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Title: Characterization of Interferon and Retroposon-like Repetitive Elements in Salmonid Fish

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Hatchery-reared salmonid fish routinely encounter stress due to handling, barging, tagging, and overcrowding. It has been demonstrated that there exists a direct correlation between stress and transient immune suppression which can last for many days in fish. Epizootic viral infections routinely appear in hatcheries and can have a devastating effect on the fish population. The major viral pathogens in salmon and trout are the fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV), and the fish birnavirus, infectious pancreatic necrosis virus (IPNV). Vaccines for these viral pathogens are under investigation; however, the fish immune system becomes virtually nonresponsive during episodes of immune suppression. It was necessary to develop a nonantibody mediated, nonimmune method for preventing viral infections.

An interferon-like substance has been described for fish which possesses antiviral activity against both IHNV and IPNV. Since interferon administered to cattle has been very effective against vesicular stomatitis virus, a cattle rhabdovirus, an examination of interferon-like activity in fish was

initiated. We report here the establishment of in vitro interferon assays. In addition, the salmonid genome contains a multigene family of β-interferon-like genes, much like those in the bovine, equine and porcine genome. The rainbow trout interferon-like genes were found to be inducible in a manner which parallels those seen with bovine and human interferons.

In addition to the multigene interferon-like family, it was found that rainbow trout also contain a retroposon multigene family. Retroposons are repetitive elements which appear to have arisen by a reverse transcription event. Two L1 like repetitive elements have been cloned, one of which contains a Drosophila retroposon polymerase sequences never before described for salmonid fish. A number of retroviruses have been described in fish including the walleye dermal sarcoma virus and the Atlantic salmon swimbladder sarcoma virus. Interferon shows prophylactic promise both in vivo and in vitro against the human retrovirus, HIV. Therefore, research into fish interferon may be even more important if it demonstrates not only anti-IHNV and anti-IPNV, but also anti-fish retrovirus properties.

# Characterization of Interferon and Retroposon-like Repetitive Elements in Salmonid Fish

by

Leslie A. Tengelsen

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Redacted for Privacy

Chairman of Department of Microbiology

Redacted for Privacy

Dean of Graduate School

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Typed by Leslie A. Tengelsen for herself

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What was popularly described as phenomenology in the 50's and 60's has now become my research focus. Is that concept meaningful?! I would like to think that my years as a scientist and an artist have given me the ability to understand biological processes with a unique agenda.

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## INTRODUCTION

Aquaculture of salmonid fish species is an important industry in the Pacific Northwest and viral diseases of salmon and trout have had a serious impact on the growth of this industry. The American diet continues to shift from red meat to leaner chicken and fish and per capita consumption of fish is predicted to reach 30 lb/capita/year by the year 2000. This is twice the consumption determined in 1989 at 15 lb/capita/year, and is a strong indication that the demands on aquaculture will continue to increase (Buyers Guide, 1990, Aquaculture Magazine). Since many aquaculture facilities do not have pathogen free water available, it is virtually impossible to prevent viral epizootic outbreaks. It is important to develop comprehensive, cost-effective prophylactic programs to limit virus related losses. One way to do this is to develop vaccines against the most serious viral pathogens- infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, and infectious pancreatic necrosis virus (IPNV), a fish birnavirus.

Vaccines alone, although effective in certain circumstances, can be ineffective during times of stress. Hatchery-reared fish commonly encounter stress inducers such as handling, barging, tagging, and overcrowding. These stress factors have been shown to be directly linked to periods of marked immune suppression. In these circumstances, it is believed that the non-immune antiviral substance, interferon (IFN), would be a logical partner to vaccination for antiviral prophylaxis. It has been shown in mammalian systems and in early fish IFN work that IFN activity reduces the infectivity

of both rhabdoviruses and birnaviruses, so this course of investigation appears valid.

The studies reported here describe the molecular and biological features of a salmonid IFN-like substance. The ultimate goal of this study is to determine whether the administration of fish IFN may be useful as an economically sound part of good hatchery management.

In addition, information regarding the genetic makeup of the rainbow trout genome has been elucidated. In an attempt to isolate rainbow trout IFN genes from a lambda-based rainbow trout genomic library, retroposon repetitive elements were discovered. It has been reported that the rainbow trout genome is comprised of 84% repetitive elements. We report here the discovery of retroviral-like polymerase sequences embedded within the fish genome. Pitha (1991) reports that in vivo and in vitro evidence indicates that endogenous human IFN may be important in limiting HIV spread, replication and concommitant opportunistic infections. Retroviruses such as the walleye dermal sarcoma virus (Walker, 1947) and Atlantic salmon swimbladder sarcoma virus (Duncan, 1978) have been described in fish; therefore, the importance of studying fish IFN and the prophylactic uses of fish IFN may be even greater than originally envisioned.

# LITERATURE REVIEW

## INTERFERON

In 1957, Isaacs and Lindenman described IFNs as a group of proteins and glycoproteins that could inhibit the growth of a wide range of viruses. Interferons have since been described in all mammals investigated including the brown lemur, blackbuck, and gray seal (Wilson et al., 1983). There is some biological and molecular evidence that IFN exists in bony fish ( see FISH INTERFERON BACKGROUND section) and other preliminary evidence indicates that IFN may even exist in plants (tobacco); (Edelbaum et al., 1990). To date, no evidence has been reported demonstrating any IFN moiety in invertebrates.

Sehgal and Sagar (1980) discussed the fact that viral pathology was more dramatic in animals treated with antibodies against IFN than in untreated infected animals. Thus, IFNs are thought to play an important role in the early nonspecific defense against viruses.

In the sixties, investigators discovered that some IFNs were more than just antiviral substances (see Pestka, 1987 review). Jounger and Stinebring (1964) detected IFN in the serum of chickens injected with the Brucella abortus bacterium. Thus, IFN also stimulated antimicrobial activity. Investigators from the 1960's through the 1980's collectively demonstrated that IFNs played a role in various cellular functions including the modulation of immune responsiveness and regulation of cellular growth. The pharmaceutical potential of IFNs includes not only antiviral possibilities, but uses in cancer chemotherapy, immune modulation, and antimicrobial chemotherapy. Table 1 lists many of the current biological activities attributable to IFNs.

Table 1. Interferon inducible biological activities.

ANTIVIRAL- RNA and protein synthesis inhibition

ANTIPROLIFERATIVE: cell growth is inhibited
-intracellular parasites such as toxoplasma and plasmodium are inhibited

STIMULATE: phagocytosis
-tumor cell differentiation
-tumor necrosis factor activity
-myoblast and fibroblast differentiation

IMMUNOMODULATE: stimulate macrophage cytotoxicity
-toward tumor cells and virus infected cells
-B-cells are stimulated to produce antibody
-NK cell activity is stimulated

Table 1.

Stringfellow, in a 1980 overview, described the three main antiviral properties a substance must possess to be an IFN:

1. inactivation of antiviral activities possible by treatment with proteolytic enzymes but not nucleases;
2. antiviral properties arise as inducible intracellular activities mediated by an IFN receptor-IFN interaction; and
3. the IFN-like substance must be active against a wide variety of viruses.

Standard IFN nomenclature is shown in Table 2. IFNs are classified as either type 1 or type 2.

Type 1 IFNs are represented by both alpha (α) and beta (β) IFN. There are at least 15 different α-IFNs located at 15 unique gene loci in man. β-IFNs have traditionally been thought to be represented by a single gene; however, information to the contrary appears in some select species. There are between 1 and 5 human β-IFN genes; the number is unclear at this time. Ungulates possess a β-IFN multigene family (Wilson et al., 1983) as do the equine and porcine species (Leung et al., 1984). Type 1 IFNs (with the exception of human $\beta_2$-IFN ) possess no introns. α-IFNs are not glycosylated while β-IFNs are. Both type 1 IFNs are stable to pH 2 and stable when held at 56°C for 30 min.

Gamma or immune IFN (γ–IFN) is also known as type 2 IFN and there appears to be only one γ–IFN gene in humans (Lengyel, 1986). Unlike the type 1 IFNs, there are three introns located in the type 2 γ-IFN. The protein itself is glycosylated, labile at pH 2, and heat instable at temperatures above 56°C for 30 min.

Table 2. Interferon species, their inducers and the cells producing each type of interferon.

| IFN type | | Inducer | Secreting cell type |
|---|---|---|---|
| 1 | α | nucleic acid | leukocytes<br>lymphoblastoid cells |
| | β | nucleic acid | fibroblasts |
| 2 | γ | antigenic stimulus | macrophage stimulated T-cells |

Table 2.

Recently, investigators have described a variety of novel IFN activities which may be represented by as yet unmapped IFNs. The first is known as "endogenous IFN". This cell product was found expressed in physiologic amounts by uninduced cells in culture and varied in strength over the cell cycle (Friedman-Einat et al., 1982). This IFN-like substance demonstrated antiviral and cell growth inhibitory activity and was thought to be a novel IFN by the kind of activity demonstrated. An acid-labile α-IFN was described in serum samples from patients suffering from systemic lupus erythematosus (Preble et al., 1982). Placental IFN was recently described as an antiviral activity not associated with virus infection. This substance was found in a majority of amniotic fluid samples taken from the 37th week of pregnancy on. The function of placental IFN is unknown but investigators noted that no known infectious agents were present at the time of sampling. Thus, placental IFN appears to be a constituitively expressed nonimmune form of fetal protection (Duc-Goiran et al., 1985). Another IFN is readily induced by growth factors. This IFN has been given the name $\beta_2$-IFN (Sehgal and Sager 1980; Kohase et al., 1986; Sehgal et al., 1987) because it possesses cross-reacting epitopes with the more commonly studied β-IFN and can be induced from the same cells. Joklik (1990) stated that $\beta_2$-IFN possessed weak antiviral activity but was an active cytokine/lymphokine. $\beta_2$-IFN is also known as interleukin-6 (IL-6) (Ray et al., 1988). Finally, two IFN-like glycoproteins were identified in tobacco (Edelbaum et al., 1990). A human β-IFN monoclonal antibody cross-reacted with two glycoproteins, gp-22 and gp-35 from tobacco. These proteins were shown to possess antiviral activity against tobacco mosaic virus.

INDUCTION OF INTERFERON

IFN is not generally thought to be constitutively expressed in quantities that can elicit a strong response; rather, IFNs are inducible. Type 1 IFNs, α– and β–, are readily induced by class-A inducers (reviewed by Baron et al., 1984). Class A IFN inducers are potent and they contain nucleic acid. RNA viruses are good IFN inducers, with the myxoviruses being the most effective. DNA viruses, with the exception of vaccinia virus, are poor IFN inducers. It is believed that a dsRNA intermediate of viral replication is the signal to activate the antiviral arm of IFN. Marcus and Sekellick (1977) demonstrated that one defective-interfering particle of vesicular stomatitis virus (VSV) could induce IFN production. An extremely useful commercially available IFN inducer is the synthetic dsRNA polymer, polyinosinic-polycytidylic acid or poly I:C (Lampson et al., 1981).

Increased levels of human β-IFN mRNA are seen following induction, due to increased gene transcription. IFN message concentrations increased by two logs after 3 h of induction with poly I:C. An increase in protein activity paralleled the mRNA production with antiviral activity peaking by 6 h postinduction (Fujita et al., 1982). Whittmore and Maniatis (1990) investigated the rapid post-induction drop in β-IFN mRNA concentration. They found that the mRNA reduction was due to a combination of factors; mainly repression of transcription and rapid message turnover. In the presence of the protein synthesis inhibitor, cycloheximide, IFN mRNA became more stable. Thus, a repressor appeared to be induced by the virus as well.

Class-B inducers elicit a weak IFN response from α– and β-IFNs following target cell exposure. Representatives of this class include microbes and microbial products, some chemicals such as polyvinyl sulfate, and low

molecular weight products such as kanamycin and basic dyes (Baron et al., 1984).

γ-IFN is induced by a variety of immunostimulants. Antigens can be effective as γ-IFN inducers if the immune cell has already been sensitized. An antibody responding to the OKT3 antigen on T-lymphocytes will stimulate γ-IFN production while antibodies against OKT4 or OKT8 on T-helper cells and T-suppressor cells, respectively, do not elicit an IFN response. Tumor cells and mitogens such as PHA, Con A and LPS are also good inducers of IFN (Baron et al., 1984).

PRIMING

Priming is a phenomenon where cells are exposed to low levels of IFN prior to IFN induction by other means, such as poly I:C (Abreu et al., 1979). Priming enhances the production of IFN over induction alone. The current theory is that IFN, in low priming concentrations, stimulates the production of transcription initiation factors. It is interesting to note that poly I:C induction of an IFN response varies from cell line to cell line. This suggests that these factors may be limiting in some cell lines which are semiresistant to induction. This theory is supported by the finding that priming is ineffectual in cells where protein synthesis is inhibited (Fujita et al., 1981). Thus, priming appears to stimulate the translation of a novel gene product necessary for enhanced IFN gene transcription.

Fujita et al. (1981) investigated priming in L-cells. HeLa cells are an example of a poorly inducible cell line. Pre-treatment of HeLa cells with β-IFN and the subsequent induction with poly I:C yielded a two-fold greater IFN response over cells which were only treated with poly I:C. Enoch et al. (1986) felt that these results indicated that a trans-acting factor was involved and was

induced by IFN itself. It was possible that this factor was limiting in those cell lines which were poorly inducible. Dron et al. (1990) demonstrated that treatment of HeLa cells with hu-α-IFN prior to induction with poly I:C yielded an 8- to 100-fold increase in β-IFN mRNA. Dron et al. investigated chloramphenicol acetyltransferase (CAT) activity in HeLa cells transfected with a plasmid containing the CAT gene. This CAT gene was under the control of a β-IFN promoter. The promoter corresponded to a fragment spanning positions -91 to -62 with respect to the IFN trancription initiation site. Plasmids were constructed with the promoter and CAT gene in the correct and the opposite orientations. IFN priming of these transfected HeLa cells yielded increased levels of CAT activity upon subsequent poly I:C induction with both plasmid constructions. The authors also noted that IFN treatment alone without any induction had no effect on plasmid constructions containing either this 30 bp promoter fragment or an entire IFN promoter.

RECEPTORS

Interferons elicit their effects on target cells via a receptor ligand interaction. The IFN molecule binds with specific receptors on the cell surface in a narrow, species-specific manner. Interferon enters cells via receptor-mediated endocytosis. The signal is relayed to the nucleus where specific IFN-inducible genes are then transcribed and these gene products then carry out the biological effects of IFN (Aguet, 1980). The signal transmission between the cell membrane to the level of transcription initiation is unknown at this time.

Experimental evidence indicates that IFNs must utilize the receptor to elicit the appropriate biological response. Higashi and Sokawa (1982) microinjected purified IFN directly into the cytoplasm of target cells. They

found a total lack of characteristic IFN-induced biological activity after intracytoplasmic injection. Riviere et al. (1990) microinjected 20,000 α- and β-IFN molecules directly into the nucleus of mouse L-cells. Reviere indicated that this amount of IFN was sufficient to elicit an antiviral response from cells bathed in IFN at this level in culture media. The L-cells were challenged with either vesicular stomatitis virus or Semliki Forest virus 3, 6, or 24 h following nuclear injection. The results indicated again that there was no antiviral activity induced. Thus, IFN added to the cytoplasm or the nucleus could not elicit antiviral activity; IFN must use the receptor to generate any characteristic activities.

