The EMBO Journal vol.14 no.10 pp.2307–2316, 1995

Embryonic tissue differentiation in *Caenorhabditis elegans* requires *dif-1*, a gene homologous to mitochondrial solute carriers

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Communicated by J.Hodgkin

The *dif-1* gene was identified in a general screen for maternal-effect embryonic lethal (Mel) mutants. *dif-1* mutant embryos complete gastrulation and embryonic cell division normally, but then arrest development with only a small amount of tissue differentiation. Either maternal or zygotic *dif-1* activity is sufficient for wild-type development. The temperature-sensitive period of a cold-sensitive *dif-1* mutant shows that *dif-1* activity is essential only for 3 h, corresponding to the major period of embryonic tissue differentiation, and is not required post-embryonically. The results point to a role for *dif-1* in the maintenance of tissue differentiation in the developing embryo, but not for its initiation. Cloning and sequencing of the *dif-1* gene revealed that its product is homologous to proteins in the mitochondrial carrier family. Although *dif-1* activity is required only during embryogenesis, *dif-1* RNA is expressed at all stages of development. *In situ* hybridization to embryos showed that *dif-1* RNA is initially present in all cells of the embryo; this most likely corresponds to maternal *dif-1* RNA. Later, the presumable zygotic *dif-1* RNA is found only in the gut and hypodermis of the embryo. This tissue-specific expression raises the possibility that the *dif-1* protein acts non-cell autonomously and that some communication or molecular transport dependent on DIF-1 takes place during embryonic tissue differentiation. *dif-1* is the first mitochondrial carrier homologue known to be needed specifically for a developmental process.

Key words: *Caenorhabditis elegans*/cold sensitivity/*dif-1*/differentiation/embryonic morphogenesis mitochondrial transporter

Introduction

During embryonic development many specialized processes occur. The fertilized egg undergoes rapid cell divisions, cells become committed to express particular fates, cell rearrangements occur and, finally, tissues and organs are made. One way to find genes that act specifically during embryogenesis is to isolate maternal-effect embryonic lethal (Mel) mutants. This type of mutant can identify genes with maternally contributed protein or RNA products that are required during embryogenesis. In *Caenorhabditis elegans*, maternal-effect genes have been shown to be required for meiosis, early cytoplasmic organization, blastomere identity and the establishment of axes (reviewed in Strome and Wood, 1989; Mains, 1992; Priess, 1994; Wood and Edgar, 1994). All these genes act during the early part of embryogenesis. To try to identify the type and range of maternal gene activities needed for embryogenesis and to ask whether any maternal products are required later in embryogenesis, a collection of Mel mutants was isolated and analysed. Among 50 mutants isolated, two allelic mutations were identified that resulted in normal development through the end of gastrulation, but then arrest with little tissue differentiation. A phenotypic analysis of mutants of this gene, *dif-1*, and its molecular characterization are presented. The *dif-1* gene encodes a putative mitochondrial solute carrier that is required only during the major period of embryonic tissue differentiation.

Results

Isolation of dif-1 mutants

In a general screen for Mel mutants, two allelic mutations (*e2562* and *e2577*) were identified that resulted in developmental arrest with a severe reduction in the differentiation of all tissues, after apparently complete cell division and gastrulation. This gene was named *dif-1* (for differentiation defective); it maps at position +3.4 on chromosome IV. In this paper, homozygous *dif-1* embryos that are laid by homozygous mutant mothers will be called *dif-1* mutant embryos. Either maternal or zygotic *dif-1* product is sufficient for wild-type development: *dif-1* homozygous embryos laid by *dif-1*/+ mothers are completely wild-type, and mating *dif-1* homozygous mutant mothers with *dif-1*/+ males only rescues the embryonic lethality of *dif-1*/+ embryos. This suggests that *dif-1* activity can be provided as either protein or RNA by the mother, or by transcription in the embryo.

The alleles *e2562* and *e2577* appear to be null mutations. The phenotypes of homozygotes are identical to each other and to those of animals carrying either allele over a deficiency (see Materials and methods). In addition, the molecular lesion caused by *e2562* confirms that this mutation should abolish all *dif-1* activity (see below). Unless otherwise stated, the phenotypic analysis describes that of the *dif-1* mutants *e2562* or *e2577*.

dif-1 phenotype

Wild-type embryogenesis can conveniently be divided into three phases. In the first (from 1 to 28 cells), the axes of the embryo are specified and the fates of blastomeres determined (reviewed in Priess, 1994; Wood and Edgar, 1994). From 28 to 550 cells, a period of cell proliferation and gastrulation occurs, producing an organized 'ball', with cells lying in positions where they will form tissues (Sulston *et al.*, 1983); embryonic cell division is nearly complete. At this point most tissues begin to differentiate

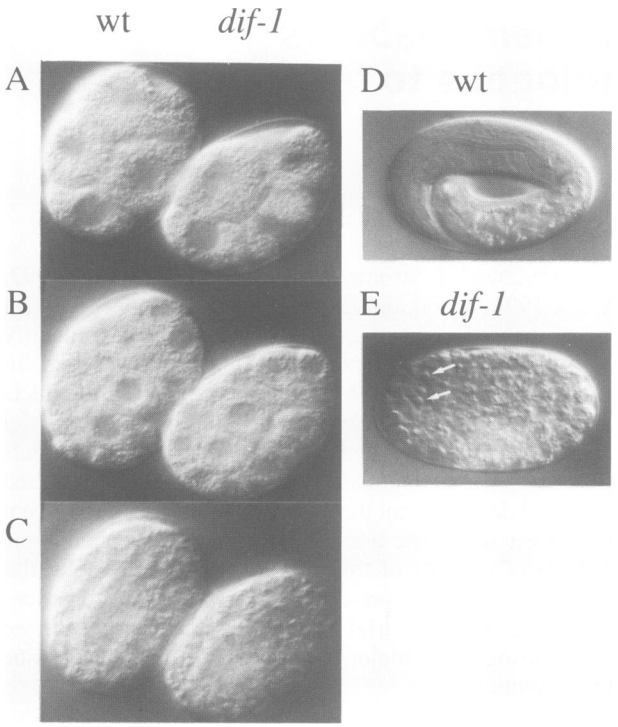

Fig. 1. *dif-1* mutant phenotype. (A−C) Simultaneous development of wild-type (left) and *dif-1* mutant (right) embryos, followed using a time lapse video recorder (Hird and White, 1993). (**A**) Six-cell embryos. (**B**) Twenty-eight-cell embryos, beginning of gastrulation. (**C**) Approximately 550 cells, end of gastrulation and cell proliferation; the *dif-1* mutant embryo is indistinguishable from wild-type. (**D**) Wild-type embryo just prior to hatching; tissues are well developed. (**E**) *dif-1* mutant embryo, terminal phenotype (8 h at 25°C); most cells look sick and numerous unengulfed cell deaths are visible (arrows).

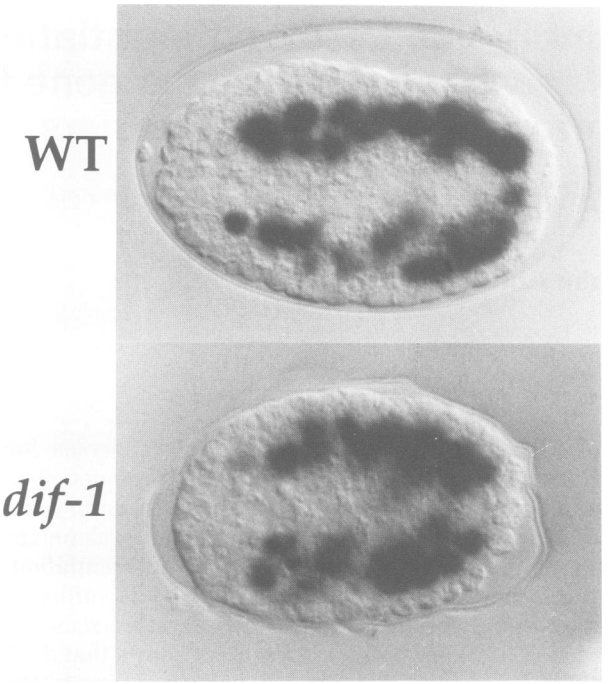

Fig. 2. *hlh-1* is expressed correctly in *dif-1* mutant embryos. An *hlh-1::lacZ* reporter gene, 221F, was used to assay the muscle cell fate [*hlh-1* is the *C.elegans* myoD homologue (Krause *et al.*, 1990)]. Embryos shown are 5 h old (at 25°C) and have completed gastrulation. Wild-type (top) and *dif-1* mutant (bottom) embryos are homozygous for 221F. Embryos were fixed and then β-galactosidase activity was detected using the substrate X-Gal.

and the ball of cells is transformed into a worm. This is a very dynamic period and obvious function can be assayed visually in many tissues. For example, the hypodermis (or epidermis) migrates over the surface to enclose the embryo and squeezes the ball of cells into a tube, the gut produces autofluorescent 'gut granules' and muscles begin to twitch (Wood, 1988). Many differentiated products can also be assayed by antibody detection. This period of embryonic tissue differentiation will be referred to as embryonic morphogenesis.

