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Identification of a serine protease gene expressed by *Myxobolus cerebralis* during development in rainbow trout *Oncorhynchus mykiss*

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ABSTRACT: Serine proteases have been recognized as key factors in parasite physiology and disease development. We have identified a serine protease gene from *Myxobolus cerebralis*, *Myx*SP-1, the myxozoan parasite causing whirling disease in salmonid fishes. The amino acid sequence, as deduced from the cDNA sequence, included a catalytic residue arrangement similar to that of the chymotrypsin family of serine proteases. A real-time TaqMan polymerase chain reaction (PCR) analysis revealed differences in the transcription levels for the chymotrypsin-like protease as found in early, intermediate, and late developmental stages of the parasite in experimentally-infected rainbow trout *Oncorhynchus mykiss*. *Myx*SP-1 transcription differed between individual tissues at each sampling point and in the same tissues over time ($p < 0.0001$). A nonradioactive mRNA *in situ* hybridization (ISH) protocol was developed to detect *Myx*SP-1 transcripts. Using a mixture of 3 digoxigenin-labeled antisense mRNA probes, *Myx*SP-1 transcription was observed in developmental stages of the parasite during the acute and chronic phases of the disease over a 240 d time period in infected rainbow trout tissues. *Myx*SP-1 transcription observed by ISH in cartilage and as associated with cartilage destruction was consistent with our real-time TaqMan PCR findings that demonstrated high levels of *Myx*SP-1 transcription during lesion development. Identifying genes encoding these enzymes and characterization of their functions can lead to the development of new chemotherapeutic protocols and vaccine approaches to control parasitic diseases.

KEY WORDS: Salmonid whirling disease · *Myxobolus cerebralis* · Serine protease · Real-time TaqMan polymerase chain reaction · mRNA *in situ* hybridization

INTRODUCTION

Myxobolus cerebralis, a myxozoan fish parasite and the causative agent of salmonid whirling disease, is responsible for severe losses in populations of wild juvenile rainbow trout *Oncorhynchus mykiss* across North America (Nehring & Walker 1996, Vincent 1996, Hedrick et al. 1998, Baldwin et al. 2000). *M. cerebralis* has a complex 2-host life cycle that alternates between a myxosporean and an actinosporean (also referred to as a triactinomyxon) spore stage that are infectious for an aquatic oligochaete *Tubifex tubifex* and salmonid fishes, respectively (Wolf & Markiw 1984, Markiw 1989, El-Matbouli et al. 1995, El-Matbouli & Hoffmann 1998). In the fish hosts, sequential presporogonic stage development occurs in the skin, nerves, and skeletal cartilage. Presporogonic stages that reach skeletal cartilage cause extensive tissue damage (lysis of chondrocytes) and disrupt osteogenesis before undergoing myxospore formation (sporogenesis). Early stages of

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sporogenesis are associated with granulomatous inflammation but myxospores that remain sequestered in areas of bone in the head, gills, and axial skeleton invoke little response from the host (MacConnell & Vincent 2002). Clinical signs of disease during the acute phase are commonly characterized by darkened skin coloration, skeletal deformities, and periods of radical whirling behavior. Drug treatments that target DNA or RNA synthesis have shown moderate success in controlling the overt signs of disease (El-Matbouli & Hoffmann 1991, Wagner 2002) but sub-clinical infections often remain, leaving trout as carriers of the pathogen. Eliminating parasite infestations from fish-rearing facilities has focused on disrupting the life cycle of the parasite by chemically disinfecting ponds and raceways and using water sources free of fishes or oligochaetes. Managing whirling disease among wild salmonid populations has been more difficult, with major efforts focused on prohibiting stocking with infected farm-reared fishes and reducing conditions that contribute to habitats that may increase the populations of susceptible oligochaetes (Beauchamp et al. 2002, Wagner 2002). Detecting *M. cerebralis* among populations of captive or wild fish stocks is a key step in controlling the spread of whirling disease. Currently, a presumptive diagnosis of whirling disease is dependent on morphological identification of myxospores released by mechanical and chemical treatments from skeletal tissues of infected trout or salmon (Markiw & Wolf 1974, O'Grodnick 1975). Detection of spores or presporogonic stages in cartilage in hematoxylin and eosin (H&E)-stained tissue sections have been used as a confirmatory test for *M. cerebralis* (Hedrick et al. 1999a, Baldwin et al. 2000). Recently developed molecular-based tests such as the polymerase chain reaction (PCR), *in situ* hybridization (ISH), and a newly developed real-time TaqMan PCR test have provided time-saving and more-sensitive alternatives to detect and quantify numbers of the parasite in fish and oligochaete tissues (Andree et al. 1998, Antonio et al. 1998, Baldwin & Myklebust 2002, Kelley et al. 2004).

Prior research has shown that susceptibility to infection and severity of disease depend on fish species, parasite dose, age and size of the fish at the time of first exposure, and water temperature (Halliday 1976, O'Grodnick 1979, Markiw 1991, 1992, Hedrick et al. 1999a,b, 2001, Vincent 2002). The life cycle of *Myxobolus cerebralis* and history of whirling disease have been well described, but the pathogenic mechanisms by which the parasite recognizes, invades, migrates, replicates, and induces host cell injury in both the fish and oligochaete hosts are largely speculative. Prior studies (El-Matbouli et al. 1995, MacConnell & Vincent 2002) have shown that presporogonic stages are involved with rapid penetration of the host epidermis, and eventual lysis and ingestion of chondrocytes. The processes of penetration, migration into and between and lysis of host cells are all processes that are potentially facilitated by proteases of parasite origin. Proteolytic enzymes such as serine protease have been identified as critical factors in lesion formation and virulence of numerous pathogenic organisms (McKerrow 1989, McKerrow et al. 1993). Protease genes are critical elements to normal parasite physiology and development (Rosenthal et al. 1988, McKerrow 1989, 1999, McKerrow et al. 1993, 1999), but also influence parasitic virulence factors and subvert host defenses (Que et al. 2003) and thus are targets for potential chemotherapeutic or anti-parasite treatments. We presume that proteases, including serine proteases, are involved in several key phases of the development of *M. cerebralis* and thereby play a role in causing whirling disease in the fish host. Identifying and characterizing protease gene(s) and their expression during development of *M. cerebralis* have been an aim of our recent studies. In this manuscript we describe the isolation and partial characterization of a serine protease gene (*Myx*SP-1) expressed by *M. cerebralis* that may underlie one of several pathogenic mechanisms by which the parasite causes disease in the salmonid host.

MATERIALS AND METHODS

Parasite purification and nucleic acid isolation. A population of known susceptible aquatic oligochaetes was maintained in aquaria for the production of the triactinomyxon stage of *Myxobolus cerebralis*. The oligochaetes were exposed to myxospores freshly isolated (Markiw & Wolf 1974) from experimentally infected rainbow trout as previously described (Andree et al. 1998). Beginning at 90 d post-exposure, triactinomyxons released in the aquarium water were harvested and counted (Markiw 1989, Andree et al. 1998). Purified triactinomyxons were stored at 4°C prior to their use for fish exposures or nucleic acid isolation. Genomic DNA (gDNA) was isolated from the triactinomyxon spores using QIAamp® DNA Mini Kit (Qiagen). For RNA purification, 1 ml of RNA Later (Ambion) was added to the purified triactinomyxons to preserve RNA templates. Total RNA was extracted from the purified triactinomyxons using RNeasy® Mini Kit (Qiagen).

