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Segmental expression of an *engrailed*-class gene during early development and neurogenesis in an annelid

CATHY J. WEDEEN and DAVID A. WEISBLAT

Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA

Summary

***ht-en* protein, an annelid homolog of the *Drosophila engrailed* protein, is expressed during both early development and neurogenesis in embryos of the leech, *Helobdella triserialis*. In *Helobdella* as in *Drosophila*, early expression is in segmentally iterated stripes of cells within the posterior portion of the segment and later expression is in cells of the segmental ganglia. These findings suggest that dual expression of an *en*-class gene was present in a common ancestor of annelids and arthropods.**

Key words: *engrailed*, segmentation, leech, annelid, *Helobdella triserialis*, homeobox.

Introduction

Although the segmental body plans of such protostomes as annelids, arthropods and onychophorans are similar, there are wide differences in the cellular mechanisms by which segments form in embryos of these phyla (Anderson, 1973) and some authors have proposed that segmentation arose independently among them (Barnes *et al.* 1988). The process of segmentation has been analyzed extensively in arthropods, particularly by applying genetic and molecular techniques to *Drosophila melanogaster*. In this insect, regulatory networks of genes establish proper segment number, polarity and identity during early development. Many of these genes encode proteins containing homeodomains, highly conserved (approximately 60 aa) DNA-binding domains necessary for regulating gene transcription (Akam, 1987; Ingham, 1988 for reviews). Some of these genes exhibit what we shall refer to here as 'dual expression', meaning that, in addition to being expressed during early development, they are also expressed later, in overlapping subsets of cells within the nervous system (see Akam, 1987; Doe and Scott, 1988 for reviews).

One such homeodomain-containing protein that shows dual expression in *Drosophila* is encoded by the *engrailed* (*en*) gene (Fjose *et al.* 1985; Poole *et al.* 1985). In early development, *en* expression is detected in segmentally iterated circumferential stripes of cells where it is required to specify the identity of the posterior 'compartment' of each segment (Garcia-Bellido *et al.* 1973; Garcia-Bellido, 1975; Kornberg, 1981*a*,*b*; Lawrence and Struhl, 1982; Fjose *et al.* 1985; Poole *et al.* 1985; Kornberg *et al.* 1985; DiNardo *et al.* 1985; Lawrence and Morata, 1986; Brower, 1986). Later, during neurogenesis, *en* is expressed in a subset of segmentally iterated neurons (Brower, 1986; Patel *et al.* 1989*a*).

Homologs of *en* have been characterized in various arthropod, echinoderm and chordate species (Joyner and Martin, 1987; Dolecki and Humphries, 1988; Gardner *et al.* 1988; Fjose *et al.* 1988; Patel *et al.* 1989*b*; Wedeen *et al.* 1991; Hemmati-Brivanlou *et al.* 1991). While expression of *en* during neurogenesis has been reported in all three of these phyla, the earlier embryonic expression of *en* during segmentation has been observed only in arthropods. Therefore, it has been proposed that the ancestral role for *en* was in neural specification and that only following the phylogenetic separation of the arthropods did it acquire a role in segmentation (Patel *et al.* 1989*a*).

To test this proposal, further study of *en* homologs in segmented protostomes other than arthropods is required. We have chosen to examine the expression of a homolog of *en* (*ht-en*) in embryos of *Helobdella triserialis*, a glossiphoniid leech, because embryogenesis has been described in some detail at the cellular level in this annelid (Weisblat *et al.* 1989; Shankland, 1991, for reviews). Here we present evidence showing that *ht-en* is expressed both in segmentally iterated stripes during early development and in a segmentally iterated subset of neurons during neurogenesis. We conclude from these data that, contrary to prior proposals, a dual expression pattern for *en* class genes was present in a common ancestor of annelids and arthropods. It thus seems likely that this common ancestor of annleids and arthropods was already segmented or at least had some serially iterated components in its body plan.

Materials and methods

Animals

Helobdella triserialis embryos were obtained from a laboratory breeding colony, periodically supplemented with leeches collected from lakes within Golden Gate Park, San Francisco. The colony was maintained and embryos were reared as described by Blair and Weisblat (1984) and Wedeen *et al.* (1990). Embryos were staged by the conventions of Fernandez (1980), as modified by Bissen and Weisblat (1989).

Recombinant clones

The subclones, pfhten1x and pfhten4x (fusions of one or four tandem copies, respectively, of the *ht-en* homeobox region with the *E. coli lacZ* gene) and phten4x–trpE (a fusion of four tandem copies of the *ht-en* homeobox region with the *E. coli* anthranilate synthase gene) were generated in the following way. First, a construct, phten1x0.24Sp+(KS), was made by cloning a 242 bp fragment (including the homeobox and the flanking 5 bp 5′ and 57 bp 3′) amplified from *ht-en* by polymerase chain reaction (PCR), into the Bluescript plus (KS) vector *via* the blunt ends of the *Spe*I site of the vector that had been 3′-extended by Klenow polymerase. Using this subclone, two tandem repeats of the fragment were constructed in frame by digesting one aliquot of the plasmid with *Xba*I and the other aliquot with *Spe*I. (The *Xba*I and *Spe*I sites are present in the polylinker flanking the insert and are not present in the insert.) The two aliquots were then ligated together. This process was repeated on the resulting subclone to generate four in-frame copies of the *ht-en* homeobox region. Each copy coded for the following amino acid sequence from *ht-en*: - DEKRPRTAFTGDQLARLKREFSENKYLTEQRRTCLAKELNLNESQIKIWFQNKRAKMKKASGVKNQLALQLMAQGLYNHS (Wedeen *et al.* 1991), preceded by 4 additional amino acids, LELE, due to the contribution of the polylinker sequences from the Bluescript plasmid. The four tandem repeats were then removed from the Bluescript vector by digestion with *Xba*I and *Hin*dIII and ligated either into the *Xba*I and *Hin*dIII sites of pUR278 (Ruther and Muller-Hill, 1983) to generate a fusion with the *lacZ* gene (pfhten4x) or into the *Xba*I and *Hin*dIII sites of pATH10 (Koerner *et al.* 1990) to generate a fusion with the bacterial anthranilate synthase gene (phten4x–trpE). The *lacZ* fusion construct, pfen1x was obtained by subcloning the *Xba*I–*Hin*dIII fragment from phten1×0.24Sp+(KS) into the *Xba*I and *Hin*dIII sites of the pUR278 vector (Ruther and Muller-Hill, 1983).

