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Differential expression and intrachromosomal evolution of the sghC1q genes in zebrafish (*Danio rerio*)

Tristan M. Carland [a], Jeffrey B. Locke [a,b], Victor Nizet [a,b,c], Lena Gerwick [a,*]

[a] Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA, USA
[b] Department of Pediatrics, University of California, San Diego, La Jolla, CA, USA
[c] Skaggs School of Pharmacy & Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA, USA

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ABSTRACT

The secreted globular head C1q (*sghC1q*) genes can be characterized as a family of genetic loci encoding signal peptides followed by single complement component 1q globular (gC1q) motifs. Members of this family have been referred to as precerebellin-like (Cblnl), C1q-like or ovary specific C1q-like factors, and are transcribed in response to infection and/or during early development. This study was primarily undertaken to identify the zebrafish *sghC1q* (or *DrsghC1q*) genes that increase their transcription in response to infection and to examine their transcriptional patterns during early development. Twenty *sghC1q* genes were found in the zebrafish (*Danio rerio*) genome (Zv9). Two of *the examined twenty genes* showed significant up-regulation within 24 h of infection with the fish pathogen *Streptococcus iniae,* and eleven of the examined twenty were expressed during early development. Due to the clustered nature of these genes on chromosomes two and seven, intrachromosomal duplication events are hypothesized and explored.

1. Introduction

The immediate innate immune response (acute phase response) has been characterized to a certain extent in humans and mice (Murphy et al., 2008), and is beginning to be explored in several fish species through proteomic and transcriptional studies. These fish species include rainbow trout (*Oncorhyncus mykiss*) (Gerwick et al., 2007; Raida and Buchmann, 2009), catfish (*Ictaluras punctatus*) (Peatman et al., 2007), zebrafish (*Danio rerio*) (Hegedus et al., 2009), turbot (*Scophthalmus maximus*) (Pardo et al., 2008), Japanese flounder (*Paralichthys olivaceus*) (Dumrongphol et al., 2009), carp (*Cyprinus carpio*) (Gonzalez et al., 2007), tilapia (*Oreochromis mossambicus*) (Ndong et al., 2007), large yellow croaker (*Pseudosciaena crocea*) (Yan et al., 2009) and hybrid striped bass (*Morone saxatilis x M. chrysops*) (Pasnik and Smith, 2006) among others. Changes in gene transcription occur soon after infection, and as such, the newly transcribed genes can be considered to encode acute phase proteins. The acute phase response is an organism's response to physiological insult (infection, injury, etc.), or is alternatively defined as any time during which the organism is not in homeostasis (Bayne and Gerwick, 2001). Acute phase proteins are those experiencing altered synthesis during the onset of non-homeostasis, as measured by changes in transcript or protein abundance. The onset of the altered transcription is likely variable between different acute phase genes. However, numerous studies have found transcription of these genes to be activated and peaking within the first 8–24 h post infection (hpi), sometimes lasting for days, though the response tends to subside within 24–48 h (Baumann and Gauldie, 1994; Cray et al., 2009). Many acute phase proteins are secreted from the liver into the plasma (vertebrates) or from the hepatopancreas or equivalent organ into the hemolymph (invertebrates). In previous studies the hepcidin gene rapidly increased its transcription between 4 and 48 hpi (Bayne et al., 2001; Lauth et al., 2005), haptoglobin's transcription increased by 12 hpi (Giffen et al., 2003; Quaye, 2008), and mannose-binding lectin was detected for multiple days with the transcription only occurring in the liver (Sastry et al., 1991). In addition, several complement components, transferrin and the precerebellin-like protein (*Cblnl*) transcripts have been detected at 24 hpi (Peatman et al., 2007; Gerwick et al., 2007). All these examples fit the definition of being acute phase genes (Baumann and Gauldie, 1994).

The Cblnl protein was first discovered as an acute phase protein in rainbow trout (Gerwick et al., 2000). This protein was so named

Abbreviations: hpi, Hours Post-Infection; Cbln, Precerebellin; Cblnl, Precerebellin-like; gC1q, globular domain of Complement component 1 (sub-component q); sghC1q, secreted globular head C1q; DrsghC1q, *Danio rerio* sghC1q genes/proteins; CFU, colony forming units; TGIP, the gene indices project; NCBI, National Center for Biotechnology Information; EST, expressed sequence tag; PSI-BLAST, position specific iterated – basic local alignment search tool; BLAT, BLAST-like alignment tool; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

* Corresponding author. Address: 9500 Gilman Drive MC0212, La Jolla, CA 92037, USA. Tel.: +1 858 534 0566; fax: +1 858 534 0576.

E-mail addresses: tcarland@ucsd.edu (T.M. Carland), jlocke@triusrx.com (J.B. Locke), vnizet@ucsd.edu (V. Nizet), lgerwick@ucsd.edu (L. Gerwick).

