

A Gene Located at 56F1-2 in *Drosophila melanogaster* Encodes a Novel Metazoan β -like Subunit of Casein Kinase II

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***Drosophila melanogaster* casein kinase II (DmCKII) is composed of catalytic α and regulatory β subunits associated as an $\alpha_2\beta_2$ heterotetramer. Using the two-hybrid system, we have screened a *Drosophila* embryo cDNA library for proteins that interact with DmCKII α . One of the cDNAs encodes a novel β -like polypeptide, which we designate β' . *In situ* hybridization localizes the corresponding gene to 56F1-2, a site distinct from that of both the β gene and the *Stellate* family of β -like sequences. The predicted sequence of β' is more closely related to the β subunit of *Drosophila* and other metazoans than to the *Stellate* family of proteins, suggesting that it is a second regulatory subunit. *In vitro* reconstitution studies show that a GST- β' fusion protein associates with the α subunit to generate a tetrameric complex with regulatory properties similar to those of the native $\alpha_2\beta_2$ holoenzyme. The data are consistent with the proposed role of the β' subunit as an integral component of the holoenzyme.**

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Casein kinase II (CKII) is a highly conserved, pleiotropic protein kinase that is ubiquitous in eukaryotes [1, 2]. The enzyme is essential for viability in fungi [3] and capable of functioning as an oncogene in mammals [4]. CKII from most sources is composed of catalytic α and regulatory β subunits that combine to form an $\alpha_2\beta_2$ holoenzyme. With the exception of *Drosophila melanogaster* [5], *Caenorhabditis elegans* [6], and *Schizosaccharomyces pombe* [7], most organisms contain two α polypeptides, α and α' , that are encoded by distinct genes. In contrast, β subunit heterogeneity has so far been reported only in *Saccharomyces cerevisiae* [8] and *Arabidopsis thaliana* [9].

Comparisons between expressed α subunit and native or reconstituted holoenzyme have revealed that the β subunit plays a complex role in regulating the basal catalytic activity of the α subunit [10–13]. On the one hand, the β subunit stabilizes the α subunit against proteolysis and thermal denaturation and stimulates its activity approximately 5-fold against most substrates; on the other, it negatively regulates phosphorylation of selected substrates, notably calmodulin [14, 15]. The β subunit also mediates stimulation of CKII by polybasic compounds such as polylysine and protamine. An internal acidic region within the β subunit (residues 55–64 of human β) is necessary for both down-regulation by the β subunit and activation by polycations [16]. The β subunit is also subject to autophosphorylation, catalyzed by α [17], and this reaction is also affected by mutations within the acidic region [18].

D. melanogaster is unusual in possessing tandemly repeated sequences that share homology with the CKII β subunit gene. These sequences are the *Stellate* (*Ste*) genes and the *Suppressor-of-Stellate* (*Su(Ste)*) or *crystal* (*cry*) genes. *Ste* repeats are located in two X-linked clusters (one at 12E1-2 and the other in heterochromatin) and encode polypeptides structurally related to the β subunit [19–21]; *Su(Ste)* repeats are located on the Y chromosome but appear to be pseudogenes [22]. Loss of *Su(Ste)* function (for example, in an XO male) results in testis-specific overexpression of *Ste*, accumulation of the *Ste* product as star- or needle-shaped crystals in the nuclei and cytoplasm of primary spermatocytes, and male sterility [20, 23]. These and related results suggest that a balance between *Ste* and *Su(Ste)* copy number is required for normal gametogenesis [20], possibly because of an effect of *Su(Ste)* on the processing and/or stability of *Ste* transcripts [19, 24]. Hurst [25] has proposed that *Ste* arose as a meiotic driver, though this proposal and the underlying mechanism remain controversial [26, 27]. Purified *Stellate* protein ex-

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pressed in *E. coli* interacts weakly with the α subunit *in vitro* and stimulates its activity against a peptide substrate [23], but the biochemical role, if any, of the Stellate protein *in vivo* remains to be defined.

Recently, a single-copy homolog of the CKII β subunit gene has also been identified in *D. melanogaster*. This gene is more closely related to *Ste* and *Su(Ste)* than it is to the β subunit gene itself and has been named *Suppressor-of-Stellate-Like (SSL)* [28]. The gene is located at 60D1-2 on the second chromosome (the β gene itself is located at 10E1-2 on the X chromosome; C. Glover, unpublished). Kalmykova *et al.* [28] propose that SSL arose from the β subunit gene, possibly with the involvement of reverse-transcriptase, and then gave rise to both the *Ste* and *Su(Ste)* clusters. *SSL* expression is also restricted to the adult testis but does not exhibit the XO-dependence observed with *Ste*. Although the predicted SSL protein shares most of the conserved features present in bona fide β subunits, the biochemical function of the protein has not yet been analyzed *in vitro*.

