Mx mRNA expression and RFLP analysis of rainbow trout *Oncorhynchus mykiss* genetic crosses selected for susceptibility or resistance to IHNV

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ABSTRACT· Three interferon-inducible Mx genes have been identified in rainbow trout *Oncorhynchus mykiss* and their roles in virus resistance have yet to be determined. In mice, expression of the Mx1 protein is associated with resistance to influenza virus. We report a study to determine whether there was a correlation between the expression of Mx in rainbow trout and resistance to a fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV). A comparison of Mx mRNA expression was made between different families of cultured rainbow trout selected for resistance or for susceptibility to IHNV. A trout-specific Mx cDNA gene probe was used to determine whether there was a correlation between Mx mRNA expression and resistance to the lethal effects of IHNV infection. Approximately 99% of trout injected with a highly virulent strain of the fish rhabdovirus, IHNV, were able to express full length Mx mRNA at 48 h post infection. This is markedly different from the expression of truncated, non-functional Mx mRNA found in most laboratory strains of mice, and the ability of only 25% of wild mice to express functional Mx protein. A restriction fragment length polymorphism (RFLP) assay was developed to compare the Mx locus between individual fish and between rainbow trout genetic crosses bred for IHNV resistance or susceptibility. The assay was able to discriminate 7 distinct RFLP patterns in the rainbow trout crosses. One cross was identified that showed a correlation between homozygosity at the Mx locus and greater susceptibility to IHN-caused mortality.

KEY WORDS: Mx protein · *Oncorhynchus mykiss* · Interferon response · RFLP

INTRODUCTION

In vertebrates, the first line of defense against viral infection is the production of interferon (IFN) in virus-infected cells. The IFNs then induce the production of antiviral proteins or IFN-regulated proteins (IRPs) that actually produce virus resistance in the neighboring uninfected cells (for review see Samuel 1991). The Mx family of IRPs are 70 to 80 kD in size and are expressed in either the nucleus or cytoplasm of cells treated with Type I IFNs, IFN α or IFN β. Some members of this family have been shown to confer resistance to the replication of specific viruses in mice and human cells. For example, the replication of influenza viruses (Staeheli et al. 1986, Frese et al. 1995, Thimme et al. 1995), the

rhabdovirus vesicular stomatitis virus (VSV) (Meier et al. 1990, Staeheli & Pavlovic 1991, Zurcher et al. 1992), the measles paramyxovirus (Schneider-Schaulies et al. 1994), and members of the bunyaviridae (Frese et al. 1996) are inhibited by specific Mx proteins. The mechanism for this inhibition is not completely understood; however, it is thought that Mx proteins may interact with viral polymerases (Schwemmle et al. 1995, Frese et al. 1996).

Studies of the murine Mx proteins have shown that Mx+ and Mx– strains of mice are found in both laboratory and wild mice populations. The Mx+ phenotype is inherited as a single dominant autosomal trait (Lindenmann 1964). Upon Type I IFN induction, Mx+ mice produce a 72 kD protein whose expression confers resistance to influenza virus at doses that kill Mx– mice (Horisberger 1983, Staeheli et al. 1986). The Mx– phe-

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notype in some strains is due to a deletion that results in a Mx mRNA with 424 nucleotides (nt) missing from its 3' end and the production of a truncated Mx protein. Curiously, most inbred laboratory mouse strains are Mx– (Staeheli et al. 1988), and the Mx– allele occurs at about 50% in wild populations of mice (Haller et al. 1987). The selective advantage of Mx– alleles is unclear. However, the clear relationship between resistance to virus infection and the Mx+ genotype prompted us to examine rainbow trout for the same correlation.