The presence of receptors for α-, β-, and γ-IFN on the surface of target cells was proven as early as 1982 by Joshi et al. To identify the location and usage of cell receptors, cross-linking and competition assays were performed. Purified human α-, β-, and γ- IFNs were radiolabeled with $^{125}$I-methionine. Bifunctional cross-linking reagents were used to bind and cross-link $^{125}$I labeled IFNs to cell surfaces (Gupta et al., 1985). The affinity of binding was tested for each IFN type in a competitive binding assay by varying concentrations of homologous unlabeled IFNs. Following cross-linking, membrane fractions were analyzed by SDS-poly acrylamide gel electrophoresis (SDS-PAGE) to determine whether any complexes had formed (Joshi et al., 1982; Sarkar and Gupta., 1984; Raziuddin et al., 1984). The hu-α-IFN receptor was found to interact with radiolabeled α-IFN to form a 150 kDa complex. This complex could be immunoprecipitated with anti-α-IFN but not by preimmune sera (Gupta et al., 1985). Interestingly, nonradioactive hu-α-IFN and hu-β-IFN both blocked the formation of the 150 kDa complex, while hu-γ-IFN did not. This finding led investigators to

conclude that type 1 IFNs both used the same receptor while type 2 IFN did not. Similarly, the binding of $^{125}I$ -labeled hu-γ-IFN to cell-surface receptors led to the formation of a 105 kDa ± 5 kDa complex when analyzed by SDS-PAGE. Complex formation was blocked by nonradioactive hu-γ-IFN but not by hu-α-IFN or hu-β-IFN. Thus, it appeared that hu-γ-IFN bound to a unique receptor.

Evidence has accumulated for human chromosome 21 as the carrier of the IFN-α/β receptor gene(s). Mouse/human hybrid cells were created with three copies of human chromosome 21 (Gupta et al., 1985). These cells, WA17 cells, when treated with $^{125}I$-labeled hu-α-IFN formed the 150 kDa complex previously described; the A9 parental mouse cells did not. Mouse/human hybrid cell lines containing chromosomes other than #21 also did not form this complex.

Uze et al. (1990) cloned and sequenced the cDNA for the human α-IFN receptor. This sequence was compared with the previously published human γ-IFN receptor sequence (Aguet et al., 1988) and was found to contain no sequence similarities. Bazan (1990) noted that there were evolutionary similarities between these receptors in their secondary structure. Type 1 receptors possess binding domains thought to possess two regions with preferential affinity for either the α-IFN or β-IFN so that the receptor can accomodate both type 1 IFNs. Arnheiter et al. (1983) demonstrated that the amino-terminus of $\alpha_2$-IFN contained the ligand for the membrane bound IFN receptor. Epitopes for receptor binding were mapped to common N-terminal regions in both α- and β-IFN and γ-IFN (Bazan, 1990). Type 1 and 2 receptors appear to share regions whose three dimensional structures were rich in β-turns. The current theory is that, although there is no evidence that

α/β-IFN can compete for binding to γ-IFN receptors and vice-versa, there are similar regions of antiparallel helical bundles in these conserved N-terminal ends which could bind to similarly shaped receptors (Bazan, 1990).

ANTIVIRAL ACTIVITIES INDUCED BY INTERFERON

Interferons typically elicit a variety of responses from the same cell. A "fingerprint" of a specific IFN includes the ratio of antiviral to antiproliferative activities induced by the IFN. This has been one descriptive way to separate IFN species.

Antiviral effects of IFNs vary. The mode of action generally involves either inhibition of viral protein synthesis or degradation of viral RNA. Inhibition of protein synthesis is cytotoxic and may result in the sacrifice of the infected cell. Inhibition of viral mRNA or intermediate dsRNA is less toxic to the host cell, but creates a condition where immature virus particles could establish persistence (Joklik, 1990).

The nature of the infecting virus ultimately directs the outcome of that infection. Two of the factors involved in the success of the viral infection include nucleic acid type and multiplicity of infection. RNA viruses are classically good inducers of IFN while DNA viruses, with the exception of vaccinia virus, are relatively poor inducers of IFN. Joklik (1990) described some of his early vaccinia work from 1966. In this work, the investigator described the outcome of vaccinia virus-nfected L-cells following IFN treatment. L-cells, not pretreated with IFN (unprimed), demonstrated a marked and rapid dissociation of the host message from resident ribosomes. The virus mRNA was then able to form viral polyribosomes and direct the production of virus-encoded proteins. The investigators postulated that priming of these cells with IFN prior to vaccinia infection caused a different

biological response. The host polyribosomes still rapidly disaggregated from host mRNA; however, the vaccinia virus nascent message was unable to form viral polyribosomes resulting in an inhibition of vaccinia protein production and ultimately, viral replication. In this case, viral message was produced but translation was impeded. The host cell generally died due to the universal inhibition of protein synthesis and viral dissemination was curtailed. The phenomenon was also described for VSV (rhabdovirus) and influenza virus (myxovirus) by Repik et al. (1974), reovirus (Wiebe and Joklik, 1975), and SV40 (papovavirus; Jakobson et al., 1977).

The following three inducible antiviral mechanisms are the best described activities of IFN in the literature. They are: 1) a specific inducible protein kinase, 2) 2,5-oligoadenylate synthetase, which is linked to the activation of a latent ribonuclease RNase L, and 3) the Mx protein, which is effective against influenza virus.

Protein Kinase Induction

Kerr et al. (1974) first observed that dsRNA inhibited protein synthesis. This inhibition was brought about by a dsRNA and/or IFN inducible protein kinase (Hovanessian et al., 1986). The protein kinase, DA1, possessed two distinct kinase activities. It was found to be responsible for the endogenous phosphorylation of a 67 kDa protein subunit of the kinase itself and it phosphorylated the small $\alpha$-subunit of elongation initiation factor eIF-2. The 67 kDa subunit was activated by phosphorylation and was thought to exist in a latent nonphosphorylated form. The phosphorylation of eIF-2 effectively inhibited the protein synthetic activities attributed to the initiation factor. The concentration of this protein kinase increased 20-fold in IFN-treated cells. Since the phosphorylation of eIF-2 halted all protein synthesis, the host as

well as the virus was affected.

Vaccinia virus has been shown to be capable of escaping the effects of IFN by inhibiting DA1 (Younger and Whitaker-Dowling, 1985). The structure of the factor responsible for the inhibition of DA1 has not been determined, but is known as a specific kinase inhibitory factor. The vaccinia-induced kinase inhibitor can rescue other viruses. When IFN primed cells were coinfected with vaccinia and VSV, VSV was able to replicate and cause cytopathic effects (CPE). This has also been shown for co-infection of vaccinia virus with picornaviruses and herpes simplex virus. Influenza, like vaccinia, is also able to avoid antiviral inactivation by DA1 by a method not yet determined.

The 2,5-Oligoadenylate/RNase L System

Ribonuclease activity is another inducible mechanism of IFN action. The pathway known as 2,5-oligoadenylate synthetase/RNase L is dependent on dsRNA for its activity.

Research by Kerr et al. (1978) identified an IFN-inducible enzyme oligoadenylate synthetase. They also isolated heat stable oligonucleotides with unique 2' to 5' phosphodiester linkages that were derived from the reaction: (n + 1) ATP + synthetase ------> (2'-5') pppA(pA)n + n-pyrophosphate where n ranged from 1-15. This product was referred to as 2,5-$(A)_n$.

The formation of small 2,5-$(A)_n$ oligomers led to the activation of another enzyme, RNase L (latent), also known as RNase DS because of its dsRNA degradation capabilities (Meegan and Marcus, 1989). Ribosomal RNA is known to be a casualty in the degradative path of RNase L. It binds reversibly to the 2,5-$(A)_n$ to make an RNase L/2,5-$(A)_n$ complex. The RNase L recognizes 5'ppp and free 2'-OH groups at the terminus of its target and, if the substrate is not $(A)_{16}$ or greater, then the ribonuclease is no longer active.

Naturally occurring dsRNA is found during the replication of (+) single-strand RNA viruses such as the picornaviruses (Chebath et al., 1987), togaviruses, coronaviruses, and caliciviruses. Double-strand RNA viruses include reoviruses and birnaviruses. These may be the most likely targets for this kind of antiviral activity (Joklik, 1990). There is speculation that other viruses sensitive to IFN, such as the DNA virus vaccinia, may contain a transient double-stranded RNA phase which has not yet been described. It has been found that two viruses, encephalomyocarditis virus (a picornavirus) and herpes simplex virus, are able to inhibit RNase L (Joklik, 1990). In HeLa cells, α-IFN will induce the synthetase but β-IFN will not. Even though these IFNs share the same receptor, they do not necessarily induce the same antiviral response.

Mx Gene Product

A third inducible antiviral substance is known as the Mx gene product. In 1962, Lindemann described a unique protein induced by type 1 IFNs but not type 2, which could selectively inhibit influenza virus replication. The Mx gene was first described in the mouse. Horesberger et al. (1983) carried out experiments on the genetics of Mx activity in mice. The Mx gene was found to encode a product which provided a natural mode of early protection against influenza virus in response to IFN. Mice from the strain A2G are homozygous Mx/Mx and able to withstand a lethal infection of influenza virus. These mice were crossed with BALB/c mice which lack both Mx alleles and are highly sensitive to influenza infection. Each crossed generation was tested for its sensitivity to influenza infection. The survivors of influenza infection were backcrossed again and again. Congenic embryo cells from 11th generation backcross males were tested for their sensitivity to IFN. The cells from the

backcrossed population were able to withstand influenza infection. The investigators were able to determine that a unique 72.5 kDa protein was produced in the backcrossed cells that was not found in the original BALB/c cells. The maximal production of this protein occurred at 4 h following the exposure to IFN. Actinomycin D was able to totally block the production of the unique protein, indicating the necessity for mRNA synthesis.

The 72.5 kDa mouse protein product is nucleophilic, accumulating in the nucleus of cells activated by virus infection or induction with dsRNA. The Mx gene product is capable of inhibiting both influenza A and B. When antibodies to the mouse Mx gene product were microinjected into mouse cells, the treated cells were unable to mount an anti-influenza state. Thus, the Mx gene product was responsible for the antiviral activities against influenza.

Staeheli and Haller (1985) described the characterization of a human Mx counterpart. This gene product possessed antigenic characteristics similar to mouse Mx. The human Mx counterpart appeared to function within the cytoplasm, unlike the murine Mx. This protein also cross-reacted with mouse Mx antisera and could be purified by affinity chromatography. The human protein had a molecular weight of 78 kDa, similar in size to the mouse Mx protein. Yet, the human Mx protein did not confer an anti-influenza state to IFN-induced cells. Anti-Mx monoclonal antibodies, microinjected into human cells (Weitz et al., 1988), did not alter the cells' susceptibility to influenza infection. Therefore, the human Mx gene product did not appear to play a large role in protecting human cells against influenza infection.

The gene loci in mice had been shown to consist of two genes, Mx1 and Mx2. Mx1 was 90% homologous to Mx2 in the 5' region. However, the Mx2, found in BALB/c and CBA laboratory mice, contained many mutations

and no message was found upon induction (Staeheli and Sutcliffe, 1988).

The specific antiviral activity attributable to Mx has not yet been established. All Mx sequences examined to date contained three consensus GTP binding sequences identified by sequence alignments (Horesberger et al., 1990). Future studies include testing this product for binding to nucleotides or nucleic acid.

It is important to point out that there are still many unanswered questions regarding all the antiviral proteins examined which are inducible by IFN. All proteins mentioned have demonstrated biological activity that will vary by cell type, infecting virus, and inducer. Not all of the mechanisms of their actions have been uncovered. In some cases, the protein kinase or the synthetase will be present but not elicit an antiviral response. There clearly are more signals to the IFN-inducible system yet to be elucidated.

BETA INTERFERON GENE STRUCTURE

Beta IFNs were long thought to be represented by a single gene moiety in all systems investigated (Pestka et al., 1987). In 1984, Sehgal and May isolated 22 independent clones of a β-IFN from a human lambda library and mapped two of these: lambda B3, to human chromosome 2 and lambda B4, to human chromosome 4. Subsequent studies have suggested that there were "at least" 2 and possibly as many as 5 β-IFN genes. The human-$\beta_1$-IFN has been located on chromosome 9 (Owerbach et al., 1981) and the human $\beta_2$-IFN has been located on chromosome 7 (Sehgal et al., 1986).

Interferons are created as pre-proteins with an N-terminal signal peptide, for secretion. The signal peptide is subsequently cleaved to produce a mature IFN molecule. All IFNs, both type 1 and type 2, possess this signal

sequence which is approximately 20 to 23 amino acids in length (for review see Joklik,1990).

Human $\beta_1$-IFN is an intronless gene in all systems described to date, encoding a 166 amino acid gene product with a 21 amino acid signal sequence, as depicted in Figure 1. The $\beta_2$-IFN gene is unusual, as a type 1 IFN, in that it is comprised of 4 exons. The gene product contains a signal sequence of 28 amino acids and is 184 amino acids in length following cleavage (Sehgal et al., 1987).

The DNA encoding the $\beta_2$-IFN gene did not cross-hybridize with $\beta_1$-IFN nucleic acid. Interestingly, polyclonal and monoclonal antibodies to $\beta_1$-IFN neutralize the antiviral effects of $\beta_2$-IFN (Zilberstein et al., 1987). $\beta_2$-IFN was described as a translationally active IFN when programmed into Xenopus laevis oocytes. The mRNA of $\beta_2$-IFN differs from $\beta_1$-IFN RNA by 400 nt. Cytokines affecting cell proliferation such as platelet-derived growth factor (PDGF), IL-1 and β-IFN all increase the expression of $\beta_2$-IFN in human fibroblasts (Sehgal and May, 1986). Beta-2 IFN is inducible by tumor necrosis factor (TNF) and appears to mediate the antiviral and mitogenic activities of TNF in fibroblasts.

These interactions and functions were dissected by the use of antibodies to $\beta_2$-IFN. The $\beta_2$-IFN protein is also known as interleukin-6 (IL-6) (Ray et al., 1988).

Type 1 IFNs possess internal sequence duplications suggesting that they evolved via a gene duplication from a smaller related gene. Peter Sellers (1980) of Rockefeller University, developed a metric analysis

Figure 1. An example of β-IFN gene structure. Human $\beta_1$-IFN possesses a 21 amino acid signal sequence on the amino terminus of the 166 amino acid protein.

