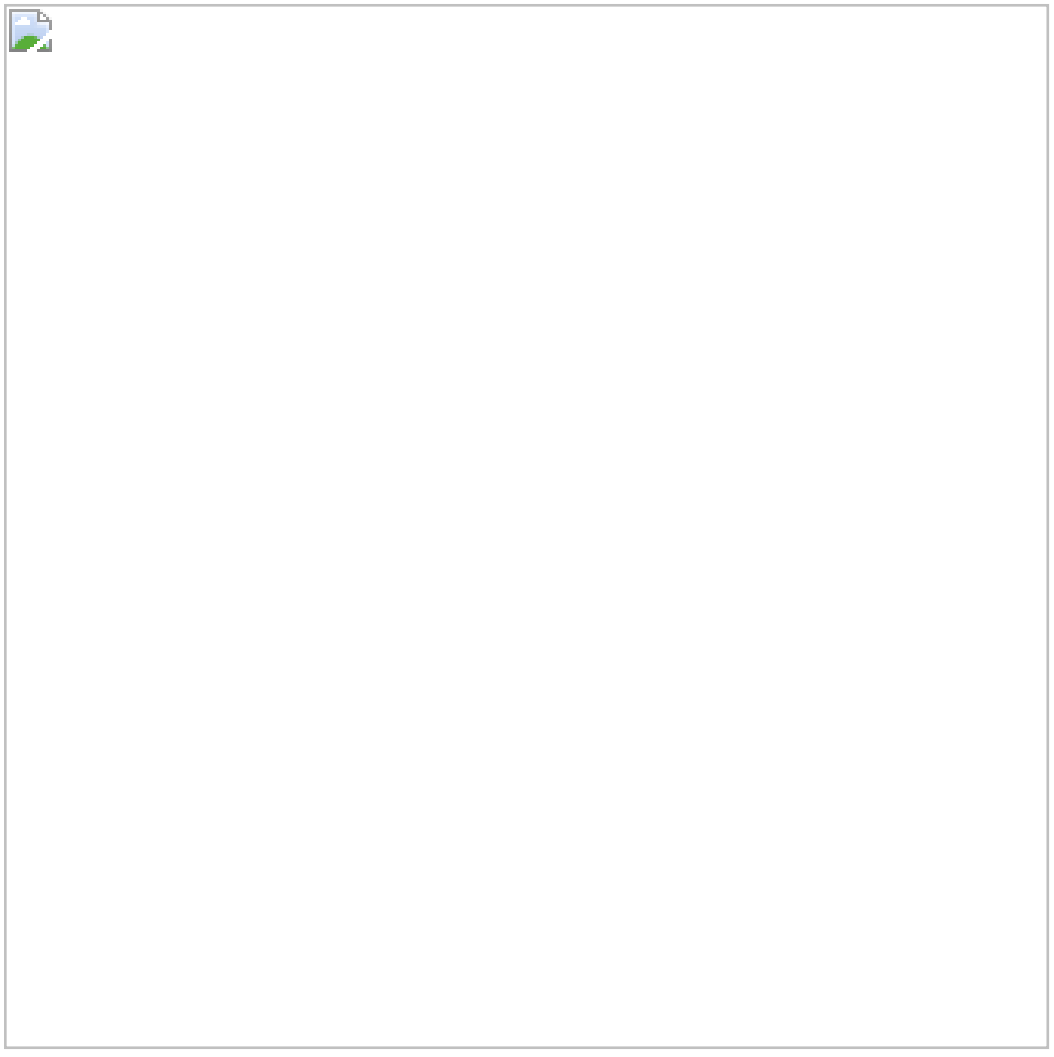

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Molecular Cloning of Bone Morphogenetic Protein (BMP) Gene from the Planarian *Dugesia japonica*

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ABSTRACT—BMP (Bone Morphogenetic Protein) acts as a morphogen for dorso-ventral patterning and organogenesis in both vertebrate and invertebrate development. A cDNA encoding BMP (named *Djbmp*) has been cloned and sequenced from the planarian *Dugesia japonica*. The mature form of DjBMP which was deduced from the cDNA sequence was composed of 114 amino acid residues. The position of seven cysteine residues of the mature DjBMP was highly conserved among the TGF-β superfamily. DjBMP had high similarity to human BMP-2A (50% amino acid identity), BMP-4 (49%) and *Drosophila decapentaplegic* protein (48%), indicating that DjBMP belongs to DVR (decapentaplegic-Vg1-related) group. The expression pattern in intact and regenerating planarians revealed by whole mount *in situ* hybridization suggested that the DjBMP plays a role not only in dorso-ventral but also in mid-lateral body patterning.