Experimental fish and tissue collection. We exposed 7 wk post-hatch rainbow trout (n = 232) to 20 000 triactinomyxons per fish in 450 ml of 15°C water without aeration for 30 min. An equal number of trout was used as a control group from the same egg lot, and was

maintained under the same conditions but not exposed to the parasite. Exposed and unexposed fish were maintained in separate 20 l aquaria receiving 15°C well water at 2 l min^{-1} and fed once daily with a commercial trout diet. The sample points included: time zero, 5 min, 2 h, 4 d, 10 d, 24 d, 54 d, and 240 d post-exposure. At each time point, 6 fish were euthanized with 500 mg l^{-1} ethyl *p*-aminobenzoate (Sigma). Using sterile techniques, approximately 20 to 50 mg of tissue from each of the caudal fin, dorsal fin, gill, muscle, spinal column, and head (near the ventral calvarium) were placed separately into sterile 1.5 ml centrifuge tubes and immediately placed on dry ice and stored at –80°C.

Tissue fixation and histological preparation. At each of the sampled time points, 6 fish were preserved in Davidson's fixative for 48 h and then transferred to 70% ethanol. After fixation, the fish were processed for standard paraffin embedding, and then sectioned (5–6 µm) for mRNA ISH and H&E staining.

PCR amplification with degenerate serine protease primers. A degenerate primer set (Sakanari et al. 1989) was modified to amplify protease gene fragments from gDNA of the triactinomyxon stage of *Myxobolus cerebralis* by PCR. The sequence of the degenerate primers are: serine protease forward primer: 5′-TTG GTI GTI ACI GCI GCI CA(C/T) TG–3′, serine protease reverse primer 5′-A(A/G)I GGI CCI CCI (A/T)(C/G)(A/G) CT(T/G) CC-3′. A 50 µl PCR mixture was prepared that contained Advantage®-GC 2 PCR reagents (Clontech Laboratories) as follows: 1X PCR buffer (40 mM Tricine-KOH, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 5% dimethyl sulfoxide, 3.75 µg ml^{-1} bovine serum albumin (BSA), 0.0005% Nonidet P-40, 0.0005% Tween-20), 1 M GC-Melt, 200 µM deoxynucleoside triphosphates (dNTPs), 1X Advantage-GC 2 polymerase mix (1.0% glycerol, 0.3 mM Tris-HCl (pH 8.0), 1.5 mM KCl, 1.0 µM EDTA), 40 pmol of each primer and 100 ng of gDNA. The mixture was subjected to 35 cycles of amplification (95°C for 3 min, 94°C for 1 min, 48°C for 1 min, 72°C for 2 min followed by an extension at 72°C for 10 min). After amplification, 10 µl of the PCR sample were visualized on a 1.5% agarose gel. An 810 bp amplicon was excised from the gel and purified using QiAquick® Extraction Kit (Qiagen) and cloned using the TOPO TA Cloning Kit (Invitrogen Corporation). Transformed cells were screened using PCR (protocol described above) and cells containing the desired plasmid were isolated using the QiAprep® Spin Miniprep Kit (Qiagen). Purified plasmid was sequenced using the SP6 and T7 promoter primers with an ABI 377 automated sequencer (Applied Biosystems). Sequences were aligned using the computer program MacDNASIS, version 3.7 (MiraiBio.).

Specific serine protease PCR amplification. Specific primers (see Table 1) designed with the software Amplify (University of Wisconsin Genetics, Madison, WI) were used to amplify *Myx*SP-1 gene fragments from cDNA templates. Utilizing 300 ng of total RNA, cDNA was synthesized using Omniscript reverse transcriptase (Qiagen) following the manufacturer's protocol. A 50 µl reaction mixture containing 1X PCR buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 400 µM deoxynucleoside triphosphate, 0.5 U Taq polymerase (Invitrogen), 5 µM tetramethyl ammonium chloride, 40 pmol of each primer and 250 ng of cDNA was subjected to 35 cycles of amplification (95°C for 3 min, 94°C for 1 min, 58°C for 1 min, 72°C for 2 min followed by an extension at 72°C for 10 min). PCR products were visualized, cloned, and sequenced in the same manner as described above.

5′ and 3′ rapid amplification of cDNA ends (RACE). The 5′ and 3′ ends of *Myx*SP-1 were amplified using a SMART™ RACE cDNA Amplification Kit (Clontech). First-strand 5′ and 3′ cDNA templates were produced following the manufacturer's protocol. The gene specific primers for RACE amplification were: $5'RC_{Ser}$: 5′-CCT TGG CTA AAT CAA GTC CTG CAC-3′; and $3'RC_{Ser}$: 5′-CTC GTA AGA GCA GAG TCA AGA TCC-3′. The RACE PCR reaction (50 µl) contained 1X Advantage 2 PCR buffer (40 mM Tricine-KOH, 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 µg ml^{-1} BSA, 0.005% Tween-20, 0.005% Nonidet-P40), 200 µM dNTPs, 1X Advantage 2 polymerase mix (1.0% glycerol, 0.3 mM Tris-HCl (pH 8.0), 1.5 mM KCl, 1.0 µM EDTA), 40 pmol of each primer and 200 ng of 5′ or 3′ cDNA templates. RACE amplification consisted of a denaturation step for 5 min at 95°C followed by 5 cycles of amplification (95°C for 3 min, 94°C for 30 s, 75°C for 1 min, 72°C for 2 min) and 30 cycles of amplification (94°C for 30 s, 68°C for 1 min, 72°C for 2 min followed by an extension at 72°C for 5 min). The PCR samples were visualized, purified, cloned, and sequenced as described previously.

Real-time TaqMan PCR systems. Using the 18S ribosomal RNA (rRNA) and *Myx*SP-1 genes of *Myxobolus cerebralis*, and the internal transcribed spacer-2 (ITS-2) gene of rainbow trout, 2 primers and an internal fluorescent-labeled probe (5′ end, reporter dye FAM; 3′ end, quencher dye TAMRA) were designed using Primer Express software (Applied Biosystems) (Table 1). TaqMan systems for both the 18S rRNA sequence for *M. cerebralis* (GenBank Accession No. AF115253) and the ITS-2 sequence for *Oncorhynchus mykiss* (AF170543) provided endogenous controls for the parasite and trout tissue, respectively. The length of the PCR products was held very short (between 75 and 120 bp) to enable high amplification efficiencies.

Table 1. *Myxobolus cerebralis* infecting *Oncorhynchus mykiss*. Sequence for PCR primers and TaqMan probes specific for *O. mykiss* ITS-2 gene, and *M. cerebralis* 18S rDNA gene and *Myx*SP-1 protease gene. F: forward primer; R: reverse primer

Target	Primers and probes	Sequence (5′→3′)	Product length (bp)
ITS-2	tITS2.2 F[a]	TCATCAATCGGAACCTCTGG	120
	tITS2.157 R[a]	AAGGAAGAGCGCACGGG	
	tITS2.75 Probe[a]	CACCGAGCCGTCCTGGTCTGAACTTA	
18S rRNA	Myx18-909 F[a]	CTTTGACTGAATGTTATTCAGTTACAGCA	88
	Myx18-996 R[a]	GCGGTCTGGGCAAATGC	
	Myx18-953 Probe[a]	ACCGGCCAAGGACTAACGAATGCG	
*Myx*SP-1	SP4812-266 F[a]	GCCATTGAATTTGACTTTGGATTA	75
	SP4812-338 R[a]	ACCATTCATGTAAGCCCGAACT	
	SP4812-291 Probe[a]	TCGAAGCCTTGACCATCTTTTGGCC	
	SP4812-124 F[b]	GGCGAACGAAGGACCAATCTTTTC	171
	SP4812-130 R[b]	GCCTGGAAAACACCGCAGAGATC	
	SP4812-127 F[b]	CCAAGTTCGGGCTTACATGAATGGTC	219
	SP4812-134 R[b]	CAAAGGATCTTGACTCTGCTCTTAC	
	SP4812-246 F[b]	GCAAATGGCACTCTTGATTGCATCAG	189
	SP4812-252 R[b]	CATCTACCAAAGTGTTACAGGTGTTGTC	
	SP4812-115 F	CCTAAGAAGTTCATCAATAGCGAG	3889
	SP4812-255 R	TTTTTTTTGAGTTGAGGTTGCCG	
	SP4812-228 F	GAAGTCCTGGCATTTTGAGCATATTAC	1735
	SP4812-238 R	CCCCTAAACGTTGAATACACGCATTAC	
	SP4812-135 F	GGCCGAAAGTCGTCAACTGTG	1386
	SP4812-231 R	CGTTCTCTTGCAGTTCCCTGTGGC	
	SP4812-01 F	CCGTACTAGAAAAGGGAGCC	541
	SP4812-05 R	GGTCCGCTCTCATCTTCTC	

