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Original Article

Synergistic action of *HNF-3* and *Brachyury* in the notochord differentiation of ascidian embryos

YOSHIE SHIMAUCHI, SHOTA CHIBA and NORI SATOH*

Department of Zoology, Graduate School of Science, Kyoto University, Sakyo-Ku, Japan

ABSTRACT In vertebrate embryos, the class I subtype forkhead domain gene *HNF-3* is essential for the formation of the endoderm, notochord and overlying ventral neural tube. In ascidian embryos, *Brachyury* is involved in the formation of the notochord. Although the results of previous studies imply a role of *HNF-3* in notochord differentiation in ascidian embryos, no experiments have been carried out to address this issue directly. Therefore the present study examined the developmental role of *HNF-3* in ascidian notochord differentiation. When embryos were injected with a low dose of *HNF-3* mRNA, their tails were shortened and when embryos were injected with a high dose of *HNF-3* mRNA, which was enough to inhibit differentiation of epidermis and muscle, no obvious ectopic differentiation of endoderm or notochord cells was observed. However, co-injection of *HNF-3* mRNA along with *Brachyury* mRNA resulted in ectopic differentiation of notochord cells in the animal hemisphere, suggesting that *HNF-3* acts synergistically with *Brachyury* in ascidian notochord differentiation. Notochord differentiation of the A-line precursor cells depends on inducing signal(s) from endodermal cells, which can be mimicked by bFGF treatment. Treatment of notochord precursor cells isolated from the 32-cell stage embryos with bFGF resulted in upregulation of both the *HNF-3* and *Brachyury* genes.

KEY WORDS: *ascidians, notochord differentiation, forkhead/HNF-3, Brachyury, FGF signal*

Introduction

We are interested in molecular mechanisms underlying notochord formation in chordate embryos (Satoh and Jeffery, 1995; Takahashi *et al.*, 1999; Hotta *et al.*, 2000; Suzuki and Satoh, 2000). The notochord of the ascidian tadpole larva is composed of 40 cells aligned longitudinally in a single row along the midline of the tail. Among these 40 cells, the anterior 32 cells are derived from a pair of A4.1 (anterior-vegetal) blastomeres of the bilaterally symmetrical 8-cell stage embryo, and the posterior 8 cells arise from B4.1 (posterior-vegetal) blastomeres (reviewed by Satoh, 1994). In *Halocynthia roretzi* embryos, specification of the A-line notochord cells occurs at the late 32-cell stage as a result of an inductive influence from vegetal blastomeres, which include the primordial endodermal blastomeres and the presumptive-notochord blastomeres themselves (Nakatani and Nishida, 1994). When the notochord precursor cells are isolated from embryos in the early phase of the 32-cell stage, they are not able to differentiate into notochord, while isolation in the late phase of the 32-cell stage produces blastomeres capable of notochord differentiation. If the notochord precursor cells isolated in the early phase of the 32-cell stage are treated with human recombinant bFGF, the cells differentiate into notochord cells, while activin fails to induce the notochord cell differentiation (Nakatani *et al.*, 1996). A cytoplasmic protein, Ras, is involved in a signaling pathway activated by FGF receptors (Satoh *et al.*, 1992; Pawson, 1995). Nakatani and Nishida (1997) showed that injection of a dominant-negative form of human Ras protein into fertilized *Halocynthia* eggs inhibits the notochord formation. Recently, a cDNA clone for the *Halocynthia* FGF receptor (*HrFGFR*) was isolated, and it was shown that *HrFGFR* mRNA is expressed maternally (Kamei *et al.*, 2000; Shimauchi *et al.,* 2001). When synthetic mRNA encoding a dominant-negative form of HrFGFR was injected into fertilized *Halocynthia* eggs, notochord differentiation was inhibited (Shimauchi *et al.*, 2001). These results strongly suggest that differentiation of notochord cells in *Halocynthia* embryos is induced by a molecule that is involved in FGF signaling transduction.

Abbreviations used in this paper: bFGF, basic fibroblast growth factor; HNF, hepatocyte nuclear factor.

***Address correspondence to:** Dr. Nori Satoh. Department of Zoology, Graduate School of Science, Kyoto University, Sakyo-Ku, Kyoto 606-8502, Japan. Fax: +81-75-705-1113. e-mail: satoh@ascidian.zool.kyoto-u.ac.jp

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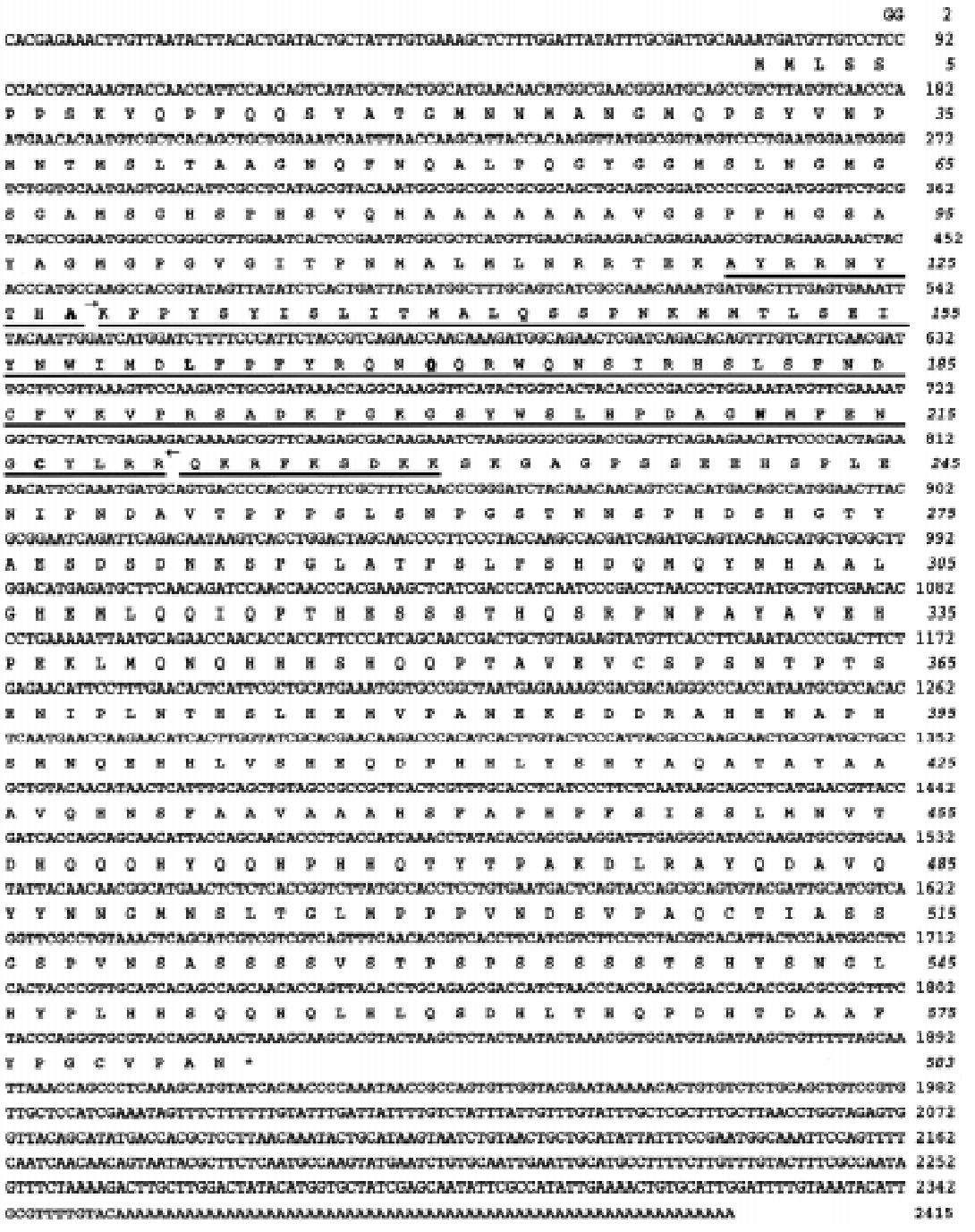

