

MULTIPLE ACTINS IN *DROSOPHILA MELANOGASTER*

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ABSTRACT

The tissue and developmental specificities of the three *Drosophila* isoactins, originally identified in primary myogenic cultures and in the permanent Schneider L-2 cell line, have been investigated. Of these three isoactins (I, II, and III), actins I and II are stable and actin III is unstable. Two-dimensional polyacrylamide gel electrophoretic analyses of total cellular extracts after 1-h [³⁵S]methionine pulses were performed on a large variety of embryonic, larval, and adult muscle and nonmuscle tissues. The results suggest that isoactins II and III are generalized cellular actins found in all *Drosophila* cell types. Actin I, on the other hand, is muscle-associated and is found exclusively in supercontractile muscle (such as larval body wall and larval and adult viscera) including primary myogenic cell cultures. Although actin I synthesis is not detectable during very early embryogenesis, it is detectable by 25 h and actin I is a major stable actin in all larval muscle tissues. Actin I is synthesized in reduced amounts relative to the other actins in late third instar larvae but is again a major product of actin synthesis in the adult abdomen. A stable actin species with the same pI as actin III has been identified in the adult thorax and appears to be unique to flight muscle tissue. This new stable form of thoracic actin may be the result of a stabilization of the actin III found in other tissues or may be an entirely separate gene product.

KEY WORDS multiple actins · muscle · myogenesis · *Drosophila melanogaster* · two-dimensional gel electrophoresis

Actin is a highly abundant protein in both muscle and nonmuscle cells and has been implicated in a large variety of cellular functions. In muscle cells, actin is a major component of myofibrils and participates directly in muscle contraction. In non-muscle tissue and in unicellular organisms, actin can represent as much as 20–30% of the total soluble protein. It exists in these cells in the form of 60-Å-diameter filaments, as well as in its subunit form, G-actin. The actin microfilaments, both nu-

clear and cytoplasmic, are thought to participate in a diverse range of generalized cellular functions including cytokinesis, pseudopod formation, cytoskeletal support, chromosome movement, and cell motility (7, 9).

Recently it has been shown that actin is not a single protein but consists of several molecular species (isoactins). This suggests that the various isoactins might have different functions. The isoactins have the same molecular weights when electrophoresed in SDS polyacrylamide gels but differ slightly in overall net charge as determined by isoelectric focusing gels or urea SDS polyacrylamide gels (4, 8, 16, 21, 24). These differences in

net charge probably reflect a small number of differences in amino acid sequence (2, 12) and/or in the degree of post-translational modification. Indeed, it has been reported that muscle and non-muscle actins differ in their N-terminal amino acids and that this N-terminal difference alone could account for the slight difference in net charge observed by isoelectric focusing (23).

Actin heterogeneity has been most thoroughly studied in a number of vertebrates. However, recent studies of *Drosophila* myogenic cell cultures have indicated a heterogeneity similar to that found in vertebrates. Three actin species, designated I, II, and III (in order of increasing basicity), have been identified in primary myogenic cell cultures of *Drosophila melanogaster* (22). These isoactins have the same molecular weight as chick skeletal muscle actin, show over 50% sequence homology with chick skeletal muscle actin, and differ only slightly in isoelectric point. The pI's of actins I, II, and III are 5.70, 5.77, and 5.84, respectively. Moreover, actins I and II are stable, while actin III does not appear to accumulate in these myogenic cultures. Both actins II and III show fairly constant levels of synthesis throughout myogenesis in culture. Actin I, on the other hand, is synthesized and accumulated at a steadily increasing rate during myogenesis in cultured cells, showing at least a fourfold increase in synthesis between prefusion and postfusion stages of development.

Actin synthesis in the permanent Schneider L-2 cell line, derived from *Drosophila* embryos, has also been studied (22). Only actins II and III are synthesized by the Schneider line and, of these two species, actin II is stable while actin III turns over rapidly.