The cell positions and timing of divisions in *dif-1* mutant embryos were carefully followed to the 50-cell stage and found to be identical to those of wild-type embryos. Further cell division and gastrulation, monitored by overall organization and cell number, are also indistinguishable from those of wild-type up to the final round of cell division (Figure 1A−C); *dif-1* mutants are slightly slower to complete the final round of general cell division (not shown). Whereas wild-type embryos undergo embryonic morphogenesis at this point, *dif-1* mutant embryos soon arrest development with little tissue differentiation (Figure 1D and E). Cell positions become slightly abnormal as *dif-1* mutant embryos appear to begin embryonic morphogenesis before arresting development completely. Within a few hours of arrest, the cells of *dif-1* mutant embryos begin to degenerate and look necrotic (Figure 1E). This is unusual as the cells of most embryonic lethal mutants appear healthy looking for at least the length of normal embryogenesis (unpublished observation). To investigate whether inhibition of programmed cell death would suppress the lethality of *dif-1* mutants, double mutants were constructed between *dif-1* and *ced-4*; mutations in *ced-4* prevent all programmed cell deaths (Ellis and Horvitz, 1986). *dif-1; ced-4* double mutants are still lethal (not shown; see Materials and methods), suggesting that the lethality of *dif-1* mutants is not due to activation of the programmed cell death pathway.

To ask whether cell fates are specified normally in *dif-1* mutant embryos, two cell type markers first expressed during gastrulation (prior to embryonic differentiation) were assayed. Expression of *hlh-1* was used to assay commitment to the muscle cell fate [*hlh-1* is the *C.elegans* myoD homologue (Krause *et al.*, 1990)] and *lin-26* the hypodermal fate (Labouesse *et al.*, 1994; M.Labouesse, personal communication). Both these markers are expressed at the correct time and place during gastrulation in *dif-1* mutant embryos (Figure 2 and data not shown). This suggests that cell fates in *dif-1* mutants are correctly specified but cannot be properly executed.

To examine differentiation more carefully in *dif-1* mutant embryos, antibodies that stain early tissue differentiation products were used. These antibodies assayed many tissue types: body wall muscle, pharyngeal muscle, gut and hypodermis. For all antibodies tested, a small amount of staining was detected in *dif-1* mutants, but was far reduced compared with wild-type animals (Figure 3). Most *dif-1* embryos also produced autofluorescent gut granules but these were also reduced in intensity compared with wild-type (not shown). Because most tissues are able

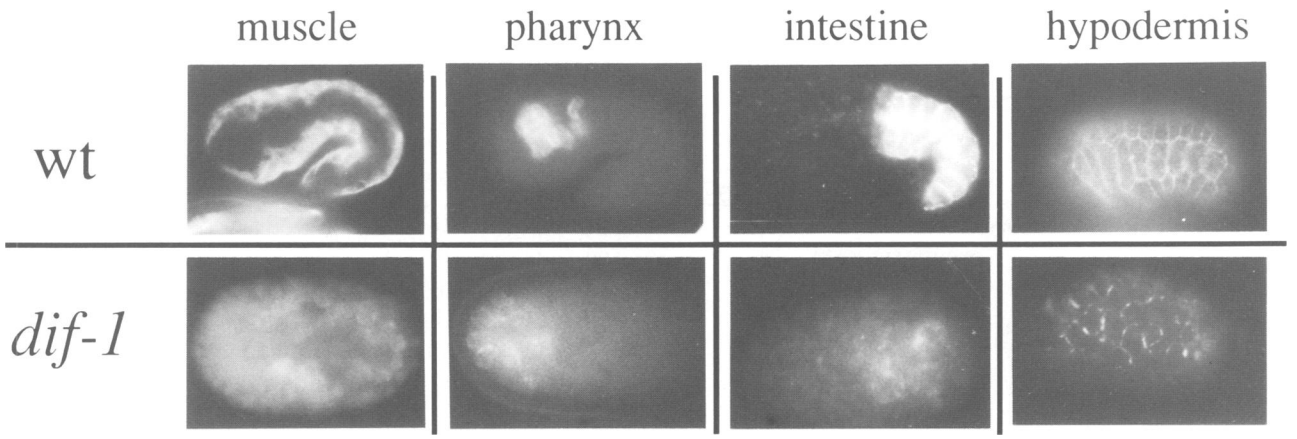

Fig. 3. *dif-1* mutant embryos have greatly reduced tissue differentiation. Differentiated products were detected by indirect immunofluorescence using antibodies to body wall myosin [5.8.1 (Miller *et al.*, 1983)], pharyngeal muscle [3NB12 (Okamoto and Thomson, 1985; Priess and Thomson, 1987)], gut [1CB4 (Okamoto and Thomson, 1985)] and hypodermal adherens junctions [MH27 (Francis and Waterston, 1991)]. The photographs of *dif-1* mutant embryos have been overexposed to show the small amount of staining that is detectable. In all cases *dif-1* mutant embryos had greatly reduced staining compared with wild-type animals. Embryos were allowed to develop for 6–8 h at 25°C (or 12–16 h at 15°C) before fixing.

to express some final differentiation products, *dif-1* activity is not needed for the onset of tissue differentiation, but rather is required for its continuation. Consistent with this, two markers of late tissue differentiation, pharyngeal myosin [assayed with antibody 9.2.1 (Miller *et al.*, 1983)] and a hypodermal seam antigen [assayed with antibody NE2/1B4–14 (Schnabel, 1991)], are not expressed in *dif-1* mutant embryos (not shown).

dif-1 activity is required for only 3 h of embryogenesis

Three additional alleles of *dif-1* were isolated in a non-complementation screen (see Materials and methods). One of these new alleles (*e2591*) is cold sensitive (*cs*). The phenotype of *e2591(cs)* at the restrictive temperature (15°C) is very similar to that of the putative null alleles (*e2562* and *e2577*), but at the permissive temperature (25°C) some embryos hatch and grow to adulthood. Some of these survivors hatch as dumpy (shorter and fatter than wild-type) and slightly ill animals, but most appear wild-type and are fertile.