Fusion proteins

Betagalactosidase fusions

LacZ fusion constructs were transformed into strain, XL1 (Stratagene). They were grown as 500 ml cultures at 220 revs min^{-1} at 37 °C to an O.D. 660 of 0.6, at which time, isopropyl β, D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. They were grown for an additional 2 h and then harvested.

Fusion proteins were purified as follows. The 500 ml culture was resuspended in 20 ml of 50 mM Tris–HCl, pH 7.5, 50 mM Tris Base, 25 mM EDTA, pH 8.0, 20 % sucrose, 1 mg ml^{-1} lysozyme, and incubated on ice for 1 h. 20 ml of 1 % Triton X-100 was added rapidly and the cells were frozen at −70 °C. Upon thawing, cells were sonicated to reduce the viscosity and insoluble proteins were pelleted at 4 °C in an SS34 rotor at 10 000 revs min^{-1} for 20 min. The pellet was resuspended by douncing in 6 ml 100 mM Tris–HCl 7.5, 0.1 % Triton X-100. The insoluble fraction was again pelleted and resuspended in 6 ml 100 mM Tris–HCl 7.5, 4 M Urea. The process was repeated, resuspending the pellet in 50 mM Tris–HCl, pH 7.5, 8 M Urea. This final fraction was found to be highly enriched for the *lacZ* fusion protein.

Anthranilate synthase *fusions*

The trpE fusion construct was grown as recommended by Koerner *et al.* (1990). The trpE fusion protein generated from the phten4x–trpE construct was soluble in 50 mM Tris–HCl, pH 7.5, 50 mM Tris Base, 25 mM EDTA, pH 8.0. 20,% sucrose, 1 mg ml^{-1} lysozyme and all subsequent solutions. Therefore, the protein was purified by electroelution from SDS–polyacrylamide gels.

Antibodies

A dose of 0.5 mg of fusion protein generated from the pfhten4x construct was injected into a rabbit. After 2 weeks a booster injection of 0.25 mg was given. On a schedule of every two weeks the rabbit was alternately bled or boosted, until 4 bleeds were obtained.

Affinity purification of antibodies

A fusion protein containing 4 copies of the *ht-en* sequence fused in frame to one copy of the *E. coli* anthranilate synthase gene (phten4x–trpE) was used to affinity purify antibodies specific for the *ht-en* homeodomain region.

The en4x–trpE protein was purified by electroelution from an acrylamide gel slice and was coupled to an affigel-10 column (BioRad). Antibodies were bound to the column in PBS. Nonspecific antibodies were washed from the column with 10 mM Tris, pH 7.5 and specific antibodies were eluted in 100 mM glycine, pH 4.0. The partially purified antiserum was then dialyzed against PBS overnight at 4 °C. Anti-βgal antibodies were further removed by passing the antisera over an affinity column which had coupled to it the βgal protein obtained from inducing the pUR278 vector and purified in the manner described above for βgal fusions. The IgG component of the purified antisera was concentrated to [30 μg ml^{-1}] by binding to and eluting it from a protein A column (BioRad).

Antibody competition with bacterial proteins

A saturated culture of *E. coli*, strain XL-1, was harvested by centrifugation, resuspended in 1/10 original volume with water and lysed by sonication at 0 °C. An aliquot of lysate was boiled 3 min and combined with an equal volume of non-heat treated lysate. An aliquot of this mixture was agitated for 48 h at 4 °C with 1/5 volume of affinity purified *αht-en*. Solid debris was precipitated by centrifugation and the supernatant was used at the appropriate dilution on western blots and embryos.

Western blot analysis

Westerns were blocked overnight at 4 °C in western blocking buffer (1 % bovine serum albumin (Sigma no. A-7906), 2 % nonfat dry milk (Carnation), 1 mM $MgCl_2$, 25 mM Tris (pH 8), 137 mM NaCl, 2.7 mM KCl). Westerns were reacted at 4 °C overnight with primary antibody (either a 50-fold dilution of *αht-en* or 2-fold dilution of mAb4D9 supernatant) and for one hour with 500-fold dilution of horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody (GAR-HRP; Cal Biochem) or 2000-fold dilution of horseradish-peroxidase-conjugated goat anti-mouse secondary antibody (GAM-HRP; Jackson Immunoresearch Lab), respectively. Nonspecifically bound antibody was removed by washing after each antibody treatment with six washes of blocking buffer for 10 min each. The HRP immunodetection was done in the presence of 0.03 % cobalt and nickel ions (Adams, 1981).

Lineage tracing

Rhodamine dextran amine (RDA) lineage tracer (50 mg ml^{-1} in 0.2 % KCl) was pressure injected as described by Weisblat *et al.* (1980*b*) into individual teloblasts at stage 7.

α-hten antibody staining of embryos

All embryos were fixed for 1 h in 4 % formaldehyde, 50 mM cacodylic acid (pH 7.4) and immediately transferred to PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4) where the vitelline membranes were dissected off unhatched embryos (Fig. 3). Stage 11 embryos (Fig. 4) were transferred from PBS to 4 units ml^{-1} chitinase (Sigma C1525 from *Streptomyces griseus*; 100 units ml^{-1} stock prepared in 75 mM Hepes, pH 7.5/130 mM NaCl) diluted in 100 mM Hepes (pH 8), where they incubated at room temperature for 24 h. Chitinase-treated embryos were rinsed in PBS. All embryos were then incubated at least 12 h in block (2 % BSA, 2 % normal goat serum, 0.5 % triton X-100, 1×PBS) before adding the primary antibody.

