Ci-opsin1, a vertebrate-type opsin gene, expressed in the larval ocellus of the ascidian *Ciona intestinalis*[1]

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Abstract **A novel gene encoding visual pigment, *Ci-opsin1*, was identified in a primitive chordate, the ascidian, *Ciona intestinalis*. Molecular phylogenetic analysis and the exon–intron organization suggest that Ci-opsin1 is closely related to the retinal and pineal opsins of vertebrates. During embryogenesis, *Ci-opsin1* transcripts were first detected in part of the brain of mid tailbud embryos; its expression was confined to photoreceptor cells of the ocellus (eye spot) in the larval brain as development proceeded. These results suggest a common descent of the ascidian ocellus and the vertebrate eyes. The ocellus of ascidian larvae may represent an ancestral state of the vertebrate eye.**

Key words: Ascidian; Eye; Evolution; Opsin; Photoreceptor cell; Pineal organ

1. Introduction

Ascidians (sea squirts) are lower chordates and their simple tadpole-like larvae share a basic body plan with vertebrates [1]. The ascidian tadpole is organized into a trunk and a tail. The trunk contains a dorsal brain, endoderm, epidermis, and mesenchyme. The tail contains a notochord flanked by the spinal cord, endodermal strand, and three rows of striated muscle cells. The non-feeding larva is a dispersal phase. During metamorphosis, the larval tail is destroyed, and the adult tissues and organs differentiate from precursors in the trunk. The adult ascidian is a sessile organism with little resemblance to vertebrates.

The brain of the ascidian larva contains two sensory organs with a melanocyte: a gravity sense organ (otolith) and an eye spot (ocellus). In the ascidian *Ciona intestinalis*, the ocellus consists of three lens cells, one pigment cup cell, and about 20 photoreceptor cells [2]. Similarity in morphology and physiology of photoreceptor cells has been recognized between the ascidian ocellus and the vertebrate eye [3,4]. However, so far no molecular evidence has been reported to suggest a common descent of the ascidian ocellus and the vertebrate eye.

We have previously shown that the action spectrum of photic behavior of ascidian larvae was similar to the absorption spectrum of human rhodopsin [5]. The localization of visual pigment in the larval ocellus of the ascidian *Halocynthia roretizi* was demonstrated by the retinal protein imaging method [6]. However, the primary structure of the visual pigment in the ascidian ocellus is not known. Here we report the primary structure and mRNA expression patterns of Ci-opsin1, a homologue of the vertebrate visual pigment (opsin), which is present in the ocellus of *C. intestinalis* larvae. Our results, together with previous observations on the photic behavior of the larvae, suggest that the ocellus of ascidian larvae represents an ancestral state of the vertebrate eye.

2. Materials and methods

2.1. Animals and embryos

Mature adults of *C. intestinalis* were collected and maintained as described [5]. Eggs and sperm were obtained surgically from gonoducts and fertilized in vitro. Embryos were allowed to develop at 18°C.

2.2. Cloning and sequencing of Ci-opsin1 cDNA

In a project analyzing expressed sequence tags (EST) of *C. intestinalis* larvae [7], we found a *Ci-opsin1* cDNA clone by a BLAST search [8]. This clone was interrupted by two intron sequences (Fig. 1, codons 194 and 268/269). Full-length cDNAs without introns were amplified from larval poly(A)$^+$ RNA by RT-PCR. Total RNA and poly(A)$^+$ RNA were prepared from *C. intestinalis* larvae using Isogen (Nippon Gene, Japan) and Oligotex-dT30 Super (Roche Diagnostics, Japan), respectively, according to the manufacturer's protocols. 1 μg of poly(A)$^+$ RNA was reverse-transcribed with an oligo(dT) primer using SuperScript II reverse transcriptase (Life Technologies). The *Ci-opsin1* cDNA was amplified from the first strand cDNA by PCR using a thermostable DNA polymerase with proofreading activity (TaKaRa LA Taq; Takara Shuzo, Japan) and a pair of gene-specific oligonucleotide primers (5′-AAACGCAGCATTATGAATCAGTG-3′ and 5′-GTCAAGACTTTTTATTTTCAGAATATATAG-3′) synthesized based on the 5′- and 3′-terminal sequences of the EST clone. The PCR products were cloned into pBluescriptII SK(+) (Stratagene, La Jolla, CA, USA). Nucleotide sequences were determined on both strands by the cycle sequencing method with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. Analysis of exon–intron organization of the Ci-opsin1 gene

