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Group B *Sox* Genes That Contribute to Specification of the Vertebrate Brain are Expressed in the Apical Organ and Ciliary Bands of Hemichordate Larvae

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ABSTRACT—We have identified and characterized the sequence and expression of two Group B *Sox* genes in the acorn worm, *Ptychodera flava*. One sequence represents a Group B1 *Sox* gene and is designated *Pf-SoxB1*; the other is a Group B2 *Sox* gene and is designated *Pf-SoxB2*. Both genes encode polypeptides with an HMG domain in the N-terminal half. Whole-mount *in situ* hybridization to embryonic and larval stages of *P. flava* shows that the two genes are expressed in rather similar patterns at these stages. Expression is first detected in the cells of the blastula and subsequently localizes to the ectoderm during gastrulation. As the mouth forms, expression becomes concentrated in the stomodeum region. During morphogenesis of the tornaria larva, expression in the stomodeal ectoderm remains prominent around the mouth and under the oral hood. Later the genes are prominently upregulated in the ciliary bands and the apical organ. These results provide additional evidence that genes playing essential roles in the formation of the chordate dorsal central nervous system function in the development of the ciliary bands and apical organ, neural structures of this non-chordate deuterostome larva.

Key words: hemichordate, Group B *Sox* genes, embryonic expression, ciliary bands, apical organ

INTRODUCTION

Many aspects of the evolution of the chordate body plan from primordial bilaterians are yet to be delineated or understood. One of the most interesting questions is the evolutionary foundations of the dorsal nerve chord that has produced the complex central nervous systems of higher vertebrates. Phylogenetically, chordates are deuterostomes, a superphylum that according to present molecular phylogeny consists of echinoderms and hemichordates as well as the chordates. Recent studies using comparison of 18S rDNA sequences strongly support the association of these phyla in the deuterostomes and assign other phyla traditionally linked to deuterostomes to different groups (Wada and Satoh, 1994; Turbeville *et al*., 1994; Aguinaldo *et al*., 1997; Wada, 1998; Knoll and Carroll, 1999; Cameron *et al*., 2000). We have been studying the expression of homologues of vertebrate brain-specific genes in hemichordates and echinoderms to gain clues concerning the evolution of the vertebrate brain (Tagawa *et al*., 2001).

The first vertebrate brain gene we examined was *T-brain*, a gene that is involved in the patterning of the vertebrate forebrain (Bulfone *et al*., 1995; Ryan *et al*., 1998). The *T-brain* homologue of the acorn worm *Ptychodera flava* (*Pf-Tbrain*) is expressed in the apical organ, suggesting a link between the apical organ of the deuterostome larvae and the forebrain of vertebrates (Tagawa *et al*., 2000). This result provides the first molecular support for the hypothesis that the apical organ of dipleurula-type larvae is related to structures that provide the evolutionary substrate for the vertebrate forebrain (Lacalli, 1996)

Another brain-related gene that we have studied is *otx*, a gene essential for the development of the vertebrate anterior brain. Its homologues in *P. flava* (*Pf-otx*; Harada *et al*., 2000) and the sea cucumber *Stichopus japonicus* (*Sj-otx*; Shoguchi *et al*., 2000) are expressed in the both the preoral and postoral ciliary bands on the ventral or oral face of the larvae from both of these species. Shoguchi *et al.* (2000) followed *Sj-otx* expression from fertilized eggs through the auricularia larva and doliolaria larva stages to young juveniles. The *Sj-otx* is expressed in the components of ciliary bands that give rise to the oral side of juvenile holothurians.

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These results on the expression of vertebrate brain-specific genes in the ciliary bands of hemichordate and echinoderm larva highlight Garstang's idea of more than 70 years ago (Garstang, 1928) that there is an evolutionary relationship between the ciliary bands of dipleurula larvae and the chordate dorsal nerve chord. He proposed that the chordate dorsal nerve chord arose by rolling up of the ciliary bands on the dorsal side of the larva much as occurs during the formation of the dorsal hollow nerve cord of the juvenile acorn worm (Morgan, 1894).

We decided to examine in hemichordates homologues of other genes important in vertebrate brain development. In the present study, we have focused on the *Sox* (*SRY*-related box) gene family. Sox transcription factors contain a highly conserved ~80 amino acid DNA-binding domain (Sox box) related to those of high mobility group (HMG) proteins. Members of the HMG superfamily bind AT-rich motifs and regulate gene transcription (Grosschedl *et al.*, 1994; Pevny and Lovell-Badge, 1997). Members of the Sox transcription factor family play pivotal developmental roles in a number of organ systems (reviewed by Pevny and Lovell-Badge, 1997).

Sox family genes in vertebrates are classified into groups, presently lettered from A to J based on sequence similarity of the encoded HMG domain (Bowles *et al.*, 2000). The Group B *Sox* genes are further subdivided into Group B1 genes (*Sox1*, *Sox2*, and *Sox3* of human, mouse and chick) and Group B2 genes (*Sox14* and *Sox21* of mouse and *zf-Sox19* of zebrafish) (Uchikawa *et al.*, 1999). Analyses of early embryos have revealed that *Sox2* and *Sox3* are widely expressed in the nervous system in the chicken and *Xenopus* (Uwanogho *et al.*, 1995; Mizuseki *et al.*, 1998). *Sox2* gene expression is correlated with neural competence of the ectoderm (Streit *et al.*, 1997; Mizuseki *et al.*, 1998). *Sox14* and *Sox21* of chick and *zfSox19* of zebrafish are also expressed in the nervous system of embryos (Rex *et al.*, 1997; Uchikawa *et al.*, 1999; Vriz *et al.*, 1996). In addition one group B1, *SoxNeuro/dr-SoxB1*, and one group B2, *Dichaete/Fish/Sox70D/dr-SoxB2.1*, *Sox* genes are expressed in the developing *Drosophila* nervous system (Nambu and Nambu, 1996; Russell *et al.*, 1996; Cremazy *et al.*, 2000).

