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Deficiency of protein phosphatase 2A uncouples the nuclear and centrosome cycles and prevents attachment of microtubules to the kinetochore in *Drosophila microtubule star* (*mts*) embryos

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SUMMARY

A *Drosophila* strain, carrying a *P[lacW]* element in the promoter of the protein phosphatase 2A (PP2A) catalytic subunit gene at chromosomal location 28D, has been identified using plasmid rescue of the P element and adjoining genomic DNA in *Escherichia coli*. Reversion mutagenesis was employed to demonstrate that the observed phenotype of the *Drosophila* strain was due to a single *P[lacW]* element insertion at 28D and to create three deficiency strains at this locus. *Drosophila* heterozygous for *P[lacW]28D* have reduced levels of PP2A mRNA and reduced PP2A catalytic activity against four different substrates compared to wild type, while homozygotes are deduced to have approximately 20% of wild-type PP2A activity. *P[lacW]28D* homozygotes, termed *microtubule star* (*mts*), die in embryogenesis around the time of cellularisation, exhibiting overcondensed chromatin and a block in mitosis between prophase and the initiation of anaphase. Multiple centrosomes are visible in cellularised embryos, suggesting that PP2A may play a role in coupling the nuclear and centrosome cycles. When embryos arrest just prior to cellularisation, disorganised elongated arrays of microtubules radiate from centrosomes in all directions, but they are rarely associated with any DNA, suggesting that PP2A is required for the attachment of microtubules to chromosomal DNA at the kinetochore.

Key words: Protein phosphatase, *Drosophila*, Mitosis, Centrosome, Microtubule, Kinetochore

INTRODUCTION

One of the most abundant protein phosphatases mediating the regulation of many eukaryotic cellular functions via the dephosphorylation of serine and threonine residues is protein phosphatase 2A (PP2A). This enzyme has a trimeric structure comprising a 36 kDa catalytic subunit (C), a tightly bound 65 kDa regulatory subunit (A) and a third subunit (B) that can vary in structure and range in size from 52-130 kDa. The substrate specificity and activity of the A-C dimer is modulated by interaction with the B subunit (Cohen, 1989; Wera and Hemmings, 1995).

The functional roles of PP2A are difficult to analyse biochemically since PP2A will dephosphorylate a wide range of substrates in vitro which it probably does not act on in vivo. Genetic approaches in lower eukaryotes are therefore crucial to the analysis of PP2A function in vivo and several mutational studies indicate functional role(s) for PP2A in cell division. In both *Saccharomyces cerevisiae* (Sneddon et al., 1990; Ronne et al., 1991) and *Schizosaccharomyces pombe* (Kinoshita et al., 1990) PP2A catalytic subunit performs an essential function. *S. cerevisiae*, deleted for three genes encoding PP2A (*PPH21*, *PPH22* and *PPH3*) and carrying a mutant temperature sensitive *pph2* gene show a block at the G_2/M stage of the cell cycle at the restrictive temperature. Since this block is partially overcome by overproduction of a B-type cyclin, PP2A may provide an essential function by positively affecting cdc2 kinase (Lin and Arndt, 1995). In contrast, *S. pombe* strains disrupted in *ppa2*, one of two genes encoding PP2A, divide at a smaller cell size than wild type (Kinoshita et al., 1990), while studies in *Xenopus* egg extracts show that inhibition of PP2A activity with okadaic acid leads to activation of cdc2 kinase activity (Felix et al., 1990), suggesting that PP2A negatively regulates cdc2 kinase. The negative regulation could operate through PP2A inhibition of cdc25 phosphatase which dephosphorylates cdc2 kinase (Kumagai and Dunphy, 1992). The opposite phenotypes obtained in budding and fission yeast PP2A mutants make an analysis of the effect of PP2A deficiency on the cell cycle in *Drosophila* relevant. Although very recently, *Drosophila* heterozygous for a deficiency of PP2A catalytic subunit have been investigated, these studies did not analyse effects on cell division, but demonstrated that partial deficiency of PP2A affects the Ras1/Raf MAP kinase signal transduction cascade and thereby influences photoreceptor cell fate (Wassarman et al., 1996).

Mutants of the A regulatory subunit of PP2A have been described in yeasts. *S. cerevisiae TPD3*, which encodes a homologue of the mammalian PP2A A subunit, was identified as a regulator of tRNA synthesis (van Zyl et al., 1992). Although it is not essential for viability, at low temperatures the *tpd3* mutant becomes multibudded and multinucleate, probably as a result of defects in cytokinesis. In *S. pombe*, an A subunit homologue encoded by the *paa1*$^+$ gene was essential for viability and cells disrupted for *paa1* were blocked in cytokinesis and showed anomalous actin and microtubule distributions (Kinoshita et al., 1996).

Homologues of the mammalian B subunit are non-essential for viability in yeasts. Based on the heterogeneity of the B subunit in mammals, it is not surprising that at least two exist in *S. cerevisiae* (Evangelista et al., 1996). One of these, *CDC55*, when mutated displays defects in morphology, forming abnormally elongated buds and a delay in septation and/or cell separation (Healy et al., 1991). *S. pombe* mutants deleted for *pab1*, encoding a B subunit homologue, displayed altered cell morphology, anomalous distribution of actin and microtubules and a delay in cytokinesis at restrictive temperatures (Kinoshita et al., 1996). In *Drosophila*, different P element insertions at 85E in a PP2A B subunit gene give rise to *abnormal anaphase resolution* (*aar*^{1}) and *twins*^{P} mutants, both of which display mitotic abnormalities in anaphase (Gomes et al., 1993; Mayer-Jaekel et al., 1993). *twins*^{P} mutants also show partial pattern duplications which arise from defects in cell fate determination (Uemura et al., 1993; Shiomi et al., 1994).

Analysis of genetic deficiencies of PP2A subunits in different organisms has identified functional roles of PP2A in cell division and other processes such as transcription and cell fate determination. From analysis of a *Drosophila* strain carrying a P element in the PP2A catalytic subunit gene at 28D, we show here that deficiency of PP2A causes a block in mitosis and, distinct from genetic analyses in budding and fission yeasts, uncouples the centrosome cycle from the nuclear cycle and prevents the attachment of microtubules to the kinetochore.

MATERIALS AND METHODS

Drosophila strains and maintenance of *Drosophila* stocks

The Szeged collection of lethal *P[lacW]* element insertions on the 2nd chromosome of *Drosophila melanogaster* (Török et al., 1993) was kindly provided by Istvan Kiss. All strains in this collection including l(2)k09822 had the genotype *y w*; *P[lacW]/CyO*; *+/+*. The *P[lacW]* contains the P element transposase-*lacZ* fusion gene, the mini-*white* marker gene, a bacterial origin of replication, the β-lactamase gene and polylinkers facilitating cloning by plasmid rescue. Strain *w*; *Sp*^{1}*/Cyo*; *Sb*^{1} *P[ry*^{506} *Δ2-3]*$_{99B}$*/TM6B* was used as a source of transposase (Robertson et al., 1988). The *y*$^+$ *CyO* balancer chromosome carrying a *P[y*$^+$*]* insertion on the original *CyO* is described by Török et al. (1993). Other genetic markers and chromosomes are detailed by Lindsley and Zimm (1992). *Drosophila* were maintained on yeast/glucose media and all experiments were performed at 25°C unless otherwise stated.

Analysis of the Szeged collection of lethal *P[lacW]* element insertions and examination of plasmid 1393 and genomic DNA from strain 1(2)k09822

The Szeged collection of lethal *P[lacW]* element insertions on the 2nd chromosome of *D. melanogaster* (Török et al., 1993) was analysed for the presence of the *P[lacW]* element in or near the PP2A 28D gene as described by Guo et al. (1996). Since the *P[lacW]* transposon contains a plasmid replicon, it can be rescued together with adjoining genomic DNA in *E. coli*. A collection of 1836 independently rescued plasmids containing genomic DNA flanking the sites of transposon insertion was examined in batches with a 1.2 kb *Drosophila* cDNA probe encoding the catalytic subunit of PP2A (Orgad et al., 1990). By successive subdivision of the hybridising pool, plasmid 1393 originating from *Drosophila* strain l(2)k09822 was found to contain PP2A hybridising sequences. Plasmid 1393 was cultured in *E. coli* DH5α and the plasmid DNA purified for sequencing by CsCl density gradient centrifugation. Genomic DNA was prepared essentially as described by Ashburner (1989). A 1 ng sample of plasmid 1393 DNA was analysed in a polymerase chain reaction (PCR) using Taq polymerase (Life Technologies, Paisley, UK) according to the supplier's protocol with a P element primer 181G (5′ CGACGGGACCACCTTATGTTATTTCATCATG 3′) and a PP2A primer AB1025 (5′ CGAACTTGTGTCTCTGTCAACTG 3′) each at a final concentration of 0.5 μM. The same primer combination was used with 10 ng of genomic DNA to check the position of the P element in strain l(2)k09822 and to show the excision of the P element from deficiency strains. The PCR fragment and plasmid 1393 were sequenced using the P element and PP2A specific primers, the DNA sequencing being performed on the Applied Biosystems 373A DNA sequencer using Taq dye terminator cycle sequencing.

