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A *nanos* homolog in leech

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SUMMARY

From the glossiphoniid leech *Helobdella robusta*, we have cloned and determined the complete coding sequence of *Hro-nos*, a gene homologous to the *nanos* gene from *Drosophila melanogaster*. Developmental northern blots show that *Hro-nos*, like *nanos*, is a maternal transcript that decays rapidly during early development. A polyclonal antiserum raised against the HRO-NOS protein was used in developmental western blots and for immunostaining leech embryos of different developmental stages. The HRO-NOS protein is first detectable in 2-cell embryos (4-6 hours of development) and exhibits a transient expression peaking during fourth cleavage (9-12 cells; 8-14 hours of development). The HRO-NOS protein exhibits a graded distribution along the primary embryonic axis and is partitioned unequally between the sister cells DNOPQ and DM, progeny of macromere D′ at fourth cleavage: DNOPQ is the segmental ectoderm precursor cell and exhibits levels of HRO-NOS protein that are at least two-fold higher than in cell DM, the segmental mesoderm precursor cell. The observed expression pattern suggests that *Hro-nos* plays a role in the decision between ectodermal and mesodermal cell fates in leech.

Key words: *Helobdella robusta*, leech, *nanos*, cell fate determinant

INTRODUCTION

The establishment of embryonic polarity is a fundamental event in development because the asymmetric distributions of cells or cellular components that arise in the embryo underlie the formation of the definitive body axes. Moreover, in many species, polarity present in the egg or zygote is associated with the initial segregation of developmental potential. In *Drosophila melanogaster*, for example, where the nuclei of segmental founder cells acquire their fates within a syncytium, the formation of the anteroposterior axis is controlled in part by two maternal genes, *nanos* (*nos*) and *bicoid* (*bcd*) (for review, see St. Johnston and Nusslein-Volhard, 1992). The *nos* and *bcd* mRNAs are localized during oogenesis to the posterior and anterior poles of the egg, respectively (reviewed in Micklem, 1995). The BCD protein diffuses from its site of translation at the anterior pole, forming an anteroposterior gradient, while NOS protein diffuses from the site of its translation at the posterior pole, forming a posteroanterior gradient. These two protein gradients act antagonistically to regulate expression of the gene *hunchback* (*hb*) and thence the differential expression of other interacting regulatory molecules along the length of the embryo; in particular, NOS protein (acting with PUMILIO; Barker et al., 1992; Murata and Wharton, 1995) represses translation of the uniformly distributed *hb* RNA posteriorly (Tautz, 1988; Tautz and Pfeifle, 1989) through regulatory elements in the *hb* 3′ UTR, designated '*nanos*-response elements' (NRE's; Wharton and Struhl, 1991). The ensuing cascade of patterning events, dominated in its early stages by the diffusion of transcription factors within the syncytium, gradually subdivides the embryo into metameric body regions or segments, which arise more or less simultaneously along the length of the embryo (Driever and Nusslein-Volhard, 1988; Struhl et al., 1989; Hulskamp et al., 1990; Irish et al., 1989; Gavis and Lehman, 1992; Struhl et al., 1992; reviewed by Hulskamp and Tautz, 1991, and St. Johnston and Nusslein-Volhard, 1992).

It is not surprising that the role of *nos* in establishing early embryonic polarity appears to be conserved among other Dipteran species that develop via syncytial blastule (Curtis et al., 1995). But there is also evidence that *nos*-class genes function in early development of embryos undergoing holoblastic cleavages. For example, a *nos*-class gene designated *Xcat-2* has been cloned from *Xenopus*; its transcript is localized to the vegetal pole of oocytes (Mosquera et al., 1993; Forristall et al., 1995; Zhou and King, 1996). Furthermore, both the developmental role and the translational regulation of the *hunchback* gene in *Drosophila* is paralleled by that of the maternal *glp-1* gene in *C. elegans*: like hunchback, *glp-1* is translationally regulated, contains NRE-like sequences in its 3′ UTR and is asymmetrically expressed in the early embryo (Evans et al., 1994). This was taken as evidence for the presence of a *nanos*-like gene in that species. Taken together, these observations have led to the proposal that the non-uniform distribution of a NANOS-class protein, resulting from the cortical association of its mRNA at one embryonic pole, is an ancient mechanism for creating asymmetric patterns of gene expression in early embryos (Curtis, 1994; Kimble, 1994).

A critical test of this hypothesis is to determine if *nanos* homologs play a role in establishing embryonic polarity in other animals. To this end, we seek to determine the developmental function of the *nanos*-class gene in embryos of glossiphoniid leeches (phylum Annelida) such as *Helobdella robusta*, whose development features holoblastic cleavages and

