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Review

Neuroendocrinology of protochordates: Insights from *Ciona* genomics ☆

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Abstract

The genome for two species of *Ciona* is available making these tunicates excellent models for studies on the evolution of the chordates. In this review most of the data is from *Ciona intestinalis*, as the annotation of the *C. savignyi* genome is not yet available. The phylogenetic position of tunicates at the origin of the chordates and the nature of the genome before expansion in vertebrates allows tunicates to be used as a touchstone for understanding genes that either preceded or arose in vertebrates. A comparison of *Ciona*, a sea squirt, to other model organisms such as a nematode, fruit fly, zebrafish, frog, chicken and mouse shows that *Ciona* has many useful traits including accessibility for embryological, lineage tracing, forward genetics, and loss- or gain-of-function experiments. For neuroendocrine studies, these traits are important for determining gene function, whereas the availability of the genome is critical for identification of ligands, receptors, transcription factors and signaling pathways. Four major neurohormones and their receptors have been identified by cloning and to some extent by function in *Ciona*: gonadotropin-releasing hormone, insulin, insulin-like growth factor, and cionin, a member of the CCK/gastrin family. The simplicity of tunicates should be an advantage in searching for novel functions for these hormones. Other neuroendocrine components that have been annotated in the genome are a multitude of receptors, which are available for cloning, expression and functional studies.

Keywords: *Ciona intestinalis*; Cionin; Gonadotropin-releasing hormone (GnRH); Insulin; Insulin-like growth factor (IGF); Neuroendocrinology; Hormone receptors; Tunicates

Contents

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1. Introduction

The genomes have been sequenced for two protochordate species, *Ciona intestinalis* (Fig. 1) and *Ciona savignyi*. The first draft of the genome for *C. intestinalis* has been published and annotated (Dehal et al., 2002) (http://www.jgi.doe.gov/ciona). The sequence of the genome for the closely related species, *C. savignyi*, has been released although not published (http://www.broad.mit.edu/annotation/ciona). This review is almost exclusively on *C. intestinalis* rather than *C. savignyi* because the genome of the latter is not yet annotated. The importance of this achievement is related in part to understanding the origin of the vertebrates from an ancestral chordate. Several hypotheses involving ancestral tunicates have been suggested for the evolutionary events leading to early vertebrates (see Gee, 1996; Lacalli, 2005). The suggestions are based in part on the resemblance of the tunicate larva to the vertebrate tadpole and on the similar development and body plan of the tunicate larva and chordate embryos. Despite such observations, the identity of the ancestor and its swimming ability remain unknown. The complexity of the vertebrates compared to the tunicates or cephalochordates (amphioxus) appears to have developed as a result of expansion of the genome through duplications in early vertebrates (Holland et al., 1994; Furlong and Holland, 2002). The mechanism of expansion is controversial as to whether whole-genome duplication or segmental duplications occurred. However, the observation that invertebrates have one *Hox* cluster, whereas mouse and human have four clusters gave rise to the inference that there were "two rounds of extensive gene duplication (perhaps by complete or partial tetraploidization)" (Holland et al., 1994). In further support for this theory, many regions of the vertebrate genome, such as the major histocompatibility complex (MHC), have expanded compared to invertebrates. However, other evidence suggests that smaller duplications may have been the driving factor in explaining the origin of vertebrates (Spring, 2002).

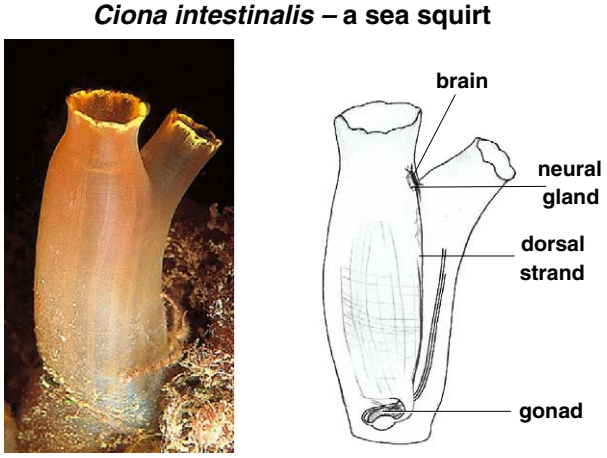


Fig. 1. *Ciona intestinalis* is shown as a photo (by Lukk Vindu) and as a drawing.

Another issue in considering tunicates as a model in evolution is that modern tunicates, both larva and adult, appear to be highly derived (Lacalli, 2005). The modern sessile asymmetrical adult may be derived from a motile, bilaterally symmetrical ancestor. Research on the organization of *Hox* genes has been very helpful in deducing evolutionary changes that have occurred in the tunicate lineage. All bilateral invertebrates have one cluster of *Hox* genes, whereas mammals have four clusters, which are likely to be duplications of the single cluster at a time near the origin of vertebrates (Holland et al., 1994). The order of the *Hox* genes (13 are identified) on the chromosome has been shown to influence the anterior–posterior axis of the body. The putative chordate ancestor is thought to have had one *Hox* cluster with 13 genes and indeed, the sea urchin (Echinodermata) and amphioxus (Cephalochordata) have relatively intact *Hox* gene clusters. However, early in the tunicate lineage there was probably a breakage of the *Hox* gene cluster and extensive shuffling of the genome (Ikuta and Saiga, 2005). As a result, the single *Hox* cluster of genes in *Ciona* (both *C. intestinalis* and *C. savignyi*) is split between two chromosomes and four genes (*Ci-Hox7*, *8*, *9*, *11*) are lost. Also, the remaining *Ciona Hox* genes have long distances between each other and the genes are expressed in an unusual spatial pattern. These changes in the tunicates after separation from the lineage leading to amphioxus and vertebrates may be responsible for the simplification of the larva and unusual body plan and sessile nature of adults. The disruption of the *Hox* cluster and genome in tunicates suggests their ancestor had a different body plan.

As a model, *Ciona* still appears to be very useful, provided one understands the changes that have occurred in modern *Ciona*. One reason for selecting the tunicates for genome sequencing was their phylogenetic position as both an

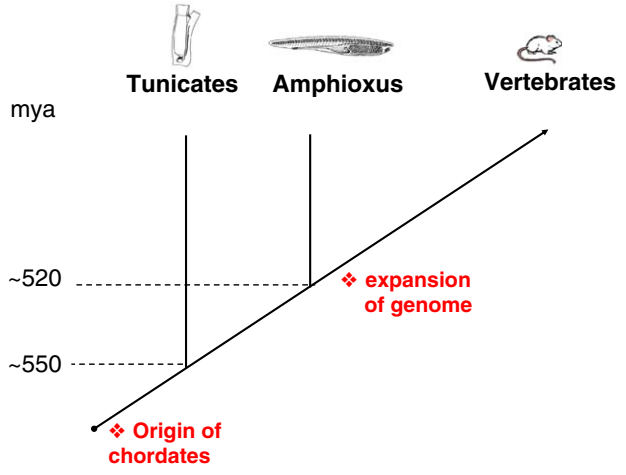


Fig. 2. Phylogeny of the phylum Chordata. *Ciona intestinalis* is a member of the subphylum Tunicata, which is the most basal of the chordates. Amphioxus is a member of the subphylum Cephalochordata, whereas the vertebrates belong to the subphylum Vertebrata. Both the tunicates and cephalochordates are considered to be invertebrates. The expansion of the genome is thought to have occurred in early vertebrates by genome duplication or by large-scale gene duplications. (mya, million years ago).

invertebrate and basal chordate. Also, although tunicates may have had some lineage specific duplications, genome-wide duplications did not alter the single copy of most genes. An understanding of the modern tunicate genome, even though it is ~550 million years removed from the prevertebrate ancestors (Dehal et al., 2002), should illuminate essential genes in the chordate foundation prior to expansion of the genome (Fig. 2).

2. *C. intestinalis* as a model organism for the study of evolution

The tunicates have been identified as chordates by the presence of a dorsal nerve cord, a notochord and pharyngeal slits. *C. intestinalis* is a member of one subgroup of tunicates, the ascidians or sea squirts. Like most tunicates, *C. intestinalis* is small, has rapid development and a short life span of only a few months, all of which make it a useful experimental model. Even more important is that the genome is small and compact. There are an estimated 16,000 genes (14 chromosomes), which is about half of the number of genes in the human genome. The small amount of DNA between genes in tunicates has resulted in a total tunicate genome with only 5% of the number of nucleotides compared with humans (Dehal et al., 2002). The compact tunicate genome with small intergenic regions makes it easier to identify regulatory regions for the tunicate genes compared to larger genomes, which is a considerable advantage. On the down side, high polymorphism of 1.2 % between alleles in single individuals has been reported in *C. intestinalis* (Dehal et al., 2002) and 4.6% in *C. savignyi*, so that the latter genome had to be assembled in separate haplotypes unlike other genomes (Vinson et al., 2005).

As a model system, *C. intestinalis* can be compared to other major models in terms of its characteristics and whether it is amenable for different experimental approaches. This is essential if gene function is to be studied. The other major models include a nematode worm (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), zebrafish (*Danio rerio*), African clawed frog (*Xenopus laevis*), chicken (*Gallus gallus*) and mouse (*Mus musculus*). These models have been previously compared (Stern, 2005), but here we add *C. intestinalis* to show that this species has many useful features that allow experimentalists to test the function of individual genes (Table 1). An understanding of the protochordates depends heavily on identifying individual genes, comparing their encoded proteins with homologs in other species, and determining their functions. The advantage of using tunicates is that for most genes there is only a single copy and very few genes that are redundant. It is this feature that allows us to deduce the basic set of genes in a model system prior to the expansion of the genome in early vertebrates.