Fig. 1. Nucleotide and predicted amino acid sequences of a cDNA clone for the *Cs-HNF3* gene. *The insert of the cDNA encompasses 2415 bp. The ATG at position 78-80 represents the putative start codon of the Cs-HNF3-encoded protein. An asterisk indicates the putative termination codon. The predicted Cs-HNF3 protein consists of 583 amino acids. The forkhead domain is underlined. Amino acid residues characteristic of the class I subfamily are shown by bold letters. Arrows indicate the amino acid residues used for molecular phylogenetic analysis (Fig. 2). The nucleotide sequence data of Cs-HNF3 will appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Databases with the Accession No. AB049587.*

Genetic analysis in zebrafish and mice have identified several genes involved in specification and subsequent differentiation of notochord cells, including *Brachyury* (Herrmann *et al.*, 1990; Schulte-Merker *et al.*, 1994; Wilson *et al.*, 1995), *forkhead/HNF-3β* (Ang and Rossant, 1994; Weinstein *et al.*, 1994), and *floating head* (Talbot *et al.*, 1995). An ascidian *Brachyury* gene (*HrBra* or *As-T* of *H. roretzi* and *Ci-Bra* of *Ciona intestinalis*) is expressed exclusively in notochord cells (Yasuo and Satoh, 1993; Corbo *et al.,* 1997a). The timing of initiation of *HrBra* expression coincides with that of the developmental fate restriction of the primordial notochord cells (Yasuo and Satoh, 1993, 1994). *HrBra* expression in A-line precursors is initiated by the notochord-inducing signal (Nakatani *et al.*, 1996). Injection of *HrBra* mRNA into fertilized *Halocynthia* eggs leads to the differentiation of notochord cells in the absence of inducing signal(s) from neighboring cells and causes fate changes of non-notochord cells into notochord (Yasuo and Satoh, 1998). In addition, it is highly likely that Brachyury protein of *Ciona* embryos activates more than 20 notochord-specific structural genes that are involved in the formation of the notochord (Takahashi *et al.*, 1999; Hotta *et al.*, 2000). These data indicate that the *Brachyury* gene plays a fundamental role in notochord formation in ascidian embryos.

Due to the remarkable function of the ascidian *Brachyury* in the formation of the notochord, we have in the past overemphasized its importance (e.g., Satoh *et al.*, 1999). However, when the results of

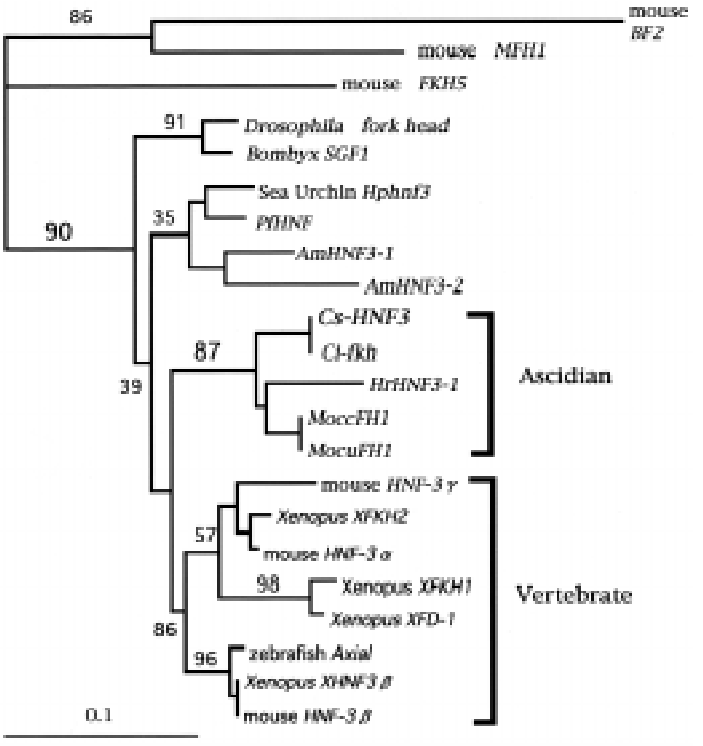

Fig. 2. Molecular phylogenetic tree of class I *forkhead/HNF-3* subfamily genes. *This tree was constructed by comparison of 93 amino acid residues of the forkhead domain by the neighbor-joining method (Saitou and Nei, 1987). The numbers at the branches are bootstrap values that indicate confidence in the topology of the tree. This method produces an unrooted tree. However, we confirmed the root position by using mouse BF2, MFH1 and FKH5 as outgroups. Branch length is proportional to the number of amino acid substitutions; the scale bar indicates 0.1 amino acid substitutions per position in the sequences.*

previous experiments are examined more carefully, it becomes clear that the expression of the *forkhead/HNF-3* gene is essential as a prerequisite for *Brachyury* to function to promote notochord differentiation. Namely, competent blastomeres whose fate can be changed into notochord by ectopic expression of *HrBra* or *Ci-Bra* are those of the endoderm and nerve cord, but presumptive epidermal cells and muscle cells do not respond to ectopic expression of *HrBra* or *Ci-Bra* to form notochord cells (Yasuo and Satoh, 1998; Takahashi *et al.,* 1999). The ascidian *HNF-3* gene is expressed from the 16-cell stage in lineages that give rise to endodermal cells, notochord cells and nerve cord cells (Shimauchi *et al.*, 1997; Corbo *et al.*, 1997b) but not in lineages that form epidermis and muscle.

