Drosophila *crinkled*, Mutations of Which Disrupt Morphogenesis and Cause Lethality, Encodes Fly Myosin VIIA

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ABSTRACT

Myosin VIIIs provide motor function for a wide range of eukaryotic processes. We demonstrate that mutations in *crinkled* (*ck*) disrupt the Drosophila myosin VIIA heavy chain. The *ck*/myoVIIA protein is present at a low level throughout fly development and at the same level in heads, thoraxes, and abdomens. Severe *ck* alleles, likely to be molecular nulls, die as embryos or larvae, but all allelic combinations tested thus far yield a small fraction of adult “escapers” that are weak and infertile. Scanning electron microscopy shows that escapers have defects in bristles and hairs, indicating that this motor protein plays a role in the structure of the actin cytoskeleton. We generate a homology model for the structure of the *ck*/myosin VIIA head that indicates myosin VIIAs, like myosin IIs, have a spectrin-like, SH3 subdomain fronting their N terminus. In addition, we establish that the two myosin VIIA FERM repeats share high sequence similarity with only the first two subdomains of the three-lobed structure that is typical of canonical FERM domains. Nevertheless, the ~100 and ~75 amino acids that follow the first two lobes of the first and second FERM domains are highly conserved among myosin VIIIs, suggesting that they compose a conserved myosin tail homology 7 (MyTH7) domain that may be an integral part of the FERM domain or may function independently of it. Together, our data suggest a key role for *ck*/myoVIIA in the formation of cellular projections and other actin-based functions required for viability.

MYOSIN VIIIs are actin-based motor proteins essential for a variety of biological processes (Chen et al. 1996; Hodge and Cope 2000; Yamashita et al. 2000; Berg et al. 2001; Redowicz 2002; Tzolovsky et al. 2002; Ahmed et al. 2003 and references therein). In vertebrates, they play a key role in sensory perception: defects in myosin VIIA lead to deafness and blindness in humans, retinal defects and deafness in mice, and aberrant auditory and vestibular function in zebrafish. The cellular basis of these phenotypes suggests that the defects are the consequence of aberrant actin cytoskeleton function. Moreover, the tissue-specific expression pattern of myosin VIIA correlates well with the phenotypes observed. Biochemical experiments on purified or recombiant proteins show that the myosin VIIAs have plus (barbed) end-directed motor activity on actin filaments and a characteristic actin-activated ATPase activity (Udovichenko et al. 2002; Inoue and Ikebe 2003). Structurally, the myosin VIIA heavy chain is well conserved and the various domains provide for motor- and cargo-binding functions. The myosin VIIA head and neck are composed of a conserved N-terminal region (~60 amino acids), a characteristic motor domain, and four to five isoleucine-glutamine (IQ) motifs that bind calmodulin (Cheney and Mooseker 1992; Todorov et al. 2001) and/or specific light chains. The myosin VIIA tail begins with a short sequence predicted to form an alpha-helical coiled-coil that may contribute to dimerization. The remainder of the tail consists of a tandem repeat of myosin tail homology 4 (MyTH4) domains and partial four-point 1, ezrin, radixin, and moesin (FERM) domains (see below) that are separated by an SH3 subdomain and are thought to mediate dimerization and binding to other proteins or cargo.

Myosin VIIAs are part of a myosin subfamily that is conserved phylogenetically in metazoa and amoebozoa (Dictystelium) but is lacking in sequenced fungal and plant species. The subfamily includes myosin VIIA, myosin VIIB, myosin VII (from species that do not have distinct myosin VIIA and myosin VIIB genes, see below and discussion), myosin X, and myosin XV. In vertebrates and insects, the closely related myosin VIIB heavy chain is encoded by distinct, myosin VIIB genes (see references above and Chen et al. 2001) that have not been characterized extensively. The fly myoVIIB gene is encoded by a transcription unit at polytene location 28B. The VIIB heavy chains share clear sequence similarity throughout the heavy chain, but lack a region pre-
dicted to form a coiled-coil. No pathologies associated with myosin VIIA have so far been discovered in any species. Caenorhabditis elegans and Dictyostelium discoideum have a single myosin VII heavy chain gene (not distinct VIIA and VIIIB forms). MyoI encodes the D. discoideum myosin VII: it is essential for the initial steps of cell adhesion that contribute to phagocytosis, cell-cell interactions, translocation across a substrate, and the formation of filopodia (Tittus 1999; Tuxworth et al. 2001).

Interestingly, recent analysis of vertebrate myosin VIIA mutants suggests that it is not required for the early adhesion events in phagocytosis (Gibbs et al. 2003). Nevertheless it may play an important role in linking adjacent stericilia in hair cells (Kussel-Andermann et al. 2000). Thus far, no mutants are available for the worm hum-6/myoVII. Myosin X and XV, like members of the myosin VII subfamily, include tails with one or more FERM domains. Flies have a myosin XV encoded by a transcription unit at polytene location 10A, but they do not have a myosin X, which may have some overlap in function with myosin VIs in vertebrates (Yamashita et al. 2000; Berg et al. 2001; Tuxworth et al. 2001; Tzolovsky et al. 2002).

