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Molecular characterization of mutant actin genes which induce heat-shock proteins in *Drosophila* flight muscles

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Heat-shock proteins (hsps) are constitutively induced by the mutant actins in the *Drosophila* indirect flight muscles (IFM). We compared primary structures of the mutant actin genes (KM75 and HH5) which induce hsps and of the non-inducing alleles (KM129 and KM88). The KM75 actin has lost 20 amino acids at the C-terminus. The HH5 actin has only one amino acid substitution, from Gly-336 to Ser. In KM129, the C-terminal part of actin is replaced by novel amino acids. KM88 is a null allele, with an amber mutation early in the coding region of the mutated actin gene. Although all of the KM75, HH5 and KM129 actins have defects near the C-terminus, only hsp-inducing mutant actins cause enlargement of the IFM nuclei as well as a disruption of myofibrils even in the presence of two copies of the normal genes. We further consider the underlying mechanisms linking these features of the hsp-inducing alleles.

Key words: actin gene/heat shock protein/*Drosophila*

Introduction

We have reported four dominant flightless mutants (KM75, KM88, KM129 and HH5) of *Drosophila melanogaster* with defective *act88F* actin genes which would normally encode actin III in the indirect flight muscles (IFM) (Hiromi and Hotta, 1985). In KM88, no actin III is detected. In KM75 and KM129, actin-related proteins p42 and p38 are detected respectively (Hiromi and Hotta, 1985). Among them, only in KM75 and HH5 are the heat-shock proteins (hsps) constitutively induced at low temperatures (24°C) in the IFM.

We have shown by the P-element mediated transformation method that the mutations in the *act88F* gene are the primary cause of the abnormal hsp synthesis in these mutants (Hiromi *et al.*, 1986). Hsps are known to be induced by various agents which impose stress on cells such as heat, anoxia, ionophores and inhibitors of oxidative phosphorylation (for review, see Ashburner and Bonner, 1979). The destruction of the myofibrillar structure is common among all the *act88F* mutants, while only two of them induce hsps. Therefore, the stress imposed by the myofibrillar destruction cannot be the cause of the abnormal expression of hsps in these mutants.

The first aim of our study is to elucidate whether a common molecular feature exists in these hsp-inducing mutant actins and to obtain a clue for solving the mechanism of the hsp induction. For this purpose, we first identified the mutational sites of these mutant *act88F* genes followed by examination of the effect of these mutations on the IFM morphology; myofibrillar structures and the nuclear morphology.

The second aim of this paper is to find out why KM88 is a null mutant. We analyzed the accumulation of the *act88F* gene transcripts to determine whether the defect lies in the transcriptional or in the translational process. Then we compared the DNA sequence of the coding and flanking regions necessary for normal gene expression (Hiromi *et al.*, 1986) between KM88 and the normal *act88F* gene. We found that polyadenylated transcripts from the *act88F* gene are reduced remarkably by the amber mutation near the amino terminus of the coding region.

Results

Characterization of the normal act88F gene

We cloned the *act88F* gene of the wild strain (Canton-S, abbreviated as CS), from which all our mutants were derived, and determined the complete DNA sequence of the coding and flanking regions, to check if there were polymorphic changes from the published sequence data. The restriction map of the *act88F* gene is shown in Figure 1. Our results are identical with the sequence data of the mutant *raised* and CS reported by Mahaffey *et al.* (1985).

The sequence of the 770 nucleotides upstream from the translation initiation codon is shown in Figure 2. To determine the transcription initiation site, we performed a primer extension analysis. As a primer, we used a chemically synthesized 17-mer, which is complementary to the region near the translation start site of the *act88F* mRNA (Figure 3a). According to published data (Fyrberg *et al.*, 1982), this region is not homologous to other actin genes. 5′-^{32}P-labeled primer was hybridized to pupal poly(A)$^+$ RNA, and extended by reverse transcriptase. The lengths of the reaction products were 121 and 120 bp (Figure 3b). Since the first 5′ nucleotide of the mRNA is generally capped and difficult to reverse-transcribe from, the 120-bp product was considered to be incomplete by one base. However, as this was a major product, we recovered it and determined its nucleotide sequence by the Maxam-Gilbert method (Figure 3c). Comparing the sequence with that of the 5′ flanking region of the *act88F* gene (Figure 2), we found a 552-bp intron starting from

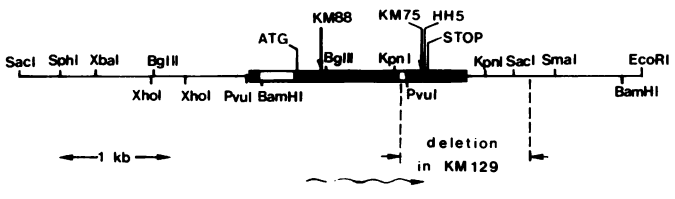

Fig. 1. Restriction map of the *act88F* gene. The restriction map of the *act88F* gene is shown. Exons and introns are indicated by heavy and open boxes respectively. The direction of transcription is shown by a wavy arrow. The mutation sites of KM88, KM75 and HH5 are indicated by arrows. The break points of the deletion in KM129 are indicated by broken lines.

Fig. 2. The nucleotide sequence of the 5′-flanking regions of the *act88F* gene. The nucleotide just before the first Met codon is numbered as −1. The region of the first intron is in a shaded box. The cap site and the first Met are marked. A TATA box and a CAT box are indicated by underlines.