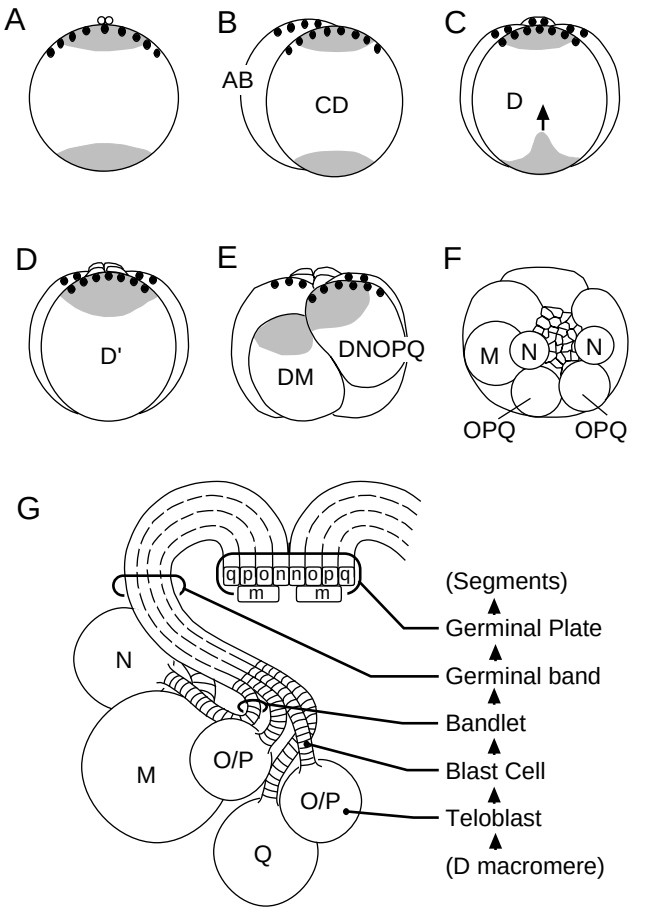

Fig. 1. Schematic summary of *H.robusta* development with emphasis on events leading to ectodermal and mesodermal fate segregation. (A-E) Equatorial views of early cleavage stages, showing the distribution of teloplasm (shaded regions) and the cortically associated ectodermal determinants (filled circles) postulated by Nelson and Weisblat (1992); animal pole is up. (A) Zygote (stage 1); after polar bodies (open circles) are extruded, teloplasm (shaded regions) forms at the animal and vegetal poles. (B) 2-cell embryo (stage 2); first cleavage is moderately unequal and the larger cell, CD, inherits most of the teloplasm. (C) 4-cell embryo (stage 3); the cleavage of CD is also unequal and the larger cell, D, inherits most of the teloplasm; during this stage, vegetal teloplasm migrates (arrow) toward the animal pole, joining with the animal teloplasm. (D) 8-cell embryo (stage 4a); third cleavage is highly unequal and produces quartets of animal micromeres and vegetal macromeres; teloplasm is located at the animal end of macromere D′. (E) 12-cell embryo (stage 4b); DNOPQ and DM each inherit a mixture of animal and vegetal teloplasm from macromere D′. Four of the seven micromeres at the animal pole are shown. (F) Stage 6a embryo, viewed from animal pole; DNOPQ has given rise to two N teloblasts, two OPQ proteloblasts, plus additional micromeres. DM has given rise to two M teloblasts (only one of which is visible from this view), plus additional micromeres. By stage 7 (not shown), the embryo contains four bilateral pairs of ectoteloblasts (the N, O/P, O/P and Q teloblasts), one bilateral pair of mesoteloblasts (the M teloblats), 25 micromeres and three macromeres. (G) Partial view of a stage 8 embryo, showing the left side teloblasts and their progeny. Each teloblast produces a column, or bandlet, of blast cells. Ipsilateral bandlets merge, forming left and right germinal bands; these coalesce anteroposteriorly along the ventral midline (top of figure) into the germinal plate, from which definitive segmental tissues arise. (Adapted from Nelson and Weisblat, 1992).

stereotyped cell lineages and cell fates (Fig. 1; reviewed in Irvine and Martindale, 1996, and Wedeen, 1995). For our present work, the following aspects of polarity and cell fate determination in early leech development are relevant. Segmental mesodermal and ectoderm arise from the D′ macromere of the 8-cell embryo, which is determined to be different from the A′, B′ and C′ macromeres by its inheritance of yolk-deficient cytoplasm (teloplasm) that arises in the zygote. At fourth cleavage, an obliquely equatorial division of macromere D′ separates the ectodermal and mesodermal lineages. The animal daughter of this division, cell DNOPQ, is the ectodermal precursor; the vegetal daughter, cell DM, is the mesodermal precursor. Based on cytoplasmic extrusion and cleavage plane alteration experiments, Nelson and Weisblat (1991, 1992) have proposed the existence of a cortically associated determinant in the animal hemisphere that, in association with teloplasm inherited by the D quadrant cells, governs the ectodermal fate adopted by cell DNOPQ.

Further divisions of cells DM and DNOPQ yield a set of embryonic stem cells (teloblasts), which produce segmental founder cells (blast cells; see Fig. 1) one by one in a strict anteroposterior progression over the course of many hours. The teloblasts therefore constitute a posterior growth zone like that seen in arthropods such as crayfish (Dohle and Scholtz, 1988; Scholtz and Dohle, 1996). Weisblat et al. (1994) proposed that a temporal activity gradient of a factor contained within individual teloblasts could initiate the assignment of segmental identities to individual blast cells in leech, just as the spatial activity gradients of BCD, NOS and HB proteins initiate the assignment of segmental identities within the syncytial blastoderm of *Drosophila*.

Here we report the cloning of *Hro-nos*, a leech homolog to the *Drosophila* gene *nanos* and show that *Hro-nos* mRNA is a maternal transcript. HRO-NOS protein is expressed preferentially within the ectodermal precursor cell, DNOPQ, and declines gradually during the period of blast cell production, consistent with a role in the establishment of polarity in the leech embryo.

MATERIALS AND METHODS

Embryos

Embryos of *Helobdella robusta* (Shankland et al., 1992) were obtained from a laboratory colony. Standard culture conditions (Blair and Weisblat, 1984), staging criteria (Fernandez, 1980) and injection procedures (Weisblat et al., 1984) were used.

Polymerase Chain Reaction

To amplify *Hro-nos* sequences, the following PCR primers were used (written in 5′ to 3′ orientation with redundant nucleotides in parentheses; the corresponding NANOS amino acid sequences are in brackets; *Eco*RI and *Bam*HI sites are underlined):

nanos1: CGGAATTCCGTG(CT)GTITT(CT)TG(TC)(GAC)AGI-AA(CT)AA [CVFCENN]
nanos2: CGGGATCCCGGG(GA)CA(GA)TA(TC)TTIA(TC)IGT-(GA)TG [HTIKYCP].

PCR conditions were: 50 mM KCl, 10 mM Tris, pH 9.0, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each dNTP, 0.8 uM each primer, 1 μg *H. robusta* genomic DNA, 1 unit Taq polymerase (Promega) in a 50 μl volume with mineral oil overlay. Cycling was 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 60 seconds, 35 cycles. The

amplified DNA was isolated from an agarose gel, digested with *Bam*HI and *Eco*RI then cloned into the *Bam*HI-*Eco*RI sites of pBS KS+.

Library screening and sequencing

A *H. robusta* stage 1-6 cDNA library (kindly provided by D. Isaksen) in the Lambda ZAPII vector (Stratagene), was screened using the PCR-amplified *Hro-nos* fragment labeled with ^{32}P-dCTP by random hexamer priming. Hybridization was done at 68°C in 5× SSC, 0.5% SDS, 40 mM phosphate buffer, pH 7.5 and 100 μg/ml herring sperm DNA. Washes were in 0.1× SSC, 0.1% SDS at 68°C. pBlueScript SK(–) phagemids were excised in vivo according to the manufacturer's instructions. A continuous *Hro-nos* open reading frame of 744 bp, and some flanking sequence (180 bp 5′UTR and 434 bp 3′UTR), was determined and found to be identical for both inserts. This sequence has been submitted to the GenBank database [accession number U85192].

