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Evolutionary Conservation of the Bone Morphogenetic Protein 2/4 Gene between Diploblastic and Triploblastic Metazoans

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Sheng-Ping L. Hwang, Chaolun Allen Chen, Mou-Yung Peng and Chang-Po Chen (2003) Evolutionary conservation of the bone morphogenetic protein 2/4 gene between diploblastic and triploblastic metazoans. *Zoological Studies* 42 (1):227-234. Bone morphogenetic protein 4 (BMP4) is a multifunctional regulator during both vertebrate and invertebrate development. BMP4 and DPP (*Drosophila* homolog) have been shown to regulate the dorsal-ventral patterning in both vertebrates and *Drosophila*. These results infer that *BMP4* homologs possibly only exist in the Bilateria. In this report, we show the presence of *BMP4* homologs in 4 coral species including *Acropora muricata*, *A. digitifera*, *Favia favus*, and *Platygyra sinensis* and identify the *BMP2/4* gene in an actiniarian, *Actina equina*. All of these species belong to the Radiata and represent the basal animal group in the evolution of the metazoans. Phylogenetic analyses further suggest that the *BMP2/4* gene identified in *A. equina* is likely the most ancient one, and that the appearance of the *BMP2/4* gene is not only connected to bilateral symmetry, but may also be closely related to the evolution of the Metazoa.

http://www.sinica.edu.tw/zool/zoolstud/41.1/227.pdf

Key words: *BMP2/4* gene, Sea anemone, Coral, Phylogeny.

Bone morphogenetic protein 4 (BMP4) belongs to the transforming growth factor β (TGF-β) supergene family (Kingsley 1994). BMPs including BMP4 are synthesized as precursor proteins. The mature C-terminal domain is generated through protease cleavage on dibasic signaling amino acids (Ozkaynak et al. 1992). Seven conserved cysteine residues have been found in the mature domain, and they are responsible for intra- and inter-molecule disulfide bond formation (Griffith et al. 1996).

BMPs including BMP4 have been found to be multifunctional regulators during both vertebrate and invertebrate development. BMP4 has been shown to play important roles in establishment of the basic embryonic body plan, in morphogenesis, apoptosis, and the development of organs and tissues (Hogan 1996). For instance, BMP4 functions in ventral mesoderm induction and patterning in *Xenopus* (Hemmati-Brivanlou and Thomsen 1995). The ventralizing activity of BMP4 is antagonized by organizer factors (e.g., chordin) which promote the formation of dorsal mesoderm structures (Sasai et al. 1994). It has been shown that chordin binds directly to BMP4 and sequesters it from binding to its receptor (Piccolo et al. 1996). Conversely, xolloid, a metalloprotease, regulates the amount of free BMP4 through direct cleavage of chordin (Piccolo et al. 1997). Analogously, a similar set of protein homologs (tolloid, SOG, and DPP) regulates the dorsal-ventral patterning in *Drosophila* embryos (Holley et al. 1995, Marques et al. 1997). Thus, a conserved molecular mechanism involving BMP4/DPP has been shown to regulate dorsal-ventral patterning from *Drosophila* to *Xenopus* (De Robertis and Sasai 1996, Ferguson 1996, Graff 1997, Mullins 1998, Schier and Talbot 1998).

Most of the molecular evidence on dorsal-ventral patterning comes from 2 very distantly related groups of triploblastic and bilaterally symmetrical organisms: the arthropods and verte-

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brates. Based on these data, a hypothetical animal (*Urbitlateria*) which possesses several characteristics including a conserved system of dorsal-ventral patterning provided by SOG/chordin and DPP/BMP4 was proposed to be the common ancestor of the Bilateria (De Robertis and Sasai 1996). The discovery of *BMP4* homologs in sea urchins has recently created a good subject through which to evaluate this proposal (Hwang et al. 1999, Angerer et al. 2000). Although echinoderms possess a radially symmetrical body plan in the adult stage, their larvae are bilaterally symmetrical, and possess both an animal-vegetal axis and an aboral-oral axis (Ettensohn and Ingersoll 1992, Jeffery 1992). Results from ectopical expression of *BMP2/4* in sea urchin embryos suggest that sea urchin BMP2/4 is an animalizing factor and regulates the ectoderm-endoderm boundary along the animal-vegetal axis. In addition, it specifies the differentiation of epidermal cells within the ectoderm along the oral-aboral axis (Angerer and Angerer 1999, Angerer et al. 2000). Thus, it has been proposed that the animal-vegetal axis in the sea urchin is analogous to the dorsal-ventral axis in vertebrates (Angerer et al. 2000).