The crinkled (ck) locus has been studied intermittently for the past 70–80 years (Gubb et al. 1984; Ashburner et al. 1999), and genome sequence analysis suggested that it was likely to encode myosin VIIA (Ashburner et al. 1999). A mutation in ck was first identified by Bridges in the 1930s, but the allele was lost (Bridges and Brehme 1944). Detailed studies on the region around Adh identified a number of alleles in the l(2)br27 complement group with phenotypes very similar to those described by Bridges for ck, so ck and l(2)br27 were deemed allelic (Gubb et al. 1984). A number of phenotypes attributable to mutations at the ck locus have been described [other synonyms for ck are listed in FlyBase (FlyBase Consortium 2003)]. Severe mutations in ck are lethal or semilethal, with a small fraction of homozygous [ck/ck or hemizygous, ck/Df(ck-)] flies reaching adulthood (<0.5–5%, so-called “escapers”). Adult escapers of these lethal alleles show common expressivity of characteristic defects that include stubby microchaetae; short, multiple setae that are frequently branched; short aristae that are more highly branched than normal; and wavy and crumpled wings. In the context of specification of asymmetric cytoskeletal organization for planar polarity, crinkled suppresses both frizzled gain-of-function and dishevelled loss-of-function mutations (Winter et al. 2001). More recently, ck homozygotes were shown to have aristae that are abnormal in morphology (He and Adler 2002).

Here we formally demonstrate that the myosin VIIA heavy chain is encoded by the ck locus (Ashburner et al. 1999). We demonstrate that the ck/myoVIIA transcript is differentially spliced and show that protein is present at a low level throughout development and in different body parts. We establish that severe alleles die as embryos and document new phenotypes in escapers that are consistent with disruption of actin cytoskeletal dynamics. In addition, we propose a homology model for the structure of the ck/myoVIIA head that indicates that the N-terminal 55 amino acids constitute a spectrin-like SH3 subdomain comparable to that seen in myosin-II heavy chains. Finally, we use sequence comparisons to show that only the first two of the three lobes of each of the FERM domains is well conserved with other FERM domain proteins. Sequences that follow lobe 2 are highly conserved among myosin VIIs and show only weak sequence similarity with other proteins. We refer to these sequences as a MyTH7 domain.

MATERIALS AND METHODS

Fly husbandry and stocks: Flies were raised and crossed at 22° or 25° on standard yeast-molasses-agar medium using standard methods (Roberts 1998). EP(2)2051 was obtained from Janos Szidonya at the Szeged stock center and BG00682 from the Baylor/Berkeley Drosophila Genome Project (BDGP) Gene Disruption Project. Other ck alleles came from the stock collection at the Department of Genetics, University of Cambridge, United Kingdom. All other mutations and deletions used in this study are fully described in FlyBase (FlyBase Consortium 2003).

Genetic mapping: Mutant alleles were mapped to ck by crossing to deletion stocks and by crossing ck alleles inter se. Adult flies prepared for SEM analysis were from crosses of ck alleles to the large chromosomal deletion Df(2L)jos29 (SB1–3; SB66).

Verification of P[PZ]07130 as a ck allele: Deletions with one breakpoint at the insertion site of the P element were isolated using the P element transposase-mediated “male recombination” technique described elsewhere (Preston et al. 1996). Putative deletions were recognized by exchange between the flanking markers dumpy (dp), black (b) and cinnabar (cn), brown (bw). The cross scheme was as follows: male P[PZ]07130, ry+/CyO flies were crossed to females of the genotype dp b cn bw; Df, D2-3/TM6B; individual male flies of the genotype P[PZ]07130, ry+/dp b cn bw; Df, D2-3/+ were recovered and crossed to female CyO, dp+/b pr cn bw/Gla flies. Exceptional progeny of the genotype dp b P[PZ]07130/CyO, dp+/b pr cn bw indicated duplications extending proximally or deletions extending distally from the insertion site; those of the genotype P[PZ]07130 cn bw/CyO, dp+/b pr cn bw indicated duplications extending distally or deletions extending proximally from the insertion site.

Identification of Drosophila myosin VIIA: Standard methods were used for molecular biology throughout this study unless specified (Sambrook et al. 1989). Degenerate primers designed to amplify conserved sequences from myosin heavy chains but not the three fly myosin genes whose sequences were available at the time (muscle myosin II heavy chain, zipper non-muscle myosin II heavy chain, and ninac/myosin III heavy chain) were used to amplify DNA from a fly head cDNA library (Ittii et al. 1985), from an embryonic cDNA library (Brown and Kafatos 1988), and from a Keo cell line cDNA library in agt11 (origin unknown).

cr/myoVIIA cDNA: A nearly full-length cr/myoVIIA cDNA (L10736) was identified by the BDGP. cDNA from GCpl prepared plasmid DNA and PCR product derived from genomic DNA isolated from heterozygous or homozygous mutant flies and amplified with custom gene-specific primers (IDT, Coralville, IA or Gibco BRL, Gaithersburg, MD) were sequenced. Raw sequences were viewed with EditView (Perkin Elmer, Wellesley, MA) and differences between sequences from
two templates in the same fly (i.e., from the mutant and balancer chromosomes) gave two peaks at approximately half height. Sequences were assembled and analyzed with Sequencher (Gene Codes, Ann Arbor, MI) or SeqMan (DNASTar, Madison, WI). All differences were verified by sequencing homozygous mutant (identified in the progeny of heterozygous flies through the absence of a GFP-marked balancer chromosome) or hemizygous mutant escapers (ck\(^+/\)Df(2L)josp29). Alterations that affected changes in protein coding were verified by sequencing myoVIIA, which can be downloaded for viewing at the Kiehart Lab website (http://www.biology.duke.edu/kiehartlab/) using Rasmol or SwissPdb Viewer.

