Cloning, Structure, and Function of Two Rainbow Trout Bf Molecules[1,2]

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The factor B (Bf) and C2 complement genes are closely linked within the MHC class III region and are thought to have arisen by gene duplication from a single gene encoding an ancestral molecule; the animal phyla in which this duplication event took place is unknown. Two teleost fish, (zebrafish and medaka fish) have each been shown to possess only a single molecule that shows an equivalent degree of similarity to mammalian Bf and C2. In contrast, here we present the characterization of two factor B molecules (Bf-1 and Bf-2) in another teleost fish (the rainbow trout) that are about 9% more similar to mammalian factor B than C2, yet play a role in both alternative and classical pathways of complement activation. The full lengths of Bf-1 and Bf-2 cDNAs are 2509 and 2560 bp, respectively, and their deduced amino acid sequences are 75% identical. Both trout Bf genes are mainly expressed in liver and appear to be single-copy genes. The isolated Bf-1 and Bf-2 proteins are able to form the alternative pathway C3 convertase and are cleaved (in the presence of purified trout C3, trout factor D, and Mg^{2+}EGTA) into Ba- and Bb-like fragments in a manner similar to that seen for mammalian factor B. The most remarkable feature of trout Bf-2 is its ability to restore the hemolytic activity of trout Bf-depleted serum through both the alternative and classical pathways; whether Bf-1 possess similar activity is unclear at present. ***The Journal of Immunology*****, 1998, 161: 4106–4114.**

The complement (C′) system includes a group of plasma proteins and cell receptors that play a crucial role in nonspecific and specific immune response pathways. The complement system can be activated through three different pathways: the classical, alternative, and lectin pathways. Invertebrates such as equinoderms (1) and tunicates (2) have recently been shown to contain complement molecules. Lamprey, the most ancient vertebrate, has a primitive complement system represented only by the alternative pathway (3, 4). However, with the appearance of Igs in cartilaginous fish (5, 6), all the rest of animal groups, from teleost fish to mammals, appear to have in addition to the alternative pathway, the classical pathway. Thus, the classical pathway seems to be phylogenetically the most modern of the three pathways (7); however, it is not clear whether the lectin or the alternative pathway was the first to emerge.

Complement activation through any of the three pathways results in the proteolytic cleavage of C3 to C3b and C3a, a reaction that is mediated by the C3 convertase. In the alternative pathway, factor B serves as the catalytic subunit of C3 convertase (8–10); in the classical pathway, this role is played by C2 (11, 12). In mammals, factor B and C2 share extensive amino acid homology; they have the same exon and intron organization and are located in tandem on the same chromosome (13, 14) within the mammalian MHC class III region. For these reasons, the two proteins are thought to have originated by gene duplication from an ancestral molecule (15). It is at present unclear in which animal phyla the duplication event took place.

The factor B protein has been purified only from mammals and birds (chicken). A partial amino acid sequence of chicken factor B was roughly equal in similarity to human and mouse factor B and C2 (16). In addition, the protein seemed to participate in both classical and alternative pathways of complement activation; nevertheless, the studies on the classical pathway in chicken were not clearly defined. It was suggested that the factor B protein in chicken is derived from a presumed common ancestral form of mammalian factor B and C2. In contrast to this situation, two genes encoding factor B-like molecules have been cloned from the amphibian (*Xenopus*). The two *Xenopus* molecules, designated Bf A (17) and Bf B (18), showed more sequence similarity to factor B than to C2 (40 and 30% identities to mouse factor B and C2, respectively) and consequently were considered to be factor B molecules. It was therefore hypothesized that the Bf/C2 gene duplication from a common ancestor occurred before the mammalian/amphibian divergence (17, 18). One gene encoding for a factor B-like molecule has recently been cloned from two teleost species, medaka fish (19) and zebrafish (20). Each of these molecules showed equal sequence similarity to factor B and C2 from mammals, and no additional factor B/C2-like molecules were found. In both cases, the factor B-like molecules were thought to function as both C2 and factor B; however, (as with *Xenopus* factor B), no functional studies of the proteins encoded by those genes were conducted to test this hypothesis. Functional studies have indicated the presence of both the classical and alternative pathways in teleost fish such as trout, a finding that suggests that these fish have both factor B and C2 molecules (21, 22). In

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[2] The sequences described in this paper have been deposited in the GenBank database under accession numbers AF089860 to AF089861.

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this case, the Bf/C2 gene duplication would have predated the appearance of these fish.

Teleost fish have been shown to contain multiple forms of the third component of complement (C3)[4] (23–25), which have been suggested to play an important role in generating immune diversity in fish (26). In the present study we demonstrate that trout, in addition to having multiple C3 genes, also possess two factor Bf/C2 genes. We have characterized their protein products and demonstrated that teleost fish contain a unique molecule that is involved in the activation of both alternative and classical pathways. Furthermore, we have succeeded in characterizing and reconstituting the trout alternative pathway with purified trout complement components (Bf, Df, and C3).

Materials and Methods

Fish and serum

Rainbow trout (*Onchorhynchus mykiss*) were obtained from Landenberg trout farm (Philadelphia, PA) and Limestone Trout Farm (Reading, PA). Blood was collected with a syringe from the caudal artery, and serum was obtained by incubating the blood at 4°C for 4 h. The serum was separated by centrifugation at 2000 × *g* for 10 min. Serum was stored at −70°C.

Determination of trout Bf-1 and Bf-2 cDNA sequences and phylogenetic analysis

Primers based on a partial trout cDNA sequence (clone RT-L72, accession no. T23101, NID: g505992) with similarity to factor B and C2 from other species were designed, and a clone with an identical sequence to the RT-L72 sequence was obtained by RT-PCR from trout liver RNA. This PCR product was subcloned into the PCRTMII vector using the TA cloning kit (Invitrogen, San Diego, CA) and was used to screen a λgt11 trout liver cDNA library. The full-length cDNA clone obtained was designated Bf-1.

To obtain a full-length cDNA clone encoding Bf-2, we first produced a partial cDNA clone by RT-PCR using degenerate primers based on the internal protein sequence of the Bf-2 protein. We used primers P5F (5′-(C,T)T(A,G,C,T)GA(C,T)AA(C,T)TT(C,T)AA(C,T)-3′) and P3R (5′-AT(A,T,C,G)CC(A,G)TA(A,G)TT(A,T,C,G)GG(A,G)TG-3′) and the PCR conditions described previously (23). The product obtained had the expected molecular size of 0.6 kb, and its deduced amino acid sequence showed high similarity to the corresponding region of trout Bf-1 and of factor B and C2 molecules from other species (data not shown). The PCR product was used to screen a λgt11 trout liver cDNA library, and a full cDNA clone encoding Bf-2 was obtained. Nucleotide sequences were determined by the Sanger method (27). The deduced amino acid sequences of Bf-1 and Bf-2 as well as all available factor B and C2 were aligned using the Clustal W program (28), and the resulting alignments were manually corrected. The obtained alignment (Fig. 1) was used to calculate Poisson-corrected distance matrexes to construct trees by the neighbor-joining method (29).

