord and the supraoesophangial ganglion could be detected. In addition, we observed elevated transcript levels in the gonads (Figure 8, C and D).

DISCUSSION

This paper reports the molecular cloning of the *Drosophila* catalytic and PR65 subunits of PP2A and the developmental expression of the transcripts encoding both these subunits. The PR65 protein is composed of an imperfect repeating units structure that is highly conserved from *Drosophila* to humans (Figure 3). The consensus sequence previously defined for the residues conserved between the repeats in mammalian PR65 isoforms (Hemmings *et al.*, 1990) remains valid for the *Drosophila* protein. However, in several cases there were

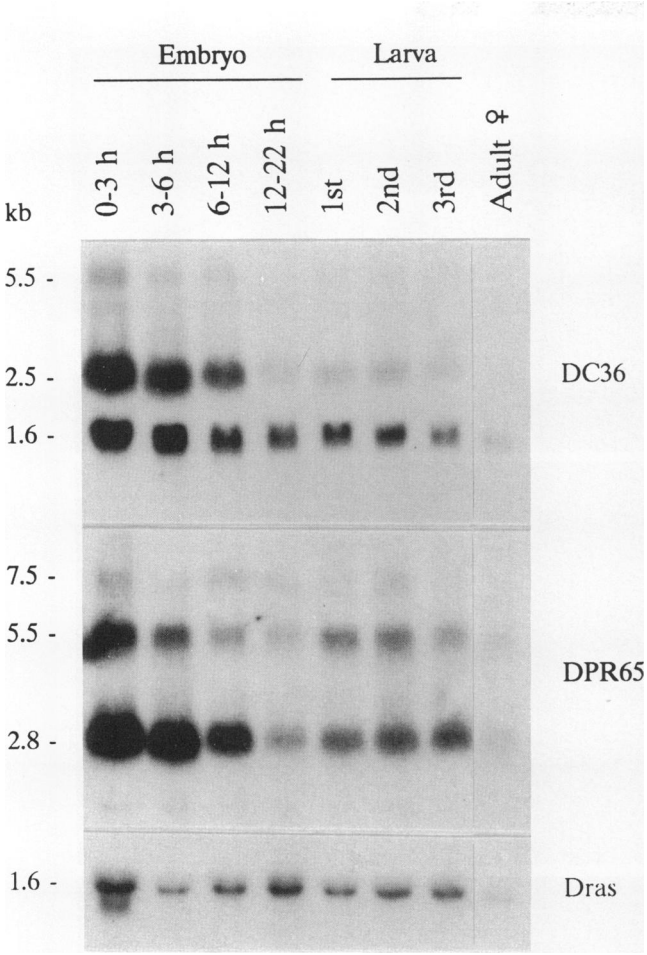

Figure 5. Northern blot analysis of *Drosophila* PP2A catalytic and PR65 subunit mRNAs (DC36 and DPR65). Total *Drosophila* wild-type RNA (20 μg) was fractionated on 0.8% agarose formaldehyde gel and transferred to Zeta-Probe nylon membrane. The probes used are indicated on the right and the estimated transcript sizes on the left. The DPC-7 cDNA insert was labeled to a specific radioactivity of 1.6×10^9 cpm/μg, the DP65-14 cDNA to 1.5×10^9 cpm/μg, and the *Drosophila ras* (Dras) fragment to 1.2×10^9 cpm/μg. Filters were washed in 15 mM sodium chloride, 1.5 mM sodium citrate, pH 7.0/0.1% SDS at 60°C for three times 30 min and exposed for 3 d for DPC-7 and DPR65-14 and for 11 d for Dras, at −70°C with two intensifying screens.