In view of these cell culture analyses, it was suggested that actins II and III might be generalized cellular actins found in all *Drosophila* cell types, while actin I might be a muscle-associated actin species (22). To probe the significance of this actin heterogeneity in *Drosophila* and to further gain some insight into possible developmental controls of actin synthesis, we have analyzed the tissue specificity of the multiple actins in embryonic, larval, and adult stages of *Drosophila melanogaster*.

MATERIALS AND METHODS

Tissue Labeling and Sample Preparation

All tissues used were derived from the P₂ line of *Drosophila melanogaster* Oregon-R (22). Larval brains,

imaginal disks, salivary glands, and body wall tissue were obtained by dissection of mature third instar larvae. The larvae were immersed during the dissections in a drop of Ringer's solution (17) on a glass depression slide. Thoracic and abdominal specimens were obtained from anesthetized adults of both sexes. The thoraces were cleared of wings and legs. The tissues excised were immediately plunged into Ringer's solution to prevent desiccation before labeling. First instar larvae were grown in sterile growth medium, from dechorionated, surface-sterilized eggs, to reduce the bacterial flora of their guts. The larvae were slit open with a tungsten needle before labeling to increase contact with the radioactive environment.

In all cases, the tissues were labeled for 1 h at 26°C with 4 mCi/ml [³⁵S]methionine (Amersham Corp., Arlington Heights, Ill.) in methionine-free growth medium (19), in the presence of 100 µg/ml gentamycin.

Permeabilization of Embryos

Gastrula-stage (3.5- to 4-h) and 15-h embryos were rinsed free of yeast, dechorionated in a 1:1 Chlorox/95% ethanol solution (30-60 s), and then rinsed several times in Ringer's solution. Embryos of the desired developmental stage were transferred with forceps into a small (5 mm diameter) copper-mesh basket lined with nylon net. The basket, after degreasing in ether, was then dipped into heptane (11) for 1-2 min, and immediately transferred to growth medium for several rinses. Permeability was assayed in parallel controls by the uptake of acridine orange, which was not absorbed by dechorionated, nonpermeabilized embryos. Pulse-labelings were performed as described above.

Two-Dimensional Polacrylamide

Gel Electrophoresis

Tissue samples in 25- to 50-µl volumes were subjected to two-dimensional gel electrophoresis according to O'Farrell (14) as described previously (22). The first-dimension isoelectric focusing gels consisted of pH gradients of 5-7. The second-dimension slab gel consisted of 12% polyacrylamide. Electrophoresis was performed according to Laemmli (10). Coomassie Blue staining and fluorography have been described (1, 21).

RESULTS

An Analysis of Multiple Actins in Drosophila Nonmuscle Cell Types

Actins II and III are the sole actins produced by the Schneider L-2 cell line and are the predominant actins in prefusion myoblast cultures (22). These observations suggested that species II and III might be ubiquitous cellular forms of actin. Actin I, while not produced in the Schneider line,

undergoes at least a fourfold increase in relative rate of synthesis during myogenesis in primary culture (22) to become a major product of synthesis in mature myotubes. The actin profiles of pre- and postfusion *Drosophila* myogenic cultures are shown in Fig. 1 *A* and 1 *B*.

This analysis has now been extended to larval brain, imaginal disks, and salivary glands. Each of these tissues was pulse-labeled with [³⁵S]methionine for 1 h. The proteins were extracted and analysed by two-dimensional polyacrylamide gel electrophoresis. Fluorograms of these protein samples are shown in Fig. 2 *A–C*. As is the case with the Schneider cell line, it can be seen that only actins II and III are synthesized in these nonmuscle tissues and that only actin II accumulates as Coomassie Blue-stained material (Fig. 2 *D*). The unstable nature of actin III, as suggested by its lack of associated stain, has been a consistent observation in all larval cell types examined as well as in the Schneider cell line.

