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Expression and functional analysis of *Cititf1*, an ascidian *NK-2* class gene, suggest its role in endoderm development

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SUMMARY

In solitary ascidians the fate of endoderm is determined at a very early stage of development and depends on cytoplasmic factors whose nature has not been determined. We have isolated a member of the *NK-2* gene family, *Cititf1*, from the ascidian *Ciona intestinalis*, showing high sequence homology to mammalian *TITF1*. The *Cititf1* gene was expressed in all endodermal precursors at the pregastrula and gastrula stages, and is thus the first specific regulatory endodermal marker to be isolated from an ascidian. *Cititf1* expression was downregulated at the end of gastrulation to reappear at middle tailbud and larval stages in the most anterior and ventral parts of head endoderm, regions which give rise, after metamorphosis, to the adult endostyle, where *Cititf1* mRNA was still present. Microinjection of *Cititf1* mRNA into fertilized eggs resulted in tadpole larvae with abnormalities in head-trunk development consequent to the formation of excess endoderm, perhaps due to recruitment of notochord precursors to an endodermal fate. These data suggest that *Cititf1* plays an important role in normal endoderm differentiation during ascidian embryogenesis.

Key words: *Ciona intestinalis*, *Cititf1*, Endoderm development, Embryonic expression, Endostyle, *NK-2* class, Homeobox, Overexpression

INTRODUCTION

A very important aspect of development in chordates is the commitment of a distinct cell population to form the three germ layers. While considerable information has accumulated about factors involved in mesoderm and neuroectoderm formation (Smith, 1989; Kimelman et al., 1992; Kessler and Melton, 1994; Slack, 1994; Lemaire and Kodjabakian, 1996; Heasman, 1997), little is known about the mechanism responsible for endoderm differentiation. In solitary ascidians the developmental fate of endoderm is determined at a very early stage of embryogenesis (Conklin, 1905; Nishida and Satoh, 1983, 1985; Nishida, 1987). Transplantation of cytoplasmic fragments demonstrated the presence of cytoplasmic factors that are responsible for determination of endodermal cell fate (Nishida, 1993). However, the molecular identity of the maternal factors and their mechanism of action remains to be elucidated.

Essentially all of the endodermal cells in an ascidian larva are derived from the two pairs of vegetal blastomeres of the eight-cell embryo. After three cell divisions, at the 64-cell stage, the endodermal fate is restricted to five blastomere pairs that give rise to approximately 500 endoderm cells of the larva, located in both the trunk and the tail (endodermal strand) (Nishida, 1987). After metamorphosis, the cephalic endoderm of ascidian larvae forms a specialized organ, the endostyle, present in the ventral wall of the adult pharynx. The ascidian endostyle has been proposed to be the precursor of the thyroid gland of the vertebrates (Dohrn, 1886). Indeed, specific endostyle cells have been shown to concentrate radioiodine from sea water (Barrington, 1957), and to produce monoiodiotyrosine, diiodiotyrosine and tyroxine (Roche et al., 1959; Kennedy, 1966; Dunn, 1974).

To obtain insights into the molecular mechanisms responsible for the formation of endodermal cell types and to further investigate the presence of a thyroid-like structure in protochordates, we searched for *NK-2*-like homeobox-containing genes in the ascidian *Ciona intestinalis*. The *NK-2* type genes encode a subclass of homeodomain-containing proteins, which have been identified in many distantly related species ranging from *Drosophila* to man. Four *NK*-type genes have been isolated from *Drosophila* (Kim and Nirenberg, 1989) and the encoded proteins were classified by Burglin into two new homeodomain protein classes, NK-1 and NK-2 (Burglin, 1994). Several genes encoding an NK-2-like homeodomain have been isolated from vertebrates, planaria and leech. TTF-1 was the first vertebrate transcription factor containing an NK-2-like homedomain to be cloned. TTF-1,

whose genetic locus has been entitled *TITF1* (human) and *titf1* (mouse), is involved in thyroid-specific gene expression. Expression studies have demonstrated that *titf1* mRNA, in rat and mice, is restricted to the thyroid, lung and some areas of the foetal forebrain (Lazzaro et al., 1991). Mice homozygous for the disrupted *titf1* gene lack the thyroid and lung parenchyma (Kimura et al., 1996), demonstrating that TTF-1 is required not only for the regulation of tissue-specific gene expression but it is also involved in the organogenesis of thyroid, lung and some areas of the forebrain.

In this study we report the isolation of a cDNA, coding for an NK-2-like protein with high sequence homology to mammalian TTF-1, from *Ciona intestinalis*. By analogy with the mammalian counterpart, we have named the protein CiTTF-1 and the genetic locus *Cititf1*. The mRNA encoding CiTTF-1 was expressed in a complex pattern during embryonic development. It was first found in all the endodermal precursors at the pregastrula and gastrula stages, and thus represents the first early marker for the endodermal lineage in ascidians. At the end of gastrulation the *Cititf1* mRNA became undetectable but reappeared later, at middle tailbud and at swimming larva stages, in the most anterior and ventral parts of head endoderm. After metamorphosis, these parts of the larval endoderm give rise to the adult endostyle, where *CiTTF-1* mRNA is still present. This later expression could be the counterpart of TTF-1 expression during thyroid development in mammals. A reporter construct containing the *Cititf1* regulatory region fused to the β-galactosidase gene showed an expression pattern that was virtually identical to the endogenous gene, demonstrating that CiTTF-1 expression is regulated at the transcriptional level. Moreover, we have demonstrated an important role for CiTTF-1 in endoderm differentiation, since microinjection of synthetic mRNA into fertilized eggs resulted in the development of tadpole larvae with abnormalities in trunk-tail development consequent to the formation of excess endoderm.

MATERIALS AND METHODS

Animals and embryos

Adult *Ciona intestinalis* were collected in the Bay of Naples by the fishing service of the Stazione Zoologica. Naturally spawned eggs were fertilized in vitro with a suspension of sperm, and fertilized eggs were raised in filtered sea water at 18-20°C. Tadpole larvae hatched about 18-20 hours after fertilization. Samples at appropriate stages were collected by low speed centrifugation and were fixed for whole-mount in situ hybridization or were frozen for RNA extraction.

Preparation of poly(A)$^+$ RNA

Total RNA from embryos at various stages was prepared essentially according to the method of Chomczynski and Sacchi (1987). Poly(A)$^+$ was purified by oligo(dT)-cellulose chromatography (Sambrook et al., 1989).

Isolation and sequencing of cDNA clone

cDNA was synthesized from poly(A)$^+$ RNA extracted from *Ciona* larvae using the ZAP cDNA synthesis kit (Stratagene) and cloned in the UNI-ZAP XR-VECTOR System (Stratagene). The ligated material were packaged in vitro using the packaging System (Stratagene).

The probe used to screen the library was *CiHB1*, a 384-fold degenerate oligonucleotide (5′-AARATYTGGTTYCARAA-YMGVMG, where M=A or C; R=A or G; V=A or C or G; Y=C or T) (Di Gregorio et al., 1995) coding for the most conserved amino acid sequence of the helix III of *Antennapedia*-type homeodomain. Hybridization of phage lifts were carried out as previously described (Di Gregorio et al., 1995). The cDNA inserts from positive clones were rescued as Bluescript plasmids (SK+) by helper phages mediated in vivo excision, as described by the manufacturer (Stratagene). The directionated inserts (5′*Eco*RI-3′*Xho*I) were sequenced on both strands by the dideoxynucleotide procedure (Sanger and Coulson, 1975).

Deduced homeodomain sequences were compared with translation in reading frames of the collection sequences contained in the GenBank and EMBL databases by using the TFasta program from the GCG package (Pearson and Lipman, 1988).

Northern blotting

Poly(A)$^+$ RNAs (8 μg per lane) were separated by electrophoresis on a 1% agarose gel containing 2.2 M formaldehyde and blotted on nylon filters (Hybond N, Amersham, UK) using 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.6) as transferring buffer. Blots were hybridized to *Cititf1* probe at 65°C for 16 hours in a buffer containing 6× SSC, 5× Denhardt's solution, 0.1% SDS (sodium dodecyl sulfate), 100 μg/ml denatured and sonicated salmon sperm, washed with 2× SSC, 0.1% SDS once (15 minutes) at room temperature and at 65°C three times for 15 minutes each. Blots were exposed to X-ray film (Kodak AR) for 5 hours. Labeling of *Cititf1* probes was performed with [α-^{32}P]dATP (3000 Ci/mmol; 10 μCi/ml, Amersham, UK) using a random priming labeling reaction to a final specific activity of 2×10^9 cpm/μg.

