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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, HrGαn, 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, HrGαn 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 HrGαn defines a novel subclass within G_i class of G protein α subunits. The zygotic expression of HrGαn 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 HrGαn became restricted and was observed in the muscle, mesenchyme and a part of trunk lateral cells in tailbud embryos. With HrGαn-GFP fusion-gene construct it was showed that the genomic fragment containing 2674 bp upstream of the putative translation start site of HrGαn 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 HrGαn 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...
(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 HrG α, 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 HrG α defines a novel subclass within G protein α subunits of G 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 HrG α-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 Aoi-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 KKW(HE)CF and a reverse primer, G-domain corresponding to amino acid sequence FLNKKD (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 (I.ZAPII, 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, Upplala, Sweden). DNA sequencing (Shimadzu DSQ 1000L, Shimadzu, Kyoto, Japan).

Isolation of cDNA clones

A cDNA library of tadpole larvae of H. roretzi was screened with random primed 32P-labeled PCR-HrG α (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 denaturated 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 1.0×105 plaques we obtained only one positive plaque containing HrG α-1 (Fig. 1A), which lacks C-terminal sequence. We further screened the cDNA library (1.2×105 plaques) with HrG α-1 as a probe, and obtained a new positive plaque containing HrG α-2 (Fig. 1A).

Northern blot analysis

Total RNA was prepared with standard CsCl-ultracentrifugation methods (Ausbel 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, Upplala, Sweden). The membrane was cross-linked by UV cross-linker (CL-1000 Ultra Cross Linker, UVP, San Gabriel, USA). Hybridization was carried out by the same procedure as described under “Isolation of cDNA clones”, with 32P-labeled HrG α-3 probe, Hinc II - Pst I fragment of HrG α-2 (Fig. 1A). The washed membranes were analyzed with an imaging plate (LAS 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, Hind III  or Pst I, electophoresed 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 (LAS 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 anti-sense 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 HrG α

The genomic library of H. roretzi (kind gift from Dr. N. Satoh, Kyoto University) was screened with 32P-labeled HrG α-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 HrG α was obtained with PCR using a combination of a gene-specific (TTTATAGTCGATTCCGAGC; nt 354-373 for HrG α-1 probe) 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 deletion products (Deletion Kit for Kilo-Sequence, Takara, Japan). A 3.5 kb fragment was also cloned into pT7blue vector (Stratagene, La Jolla, USA) (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 HrG α. 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 DNA sequencer (Shimadzu DSQ 1000L, Shimadzu, Kyoto, Japan).
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 HrGαn

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; 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 HrGαn. (A): Schematic representation and restriction map of HrGαn. 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 HrGαn 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 HrGαn with that of G protein α subunits of Go and Gi 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 Go (M60162), Gi3 (J03238), Drosophila Go (M29731), Gi (M23094), Octopus Go (AB025781) and Gi (AB025780). The nucleotide sequence of HrGαn is available at DDBJ/EMBL/GenBank with an accession number AB022098.
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 HrGαn (Halocynthia roretzi G protein α subunit in n subclass).

HrGαn defines a novel subclass within G protein α subunits of G class

Although the amino acid sequence of the guanine nucleotide pocket of HrGαn 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 Gi class, Gαi. The candidate sequence for N-terminal myristoylation (MGxxx(S/T)), characteristic for Gαi (Chen and Manning, 2001), was conserved in N-terminal region of HrGαn, indicating that the HrGαn belongs to Gαi class (Fig. 1B).

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

To investigate evolutionary relationships between HrGαn 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

![Figure 2](https://bioone.org/journals/Zoological-Science/fig/2)

**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 pseudoreplications by the neighbor-joining method. Sources of sequence information: Human (Homo) Gα (M60162), Gβ (J03238), Gγ (X15088), Gαi (D10384), Gαi2 (J03260), Gαj (L01694), Gαq (M69013), Gαs (M21142), Gαz (L10665), Drosophila (Dro) Gα (M29731), Gα (M23094), Gαi (U31092), Gαs1 (M23233), Octopus (Oct) Gα (AB025781), Gα (AB025780), Gα (AB025782), and Gα (AB025783).
HrGαn is most closely related to Gαi and that it should be a member of Gαi class. Within the Gαi class, however, HrGαn 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 BamHI, EcoRI, or HindIII. In the digests with PstI 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 HrGαn is a single copy gene.