Northern blotting

Samples of total RNA were prepared by homogenizing and digesting 50 oocytes (surgically removed from pregnant leeches) or embryos in 400 μl of 200 μg/ml proteinase K (Gibco), 0.5% SDS, 50 mM Tris, pH 7.5, 5 mM EDTA, pH 8.0, 50 mM NaCl for 40 minutes at 37°C, followed by two phenol extractions then precipitation with 0.1 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of ethanol at –80°C. The RNA was collected by centrifugation, air dried and then resuspended in 10 μl of 1.5% Ficoll, 25% formamide, 1 M formaldehyde and 0.25% bromophenol blue. The RNA samples were separated according to size through a submerged 1% agarose/2.2 M formaldehyde gel in 0.1 M MOPS, pH 7.0, 40 mM sodium acetate, 5 mM EDTA, pH 8.0, stained with ethidium bromide, photographed, and then transferred to a nitrocellulose membrane, which was baked for 1 hour at 80°C prior to hybridization.

Probe synthesis

A 643 bp *Kpn*I-*Pvu*II fragment encoding the carboxyl 157 amino acids and 172 nucleotides of the 3′UTR was subcloned into the corresponding sites of pBS KS (+) to generate pnanosKP. 1 μg of pnanosKP was digested with *Hin*dII, gel purified then used as template in a T7 driven in vitro transcription reaction to generate an antisense riboprobe covering the carboxyl 125 amino acids and 172 nucleotides of the 3′UTR. The reaction contained: 4 μl DNA template, 4 μl 5× transcription buffer (Stratagene), 2 μl 100 mM DTT, 1 μl RNAsin (40 U/μl; Boehringer), 1 μl 10 mM GTP, 1 μl 10 mM ATP, 1 μl 10 mM CTP, 1 μl 200 μM UTP, 5 μl α-^{32}P-UTP (50 μCi/800 Ci/mmol) and 1 μl T7 polymerase (5 U/μl; Promega). After 30 minutes at 40-42°C, another 1 μl of T7 polymerase was added to the reaction, which was allowed to proceed again for 30 minutes before the addition of 1 μl of 200 uM cold UTP and further reaction for 30 minutes. The template was digested away by the addition of 2 μl of RNAse-free DNAseI (10 U/μl; Boehringer) and 10 minute incubation at 37°C. Typically ~50 ng of labeled probe was synthesized with a specific activity of ~1.5×10^9 cts/minute/μg. The nitrocellulose membrane was pre-hybridized for 2 hours at 65°C in 50% formamide, 5× SSC, 0.1% SDS, 250 μg/ml herring sperm DNA, 8× Denhart's solution, 10 mM EDTA, 25 mM Tris, pH 8.0, 250 μg/ml torula RNA, then hybridized overnight at 64°C in the same solution but containing 2×10^6 cts/minute/ml of probe. Washes were: twice 10 minutes room temperature in 2× SSC, 0.1% SDS, then twice 30 minutes at 64°C in 0.1× SSC, 0.1% SDS. For quantification, the blot was analyzed using a phosphoimager (Molecular Dynamics). A 24 hour exposure on a Fuji Medical RX X-ray film was used for documentation.

Expression plasmid construction and injection

Approximately 2.5 kb of genomic sequence upstream of the *H. triserialis* EF1α gene (kindly provided by R. Streck) was subcloned as an *Eco*RI-*Xho*I fragment into the corresponding sites of pNASSβ (Clonetech) to generate pEF1NASS, a β-galactosidase (β-GAL) expression construct. Preliminary experiments revealed that injection of ~20-100 pl of pEF1NASS (4 μg/ul) in the M or N teloblasts leads to detectable β-GAL expression within 6 hours, peaking at 24-36 hours and lasting up to at least 240 hours after injection. The entire *Hro-nos* coding sequence was introduced in place of the *β-GAL* gene in pEF1NASS to generate the *Hro-nos* expression construct, pEF1NANOS. Injection of 20-100 pl of 4 μg/μl pEF1NANOS in the N teloblasts of stage 6a embryos was used as positive control in western blotting and immunostaining experiments.

Antibody production and purification

The sequence encoding the carboxyl 165 aa of the HRO-NOS protein was subcloned with the help of an adaptor into the *Eco*RI site of pGEX-KG to generate pnanosAb, a plasmid encoding a GST-HRO-NOS fusion protein. The amino sequence at the fusion border reads (HRO-NOS amino acids are underlined): ...GGIL*GMNNNKSS*... IPTG induction of DH5α cells harboring the pnanosAb construct led to the expression of the fusion protein, most of which was confined to inclusion bodies. Immunogen was purified from the soluble fraction using glutathione agarose beads according to the method of Guan and Dixon (1991). The HRO-NOS part of the fusion protein was cleaved from the beads using thrombin digestion and some 2,500 μg of the HRO-NOS portion was in this way purified. One rabbit (Babco) was initially immunized with 400 μg of purified HRO-NOS fragment, followed by 4 boosts of 200 μg at 30 days intervals. Serum was collected on day 117 and affinity purified by using a column containing 500 μg of purified HRO-NOS fragment coupled to 250 μl of Affi-Gel 10 (Bio-Rad). This antibody preparation recognized predominantly the purified HRO-NOS fragment with a sensitivity of ~5 ng, and reacted weakly against *E. coli* proteins as assayed by immunoblots of purified proteins and *E. coli* lysates (data not shown).

Western blotting

Whole-embryo protein samples were prepared from *H. robusta* embryos at selected stages, using 50 embryos for each sample. Settled embryos were homogenized in 100 μl of SDS-PAGE sample buffer containing 5% β-mercaptoethanol and heated to 95°C for 5 minutes. Proteins were then selectively precipitated in a methanol-chloroform-water mixture according to Wessel and Flügge (1984) then resuspended in SDS-PAGE sample buffer, boiled 3 minutes and separated by SDS-PAGE on a 12% gel and electroblotted to nitrocellulose. The blot was blocked 30 minutes in PBS/5% normal goat serum (NGS), hybridized 1 hour with $1{:}10^3$ dilution of the affinity-purified anti-HRO-NOS antibody in PBS/0.1% Tween-20 (PBT), washed three times 10 minutes in PBT, hybridized for 1 hour with a $1{:}10^4$ dilution of a horseradish peroxidase (HRP)-conjugated goat anti-rabbit (Jackson ImmunoResearch) secondary antibody in PBT and then washed as above before detection using chemiluminescence reagents (NEN). For quantitation purposes, a I^{125}-goat-anti-rabbit (ICN) was used at 0.5 μCi/ml as secondary antibody and the results analyzed using a phosphoimager (Molecular Dynamics). In some experiments, individual cells were isolated and pooled for western blot analysis; for this purpose, embryos were kept in 25% propylene-glycol for 10-20 minutes to facilitate dissection (Astrow et al., 1987) and the isolated cells were drained of excess fluid, resuspended in loading buffer, boiled and immediately loaded on a SDS-PAGE gel.

