J Mol Evol (1997) 44:371–377

JOURNAL OF MOLECULAR EVOLUTION

Complete Sequence of *SpHox8* and Its Linkage in the Single *Hox* Gene Cluster of *Strongylocentrotus purpuratus*

Pedro Martinez, Jason C. Lee, Eric H. Davidson

Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125, USA

Received: 7 November 1996 / Accepted: 4 December 1996

Abstract. *SpHox8* is the paralog group 8 *Hox* gene of *Strongylocentrotus purpuratus.* This identification follows from an analysis of the sequence of the complete open reading frame of a late-gastrula-stage cDNA clone; from direct linkage to adjacent *Hox* genes in a fosmid contig; and from blot hybridizations carried out on pulse field gel electrophoretic separations. *SpHox8* is a single-copy gene, and there is only one *Hox* gene cluster per genome in *S. purpuratus.*

Key words: *SpHox8 — Strongylocentrotus purpuratus — Hox* gene cluster

Introduction

The *Hox* genes of echinoderms are of particular interest in both evolutionary and developmental senses. Morphological analyses indicate that echinoderms are the deuterostome sister group of hemichordates plus chordates (e.g., Peterson 1995). Current evidence indicates that in contrast to vertebrates, in ascidians (Katsuyama et al. 1995; Di Gregorio et al. 1995), in a cephalochordate (Garcia-Fernàndez and Holland 1994), and possibly in a hemichordate (Pendleton et al. 1993) there is only one *Hox* gene cluster. Arthropods also have only one *Hox* gene cluster, and the same is therefore expected of the echinoderms as well. There is, indeed, some evidence that echinoderms have a single *Hox* gene cluster, based on lack of significant variation in the sequences of fragments that include each of the *Hox* gene homeoboxes and based on the observation of a single large restriction fragment resolved by pulse field gel electrophoresis that reacts with several different *Hox* gene homeobox probes (Popodi et al. 1996). Many individual homeobox sequences that are apparently derived from *Hox* genes have been reported from several different echinoid species (Dolecki et al. 1986; DiBernardo et al. 1994; Ruddle et al. 1994; Popodi et al. 1996).

Here we describe the complete sequence of the open reading frame of a gene we identify as the paralog group 8 *Hox* gene of *Strongylocentrotus purpuratus*. A fragment of this gene including the homeobox was originally isolated from *Tripneustes gratilla* by Dolecki et al. (1986), who named it ''*TgHbox1*.'' This probe was used to isolate a genomic fragment of the same gene from *S. purpuratus*, with which *Hbox1* expression could be studied by in situ hybridization (Angerer et al. 1989). Late in the pluteus stage, *Hbox1* transcripts were found localized to the vertex of the aboral ectoderm, i.e., in the progeny of a specific aboral ectoderm founder cell, VA (Cameron et al. 1987), although earlier they have a more widespread distribution. The functional meaning of this expression pattern is so far unknown. In the following we present an analysis of the sequence of a cDNA encoding the complete Hbox1 protein and of its linkage within the single *S. purpuratus Hox* gene cluster. The combined evidence indicates that this cDNA represents an mRNA produced by the *S. purpuratus Hox8* gene (*SpHox8*).

Materials and Methods

PCR Primers. Two oligonucleotides (CT77 and CT78) representing well-conserved, canonical sequences present in Antp-class homeodomains were used to amplify homeobox fragments from the *S. purpu-*

Correspondence to: E.H. Davidson

ratus genome. A third oligonucleotide (HBO-T) represents the 3′ terminal primer for reverse transcriptase in the construction of the cDNA library from which the *SpHox8* gene described in this paper was recovered. The oligonucleotide CLO7 represents a specific sequence inside the *SpHox8* homeobox. Oligonucleotides are as follows (I—inosine monophosphate):