In an attempt to better define the physiological role of CKII, we have used the two-hybrid approach [29] to identify and characterize its physiological partners. We describe here the isolation of a novel β -like subunit, β' , not previously identified in any metazoan organism. β' is not a product of the previously described β gene [30] but is encoded by a distinct gene located at 56F1-2 on the polytene map. *In vitro* reconstitution and biochemical studies indicate that a GST- β' fusion protein can associate with the α subunit to generate a tetrameric complex with regulatory properties similar to those of native $\alpha_2\beta_2$ holoenzyme. The data are consistent with the proposed role of the β' subunit as an integral component of the holoenzyme.

MATERIALS AND METHODS

Construction of two-hybrid plasmids. All manipulations involved in construction of the two-hybrid plasmids were carried out as described [31]. DmCKII α and β were amplified by PCR using primers containing two terminal 5' bases, a restriction site, and 20 bases of exact homology. The PCR products were subcloned into the plasmid, pGBT9 (gift of S. Fields, now available from Clontech). The resulting plasmids express either Dm α or Dm β as C-terminal fusions with the DNA-binding (DB) domain (amino acids 1–147) of *S. cerevisiae* Gal4. The inserts were sequenced using the Prism Dye Terminator Cycle sequencing kit and custom primers (Applied Biosystems). Subsequently, inserts were subcloned into plasmid pGAD424 (Clontech), where proteins are expressed as C-terminal fusions with the transactivation (TA) domain (amino acids 768–881) of Gal4.

Two-hybrid interactions of CKII subunits. Yeast strain HF7C [32] was transformed with plasmids ex-

pressing CKII subunits as fusions with GAL4DB and GAL4TA in every possible pairwise combination. Transformants were tested for induction of *HIS3* on dropout medium lacking His [31] at 29°C for 4 days. In parallel, cultures were analyzed for the induction of *lacZ* [33].

Yeast two-hybrid screening and β -galactosidase assays. Yeast strain HF7C expressing GAL4DB-Dm α was used to screen a 3- to 12-h *Drosophila* embryo cDNA library (gift from S. J. Elledge) according to the Matchmaker Two-Hybrid System protocol (Clontech). A total of 2×10^6 transformants were plated on medium lacking Trp, Leu, and His [31]; and colonies exhibiting rapid growth were rescreened for expression of *lacZ* [33]. Of the 45 His⁺ colonies, 15 tested positive for *lacZ* and were therefore chosen for further analysis. Plasmids expressing GAL4TA-cDNA fusions were recovered and retransformed into HF7C alone and into HF7C expressing GAL4DB-Dm α or GAL4DB-Dm β . Those cDNAs which induced expression of the reporter genes (*HIS3* and *lacZ*) in a bait-specific manner were identified by sequencing across the GAL4TA-cDNA junction using a GAL4-specific primer. Two clones, DmA-15 and DmA-16, encoded Dm β' and were completely sequenced as described above.

In situ hybridization to polytene chromosomes. Salivary glands from third instar *D. melanogaster* larvae were isolated, dissected, and prepared for hybridization essentially as described [34]. The cDNA from clone DmA-15 was labeled by nick translation using biotinylated dUTP (Boehringer-Mannheim). Probe binding was visualized using streptavidin coupled to alkaline phosphatase, and slides were photographed at 400 \times magnification on Kodak Tmax-100 film.

Expression of GST-fusion proteins. The full-length β' cDNA was isolated by digestion of plasmid DmA-16 with *Bgl*III and subcloned into the BamHI site of the *E. coli* expression plasmid, pZEX (gift of Z. Paroush and D. Ish-Horowicz). pZEX has been modified to accommodate the multiple cloning site and reading frame of the library-encoded cDNAs, and proteins are expressed as C-terminal fusions with *Schistosoma japonicum* glutathione S-transferase (GST). Plasmids expressing GST-Dm β' were transformed into *E. coli* BL21(DE3) harboring the plasmid pTRX (gift of S. Ishi). pTRX drives expression of thioredoxin which increases the solubility and functionality of eukaryotic proteins expressed in *E. coli* [35]. Cultures (100 ml) were grown in 2 \times YTA [31] containing 150 μ g/ml ampicillin and 15 μ g/ml chloramphenicol to an OD₆₀₀ of 0.7 and induced with 1 mM isopropyl- β -D-thiogalactoside for 4 h at 22°C with vigorous shaking. All subsequent steps were conducted at 4°C. Cultures were harvested, resuspended in 10 ml phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.1% 2-mercaptoethanol, and lysed by sonication. Triton

