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Protein phosphorylation is involved in the regulation of chromatin condensation during interphase

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Loci affecting the condensation state of interphase chromatin have been previously identified from analysis of suppression and enhancement of position effect variegation (PEV) in *Drosophila*. Here we show that *Su-var(3)6* and an allelic mutant, *e078*, which both show suppression of PEV in the heterozygous state, have point mutations (Gly^{220}→Ser and Gly^{220}→Asp, respectively) in a protein phosphatase 1 catalytic subunit located at 87B (PP1 87B). The mutated glycine is conserved in all known protein serine/threonine phosphatases in the same gene family, and its substitution decreases PP1 activity. We conclude that protein dephosphorylation by PP1 87B regulates the condensation state of chromatin during interphase.

Protein phosphatase; Chromatin; Position effect variegation; Mitosis; *Drosophila*

1. INTRODUCTION

Interphase nuclei in higher eukaryotes contain euchromatin, which comprises chromosomes with a decondensed structure capable of gene expression, and heterochromatin, which consists of chromosomes or chromosomal regions with a highly condensed structure that are inactive in gene expression. Chromosomal rearrangements in *Drosophila* which move a locus from a euchromatic region and place it close to constitutive heterochromatin may result in the locus becoming refractory to transcriptional activation. If the locus in question is w^+, a gene essential for the production of red pigment in the *Drosophila* eye, the phenotypically visible result is patches of white (zero) and red pigmentation, which result from cell clones with and without heterochromatinization of the w^+ locus, respectively. The variable expression of a euchromatic gene located close to a region of heterochromatin is referred to as position effect variegation (PEV) [1,2]

Dominant mutations which suppress or enhance PEV of the w^+ locus have been identified. One dominant suppressor, *Su-var(3)6*, was mapped to cytological location 87B5–10 on the right arm of the third chromosome [3], and the semi-lethal phenotype associated with the homozygous state was found to be allelic to the lethal complementation group, *ck19* [4].

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Abbreviations: PP, protein phosphatase; PEV, position effect variegation; PCR, polymerase chain reaction.

Analysis of protein phosphatase 1 (PP1) genes in *Drosophila* showed that one isoform mapped to 87B6–12 [5]. PP1 activity was found to be reduced in three mutants of the *ck19* complementation group (*e078*, *e211* and *hs46*) and molecular analyses showed that a deletion in the promoter region of *e211* eliminated the production of the PP1 87B enzyme in this mutant [6]. In the hemizygous state, *e211* and *hs46* show a mitotic block in the third instar larval brain, but mitosis was not disrupted in the hemizygous *e078* mutant [7], which has some residual PP1 87B activity [6].

Suppression of PEV was observed in flies heterozygous for the *Su-var(3)6* and *e078* mutations, but was not examined in *e211* and *hs46* heterozygous mutants [3]. The reduced PP1 activity of the *e078* mutant suggested that PP1 87B is likely to underlie suppression of PEV in this mutant [6]. In this communication we delineate the sequences of *Su-var(3)6*, *e078* and *hs46* mutant genes, demonstrating that the known dominant suppressors of PEV, *Su-var(3)6* and *e078* both carry point mutations in the PP1 87B coding region.

2. MATERIALS AND METHODS

2.1. *Drosophila strains and DNA preparations*

Drosophila were bred at 20–25°C on standard *Drosophila* medium. Mutations used in this study are described in [3,4]. The lethal mutations, *e087* and *hs46*, were used in the hemizygous state over a large deficiency *Df(3R)E079*. Larvae with the correct phenotype were selected with the aid of *TM6B* balancer chromosome, which carries the dominant larval marker mutation, *Tubby* (*Tb*), and genomic DNA was prepared from third instar larvae according to [8]. Adult flies of the semilethal homozygous *Su-var(3)6* mutant and the wild-type strains, Oregon R and Canton S, were starved for 1 h, anaesthetised with ether, and frozen in liquid nitrogen. Genomic DNA was prepared

from these flies [8]. RNA contamination of the DNA preparations was eliminated by RNAse A treatment [9]. The DNA was precipitated in 0.3 M sodium acetate, pH 5.2, and 2 vols. ethanol and the precipitate redissolved in 10 mM Tris-HCl, 1 mM EDTA, pH 8, at a concentration of 1 mg/ml, and stored at 4°C.