C. intestinalis, which we will abbreviate as *Ciona* in this review, is known to have 2n ploidy (Table 1); this is an advantage when comparing *Ciona* genes to those in chick or mouse in order to understand whether a homolog exists and has been duplicated. Teleost fish (including zebrafish) and some amphibians (including *Xenopus*) are more complicated models compared to other vertebrates as there is evidence that they have had additional gene duplications with subsequent loss and rearrangement of genes (Amores et al., 1998; Ohno, 1999).

Functional genomics often depends on accessibility of early stages of the embryo for manipulation. *Ciona* is an excellent model for experimental embryology (Table 1). The cells produced during the cleavage of the egg (blastomeres) are large, which facilitates injections or other manipulations. Both the pregastrula and postgastrula are available for experimentation (Satoh, 1994). Extensive work has been done on lineage tracing in the embryo and larva (Conklin, 1905; Nishida, 1987; Satoh, 1994).

Ciona is amenable to forward genetics due to either spontaneous mutations or induced mutations. Due to the hermaphroditic nature of *Ciona*, it is possible to do rapid screening for recessive mutant phenotypes in only two generations (Corbo et al., 2001; Sordino et al., 2001). The method is based on self-fertilization in *Ciona*. For chemically induced mutations, *N*-ethyl1-*N*-nitrosourea (ENU) has been used to generate point mutations in *Ciona* sperm, which are used to fertilize untreated eggs. Sperm and eggs from these F1 adults can be used for self-fertilization to produce F2 tunicates. The F2 animals can be screened for a recessive mutation (homozygous mutants), and then sperm can be out-crossed to fertilize wild type eggs and examined for inherited mutations (Moody et al., 1999; Sordino et al., 2001).

Loss-of-function studies in which a specific gene is inactivated have great potential for understanding gene function in *Ciona*. Although methods are not available to disrupt or knock out a gene permanently from the germline in *Ciona*, mRNA transcribed from a specific gene can be blocked from being translated into a protein by morpholinos or the mRNA can be targeted for degradation by siRNAs (small inhibitory RNAs). Morpholinos, which are modified antisense oligonucleotides, are designed to hybridize to either the 5′untranslated region (5′

Table 1
Comparison of model organisms for study of gene function

Characteristics	*C. elegans*	*Drosophila*	Ciona	Zebrafish	*Xenopus*	Chick	Mouse
Ploidy	2n	2n	**2n**	4n-pseudo	4n-pseudo	2n	2n
Expt. embryology							
pregastrula	+/–	No	**+/–**	Yes	Yes	Yes	+/–
postgastrula	No	+/–	**+/–**	+/–	No	Yes	+/–
Single cell or							
lineage tracing	Yes	No +/–	**Yes**	Yes	Yes	Yes	+/–
Forward genetics							
Spont. mutations	Yes	Yes	**Yes**	Yes	No	Yes	Yes
Induced mutations	Yes	Yes	**Yes**	Yes	No	No	Yes
Loss of function							
(gene targeted)							
somatic cells	Yes-RNAi	Yes	**Yes-MO**	Yes-MO	Yes-MO	Yes-MO,siRNA	Yes-Cre/lox
germline	No	Yes	**No**	+/–	No	No	Yes
Gain of function							
whole embryo	No?	Yes	**Yes**	Yes	Yes	+/–	Yes
targeted in time	No?	Yes	**Yes**	No	+/–	Yes	Yes
and space							
ES cells	No	No	**No**	No	No	Yes	Yes
Genome sequenced	Yes	Yes	**Yes**	Yes	+/–	Yes	Yes
No. of genes	19 K	14 K	**16 K**			20–23 K	25–30 K

Characteristics that are important in the study of gene function related to development for seven model organisms used in analysis of evolutionary changes. The table is modified from Stern (2005), in particular by adding *Ciona intestinalis* to the models. The 'yes' responses are highlighted in blue to show that *Ciona* is a good model for the traits that are examined. The +/− symbol indicates that the approach is possible either with certain limitations or with some technical difficulties. Further details about approaches and/or difficulties can be found in Stern and Holland, 1993. The models include a nematode (*Caenorhabditis elegans*), fruit fly (*Drosophila melanogaster*), sea squirt (*Ciona intestinalis*), zebrafish (*Danio rerio*), African clawed frog (*Xenopus laevis*), chicken (*Gallus gallus*) and mouse (*Mus musculus*).

UTR) or to the region including the start codon (ATG) (Summerton and Weller, 1997; Nasevicius and Ekker, 2000; Ekker and Larson, 2001). Morpholinos injected into fertilized eggs in *Ciona* have been effective in knocking down (preventing translation) of mRNA (Satou et al., 2001); an example is knockdown of the *Mesp* gene in *C. savignyi*, which resulted in failure of the juvenile heart to develop (Satou et al., 2004). One drawback of morpholinos is that they are diluted as cells continue to divide and total cell volume increases until they are no longer effective; immunocytochemistry is used to show the period in which the protein product remains absent. Nonetheless, the knockdown method using morpholinos or siRNAs is effective in understanding the role that a specific protein plays in early development of a tunicate or another animal with accessible eggs.

Gain-of-function studies usually involve the injection or electroporation of cDNA into a fertilized egg or into specific blastomeres during development (Table 1). In *Ciona*, a stable transgenic line was created with a construct of the *Ci-Brachyury* promoter and green fluorescent protein as a reporter (Deschet et al., 2003). A homing restriction enzyme was co-injected to allow integration of the construct into the genome. The construct was shown to be inherited through at least four generations. Also, a stable transgenic line was generated in *Ciona* by microinjecting a mixture of a transposon construct and its transposase mRNA, which enhanced integration into the genome. The transgene (green fluorescent protein) and an upstream promoter were incorporated within the transposon (Sasakura et al., 2003). Another method for creating a stable transgenic line is by electroporating DNA into *Ciona* eggs; the transgenes were shown to be inherited (Matsuoka et al., 2005). An important feature for gain-of-function experiments is that specific promoters can be used in constructs to ensure that the cDNA of interest is expressed in a designated tissue at a specific time (Stern, 2005). For example, the *HNF-3*β enhancer-promoter was attached to the *Brachyury* gene in a construct that was electroporated into *Ciona* fertilized eggs. Normally *Brachyury* is expressed in the notochord, but the presence of the *HNF-3*β promoter resulted in mis-expression of the gene in the endoderm and CNS (Corbo et al., 2001). The resulting mutant was used to identify the downstream genes that were activated by overexpression of *Brachyury* in an inappropriate tissue; almost 40 notochord-specific genes were identified after overexpression by using subtractive hybridization. In another experiment, *macho-1* was mis-expressed and the formation of muscle cells in the ectopic tissues confirmed the role of *macho-1* in muscle formation (Nishida and Sawada, 2001).

To date, true embryonic stem (ES) cells have not been generated from any blastocysts except for mouse and possibly chick (Table 1; Stern, 2005). ES cells are normally obtained by dissociation of cells at the blastula stage followed by culture conditions that inhibit differentiation, but encourage cell proliferation. ES cells should have the potential to contribute to the germline as well as to all somatic cells.

In tunicates, the daughter cells (blastomeres) begin to differentiate rapidly after cleavage of the egg begins. Satoh (1994) described experiments by earlier workers in which a single cell from the two-cell stage of an embryo produced a complete embryo and adult, but both were smaller. Thereafter, individual blastomeres are not totipotent. Already at the 64-cell

stage in a different species of tunicate (*Halocynthia roretzi*), there are only 20 cells that are not yet "tissue restricted" (contribute to a single tissue type) and at the 110-cell stage there are only 16 cells that are not yet committed to a single tissue type (Nishida, 1987). However, the tunicate embryo has an advantage in that the cells are large and are all on the surface, even at the 64-cell stage, allowing injection into identified cells (Satoh, 1994).

In *Ciona*, primordial germ cells have not been isolated for experimental use, but cells in the blastula after six rounds of cleavage can be identified (blastomeres B7.6) as the ones that will likely give rise to primordial germ cells (Yamamoto and Okada, 1999; Takamura et al., 2002; Satou et al., 2004). The primordial germ cells can be detected in *Ciona* at the tailbud embryo stage, in larvae, and in juveniles that are just completing metamorphosis (Yamamoto and Okada, 1999; Takamura et al., 2002). Electron microscopy was used to identify the primordial germ cells at 12 h after settlement in the region where the tail was reabsorbed and at subsequent times up to 8 days when the gonad rudiment was established. A few days later, differentiation and separation of the ovary and testes occurred (Yamamoto and Okada, 1999). Monoclonal antibodies to the *Ciona* vasa protein, specific to germ cells, were used to detect cells beginning in the tail bud stage. The cells were further tracked sequentially into the endodermal strand cells in the larval tail, into primordial germ cells after metamorphosis, followed by movement into the gonad (Takamura et al., 2002).

Stem cells, which are long-lived, are present in *Ciona* where they have been shown to repopulate the neural ganglion (brain). In adults, neurons appeared in the regenerating ganglion at 5–7 days after ablation. Some of the new cells were 5-bromodeoxyuridine labeled, which suggested that neuronal precursor cells, possibly from the dorsal strand, had divided (Bollner et al., 1995). In a subsequent study, the same laboratory was able to show that one of the subpopulations of neuroblasts that migrate into the regenerating ganglion is one that could be immunolabeled with a specific neuropeptide, gonadotropin-releasing hormone (GnRH). The authors showed that these GnRH neuroblasts were not labeled at any stage with bromodeoxyuridine, but were likely to have been born in the dorsal strand before the ablation and then migrated to the ganglion (Bollner et al., 1997). In summary, it appears that embryonic stem cells and isolated primordial germ cells are not available for *Ciona*, but identified blastomeres are available for injection and neuronal precursor cells exist.