A requirement for *HNF-3* in notochord formation has been shown in vertebrate embryos (Ang and Rossant, 1994; Weinstein *et al.*, 1994). Cooperation of *pintallavis*, a *Xenopus HNF-3*-related gene, and *Xbra*, a *Xenopus Brachyury* gene, for notochord formation was demonstrated in frog animal pole explants, whereas each of them alone induced formation of paraxial mesoderm tissues such as muscle (O'Reilly *et al.*, 1995). All together, it is likely that during ascidian embryogenesis, the *Brachyury* gene and *HNF-3* gene interact synergistically to direct notochord differentiation. In the present study, we examined this question, and showed that coectopic expression of *HNF-3* and *Brachyury* genes can induce notochord differentiation even in epidermal cells of ascidian embryos.

Results

Isolation of the Ciona savignyi HNF-3-related gene

We have already characterized the *forkhead/HNF-3*-related gene *HrHNF3-1* of *H. roretzi* (Shimauchi *et al.*, 1997). In order to study the developmental role of the *HNF-3* gene in the notochord differentiation of ascidian embryos by means of microinjection of synthesized mRNA, we tried to clone a plasmid containing full-length *HrHNF3-1* gene. However, the growth of coliform bacilli which contained plasmids carrying inserts that included the C-terminal half of *HrHNF3-1* was inhibited, and such plasmids often showed recombination of the nucleotide sequence of the plasmid. Thus, we could not obtain a template for synthesizing *HrHNF3-1* mRNA. A cDNA clone encoding the *HNF-3*-related gene of *C. savignyi* was isolated in an attempt to obtain cell differentiation markers. We determined the entire nucleotide sequence of this cDNA (Fig. 1). The cDNA contained an insert of 2415 nucleotides encompassing a potential open reading frame of 1748 nucleotides. Its sequence predicted a protein of 583 amino acids with a forkhead domain (Fig. 1). The gene was

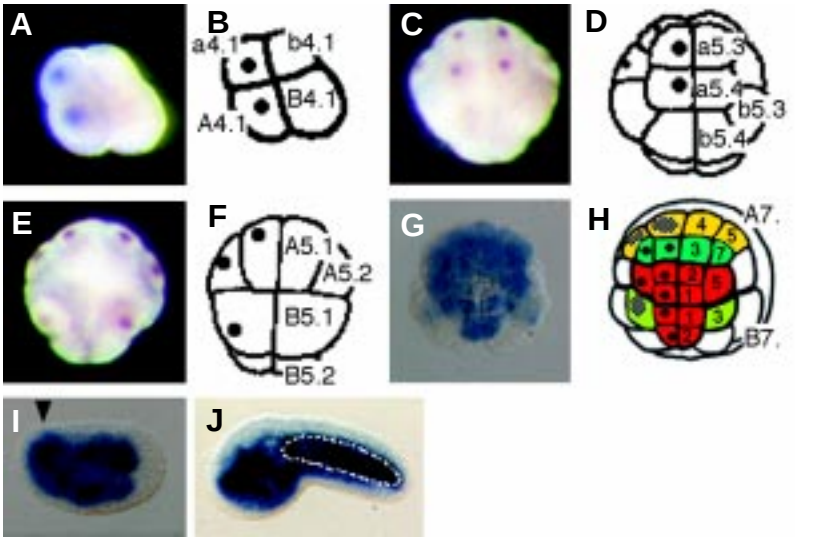

Fig. 3. Spatial expression pattern of the *Cs-HNF3* gene as revealed by whole-mount *in situ* hybridization. (A) *An 8-cell stage C. savignyi embryo; lateral view.* **(B)** *Diagrammatic drawing of A.* **(C-F)** *Sixteen-cell stage embryos, viewed from the animal pole* **(C)** *and vegetal pole* **(E)**. **(D)** *Diagrammatic drawing of C, and* **(F)** *that of E.* **(G)** *A 64-cell stage embryo, vegetal view.* **(H)** *Diagrammatic drawing of G. Primordial endodermal cells are shown in red, notochord cells in green, nerve cord cells in yellow, or mesenchyme and notochord cells in yellow-green.* **(I)** *A neurula viewed from the side. An arrowhead indicates gene expression in some of the brain cells.* **(J)** *An early tailbud embryo viewed from the side. The notochord is shown by a dotted line.*