In situ hybridization

Full-length cDNA was used as template for in vitro transcription by using Boehringer Mannheim DIG RNA labeling Kit, according to the supplier's instructions, and stored in H_2O at –80°C.

Larvae, embryos and adult juveniles were fixed with 4% paraformaldehyde in 0.5 M NaCl, 0.1 M Mops (3-N-morpholino propansulfonic acid), pH 7.5, at room temperature for 90 minutes, dehydrated in 30%, 50% and 70% ethanol (10 minutes each) and stored in 70% ethanol at –20°C. For adults the tunic was stripped off with tungsten needles prior to fixation. After dehydration, juveniles were embedded in paraffin. After a thorough wash with phosphate-buffered saline (PBS), embryos were manually deprived of the envelopes. The specimens were treated with 2 μg/ml proteinase K in PBST (PBS containing 0.1% Tween 20) (30 minutes, 37°C) and then post-fixed with 4% paraformaldehyde in PBS for 1 hour. After 1 hour in prehybridization buffer (50% formamide, 5× SSC, 50 μg/ml heparin, 50 μg/ml yeast tRNA, 5× Denhardt's solution, 0.1% Tween 20) at 48°C, embryos were allowed to hybridize with 0.25 μg/ml digoxygenin (DIG)-labeled antisense or sense transcripts. Hybridization was carried out at 48°C for 16 hours. The specimens were washed in 50% formamide, 4× SSC, 0.1% Tween 20 (2× 15 minutes, 50°C), then in 50% formamide, 2× SSC, 0.1% Tween 20 (2× 15 minutes, 50°C) and in solution A (0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.1% Tween 20) (3× 10 minutes, 37°C), then treated with 20 μg/ml RNase A in solution A (30 minutes, 37°C) and washed with 50% formamide, 2× SSC, 0.1% Tween 20 (20 minutes, 50°C), 50% formamide, 1× SSC, 0.1% Tween 20 (2×15 minutes, 50°C), 1× SSC/PBST 1:1 (15 minutes), PBST (4×5 minutes). RNA hybrids were detected immunohistochemically. After blocking in 5% Normal Sheep Serum (NSS) in PBST (30 minutes), the specimens were incubated with 1:2000 alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim) in the above buffer (overnight, 4°C). The specimens were washed with PBST four times (20 minutes each) and alkaline phosphatase buffer (APB) (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.0) (3×10 minutes). Signal detection was performed in APB containing 4.5 μl/ml NBT (nitroblue

tetrazolium salt) and 3.5 μl/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate) according to the supplier's instructions (Boehringer Mannheim DIG RNA Detection Kit). When satisfactory signals over the background were obtained, the solution was replaced with PBST.

Cell culture and transfection

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were plated at 3×10^5 cells/60 mm tissue culture dish 4-6 hours prior to transfection. Transfections were carried out by the calcium phosphate coprecipitation technique (Graham and van der Eb, 1973). After exposure to calcium phosphate-DNA precipitate for 16 hours, the cells were washed with PBS and grown for 48 hours. Luciferase activity of cell extract was determined as previously described (de Wet et al., 1987). CAT activity was determined using the Boehringer Elisa kit.

Cititf1 construct and electroporation

Fusion genes were prepared with a genomic DNA fragment containing 3.5 kb upstream from the *Cititf1* cDNA start site. This fragment was fused to β-galactosidase into pBluescript vector. The construct was electroporated into fertilized eggs according to the protocol of Corbo et al. (1997).

Preparation of synthetic capped mRNAs

Cititf1 cDNA was excised from pBluescript vector by *Bam*HI/*Afl*II digestion and subcloned into a *Bgl*II/*Eco*RI digested pBluescript RN3 vector (Lemaire et al., 1995) after partial filling-in. Control *Cititf1* cDNA, named *MCititf1*, was made by digesting *Cititf1* in pBluescript RN3 vector with *Ncol*/*Bst*XI, to excise the whole homeodomain-coding region, followed by filling-in protruding ends and religating the cDNA. As another control, a plasmid encoding green fluorescent protein (GFP) (Green Lantern™ Gibco BRL), named GL, was also constructed in the pBluescript RN3 vector. The recombinant plasmids were linearized with *Sfi*I and transcribed using MEGAscript™ T3 kit (Ambion) in the presence of $m^7G(5')ppp(5')G$ cap analog.

Microinjection of poly(A)$^+$ RNA

Poly(A)$^+$ RNA was injected according to the method described previously (Marikawa et al., 1995) using a micromanipulator (model MMO-220 Narishige Sci. Instr. Lab., Tokyo). *Ciona* eggs are enclosed by the vitelline coat or chorion, which is tough and sticky and must be removed before injection. Dechorionation was carried out by treating the eggs with a solution 1% sodium thioglycolate (Serva), 0.05% protease E (Sigma), in sea water, pH 10, for 5-10 minutes with pipetting. After this treatment eggs were transferred onto plastic dishes coated with 0.9% agarose in millipore-filtered sea water (MFSW) containing 50 μg/ml streptomycin sulfate (Calbiochem) and fertilized.

Injection pipets were prepared by pulling capillary tubes (Microcaps of Drummond Sci. Co., Broomall, PA, USA) using a microelectrode puller (Model PN-3, Narishige, Tokyo). Capillary tubes, before pulling, were siliconyzed and sterilized by baking at 180°C for 2 hours. Micropipets were filled with a 400-800 μg/ml solution of poly(A)$^+$ RNA in DEPC-treated water containing 0.5% rhodamine B isothiocyanate dextran 70S (Sigma) as a marker dye, and forced into fertilized eggs by manual pressure. The volume injected was estimated to be about 5% of the volume of eggs by the amount of the coinjected marker dye.

Cleavage arrest and alkaline phosphatase staining

To arrest the cleavage, embryos were incubated in MFSW containing 2 μg/ml cytochalasin B (Sigma). Differentiation of endoderm cells was monitored by histochemical staining for alkaline phosphatase (AP) (Hinman and Degnan, 1998). In brief, embryos and larvae were fixed at room temperature for 20 minutes in 4% (w/v) formaldehyde

A

Organism	Gene Name	1 10 20 30 40 50 ↓ 60	Percent Homology
Ascidian	**Cititf1**	**RRKRRVLFSQAQVFELERRFKQQKYLSAPEREHLAQMIHLTPTQVKIWFQNHRYKNKRSL**	**100**
Human	TiTF1	----------Y----------S----------M—QA	92
Mouse	titf1	----------Y----------S----------M—QA	92
Canine	titf1	----------Y----------S----------M—QA	92
Flatworm	Dth-2	-----I------IY----------NL-N----------C---Q	87
Leech	Lox10	-----I------IY-----R----------TF-G----------TK-SK	82
Mouse	NKx2.3	---P----------R----------SSLK--S---------R---C—QR	82
Chicken	cNKx2.3	---P----------R----------SSLK--S---------R---C—QR	82
Fish	nkx2.3	---P----------R----------STLK--S---------R---C--QR	82
Frog	XNkx2.3	---P----------R----------NSLK--S---------R---C--QR	82
Mouse	NKx2.2	K--------K--TY------R--R----------SL-R----------M—AR	80
Frog	XeNK-2	K--------K--TY------R--R----------SL-R----------M--AQ	80
Fish	nkx2.2	K--------K--TY------R--R----------SL-R----------M--AR	80
Chicken	cNkx2.5	---P---------Y----------D---NVLK--S---------R---C--QR	80
Fish	nkx2.5	---P---------Y----------D---NVLK--S---------R---C--QR	80
Frog	XNkx2.5	---P---------Y----------D---NVLK--S---------R---C--QR	80
Fish	nkx2.7	---P------T----------R-------D---LALK--S---------R---C--QR	78
Chicken	cNkx2.8	---P------T--L----------L------NVLQ--S---------R---C--QR	78
Fruitfly	NK2	K-------TK--TY------R--R----------SL-R----------T—AQ	78
Human	CSX	---P---------Y---------R-------DQ--SVLK--S---------R---C--QR	77
Flatworm	ceh-22	K-------TK--TY------RS----------AL-MQ-R----------T-K-H	75
Flatworm	Dth-1	K--------KK-IL----H-R-K----------NL-G-S----------M—AH	75
Mouse	NKx2.5	---P---------Y-----P---R-------DQ--SVLK--S---------R---C—QR	75
Mouse	Nkx2.6	Q--S---------LA--------R--T--------SALQ--S---------R---S-SQR	73
Fruitfly	NK4	K--P---------L---C--RLK---TGA---II--KLN-SA---------R---S--GD	65
Fruitfly	NK3	KKRS-AA--H----------A--R---G---SEM-KSLR--E---------R---T--KQ	63
Fruitfly	NK1	P-RA-TA-TYE-LVS--NK--TTR---VC--LN--LSLS--E---------R-T-W-KQN	48