To identify when during development *dif-1* activity is required, *e2591(cs)* mutant embryos were shifted from the restrictive to the permissive temperature and vice versa at various times during embryogenesis to determine the temperature-sensitive period (TSP) (Figure 4). Developmental times are normalized to 25°C and 0 h corresponds to a two-cell embryo. A shift to the permissive temperature at or before 5 h of embryogenesis allowed the survival of mutant embryos, but shifts after this time resulted in 100% lethality (Figure 4). Conversely, a shift to the restrictive temperature at or after 8 h of embryogenesis allowed mutant embryos to survive, but a shift before 8 h resulted in 100% lethality. These temperature shift experiments define a window of 3 h when *dif-1* activity (or synthesis) is required for survival. The beginning of the TSP (at 5 h of embryogenesis) corresponds to the start of embryonic morphogenesis. This is the time that *dif-1* null mutant embryos arrest development (see above). The TSP ends at 8 h of embryogenesis, when wild-type embryos have developed into a worm with function in most tissues, but

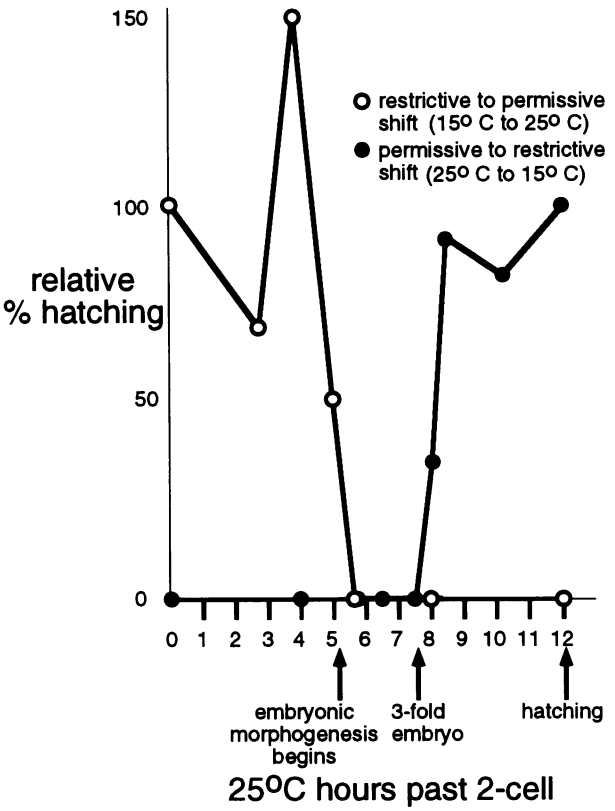

Fig. 4. Temperature-sensitive period of *dif-1(e2591)*.Time points, normalized to 25°C development, give the time embryos spent at the first temperature before being shifted (0 h = two-cell embryo). Percentage hatching is calculated as the percentage of experimental embryos that hatched relative to control (constant permissive temperature) embryos. Data and calculations are given in Materials and methods.

3 h prior to hatching. This TSP suggests that *dif-1* activity is needed only for 3 h of embryogenesis, covering the major period of embryonic tissue differentiation. No post-embryonic requirement was revealed when *dif-1[e2591(cs)]* mutants were shifted to the restrictive temperature after hatching (see Materials and methods).

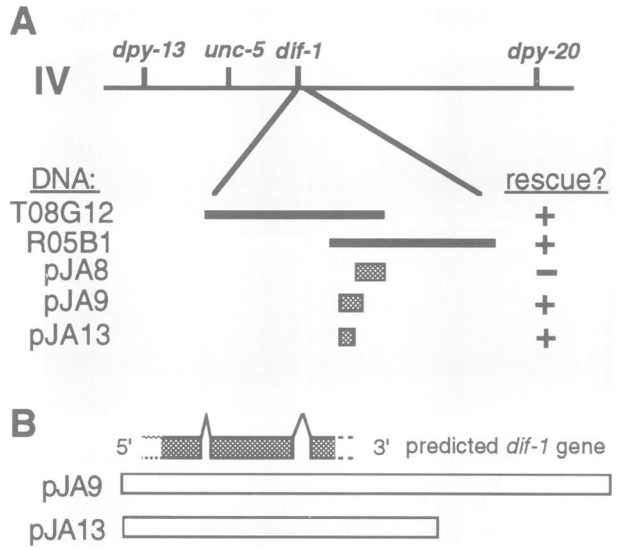

Fig. 5. *dif-1* genomic organization. (**A**) Top line, schematic diagram of part of the chromosome IV genetic map. Expanded is a region of the physical map (Coulson *et al.*, 1986) corresponding to the *dif-1* genetic region. DNAs T08G12 and R05B1 are cosmids; pJA8, pJA9 and pJA13 are plasmid subclones from regions of overlap between these cosmids; + indicates the DNA has rescuing activity, − indicates no rescuing activity. (**B**) GENEFINDER (P.Green, personal communication) prediction of the *dif-1* gene and its position within pJA9 and pJA13. Shaded is the predicted coding region; the program does not predict 5′ or 3′ untranslated regions (indicated by dashes). The orientation of *dif-1* on the physical map is not known.

In summary, *dif-1* mutant embryos develop identically to wild-type embryos until the start of embryonic morphogenesis: gastrulation is completed and cell fates appear to be properly specified with most tissues undergoing a small amount of differentiation. Development is then arrested and cells soon look visibly necrotic. Finally, *dif-1* activity is required only for 3 h of embryogenesis, corresponding to the first 3 h of embryonic morphogenesis. Taken together, these results suggest that *dif-1* activity is generally required for the continuation of embryonic tissue differentiation and may be important for the survival of cells during this time.

dif-1 is homologous to mitochondrial carriers

To understand the function of *dif-1* the gene was cloned. Cosmid clones spanning the region (Coulson *et al.*, 1986) were injected into *dif-1[e2591(cs)]* mutant mothers at the restrictive temperature to attempt to rescue the embryonic lethality. Two individual cosmids were found to have rescuing activity and this was narrowed to a 3.3 kb region of overlap between the cosmids (pJA9; Figure 5A). This region was sequenced and then the GENEFINDER program (P.Green, personal communication) was used to identify possible genes within it. Only one gene was predicted to lie within this fragment (Figure 5B). Transformation rescue with a smaller region containing this predicted gene (pJA13) further localized the *dif-1* gene. The sequence of 1.68 kb containing this gene is shown in Figure 6. Conceptual splicing and translation predicts a protein of 312 amino acids. A *dif-1* cDNA, cm04a8 (Waterston *et al.*, 1992), was recently sequenced (Runswick *et al.*, 1994) and has the splicing pattern predicted by GENEFINDER. The sequence of *dif-1* mutations (see below) confirmed that this is the *dif-1* gene.

The *dif-1* gene is homologous to proteins in the mitochondrial carrier family. These proteins are usually found in the inner mitochondrial membrane and carry solutes into and/or out of mitochondria (reviewed in Walker, 1992; Walker and Runswick, 1993). They consist of three 100-amino-acid blocks, each of which is similar to the others, and contain two predicted membrane-spanning regions and a loop region (shown in Figure 7 as 1, 2 and 3). Members of this family include the ATP/ADP carrier, phosphate carrier, oxoglutarate/malate exchanger and brown fat uncoupling protein that generates heat by uncoupling mitochondrial respiration from ATP synthesis. Figure 7 shows an alignment of the *dif-1* protein (DIF-1) with different carrier proteins; the percentage of amino acids identical with DIF-1 is given in parentheses after each name. Within a class of carriers (e.g. the ATP/ADP carriers) the percentage identity is usually fairly high (50% or greater), with characteristic residues conserved, and between members of different classes it is usually ~20–30% (Walker, 1992; Kuan and Saier, 1993; Walker and Runswick, 1993). Based on sequence comparison, DIF-1 does not belong to a class of carrier with known solute specificity (Figure 7 and data not shown). The yeast protein of unknown function YMC1 (Graf *et al.*, 1993) is the most similar to DIF-1 but has only 29% identical amino acids. Therefore, it appears that the *dif-1* gene does not belong to a previously cloned type of carrier, so the type of solute DIF-1 might transport is not obvious.

