Both Synchronous and Asynchronous Muscle Isoforms of Projectin (the Drosophila Bent Locus Product) Contain Functional Kinase Domains

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Abstract. In Drosophila, the large muscle protein, projectin, has very different localizations in synchronous and asynchronous muscles, suggesting that projectin has different functions in different muscle types. The multiple projectin isoforms are encoded by a single gene; however they differ significantly in size (as detected by gel mobility) and show differences in some peptide fragments, presumably indicating alternative splicing or termination. We now report additional sequence of the projectin gene, showing a kinase domain and flanking regions highly similar to equivalent regions of twitchin, including a possible autoinhibitory region. In spite of apparent differences in function, all isoforms of projectin have the kinase domain and all are capable of autophosphorylation in vitro. The projectin gene is in polytene region 102C/D where the bent° phenotype maps. The recessive lethality of bent° is associated with a breakpoint that removes sequence of the projectin kinase domain. We find that different alleles of the highly mutable recessive lethal complementation group, l(4)2, also have defects in different parts of the projectin sequence, both NH2-terminal and COOH-terminal to the bent° breakpoint. These alleles are therefore renamed as alleles of the bent locus. Adults heterozygous for projectin mutations show little, if any, effect of one defective gene copy, but homozygosity for any of the defects is lethal. The times of death can vary with allele. Some alleles kill the embryos, others are larval lethal. These molecular studies begin to explain why genetic studies suggested that l(4)2 was a complex (or pseudoallelic) locus.

In the past five years a new family of intracellular muscle proteins has been recognized (Benian et al., 1989). The family, which now contains about a dozen proteins, has two distinguishing features. All members, except one (Lakey et al., 1993), interact in some way with the myosin thick filament and all members contain two types of motifs, each 90–100-amino acids long. Both motifs are also found in cell-surface proteins. Motif I resembles fibronectin type III domains, while motif II resembles the immunoglobulin superfamily C2 motif (Benian et al., 1989). In each organism, one member of this new muscle protein family is significantly larger than the others. These giant proteins appear to make up a specific subfamily. In vertebrate muscle, the giant protein is called titin or connectin (Wang et al., 1979; Maruyama et al., 1981; Trinick et al., 1984). In the nematode, Caenorhabditis elegans, it is twitchin (Waterston et al., 1980; Moerman et al., 1988; Benian et al., 1989), and in both honeybee and Drosophila, it is projectin (Saide, 1981; Ayme-Southgate et al., 1991). Similar proteins, referred to as mini-titins, have also been characterized in other invertebrates (Nave and Weber, 1990; Lakey et al., 1990; Vibert et al., 1993).

While the giant proteins form a subfamily on the basis of size, they appear less homogeneous in function. It has been suggested that twitchin may have a role in regulating myosin activity. The suggestion is supported by strong genetic evidence. Mutants in the gene for twitchin (the unc-22 gene) cannot develop or sustain contraction (Waterston et al., 1980). Some also show disrupted myofibrils, though it is unclear at this time whether it is the assembly or the maintenance of the myofibrils which is disrupted. Twitchin colocalizes with myosin and mutations in twitchin can be suppressed by mutations in the myosin heavy chain (Moerman et al., 1982). In addition, the DNA sequence of the twitchin gene contains a region encoding the catalytic domain of a protein kinase resembling that of chicken smooth muscle myosin light chain kinase (csmMLCK; Benian et al., 1989). A bacterially expressed portion of twitchin re-
cently demonstrated, in vitro, a kinase activity, both towards an exogenous model substrate and in autophosphorylation (Lei et al., 1994).

Studies of titin have suggested that the function of this protein may be rather different from the function of twitchin. Titin forms a third set of muscle filaments, running parallel to the myosin and actin filaments and extending from the Z line to the M line (Wang et al., 1984; Forst et al., 1988; Funatsu et al., 1993). It has been suggested that the titin filaments may be responsible for the passive elasticity of muscle (Horovitz et al., 1986, 1989), for centering the A band and for regulating the length of the thick filaments (Whiting et al., 1989; Labeit et al., 1992). During myofibrillogenesis, titin has a pattern of expression suggesting a role in integrating the assembly of thick and thin filaments (Fulton and Isaac, 1991). Despite the apparent differences in function, titin resembles twitchin in that the titin gene also contains sequence that encodes a protein kinase domain (Labeit et al., 1992). Titin is phosphorylated in vivo (Somerville and Wang, 1988), however, no kinase activity has yet been demonstrated for this protein.

While the studies of twitchin and titin indicate that different members of this subfamily of giant proteins may have different functions, our studies of the Drosophila protein, projectin, suggest that different isoforms encoded by the same gene may have different functions. This suggestion comes from studies of the localization of projectin in different muscle types. In the highly specialized indirect flight muscles (IFM), antibody staining shows a sarcomeric distribution of projectin that is almost the reciprocal of the distribution seen in other Drosophila muscles. In the IFM, projectin is detected in the I band; in other muscles, anti-projectin antibodies stain only the A band (Vigoreaux et al., 1991).

The IFM are powerful muscles adapted for the rapid retraction of the wings in the haptonomic walk and the formation of the fruiting body. Analyses of the available alleles of the 1(4)2 chromosome region has been performed using the Laemmli system (Laemmli, 1970) in either a 4% or a 3-10% gradient SDS-polyacrylamide gel. The DNA sequence was determined using the chain termination protocol (Sanger et al., 1977). The templates were either single stranded m13 clones or double stranded pGEM subclones.