2.2. *Isolation of the mutant genes* e078 *and* Su-var(3)6 *by the polymerase chain reaction (PCR)*

Two oligonucleotides, termed A and C, identical to the 5′ non-coding region and one, termed B, complementary to the 3′ non-coding region (see Fig. 3) of the PP1 (87)B gene, were synthesized on an Applied Biosytems 381A oligonucleotide synthesizer. PCR conditions were as described in [10], except that the reaction mixtures were optimized for free magnesium ion concentration, 1 mM $MgCl_2$ being optimal for the A-B oligonucleotide combination and 2 mM for the B-C combination. The PCR reactions were performed as follows: 94°C for 3 min; 72°C (A-B oligonucleotides) or 65°C (B-C oligonucleotides) for 1 min, 72°C for 3 min, 94°C for 1 min, 30 cycles; followed by 72°C (A-B oligonucleotides) or 65°C (B-C oligonucleotides) for 5 min and then 72°C for 10 min. 10 μl of the reaction mixture was used to check the efficiency of the reaction by electrophoresis in 1% agarose gels, followed by Southern blotting. Blots were hybridised with a 0.68 kb *PP1 87B* cDNA probe (nucleotides 151–829 in Fig. 3), which was labelled with [α-^{32}P]dATP by random oligonucleotide priming [11]. Hybridization was at 60°C as described previously [5]. Washes were in 0.15 M NaCl, 30 mM sodium citrate, 0.1% SDS, pH 7, at 60°C. Radioactive bands were visualized by short (~10 min) autoradiography.

2.3. *Cloning and sequencing of* e078 *and* Su-var(3)6 *genes*

Any recessed ends of the PCR amplified DNA were filled in by adding 1 U Klenow fragment of DNA polymerase I to 90 μl PCR reaction, made 1 mM in each deoxynucleotide (dATP, dCTP, dGTP, TTP). The reaction was allowed to proceed for 30 min at room temperature and then extracted with an equal volume of phenol/chloroform/isoamyl alcohol (50:50:1). The aqueous phase was subjected to filtration on a Centricon 100 (Amicon, Stonehouse, Glos., UK) to remove the deoxynucleotides. The 5′ end of the amplified DNA was phosphorylated by T4 polynucleotide kinase and ATP [9]. The DNA was subjected to electrophoresis through a 1% agarose gel in 40 mM Tris, 5 mM sodium acetate, 1 mM EDTA buffer, pH 8, and stained with ethidium bromide. Bands of the expected size were excised and the DNA from the gel slices was purified by glass bead adsorption using Prepagene (Northumbria Biologicals Ltd, Cramlington, Northumberland, UK) and concentrated by ethanol precipitation.

The purified PCR fragments were blunt-end ligated [9] into the Bluescript plasmid pKS$^-$ (Stratagene, La Jolla, CA), which had been previously cleaved with *Eco*RV and treated with alkaline phosphatase (Boehringer-Mannheim, UK). *Epicurian Coli* SURE competent cells (Stratagene, La Jolla, CA) were transformed with the recombinant plasmids. Clones containing inserts of the expected size were selected

