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ORIGINAL ARTICLE

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Distinct expression patterns of the two T-box homologues *Brachyury* and *Tbx2/3* in the placozoan *Trichoplax adhaerens*

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Abstract *Trichoplax adhaerens* is the only species known from the phylum Placozoa with one of the simplest metazoan body plans. In the small disc-like organism an upper and a lower epithelium can be distinguished with a less compact third cell layer in between. When *Trichoplax* was first described in 1883, the relation of these three cell layers with ectoderm, endoderm and mesoderm of higher animals was discussed. Still, little is known about embryonic development of *Trichoplax*, however, genes thought to be specific for mesoderm in bilaterian animals turned out to be already present in non-bilaterians. Searching for a *Brachyury* homologue, two members of the T-box gene family were isolated from *Trichoplax*, *Brachyury* and a *Tbx2/3* homologue. The T-box genes encode a transcription factor family characterized by the DNA-binding T-box domain. T-box genes have been found in all metazoans so far investigated, but in contrast to other transcription factors such as the homeobox family, T-box genes are not present in plants or fungi. The distinct expression patterns of two T-box genes in *Trichoplax* point to non-redundant functions already present at the beginning of animal evolution. Since the expression patterns derived by in situ hybridization do not overlap with anatomical structures, it can be concluded that this simple animal has more than the four cell types described in the literature. This hidden complexity and the unresolved position in relation to Porifera, Cnidaria, Ctenophora and Bilateria highlight the necessity of the inclusion of *Trichoplax* in studies of comparative evolutionary and developmental biology.

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Introduction

Trichoplax adhaerens is an enigmatic species described for the first time by Schulze (1883), who was not able to put it in any known phyla due to its peculiar characteristics. In these early studies the main features, such as the small size of only a few millimeters with a constant thickness of 0.02 mm, no symmetry axis, no organs, a dorso-ventrally three-layered body and continuous changes in shape, were observed. The description of this new animal type in German had an immediate echo in the scientific community and an English report appeared in the first volume of Science (Minot 1883). An apparently related species was observed in Naples and named *Treptoplax reptans* by F. S. Monticelli, but since this original description it was not found again. Later, *Trichoplax* was described as the planula larva of the hydrozoan *Eleutheria krohi* (Krumbach 1907). Although this work was criticized (Schubotz 1912), the idea of *Trichoplax* as a hydrozoan larva was accepted and reported in textbooks (Hyman 1940). *Trichoplax* disappeared from the literature until 1971, when new observations suggested that *Trichoplax* is not a larva (Grell 1971; Miller 1971). *Trichoplax* is now thought to be the only species from the phylum Placozoa (Grell and Ruthmann 1991; Ruppert and Barnes 1994; Syed and Schierwater 2002).

The body structure of *Trichoplax* consists of an upper thin epithelial layer with stretched mononuclear cells, a basal epithelial layer with two cell types, column-shaped cells with cilia and round-shaped cells, called gland cells, without cilia. Between these two layers there are star-shaped cells, that form a syncytial net. *Trichoplax* move by changing their shape, similar to amoebae, due to the contraction of the microtubular system of the star-shaped cells (Thiemann and Ruthmann 1989). A nervous system seems to be missing, but with an antibody against

RFamide, which recognizes neuropeptides in many animal phyla, a few isolated cells can be detected (Schuchert 1993). The feeding system is located in the ventral layer; particles already digested by enzymes produced from the gland cells can be incorporated by endocytosis (Ruthmann et al. 1986). Apparently, *Trichoplax* is missing a structured extracellular matrix and a recognizable basal lamina (Grell and Ruthmann 1991). The epithelial cells are connected by belt desmosomes in both epithelial layers and no other type of junction has been described (Ruthmann et al. 1986). Feulgen cytometry has shown that *Trichoplax* is the species with the lowest amount of DNA of all the Metazoa (Ruthmann and Wenderoth 1975).

As Schulze noticed in 1883, the three layers of *Trichoplax* could be compared to ectoderm, mesoderm, and endoderm of higher animals, but this could be proved or disproved only by comparing developmental processes. Unfortunately, still little is known about reproduction of *Trichoplax*. In aquaria, it does it irregularly by fission. Under special circumstances it was observed to build hollow swarmer spheres, stolons and eggs developing up to the 64-cell blastula stage (Grell 1971; Ruthmann et al. 1986; Thiemann and Ruthmann 1991). Without the natural clues to the regulation of the life cycle of this peculiar animal, it will be difficult to find the relevant life stages. We could address, however, the question of whether so-called mesoderm-specific genes known from triploblasts are present in *Trichoplax* and where and eventually when they are expressed. With that aim we decided to investigate *Brachyury* and the T-box transcription factor gene family.

