Genesis of an organ: molecular analysis of the pha-1 gene

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The organisation of organ formation is still an unsolved problem. Mutations in the zygotic lethal gene pha-1 affect a late step during organ development in the nematode C. elegans. In mutant embryos all tissues in the pharynx fail to undergo terminal differentiation and morphogenesis. The expression of an early differentiation marker in pharyngeal muscle precursors is not impaired in mutant embryos, which suggests that pharynx cells still acquire their identity. Therefore the gene defines an organ-specific terminal differentiation function. We cloned and sequenced the pha-1 gene and found that the deduced protein sequence contains features characteristic of the bZIP family of transcription factors. During embryogenesis a transgenic pha-1 reporter construct is expressed tran-

INTRODUCTION

Organ formation is a fundamental aspect of animal embryogenesis and involves the behaviour of cells as a group. It has been proposed that in a first phase of organogenesis cells become determined to an organ-specific fate (restriction) and that in a second phase, an ‘expressive phase’, cells acquire cell type-specific morphological and functional characteristics (Bernfield, 1978). However, little is known about molecules or mechanisms that are involved in organ development. It is not clear whether a primordium of an organ is first specified as a whole or whether the different cell types are specified independently and then are assembled to form the organ. Genes affecting organ-specific differentiation have rarely been isolated. Mutants in two genes, cardiac lethal (Humphrey, 1972) and eyeless (Epp, 1978) in the Mexican axololl affect the differentiation of whole organs. This suggests that also in vertebrates organs are developmental units. In Drosophila a homeobox containing gene tinman was identified, which affects besides other structures heart development (Bodmer, 1993). In Drosophila it was further shown that the determination of an organ, the salivary gland, is controlled by homeotic and dorsoventral patterning genes (Panzer et al., 1992).

Mutations in the gene pha-1 specifically affect the formation of an organ, the pharynx of C. elegans (Schnabel and Schnabel, 1990 and Fig. 1). The pharynx takes up food, grinds it, adds digestive enzymes and transports it through a valve into the intestine. The pharynx contains 80 nuclei which form five tissues: muscles, epithelial cells, marginal cells, glands and nerves (Albertson and Thomson, 1976). The lineal descent of these cells is polyclonal. The pharynx is derived from the somatic founder cells AB and MS (Sulston et al., 1983). Lineages contributing to the pharynx are intermingled with lineages contributing to other tissues. A lineal restriction occurs only rather late in embryogenesis when six of the approximately nine cell divisions of the embryo are completed. In embryos homozygous mutant for pha-1, the entire pharynx fails to undergo late differentiation and morphogenesis. None of the five different pharyngeal cell types is terminally differentiated in mutant pha-1 embryos (Schnabel and Schnabel, 1990). Differentiation of cell types outside the pharynx is not affected in mutant pha-1 embryos. In contrast to maternal effect genes that act early in the embryo to specify the two pharynx producing blastomeres (Priess et al., 1987; Bowerman et al., 1992; Mello et al., 1992), the zygotic lethal gene pha-1 is exclusively required for late development of the pharynx (Schnabel and Schnabel, 1990). As in wild-type embryos, all 80 pharyngeal cells are generated and form a pharynx primordium (Schnabel and Schnabel, 1990 and Fig. 1).

pha-1 affects pharynx development after pharynx cells are committed to a specific cell fate, but before terminal differentiation of the different pharyngeal cell types occurs. This is indicated, for example, by the state of differentiation of the
pharyngeal muscle cells in pha-1 embryos. In mutant pha-1 embryos, the pharyngeal muscle precursor cells express an early cell type-specific marker, but fail to express pharyngeal muscle myosin characteristic of the terminal differentiation of pharyngeal muscle cells. pha-1 provides information that allows different cell types to function together as an organ in terminal differentiation steps and in morphogenesis. Organogenesis, which involves many tissues, requires a strict coordination of the numerous cell types involved in the process. This could be achieved by supplying a start signal.

There are two possible modes by which pha-1 might act. Either pha-1 is expressed in only a few cells located inside or outside the pharynx primordium and acts in an inductive manner to permit pharynx development, or it acts cell-autonomously in all the pharyngeal precursor cells to initiate programs required for the terminal differentiation of these cells.

Molecular analysis of a gene that has an organ-specific differentiation function may provide insight into the nature of organ development. Here, we describe the cloning and molecular identification of the pha-1 gene. We find two regions in the predicted pha-1 protein similar to the two functional domains present in bZIP transcription factors. A pha-1: lacZ hybrid gene is expressed for a short period in all the pharynx precursors present. Using extrachromosomal arrays of pha-1 DNA to generate genetically mosaic pha-1 embryos, we find evidence that pha-1 acts cell-autonomously in pharyngeal precursor cells. The data suggest that pha-1 is involved cell-autonomously in a regulatory process to initiate cellular programs for cytodifferentiation and morphogenesis of the entire organ.

MATERIALS AND METHODS

Strains and genetic methods
Methods for culturing, handling and genetic manipulation of C. elegans have been described by Brenner (1974). The standard wild-type strain N2 is that of Brenner (1974). The alleles of mutants used in this study are listed below.
Cosmid clones, lambda clones and C. elegans physical map

All cosmid and lambda clones are shown in Fig. 4 and the physical map; overlapping cosmid and lambda clones were obtained from J. Hodgkin, A. Coulson and J. Sulston (MRC Laboratory of Molecular Biology, Cambridge, England).