[a]Primers and probes used in TaqMan PCR; fluorescent dye labels: 5′-6FAM, 3′-TAMRA
[b]Primers used to amplify *in situ* hybridization probes

Real-time quantitative PCR sample preparation. Rainbow trout tissues were transferred frozen into 96 deep-well plates containing 800 µl of 1 × ABI lysis buffer (Applied Biosystems) and two 4 mm diameter grinding beads (SpexCertiprep). Tissue samples were homogenized in a GenoGrinder2000 (SpexCertiprep) for 2 min at 1500 strokes min^{-1}. After a 30 min period at 4°C, total RNA was extracted from the tissue lysates using a 6700 Automated Nucleic Acid Workstation (Applied Biosystems) according to the manufacturer's instructions.

Reverse transcriptase reaction and real-time quantitative TaqMan PCR. cDNA was synthesized using 100 U of SuperScript II (Invitrogen), 300 ng random hexadeoxyribonucleotide ($pd(N)_6$) primers, 10 U RNaseOut (Invitrogen), and 1 mM dNTPs in a final volume of 40 µl. The reverse transcription reaction proceeded for 50 min at 42°C. After addition of 10 µl of molecular grade water, the reaction was terminated by heating for 5 min to 95°C and immediately cooled on ice.

Each TaqMan PCR reaction contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM dNTPs, 0.625 U AmpliTaq gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction and 5 µl of the diluted cDNA sample in a final volume of 25 µl. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 60 s. Final quantification used a modification of the comparative cycle threshold (CT) method (User Bulletin #2, Applied Biosystems). The endogenous control for trout tissue (ITS-2) was used to normalize the *Myxobolus cerebralis* 18S transcription which, in turn, was used to normalize *Myx*SP-1 gene transcription for each tissue. Subsequently, *Myx*SP-1 transcription was calibrated against the lowest amount of *Myx*SP-1 transcription found in each tissue sampled. All normalized *Myx*SP-1 gene transcription values are reported as the n-fold difference relative to a calibrator cDNA (i.e. lowest target-gene transcription).

***In situ* hybridization**. To prevent RNA degradation, all reagents were prepared (Sambrook et al. 1989) with diethyl pyrocarbonate (DEPC) (Sigma)-treated distilled water, and all equipment was treated with RNase® AWAY (Molecular Bioproducts). Using *Myx*SP-1 gene-specific primers (Table 1), 3 cDNA

gene fragments were amplified by PCR and cloned using the methods described above. The 3 cDNA clones were used to make sense and antisense RNA probes using the DIG RNA labeling kit (Roche Applied Science) following the manufacturer's protocol.

The methods used for tissue deparaffinization, permeabilization, and prehybridization are those developed by Antonio et al. (1998), and where appropriate they were optimized for mRNA ISH. Tissue sections were prehybridized at 37°C for 2 h and slides were then rinsed in 2x saline sodium citrate (SSC; 1× SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), and briefly dried prior to applying the hybridization solution. The sense and antisense hybridization solutions were handled separately, and each solution contained 3 labeled probes for either *Myx*SP-1 sense or antisense signal detection. The components of the sense and antisense hybridization solutions were similar to the prehybridization solution, except that each probe was added at 1:320 (v:v; 0.003 pg μl^{-1}). Hybridization solutions (0.75 ml) were added to tissue sections and were simultaneously denatured at 100°C for 10 min. The tissues were covered with sterile coverslips (RPI) and then incubated overnight at 50°C in a humid chamber. Following methods reported by Antonio et al. (1998), stringency washes were performed to remove either unbound probes or nonspecific hybridization of probes.

Detection of hybridization signals. Sense and antisense probe hybridization was detected by an enzyme-catalyzed color reaction (Antonio et al. 1998). Tissue sections were incubated with substrate solution for 1.5 h at room temperature in a humid chamber. Signal intensity was examined microscopically before the reaction was stopped by submersion of slides in distilled water. Tissue morphology was enhanced against the hybridization signals by counter-staining with 0.05% aqueous Bismarck Brown Y (Sigma) for 30 s. Slides were briefly washed with xylene and permanently mounted with a coverslip using Permount adhesive (Permount). Hybridization signals for *Myx*SP-1 mRNA transcripts were observed as purple precipitates in tissue sections by light microscopy.

Statistical analysis. All statistical analyses were performed using SPSS 10.0 (SPSS). *Myx*SP-1 transcription values were log-transformed to enhance normality for posterior statistical analyses. An ANOVA model was used to compare the effects of tissue and time on *Myx*SP-1 transcription. Differences in *Myx*SP-1 transcription among sampling times were evaluated for each tissue using univariate ANOVA models followed by pairwise multiple comparisons using Tukey's Honestly Significant Difference (HSD) test to adjust for unequal sample sizes. For all the statistical analyses, $p < 0.05$ was considered significant.

RESULTS

*Myx*SP-1 sequence analysis

Amino acid sequence analysis for *Myx*SP-1 showed 3 catalytic sequences (His-54, Asp-248, Ser-310) that are characteristic for serine proteases (Hartley 1964). Additional sequence data obtained by 5′ and 3′ RACE amplification allowed a prediction of the open reading frame for *Myx*SP-1. *Myx*SP-1 contained a complete coding sequence from the initiation codon ATG (Met-1) to the stop codon TGA, and a 5′ and 3′ untranslated region (UTR) (Fig. 1; GenBank Accession No. AY275708). A determination of the initiation codon was based on Kozak's initiation consensus sequence (Kozak 1981, 1999). No introns were found based on alignments of the genomic *Myx*SP-1 sequence with the *Myx*SP-1 cDNA sequence (data not shown). The 3′ UTR of *Myx*SP-1 contained a 366 bp stretch that complemented portions of the 18S rDNA and ITS-1 genes described for *Myxobolus cerebralis* (present Fig. 1, and Andree et al. 1999). A BLASTP search (Altschul et al. 1990) revealed no detectable amino acid similarities outside the catalytic sites for *Myx*SP-1 compared to serine protease genes from other species (data not shown). However, the *Myx*SP-1 catalytic triad was aligned with catalytic regions from other eukaryotic serine proteases, and the conservation of the catalytic triad was confirmed (Fig. 2). A simple sequence comparison of the predicted mature *Myx*SP-1 protein revealed limited sequence homology (22 to 28%) with the mature serine protease sequences from selected eukaryotic organisms (Fig. 2).