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because it shared 53% amino acid sequence similarity to precerebellin (Cbln) (Urade et al., 1991), and in addition, shares 47% identity with the B – chain of the first complement component (C1qB). Precerebellin is considered a precursor to the neuropeptide cerebellin (Umrath and Silberbauer, 1967). The Cbln and Cblnl proteins differ in sequence and structure from complement component 1q (C1q), as they lack an N-terminal sequence that folds into an alpha-helical collagen structure (Carland and Gerwick, 2010). There are currently four *cbln* homologues (*cbln1-4*) in mammals. These genes (*cbln*) are largely expressed in the brain, notably during development (Wei et al., 2007; Rucinski et al., 2009), and appear to function in the formation and stabilization of synaptic contact and the control of functional synaptic plasticity between cerebellar granule cells and Purkinje cells (Hirai et al., 2005). The four genes exhibit differential expression in the mouse brain during development and in adults (Miura et al., 2006). All of these proteins are able to form homomeric and heteromeric trimers via their shared C1q domains and larger assemblies (dimers of trimers) by disulfide bonds from their respective dual cysteine residue motifs (Bao et al., 2005). It has also been shown in mice that expression of *cbln1* can modulate the trafficking of Cbln3 out of the endoplasmic reticulum (Iijima et al., 2007). The Cbln and Cblnl proteins all fit within the secreted globular head C1q (sghC1q) protein family recently reviewed by Carland and Gerwick (2010).

The complement system is an innate defense mechanism that can lead to the eradication or opsonization of pathogens and damaged tissues. Complement can be triggered by recognition of substrates by complement component 1 (C1), lectin, or C3 tick-over. C1q is an important structural and binding component of C1, a protein complex consisting of C1r, C1s, and C1q. The C1 protein complex initiates the complement cascade leading ultimately to the formation of the final pore-forming membrane attack complex. The C1q molecule itself interacts with several ligands including recognition molecules on pathogens and responds through either C1qR receptor mediated phagocytosis or via the deposition of C1r and C1s on the cell surface leading to activation of the complement cascade (Kishore and Reid, 2000). More recently C1q expression was identified in postnatal neurons and found to mediate elimination of inappropriate synaptic connections during development (Stevens et al., 2007).

Like the Cbln proteins, the Cblnl proteins have a relatively high amino acid sequence identity to C1q, due to their shared globular C1q domain (gC1q) composed of ten beta strands (Fig. 1) folding into a beta-barrel formation (Ghai et al., 2007). The *C1q* domain has been genetically conserved and replicated throughout vertebrate evolution as there exist at least 31 C1q-domain-containing proteins within the human (*Homo sapiens*) genome (Tom Tang et al., 2005), at least 52 in the zebrafish genome (Mei and Gui, 2008) and 75 in the amphioxus (*Branchiostoma floridae*) genome (Huang et al., 2008). These C1q domain-containing proteins are considered a structural family and have a diverse range of functions. Kishore et al., 2004, grouped the C1q domain proteins into three subfamilies based on their sequence similarities to the A, B or C chains found in the heterotrimeric heads of the hexameric human C1q protein. The Cblnl family of proteins fit as members of the B group (Kishore et al., 2004). We have recently proposed a more structurally based classification as a further clarification of the gC1q family (Carland and Gerwick, 2010). The sghC1q group can be identified by their: (a) tendency to contain an N-terminal signal peptide, (b) C-terminal gC1q domain, (c) size of between 100 and 300 amino acids, and (d) lack of an N-terminal collagen-like region.

Studies of proteins containing C1q domains have recently been undertaken in a few aquatic organisms. In surfperch, (*Neoditrema ransonnetii*) the protein was isolated using a fucose affinity column. The C1q domain protein in the Zhikong scallop (*Chlamys farreri*) has the capacity to bind lipopolysaccharide (Nakamura et al., 2009; Zhang et al., 2008). Transcriptional work in the mussel (*Mytilus galloprovincialis*) has revealed significant upregulation of MgC1q after infection and the likelihood of adaptive molecular features based on positive selection analyses (Gestal et al., 2010). The goldfish (*C. auratus*) ovary specific *C1q*-like (CaO*C1q*-like) protein contains a collagen region and appears to only be expressed in follicular epithelial cells (Mei et al., 2008a). The previously identified C1q-like factor from zebrafish fully fits the characteristics of the Cblnl family of proteins and it was shown to inhibit p53-mediated apoptosis during head and craniofacial development (Mei et al., 2008b).