These accumulated results establish that Group B *Sox* genes are involved in the specification and development in the vertebrate nervous system and were probably involved in neural specification in a common early ancestor of some or all of the bilaterian animals. We designed studies to identify Group B *Sox* genes conserved in hemichordates and determine their pattern of expression in embryos and tornaria larva.

MATERIALS AND METHODS

Animals and embryos

Mature adults of *Ptychodera flava* were collected from areas with shallow sandy bottoms around Oahu in December and maintained for a number of days at the Kewalo Marine Lab. Spawning was induced by temperature shift as described (Tagawa *et al.*, 1998a). Fertilized eggs were allowed to develop to the specified developmental stages at room temperature. The time table of *P. flava* development is seen in Tagawa *et al.* (1998a).

Molecular cloning

About 120 cDNA clones were randomly selected from the gastrulae cDNA library of *P. flava* constructed with λZAPII (Stratagene) (Tagawa *et al.*, 1998b), converted to plasmids and sequenced for about 500 bp at their 5' end. The selected cDNA clones with *SoxB2* similarity were completely sequenced on both strands with a BigDye Primer Cycle Sequencing Ready Reaction Kit and ABI-PRISM 377 DNA Sequencer (Perkin Elmer). The cDNA library was then screened with a random-labeled with [^{32}P]dCTP probe (Amersham) of the HMG box of *SoxB2* clone under reduced stringency conditions. Eleven clones were selected and sequenced as above. Nine clones contained inserts from the same *SoxB1* gene. The longest was sequenced completely on both strands.

Sequence comparison and molecular phylogenetic analysis

Amino acid sequences of the HMG domains of *Pf-SoxB1* and *Pf-SoxB2* gene products were deduced from their cDNA nucleotide sequences. They were aligned with other *SoxB1* subfamily members, and their molecular relationships were analyzed phylogenetically using the PHYLIP Neighbor-Joining method (ver. 3.5; Felsenstein, 1993).

Whole-mount *in situ* hybridization

Whole mount specimens were hybridized *in situ* essentially as described by Tagawa *et al.* (1998b). Briefly embryos and larvae were fixed in 4% paraformaldehyde in 0.5 M NaCl, 0.1 M MOPS, pH 7.5 on ice overnight. After a thorough wash with PBST (phosphate-buffered saline containing 0.1% Tween 20), the specimens were partially digested with 2 μg/ml proteinase K (Sigma) in PBST for 20 min at 37°C and were post-fixed with 4% paraformaldehyde in PBST for 1 hr at room temperature. After 1 hr prehybridization at 42°C the specimens were hybridized with digoxigenin (DIG)-labelled antisense probes for about 16 hr at 42°C. The hybridization buffer contained 50% formamide, 5 x Denhardt's solution, 100 mg/ml yeast RNA, 5 x SSC, and 0.1% Tween 20. Antisense and sense probes were synthesized from the full length cDNA insert following the instructions supplied with the kit (Boehringer Mannheim DIG RNA Labelling kit), and the hybridized fragments were reduced to about 200–500 nucleotides by alkaline hydrolysis. The probes were used at 0.5 μg/ml in the hybridization buffer. After hybridization, the specimens were digested with 20 mg/ml RNase A. After final wash in 50% formamide, 0.5 x SSC, 0.1% Tween 20 (20 min, 50°C), specimens were incubated with 0.5% blocking reagent (blocking buffer) in PBST at room temperature, then with 1:2000 Boehringer Mannheim alkaline-phosphatase-conjugated anti-DIG antibody in the blocking buffer, overnight at 4°C. Washes proceeded in PBST (20 min, four times), then in alkaline phosphatase buffer for 10 min twice. A signal was detected using NBT and BCIP following the supplier's instructions. The reactions were stopped in PBST after 1 to 2 hr, then the embryos were observed under a microscope. For double staining, the *Pf-HNF3* probe (Taguchi *et al.*, 2000) was labeled with fluorescein and both probes hybridized simultaneously. After developing signal with the anti-DIG reagent, the DIG antibody conjugate was removed at low pH and *Pf-HNF3* signal developed with AP coupled anti-fluorescein FAB (Boehringer-Mannheim) and detected with Fast Red TR / Napthol AS-MX using the same staining procedure described above for the NBT and BCIP substrate.

RESULTS

Isolation and characterization of cDNA clones for hemichordate Group-B *Sox* genes

cDNA clones containing sequences of two different Group-B *Sox* genes were isolated from a *P. flava* gastrula cDNA library (Tagawa *et al.*, 1998b) by EST sequencing and library screening methods. About 120 cDNA clones were randomly selected for EST sequencing from the 5' end. Of these, four clones contained HMG boxes. One had sequence similarity to a *Sry*-related gene, another to an HMG gene, and two were identical and showed sequences suggestive of Group B2 *Sox* genes. An apparently full length clone was sequenced. The insert was 1,770 bp in length with an open reading frame that encoded a peptide of 246 amino acids (Fig. 1). It was tentatively designated *Pf-SoxB2*.

We searched further for cDNA clones with Group B1 type *Sox* gene sequences. The HMG box of *Pf-SoxB2* was used as a probe to screen the *P. flava* gastrula cDNA library under reduced stringency. The screening yielded 109 positive clones. About 500 bp of the 5' end of 11 clones were sequenced and it was evident that only two distinct mRNA sequences were represented in the positive clones. Two clones were from *Pf-SoxB2* and nine appeared to be from a single *SoxB1* gene. The longest *SoxB1* clone was sequenced over both strands. The insert was 1,815 bp with an open reading frame encoding a polypeptide of 320 amino acids (Fig. 2). It was tentatively designated *Pf-SoxB1*.