Ciona has been an important model to developmental studies because of its rapid development, transparency, known cell lineage, low cell number and susceptibility to gene transfer or knock down in the embryo. These same traits make *Ciona* useful for the study of evolution because gene function can be studied by using the classical developmental approaches. In particular, forward genetics with spontaneous or induced mutations and gain- or loss-of-function experiments are critical for functional genomics in *Ciona*. Further studies comparing *Ciona* to other species will be advanced when the genomes for a hemichordate and amphioxus are completed so that gene losses or additions can be mapped more finely.

3. *C. intestinalis* as a model for neuroendocrinology studies

Dramatic changes have occurred in the nervous system during the evolution of chordates. Our basic questions are which neurohormones were present at the origin of the chordates and what were their functions. Of great curiosity is whether vertebrate-type releasing factors are present because genomic searches in *Ciona* did not detect either pituitary hormones or a full complement of enzymes needed for the synthesis of the steroids including estrogens, androgens, progestagens, mineralocorticoids and glucocorticoids (Campbell et al., 2004). To date, of the possible releasing factors, only gonadotropin-releasing hormone (GnRH) has been identified; both protein chemistry (Powell et al., 1996) and gene sequencing were used (Adams et al., 2003). Corticotropin-releasing hormone (CRH) has not been identified, but receptors have been annotated in the genome (http://www.jgi.doe.gov/ciona). Clearly the mechanism of action for GnRH in *Ciona* must be distinct from organisms with a pituitary gland.

Ciona is an interesting model for comparison of the nervous system or reproductive system with those of vertebrates, as we expect these two systems in tunicates to have retained many conserved features of the ancestral tunicates, unlike the body plan that might have changed in modern sessile tunicates compared to ancestral tunicates. Indeed, GnRH and its receptor, which are associated with reproduction, have not only been conserved but expanded in a lineage-specific manner; there are six GnRH peptides and four GnRH receptors in *Ciona*. The role of GnRH in organisms such as tunicates is of great interest because they represent a stage just prior to the development of the pituitary and sex steroids in chordates.

As a result of the sequencing of the genomes for both *C. intestinalis* and *C. savignyi*, a number of receptors have been characterized. The large receptors, especially the G-protein-coupled receptors with their seven-transmembrane hydrophobic domains, have been identified and compared to invertebrate and vertebrate receptor families, whereas it has been more difficult to find small peptides encoded within the genome. The genes of only a few *Ciona* neuropeptides have been sequenced: GnRH, insulin, insulin-like growth hormone (IGF), and cionin (a cholecystokinin/gastrin-like molecule) (see refs. in Sections 4–6 below). Of these four, only GnRH and cionin have been isolated as protein molecules. Meanwhile, in *Ciona* at least one member of each of the five G-protein-coupled receptor (GPCR) families has been identified in the genome and annotated. The GPCR families include glutamate, rhodopsin, adhesion, frizzled and secretin (GRAFS) (Fredriksson et al., 2003; Schioth and Fredriksson, 2005). Of these five families, the rhodopsin and secretin families contain the receptors important to neuroendocrinology.

Identification of the structure and function of neuropeptides and their receptors is an important focus, but so are transcription factors that regulate the ligand and receptor genes. To date, the regulatory regions in *Ciona* appear to be only a few kilobases immediately upstream from the transcriptional start site of the gene (Corbo et al., 2001), which will facilitate identification of transcription factors. The compact genome coupled with the

ease of experimentation using forward genetics, and gain- or loss-of-function should lead to an understanding of the network of hormones and receptors that are controlled by specific regulatory factors.

4. Characterization of a neurohormone system: gonadotropin-releasing hormone (GnRH)

In this review, we analyze three hormone-receptor systems that have been characterized within *Ciona*. We recognize that many hormones and receptors have been tentatively identified with immunocytochemistry using antibodies that were raised against molecules in other organisms. In an earlier review, we reported on these immunocytochemistry studies and will not repeat the information here (Sherwood et al., 2005). Rather, we focus on hormones and receptors for which the cDNA or gene have been isolated. The three neuroendocrine systems are GnRH, cionin, and insulin/insulin-like growth factor and their receptors. In addition, we discuss several other neuroendocrine receptors that have been isolated even though the ligand in *Ciona* has not been found. To some extent, the three hormone-receptor systems, in particular GnRH, have been analyzed for expression and function. To date, *Ciona* as a basal chordate has been shown to have neurohormone receptors in the G-coupled-protein group, the nuclear receptor group and the protein-kinase group. It is also clear that receptors found in vertebrates but missing in *Ciona* are not always innovations in the vertebrates; many nuclear receptors appear to have evolved in both invertebrates and vertebrates, but were selectively lost in some lineages including the tunicates (Bertrand et al., 2004).

4.1. GnRH hormones in Ciona

Gonadotropin-releasing hormone (GnRH) is a key neurohormone responsible for the control of reproductive functions in vertebrates. Currently there are 24 distinct members within the GnRH family; 14 isoforms are identified within the vertebrates and 10 within the invertebrates (Sherwood and Adams, 2005). In vertebrates, each GnRH gene loci encodes a single GnRH decapeptide (Fig. 3). GnRH gene organization for invertebrates is of particular interest as it may reveal clues to the gene structure present in the ancient chordate ancestor before the divergence of tunicates and the lineage that led to the vertebrates. Two forms of the GnRH peptide have been isolated from the tunicate, *Chelyosoma productum*, based on primary amino acid structure, but the genes are yet to be sequenced (Powell et al., 1996). Aided by the genome sequencing projects from the two sister tunicates, *C. intestinalis* and *C. savignyi*, we recently found two genes each encoding three GnRH peptides in a triplet array, giving a total of six putative GnRH peptides encoded within each genome (Fig. 3) (Adams et al., 2003). A grand total of seven novel GnRH peptides are encoded within the two genomes; each is unique from all GnRH isoforms previously identified. The fact that *Ciona* encodes the largest number of GnRH peptides found in any animal to date raises some questions as to whether the triplet-coding gene sequences

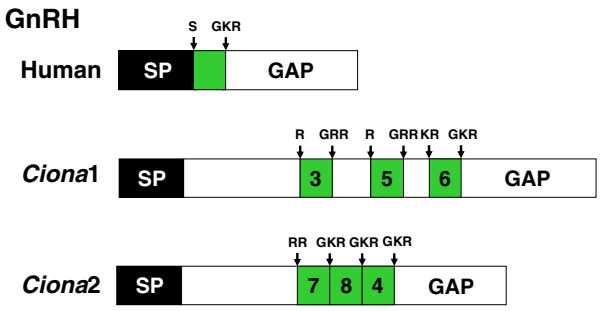


Fig. 3. A schematic diagram of precursor proteins for gonadotropin-releasing hormone (GnRH). The human and other vertebrate GnRH precursors encode only one form of GnRH (green box) in each gene. In contrast, *Ciona intestinalis* has two precursors, each encoding three distinct forms of GnRH. Each peptide has potential cleavage sites shown by an arrowhead with the amino acids that can be cleaved. SP, signal peptide; GAP, gene-associated peptide; G, glycine; K, lysine; R, arginine, S, serine.

were an innovation specific to the tunicate lineage or whether the multiple copies of GnRH in each gene were present in the chordate ancestor. Other questions are whether each unique form of GnRH has specialized functions within this seemingly simple organism or whether these triplet repeats serve in a gene dosage manner for a quick hormonal response. The exact role of GnRH within the invertebrates is still relatively uncertain. It is evident that tunicates lack a bona fide hypothalamic–pituitary–gonadal axis; missing are the genes encoding the major pituitary hormones of follicle stimulating hormone, luteinizing hormone, and many key enzymes necessary for the synthesis of steroid hormones (Campbell et al., 2004). Unlike the complex signaling network in vertebrates, GnRH may bypass the release of pituitary hormones and act directly on the gonoducts or by another pathway to induce gamete release in gravid individuals. In its natural environment, changes in photoperiod provide the cue to signal gamete release in gravid *C. intestinalis* after a 27-min latent period (Lambert and Brandt, 1967). A study by Terakado showed that an injection of a synthetic form of GnRH from *C. productum* into mature *C. intestinalis* was able to induce spawning within minutes (Terakado, 2001). In a further study, we showed that injection of each native *Ciona* GnRH was effective at inducing gamete release in gravid *C. intestinalis* (Adams et al., 2003). In *Ciona*, immunoreactive (ir)-GnRH neurons and/or their processes are localized in the blood sinus near the testis and ovary (Mackie, 1995) suggesting that GnRH may act directly or indirectly on the gonads or gonoducts to induce spawning (Terakado, 2001; Adams et al., 2003). The GnRH peptides and their receptors in tunicates have been localized to tissues not considered to be directly involved with reproductive functions. For example, sensory cells that contain immunoreactive GnRH have been localized in areas surrounding the siphons (Mackie and Wyeth, 2000) and recently we have isolated GnRH receptor transcripts from the branchial sac using RT-PCR (Tello et al., 2005). To determine if there is redundancy between the multiple forms of *Ciona* GnRH and if some functions exist outside the reproductive axis, we recently characterized the complete GnRH receptor complement within the *Ciona* genome (Tello et al., 2005).