The multifunctional nature of BMP4 leads us to believe that it may have existed before the evolution of a bilateral body plan. For example, BMP4 has also been shown to be involved in organogenesis in both vertebrates and invertebrates (Hogan 1996, Panopoulou et al. 1998). Thus, in this report, we searched for the presence of *BMP4* homologs in 4 coral species including *Acropora muricata*, *A. digitifera*, *Favia favus*, and *Platygyra sinensis* as well as in an actiniarian, *Actina equina*, representing the basal animal group (phylum Cnidaria) in the evolution of the metazoans (Bridge et al. 1995, Odorico and Miller 1997, Kim et al. 1999). We were able to show the presence of the *BMP4* homolog in the 4 coral species and obtained a *BMP2/4* gene fragment from *A. equina*. Phylogenetic analyses were conducted, and the results suggest that the *BMP2/4* gene from *A. equina* may be the most ancient one.

MATERIALS AND METHODS

Materials

Sea anemones (*Actina equina*) were collected from Aoti, northeastern Taiwan and reared in the laboratory. Sperm was collected from 4 coral species (*Acropora muricata*, *A. digitifera*, *Favia favus*, and *Platygyra sinensis*) on nights which we predicted coral would be releasing sperm in the Penghu Islands in 1998 (Chen et al. unpubl. data). All chemicals were obtained from Merck (Darmstadt, Germany) unless otherwise specified.

DNA extraction, PCR, cloning, and sequencing

The method used for DNA extraction from the sea anemone and sperm from the 4 coral species was based on Chen et al. (2000). A pair of degenerate oligonucleotides was designed according to conserved regions obtained from the alignment of the amino acid sequences of BMP2 and BMP4 homologs from various mammalian species (Hwang et al. 1996, Hwang et al. 1999). The PCR reaction mixture containing 100 pmole of each degenerate oligonucleotide, 1x replitherm DNA polymerase buffer, 5 mM $MgCl_2$, 0.2 mM dNTP, and replitherm DNA polymerase (2.5 U/100 μl, Epicentre, Madison, WI) was added to denatured DNA (2-5 μg). PCR cycles were as follows: 94°C for 1 min, 50°C for 1 min, 72°C for 1 min for 35 cycles; then 72°C for 10 min for 1 cycle, followed by soaking at 4°C. The amplified PCR products were cloned into the pGEMT vector (Promega, Madison, WI). The nucleic acid sequence was determined by the dideoxy chain-termination method (Sanger et al. 1977). The sequences have been submitted to GenBank under accession no. AY027547.

Southern blot analyses

Ten microliters of PCR products was separated on 2% 0.5 x TBE agarose gels. After photographing under UV light, the DNA was denatured, neutralized, and transferred onto nylon membranes as described in Sambrook et al. (1989). After UV cross-linking, membranes were prehybridized in hybridization buffer containing a mixture of 5 x SSC, 50% formamide, 2% blocking solution (Roche, Mannheim, Germany), 0.1% N-lauroylsarcosine, and 0.02% SDS, at 37°C for 1 h. Hybridization was conducted by replacing hybridization buffer with the same buffer but containing a denatured digoxigenin-11-dUTP (DIG)-labeled zebrafish *BMP4* DNA fragment as a probe (25 ng/ml) at 37°C overnight. Excess probes were removed by 2 washes with 2 x SSC and 0.1% SDS at room temperature and 2 washes with 0.1 x SSC and 0.1% SDS at 37°C for 15 min each. Subsequently, anti-DIG-alkaline phosphatase antibody incubation followed by AMPPD (Tropix, Bedford, MS) chemiluminescent detection

was performed based on protocols provided by the manufacturer (Roche, Mannheim, Germany).