Sequence analyses confirm that *Pf-SoxB1* and *Pf-SoxB2* are respectively Group B1 and Group B2 *Sox* genes

The identification of these clones as Group B1 and Group B2 *Sox* genes is based on comparisons of the amino acid sequences of the HMG domains. These domains constitute 79 amino acids and are found at the positions 42–120 in the open reading frame of Pf-SoxB1 (Fig. 2) and at positions 7–85 of Pf-SoxB2 (Fig. 1). Fig. 3A shows comparison of the HMG domain amino acid residues of Pf-SoxB1 and Pf-SoxB2 with those of *SoxB1* and *SoxB2* gene products of other animals.In these sequences 61 residues are identical and 13 are similar in the HMG domain in all of the proteins. The two diagnostic residues that distinguish Group B1 and Group B2 HMG domains occur at positions 2 and 78. Posi-

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   1  CACGAGCAACTGACGACGGCTAGTGATTCTAGTGCCGGAATTGGGTGAATGTATACGTTT    60
  61  TTCGGACTCTTGACAAGGCCAGTAACTTACGAAAGTCATTCTTTTGACTTGACACGTGTC   120
 121  TGTGACCGTACTGTACTACCTCATACAGTGACCGCGTTTTCAAAAAAGTGTTAAAAACAC   180
 181  GACGACAACAACATGGCCTGCAAACCAGTCGATCATGTGAAACGTCCCATGAATGCTTTC   240
   1              M  A  C  K  P  V  D  H  V  K  R  P  M  N  A  F    16
 241  ATGGTGTGGTCTCGTGGTCAGCGGCGGAAGATGGCACAGGAGAATCCCAAAATGCACAAT   300
  17  M  V  W  S  R  G  Q  R  R  K  M  A  Q  E  N  P  K  M  H  N    36
 301  TCTGAGATAAGTAAGCGACTTGGTGCCGAGTGGAAACTCTTGTCGGAGGTGGAGAAGAGA   360
  37  S  E  I  S  K  R  L  G  A  E  W  K  L  L  S  E  V  E  K  R    56
 361  CCATTTATTGACGAAGCCAAAAGACTACGAGCGTTGCATATGAAGGAACACCCTGATTAT   420
  57  P  F  I  D  E  A  K  R  L  R  A  L  H  M  K  E  H  P  D  Y    76
 421  AAGTACCGGCCAAGACGTAAACCCAAATCGCTACTGAAGAAAGACAGGTACGCATTTCCG   480
  77  K  Y  R  P  R  R  K  P  K  S  L  L  K  K  D  R  Y  A  F  P    96
 481  ATACCATGTATTCCCACCAGCGGTAACTATACCGCAACGTCGTCAGCCAACGATATATAT   540
  97  I  P  C  I  P  T  S  G  N  Y  T  A  T  S  S  A  N  D  I  Y   116
 541  AGTAGCTACTCGGCGTCGGAAAAGGTCCGCTCATACTTGCCAAACACGACGTCGCCATAC   600
 117  S  S  Y  S  A  S  E  K  V  R  S  Y  L  P  N  T  T  S  P  Y   136
 601  TCTCTGCTGGACTCTGTGAACGCGTCGGTCAACAGCAAGCTCGACGGCGGGTCGTACCCA   660
 137  S  L  L  D  S  V  N  A  S  V  N  S  K  L  D  G  G  S  Y  P   156
 661  GGTGCCAACCCTCTGTACTCGTCCCATCTCTACCCGAGTTTACCTGGCAGTGCGTTCGGA   720
 157  G  A  N  P  L  Y  S  S  H  L  Y  P  S  L  P  G  S  A  F  G   176
 721  GCGGCCTCCATCCCGACACCACATACACACACGCATCCTACGCCTGGAATGCCGGGACAG   780
 177  A  A  S  I  P  T  P  H  T  H  T  H  P  T  P  G  M  P  G  Q   196
 781  CAATACATGATGCCTTGTAATTGTTGGCAGCCAGCTCAGGATCCTCTCAGACGCCCTGTT   840
 197  Q  Y  M  M  P  C  N  C  W  Q  P  A  Q  D  P  L  R  R  P  V   216
 841  GCGTATATTCTGGTAAAACCGGACGAACACTACTCGGCGACACACCCAGCCCACCACCAC   900
 217  A  Y  I  L  V  K  P  D  E  H  Y  S  A  T  H  P  A  H  H  H   236
 901  ACGGCTCACCCGCCGCCAACATCGCACATATAGGCCTAAATGACTAGAACTGAGTAAGAC   960
 237  T  A  H  P  P  P  T  S  H  I  *                             246
 961  TTTCTATGACAATTCTAAACAAAACATTATGTTGTGTTTGACCTAGCTTTATTTTGGCTG  1020
1021  GGAATGGTTAACCTAAAGCTCACGGACACCTTAGACGCGGGATTCATTTTGTTATATGAT  1080
1081  TTCCCAAAAATGAGTTTATATATTATATATTTTCCCTTCATGGTGAAGGTGACGTGTAGC  1140
1141  TGGCCGCCACCGTATAATGTAGAAGTGTTGTTGCAATGTTTTCGATTAGAAACGATGAGA  1200
1201  TACAGCTGTTCATATAATTTCGTTTCATGATCCTAACGTTTAACTTAACCCTATCCCTCC  1260
1261  CCGATTTAGAGAGATAAAAAGACGATCAACATGATTTGTTTATCACATTCGGCTCATTTC  1320
1321  TTTGGCCGAGTTTTGTAAGATTTATTTTCAACGAGGGTTGAGGGATAAAGTCAAGTATTT  1380
1381  TTGTTTATTTTTTTAATGTTTAAAGTACTGAAATTTCCTTTTTTTCCCAAAAAGTTGATG  1440
1441  TTCACAAGCGACTTACTAATGCTGCCTTGTCACACAAGAACTAAACCAGAAAAGGACTTC  1500
1501  TAACAAATTTTATGTGTTCGAATATTTAGATAATATTTTACGAGTGTAAAGTATATTGAC  1560
1561  TTATTTTTTGGTATAAGAGGAGGTAACGACGCAGTGGATCGAAATTTCAATGTCGCAGAT  1620
1621  CAAAACGATGCATTTTTGTATTTGTTTTAGCAGTCGTTATTTAATACTGATTTTGTTTTC  1680
1681  CTGTCAAATGTAAATTGTACCGAGTCTGTTCAGTCTTTGAACTAAGTCACAGTAAAAAAA  1740
1741  CACGAGAAACTGAAAAAAAAAAAAAAAAA  1770
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Fig. 1. The nucleotide and predicted amino acid sequences of a cDNA clone for the *Pf-SoxB2* gene. The cDNA consists of 1,770 bp including 18 adenylyl residues at the 3' end. The ATG at the position 193-195 represents the putative start codon of the *Pf-SoxB2*-encoded protein. Seventy-nine amino acids constituting the HMG DNA-binding domain (positions 7-85) are shadowed. An asterisk indicates the termination codon.