Whole-mount immunostaining

Embryos were fixed for 90 minutes in 0.25 PBS/4% formaldehyde, rinsed in PBT and removed from their fertilization membranes with insect pins in PBT. Embryos were then incubated for 3-4 hours in PBS/1% Tween-20/10% normal goat serum (PTN) to block non-specific binding, followed by a 16 hours incubation in PTN with the HRO-NOS antibody (used at 1:100). Unbound primary antibody was removed by 12 hours washing in several changes of PBT. A HRP-conjugated secondary antibody (Jackson ImmunoResearch) was

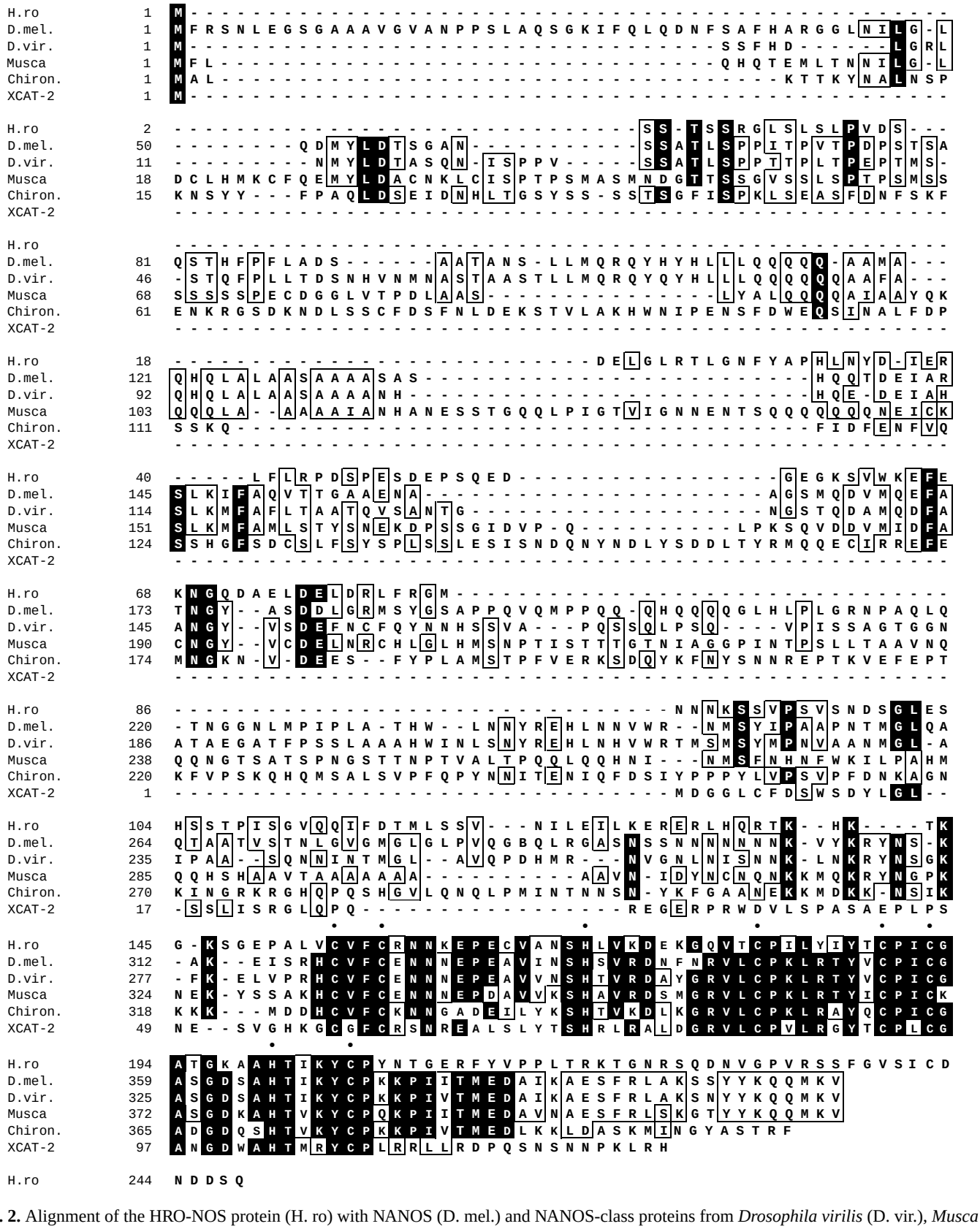

Fig. 2. Alignment of the HRO-NOS protein (H. ro) with NANOS (D. mel.) and NANOS-class proteins from *Drosophila virilis* (D. vir.), *Musca domestica* (Musca), *Chironomus samoensis* (Chiron.) and *Xenopus laevis* (XCAT-2). Dashes indicate gaps introduced to maximize the alignment. Filled boxes indicate residues that are identical among at least four of the aligned proteins. Open boxes indicate residues that are identical or conserved among at least three of the sequences. Conserved residues are considered to be: (A/V/L/I), (S/T), (R/K), (D/E), (Q/N) and (F/Y). Black dots mark the conserved C and H residues in the putative Zn finger motifs.

added to a dilution of $1{:}1.5{\times}10^3$ in PTN and the embryos were incubated overnight, followed by six 1 hour washes in PTN. HRP was visualized by reacting the embryos in a solution of 500 μg/ml diaminobenzidine, 0.01% H_2O_2 in PBS. These embryos were dehydrated by two passages in methanol and cleared in a solution of benzyl benzoate and benzyl alcohol (3:2). Embryos were examined using a Zeiss Axiophot microscope. As controls, rabbit polyclonal antisera to the rat synaptosome-associated protein of $25{\times}10^3$ M_r (SNAP-25; Hao et al., 1997) or syntaxin3 (Syn3; Gaisano et al., 1996) proteins were used as primary antibody.