B

Gene	TN Domain
Cititf1	**MSVSPKHSTTTPFSVTDILSPLEESY**
TiTF1	--M----..------S----------
NKx2.5	-FP--ALTP.-----K---NLEQQQR
XNkx2.5	-FA—VTS..-----K---NLEQHQS
cNkx2.5	-FP—V..-------K---NLEQQQQ
nkx2.5	-AMFSSQM-S-----R---NLEQNQE
cNkx2.3	-MLPSPVTS.--S--K----LEQQQQ
XNkx2.3	-MLPSPVTS.-----K---NLEQQAQ
Nkx2.3	-MLPSPVTS.-----K---NLEQQRH
nkx2.3	-MLPSPVTS.-----K---KLEQQTR
Nkx2.2	--LTNTK...-G---K---DLPDTND
XeNK2	--LTNTK...-G---K---DLPDTND
cNkx2.8	-LP.....-----E----LEQS-A
nkx2.7	-PL--VT-..-----K---KLEQQQA
vnd	-TT-AS // SG-HIS---NLAGSEL

Fig. 1. (A) Comparison of the CiTTF-1 homeodomain deduced sequence with homeodomains of *NK-2*-like genes. The 60-amino-acid homeodomain is indicated along with the gene names and species of origin on the left side. The percentage of homology is on the right side. The dashes indicate identical amino acids. The tyrosine at position 54, a specific feature of NK-2 homeodomain proteins, is indicated with an arrow. Sequences of individual genes were obtained from Harvey (1996 and references therein). (B) Comparison of the CiTTF-1 TN-Domain and surrounding deduced sequences with TN-Domains of different *NK-2* like genes, starting from the predicted N-terminal methionine. The TN-Domain is underlined. Dashes indicate identical amino acids. Dots indicate gaps in the sequence. Double slashes indicate a gap of more than 10 amino acids. Note the high homology of CiTTF-1 with TTF-1 outside the TN-domain deduced sequence.

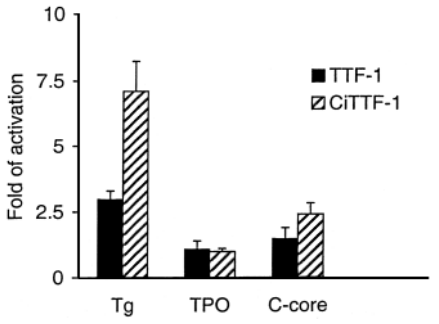

Fig. 2. CiTTF-1 transcription activity on thyroid-specific promoters in HeLa cells. 1 μg of *Cititf1* expression vector (SV-40-CiTTF1) was introduced into HeLa cells together with 5 μg of reporter constructs for either Tg, TPO or C-core (De Felice et al., 1995). The expression vector for murine CMV-Titf1 was used as control. After 48 hours, cells were lysed and assayed for CAT and luciferase activity. Fold of activation is the ratio between values obtained with and without *Cititf1* expression vector. The values represent the averages of three independent experiments ± s.d.

in 100 mM Hepes, pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA, then dehydrated in 100% ethanol and stepped into 100 mM Tris, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ (APB). Colorimetric detection for AP was initiated in APB containing 4.5 μl/ml NBT (nitroblue tetrazolium salt) and 3.5 μl/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate) and the colour was allowed to develop for approximately 5-10 minutes.

RESULTS

Isolation and sequencing of a tunicate *NK-2* related gene

We have previously described a screen for homeobox-containing genes in the ascidian *Ciona* using, as a probe, a degenerate oligonucleotide (CiHB1) coding for the most conserved region of the Hox class homeodomains (Di Gregorio et al., 1995). A cDNA library prepared from *Ciona* larvae mRNA was screened and, among several clones isolated, most

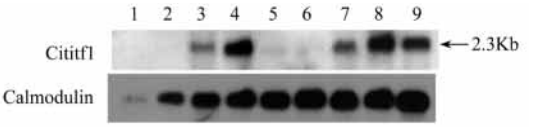

Fig. 3. Temporal expression of *Cititf1* mRNA during *Ciona* development. A northern blot containing poly(A)$^+$ RNA from (1) eggs, (2) 16-cell embryos, (3) pregastrulae, (4) gastrulae, (5) neurulae, (6) early tailbud, (7) middle tailbud, (8) late tailbud embryos and (9) larvae. Top, *Cititf1* probe. The single trancript of 2.3 kb is indicated by the arrow. Bottom, the blot was stripped and reprobed for *calmodulin* mRNA, to control for RNA loading (Di Gregorio et al., 1998).

of which contained a homeobox, we identified a cDNA insert containing an *NK-2* type homeobox. The complete nucleotide sequence of this cDNA (not shown, EMBL accession number AJ009607) demonstrated an insert length of 2301 bp, containing a 1431 bp open reading frame that starts with an ATG in position 72 and ends with a stop codon in position 1503 followed by 783 bp of untranslated trailer comprising a canonical polyadenylation signal (Bardwell et al., 1991) 12 nucleotides upstream from an $(A)_{11}$ stretch. The reading frame encoded a protein of 477 amino acids, containing a homeodomain that shared substantial homology with those of the NK-2 class. The highest similarity (92% identity) was with the homeodomain of mammalian TTF-1 (Guazzi et al., 1990), and decreased for other homeodomains of the NK-2 class, as shown in Fig. 1A. The homeodomain of the *Ciona* protein contained several residues that are unusual in other class of homeobox-containing genes, such as Leu-7, Arg-19, Ala-28, His-33 and Tyr-54. In particular Tyr-54, which has not been found in other homeodomains, is currently the most unambiguous feature of the *NK-2* class. Moreover, the *Ciona* homeodomain showed a His in position 51 that is also present in mammalian TTF-1 and other homeodomains closely related to TTF-1. Furthermore, among the several genes belonging to the *NK-2* class, CiTTF-1 showed a long stretch of homology only with mammalian TTF-1 near the amino terminus (93% of

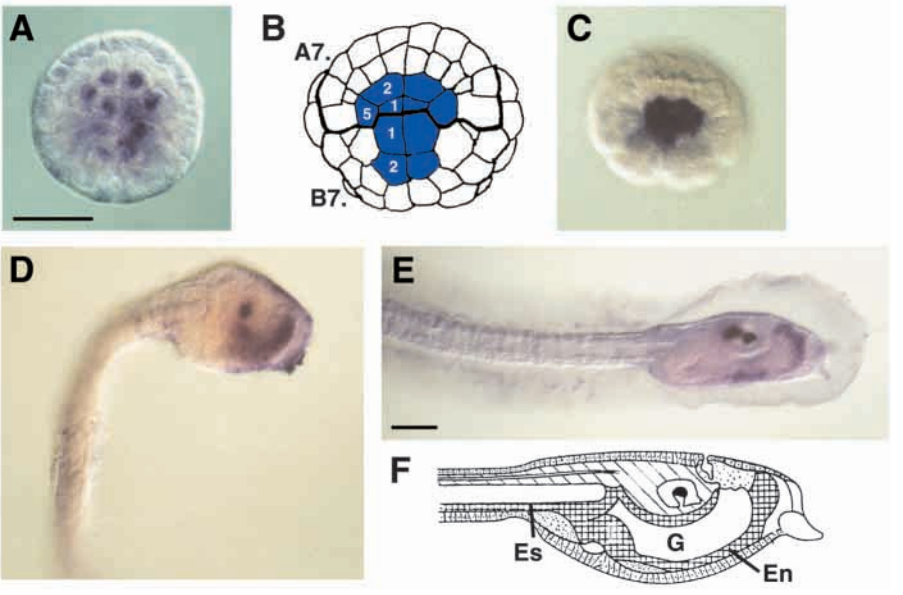

Fig. 4. Spatial expression of *Cititf1*, as revealed by whole-mount in situ hybridization with a digoxigenin-labeled antisense probe. (A) A 76-cell stage embryo viewed from the vegetal pole. Hybridization signals are seen in the endodermal precursor blastomeres A7.1, A7.2, A7.5, B7.1 and B7.2, as shown schematically in B. Expression persists during gastrulation (C), is downregulated from neurula to early tailbud stage (data not shown) and reappears at middle tailbud (D) and swimming larval (E) stages in the gut primordium (shown schematically in F). En, endoderm; Es, endodermal strand; G, gut primordium. Bars, 50 μm.