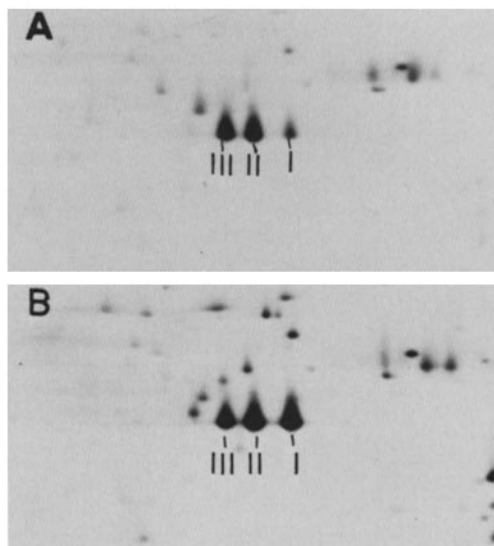


FIGURE 1 Fluorograms of proteins synthesized in pre- and postfusion primary myogenic cell cultures of *Drosophila*. A 3-h culture (*A*) and an 18-h culture (*B*) were labeled for 1 h with [³⁵S]methionine. Total cell homogenates, each containing 64,000 cpm, were analysed by two-dimensional gel electrophoresis. In all of the two-dimensional gels in this paper, the isoelectric focusing gel is run from left (OH⁻) to right (H⁺) and the SDS slab gel is run from top to bottom. Only the actin region of the fluorograms is shown. *I*, *II*, and *III* in all cases refer to *Drosophila* actins. In this experiment, fluorography was for 12 d.

An Analysis of Multiple Actins in *Drosophila* Larval and Adult

Muscle Tissue

A further study of the tissue specificity of *Drosophila* isoactins was performed with *Drosophila* larval and adult muscle tissue. Two classes of muscle tissue were analyzed: supercontractile muscle, such as larval body wall (5) as well as larval and adult gut (6, 20), and the muscle of the adult thorax which is predominantly flight muscle. The flight muscle is considerably more ordered ultrastructurally than supercontractile muscle (3, 13), though both muscle types are striated (15).

Drosophila third instar visceral muscle and body wall tissue were analyzed by two-dimensional gel electrophoresis of 1-h pulse-labeled proteins (Fig. 3). Autoradiography of ³⁵S-labeled proteins indicates synthesis of primarily actins II and III, with the amount of actin II synthesized again exceeding that of actin III (Fig. 3 *D*). This is similar to the actin profile seen in nonmuscle tissue (Fig. 2), with the exception that a low level of newly synthesized actin I can also be identified in third instar viscera and body wall (Fig. 3 *D*). An analysis of Coomassie Blue uptake by third instar supercontractile muscles reveals a substantial accumulation of actins I and II (Fig. 3 *A–C*). These results suggest that while actin I makes up a large percentage of the actin accumulated in the mature 3rd instar larva, it undergoes only a very low level of synthesis at this stage, relative to the other two actins.

Adult abdominal tissue, largely supercontractile in nature (6), also demonstrates an actin profile similar to that found in larval supercontractile muscle. Two-dimensional gels of [³⁵S]methionine-labeled abdominal tissue proteins indicated that, as in the mature larva, actin I is one of the predominant stable species in the adult abdomen (Fig. 4 *C*). Autoradiography (Fig. 4 *A*) reveals a relatively high level of actin I synthesis in the adult abdomen which is in sharp contrast to the reduced level of actin I synthesis in the mature larva. The continued synthesis of stable actin II and unstable actin III is consistent with the generalized cellular roles postulated for these actins.

Adult thoracic tissue, composed primarily of flight muscle, was labeled for 1 h with [³⁵S]methionine and analyzed by high-resolution two-dimensional gel electrophoresis of total protein extracts (Fig. 4 *B* and *D*). Little actin I synthesis or accumulation can be detected in adult thoracic tissue. The small amount of actin I seen is very likely