Germline transformation of pha-1 mutants

Microinjection of cloned DNA fragments into the gonad syncytium of C. elegans hermaphrodites was carried out according to the methods developed by Fire (1986) and Mello et al. (1991). To facilitate the identification of transgenic animals, the pRF4 plasmid encoding a dominant allele of the rol-6 gene was included as a marker for co-transformation (Kramer et al., 1990; Mello et al., 1991). The injected animals were kept at the permissive temperature (15°C) of the pha-1(e2123ts) allele. The progeny were scored for animals with a Roller phenotype (caused by co-injection of the pRF4 DNA) and individual rol-6 animals were isolated. rol-6 larvae of the F2 progeny were shifted to the non-permissive temperature of the pha-1(e2123ts) allele (25°C) and screened for viable progeny. For all transformation experiments the temperature sensitive pha-1 strain pha-1(e2123ts);lon-2(e678) was used. Rescued lines also complemented pha-1(t1001ts) and pha-1(e2468).

Genomic DNA, cDNA manipulation and sequencing

Restriction fragments from cosmid and lambda clones were subcloned into the plasmid Bluescript KS + (Stratagene). The 3 kb pha-1 amazed into the plasmid Bluescript KS + (Stratagene). The 3 kb pha-1 amplified from genomic DNA using Vent polymerase (Biolabs) and specific oligonucleotide primers. PCR products were gel purified and cloned blunt ended into pBSK KS+. Specific oligonucleotide primers were used to sequence the PCR products from two clones of different pha-1(e2123ts) isolates.

Northern blot analysis

Northern blots were prepared by electrophoresing 10 µg per lane of total RNA from staged embryos, as described in Fig. 4, on a 1.25% agarose-glyoxal gel. The RNA was transferred to a nylon membrane (Hybond N, Amersham), UV cross linked and baked for 2 hours at 80°C. The gel purified 6.6 kb genomic fragment C1 containing the pha-1 gene was labelled with 32P using the oligo-labeling procedure described by Feinberg and Vogelstein (1983). Hybridisation and washes were done at 60°C.

β-galactosidase fusion vectors

A 3.1 kb Clal-HindIII fragment from the 5’ end of the pha-1 gene was fused in the proper orientation and reading frame to the β-galactosidase fusion vector pPD22.04 to generate the plasmid pH1-ΔlacZ. The vector pPD22.04 was derived from the β-galactosidase expression vector pPD22.04 (Fire et al., 1990) by removing a 51 bp KpnI fragment encoding the SV40 nuclear localisation signal (NLS). To ensure the proper orientation and reading frame all constructs were sequenced at the fusion site. The presence of the NLS in the pha-1: lacZ constructs resulted in no detectable expression of β-galactosidase in embryos carrying extrachromosomal arrays of the construct (data not shown). To examine expression during embryogenesis, we made transformed lines carrying the pha-1: lacZ construct pH1-ΔlacZ. Six extrachromosomal transformed lines were isolated, including TG25. Two stable strains, TG25X1 and TG25X19 were derived from TG25 by gamma irradiation. All sets of strains exhibit β-galactosidase staining in the embryo.

Sequence analysis

The program motifs of the UWCWG software package was used to search the Pir, Swisprot and Patch.x databases, containing more than 120,000 entries with the following consensus of the pha-1 and bZIP proteins: (L,R)×2(N,R,K,N,T,x(V,A)×2(5,R,K,A))[-1,1000](M,I,T,L)x6L6x6L6x(L,N,C,L,R). This consensus is marked in Fig. 6 with (+). The consensus permits a spacer of up to 1000 amino acids between the basic region and the leucine zipper motifs. Only two of 100 hits were not bona fide bZIP proteins. The first was the fusion glycoprotein from human respiratory virus (Johnson and Collins, 1988). This protein contains a spacer of 64 amino acids between the ‘basic’ and the ‘leucine zipper’ motifs. The Robson Garnier algorithm, however, predicts only for two out of the 22 residues forming the putative leucine zipper an α-helical structure. The second protein was the mouse T-cell antigen receptor α-chain (TCR-ATF2) which showed a perfect match to the bZIP motifs. In this protein, the Robson Garnier algorithm only predicts for 10 out of the 22 residues an α-helical structure (Chung et al. unpublished 1993, reference EMBL: M77167).

Antibody staining of whole-mount embryos

Embryos were prepared and fixed as described by Goh and Bogaert (1991) and stained as described previously ( Schnabel and Schnabel, 1991). Fixed embryos were incubated overnight (4°C) with primary antibodies and then for 4 hours with fluorescently tagged secondary antibody at room temperature. All developmental times refer to time after the first cleavage at 20°C. Several antibodies were used in this study: 9.2.1 detects pharynx muscle myosin (Epstein, 1982); 3NB12, 21 of the 34 pharynx muscle precursor cells (Priess and Thomson, 1987); and α-IFA (Pres et al., 1981), the marginal cells in the pharynx. The antibody 48C7 (antibody collection MRC LMB, Cambridge) recognises the pharyngeal precursors, some neurons in the head and tail region and the ventral cord (Fig. 2). Embryos were fixed with the standard protocol employing methanol/acetic acid fixation. The HM-1 antiserum detects the nuclear myoD protein in body wall muscle cell precursors (Krause et al., 1990). For double staining experiments with embryos expressing β-galactosidase, a monoclonal
anti-β-galactosidase antibody (Promega) was directly labelled with FITC (Sigma) as described by Harlow and Lane (1988). The methods to stain and count body wall muscle cells are described in Schnabel (1994). Immunofluorescence microscopy was performed with a Zeiss Axiophot microscope equipped for epifluorescence. Photographs were taken on Kodak Technical Pan and developed in HC110, 1:10, for 6 minutes (400 ASA). The pictures for Fig. 2 were collected with a Hamamatsu SIT-camera using the Fluovision software of Improvision (Coventry, England).