Members of the T-box gene family have been found in all the animal phyla so far investigated (Papaioannou 2001; Technau 2001), but no T-box gene can be recognized in genomes of fungi, plants or parasitic protists. The main feature of the protein is the conserved region of about 180 amino acids, called the T-box domain, that is characteristic of the family. The T-box domain is able to recognize half of a palindromic binding site in DNA (Müller and Herrmann 1997). There is still some confusion about the classification of the five to eight subfamilies and the nomenclature in different species (Papaioannou 2001; Ruvinsky et al. 2000), but at least comparisons of amphioxus and vertebrates suggests a conservative evolution of this gene family. Among the T-box family members the best studied is the founding member *Brachyury* or *T*, which is expressed during the gastrulation in the organizer and later along the AP axis of all the bilaterians that have been investigated so far (Arendt et al. 2001; Technau 2001; Lartillot et al. 2002; Takada et al. 2002). In chordates, *Brachyury* is involved in notochord formation (Amacher et al. 2002). *Brachyury* homologues have also been identified in cnidarians, which should be distinguished from bilaterians by the absence of mesoderm. In *Hydra vulgaris*, a simple fresh-water polyp with only two cell layers, *Brachyury* is expressed in the endoderm and is associated with the formation of the hypostome (Technau and Bode 1999). In the marine hydrozoan jellyfish *Podocoryne carnea*, however, *Brachyury* is expressed in the entocodon, a third mesoderm-like layer characteristic of the medusa bud development (Spring et al. 2002). In the anthozoan sea anemone *Nematostella vectensis*, *Brachyury* is expressed around the blastopore during the early stages of development and later in the mesenteries, invaginating septae of the polypoid sea anemone (Scholz and Technau 2003).

Trichoplax or Placozoa are often missing in phylogenetic studies and discussions on the relationship of animal phyla. In some studies based on *18S rRNA* (Wainright et al. 1993; Philippe et al. 1994; Bridge et al. 1995; Odorico and Miller 1997; Collins 1998) or *28S rRNA* sequences (Christen et al. 1991) *Trichoplax* is included and appears to belong to the cnidarians or be a sister-group of cnidarians, ctenophores or bilaterians. While this manuscript was in preparation, a further discussion of *Trichoplax* phylogeny based on mitochondrial *16S rRNA* sequence and structure appeared, confirming that Placozoa are not derived Cnidaria (Ender and Schierwater 2003). Only two partial sequences of protein-coding genes have been published so far; one a fragment of the homeobox gene *Trox-2* (Schierwater and Kuhn 1998), a homologue of the ParaHox gene *Gsx*, and the other one a fragment of *Pax-B* (Gröger et al. 2000), a homologue of Pax2/5/8, both known already from a wide variety of animals including non-bilaterians and bilaterians.

With the first in situ hybridizations of *Trichoplax* we present a molecular approach to the description of this elusive animal. Genes such as *Brachyury*, *Tbx2/3*, *elongation factor-1 alpha* (*EF1a*) or actin are highly conserved and often more similar to human sequences than to invertebrate model organisms. Not so surprisingly, there are also genes such as *Secp1*, coding for a putative small secreted protein, without recognizable sequence similarities. But together, conserved and novel genes provide evidence that the repertoire of this simple animal is comparable to those of higher animals and the complex expression patterns seen with *Brachyury*, *Tbx2/3* and *Secp1* already indicate that *Trichoplax* consists of more than the four cell types described in the literature.

Materials and methods

Animals

T. adhaerens appear regularly on the glass walls of marine aquaria maintained to culture the hydrozoan jellyfish *Podocoryne carnea* (mixed culture). Animals were removed from the glass walls of the aquaria by a blow with a Pasteur pipette and transferred to a clean glass dish (clean culture). They were left to adhere to the glass surface for about 2–3 h and washed 4 times with Millipore-filtered sea water. Individual specimens were selected for longer cultures, RNA and DNA extractions or in situ hybridization experiments.

Molecular cloning and sequence comparison

Genomic DNA and total RNA were isolated from *Trichoplax* by using TriReagent (Molecular Research Center) according to the

manufacturer's recommendations. First strand cDNA was synthesized with the anchored oligo (dT) primer XT20 V [5′-GGC AGG TCC TCG TTG ACT CGA GAC $GT_{(20)}$(AGC)-3′] by using the SMART RACE cDNA Amplification Kit (Clontech). With the Smart cDNA we were able to do homology PCR, 3′ and 5′ RACE to obtain the full length of *Brachyury*, *Tbx2/3* and *EF1a* homologues of *Trichoplax*. A 113-bp *Brachyury* fragment was amplified with the set of degenerated primers TF1 and TR1 (Spring et al. 2002), followed by TF2 [5′-TT(CT) GG(AGCT) CA(CT) TGG ATG-3′] and TR2 (Spring et al. 2002); a 308-bp *Tbx2/3* fragment was amplified with the set of degenerated primes TF1 and TR1 followed by TF1 and TR2. Standard conditions were used, except that the annealing temperature was 37°C for the first PCR round of 20 cycles. In the second PCR round the annealing temperatures were 37°C for 10 cycles and 45°C for 35 cycles. A 239-bp *EF1a* fragment was amplified with the degenerated primers uEF [5′-AAG TCA GT(AGCT) GA(AG) ATG CA(CT) CA(CT) GA-3′] and uER [5′-GCA AT(AG) TG(AGCT) GC(AGCT) GT(AG) TG(AG) CA(AG) TC-3′]; for the PCR standard condition two different annealing temperatures were used, 40°C for 10 cycles and 50°C for 40 cycles. PCR products of the expected size were gel-purified with a Qiaquick column (Qiagen), subcloned in the pCRII-TOPO vector (TOPO TA cloning Dual Promoter kit, Invitrogen) and sequenced on an ABI PRISM 310 genetic analyzer (Applied Biosystems). Based on the sequences gene-specific primers were designed to carry out the 5′ and 3′ RACE as described (Yanze et al. 2001). Clones with the complete coding sequences were isolated for *Brachyury* (1,872 bp), *Tbx2/3* (2,439 bp) and *EF1a* (1,635 bp). The full-length coding sequences of *Secp1* (737 bp) and an *actin* homologue (1,351 bp) were found by random screening of clones, that were obtained by using as insert the PCR product on SMART cDNA amplified with the primers Nup1 (5′-AAG CAG TGG TAT CAA CGC AGA G-3′) and X1 (5′-GGC AGG TCC TCG TTG ACT CG-3′) subcloned in the pCRII-TOPO vector. The *Trichoplax Brachyury*, *Tbx2/3*, *Secp1*, *actin* and *EF1a* sequences described here have been submitted to the DNA databases with the accession numbers AJ549221–5, respectively. *Podocoryne EF1a* is available under AJ549292.