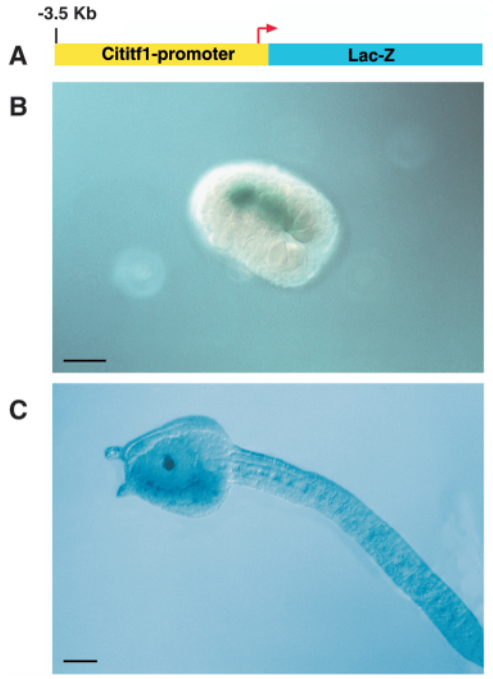

Fig. 5. Expression of *Cititf1*/*lacZ* fusion gene in electroporated embryos. The 3.5 kb *Cititf1* promoter region was fused to *lacZ* coding sequence (A) and the construct was introduced into fertilized eggs via electroporation. The electroporated embryos were allowed to develop at 18°C, collected at early neurula stage (B) and at larval stage (C) and subsequently stained with X-gal to visualize the sites of expression. (B) Vegetal view of an early neurula: most of the staining is detected in the endodermal precursor derived from one side of the embryo. (C) Lateral view of a larva: the staining is detected in the anterior/ventral endoderm of the trunk. Bars, 50 μm.

homology on 23 amino acids). This domain comprises the so-called TN-Domain identified in some genes of the *NK-2* class (Harvey, 1996) (Fig. 1B). A shorter stretch of homology (amino acids 178-192), with mammalian TTF-1 only, was localized upstream from the homeodomain (83% of homology on 15 amino acids).

Given the high similarity, both within and outside the homeodomain, we suggest that the clone that we isolated is the ascidian orthologue of mammalian *TITF1*. Thus, by analogy with the mammalian nomenclature, we have called the ascidian gene *Cititf1* and the corresponding protein product CiTTF-1.

The protein encoded by *Cititf1* is a transcription factor with the same transcriptional specificity as mammalian TTF-1

The ability of the protein encoded by *Cititf1* to transactivate both thyroglobulin (Tg) and thyroperoxidase (TPO) promoters was investigated to assess its ability to function as a transcription factor. Previously, two reporter constructs have been generated, which contained a minimal Tg or TPO promoter fused to CAT or luciferase (Luc) coding sequences. Mammalian TTF-1 binds to three sites of both promoters but efficiently activates Tg transcription, while TPO is activated very poorly, if at all (De Felice et al., 1995, and references therein). HeLa cells were cotransfected with expression vector containing the sequence encoding CiTTF1 under the control of SV40 promoter (SV40-CiTTF1) together with either the Tg or TPO reporter constructs. As shown in Fig. 2, CiTTF1 was able to specifically activate the Tg promoter with very high efficiency while the TPO promoter was not activated. To verify the specificity of the activation, a Tg promoter (C-core) mutated in one of its TTF-1 binding sites was also used

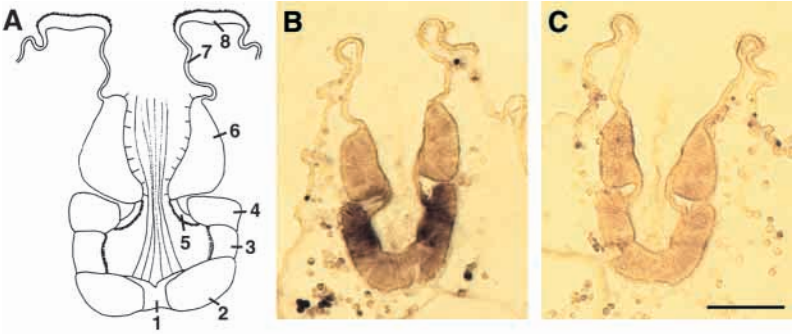

Fig. 6. Schematic representation of ascidian endostyle (A). Zones 1, 3 and 5 contain supporting elements. Zones 7 and 8 show iodine-binding activity. Zones 2, 4 and 6 secrete proteins related to digestion. (B,C) Expression of *Cititf1*, as revealed by whole-mount in situ hybridization with a digoxigenin-labeled antisense (B) or sense (C) probe, on sectioned *Ciona* endostyle. Bar, 100 μm.

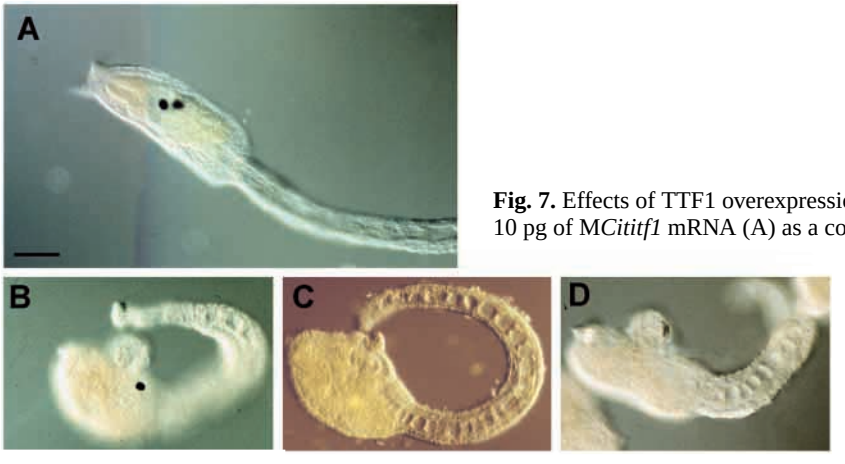

Fig. 7. Effects of TTF1 overexpression in *Ciona* larvae. Fertilized eggs were injected with 10 pg of M*Cititf1* mRNA (A) as a control or 10 pg of *Cititf1* mRNA (B-D). The control larva, injected with M*Cititf1* mRNA (A), shows a normal morphology with the brain vesicle containing two pigment cells, otolith and ocellus. In contrast the larvae injected with *Cititf1* mRNA (B-D) show variable abnormalities in head-trunk development. Present on the dorsal side is a bulb-like structure containing a pigmented region on the outermost surface. The tail is bent dorsally. Bar, 50 μm.

(Civitareale et al., 1989). In this case, both TTF-1 and CiTTF1 were unable to activate transcription. We conclude that CiTTF1 is a transcription factor with a transcriptional specificity similar to the mammalian counterpart.

Expression of *Cititf1* during *Ciona* embryogenesis

Cititf1 expression during *Ciona* embryogenesis was examined by northern blot analysis with 8 μg of poly(A)$^+$ RNA isolated from different stages of development, using the full-length cDNA as a probe (Fig. 3). Expression of *Cititf1* was first detected at the pregastrula stage, where a single transcript of 2.3 kb was observed. The expression increased at gastrula but the mRNA rapidly disappeared and no transcript was observed at neurula and early tailbud stages. At middle tailbud stage the transcript was detected again and expression was maintained throughout larval life. The length of the transcript correlated well with the length of the cDNA clone isolated, suggesting that the clone that we isolated was nearly full size.

Non-radioactive whole-mount in situ hybridization assays were carried out in order to determine the pattern of *Cititf1* expression during *Ciona* embryogenesis. A full-length cDNA was used as probe. No signal was detected with sense probe (data not shown).

No expression was detected before the 76-cell stage (data not shown). At this stage *Cititf1* was expressed exclusively in most, but not all, endoderm precursors (Fig. 4A). Specific staining was seen in blastomeres A7.1, A7.2, A7.5 and B7.1, all of which give rise to the trunk endoderm, as well as in the blastomeres B7.2 that give rise to part of the tail endoderm (Nishida, 1987). Staining in endodermal precursors persisted during gastrulation (Fig. 4C) but was completely lost by the onset of neurulation until early tailbud stage. At middle tailbud stage the transcript appeared in a subset of endodermal cells (Fig. 4D-E), merely the most anterior and ventral cells of the trunk, corresponding at the larval stage to the region where the adult endostyle originates.