Analysis of genetically mosaic pha-1 embryos

The strain TG22 was generated by microinjecting the 6.6 kb genomic fragment pC1 containing the entire pha-1 gene into the gonad syncytium of pha-1(e2123ts) animals (see above). Animals were raised at the non-permissive temperature (25 °C) of pha-1(e2123ts) and mixed stage embryos were isolated and fixed as described by Goh and Bogaert (1991). Fixed embryos were incubated overnight (4°C) with the antibodies indicated in Fig. 9 and then for 4 hours with fluorescently tagged secondary antibody at room temperature. Only embryos at terminal stages of embryonic development were scored for mosaic expression in the pharynx. As controls, 3841 terminal embryos from the wild-type strain N2, and 3809 embryos mutant for pha-1(e2123ts) were inspected with the antibody 9.2.1. The numbers for antibody αIFA were 1151 for N2 and 1680 for pha-1(e2123ts). No mosaic patterns corresponding to those described in the Results.

Fig. 2. The monoclonal antibody 48C7 stains pharyngeal cells and nerve cells in the embryo. (A) Embryo at about 150 minutes of development. Approximately 10 pharyngeal precursors stain in the anterior of the embryo. (B) At this time of development also four cells, which are very probably neuronal precursors, are stained in the posterior of the embryo. Shortly before hatching 8 cells stain in the tail of the larva (not shown). (C) Embryo at about 200 minutes of development. Approximately 27 cells stain in the pharynx primordium. The arrows point at faintly stained cells outside the pharynx primordium, which are very probably neuronal precursors. (D) Comma stage embryo (approx. 400 minutes). All pharynx cells are born. The arrows point at the cells outside the pharynx. (E,F) Pretzel stage embryo shortly before hatching. (E) The pharynx and cells in the nerve ring (arrows) are stained. (F) The arrows point to the ventral nerve cord. Bar, 10 µm.

Fig. 3. Cloning of the pha-1 gene. (A) Genetic map around the pha-1 gene on chromosome III. The left breakpoint of the deficiency eDf20 maps between vab-7 and pha-1 and was used as a starting point to clone the pha-1 gene. (B) Identification of the left eDf20 breakpoint by RFLP analysis. DNA from the indicated strains was electrophoretically separated on an agarose gel, transferred to a nitrocellulose membrane, and probed with the 32P-labelled pME6 subclone (see Methods). The probe detects an additional restriction fragment in the eDf20 DNA (arrow). Because eDf20 mutants cannot be propagated as a homozygous strain, DNA from balanced animals was analysed on this blot and therefore the wild-type fragments are detected in both lanes. (C) Identification of pha-1 by germline transformation. The cosmid clone spanning the left eDf20 breakpoint (MO2H11) and clones that mapped to the right were used for germline transformation experiments. Lines carrying the MO2H11 or CB#H8J35 DNA did not rescue the pha-1 phenotype. Injection of the lambda clone CB#H8J04 and the indicated subclones resulted in transformed lines that rescued the mutant pha-1 phenotype.
were found. The frequency for mosaics considering all embryos scored as mosaic was one in 240 for antibody 9.2.1 and one in 203 for αIFA. Therefore the probability that mosaics do not occur in the control populations is larger than 0.99.

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X73845.

RESULTS

Cloning of the pha-1 gene

To clone the pha-1 gene we have first aligned the genetic map to the physical map of C. elegans (Coulson et al., 1986, 1988) in the region of pha-1, and then identified the pha-1 gene by DNA-mediated germline transformation. pha-1 maps to the third chromosome between vab-7 and tra-1 (Schnabel and Schnabel, 1990). The deficiency eDf20 (Hodgkin, 1987) fails to complement pha-1 and tra-1, but does complement vab-7, indicating that the left breakpoint of the deficiency maps between vab-7 and pha-1 (Fig. 3A). We used cosmid clones from this region (Coulson et al., 1986, 1988) to identify the left eDf20 breakpoint through the analysis of restriction fragment length polymorphisms (RFLPs) between the eDf20 strain and the standard wild-type strain N2. One cosmid clone, MO2H11, revealed a polymorphism (data not shown). Using individual subclones from this cosmid, a 3 kb EcoRI fragment was identified that spanned the eDf20 deficiency breakpoint (Fig. 3B).

The M02H11 clone and clones that mapped to the right of this clone were injected into the syncytial gonad of temperature-sensitive eDf2(e2123ts) hermaphrodites. Larvae of the F2 progeny, positive for coinjected DNA encoding a visible marker, were shifted to the non-permissive temperature (25 °C) and screened for viable progeny. The result of the germline transformation experiment is shown in Fig. 3C. Only injection of the lambda clone CB#H8J04 resulted in F3 progeny viable at 25 °C, indicating that the pha-1 gene is contained within this lambda clone. An 8 kb BamHI/XhoI (pBX) and a smaller 6.6 kb ClaI/XhoI (pC1) genomic subclone from the CB#H8J04 clone also rescued the mutant pha-1 phenotype.

pha-1 encodes a protein that contains two motifs characteristic of the bZIP transcription factors

A single transcript of about 2.2 kb was detected on a northern blot of RNA from wild-type embryos using the 6.6 kb genomic pC1 fragment as a probe (Fig. 4). High levels of expression were detected around 3 to 5 hours of embryonic development, consistent with the genetic requirement of pha-1 at this time (Schnabel and Schnabel, 1990). To isolate pha-1 cDNA clones we constructed a cDNA library using mRNA from mixed stage embryos and screened the library with the 6.6 kb pC1 fragment as a probe (Fig. 4). High levels of expression were detected during the larval stages using the 6.6 kb genomic pC1 fragment as a probe on northern blots. Low levels of pha-1 transcript are detectable during the early stages of embryonic development (0-2 hours). At 3-5 hours of development pha-1 expression is increased, consistent with the genetic requirement of pha-1 during the larval stages was observed. Below: 28S rRNA stained with methylene blue as a control for loading equal amounts of RNA.