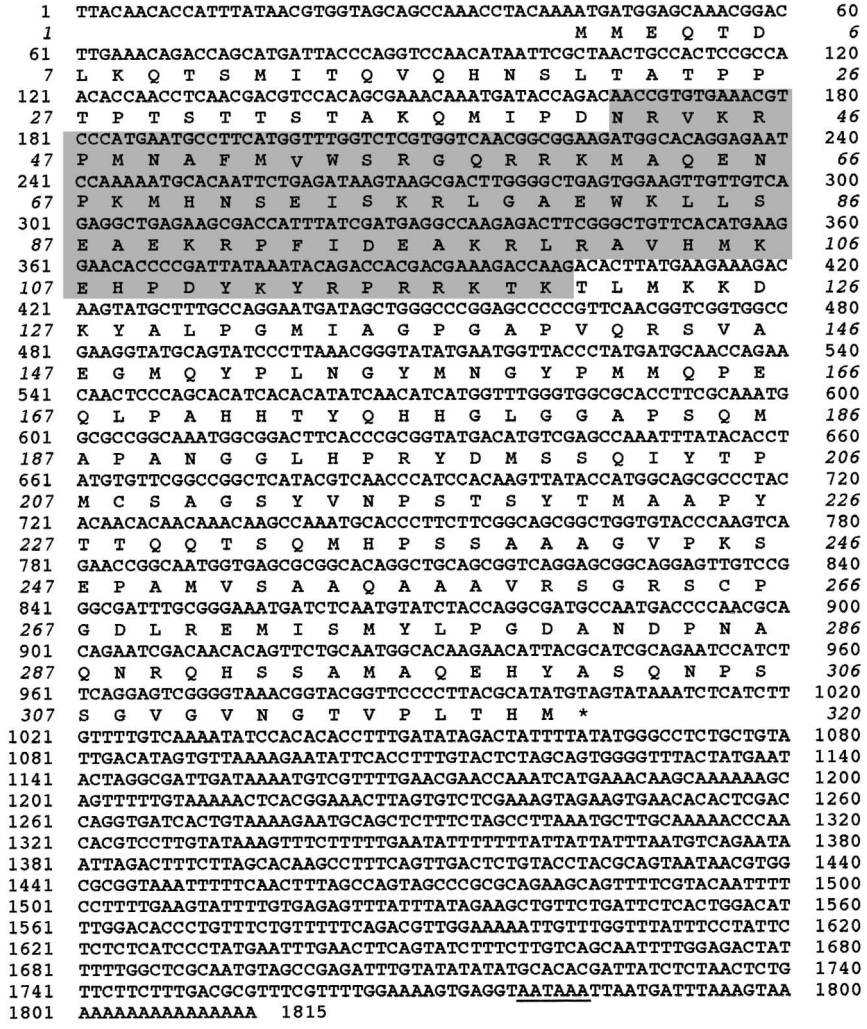

Fig. 2. The nucleotide and predicted amino acid sequences of a cDNA clone for the *Pf-SoxB1* gene. The cDNA consists of 1,815 bp including 17 adenylyl residues at the 3' end. The ATG at the position 43-45 represents the putative start codon of the *Pf-SoxB1*-encoded protein. A potential signal sequence for polyadenylation is underlined. An asterisk indicates the termination codon. Seventy-nine amino acids constituting the HMG DNA-binding domain (positions 42-120) are shadowed.

tion 2 is R in Group B1 and H in Group B2 while position 78 is T in Group B1 and P in Group B2. As marked by double underlines in Fig. 3A, these diagnostic residues are appropriately found in the respective gene products.

In a second approach, we applied the neighbor-joining algorithm (Saitou and Nei, 1987) to perform a molecular phylogenetic sorting of Pf-SoxB1 and Pf-SoxB2 with Group B *Sox* gene products using the 79 amino acids residues of the HMG-domain. As shown in Fig. 3B, the tree sorted with *Pf-SoxB1* among the Group B1 *Sox* genes while *Pf-SoxB2* is included among members of the Group B2 *Sox* genes. The same groups included sea urchin *SpSoxB1* and *SpSoxB2*, respectively (Fig. 3B; Kenny *et al*., 1999). These results establish that both Group B1 and Group B2 *Sox* gene sequences are found in cells of the gastrula stage of hemichordate embryos.

Pf-SoxB1 and *Pf-SoxB2* are expressed in the apical organ and ciliary bands of the tornaria larva

The pattern and localization of expression of *Pf-SoxB1* and *Pf-SoxB2* during embryonic and larval development were examined by whole-mount *in situ* hybridization with antisense probes. Temporal and spatial patterns of expression of *Pf-SoxB1* and *Pf-SoxB2* were similar (Fig. 4). The *Pf-SoxB1* mRNAs appear to be generally expressed at higher levels than are *Pf-SoxB2* sequences. Equivalent hybridization signals from the two genes are produced when *Pf-SoxB1* hybridized embryos and larvae are incubated with substrate for about 1/3 as long as *Pf-SoxB2* hybridized samples. *In situ* hybridization with sense probes resulted in background signals only.

