Vol. 203, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1994 Pages 523-531

AN OLIGOGENE FAMILY ENCODES ACTINS IN THE HOUSEFLY, *MUSCA DOMESTICA*

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Received July 13, 1994

Southern hybridization analyses of genomic DNA and dot-blot reconstruction experiments indicated that 5-6 members comprise the actin gene family in the housefly, *Musca domestica*. Hybridization data failed to reveal conservation between housefly and fruitfly sequences flanking actin genes at their 3' ends, in marked contrast to the high degree of conservation observed in these regions of orthologous actin genes in vertebrates. Hybridization analysis of genomic DNA isolated from individual houseflies revealed polymorphism within actin-associated sequences. A representative genomic library was constructed in lambda EMBL3 and screened using a heterologous actin coding sequence probe. Of nearly 20 recombinants isolated, 6 represented overlapping clones and the remainder proved to be inviable.

Actins, ubiquitous and highly conserved proteins of eukaryotes, participate in essential cellular functions (1) effected through the interactions of actin with a variety of actin binding proteins (2).

Studies on vertebrate actins identify the existence of three major isoforms differing primarily in N-terminal amino acid sequences (3). Two of these isoforms, beta and gamma, participate in cytoskeletal functions and co-exist in most cell types. The third consists of four slightly different alpha-actins (4), restricted to vertebrate muscle tissues: skeletal, cardiac, vascular smooth and non-vascular smooth muscle (5). Within vertebrates, actin isotypes differ in amino acid sequence by about 6%. Interspecies comparisons between corresponding actin isotypes reveal even greater amino acid sequence conservation. This marked degree of conservation probably results from the essential nature of actin functions and the need for interactions with numerous actin-binding factors.

The conservation of actin amino acid sequences across broad evolutionary distances is a reflection of the conservation in actin coding sequences (6, 7). In contrast to this conservation, other features of actin genes display remarkable variation with respect to copy number (8-11),

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0006-291X/94 $5.00

distribution on the genome (12-14), presence and position of introns (15) as well as the presence of pseudogenes (16, 17).

Actin genes are flanked on the 5' and 3' ends by sequences that are transcribed but not translated (UTRs) and contribute to the occurrence of disparate size classes of actin mRNA. The 3' UTRs appear to be gene specific (18-20), diverge at rates lower than expected for nonfunctional DNA and are conserved between orthologous actin genes in vertebrates (21, 22). These findings suggest that actin-associated 3' UTRs have some biological significance (23-27).

In contrast to the situation in vertebrates, relatively little is known concerning the structural and regulatory features of invertebrate actin genes. The present study was undertaken to isolate and characterize actin genes in the housefly, *Musca domestica* (*Md*), in order to perform a comparative analysis with those of *Drosophila melanogaster (Dm)*, in which this gene family has been characterized in detail. The housefly was chosen for study for several reasons. First, *Md* and *Dm* are distantly related, having shared their last common ancestor between 65-100 million years ago. The genome of *Dm* is organized in the long period interspersion pattern, while that of *Md* displays the short period interspersion pattern. The genome of *Md* is approximately 5X the size of the *Dm* genome, containing about 3.6X as much single copy DNA (28). The two dipterans also differ in the complexity of RNA sequences stored in their eggs (28-29). In spite of these differences, both dipterans share similar body plans and pass through similar developmental stages (30). Thus observed differences would not be expected to be imposed by developmental constraints, as may be the case, for example, with the nematodes, *C. elegans* and *Ascaris suum* (11). Lastly, the housefly is an organism with medical, veterinary and economic importance. For this reason, many studies have focussed on the physiology and genetics of the housefly, particularly in relation to the acquisition and biochemical characterization of insecticide resistance. However, there have been few studies reported on the molecular biology of this organism.

The data obtained from the present study indicate that *Md* actin is encoded by an oligogene family. There exists some restriction fragment length polymorphism associated with these genes. Southern blot hybridization analysis did not reveal sequence similarities between the 3' UTRs of the 6 *Dm* and those of *Md* actin genes, in contrast to the high degree of sequence conservation in 3' UTRs between orthologous actin genes of vertebrates.