There have been several reports characterizing IFN-like activity in fish cells *in vitro* (Gravell & Malsberger 1965, Beasley & Siegel 1967, Oie & Loh 1971, de Sena & Rio 1975, MacDonald & Kennedy 1979, Sano & Naga-kura 1982, Tengelsen et al. 1989). Further, the *in vivo* effectiveness of the trout IFN response to the fish rhab-doviruses viral hemorrhagic septicemia virus (VHSV) (de Kinkelin & Dorson 1973) and infectious hematopoi-etic necrosis virus (IHNV) (Eaton 1990) has been docu-mented. The importance of the IFN response in virus resistance in salmonid fish led us to examine the role of the IFN-regulated Mx proteins in rainbow trout *Oncorhynchus mykiss*.

We have previously reported the isolation and se-quence analysis of Mx cDNA clones encoding 3 dis-tinct Mx proteins of rainbow trout (Trobridge & Leong 1995, Trobridge et al. 1997a). On further analysis, the expression of these trout Mx mRNAs and proteins *in vivo* and in trout tissue culture cells was found to be controlled by such IFN inducers as virus infection and the polyribonucleotide, poly I:C (Trobridge & Leong 1995, Trobridge et al. 1997a,b). These clones also pro-vided useful probes to determine whether alterations in the Mx genes similar to those found in mice are also present in salmonid fish. Previous reports had shown that resistance to IHNV is a heritable trait in sockeye and chinook salmon *Oncorhynchus nerka* (McIntyre & Amend 1978). A report by Winter et al. (1980) demon-strated a correlation between a specific transferrin genotype and susceptibility to bacterial kidney dis-ease. The question was whether resistance/suscepti-bility to IHNV was correlated with a specific Mx mRNA species or restriction fragment length polymor-phisms (RFLP) pattern. In the study presented here, the genomes of distinct rainbow trout families selected for different levels of resistance to IHNV were ana-lyzed for Mx RFLP patterns. Fish from each family were also infected with IHNV and after 48 h, the Mx mRNA species were identified in northern blots.

METHODS

IHNV susceptibility crosses. Both male and female gametes were collected in individual plastic bags, oxy-genated and kept refrigerated at 4°C until fertilization. Single pair matings of Clear Springs strain rainbow trout (Buhl, Idaho, USA) were used in order to maxi-mize the diversity among the selected families within each line. After the eggs were fertilized, they were hardened in water containing a final concentration of 100 µg ml^{-1} iodophore (Western Chemical Inc., Fern-dale, WA) for 15 min and placed in small (2 l) upwelling incubators supplied with ultraviolet disinfected 15°C spring water. Surviving fry were fed a standard ration and evaluated for susceptibility to IHNV at 66 d post hatching.

Cell lines. Two fish cell lines were used for the isola-tion, propagation, and identification of IHNV used in this study: (1) the CHSE-214 cell line (ATCC CRL 1681) from chinook salmon embryos (Lannan et al. 1984) and (2) *epithelioma papulosum cyprini* (EPC) cells from common carp (Fijan et al. 1983). Both cell lines were propagated and maintained in minimal essential media (GIBCO Laboratories, Grand Island, NY) supple-mented with 2 to 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO) and buffered with 1 M HEPES and 7.5% sodium bicarbonate to pH 7.5. For routine cell propagation, the CHSE-214 cell line was incubated at 20°C and the EPC cell line at 25°C.

Viruses. Virus used in this study was a 1990 isolate of IHNV (220-90) (LaPatra et al. 1991) obtained from rain-bow trout at a commercial hatchery in the Hagerman Valley (Buhl, Idaho). A low passage isolate (2×), con-firmed to be IHNV by serum neutralization tests, was used to produce all stock virus. Virus stocks were repli-cated in CHSE-214 cells at 18°C and stored at –75°C.