−568 and terminating at −17. The transcription initiation site was located at −647. The presence of the intron in the 5′-nontranslated region was overlooked by the previous authors (Fyrberg *et al.*, 1981; Sanchez *et al.*, 1983). TATA box and CAT box-like structures were found 24 and 78 bp upstream from the cap site respectively (Figure 2).

RNA blot hybridization of mutant gene transcripts

KM88 is apparently a null allele, lacking actin III in the IFM (Hiromi and Hotta, 1985). To determine whether the defect is in the transcriptional or in the translational process, we examined poly(A)$^+$ RNA from pupae by RNA blot hybridization.

We used two kinds of single strand DNA probes (Figure 4). M105 hybridizes to the 3′-nontranslated region of the *act88F* gene transcripts. This region is shown to be specific for the *act88F* transcripts (Fyrberg *et al.*, 1983). M210 is derived from the 5′ part of the protein-coding region and should hybridize to all actin gene transcripts due to the sequence homology.

We first examined poly(A)$^+$ RNA from larvae, pupae and adults of the CS strain. With probe M210, actin transcripts of 1.7 kb were detected at the larval and pupal stages (Figure 4b), while probe M105 hybridized to a 1.7 kb fragment only at the pupal stage (Figure 4c). These results show that the *act88F* gene transcript is accumulated exclusively at the pupal stage. This is consistent with the result of the dot-blot hybridization by Fyrberg *et al.* (1983), but inconsistent with those of Sanchez *et al.* (1983) who found the *act88F* transcripts also in the larval stages.

In KM88, no transcript was detected with the *act88F* specific probe M105 (Figure 4c). Only small amounts of transcripts were detected by probe M210 (Figure 4b). These are most likely transcripts of actin genes other than the *act88F*.

In KM75 and HH5, the size and quantity of their transcripts were found to be normal. In KM129, the transcript was not detected at all with probe M105 (Figure 4c), but transcripts detected with probe M210 are normal (Figure 4b). This result

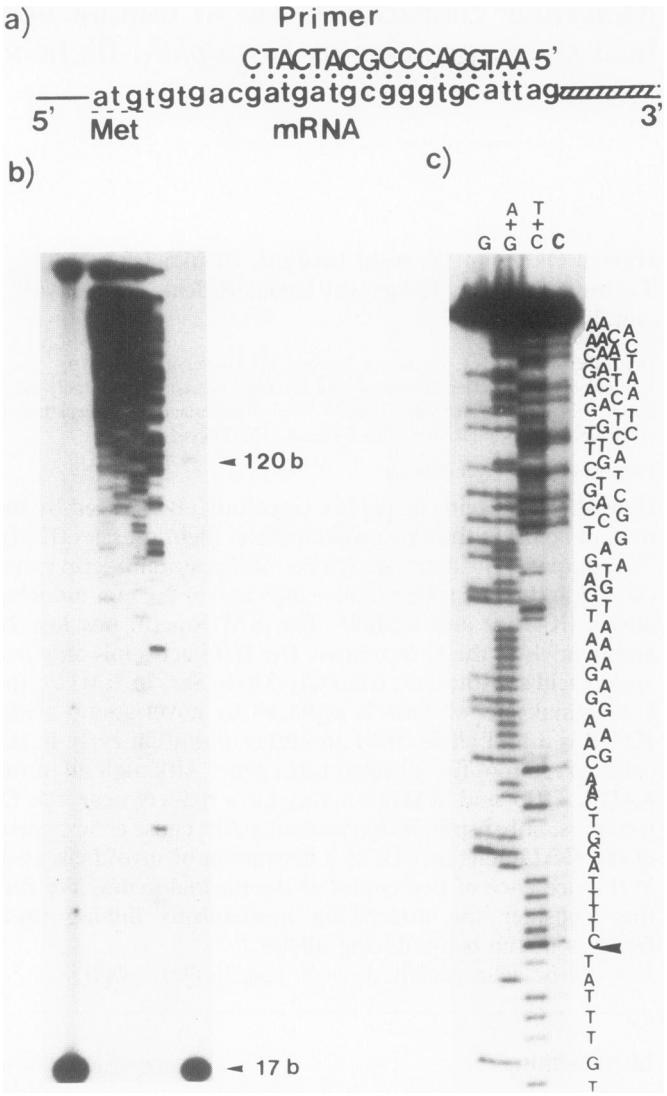

Fig. 3. Primer extension analysis of the *act88F* gene transcript. (**a**) The 28 bases from the first Met codon of the *act88F* gene transcripts are shown by lower case letters. The region hybridizing to the chemically synthesized primer (capital letters) is indicated by dots. (**b**) The result of the primer extension reaction is shown on the right-most lane. The major product is 120 bases long, and the minor product is 121 bases long. On the middle four lanes are shown ladders of known sequence as a marker. On the left-most lane is shown only the labeled primer. (**c**) The nucleotide sequence of the 120 base product. The site of junction after RNA splicing is shown by an arrow.

is consistent with the sequence data described later; KM129 having a deletion which eliminates the region complementary to probe M105.

