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ORIGINAL ARTICLE

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Formation of the chordamesoderm in the amphioxus embryo: Analysis with *Brachyury* and *fork head/HNF-3* genes

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Abstract The embryonic development of amphioxus (cephalochordates) has much in common with that of vertebrates, suggesting a close phylogenetic relationship between the two chordate groups. To gain insight into alterations in the genetic cascade that accompanied the evolution of vertebrate embryogenesis, we investigated the formation of the chordamesoderm in amphioxus embryos using the genes *Brachyury* and *fork head/HNF-3* as probes. *Am(Bb)Bra1* and *Am(Bb)Bra2* are homologues of the mouse *Brachyury* gene isolated from *Branchiostoma belcheri*. Molecular phylogenetic analysis suggests that the genes are independently duplicated in the amphioxus lineage. Both genes are initially expressed in the involuting mesoderm of the gastrula, then in the differentiating somites of neurulae, followed by the differentiating notochord and finally in the tail bud of ten-somite stage embryos. On the other hand, *Am(Bb)fkh/HNF3-1*, an amphioxus (*B. belcheri*) homologue of the *fork head/HNF-3* gene, is initially expressed in the invaginating endoderm and mesoderm, then later in the differentiating notochord and in the tail bud. With respect to these two types of genes, the formation of the notochord and tail bud in amphioxus embryos shows similarity and dissimilarity with that of the notochord and tail bud in vertebrate embryos.

Key words Chordamesoderm formation · Amphioxus embryos · *Brachyury* gene · *fork head/HNF-3* gene · Evolution of chordates

Introduction

The embryogenesis of amphioxus (the subphylum Cephalochordata) was first described around the 1880s (Kowalevsky 1876; Hatschek 1881), then Conklin (1932) and others reported more details. Despite several experimental investigations (Tung et al. 1958, 1962), the amphioxus embryo did not attract the attention of many embryologists. Recently however, studies with molecular probes have revealed the amphioxus embryo as a prototype of vertebrate embryogenesis (Holland et al. 1992, 1995). Amphioxus shares many embryological and morphological features with vertebrates, including a notochord, a dorsal nerve cord, pharyngeal gill slits and endostyle (Brusca and Brusca 1990). However, amphioxus does not develop jaws like vertebrates and migrating neural crest cells have not been described in amphioxus embryos.

Cephalochordates form one monophyletic chordate group with urochordates and vertebrates (Wada and Satoh 1994). Therefore, amphioxus occupies an important phylogenetic position in terms of understanding the origin and evolution of chordates. Moreover, Garcia-Fernàndez and Holland (1994) found that amphioxus has one Hox cluster in a genomic organization similar to that of vertebrates. The single Hox cluster in the amphioxus genome duplicated twice into four clusters during the evolution of vertebrates (reviewed by Holland et al. 1994; Holland and Garcia-Fernàndez 1996), reconfirming their position intermediate to that of vertebrates.

In this study, we examined amphioxus development, and especially the formation of the chordamesoderm, using the *Brachyury* (*T*) and *fork head/HNF-3* genes as molecular probes, because both types of genes are implicated in notochord formation. *Brachyury* is a mouse mutation that causes short-tailed mice as the heterozygous phenotypes (Chesley 1935; for reviews see Herrmann and Kispert 1994). In 1990, the gene was cloned by Herrmann et al. Since then homologues have been identified in *Xenopus* (*Xbra;* Smith et al. 1991), zebrafish (*no tail*; Schulte-Merker et al. 1992), ascidian (*As-T*; Yasuo and Satoh 1993, 1994), *Drosophila* (*Trg*; Kispert et al. 1994), chicken (Kispert et al. 1995a), sea urchin (*HpTa*; Harada et al. 1995) and amphioxus (Holland et al. 1995; Terazawa and Satoh 1995). *Brachyury* encodes a transcriptional factor (Kispert et al. 1995b). The gene is thought to have two independent roles, notochord differ-

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entiation and posterior mesoderm formation (reviewed by Herrmann 1995).

The vertebrate *fork head/HNF-3* genes are expressed in three germ layers within the midline structure, including the organizer (reviewed by Kaufmann and Knöchel 1996). In particular, mouse *HNF-3β* is required for the formation of the notochord and the floor plate of the neural tube (Sasaki and Hogan 1994; Ang and Rossant 1994; Weinstein et al. 1994). *Drosophila fork head* has important roles in development of the tip of the fore-gut and hind-gut (Weigel et al. 1989). *Fork head/HNF-3* genes are characterized by a 110 amino-acid DNA-binding domain (Weigel and Jäckle 1990; Lai et al. 1991). The genes *Pintallavis* (Ruiz i Altaba and Jessell 1992) and *axial* (Strahle et al. 1993) have been isolated from *Xenopus* and zebrafish, respectively, and more related genes have been cloned (Kaufmann and Knöchel 1996).

We report here the expression of amphioxus *Brachyury* and *fork head/HNF-3*-related genes in relation to axial mesoderm formation in the embryo. We isolated cDNA clones for two *Brachyury* genes and for a *fork head/HNF-3*-related gene, respectively. Both the *Brachyury* genes and the *fork head*-related gene were expressed in a manner similar to that of their vertebrate counterparts, suggesting that some developmental mechanisms are shared by these two groups. However, there were some differences between the two groups, suggesting important implications for the evolution of developmental mechanisms in chordates.

Materials and methods

Biological materials

Branchiostoma belcheri was collected during the spawning season (June and July) at the Ariake-Kai near the Aizu Marine Biological Station of Kumamoto University, Kyushu, Japan. Naturally spawned eggs were fertilized and raised at room temperature (RT). They developed into blastulae, gastrulae, neurulae and 10- to 11-somite stage embryos, about 4, 5, 10 and 12 h after fertilization, respectively. Neurulae hatched after about 10 h of development.

Eggs and embryos at various stages were collected by low-speed centrifugation, washed several times with filtered seawater and fixed for in situ hybridization as whole-mount specimens or frozen quickly in chilled ethanol for RNA isolation. Adults were also frozen quickly for RNA isolation. We also obtained fixed specimens from Dr. Hidetoshi Saiga of Tokyo Metropolitan University, that were collected in the vicinity of the National Institute of Oceanology, Qingtao, China.

Construction of an amphioxus cDNA library

Total RNA was extracted from embryos and adults as described by Chomczynski and Sacchi (1987) and Lemaire and Gurdon (1994). Poly(A)$^+$ RNA was purified using Oligotex-dT30 beads (Roche Japan, Tokyo, Japan) according to the manufacturer's instructions. An amphioxus cDNA library was constructed by means of the λZAP II cDNA Synthesis kit (Stratagene, La Jolla, Calif.), using a mixture of adult poly(A)$^+$ RNA and gastrula total RNA.

Polymerase chain reaction (PCR) amplification of T and fork head domains

T domain

The amino acid sequences of the T domain of *Brachyury* (*T*) gene products are highly conserved among the mouse (Herrmann et al. 1990), *Xenopus* (Smith et al. 1991), zebrafish (Schulte-Merker et al. 1992) and ascidians (Yasuo and Satoh 1994). The sense strand oligonucleotide corresponding to the amino acid sequence Y(I/V)HPDSP and the antisense strand corresponding to NPFAK(G/A)(L/F) were constructed using an automated DNA synthesizer (Applied Biosystem Inc, Fostercity, Calif.). Fragments were amplified using a cDNA library as a template, by means of 30 PCR cycles at 94°C (1 min), 50°C (2 min) and 72°C (1 min). The fragments were then purified by electrophoresis and subcloned into pBluescriptII SK(+) (Stratagene).