Determination of IHNV susceptibility. From each family, 2 groups of 50 fish (total of 100 fish) were exposed to 10^4 plaque forming units (PFU) ml^{-1} in a volume of water that was 10× the weight of the fish as previously described (Engelking & Leong 1989). Chal-lenges were conducted in closed systems for 1 h with aeration. Each group was then placed in separate 22 l aquaria receiving constant temperature (15°C), ultravi-olet disinfected, single pass water. Fish were moni-tored for mortality and fed *ad libitum* (4×) daily. Each experiment was terminated after 21 d. A minimum of 20% of each day's mortality from each aquaria were examined for bacteria and virus, as previously de-scribed (Thoesen 1994). Virus titers used to infect fish or isolated from dead fish was determined by plaque assay procedures as previously described by LaPatra et al. (1991). Virus concentrations in whole fish or kidney-spleen-liver homogenates were determined for some of the dead fish examined in each test.

Induction of Mx mRNA and preparation of tissues. From the unchallenged rainbow trout in each of the 8 groups, 25 fish were injected with 10^4 PFU ml^{-1} of IHNV and held at 15°C. At 48 h post injection, trout

were euthanized and livers removed for northern blot analysis. The trout livers were placed immediately in 1 ml Eppendorf tubes and snap frozen in liquid nitrogen. The caudal portion of each trout was also snap frozen for DNA extraction and subsequent RFLP analysis. All tissues were stored at –75°C until analyzed. The tissues were marked with a coding scheme to ensure that the northern and Southern blot analyses were conducted as blind studies.

RNA sample preparation. Livers were placed into 2 ml of STAT-60 RNA extraction reagent (Cinna-Biotech, Friendswood, TX, USA) in 14 ml polypropylene tubes and homogenized using a Brinkmann polytron PT3000 for 5 s. Following homogenization, samples were allowed to stand 5 min at room temperature, and 0.4 ml of chloroform was added. The mixture was then shaken for 15 s and allowed to stand on ice for 5 min. Samples were then centrifuged at 8000 rpm (4989 × g) for 10 min in a Sorvall SS34 rotor. The upper aqueous layer containing the RNA was removed to another tube and precipitated by adding 0.7 volumes of isopropanol. RNA was precipitated at 4°C for 30 min and the precipitate was collected by centrifugation as described above. The supernatant was removed and the RNA pellet washed 2 times in 75% ethanol and allowed to dry at room temperature. RNA pellets were resuspended in 50 µl of TE buffer and the concentration of RNA was determined by absorbance at 260 nm. An aliquot of 10 µg of total RNA was electrophoresed in agarose formaldehyde gels. Following electrophoresis, agarose gels were visualized and photographed under UV illumination and then the RNA was transferred to Nytran membranes (Schleicher and Schuell, Keene, NH). Northern blots were performed as described by Maniatis et al. (1989).

DNA sample preparation. DNA was extracted from the caudal portion of each fish used for northern blot analysis of Mx mRNA. The caudal region of each fish was put in a stomacher bag containing 5 ml of DNA digestion buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.8, 25 mM EDTA, 0.5% SDS, and 0.2 mg ml^{-1} proteinase K). The tissue was homogenized in a stomacher apparatus for 2 min followed by transfer of 4 ml of the suspension to a 5 ml tube and digestion overnight at 37°C with gentle rotation. After digestion, the DNA was extracted with phenol chloroform followed by ethanol precipitation. The quantity of DNA was determined by fluorometry and 15 µg of DNA was cut with the *Hpa*I restriction enzyme. Following digestion the DNA was precipitated and Southern blots were carried out as described by Maniatis et al. (1989) and Southern (1975) using Nytran membranes.

