ELSEVIER

FEBS Letters 339 (1994) 124–128

FEBS 13649

FEBS LETTERS

# Identification of a cDNA encoding a *Drosophila* calcium/calmodulin regulated protein phosphatase, which has its most abundant expression in the early embryo

Linda Brown, Mao Xiang Chen, Patricia T.W. Cohen*

*Medical Research Council Protein Phosphorylation Unit, Department of Biochemistry, The University, Dundee DD1 4HN, Scotland, UK*

Received 3 January 1994

**Abstract**

A 3.3 kb cDNA encoding the complete amino acid sequence of a calcium/calmodulin regulated protein phosphatase has been isolated from a *Drosophila* eye disc cDNA library. The predicted protein of 560 amino acids (molecular mass 62 kDa) is 73–78% identical to human PP2B isoforms. The cDNA hybridised to the X-chromosome at cytological position 14D1–4. Two transcripts of 3.5 kb and 3.0 kb were expressed during embryonic development, their levels being highest in the early embryo. The larger transcript was also clearly present in adult females. This pattern of expression indicates a role for calcium/calmodulin regulated protein phosphatase in embryonic development.

*Key words:* Protein phosphatase 2B; Calcineurin; cDNA sequence; Calcium; Calmodulin; *Drosophila melanogaster*

## 1. Introduction

The $Ca^{2+}$/calmodulin protein phosphatase, PP2B, (also termed calcineurin, and PPP3 in human gene nomenclature) is an enzyme which dephosphorylates serine and threonine residues, interacts with calmodulin and is dependent on calcium ions for activity (reviewed in [1]). In mammals PP2B has recently been implicated in the immune response (reviewed in [2]) where it is thought to dephosphorylate the transcription factor N-FAT leading to increased transcription of the interleukin-2 gene [3]. By the use of the immunosuppressant drugs FK506 and cyclosporin A, which are specific inhibitors of PP2B when bound to cytoplasmic binding proteins, PP2B has also been implicated in a number of processes such as the regulation of ion fluxes in renal tubule cells [4] and neutrophil chemokinesis [5]. However, the high level of PP2B in the brain (1% of the total protein as opposed to 0.05–0.1% in other tissues) suggests an important role for PP2B in the regulation of neuronal function. In vitro, PP2B will dephosphorylate a number of proteins, including the regulatory subunits of cAMP dependent protein kinase, inhibitor-1 and DARPP32, suggesting that it may antagonise the actions of cyclic AMP dependent protein kinase (reviewed in [1]). Heat shock protein 25 has also recently been reported to be dephosphorylated by PP2B [6]. In lower organisms, the function of PP2B (*CMP, CNA*) has only been examined in *S. cerevisiae*, where it is involved in the recovery of yeast cells from growth arrest induced by mating factors [7].

Mammalian PP2B is a heterodimer consisting of a 58–59 kDa catalytic subunit complexed to a 19 kDa regulatory subunit which shows 35% identity to calmodulin. Binding of $Ca^{2+}$ to the 19 kDa subunit results in a small basal activity of PP2B, while binding of $Ca^{2+}$ to calmodulin facilitates the interaction of calmodulin with the catalytic subunit with a concomitant ten fold increase of the basal enzyme activity [8]. The catalytic subunit of PP2B is a member of the family of protein phosphatases that includes PP1 and PP2A and several more newly identified protein phosphatases [9]. In order to identify novel protein phosphatases in this family, polymerase chain reactions using oligonucleotides constructed to conserved regions were employed with human, *Drosophila* and *S. cerevisiae* genomic DNA as a template [10]. Seven new protein phosphatases were identified in *Drosophila*, three of these being more closely related to PP2B than any other mammalian protein phosphatase. The PCR fragments D14, D33 and D27, each encoding 24 amino acids showed 88%, 87% and 83% amino acid identity, respectively, to human PP2B$\beta$, suggesting that they may be homologues of mammalian PP2B. The full sequence of a cDNA identical to D14 was recently presented [11], confirming D14 to be a *Drosophila* homologue of mammalian PP2B. In order to see whether D27 was a PP2B homologue or a more novel protein phosphatase, we have cloned the full length cDNA. Here we

*Corresponding author. Fax: (44) (382) 23778.