Antibody dilutions and washes between antibody incubations were done in block. Antibody incubations were carried out for at least 24 h at room temperature. Washes were for at least 6 h with hourly changes of the block solution. Embryos were incubated in a 50-fold dilution of the *αht-en* antibody, followed by a 500-fold dilution of GAR-HRP. Following the second wash, the embryos were preincubated in 5 mg ml^{-1} diaminobenzidine (DAB) in PBS for 15 min before treating with 0.03 % hydrogen peroxide with 0.03 % $NiCl_2$ and $CoCl_2$ (Adams, 1981). The color reaction was monitored under the dissecting microscope; embryos were transferred to PBS when the desired degree of staining had been achieved. The embryos were then rinsed in 100 mM Tris, pH 7.5, stained for 1 h in 1 μg ml^{-1} Hoechst in 100 mM Tris, pH 7.5, and transferred to 90 % glycerol in 100 mM Tris, pH 7.5. Embryos were transferred to 80 % glycerol/10 mM Tris, pH 7.5/ 40 mg ml^{-1} *N*-propylgalate for dissection and mounting.

Microscopy and photography

Photomicrographs were taken on a Zeiss Axiophot microscope using DIC optics and Tech Pan 2415 film at ASA 100, except for Fig. 3A which was illuminated by fluorescence and obtained from a confocal microscope (Biorad); the image was photographed from a computer moniter using Plus-X pan at ASA 125.

Results

Summary of leech development

The development of glossiphoniid leeches from the newly laid egg to the juvenile leech has been divided into 11 stages (Fernandez, 1980). The process of segmentation occurs over an extended period of time lasting from stage 7–10; segments arise and mature as shown schematically in Fig. 1, from a posterior growth zone composed of 5 bilateral pairs of individually identified stem cells called *teloblasts*. One pair of teloblasts (M) generates mesoderm and four other pairs of teloblasts (N, O/P, O/P and Q) generate ectoderm. During stage 7 and 8, each teloblast undergoes a series of several dozen unequal divisions at the rate of about 1 per hour (Wordeman, 1982), generating a column (*bandlet*) of *primary blast cells*. Ipsilateral bandlets come into parallel arrays called *germinal bands*, within which the bandlets occupy stereotyped positions and

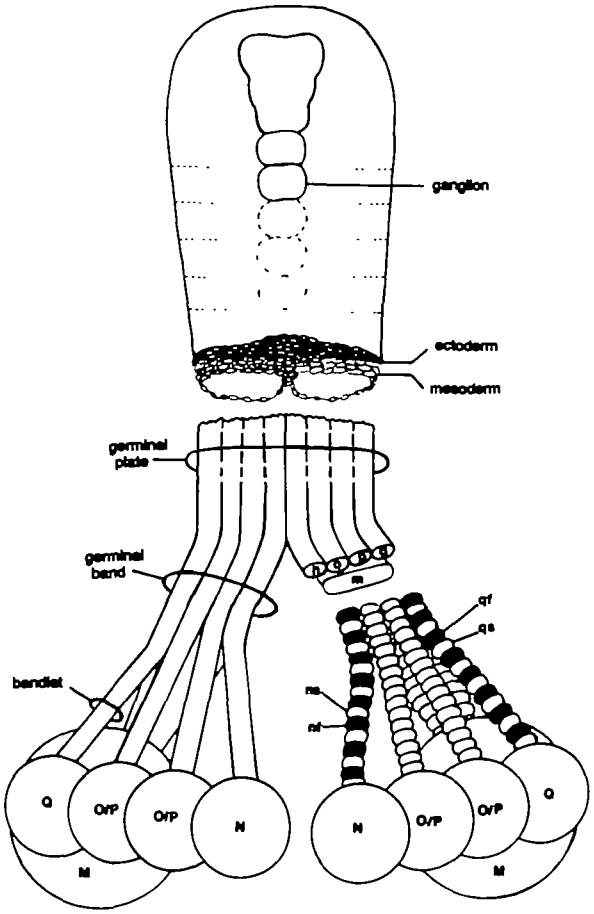

Fig. 1. Summary of leech development. A schematic summary of the development of segmental tissues, as viewed from the ventral surface. In the lower half of the figure, paired *teloblasts*, M, N, O/P, O/P and Q at the posterior end each divide repeatedly at a common budding site, giving rise to columns (or *bandlets*) of *blast cells* shown in detail only on the right. Alternate blast cells in the n and q bandlets are shaded to denote the two classes of blast cells in these bandlets. Ipsilateral bandlets join to form *germinal bands* which coalesce along the ventral midline into the *germinal plate*. A cross section of the right germinal band shows the relative positions of the ectodermal (n,o,p and q) and mesodermal (m) bandlets. Above, a cross section through the germinal plate shows the segmental mesoderm (lighter lines) and ectoderm (heavier lines). Ganglia are shown developing along the ventral midline. Horizontal dotted lines in the germinal plate indicate segment borders.

are designated m, n, o, p and q, as shown in Fig. 1. During stage 8, the two germinal bands coalesce from anterior to posterior along the ventral midline forming a structure called the *germinal plate*, from which segments arise. Ganglia first become evident in stage 9, again in a rostrocaudal progression. During stage 10, the edges of the expanding germinal plate meet along the dorsal midline, closing the body tube of the leech, and during stage 11 segmental tissues differentiate to a state approximating their mature form.

