One of the two cytoplasmic actin isoforms in *Drosophila* is essential

Cynthia R. Wagner[†‡§], Anthony P. Mahowald[†], and Kathryn G. Miller[‡]

[‡]Department of Biology, Washington University, St. Louis, MO 63130; and [†]Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637

Contributed by Anthony P. Mahowald, April 18, 2002

Actin is a highly conserved protein found in all eukaryotic organisms. Most organisms have multiple cytoplasmic actin genes that encode isoforms with slightly different amino acid sequences. These different isoforms are coexpressed in many cell types. Why organisms have multiple very similar cytoplasmic actin genes is unclear. We have addressed this question with the cytoplasmic actins in *Drosophila*, Act5C, and Act42A. These isoforms differ by only two amino acids and both genes are expressed in all cells at all times during development. We identified *P* element insertions in the *Act5C* gene that resulted in a lethal phenotype. The lethal phenotype is rescued by a transgene with a genomic fragment that includes *Act5C* regulatory and amino acid coding sequences. A hybrid transgene containing the protein coding sequence for the Act42A isoform, under the control of the regulatory regions of the *Act5C* gene, also rescues the lethality of the *Act5C* mutants. Furthermore, flies that carry only one copy each of *Act5C* and *Act42A* are viable. These results suggest the amino acid differences between these two cytoplasmic actin isoforms are not important for function and the need for increased gene dosage to provide more actin is not likely to explain the existence of multiple genes. Instead, our results suggest that regulated expression of *Act5C* is essential to the fly.

Actin is a key component of the cytoskeleton in all eukaryotic organisms. It is critical for cell movement, determination of cell shape and cell division, and it plays important roles in many other processes, including organelle transport. Actin is a highly conserved protein, with only a few amino acid sequence differences between species as evolutionarily distant as humans and slime molds. Multicellular organisms have several isoforms of cytoplasmic actin, which are coexpressed in most cell types and have very similar sequences to each other (1).

There are several possible reasons why organisms have multiple, highly similar, actin isoforms. Organisms need a large quantity of actin, and the best way to provide enough actin may be to have multiple genes. If this were the case, the amino acid sequence differences between the isoforms would likely have no functional consequences. A related explanation for multiple isoforms is that because some cells need more actin than others, multiple genes provide a mechanism for differential regulation of actin expression. In this case, the amino acid differences would not be functionally significant either, but the regulation of the expression of the genes would be critical. Finally, the small number of amino acid sequence differences may be functionally important, allowing different isoforms to have different roles in the same cell.

There are several lines of evidence that strongly suggest that different isoforms do indeed have specialized functions (1). Myogenic cells show regulated isoform-specific expression, switching from the expression of cytoplasmic to muscle isoforms. In quiescent aortic smooth muscle cells, an abundance of α-vascular smooth muscle actin isoform is produced; but when these cells begin to proliferate and migrate in culture, they express nonmuscle actins (2). Nonmuscle vertebrate actin isoforms, β and γ, cause different phenotypes when overexpressed in tissue culture cells. For example, overexpression of β-actin in C2 myoblasts leads to increased cell surface area and loss of stress fibers, whereas overexpression of γ-actin causes the cells to round up (3). It has also been demonstrated that the actin-binding protein ezrin interacts specifically with β- but not α-actin filaments (4). Although these experiments support the idea that the sequence differences among cytoplasmic actin isoforms are functionally important, there has been no definitive test of whether different cytoplasmic isoforms can functionally replace each other. In this work, we directly test whether the cytoplasmic actins in *Drosophila* are functionally unique.

In *Drosophila melanogaster*, two isoforms each of cytoplasmic actin, larval muscle actin, and adult muscle actin have been identified, all encoded by different genes (5, 6). Experiments by Fyrberg *et al.* (7) have shown that specific functions do exist for muscle-specific isoforms. Null mutations in the *88F* gene, encoding an adult muscle isoform, result in a flightless fly. Flight can be restored by germ-line transformation of a wild-type copy of the *88F* gene. A hybrid gene, containing the coding region for the 79B isoform (the other adult muscle-specific isoform), under the regulatory control of the *88F* gene also rescues the flight defect. However, substituting other actin isoforms, including the two larval muscle-specific or one of the cytoplasmic isoforms for the 88F protein, does not restore flight to the *88F* mutants. A similar experiment performed by Brault *et al.* (8) has shown that the human β-actin isoform does not fully compensate for the endogenous 88F isoform in restoring flight to *88F* mutant animals. Although they report assembly of the human β-actin isoform into thin filaments, sarcomeric organization of the indirect flight muscle is defective.

Because muscle actin participates in a uniform and relatively static (as least from a polymerization standpoint) structure, it could be argued that the result that isoforms are functionally specialized in muscle is not generally applicable.

Cytoplasmic actins, in contrast to muscle actins, participate in many different structures and interact with a large number of different proteins to form these structures. Cytoplasmic actins might be thought of as more "multifunctional" than muscle isoforms, and thus need to be less specialized. However, whether individual cytoplasmic isoforms are specialized to perform subsets of the many functions required of actin remains unanswered.