Sequence comparison and phylogenetic analyses

Sequence alignment and comparison among BMP2 and BMP4 from various vertebrate species, and BMP2/4 from various invertebrates and a sea anemone were respectively performed using the PILEUP and GAP programs from the Wisconsin Sequence Analysis Package of the Genetic Computer Group (GCG). Phylogenetic analyses were performed using PHYLIP 3.6 (Felestein 2000). Neighbor-joining (NJ) analysis was performed in the NEIGHBOR option after genetic distances were calculated based on the Dayhoff PAM model (Dayhoff et al. 1978) in the PROTDIST

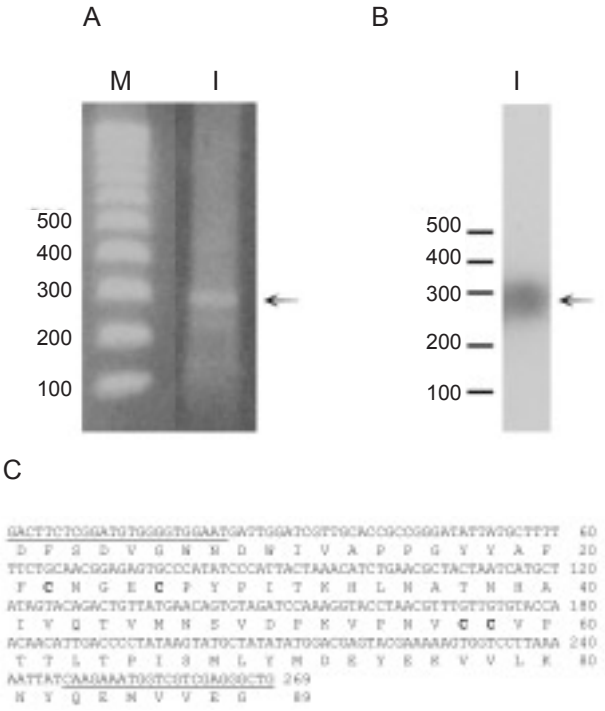

Fig. 1. Amplification of the *BMP2/4* gene fragment from the sea anemone and sequence analyses. (A) Ten microliters of PCR product was separated on a 2% agarose gel and stained with ethidium bromide. (B) The gel was denatured, neutralized, and blotted onto a nylon membrane. A DNA band with a size of 270 bp was hybridized with a DIG-labelled zebrafish *BMP4* probe. Sea anemone (*Actina equina*) (lane 1) genomic DNA was used as the template in the PCR reaction. (C) Nucleic acid and deduced amino acid sequences of sea anemone *BMP2/4* were determined. Conserved cysteine residues are shown in boldface letters. DNA sequences corresponding to primer regions are underlined. A DNA molecular weight marker (bp) is shown (lane M).