RESULTS

Hro-nos is a homolog of *nanos*

PCR amplification of genomic *H. robusta* DNA yielded a fragment encoding a putative *nos* homolog (see Materials and Methods) and an identical fragment was amplified from an *H. robusta* stage 1-6 cDNA library using the same oligonucleotides. This fragment was used to screen 100,000 plaques from the library; two independent positive phage were isolated and their inserts sequenced. Fig. 2 shows an alignment of the predicted HRO-NOS amino acid sequence with the sequences of NANOS-class proteins from four insects and *Xenopus*. This alignment reveals a high degree of conservation between the Zn finger domain of HRO-NOS and that of the other NANOS proteins: in a span of 53 amino acids ranging from the cysteine at position 154 to the phenylalanine at position 206, HRO-NOS bears 68% identity with *D. virilis*-NANOS, 66% with NANOS (*D. melanogaster*), 60% with *Musca domestica*-NANOS, 60% with *Chironomus*-NANOS and 51% with XCAT-2. The next best alignment, obtained from a Blast search of the SwissProt data bank, was with the human zinc finger protein HRX (ALL-1; Djabali et al., 1992) which showed only 23% identity over the same region and a P(N) of only 0.068 (compared to the P(N) of $1.1{\times}10^{-23}$ for NANOS. Southern blot analysis suggests that *Hro-nos* is a single copy gene (data not shown).

In addition to the Zn finger domain, several small regions of homology exist among the NANOS-class proteins of the invertebrates that are not present in the *Xenopus* gene (Fig. 2). Most notable among these is a serine-threonine-rich region at the amino end of the protein; none of these motifs gave high scores to other known protein sequences in computerized homology searches.

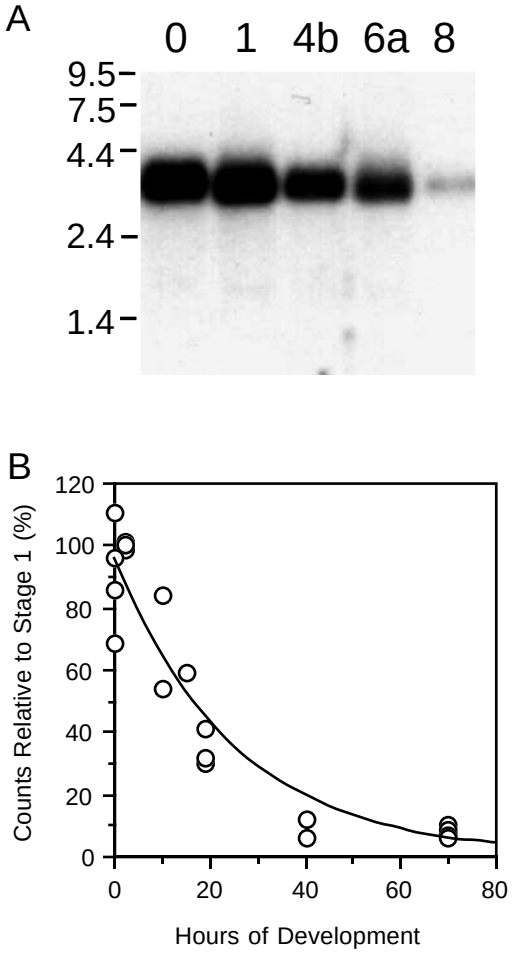

Fig. 3. Developmental northern blot analysis of the *Hro-nos* transcript. (A) RNA samples were collected from 50 embryos for each stage indicated (top), and probed with a *Hro-nos* specific probe. Positions of the RNA molecular weight standards are indicated, in kb, at the left. (B) The amount of radioactivity in each band was assessed using a phosphoimager and the results of several experiments, each standardized against stage 1 embryos, were plotted over time. Stages of development correspond to the following time points: oocytes (0 hours), stage 1 (2 hours), stage 4b (10 hours), stage 5 (15 hours), stage 6a (19 hours), stage 7 (40 hours) and stage 8 (70 hours). The solid line represents the best fit exponential curve as would be expected if the *Hro-nos* RNA is uniformly degraded and if no zygotic transcription of *Hro-nos* occurs.

Hro-nos is a maternal transcript

Northern blot analysis of *Hro-nos* (Fig. 3) revealed a single transcript that is approximately 3 kb in length. Since the open reading frame comprises only 744 bp, we conclude that the mature *Hro-nos* transcript contains approximately 2 kb of noncoding sequence.

The developmental northern blot also showed that the *Hro-nos* transcript is present at its highest levels in oocytes and stage 1 embryos. Its abundance declines to ~30% of the initial value by stage 6a, after roughly 18 hours of development (i.e. after zygote deposition). The *Hro-nos* transcript also persists at nearly 10% of the initial value up to at least mid stage 8, by

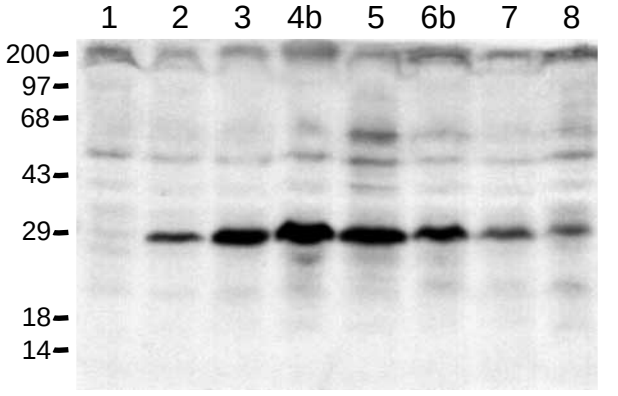

Fig. 4. Developmental western blot analysis of the HRO-NOS protein. Protein samples were collected from 50 embryos at stages indicated (top) and HRO-NOS protein was detected with a rabbit polyclonal HRO-NOS antibody. Positions of molecular weight standards are indicated, $\times10^{-3}$, at the left.