In *Drosophila*, cytoplasmic actin is supplied by Act5C and Act42A (9, 10). There are only two amino acid differences between Act5C and Act42A (see *Results*). Each substitution is conservative in nature (A261S and I331V), and neither is located in a region of the actin molecule known to interact with actin-binding proteins (11). mRNAs for both *Act5C* and *Act42A* are maternally loaded and initially, transcripts for both mRNAs are evenly distributed throughout the early embryo (10). Zygotic expression of both genes occurs throughout embryonic development in all cells. The relative levels of transcripts for the two genes vary in particular tissues: *Act5C* mRNA levels are highest in the devel-

Abbreviation: GFP, green fluorescent protein.

[§]To whom reprint requests should be sent at the present address: Department of Biological Sciences, Goucher College, 1021 Dulaney Valley Road, Baltimore, MD 21204. E-mail: cwagner@goucher.edu.

CELL BIOLOGY

oping brain, whereas *Act42A* mRNA levels are most abundant in the developing gonad and portions of the intestine (10).

We used *Drosophila* as a genetically tractable model system to study the function of cytoplasmic actin. We asked whether the two cytoplasmic isoforms have different functions, and if so, were these specialized functions the result of amino acid sequence differences, the different expression patterns of the two genes, or a gene dosage effect. To differentiate between the three possibilities, we identified mutations in the *Act5C* gene. These mutations cause a lethal phenotype, suggesting that Act5C does have a unique function. We demonstrate that the lethal phenotype of the mutants can be rescued with a hybrid gene containing the protein-coding region of the *Act42A* gene under the control of the regulatory regions of the *Act5C* gene. This result establishes that the amino acid differences between the two isoforms are not functionally important. Manipulation of the gene dosage of both genes indicates that it is unlikely that multiple actin genes are required merely to provide enough actin globally. These results indicate that the pattern of expression of the Act5C protein is important for its essential function, and suggest that some tissues in which Act5C is expressed at high levels cannot develop or function normally with only the actin provided by Act42A.

Materials and Methods

P Element Mutagenesis to Isolate Act5C Mutations. A local *P* element mobilization screen (12) was performed with *fs(1)ph*$^{8-29B}$ flies that have a *P* lArB element inserted seven kb downstream of the start site of transcription of the *Act5C* gene (13). Fifty X chromosome lethals were isolated and four *Act5C* lethal alleles were identified. Those lethal mutations that were rescued by an *Act5C* transgene were subjected to inverse PCR to confirm the insertion of a *P* element near the gene.

Determination of Lethal Phase. To determine when the *Act5C* mutants died, we placed each of the four mutant chromosomes in *trans* to an X chromosome balancer that carried a green fluorescent protein (GFP) marker. Each mutant line was allowed to lay eggs overnight, and mutant larvae were selected by lack of GFP fluorescence. The homozygous mutant larvae were transferred to new grape plates and examined each day.

Northern Analysis. To determine the amount of residual *Act5C* transcript present in larvae, all four *Act5C*^{P} alleles were placed over a GFP X chromosome balancer, hemizygous mutant larvae were selected by lack of GFP fluorescence, and RNA was isolated (14) from these larvae.

To determine the amount of transcript produced by the different *Act5C* transgenes, the different transgenes were placed in the background of a lethal deficiency [*y w l (1)Z76B*] whose distal breakpoint deletes DNA containing the two most 3′ polyadenylation signals of *Act5C*. Males were recovered by introducing a *P* element, *P[YES BC]*, which rescues the lethality caused by the *Df* but does not restore the missing *Act5C* mRNAs. These males had the following genotype: *y w l (1)76B;P[Act5C]; P[YES BC]*. Adult males were collected and RNA-prepared.

In both cases, total RNA was electrophoresed, transferred to Nytran (Schleicher & Schuell), and the filter was hybridized to an *Act5C*-specific probe, pA1 (15), which recognizes all three size classes of *Act5C* transcripts. In addition, both filters were probed with a ^{32}P-labeled *RP49* probe to ensure that equal amounts of RNA were loaded.

Construction of Transgenes and Generation of Transgenic Lines. All transgenes were constructed with standard DNA molecular cloning techniques (16). In the case of the *Act5C-42A* transgene and the *H264Q* transgene, a 3.1-kb *Xba*I (isolated from ϕPCR-A) "cassette" was used as a template for *in vitro* mutagenesis reactions with the Chameleon kit (Stratagene) to change the appropriate amino acids present in each transgene. The changes were verified by sequencing with primers specific to the *Act5C* gene.

All transgene constructs were injected into *w;[Δ2-3 ry*$^{+}$*]Sb ry/TM3* flies (17), injected survivors were mated to *yw67c23* flies, and transformants in the resulting progeny were identified by red eye color. Stable transformed lines were made by removing the *[Δ2-3 ry*$^{+}$*]Sb ry* chromosome and using appropriate balancers for each transformed line.

Genomic DNA and Reverse Transcription (RT)-PCR Sequencing. Genomic DNA or total RNA was isolated from either *IsoA* or *OreR* adult flies as described by Jowett (14). Genomic DNA was used as a template for amplifying the coding regions of either *Act5C* or *Act42A* genes. The RNA was used to generate cDNAs with an RT-PCR kit (Stratagene). These single-stranded cDNAs were the templates in PCR designed to amplify either *Act5C* or *Act42A* cDNAs by using primers specific for each gene. The amplified products were electrophoresed, gel purified with the Qiaquick extraction kit (Qiagen, Chatsworth, CA), and sequenced directly with gene-specific primers.