INTRODUCTION

Transforming growth factor-β (TGF-β) superfamily is a large group including biological active proteins such as activin, inhibin, growth/differentiation factor (GDF) and bone morphogenetic proteins (BMPs) (Horgan *et al*., 1994; Kingsley, 1994). One of the members, BMP-4, was found to act as a morphogen for ventralization in vertebrate development (Dale *et al*., 1992; Jones *et al*., 1992; Dosch *et al*., 1997). In *Drosophila*, *decapentaplegic* (*dpp*) gene which encodes a BMP-4 homologue has been known to play a key role in determination of dorsalization during the early development (Irish and Gelbart, 1987). The idea that the dorso-ventral (D-V) axis in deuterostomes including vertebrate corresponds to the ventro-dorsal axis in protostomes including arthropod is supported by molecular evidence that DPP and BMP-4 are functionally similar in inducing ventral structure in frog (deuterostome) and dorsal structure in fly (protostome), respectively (Holley *et al*., 1995; Horgan, 1995; De Robertis and Sasai, 1996).

The freshwater planarian *Dugesia japonica*, belonging to the Phylum Plathelminthes has powerful activity for regeneration (for review; Baguñà *et al*., 1994). We are interested in how body plan is established during the planarian regeneration. As for the antero-posterior axis in planarians, the expression of Hox/HOM-C genes may be important (Orii *et al*., in preparation). On the other hand, we have no information on their D-V patterning. In this study, we focused on a planarian BMP-4/DPP homologue as a candidate molecule in D-V patterning. The molecular cloning of the BMP gene and its expression in intact and regenerating planarians are reported.

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MATERIALS AND METHODS

Organisms

All planarians in this study were derived from one worm of *Dugesia japonica* collected in Irima river in Gifu, Japan and maintained clonally in our laboratory (clonal population GI) (Orii *et al*., 1993). Amputated worms were regenerated in autoclaved tap water at about 22°C.

cDNA cloning and sequencing

A cDNA library (4×10^6 in size) was constructed from poly A^+ RNA of whole worms using λZAPII vector (Umesono *et al*., 1997). The mixture (10 μl) for PCR (polymerase chain reaction) contained 1 × Taq buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), Takara Taq polymerase (Takara, 0.025 U/μl), dNTPs (0.25 mM each), *D. japonica* total DNA (about 40 ng/μl) and a set of degenerate primers (1 pmole/μl each): a forward primer 5'-GGITGG(A/G/C)AIGA (C/T)TGG(G/A)TIGCICC-3' and a reverse primer 5'-ACIA(G/A)IGT (T/C)TGIA(C/G)IA(T/C)IGC(G/A)TG(G/A)TT-3' corresponding to amino acids GW(N/D/Q)DW(I/V)(I/V)AP and NHA(I/V)VQTLV, respectively. The reaction conditions were as follows: an initial denaturation at 94°C for 1min followed by 40 cycles of 94°C for 1min, 45°C for 1min, 72°C for 1 min and a final elongation step at 72°C for 5 min. The PCR product (about 120 bp) was extracted from 6% acrylamide gel, amplified again, cloned into pT7blue T vector (Novagen) and sequenced. From the sequence of the PCR product corresponding to BMP, we designated a specific forward primer 5'-CGTATCCGTTATC-AGATAATTTTAA-3' (arrow in Fig.1) and screened the cDNA library by PCR with the primer and M13 universal primer 5'-CGCCAGGGTTT-TCCCAGTCACGAC-3' (Watanabe *et al*., 1997). Out of 11 positives, one clone carrying the longest insert (pDjBMP17) has been sequenced as to both strands by using Thermo Sequenase cycle sequencing kit (Amersham) and automatic DNA sequencer DSQ1000L (Shimadzu).