Nucleotide and deduced amino acid sequences were analyzed by using the GCG software package. BLAST searches (Altschul et al. 1997) were performed on the BLAST network service at the NCBI (http://www.ncbi.nlm.nih.gov). Sequences from representative species were retrieved from BLAST searches with *Trichoplax* and human homologues; a detailed list of accession numbers and the alignments are available on request. Multiple sequence alignments and phylogenetic trees based on the neighbor-joining method were generated with Clustal X (Jeanmougin et al. 1998) and maximum likelihood trees calculated with TREE-PUZZLE (Schmidt et al. 2002).

In situ hybridization

Whole-mount in situ hybridization experiments were carried out with adaptations to the protocol described for *Podocoryne carnea* (Yanze et al. 2001; Spring et al. 2002). Animals were fixed overnight at 4°C in Lavdowsky fixative supplemented with 0.2% glutaraldehyde; washed 3 times for 30 min in PBST (0.1% Tween20 in PBS); underwent a stepwise increase of the concentration from 15%, 30%, 45%, 60%, 75%, 90% and 100% of step-HB (5x SSC; 50% formamide; 50 mg/l heparin; 0.1% Tween20) in 5x SSC, shaking at each step for 10 min at room temperature (RT); prehybridized in hybridization buffer [HB; 5x SSC; 50% formamide; 50 mg/l heparin; 100 mg/l tRNA from *E. coli* (Sigma type XXI R4251); 0.1% Tween20] for 1 h at 50°C; hybridized with DIG-labeled antisense probe (approximately 20 ng/ml HB) overnight at 50°C; washed 2 times for 20 min at 50°C with WS1 (5x SSC; 50% formamide; 0.1% Tween20), WS2 (2x SSC; 50% formamide; 0.1% Tween20), WS3 (2x SSC; 0.1% Tween20) and WS4 (0.2x SSC; 0.1% Tween20); incubated in PBST for 5 min, in blocking solution (1% blocking reagent; 100 mM maleic acid pH 7.5; 150 mM NaCl) for 1 h at RT and with anti-DIG-AP Fab fragments 1:5,000 in blocking solution overnight at 4°C; washed 3 times with PBST and equilibrated in TMNT (100 mM Tris-HCl, pH 8; 50 mM $MgCl_2$; 100 mM NaCl; 0.1% Tween20) for 30 min at RT. Detection was done by incubation of the specimens in TMN (100 mM Tris-HCl, pH 8; 50 mM $MgCl_2$; 100 mM NaCl) with 340 mg/l NBT and 175 mg/l BCIP. When the chromogenic reaction was ready (2–4 h), it was stopped by washing the specimens in PBST. DIG-RNA-labeled probes were synthesized according to the manufacture's recommendation (DIG-RNA-labeling, Roche). The DNA fragments for *Brachyury* and *Tbx2/3* probes were amplified with primers designed outside the T-box domain as follows: TBra F6 (5′-GTC GAC ATC AAC GCC ATT AGG-3′) and TBra R4 (5′-TAC TTT GCT ACT TGT TTG ATA-3′), TTbx F4 (5′-GAC ATG TGC AGA AGT AAC TAA AGG-3′) and TTbx R7 (5′-TTC TTG AGT ACG GAA TTT TCT CG-3′). These fragments were subcloned in the pCRII-TOPO vector (TOPO TA cloning Dual Promoter kit, Invitrogen) and used as template for the probe synthesis. The equivalent was done for *Secp1* using the primer set TSec1 F1 (5′-TAA CTG TAA GGA CTG AAA AAT-3′) and TSec1 R1 (5′-TTC GAA ATC CTT ATC GTG AAA C-3′).

