

## The *held out wings (how)* *Drosophila* gene encodes a putative RNA-binding protein involved in the control of muscular and cardiac activity

Stéphane Zaffran, Martine Astier, Danielle Gratecos and Michel Sémériva\*

Laboratoire de Génétique et Physiologie du Développement, UMR 9943 CNRS-Université, IBDM CNRS-INSERM, Université de la Méditerranée, Campus de Luminy Case 907, 13288 Marseille Cedex 9, France

\*Author for correspondence (e-mail: semeriva@lgpd.univ-mrs.fr)

### SUMMARY

In an attempt to identify genes that are involved in *Drosophila* embryonic cardiac development, we have cloned and characterized a gene whose function is required late in embryogenesis to control heart rate and muscular activity. This gene has been named *held out wings (how)* because hypomorphic mutant alleles produce adult animals that have lost their ability to fly and that keep their wings horizontal at a 90° angle from the body axis. In contrast to the late phenotype observed in null mutants, the *How* protein is expressed early in the invaginating mesoderm and this expression is apparently under the control of *twist*. When the different mesodermal lineages segregate, the expression of *How* becomes restricted to the myogenic lineage, including the cardioblasts and probably all the

myoblasts. Antibodies directed against the protein demonstrate that *How* is localized to the nucleus. *how* encodes a protein containing one KH-domain which has been implicated in binding RNA. *how* is highly related to the mouse *quaking* gene which plays a role at least in myelination and that could serve to link a signal transduction pathway to the control of mRNA metabolism. The properties of the *how* gene described herein suggest that this gene participates in the control of expression of as yet unidentified target mRNAs coding for proteins essential to cardiac and muscular activity.

Key words: *Drosophila*, heart development, myogenesis, KH-domain, *held-out-wings*

### INTRODUCTION

During the past 2 to 3 years, and particularly due to recent studies in *Drosophila*, important informations have been gained on the regulatory mechanisms that underlie heart formation and acquisition of function (Bate, 1993; Bodmer, 1995; Zaffran et al., 1995; Lyons, 1996). In the fruit fly, construction of the heart, or dorsal vessel, is completed by the end of embryogenesis and the newly built organ is fully functional (Bate, 1993). It is composed of essentially two types of cells: the cardinal cells, which are muscular cells responsible for the contractile activity of the heart, and the pericardial cells, which are compared to nephrocytes and which do not participate in the cardiac function proper. The cardinal cells are arranged as a tube composed of two rows of cells bordering the lumen. The dorsal vessel extends from the posterior region of the cerebral hemispheres to the last abdominal segment, beneath the dorsal epidermis at the dorsal midline (Bate, 1993; Ruggendorf et al., 1994) to which it is anchored by seven pairs of alary muscles.

The heart cells originate from the dorsal crest of the mesoderm and they require inductive instructions from the dorsal ectoderm for both determination and differentiation (Staehling-Hampton et al., 1994; Frasch, 1995; Park et al., 1996; Wu et al., 1995; Baylies et al., 1995; Lawrence et al., 1995). These signals, including diffusible proteins such as Wingless and Decapentaplegic, activate and maintain in the dorsal mesoderm the expression of the homeobox-containing

gene *tinman* whose activity is necessary for the formation of the heart, the visceral mesoderm and some body wall muscles (Bodmer et al., 1990; Azpiazu and Frasch, 1993; Bodmer, 1993).

The early events underlying the development of the cardiac tube in invertebrates appear remarkably well conserved in vertebrates, despite obvious differences in adult heart forms (Bodmer, 1995; Lyons, 1996). The precursor cells arise from the anterior plaque of the lateral mesoderm on each side of the embryo. Later, they organize themselves in two rows of cells which, as in insects, ultimately join to form the cardiac tube in the middle of the ventral side (note that it is not the dorsal side since insects and vertebrates have inverted dorsoventral axes) in close proximity to the epidermis. Inductive signals transmitted through different germ layers have been involved, in vertebrates as well, in embryonic formation of the heart. Some of these signals, however, emanate from the endoderm in vertebrates, whereas they arise from the ectoderm in *Drosophila* (see for review, Lyons, 1996). Finally, the fundamental pharmacological properties of the heart rate appear comparable (Gu and Singh, 1995).

Such a morphological similarity in vertebrates and invertebrates embryogenesis is mirrored at the molecular and genetic levels. *tinman*-related genes such as *Nkx 2.5* and *Nkx 2.3* have been identified in vertebrates and their expression has been predominantly found in the developing heart. Functional disruption of *Nkx 2.5* in mice causes strong defects in embryonic

heart development (Lyons, 1996) and perturbation of *tinman* homologous function in other vertebrates leads to similar phenotypes (see for review, Harvey, 1996).

To gain a better understanding of the different aspects of heart differentiation, we are attempting to identify and characterize *Drosophila* genes that could play a role in these various processes. At gastrulation, mesodermal cells are not committed and their fate towards different mesodermal derivatives is progressively acquired with respect to their position in the embryo and inductive inputs (Bate, 1993). Genes responsible for the heart formation could also be expressed in other tissues from mesodermal origin and be involved in more general processes preceding the segregation of the different mesodermal lineages. The most specific heart markers such as *Gox*, *EC11* (Zaffran et al., 1995) or *disconnected* (Lee et al., 1991) are expressed after the segregation of mesodermal lineages and the choice between cardioblasts and pericardial cells. As a consequence, one can assume that only a few mutations will specifically affect heart formation without producing other phenotypes due to earlier events that will mask the heart phenotype.

These considerations have led us to select an enhancer trap line, 1A122 (Perrimon et al., 1991), in which the reporter gene is expressed in the cardioblasts but, also, in other derivatives of the mesoderm. Its early expression in the invaginating mesoderm could reflect its participation in the early steps of heart formation and of the other muscle derivatives. Moreover, its expression becomes, later during development, restricted to the myogenic lineage suggesting a potential role in that step that allows a choice for the mesodermal cells to evolve towards a muscular or a non-muscular fate.

This gene has been cloned and has been called *held out wings* (*how*) because hypomorphic alleles produce individuals that do not fly and keep their wings extended horizontal at a 90° angle from the body axis. Surprisingly enough, *how* does not seem to be required for the heart or muscles formation despite its early expression but rather for the activity of the differentiated tissues. *How* encodes a putative RNA-binding protein containing a KH-domain that could participate in some aspect of gene regulation to insure tissue-specific control of the expression of gene products required for correct muscular and cardiac activity.

## MATERIALS AND METHODS

### (1) DNA techniques

Standard molecular biology methods were used (Sambrook et al., 1989). Genomic DNAs flanking the enhancer trap P-elements were isolated by using the plasmid rescue technique. The largest plasmid rescue clone, a region of 3.5 kb flanking the 1A122 P-element (p1A122XhoI), was used to screen an EMBL3 Canton-S genomic library (Clontech Laboratories, Inc.) and a Canton-S 4-8 hours embryonic cDNA library (Brown and Kafatos, 1988). Fragments of genomic and cDNA clones were subcloned into pBluescript (Stratagene) and mapped by using restriction enzymes. All the clones obtained mapped to position 93E-F. Both strands of the largest cDNA clone pRX5 and the 3.5 kb genomic fragment obtained from plasmid rescue were sequenced by Genome Express (Grenoble, France). EMBL Database Library accession number: U 72 331.

### (2) In situ hybridization on whole-mount embryos

Digoxigenin (DIG)-labelled antisense or sense RNA probes were

generated from DNA with T3 or T7 RNA polymerase (Promega) and DIG-UTP (Boehringer) and were used for whole-mount in situ hybridization of fixed staged embryos as described in Vincent et al. (1994). The DIG-labelled RNA probes were detected with the aid of a preadsorbed anti-DIG antibody coupled to alkaline phosphatase (Boehringer) and NBT/BCIP as substrate. The embryos were mounted in Geltol medium (Immunotech, France) for further observation.