4.2. GnRH receptors in Ciona

The draft sequence of the genome for *C. intestinalis* revealed four putative GnRH receptors (Dehal et al., 2002). Kusakabe et al. (2002) isolated two GnRH receptor transcripts from the neural complex of *C. intestinalis* and found the transcripts corresponded to a 23 kb chromosomal region where the two genes were arranged in tandem. We have recently isolated each of the four receptor transcripts (Fig. 4) and assayed each receptor for activation after incubation with each of the native *Ciona* GnRH peptides (Tello et al., 2005). Whereas Ci-GnRHRs1-3 increased cAMP after stimulation by each form of *Ciona* GnRH, only Ci-GnRHR1 increased IP accumulation and only in response to tunicate GnRH-6. The shorter fourth GnRH receptor was unable to stimulate any ligand-induced accumulation of either cAMP or inositol phosphates. The peptides encoded in the *Ci-gnrh1* gene were more potent in activating Ci-GnRHR1 and Ci-GnRHR3 than the peptides encoded in *Ci-gnrh2* gene. In contrast, the peptides encoded in the *Ci-gnrh2* gene (tunicate GnRH-7 and -8) were more potent than the peptides from gene1 with the receptor Ci-GnRHR2, although significance was not established. Another interesting finding was that the three tunicate GnRHs with the highest potency for Ci-GnRHR3 were the same peptides (at high concentrations) that activated the human GnRH1 receptor (Adams et al., 2003). Even GnRH-2 (aka chicken GnRH-II), a conserved form of GnRH found from cartilaginous fish to humans, was highly effective at receptor-induced cAMP accumulation in all three active *Ciona* GnRH receptors. Unique stimulation profiles for each receptor indicate that each may have distinct functions within *Ciona*. The delineation of the physiological function of each *Ciona* GnRH receptor may reveal clues to the origin of all GnRH receptor subtypes and may uncover functions that are masked in vertebrates.

4.3. Comparison of GnRH system in Ciona with other models

When GnRH signaling components are compared to most other model animals, *Ciona* has a higher number of both ligand and receptor genes. Human, chicken and zebrafish all possess two intact GnRH genes, each encoding a single GnRH decapeptide (Seeburg and Adelman, 1984; Dunn et al., 1993; White et al., 1998; Steven et al., 2003; Morgan and Millar, 2004). Humans possess a type I 'tail-less' GnRH receptor and they encode a type II receptor that may not be functional due to the lack of a Met translation start codon and a frame-shift mutation leading to a premature stop codon (Millar et al., 2004). One GnRH receptor transcript has been isolated and characterized from the chicken (Sun et al., 2001) and another has been identified in the newly sequenced genome (see Ensembl). It appears that there are three full-length GnRH receptors encoded within the zebrafish genome with a fourth truncated pseudogene (Lethimonier et al., 2004). In amphibians, there are three distinct *gnrh* genes possessed by frogs, with a gene for *gnrh*-1 (aka mammalian *gnrh*) identified in the African clawed frog (*X. laevis*) (Hayes et al., 1994), a gene for *gnrh*-2 sequenced from bullfrog (*Rana catesbeiana*) (Wang et al., 2001b) and a novel *gnrh* gene for a frog-specific precursor isolated from the brown frog (*Rana dybowskii*) (Yoo et al., 2000). A single *gnrh* receptor transcript was isolated from *X. laevis* (Troskie et al., 2000), but three different types were found in the bullfrog (Wang et al., 2001a).

The mouse presents an interesting exception to the rule for GnRH and its receptors. Mice have a single gene encoding

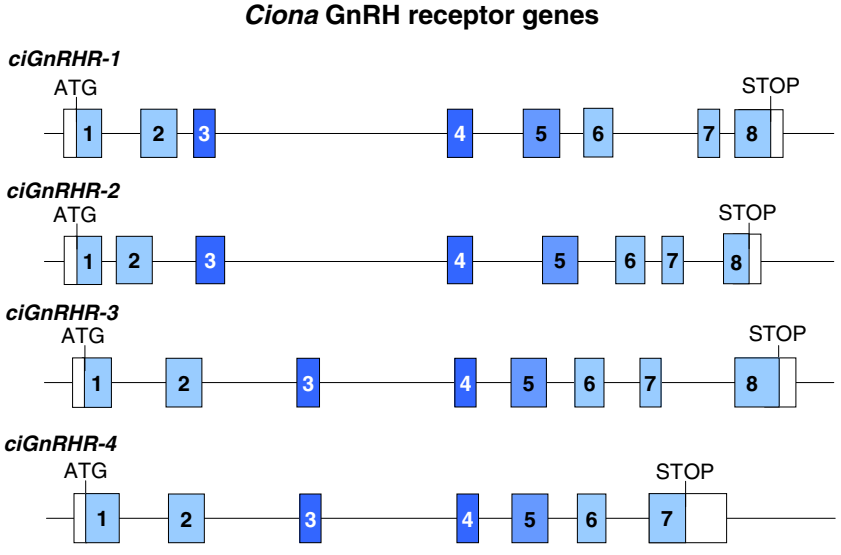


Fig. 4. Four gonadotropin-releasing hormone (GnRH) receptor genes cloned from *Ciona intestinalis*. The boxes with numbers inside represent exons. The dark blue exons with white numbers are tightly conserved in length; the medium blue exons are less conserved and the light blue exons are the least conserved among the four receptors. Receptors 1–3 have been expressed in monkey COS-7 cells and shown to be activated by the six *Ciona* GnRH peptides and by the vertebrate GnRH2. Each of these receptors has 8 exons. Receptor 4 has been expressed in COS-7 cells, but is not activated by *Ciona* GnRHs or GnRH2 via the IP or cAMP paths. This receptor is missing exon 8, which codes for most of the intracellular tail. *Ci*, *Ciona*; GnRHR, GnRH receptor; ATG, nucleotides coding for the translation start site; STOP, the site at which a nucleotide codon signals the end of translation.

GnRH-1 but the locus for GnRH-2 is classified as a pseudogene (Morgan and Millar, 2004) due to a premature stop codon. The mouse also encodes a single type I GnRH receptor with the type II receptor gene absent. It is not clear how the mouse is affected by the loss of GnRH-2 and its receptor, both of which have been conserved in most jawed vertebrates. Also, a few other mammals such as the sheep lack a type II receptor.

GnRH gene homologs were not found in either the genomes of *C. elegans* or *D. melanogaster* (see http://www.ensembl.org/), but genes with some GnRH receptor-like homology are present in both species (Hauser et al., 1998; Millar et al., 2004). Staubli et al., 2002 showed that the fruit fly receptor, which is structurally related to GnRH receptors, did not respond to GnRH but was activated by adipokinetic hormone, a peptide with ends similar to GnRH at the N terminus (pGlu) and C terminus (Gly-NH_2). The absence of GnRH and any true cognate receptors in these two protostome genomes suggests that a functioning GnRH ligand-receptor complement is not present in these specific lineages. However, a unique 12-amino-acid GnRH peptide was identified in the invertebrate octopus (Iwakoshi et al., 2002), another protostome. Screening and comparison of homologous GnRH genes in hemichordates and echinoderms will be of great interest to clarify whether functioning GnRH ligands and receptors are present throughout the deuterostomes.

5. Characterization of a neurohormone system: cionin (CCK/gastrin family)

Cionin is a neuropeptide that was isolated as a protein from the neural ganglion of *Ciona* (Johnsen and Rehfeld, 1990) and has been localized in large nerve cells in the ganglion (Thorndyke and Dockray, 1986). However, cionin is a classical brain-gut peptide as shown by expression of both protein and cDNA in these two tissues (Monstein et al., 1993). Cionin is thought to be the common ancestor of both cholecystokinin (CCK) and gastrin because of a shared sequence of four amino acids at the C terminus of the peptide.

5.1. Cionin hormone in Ciona

Cionin is a peptide of eight amino acids that was purified from the neural ganglion (brain) of *C. intestinalis* and sequenced (Johnsen and Rehfeld, 1990). Cionin is clearly related to CCK and gastrin because the last four amino acids (Trp–Met–Asp–Phe-NH_2) are identical in all three peptides. In contrast, cionin has two sulfated tyrosines in positions 6 and 7 (counting from the conserved C terminus), whereas CCK and gastrin each have one sulfated tyrosine.

Cionin cDNA was sequenced from *Ciona* as a prepro-cionin of 128 amino acids (Fig. 5). Included in this precursor is a signal peptide (20 amino acids) and cionin (8 amino acids) along with a processing site at each end of the peptide (Monstein et al., 1993). Later, the gene was cloned and sequenced (Monstein, 1995). Unlike GnRH, only a single peptide is encoded in the cionin precursor. The function of cionin in the brain is speculated to be control of body wall and siphon movements (Thorndyke and Dockray, 1986; Johnsen and Rehfeld, 1990).

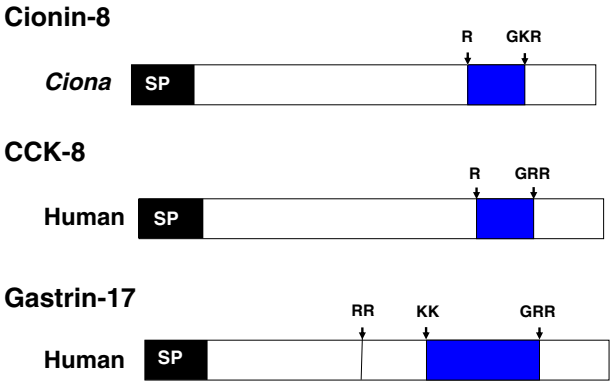


Fig. 5. A schematic diagram of the precursor proteins for cionin, cholecystokinin (CCK) and gastrin. The *Ciona* member of the family, cionin, is eight amino acids in length and shares four terminal amino acids with the vertebrate peptides of CCK and gastrin. Gastrin does not have a cleavage site to generate an eight amino acid peptide, but has other cleavage sites to produce 14, 17, 34 and longer gastrin peptides. Each peptide is amidated as shown by the sequence of amino acids (GKR or GRR) at the C-terminal end. SP, signal peptide; G, glycine; K, lysine; R, arginine.