After in situ hybridization some samples were processed for sectioning, which involved: postfixation in 4% paraformaldehyde at RT overnight; dehydration by incubation in ethanol 30%, 50%, 80%, 90%, 95% and 100% for 1 h at RT, respectively; incubation in 50% ethanol/histo-clear overnight at RT; incubation in histo-clear for 20 min at 58°C; transfer to 50% histo-clear/parablast for 1 h at 58°C; embedding twice in parablast, the first time 1 h at 58°C and the second time overnight at 58°C; hardening in a 14°C water bath for 10 min; mounting on block carrier; cutting by microtome in slices of 10 μm (Microm HM 360); stretching sections on water at 37°C for 1 h; mounting sections on slides previously washed in 50% ethanol/ether and dried for 1 h; washing the slides in histo-clear for 10 min at RT; mounting permanently with DPX (Fluka); covering with cover slides; hardening overnight at RT.

Results and discussion

Highly conserved *Brachyury* and *Tbx2/3* homologues from *Trichoplax*

Two distinct T-box family members were isolated from *Trichoplax* by homology PCR. One is clearly a member of the *Brachyury* subfamily and the other a member of the *Tbx2/3* subfamily that was previously known only from bilaterian animals. *Trichoplax Brachyury* is 70–80% identical to *Brachyury* subfamily members and less than 55% identical to other subfamilies in the T-box domain, while little similarity can be detected outside of the T-box domain (Fig. 1A). The conservation of the amino acid residues involved in DNA binding and dimerization suggest that *Trichoplax* Brachyury also binds the typical consensus sequence as a dimer as determined by crystallographic studies (Müller and Herrmann 1997). Transactivation and repression domains were defined in the C-terminal half of mouse Brachyury (Kispert et al. 1995). Although there is little sequence conservation in the C-terminal part of Brachyury from *Trichoplax* to *Drosophila* or vertebrates, there are at least three conserved motifs containing proline, tyrosine or tryptophane (PY/W) in human and *Trichoplax* Brachyury (Fig. 1A).

Trichoplax Tbx2/3 is 60–70% identical to Tbx2/3 subfamily members and up to 60% identical to Tbx4/5 and less-related subfamilies (Fig. 1B). The complexity of the T-box family in higher animals is best seen in the