Fork head (Fkh) domain

The amino acid sequences of the Fkh domain, especially of *fork head/HNF-3* genes, are also highly conserved among the rat (Lai et al. 1991, 1993), *Xenopus* (Ruiz i Altaba and Jessell 1992; Ruiz i Altaba et al. 1993), zebrafish (Strahle et al. 1993) and *Drosophila* (Weigel et al. 1989). We prepared sense and antisense oligonucleotides corresponding to HAKPPYS and (F/Y)W(A/T)LHP, respectively. Thirty PCR cycles of 1 min at 94°C, 2 min at 42°C, and 1 min at 72°C followed.

Isolation of cDNA clones

Brachyury *(*T*) genes*

The candidate cDNA fragment was random-prime labelled with [^{32}P]-dCTP. Using this probe, we screened 3.0 × 10^5 pfu of the amphioxus cDNA library under high-stringency conditions as follows: hybridization in 5 × SSPE, 0.1% sodium dodecyl sulphate (SDS), 1 × Denhardt's solution, 100 μg/ml denaturated salmon sperm DNA, 50% formamide at 42°C for 12 h followed by two washes in 2 × SSC containing 0.1% SDS at 55°C for 15 min and one in 1 × SSC containing 0.1% SDS at 55°C for 10 min. We obtained two cDNA clones and subcloned them into pBluescriptII SK(+). Both strands of the clones were determined using an ABI PRISM auto sequencer with a dye primer cycle sequencing kit (Perkin Elmer).

Fork head/HNF-3 *related genes*

We screened 3.0 × 10^5 pfu of the cDNA library under the high stringency conditions described above. This procedure yielded three cDNA clones that partial sequencing showed were identical. Therefore, we subcloned and sequenced the longest of them.

Sequence comparisons and molecular phylogenic analysis

Amino acid sequences of *Brachyury* gene products or those of *fork head/HNF-3*-related gene products were aligned and gaps were introduced for maximal similarity. Molecular phylogenetic relationships among the gene products were estimated by means of Neighbor-Joining (Saitou and Nei 1987) using the PHYLIP ver 3.5c package (Felsenstein 1993). The degree of support for internal branches of each tree was assessed by bootstrap resampling of the data (Felsenstein 1985).

In situ hybridization

Whole-mount in situ hybridization proceeded essentially as described by Holland et al. (1992). Embryos were fixed in 4% para-

formaldehyde in 0.1 M MOPS (pH 7.5), 0.5 M NaCl at 4°C overnight. Pre-hatched embryos were peeled off the fertilization membrane with sharpened tungsten needles. RNA probes were synthesized following the instructions supplied with the kit (Boehringer Mannheim DIG RNA labelling kit), then brought to a uniform length of about 500 bp by alkaline hydrolysis. Templates for the probes were full-length cDNAs of each clone. The reacted specimens were observed using an Olympus BX60 microscope and photographed in 80% glycerol in PBT. Some specimens were sectioned after ethanol dehydration and embedded in polyester wax (BDH Laboratory Supplies).

Results

Isolation and characterization of cDNA clones for the *Brachyury* (*T*) genes of the amphioxus *B. belcheri*

Using degenerate oligonucleotides primers, we amplified PCR fragments of about 300 bp from a *B. belcheri* cDNA library. The fragments contained sequences that were conserved in vertebrate and invertebrate *Brachyury* genes. Using the PCR fragments as a probe, we screened the library and isolated two cDNA clones (partial sequence of one of them was previously described in a report by Terazawa and Satoh 1995). The longer cDNA clone consisted of a 3056-bp insert that contained a single 1341-bp open reading frame encoding 447 amino acids (Fig. 1; the sequence will appear under the DDBJ/EMBL/GenBank accession number D84219). The insert of the shorter clone was 1939 bp long, with a single 1320-bp open reading frame encoding 440 amino acids (Fig. 1; the sequence will appear under the DDBJ/EMBL/GenBank accession number D84220). As shown in Fig. 1, the predicted amino acid sequences of these clones shared a highly conserved T domain in the N terminal region with mouse Brachyury (T) (Herrmann et al. 1990). Therefore, we named the former gene, *Am(Bb)Bra1* [*Am*phioxus (*B. b*elcheri) *Bra*chyury gene *1*] and the latter gene, *Am(Bb)Bra2* [*Am*phioxus (*B. b*elcheri) *Bra*chyury gene *2*].

Am(Bb)Bra1 and *Am(Bb)Bra2* relationships with other *Brachyury* genes

Holland et al. (1995) have isolated cDNA clones for two closely related amphioxus *Brachyury* genes, *AmBra1* and *AmBra2* from *B. floridae*. As shown in Fig. 1, the amino acid sequences of Am(Bb)Bra1 and Am(Bb)Bra2 showed a high degree of similarity with AmBra1 and AmBra2, respectively, over the entire amino-acid coding region of the polypeptides. To clarify the relationships between the four amphioxus *Brachyury* genes, we constructed a molecular phylogenetic tree of the chordate *Brachyury* gene products taking ascidian *Brachyury* (*As-T*) as an outgroup. When we used 174 confidently aligned sites of the T domain, the 4 amphioxus genes, *Am(Bb)Bra1*, *Am(Bb)Bra2*, *AmBra1* and *AmBra2*, formed a discrete group (data not shown). However, the internal branch of the 4 amphioxus Brachyury polypeptides was not clear. We therefore constructed another tree using 350 sites including the T domain and the following C-terminal region. Mouse T, chick T and Xbra were taken as the outgroup. As shown in Fig. 2, Am(Bb)Bra1 and Am(Bb)Bra2 formed a discrete group with AmBra1 and AmBra2, respectively. The branching of the 4 gene products was supported by 100% bootstrapping. This tree therefore supported the notion suggested by Holland et