Distribution of *Myx*SP-1 transcripts across tissues and sampling time

Differences in the transcription of *Myx*SP-1 were found between individual tissues during the course of this study (Fig. 3). The overall comparison for the effects of tissues on the log-transformed *Myx*SP-1 transcription over time was highly significant ($p < 0.0001$). No protease transcription was found for tissues collected from control fish (uninfected). At 5 min post-exposure, mean *Myx*SP-1 transcription values found in the gill were nearly 10-fold greater than levels detected in the caudal fin. As expected *Myx*SP-1 transcription was not detected in the spine and head tissues at 5 min post exposure. *Myx*SP-1 transcription peaked at 2 h post exposure in the gill and dorsal fin epithelial tissues. The first indication of protease transcription was observed in the spine and head tissues at 2 h post exposure. Mean *Myx*SP-1 values observed in spinal tissues at 4 d post exposure increased nearly 10-fold from lev-

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-555 ACGCGGGCAGCACGTTACAGAATTCGTCTTAAATTCGAATCCAGGAAAGTCAGAGAGATC
-495 CCTAAGAAGTTCATCAATAGCGAGATTGAAAATGCAAGAGGGGAAACGGGACAACCTTGT
-435 CTGATGCCTCTTTTGAGTGACACAGTATTTTTTGATACGAAATTGTCAAATGAAGTTTGT
-375 CCCCCGGAGAACATGTCTGAGATAAGAGAGGCGAACGAAGGACCAATCTTTTCTTCAATT
-315 GAAAAAAGAAGGGCAGAGTGCATCCACCTAGATCGAAGTGCCTTGCGAAAGTCGACAAAG
-255 AGCAACAGCGGCTTTACACTTCATCGAACGTTGAGTTTGGAAGATAAGACTCTAAGATCT
-195 CTGCGGTGTTTTCCAGGCAGCCATCCACCTTCGAGGAAACCACGCAGAGACTTGTGGAGA
-135 AAGTTTGGCGACAAGAGAACAAGTCTATCAGCTAGGAGGCTCACTGAAGAATTCGATAGA
-75  GAACATCTTGTGATTGAGATCGGCCGAAAGTCGTCAACTGTGGAGGGGGAAGAATCCTTA
-15  GATTTTGGAATAAGGATGGTATTGGATAGTTTCAAAAAGGAAGGAATAGCTCTCTCAGCC
                  M  V  L  D  S  F  K  K  E  G  I  A  L  S  A  15
46   AGAAAACGGTTGAGGAGACATGTTAGAGCAGACAATGCCAGGTGTGTCTGGAGACGAGCC
      R  K  R  L  R  R  H  V  R  A  D  N  A  R  C  V  W  R  R  A  35
106  AAAGAAACGAGTTGCACCAGATATACCATCAGGGCCAGTCGAAGTTGTTGGGCTCATTGC
      K  E  T  S  C  T  R  Y  T  I  R  A  S  R  S  C  W  A  H• C  55
166  ATTGACTTTATTAAATACATCCGTACTAGAAAAGGGAGCCATAAGATCAGGAAATTTGTC
      I  D  F  I  K  Y  I  R  T  R  K  G  S  H  K  I  R  K  F  V  75
226  AGAGAAACAGATTCAAGAAGACTGAAGAGACATAAGTTGAATCTCAGAGGGCACATTCCA
      R  E  T  D  S  R  R  L  K  R  H  K  L  N  L  R  G  H  I  P  95
286  GAGAGAATTATAGTCGACGAGCAATCTCCTCGGACGAGCATGTACACTTTGTATTGTTCC
      E  R  I  I  V  D  E  Q  S  P  R  T  S  M  Y  T  L  Y  C  S  115
346  GATTTTTGGGTTATGAGTTCAAAAGCCCGTGCAGGACTTGATTTAGCCAAGGAAATTGAG
      D  F  W  V  M  S  S  K  A  R  A  G  L  D  L  A  K  E  I  E  135
406  TATGATTGGGCCATTGAATTTGACTTTGGATTATTCGAAGCCTTGACCATCTTTTGGCCA
      Y  D  W  A  I  E  F  D  F  G  L  F  E  A  L  T  I  F  W  P  155
466  AGTTCGGGCTTACATGAATGGTCGTACGACAGGAAAGGCCGAGTCTTTGAGAGCTCTTTT
      S  S  G  L  H  E  W  S  Y  D  R  K  G  R  V  F  E  S  S  F  175
526  TTCGCTGAACGCTTCCTAGCCTTACGTTTGTGGAATGAGAGGACTGGATTGGACCTTGAG
      F  A  E  R  F  L  A  L  R  L  W  N  E  R  T  G  L  D  L  E  195
586  ACTGACAGGAGCGACTCTACTGCCAAAACCGACGTTTCCCAGCTACCAAAAGTTCTTTCG
      T  D  R  S  D  S  T  A  K  T  D  V  S  Q  L  P  K  V  L  S  215
646  AGAAACCTCGTAAGAGCAGAGTCAAGATCCTTTGAGTCGATGGTTGATAGAGAAGCCAGA
      R  N  L  V  R  A  E  S  R  S  F  E  S  M  V  D  R  E  A  R  235
706  GAGAAGATGAGAGCGGACCTTGAGGCTGACGAACTTGACACTGCGCTTTTCTGGCATTTA
      E  K  M  R  A  D  L  E  A  D  E  L  D• T  A  L  F  W  H  L  255
766  AGTGGAATATTAGAAGGGAAAAATGGATCGGAGACTGTGATCAACGGGCCCCTGAGAGGG
      S  G  I  L  E  G  K  N  G  S  E  T  V  I  N  G  P  L  R  G  275
826  GACCTGAGAATTCCATATGTGTTTTGGAGTTTGATAGATTCGCAGCTATTAGACCGACGA
      D  L  R  I  P  Y  V  F  W  S  L  I  D  S  Q  L  L  D  R  R  295
886  TTAGAAGATGAAACTTCTTTTACTCTGGGTGCGATAATCTCAAGTGGCAGTGACGAGCGT
      L  E  D  E  T  S  F  T  L  G  A  I  I  S  S• G  S  D  E  R  315
946  GAAAGGGCTCCAGAACTTGAAACCGACTCGTCCGATGATTCATTATCCAGAGGAAGTGGA
      E  R  A  P  E  L  E  T  D  S  S  D  D  S  L  S  R  G  S  G  335
1006 ACGACACGCGACAGCGCTTGGATTTTTTCTGGAGTAGAGACTATAATTGGACGTGAATTA
      T  T  R  D  S  A  W  I  F  S  G  V  E  T  I  I  G  R  E  L  355
1066 TTGATTTCCTTGGAGATATCCTTAGAGACCGGCACGAGAAAAGGAGAGGGAGAAGGGACC
      L  I  S  L  E  I  S  L  E  T  G  T  R  K  G  E  G  E  G  T  375
1126 TTGCTAGACTTTTCTTTCTTCACAGACTTGGTCTGAGATTGGCAGGACTGGAAGTGTATT
      L  L  D  F  S  F  F  T  D  L  V  *                          386
1186 GAGAAATCTTTCAGGTTGTTAGATTTGAAGTCCTGGCATTTTGAGCATATTACAAGCACT
1246 TTACACTTTATGAACATTAATGAAATGGTCTCTTGCAAGTATCGAGTTGAGTTAAATGAG
1306 AGCCACAGGGAACTGCAAGAGAACGAGGCCCTGTGGAATTGGAATAAAAGTAGAGGAAAT
1366 TTGATTATCCATTACTATAGGAAGAAAAGCCGCCAAAGCCGAGTAAACCACATCAACATG
1426 ATTGACTTCGGACTGCTTCGTCGGGGCATTAAGGTTTTCAGGCTGAGTAGGATTCACGGG
1486 TTGAGATGGGGGCATGATTTGAATTTTCCCAAGAAAATTGACTTTTTTTCCCCGATGACT
1546 TACGAAATTGAGATTCGGTCAGAGGGGACCTTTGATTTATTTCTGTCACGGGACGGTAAA
1606 ATTTTTTGGTCTGCCGGGCTGGGGAGAGAAAAACATCTGAGACGTTTTTTGGAAAATTAT
1666 TTGTGGCGGCAGGCTTTGAGCCTAGAAAGCCCTAAGTCAGGATTAACAACAGGCACATGC
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Fig. 1 (above and facing page.) Nucleotide and deduced protein sequence of *Myx*SP-1. Numbers on left denote nucleotide sequence, those on right amino acid sequence. Amino acid sequence starts with the methionine (Met-1) residue and is represented by the single-letter code below the nucleotide sequence. Residues in boldface correspond to relatively conserved catalytic motifs for serine proteases. Catalytic active site residues His-54, Asp-248, and Ser-310 are in bold and designated by dots (•). Underlined sequence significantly complements a conserved portion of the 18S rRNA and ITS-2 gene of *Myxobolus cerebralis*. Stop codon is indicated by an asterisk *