### (3) Northern blot analysis

Total RNA was prepared from 0-2 hours, 2-8 hours and 12-20 hours old embryos, 1st instar larvae, and male and female adults using TRIzol reagent (GIBCO-BRL). Poly(A)-rich mRNA was affinity-purified using a mRNA purification kit (Pharmacia Biotech). Poly(A)-rich mRNAs (5 µg per lane) were separated in 1% agarose gels under denaturing conditions, blotted onto a nitrocellulose filter (Hybond-C extra, Amersham) and probed with DNA radiolabelled with the random priming procedure (Megaprime DNA-labelling system, Amersham) according to standard protocols (Sambrook et al., 1989).

### (4) Generation of antibodies

A *Pst*I-*Ssp*I 3 kb fragment from the *how* cDNA encoding amino acids 14-405 was inserted into the QIA express pQE31 vector (Qiagen Inc.). The protein was purified on a Ni-NTA column under the conditions proposed for small-scale purification of insoluble proteins including 6 M GuCl and elution with 500 mM imidazole. After dialysis, the protein was used to immunize rats following standard protocols.

Polyacrylamide gel electrophoresis under denaturing conditions and Western blotting procedures were as described by Towbin et al. (1979). Revelation of the blots was carried out by using secondary antibodies coupled to alkaline phosphatase (Promega).

### (5) Antibody staining of whole-mount embryos

Embryos were fixed and stained with antibodies according to the protocol described by Ashburner (1989).  $\beta$ -galactosidase in embryos was detected by using either a mouse (Promega) or, in double-staining experiments, a rabbit (Cappel) anti- $\beta$  galactosidase antibody. Secondary antibodies (Immunotech, France or Vector labs for anti-rat antibodies) were coupled to alkaline phosphatase or to peroxidase and were used at a 1:1000 dilution. All primary antibodies were preadsorbed on embryos before use. The anti-*How* antibody was routinely diluted 2000-fold. The embryos were mounted in Permount medium for observation under the microscope.

### (6) Generation and analysis of mutations

In situ hybridization on polytene chromosomes was performed as previously described. The Df(3R)e-BS2, Df(3R)e-Gp4, Df(3R)-GC14, Df(3R)e-N19 and Df(3R)e-F1 deficiencies for the mapping of the *how* mutation were obtained from the Bloomington *Drosophila* Stock Center. The 1A122 insertion mapped inside the region uncovered by Df(3R)e-BS2 or Df(3R)e-N19 and outside the regions uncovered by the other three.

The enhancer trap P-element (P[ry<sup>+</sup>, *lacZ*]) of line 1A122 was mobilized in a cross with flies carrying a stable source of transposase, the P[ry<sup>+</sup>,  $\Delta$ 2-3] chromosome (Robertson et al., 1988). A set of lethal excisions, *how*<sup>13</sup>, *how*<sup>15</sup> and *how*<sup>18</sup>, was isolated that failed to complement the deficiency Df(3R)e-BS2. Mutant chromosomes were placed over marked balancer chromosomes carrying a *lacZ* marker (TM3Sb, P[Deformed-*lacZ*]) that serve to identify in antibody staining experiments mutant embryos.

An additional lethal mutation was associated to the original 1A122 3rd chromosome since revertants obtained by accurate excision of the P-element were fully viable on Df(3R)e-BS2 but lethal when homozygous. Cleaning of the initial chromosome while preserving the mutation has been carried out by recombination with a *ru*, *h*, *th*, *st*, *sr*, *cu*, *e*, *ca* homozygous viable chromosome. The efficiency of the cleaning was checked by the viability of the recombinant flies over the revertants obtained by jump-start. Five recombination events have

been kept for each one of the *how* mutation (*how*<sup>13</sup>, *how*<sup>15</sup>, *how*<sup>18</sup> and 1A122).

Southern analyses were performed on the DNA from the *how* lines that was isolated from heterozygous mutant adults and control lines (Oregon R, 1A122 and Df(3R)e-BS2). Although the DNA preparation from adults contained a mixture of both wild-type (balancer) and mutant DNA, the new restriction fragments generated by deletions in the mutant were easily identified.

### (7) Germ-line clone analysis

Generation of germ-line clones was as described in Chou and Perrimon (1992). The P (*ry*<sup>+</sup>, neo-FRT) 82B, *ry*<sup>506</sup> chromosome was recombined with a chromosome bearing the *how*<sup>18</sup> mutation. The recombined chromosomes were checked for both their ability to confer neomycin resistance and lethality brought about by the *how*<sup>18</sup> mutation in *trans* of a deficiency of the locus. The analyses were carried out on two individual recombinant chromosomes and gave identical results.

Late 3rd instar larvae derived from mating P (*ry*<sup>+</sup>, neo-FRT) 82B/*how*<sup>18</sup> / TM3 males to w/P (*ry*<sup>+</sup>, *hs* FLP); P (*ry*<sup>+</sup>, neo-FRT) 82B, P (w<sup>+</sup>, *ovo*<sup>D1</sup>) 3R1, P (w<sup>+</sup>, *ovo*<sup>D1</sup>) / TM3 females were heat shocked for 2 hours at 38°C.

The adult females FRT *ovo*<sup>D1</sup> / FRT *how*<sup>18</sup> crossed with wild-type males laid eggs that developed into normal adult flies. The maternal expression of *how*<sup>18</sup> is therefore dispensable. When crossed with Df(3R)e-BS2 / TM3 males, the females produced eggs of *how*<sup>18</sup> / Df(3R)e-BS2 genotype; although deprived of the maternal contribution to the *how* expression, these embryos did not show any additional phenotype when compared to *how*<sup>18</sup> / Df(3R)e-BS2 embryos produced zygotically. Therefore, the maternal expression of *how* does not contribute to the *how* embryonic function. All the FRT *ovo*<sup>D1</sup> / FRT *how*<sup>18</sup> females had at least one apparently wild-type ovariole per ovary with an average of 6-7 developing ovarioles per ovary.

### (8) Observation of living embryos

Embryos were dechorionated manually, fixed with glue to a microscope slide and covered with a drop of mineral oil. Mutant embryos were defined as being not hatching embryos and not TM3 embryos (abnormal trachea). Heart rate was measured under a phase-contrast microscope by counting the contractions. In a typical experiment, 20 mutant embryos were observed. The heart rate, in mutant and wild-type embryos, can vary as much as twofold among embryos. The rate reaches a steady-state level in the larvae.

## RESULTS

### (1) Identification and cloning of the *held out wings* gene.

The pattern of expression of the  $\beta$ -galactosidase reporter gene for the *Drosophila* 1A122 enhancer trap line (Perrimon et al. 1991) is shown in Fig. 1. At the onset of gastrulation,  $\beta$ -galactosidase was expressed in the presumptive mesoderm territory (Fig. 1A) and during germ-band retraction into the mesoderm (Fig. 1B). Later, this expression became restricted exclusively to the myogenic cells whose determination into different lineages within the mesoderm was already accomplished (Fig. 1C). From that stage on,  $\beta$ -galactosidase was revealed in precursors of the somatic (Fig. 1C) and pharyngeal muscles, the cardiac cells (Fig. 1D) and the visceral muscles. It was never detected in cells that do not differentiate into muscular derivatives such as, for example, the fat body or the pericardial cells. At the onset of stage 14 and later, the muscle attachment sites, which are from ectodermal origin, became also strongly labelled (Fig. 1D).

By in situ hybridization on polytene chromosomes and genetic mapping with deficiencies (see Materials and methods), the 1A122 insertion has been precisely located on the right arm of chromosome III in 93F, in a position distal to S59 and E2F. Two other enhancer trap lines BI93F (Ruohola et al. 1991) and 24B GAL 4 (Brand and Perrimon, 1993) map in the same region and their reporter genes displayed the same embryonic pattern of expression as 1A122.