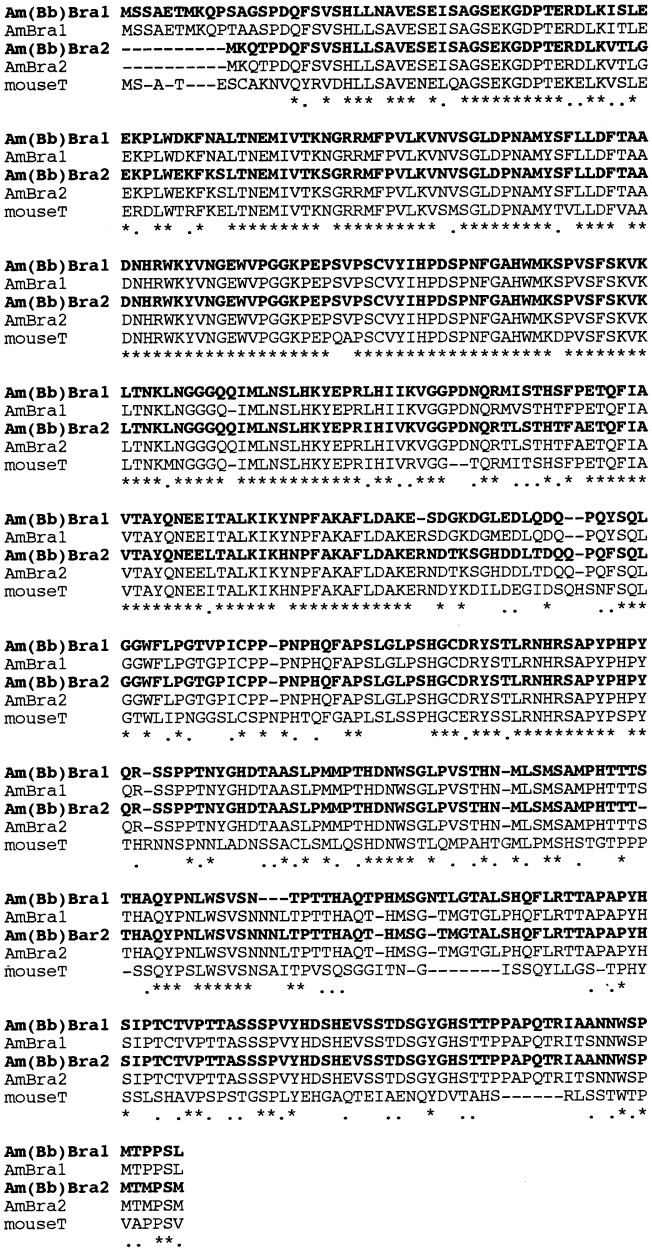

Fig. 1 A comparison of the amino acid sequences of four amphioxus *Brachyury* gene products with that of mouse *Brachyury* (*T*). *Asterisks* indicate sequence identity among five gene products, whilst *dots* indicate that of three or four proteins. Gaps (-) are inserted for maximal similarity. AmBra1 and AmBra2 are *Brachyury* gene products of the amphioxus *B. floridae* reported by Holland et al. (1995); mouse T was from Herrmann et al. (1990)

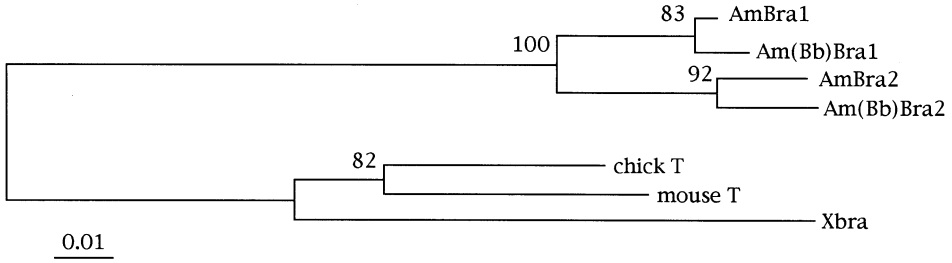

Fig. 2 Relationships among four amphioxus T products. Am(Bb)Bra1 and Am(Bb)Bra2 of the Asian amphioxus (*B. belcheri*) were characterized in this study and AmBra1 and AmBra2 were determined by Holland et al. (1995) from a floridian (*B. floridae*). The mouse T, chick T and Xbra were taken as outgroups. Three hundred and fifty amino acid sites of the T domain and the following C-terminal of each protein were analysed. The tree was constructed based on Neighbor-Joining, and assessed by bootstrapping (100 times; percentages are indicated by numbers; 0.01, unit of distance). Sources: mouse T, Herrmann et al. (1990); chick T, Kispert et al. (1995); Xbra, Smith et al. (1991)

al. (1995), that a single ancestral *Brachyury* (*T*) gene independently duplicated in the amphioxus lineage.

Expression of *Am(Bb)Bra1* and *Am(Bb)Bra2* during *B. belcheri* embryogenesis

We examined the spatial expression of the *Am(Bb)Bra1* and *Am(Bb)Bra2* genes by in situ hybridization of whole-mount specimens (Fig. 3). Because we could not generate specific probes from the 3' untranslated regions of these genes, RNA probes were generated from full-length cDNAs as templates. Both genes showed a very similar spatial expression pattern. We think that detected signals may be a summation of expression of the two genes. Here, we only describe the results of *Am(Bb)Bra2*. The sense probes did not generate hybridization signals above the background level (data not shown).

In situ hybridization could not be applied to embryos at the cleavage and blastula stages because they collapsed during the manipulations. At the mid-gastrula stage, hybridization signals were evident in the involuting cells (Fig. 3A, B). When viewed from the blastopore (Fig. 3A), the dorsal side of the blastopore was flattened and the signals were more intense there than at the ventral side (Fig. 3A, C). As embryos developed from the late-gastrula to the early-neurula stage, the dorsal side was further flattened (Fig. 3D). The signals were intense at the presumptive somite region, which contained one or two pairs of bands in the dorsal view (Fig. 3E). In neurulae, the neural plate was folded off from the ectoderm (Fig. 3G). *Am(Bb)Bra2* was expressed in the paraxial mesoderm (Fig. 3H). From the lateral view (Fig. 3I), *Am(Bb)Bra2* was expressed in the presumptive somite region as three pairs of bands. In addition, signals became evident in the presumptive notochord region (Fig. 3H, I). However, at the 10- to 11-somite stage (Fig. 3J), signals became undetectable in the somite. In contrast, signals were evident in the notochord at this stage (Fig. 3J) and intense in the anterior tip of the notochord as well as the tail bud (Fig. 3 K).

Isolation and characterization of a cDNA clone for an amphioxus *fork head/HNF-3*-related gene

The *fork head/HNF-3* genes encode transcription factors (Weigel et al. 1989; Weigel and Jäckle 1990) that form a large family sharing the fork head (Fkh) domain (Kaufmann and Knöchel 1996). We generated a cDNA fragment of about 250 bp using a pair of degenerate oligonucleotide primers and used it as a probe to screen a *B. belcheri* cDNA library. Partial sequencing showed that the three isolated clones were identical. Therefore, we further analysed the longest. The insert consisted of 2366 bp containing a single 1215-bp open reading frame that encoded a polypeptide of 405 amino acids (Fig. 4; the sequence will appear under the DDBJ/EMBL/GenBank accession number D84221). The predicted amino acid sequence shared a highly conserved Fkh domain (Fig. 4) within the N-terminal half and the transactivation site of the partial C-terminal half of this clone with mouse *HNF-3*α, β, and γ genes (Kaestner et al. 1994), *Xenopus Pintallavis* (Ruiz i Altaba and Jessell 1992) and zebrafish *axial* (Strahle et al. 1993; Fig. 4). We therefore

Fig. 3A–K The spatial expression of *Am(Bb)Bra2*, as revealed by whole-mount in situ hybridization with a digoxigenin-labelled antisense probe. Embryos are at the mid gastrula (**A**—**C**), late gastrula (**D**—**F**), neurula (**G**—**H**) and 10- to 11-somite stages (**J**, **K**), about 6, 9, 10 and 12 h after fertilization, respectively. **A** A mid gastrula, viewed from the blastopore (*bp*), showing hybridization signals in cells surrounding the blastopore (*d* dorsal, *v* ventral side of the embryo). **B** Dorsal view of the same embryo showing intense signals in cells involuting into the archenteron (*ar*; *a* anterior, *p* posterior, *ect* ectoderm, *en* endoderm of the embryo). **C** A mid gastrula viewed from the lateral side, showing signals in involuting cells. **D** A late gastrula viewed from the blastopore. More intensive signals are detected around both shoulders (*arrows*) of the flattened dorsal side of the embryo. **E** The same embryo viewed from the dorsal side. Transcripts are evident in the involuting cells and in a pair of bands in the paraxial mesoderm (*arrows*). **F** Lateral view of a late gastrula. The dorsal side is flattened and emits stronger signals than that of the ventral side. **G** A neurula viewed from the blastopore. Non-neural ectoderm is beginning to close (*arrowheads*). **H**, **I** The same embryo, dorsal (**H**) and lateral (**I**) views. **H** Signals are now detected in the presumptive notochord (*nc*) in addition to the somite (*so*) region (*ms, muscle*). Signals of the presumptive somite appear as bands (*arrows in* **I**). **J**, **K** An embryo at the somatic stage. Transcripts are evident in the notochord and tail bud (*nt* neural tube; *scale bar* 25 μm for all panels)

