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A Novel G Protein α Subunit in Embryo of the Ascidian, *Halocynthia roretzi*

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ABSTRACT—A cDNA clone encoding a novel G protein α subunit, *HrGα$_n$* was isolated from the larvae of ascidian, *Halocynthia roretzi*. In contrast with overall amino acid identity (63%) with G protein α subunit of G_i or G_o subclass, HrGα$_n$ has a unique amino acid sequence, which lacks a residue for pertussis toxin substrate, but retains for cholera toxin substrate for ADP-ribosylation. The sequence characteristics and molecular phylogenetic analysis suggest that HrGα$_n$ defines a novel subclass within G_i class of G protein α subunits. The zygotic expression of *HrGα$_n$* was first detected at the 64-cell stage and observed in all blastomeres except for B7.4, B7.5 and B7.6 cells till the 110-cell stage. As progress of the developmental stages, the expression of *HrGα$_n$* became restricted and was observed in the muscle, mesenchyme and a part of trunk lateral cells in tailbud embryos. With HrGα$_n$-GFP fusion-gene construct it was showed that the genomic fragment containing 2674 bp upstream of the putative translation start site of *HrGα$_n$* contained the regulatory sequence responsible for the expression in the muscle and mesenchyme cells, and that the regulatory sequence functioned also in *Ciona intestinalis*. Our results suggest a possible involvement of HrGα$_n$ in the signaling system regulates the cell fate during the embryogenesis of the ascidian.

Key words: G protein, ascidian, *in situ* hybridization, GFP, 5'-flanking sequence

INTRODUCTION

Ascidians are lower chordates, but newly hatched tadpole larvae share a basic body plan with vertebrates. The close relationship between protochordates and the vertebrates, classically based on similarities in embryological development and the ascidian larval structural plan, has received recent support from molecular studies (Satoh and Jeffery, 1995; Corbo *et al*., 2001; Meinertzhagen and Okamura, 2001). During embryogenesis of the ascidian the cell cleavage pattern is invariant and the cell lineage is well characterized (Nishida, 1986, 1987; Nicol and Meinertzhagen, 1988). With these advantages, the ascidian embryo is an appropriate model system for studying the molecular mechanism of the embryonic cell specification, which includes a regulatory mechanism of a specific gene expression and a signal transduction system participating in the cell-cell interactions (Satoh, 2001; Jeffery, 2001; Nishida, 2002).

Here we focused our attention on heterotrimeric G proteins, which form a superfamily of signal transducing molecules from cell surface receptor to cytoplasmic effector (Hamm and Gilchrist, 1996; Hamm, 1998; Wilkie, 2000). The α subunits of G protein are primarily responsible for specificity to the coupling-effector molecule. They are divided into four classes (G_i, G_s, G_q and G_{12}) according to their amino acid sequence identity and the each class is composed of several subclasses; for example, G_i class is composed of G_i, G_t, G_o, and G_z subclasses. The expression pattern of G protein α subunit gene was studied in the early developmental stages in mouse (Allworth *et al*., 1990), *Xenopus* (Otte *et al*., 1992), and *Drosophila* (Wolfgang *et al*., 1991), and involvement of G proteins in embryogenesis was suggested. The Wnt pathway is one of the major signaling systems, which control the development of tissues and organs (Kuehl *et al*., 2000, Huelsken and Birchmeier, 2001). Frizzleds, cell surface Wnt-receptors, were considered as members of the G protein-coupled receptors

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Note: The nucleotide sequences reported in this paper have been deposited in the DDBL/EMBL/GenBank databases under the accession numbers AB022098 and AB083069

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(GPCR), because they possess seven transmembrane spanning domain. Recently it was established that Frizzleds are really members of GPCR and couple with G protein (Wu *et al*., 2000; Liu *et al*., 2001). In embryos of *Ciona savignyi* nuclear accumulation of β-catenin, an essential component of the Wnt signaling pathway, was reported as a most probable first step of endodermal cell specification and one of the genes downstream of β-catenin was found to be responsible for the endoderm differentiation (Imai *et al*., 2000; Satou *et al*., 2001).

In this paper, we describe the isolation of *HrGα$_n$*, a cDNA encoding a novel G protein α subunit, from cDNA library of tadpole larvae of a solitary ascidian, *Halocynthia roretzi*. Based on the comparison of the deduced amino acid sequence and molecular phylogenetic analysis, we concluded that HrGα$_n$ defines a novel subclass within G protein α subunits of G$_i$ class. The spatio-temporal expression pattern was studied by northern blot analysis and whole-mount *in situ* hybridization. The genomic fragment containing the regulatory sequence responsible for the site-specific expression in tailbud embryos was obtained from *H. roretzi*, and was shown to function also in *Ciona intestinalis*. Based on these findings, a possible involvement of HrGα$_n$-signaling in embryogenesis of the ascidian was discussed.

MATERIALS AND METHODS

Animals, Eggs and Embryos

Halocynthia roretzi were purchased from fishermen at Wakkanai, Hokkaido, Japan, and transported to the Cell Biology Center of Himeji Institute of Technology, where animals were kept at 5°C under the constant illumination until use. Naturally spawned eggs were collected with a mesh of an appropriate pore size (200 μm), were fertilized with a suspension of non-self sperm and were incubated in artificial seawater (Aquamarine, Senju, Osaka, Japan) at 13°C. At this temperature, embryos developed into 64-cells, gastrulae, and early tailbud stages about 6, 10, and 20 hr after fertilization, respectively. At 35 hr after fertilization tadpole larvae hatched. Removal of the chorion was done mechanically with sharpened tungsten needles.

Ciona intestinalis were collected from several locations of Aioi-bay, Hyogo, Japan. Handling of eggs and embryos were according to Nakagawa *et al*. (1999).

Polymerase Chain Reaction (PCR)

Degenerated PCR primers were designed according to the conserved amino acid motifs of G protein α subunits. A forward primer was directed against the C-domain corresponding to amino acid sequence KKWI(H/Q)CF and a reverse primer, G-domain corresponding to amino acid sequence LFLNKKD (GF22 and GR22, respectively) (Iwasa *et al*., 2000, 2001). Polymerase chain reactions were performed with 1 μl aliquots of cDNA library of tadpole larvae of *H. roretzi* (λZAPII, Stratagene, La Jolla, USA; kind gift from Dr. N. Satoh, Kyoto University) in 15 μL reactions. PCR products of the expected size (*ca*. 200 bp) were subcloned in pT7blue vector (Stratagene, La Jolla, USA). Nucleotide sequences were determined for both strands with the primer-labeled cycle sequencing kits (Amersham Pharmacia Biotech, Uppsala, Sweden) and an automatic DNA sequencer (Shimadzu DSQ 1000L, Shimadzu, Kyoto, Japan).

Isolation of cDNA clones

A cDNA library of tadpole larvae of *H. roretzi* was screened with random primed ^{32}P-labeled PCR-HrGα (Fig. 1A) probe (Megaprime DNA labeling system, Amersham Pharmacia Biotech, Uppsala, Sweden) by hybridization in 45% formamide, 6 × SSC, 1% SDS, 5 × Denhardt's solution, 20 μg/ml denatured salmon sperm DNA at 42°C overnight. Afterwards, the filters were washed twice in 2 × SSPE, 0.1% SDS for 10 min at RT, and twice in 1 × SSPE, 0.1% SDS for 15 min at 65°C. Among 1.0×10^5 plaques we obtained only one positive plaque containing HrGα-1 (Fig. 1A), which lacks C-terminal sequence. We further screened the cDNA library (1.2×10^5 plaques) with HrGα-1 as a probe, and obtained a new positive plaque containing HrGα-2 (Fig. 1A).