Electroporation is very useful for introducing reporter DNA constructs into *Ciona* eggs (Corbo et al., 1997). To test whether the observed expression of *Cititf1* was due to transcriptional regulation, we cloned the *Cititf1* gene from a *Ciona* genomic library, using the entire cDNA as a probe, and determined the transcription start site by RNase protection assay (not shown). A 3.5 kb *Cititf1* promoter region was then fused to a *lacZ* reporter. The *Cititf1/lacZ* construct was introduced into embryos via electroporation at a concentration of 50 μg/ml. The electroporated embryos were grown at 18°C and analyzed at gastrula, neurula and swimming larval stages (Fig. 5).

We did not observe expression of the reporter at gastrula stage (not shown), while at neurula, β-gal staining was detected in the endodermal precursors. This delay in the expression of the reporter gene, compared to the detection of endogenous mRNA, has been observed before and could be due to the time necessary to accumulate enough functional β-gal protein (Corbo et al., 1997). The embryo in Fig. 5B showed expression only in one side, suggesting that the transgene was inherited in one of the two blastomeres after the first cell cycle as previously reported (Corbo et al., 1997). Expression of the reporter in swimming larvae matched the pattern of endogenous *Cititf1* expression, as β-gal staining was detected in the antero-ventral region of the trunk endoderm. We concluded that the promoter fragment that we had cloned recapitulates *Cititf1* expression and that the control of *Cititf1* expression is largely due to transcriptional regulation.

Expression of *Cititf1* in the adult endostyle

In order to verify if *Cititf1* was expressed in the adult endostyle, in situ hybridization on sectioned young adults were carried out. The general organization of the ascidian endostyle is depicted in Fig. 6A. The cells of this organ are differentiated into eight or nine stripes that run parallel to one other in longitudinal orientation. The cells of each zone are highly specialized in morphology and function. The cells of zone 7 and 8 have an iodine-binding activity. The cells of zone 2, 4 and 6 are believed to secrete proteins related to digestion. The cells of zone 1, 3 and 5 are considered as supporting elements but their function is still unclear. *Cititf1* mRNA was detected specifically in zone 3-4 of the adult endostyle (Fig. 6B).

Overexpression of *Cititf1* affects trunk development

The characteristic distribution of *Cititf1* mRNA during early stages of development suggests a significant role of this gene in endodermal differentiation. To explore its putative function in the ascidian development, we performed a series of microinjection experiments. We injected into fertilized eggs 10 pg of *Cititf1* mRNA or, as controls, *MCititf1* mRNA, a mutated mRNA encoding a protein lacking its DNA binding domain, or 15 pg of mRNA encoding the green fluorescent protein (GFP). In each experiment we scored for normal or visibly altered embryos both at gastrula and at larval stages (Table 1). No anomalies were detected at gastrula stage, while at larval stage the injection of *Cititf1* mRNA induced a visible and specific alteration in most of the injected embryos (see

Table 1. Effects of *Cititf1* overexpression on development of Ciona embryos

	Gastrula stage		Larva stage	
	Normal/Total	%	Normal/Total	%
Exp. 1				
Control (no injection)	37/52	71	37/37	100
Control (+GFP, 15 pg)	9/14	64	7/9	77
Cititf1, 10 pg	9/15	60	0/9	0
Exp. 2				
Control (no injection)	20/28	71	20/20	100
Control (+GFP, 15 pg)	13/21	61	9/13	69
Cititf1, 10 pg	8/15	53	0/8	0
Exp. 3				
Control (no injection)	56/79	71	28/56	50
Control (+MCititf1, 10 pg)	37/59	63	23/37	62
Cititf1, 10 pg	38/74	52	4/38	10
Exp. 4				
Control (no injection)	108/135	80	94/108	87
Control (+MCititf1, 10 pg)	35/45	78	29/35	83
Cititf1, 10 pg	45/56	80	2/45	2
Exp. 5				
Control (no injection)	50/70	71	44/50	88
Control (+MCititf1, 10 pg)	27/39	69	24/27	89
Cititf1, 10 pg	43/67	64	2/43	4

In five separate experiments (1-5) the numbers of normal or visibly altered embryos were scored at gastrula and larval stages, and the percentage of normal embryos is indicated at both stages.

Control eggs were injected either with GFP or MCititf1 mRNA.

below). In contrast, control mRNAs did not induce any significant aberrations in addition to those caused by the injection procedure itself, which were largely represented by embryos arrested in their development (data not shown). Otherwise, eggs from control injections developed into morphologically normal tadpoles (Fig. 7A). The translation efficiency of injected *GFP* mRNA was assessed by fluorescence microscopy, showing that the protein was translated and evenly distributed throughout the embryos (data not shown).

Injection of 10 pg of *Cititf1* mRNA produced visible abnormalities in head-trunk development compared to the control (Fig. 7B-D). The larvae showed an aberrant development of the head and the presence of a bulb-like structure, on the dorsal side, with a pigmented region positioned at its outermost external surface. The tail, shorter than normal, was bent dorsally.

Sections of *Ciona* larvae permitted a more detailed analysis of this phenotype (Fig. 8). The trunk region of *Ciona* larva is surrounded by epidermis and contains endoderm, mesenchyme and the brain vescicle, with the pigmented otolith and the ocellus (Fig. 8A,D). Mid-sagittal and trasverse sections of *Ciona* larvae, injected with *MCititf1*, showed that the distribution of cells in the trunk was the same as that in normal, uninjected embryos (Fig. 8B,E)

In contrast, trunk organization was clearly altered in *Ciona* larvae injected with *Cititf1* mRNA (Fig. 8C,F), particularly the presence of a large mass of endodermal cells, which completely filled the trunk region, and the absence of the brain vesicle, which seemed to be displaced on the dorsal side and appeared like a bulb filled with endoderm-mesenchyme intrusion. The brain of normal *Ciona* larvae contains two sensory pigmented organs, the otolith and the ocellus. In larvae injected with *Cititf1* mRNA, a pigmented region was present on the outermost surface of the bulb. The tail was shorter than normal and bent. We concluded that the overexpression of a functional CiTTF1 protein causes a substantial alteration in development, resulting in higher amounts of endoderm in the trunk region and in an altered tail morphology.

Endoderm territories in *Cititf1* overexpressing larvae were compared to that of control larvae, by histochemical detection of alkaline phosphatase (AP). In control larvae, the staining was detected in the endoderm cells present in the trunk region and in the notochord cells at the tip of the tail, as already demonstrated (Whittaker, 1977) (Fig. 9A). In *Cititf1* overexpressing larvae, AP activity was present in the trunk region but its domain was extended into the bulb-like structure, on the dorsal side (Fig. 9B). These data demonstrate that the bulb-like structure was filled with cells showing endoderm features. The tip of the tail was stained as in the control embryos.

Endoderm territories were also examined by AP detection at an earlier stage of development, taking advantage of cleavage-arrested embryos at the 110-cell stage. It has been demonstrated that, at the 110-cell stage, most of the blastomeres are already fate-restricted (Conklin, 1905; Nicol and Meinertzhagen, 1988; Nishida, 1987; Ortolani, 1971; Satoh et al., 1996). If the embryos are cleavage-arrested at this stage and reared until the control embryos reach the larval stage, each blastomere expresses markers specific for the tissue originating from its descendents (Crowther and Whittaker, 1983; Whittaker, 1977). Normal and *Cititf1* mRNA injected embryos were arrested at the 110-cell stage, reared until controls reached larval stage and then the endoderm cell differentiation was analyzed by AP activity. In control embryos AP activity was localized exclusively in the endodermal precursors (compare Fig. 9C to D,E), whereas in embryos injected with *Cititf1* mRNA the expression domain of AP was expanded anteriorly into the blastomeres of the notochord lineage (compare Fig. 9F,G to D,E). This finding suggests that the excess endoderm present in the trunk was derived from notochord blastomeres, whose fate was changed by ectopic *Cititf1* expression.

DISCUSSION

Cititf1 is an ascidian hortologue of mammalian TTF1

In this report we describe the isolation and characterization of one *NK-2* class gene from the ascidian *Ciona intestinalis*. Structural comparison of the predicted CiTTF-1 protein with previously reported Nkx-2 family members revealed a high degree of homology with mammalian TTF-1, not only in the homeodomain but also in two stretches of amino acids upstream from the homeodomain. One of them comprises the so-called TN-Domain. This region, that is near the amino terminus of both TTF-1 and CiTTF1 proteins, has been suggested to be a transcriptional activating domain that is able to function as activator or repressor, depending on the promoter arrangement (De Felice et al., 1995).