Rescue Analysis. Males carrying the different transgenes were crossed to females of the genotype *ywAct5C*^{P}*/FM6;+/+;+/+*. Rescue was determined by the presence of non-Bar, red-eyed male progeny. All four *Act5C P* element mutations were tested for rescue with the different transgenes.

Gene Dosage Studies. Females with the genotype *ywAct5C*^{P*} were mated to *Df(2R)nap1/In(2LR)Gla Dp(2:2)BGwg*$^{Gla-1}$ or *Df(2R)nap9/In(2LR)Gla Dp(2:2)BGwg*$^{Gla-1}$ males. Both are heterozygous for the *Act42A* gene. Each *Act5C P* element allele was mated to each deficiency. The number of *ywAct5C*^{P*}*/+;+/Df(2R)nap1* or *ywAct5C*^{P*}*/+;+/Df(2R)nap9* female progeny was compared with the number of *ywAct5C*^{P*}*/+;+/In(2LR)Gla Dp(2:2)BGwg*$^{Gla-1}$ female progeny.

Results

P Element Insertions Upstream of the *Act5C* Transcription Start Site Are Lethal. To obtain *Act5C* mutations that would permit us to examine the reasons that animals have multiple actin genes, we performed a local mobilization screen to obtain *P* element insertions within the *Act5C* gene. Our parental line, *fs (1)ph*$^{8-29B}$, was one in which a *P* element insertion resided ≈7.5 kb downstream of the transcription start site of the *Act5C* gene (Fig. 1). The presence of this *P* element, *fs (1)ph*$^{8-29B}$, causes a female sterile phenotype (13), but *Act5C* mRNA levels are unaffected (C.R.W., data not shown). We mobilized the parental *P* element and, because *Act5C* is on the X chromosome, we collected flies that had a lethal phenotype with respect to the X chromosome. Females heterozygous for the lethal chromosome in *trans* to an X chromosome balancer were collected, DNA was isolated, and genomic Southern analysis was performed to identify insertions in the *Act5C* region (data not shown). We identified four such lines, each with a *P* element insertion within a 200-bp region ≈3 kb upstream of the start site of transcription of the *Act5C* gene (Fig. 1). All four lines also carry a copy of the original *P* element insert, and one line has a total of three *P* element insertions (data not shown).

All four *Act5C P* element mutant alleles die as first instar larvae. Shortly after hatching the mutant larvae are indistinguishable from wild type with regards to movement, but they soon appear noticeably sluggish and die within 4 days after egg hatching without growing substantially or molting.

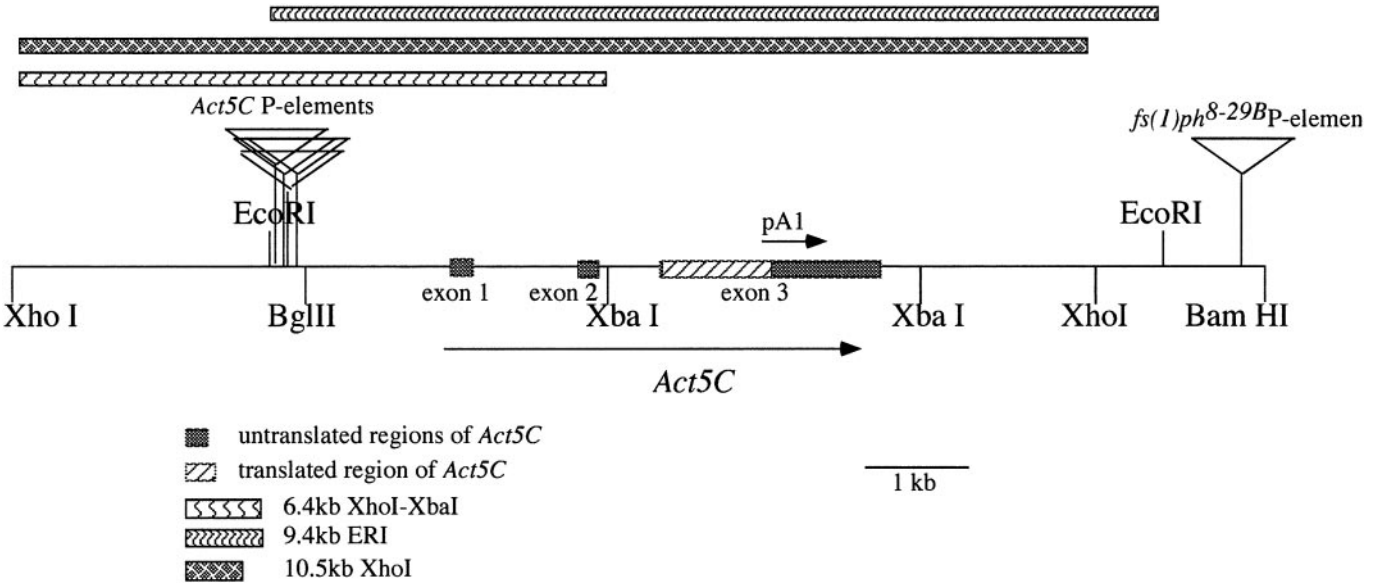

Fig. 1. Map of the *Act5C* region. Shown are the *Act5C* transcription unit, the location of *P* element insertions (original and *Act5C*), DNA included in transgene constructs, and an *Act5C*-specific probe, pA1. The 6.4-kb *Xba*I-*Xho*I DNA fragment was used as a probe for the Southern blots. All four *Act5C P* elements map within the ~200-by *Eco*RI-*Bgl*II fragment.