Nucleotide sequences of *C. intestinalis* genomic DNA fragments which significantly matched up to the *Ci-opsin1* cDNA sequence were found by a BLAST search against the database (http://www.jgi.doe.gov/programs/ciona.htm) of the *C. intestinalis* genome project by the Joint Genome Institute (JGI, Walnut Creek, CA, USA). Intron

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[1] The nucleotide sequence reported in this paper has been submitted to DDBJ/GenBank/EMBL nucleotide databases under the accession number AB058682.
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positions were determined by aligning the genomic DNA sequences with the *Ci-opsin1* cDNA sequence.

2.4. Molecular phylogenetic analysis

Amino acid sequences of various animal opsins were aligned and a neighbor-joining tree was constructed using the ClustalW program [9]. Because N- and C-terminal regions were highly variable in length and sequence, they were excluded from the analysis. The region between amino acid positions 34 and 355 of Ci-opsin1 and the corresponding regions of other opsins were subjected to phylogenetic analysis. Evolutionary distances were estimated using Kimura's empirical method. Sequences used are: *Homo sapiens* opsins (rhodopsin U49742, blue M13295, green K03490, red M13300, encephalopsin NM_014322), *Gallus gallus* opsins (green M92038, blue M92037, violet M92039, pinopsin U15762), *Bufo japonicus* pinopsin AF200433, *Xenopus laevis* melanopsin AF014797, *Carassius auratus* blue L11864, *Salmo salar* VA AF001499, *Danio rerio* VAL AB035276, *Ictalurus punctatus* parapinopsin AF028014, *Lampetra japonica* rhodopsin M63632, *Octopus dofleini* rhodopsin X07797, *Patinopecten yessoensis* opsins (SCOP1 AB006454, SCOP2 AB006455), *Drosophila melanogaster* Rh1 K02315, *Limulus polyphemus* opsin L03781.

2.5. Whole-mount in situ hybridization

A full-length *Ci-opsin1* cDNA clone was used as a template to synthesize a digoxigenin-labeled antisense DNA or RNA probe and whole-mount in situ hybridization was carried out as described previously [7,10].

3. Results and discussion

We isolated cDNA clones showing significant homology to vertebrate opsins from *C. intestinalis* larvae. The corresponding gene was named *Ci-opsin1*. The *Ci-opsin1* cDNA encodes a putative opsin protein of 390 amino acids. Amino acid comparison (Fig. 1) and phylogenetic analysis (Fig. 2) revealed that Ci-opsin1 is more closely related to the vertebrate