Fig. 3B. Hind III and Ssp I digestion of lambda cosmids. The rescuing 6.6 kb genomic fragment contains the entire pha-1 gene. The rescuing 6.6 kb fragment pC1 extends from the ClaI site to the Sau3AI site and does not include the 442 bp from the 3′ untranslated region of the pha-1 transcript (see Methods). This portion of the gene appears to be dispensable to rescue the mutant pha-1 phenotype. The primary transcript spans about 5 kb of genomic sequence. The boundaries of the 5 exons were deduced from DNA sequencing analysis of the 2.2 kb full-length cDNA, pm2, and the rescuing 6.6 kb genomic fragment. The first six nucleotides at the 5′ end of the 2.2 kb pm2 cDNA are homologous to the 22 bp SL1 trans-spliced leader RNA. This 22 bp sequence has been found at the 5′ end of about 15% of C. elegans messages (Krause and Hirsh, 1986; Bektesh et al., 1988). The pm2 cDNA extends 5′ exactly to a putative trans-spliced acceptor site present in the genomic sequence (data not shown). At the 3′ end of the pm2 cDNA clone, multiple potential polyadenylation signals and a poly(A)′ tail are present (see Fig. 6).

Fig. 4. Northern analysis of the pha-1 transcript. A single transcript is detected at different stages of development using the 6.6 kb genomic fragment that rescues the pha-1 phenotype as a probe on northern blots. Low level of pha-1 transcript is detectable during the early stages of embryonic development (0-2 hours). At 3-5 hours of development pha-1 expression is increased, consistent with the temperature-sensitive period of pha-1 (e2123ts) (Schnabel and Schnabel, 1990). The mRNA is also expressed at lower levels in larval stages. No genetic requirement of pha-1 during the larval stages was observed. Below: 28S rRNA stained with methylene blue as a control for loading equal amounts of RNA.

Fig. 5. Genomic structure of the pha-1 gene. The structure of the pha-1 gene as revealed by comparing genomic and cDNA sequences. A 7 kb Clal/SspI genomic fragment contains the entire pha-1 gene. The rescuing 6.6 kb fragment pC1 extends from the ClaI site to the Sau3AI site and does not include the 442 bp from the 3′ untranslated region of the pha-1 transcript (see Methods). This portion of the gene appears to be dispensable to rescue the mutant pha-1 phenotype. The primary transcript spans about 5 kb of genomic sequence. The boundaries of the 5 exons were deduced from DNA sequencing analysis of the 2.2 kb full-length cDNA, pm2, and the rescuing 6.6 kb genomic fragment. The first six nucleotides at the 5′ end of the 2.2 kb pm2 cDNA are homologous to the 22 bp SL1 trans-spliced leader RNA. This 22 bp sequence has been found at the 5′ end of about 15% of C. elegans messages (Krause and Hirsh, 1986; Bektesh et al., 1988). The pm2 cDNA extends 5′ exactly to a putative trans-spliced acceptor site present in the genomic sequence (data not shown). At the 3′ end of the pm2 cDNA clone, multiple potential polyadenylation signals and a poly(A)′ tail are present (see Fig. 6).
Fig. 5). The sequences of the 6.6 kb genomic subclone and the full-length 2.2 kb cDNA predict a transcript that includes 5 exons (Fig. 5). The single large open reading frame translates conceptually into a protein of 491 amino acids with a predicted molecular weight of 57 kd.

To confirm that the isolated cDNAs correspond to the genetically defined pha-1 locus, we cloned and sequenced the strong temperature-sensitive allele e2123ts. The open reading frame contains a single point mutation (G to A transition) at position 537 of the DNA sequence, changing a cysteine to a tyrosine residue (Fig. 6A). This confirms the identity of the pha-1 gene.

The pha-1 cDNA sequence and the amino acid sequence of the encoded protein are shown in Fig. 6A. The most striking feature of the pha-1 protein sequence is a putative leucine zipper motif and a region with significant similarity to the basic region characteristic of the bZIP family of transcription factors (for review see Pathak and Sigler, 1992). This class includes the proto-oncogenes c-jun (Sakai et al., 1989) and c-fos (Curran et al., 1987), and the yeast transcription factor GCN4 (Vogt et al., 1987). The basic region has also been found in putative transcription factors, which in contrast to the bZIP proteins does not contain the leucine zipper, for example the skn-1 gene product of C. elegans (Bowerman et al., 1993) and the zeste (Mansukhani et al., 1988) and mastermind (Smoller et al., 1990) gene products of D. melanogaster.

The amino acid sequence of the basic region and the putative leucine zipper motif of pha-1 are aligned for comparison with sequences from bZIP transcription factors, which in contrast to the bZIP proteins do not contain the leucine zipper, for example the skn-1 gene product of C. elegans (Bowerman et al., 1993) and the zeste (Mansukhani et al., 1988) and mastermind (Smoller et al., 1990) gene products of D. melanogaster.