Identification of the mutation site in KM88

We showed that a 4.05-kb *Xba*I-*Sac*I fragment containing the CS *act88F* gene functions normally when introduced into the genome by P-element mediated transformation (Hiromi *et al.*, 1986). To identify the mutation site responsible for the lack of the *act88F* gene transcript in KM88, we cloned the mutant gene and compared the whole nucleotide sequence of the 5-kb *Sac*I-*Sac*I fragment which includes the coding region and the 5′ and 3′ flanking sequences sufficient for normal gene expression. Only a single base change was found in this region of KM88; TGG (Trp 79) being converted to an amber stop codon (TAG) in KM88

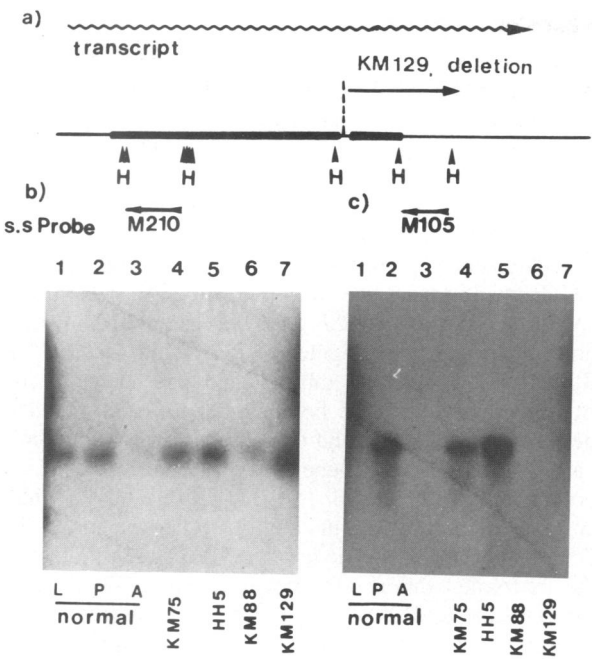

Fig. 4. RNA blot hybridization. (**a**) The regions complementary to single strand probe M105 or M210 are shown. *Hpa*II sites (H) are indicated by arrowheads. (**b**), (**c**) RNA blot hybridization of CS larvae (**lane 1**), CS pupae (**lane 2**), CS adults (**lane 3**), KM75 pupae (**lane 4**), HH5 pupae (**lane 5**), KM88 pupae (**lane 6**), KM129 pupae (**lane 7**). Larvae are a mixture of the second and third instar, and pupae are a mixture of all stages. M210 and M105 were used as probes respectively, in (**b**) and in (**c**). The same amount (10 μg) of poly(A)$^+$ RNA was loaded in all lanes.

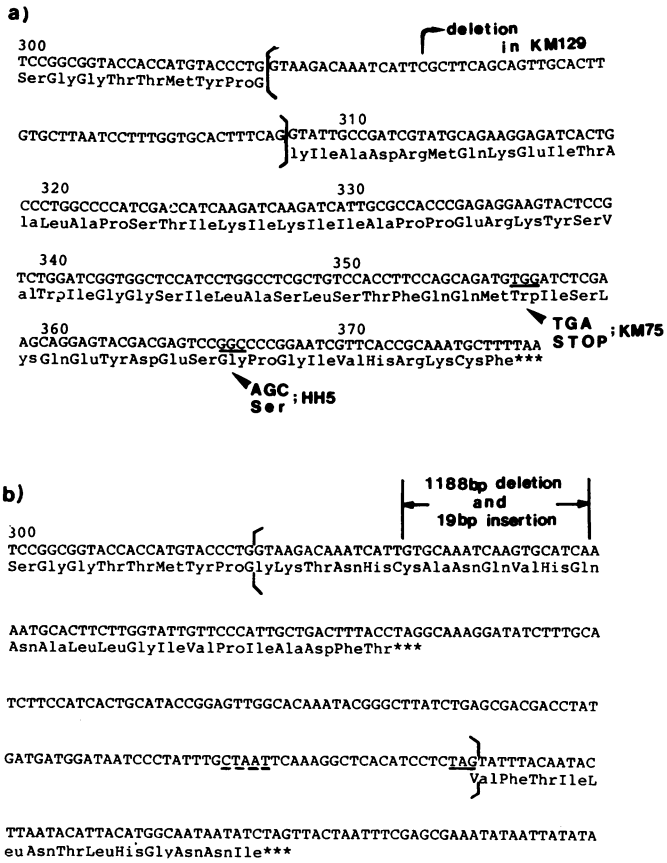

Fig. 5. The mutational sites of the *act88F* gene in KM75, KM129 and HH5. (**a**) Nucleotide and corresponding amino acid sequence of the *act88F* gene of the CS strain maintained in our laboratory is shown from Ser-300. The second intron is demarcated by parentheses. The stop codon is indicated by asterisks. Mutational changes of KM75 and HH5, and the deletion start site of KM129 are indicated. (**b**) Nucleotide sequence of the KM129 *act88F* gene covering the deletion site. The candidates for the 3′ splice site and 3′ splice signal (see text) are indicated by solid and broken underlines respectively. Deduced amino acid sequences according to the two possibilities described in the text are shown below. The 19-bp insertion of unknown origin is indicated. Stop codons are indicated by asterisks.

(Figure 1). Therefore, we conclude that this amber mutation is the cause of the reduction of the polyadenylated form of the mutant transcript to below the detectable level.