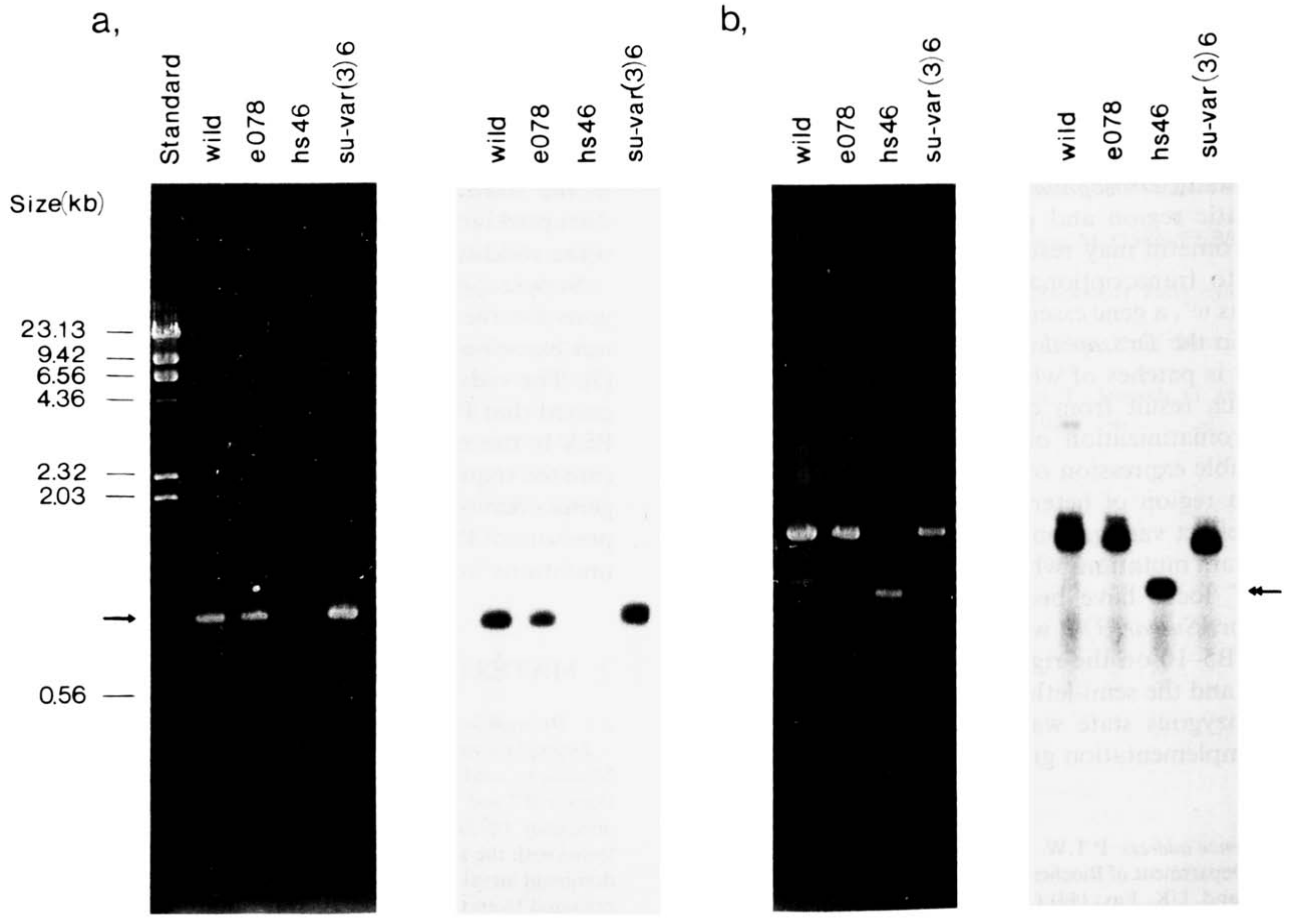

Fig. 1. PCR amplification of the *PP1 87B* gene. Left panels show the ethidium bromide-stained products after electrophoresis in a 1% agarose gel. Right panels show Southern blots of the same gel hybridized with the 0.68 kb *PP1 87B* coding region probe. (a) A + B oligonucleotide combination. The arrow indicates the 0.95 kb band that is missing from the mutant, *hs46*. (b) B + C oligonucleotide combination. The double headed arrow shows the 1.05 kb band that is amplified from the mutant, *hs46*.