Genomic DNA was analysed by restriction enzyme digestion and Southern blotting of DNA on Hybond N$^+$ membranes (Amersham, Little Chalfont, UK) as described by the suppliers. The DNA was hybridised with PP2A cDNA probes labelled with [α-^{32}P]dATP (Feinberg and Vogelstein, 1984), which included the 1.2 kb full length PP2A cDNA (Orgad et al., 1990), a 5′UTR/N-terminal coding region probe (nucleotides −189 to 198) and a C-terminal coding region/3′UTR probe (nucleotides 434 to 1,115).

Determination of lethal phase of l(2)k09822 stock

The original *CyO* balancer was replaced with a modified *CyO* balancer marked with a copy of *y*$^+$. Embryos were collected overnight from *y w*; *P[lacW]/y*$^+$ *CyO* females crossed with *y w*; *P[lacW]/y*$^+$ *CyO* males. These embryos were laid out on a large apple juice agar plate in 15 rows of 10 embryos and incubated at 25°C. At regular intervals over a 48 hour period the plate was examined to determine how many larvae had hatched. The phenotype of the larvae was determined by examination of their mouth hooks, *y* larvae possessing golden brown mouth hooks while *y*$^+$ larvae have brown/black mouth hooks.

Northern blot analysis of mRNA

Total RNA was prepared from adult Oregon R *Drosophila*, strain l(2)k09822 (*y w*; *P[lacW]/CyO*; *+/+*) and 2nd chromosome deficiency strains (*y w; Df(2)/CyO; +/+*) and analysed by northern blotting as described by Armstrong et al., (1995) using the 1.2 kb full length PP2A cDNA probe, radiolabelled as described above. The nylon membrane was washed at high stringency in 0.1% SDS, 15 mM NaCl, 1.5 mM sodium citrate, pH 7, at 65°C. The PP2A hybridisation signals were quantified using a Phosphorimager (Molecular Dynamics, Sunningvale, CA, USA). The PP2A probe was then removed by immersion of the membrane in 0.5% SDS at 100°C, followed by cooling of the SDS to room temperature. Removal of the probe was checked by overnight exposure of the membrane to X-ray film at −80°C. The membrane was hybridised with a control probe, full length PP1 87B cDNA (Dombrádi et al., 1989), washed at high stringency and the hybridisation signals analysed as for the PP2A probe.

Protein phosphatase assays

Drosophila extracts were prepared by homogenising 10 flies in a 1.5 ml microcentrifuge tube with a micropestle in 0.4 ml 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% glycerol, 0.1% 2-mercaptoethanol, 0.03% Brij 35, 1 mM benzamidine, 0.1 mM phenylmethylsulphonyl fluoride and 0.1 mM *N*-tosyl-*L*-phenylalanine chloromethyl ketone, and centrifuging at 21,000 ***g*** for 30 minutes at

4°C. The supernatant was used for protein phosphatase assays and the protein concentration was determined by Coomassie Protein Assay reagent (Pierce, Rockford, IL, USA). Rabbit skeletal muscle glycogen phosphorylase (prepared by Mr M. Doherty) was ^{32}P-labelled by phosphorylase kinase (prepared by Miss F. Douglas) to a stoichiometry of 1 mol phosphate per mol subunit (Cohen et al., 1988). Cyclic AMP-dependent protein kinase (prepared by Dr L. MacDougall) was used to phosphorylate partially hydrolysed bovine casein to 3.5 nmol phosphate per mg (McGowan and Cohen, 1987). p34^{cdc2} was affinity purified from *Xenopus* extracts (prepared with the help of Dr C. Smythe and Mr C. Jones) using a glutathione S-transferase-cyclin B fusion protein (Pfaller et al., 1991). Histone H1 (Life Technologies, Paisley, UK) and caldesmon (prepared by Dr P. Ferrigno) were phosphorylated with p34^{cdc2}/cyclinB to 3 mol phosphate per mol and 1.4 mol phosphate per mol, respectively (Ferrigno et al., 1993). The specific radioactivity of the ATP used in the phosphorylations was 10^6 cpm/nmol. Protein phosphatase assays with phosphorylase and casein were performed in the absence of divalent cations as described by Cohen et al. (1988). The dephosphorylation of histone H1 and caldesmon was carried out in an identical manner except that after terminating the reactions with 20% trichloracetic acid, the released phosphate was quantified with acid molybdate and extraction with organic solvents (Ferrigno et al., 1993). The substrate concentrations in the assays were 10 μM for phosphorylase, 6 μM for casein and 2 μM for histone H1 and caldesmon. One unit of activity is the amount of enzyme which catalyses the release of 1 μmol of [^{32}P]phosphate per minute.

Immunostaining of *Drosophila* embryos

Embryos were collected from adult *Drosophila* females over 30 minute periods onto apple juice agar plates and aged for the desired period of time at 25°C. The embryos were washed from the agar plate with tap water, dechorionated with 100% chloros (Ellis and Everard, Bradford, UK) for 4 minutes, washed thoroughly in tap water and fixed by one of two protocols: (1) embryos to be stained with anti-α-tyrosinated-tubulin antibodies were fixed in 38% formaldehyde and heptane in a ratio of 2:5 by shaking vigorously for 2 minutes and then with continuous mixing for 15 minutes at room temperature. (2) Embryos to be stained with an antibody against non-microtubule associated epitopes were fixed in 4% paraformaldehyde in 10 mM potassium phosphate buffer, pH 6.8, 45 mM KCl, 15 mM NaCl, and heptane in a ratio of 3:5 with continuous mixing at 4°C overnight. The fixed embryos were washed in phosphate buffered saline + 0.1% Tween-20 (PBST) at 4°C overnight to remove any residual heptane.

Embryos were stained for β-galactosidase to identify *P[lacW]* homozygous embryos by incubation in 0.28% X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) in 7.2 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, 150 mM NaCl, 1 mM $MgCl_2$, 3.05 mM $K_3(Fe(CN)_6)$, 3.05 mM $K_4(Fe(CN)_6)$, pH 7.2, for 6 hours at 37°C.

After removing the X-gal staining solution and washing in PBST, the vitelline membrane was removed from the embryos by addition of 1 volume fresh *n*-heptane followed by 1 volume methanol and shaken vigorously for 1 minute. The heptane/methanol was removed, fresh methanol was added and they were allowed to stand for 10 minutes. The methanol was replaced with PBST and the embryos rehydrated for 10 minutes, followed by 2× 15 minute washes in PBST.