***Act5C* mRNA Levels Are Affected by the Presence of the *P* Element Insertions Upstream of the *Act5C* Gene.** We collected hemizygous mutant larvae and measured *Act5C* mRNA abundance in each of the *P* element lines as compared with wild type. As shown by Northern analysis (Fig. 2), levels of *Act5C* mRNAs are severely reduced in all four of the *P* element insertion lines. *Act5C* mRNA is maternally loaded into the oocyte and we believe the small amount of *Act5C* mRNA seen in the *P* element insertion lines reflects residual maternally loaded *Act5C* mRNA.

The Lethal Phenotype of the *P* Element Insertions Can Be Rescued by Introducing a Wild-Type Copy of the *Act5C* Gene. To demonstrate that the lethal phenotype of these *P* element insertions is the result of loss of Act5C function, we introduced a wild-type copy of the *Act5C* gene into the mutant background and assayed for rescue of the lethality.

Two different rescue constructs, 9.4*Eco*RI Act5C and 10.5*Xho*I Act5C (Fig. 1), were transformed into flies by means of *P* element-mediated transformation and stably transformed lines were obtained. We assayed for *Act5C* expression from the transgenes by crossing the transgenes into a line of flies that express only the 1.8-kb transcripts of *Act5C* (for details on the strain, see *Materials and Methods*). As shown in Fig. 3 (lanes 3–6), three independent lines carrying the 10.5*Xho*I Act5C transgene and one line carrying the 9.4*Eco*RI Act5C transgene express the 2.0-kb and to a lesser extent, the 2.3-kb transcripts of the *Act5C* gene. These transcripts are the result of *Act5C* transcription from the transgene.

We then tested the ability of these four transgene insertions to rescue the lethal phenotype of the four *Act5C P* element mutant alleles. All four Act5C transgene insertions rescued the lethal phenotype of the *Act5C P* element mutant alleles. Quantitative data for rescue efficiency of two mutant lines are shown in Table 1. If the wild-type transgene fully rescues the lethality of the mutants, we would expect the number of males carrying an X

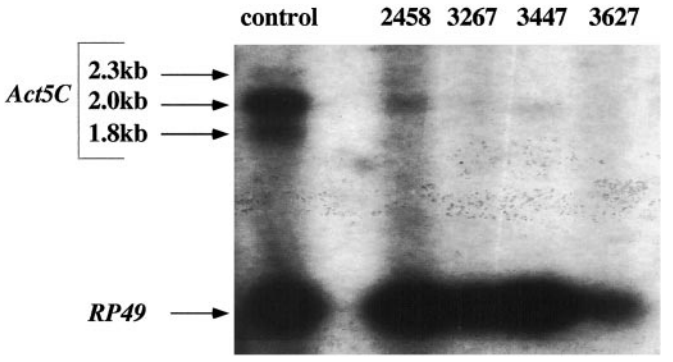

Fig. 2. Northern blot analysis of *Act5C* mutants. Mutant alleles were placed in *trans* to a GFP X chromosome balancer, and fluorescent and nonfluorescent first instar larvae were collected separately. Total RNA was isolated from pools for each mutant line. To determine the amount of *Act5C* transcript, an *Act5C*-specific probe, pA1, was used. *RP49* is a loading control and demonstrates approximately equal loading in all lanes.

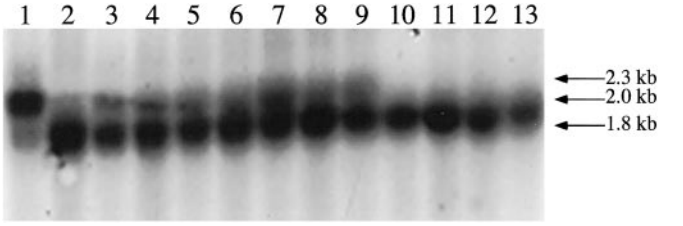

Fig. 3. Northern blot analysis to determine whether *Act5C* transgenes are expressed. Transgenes were placed in a genetic background [*y w l (1)76Z; YES BC*] in which only the 1.8-kb message of *Act5C* is made (lane 2). Males with the correct genotype were collected and total RNA was isolated. The *Act5C*-specific probe, pA1, was used to detect expression of the transgenes by the presence of the 2.0-kb message. Genotypes of the flies used to isolate RNA were as follows: lane 1, wild type; lane 2, *y w l(1)76Z; YES BC*; lane 3, 9.4*EcoRI Act5C* (Act5C wild-type transgene); lane 4, 10.5*XhoI Act5C-D* (Act5C wild-type transgene); lane 5, 10.5*XhoI Act5C-G* (Act5C wild-type transgene); lane 6, 10.5*XhoI Act5C-H* (Act5C wild-type transgene); lane 7, *Act5C-42A-D*$_2$; lane 8, *Act5C-42A-K*$_1$; lane 9, *Act5C-42A-JJ*$_1$; lane 10, *Act5C-42A-AA*$_3$; lanes 11–13, three different *BglII* transgenes with a premature stop at amino acid 90 in the Act5C protein. *RP49* was used as a control to demonstrate that the lanes were loaded similarly (data not shown). Note that the total amount of *Act5C* mRNA is unaffected by the deletion of the 3′ sequences (compare lanes 1 and 2).