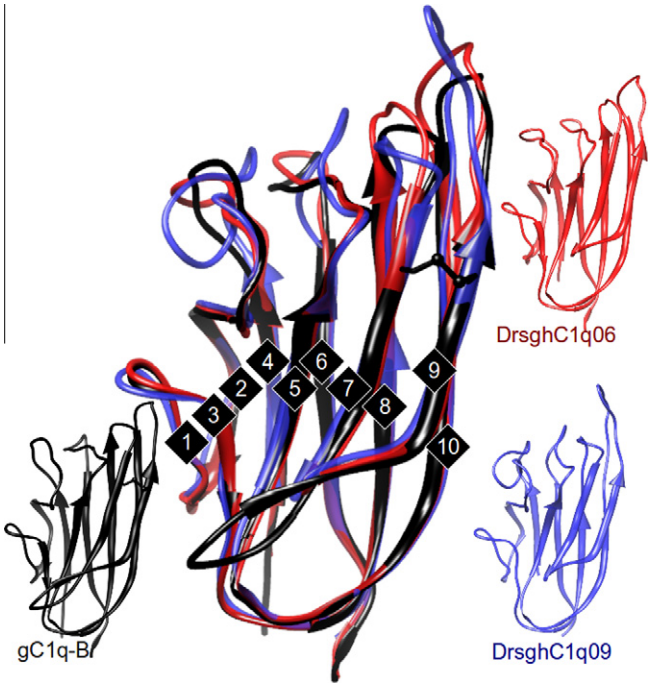

Fig. 1. DrsghC1q Modeling. The models seen here are a combination of chain B of the globular portion of mammalian C1q (gC1q-B in black) as determined by X-ray crystallography and computational predictions of the structures for sghC1q06 (red) and 09 (blue). The large model is a combination of the three, illustrating the conservation of the ten beta-strands (numbered in black). Also shown in black is the disulfide bond known to mammalian C1q and some of the *DrsghC1q* genes. Graphics developed in the Chimera viewer (Pettersen et al., 2004). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Zebrafish was chosen as a model organism for further functional studies of the sghC1q genes due to its extensive utility in functional genomic studies (Yoder et al., 2002) and as a model for studying extensive gene duplication events. In order to characterize the sghC1q genes upregulated in zebrafish during the inflammatory response, we identified twenty genes in the zebrafish genome that fit the description of an sghC1q gene (Carland and Gerwick, 2010). These *twenty* genes were explored by transcriptional profiling during two different physiological conditions; the first in response to an inflammatory stimulus and the second during early development. Our study of the *sghC1q* genes includes the four previously identified *cbln* genes (Mei and Gui, 2008) as well as the previously studied *C1q*-like gene (Mei et al., 2008b).

2. Materials and methods

2.1. Zebrafish maintenance

Adult zebrafish (*Danio rerio*) were purchased from Aquatica Tropicals (Plant City, FL) and maintained at 28 °C in a three tier

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Table Top Rack (Aquaneering Inc. San Diego, CA) following standard husbandry procedures for care and feeding (Westerfield, 1995). When not breeding, male and female fish were cohabitated to prevent harmful overproduction of eggs. Fish were fed a dry flake mix (57% Aquatox Flake, 19% Spirulina Flake, 8% Hikari Micropellet, 8% Cyclop-eeze, 4% Golden Pearl 300–500 and 4% Golden Pearl 500–800) and freshly grown brine shrimp two to three times daily.

2.2. Zebrafish breeding and egg collection

On the day prior to breeding, male and female fish were recollected and placed in a standard 2-way fish breeder (Petsmart, Inc.) in the afternoon and kept separated by a clear plastic divider during the night without tank water system flow. At daybreak (at least 16 h after separation) the divider was removed and fish were given privacy for 1 h. The adult fish were then removed and the eggs were collected and sorted with plastic transfer pipettes (VWR, Cat.# 16001-174) and considered $T = 0$. Eggs were kept in a salt solution with 0.001% methylene blue (Westerfield, 1995). Eggs were collected at 12, 24, 36 and 48 hpf and placed in 1.5 ml microcentrifuge tubes with 100 μl TRIZol ® Reagent (Invitrogen Cat#15596-026). The collected eggs were flash frozen in liquid nitrogen followed by storage at −80 °C. This experiment was repeated a second time.

2.3. Bacteria culture, strain and preparation

Streptococcus iniae strain K288 was isolated from the brain of a diseased hybrid striped bass at the Kent SeaTech aquaculture facility in Mecca, California (Buchanan et al., 2005). *S. iniae* was grown at 30 °C in Todd Hewitt Broth (THB) or on Todd Hewitt Agar (THA) (Hardy Diagnostics). Prior to injection, an overnight culture of *S. iniae* was diluted 1:10 in fresh THB and grown to mid-log phase (optical density, $OD_{600} = 0.40$). A 1.0 ml aliquot of the culture was centrifuged at 3500×*g* for 5 min, washed once in an equal volume of phosphate buffered saline (PBS) (Gibco) (no calcium or magnesium), pelleted, and resuspended in PBS. Bacteria were then diluted in PBS to a final concentration of 3.5×10^5 CFU/ml and held on ice until injected.