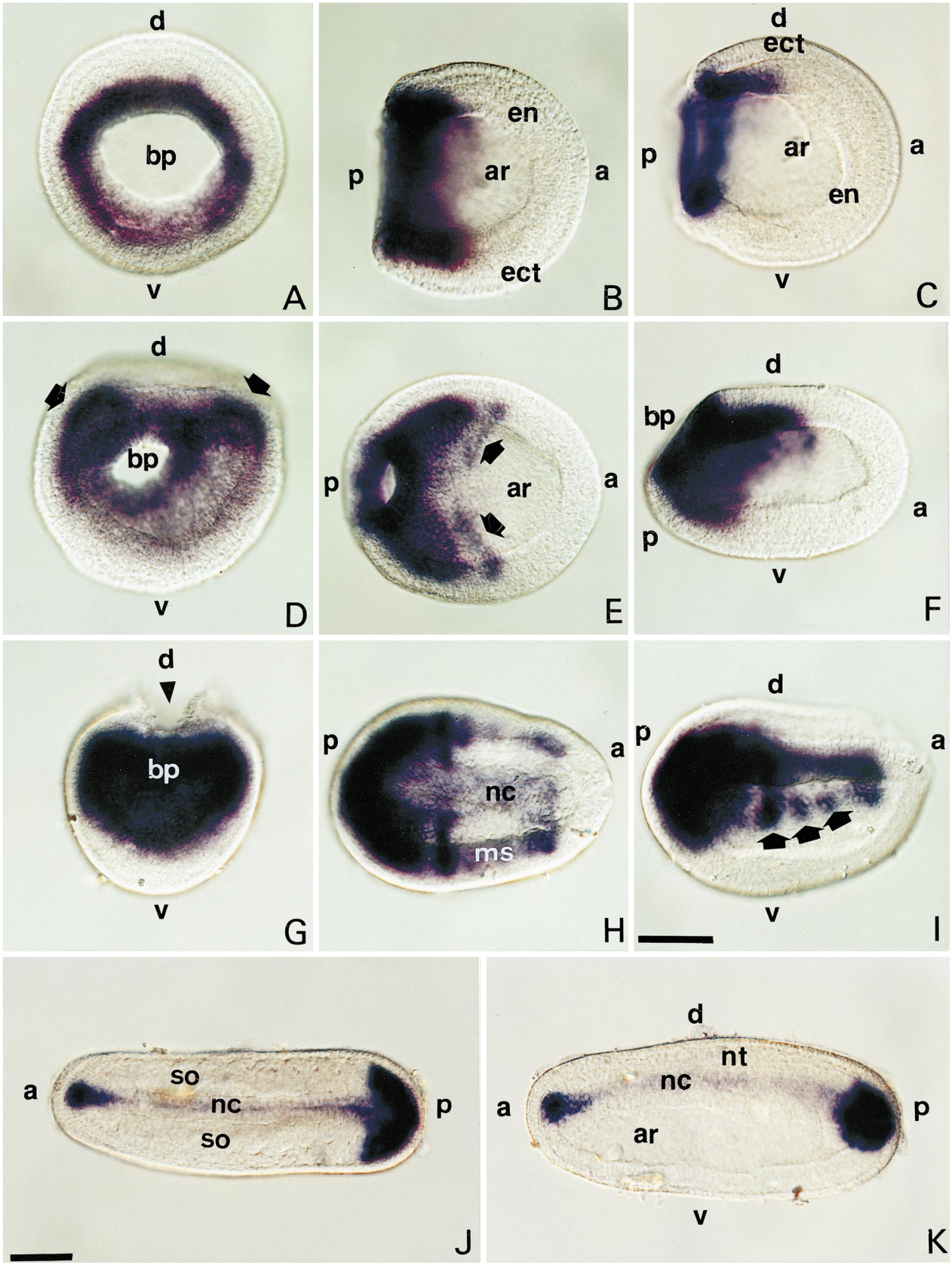

Fig. 3 for legend see page 4

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Am(Bb)fkh/HNF3-1 MLSAPKGYPTG----T---------TMNTMGMNTMSSMGG-MNHA-TYTG
HNF3-alpha       MLGTVKMEGHE-SNDWNSYYADTQEAYSSVPVSNMNSGLGSMNSMNTYMT
HNF3-beta        MLGAVKMEGLE-PSDWSSYYA---EPEGYSSVSNMNAGLG-MNGMNTYMS
HNF3-gamma       MLGSVKMEAHD-LAEWSYYPE---AGEVYSPVNPVPTMAP----LNSYMT
pintallavis      MLNRVKLEIKD-PMDWNTMYQ---ENEMYSGIHNMTNVLP----SNSFLP
axial            MLGAVKMEGHEHAADWSTYYG---EPECYTSVSNMNTGLG-MNSMNTYMT
                 **   *                          .

Am(Bb)fkh/HNF3-1 TGP--------------VNPGAYSASAYSGTMNGMGTGYPAAGGMG----
HNF3-alpha       MNTMTTSGNMTPASFNMSYANTGLGAGLSPGAVAGMPGASAGAMNS--MT
HNF3-beta        MSAAAMGGGSGNMSAGSMNMSSYVGAGMSPSLAGMSPGAGAMAGMSGSAG
HNF3-gamma       LNP---------------LSSPYPPGGLQASPLPTGPLAPPAPTAPLGP-
pintallavis      NDVSTV-----------TTSMPYMSNGLPGPVTSIQGNIGSLGSMPQG--
axial            MSGMSS---TANMTAANTMNMSYVNTGMSPSMTGMSPGTGAMAGMG----
                                  .   .

Am(Bb)fkh/HNF3-1 ----------------GLQSYPAGSVNAMGTMQTMNN---------MALNRN
HNF3-alpha       AAGVTAMGTALSPGGMGSMGAQPVTSMNGLGPYAAAMNPCMSPMAYAPSN
HNF3-beta        AAGVAGMGPHLSP-SLSPLGGQAAGAMGGLAPYANMN--SMSPMYGQ-AG
HNF3-gamma       --------------TFPSLGTGGS-TGGSASGYVAPG-------PGL---
pintallavis      --------------MVGSLAPPPS-TAAYPLGYCQ----------GE---
axial            -AGMTGMSAALSP-TMSPMAAQAP-SMNALTSYSNMN--AMSPMYGQ-SN
                                        .

