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EXPRESSION NOTE

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Expression patterns of *musashi* homologs of the ascidians, *Halocynthia roretzi* and *Ciona intestinalis*

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Abstract The gene family encoding RNA-binding proteins includes important regulators involved in the neurogenesis in both protostomes and deuterostomes. We isolated cDNAs of the ascidian homolog of one of the RNA-binding proteins, MUSASHI, from *Halocynthia roretzi* and *Ciona intestinalis*. The predicted amino acid sequences contained two RNA-recognition and RNA-binding motifs in the N-terminus and an ascidian-specific YG-rich domain in the C-terminus. Maternal transcripts of *musashi* were ubiquitous in early cleavage-stage embryos. Ascidian *musashi* had three domains of zygotic expression: the brain, nerve cord, and mesenchyma. The temporal order of the onset in these domains was highly divergent between the two species of ascidian examined.

Key words Ascidian · Musashi · RNA-binding protein

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The sequence reported in this paper has been deposited in the GenBank data base (accession nos. AB030275 and AB0302233).

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The central nervous system (CNS) of the tadpole larva of ascidians, a basal group in the phylum Chordata, is formed by the rolling of a neural plate into a neural tube. This CNS feature, shared by other chordates, has drawn biologists' interest for more than a century (Kowalevsky 1866). The larval CNS is composed of about 330 cells (Monroy 1979), including 215 in the brain, 50 in the tail ganglion, 65 of the caudal nerve cord (spinal cord), and 40–50 in the future adult CNS (Nicol and Meinertzhagen 1991). The ascidian CNS offers several advantages for studying the induction and differentiation of the CNS (for review see Satoh 1994). To classify the brain cells using molecular markers we isolated *musashi* homologs from two ascidians, *Halocynthia roretzi* from the order Pleurogona, and *Ciona intestinalis* from the order Enterogona. MUSASHI (MSI) is an RNA-binding protein specifically expressed in neural tissues in *Drosophila* (Nakamura et al. 1994) and mammals (Sakakibara et al. 1996).

Isolated cDNAs had 1339 bp for *Halocynthia musashi* (*HrMsi*) and 1351 bp for *Ciona musashi* (*CiMsi*). These have the potential to encode proteins of 335 and 393 amino acids, respectively. A genomic Southern blot suggested that the *HrMsi* gene is present as a single copy per haploid *Halocynthia* genome (data not shown). As shown in Fig. 1B, a northern blot analysis showed that a major *HrMsi* transcript 1.3 kb in length, which corresponds to the cloned cDNA, was abundant throughout embryogenesis, and a minor transcript of 2.3 kb, which may be an alternatively spliced product, was detected after the neurula stage. The amount of transcripts seemed to be nearly constant throughout embryogenesis in both species. Semiquantitative reverse transcriptase polymerase chain reaction analysis showed that this is also the case in *Ciona* embryos (Fig. 1A).

Figure 2 shows an alignment of the two ascidian proteins. Predicted amino acid sequences of ascidian MSI also contain two domains known as RNA-recognition and RNA-binding motifs (RRMs; Nakamura et al. 1994). These domains were well conserved beyond species and phyla: 60.8% for *Halocynthia*/*Ciona*, 53.9% for *Halocynthia*/mouse, and 48.4% for *Ciona*/mouse. The overall

sequence similarity between the two ascidian species was 47.0%, whereas that between *Halocynthia* and mouse was 40.9%, and that between *Ciona* and mouse was 41.1%. Unexpectedly, the sequence similarity between the two ascidian species was in the same range as those between mouse and ascidians. These values may reflect a further phylogenetic distance of the two ascidian species than generally accepted. An alanine-rich sequence in the C-terminus reported to be conserved among MUSASHI proteins was not found in the two ascidian proteins. Instead, they shared a unique tyrosine/glycine-rich sequence in the C-terminus, although its function is unknown (Fig. 2).