Results from whole-mount *in situ* hybridization of *Pf-SoxB1* in early embryos and tornaria larvae are shown in Fig. 4A–E. Weak hybridization signal is first evident in the blastula at about 12 hr after fertilization. The signal occurs

A

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Group B1 Sox          1       10        20        30        40        50        60        70        79
PfSoxB1               NRVKRPMNAFMVWSRGQRRKMAQENPKMHNSEISKRLGAEWKLLSEAEKRPFIDEAKRLRAVHMKEHPDYKYRPRRKTK
SpSoxB1(Sea urchin)   DRVKRPMNAFMVWSRGQRRKLSQENPKMHNSEISKRLGAEWKLLSETEKRPFIDEAKRLRAVHMKEHPDYKYRPRRKTK
Sox 1(Chick)          DRVKRPMNAFMVWSRGQRRKMAQENPKMHNSEISKRLGAEWKVMSEAEKRPFIDEAKRLRALHMKEHPDYKYRPRRKTK
Sox 2(Chick)          DRVKRPMNAFMVWSRGQRRKMAQENPKMHNSEISKRLGAEWKLLSEAEKRPFIDEAKRLRALHMKEHPDYKYRPRRKTK
Sox 3(Chick)          DRVKRPMNAFMVWSRGQRRKMAQENPKMHNSEISKRLGADWKLLSDAEKRPFIDEAKRLRAVHMKEYPDYKYRPRRKTK
SoxNeuro(Drosophila)  DRVKRPMNAFMVWSRGQRRKMASDNPKMHNSEISKRLGAQWKDLSESEKRPFIDEAKRLRAVHMKEHPDYKYRPRRKTK
                       =                                                                         =
                       =                                                                         =
Group B2 Sox
PfSoxB2               DHVKRPMNAFMVWSRGQRRKMAQENPKMHNSEISKRLGAEWKLLSEVEKRPFIDEAKRLRALHMKEHPDYKYRPRRKPK
SpSoxB2(Sea urchin)   DHVKRPMNAFMVWSRGQRRKLAQENPKMHNSEISKRLGAEWKLLSEDDKRPFIDEAKRLRALHMKEHPDYKYRPRRKPK
Sox14(Chick)          DHIKRPMNAFMVWSRGQRRKMAQENPKMHNSEISKRLGAEWKLLSEAEKRPYIDEAKRLRAQHMKEHPDYKYRPRRKPK
Sox21(Chick)          DHVKRPMNAFMVWSRAQRRKMAQENPKMHNSEISKRLGAEWKLLSEAEKRPFIDEAKRLRAMHMKEHPDYKYRPRRKPK
                      ...************.****.. .***************.** .*. .***.********* **** **********.*
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B

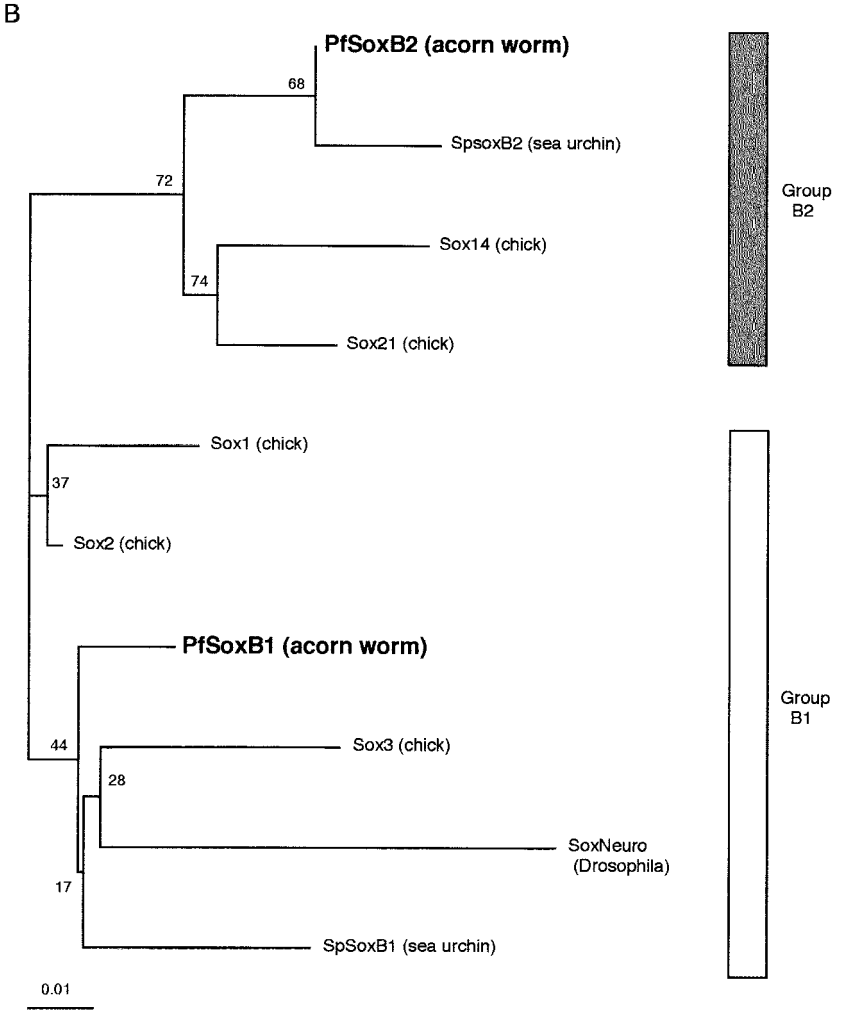

Fig. 3. Comparison of Pf-SoxB1 and Pf-SoxB2 amino acid sequences to other Group B *Sox* gene products. **(A)** An alignment of amino acid sequences of the HMG box of (a) Pf-SoxB1 and (b) Pf-SoxB2 with those of other Group B *Sox* gene members. Asterisks indicate identities of amino acids among all members, while dots show similarities of amino acids. Two residues at positions 2 and 78, shown by double-underlines, provide diagnostic differences between groups B1 and B2 *Sox* genes. **(B)** Molecular phylogenetic analysis of relationships of the HMG box amino acid sequences of *Pf-SoxB1* and *Pf-SoxB2* with *Sox* gene members as deduced using the Neighbor-Joining method. Branch lengths are proportional to evolutionary distance corrected for multiple substitutions with the scale denoting 0.01 amino acid substitutions per site. The numbers indicate the relative robustness of each node as assessed by bootstrap analysis (100 replications).

