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# *Drosophila* protein phosphatase V functionally complements a *SIT4* mutant in *Saccharomyces cerevisiae* and its amino-terminal region can confer this complementation to a heterologous phosphatase catalytic domain

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**The sequence of a *Drosophila melanogaster* cDNA encoding a novel 35 kDa protein serine/threonine phosphatase, termed *PPV*, is presented. PPV is 40-41% identical to *Drosophila* PP1, 53% identical to *Drosophila* PP2A and 63% identical to *Saccharomyces cerevisiae* SIT4. Complementation studies demonstrated that PPV can functionally rescue a temperature sensitive mutant of *SIT4*, a protein phosphatase required for the $G_1$ to S transition of the cell cycle. When placed under the *SIT4* promoter, *PPV* cDNA is able to replace the *SIT4* gene in *S.cerevisiae*. The amino-terminal domain of PPV fused to another phosphatase catalytic region (PP1) also rescues the temperature sensitive *SIT4* mutant and the SIT4 deletion mutant, implicating this region in binding to regulatory subunits and/or altering specificity. In *Drosophila*, a substantial transient increase in both PPV mRNA and protein occurs in late syncitial and early cellular blastoderm embryos. At the latter stage PPV is localized to the cytoplasm of cells at the cortex. This increase in PPV correlates with introduction of the $G_2$ phase of the cell cycle, elevated zygotic transcription and cellularization, indicating that PPV may play a role in one or more of these processes.**

*Key words:* cell cycle/cellularization/*Drosophila*/protein phosphatase/regulation of transcription

## Introduction

Reversible phosphorylation on serine and threonine residues is now recognized as a major mechanism by which many intracellular processes are controlled, such as signal integration (Cohen, 1992), transcription (Meek and Street, 1992) and cell division (Yanagida *et al.*, 1992). A diverse array of protein serine/threonine kinases have been characterized (Hunter, 1991) and it has become evident over the last few years that there is also a large family of protein serine/threonine phosphatases which reverse the actions of these kinases (Cohen *et al.*, 1990; Chen *et al.*, 1992). Four types of protein serine/threonine phosphatase activity were originally distinguished, PP1, PP2A, PP2B and PP2C, by enzymatic criteria (Cohen, 1989) and molecular cloning has revealed that they fall into two structurally distinct families: the PP1/2A/2B family and the PP2C family (Cohen *et al.*, 1990).

*In vitro*, the isolated catalytic subunits of the type 1 and type 2A protein phosphatases have broad and overlapping substrate specificities. Tight control of these activities *in vivo* is believed to be achieved by reversible binding to targeting subunits which not only direct the catalytic subunits to particular locations but also modify their catalytic properties (Hubbard and Cohen, 1993). Among the best characterized targeting subunit is the G subunit, which directs the PP1 catalytic subunit to glycogen particles where it mediates the hormonal regulation of glycogen metabolism (Dent *et al.*, 1990). More recently, further targeting subunits have been identified in skeletal and smooth muscles, which target PP1 to the myofibrils and enhance the myosin dephosphorylating activity of the PP1 catalytic subunit while suppressing the dephosphorylation of glycogen metabolizing enzymes (Alessi *et al.*, 1992; Dent *et al.*, 1992).

The PP2A catalytic subunit is also complexed to other proteins *in vivo*. The catalytic subunit can bind to the A subunit, through which it then binds to one of a number of B subunits. Although there is currently no evidence that the the A and B subunits have a targeting function, their presence modifies the catalytic activity of PP2A towards various substrates (reviewed in Cohen, 1989; Ferrigno *et al.*, 1993). In addition, the A subunit binds to the SV40, polyoma and BK virus small T antigens and polyoma middle T antigen in infected cells, resulting in the formation of T antigen-A subunit- PP2A catalytic subunit complexes (Mumby and Walter, 1991).

The catalytic subunits of mammalian PP1 and PP2A are ~40% identical in amino acid sequence (Berndt *et al.*, 1987) and both catalytic subunits have remained remarkably conserved through evolution (Cohen, 1990). However, molecular cloning has demonstrated the existence of a number of novel PP1/PP2A-like catalytic subunits including rabbit PPX (Brewis *et al.*, 1993), *Drosophila melanogaster* PPY (Dombrádi *et al.*, 1989b), *Saccharomyces cerevisiae* PPZ (da Cruz e Silva *et al.*, 1991; Posas *et al.*, 1992; Hughes *et al.*, 1993) and PPG (Posas *et al.*, 1993). The physiological substrates of these novel enzymes are unknown.

Among the best characterized of the novel protein phosphatases in *S.cerevisiae* is SIT4, which was originally identified from a gene in which mutations increased *HIS4* transcription in the absence of three transcription factors, *BAS1*, *BAS2* and *GCN4* (Arndt *et al.*, 1989). In certain genetic backgrounds, an allele of *SIT4*, *sit4-102*, causes a temperature sensitive cell cycle arrest in late $G_1$, at about the time when SIT4 becomes complexed with two high molecular weight phosphoproteins (Sutton *et al.*, 1991a). The late $G_1$ arrest is due to the requirement for SIT4 in the accumulation of the $G_1$ cyclin mRNAs, an effect mediated at least in part via the transcription factor *SWI4* (Fernandez-Sarabia *et al.*, 1992). SIT4 is also required for at least one other function in late $G_1$ as evidenced by the fact that in *sit4* mutants, constitutive expression of the $G_1$ cyclin CLN2 results in DNA synthesis without bud emergence (Fernandez-Sarabia *et al.*, 1992).

Here we report the isolation of a cDNA encoding a novel

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protein phosphatase from *D.melanogaster*, termed PPV, which is maximally expressed at the periphery of the blastoderm embryo with a cytoplasmic distribution. We show that PPV is a functional homologue of *S.cerevisiae* SIT4 and that the amino-terminal domain of PPV can convert a PP1 catalytic subunit into an enzyme with the *in vivo* function of SIT4.

## Results

### *Isolation and cDNA sequence of PPV*

Screening 90 000 plaque forming units of a *Drosophila* head cDNA library at moderate stringency with a *Drosophila PP1 87B* probe yielded 92 positively hybridizing plaques. These clones were screened with a panel of oligonucleotides complementary to known *Drosophila* protein phosphatases. Two clones hybridizing positively with the *PP1 87B* cDNA probe, but not with any of the specific oligonucleotide probes, contained inserts of 1.5 kb whose 5′ and 3′ terminal 300 nucleotides were identical. The nucleotide sequence of one clone was completely elucidated (nucleotides 39–1596, Figure 1). Sequence analysis showed the presence of a single large open reading frame (ORF), which displayed considerable similarity to the amino acid sequences of type 1 and type 2A protein phosphatases and was termed protein phosphatase V (PPV). However, the cDNA appeared to be truncated at the 5′ end when compared with other protein phosphatases and lacked a methionine codon in a sequence context suitable for translation initiation. Therefore, a cDNA library derived from *Drosophila* eye imaginal discs was screened with oligonucleotides complementary to sequences at the 5′ end of the ORF. One double positive clone was isolated containing a 1.6 kb insert which was sequenced from both strands (Figure 1). Nucleotides 39–1596 were identical to those of the previous clone, but the ORF was extended at the 5′ terminus.