Identification of mutational sites in KM75, HH5 and KM129

To reveal whether a common molecular feature exists among the mutations that cause the hsp induction, we cloned the KM75, KM129 and HH5 mutant *act88F* genes. Since no gross alteration was found in KM75 or HH5 *act88F* genes by restriction mapping, we compared them with the normal gene by their nucleotide sequences. By P-element mediated transformation with chimeric actin genes, it was shown that mutation changes in the KM75 and HH5 *act88F* genes responsible for hsp induction must reside within the 0.8-kb *Kpn*I fragment (Figure 1) including the 3′ end of the gene (Hiromi *et al.*, 1986). Comparison of the mutant *act88F* sequences with the normal one revealed that both alleles have single base alterations in this fragment. The KM75 *act88F* gene has a change in Trp 356 (TGG) to a stop codon (TGA) (Figures 1, 5a). Therefore, the KM75 actin must be 20 amino acids shorter. This is consistent with the protein analysis (Hiromi and Hotta, 1985). We also determined the entire nucleotide sequence of the KM75 *act88F* gene, but no changes other than the one described above were found. Karlik *et al.* (1984) have recently analyzed the same mutant, and they reached the same conclusion on the basis of partial sequence data. In the other hsp-inducing allele, HH5, Gly 366 (GGC) was found to be converted to Ser (AGC) (Figures 1, 5a). This change should not make any detectable alteration in the isoelectric point or the molecular weight of the gene product. This is consistent with our previous analysis at the protein level (Hiromi and Hotta, 1985). Combined with the results of the P-element mediated transformation experiments, our experiments prove that these changes in the actin sequence are the causes of hsp induction and the myofibrillar disruption in KM75 and HH5.

From the restriction map analysis, we found that the KM129 *act88F* gene has a 1.2-kb deletion between the *Kpn*I site within the coding region and the *Sma*I site (Figure 1). Therefore we determined the nucleotide sequence in this region for KM129. The KM129 deletion turned out to be 1188 bp long starting from the 16th nucleotide in the second intron, and ending at 936 bp downstream from the translation termination codon (Figure 5a and b). This mutation deletes part of the second intron, the third exon and the 3′ flanking sequence (Figure 1). At the site of the deletion, 19 nucleotides of unknown origin are inserted (Figure 5b). Two alternative possibilities exist as to how this mutant gene transcript is processed. The 5′ consensus sequence of the second intron might be neglected and translation might continue into the intron without splicing. In this case, 25 novel amino acids would replace the normal 67 amino acids at the carboxy terminus (C terminus). This new protein is expected to be ~39 kd. Alternatively, the second intron might be spliced out utilizing a new 3′ splice site downstream from the deleted region. We find a candidate

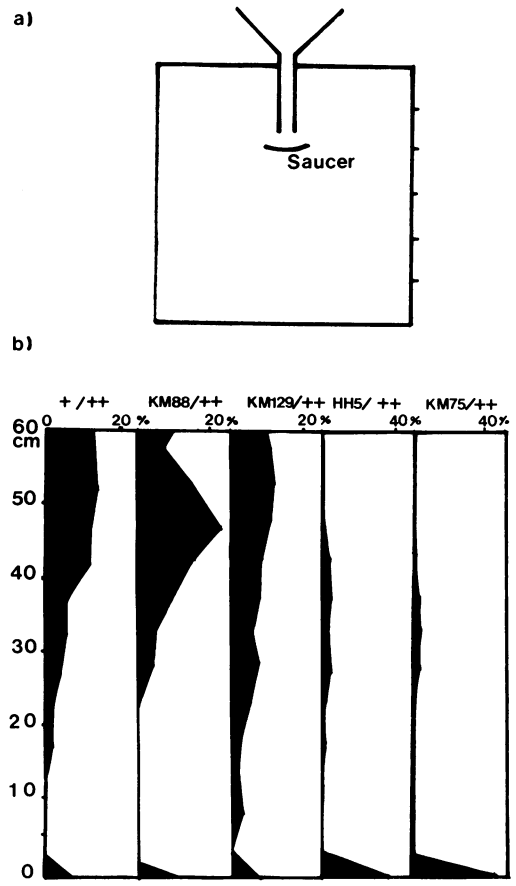

Fig. 6. Flight ability tests of transformants. (**a**) Flight tester. The flight tester is a column of 40 cm diameter and 60 cm height made of a 0.5 mm thick transparent plastic board. The top and the bottom are covered with transparent boards. At the center of the top board is inserted a funnel with a duct 17 cm long below the top board. A saucer of 4 cm diameter is hung 3 cm below the funnel. Inside walls of the apparatus are coated with liquid paraffin. (**b**) The flight ability of the transformants with an ectopic normal *act88F* gene in addition to one normal and one mutant gene at the 88F locus. Distributions of transformants trapped inside the wall are shown by percentage. The number of flies tested was 182, 255, 127, 365 and 244 for +/++, KM88/++, KM129/++, HH5/++ and KM75/++ respectively (abbreviations, see text). 3% or 6.6% of HH5/++ or KM75/++ respectively still remained on the saucer 3 min after they were released.

with a consensus sequence $[(\frac{T}{C})_n$-N-$\frac{T}{C}$-A-G] (for reviews see Breathnach and Chambon, 1981; Mount, 1982) for the new 3′ splice site (Figure 5b). The consensus sequence (C-T-A-A-T) for the *Drosophila* 3′ splice signal (Keller and Noon, 1984) is located 19 bp upstream from this site. In this case, 13 novel amino acids would replace the 67 normal C-terminal amino acids. This change would also make a new smaller protein (~38 kd). Since we found that the KM129 mutant actin migrates with apparent molecular weight of ~38 kd, we cannot distinguish between these two possibilities. In either case, novel amino acids would replace the C-terminal portion of the normal actin molecule.

Disruptive effect of mutant actins on myofibrils

Heterozygotes of the null mutant KM88 are flightless, and have defective myofibrils. This shows that a single dose of the normal *act88F* gene is insufficient for normal myofibrillar formation. Therefore we cannot clearly discriminate whether KM75, KM129 and HH5 mutant actins are merely 'hypomorphic' (loss of function) or 'antimorphic' (positively destroying the myofibrils), just by observing heterozygotes with only one normal and one mutant *act88F* gene.