Segments arise in a highly determinate manner from

the stereotyped divisions of the blast cells within the germinal bands and germinal plate; older blast cells in each bandlet contribute to more anterior segments, which accounts for the pronounced rostrocaudal temporal gradient seen throughout development (Braun and Stent, 1989). Each bandlet contributes a distinct subset of segmentally iterated neurons and non-neuronal progeny to the mature leech (Weisblat *et al.* 1984); each m, o and p blast cell generates a full segment's worth (*segmental complement*) of definitive progeny for its cell line, although individual clones interdigitate and are distributed over more than one segment (Weisblat and Shankland, 1985). In the N (and Q) cell lines, *two* classes of blast cells, nf and ns (and qf and qs), arise in exact alternation, each contributing a specific subset of cells to a segmental complement of definitive N (or Q) progeny (Weisblat *et al.* 1984; Zackson, 1984; Bissen and Weisblat, 1987). Within the N cell line in particular, the anterior portion of a segment is generated almost entirely from ns-derived progeny, whereas the posterior portion is generated almost entirely from the nf-derived progeny (Bissen and Weisblat, 1987). The nf and ns clones within a segment intermingle slightly, but do not extend beyond the segment borders (Braun and Stent, 1989).

An en *homolog in* Helobdella

We have previously reported the cloning of an *en* homolog from *Helobdella* and the sequencing of the homeobox and 3′ region of this gene (Wedeen *et al.* 1991). In several species in which *en* class genes have been studied, two *en* class genes have been identified (Poole *et al.* 1985; Joyner and Martin, 1987; Gardner *et al.* 1988; Fjose *et al.* 1988; Walldorf *et al.* 1989; Hemmati-Brivanlou *et al.* 1991). Exceptions are sea urchin and grasshopper, in each of which only one *en* homolog has been detected (Dolecki and Humphries, 1988; Patel *et al.* 1989*a*). In previous experiments, a single *en* class gene was detected in *Helobdella* using a *Drosophila en* homeobox fragment as a low stringency hybridization probe (Wedeen *et al.* 1990). Attempts to identify additional *en* class genes in the leech have been unfruitful: (1) screening 10 genome equivalents of a leech genomic library using low stringency conditions with an *en* homeobox probe yielded 10 recombinants, all with the same *en* homeobox cross-hybridizing region (Wedeen *et al.* 1991); (2) low stringency hybridization of a homeobox-containing fragment from *ht-en* to Southern blots of *Helobdella* and *Drosophila* DNAs identified 2 *en* class genes in *Drosophila* DNA and intense bands corresponding to *ht-en* in *Helobdella*. Additional faint bands in *Helobdella* DNA were detected by the *ht-en* probe under low, but not high, stringency conditions. These bands might correspond to other homeobox-containing genes or to additional *en* class genes. However, in the latter case, the *Helobdella en* class genes would be far more divergent from each other than are any known pairs of intraspecific *en* homologs (Poole *et al.* 1985; Joyner and Martin, 1987; Walldorf *et al.* 1989). Although we cannot definitively rule out the existence of a second *en* class gene in *Helobdella* on the basis of these negative results, there is no evidence for this possibility.

Polyclonal antibodies against an ht-en/*βgal fusion protein*

A region of *ht-en* that is highly conserved among *en* class genes (the homeobox plus the flanking 5′ 2 bp and 3′ 57 bp) was amplified by polymerase chain reaction and four tandem repeats of this sequence (*ht-en*4x) were cloned in frame with the *E. coli* β-galactosidase (βgal) gene (pUR278; Ruther and Muller-Hill, 1983) and with the *E. coli* anthranilate synthase (trpE) gene (pATH21; Koerner *et al.* 1990). The βgal fusion protein was induced, purified from the *E. coli* extract by the procedure of Ruther and Muller-Hill (1983) and injected into a rabbit. The iterated *ht-en* sequence was employed to bias the rabbit's immune response toward the *ht-en* portion of the fusion protein (R. Kostriken, personal communication). Rabbit polyclonal antibodies directed against *ht-en* were affinity purified from the crude antiserum. Antibodies specific for *ht-en* were selected by affinity to the trpE fusion protein that had been purified by electroelution from an SDS–polyacrylamide gel (SDS–PAG) and bound to an affinity column (Affigel, Biorad). Anti-βgal antibodies were removed from the antiserum by running it over a second affinity column to which proteins from a crude bacterial lysate containing the βgal protein (induced pUR278; Ruther and Muller-Hill, 1983) had been bound. The purified antibody was designated *αht-en*.

In previous studies, a monoclonal antibody, mAb4D9, directed against a highly conserved portion of the *Drosophila inv* homeodomain had been used to determine the expression pattern of a putative *en* homolog in the leech (Weisblat *et al.* 1989; Patel *et al.* 1989*b*). We compared the specificities and affinities of mAb4D9 and *αht-en* on immunoblots of electrophoretic gels run with matched concentrations of crude extracts of *E. coli* strains bearing βgal alone or fusions of βgal with a single copy of the homeodomain and 3′ conserved region of either *en* (*en*/βgal) or *ht-en* (*ht-en*/βgal) (Fig. 3A). The *Drosophila* and *Helobdella* fusion proteins were of nearly equal size and serial 10-fold dilutions of each were electrophoresed in neighboring lanes. mAb4D9 detected *ht-en*/βgal with less than 1/1000 the sensitivity with which it detected *en*/βgal, a degree of cross-reactivity equivalent to background. By contrast, *αht-en* identified both the fusion proteins, but demonstrated approximately 10-fold higher affinity for *ht-en*/βgal than for *en*/βgal (see lanes 7 and 10, Fig. 3B). Thus, we believe that the antigen detected by mAb4D9 in leech embryos is not *ht-en*, but rather some other antigen (possibly another homeodomain protein).