Am(Bb)fkh/HNF3-1 AIAERE------KAYRRSYTHAKPPYSYISLITMSIQSSPNKMVTLAEIY
HNF3-alpha       LGRSRAGGGGDAKTFKRSYPHAKPPYSYISLITMAIQQAPSKMLTLSEIY
HNF3-beta        LSRARD-----PKTYRRSYTHAKPPYSYISLITMAIQQSPNKMLTLSEIY
HNF3-gamma       -VHGKEM----AKGYRRPLAHAKPPYSYISLITMAIQQAPGKMLTLSEIY
pintallavis       SEFQRD----PRTYRRNYSHAKPPYSYISLITMAIQQAPNKMMTLNEIY
axial            INRSRD-----PKTYRRSYTHAKPPYSYISLITMAIQQSPSKMLTLSEIY
                     .       . ..*   **************.** .* **.** ***

Am(Bb)fkh/HNF3-1 QFIMDLFPYYRQNQQRWQNSIRHSLSFNDCFVKVPRTPDRPGKGSYWTLH
HNF3-alpha       QWIMDLFPYYRQNQQRWQNSIRHSLSFNDCFVKVARSPDKPGKGSYWTLH
HNF3-beta        QWIMDLFPFYRQNQQRWQNSIRHSLSFNDCFLKVPRSPDKPGKGSFWTLH
HNF3-gamma       QWIMDLFPYYRENQQRWQNSIRHSLSFNDCFVKVARSPDKPGKGSYWALH
pintallavis      QWIIDLFPYYRQNQQRWQNSIRHSLSFNDCFVKVPRSPEKPGKGSYWTLH
axial            QWIMDLFPFYRQNQQRWQNSIRHSLSFNDCFLKVPRSPDKPGKGSFWTLH
                 * *.****.**.********************.** *.*..*****.*.**

Am(Bb)fkh/HNF3-1 PEAGNMFENGCYLRRQKRFKCEKKLAMKMAQQQSARDTPNPGTENTAVAP
HNF3-alpha       PDSGNMFENGCYLRRQKRFKCEKQPGA----GGGSGGGGSKGGPESRKDP
HNF3-beta        PDSGNMFENGCYLRRQKRFKCEKQLAL-----KEAAGAASSGGKKT----
HNF3-gamma       PSSGNMFENGCYLRRQKRFKLEEKAKK----GNSATSASRNG--------
pintallavis      PESGNMFENGCYLRRQKRFKCER-SKS----GEGEKKVNKPGEE------
axial            PDSGNMFENGCYLRRQKRFKCDKKLS------KDPSRKTSEGG-------
                 *..*****************...                *

Am(Bb)fkh/HNF3-1 TTSPEAASTPTTTSNGASTLQPLQPINTPSPNPQEQ---QQHQHHQHQQQ
HNF3-alpha       SGPGNPSAESPLHWGVHGKASQLEGAPAPGPAASPQTLDHSGATATGGAS
HNF3-beta        -APGSQASQAQLGEAAGSASETPAGTESPHSSASP-----CQEHKRGGLS
HNF3-gamma       --TAGSATS--------------ATTTAATAVTSP-----AQPQPTPSEP
pintallavis      --TGGNLKENPL---G---YDDCSSSRSPQAAVND-----GGRDSTGSSI
axial            -----------S---N-SSSESCNGNESPHSNSS------SNELKR-SLS
                                                .

Am(Bb)fkh/HNF3-1 HQQQPPVQTTQPQDMQQHAQQHQGLPARP--IPQQSSLPMSMGGYFSPEH
HNF3-alpha       ELKSPASSSAPPI-----SSGPGALASVPPSHPAHGLAP-----HESQLH
HNF3-beta        ELKGAPASALSPP-------EPAPSPGQQQQAAAHLLGPPHHPGLPPEAH
HNF3-gamma       EAQSGDDVGGLDC-----ASPPSSTP-----YFSGLELPGELKLD-----
pintallavis      HQACGSSPVGLSP-----TSEQAGTASQLM-YPLGLSNDGYLGLVGEDVH
axial            DMK--SGQGLSP--------DHAASPTSQ---AQHLLAQ-HHSVLAHEGH

Am(Bb)fkh/HNF3-1 LR--AAHGFTHPFSISNLMSQE------------HKPDLKEYAAMG--YS
HNF3-alpha       LKGDPHYSFNHPFSINNLMSSSE---------QQHKLDFKAYEQALQ-YS
HNF3-beta        LKPEHHYAFNHPFSINNLMSSEQQHHHSHHHHQPHKMDLKAYEQVMHYPG
HNF3-gamma       ----APYNFNHPFSINNLMSEQTS--------TPSKLDVGFG-----NYS
pintallavis      LK-HDPFSGRHPFSITQLMSSEQDQT------YANKMEMCPTTDHLVHYS
axial            LKPEHHYSFNHPFSINNLMSSEQ---------QHHKMDLKTYEQVMH-Y-
                        ..******..***              .*..          ..

Am(Bb)fkh/HNF3-1 GYNSMSPTGVPKTTMSMDS--------MGTDYYQGYVPQHSQPSSL
HNF3-alpha       PYGATLPASLPLGSASVATRSPIEPSALEPAYYQGVYSRPVLNTS-
HNF3-beta        GYGSPMPGSLAMGPVTNKAGLDASPLAADTSYYQGVYSRPIMNSS-
HNF3-gamma       GYGAE---------------SGE-----PGVYYQSLYSRSLLNAS-
pintallavis      ---SDYHNMASKNGLDMQTSSST-----DNGYYANMYSRPIL-SSL
axial            GYGSPMAGTLSMGSMASKAGLDS-P---DTSYYQGV----------
                 ...                          **.
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Fig. 4 A comparison of the amino-acid sequences of Am(Bb)fkh/HNF3-1 with those of mouse HNF-3α, β, γ (Kaestner et al. 1994), *Xenopus* Pintallavis (Ruiz i Altaba and Jessell 1992) and zebrafish axial (Strahle et al. 1993). *Asterisks* indicate sequence identity, whilst *dots* indicate similar amino-acids in four or five proteins. Gaps (-) were inserted for maximal similarity)

designated this gene as *Am(Bb)fkh/HNF3-1* (*Am*phioxus *B. b*elcheri *f*ork *h*ead/*HNF-3*-related gene *1*).

The relationship of *Am(Bb)fkh/HNF3-1* with other Fkh domain genes

To understand the relationship of *Am(Bb)fkh/HNF3-1* to other members of the family, we constructed a molecular

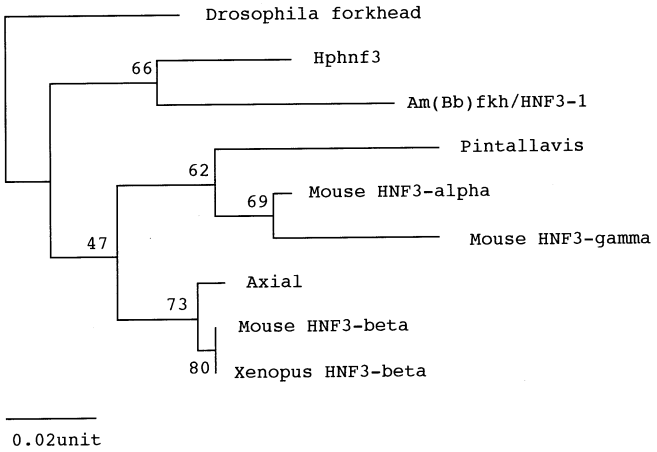

Fig. 5 Relationship of Am(Bb)fkh/HNF3-1 to other fork head-related gene products. The molecular phylogenetic tree was obtained by Neighbor-Joining, using 86 amino-acid sites of the fork head domains. Percentages of 100 bootstrap operations are indicated on each branch (0.01, unit of distance). The sources are mouse HNF-3α, β, γ, Kaestner et al. (1994); *Xenopus* HNF-3β, Pintallavis, Ruiz i Altaba and Jessell (1992), Ruiz i Altaba et al. (1993); zebrafish axial, Strahle et al. (1993); *Drosophila* fork head, Weigel et al. (1989); and sea urchin Hpfkh, Harada et al. (1996)