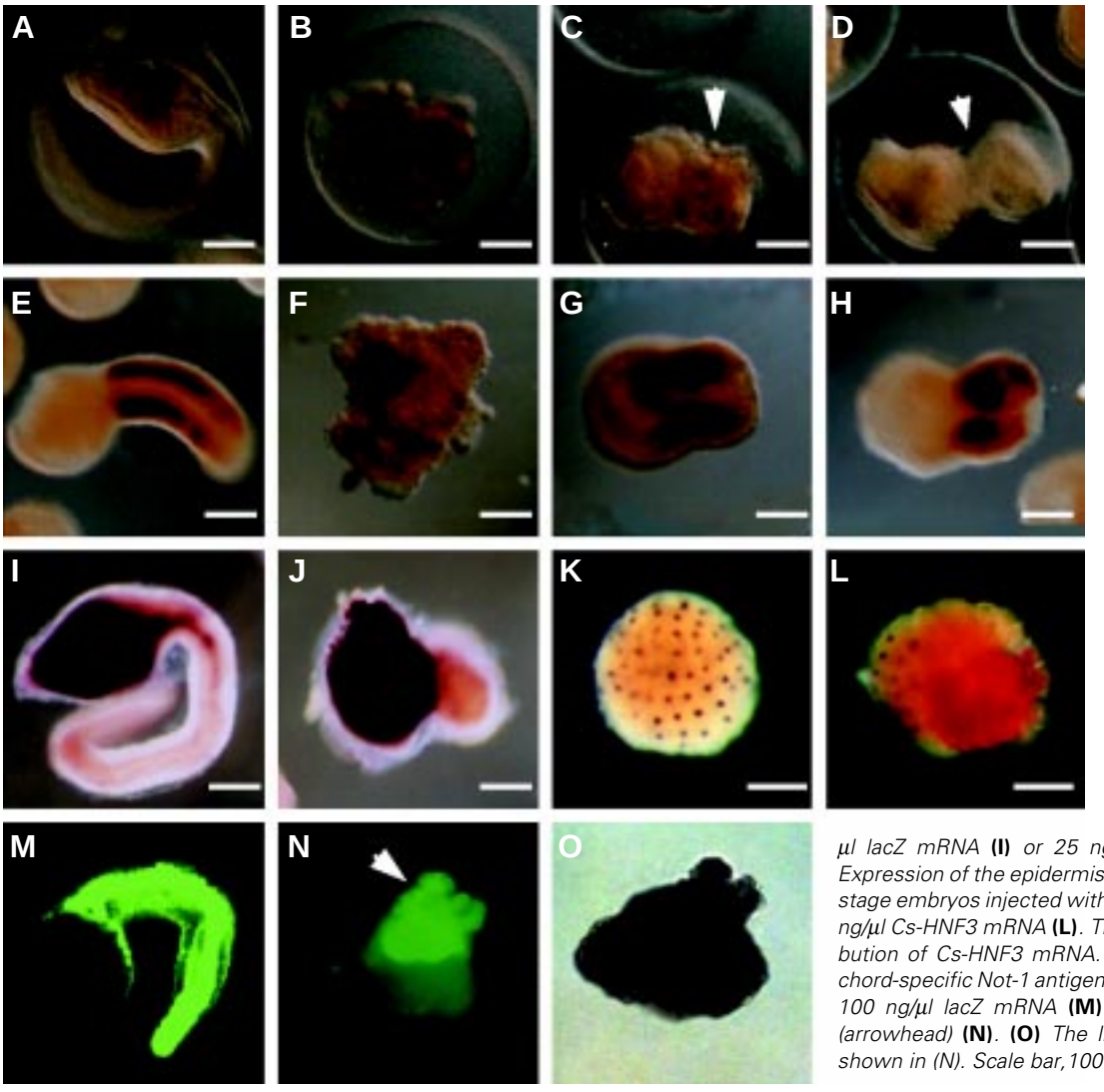

Fig. 4. Effects of over-expression of *Cs-HNF3* by injection of its synthetic mRNA into fertilized *Halocynthia* eggs. **(A)** *A control embryo injected with lacZ mRNA at a concentration of 100 ng/μl.* **(B-D)** *Embryos injected with Cs-HNF3 mRNA at a concentration of 100 ng/μl* **(B)**, *25 ng/μl* **(C)** *and 10 ng/μl* **(D)**. *The arrowhead in (C) indicates protruding cells which were not enveloped by epidermis, and that in (D) shows epidermal sheet which fails to envelop around the area.* **(E-H)** *Expression of muscle-specific AChE.* **(E)** *A control embryo injected with lacZ mRNA at a concentration of 100 ng/μl. Experimental embryos injected with 100 ng/μl* **(F)**, *25 ng/μl* **(G)** *and 10 ng/μl* **(H)** *Cs-HNF3 mRNA.* **(I,J)** *Expression of endoderm-specific AP activity in embryos injected with 100 ng/μl lacZ mRNA* **(I)** *or 25 ng/μl Cs-HNF3 mRNA* **(J)**. **(K,L)** *Expression of the epidermis-specific HrEpiC gene in 110-cell stage embryos injected with 100 ng/μl lacZ mRNA* **(K)** *or 100 ng/μl Cs-HNF3 mRNA* **(L)**. *The red signal indicates the distribution of Cs-HNF3 mRNA.* **(M-O)** *Expression of the notochord-specific Not-1 antigen in tailbud embryos injected with 100 ng/μl lacZ mRNA* **(M)** *or 100 ng/μl Cs-HNF3 mRNA (arrowhead)* **(N)**. **(O)** *The light micrograph of the embryo shown in (N). Scale bar, 100 μm.*

named *Cs-HNF3* (*Ciona savignyi forkhead/HNF3*-related gene). Ascidian *HNF-3* related genes have already been isolated from other species, *Ci-fkh* of *Ciona intestinalis* (Corbo *et al.*, 1997b); *MoccFH1* and *MocuFH1* of *Molgula occulta* and *M. oculata* (Olsen and Jeffery, 1997; Olsen *et al.*, 1999), and *HrHNF3-1* of *H. roretzi* (Shimauchi *et al.*, 1997). The predicted amino acid sequence of the forkhead domain of *Cs-HNF3* shows 98% identity to that of *Ci-fkh*, and 89% identity to that of *HrHNF3-1*.

The class I *forkhead* genes are characterized by a set of shared amino acid residues, A at position 9 of the domain, L at 43, Q at 51, N at 92, and C at 98 (Kaufmann and Knochel, 1996). Figure 1 shows that all of these characteristic amino acid residues are conserved in Cs-HNF3, suggesting that *Cs-HNF3* is a member of the class I genes. To confirm further that *Cs-HNF3* is a member of the class I *forkhead/HNF-3*-related genes, we constructed a molecular phylogenetic tree based on comparison of the amino acid sequences of the forkhead domain (Fig. 1, indicated by arrows). The tree showed that *Cs-HNF3* is a member of the class I forkhead/HNF-3 superfamily which contains the other ascidian *forkhead/HNF-3* genes (Fig. 2). The clade of the ascidian *HNF-3*-related genes was supported by a bootstrap value of 87% (Fig. 2).

In situ hybridization revealed that *Cs-HNF3* was expressed predominantly in blastomeres of lineages giving rise to endoderm, notochord and nerve cord (Fig. 3), a pattern similar to that of *HrHNF3-1* expression. The *Cs-HNF3* expression commenced as early as the 8-cell stage in the anterior blastomeres (a4.2 and A4.1 pairs: Fig. 3 A,B). In ascidian embryos, zygotic expression of certain genes is first detected in the nucleus by *in situ* hybridization (e.g., Yasuo and Satoh, 1993). At the 16-cell stage, the hybridization signal was observed in the anterior animal blastomeres (a5.3 and a5.4 pairs: Fig. 3 C,D) and anterior vegetal blastomeres (A5.1, A5.2 and B5.1: Fig. 3 E,F). *Cs-HNF3* expression in the animal blastomeres was transient, and was downregulated by the 64-cell stage. At the 64-cell stage, *Cs-HNF3* was expressed in blastomeres which give rise to endoderm (red in Fig. 3H), notochord (green in Fig. 3H), nerve cord (yellow), or mesenchyme and notochord (yellow-green) in the vegetal hemisphere (Fig. 3 G,H), while no signal was observed in the animal blastomeres. As development proceeded, mesenchyme expression of *Cs-HNF3* was downregulated, and at the neurula stage, the hybridization signal was observed in cells of the brain (Fig. 3I, arrowhead) and the nerve cord in addition to notochord

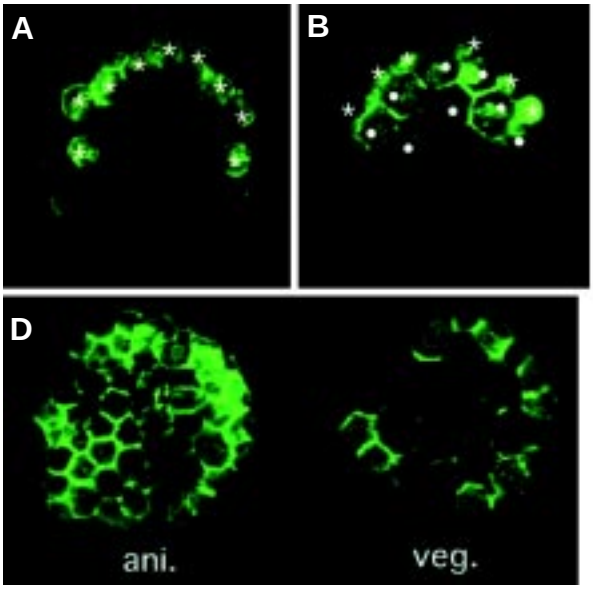

Fig. 5. Expression of the notochord-specific Not-1 antigen in *Halocynthia* embryos injected with *Cs-HNF3* and/or *HrBra* mRNA. *Embryos were cleavage-arrested at the 110-cell stage and cultured for 10 h before fixation.* **(A)** *A control lacZ-injected embryo (100 ng/μl mRNA), vegetal view. Asterisks indicate notochord progenitor cells.* **(B)** *HrBra-injected embryo (100 ng/μl mRNA), vegetal view. Dots show Not-1-positive endodermal-lineage cells.* **(C)** *Cs-HNF3-overexpressing embryo (100 ng/μl mRNA), vegetal view.* **(D)** *An embryo co-injected with 100 ng/μl Cs-HNF3 and 100 ng/μl HrBra mRNAs, animal pole view (ani) and vegetal pole view (veg). Animal blastomeres show Not-1 antigen expression.*

cells and endodermal cells. The expression pattern was retained at the early tailbud stage (Fig. 3J).