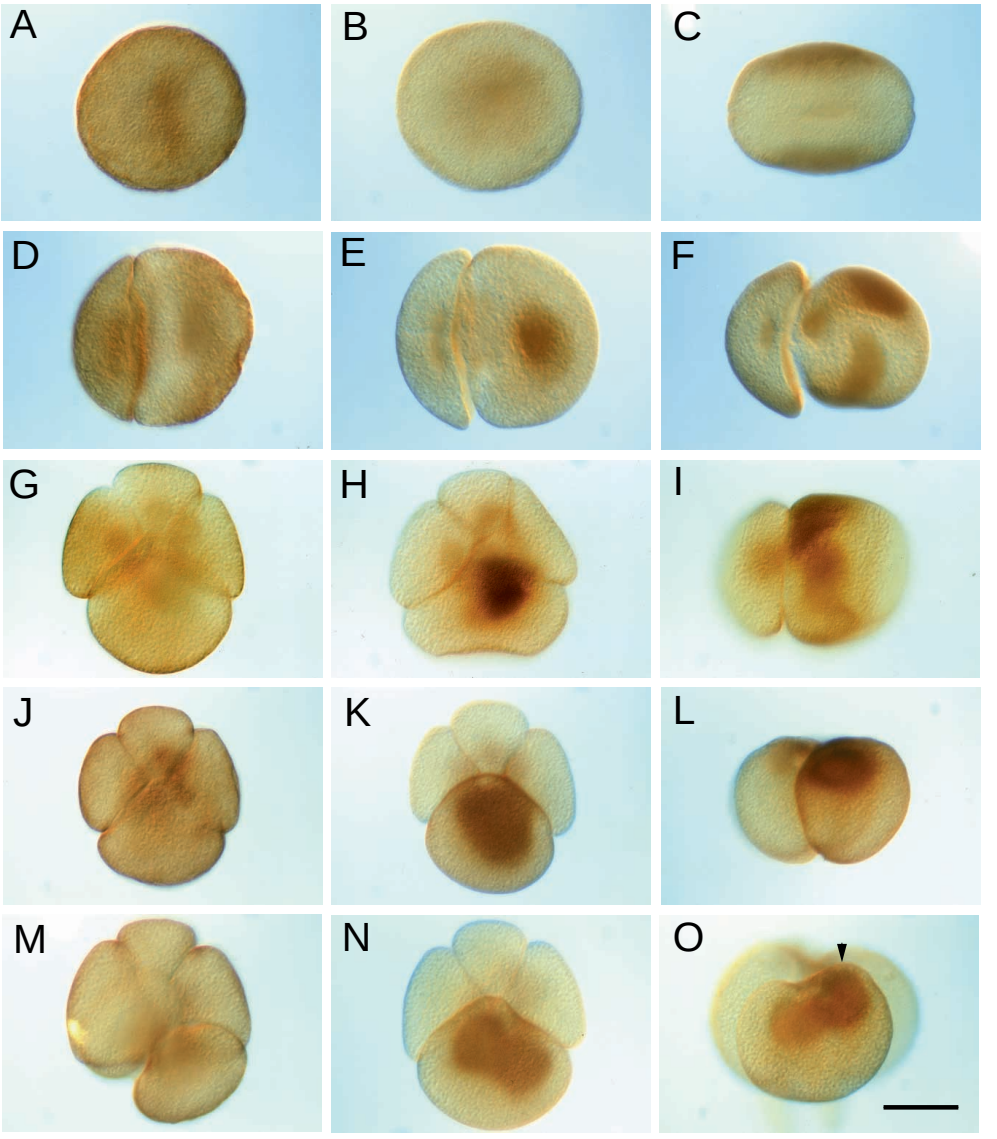

Fig. 5. Whole-mount immunostaining detection of HRO-NOS protein at developmental stages 1 to early 4b. The left column shows animal views of embryos stained with the control Syn3 antibody. The central and right columns show animal and equatorial views, respectively, of embryos stained with the HRO-NOS antibody. (A-C) Stage 1 embryos; (D-F) stage 2 embryos; (G-I) stage 3 embryos); (J-L) stage 4a embryos; (M-O) early stage 4b embryos in which the cytokinesis of cell D′ is not yet complete; arrowhead in O indicates the animal half of cell D′, which stains strongest for HRO-NOS. Scale bar, 200 µm.

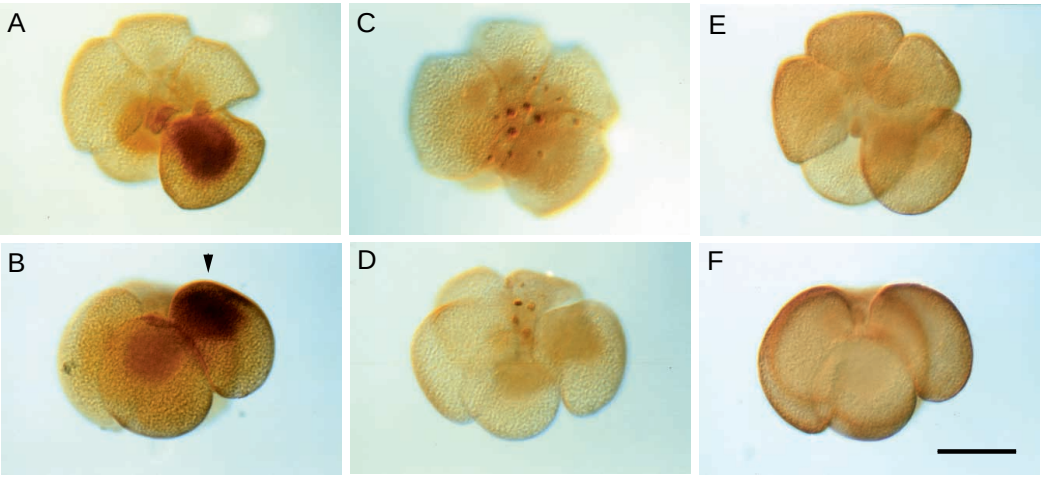

Fig. 6. Immunostaining of stage 4b embryos. Top and lower rows show animal and equatorial views, respectively. (A,B) Immunostaining using the HRO-NOS antibody; arrowhead in B indicates cell DNOPQ, in which HRO-NOS staining is strongest. (C,D) Immunostaining using a control SNAP-25 polyclonal antibody, which fortuitously stains nuclei in *H. robusta* embryos. (E,F) Immunostaining using the control Syn3 antibody. Scale bar, 200 µm.

which time the embryos are some 70 hours old and the coalescence of the germinal bands into a germinal plate is half completed (see Fig. 1). Phosphoimager quantitation of several developmental northern blots showed that the *Hro-nos* transcript has a half-life of approximately 15 hours (Fig. 1B). Extensive experimentation failed to yield an in situ hybridization protocol that reliably distinguished signal (antisense probe) from background (sense and heterologous probes); hence we have no information regarding the distribution of the *Hro-nos* transcripts within the embryos.

HRO-NOS protein accumulates preferentially within the ectodermal proteloblast

A developmental western blot analysis of HRO-NOS protein (Fig. 4) revealed that the protein migrates in SDS-PAGE gels with an apparent relative molecular mass of 29-30×10^3 M_r, in accord with that predicted from the cDNA sequence. In contrast to *Hro-nos* mRNA, the protein was first detected in stage 2 embryos (4-6 hours of development) and exhibited a transient expression peaking at stages 4b (8-12 hours), by which time the transcript level had already declined significantly. Like its mRNA, however, HRO-NOS protein persists at low levels up to at least mid stage 8. Other western blot analyses have revealed that, by stage 7 (45 hours), HRO-NOS protein has already declined to levels indistinguishable from those found in mid stage 8 embryos (data not shown).