conservative amino acid substitutions. A few residues show extreme conservation, occurring in ≥11 of the repeat units: proline at position 3, aspartic acid at position 11, and a valine/arginine pair at positions 16/17 of the repeats. Altogether there are eight positions where hydrophobic amino acids dominate, seven positions with polar amino acids, and five positions with charged amino acids (see consensus line of Figure 3). There is a clustering of polar and charged residues between position 9 and 21 of all repeats, whereas at the beginning of the repeats hydrophobic residues dominate. The function of the repeating units remains to be elucidated. PR65 is the only subunit that is tightly associated with the catalytic subunit in all purified holoenzymes (Hendrix, Mayer-Jaekel, Goris, Hofsteenge, Merlevede, and Hemmings, unpublished data), and it appears likely that it is involved in modulation of activity and/or substrate specificity of the catalytic subunit. The PR65 subunit also interacts with the other regulatory subunits and appears to be the subunit that is responsible for the interaction of PP2A with the small and middle T antigens of polyoma virus (Pallas *et al.*, 1990) and the small t antigen of SV40 virus (Joshi and Rundell, 1990; Yang *et al.*, 1991). Thus, the unique repeating structure of PR65 may be important for the interactions with these different proteins. Interestingly, the molecular cloning of a novel regulatory subunit of PP1, termed *sds*22, from *Schizosaccharomyces pombe* (Ohkura and Yanagida, 1991) has revealed a protein with another kind of leucine-rich repeating structure. It will be interesting to elucidate how these structural features contribute to subunit interaction in protein phosphatase holoenzymes.

The *Drosophila* PR65 subunit shows 74.5 and 70.4% identity with the mammalian α and β isoforms, which themselves are 85.6% homologous (Hemmings *et al.*, 1990). Therefore, the *Drosophila* protein cannot be identified as a strict homolog of either mammalian isoform. Thus, it is possible that the duplication of the PR65 gene occurred after the divergence of arthropods and chordates. The sequence conservation of the PR65 subunit is comparable with those of mammalian and *Drosophila* protein kinases, such as the cAMP-dependent protein kinase (82%) (Kalderon and Rubin, 1988), GSK-3 (75%) (Bourouis *et al.*, 1990; Siegfried *et al.*, 1990b; Woodgett, 1990), or $p34^{cdc2}$ (60%) (Jimenez *et al.*, 1990; Lehner and O'Farrell, 1990). Analysis of the predicted amino acid sequence of the 55-kDa subunit of PP2A from *Drosophila* (Mayer-Jaekel, unpublished data) revealed ~78% identity to the two human isoforms (Mayer *et al.*, 1991a). However, a much higher degree of conservation was found for the catalytic subunits of PP1α and PP2A from mammals and *Drosophila* (92 and 94% identity, respectively) (Dombradi *et al.*, 1989; Orgad *et al.*, 1990).

The genes encoding the *Drosophila* catalytic and PR65 subunits are both located on the left arm of the second chromosome at 28D and 29B1,2, respectively (Orgad *et al.*, 1990; this study), which is a relatively poorly characterized region of the *Drosophila* genome. Identification of a single locus for each PP2A subunit indicates that the *Drosophila* genome contains only a single gene for the respective proteins. This might suggest that there is a certain redundancy in the PP2A subunit isoforms detected in other species, which could also serve as a backup system. In yeast, where two isoforms of the PP2A catalytic subunit are present, cells carrying only one PP2A catalytic subunit isoform have a smaller cell size but are still viable, whereas double negative mutants are lethal (Kinoshita *et al.*, 1990). This shows at least a partial redundancy of the isoforms in these cells.

Northern blot analysis of the catalytic and PR65 subunit transcripts revealed high expression of the major