Northern blot analysis

Total RNA was prepared with standard CsCl-ultracentrifugation methods (Ausubel *et al*., 1987) from eggs or embryos of the desired stage. The total RNA (10 μg) was separated on 0.8% agarose-formamide gel and transferred to nylon membrane (Hybond N$^+$, Amersham Pharmacia Biotech, Uppsala, Sweden). The membrane was cross-linked by UV cross-linker (CL-1000 Ultra Violet Cross Linker, UVP, San Gabriel, USA). Hybridization was carried out by the same procedure as described under "Isolation of cDNA clones", with ^{32}P-labeled HrGα-3' probe, *Hin*c II - *Pst* I fragment of HrGα-2 (Fig. 1A). The washed membranes were analyzed with an imaging plate (BAS 2000, Fuji Film, Tokyo, Japan).

Southern blot analysis

Chromosomal DNA from sperm of *H. roretzi* was prepared according to a standard procedure (Davis *et al*., 1986). Ten μg of the genomic DNA was digested with *Eco* RI, *Bam* HI, *Hin*d III or *Pst* I, electrophoresed on 0.7% agarose gel, and then transferred to nylon membranes. The membranes were hybridized, washed as described under "Isolation of cDNA clones", and then analyzed with an imaging plate (BAS 2000, Fuji Film, Tokyo, Japan).

In situ hybridization

Whole-mount specimens at various developmental stages of *H. roretzi* were hybridized *in situ* using Digoxigenin (DIG) -labeled antisense and sense RNA probes (DIG RNA labeling Kit, Roche, Japan). The procedures were mainly according to Yasuo and Satoh (1994).

Cloning of 5'-flanking sequence of *HrGα$_n$*

The genomic library of *H. roretzi* (kind gift from Dr. N. Satoh, Kyoto University) was screened with ^{32}P-labeled HrGα-1 probe using standard procedures (Davis *et al*., 1986). The phage DNA was prepared from the cloned phage and used as a template for the following PCR amplification. The 5'-flanking sequence of *HrGα$_n$* was obtained with PCR using a combination of a gene-specific (CTTCATAGTCGATTCCGAGC; nt 354-373 for *HrGα$_n$*) and a vector-specific primer (T3 or T7 primer, Toyobo, Osaka, Japan). PCR was performed for 30 cycles of 1 min template denaturation at 95°C, 1 min primer annealing at 50°C, 5 min primer extension at 72°C with Expand High Fidelity PCR system (Roche, Tokyo, Japan). The longest PCR fragment (ca. 6.0 kb) was cloned into pT7blue vector (Stratagene, La Jolla, USA) (Gn6.0-pT7b) and the nucleotide sequence was determined in both strands with a series of deletion products (Deletion Kit for Kilo-Sequence, Takara, Kusatsu, Japan). A 3.5 kb fragment was also cloned into pT7blue vector (Gn3.5-pT7b) and was used for preparation of GFP-fusion construct.

Fusion gene construct and microinjection

Fusion construct was prepared using Gn3.5-pT7b plasmid, which contains 2674 bp upstream and 244 bp downstream sequence from the putative translation start-site of *HrGα$_n$*. Gn3.5-pT7b was double-digested with *Sac* I and *Bam* HI, and then fused with a DNA fragment encoding GFP (a *Sac* I-*Bam* HI fragment of

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pAcPr-GFP, kind gift from Dr. Y. Okamura, NIPS). The resulted fusion construct was pGn3.5-GFP. The *Sac* I-*Bam* HI fragment of the pAcPr-GFP was cloned into pT7blue vector and the construct, (-)-GFP, was used as the reference. Microinjection of the fusion construct into fertilized eggs of *H. roretzi* was performed according to Takahashi *et al*. (1999). In the case of *C. intestinalis*, the fusion construct was electroporated into fertilized eggs according to Corbo *et al*. (1997). The expression of *GFP* was observed by fluorescent-stereomicroscopy (SZX-12, Olympus, Tokyo, Japan).

RESULTS

Isolation of cDNA clones encoding HrGα$_n$

We obtained a cDNA fragment PCR-HrGα showing high similarity (76%) in the deduced amino acid sequence to that of rat G protein α subunit of G$_i$ class (Fig. 1A). Two cDNA inserts, HrGα-1 and HrGα-2 were obtained by screening of a cDNA library of tadpole larvae of *H. roretzi* as described in Materials and Methods. HrGα-1 (927 bp) lacked C-terminal sequence. HrGα-2 (2397 bp) contained C-terminal and 3'-noncoding sequence with poly(A) tract, which overlapped 612 bp with HrGα-1. An open reading frame encoding 357 amino acids (calculated molecular mass: 40532 Da.) was deduced from the nucleotide sequences of HrGα-1 and -2. Three possible polyadenylation signals (AATAAA) are found at 1626-1631, 2043-2048 and 2629-2634. The nucleotides at 1895-1992 (TTTTTAT) are the sequence that activates polyadenylation of messages during maturation in *Xenopus* oocyte (Fox *et al*., 1989).