Preparation of Mx probe. The rainbow trout RBTMx2 (Trobridge & Leong 1997a) cDNA clone was used to generate digoxigenin-labeled probes following the method of Lanzillo (1991). Briefly 2 primers, ME 151 (5' ATTATGAGGAGAAGGTGCGT 3'), and ME 204 (5' CGATCTTAGTCTTTGCTGTTTTC 3') were used initially to PCR amplify a fragment from 0.1 pg of the RBTMx2 clone plasmid DNA. The reaction was performed for 1 step at 1.5 min at 95°C, followed by 30 cycles of 95°C for 1.5 min, 55°C for 2 min, and 72°C for 1 min, and a final step of 72°C for 2 min. The cycles were performed with final concentrations of 0.5 mM each primer, 200 mM each dNTP and 2.5 U Taq polymerase in standard PCR buffer (Promega, Madison, WI). The initial PCR product was diluted in TE to 20 pg and used as the template to generate an internal PCR fragment in which digoxigenin DNA labeling mix (Boehringer Mannheim, Mannheim, Germany) were substituted for dNTPs. The reaction was performed with the primers ME 140 (5' AGAAGGACCTTGCGC-TGC 3') and ME 201 (5' CAAGTTGATTGTTTCTTG-CTTTG 3') for 1 step of 95°C for 1.5 min, and then 35 cycles of 95°C for 1.5 min, 56°C for 2 min, and 72°C for 2 min, and a final cycle of 72°C for 5 min. The amplification was performed with final concentrations of 0.5 mM each primer, 2.5 U Taq polymerase in standard PCR buffer (Promega). The final concentrations of dNTPs were: 100 mM each of dATP, dCTP, and dGTP, 65 mM dTTP, and 35 mM DIG-11-dUTP. This resulted in a digoxigenin labeled 448 nt fragment of the RBTMx2 gene corresponding to nt 139 to nt 587 of the RBTMx2 cDNA (Genbank Acc. U47945). This is a conserved 5' region of the Mx gene that is found in all 3 Mx cDNA clones.

Northern and Southern blot detection. For all blots, the hybridization and chemiluminescent detection were performed using the methods of Holtke et al. (1992) with some modification in the hybridization and washing temperatures. Hybridization was carried out at 42°C. The hybridization solution was 50% formamide with 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na citrate), 2% wt/vol blocking solution (Boehringer), 0.1% (wt/vol) N-lauryl sarkosine and 0.02% (wt/vol) SDS. Membranes were washed twice for 15 min at room temperature with 2× SSC, 0.1% SDS, followed by 2 washes of 15 min each at 55°C with 0.1× SSC, 0.15% SDS.

RESULTS

Challenge data from IHNV susceptibility crosses

The level of resistance to IHNV infection in the progeny of selected rainbow trout crosses was assessed using standard challenge procedures. The low susceptibility crosses resulted in significantly lower cumulative percent mortalities than the 4 groups of high sus-

Table 1. Comparison of rainbow trout groups selected for low and high susceptibility to IHNV. Cumulative percent mortality (CPM), mean number of days to death (MDD) and restriction fragment length polymorphism determined heterozygosity (RFLP Het) measurements were made for 66 d old rainbow trout *Oncorhynchus mykiss* selected for low and high susceptibility crosses after waterborne exposure to IHNV

Group	Size[a] (g)	CPM	MDD	RFLP Het[b]	Group	Size[a] (g)	CPM	MDD	RFLP Het[b]
		Low susceptibility crosses					**High susceptibility crosses**		
A	0.9	23% (23/99)	13.1	2/24 a15:b9	C	1.0	72% (72/100)	11.6	3/10 b4:c5:d1
B	1.1	34% (34/100)	12.1	3/22 a4:c15:d3	E	1.1	89% (91/102)	9.3	1/17 c17
D	1.2	40% (42/105)	10.3	2/3 b1:c2	G	0.9	55% (59/108)	10.2	3/15 b1:c10:e4
F	0.8	43% (43/101)	10.8	2/19 d13:g6	H	0.9	67% (66/99)	11.6	3/7 d3:f3:g1

[a]Average weight of the fish
[b]Heterozygosity in RFLP patterns is indicated as the number of different RFLP patterns per number of fish analyzed and the RFLP patterns a to g, followed by number of fish with that pattern. Patterns are specified in Fig. 3

ceptibility crosses as determined by Student's *t*-test (p = 0.03). The mean days to death and average size of the fish were not significantly different between the 2 groups, p = 0.34 and p = 0.80 respectively (Table 1).