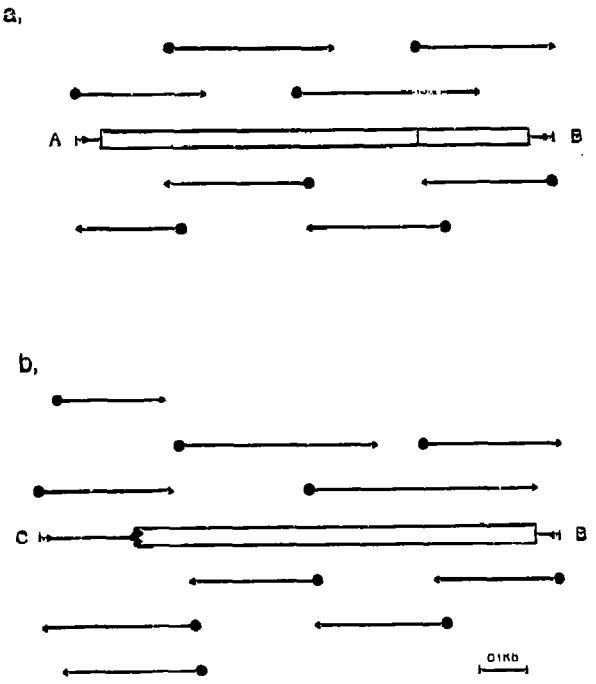

Fig. 2. PCR and sequencing strategies for the characterization of the wild-type and mutant *PP1 87B* genes. Open bars represent the coding region and lines the non-coding regions. The 5' end is at the left. Arrows show the length and direction of sequences obtained with synthetic nucleotide primers. (a) Oregon R and Canton S (wild-type), *Su-var(3)6* and *e078* (mutants). The vertical line in the open bar shows the position of the mutations in *Su-var(3)6* and *e087*. (b) *hs46* mutant. The zigzag line at the start of the open bar indicates the position of the deletion.

and purified by CsCl centrifugation. Double-stranded DNA sequencing was performed on both strands by the dideoxy chain-termination method [12] using oligonucleotide primers (see Fig. 2) and Sequenase version 2.0 DNA polymerase (US Biochemical Corp. Cleveland, OH). Compressions were resolved by replacing dGTP with 8-deaza dGTP in the sequencing reaction [13].

3. RESULTS

3.1. *Molecular analyses of wild-type genes*

Amplification of Canton S and Oregon R DNA by PCR produced a 0.95 kb fragment using oligonucleotide primers A and B, and a 1.5 kb fragment using primers B and C (Fig. 1) as expected from the PP1 87B sequence. The sequences of the cloned Canton S and Oregon R 0.95 kb fragments were identical to that determined previously for the *PP1 87B* gene [6] (Figs. 2 and 3).

3.2. *Molecular analyses of mutations in* Su-var(3)6, e078 *and* hs46

PCR amplification of *Su-var(3)6* and *e078* DNA with both sets of primers gave DNA fragments of the same size as those obtained with wild-type DNA. In contrast, no PCR product was observed with *hs46* DNA using primers A and B, while the production of a 1.05 kb fragment with primers B and C indicated that a 0.45 kb deletion was present in the PP1 87B gene of the *hs46* mutant. Hybridization with a PP1 87B probe (Fig. 1) confirmed that the PCR fragments were derived from this gene.

Sequencing (Fig. 2) of the cloned 0.95 kb fragment of both the *Su-var(3)6* and *e078* and the 1.05 kb fragment of *hs46* produced the results shown in Fig. 3. In the *hs46* mutant a 0.474 kb deletion removes the TATA box and the start of the PP1 87B coding region. In *Su-var(3)6* and *e078*, G-to-A point mutations in the first and second base of the same codon change Gly^{220} to Ser and Asp, respectively. In order to confirm that these single base changes did not arise from PCR error in a single clone, we carried out restriction analysis of the PCR fragments with *Sac*II, since the *Su-var(3)6* and *e078* mutations eliminate a *Sac*II site present in the wild-type PP1 87B gene. The results demonstrated that the *Su-var(3)6* and *e078* PCR products could not be cleaved, while the wild-type PCR fragment could be digested with *Sac*II (data not shown).

4. DISCUSSION

4.1. *Defects in the* PP1 87B *gene underlie suppression of PEV*

Analyses of the alleles *Su-var(3)6* and *e078* which exhibit suppression of PEV in *Drosophila* show that they both have mutations in the PP1 87B coding region. Since the deficiency, *Df(3R)E079*, also shows suppression of PEV [3] and carries a deletion for a number of genes, including PP1 87B [7], molecular defects of PP1 87B correlate with suppression of PEV in three mutants (Table I).