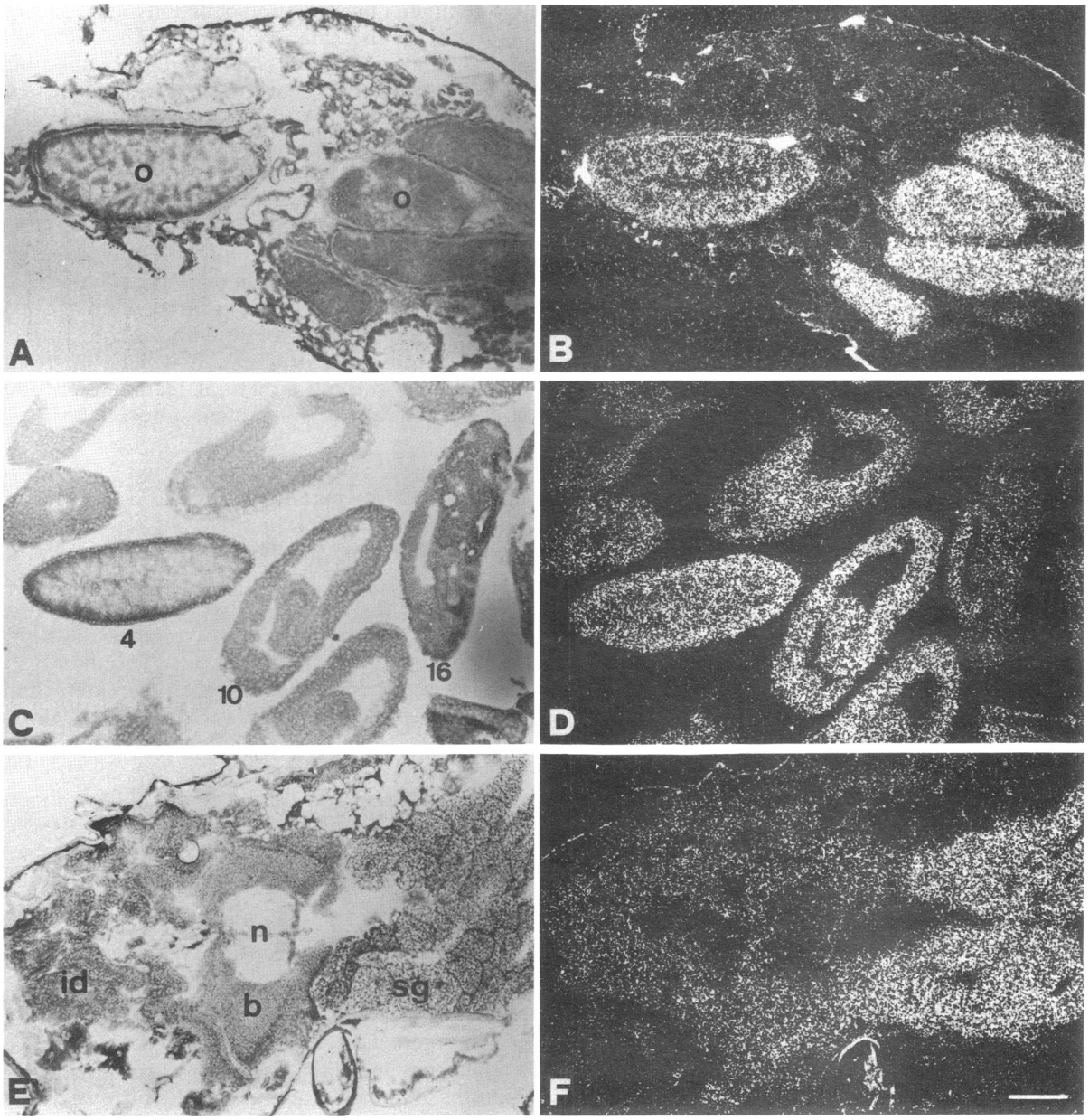

Figure 6. In situ localization of PP2A catalytic subunit mRNA expression during *Drosophila* development. Cryostat sections of different developmental stages were hybridized with ^{3}H-labeled DPC-7 cDNA probe and exposed for 4 wk. Bright-field (left) and corresponding dark-field (right) micrographs are shown. (A and B) Section through an adult female abdomen; o, oocyte. (C and D) Sections through different developmental stages as defined by Campos-Ortega and Hartenstein (1985): 4, stage 4, 2 h; 10, stage 10, 5 h; 16, stage 16, 15 h. (E and F) Section through third instar larvae: b, brain; n, neuropile; id, imaginal disk; sg, salivary gland. The bar in F corresponds to 100 μm.

transcripts during early embryonic development, whereas the later stages all show lower levels.

For the catalytic subunit we observed a complex expression pattern of three transcripts of ~1.6, 2.5, and 5.5 kb. The 1.6 kb transcript appears to be expressed at high levels throughout development with about a three-fold variation between early embryo and larvae and was the predominant form in adult flies. In contrast, the 2.5 kb transcript was only found at high levels in early embryo and was reduced 20-fold in larvae and adults. The developmental pattern of the catalytic subunit mRNA expression is more complex than that observed by Orgad *et al.* (1990). This difference might be attributed to either the antisense probe used, which apparently only detects the 3′ noncoding region unique to the 2.5 kb transcript, or the absence of the 5.5 kb transcript in poly(A)$^{+}$ RNA.