To confirm that this gene is *dif-1* and to ask whether the strong alleles result in complete loss of *dif-1* function, three *dif-1* mutations were sequenced. One putative null mutation (*e2562*) is an 11 bp deletion that results in a frameshift that truncates the *dif-1* coding region (Figure 6, nucleotides changed or deleted by mutation are underlined; Figure 7, amino acid changes are indicated with arrows). The second null class mutation, *e2577*, causes a substitution of glutamate for glycine near the end of the second membrane-spanning region. The finding that the *e2562* mutation truncates over a third of the coding region suggests that this mutation should indeed abolish all *dif-1* activity. Because *e2577* causes a phenotype similar to that of *e2562*, it probably also results in the loss of all *dif-1* activity. The cold-sensitive allele *e2591* causes a glycine to lysine change within the first predicted membrane-spanning region. Since *e2591* and *e2577* introduce charged residues within or near the end of predicted membrane-spanning regions, these changes may grossly affect the structure of the protein. Alternatively, they may identify residues important for DIF-1 function.

dif-1 RNA is expressed throughout development

To find out when *dif-1* RNA is expressed, a Northern blot containing mRNA from staged animals was performed. Figure 8 shows that *dif-1* RNA is ~1.2 kb long and is found at all stages of development at approximately equivalent levels. *dif-1* RNA is also found in a mutant strain lacking a germline (*glp-4*; Beanan and Strome, 1992; Figure 8), indicating that *dif-1* RNA is not germline specific. The expression of *dif-1* RNA throughout development suggests that *dif-1* activity might be present at

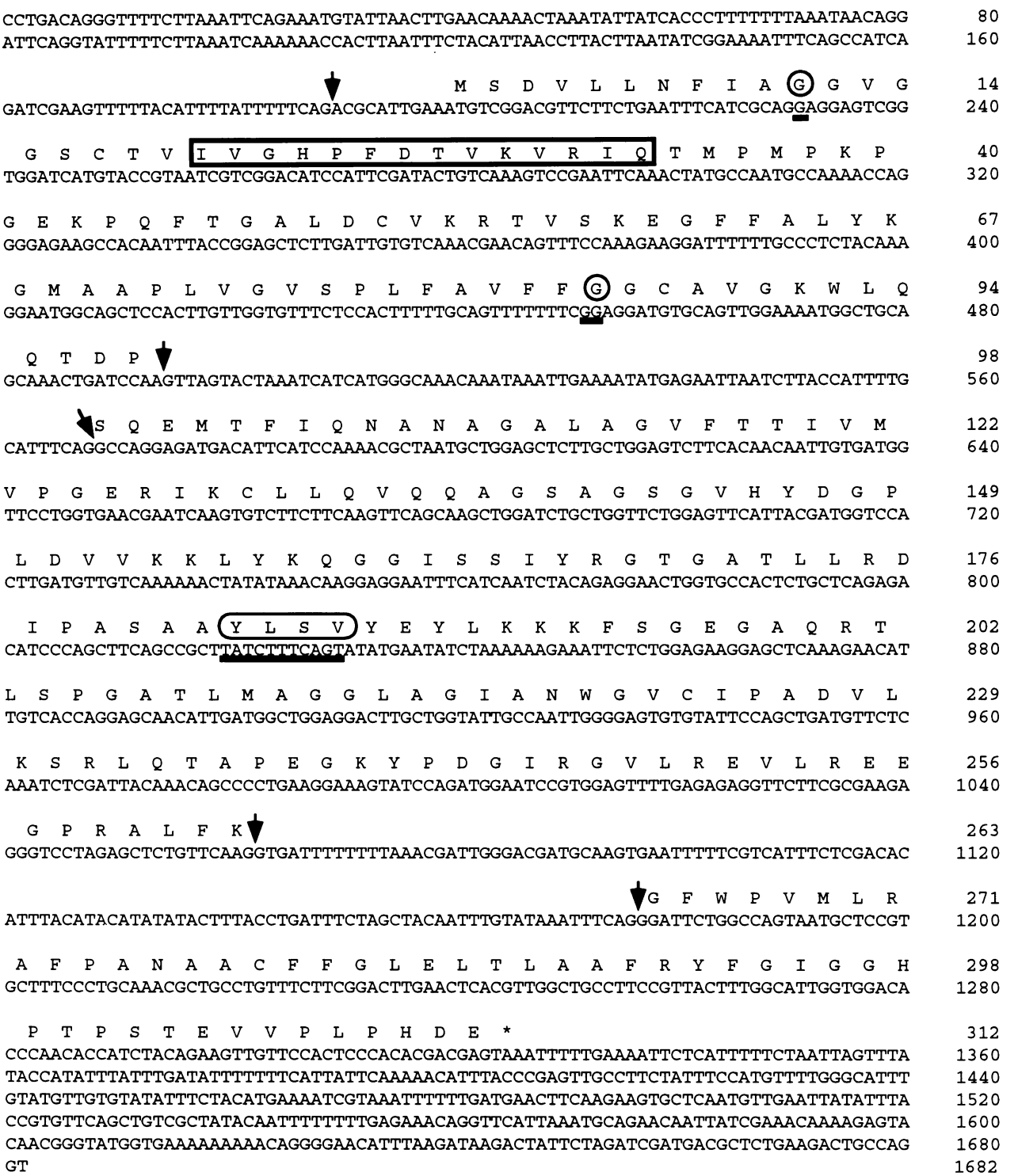

Fig. 6. Sequence of *dif-1* genomic DNA. Predicted translation is shown above the DNA sequence. Arrows point to predicted 5′ and 3′ splice sites; the first arrow indicates the site of trans-splicing to leader SL1 (Krause and Hirsh, 1987). SL1 was found at this position on cDNA cm04a8, which was partially sequenced by Waterston *et al.* (1992). The entire splicing pattern is confirmed by the recently reported complete sequence of cm04a8 (Runswick *et al.*, 1994). The nucleotides changed by *dif-1* mutations are underlined and the altered amino acids are circled; *e2591*: G (nt 231) to A → G (aa 11) to K; *e2577*: GG (nt 462–463) to AA → G (aa 85) to E; *e2562*:: TATCTTTCAGT (nt 820–830) deleted → frameshift resulting in a stop codon after one additional amino acid (isoleucine). Boxed is the mitochondrial carrier signature sequence (Kuan and Saier, 1993). The mutations *e2562* and *e2577* were isolated after UV mutagenesis, which is thought to induce mutation by faulty repair after dimerization of thymine residues (Friedberg, 1985); the 11 nucleotides deleted in e2562 contain three T residues, and the two guanine residues altered in e2577 closely follow a run of thymines. EMBL accession number for the *dif-1* genomic sequence: Z48240.

all times, even though it is only required for 3 h of embryogenesis (Figure 4). This raises the possibility that *dif-1* is essential only during embryogenesis because it is redundant post-embryonically. A Southern blot shows that under low stringency conditions, only the *dif-1* gene is detected (Figure 9), indicating that *dif-1* has no close