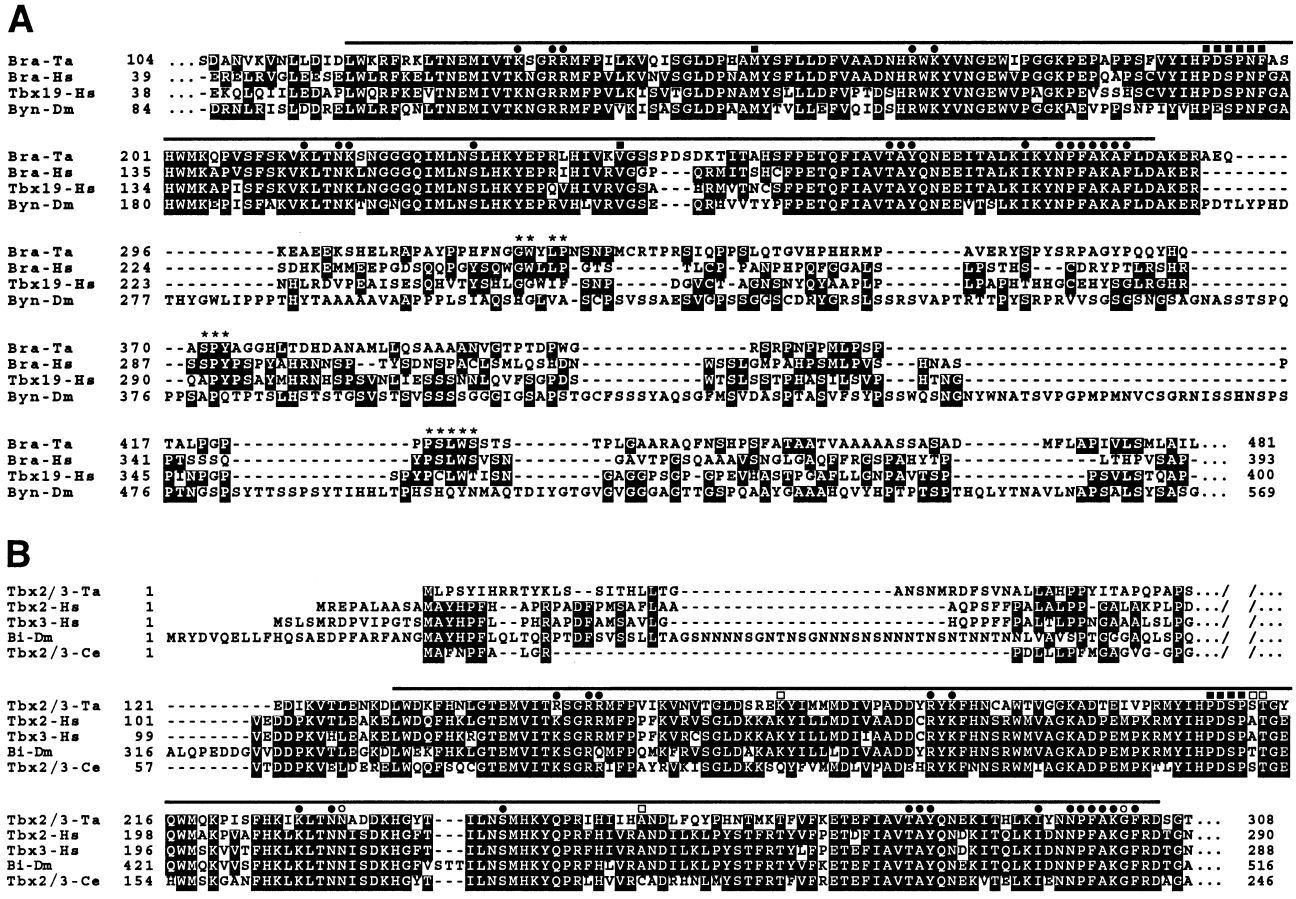

Fig. 1A, B Comparisons of *Trichoplax* Brachyury and Tbx2/3 with subfamily members. **A** The alignment of Brachyury protein sequences illustrates the high similarity in the T-box domain in comparison with the low similarity in the rest of the proteins. The T-box domain is labeled by *over-lining*, the residues involved in DNA-binding are indicated with a *circle* and the residues involved in dimerization are indicated with a *square* (Müller and Herrmann 1997). Three conserved motifs with PW/Y residues in *Trichoplax* and human *Brachyury*, but not *Drosophila*, are highlighted with *asterisks* in the C-terminal part. **B** For Tbx2/3 the N-terminal part and the T-box domain are shown. The T-box domain, the residues involved in DNA-binding and dimerization are labeled as in **A**. *Empty circles* and *empty rectangles* indicate critical residues not conserved between Brachyury and Tbx2/3 families. The N-terminal end of *Trichoplax* Tbx2/3 clearly lacks the MAYHPF-motif conserved in human and *Drosophila* (*Ta Trichoplax adhaerens*, *Hs Homo sapiens*, *Dm Drosophila melanogaster*, *Ce Caenorhabditis elegans*)

comparison of amphioxus family members to the corresponding vertebrate and invertebrate homologues (Ruvinsky et al. 2000). In these analyses the Tbx2/3 subfamily is most related to the Tbx4/5 subfamily specific for vertebrates and amphioxus and less related to the Tbx1/10, Tbx15/18/22 or Tbx20 subfamilies. These five subfamilies seem to be as similar as the Brachyury and Eomes subfamilies, while the Tbx6 subfamily appears to be more diverged.

Comparison of the two known T-box domain structures of *Xenopus* Brachyury and human TBX3 explains some of the differences observed with different subfamilies (Coll et al. 2002). TBX3 does not need to dimerize to bind DNA and, consistent with this, some of the residues required for Brachyury dimerization are not conserved in TBX3, and three out of four of these residues are identical in *Trichoplax* and human Tbx2/3 subfamily members (Fig. 1B). All residues involved in DNA binding are identical between *Trichoplax* and human Tbx2/3 subfamily members. The C-terminal part does not show any significant similarity with other members of the subfamily, although this region should contain the activator and repressor activities of the protein (Paxton et al. 2002). There is residual sequence similarity at the N-terminal end of *Trichoplax* Tbx2/3 with other subfamily members, but the otherwise highly conserved MAYHPF motif is absent (Fig. 1B).

Divergent evolution of *Trichoplax* T-box and reference genes

Phylogenetic analyses with the neighbor-joining method and the maximum likelihood method indicate that both T-box homologues of *Trichoplax* belong to well-defined subfamilies, *Brachyury* and *Tbx2/3*, respectively (Fig. 2A, B). Interestingly, *Trichoplax Brachyury* appears to be even more similar to amphioxus and vertebrate than to