Genomic DNA flanking the P-element in the 1A122 line was isolated by using the plasmid rescue technique and, with the aid of a chromosome walk, a region spanning 50 kb was cloned (Fig. 2A). Two transcription units (*how* and *hel93F*) were identified in this region and a nearly full-length cDNA was isolated for each one of them. *hel93F*, which probably does not correspond to the resident gene revealed by the expression pattern of the 1A122 line, will be described elsewhere. The longest cDNA clone isolated for the *how* transcription unit (4.0 kb: pRX5) recognized two mRNAs of 4.4 kb and 3.6 kb, respectively (Fig. 2E). The transcription of the 3.6 kb species was strictly maternal whereas that of the 4.4 kb transcript was exclusively zygotic and especially important during the second half of embryogenesis (Fig. 2E). The pattern of expression of the 4.4 kb mRNA in embryos coincided with that of the  $\beta$ -galactosidase in the original enhancer trap line. A strong expression was also observed in early embryos persistent in all cells at gastrulation in good agreement with the presence of a maternal transcript (Fig. 1E). The use of different probes distributed along the total length of the cDNA has shown that the two transcripts differed in their 3'-UTR, the maternal transcript lacking around 800 bp in its 3'-end. Two polyadenylation signals at positions 3068 and 3152 in the pRX5 cDNA might be used as premature stops for the transcription of the maternal mRNA and explain its shortened size.

A partial genomic structure of the *how* gene is outlined in Fig. 2C and shows that the transcription unit is divided into at least 4 exons. Given the length of the zygotic mRNA in northern blot analyses and assuming about a hundred bp for the poly(A) tail, it is possible that the 5'-untranslated region of the gene extends farther upstream ( $\approx$ 200 bp). Locations of the three P-elements (1A122, BI93F and 24B) have respectively been ascribed by PCR and sequencing to a site within the first intron for the first one and to a few hundred bp upstream of the putative transcription start site for the second and the third ones.

### (2) *how* encodes a protein homologous to the mouse Quaking protein, which contains one KH-domain

The general organization of the *how* mRNA is schematized in Fig. 3A. It is composed of a 1.2 kb open reading frame that predicts a protein sequence comprising 404 amino acids, a short incomplete (see above) 5'-UTR (250 bp) and a very long 3'-UTR (2.5 kb) containing a polyadenylation site and a poly(A) tail.

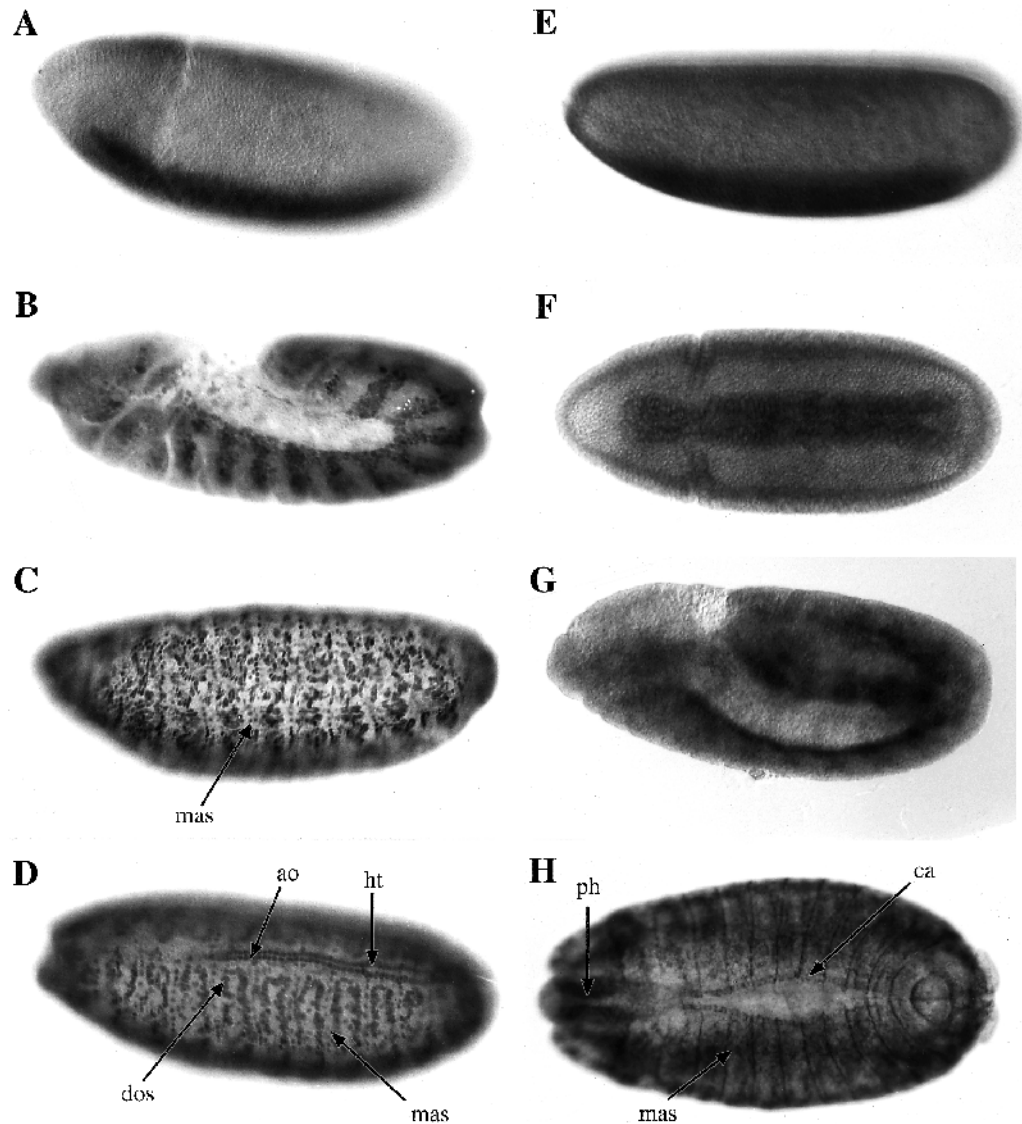
The conceptual protein sequence displayed a significant score of homology with the general family of hnRNP K-related proteins containing KH-domains. These proteins can bind RNA and have been implicated in RNA processing, transport or translation (Siomi et al., 1993; Gibson et al., 1993; Musco et al., 1996). The sequence of How showed the highest similarity to the sequence of the product of the mouse *quaking*

gene, which is involved in the myelination of brain nervous fibers and probably also in embryogenesis (Ebersole et al., 1996), suggesting that *how* might be the *Drosophila quaking* homolog. *quaking* is a member of a subfamily of KH-domain-containing proteins which appear to link a signal transduction pathway to RNA metabolism and that have been named Signal Transduction and Activation of RNA (STAR) (Ebersole et al., 1996). In this subfamily, the proteins contain only one KH-domain and it includes the Sam68 mouse protein and its human homolog p62, which play a role during mitosis (Lock et al., 1996), and *C.elegans* Gld-1, which behaves as a tumor suppressor gene in the germ line (Jones and Schedl, 1995) (Fig. 3B).

Based on recent three-dimensional structure observations, the classical KH-domain has been enlarged to a maxi KH-domain (Musco et al., 1996) and comparison of these domains among the STAR subfamily members revealed 76% identity between How and Gld-1 and 83% between How and Qk-1, values that can reach 93% by taking into account conservative changes. This similarity extends outside the KH-domain to a N-terminal region (QUAI domain (Ebersole et al., 1996) in Qk-1 and part of the GSG domain in Gld-1 (Jones and Schedl, 1995)) and also to a C-terminal domain (CGA or QUAI domains respectively in Gld-1 and Qk-1). The How C-terminal end is rich in proline residues and five tyrosine residues are invariant in How and Qk-1. This region could also contain, as in Sam68, SH2-binding sites (Taylor and Shalloway, 1994; Fumagalli et al., 1994). From these comparisons, it clearly appeared that How was more closely related to Qk-1 than to any other member of the family (See Fig. 3C). By contrast, a How N-terminal region rich in alanine and

glutamine residues had no counterpart in Qk-1. Such regions have previously been involved in the repression of transcription in *Drosophila* (see for review Hanna-Rose and Hansen, 1996).