Northern blot analysis

From the unchallenged rainbow trout in each of the 8 groups, 25 fish were injected with the 220-90 isolate of IHNV and analyzed for Mx mRNA expression 48 h post-injection. Trout were challenged by injection in order to maintain a uniform time of induction for Mx expression. Ethidium bromide staining of the electrophoresed total liver RNA showed that almost all samples (183/186) contained high quality RNA, suitable for northern blot analysis. Northern blot analysis showed that in all cases no truncated mRNA species were detected, as would be evidenced by increased electrophoretic mobility. Approximately 99% (181/183) of the fish with high quality RNA were able to express Mx mRNA of the correct size. The intensity of the Mx northern blot Mx bands varied, but this may be accounted for by the differences in IHNV induction of the Mx mRNA, the differences in RNA extraction efficiency and purity of each RNA sample (Fig. 1). The uniformity of Mx induction was particularly striking in that injection of this virulent strain of IHNV was able to induce Mx mRNA in 99% of the analyzed fish at 48 h. As such, the northern blot analysis did not identify any groups of fish that had a predominant Mx– phenotype as evidenced by an inability to express Mx mRNA of the correct size.

Also of interest was the finding that some extractions did not contain appreciable amounts of ribosomal RNA.

However, some of these lanes contained other bands (e.g. Fig. 1, lane 2). It was not determined what these bands were, but we speculate that these other bands may be IHNV viral RNA representing an extreme in-
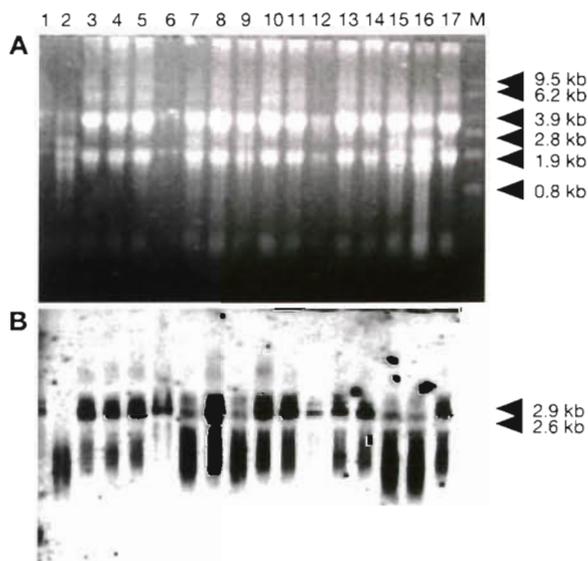