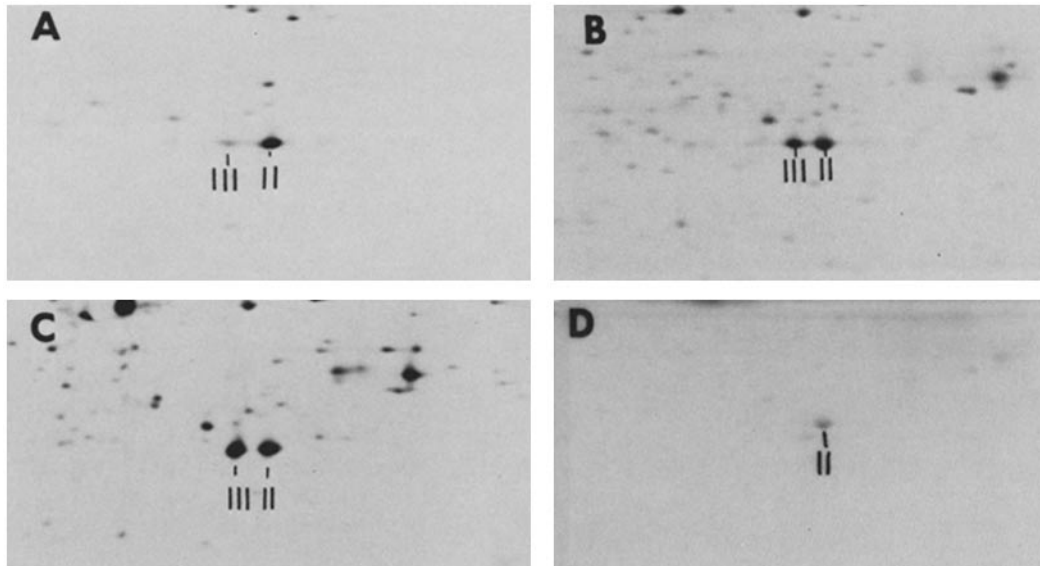


FIGURE 2 An analysis of actin synthesis (*A*, *B*, and *C*) and accumulation (*D*) in *Drosophila* third instar nonmuscle tissues. After a 1-h pulse with [³⁵S]methionine, two-dimensional gel electrophoresis was performed as described in Fig. 1 and Materials and Methods using total protein extracts. Fluorograms of (*A*) larval salivary glands, (*B*) larval brain, and (*C*) larval imaginal disks are shown. 50,000–100,000 cpm were loaded onto each gel, and fluorography was for 2–5 d. The Coomassie Blue-stained gel (*D*) of larval brain protein extract shows the pattern of staining in the actin region which is typical of all three of these tissues.

accounted for by the traces of upper digestive tract visceral muscle which are difficult to remove from the thorax during dissection. The observed synthesis of the stable generalized actin II is consistent with the analyses of other cell types. In addition, however, a new major stable thoracic actin is observed which co-electrophoreses with actin III. This is the only tissue in which a stable actin of pI 5.84 has been identified. In all other cell types studied, the isoactin of pI 5.84 is unstable and cannot be detected by Coomassie Blue staining of electrophoretic gels. The fact that a stable actin species co-electrophoresing with actin III is synthesized by the adult thorax makes it difficult to establish whether the unstable generalized actin III is also being produced.

Actin I Synthesis during Embryonic and Larval Development

A high degree of tissue specificity has been demonstrated for actin I. It is not surprising therefore to find that actin I synthesis appears to be under tight developmental control. Two-dimensional electrophoretic analysis was made of the actin profile of a variety of embryonic and larval

developmental stages, after a 1-h pulse with [³⁵S]methionine (Fig. 5). Actin I cannot be identified in *Drosophila gastrulae* (3.5- to 4-h) (Fig. 5*A*) or 15-h embryos (Fig. 5*B*), but is detected in the first instar larva (25 h) (Fig. 5*C*) where it is synthesized at a moderately high rate and accumulates. In late third instar larval muscle tissue, both visceral muscle (Fig. 3*D*) and body wall (Fig. 5*D*), actin I synthesis is greatly reduced relative to synthesis of actins II and III. Despite the apparent low level of actin I synthesis in muscle tissues of the mature larva, a substantial accumulation of actin I does exist at this stage, and in fact it makes up a significant percentage of the actin identified in the mature larva by uptake of Coomassie Blue stain.

