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Expression of complement components coincides with early patterning and organogenesis in *Xenopus laevis*

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ABSTRACT The complement system is the central component of innate immunity and an important player in the adaptive immunity of vertebrates. We analyzed the expression patterns of several key members of the complement cascade during *Xenopus* development. We found extensive expression of these molecules already during gastrula/early neurula stage. Remarkably, several genes also showed an organ-specific expression pattern during early organogenesis. Early expression is notable for two different expression patterns in the neuroectoderm. In one group, there is early strong neural plate and neural precursor expression. This is the case of *properdin, C1qA, C3* and *C9*. The second pattern, seen with *C1qR* and *C6,* is noteworthy for its expression at the periphery of the neural plate, in the presumptive neural crest. Two genes stand out for their predominantly mesodermal expression. *C3aR,* the message for the cognate receptor for C3 in the complement cascade, is expressed at the same time as *C3,* but in a complementary, reciprocal pattern in the mesoderm. *C1qA* expression also predominates in somites, pronephros, visceral mesoderm and ventral blood islands. Finally, several genes are characterized by later expression in developing organs. *C1qR* displays a reticular pattern consistent with expression in the developing vasculature. The late expression of *C1qA* and *C3bC4b* is strongest in the pronephros. Finally, the expression of *properdin* in the hindbrain and in the developing lens are novel findings. The expression patterns of these molecules suggest that these components of the complement system may have in *Xenopus* a so far undefined developmental role.

KEY WORDS: *complement, organogenesis, patterning, Xenopus*

Introduction

The complement system is the central component of innate immunity and an important player in the adaptive immunity of vertebrates. It is an ancestral system of soluble factors, cell-bound receptors, and numerous soluble and cell-bound regulators, including several proteases. It functions largely as a zymogen cascade whereby each protein serves as an enzyme precursor for the next step of the cascade. In host defense, the initial activation is understood to occur in one of three ways: by contact with immunoglobulins bound to a pathogen (classical pathway), by binding to bacteria with mannose-containing surface polysaccharides (lectin pathway), or by autologous activation (alternative pathway). C3 is the convergence point of all three pathways, and is upstream of the lytic pathway which is the downstream cascade leading to lysis of the offending agent or cell. In addition to interactions with other complement proteins, C3 and other members of the complement system interact with extracellular matrix proteins such as fibronectin and integrins (Hautanen and Keski-Oja, 1983, Lambris, 1993, Leivo and Engvall, 1986).

Because of the central role of the complement system in innate immunity, expression studies of individual components of the complement system have been primarily performed on the backdrop of the development and function of innate immunity (Ellingsen *et al.*, 2005, Gongora *et al.*, 1998, Kato *et al.*, 2004, Lovoll *et al.*, 2007, Lovoll *et al.*, 2006, Mastellos and Lambris, 2002). However,

Abbreviations used in this paper: C1qA, complement component 1, subcomponent q, alpha polypeptide; C1qR, complement component 1, subcomponent q, receptor; C3, complement component 3; C3aR, complement component 3, anaphylatoxin receptor; C9, complement component 9; MAC, membrane attack complex.

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there is evidence suggesting that the function of these molecules is not strictly limited to immunity (Mastellos and Lambris, 2002) (Mastellos *et al.*, 2005). For example, in urodeles, *C3* is expressed in myocytes of the regenerating limb (Del Rio-Tsonis *et al.*, 1998). In addition, C3a, C3b, C3aR, C5a and C5aR all participate in liver regeneration in mammals (DeAngelis *et al.*, 2006, Markiewski *et al.*, 2004, Mastellos *et al.*, 2001, Strey *et al.*, 2003). The C3aR receptor has also been shown to participate in the homing of hematopoietic progenitor cells in mouse (Reca *et al.*, 2003). Furthermore, homologues of the complement cascade in invertebrates are known to participate in developmental processes. For example, the C2/B-like protease gastrulation defective, is involved in early dorso-ventral patterning of the *Drosophila* embryo (DeLotto, 2001). These findings are of interest for two reasons. First, regeneration is commonly accepted to recapitulate developmental paradigms. Second, they illustrate that complement components are expressed by cells not commonly thought to be part of the immune system.

In spite of compelling functional data in regeneration models and developmental data from invertebrates, little is known of the role and expression of complement in the developing vertebrate embryo. In mammals, it is generally accepted that complement components are largely synthesized by the liver, white blood cells and endothelial cells. There is limited evidence from studies in fish that complement is expressed during embryonic development, although most of the published reports examined protein expression or gene expression in the whole embryo, with little focus on timing and organ-specificity. There are a few reports of complement components isolated from *Xenopus laevis* and *tropicalis* screens showing expression in tailbud and early larval embryo (Changkyun Park *et al.*, 2007, Costa *et al.*, 2003, Pollet *et al.*, 2005), but we are not aware of any systematic analysis of gene expression of complement components during early vertebrate development. Based on our finding that in *Xenopus C3* mRNA expression was conserved from the neurula stage endoderm to the adult liver, we aimed to examine the developmental expression of other complement genes. We hypothesized that expression analysis of this evolutionarily conserved cascade may be suggestive of a previously unrecognized developmental role. Here, we report that in *Xenopus laevis,* several of the complement genes are expressed during early patterning, largely in the neural precursors and mesoderm, and later during organogenesis in such organs as the kidney, intestine, brain and lens.

Results

In immunity, the complement system functions as a cascade starting with C1q binding to pathogens, and culminating in the formation of the membrane attack complex (C6-C9). The relationship between complement genes during development is unknown. However, for the purposes of presenting our results, we have chosen to report our findings in the order of the known cascade.

C1qA *is expressed in multiple mesodermal derivatives*

C1qA encodes for one of the three domains comprising the soluble C1q. In the complement cascade, C1q is an upstream component of the classical pathway of complement activation. Because of similarities in gene structure and function, C1q proteins are considered part of the tumor necrosis factor family of signaling molecules (Kishore and Reid, 2000). They also share structural similarities with mannan-binding lectins known both for their role in the lectin-pathway of complement activation and for their conserved role in molecule recognition (Petersen *et al.*, 2001). Two major functions have been ascribed to C1q. First, it plays a key role in the recognition of immune complexes. Second, it is a potent chemoattractant for inflammatory cells (Vegh *et al.*, 2006). There are no functional or descriptive studies to date examining the expression or function of C1q in lower vertebrates or invertebrates.