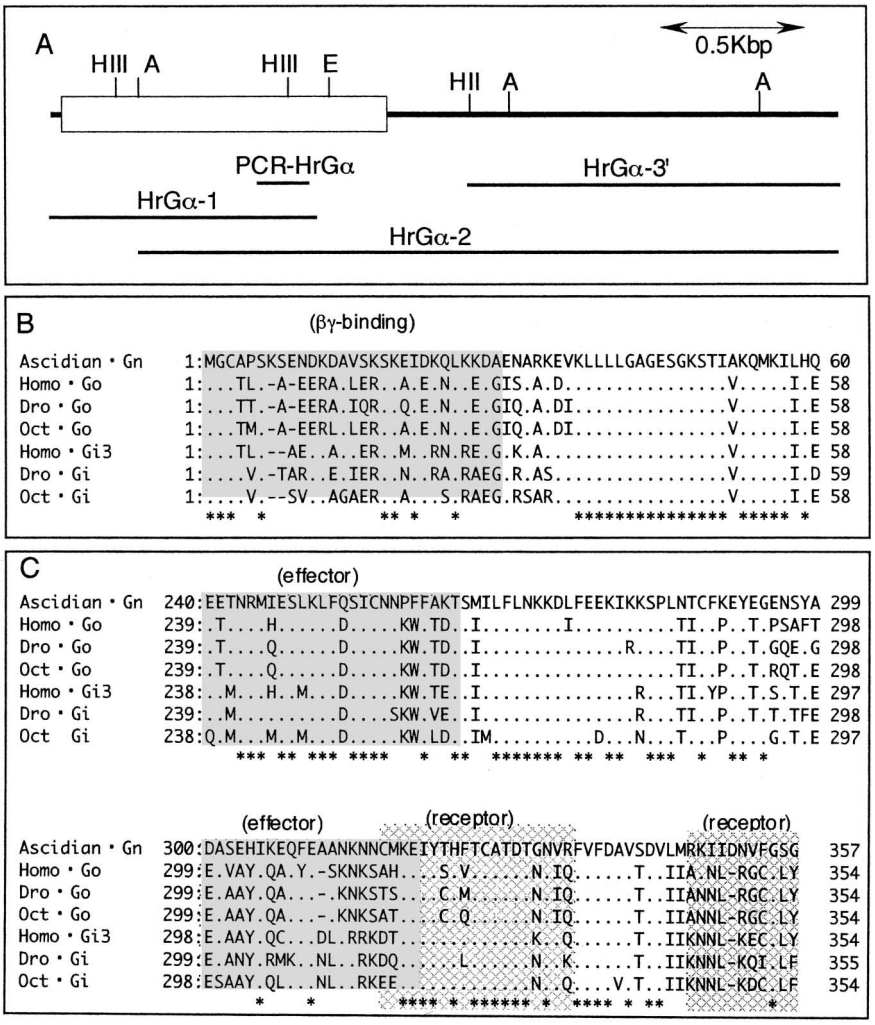

Fig. 1. Molecular characterization of HrGα$_n$. (A): Schematic representation and restriction map of *HrGα$_n$*. Open box represents protein-encoding region. Straight line represents 5' and 3'-untranslated sequence. The PCR clone, PCR-HrGα, and two cDNA clones, HrGα-1 and HrGα-2, are shown underneath. HrGa-3' is the *Hin*c II - *Pst* I fragment of the 3'-noncoding region of *HrGα$_n$* and was used as a probe for the northern blot analysis. A; *Acc* I, E; *Eco* RI, HII; *Hin*c II, HIII; *Hin*d III. (B) and (C): Comparison of the amino acid sequence of N-terminal region (B) and that of C-terminal region (C) of HrGα$_n$ with that of G protein α subunits of G$_o$ and G$_i$ subclass of human (Homo), *Drosophila* (Dro) and *Octopus* (Oct). Dots represent identical amino acids, and dashes, introduced gaps. The asterisks indicate the conserved amino acids among all sequences. The location of interaction sites with Gβγ subunit or effector, or receptor are indicated above the sequences. Sources of sequence information: Human G$_o$ (M60162), G$_{i3}$ (J03238), *Drosophila* G$_o$ (M29731), G$_i$ (M23094), *Octopus* G$_o$ (AB025781) and G$_i$ (AB025780). The nucleotide sequence of *HrGα$_n$* is available at DDBJ/EMBL/GenBank with an accession number AB022098.

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The well-conserved amino acid motifs of G protein α-subunit, domains A, C, and G of the guanine nucleotide-pocket were found in the deduced amino acid sequence. The A domain is a binding site of phosphate of guanine nucleotide (Dever *et al.*, 1987). The consensus sequence GXXXSGKS is found G42 - S49. The G domain, switch II region, undergoes a conformational change upon binding GTP (Mazzoni and Hamm, 1993). The consensus sequence (DVGGQR) is found at D202 - R207. The C domain stabilizes the guanine base of GTP through hydrogen bonds to guanine ring nitrogen (Gupta *et al.*, 1992). The deduced amino acid sequence of L268 - D274 is identical to the consensus sequence LFLNKXD. Thus, the cDNA clone is possibly translated as a functional G protein α subunit. We tentatively designated the G protein α subunit of ascidian larvae as HrGα_n (*Halocynthia roretzi* G protein α subunit in n subclass).

HrGα_n defines a novel subclass within G protein α subunits of G$_i$ class

Although the amino acid sequence of the guanine nucleotide pocket of HrGα_n was highly conserved, overall amino acid sequence exhibited a rather low degree of identity with any classes of the human G protein α subunit (63–42%). It showed the highest similarity to G protein α subunits of G$_i$ class, Gα_i. The candidate sequence for N-terminal myristoylation (MGxxx(S/T)), characteristic for Gα_i (Chen and Manning, 2001), was conserved in N-terminal region of HrGα_n, indicating that the HrGα_n belongs to Gα_i class (Fig. 1B).

Detailed comparison of amino acid sequences among those belong Gα_i suggests that HrGα_n should be classified in a novel subclass within Gα_i class on the following criteria. At first, almost all G protein α subunits of G$_i$ class are substrates of pertussis toxin; a cystein, four residues away from the C-terminus, is ADP-ribosylated. HrGα_n lacked the cystein residue at this position. An arginine residue for a possible substrate of another bacterial toxin, cholera toxin, was present in HrGα_n (R180). Additionally, the sequence of C-terminal 13 amino acids of HrGα_n was completely different from that of any other members of Gα_i class (G$_i$, G$_o$, G$_t$, and G$_z$ subclass) (Fig. 1C).

To investigate evolutionary relationships between HrGα_n and known metazoan Gα isoforms, phylogenetic tree was constructed with the neighbor-joining method (Saitou and Nei, 1987)(Fig. 2). The phylogenetic tree reveals that