The mutations in *Su-var(3)6* and *e078* affect the same glycine residue in PP1 87B which is known to be conserved in all protein serine/threonine phosphatases of the PP1/PP2A/PP2B family [15] (Fig. 4). The non-conservative substitutions of the glycine for serine and aspartic acid would therefore be expected to affect the function of PP1 87B, and indeed deficient activity has been measured for *e078* [6]. Although four genes for PP1 have been identified at cytological locations, 9C, 13C, 87B and 96A [5,16], *PP1 87B* is thought to provide the major contribution to the PP1 activity at all stages of the *Drosophila* life cycle. Two *PP1 87B* transcripts (1.6 and 2.5 kb) are abundant at all development stages, and from analysis of the null mutant, *e211*, with a >6 kb deletion removing promoter elements, the *PP1 87B* locus was estimated to contribute ~80% of the total PP1 activity in third instar larvae [6]. This data is confirmed by the demonstration that *hs46* contains a 0.474 kb deletion affecting the 5' non-coding and part of the coding region (Fig. 3) and explaining the low level production of a smaller mRNA than the wild-type [7]. However, since the sequence shows that the initiating AUG is deleted and no other in-frame methionine

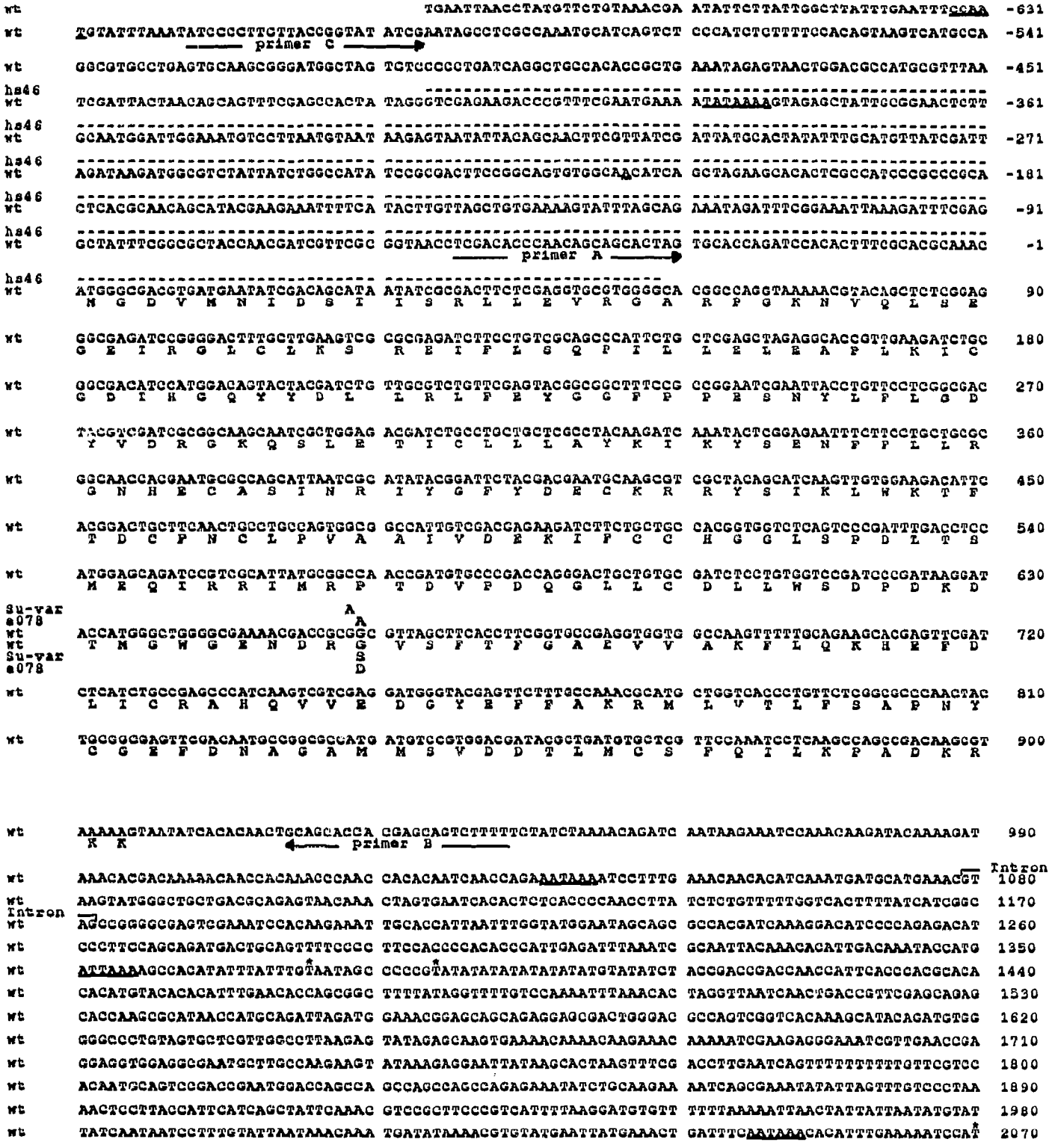

Fig. 3. *Su-var(3)6*, *e078* and *hs46* mutations in the *PP1 87B* gene of *Drosophila*. In *Su-var(3)6* there is a G-to-A point mutation at nucleotide 658 which changes Gly^{220} to Ser. In *e078* there is a G-to-A point mutation at nucleotide 659 which changes Gly^{220} to Asp. In *hs46* 474 nucleotides are deleted from positions −416, −417 or −418 to +58, +57 or +56 of the wild-type gene. The sequence of the wild-type *PP1 87B* gene was extended in the 3′ non-coding region by 843 nucleotides over that presented in [6]. Sequence analysis of nine cDNAs from a *Drosophila* embryonic library [14] showed that the longest 5′ non-coding region started at nucleotide −216 (underlined) and identified three poly(A) addition sites at nucleotides 1,374, 1,386 and 2,070 (marked with *). Putative promoter sites are underlined in the 5′ non-coding region and putative polyadenylation signals are underlined in the 3′ non-coding region. The section at nucleotides 1,078–1,172 was present in the gene sequence in [6] but not in any cDNA analyzed and is therefore identified as an intron.