Southern blot hybridization

D. japonica total DNA (10 μg) was digested with *Eco*RI or *Spe*I, subjected to 0.8% agarose gel electrophoresis, and blotted on Hybond N^+ membrane (Amersham). The membrane were prehybridized in 6 × SSC, 5 × Denhardt's solution, 1%SDS, 100 μg/ml salmon testes DNA at 65°C for 1 hr and hybridized in the same solution with probe. pDjBMP17 DNA was labeled by using random primed labeling kit (Takara) with α-[^{32}P]-dCTP (Amersham, ~3000 Ci/mmol) and used as the hybridization probe. The membrane was washed twice in 2 × SSC, 0.1% SDS at 65°C for 30 min and exposed to film with an intensifying screen.

Whole mount *in situ* hybridization

The digoxygenin (DIG) labeled anti-sense and sense RNA probes were synthesized with T7 RNA polymerase and T3 RNA polymerase, respectively, and used for hybridization without alkaline hydrolysis (Umesono *et al.*, 1997; Agata *et al.*, 1998).

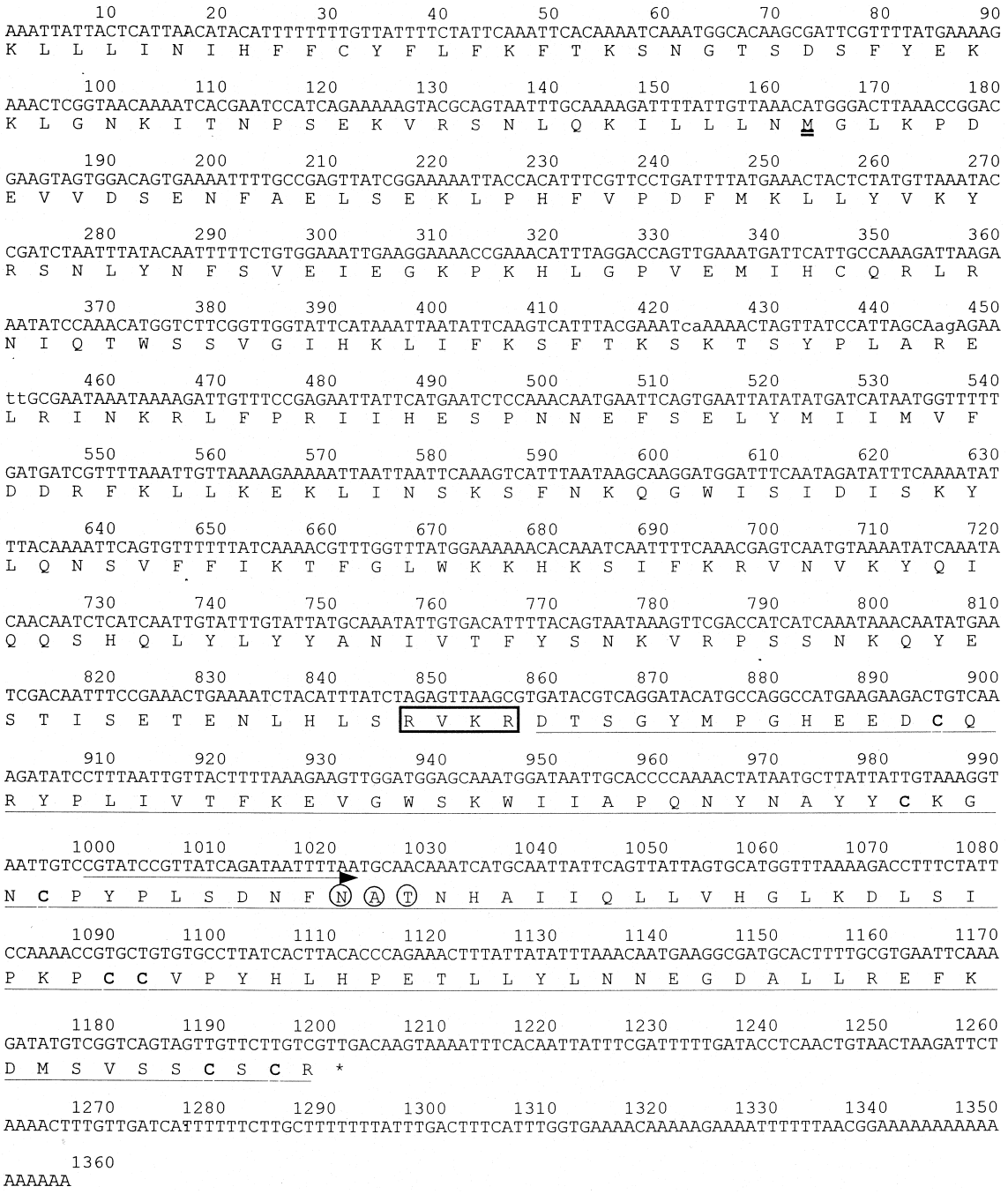

Fig. 1. Nucleotide sequence of *Djbmp* cDNA (upper) and the deduced amino acid sequence (lower). The first methionine is double underlined. Seven cysteine residues conserved among the members of TGF-β superfamily are in bold. The box shows the consensus sequence of proteolytic cleavage. The predicted mature form is underlined. The potential asparagine-linked glycosylation sites are circled. The arrow shows the specific primer used for PCR screening of the cDNA library. The amino acids are shown with single letter abbreviations.

RESULTS AND DISCUSSION

We have aligned many members of TGF-β superfamily to designate a set of degenerate primers for PCR (see Materials and Methods). Using the primers, PCR was performed with *D. japonica* total DNA. Cloning and sequencing analysis revealed that only one PCR fragment had similarity with BMP genes. A cDNA library was screened by the stepwise dilution method with a specific primer designed for the sequence of PCR product (Watanabe *et al*., 1997). The positive cDNA clone with the longest insert (pDjBMP17) was sequenced (Fig. 1). The longest open reading frame (ORF) from the 5' end of the insert to the stop codon (nucleotide 1201-1203) was found. Although