The basic region of bZIP proteins contains a single point mutation (G to A transition) at position 537 of the DNA sequence, changing a cysteine to a tyrosine residue (Fig. 6A). This striking feature of the pha-1 protein is conserved in itself. Recently the crystal structure of the entire bZIP element of the GCN4 protein bound to DNA was determined (Ellenberger et al., 1992). A relatively small number of specific contacts, organised by a simple structural motif, is sufficient for sequence-specific DNA binding by the GCN4 protein.
Three of the five residues that make these specific contacts to the DNA are conserved or similar in the basic region of the pha-1 protein. The presence of two leucine zipper domains characteristic for bZIP transcription factors separated by 106 amino acids. In a related class of transcription factors, the basic helix-loop-helix leucine zipper (bHLH-ZIP), the basic region and the leucine zipper domain are adjacent to the basic region, these two domains in the pha-1 protein do not contain any known structural motif. The pha-1 protein might encode a transcriptional regulator with a novel protein structure.

The pha-1 gene is expressed transiently in all pharyngeal precursor cells of the embryo

To determine the spatial and temporal expression pattern of pha-1 in the embryo we ‘tagged’ the pha-1 protein with the E. coli gene encoding β-galactosidase (lacZ) by constructing a hybrid gene (see Methods). The expression pattern of the hybrid gene was analysed in two strains where the pha-1:lacZ gene was integrated into the C. elegans genome and in strains that carried the hybrid DNA as extrachromosomal arrays (see Methods). Since the hybrid construct contains the pha-1 promoter region that is sufficient to rescue the mutant pha-1 phenotype, the resulting expression pattern of the lacZ reporter very likely reflects the endogenous pha-1 expression pattern. The expression of the reporter gene was analysed by immunofluorescence staining with anti-β-galactosidase antibodies in conjunction with pharynx-precursor-specific or body wall muscle specific antibodies to allow the identification of the cells stained in the embryo. The first expression of the pha-1:lacZ hybrid gene is detectable at the 100 cell stage of embryogenesis (130 minutes of embryonic development; all developmental times refer to time after the first cleavage at 20°C) in cells that have positions in the embryo corresponding to the pharyngeal and body wall muscle precursors (Fig. 7A,B). Expression of the pha-1:lacZ hybrid gene at around 200 minutes of development is detected in 25±2 cells (± s.d., n = 10) that form a cylinder in the anterior part of the embryo (Fig. 6).
Fig. 7. Expression of a pha-1::lacZ hybrid protein during embryogenesis. (A-J) Immunofluorescence of pha-1::lacZ expressing embryos of different stages double stained with anti-β-galactosidase (left column) and cell type-specific antibodies (right column). All embryos are arranged with the anterior to the left. (A) Expression of the pha-1::lacZ hybrid protein is first detectable at about 130 minutes of embryonic development in some cells that have positions similar to descendants of the pharynx producing blastomere MS and AB (arrowheads) and all body wall muscle cell precursors at the posterior pole (arrows). (B) Staining of the same embryo with the myoD-specific antiserum HM-1. The arrows point towards cells that express the pha-1::lacZ hybrid and the myoD protein, indicating that these cells are the body wall muscle cell precursors. (C) Around 200 minutes of embryonic development the pha-1::lacZ protein is expressed in pharyngeal precursors at the anterior pole of the embryo and in body wall muscle cell precursors that form a U-shaped structure in the posterior half of the embryo. The β-galactosidase-positive cells at the anterior pole of the embryo have positions similar to the pharyngeal precursors present at that time of development. (D) Positions of the AB- or MS-derived pharyngeal precursors that will contribute to pharyngeal cells (open circles) and C- and D-derived myoblasts (filled circles; adopted from Sulston, 1983). At this time of development 26 pharyngeal precursors are present in the embryo that will contribute to 77 of the 80 embryonic pharyngeal cells (Fig. 8). (E,F) Embryo of the same stage as in C, stained with a monoclonal antibody against β-galactosidase (E) and with the 48C7 antibody that stains pharyngeal precursors (F). (G,H) Embryo at about 400 minutes of embryonic development stained with a monoclonal antibody against β-galactosidase (G) and with the HM-1 antibody that recognises body wall muscle cell precursors. The pha-1::lacZ hybrid protein (G) is still expressed in the body wall muscle cell precursors. (H) Few cells in the presumptive pharynx region express the hybrid protein (not shown). (I,J) Embryo at 450 minutes, shortly after morphogenesis has started, stained with a monoclonal antibody against β-galactosidase (I) and with the 3NB12 antibody that stains pharyngeal precursors (J). No expression of the pha-1 hybrid protein is detectable in the pharyngeal cells. (K-N) Analysis of body wall muscle differentiation in pha-1 mutants. Embryo mutant for the allele e2123ts raised at the nonpermissive temperature. (K) Normarski micrograph of the embryo. The arrows point at the undifferentiated pharynx primordium. (L) Immunofluorescence micrograph of the embryo. The body wall muscle is differentiated normally in the embryo (mAb NE8 4C6.3; see Schnabel (1994) for a description of the antibody). (M-N) Two focal layers of an embryo that is homozygous for a small deficiency Df(2L)8 deleting the pha-1 gene (Schnabel and Schnabel, 1990). The staining with the mAb NE8 4C6.3 shows that the body wall muscle has differentiated normally in the absence of pha-1 activity. Bar, 10 µm.
These *pha-1*:lacZ expressing cells at the anterior pole of the embryo also stained with the 48C7 antibody, which is specific to the pharyngeal precursors (Fig. 7E,F). The position and the immunohistochemical double staining of the *pha-1*:lacZ expressing cells at the anterior pole of the embryo show that these cells are the pharyngeal precursors. At this developmental stage the 27 AB- or MS-derived precursors of the pharynx form a central cylinder in front of the gut precursors in the anterior half of the embryo (Sulston, 1983; Fig. 7D). The lineage of most of these cells (26 of 27) is restricted to pharyngeal fate and only the MSaaa cell produces pharyngeal cells and non-pharyngeal cells (two neurons of the ring ganglion, Fig. 8).