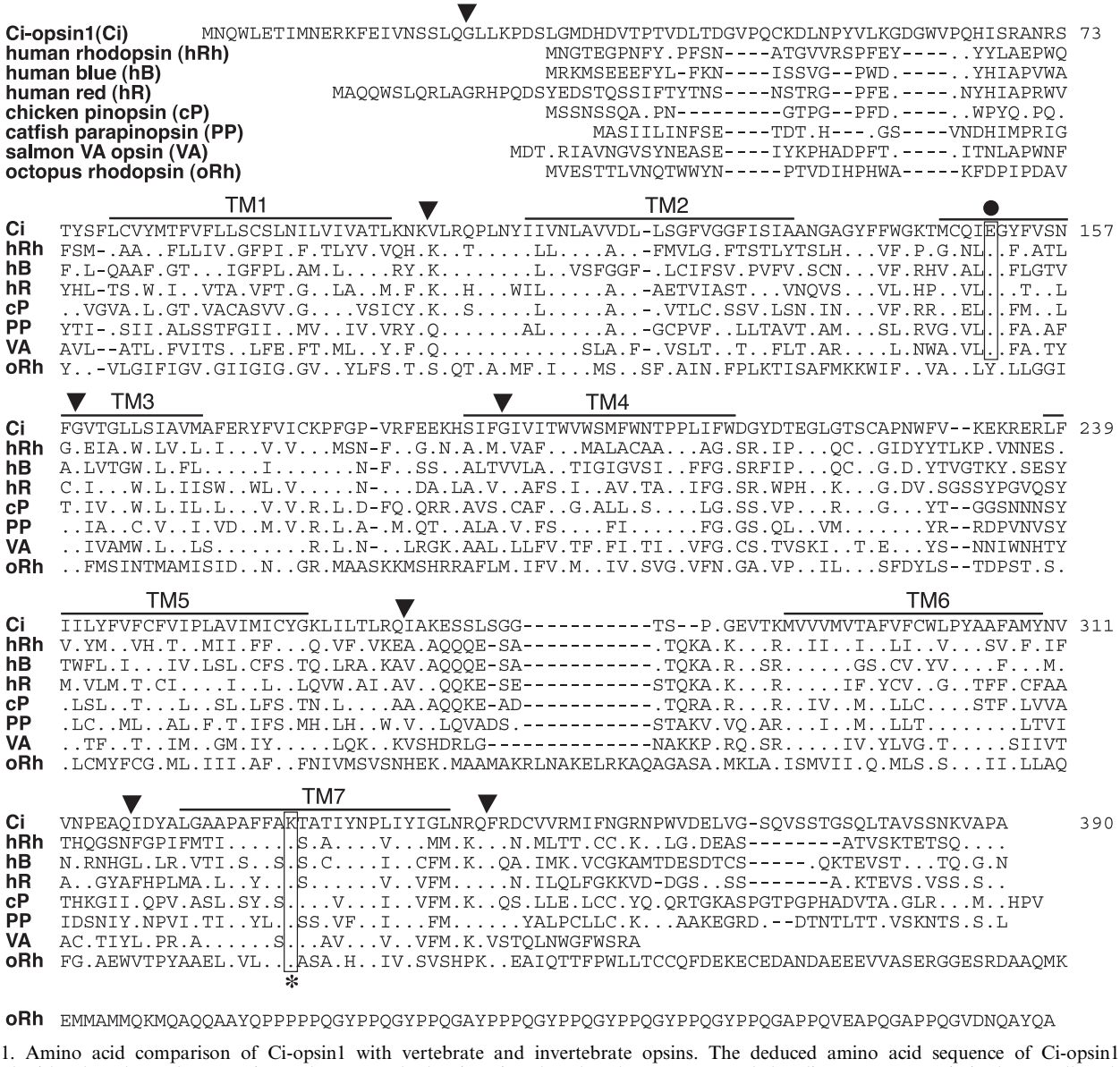

Fig. 1. Amino acid comparison of Ci-opsin1 with vertebrate and invertebrate opsins. The deduced amino acid sequence of Ci-opsin1 was aligned with selected vertebrate opsins and octopus rhodopsin using the ClustalW program and the alignment was optimized manually. The entire sequence of Ci-opsin1 is shown. The dots represent amino acids identical to those of Ci-opsin1 and the letters represent variable positions in other opsins. Dashes indicate gaps introduced in the sequence to optimize the alignment. The putative transmembrane domains (TM1–TM7) are indicated by horizontal lines. The glutamate counterion (solid circle) and the Schiff base lysine (asterisk) are boxed. Triangles indicate positions of introns in the *Ci-opsin1* gene.