The peak of expression at stage 4b coincides with the cleavage of macromere D′ to form cells DM and DNOPQ. These cells are differentially committed to be precursors of segmental mesoderm and ectoderm, respectively, at the time of their birth (Nelson and Weisblat, 1992). Immunostaining of intact embryos with HRO-NOS antibody suggested that HRO-NOS may be involved in this process. In stage 1 embryos, in which no HRO-NOS protein was detected by western blots, the animal and vegetal pools of teloplasm stained slightly with both HRO-NOS antibody and with a control antibody (Fig. 5A-C), which we therefore interpret as background staining. In stage 2 embryos, it was also difficult to discern HRO-NOS staining above background levels (Fig. 5D-F), but polar views

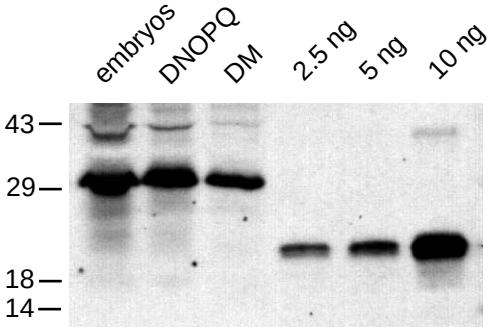

Fig. 7. Western blot analysis of dissected stage 4b embryos probed with HRO-NOS antibody. The first three lanes from the left represent samples prepared from 50 intact embryos, 50 dissected DNOPQ cells, and 50 dissected DM cells, respectively. The next three lanes contain 2.5, 5 and 10 ng, respectively, of truncated HRO-NOS expressed in and purified from bacteria.

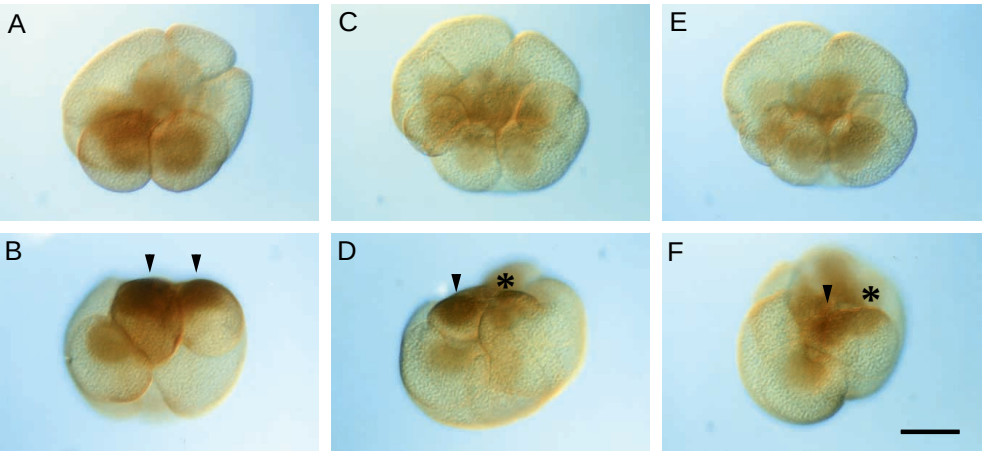

Fig. 8. Immunostaining of stage 5 and 6b embryos using the HRO-NOS antibody. Top and lower rows show animal and equatorial views, respectively. (A,B) Stage 5 embryos; arrowheads in B indicate the two NOPQ cells, in which HRO-NOS staining is strongest. (C-F) Stage 6a embryos; arrowhead and asterisk indicate cells N-left and OPQ-left, respectively. Scale bar, 200 μm.

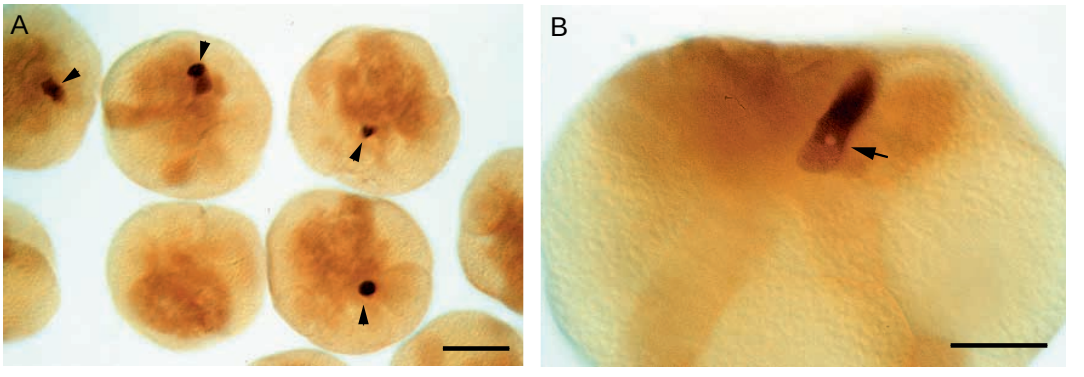

Fig. 9. Immunostaining of HRO-NOS protein in stage 7 embryos that were microinjected, 24 hours earlier, with the pEF1NANOS plasmid. (A) Arrowheads indicate cells that are strongly staining for HRO-NOS. (B) Arrow indicates a nucleus in focus, from which HRO-NOS protein is excluded. Scale bar, 200 μm (A); 100 μm (B).

suggested that the protein may be starting to accumulate, congruent with the western blot data; if so, it appears to be concentrated in teloplasm.

The first definitive immunostaining was seen in blastomere D of the stage 3 embryo (Fig. 5G-I). Equatorial views showed slightly higher staining in the animal pool of teloplasm at this stage. By stage 4a, the HRO-NOS staining was nearing peak levels and was confined to the the large pool of teloplasm located at the animal pole of cell D′ (Fig. 5J-L). As described previously (Holton et al., 1989), most of the vegetal teloplasm moves toward the animal pole and merges with the animal teloplasm during stages 3-4a. The teloplasm is redivided at stage 4b as macromere D′ cleaves to form cells DM and DNOPQ. Midway through this cytokinesis, and while the two pools of teloplasm are still contiguous, the animal pool of teloplasm exhibited higher staining than the vegetal pool, thus forming an intracellular protein gradient (Fig. 5M-O). Upon completion of this division, at stage 4b, which corresponds to the peak of HRO-NOS expression as assessed by western blot, immunostaining with HRO-NOS antibody remained strong in the teloplasm of cell DNOPQ, while staining in DM was only slightly above background (Fig. 6).