Northern and Southern blot analyses

A portion of 300 bp was amplified from both Bf-1 and Bf-2 cDNAs via PCR using Bf-1- and Bf-2-specific primers (Bf-1 sense primer, 5′-TAGC CCTGAAAACACAATGG-3′ (nucleotides 1086–1106); Bf-1 antisense primer, 5′-TCCGTGCCATCCAGGGGTAT-3′ (nucleotides 1377–1397); Bf-2 sense primer, 5′-CATCCCTTGCACCAAGGTAA-3′ (nucleotides 1759–1779); Bf-2 antisense primer, 5′-TAGTAGGTTGACGACCAC CCG-3′ (nucleotides 2040–2051). Numbers in parentheses refer to trout Bf-1 and trout Bf-2 with GenBank accession numbers AF089861 and AF08960, respectively. The amplified products were purified using Qiaquick spin columns (Qiagen, Basel, Switzerland) and randomly primed (BRL, Gaithersburg, MD) with [^{32}P]dCTP (Amersham, Arlington Heights, IL). Nonincorporated nucleotides were removed using G-50 spin columns (BMB, Rotkrevz, Switzerland), and the labeled fragments were then used as homologous probes for Northern and Southern blot analyses as described previously (30, 31).

Peptide synthesis

An 18-amino acid peptide ($TBf_1^{725–743}$) corresponding to the C-terminal portion (KYLGNDTEDYQPLEFLEN) of the deduced amino acid sequence of the RT-L72 clone that aligned with the C-terminal part of Bf and C2 molecules from other species was synthesized using an Applied Biosystems 430A peptide synthesizer (Foster City, CA) as described previously (32). A 14-amino acid peptide ($TBf_1^{2–15}$) derived from the deduced amino acid sequence of Bf-1 corresponding to the N-terminal part of the molecule (RREWAWEGGSYTLT) was also synthesized.

Ab production

The synthesized peptides were coupled to keyhole limpet hemocyanin by the glutaraldehyde method (33) and used to raise Abs in rabbits. The Abs were purified by affinity chromatography, using synthetic peptides coupled to cyanogen bromide-activated Sepharose (Pharmacia, Piscataway, NJ). Polyclonal antisera against trout Bf-2 and against two trout protein contaminants from a post Mono-P Bf-2 preparation (65 and 23 kDa) were generated in rabbits by immunization with the SDS-PAGE-purified molecules (described below).

Purification of trout C3 isoforms, Bf-1, Bf-2, and Df

The trout C3 isoforms were purified as described previously (23). To purify trout Bf-2, trout serum (40 ml) was precipitated with 16% polyethylene glycol (PEG) at 4°C for 30 min in the presence of 20 mM EDTA, 10 mM benzamidine, and 1 mM PMSF and then centrifuged (15,000 × *g*, 20 min). The resulting supernatant was brought to 25% $(NH_4)_2SO_4$, and the liquid phase containing PEG was removed. The remaining liquid phase containing the trout proteins was precipitated sequentially with 45, 60, and 75% $(NH_4)_2SO_4$ at 4°C for 30 min; all incubations were followed by centrifugation at 15,000 × *g* for 20 min. The pellet from the final precipitation was resuspended in 10 mM sodium phosphate buffer, pH 7.5, loaded onto a DEAE 40 HR (6.5 × 5.0 cm) anion exchange column (Millipore, Bedford, MA) equilibrated in the same buffer, and eluted with a linear salt gradient (0–500 mM NaCl). Bf-2 was identified by immunoblotting with the immunoaffinity-purified anti-$TBf_1^{725–743}$ Ab and with the polyclonal anti-trout Bf-2 obtained after the Bf-2 purification. The immunoreactive fractions were pooled and concentrated with Amicon filters (30-kDa cut-off; Amicon, Beverley, MA), then exchanged into 25 mM imidazole buffer, pH 6.2, by passage over a PD10 column (Pharmacia). The sample was applied to a Mono P 10/10 isoelectric focusing column (Pharmacia) equilibrated in imidazole buffer, and eluted with a pH gradient (4.2–6.2) with polyampholites. The Bf-2 preparation contained some contaminants that were removed by preparing polyclonal Abs against two contaminating proteins (65 and 23 kDa). This reagent was used in affinity chromatography to obtain a homogeneous preparation of Bf-2.

Trout Bf-1 was partially purified from trout serum by a single precipitation with 45% PEG and anion (Mono Q HR 5/5) and cation (Mono S HR 5/5) chromatography. Bf-1 was identified with the anti-$TBf_1^{2–15}$ Ab.

Trout Df was purified by gel filtration chromatography on a Superose 12 column. Fractions were concentrated 10-fold in Centricon filters (10-kDa cut-off) and tested for their ability to mediate the cleavage of purified trout Bf-2 into Bb and Ba fragments in the presence of purified trout C3-1 and a buffer containing Mg^{2+}EGTA. The fractions mediating cleavage were reapplied to the Superose 12 column. Purification of each of these trout proteins was monitored by SDS-PAGE and by immunoblotting using Abs specifically recognizing the individual proteins. The concentrations of trout Bf-1, Bf-2, and Df in serum were determined as described previously (23).