Fig. 1. Expression of Mx mRNA in rainbow trout Group B after injection with IHNV. Rainbow trout were injected intraperitoneally with the 220-90 strain of IHNV, and 48 h post-injection trout were euthanized; the liver tissue was excised and frozen in liquid nitrogen. RNA was extracted and 10 µg of total RNA was analyzed by northern blot using the 448 nt digoxigenin-labeled gene probe. Lanes 1 to 17 are liver total RNA from the IHNV low susceptibility Group B trout. Lane M is the RNA molecular weight standards. (A) Agarose gel under UV illumination. (B) Corresponding northern blot showing expression of the 2.9 and 2.6 kb transcript sizes of Mx mRNA. Lane 2 shows degradation of rRNA and other predominant RNA bands
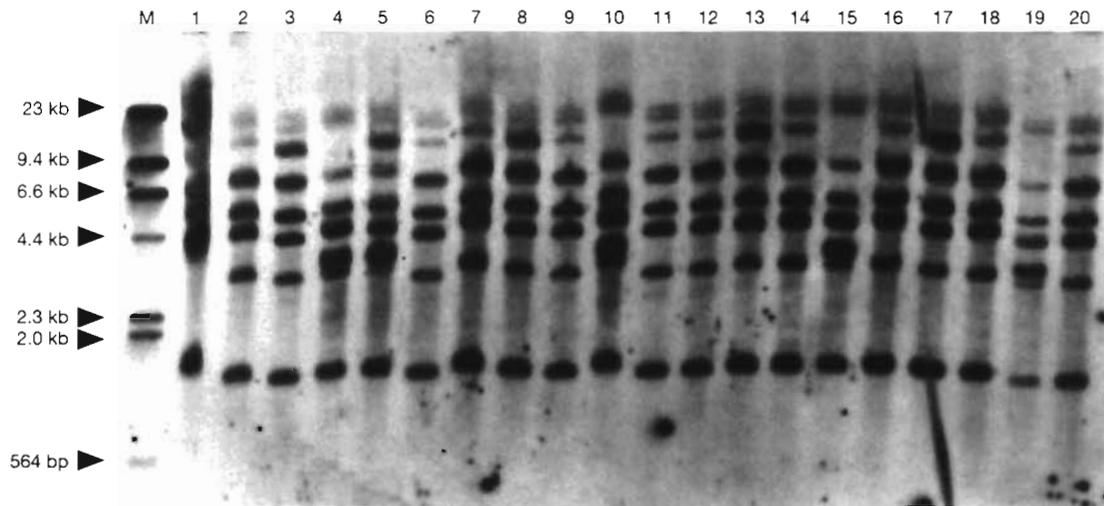
Fig. 2. Mx RFLP analysis of rainbow trout from Group B. DNA was extracted from the caudal portion of rainbow trout fry, digested with the *Hpa*I restriction enzyme, and analyzed by Southern blotting. Specific hybridization of Mx sequences was detected with a 448 nt digoxigenin-labeled Mx gene probe. Lane M is the *Hind*III digested, digoxigenin-labeled lambda DNA molecular weight markers. Lanes 1 to 17 are trout from the IHNV low susceptibility Group B. The polymorphism patterns a, c, and d were represented in this group as follows: RFLP pattern a—lanes 4, 10, 15, and 19; RFLP pattern c—lanes 2, 3, 6, 7, 8, 9, 11, 12, 13, 14, 16, 17, 18, and 20; pattern d—lanes 1 and 5

fection of the liver in these samples. Generally fish infected with IHNV at 66 d post-hatching do not show signs of the disease until 3 to 4 d after infection. However, the virus mRNA and proteins are being synthesized at a very high rate at this time.

Southern blot analysis

Although the northern blot analysis did not show any groups of fish with an Mx– phenotype, Southern blot analysis was performed to examine the RFLP patterns (Fig. 2). RFLP patterns were scored to give 7 unique patterns (a to g) (Fig. 3) among the 8 groups of rainbow trout crosses (A to H). For some groups the quality of the DNA extracted was poor and limited the number of samples available for RFLP analysis. It was interesting to note that a comparison of the RFLP patterns in terms of the heterozygosity of the Mx locus for each group varied. Further, the group with the highest mortality had the least heterozygosity at the Mx locus with only 1 RFLP pattern per 17 fish examined (Table 1).

DISCUSSION

The Mx proteins of mammals are specifically and transiently induced by IFN to high levels of expression. Some members of this protein family are efficient inhibitors of viral replication of negative stranded RNA viruses. Extensive studies have shown clearly that the

murine Mx1 protein confers resistance to influenza virus challenge and Mx– mice with deletions in the Mx1 gene are very susceptible to lethal influenza infection. These Mx– alleles occur rather frequently in both wild and laboratory mice strains and the absence of the appropriate Mx mRNA can be used as an indicator of sensitivity to influenza virus infection. We chose
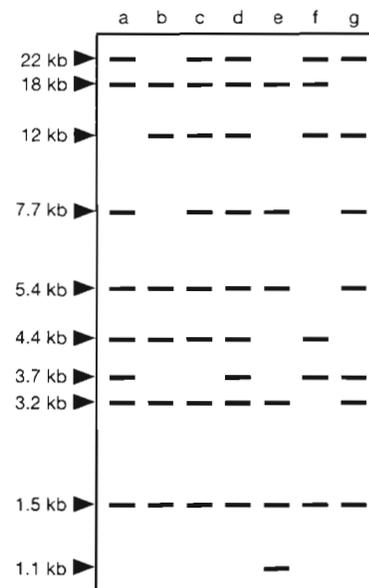