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1726 CGGTTGAACACATGGTGTTCAAGGCTGAGTCTGGGTTATTTACTGAGGACGAGTAGTGAA
1786 CGCTGGAAGCACGAGAATCTTGTAGGGAAATTGTTTCGGGATAGCTCGGCAACTTTTCCA
1846 ACGATAGAACATAGCGAACTTTGGGCAGAACCCAACACAATACTAAGATTGGCCACTCCC
1906 TTGAAGCAAGGCGGAGATATCCGCTTGACGCAAGTTGTTAAATTTGAGGCCATTGTTGAG
1966 AGAGAATTGAGGAGCCGTGAGGCGGTGAGTTTTGGCTCATATTTTGCGCGCCTGACGGGG
2026 GTCGAATTATTGGAAGAGGGTAAGGCGGTAGGCGTAGATGCGCCACGGGAATTATCAAAC
2086 ATCTTGTTGTTTGACTTTTTTCGCGCCATAATATGTATGCGGTTTAGGTCGATTTATCTA
2146  AGTATACTTATCAAAAAAAATTCTGGGAATTCCGGTTTTTTTTCAGGAGTAAAATTAAAC
2206  GCACTGACCACTAGAAATTGTGATGCCCTAAGATGTCCTGGGCTGCACGCGCGCTACAAT
2266  GATGGTGACAGCGAGTTTCTAGGTCGAGAGACCTGGCAATCTTGTAATCATCATCGTGAT
2326  GGGATTGACCATTGTAATTTGGTCATGAATAGGAATTCCTGTAGGCGCACTTTATTAGAG
2386  TGTGCCGAACGAGTCCCTGCCCTTTGTACACACCGCCCGTCACTACTACCGAGTGAATCG
2446  TGTCATGATGCCTTGGGACCGGACGTATTTGGAGCTGCAAGGCTTGAAATACGCTGGGAT
2506  CGATGTAAAATGGTGCAATTTCGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAA
2566  CCTGCGGAAGGATCATTGACGTTTACACAAACATTTTGTACGTTGTACGTATTATTCGAG
2626  AAATTACATCTGTTGCTGAAGCTGTGTCTCAGACTAGTTGAGGAGAGTCGCATTACGAGA
2686  AAGCATTCTTCTAGTCGCTTCAGCAACACAGAGAAATCAAGACGACCTCCACTTTTAAAT
2746  ATAATCACTGAGAGTAGACTACTTATTTCGTCTATTCTGTTCGACATTGATGAGACAATA
2806  GTGAACTTTAGAGTAATATTAGTCTTTGTTTGGGAATTTTTAATCGTGTTGTATAAAGCG
2866  GTTGTGTTTATAAATGTGAATGATAAGTATTTCCTGTTCGTTGGGTATGTGTGTAATGCG
2926  TGTATTCAACGTTTAGGGGTGCTCTATTTTCACAGAGTGAGATTTTACGAGTTGATATAT
2986  TCAATAACCATTAACGGTGGATCACTTGGCTCGAGGACGATGAAGAACGTGGCAAATGCG
3046  ATAAGTGATGCGATTCGCAAGCCTAGTGAGTCATCAAGTTTGAACGCAAATGGCACTCTT
3106  GATTGCATCAGGAGTATGTCTGGTTGGGGTTGTTTTTTGAGATACGTCACACGGTTGGCT
3166  TTGCAATTGATGTGCGAATATACACTAGTTGTATATAGAGTTGGGTGTGAAACCGAAGCG
3226  CGGGTTCTCATCTCAAATGGATTGGACAACACCTGTAACACTTTGGTAGATGTATTAATT
3286  GTTGAATTTGTTGGTGAAAACTTTAGAGTACAACAATGTGTTGCACCTATTAAAAGATAC
3346  AGTAAGTTTATAATTATAAATTTACGGCAACCTCAACTCA25
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Fig. 1 (continued)

els detected earlier at 2 h post exposure. The *Myx*SP-1 transcription found in the nerve tissues of the spine at 4 d was 12-fold greater than that of the caudal fin and head tissues. At 10 d, mean levels of *Myx*SP-1 transcription followed a general decreasing trend in the gill and dorsal fin tissues, albeit transcription remained greater than those levels detected in the remaining tissues. Mean *Myx*SP-1 transcription measurements in spine tissues at 10 d post exposure decreased 8-fold from prior transcription levels observed at 4 d post exposure. There was at least a 15-fold increase in *Myx*SP-1 transcription found at 24 d in the spine compared to protease transcription detected in the caudal fin. At 54 d post exposure, relatively high levels of protease transcription remained in the spine but the overall protease transcription in all tissues sampled exhibited a decreasing trend. At 240 d, protease transcription was observed in the spine and head but in no other tissues.

*Myx*SP-1 transcription changed significantly ($p < 0.0001$) over the sampling period for all tissues except for muscle ($p = 0.179$; Fig. 3). *Myx*SP-1 transcription at 2 h in the caudal fin was significantly greater than that for all other time points. *Myx*SP-1 transcription measured at 2 h in the dorsal fin was significantly greater

Myxozoan *(Myxobolus cerebralis)*	RSCWA**H**CID	DEL**D**TALFWH	LGAIIS**S**GSD
Cnidarian *(Aurelia aurita)* – Type s1A serine protease	ILTAS**H**VCV	LTN**D**IALIKL	ITAPGD**S**GGP
Nematode *(Trichinella spiralis)* – stage-specific serine protease	VVTAA**H**CVQ	LHN**D**IAVLEL	DSCNGD**S**GGP
Trematode *(Schistosoma mansoni)* – elastase 2a	VLTAG**H**CVC	SGF**D**IALTML	ITAPGD**S**GGP
Insect *(Anopheles gambiae)* – trypsin-like protease	ILTAA**H**CID	NNY**D**IALELE	GTCRND**S**GGP
Crustacean *(Panulirus argus)* – trypsin-like protease	VLTAA**H**CVD	VDN**D**MALLRL	DSCQGD**S**GGP
Frog *(Xenopus laevis)* – epidermis-specific serine protease	VMTAA**H**CID	SSG**D**IALIEL	DACQGD**S**GGP
Mouse *(Mus musculus)* – Elastase 1	VMTAA**H**CVD	AGY**D**IALLRL	SGCQGD**S**GGP
Rat *(Rattus norvegicus)* – chymotrypsin-like	VVTAA**H**CKV	MNN**D**LTLLKL	SSCQGD**S**GGP
Human *(Homo sapiens)* – plasminogen	VLTAA**H**CLE	TRK**D**IALLKL	DSCQGD**S**GGP

Fig. 2. Conserved residues of catalytic triad for serine proteases (boldface) and regions flanking the active sites for comparison. Amino acid sequences surrounding the catalytic active site of *Myx*SP-1 from *Myxobolus cerebralis* were aligned to serine proteases from a cnidarian (*Aurelia aurita*; AAO12214), nematode (*Trichinella spiralis*; AAD09211), trematode (*Schistosoma mansoni*; AAM43941), insect (*Anopheles gambiae*; S40003), crustacean (*Panulirus argus*; AAK48894), frog (*Xenopus laevis*, BAA84941), mouse (*Mus musculus*; BAB25008), rat (*Rattus norvegicus*; NP_446461), and human (*Homo sapiens*; NP_000292). Amino acids are in single-letter code