In *Halocynthia* in situ hybridization signals were uniformly detected in the unfertilized egg (data not shown). Maternal transcripts remained ubiquitous until the 16-cell stage and then gradually disappeared. During early cleavage stages the signals in the vegetal hemisphere became undetectable first, and then those in the animal hemisphere became weaker (see Fig. 3A, for example). From the gastrula stage onward the maternal signal was low in intensity everywhere. As shown in Fig. 3A, zygotic expression of *HrMsi* started in A7.4, A7.8 and b6.5 in the 44-cell embryo. These are blastomeres containing neural lineages which give rise to the brain stem and/or the nerve cord (Nishida 1987). Among their descendants the neural lineage cells continued to express the *HrMsi* gene until the gastrula stage (Fig. 3B), and then the signals disappeared until the neurula stage. At the early neural plate stage (approximately 218 cells) cells in the anterior neural plate, which were derived from the a-line cells, started to express *HrMsi* (Fig. 3C). The a-line cells of the neural plate at this stage comprise four rows of cells: the two posterior rows gave rise to brain, while the two anterior rows became pulps and epidermis (Nishida 1987). The two rows of brain-lineage cells were daughter blastomeres of a8.25, a8.17, and a8.19 from lateral to central.

As shown in Fig. 3C, at first a posterior row of four cells in the neural plate in the early neural plate stage started to express *HrMsi*. Judging from their position, they are likely to be descendants of the a8.17 and the a8.19, that is, the stained row of cells were considered to be a part of the neural plate III (a9.33 and a9.37) region, according to the description by Nicol and Meinertzhagen (1988). Then a part of the neural plate IV (a9.34 and a9.38) regions started to express the gene (data not shown). However, none of the a8.25-derived brain-lineage cells expressed the *msi* gene. In the late neural plate stage four lines of *HrMsi*-positive cells, which were descendants of a9.33, a9.37, a9.34, and a9.38, ran anteriorly in the neural plate as a region of the brain expanded to the anterior direction, showing that all brain-lineage cells except the descendants of the a8.25 in the neural plate expressed *HrMsi* (Fig. 3D). This may show a property of the a8.25-derived brain-lineage cells which was different from other brain cells: the pigment cells of the sensory organs descended from the a8.25 pair, for example (Nishida 1987). At the neurula stage an additional expression was observed in two rows of nerve cord cells along the neural fold (Fig. 3D). Mesenchyme also started to show a signal at the late

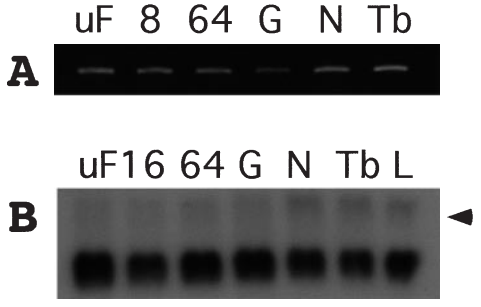

Fig. 1A,B Temporal expression patterns of *msi* genes of two ascidian species. *Arrowhead* minor 2.3-kb mRNA. **A** RT-PCR analysis of *CiMsi*. **B** Northern blot analysis of *HrMsi*. Analyzed were RNA extracted from unfertilized eggs (*uF*); *8* 8-cell embryos; *16* 16-cell embryos; *64* 64-cell embryos; *G* gastrulae; *N* neurulae; *Tb* tailbud embryos; *L* swimming larvae