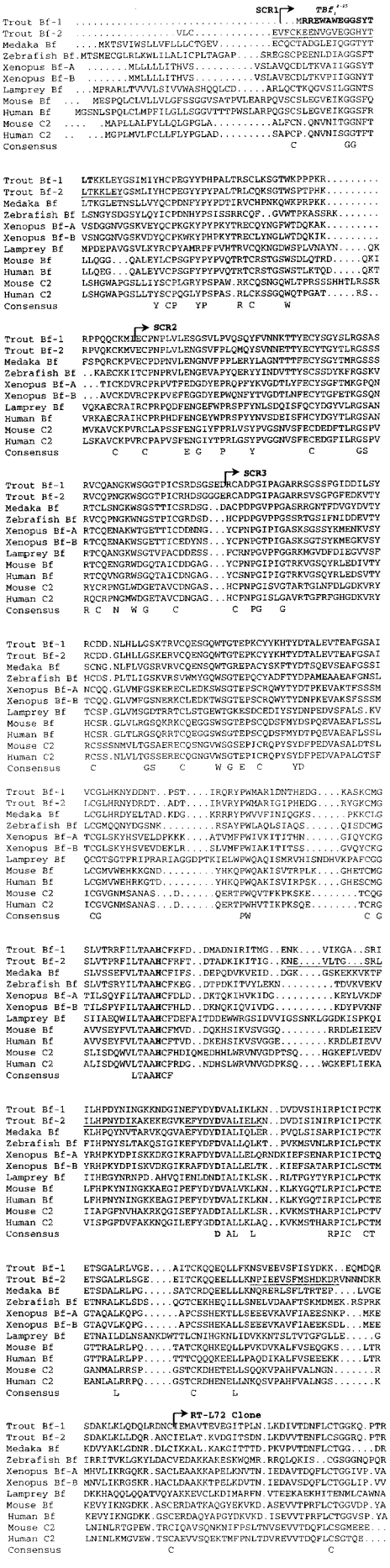
Protein sequencing

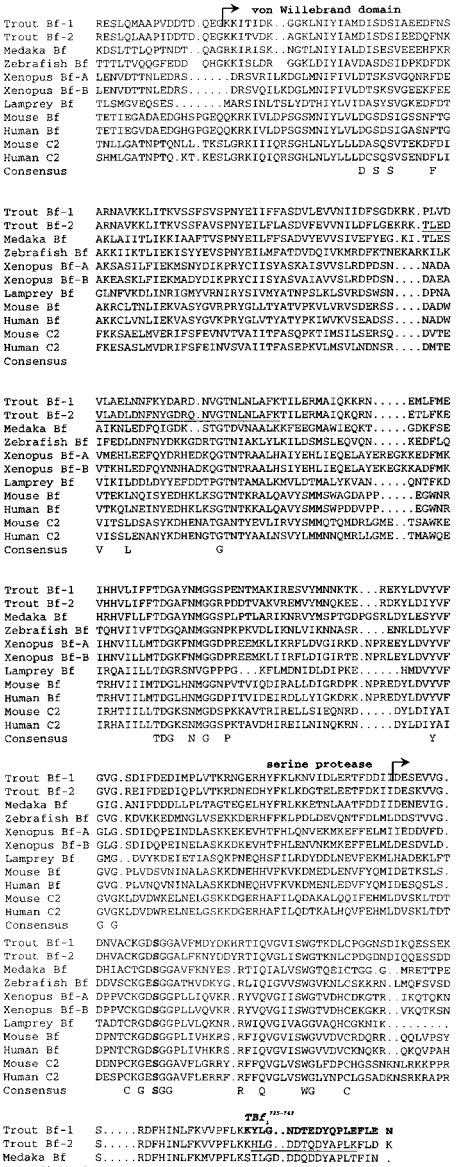
N-terminal sequences were obtained by subjecting the purified molecules to electrophoresis, followed by electroblotting onto ProBlott membranes (Applied Biosystems). A modified version of the method of Matsudaira (34) was used for sequencing, as described previously (35). The electroblotted proteins were subjected to Edman degradation, using an Applied Biosystem 473A Protein Sequencer. The internal protein sequence of trout Bf was obtained by digesting the protein with the endoproteinase Lys-C from *Lysobacter enzymogenes* (Boehringer Mannheim, Indianapolis, IN) (35).

Trout antiserum against sheep E

Abs against sheep E were generated by immunizing rainbow trout (200–300 g) i.p. with a suspension (0.5 ml) of washed sheep E (5×10^8) mixed (1/1) in CFA. Thereafter, trout were injected weekly for 4 wk with a suspension (0.5 ml) of washed sheep E (5×10^8) mixed (1:1) in IFA. Fish were bled 1 wk after the last injection.

[4] Abbreviations used in this paper: C3, third component of complement; PEG, polyethylene glycol; RaRBC, rabbit E; VBS, veronal-buffered saline.





Complement assays

Factor Bf-1/Bf-2 activities were measured by two different assays: 1) formation of fluid phase C3bBb convertase and 2) reconstitution of hemolytic activity of Bf-1/Bf-2-depleted trout serum with purified Bf-2.

To determine whether the Bf-1/Bf-2 and Df molecules mediate cleavages homologous to those mediated by their mammalian counterparts, we reconstituted the trout alternative pathway of complement activation with purified trout C3, Bf-2 (or partially purified Bf-1) and Df. For each experiment 2 μg of C3, 1 μg of Bf-2, and 0.02 μg of Df were used. The three proteins or reaction mixtures containing only two of the proteins were incubated at 20°C for 40 min in the presence of Mg^{2+}EGTA or EDTA. Cleavage of the C3 and Bf-2 molecules was studied by resolving the reaction mixtures on 9% SDS-PAGE under nonreducing and reducing conditions and staining with Coomassie blue. In the case of Bf-1, its cleavage to Bb and Ba was detected by Western blotting using the anti-Bf-1 Abs.

The hemolytic activity of trout serum or Bf-depleted trout was measured using rabbit E (RaRBC) and sensitized SRBC; SRBC were sensitized with trout anti-SRBC as described previously (21). Bf-1- and Bf-2-depleted trout serum was generated using a polyclonal anti-Bf-2 Ab; this Ab cross-reacts with both Bf-1 and Bf-2. The Ig fraction of this antiserum was purified and covalently coupled to cyanogen bromide-activated Sepharose. Trout serum (0.2 ml) was made 10 mM in EDTA and passed three times over the anti-Bf column. Bound material was eluted with 0.2 M glycine buffer, pH 2.6; the column was then re-equilibrated in PBS-EDTA, and the partially depleted Bf trout serum was again passed over the column. As a control, a second sample of trout serum was fractionated on a similar Sepharose column coupled with an unrelated Ab. The eluted samples were concentrated to one-half of their original volume, reconstituted with either 20 mM Ca^{2+} and Mg^{2+} or 20 mM Mg^{2+}EGTA, and tested for hemolytic activity mediated through the classical and alternative pathways, respectively. RaRBC and SRBC were washed four times in veronal-buffered saline (VBS) and resuspended in VBS containing 10 mM Mg^{2+}EGTA VBS (Mg^{2+}EGTA-VBS) or 5 mM $Ca^{2+}Mg^{2+}$ ($Ca^{2+}Mg^{2+}$-VBS) to give a concentration of 2.5×10^8 cells/ml. For the classical pathway assays, ShRBC were sensitized with trout anti-ShRBC as described previously (21). Sensitized ShRBC (7 μl) were then added to 25 μl of serially diluted trout serum in $Ca^{2+}Mg^{2+}$-VBS. For alternative pathway assays, 7 μl of RaRBC were added to 25 μl of serially diluted trout serum in Mg^{2+}EGTA-VBS. The reaction mixtures were incubated at room temperature for 40 min with shaking, and the reaction was stopped by adding 50 μl of VBS containing 40 mM EDTA. The extent of hemolysis was estimated by measuring the OD of the supernatant at 414 nm. The reciprocal of the serum dilution causing 50% lysis of RBC was designated the ACH_{50} or CH_{50} titer; results were presented as ACH_{50} and CH_{50} units per ml for the alternative and classical pathways, respectively, and were calculated as described previously (21, 36). To determine whether trout Bf-2 could restore the hemolytic activity of the Bf-depleted serum, various concentrations of purified Bf-2 were mixed with the depleted serum and serially diluted; hemolytic activity was assessed as described previously.