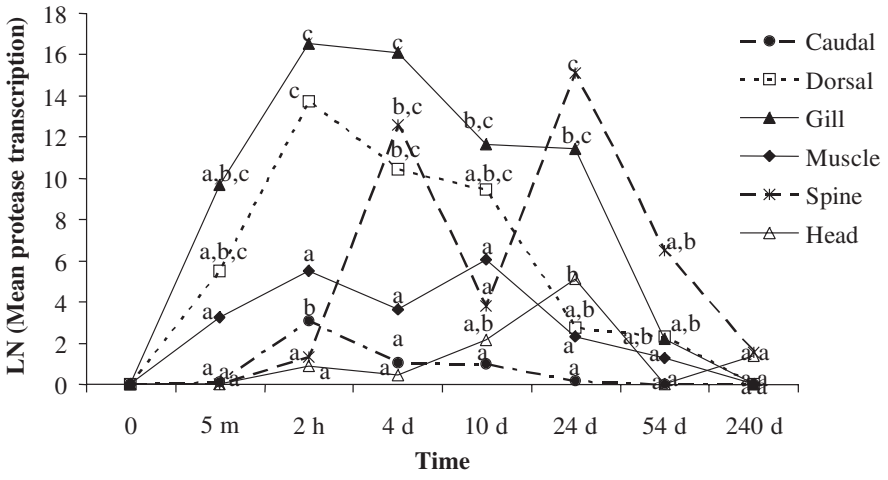

Fig. 3. *Myxobolus cerebralis* infecting *Oncorhynchus mykiss*. Transcription levels for *Myx*SP-1 during *M. cerebralis* development in rainbow trout tissues over 240 d time course measured by real-time TaqMan PCR. Results represent natural log (LN) mean of normalized linear amounts of *Myx*SP-1 transcription relative to the weakest protease signal (also referred to as the calibrator) in each tissue. Differences in *Myx*SP-1 transcription among sampling times for each tissue are represented by superscripts a,b,c, whereby mean protease transcription for each tissue with same superscript(s) was not significant different at 95% confidence level

than transcription detected at 24, 54, and 240 d post exposure. *Myx*SP-1 transcription in gill tissues at 2 h and 4 d differed significantly from levels measured at 54 and 240 d post exposure. At 24 d, *Myx*SP-1 levels measured from head tissues were significantly greater than those at earlier or later time points. Also, *Myx*SP-1 levels measured at 24 d from the spine were significantly greater than transcription levels for all other time points but 4 d.

Location of *Myx*SP-1 transcription in rainbow trout

Using the mRNA ISH test, *Myx*SP-1 transcription was observed associated with all developmental stages seen in the tissues of infected fish over a 240 d time period. A combination of 3 antisense probes yielded positive signals associated with sporoplasm aggregates infecting host epithelium at 5 min and 2 h post exposure (Fig. 4B). *Myx*SP-1 antisense probes bound to developmental stages were found throughout the peripheral and central nervous system (CNS) tissues at 4 d (Fig. 4D) and 10 d (Fig. 4F) post exposure. Parasite stages at these time points were elongated, spindle-shaped, and associated with no host-tissue reaction. Also at 10 d post exposure, *Myx*SP-1 antisense signals were associated with presporogonic stages amassed in close proximity to chondrocytes in the cartilage of the head (Fig. 4F) and spine. There was no host-cell response to parasites in this location at this time point, as observed by histopathology. However, by 24 d post exposure, *Myx*SP-1 antisense signals were commonly associated with presporogonic stages in distinct areas of host-tissue degeneration characterized by stages infiltrating and lysing cartilage of the head (Fig. 5B). Positive *Myx*SP-1 transcription detected at 54 and 240 d post exposure was from presporogonic stages frequently associated with severe cartilage damage characterized by lysis and degeneration of chondrocytes in the head (Fig. 5D,F). No positive signals were observed using the control *Myx*SP-1 sense hybridization solution with infected fish tissues (Fig. 5G). In addition, no positive signals were observed in tissues from control uninfected fish with either the sense or antisense *Myx*SP-1 hybridization solutions (data not shown).

DISCUSSION

The identification and initial characterization of a serine protease gene for *Myxobolus cerebralis* is the first we are aware from a member of the phylum Myxozoa. Previous reports have shown other myxozoan fish parasites are capable of producing proteolytic enzymes responsible for tissue deterioration and possibly parasite encystation (Bilinski et al. 1984, Martone et al. 1999). However, these prior studies did not report nucleotide or protein sequence data along with the described protease activity. Under the conditions and time points tested, our real-time TaqMan PCR analysis provided a relative assessment for *Myx*SP-1 mRNA transcription. Furthermore, our mRNA ISH test demonstrated the ability to visually detect *Myx*SP-1