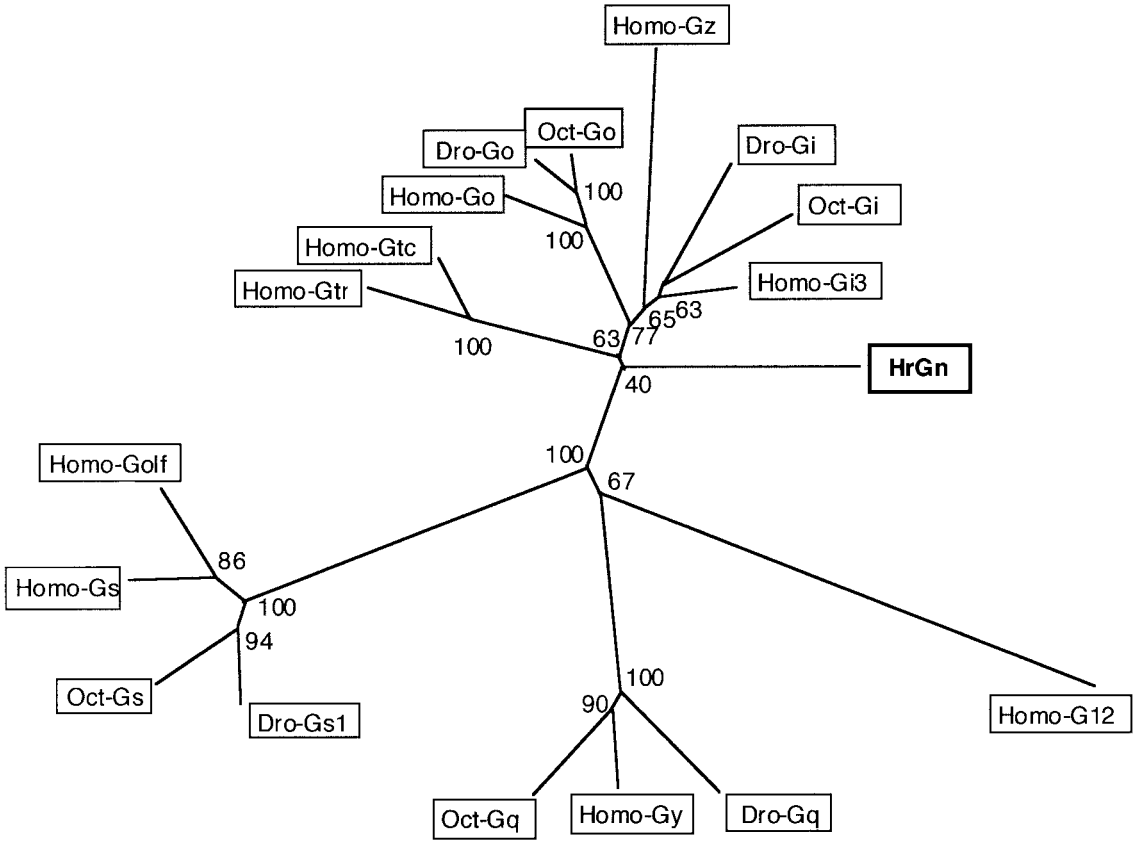

Fig. 2. A molecular phylogenetic tree constructed by the neighbor-joining method (Saitou and Nei, 1987). Numbers at each branch indicate the percentage of times a node was supported in 1,000 bootstrap psuedoreplications by the neighbor-joining method. Sources of sequence information: Human (Homo) G$_o$ (M60162), G$_{i3}$ (J03238), G$_{tr}$ (X15088), G$_{tc}$ (D10384), G$_z$ (J03260), G$_{12}$ (L01694), G$_y$ (M69013), G$_s$ (M21142), G$_{olf}$ (L10665), *Drosophila* (Dro) G$_o$ (M29731), G$_i$ (M23094), G$_q$ (U31092), G$_{s1}$ (M23233), *Octopus* (Oct) G$_o$ (AB025781), G$_i$ (AB025780), G$_q$ (AB025782), and G$_s$ (AB025783).

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HrGα_n is most closely related to Gα_i and that it should be a member of Gα_i class. Within the Gα_i class, however, HrGα_n was fairly diverged from any subclasses, G_i, G_o, G_t, and G_z.

Southern blot analysis of the ascidian genomic DNA

Southern blot analysis of ascidian genomic DNA was performed with HrGα-1 as a probe. As shown in Fig. 3A, the HrGα-1 probe detected a single band in the genomic DNA digested with *Bam* HI, *Eco* RI, or *Hin*d III. In the digests with *Pst* I the main band was observed at about 15 kb and an additional weak band was observed at about 1.9 kb. Judging from these results, it is very likely that *HrGα_n* is a single copy gene.

Spatio-temporal expression pattern of the *HrGα_n* transcripts

To examine the temporal expression of *HrGα_n* during ascidian embryogenesis, northern blot analysis was performed with eight different developmental stages from the unfertilized egg up to the tadpole larva with ^{32}P-labeled HrGα-3' as a probe. The signal of *HrGα_n* was observed at about 3.2 kb in the unfertilized egg indicating presence of maternal mRNAs of *HrGα_n* (lane 1 in Fig. 3B). The signal became slightly weaker as the progress of development until the 64-cell stage. At the 64-cell stage, the transcripts gave more clear signal (lane 5 in Fig. 3B) and intensity of the signal increased in the later stages, indicating the zygotic expression starts from the 64-cell stage up to tadpole larvae (lanes 5 - 8 in Fig. 3B). After an overnight exposure of the hybridized membrane to the BAS analyzer, two additional bands of 2.5 and 1.8 kb were detected after gastrula stage (data not shown). These three types of mRNAs different in size probably correspond to three possible polyadenylation sites found in HrGα-2. The expression of *HrGα_n* in the adult tissues was also investigated (Fig. 3C). The clear signal of *HrGα_n* was detected at about 3.2 kb in all tissues examined. The expression in the branchial sac, ganglion and intestine were rather higher than in stomach and ovary. Thus, *HrGα_n* is expressed in both larvae and adult tissues.

The spatial distribution of expression of *HrGα_n* was investigated in developing embryos with whole-mount *in situ* hybridization. The maternal mRNAs of *HrGα_n* were detected evenly in the entire region of the cytoplasm of the unfertilized egg (data not shown). As shown in Fig. 4A - D, no obvious difference in the signal was observed in each blastomere in the 16-cell and 32-cell stage, indicating that the maternal mRNAs were divided evenly to each blastomere.