We showed that the introduction of two doses of the KM75 or HH5 mutant *act88F* gene by the P-element mediated method renders the individuals flightless even when they possess two doses of the normal gene (Hiromi *et al.*, 1986). This suggests that their mutant actins are antimorphic. We further examined whether KM129 actin, which has its defect near the C-terminus as KM75 or HH5 actins have but does not induce hsps, is also antimorphic. For this purpose, we utilized the transformant fly P[*ry*,CSB] transformant line No.4 (Hiromi *et al.*, 1986) in which one additional normal *act88F* gene was integrated in the third chromosome. The homozygotes of this strain: *act88F*$^+$ P[*ry*, CSB]/*act88F*$^+$ P[*ry*,CSB] (abbreviated as ++/++ in this paper) were mated with the homozygotes of each mutant. The progeny have one mutant and one normal gene at the 88F loci and an additional normal gene at the ectopic locus.

As shown in Figure 6b, CS/++, KM129/++ and KM88/++ show almost normal flight ability, whereas most of the KM75/++ and HH5/++ do not fly. Phase contrast micrographs of the macerated thorax are shown in Figure 7. In +/++, KM88/++ and KM129/++, a number of long free myofibrils are present. In KM 75/++ and HH5/++, however, such free myofibrils are rarely found. When present, they are generally short and fragmented.

Therefore, we conclude that the KM129 mutant actins disrupt myofibril assembly to a much lesser extent than those of KM75 and HH5.

Nuclear morphology of mutant IFM

Another remarkable feature unique to the hsp-inducing alleles (KM75 and HH5) is that the IFM nuclei of these alleles are irregularly deformed and swollen. IFM nuclei were observed by the indirect immunofluorescence method, using a monoclonal antibody (MAb8C5), which specifically binds to all the nuclei (Fujita *et al.*, 1982). Nuclei of the normal IFM are ellipsoidal (6 μm × 2 μm) with a long axis aligned along the myofibrils (Figure 8a). IFM nuclei of KM75, on the other hand, are enlarged to become spheroidal with a diameter of 4.5−7 μm (Figure 8b). These nuclei are stained homogeneously with toluidine blue, and are devoid of the heterochromatin-like condensed structures (Figure 8g). The nuclear enlargement is observed only in the IFM, but not in any other tissues including tubular muscles adjacent to the IFM.

A similar but less prominent morphological change is observed also in the IFM of HH5 (Figure 8f). In this strain, the heterochromatin-like structures are present, but appear to be localized near the nuclear surface. These morphological abnormalities are never observed in the IFM of alleles which do not induce hsps such as KM88 or KM129 (Figure 8d and e). Since IFM myofibrils in KM88 are also disorganized, the abnormal nuclear enlargement in KM75 and HH5 cannot be explained by the mere absence of myofibrils. It is also unlikely to be due to the presence of hsps, since such a nuclear enlargement is not observed in the normal IFM under the heat-shocked condition (data not shown).

Discussion

The amber mutation early in the coding region of the act88F gene reduces the accumulation of its transcripts

The prominent decrease in the polyadenylated form of the KM88 *act88F* transcript is caused by the amber mutation of the codon which would normally encode Trp 79.

