Hox/Wnt interact to pattern the primary body axis of an anthozoan cnidarian prior to gastrulation

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Abstract

Hox gene transcription factors are important regulators of positional identity along the anterior-posterior axis in bilaterian animals. Cnidarians (e.g. sea anemones, corals and hydroids) are the sister group to the Bilateria and possess genes related to both anterior and central/posterior class Hox genes. Here we report a previously unrecognized domain of Hox expression in the starlet sea anemone, Nematostella vectensis, beginning at early blastula stages. We explore the relationship of two opposing Hox genes (NvAx6/NvAx1) expressed on each side of the blastula during early development. Functional perturbation reveals that NvAx6 and NvAx1 not only regulate their respective expression domains, but also interact with Wnt genes to pattern the entire oral-aboral axis. These findings suggest an ancient link between Hox/Wnt patterning during axis formation and indicate that oral-aboral domains are likely established during blastula formation in anthozoan cnidarians.

Procedure

Phenol-chloroform RNA isolation from Nematostella

1. Collect embryos or small polyps by swirling them around in the dish, pipetting off garbage, and pipetting embryos into 1.5 ml centrifuge tube,

minimizing water. Pipette off excess water.

2. In the hood, add 1 mL Trizol to each tube of embryos. Swirl to dissolve; vortex lightly if needed. Make sure all embryos are dissolved. If extracting

from small polyps, start with 0.5 mL Trizol, homogenize, then add another

0.5 mL Trizol.

3. Spin down heavy phase lock tubes, keeping centrifuge at 4°C. Transfer the phenol containing dissolved embryos into the heavy phase lock tubes.
4. Add 200 µL chloroform to each tube and shake well for 15 seconds.
5. Incubate 10 minutes on ice.
6. Spin down at max speed for 15 minutes at 4°C.
7. Spin down new, empty phase lock tubes for 1 minute. Transfer the aqueous phase to

the new phase lock tubes.

8. Add 600 µL phenol-chloroform-isoamyl-alcohol to each tube and shake for 15 seconds.
9. Incubate on ice for 5 minutes.
10. Spin down at max speed 15 minutes at 4°C.
11. Transfer aqueous phase to new, clean 1.5 mL tube. Use a barrier tip and be very careful the tube does not touch the bench.
12. Add 1 µl glycogen.
13. Add 500 µl isopropanol, shake, and incubate at room temperature 10-20 minutes.
14. Spin at max speed for 15 minutes at 4°C.
15. Remove liquid being careful to avoid pellet. Spin again for 10 seconds and remove more liquid.
16. Add ~890 µl RNase-free 70% EtOH stored at -20°C. Vortex.
17. Remove liquid, avoiding pellet. Spin down briefly and remove liquid again.
18. Repeat (16) & (17).
19. Let pellet dry thoroughly and add 10µl RNase free water.

PART I: Select primers

• Ideal probes are the entire length of your gene. If the gene is >2 kb, the probe can be a 1-2 kb fragment of the gene. Probes should be at least 1 kb.

• Using Geneious, select 200-250 bp at the beginning of your gene sequence. Use “Design primers” and select forward primer. Primer should be:

o length: 20-27 bp; optimal 22 bp

o Tm: 57-63°C; optimal 60°C

o GC: 40-60%; optimal 50%

• Repeat for last 200-250 bp of gene, using “Design primers” and selecting

reverse primer.

• Blast primers against the species genome if possible to make sure they hit only your target gene.

• Check for primer dimers using online tools.

PART II: Make template cDNA

Materials

• Trizol reagent

• chloroform

• phenol/chloroform/isoamyl alcohol

• glycogen

• isopropanol

• RNAse-free 70% EtOH

• Advantage RT-for-PCR cDNA synthesis kit

1. Isolate RNA using Trizol reagent protocol.
2. Collect embryos or small polyps by swirling them around in the dish, pipetting off garbage, and pipetting embryos into 1.5 ml centrifuge tube, minimizing water. Pipette off excess water.
2. In the hood, add 1 mL Trizol to each tube of embryos. Swirl to dissolve; vortex lightly if needed. Make sure all embryos are dissolved. If extracting from small polyps, start with 0.5 mL Trizol, homogenize, then add another 0.5 mL Trizol.
3. Spin down heavy phase lock tubes, keeping centrifuge at 4°C. Transfer the phenol containing dissolved embryos into the heavy phase lock tubes.
4. Add 200 µL chloroform to each tube and shake well for 15 seconds.
5. Incubate 10 minutes on ice.
6. Spin down at max speed for 15 minutes at 4°C. Riboprobe synthesis for in situ