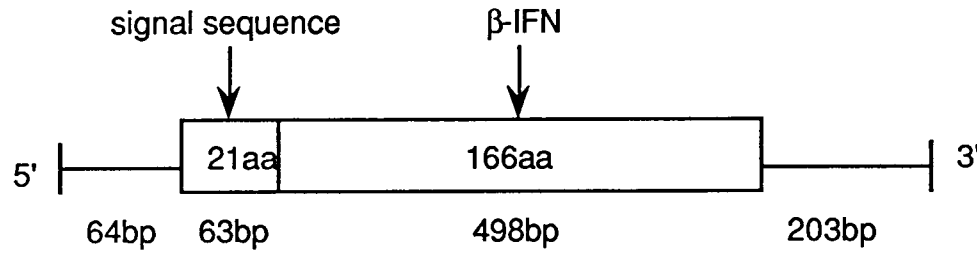


Figure 1.

algorithm for comparing the relatedness of two nucleotide sequences. The analysis led to the recognition of a number of internal patterns including inverted repeats, direct repeats, and palindromes (Erickson et al., 1984). Figure 2 shows the 5' amino acid positions which are duplicated in the same positions within the 3' half of the gene, demonstrating redundancy. Using metric analysis, Sehgal and May (1984) were able to show that human $\beta_1$-IFN possessed two major repeats of approximately 300 bp and the repeats possessed 45% similarity at the nucleotide level. The investigators also found that human $\alpha$-IFN demonstrated a similar internal duplication of approximately 300 bp with 45% similarity at the nucleotide level. In addition, the results of metric analysis of human $\alpha$- and $\beta$-IFNs demonstrated that they shared 56% nucleotide homology with each other. Murine $\alpha$- and $\beta$-IFNs also contained large internal duplications. Further metric analysis revealed that each duplicated segment itself contained smaller 80 bp duplications (Erickson et al., 1984). In 1983, May et al. reported that a poly I:C induced message of approximately 350 nucleotides (nt) from human fibroblasts was sufficient to encode a biologically active IFN when injected into Xenopus laevis oocytes. A thermolysin cleavage product of $\alpha_2$-IFN corresponding to the amino-terminal 110 amino acids, or 330 bp, was also found to be biologically active (Ackerman et al., 1984). This supports the fact that the gene appears to be redundant. Investigators have not yet determined whether the carboxy terminus half of the IFN gene is biologically active. With such striking duplications and the relatedness between $\alpha$-IFN and $\beta$-IFN, Sehgal and May stated that it was no wonder that they shared the same cell surface receptor (see RECEPTORS section).

Human β-IFN was first cloned in 1979 by Taniguchi et al. The kinetics of IFN production had to be determined before the initial isolation of the IFN gene could be carried out . Human fibroblasts were induced by dsRNA to produce IFN mRNA and the RNA produced was characterized for its capacity to produce an antiviral substance when microinjected into Xenopus laevis oocytes. The information gained from this experiment allowed the researchers to select the appropriate time post-induction for mRNA isolation. The mRNA was selected from total RNA by chromatography on oligo-dT affinity columns. The mRNA was then reverse transcribed into cDNA and cloned.

A model for $\beta_1$-IFN gene organization and control has been developed (Zinn and Maniatis, 1986). The IFN gene regulatory element (IRE), a region 5' to the transcription initiation site, appears to be under the control of two repressor molecules (Goodbourne et al., 1985). Gene transcription required the removal of these repressors, each of which bound to separate sites in the IRE. One site at -167 to -94 was thought to be a negative control sequence as the deletion of this region improved expression by 10-fold. One of the repressor molecules appeared to bind here. A second site was located between -68 to -38 which, in itself could be divided into two sections; the 5' half appeared to have enhancer capabilities while the 3' half appeared to contain an element for the second repressor molecule, which negatively controlled the enhancer (Goodbourne et al., 1986). During induction a transcription factor bound to the enhancer, the repressor molecules dissociated, and transcription began (Goodbourne et al., 1985; Keller and Maniatis, 1988). Investigators have found that the molecular control elements

Figure 2. Single-letter amino acid code for the amino terminus of human $\beta_1$-IFN including the 21 amino acid signal sequence. The bold/underlined letters represent amino acid positions which are duplicated in the same positions within the 3' half of the gene demonstrating redundancy.

5'- M**T** N K C L **L** Q I A L L L C F S T T A L S M S Y N

**L** L G F L Q R S S N F Q C Q K L **L** W Q L N **G R** L E

Y C **L K** D R M N F D I P E E **I** K Q L Q Q F Q K E D A

A L T I Y E M **L** Q **N**.....

Figure 2.

appear to vary in different cell lines. Dinter and Hauser (1987) found that, in mouse L-cells, 111 nt were required upstream from the transcription initiation site, while Goodbourne et al. (1988) found that 77 nt were required upstream for full transcription. Goodbourne et al. also described the enhancer and second negative regulatory domain. The current model describes a 5' IRE which contains two positive regulatory domains and one negative regulatory domain. These areas are called PRD1, PRD2, and NRD1, respectively (Lenardo et al., 1989; see Figure 3).

Activation of the $\beta_1$-IFN gene is dependent on a trans-acting factor, which is itself IFN inducible (Enoch et al. 1986). In certain cell lines induction of IFN activity is poor. HeLa cells induced with poly I:C or virus were found to be very inefficient in their ability to mount an antiviral response (Enoch et al., 1986). However, if they primed these cells with IFN in the presence of cycloheximide, they found a 200-fold increase in antiviral activity. Cycloheximide is a potent inhibitor of protein synthesis and presumably acts by stabilizing the $\beta_1$-IFN mRNA which leads to superinduction. Protein synthesis is not required to induce IFN mRNA (Ray and Pitha, 1983). Therefore, this trans-acting substance is only produced in low levels in unprimed HeLa cells and requires priming in order to be expressed.

PRD2 has been shown to bind a nuclear factor, PRD2-BF. Sequence analysis of the factor PRD2-BF demonstrates conservation with the mammalian transcription factor NF-kB. Sequence analysis of PRD2 revealed a strong conservation with NF-kB binding sites within the MHC class I H-2K gene and the Ig k enhancer (Lenardo et al., 1989). In fact, in vitro experiments revealed that NF-kB bound PRD2 (Lenardo et al., 1989). Lenardo et al. speculated that PRD2 was also an enhancer site. NF-kB has been

Figure 3. The nucleotide sequence of the 5' upstream regulatory elements affecting β-IFN induction are shown here. The elements include the IRE (IFN gene regulatory element), PRD1 (positive regulatory domain 1), PRD2 (positive regulatory domain 2), and NRD1 ( Negative regulatory domain 1). The highlighted sequences bind the trans-acting factor NF-κβ.

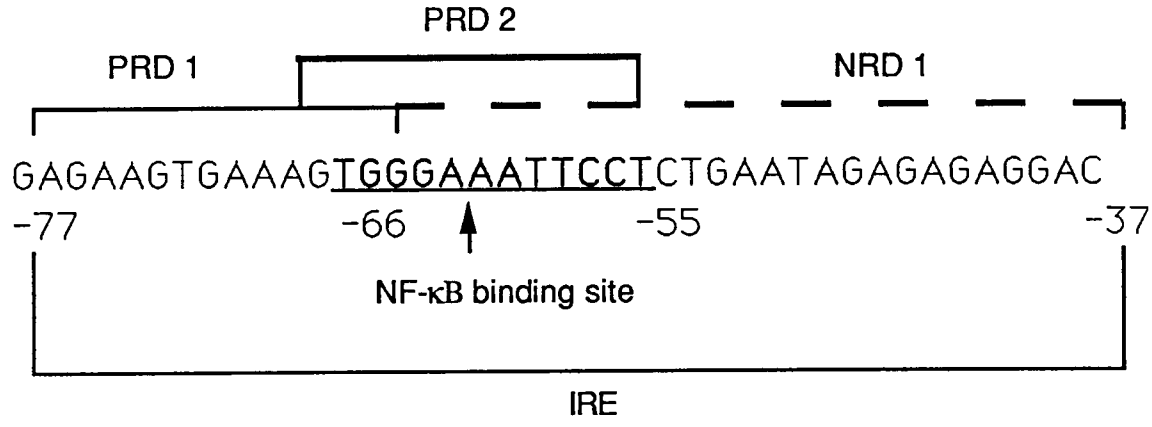
PRD 2
PRD 1
NRD 1
GAGAAGTGAAAGTGGGAAATTCCTCTGAATAGAGAGAGGAC
-77
-66
-55
-37
NF-κB binding site
IRE

Figure 3.

described as a trans-acting factor which is independent of new protein synthesis (Lenardo and Baltimore, 1989). What is required for its activation is a removal of an inhibitory factor which then allows the factor to pass from the cytosol to the nucleus to effect its trans-acting functions. Experiments with cytoplasmic extracts treated with dissociating chemicals such as deoxycholate yielded high NF-kB activity. This result demonstrated the presence of a binding inhibitory factor. Treatment of cells with phorbol esters stimulated the production of protein kinase-C (PKC). Investigators were able to determine that PKC leads to the dissociation of NF-kB from the cytoplasmic inhibitor by phosphorylating the inhibitor and allowing the subsequent migration of NF-kB to the nucleus as a signal transducer (Ghosh and Baltimore, 1990).

EVOLUTION OF BETA-INTERFERON GENES

In 1961, Clem et al. described the possibility that primitive poikilothermic vertebrates had developed the capacity to produce IFN and that this mechanism must have evolved early as a nonspecific defense.

Human α- and β-IFN possess 45% similarity at the nucleotide level. The amount of divergence of α-IFN nucleotide sequences from β-IFN sequences led some investigators to believe that type 1 IFNs emerged from a common proto-α/β IFN ancestral gene. The proto-α/β IFN was in turn thought to have arisen from a short 300 bp duplication to yield the ~600 bp full length coding region. This divergence was thought to have occurred approximately 300 million to 400 million years ago (Pestka et al., 1987; Wilson et al., 1983). This was also roughly the predicted timeline for divergence of mammals from reptiles, birds, fish, and amphibia (Henco et al., 1985). Pestka et al. (1987 ) felt that fish diverged with only the one type, β-IFN , and this hypothesis was supported by Wilson et al. (1983) (see FISH INTERFERON BACKGROUND

section), who showed that a hu-β-IFN gene probe would hybridize very weakly in Southern blots to the DNA derived from perch (Perca fluviatilis), minnow (Phoxinus phoxinus), dace (Leuciscus leuciscus) and stone loach (Noemacheilus barbatulus). A hu-α-IFN gene probe did not show similar hybridization.

Wilson et al. (1983) examined the DNA of a wide variety of animals for the presence of β-IFN-like sequences with a hu-β-IFN derived probe. Most mammalian species possessed only one or two hybridizing fragments corresponding to β-IFN. However, an anomalous hybridization pattern was noted in ungulates; the blackbuck (Antilope cervicapra) and the cow (Bos taurus). The DNA from these animals were found to contain 9 to 11 hybridizing fragments which indicated that ungulates contained a multigene family of β-IFN genes. The finding suggested that the β–IFN gene was duplicated specifically in the ungulates following their divergence from other mammals somewhere between 85 and 20 million years ago. Similarly, Leung et al. (1984) described multiple hybridizing bands in a Southern blot containing endonuclease restricted equine and porcine genomic DNA, indicating that these species also contained a multigene β-IFN family.

FISH INTERFERON BACKGROUND

In vitro production of an IFN-like substance was demonstrated as early as 1965 by Gravell and Malsberger, in fathead minnow cell culture (FHM) (Pimephalas promelas). The investigators examined the temperature dependent production of an antiviral substance secreted into the supernatant by cells infected with infectious pancreatic necrosis virus (IPNV), a fish birnavirus. The antiviral substance was secreted at 23°C but not at 34°C. This substance could withstand 37°C for 2 h in the test tube and still yield

an antiviral effect on homologous cell cultures. Antiviral activity, thought to be IFN, was measured by a plaque reduction assay where units of IFN activity were described as the inverse of the dilution of sera where 50% of input virus was inhibited, i.e. plaque reduction (Wagner, 1961).

Watson et al. (1954) observed a decrease in infectious hematopoietic necrosis virus (IHNV)-induced mortality in sockeye salmon (Oncorhynchus nerka) when the fish were reared at 20°C rather than 15.5°C. Infectious hematopoietic necrosis virus (IHNV) is a fish rhabdovirus. Amend (1970) examined the control of IHNV by temperature fluctuation. Amend held sockeye salmon at 12°C and segregated experimental groups at 16, 18, and 20°C to determine what temperature yielded the best anti-IHNV activity (the least mortality). At 18 and 20°C, the fish in these groups exhibited a mortality of less than 10% while 12 or 16°C experimental fish groups were killed by IHNV up to 100%. Timing was critical as an increase in temperature was most useful within 24 h of the initiation of an IHNV outbreak, while the delay of temperature treatment by one week increased the average mortality to nearly 50%. Amend stated that the reason for the decreased mortality was not known. IHNV was known to be viable at 28°C, so heat sensitivity was not thought to be the cause of decreased pathogenicity. The investigator also felt that the biological response was not immune; i.e. the antiviral activity was seen too rapidly to be a humoral response, and infection was restored rapidly when the temperature was returned to 15.5°C. They postulated, in conjunction with the Gravell and Malsberger (1965) findings, that the fish were producing an IFN-like substance. Sixteen months following the initial heat treatment, Amend was unable to reculture any IHNV isolates from the 18 and 20°C treated fish populations; however, some of the fish developed scoliosis

pathognemonic for IHNV survival).

De Kinkelin and Dorson (1973) described the same temperature related phenomenon in rainbow trout infected with viral hemorhaggic septicemia (VHS/Egtved virus), another fish rhabdovirus. The investigators noted a decrease in virus-induced mortality when the temperature was elevated from 12°C to 15°C. VHS grows well at 15°C in vitro, so IFN was again suspected as the antiviral substance responsible for the temperature dependent antiviral activity seen.

Coho salmon (Oncorhynchus kisutch) have been shown to be relatively resistant to IHNV infection while rainbow trout are extremely susceptible. Busch et al. (1985) demonstrated that rainbow trout x coho triploid hybrid crosses gained significant resistance to IHNV. IHNV induced mortality dropped by 74.2% from normal diploid rainbow trout controls. It will be of interest in their future experiments to determine whether coho contain extra copies of IFN or possess biological activities missing in other salmonids.

Fish have been shown to possess Mx-related sequences in their genome (Staeheli et al., 1989). The fish species examined were perch (Perca fluviatilis) and rainbow trout (Oncorhynchus mykiss). Genomic DNA was cloned and the subsequent recombinant library was probed with an Mx DNA probe. Sequence analysis of positive clones revealed a 60 to 84% similarity at the nucleotide level when comparing exons from fish to exons in mouse. Intron sequences demonstrated little or no similarity between fish and mouse. Northern analysis revealed that following 18 h of poly I:C induction, two novel fish RNA species of 2.5 and 2.0 kbp, hybridizing to the Mx probe, emerged. These species were not seen in the uninduced control fish. Myxoviruses have been isolated from fish (Winton et al., 1985). The presence of an inducible

Mx-like gene product with similar kinetics of induction to the mouse Mx gene product suggests that the Mx protein may play an antiviral role in fish.