phylogenetic tree by the Neighbor-Joining method. Eighty-six confidently aligned amino acid sites of the Fkh domain were used in the analysis. The tree suggested that *Am(Bb)fkh/HNF3-1* forms a discrete subgroup with the class I genes classified by Sasaki and Hogan (1993) that includes mouse *HNF-3α, β, γ, Xenopus Pintallavis* and *HNF-3β*, zebrafish *axial* and *Drosophila fork head* (data not shown). To clarify further the relationships of *Am(Bb)fkh/HNF3-1* to other class I members of the family, a hundred confidently aligned amino acid sites of the Fkh domain were used for the analysis. The tree shown in Fig. 5 suggests that *Am(Bb)fkh/HNF3-1* forms a subgroup with *Hphnf3*, a sea urchin homologue (Harada et al. 1996). These two invertebrate genes are closer to each other than to any single vertebrate *HNF-3* gene. The tree also suggests two separate duplications in the lineage leading to vertebrates, the first including *HNF-3β* and *axial* and the other including *HNF-3α, HNF-3γ* and *Pintallavis*.

Spatial expression of *Am(Bb)fkh/HNF3-1* during amphioxus development

Zygotic transcripts of mouse *HNF-3β* (Sasaki and Hogan 1993) or *Xenopus Pintallavis* (Ruiz i Altaba and Jessell 1992) first appear in the organizer region immediately before gastrulation is initiated. However, we could not apply in situ hybridization to amphioxus embryos at earlier stages because they collapsed during the procedure. At the mid-gastrula stage, we distinguished the dorsal and ventral side of the embryo from the shape of the blastopore. We detected weak signals (after 72 h of reaction) in the most anterior endoderm and involuting cells at the dorsal side of the blastopore (Fig. 6A, B). As the

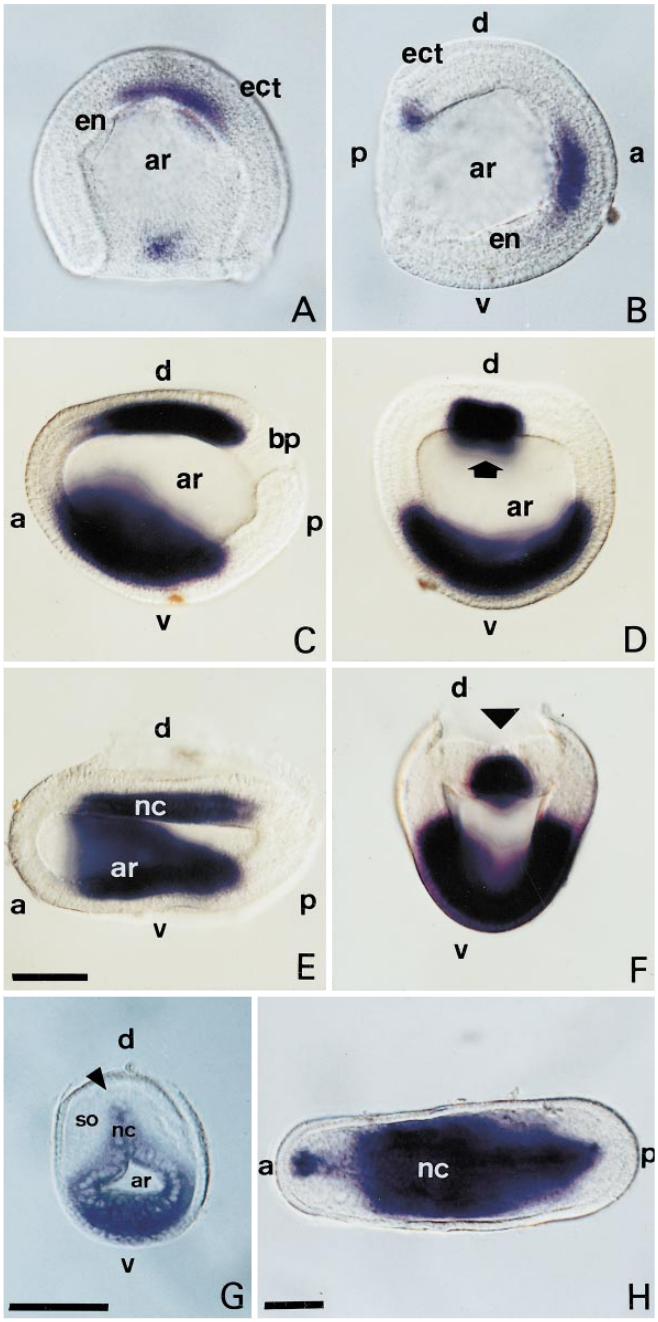

Fig. 6A–H The spatial localization of *Am(Bb)fkh/HNF3-1* transcripts in amphioxus embryos. The development stages are the same as those shown in **Fig. 3**. **A** Dorsal and **B** lateral views of a mid gastrula, showing hybridization signals in the dorsal lip of the blastopore and the anterior-most region of the involuted endoderm (*ar* archenteron; *a* anterior, *p* posterior, *d* dorsal, *v* ventral, *en* endoderm, *ect* ectoderm of the embryo). **C** Lateral and **D** anterior views of a late gastrula. Signals are seen in the presumptive notochord (*nc*, *arrow* in **D**) and in the presumptive endoderm (*ar* archenteron). **E**, **F** A neurula from lateral (**E**) and anterior (**F**) views. The *arrowhead* in **F** indicates elimination of the neural plate. Transcripts are localized in a similar way to those of the late gastrula. **H** Dorsal view of the 10- to 11-somite stage and its transverse section (**G**). Signals are evident in the notochord (*nc*) and the archenteron, but not in the neural tube (*arrowhead* in **G,** *so* somite; *scale bar* 25 μm for all panels)

embryo developed into late-gastrulae or early-neurulae, intense signals were detected in the axial mesoderm of the presumptive notochord region (Fig. 6C, D). In addition, intense signals were evident in the endoderm cells of the archenteron floor (Fig. 6D); the signals were stronger in its anterior region and weaker in its posterior region (Fig. 6C). In the embryo shown in Fig. 6F, the neural plate was folded off from the non-neural ectoderm. At this stage, signals in the axial mesoderm and endoderm were retained (Fig. 6E, F) but became undetectable from the neural plate. At the 10- to 11-somite stage, *Am(Bb)fkh/HNF3-1* was expressed in the notochord cells and in cells of the archenteron floor (Fig. 6H) but apparently not in the neural tube in transverse sections (Fig. 6G).

Expressions of *Am(Bb)Bra1*, *Am(Bb)Bra2* and *Am(Bb)fkh/HNF3-1* and elongation of the embryo

At the somite stage, the amphioxus embryo elongates intensively along the anteroposterior axis. We examined this phenomenon in detail in relation to expression of the *Am(Bb)Bra* and *Am(Bb)fkh/HNF-3* genes. Samples were also sectioned to examine gene expression inside the embryo (Fig. 7).

The *Am(Bb)Bra2* (Fig. 7A, D) and *Am(Bb)fkh/HNF3-1* (Fig. 7F) genes were both expressed in the anterior notochord. As evident in sections, both signals were found up to the anterior tip of the notochord (Fig. 7B, G). This is discussed below, since this expression has not been found in vertebrate counterparts.