CELL BIOLOGY

Table 1. Rescue of two *Act5C* mutant alleles by using various transgenes

	ywact5C^{3447}	*ywact5C*^{3627}
yw;10.5XhoI Act5C-D	54% (188)	46% (348)
yw;9.4EcoRI Act5C	61% (141)	64% (173)
yw; Act5C-42A-D$_2$	55% (200)	54% (368)
yw; Act5C-42A-AA$_3$	0.03% (58)	0.018% (56)
yw;H264Q #9-3	63% (65)	47% (92)
yw;H264Q #16	53% (94)	34% (83)

The number shown is the percent of males carrying an *Act5C* mutant X chromosome out of total males, shown in parentheses. The transgenic lines were homozygous, therefore every male offspring should receive a copy of the transgene. 10.5*XhoI Act5C-D* and 9.4*EcoRI Act5C* are wild-type transgenes; *Act5C-42A-D*$_2$ (expressed) and *Act5C-42A-AA*$_3$ (not expressed, see text) are transgenes with *Act5C* control regions and *Act42A* protein sequences. *H264Q #9-3* and *H264Q #16* are transgenes with the H at 264 in ϕPCR-A corrected to Q.

chromosome with the *Act5C P* element mutation should equal the number of males carrying nonmutant X chromosomes (balancer). We expected 50% of all males to be rescued (our values range from 46 to 64%). Despite the fact that the rescue efficiency was somewhat variable for different transgenes rescuing different mutant lines, the transgenes have strong rescuing ability. This rescue supports the conclusion that the mutant alleles are indeed *Act5C* mutations.

We also constructed a transgene, *H264Q*, in which we "corrected" the amino acid histidine at position 264 to glutamine. This change corrects an apparent cloning artifact in the *Act5C* ϕDNA clone isolated previously (see next section). As seen in Table 1, two independent transgene insertions (*H264Q #9-3* and *H264Q #6*) rescue the *Act5C* mutants but not significantly better than the transgenes that encode histidine at position 264.

To be sure that expression of Act5C protein was required, the 10.5*Xho*I Act5C construct was modified to introduce a premature stop codon at amino acid 95 of the Act5C protein (Δ*Bgl*II transgene). Three stably transformed lines were obtained and were tested for their *Act5C* expression (Fig. 3, lanes 11–13). In contrast to our result with the 9.4*Eco*RI Act5C and 10.5*Xho*I Act5C transgenic lines, we do not see expression of the 2.0- or 2.3-kb transcripts in these lines. It is likely that because the construct introduces a premature stop codon, the transcript is unstable. The Δ*Bgl*II transgenes were tested for their ability to rescue the lethal phenotype of the *Act5C P* element mutant alleles and all three failed to rescue the lethal phenotype of the *P* element insertion lines.

Because this region of the genome is very tightly packed with ORFs, we wanted to be sure that the observed rescue was the result of *Act5C* expression and not expression of other ORFs on our transgenes. A transgene containing a 6.4-kb *Xho*I-*Xba*I fragment, including *Act5C* upstream regulatory regions and the first two *Act5C* exons, was constructed (Fig. 1). Six transgenic chromosomes carrying this construct were tested for their ability to rescue the *Act5C P* element mutant alleles and all six failed to rescue the lethal phenotype of the *P* element insertion lines.

A gene encoding a protein with homology to the Male-sterility 2 protein in *Arabidopsis* (18) lies just 3′ of *Act5C* (unpublished results). To exclude the possibility that the *P* element insertions affect this gene and are responsible for the lethal phenotype, a transgene containing a 3.3-kb *Sal*I-*Bam*HI fragment was constructed. This 3.3-kb *Sal*I-*Bam*HI transgene rescues flies with lethal mutations (generated in the screen in which the *Act5C* mutations were isolated) that fall into in a different complementation group. Five independent insertions of this transgene failed to rescue the *Act5C P* element mutant alleles. These data indicate that alterations in the gene just 3′ to *Act5C* are not responsible for the lethal defects in the *Act5C P* element insertion alleles.

In sum, the *Act5C* lethal *P* element insertion lines fail to accumulate *Act5C* mRNA, are rescued by transgenes carrying a functional *Act5C* gene, and are not rescued by other transgenes that include all of the sequences from the region but do not express *Act5C*. These data argue that we have identified mutations in the *Act5C* gene and that loss of *Act5C* function is lethal to the fly.