αht-en did not react with bacterial βgal either in the marker lane or in the induced crude extract (Fig. 2B, lane 6), but did detect a single low molecular weight bacterial protein (lanes 6–10). Thus, to further test the specificity of this antibody and to insure that the antibacterial immunoreactivity would not generate a spurious pattern of staining in leech embryos, we

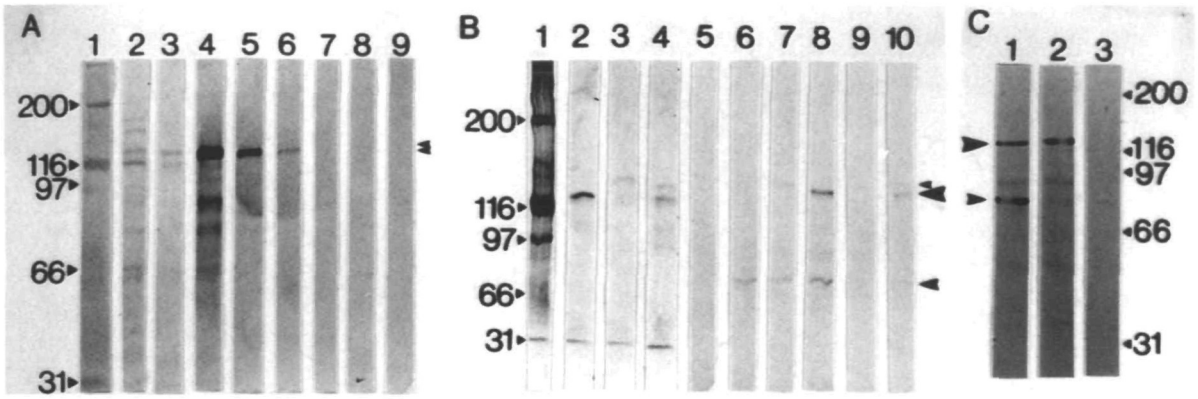

Fig. 2. Specificity of *α-ht-en* and mAb4D9 on immunoblots of *E. coli* extracts. *E. coli* strain XL1 was transformed with pUR278 (Ruther and Muller-Hill, 1983), enHD (Desplan *et al.* 1985), or pfht-en1x (see Experimental Procedures). Herein, these are referred to as βgal, *en*/βgal, and *ht-en*/βgal, respectively. The *lacZ* promoter was induced as described in Experimental Procedures. Crude extracts of the cultures were electrophoresed on 8% (A and C) and 6% (B) SDS–PAGs and transferred to nitrocellulose. (A) Relative affinity of mAb4D9 for *en*/βgal and *ht-en*/βgal. Lane 1, molecular weight standards, as indicated in the left margin. Lane 2, *en*/βgal. Lane 3, *ht-en*/βgal. Lane 4, a 10-fold dilution of the sample run in lane 2. Lanes 5–8, further serial 10-fold dilutions of *en*/βgal. Lane 9, identical to lane 3. Lanes 1–3 were stained for total protein using india ink stain. Lanes 4–9 were treated with mAb4D9 primary and horseradish-peroxidase-conjugated goat anti-mouse secondary antibody (GAM-HRP). The relative positions of the *en*/βgal and *ht-en*/βgal fusion proteins are shown by the upper and lower arrowheads, respectively, in the right margin. A 1000-fold dilution of *en*/βgal fusion protein (lane 6) is clearly identified by the mAb4D9 antibody. Staining specific to the *ht-en*/βgal fusion (lane 9) is not distinguishable from background. (B) Relative affinity of *α-ht-en* for *ht-en*/βgal and *en*/βgal. Lanes 1 and 5, molecular weight standards as indicated in the left margin. Lanes 2 and 6, βgal. Lanes 3, 7 and 9, *en*/βgal); lane 9 is a 10-fold dilution of protein relative to lanes 3 and 7. Lanes 4, 8 and 10, *ht-en*/βgal; lane 10 is a 10-fold dilution of protein relative to lane 4 and 8. Lanes 1–4 were stained for total protein using india ink stain. In lane 4, a doublet is observed, the lower band of which comigrates with βgal in lane 2. The upper band represents the *ht-en*/βgal fusion (large arrowhead in right margin). The slightly higher molecular weight band migrating in lane 3 is the *en*/βgal fusion protein (small arrowhead in right margin). Lanes 5–10 were treated with *α-ht-en* and HRP-conjugated goat anti-rabbit secondary (GAR-HRP). In each of the crude extracts, an approximately 80×10^3 M_r bacterial protein is recognized by *α-ht-en*. In addition, the *en*/βgal fusion protein is recognized in lane 7 and, with about 10-fold higher efficiency, the *ht-en*/βgal protein is recognized in lane 8. (C) Competition for *α-ht-en* using bacterial extracts. Lanes 1–3, *ht-en*/βgal. Molecular weights are designated in the right margin. Lane 1 was treated with *α-ht-en*. Lane 2 was treated with *α-ht-en* that had been pre-incubated 48 h (4°C) with a 1:1 mix of native and denatured *E. coli* proteins from strain XL-1; lane 3 with *α-ht-en* that had been preincubated 48 h with a 1:1 mix of native and denatured *E. coli* proteins from XL-1 that had been transformed with pf*ht-en*4x and induced to produce the *ht-en*4x/βgal fusion protein. All lanes were treated with GAR-HRP. In the left margin the upper (large) arrowhead indicates the position of *ht-en*/βgal and the lower (small) arrowhead indicates the position of the approximately 80×10^3 M_r bacterial protein to which *α-ht-en* cross reacts.

performed the following competition experiments. Crude bacterial lysate from *E. coli* strains containing either βgal or the *ht-en*4x/βgal fusion protein was incubated for 48 h with the purified antibody. After centrifugation, the supernatant was applied at 1:50 final dilution of the antibody to *Helobdella* embryos and to an immunoblot of a crude *ht-en*/βgal lysate. The *α-ht-en* preincubated with *ht-en*4x/βgal-containing lysate failed to stain the immunoblot (Fig. 2C, lane 3) and also failed to stain the embryos (not illustrated). The *α-ht-en* preincubated with lysate containing unfused βgal failed to stain the bacterial protein on the immunoblot, but did recognize *ht-en*/βgal (Fig. 2C, lane 2) and also revealed patterns of immunoreactive nuclei in embryos identical to those described below. Together, these results indicate that *α-ht-en* specifically reveals the expression pattern of *ht-en* in *Helobdella* embryos. However, conventional western blot analysis using *α-ht-en* on extracts of *Helobdella* embryos failed to show any bands (our unpublished observations) presumably due to the small relative amount of *ht-en* protein. Thus, we cannot formally exclude the possibility that *α-ht-en* recognizes more than one protein in embryos.

ht-en *expression during early development and neurogenesis*

Throughout development, *ht-en* expression, as revealed by immunostaining with *α-ht-en*, is bilaterally symmetric and localized to the nuclei of immunoreactive cells (Figs 3,4), in accord with the postulated role of *en* class proteins as transcription factors. The earliest detected expression of *ht-en* occurs in the germinal bands during late stage 7. Through mid-stage 8, expression occurs as a dynamic series of segmentally iterated patterns, the details of which remain to be determined.