2.4. Bacterial infection and zebrafish liver collection

Male zebrafish were placed in independent challenge tanks and allowed to acclimate for one week. The separate tanks had the same water parameters and the fish were given a reduced feed cycle (once daily). Fish were anesthetized in Tricaine (3-aminobenzoic acid ethyl ester, Sigma–Aldrich, St. Louis, MO) and challenged via intraperitoneal injection of 10 μl of *S. iniae* (3.5×10^3 CFU) or PBS (control) using a 0.3 cc syringe and 29 g needle as previously described (Phelps et al., 2009). At 12 and 24 h after the injection fish were snap frozen in liquid nitrogen and their frozen livers removed. Three livers were pooled for each sample in 200 μl of TRIZol ® Reagent and kept frozen in liquid nitrogen. Each time point had two biological replicates and the entire experiment was repeated once. After injection the CFU were confirmed through serial dilution of the starting inocula and plating on THA. Fish challenges were carried out in an AAALAC certified facility following IACUC approved protocols.

2.5. Gene discovery and annotation

Using the previously characterized trout *cblnl* protein sequence [NCBI: NP_001117737] as the query, tBLASTn and BLASTp alignments were performed against the zebrafish data available from TGIP (webref) and at NCBI (webref) as well as PSI-BLAST alignments (NCBI only). The resulting sequences (>500) were built into contiguous sequences with CAP3 (Huang and Madan, 1999) where appropriate and their ORFs extracted via web-service pipeline EST Keeper (Carland and Gerwick, unpublished). The best alignments were screened for size (<300 AA), absence or presence of a signal peptide using the program SignalP (Emanuelsson et al., 2007), and the presence of the beta-strand motifs that characterize this family via protein modeling (detailed below). The locations of the genes on their corresponding chromosomes were discerned using BLAT and BLASTn alignments against the Zv9 version of the zebrafish genome from Ensembl (webref).

2.6. Primer design

Full-length cDNA sequences and their corresponding genomic sequences aligned in Spidey (webref) to determine exon location to guide primer design. Primer3 v. 0.4.0 (webref) was used to design primers that would cross or span intron/exon boundaries with amplicon sizes between 80 and 120 bp. Primers were obtained from Sigma–Aldrich.

2.7. Phylogenetic analysis

All of the *sghC1q* genes from zebrafish and one from amphioxus (as an out-group) were aligned by codon using MUSCLE (Edgar, 2004) and loaded into TOPALi (Milne et al., 2009) for subsequent model selection testing and Bayesian tree phylogenetic analysis (Ronquist and Huelsenbeck, 2003; Anisimova and Kosiol, 2009). Codon substitution model settings (obtained from model selection testing) are as follows: 1000,000 generations, 65% burn in, HKY substitution model for codon position one with gamma and invariable sites, HKY substitution model again for codon position two with gamma and without invariable sites, K80 substitution model for codon position three without gamma or invariable sites, no parameter linking across codon positions.

2.8. Protein modeling

Protein models were generated through submission of amino acid sequences to the M4T modeling server (Fernandez-Fuentes et al., 2007) with analysis performed using default settings. Models are displayed using the UCSF Chimera package (Pettersen et al., 2004).

2.9. RNA isolation and cDNA synthesis

The embryonic and liver tissues were homogenized by manual force using a plastic pestle (VWR Cat# KT749520-0500) in a 1.5 ml microcentrifuge tube and 200 μl of TRIZol. Total RNA was isolated from the tissue following the TRIZol protocol for isolating RNA from animal tissue (Invitrogen, Carlsbad, CA) followed by cDNA synthesis using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA). Total RNA (800 ng/sample) were incubated with Superscript III at 55 °C for 1 h following manufacturer's protocol for First Strand Synthesis.

2.10. Semi-quantitative Reverse Transcriptase PCR

Upon obtaining cDNA samples corresponding to the embryonic time points collected, PCR reactions were performed using all combinations of embryonic time point derived cDNA and *cblnl* primer pairs. PCR reactions were set up as follows: 8 μl 2x MasterMix (Promega, Cat #PAM7505), 0.5 μl of 25 mM Mg^{2+}, 0.5 μl of both forward and reverse primers at 20 μM each, 1 μl cDNA template (100 ng/μl) at 1:50 dilution and water for a final reaction volume of 20 μl. The subsequent PCR reaction (Eppendorf gradient

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thermocycler) was programmed to run at 95 °C for 3 min followed by 32 cycles of 95 °C for 15 s, annealing of 57 °C for 15 s and extension of 72 °C for 15 s, with a final elongation step of 72 °C for 2 min. Gel electrophoresis was performed with 5 μl of each PCR product mixed with 1.5 μl 5x Loading Dye in a 1% agarose gel stained with 1x SYBR Gold (Invitrogen, Carlsbad, CA) at 90 Volts for 55 min in sodium borate buffer. All resulting amplicons were sequence verified.