As soon as these 27 pharynx precursor cells divide (230-250 minutes), expression of the *pha-1*:lacZ gene in the pharyngeal precursors ceases and only a few cells in the presumptive pharynx region express the hybrid protein. At 420 minutes of development (2 fold stage) no *pha-1*:lacZ gene expression in the pharyngeal precursor cells is detectable (Fig. 7I,J). These results indicate that the *pha-1* hybrid gene is expressed for a short time period in all pharyngeal precursor cells. This temporal expression pattern of the hybrid gene in the pharyngeal precursors corresponds to the genetic requirement of *pha-1* at this time of embryonic development as determined by temperature shift experiments (Schnabel and Schnabel, 1990).

We also observed *pha-1*:lacZ expression in body wall muscle cell precursors (Fig. 7). Expression of the *pha-1* hybrid protein starts early in all body wall muscle cell precursors and is detectable throughout embryonic development in these cells. (Fig. 7). However, an immunochemical analysis (Fig. 7K-N) shows that body wall muscle differentiates in *pha-1* embryos homozygous for the strong allele e2123ts or homozygous for the small deficiency tDf2 deleting the gene (Schnabel and Schnabel, 1990). Counting of the body wall muscle cells in embryos mutant for e2123ts revealed that the embryos contain the normal number of 81 body wall muscle cells (80±2; ± s.d., n=10). We take this as a strong indication that *pha-1* expression is not essential in body wall muscle precursor cells as far as we can tell by our analyses.

**Mosaic analysis of *pha-1***

Since *pha-1* is expressed in all pharyngeal precursors and has similarities to the bZIP transcription factors it seems likely that *pha-1* functions in a cell-autonomous manner. We analysed pharyngeal development in genetically mosaic *pha-1* embryos to test this directly and to determine if the gene is required in all cells of the pharynx primordium. To generate genetic mosaics we used a strain (e2123ts) that carries extrachromosomal arrays of the 6.6 kb *pha-1* genomic fragment, pC1 (e2123ts; pC1, called TG 22). In *C. elegans* these arrays are lost spontaneously in the soma like free extrachromosomal duplications (Herman, 1984; Stinchcomb et al., 1985). Loss of the array carrying the wild-type copy of the *pha-1* gene in a *pha-1* background results in mutant *pha-1* cells. However, there is no visible marker available to score embryonic cells for the presence of the extrachromosomal array complementing the *pha-1* mutation. Therefore the analysis depends on the reasonable assumption that partially undifferentiated pharynges in embryos from this strain are due to the somatic loss of the extrachromosomal array.

The knowledge of embryonic lineage allows one to analyse the mosaic animals in detail, as described below. Several different patterns of differentiation in the pharynx can be expected in mosaic embryos depending on the manner in which *pha-1* functions. A non-autonomous requirement for *pha-1* activity in one inductive centre would result in the absence of mosaically differentiated embryos. Loss of the construct from cells outside the centre would not result in a pharyngeal defect; loss from cells in the centre would result in a phenotype indistinguishable from the complete loss of the array in the germ line of the parent, which occurs in approximately 50% of all animals. If more than one inductive centre is required, embryos would display mosaic patches not following the lineage boundaries. In contrast, an autonomous requirement for the gene activity would be indicated if the mosaic patterns respect the lineage boundaries, and that is what we indeed observed.

The pharynx is derived from two somatic founder cells, AB and MS (Fig. 8; the pharyngeal lineage is described in Sulston et al., 1983; the anatomy of the pharynx in Albertson and
Thomson, 1976). If the loss of the extrachromosomal array occurred early in development large parts of the pharynx would be undifferentiated. Single undifferentiated cells in an otherwise fully differentiated pharynx or single differentiated cells in a completely undifferentiated pharynx are expected to be found if pha-1 acts cell-autonomously but are not expected to be found if the gene acts non-autonomously.

Two monoclonal antibodies indicating terminal differentiation of pharyngeal cells were used in the mosaic analysis. Monoclonal antibody 9.2.1 specifically stains the pharynx muscle myosin (Epstein, 1982). The differentiated pharynx contains 20 muscle cells formed by 37 nuclei. Only six of these cells are mononucleate. This antibody is especially suited to identify large patches of muscles missing in a mosaic embryo. The second antibody, αIFA (Pruss et al., 1981), stains the seven marginal cells in the pharynx, six of which are mononucleate. Three cells each are in the anterior (3× mc1) and in the middle region (3× mc2) of the pharynx. Only the most posterior marginal cell spanning the posterior bulb is a syncytium containing three nuclei (3× mc3). This antibody is especially suited to score the loss of single marginal cells.

The antibody 9.2.1 was used to screen 7681 terminally differentiated embryos from the strain TG22 for mosaic patterns. Sixteen embryos were scored to be mosaic, which corresponds to a frequency of one mosaic embryo in 460. In nine embryos one, two or three nuclei were missing in the staining pattern. It was, however, not possible to identify the missing nuclei with certainty since the pharynges were rearranged (not shown). In seven embryos large parts of the pharynx did not express the pharynx myosin. In two animals each, either the complete AB or MS contribution to the muscle appeared undifferentiated. In Fig. 9B,C an embryo is shown where the MS contribution in the posterior half of the pharynx is differentiated, whereas the anterior half corresponding to the AB derived contribution is undifferentiated. In three animals the posterior half of the pharynx corresponding to the MS derived contribution contained the full equivalent of 18 nuclei surrounded with antibody 9.2.1 stained cytoplasm. The anterior half of these pharynges contained only four cells with stained cytoplasm indicating that part of the AB lineage was undifferentiated (Fig. 9E, F). The AB lineage produces 19 pharyngeal muscle nuclei, five derived from ABalp and 14 derived from ABara. The staining pattern of these pharynges is consistent with the pattern expected if the extrachromosomal array was lost in the ABara lineage, although one cell of the five expected could not be detected, which could be due to a partial syncytium formation. In the example shown in Fig. 9E,F one of these four stained cells resides in a completely undifferentiated environment, which is consistent with the notion that pha-1 acts completely autonomously in this cell. In another screen involving a large number of animals, which was especially carried out to detect animals with easily scorable phenotypes, two more animals were found with one of the six mononucleated cells missing (Fig. 9D). Thus, single muscle cells may be undifferentiated in an otherwise differentiated environment.