X-100 was added to a final concentration of 1% and mixed for 1 h at 4°C. Insoluble material was removed by centrifugation, and the supernatant was passed twice through a column containing 1 ml of glutathione-Sepharose 4B (Pharmacia). The column was washed with 10 bed volumes of PBS, and bound protein was eluted with 5 ml of 100 mM reduced glutathione in 50 mM Tris, pH 8.0. The eluted protein was concentrated and exchanged into storage buffer (50 mM Tris, pH 8.0, 0.5 mM EDTA, 10% glycerol, 200 mM NaCl, 1 mM PMSF) using a Centricon apparatus (Amicon). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the concentration and purity were estimated by densitometry of Coomassie blue-stained gels relative to known standards.

Association of GST-Dm β' with Dm α . Monomeric Dm α was purified from a yeast expression system as previously reported [10]. Aliquots corresponding to 10 μ g of Dm α were mixed with purified GST or GST-Dm β' and incubated overnight at 4°C in storage buffer. Subsequently the entire mixture was loaded onto preformed 10-30% linear glycerol gradients and centrifuged at 38,000 rpm for 60 h in a SW41 rotor (Beckman). Aliquots of each gradient fraction were assayed for CKII activity using casein as a substrate [5], and the position of polypeptides within the gradient was determined by SDS-PAGE followed by silver-staining. Carbonic anhydrase, bovine serum albumin, and amylase were used as sedimentation standards. The migration of the latter proteins was determined by analyzing gradient fractions by SDS-PAGE followed by staining with Coomassie blue.

Phosphorylation of calmodulin. Aliquots of gradient fractions containing equivalent amounts of catalytic subunit either as monomeric α subunit or as α_2 (GST-Dm β')₂ holoenzyme were used to phosphorylate calmodulin essentially as described [14]. Samples were separated by SDS-PAGE and stained with Coomassie blue, and the radioactivity incorporated in calmodulin was determined by phosphorimaging (Molecular Dynamics).

RESULTS

Interaction of CKII subunits. We first sought to establish that DmCKII α and β subunits expressed as fusions with either GAL4DB or GAL4TA interact in the yeast two-hybrid system. As expected, transformants expressing GAL4DB-Dm α/β or GAL4TA-Dm α/β alone did not induce expression of the reporters *HIS3* and *lacZ* (Fig. 1). However, coexpressing GAL4DB-Dm α and GAL4TA-Dm β resulted in the induction of both reporters, suggesting that DmCKII α and β interact in the two-hybrid assay, presumably reflecting formation of the $\alpha_2\beta_2$ holoenzyme. Identical results were observed in the reverse orientation, i.e., GAL4DB-Dm β

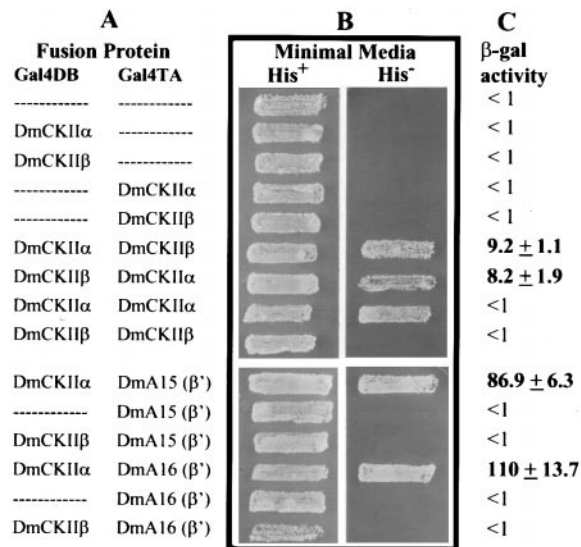


FIG. 1. Subunit interactions of DmCKII and isolation of Dm β' . (A) *S. cerevisiae* strain HF7C was transformed with plasmids expressing the indicated fusions between the DmCKII α or β subunits and GAL4DB or GAL4TA. Untransformed HF7C was used as a control (a dash indicates the absence of a plasmid). Following growth in minimal (Leu⁻, Trp⁻) medium, induction of the two-hybrid reporter genes, *HIS3* and *lacZ*, was assayed as described under Materials and Methods. (B) Growth on His⁺ and His⁻ medium for 4 days at 29°C. (C) Induction of *lacZ*, assayed as β -galactosidase activity. Activity is expressed in Miller Units, and the mean and standard deviation of three replicate assays is shown.

and GAL4TA-Dm α , indicating that this interaction is not Gal4 domain-dependent. These data are in agreement with two-hybrid analysis of human CKII [36–38].