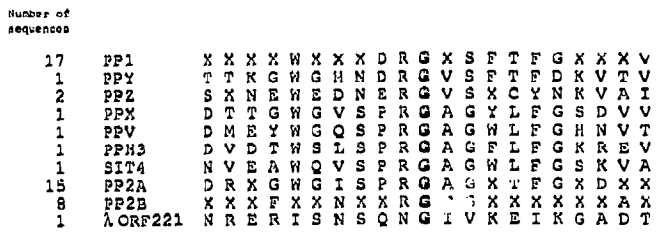

Fig. 4. Conservation of Gly^{220} in protein serine/threonine phosphatases. Sequences are from [15] and the Swissprotein and National Biomedical Research Foundation databases. The conserved G (in bold) is residue 222 in rabbit PP1a [15]. X is a variable amino acid between isoforms or species.

codon occurs until amino acid 181 of PP1 87B (Fig. 3), the mRNA cannot be translated to produce an active PP1. Therefore *hs46*, like *e211*, is a null mutant of PP1 87B, and the residual PP1 activity measured in these mutants can be attributed to the other PP1 isoforms. The data summarized in Table I would predict that *e211* and *hs46* should also show suppression of PEV in the heterozygous state.

4.2. *The PP1 87B gene product serves at least three functions in vivo*

In the hemizygous state the null mutants, *e211* and *hs46*, show a block in mitosis which can be seen in the brain at the third instar larval stage of development. Sister chromatids fail to separate and remain overcondensed [7]. The hemizygous mutant, *e078*, which synthesizes a PP1 gene product that exhibits low activity [6], is not blocked in mitosis [7]. These results imply either that a low level of PP1 87B activity is sufficient for mitosis to proceed, or that in mutants where the PP1 87B catalytic subunit is not synthesized (*e211* and *hs46*) excess regulatory subunits (which would normally bind to PP1 87B catalytic subunit) inhibit the function of the other PP1 catalytic subunits or possibly other proteins.

The *e078* hemizygous mutant dies before the completion of pupation, despite the fact that it is not blocked in mitosis. The lethal phenotype was rescued by a 6.5 kb genomic fragment encoding *PP1 87B*, but not by the same genomic fragment disrupted in the *PP1 87B* coding region [7]. Since sequence analysis showed only a single point mutation in the *PP1 87B* coding region (Fig. 3), it is clear that PP1 must serve a third cellular function besides its role in mitosis and modification of heterochromatin structure.