Persistence in Fish

In 1961 Clem et al. described the emergence of what was termed the grunt fin agent in cultured grunt fin cells, Haemulon sciurus. This agent caused CPE in these cells following the 65th passage, which is more than a year after cell line initiation. It was postulated that the cultured grunt fin cells harbored an agent in a latent or subclinically persistent state. Beasley et al. (1966) felt that this persistent carrier state was mediated by endogenous IFN. In fact the supernatant fluids from carrier cells, when applied to grunt fin cells, effectively inhibited CPE and virus replication of IPNV. Oie and Loh (1971) also described IFN production in marine cell culture with similar results. Oie and Loh examined FHM cells infected with fish reovirus type 2 and demonstrated the presence of an antiviral substance, confirming Gravell and Malsbergers findings (1965) that antiviral activity was not solely due to temperature fluctuations.

Sigel (1966) described some of the IFN-like properties demonstrated by this substance:

1. the retention of antiviral activity in the supernatant after centrifugation at 100,000 g,
2. thermostability following 30 min at 37°C,
3. resistance to pH 2.0 , and
4. species specificity, working only on homologous GF cells and not on heterologous cells from other species of fish.

Hedrick and Fryer (1981) examined persistent IPNV infection of three salmonid cell lines. They established persistent virus-shedding cell lines with a concomitant lack of CPE in chinook salmon embryo cells (CHSE-214), steelhead trout embryo cells (STE-137), and rainbow trout cells (RTG-2). Apparent IFN-like activity was undetectable in the culture fluids of both the CHSE-214 and STE-137 cells while the RTG-2 cells contained an antiviral substance thought to be IFN.

In vivo experiments were performed to determine the duration of production of the antiviral substance in fish serum. Rainbow trout were injected with VHS by de Kinkelin and Dorson (1973). $5 \times 10^6$ plaque forming units (PFU) of VHS were injected intraperitoneally into ~80 g rainbow trout. At three, nine, and fourteen days following the initial injection, fish were bled and their serum tested for antiviral activity. Sera was heated initially to 37°C for 2 h to inactivate any virus. RTG-2 cells were then overlaid with fish serum in normal growth media from 1:50 to 1:20,000 dilutions for 16 h. The cells were then washed with Tris-buffered saline and subsequently challenged with 100 PFU of IPNV and observed for CPE. The IFN units were determined by plaque reduction assay as described above. Serum collected on day three was determined to contain the most potent antiviral activity while day 14 serum IFN activity approached zero. Serum from five trout bled on day three post-infection demonstrated IFN titers ranging from 100 to 2750 IFN units/ml and controls contained no detectable antiviral activity. The extreme variability of IFN units, from one trout to another, was thought to be due to genetic heterogeneity (de Kinkelin and Dorson, 1973).

Biochemical and biophysical properties of fish IFN were determined from pooled sera from day three bleedings. The results are listed below:

1. broad antiviral activity (RTG-2 cells were protected from IPNV and IHNV),
2. species specificity (15x more effective on RTG-2 cells than FHM cells),
3. no loss of activity after sedimentation at 100,000 x g for 3 h,
4. thermostable following 30 min at 56°C,
5. stable at pH 2, and
6. trypsin-labile and RNase-resistant.

By 1975, investigators were attempting to partially purify and characterize an RTG-2 cell-derived IFN-like substance. De Sena and Rio (1975) described properties shared by this rainbow trout IFN-like substance and higher animals. The partial purification scheme began with tissue culture supernatants collected from IPNV-induced RTG-2 cells. The supernatant was clarified at 70 000 x g for 2 h following an acidification step to pH 2 for 48 h. The supernatant was then further purified by gel chromatography on Sephadex G-150. Fractions were tested for IFN activity by the neutral red dye uptake method of Finter (1969). Positive fractions were pooled and then placed on a CM-Sephadex (C-50) column. These fractions were then tested for IFN activity by plaque reduction. The final partially purified substance was found to be:

1. heat stable at 40 and 56°C 30 min,
2. stable to pH. 2,
3. exhibited species specificity; more active in RTG-2 cells than SJU-1 cells (Goldfish cell line),
4. sensitive to trypsin treatment and resistant to ribonuclease and deoxyribonuclease activity, and

5. non-specific antiviral activity, effective against both IPNV and IHNV.

The investigators determined that the antiviral substance possessed a molecular weight of 94 kDa.

Further physicochemical characteristics of rainbow trout IFN from live fish were elucidated by Dorson et al. (1975). Protocols for partial purification were similar to De Sena and Rio (1975). Dorson et al. determined that the rainbow trout IFN protein existed as a 26 kDa polypeptide with a sedimentation constant of 2.3S. They have reported that these values fall within normal values for IFNs of higher mammals. There is a discrepancy between the molecular weight determinations of De Sena and Rio and Dorson et al. and may be a reflection of the different purification schemes.

Interferons with differing molecular weights were being described in the late 1960's and early 1970's. These species were found to be of two general sizes, those weighing approximately 100 kDa and those falling in the range between 20 and 40 kDa. It was later determined that under dissociating salt conditions, the high molecular weight species would disaggregate into the smaller forms which were biologically active, thus indicating the presence of high molecular weight multimeric forms of the smaller species (Stringfellow, 1980). This may explain the observed disparity in molecular weight found by the early fish IFN investigators using similar purification schemes.

MOLECULAR ANALYSIS OF FISH INTERFERONS

Some preliminary molecular information on fish IFN has been available since 1983. Wilson et al. (1983) carried out a hybridization "survey" of vertebrate IFN gene families using human $\alpha$- and $\beta$-IFN DNA as the heterologous probe. They concluded that perch (Perca fluviatillis), minnow,

(Phoxinus phoxinus), dace (Leuciscus leuciscus) and stone loach (Neomacheilus barbatulus) all possessed weakly hybridizing genomic sequences under low stringency conditions: 3x SSC, 60°C for hybridization and 3x SSC for subsequent washing (0.45 M NaCl, 0.075 M NaCitrate). The faintly hybridizing genomic fragment was approximately a 3.5 kbp EcoR1 digestion product. Wilson et al. (1983) postulated that the bony fishes may possess only one IFN-like gene of the α/β type, due to the hybridization frequency. The resulting bands were very difficult to see. It appears difficult to draw any conclusions on the size of the gene family from this paper.

Dehlin et al. (1987) described the discovery of an IFN-like gene from zebrafish. The investigators were able to isolate three positive plaques/600,000 plaques from a zebrafish genomic library by screening with the coding regions of both the human α-IFN and β-IFN. The frequency of hybridization in this case again reflects the possibility of a single copy gene. The information, in abstract form, did not include any sequence or other molecular information on this gene. No other molecular information has been published to date on salmonid IFN.

REPETITIVE ELEMENTS

A large number of nonfunctional genes and repetitive elements are found within mammalian genomes. They are thought to arise in two ways; by recombination where the duplicated gene acquires defects generating pseudogenes, or by retroposition where a cDNA copy of RNA is dispersed within the genome (Rogers, 1985; Weiner et al., 1986). Reverse transcription has been established in such diverse groups as humans, insects, and yeast (Baltimore, 1985) and may represent a hallmark of genetic reorganization and pseudogene genesis. Some of these elements appear to have arisen from a

reverse transcription event in that they appear more like a cDNA than DNA, lacking introns. These retroposition events give rise to retroposons, including retropseudogenes and short and long interspersed repetitive elements which can be present in as many as 500,000 copies per genome (Brosius, 1991). A number of examples of transcribed retroposons have been summarized in the review by Brosius (1991) and include an insulin type 1 gene in mice, pyruvate dehydrogenase E1a subunit gene in mice and humans and N-myc2 oncogene in woodchucks.

Retrotransposons are nonretroviral transposed elements found to share homology with retroviruses (Baltimore, 1985). They are loosely characterized by their poly A 3' tracts, lack of introns, polymerase-like sequences and repeat elements. These retroposons are organized like retroviruses and their presence within genomes indicates the act of nonretroviral or retroviral assisted reverse transcription and integration.

One of the most common types of retroposons are the long interspersed elements known as Line-1 (L1) elements or LINES. L1 elements are an average of 6 kbp long, but 5' truncated versions are common, with the 3' poly A tract and repeat short flanking repeats the most frequently found forms (Rogers, 1986). Complete L1 elements contain two open reading frames whose primary sequence can vary depending on the amount of point mutations and frame shifts found in them. In general, these open reading frames resemble gag-pol retroviral sequences and organization (Loeb et al., 1986).

Historically, retroposons have been viewed as garbage littering the genome with worthless information. However, researchers are beginning to believe that retroposons may actually play a role in the evolutionary process.

This is especially true if they insert regulatory elements upstream altering the expression of the resident genes or are transcriptionally active themselves (Brosius, 1991).

# CHAPTER ONE

## Variation in Fish Interferon-like Activity: Cell Line Production and IHN Virus Isolate Sensitivity

Leslie A. Tengelsen, Eric Anderson, and Jo-Ann C. Leong

Department of Microbiology
Oregon State University
Corvallis, OR 97331-3804

# ABSTRACT

The induction and assay of fish IFN was carried out with several salmonid fish cell lines. Cells were treated with poly I:C and the subsequent induction of IFN activity in vitro was measured. Four IHNV isolates from different fish species were tested for their sensitivity to the IFN-like activity produced by RTG-2 cells. The isolates included Lake Auke (LA) from sockeye salmon, Round Butte-1 (RB-1) from steelhead salmon, 039-82 from rainbow trout, and Coleman-3 (CO-3) from chinook salmon (Hsu et al., 1986). The Lake Auke strain was the most sensitive to the antiviral effects of IFN. The viral isolate 039-82 was then tested for its sensitivity to IFN-like activities in four fish cell lines: RTG-2, KO6, SSE-5 and STE-137. SSE-5 cells were the most effective in reducing the titer of the 039-82 strain of IHNV.

# INTRODUCTION

Interferon-like activity produced by fish cells has been examined in a number of fish cell lines (de Kinkelin and Dorson., 1973; De Sena and Rio, 1975). The activity is characterized by the induction of resistance in cells to viral infection and by its resistance to acid treatment (pH 2.0), resistance to deoxyribonuclease and ribonuclease digestion, and sensitivity to trypsin digestion. The activity can be induced by exposure to inactivated virus, double-stranded RNA or synthetic polyribonucleotide polymers such as poly inosinic: poly cytidylic acid (poly I:C) (Trapman, 1979). We have characterized the production of this activity in a number of different cell lines and we have also determined that there are marked differences in the sensitivity of virus isolates to this activity.

# MATERIALS AND METHODS

## Cell lines

Cell lines from rainbow trout gonad (RTG-2; Oncorhynchus mykiss; Wolf and Quimby,1962), kokanee embryo (KO6; Oncorhynchus nerka; Lannan et al., 1984), sockeye salmon embryo (SSE-5; Oncorhynchus nerka; Nims et al., 1970), and steelhead trout embryo (STE-137; Oncorhynchus mykiss; Fryer et al., 1965) were obtained from J. L. Fryer (Oregon State University, Corvallis, Oregon) and tested for IFN-like activity. The cells were plated onto 96-well microtiter dishes and allowed to grow until they attained an 80% confluent monolayer. All cells were maintained in Minimal Essential Media (MEM) containing 5 or 10% fetal calf serum. At the time of treatment with the IFN inducer, poly I:C (Boehringer-Mannheim), the media was removed and 200 µl of poly I:C (6 µg/ml in MEM-0% FCS) were added to each well. Control

cells received MEM-0% FCS alone. After 24 h, the inducer was removed by two washes for 5 min each with Tris-buffered saline. Following the wash, 100 µl of infectious hematopoietic necrosis virus (IHNV) in MEM containing 5% FCS were added to the cells from a ten-fold dilution series which ranged from $10^{-1}$ to $10^{-6}$. Interferon-like activity was measured by the difference in virus titer in control, untreated cells vs. poly I:C treated cells.

## RESULTS

Differences in the viral induced cytopathic effect (CPE) between poly I:C treated and untreated cells is clearly demonstrated in Figure I.1. In plate A, the darkly staining wells indicate cells that have not been destroyed by virus infection and the apparent viral titer for the IFN-induced cells was $5.57 \times 10^1$ $TCID_{50}$/ml (tissue culture infective dose 50%). In plate B of Figure I.1, the clear wells represent those wells where the monolayer has been destroyed by the virus infection and the viral titer for these control cells was $5.57 \times 10^6$ $TCID_{50}$/ml. Thus, a measure of the IFN-like activity was taken as the difference (protection) between the log of the virus titers for treated and untreated cells, a four log difference in this example.

Four IHNV isolates from different fish species were tested for their sensitivity to the IFN-like activity produced by RTG-2 cells. The isolates included Lake Auke (LA) from sockeye salmon, Round Butte-1 (RB1) from steelhead salmon, 039-82 from rainbow trout, and Coleman-3 (CO3) from chinook salmon (Hsu et al., 1986). The results of these studies are presented schematically in Figure I.2. Each bar represents the mean of three assays which compared the viral titer for a particular IHNV isolate in cells treated and untreated with poly I:C. The difference is plotted as the mean log difference:

Figure I.1. KO6 cells were challenged with the IHNV strain 039-82. This figure illustrates the differences in virus-induced CPE in cells that were treated with polyI:C and control untreated cells. A.) poly I:C, B.) control.

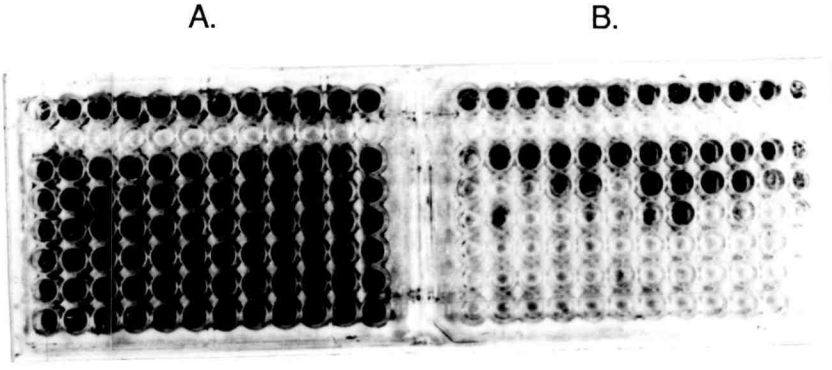


Figure I.1.

Figure I.2. Bar graph representing the mean log difference in $TCID_{50}$/ml results of CPE reduction assays seen in each experimental cell type and IHNV isolate combination between control and poly I:C treated cells. Cell lines and virus isolates are: 1.) STE-137/039-82, 2.) SSE-5/039-82, 3.) KO6/039-82, 4.) RTG-2/039-82, 5.) RTG-2/RB1, 6.) RTG-2/CO3, 7.) RTG-2/LA.