Transformants coexpressing GAL4DB-Dm α and GAL4TA-Dm α exhibited growth in the absence of His, and although the levels of *lacZ* were undetectable in the liquid assay (Fig. 1), a weak blue color was observed in the filter assay (data not shown). While the interaction is clearly weaker than the α - β interaction, the isolation of Dm α in a random screen with Dm α as bait (see below), confirms that the interaction is real. At face value these data indicate either formation of a Dm α dimer, a result at odds with two-hybrid analysis of human CKII α [36–38], or a bridging interaction involving the endogenous yeast β subunits. Consistent with the latter possibility, Dm α exhibits a two-hybrid interaction with either β subunit of *S. cerevisiae* CKII when tested explicitly (unpublished results).

Surprisingly, interaction of Dm β with itself could not be detected with either reporter (Fig. 1) or with the β -galactosidase filter assay (data not shown). This result contrasts with two-hybrid results using human CKII β that suggest the formation of a β - β dimer [36–38]. We have previously observed that expression of Dm β in *S. cerevisiae* does not lead to the accumulation of detectable immunoreactive material unless the protein is co-expressed with Dm α [10]. Thus, our inability

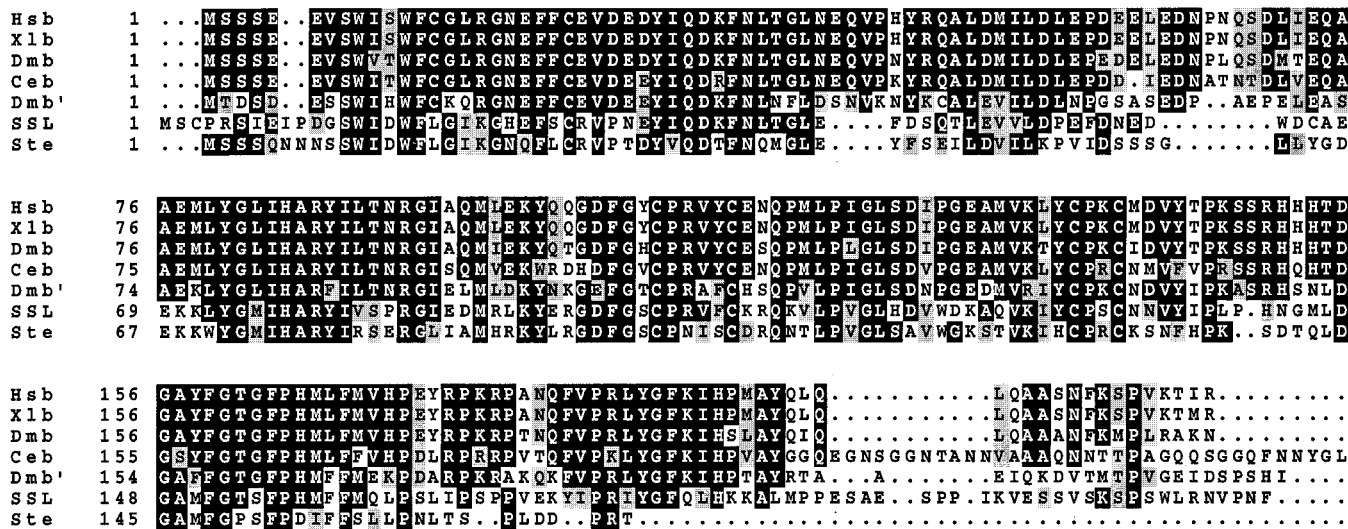


FIG. 2. Alignment of animal CKII β subunits and the β -like polypeptides encoded by *D. melanogaster* *Stellate* and *SSL*. Sequences shown and their GenBank accession numbers are *Homo sapiens* β (X16312), *Xenopus laevis* β (X62376), *Caenorhabditis elegans* β (M73827), *D. melanogaster* β (M16535), *D. melanogaster* β' (U51209), *D. melanogaster* *Stellate* (X15899), and *D. melanogaster* *SSL* (L49382). Bovine, rat, and chicken β sequences are not shown explicitly, as they are identical to human β . Dashes indicate gaps introduced to maintain alignment. Identical residues are highlighted in black, and conservative replacements are shaded.

to detect a β - β interaction may reflect the instability of *Dm* β in yeast.

The analysis of the interactions among the *Dm*CKII subunits confirms that the fusion proteins are expressed in a functional form and establishes which subunits are potentially detectable in a random screen employing *Dm* α .