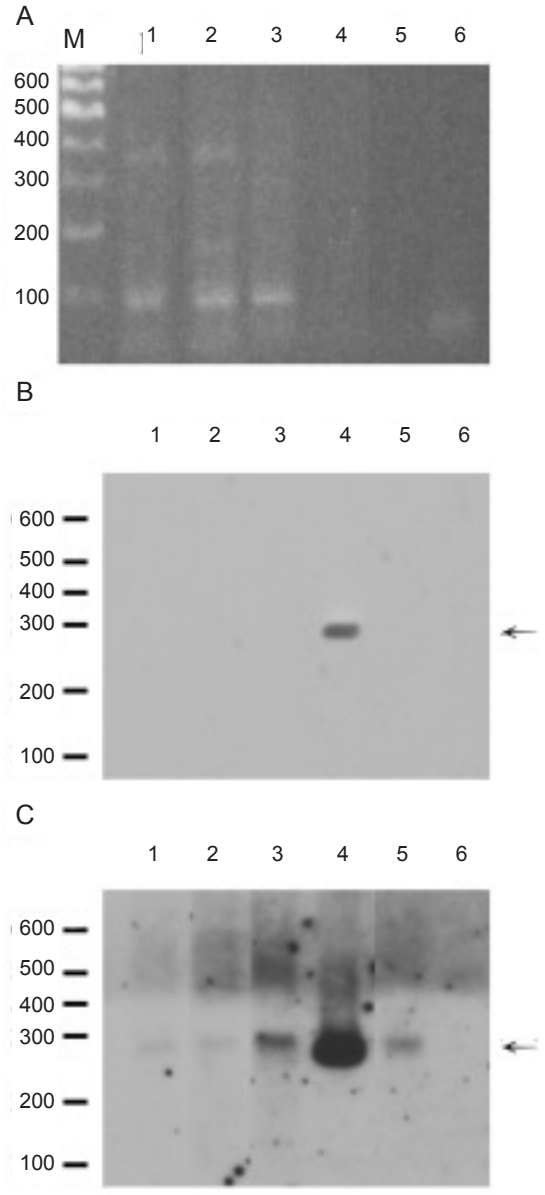

Fig. 2. Amplification of *BMP2/4* gene fragments from 4 coral species and Southern blot analyses. (A) Ten microliters of PCR products from a reaction mixture containing 50 ng of *Acropora muricata* DNA (lane 1), 50 ng of *A. digitifera* DNA (lane 2), 50 ng of *Favia favus* DNA (lane 3), 500 ng of *F. favus* DNA (lane 4), and 50 ng of *Platygyra sinensis* DNA (lane 5) was separated on a 2% agarose gel and stained with ethidium bromide. (B) The gel was denatured, neutralized, and blotted onto a nylon membrane. The membrane was hybridized with a DIG-labelled zebrafish *BMP4* probe. After CDP-star chemiluminescent treatment, the membrane was exposed to x-ray film for 5 min and developed. (C) The same membrane as (B) but exposed to x-ray film for 30 min. Black round spots (or dots) were generated by a static reaction to the x-ray film. Arrows indicate the expected 270-bp DNA bands hybridized with the zebrafish *BMP4* probe. PCR control was conducted using dH_2O as a template (lane 6). A DNA molecular weight marker (bp) is shown (lane M).

option. The robustness of the NJ phylogenies was assessed by 1000 bootstrap replicates using the SEQBOOT and CONSENSE options.

RESULTS

Using the conserved degenerate primer pair, a single DNA band with a size of approximately 270 bp was detected when genomic DNA isolated from a sea anemone (*Actina equina*) was used as the template (Fig. 1A, lane 1). The amplified PCR products were hybridized with a zebrafish *BMP4* probe in the Southern blot analyses (Fig. 1B). This result indicated that the amplified DNA fragment is a *BMP4* homolog fragment from the sea anemone (named *AeBMP2/4*). We also conducted PCR using the same primer pair and genomic DNA isolated from 4 coral species (*Acropora muricata*, *A. digitifera*, *Favia favus*, and *Platygyra sinensis*) as templates. The expected 270-bp amplified DNA fragments were not observed when agarose gels were stained with ethidium bromide, probably due to the low yield of PCR products (Fig. 2A). Subsequent Southern blot analyses showed that a DNA band with a size of approximately 270 bp hybridized with the zebrafish *BMP4* probe when *F. favus* genomic DNA was used as the template (Fig. 2B, lane 4). With a longer exposure time, 270-bp DNA bands of different intensities were detected in respective PCR reaction mixtures using genomic DNA from 4 different coral species as templates (Fig. 2C). These results indicate that the *BMP4* homolog may also be present in corals.

Sequence analyses of the *BMP2/4* gene fragment from *Actina equina* further confirmed the results of Southern blotting (Fig. 1C). The deduced amino acid sequence of the *BMP2/4*