Primers	Derived from	Sequence
CT77	Conserved homeodomain peptide: LELEKEF	5′CGGATCC(T/C)TIGA(A/G)(T/C)TIGA(A/G)AA(A/G)GA(A/G)T
CT78	Conserved homeodomain peptide: KIWFQNRRM	5′-GGAATTCATIC(G/T)IC(G/T)(A/G)TT(T/C)TG(A/G)AACCAIAT(T/C)T
HBO-T	Cloning primer	5′-GAGAGAACTAGTCTCGAG(T)$_{18}$
HBO	Cloning primer	5′-GAGAGAACTAGTCTCGAG
CLO7	SpHox8 specific primer	5′-GAAGAAGACGAATCGAACTCAGTCATCTCCTCGG

PCR Amplification and Cloning of SpHox8 *Homeobox and 3′ Terminal Sequences. S. purpuratus* cDNA was reacted with primers CT77 and CT78 and subjected to reverse transcriptase PCR. Three micrograms of RNA from *S. purpuratus* 50-h embryos (late gastrula stage) supported the first-strand cDNA synthesis, using M-MLV reverse transcriptase, and the oligonucleotide HBO-T as a primer. Amplification of *Hox*-class homeobox fragments from this cDNA was accomplished by using the degenerate oligonucleotides CT77 and CT78. We performed 35 PCR cycles at 94°C, 1 min; 40°C, 1.5 min; 72°C, 2 min; plus a final extension of 20 min at 72°C. The homeobox products were gel purified and subcloned into the pCR vector (Invitrogen). To obtain adjacent 3′ sequences of this gene, 3 μg of poly(A) RNA from 50-h *S. purpuratus* embryos were transcribed to cDNA with M-MLV reverse transcriptase, using oligonucleotide HBO-T as primer. The 3′ region of *SpHox8* was amplified from this cDNA by PCR, using the oligonucleotides HBO and CLO7 as primers. Amplification was accomplished in 35 cycles at 94°C, 1 min; 50°C, 1.5 min; 72°C, 3 min; with a final extension of 20 min at 72°C. The sample was displayed by electrophoresis on a 1% agarose gel and then transferred to a nylon filter (Hybond N, Amersham). To detect homeobox-containing fragments, oligonucleotide CT78 was labeled at the 5′ terminus with T4 kinase and used as a probe. Positive fragments were eluted from a preparative gel and cloned into the vector pGEM-T (Promega). After purification these clones were sequenced with a Sequenase kit (USB).

Isolation of cDNA Clones. A riboprobe prepared from clone pH1UT (see Fig. 1A) was used as a probe to screen a 50-h gastrula-stage embryo cDNA library constructed in the λZAPII vector (Stratagene). Hybridization of filters containing a total of 3×10^5 clones was carried out for 16 h at 42°C in 50 mM Tris-HCl, pH 7.5; 0.1% sodium pyrophosphate; 1% SDS; 1× Denhardt's solution; 5× SSPE, 50% formamide; 100 μg/ml denatured salmon sperm DNA; and 10 μg/ml yeast RNA. The filters were washed to a criterion of 0.2× SSC; 0.2% SDS, at 65°C. SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, pH 7.4, 10 mM EDTA, pH 7.4; and SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0. Positive cDNA clones were rescued in Bluescript SK (+). The longest clone, pH1.8 (see Fig. 1A), was sequenced by the exonuclease III deletion method, using an ABI 373 automated sequencer.

Genome Blot and RNA Gel Blot Hybridization. For genome blot hybridizations samples of 10 μg of genomic DNA derived from sperm of three different individuals were digested to completion with the enzyme *Eco*RI. Following electrophoresis in a 1% agarose gel the samples were transferred by blotting overnight to a nylon filter (Hybond N, Amersham). The filter was hybridized using the insert of the