The *PPV* cDNA contained a single large ORF encoding a protein of 303 amino acids with a predicted molecular mass of 34.7 kDa. The sequences adjacent to the assigned initiating methionine exhibit close correlation with the *Drosophila* consensus initiation sequence as determined by Cavener (1987). Sequence of the gene (data not shown) identified an in frame stop codon 12 nucleotides 5′ to the start of the sequence in Figure 1 with no motifs that indicated the presence of an intron–exon boundary in this region, corroborating our assignment of the initiating methionine

```
-25                                                                           GTGGATTTCACAGGCAGCGGGCATC    -1
   1 ATGGGCGACGTGGACAAGTGGATAGAAGACGTGAAGAAATGCAAGTACCTGCCGGAGAACGAGCTGAAGAAGCTATGCGAGATGGTCTGC    90
   1  M  G  D  V  D  K  W  I  E  D  V  K  K  C  K  Y  L  P  E  N  E  L  K  K  L  C  E  M  V  C    30

  91 GATATCCTCCTGGAGGAGACGAACATCCTGCCCGTGAGCACGCCCGTCACCGTTTGCGGTGACATCCATGGCCAGTTCTACGACCTGGAG   180
  31  D  I  L  L  E  E  T  N  I  L  P  V  S  T  P  V  T  V  C  G  D  I  H  G  Q  F  Y  D  L  E    60

 181 CAGCTGTTCCGCACTGGAGGCCAGGTGCCGCATACCAACTACATATTCATGGGCGACTTCGTGGACAGGGGCTACTACTCGCTGGAGACA   270
  61  Q  L  F  R  T  G  G  Q  V  P  H  T  N  Y  I  F  M  G  D  F  V  D  R  G  Y  Y  S  L  E  T    90

 271 TTCACCAGACTGCTCACGCTGAAGGCGCGCTATCCCAGCCGGATCACCCTGCTGCGCGGCAACCACGAAACGCGGCAGATCACCAAGGTG   360
  91  F  T  R  L  L  T  L  K  A  R  Y  P  S  R  I  T  L  L  R  G  N  H  E  T  R  Q  I  T  K  V   120

 361 TACGGATTCTTTGACGAGTGCTTCAGCAAGTACGGCAATGCCAATGGCTGGAAATACTGCTGCAAGGTCTTCGATTTGCTTACCATCGCT   450
 121  Y  G  F  F  D  E  C  F  S  K  Y  G  N  A  N  G  W  K  Y  C  C  K  V  F  D  L  L  T  I  A   150

 451 GCCATCATCGACGAGGAGGTGTTGTGCGTGCACGGTGGCCTGAGTCCCGAGATCATTACACTGGACCAGATCAGGACGATCGATCGCAAC   540
 151  A  I  I  D  E  E  V  L  C  V  H  G  G  L  S  P  E  I  I  T  L  D  Q  I  R  T  I  D  R  N   180

 541 GGCGAGATACCGTACAAGGGTGCCTTCTGCGATCTGGTGTGGTCCGATCCCGAGGACATGGAGTACTGGGGTCAGAGTCCGCGCGGCGCT   630
 181  G  E  I  P  Y  K  G  A  F  C  D  L  V  W  S  D  P  E  D  M  E  Y  W  G  Q  S  P  R  G  A   210

 631 GGCTGGCTGTTTGGCCACAATGTGACCAAGGATTTTATGGCGATCAATAATCTGAACCTCATATGCCGAGCACATCAGCTGGTCAACGAG   720
 211  G  W  L  F  G  H  N  V  T  K  D  F  M  A  I  N  N  L  N  L  I  C  R  A  H  Q  L  V  N  E   240

 721 GGCATCAAGTATATGTTTGATGGCAAACTGGTGACCGTGTGGTCGGCGCCAAACTATTGCTATCGCTGCGGCAACGTTGCCGCCATTCTT   810
 241  G  I  K  Y  M  F  D  G  K  L  V  T  V  W  S  A  P  N  Y  C  Y  R  C  G  N  V  A  A  I  L   270

 811 AGCTTCGAAACGGCGGAGAAGCGGCAGACGAAGATCTTCCTAGCGGTTCCGGATGCGGAGCGTGTGATACCCAAGCAGAACACAACGCCC   900
 271  S  F  E  T  A  E  K  R  Q  T  K  I  F  L  A  V  P  D  A  E  R  V  I  P  K  Q  N  T  T  P   300

 901 TACTTCCTGTAAAACGTTCTGATGTGATGCGATGTGAAGTGATGCAACGCAATACTATACCACATACCATACCACATACCGATCCAAAAC   990
 301  Y  F  L  *                                                                                 303

 991 GATCCGCCCACTGTTCCGGCATCGTGGCTAATAATCATATACGCTTGTGGAGAGCAGAGCGGAGTGCAGCCTTCGACTGACTGATCCGTT  1080
1081 TCATTTGACCACCCATGAACTCAACGCCTTAAACTCAAAACGTGCGCAACGAGCACCCCAAGCATAGCAAACACTCAGAACAGCAAACAC  1170
1171 AAGCCACACACACCACACACACACCTGTAACTTGTAATTTTCGTCTCTTTTTTGTATGGCAAAAACACATATCTAATTCTTAAGAGCACA  1260
1261 GTTAAAAAAAAACTTTGTTATTAGTTTCCGGCAAGTCCAAAGAGAGGGGAACACGTGAATTGATTTCTTTAAGTTGTATATCCAAGTATT  1350
1351 GTATTTACTGATAACGGATGCTAATGGAAGCAAGAAGTATATAGAGATTCACTTTGTTATTTTGTATTCCAAGCCTACATATTTATTTCT  1440
1441 GTGAACAAACACAAAAATCATATACTTTGTACATTTTGTATTAGTAATGCTTTGAAGGAAATAAGCAAGAATAACTAAGAATAACCCAAT  1530
1531 TGTGTGATCCCGATCAAAACGATATGATTAAAACAAAAAAGACAAAAAAAAAAAAAAAAAAAAAAAAA                         1599
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**Fig. 1.** Nucleotide and predicted amino acid sequence of the *PPV* cDNA. Nucleotides are numbered from the ATG encoding the putative initiating methionine. Amino acids conserved in all members of the PP1/PP2A/PP2B family are underlined. The termination codon is indicated by an asterisk and the potential polyadenylation signal is double underlined. The sequence of the genomic clone was identical to the cDNA except for nucleotide 675 (C) which was T in the genomic sequence; this change does not result in any amino acid difference. Arrowheads indicate the positions of the two introns in the coding region of the *PPV* gene.

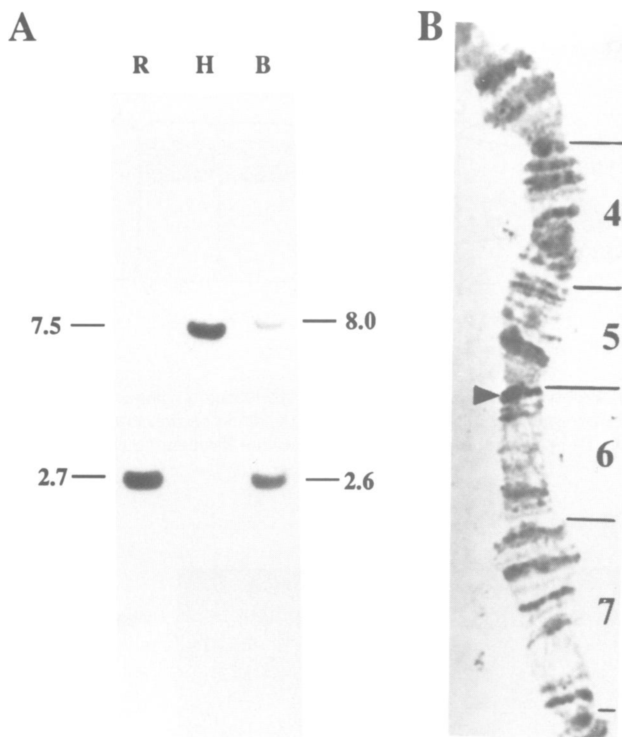