Alignment between projectin, twitchin, and csmMLCK sequences was performed manually. The database search for identities to the projectin unique regions was carried out using the BLAST program (Altschul et al., 1990). The very different sarcomeric localizations of projectin strongly suggest that the role of projectin in asynchronous muscles differs from its role in synchronous muscles. It has been proposed that in the IFM, where projectin is found in filaments connecting the thick filaments with the Z disk, projectin has a structural role that might include facilitating stretch activation and participating in the specialized domain of transduction of tension to the myofibrils (Jewell and Ruegg, 1966; Wray, 1979; Squire, 1992). Because they lack a 1:1 relationship between nerve stimulation and contraction, the IFM are referred to as asynchronous muscles. The other Drosophila muscles, like most striated muscles in other organisms, undergo one contraction per nerve impulse and are classified as synchronous muscles (reviewed in Crossley, 1978).

Protein Isolation

Muscle extracts were prepared from various adult body parts as well as larval body wall, as previously described (Vigoreaux et al., 1991). IFM myofibrils were isolated from 0.5 g of adult Drosophila flies, as previously reported (Saide, 1981). IFM dissection from acetone-dried flies was as described (Mogami et al., 1982). Protein electrophoresis was carried out using the Laemmli system (Laemmli, 1970) in either a 4% or a 3-10% gradient SDS-polyacrylamide gel.

Immunoprecipitation

Approximately 3 mg of honeybee leg muscles were frozen in liquid N2 and lyophilized. They were then homogenized in modified Hasselbach-Schneider (HS) solution (1 M KCl, 1 mM MgCl2, 10 mM Na Pyrophosphate, 20 mM Na Phosphate, pH 6.5) containing various protease inhibitors. The extract was centrifuged at 4000 g for 20 min and the supernatant passed through a 0.45-μm pore size filter. The filtrate was mixed with 20 μl of ascites fluids containing either anti-projectin monoclonal antibodies (P and P3) or a control monoclonal antibody of unknown specificity. The antigen-antibody complex was ultimately precipitated with Sepharose 4B beads coupled with anti-mouse IgG (Organon Teknika Corp., Malvern, PA).
**Autophosphorylation, Kinase Assay, and Western Blotting**

The autophosphorylation assay was conducted as previously described (Cave and Carlson, 1989) except for the final washes. Instead of the 30 mM HEPES washes, the filters were processed directly from the γ32P-ATP incubation to the Western blot protocol (Towbin et al., 1979). The TBST (1× TBST: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) was resuspended in kinase assay buffer (50 mM NaCl, 5 mM MgCl2, 10 mM Tris, pH 7.4) and incubated in the presence of 25 μCi of γ32P-ATP. After incubation at room temperature for 15 min, the reaction was terminated, analyzed by polyacrylamide gel electrophoresis using a 12–20% gradient gel and autoradiographed.

Western blot identification of projectin used various previously described monoclonal antibodies against projectin (Saïde et al., 1989) as well as a polyclonal antibody directed against the kinase domain of twitchin. The filter was later reprobed with monoclonal antibodies against α-actinin and myosin heavy chain. The secondary antibodies used (anti-mouse or anti-rabbit) were coupled with alkaline phosphatase and the enzyme activity detected using NBT and BCIP substrates (Promega Corp., Madison, WI). In later stages, the enhanced chemiluminescence system (ECL; Amersham Corp., Arlington Heights, IL) was also used.

**Genetic Analysis**

All the mutant stocks described in this study were obtained from the Drosophila Stock Centers (Bowing Green, OH and Bloomington, IN). All mutations were described in Lindsley and Zimm (1992) where the 1(4)2 complementation group was renamed 1(4)02Dca. Our analysis now shows that this complementation group is allelic to bent and the alleles have been renamed. The designation of Hochman (1971) have been retained so that the designation of the 1(4)102CDa. Our analysis now shows that this complementation group is allelic to bent and the alleles have been renamed. Some stocks were outcrossed to a different balancer chromosome designation was supported by staining Drosophila muscles with antibody directed against twitchin (Vigoreaux et al., 1991). We therefore used primers based on the most conserved regions of motif I from twitchin to amplify a segment of Drosophila DNA by PCR (Ayme-Southgate et al., 1991). The PCR clone was then used to select clones from a λ phage library of Drosophila DNA and to initiate a walk through the projectin gene. The ~2.8-kb sequence obtained in the earlier study confirmed that projectin had motifs I and II as seen in twitchin (Fig. 1, blocks 1 and 2) and tin and that these motifs were in the same regular I-II pattern seen in twitchin (Benian et al., 1989). Comparisons with twitchin suggested the projectin sequence in our first report should be located upstream of a kinase domain, if such a domain existed in the Drosophila protein.

It was of interest to know whether the similarity between projectin and twitchin included a kinase domain. We have now been able to find a projectin kinase domain by cross-hybridization with a DNA fragment from the kinase domain of twitchin. The twitchin fragment was used in low-stringency hybridization to the lambdaphages which formed a 30-kbp walk in the projectin gene region. As expected, the region identified by the cross-hybridization was several kbp downstream of the previously sequenced portion of the projectin gene (Fig. 1). This is the only region of cross-hybridization which a 30-kbp walk in the projectin gene region. As expected, the region identified by the cross-hybridization was several kbp downstream of the previously sequenced portion of the projectin gene (Fig. 1). This is the only region of cross-hybridization within the 30 kbp of cloned sequence which we believe contains the 3' end of the projectin gene (see below). Published amino acid comparison between different kinase domains shows the level of identity varies along the length of the kinase region, the similarity being somewhat higher at the NH2-terminal part compared to the COOH-terminal region (Hanks et al., 1988). It is therefore possible that our cross-hybridization signal comes entirely from the NH2-terminal portion of the twitchin kinase.