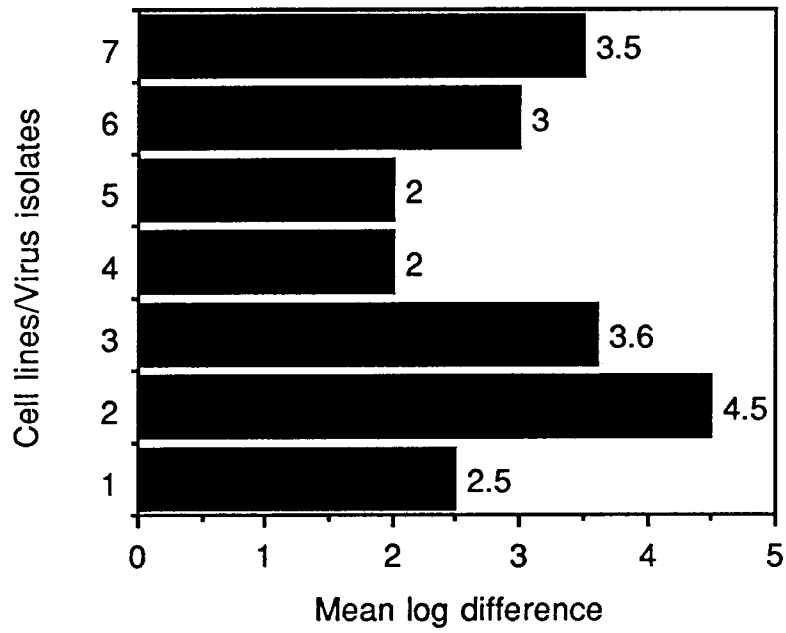
Cell lines/Virus isolates
7
6
5
4
3
2
1
3.5
3
2
2
3.6
4.5
2.5
0
1
2
3
4
5
Mean log difference

Figure I.2.

the larger the number, the more sensitive is the virus to the induced IFN-like activity. The LA virus was the most sensitive isolate and a 3.5 log difference was found in cells treated with the IFN inducer. Nearly as sensitive was the CO3 isolate with a mean log difference of 3. The virus isolates RB1 and 039-82 were less sensitive to the induced activity with only a 2 log reduction in virus titer observed. The titers for each of the isolates were $1 \times 10^6$ $TCID_{50}$/ml for LA; $6.85 \times 10^5$ $TCID_{50}$/ml for RB-1; $5.6 \times 10^5$ $TCID_{50}$/ml for 039-82; and $3.9 \times 10^5$ $TCID_{50}$/ml for CO3.

The same viral isolate, 039-82, was then tested for its sensitivity to IFN-like antiviral activities in the four cell lines. This experiment was designed to determine whether there was any variation in the level of IFN-like activity induced in the different cell lines. Treated SSE-5 cells were the most effective in reducing the titer of 039-82; the mean log difference was 4.5. In the other O. nerka line KO6, the mean log difference was also substantial at 3.6. However, poly I:C treatment of the O. mykiss cell lines STE-137 and RTG-2, did not result in the same level of protection; there was only a 2.5 and 2 log reduction in virus titer, respectively. It is possible that 039-82, which was isolated from rainbow trout, has adapted to grow in cells of this species and is more resistant to the IFN-like activity produced by these cells.

## DISCUSSION

An IFN-like activity in four different cell lines has been induced by treatment with poly I:C, a potent IFN inducer. We observed differences in the sensitivity of a single virus isolate of IHNV, 039-82, to the antiviral activity induced in the four cell lines. The results of this study suggest that SSE-5 cells would be the cell line of choice to study IFN induction with 039-82. There was considerable variation in the level of activity that was induced among

these cells. RTG-2 cells were then tested for their relative ability to produce antiviral activity against four IHNV strains; Lake Auke, Coleman-3, 039-82 and Round Butte-1. We determined that the RTG-2 cell IFN-like substance was the most effective against the Lake Auke isolate of IHNV. The fish IFN-like substance is capable of protecting fish cells against the cytopathic effects of IHNV infection. In the future it will be necessary to determine whether the antiviral activity is due to the poly I:C or the IFN-like substance.

## ACKNOWLEDGEMENTS

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# CHAPTER TWO

## Salmonid in vivo Interferon Production and Evaluation of Homologous and Heterologous Cell Culture IFN Assays

Leslie A. Tengelsen and Jo-Ann C. Leong

Department of Microbiology
Oregon State University
Corvallis, OR 97331-3804

# ABSTRACT

Rainbow trout, coho salmon, and chinook salmon were induced in vivo to produce an IFN-like substance by injection with the IFN inducer, poly I:C. The fish cell lines: rainbow trout gonad-2 (RTG-2), coho salmon embryo-119 (CSE-119), chinook salmon embryo-114 (CHSE-114), and chinook salmon embryo-214 (CHSE-214) were examined for their responsiveness to exogenously applied induced fish serum. The respnsiveness was determined by the presence of antiviral acitivty against the fish rhabdovirus, infectious hematopoietic necrosis virus (IHNV). All cell lines, excluding CHSE-214, responded to the exogenously applied serum from poly I:C treated fish. The cell lines, RTG-2 and CSE-119, were induced by both homologous and heterologous serum to develop an antiviral state. The CHSE-214 cells were completely non-responsive to exogenously applied serum. In addition CHSE-214 cells, unlike CHSE-214 cells, were unable to produce any detectable endogenous IFN-like substance when induced with poly I:C. The IFN-like substance induced in vivo in rainbow trout and in vitro in RTG-2 cells, in response to poly I:C, was examined by western blot analysis. The molecular weight observed was approximately 40 kDa in size from both sources.

## INTRODUCTION

Interferon (IFN) activity induced in fish cells has been reported by a number of investigators. Beasley and Sigel (1967) were the first to report in vitro fish IFN-like activity in tissue cultured grunt fin cells. Subsequently, IFN-like activity has been found in fathead minnow (Oie and Loh, 1971), swordtail (Kelly and Loh, 1973), goldfish (Shea and Berry, 1984) carp (de Kinkelin et al., 1982), and rainbow trout cells (De Sena and Rio, 1975). The production of salmonid fish IFN-like activity has also been documented in vivo by de Kinkelin and Dorson (1973), Dorson et al. (1975), and Eaton (1990). The IFN-like activity of the fish serum substance was characteristic of mammalian type1 IFNs, demonstrating antiviral activity as well as heat and acid pH stability. In addition, Wilson et al. (1983) found that a human β-IFN gene probe hybridized to the DNA of four different teleost species under low hybridization stringencies. This result suggested a fish IFN with homology to human β-IFN (fibroblast) existed. Rainbow trout (Oncorhynchus mykiss), coho salmon (O. kisutch) and chinook salmon (O. tshawytscha) were evaluated for their ability to secrete an IFN-like substance into their blood system following intraperitoneal injections of the IFN inducer, poly I:C. This report describes the development of an in vitro assay for evaluating salmonid fish IFN-like activity. In addition we examine the molecular weight of the rainbow trout in vivo and in vitro induced IFN-like substance by western blot analysis.

## MATERIALS AND METHODS

### Fish

Chinook salmon, coho salmon, and rainbow trout, which weighed on average 35g, were held at the Salmon Disease Laboratory at Oregon State

University. Each fish was injected intraperitoneally with 40 µg/g of poly I:C in 100 µl of sterile STE (10 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM EDTA). Control fish were injected with 100 µl of STE alone. Fish were returned to their tanks and held for three days at 12°C in specific pathogen free (SPF) water. Previous studies (Eaton, personal communication; de Kinkelin and Dorson, 1973) determined that fish IFN was maximally produced at 3 days post-injection so the experiments described here were all based on IFN activity detected three days post-injection. At three days post-injection, fish were lightly anesthetized in benzocaine and bled from the tail. The serum was separated from the red blood cell clot by microcentrifugation for 5 min and then treated at 56°C for 30 min to destroy endogenous complement. The serum samples were stored at 4°C (de Kinkelin and Dorson, 1973).

IFN Assay

Rainbow trout gonad-2 cells (RTG-2) cells were examined for their ability to mount an antiviral response when exposed to homologous and heterologous IFN-like substances produced by rainbow trout, coho salmon and chinook salmon. Additionally, coho serum was tested on cultured coho cells, CSE-119. Rainbow trout serum was also tested on two chinook cell lines, CHSE-114 and CHSE-214, for IFN-like activity. All cells used were originally received from C. Lannan at the Mark Hatfield Marine Science Center, Newport, Oregon. Cells were seeded in 96-well microtiter plates to confluency. Fish serum samples from above were serially diluted 2-fold to 1:2450 in minimum essential media (MEM) containing no fetal calf serum (0% FCS). Cell monolayers were washed one time with MEM-0% FCS and exposed to the serum dilutions. The diluted serum was kept on the monolayers for 24 h at 18°C. Subsequently, the cells were rinsed once

withTris-buffered saline and exposed to 100 µl of a solution containing a constant 5.6 x $10^5$ $TCID_{50}$/ml (tissue culture infective dose-50% per ml) of IHNV, 039-82 isolate, for 1 h. Following adsorption, the virus was removed and the cells were overlayed with MEM containing 5% FCS. The cells were observed for cytopathic effect (CPE) which was seen as a rounding up and subsequent loss of cells from the monolayer. The assay was read at 10-14 days for most of the cell lines with the exception of CSE-119 which required 18 days. Average IFN units were calculated as follows: the reciprocal of the serum dilutions which yielded 50% reduction in CPE when compared to controls, were recorded as units of IFN-like activity.

Induction with Poly I:C

The CHSE-114 and CHSE-214 cells are mixed fibroblastic cells. It was of interest to determine which chinook cell line, if any, would be both responsive to exogeously applied IFN and also be capable of producing their own endogenous IFN upon induction. Cells were grown to 80% confluency in 96-well microtiter dishes. Cells were maintained in MEM-5% FCS with penicillin, streptomycin, and L-glutamine. The media was removed and replaced with MEM-0% FCS containing 10 µg/ml poly I:C in the experimental groups or media alone in the control groups. Plates were held at 18°C for 1 h and the media was then removed. The cell layer was washed twice with sterile phosphate buffered saline (PBS) and replenished with MEM-5% FCS containing a ten-fold serial dilution series which ranged from $10^{-1}$ to $10^{-6}$ of the Round Butte-1 isolate of IHNV. The plates were then observed for the presence of CPE for 10-14 days. Experiments were done in triplicate. IFN-like activity was measured as described above.

Western Blot

Cultured rainbow trout cells (RTG-2) were induced, in the presence of poly I:C, to secrete an IFN-like substance into the surrounding culture media. The fish IFN-like protein was then examined for antigenicity and size by western blot analysis.

The supernatants were prepared according to the protocol of Frances and Lehman (1989). Two 150 cm2 disposable tissue culture flasks (Corning) per experimental condition were seeded with RTG-2 cells and grown to 80% confluency. The cells were then washed with sterile phosphate buffered saline (PBS) twice and re-fed with 30 mls of MEM-0% FCS containing 50 ug/ml poly I:C and 200 ug/ml DEAE-dextran. The control flasks received MEM-0% FCS. The cells were held at 18°C for 2 h. Cells were then washed two times with sterile PBS and re-fed with 10 mls of MEM-0% FCS containing no inducer and held at 18°C for 18 h. Supernatant was then placed in sterile 15 ml conical tubes and clarified by centrifugation for 15 minutes at 3000 rpm. The supernatant was collected in fresh 15 ml conical tubes.

The induction of rainbow trout fish serum was as described above. The serum was treated in the same manner as the cell supernatants from this step forward.

To precipitate the proteins contained within both the supernatant and the fish serum samples, 800 µl of -20°C methanol was added to 500 µl of sample in a 1.5 ml eppendorf tube, as described by Wessel and Flugge (1984). The samples were then held at -20°C for 30 minutes and subsequently microfuged for 15 minutes at 15,000 rpm. The sample supernatants were decanted and the pellets subsequently washed once with 800 µl of fresh cold methanol. The tubes were microfuged for 1 minute and

the supernatants again decanted. The pellets were briefly dried and resuspended in 15 µl of water each. Duplicate serum pellets of 15 µl each were made; one set was clarified by a 5 min microcentrifugation step at 15,000 rpm while the second set was left unclarified. The protein concentration was determined by the BioRad protein assay (Bradford, 1976). The proteins were aliquoted into 10 ug samples and fractionated by denaturing SDS-polyacrylamide gel electrophoresis (Laemmli, 1970; Maniatis et al., 1982). The proteins were then transferred to a nylon membrane in the presence of Towbin buffer for subsequent western blot analysis (Maniatis et al., 1982).

The western immunoblot was carried out as follows: The nylon membrane was soaked in PBS/tween for 30 minutes (0.05% Tween-20 in PBS), washed for 30 minutes in PBS/Brij (0.1% Brij-58 in PBS), and subsequently washed for 5 minutes in water. The primary antibody, MONA, was a polyclonal anti-α–, anti-β-IFN sheep-antimouse antibody provided by Dr. Marasco (Pennsylvania Medical College, King of Prussia, PA). The primary antibody was originally produced by Paucker and Dalton (1980). The primary antibody was diluted 1:500 in NETG/NP-40 (0.05% NP-40 in 1x NETG: 5x NETG =750 mM NaCl, 25 mM EDTA, 250 mM Tris pH 7.4, 1.25% gelatin) and was applied to the nylon blot for 9 h. The blot was then washed 3x, 1 min each time in 1/100 NETG/TX/SDS (NETG, 0.5% Triton X-100 and 0.1% SDS), 2x 5 min in 1x NETG/TX/SDS, 3x in 1/100 dilution of NETG/TX/SDS, and 2x 5 min in water. The second antibody used was rabbit anti-goat antisera conjugated to the colorimetric substance horse radish peroxidase (HRP) (Boehringer-Mannheim). A 1:1000 dilution of the second antibody in NETG/NP40 was applied to the blot for 1.5 hours. The blot was

then washed as described above and developed. To develop the color reaction, 10 mg of the horse radish peroxidase substrate, 4-chloro-1-napthol (4CN) (Sigma), was initially mixed with 1 ml of ethanol and then combined with 100 mls of water and 100 µl of hydrogen peroxidase. Following the last wash step above, the water was removed and the developement solution was added to the blot. When the color change had gone to completion the reaction was stopped with water. The blot was then air-dried and photographed.

## RESULTS

Rainbow trout cells (RTG-2) responded to homologous and heterologous serum by demonstrating enhanced antiviral activity. The control uninduced and experimental poly I:C induced rainbow trout, coho salmon, and chinook salmon fish serum was applied to RTG-2 cells in tissue culture and the results are shown in Table II.1. Rainbow trout serum IFN-like activity varied between individual fish samples (320 U/ml to 1920 U/ml) when tested on homologous RTG-2 cells. RTG-2 cells also responded to both coho and chinook salmon heterologous serum (Table II.1). Control serum samples possessed undetectable amounts of activity in all cases except one where 160U/ml of activity were found. Coho serum contained an apparent 320 to 640 U/ml of activity while chinook serum demonstrated between 240 and 480 U/ml on RTG-2 cells. Table II.2 displays the results of homologous coho salmon serum on coho CSE-119 cells ranging from 320 U/ml to 1280 U/ml between individuals tested. The untreated fish possessed IFN background activity from 0 to 160 U/ml.