**Fig. 2.** Characterization of the *PPV* gene. (**A**) Total genomic DNA from *D.melanogaster* strain Oregon R was digested with *Eco*RI (R), *Hin*dIII (H) or *Bgl*II (B). The DNA fragments were separated by agarose gel electrophoresis, transferred to Hybond N+ by Southern blotting and hybridized with full-length *PPV* cDNA. The fragment sizes indicated are in kilobases. (**B**) The biotin-labelled *PPV* cDNA was hybridized to polytene chromosomes from third instar larvae. A single site of hybridization was detected at cytological position 6A1-2 on the X chromosome. This site was consistently observed in more than five different preparations.

codon. The 3′ non-coding region is 690 nucleotides in length and following the stop codon there are additional in frame termination signals starting at positions 925 and 950. The clone terminates in a poly(A) tract and, although lacking the consensus polyadenylation signal, contains the motif ATTAAA, commencing 17 nucleotides from the start of the poly(A) tail which can direct polyadenylation (Wickens, 1990).

### Chromosomal localization and expression of PPV

Southern blot analysis (Figure 2A) of *Drosophila* genomic DNA using the 1.6 kb cDNA as probe indicated that *PPV* is encoded by a single copy gene. This result is corroborated by hybridization of the *PPV* cDNA to polytene chromosomes; only a single site of hybridization was found, at cytological position 6A1-2 on the X chromosome (Figure 2B).

Northern blot analysis utilizing poly(A)+ RNA isolated from *Drosophila* at different developmental stages revealed that the cDNA hybridized to a single 2.0 kb polyadenylated species which was most abundant in 0–4 h old embryos (Figure 3A). Prolonged exposure showed *PPV* to be present in all of the other developmental stages and in adults of both sexes at a low level (data not shown). This result is consistent with the fact that the *PPV* cDNA was isolated from libraries derived from non-embryonic tissues. As a control for the possible variation in the amount of RNA loaded, the same blot is shown after removal of the *PPV* cDNA probe and hybridization with a cDNA probe specific for *PP1 87B* (Figure 3B), which is present at a constant level during *Drosophila* development (Dombrádi *et al.*, 1989a). In order to study the developmental expression of *PPV* in greater detail, Northern blots were performed using poly(A)+ RNA isolated from 0–2 and 2–4 h old embryos. Figure 3C shows that *PPV* is expressed at a higher level in 2–4 h

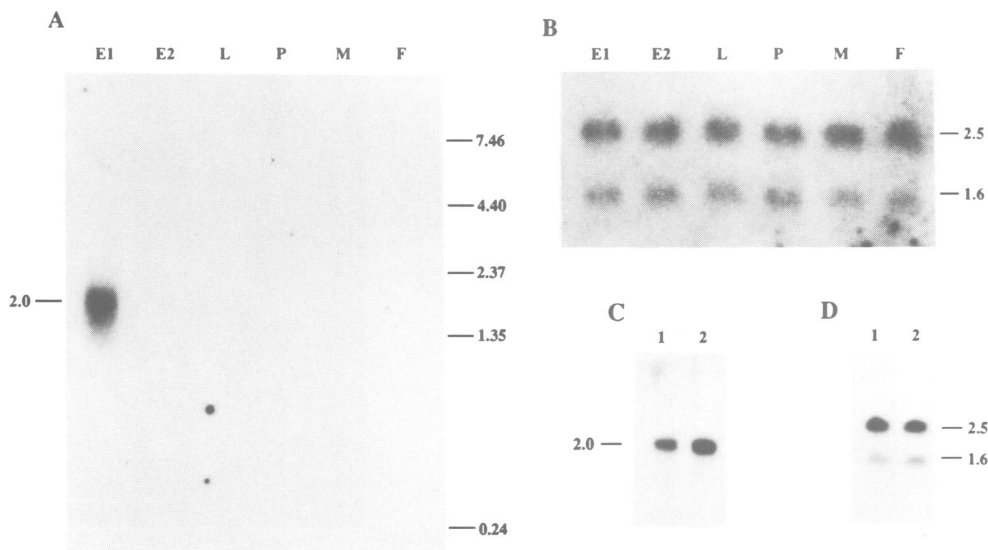


**Fig. 3.** *PPV* mRNA levels during the *Drosophila* life cycle. (**A**) Poly(A)+ RNA from different developmental stages was analysed, after electrophoresis and transfer to Hybond N membranes, by hybridization with the full-length *PPV* cDNA. E1, 0–4 h old embryos; E2, 4–24 h old embryos; L, larvae; P, pupae; M, male adult flies; F, female adult flies. (**B**) To control for variation in the amount of RNA loaded, the *PPV* cDNA probe was stripped from the blot shown in panel A and the blot hybridized with the *PP1 87B* cDNA. This mRNA is expressed at a constant level during *Drosophila* development (Dombrádi *et al.*, 1989a). (**C** and **D**) Poly(A)+ RNA, isolated from 0–2 h (1) or 2–4 h (2) old embryos maintained at 25°C and hybridized with either *PPV* or *PP1 87B* cDNA, respectively. The sizes of the transcripts are in kilobases and the positions of migration of RNA molecular weight markers are shown to the right of panel A.

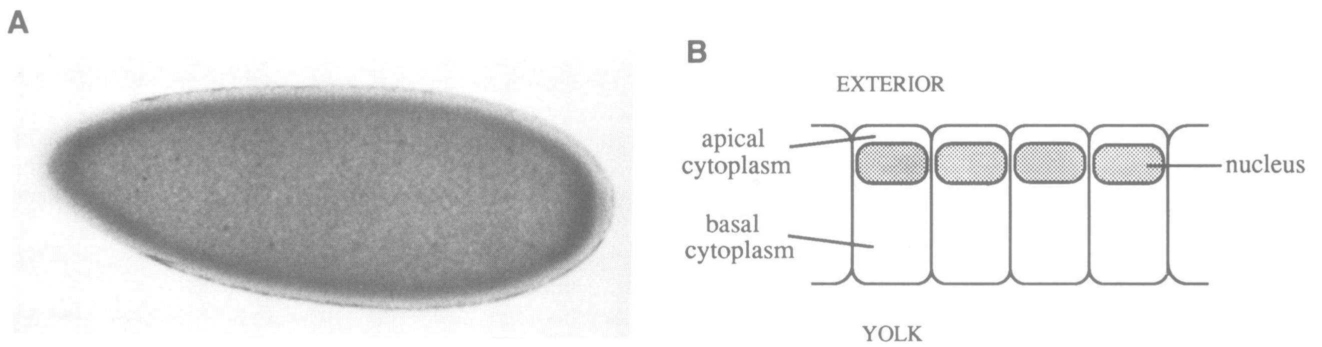


**Fig. 4.** Spatial distribution of *PPV* mRNA in a cellular blastoderm embryo. (**A**) Embryos were analysed by *in situ* hybridization using a digoxygenin-labelled full-length *PPV* cRNA probe, binding being detected indirectly via anti-digoxygenin antibodies conjugated to alkaline phosphatase. The embryo is orientated with the anterior on the left and the dorsal side uppermost. (**B**) Schematic diagram showing a section through the embryonic cortex.

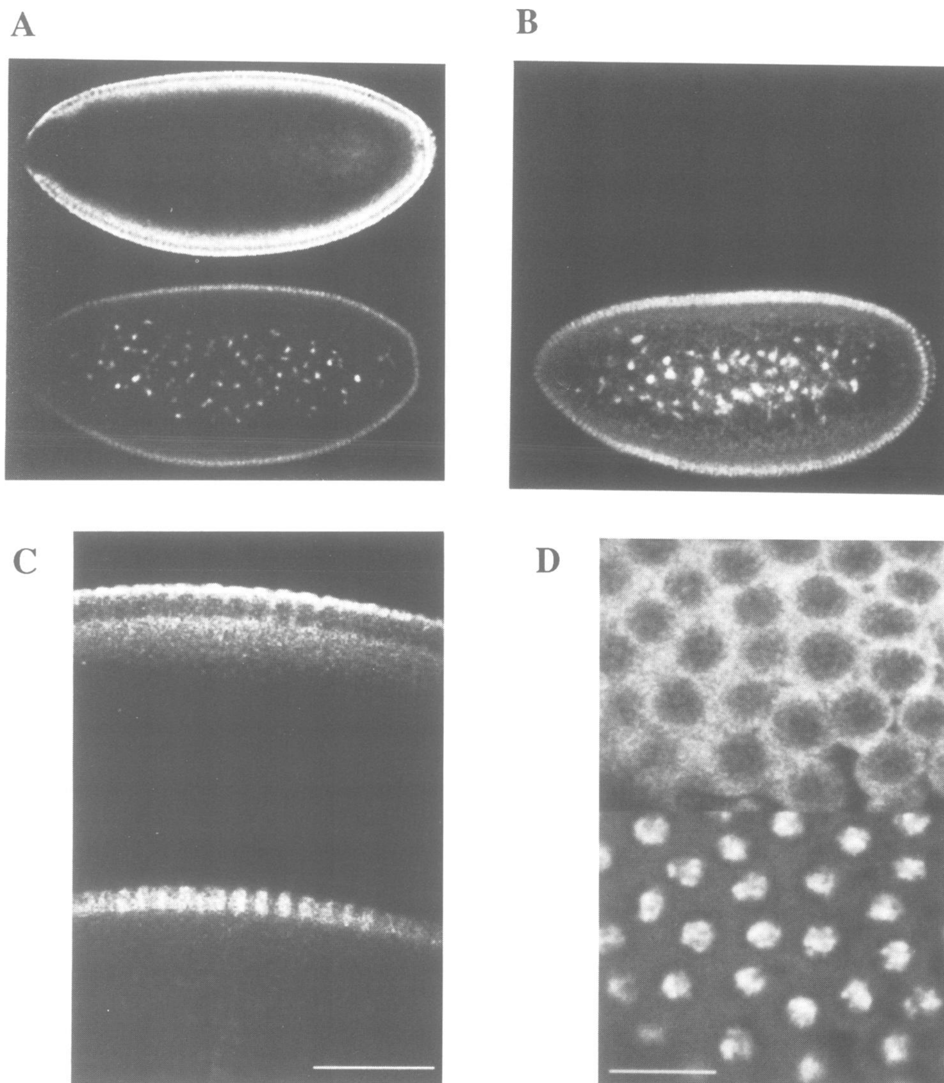