All of the data described above clearly indicate that *Cs-HNF3* is the orthologue of *HrHNF3-1*. Therefore, we used *Cs-HNF3* to study the role of *HNF-3* gene in ascidian notochord differentiation.

Effects of overexpression of the forkhead/HNF-3 gene

With *Cs-HNF3* cDNA as probe, we examined the effects of overexpression of the *HNF-3* gene on ascidian embryogenesis. We used *Halocynthia* eggs and embryos by following reasons. Firstly, an antibody against Not-1 antigen which is a notochord specific marker in *Halocynthia* embryos does not cross-react with notochord cells of *Ciona* embryos. Secondly, *Halocynthia* embryo is large and convenient for blastomere manipulation, so we can isolate notochord precursor cells from the 32-cell stage embryos. Fertilized *H. roretzi* eggs were injected with *Cs-HNF3* synthetic mRNA at a concentration of 100 ng/μl, 25 ng/μl or 10 ng/μl. Control eggs injected with 100 ng/μl *lacZ* mRNA developed into larvae with normal morphology (Fig. 4A). However, eggs injected with *Cs-HNF3* mRNA developed into larvae with disordered morphology in a concentration-dependent manner (Fig. 4 B-D). Embryos developed from eggs injected with 100 ng/μl of *Cs-HNF3* mRNA failed to show normal gastrulation movement (Fig. 4B). Embryos developed from eggs injected with 25 ng/μl of *Cs-HNF3* mRNA underwent gastrulation, but the epibolic movement of animal blastomeres was insufficient and otolith and ocellus did not develop (Fig. 4C). In embryos injected with 10 ng/μl of *Cs-HNF3* mRNA, the head region was very nearly normal and in 27% embryos (n = 11) otolith and ocellus was observed (data not shown); however, the tail did not elongate (Fig. 4D). The failure of otolith and ocellus development is likely a secondary effect caused by incomplete morphogenesis. In many cases, the epidermis failed to surround the embryo completely, leaving embryonic regions not covered by epidermis (arrowheads in Fig. 4 C,D).

Differentiation of muscle, endoderm, notochord and epidermis was examined in *Halocynthia* embryos overexpressing *Cs-HNF3* by monitoring the expression of differentiation markers. Acetylcholinesterase (AChE), which is a marker of muscle cells (Fig. 4E), was expressed in the posterior region of embryos injected with a lower dose of *Cs-HNF3* mRNA (Fig. 4 G,H). However, when a high dose of *Cs-HNF3* mRNA was injected, the expression of AChE was suppressed (Fig. 4F), indicating that *HNF-3* overexpression affects muscle cell differentiation.

Alkaline phosphatase (AP) activity, a marker of endoderm differentiation (Fig. 4I) was detected in embryos developed from eggs injected with a high dose of *Cs-HNF3* mRNA (Fig. 4J). To examine further whether overexpression of *Cs-HNF3* leads to ectopic endoderm differentiation, we took advantage of cleavage-arrested embryos. When cytochalasin B is applied, the cytokinesis of ascidian embryos is blocked, but the division-arrested blastomeres continue the process of differentiation and eventually express tissue-specific markers in the appropriate cell lineages (Whittaker, 1973). Injected embryos were arrested with cytochalasin B at the 110-cell stage and then cultured until the equivalent of the middle-tailbud stage. When AP activity was examined in *Cs-HNF3* mRNA-injected and cytokinesis-arrested embryos, it became evident that ectopic AP expression very scarcely occurred (data not shown).

Notochord-specific Not-1 antigen was also expressed in embryos injected with a high dose of *Cs-HNF3* mRNA. As shown in Fig. 4, plates M-O, overexpression of the *Cs-HNF3* gene did not cause differentiation of extra notochord cells.

HrEpiC gene expression, which is a marker of epidermal cells (Fig. 4K), was inhibited by injection of a high dose of *Cs-HNF3* mRNA (Fig. 4L). The red staining shown in Fig. 4K is the hybridization signal of *Cs-HNF3*, which allows visualization of the injected *Cs-HNF3* mRNA. In this case, the injected mRNA was inherited by the progenitor cells of the right half blastomere during the first cleavage. *HrEpiC* expression was inhibited in the half that received *Cs-HNF3* mRNA.

All of the above data suggest that overexpression of *Cs-HNF3* causes downregulation of an epidermis-specific gene and muscle AChE activity, but that *Cs-HNF3* alone does not affect the differentiation of endodermal cells or notochord cells.

Co-injection of Cs-HNF3 and HrBra mRNAs causes ectopic differentiation of notochord cells

We then examined the possibility of synergistic action of *HNF-3* and *Brachyury* in the ascidian notochord cell differentiation. Synthetic mRNAs of both genes were solely or co-injected into fertilized *Halocynthia* eggs, and cytokinesis of the embryos was arrested at the 110-cell stage. Control cleavage-arrested embryos which developed from eggs injected with *lacZ* mRNA expressed Not-1 antigen in 10 primordial notochord cells (Fig. 5A). This pattern is the same as that of *HrBra* expression in normal 110-cell stage embryos (Yasuo and Satoh, 1993). In embryos injected with *HrBra* mRNA, Not-1 antigen expression was detected not only in the 10 primordial notochord cells but also in cells of endoderm lineage (Fig. 5B). This confirms the result of experiments done by Yasuo and Satoh (1998). However, no blastomeres of the animal hemisphere expressed Not-1 antigen. The number of Not-1 positive cells did not change markedly in embryos injected with *Cs-HNF3* mRNA alone (Fig. 5C). Because the cellular arrangement was disorganized in *Cs-HNF3* mRNA-injected embryos, it was difficult to determine the exact number and position of Not-1 antigen-positive cells, but it was evident that no blastomeres of the animal hemisphere expressed Not-1 antigen.