**RT-PCR and 5’ RACE:** Total RNA from overnight collections of \(w^{1118}\) embryos was prepared by standard methods and used as template for RT-PCR (using the One-Step RT-PCR kit; Qiagen, Valencia, CA) or 5’ RACE (using the FirstChoice RLM-RACE kit; Ambion, Austin, TX). Despecific primers were used for both RT-PCR and RACE and products were TA cloned (Qiagen PCR cloning kit) into DH5\(\alpha\) cells. DNA from randomly picked colonies was prepared by standard methods and subjected to automated sequencing.

**Lethal phase analysis:** Brief egg collections were made on grape plates with yeast paste from small population cages by standard methods (Wieschaus and Nüsslein-Volhard 1998). Embryos were dechorionated, transferred to a grid marked on a new plate, and overlaid with a 1:1 mixture of Halocarbon 27 and 700 oils (Sigma/Aldrich, St. Louis). A dissecting microscope was used to select embryos that were undergoing cellularization and/or gastrulation. Hatch rates were determined after 36 hr. Larvae were collected, counted, and transferred to a new food vial. After 5–7 days the number of larvae that had formed pupae was counted.

**SEM:** Adult flies were fixed in 70% ethanol for several hours, dehydrated into 100% ethanol, and then critically point dried in CO\(_2\) by methods recommended by the manufacturer of the critical point dryer (Ted Pella, Redding, CA). Samples were coated with 60% Au, 40% Pd, with a Hummer V sputter coater (Anatech, Springfield, VA), and then observed with a Philips ELC Plus (Amersham, Piscataway, NJ) or Super Signal West Amido using standard methods (Anatech, Springfield, VA), and then observed with a Philips ELC Plus (Amersham, Piscataway, NJ) or Super Signal West Amido using standard methods.

**RESULTS**

**Fly myosin VIIA:** We cloned myosin VIIA from flies using a PCR strategy designed to recover unconventional myosins. We recovered a partial cDNA encoding novel sequences similar to myosin heavy chain motor domains (Chen et al. 1991)—this partial clone became the founding member of the myosin VII subfamily of motor proteins (Cheney et al. 1993; Moosker and Cheney 1995). Positional cloning of a human Usher syndrome type 1B and of the Shaker defect in mouse characterized a full-length myosin VII cDNA and its gene (Chen et al. 1996; Weil et al. 1996; Kelley et al. 1997 and references therein). We recovered additional cDNA and genomic sequences and performed polytene in situ to show that this gene mapped to chromosomal location 35B (not shown). The physical map provided by subsequent sequencing of the Drosophila genome through the region (Ashburner et al. 1999) was compared to the genetic map and indicated that myosin VIIA likely corresponded to the \(ck\) gene.
**crinkled encodes myoVIIA:** Sequence analysis, reversion analysis, and fine-scale genetic mapping confirm that the myoVIIA transcription unit corresponds to the locus disrupted by *ck* mutations. An overview of the transcription unit, its relationship to other genes in the region, and to the orthologous transcription units from *D. pseudoobscura* and *Anopheles gambiae* appears in Figure 1. The domain structure of *ck/myoVIIA* appears as a schematic (Figure 1H) and on a sequence alignment with human myosin VIIA and worm myosin VII (Figure 2). An alignment with the two insect orthologs is shown in supplementary Figure 1 (http://www.genetics.org/supplemental/).

**Lesions in the *ck/myoVIIA* open reading frame characterize *ck* mutations:** Sequence analysis of genomic DNA purified from hemizygous escaper adults [*ck*1, *ck*13, *ck*4, and *ck*19/*Df(ck]] show nonsense mutations (premature stop codons) in *ck* (Leu1445Stop, truncates upstream of the first FERM domain) and *ck*13 (Arg768Stop, truncates in the light chain-binding IQ domain; see Figure 2). In addition, we found missense mutations in *ck*19 and *ck*4 that alter highly conserved sequences in the C-terminal, 20-kD subdomain of the myosin motor (Pro684Leu) and in the P-loop, polyphosphate binding sequence, GESGAGKT (Gly156-Glu; discussed below), respectively. Our sequencing effort also identified two insertional polymorphisms in various *ck* and control stocks that we sequenced (Figure 1G).

The locations of two *P*element insertion mutations near transcription start [*P(PZ)07130*, *BG00682*] and a third *P*element insert in the middle of intron 1 [*EP(2)2051*], all of which fail to complement *ck* alleles, are shown in Figure 1F. In *trans* with the large *ck* deletion *Df(2L)osp29*, they show characteristic *ck* phenotypes.

**Fine-scale genetic mapping of *P(PZ)07130* to *ck***: Fine-scale genetic mapping shows that *P(PZ)07130* maps to *ck* and not to adjacent loci. We isolated *ck* deletions using the *P*element transposase-mediated male recombination technique (PRESTON et al. 1996). These deletions usually retain the original insertion, extend either distally or proximally from the insertion site, and are recognized by exchange between flanking markers. From 14,545 progeny we selected 28 independent recombinants between the flanking markers (14 *dp b* and 14 *cn bw*). The 14 *dp b* recombinants could be either deletions extending distally or duplications extending proximally. Two were deletions affecting loci distal of *ck*. They were both lethal over severe *ck* alleles. The 14 *cn bw* recombinants could be either deletions extending proximally or duplications extending distally. Two were deletions affecting loci proximal of *ck*. These had very weak *ck* phenotypes over *ck* alleles [similar to the original *P(PZ)07130*]. Two further recombinants did not affect other loci but were weak *ck* alleles. Thus, the *P* elements map molecularly to myosin VIIA sequence and genetically to the *ck* locus.