Results

Cloning and sequence analysis of trout Bf-1 and Bf-2

The full-length Bf-1 and Bf-2 cDNAs that we isolated were 2509 and 2560 bp in length, respectively, and encoded proteins of 743 and 749 amino acids. Both molecules had a domain structure similar to those of factor B and C2 molecules from other species. The Bf-1 and Bf-2 molecules consisted of three short consensus repeats at the N-terminus, a von Willebrand domain, and a serine protease domain at the C-terminus (Fig. 1). The distribution of the cysteine residues was highly conserved, except for one cysteine in the von Willebrand domain that is present in human and mouse factor B, but is not present in any other known factor B/C2 molecules. Residues His, Asp, and Ser, which are located at the active center of the serine protease domain, were also conserved (Fig. 1).

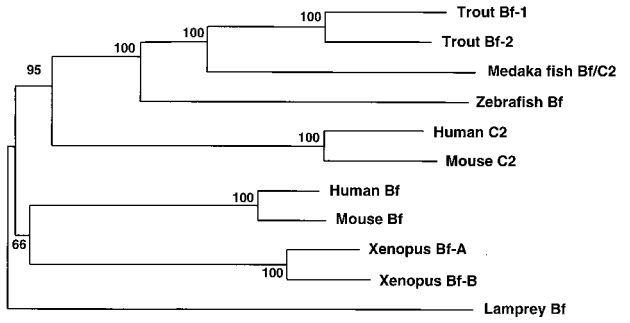


FIGURE 2. Phylogenetic tree of factor B and C2 protein sequences. The tree was generated by the neighbor-joining method, based on the entire sequences and the alignment of Figure 1. Numbers on the branches show the percent recovery in 1000 bootstrap replications.

The deduced amino acid sequences of Bf-1 and Bf-2 showed more similarity to human and mouse factor B than to C2 molecules. Trout Bf-1/Bf-2 showed amino acid identities of 38/39% to human factor B, 30/31% to human C2, 37/38% to mouse factor B, 28/31% to mouse C2, 34/33% to *Xenopus* factor B-A, 33/32% to *Xenopus* factor B-B, 50/51% to medaka fish factor Bf/C2, 42/43% to zebrafish factor B, and 16/22% to lamprey factor B. Trout Bf-1 was 75% identical with Bf-2. Thus, the trout Bf sequences showed about 9% more similarity to mammalian factor B than to C2. In contrast, the factor B/C2 sequences from the medaka fish and zebrafish were equally similar to mammalian factor B and mammalian C2 (19, 20). The situation in the case of the trout factor B molecules was very similar to that in *Xenopus*, which also contains two factor B molecules that show about 10% more similarity to mammalian factor B than to C2 (17, 18). The amino acid differences between trout Bf-1 and Bf-2 were scattered throughout the entire sequence, indicating that the two molecules are not generated by differential processing of transcripts from a single gene, but are the products of two distinct genes.

A phylogenetic tree generated from all available factor B and C2 sequences showed that despite the higher sequence similarity of trout Bf-1 and Bf-2 to mammalian factor B than to C2, both trout molecules clustered with the mammalian C2 sequences, as was the case also for the medaka fish and zebrafish factor B molecules (Fig. 2). The *Xenopus* factor B sequences clustered instead with those of mammalian factor B. Lamprey factor B appeared as an outgroup.

Northern and Southern blotting analyses

The tissue-specific mRNA expression of Bf-1 and Bf-2 in trout was investigated by Northern blot hybridization. Expression of trout Bf-1 (3 kb) and Bf-2 (2.7 kb) is limited to the liver (Fig. 3, *A* and *B*), although slight expression of Bf-1, but not Bf-2, was detected in the intestine with prolonged exposure (data not shown). Interestingly, both Bf-1 and Bf-2 express high levels of message, in contrast to the low levels of Bf-1 protein expression (2–4 μg/ml) compared with that for Bf-2 (300–400 μg/ml). These results suggest a possible post-transcriptional or translational mechanism

FIGURE 1. Alignment of trout Bf-1 and trout Bf-2 with known factor B and C2 molecules. The amino acid sequence of trout Bf-2 (underlined residues) was obtained by N-terminal sequencing of the molecule and of peptides generated using the endoproteinase Lys C. Bolded trout Bf-1 residues indicate the peptides synthesized (TBf_1^{2-15} and $TBf_1^{725-743}$) for Ab production. Bolded residues H, D, and S show conserved residues at the active center of the serine proteases. The short consensus repeat (SCR), von Willebrand, and serine protease domains are indicated. Dots indicate gaps introduced for maximum sequence alignment.