*Abbreviations:* PP, protein phosphatase; PCR, polymerase chain reaction


*SSDI* 0014-5793(94)00033-R

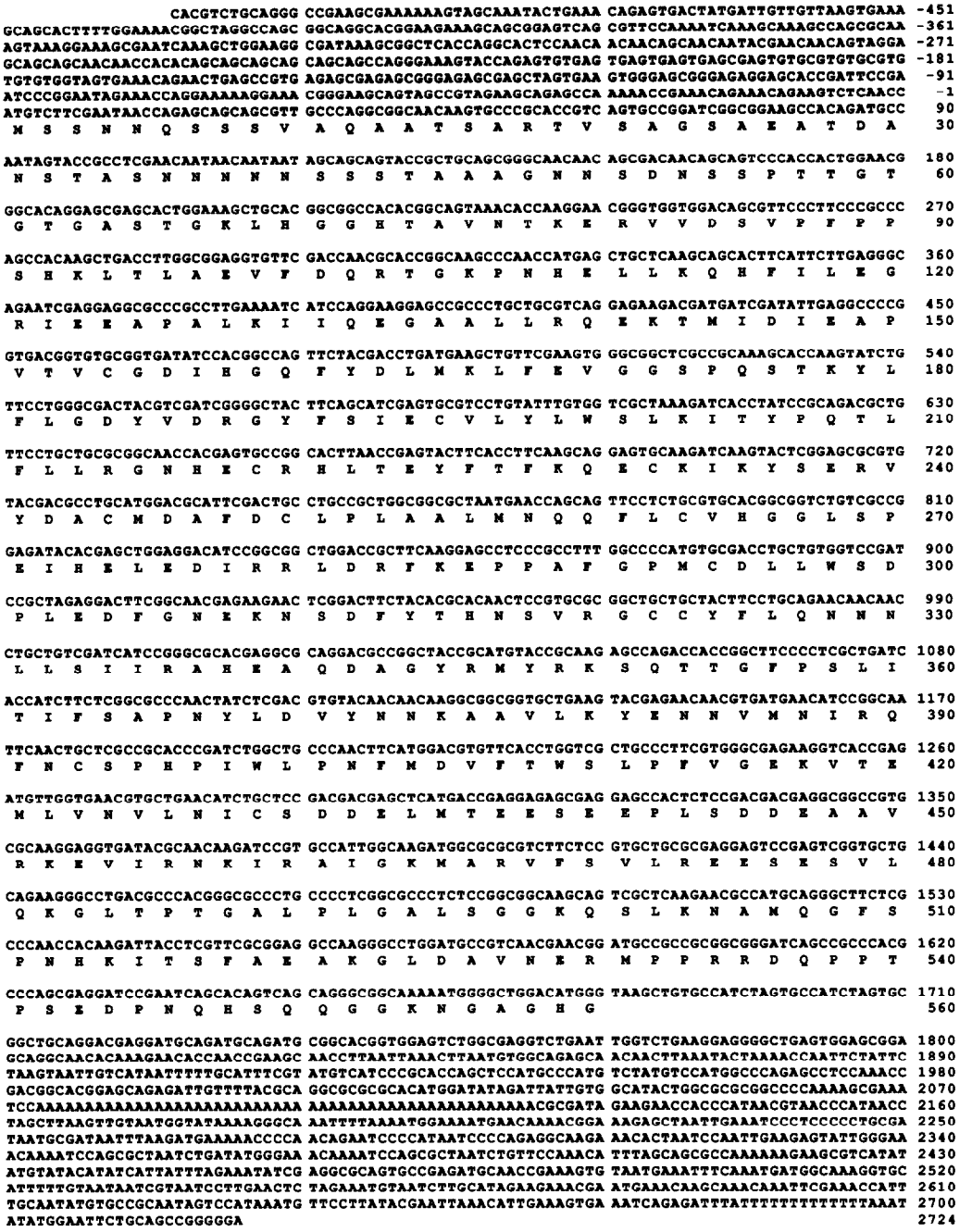

Fig. 1. Nucleotide and predicted amino acid sequence of *Drosophila* protein phosphatase 2B cDNA.

present the complete sequence of D27 demonstrating that it is indeed a PP2B isoform. Analysis of the expression of this new isoform during development shows it to be most abundant in the early embryo, indicating that PP2B is likely to play a role in embryonic development.