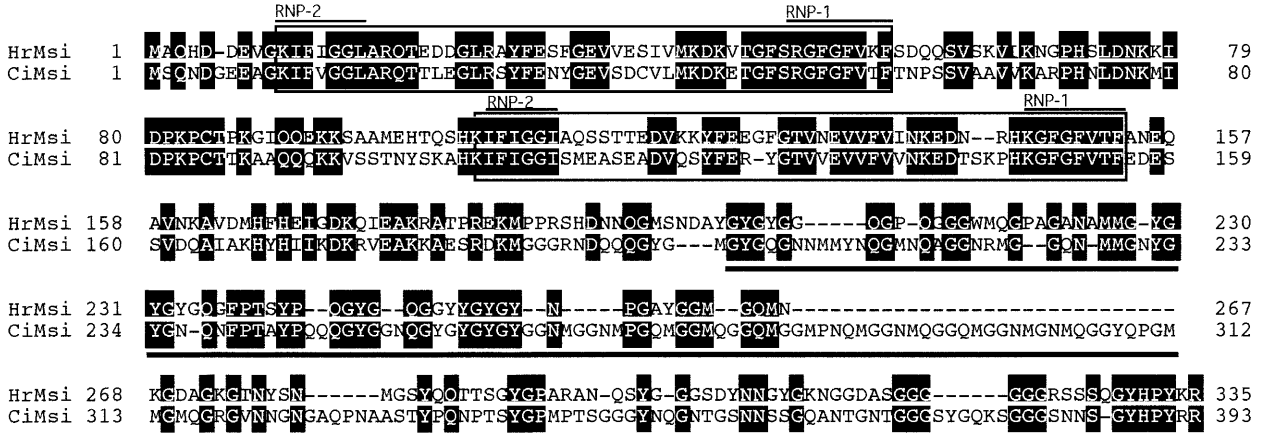

Fig. 2 Comparisons of the amino acid sequences of ascidian *msi* genes. An alignment of amino acid sequences of HrMsi (accession no. AB030275) and CiMsi (accession no. AB030233). *Boxed* two RNA-recognition motifs (*RRMs*). *Highlighted* Amino acids that are identical between the two sequences; *underlined* the ascidian-specific YG-rich domain conserved in the C-termini