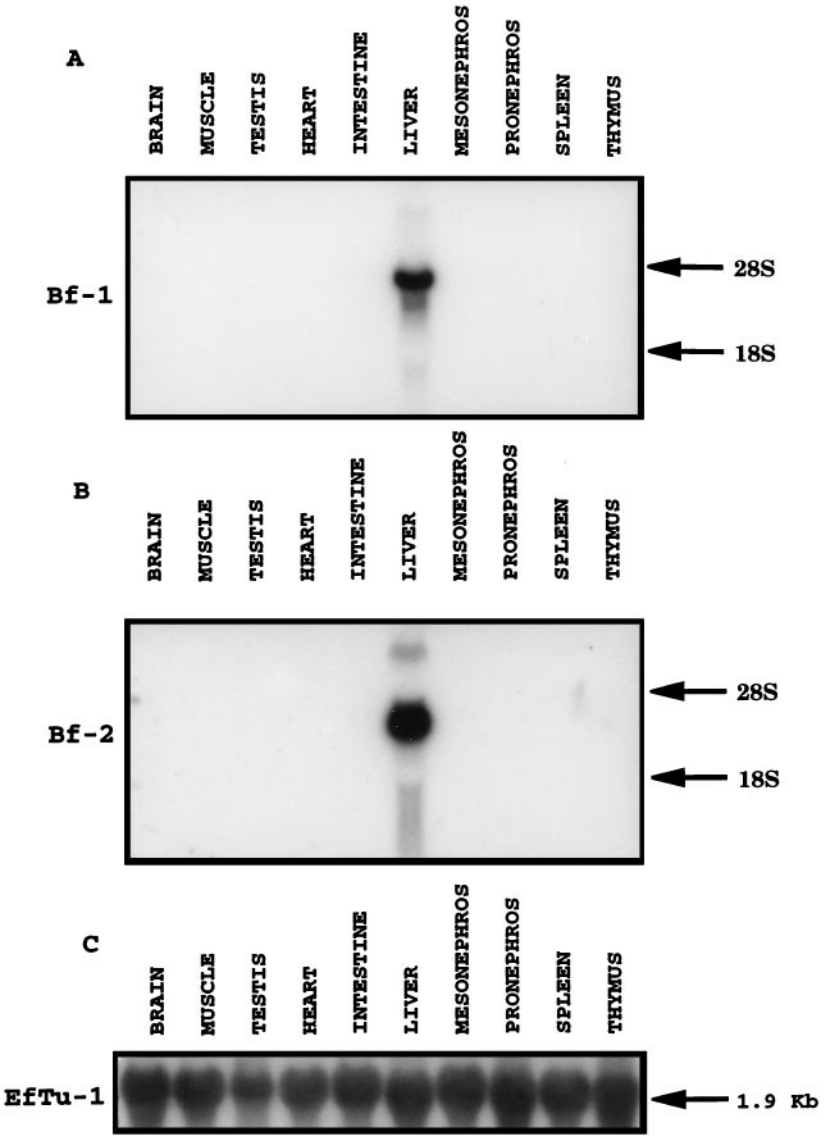


FIGURE 3. Tissue-specific expression of Bf-1 (*A*), Bf-2 (*B*), and EfTu-1 (*C*) by Northern blot. Fifteen micrograms of total RNA from specified tissues of 1-yr-old rainbow trout were electrophoresed, blotted to nylon, and sequentially hybridized and stripped with probes corresponding to trout Bf-1 (*A*), Bf-2 (*B*), and EfTu-1 (*C*). Strong expression of Bf-1 (~3 kb) and Bf-2 (~2.7 kb) was found primarily within the liver (1-day exposure), but with prolonged exposure (7 days) weak intestinal expression of Bf-1, but not Bf-2, was observed.

for the differential protein expression observed in the serum. Relative equivalency of loading was verified by reprobing the Northern blot with a trout housekeeping gene (EfTu-1; Fig. 3*C*).

We next determined whether Bf-1 and Bf-2 are present as single or multiple copies within the trout genome by Southern blot analysis using the same probes as those for the Northern blots. Only one hybridizing band was observed for Bf-1 (Fig. 4*A*) and Bf-2 (Fig. 4*B*) using three different restriction enzymes (*Hin*dIII, *Eco*RI, and *Eco*RV), suggesting that both are single-copy genes in trout. However, two of the four siblings for the Bf-2 analysis showed slight polymorphism for *Hin*dIII (Fig. 4*B*), which is most likely due to allelic variants of this gene. In contrast, Bf-1 displayed no polymorphism for the enzymes or siblings used in this analysis.

Isolation and characterization of trout Bf-1, Bf-2, and factor D

An immunoaffinity-purified Ab recognizing residues 725 to 743 of Bf-1 (corresponding to the C-terminal part of the deduced amino acid sequence of the RT-L72 clone) was used to identify Bf-2 in trout serum. This Ab cross-reacted to a low degree with an 81-kDa protein in trout serum (data not shown) that we designated trout Bf-2 because its N-terminal amino acid sequence differed from the deduced N-terminal sequence of trout Bf-1. The reactivity of this anti-peptide Ab with Bf-2 reflected the high sequence similarity of Bf-1 and Bf-2 in the region spanned by the peptide (Fig. 1).

The resulting preparation obtained from the precipitation of trout serum with PEG and ammonium sulfate contained mainly fish albumin and hemoglobin along with the trout Bf-2 protein. Half of the fish albumin and hemoglobin could be separated from Bf-2 by anion exchange chromatography. Thereafter, isoelectric focussing was very effective in removing most of the albumin and hemoglobin from the Bf-2-containing fractions (Fig. 5). The remaining contaminants were removed by affinity chromatography, and the Bf-2 protein was purified to homogeneity (>95% pure as judged by SDS-PAGE and Coomassie blue staining; Fig. 6, *lane 3*). In addition, N-terminal sequencing of the purified Bf-2 molecule gave a single sequence, suggesting that the Bf-2 preparation was homogeneous. Moreover, the anti-TBf_1^{2-15} peptide Ab, which specifically recognized Bf-1 (see below), did not react to any extent with the purified Bf-2 (data not shown). Bf-2 was present in trout serum at 300 to 400 μg/ml.

Internal protein sequence obtained for trout Bf-2 confirmed that the purified molecule was indeed distinct from Bf-1; this sequence then served as the basis for designing degenerate primers for use in cloning the Bf-2 gene. N-terminal and internal amino acid sequences obtained