For antibody and DNA staining, embryos were incubated as follows with continuous mixing: 10% sheep serum and 2.5 mg/ml RNase A in PBST was used to block non-specific binding of antibodies for >30 minutes at room temperature. Incubation with the primary antibody in PBST containing 10% sheep serum was carried out at 4°C overnight. The embryos were washed at least 3 times for 15 minutes in PBST. Embryos were then incubated with the secondary antibody conjugated to a fluorescent dye, which was either fluorescein isothiocyanate (FITC) or Texas Red, in PBST overnight at 4°C. The embryos were again washed at least 3 times in PBST and once in PBS only. Propidium iodide was added at a final concentration of 1 μg/ml in PBS and the embryos incubated for 20 minutes, before finally washing for 15 minutes in PBS. The embryos were mounted in approximately 50 μl of 85% glycerol/2.5% *N*-propylgallate onto silicon coated coverslips, and then examined using a Bio-Rad MRC-600 laser scanning confocal microscopy. Rat anti-tyrosinated-α-tubulin YL1/2 (Sera-Lab Ltd, Sussex, UK) coupled to goat anti-rat FITC-conjugated IgG (Jackson ImmunoResearch Laboratories Inc.,

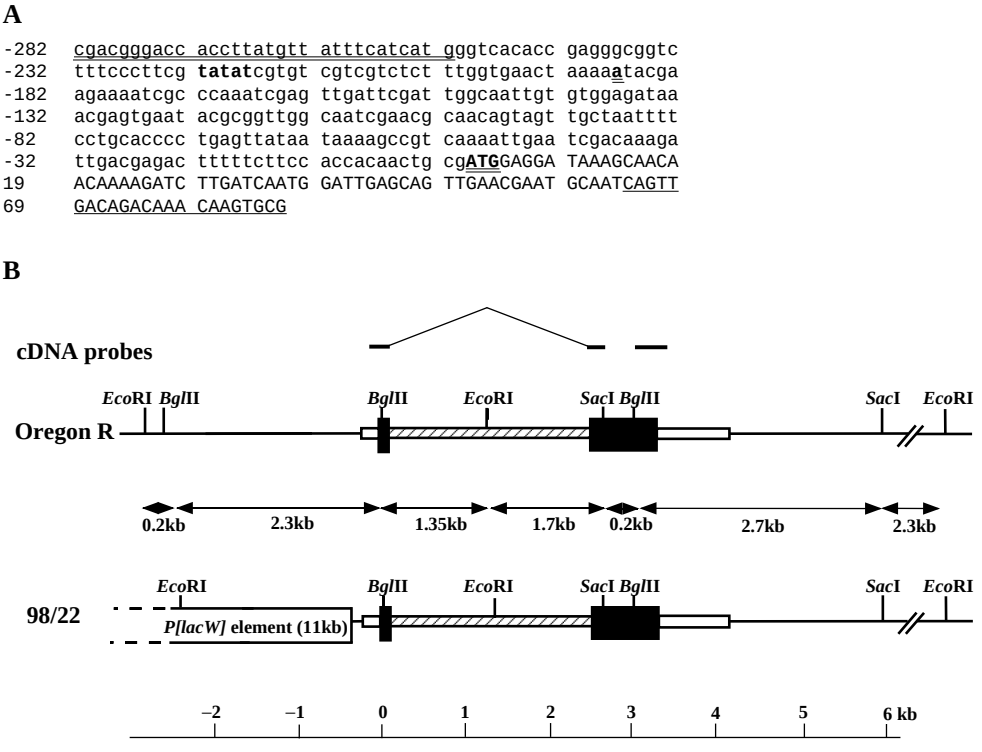

Fig. 1. (A) The sequence of the promoter and 5′ untranslated region of the PP2A gene at chromosomal location 28D. The *P* element primer sequence is double underlined and the PP2A specific primer sequence single underlined. The translated region of the PP2A gene is in upper case and the initiating ATG is in bold type and double underlined. The nontranslated sequences are in lower case, a putative TATA box is in bold type and the start of the cDNA (Orgad et al., 1990) is bold type and double underlined. (B) Map of the PP2A gene locus at 28D with and without the *P[lacW]* element. The 5′ and 3′ untranslated regions are depicted as open boxes, the coding exons as black boxes, the intron sequence as a hatched box and the nontranscribed sequences as lines. Restriction sites and fragment sizes are indicated.

West Baltimore Pike, PA, USA) was used for microtubule staining, rabbit Rb188 antibody raised against CP190 (Whitfield et al., 1995) coupled to goat anti-rabbit Texas Red or FITC-conjugated IgG (Jackson ImmunoResearch) was used to localise centrosomes. Actin was stained with phalloidin coupled to FITC (Sigma, Poole, UK).

RESULTS

Identification of a *Drosophila* strain (l(2)k09822) carrying a P element in the PP2A catalytic subunit gene at 28D

Analysis of 1,836 plasmids derived from the Szeged collection of lethal *P[lacW]* element insertions on the 2nd chromosome of *Drosophila melanogaster* (Török et al., 1993) showed that a single plasmid possessed part of the PP2A gene located at 28D. The plasmid collection was analysed by Southern blotting of all the plasmids in batches, in the following sequence: (1) 18 lanes, each containing 100 plasmids; (2) 10 lanes, each containing 10 plasmids; and lastly (3) 10 lanes each containing a single plasmid. Hybridisation of the blots with ^{32}P-labelled *Drosophila* PP2A cDNA (Orgad et al., 1990) led to the isolation of number 1393 as the plasmid containing the PP2A gene sequences.

Analysis of plasmid 1393 by PCR using primers specific to the *P[lacW]* element (181G) and to the PP2A gene at position nucleotides 64-86 (AB 1025) produced a 368 bp fragment indicating that the *P[lacW]* element had inserted 251 bp upstream of the initiating ATG. An identical fragment was obtained when the same PCR was carried out on strain l(2)k09822, the fly stock from which the 1393 plasmid had been obtained. The sequence in the PP2A gene 3′ to the P element insertion conforms to the consensus insertion site for P elements (O'Hare and Rubin, 1983). The PCR fragment (Fig. 1A) contains a TATA box 30 bp upstream of the start of the cDNA but no other upstream regulatory elements, suggesting that the *P[lacW]* element disrupts the promoter of PP2A. The genomic sequences adjoining the insertion site of the *P[lacW]* element were examined by sequencing of the 1393 plasmid, which revealed that 102 bp 3′ of the initiating ATG were identical with PP2A cDNA. Thereafter the sequences diverged, with the genomic DNA possessing a sequence almost identical to the consensus sequence determined for 5′ intron splicing in *Drosophila* (Shapiro and Senapathy, 1987), indicating the presence of an intron. Further sequencing into the intron did not reach the 3′ end. Southern blotting of restriction digests of genomic DNA from wild-type and l(2)k09822 *Drosophila* strains indicated the PP2A genomic structure shown in Fig. 1B.

Inspection of the l(2)k09822 heterozygous *Drosophila* suggests that the *P[lacW]* element has inserted under a weak enhancer, because X-gal staining of embryos laid by heterozygous females crossed with heterozygous males shows

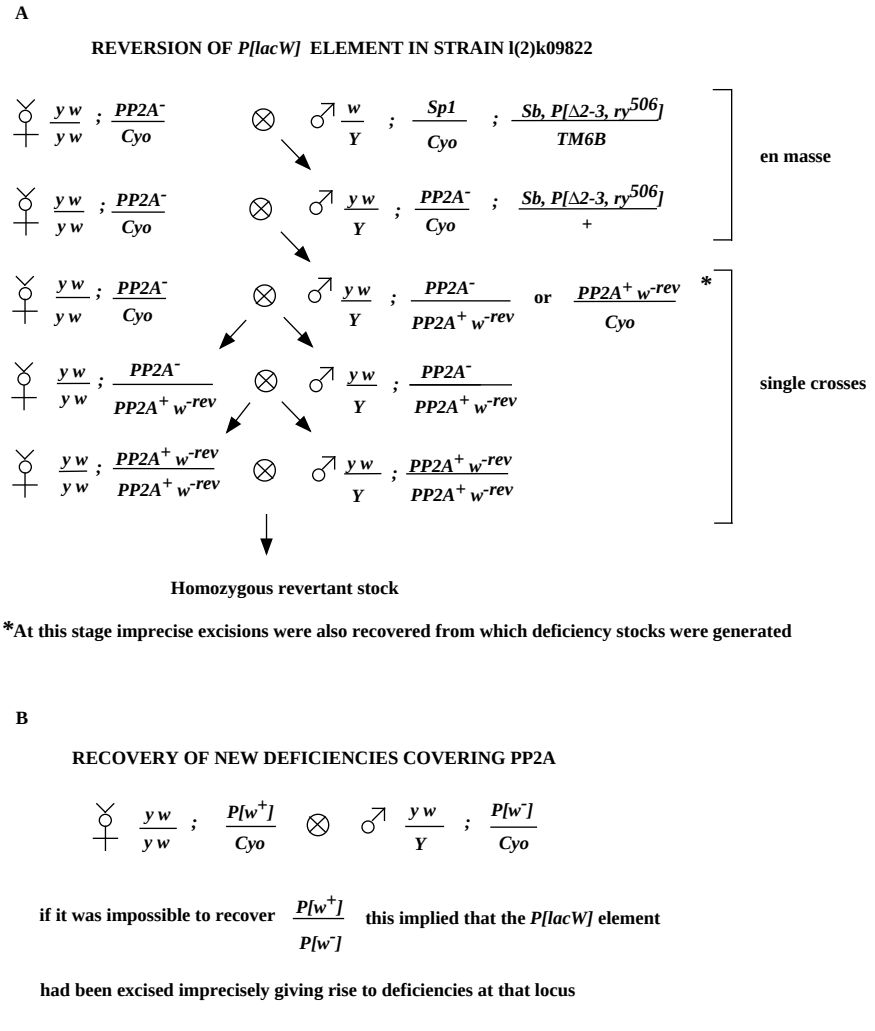

Fig. 2. (A) Scheme for isolation of viable revertants by mobilisation of the *P[lacW]* element in the *PP2A* gene at 28D. *PP2A*$^{-}$ is the *PP2A* gene carrying the *P[lacW]* element. *PP2A*$^{+}w^{-rev}$ is a revertant allele which has lost the *P[lacW]* element and regained the wildtype *PP2A* gene. (B) Scheme for isolation of imprecise excisions of the *P[lacW]* element (i.e. generation of deficiency strains which carry a deletion at 28D). *P[w*$^{+}$*]* stands for the *P[lacW]* transposon, which carries the w^{+} marker gene.