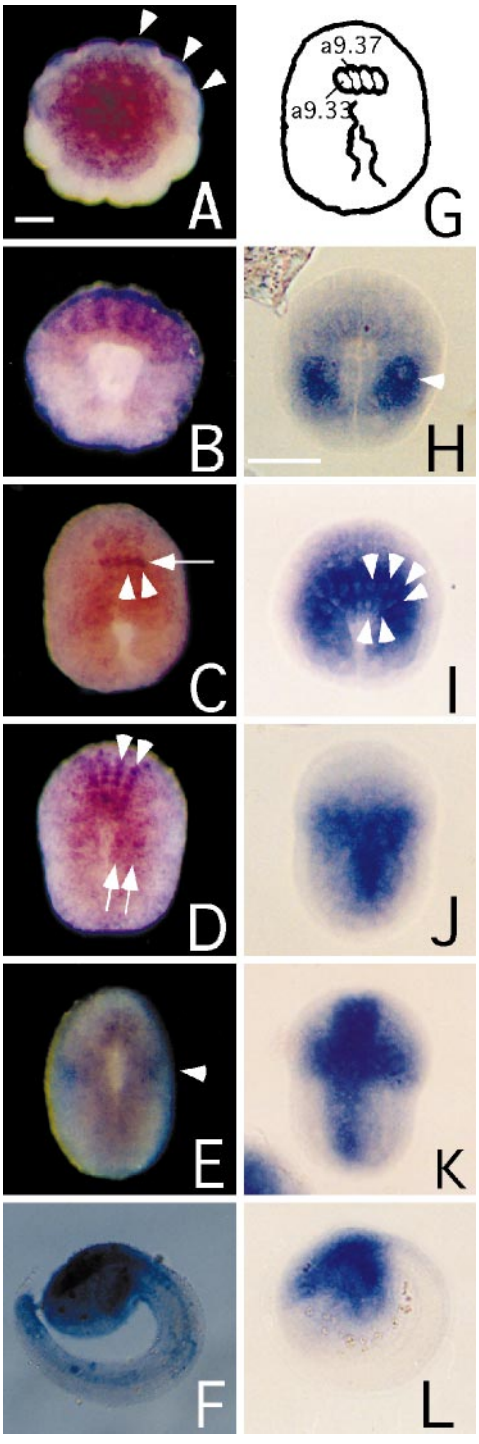

Fig. 3A–L Temporal and spatial expression patterns of *HrMsi* and *CiMsi* revealed by whole-mount in situ hybridization. **A**–**F** Expression of *HrMs* in *Halocynthia roretzi* embryos. **A** A 44-cell embryo, animal view. *Left* Blastomeres expressing *HrMsi* (*arrowheads*). **B** A gastrula, vegetal view. **C** An early neural plate embryo, dorsal view. *Right* Two brain precursor cells expressing *HrMsi* (*arrowheads*). **D** A late neural plate embryo, dorsal view. *Right* Two lines of neural plate cells expressing *HrMsi* (*arrowheads*); *arrows* nerve cord cells expressing *HrMsi*. **E** A middle neurula, dorsal view. *Arrowhead* Mesenchyme expressing *HrMsi*. **F** A middle tailbud embryo, lateral view. **G** A schematic drawing of C. **H**–**L** Expression of *CiMsi* in *Ciona intestinalis* embryos. After visualization of the hybridization, all specimens of *Ciona* were dehydrated and rendered transparent with a 1:2 mixture (v/v) of benzyl alcohol and benzyl benzoate. **H** Gastrula, vegetal view. *Arrowhead* Mesenchyma expressing *CiMsi*. **I** An initial neural plate embryo, dorsal view. *Arrowheads* Nerve cord precursor cells expressing *CiMsi*. **J** A late neurula, dorsal view. **K** An initial tailbud embryo, dorsal view. **L** A middle tailbud embryo, lateral view. **A**–**J** Anterior is *on the top*. **F**,**L** Anterior is *on the left. Scale bar* 50 μm

neurula stage (Fig. 3E). The signals in the neural fold disappeared in a posterior-to-anterior manner as tail elongation proceeded. Hence by the tailbud stage the signal was confined to a large part of the brain and the mesenchyme, with no signal in the posterior nerve cord, pulps or epidermis (Fig. 3F). The expression in the brain was observed until hatching of embryos, and then the signal in the brain became undetectable in swimming larvae (data not shown).

In *Ciona* maternal transcripts were also detected uniformly in cleavage-stage embryos, and disappeared by the gastrula stage. The first zygotic expression was detected in mesenchyme lineage blastomeres in the gastrula stage (Fig. 3H). The nerve cord precursor cells started to strongly express *CiMsi* at the early neural plate stage (Fig. 3I). The expression was observed in the newly formed nerve cord cells in the late neurula stage (Fig. 3J). Shortly after the initial tailbud stage (Fig. 3K) *CiMsi* started to be expressed in the brain while the expression of *CiMsi* in the nerve cord became faint. In the middle tailbud stage (Fig. 3L) the expression of *CiMsi* disappeared in the nerve cord and was restricted to the brain and the mesenchyme. The expression in the brain was observed until hatching of embryos (data not shown). The strong expression in the mesenchyme of *CiMsi* was constantly observed until the tailbud stage.

In both species maternal transcripts were ubiquitous and disappeared by gastrulation. Zygotic expression of ascidian *msi* was detected in the brain, nerve cord, and mesenchyme, although the temporal order of onset expressed in these domains differed between the species. For example, the period of expression in the mesenchymal lineage was relatively longer in *Ciona* embryos than in *Halocynthia* embryos. Transient expression in nerve cord precursor cells observed in the gastrula of *Halocynthia* embryos was not observed in *Ciona* embryos. In both species the signals in the nerve cord disappeared in the early-to-middle tailbud stages, and the signals in the brain became undetectable in swimming larvae. The time of the disappearance of the transcripts seemed to be associated with the closure of the neural tube.

In *Drosophila* Msi functions in the process of asymmetric cell divisions to produce four different neural cell types from a single mother cell (Nakamura et al. 1994), while mouse msi-1 is exclusively expressed in undifferentiated neural stem cells and involves in the differentiation of neurons and glial cells (Sakakibara et al. 1996). Ascidian *msi* is expressed in the brain and the nerve

cord, and the signal disappears in a posterior-to-anterior manner as tail elongation proceeds. This embryonic expression pattern of the *musashi* homolog of ascidians is similar to that in the CNS of mammals. This suggests that ascidian MSI functions to form a neural tube from a neural plate and regulates neural cell differentiation in the similar way to the *msi* gene homologs of vertebrates.

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