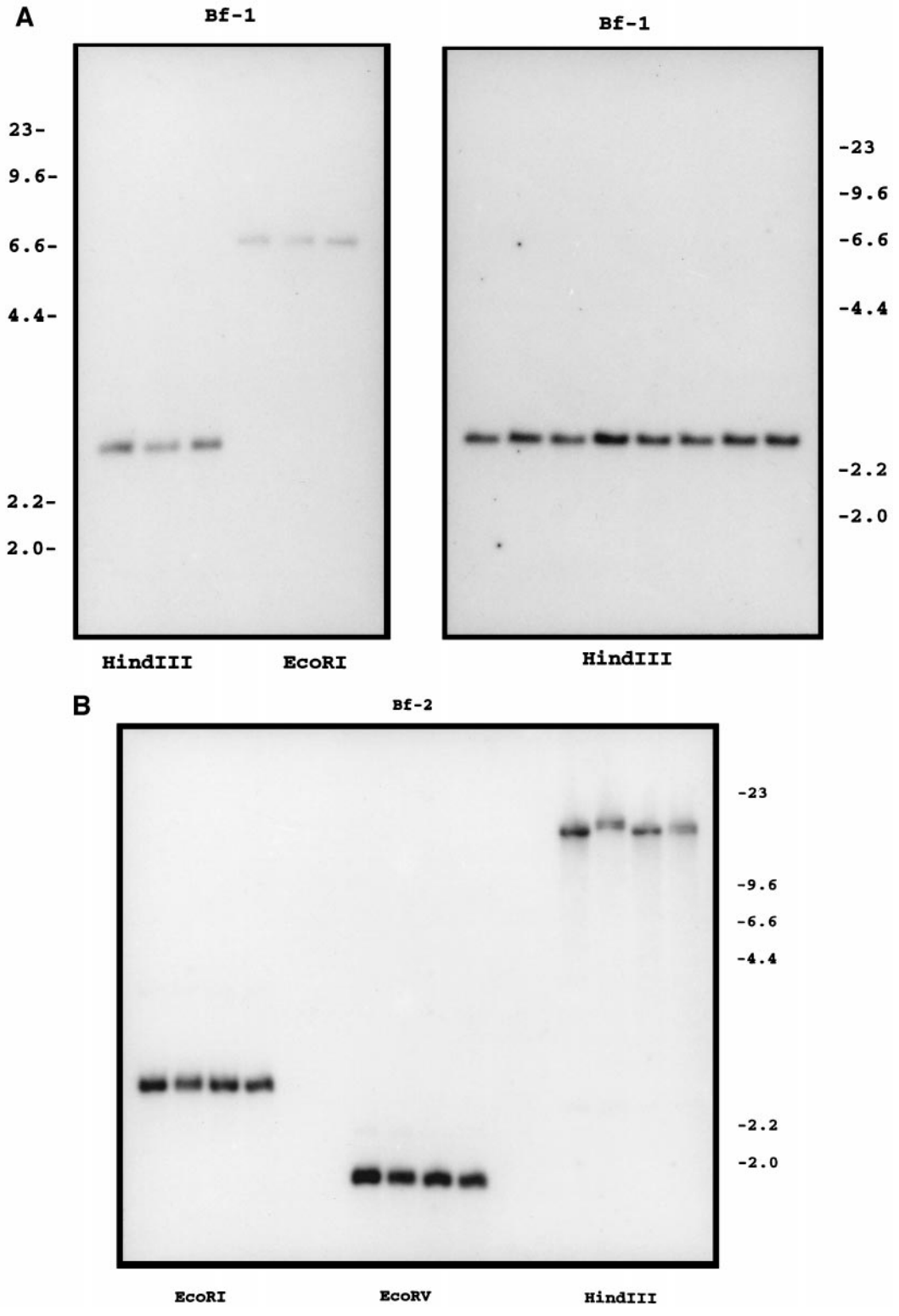


FIGURE 4. Bf-1 and Bf-2 gene copy number. *A*, Trout genomic DNA was digested with *Hin*dIII and *Eco*RI, transferred to nylon, and hybridized with the trout Bf-1 probe. The *left panel* contains three siblings of European origin, and the *right panel* contains eight siblings from North America. The single bands found at about 2.8 kb (*Hin*dIII) and about 6.8 kb (*Eco*RI) suggest that Bf-1 is a single-copy gene within rainbow trout. *B*, Southern blot analysis of trout genomic DNA from four siblings (European origin) hybridized with the trout Bf-2 probe. Single bands at approximately 3 kb (*Eco*RI), approximately 1.8 kb (*Eco*RV), and approximately 16 kb (*Hin*dIII) suggest that Bf-2 is also present as a single-copy gene within rainbow trout. Positions of DNA standards in kilobases are shown on both sides of *A* and on the *right* of *B*.

for Bf-2 confirmed that the cDNA clone we had isolated encoded the Bf-2 protein (Fig. 1).

The anti-TBf_1^{2-15} peptide Ab was used to isolate Bf-1 from trout serum. The sequence spanned by the peptide was very different from the corresponding Bf-2 sequence (Fig. 1). Consequently, the Ab we raised against TBf_1^{2-15} was unable to recognize Bf-2, but did recognize a molecule of a similar size (data not shown). This molecule was partially purified and was shown to

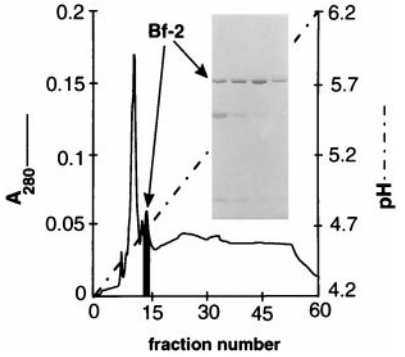


FIGURE 5. Elution profile of Bf-2 from a Mono P 10/10 column. Fractions containing Bf-2 from the DEAE 40 HR column were pooled and applied to a Mono P 10/10 isoelectric focusing column (Pharmacia) equilibrated in imidazole buffer and eluted with a pH gradient (4.2–6.2) with polyampholites. Protein was monitored by absorbance at 280 nm. The shaded peak represents the fractions containing Bf-2.

react much more strongly with the anti-$TBf_1^{725–743}$ Ab than did the Bf-2 protein, suggesting that the partially purified molecule was indeed Bf-1. A polyclonal Ab raised against trout Bf-2 also strongly reacted with Bf-1, probably because of the high sequence similarity between Bf-1 and Bf-2 (data not shown). All of the individual fish analyzed for the presence of Bf-1 using the anti-$TBf_1^{2–15}$ and the anti-$TBf_1^{725–743}$ Abs were positive, suggesting that Bf-1 is present in all fish and that the putative Bf-1 that we have identified is not a polymorphic form of Bf-2. A rough calculation indicated that the serum concentration of Bf-1 was very low (~2–4 μg/ml).

Trout factor D (Df) was purified to >90% homogeneity as judged by SDS-PAGE and Coomassie blue staining (Fig. 6, *lane 4*). The molecular size of trout Df was 24 kDa, consistent with the size of factor D molecules from all other species analyzed to date. Its concentration in serum was 25 to 50 μg/ml; this value was significantly higher than that in the teleost fish *Cyprinus carpio* (common carp; 6 μg/ml) (37) or in humans (1 μg/ml) (38), although it was roughly similar to the values in mice (50–100 μg/ml) (39). The N-terminal amino acid sequence of trout factor D was highly similar to that of factor D from other species (15/3 and 13/5, identical/different residues to the corresponding sequenced residues in carp and mouse, respectively; Fig. 7). The residues that are conserved in these three species were also conserved in trout Df, indicating that the purified protein was indeed factor D.