5.2. Cionin receptor in Ciona

A cionin receptor has been tentatively identified after cloning of the cDNA from the gastrointestinal tract of *Ciona* (Nilsson et al., 2003). The gene that encodes the cionin receptor would be translated to a 526 amino acid protein. The deduced amino acid sequence indicated the receptor was a seven transmembrane molecule with 35–40% amino acid identity with mammalian CCK1 and CCK2 receptors and chicken and *Xenopus* CCK receptors (Nilsson et al., 2003). The *Ciona* receptor cDNA sequence was confirmed by comparison to that of the annotated gene in the draft genome of *C. intestinalis*. Radiolabeled CCK-8 did not bind sufficiently well to the cionin receptor expressed in COS-7 cells for competitive binding studies (Nilsson et al., 2003). Further characterization of the putative cionin receptor with the [^{125}I]-cionin will be necessary when the radiolabeled ligand becomes available. Nilsson proposed that the cionin receptor might be an ortholog of the vertebrate cholecystokinin receptors based on phylogenetic analysis.

Although more evidence is needed to show that cionin binds to its putative receptor and activates specific signaling pathways, cionin does bind to certain types of CCK receptors in vertebrates and has high biological activity. For example, synthetic cionin and sulfated CCK were equally potent in causing release of somatostatin from the stomach (dog) or contraction of gall bladder strips isolated from either a mammal (pig) or fish (rainbow trout, *Oncorhynchus mykiss*); both peptides had equal binding affinity for receptors in mammalian gall bladders (Schjoldager et al., 1991, 1995).

5.3. Comparison of cionin system with other models

The ancient CCK–gastrin family, which includes cionin, is always identified by the four amino acids (Trp–Met–Asp–Phe-NH_2) at the C-terminal end of the hormone as this region is required for biological activity. The functions of CCK and

gastrin in vertebrates are many. For example, CCK in the intestine can regulate gall bladder contractions and pancreatic enzyme secretion (Johnsen, 1998; Boushey and Drucker, 2003). CCK in the brain (cerebral cortex and limbic system) affects memory, sleep, sexual behavior, anxiety and food intake (Ganong, 2005; Boushey and Drucker, 2003). Gastrin is found not only in the stomach and intestine, where it stimulates gastric acid and pepsin release, but also is in the brain (hypothalamus, medulla oblongata, cerebellum) and pituitary, where the functions are unknown (Rehfeld, 1978, 1991; Johnsen, 1998; Ganong, 2005).

Members of this family have been identified by structure in many chordates including tunicates, fishes, amphibians, reptiles, birds and mammals (Johnsen, 1998). Johnsen suggests that a duplication of the gene in vertebrates led to two lines of peptides, CCK and gastrin, at least as early as cartilaginous fish. In contrast, tunicates such as *Ciona* have only a single gene encoding one hormone.

A comparison of cionin with vertebrate homologs shows that an extension of the N terminus can occur for both CCK and gastrin. In humans, the predominant CCK fragments are 14, 17 and 34 amino acids (Ganong, 2005); the N-terminal cleavage points for the latter two fragments are shown (Fig. 5). Also, the genes have been isolated for CCK and gastrin in a number of vertebrates (Johnsen, 1998). Of some interest is the change in the position of the sulfated tyrosine group counting from the C terminal; it is in position 7 in both vertebrate CCK and nonmammalian gastrin, but the group changes to position 6 in mammalian gastrin. In the ancestral cionin, both positions 6 and 7 have sulfated tyrosines.

In birds, reptiles, amphibians and fish, there is variation in the length of the N-terminal extension, in the degree of sulfation and even in the number of gene copies in certain tetraploid animals such as *Xenopus* and some fish. An interesting aspect of the CCK–gastrin family is a new member, caerulein that is in the skin glands in some amphibians. The molecule retains the C-terminal tetrapeptide and sulfated tyrosine in position 7, but is considered to be a side branch in the evolutionary story because it was only found in frog skin (Johnsen, 1998).

Nonchordate invertebrates do not have molecules that retain the C-terminal tetrapeptide that is considered the biological core of CCK–gastrin–cionin family. The insect sulfakinin peptides are present in a number of species including *Drosophila*. They have only two of four amino acids that are identical to those of the crucial tetrapeptide, although sulfakinins do have sulfated tyrosine in position 6 counting from the C terminus (see Conlon et al., 1988). In has been noted that sulfakinins share three of their final amino acids with FMRFamide, which adds to the hypothesis that sulfakinins are a separate family compared with that of CCK–gastrin (Johnsen, 1998). Another peptide family isolated from snails was thought to belong to the CCK–gastrin family. However, the structure shows that LymnaDFamides (isolated from *Lymnaea*) only share the final two amino acids (Asp–Phe-NH_2) with the CCK–gastrin family and hence are separate. In conclusion, cionin, CCK and gastrin are neuropeptides of the brain-gut variety, but are confined to chordates based on current evidence.

The receptors for cionin, CCK and gastrin bear a number of similarities as all are 7-transmembrane receptors. In *Ciona* only a single copy of the cionin receptor exists and is thought to be the common ancestor of the CCK1 and CCK2 receptors found in mammals. CCK1 receptor is found in peripheral tissues, whereas the CCK2 receptor, which binds both CCK and gastrin, is found in the central nervous system. Hence the gastrin receptor is identical to the CCK2 receptor. The single CCK receptors found in chicken and *Xenopus* resemble the mammalian CCK2 receptor closely. The two identified CCK receptors in fish (puffer fish, *Fugu rubripes*) appear to be orthologs of mammalian CCK1 and CCK2 receptors, respectively (Nilsson et al., 2003). There are three identified CCK-like receptors in *Drosophila*; one of which is a sulfakinin receptor. None of the three forms a clade with *Ciona* and vertebrate CCK receptors, leaving them in a category of "distantly related receptors" (Nilsson et al., 2003).

6. Characterization of a neurohormone system: insulin and IGF

The insulin superfamily of peptide hormones is composed of members regulating key aspects of several physiological processes. Members of the superfamily have been identified in a wide range of metazoans including protostomes (mollusks, nematodes and insects), protochordates, and chordates. In vertebrates, insulin is recognized for its metabolic role in glucose uptake after meals; the insulin growth factors (IGFs) have a variety of mitogenic functions; and the relaxins are best known for preparation of the birth canal for parturition. Also, the insect insulin-related peptides (IRPs) have been implicated in carbohydrate metabolism and ovarian development (for a review see Claeys et al., 2002). The role of insulin superfamily members in the regulation of lifespan has been established in a wide variety of species, most notably *C. elegans*.

6.1. Insulin and IGF in Ciona

A partial IGF-like sequence was procured from the genome of *C. intestinalis* (Satou et al., 2003). The cDNA of this transcript as well as a putative insulin-like molecule has been isolated and sequenced from *C. intestinalis* (Roch and Sherwood, unpubl.). The preproinsulin-like molecule encodes a peptide with distinct B-, C- and A-domains (Fig. 6). The B- and A-domains together share approximately 31% amino acid identity with the human insulin B-domain and A-domain. This sequence appears more derived compared with the only other previously characterized tunicate insulin, isolated from *C. productum*, which retains 64% identity with the B- and A-chains of human insulin (McRory and Sherwood, 1997). However, all six of the conserved cysteines which are critical for proper disulfide bridging in the insulin superfamily (Conlon, 2001) are found in B- and A-chains of the *Ciona* insulin-like peptide. It appears that the minimum complement of residues necessary to form the tertiary structure typical of vertebrate insulins (McRory and Sherwood, 1997) has been retained in the *Ciona* insulin-like peptide. Also, the *Ciona* peptide contains

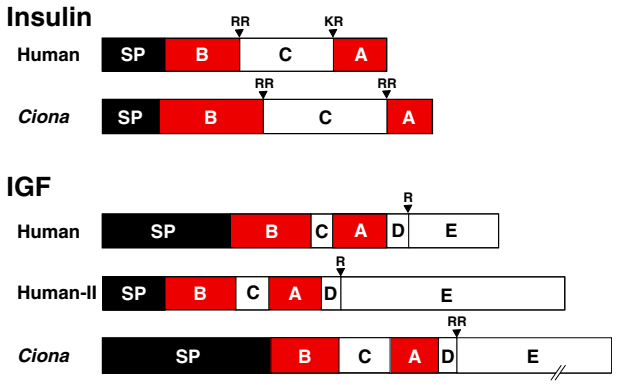


Fig. 6. A schematic diagram of the precursor proteins for insulin and insulin-like growth factor (IGF). The B and A chains in each precursor are conserved more highly than the C, D or E domains. In insulin, the C chain is cleaved from the human and other vertebrate molecules and is potentially cleaved from the *Ciona* as the appropriate cleavage sites (RR) are present. In IGF molecules, the C and D domains are retained in the active hormone, but the E domain is cleaved in the human forms. The E domain in the *Ciona* IGF is thought to be cleaved as the cleavage site of RR is present. SP, signal peptide; human II, human IGF-II.

residues thought to be critical in receptor binding (Kristensen et al., 1997).

The *Ciona* prepro-IGF-like molecule contains the requisite B-, C-, A-, D- and E-domains found in vertebrate IGF molecules (Fig. 6). If taken together, the conserved B and A domains retain an amino acid identity of about 28% with human IGF-I and IGF-II. An IGF molecule was previously characterized in *C. productum* and again its identity with the human IGF-I and IGF-II B-, A- and D-domains is higher; 40% overall. It is worth noting that the unusual fourteen amino acid repeat in the *Chelyosoma* IGF B-chain is not present in the *Ciona* peptide (Fig. 6). Similar to the insulin peptide, the IGF-like molecule still retains the six conserved cysteine residues and additional residues necessary to form the structure common to members of the insulin superfamily.

6.2. Insulin and IGF receptors in Ciona

A putative insulin/IGF receptor has been identified from the *C. intestinalis* genome (Satou et al., 2003) and is currently being sequenced (Roch and Sherwood, unpubl.). Although the receptor shows low amino acid similarity to its human counterpart (approximately 20%), it appears to retain all of the major structural elements found in other insulin receptors (Ullrich et al., 1986). In addition, an IGF-II/cation-independent mannose-6-phosphate (IGFII/M6P) receptor has been annotated in the genome. As the IGF-II receptor is thought to act as a 'clearance' receptor in other species, the single insulin/IGF receptor appears to be the only binding receptor for both insulin superfamily peptides in *Ciona*.