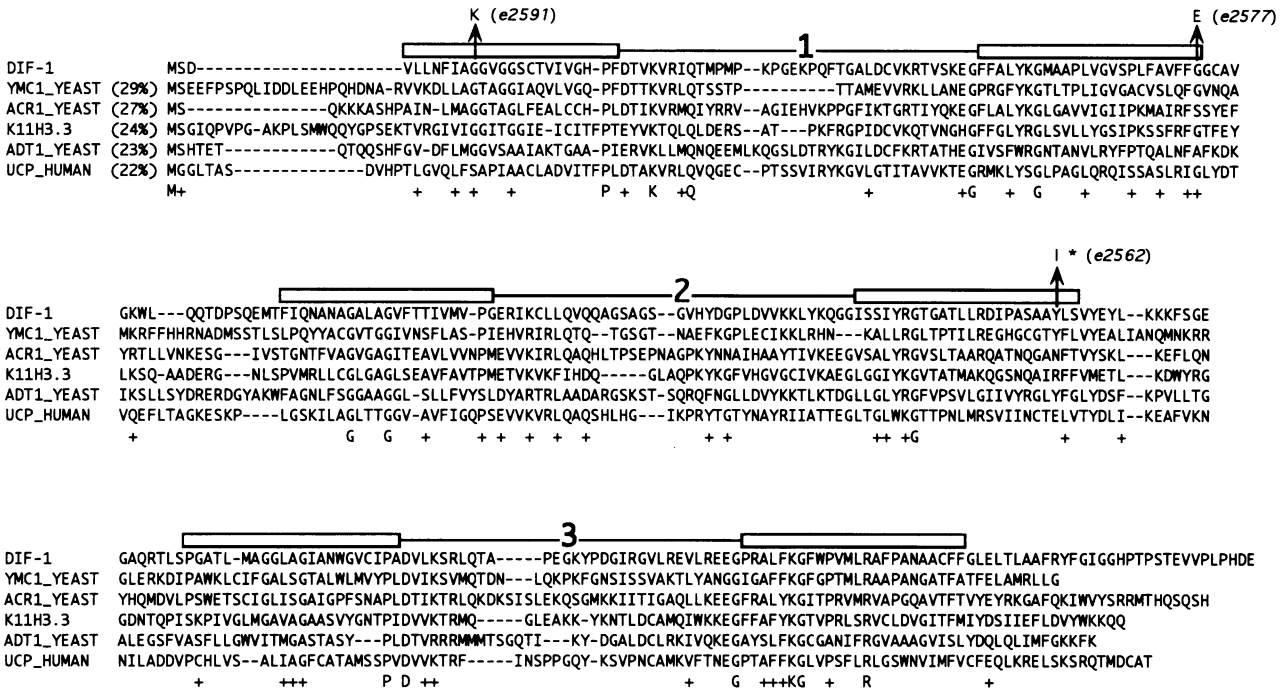

Fig. 7. Alignment of the predicted *dif-1* protein with mitochondrial carriers. Shown is a slightly modified CLUSTAL V alignment (Higgins, 1994). The percentage amino acids identical with DIF-1 are given in parentheses after the name. The open boxes above the alignment indicate hydrophobic regions that are predicted to be membrane spanning; these boxes and the thin lines connecting them (1, 2 and 3) show the three blocks of amino acids that are homologous to each other. Amino acids identical in all the proteins are shown below the alignment; + indicates similar amino acids. YMC1 (Graf *et al.*, 1993) and ACR1 (Fernandez *et al.*, 1994) are yeast carriers of unknown solute specificity, ADT1_yeast (Adrian *et al.*, 1986) is a yeast ATP/ADP carrier, K11H3.3 is a predicted *C.elegans* gene (Wilson *et al.*, 1994), most similar to citrate carriers, and UCP_human (Thomas and Ricquier, 1990) is a human brown fat uncoupling protein. Arrows indicate amino acids altered by *dif-1* mutations.

homologues. It is possible, however, that a gene with a redundant activity exists but is not sufficiently similar to be detected by this method.

dif-1 RNA is found in the embryonic gut and hypodermis

Since *dif-1* is required for the continued differentiation of all tissues, it was expected to be expressed in all cells of the embryo. However, *in situ* hybridization revealed tissue-specific expression of *dif-1* RNA. In one- to 28-cell embryos, *dif-1* RNA is found in all cells and this probably represents maternal *dif-1* RNA. The staining then decays in all but the gut progenitor cells (Figure 10C); gut expression continues throughout embryogenesis (Figure 10E, F and I and data not shown). At about the 200-cell stage, *dif-1* RNA is detected in the hypodermal cells (Figure 10D) and this persists at least until hypodermal cells begin to enclose the embryo (Figure 10G and H). It is not clear whether the hypodermal expression continues after this time.

Development can proceed with either maternal or zygotic *dif-1* activity. The *dif-1* expression in the hypodermis occurs after the decay of *dif-1* RNA in all but the gut precursor cells, so this must be zygotically transcribed. The later gut expression is probably also zygotic since it becomes stronger at the 28-cell stage (Figure 10B). All tissues require *dif-1* activity for differentiation but zygotic *dif-1* RNA appears to be expressed only in the gut and hypodermis. This suggests that *dif-1* can act non-autonomously to allow tissues that do not express it to develop properly. Alternatively, there may be a low level

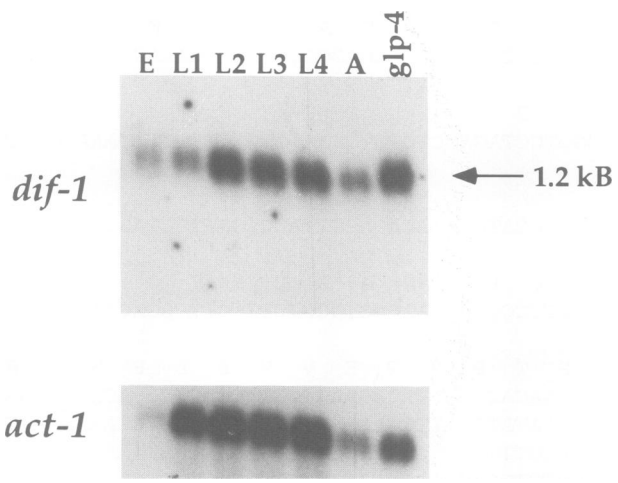

Fig. 8. *dif-1* RNA is expressed throughout development. Northern blot of *C.elegans* poly(A)$^+$ RNA hybridized with probes to *dif-1* (top) and *act-1* (bottom) as a loading control; stages are indicated above the lanes. E, embryos; A, adults. *glp-4* RNA was obtained from adult hermaphrodites raised at the restrictive temperature; *glp-4* animals have a greatly reduced germline and therefore essentially contain only somatic tissue (Beanan and Strome, 1992).

of zygotic *dif-1* RNA in all embryonic cells not detected by the *in situ* hybridization.

Discussion

A new *C.elegans* gene, *dif-1*, has been identified, mutations in which result in the maternal-effect loss of embryonic

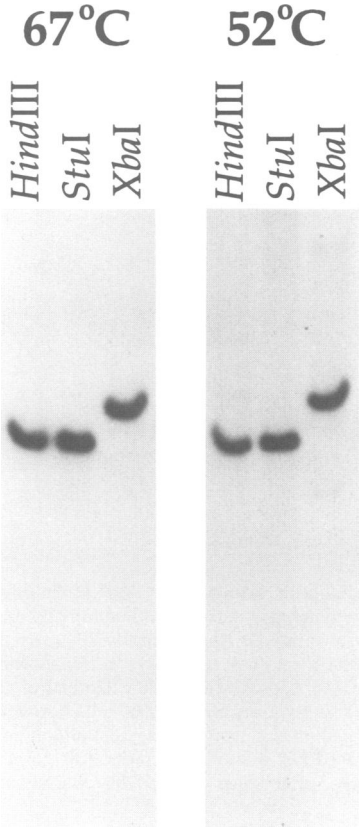

Fig. 9. *dif-1* is a single copy gene. *Caenorhabditis elegans* genomic DNA was digested with the restriction enzymes indicated and the blots were probed with *dif-1* DNA at either 67°C (high stringency) or 52°C (low stringency). Only *dif-1* DNA is detected at either temperature. There are no *Hin*dIII or *Stu*I restriction sites within the *dif-1* gene, but *Xba*I cuts 30 bp from the df7 end of the probe; below the major band a faint band is visible in the *Xba*I lane of the 52°C blot.

tissue differentiation. *dif-1* activity is essential only during a 3 h period of embryonic morphogenesis and is dispensable at other times of development. Molecular analyses showed that the *dif-1* protein is a member of the mitochondrial carrier family and may act within the embryonic gut and/or hypodermis. Below, *dif-1* is compared with other carriers and possible functions for *dif-1* are discussed.

Mitochondrial carrier proteins transport solutes across the inner mitochondrial membrane (reviewed in Walker, 1992; Walker and Runswick, 1993). They form a family with similar structural organization but relatively little sequence conservation. Across all family members only a few residues are invariant, but they share a 3-fold repeat of ~100 amino acids, each containing two membrane-spanning regions. There are now sequences for ~14 distinct types of carrier but the solute transported is known for only five of them: the ATP/ADP carrier, the phosphate carrier, the oxoglutarate carrier, the brown fat uncoupling protein and the tricarboxylate carrier (Kaplan *et al.*, 1993; Walker and Runswick, 1993). Recently, *C.elegans* cDNAs for five mitochondrial carriers were sequenced (Runswick *et al.*, 1994); these identified ATP/ADP, phosphate and oxoglutarate carriers, as well as a cDNA corresponding to *dif-1* (cm04a8) that had been isolated in a survey of expressed genes (Waterston *et al.*, 1992). The *dif-1* protein, however, does not fall into a group with known solute specificity and DIF-1 is not sufficiently similar to any carrier of unknown solute specificity to place it in a particular class. Therefore, it appears that DIF-1 defines a new class of carrier.