Cotransfection studies in HeLa cells have shown that CiTTF-1, as the mammalian TTF-1, was able to activate the transcription of the thyroglobulin promoter even better than its mammalian counterpart. Like the mammalian TTF-1, CiTTF-1 was unable, under the conditions tested, to activate the thyroperoxidase promoter. Even though the number and spacing of binding sites are quite similar in the regulatory region of Tg and TPO promoters, the orientation of the binding sites and their affinity towards TTF-1 are not identical, suggesting that CiTTF-1 activity depends on the promoter architecture. The similarities in sequence and transcriptional activity with the mammalian TTF-1 strongly suggest that CiTTF-1 is the ascidian orthologue of mammalian TTF-1.

Expression pattern of *Cititf1* in ascidian embryos

Spatio-temporal expression pattern of *Cititf1*, studied by northern blot, showed a biphasic expression of *Cititf1* during the development of the ascidian embryos. *Cititf1* was first expressed at the pregastrula stage (64-76 cells) and its mRNA increased until the gastrula stage. No transcript was detected at the neurula stage, while expression was restored in swimming larvae.

The endodermal fate of early blastomeres and their differentiation in tunicates has been studied by injection of tracer enzymes and by the expression of alkaline phosphatase, which is, in certain species, an unequivocal indication of endodermal differentiation. Culture of isolated blastomeres, derived from 8- or 16-cell embryos, has shown that only

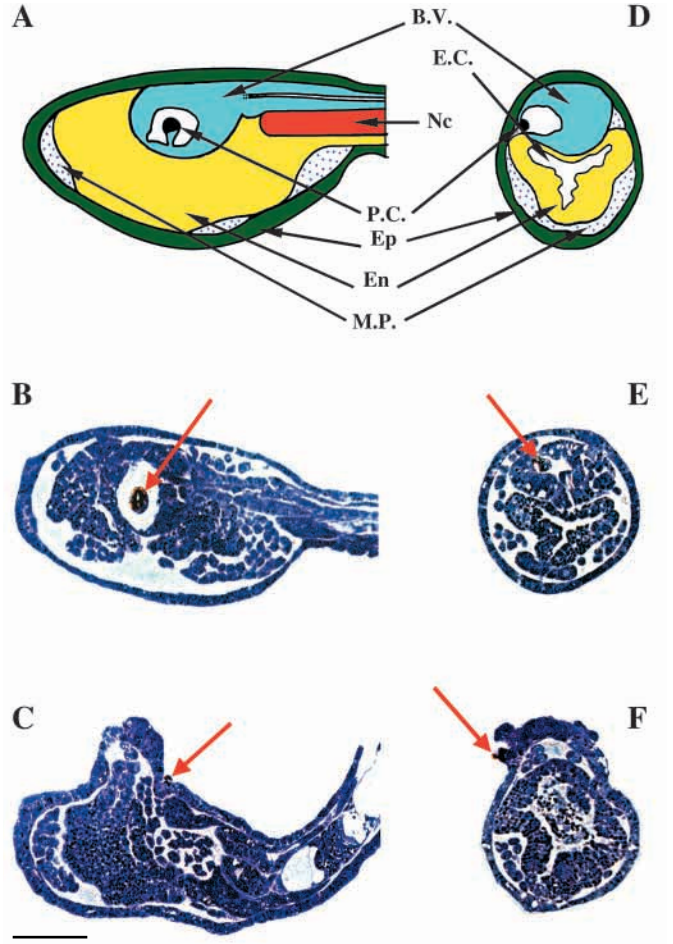

Fig. 8. Mid-sagittal sections (B,C) and transverse sections at the level of the sensory vesicle (E,F) of *Ciona* larvae injected with M*Cititf1* mRNA (B,E) as a control, or *Cititf1* mRNA (C,F). Tissue organization is shown in (A) and (D). Dorsal is on the top. BV, brain vesicle; EC, endodermal cavity; Nc, notochord; PC, pigmented cell; Ep, epidermis; En, endoderm; MP, mesenchyme pockets. Note the increased mass of the endodermal cells, the absence of the brain vesicle and the presence of an extroflected vesicle in C and F only. Red arrows indicate a pigmented region that is normally included in the brain vescicle. Bar, 50 μm.

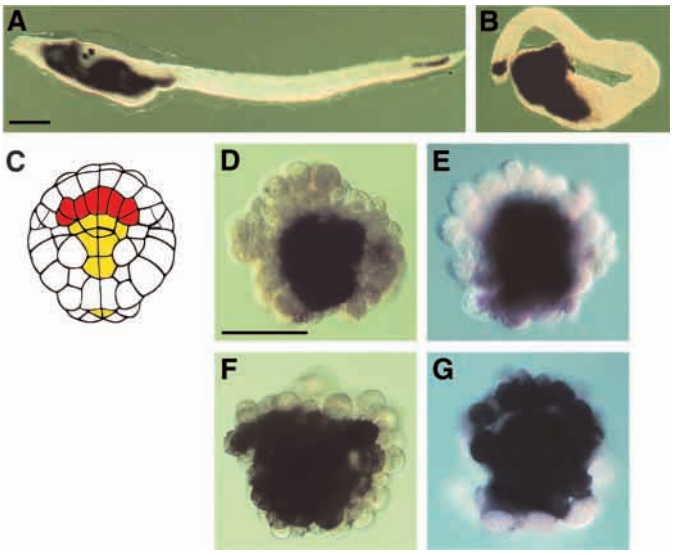

Fig. 9. Alkaline phosphatase (AP) expression in *Ciona* larvae and cleavage-arrested embryos. In control larvae (injected with M*Cititf1* mRNA) the staining is localized in the endoderm cells of the trunk and in the tip of the tail (A). In the larvae injected with *Cititf1* mRNA (B) AP activity is present in the whole trunk region and its domain is extended into the bulb-like structure on the dorsal side. The tip of the tail is stained like in the control. (C) Schematic representation of endodermal precursor blastomeres (shown in yellow) and notochord precursors of primary lineage (shown in red) at the 110-cell stage. Control embryos (injected with M*Cititf1*) arrested at the 110-cell stage and reared in MFSW until the untreated embryos reach the larval stage (D,E). AP activity is localized exclusively in the endoderm precursors, as shown in C. (F,G) Embryos injected with *Cititf1*, arrested at the 110-cell stage and reared in MFSW until the untreated embryos reach the larval stage. The expression domain of AP is expanded into the blastomeres of notochord lineage as shown in C. Bars, 50 μm.

vegetal pole blastomeres become positive to staining specific for alkaline phosphatase (Whittaker, 1990). However, other cell types also derive from the vegetal pole blastomere of early tunicate embryos. Injection of tracer enzymes in individual blastomeres of the 64-cell *Halocynthia roretzi* embryo have demonstrated that most of the larval endodermal cells are derived from blastomeres A7.1, A7.2, A7.5, B7.1 and B7.2. All of these endodermal precursors are fate-restricted at this stage, their descendants being exclusively in the endoderm of either the trunk (A7.1, A7.2, A7.5, B7.1) or the tail (B7.2) (Nishida, 1987). Whole-mount in situ hybridization experiments carried out on *Ciona* embryos showed that *Cititf1* was expressed at the 76-cell stage exclusively in blastomeres A7.1, A7.2, A7.5, B7.1 and B7.2. *Cititf1* expression was one stage delayed compared to the stage when most of the endodermal precursors become fate-restricted (64-cell stage). Since cellular divisions do not involve endodermal blastomeres between the 64- and 76-cell stages, the lag could be explained by the time required to accumulate enough *Cititf1* mRNA to be detectable by in situ hybridization techniques.

Cititf1 is the first specific molecular marker to be isolated from the ascidians at this early stage of endodermal differentiation. Other *NK-2* genes have been shown to be expressed in overlapping domains in the ventral endoderm and its derivatives, like the thyroid gland and the lung (Nardelli-Haeflinger and Shankland, 1993; Lee et al., 1996; Reecy et al., 1997), but they are expressed later than *Cititf1* during the embryogenesis and in restricted domains of the endoderm. Little is known about the genes that govern the development of the endoderm in vertebrates. A forkhead gene *HNF3* has been shown to be expressed in the mouse in the anterior portion of the primitive streak at the onset of gastrulation in a region where the definitive endoderm first arises (Ang et al., 1993). Recently, two HMG domain-containing genes, *Xsox17*α and β, have been clearly shown to be involved in the early differentiation of endoderm in *Xenopus* embryo (Hudson et al., 1997). *Xsox17*α and β are expressed in the presumptive endoderm starting from the beginning of gastrulation, a pattern that clearly resembles that of *Cititf1* expression, suggesting a

similar role for the ascidian gene in the endodermal differentiation.