**Reversion analysis:** To investigate further the relationship between *P(PZ)07130* and the *ck* locus, we performed reversion analysis. Of 54 transposase-induced excisions, 34 reverted to wild type and complemented severe *ck* alleles: they are likely the consequence of precise *P* element excisions. In contrast, 20 excisions failed to complement other *ck* alleles, 17 of which had a phenotype stronger than the original *P* insertion allele. These lines were likely the consequence of small deletions that removed part of the *ck* transcription unit. None of these more severe alleles were lethals, nor were they deletions of adjacent loci as demonstrated genetically. The *P(PZ)07130* insertion is not the cause of lethality in this chromosome: hemizygotes of this chromosome [in *trans* to *Df(2L)osp29*, a deficiency that removes the *ck* locus] or *transheterozygotes* with severe *ck* alleles are viable, suggesting that the lethality is due to another lesion on the chromosome.

**Phenotype of developmental arrest:** To evaluate when *ck/myoVIIA* function is required in development, we

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**Figure 1.**—A schematic overview of genomic organization at the *crinkled* locus at polytene location 35B shows *ck/myoVIIA* transcription units from *Drosophila melanogaster*, *D. pseudoobscura*, and *Anopheles gambiae* and indicates significant differences in exon/intron structure. (A) Numbers provide a scale in nt and increase in the direction of crinkled transcription (“0” was chosen early in the project at a site predicted to be close to transcription start—its location is therefore arbitrary). Transcription start, identified by 5’ RACE, is at nt 663. This origin corresponds to nt 58301 in accession no. AE003646 from the *Drosophila* genome project (in which numbers decrease in the direction of *ck* transcription). Adjacent genes *TfIIS* and *Suppressor of Hairless* are also shown. (B) An enlarged view of exons 1 and 2 from *D. melanogaster* shows differential splicing at the first intron. (C) Domain structure of *ck/myoVIIA* protein mapped onto the exon/intron structure of the *D. melanogaster* gene. (D and E) Exons and introns in the *D. pseudoobscura* and *A. gambiae* *ck/myoVIIA* genes are shown (compare to A). These species last shared a common ancestor with *D. melanogaster* 25 million and 250 million years ago, respectively (Russo et al. 1995; Zdobnov et al. 2002). In *D. pseudoobscura*, a single exon 8 replaces exons 8 and 9 of *D. melanogaster*. In contrast, in *A. gambiae*, the exons corresponding to the *melanogaster* exons 4, 5, and 6, and part of 7 are “fused” into exon 4. Likewise, parts of the *melanogaster* exons 8 and 9 are fused to make the *A. gambiae* exon 7. (F) Sequence flanking three *P* element-induced alleles of *ck*. Sequence in black is the 8-bp target sequence that is duplicated upon *P* insertion. Note that the target sites for *BG00682* and *P(PZ)07130* are directly adjacent to one another (shared sequence is underlined). (G) Insertional polymorphisms in *D. melanogaster* exon 1 and intron 11. (H) Schematic of the *ck/myoVIIA* protein outlines the overall structure of the protein and is color coded using the scheme in Figure 2. The schematic is drawn approximately to scale: the area of each “domain” is roughly proportional to the number of amino acids in that domain. Contact between dimerized heavy chains is shown at the coiled-coil region, the FERM domains, and the tail SH3 domain because those domains are thought to mediate protein--protein interactions. It is important to note that no evidence, either for or against such interactions, exists. Similarly, the MyTH4 and MyTH7 domains may also contribute to intradimer interactions. Rings drawn around the IQ motif region represent light chains.
Figure 2.—Outlines of the domain structure of *ck*/myosin VIIA are superimposed on a sequence alignment that compares *ck*/myoVIIA from *D. melanogaster* and *D. pseudoobscura* to myosin VIIA from humans and *hum6*/myoVII from *C. elegans*. Numbers indicate residue number in *D. melanogaster*. Domains were identified by searching the conserved domain database with reverse position-specific BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and/or correspond to the position of comparable domains previously identified in human myosin VIIA (Chen et al. 1996). Additional features are described in the text.
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investigated when the most severe ck/myoVIIA mutant animals die as hemizygotes. Nearly all ck13 mutants die as embryos and most ck7 mutants die as larvae, suggesting an acute need for ck/myoVIIA function in both stages.

We have not yet identified any morphological defects that correlate with these lethal phenotypes. All combinations of ck alleles yield some adult escapers, demonstrating that ck is not absolutely essential for viability. Nevertheless, for all intents and purposes, ck is essential: all emerging adults show a variety of morphological defects (described below) and fail to live very long. We have tested a small number of escapers for fertility and find that hemizygous males and females of the severe ck alleles are not fertile (ck7, ck13, and ck16). In all, 15 EMS-induced alleles of ck, plus 4 insertional alleles (3 P-element insertions and 1 due to the insertion of the complex element TE36) have been characterized genetically. Their hemizygous viabilities vary between 0.1 and 20% (the 3 P-element alleles are all weak by this criterion), but all hemizygous escapers have a typically crinkled phenotype.

**Phenotypes of escapers:** To understand the function of ck/myoVIIA better, we examined hairs, bristles, and aristae in escaper, hemizygous ck flies by scanning electron microscopy (SEM, Figures 3 and 4). We confirmed that these ck/myoVIIA mutant flies had wispy aristae (previously described as "feathery," Figure 4) and had aberrant wing hairs (setae) and bristles (chaetae) as described previously (Gubb et al. 1984; He and Adler 2002).