Musco et al. (1996) have proposed that any KH-domain



**Fig. 1.** Expression of  $\beta$ -galactosidase reporter gene from the 1A122 line and of the *how* mRNA during embryogenesis. (A-D) Whole-mount 1A122 embryos stained for  $\beta$ -galactosidase. (A) The expression is first observed at early gastrulation in the invaginating mesodermal cells. (B) During germ-band retraction, the reporter gene is expressed in the developing mesoderm of a stage 12 embryo. (C) Lateral view of a stage 15 embryo showing that all the nuclei of the somatic muscles are labelled.  $\beta$ -galactosidase is also expressed in attachment sites of the muscles to the epidermis (mas). (D) Dorsal view of a stage 15 embryo showing the labelling of the cardiac cells in the aorta (ao) and in the heart (ht). dos, dorsal somatic muscles; mas, muscle attachment sites. (E-H) In situ hybridization of whole-mount embryos with an antisense *how* mRNA probe. (E) The probe reveals the maternal mRNA, which is ubiquitously expressed and which is not detected in the  $\beta$ -galactosidase expression pattern of the 1A122 line (compare E to A). Activation of the zygotic expression is apparent in the invaginating mesoderm. (F) The expression domain in the ventral region overlaps at gastrulation the domain of the cells of the ventral furrow that will give rise to the mesodermal cells. (G) A stage 11 embryo. The expression is concentrated in the developing mesoderm. A weak ubiquitous maternal expression is still visible. (H) Dorsal view of a stage 14 embryo. ca, cardiac cells; ph, pharyngeal muscles; mas, muscle attachment sites to the epidermis. In all the views, anterior is left and dorsal is up.

could adopt a three-dimensional structure organized as a succession of  $\beta$ -sheets and  $\alpha$ -helices in the order  $\beta\alpha\alpha\beta\beta\alpha$ . By using the SOPMA method for secondary structure prediction (Geourgon and Delaage, 1994), we have shown that the maxi KH-domain of How could also adopt such a configuration. Two loops (residues 145-153 and 185-202) linking  $\beta$ 1 to  $\alpha$ 1 and  $\beta$ 2 to  $\alpha$ 3 are longer within the STAR family members How, Qk-1, Gld-1 and Sam68 than within the general family and this larger length is conserved among the 4 proteins. These loops could in fact have modulated their dimension to accommodate interactions with ligands specific for each subfamily.

In terms of structure-function relationships, it is noteworthy that all the point mutations affecting Gld-1 or Qk-1 functions essentially concerned residues that are fully conserved in How (Fig. 3B).

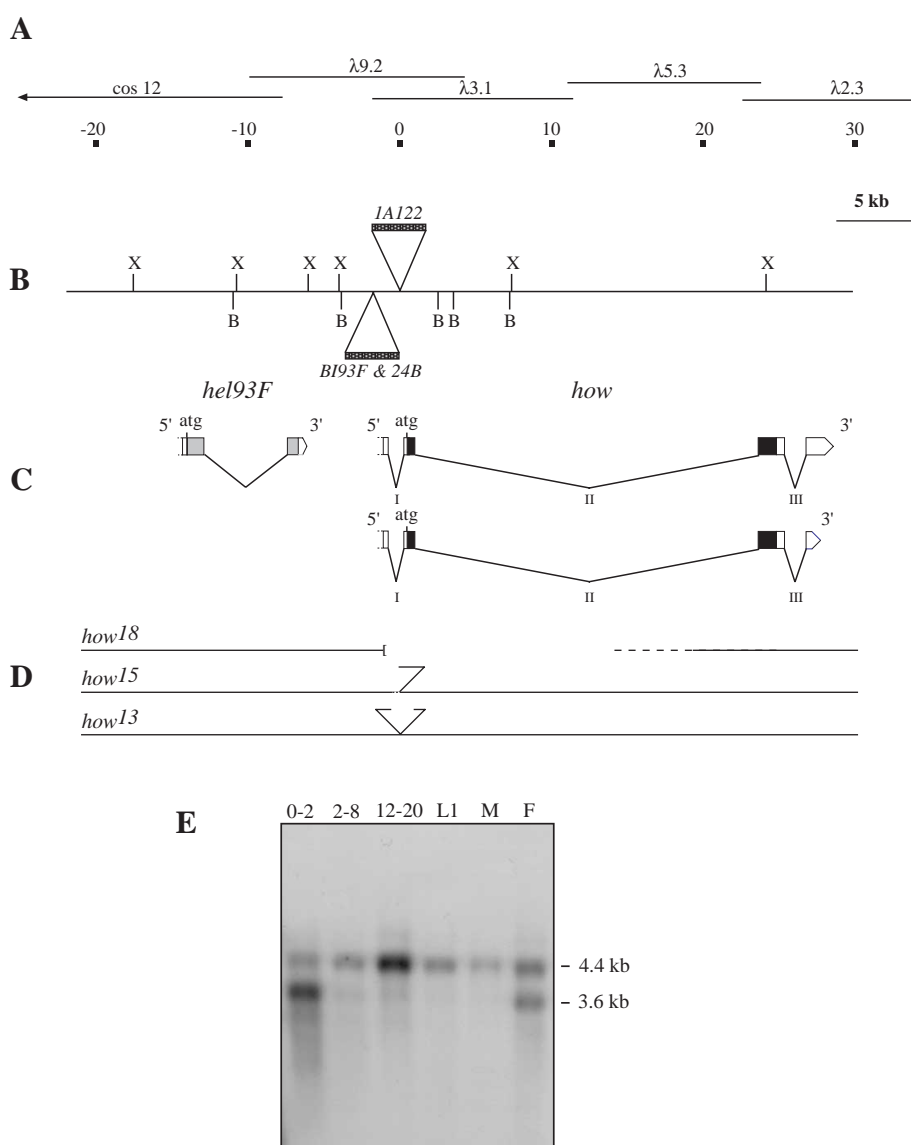
The *qk-1*, *gld-1* and *how* mRNAs all possess long 3'-UTR (2.5 kb) sequences that do not share any obvious similarities.

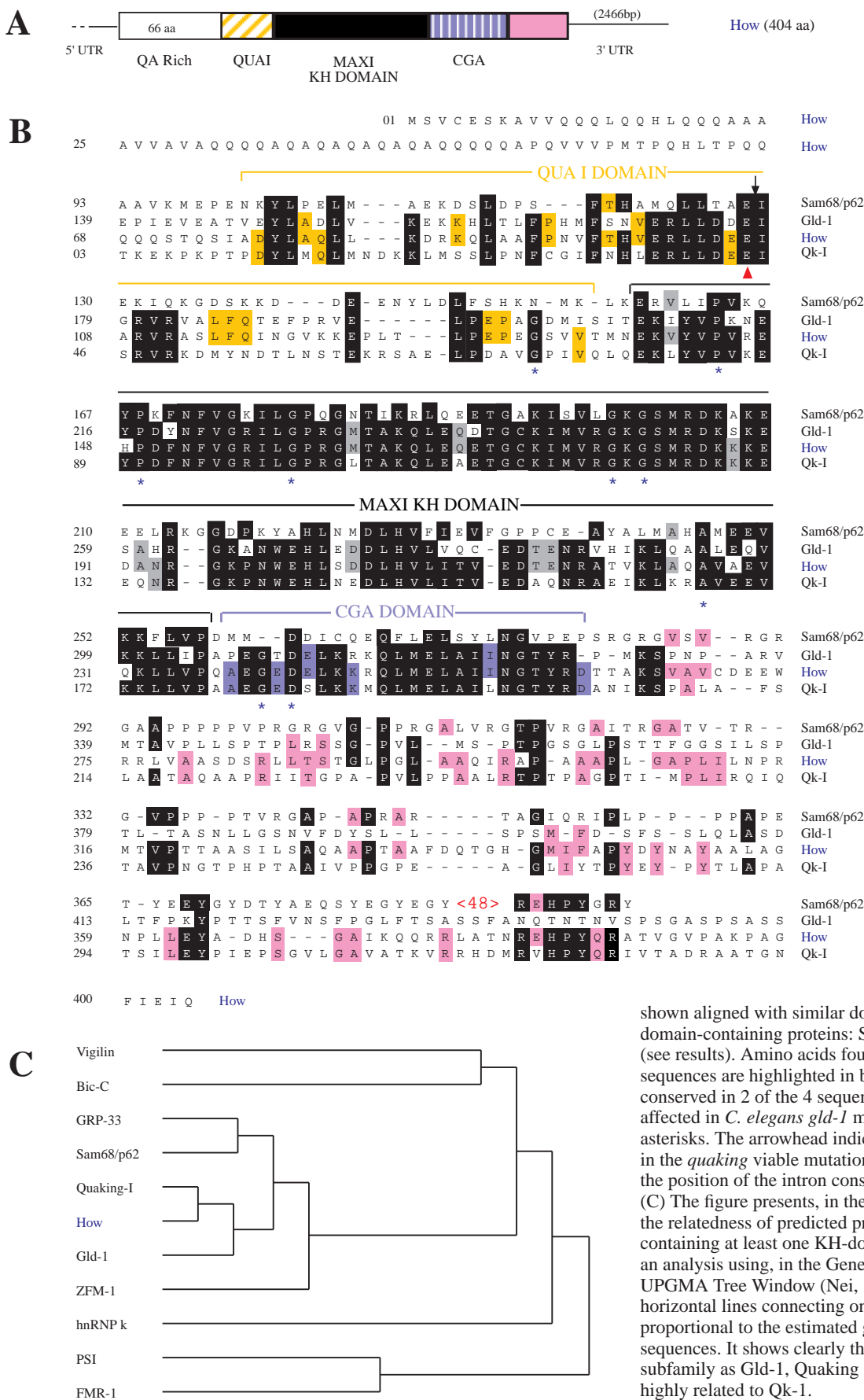
Finally, the genomic organization of the *how* gene has not yet been completely elucidated but the position of three introns has been established with precision. The first intron is located at the same position as in *gld-1* (Jones and Schedl, 1995) and in *qk-1* (Artzt, personal communication) interrupting a codon translated in a Glu residue (position 57 in How) in the three proteins (Fig. 3B).