## 2. Materials and methods

### 2.1. Isolation of cDNAs

A *Drosophila melanogaster* (Oregon R) eye disc cDNA library constructed in λgt10 (kindly provided by D. Kalderon and G.M. Rubin [12]) was screened in *E. coli* C600 Hfl on duplicate nitrocellulose filters. The 120 base pair PCR fragment, D27, derived from *Drosophila* genomic DNA using degenerate oligonucleotides based on conserved regions of the PP1/PP2A/PP2B family [10] was used as a probe. It was labelled with [α-$^{32}$P]dATP as in [13] using the degenerate oligonucleotides at a concentration of 0.3 μM in place of random hexanucleotides. The probe was purified by spun column chromatography [14]. Hybridisation was carried out in 0.9 M NaCl, 0.09 M sodium citrate, pH 7.0, 0.1% polvinylpyrrolidone, 0.1% Ficoll 400, 0.1% bovine serum albumin, 0.5% sodium dodecyl sulphate, 0.1 mg/ml heat-denatured herring sperm DNA at 65°C overnight and filters washed under stringent conditions in 15 mM NaCl, 1.5 mM sodium citrate pH 7.0, 0.1% SDS at 65°C. Recombinant phage that hybridised to the probe were purified by CsCl density centrifugation and phage DNA was purified by formamide extraction [14].