So far, proteins in the mitochondrial carrier superfamily have been found almost exclusively at the inner mitochondrial membrane, so it is likely that DIF-1 will also be found there. The exception is the yeast protein PMP47, which is found in peroxisomes (Jank *et al.*, 1993). However, PMP47 is also the only member of the family that does not have a perfect signature sequence {[(LIVSAT) X_3 (LIVF) (DE)×(LIVTA) (KR) (LIVTCN) (LIVMSQ) (QM)]; Kuan and Saier, 1993} and it is much larger than other mitochondrial carrier proteins (47 versus ~32 kDa). DIF-1 has this signature sequence (boxed in Figure 6) and is of normal size, placing it in the group of carriers known to reside in mitochondria.

The sequence of two *dif-1* mutations may give some insight into regions important for its function. The cold-sensitive mutation *e2591* substitutes a lysine residue for a glycine within the first membrane-spanning region. In the yeast ATP/ADP carrier AAC2, mutation of the corresponding glycine to a cysteine is a second site revertant of a null mutation predicted to lie in a matrix loop (Nelson and Douglas, 1993; Nelson *et al.*, 1993). From the analysis of this and other revertant mutations the authors suggest that this glycine residue (and other residues at this position in the membrane) is important for the structure of the membrane-spanning helix. By analogy, the *e2591* mutation may abolish the activity of DIF-1 at low temperature by causing a structural change in the first membrane-spanning helix. Interestingly, a cold-sensitive mutation in the *Drosophila* membrane protein *frizzled* also changes a glycine in a predicted membrane-spanning region to a basic amino acid (Adler *et al.*, 1994). The null mutation *e2577* substitutes a glutamate for a glycine at or near the end of the second membrane-spanning region. This residue may have a specific functional role or, alternatively, the mutation could generally affect the structure of DIF-1.

Several observations argue that *dif-1* activity is required only during embryonic tissue differentiation. First, as is common for gene products required only during embryogenesis, either maternal or zygotic *dif-1* activity is sufficient for survival. In addition, homozygous *dif-1* null mutant progeny from *dif-1/+* mothers are completely wild-type. Second, *dif-1* null mutant embryos develop like wild-type until the start of embryonic morphogenesis and then development is arrested. This suggests that development prior to this time does not require DIF-1. Third, the TSP of *e2591(cs)* mutants starts at the beginning of embryonic morphogenesis and lasts for 3 h, after the major period of embryonic tissue differentiation is complete. The arrest of *dif-1* null mutant embryos corresponds to the beginning of the TSP, arguing that the *e2591(cs)* mutant protein completely lacks function at the restrictive temperature. Finally, there appears to be no post-embryonic requirement for *dif-1* activity. Homozygous *e2591(cs)* mutant embryos laid by homozygous mutant mothers grow to adulthood if shifted to the restrictive temperature after the TSP. Although *dif-1* activity is required only for 3 h of embryogenesis, *dif-1* RNA is

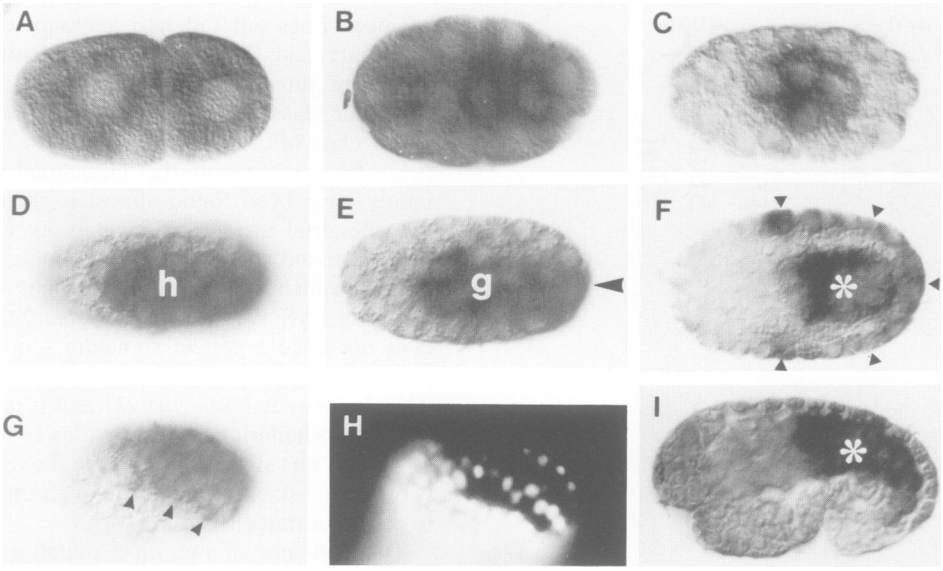

Fig. 10. Localization of *dif-1* RNA during embryogenesis. *dif-1* RNA was detected with a digoxigenin labelled *dif-1* DNA probe using the method of Seydoux and Fire (1994). (**A**) Two-cell embryo and (**B**) 28-cell embryo; *dif-1* RNA (probably maternally derived) is found in all cells of early embryos. (**C**) Fifty-eight-cell stage, the first probable zygotic expression is in four gut precursor cells. (**D**) Approximately 200 cells, *dif-1* RNA is now detected in hypodermal precursor cells (h) on the dorsal surface of the embryo. (**E**) Central focal plane of embryo in (D) showing *dif-1* RNA in gut cells (g), arrowheads at posterior end indicate hypodermal cells visible in this focal plane. (**F**) Approximately 550 cells (end of gastrulation and cell proliferation), central focal plane, ventral side up; *dif-1* RNA is still only detected in gut (asterisk) and hypodermal cells (arrowheads at edge). (**G**) Lateral view of *dif-1* RNA detected in hypodermal cells; arrowheads show the line of hypodermal cells migrating ventrally to enclose the embryo. (**H**) DAPI staining of embryo in (G) to visualize nuclei. (**I**) Early morphogenesis; *dif-1* RNA is strongly expressed in the gut but hypodermal staining has apparently weakened. During morphogenesis RNA detection was more variable and it is not clear whether the hypodermal expression continues.

present throughout development, suggesting that *dif-1* activity may be continuously present. It is possible that DIF-1 does function post-embryonically, but that this function is not essential. As *dif-1* is a single copy gene, DIF-1 may have an activity that is distinct from that of other proteins.

The localization of *dif-1* RNA to the embryonic gut and hypodermis is surprising given that all tissues fail to differentiate properly in *dif-1* mutants. In early embryos, *dif-1* RNA is found in all cells and this most likely represents maternal *dif-1* RNA. The disappearance of this RNA from all but the gut precursor cells, and the subsequent stronger gut staining and hypodermal expression, suggests that this later *dif-1* RNA is transcribed from the zygotic genome. Genetic experiments showed that either maternal or zygotic *dif-1* activity is sufficient for development. This suggests that the gut and hypodermal expression of *dif-1* RNA is sufficient for the differentiation of all tissues. If this is the case then *dif-1* can act in a non-cell autonomous manner, and either directly or indirectly allows some molecular transport or communication between cells. However, it is not yet known whether maternal *dif-1* activity normally functions in all embryonic cells or only in the gut and hypodermis.

What is the function of DIF-1? Because it is unlike carriers with known solute specificity, it is impossible to predict what solute it might carry. The finding that cells of *dif-1* mutant embryos become necrotic soon after arrest and the probable location of DIF-1 in mitochondria raise the possibility that DIF-1 may be required for cell survival by aiding in embryonic metabolism. For example, there might be a substrate that is utilized specifically during high-energy usage or at a certain time in development that requires transport into mitochondria. As yet, little is known about embryonic metabolism in *C.elegans*. However, there is a precedent for a protein in the mitochondrial carrier family being essential for specialized metabolism. The ACR1 protein of *Saccharomyces cerevisiae* is required for growth on ethanol or acetate, but not for fermentation (Fernandez *et al.*, 1994). An alternative idea is that DIF-1 is required to prevent the production or transport of a cell-poisoning toxin. Finally, a more interesting possibility is that DIF-1 is needed for production of a differentiation signal.