### (3) *how* codes for a nuclear protein whose expression in the mesoderm depends on *twist* activity

Antibodies raised against the How protein recognized on western blots a single band of  $44 \times 10^3 M_r$  and on whole-mount

**Fig. 2.** The *how* locus. (A) Physical map showing the overlapping genomic  $\lambda$  phages that covered the 93F region. (B) The location of three enhancer trap P-elements 1A122 (Perrimon et al., 1991), BI93F (Ruohola et al., 1991) and 24B (Brand and Perrimon, 1993) is indicated by triangles and some restriction enzymes sites are shown. X, *Xho*I; B, *Bam*HI. This map has not been oriented with respect to the centromere. (C) Structure of two cDNAs in the genomic region. The left transcription unit corresponds to *hel93F* whereas the right transcription unit corresponds to *how*. The boxes represent the cDNA sequences with open boxes indicating untranslated sequences and filled boxes indicating coding sequences. The intron-exon structure of *how* is not yet completely elucidated but the three introns schematized on this figure have been precisely located. The 1A122 transposon is inserted inside the first intron while BI93F and 24B are located upstream of the 5'-end of the cDNA. Around 200 bp may be missing upstream of that 5'-end. (D) Deletions in three lethal mutations generated by imprecise excision of the homozygous lethal 1A122 insertion. In *how*<sup>18</sup>, the first two exons have been deleted. In *how*<sup>13</sup> and *how*<sup>15</sup>, part of the transposon is still present. In *how*<sup>15</sup>, a few nucleotides have been removed from intron I. By contrast, a deletion of genomic DNA in *how*<sup>13</sup> is unlikely. Only a central part of the transposon seems to be deleted. In *how*<sup>18</sup>, the breakpoint has not accurately been determined in the second intron but it is situated upstream of the 3rd exon. In the 5' side, the breakpoint is situated inside the first exon. (E) Developmental northern blot probed with the *how* cDNA. 5  $\mu$ g of poly(A)-rich mRNA extracted from 0-2 hours (lane 0-2), 2-8 hours (lane 2-8), 12-20 hours (lane 12-20) embryos, first instar larvae (lane L1), males (lane M) and females (lane F) were loaded on the gel. The blot was probed with the full-length pRX5 cDNA and also with Rf49 mRNA to assess the amount of RNA in each lane (not shown). The maternal transcript visible in 0-2 hours old embryos and in adult females rapidly disappears during embryogenesis. The zygotic mRNA is especially abundant during mid-embryogenesis. A low level of expression of this latter transcript in 0-2 hours embryos is likely due to a contamination by older embryos. 24 hours exposure under magnifying screen.





**Fig. 3.** Sequence of the How protein and its alignment to three other proteins. (A) The *how* cDNA contains an ORF of 404 amino acids. The putative protein is composed of 5 domains: a 66 amino acid QA-rich domain with no homology with the other proteins of the subfamily; a central region with a high degree of conservation composed of a QUA1 domain (Ebersole et al., 1996) and of the maxi KH-domain (Musco et al., 1996); a part of the CGA domain (Jones and Schedl, 1995) and a C-terminal region with weak homologies with the other three proteins of the subfamily. (B) The central domain of the How protein is

shown aligned with similar domains in a subfamily of KH-domain-containing proteins: Sam 68/p62, Gld-1 and Qk-1 (see results). Amino acids found in at least 3 of the 4 sequences are highlighted in black squares. Amino acids conserved in 2 of the 4 sequences are shaded. Amino acids affected in *C. elegans gld-1* mutants are marked with asterisks. The arrowhead indicates the amino acid modified in the *quaking* viable mutation. The vertical arrow shows the position of the intron conserved in *gld-1*, *qk-1* and *how*. (C) The figure presents, in the form of an evolutionary tree, the relatedness of predicted proteins encoded by genes containing at least one KH-domain. This result is based on an analysis using, in the Gene Works program, the UPGMA Tree Window (Nei, 1987). The length of the horizontal lines connecting one sequence to another is proportional to the estimated genetic distance between the sequences. It shows clearly that How belongs to the same subfamily as Gld-1, Quaking and Sam 68 and is most highly related to Qk-1.