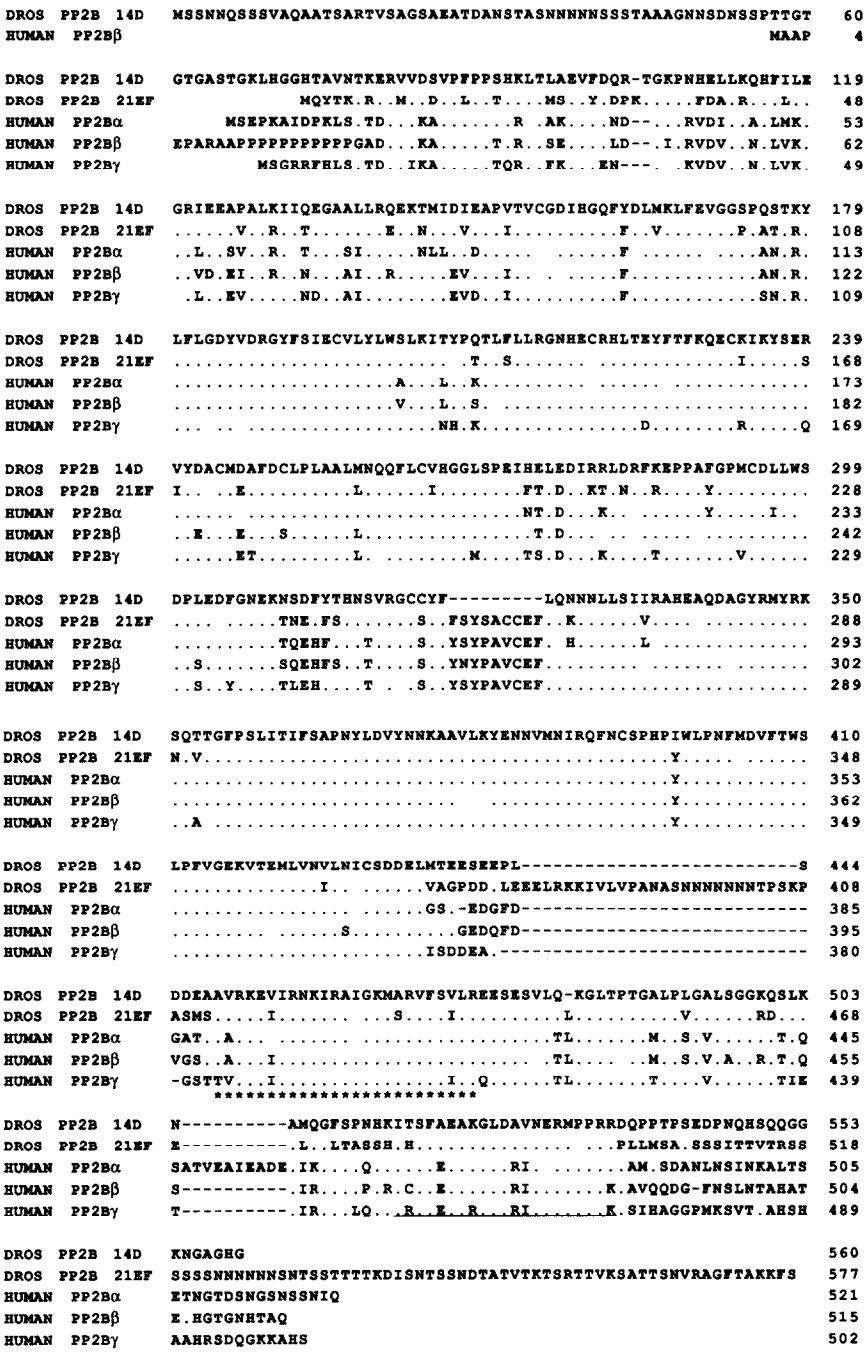


Fig. 2. Comparison of *Drosophila* PP2B 14D with *Drosophila* PP2B 21 EF [11] and human PP2B$\alpha_1$ [22], PP2B$\beta_3$ [24] and PP2B$\gamma$ [29]. The conventions for the alternatively spliced forms are as described in [24]. Positions of identity between *Drosophila* PP2B 14D and other PP2B sequences are indicated by periods. The region underlined with asterisks is the calmodulin binding domain [20] and that with a single underline corresponds to the autoinhibitory domain [21].

### 2.2. Subcloning and sequence analysis

The recombinant DNA was digested with *Eco*RI to release a 3.3 kb insert which was subcloned into Bluescript pKS M13$^+$ vector (Stratagene, La Jolla, CA). This subclone was cleaved with *Pst*I releasing five fragments which were subcloned separately into Bluescript. Sequencing was performed either using an Applied Biosystems Inc automatic DNA sequencer model 373A or manually using [$\alpha$-$^{35}$S]dATP and Sequenase Version 2.0 (US Biochemical Corp.) and Bluescript or specific oligonucleotide primers (synthesized by A.I.H. Murchie, University of Dundee).

### 2.3. RNA preparation and Northern blot analysis

Total RNA was isolated as described in [15] from early embryos (0–4 h), late embryos (4–24 h), larvae, pupae, male and female adults of *D. melanogaster*, stored at −80°C. Poly(A)-rich mRNA was isolated by selection on oligo d(T) cellulose (Sigma) according to [16]. 5 $\mu$g of poly(A)-rich mRNA was loaded on a denaturing gel containing 1% agarose, 6% formaldehyde, 0.02 M morpholinopropane sulphonic acid, pH 7, 0.5 mM sodium acetate and 0.1 mM EDTA as described in [14]. The RNA was blotted on to Hybond N (Amersham, Bucks, UK) according to the manufacturers instructions. Hybridisation was in

0.9 M NaCl, 0.09 M sodium citrate, pH 7.0, 50% formamide, 0.04% polvinylpyrrolidone, 0.04% Ficoll 400, 0.04% bovine serum albumin, 0.5% sodium dodecyl sulphate, 0.25 mg/ml heat-denatured herring sperm DNA and 10% dextran sulphate at 47°C. The blot was washed in 15 mM NaCl, 1.5 mM sodium citrate pH 7.0, 0.1% SDS at 65°C. The probe was an *Eco*RI/*Sac*I restriction fragment of the PP2B cDNA (nt−523 to 1,301) labelled to high specific activity using a random primed DNA labelling kit (Boehringer, Mannheim). To control for variation in loading of the poly(A)-rich RNA the same blot was hybridised with a 1.2 kb *Drosophila* PP1 87B cDNA [15].