The Lethal Phenotype of the *P* Element Insertions Can Be Rescued by Introducing a Hybrid *Act5C-42A* Gene. We wanted to determine whether the amino acid differences between the Act5C protein and the Act42A protein were essential for the Act5C protein function. We constructed a hybrid transgene that produces a protein identical to *Act42A* gene but under the control of the regulatory regions of the *Act5C* gene. We obtained four independent transformed lines carrying this hybrid construct. We assayed flies carrying these transgenes for transgene expression in the background where only a 1.8-kb transcript is seen from the endogenous *Act5C* gene (Fig. 3, lanes 7–10). In three of the lines we detected a 2.0-kb and to a lesser extent, a 2.3-kb transcript, which are characteristic of the *Act5C* gene that must be derived from the transgene. One line did not show the expression of these transcripts, indicating the transgene sequences were not expressed. We then tested the ability of these four independent transgene insertions to rescue the lethal phenotype of the four *Act5C P* element mutant alleles. The three transgene insertions that restored expression of the three *Act5C* transcript classes rescued the lethal phenotype of the *Act5C P* element mutant alleles. The one transgene insertion (*Act5C-42A*$_{AA3}$), in which the *Act5C* expression pattern was not restored, did not rescue the lethal phenotype (Table 1).

It was possible that, although the Act42A isoform rescued lethality to some extent, this isoform did not function as well as the Act5C isoform. To test this hypothesis, we counted the number of rescued animals with each independent insertion of each transgene. Although there was some variation among different lines in rescue efficiency, the rescue of lethality was quantitatively similar with transgenes carrying *Act5C* or *Act42A* coding sequences (Table 1). These data indicate that the amino acid differences between the two cytoplasmic isoforms in *Drosophila* are not functionally important.

The Act5C and Act42A Proteins Differ by Two Amino Acids. In the process of sequencing the various transgenes we constructed, we noticed that the amino acid sequence in the clone (ϕPCR-A) we used in all our experiments differed from those published in the literature. Published sequence data reported differences of either five or seven amino acids between the two cytoplasmic actins (6, 7, 15). Because the actual isoform amino acid sequence differences were critical for our experiments, we resequenced relevant regions of the genomic DNA and cDNAs for both *Act5C* and *Act42A* from two *Drosophila* strains (OreR and IsoA). We detected no strain differences, and the sequences we obtained were identical for each gene whether we used genomic DNA or cDNAs. We observed only two amino acid differences between Act5C and Act42A: an alanine (5C) or serine (42A) at amino acid 261 and an isoleucine (5C) or valine (42A) at amino acid 331 (Fig. 4). In the ϕPCR-A clone (5C) there is a histidine at amino acid 264. This residue is a glutamine in the sequence from both genomic DNA and cDNA, suggesting the histidine in the cloned DNA (ϕPCR) resulted from a cloning artifact. A glutamine at amino acid 264 is present in all of the other actin isoforms in *Drosophila*. Our sequence data for the *Act5C* and *Act42A* genes are in complete agreement with the recent BDGP sequences for these two genes (19).

```
act5C       1 MCDEEVAALVVDNGSGMCKAGFAGDDAPRAVFPSIVGRPRHQGVMVGMGQKDSYVGDEAQ
act42A      1 ............................................................
human β     1 M-.DDI.....................................................
human γ     1 M-E..I....I................................................

act5C      60 SKRGILTLKYPIEHGIVTNWDDMEKIWHHTFYNELRVAPEEHPVLLTEAPLNPKANREKM
act42A     60 ............................................................
human β    61 ............................................................
human γ    61 ............................................................

act5C     120 TQIMFETFNTPAMYVAIQAVLSLYASGRTTGIVLDSGDGVSHTVPIYEGYALPHAILRLD
act42A    120 ............................................................
human β   121 ...............................M......T...................
human γ   121 ...............................M......T...................

act5C     180 LAGRDLTDYLMKILTERGYSFTTTAEREIVRDIKEKLCYVALDFEQEMATAASSSSLEKS
act42A    180 ............................................................
human β   181 ............................................................
human γ   181 ............................................................

act5C     240 YELPDGQVITIGNERFRCPEALFQPSFLGMEACGIHETTYNSIMKCDVDIRKDLYANTVL
act42A    240 ....................S.......................................
human β   241 ...............................S.......F...................
human γ   241 ...............................S.......F...................

act5C     300 SGGTTMYPGIADRMQKEITALAPSTMKIKIIAPPERKYSVWIGGSILASLSTFQQMWISK
act42A    300 ..............................V.............................
human β   301 ............................................................
human γ   301 ............................................................

act5C     360 QEYDESGPSIVHRKCF
act42A    360 ................
human β   361 ................
```

Fig. 4. Alignment of the *Drosophila* Act5C, *Drosophila* Act42A, human β, and human γ protein sequences. The consensus sequence is the *Drosophila* Act5C protein, and differences in amino acids as compared to Act5C are shown.

The Expression of *Act5C* Is Crucial to the Fly. Because the *Act5C-42A* transgene rescued the lethal phenotype of the *Act5C* mutants, we know it is not the difference in amino acid sequences between the two cytoplasmic actin isoforms that is essential to the fly. However, two possibilities exist as to why viability is restored. The fly may need expression of *Act5C* in specific cells at specific times during development. Alternatively, it is possible that the total gene dosage of cytoplasmic actin is important. In the latter case, flies mutant for *Act5C* would die, because they have only two copies of cytoplasmic actin genes (two copies of *Act42A*) and the level of actin expression is simply below a critical threshold. The flies carrying the *Act5C* transgenes might live, because by supplying cytoplasmic actin from the transgene, a critical level of total cytoplasmic actin is once again present. To test for this sort of gene dosage effect, we constructed flies that had only one copy of *Act5C* and one copy of *Act42A*. We reasoned that if the regulated expression of the *Act5C* gene is essential to the fly, flies carrying one copy of each cytoplasmic actin gene should live, even though they have only two cytoplasmic actin genes. On the other hand, if a critical supply of cytoplasmic actin is required, we predicted that flies carrying only one copy of each cytoplasmic actin gene (i.e., two total copies, like the *Act5C* mutants) should not make enough actin and, therefore, die.

