JOURNAL OF VIROLOGY, Dec. 1994, p. 8447–8453
0022-538X/94/$04.00+0

Vol. 68, No. 12

Neutralization-Resistant Variants of Infectious Hematopoietic Necrosis Virus Have Altered Virulence and Tissue Tropism†

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Received 17 June 1994/Accepted 2 September 1994

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that causes an acute disease in salmon and trout. In this study, a correlation between changes in tissue tropism and specific changes in the virus genome appeared to be made by examining four IHNV neutralization-resistant variants (RB-1, RB-2, RB-3, and RB-4) that had been selected with the glycoprotein (G)-specific monoclonal antibody RB/B5. These variants were compared with the parental strain (RB-76) for their virulence and pathogenicity in rainbow trout after waterborne challenge. Variants RB-2, RB-3, and RB-4 were only slightly attenuated and showed distributions of viral antigen in the livers and hematopoietic tissues of infected fish similar to those of the parental strain. Variant RB-1, however, was highly attenuated and the tissue distribution of viral antigen in RB-1-infected fish was markedly different, with more viral antigen in brain tissue. The sequences of the G genes of all four variants and RB-76 were determined. No significant changes were found for the slightly attenuated variants, but RB-1 G had two changes at amino acids 78 and 218 that dramatically altered its predicted secondary structure. These changes are thought to be responsible for the altered tissue tropism of the virus. Thus, IHNV G, like that of rabies virus and vesicular stomatitis virus, plays an integral part in the pathogenesis of viral infection.

Infectious hematopoietic necrosis virus (IHNV) is a rhabdovirus that kills salmonid fish, including rainbow trout, chinook, sockeye, and Atlantic salmon. The disease process is associated with massive destruction of hematopoietic tissue in the anterior kidney. The progression of virus infection has been carefully described elsewhere (9), but the roles of different viral proteins in the pathogenic process are not clear. For the mammalian rhabdovirus rabies virus (RV), glycoprotein (G) was found to be a critical determinant in pathogenicity (1, 7, 8, 21, 23). A single change in one amino acid of RV G resulted in the complete loss of pathogenicity because of altered movement of the virus to the brain (13, 16, 17) after virus inoculation or because of the infection of fewer neurons in the brain upon intracranial inoculation (30). For IHNV, one escape variant from a G-specific neutralizing monoclonal antibody (MAb) was found to have reduced pathogenicity in fish (22). However, that study did not establish the basis for the reduced pathogenicity or make any correlation with specific changes in G.

In this study, the parental IHNV isolate, RB-76, and four variants selected for resistance to the neutralizing MAb RB/B5 were examined for pathogenicity, antigen distribution, and G gene sequence. Fish were infected by noninvasive, waterborne exposure to the virus. All escape variants were found to have some reduced pathogenicity in fish, but variant RB-1 was found to be highly attenuated. Also, variant RB-1 was found to have an altered distribution of viral antigen in moribund fish. When the G gene of this variant was sequenced, changes in the deduced amino acid sequence that significantly altered the predicted secondary structure of the protein were found. These observations suggest that IHNV G governs the tissue tropism of the virus and that the altered tissue tropism results in reduced pathogenicity.

The parental IHNV isolate, RB-76, had been obtained from moribund steelhead fry sampled during an epizootic at Round Butte Hatchery, Oregon, in 1976. To produce stocks of RB-76, virus was grown on epithelioma papillosum cyprini cells as previously described (22). Stocks of the viral variants were prepared after incubation with MAb RB/B5 for 1 h prior to adsorption to the epithelioma papillosum cyprini monolayer (22). For sequence analysis, variants were continuously grown in the presence of the MAb.

The virulence of the four variants and the parental virus was determined in vivo at two different virus challenge doses. All experiments were carried out in triplicate with three tanks of 100 rainbow trout fry (0.5 g) exposed to either 10^3 or 10^5 PFU/ml by static immersion for 6 h at 13°C. The viral cause of death in these fish was confirmed by coagglutination assay for viral antigen (2). This study revealed that all the variants were attenuated at the lower challenge dose of 10^3 PFU/ml (Fig. 1A). However, at the higher challenge dose of 10^5 PFU/ml, variants RB-2, RB-3, and RB-4 were found to be nearly as virulent as the parental isolate (Fig. 1B). Variant RB-1 remained attenuated at the higher challenge dose.