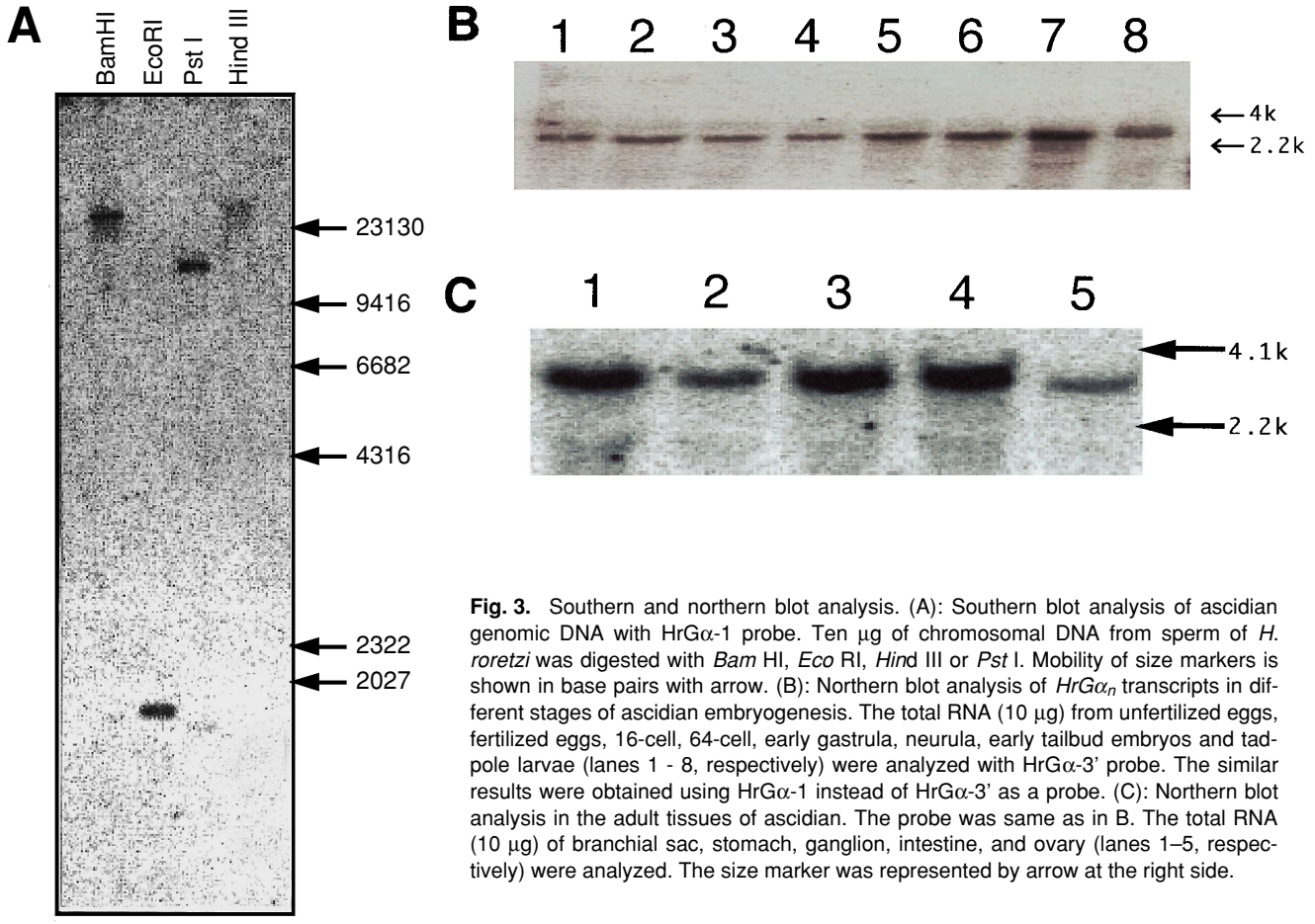

Fig. 3. Southern and northern blot analysis. (A): Southern blot analysis of ascidian genomic DNA with HrGα-1 probe. Ten μg of chromosomal DNA from sperm of *H. roretzi* was digested with *Bam* HI, *Eco* RI, *Hin*d III or *Pst* I. Mobility of size markers is shown in base pairs with arrow. (B): Northern blot analysis of *HrGα_n* transcripts in different stages of ascidian embryogenesis. The total RNA (10 μg) from unfertilized eggs, fertilized eggs, 16-cell, 64-cell, early gastrula, neurula, early tailbud embryos and tadpole larvae (lanes 1 - 8, respectively) were analyzed with HrGα-3' probe. The similar results were obtained using HrGα-1 instead of HrGα-3' as a probe. (C): Northern blot analysis in the adult tissues of ascidian. The probe was same as in B. The total RNA (10 μg) of branchial sac, stomach, ganglion, intestine, and ovary (lanes 1–5, respectively) were analyzed. The size marker was represented by arrow at the right side.

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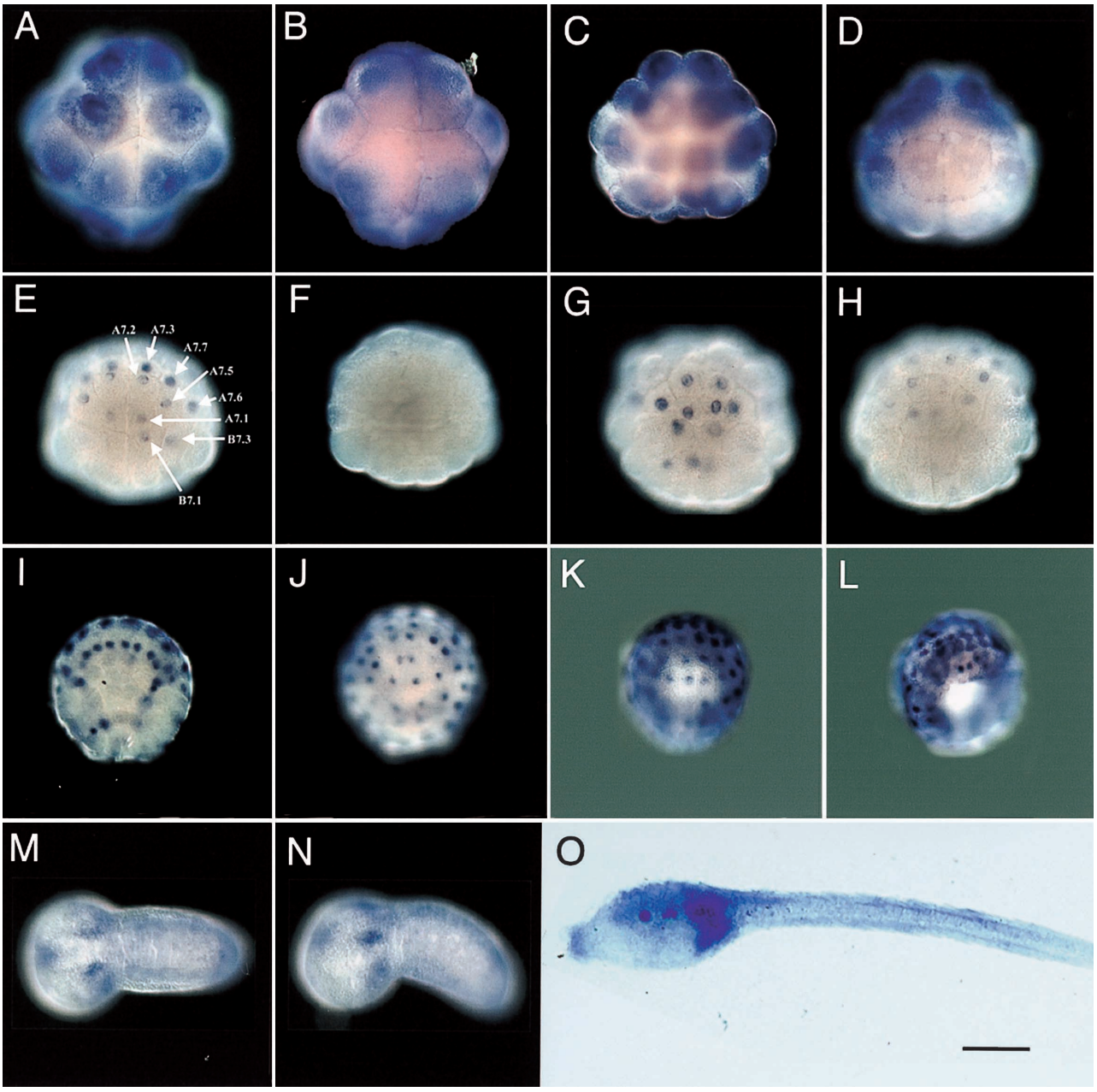

Fig. 4. Whole-mount *in situ* hybridization of developing embryos with a Digoxigenin-labeled antisense probe for *HrG*α_n message. (A) and (B): A 16-cell embryo (animal view and vegetal view, respectively). (C) and (D): A 32-cell embryo (animal view and vegetal view, respectively). (E) and (F): A 64-cell embryo (animal view and vegetal view, respectively). (G) and (H): A 76-cell embryo (animal view and vegetal view, respectively). (I) and (J): A 110-cell embryo (animal view and vegetal view, respectively). (K) and (L): Gastrula (animal view and vegetal view, respectively). (M) and (N): Early tailbud embryo, dorsal and lateral view, respectively. (O): A tadpole larva. Scale bar=100 μm.

The observed temporal expression patterns of *HrG*α_n from the 64-cell to 110-cell stage were summarized in Fig. 5. At the 64-cell stage embryo, zygotic expression of *HrG*α_n was first detected in the nuclei of all A-line cells and two B-line cells (B7.1 and B7.3) (Fig. 4E and F). No signal was observed in a- and b-line cells at this stage. In the later developmental stages (the 76-cell and 110-cell stage), the expression was observed in all A- and a-line cells except for A7.1 and A7.2 cells, which showed the signal at the 64-cell and 76-cell stage and did not at the 110-cell stage. The daughter cells of A7.3, A7.4, A7.7 and A7.8 cells did not showed the expression signal at the 76-cell stage, but showed at the 110-cell stage. In the B-line cells, only four pairs of blastomeres showed the signal during the stages. The B7.1 and B7.2 cells showed the expression signal at the 64-cell and/or 76-cell stage, but not at the 110-cell stage. They develop into endoderm or endodermal strand. The B7.3 cell showed the expression at the 64-cell stage, but two daughter cells did not at the 76-cell stage. At the 110-cell stage, one of the daughter cell, the B8.5 cell, showed