C1qA is largely expressed in mesodermal tissues of the developing *Xenopus* embryo. In *Xenopus*, the mesoderm forms during gastrulation. It gives rise to the axial mesoderm and the lateral plate mesoderm. The axial mesoderm gives rise to notochord and somites. The lateral plate mesoderm, in turn, gives rise

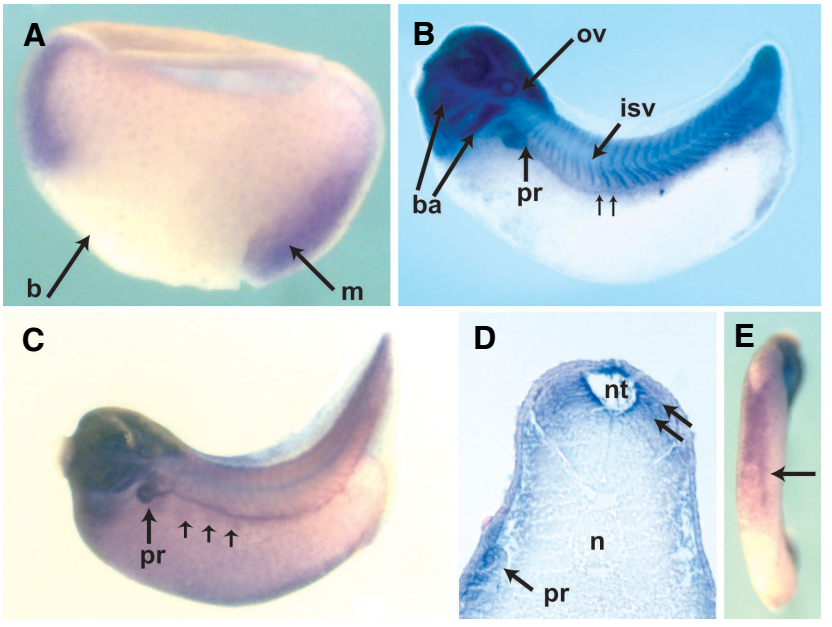


Fig. 1. Developmental expression of *C1qA*. (A) *Bissected gastrula. Dorsal is to the right. Expression is restricted to the marginal zone mesoderm indicated by the arrow; (b) blastopore, (m) mesoderm.* **(B)** *Stage 28 embryo. Anterior is to the left and dorsal to the top. There is strong expression in the cephalic structures, especially in the branchial arches (ba) and in the otic vesicle (ov). Expression in the intersomitic veins is visible (isv). Pronephric expression has begun (pr). The small arrows indicate expression in the pronephric duct.* **(C)** *Stage 35 embryo shows detailed expression in pronephros (pr) and duct. At this stage, expression in the lateral muscle presursors (small arrows) is visible ventral to the duct.* **(D)** *Transverse section through an age-matched embryo to (C). The section is at the level of the pronephros. Dorsal is to the top. Somitic expression is most pronounced in the cells adjacent to the neural tube (nt) as indicated by the small arrows, and in the pronephros (pr). There is no staining in the notochord (n).* **(E)** *Ventral view of a stage 35 embryo reveals staining consistent with ventral blood island expression (arrow).*

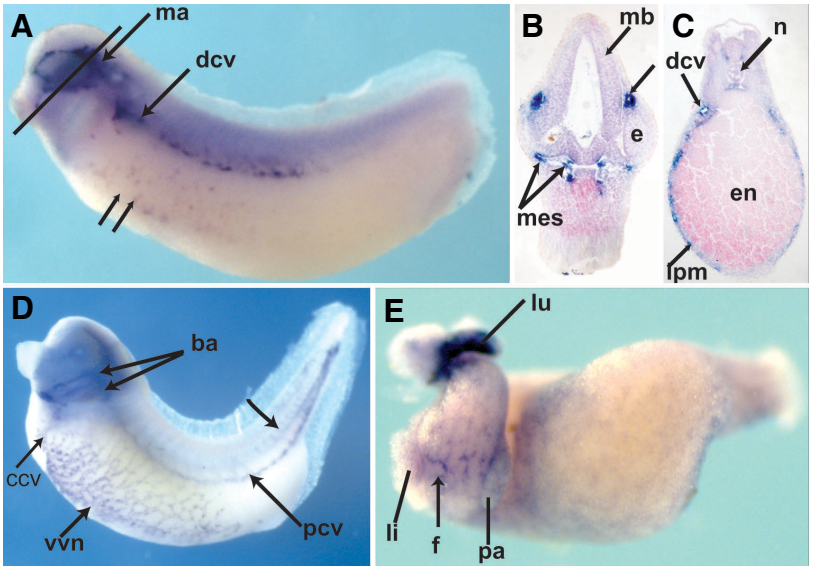


Fig. 2. Developmental expression of *C1qR*. *Anterior is to the left and dorsal to the top unless otherwise specified.* **(A)** *Tailbud embryo. Marked expression in the mandibular segment of the neural crest is indicated (ma). Double arrows indicate punctate ventral staining consistent with early vascular precursor expression. Expression in the dorsal cardinal vein is indicated (dcv).* **(B)** *Transverse section through the head of embryo in (A) (solid black line). Dorsal is to the top. The developing mesencephalon or mid-brain is labeled (mb). The small arrow to the right of the figure points to mandibular segment expression, dorsal to the developing eye (e). Expression in the head mesenchyme is shown, consistent with neural crest expression.* **(C)** *Section through the trunk of a tailbud embryo (shown in D). Dorsal is to the top. The dorsal cardinal vein is indicated (dcv). Small structures with a lumen in the lateral plate mesoderm (lpm) are consistent with expression in the developing vessels of vitelline network (vvn); (en) endoderm, (n) notochord.* **(D)** *Tailbud embryo showing expression throughout the developing vasculature. In addition to the ventral vitelline network, both the common (ccv) and the posterior (pcv) cardinal veins show strong expression. There is some expression in the intersomitic veins as indicated by the small arrow. Expression in the branchial arches is labeled (ba).* **(E)** *Expression in the vasculature of the larval gut. The reticular pattern of the developing splanchnic vasculature is indicated in the foregut (f) but visible throughout, including in the liver and pancreatic buds. Expression is strongest in the lung bud. (li) liver, (lu) lung, (pa) pancreas.*