In contrast, when eggs were co-injected with *Cs-HNF3* and *HrBra* mRNAs, the resultant embryos showed Not-1 expression not only in vegetal blastomeres but also in animal blastomeres. As shown in Fig. 5D, half of embryos co-injected with *Cs-HNF3* and *HrBra* mRNAs showed an increased number of cells expressing Not-1 antigen. These results strongly suggest that *HNF-3* acts synergistically together with *Brachyury* in the differentiation of the ascidian notochord cells.

bFGF upregulates both HrBra and HNF-3 gene expression in A-line notochord cells

Specification of the A-line presumptive notochord cells depends on inducing signal(s) emanating from adjoining endodermal cells in the late phase of the 32-cell stage (Nakatani and Nishida, 1994). This signal cascade promotes the *HrBra* expression in A-line primordial notochord blastomeres, and bFGF is able to mimic the notochord induction (Nakatani *et al.*, 1996). The A-line presumptive notochord cells at the 32-cell stage, A6.2 and A6.4, are not restricted to notochordal fate, but rather have the potential to form notochord and nerve cord cells (Fig. 6A). bFGF treatment of isolated A-line-presumptive notochord blastomeres induces formation of partial embryos in which all cells are converted to the notochord fate (Nakatani *et al.*, 1996).

To study the effects of bFGF on *HrHNF3-1* gene expression in presumptive notochord blastomeres, we isolated A6.2 and A6.4 blastomeres from the 32-cell stage and cultured them until the 110-cell stage. The cells divided twice to form partial embryos, each consisting of 4 cells. The specimens were fixed to detect *HrHNF3-1* gene expression by means of *in situ* hybridization. As shown in Fig. 6B, *HrHNF3-1* gene expression was detected in partial embryos without bFGF treatment. We observed each partial embryo in detail, and found that most of them expressed *HrHNF3-1* in one or two cells (58.3%). When isolated blastomeres were treated with bFGF at the concentration 0.2 ng/ml, the overall proportion of partial embryos with an *HrHNF3-1* hybridization signal did not change, but the proportion of partial embryos expressing *HrHNF3-1* in 3 or 4 cells was increased. This change suggests that the

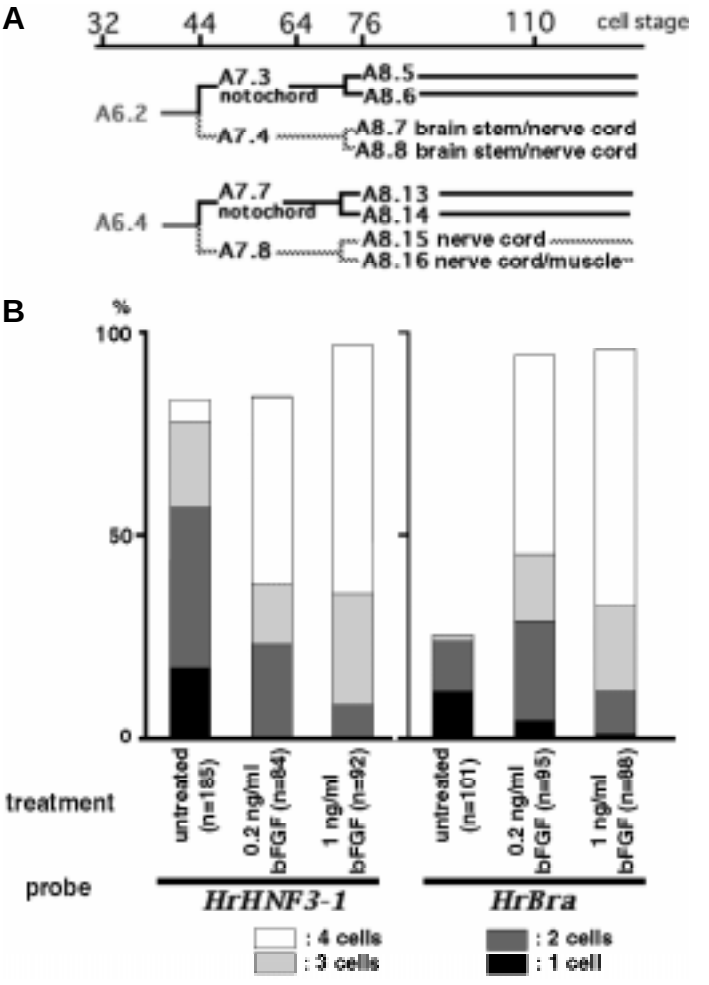

Fig. 6. Activation of *HrHNF3-1* and *HrBra* expression in partial embryos originated from isolated A6.2 or A6.4 cells by treatment with bFGF. (A) *Lineages of A6.2 and A6.4 cells in the 32-cell stage embryo. When isolated, they give rise to partial embryos which contain 2 notochord cells.* **(B)** *Treatment of isolated A6.2 or A6.4 with bFGF activates expression of HrHNF3-1 and HrBra genes.*

HrHNF3-1 gene expression is upregulated not only in primordial notochord cells, but also in primordial nerve cord cells derived from A6.2 and A6.4 blastomeres. When isolated blastomeres were treated with a higher concentration of bFGF (1 ng/ml), the proportion of partial embryos expressing *HrHNF3-1* gene in all 4 cells was increased (Fig. 6B).

We also examined *HrBra* expression in bFGF-treated blastomeres. Without bFGF treatment, only one-quarter of the partial embryos showed *HrBra* expression (Fig. 6B). However, with bFGF treatment, 95% of the partial embryos showed an *HrBra* hybridization signal (Fig. 6B). When isolated blastomeres were treated with bFGF at the concentration of 0.2 ng/ml, the proportion of partial embryos expressing the *HrBra* gene in 3 or 4 cells was 69%, and when isolated blastomeres were treated with 1 ng/ml bFGF, the proportion was increased to 88%.

These results suggest that bFGF upregulates the expression of both the *HNF-3* and *Brachyury* genes in *Halocynthia* embryos.

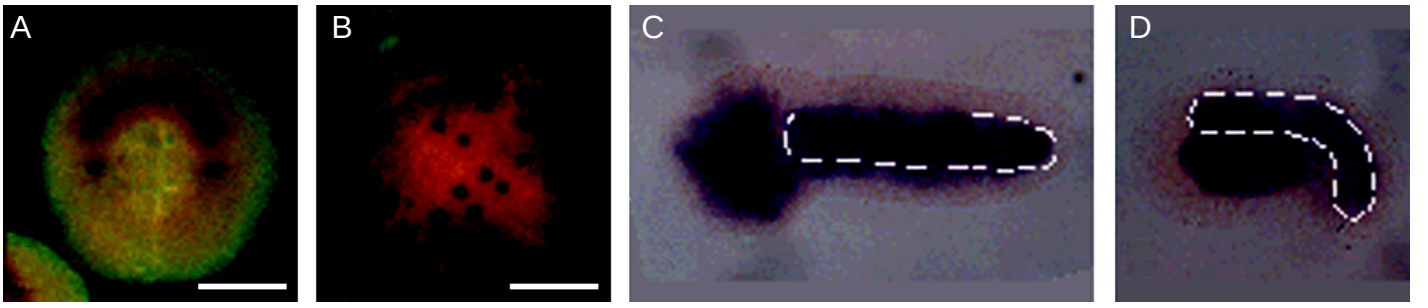

Fig. 7. *Cs-HNF3* upregulates *HrBra* expression. (A,B) *Expression of the HrBra gene in Halocynthia embryos injected with lacZ* **(A)** *or Cs-HNF3 mRNA* **(B)**. *Embryos were fixed at the 110-cell stage. The red signal indicates the distribution of Cs-HNF3 mRNA.* **(C,D)** *Expression of HrHNF3-1 in early tailbud stage Halocynthia embryos injected with lacZ* **(C)** *or HrBra mRNA* **(D)**. *The dotted lines in (C) and (D) indicate the placement of the notochord.*