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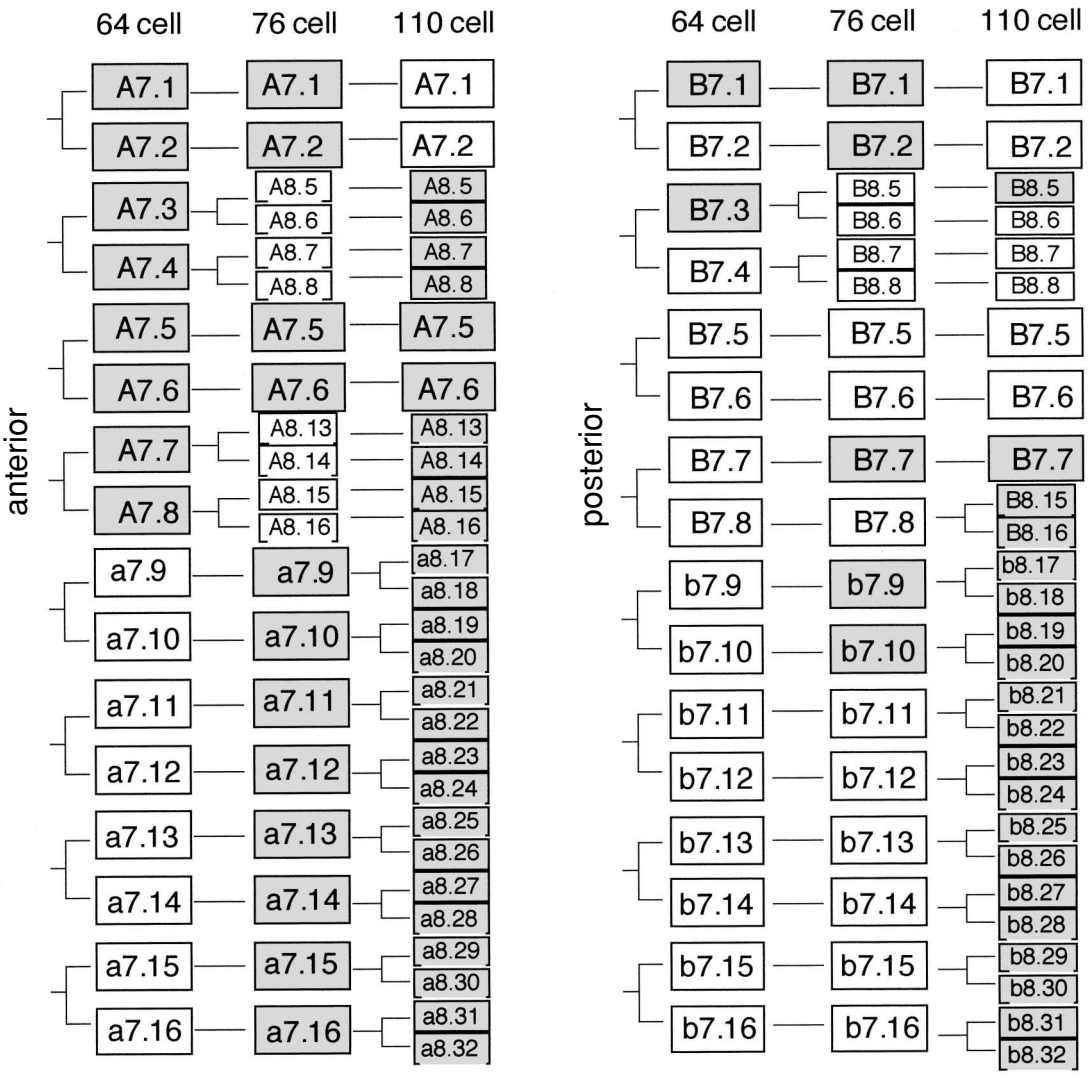

Fig. 5. Zygotic expression of *HrGα_n* at the 64-cell, 76-cell and 110-cell stage as summarized referring to the cell lineage. The names of blastomeres where the expression of *HrGα_n* was detected are presented in shaded boxes. The cell lineage is according to Nishida (1987).

the expression, but another daughter cell, the B8.6 cell, did not. Their cell fate was determined at this stage. The B8.5 cell develops into the mesenchyme and the B8.6 cell develops into the notochord. The B7.7 cell showed the expression at the 76-cell and 110-cell stage. The B7.7 cell develops into mesenchyme. The other cells (B7.4, B7.5, B7.6 and B7.8 cells) and their descendants did not show the expression at these stages. They give rise to muscle, endoderm or endodermal strand. The expression in the b-line cells was observed at the 110-cell stage. Exceptions were the case in b7.9 and b7.10 cells (containing the muscle, spinal cord, endodermal strand, epidermis and brain stem lineage cells), which showed expression in both the 76-cell and 110-cell stage.

In the early tailbud stage, the expression was detected in the muscle cells of the tail and the ventral portion of the mesenchyme. A weak expression was observed in a dorsal region of the trunk, some of trunk lateral cells and also several cells of the brain stem (Fig. 4M and N). In a tadpole larva, a strong expression of *HrGα_n* was observed in mesenchyme cells of the posterior trunk region and a weak expression was observed in a dorsal region of the trunk (Fig. 4O).

The 5'-flanking sequence of *HrGα_n*

In order to analyze the regulatory sequence responsible for observed spatio-temporal expression of *HrGα_n*, we isolated the 5'-flanking sequence of *HrGα_n* from a genomic library of *H. roretzi*. The longest PCR fragment (Gn6.0-pT7b) contains 5478 bp upstream of the putative translation start site and 244bp downstream (Fig. 6A). The possible ORFs longer than 200 bp were searched in the upstream sequence. Seven ORFs (201–339 bp) were found in *cis*-direction to *HrGα_n* and four (201–339 bp) in *trans*-direction. The amino acid sequences similar to those deduced from the putative ORFs were searched against GenBank database and we did not find out any sequence with high similarity. The results suggest that there is no gene in the