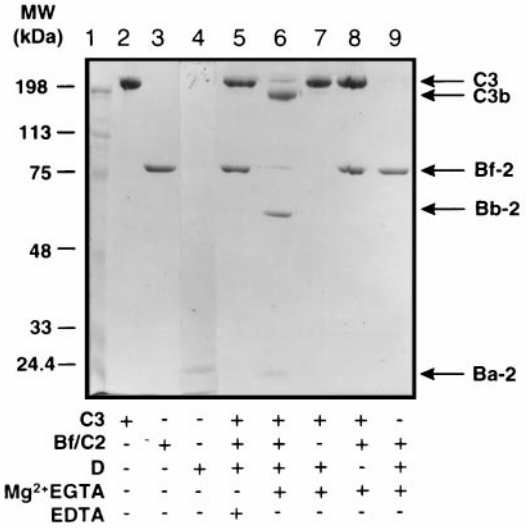


FIGURE 6. Formation of fluid phase alternative pathway C3 convertase with purified trout C3-1, Bf-2, and D proteins. Trout C3-1 (2 μg), Bf-2 (1 μg), and trout factor D (0.02 μg) were incubated together in the presence of EDTA or Mg^{2+}EGTA. Reaction mixtures were incubated for 40 min at room temperature (~20°C), electrophoresed on 7.5% SDS-PAGE under nonreducing conditions, and stained with Coomassie blue.

```
                1                   20
                |                   |
Carp D      - ITGGNEAVAXSXPYMASLQV
Mouse D     - *L**Q**A*HAR*****V*V
Trout D     - ****R**E*H*R********
```

FIGURE 7. N-terminal amino acid sequence comparison of trout, carp, and mouse factor D. X indicates unidentified residues, and asterisks denote residues identical with those of carp factor D.

Functional activities of trout Bf-1 and Bf-2 proteins

Reconstitution of the trout alternative pathway. The ability of Bf-1 and Bf-2 to participate in the formation of fluid phase C3bBb convertase was assessed using purified trout C3 and Df. To date, reconstitution of the alternative pathway with purified components has only been achieved in mammalian species. We were able to reconstitute the trout alternative pathway with purified trout complement components (C3-1, Bf-2, and Df; Fig. 6). As previously observed in mammals, trout C3-1 and trout Bf-2 in the presence of trout Df and EDTA remained uncleaved (Fig. 6, *lane 5*); however, in the presence of Mg^{2+}EGTA, C3-1 was cleaved to C3b, and Bf-2 was cleaved to yield fragments homologous to mammalian Bb and Ba (Fig. 6, *lane 6*) (40). These data are consistent with the requirement for Mg^{2+} that is seen when trout serum is used to lyse rabbit RBC through the alternative pathway (21, 41). Furthermore, combinations of C3-1 and Df (Fig. 6, *lane 7*), C3-1 and Bf-2 (Fig. 6, *lane 8*), and Bf-2 and Df (Fig. 6, *lane 9*) did not lead to cleavage of Bf-2 or C3-1 in the presence of Mg^{2+}EGTA, indicating that Df, Bf-2, and C3-1 preparations were not cross-contaminated. Trout C3-3 and C3-4 also formed the alternative pathway C3 convertase with trout Bf-2 in the presence of Df and Mg^{2+}EGTA (data not shown), indicating that trout Bf-2 was capable of interacting with all trout C3s. In contrast to the situation in humans (42), trout Df was not the limiting factor in the fish system, since as little as the equivalent of 0.2 μg/ml of Df was capable of cleaving the same amount of Bf-2 as a physiologic concentration of Df (25–50 μg/ml) in trout serum (data not shown). In similar experiments a partially purified preparation of Bf-1 was cleaved in the presence of trout C3, factor D, and Mg^{2+}EGTA, implying that Bf-1 can also act as a factor B molecule (data not shown).

Role of trout Bf-2 in complement activation

The involvement of the Bf-2 protein in the hemolytic activity of trout serum via both classical and alternative pathways was assessed using Bf-depleted serum. The polyclonal anti-Bf-2 Ab was coupled to Sepharose and used to deplete Bf-2 as well as Bf-1 from trout serum. To our surprise this immunodepletion abolished the hemolytic activity of the serum through both alternative and classical pathways, suggesting that trout Bf-1 and Bf-2 were involved in either the alternative or the classical pathway or both. Addition of purified Bf-2 to the depleted serum restored both classical and alternative pathway activities (Fig. 8), suggesting that trout Bf-2 may represent an ancestral molecule that has both Bf and C2 functions. Trout-mediated lysis of SRBC through the alternative pathway is negligible, and sensitization of SRBC with trout Abs did not contribute to any significant lysis through the alternative pathway

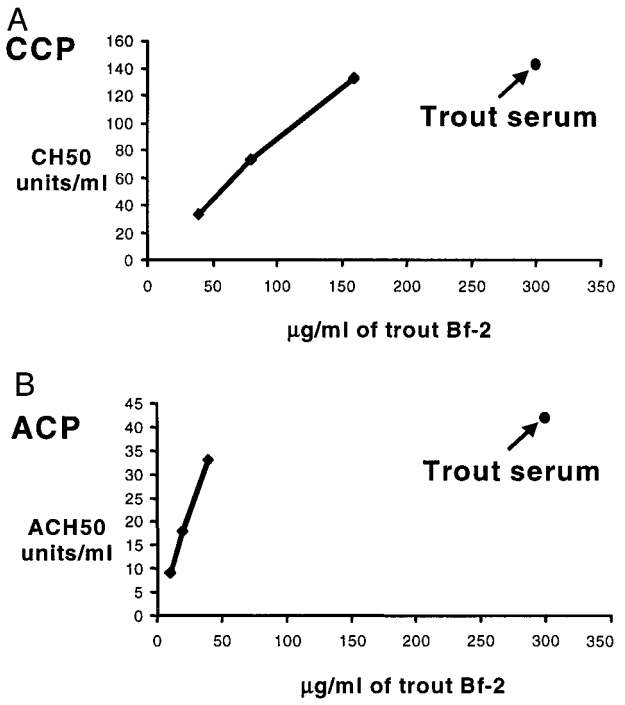


FIGURE 8. Lysis of sensitized SRBC (*A*) and RaRBC (*B*) with Bf-depleted trout serum reconstituted with purified Bf-2. Trout serum was depleted of Bf-1 and Bf-2 by affinity chromatography, and its hemolytic activity through the classical pathway (CCP) or the alternative pathway (ACP) was then restored by adding various amounts of purified Bf-2. ●, The amount of Bf-2 present in normal trout serum (*x*-axis) plotted against the hemolytic activity of normal trout serum (*y*-axis) through the alternative (*B*) or classical (B) pathway.