To verify these immunostaining results, we performed western blot analyses on pools of dissected DM and DNOPQ cells (Fig. 7). Quantitation using a I^{125}-secondary antibody and phosphoimager analysis allowed us to make the conservative estimate that DNOPQ contains at least 2-fold more HRO-NOS protein than cell DM. Similar experiments also showed that cell DNOPQ contains at least 15-fold more HRO-NOS protein than cells A′, B′ or C′ (data not shown).

In subsequent stages, the HRO-NOS staining weakens rapidly, as predicted from the western blot; staining seemed to persist preferentially in the NOPQ cells relative to the M teloblasts at stage 5 (Fig. 8A-B) and in the N teloblasts relative to the M or OPQ cells at stage 6a (Fig. 8C-D), though this staining was often very weak in stage 6a (Fig. 8E-F). By stages 7-8, it had fallen to levels that were not clearly distinguishable from background, even though persistence of the HRO-NOS protein well into stage 8 is indicated by the western blots (Fig. 4).

As a positive control for HRO-NOS immunostaining, stage 6a embryos were microinjected in both N teloblasts with pEF1NANOS, a *Hro-nos* expression plasmid (see Materials and Methods), then allowed to develop for 24 hours, i.e. to stage 7, prior to fixation and immunostaining (Fig. 9). As occurs in other systems, plasmid-based transient expression resulted in developmental abnormalities and a mosaic expression pattern of HRO-NOS. Similar abnormalities and mosaic expression were observed even when green fluorescent protein or β-galactosidase were used as protein reporters of expression (data not shown). The HRO-NOS protein was excluded from the nuclei of cells expressing the microinjected pEF1NANOS plasmid (Fig. 9B). Weaker staining of bandlets and micromeres was obtained with both control and HRO-NOS antibodies and is therefore interpreted as background signal in stage 7 embryos.

DISCUSSION

Hro-nos as a possible ectodermal determinant

Three major conclusions may be drawn from the data presented here. First, there is a store of maternal *Hro-nos* transcripts in the oocyte that is degraded during early cleavages. Second, the HRO-NOS protein expression detectable by immunostaining is both temporally and spatially regulated, with highest expression restricted to the ectodermal precursor cell at stage 4b. Third, some *Hro-nos* mRNA and HRO-NOS protein persists until at least mid stage 8, but at much lower levels than in early development.

The HRO-NOS protein expression pattern suggests a role in the assignment of distinct ectodermal and mesodermal fates to sister cells DM and DNOPQ at fourth cleavage. Both immunostaining and western blot data from dissected embryos indicate that HRO-NOS protein is abundant in the DNOPQ ectodermal precursor cell, and present at low levels in the sister cell DM, the mesodermal precursor cell. In light of previous observation that cells DM and DNOPQ are committed to different fates as soon as they are born (Nelson and Weisblat, 1992), it is significant that these immunostaining differences were evident prior to the completion of cytokinesis.

Comparison with *nanos*-class genes in other animals

The simplest interpretation for the temporal regulation of *Hro-nos* mRNA and protein expression is that the maternal transcript becomes available for translation and degradation after fertilization, as in *Drosophila*. Also by analogy with *Drosophila*, a compelling explanation for the spatial distribution of HRO-NOS protein is that the maternal *Hro-nos* transcripts are cortically associated near the animal pole of the *Helobdella* oocytes and embryos, so that, upon division of cell D′, most of this RNA is inherited and translated in the animal daughter, cell DNOPQ. Alternatively, the *Hro-nos* mRNA may be evenly distributed throughout the leech egg but with its translation spatially restricted to regions near the animal cortex. This would also generate polarized HRO-NOS protein expression; mounting evidence suggests that spatially restricted translation is crucial for proper *nos* function in *Drosophila* (Dahanukar and Wharton, 1996; Gavis et al., 1996; Smibert et al., 1996). In situ hybridization is obviously the direct approach to resolve the issue of *Hro-nos* RNA localization in leech embryos. Unfortunately, we have yet to find an in situ hybridization protocol that reliably distinguished signal from background during the stages of interest here.

The explanations offered above for the preferential expression of HRO-NOS in cell DNOPQ are not complete, since the A′, B′ and C′ macromeres also inherit animal cortex, yet do not express high levels of HRO-NOS. The accumulation of protein in DNOPQ relative to these endodermal precursors might be accounted for by differential activation of translation in accord with the differential inheritance of teloplasm, which is enriched in organelles (Fernandez and Stent, 1980; Fernandez and Olea, 1982), presumably including protein translation apparatus. Teloplasm-dependent translation of a cortically associated maternal mRNA fits well with the behavior expected for the leech ectodermal determinant postulated by Nelson and Weisblat (1992).

We have also observed that both the *Hro-nos* RNA and HRO-NOS protein persist until at least mid-stage 8 of development. In *Drosophila*, low levels of NANOS protein also persist beyond early development; the expression of NANOS is restricted to germ cells and is necessary for proper germ-line

formation (Kobayashi et al., 1996). Little is known about the origins of germ-line cells in annelids, and thus it would be interesting if the expression of HRO-NOS is similarly restricted in *Helobdella*. Alternatively, the gradually declining levels of HRO-NOS during stages 7-8 is not inconsistent with the notion (Weisblat et al., 1994) of a temporally graded intracellular factor in teloblasts that would initiate the assignment of segment-specific identities to individual blast cells.

Our findings regarding both the transient early timing and the spatially localized expression of HRO-NOS in leech embryos support the hypothesis that a *nos*-class gene was part of an ancient mechanism for establishing early embryonic polarity; it appears that this gene has been co-opted in the course of evolutionary tinkering to play different roles in different embryos. In *Drosophila*, NOS functions in conjunction with the PUMILIO (PUM) protein. In vitro, PUM binds the NRE found in the *hb* mRNA and probably acts in vivo by recognizing the NRE, then recruiting NOS (Barker et al, 1992; Murata and Wharton, 1995). While no *pum* homolog has been reported in leech, the existence of a yeast homolog (Coglievina et al, 1995) suggests that a similar gene exists in leech. Functional characterization of a leech *pum* and other genes upstream and downstream of *Hro-nos* should eventually permit inferences as to which aspects of the *nos* gene pathway constitute the ancient mechanism for establishing early embryonic polarity.

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