**Fig. 5.** Analysis of PPV protein distribution in the embryo. The upper halves of panels A, C and D show the distribution of PPV in whole mount embryos detected by indirect immunofluoresence using affinity purified anti-peptide antibodies and fluorescein-conjugated secondary antibodies. The upper half of panel B shows a whole mount embryo treated in an identical manner except that the anti-PPV antibody was preadsorbed using the antigenic peptide coupled to Affigel 15. The lower halves of all panels show the same fields stained with propidium iodide to visualize DNA. Panels A and B show optical sections through cellular blastoderm embryos. Both embryos are orientated with the anterior on the left and the dorsal side uppermost. (**C**) Shows a higher magnification of an optical section through the ventral surface of the embryo in (A) and (**D**) shows a transverse section through the cortical nuclei of a cellular blastoderm embryo. The scale bars are 20 μm in (C) and 10 μm in (D). The photographs were obtained using the Bio-Rad MRC 600 scanning laser confocal microscope.

embryos. Quantification by densitometric scanning demonstrated that the 2–4 h embryos expressed approximately twice as much *PPV* mRNA as the 0–2 h embryos. The timing of increased expression indicates that the majority of the *PPV* mRNA is zygotically transcribed, consistent with the observation that the level of this mRNA in adult females is similar to that observed in adult males (data not shown).

The distribution of *PPV* mRNA in early embryos was analysed by *in situ* hybridization using antisense cRNA probes. In early syncitial embryos, *PPV* mRNA was barely detectable over control hybridizations performed in parallel with a sense *PPV* cRNA probe (data not shown). However, after the cortical nuclear migration, the level of *PPV* transcripts had increased significantly and was clearly detectable over non-specific staining. The *PPV* mRNA was concentrated in the cytoplasm and excluded from the nuclear region (Figure 4). Later embryos displayed less specific staining (data not shown) indicating either degradation of the message or redistribution throughout the embryo.

In order to investigate whether the PPV protein had the same spatial and temporal distribution as *PPV* mRNA, we raised antibodies to a synthetic peptide comprising amino acids 285–300. The sequence of this region is distinct from that of all other known *Drosophila* protein phosphatases. Immunoblotting with affinity purified antibodies showed that they were specific to PPV and did not cross-react with up to 5 μg of either PP1 or PP2A purified from rabbit skeletal muscle, *Drosophila* PPY or rabbit PPX expressed in baculovirus (data not shown). The antibodies were used to analyse the distribution of PPV in embryos, binding being detected via an anti-rabbit IgG–fluorescein conjugate. The pattern of antibody staining was similar to that observed for the *PPV* mRNA. A transient increase in the expression of PPV protein was found in late syncitial and early cellular blastoderm embryos. PPV was excluded from nuclei and found in the surrounding cytoplasm, both apical and basal to the nuclei (Figure 5). The level of PPV protein, like the *PPV* mRNA, declined rapidly after gastrulation commenced (data not shown). Control experiments in which the PPV antibodies were preadsorbed against the synthetic peptide showed no specific staining of embryos.

### *PPV is most closely related in sequence to S.cerevisiae SIT4*

The amino acid sequence of PPV exhibits 40–41% identity to the type 1 protein phosphatases found in *D.melanogaster* (Dombrádi *et al.*, 1990a, 1993) and 53% identity to *Drosophila* PP2A (Orgad *et al.*, 1990). Thus, PPV belongs to the type 2A subfamily. In order to understand the sequence relationships between the various members of the type 2A family, we constructed a phylogenetic tree of these protein phosphatases from mammals, *Drosophila* and yeast (Figure 6) by the progressive alignment procedure of Feng and Doolittle (1990). On the basis of this comparison, the type 2A protein phosphatases fall into two groups, the classical PP2As and the novel PP2A-like protein phosphatases. The latter can be further subdivided into three subgroups: PPX/PPH3, PPG and PPV/SIT4. The more recently published *S.pombe* ppe1$^+$ (Shimanuki *et al.*, 1993; Matsumoto and Beach, 1993) falls into the PPV/SIT4 subgroup (Figure 6A).

### *PPV can functionally complement a SIT4 mutation*

The sequence similarity of PPV to SIT4 prompted us to test whether overexpression of *PPV* would suppress the temperature sensitive growth phenotype of *S.cerevisiae* strain CY249. This haploid strain has a genomic deletion of *SIT4* and carries the *sit4-102* allele on the plasmid YCp50. *sit4-102* contains a mutation within the coding region of the gene (glutamic acid 38 to lysine, Sutton *et al.*, 1991b) which leads to arrest at the $G_1$–S transition of the cell cycle at 37°C

A

5 units

R. PP2A α
R. PP2A β
D. PP2A
pp2a1$^+$
pp2a2$^+$
PPH21
PPH22
R.PPX
PPH3
D.PPV
ppe1$^+$
SIT4
PPG

B