A comparison of the tissue distribution of IHNV nucleocapsid antigen in fish infected with the parental virus or a variant virus was made by immunohistochemical staining of whole fish tissue sections as described by Drolet et al. (9). Only moribund fish exhibiting skin darkening, exopthalmia, and aberrant swimming at 14 to 16 days postexposure were selected for this study. The relative amounts of IHNV in tissues were scored by red staining intensity produced by alkaline phosphatase-linked antibody detection of anti-nucleocapsid MAb 14D bound to

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† This is Oregon Agriculture Experiment Station technical paper 10575.

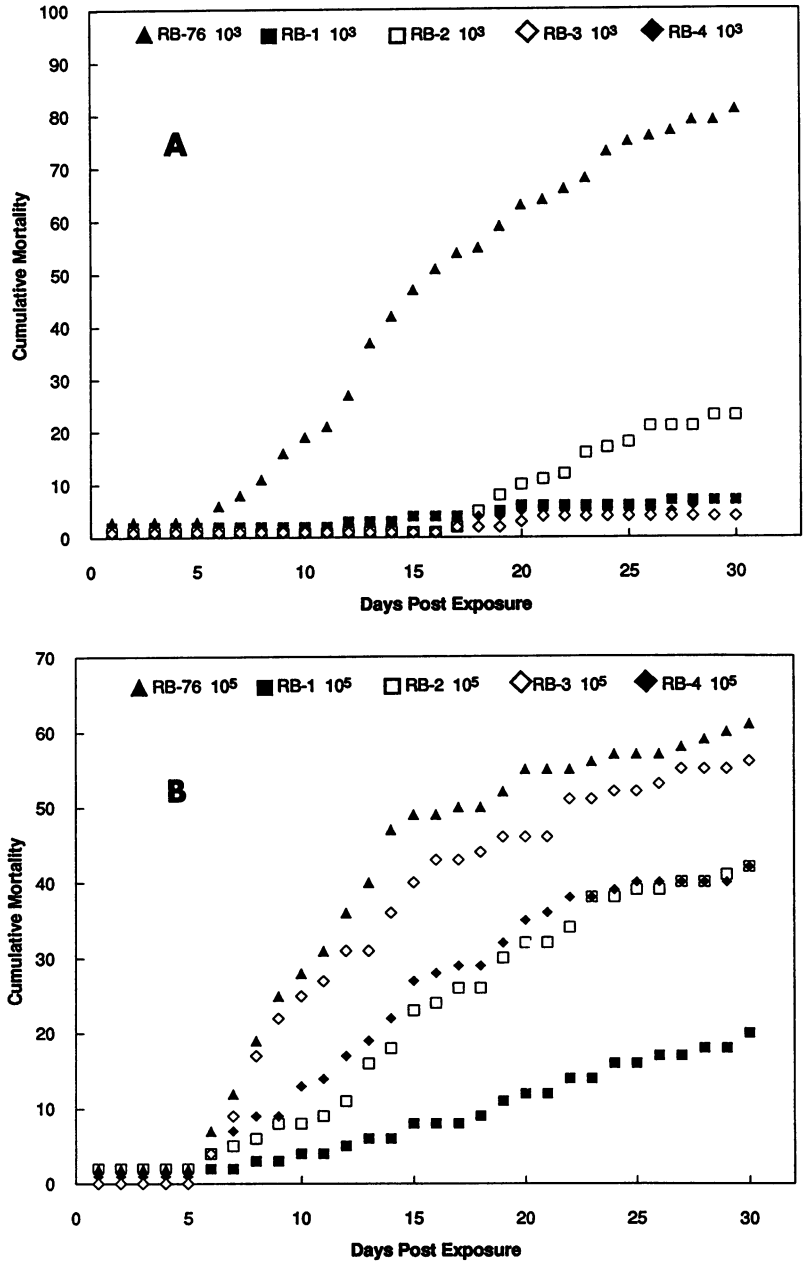


FIG. 1. Percent cumulative mortalities of rainbow trout challenged with IHNV strain RB-76 and MAb-resistant variants RB-1, RB-2, RB-3, and RB-4. Triplicate tanks of 100 fish each were infected with a challenge dose of 10^3 (A) or 10^5 PFU/ml (B). Thus, each virus was tested in a total of six tanks. The average percent cumulative mortalities for each virus isolate is shown on the ordinate and plotted against days postexposure. The range did not vary significantly between tanks and thus is not shown.