6.3. Insulin and IGF systems in model organisms

All vertebrate lineages more recent than the agnathans (jawless fishes) retain two distinct IGF moieties, IGF-I and IGF-II, in addition to distinct insulin peptides. Although the agnathan IGF appears to be equally homologous to IGF-I and IGF-II, the length of its signal peptide and its exclusive liver expression suggests it may be more closely related to IGF-I (Nagamatsu et al., 1991). The tetraploid *Xenopus*, as well as the mouse, have two copies of insulin, whereas humans possess one (Shuldiner et al., 1989; Wentworth et al., 1986; Bell et al., 1980). A search of the zebrafish genome yielded two insulin genes which have yet to be isolated (Irwin, 2004). The vertebrate ligands are well conserved and all possess the six conserved cysteine residues necessary for disulfide bridging along with identical sites for receptor binding (Conlon, 2001). In addition, the jawed vertebrates possess a single copy of the IGF-I and IGF-II genes, except for *Xenopus* and zebrafish which retain two copies of IGF-I (Shuldiner et al., 1990; Chen et al., 2001). The IGF-II genes for *Xenopus* and zebrafish are currently uncharacterized. However, a search of their sequence databases yielded a single copy of each gene, in addition to a novel IGF-like isoform for *Xenopus* (Roch and Sherwood, unpubl.). The human, mouse and chicken IGF-I and IGF-II genes are alternatively spliced to produce multiple distinct mRNA variants (Rotwein et al., 1986; Bell et al., 1986; de Pagter-Holthuizen et al., 1988; Rotwein and Hall, 1990; Fawcett and Bulfield, 1990; Darling and Brickell, 1996).

Within the invertebrates many copies of insulin-like genes can be found. A search of the *Drosophila* genome yielded seven insulin-like sequences with features common to their vertebrate counterparts including the prepro-insulin domain structure and the conserved cysteine residues (Brogiolo et al., 2001). The *C. elegans* genome contains 37 insulin-like sequences, however, many of these possess unusual features including extra or deleted cysteine residues, and as many as 22 may be pseudogenes (Pierce et al., 2001). Further expression analysis studies will be required to determine the genuine complement of insulin-like molecules.

Single copies of the insulin receptor gene are found in mammals which can be alternatively spliced into two isoforms (with or without the 11th exon) and a single isoform of the IGF-I receptor is known (Seino and Bell, 1989; Abbott et al., 1992). Partial fragments of a chicken insulin receptor and a full IGF-I receptor have been sequenced (Holtzenberger et al., 1996; Kato et al., 2000). In *Xenopus*, a single copy of the IGF-I receptor gene has been isolated (Zhu et al., 1998) and an insulin receptor-like gene can be found in the genome (Roch and Sherwood, unpubl.). Zebrafish has two copies each of the insulin and IGF-I receptors (Maures et al., 2002). The IGF-II/M6P receptor gene has been found as a single copy in humans and mice (Morgan et al., 1987; Szebenyi and Rotwein, 1994). Chickens and *Xenopus* both possess cation-independent mannose-6-phosphate receptors, but interestingly these receptors do not seem to bind IGF-II (Clairmont and Czech, 1989). Although a homologous receptor has not yet been characterized in zebrafish, a search of the genome sequence yielded two putative M6P receptor sequences.

Two isoforms of an insulin receptor homolog have been characterized in *Drosophila* with equal amino acid identity to the human insulin and IGF-I receptors (Fernandez et al., 1995). One of these isoforms retains a structure similar to the vertebrate receptors while the other has an extended cytoplasmic C-

Table 2
Gene model identifiers and/or accession numbers for ligands and receptors (R) in *Ciona intestinalis*

Gene	Gene model identifier	Genbank accession #
AdrenergicR Alpha-I	ci0100131416	
AdrenergicR Alpha-I	ci0100131572	
AdrenergicR Alpha-I	ci0100131143	
AdrenergicR Alpha-II	ci0100132133	
AdrenergicR Alpha-II	ci0100146328	
AdrenergicR Alpha-II	ci0100142398	
AdrenergicR Beta-II	ci0100130320	
AdrenergicR Beta-II	ci0100137803	
AdrenergicR Beta-II	ci0100130090	
AdrenergicR Beta-III	ci0100153326	
Angiotensin IIR Type 1	ci0100147553	
Angiotensin IIR Type 2	ci0100143330	
CalcitoninR	ci0100141557	
CannabinoidR1	ci0100149095	
CannabinoidR1	ci0100131120	
CannabinoidR1	ci0100131828	
ChemokineR C-C motif	ci0100142526	
ChemokineR C-C motif	ci0100130858	
ChemokineR C-C motif	ci0100141574	
ChemokineR C motif	ci0100137436	
Cionin		NM_001032539
CioninR	ci0100139179	NM_001032773
CionnR	ci0100144874	
Corticotropin Releasing FactorR	ci0100145837	
Corticotropin Releasing FactorR	ci0100145584	
DopamineR	ci0100146763	
DopamineR	ci0100130445	
EndothelinR	ci0100153613	
Epidermal Growth FactorR	ci0100134399	
Epidermal Growth FactorR	ci0100142360	
EphrinR Type A4	ci0100152405	
EphrinR Type A4	ci0100141382	
EphrinR Type A4	ci0100136974	
EphrinR Type A7	ci0100149186	
EphrinR Type A7	ci0100136022	
EphrinR Type B3	ci0100137109	
Fibroblast Growth FactorR	ci0100136189	
Fibroblast Growth FactorR	ci0100148947	
GalaninR	ci0100133550	
GalaninR	ci0100130986	
GalaninR	ci0100131794	
Glucagon-like PeptideR	ci0100145281	
Glucagon-like PeptideR	ci0100145252	
Glucagon-like PeptideR	ci0100141310	
GnRH-I	ci0100133548	AY204706, AY204707
GnRH-II	ci0100138973	AY204708, AY204709
GnRHR1	ci0100152622	AB103333, AY742888
GnRHR2	ci0100153146	AB103334, AY742889
GnRHR3	ci0100133065	AY742890
GnRHR4	ci0100134571	AY742891
Growth Hormone SecretagogueR	ci0100143040	
Growth Hormone SecretagogueR	ci0100147236	
Growth Hormone SecretagogueR	ci0100133464	
Growth Hormone SecretagogueR	ci0100137397	
Insulin Growth Factor	ci0100145885	
Insulin Growth Factor-IIR	ci0100133179	
Insulin receptor (part 1)	ci0100154612	
Insulin receptor (part 2)	ci0100142729	
MelanocortinR	ci0100143146	
MelanocortinR	ci0100132478	
MelatoninR Type 1	ci0100136041	
MelatoninR Type 1	ci0100132083	
MelatoninR Type 1	ci0100153785	
MelatoninR Type 1	ci0100131681	
Nerve Growth FactorR	ci0100144101	
Nerve Growth FactorR	ci0100133112	
Nerve Growth FactorR	ci0100141751	
Nerve Growth FactorR	ci0100142986	
NeurotensinR	ci0100143093	
OpioidR Type Mu	ci0100150689	
OpioidR Type Mu	ci0100140366	
OpioidR Type Mu	ci0100150840	
Opsin1	ci0100143990	
Opsin	ci0100132620	
Opsin	ci0100131351	
Parathyroid HormoneR1	ci0100139945	
Parathyroid HormoneR2	ci0100151327	
ProstaglandinER Type EP4	ci0100153844	
ProstaglandinER Type EP4	ci0100147407	
Serotonin 5-HT-1R	ci0100137935	
Serotonin 5-HT-1R	ci0100144199	
SerotoninR 5-HT-2R	ci0100134145	
Serotonin 5-HT-6R	ci0100145160	
Serotonin 5-HT-7R	ci0100140881	
Serotonin 5-HT-7R	ci0100148112	
Serotonin 5-HT-7R	ci0100131826	
Serotonin 5-HT-7R	ci0100148350	
Somatostatin and Angiotensin-likeR	ci0100143362	
Somatostatin and Angiotensin-likeR	ci0100143923	
SomatostatinR	ci0100153327	
SomatostatinR2	ci0100145437	
SomatostatinR2	ci0100131140	
SomatostatinR2	ci0100141702	
SomatostatinR2	ci0100146266	
SomatostatinR2	ci0100143261	
SomatostatinR2	ci0100148068	
SomatostatinR3	ci0100133606	
SomatostatinR3	ci0100135665	
SomatostatinR4	ci0100144360	
SomatostatinR5	ci0100143057	
SomatostatinR5	ci0100141602	
Transforming Growth FactorR Beta Type 1	ci0100144586	
Transforming Growth FactorR Beta Type 2	ci0100135103	
Vacular Endothelial Growth Factor	ci0100130196	

terminal tail believed to function similarly to the insulin receptor substrates (Fernandez et al., 1995). *C. elegans* retains the insulin-like receptor Daf-2, recognized for its role in the regulation of longevity (Kimura et al., 1997). It too contains a C-terminal extension that may function in place of an insulin receptor substrate (Kimura et al., 1997).

7. Other characterized neurohormone receptors

The *Ciona* genome contains G-protein-coupled receptors (GPCR) along with tyrosine kinase and nuclear receptors. In

addition to the *Ciona* GPCRs characterized and mentioned above (cionin receptor and GnRH receptors) there are a number that have been annotated in the genome. To date, tyrosine kinase receptors have not been functionally characterized for *Ciona*, whereas a few nuclear receptors have been characterized.