The region of the projectin gene identified by the twitchin kinase DNA probe encodes an amino acid sequence with all the characteristics of the catalytic core of serine/threonine kinases (Fig. 2, characteristic amino acids are marked by stars; Hanks et al., 1988). The sequence analysis implies that a small intron of 63 bp is present between the codons for amino acid 82 and 83 of the kinase domain. The twitchin kinase domain also contains a small intron but in a different position (Benian et al., 1989). There is a second, larger, intron interrupting the projectin kinase domain at amino acid position 196. Using an RNA-PCR approach (Southgate, R., unpublished results), we have localized the remainder of the kinase domain on the genomic walk. The sequence of
the position of the kinase domain. The two introns detected in the kinase domain are not indicated. Our new sequence information shows that the region between the core region and the kinase domains consists of more diverged motifs I and II. Short vertical lines indicate the EcoRI sites. The arrow underneath the map represents the direction of transcription. The detectable sequence disruptions in the mutants are indicated on the map as follows: *x, bent°; *, be-°; ▼, be-°; and ▶, Df(4)M62f. The vertical lines underneath the map represent the position of the two PstI restriction sites involved in the Southern analysis of Fig. 6. The double-headed arrows underneath the map represent the position and extent of the DNA probes used in Southern analysis presented in Figs. 5, 6, and 7. Probes A and B are probes A and B of Fig. 5. Probe C is used for the Southern analysis in Fig. 6 and probe D for the Southern analysis in Fig. 7. The boxes (numbered 1 through 4) below the line mark the regions for which the genomic DNA sequence is available. The detailed arrangement of motifs I and II, from boxes 2, 3, and 4, is presented in Fig. 3. The sequence of the kinase domain and its flanking regions is given in Fig. 2.

Projectin Shows Significant Similarity to Twitchin in the Kinase Domain and Its Putative Autoinhibitory Region

When the kinase domains of projectin, twitchin, titin, and csmMLCK are compared (Fig. 2), projectin is seen as most similar to twitchin. The kinase domains of projectin and twitchin show 61% identity with no gap over a span of 261-amino acid residues.

In twitchin there is a unique amino acid sequence immediately COOH-terminal of the kinase domain, before the start of the next motif II (Benian et al., 1989). The alignment of the twitchin and projectin sequences in this region shows the conservation of 24 residues out of 65 (Fig. 2). The identity is mostly localized between amino acid 35 and 50. Recently, Lei et al. (1994) have shown that these 60 to 65 amino acids act as an autoinhibitor in in vitro protein kinase assays of bacterially expressed twitchin kinase domain. This region is not conserved with either titin or csmMLCK.

Sequence NH2-terminal to the Kinase Catalytic Core Is Well Conserved between Projectin and Twitchin

Immediately adjacent to the kinase domain, on the NH2-terminal side, the identity between projectin and twitchin

Figure 2. Amino acid sequence comparison of the kinase regions of projectin, twitchin, titin, and smooth muscle myosin light chain kinase. Aligned sequences from projectin (this study), twitchin (Benian et al., 1989), chicken smooth muscle myosin light chain kinase (as reported in Benian et al., 1989), and titin (Labeit et al., 1992). Amino acids shared by projectin and any of the other proteins are shaded. The star (*) indicates amino acids conserved in the different functional domains of protein kinases (Hanks et al., 1988). These sequence data are available from GenBank/EMBL/DDBJ under accession numbers L35899 and L35900.
extends into a unique region. The conservation between projectin and twitchin, is especially high for the last 22 amino acids just before the start of the kinase catalytic core. It is interesting that neither titin or csmMLCK show similar conservation and a database search shows no other significant identity between this region and any other known proteins, apart from twitchin itself.

In addition this region just before the kinase domain shows an alteration of the regular I-I-II pattern of motifs seen throughout the available projectin sequence (Fig. 3). Twitchin shows a similar variation in the pattern of motifs upstream of the kinase domain (Benian et al., 1989; see Fig. 3) and so does titin (Labet et al., 1992).

**All Projectin Isoforms Are Capable of Kinase Activity**

The identification of a kinase domain in the projectin sequence extends the similarity between at least one projectin isoform and twitchin but leaves open the possibility that some projectin isoforms, such as the IFM isoform may lack the domain. To investigate this question we have tested projectin from different muscles for kinase activity. IFM preparations were made in two ways, by dissection of IFM from acetone-dried adult flies (Mogami et al., 1982) and by bulk preparation of myofibrils from whole adults (Saide, 1981). The protocol used for bulk preparation yields nearly pure IFM myofibrils, as judged by light microscopy, by the presence of the IFM-specific protein, flightin (Vigoreaux et al., 1993) and by the absence of the synchronous muscle-specific protein, mp20 (Ayme-Southgate et al., 1989; and data not shown). Synchronous muscle preparations were made from adult legs, head, and larval body walls. In each case the purity of the preparation could be assessed by electrophoresis in the appropriate gel system, since muscle type-specific projectin isoforms differ in mobility.