We observed four additional phenotypes (Figure 3). First, SEMs show that escapers have odd projections emanating from the base of macrochaetae on their thoraxes, perpendicular to the long axis of the bristle (Figure 3, A' and A''). Second, both macrochaetae and microchaetae are stubby, branched, frequently twisted (Figure 4, A vs. A'), and not uniformly (or smoothly) tapered. These bristle morphologies appear related to the third phenotype: deep grooves that are curved and fused characterize ck mutant bristles (compare macro-
shown). The serum fails to detect ck7 in adult escapers of ck13 vs. ck7 in wild type; compare setae in Figure 3, regarding the severity of this antiserum. Because the antiserum was raised against ck13 vs. chaetae in Figure 3, A gaster hairs tend to split into two to three hairs that are fre-
table. The wavy band in the thorax sample is due to the high abundance of muscle myosin that migrates just below the to distal direction, and is differentially spliced. Due to the large size of the fi rst two introns, the first 0.2–0.4 kb of cDNA (depending on splicing) spans 5.4 kb of genomic DNA. The remainder of the cDNA is more compactly organized, such that the 6784 bp of cDNA spans 7419 bp of genomic DNA and is interrupted by 9 introns of close to minimal length. The structure of the transcription unit and the protein that it encodes is detailed in Figure 5, both with premature stop codons), thereby verifying specificity of this antisera. Because the antisera was raised against an internal fragment of ck/myoVIIA, it is possible that in the ckD allele a stable, N-terminal fragment of protein is made and retains partial function. Our data suggest that no ck protein is made (however, see discussion regarding the severity of ckD1 vs. ckD alleles based on phenotype of arrest).

ck/myoVIIA transcription unit: We sequenced a nearly full-length ck cDNA and aligned it with Drosophila melanogaster genomic sequence (Release 3). Exon-intron boundaries and the overall organization of the transcription unit are shown in Figure 1 and supplemental Figure 1. ck lies ~1.8 kb proximal of the Suppressor of Hairless transcription unit and 1.4 kb distal to the TfIIS transcription unit on the left arm of the second chromosome. It is encoded by a 12.8-kb transcription unit [transcription start to poly(A) addition site] that includes 12 exons and 11 introns (Figure 1 and Table 1). It makes a 7.0-
to 7.2-kb mature transcript, is transcribed in a proximal to distal direction, and is differentially spliced. Due to the large size of the first two introns, the first 0.2–0.4 kb of cDNA (depending on splicing) spans 5.4 kb of genomic DNA. The remainder of the cDNA is more compactly organized, such that the 6784 bp of cDNA spans 7419 bp of genomic DNA and is interrupted by 9 introns of close to minimal length. The structure of the transcription unit and the protein that it encodes is detailed in supplemental material at http://www.genetics.org/supplemental/. Remarkably similar ck/myoVIIA proteins (see below) are encoded by orthologous genes in both D. pseudoobscura and the mosquito, A. gambiae (Holt et al. 2002), but the distribution of exons and introns is not preserved (Figure 1).

ck/myoVIIA protein organization: All the D. melano-
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**gaster ck/myoVIIA transcripts include a 6501-bp open reading frame that encodes a 250-kD protein (Figures 1 and 2). The size of this ORF is consistent with the ck/myoVIIA band seen on immunoblots (Figure 5). The relationship between ck/myoVIIA, its ortholog from humans (61.7% identical), the single myosin VII found in *C. elegans* (58.8% identical), and its orthologs from other insects is shown in sequence alignments (Figure 2 and supplemental Figure 1). A consensus sequence indicates the shared amino acid if two or more sequences match, an x if there is no match, and a blank if there is a gap. A corresponding bar color codes the alignment: regions of sequence identity are shown in red (perfect match), sequence similarity is in green (two of the three residues match), no match is in blue, and a gap is blank. Boxed sequences indicate various domains that are shared between ck/myoVIIA and other proteins. Sequence motifs in the myosin head are shaded and labeled and are based on detailed modeling of the 3-D structure of the ck/myoVIIA head described below. Also shown in red text, in and above the alignments, are the 40 amino acids that distinguish the *D. melanogaster* ck/myoVIIA from the *D. pseudoobscura* ck/myoVIIA (the amino acid shown above the alignment is the one from *pseudoobscura*, the two proteins are 98.2% identical). For comparison, the *A. gambiae* protein is 88.1% identical (supplementary Figure 1). Hot spots for amino acid substitutions exist in several locations.

**Myosin head domain:** The myosin VIIA head, extending from the N terminus through the motor domain, shows remarkable sequence identity with its human myosin VIIA ortholog and the single myosin VIIIs from *C. elegans* and *D. discoideum*. There is considerable sequence identity to heads from other myosin superfamily members, including the class I and II myosins, for which crystal structures are available (36–45% identity in the myosin head). This prompted us to generate a 3-D homology model of the ck/myoVIIA head (Figures 6 and 7). The model satisfies most known physical constraints for well-resolved X-ray structures, with a Ramachandran Z-score of −1.687, no Ramachandran outliers, and an average packing Z-score of −1.008. Thus it is a useful working model for the structure of ck/myoVIIA in the absence of a crystal structure. Our confidence in the model is low in the regions, usually variable loops, that are poorly or not resolved in the template structures. The overall topology of our model is similar to that of myosin I and II heads, yet distinct differences are likely to account for the unique properties of myosin VIIA.