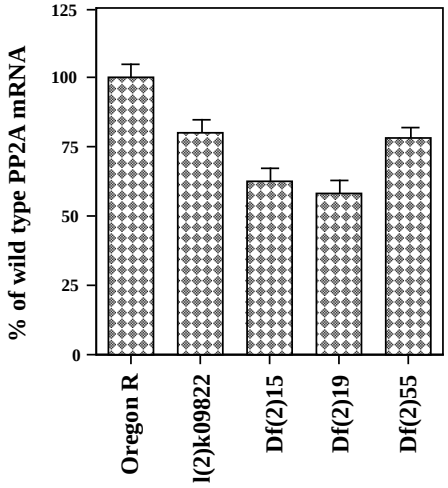

Fig. 3. PP2A messenger RNA levels in wild type (Oregon R), strain l(2)k09822 and deficiency strains Df(2)15, Df(2)19 and Df(2)55 *Drosophila*. Total RNA was analysed by northern blotting using a ^{32}P-labelled PP2A cDNA probe, followed by a ^{32}P-labelled PP1 87B cDNA probe as a control. The hybridisation signals were quantified using a phosphorimager and the value for each strain is presented as a ratio of PP2A/PP1 87B signals. Each value is the average of 4 determinations. The levels in PP2A deficient strains are presented relative to that in Oregon R which is taken as 100%. Bars indicate s.e.m.

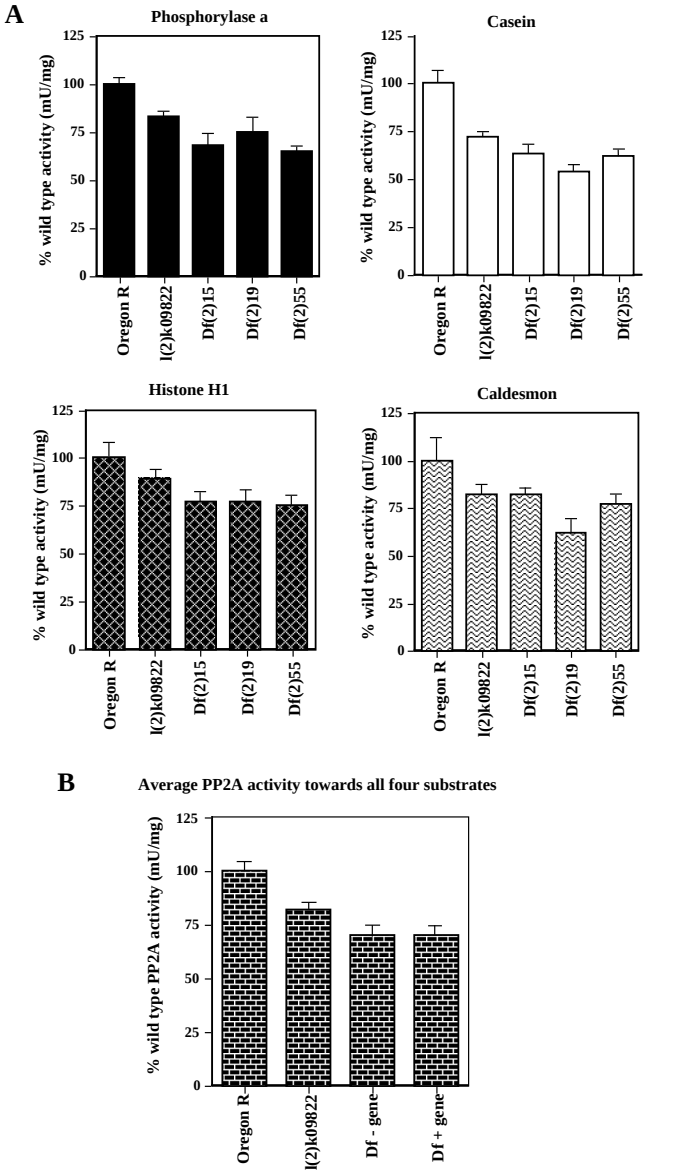

Fig. 4. (A) Protein phosphatase 2A activities in wild type (Oregon R), strain l(2)k09822 and deficiency strains Df(2)15, Df(2)19 and Df(2)55 *Drosophila*. Protein phosphatase activities were determined towards the indicated substrates in fly extracts in the presence of inhibitor 2 to inhibit protein phosphatase 1. Each activity presented is an average of duplicate determinations on 3 extracts. The activities in PP2A deficient strains are presented relative to those in Oregon R which are taken as 100%. Bars indicate s.e.m. (B) Average activity of PP2A towards all four substrates in wild type (Oregon R), strain l(2)k09822 and deficiency strains. Df-gene is Df(2)55, which has only one copy of the PP2A gene. Df +gene is an average of the values for Df(2)15 and Df(2)19, which have one wild-type and one mutant PP2A gene. Bars indicate s.e.m.

that dark blue/black crystals produced by high levels of β-galactosidase can only be seen in embryos homozygous for the *P[lacW]* element and not in heterozygous embryos. In addition, it was found that the eye colour of the heterozygous flies is very pale yellow on eclosion and only gradually darkens to orange. Red, wild-type w^+ eye colour is not seen.

Lethality is caused by insertion of a single *P[lacW]* element in strain l(2)k09822

The *P[lacW]* element was shown to be responsible for the lethality of the l(2)k09822 *(y w/ y w; PP2A$^-$/CyO; +/+)* strain by carrying out reversion mutagenesis. The *P[lacW]* element was mobilised by the introduction of the exogenous transposase source *Δ2-3*, and flies recovered that were viable even though they carried the original *P[lacW]* chromosome and did not carry the *CyO* chromosome (Fig. 2A). These flies must therefore be heterozygous for a reverted chromosome from which the *P[lacW]* element has been precisely excised or sufficiently deleted to minimise its effects. Homozygous reverted strains were generated, in which white eye colour was interpreted as loss of the *P[lacW]* element. The viability of this strain demonstrates that the lethality of strain l(2)k09822 is due to the presence of the *P[lacW]* element and not due to a spurious lethal mutation elsewhere in the genome.

During the reversion mutagenesis 2nd chromosome deficiencies were also recovered. These flies had lost the P element but were inviable in heterozygous combination with the original *P[lacW]* chromosome, presumably because of excision of some genomic DNA with the P element (Fig. 2B). From a total of 6,500 flies screened, precise reversion of the *P[lacW]* phenotype was obtained in 65 cases (1%) and presumed imprecise excision in 3 cases (0.05%). Southern blotting with cDNA probes (Fig. 1B) of genomic DNA from flies carrying the imprecise excisions, termed deficiencies Df(2)15 (*y w; Df(2)28D-15/CyO; +;+*), Df(2)19 (*y w; Df(2)28D-19/CyO; +;+*) and Df(2)55 (*y w; Df(2)28D-55/CyO; +;+*); showed that Df(2)15 and Df(2)19 exhibit a pattern similar to that of the original l(2)k09822 parent, whereas

Df(2)55 is comparable with Oregon R. This can be explained if Df(2)15 and Df(2)19 have both lost the region of the *P[lacW]* element carrying the w^+ gene, but retained part of the *P[lacW]* element 3′ to w^+. The remaining *P[lacW]* element sequence will contain the same *EcoR*1 site as l(2)k09822 and hence the patterns are identical. In Df(2)55 all of the *P[lacW]* element and the whole of the coding region was removed resulting in the flies being hemizygous for PP2A and hence the genomic pattern is returned to that of the wild type.