Cs-HNF3 upregulates HrBra expression

As mentioned above, ascidian *HNF-3* and *Brachyury* act synergistically in notochord cell differentiation, and treatment of embryos with bFGF upregulates expression of both genes. In order to examine genetic interaction of the genes, both encoding transcription factors, we examined whether *Cs-HNF3* overexpression upregulates *HrBra* expression or whether *HrBra* overexpression upregulates *HrHNF3-1* expression. Whole-mount *in situ* hybridization of *HrBra* was carried out in embryos overexpressing *Cs-HNF3*. As shown in Fig. 7B, *HrBra* was ectopically expressed in *Cs-HNF3* mRNA-injected gastrulae. This result was confirmed at the 64-cell, early gastrula and neurula stages.

Subsequent to the initiation of expression of *HrBra*, both *HrBra* and *HNF-3* genes continue to be expressed in notochord cells. To examine the positive feedback loop of the expression of the two genes, we examined the effects of *HrBra* overexpression on *HrHNF3-1* expression. *HrBra* overexpression did not promote ectopic expression of *HrHNF3-1* gene, more like seemed slightly inhibit *HrHNF3-1* expression in primordial notochord cells, neither in the 110-cell stage (data not shown) nor in the early tailbud stage (Fig. 7 C,D).

Discussion

Developmental role of the ascidian HNF-3 gene

In ascidian embryos, overexpression of genes through injection of synthesized mRNA has been performed with the aim of analyzing the developmental role of those genes in embryogenesis (e.g., Yasuo and Satoh, 1998; Yoshida *et al.*, 1998; Imai *et al.*, 2000). Some transcription factor genes have shown tissue-inducing activity by this technique. For example, overexpression of *As-T2* (a *Tbx6*-related gene of *H. roretzi*) promotes cell fate change of presumptive epidermal cells into muscle cells (Mitani *et al.*, 1999), while injection of *β-catenin* mRNA induces ectopic differentiation of endodermal cells in the presumptive epidermal cells and notochord cells (Imai *et al.*, 2000). In the present study, we obtained overexpression of *Cs-HNF3* by injection of synthesized mRNA into fertilized *Halocynthia* eggs to examine the developmental role of the *HNF-3* gene in notochord cell differentiation. Injection of a high dose of *Cs-HNF3* mRNA inhibited the expression of an epidermis-specific gene and muscle AChE, but the differentiation of endodermal cells and notochord cells was not greatly disturbed. However, morphogenetic movements were clearly suppressed. In addition, although embryos which developed from eggs injected with a low dose of *Cs-HNF3* mRNA showed proper head formation and expression of muscle AChE, their tail was shortened, and some embryonic regions were not surrounded by epidermal cells completely. These results suggest that the developmental role of the ascidian *HNF-3* gene is more related to morphogenetic movements than to tissue differentiation. Previous studies examined the developmental role of the ascidian *HNF-3* gene by mean of antisense oligodeoxynucleotide (ODN) treatment in another species of ascidian, *Molgula oculata* (Olsen and Jeffery, 1997; Olsen *et al.*, 1999). The ODN treatment caused suppression of *HNF-3* (*MocuFH1*) gene expression during gastrulation, which in turn resulted in incomplete gastrulation movement or failure of axis formation during gastrulation, and thus severely affected embryos were formed without notochord formation. In the present study, we also examined the role of *HrHNF3-1* by ODN treatment but failed to obtain specific effects of *HrHNF3-1* ODNs. However, these down-regulation and overexpression experiments suggest that the ascidian *HNF-3* gene plays an important role in gastrulation and axis formation.

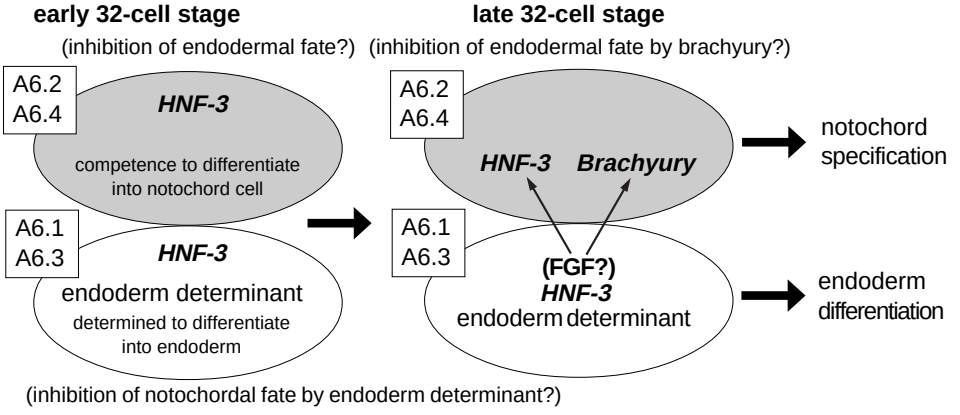

Fig. 8. Hypothetical scenario regarding the developmental functions of the FGF signal and the genes encoding *HNF-3* and *Brachyury* in A-line presumptive endodermal cells and notochord cells. *In Ciona intestinalis and C. savignyi embryos, it is reported that β-catenin has the endoderm determinant activity and inhibits notochord differentiation and Brachyury expression (Imai et al., 2000).*

Yasuo and Satoh (1998) showed that cells of the presumptive endoderm and a part of the nerve cord have competence to differentiate into notochord cells when *HrBra* is overexpressed. These blastomeres also express *HrHNF3-1*. In the present study, we demonstrated that *HNF-3* and *Brachyury* act synergistically to direct notochord differentiation during ascidian embryogenesis. This result confirms the findings of previous experiments in *Xenopus* embryos; in *Xenopus* embryos, co-injection of *pintallavis* (*HNF-3*) and *Xbra* can induce notochord differentiation in animal cap cells, although neither *pintallavis* nor *XBra* alone can induce notochord differentiation in the animal cap system (O'Reilly *et al*., 1995). Although the *Xenopus* animal cap assay is more artificial than overexpression of genes in ascidian embryos, the synergistic action of these genes on notochord differentiation seems to be conserved among chordates.