The chinook cell lines CHSE-114 and CHSE-214 differed in their responses to exogenously applied IFN (Table II.3). CHSE-214 cells

Table II.1. Assays for fish serum IFN on RTG-2 cells. Fish were treated with 40 µg/g poly I:C by intraperitoneal injection. Fish serum was harvested and analyzed for the presence of IFN-like activity. The IFN units were calculated as the reciprocal of the serum dilution which produced a 50% reduction in CPE when cells were challenged with $5 \times 10^2$ $TCID_{50}$/ml units of IHNV. IFN assays were carried out in triplicate.

A. Poly I:C Treated Fish:

| Rainbow Trout | | Coho Salmon | | Chinook Salmon | |
|---|---|---|---|---|---|
| Fish # | IFN units | Fish # | IFN units | Fish # | IFN units |
| 1 | 480 | 1 | 240 | 1 | 240 |
| 2 | 640 | 2 | 480 | 2 | 480 |
| 3 | 640 | 3 | 640 | 3 | 480 |
| 4 | 320 | 4 | 320 | 4 | 320 |
| 5 | 1280 | | | | |
| 6 | 1066 | | | | |
| 7 | 1280 | | | | |
| 8 | 960 | | | | |
| 9 | 1920 | | | | |
| 10 | 1280 | | | | |

B. Control, Untreated Fish:

| Rainbow Trout | | Coho Salmon | | Chinook Salmon | |
|---|---|---|---|---|---|
| Fish # | IFN units | Fish # | IFN units | Fish # | IFN units |
| 1 | 0 | 1 | 0 | 1 | 0 |
| 2 | 0 | 2 | 0 | | |
| 3 | 160 | | | | |
| 4 | 0 | | | | |

Table II.1.

Table II.2. Assay for fish serum on CSE-119 cells. Fish were treated with 40 µg/g poly I:C by intraperitoneal injection. The IFN units were calculated as the reciprocal of the serum dilution which produced a 50% reduction in the CPE when the cells were challenged with 5 x $10^2$ $TCID_{50}$/ml units of IHNV. IFN units represent the average of 3 assays. Because the coho salmon cells CSE-119 are very resistant to IHNV induced CPE, these assays were held for 18 days before the serum IFN titer was determined.

A. Poly I:C Treated Fish:

| Coho Salmon | |
|---|---|
| Fish # | IFN Units |
| 1 | 1280 |
| 2 | 1280 |
| 3 | 640 |
| 4 | 320 |
| 5 | 320 |

B. Control, Untreated Fish:

| Coho Salmon | |
|---|---|
| Fish # | IFN Units |
| 1 | 160 |
| 2 | 0 |
| 3 | 0 |

Table II.2.

Table II.3. Assay for fish serum IFN on two chinook salmon cell lines; CHSE-114 and CHSE-214 cells. Fish were treated with 40 μg/g poly I:C by interaperitoneal injection. The IFN units were calculated at the reciprocal of the serum dilution which produced a 50% reduction in the CPE when the cells were challenged with 5 x $10^2$ $TCID_{50}$/ml units of IHNV. IFN assays were carried out in triplicate.

A. CHSE-114:

| Rainbow Trout | | Coho Salmon | | Chinook Salmon | |
|---|---|---|---|---|---|
| Fish # | IFN units | Fish # | IFN units | Fish # | IFN units |
| 1 | 1280 | 1 | 240 | 1 | not tested |
| 2 | 640 | | | | |
| 3 | 320 | | | | |
| 4 | 320 | | | | |

B. CHSE-214:

| Rainbow Trout | | Coho Salmon | | Chinook Salmon | |
|---|---|---|---|---|---|
| Fish # | IFN units | Fish # | IFN units | Fish # | IFN units |
| 1 | 0 | 1 | 0 | 1 | 0 |
| 2 | 0 | 2 | 0 | 2 | 0 |
| 3 | 0 | 3 | 0 | 3 | 0 |
| 4 | 0 | 4 | 0 | 4 | 0 |
| 5 | 0 | 5 | 0 | 5 | 0 |
| 6 | 0 | 6 | 0 | | |
| 7 | 0 | 7 | 0 | | |
| 8 | 0 | | | | |

Table II.3.

Figure II.1. Comparison of cell line ability to reduce IHNV infection titers following poly I:C induction of IFN-like activity. 1.) CHSE-214 (controls), 2.) CHSE-214 (poly I:C-induced), 3.) CHSE-114 (controls), 4.) CHSE-114 (poly I:C-induced). All cultures were infected by the Round Butte-1 strain of IHNV.

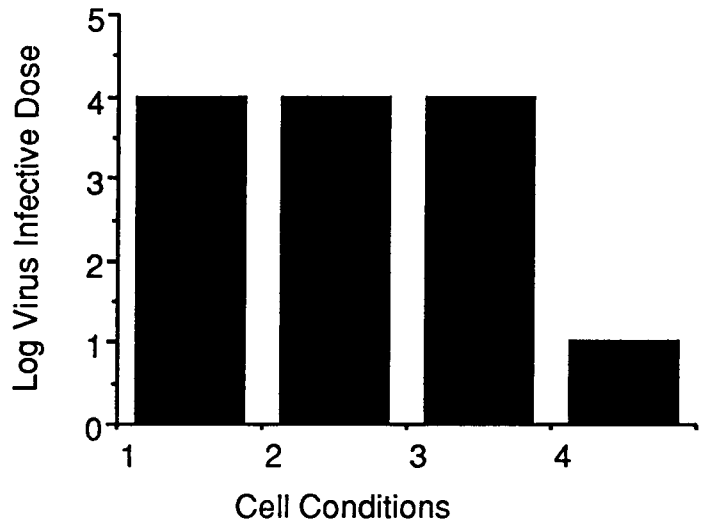



Figure II.1.

Figure II.2. Western blot analysis of proteins found in induced and uninduced rainbow trout serum and cultured rainbow trout cell (RTG-2) supernatants. 1.) RTG-2 cell supernatant; uninduced, 2.) poly I:C/DEAE-dextran induced RTG-2 cells, 3.) control fish serum, clarified, 4.) poly I:C induced fish serum, clarified, 5.) control fish serum, 6.) poly I:C induced fish serum, 7.) MEM-O% control, no cells, M.) prestained molecular weight markers. The arrowhead marks the cross-reactive protein in the experimental lanes.

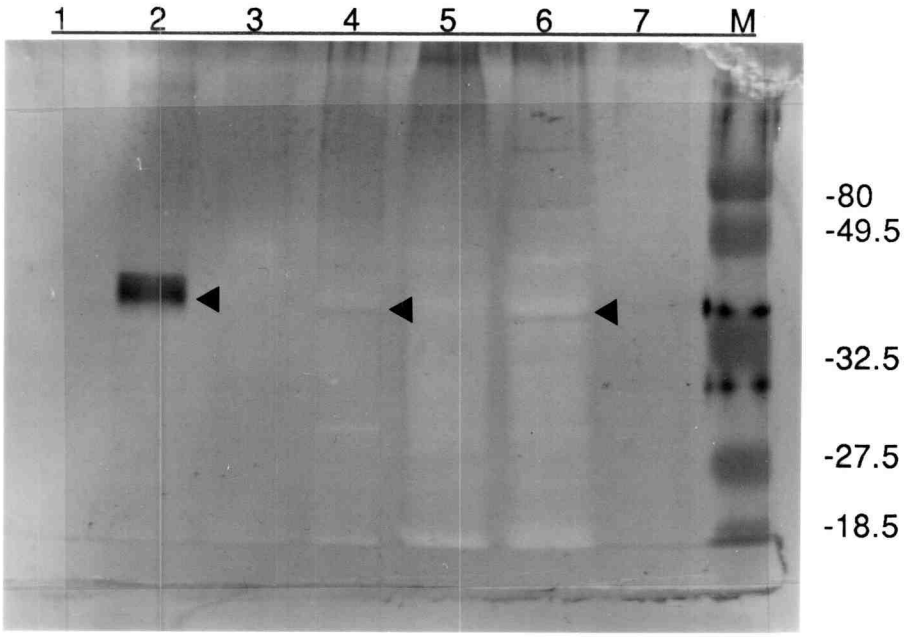



Figure II.2.

demonstrated no detectable change in antiviral activity when treated with either rainbow trout, coho, or chinook serum. CHSE-114 cells responded well, with IFN-like titers ranging from 160 U/ml to1280 U/ml with the heterologous rainbow trout serum and 1280U/ml from the coho individual tested. Chinook serum was not tested.

Endogenous chinook cell IFN-like activity was examined in two chinook cell lines; CHSE-114 and CHSE-214. Activity was measured as a 50% reduction in IHNV infectivity (tissue culture infective dose or $TCID_{50/ml}$). Figure II.1 contains a bar graph depicting the reduction of IHNV (Round Butte-1 strain) for both cell lines. When the two chinook salmon cell lines, CHSE-114 and CHSE-214, were compared for IFN production upon poly I:C induction there was a marked difference in the titer of the same dilutions of IHNV plated on the cells; $3.2 \times 10^4$ $TCID_{50}$/ml of CHSE-214 vs $4.7 \times 10^1$ on CHSE-114.

Western blot

Proteins precipitated from both control uninduced and poly I:C induced rainbow trout serum and cultured rainbow trout cell (RTG-2) supernatants were fractionated by SDS-PAGE and analyzed by western immunoblot. Figure II.2 represents the banding pattern acheived. A 40 kDa broad protein band was found only in the induced serum and cell supernatant lanes. The colorimetric change was more pronounced in the cell supernatant lane than in the fish serum lanes. It may be that the actual IFN-specific bands were not as well represented in the serum samples because the amount of total protein is greater in serum than in MEM-0% FCS.

# DISCUSSION

Infectious hematopoietic necrosis virus (IHNV) is a fish rhabdovirus utilized in these studies and represents an economically important pathogen. Bukholm et al. (1989) demonstrated that IFN treatment reduced endocytosis of the mammalian rhabdovirus, vesicular stomatitis virus (VSV). It has been demonstrated that IFN can restrict the growth of VSV at many levels in the virus replication process. These stages include penetration, primary transcription, cap methylation, protein synthesis and assembly (Bukholm, 1989). However, it has been reported that there are variations in cell line responsiveness to the receptor-mediated IFN induction of these antiviral activities. For example, mouse L-cell IFN can block VSV at the level of protein synthesis, and human L-Youngner cell IFN can block VSV uptake and subsequent primary transcription. Human L-Lewis cells are readily infected with VSV, however maturation of the virus is blocked by the activities of IFN (Whitaker-Dowling, 1983). Thus, we were interested in examining more closely IFN production by three salmonid species: Oncorhynchus mykiss, O. kisutch, and O. tshawytscha, and the variability of expressed antiviral activity between available established cell lines in response to exogenously applied homologous and heterologous fish serum, against the fish rhabdovirus IHNV.

In vitro assay of antiviral activity in fish serum has been determined by Eaton (1990) not to be due to any antibody mediated activity, but due to some other inducible substance in fish serum. This substance has been evaluated in the past, using crude extracts (Dorson et al., 1975; De Sena and Rio, 1975), for biophysical characteristics typical of mammalian IFNs and appears to possess many similar characteristics to known IFNs.

Mammalian IFNs are measured with respect to international IFN standard units which researchers can obtain and use as a standardization tool. There are no international units to standardize fish IFN-like activity; thus, all IFN-like units measured and described here are based on a relationship between control and experimental observations. The predicted IFN units in each figure represent an average of the IFN units found in assays that were conducted a minumum of two and a maximum of three times for each fish serum sample. No assay result varied more than one dilution in activity for each fish sample over the duplicate assay.

As the fish available were not clones, it was of interest to observe the individual variation in IFN production between individuals. To test for the presence of IFN in the serum of rainbow trout, coho salmon, and chinook salmon, a fish cell cytopathic-reduction in vitro assay was tailored using RTG-2 ,CSE-119 , CHSE-114 and CHSE-214 cells. It is clear that a rainbow trout IFN-like substance is active on RTG-2 cells as well as CHSE-114 cells against IHNV. The coho salmon IFN-like substance is active on RTG-2, CHSE-114, and CSE-119 cells. In addition, the chinook IFN-like substance could be assayed on RTG-2 cells. The data suggests that the salmonid fish species examined here secrete a poly I:C-inducible IFN-like substance into their bloodstream which segregates with the serum fraction. These IFN-like substances appear to have broad species specificity, but that there is a large variation among individuals and across species.

Previous investigators have recorded the inablility of the CHSE-214 line to respond to exogenous IFN (MacDonald and Kennedy, 1979; Bill Eaton, personal communication). It was of interest to determine whether it was the species of fish or rather the particular cell line, CHSE-214; routinely used in

cell culture, which was unable to produce endogenous IFN or respond detectably to exogenously applied IFN. In this study, we were able to demonstrate that the two chinook cell lines CHSE-114 and CHSE-214 varied dramatically in their abilities to both respond to exogenously applied IFN and produce their own endogenous IFN. CHSE-114 cells were able to both mount an anti-IHNV response in the presence of exogenously applied IFN and were able to successfully produce endogenous poly I:C inducible IFN. CHSE-214 cells were not able to do either task. Clearly, there is some mechanism in the CHSE-114 line that is missing from the CHSE-214 cell line. We have confirmed the findings of Eaton (1990) that CHSE-214 cells are refractory to induction of IFN activity with poly I:C and have also demonstrated that the alternatively available chinook cell line, CHSE-114 could be a viable choice for chinook IFN studies. It was demonstrated here that it was not the chinook species but rather the particular cell line isolate that was resistant or unable to respond to the inducing activities of IFN.

The polyclonal antibody, MONA, against mouse type 1 IFNs, is able to cross-react in a limited manner with human IFN (Paucker and Dalton, 1980). Classically, antisera raised against IFNs from one species do not readily cross-react with IFNs produced in other species. There are exceptions, as anti-mouse IFN will cross-react with human IFN (Havell, 1979). It was of interest to test whether MONA could detect IFN from both poly I:C induced rainbow trout serum and poly I:C induced rainbow trout cell culture supernatants by western blot.

In this study a strong doublet of approximately 40 kDa was found only in the induced cell supernatant lane. A single band of approximately 40 kDa was found in both the clarified and unclarified induced serum lanes. No

bands were found in any of the control lanes. It is unclear that this represents fish IFN at this point as the size is different from those reported for other IFNs which generally range between 18 and 30 kDa. Fish IFN has been reported to range in size from 26 kDa to 94 kDa (Dorson et al., 1975; De Sena and Rio, 1975) therefore its actual size remains a mystery. The evidence presented here demonstrates the presence of an inducible protein which cross-reacts with the polyclonal sheep anti-mouse antibody, MONA, raised against both type 1 IFNs; $\alpha$ and $\beta$. This cross-species immunoreactivity is uncommon, but may demonstrate the presence of a specific inducible fish IFN-like protein.