By late stage 8, clones of cells derived from primary mesodermal (m) blast cells in the anterior portion of the germinal plate, which is developmentally more advanced, have formed hollow blocks of tissues corresponding to hemisomites (Zackson, 1982). Within the ectoderm, aggregations of cells are starting to become

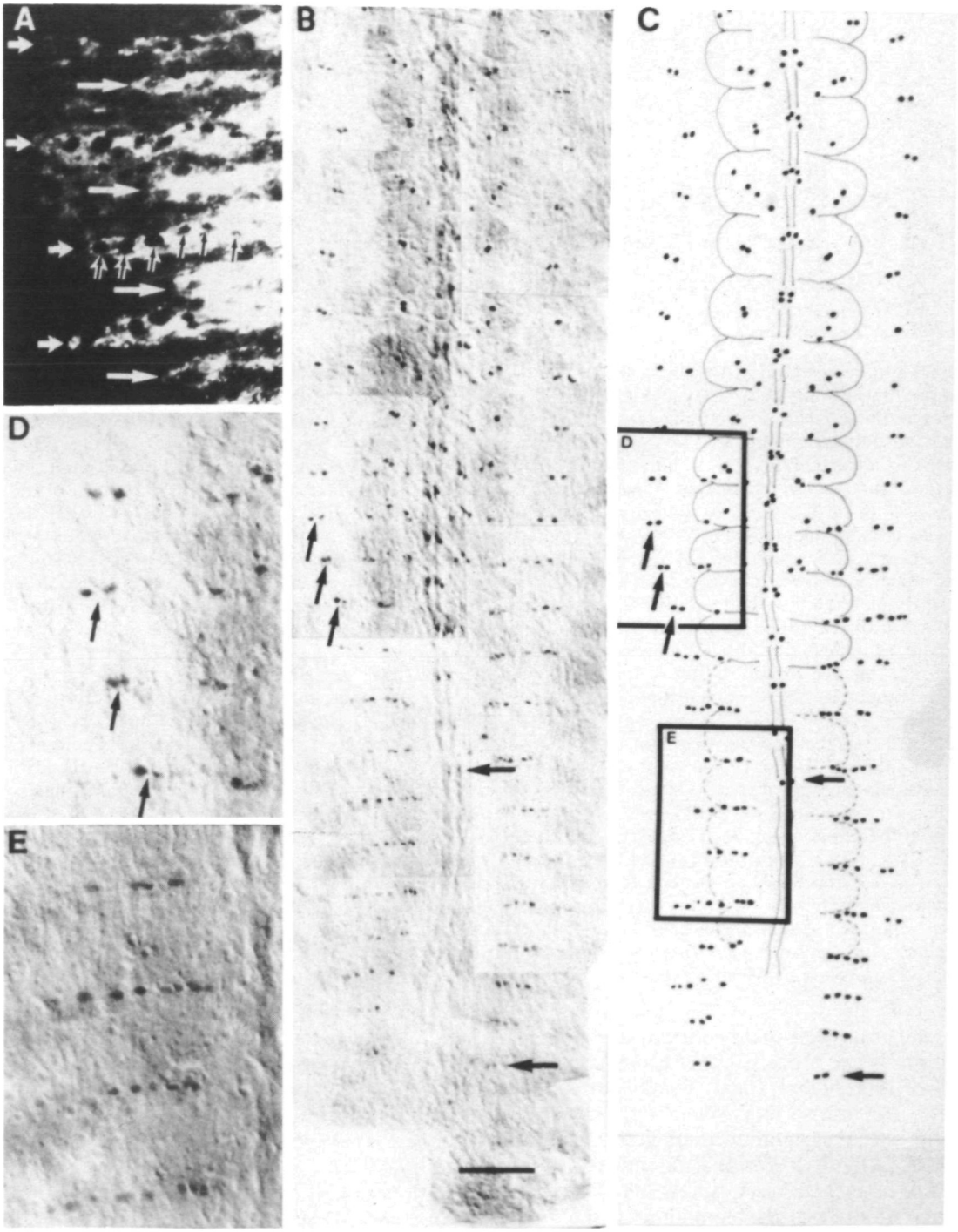

Fig. 3. *Ht-en* expression, stage 9. (A) Fluorescence photomicrograph of four half segments from a stage 9 embryo in which an N teloblast was microinjected (Weisblat *et al.* 1978) with lysinated rhodamine dextran (RDA) at early stage 7. Anterior is up, ventral midline at right. Tracer-labeled cells appear white; *ht-en* positive nuclei (black, indicated in one segment by black-on-white arrows) arise within lobes of cells derived from nf blast cells (short arrows) and not within those derived from ns blast cells (long arrows). (B,C) Bright-field photomontage and tracing of 22 segments in a dissected germinal plate. Boxes in C indicate fields shown at higher magnification in D and E. Anterior is up, ventral midline at center. Ganglia developing along ventral midline are designated by a solid outline; areas where ganglia will soon form are designated by a dotted outline. Posteriorly, development is least advanced; no *ht-en* expression is detected. Stripes first appear as 2 bilateral pairs of nuclei/segment (bottom arrow). More anteriorly, additional nuclei stain; when segmental ganglia appear, the boundaries between them are aligned with the stripes (see E). Concomitantly, expression disappears within the stripe except for two cells (nz1, nz2; left arrows and D) and new expression appears in ganglionic nuclei, first in two pairs of cells at ventral midline (upper right arrow). Still more anteriorly, additional ganglionic nuclei express *ht-en*. Slight deviations from bilateral symmetry in the staining pattern are not consistent from embryo to embryo and we attribute them to developmental noise. Scale bar, 26 μ in A, 50 μ in B, 23 μ in D and E.