The PR65 probe recognized a major transcript of 2.8 kb and minor ones of 5.5 and 7.5 kb. After washing at increased stringency, the ratio of the hybridization sig-

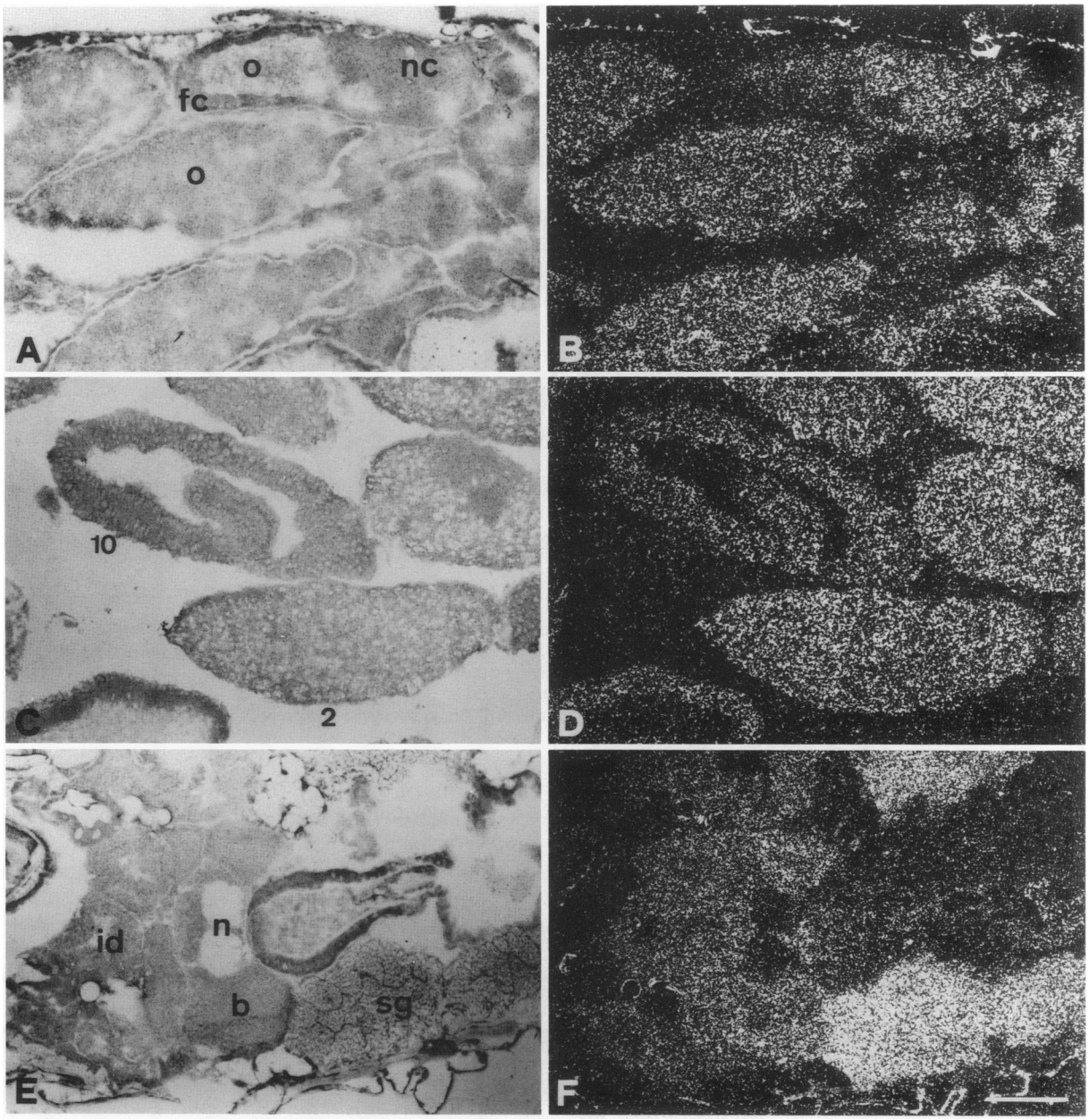

Figure 7. In situ localization of PP2A PR65 subunit mRNA expression during *Drosophila* development. Cryostat sections of different developmental stages were hybridized with a ^{3}H-labeled PR65 cDNA fragment assembled from DPR65-16-1 and DPR65-16-2 and exposed for 4 wk. Bright-field (left) and corresponding dark-field (right) micrographs are shown. (A and B) Section through an adult female abdomen: o, oocyte; nc, nurse cells; fc, follicle cells. (C and D) Sections through different developmental stages as defined by Campos-Ortega and Hartenstein (1985): 2, stage 2 embryo, 1 h; 10, stage 10, 5 h. (E and F) Section through third instar larvae: b, brain; n, neuropile; id, imaginal disk; sg, salivary gland. The bar in F corresponds to 100 μm.

nals remained constant, suggesting that the multiple transcripts were derived from the same gene. It is possible that the larger transcripts are processed partially because they were not detected in poly(A)$^+$ enriched RNA from another RNA preparation.

The transcripts encoding the catalytic and PR65 subunit apparently colocalize during all developmental stages. This is consistent with the fact that these two proteins are the "core subunits" of all characterized PP2A holoenzymes (Hendrix, Mayer-Jaekel, Goris, Hofsteenge, Merlevede, and Hemmings, unpublished data). Low amounts of the transcripts could be detected in all tissues throughout development, which is in agreement with the multiple functions PP2A plays in cellular metabolism. However, there also appears to be a specific localized requirement for high amounts of the PP2A core subunits because both tissue and temporal specific changes in the transcript levels were observed.