The *P[lacW]* element insertion in l(2)k09822 *Drosophila* reduces the level of PP2A mRNA and protein phosphatase activity

Evidence that the insertion of the *P[lacW]* element 251 bp 5′ of the PP2A initiating ATG affects the level of PP2A mRNA in *Drosophila* comes from northern blot analysis of Oregon R, l(2)k09822 and 2nd chromosome deficiencies derived from the latter. The ratio of the level of PP2A mRNA to the level of PP1 87B mRNA was found to be 20% lower than wild type in the l(2)k09822 strain and 40% lower than wild type in Df(2)15 and Df(2)19 strains (Fig. 3). Since l(2)k09822, Df(2)15 and Df(2)19 strains are heterozygous for the mutant PP2A gene and the chromosome balancer CyO, these results demonstrate that the PP2A mRNA level is considerably reduced by mutation of the PP2A gene 251 bp 5′ of the initiating ATG.

PP2A activities in these strains were examined using the ^{32}P-labelled substrates, phosphorylase, casein, histone H1 and caldesmon. PP2A activity was taken as the activity inhibited by 2 nM okadaic acid after PP1 activity had been inhibited by 200 nM inhibitor-2, a specific PP1 inhibitor. The total phosphatase activity in extracts was completely inhibited by 2 nM okadaic acid and 200 nM inhibitor-2. The data show that in heterozygotes for the *P[lacW]* element (strain l(2)k09822), PP2A activity was reduced to 80% of the wild-type level, while in heterozygous deficiencies PP2A activity was reduced to 70% (Fig. 4A and B). No clear differences were discerned between the values for different substrates. Since PP2A activity is higher than 50% in Df(55) which is likely to have only one copy of the PP2A gene, the results indicate that a compensatory increase in PP2A activity from a single active gene can occur. If a similar

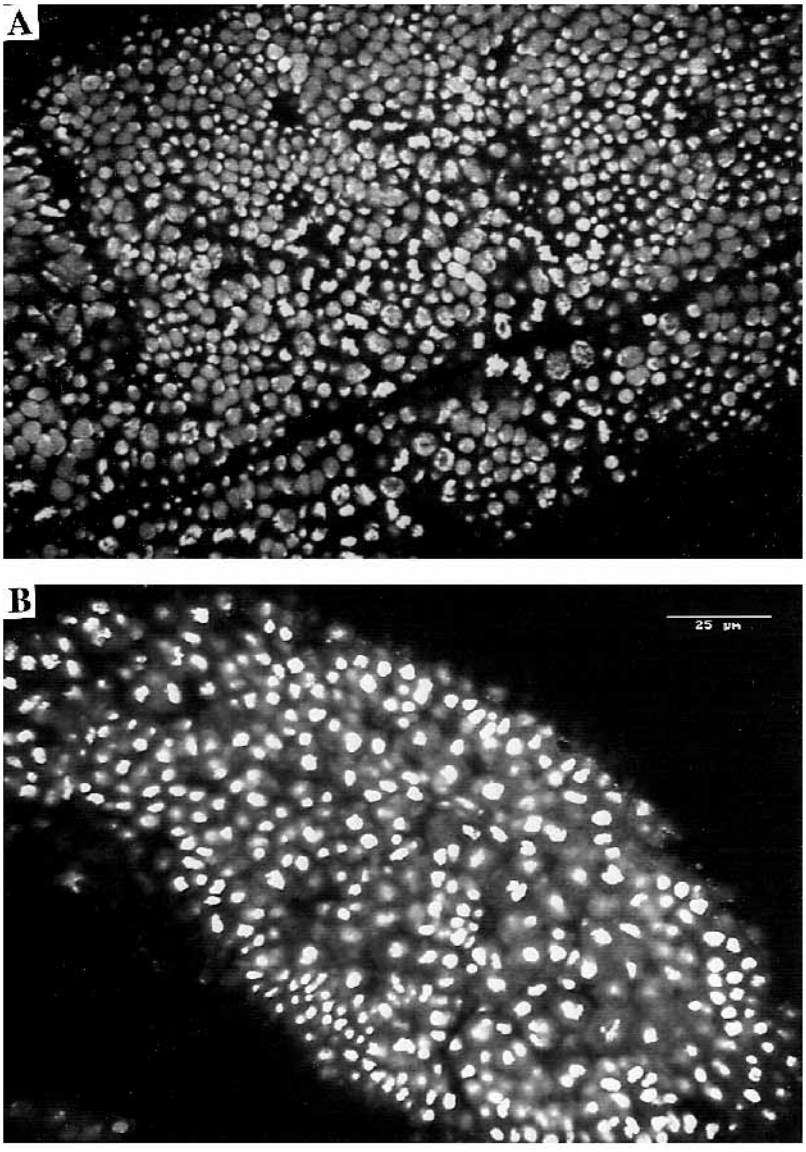

Fig. 5. Indirect immunofluorescence staining of *mts* *Drosophila* embryos homozygous for the *P[lacW]28D* element and wild-type (Oregon R) embryos. Six hour cellularised embryos were fixed and stained for DNA (white) with propidium iodide. Fluorescence micrographs were taken with a confocal microscope. (A) Wild-type embryo showing the ventral furrow running from left to right and cells in different stages of the cell cycle. (B) *mts* embryo, showing retarded development and all nuclei with highly condensed DNA.

compensatory increase in PP2A activity from the single wild-type gene occurs in strain l(2)k09822, then this would cause a rise from 50% to 70% and suggest that the PP2A activity derived from the *P[lacW]* carrying PP2A gene in strain l(2)k09822 is approximately 10%. *Drosophila* homozygous for the *P[lacW]* element in the PP2A gene may therefore be deduced to have only approximately 20% of wild-type activity.

Determination of the lethal stage of the mutation

The lethal phase of strain l(2)k09822 was determined by Istvan Kiss and colleagues to occur between embryogenesis and the early 3rd instar larval stage (Török et al., 1993). In order to study the phenotype of flies homozygous for the *P[lacW]* insertion it was necessary to more accurately determine the lethal stage. The *CyO* balancer in l(2)k09822 was replaced with *CyO* marked with y^+*(yellow*$^+$*)*. Thus, since the parent strain was homozygous for *y w*, only flies homozygous for the *P[lacW]* element would remain *y (yellow)*. *y w*; *P[lacW]/*y^+, *CyO* heterozygotes would exhibit a y^+ phenotype. In early larvae, the *y* phenotype is manifested as golden brown mouth hooks, while the y^+ phenotype is characterised by brown/black mouth hooks. On examination of 150 embryos laid by *y w*; *P[lacW]/*y^+ *CyO* females crossed with *y w*; *P[lacW]/*y^+, *CyO* males, 67 embryos were observed to hatch into larvae but 83 arrested during embryogenesis. None of the hatched larvae were observed to have golden brown (y) mouth hooks but exhibited wild-type colouring of this organ. If the *P[lacW]* element caused embryonic lethality equal numbers of hatching and dying embryos would be expected, because the *CyO/CyO* homozygous phenotype is also embryonic lethal at 25°C. Since the ratio of 67 hatching:83 dying is close to the expected 1:1 and all of the hatched embryos had the phenotype of *P[lacW]/*y^+ *CyO* larvae (brown/black mouth hooks), the data indicate that the *P[lacW]* element insertion can be concluded to cause embryonic lethality.