The antibody αIFA was used to screen 1420 terminally differentiated embryos. In two embryos only two cells in the posterior pharynx were stained corresponding to a loss of the array in the AB lineage. Two of the 9 marginal cell nuclei are derived from MS. In 4 mosaic embryos one of the 6 mononucleated cells was missing. In two embryos one of the mc2 cells was missing, which corresponds to a loss of the array in either of the lineages ABarapa, ABarapp or ABarapp (Fig. 9K,L). In one embryo one mc1 cell was missing, which corresponds to a loss of the array in either the ABarapa or ABaralp lineages. In one embryo one mc1 cell and one mc3 nucleus in the posterior pharynx were missing. This pattern corresponds to a loss of the array in the lineage ABaralp. The frequency of mosaic staining patterns was one in 203 embryos.

As a control, a large number of wild-type N2 embryos (n=3841 for antibody 9.2.1, n=1151 for antibody αIFA) and mutant pha-1 (e2123ts) embryos (n=3809 for antibody 9.2.1, n=1680 for antibody αIFA) were screened for similar staining patterns. No embryos with mosaic staining patterns were found in N2 embryos and none like those described above were found in pha-1 embryos (all P>0.99). However, in 3% of the terminal pha-1 (e2123ts) embryos one cell or a few isolated cells scattered throughout the whole pharynx stained with the antibody 9.2.1 (Fig. 9G,H). With the antibody αIFA, 0.3% of the pha-1 mutant embryos contained one or three cells that expressed the marker.

The exact knowledge of the pharyngeal lineage (Sulston et al., 1983) allows prediction of the expected mosaic patterns if an autonomously acting gene activity is lost in different parts of the pharyngeal lineage at different developmental stages. The observed mosaic patterns match the patterns predicted by the lineage and are thus consistent with a cell-autonomous requirement for pha-1 activity to initiate terminal differentiation and morphogenesis in the pharynx. The notion that pha-1 acts cell-autonomously is also supported by the fact that isolated pharyngeal cells surrounded by an undifferentiated environment can undergo terminal differentiation and vice versa.

DISCUSSION

Mutations in the zygotic lethal gene pha-1 block pharynx development in an organ-specific manner. In mutant pha-1 embryos the pharynx fails to undergo morphogenesis and the different pharyngeal cell types, although committed to their fates, do not undergo terminal differentiation (Schnabel and Schnabel, 1990 and Fig. 1). The entire pha-1 gene is contained within a 6.6 kb genomic fragment sufficient to rescue pha-1 mutants phenotypically.

The pha-1 gene may encode a transcriptional regulator

The similarity of the predicted pha-1 protein sequence to the basic region of bZIP transcription factors (Fig. 6) suggests that the pha-1 gene product encodes a transcriptional regulator. The protein sequence further contains a putative leucine zipper motif. In contrast to the bZIP proteins, these two domains are not adjacent to each other in the pha-1 protein, but are separated by a ‘spacer’ of 106 amino acids, which does not contain any known structural motifs. Leucine-zipper-containing transcription factors belong to a growing family of proteins with different combinations of DNA binding and dimerisation elements (Pathak and Sigler, 1992). In most of these proteins the functional subdomains are adjacent to each other. However, some members have variation in the spacing between the individual domains (Pathak and Sigler, 1992; Williams and Tjian,
Fig. 9. Extrachromosomal pha-1 DNA causes terminal differentiation of pharyngeal cells in a mosaic like manner in pha-1− embryos. Strains carrying extrachromosomal arrays of microinjected DNA can be used to generate genetically mosaic embryos, since the arrays are lost spontaneously during mitotic divisions and thus homozygously mutant cells are created in the embryo. Wild-type (A,I,J), mosaic pha-1 embryos (B-F,K,L) derived from the strain TG22(e2123ts;pC1) and e2123ts (G,H) embryos were stained with two antibodies that recognise markers characteristic of the terminal differentiation of two pharyngeal cell types. (A-H) Embryos stained with the pharynx muscle myosin specific antibody 9.2.1 (Epstein et al., 1982). (A) Wild-type embryo. (B-F) Mosaic pha-1 embryos derived from the strain TG22(e2123ts;pC1) stained with 9.2.1 antibody. (B) Mosaic embryo where only the MS derived pharyngeal cells express the terminal marker. (C) The same embryo shown in a double exposure with Nomarski and fluorescence optics. An undifferentiated group of pharynx cells, the AB derived contribution (small arrows) is located in front of the MS derived contribution to the pharynx, which expresses pharyngeal myosin (large arrow). (D) Mosaic embryo with a fully differentiated pharynx. One of the three cells in the posterior bulb normally staining very brightly is not expressing the marker. Thus a single cell is not terminally differentiated in a differentiated environment. (E,F) Two focal planes of a mosaic pharynx. At the tip of the pharynx three cells are staining (small arrow). About midway through the pharynx an isolated cell is expressing the marker (arrowhead). Thus isolated or very small groups of cells are able to express the marker. MS-derived muscle cells are expressing the marker. This mosaic pattern is consistent with a loss of the extrachromosomal array in ABara. (G,H) Two examples of the rare e2123ts embryos, which expressed the marker (see Results). In one embryo a single cell expressed the marker (G). In the other embryo, 9 cells scattered through the whole pharynx were expressing the marker (H). The expression of the terminal marker may be the result of a spurious pha-1 activity in pharyngeal precursors. These stainings are also an indication that the gene acts cell-autonomously. (I,J) Two focal planes of a wild-type embryo at the end of embryogenesis stained with the monoclonal antibody αIFA (Pruss et al., 1981) recognising intermediate filaments in the marginal cells of the pharynx. (K,L) Two focal planes of a mosaic pha-1 embryo derived from the strain TG22(e2123ts;pC1) stained with αIFA. Two of the three rows of marginal cells are complete in this embryo (small arrows). The third row is incomplete (large arrow), there is a gap between the anterior and the posterior bulb of the pharynx. This pattern is consistent with the lack of terminal differentiation in one of the three marginal cells called mc2. Bar, 10 µm.
Molecular analysis of the yeast GCN4 protein indicates that altering the highly conserved spacing between the basic domain and the leucine zipper domain by insertion of integral numbers of \( \alpha \)-helical turns does not affect GCN4 function (Pu and Struhl, 1991). Recently, a mammalian tissue-specific transcription factor, Olf-1, was isolated, which has a dimerisation domain located more than 350 amino acids apart from the DNA binding motif (Wang and Reed, 1993). It is possible that pha-1, like Olf-1, encodes a transcription factor with an unusual spacing between the DNA binding and dimerisation domain.