Fig. 3. Representative Mx RFLP patterns. Seven RFLP patterns designated a to g were seen in the 8 groups of trout crosses A to H. Band sizes for each RFLP pattern are shown

to examine trout Mx expression at a commercial trout farm to determine whether a difference in Mx expression would correlate with susceptibility to IHNV infection in rainbow trout. The expression of Mx mRNA and the corresponding RFLP patterns at the Mx locus were examined in trout crosses known to have differences in susceptibility to IHNV.

Approximately 99% of 183 rainbow trout examined were able to express Mx mRNA of the correct size at 48 h post injection with a virulent isolate of IHNV. The ability to express Mx mRNA of the correct size demonstrated that if there was a defect in Mx expression for some rainbow trout, it was not due to a deletion resulting in a truncated mRNA. It should be noted that a point mutation, resulting in a truncated Mx protein would not be detected by this assay.

In a previous report, a single exon probe was used to map the Mx RFLP patterns in the trout genome after digestion with BamHI, and EcoR1 (Trobridge & Leong 1995). We subsequently found that by using the restriction enzyme HpaI, polymorphism differences between individuals of different trout stocks were more easily detected. The 448 nt probe used in the study reported here spans the region from putative exons 3 to 6 of the rainbow trout Mx gene and yielded a higher number of bands than the single exon probe (data not shown). The murine Mus musculus and perch Perca fluviatis Mx genes have conserved exon-intron boundaries between Mx exons 3 to 8 (Staeheli et al. 1989) so it was likely that the trout would also have these conserved junctions. As such, the HpaI-based Mx RFLP assay reported here relies on the differences in HpaI sites within the introns of the Mx locus to differentiate Mx loci. The HpaI enzyme, more than likely, produces a higher degree of resolution because of the HpaI repeats in the introns found in the rainbow trout genome (Murata et al. 1993).

Because no gross defects in the ability of certain trout crosses to express Mx mRNA were observed it was not possible to identify any RFLP marker for a defect in Mx expression. However, RFLP analysis of rainbow trout using the Mx probe allowed us to examine the relative heterogeneity of the trout crosses at the Mx locus. The heterogeneity, as determined by the number of RFLP patterns per fish in each cross, varied from 1 RFLP pattern per 17 samples to 3 per 7 samples. Interestingly, the high IHNV susceptibility cross, Group E, had the highest cumulative mortality (89%) and the lowest heterozygosity (1 RFLP pattern per 17 fish) at the Mx locus. A correlation between disease resistance and heterozygosity at a single loci has been demonstrated in humans and pigeons (Allison 1955, Frelinger 1972). Disease resistance and enzyme heterozygosity at 9 polymorphic loci has also been shown for rainbow trout Oncorhynchus mykiss chal-

lenged with bacterial gill disease (Ferguson & Drahushchak 1990). The potential for the Mx gene probe to differentiate heterozygosity relevant to IHNV resistance warrants further study. It is possible that heterozygosity at the Mx locus is important for IHNV resistance, and the Mx gene probe may be an important disease resistance marker in terms of maintaining heterozygosity in cultured trout populations. It should be possible to develop PCR primers to the trout Mx exons. This would allow development of a PCR HpaI I fragment based polymorphism analysis with reduced labor requirements. Such an assay may be useful in a selective breeding program designed to enhance resistance to IHNV.

We have shown here that the differences in IHNV susceptibility of selected rainbow trout crosses within a trout farm are not correlated with the ability to express full length Mx mRNA. In addition, we have developed an assay to determine heterozygosity at the Mx locus that can differentiate individuals within cultured trout populations.

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