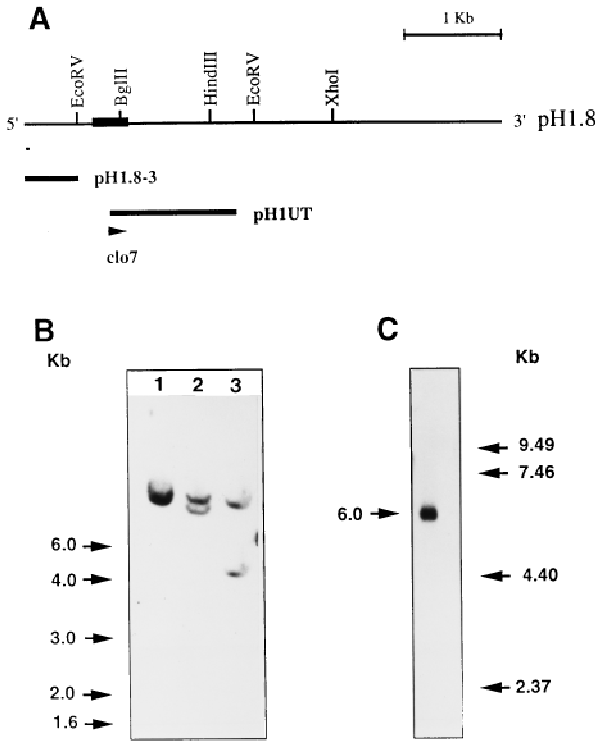

Fig. 1. Map of cDNA clone and probes, and gel blot hybridizations. **A** Restriction map of cDNA clone pH1.8. The *black box* represents the homeodomain. Clone pH1UT was generated by PCR as described in text and pH1.8–3 is a subclone (see Materials and Methods for details). **B** Genome blot hybridizations. DNA from sperm of three different individuals was digested with the enzyme *Eco*RI, electrophoresed on a 1% agarose gel, and transferred to a Hybond N filter. The filter was hybridized with ^{32}P-labeled pH1.8–3 probe. The gel ran slightly faster on one side than the other and all three top bands are the same size. **C** RNA gel blot hybridization. Poly(A) RNA from 50-h *S. purpuratus* embryos was electrophoresed on a 1% agarose/formaldehyde gel and transferred to a Hybond N filter. The filter was then hybridized with a ^{32}P-labeled pH1.8–3 probe.

subclone pH1.8–3 as a probe (see Fig. 1). The probe was labeled by random priming. Hybridization was carried out at 65°C in a buffer containing 5× SSC, 5× Denhardt's, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. After hybridization, the membrane was washed to a final criterion of 0.2× SSC, 0.2% SDS at 65°C.

For RNA gel blot hybridizations, 1 μg of poly(A) RNA was fractionated by electrophoresis on a 1% agarose-formaldehyde gel and then transferred overnight to a nylon membrane (Hybond N, Amersham). The membrane was hybridized with the same probe as above and under the same conditions. The membranes were washed to a final criterion condition of 0.2× SSC, 0.2% SDS at 67°C.

Pulse Field Gel Electrophoresis of S. purpuratus *DNA.* Sperm cells were obtained by electrical stimulation of adult male sea urchins. Cells were collected in sterile tubes and diluted to 2×10^8 cells/ml in filtered seawater. An equal volume of 2% low-melting InCert agarose (FMC BioProducts) dissolved in the same buffer was added, and after mixing poured in 10-ml-volume methacrylate molds and left on ice until a hard plug was obtained. The embedded cells were lysed and protein digested by incubation for 24 h at 50°C in 0.5 M EDTA, pH 8.0, 1% lauryl sarcosine, 5 mM DTT, and 2 mg/ml proteinase K (Birren and Lai 1993). The DNA was then digested with appropriate enzymes and run for 20 h in a 1% SeaKem LE agarose gel (FMC BioProducts) at 14°C using 0.5× TBE buffer. TBE is 0.089 M Tris-borate, 0.089 M boric

acid, 0.002 M EDTA. Electrophoresis was performed using the CHEF-DRIII System (BioRad Laboratories) at 6 V/cm and with a 75-s pulse. Following electrophoresis, DNA was transferred to nylon filters using conventional methods (Sambrook et al. 1989) and then hybridized using gene-specific probes as described above for genome blots.