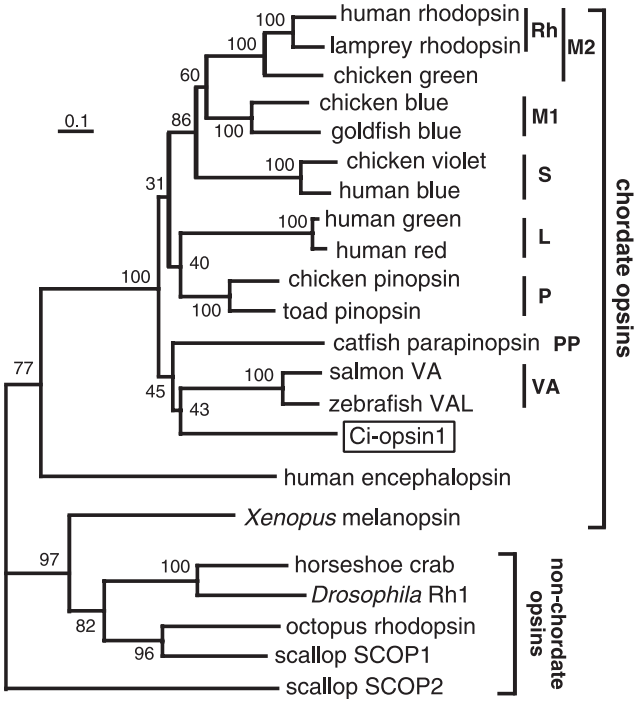

Fig. 2. Phylogenetic tree of animal opsins. The phylogenetic tree was inferred from amino acid sequences by the neighbor-joining method [29]. Scale bar indicates 0.1 amino acid replacements per site. Numbers at nodes are bootstrap values based on 1000 replicates. Major groups of known visual pigments [30] are indicated at the right of the corresponding branches: L, red–green opsin; M1, blue opsin; M2, green opsin and rhodopsin; P, pinopsin; PP, parapinopsin; Rh, rhodopsin; S, violet–UV opsin; VA, vertebrate ancient opsin.
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visual pigments than to those of invertebrates. Clustering of Ci-opsin1 with the vertebrate retinal and pineal opsins is supported by 100% of bootstrap value (Fig. 2). The exon–intron organization of the *Ci-opsin1* gene was determined by aligning the cDNA sequence with genomic DNA sequences obtained from the JGI *Ciona* genome project database. Seven introns (codons 23, 104/105, 159, 194 268/269, 317/318, and 347/348) interrupt the coding region of *Ci-opsin1* (Fig. 1). Among them, three introns (codons 159, 268/269, and 347/348) are present at positions identical to those of vertebrate opsin genes [11], further supporting a close relationship between Ci-opsin1 and vertebrate opsins.

The deduced amino acid sequence of Ci-opsin1 exhibits features characteristic of functional opsins (Fig. 1). Ci-opsin1 has a lysine at position 331, corresponding to 296 in bovine rhodopsin, the site of the Schiff base linkage with 11-*cis*-retinal [12]. Glutamate 151 (corresponding to 113 in bovine rhodopsin) is the putative counterion to the protonated Schiff base conserved in vertebrate opsins [13], while the corresponding residue in invertebrate opsins is an aromatic residue [14,15].

We determined the expression patterns of *Ci-opsin1* in embryos and larvae by whole-mount in situ hybridization (Fig. 3). In addition to an RNA probe (Fig. 3A–F, which is commonly used in this kind of analysis, we also used a single-