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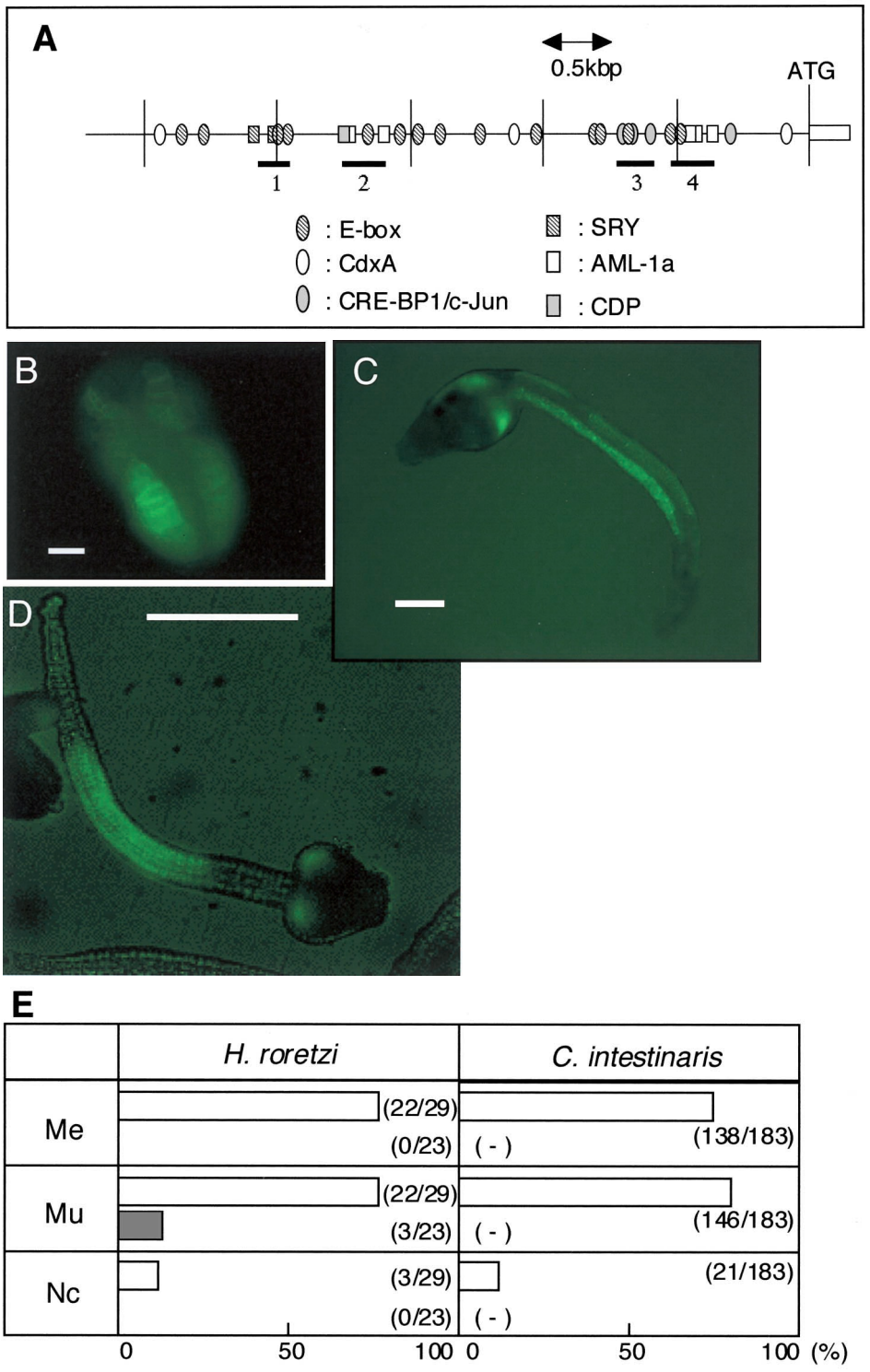

Fig. 6. (A): Schematic representation of sequence motifs in the 5'-flanking sequence of *HrGα$_n$* gene. Putative binding sites for transcription factors were searched against the TRANSFAC database using TRANSFAC Find (Heinemeyer *et al.*, 1999) and also with Genetyx Mac (Genetyx, Tokyo, Japan). The thick lines under the sequence indicate the clusters of the potential binding motif(s) and E-box motif. (B) and (C): Expression of *HrGα$_n$-GFP* fusion genes. pGn3.5-GFP was injected in fertilized egg of *H. roretzi*. The expression of *GFP* in the early tailbud embryo (B) and the hatched larva (C). (D): 3.5Gn-GFP was introduced via electroporation into fertilized eggs of *Ciona intestinalis*. GFP expression was observed in muscle and mesenchyme cells of hatched larva. Scale bar; 100 μm. (E): Frequency of embryos with GFP expression, which developed from eggs carrying *HrGα$_n$-GFP* fusion constructs. The sites of the expression were shown on the left (Me; mesenchyme, Mu; muscle, Nc; notochord). Open boxes represent the results of 3.5Gn-GFP. The shaded box represents the result of injection of (–)-GFP. Numerals in parentheses indicate number of positive embryos relative to total embryos examined. (–); Not tested. The nucleotide sequence of the 5'-flanking sequence of *HrGα$_n$* gene is available at DDBJ/EMBL/ GenBank with an accession number AB083069.

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upstream sequence of 5478 bp.

The sequence motifs of the potential binding site for transcription factors in the 5'-flanking sequence were searched against the TRANSFAC database (Heinemeyer *et al*., 1999). The results were summarized in Fig. 6A. We found 15 E-box motifs (CANNTG) at -4713, -4529, -4022, -3909, -3359, -3094, -2944, -2795, -2444, -2072, -1792, -1609, -1404, -1013, and -990 bp from the putative translation start site of *HrGα_n*. There were four clusters of the potential binding motif(s) and E-box motif. We tentatively designated them as Cluster 1–4 (thick lines in Fig. 6A). Cluster 1 (-4106 to -3909) consisted of two SRY motifs (-4160 and -4004) and two E-box motifs (-4022 and -3909). Cluster 2 (-3482 to -3203) consisted of one CDP motif (-3482), two AML-1a motifs (-3445 and -3203) and an E-box motif (-3359). AML-1a motif is a potential binding site of AML-1a protein, a central 118 amino acid domain of which shows 69% homology to the *Drosophila* pair-rule gene, *runt* (Meyers *et al*., 1993). CDP motif is a potential binding site of Cut-like homeodomain protein (Harada *et al*., 1995). Thus, Cluster 2 consisted of potential binding sites for proteins known to regulate cellular development. Cluster 3 (-1404 to -1180) consisted of two CREB motifs (-1310 and -1180), a CRE-BP1/c-Jun motif (-1403) and an E-box motif (-1404). Cluster 4 (-1013 to -729) consisted of two E-box motifs (-1013 and -990) and three AML-1a motifs (-883, -863 and -729). There were three CdxA motifs at -4880, -2235 and -148bp, but they do not form a cluster with the other motifs.

Expression of the fusion construct in embryos of *H. roretzi* and *C. intestinalis*

To analyze the regulatory activity of the 5'-flanking region of *HrGα_n*, we constructed a fusion gene of the 5'-flanking sequence of *HrGα_n* and the DNA fragment encoding GFP, and injected them into fertilized eggs of *H. roretzi*. The construct, pGn3.5-GFP, contains 2674 bp upstream of the putative translation start site and 244bp downstream of *HrGα_n*. The upstream-sequence of 2674 bp directs expression of *GFP* gene in a pattern that is virtually identical to the endogenous gene. The fluorescence from the GFP was first observed in the initial tailbud stage (Fig 6B), suggesting the expression in the earlier stages was too weak to detect the fluorescence. The fluorescence from GFP was observed primarily in the muscle and mesenchyme cells (Fig. 6B and C). In a few embryos, the expression of *GFP* was observed in the notochord or posterior sensory vesicles (Fig. 6C). The injection of the fusion construct without the 5'-flanking sequence of *HrGα_n* ((–)-GFP) resulted in few expression of *GFP* with three exceptions observed in the muscle cells (Fig. 6E, left).

The electroporation of the pGn3.5-GFP into the fertilized eggs of *C. intestinaris* resulted in the expression of *GFP* in the muscle and mesenchyme cells (Fig. 6D). The expression pattern was same as in *H. roretzi*. Small numbers of the embryos showed an ectopic expression of *GFP* in the notochord or brain stem (Fig. 6E, right).