tissues. The staining intensity was rated on a scale of 1 to 5, from lightest to darkest staining. Five fish infected with RB-76 and RB-4, two infected with RB-1, and three infected with RB-2 and RB-3 were examined. At least five whole body serial sections for each fish were stained for virus. Because RB-1 was highly attenuated, only two fish exhibited the initial signs of infection in 14 to 16 days.

All fish showed signs of infection by heavy staining in the spleen, kidney, gills, stomach, pyloric caeca, pancreas, and intestine, confirming the findings of Drolet et al. (9). When liver and brain tissues were examined, there were distinct differences observed with parental and variant viruses. The parental isolate, RB-76, and variants RB-2, RB-3, and RB-4 produced dark staining in livers and very little staining in brains. The staining pattern was dramatically different for the highly attenuated variant, RB-1; there was dark, multifocal staining in brain tissues and weak staining in livers.

The differences in staining for brain and liver tissues from RB-76- and RB-1-infected fish are shown in Fig. 2. Since each slide contained a sagittal section of the entire fish, it was possible to examine all of the tissues that had been stained at the same time from one fish. Thus, the relative staining of liver

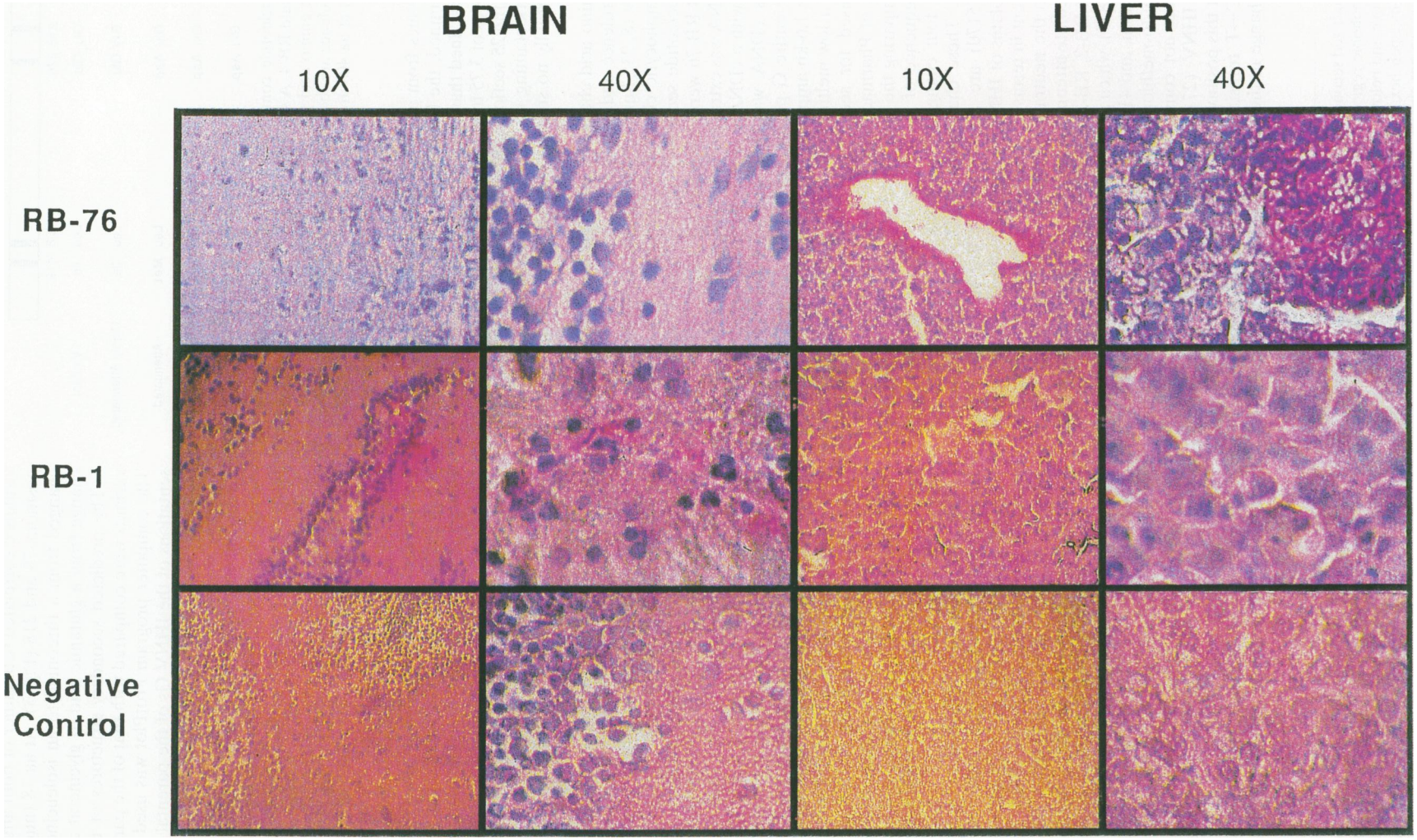
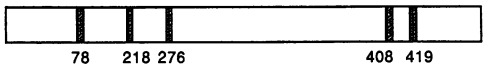