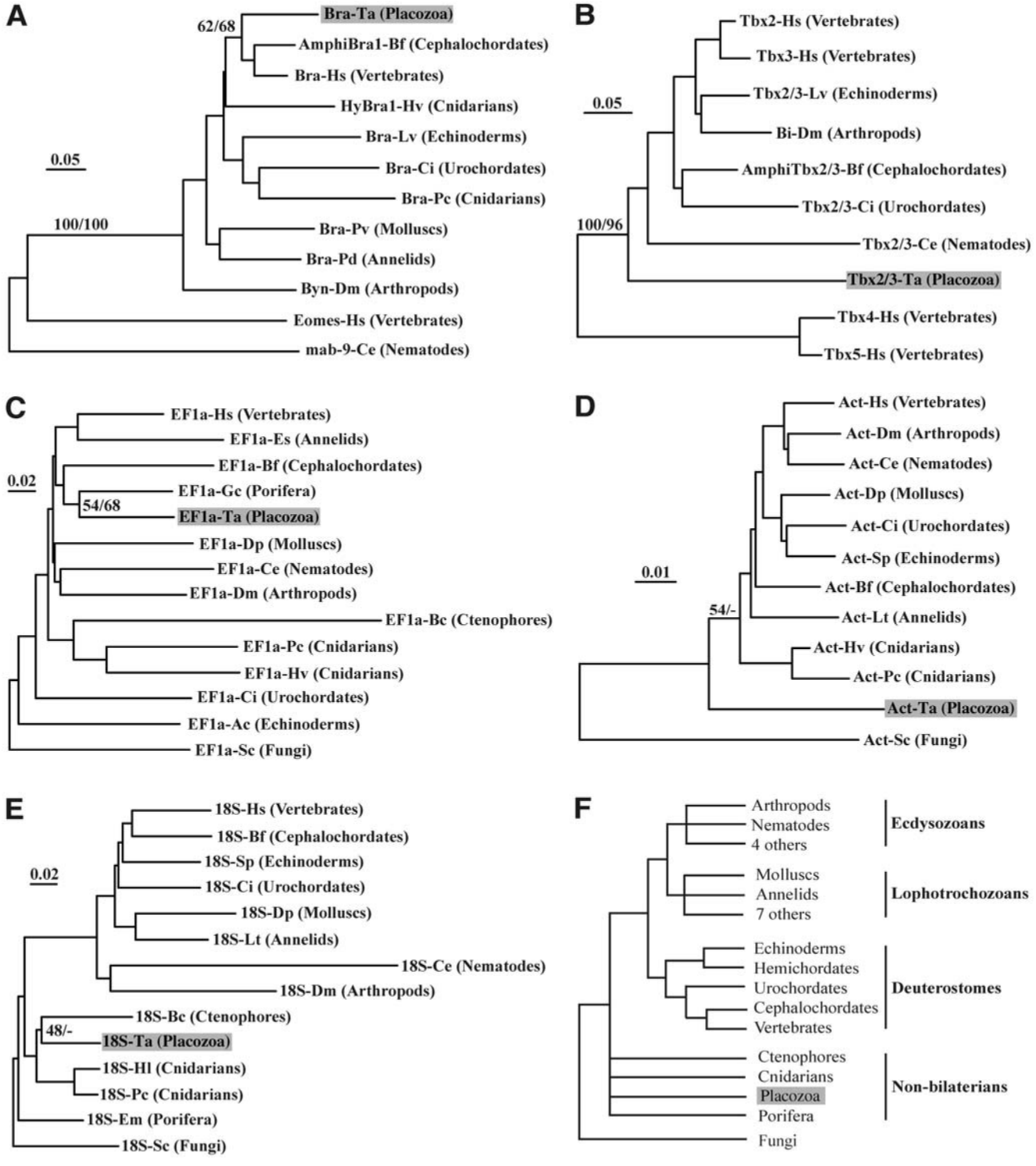

Fig. 2A–F Phylogenetic trees of Brachyury, Tbx2/3 and reference sequences. **A** *Trichoplax* Brachyury is well placed within the Brachyury subfamily. The apparent grouping with amphioxus and human Brachyury is only weakly supported. **B** *Trichoplax* Tbx2/3 is at the base of all known Tbx2/3 subfamily members but clearly separated from other subfamilies. **C** *Trichoplax* EF1a groups with a sponge, but not with other non-bilaterians. **D** *Trichoplax* actin appears at the base of all animal actins. **E** *Trichoplax* 18S rRNA forms a weak clade with ctenophores within other non-bilaterians. **F** Simplified phylogeny of the animal kingdom (after Holland 1999). *Numbers on the branches* indicate the percentage of 1,000 bootstrap replicates that support the topology shown calculated with Clustal X or TREE-PUZZLE, respectively. *Bars* represent the number of substitutions per site. Human EOMES and *C. elegans* mab-9 were included as outgroups for the Brachyury subfamily and TBX4 and TBX5 for the Tbx2/3 subfamily, respectively. For the other genes, yeast homologues were used for rooting the trees (*Ta Trichoplax adhaerens*, *Bf Branchiostoma floridae*, *Hs Homo sapiens*, *Hv Hydra vulgaris*, *Lv Lytechinus variegatus*, *Ci Ciona intestinalis*, *Pc Podocoryne carnea*, *Pv Patella vulgata*, *Pd Platynereis dumerilii*, *Dm Drosophila melanogaster*, *Ce Caenorhabditis elegans*, *Es Enchytraeus* sp., *Gc Geodia cydonium*, *Bc Beroe cucumis*, *Ac Anthocidaris crassispina*, *Sc Saccharomyces cerevisiae*, *Dp Dreissena polymorpha*, *Sp Strongylocentrotus purpuratus*, *Lt Lumbricus terrestris*, *Hl Hydra littoralis*, *Em Ephydatia muelleri*)

other non-bilaterian homologues. But the positioning of the urochordate *Ciona* with the cnidarian *Podocoryne*, and not with another cnidarian, *Hydra*, also indicates that this Brachyury tree is not reflecting the evolutionary tree correctly. It should be noted that in spite of the apparently high conservation of *Brachyury* from *Trichoplax* to humans there is no clear orthologue identifiable in the model organism *Caenorhabditis elegans*. There are even more, although highly derived, T-box genes found in the complete genome sequence and a Brachyury-like function might be taken over by *mab-9*, the *Tbx20* homologue of *C. elegans* (Woolard and Hodgkin 2000).

The Tbx2/3 tree indicates that the *Trichoplax* homologue belongs to this subfamily, even to the exclusion of the Tbx4/5 subfamily only found in chordates (Fig. 2B). *Trichoplax* Tbx2/3 appears at the base of the subfamily, but it should be noted that here also *Ciona* appears to be highly derived and non-bilaterian examples are missing. Besides Brachyury subfamily members, no T-box genes have been described so far from non-bilaterian animals.