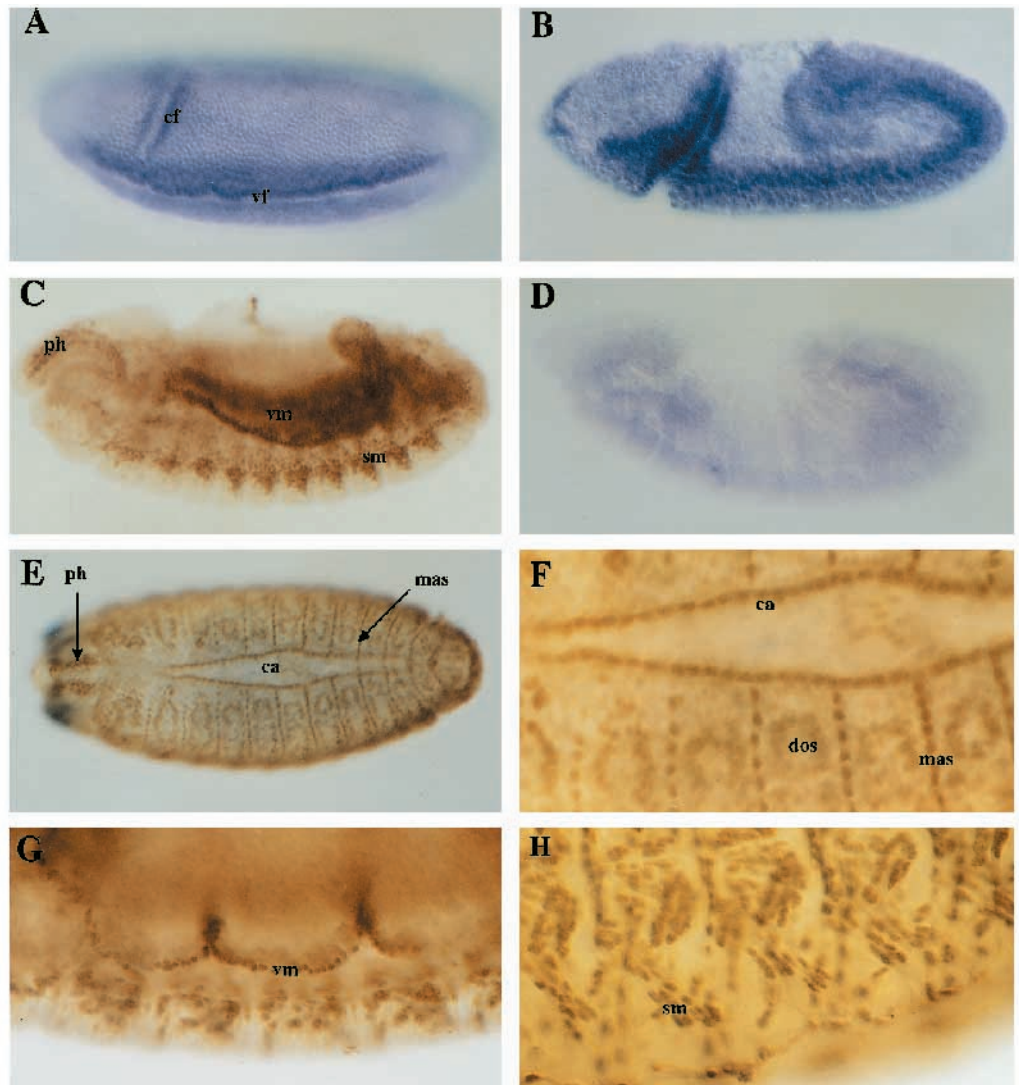
embryos displayed a pattern of staining indistinguishable from that of  $\beta$ -galactosidase in the 1A122 line (Fig. 4A-C). No such staining could be detected in an embryo bearing a *Df(3R)e-BS2* deficiency which uncovers the *how* locus supporting the specificity of the antibody (Fig. 4D). In addition, the labelling by the antibody was clearly concentrated in the nucleus at all developmental stages (Fig. 4E,H,M), suggesting that How exerts its function in that sub-cellular fraction as do most of the KH-domain-containing proteins (Dreyfuss et al., 1993). In larvae, the protein was detected in all the nuclei of the muscle fibers and in those of the *twist*-positive cells associated with the motoneurons (Bate et al., 1991) (Fig. 5). In imaginal discs, ad epithelial cells, which are the precursor cells of some of the adult muscles, were also labelled (not shown) as already described in the case of the 24B *GAL4* line (Roote and Zusman, 1996). The expression of *how* was maintained in the adult with the same specificity as in the embryo or in the larvae. Fig. 5B and C shows the staining of the nuclei of the tendon cells that attach the flight muscle fibers to the dorsal cuticle of the thorax. Finally, during oogenesis, the protein was revealed in the follicular stalk cells that join the different egg chambers in the ovaries (not shown).

In spite of the strong maternal expression of the 3.6 kb transcript, no protein was detected before the onset of gastrulation suggesting that the maternally supplied mRNA was either not translated or that the translated protein was highly unstable.

The expression of *how* was not affected in a *D-mef2* mutant in which late differentiation stages of muscular cells was altered. Likewise, the early expression of *how* in presumptive mesoderm territory was not modified either in *snail* mutants or in *tinman* mutants. The *tinman* gene is expressed primarily in mesoderm but, later in embryogenesis, its mRNA is

present only in structures derived from the dorsal mesoderm (Azpiazu and Frasch, 1993; Bodmer, 1993). Since, in *tinman* mutants, these tissues do not form, it could not be ascertained whether *tinman* is necessary for the expression of *how* in cardiac, visceral and dorsal muscles.

In contrast, the early expression of *how* in the presumptive mesodermal cells was completely abolished in homozygous



**Fig. 4.** *How* protein expression. Whole-mount embryos were treated with anti-How antibody and stained with secondary antibodies coupled either to alkaline phosphatase (A, B, D) or to peroxidase (C, E, F, G, H). (A) The first detectable expression occurs in the cells of the ventral furrow (vf) and, as in the case of the 1A122 line, no maternal expression is observed (compare to Fig. 1A). A significant level of expression is visible on each side of the cephalic furrow (arrowhead), in contrast to the pattern obtained by labelling either with antisense mRNA or with anti- $\beta$ -galactosidase in 1A122. (B) In the early stages of gastrulation, the staining is mainly restricted to nuclei from ventral mesoderm but some expression around the cephalic furrow (arrowhead) as well as in the anterior region of the head mesoderm can be detected (C) Lateral view of a stage 14 embryo. sm, somatic mesoderm; ph, pharyngeal mesoderm; vm, visceral mesoderm; hg, hindgut mesoderm. (D) A homozygous *Df(3R)e-BS2* embryo slightly younger than the embryo in C is not stained at all by the anti-How antibody. (E) Dorsal view of a late stage 14 embryo. The embryo is double-stained to reveal in the anterior region of the head the Deformed *lacZ* expression associated to the TM3 balancer to identify mutant embryos: ca, cardioblasts; mas, muscle attachment sites. (F) Enlargement of the embryo in E to show the nuclear staining in cardioblasts (ca), in the muscle attachment sites (mas) and in dorsal muscles (dos). (G) Staining of the nuclei in the visceral mesoderm (vm) in a late stage 15 embryo and (H) in all the nuclei of the somatic muscles (sm).

*twist* mutants (Fig. 6), while its late expression in the attachment sites was maintained. A direct role for *twist* in the mesodermal expression of *how* was further suggested by the existence of four putative Twist binding-sites (E-box, CANNTG, Ip et al., 1992) in a 80 bp genomic fragment located near the insertion sites of BI 93F and 24B GAL4 (not shown). Also consistent with this hypothesis is the fact that How was expressed in the precursor cells of adult muscles that re-express *twist*. (Fig. 5A).

#### (4) Characterization of mutations generated in the *how* gene

Mutations in the *how* gene were generated by P-element excision from the 1A122 original enhancer trap line and selection for lethality. Precise excisions were able to revert the lethal phenotype prevailing in the 1A122 line confirming that the mutation was due to the insertion. Three lethal excisions, *how*<sup>13</sup>, *how*<sup>15</sup> and *how*<sup>18</sup> failed to complement the deficiency Df(3R)e-BS2. The initial 1A122 insertion as well as *how*<sup>18</sup> were homozygous embryonic lethal.

In *how*<sup>18</sup>, exon I, intron I and exon II were deleted and the distal breakpoint of the deficiency fell somewhere within the large intron II (Fig. 2D). The proximal breakpoint was located 150 bp upstream of the first nucleotide of the cDNA pRX5. In situ hybridization analyses revealed the presence in this mutant of both the maternal and zygotic mRNA. Their respective size as probed by northern blot, however, was shortened by 700 bp, which accounted for the lengths of exon I and exon II. These observations suggest that the transcription start site, as well as the tissue-specific enhancer elements, have not been affected in the *how*<sup>18</sup> mutation. The ATG was deleted in *how*<sup>18</sup> and, indeed, no protein was synthesized as demonstrated by western blots and immunohistochemistry experiments. The original insertion 1A122 is homozygous embryonic lethal and does not produce any detectable How protein.

In both *how*<sup>13</sup> and *how*<sup>15</sup>, at least part of the initial transposon was still present (Fig. 2D). Intron I, in which is located the 1A122 insertion, was full length in *how*<sup>13</sup> and was deleted from only a small region in *how*<sup>15</sup>. The transcribed genomic region was not affected in either mutant. It was assumed that the level of expression of *how* was probably affected in these different alleles, but no attempts have been made to quantify the effects.

*how*<sup>13</sup> and *how*<sup>15</sup> in *trans* of 1A122 were viable and produced fertile healthy adults that were, however, unable to fly and held their wings horizontal at a 90° angle from the body axis. Although some of the homozygous animals died as pupae, all the adults that hatched were characterized by this typical phenotype indicating a full penetrance.