the first methionine in the ORF was found at nucleotide 163-165, it did not seem to be the initiation codon, because the upstream region of the methionine of the ORF did not contain any stop codons in frame and the N-terminal region of the deduced protein translated from the methionine did not contain many hydrophobic amino acid redisues, which may serve as signal sequence of secreted peptide such as BMP precursor (Nishimatsu *et al*., 1992). As the deduced protein was similar to the members of BMP group on the whole, we designated it DjBMP (*Dugesia japonica* BMP). In comparison with other proteins belonging to TGF-β superfamily, the pro-protein of DjBMP might be processed into mature form by cleavage at the carboxyl end of R (amino acid 286) of RVKR corresponding to the cleavage consensus sequence RXXR (Panganiban *et al*., 1990). The position of seven cysteine residues conserved among the members of TGF-β superfamily was also confirmed in the protein. Figure 2 shows the comparison of mature protein of DjBMP with other TGF-β related proteins from the first cysteine to the carboxyl terminal. In this region, DjBMP protein was similar to human BMP-2A (50% amino acid identity), BMP-4 (49%), sea urchin univin (50%), *Drosophila* DPP (48%), *Drosophila* 60A (47%), human BMP-5 (44%), TGF-β1(33%) and inhibin-βa (33%). A putative N-linked glycosylation site (NXT/S) was found at amino acid 341-343 (NAT), whose position was also conserved among 60A and DPP subclasses. These results indicate that DjBMP belongs to DVR (decapentaplegic-Vg1-related) group of TGF-β superfamily (Kingsley, 1994). It was very difficult to classify DjBMP more precisely even by the phylogenetic sequence analysis with Genetics Computer Group (GCG) program (Madison, Wisconsin), because the sequences of members of TGF-β superfamily are various for their length.

Genomic Southern hybridization probed with the *Djbmp* cDNA revealed that the *Djbmp* was a single copy (Fig. 3). PCR using genomic DNA as a template indicated that there was no intron in the region encoding mature protein as well as vertebrate BMP-4 gene (Kurihara *et al*., 1993) (data not shown).