# CHAPTER THREE

## Molecular Biology of Salmonid IFN

Leslie A. Tengelsen and Jo-Ann C. Leong

Department of Microbiology

Oregon State University

Corvallis, OR 97331-3804

## ABSTRACT

Fish have been reported to possess proteins with IFN-like activities (Oie and Loh, 1971; deKinkelin and Dorson, 1973; De Sena and Rio, 1975; Hedrick and Fryer, 1981). Hybridization analysis of vertebrate gene families using α- and β-IFN DNA as heterologous probes demonstrated the presence of IFN gene-homologs within a variety of fish species. We report the presence of a β-IFN type gene family in rainbow trout (Oncorhynchus mykiss), coho salmon (O. kisutch) and chinook salmon (O. tshawytscha). In addition, we report molecular evidence supporting the presence of a fish β-IFN-like gene by RNA blotting and polymerase chain reaction (PCR). Fish IFN-like RNA transcription examined here appears to be inducible in the same manner as bovine and human β-IFN; in the presence of the IFN inducer poly I:C, DEAE-dextran and cycloheximide.

# INTRODUCTION

Interferons, as described by Isaacs and Lindenman in 1957, are proteins and glycoproteins that inhibit the growth of a large number of viruses. The antiviral activities can effect both RNA and protein synthesis. Since 1957, IFNs have also been shown to possess antimicrobial activities, immune modulatory effects, and cell growth regulatory activities implicated in the reduction of some forms of cancer (Jounger and Stinebring, 1964; Pestka, 1987).

Interferons have been found in all vertebrates described to date (Wilson, 1983). Some preliminary molecular information on fish IFN has been available since 1983. Wilson et al. (1983) carried out a hybridization survey of vertebrate IFN gene families using human α- and β-IFN DNA as heterologous probes. Wilson et al. found that perch (Perca fluviatilis), minnow (Phoxinus phoxinus), dace (Leuciscus leuciscus), and stone loach (Neomacheilus barbatulus) all possessed weakly hybridizing genomic sequences, under low stringency conditions. Wilson et al. postulated that the "bony fishes" may possess only one IFN gene of the α/β type, due to the hybridization frequency. Dehlin et al. (1987) described an IFN-like gene in zebrafish. The investigators were able to isolate three positive plaques from a 600,000 plaque zebrafish genomic library by screening with the coding regions from both the human $\alpha_1$-and $\beta_1$-IFN. The frequency of hybridization in this case supported the possibility of a single copy gene. No other molecular information has been published to date on salmonid IFN.

The Investigation reported here describes the presence of IFN-like sequences within three Oncorhynchus genomes: O. mykiss, O. kisutch, and O.

tshcwytscha. In addition, we report preliminary studies on the conditions for induction of this fish IFN-like gene.

## MATERIALS AND METHODS

### Preparation of Genomic DNA

All genomic DNA was prepared from fresh frozen liver as described in 'Current Protocols in Molecular Biology' (1987) and by Gros-Bellard et al. (1972). Bovine liver was obtained from the Oregon State University Meat Lab. Fish were obtained from the Salmon Disease Laboratory at Oregon State University. Bovine liver was acquired and quick-frozen in powdered dry ice. Salmonid liver also was quick-frozen in dry ice following dissection. Salmon species sampled included rainbow trout (Oncorhynchus mykiss), chinook salmon (O. tshawytscha), and coho salmon (O. kisutch). In all cases, the gallbladder was removed from the liver tissue as it contains high levels of degradative enzymes. One g of liver from each source provided the starting material for the isolation of genomic DNA.

Frozen tissue was ground to a fine powder in a chilled mortar and pestle. Protein was digested away by resuspending the powdered tissue in 1ml of digestion buffer/100 mg of tissue (100 mM NaCL, 10 mM Tris-Cl (pH 8.0), 25 mM EDTA (pH 8.0), 0.5% SDS, and 0.1 mg/ml proteinase K) and shaking at 50°C for 18 h in a sterile 30 ml Corex tube. This released the nucleic acid creating a highly viscous solution. Nucleic acid was then isolated from the digested tissue by phenol-chloroform-isoamyl alcohol (IAA) (25:24:1) extraction. The resulting aqueous layer contained DNA. The aqueous layer was then transferred to a new Corex tube and combined with 1/2 volume of 7.5 M ammonium acetate (pH 7.5), and 2 volumes of chilled 95% EtOH. The

long strands of precipitated DNA were removed with a sterile glass rod. The DNA was placed in a new sterile 15 ml Corex tube and rinsed with 2 ml of chilled 70% EtOH. The tubes were centrifuged for 5 min at 5000 rpm and the EtOH was then removed. The DNA pellet was air dried and gently resuspended in TE buffer. The DNA was stored at 4°C.

Genomic Southern Blot

Ten µg of genomic DNA was digested with either BamH1 or Hind III for Southern blot analysis (Southern, 1975). Genomic DNA from bovine, rainbow trout, chinook, and coho DNA were digested with restriction endonucleases to completion at 37°C. DNA was then phenol-chloroform extracted, chloroform extracted, and ethanol precipitated in the presence of 0.3 M NaOAc (pH 5.2). The resulting pellet of DNA was then resuspended in TE and 1x agarose gel loading buffer (6x: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll type-400 in water), heated to 65°C for 5 min, chilled on ice for 2 min, and separated through a 0.8% agarose gel. The DNA was stained with ethidium bromide, 0.5 µg/ml, and photographed with a ruler to determine a size/distance ratio. The gel was then placed in a denaturation bath (0.5 M NaOH, 1.5 M NaCl) and gently rocked for 30 min. The gel was subsequently neutralized for 30 min in a neutralization bath (0.5 M Tris (pH 7.4), 3 M NaCl).

The DNA was transferred to a nylon filter (Nytran™; Schleicher and Schuell) for hybridization analysis. The DNA transfer was performed as described by Maniatis et al. (1982) in the presence of 10x SSC. Following transfer, the blotting paper was gently removed, the filter was then lifted off the flattened gel and air-dried for 30 min. The membrane was then baked at 80°C under vacuum for 2 h and prehybrided in hybridization buffer at 42°C (5x SSC, 40% formamide, 0.1% N-Lauryl sarcosine, 0.02% SDS, and 4%

GENIUS™ blocking reagent (Boehringer Mannheim)) for 1 h.

Human $\beta_1$-IFN Probe Preparation

Human $\beta_1$-IFN (received from Dr. David Leung, Genentech) was subcloned into pUC18 at the Pst 1 site (Figure III.1). For hybridization purposes, the Pst 1-Bgl II fragment of human $\beta_1$-IFN was gel isolated from a 1.3% low melting temperature preparative agarose gel and purified from the agarose by GENECLEAN II™, according to manufacturers instructions (Bio101, Inc.). The 600 bp fragment was then radiolabeled with a $^{32}$P-dCTP using a random priming/klenow kit (Boehringer-Mannheim), to a total of $1 \times 10^9$ counts per min/μg. The labeled fragment was separated from free, unincorporated nucleotides by gel filtration through a Sephadex G-50 Nick column (Pharmacia). The labeled fragment was heated in a boiling water bath for 5 min and added to a 50 ml conical tube containing 25 ml of 42°C hybridization buffer (the same as the pre-hybridization mixture). The prehybridization mixture was removed from the Seal-a-Meal™ bag above, and replaced with the radioactive hybridization mix. The bag was resealed and held at 42°C for 18 h. The membrane was washed twice with 2xSSC:1% SDS at room temperature for 15 min each time, followed by a wash in 1x SSC:0.1% SDS at room temperature and finally, a 15 min wash with 0.1xSSC, 0.1% SDS at 42°C. The membrane was then placed between Saranwrap™ and exposed to XAR-X-ray film.

RNA

In vitro RNA was harvested from rainbow trout gonad-2 fibroblast cells (RTG-2) following the production of IFN-like RNA under a variety of inducing conditions. RTG-2 cells were obtained from C. Lannan (Mark Hatfield Marine Science Center, Newport, Oregon) and were maintained in minimal essential

media containing 5% fetal calf serum (MEM-5%), penicillin-streptomycin (5 ml stock solution/500 ml), L-glutamine (5 ml of 200 mM/500 ml), and sodium bicarbonate (pH 7.5) at 18°C. Cells were grown to 95% confluency in T-150 $mm^2$ flasks in 35 ml of media. The inducer, poly I:C, was prepared from a 2.5 mg/ml water, filter-sterilized stock; cycloheximide was prepared from a 10 mg/ml water filter-sterilized stock; and DEAE-dextran was prepared from a 10 mg/ml water filter-sterilized stock. Each inducing condition was carried out in duplicate and the isolated RNA was subsequently pooled prior to analysis. Cellular IFN-like RNA was induced as follows. At time zero ($T_0$), when cells had reached 95% confluency as described above, media was removed from the flasks and the cells washed twice with 20 ml of MEM-0% FCS. The media was replaced with 20 ml of MEM-0% FCS according to the following set of parameters: 1.) MEM-0% FCS, 2.) MEM-0% FCS + 10 μg/ml poly I:C, 3.) MEM-0% FCS + 10 μg/ml poly I:C + 200 μg/ml DEAE-Dextran, 4.) MEM-0% FCS + 50 μg/ml cycloheximide (CHX), 5.) MEM-0% FCS + 10 μg/ml poly I:C + 50 μg/ml CHX, and 6.) MEM-0% FCS + 10 μg/ml poly I:C + 200 μg/ml DEAE-Dextran + 50 μg/ml CHX. The flasks were placed in an 18°C incubator for 3 h (for conditions A, B, and C) or 5 h for all conditions (duplicates of A, B, and C were run for the longer time). Following the incubation time, the media was removed. The monolayers were washed twice, as described above, with MEM-0% FCS. RNA was prepared using the guanidinium isothiocyanate, phenol, β-mercaptoethanol RNAzol kit (CINNA/BIOTECX). Two ml of RNAzol were added to each monolayer which, according to the manufacturer's instructions, liberated the cellular RNA. Duplicate flask contents were pooled and RNA precipitated with an equal volume of cold isopropanol at -20°C for 45 min. RNA was pelleted at

12,000 rpm for 15 min and washed once with 5 ml of 75% EtOH in DEPC-treated water to inhibit any RNAses present. The pellet was briefly air-dried and resuspended in 150 µl of DEPC-treated water containing 1 mM EDTA. RNA sample concentrations were determined by $O.D._{280}$ readings. Aliquots of 50 µg each were stored in the presence of 0.3 M NaOAc (pH 5.2) and 95% EtOH. Samples were analyzed for their content by dot blot.

Dot blot analysis was performed as described (Maniatis et al., 1982). Samples were microfuged to pellet the RNA from its storage condition in EtOH, and washed once with 100 µl of 75% EtOH/DEPC-treated water. The samples were briefly air dried and resuspended in 10 µl of DEPC-water. RNA was immobilized onto Nytran™ (Schleicher and Schuell) with the aid of a dot blot manifold suction device (Schleicher and Schuell). Nytran™ was wetted briefly in DEPC-treated water and then soaked in 20X SSC/DEPC-water for 1 h at room temperature. Concurrently, the manifold suction device was cleaned with 0.1N NaOH/DEPC-water and then rinsed in DEPC-water. Two sheets of Whatman 3mm filter paper were prewetted with 20X SSC/DEPC-water. The manifold suction device was assembled according to manufacturer's recommendations. The wells were rinsed with 200 µl of 10X SSC/DEPC-water which was passed through the wells by suction. The wells were then refilled with 10X SSC/DEPC-water and allowed to sit while the RNA was prepared for immobilization. The RNA, in 10 µl of DEPC-water described above, was combined with 20 µl of 100% deionized colorless formamide, 7 µl of formaldehyde (37%, the pH must be greater than 4.0), and 2 µl of 20X SSC. This mixture was incubated at 68°C for 15 min and cooled on ice for 5 min. Two volumes (80 µl) of 20X SSC were added to each sample. The 10X SSC which filled the wells was drawn through the

nitrocellulose by suction. The suction was turned off and the RNA was then applied to designated wells within the manifold suction device as shown in Figure III.3. RNA was distributed in 20, 10, and 5 μg aliquots. Samples were drawn onto the membrane by suction and subsequently rinsed by passing 2 ml of 10X SSC through. Once the wells appeared dried, the device was then disassembled and the membrane air dried, sample side up, and subsequently baked for 2 h at 80°C in vacuo. The filter was then probed for the presence of sequences homologous to human $\beta_1$-IFN as described above in the Southern blot section.

In vivo production of IFN-specific RNA was examined. RNA was isolated from two separate size/age groups of fish under control and IFN-inducing conditions, using poly I:C as the inducer. Poly I:C was prepared in filter sterilized STE (10 mM Tris, pH 8.0, 100 mM NaCl, and 1 mM EDTA) from a 10 mg/ml stock solution such that the total dosage was delivered in no more volume than 200 μl/fish. Control fish were injected with STE alone. Poly I:C was delivered as a 50 μg /g body weight dosage injected intraperitoneally into experimental fish from each size group while fish were lightly anesthetized. Experimental group sizes were on average either 15 g or 600 g; 7 fish for the 15 g size group for each the poly I:C and control groups and 3 fish each for the 600 g fish poly I:C and control groups. On day three following injection, the animals were terminated by anesthetization in 10% benzocaine and pithed. The livers were rapidly removed and the gallbladders dissected away from the solid liver tissue to reduce the presence of degradative enzymes. The livers were quick-frozen on dry ice and kept frozen prior to RNA extraction. Liver tissue was pooled from each experimental group and RNA was subsequently extracted by homogenization and RNAzol extraction as described above. The

RNA was stored at -20°C in the presence of 0.3 M NaOAc (pH 4.8), and 2 volumes of 100% ethanol. The yield was approximately 1.5 mg of total RNA per 300 mg of starting tissue.

Northern analysis of O. mykiss RNA was carried out by using a denaturing formaldehyde/agarose gel (Lehrach et al., 1977; Ausubel et al., 1987; Maniatis et al.,1982). RNA samples, kept at -20°C in the presence of 0.3 M NaOAc (pH 4.8), and 2 volumes of 100 % ethanol (see RNA preparation by RNAZOL above), were pelleted by microcentrifugation for 10 min and briefly air dried. The RNA was then resuspended in 20 µl of formaldehyde/formamide loading buffer (Maniatis et al., 1982), heated to 95°C for 2 min to denature the RNA, and loaded into gel wells. A control lane containing ribosomal RNA as markers was loaded in the outside well. The RNA was electrophoresed for 5 h at 75 volts or until the bromphenol blue dye traveled 3/4 of the distance through the gel. The gel was rinsed in several changes of distilled water to remove excess formaldehyde. The gel was then soaked, while gently rocking, in 10x SSC for 45 min. The RNA was then transfered to a nylon membrane (Nytran™, Schleicher and Schuell) in the presence of 20x SSC by the wicking technique previously described for Southern transfers (Maniatis et al., 1982). The filter was not rinsed after transfer. Following 30 min of air-drying, the membrane was baked in vacuo at 80°C for 2 h. The membrane was then hybridized to a radioactive $\beta_1$-IFN probe as described above for Southern hybridization and exposed to film for two weeks.