No gross abnormalities could be recognized by staining homozygous *how*<sup>18</sup> embryos with a collection of antibodies or by observation of the muscles under polarized light. The number and morphology of muscles were identical to those of wild-type embryos, and the gut, the heart and the pharynx were formed normally. These embryos expressed *D-mef2* required for the synthesis of muscle-specific proteins (Bour et al., 1995; Lilly et al., 1995), myosin and also *twist* in late stages of embryogenesis when it is present in adult muscles precursors (Bate et al., 1991). Muscles were innervated by motoneurons in appropriate locations as assessed by a staining of Fas II, which was wild type in its pattern (Grenningloh et al., 1991), and the attachment sites were properly differentiated as judged

from their reaction with an antibody directed against Groovin (Volk and Vijayraghavan, 1994). Hence the developmental process of making muscle, that is setting cells aside to become a particular derivative, organizing these cells and, in the case of the somatic muscles, selecting founder cells, fusing and making myosin is normal in *how* mutants. As discussed below, it is the actual functioning of these tissues that is disturbed.

A more precise study of the events leading to lethality in *how*<sup>18</sup> showed that the development of mutant embryos progressed without difficulties until late stages but hatching failed to occur. In spite of an apparent normal morphology, the embryos did not behave with the characteristic coordinated muscular movements associated with the latest stages of embryogenesis (Broadie and Bate, 1993). In particular, the head was immobile in mutant embryos ready to hatch. Nevertheless, late embryos and non-hatched larvae were still animated by light contractions more than 48 hours after egg-laying.

We have centered our observations on the heart. Even though the development of this organ appeared normal, the heart rate was considerably slower than that of a wild-type animal. Heart pulsations in *Drosophila* start 16 hours after fertilization and their number increase to a value of 60 per minute at the end of embryogenesis to level off at 150 per minute in 3rd instar larvae (Miller, 1974; Gu and Singh, 1995). In mutant *how*<sup>18</sup> embryos, the heart contractions were of very small amplitude, appearing as small palpitations rather than as the constriction movements that occur in the wild-type cardiac myoendothelium. The heart of mutant embryos began to contract according to a proper schedule and its rate progressively reached 20-30 pulsations per minute. However, it never exceeded this value and decreased later (after around 30 hours of development at 25°C). Interestingly, in the heart region of the dorsal vessel, the lumen was very narrow indicating either that it was blocked in a contracted state or that the complete differentiation of the heart was not effective in *how*<sup>18</sup> mutants.

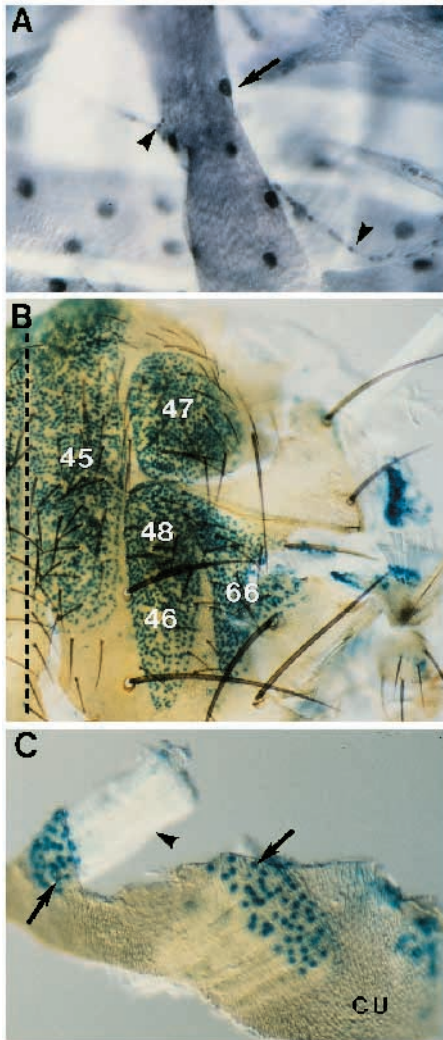
## DISCUSSION

### Expression of *how* in the mesodermal and myogenic lineages

An important aspect that emerges from the study of the expression of the *how* gene is its early expression in the presumptive mesoderm under the control of *twist* while its function is needed later in embryogenesis.

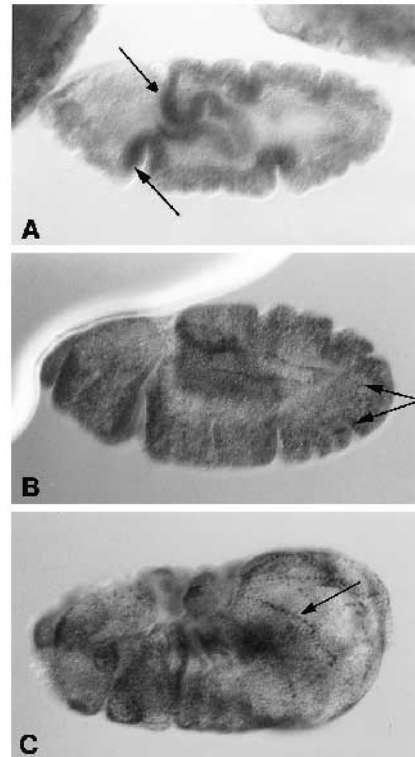
In general, one assumes a strict correlation between the period of expression of a gene and the period during which its activity is necessary. This is, for example, the case for genes, such as *twist* or *tinman*, which are expressed very early in the mesoderm and are required for its formation or for the determination of a subset of mesodermal derivatives (Thisse et al., 1988; Azpiazu and Frasch, 1993; Bodmer, 1993). Conversely, genes expressed later, at the onset of organogenesis, direct the synthesis of components specific for differentiated muscle cells (muscle fiber proteins, neuromuscular junction proteins, ion channels etc...).

At least two genes *D-mef2* (Lilly et al., 1994; N'guyen et al., 1994; Taylor et al., 1995) and *how* (this work) appear to contradict this scenario. In *D-mef2* mutants, the muscle cells have a normal development but a number of muscle structural



**Fig. 5.** Expression of How in larvae and adults. (A) How antibodies labelled the large polyploid nuclei of the muscle fibers (arrow) in a third instar larva. Small nuclei, associated with nerves, were also stained (arrowheads) and are likely to correspond to the nuclei of *twist*-expressing cells (Bate et al., 1991). Flat preparation observed under Nomarski optics and showing muscle 8 in abdominal segment A4. Anterior to the right, dorsal up. (B) Dorsal view of a thorax from a 1A122 adult stained for  $\beta$ -galactosidase activity. The nuclei of the muscle attachment cells on the epidermis, stained in blue, delineate the muscle fibers of the different flight muscles which are inserted behind the dorsal cuticle. Right half of the thorax is shown: indirect muscles (dorsolateral muscle 45, dorsoventral muscles 46, 47, 48) and mesothoracic tergal depressor of trochanter, 66 (Bate, 1993). Anterior is up. The median axis is materialized by the dotted line. (C) Dissected indirect muscles (arrowhead) from a 1A122 fly showing the nuclei of the cells associated with the extremities of the muscle fibers and which are also attached to the epidermis (arrows). (cu), cuticle.

proteins is not synthesized (Lilly et al., 1995; Bour et al., 1995). *D-mef2* is expressed very early in the invaginating mesoderm under direct activation by *twist*, but no obvious function is associated with this early expression. The same situation prevails in the case of *how* whose function is probably required even later since normal and partially functional myofibers are present in null mutants. Two arguments, the lack



**Fig. 6.** How expression in *twist* and *snail* mutants. (A) In a *twist* mutant embryo, no How protein is detected, except around the folds of the cephalic furrow (arrows). This expression, also observed in the wild type (Fig. 3B), is not under the control of *twist*. (B) In a *snail* mutant embryo, a faint labelling is maintained at stage 10 in the nuclei of the primordia of the mesoderm that have not invaginated (arrows). These cells stand as ectodermal cells. (C) In a late *snail* embryo, the ectodermal cells responsible for the attachment of the muscles are labelled (arrows). These cells are also labelled in a *twist* mutant (not shown). This result confirms that the development of muscle attachment cells does not rely on a normal development of the muscles. In all panels, anti-How antibody has been revealed with a horseradish peroxidase-coupled secondary antibody.

of expression of *how* in *twist* mutants and the existence of a cluster of four E-boxes in the 5'-region of the *how* gene, favour a direct control by *twist* of the early expression of *how*. Further experiments are in progress to assess this point. An early requirement for the function of these two genes may have escaped the scrutiny of phenotypic analyses and experiments are actually carried out in that direction in the case of *how*. Alternatively, such a strategy may ensure the specificity of mesodermal expression of these genes through a positive control by *twist* and their presence prior to the segregation between the different lineages might be a prerequisite for their correct expression later in development.