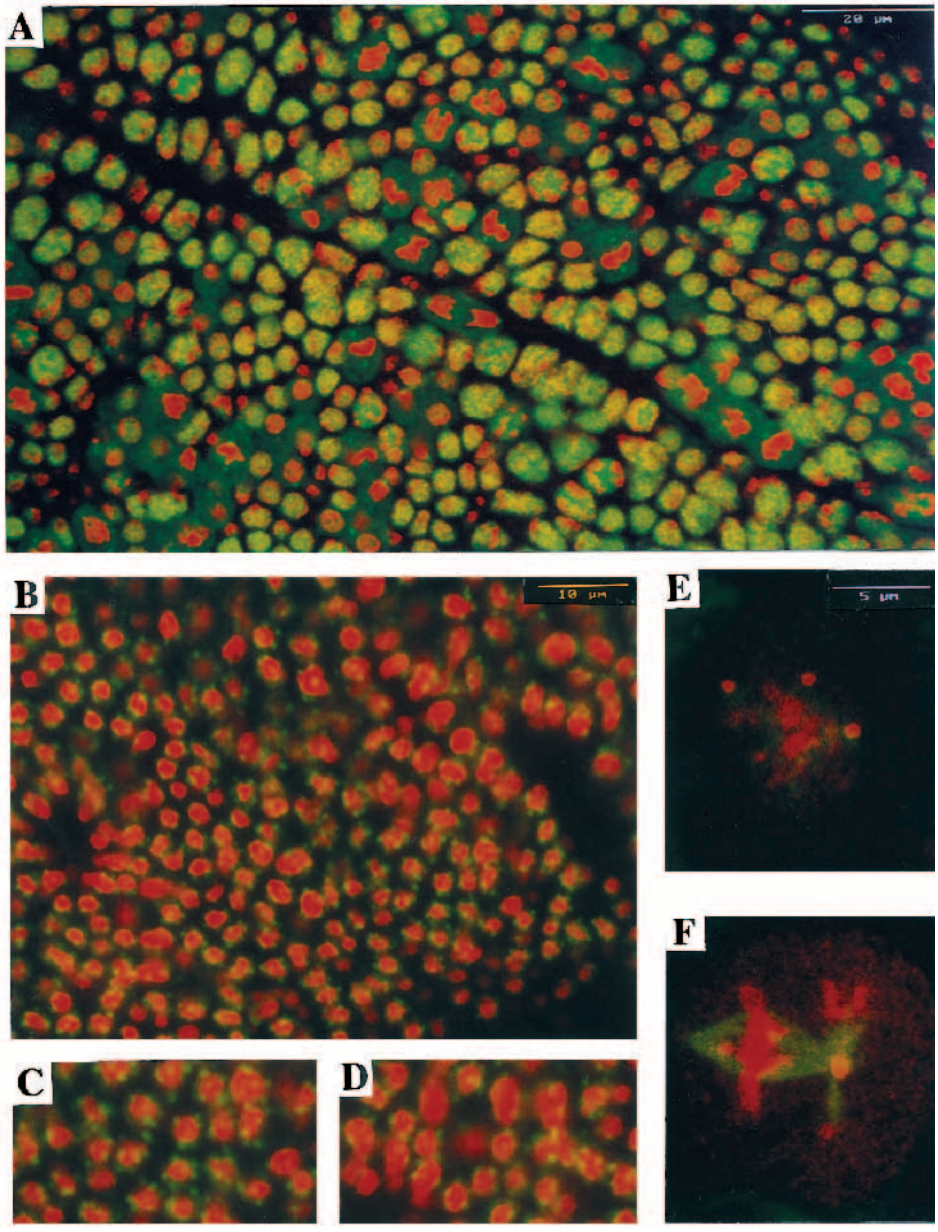

Fig. 6. (A) Wild-type embryos stained for DNA (red) and centrosomes with anti-CP190 antibody (green). CP190 is only associated with centrosomes during mitosis and is nuclear during interphase. Therefore interphase nuclei appear bright green or yellow, since CP190 colocalises with the DNA. The ventral furrow is visible and cells can be seen in different stages of the cell cycle. (B) *mts* embryo stained as in A showing multiple centrosomes surrounding the DNA in each cell. (C and D) Higher magnification of parts of panel B. (E and F) *mts* embryos stained for DNA (red), centrosomes with anti-CP190 antibody (red) and tubulin (green) showing abnormal mitoses.

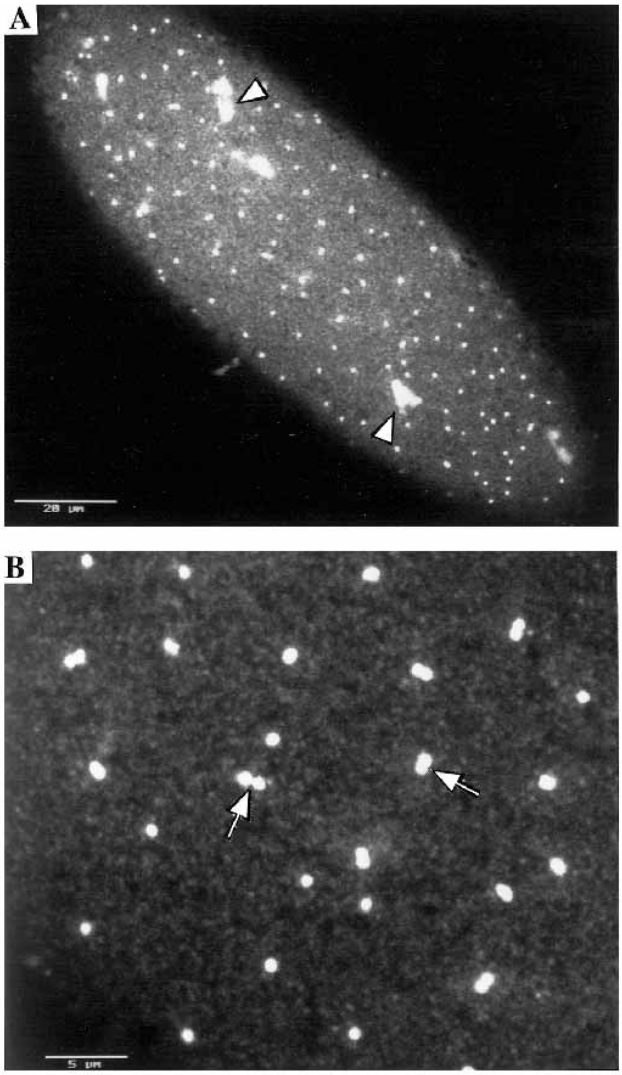

Fig. 7. Six hour *mts* embryos which had arrested prior to cellularisation (A) stained for centrosomes with anti-CP190 (white) and DNA (white, indicated by arrowheads). (B) Higher magnification of part of A showing that most centrosomes are no longer associated with DNA but are continuing to duplicate (indicated by arrows).

Examination of the embryonic phenotype of *Drosophila* homozygous for the *P[lacW]* in the PP2A gene

The insertion of the *P[lacW]* element in strain l(2)k09822 resulted in embryonic death of the *P[lacW]* homozygotes, termed *microtubule star* (*mts*). Hence the phenotype of the latter was examined during early embryogenesis. Embryos were collected for 30 minutes at 25°C onto apple juice agar plates and allowed to develop for different lengths of time at 25°C. By 6 hours (mitotic division cycle 15-16) clear phenotypic abnormalities were evident, which might be expected since the maternal deposit of mRNA present in syncitial and blastoderm embryos should be exhausted by this time necessitating reliance on zygotic transcription for the provision of essential transcripts. Embryos were therefore initially analysed at this stage. In the wild-type embryo, cellularisation has taken place, cells of the ventral epidermis being in mitosis 15 and cells of the dorsal epidermis in mitosis 16. The germband is undergoing elongation and many structures are identifiable such as the tracheal pits, and the ventral and cephalic furrows. The epidermis of the embryo is easily penetrated by antibodies and can be clearly visualised under a confocal microscope.

After removal of the chorion and fixation, 6 hour *mts* embryos were identified by staining with X-gal. As described above, the dark blue/black crystals produced by β-galactosidase could only be seen in *mts* embryos, the single copy of *lacZ* under a weak enhancer being insufficient to produce staining in heterozygotes. After thorough washing, *mts* embryos were stained for DNA and examined with tyrosinated α-tubulin antibodies to reveal the mitotic spindle and CP190 (centrosomal protein 190), a protein that is a component of the centrosome during mitosis but is associated with specific sites on chromatin during interphase (Whitfield et al., 1995).

Compared to wild-type embryos, *mts* embryos were retarded in development of differentiated structures (Fig. 5A and B). The most prominent phenotype seen was that, unlike wild-type embryos which had cells in different stages of mitosis with the majority being in interphase (Fig. 6A), virtually all cells in the *mts* embryos were arrested in mitosis, with highly condensed DNA but without the formation of a metaphase plate or a mitotic spindle (Fig. 6B). Although the chromosomal DNA in some cells of *mts* embryos was dumbbell-shaped or elongated, no cells appeared to have successfully completed anaphase and a few cells had aberrantly condensed DNA and multipolar nuclei, suggestive of a previously unsuccessful cell division (Fig. 6B and D). Sometimes in *mts* embryos, cells are seen in metaphase with aberrant spindle formation, where they appear to have entered a second mitosis before completion of the first (Fig. 6E and F) or very occasionally in anaphase with one or more lagging chromatids (data not shown). However, in most cells of the *mts* embryos anti-tubulin antibodies showed disorganised tubulin structures and a diffuse localisation of tubulin (data not shown).