When we examined the tail bud region, *Am(Bb)Bra2* was expressed in the tip of the notochord, of the somite mesoderm and of the archenteron (Fig. 7C, E). In addition, signals were detected in inner ectoderm cells of the tip region (Fig. 7E). On the other hand, *Am(Bb)fkh/HNF3-1* was expressed in the tips of the notochord and in cells of the archenteron floor and roof but not in cells of the lateral archenteron (Fig. 7H).

Discussion

Similarity and dissimilarity between *Brachyury* genes of amphioxus and of other chordates

The T-domain genes encode a family of transcription factors (Kispert et al. 1995b). In this study, we isolated the *T*-related genes, *Am(Bb)Bra1* and *Am(Bb)Bra2*, from the amphioxus *B. belcheri*. A molecular phylogenetic analysis demonstrated that both genes are homologues of mouse *Brachyury*. Therefore, they might have independently duplicated in the amphioxus lineage as pointed out by Holland et al. (1995), as *AmBra1* and *AmBra2*. Their position in the tree seems to reflect the evolutionary history of cephalochordates.

The results of the in situ hybridization showed that *Am(Bb)Bra1* and *Am(Bb)Bra2* are expressed in involuting mesodermal cells of the gastrulae, then in the noto-

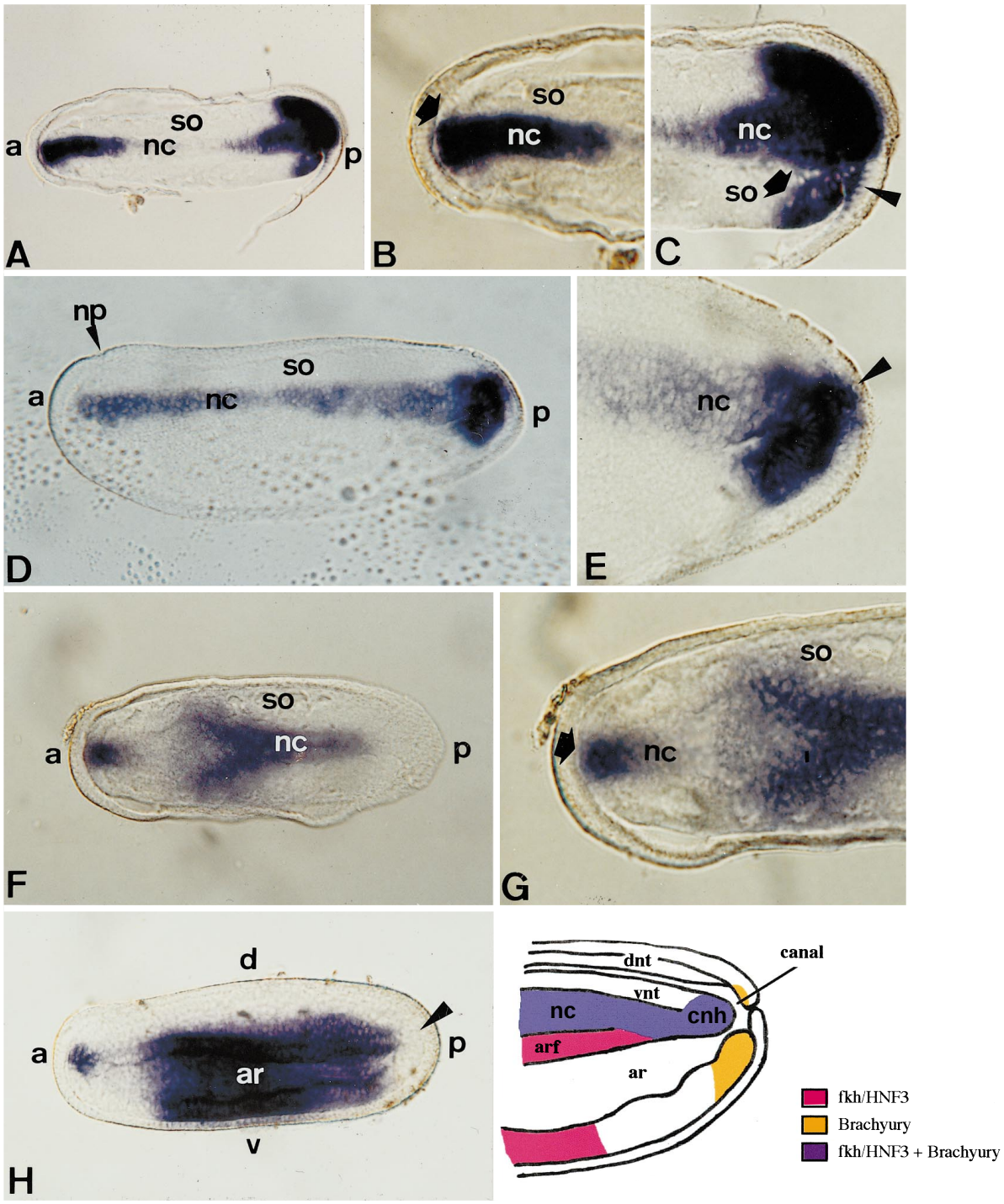

Fig. 7A–H Sections of 10- to 11-somite stage embryos showing the distribution of *Am(Bb)Bra2* and *Am(Bb)fkh/HNF3-1* transcripts. **A–E** *Am(Bb)Bra* expression. **A** Horizontal section, **B** anterior (*a*) and **C** posterior (*p*) magnification. **B** Signals are also evident at the anterior end of the notochord (*arrow*). **C** Signals are evident in the posterior tip of pre-somite mesoderm (*arrow*) and in the post-anal ectoderm (*arrowhead*; *so* somite, *nc* notochord of the embryo). **D** A vertical section and the posterior region are enlarged in **E**. **D** The neuropore (*np*, *arrowhead*) can be seen in the section, suggesting that the embryo was cut along the midline of the embryo. **E** *Am(Bb)Bra2* signals are located in the posterior tips of the all three germ layers (chordoneural hinge, archenteron and post-anal ectoderm, *arrowhead*). **F–G** *Am(Bb)fkh/HNF3-1* expression. **F** Horizontal section and its magnification (**G**). **G** Signals are detected in the anterior tip of the notochord (*arrow*). **H** Vertical section. Signals are absent from the posterior tip of the archenteron (*ar*) and the post-anal ectoderm (*arrowhead*; *d* dorsal, *v* ventral regions of the embryo). All of the pictures are summarized in the drawing at the *bottom right* (*pink Am(Bb)fkh/HNF3-1*, *orange Am(Bb)Bra2*, *red Am(Bb)Bra2* plus *Am(Bb)fkh/HNF3-1*, *canal* neurenteric canal, *arf* archenteron roof, *cnh* chordoneural hinge, *dnt* dorsal neural tube, *vnt* ventral neural tube)

chord and tail bud of the neurulae and somite-stage embryos. This pattern of *Am(Bb)Bra* expression corresponds to that of vertebrate *Brachyury* genes (Herrmann 1995). However, the role of amphioxus *Brachyury* genes does not always seem identical to that of vertebrates. In the middle of vertebrate gastrulation, the *Brachyury* gene is expressed in involuting cells and in the presumptive notochord region. However, in amphioxus, we did not detect *Brachyury* gene expression in the presumptive notochord of the gastrulae (Fig. 3E; Terazawa and Satoh 1995). This is also true of *B. floridae* (Holland et al. 1995). *Am(Bb)Bra2* expression in the notochord becomes detectable at the neurula stage when the embryo begins to elongate after gastrulation (Fig. 3H). In vertebrate embryos, *Brachyury* gene expression in the anterior notochord quickly disappears (Herrmann 1995); in contrast, it remains in amphioxus embryos (Fig. 3J, K). Amphioxus adults have a notochord extending to the anterior end of the head and the notochord contains actin or paramyosin filaments (Flood 1975). Of interest is the expression of the genes in the paraxial mesoderm of the presumptive somite (Fig. 3E, I). The expression was synchronized with somite band formation, suggesting roles for the genes in the formation of somites. A role for *Brachyury* genes in vertebrate somite formation has not been described.