**Spatio-temporal expression pattern of the HrGαn transcripts**

To examine the temporal expression pattern of HrGαn during ascidian embryogenesis, northern blot analysis was performed with eight different developmental stages from the unfertilized egg up to the tadpole larva with HrGα-3' as a probe. The signal of HrGαn was observed at about 3.2 kb in the unfertilized egg indicating presence of maternal mRNAs of HrGαn (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 HrGαn in the adult tissues was also investigated (Fig. 3C). The clear signal of HrGαn 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, HrGαn is expressed in both larvae and adult tissues.

The spatial distribution of expression of HrGαn was investigated in developing embryos with whole-mount in situ hybridization. The maternal mRNAs of HrGαn 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.

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**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 BamHI, EcoRI, HindIII or PstI. Mobility of size markers is shown in base pairs with arrow. (B): Northern blot analysis of HrGαn 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.
The observed temporal expression patterns of HrGαn from the 64-cell to 110-cell stage were summarized in Fig. 5. At the 64-cell stage embryo, zygotic expression of HrGαn 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

![Fig. 4. Whole-mount in situ hybridization of developing embryos with a Digoxigenin-labeled antisense probe for HrGαn 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.](https://bioone.org/journals/Zoological-Science)
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 HrGαn 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 HrGαn**

In order to analyze the regulatory sequence responsible for observed spatio-temporal expression of HrGαn, we isolated the 5'-flanking sequence of HrGαn 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 HrGαn 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. 5.** Zygotic expression of HrGαn 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 HrGαn was detected are presented in shaded boxes. The cell lineage is according to Nishida (1987).
Fig. 6. (A): Schematic representation of sequence motifs in the 5'-flanking sequence of *HrGαn* 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 *HrGαn*-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 *HrGαn*-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 *HrGαn* gene is available at DDBJ/EMBL/GenBank with an accession number AB083069.
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 HrGαn. 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 Cαn protein, a central 118 amino acid domain of which shows 69% homology to the 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 one CDP motif (-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 *HrGαn*, we constructed a fusion gene of the 5′-flanking sequence of *HrGαn* 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 *HrGαn*. 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 *HrGαn* (-GFP) resulted in fewer 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. intestinalis* 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, HrGαn, of ascidian tadpole larvae. The deduced amino acid sequence of HrGαn (357 amino acids) showed higher similarity to human Gαi and Gαo. There was a candidate sequence for palmitoylation in the N-terminal region of HrGαn, which is conserved in Gαi (Chen and Manning, 2001). The phylogenetic tree constructed based on the deduced amino acid sequence clearly showed that HrGαn is a member of Gαi. However, HrGαn was unique among those belong to Gαi in the following criteria. First of all, the deduced amino acid sequence of HrGαn 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 Gαi. HrGαn, however, possessed an arginine residue positioned at 180 (R180), which is a possible substrate of cholera toxin, and ADP-ribosylated in Gαo and Gαn. Second, the amino acid sequence of C-terminal region was extensively different from that of the other Gαi (Fig 1C). Third, HrGαn was fairly diverged from any other members of Gαi in the phylogenetic tree (Fig. 2). Based on these results we concluded that HrGαn defines a novel subclass in G protein α subunits of Gα class.

The zygotic expression of HrGαn was first observed at the 64-cell stage. From the 64-cell to 110-cell stage, a transient expression of HrGαn was observed in all cells except B7.4, B7.5 and B7.6 cells. An interesting expression pattern of HrGαn was observed in the case of the B7.3 cell. The B7.3 cell showed expression of HrGαn 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 HrGαn 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 HrGαn is involved in promotion of mesenchyme formation. Concerning about this issue the expression pattern of HrGαn 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 HrGαn 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 HrGαn in the mesenchyme cells con-
tined 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 HrGαn from the 64-cell to 110-cell stage. These results showed that expression of HrGαn was observed in mesenchyme and their precursor cells, suggesting another possible role of HrGαn, 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 HrGαn 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 (TAGCGAT), E-box/Box B (CAGTGGCGC), GATA binding site (TGATAG), and Box T1/T2 (Box T; CTTCTTCTTCA) 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 sequence contained in pGn3.5-GFP for these motifs and their precursor cells, suggesting another possible role of HrGαn, 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.

Four clusters of the sequence motifs of the potential binding site for transcription factors were found in the 5'-flanking sequence of HrGαn (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 CDK 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 HrGαn gene is remained to be elucidated.

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