What is now necessary for understanding the function of DIF-1 is to know what solute it transports. Recently, it has been shown that specific transport of solutes by carrier proteins can be achieved *in vitro* using protein made in bacteria (Fiermonte *et al.*, 1993). Assays of this type could determine the solute specificity of DIF-1 and bring insight into its role in development.

Materials and methods

Genetic analyses and isolation of mutants

Worms were cultured as described (Brenner, 1974). The following strains (or mutations) were used: N2 (Bristol) wild-type strain. LGIV: *unc-5(e53), bli-6(sc16), dpy-20(e1282), unc-44(e362), smg-3(r929), deb-1(st555), unc-24(e138), dif-2(e2576), egl-23(n601sd)*. LGIV and V: +/ *nT1(let(−) unc(n754sd))* (IV); + / *nT1* (V) [translocation balancer hereafter called *nT1(dom)*], *nDf41/nT1(dom)*. These strains are described in Hodgkin *et al.* (1988) and in the *Caenorhabditis* Genetics Center data releases.

Two alleles of *dif-1* were isolated after UV mutagenesis of *egl-23* hermaphrodites, using a previously described screen to isolate Mel mutants (Kemphues *et al.*, 1988). *egl-23(n601sd)* hermaphrodites cannot lay eggs, so their progeny hatch inside and devour them. If a Mel mutation is induced, the *egl-23(n601sd)* hermaphrodites will live since

all their progeny die. The Mel mutation can be recovered from a heterozygous sibling. UV mutagenesis was accomplished by suspending a Petri plate containing L4 worms and young adults over a short-wave UV light box (UVP, incorporated 302 nm) at a height of 14 cm and irradiating for 20 s. After mutagenesis, *egl-23(n601sd)* hermaphrodites were picked to individual plates and allowed to produce self-progeny F1 worms. These F1s were picked to individual agar-filled microtitre wells and the wells were scored for the presence of live adults 5 days later (at 22°C). A total of 50 Mel mutants were recovered from 7127 F1s cloned.

The *dif-1* gene was genetically mapped as follows. First, it was determined to lie on chromosome IV by virtue of its linkage to *egl-23(n601)*. Three- and four-factor mapping then placed *dif-1* at position +3.4 on IV. From a parent of the genotype *dif-1(e2562)/unc-44 (e362) deb-1(st555)*, 16/16 Unc non-Deb recombinants carried *dif-1(e2562)*, placing *dif-1* to the right of *unc-44*, near or right of *deb-1*. From a parent of the genotype *dif-1(e2562)/unc-44(e362) smg-3(r929) unc-24(e138)*, 10/30 Unc-24 non-Unc-44 recombinants carried *dif-1(e2562)*, placing *dif-1* approximately two-thirds of the way between *unc-44* and *unc-24*. Using the physical location of *unc-44* and *unc-24*, and estimating the size of yeast artificial chromosomes (YACs) that bridge cosmid contigs, a probable location for *dif-1* on the *C.elegans* physical map (Coulson *et al.*, 1986) was determined. Cosmids from C07A3 to R05B1 were used in pools for initial transformation rescue of *dif-1(e2591)* (see below).

To obtain more alleles of *dif-1* two different non-complementation screens were carried out. In the first, *unc-5(e53) dif-1(e2577)/nT1(dom)* hermaphrodites were crossed with *dpy-20(e1282)* males that had been mutagenized with ethylmethanesulfonate (ems) (Wood, 1988). Non-Unc cross progeny [all of which should carry *dif-1(e2577)*] were cloned to individual plates and scored for the presence of 100% dead embryos. Candidates were crossed with N2 males to recover new mutations. From 1462 F1 worms cloned, one new *dif-1* allele, *e2591*, was recovered. In the second screen, *unc-5(e53) dif-1(e2562) dif-2(e2576) dpy-20(e1282)/nT1(dom)* hermaphrodites were crossed with ems-mutagenized N2 males. From 1514 F1 worms cloned, two new *dif-1* alleles were isolated (*e2597* and *e2598*). All three new alleles were outcrossed against N2 at least four times and the *dpy-20(e1282)* mutation was separated from *dif-1*(e2591) by recombination before analysis. The new alleles failed to complement *e2562* on retesting after outcrossing. The *dif-1* alleles *e2597* and *e2598* are slightly weaker than the null alleles (*e2562* and *e2577*). Although 0/300 *e2597* or *e2598* embryos hatched at either 15 or 25°C, 4/43 *e2597* embryos and 2/49 *e2598* embryos developed to the 2-fold stage before arresting at 15°C. Null mutant embryos never develop to the 2-fold stage (zero out of many hundreds).

To test whether the deficiency *nDf41* removed *dif-1*, *e2562/+* males were crossed into *nDf41/nT1(dom)* hermaphrodites and non-Unc cross progeny picked; half of these should be *+/nDf41* and half *e2562/nDf41*. These cross progeny were indistinguishable from each other and all grew to adulthood; 22/42 were *e2562/nDf41* and laid only dead eggs (maternal effect lethality); 20/42 were *+/nDf41* and laid both live and dead eggs. The phenotypes of the dead embryos laid by *e2562/nDf41* mothers were very similar to those laid by *e2562* homozygotes, but some were slightly more disorganized. These were probably homozygous for the *nDf41* deficiency. *nDf41/nDf41* laid by *nDf41/+* mothers are severely disorganized, have slightly less than the normal number of cells and possibly have defects during gastrulation (not shown).

To test whether *ced-4* would suppress the *dif-1* phenotype, 40 Lon progeny from animals of the genotype *ced-4(n1162) lon-1(e1920)/ + +* ; *dif-1(e2562)/ +* were picked to individual plates. Six out of 40 were Mel and laid dead eggs with a *dif-1* phenotype.

Embryos were observed and development recorded using a time lapse video recording system (Hird and White, 1993). Cell lineages were determined from recordings. Photographs were taken either from the monitor or directly through the microscope.

Antibody and lacZ stainings

Embryos were isolated by cutting open gravid hermaphrodites in M9 buffer (Wood, 1988) and transferring them to poly-L-lysine (Sigma) coated slides; coverslips were applied and the slides put in a humidified chamber to allow embryos to develop to the desired age. Liquid was then removed using a wick until slight pressure was exerted, and the slides were placed on a metal block embedded in dry ice and left for at least 10 min. Antibody stainings were carried out essentially as detailed in Albertson (1984). All primary antibodies were diluted in PBS containing 0.2% Tween-20 (PBST) before use: monoclonal antibodies 1CB4 and 3NB12 (Okamoto and Thomson, 1985) in cell serum were used at 1:2, monoclonal antibody MH27 (Francis and Waterston, 1991) was used at 1:300, monoclonal antibody 5.8.1 (Miller *et al.*, 1983) at 1:1000 and anti-LIN-26 polyclonal rabbit serum (M.Labouesse, personal communication) at 1:1000. Antibody stainings of *dif-1* mutants usually contained a second primary antibody that was known to give a signal, as a control [either anti-LIN-26 or YL1/2 (Kilmartin *et al.*, 1982), an antitubulin antibody that cross-reacts with *C.elegans* (Albertson, 1984)].

To stain strains containing the *hlh-1::lacZ* fusion gene 221F (Krause *et al.*, 1990), embryos were obtained and frozen as above, fixed in acetone at −20°C for 10 min, partially rehydrated in 70% acetone at room temperature and then air dried. Staining solution containing X-Gal (Fire *et al.*, 1990) and a coverslip were applied and the slides incubated in a humidified chamber at 37°C until staining reached the desired intensity. Slides were washed once in PBST before being mounted. During gastrulation, up to the beginning of embryonic morphogenesis, expression of *hlh-1−lacZ* in *dif-1* mutants was identical to that in wild-type individuals.