Isolation of cDNAs encoding Dm β' . The yeast strain HF7C expressing GAL4DB-*Dm* α was used to screen a 3- to 12-h *D. melanogaster* embryo cDNA library. From approximately 1×10^6 transformants, we recovered 15 clones that activated transcription of *HIS3* and *lacZ*. All 15 clones induced the two reporter genes only when co-transformed with *Dm* α (data not shown). Sequencing of the GAL4TA-cDNA fusion junctions revealed that 7 of the clones encode *Dm* β , 1 encodes *Dm* α , 2 (*Dm*A-15 and *Dm*A-16) encode a *Dm* β -like subunit which we call *Dm* β' (see below), and the rest encode novel proteins that will be described elsewhere.

Dm β' interacts strongly with *Dm* α but not with *Dm* β (Fig. 1), demonstrating that this interaction is specific for the α subunit and corroborating our inability to detect β - β interactions (see above). Furthermore, neither reporter was expressed using GAL4TA-*Dm* β' alone. The levels of *lacZ* induction observed with *Dm* α + *Dm* β' are approximately 10-fold higher than those observed with *Dm* α + *Dm* β (Fig. 1). Whether this reflects a stronger interaction of *Dm* α with *Dm* β' than with *Dm* β cannot be determined from the available data since the stability of the fusion proteins and other factors may influence expression of the reporters used in the two-hybrid assay.

Sequencing of the two β' -encoding cDNAs, *Dm*A-15 and *Dm*A-16, revealed that the latter contains an apparently full-length protein coding region. This cDNA (GenBank Accession No. U51209) is 763 bp long and contains an open reading frame of 660 bp. The putative initiation codon is located within the context AGC-TATGA, which is a reasonable match to the start-site consensus for *D. melanogaster* [39]. The absence of a poly-A tail as well as a poly-A addition signal suggests that the cDNA is truncated within the 3' untranslated region. Conceptual translation of the open reading frame yields a predicted polypeptide of 219 amino acids with a calculated molecular mass of 24,966 Da, a value similar to that of *Dm* β (24,700 Da) [30].

Relationship to other β subunit sequences. An alignment of *Dm* β' with metazoan β subunits and the β -like polypeptides encoded by the *D. melanogaster* *Ste* and *SSL* genes is shown in Fig. 2. *Dm* β' appears to contain all of the major features conserved among β subunits of other organisms, including the N-terminal autophosphorylation site, the internal acidic region, and the invariant sequence CPxxC-x₂₂-CPxC, proposed to represent a metal-binding motif [40]. However, not all of these features are perfectly conserved. The autophosphorylation site of β' differs at several positions from that of metazoan β subunits and in particular contains a Thr in place of Ser at position 2. Autophosphorylation of human CKII results in the phosphorylation of the β subunit at Ser2 [17], and its replacement by Thr in *Dm* β' would not be expected to affect this reaction. While 7/10 residues in the acidic domain (residues Asp55-Asp64 of the human sequence, see Fig. 2) are Asp/Glu in mammalian and *Drosophila* β , only 3/10

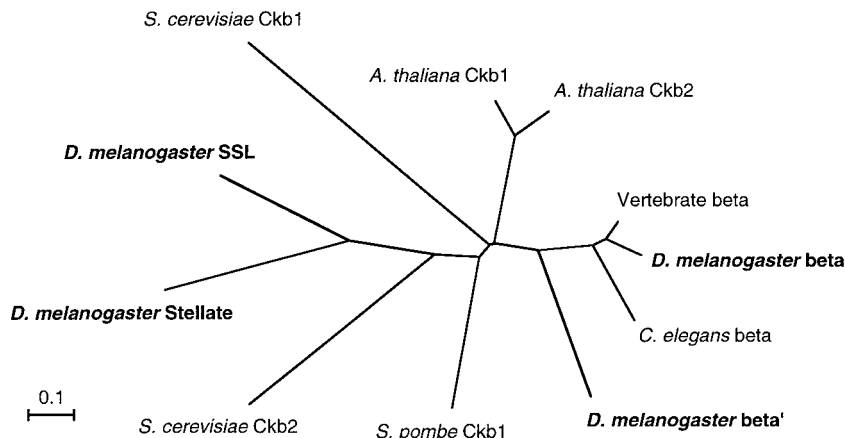


FIG. 3. Unrooted phylogenetic tree of the CKII β subunit. The deduced amino acid sequences of all available β subunits and β -like polypeptides were aligned using the PILEUP program of the GCG suite (Genetics Computer Group, Madison, WI) with a gap weight of 3.0 and a gap length weight of 0.1. The program DISTANCES was used to calculate phylogenetic distances by the method of Kimura, and GROWTREE was used to construct an unrooted phylogenetic tree via the neighbor-joining method. The scale bar indicates 0.1 amino acid replacements per site.

are acidic in Dm β' , 5–6 (depending upon the alignment) in SSL, and 1 in Stellate.