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Consensus          DWIVAPPGYQAFYCHGECPFPLADHLNSTNHAIVQTLVNSVNP-SVPKACCVPTELSAISMLYLDEYEKVVLKNY
                              *   *                                  **
HBMP2              .........H.................................S-KI.....................N........
MBMP2              .........H.................................S-KI.....................N........
XBMP2              .........H.................................T-NI.....................N........
ZBMP2              .........H....Q.............TN.M.........S-NI.R......D..PV.L.......R.I....
HBMP4              ................D..........................S-.I.....................D.......
MBMP4              ................D..........................S-.I.....................D.......
XBMP4              ................D..........................S-.I.....................D.......
ZBMP4              ...........Y...............................T-NI....................TDR......
AmphiBMP2/4        ...........Y.................................IA.........D..P......N.NDQ......
PfBMP2/4           .........N....D.......................H..KASA..Q.........P.........D..I....
HrBMPb             ......H..H....N........EYM.A..............D.SLT..P.........P.A...V..C.L....T.
LvBMP2/4           ......A....Y...........E...T...............AL......G.......................
SpBMP2/4           ......A....Y..R........E...T...............AL.............................
TgBNP2/4           ......A....Y...........E...T...............AL.............................
ArBMP2/4           ......A....Y..Q.......V....A.............AS.QLA........D..P........SDA.I....
AtBMP2/4           S.....A....Y..Q.......V....A.............AS.QLA........D.........DSDS.I.R..
SoBMP2/4           ......S....HF.E.K.L..IS.Y..T.............M.NKI.R.........P.....V..Q......T.
HtBMP2/4           ......S..N......D......H..................SA...........T.........WD.......
DBL-1              ...M..K..D.YQ.Q.S..N.MPAQ..A.....I.S.LH.LR.DE..PP.....T.PL.I..M.VDKVI.IRE.
DPP                ......L..D.Y....K........F......V......NM..GK.........Q.DSVA....NDQST......
AeBMP2/4(France)   .........Y..F.N....Y.ITK...A........VM...D.-K..NV.....T.TP.....M..........
AeBMP2/4(Taiwan)   .........Y..F.N....Y.ITK...A........VM...D.-K..NV.....T.TP.....M..........
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Fig. 3. Alignment of deduced amino acids for *BMP2* and *BMP4* from various vertebrate organisms and *BMP2/4* from various invertebrates. Amino acid residues that are identical to the consensus sequence are shown as dots. The positions of the 4 conserved cysteine residues are indicated by stars. Sequences used are: human *BMP2* (*HBMP2*), *BMP4* (*HBMP4*; Wozney et al. 1988); mouse *BMP2* (*MBMP2*; Feng et al. 1994); mouse *BMP4* (*MBMP4*; X56848); *Xenopus BMP2* (*XBMP2*; Plessow et al. 1991); *Xenopus BMP4* (*XBMP4*; Nishimatsu et al. 1992); zebrafish *BMP2* (*ZBMP2*; Martinez-Barbera et al. 1997); zebrafish *BMP4* (*ZBMP4*; Hwang et al. 1997); amphioxus *BMP2/4* (*AmphiBMP2/4*; Panopoulou et al. 1998); hemichordata (*Ptychodera flava*) *BMP2/4* (*PfBMP2/4*; AB028219); ascidian *BMP2/4* (*HrBMPb*; Miya et al. 1997); sea urchin (*Lytechinus variegatus*) *BMP2/4* (*LvBMP2/4*; Angerer et al. 2000); sea urchin (*Strongylocentrotus purpuratus*) *BMP2/4* (*SpBMP2/4*; Angerer et al. 2000); sea urchin (*Tripneustes gratilla*) *BMP2/4* (*TgBMP2/4*; Hwang et al. 1999); starfish (*Asterias rubens*) *BMP2/4* (*ArBMP2/4*; AJ251826); starfish (*Archaster typicus*) *BMP2/4* (*AtBMP2/4*; AF334705); mollusc (*Haliotis tuberculata*) *BMP2/4* (*HtBMP2/4*; AJ251823); mollusc (*Sepia officinalis*) *BMP2/4* (*SoBMP2/4*; AJ251824); nematode (*Caenorhabditis elegans*) *BMP2/4* (*DBL-1*; Suzuki et al. 1999); fruit fly (*Drosophila melanogaster*) *BMP2/4* (*DPP*; Padgett et al. 1987); and sea anemone (*Actinia equina*) *BMP2/4* (France) (*AeBMP2/4*; AJ2511825 and this report).

gene fragment from *A. equina* comprised 2/3 of the processed mature domain including 4 of 7 conserved cysteine residues. At the time we were analyzing our data, a similar sequence (AJ251825) appeared in GenBank, so we added this sequence in subsequent sequence comparisons. When comparing our sequence with that of GenBank, 6 differences appeared in the nucleic acid sequence, but the deduced amino acid sequence remained the same. In addition, the position of the designed degenerate primer pairs differed between our sequence and that of GenBank. Sequence comparison showed that *A. equina* BMP2/4 shared high amino acid sequence similarity ranging from 62% to 81% with those of BMP2 and BMP4 from various vertebrates and BMP2/4 from various invertebrate organisms (Fig. 3).