2.11. Quantitative Reverse Transcriptase PCR (qRT-PCR)

After validating primers and optimizing PCR conditions by use of agarose gel electrophoresis (as mentioned above) to ascertain that amplification yielded a single product of the predicted size, qPCR reactions were set up as follows: 10 μl of SYBR® Premix Ex Taq™, 0.4 μl of a 50x ROXII™ solution (Cat#RR041A, Takara Mirus Bio Inc.), with primers at 200 nM final concentration, 1 μl cDNA template at 1:50 dilution, and dH_2O for a final reaction volume of 20 μl. An MX3000p qPCR thermocycler (Stratagene) was programmed to run at 95 °C for 30 s, 40 cycles of 95 °C for 15 s, annealing of ~53 °C for 15 s and extension of 72 °C for 15 s. The qRT-PCR protocol for each target gene was validated by melting curve analysis to ensure the absence of primer-dimers or other unwanted amplicons. The relative expression levels of the *sghC1q* transcripts were calculated by using the delta delta cT method and normalized by the expression level of *ef1a*. The MxPro v4 (Stratagene) software package was used to analyze raw data and OpenOffice.org Calc (Sun Microsystems, Inc.) was used to perform Student's *t*-tests of the means from infected and control livers and to graph the resulting fold changes following established protocols (Schmittgen and Livak, 2008).

3. Results and discussion

3.1. Intrachromosomal duplications as deduced by wide expansion, phylogeny and conserved predicted protein structure

The databases of zebrafish cDNAs and ESTs available at The Gene Indices Project (TGIP) website (Lee et al., 2005) were investigated using the tBLASTn and BLASTp algorithms (Altschul et al., 1990) using the sequence encoding the conserved C1q domain from the trout Cblnl sequence (Gerwick et al., 2000) and the zebrafish Cbln sequences identified by (Mei and Gui, 2008) as a queries. All of the identified transcripts that fit the criteria for potentially encoding a secreted globular head C1q (sghC1q) protein (Carland and Gerwick, 2010) were kept. Next, the NCBI non-redundant (NR) and expressed sequence tag (EST) datasets were similarly queried. From these searches, 597 sequences of varying similarity were obtained. Many of these represented partial duplicate ESTs so a contiguous sequence building algorithm (Huang and Madan, 1999) was employed to combine any exact duplicates, and then their open reading frames (ORFs) were extracted by a custom Perl script and this process was repeated using the EST Keeper program (Carland and Gerwick, unpublished). This process brought the number of sequences down to 45, consisting largely of the 52 C1qDC found previously (Mei and Gui, 2008). After careful removal of sequences encoding a collagen motif, sequences without a signal peptide motif, sequences including introns, and sequences that were deemed too long (greater than 300 amino acids), the candidate list narrowed to 21 sequences that could be accurately located on the zebrafish genome using the BLAT algorithm (Kent, 2002). One of these sequences appeared to be an alternative splice variant of another, thus leaving twenty genetic loci that were identified in the zebrafish genome. The TC number of each gene from TGIP (webref), their Ensembl identification number, their name in any previous studies and any NCBI accession numbers are listed in Table 1 along with their basic attributes and proposed formal family names. To bring order and clarity to the many different genes encountered, acronym based names are assigned to each of the genes that exist in the genome. For example, the formal family name of Cbln1 [NCBI:gi50540102] is DrsghC1q01, the Dr is for *Danio rerio* and will not be a necessary part of the gene/protein name for the remainder of this text; sghC1q01 will suffice.

The degree of amino acid sequence identity among the sghC1q proteins ranges from 23% to 95%. Despite this wide range of identity, the family members maintain a preserved predicted ten stand beta-barrel configuration (Fig. 1). To illustrate the relationships of these genes, a Bayesian codon phylogram was chosen as it can take into account prior distributions related to both the sequential and biochemical relatedness of amino acids and codon information (Ronquist and Huelsenbeck, 2003; Anisimova and Kosiol, 2009). The ensuing phylogram (Fig. 2a) has three groups of proteins that clade according to their location on the same chromosome. Two of these groups are on chromosome two but occur at distant reaches of that chromosome (Fig. 2c). Within the clustered regions on the chromosomes, the *sghC1q* genes can be found close together and surrounded by pseudo-genes copies or gene-fragments of themselves (not shown). The relatedness of the clustered genes on these chromosomes and this arrangement of gene-fragments and pseudo-genes are indicative of intrachromosomal gene duplication events (Peatman et al., 2007; Bennetzen, 2007). Additionally, there has been almost no mention of splice variants in this gene family. Only *sghC1q05* appears to have an immediately identifiable splice variant in the form of an alternate donor site within the first exon. It has been observed that while alternative splicing may occur in more than 50% of mammalian genes, it is less frequent among genes that have been recently duplicated (Su et al., 2006). Barely any of the twenty genes in this family (in zebrafish) appear to undergo alternative splicing, thus it is likely that the duplications that created this family are relatively recent. In addition, polymorphisms have been noted in certain EST datasets for these genes making the need for careful analysis of the different sequences even greater.