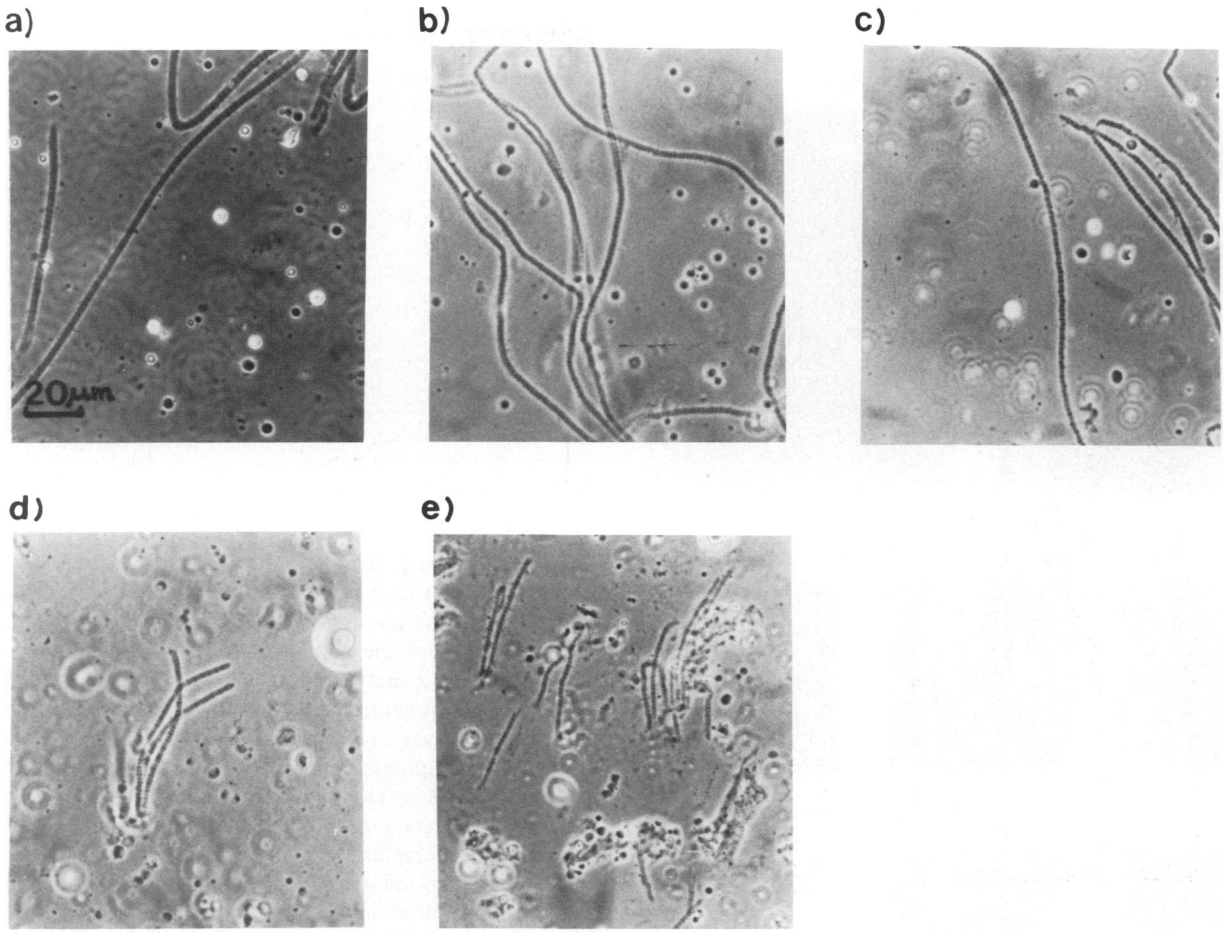

Fig. 7. Phase contrast micrographs of the macerated IFM. A number of free myofibrils are observed in +/++ (**a**), KM88/++ (**b**), KM129/++ (**c**). In HH5/++ (**e**) and KM75/++ (**f**), on the other hand, such free myofibrils are rarely found. If present, they are short and fragmented. Bar = 20 μm.

In the yeast *URA3* gene, amber mutations near the N terminus of the coding region are known to impair the stability of its transcript (Losson and Lacroute, 1979). Therefore, the lack of the *act88F* mRNA accumulation in KM88 is likely due to its accelerated degradation. Frameshift mutations which generate a new nonsense codon early in the coding region of the mouse immunoglobulin heavy chain gene also remarkably reduce the μ-mRNA (Baumann *et al.*, 1985).

Since we have not yet measured the rate of mRNA synthesis nor quantitated the non-polyadenylated form of the transcript, the possibility still remains that transcription of the KM88 *act88F* gene is inactivated or prematurely terminated.

Hsp-inducing mutant actins are antimorphic with respect to myofibrillar formation

There is a correlation between the hsp-inducing ability of mutant actins and their antimorphic effect on myofibril assembly; the two hsp-inducing alleles (KM75 and HH5) are antimorphic, whereas the hsp-noninducing allele (KM129) appears to be hypomorphic (Table I).

All hsp-inducing actins we have analyzed have turned out to have their defects near the C terminus. Chemical cross-linking studies with rabbit skeletal actin have shown that one of the actin-actin binding sites is close to the C terminus (Sutoh, 1984; Elzinga and Phelan, 1984). Therefore, it is possible that the mutant actins have defects in one binding site and cannot polymerize normally. From the antimorphic property of these mutants, we further propose a hypothesis that these mutant actins interfere with the polymerization of normal actins. In *Physarum,* proteins with an actin-related structure, such as fragmin, Cap42(a) and Cap42(b), bind to the fast-growing end of F-actin and inhibit its further elongation (Maruta *et al.*, 1984). KM75 and HH5 mutant actins may disrupt myofibrils in a similar manner. Or they may be incorporated into thin filaments with normal actins but make thin filaments fragile.

The hsp-noninducing allele (KM129) also lacks the C terminal part including the putative actin-actin binding site. However, this mutant actin has little disruptive effect on the myofibrillar organization. A relatively large deletion of the C terminus (67 amino acids as compared with 20 in KM75) cannot alone account for this property, since an *in vitro* mutated actin, whose C-terminal 72 amino acids were replaced by only two new ones, also shows an antimorphic property and induces hsps constitutively (Hiromi *et al.*, 1986).

The unique feature of the KM129 actin is that a long stretch of novel amino acids have replaced the C-terminal portion of the normal actin. These irrelevant amino acids may sterically hinder the KM129 mutant actin from interacting with other normal actins. This could explain why KM129 actin does not affect the myofibril organization in the transformants.