TSP determination

Embryos were isolated by cutting gravid *e2591* hermaphrodites open in egg salts (5 mM HEPES, pH 7.2, 110 mM NaCl, 4 mM KCl, 5 mM $MgCl_2$). Two-cell stage embryos were transferred to agar plates pre-incubated at the appropriate temperature. Embryos were incubated for various times at either the permissive (25°C) or the restrictive (15°C) temperature and then shifted to the other one. Hatching was scored 24 h later; any hatched animals invariably survived to adulthood. For each time point, control two-cell embryos were isolated alternately with experimental embryos and left at the permissive temperature at all times, as survival varied from 10 to 85%. This variability is probably due to the extreme sensitivity of *e2591(cs)* mutants to temperatures below 25°C, as almost no hatching occurs at 24°C. The experimental percentage hatching shown in Figure 4 is calculated as the percentage of shifted animals hatching relative to controls at the continuous permissive temperature. The times in Figure 4 are normalized to 25°C development. Following are the data for each time point in the format: hours before shifting (normalized to 25°C time): number hatched/total number experimental embryos (experimental percentage hatched), number hatched/total number control embryos (control percentage hatched), experimental percentage hatched/control percentage hatched = reported % hatched. Data for the shift from permissive to restrictive temperature: 4 h: 0/18 (0%), 7/16 (44%), 0/44 = 0%; 5.75 h: 0/13 (0%), 7/14 (50%), 0/50 = 0%; 6.5 h: 0/15 (0%), 10/16 (62%), 0/62 = 0%; 7.5 h: 0/18 (0%), 4/16 (25%), 0/25 = 0%; 8 h: 5/19 (26%), 15/19 (79%), 26/79 = 33%; 8.5 h: 2/21 (9.5%), 2/19 (10.5%), 9.5/10.5 = 90%; 10.25 h: 11/17 (65%), 14/17 (82%), 65/82 = 79%. Data for the shift from restrictive to permissive temperature: 2.8 h: 5/11 (45%), 7/10 (70%), 45/70 = 64%; 3.75 h: 5/13 (38%), 4/16 (25%), 38/25 = 152%; 5 h: 1/14 (7%), 2/15 (13%), 7/13 = 54%; 5.7 h: 0/13 (0%), 6/14 (43%), 0/43 = 0%; 8 h: 0/11 (0%), 7/12 (58%), 0/58 = 0%. At the 0 h time points, there is 0% hatching if always at the restrictive temperature (no embryos in many hundreds have hatched) and by definition 100% hatching if shifted immediately to the permissive temperature. In one experiment, 7/37 (19%) hatched when grown at the restrictive temperature and shifted to the permissive temperature at the two-cell stage; this is within the range of variation of *e2591(cs)*. The 12 h permissive to restrictive temperature time point is at hatching; 100% of hatched embryos grow to adulthood if kept at the restrictive temperature during the remainder of development, although animals are sometimes dumpy (26% ; n = 89) and appear slightly ill. This phenotype is also seen in *e2591(cs)* mutant embryos grown continuously at the permissive temperature; this is usually evident at hatching and probably reflects slightly defective embryogenesis due to reduced *dif-1* activity.

Molecular analyses

Three pools of six cosmids each were originally injected into *e2591* to attempt to rescue the embryonic lethality at the restrictive temperature. Transformation rescue procedures were as described (Fire *et al.*, 1990; Mello *et al.*, 1991); injection DNA contained 10 μg/ml of each cosmid or plasmid of interest and 100 μg/ml of the pRF4 plasmid [containing a *rol-6(d)* gene; Mello *et al.*, 1991] as a coinjection marker in 10 mM Tris−HCl, pH 7.5, 0.1 mM EDTA. One pool (containing cosmids K11C12, D1074, T04E4, C31B5, T08G12, R05B1) was successful (31 rescued progeny were obtained from 17 injections) and some individual cosmids from this pool were then tested. Cosmids T08G12 (16 rescued from 17 injected) and R05B1 (three rescued from seven injected) rescued *e2591* but C31B5 (0 rescued from 13 injected) did not. After restriction mapping of T08G12 and R05B1, two fragments of DNA in the overlap

region were subcloned into pBSKS− (Stratagene). pJA8 contains a 5-kb *Sac*I fragment and pJA9 contains a 3.3-kb *Stu*I fragment. pJA9 has rescuing activity (13 rescued from 18 injected) but pJA8 does not (0 rescued from 10 injected).

pJA9 was sequenced on one strand by making nested deletions (Yanisch-Perron *et al.*, 1985); the Sequenase 2.0 kit (USB) was used for all sequencing. Sequence assembly and analysis was undertaken using the Staden package (Staden, 1994). The GENEFINDER program (P.Green, personal communication) was then used to look for potential genes and splicing patterns. Only one gene was predicted to lie within this 3.3 kb of DNA. To narrow further the *dif-1* gene, a deletion of pJA9 having 1.8 kb containing this gene (pJA13) was tested and found to have rescuing activity (six rescued from 12 injected). The sequence of 1.68 kb of this DNA was completed on both strands and is shown in Figure 6.

To sequence *dif-1* mutations, 10 homozygous *dif-1* mutant mothers were picked into 1× PCR reaction buffer (Promega) containing 100 μg/ml proteinase K (Sigma) and incubated at 60°C for 60 min, then at 95°C for 15 min. PCR reaction components were added [final concentration: 1× PCR buffer (Promega), 0.2 mM dNTPs (Pharmacia), 5 μg/ml oligonucleotides] and a fragment of 1.6 kb containing the entire *dif-1* gene was amplified by PCR in 35 cycles using oligonucleotides df2 (nt 190–209: 5′-ACGCATTGAAATGTCGGACG-3′) and df7 (nt 1682–1663: 5′-ACCTGGCAGTCTTCAGAGCG-3′). This fragment was cloned into pBSKS- and the sequence obtained using *dif-1* specific primers. For each allele, mutant DNA was cloned from two independent PCR reactions. The entire coding region was sequenced from one clone and changes were confirmed by sequencing the independent clone.

For the Northern blot ~1 μg of poly(A)$^+$ RNA was used per lane, except for embryonic, L1 and adult RNA, where slightly less was used. Preparation of poly(A)$^+$ RNA, formaldehyde–agarose electrophoresis and blotting were carried out as described (Rosenquist and Kimble, 1988), except that Hybond membrane (Amersham) was used. The hybridization buffer contained 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS and 200 μg/ml sheared salmon sperm DNA. The probe was a DNA fragment containing the entire *dif-1* gene, PCR amplified from pJA9 using oligonucleotides df2 and df7, and labelled with [^{32}P]dCTP using the Pharmacia oligolabelling kit; hybridization was at 50°C. The Northern blot was reprobed with labelled *act-1* DNA (Files *et al.*, 1983); this was the insert from plasmid pT7/T3–18–103, which contains *act-1* specific DNA (a gift from M.Krause).

For the Southern blots, 4 μg mixed-stage N2 DNA was digested with *Hin*dIII, *Stu*I or *Xba*I and loaded twice on a 0.7% agarose gel. The gel was blotted to Hybond (Amersham); one side was hybridized at 67°C (for high stringency) and the other at 52°C (for low stringency). The probe and hybridization buffer were as for the Northern blot (above), except that formamide was omitted.

In situ hybridization was performed according to Seydoux and Fire (1994). The probe was a digoxigenin dUTP-labelled df2/df7 PCR product.

Acknowledgements

I thank B.Cali, S.Hird, M.Krause, J.Priess and J.Waddle for providing strains, A.Coulson for cosmids, M.Krause for the *act-1* plasmid, G.Seydoux for the *in situ* hybridization protocol and M.Labouesse for anti-LIN-26 antibody. I also thank P.Green for the use of GENEFINDER and M.Labouesse for communicating unpublished results. Some nematode strains used in this work were provided by the *Caenorhabditis* Genetics Center, funded by the NIH National Center for Research Resources (NCRR). I also thank J.Walker for helpful discussions about mitochondrial carriers and R.Durbin for help with genefinding. I am grateful to M.Bretscher, R.Durbin, S.Hird and J.Hodgkin for critical comments on the manuscript. J.White and J.Hodgkin generously provided laboratory space. This work was supported by fellowships from the NIH (GM14155) and the Human Frontiers in Science Program Organization.

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Received on November 25, 1994; revised on February 10, 1995