*2.4. In situ hybridisation to polytene chromosomes*

Polytene chromosomes were isolated from the salivary glands of *D. melanogaster* Oregon R third instar larvae and fixed [17] with modifications as described in [18]. They were hybridised with the *Eco*RI/*Sac*I PP2B cDNA fragment (nt −523 to 1,301), labelled with biotin-16-dUTP using random priming (Boehringer Mannheim). The signal was detected by complexing with a streptavidin–alkaline phosphatase conjugate followed by colour development.

## 3. Results

### *3.1. cDNA sequence and chromosomal location*

Two clones in $10^5$ plaque forming units of a *Drosophila* eye disc cDNA library hybridised to the PCR fragment D27. One of these was purified to homogeneity and the insert contained three *Eco*RI fragments of 3.3 kb, 0.5 kb and 0.35 kb. Since the 3.3 kb DNA fragment hybridised to the D27 PCR fragment, it was subcloned into the Bluescript vector and sequenced. The DNA encoded a protein of 560 amino acids (Fig. 1). The 5′ noncoding region has stop codons in all reading frames preceding the proposed starting methionine and the 3′ noncoding region has additional stop codons following the stop codon at 1,681 in all reading frames. The predicted molecular mass of the encoded protein is 62 kDa, assuming that there are no posttranslational modifications. The deduced amino acid sequence contains all of the regions that are conserved among other members of the PP1/PP2A/PP2B family [19] and contains a putative calmodulin binding site [20] and a putative autoinhibitory domain [21] very similar to those in other PP2B catalytic subunits (Fig. 2). It shows 77% amino acid identity to the *Drosophila* PP2B encoded at locus 21EF [11], 78% identity to human PP2Bα [22], 73–75% to human PP2B$\beta_1$, PP2B$\beta_2$ [23] and PP2B$\beta_3$[24] and 76% to human PP2Bγ [29]. An 1.824 kb *Eco*RI/*Sac*I of the D27 3.3 kb cDNA encompassing the 5′ noncoding region hybridises in situ to polytene chromosomes at a single locus on the X-chromosome at cytological position 14D1-4 (Fig. 3)

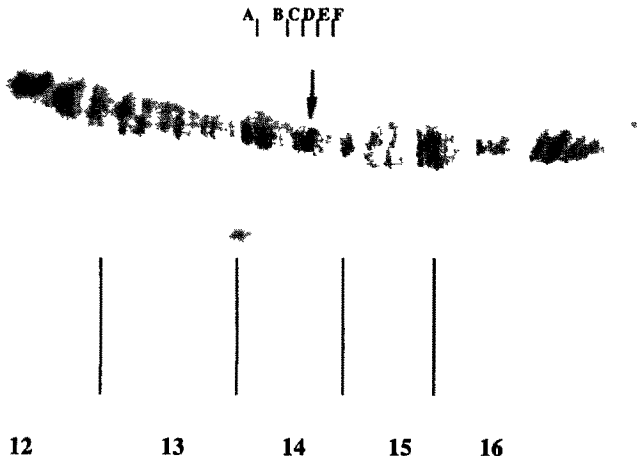


Fig. 3. Localisation of *Drosophila* PP2B by in situ hybridisation to salivary gland polytene chromosomes. The arrow indicates the site of hybridisation at 14D1–4 on the X chromosome.