FIG. 2. Immunohistochemical staining of infected fish tissue. Tissues of fish infected with the variant RB-1 and parental RB-76 were stained with MAb 14D to the nucleocapsid as previously described (10). Red staining in tissues indicates the presence of virus. These immunohistochemical sections are from a single fish exposed to RB-76 or RB-1. Brain and liver tissues were examined at low and high magnifications, ×10 and ×40, respectively. Tissues from a fish that was not exposed to virus were used as a negative control.



	78	218	276		408	419	
Parental	Thr	Glu	Glu		Leu	Ile	Virulent
RB-1	**Ile**	**Gly**	Glu		Leu	Ile	Highly Attenuated
RB-2	Thr	Glu	**Asp**		Leu	**Met**	Attenuated
RB-3	Thr	Glu	**Asp**	(CTG->TTG)	Leu	**Met**	Attenuated
RB-4	Thr	Glu	**Asp**	(CTG->TTG)	Leu	**Met**	Attenuated

FIG. 3. Nucleotide comparison of the G genes of RB-76, RB-1, RB-2, RB-3, and RB-4. A schematic diagram of the G gene of RB-76 is shown. The amino acid substitutions in variants that may result in changes in virulence are shown in boldfaced type. The silent mutations (CTG→TTG) at aa 408 are shown.

and brain tissues from any fish could be compared. In the 10 sections examined, the brain tissue of RB-1-infected fish was more darkly stained than liver tissue, with relative red staining intensity scores of 3.75 in brain tissue versus 2.25 in liver tissue. Conversely, the 25 sections examined from RB-76-infected fish had very dark staining in the liver, with an intensity score of 4.53, and virtually no staining of brain tissue, with a score of 1.52.

Single amino acid changes in viral Gs have been shown to affect the virulence and pathogenesis of a number of viruses, including RV (8, 23), human immunodeficiency virus (4, 12, 24, 26), and lymphocytic choriomeningitis virus (19). Thus, the complete nucleotide sequences of the G genes of the IHNV variants and RB-76 were determined by direct PCR sequencing. Total RNA was extracted from cells, and a cDNA copy was synthesized with a cDNA synthesis kit (Invitrogen, San Diego, Calif.). This cDNA was then amplified with primers that spanned the entire G protein gene (from 5′ to 3′ noncoding region). The 1.6-kb amplified product was purified by electrophoresis on a low-melting-point agarose gel, and at least 100 ng/μl was used for sequencing at the core facility of the Department of Pharmacology, University of Washington.