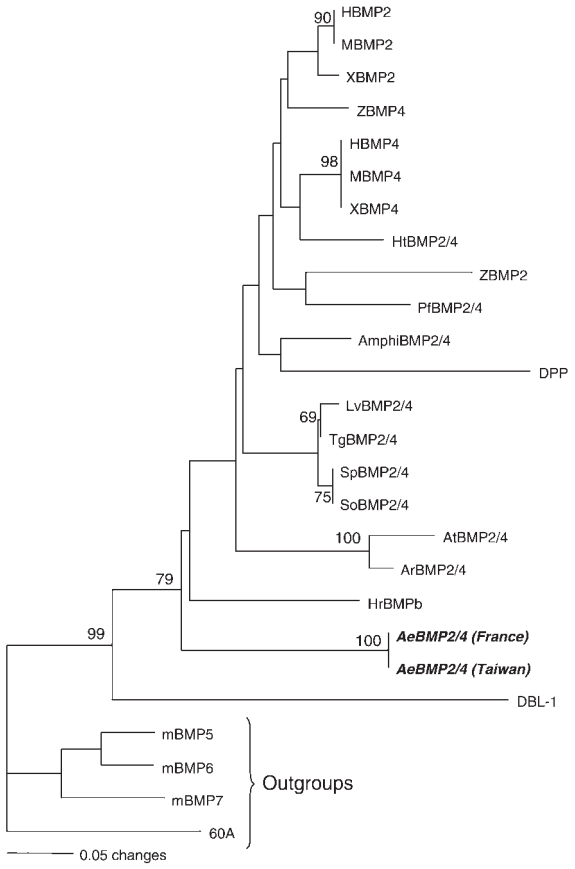

Fig. 4. Phylogenetic analysis of BMP2 and BMP4 from various vertebrates, BMP2/4 from various invertebrate organisms, and an additional 4 TGF-β growth factors. BMP2 and BMP4 from various vertebrate organisms and BMP2/4 from various invertebrates used for the phylogenetic analyses are the same as those described in figure 3. Sequences used as outgroup were mouse *BMP5* (*MBMP5*; King et al. 1994); mouse *BMP6* (*MBMP6*; Lyons et al. 1989); mouse *BMP7* (*MBMP7*; Ozkaynak et al. 1991); and *Drosophila 60A* (Wharton et al. 1991). Bootstrap values > 68% are shown above the branches.

To further understand the evolutionary relationships among AeBMP2/4 and BMP2/4 from various invertebrates, and BMP2 and BMP4 from various vertebrate organisms, a rooted phylogenetic tree was constructed using the deduced amino acid sequence comprising 2/3 of the mature domain (Fig. 4). Four TGF-β sequences including mouse BMP5, BMP6, BMP7, and *Drosophila* 60A were used as outgroups in the phylogenetic analyses. The results show that AeBMP2/4 is located at the basal position of the phylogenetic tree separated from the other triploblastic group with 79% bootstrap support. Nematode DBL-1 is separated from both the triploblastic group and AeBMP2/4 due to the long-branch attraction effect. Within the triploblastic group, BMP2/4 from an ascidian (*Halocynthia roretzi*) diverges first. BMP2/4 from 2 species of starfish (*Archaster typicus* and *Asterias rubens*) diverge as separate branches with 100% bootstrap support. Three species of sea urchins (*Tripneustes gratilla*, *Lytichinus variegatus*, and *Stongylocentrotus purpuratus*) and BMP2/4 from a mollusk (*Sepia officinalis*) diverge as separate branches without significant bootstrap values. BMP2/4 from amphioxus (*Branchiostoma floridae*) and DPP from the fruit fly (*Drosophila melanogaster*) as well as BMP2/4 from a hemichordata (*Ptychodera flava*) and zebrafish BMP2 are grouped as separated branches, respectively. BMP2/4 from a mollusc (*Haliotis tuberculata*) is grouped with the vertebrate BMP4, but the bootstrap support is not significant. In the vertebrate clade, gene duplication occurred and formed the *BMP2* and *BMP4* genes. BMP4 and BMP2 from the vertebrates, the human, mouse, and *Xenopus*, are grouped together as separate respective branches. However, zebrafish BMP4 is grouped with vertebrate BMP2. The variation observed in vertebrate BMP2 and BMP4 branches is caused by the long-branch attraction effect of 2 invertebrate sequences (nematode DBL-1 and *Drosophila* DPP) as well as high conservation of amino acid sequences within the compared mature domain of BMP2 and BMP4 from vertebrates.