```
ppe1                                 M F D L D E W I A T V   11
SIT4                             M V S R G P D E W L E T I   13
PPV                                  M G D V D K W I E D V   11
PP2A                     M E D K A T T K D L D Q W I E Q L   17
PP1 87B    M G D V M N I D S I I S R L L E V R G A R P   22

ppe1       R K C K Y L P E H Q L K R L C E M V K V I L   33
SIT4       K K C Q A L T E N E M K Q L C E M V K E L L   35
PPV        K K C K Y L P E N E L K K L C E M V C D I L   33
PP2A       N E C N Q L T E T Q V R T L C D K A K E I L   39
PP1 87B    G K N V Q L S E G E I R G L C L K S R E I F   44

ppe1       M E E S N I Q P V R T P V T V C G D I H G Q   55
SIT4       M E E S N I Q P V Q T P V T V C G D I H G Q   57
PPV        L E E T N I L P V S T P V T V C G D I H G Q   55
PP2A       S K E S N V Q E V K C P V T V C G D V H G Q   61
PP1 87B    L S Q P I L L E L E A P L K I C G D I H G Q   66
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**Fig. 6.** Relationship of PPV to other protein serine/threonine phosphatases. (**A**) Phylogenetic tree constructed using the progressive alignment programme of Feng and Doolittle (1990). The scale bar indicates evolutionary distance units. The distance is derived according to the Poisson relationship D = −lnS where S is a measure of the similarity between sequences. R. PP2Aα, R. PP2Aβ (da Cruz e Silva and Cohen, 1987) and R.PPX (Brewis *et al.*, 1993) are rabbit sequences; D. PP2A (Orgad *et al.*, 1990) and D.PPV (this paper) are *Drosophila* sequences; pp2a1$^+$, pp2a2$^+$ (Kinoshita *et al.*, 1990) and ppe1$^+$ (Shimanuki *et al.*, 1993) are from *S.pombe*; PPH21 and PPH22 (mammalian PP2Aα and PP2Aβ homologues, Sneddon *et al.*, 1990), PPH3 (Ronne *et al.*, 1990), SIT4 (Arndt *et al.*, 1989) and PPG (Posas *et al.*, 1993) are from *S.cerevisiae*. (**B**) Comparison of the amino-termini of PPV. SIT4 and ppe1 with *Drosophila* PP1 and PP2A. The boxed amino acids are residues common to PPV, SIT4 and ppe1 but absent from other known *Drosophila*, *S.cerevisiae* or *S.pombe* protein serine/threonine phosphatases. The residues in the stippled boxes denote a motif which is conserved between all members of the PP1/PP2A/PP2B family.

(Sutton *et al.*, 1991a). The 1.6 kb *PPV* cDNA was cloned into the multicopy expression vector pADH (which possesses a *LEU2* selectable marker) so that *PPV* expression was driven by the *ADH1* promoter. Transformants of CY249 were tested for the ability to grow at 37°C. Only *PPV* was able to complement the mutation so as to permit growth at the restrictive temperature (Table I). It should be noted that both *Drosophila PP2A* and *PPX* appeared to be lethal to *S.cerevisiae* under this expression regime; no transformants were obtained, even when batches of the same competent cells produced transformants when treated with either *PPV* in the vector or vector alone.

As the *ADH1* promoter utilized above is a strong, constitutive promoter, we tested the ability of *PPV* to rescue the temperature sensitivity of CY249 when expressed under the control of the *SIT4* promoter itself. The construct created (Figure 7A) comprised the *SIT4* promoter, the first four codons of *SIT4*, the *PPV* coding region (minus its first four codons) and the *SIT4* 3' non-coding region in the single copy vector YCp50; in addition the *URA3* gene of YCp50 was replaced with the *LEU2* gene to provide an appropriate selectable marker. After introduction of this construct into CY249, transformants were selected and tested for their ability to grow at 37°C. *PPV* was again able to suppress the growth arrest phenotype (Table I).

**Table I.** Rescue of the temperature sensitive mutation *sit4-102*

| Construct | Transformation of CY249 | Growth at restrictive temperature |
|---|---|---|
| pADH | + | − |
| pADH-V | + | + |
| pADH-V reverse | + | − |
| pADH-X | − | ND |
| pADH-2A | − | ND |
| pADH-13C | + | − |
| PPH21 | + | − |
| YCp50-SIT4:V | + | + |

Plasmids were constructed as described in Materials and methods and used to transform *S.cerevisiae* strain CY249. Transformants were tested for the ability to grow at 37°C. Overexpression of PPH21 was shown by Sutton *et al.* (1991a) to be ineffective in suppressing the temperature sensitivity of any *sit4* strain. ND, not determined.

Since *sit4-102* was expressed from the plasmid YCp50 which contains the *URA3* selectable marker we were able to test whether *PPV* could completely replace the *SIT4* gene by growing transformants on medium containing 5-fluoro-orotic acid (FOA); this compound is metabolized by the product of the *URA3* gene to produce 5-fluorouracil, a toxic metabolite. Thus, growth on medium containing FOA and uracil enables strains to be cured of *URA3*-containing plasmids (Guthrie and Fink, 1991). Expression of *PPV* from either the *ADH1* or *SIT4* promoters permitted growth on medium containing FOA, demonstrating that *PPV* can completely replace the otherwise essential *SIT4* gene in this strain (Figure 7B).

#### *Importance of the amino-terminal domain of PPV*

Alignment of PPV and SIT4 with *D.melanogaster* type 1 and type 2A phosphatases revealed amino acid identities which were common to PPV and SIT4 but absent from other protein phosphatases. The amino-terminal region was the only domain in which these unique identities were more than simply isolated amino acids (Figure 6B). In order to test whether the ability of *PPV* to rescue the *sit4-102* allele was conferred by the amino-terminal region with sequence similarity to SIT4, we constructed chimeric protein phosphatases. Since both *Drosophila PP2A* and *PPX* were apparently lethal using the *ADH1* expression system, an isoform of *Drosophila* PP1, termed PP1 13C (Dombrádi *et al.*, 1993), was used to construct the chimeric protein phosphatases. PP1 13C has been shown to encode a functional protein phosphatase, which is inhibited by inhibitor 2 and is 94% identical at the amino acid level to the better characterized PP1 87B (Dombrádi *et al.*, 1990b, 1993). Two different chimeras were constructed in pADH (Figure 8A); the amino-terminal domain of *PPV* ligated to the catalytic domain of *PP1 13C* (termed *V:13C*) and the amino-terminal domain of *PP1 13C* ligated to the catalytic domain of *PPV*

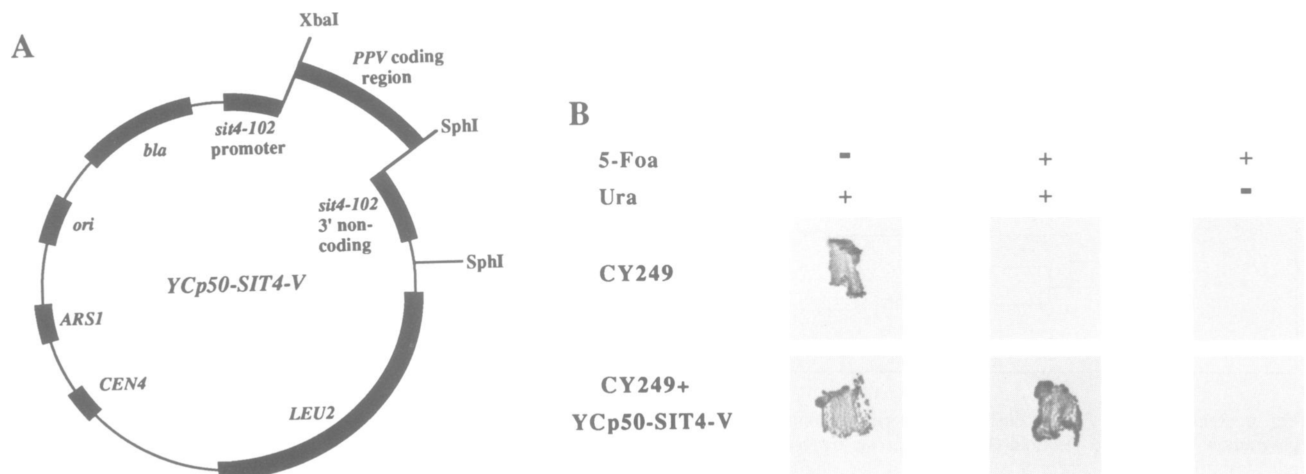


**Fig. 7.** Expression of *PPV* from the *SIT4* promoter using a single copy plasmid abolishes the requirement for endogenous *SIT4*. (**A**) Schematic diagram showing the construct YCp50-SIT4-V. The plasmid is based on YCp50 except that the *URA3* gene has been replaced with the *LEU2* selectable marker. (**B**) *S.cerevisiae* strain CY249 (which contains *sit4-102* on the YCp50 plasmid) was transformed with YCp50-SIT4-V. Transformants were grown on medium containing FOA (5-Foa) and uracil (Ura). Colonies were replated onto medium containing FOA and/or uracil and their ability to grow compared with that of the untransformed CY249. The inability of the *PPV* transformed yeast to grow in the absence of Ura indicates that the *URA3*/*sit4-102*-containing plasmid in CY249 had been lost.