**ck/myoVIIA has an N-terminal SH3 subdomain:** Like myosin II, the ck/myoVIIA head predicted by our homology model has a N-terminal spectrin-like SH3 subdomain formed by amino acid residues Tyr 9 to Gln 63 (Figure 6). This feature of myosin VIIA has previously been overlooked in the primary sequence alignments used to evaluate domain structure. In myosin II, this region forms a structural unit independent of the rest of the head, similar to the SH3 subdomain of spectrin (*Rayment et al. 1993; Dominguez et al. 1998*; overall structure of the myosin head reviewed in *Geeves and Holmes 1999*; *Houdusse and Sweeney 2001*). The primary sequence of this subdomain is well conserved between ck, its human ortholog, and the worm myosin VII (but not the Dictyostelium myosin VII).
Figure 7.—The α-carbon backbone of the N-terminal SH3 subdomain predicted by our model and compared to scallop muscle myosin II (chrome yellow) and chicken smooth muscle myosin (peach). The fit is excellent except where there are additional residues in ck/myoVIIA compared to the reference structures (three additional residues at Pro-14 to Gly-17 and one additional residue at Phe-20 to Asp-21). Shown are ck/myoVIIA residues Tyr-9 to Gln-63, chicken smooth muscle myosin Leu-34 to Ser-84, and scallop muscle myosin II Ser-37 to Gln-83.

Motor domain: Our model predicts the traditional division of the myosin head into distinct subdomains, seen in the structures of all myosin heads solved to date and based on limited proteolytic cleavage of myosin II into N-terminal 25-kD, middle 50-kD, and a C-terminal 20-kD fragments. As expected, it suggests that the motor core of the ck/myoVIIA head, which includes the nucleotide-binding pocket (γ-phosphate-binding P-loop of the 25-kD subdomain) and switch I and II of the upper 50-kD subdomain, is conserved in structure. Regions of sequence divergence correspond mainly to flexible surface loops, some of which participate in actin binding, and “hinges” or “joints” between conserved elements of the motor core (Figure 1). Finally, the C-terminal subdomain comprises the “converter,” believed to amplify the relatively small conformational changes in the motor core to drive movement of the lever arm that extends through the neck (and includes the light-chain binding, IQ repeats). The conformations of the joints and of the converter in our model are characteristic of the ADP·P$_\text{i}$-bound state or state II (Houdusse et al. 1999). Our homology model allows interpretation of the structural ramifications of amino acid replacements in ck/myoVIIA mutations (see discussion).

ck/myoVIIA tail and the myosin tail homology (MyTH7) domain: Most of the remainder of the protein is remarkably similar to its human ortholog and worm homolog. A sequence predicted to form a coiled-coil is considerably shorter than the corresponding region in vertebrate myosin VIIAs and even in vertebrates the role of this region in dimerization has been called in question (Inoue and Ikebe 2003). Following the putative coiled-coil region, two modules, each of which contains a MyTH4 domain and a FERM domain, appear in a tandemly repeated fashion (Figures 1H and 2, supplemental Figure 1). The two modules are separated by an SH3 domain (distinct from the spectrin-like SH3 domain at the very N terminus of the ck/myoVIIA heavy chain). Since original alignments were performed (e.g., Chen et al. 1996), the crystal structures of the ezrin, radixin, and moesin FERM domains showed that FERM domains have a cloverleaf like structure, consisting of three subdomains or lobes, each containing ~100 amino acids (Pearson et al. 2000; Hamada et al. 2003; Smith et al. 2003 and references therein). Only the first ~200 amino acids (i.e., the first two lobes) of the ck/myoVIIA FERM domain are well conserved with FERM domains from other proteins. Nevertheless, sequences immediately following these two partial FERM domains are highly conserved among myosin VIIAs, myosin VII, and myosin VIIIIs. Indeed, among these subclasses of motor proteins, these stretches are as conserved as or more highly conserved than the two lobes of the FERM domain that directly precede them (vs. the Anopheles, human, and C. elegans ck/myoVIIAs; see Table 2). Due to this remarkable conservation, we refer to the ~100-amino-acid stretch following ck/myoVIIA FERM 1 and the ~75-amino-acid stretch following FERM 2 as MyTH7 domains (see Figure 1H and discussion).

Conserved sequence 3’ of the poly(A) addition site: 3’ of the site at which poly(A) is added, a sequence that is remarkably conserved between D. melanogaster and D. pseudoscura (94.2% identical over 119 bp) suggests that there may be a gene that encodes a micro-RNA (Ambros 2003; Lai et al. 2003). This sequence is not conserved in A. gambiae and shows less secondary structure than many of the micro-RNAs identified through genomic strategies in D. melanogaster (Lai et al. 2003). Experimental analysis will be required to evaluate the significance of the conservation in this region.

DISCUSSION

Here, through a combination of molecular and genetic analysis, we provide formal proof that the fly myosin VIIA heavy chain is encoded by the crinkled locus. In addition, we establish that two severe alleles (molecular nulls that cannot encode more than a fraction of the myosin VIIA motor domain) die as embryos and larvae, although all alleles show a small number of escapers, animals that can survive to adulthood without zygotically encoded ck/myoVIIA. These escapers are severely compromised—we have been unable to set up a homozygous stock. We demonstrate that the ck/myoVIIA pro-
tein is present at low abundance throughout development. While we have been unable to identify a function for crk/myoVIIA that is required for embryonic and larval viability, we document phenotypes that confirm and extend older observations on crk phenotypes: bristles and hairs (chaetae and setae) have aberrant morphologies and/or distributions.