On the basis of morphological features the placozoan *Trichoplax* has always been thought to be a basal animal and was often placed between sponges and the other animals. In the last decade, with the rise of molecular phylogenetics, several analyses were carried out with the *18S rRNA* gene to determine the relationship of animal phyla. All possible combinations of grouping *Trichoplax* with other non-bilaterians were reported. *Trichoplax* has been suggested to be a sister group of cnidarians (Wainright et al. 1993; Philippe et al. 1994) or it has been suggested to belong to the cnidarians itself (Bridge et al. 1995). Moreover, *Trichoplax* has been grouped with ctenophores, as a sister group of cnidarians (Odorico and Miller 1997) and, most recently, as a sister group of the bilaterians, to the exclusion of cnidarians, ctenophores and sponges (Collins 1998, 2002).

Since phylogenetic analysis of Brachyury does not conform to any reasonable expectation and for Tbx2/3 there are no other non-bilaterian sequences available, we performed comparable analyses with some well-studied reference genes such as *EF1a*, *actin* and *18S rRNA*. *Trichoplax EF1a*, *actin* and the ends of the previously published *18S rRNA* (Wainright et al. 1993) were sequenced in this study for quality control reasons. Unfortunately, in spite of many phylogenetic studies with these genes, there are still no complete actin sequences from sponges or ctenophores available. Representative species were selected from the ecdysozoan and lophotrochozoan subgroups of the protostomes and from deuterostomes to fit with the available data from T-box genes.

In the EF1a tree *Trichoplax* groups with a sponge with low bootstrap values (Fig. 2C). It could make sense to think of Porifera and Placozoa as the simplest multicellular animals, but this group should then be at the base of the tree and not a sister-group to amphioxus in the middle of badly sorted bilaterian and non-bilaterian homologues. In the actin tree *Trichoplax* appears basal to cnidarians and bilaterians, but the lack of actin sequences from sponges and ctenophores does not allow the resolution of the relationship within non-bilaterian phyla (Fig. 2D). In a *18S rRNA* tree with a comparable set of species *Trichoplax* is placed in a group with the other non-bilaterians (Fig. 2E). In contrast to more extensive studies (Collins 1998, 2002), however, *Trichoplax* appears here as a sister-group to ctenophores and not to bilaterians. This indicates that the choice of species and number of different species within a phylum are important, which should also be considered when collecting additional data for T-box genes for improved phylogenetic studies.

The data that are presently available indicate that *Brachyury* and *EF1a* evolved more irregularly than *Tbx2/3*, *actin* and *18S rRNA*. Whether this is correlated with different functional conservation of *Brachyury* versus *Tbx2/3* is difficult to judge when considering the apparent differences in reference genes such as *EF1a* versus *actin* or *18S rRNA*. *Trichoplax* homologues group with chordates, sponges, ctenophores or at the base of all animals depending on the gene and the species available suggesting at least that Placozoa are an independent phylum. Since *Trichoplax* is the only species of the phylum it is prone to groupings with other outsiders due to long-branch attraction. The best way to summarize the knowledge on the phylogenetic position of non-bilaterians and *Trichoplax* is still an unresolved tree (Fig. 2F).

Distinct expression of *Brachyury* and *Tbx2/3*

In situ hybridization revealed that *Trichoplax Brachyury* is expressed irregularly in individual animals in a few cells or groups of cells (Fig. 3A–D). While some small animals almost lack expression, *Brachyury* expression is localized to the edge of potential outgrowth zones in larger animals (Fig. 3D). It has not yet been possible to resolve the position of the signals in the three cell layers in sections. Although such an irregular staining could also be due to artefacts, no other probes, sense or anti-sense, showed a comparable staining pattern. The increased staining in large, bifurcated animals might be correlated with organiser activity of the *Brachyury* gene. Even more interesting would be the expression during embryonic development. Unfortunately, neither vegetative fission nor embryonic development have been observed under clean culture conditions.

Trichoplax Tbx2/3 is expressed in a very different pattern with strong signals at the periphery of attached animals (Fig. 3E, F). In sections of a specimen that was not attached completely to the substrate, a differential *Tbx2/3* expression between the attached part and the floating part can be distinguished (Fig. 3G, H). The part of the body that was attached showed a strong signal in both the ventral and dorsal layers while the floating region had a signal only in the thin ventral layer (Fig. 3H).

Reference genes such as *actin* give a strong ubiquitous signal in comparable experiments (not shown). A more useful control was found with the gene coding for the putative small secreted protein *Secp1*, which gave a clear pattern of expression by in situ hybridization, appearing