Maroto et al. (1992) have shown that projectin from crab can undergo autophosphorylation in vitro and that Drosophila projectin is phosphorylated in vivo. The authors did not investigate the kinase activity of different Drosophila muscle type-specific isoforms of projectin. We have carried out these experiments by performing an in vitro autophosphorylation assay together with antibody identification of projectin on the same Western blot. The autophosphorylation was detected by autoradiography and the antibody binding was detected by a colorimetric reaction. In all cases the band on the autoradiograph and the band from the colorimetric reaction coincide exactly. Fig. 4 demonstrates clearly that projectin extracted from a variety of muscles, including the IFM, is capable of autophosphorylation in vitro. In the autophosphorylation reaction projectin is not the only labeled protein. Some smaller proteins also autophosphorylate, as might be expected but none of these are detected by antibodies against projectin (Fig. 4 B).

The polyacrylamide gradient gels used in these experiments contain many other muscle proteins as judged by Coomassie blue staining of equivalent gel lanes and antibody localization of myosin heavy chain and α-actinin on the filters used for the kinase assay (stars in Fig. 4 A indicate positions detected by antibody reaction subsequent to the anti-projectin staining). Neither myosin heavy chain, nor α-actinin, two very abundant muscle proteins, are labeled in the autophosphorylation assay, eliminating the possibility that labeling seen for projectin in the assay is due to non-specific sticking of radiolabeled γ-ATP to an abundant protein.

The in vitro studies show clearly that isoforms of projectin from both synchronous and asynchronous muscle are capable of autophosphorylation and thus have kinase domains which are potentially functional. This result is supported by experiments in which the filters were probed with antibody against the kinase domain of twitchin. All isoforms were recognized by this antibody (data not shown). The inclusion of the kinase domain in both projectin isoforms is also supported by data from RNA in situ hybridization on adult sections. An anti-sense RNA probe including the sequence for the kinase domain specifically detects transcripts on both synchronous and asynchronous muscles but not other tissues (data not shown).

The honeybee has sarcomeric distributions of projectin identical to those seen in Drosophila (Saide et al., 1990). Projectin, immunopurified from synchronous honeybee leg muscles, is capable of in vitro phosphorylation, as in Drosophila (Fig. 4 C). Preliminary results from immunoblots analysis with the twitchin anti-kinase antibody and from the kinase filter assay indicate that honeybee IFM projectin also has a kinase domain capable of autophosphorylation.

**The Bent° Rearrangement Removes the Kinase Domain from the Projectin Sequence**

We have mapped the projectin coding sequence to polytene region 102C/D (Ayme-Southgate et al., 1991). This region contains a recessive lethal complementation group l(4)2 and the bent° mutation. The bent° mutation was originally isolated because heterozygous flies held their wings in a distinctive position (a "bent" position). The phenotype suggests that bent° might represent a mutation in the projectin gene since similar phenotypes affecting wing position have been linked with mutations in other muscle proteins (Mahaffey et al., 1985; Beall and Fyrberg, 1991). The abnormal positions
was immunopurified from honeybee leg muscles with monoclonal antibodies and used in an in vitro kinase assay with \( \text{\textsuperscript{32}}P-\text{ATP} \). Products of label to protein is very low in this band. It could represent either a low level of phosphorylation of the heavy chain or trapping of histone.

The band just above the histories co-runs with the heavy chain of the monoclonal antibody used to immunoprecipitate projectin. The ratio with the antibody localization. (B) A filter assay similar to that in A is shown. (Lane 1) Purified IFM as in A. (Lane 2) Extracts of legs. (Lane 3) Extracts of adult heads. Arrows indicate isoforms. This gel has not separated the isoforms as well as has the gel in A. Projectin was immunopurified from honeybee leg muscles with monoclonal antibodies and used in an in vitro kinase assay with \( \gamma\text{\textsuperscript{32}}P-\text{ATP} \). Products of the reactions were run on a gradient polyacrylamide-SDS gel, stained, dried, and autoradiographed. The arrow indicates the position of projectin. The two lowest bands of labeled proteins run at the positions of the histones which were added as potential kinase substrates. The band just above the histones coruns with the heavy chain of the monoclonal antibody used to immunoprecipitate projectin. The ratio of label to protein is very low in this band. It could represent either a low level of phosphorylation of the heavy chain or trapping of histone.

suggest that some thoracic muscles may be more sensitive to a 50% reduction in amount of muscle proteins than other muscles. It is also possible that these muscles may be more sensitive to the presence of the product of the defective allele. Unfortunately flies no longer show the dominant phenotype of bent\(^p\), presumably because the stock has accumulated modifying genes in the many years since the original isolation. Although the dominant wing position phenotype has been lost, the bent\(^p\) chromosome still has a recessive lethal phenotype. As discussed below, this phenotype is due to a disruption which removes the kinase domain and other COOH-terminal sequences from the projectin gene. We also show that this bent\(^p\) disruption is within the sequence of the \( l(4)2 \) complementation group and that different disruptions in the projectin sequence are associated with different lethal periods.

The bent\(^p\) mutation was originally described as a deletion, probably because the bent\(^p\) chromosome failed to complement two complementation groups in the 102CD region. \( l(4)2 \) and \( l(4)23 \) (Hochman, 1974). Our complementation tests are entirely consistent with the original data; the bent\(^p\) chromosome fails to complement any of the \( l(4)2 \) alleles that are still available (Table I). Unfortunately all alleles of the second complementation group, \( l(4)23 \), have been lost so we cannot reconfirm its interaction with bent\(^p\).