(termed *13C:V*). All constructs were verified by sequence analysis, cloned under the *ADH1* promoter and transformed into CY249. Recombinant *S.cerevisiae* were then replated at the non-permissive temperature. As shown in Figure 8B, only the chimera with the amino-terminal domain of *PPV* was able to permit growth at the restrictive temperature, clearly demonstrating that this region of *PPV* is essential for the rescue of *sit4-102*. The rate of growth permitted by the *V:13C* chimera was similar to that observed with intact *PPV*. In addition, counter selection on medium containing FOA and uracil to remove the *URA3*-containing YCp50 plasmid carying the *sit4-102* allele was performed. This showed that the *V:13C* chimera expressed from the *ADH1* promoter could rescue the strain carrying a deletion of the *SIT4* gene and no *sit4* mutant allele (data not shown).

## Discussion

### *PPV is a protein serine/threonine phosphatase and a functional homologue of SIT4*

The *Drosophila* cDNA sequence presented here encodes a protein, termed PPV, which has sequence similarities to the family of protein serine/threonine phosphatases that includes PP1, PP2A and PP2B. Amino acid motifs invariant among all protein phosphatases from mammals to bacteriophage and predicted to be essential for catalytic activity (Cohen and Cohen, 1989) are also found in PPV (Figure 1). It is therefore likely that PPV is a functional protein serine/threonine phosphatase.

PPV exhibits most amino acid similarities to members of the PP2A subfamily (Figure 6A), showing 51–55% identity to mammalian, *Drosophila* and *S.cerevisiae* PP2A. The sequence similarities of *Drosophila* PPV to *S.cerevisiae* PPH3 (58% identity) and SIT4 (63% identity) are somewhat higher, suggesting that PPV may be the *Drosophila* homologue of *S.cerevisiae* SIT4. In order to examine this possibility, we showed that PPV functionally complemented a lethal temperature sensitive allele of the *SIT4* gene, *sit4-102*, permitting growth at the restrictive temperature. No other phosphatases tested showed complementation. Moreover, *PPV* cDNA was able to replace completely the *SIT4* gene when provided in single copy under the control of the *SIT4* promoter.

### *The amino-terminal domain of PPV confers the ability to rescue a SIT4 mutant onto a heterologous phosphatase catalytic domain*

A chimera with the amino-terminal domain of PPV and the middle and carboxy-terminal region of PP1 13C (V:13C) rescued the SIT4 mutant, whereas the converse construct (13C:V) did not, pointing to the importance of the amino-terminal domain in conferring specificity of function. Studies in yeast and *Aspergillus* have demonstrated that *PP2A* genes cannot complement mutations in *PP1* genes and vice versa (Kinoshita *et al.*, 1990; Doonan *et al.*, 1991; Shimanuki *et al.*, 1993). Therefore it is remarkable that although PPV and SIT4 are members of the PP2A subfamily, the amino-terminal domain of PPV can rescue a SIT4 mutant when fused to a PP1 catalytic domain. This rescue is not due to interaction of the PPV amino-terminal domain with the endogenous sit4-102 mutant protein, since the V:13C chimera was able to rescue the strain lacking *sit4-102* and carrying a deletion of the chromosomal *SIT4* gene. It will clearly be of considerable interest to see whether fusion of the amino-terminal domain of PP2A to the catalytic domain of PP1 can complement a PP2A mutation.

The amino-terminus of PPV is encoded by a section of the *PPV* gene separated from the remainder of the *PPV* coding sequence by an intron 3′ of the conserved histidine–glycine amino acid doublet (residues 53 and 54 in Figure 1). Since introns often divide proteins into modular domains (Gilbert *et al.*, 1986), this finding supports the view that the amino-terminus of PPV may have a specific function. There are several mechanisms by which the amino-terminal

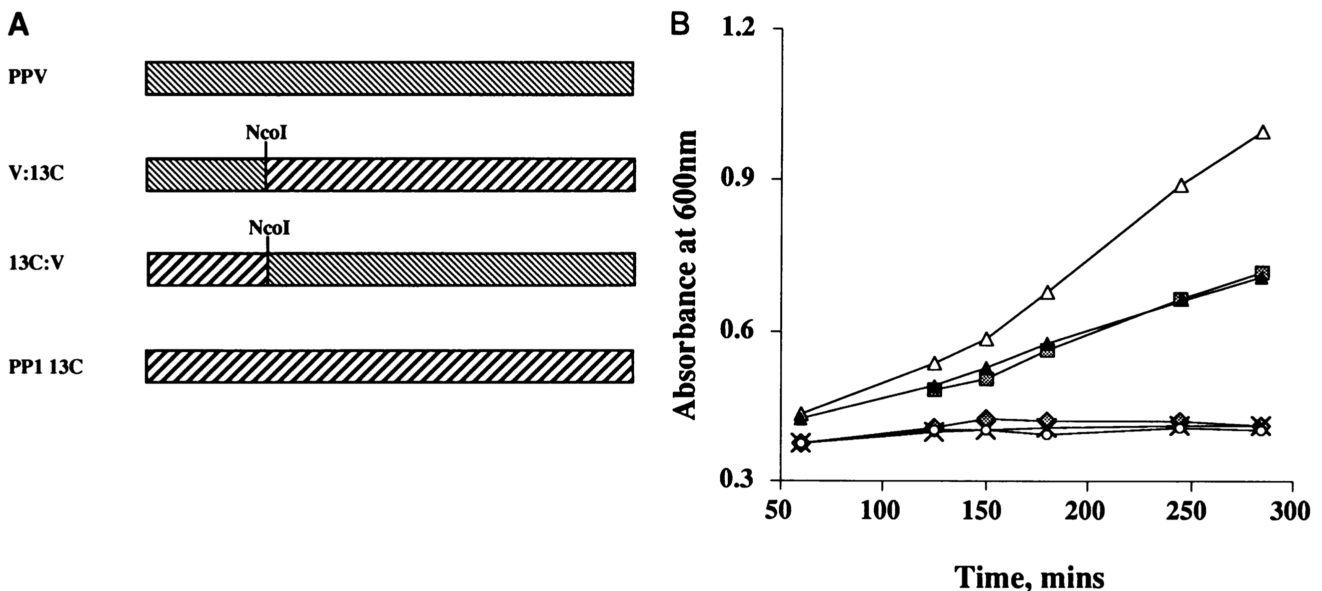


**Fig. 8.** The amino-terminal domain of PPV confers the ability to rescue the *SIT4* mutation onto a heterologous catalytic subunit. (**A**) Schematic diagram showing the derivation of the chimeric phosphatases. Heterologous domains were joined using an *Nco*I site which was common to the two catalytic subunits and cleaved immediately 5′ of the His codon in the conserved GDXHG motif (where X represents any amino acid). These constructs were cloned under the *ADH1* promoter (see Materials and methods). (**B**) Growth rates at 37°C were determined for the strain carrying the *sit4-102* mutation (CY249) when transformed with either *pADH* (○), *pADH-PPV* (▲), *pADH-PP1 13C* (◆), *pADH V:13C* (▦) or *pADH 13C:V* (×). The growth rate of the isogenic parental strain AY926 (△) is also shown.

domain of PPV might change the specificity of PP1 to that of PPV. First, the amino-terminal domain per se may convert the associated catalytic domain from type 1 to PPV (or type 2A-like) specificity. Secondly, the amino-terminal domain may be responsible for binding to an accessory subunit that targets the enzyme to its correct location. Thirdly, the amino-terminal domain may bind to a regulatory subunit that changes the specificity of the protein phosphatase catalytic domain. The latter two possibilities are perhaps more likely in that regulatory subunits have been shown to target and/or alter the specificity of other protein phosphatases (see Introduction). However, the cytoplasmic location of PPV does not indicate specific subcellular targeting (Figures 4 and 5). Therefore, the amino-terminal domain of PPV may be responsible for binding one or more regulatory subunits, which create very specific substrate preferences for this enzyme. In *S.cerevisiae*, the two high molecular weight phosphoproteins with which SIT4 associates at its execution point in late $G_1$ of the cell cycle (Sutton *et al.*, 1991a) may indeed be such regulatory proteins.