(data not shown), indicating that Abs do not play a role in the activation of the alternative pathway. Reconstitution of the hemolytic activity was dose dependent (Fig. 8), and the concentration of Bf-2 needed to fully restore the hemolytic activity (40 and 160 μg/ml for the alternative and classical pathways, respectively) was less than that of Bf-2 in serum (300–400 μg/ml), indicating that Bf-2 is in excess.

Discussion

In the present study we have demonstrated that in contrast to two other teleost fish (the zebrafish and the medaka fish) (19, 20), trout contain two genes encoding Bf-1 and Bf-2 and possess a molecule (trout Bf-2) that appears to be required for both classical and alternative pathway activities. Both Bf-1 and Bf-2 molecules are likely to be the product of a single gene, as shown by Southern blot analysis (Fig. 4, *A* and *B*). The two molecules are expressed mainly in liver, as is the case in higher animal species, although Bf-1 appears to be expressed in intestine also (Fig. 3); the functional significance of expressing Bf-1 in the intestine is unknown at the moment. Interestingly, both Bf-1 and Bf-2 show high levels of mRNA expression; this contrasts with the very low expression of Bf-1 (2–4 μg/ml) compared with Bf-2 (300–400 μg/ml) at the protein level. The mRNA of both Bf-1 and Bf-2 are different in size and are within the size range found for factor B of other animal species. The higher similarity of trout Bf-2 to mammalian factor B molecules than to C2 might suggest that trout Bf-2 would function only as a factor B molecule, in contrast to the dual role that it appears to play. This higher similarity may reflect the faster rate of evolution of C2 compared with that of factor B (20). However, this situation differs from that in the medaka fish and zebrafish, whose factor B/C2 molecules show equal similarity to mammalian factor B and C2 molecules. Seeger et al. have postulated that this equal similarity in medaka fish reflects the fact that the substitutions in these molecules have reached a saturation point (20). If this were the case, however, the trout Bf molecules would also be expected to show equal similarity to mammalian factor B and C2. As Seeger and co-workers have observed, these considerations make it difficult to draw any significant conclusions from the phylogenetic tree (20). For example, it was unexpected to find that trout Bf molecules clustered with mammalian C2 molecules (Fig. 2) when they show about 9% more similarity to mammalian factor B than C2, whereas the *Xenopus* factor B molecules (which are ~10% more similar to mammalian factor B than C2) do cluster, as expected, with mammalian factor B. Furthermore, trout Bf-1 and Bf-2 appear to be more similar to mammalian factor B than C2, since they both have 11 charged residues in the area that aligns with the exon 15-encoded region from medaka fish. The number of charged residues differs greatly between mammalian factor B and C2 in the exon 15-encoded region (human and mouse Bf contain 15 and 12, whereas C2 contains only 6); this difference may be related to the functional differences between Bf and C2 (43). Consequently, from the phylogenetic analysis of the primary sequences of trout Bf-1 and Bf-2, it was not possible to deduce whether these molecules were factor B or C2. Therefore, the only way to determine whether these molecules represented Bf or C2 molecules was by analyzing their functions.

Our results suggest that the purified trout Bf-2 molecule can function in both alternative and classical pathways of complement activation. Bf-2 was able to reconstitute the alternative pathway in the presence of purified trout C3-1 and factor D (Fig. 6). In this experiment, trout Bf-2 behaved like a mammalian factor B, in that it was able to form the alternative pathway C3 convertase and be cleaved to Bb and Ba fragments in the presence of a buffer containing Mg^{2+}EGTA. Furthermore, Bf-2 fully reconstituted the hemolytic activity of the Bf-depleted trout serum through the alternative and classical pathways (Fig. 8). It is interesting that the amount of Bf-2 required to restore the classical pathway was about fourfold higher than that needed to restore the alternative pathway; this difference might reflect the higher titers of the trout classical pathways (three- to fourfold higher) than those of the alternative pathway. The fact that both trout Bf-2 and Df are present in significantly higher serum concentrations than are human Bf and Df and that both trout proteins appear to be functionally in excess could explain why the hemolytic titers of the fish alternative pathway are 5 to 10 times higher than those in humans. Whether Bf-1 also works through the classical pathway is unknown at present, because the very low concentration of Bf-1 in serum did not allow us to obtain a pure Bf-1 preparation. To confirm whether Bf-2 functions as both Bf and C2, trout C4 and C1 will have to be purified to analyze the requirement of Bf-2 in the formation of the classical pathway convertase. This work is currently in progress in our laboratory.

Our data are in agreement with predictions of Kuroda et al. (19) and Seeger et al. (20), who have suggested that teleost fish might contain a molecule functioning as both factor B and C2, since they were unable to assign their fish (medaka fish and zebrafish) sequences to either factor B or C2. Nevertheless, Kuroda et al. (20) suggested that if medaka fish had a molecule that played a dual role, then the split between Bf and C2 would have had to happen after the divergence of teleosts but before the divergence of amphibians from a common vertebrate ancestor, since they had previously found that *Xenopus* contains two factor B molecules that are identified as Bf on the basis of their higher similarity (~10% more similar) to mammalian factor B than to C2 molecules (17,

18). In contrast, our results closely resembled those obtained in *Xenopus*. As in the case of *Xenopus*, trout appear to have two molecules that show about 9% more similarity to mammalian factor B than to C2, yet one of the two molecules (Bf-2) appears to function in both classical and alternative pathways of complement activation. Therefore, it is possible that the *Xenopus* Bfs could also assume the roles of both factor B and C2. This situation would imply that the split between factor B and C2 happened after the divergence of the amphibians from a common vertebrate ancestor.

It is interesting that in addition to having multiple forms of C3 (23, 44), trout also contain (in contrast to medaka fish and zebrafish) at least two factor B molecules. Our results cannot exclude the possibility that additional Bf isoforms are present or that a C2-like molecule exists in trout serum. However, trout Bf-2 alone (the most abundant trout Bf) was sufficient to completely reconstitute the hemolytic activity of trout serum through the alternative or classical pathway, even at lower concentrations than those present in serum. This finding suggests that a C2-like molecule may not be required, and therefore it is probably not present in the trout. The significance of trout Bf-1 remains unknown, although we have shown that it is present in serum at very low concentrations, and it can be cleaved at least through the alternative pathway; its role in the classical pathway is currently under investigation.

Our findings suggest that before the divergence of C2 and factor B from a common ancestor, a molecule existed that was able to function in both alternative and classical pathways. The need for higher evolved animals to have two separate molecules is uncertain; however, one could speculate that the system could be better regulated if each pathway was dependent on a distinct molecule, instead of both pathways relying upon a common Bf/C2 molecule.

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