We used the highly conserved sequence among different myosin VIIs to investigate the structure of crk/myoVIIA in two ways. First, we generated a homology model of the crk/myoVIIA head on the basis of solved structures of myosin I and IIs and show that myosin VIIs have a heretofore unnoticed N-terminal, spectrin-like SH3 subdomain. We used the model to hypothesize the effect of specific amino acid substitutions that characterize sequenced mutants. In addition, we compared the sequence of the melanogaster crk/myoVIIA tail to its orthologs from another D. pseudoobscura, mosquito, and humans and to a myosin VII homolog from worms. We identified two highly conserved protein repeats that are shared by myosin VIIs, VIAs, and VIIBs. We refer to these conserved repeats as MyTH7 domains. One possibility is that the two MyTH7 domains fold to form a specialized FERM lobe 3 subdomain. Another alternative is that the MyTH7 domain forms a distinct structure that folds and functions independently of the first two lobes of the FERM domain. Clearly, both structure and structure/function analysis of the crk/myoVIIA tail will be required to distinguish among these possibilities. Together our studies provide an essential step in the characterization of this motor protein in flies.

Animals homozygous or hemizygous for severe crk mutants almost all die as embryos or in early larval stages. Nevertheless, a small fraction of so-called escapers emerge as adults, indicating that those individuals that persist through an acute, early period in ontogeny, during which crk/myoVIIA is all but essential, can survive through the remaining stages of development. Such escapers have defects in the distribution and morphology of hairs and the morphology of bristles all over their bodies, fail to live very long, and are infertile. Together, these observations demonstrate that myosin VIIA plays a more extensive role in flies than in vertebrates, where defects in myosin VIIA cause aberrant sensory perception but appear to have little or no effect on viability per se (of course from a Darwinian perspective, most vertebrates whose hearing and vision are defective will be far from fit). A simple explanation for this discrepancy may be differences in the pattern of expression of myosin VIIA in flies and vertebrates. In flies, the distribution of mutant phenotypes, immunoblot analysis, and preliminary antibody and RNA in situ studies (data not shown) all point to an expression pattern of crk/myoVIIA that is widespread, if not “ubiquitous.” In contrast, the tissues affected by defects in vertebrate myosin VIIA, cochlea, retina, lung, and testis are commensurate with an expression pattern that is restricted to these tissues and the kidneys. Previous investigators hypothesized that a lack of phenotype in kidney might be attributed to redundant function supplied by additional myosin superfamily members (Hasson et al. 1995). In addition to the widespread role for crk/myoVIIA in epithelial cell function (demonstrated by defective patterns of setae on all body parts), it is interesting to note that myosin VIIA also plays a role in fly sensory perception: there are defects in macrochaetae formation (bristles are sensory structures in flies) and crk/myoVIIA is required for hearing in flies (S. V. Tod and D. F. Eberl, personal communication and Tod et al. 2003). By comparing the function of crk/myoVIIA in embryos—where lethality due to specific effects on sensory cells is unlikely—to the function of crk/myoVIIA in sensory perception, we may well gain insight into the chemomechanical constraints that define the niche in which this motor protein functions.

Another explanation for different roles of myosin VIIA in flies and vertebrates may be redundancy that allows other myosins to compensate for defects in myosin VIIA in vertebrates but not in flies. Indeed, humans, mice, and flies have genes that encode distinct myosin VIIBs and all have a myosin XV, a more distantly related myosin whose tail nevertheless includes two MyTH4 domains and a single FERM domain and is therefore more closely related to this class than to other myosin classes

### Table 2

<table>
<thead>
<tr>
<th>Domain protein</th>
<th>MyTH4-2 (%)</th>
<th>FERM-1 lobes 1 and 2 (%)</th>
<th>MyTH7-1 (%)</th>
<th>FERM-2 lobes 1 and 2 (%)</th>
<th>MyTH7-2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anopheles VIIA</td>
<td>87/95</td>
<td>91/97</td>
<td>97/98</td>
<td>89/97</td>
<td>97/98</td>
</tr>
<tr>
<td>Human VIIA</td>
<td>60/76</td>
<td>72/86</td>
<td>72/86</td>
<td>72/85</td>
<td>90/96</td>
</tr>
<tr>
<td>C. elegans VII</td>
<td>59/73</td>
<td>63/81</td>
<td>64/79</td>
<td>54/70</td>
<td>56/80</td>
</tr>
<tr>
<td>Dicytostelium VII</td>
<td>26/48</td>
<td>28/52</td>
<td>22/42</td>
<td>19/42</td>
<td>23/45</td>
</tr>
<tr>
<td>Fly VII B</td>
<td>40/56</td>
<td>41/61</td>
<td>30/54</td>
<td>42/66</td>
<td>50/69</td>
</tr>
<tr>
<td>Fly XV</td>
<td>32/43</td>
<td>47/61</td>
<td>NA</td>
<td>26/51</td>
<td>26/46</td>
</tr>
</tbody>
</table>