to the visceral mesoderm, the kidney and blood precursors. The pronephros gives rise to the kidney, a highly vascularized organ which is also active in blood formation in the adult (Brandli, 1999). Expression of *C1qA* begins at gastrula stage in the mesoderm (Fig. 1A). By neurula stage, expression is most noticeable in the anterior neural plate (not shown). As development proceeds there is continued strong expression in cephalic structures, which we observed through the late tadpole stages (Fig. 1B). Additionally, as the embryo begins to elongate expression appears in the somites around stage 22-25, which is best appreciated in the tailbud embryo (Fig. 1B) and then begins to fade by tadpole stage (Fig. 1C). Expression in the developing kidney is also first noticeable in the tailbud embryo. Pronephric expression is best seen a few hours later in the stage 35-37 embryo in which the pronephros is clearly visible, as is the duct (Fig. 1C). In tandem with the pronephric expression, a subset of cells, grouped in discrete islands just ventral to the duct express *C1qA*. This pattern mimicks the expression of the transcription factor *xFoxK1* in lateral muscle precursors as reported by Pohl and Knochel (Pohl and Knochel, 2004) (Fig. 1C). A section through an age-matched embryo reveals residual staining in the somites and tubular expression in the pronephros (Fig. 1D). Concurrently, strong expression in the ventral blood islands is noticeable (Fig. 1E). Finally, there is expression in the mesoderm of the larval gut, resembling expression of the mesodermal transcription factor *FoxF1* (Tseng *et al.*, 2004). In this tissue, expression proceeds in a cranio-caudal fashion, temporally beginning in the mesoderm of the foregut around stage 42, and then progressing throughout the mesoderm of the primitive intestine by stage 43-44 (not shown).

C1qR *is expressed in the developing vasculature*

In mammals, C1qR is a membrane-associated receptor which is expressed by many different cell-types including peripheral white blood cells, fibroblasts and endothelial cells. C1qR interacts with the first component of complement C1 in part by binding to its collagen-like domains (Ghebrehiwet, 1989, Peerschke *et al.*, 1993). C1qR has diverse functions in different adult cell types. These include leucocyte chemotaxis and calcium release (Bordin *et al.*, 1990, Eggleton *et al.*, 1994, Fusi *et al.*, 1991, Ghebrehiwet *et al.*, 1990, Ghebrehiwet *et al.*, 1992, Peerschke and Ghebrehiwet, 1990, Peerschke and Ghebrehiwet, 1990, Peerschke *et al.*, 1993, Vegh *et al.*, 2006). Although *C1qR* was recently identified in a screen for mesenchymal genes in the developing mouse intestine (Li *et al.*, 2007), there are no functional or expression studies to date investigating C1qR in lower vertebrates or invertebrates.

In the *Xenopus* embryo, the expression of *C1qR* is most remarkable for its expression in the developing vasculature (Fig. 2). Briefly, there are two phases of vascular development in *Xenopus*. The first, called vasculogenesis, consists in the formation of vessels from vascular precursors. The next phase, called angiogenesis, consists in the outgrowth of vessels from these founder vessels (Cox *et al.*, 2006). *C1qR* expression is first noted during the early development of the dorsal cardinal vein of the tailbud embryo (Fig. 2A). Punctate staining in the ventral portion of the embryo suggests early expression in ventral vascular precursors (Fig. 2A). Later this expression evolves into a reticular pattern observed in the ventral region of the tadpole (Fig. 2D), mimicking the expression of other vascular markers such as *X-msr*, *Xl-fli*, *Dab2*, *Apelin*, *Ami* and *Xl Erg* (Baltzinger *et al.*, 1999, Cheong *et al.*, 2006, Cox *et al.*, 2006, Devic *et al.*, 1996, Inui and Asashima, 2006, Meyer *et al.*, 1995, Meyer *et al.*, 1993). Expression of *C1qR* is noted in the visceral mesoderm starting at stage 35 (Fig. 2C). The expression appears to localize to cells surrounding small lumina, consistent with a vascular expression (Fig. 2C). In the isolated larval gut, the reticular expression is also noticeable in the intestinal precursors and in the organ buds of the liver and pancreas (Fig. 2E). Additionally, strong expression is noted in the lung buds (Fig. 2E). Interestingly, the recently-isolated *Ami* which has a very similar expression pattern, is homologous to the human soluble complement inhibitor Factor D (Inui and Asashima,

2006). However, sequence analysis did not reveal any significant similarity between C1qR and Ami (AB238233).

In addition, *C1qR* expression is also noticeable in the developing neural crest. The neural crest is a uniquely vertebrate cell type that arises from the peripheral regions of the neural plate. Neural crest cells are characterized by their ability to migrate long distances and contribute to many organs: cranial structures, somites, adrenal medulla and pronephros, pigment cells, fins, peripheral nervous system and enteric ganglia. At tailbud stage, expression is remarkably confined to the mandibular segment of the neural crest, outlining the developing eye and otic vesicle (Fig. 2A). A section through the head at this stage shows focal expression in the head mesenchyme, similar to the expression of established migratory neural crest markers such *twist*, *slug*, *FoxD3* and *Inca* (Fig. 2B) (Dirksen *et al.*, 1993, Hopwood and Gurdon, 1991, Luo *et al.*, 2007, Mayor *et al.*, 1995). By tadpole stage, mandibular segment expression has ceased; in turn, expression is noted in the branchial arches corresponding to the hyoid and branchial segments of the neural crest (Fig. 2D). Expression of vascular markers in the branchial arches has been shown previously for several genes involved in vasculogenesis (Baltzinger *et al.*, 1999, Cheong *et al.*, 2006, Cox *et al.*, 2006, Devic *et al.*, 1996, Inui and Asashima, 2006, Meyer *et al.*, 1995).