DISCUSSION

In this study, we described a novel G protein α subunit, HrGα_n, of ascidian tadpole larvae. The deduced amino acid sequence of *HrGα_n* (357 amino acids) showed higher similarity to human G_i and G_o. There was a candidate sequence for palmitoylation in the N-terminal region of HrGα_n, which is conserved in Gα_i (Chen and Manning, 2001). The phylogenetic tree constructed based on the deduced amino acid sequence clearly showed that HrGα_n is a member of Gα_i. However, HrGα_n was unique among those belong to Gα_i in the following criteria. First of all, the deduced amino acid sequence of HrGα_n lacked the cysteine residue in its C-terminal region. The cysteine residue is a substrate of ADP-ribosylation by pertussis toxin, which is conserved in almost all Gα_i. HrGα_n, however, possessed an arginine residue positioned at 180 (R180), which is a possible substrate of cholera toxin, and ADP-ribosylated in Gα_t and Gα_s. Second, the amino acid sequence of C-terminal region was extensively different from that of the other Gα_i (Fig. 1C). Third, HrGα_n was fairly diverged from any other members of Gα_i in the phylogenetic tree (Fig. 2). Based on these results we concluded that HrGα_n defines a novel subclass in G protein α subunits of G_i class.

The zygotic expression of *HrGα_n* was first observed at the 64-cell stage. From the 64-cell to 110-cell stage, a transient expression of *HrGα_n* was observed in all cells except B7.4, B7.5 and B7.6 cells. An interesting expression pattern of *HrGα_n* was observed in the case of the B7.3 cell. The B7.3 cell showed expression of *HrGα_n* in the 64-cell stage, but two daughter cells did not in the 76-cell stage. At the 110-cell stage, one of the daughter cell, the B8.5 cell, showed the expression, but another daughter cell, the B8.6 cell, did not. Their cell fate was determined at this stage. The B8.5 cell develops into the mesenchyme and the B8.6 cell develops into the notochord. The results suggest a possibility that expression of *HrGα_n* is related to determination of their cell fate. Kim and Nishida found that formation of mesenchyme cells requires cellular interaction that suppresses muscle fate in the mesenchyme precursor blastomeres (Kim and Nishida, 1999). They further mentioned about two possibilities about the formation of mesenchyme cells. One is that suppression of muscle fate is enough to allow cells to choose a mesenchyme pathway and another is that suppression of the muscle fate and promotion of mesenchyme formation are distinct processes. If the latter is the case, it seems likely that *HrGα_n* is involved in promotion of mesenchyme formation. Concerning about this issue the expression pattern of *HrGα_n* in daughter cells of the B6.4 cell should be noted. The B7.8 cell, a daughter cell of the B6.4 cell, gives rise to muscle cells and another daughter cell (B7.7), to mesenchyme cells. The expression of *HrGα_n* was not observed in the B7.8 cell, but observed in the B7.7 cell in the 76-cell and 110-cell stage.

The expression of *HrGα_n* in the mesenchyme cells con-

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tinued and became stronger in the tadpole larva (Fig.4M–O). In tadpole larvae the strong expression was also observed in trunk lateral cells (TLC). The TLC is one of the mesodermal tissues of a larva and categorize as a kind of embryonic mesenchyme cell (Satoh, 1994). The A7.6 cell, only one TLC precursor cell, showed expression of *HrGα$_n$* from the 64-cell to 110-cell stage. These results showed that expression of *HrGα$_n$* was observed in mesenchyme and their precursor cells, suggesting another possible role of HrGα$_n$, maintenance of mesenchyme cells. The physiological meaning of the transient expression in the other cells from the 64-cell to 110-cell stage is remained to be elucidated.

The expression of pGn3.5-GFP in embryos of *H. roretzi* was observed in a pattern that is virtually identical to the endogenous gene, in the muscle and mesenchyme cells in the early tailbud stage. The results indicate that the upstream-sequence of 2674 bp contains a regulatory sequence responsible for expression of *HrGα$_n$* in the muscle and mesenchyme cells. Araki and Satoh investigated the regulatory sequence for muscle specific expression of *HrMHC1* (*H. roretzi* muscle myosin heavy-chain) gene, compared it with that of *HrMA4* (*H. roretzi* muscle actin) gene and found several common motifs between them (Araki and Satoh, 1996). They were Box A (TACGAAT), E-box/Box B (CAGTTGCGC), GATA binding site (TGATAG), and Box T1/T2 (Box T; TTTTTTCTTTCA) and their results suggest that the BoxT1/T2 is critical for the promoter activity of the *HrMHC1* gene. Because the expression of pGn3.5-GFP was observed in the muscle cells, we searched in the 5'-flanking sequence contained in pGn3.5-GFP for these motifs and found one GATA binding site (TGATAA) at -721 and one Box T1 (TTTTTTC) at -2347 in *HrGα$_n$*. The box T1 found in the upstream region of *HrGα$_n$* was not so proximal as the Box T1/T2 found in the upstream region of *HrMHC1* gene. In more upstream region than that contained in pGn3.5-GFP two potential GATA binding site (WGATAR) existed in Cluster 2 (-3482 to -3203). They were overlapped in the sequence, AGATAGATAG, between -3260 and -3251. Thus, the regulatory mechanism for expression in the muscle cells of the *HrGα$_n$* gene would be rather different from that of the *HrMHC1* gene. The muscle-expression of pGn3.5-GFP was also observed in electroporated embryos of *C. intestinalis*, suggesting that the regulatory mechanism seems common in *H. roretzi* and *C. intestinalis*. In the case of notochord specific expression, p(-289)As-T/lacZ, a construct of the 5'-flanking region of *As-T* gene of *H. roretzi* and the reporter gene (*lacZ*), showed notochord specific expression in *Ciona* embryos (Takahashi *et al*., 1999). However, the minimal promoter system for the notochord-specific expression seems different between *As-T* and *Ci-Bra*. The present results suggest that the upstream-sequence of 2674 bp of *HrGα$_n$* contains a regulatory sequence responsible for expression of *GFP* gene in the muscle and mesenchyme cells in *H. roretzi* and also in *C. intestinalis*. The minimal promoter system for *HrGα$_n$* expression should be elucidated for more detailed consideration.

Four clusters of the sequence motifs of the potential binding site for transcription factors were found in the 5'-flanking sequence of *HrGα$_n$* (Fig. 6A). Two of them, Clusters 2 and 4 consisted of sequence motifs for AML-1a, CDP, E-box and/or GATA. Cluster 3 consisted of sequence motifs for CREB and E-box. Cluster 1 consisted of sequence motifs for CDP and E-box. The homologues of the transcription factors possibly bind to these sequence motifs are found in the EST library of *C. intestinalis* (Satou *et al*., 2002), suggesting that these transcriptional factors are involved in regulation of gene expression in *C. intestinalis*. Whether or not these transcriptional factors function in *H. roretzi* and regulate the expression of *HrGα$_n$* gene is remained to be elucidated.

The clarification of the physiological function of HrGα$_n$, its effector(s) and receptor(s) is also remained to be elucidated. An *HrGα$_n$*-homologue was recently found from planarian central nervous system, which is specifically expressed in the distal part of brain lateral branches (Cebria *et al*., 2002). Unfortunately the physiological role of the gene product was not reported and the homologue belongs to the same subclass is not found yet from the other animals. Thus, we cannot expect physiological function of HrGα$_n$ by analogy. On this issue it should be noted that the amino acid sequence of HrGα$_n$ was quite unique in its C-terminal region, which is a possible interaction site of G protein α subunit with its receptor molecule (Hamm *et al*., 1988; Nishimura *et al*., 1998; Gilchrist *et al*., 2002). It can be expected that HrGα$_n$ couple with a novel receptor.

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