We performed a genetic cross that would allow us to obtain flies that carry one copy of *Act5C* and one copy of *Act42A* to test whether this combination was viable (see *Materials and Methods* for details). In all crosses to generate such animals, the number of flies doubly hemizygous for *Act5C* and *Act42A* was not significantly different from those only hemizygous for *Act42A*. Because we were able to obtain doubly hemizygous flies, reduced cytoplasmic actin gene dosage, *per se*, is not likely to be the reason that *Act5C* mutants die.

Discussion

We have generated mutations in one of the two cytoplasmic actin genes in *Drosophila*, *Act5C*. Mutations in this gene are lethal, indicating that this gene's function is not redundant with that of the other cytoplasmic isoform, *Act42A*, with which it coexpressed in all cell types. Our substitution of Act42A amino acid coding sequences for Act5C in the rescuing transgene conclusively demonstrates that amino acid sequence differences between the two isoforms are not functionally important. Rather, our results are most consistent with a requirement for *Act5C* gene expression in particular cells to supply enough actin for their normal function. In other words, it is the regulated temporal and spatial pattern of expression of the *Act5C* gene that is important for its unique role in flies.

It is known that mutations in the single actin gene in budding yeast are lethal (20), but this result is expected, because there is only one gene encoding actin in the yeast genome and actin is known to play many important roles in cells. However, multicellular organisms have multiple actin genes. Some of these genes are known to be specific to certain tissues, like muscle, and when mutated, cause defects in those tissues. However, in every organism where it has been studied, there are also several cytoplasmic actin isoforms and these isoforms are coexpressed in many different cell types of the organism. Because there have been no previous reports of cytoplasmic actin mutations in an animal, it has been unclear, until this work, whether mutations in one of the coexpressed genes would cause a phenotype. It seemed reasonable to expect that loss of the expression of one gene might have no detrimental effect because of the redundant expression of the remaining gene(s). The isolation of *Act5C* lethal mutations demonstrates that, at least in *Drosophila*, the cytoplasmic isoform genes are not redundant. However, our data that Act42A amino acid coding sequences expressed in the *Act5C* pattern rescue *Act5C* mutants suggest that it is the expression pattern of *Act5C*, rather than a unique function of the Act5C amino acid sequence, that is important.

Given the similarity of the *Drosophila* cytoplasmic isoforms, it is perhaps not surprising that Act42A coding sequences can replace Act5C coding sequences in rescuing the lethality of *Act5C* mutants and provide full function. We do not see any apparent morphological defects in *Act5C* mutants rescued with the hybrid transgene, although it is possible there are subtle phenotypes we have not yet detected.

By contrast, the two cytoplasmic actin isoforms in vertebrates β and γ, which differ by only four amino acids (Fig. 4), do not seem to be functionally identical. Overexpression of each isoform in tissue culture cells results in distinct changes to their cell morphology or cytoarchitecture (3). Further, biochemical interactions of the two isoforms with particular actin binding proteins are also different (4). These data have been interpreted to mean that the isoforms have distinct functions caused by the amino acid differences between them. N-terminal residues 1–4, where the differences between β and γ are found, participate in interactions with actin-binding proteins (11). Amino acid differences in this region are more likely to affect function than those seen in the *Drosophila* isoforms, which fall into regions that have not been shown to participate in interactions with ABP. Thus, vertebrate isoforms may be functionally specialized even though those in *Drosophila* are not. However, the methods of functional assay used in the vertebrate experiments are quite different from mutational/substitution experiments such as we have performed. The phenotype of mutants of the vertebrate cytoplasmic actin genes might prove helpful in understanding the true differences among highly similar isoforms.

In these experiments we have also tested whether increased gene dosage is an important reason that there are multiple actin genes in multicellular organisms. The viability of flies carrying only one copy each of *Act5C* and *Act42A* strongly suggests that this is not the case. Our data that actin gene dosage is not critical might be explained by the idea that actin protein levels are maintained through autoregulation of actin gene transcription (21). If the levels of monomeric pools of G-actin are high, actin mRNA is degraded. If monomeric pools of G-actin are low, actin mRNA synthesis is increased (21). We see evidence of such autoregulation in flies whose two most 3′ polyadenylation signals of *Act5C* are deleted. In these flies, only the 1.8-kb-size class of *Act5C* mRNA is produced and its level is comparable to the levels of the 2.0-kb-size class seen in wild-type flies (see Fig. 3, lanes 1 and 2). Unfortunately, we cannot determine how much

of the total cytoplasmic actin is either Act5C or Act42A, because no isoform-specific Abs are available.