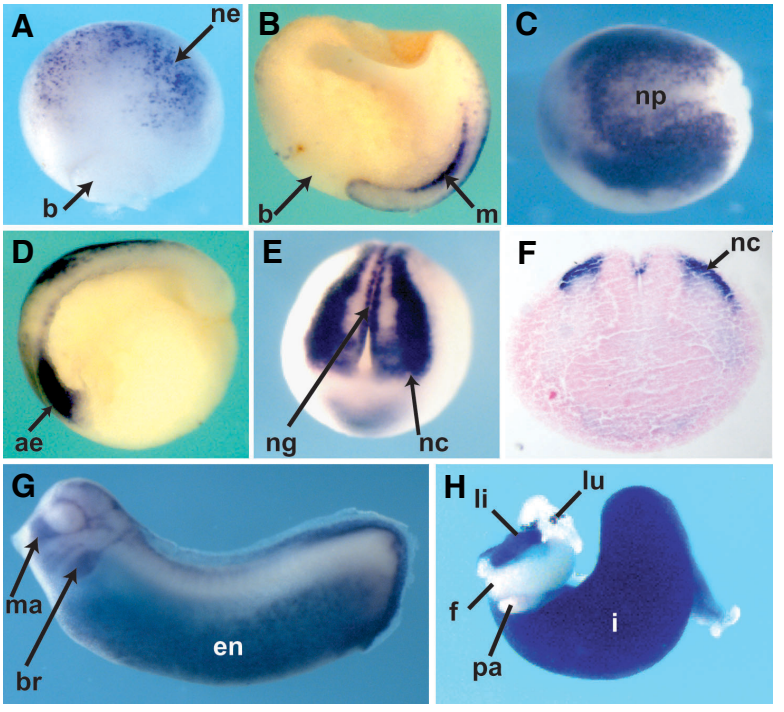


Fig. 3. Developmental expression of *C3* from gastrula to organ bud stage. (A) *Dorsal view of stage 10 embryo. Anterior is to the top. There is early, punctate expression of* C3 *in the neuroectoderm (ne); (b) blastopore.* **(B)** *Midline view of a bisected gastrula; dorsal is to the right.* C3 *is expressed in dorsal ectoderm and dorsal mesoderm (m).* **(C)** *Dorsal view of stage 13 embryo shows complex expression pattern in and around the neural plate (np). Anterior is to the left.* **(D)** *Sagital view of early neurula embryo shows expression in anterior endoderm (ae) and continued expression in neuroectoderm at the top. Anterior is to the left and dorsal to the top.* **(E)** *Anterior view of stage 17 embryo showing defined expression in both neural crest (nc) and neural groove (ng). Dorsal is to the top.* **(F)** *Frontal section through the embryo in (E) shows gene expression in neural crest (nc). Dorsal is to the top.* **(G)** *Stage 27 embryo shows expression in the mandibular and branchial segments of anterior neural crest. Hyoid segment is visible between the mandibular (ma) and branchial segments (br). At this stage there is strong endodermal expression (en).* **(H)** *Isolated gut tube from stage 42 embryo shows strong expression in liver and presumptive intestine, with sparing of foregut and pancreas. (f) foregut, (i) presumptive intestine, (li) liver, (lu) lung, (pa) pancreas.*

Complement factor C3

One of the central components of the complement system is C3. It is a large protein, composed of multiple functional domains and binding sites, including motifs that recognize fibronectin and integrins. Its function is regulated by proteases, which induce conformational changes. It exerts its many functions mostly through its two active fragments, C3a anaphylatoxin, a potent chemoattractant, and C3b, which contains many binding sites for interacting with other complement and cell surface proteins (Hautanen and Keski-Oja, 1983, Janssen *et al.*, 2005). In humans, C3 is synthesized in the liver and macrophages. In teleost fish, *C3* mRNA has been found in developing neural tissue and gastrointestinal tract (Lange *et al.*, 2004, Lange *et al.*, 2004).

We initially isolated a 5 kb long *C3* cDNA clone in a screen designed to isolate endoderm-specific genes. The sequence showed 100% identity to the previously isolated *C3 Xenopus gilli* (U19253) and 97% identity to the 900bp partial coding sequence Unigene XI.55075, LOC398666.

In situ hybridization analysis reveals that *C3* has an intricate pattern of expression that shows a distinct temporo-spatial regulation. The expression pattern is remarkable for its early expression in the neural plate, followed by an intense neural crest and endodermal expression in the gastrointestinal precursor cells, confirming the findings of others (Costa *et al.*, 2003, Pollet *et al.*, 2005).

By *in situ* hybridization, we can detect expression of *C3* in the dorsal region of early gastrulae, in a wide, crescent shaped area (Fig. 3A). While most of the expression is in the superficial layer of the neural plate, there is also expression in the dorsal mesoderm (Fig. 3B). At this stage, a few cells on the ventral side of the blastopore selectively express *C3* (not shown). By stage 13, expression in the neuroectoderm is remarkably complex. A population of cells organized in the characteristic crescent shape of the neural folds display solid *C3* expression. Medial to the crescent shaped area, in the central region of the neural plate, there is a mosaic expression of *C3* (Fig. 3C). At this stage, in cells anterior to the neural plate, an area destined to form the cement gland and the placodal structures, cease expressing *C3*. However, a cross section of the embryo reveals an intense transcription of *C3* in the prospective pharyngeal endoderm (Fig. 3D). During neurulation, *C3* expression in the dorsal region of the embryos is progressively restricted to the neural crest cells (Fig. 3E, F). At the same time, *C3* expression appears in the endoderm, in the presumptive intestinal cells (Fig. 3D,E), as has been previously reported (Costa *et al.*, 2003). By stage 22, the neural crest expression pattern mimicked that of the known migratory

neural crest markers (Devic *et al.*, 1996, Dirksen *et al.*, 1993, Hopwood and Gurdon, 1991, Luo *et al.*, 2007, Mayor *et al.*, 1995). Following neural tube closure, in the tailbud stages, we observed strong expression of *C3* in the anterior migrating neural crest - in the mandibular, hyoid, and branchial segments (Fig. 3G). Although Costa *et al.* had shown late neural crest expression, the early pattern in the neural plate and neural crest had not previously been analyzed in detail. Indeed, the mandibular, hyoid, and branchial segments of the cranial migratory neural crest show high levels of *C3* expression. In addition, expression in the neural groove and later in the dorsal fin suggests that trunk neural crest cells also express *C3*.

In addition, as expected, intense *C3* expression is observed in the bulk of the endoderm (Fig. 3G) (Costa *et al.*, 2003, Pollet *et al.*, 2005). Late expression in the larval gut is remarkable for its strong expression in the endoderm of intestinal precursors and in the early liver diverticulum, but not in the remainder of the foregut (Fig. 3H).

Expression pattern of C3aR

In the complement cascade as it is understood in immunity, one of the ways C3 signals is through the binding of C3a to the G-protein coupled transmembrane receptor C3aR. In mammals, *C3aR* is expressed in myeloid, non-myeloid, and endothelial cells (Morikis, 2005). It has also been identified on glial cells (Nataf *et al.*, 1999). C3aR has been implicated in multiple cellular processes, among them leucocyte chemotaxis, vascular adhesion and smooth muscle contraction (Morikis, 2005). In light of the expression pattern of *C3*, we sought to examine the developmental expression of its cognate receptor. *C3aR* expression is markedly different from the expression of its ligand *C3*.

First, at gastrula stage, we observe a strong expression in the mesoderm (Fig. 4A, B). During neurulation, additional diffuse expression of *C3aR* appears in the neural plate (not shown). By early tailbud stage, *C3aR* expression is present in the branchial segment of the cranial neural crest, in the developing eye and in the otic placode (Fig. 4C).

In the trunk, expression of *C3aR* is observed in a triangle surrounding the presumptive liver bud (Fig. 4D). A section through this area reveals that most of the expression is in the developing visceral mesoderm (Fig. 4G). This mesodermal expression extends throughout the developing gastrointestinal tract of the larval stage embryo (Fig. 4F and 4H).

Finally, the ventral blood islands, also mesodermal in origin, expressed *C3aR* starting at early tailbud stage (Fig. 4C), but is most noticeable in the tadpole (Fig. 4E). The VBI are derived from lateral plate mesoderm and are the site of embryonic hematopoiesis (Walmsley *et al.*, 2002).