MATERIALS AND METHODS

DNA Isolations. Genomic DNA was isolated from *Md* pupae by the method of Blin and Stafford (31). DNA from individual adult flies was isolated according to Jowett (32).

Southern and Dot-Blot Hybridizations. Genomic DNAs were digested with restriction endonuclease Eco RI and analyzed by Southern blot hybridization (33) using a ^{32}P-labelled 0.8 kb *Dm* actin coding sequence probe (25). Restriction enzymes were routinely obtained from Gibco/BRL (Gaithersburg, MD) or Promega (Madison, WI). Digestions were performed using conditions recommended by the manufacturers.

For the dot-blot genomic reconstruction experiment, an *Md* actin clone, lambda C9P, was diluted to mass amounts representing 0.5 to 6 copy equivalents. The samples were denatured in NaOH, applied to nylon membrane and hybridized to the *Dm* actin probe. Resulting autoradiograms were scanned at 500nm using a Beckman DU-8 spectrophotometer.

Genomic Library Construction. A housefly genomic library was constructed in the lambda replacement vector EMBL3 according to the method of Frischauf *et al.* (34). Vector DNA was prepared for cloning essentially by the protocol supplied by the manufacturer (Promega,

Madison, WI). Recombinant lambda chromosomes were packaged *in vitro* using the Packagene system (Promega). The housefly genomic library was screened unamplified at 55°C by the method of Benton and Davis (35).

Isolation and Restriction Enzyme Mapping of Recombinant Clones. *Md* genomic library was screened under low stringency conditions with the *Dm* actin probe. Phage DNAs from plaque-purified clones were isolated essentially by the method of Vande Woude *et al.*, (36). Restriction enzyme maps of recombinant lambda phage clones were generated by single and double digestions with a battery of restriction enzymes.

Analysis of Actin-Associated 3'UTRs. Actin-associated 3' UTRs from the six *Dm* actin genes (20) were used as probes in hybridization to *Md* genomic DNA under high or low stringency conditions.

RESULTS

Genomic Southern blot hybridization. To visualize actin sequences in the housefly genome and to obtain an initial estimate of their multiplicity, a genomic Southern blot hybridization was performed. Digestion with Eco RI produced seven hybridizing bands of approximately 23, 9.1, 8.3, 7.1, 5.7, 4.6, and 2.7 kilobases (kb) as shown in Fig.1. The 2.7 kb fragment yielded an extremely intense hybridization signal.

Genomic reconstruction experiment. To obtain a more accurate estimate of the copy number of *Md* actin genes, a quantitative dot blot genomic reconstruction experiment was performed. Preliminary quantitative studies indicated that the housefly harbored less than 7.5 actin genes per haploid genome. Therefore, amounts of DNA from the lambda clone C9P representing 0.5 to 6 copy equivalents were loaded in the reconstruction series on the nylon membrane. In order to make the copy number determination, duplicate samples each containing 1 μg of *Md* genomic DNA were loaded. An additional sample, containing 0.5 μg of wild type lambda phage DNA was also dot-blotted onto the filter in order to determine the extent to which probe DNA hybridized in a DNA-dependent, nonspecific manner.

In Fig. 2A is the autoradiogram resulting from the reconstruction series aligned with its scan. The absorbance peaks were integrated and plotted (inset in Panel A) and indicate a linear relationship between copy equivalents and autoradiographic intensities. The three dots in Fig. 2B represent 1, 1 and 0.25 μg of *Md* genomic DNA aligned with a scan of the dots. The 1 μg duplicates gave areas of 1.14 and 0.99, respectively. These results indicate that there are five to six actin genes per housefly haploid genome.

Genomic library construction and restriction endonuclease maps of recombinant phage clones. To isolate housefly actin sequences for further study, a genomic library was constructed in λ EMBL3. The number of recombinants produced, $2.47X10^5$, was representative of the housefly genome (37).