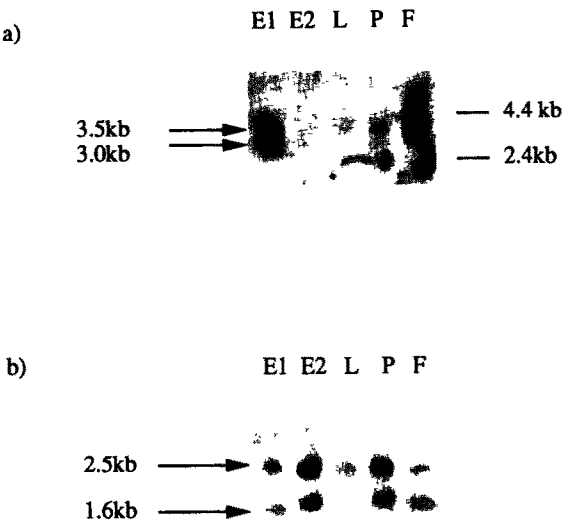


Fig. 4. Expression of protein phosphatase 2B 14D during the *Drosophila* life cycle. E1, 0–4 h embryo; E2 4–24 h embryo; L, larva; P, pupa; F, adult female; M, adult male.(a) Northern blot hybridised with an *Eco*RI/*Sac*I fragment of *Drosophila* PP2B 14D cDNA, comprising 523 nucleotides of 5′ noncoding region and the region encoding aminoacids 1–434. (b) The same blot stripped and rehybridised with a *Drosophila* PP1 87B cDNA, which is present at a constant proportion of the total poly(A)-rich RNA throughout development [14].

### *3.2. Expression of Drosophila $Ca^{2+}$/calmodulin regulated protein phosphatase gene located at 14D1–4*

The 1.824 kb *Eco*RI/*Sac*I of the *Drosophila* PP2B cDNA recognised two transcripts of 3.5 kb and 3.0 kb in the 0–4 h embryo under stringent hybridisation conditions (Fig. 4), similar to those that led to the hybridisation of the same probe to the single location at 14D1–4 on polytene chromosomes. The levels of these transcripts had declined in later (4–24 h) embryos and the smaller transcript was not visible at any later developmental stages. The larger transcript was clearly visible in adult females, but was present only at low levels in males and extremely low levels in larvae and pupae, indicating that the calcium/calmodulin regulated protein phosphatase encoded at 14D1–4 is mainly an embryonic form of PP2B.

## 4. Discussion

Three isoforms of the $Ca^{2+}$/calmodulin regulated

phosphatase have been identified in mammals PP2Bα and PP2Bβ in man [22–24], mouse [25,26] and rat [27,28], and a testis specific form, PP2Bγ, in man [29] and mouse [30]. There is as yet no evidence for an embryonic form of PP2B in mammals, although PP2Bβ was reported to be low in all adult tissues and was not examined in embryos [23]. From the studies presented here, *Drosophila* possess a form of PP2B encoded by a gene at chromosomal location 14D that is most highly expressed in the early embryo. *Drosophila* also possess at least two further isoforms of PP2B. The level of the transcript from the PP2B gene located at 21EF was undetectable on a Northern blot at all developmental stages and a third isoform is reported to be highest in adult tissues [11].

The diversity of structure of mammalian PP2B is increased by alternative splicing, which gives rise to α1, α2, α3 isoforms and β1, β2, β3 isoforms. Since the spliced forms differ in the region close to the autoinhibitory domain, they may be functionally distinct. The presence of two transcripts for PP2B 14D must result from alternative splicing of the mRNA, but it is not known whether this results in alteration of the protein structure. If it does then the smaller transcript encodes a embryonic specific PP2B, since it was absent from all other developmental stages. *Drosophila* PP2B 14D and PP2B 21EF show 73–78% and 77% amino acid identity to human PP2B, respectively, demonstrating that the PP2B structure has been highly conserved throughout evolution, although less so than PP1 and PP2A (which show 92–94% amino acid identity between *Drosophila* and mammals [15,31]). However, PP2B 14D is rather different from mammalian PP2B enzymes in that the N-terminus is longer by >56 amino acids and a ten amino acid deletion is present after residue 325, suggesting that these regions may serve a distinct roles (Fig. 2). In addition, amino acids 323 (Cys) and 398 (Ile) are Ser and Tyr, respectively in all other PP2B structures examined so far. Mapping the embryonic $Ca^{2+}$/calmodulin regulated phosphatase to 14D on the X-chromosome allows a genetic approach to analyse the function of embryonic PP2B in *Drosophila*.

*Acknowledgements:* We are grateful to Christopher Armstrong for the Northern blot shown in Fig. 3. The work was funded by the Medical Research Council, London. LB is the recipient of a Medical Research Council studentship.