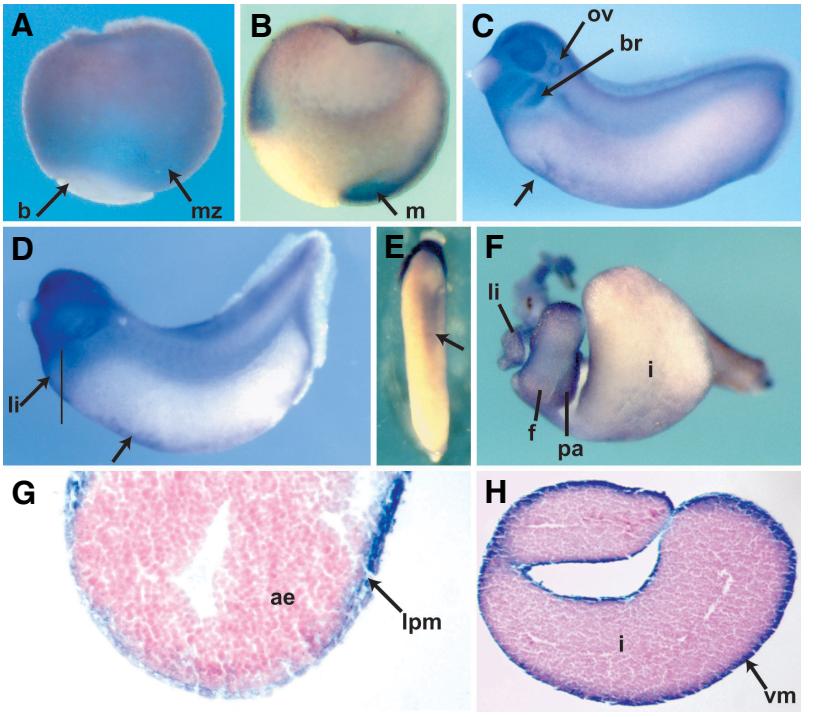
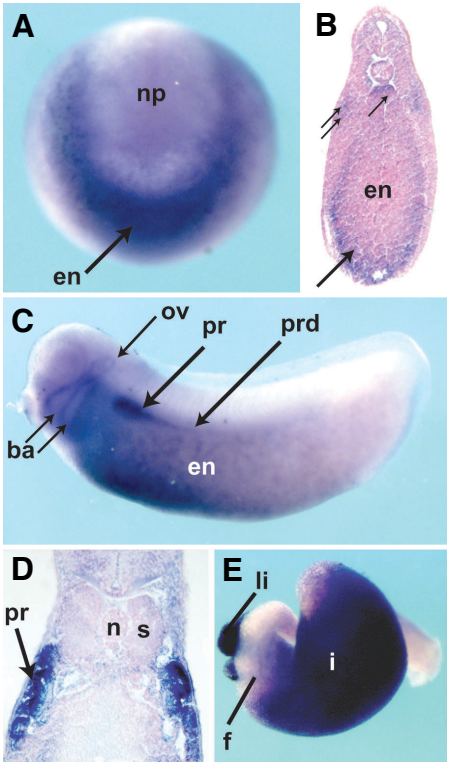


Fig. 4. Developmental expression of *C3aR* from gastrula to organ bud stage. (A) *Whole mount* in situ *hybridization on a stage 10 gastrula showing expression predominantly in the marginal zone (mz); (b) blastopore.* **(B)** *Bissecting the gastrula revealed that the expression is mesodermal (m). In both (A,B) dorsal is to the right.* **(C)** *Stage 25 embryo showing expression in both the branchial segment of the neural crest (br) and in the otic vesicle (ov). Anterior is to the left, dorsal to the top. The arrow indicates early ventral blood island expression (VBI).* **(D)** *Stage 32 embryo. In addition to strong expression in cephalic structures, the presumptive liver area (li) is surrounded by cells expressing* C3aR *message. Arrow shows VBI expression.* **(E)** *Ventral view of same stage 32 embryo showing detail of VBI expression (arrow); head is to the top.* **(F)** *Isolated gut tube showing* C3aR *expression throughout the foregut (f) and presumptive intestine (i), (pa) pancreas. Anterior is to the left.* **(G)** *A section through the presumptive liver area (indicated in D) shows expression in lateral plate mesoderm (lpm) surrounding the anterior endoderm (ae).* **(H)** *Section through isolated gut showing staining in visceral mesoderm (vm), surrounding the presumptive intestine.*

C3bC4b inactivator (Factor I) is expressed in the pronephros

C3bC4b inactivator, also known as Factor I, is a regulatory protein for both the classical and alternative pathways of complement activation. Human factor I contains motifs very similar to the heavy chain of the low density lipo-protein receptor (LDLr) (Stanley *et al.*, 1986). Other regions of the molecule show similarities to both epidermal growth factor and alphafeotoprotein (Catterall *et al.*, 1987). Factor I has been isolated from *Xenopus* liver, and its immune function in *Xenopus* serum analyzed. The main difference between human and *Xenopus* Factor I is the presence of an additional 87 base pair domain in the amphibian (Kunnath-Muglia *et al.*, 1993). To date, its gene expression pattern in the vertebrate embryo is unknown.

Embryonic expression begins in the neurula. Its expression is crescent-shaped lateral and ventral to the neural plate (Fig. 5A) and in the closing blastopore (not shown). *In situ* on a sectioned, early tailbud embryo reveals that *C3bC4b* is expressed at the periphery the developing endoderm, including in the cells of the archenteron roof (Fig. 5B). In the tailbud embryo, endodermal expression expands caudally before localizing to the liver and presumptive intestine in the larval gut tube (Fig. 5C). During the development of the endoderm into a gut, expression progresses radially, from the outside in, to finally express throughout the endoderm of the developing intestine (not shown). Similar to other