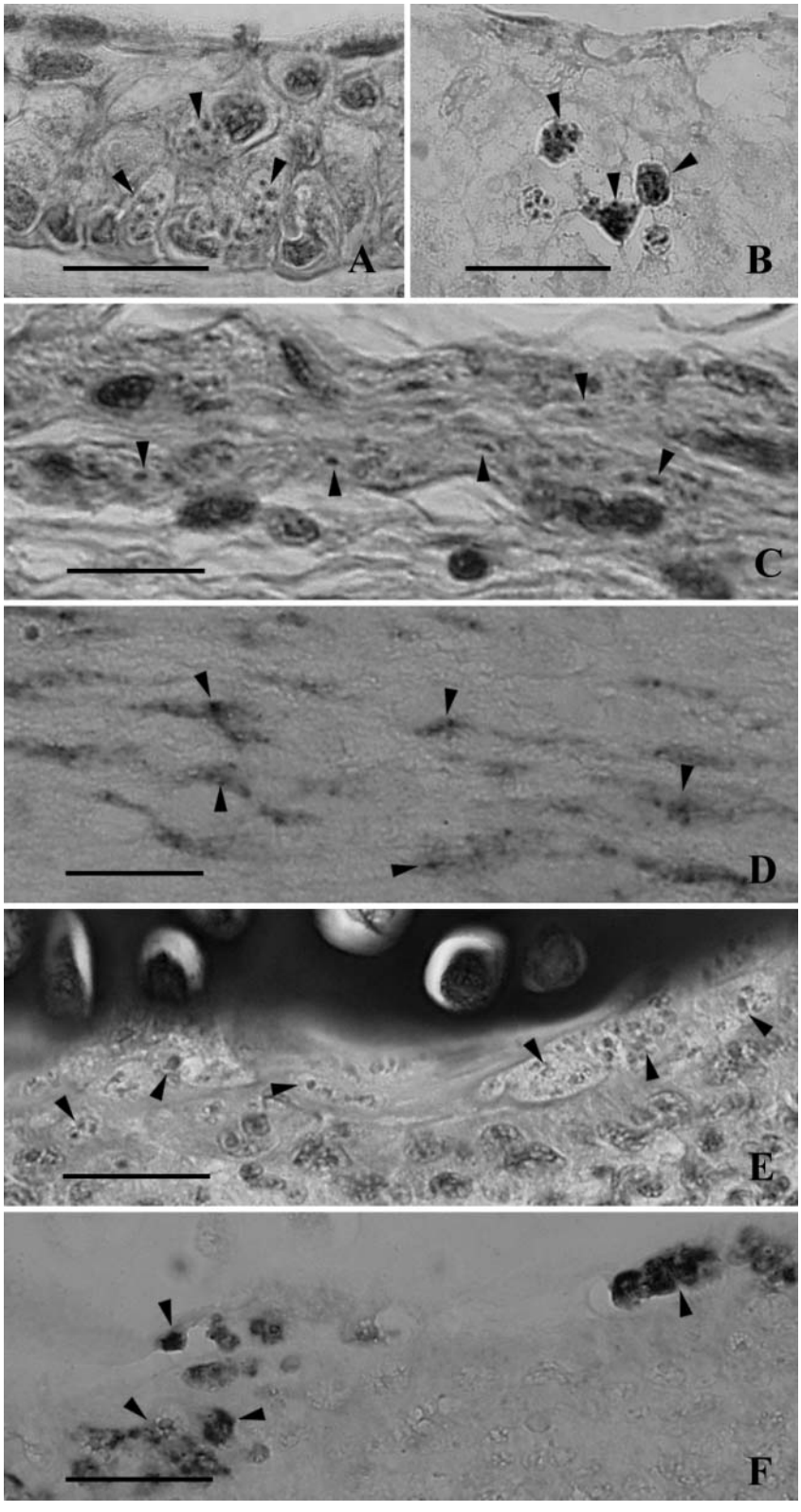

Fig. 4. *Myxobolus cerebralis* infecting *Oncorhynchus mykiss*. Detection of *Myx*SP-1 transcription from presporogonic stages of *M. cerebralis* in experimentally infected rainbow trout tissue-sections. At 2 h post-exposure: (A) hematoxylin and eosin (H&E) stain shows presporogonic stages that have infected caudal fin epithelial cells and (B) similar stages expressing *Myx*SP-1 found by mRNA *in situ* hybridization. At 4 d post-exposure: (C) H&E shows spindle-shaped and elongated development stages located in the central nervous system and (D) *Myx*SP-1 transcription. At 10 d post-exposure: (E) H&E aggregates of presporogonic stages near cartilage of the head and (F) indicates *Myx*SP-1 transcription. Arrowheads indicate presporogonic stages. Scale bars = 25 µm (A,B), 12.5 µm (C,D), 25 µm (E,F)

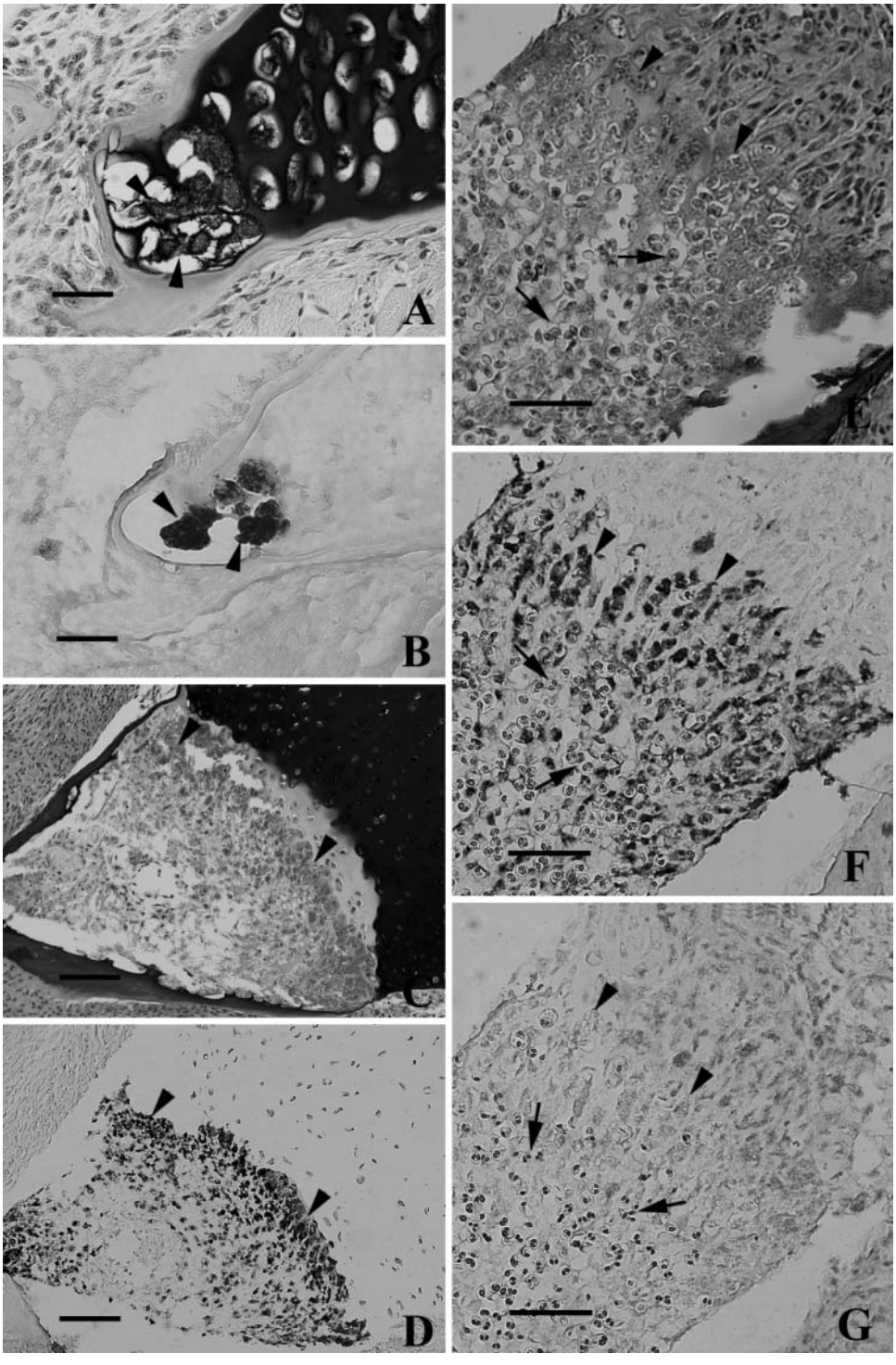

Fig. 5. *Myxobolus cerebralis* infecting *Oncorhynchus mykiss*. Detection of *Myx*SP-1 in experimentally infected rainbow trout tissue-sections with presporogonic and sporogonic stages of *M. cerebralis*. At 24 d post-exposure: (A) hematoxylin and eosin (H&E) stain shows focal areas of presporogonic stages infecting cranial cartilage and (B) *Myx*SP-1 transcription. At 54 d post-exposure: (C) H&E stain shows tissue sections of cartilage in the head with numerous parasites, and (D) expression of *Myx*SP-1 in association with zones of chondrocyte lysis and degeneration. At 240 d post-exposure: (E) H&E stain shows tissue section of cartilage near the ventral calvarium infected with sporogonic stages, and (F) presporogonic stages expressing *Myx*SP-1. (G) mRNA *in situ* control test indicating no hybridization signal when infected tissues were probed with *Myx*SP-1 sense probes. Arrowheads indicate presporogonic stages and arrows show sporogonic (also referred to as myxospores) stages. Scale bars = 25 µm (A,B), 100 µm (C,D), 50 µm (E–G)

transcription associated with developmental stages found in epithelial and nerve tissues, and in conjunction with lesions in the cartilage of experimentally infected rainbow trout.

Although no significant protease sequence identities were found outside the catalytic sites, the *Myx*SP-1 protein sequence did contain the conserved catalytic triad and surrounding amino acid residues that are characteristic for serine proteases (present Fig. 2, and Rawlings & Barrett 1994, Barrett & Rawlings 1995). In addition, Rawlings & Barrett (1994) reported that a key aspect of the evolutionary division of serine proteases is based on the linear sequence arrangement of the catalytic residues. The linear sequence data for the catalytic triad of *Myx*SP-1 (His-54, Asp-248, Ser-310) indicates an evolutionary affinity with chymotrypsin-like serine proteases. Unexpectedly, we found that the 3′ UTR of *Myx*SP-1 contains a 366 bp region that significantly complemented that found with the 18S rDNA and ITS-1 genes for *Myxobolus cerebralis*. The insertion of eukaryotic 18S rDNA sequence within serine protease genes has been previously reported for *Schistosoma mansoni* (Newport et al. 1988), but the significance and function of the rDNA insertion is not known. Similarly, the function of the rDNA sequence found in the *Myx*SP-1 gene remains unclear, but the location of the sequence after the stop codon and in the 3′ UTR suggests that the 18S rDNA and ITS-1 inserts are pseudogenes. Future studies will be needed to examine how specific sequence characteristics for *Myx*SP-1 play a role in protein structure, function, and substrate specificities.