To investigate the expression pattern of the gene, whole mount *in situ* hybridization was performed in intact worms (Fig. 4A, B and C). The *Djbmp* was expressed, though very weakly, in dorsal cells (Fig. 4A and B). No signal was detected with sense probe (data not shown). We never observed any signals in the ventral side (Fig. 4C). The expression was stronger in the medial region. There has been no report of morphologically special cells whose distribution is the same as that of *Djbmp* expressing cells. Unfortunately, we could not identify what kind of cells expressed *Djbmp*, because of the sensitivity of our *in situ* hybridization method on paraffin embedded sections. In addition to sequence comparison (Fig. 2), the expression pattern suggests that DjBMP may be a homologue of DPP/BMP-4 in *Drosophila* and vertebrates. DjBMP may play a key role as a dorsal forming or anti-neurogenic factor in the planarian as well as DPP in *Drosophila* (Sasai *et al*., 1995; Wilson and Hemmati-Brivanlou, 1995). To elucidate the role of DjBMP in D-V patterning, one should investigate the expression pattern of *Djbmp* during regeneration of dorsal and ventral parts. However, it is impossible to divide a flat worm into dorsal and ventral pieces.

The expression along the dorso-medial region suggests that DjBMP also plays some role in mid-lateral patterning of the dorsal side. The planarian *Dugesia japonica* has high regeneration ability and a piece of the body can regenerate completely. The left or right marginal fragment without medial region can also regenerate to a whole animal. We investigated the expression pattern of *Djbmp* during regeneration from the lateral piece without the cells expressing *Djbmp*. One day after amputation, the cells expressing *Djbmp* appeared dispersedly and very weakly only on the dorsal side (Fig. 4D). On the second or third day, the positive cells were distributed mainly in the dorso-medial region (Fig. 4E). It was not until five days after amputation that a pair of small eyes and a pharynx appeared, indicating the formation of the medial structure. *Djbmp* expressing cells were distributed mainly on the curved dorso-medial region at this stage. Seven days after amputation, distinct eyes and pharynx were regenerated and the expressing cells were more clearly distributed on the dorso-medial region (Fig. 4F). The expression pattern is represented schematically in Fig. 4G. The expression before differentiation of bilateral eyes suggests that DjBMP is involved in mid-lateral patterning during regeneration. As we could not monitor the behavior of single cells during regeneration, we do not know if the expressing cells move into the midline during regeneration or not. The change of expression pattern during regeneration suggests that dynamic morphogenesis occurs throughout the remaining tissue after amputation rather than in the restricted regenerating region called 'blastema'. In other words, the planarian regeneration could be referred to as 'morphallaxis' rather than 'epimorhosis'. Furthermore, the quick response of the expression after amputation (after only 1day) also suggests that it may be involved in initial D-V patterning which occurs before dorsal cell differentiation.

It is interesting that the *Djbmp* was expressed in both intact and regenerating worms, because *dpp*/*Bmp-4* gene is expressed and functions in early embryogenesis of fly and vertebrates. It is probable that *Djbmp* is expressed in early embryogenesis as well as regeneration. If so, the mechanism of body planning in early embryogenesis may be maintained and utilized for regeneration in planarians. Furthermore, the