Pairwise comparisons among the sequences aligned in Fig. 2 indicate that Dm β' is less closely related to metazoan β subunits than is Dm β . For example, Dm β' is only 59% identical to human β , whereas Dm β is 88% identical. Nevertheless, among the β -related sequences in *Drosophila*, Dm β is clearly the closest relative. Dm β' is 60% identical to Dm β but only 46 and 39% identical to SSL and Stellate, respectively (Stellate and SSL are themselves 53% identical). To analyze more rigorously the position of Dm β' within the β family, we used a neighbor-joining algorithm to construct an unrooted phylogenetic tree of all available β sequences (Fig. 3). The analysis reveals that Dm β' is most closely related to the β subunit of metazoan species. In contrast, Stellate and SSL are located on a distinct and distantly related branch of the β tree.

In situ hybridization to polytene chromosomes. The cytological location of the Dm β' gene was determined by *in situ* hybridization to polytene chromosomes. Using the DmA-15 cDNA as a probe, the β' gene was localized to 56F1-2 on the second chromosome (Fig. 4). This site of hybridization is distinct from those of the β gene (10E1-2), SSL (60D1-2), and the X- and Y-linked *Ste* and *Su(Ste)* arrays. This result confirms that the β' isoform is encoded by a unique gene. No additional sites of hybridization were observed for Dm β' .

Association of Dm α and GST-Dm β' . It has been previously shown that subunits of CKII expressed and purified from *E. coli* associate to form an $\alpha_2\beta_2$ holoenzyme with properties similar to those of the native holoenzyme [13]. To determine whether Dm β' is competent to associate with the α subunit to form an $\alpha_2\beta'_2$ holoenzyme, the β' subunit was expressed as a GST-

fusion protein and purified by affinity chromatography on glutathione-agarose. The purified fusion protein (or GST as a control) was then incubated with purified Dm α , and potential complexes were detected by glycerol gradient centrifugation. As shown in Fig. 4, Dm α and GST yielded a single peak of kinase activity with a mobility equal to that of free α subunit, indicating the absence of an interaction between the α subunit and GST. In contrast, a mixture containing Dm α and GST-Dm β' yielded two peaks of kinase activity, the first corresponding to free α subunit and the second having an apparent molecular mass of 180,000 Da. The latter size is consistent with the formation of an $\alpha_2(\text{GST-}\beta')_2$ holoenzyme (predicted molecular mass, 200,000 Da). That the second peak of activity reflects formation of a complex of Dm α and GST-Dm β' was confirmed by SDS-PAGE analysis of gradient fractions (data not shown). We estimate that approximately 40% of the available α subunit was converted to holoenzyme in this experiment. Given that Dm α and GST-Dm β' were added in approximately stoichiometric amounts at relatively low concentration (<100 $\mu\text{g/ml}$), formation of a stable tetrameric complex appears to be relatively efficient under the conditions employed.

Phosphorylation of calmodulin. We and others have shown that the β subunit inhibits the ability of the α subunit to phosphorylate calmodulin and that this inhibition can be overcome by the polybasic activators, polylysine and protamine, but not spermine [14, 15]. We asked whether the presence of the β' subunit in the $\alpha_2(\text{GST-}\beta')_2$ holoenzyme similarly inhibits phosphorylation of calmodulin. As shown in Fig. 5B, calmodulin is weakly phosphorylated in the presence of free α subunit (lane 5), as reported previously, but the activity of an identical amount of α subunit present as