Prior studies (Andree et al. 1998, Antonio et al. 1998, Baldwin & Myklebust 2002, Schisler et al. 2001) have reported effective molecular methods for detecting *Myxobolus cerebralis*, but these tests provided no biological explanation for parasite activity during disease development. Using *Myx*SP-1 as the target gene, our real-time TaqMan PCR test detected protease transcription from early, intermediate, and late developmental stages of the parasite (Fig. 3). The spatial and temporal appearance of parasite stages in our study were consistent with an in-depth light and electron microscopic study of the sequential development of *M. cerebralis* in rainbow trout tissues by El-Matbouli et al. (1995) and trials detecting presporogonic and sporogonic stages using newly developed molecular-based assays (Andree et al. 1998, Antonio et al. 1998, 1999). From 5 min to 10 d post exposure, the mean values for *Myx*SP-1 transcription in the gills were greater than those found in any other location susceptible to parasite invasion. The respiratory epithelium is a common site for host-tissue invasion and trapping of triactinomyxons by gill filaments or gill rakers may have increased the number of parasites infecting the gill tissues during exposure. The consistently high level of protease transcription found in gill tissues up to 24 d post exposure was probably a function of a heavy exposure and numerous parasites residing in the nerve and cartilage tissues of the gills before the onset of sporogenesis. We found a significant increase in protease transcription at 2 h post exposure from the gill and dorsal fin compared to other host tissues at this time point (Fig. 3). The high level of *Myx*SP-1 expression in the gill and dorsal fin may reflect a role of this enzyme in allowing presporogonic cell-doublet stages to penetrate additional epithelial cells or for the intercellular parasite stages to migrate deeper into host tissues of the subcutis to reach peripheral nerves (El-Matbouli et al. 1995). From 2 h to 240 d post exposure, the decreasing trend for *Myx*SP-1 transcription found in the caudal and dorsal fins, gills, and muscle tissues suggests that presporogonic stages once present in epithelial tissues advanced on to the nerves and cartilage, as found in the spine and head of the fish (Fig. 3). El-Matbouli et al. (1995) demonstrated that between 4 and 24 d post exposure the presporogonic stages are less frequently observed in epithelial tissues and are more abundant in peripheral, and then central nerves, and eventually the cartilage. The significant decrease in protease transcription at 10 d post exposure may suggest that *Myx*SP-1 plays a minor role during presporogonic replication in the CNS.

However, other significant changes in the temporal (4 to 24 d) transcription of *Myx*SP-1 in the spine and head (regions of greater nerve and cartilage concentrations) may be a function of enzymatic activities involved with penetration of nervous tissues, interaxonal migration, egress and the lysis of cartilage. Presporogonic stages found in the central nervous system beginning at 4 d post exposure possess distinct pseudopodia-like cytoplasmic protrusions (El-Matbouli et al. 1995). These pseudopodia-like protrusions combined with significant levels of *Myx*SP-1 transcription may allow presporogonic stages to migrate through host tissues and later intracellular digestion of proteins from lysed host cells. The formation of mature spores (myxospores) in fish tissues may require between 52 and 90 d at water temperatures of 15°C (Halliday 1973). Under our test conditions, sporogenesis was in the late stages by 240 d post exposure and this was consistent with greater *Myx*SP-1 transcription from the spine and head, and less or none in other tissues examined (Fig. 3). Additional studies that examine protease transcription from more lightly infected fish and from different fish species are warranted. These investigations could quantify the presences of *Myx*SP-1 in relation to differences in the pathogenesis of disease in resistant compared to susceptible fish hosts.

The mRNA ISH protocol reported here detected *Myx*SP-1 transcription from all presporogonic stages observed in fixed tissues of *Myxobolus cerebralis*-infected trout. Previous ISH studies have examined the utility of different fixatives for detecting parasitic rDNA in infected fish tissues (Antonio et al. 1998, 1999, Frasca et al. 1999, Morris et al. 1999, Sanchez et al. 1999, Jones et al. 2003). Our mRNA ISH test showed that fish tissues fixed for 48 h with Davidson's solution sufficiently inactivated RNases without damaging tissue morphology or probe hybridization. The digoxigenin labeling of *Myx*SP-1 probes and a previously described immunohistochemical detection protocol (Antonio et al. 1998) provided a sensitive nonradioactive method for identifying parasitic protease transcription in moderately to heavily infected rainbow trout. Visually detecting *Myx*SP-1 transcription within infected fish tissues supports our real-time PCR data and, more importantly, supports the spatial distribution of developmental stages as previously described by El-Matbouli et al. (1995) and Antonio et al. (1998, 1999). Although the rDNA ISH assay described by Antonio et al. (1998, 1999) accurately detected developmental stages from infected rainbow trout tissues, it provided no explanation for presporogonic biological activity along with lesion formation. We observed strong *Myx*SP-1 hybridization signals from early developmental stages of the parasite (5 min to 2 h post exposure) in epithelial tissues. The expression of *Myx*SP-1 during initial host epithelial cell invasion strongly implicates this enzyme as a possible catalytic mechanism used to break down extracellular matrix proteins that would allow sporoplasm aggregates and free sporoplasm cells of *M. cerebralis* to migrate between or invade host epithelial cells. A significant advantage of the mRNA ISH test was the straightforward recognition (Fig. 4D,F) of developmental stages from 4 to 10 d post exposure that might otherwise be difficult to identify by rDNA ISH or light microscopy using traditional tissue stains. *Myx*SP-1 transcription detected presporogonic stages at 4 to 10 d post exposure that were accompanied by host cellular injury or reaction in peripheral nerves or the CNS. As in the epidermis and subcutis, the expression of *Myx*SP-1 may facilitate migration through nerve cells. At 54 and 240 d post exposure, when most parasite stages have reached the cartilage, *Myx*SP-1 transcription was associated with presporogonic stages involved in the induction of severe host-tissue degeneration (Fig. 5D,F). At these later time points, lesions in host tissues were characterized by severe cartilage degradation and necrosis (Fig. 5C,E). The detection of protease transcription by ISH in the cartilage and associated with cartilage destruction was consistent with our real-time TaqMan PCR findings that demonstrated high levels of *Myx*SP-1 transcription during lesion development.

Our studies describe the first serine protease gene (*Myx*SP-1) from a member of the phylum Myxozoa, and in particular from the salmonid parasite *Myxobolus cerebralis*. The conserved catalytic triad of *Myx*SP-1 is linearly arranged, suggesting functions similar to that of chymotrypsin-like serine proteases. The chymotrypsin-like serine proteases secreted by the human pathogen *Schistosoma mansoni* are responsible for parasite penetration of skin and passage through the extracellular matrix of host tissues (Keene et al. 1983, McKerrow et al. 1983, 1985, Newport et al. 1988, Salter et al. 2000). We suspect that *Myx*SP-1 serves a similar role for *M. cerebralis* in the fish host. Both real-time TaqMan PCR and mRNA ISH provided evidence linking *in vivo* expression of the *Myx*SP-1 gene with key features of the pathogenesis of whirling disease.

Additional studies that further link protease expression with key pathological features of whirling disease are needed. These include examining dose responses, substrate-specificities and protease inhibitors on activity of *Myx*SP-1. Also, studies that demonstrate that parentally delivered anti- *Myx*SP-1 antibodies prepared to recombinant or synthetically made protein that alter the pathogenesis of the disease in rainbow trout are warranted. Lastly, exploitation of unique amino acid motifs of *Myx*SP-1 as targets for chemotherapeutics or anti-parasite vaccine development should be pursued.

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