7.1. G-protein-coupled receptors

This superfamily of receptors includes a large number of receptors identified from yeast to humans. Tunicates such as *Ciona* share with vertebrates five major families of G-protein-coupled receptors (GPCR): rhodopsin, secretin, glutamate, adhesion and frizzled (Schioth and Fredriksson, 2005). *Ciona* lacks the vertebrate receptor families known as taste 2, OA1 and vomeronasal. Within the five major families in *Ciona*, the rhodopsin and secretin families are the most relevant for neuroendocrine hormones, as we are not including neurotransmitters in this review. Each receptor shares a common structure with seven transmembrane (TM) domains containing hydrophobic amino acids that form α helices, one in each domain. Variation among receptors tends to be in the extracellular N-terminal region, in the intracellular C-terminal region and the connecting regions of three extracellular loops (ECL) and three intracellular loops (ICL). Also, each receptor can couple with one or more G proteins to activate (or inhibit) a cascade of events in different signaling pathways.

The rhodopsin family is the largest of the five GPCR families and has been divided into 13 groups (or branches). *Ciona* has receptors in 9 of these groups, but not all contain neurohormone receptors. The rhodopsin family can be distinguished from the other four families by a short N terminus. Ligands, including the neurohormones that bind to these receptors, are thought to bind in a pocket among the transmembrane regions of the receptor with only a small component of binding to the N terminus. In addition, there are sequences within the rhodopsin receptors that identify them such as the sequence within TM7 (NSxxNPxxY) and the one at the junction between TM3 and ICL2 (DRY motif or D(E)-R-Y(F)).

Ciona has a number of receptors that could be considered as possibly having a neuroendocrine ligand. To date these receptors have been identified primarily in the genome (http://www.jgi.doe.gov/ciona) where they are annotated as to their sequence and their percent identity with receptors sequenced from other species, both vertebrate and invertebrate. From this resource (Table 2) the following receptors are annotated in the different rhodopsin receptor groups. Group one, the amine group, in *Ciona* includes annotated adrenergic receptors of both α and β subtypes, dopamine and serotonin receptors. Some of these molecules are considered to be neurotransmitters, but adrenaline and noradrenaline are considered to be both transmitters and hormones and so will be discussed below. Group two, the peptide group, includes receptors for GnRH and cionin (both isolated as cDNA), and annotated receptors for endothelin, growth hormone secretagogue, and neurotensin. Both melanocortin and cannabinoid receptors (group 3) are annotated in the *Ciona* genome. In the opsin group (group 4), there is an annotation for 'vertebrate ancient opsin' or pineal gland-specific opsin. Also, three *Ciona* opsins have been identified by cDNA sequence and localized in larval photoreceptor or brain cells by in situ hybridization or immunohistochemistry (Tsuda et al., 2003). In the somatostatin-opioid-galanin group (group 5), several receptor types have been annotated for somatostatin, and at least one opioid and one galanin receptor have been noted for *Ciona*. Also annotated are melatonin receptors (group 7). A prostaglandin receptor (group 8), chemokine receptors and an angiotensin receptor (group 9) are also noted in the *Ciona* genome. Of all these receptors, only GnRH receptors have been confirmed by cDNA and functional studies; cionin receptors are verified by cDNA sequence only (see Section 5.2); and the presence of adrenergic receptors has been supported by physiological and pharmacological studies (see below).

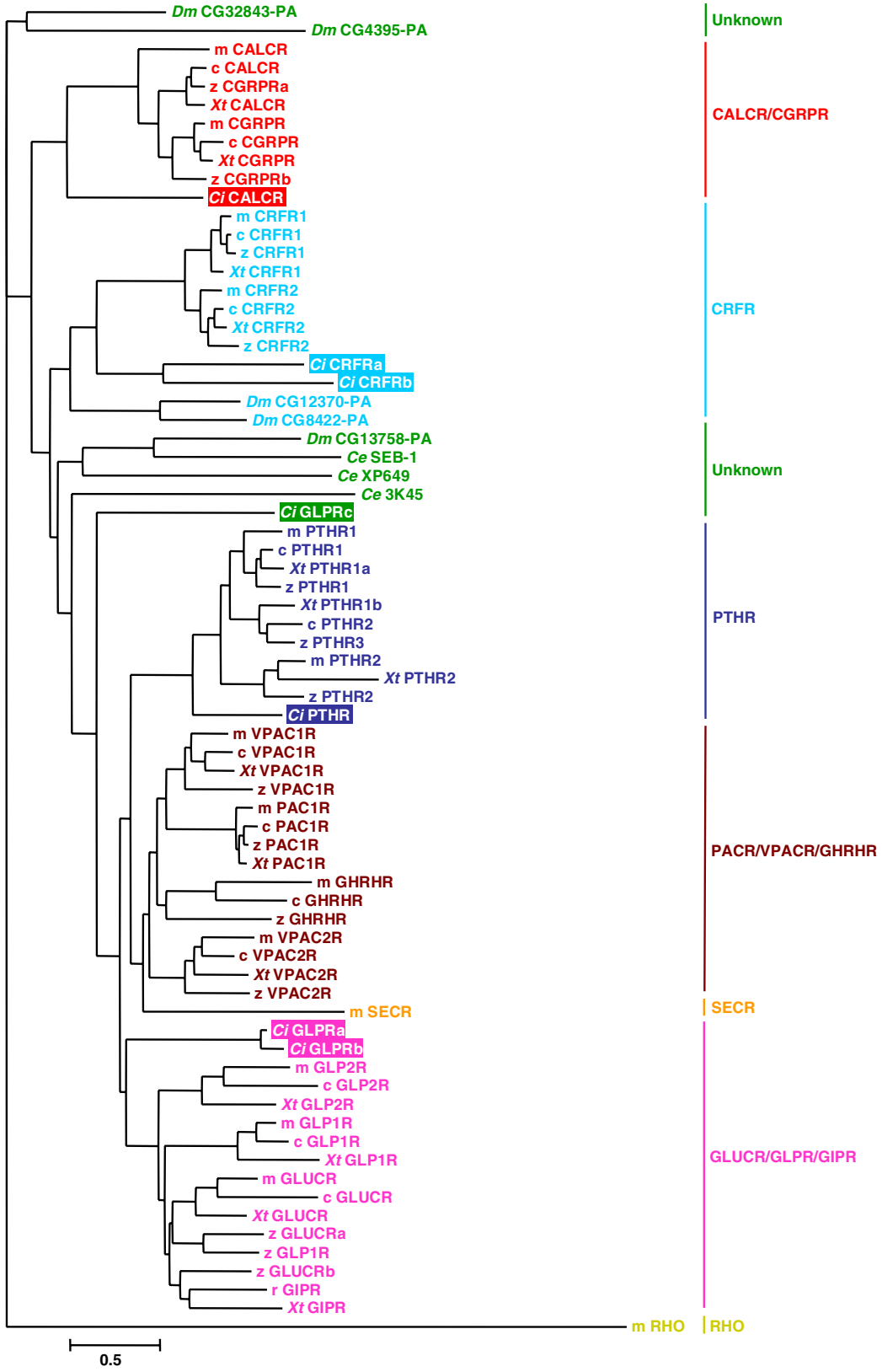
The β-adrenergic receptor (β-AR) has been deduced in experiments that show adrenaline and noradrenaline can accelerate the onset of metamorphosis in *C. savignyi* larvae (Kimura et al., 2003). As well, an agonist (isoproterenol) of the β-AR induced tail resorption. Further evidence was the demonstration that antagonists of the β-AR (propranolol) and β_1-AR (metoprolol) inhibited noradrenaline-induced tail resorption. In contrast, an antagonist of the β_2-AR (butoxamine) or of the α-AR (phentolamine) did not inhibit tail resorption in response to noradrenaline. Finally, immunocytochemistry was used in *C. savignyi* larvae to show cross-reactivity of specific antibodies with the β_1-AR or with dopamine β-hydroxylase enzyme, which is needed in the synthesis of adrenaline and noradrenaline. These functional data are important in understanding the role of the hormones and receptors, and emphasize the need to isolate and sequence the receptors.

Another seven-transmembrane receptor that appears to be specific to the tunicate lineage is *Ciona* neural tube specific gene (*Ci-Nut*). Of great interest is that *Ci-Nut* mRNA is present in the gastrula stage in six cells that will give rise to the entire brain. As these cells divide, eventually all neural tube cells express *Ci-Nut* mRNA in the neurula (Etani and Nishikata, 2002). Although the ligand is not yet identified, Etani and Nishikata (2002) suggest that the CiNut receptor may have an important role in the morphogenesis of the neural tube.

The secretin family of GPCR receptors is recognized by ligand-binding N termini which contain conserved cysteine bridges (Fredriksson et al., 2003). By genomic analysis, *Ciona* appears to retain several of the secretin-family receptors found in genomes of the other model organisms discussed here. These include receptors for corticotropin-releasing hormone, calcitonin or calcitonin gene-related peptide, parathyroid hormone, and glucagon or glucagon-like peptides (Fig. 7). Cardoso et al. (2005) also analyzed the *Ciona* receptors within the secretin family but their study used comparisons to somewhat different organisms (Cardoso et al., 2005). We found that the *Ciona* genome does not appear to contain receptors for glucose-dependent insulinotropic peptide (GIP, also known as gastric inhibitory peptide), growth hormone-releasing hormone, pituitary adenylate cyclase-activating peptide (PACAP), vasoactive intestinal peptide I or II or secretin.

7.2. Tyrosine kinase receptors

A number of putative receptors with a tyrosine kinase domain have been deduced and annotated in the *Ciona* genome (Satou et al., 2003). Some of the *Ciona* receptors included in this group are epidermal growth factor receptor (EGF-R); transforming growth factor receptor (TGF-R, β type 1 and 2); fibroblast growth factor receptor (FGF-R); ephrin receptors with



6 family members; vascular endothelial growth factor receptor (VEGF-R); and nerve growth factor receptor (NGF-R). The neuroendocrine receptor that has been cloned in *Ciona* is the insulin/IGF receptor, which was discussed in Section 6.2.