The library was screened without prior amplification in order to minimize duplication of unique phages and to decrease the probability of losing recombinants due to viability differences. Of 17 clones initially isolated, only six were successfully propagated. Restriction enzyme mapping of these six recombinants revealed 5 of them to be overlapping clones of λC9P, the restriction map of which is shown in Fig. 3.

Conservation of actin-associated 3' UTRs between *Md* and *Dm*. The extent to which actin-associated 3' UTRs are conserved between *Dm* and *Md* was investigated in order to

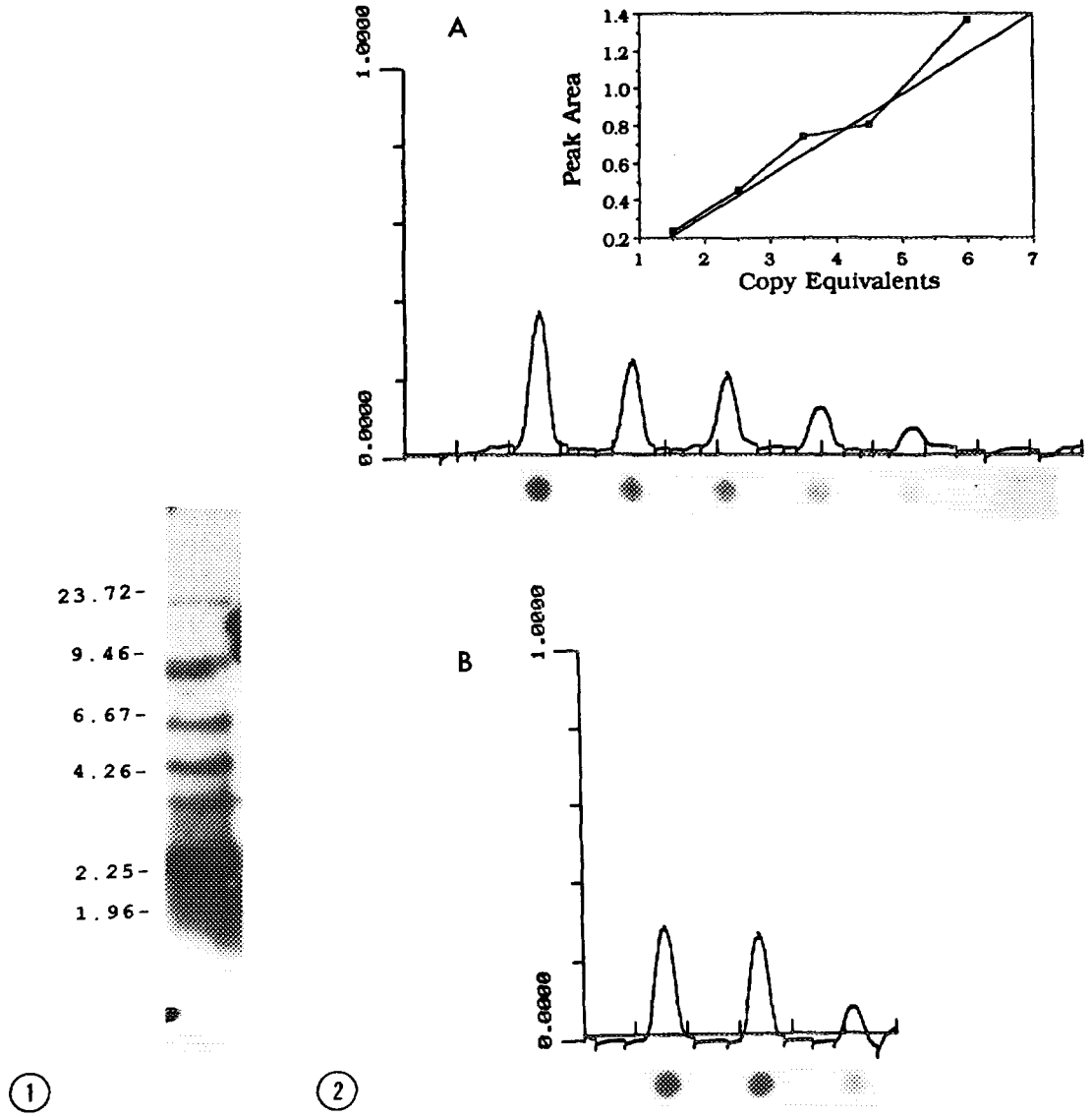

Figure 1. Genomic Southern Blot Analysis of *Md* DNA. Six micrograms of *Md* genomic DNA were digested with Eco RI restriction endonuclease and electrophoresed through a 0.7% agarose gel. The fractionated DNA was transferred to nylon membrane by capillary blotting and baked at 80°C *in vacuo* for two hr. Prehybridization was for 16 hr and hybridization for 48 hr at 55°C with a ^{32}P-labelled *Dm* actin coding sequence probe. The filter was washed five times for 20 min each in 2X SSC containing 0.1% SDS and 0.1% sodium pyrophosphate at 55°C, followed by a 20 min wash in 2X SSC at room temperature. The filter was exposed to X-ray film with two intensifying screens at -70°C for seven days. Size standard used was Hind III-digested lambda DNA; sizes are indicated in kilobases.