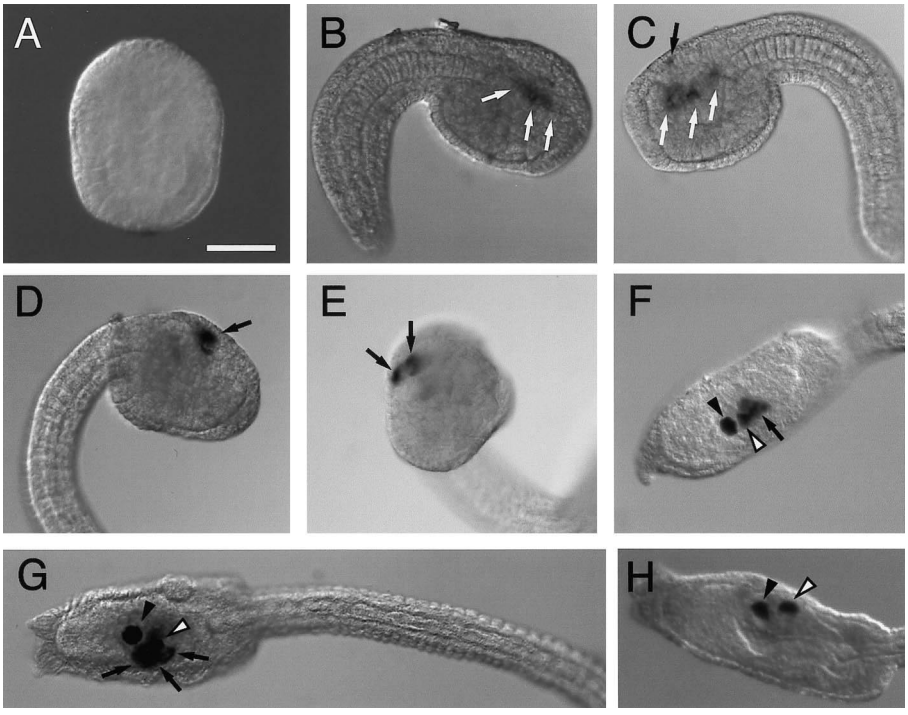

Fig. 3. Expression of *Ci-opsin1* in *C. intestinalis* embryos and larvae. *Ci-opsin1* mRNA was detected by whole-mount in situ hybridization using a digoxigenin-labeled RNA (A–F) or DNA (G) probe. A: A neurula. B and C: Mid tailbud embryos showing *Ci-opsin1* expression in the ventral part (white arrows) and dorsal part (black arrow) of the brain. D and E: Late tailbud embryos. *Ci-opsin1*-expressing cells (black arrows) form two clusters sitting dorsal to the pigment cell. F–H: Hatched larvae. White arrowheads indicate the pigment cup cell of the ocellus and black arrowheads indicate the pigment cell of the otolith (gravity sense organ). *Ci-opsin1* expression is restricted to the photoreceptor cells (black arrows in F and G) of the ocellus. H: A control embryo hybridized with an unrelated probe (*C. intestinalis* EST clone #00754 [7]) showing no hybridization signals in the photoreceptor cells. Scale bar, 50 μm.

stranded DNA probe (Fig. 3G) synthesized by the PCR-based method [7], which allows easier preparation and handling of probes. The two types of probes gave the same results, demonstrating that DNA probes are useful for whole-mount in situ hybridization of ascidian embryos.

The expression of *Ci-opsin1* was not detected in the neurula and earlier stages (Fig. 3A). *Ci-opsin1* mRNA first appeared in the ventral part of the developing brain at the mid tailbud stage (Fig. 3B,C). In older embryos at the mid tailbud stage, a small number of dorsal cells were also stained (Fig. 3C). When melanization of the pigment cell began in late tailbud embryos, *Ci-opsin1* expression was restricted to cells dorsal to the pigment cell (Fig. 3D,E). Two laterally adjacent clusters of cells expressed *Ci-opsin1* mRNA (Fig. 3E). At the late tailbud stage, *Ci-opsin1* expression was not evident in the ventral part of the brain (Fig. 3D,E). Therefore, the ventral *Ci-opsin1* expression observed in mid tailbud embryos may be transient and only the dorsal *Ci-opsin1*-positive cells may become photoreceptor cells. In the swimming larva, *Ci-opsin1* expression is restricted to photoreceptor cells of the ocellus (Fig. 3F–H). These results suggest that *Ci-opsin1* is a visual pigment of the ascidian ocellus.

The pigment cells of the ocellus and otolith comprise a developmental 'equivalence group' [16–18]. A bilateral pair of cells in the blastula gives rise to the otolith and ocellus melanocytes in a complementary manner: one cell develops into an ocellus pigment cell and the other cell develops into an otolith pigment cell. Because of lack of differentiation markers, however, cell lineage and developmental mechanisms of photoreceptor cells of the ocellus remain unclear. The present study suggests that *Ci-opsin1* can be used as a differentiation marker for photoreceptor cells. At the late tailbud stage, two clusters of cells expressing *Ci-opsin1* locate side by side roughly bilaterally, suggesting that, unlike the pigment cell, both left and right lineages of cells contribute to formation of the retina.

The vertebrate retina contains several types of opsin in visual cells; up to five different opsins are expressed in some retinas. In addition to the visual cells of the retina, recent studies have identified a number of novel opsins in several different sites of the vertebrate brain, including horizontal cells and amacrine cells of the retina [19], the median eye (pineal complex) [20–23], and deep regions of the brain [24,25]. Our data strongly suggest that the opsins of the ascidian ocellus and the vertebrate eye and brain are derived from an ancestral opsin present in the common ancestor of extant chordates. Therefore, the diverse opsin family may have evolved in accordance with the increase of complexity and diversity of brain-derived photoreceptors, including the lateral eye and the median eye, during early vertebrate evolution from an ancestor that had a simple eye in the brain similar to the ocellus of ascidian tadpoles.