DISCUSSION

The pattern of actin tissue specificity in *Drosophila* appears to be a complex one. Two actins, II and III, the latter being unstable, are identifiable in all *Drosophila* cell types studied, including primary myogenic cultures and a permanent undifferentiated cell line, as well as both muscle and non-muscle tissues of embryonic, larval, and adult

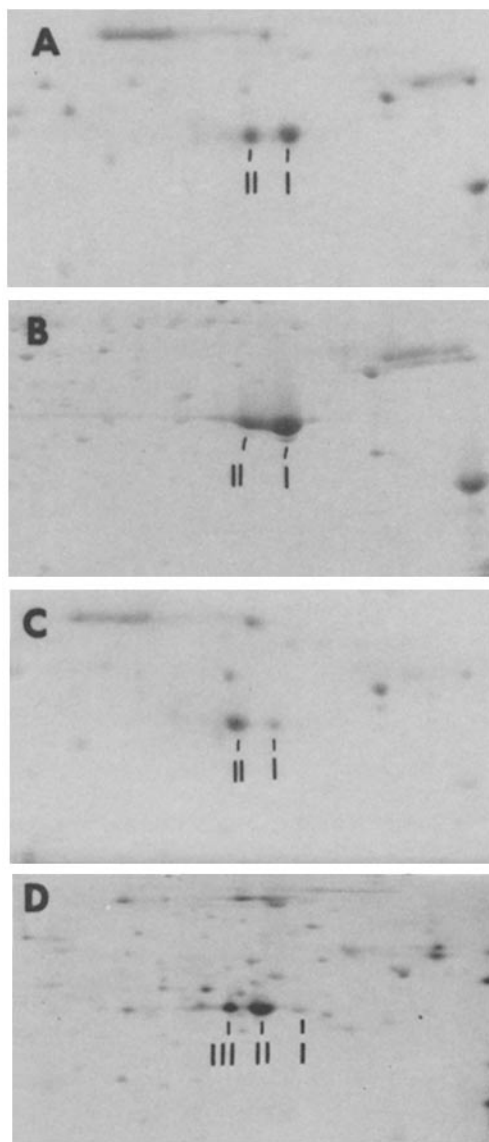


FIGURE 3 An analysis of actin accumulation (*A*, *B*, and *C*) and synthesis (*D*) in late third instar muscle-containing tissues. The tissues were labeled for 1 h with [35 S]methionine, and the total protein extracts were subjected to two-dimensional gel electrophoresis. The Coomassie Blue-stained gels of (*A*) whole third instar larvae, (*B*) third instar body wall tissue, and (*C*) third instar viscera are shown. The autoradiograph (*D*) of third instar visceral proteins shows the pattern of [35 S]methionine incorporation into actin which is characteristic of all three of these tissues. Approximately 100,000 cpm were electrophoresed, and autoradiography was for 12 d.

stages. The wide tissue distribution of these two isoactins supports the hypothesis that actins II and III are generalized cellular actins which are probably involved in a large variety of cell functions such as motility, cytoskeletal support, pseudopod formation, and chromosome movement. The unstable nature of actin III is an interesting phenomenon, though one not exclusive to *Drosophila*. Unstable actins have been reported in the rat (8), and our preliminary experiments suggest that the unstable *Drosophila* actin III may actually be an unacetylated precursor to actin II.

While actins II and III are found in all *Drosophila* cell types studied, actin I appears only in muscle tissue, both in myogenic culture and in vivo. In the tissue analyses reported here, actin I was found only in tissues containing supercontractile muscle. Therefore the presence of actin I in primary myogenic cultures of *Drosophila* suggests that this myogenic culture system is of a supercontractile nature, perhaps resembling larval skeletal muscle. Both larval skeletal muscle and mature myogenic cultures contain multinucleate myotubes. Moreover, preliminary electron microscope analysis of these myogenic cultures has revealed a highly disordered myosin array (unpublished observations) characteristic of supercontractile muscle. Earlier ultrastructural studies are consistent with this hypothesis (18), and a recent electron microscope analysis appears to confirm this identification of the *Drosophila* myogenic culture system as larval skeletal muscle (2).