Results and Discussion

Isolation of cDNA Clones

A cDNA clone encoding the complete open reading frame of the gene we identify below as *SpHox8* was isolated in three successive stages, as described in detail in Materials and Methods. Briefly, RT-PCR was first carried out on 50-h (late gastrula) poly(A) RNA, using canonical Antp-class *Hox* homeobox primers. The sequence of one of the PCR products obtained revealed it to include a homeobox identical to that of *TgHbox1* (Dolecki et al. 1986; Angerer et al. 1989). To obtain a probe that would identity transcripts of this gene exclusively, we next cloned a DNA fragment that was amplified by PCR between a primer specific to the *Hbox1* sequence and a sequence tag introduced in the primer at the 3′ terminus of a cDNA preparation (RACE protocol). This probe was cloned and it was used in turn to screen a 50-h cDNA library. We selected the longest of 15 positive clones, clone pH1.8, which, as indicated in Fig. 1A, carries a 5-kb insert. Genome and RNA blot hybridizations were carried out, using as probe a subclone, pH1.8–3. As shown in Fig. 1A, pH1.8–3 includes only sequences outside the homeobox, thereby reducing the possibility of cross-reaction with other homeobox-containing genes.

The gene represented by clone pH1.8 is clearly single copy, as was also concluded for the genomic *SpHbox1* fragment isolated by Angerer et al. (1989). Figure 1B shows the results of genome blot hybridizations carried out with three different individual sea urchin DNAs. Individual 1 is homozygous (AA), while individual 2 is heterozygous, sharing one allele with individual 1 (AB). Individual 3 is also heterozygous, sharing allele (A) with the other individuals, but displaying a different second allele (AC). The RNA gel blot shown in Fig. 1C indicates a single 6-kb transcript in 50-h embryo RNA, as reported earlier by Angerer et al. (1989) for their *Hbox1* probe.

Structure of the Implied Protein and its Sequence Relationships

The entire coding sequence of cDNA clone pH1.8 is shown in Fig. 2A. The ATG assigned as the translation initiation codon is the first that is in-frame with the sequence encoding the homeodomain and that is not followed shortly by stop codons. The immediately preceding sequence conforms well with the Kozak (1986) consensus sequence for translation initiation. The open reading frame codes for a protein of 308 residues. Several features of the sequence are highlighted in Fig. 2A. At the N-terminus of the protein there is a ten-amino-acid sequence element shown (boxed in Fig. 2A; see Fig. 2B), which is very similar to the equivalent regions of chordate HOX proteins belonging to paralog groups 5–8. This comparison is shown in Fig. 3A. The orthologous *Drosophila* genes share only a few residues in this region, and no function has been described for the sequences listed in Fig. 3B for any metazoan *Hox* gene.

As indicated in the diagram shown in Fig. 2B, following the conserved N-terminal peptide there is a region in which serine (26%), proline (11%), and threonine (9%) are highly represented. Ser/Pro/Trh-rich sequences are found in the activation domains of several transcription factors (Gavis and Hogness 1991; Carman and Monroe 1995; Radtke et al. 1995). Gavis and Hogness (1991) demonstrated that Ser residues in the N-terminal region of the *Drosophila* UBX protein are phosphorylated in vivo, and they suggest that phosphorylation could create an acid domain or an amphipathic α-helix (Ptashne 1988), which might accelerate the interactions of these proteins with the basal transcription apparatus.

The predicted amino acid sequence also contains a highly conserved hexapeptide (GYPWMP; boxed in Fig. 2A), which has been found in most HOX proteins (Mavilio et al. 1986). Chang et al. (1995) showed that this sequence promotes cooperative binding of HOX proteins to target DNA by interactions with members of the Pbx family of homeodomain proteins. Twelve amino acids downstream of the hexapeptide is located the putative DNA-binding domain of the protein, i.e., the homeodomain (underlined in Fig. 2A; see Fig. 2B). This homeodomain obviously belongs to the HOX class of homeodomain proteins.