hybridization

7. Spin down new, empty phase lock tubes for 1 minute. Transfer the aqueous phase to the new phase lock tubes.
8. Add 600 µL phenol-chloroform-isoamyl-alcohol to each tube and shake for 15 seconds.
9. Incubate on ice for 5 minutes.
10. Spin down at max speed 15 minutes at 4°C.
11. Transfer aqueous phase to new, clean 1.5 mL tube. Use a barrier tip and be very careful the tube does not touch the bench.
12. Add 1 µl glycogen.
13. Add 500 µl isopropanol, shake, and incubate at room temperature 10-20 minutes.
14. Spin at max speed for 15 minutes at 4°C.
15. Remove liquid being careful to avoid pellet. Spin again for 10 seconds and remove more liquid.
16. Add ~890 µl RNase-free 70% EtOH stored at -20°C. Vortex.
17. Remove liquid, avoiding pellet. Spin down briefly and remove liquid again.
18. Repeat (16) & (17).
19. Let pellet dry thoroughly and add 10µl RNase free water.
20. Make cDNA using Advantage RT-for-qPCR kit. Follow published protocol (below). Use 1 µg RNA.

PART III: Clone probe sequence into plasmid

Materials

Day 1

• Taq polymerase and 10x buffer

• dNTPs (10 mM)

• QiaQuick gel purification kit

• isopropanol

• pGEM-T plasmid ligation kit

Day 2

• DH5-alpha (competent bacterial cell line)

• LB liquid medium

• LB/ampicillin agarose plates

Day 3

• LB liquid medium with ampicillin

• Sp6 and T7 primers

Riboprobe synthesis for in situ hybridization

Day4

• 5 Prime Fast Plasmid kit

• 50% glycerol

-DAY 1-

3. Amplify the probe sequence from cDNA using selected primers. Reaction:

10x reaction buffer 5 µl

dNTPs (10 mM) 0.5 µl

Taq polymerase 0.5 µl

nuclease-free water 41 µl

forward primer (10 µM) 1.0 µl

reverse primer (10 µM) 1.0 µl

template cDNA 1.0 µl

TOTAL 50 µl

Amplify 40 cycles.

4. Run the PCR product on a 1% agarose gel. Extract the correct band using a QiaQuick

gel purification kit (Protocol below from D.K. Simmons)

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a colorless tube. Add 400µl of Buffer QG to 1 volume of gel (100 mg gel ~ 100 µl). The maximum amount of gel slice

per spin column is 400 mg. (or just add 400 µl) For >2% agarose gels,

add 6 volumes Buffer QG.

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min during incubation to help

dissolve the gel.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose). If

the color of the mixture is orange or violet, add 10 µl 3 M sodium

acetate, pH 5.0, and mix. The color of the mixture will turn to

yellow.(~400ul)

5. Add 1 gel volume of isopropanol to the sample and mix by inverting. (~133ul)
6. Place a MinElute spin column in a provided 2 ml collection
7. Apply sample to the MinElute column and centrifuge for 1 min or Discard flow-through and place the MinElute column back into the

same collection tube. For sample volumes of more than 800 µl, simply

load and spin again.

8. Add 500 µl Buffer QG to the MinElute column and centrifuge for 1 min. Discard flow-through and place the MinElute column back into the

same collection tube.

9. Add 750 µl Buffer PE to MinElute column and centrifuge for 1 min Discard flow-through and place the MinElute column back into the

same collection tube.

Note: If the DNA will be used for salt-sensitive applications, such as direct sequencing and blunt-ended ligation, let the column stand 2–5 min after addition of Buffer PE.

10. Centrifuge the column in a 2 ml collection tube (provided) for 1 min. Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

11. Place each MinElute column into a clean 1.5 ml microcentrifuge tube. To elute DNA, add 10 µl water to the center of the MinElute membrane. (Ensure that the elution buffer is dispensed directly onto the membrane for complete elution of bound DNA.) Let the column stand for 1 min, and then centrifuge the column for 1 min.

5. Ligate the fragment into the pGEM-T vector plasmid. (Protocol below from D.K. Simmons)

1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes.

2. Set up ligation reactions as described below. Note: Use 0.5ml tubes known to have low DNA-binding capacity (e.g., VWR Cat.# 20170-310). Vortex the 2X Rapid Ligation Buffer vigorously before each use.

3. Mix the reactions by pipetting. Incubate the reactions for 1 hour at room temperature.

4. Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 16°C.

Reaction Component Standard Reaction 1/2 Rxn ¾ Rxn

2X Rapid Ligation Buffer, T4 DNA

Ligase 5ul 2.5ul

3.75ul

pGEM®-T or pGEM®-T Easy Vector

(50ng) 1ul 0.5ul

0.75ul

PCR product Xul (3ul) X(1.5ul) X(2.25ul)

T4 DNA Ligase (3 Weiss units/µl) 1ul 0.5ul 0.75ul

nuclease-free water to a final volume

of 10ul 5ul

7.5ul

Note: if >1kb think about using 25ng of vector instead, due to the lowered ratio.