## References

[1] Cohen, P. (1989) Annu. Rev. Biochem. 58, 453–508.
[2] Schreiber, S.L. (1992) Cell 70, 365–368.
[3] Jain, J., McCaffrey, P.G., Milner, Z., Kerppola, T.K., Lambert, J.N., Verdine, G L., Curran, T. and Rao, A. (1993) Nature 365, 352–355.
[4] Aperia, A., Ibarra, F., Svensson, L.-B., Klee, C. and Greengard, P. (1992) Proc. Natl. Aacd. Sci. USA 89, 7394–7397.
[5] Hendey, B., Klee, C.B. and Maxfield, F.R. (1992) Science 258, 296–299.
[6] Gaestel, M., Benndorf, R., Hayess, K., Priemer, E. and Engel, K. (1992) J. Biol. Chem. 267, 21607–21611.
[7] Cyert, M.S., Kunisawa, R., Kaim, D. and Thorner, J. (1991) Proc. Natl. Acad. Sci. USA 88, 7376–7380.
[8] Guerini, D. and Klee, C.B. (1991) Adv. Prot. Phosphatases 6, 391–410.
[9] Cohen, P.T.W., Brewis, N.D., Hughes, V. and Mann, D.J. (1990) FEBS Lett. 268, 355–359.
[10] Chen, M.X., Chen, Y.H. and Cohen, P.T.W. (1992) FEBS Lett. 306, 54–58.
[11] Guerini, D., Montell, C and Klee, C.B. (1992) J. Biol. Chem. 267, 2254–22549.
[12] Kalderon, D. and Rubin, G.M. (1989) J. Biol. Chem. 264, 10738–10748.
[13] Feinberg, A.P. and Vogelstein, B. (1984) Anal. Biochem. 137, 266–267.
[14] Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, 2nd edn., 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[15] Dombrádi, V., Axton, J.M., Glover, D.M. and Cohen, P.T.W. (1989) Eur. J. Biochem. 183, 603–610.
[16] Jacobson, A. (1987) Methods Enzymol. 152, 254–261.
[17] Ashburner, M., *Drosophila*: a Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[18] Dombrádi, V., Mann, D.J. Saunders, R.C. and Cohen, P.T.W. (1993) Eur. J. Biochem. 212, 177–183.
[19] Cohen, P.T.W (1991) Methods Enzymol. 201, 398–408.
[20] Kincaid, R.L., Nightingale, M.S. and Martin, B.M. (1988) Proc. Natl. Acad. Sci. USA 85, 8983–8987.
[21] Hashimoto, Y., Perrino, B.A. and Soderling, T.R. (1990) J. Biol. Chem. 265, 1924–1927.
[22] Muramatsu, T and Kincaid, R.L. (1993) Biochim. Biophys Acta 1178, 117–120.
[23] Guerini, D. and Klee, C.B. (1989) Proc. Natl. Acad. Sci. USA 86, 9183–9187
[24] McPartlin, A.E., Barker, H.M. and Cohen, P.T. (1991) Biochim. Biophys. Acta 1088, 308–10.
[25] Kincaid, R L., Rathna Giri, P., Higuchi, S., Tamura, J., Dixon, S.C., Marieta, C.A., Amorese, D A and Martin, B.M (1990) J. Biol. Chem. 265, 11312–11319
[26] Rathna Giri, P., Higuchi, S. and Kincaid, R.L. (1991) Biochim Biophys. Res. Comm. 181, 252–258.
[27] Ito, A., Hashimoto, T., Hirai, M., Takeda, T., Shuntoh, H., Kuno, T. and Tanaka, C. (1989) Biochem. Biophys. Res. Comm. 163, 1492–1497.
[28] Kuno, T., Takeda, T., Hirai, M., Ito, A., Mukai, H. and Tanaka, C. (1989) Biochem. Biophys. Res. Comm. 165, 1352–1358.
[29] Muramatsu, T. and Kincaid, R.L. (1992) Biochem. Biophys. Res. Comm. 188, 265–271.
[30] Muramatsu, T., Giri, P.R., Higuchi, S. and Kincaid, R.L. (1992) Proc. Natl. Acad. Sci. USA 89, 529–533.
[31] Orgad, S., Brewis, N.D., Alphey, L., Axton, J.M., Dudai, Y. and Cohen, P.T.W. (1990) FEBS lett. 275, 44–48.