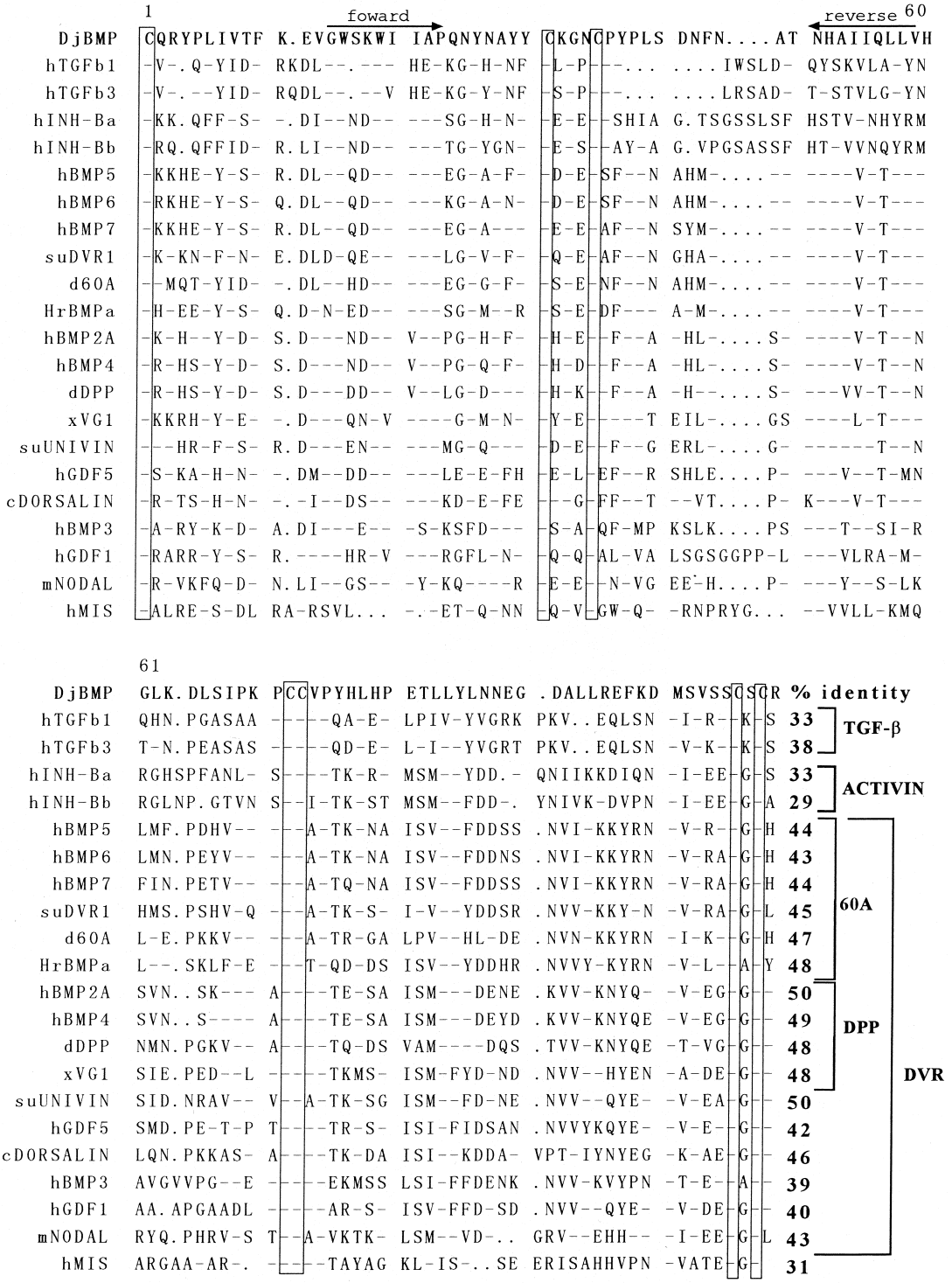

Fig. 2. A comparison of the amino acid sequence of the DjBMP with other members of TGF-β superfamily. Primers and regions used for PCR are shown. All sequence data were obtained from DNA data bank of Japan. Gaps in the alignment are represented by a dots. Amino acid residues identical to DjBMP are represented by a dash. Accession numbers are as follows: human TGF-β1 (hTGFb1), Swiss-Prot/P01137; human TGF-β3 (hTGFb3), Swiss-Prot/P10600; human inhibin-βa (hINH-Ba), PIR/B24248; human inhibin-βb (hINH-Bb), Swiss-Prot/P09529; human BMP-2A (hBMP2A), PIR/B37278; human BMP-3 (hBMP3), Swiss-Prot/P12645; human BMP-4 (hBMP4), PIR/C37278; human BMP-5 (hBMP5), PIR/A39263; human BMP-6 (hBMP6), PIR/B39263; human BMP-7 (hBMP7), PIR/C39263; human GDF-1 (hGDF1), PIR/C39364; human GDF-5 (hGDF5), PIR/JC2347; human Mullerian inhibiting substance (hMIS), PIR/A01397; *Drosophila* dpp (dDPP), PIR/A26158; *Drosophila* 60A (d60A), PIR/A43918; *Xenopus* Vg-1 (xVG1), PIR/A29619; sea urchin DVR-1 (suDVR1), PIR/S52408; sea urchin univin (suUNIVIN), GenBank/U10533; chicken dorsalin (cDORSALIN), GenBank/L12032; mouse nodal (mNODAL), PIR/S29718; ascidian BMPa (HrBMPa), Miya *et al.*, 1996.