Figure 2. Dot-blot Genomic Reconstruction Experiment. DNAs were denatured and applied to a nylon membrane. The probe was the *Dm* actin coding sequence probe. Autoradiographic exposure was for 24 hr at -70°C with one intensifying screen. Panel A: Autoradiogram and scan resulting from genomic reconstruction series in which mass amounts of phage clone C9P equivalent to 6, 4.5, 3.5, 2.5, 1.5 and 0.5 actin gene copies per haploid genome were dot-blotted. Panel B shows the autoradiogram and scan resulting from hybridization of 1, 1 and 0.25 μg of *Md* genomic DNA with probe DNA. In the inset on Panel A, the peak areas of the reconstruction series were plotted to compare with the genomic DNA series. Scans of the autoradiogram were at a wavelength of 500nm.

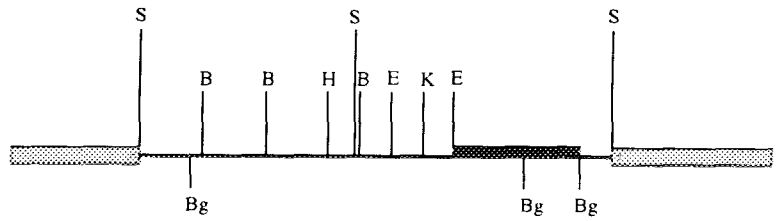

Figure 3. Restriction enzyme map of actin-containing recombinant clone λC9P. 1μg of phage DNA was used in single and double digestions with Bam HI, Bgl II, Eco RI, Hind III, Kpn I and Sal I. Actin coding sequences were visualized by hybridization of digested DNAs with the *Dm* actin probe. B=Bam HI; Bg=Bgl II; E=Eco RI; H=Hind III; K=KpnI; S=Sal I. A size calibration is given in kilobases. The thin line indicates *Md* insert DNA. Blackened box indicates fragment that hybridizes with actin probe; shaded boxes represent the left and right arms of the lambda chromosome.

study possible regulatory roles of 3' UTRs in dipterans. Bgl II-digested *Md* genomic DNAs did not cross-hybridize with the six *Dm* actin-associated 3' UTR probes under high or low stringency conditions (data not shown). Partial sequencing of one of the *Md* actin 3' UTRs failed to reveal any significant sequence similarities when compared to available sequences of *Dm* actin-associated 3' UTRs (Hadden and Sodja, unpublished observation).