In wild-type embryos, two centrosomes are located at either end of the mitotic spindle in metaphase until the end of telophase. The most striking feature of all cells of *mts* embryos was that they possessed multiple centrosomes, with usually more than four and often upwards of eight centrosomes being visible (Fig. 6B and C). In wild-type cells CP190 is known to relocate from the centrosome to chromatin during late anaphase and early telophase (Fig. 6A). Since in the *mts* embryos seen here, CP190 remains clearly associated with the multiple centrosomes, the nuclei must be arrested in anaphase A or an earlier stage of mitosis. In contrast, the centrosome cycle is clearly able to continue. In a few *mts* embryos, development had arrested before cellularisation. Inspection of these syncitial *mts* embryos showed that centrosomes could be seen at the periphery of the embryo and were continuing to duplicate but had no associated chromosomal DNA (Fig. 7A and B).

Examination of the syncitial *mts* embryos with anti-tubulin antibodies revealed disorganised arrangements of microtubules radiating from centrosomes in all directions (Fig. 8C-E). A direct comparison of the length of microtubules in *mts* and wild-type embryos is difficult because *mts* embryos are delayed in development and the microtubules present are disorganised. Nevertheless, Fig. 8E shows that microtubules in *mts* embryos seem to be longer than normal (compare the

length of microtubules in 6 hour *mts* embryos in Fig. 8E with the mitotic spindles of 2 and 6 hour wild-type embryos in Fig. 8A and B). It should also be noted that the embryos were *not* treated with any microtubule stabilising agent such taxol prior to fixation.

Although the actin cytoskeleton is perturbed in *S. cerevisiae* mutants and the normal polar distributions of actin are lost in *S. pombe* mutants which lack normal PP2A function, immunolocalisation of actin in cellularised *Drosophila mts* embryos did not reveal any abnormalities of the actin cytoskeleton (data not shown).

DISCUSSION

PP2A is required for the progression through mitosis in *Drosophila*

We have identified a *Drosophila* strain (l(2)k09822) carrying a *P[lacW]* element in the promoter of the single PP2A catalytic subunit gene at chromosomal location 28D and shown that lethality in this stock is due to the *P[lacW]* element by reversion mutagenesis. *mts* embryos homozygous for the *P[lacW]28D* element die around the time of cellularisation. The development of cellularised *mts* embryos arrests with all cells blocked in mid-mitosis, having highly condensed DNA but with no metaphase plate or mitotic spindle, indicating a requirement for PP2A at this stage of mitosis. Since a few cells show multipolar nuclei, where the DNA has replicated but no cytokinesis has occurred and CP190 is entirely centrosomal (Whitfield et al., 1995), the block appears to be between prophase and the initiation of anaphase. This would be consistent with studies in *S. cerevisiae*, which show that a mutant of PP2A catalytic subunit causes a block in G_2/M phase (Lin and Arndt, 1995) but opposite to fission yeast, *S. pombe*, where deficiency of PP2A catalytic subunit appears to negatively regulate mitosis (Kinoshita et al., 1990). The differences in the effects of deficiency of PP2A catalytic subunit do not appear to reside in the different levels of residual PP2A activity in these mutants. *mts Drosophila* embryos are deduced to have approximately 20% of wild-type activity (see Results), while *S. pombe* strains carrying a deletion of the $ppa2^+$ gene also show a substantial (but not precisely quantitated) reduction in the PP2A activity. In contrast, *S. cerevisiae* deleted for PP2A genes and carrying a mutant *pph21-102* allele show little difference from wild-type PP2A activity at the restrictive temperature, although it is possible that more variation may be discernible with different substrates.

aar^1 and $twins^P$ mutants affecting the B subunit of PP2A in *Drosophila* die during pupariation, abnormal mitoses occurring in 3rd instar larval brains (Gomes et al., 1993; Mayer-Jaekel et al., 1993). The proportion of cells in metaphase and anaphase in aar^1 homozygotes is elevated, suggesting that the mutation delays progression through mitosis. DNA is hypercondensed and chromosomes fail to separate correctly giving rise to lagging chromatids and chromosome bridges, in which one or more chromatids is left at the midzone during anaphase. The PP2A activity of aar^1 and $twins^P$ homozygotes was significantly reduced against substrates phosphorylated by $p34^{cdc2}$/cyclin B kinase, although not against substrates phosphorylated by other kinases, suggesting that the B subunit enhances the dephosphorylation of $p34^{cdc2}$ phosphorylated substrates (Mayer-Jaekel et al., 1994). In contrast, the l(2)k09822, Df(2)15, Df(2)19 and Df(2)55 mutants showed a similar reduction of PP2A activity against four substrates phosphorylated by three different kinases, including $p34^{cdc2}$. However, a reduction in the level of the catalytic subunit might not be expected to influence the activity of different PP2A complexes differentially if the binding affinities of regulatory subunits for the catalytic subunit are of the same order of magnitude. *mts*, $twins^P$ and aar^1 mutants are all deficient in PP2A activity against $p34^{cdc2}$ phosphorylated substrates and all show a block or delay in mitosis. The PP2A activity in *mts* embryos is deduced to be 20% of wild type against histone H1 and caldesmon, while in the $twins^P$ mutant it was 27% against histone H1 and 14% against caldesmon. Since these values are similar, the more severe mitotic phenotype in the *mts* mutant suggests that PP2A has site(s) of action in mitosis additional to that affected in $twins^P$ and aar^1 mutants. Unlike the situation in *S. pombe* there is no evidence that any of these negatively regulates mitosis.

PP2A is required for the interaction of microtubules with chromosomal DNA and the stabilisation or elongation of microtubules

Analysis of *Drosophila mts* embryos which have arrested just prior to cellularisation shows disorganised arrays of microtubules of differing lengths emanating from centrosomes in all directions. We therefore termed this mutant *microtubule star* (*mts*). Centrosomes are able to nucleate microtubules which can grow *in mts* embryos to a length that *appears to exceed* that of microtubules *in wild-type embryos of comparable development*. However, in *mts* embryos the microtubules are rarely associated with any DNA, suggesting that arrest of embryonic development has occurred because the microtubules are incapable of interaction with chromosomal DNA or cannot be organised or stabilised correctly to undergo this process. In mammals, several proteins have been implicated in the attachment of microtubules to the kinetochore on chromosomal DNA, including cytoplasmic dynein (Pfarr et al., 1990; Steuer et al., 1990), centromeric protein E (CENP-E; Yen et al., 1991) and mitotic centromere associated kinesin (Wordeman and Mitchison, 1995). In *Xenopus*, a kinesin related protein XKCM1 has also recently been identified at mitotic centromeres and spindle pole bodies (Walczak et al., 1996). Depletion of XKCM1 in *Xenopus* embryos leads to the formation of centrosomes radiating abnormally long microtubules. It is suggested that XKCM1 walks along the microtubules from the centrosome towards the plus end of the growing microtubule where it triggers a catastrophe. CENP-E might act as a kinetochore coupler to the depolymerising microtubule and the chromosome would then move back towards the pole concomitant with microtubule depolymerisation (Lombillo et al., 1995; Walczak et al., 1996). Like many motor proteins, CENP-E (Liao and Yen, 1994) and cytoplasmic dynein (Dillman and Pfister, 1994) have been reported to undergo phosphorylation which may regulate their function. The mitotic defects seen in the aar^1 and $twins^P$ *Drosophila* mutants could also be explained by a defect in attachment to the kinetochore. Since the B subunit is not absent in these mutants, but present at reduced levels, there may be virtually sufficient activity to attach chromosomal DNA to the mitotic spindle, such that defects