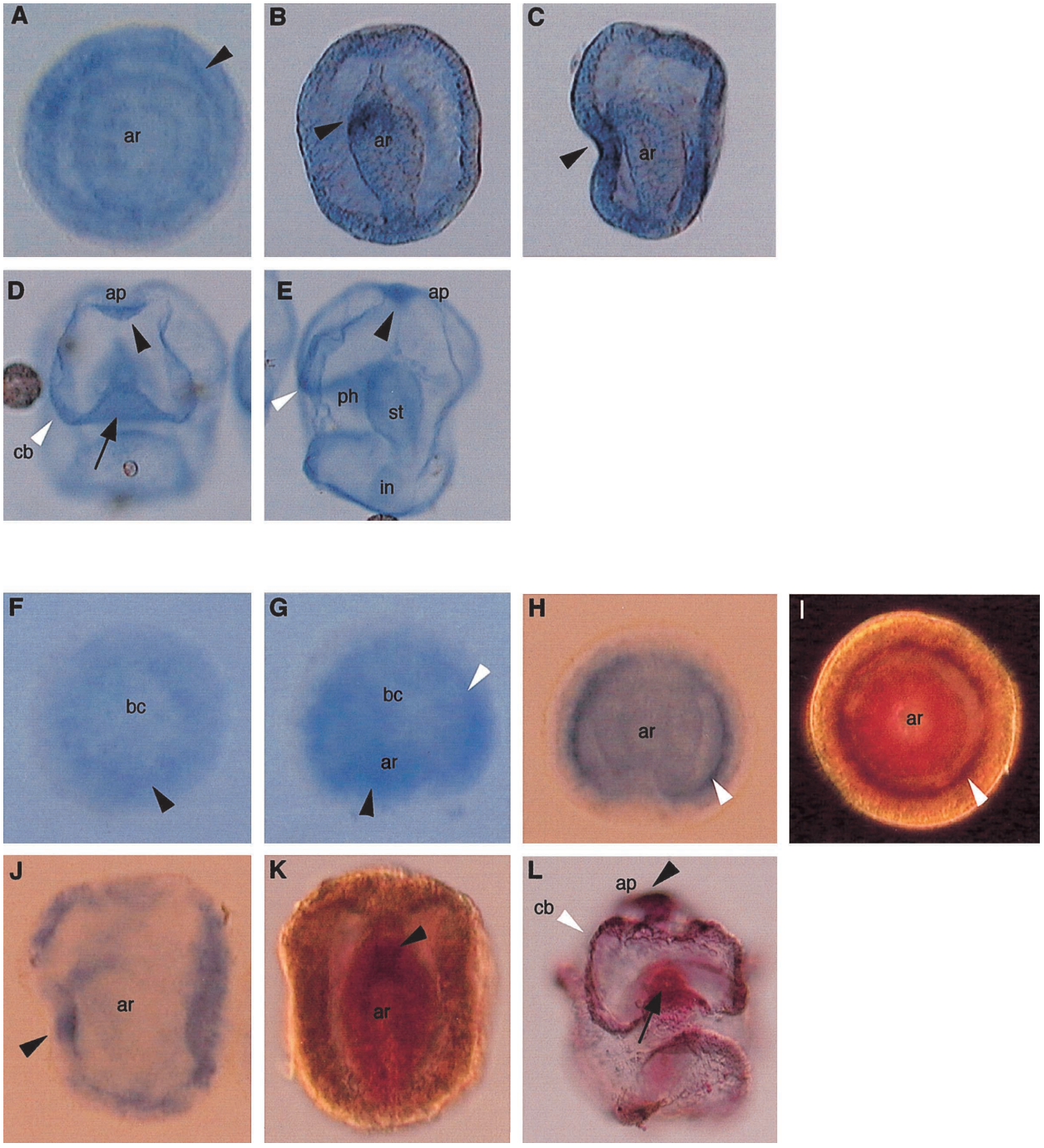

Fig. 4. Embryonic expression of *Pf-SoxB1* and *Pf-SoxB2*, as revealed by whole-mount *in situ* hybridization with a DIG-labeled antisense probe. **A–E** Spatial expression of *Pf-SoxB1*. **A** An early gastrula at 18 hr after fertilization, vegetal pole view. *Pf-SoxB1* hybridization signal is seen in cells at the pole facing the blastocoel (arrowhead). **B, C** A late gastrula at 43 hr of development, **B** lateral view and **C** frontal view. Weak signal is seen in the entire ectoderm while strong signal is evident the stomodeum region (arrowhead). **D, E** A 4-day-old tornaria larva, **D** frontal view and **E** lateral view. The hybridization signals are seen in the apical organ (black arrowhead), ciliary bands (white arrowhead) and in the ectoderm under the preoral hood and around the mouth (arrow). **F–L** Expression of *Pf-SoxB2*. **F** A blastula at 12 hr after fertilization, lateral view. Weak hybridization signal is seen in ectoderm cells facing the blastocoel (arrowhead). **G** An early gastrula at 18 hr after fertilization, lateral view, showing the signal in the ectoderm (white arrowhead) and archenteron invagination region (black arrowhead). **H, I** A middle gastrula at 21 hr of fertilization, **H** lateral view and **I** vegetal pole view. **J, K** A late gastrula at 39 hr of development, **J** lateral view and **K** frontal view. Strong signal is evident in the stomodeum region (arrowhead). **L** A 4-day-old tornaria larva, frontal view. Signals are seen in the ectoderm under the preoral hood and around the mouth (arrow region), the apical organ (black arrowhead) and ciliary bands (white arrowhead). In **I**, **K** and **L**, double *in situ* hybridization was performed to compare the expression of *Pf-HNF3* expression (red, arrow in **L**) with the expression of *Pf-SoxB2* (purple). *Pf-HNF3* is expressed in the archenteron. ap, apical organ; ar, archenteron; bc, blastocoel; cb, ciliary bands; in, intestine; ph, pharynx; and st, stomach.

in the basal regions of the cells facing the blastocoel and this signal becomes more prominent in all of the cells as gastrulation is initiated (Fig. 4A). The signal in the archenteron cells wanes and disappears as gastrulation proceeds. During mouth formation, the signal in the stomodeal ectoderm upregulates while expression in the remainder of the ectodermal cells is extinguished. The upregulated, strong signal in the stomodeal ectodermal cells appears throughout the cell bodies (Fig. 4B, C) and continues under the oral hood and around the mouth of the tornaria. As the tornaria develops, strong signals appear in the apical organ (black arrowhead of Fig. 4D, E) and ciliary bands (white arrowhead of Fig. 4D, E). This pattern of signals showing expression on the ventral surface of the oral hood above the mouth and extending around the mouth as well as in the apical organ and the ciliary bands continues until at least the 8-day larva.