DISCUSSION

In the present study, we show the potential existence of *BMP4* homologs in 4 coral species and obtained a *BMP2/4* gene fragment from sea anemone *Actina equina*. Sequence comparison showed that *A. equina* BMP2/4 shares high amino

acid sequence similarity with BMP2 and BMP4 from various vertebrates and BMP2/4 from various invertebrate organisms. In the partial sequence of sea anemone BMP2/4, four of 7 conserved cysteine residues that have been shown to be involved in inter- and intra-molecular disulfide bond formation were also located at the correct positions. This result suggests that the mature domain of sea anemone BMP2/4 folds into similar secondary and 3-D structures as illustrated in Griffith et al. (1996).

The phylogenetic tree constructed using BMP2/4, BMP2, and BMP4 from various organisms is similar to those based on morphology or on ribosomal RNA-molecular data, but with some variations (Adoutte et al. 2000). The variation in branching order within BMP2/4 from invertebrates such as hemichordata, amphioxus, ascidian, mollusc, fruit fly, and nematode may indicate an early divergence due to the multifunctional nature of BMP2/4 in invertebrate evolution (Table 1). Through comparative analyses of early development in all metazoan phyla, a micrometazoan ancestor similar to extant invertebrate free-living larvae that use type 1 embryogenesis has been proposed (Davidson et al. 1995, Peterson and Davidson 2000). In addition, these micrometazoan ancestors contain some set-aside cells as rudiments for growth, pattern formation, and production of additional useful structures. These set-aside cells develop regulatory genetic mechanisms like the *Hox* gene complex to further generate different phyletic body plans (Peterson et al. 1997, Arenas-Mena et al. 2000, Peterson et al. 2000). Since *BMP4* is expressed in embryos of different organisms, it may regulate gene expression of the *Hox* complex in set-aside cells and allow them to further build up the adult body plans of the major animal phyla.

We also tried to identify *BMP4* homologs in sponges and yeast using the same pair of degenerate primers but obtained no PCR products from the genomic DNA of 2 species of sponges (*Paratetilla* sp. and *Callyspongia* sp.) or a budding yeast (*Saccharomyces cerevisiae*) (data not shown). In addition, we searched the yeast genomic sequence and were unable to detect the presence of any similar *BMP4* homolog sequences. Although the possibility that sequences of *BMP4* in sponge which greatly diverge from other *BMP2/4* homologs may exist, this additional information is in accordance with the current concept of metazoan evolution based on the 5'-end of the 23S-like rDNA, the 18S rDNA sequence, mitochondrial 16S rDNA sequence, mitochondrial genomic structure, and morphological traits (Bridge et al. 1995, Odorico and Miller 1997, Kim et al. 1999). These reports all place

Table 1. Diverse functions of *BMP2/4* in invertebrates

Organism	Functions	Reference
Amphioxus (*Branchiostoma floridae*)	Has patterning roles in the ectodermal-dorsoventral axis. May be involved in somite evagination, tail bud growth, pharyngeal differentiation, hindgut regionalization, differentiation of olfactory epithelium, patterning of the anterior central nervous system, and establishment of the heart primordium.	Panopoulou et al. (1998)
Ascidian (*Halocynthia oretzi*)	Functions as a neural inhibitor and an epidermal inducer.	Miya et al. (1997)
Sea urchins (*Lytechinus variegatus, Strongylocentrotus purpuratus*)	Influences cell fate between epidermal and nonepidermal differentiation within the ectoderm. Establishes the ectoderm/endoderm boundary along the animal-vegetal axis.	Angerer et al. (2000)
Fruit fly (*Drosophila melanogaster*)	Has patterning roles in both the ectodermal and mesodermal dorsal-ventral axes. Has a patterning role in larval midgut morphogenesis. Controls wing size.	Padgett et al. (1987)
Nematode (*Caenorhabditis elegans*)	Is expressed in neurons and acts as a dose-dependent regulator of body size and male tail patterning.	Suzuki et al. (1999)

anthozoans (a class in the phylum Cnidaria) at the base of metazoan phylogeny while regarding other simple multicellular animals without true tissue organization, such as the porifera, choanozoa, ctenophora, and placozoa, as independent evolutionary branches originating from unrelated protozoan ancestors. Overall, our results suggest the potential existence of a *BMP4* homolog in coral and that the obtained *BMP2/4* gene in *Actina equina* may be the most ancient one. Thus, the appearance of the *BMP2/4* gene may not only be connected to bilateral symmetry, but may also be closely related to the evolution of the Metazoa.

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