Genomic Southern hybridization analyses of the bent\(^p\)/ci\(^p\) stock show the presence of a disruption within the projectin gene (Fig. 1). To determine that the rearrangement originates from the bent\(^p\) chromosome and not the balancer ci\(^p\) chromosome, the bent\(^p\) chromosome was outcrossed to either a wild type chromosome (by crossing with Oregon R flies) or to another balancer, ey\(^n\) (by crossing with ey\(^n\)/ci\(^p\)). In both cases the change in restriction fragment pattern, originally identified in the bent\(^n\)/ci\(^p\) stock, segregates with the non-ci\(^p\) progeny. Using combinations of restriction digests and progressively smaller probes, we have localized one breakpoint of the bent\(^n\) rearrangement to the projectin sequence within, or just NH\(_2\)-terminal to, the kinase domain. An example of the experiments used for this mapping is presented in Fig. 5. These hybridization analyses also show that the bent\(^n\) mutation is not a deletion, as originally thought. Instead, se-

| Table I. Results of Complementation Testing in the 102CD Region |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | \( \text{bt}^0 \) | \( \text{bt}^{+n} \) | \( \text{bt}^{+b} \) | \( \text{bt}^{+t} \) | \( \text{Df}(4)\text{M}^{12f} \) |
| \( \text{bt}^0 \) | -               | -               | -               | -               | +               |
| \( \text{bt}^{+n} \) | -               | -               | -               | -               | +               |
| \( \text{bt}^{+b} \) | -               | +               | -               | -               | +               |
| \( \text{bt}^{+t} \) | -               | -               | -               | +               | +               |
| \( \text{bt}^{-x} \) | -               | -               | -               | -               | -               |

Crosses were performed using two mutant stocks over the same balancer (ci\(^p\) or ey\(^n\)) and complementation was determined by the presence of adult offspring that did not carry the parental balancer. Complementation was considered positive if 1/3 of the surviving adults did not show the balancer dominant phenotype. These flies were therefore heterozygous for the two mutations being tested. In the crosses showing no complementation, all adult flies displayed the parental balancer phenotype and were therefore homozygous for the balancer chromosome. No cases of partial complementation were detected. The \( \text{bt}^{+n} \) and \( \text{bt}^{+t} \) alleles complement each other; however both are homozygous lethal and fail to complement all other \( \text{bt} \) alleles. This example of inter-allelic complementation was first reported by Hochman (1971). D(4)M\(^{12f}\) complements all \( \text{bt} \) alleles. However the 3' end of the cloned projectin contig is rearranged in the D(4)M\(^{12f}\) chromosome. This rearrangement, together with the complementation, defines the 3' end of the projectin transcription unit.
Localization of the bent° breakpoint in the projectin gene. An autoradiograph of a Southern blot hybridization of wild type Oregon R and bent°/ci° (M) flies, probed with the DNA fragments indicated below the restriction map, is shown. Two identical sets of filters were each hybridized with either probe A (the three filters on the left) or probe B (the three filters on the right). As indicated below the top restriction map, the DNA fragment for probe A is completely included in the DNA fragment for probe B. Numbers below each set of filters indicate the restriction enzyme used to cleave the two DNA samples. The position of the cleavage sites is indicated on the restriction map below. Enzymes were: 1, AvaI; 2, HindIII; and 3, EcoRI. The arrow points to a new band detected by probe B but not A. From this and similar experiments we conclude that the bent° chromosome has been rearranged from the map shown on top to that shown on the bottom (see Fig. 1 for position within the projectin gene). The identity of the DNA after the breakpoint (indicated by a wavy vertical line) is unknown and therefore drawn as a dashed line.

The most probable rearrangement is an inversion, although a relatively large insertion is also possible. In any case, the early studies showing non-complementation with a second complementation group, the lost l(4)23 group, are most easily interpreted if there is a second breakpoint affecting l(4)23. Whatever the aberration, the DNA region involved is not large enough to produce detectable changes in the polytene banding pattern of the bent° chromosome (data not shown; Hochman, 1971).

Loss of the Kinase Domain and More COOH-terminal Sequences of Projectin Is Lethal after Muscle Contractions Begin

The genomic analyses show clearly that the kinase domain and the more COOH-terminal sequences are removed from the projectin gene in the bent° chromosome. Our phenotypic analyses show that homozygosity for this loss does not prevent muscle contraction but that homozygous embryos die just before hatching, giving the impression that they were unable to emerge from the chorion and vitelline membrane. Presumably they lack sufficient muscle activity or coordination for this final exertion. This phenotype is reminiscent of phenotypes observed for embryonic lethal mutants in other muscle protein-specific genes (for example, myosin heavy chain mutant mhc-1; O'Donnell and Bernstein, 1988).

The recessive lethality of the bent° chromosome was studied in homozygous mutant progeny from heterozygous parents. Because the bent° chromosome must be carried in stock as a heterozygote with a balancer chromosome
also has a recessive lethal allele, half the dead progeny of these crosses will be homozygous for the balancer rather than the mutated projectin gene. Therefore it is necessary to find ways to distinguish between the two classes of dead progeny. This was accomplished by using two different balancer chromosomes, the ci° and the ey° balancers. Animals homozygous for the ci° chromosome die as embryos; animals homozygous for ey° die as larvae or pupae. Stocks balanced by the ey° chromosome were, therefore, used to test for embryonic lethality; stocks balanced by the ci° chromosome were used to test for larval lethality.

Our studies showed that the bent° homozygotes died as late embryos, well after the formation of muscle. Muscle contractions could be seen in all embryos of the bent°/ey° stock, including those that did not hatch. To confirm that the dead embryos from this stock were homozygous for the bent° chromosome, DNA from dead embryos was analyzed by restriction mapping. An example of such analysis is given in Fig. 6. The dead embryos are homozygous for restriction fragments that characterize the bent° chromosome.