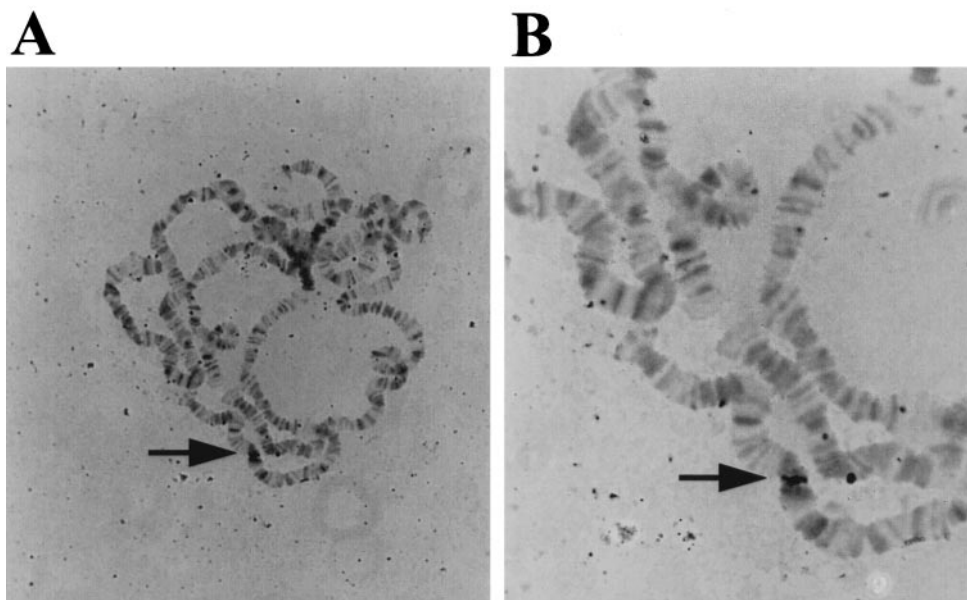


FIG. 4. *In situ* hybridization to polytene chromosomes. The DmA-15 cDNA encoding Dm β' was used to determine the cytological location of the Dm β' gene on the polytene chromosome map. The probe was labeled with biotin-dUTP, hybridized to polytene chromosomes of third instar salivary glands, and visualized with streptavidin-coupled alkaline phosphatase. (A) *In situ* hybridization to a complete chromosome set; (B) enlargement of the area of hybridization shown in A. The site of hybridization is indicated by an arrow.

tetrameric complexes with GST-Dm β' is significantly inhibited against this substrate (lane 9). Furthermore, the addition of polylysine (lane 11) and protamine (lane 12) but not spermine (lane 10) overcomes the inhibition

of calmodulin phosphorylation and in fact results in a dramatic stimulation of activity, relative to that of free α subunit. All of this behavior closely parallels that of $\alpha_2\beta_2$ holoenzyme [14]. No phosphorylation of calmodu-

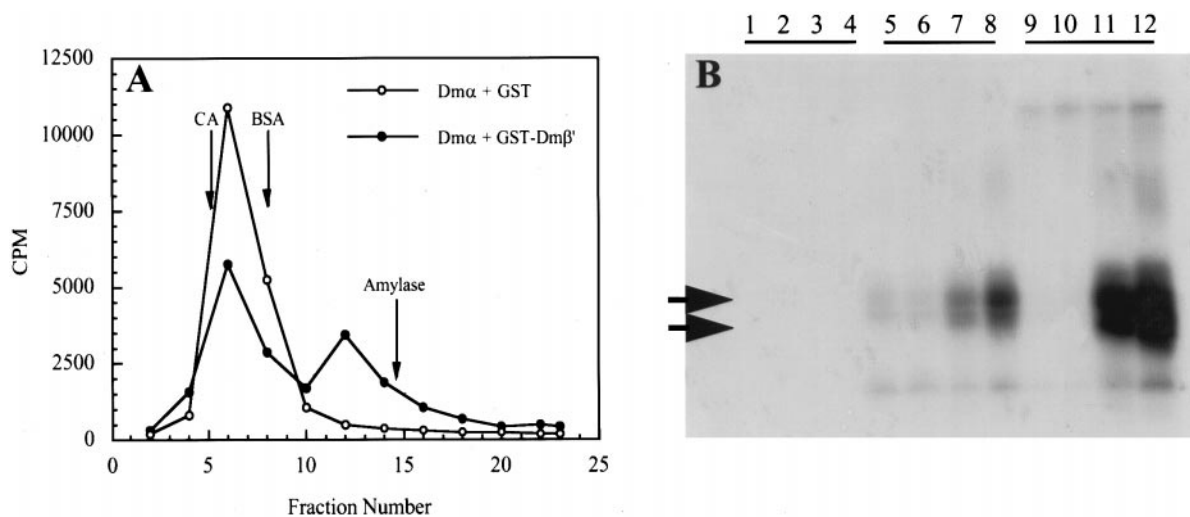


FIG. 5. Purification of $\alpha_2(\text{GST-Dm}\beta')$ holoenzyme and phosphorylation of calmodulin. (A) GST-Dm β' was expressed in *E. coli* and purified as described under Materials and Methods. Purified Dm α and GST or GST-Dm β' were allowed to reassociate, and the mixtures were resolved by sedimentation in glycerol gradients. Gradient fractions were assayed for kinase activity using casein as a substrate. The sedimentation positions of the protein standards, carbonic anhydrase (CA), bovine serum albumin (BSA), and amylase, are indicated. (B) Fractions 6 (Dm α) and 12 (holoenzyme) from the gradient shown in panel A were assayed for kinase activity against calmodulin in the presence or absence of basic effectors. Samples were resolved by SDS-PAGE, and radioactivity was visualized by phosphorimaging. Lanes 1-4, no enzyme control; lanes 5-8, Dm α ; lanes 9 and 10, $\alpha_2(\text{GST-Dm}\beta')$ holoenzyme. Samples were phosphorylated in the absence of any effector (lanes 1, 5, and 9) or in the presence of 100 $\mu\text{g/ml}$ spermine (lanes 2, 6, and 10) or 100 $\mu\text{g/ml}$ poly-(D,L)-lysine (lanes 3, 7, and 11), or 125 $\mu\text{g/ml}$ protamine (lanes 4, 8, and 12). The position of calmodulin, which runs as a doublet, is indicated by the arrows.