Even if the early expression of *how* (or *D-mef2*) is mainly due to a positive control by *twist*, as already suggested for *D-mef2*, other transcription factors are probably needed for the maintenance of their expression, (Taylor et al., 1995; Taylor, 1995). *twist* expression is switched off rapidly during embryogenesis to become hardly detectable when organogenesis begins (Bate et al., 1991; Baylies and Bate, 1996). In addition, high concentrations of *twist* have deleterious effects on heart and visceral muscles differentiation (Baylies and Bate, 1996).

The nature of these putative factors is totally unknown at the moment. Likewise, it is necessary to speculate on the existence of other factors which, at least in the case of *D-mef2* (no targets of *how* have yet been characterized), would silence the transcriptional activation of genes specific for differentiated muscles from gastrulation stage to the onset of organogenesis when the expression of muscular proteins becomes apparent.

Another interesting aspect of *how* expression concerns its restriction to the myogenic lineage leading to an ubiquitous expression in the whole mesoderm. This property is shared by other genes, such as *D-mef2*,  $\beta$ -*tubulin* (Leiss et al., 1988) and *DFR1* (Shishido et al., 1993), and, probably, does not originate from a specific degradation of the mRNA in the non-myogenic cells and a constant transcription in the myogenic cells. This kind of mechanism would rely upon special features of mRNA structure. No evidence, however, supports this hypothesis since the mRNA for  $\beta$ -galactosidase in the *how* enhancer trap line and the *how* mRNA seem to behave in the same way. In contrast, a dynamic expression of *how* is observed during embryogenesis (Fig. 1): at gastrulation, *how* expression is activated in apparently all the mesodermal precursor cells. Then, it slowly declines along with successive cell divisions and mesoderm spreading on the lateral ectoderm. At the end of germ-band retraction, an intense re-expression is observed only in the future muscle cells. The final pattern of *how* expression in late embryogenesis is probably due to such a specific activation in myogenic cells.

How does segregation between the myogenic and non-myogenic lineages occur during the first half of embryogenesis? Asymmetric cleavages during mesodermal cell divisions could be invoked to explain an asymmetric location of mRNAs in the progenitor cells. Alternatively, a progressive restriction of competence of mesodermal cells could arise from lateral inhibition in a manner similar to that occurring in the early stages of neurogenesis. Genes involved in these processes in neurogenesis seem to play a similar role in the mesodermal cells in *Drosophila* (Corbin et al., 1991; Bate et al., 1993; Carmena et al., 1995) or in vertebrates (Kopan et al., 1994). In the same line, the choice among heart precursor cells between cardiac and pericardial cells appears to be controlled by *Notch* and other neurogenic genes (Hartenstein et al., 1992; Zaffran et al., 1995). For example, in temperature-sensitive *Notch* mutants more cells express *how* than in the wild type and consequently fewer pericardial cells seem to be formed.

### Homology of *how* with the mouse *quaking* gene suggests a role in RNA metabolism

KH-domain-containing proteins exert their function in close association to RNA (see Musco et al., 1996 and ref. therein). In prokaryotes, direct binding to RNA has been demonstrated for the polynucleotide phosphorylase (PNP) from *E. coli*, the ribosomal protein S3 and NusA, a transcription elongation factor. In eukaryotes, Mer1 in yeast and Psi in *Drosophila* have been directly involved in RNA splicing and  $\alpha$ -CP1 and  $\alpha$ -CP2 have been recognized as constituents of the  $\alpha$ -globin messenger RNP stability complex. As a component of hnRNP, hnRNP K may participate in the processing and transport of pre-mRNA and the function of FMR1 is linked to the fragile X syndrome.

How belongs to the STAR subfamily of KH-proteins whose role is probably to couple a signal transduction pathway to

some aspect of RNA metabolism. The RNA targets as well as the pathway have not yet been deciphered. Two arguments suggest that How is the ortholog of Qk-1: a strong homology in their sequences, within and outside the maxi KH-domain, and cross-reactivity of an antiserum directed against a mouse Quaking decapeptide issued from a region of the C-terminal domain that is conserved in How. This antiserum recognizes in *Drosophila* embryos only one antigen on western blots and labels whole-mount embryos with a pattern of expression superimposable to that observed with an antiserum directed against How (not shown). Particularly, no staining is detected in cells unlabelled with the specific anti-How antibody. This result suggests that only one protein belonging to the Quaking family is present in *Drosophila* embryos. Work is in progress to rescue *how* mutants with the *quaking* gene. A viable mutant for *quaking* presents defects in splicing of the mRNA for myelin that relate the function of this gene to processing of mRNA (Fujita et al., 1988). If How is the ortholog of Qk-1, it is tempting to postulate for the fly protein a function in RNA processing. A genetic approach will be used in *Drosophila* to identify targets for the *how* function and eventually effectors regulating the *how* activity, for example, in relation to the STAR transduction pathway.

Phenotypes resulting from mutations in the two genes are, however, not comparable, which is in conflict with the high level of structure conservation. *how* is never expressed in the nervous system and nervous fibers are not myelinated in insects. Embryonic lethal mutations have been obtained in the *quaking* gene (Shedlovsky et al., 1988) and, even though the associated phenotypes have not yet been analyzed in detail, gross abnormalities have been reported at the 15–26 somites stage, long before the onset of myelination. On the contrary, *qk-1* is transiently expressed in the heart during embryogenesis, thus raising the possibility that *qk-1* and *how* exert their function, at least partially, in common territories.

### The function of *how*

Two classes of mutations in the *how* gene have been analyzed in this work: two hypomorphic alleles *how*<sup>13</sup> and *how*<sup>15</sup> and one amorphic allele *how*<sup>18</sup>. A complete lack of function of *how* can be invoked to account for the phenotype of the *how*<sup>18</sup> mutation. At least within the limits of the techniques used, no protein could be detected in homozygous *how*<sup>18</sup> embryos. Also, since the maternal mRNA is not translated during embryogenesis, it does not contribute to the overall expression of *how*. Furthermore, homozygous *how*<sup>18</sup> germ-line clones give rise to embryos that do not display any additional phenotypes (see Materials and methods).

The study presented in this article has been particularly focused on the mutant phenotype related to the development and the acquisition of embryonic cardiac activity. Even though heart formation is normal, heart rate is dramatically slowed down in the so-called heart region as compared to a wild-type animal, suggesting that *how* is involved in the steps that lead to the acquisition of the heart larval function.

In *how* mutants, reduced muscular activity also prevails in the body wall muscles of embryos and in adult flight muscles and such similar phenotypes favour the hypothesis of a common function for *how* in these different tissues. The How protein cannot be a structural or even a functional component per se in the muscle cell. Its nuclear localization and its

potential affinity for the RNA rather provide arguments for considering *how* as a likely candidate to control the expression of the other components. In the same line, the sequence data support the hypothesis that the *how* activity is itself regulated in response to a signal transduction cascade. The signal might not be of developmental importance but may rather be related to the functioning of mature muscles.

The time-specificity of *how* expression could allow the tissular expression of specific muscular protein isoforms. For example, in *Drosophila*, at least 13 different myosin heavy-chain isoforms can be generated from a single gene and it has been recently reported that a specific embryonic isoform was not functionally equivalent to the isoform normally produced in the indirect flight adult muscle (Wells et al., 1996). Likewise, the electrical properties of a *Drosophila* tissue result from a combination of different isoforms of ionic channels that are synthesized with a specific time table and unique electrophysiological properties that could be controlled by the activity of *how* (Iverson and Rudy, 1990; Lagrutta et al., 1994; Becker et al., 1995). Additional experiments including the characterization of potential *how* targets are needed to assess its participation in such processes as well as physiological more detailed informations.

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