After the gastrula stage the expression of *Cititf1* became undetectable, as shown both by northern blot analysis and in situ hybridization experiments, and it remained absent during the neurula and early tailbud stages. Later, starting from middle tailbud stage, *Cititf1* was turned-on again in a subset of endodermal cells. In the tailbud embryo the endoderm can be subdivided into gut primordium in the trunk and endodermal strand, a single strand of cells, in the tail. At this stage *Cititf1* was expressed only in the anterior and ventral regions of the gut primordium. Promoter fusion analysis confirmed the expression pattern of *Cititf1* in the gut primordium at the larval stage. A 3.5 kb sequence from *Cititf1* 5′ regulatory region was able to drive the tissue-specific spatial expression of reporter genes.

The antero-ventral part of the trunk endoderm, where *Cititf1* was expressed, gives rise after metamorphosis to the endostyle, a specialized organ present in the ventral wall of the pharynx in adult tunicates. Several lines of evidence support the homology, initially proposed on the basis of morphological criteria (Dohrn, 1886), between the endostyle and the thyroid gland of vertebrates (Salvatore, 1969; Ogasawara et al., 1996 and references therein). The structural and functional homology of CiTTF-1 with mammalian TTF-1, which has an important role in thyroid morphogenesis and differentiation, prompted us to test whether *Cititf1* was also expressed in the endostyle of *Ciona* adults. In situ hybridization of sectioned young adults (1 month old) demonstrated that *Cititf1* was expressed in region 3-4 of the endostyle. Even though this is not the region demonstrated to bind iodine (Olsson, 1963), *Cititf1* expression in the endostyle supports the suggestion that this organ is homologous to the vertebrate thyroid gland.

The potential role of *Cititf1* in ascidian development

In the ascidian embryo, the neural tube formation progresses in a fashion similar to that of a vertebrate. Neurulation is accomplished by folding of the presumptive neural cells and proceeds from the posterior to the anterior end. At the stage when the future larval tail is elongating (tailbud) the tube is brought under the epidermis and becomes completely closed. The anterior portion forms the brain vesicle with a spacious cavity where the brain sensory organs, including the pigmented cells of otolith and ocellus, differentiate.

When *Cititf1* mRNA was injected into *Ciona* eggs, trunk and tail development were affected. The tail was shorter than normal and bent. The trunk region seemed to be completely occupied by a mass of endodermal cells, lacked the brain vesicle and presented, on the dorsal side, a hollow bulb with a pigmented region on the outermost surface.

The analysis of endoderm territories by alkaline phosphatase detection in control and *Cititf1* overexpressing larvae showed that: (1) in control larvae the staining reflected the endoderm cells present in the trunk region; (2) in *Cititf*1 overexpressing larvae the staining was localized not only in the whole trunk region but was extended in the bulb like structure.

We suggest that, in *Cititf1* overexpressing larvae, the bulb is the brain vesicle, since its position is at the site where, in normal larvae, a proper brain vesicle is formed inside the trunk. The presence of a pigmented region, normally located in the brain at the base of the bulb, and the presence of cells with endoderm features in the bulb, support the possibility that the increased mass of endodermal cells in the trunk could have displaced the brain vesicle from its normal site, preventing its internalization.

The overall phenotype of *Ciona* larvae overexpressing *Cititf1* could be the consequence of an interference exerted by *Cititf1* on the differentiation of blastomeres of other lineages. This hypothesis is supported by the analysis of the cleavage-arrested embryos. Ascidian embryos, which are cleavage-arrested at various early stages with cytochalasin B, eventually express alkaline phosphatase only in those cells that are known to be of endodermal lineage (Whittaker, 1977; Satoh, 1982). We decided to arrest the embryos at the 110-cell stage, approximately 45 minutes after the endogenous *Cititf1* was first detected by in situ hybridization, at the 76-cell stage. The embryos were reared and analyzed by alkaline phosphatase staining when the control embryos reached the larval stage. In uninjected or control RNA-injected embryos, only blastomeres known to be precursors of endoderm (Nishida, 1987) showed AP staining. Conversely, in *Cititf1* injected embryos, AP staining was extended in blastomeres fated to become notochord. It has been demonstrated that tail elongation depends on the formation of notochord. Embryos that lack notochord cells form rudimentary tails which do not elongate (Reverberi et al., 1960; Miyamoto and Crowther, 1985). Our results suggest that *Cititf1* overexpression could affect the differentiation of blastomeres of notochord lineage, recruiting them to the endoderm lineage. This effect could in turn be responsible, at the larval stage, for the overall phenotype, which is characterized by a short tail and a trunk completely filled by cells with endoderm features.

Notochord precursors of primary lineage are clonally restricted at the 64-cell stage, and this event is accompanied by the concomitant detection of *As*-T gene expression (the ascidian *brachyury*) (Yasuo and Satoh, 1993, 1994; Nakatani and Nishida, 1994). Moreover when the *As-T* gene is overexpressed in whole embryos by synthetic *As*-T mRNA injection into *Halocynthia* eggs, the fate of endodermal blastomeres is changed to become notochord cells (Yasuo and Satoh, 1998). On the other hand *Cititf1* was first detected, by in situ hybridization techniques, at the 76-cell stage, just one cell division after most of the endodermal blastomeres undergo fate restriction. If *Cititf1* was expressed at earlier stages by synthetic *Cititf1* mRNA injection into *Ciona* eggs, notochord lineage blastomeres, in cleavage-arrested embryos, expressed the endoderm-specific marker alkaline phosphatase.

The switch from notochord to endoderm fate induced by *Cititf1* parallels the reverse change (endoderm to notochord fate) induced by *As*-T. Taken together these data suggest that the blastomeres of endoderm and notochord lineages have the potential to differentiate in either direction and that the final differentiation is induced by the appearance of either *As*-T (for notochord) or *Cititf1* (for endoderm).

Cititf1 represents the first homeobox containing gene, of the *NK-2* class, to be shown to exert an effect on endoderm development and is the first specific regulatory endodermal

marker to be isolated from an ascidian. *Cititf1* seems to play additional important roles later during endoderm patterning and endostyle differentiation. *Cititf1* will be instrumental in elucidating these processes.

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REFERENCES

Ang, S. L., Wierda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J. and Zaret, K. S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/forkhead proteins. *Development* **119**, 1301-1315.

Bardwell, V. J., Wickens, M., Bienroth, S., Keller, W., Sproat, B. S. and Lamond, A. I. (1991). Site-directed ribose methylation identifies 2′-OH groups in polyadenylation substrates critical for AAUAAA recognition and poly(A) addition. *Cell* **65**, 125-133.

Barrington, E. J. W. (1957). The distribution and significance of organically bound iodine in the ascidian *Ciona intestinalis. J. Mar. Biol. Ass. UK* **36**, 1-16.

Burglin, T. R. (1994). A Comprehensive Classification of Homeobox Genes. In *A Guidebook for Homeobox Genes* (ed. D. Duboule), pp. 27-71. Oxford, Oxford University Press

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Civitareale, D., Lonigro, R., Sinclair, A. J. and Di Lauro, R. (1989). A thyroid-specific nuclear protein essential for tissue-specific expression of the thyroglobulin promoter. *EMBO J.* **8**, 2537-2542.

Conklin, E. G. (1905). The organization and cell lineage of the ascidian egg. *J. Acad. Nat. Sci. (Phila.)* **13**, 1-119.

Corbo, J. C., Levine, M. and Zeller, R. W. (1997). Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis. Development* **124**, 589-602.

Crowther, R. J. and Whittaker, J. R. (1983). Developmental autonomy of muscle fine structure in muscle lineage cells of ascidian embryos. *Dev. Biol.* **96**, 1-10.

De Felice, M., Damante, G., Zannini, M., Francis-Lang, H. and Di Lauro, R. (1995). Redundant domains contribute to the transcriptional activity of the thyroid transcription factor 1. *J. Biol. Chem.* **270**, 26649-26656.

de Wet, J., R., Wood, K., V., Deluca, M., Heliski, D. R. and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**, 725-737.