In contrast to these two chordate groups, the ascidian (urochordates) *Brachyury* gene (*As-T*) is expressed only in cells that are destined to differentiate into the notochord (Yasuo and Satoh 1993, 1994). Urochordates are regarded as a more basal type of chordate. The vertebrate *Brachyury* genes are thought to have two roles, namely notochord differentiation and posterior mesoderm formation associated with cell surface activities (Herrmann 1995). It has been shown recently that the ascidian genome contains another T-domain gene (*As-T2*) that is expressed in differentiating muscle and the tail tip of the embryo (Yasuo et al. 1996). Interestingly, the combined pattern of spatial expression of *As-T* and *As-T2* appears to correspond to that of a single vertebrate *Brachyury* gene.

Similarity and dissimilarity between amphioxus *fork head/HNF-3* and vertebrate *HNF-3* gene expression

The Fkh domain is a 110-amino acid DNA-binding motif (Weigel and Jäckle 1990), and related genes are divided into several subtypes, depending on sequence similarity (Sasaki and Hogan 1993; Kaufmann and Knöchel 1996). The molecular phylogenetic tree indicated that *Am(Bb)fkh/HNF3-1* is homologous to mouse *HNF-3* and *Xenopus Pintallavis* (Fig. 5). It is uncertain whether or not there is another gene belonging to this subgroup in the *B. belcheri* genome, because the *B. floridae* genome contains two *HNF-3* genes (Sebastian Shimeld, personal communication).

Am(Bb)fkh/HNF3-1 expression was first detected in the dorsal lip of the blastopore and in part of the endoderm of gastrulae (Fig. 6B). This spatial *Am(Bb)fkh/HNF3-1* expression is compatible with that of *Pintallavis* in the organizer and presumptive endoderm in *Xenopus* embryos and with *Xenopus HNF-3β* in the endoderm (Ruiz i Altaba and Jessell 1992; Ruiz i Altaba et al. 1993). Furthermore, *Am(Bb)fkh/HNF3-1* expression resembles that of *Xenopus HNF-3β* in that both are found only in the anterior endoderm. In amphioxus embryos, no hybridization signals were detected in the posterior region of the archenteron (Fig. 7H), although the stage at which *Xenopus HNF-3β* expression begins is later than that of amphioxus (Ruiz i Altaba et al. 1993). On the other hand, mouse *HNF-3β* is expressed throughout the gut (Sasaki and Hogan 1993).

There are some differences in gene expression in the midline of the neural region. In *Xenopus*, both *HNF-3β* and *Pintallavis* are expressed in cells of the midline of the three germ layers (Ruiz i Altaba and Jessell 1992; Ruiz i Altaba et al. 1993). In addition, their expression in the notochord and floor plate is associated with the competence of notochord induction, because ectopic expression of mouse *HNF-3β* in the neural tube induces floor-plate-like structures (Sasaki and Hogan 1994). From a phylogenetic viewpoint, a common ancestor of cephalochordates and vertebrates is defined by the differentiation of the neural tube into inner and outer layers with separate dorsal and ventral innervation (Brusca and Brusca 1990). Therefore, the amphioxus neural tube may develop by a similar underlying mechanism as that of vertebrates and thus, *Am(Bb)fkh/HNF3-1* should be similarly expressed in the neural tube. Nevertheless, we did not detect this (Fig. 6G). It is unlikely that expression of *fork head/HNF-3*-related genes in the floor plate evolved later in the lineage to the vertebrates, because the ascidian *fork head/HNF-3* related gene is expressed in the neural lineage (Shimauchi et al., unpublished data). Amphioxus neural development is different from that of vertebrates because, in amphioxus, the neural plate is not eliminated from the non-neural ectoderm and the tube is synchronously formed (Fig. 6F; Hirakow and Kajita 1994). Moreover, motor-neuron-like cells would exist but their innervation differs between amphioxus and vertebrates (Fritzsch and Northcutt 1993). *Am(Bb)fkh/HNF3-1* expression may be affected by these differences, although there is no direct evidence at present. Another possibility is that other *fork head/HNF-3* genes belonging to the same subtype exist in amphioxus and the summation of these genes, including *Am(Bb)fkh/HNF3-1*, is equivalent to *HNF-3β* and others, as suggested by Ruiz i Altaba et al. (1993).

Expression of *Am(Bb)Bra1*, *Am(Bb)Bra2* and *Am(Bb)fkh/HNF3-1* in secondary development

As reviewed by Griffith et al. (1992), it has been suggested that morphogenetic processes during vertebrate development are separated into the establishment of the three classical germ layers (endoderm, mesoderm and

ectoderm) through gastrulation and the formation of the tail bud that generates three germ layers from an undifferentiated cell mass. Moreover, many genes, including *Brachyury* (*T*), *Pintallavis* and *Xnot* (Talbot et al. 1995) continue to be expressed in these regions. These genes are thought to be required for cell differentiation or growth (De Robertis et al. 1994). In addition, the tail bud feature binds various chordates into one common group because all chordates have tails at the larval stage (Satoh and Jeffery 1995).

As reported by Gont et al. (1993), *Xbra* is expressed in the chordoneural hinge, the notochord, posterior wall of neurenteric canal and cells of the spinal cord. They suggested that tail formation is a continuation of gastrulation. Amphioxus also has a neurenteric canal, which is of the same structure as the vertebrate counterpart (Conklin 1932). Therefore, tail formation may proceed in a similar manner to that in vertebrates and *Am(Bb)Bra2* is expressed in the chordoneural hinge and ectoderm posterior to the canal. In addition to these two regions, *Am(Bb)Bra2* was expressed in the posterior end of the archenteron (Fig. 7). Therefore, amphioxus *Brachyury* genes are expressed in the three germ layers. This correlates with the fact that zebrafish *ntl* expression is not restricted to mesoderm precursor cells alone (Schulte-Merker et al. 1992). However, *Am(Bb)fkh/HNF3-1* is not expressed in the posterior end of the archenteron and little is expressed in the notochord and the following chordoneural hinge. Therefore, it is unlikely that *Am(Bb)fkh/ HNF3-1* functions like *Pintallavis* or mouse *HNF-3β* in tail bud formation.

Am(Bb)Bra1, *Am(Bb)Bra2* and *Am(Bb)fkh/HNF3-1* are expressed in the most rostral region of the notochord (Fig. 7B, G). As discussed by Holland et al. (1995), this region seems to differ from the more caudal region of the notochord. The notochord runs along the anteroposterior axis and bears the driving force of body elongation. Therefore, the expression of these three genes may reflect their function in this region.

In conclusion, our characterization of amphioxus *Am(Bb)Bra1*, *Am(Bb)Bra2* and *Am(Bb)fkh/HNF3-1* genes and their expression in the chordamesodermal region will provide useful information towards understanding the molecular basis of amphioxus development, as it indicated that they retain fundamental features of vertebrate ancestors.

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