In Fig. 3B the homeodomain sequence of clone pH1.8, which as noted above is identical to that earlier termed Hbox1 (Dolecki et al. 1986; Angerer et al. 1989), is compared with mouse, amphioxus (*Branchiostoma floridae*), and hemichordate (*Saccoglossus kowalevskii*) *Hox* gene homeoboxes of paralog groups 5–9. The pH1.8/*Hbox1* sequence is >80% identical will all of the group 5–8 sequences, although significantly less so with the paralog group 9 sequence. However, in the diagnostic region at the N-terminal end of the homeobox the sequence differs more from the group 5 sequences than from the group 6–8 sequences. But neither this comparison nor that shown in Fig. 3A suffices to indicate unequivocally whether the pH1.8/*SpHox8* sequence is to be assigned to paralog group 6, 7, or 8.

Assignment to Paralog Group 8

We have isolated a contig consisting of three genomic clones (fosmids) that overlap, each of which bears a

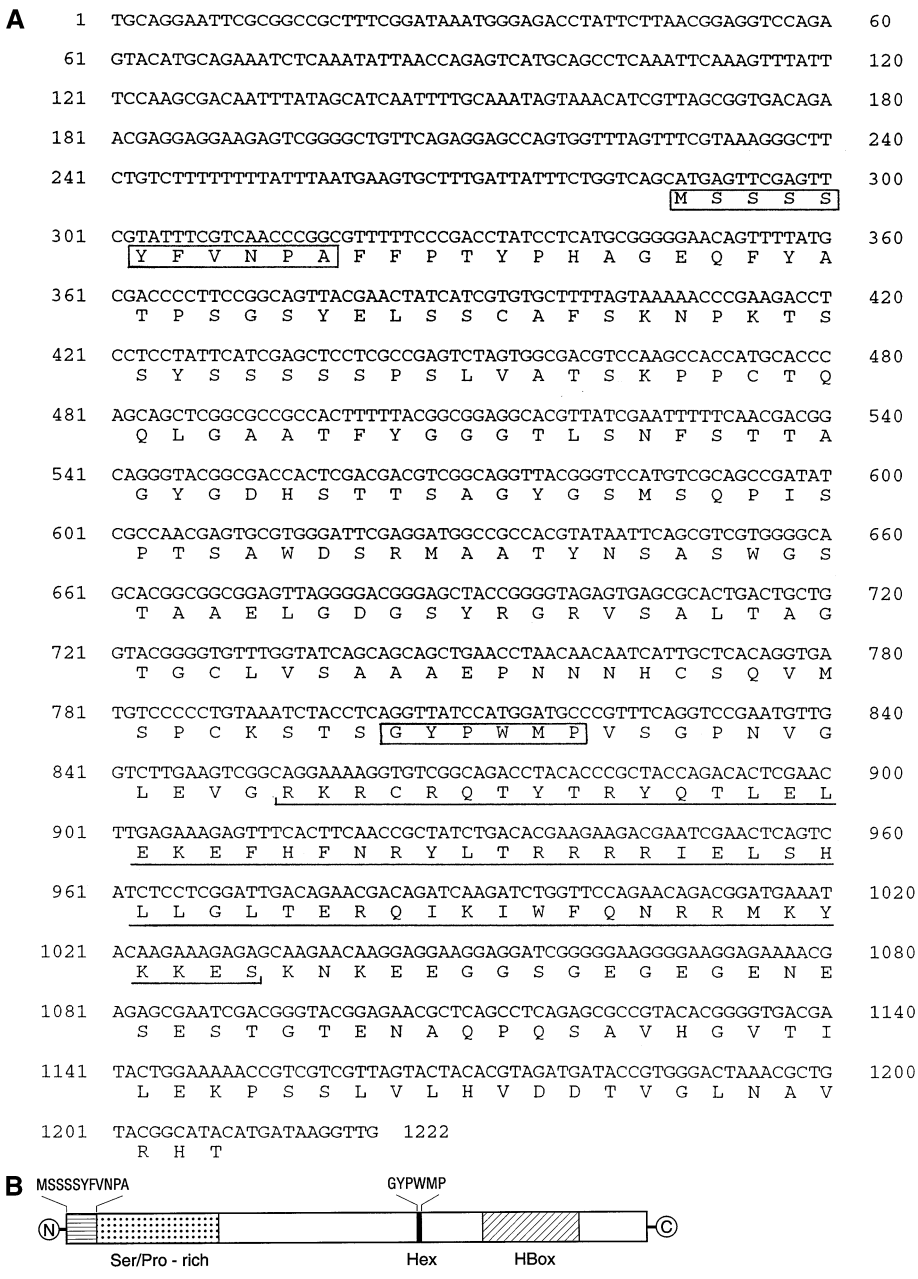

Fig. 2. A Nucleotide and translated amino acid sequences of clone pH1.8 (i.e., *SpHox8*). The homeobox is *underlined.* Conserved N-terminal and hexapeptide sequences are *boxed.* The nucleotide sequence reported here will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases with the accession number D85419. **B** Structure of encoded protein. The coding region can be subdivided into four domains: N-terminal conserved sequence (*box with horizontal lines*); Ser/Pro-rich domain (*dotted box*); conserved hexapeptide (Hex, *solid box*); and homeobox (Hbox, *hatched*).

single *Hox* gene (data not shown). The homeobox sequences of these three *Hox* genes, like that of pH1.8/*Hbox1,* can all be assigned to paralog groups 6–8, as will be presented elsewhere. The temporary laboratory names of the other two genes are *SpHbox3* and *SpHbox6,* and they are linked in the fosmid contig so that *SpHbox6* is in the middle. With respect to the ''anterior'' end of the *Hox* gene cluster, the order of these genes could be either 3-6-1 or 1-6-3 (where left denotes ''anterior''). The order is shown to be 3-6-1 by the experiment reproduced in Fig. 4.

Sperm DNA was digested with appropriate enzymes under conditions producing very large fragments. These were resolved by pulse field gel electrophoresis and blotted with probes specific for various *Hox* gene homeoboxes (see Materials and Methods and legend). Figure 4A shows a blot reacted with a probe for a gene assigned by sequence to paralog group 4 or 5 (PG 4/5 gene). The *Bsc*pI digest displays a reactive band that is