The timing and pattern of embryonic expression of *Pf-SoxB2*, as shown in Fig. 4F-L, is not distinguishable from the expression of *Pf-SoxB1*. Figure 4F shows the result from a whole-mount *in situ* hybridization of a *Pf-SoxB2* antisense probe to a blastula stage embryo fixed at 12 hr after fertilization. A weak signal is exhibited in the basal region of the cells around the blastocoel (Fig. 4F, arrowhead). As shown in Fig. 4G for an 18-hr early gastrula and Fig. 4H, I for a 21-hr middle gastrula, the signal in the base of the cells becomes more prominent in ectodermal cells as gastrulation proceeds. After mid gastrulation, the signals in the base of the ectodermal cells gradually wane. But as with the *Pf-SoxB1* gene, at the late gastrula stages the cells in the region of ectoderm that will form the stomodeum develop a strong signal throughout their cell bodies (Fig. 4J, K). As with *Pf-SoxB1*, the expression of *Pf-SoxB2* in the stomodeal ectoderm cells continues as a zone of expression under the oral hood and around the mouth.

Tornaria larvae reacted to *Pf-SoxB2* probe show signals in the ectoderm under the preoral hood and around the mouth (Fig. 4L, arrow region) as well as in the apical organ (Fig. 4L, black arrowhead) and ciliary bands (Fig. 4L, white arrowhead). As with *Pf-SoxB1*, this expression of *Pf-SoxB2* in these tissues continued until at least the 8-day tornaria larva.

In the forming tornaria, the expression of the *HNF-3/forkhead* gene of *P. flava*, *Pf-HNF3*, is ultimately focussed in the endodermal cells that join the ectoderm to form the stomodeum (Taguchi *et al*., 2000). To delineate the spatial relationships between the endodermal expression of *Pf-HNF3* and the ectodermal expression of *Pf-SoxB2* in the stomodeal region, we performed double *in situ* hybridization with antisense probes from both genes. Archenteron expression of *Pf-HNF3*, as shown by red staining, is confirmed in late gastrula by this approach as shown in a vegetal view of the gastrula in Fig. 4I. When the double stained late gastrula preparations are viewed laterally or frontally (Fig. 4K, arrowhead), it is clear that the ectodermal expression of *Pf-SoxB2*, shown by purple staining, appears around the mouth and does not overlap the endodermal expression of *Pf-HNF3*, as shown by red signal, is in the pharynx (Fig. 4K). These genes appear to delineate the cells of ectodermal and endodermal origin respectively in the region of the mouth and pharynx in the tornaria larvae (Fig. 4L, arrow).

DISCUSSION

In this work, we add another example to evidence that genes implicated in the development of the vertebrate brain and nerve chord are expressed in the apical sensory organ and ciliary bands of dipleurula-type deuterostome larvae. We have isolated two *P. flava* homologues of Group B *Sox* genes, *Pf-SoxB1* and *Pf-SoxB2*, and have shown that these homologues are both expressed in the apical organ and the ciliary bands of the tornaria. Previously, we described expression of *T-brain*, a mammalian forebrain-specific gene, in the apical organ in a hemichordate tornaria (Tagawa *et al*., 2000). As well, we found that homologues of *otx*, a gene important in anterior brain development in vertebrates, are expressed in the ventral or oral ciliary bands of hemichordate and echinoderm larvae (Harada *et al*., 2000; Shoguchi *et al*., 2000). The cases of the *Sox* genes reported here represent the first examples of nervous system genes expressed simultaneously in both of these hemichordate larval structures.

The *Pf-SoxB1* and *Pf-SoxB2* sequences show the highest similarity to the sea urchin counterparts *SpSoxB1* and *SpSoxB2* derived from *Strongylocentrotus purpuratus* (Kenny *et al*., 1999). The study of the expression of the sea urchin genes has focussed on the expression of *SpSoxB1* during early stages. The gene is expressed maternally and zygotically. The original uniform distribution becomes asymmetric at fourth cleavage when the protein is reduced in the vegetal micromeres.

The expression of the Group B1 genes, *Sox2* and *Sox3*, in early chick embryos is also correlated with the specification of the ectoderm as well as establishment of neural competence in these cells (Streit *et al*., 1997; Mizuseki *et al*., 1998). *Sox2* (group B1) expression increases dramatically as neural ectoderm is established. The appearance of *Sox2* in neural ectoderm represents one of the earliest molecular responses to neural induction documented so far (Rex *et al*., 1997). *Sox3* (also group B1) in the chick embryo is expressed throughout the ectoderm that is competent to form nervous tissue before neural induction. The expression of *Sox3* is lost from cells as they undergo gastrulation to form non-ectodermal tissues. The transcription factor, Brachyury, is produced in cells about to undergo gastrulation a short time before *Sox3* transcripts are lost. Therefore, *Brachyury* expression may act functionally upstream of *Sox3* down regulation. *Sox3* expression is also lost from non-neural ectoderm shortly after the neural plate becomes morphologically apparent.

Sox2 function is required for neural differentiation of early *Xenopus* ectoderm. Microinjection of dominant-nega-

tive forms of *Sox2* (dn*Sox2*) mRNA inhibits neural differentiation of animal caps caused by attenuation of BMP signals (Kishi *et al*., 2000). Expression of dn*Sox2* in developing embryos suppresses expression of N-CAM and regional neural markers. Analysis of the temporal requirement of *Sox2*-mediated signaling using an inducible dn*Sox2* construct fused to the ligand-binding domain of the glucocorticoid receptor shows that attenuation of *Sox2* function beginning at late blastula or late gastrula stage onwards causes an inhibition of neural differentiation in animal caps and in whole embryos. Additionally, dn*Sox2*-injected cells that fail to differentiate into neural tissues are not able to adopt epidermal cell fates. These data suggest that *Sox2* gene products are essential for early neuroectoderm cells to consolidate their neural identity during secondary steps of neural differentiation.