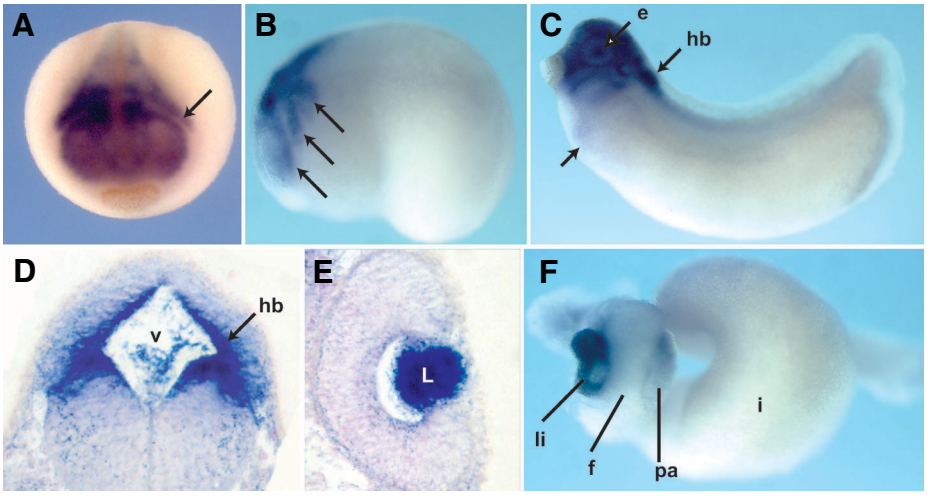


Fig. 5 (Left). Developmental expression of *C3bC4bi*. **(A)** *Anterior view of a neurula stage embryo shows strong expression in the endoderm (en) lateral and ventral to the neural plate (np).* **(B)** *Transverse section of an early tailbud embryo in the presumptive trunk area. Dorsal is to the top. Expression is in the peripheral endoderm (large arrow) and in the archenteron roof (small arrow). Double arrows indicate early expression in presumptive kidney domains.* **(C)** *Tailbud embryo. Anterior is to the left. Endodermal expression is more extensive although not yet uniform. Expression in branchial arches (ba), the otic vesicle (ov), the pronephros (pr), and the pronephric duct (prd) are indicated.* **(D)** *Section through the proximal trunk of a tailbud embryo highlighting strong expression in the developing kidney. Dorsal is to the top; (n) notochord, (s) somite.* **(E)** *Isolated larval gut reveals strong expression in presumptive intestine (i) and liver (li), but no expression in foregut (f) and distal most segment of the developing intestine.*

Fig. 6 (Right). Developmental expression of *Properdin*. *Anterior is to the left and dorsal to the top unless otherwise specified.* **(A)** *Anterior view of a neurula stage embryo. Arrow indicates neural crest expression.* **(B)** *Late neurula/early tadpole stage embryo. Arrows indicate expression in mandibular, hyoid and mandibular segments of anterior neural crest (from anterior to posterior).* **(C)** *Tailbud embryo shows strong cephalic expression. Intense expression in developing eye (e) and hindbrain (hb) are indicated. The ventral arrow highlights beginning expression in the anterior endoderm.* **(D)** *Cross section through the hindbrain of the embryo in (C). Dorsal is to the top. Expression is limited to the dorsal most part of the neural tube, in the periventricular cells of the hindbrain; (v) ventricle.* **(E)** *Section through the developing eye of a tailbud embryo shows strong expression in the lens. Anterior is to the right.* **(F)** *In the isolated larval gut expression is confined to the liver bud only (li); (f) foregut, (i) presumptive intestine, (pa) pancreas.*

complement components such as *C3* and *C9* expressed in the endoderm, the expression is limited to the liver and presumptive intestine, with little or no expression in the foregut (Fig. 5E). Expression is also visible in the developing branchial arches of the tailbud embryo. Based on the early endodermal expression, it is probable that the expression in the branchial arches is in cells derived from pharyngeal endoderm (Fig. 5C). Together with the neural crest, which expresses *C3*, *C9* and *C1qR*, the pharyngeal endoderm is important in the development of the branchial arches (Graham *et al.*, 2005).

The distinguishing feature of *C3bC4b* expression in the *Xenopus* embryo is the appearance of a message in the pronephros of the tailbud embryo (Fig. 5B, 5C). The pronephros, which is mesodermal in origin, gives rise to the kidney. It is composed of two basic units: the tubules and collecting duct, and the glomus which is vascular in origin. At tadpole stage, expression is noticeable in the developing tubules of the pronephros and the proximal duct (Fig. 5C, D).

Properdin is expressed in the developing neural tube and lens

Properdin is a regulatory protein of the alternative pathway of complement activation and the only positive regulator of the complement system (Perkins, 2005). The role of properdin in the human complement cascade is stabilization of the C3 convertase. C3 convertase catalyzes the breakdown of C3 into its active components C3a and C3b. Therefore, properdin serves as a positive regulator of C3 activity (Fijen *et al.*, 1999). The structure of properdin contains six thrombospondin domains (TSR), like many other complement components (Perkins, 2005). TSRs are involved in binding to other molecular structures. Absence of or defects in properdin synthesis is rare, but associated with life-threatening clinical conditions (Fijen *et al.*, 1999). In humans, properdin is synthesized by peripheral white blood cells, hepatocytes, and astrocytes, which contribute to the formation of the blood-brain barrier. There are no descriptive or functional studies examining the role of properdin in lower vertebrates.

Similar to the expression of other members of the complement system in the *Xenopus* embryo, *properdin* mRNA is noticeable early in the neural crest (Fig. 6A, B) and later in the liver precursors and liver bud (Fig. 6C, F). However, it displays a remarkably different expression pattern starting in the tailbud embryo at which time expression is confined to cephalic structures (Fig. 6C). At this stage, strong expression is noted throughout the head, including the eye, otic vesicle, and presumptive hindbrain, but excluding the cement gland (Fig. 6C). In the tadpole, cephalic expression is