3.2. Differential expression in response to infection and during early development

Experiments to study the potential differential regulation of the *sghC1q* genes were conducted in two ways. Firstly, to determine which (if any) of the identified sghC1q genes would be transcribed during an acute infection adult male zebrafish were injected intraperitoneally with 3500 colony-forming units of the aquatic bacterial pathogen *Streptococcus iniae* (*S. iniae*). Only adult males were used in an effort to reduce potential gender specific variation in transcription. The fish were euthanized at 12 and 24 hpi, as was done in previous experiments (Gerwick et al., 2007), and the livers removed for subsequent RNA extraction and qRT-PCR analysis. Of the twenty *sghC1q* genes examined, only the transcripts from the genes corresponding to *sghC1q08* and *sghC1q09* were significantly ($p < 0.05$) up-regulated at 23- and 13-fold respectively by 24 h post-infection (Fig. 3). Previous studied *sghC1q05a* (*C1q-like*) indicated the presence of the transcript at 12 and 24 h post infection in embryos, however no significant difference between the treatment and the control fish were seen. (*p*-values of 0.19 and 0.90). These results neither confirm nor discount previous studies (Mei et al., 2008b) that found this gene to reach its maximum up-regulation at 4 h post infection in zebrafish embryos. Regulation of gene *sghC1q05b* was similarly inconsequential yet notably different from its alternate splice variant. Gene *sghC1q01* exhibited significant down-regulation 24 h post infection. The expression patterns of the other *sghC1q* genes were highly variable between individuals and generally exhibited down-regulation (not shown). Due to the

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Table 1
Directory of the *DrsghC1q* genes according to Zv9. This table contains the proposed formal family names of the *sghC1q* genes in zebrafish as well as a listing of their previous names and identifiers from Ensembl and NCBI. Also shown are the chromosomes and number of exons of the genes.

Naming		Chr	Exons	Ensembl	NCBI
Formal	Previous names				
DrsghC1q01	Cbln1	18	3	ENSDARG00000057296	50540101
DrsghC1q02	Cbln2a	2	3	ENSDARG00000074601	190337513, 192452522
DrsghC1q03	Cbln2b	24	3	ENSDARG00000077151	148922966
DrsghC1q04	Cbln4	23	3	ENSDARG00000061240	160333645
DrsghC1q05a, DrsghC1q05b	C1ql1, TC312919	2	4	ENSDARG00000053802	165970362, 187960124
DrsghC1q06	TC316425	7	3	ENSDARG00000035718	292616584, 166796410
DrsghC1q07	TC321847	2	3	ENSDARG00000095040	42406703, 47776018
DrsghC1q08	TC310692	7	3	ENSDARG00000019294	158534006
DrsghC1q09	TC326038	2	3	ENSDARG00000030254	92097198, 186910331, 213624837, 213624839
DrsghC1q10	TC310182	2	3	ENSDARG00000023157	70887628, 94536921, 120538649, 124001534
DrsghC1q11	C1q4L, TC341552	7	3	ENSDARG00000086654	146350791, 292615669
DrsghC1q12		2	3	ENSDARG00000068232	24459827, 42406648
DrsghC1q13		2	4	ENSDARG00000026904	158254221, 176866358
DrsghC1q14		2	4	ENSDARG00000053845	26984632, 78183339
DrsghC1q15		24	6	ENSDARG00000091278	42406703, 47776018
DrsghC1q16		2	3	ENSDARG00000088624	157154298, 292616586
DrsghC1q17		2	5	ENSDARG00000088911	32362346, 90954827, 117957400
DrsghC1q18		7	4	ENSDARG00000090969	1888528553, 292621116
DrsghC1q19		10	3		57163716, 83308939, 292613885, 46935016
DrsghC1q20		15	3	ENSDARG00000087476	188528553, 189528471