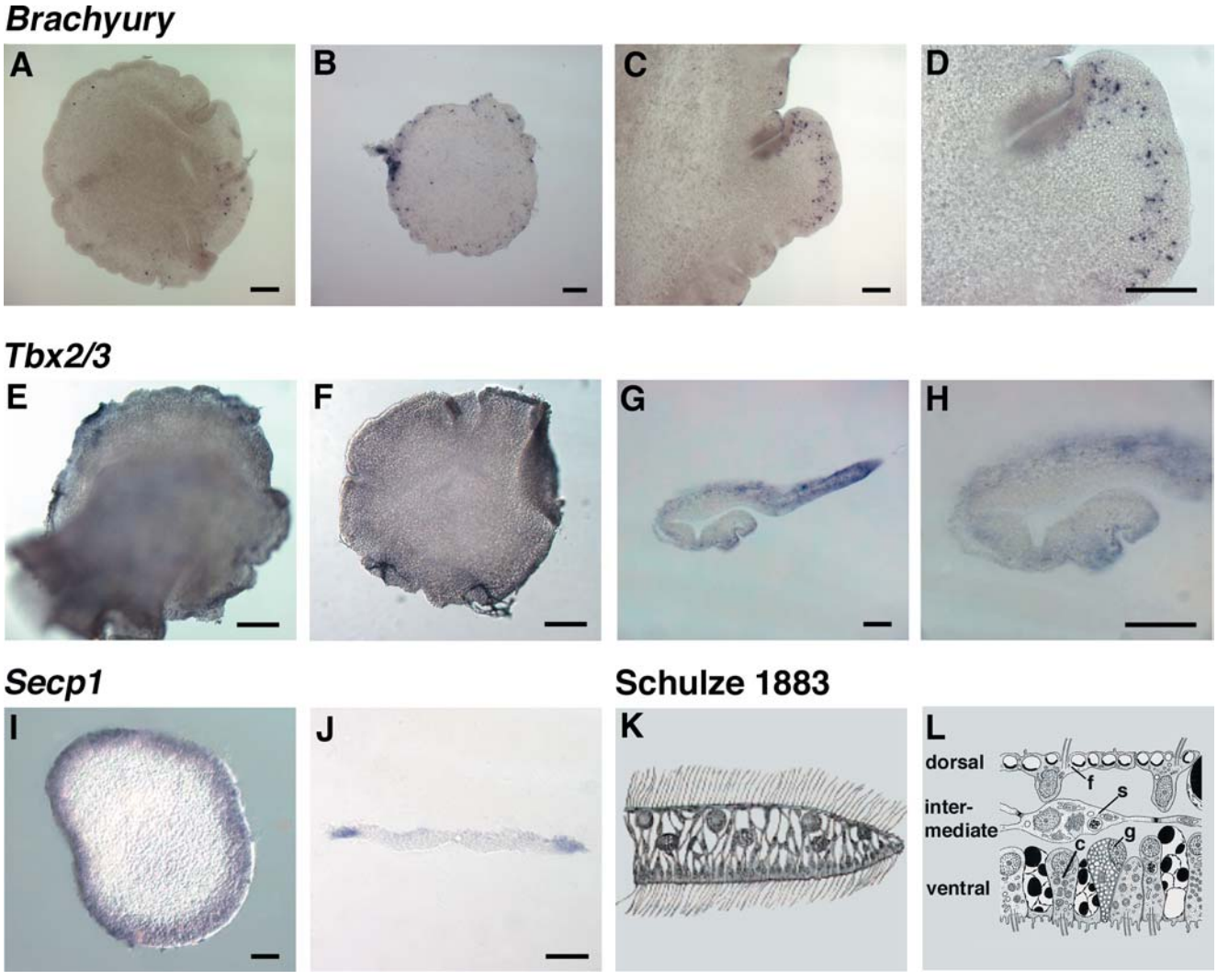

Fig. 3A–L Expression analysis by in situ hybridization of *Trichoplax* genes. *Brachyury* is expressed in a few isolated cells, mainly near the edge of the animal. **A**, **B** Small round animals; **C** larger bifurcated animal with potential outgrowth zone; **D** higher magnification of **C**. **E**, **F** *Tbx2/3* is expressed at the periphery of the animal, but not in all cells; **G** section of **E** shows the differential expression between the body region attached to the substrate and the floating body region; **H** higher magnification of **G**, in the floating region the *Tbx2/3* gene is mainly expressed in cells of the dorsal layer. **I** The gene for the putative secreted protein *Secp1* is expressed in a ring-shaped pattern near the edge of the animal. **J** Section of **I;** *Secp1* appears to be expressed in all three layers along the ring. **K** Sketch of the body structure of *Trichoplax* at an enlargement of ×300 (Schulze 1883). **L** Scheme according to Grell and Ruthmann (1991) with flat epithelial cells (*f*) in the dorsal layer, columnar epithelial (*c*) and non-flagellated gland cells (*g*) in the ventral layer and star-shaped cells (*s*) in the intermediate layer. The *bars* correspond to 0.3 mm

as a well-defined ring-shaped signal that covered the edge of the animal (Fig. 3I). In sections, *Secp1* appears to be expressed in all three body layers (Fig. 3J). Analyses of the 150-amino acid sequence of Secp1 reveals an N-terminal signal peptide of 19 residues with a potential signal cleavage site. The mature protein of 131 residues consists of 76 charged amino acids and shows no significant sequence similarity to known proteins.

Only four types of cells have been described in *Trichoplax* (Fig. 3K, L). From the expression patterns of *Trichoplax Brachyury*, *Tbx2/3* and *Secp1* we can conclude that there are at least three different kinds of cells with different gene expression profiles which do not coincide with the morphologically distinguishable cell types. This hidden anatomical complexity and the clear presence of homologues of the Brachyury and Tbx2/3 subfamilies in *Trichoplax*, suggest that the common ancestor of bilaterians and more primitive animals was more complex than previously anticipated.

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