When comparing the amino acid sequence of RB-76 to the published sequence of Koener et al. for the IHNV isolate RB-75 (15), four changes at positions 91, 221, 288, and 419 were found. These changes in RB-76 (GenBank accession number U15170) are also found in the sequences of 12 wild-type isolates of IHNV (27) and are therefore not considered significant in terms of this study. Changes associated with resistance to the neutralizing effects of the RB/B5 MAb and with intermediate attenuation were located at amino acids (aa) 276 and 419. RB-2, RB-3, and RB-4 showed changes at aa 276 (nucleotide 875), which gave rise to a glutamic acid-to-aspartic acid substitution, and 419 (nucleotide 1305), which resulted in an isoleucine-to-methionine substitution (Fig. 3). The change at aa 419 was not considered significant since 12 wild-type isolates of IHNV (27) contained methionine rather than isoleucine at this position. The RB-3 and RB-4 isolates had an additional C→T transition at nucleotide 1222 that did not result in a change in the amino acid (L). In RB-1, the loss of virulence correlated with either or both amino acid changes at positions 78 and 218 (Fig. 3). At aa 78 (nucleotide 281) RB-1 changed from a threonine to an isoleucine, and there was a change from a glutamic acid to glycine at aa 218.

The predicted secondary structures of the Gs of the four variants were compared with that of the parental virus (Fig. 4). The computer program ProtPlot was used to generate representations of the IHNV Gs of the parental and variant viruses from the derived amino acid sequence of each G gene (28). The rules established by Chou and Fasman (5, 6) were used to predict the secondary structure of each protein. The probabilities of occurrences of helix (*Pa*), pleated sheet (*Pb*), and beta turns (*Pt*) were evaluated under stringent conditions ($Pt > 5.0 \times 10^{-5}$ or $Pt > 7.5 \times 10^{-5}$; $Pt > Pa$ and $Pt > Pb$). An analysis of hydrophilic and hydrophobic regions in each protein was performed by calculating the average hydrophobicity or hydrophilicity at a given residue and the next five residues as described by Hopp and Wood (10). No significant differences were observed when comparing the G structures of RB-2, RB-3, and RB-4 with that of RB-76 (Fig. 4A). The amino acid change at position 276 from a glutamine to an aspartic acid resulted in reduced overall hydrophobicity in this region (structure not shown). The amino acid change at position 419 had no effect on either hydropathicity or calculated secondary structure. In contrast, the predicted secondary structure of RB-1 G was significantly different as a result of the change from glutamic acid to glycine at position 218 (Fig. 4B). This change from an acidic (hydrophilic) to an ambivalent amino acid introduced an additional beta turn in this region. The amino acid change at RB-1 aa 78 (threonine to isoleucine) also generated a more hydrophobic region.

In summary, a correlation between the amino acid changes in IHNV G and changes in virus virulence and viral antigen distribution in fish was made. A single change from glutamic acid to glycine at position 218 in avirulent RB-1 G introduced an additional beta turn in the molecule. This change and/or an amino acid change at position 78 in RB-1 G was correlated with an apparent change in the distribution of the virus in fish. Viral antigen was subsequently prominently detected in brain tissue but only weakly in liver tissue from RB-1-infected fish. This amino acid substitution was located in an area of IHNV G (aa 64 to 83) shown to be highly conserved among 12 different wild-type isolates (27).

The differences in distribution of viral antigen for RB-1-infected fish are striking since studies of the progression of virus infection in rainbow trout fry with wild-type virus have shown that brain tissue is involved only late in infection (9). With the highly virulent strain of IHNV RB-83, all fish showing positive brain tissue were found to have very darkly stained liver tissue (8a). There were fish that did not show any staining in brain tissue despite dark staining of liver tissue; but the converse observation, i.e., dark staining in brain tissue and no staining in liver tissue, was never made for fish during the first 30 days of infection. Thus, the findings presented here suggest that an altered IHNV G may affect viral pathogenesis by changing its tissue tropism.