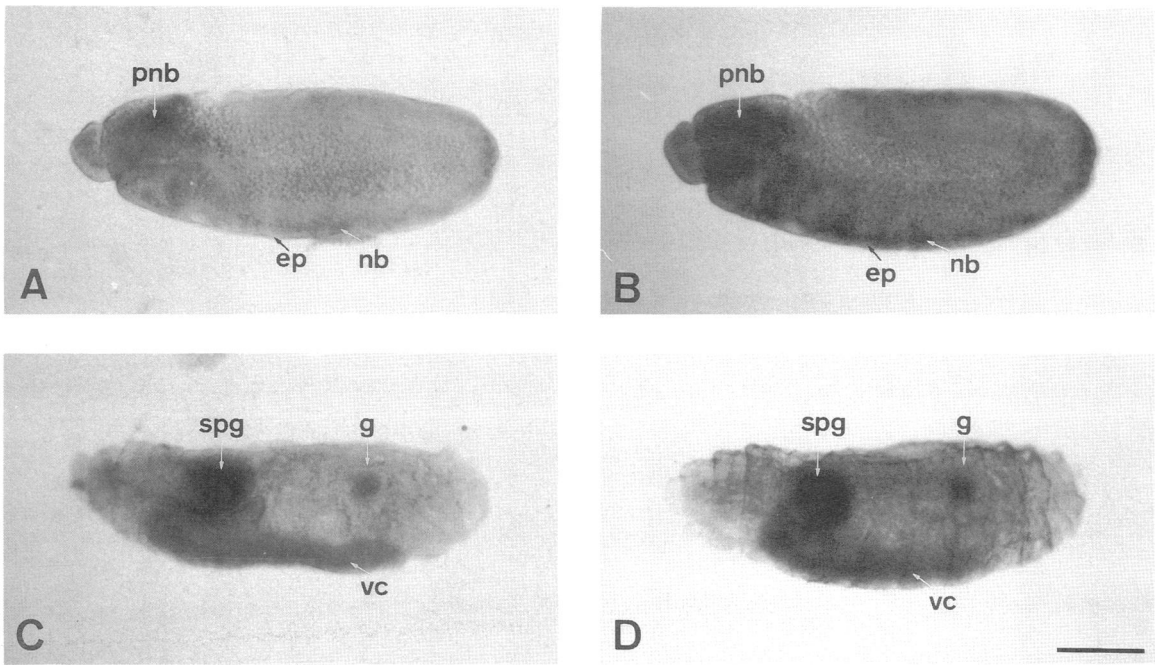

Figure 8. Whole-mount in situ analysis of PP2A catalytic and PR65 mRNA expression during embryogenesis. Whole-mount embryos were hybridized with digoxygenin-labeled DPC-7 cDNA (A and C) or the 2.2 kb fragment assembled from DPR65-16-1 and DPR65-16-2 (B and D). Alkaline phosphatase reaction was performed for 30 and 60 min for the catalytic and PR65 subunit probes, respectively. (A and B) Stage 10/11 embryos; (C and D) stage 16/17 embryos (according to Campos-Ortega and Hartenstein, 1985): ep, epidermis; g, gonads; nb, neuroblasts; pnb, procephalic neuroblasts; spg, supraoesophangial ganglion; vc, ventral cord. All embryos are shown with the anterior to the left and dorsal to the top. The bar in D corresponds to 100 μm.

This presumably reflects different promoter activities and/or differences in mRNA stability in the various tissues. It is also possible that the different transcript forms detected by Northern blot analysis show distinctive distribution patterns.

In the early syncytial phase of embryonal development, high amounts of the PP2A mRNAs were present that were provided maternally, as could be seen by the high transcript levels in nurse cells and oocytes. Interestingly, the transcripts encoding the 55-kDa regulatory subunit of *Drosophila* PP2A are also very abundant in early embryos (Mayer-Jaekel, unpublished data). This might suggest a specific role of the trimeric PP2A holoenzyme during this phase of rapid nuclear divisions. The elevated levels of the transcripts encoding the two PP2A subunits in imaginal discs, the gonads, and the developing nervous system could also reflect the involvement of PP2A in the ongoing cell divisions in these structures. The PP2A transcript levels were high in the developing nervous system, starting from the onset of neuroblast formation throughout embryogenesis. These data indicate that PP2A is involved in neurogenesis. In third instar larvae, the expression was high in the brain lobes but much lower in the neuropile, and in adults a less pronounced expression in the brain could still be detected. This indicates that the neuronal expression is not restricted to the developing brain, suggesting a more general role in neuronal functions. Interestingly, the mutations of the PP1 87B locus lead to defects in larval neuroblast development and in imaginal cell proliferation (Axton *et al.