The supercontractile nature of larval skeletal and larval and adult visceral muscle results in an ultrastructure which is strikingly different from that of insect flight muscle, although both classes of muscle are striated. In addition, unlike supercontractile muscle, adult thoracic muscle does not synthesize actin I in significant amounts. Instead, the major actin produced by thoracic tissue is a stable species with a pI of 5.84, the same isoelectric point as that of the unstable generalized actin III. This is an intriguing result for two reasons. First, the stable thoracic actin, like actin I, shows a very limited tissue distribution. The generalized actins of *Drosophila*, on the one hand, are found in all *Drosophila* cell types. Each of the muscle-associated actins, on the other hand, is restricted to a particular and distinct muscle type. Actin I appears almost exclusively in supercontractile muscle, while a stable "actin III" is found only in adult flight muscle, a musculature distinct in morphol-

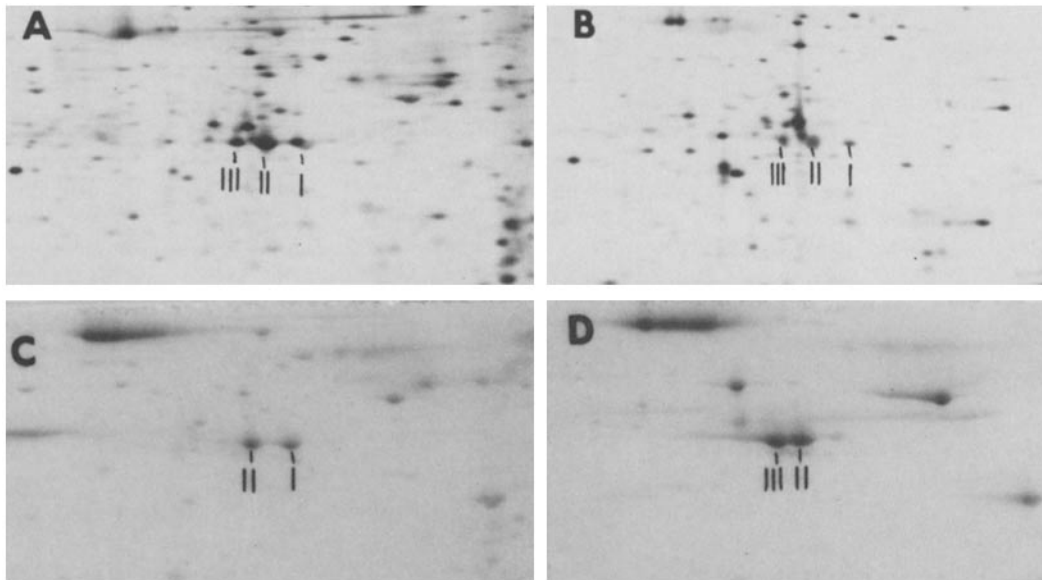


FIGURE 4 An analysis of actin synthesis (*A* and *B*) and accumulation (*C* and *D*) in adult abdominal and thoracic tissues. After a 1-h pulse with [³⁵S]methionine, two-dimensional gel electrophoresis was performed using total protein extracts. Autoradiographs of (*A*) adult abdominal tissue and (*B*) adult thoracic tissue are shown. Approximately 130,000 cpm were loaded onto each gel, and autoradiography was for 14 d. The Coomassie Blue-stained gels from which the above autoradiographs were taken are also shown: (*C*) adult abdominal tissue and (*D*) adult thoracic tissue.

ogy and physiology from supercontractile muscle. The second interesting feature is that the adult thorax produces a muscle-associated stable actin with the same isoelectric point as a highly unstable generalized cellular actin. An important question therefore is whether this stable thoracic actin represents a separate gene product differing in amino acid sequence from the unstable actin III but indistinguishable in the isoelectric focusing dimension, or whether it is merely a modified stabilized form of actin III. Both possibilities would necessitate a high degree of genetic control. In the first case, a distinct gene for a stable thoracic actin would be expressed only in the thorax of the mature adult. In the alternate case, one would have to postulate a tissue-specific stabilization of an existing unstable generalized actin, and its utilization for a specialized function.