**pha-1 is expressed in the pharyngeal precursor cells**

All pharyngeal precursor cells are affected by mutations in the pha-1 gene and fail to undergo morphogenesis and terminal differentiation, independent of the lineage the precursors originate from (Fig. 8). Correspondingly, the *pha-1: lacZ* hybrid gene is expressed in all organ precursor cells present at 200 minutes of embryonic development and *pha-1* expression is not restricted to cells of a specific pharyngeal cell-type or an individual lineage. This indicates that the organ-specific function of *pha-1* is reflected by the *pha-1: lacZ* expression pattern.

Although the *pha-1: lacZ* protein is expressed in the body wall muscle precursor cells throughout development, no genetic requirement for the initiation of the terminal differentiation of body wall muscle cells is observed in mutant *pha-1* embryos (Fig. 7; Schnabel and Schnabel, 1990). Even in the complete absence of *pha-1* activity embryos exhibit the typical body wall muscle twitching at the end of embryogenesis and show a normal number of body wall muscle cells stained with a muscle-specific antibody. We cannot exclude the possibility that the *pha-1* promoter used in the *pha-1: lacZ* construct lacks a muscle cell precursor specific silencer element. Alternatively, it is possible that *pha-1* is expressed normally but that its function is not essential in body wall muscle precursor cells. It is also not excluded that the reporter construct fails to show the expression in other cells of the embryo where the gene is normally expressed, however, the *pha-1* mutant phenotype suggests that the expression would not be essential in any other structure outside the pharynx as far as we can tell from our analyses. In summary the gene affects diverse tissues but only within the functional unit of an organ. The gene appears to be expressed in those embryonic cells that are just restricted to form the pharynx. It appears that in this case not tissues but the organ is the unit of the regulation for the differentiation of structures in the embryo.

**Does pha-1 initiate terminal differentiation and morphogenesis of the pharynx in a cell-autonomous fashion?**

The observation that *pha-1* is expressed in the pharyngeal precursor cells suggests that it acts cell-autonomously in these cells. However, regulatory gene products have been observed that are expressed ubiquitously but act cell-autonomously only in a subset of cells. For example, the *sevenless* gene encodes a receptor tyrosine kinase that is expressed on the cell surface of many precursor cells in the developing retina of the *Drosophila* eye (Hafen et al., 1987; Tomlinson et al., 1987), but is only required in the R7 precursor cell for proper R7 development (Tomlinson and Ready, 1987). To address the question of whether *pha-1* activity is required cell-autonomously in the pharyngeal precursor cells, we analysed pharynx development in genetically mosaic *pha-1* embryos. Although we did not use a genetic marker linked to the *pha-1* gene to analyse mosaic *pha-1* embryos the observed mosaic staining patterns analysed with two markers for terminal differentiation are consistent with the notion that *pha-1* acts cell-autonomously in the pharyngeal precursor cells.

**Potential role of pha-1 in organ formation**

The genetic requirement of *pha-1* for pharynx development shows that a single gene function is required to initiate organ differentiation and morphogenesis. The temporal expression pattern of the *pha-1* hybrid gene in the embryo and the temperature-sensitive period of *pha-1* (Schnabel and Schnabel, 1990) indicate that *pha-1* functions at a early stage of embryonic development. *pha-1* expression ceases prior to the first indication of pharyngeal cell-type specification. However, specification of at least the pharyngeal muscles is independent of *pha-1* (see Introduction). This suggests that terminal differentiation and morphogenesis of an organ is independent of, and occurs parallel to, the initial cell type specification. We therefore propose that the *pha-1* gene product initiates or acts early in a regulatory pathway that controls morphogenesis and differentiation of the pharynx. Both processes appear to be coordinated and both are affected by *pha-1*. The *pha-1* gene product may act as a transcriptional regulator in the pharyngeal precursor cells to turn on programs required in all cell types of the organ to undergo morphogenesis and terminal differentiation.

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