#### Potential in vivo roles for PPV

The pattern of expression of PPV in the *Drosophila* embryo indicates its involvement in processes which may parallel the functions of SIT4 in *S.cerevisiae*. During *Drosophila* embryonic development, transcription commences at about the tenth mitotic cycle and increases to a maximum during cycle 14, the time when cellularization occurs (Anderson and Lengyel, 1979, 1980). The pattern of PPV expression in the *Drosophila* embryo correlates with this temporal variation in the level of transcription. A potential role for PPV in the regulation of transcription is supported by analysis of mutations in the *SIT4* gene, which cause changes in the expression of a diverse group of genes (Arndt *et al.*, 1989). The extranuclear location of PPV does not preclude a transcriptional role since SIT4 has been shown to have a cytoplasmic distribution (Sutton *et al.*, 1991a).

The period of maximal expression of PPV also correlates with the process of cellularization. PPV may be important in regulating the membrane movements involved in this event, in a manner analogous to the requirement for SIT4 in bud formation in *S.cerevisiae* (Fernandez-Sarabia *et al.*, 1992). This could, of course, be via the regulation of transcription of genes involved in cellularization. The cellularization genes *nullo* and *serendipity* $\alpha$ exhibit a transient increase in expression between nuclear cycles 11 and 14 (Rose and Wieschaus, 1992).

Whether PPV performs a role in the $G_1$ phase of the *Drosophila* cell cycle similar to the SIT4 regulation of $G_1$ cyclin mRNA expression in *S.cerevisiae* (Fernandez-Sarabia *et al.*, 1992) is unclear. In the first 13 nuclear division cycles in the *Drosophila* embryo there is a rapid oscillation between DNA synthesis and mitosis without intervening gap phases. At cycle 14, when cellularization occurs, the $G_2$ phase is introduced. The $G_1$ phase is either absent or extremely short at cycle 14 and 15 and is only added or greatly lengthened after cycle 16, the period when germ band extension occurs (Edgar and O'Farrell, 1990). *PPV* mRNA and protein peak just before cellularization and their levels are declining at the period of germ band extension. Therefore the maximum levels of PPV are significantly earlier than the appearance of the $G_1$ phase of the cell cycle. However, it could be argued that maximal levels of PPV might not necessarily be required for the transcription of cyclin genes and are only necessary for the transcription of certain other genes at the time of cellularization. Nevertheless the only *Drosophila* homologue of the *S.cerevisiae* $G_1$ cyclins which has been reported, termed cyclin C (Léopold and O'Farrell, 1991) or CLNDm (Lahue *et al.*, 1991), is encoded by an mRNA that is maternally deposited in the embryo and the message declines as embryogenesis proceeds. Thus, there is no evidence for the transcriptional regulation of this $G_1$ cyclin gene in a cell cycle-dependent manner in *Drosophila* embryos (Lahue *et al.*, 1991; Léopold and O'Farrell, 1991). PPV could therefore only exert an effect on this $G_1$ cyclin post-translationally, by changing its level of phosphorylation.

Recently, Shimanuki *et al.* (1993) have described the *Schizosaccharomyces pombe ppe1*$^+$ gene which is structurally similar to *SIT4* and *PPV*. The conserved residues which are common to the amino-terminal region PPV and SIT4 are also found in ppe1 (Figure 6B). However, ppe1 appears to have distinct functions from SIT4, in particular its deletion alters cell shape and causes a cold sensitive $G_2$ arrest (unlike the $G_1$ block observed with *sit4-102*). While screening for suppressors of a pim1 mutant, Matsumoto and Beach (1993) also isolated *ppe1*$^+$. The pim1 mutant caused uncoupling of mitosis from the completion of DNA replication, implicating ppe1 in the mitotic checkpoint between S and M phases. Since the transient increase in PPV expression occurs just prior to the introduction of $G_2$ in the *Drosophila* embryo, it is possible that PPV regulates the initiation of this gap phase. It is clearly important to define the role of PPV in the regulation of the cell cycle and gene transcription and to this end we are currently attempting to define mutations in the *Drosophila PPV* gene.

## Materials and methods

#### General techniques and isolation of DNA clones

General molecular biological procedures were performed as described by Sambrook *et al.* (1989). cDNA probes were labelled by random priming (Feinberg and Vogelstein, 1984) with either [$\alpha$-$^{32}$P]dATP (Amersham International) or biotin 16-dUTP (Boehringer Mannheim). Oligonucleotides were labelled with [$\gamma$-$^{32}$P]ATP and polynucleotide kinase. Sense and antisense cRNA probes were synthesized using either T7 or T3 RNA polymerase and digoxygenin 11-UTP (Boehringer Mannheim) from the full-length *PPV* cDNA in the pKS$^+$ vector cut with *Bam*HI or *Hin*dIII respectively.

A λgt 10 *D.melanogaster* head cDNA library (Kalderon and Rubin, 1989) was screened with the 0.68 kb *Xho*I–*Nae*I cDNA fragment coding for amino acids 51–277 of *Drosophila PP1 87B*. Hybridization was performed as described by Dombrádi *et al.* (1989a) and the filters were washed in 2 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate pH 7.0) at 60°C. The same library was screened with the following oligonucleotides: 5′-TCCGTGGACGATACGCTGATGTGC-3′, a *Drosophila PP1 87B*-specific probe in 6 × SSC at 60°C; 5′-TGAACCGCCACGAGCT-GGACTTGAT-3′, a *PP1 9C*-specific probe in 6 × SSC at 60°C; 5′-GCG-GGATCTAAATCACACCACCAAAGGC-3′, a *PPY*-specific probe in 6 × SSC at 55°C. Bacteriophage, positive with cDNA but negative with the oligonucleotide probes, were purified by CsCl centrifugation. The DNA was isolated and digested with *Eco*RI and the released cDNA insert was subcloned into the Bluescript pKS$^+$ vector (Stratagene). Sequencing was performed on double-stranded, CsCl-purified DNA using [$\alpha$-$^{35}$S]dATP (Amersham International), Sequenase version 2.0 (United States Biochemical Corp.) and 7-deaza-2′-dGTP to resolve compressions (Mizusawa *et al.*, 1986). A partial length *PPV* cDNA was isolated from this screen.

In order to obtain the complete coding sequence, a λgt 10 *D.melanogaster* eye imaginal disc cDNA library (Kalderon and Rubin, 1989) was screened with oligonucleotides complementary to nucleotides 68–87 and 414–433 of the *PPV* sequence (Figure 1). Hybridization was performed as described by Dombrádi *et al.* (1989a) at 45°C and filters were washed in 6 × SSC and 0.1% SDS at 55°C prior to autoradiography. In addition, genomic clones

were isolated from *D.melanogaster* (Canton S strain) genomic library (Clontech) by screening with the random primed *PPV* cDNA and an oligonucleotide complementary to nucleotides 12–34 (Figure 1). Phage DNA was digested with various restriction enzymes. The DNA fragments were resolved by agarose gel electrophoresis and examined after Southern blotting. A 3 kb *Eco*RI fragment hybridizing with the *PPV* cDNA was cloned into pKS$^+$ for sequence analysis.

### Southern, Northern and in situ hybridization

Genomic DNA was isolated from *D.melanogaster* strain Oregon R as described by Ashburner (1989) and Southern blots were performed according to Lehner and O'Farrell (1989).