7.3. Nuclear receptors

Nuclear receptors are a class of ligand-induced transcriptional regulators of genes that control many diverse functions such as growth, development, differentiation, metabolism, reproduction, and homeostasis. The most striking observation for endocrinology is that *Ciona* does not appear to synthesize either "classical" steroid hormones or their corresponding steroid receptors, which includes estrogen receptors, androgen receptors, progesterone receptors, glucocorticoid receptors, and mineralocorticoid receptors (for a review, see Bertrand et al., 2004; Campbell et al., 2004). Likewise, orthologs have not been found in the genome for either *Drosophila* or *C. elegans*. However, the presence of an estrogen receptor in mollusks lends support to the notion that *Ciona*, *Drosophila*, and *C. elegans* have secondarily lost these components for steroid signaling.

A screen of the *Ciona* genome has revealed 17 genes that encode nuclear receptor family members (Yagi et al., 2003). An examination of *Ciona's* nuclear receptors suggests that receptors such as the thyroid hormone receptor, retinoic acid receptor and retinoid X receptor may be transcription factors for certain neurohormones. Also, other factors may be involved, but are not yet characterized for possible roles in neurohormone transcription.

Very few of the *Ciona* nuclear receptors have been characterized. The *Ciona* ortholog of the thyroid hormone receptor (*Ci-nuclear receptor 1*) was cloned and expressed; it lacked the ability to bind triiodothyronine, probably due to poor homology in the ligand binding domain compared with vertebrate sequences (Carosa et al., 1998). Clones for *Ciona* retinoic acid receptor and retinoid X receptor were isolated (Nagatomo et al., 2003); mRNA for the former was detected in the anterior ectoderm and endoderm during gastrulation, whereas mRNA for the latter was ubiquitously expressed. Two gene products were found for the vitamin D receptor clade, although the second receptor was not found as an expressed sequence tag (EST). Another nuclear receptor, *tailless*, was present within the *C. intestinalis* genome, but transcriptional products were not shown as ESTs and the lack of certain domains suggested the receptor was not functional (Yagi et al., 2003).

8. Conclusion

The first phase of probing the *Ciona* genome has revealed a wealth of molecules that have been matched by amino acids and nucleotides to possible homologs from other genomes from yeast to human. For neuroendocrinology, the most startling results for *Ciona* were that pituitary hormones were not identified in the genome and only a partial set of enzymes were present for the synthesis of steroids. Likewise, the nuclear receptors for steroids were missing (Campbell et al., 2004). The immediate question is whether there are neuropeptide releasing factors in *Ciona* and the nature of their action.

The most fruitful searches for molecules related to the *Ciona* neuroendocrine system have been for receptors. A number of receptors have been annotated in the three major categories of G-protein-coupled receptors, tyrosine kinase receptors and nuclear receptors. In the future, receptors of interest that have been annotated can be cloned, expressed and tested with specific neuropeptides for binding, activation and signaling pathways. The present concern is the need for a *Ciona* cell line in which receptors can be expressed and tested for activity. At present, cell lines from other species, especially the green monkey kidney cells (COS-7), are used for testing *Ciona* receptors.

The genome has been less forthcoming for identification of ligands than for receptors. Although six GnRH peptides, cionin, insulin and IGF have been annotated and cloned, other neurohormones have not been reported. Listed above in Section 7 are a number of receptors in the rhodopsin family and yet homologs for neuropeptide Y, neurokinin, vasotocin, somatostatin, endorphin, galanin and others have not been cloned. Also, the secretin-like receptors include glucagon-like peptides-1 and -2, corticotropin-releasing hormone, parathyroid hormone, and calcitonin, but again the peptides have not been identified.

The second phase of molecular studies with *Ciona* is functional genomics. This is a critical area for future studies in neuroendocrinology. If GnRH is taken as an example, a next step would be to use in situ hybridization to verify the immunocytochemistry results and to show whether genes *gnrh*-1 or *gnrh*-2 are expressed in various locations such as the nerve net around the dorsal strand or in primary sensory cells. A further step would be to determine the location of the GnRH receptors, as above. Knowledge of receptor location reveals the target tissues for GnRH, which can be used in designing experiments to study the effect of peptide injection or gene knockdown. Each ligand or receptor gene can be knocked down

Fig. 7. Phylogenetic tree of secretin family of receptors in *Ciona* compared with those in the model species discussed in the current review: mouse (m), rat (r), chicken (c), *Xenopus tropicalis* (Xt), zebrafish (z), *Ciona intestinalis* (Ci), *Drosophila melanogaster* (Dm) and *Caenorhabditis elegans* (Ce). Sequences were aligned in the region between transmembrane domains 1 and 7 using ClustalW; an unrooted neighbor joining tree was constructed with mouse rhodopsin as an outgroup. The NCBI genbank database, the TIGR gene indices and the JGI genome database for *Xenopus tropicalis* and *Ciona intestinalis* were mined for sequence data. Because many sequences from *C. intestinalis*, *X. tropicalis*, chicken and zebrafish were taken from gene prediction models, a few sequences displayed low homology to their putative homologs and were grouped differently. Rat GIPR was substituted for mouse GIPR, as a complete receptor sequence was not available. As well, the zebrafish CRFR2 receptor sequence was truncated at transmembrane domain four. Two PTHR gene models were present in the genome database of *C. intestinalis* but we think they are identical duplicates from redundant scaffolds. The receptors are CALCR, calcitonin receptor; CGRPR, calcitonin gene related peptide receptor; CRFR, corticotropin-releasing factor receptor; GHRHR, growth hormone-releasing hormone receptor; GIPR, glucose-dependent, insulinotropic peptide receptor; GLP1R and GLP2R, glucagon-like peptide-1 or -2 receptors; GLUCR, glucagon receptor; PAC1R, pituitary adenylate cyclase-activating polypeptide-1 receptor; PTHR, parathyroid hormone receptor; SECR, secretin receptor; VPAC1R and VPAC2R, vasoactive intestinal polypeptide and PACAP receptors-1 and -2.

individually. Another approach is gain-of-function experiments in which transgenic *Ciona* carry a stably transfected gene for expression in normal or ectopic locations. *Ciona* offers great potential for detecting novel functions for GnRH that may be masked in a complex vertebrate.

Included in this phase are studies to understand the regulation of the neuroendocrine hormones and receptors, especially at the transcriptional level. Because the promoter-enhancer regions of each *Ciona* gene are relatively short, transcription factors and their nuclear binding sites are expected to be easier to identify than in most vertebrates. Large-scale projects to study regulation of *Ciona* genes are suggested in which "tissue-specific enhancers are mapped for every gene, and the regulation of every enhancer is understood at the level of interacting regulatory factors acting downstream of defined signaling pathways" (Corbo et al., 2001). A web site (http://www.hoya.hgc.jp) has been initiated recently to store information on the characterization of *cis*-regulatory elements in *Ciona*.

A third phase of investigation could be further bioinformatics comparing the *Ciona* genome to newly sequenced genomes. Amphioxus and an echinoderm are currently being sequenced, whereas the sequencing process for a hemichordate is at an earlier stage. Comparisons to these organisms, which bracket *Ciona* phylogenetically, will help to further identify molecules that are lineage specific to *Ciona* from ones that are homologs in other species.

Databases are being assembled that integrate sequence data with structural, functional and localization data for proteins (InterPro, European Bioinformatics Institute, Cambridge, UK). The addition of functional data is important and will further link *Ciona* with both invertebrate and vertebrate genomics. It is clear that *Ciona* is vital in understanding the evolution of the chordates and the origin and novel functions of neuropeptides.

Finally, it is important to emphasize the biological advantages of understanding the *C. intestinalis* and *C. savignyi* genomes. The phylogenetic position of these basal chordates is unique in that they appeared in evolution close before the vertebrates. Comparative sequence analysis of genomes that include tunicates can be used to identify genetic changes that occurred at the transition between invertebrates and vertebrates. These evolutionary changes can be partially deduced by comparing the genomes of *Drosophila* and *C. elegans* to human. However, *Ciona* is closer to humans than other invertebrates and so the advantage is clear in detecting genes that are specific to the vertebrate lineage. Analysis of the *C. intestinalis* genome shows that about 60% of *Ciona* predicted genes have homologs in *Drosophila* and/or *C. elegans*; about 20% of *Ciona* genes are specific to its own lineage; and about 17% of *Ciona* genes are shared only with vertebrates and not with fruit fly or nematode (Dehal et al., 2002). Two of the most important results from comparative sequence analysis of vertebrates and tunicates are that vertebrates had duplications of the genome early in their evolution and had major developments in the nervous and immune systems (Dehal et al., 2002). Analysis of the *Ciona* genome showed that "at least one member of most vertebrate gene families" is present in *Ciona* (Cooper and Sidow, 2003). Knowledge of the *Ciona* genome is a further advantage as *Ciona* is a good model for embryological studies in that there is a strong similarity in the presence of a notochord and dorsal neural tube between tunicate larvae and vertebrate embryos. The *Ciona* model outlined in Table 1 is an example of the interface between evolution and development. Experimentation on the embryo can be used to deduce the function of a number of genes in *Ciona* and determine if the function is conserved in evolution. Also, comparative sequence analysis has provided a major advantage in identifying regulatory regions, both coding and noncoding. The *Ciona* genome is only 1/20 the size of the human genome, although there are about half the number of genes (Dehal et al., 2002). This dense organization in the *Ciona* genome means there is less distance between genes and more compact regulatory regions. Genome wide searches for noncoding regulatory regions have been successful in both *C. intestinalis* and *C savignyi* (Johnson et al., 2004, 2005). The *Ciona* genome has provided a significant resource for understanding the evolution of the neuroendocrine system and the origin of the vertebrates.

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