Polymorphisms in *Md* actin-associated sequences. The observations that the *Md* actin clones share many of the mapped restriction sites, prompted us to seek an estimate of polymorphism in housefly actin or actin-associated sequences. Genomic Southern blot hybridizations were performed using DNA isolated from individual flies. Fig. 4A shows the ethidium bromide stained gel, which indicates that two individuals (3 and 5) appear to have "satellite-like" sequences as suggested by the presence of eight strongly staining bands in the 1-4.5 kb range. In these samples there is an apparent decrease in the amount of both RNA and genomic DNA isolated. In several repetitions of this experiment, about 20% of the individual fly DNAs display this satellite complex. DNAs from individuals 1, 2 and 7 reveal the presence of some of these intense bands present in flies 3 and 5, although in much lower abundance.

Fig. 4B displays the autoradiogram obtained from the hybridization of individual fly DNAs. Three prominent hybridization patterns are evident. In the more common pattern (individuals 1, 2 and 10), seven bands of 23, 9.1, 8.3, 7.1, 5.7, 4.6, and 2.7 kb are visible. In the less common pattern, seen in individual 7, seven bands are observed. In this case, fragments of 23, 9.1, 8.2, 7.0, 5.4, 2.5 and 2.2 kb hybridize with the actin probe. Individual 8 displays the more common pattern, with the exception that the 4.5 kb fragment is absent. Clear hybridization patterns are not visible for 5 of the flies tested. The DNA from individual 9 appears to have been degraded during the isolation procedure. Lanes 3 and 5 contain DNA from flies in which the "satellite" DNA was isolated. The amount of DNA isolated from these two flies, with the exception of the intensely staining bands, appears to be relatively small. Amounts of DNA isolated from individuals four and six also appear to be too small to display intense hybridization signals. Although not apparent in the figure, individuals 3, 4 and 5 appear to display the same hybridization

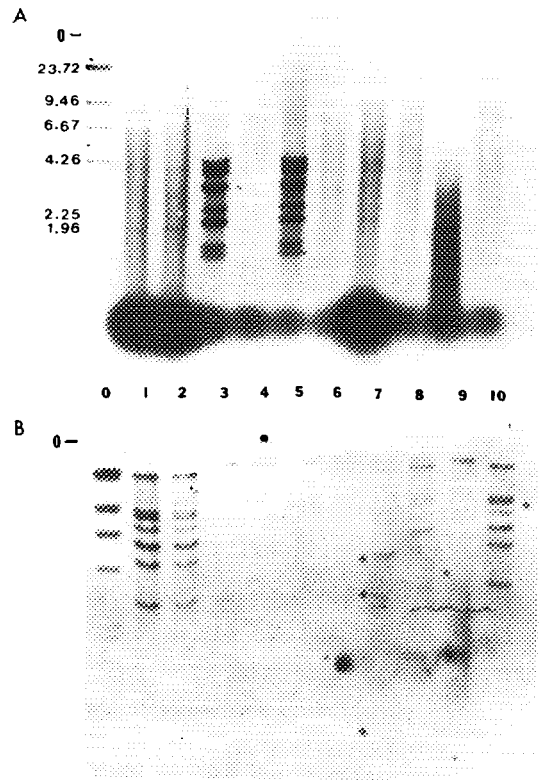

Figure 4. Analysis of DNA isolated from individual flies. Panel A: ethidium bromide-stained mass display of Eco RI-digested DNAs from 10 individual houseflies electrophoresed through 0.7% agarose. Panel B: autoradiogram resulting from hybridization of individual fly DNAs immobilized on nylon membrane with the *Dm* actin probe. Asterisks indicate polymorphic bands. Hind III-digested lambda DNA served as size markers, indicated on the figure in kilobases.

pattern as flies 1, 2 and 10. A pattern is not evident in DNA from individual 6. The results also show that none of the DNA fragments strongly stained with ethidium bromide (Panel A) harbor actin sequences.

DISCUSSION

In order to better understand the evolution of multigene families and regulation of their members, the present study of actin genes in the housefly was initiated. To date, the only other dipteran in which this gene family has been studied in detail is *Drosophila melanogaster*.