This study of the bent° homozygotes showed that absence of intact projectin does not prevent muscle from undergoing the spontaneous contractions that start in wild type embryos soon after muscle formation begins. However, the study does not allow us to conclude that the bent° deleted projectin is unable to support the exertions of hatching causing the embryonic lethality, since the bent° chromosome also fails to complement l(4)23 mutations. We have been able to eliminate the possibility that l(4)23 was responsible for the embryonic death by studying the time of death of bent°/bt° heterozygotes. As discussed below, bt° is a mutant allele of the projectin gene and does not complement the bent° mutation. Thus the death of the bent°/bt° heterozygote should reflect the time at which projectin became essential. Heterozygotes were produced by crossing bent°/ey° with bt°/ci°. Because the parents had different balancer chromosomes, only the bent°/bt° progeny are expected to die. If the bent° projectin could not support hatching, these transheterozygous progeny should die as embryos. As expected ~25% of the progeny died and all died as embryos. Thus we conclude that the bent° homozygotes failed to survive to hatching because of the disruption of the projectin gene.

**Mutant Alleles of the l(4)2 Complementation Group Have Disruptions of the Projectin Sequence both 5' and 3' of the Bent° Breakpoint**

The bent° chromosome fails to complement alleles of two complementation groups, l(4)2 and l(4)23. Although all alleles of the l(4)23 group have been lost, four alleles of l(4)2 are still available. Luckily, it appears that the l(4)2 complementation group is the one corresponding to the projectin locus and the l(4)2 locus has been renamed the bt (bent) locus. Two of the four available bt alleles have alterations in the projectin region that are large enough to produce detectable changes in restriction fragments. The bt° allele has a small insertion (Fig. 7) in the core region of the projectin gene, several kbp 5' of the kinase domain (Fig. 1). This region was sequenced in our initial study and shown to contain a regular arrangement of motifs I and II (Ayme-Southgate et al., 1991). This insertion has also been characterized by Fyrberg et al. (1992). It consists of 141 bp of sequence that appears to have inserted by retrotransposition. The insertion contains multiple stop codons in all frames and should result in a truncated, probably unstable protein, unless the insertion is spliced out of the mRNA.

The second bt allele with a detectable rearrangement is bt°. The pattern of restriction fragments generated by this mutation suggests that the rearrangement is either a large insertion or an inversion (data not shown). The rearrangement maps 3' of the kinase domain (Fig. 1). This region was sequenced in our initial study and shown to contain a regular arrangement of motifs I and II (Ayame-Southgate et al., 1991). This insertion has also been characterized by Fyrberg et al. (1992). It consists of 141 bp of sequence that...
We have repeated the studies of the times of death and our results confirm those of Hochman. The studies were performed as described for the bent0 mutation. Both bt-a and bt-c homozygotes die as late embryos. The other two alleles, bt-a and bt-c, die sometime during the larval stage. The bt-a homozygotes never showed any of the spontaneous contractions that start in normal embryos soon after muscle formation begins (Steitz, M., personal communication).

**The Extent and Orientation of the Projectin Transcription Unit**

The bt-c breakpoint is the most 3' disruption within the projectin gene and thus defines the minimal extent of the gene towards the 3' end (see Fig. 1). Deficiencies Df(4)M60a and Df(4) M60b were tested for complementation to bent0 and the bt alleles. Both DF(4)M60ab and DF(4) M60b complemented all projectin mutants (Table I). In spite of this, the Df(4)M60ab chromosome has a detectable rearrangement very near the 3' end of the cloned contig of sequence containing the projectin gene. The Df(4)M60ab breakpoint lies ~5-6 kbp 3' of the bt-c rearrangement. Restriction mapping suggests that the Df(4)M60ab chromosome, like bent0, is rearranged rather than deleted. Since Df(4)M60ab complements bent0 and all the bt alleles, it seems likely that this breakpoint lies outside the projectin gene and allows us to define the maximum extent of the locus on the 3' side. Because Df(4)M60ab maps to 10IE:102B, projectin must be transcribed toward the centromere.

**Discussion**

**Kinase Domains in Projectin**

Projectin is the first muscle structural protein that has been found to have dramatically different sarcomeric distributions in different muscle types. These localizations suggest that the protein can be adapted to more than one function. The suggestion is supported by evidence that apparently homologous proteins from other species, titin and twitchin, may have different functions. Despite the differences in function, all projectin isoforms are encoded by a single gene (Ayme-Southgate et al., 1991). The products of this gene must be differentially spliced and/or terminated because projectin has escaped detection. It is also worth noting that they are 60 % sequence identity. A domain with less homology would necessarily show that all have the same kinase domain. We have assayed the cloned contig which we believe contains the complete 3' end of the projectin sequence. The sequence reported here is the only one within the ~10 kbp of DNA which is capable of cross-hybridization with twitchin kinase sequences. Thus there may be only one kinase domain in the gene although we note that even the most similar region of this kinase sequence, the NH2-terminal domain, has only 60% sequence identity. A domain with less homology would have escaped detection. It is also worth noting that they are two introns within the projectin kinase domain. Their presence raises the intriguing possibility that alternative splicing to a less conserved sequence could yield an isoform with potentially different substrates and/or regulation.