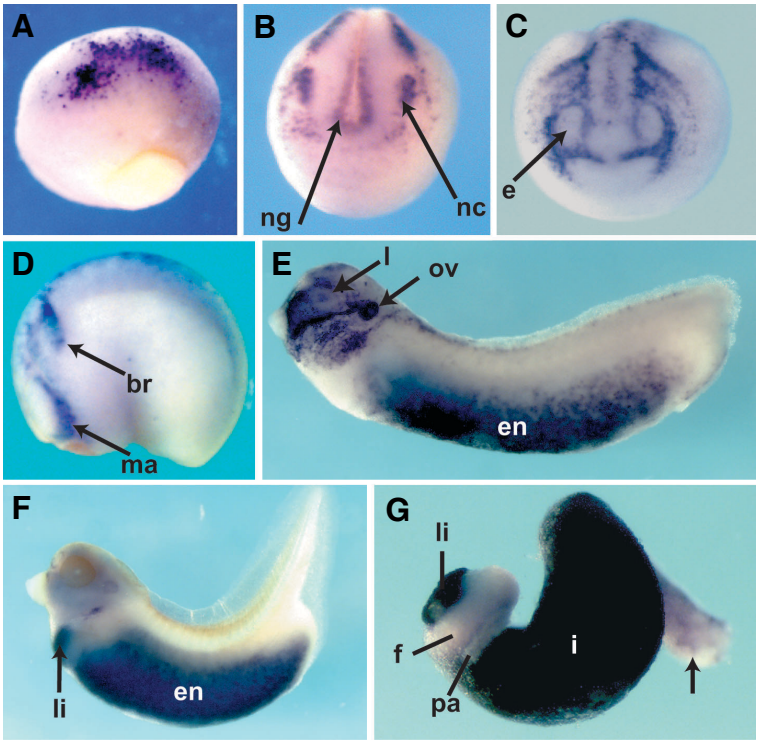


Fig. 7. Developmental expression of *C9* from gastrula to organ bud stage. *Anterior is to the left and dorsal to the top unless otherwise indicated.* **(A)** *Dorsal view of stage 10 embryo showing early spotted expression dorsal ectoderm. Anterior is to the top.* **(B)** *Anterior view of stage 17 embryo showing discrete neural crest (nc) and neural groove expression (ng).* **(C)** *Anterior view of stage 19-20 embryo. The three segments of the anterior neural crest show discrete expression at this stage. Mandibular segment expression surrounds the presumptive eye field (e).* **(D)** *Lateral view of stage 22 embryo shows expression in mandibular (ma) and branchial segments (br) of anterior migrating neural crest. There is discrete hyoid expression noticeable between those two segments.* **(E)** *Stage 27 embryo. Neural crest expression is seen in all three segments, as well as in the otic vesicle (ov), the lens (l), and the endoderm (en).* **(F)** *Stage 35 embryo shows strong liver and endodermal expression.* **(G)** *Isolated gut tube shows liver (li) and intestinal expression sparing the foregut (f), but no distal intestinal (proctodeum) expression (arrow); (lu) lung, (pa) pancreatic bud.*

confined to the hindbrain and the lens (Fig. 6D, E). In the developing hindbrain or rhombencephalon, expression is limited to the periventricular tissue (Fig. 6D), and extends caudally to the proximal neural tube (not shown).

Expression pattern of C9

C9 complement factor is the most abundant protein of the membrane attack complex (MAC), which is the terminal, cytolytic component of the complement system as it is understood in immunity. Akin to its MAC partners, C9 is made of multiple building-blocks including thrombospondin, low density lipoprotein receptor, epidermal growth factor, and perforin domains (Perkins, 2005). The C5b-9 (MAC) complex has been shown to participate in cellular proliferation of endothelial cells via the extracellular signal-regulated kinase (Perkins) and to induce cytoskeletal changes in a model of glomerular epithelial injury (Cybulsky *et al.*, 2005, Fosbrink *et al.*, 2006). C9 deficiency has been associated with an increased risk of infection and with post-ischemic injury (Liu *et al.*, 1998, Rzepecka-Wozniak *et al.*, 2006, Zoppi *et al.*, 1990). C9 was formerly identified in an expression screen, and the sequence we used to generate the antisense riboprobe was 100% identical to the one previously reported (Pollet *et al.*, 2005). However, previous analysis focused on the tailbud embryo, and we focused on the neurula stage.

Expression of *C9* as analyzed by situ hybridization shows a strong resemblance with *C3* expression during early stages of development. However, there are also some important differences. Expression of *C9* begins in gastrula stage embryos, in the presumptive neural plate (Fig. 7A). During neurulation, there is a discrete expression of *C9* in the neural crest cells and the neural groove (Fig. 7B). Cranial expression of *C9* is remarkable for a very fine linear pattern along mandibular, hyoid, and branchial segments of the cranial migrating neural crest (Fig. 7C, D). The expression is punctate, suggesting that only a subset of the migrating neural crest cells is transcribing this gene. By tailbud stage, expression in the migrating neural crest cells is strongest in the mandibular segment (Fig. 7E). Expression at this stage is distinct from the expression pattern of *C3*: it is characterized by a fine, mesh-like reticular pattern between the different neural crest segments. In the head of the tadpoles, additional intense expression is observed in the otic vesicle with a weaker expression in the developing lens.

In the endoderm, unlike *C3*, expression in the anterior endoderm is not noted until stage 19-20 (not shown). Endodermal expression in the tailbud embryo is noticeable in the peripheral endoderm, confirming the findings of Pollet *et al.* (Pollet *et al.*, 2005) and similar to the expression of *C3bC4b* at the same stage. In the isolated larval gut, expression mimics *C3* with *C9* transcripts observed in the liver and developing intestines, but not in the foregut and pancreas. At this stage, unlike *C3,* which is expressed throughout the gut, the *C9* transcripts

TABLE 1

SUMMARY OF ANALYSIS OF EARLY COMPLEMENT EXPRESSION IN *XENOPUS LAEVIS*

Gene	Accession No	Expression Pattern
C1s	BG811630 BE509150	No noticeable expression Notochord expression (*).
CR2	BM261540	Head and somite expression at st 35.
C4	BM180922	Liver expression stage 42.
C5a	BF048379	Diffuse, faint neural plate and head expression through st 35
C6	BQ734761	No neural plate staining, strong endodermal expression, similar to C3bC4b, no pronephros expression.
C8	BE509028 BE508140	Liver expression in tailbud embryo (*).
Carboxypeptidase N	BM180235	Liver expression at stage 42.
CD46	BG160519	Faint expression in neural plate and head through st 35.
CD59	BG814148	Faint expression in neural plate and head through st 35.
Complement Factor H	BC046950	Neural crest expression starting from neurula to tadpole stage. Endodermal expression from st 30 (*). Eye expression from stage 32.

(*) indicates that the expression pattern was reported by Pollet *et al.* (Pollet *et al.* 2005)

are not found in the caudal-most portion of the developing GI tract, or proctodeum, again resembling the findings for *C3bC4b*.

Expression analysis of other members of the complement system

Using BLAST searches, we identified *Xenopus* ESTs corresponding to other genes of the vertebrate complement system and performed in *situ analysis* for all of these. For the sake of completion they are listed in Table I. However, only those with an expression pattern of potential developmental interest are analyzed in detail in the figures.

Discussion

In summary, we show the developmental expression patterns of several of the major complement genes during early development of *Xenopus laevis*. The expression of each component was remarkable for some degree of tissue- or organ-specificity, often in organs not typically known for a role in immunity. To date, the most convincing data in support of a developmental function for complement is the known role of certain complement factors in hematopoietic cell migration. In fact, both the C1q-C1qR and the C3-C3aR pairs have been shown to participate in the migration and homing of hematopoietic lineages in mammals (Reca *et al.*, 2003, Vegh *et al.*, 2006). Consistent with these functional studies, both *C1qA* and *C3aR* expression is noted in the ventral blood islands of the developing *Xenopus* embryo. Ventral blood islands are the site of embryonic blood formation. From a developmental perspective, these findings are important because migration is a critical process in embryonic patterning and organogenesis.