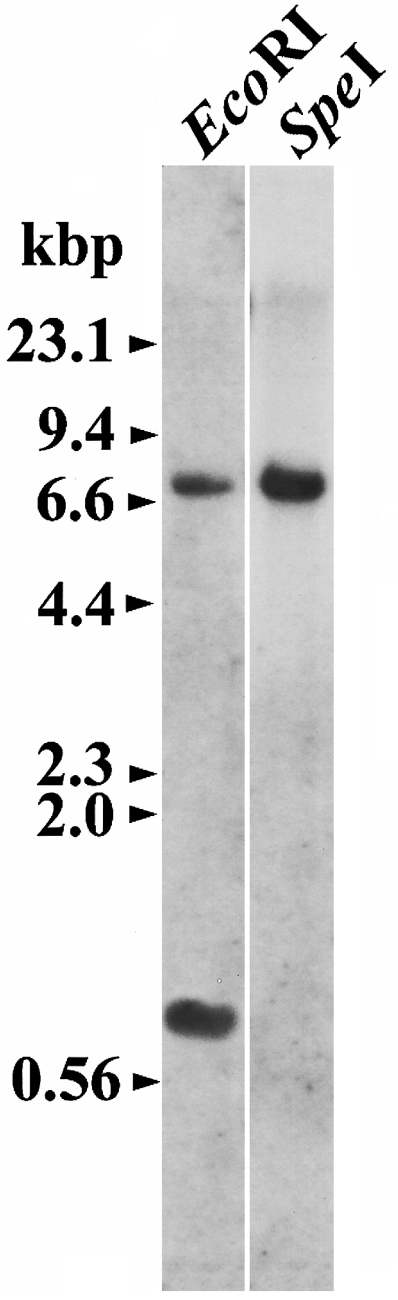

Fig. 3. Southern blot hybridization. Molecular size (*Hin*dIII digested λDNA) is shown on the left.

expression in intact worm suggests that the molecule is important in maintenance of body plan and that the cellular and molecular events during regeneration themselves occur constantly in an intact body to maintain the body plan. This was also supported by the expression pattern of Hox/HOM-C genes along the antero-posterior axis of regenerating and intact body (Orii *et al*., in preparation).

Recently, De Robertis and Sasai (1996) proposed a hypothetical ancestral and primitive bilateral animal, *Urbilateria*, from which the arthropod and the chordate lineages diverged 600 million years ago, with Hox gene complexes, D-V patterning system by *sog* (*short gastrulation*)/*chordin* and *dpp*/*Bmp-4* and so on. In accordance with recent molecular evolutionary studies, the *Urbilateria* was divided into two groups, the *Deuterostomia* and the *Protostomia*. The *Protostomia* was further subdivided into the *Lophotrochozoa* and the *Ecdysozoa* during the evolution (for review; Balavoine and Adoutte, 1998). The Plathelminthes including *Dugesia japonica* has simple body plan and is grouped into the *Lophotrochozoa*. In this paper, we showed that the Plathelminthes also has BMP gene as well as the *Deuterostomia* (vertebrates *etc*.) and the *Ecdysozoa* (arthropoda, nematoda *etc*.). In addition to Hox gene complexes and *Djbmp*, the presence of *DjotxA*, *DjotxB* and *Djotp* which are planarian homologues to *orthodenticle* and *orthopedia* in *Drosophila* (Umesono *et al*., 1997 ; Umesono *et al*., 1998), strongly suggests that the basic body plan of the *Bilateria* including *Deuterostomia*, *Ecdysozoa* and *Lophotrochozoa*, are common. To date, the presence of BMP gene has not been reported in radiata hydrozoan such as hydra. BMP gene may be related to the establishment of D-V axis in animal evolution.

We have no information on other BMP genes in the planarian. However, it has been suggested that BMP-4 forms a heterodimer with BMP-7 to function in mesoderm induction in *Xenopus* (Suzuki *et al*., 1997) and that DPP acts to establish D-V pattern by forming a heterodimer with SCREW, a member of 60A subfamily, in *Drosophila* (Arora *et al*., 1994). DjBMP may also act with another unidentified BMP-like member in the planarian. In higher organisms, *tolloid*/*Bmp-1* gene product also regulates D-V patterning in relation to DPP/BMP and SOG/Chordin proteins (Marqués *et al*., 1997; Piccolo *et al*., 1997). To search for and analyze such molecules in planarians may help us to understand the evolution of the common mechanism of body patterning in the *Bilateria*.

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The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB010966.