**Genetic Analysis of the Projectin Gene**

In his extensive analysis of genes on chromosome 4, Hochman (1974) noted that "The evidence of a high incidence of mutation, partial complementation, and divergent developmental effects leads to the conclusion that l(4)2 is a complex (or pseudoalletic) locus". We can now understand this conclusion in terms of a giant protein with different isoforms that probably become critical, qualitatively or quantitatively, at different points in development. Based on the size of the projectin protein, the coding region for the projectin gene can be calculated to span at least 22 kbp of genomic DNA (not including introns or alternative exons). For comparison, the size of twitchin mRNA, based on the complete coding sequence, has been determined to be 21.6 kbp (Benian et al., 1993). On SDS-polyacrylamide gels, all projectin isoforms run more slowly than purified twitchin. Given the size of the projectin coding region it is not surprising that the l(4)2 complementation group had 35 alleles, more than twice the number in the next largest group in Hochman's mutational study.
Genetic analysis of protein function, one of the major advantages of Drosophila as an experimental system, is especially difficult for projectin. The sheer size of the gene eliminates hopes of reverse genetics with P element transposition. In addition, analyses of genes on chromosome 4 present a special problem since there is no recombination of this tiny chromosome. Therefore it is not possible to separate two mutations, if both occur on the same chromosome. Thus conclusions about any particular allele must be qualified by the possibility that the molecular defect in the projectin coding region may not be the defect producing the phenotype. For this reason, our conclusions depend on the consistent picture that we have derived from multiple cross-comparisons of all mutations and rearrangements mapping to 102C/D. Our conclusion that the IO/42 complementation group corresponds to the projectin gene is based on the evidence that at least one, and probably two, of the four I(4)2 alleles has a detectable disruption within the projectin coding region. In addition all four alleles fail to complement the bent° chromosome which also has a breakpoint in the projectin coding region.

The complex phenotypes that led Hochman (1974) to conclude that I(4)2 might be a pseudoallelic locus appear to be due to different disruptions within the giant projectin molecule. The three mutations that we have mapped in the projectin gene have quite different phenotypic effects. Both the brt° and the bent° mutations lead to death very late in embryogenesis but brt° homoyzogotes never display any muscle contractions while bent° homozygotes show apparently normal contractions in the early embryo. The third mutation, brt°, leads to death in the larval stages. For these three mutations it appears that the severity of the defect correlates with the nature and position of the defect along the projectin gene.

The brt° insertion in the core region of the protein should produce a severely truncated protein. This protein could be unstable or, if stable and assembled into the contractile apparatus, it could seriously compromise contraction. The bent° breakpoint is much closer to the COOH-terminus of projectin and deletes part or all of the kinase, plus more terminal regions. However, if stable, the bent° protein would contain the entire central core of motifs I and II. Embryonic muscles are synchronous muscles, where we postulate that projectin has a regulatory role. If the postulation is correct, the bent° mutation might affect the efficiency of contraction rather than affecting the contractions themselves. In this case, the defect in projectin might prove lethal only when the embryo had to undergo the exertions of hatching. This phenotype is reminiscent of unc-22 mutants in the nematode. These mutants in the gene for twitchin show muscle contractions but also show twitching and disruption of muscle structure (Waterston et al., 1980). The brt° breakpoint, on the other hand, is very far toward the 5' end of the projectin gene. It remains to be determined whether the defect is in a region that is translated or even transcribed. Since brt° homoyzogotes do not die until the larval stage is reached, it is possible that the defect involves an exon important only in one or more muscles necessary for larval life. Alternatively, if the region of the breakpoint is not part of the projectin coding region it might be involved in muscle-specific regulation in a larval muscle.

Hochman (1974) reported that a subset of the I(4)2 alleles were able to complement other I(4)2 alleles. Two of the complementing alleles, bt°-a and brt°-f, are still available. They still complement for viability and we find that they also appear to have normal flight ability (unpublished observations). In spite of this complementation, both brt°-a and brt°-f are homozygous lethal and both fail to complement all other projectin mutations. Neither allele has an easily detectable disruption in the region that we have analyzed; however small insertions or deletions and point mutations would not have been detected. Allele-specific complementation, like that displayed by brt°-a and brt°-f, can occur in several ways. If projectin assembles as an oligomer, the two different mutant proteins might compensate for each other in this process. A second possible explanation is suggested by the observation that brt° is embryonic lethal whereas brt°-a is larval lethal. If these two different times of death reflect failures in muscles using alternatively spliced exons, the complementation of the alleles is easily understood.

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References

Achtschel, S. F., W. Gish, W. Miller, E. W. Meyers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.

Ashburner, M. 1989. Drosophila: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ayme-Southgate, A., P. Lasko, C. French, and M. L. Pardue. 1989. Characterization of the gene for mpaD: a Drosophila muscle protein that is found in asynchronous oscillatory flight muscle. J. Cell Biol. 108:521–531.

Ayme-Southgate, A., J. O. Vigoreaux, G. M. Benian, and M. L. Pardue. 1991. Drosophila has a twitchin/titin-related gene that appears to encode projectin. Proc. Natl. Acad. Sci. USA. 88:7973–7977.

Beall, C. J., and E. A. Fyrberg. 1991. Muscle abnormalities in Drosophila melanogaster heldup mutants are caused by missing or aberrant troponin-1 isoforms. J. Cell Biol. 114:941–951.

Benian, G. M., J. E. Kiff, N. Neckelmann, D. G. Moerman, and R. H. Waterston. 1989. Sequence of an unusually large protein implicated in regulation of myosin activity in C. elegans. Nature (Lond.). 342:45–50.