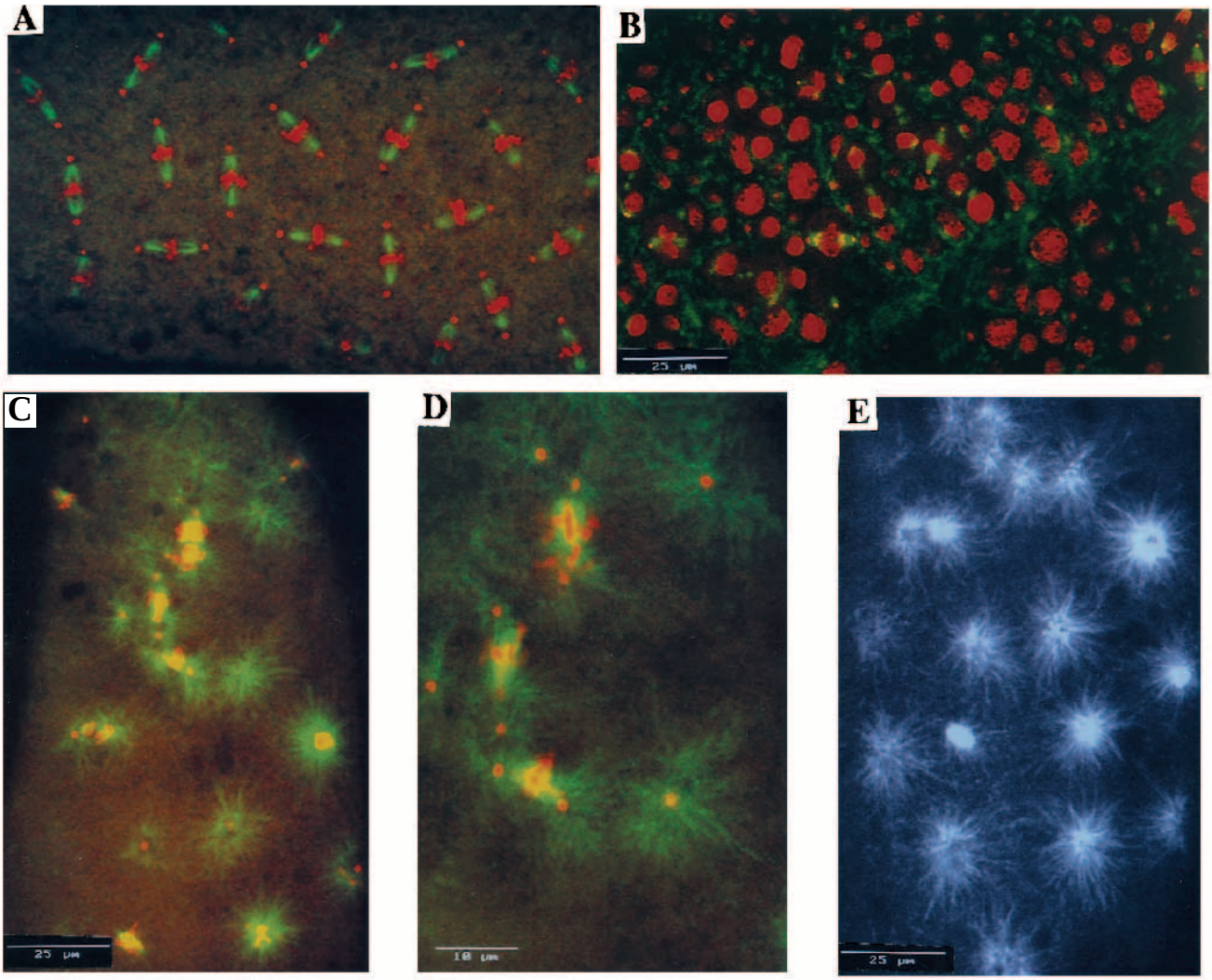

Fig. 8. (A) A 2 hour, and (B) a 6 hour wild-type embryo stained for tubulin (green) and DNA (red) and CP190 (red); (C) a 6 hour *mts* embryo stained as in A and B. (D) Higher magnification of part of C: these show centrosomes which have nucleated disorganised microtubules often in the absence of DNA; (E) a 6 hour *mts* embryo stained for tubulin (white) to show the elongated microtubules.

in the separation of chromosomes are seen mainly at a later stage of mitosis (i.e. anaphase) rather than in metaphase as seen in *mts* embryos.

Phosphorylation is also important in the stabilisation and organisation of microtubules. The severe effects of PP2A deficiency in *mts* embryos suggests that PP2A may dephosphorylate more than one target in the microtubule associated processes. In *cellularised* mutant embryos, no organised mitotic spindle was visible, anti-tubulin antibodies detecting only disorganised tubulin structures and a diffuse localisation of tubulin. This distribution of tubulin suggests that PP2A may organise/stabilise microtubules as well as being involved in attachment of microtubules to the kinetochore. Several microtubule associated proteins (MAPs) thought to be involved in stabilisation of microtubules are phosphorylated; for example MAP4 is phosphorylated during mitosis with concomitant dissociation from microtubules (Faruki and Karsenti, 1994) and XMAP230 is phosphorylated in *Xenopus* mitotic egg extracts (Andersen et al., 1994). Stathmin/oncoprotein 18 which will regulate microtubule polymerisation dynamics in *Xenopus* egg extracts (Belmont and Mitchison, 1996) is phosphorylated on four sites in mammalian cells in vivo (Beretta et al., 1993). A number of protein kinases, such as $p34^{cdc2}$, are known to associate with microtubules. Heterotrimeric PP2A has also been reported to be associated with the microtubule network in mammalian cells (Sontag et al., 1995). In *S. cerevisiae* mutant for the PP2A catalytic subunit 11% of cells were seen with a shortened mitotic spindle at the restrictive temperature. Microtubule distribution was also anomalous in *S. pombe* cells deleted for the A or B regulatory subunits of PP2A, cytokinesis being blocked or delayed, respectively. However, elongated microtubules as seen in the syncitial arrested *Drosophila mts* embryos, which implicate PP2A in attachment of microtubules to the kinetochore, have not been observed in yeast PP2A mutants.

PP2A may play a role in the coordinated regulation of the nuclear and centrosome cycles

The cells of *Drosophila mts* embryos arrest in mid-mitosis and several centrosomes, often more than eight, encircling the

chromatin are visible in a single cell. The presence of more than two centrosomes per nucleus indicates that although the cells failed to separate their chromosomes during a previous mitosis, the centrosome cycle was unaffected causing the nuclei to become poly-centrosomic in subsequent mitoses. This could arise because the nuclear cycle is blocked by deficiency of PP2A, while centrosome replication is unaffected by PP2A activity and is therefore able to continue. Alternatively PP2A may negatively regulate the centrosome cycle while positively regulating cytokinesis. The uncoupling of the nuclear cycle and the centrosome cycle in the *mts* mutant by deficiency of PP2A catalytic subunit is distinct from the situation in *S. cerevisiae* and *S. pombe* PP2A mutants in which multiple centrosomes per cell are not found. *Drosophila* mutants of the B subunit of PP2A (*aar*^{1} and *twins*^{P}) also show no apparent defects in the number or location of centrosomes, although cytokinesis is retarded. This indicates that complexes of PP2A other than those with the 55 kDa B subunit are responsible for the uncoupling, indicating at least three points of action of PP2A during mitosis. While one target is p34^{cdc2} kinase and the second a protein(s) involved in the connection of microtubules to chromosomal DNA, the third would co-ordinate the nuclear and centrosome cycles.

Although uncoupling of the nuclear and centrosome cycles is rarely observed in eukaryotes, sea urchin eggs and *Xenopus* blastulae, which have been treated with cycloheximide, undergo centrosome duplication in the absence of cytokinesis and spindle assembly (Gard et al., 1990; Sluder et al., 1990). Certain mutants in *Drosophila* which block mitosis in the early syncitial embryo, such as *gnu* (Freeman et al., 1986), have defects in the co-ordination of nuclear and centrosome cycles and exhibit multiple centrosomes per nucleus. However, this phenomenon has been attributed to the fact that in the early syncitial embryo the nuclear cycles are very rapid and do not possess the G_1 and G_2 phases of the eukaryotic cell cycle (Glover, 1992). In the case of the *mts* mutant, multiple chromosomes per nuclei are visible *after* cellularisation and there is no evidence of multiple centrosomes per nuclei in the syncitial embryo. To our knowledge PP2A is the first protein to be identified, deficiency of which uncouples the centrosome cycle from the nuclear cycle in *cellularised* embryos, suggesting that PP2A may play a role in the co-ordination of nuclear and centrosome cycles.

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