lin was observed in the absence of CKII (lanes 1–4), and spermine, polylysine, and protamine had only modest effects on free α (lanes 6–8). The weak stimulation of free α by polylysine (lane 7) and protamine (lane 8) is consistent with earlier results and may reflect direct interaction with the α subunit and/or interactions with the substrate [14, 15]. Collectively, these studies demonstrate that Dm β' is capable of associating with the α subunit *in vitro* and regulating its activity, both positively and negatively.

DISCUSSION

Using the yeast two-hybrid system, we have isolated cDNAs encoding a new β -related polypeptide in *Drosophila melanogaster*. Several lines of evidence suggest that this polypeptide represents a novel β' subunit of CKII rather than a component of the *Drosophila* Stellate/SSL complex. First, sequence comparisons and phylogenetic analysis indicate that Dm β' is most closely related to Dm β and other metazoan β subunits and only distantly related to Stellate or SSL. Second, Dm β' contains the essential structural features of a β subunit, including the autophosphorylation site, acidic region, and potential metal-binding motif. Third, Dm β' associates efficiently with Dm α *in vitro* to form a tetrameric holoenzyme. The strong interaction between Dm α and Dm β' in the two-hybrid system suggests that these proteins interact *in vivo* as well. Fourth, Dm β' silences the ability of the α subunit to phosphorylate calmodulin and mediates the activating effects of polybasic effectors such as polylysine. Fifth, cDNA clones encoding Dm β' are readily isolated from a 3- to 12-h embryo cDNA library, implying that Dm β' is significantly expressed during this developmental stage. In contrast, Stellate and SSL are more distantly related to true β subunits. In addition, Stellate interacts only weakly with α *in vitro*, and the complex does not respond to polylysine [23]. The latter is consistent with the absence of an acidic region in the Stellate polypeptide. Perhaps most importantly, the expression of the entire Stellate-related family, *Ste*, *Su(Ste)*, and *SSL*, appears to be confined to the testis of adult males [19, 28].

Biochemical data collected thus far suggest that Dm β and β' are functionally similar. In particular, although the acidic region of Dm β' has a significantly reduced negative charge density relative to β , our *in vitro* results show that Dm β' can both negatively regulate calmodulin phosphorylation and mediate activation by polylysine. Nevertheless, it is possible that Dm β' possesses subtle functional differences relative to Dm β that were not detected in our assays. Additional biochemical studies will be required to address this issue.

The spatial and temporal expression patterns of the Dm β and β' subunits remain to be defined. CKII has

been purified to homogeneity from 6- to 18-h embryos and is very abundant at this stage [5], but the β subunit(s) present in this material is N-terminally blocked and has not been sequenced. As noted above, the β' subunit appears to be expressed in embryos, and similar logic applies to the β subunit as well [30]; this report). If expression of the two isoforms occurs in the same embryonic cell type(s), the possibility of $\alpha_2\beta\beta'$ tetramers in addition to $\alpha_2\beta_2$ and $\alpha_2\beta'_2$ tetramers will need to be considered, analogous to the $\alpha_2\beta_2$, $\alpha'_2\beta_2$ and $\alpha\alpha'\beta_2$ tetramers already documented for mammalian CKII [37, 41]. Such heterotetramers may have unique regulatory properties. Northern analysis, *in situ* hybridization, and immunolocalization will be required to define the temporal and spatial expression of both isoforms.

Three species are now known to contain two β subunit genes: *S. cerevisiae*, *A. thaliana*, and *D. melanogaster*, representing fungi, plants, and animals, respectively. The phylogenetic analysis shown in Fig. 3 provides no evidence that the β and β' sequences constitute distinct orthologous families, but rather suggests that independent gene duplication events have occurred in different lines [42]. Nevertheless, the position of Dm β' in the tree shown in Fig. 3 raises the possibility that an orthologous β' gene may exist among metazoan species. Given the sequence divergence between Dm β and β' , it is possible that the probes and primers typically used to isolate β subunit genes might not have detected such a β' gene.

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