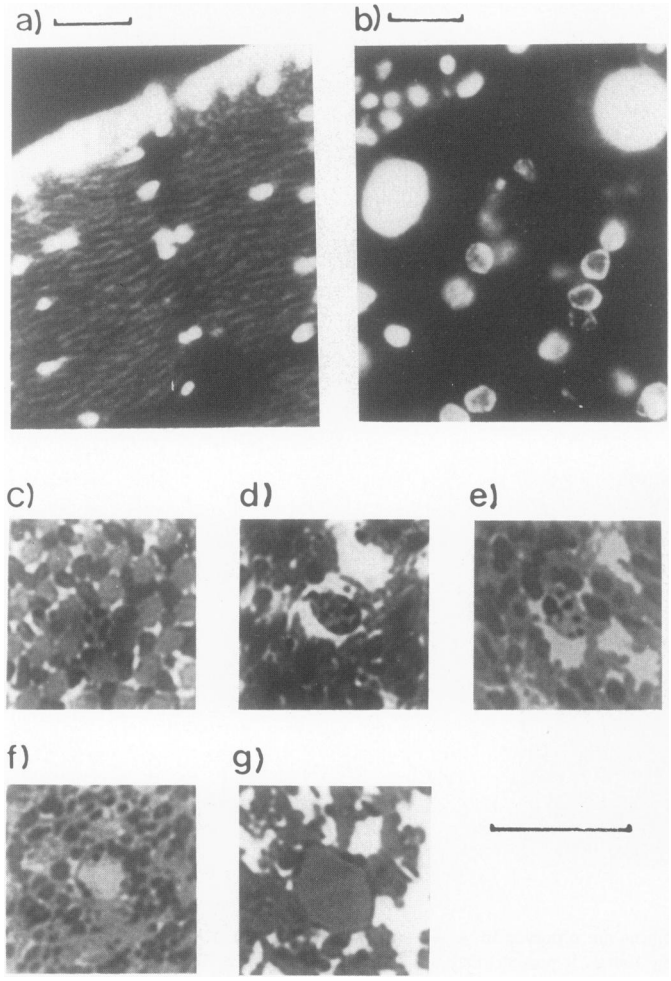

Fig. 8. Morphology of the IFM nuclei. Cryostat sections of the normal (**a**) and KM75 (**b**) thoraces were stained with a nuclear-specific monoclonal antibody MAb8C5 (Fujita *et al.*, 1982). The IFM nuclei of KM75 are abnormally enlarged in comparison with those in the normal muscle. In contrast to the homogeneous staining patterns of the normal IFM nuclei, the surface appears to be preferentially stained in KM75. The nuclear abnormality is restricted to the IFM. The nuclei of the cuticular cells, seen in the uppermost part of the figures are not deformed in KM75. Two large round structures beneath the cuticle in (**b**) are nuclei of the fat body cells. Bar = 20 μm. The typical IFM nuclei stained with toluidine blue are shown for normal (**c**), KM88 (**d**), KM129 (**e**), HH5 (**f**) and KM75 (**g**) respectively. Bar = 10 μm. In the normal IFM, nuclei appear small, round and condensed. In contrast, the IFM nuclei of HH5 and KM75 are enlarged, and their matrices appear homogeneous. The IFM nuclei of KM88 and KM129 appear somewhat oval because of their irregular orientations in the sections.

Possible mechanisms of hsp induction by the mutant actins

The hsp genes have been intensively investigated as a model to study differential gene regulation. The presence of a factor which interacts with the hsp gene promoter has been demonstrated by biochemical methods (Wu, 1984; Parker and Topol, 1984). Such a factor is thought to be involved in the final step of the sequential events leading to hsp gene expression. A genetic approach has also been carried out to select mutants affecting hsp inducibility and to identify the genes controlling the intermediate steps (Bonner *et al.*, 1984).

Table I. Summary of the characters of each mutant allele

	Variant actin	*act88F* mRNA	Hsp induction	Molecular lesions	Flight M/+[a]	Flight M/++[b]	Nuclei
KM88	None	−	−	Trp 79 →Amber	−	+	normal
KM129	p38	+	−	Substitution of C terminus	−	+	normal
KM75	p42	+	+	Trp 356 →Opar	−	−	enlarged
HH5	+[c]	+	+	Gly 366 →Ser	−	−	enlarged

[a]M/+; heterozygotes with one normal and one mutant actin gene.
[b]M/++; transformants with two normal and one mutant actin genes.
[c]HH5 mutant actin co-migrates with normal actin III.

Although the hsp-inducing ability of mutant actins shows a good correlation with their antimorphic property on the myofibrillar organization, the disruption of myofibrils *per se* cannot be the primary cause of the hsp induction, since a number of IFM mutants having a similar phenotype do not induce hsps (Mogami *et al.*, 1981; Mogami and Hotta, 1981). Recently, actins in the nuclear matrix are suggested to have a close interaction with the general transcriptional machinery. Injection of fragmin or anti-actin antibodies into the nucleus of amphibian oocytes has been reported to induce a retraction of lampbrush chromosomes and to inhibit transcription in general (Scheer *et al.*, 1984).

As to the hsp-inducing mechanism of our mutant actins, we consider the following possibilities. One possibility is that the mutant actins act on the hsp genes indirectly via some intervening factors. As actin can diffuse into the nucleus (Rubin *et al.*, 1976; De Robertis *et al.*, 1978), the mutant actins may also interact with nuclear actins. The abnormal nuclear enlargement observed only in the hsp-inducing alleles may be caused by disruption of the nuclear actin framework by the antimorphic actins. The breakdown of the nuclear framework may act indirectly to induce hsps in a similar manner to other cellular stresses.

Another possibility is that actins interact directly with the hsp genes. Egly *et al.* (1984) suggested that actin is one of the transcription initiation factors. The mutant actins themselves, or the normal form of G-actins which have detached from the disrupted nuclear or cytoplasmic framework, may directly activate the hsp genes.

It is not known whether the induction of hsps by the mutant actin is a phenomenon specific to the *Drosophila* IFM. The hsp genes are well known to have highly conserved regulatory systems (Pelham, 1982; Pelham and Bienz, 1982; Bienz and Pelham, 1982). Moreover, actin and hsp proteins are also conservative. Therefore, if actins are critically involved in such a regulatory mechanism, the mutant actins will induce hsps when expressed in cells other than *Drosophila* IFM. Actin gene mutations of *Caenorhabditis elegans* have been identified recently (Waterston *et al.*, 1984; Landel *et al.*, 1984). Some of them are suggested to be antimorphic. In the human fibroblast cell line, conversion of Gly 244 to Asp in β-actin is known to transform the cell into malignancy and to disrupt cytoskeletons (Leavitt and Kakunaga, 1980; Vanderkerckhove *et al.*, 1980; Leavitt *et al.*, 1984). It is of interest to determine whether hsps are induced in such cases.