Entries indicate percentage identity/percentage similarity, allowing conservative substitutions. Numbers were generated with BLASTP using a BLOSUM 62 matrix.
Interestingly, while defects in myosin VIIB have not been described or associated with disease loci, defects in myosin XV cause deafness in humans and mice, suggesting that myosin VIIA and XV may have some (although clearly not completely) overlapping function (reviewed in Friedman et al. 1999; Redowicz 2002). Overall, sequence comparisons between vertebrate and Drosophila myosin VIIIs and XVs suggest that all three of these myosin heavy chain subfamily members were apparently present in the last common ancestor of these organisms (0.6 and 1.2 billion years ago; Benton and Ayala 2003). At this time our observations cannot distinguish between a model in which a common set of functions is performed by a subset of myosin motors or that evolution has called upon fly ch/myoVIIA to perform functions that are distinct from those required in vertebrates. A complete understanding of how these FERM domain myosins contribute to biological function may require strategies designed to affect all three loci simultaneously. The unique molecular genetic tools available in flies, our ability to compare mutagenized ch/myoVIIA function in vitro and in vivo, and the identification of two other myosins in the ch/myoVIIA subfamily (VIIB and XV) suggest that analysis of myosin VIIA function in this system will be particularly rewarding. Moreover, comparing myosin VIIA function in flies and vertebrates to its function in systems that have a single myosin VII isoform may provide further insight into the evolution of this subfamily of the myosin motors.

Our observations indicate that ch/myoVIIA plays an important role in positioning the actin prehairs and bundles that give rise to bristles and hairs. The grooves in bristles are formed during development by bundles of actin filaments that function as struts during bristle formation (see Tilney et al. 2003 and references therein). The multiple body and wing setae phenotype observed suggests that ch/myoVIIA contributes to the distribution and/or integrity of microvillus-like prehairs that have been best studied in wings (Wong and Adler 1993; Turner and Adler 1998). Turner and Adler (1998) observed that the multiple wing hair phenotype of ch mutants could be phenocopied by low doses of cytochalasin. Why the absence of a motor protein should mimic the effects of a drug that presumably inhibits F-actin assembly raises a mystery. Analysis of the ontogeny of the ch/myoVIIA phenotype during hair development and analysis of epistasis with other genes that participate in the process may well provide insight into the mechanisms by which ch/myoVIIA functions in this process.

Our homology model of the ch/myoVIIA head facilitates analysis of the defects caused by various missense mutations. In ch10, Pro-624 is replaced by Leu. Pro-624 is located in the 20-kD subdomain, N-terminal to the SH-1 helix and the converter. This proline residue is conserved in the representative myosin VIIIs shown in Figure 2, other myosin VIIIs for which sequence in the region is available, and in 120 of the 143 myosins shown on the Myosin Home Page (http://www.mrc-lmb.cam.ac.uk/myosin/trees/trees.html). Its replacement with Leu is expected to alter the trajectory of the polypeptide backbone. In addition, it is expected to affect the stereochemistry of the hydrophobic interface between the 20-kD subdomain and the HP helix that extends into the relay element (Figure 2). This interface contributes to the rigidity of the relay that is crucial to the positioning of the converter and, as a consequence, to the overall ability of the ch/myoVIIA motor domain to produce movement.

The ch10 lesion disrupts the phosphate-binding loop that is shared by myosins and other polyphosphate-binding proteins by replacing Gly-156 with Glu. Our model predicts that the disruption of this highly conserved GESGAGKT sequence may stabilize the loop against conformational changes, which would be highly deleterious to motor activity.

The model also makes interesting predictions regarding the detailed structure of the ch/myoVIIA motor domain. For example, it confirms that the junctions between the 25-kD and the upper 50-kD subdomains (loop 1, Gly-178 to Trp-182) and between the lower 50-kD and the 20-kD subdomains (loop 2, Ile-586 to Pro-602) are both short and compact. Loop 1 affects the rate of ADP release by Dictyostelium myosin II (Murphy and Spudich 1998), while loop 2 affects the actin-binding affinity and maximum ATPase rate of this myosin (Murphy and Spudich 1999). These loops vary considerably in length and composition in different myosins, giving rise to functional differences between myosins from different classes and species. The model allows the intelligent engineering of site-directed mutations to probe the function of these loops in ch/myoVIIA. Although the elucidation of the precise orientation of residues will require the high resolution of X-ray crystal structures of the myosin head in various nucleotide states and appropriate EM studies of the acto-myosin VIIA complex, the homology-modeling approach demonstrates its utility in the interpretation of molecular lesions in mutant ch/myoVIIAs and suggests interesting targets for future functional studies.

Analysis of the two nonsense mutations suggests that in these alleles a small fragment of the ch/myoVIIA protein is synthesized. The ch12 mutation (hemizygous animals die as embryos) is more severe than the ch1 mutation (hemizygous animals die as larvae). ch12 encodes an open reading frame that ends in the middle of the ch/myoVIIA IQ domain and is 677 codons shorter that the ch open reading frame. We would expect that mRNA instability, due to nonsense-mediated decay (Wagner and Lykke-Andersen 2002; Gatfield et al. 2003), would render both alleles equivalent in severity, yet they show different lethal phases. One possible explanation is that the longer fragment has residual ch/myoVIIA that can substitute, in part, for wild-type function. Alternatively, the longer mutant protein may better stabilize the maternal load of
wild-type ck/myoVIIA, thereby increasing ck/myoVIIA activity in the ck mutant flies. Our data do not distinguish between the two possibilities.

Our studies provide the data that establish that crinkled encodes fly myosin VIIA, detail the structure of the ck transcription unit, identify the molecular lesion in four ck alleles, establish the phenotype of arrest of severe ck alleles, and document new defects in setae and chaetae morphology in ck escapers. Homology modeling and sequence comparisons identify a spectrin-like SH3 domain comparable to that seen in myosin IIIs and show highly conserved sequences that we have dubbed MyTH7 domains. Together, they provide essential groundwork for additional studies on the function of this important motor molecule in development and morphogenesis.

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LITERATURE CITED


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