Benian, G. M., S. W. L'Hernault, and M. E. Morris. 1993. Additional sequence complexity in the muscle gene, unc-22, and its encoded protein, twitchin of Caenorhabditis elegans, Genetica. 134:1097–1104.

Celenza, J. L., and M. Carlson. 1989. Mutational analysis of the Saccharomyces cerevisiae SNF1 protein kinase and evidence for functional interaction with the SNF4 protein. Mol. Cell. Biol. 9:5034–5044.

Crossley, A. C. 1978. The morphology and development of the Drosophila muscular system. In The Genetics and Biology of Drosophila. Vol. 2B. M. Ashburner and T. R. F. Wright, editors. Academic Press, London. 499–560.

Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6.

Fulton, A. B., and W. B. Isaacs. 1991. Titin, a huge, elastic sarcomeric protein with a probable role in morphogenesis. BioEssays. 13:157–161.

Funatsu, T., E. Kono, H. Higuchi, S. Kimura, S. Ishiwata, T. Yoshikawa, K. Maruyama, and T. Shoichiro. 1993. Elastic filaments in situ in cardiac muscle: deep-tet replica analysis in combination with selective removal of actin and myosin filaments. J. Cell Biol. 120:711–724.

Furt, D. O., M. Osborn, R. Nave, and K. Weber. 1988. The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line. J. Cell Biol. 106:1563–1572.

Fyrberg, C. C., S. Labeit, B. Bullard, K. Leonard, and E. A. Fyrberg. 1992. Drosophila projectin: relatedness to titin and twitchin and correlation with lethal (4)102C/D and bent-Dominant mutants. Proc. R. Soc. Lond. B. 249:33–40.

Hanks, S. K., A. M. Quinn, and T. Hunter. 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science.
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Mogami, K., S. C. Fujita, and Y. Hotta. 1982. Identification of Drosophila Indirect flight muscle myofibrillar proteins by means of two-dimensional electrophoresis. J. Biochem. 91:643-650.

Nave, R., and K. Weber. 1990. A myofibrillar protein of insect muscle related to vertebrate titin connects Z band and A band; purification and molecular characterization of invertebrate mini-titin. J. Cell Sci. 95:535-544.

O'Donnell, P. T., and S. I. Bernstein. 1988. Molecular and ultrastructural defects in a Drosophila myosin heavy chain mutant: differential effects on muscle function produced by similar thick filament abnormalities. J. Cell Biol. 107:2601-2612.

Pringle, J. R. S. 1978. Stretch activation of muscle: function and mechanism. Proc. Roy. Soc. Lond. 201:107-130.

Saide, J. D. 1981. Identification of a connecting filament protein in insect fibrillar flight muscle. J. Mol. Biol. 153:661-679.

Saide, J. D., S. Chin-Bow, J. Hogan-Sheldon, L. Busquets-Turner, J. O. Vigoreaux, K. Valgeirsdottir, and M. L. Pardue. 1989. Characterization of components of Z-bands in the fibrillar flight muscle of Drosophila melanogaster. J. Cell Biol. 109:2157-2167.

Saide, J. D., S. Chin-Bow, J. Hogan-Sheldon, and L. Busquets-Turner. 1990. Z-band proteins in the flight muscle and leg muscle of the honeybee. J. Muscle Res. Cell Motil. 11:125-136.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sanger, F., S. N. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463-5467.

Sommerville, L. L., and K. Wang. 1988. Sarcomere matrix of striated muscle: in vivo phosphorylation of titin and nebulin in mouse diaphragm muscle. Arch. Biochem. Biophys. 262:118-129.

Squire, J. M. 1992. Muscle filament lattices and stretch activation: the match-mismatch model reassessed. J. Muscle Res. Cell Motil. 13:183-189.

Towbin, H., T. Staszehin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheets: procedure and some application. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

Trinick, J., P. Knight, and A. Whiting. 1984. Purification and properties of native titin. J. Mol. Biol. 180:331-356.

Vibert, P., S. M. Edelstein, L. Castellani, and B. W. Elliott. 1993. Mini-titins in striated and smooth molluscan muscles: structure, location and immunological crossreactivity. J. Muscle Res. Cell Motil. 12:340-354.

Vigoreaux, J. O., and J. D. Saide. 1990. Structurally different Drosophila striated muscles utilize distinct variants of Z-band associated proteins. J. Muscle Res. Cell Motil. 12:340-354.

Vigoreaux, J. O., J. D. Saide, K. Valgeirsdottir, and M. L. Pardue. 1993. Flightin, a novel myofibrillar protein of Drosophila stretch-activated muscles. J. Cell Biol. 121:587-598.

Wang, K., J. McClure, and A. Tu. 1979. Titin: major myofibrillar components of striated muscle. Proc. Natl. Acad. Sci. USA. 76:3698-3702.

Wang, K., R. Ramirez-Mitchell, and D. Palter. 1984. Titin is an extraordinarily long, flexible and slender myofibrillar protein. Proc. Natl. Acad. Sci. USA. 81:3685-3689.

Waterson, R. H., J. N. Thomson, and S. Brenner. 1980. Mutants with altered muscle structure in Caenorhabditis elegans. Dev. Biol. 77:271-302.

Whiting, A., J. Wardale, and J. Trinick. 1989. Does titin regulate the length of muscle thick filaments? J. Mol. Biol. 205:263-268.

Wray, J. S. 1979. Filament geometry and the activation of insect flight muscle. Nature (Lond.). 280:325-326.