Materials and methods

Drosophila strains and mutations

The CS strain was used as a wild type. All the mutants (KM75, KM129, KM88 and HH5) were isolated by the dominant flightless character after mutagenesis with ethylmethane sulfonate (Mogami and Hotta, 1981; Hiromi and Hotta, 1985). Transformant line P[*ry*,CSB].4 with duplicated normal *act88F* genes on the third chromosome was made by introducing the normal *act88F* gene into the *rosy*^{506} strain by P-element mediated transformation (Hiromi *et al.*, 1986).

Flight ability test

With the original type of flight tester described in Koana and Hotta (1978), flies with insufficient power to fly up were still able to glide down and occasionally be trapped at a higher position in the column. This was because the diameter of the tester column was too small and flies were released directly from its top. To score more clearly flies with intermediate flight ability, an improved version was devised as illustrated in Figure 6a. We used a column of a larger diameter and allowed the flies to take off from within the column instead of just releasing them from the top.

Flight ability was tested five days after eclosion. Flies were put into a funnel, and they fell down through a plastic tube onto a saucer from which they took off voluntarily. When they bumped into a wall, they were trapped on the spot, since all the inner surface was coated with liquid paraffin. Three minutes after release, the number of flies trapped was counted in 5-cm intervals from bottom to top. When more than half of the flies of a strain could reach the wall above the saucer, we regarded them as having normal flight ability. All tests were carreid out at room temperature with illumination from above the apparatus.

Maceration of the IFM

Maceration of the IFM was performed according to the method of Hotta and Benzer (1973). An adult female was anesthetized with diethylether 5 days after eclosion. The thorax was isolated with a razor blade, and macerated with fine needles in *Drosophila* Ringer solution (0.65% NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 3 mM $NaHCO_3$). Macerated IFM was observed by phase-contrast microscopy.

Molecular cloning of the normal and mutant act88F genes

Genomic DNA of each strain was purified from 10 g ($\sim 10^4$) adult flies according to the method of Suzuki *et al.* (1972) except that flies frozen by liquid nitrogen were crushed with Freezer-Mill (Spex). DNA was digested completely with *Eco*RI and size fractionated by sucrose density gradient centrifugation. Fragments larger than 10 kb were recovered, ligated with right and left arms of Charon4A or EMBL4, and packaged *in vitro*. The library was screened with nick-translated probes K1b and Hd19 which were kindly provided by Dr S.L.Tobin (Tobin *et al.*, 1980). K1b hybridizes to the 3′ flanking region of the *act88F* gene, while Hd19 contains the entire coding region of the *act5C* gene. Clones containing a 15-kb *Eco*RI fragment hybridizing to both probes were selected as *act88F* gene clones in CS, KM88, KM75 and HH5. In KM129, clones slected by the same criteria contained a 1.2-kb shorter insert. All procedures were carried out according to Maniatis *et al.* (1982). For further analyses, cloned genomic DNA was subcloned into pBR322, pAT153 pUC18 or pUC19.

DNA and RNA blot hybridization and primer extension

DNA blot hybridization was carried out according to the standard method (Southern, 1975). RNA was purified from a mixture of second and third instar larvae, a mixture of all stage pupae, or a mixture of 4- to 7-day-old adults, according to the method of Chirgwin *et al.* (1979). Poly(A)$^+$ RNA was selected by oligo(dT)-cellulose column chromatography. Agarose gel electrophoresis and blotting onto nitrocellulose filters of poly(A)$^+$ RNA was carried out according to Thomas (1980). Single-strand specific probes M105 and M210 were made during our shotgun DNA sequencing. Probes were labeled with ^{32}P and hybridized with the blotted filter according to Messing (1983). Primer extension was performed according to Nathans and Hogness (1983), using chemically synthesized 17-mer as a primer. Reaction products were recovered and sequenced by the method of Maxam and Gilbert (1977).

Determination of DNA sequences

A 3.5-kb *Bam*HI fragment which contains the whole coding region of the CS or KM75 *act88F* gene was digested with *Taq*I or *Hpa*II. The digests were cloned into M13mp10, and sequenced by the dideoxy method (Messing, 1983). The data was put into a microcmputer and the whole sequence reconstructed with the software developed by Mitsui Knowledge Industry Co. Ltd.

The following *act88F* gene fragments were subcloned into pUC19; *Kpn*I−*Sma*I fragment for KM129, *Kpn*I−*Kpn*I fragment for HH5 and *Kpn*I−*Sac*I fragment for CS. Their sequences were directly determined by the modified dideoxy method of Hattori and Sakaki (personal communication).

Flanking regions of the CS *act88F* gene and the 5-kb *Sac*I−*Sac*I fragment of the KM88 *act88F* gene were also sequenced by the same method in combination with a 'kilosequencing method'. A 5-kb *Sac*I−*Sac*I fragment was inserted into pUC18. A set of plasmids which had variable length deletions from the primer binding site were systematically made according to the method of Frischauf *et al.* (1980). DNA sequence determination was carried out beginning with the largest plasmid to the smaller ones so that overlapping sequence data was obtained.

Observations of the IFM nuclei

The monoclonal antibody MAb8C5 was provided by Dr S.C.Fujita, and immunohistochemistry was performed according to Fujita *et al.* (1982).

For closer observations of the IFM nuclei, thoraces of 4- or 5-day-old adult flies were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde with 0.08 M cacodylate buffer (pH 7.4). They were postfixed in 2% osmium tetroxide, and were embedded in Epon. Sections were stained with 1% toluidine blue, and observed with a Zeiss light microscope.

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