Although the block in mitosis and suppression of PEV are caused by mutations in the same gene and affect chromosome condensation, the two functions are distinct. The mitotic block is observed when the maternally provided PP1 87B has been degraded and the *PP1 87B* gene in the larvae is non-functional. Suppression of PEV is observed in heterozygous adult flies where the partially decreased PP1 activity affects transcription during interphase. In addition, deficiency of PP1 activity causes chromosomes to become overcondensed in mitosis, but in interphase, lowering of PP1 activity underlying suppression of PEV causes a chromosomal region to be less condensed. The PP1 substrates must therefore be different in the two cellular functions and have not yet been identified. However, it is of interest that another suppressor of PEV is involved in the deacetylation of histone H4 [17], suggesting that post-translational modification of proteins (by acetylation and phosphorylation) may be a general mechanism for influencing chromosome condensation during interphase.

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Table I

Protein phosphatase 1 87B mutants in *Drosophila*

Name	Mutation in *PP1 87B* gene	Position effect variegation in heterozygote [3]	PP1 activity in hemizygote [6] (% of wild type)	Mitosis in hemizygote [7]	Lethality rescued by *PP1 87B* gene [7]
e078	point mutation G220→D	suppressed	35**	normal	yes
Su-var(3)6	point mutation G220→S	suppressed	ND	ND	ND
hs46	translation start deleted	ND	20*	blocked	yes
e211	promoter deleted	ND	20*	blocked	yes
Df(3R)E079	gene deleted	suppressed	NA	NA	NA

ND, not determined; NA, not applicable.
*In *hs46* and *e211* the PP1 87B isoform is not synthesized (see text) and the residual 20% activity arises from one or more of the other PP1 isoforms.
**In *e087*, a mutant PP1 87B isoform is synthesized, which therefore must possess 15% of wild-type PP1 activity, so that the total *e078* PP1 activity is 35%.

gary. We are grateful to János Gausz for *Su-var(3)6* genomic DNA and Myles Axton for isolation of *e078* and *ls46* genomic DNA and cDNAs used to extend the 3' non-coding sequence of the wild-type *PP1 87B* DNA. We thank Alastair I.H. Murchie for the synthesis of oligonucleotides.

REFERENCES

[1] Eissenberg, J.C. (1989) BioEssays 11, 14–17.
[2] Spralding, A.C. and Karpen, G.H. (1990) Genetics 126, 779–784.
[3] Reuter, G., Gausz, J., Gyurkovics, H., Friede, B., Bang, R., Spierer, A., Hall, L.M.C. and Spierer, P. (1987) Mol. Gen. Genet. 210, 429–436.
[4] Gausz, J., Gyurkovics, H., Bencze, G., Awad, A.A.M., Holden, J.J. and Ish-Horowitz, D. (1981) Genetics 98, 775–789.
[5] Dombrádi, V., Axton, J.M., Glover D.M. and Cohen, P.T.W. (1989) Eur. J. Biochem. 183, 603–610.
[6] Dombrádi, V., Axton, J.M., Barker, H.M. and Cohen, P.T.W. (1990) FEBS Lett. 275, 39–43.
[7] Axton, J.M., Dombrádi, V., Cohen, P.T.W and Glover, D.M. (1990) Cell 63, 33–46.
[8] Ashburner, M. (1989) in: *Drosophila*: A Laboratory Manual, pp. 106–109, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
[9] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
[10] Chen, M.X. and Cohen, P.T.W. (1992) in: Protein Phosphorylation: A Practical Approach (D.G. Hardie ed.) IRL Press (in press).
[11] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13 and addendum (1984) 137, 266–267.
[12] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[13] Mizusawa, S., Nichimura, S. and Seela, F. (1986) Nucleic Acids Res. 14, 1319–1324.
[14] Brown, N.H. and Kafatos, F.C. (1988) J. Mol. Biol. 302, 425–437.
[15] Cohen, P.T.W., Brewis, N.D., Hughes, V. and Mann, D.J. (1990) FEBS Lett. 268, 355–359.
[16] Dombrádi, V., Axton, J.M., Brewis, N.D., da Cruz e Silva, E.F., Alphey, L. and Cohen, P.T.W. (1990) Eur. J. Biochem. 194, 739–745.
[17] Dorn, R., Heymann, S., Lindigkeit, R. and Reuter, G. (1986) Chromosoma 93, 398–403.