only about 35 kb in length (leftward arrow). Figure 4B shows that the *SpHbox3* probe also reacts with this same band (as well as with several larger bands that also display reaction in Fig. 4A). However, *SpHbox1* reacts only with different *Bsc*pI bands, as illustrated in Fig. 4C. Therefore, the order is either (''anterior'') 4/5-3-6-1 or (''anterior'') 1-6-3-4/5. The latter is of course excluded since all three of the genes in question, i.e., Hbox1, 3, and 6, belong to groups 6–8, which cannot lie on the ''anterior'' side of the PG4/5 gene. We conclude that the orientation of the cluster is paralog group 4/5-*SpHbox3*-*SpHbox6*-*SpHbox1*. This places *SpHbox1* at the ''posterior'' end of our set of three linked paralog group 6–8

A

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SpHox8          MSSSSYFVNPA
HoxD8             M-------L     PG8
HoxC8             M-------L     PG8
HoxA7             M----Y--AL    PG7
HoxB7             M--L-YA-AL    PG7
HoxC6             MN---T--S     PG6
HoxB6             M------ST     PG6
HoxB5             M------SF     PG5
HoxA5             M------SF     PG5
HoxB4           MAM--FLI-SN     PG4
HoxA1             MDNARM-SF     PG1
HoxB2           MNFEFEREIGF     PG2
HoxB3           MQKAT-YD-T-     PG3
HoxD3           MQKAA-YE--G     PG3
HoxB9           M-I-GTLSSYY     PG9
HoxA10          M-C-ESPAANS     PG10
HoxA11          MMDFDERGP       PG11
Antp              MTMST-NC
Ubx               MN---EQAS
Abd-A             MYP-VS-HP
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Fig. 3. Comparisons between elements of the pH1.8/*Hox8* protein sequence and equivalent regions of various *Hox/HOM-C* genes. **A** N-terminal peptides. The decapeptide with which the protein encoded by pH 1.8 begins is shown at the top, and aligned with it are the corresponding N-terminal peptides of mouse *Hox* genes. At the bottom are the N-terminal peptides of the three relevant *Drosophila HOM-C* genes. *PG* denotes paralog group. **B** Alignment of the pH1.8/*Hox8* homeobox sequence with homeoboxes of hemichordate, amphioxus, and mouse *Hox* genes belonging to paralog groups 5–9. Only fragments of the hemichordate homeobox sequence are available (Pendleton et al. 1993). Amphioxus sequences are from Garcia-Fernandez and Holland (1994), and the mouse and *Drosophila* sequences are taken from Gehring et al. (1994). Sequences of the *Drosophila Antp, Ubx,* and *AbdA* genes are also shown. *Dashes* indicate amino acid identities, and percent identity overall is listed in the *right-hand column.*