The vertebrate HNF-3 protein is considered to be involved in 'genetic potentiation', which means that regulatory factors are enabled to occupy their binding sites in target gene chromatin, as a discrete step that occurs prior to transcriptional activation (Zaret, 1999). The ascidian *HNF-3* gene seems also to possess the 'genetic potentiation' activity, because it alone cannot activate tissue differentiation, although it is necessary for the notochord differentiation. It is possible that *HNF-3* is necessary for 'genetic potentiation' for notochord cell specification, and *Brachyury* can work as a notochord inducer on such a genetic background. More detailed studies, including *in vivo* footprinting of HNF-3 protein and identification of any cofactors, such as GATA, in ascidian embryonic cells will be required.

Gene expression and notochord specification

Here we demonstrated that bFGF activates not only *HrBra* but also *HrHNF3-1* expression in notochord precursor cells (Fig. 6). We also showed that *Cs-HNF3* upregulates *HrBra* expression in notochord cells after *HrBra* is expressed at the 64-cell stage, whereas *HrBra* does not upregulate *HrHNF3-1* expression (Fig. 7). In spite of the fact that *Cs-HNF3* overexpression induces ectopic expression of *HrBra*, it does not promote marked ectopic differentiation of notochord cells (Figs. 4 and 5). It is possible that *HNF-3* promotes endodermal cell differentiation by activating genes responsible for the endodermal fate. As shown by Wada and Saiga (1999) and by Imai *et al.* (2000), cells with endodermal fate have activity to compete and inhibit notochord differentiation. Thus, it is possible that the *HNF-3* gene induces not only *Brachyury* expression but also expression of some other genes that are involved in defining endodermal fate, which in turn inhibits *Brachyury* activity or notochord differentiation. Isolated A-line presumptive endodermal blastomeres and notochord blastomeres autonomously express the *HrHNF3-1* gene (Shimauchi *et al*., 1997). If *HrHNF3-1* gene expression induces *HrBra* expression in notochord precursor cells in the absence of the notochord-inducing signal, it might lead to notochord differentiation in cell autonomous manner. But, in practice, a notochord-inducing signal is essential for notochord differentiation and *HrBra* expression. Therefore, autonomous *HrHNF3-1* expression seems not to be sufficient to activate *HrBra* expression or notochord differentiation. As summarized in Fig. 8, all of these data taken together suggest that, before the 64-cell stage when notochord fate is defined, A-line notochord precursors have competence which rules out endodermal differentiation and allows them to differentiate to notochord. The latter activity may be responsible for *HrHNF3-1* gene expression. Then A-line notochord precursor cells receive a notochord-inducing signal, which in turn activates expression of genes necessary for notochord formation, such as *HrBra* and *HrHNF3-1*.

Materials and Methods

Ascidian eggs and embryos

Naturally spawned eggs of *Halocynthia roretzi* were fertilized with a suspension of sperm from another individual. Fertilized eggs were raised at 13°C in Millipore-filtered (pore size 0.45 μm) seawater (MFSW) that contained 50 μg/ml streptomycin sulfate. Tadpole larvae hatched approximately 38 h after fertilization.

Ciona savignyi adults were maintained under constant light to induce oocyte maturation. Eggs and sperm were obtained surgically from the gonoduct. After insemination, eggs were reared at 18°C in MFSW. Tadpole larvae hatched at about 18 h of development. Embryos at appropriate stages were fixed for *in situ* hybridization.

Isolation and characterization of cDNA clones for Ciona savignyi forkhead/HNF-3-related gene

We obtained a cDNA clone containing the forkhead domain during a research project to obtain cell differentiation markers of *C. savignyi* embryos (Chiba *et al*., 1998). The nucleotide sequence of the clone was completely determined for both strands with a Big-Dye Primer Cycle Sequencing Ready Reaction kit and an ABI PRISM 377 DNA sequencer (Perkin Elmer, Norwalk, CT, USA).

Molecular phylogeny

Amino acid sequences of the forkhead superfamily gene products were aligned with a SeqApp 1.9 manual aligner for Macintosh (Gilbert, 1993). Ninety-three confidently aligned residues of the forkhead domain were used for estimating relationships of the proteins by means of neighbor-joining (Saitou and Nei, 1987) using the PHYLIP ver. 3.5c software package (Felsenstein, 1993). The distance matrix was constructed according to the phylogeny, which was assessed by bootstrap resampling of the data (Felsenstein, 1985).

In situ hybridization

In situ hybridization of whole-mount specimens was carried out using digoxigenin-labeled antisense probes as described previously (Yasuo and Satoh, 1994). RNA probes were prepared with a DIG RNA labeling kit (Boehringer Mannheim, Heidelberg, Germany). Control embryos hybridized with a sense probe did not show signals above background. In this study, expression of *Cs-HNF3*, *HrEpiC, HrHNF3-1* and *HrBra* was examined by this method.

Microinjection of synthetic capped RNA

Cs-HNF3 cDNA was subcloned into the *Eco*RI site of pBluescript RN3 vector (Lemaire *et al*., 1995). The subcloned plasmid was linearized by *Asp*718, and synthesized capped mRNA was made using a Megascript T3 kit (Ambion, Austin, TX, USA) according to Wada *et al.* (1997). Synthesized RNA was injected into fertilized eggs as described previously (Hikosaka *et al*., 1992).

Detection of tissue-specific differentiation markers

Differentiation of endodermal cells was assessed by histochemical detection of alkaline phosphatase (AP) activity following the method of Whittaker and Meedel (1989). 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) was used as the substrate for the AP reaction. Differentiation of muscle cells was examined by histochemical reaction of acetylcholinesterase (AChE) following the method of Karnovsky and Roots (1964).

Notochord cell differentiation was monitored with monoclonal antibody 5F1D5, which specifically recognizes a notochord-specific antigen, Not-1

(Nishikata and Satoh, 1990). Indirect immunochemical staining was carried out using TSA^R-DIRECT (NEN^R Life Science Products, Inc., Boston, USA) according to the instructions supplied with the kit.

The expression of an epidermis-specific gene, *HrEpiC* (Ishida *et al.*, 1996), and the *Brachyury* gene *HrBra* (Yasuo and Satoh, 1993) of *Halocynthia* embryos was detected by whole-mount *in situ* hybridization.

Cleavage-arrest

To determine the identity of embryonic cells exhibiting differentiation markers, we took advantage of cleavage-arrested embryos (Whittaker, 1973). Cleavages of the 110-cell stage embryos were arrested by immersing them in MFSW containing 4 μg/ml cytochalasin B. They were cultured for 15 h and then fixed for examination of differentiation markers.

Blastomere isolation and bFGF treatment

Fertilized eggs were dechorionated by treatment with seawater containing 0.05% actinase E (Kaken, Pharmaceutical, Co. Ltd., Tokyo, Japan) and 1% sodium thioglycolate (Wako Pure Chemical Industries, Osaka, Japan). Naked eggs were incubated in 1% agar-coated plastic dishes containing MFSW. Identified blastomeres were isolated from the embryos with a fine glass needle under a stereo microscope (Olympus, SZH10). Isolated blastomeres were cultured separately with or without human recombinant bFGF (Amersham, Buckinghamshire, UK).

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