Genomic Southern blot hybridizations between Eco RI digested *Md* DNA and *Dm* actin coding sequence probe yielded seven hybridizing fragments. This result as well as those from the dot-blot genomic reconstruction experiment indicate that the housefly harbors five to six actin genes per haploid genome. Thus, actins are encoded by a moderately-sized multigene family in the housefly, as is the case in *Dm*, in which this gene family consists of 6 members. The differences in genome size and organization between *Md* and *Dm* are not reflected in the sizes of their actin

gene families. A similar number of actin genes might be expected in this case since *Md* and *Dm* pass through similar developmental stages and share similar body plans.

To study the actin genes of *Md* in greater detail, a recombinant library representative of the housefly genome was constructed and 17 actin-bearing phage clones were isolated. All of these were difficult to propagate and only 6 remained viable through the plaque purification protocol. Despite attempts to optimize culture conditions, they grew poorly; once sufficient amounts of their DNAs were isolated for restriction enzyme analysis, they were shown to represent overlapping sequences. The restriction enzyme map of λC9P shares no striking similarities with any of the *Dm* actin recombinants (38).

To determine whether actin gene-associated polymorphism could be detected, DNA from individual houseflies was analyzed by Southern hybridization. In several replications of the experiment, DNA from about 20% of the flies possessed eight extremely prominent DNA bands, likely to represent "satellite-like" sequences. Satellite DNAs, common in eukaryotic genomes and of low sequence complexity, are often separated from the bulk of the genomic DNA by CsCl gradient centrifugation. This step is omitted in isolation of DNA from individual flies; hence it is possible that in some individuals the satellite sequences are retained.

Actin-associated polymorphism was observed in *Md* as it has been in *Ascaris suum* (11) and several other species. Three prominent patterns were discernable in Eco RI digested genomic DNA from 10 individuals. In the most common pattern, the two smallest actin-hybridizing fragments were 4.5 and 2.7 kb in length. In one individual, these fragments appeared to be supplanted by 2.5 and 2.2 kb bands. This observation explained that the intensely hybridizing band seen in the genomic blot using DNA isolated from a pool of houseflies (Fig. 1, Lane 1) represented fragments of 2.7, 2.5 and 2.2 kb that were not resolved in that experiment. It is likely that additional restriction fragment length polymorphisms would be detected using other restriction enzymes or probes.

It was of interest to determine whether actin-associated 3' UTRs were conserved between *Md* and *Dm*. Based on extensive studies with vertebrate actin genes, conservation of 3' UTR sequences between these dipteran species might have allowed a rapid way to assign functional orthology to the genes as well as suggesting functional significance of the 3' UTRs. *Dm* 3' UTR hybridization to *Md* genomic DNA failed to detect stable hybrid formation under high or low stringency conditions. Nucleotide sequence determination of an *Md* actin 3' UTR reveals no similarities with *Dm* actin 3' UTRs (Hadden and Sodja, unpublished observation). Direct sequencing of each of the actin genes from *Md* would establish whether the preceding data accurately reflect absence of significant sequence similarities. If so, this observation would represent a departure from the situation in vertebrate actin genes, where a high degree of sequence conservation exists in the 3' ends of orthologous actin genes in widely divergent species. This may suggest different, if any, involvement of these regions in actin gene expression in Dipterans.

Continuing efforts are directed toward a more detailed characterization of *Md* actin genes in order to study their expression. Preliminary Northern blot hybridization of poly A+ RNA isolated from *Md* embryos, larvae, pupae and adults with an actin coding sequence probe suggests that a single size transcript is expressed throughout these stages (Plieth and Sodja, unpublished observation). In the absence of gene-specific probes we cannot determine which of the genes

contribute to the transcript detected. Future studies will also analyze putative regulatory regions found upstream from the coding sequence.

ACKNOWLEDGMENTS

This research was supported by grants from the American Heart Association of Michigan and the National Institutes of Health (GM08167-14).

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