%Identity **B**

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SpHox8          RKRCRQTYTRYQTLELEKEFHFNRYLTRRRRIELSHLLGLTERQIKIWFQNRRMKYKKES

h(e7)                           -------------IA-A---S------
Amphihox-5      N--T-TA-------------------------IA-A-C----------------W---N      83
mHoxA5          G--A-TA-------------------------IA-A-C-S--------------W--DN     80
mHoxC5          G--S--S-------------------------IANN-C-N--------------W--D-     82

h(f3)                           -------------IA-T----------
Amphihox-6      K--G-------------------K----K----IA--------------------W---N     87
mHoxA6          GR-G-------------------------IANA-C----------------W---N      83
mHoxB6          GR-G-----------------Y----------IA-A-C----------------W----     85
mHoxC6          -R-G--I-S-------------------IANA-C----------------W----        83
Antp            ---G-------------------------IA-A-C----------------W---N      88

h(g12)                          -------------IA-A----------
Amphihox-7      ---G-------------------K---------IA-A-C----------------W---N     87
mHoxA7          ---G-------------------------IA-A-C----------------W---H      88
mHoxB7          ---G-----------------Y----------IA-T-C----------------W---N     87
Ubx             -R-G-----------------Y-H---------MA-A-C----------------L---I     83

h(h2)                           -Y-------------------------
Amphihox-8      -R-G----S--------------K---------IA-A------------------L---A     85
mHoxB8          ---G----S----------L--P----K----V--A-------V----------W---N     85
mHoxC8          -RSG----S----------L--P----K----V--A-------V----------W---N     80
mHoxD8          ---G----S-F--------L--P----K----V--T-A-----V----------W---N     80
Abd-A           ---G------F----------------------IA-A-C----------------W---L     87

mHoxB9          SRKK-CP--K----------L--M----D--H-VAR--N-S---V----------M--MN     67
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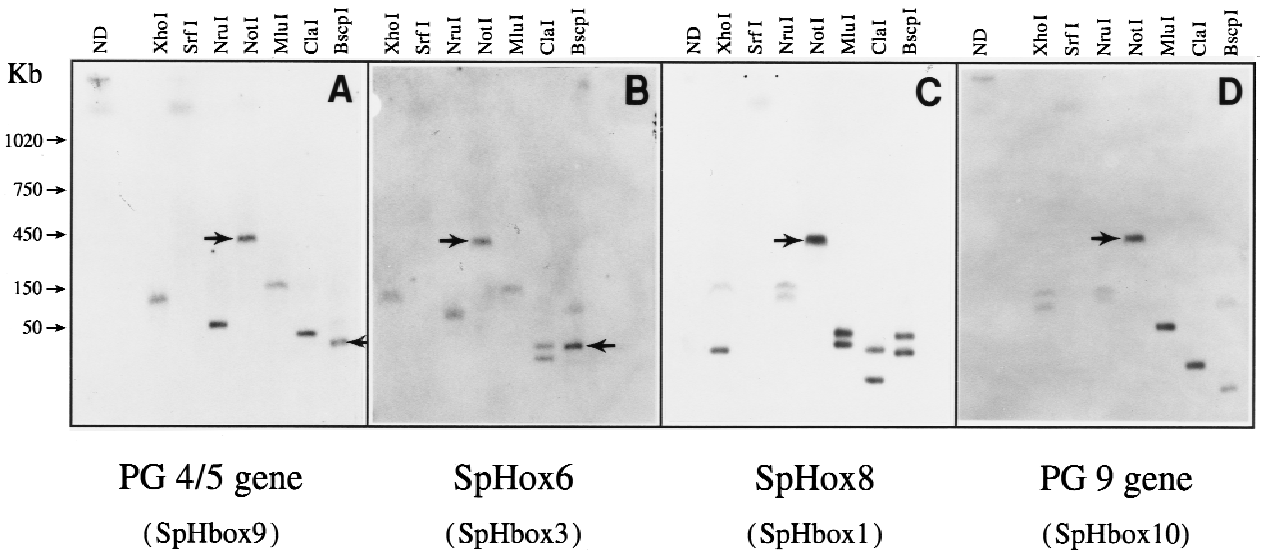