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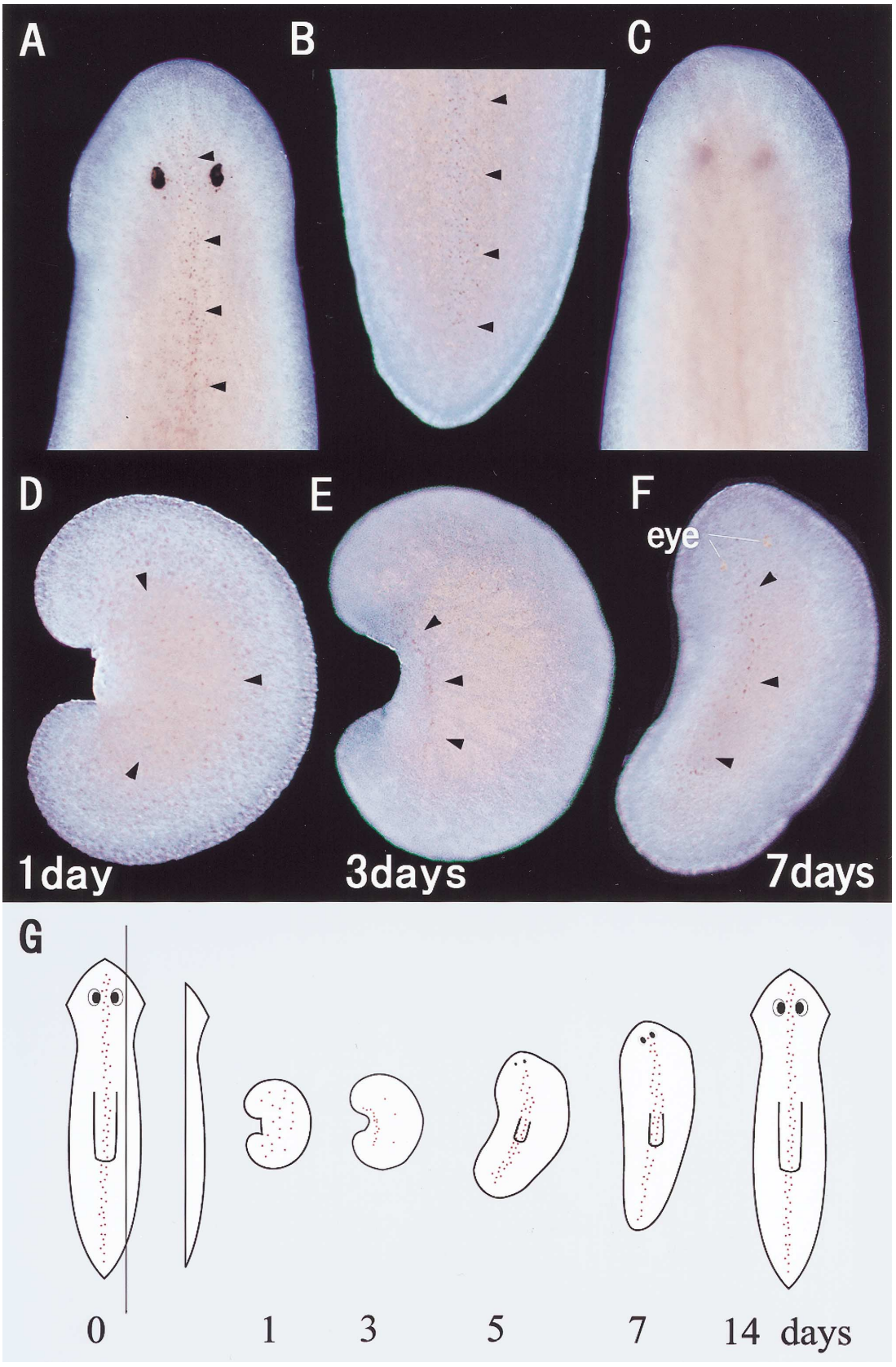

Fig. 4. Whole mount *in situ* hybridization. Anterior dorsal (**A**), posterior dorsal (**B**) and anterior ventral (**C**) view of intact worm. Dorsal view of regenerating marginal piece 1 day (**D**), 3 days (**E**) and 7 days (**F**) after amputation. The region expressing *Djbmp* is shown by the arrowheads. Schematic representation of expression of *Djbmp* during regeneration of right marginal piece (**G**). Dots show the expression of *Djbmp*.

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