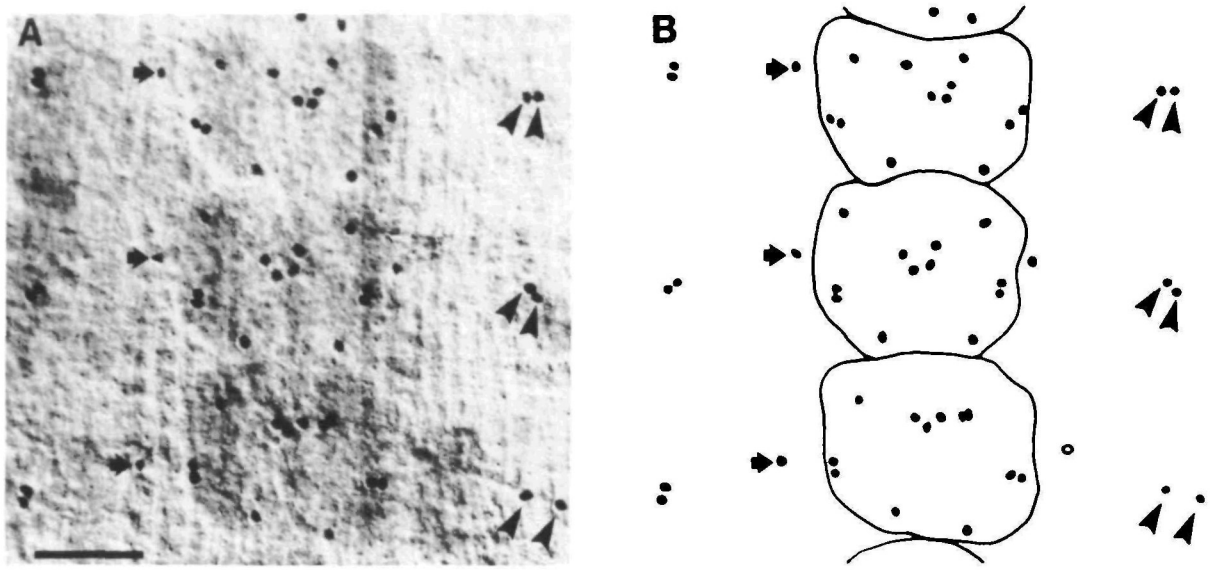

Fig. 4. *Ht-en* expression in stage 11 nervous system. (A) Photomontage (DIC optics) of three midbody ganglia and surrounding body wall from a dissected stage 11 embryo treated with *α-ht-en* and GAR-HRP to identify 6 pairs of ganglionic cells and 3 pairs of peripheral cells expressing *ht-en*. The N-derived peripheral neurons, nz1 and nz2 are designated on the right side of the embryo with arrowheads. Another pair of presumptive peripheral neurons, located just lateral to each ganglion and not derived from the N cell line are designated on the left side by arrows; the righthand member of this pair is not visible in the anterior segment and is out of focus in the posterior segment (see B). Scale bar, 50 μ. (B) Tracing of A. Ganglia are outlined. *ht-en*-positive cells are indicated as black circles.

apparent, but ganglia are not yet evident (Fernandez, 1980; Torrence and Stuart, 1986). Within these anterior segments, *ht-en* immunoreactive nuclei appear as segmentally iterated transverse stripes. In each segment, the stripe extends across the central portion of the germinal plate, with a discontinuity at the ventral midline. By early stage 9, in accord with the rostrocaudal progression of development, the stripes are present in posterior segments. The subsequent temporal progression of *ht-en* expression can be inferred by observing more anterior, developmentally more advanced segments (Fig. 3). [Each segment is about 2 h more advanced than the one posterior to it (Braun and Stent, 1989).] Several conclusions emerge from examining such preparations:

(1) When it first becomes evident, the stripe consists of 2–3 nuclei on either side of the midline (Fig. 3B and C). Over the next few hours, additional nuclei become immunoreactive, so that the stripe eventually contains 6–7 nuclei on either side of the midline (Fig. 3E).

(2) When segmental ganglia first become apparent, the boundaries between adjacent ganglia are aligned with the stripes (Fig. 3B and C).

(3) As development progresses, *ht-en* disappears in all but two nuclei on each side, which move laterally as the germinal plate expands (Fig. 3D).

(4) As the stripes fade, new *ht-en* expression appears in a subset of ganglionic cells, in a stereotyped spatiotemporal pattern, starting with a pair of anteromedial *ht-en*-positive nuclei within the developing ganglion (Figs 3B and C). Within a few hours, a second pair becomes detectable, just dorsal to the first. Soon thereafter, two more pairs of ventral nuclei begin expressing *ht-en* near the lateral margins of the ganglion; at about the same time, a fifth posteromedial pair appears. Finally, after several more hours, two pairs of anterodorsal nuclei begin to express *ht-en*. Except for the loss of expression by one of the anterodorsal pairs, the resultant pattern of *ht-en* expression in the midbody ganglia (Fig. 4) is maintained throughout advanced stages of gangliogenesis, as late as stage 11. A few extraganglionic cells also express *ht-en* in stage 11.

Lineage analysis of ht-en *expressing cells*

To ascertain the embryonic origin(s) of the stripes of nuclei expressing *ht-en*, fluoresecent lineage tracer was injected into one N teloblast of a series of embryos at early stage 7 (see Fig. 1). At early stage 9, the embryos were fixed and processed to reveal *ht-en* immunoreactivity. The clones of nf- and ns-derived progeny have not yet intermingled at this stage and form two transverse lobes of cells, extending laterally to greater and lesser extents, respectively (Fig. 3A; Weisblat *et al.* 1980*a*; Zackson, 1984). These embryos demonstrate that the stripe of *ht-en*-positive (black) nuclei is derived entirely from the nf blast cell, whose definitive progeny contribute to the posterior half of the segment.