Total RNA was isolated from various *Drosophila* developmental stages and poly(A)$^+$ RNA selected as described in Dombrádi *et al.* (1989a). Northern blots were performed by the formaldehyde method of Lehrach *et al.* (1977) and probed with the full-length *PPV* cDNA. Hybridization was performed at 65°C as for the library screens, blots being washed for 60 min in 0.1× SSC and 0.1% SDS at 65°C. After autoradiography, the probe was removed by placing the blot in 1% (v/v) glycerol at 85°C for 5 min. The blot was then rehybridized with the 0.17 kb *Eco*RI–*Xho*I fragment in the 5′ non-coding region of the *Drosophila PP1 87B* cDNA after having confirmed complete removal of the previous signal. Hybridization conditions and washing stringency for *PP1 87B* were as for *PPV*.

Polytene chromosome preparations obtained from the salivary glands of female *D.melanogaster* (Oregon R strain) third instar larvae were hybridized with biotin-labelled, full-length *PPV* cDNA as described in Dombrádi *et al.* (1993). *In situ* hybridization to RNA in whole mount *Drosophila* embryos was performed using digoxygenin-labelled cRNA probes (Dalby and Glover, 1992). After colour development had been terminated, nuclei were stained with Hoescht 33258 (10 μg/ml for 20 min). Embryos were then dehydrated in an ethanol series and mounted in Gary's Magic Mountant (Ashburner, 1989). Embryos were examined using an Olympus BH-2 microscope.

### Yeast strains and methodology

*S.cerevisiae* strains used were CY249 [*MATα ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1 ssd1-d2 sit4::HIS3* (*sit4-102* on YCp50); Sutton *et al.*, 1991a] and the parental strain AY926 (*MATa ade2-1 his3-11,15 leu2-3,112 can1-100 ura3-1 trp1-1*). Basic yeast methods and media were as described by Sherman *et al.* (1986). Plasmid DNA was recovered from *S.cerevisiae* by the method of Hoffman and Winston (1987). Transformation of yeast was as described by Ito *et al.* (1983).

### Plasmid constructions

In the following constructions a restriction site with protruding single strand termini is referred to as being 'blunt', when the cohesive ends had been filled in using the Klenow fragment of DNA polymerase and excess dNTPs (Sambrook *et al.*, 1989). If multiple products were expected from a restriction digestion, the DNA was separated by agarose gel electrophoresis and the appropriate band cut from the gel. The DNA was electroeluted from the gel slice using a Biotrap (Schleicher and Schuell) and precipitated from the eluate. All constructs were verified by sequencing. The pADH yeast/bacterial shuttle vector contains a multiple cloning site downstream of the *ADH1* promoter; the plasmid also carries the 2μ origin of replication and the *LEU2* selectable marker.

*pADH-PPV and pADH-PPV reverse.* The entire *PPV* cDNA sequence in pKS$^+$ was released with *Bam*HI blunt and *Sal*I. This *PPV* fragment was ligated into pADH which had been digested with *Hin*dIII blunt and *Sal*I to give *pADH-PPV*. In order to obtain *PPV* in the opposite orientation under the *ADH1* promoter (*pADH-PPV* reverse), *PPV* in pKS$^+$ was cut with *Hin*dIII and *Sac*I and cloned into pADH cut with the same enzymes.

*pADH-PP1 13C.* Using PCR, the *PP1 13C* genomic clone (Dombrádi *et al.*, 1993) as template and the oligonucleotides GCGCCATATGGCGGAGG-TTCTCAATTT (sense) and GCGCAAGCTTACTTCTTGCGCTTCTCGA (antisense), the *PP1 13C* coding region was amplified. The product was cut with *Nde*I and *Hin*dIII and the fragment cloned into pT7.7 digested with the same enzymes. The resultant plasmid was digested with *Nde*I blunt and *Hin*dIII. This fragment was ligated into pADH which had been digested with *Hin*dIII and *Sal*I blunt.

*pADH-V:13C. PPV* in pKS$^+$ was digested with *Nco*I (which cleaves after nucleotide 156 in PPV, Figure 1) and *Hin*dIII (in pKS$^+$). *PP1 13C* in pT7.7 was digested with *Nco*I and *Hin*dIII and cloned into the electroeluted vector fragment to yield *V:13C* in pKS$^+$. *V:13C* was then cloned into the pADH vector exactly as described for *pADH-PPV*.

*pADH-13C:V. PPV* in pKS$^+$ was digested with *Sma*I (in pKS$^+$) and *Nco*I. *PP1 13C* in pT7.7 was digested with *Nde*I blunt and *Nco*I and cloned into the electroeluted vector fragment to yield *13C:V* in pKS$^+$. *13C:V* was then cloned into the pADH vector exactly as described for *pADH-PPV*.

*YCp50-SIT4-V* (Figure 7A). Using PCR, *PPV* in pKS$^+$ as template and the oligonucleotides GCGCTCTAGAGACAAGTGGATAGAAGACGTG (sense) and GCGCGCATGCGAACGTTTACAGGAAGTAGG (antisense), a 0.9 kb fragment was amplified which introduced restriction sites at the third codon of *PPV* and 8 bp 3′ of the termination codon. The product was cut with *Xba*I and *Sph*I and the fragment cloned into *sit4-102* in YCp50 digested with the same enzymes. This strategy caused the loss of the 3′ non-coding region of the *sit4-102* gene due to the presence of a *Sph*I site in YCp50. This fragment was released from *sit4-102* in YCp50 by digestion with *Sph*I and cloned into the *SIT4-V* construct digested with the same enzyme. Recombinants with the desired orientation of the cloned fragment were determined by sequencing. The plasmid YDpL was cut with *Bam*HI blunt to release the *LEU2* gene. The *LEU2* selectable marker was cloned into the *SIT4-V* construct which had been cut with *Sma*I and *Sal*I blunt, this digestion causing the removal of the *URA3* selectable marker from the YCp50-based construct.

*pADH-2A* and *pADH-X.* The *PP2A* coding region was amplified using PCR with the *Drosophila PP2A* cDNA clone (Orgad *et al.*, 1990) as template and the oligonucleotides GCGCCATATGGAGGATAAAGCAACAAC (sense) and GCGCAAGCTTAAAGGAAATAATCGGGTG (antisense). The product was cut with *Nde*I and *Hin*dIII and the fragment cloned into pT7.7 digested with the same enzymes. The resultant plasmid was digested with *Nde*I blunt and *Hin*dIII blunt. This fragment was ligated into pADH which had been digested with *Hin*dIII blunt. The insert orientation was verified by sequencing.

*Drosophila PPX* cDNA (N.D.Brewis and P.T.W.Cohen, unpublished results) in pKS$^+$ was cleaved with *Bam*HI blunt and *Sal*I and cloned into pADH cut with *Hin*dIII blunt and *Sal*I.

### Immunological methods

Antibodies were raised in rabbits against the synthetic peptide AVPDAERVIPKQNTTP corresponding to amino acids 285–300 of PPV. The peptide was synthesized using standard tertiary butoxy carbonyl chemistry and coupled to BSA as described in Brewis *et al.* (1993). Immunization, antibody collection and serum preparation were performed according to Harlow and Lane (1988). Anti-PPV antiserum was affinity purified using the PPV peptide coupled to Affigel 15 (Bio-Rad) as described by Brewis *et al.* (1993). Immunoblotting on to nitrocellulose (BA 85, Schleicher and Schuell) was carried out according to MacDougall *et al.* (1989). Membranes were blocked by an overnight incubation in 4% Marvel dried milk powder (Premier Brands) in TBS. Blots were then incubated with PPV antiserum (1 μg/ml) in 1% Marvel in TBS for 3 h. After thorough washing, antibody binding was detected using a Vector Labs ABC Kit utilizing horseradish peroxidase conjugated to streptavidin followed by fluorography with enhanced chemiluminescence (Amersham International). Antibody analysis of whole embryos was performed as described by Klämbt *et al.* (1991), antibody binding being detected via fluorescein-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories). Embryos were viewed using a Bio-Rad MRC 600 scanning laser confocal microscope.

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