*, 1990). The increased mRNA expression in the *Drosophila* nervous system is also consistent with the high expression levels of PP2A subunit transcripts in the mammalian brain (Khew-Goodall and Hemmings, 1988; Mayer *et al.*, 1991b) and the immunolocalization of the catalytic subunit in neurons of all brain regions (Saitoh *et al.*, 1989).

The analysis of yeast mutants in the catalytic subunit of PP2A (Kinoshita *et al.*, 1990) and the effects of the protein phosphatase inhibitor okadaic acid on oocyte maturation (Goris *et al.*, 1989; Felix *et al.*, 1990) indicate that the enzyme exhibits a negative control on the entry into mitosis. The activation of the $p34^{cdc2}$ protein kinase, essential for entry into mitosis, requires tyrosine dephosphorylation by $p80^{cdc25}$ (*string* in *Drosophila*) on a key residue in the ATP binding site. Because both positive and negative regulation are required for a correct timing of mitosis, it may be that specific holoenzyme forms of PP2A indirectly participate in $p34^{cdc2}$ protein kinase regulation. Moreover, recent data from Gautier *et al.* (1991) show that *string* and PP2A act at different steps of $p34^{cdc2}$ activation. This agrees with the recent observation that a negative regulator of $p34^{cdc2}$ kinase from *Xenopus* oocytes was found to be a novel form of PP2A (Lee *et al.*, 1991).

It will be interesting to elucidate the specific targets of PP2A in the different tissues with elevated expression

levels and also to see whether specific PP2A holoenzymes, containing additional regulatory subunits, are involved. The molecular complexity of the *Drosophila* genes encoding the other regulatory subunits of PP2A, the 55- and 72-kDa subunits, is currently under investigation. The availability of cDNA clones encoding the different subunits of *Drosophila* PP2A will enable the analysis of the regulation and function of this enzyme during development and may also provide some clues regarding its function in mammalian systems.

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