In the present study, we found that the hemichordate Group B *Sox* genes, *Pf-SoxB1* and *Pf-SoxB2*, are expressed in the early ectoderm of blastulae and gastrulae. This early ectoderm expression in hemichordate could play a similar role in specification of the neuroectoderm. In addition to ectoderm, Group B *Sox* genes are dramatically implicated in the specification and differentiation of the brain and central nervous system of the vertebrates (as well as in the development of multiple other structures). *Pf-SoxB1* is a hemichordate Group B1 homologue of *Sox2* and *Sox3* that are both expressed in the immature neural epithelium of the entire CNS of HH stage 10 to 34 chick embryos. These genes are widely expressed as the nervous system develops in the chicken and *Xenopus* (Uwanogho *et al*., 1995; Mizuseki *et al*., 1998).

Pf-SoxB2 is a hemichordate Group B2 orthologue of *Sox14* and *Sox21*. In mouse and chick, *Sox14* expression is restricted to a limited population of neurons in the developing brain and spinal cord of both species. *Sox14* marks a subset of interneurons at a defined dorsoventral position adjacent to ventral motor neurons in the spinal cord (Hargrave *et al*., 2000). *Sox21* of zebrafish is expressed in the forebrain, midbrain and hindbrain, but maximally at the midbrain-hindbrain junction (Rimini *et al*., 1999)

When linked to the GAL4 DNA binding domain, these Group B2 proteins, *Sox21* and *Sox14* inhibited activation of the d1-crystallin DC5 enhancer by the Group B1 proteins, Sox1 or Sox2 (Uchikawa *et al*., 1999). This suggests that the Group B2 Sox proteins may be repressors of group B1 *Sox* genes. Since the activating B1 *Sox* genes and repressing B2 *Sox* genes display interesting overlaps of expression domains in developing tissues such as the optic tectum, spinal cord, inner ear, alimentary tract and branchial arches, this interaction could play a variety of important and subtle roles in regulating development (Uchikawa *et al*., 1999). In the context of our observations in hemichordates, we observe overlapping expression of the B1 and B2 homologues and it is interesting to speculate about possible opposing regulatory activities by the proteins from these two classes of genes.

In *Drosophila*, the gene now named *dr-Sox B2.1* (Bowles *et al*., 2000), and variously designated *Dichaete*, *Sox70D* or *fish-hook*, is essential for central nervous system development. Null *dr-Sox B2.1* mutants show the loss of specific neurons in the brain, fusion of adjacent ventral nerve chord ganglia and aberrant axon scaffold organization (Nambu and Nambu, 1996). During development, the gene has a dynamic expression profile in the forming central nervous system and has an especially intriguing pattern of expression associated with the ventral midline (Soriano and Russell, 1998). The *Drosophila* Group B1 *Sox* gene first designated *SoxNeuro* and recently renamed *dr-SoxB1* also has a highly dynamic pattern of expression during development (Cremazy *et al*., 2000). Early in development the gene is expressed in the presumptive ventro-lateral neuroectoderm. During gastrulation expression occurs in the cephalic and ventral neurogenic regions and then remains associated with the developing central nervous system.

We observed a strong *in situ* hybridization signal in the ectoderm under the oral hood and around the mouth of the hemichordate larvae. To our knowledge, there is no vertebrate correlate to this early expression of the group B *Sox* genes during mouth formation. Although there is no direct description of the transition of marked structures in this region from tornaria to adult worm, this region of the tornaria gives rise to the preoral ciliary organ of the adult. This organ was first well described by Brambell and Cole (1939) who believed it had a sensory function. Subsequently, this organ has been shown to be highly innervated as would be expected of a structure with a neural function (Knight-Jones, 1952). Thus, the *Sox* B gene expression under the preoral hood could mark another region developing neural function (Brambell and Cole, 1939).

The present finding supports the notion that genes responsible for the formation of vertebrate CNS are expressed in the ciliary bands and apical organ of tornaria larvae. The apical organ and the ciliary bands are both neural organs of the larval ectoderm. The apical organ contains serotonergic nerve cells and the ciliated epithelial cells of the ciliary bands produce axons that run in fiber tracts along the bases of the epithelial cells of the bands. These cells represent the very primitive nervous system of the larvae and could be considered logical substrates for the evolution of the vertebrate brain (Garstang, 1928; Lacalli, 1996), if it is established that vertebrate ancestors actually included a form related to these swimming larval stages.

With these results, it seems reasonable to propose that genes used for the formation of complex vertebrate CNS are likely to be expressed in the ciliary bands and/or the apical organ of invertebrate larvae. However, this does not necessarily mean that the ciliary bands and the apical organ of hemichordate larvae are orthologues of the chordate larval and adult CNS. Instead, it may be that when the two groups of deuterostomes evolved from their common ancestor, the same genes were used to determine their nervous systems. There may have been strong evolutionary constraint in the

usage of developmental genes in the formation of the nerve cells during evolution of the different groups of deuterostome. Because *Sox* group B genes are used in the formation of *Drosophila* CNS, this notion may be expanded to the entire metazoans. Therefore, it is an intriguing subject of future research to explore in detail the molecular machinery for the utilization of these genes in the formation of the nervous system in the different animal groups.

There are a number of very basic questions concerning the expression of these brain-specific genes in deuterostome larvae that remain to be examined. The hemichordate tornaria larva swims for an extended period of pelagic life before it metamorphoses in a worm and settles to the sandy bottom. The worm has a both major dorsal nerve cord and a larger ventral nerve cord (e.g. Brusca and Brusca, 1990). It will be very interesting to ascertain the expression of *Pf-Otx*, *Pf-Tbrain* and *Pf-SoxB* genes during the formation of the adult nervous system as the tornaria larva transitions to the juvenile worm.

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