Di Gregorio, A., Spagnuolo, A., Ristoratore, F., Pischetola, M., Aniello, F., Branno, M., Cariello, L. and Di Lauro, R. (1995). Cloning of ascidian homeobox genes provides evidence for a primordial chordate cluster. *Gene* **156**, 253-257.

Di Gregorio, A., Villani, M. G., Locascio, A., Ristoratore, F., Aniello, F. and Branno, M. (1998). Developmental regulation and tissue specific localization of *calmodulin* mRNA in the protochordate *Ciona intestinalis. Dev. Growth Differ.* **40**, 387-394.

Dohrn, A. (1886). Thyroidea bei petromyzon, amphioxus und den tunicaten. *Mitt. zool. Stat. Neapel* **6**, 49.

Dunn, A. D. (1974). Ultrastructural autoradiografy and cytochemistry of the iodine-binding cells in the ascidians endostyle. *J. Exp. Zool.* **188,** 103-123.

Graham, F. L. and van der Eb, A., J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456-467.

Guazzi, S., Price, M., De Felice, M., Damante, G., Mattei, M. G. and Di Lauro, R. (1990). Thyroid nuclear factor 1 (TTF-1) contains a homeodomain and displays a novel DNA binding specificity. *EMBO J.* **9**, 3631-3639.

Harvey, R. P. (1996). *NK-2* homeobox genes and heart development. *Dev. Biol.* **178**, 203-216.

Heasman, J. (1997). Patterning the *Xenopus* blastula. *Development* **124**, 4179-4191.

Hinman, V. F. and Degnan, B. M. (1998). Retinoic acid disrupts anterior ectodermal and endodermal development in ascidian larvae and postlarvae. *Dev. Genes Evol.* **208**, 336-345.

Hudson, C., Clements, D., Friday, R., V., Stott, D. and Woodland, R. (1997). *Xsox17*α and -β mediate endoderm formation in *Xenopus. Cell* **91**, 397-405.

Kennedy, G. R. (1966). The distribution and nature of iodine compounds in ascidians. *Gen. Comp. Endocrinol.* **7**, 500-511.

Kessler, D. S. and Melton, D. A. (1994). Vertebrate embryonic induction: mesodermal and neural patterning. *Science* **266**, 596-604.

Kim, Y. and Nirenberg, M. (1989). Drosophila *NK*-homeobox genes. *Proc. Natl. Acad. Sci. USA* **86**, 7716-7720.

Kimelman, D., Christian, J. L. and Moon, R. T. (1992). Synergistic principles of development: overlapping patterning systems in *Xenopus* mesoderm induction. *Development* **116**, 1-9.

Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M. and Gonzales, F. J. (1996). The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* **10**, 60-69.

Lazzaro, D., Price, M., De Felice, M. and Di Lauro, R. (1991). The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development* **113**, 1093-1104.

Lee, K. H., Xu, Q. and Breitbart, R. E. (1996). A new *tinman*-related gene, *nkx2.7*, anticipates the expression of *nkx2.5* and *nkx2.3* in zebrafish heart and pharyngeal endoderm. *Dev. Biol.* **180**, 722-731.

Lemaire, P., Garrett, N. and Gurdon, J. B. (1995). Expression and cloning of *Siamois*, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* **81**, 85-94.

Lemaire, P. and Kodjabakian, L. (1996). The vertebrate organizer: structure and molecules. *Trends Genet.* **12**, 525-531.

Marikawa, Y., Yoshida, S. and Satoh, N. (1995). Muscle determinants in the ascidian egg are inactivated by UV irradiation and the inactivation is partially rescued by injection of maternal mRNAs. *Roux's Arch. Dev. Biol.* **204**, 180-186.

Miyamoto, D. M. and Crowther, R. J. (1985). Formation of the notochord in living ascidian embryos. *J. Embryol. exp. Morph.* **86**, 1-17.

Nakatani, Y. and Nishida, H. (1994). Induction of notochord during ascidian embryogenesis. *Dev. Biol.* **166**, 289-299.

Nardelli-Haeflinger, D. and Shankland, M. (1993). *Lox10*, a member of the *NK-2* homeobox gene class, is expressed in a segmental pattern in the endoderm and in the cephalic nervous system of the leech *Helobdella. Development* **118**, 877-892.

Nicol, D. R. and Meinertzhagen, I. A. (1988). Development of the central nervous system of the larva of the ascidian, *Ciona intestinalis* L. II. Neural plate morphogenesis and cell lineages during neurulation. *Dev. Biol.* **130**, 737-766.

Nishida, H. (1987). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. III. Up to the tissue-restricted stage. *Dev. Biol.* **121**, 526-541.

Nishida, H. (1993). Localized regions of egg cytoplasm that promote expression of endoderm-specific alkaline phosphatase in embryos of the ascidian *Halocynthia roretzi. Development* **118**, 1-7.

Nishida, H. and Satoh, N. (1983). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. I. Up to the eight-cell stage. *Dev. Biol.* **99**, 382-394.

Nishida, H. and Satoh, N. (1985). Cell lineage analysis in ascidian embryos by intracellular injection of a tracer enzyme. II. The 16- and 32-cell stages. *Dev. Biol.* **110**, 440-454.

Ogasawara, M., Tanaka, K. J., Makabe, K. W. and Satoh, N. (1996). Expression of endostyle-specific genes in the ascidian *Halocynthia roretzi. Dev. Genes Evol.* **206**, 227-235.

Olsson, R. (1963). Endostyles and endostylar secretions: a comparative histochemical study. *Acta Zool.* **44**, 299-329.

Ortolani, G. (1971). Sul cell-lineage delle ascidie. *Boll. Zool.* **38**, 85-88.

Pearson, W. R. and Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA* **85**, 2444-2448.

Reecy, J., M, Yamada, M., Cummings, K., Sosic, D., Chen, C. Y., Eichele, G., Olson, E., N. and Schwartz, R., J. (1997). Chicken *NKx-2.8*: a novel homeobox gene expressed in early heart progenitor cells and pharyngeal pouch-2 and -3 endoderm. *Dev. Biol.* **188**, 295-311.

Reverberi, G., Ortolani, G. and Farinella-Ferruzza, N. (1960). The causal formation of the brain in ascidian larva. *Acta Embryol. Morph. Exp.* **3**, 296-336.

Roche, J., Salvatore, G., Rametta, G. and Varrone, S. (1959). Sur la presence d'hormone thyridiennes (3,5,3′-triiodiothyronine et thyroxine) chez un tunicier (*Ciona intestinalis*). *C. R. Soc. Biol. Paris* **153**, 1751-1757.

Salvatore, G. (1969). Thyroid hormone biosynthesis in Agnatha and Protochordata. *Gen. Comp. Endocrinol. Suppl.* **2**, 535-550.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). *Molecular cloning: A Laboratory Manual*, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY.

Sanger, F. and Coulson, A. R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J. Mol. Biol.* **94**, 441-448.

Satoh, N. (1982). DNA replication is required for tissue-specific enzyme development in ascidian embryos. *Differentiation* **21**, 37-40.

Satoh, N., Makabe, K. W., Katsuyama, Y., Wada, S. and Saiga, H. (1996). The ascidian embryo: an experimental system for studying circuitry for embryonic cell specification and morphogenesis. *Dev. Growth Differ.* **38**, 325-340.

Slack, J. M. W. (1994). Inducing factors in *Xenopus* early embryos. *Curr. Biol.* **4**, 116-126.

Smith, J. C. (1989). Mesoderm induction and mesoderm-inducing factors in early amphibian development. *Development* **105**, 665-677.

Whittaker, J. R. (1990). Determination of alkaline phosphatase expression in endodermal cell lineages of an ascidian embryo. *Biol. Bull.* **178**, 222-230.

Whittaker, J. R. (1977). Segregation during cleavage of a factor determining endodermal alkaline phosphatase development in ascidian embryos. *J. Exp. Zool.* **202**, 139-154.

Yasuo, H. and Satoh, N. (1993). Function of vertebrate T gene. *Nature* **354**, 582-583

Yasuo, H. and Satoh, N. (1994). An ascidian homologue of the mouse *brachyury (T*) gene is expressed exclusively in notochord cells at the fate restricted stage. *Dev. Growth Differ.* **36**, 9-18.

Yasuo, H. and Satoh, N. (1998). Conservation of the developmental role of *brachyury* in notochord formation in a urochordate, the ascidian *Halocynthia roretzi*. *Dev. Biol*. **200**, 158-170.