It is quite apparent that *Drosophila* actin synthesis is highly regulated. This raises the question as to how many genes there are coding for actin. As was mentioned earlier, actins II and III (unstable) may represent products of the same gene, if indeed a precursor-product relationship exists be-

tween them. The stable thoracic actin of pI 5.84, on the other hand, may be the product of a separate gene, or may actually be a stabilized product of the same gene encoding actin III. Actin I, by similar reasoning, could be encoded by a separate, independently regulated gene or could represent a post-translationally modified form of either actin II or III. Consequently a determination of the number of different actin genes will have to await a more detailed study of their amino acid sequence.

Multiple actins seem to be the rule in higher eukaryotes, such as vertebrates (4, 8, 16, 21, 23, 24) and higher invertebrates (e.g. dipterans), all of which possess specialized muscle cells. The occurrence of multiple actins raises several interesting evolutionary questions. In each group of organisms, the different types of muscle tissue have different isoactin species. It is tempting to speculate that during evolution of multicellular organisms and the subsequent determination of muscle tissue, different forms of actin evolved to meet the specific structural and functional requirements of a large variety of muscle and nonmuscle cell types.

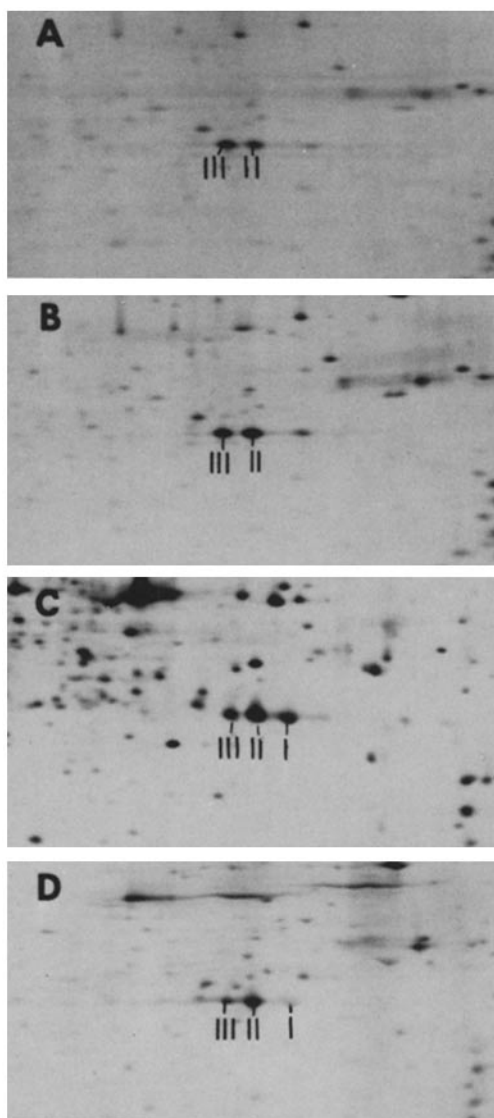


FIGURE 5 Fluorograms of [^{35}S]methionine-labeled proteins synthesized during a 1-h pulse by (A) gastrula-stage embryos (3.5–4 h), (B) 15-h embryos, (C) first instar larvae (25 h), and (D) mature third instar body wall tissue. Approximately 100,000–150,000 cpm were electrophoresed in each case, and fluorography was for 5–14 d.

This may have represented a very economical way to regulate the utilization of a single protein species for a large variety of cellular functions. It is also possible, however, that as a result of the increasing multi-functionality of actin, a special need may have arisen during evolution to more precisely regulate the amount of actin synthesized during different phases of cell growth and differentiation.

To meet this specialized requirement, one can postulate the evolution of several actin genes, fine-tuned to meet the diverse needs of a broad range of tissues during different growth and developmental stages.

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