Similar double label experiments on embryos at stages 10–11 reveal that a variety of ganglionic and peripheral neurons derived from both nf and ns blast cells express *ht-en*. In particular, the ganglionic *ht-en*-positive cells at the anterior ventral midline arise from ns blast cells (data not shown). In addition, the 2 *ht-en*-positive peripheral nuclei that arise from the nf-derived stripe and persist in expression (Fig. 4) become cells

nz1 and nz2, two peripheral neurons already identified as being derived from the nf blast cells (Braun and Stent, 1989). The late expression of *ht-en* is not confined to the N cell line, however, since additional *ht-en*-positive nuclei are observed by stage 11 near two epidermal specializations known as cell florets 3 and 6 (not shown), structures arising exclusively from the P and Q cell lines, respectively (Braun and Stent, 1989). Whether or not these cells are neurons remains to be determined.

Discussion

Patel *et al.* (1989*a*) described a monoclonal antibody, mAb4D9, which recognizes an 11 amino acid epitope within the homeodomain of the *Drosophila invected* (*inv*) gene product. This region of the *inv* homeodomain is identical to the corresponding region of the *Drosophila en* protein and is highly conserved in several other *en* class proteins. Therefore, mAb4D9 has been used to infer the expression pattern of *en* homologs in numerous species (Patel *et al.* 1989*a*; Hemmati-Brivanlou *et al.* 1989; Fleig, 1990; Whitington *et al.* 1991). In *Helobdella* embryos, mAb4D9 stains neurons in the second, third and fourth fused neuromeres of the subesophageal ganglion, a pattern more reminiscent of vertebrate than of arthropod staining patterns (Patel *et al.* 1989*a*; Hemmati-Brivanlou *et al.* 1989; Davis *et al.* 1991), and consistent with the notion of Field *et al.* (1988) that annelids and arthropods are not phyletically close (Patel *et al.* 1989*a*).

However, the *ht-en* sequence differs from that of *en* and *inv* by two amino acids in the mAb4D9 epitope. One of these differences (asparagine in *Helobdella versus* glycine in *Drosophila*) occurs at a residue that is critical for antibody binding, as evidenced by the finding that mAb4D9 does not cross react with the sea urchin or mouse *en* homologs, which have threonine and serine, respectively, rather than glycine at this position (Patel *et al.* 1989*a*). We have shown here that mAb4D9 recognizes the *Drosophila en* homeodomain with more than 1000-fold greater sensitivity than it recognizes the corresponding portion of *Helobdella ht-en*, as judged by immunoblots of fusion proteins. Based on genomic Southern blot analysis and library screening, it appears that *ht-en* is the only *en* class gene in *Helobdella*. These results suggest that mAb4D9 does not recognize an *en* class protein in *Helobdella*. The expression pattern observed with this monoclonal, may be that of a different homeodomain-containing protein. But in any case, it seems clear that the polyclonal antibody described here reveals the true pattern of *ht-en* expression in *Helobdella*.

It is hard to make meaningful comparisons between the timing of *en* expression in *Drosophila* and that of *ht-en* in *Helobdella* because the segmentation processes of *Helobdella* and *Drosophila* differ extensively at the cellular level. For example, identifiable founder cells for hemisomites in *Helobdella* arise sequentially beginning roughly 15 h after egg deposition, with the 8th cell cycle, as primary m blast cells which immediately begin generating morphologically distinct clumps of cells, before gastrulation has even begun (Zackson, 1982). In *Drosophila* by contrast, morphologically distinct segmental structures arise in the mesoderm about 3.5–4.5 h after fertilization, but this is during the 15th cell cycle, by which time gastrulation is well under way (Campos-Ortega and Hartenstein, 1985). Thus, although embryogenesis proceeds at a much slower rate in *Helobdella* than in *Drosophila*, morphogenetic aspects of segmentation in leech are markedly advanced in terms of cell generations.

Because the segmentation processes are so different in *Drosophila* and *Helobdella*, it is difficult to identify homologies in these processes. However, in the early *Drosophila* embryo, the interface between the *en*-expressing cells and neighboring cells is necessary to establish the segmental and parasegmental borders (Martinez-Arias *et al.* 1988). Although *en* eventually becomes expressed in several rows of cells per segment, in theory such an interface could be achieved by a single row of *en*-expressing cells, as we observe for *ht-en* in *Helobdella*. Thus, although we do not yet understand *ht-en* function in the leech, it may be that the transient expression of *ht-en* in a segmentally iterated stripe is necessary to delineate the border between developing ganglia and/or other segmental structures.

The pattern of *en* class protein expression reported here for *Helobdella*, including both segmentally iterated stripes before ganglion formation and a segmentally iterated subset of presumptive neurons later in development, is strikingly reminiscent of the patterns of expression of *en* class proteins previously described for arthropods (DiNardo *et al.* 1985; Brower, 1986; Patel *et al.* 1989*a*; Fleig, 1990). We interpret these similarities as indicating that a pattern of dual expression of an *en* class protein was already present in a common ancestor of annelids and arthropods. From this, we predict that such dual expression patterns of *en* homologs will also be found in other segmented protostome phyla and perhaps in molluscs as well. Moreover, the fact that *ht-en* is expressed during segmentation by cells descended from just the nf (posterior) class of n blast cells suggests that the anterior/posterior compartments of *Drosophila* and the nf/ns blast cells in *Helobdella* may both derive from a segmental subdivision that was present in the common ancestor, consistent with the previously proposed notion of homology between the ns and nf blast cells in leech and the anterior and posterior compartments of arthropods (Bissen and Weisblat, 1987).

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