In addition, several of the complement genes analyzed are expressed in other cell-types known for their migratory properties. For example, the neural crest cells, which are characterized by their ability to migrate long distances, express several of the complement genes: *C1qA*, *C1qR*, *C3*, *C3aR*, *Properdin*, and *C9*. Together with the ventral blood island data, this expression pattern raises the question of the potential mechanism. Complement proteins are known both for their ability to bind extracellular matrix proteins, and for their proteolytic activities. Thus, one possibility is that complement proteins bind to extracellular matrix proteins such as fibronectin, thereby facilitating cell movement. Alternatively, if complement proteins are also secreted during early development, they could participate in the extra-cellular release of growth factors either by cleaving inactive precursors or releasing growth factors from the extracellular matrix. Indeed, it was recently shown in *Xenopus* that the secreted serine protease xHtrA1, has a very similar expression pattern to several of the complement components analyzed, namely in the anterior neural plate, presumptive forebrain, neural folds, and branchial arches (Hou *et al.*, 2007). xHtrA1 causes cleavage of extracellular matrix proteoglycans thereby regulating the diffusion of the secreted ligand FGF4 (Hou *et al.*, 2007).

The second pattern found in this cohort of complement genes is expression in vascular structures. *C1qR* is expressed in the developing vasculature and is visible in the vessels of the developing liver and lung buds. This expression pattern is significant for two reasons. First, the C1q-C1qR pair has been shown to participate in hematopoietic cell homing in mammalian models. Second, endothelial-endodermal interactions are known to be essential in the development of highly vascularized organs such as the lung and liver (Del Moral *et al.*, 2006, Lammert *et al.*, 2003). Finally, *C1qA* and *C3bC4b* are expressed in the pronephros, another highly vascularized organ.

Third, the expression of *C1qA* and *C3aR* in the visceral mesoderm of the gut resembles the expression of the visceral mesoderm transcription factor *FoxF1* in the larval gut (Tseng *et al.*, 2004). The visceral mesoderm is the layer of cells surrounding the developing intestinal epithelium which will give rise to the intestinal smooth muscle layer and the mesenchyme. The visceral mesoderm is commonly accepted to drive the elongation of the developing gut (Roberts, 2000). Interestingly, *C3aR* is expressed in the visceral mesoderm at the same time as *C3* is transcribed in the adjacent endoderm, suggesting that these two molecules may function as a pair both in immunity and during the development of the gastrointestinal tract.

Finally, *properdin* expression is remarkable in the hindbrain and lens of the tailbud embryo. Other molecules with thrombospondin repeats, such as prothrombin and thrombin, have been shown to participate in glial cell proliferation and migration in the extracellular matrix (Krem and Di Cera, 2002), largely in disease states. Besides the homology with these molecules, the significance of our findings is unclear. It is possible that understanding their role in disease could orient research examining their role in development.

Taken together, these data are compelling for a previously unrecognized role for complement components during early patterning and organogenesis in lower vertebrates. The following observations support this hypothesis. First, their expression is noted very early in development, long before metamorphosis, the stage of onset of mature immune function in *Xenopus*. Second, expression of functional pairs occurs in different, sometimes complementary, tissues, rather than in the same tissue. This finding suggests that their role during development may not require expression at the same place and time, which is the typical paradigm in immunity. Instead, reciprocal expression patterns may be necessary to exert novel patterning functions. For example, *C3* is expressed in the endoderm at the same time as *C3aR* is expressed in the visceral mesoderm, two tissues known to require reciprocal signaling for their development and maintenance. Finally, tight spatial and temporal control of the expression of the different complement genes further supports a developmental role. For example, although *C3* and *C9* expression closely resemble each other, *C9* expression begins later than *C3* in the anterior endoderm, and appears not to extend to the distal most portion of the larval gut.

Although our results suggest that complement genes may have novel patterning functions, caution should be used when extrapolating from lower vertebrates to mammals. Indeed, several key members of the complement cascade have been knocked out in murine studies without a developmental phenotype (Mastellos *et al.*, 2001, Strey *et al.*, 2003). In light of our findings, these data suggest that a functional redundancy between members of the complement cascade may exist in mammals. Also, all of the functionally important complement genes in mammals do not seem to have an early developmental expression in *Xenopus*. For example, we were unable to identify an expression pattern for the *C5 / C5aR* pair during the stages of development examined, although it is an important component of the immune cascade.

In conclusion, we have shown in *Xenopus* the detailed embryological expression pattern of several components of the complement system, all of which were remarkable for some degree of tissue- or organ-specificity in organs not always involved in immunity. The significance of these findings is unclear and warrants further, functional studies.

Materials and Methods

Embryos

Embryos were generated by *in vitro* fertilization according to conventional methods. Embryos were cultured in 0.1xMMR at room temperature or in a 16°C incubator. Developmental stages were determined according to Nieuwkoop and Faber (Nieuwkoop, 1967). Embryos were fixed at different developmental stages according to previously described methods (Harland, 1991).

Isolation of C3

An adult liver-specific cDNA was hybridized first to a stage 13 cDNA library. Multiple copies of *C3* were isolated using this approach. The largest clone, 5kb in length, was inserted into pBS.

Identification of other complement clones in Xenopus laevis

Using BLAST searches, we identified expressed sequence tags (ESTs) with significant similarity to the human complement components reported. The corresponding I.M.A.G.E clones were obtained from A.T.C.C. DIG-labeled antisense riboprobes were generated according to conventional methods using Ambion MegaScript kit for *in vitro* transcription from the EST plasmid. pCMV-C1qA (BE507776) SalI, T7, pCMV-C1qR (BC111511) SalI, T7, pCMV-C3bC4bi (BI315342), pCMV-C6 (BC042265), SalI, T7, pCMV-Properdin (BM192350) SalI, T7 pBS-XC3 Kpn1, T7; pCMV-XC9 (BM180706) Kpn1, T7; pCS108-C3aR (CX430718) Sal1, T3.

In situ *hybridization*

In situ hybridization was performed as previously described (Harland, 1991). 65°C incubation was performed in a water bath. BM Purple (Roche) was used for the chromogenic reaction. *In situ* hybridization on isolated gut tubes were performed as previously described (Chalmers and Slack, 1998). *In situ* hybridization on bisected embryos was performed to show deep staining (Lee *et al.*, 2001, Sive, 2000). After whole-mount *in situ* hybridization, pigmented embryos were bleached in a 1% hydrogen peroxide, 5% formamide, 0.5X SSC.

Sections

Following *in situ* hybridization, embryos were dehydrated in ethanol, embedded in paraffin, and sectioned every 12-16 μm. Eosin was used for counterstaining when appropriate.

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