There are striking similarities between young frog tadpoles and ascidian larvae in photophobic response of swimming behavior. Young *Xenopus laevis* tadpoles start to swim when the illumination is dimmed, and this response is mediated by the pineal eye [26,27]. Our previous studies demonstrated a very similar response of *Ciona* larvae to a sudden decrease in light intensity [5,28]. The action spectrum suggests that a retinal protein(s) in the ocellus is responsible for this response. The median eye of vertebrates and the ocellus of ascidian larvae both develop from the dorsal midline of the brain. Considering similarities in opsin sequence, developmental origin, and behavioral function, the median eye of lower vertebrates and the ascidian ocellus may represent ancestral states of the chordate photoreceptor organ.

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References

[1] Satoh, N. (1994) Developmental Biology of Ascidians, Cambridge University Press, Cambridge.
[2] Nicol, D. and Meinerzhagen, I.A. (1991) J. Comp. Neurol. 309, 415–429.
[3] Eakin, R.M. and Kuda, A. (1971) Z. Zellforsch. 112, 287–312.
[4] Gorman, A.F.L., McReynolds, J.S. and Barnes, S.N. (1971) Science 172, 1052–1054.
[5] Nakagawa, M., Miyamoto, T., Ohkuma, M. and Tsuda, M. (1999) Photochem. Photobiol. 70, 359–362.
[6] Ohkuma, M. and Tsuda, M. (2000) Zool. Sci. 483, 143–148.
[7] Satou, Y., Takatori, N., Fujiwara, S., Nishikata, T., Saiga, H., Kusakabe, T., Shin-i, T., Kohara, Y. and Satoh, N. (2001) Gene, in press.
[8] Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[9] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acids Res. 22, 4673–4680.
[10] Satou, Y., Araki, I., Kusakabe, T. and Satoh, N. (1995) Dev. Growth Differ. 37, 319–327.
[11] Yokoyama, S. (2000) Prog. Retin. Eye Res. 19, 385–419.
[12] Mullen, E. and Akhtar, M. (1981) FEBS Lett. 132, 261–264.
[13] Sakmar, T.P., Franke, R.R. and Khorana, H.G. (1989) Proc. Natl. Acad. Sci. USA 86, 8309–8313.
[14] Ovchinnikov, Yu.A., Abdulaev, N.G., Zolotarev, A.S., Artamonov, I.D., Bespalov, I.A., Dergachev, A.E. and Tsuda, M. (1988) FEBS Lett. 232, 69–72.
[15] Nakagawa, M., Iwasa, T., Kikkawa, S., Tsuda, M. and Ebrey, T.G. (1999) Proc. Natl. Acad. Sci. USA 96, 6189–6192.
[16] Nishida, H. and Satoh, N. (1985) Dev. Biol. 110, 440–454.
[17] Nishida, H. (1987) Dev. Biol. 121, 526–541.
[18] Nishida, H. and Satoh, N. (1989) Dev. Biol. 132, 355–367.
[19] Soni, B.G., Philp, A.R., Foster, R.G. and Knox, B.E. (1998) Nature 394, 27–28.
[20] Okano, T., Yoshizawa, T. and Fukada, Y. (1994) Nature 372, 94–97.
[21] Max, M., McKinnon, P.J., Seidenman, K.J., Barrett, R.K., Applebury, M.L., Takahashi, J.S. and Margolskee, R.F. (1995) Science 267, 1502–1506.
[22] Kawamura, S. and Yokoyama, S. (1996) Gene 182, 213–214.
[23] Blackshaw, S. and Snyder, S.H. (1997) J. Neurosci. 17, 8083–8092.
[24] Yoshikawa, T., Okano, T., Oishi, T. and Fukada, Y. (1998) FEBS Lett. 424, 69–72.
[25] Blackshaw, S. and Snyder, S.H. (1999) J. Neurosci. 19, 3681–3690.
[26] Roberts, A. (1978) Nature 273, 774–775.
[27] Foster, R.G. and Roberts, A. (1982) J. Comp. Physiol. 145, 413–419.
[28] Tsuda, M., Kawakami, I., Miyamoto, T., Nakagawa, M., Shiraishi, S. and Gouda, M. (2001) in: The Biology of Ascidians (Sawada, H., Yokosawa, H. and Lambert, C.C., Eds.), pp. 153–157, Springer-Verlag, Tokyo.
[29] Saitou, N. and Nei, M. (1987) Mol. Biol. Evol. 4, 406–425.
[30] Okano, T. and Fukada, Y. (2000) Methods Enzymol. 316, 278–316.