-DAY 2-

6. Transform the ligation product into competent bacteria (we use DH5-alpha), and grow colonies overnight. (Protocol below from D.K. Simmons)

1. Add 2µl of each ligation to a 1.5ml microcentrifuge tube on ice
2. Remove tube(s) of frozen JM109 High Efficiency Competent Cells from storage and place in an ice bath until just thawed (about 5 minutes). Mix

the cells by gently flicking the tube. Avoid excessive pipetting, as the

competent cells are extremely fragile.

3. Carefully transfer 50µl of cells into each tube prepared in Step 2 (use 100µl of cells for determination of transformation efficiency).
4. Gently flick the tubes to mix and place them on ice for 20 minutes.
5. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C (do not shake).
6. Immediately return the tubes to ice for 2 minutes.
7. Add 460µl room-temperature LB medium to the tubes containing cells transformed

with ligation reactions and 900µl to the tube containing cells transformed with uncut plasmid

8. Incubate for 1.5 hours at 37°C with shaking (~150rpm).
9. Plate 100µl of each transformation culture onto LB/ampicillin plates. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1,000 × g for 10 minutes, resuspended in 200µl of LB medium, and 100µl plated on each of two plates.
10. Incubate the plates overnight (16–24 hours) at 37°C. If 100µl is plated, approximately 100 colonies per plate are routinely seen using competent cells that are 1 × 108cfu/µg DNA. Use of ultra-high- efficiency competent cells may result in a higher number of background colonies. Longer incubations or storage of plates at 4°C (after 37°C overnight incubation) may be used to facilitate blue color development.

-DAY 3-

7. Perform colony PCR to determine which colonies contain PCR fragment. Reaction:

10x reaction buffer 2.5 µl

dNTPs (10 mM) 0.25 µl

Taq polymerase 0.5 µl

nuclease-free water 19.75 µl

Sp6 primer (10 µM) 1.0 µl

T7 primer (10 µM) 1.0 µl

TOTAL 25 µl

Touch a pipette tip to a colony, then dip it into the PCR solution.

Amplify for 40 cycles.

8. Set up liquid cultures for colonies containing PCR fragment.
9. Pour 3 ml LB into a glass culture tube.

10. Touch a pipette tip to the chosen colony, then drop it into the LB in the tube.
3. Incubate overnight with shaking. -DAY 4-
9. Miniprep liquid cultures using 5 Prime Fast Plasmid kit. Simultaneously make glycerol stocks of the lines.
1. Chill the Complete Lysis Solution on ice.
2. Pellet 1.5 ml of fresh bacterial culture at maximum speed (at least 12,000 x g or 13,000 rpm) for 1 minute in the provided 2 ml Culture Tube.
3. Remove medium by decanting, taking care not to disturb bacterial pellet.
4. Add 400ul of ICE-COLD Complete Lysis Solution. The Complete Lysis Solution MUST be ice-cold (0 –4°C) to obtain maximum yield.
5. Mix thoroughly by constant vortexing at the highest setting for a full 30 seconds. This step is critical for obtaining maximum yield.
6. Incubate the lysate at room temperature for 3 minutes. (add 125ul of Isopropanol)
7. Transfer the lysate to a Spin Column Assembly by decanting or pipetting.
8. Centrifuge the Spin Column Assembly for 30– 60 seconds at maximum speed.
9. Add 400 ul of DILUTED Wash Buffer to the Spin Column Assembly.
10. Centrifuge the Spin Column Assembly for 30–60 seconds at maximum speed.
11. Remove the Spin Column from the centrifuge and decant the filtrate from the Waste Tube. Place the Spin Column back into the Waste Tube

and return it to the centrifuge.

12. Centrifuge at maximum speed for 1 minute to dry the Spin Column.
13. Transfer the Spin Column into a Collection Tube.
14. Add 50 ul of Elution Buffer directly to the center of the Spin Column membrane and cap the Collection Tube over the Spin Column.
15. Centrifuge at maximum speed for 30 – 60 seconds.

16. Remove and discard the Spin Column.

17. The eluted DNA can be used immediately for downstream applications or stored at -20°C.

10. Sequence miniprep results to ensure plasmid insertion is correct. Save glycerol stocks of bacteria containing correct plasmid.

Microinjection of mRNA or morpholinos for reverse genetic analysis in the starlet sea anemone, Nematostella vectensis

by Michael J Layden, Eric Röttinger, Francis S Wolenski, +2
Nature Protocols (22 February, 2018)

Characterizing the spatiotemporal expression of RNAs and proteins in the starlet sea anemone, Nematostella vectensis

by Francis S Wolenski, Michael J Layden, Mark Q Martindale, +2
Nature Protocols (18 April, 2013)