The requirement for flies to have at least one copy of *Act5C* to survive might be explained by differential mRNA stability. *Act42A* transcripts are turned over at a higher rate than *Act5C* transcripts (S. Tobin, personal communication), and therefore, the amount of transcript from each gene that accumulates is different. Achieving a sufficiently high level of accumulated transcript to provide enough actin in some tissues may be impossible with only Act42A transcripts. However, whether it is transcription pattern or message stability that is critical, our conclusion remains the same: there are at least some (and maybe all) tissues that require the expression of the *Act5C* gene.

One aspect of differential "regulation" that we have not tested in our experiments is the possible importance of the 3′ untranslated region (UTR). Some actin messages contain "addresses" or "zip codes" for localization within the cell, which are important for actin function (22, 23). It is possible that *Act5C* messages, through their 3′ UTRs, are targeted to a different subcellular location than are the *Act42A* messages and that this subcellular localization is critical. This phenomenon falls into the category of "regulated expression," not isoform-specific functions or gene dosage effects.

These actin mutants establish that the two cytoplasmic actin isoforms in *Drosophila* are not redundant and provide us with the opportunity to study actin cytoskeletal function in an animal with many different cell types and actin structures with a genetic approach. In addition, further studies of actin isoform specificity with transgenes encoding muscle-specific isoforms and isoforms from evolutionarily distant organisms will reveal what sequence changes can be tolerated and still permit normal actin interactions and functions in the many different cell types in multicellular organisms.

We thank members of the K.G.M., A.P.M., and Shearn labs, past and present, for stimulating discussions and those in the labs who critically read the manuscript. Special thanks to Oname Burlingame, Michael Wang, Mary Fournier, and Ari Kaplan for their assistance as undergraduate research students. We thank Carol Myers for isolating the *P* element line *fs(1)ph*$^{8-28B}$ we used to generate the *Act5C* mutants. C.R.W. also thanks Allen Shearn for generously providing space. We also thank Sally Tobin for providing us with many reagents and helpful advice. This work was supported by a National Institutes of Health training grant–Univ. of Chicago (to C.R.W.), a Muscular Dystrophy Association postdoctoral fellowship (to C.R.W.), an American Heart Association–Missouri chapter grant (to K.G.M.), and National Institutes of Health Grants R01-43607 (to K.G.M.) and R01-17608 (to A.P.M.).

1. Herman, I. M. (1993) *Curr. Opin. Cell Biol.* **5,** 48–55.
2. Barja, F., Coughlin, C., Berlin, D. & Gabbiani, G. (1986) Lab. Invest. **55,** 226–233.
3. Schezov, G., Lloyd, C. & Gunning, P. (1992) *J. Cell Biol.* **117,** 775–785.
4. Shuster, C. B. & Herman, I. M. (1995) *J. Cell Biol.* **128,** 837–848.
5. Fyrberg, E. A., Kindle, K. L. & Davidson, N. (1980) *Cell* **19,** 365–378.
6. Fyrberg, E. A., Bond, B. J., Hershey, N. D., Mixter, K. S. & Davidson, N. (1981) *Cell* **24,** 107–116.
7. Fyrberg, E. A., Fyrberg, C. C., Biggs, J. R., Saville, D., Beall, C. J. & Ketchum, A. (1998) *Biochem. Genet.* **36,** 271–287.
8. Brault, V., Reedy, M. C., Sauder, U., Kammerer, R. A., Aebi, U. & Schoenenberger, C. (1999) *J. Cell Sci.* **112,** 3627–3639.
9. Fyrberg, E. A., Mahaffey, J. W., Bond, B. J. & Davidson, N. (1983) *Cell* **33,** 115–123.
10. Tobin, S. L., Cook, P. J. & Burn, T. C. (1990) *Dev. Genet.* **11,** 15–26.
11. Kabsch, W. & Vandekerckhove, J. (1992) *Annu. Rev. Biophys. Biomol. Struct.* **21,** 49–76.
12. Zhang, P. & Spradling, A. C. (1993) *Genetics* **133,** 361–373.
13. Myer, C. (1994) Ph.D. thesis (Case-Western Reserve Univ., Cleveland).
14. Jowett, T. (1986) in *Drosophila: A Practical Approach*, ed. Roberts, D. B. (IRL, Oxford), pp. 275–286.
15. Burn, T. C., Vigoreaux, J. O. & Tobin, S. L. (1989) *Dev. Biol.* **131,** 345–355.
16. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY).
17. Rubin, G. M. & Spradling, A. C. (1982) *Science* **218,** 348–353.
18. Aarts, M. G., Dirkse, W. G., Stiekema, W. J. & Pereira, A. (1993) *Nature (London)* **363,** 715–717.
19. Rubin, G. M., Hong, L., Brokstein, P., Evans-Holm, M., Frise, E., Stapleton, M. & Harvey, D. A. (2000) *Science* **287,** 2222–2224.
20. Shortle, D., Haber, J. E. & Botstein, D. (1982) *Science* **217,** 371–373.
21. Lyubimova, A., Bershadsky, A. D. & Ben-Ze'ev, A. (1997) *J. Cell. Biochem.* **65,** 469–478.
22. Taneja, K. L. & Singer, R. H. (1990) *J. Cell. Biochem.* **44,** 241–252.
23. Kislauskis, E. H. & Singer, R. H. (1992) *Curr. Opin. Cell Biol.* **4,** 975–978.