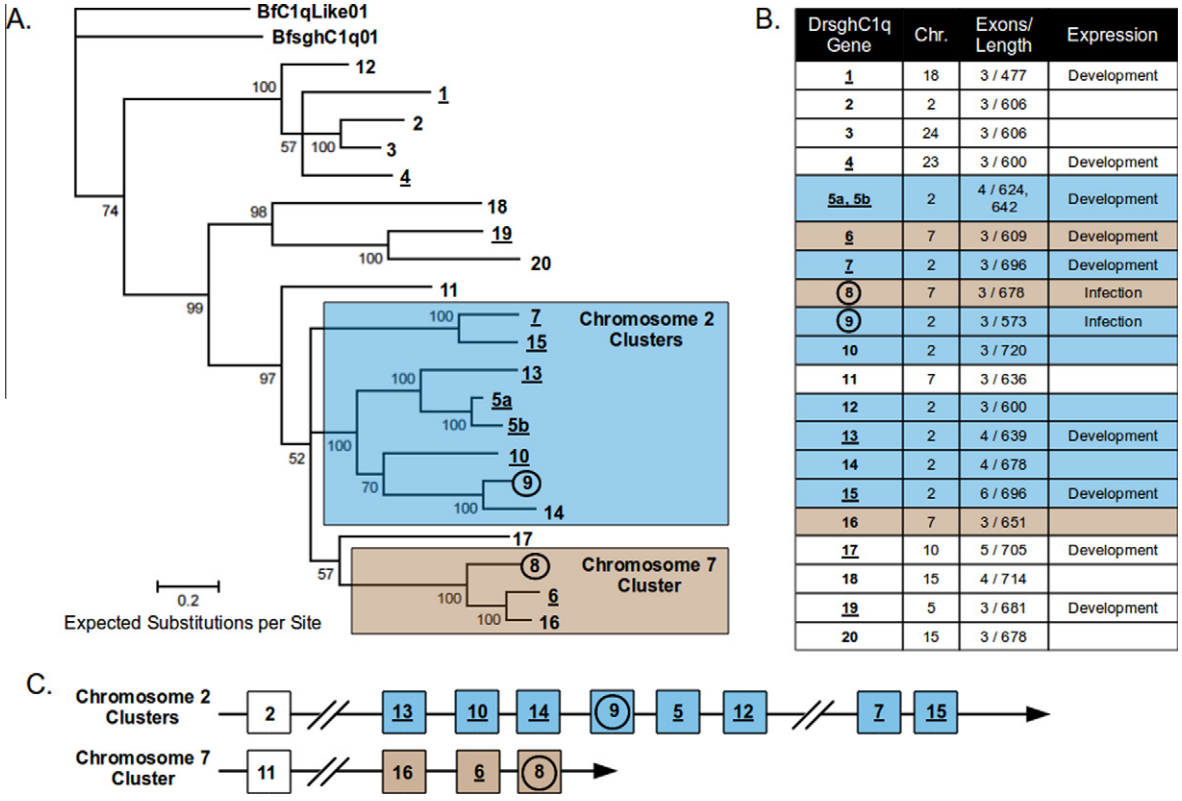

Fig. 2. Phylogeny and chromosomal clusters of the sghC1q genes in Zv9. This figure illustrates the clustered nature of the sghC1q genes, particularly on chromosomes two and seven. The circled genes indicated up-regulation during inflammation and the underlines genes indicated transcription during early development. (A) The phylogram depicts the evolutionary relationships of this family with shaded portions illustrating the chromosomal clusters. Bf indicates *Brachiostoma floridae* which was used as the out-group for the analysis. (B) Table of all of the chromosomal locations of the *DrsghC1q* genes, complete with their exon counts, lengths, and expressions found in this study. (C) Graphical representation of the chromosomal clusters on two and seven, illustrating the clustered nature of the clades even apart from other clustered clades on the same chromosome (in the case of chromosome 2).

high sequence identity of the sghC1q transcripts, all amplicons were verified by direct sequencing to ensure that no cross priming occurred during the PCR reactions.

Secondly, to examine the potential absence or presence of the identified sghC1q transcripts during early development, zebrafish embryos were collected at 0, 12, 24, 36 and 48 h post fertilization; followed by RNA extraction and semi-quantitative PCR. Of the twenty genes examined, a total of eleven *sghC1q* genes (1, 3, 4, 5a/b, 6, 7, 13, 14, 15, 17, 19) exhibited expression within 48 h of fertilization (Fig. 4). Gene *sghC1q05* has two splice variants, both

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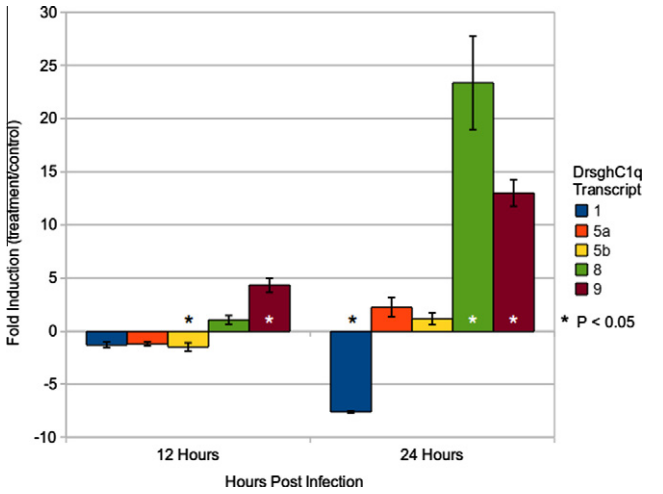

Fig. 3. *DrsghC1q* expression during infection of *D. rerio* with *S. iniae*. Fold induction results from qRT-PCR on non-infected vs. infected liver RNA at 12 and 24 h post-infection with *S. iniae* for five genes of interest. Twenty *DrsghC1q* genes were found in the zebrafish genome and qRT-PCR was performed on all them but only *sghC1q01* (blue), *sghC1q05b* (yellow), *sghC1q08* (green), and *sghC1q09* (red) exhibited significant regulation during these times (data from the other genes not shown). *Hepcidin* (not shown) was chosen as a positive control and it was up-regulated four and forty fold at 12 h and 24 h respectively. Only *sghC1q09* exhibited up-regulation at 12 h post infection while the others exhibited regulation at 24 h (all up-regulation except for *Cbln1*). ∗ indicates significance at a *p*-value < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