Fig. 4. Autoradiographs of blot hybridizations carried out on pulse field electrophoretic displays of *S. purpuratus* genomic DNA digested alternatively with seven different restriction enzymes. DNA from sperm of a single individual was used for this experiment. Procedures are described in Materials and Methods. The same blot was used for all four hybridizations, after stripping of previously bound radioactivity. Probes were obtained from regions of *Hox* genes excluding the homeobox in each case. A complete description of the *S. purpuratus Hox* gene cluster, and gene sequences, including probes, will be presented elsewhere. **A** Probes from a *Hox* gene assigned unequivocally by sequence to paralogous group 4 or 5, previously known as *SpHbox9*. **B** Probes from *Hox* gene previously known as *SpHbox3*, which, as desribed in text, is the *SpHox6* gene. **C** *SpHox8* probe, i.e., subclone pH1.8–3. **D** Probe from a *Hox* gene previously known as *SpHbox10* which by sequence is unequivocally a paralog group 9 gene.

Hox genes. On this basis *SpHbox1* is the paralog group 8 gene of *S. purpuratus.* This conclusion might require modification if there are more than three genes in the *S. purpuratus* genome in the paralog group 6–8 cluster, but our unpublished mapping and linkage data render this a very unlikely possibility. We note that on an entirely independent basis, in a phylogenetic sequence analysis, Popodi et al. (1996) hypothesized that the *Hbox1* gene is to be assigned to paralog group 8. We henceforth refer to the *S. purpuratus* gene encoded by clone pH1.8, and that previously designated *SpHbox1*, as *SpHox8.* It follows that the gene formerly known as *SpHbox6* is probably the *SpHox7* gene (not included in Fig. 4), and the gene formerly known as *SpHbox3* is probably the *SpHox6* gene.

SpHox8 Is Linked Within a Large, Single-Copy *Hox* Gene Cluster

Figure 4C and D shows that *SpHox8* is also linked to a paralog group 9 gene (rightward arrow). The same fragment reacts with the paralog group 4/5 gene, as shown in Fig. 4A. Therefore, this fragment, about 400 kb in length, includes a cluster of at least five *Hox* genes. An important additional conclusion from Fig. 4 is that this cluster occurs only once per haploid genome. Of all the bands shown in Fig. 4(A–D), 18 appear to be homozygous for sequence within the two terminal restriction sites and eight are heterozygous at least one site. This is typical and expected for a single-copy sequence element terminated by restriction sites, given the 4–5% sequence polymorphism of the *S. purpuratus* genome (Britten et al. 1978; R.A. Cameron, unpublished data). The large number of single, i.e., homozygous, bands precludes the possibility that there is more than one *Hox* gene cluster.

In summary, we have identified the paralog group 8 *Hox* gene of the *S. purpuratus Hox* gene cluster, demonstrated its linkage to other *Hox* genes, and determined the complete open reading frame. This information will now permit a detailed exploration of *SpHox8* function in echinoid development.

Acknowledgments. It is a pleasure to acknowledge the assistance of Dr. Tom Wilkie in the design of oligonucleotides and of Valeria Mancino with pulse field gel electrophoesis. We are grateful to Drs. Kevin Peterson and Roy Britten of this Institute for reviews of drafts of the manuscript. This work was supported by NICHHD grant 05753 and by the Stowers Institute for Medical Research.

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