The role of viral Gs in pathogenesis has been investigated for a number of virus-host systems. For RV, MAb neutraliza-

FIG. 4. Predicted secondary structure of IHNV G from RB-76 (A) and RB-1 (B). Open circles indicate hydrophilic regions, while shaded circles indicate hydrophobic regions. The radius is proportional to the average of five amino acids. Rapid zigzags are beta sheets with alpha carbons alternating above and below the chain. Undulating straight lines represent random coils, and sine waves are alpha helices. Glycosylated residues are indicated by open hexagons. The predicted secondary structures of Gs from RB-2, RB-3, and RB-4 are essentially unchanged from that of RB-76 and thus are not shown.


A
RB-76 Glycoprotein
NH2
218
276
419
COOH
B
RB-1 Glycoprotein
NH2
218
276
419
COOH

tion-resistant mutants to two different sites, designated site II (aa 34 to 42 and 198 to 203) and site III (aa 330 to 338), were found to have reduced virulence (1, 8, 17, 21, 23). In one class of mutants, the complete loss of pathogenicity was correlated with a single amino acid change at G position 333 from arginine to isoleucine or glutamine (8, 23). The change in pathogenicity has been attributed to changes in the distribution of RV antigen which may have affected the movement of the virus to the brain (16, 17). Other studies have suggested that after direct inoculation of the virus onto the brain surface, the attenuated variant infected fewer neurons in the brain; the topographic distribution and time of appearance of the variant virus in the brain were unaltered (13, 30).

Similar studies with human immunodeficiency virus (3, 4, 12, 24, 26), influenza virus C (25), and lymphocytic choriomeningitis virus (19) have shown that single amino acid changes in the viral G affect the viral tropism. In the lymphocytic choriomeningitis virus study, there was also a single amino acid change in the viral polymerase. This study emphasizes the fact that genes other than the envelope G genes may be important determinants of cell tropism. Thus, although the data presented here suggest that changes in the IHNV G gene are likely to affect viral virulence and tropism, the possibility that changes elsewhere in the genome are also involved has not been excluded.

At least five antigenic regions in IHNV G have been defined by sequence analysis of variants resistant to neutralizing MAbs and by immunization with specific peptides derived from different regions of the protein (11, 20, 29). In this study and others, the antigenic regions have been mapped to aa 78 to 81, 218 to 232, 272 to 276, 301 to 325, and 419 to 444 and all contained linear neutralization epitopes, with the exception of the antigenic region at aa 218 to 232 (11). The neutralizing domains delineated for IHNV are generally located in the same region as those for vesicular stomatitis virus G, which has linear epitopes in the regions aa 80 to 183 and 286 to 428 (14), and RV G, with a nonlinear neutralizing domain spanning aa 34 to 42 and 198 to 203 and a linear epitope at 330 to 338 (1, 17, 23). In RV, the amino acid positions are numbered for the mature protein 20 amino acids downstream from the initial methionine residue, whereas the IHNV and vesicular stomatitis virus G positions are numbered to include the signal peptide sequence. The apparent similarity in neutralizing domains for these three viruses suggests that the tertiary structure of the rhabdovirus G is conserved. However, confirmation of this similarity awaits X-ray crystallographic studies.

We thank James Kwon of the Department of Pharmacology, University of Washington, for assistance in operating the ABI 373 DNA sequencing apparatus and Linda Bootland and Barbara Drolet for technical assistance with fish and immunohistochemistry. We gratefully acknowledge Gael Kurath and Eric Anderson for helpful discussions.

Portions of this work were supported by the USDA Cooperative State Research Service through the Western Regional Aquaculture Center under agreements 89-38500-4287, 90-38500-5025, 91-38500-6078, and 92-38500-7195 for project 9208044-1 and by USDA Cooperative State Research Service grants 92-37204-7997, 91-34123-6124, and 92-37204-7997. In addition, this research was supported by grants from Bonneville Power Administration contract DE-FG79-89BP 92431, project 88-152, and by the Oregon Sea Grant College Program, Oregon State University, with funds from the National Oceanic and Atmospheric Administration, Office of Sea Grant, under grant NA89 AA-D-SG108, project R/FSD-16.

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