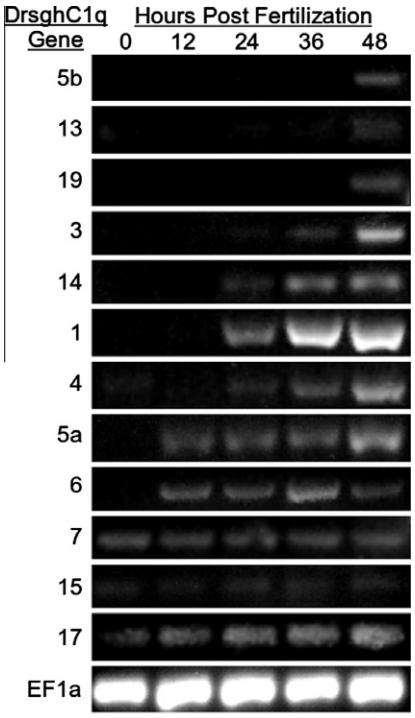

Fig. 4. *DrsghC1q* expression during early development of *D. rerio*. A series of electrophoresis gels depicting PCR amplification performed on cDNA obtained from reverse transcription of RNA taken 0, 12, 24, 36 and 48 h post fertilization (HPF) of zebrafish embryos. *EF1a* was used as a reference gene and *hepcidin* as a negative control (not shown). Of the twenty genes examined, eleven *sghC1q* genes were expressed, one of which showed expression of both of its alternative splice variants (*sghC1q05a* and *sghC1q05b*).

of which were expressed during development though at different times. *SghC1q* genes 7, 15, and 17 were transcribed throughout the first 48 h of development and may represent maternally transferred transcripts. This correlates in function with a finding in goldfish (Mei et al., 2008a) where a C1q-like protein (*Cag*OC1q-like) was discharged from maternal ovaries into egg envelopes. Genes *sghC1q05a* and *sghC1q06* begin to be expressed starting around 12 h post fertilization, corresponding to the early segmentation period (6-somite). This finding matches previous observations of *sghC1q05a* (C1q-like) by Mei and colleagues (2008b). Interestingly, *sghC1q05b* exhibited a different pattern from its splice variant. Genes *sghC1q01* and *sghC1q04* (which correspond to *cbln1* and *cbln4* respectively) as well as *sghC1q14* appear to begin transcription around 24 h post fertilization corresponding with the transition to the pharyngula period. Genes *sghC1q5b*, *13*, *19*, and *3* are transcribed at 36 to 48 h after fertilization. Hepcidin, a known acute phase protein was used as a negative control (data not shown) because this gene is expected to show little or no expression, unless the embryos were subjected to bacterial infection (Lauth et al., 2005; Gerwick et al., 2007). The *ef1*α gene was used as the reference gene and it exhibited constant expression for all of the embryonic time points collected. Again, due to the high sequence identity found among the sghC1q transcripts, all amplicons were verified by direct sequencing to ensure that no cross priming occurred during the PCR reactions.

Expression patterns in all instances did not appear to correlate with chromosomal location or any other basic characteristics of the genes (Fig. 2). An interesting finding is that the *sghC1q* genes were either transcribed during early development or during the response to infection but never during both of these two physiological conditions. This observation contrasts with findings in mice (Stevens et al., 2007) and in zebrafish (Mei et al., 2008b) where C1q and *sghC1q05a* do appear to function during both of these physiological conditions. We postulate that the zebrafish *sghC1q* gene duplications could have allowed the genes to become sub-functionalized to the point of participating in one or the other of the two physiological responses. Alternatively, the ability for *sghC1q* genes to act during both conditions may have simply evolved in other organisms separately. Genes *sghC1q05, 8* and *9* that were upregulated during the inflammatory response are now being further studied to determine what function the encoded proteins have during the inflammatory response.

4. Conclusions

The *sghC1q* genes have radiated intrachromosomally in the zebrafish genome, primarily on chromosomes two and seven. The expression patterns found in this study for the sghC1q genes do not correlate with the chromosomal locations of these genes, meaning that the functions of these genes likely changed in a manner unrelated to their evolutionary radiation. We have shown that the *sghC1q* genes identified are expressed during the innate response to infection and/or early development. These findings, coupled with the expansive radiation of these genes in zebrafish, lead us to hypothesize that the dual functionality has been lost in favor of subfunctionalization. Pairs of *sghC1q* genes that display the same temporal expression pattern can be found during early development as well as during the response to infection. These co-expressed genes may operate in concert, forming multimeric protein complexes, much like C1q or Cbln (Bao et al., 2005); however, this remains to be examined.

Conflict of Interest

None declared.

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