Polymerase Chain Reaction

Due to the cross-species amplification required of this experiment it was prudent to design flanking oligonucleotides corresponding to regions of

the human $\beta_1$-IFN containing the most contiguous, least degenerative set of codons. An attempt to determine fish codon preference bias was carried out in order to incorporate that information into the final construction of the flanking primers. The three published fish sequences used for this determination were: 1.) a protamine gene (Dixon et al.,1981), 2.) the rainbow trout growth hormone gene (Agellon and Chen, 1986), and 3.) the rainbow trout Mx gene (Staeheli et al.,1989).

Regions were located within the human $\beta_1$-IFN open reading frame using the above parameters. The oligonucleotides #66 and #67 were 21nt long corresponding to 7 amino acids. Both oligonucleotides were synthesized by Dr. R. McParland at the Oregon State University Center for Gene Research/Central Services facility. Both oligonucleotides were subsequently gel purified from a 20% acrylamide gel in the presence of 1x TBBE buffer. Gel purification was carried out by the "crush and soak" method (Maniatis et al., 1982). The major oligonucleotide band was excised by shadowing with a long wave hand held U.V. source. The $O.D._{260}$ was determined for each purified oligonucleotide and the concentration was adjusted to 50 µm. The polymerase chain reaction (PCR) was comprised of 100 ng of template DNA (rainbow trout genomic DNA as prepared in the genomic Southern section), 0.5% NP40, 10% 10x PCR buffer (Promega), dNTPs (2 mM each), 2 µl of a 50 µM solution of each primer, and water to a final volume of 99 µl. The microfuge tube was held at 95°C for 5 min to destroy any endogenous enzyme activity and placed on ice for 1 min. Taq polymerase, the thermostabile DNA polymerase from Thermus aquaticus (Promega), was added to a final concentration of 1 unit and 75 µl of mineral oil was gently overlaid over the reaction mixture. The PCR reaction was then carried out in a

COY™ tempcycler as follows: denaturation at 94°C for 1 min, hybridization at 50°C for 2 min and elongation at 72°C for 5 min for 35 cycles. Magnesium chloride was then added to a final concentration of 10 mM and 2 units of Klenow were added to create a blunt or flush end for blunt-end cloning. The blunt-ended PCR product was then gel-purified from a 1.2% agarose gel by the Geneclean method (Bio101, Inc.). The fragment was subsequently ligated to 15 ng of Sma1 digested pUC18 vector by standard blunt end ligation procedures (Maniatis et al., 1982). The final cloned product was then sequenced by the dideoxy-chain termination method of Sanger (1975) using the Sequenase™ kit (United States Biochemical Company).

## RESULTS

### Genomic Southern

The probe (Figure III.1) hybridized to a major band of 3.9 kbp in bovine DNA digested with BamH1 (Figure III. 2). Rainbow trout digested with BamH1 yielded three bands; 1.0, 2.0, and 4.2 kbp. The same DNA digested with Hind III demonstrated two bands at 4.0 and 4.3 kbp upon hybridization. Chinook DNA of 0.6, 1.65,1.8, and 4.1 kbp hybridized to the human probe when digested with BamH1. Hind III digestion of this DNA yielded 2.2, 3.8, and 4.1 kbp fragments respectively. Coho DNA hybridized at 1.2, 2.4, and 3.8 kbp from BamH1 fragmentation, while the Hind III digestion led to a major hybridizing band at 2.3 kbp and a minor band at 1.7 kbp.

Figure III.1. A schematic representation of pFIF subcloning. The Pst1-Pst1 fragment was subcloned from the tetracycline resistant pBR322 plasmid from Genentech into the Pst1 site of the ampicillan resistant pUC-18 plasmid from Bethesda Research Laboratories. Hybridization analyses were subsequently carried out using the Pst1 to Pst1 fragment shown. A.) signal sequence, B.) coding region, C.) 3' non-coding region.

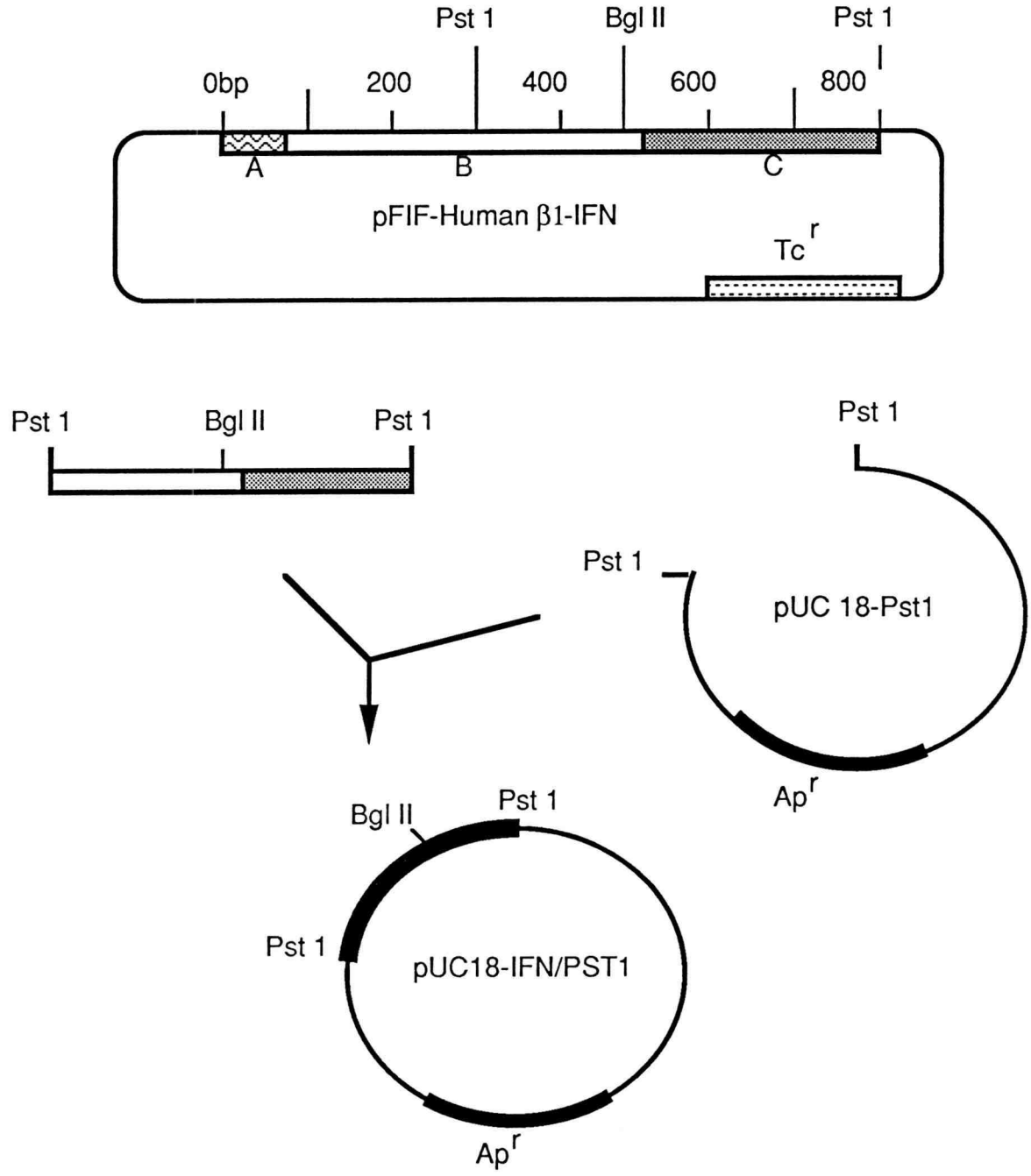

Pst 1
Bgl II
Pst 1
0bp
200
400
600
800
A
B
C
pFIF-Human β1-IFN
Tc r
Pst 1
Bgl II
Pst 1
Pst 1
pUC 18-Pst1
Ap r
Bgl II
Pst 1
Pst 1
pUC18-IFN/PST1
Ap r

Figure III.1

Figure III.2. Genomic DNA from bovine, coho, and rainbow trout was digested with BamH1 and HindIII, separated on a 1% agarose gel in 1x TAE buffer, and probed with human β-IFN gene sequences (see Figure III.1). The resulting banding pattern is shown here. The lane designations are M, molecular weight markers comprised of Lambda DNA digested with HindIII, 1.) bovine/HindIII, 2.) rainbow trout/BamH1, 3.) rainbow trout/HindIII, 4.) chinook/BamH1, 5.) chinook/HindIII, 6.) coho/BamH1, and 7.) coho/HindIII.

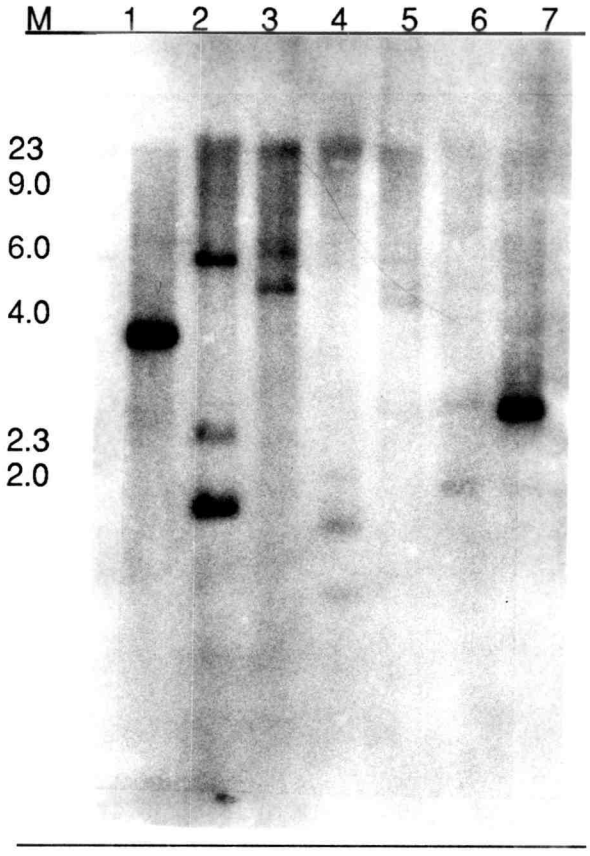



Figure III.2

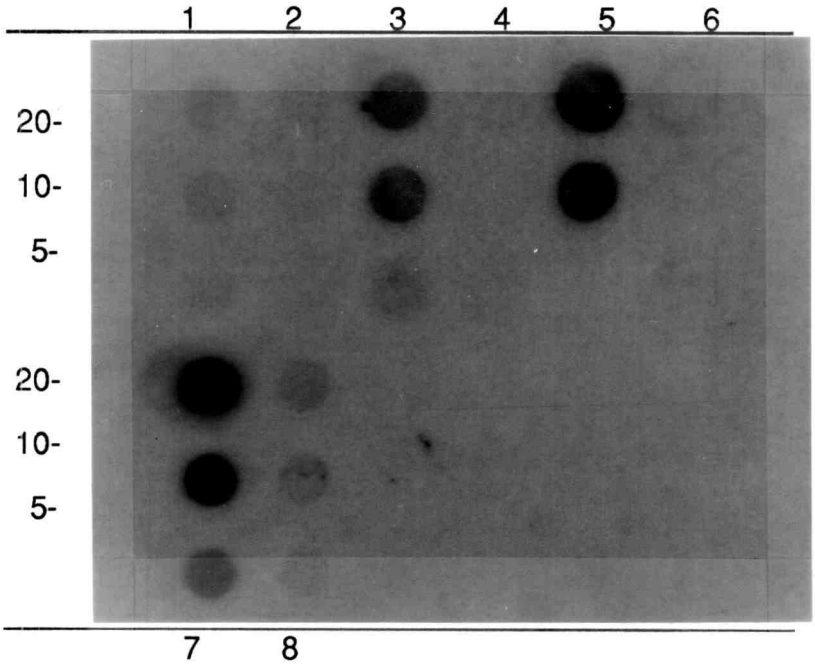
In Vitro RNA Analysis

Cellular inducible IFN-like RNA was shown, by dot blot analysis, to hybridize to human DNA derived from the $\beta_1$-IFN open reading frame. Each induction condition was examined with 20, 10, and 5 µg amounts of RNA spotted onto the filter (Figure III.3). The relative increase in the amount of hybridizable RNA when compared to control uninduced RNA, was quantified by densitometric scanning with a BioRad model 620 densitometer (Figure III.3). Densitometric units of area for each 5, 10, and 20 µg spot were pooled for each condition and averaged. These average scores were then compared to the control pooled score. The results are a fold-yield increase over controls.

Uninduced RNA harvested at either 3 or 5 h post exposure to MEM-0% FCS, as a negative control, possessed no $\beta_1$-IFN specific nucleic acid detectable under these conditions. Hybridization was also negligible following cycloheximide treatment. Rosztoczy et al. (1971) published that DEAE-dextran treatment alone had no effect on RNA induction and was not run here. The RTG-2 cells exposed to poly I:C harvested at 3 and 5 h yielded a negligible increase in detectable RNA over the controls as well. Results indicated that RNA produced in the presence of poly I:C plus the protein synthesis inhibitor cycloheximide was induced 0.3-fold over controls and poly I:C alone. RNA induced by poly I:C/DEAE-dextran harvested at 3 h showed a 2.8-fold increase over controls, while at 5 h that score increased to a 6.8-fold yield. Finally, the combination of poly I:C, DEAE-dextran and cycloheximide yielded the greatest number with a 8.7-fold increase over control RNA at 5 h. Figure III.4 demonstrates graphically the kinetics of fish IFN transcription which, at least at early times post-induction, parallels transcription patterns found when mammalian IFNs are induced.

Figure III.3. A dot blot of RTG-2 cell RNA induced under a variety of conditions is shown here. A. An autoradiograph of the dot blot results is shown here. The designated wells include: 1.) control RTG-2 cells, 2.) poly I:C induced RNA harvested at 3 h post-induction, 3.) poly I:C/DEAE-dextran induced RNA harvested at 3 h post-induction, 4.) poly I:C induced RNA harvested at 5 h post-induction, 5.) poly I:C/DEAE-dextran induced RNA harvested at 5 h post-induction, 6.) cycloheximide treated cellular RNA harvested at 3 h post-exposure, 7.) poly I:C/DEAE-dextran/cycloheximide treated cellular RNA harvested at 5 h post-induction, and 8.) poly I:C/DEAE-dextran/cycloheximide treated cellular RNA harvested at 3 h post-induction. Each condition contains 20, 10, or 5 ug of whole-cell RNA. The blot was probed with the human β-IFN Pst1 fragment (see Figure III.1). B. Quantitation of the dot blot analysis was carried out using the BioRad model 620 densitometer. Quantitation was based on the average of the optical density peak areas for the three dots per condition. The horizontal axis represents induction conditions and the time of RNA harvest post-induction. The vertical axis represents fold induction compared to the control RNA. The lanes are designated 1.) control, 2.) cycloheximide, 3.) poly I:C, 3 h, 4.) poly I:C, 5 h, 5.) poly I:C/DEAE-dextran, 3 h, 6.) poly I:C/DEAE-dextran, 5 h, 7.) poly I:C/DEAE-dextran/cycloheximide, 3 h, and 8.) poly I:C/DEAE-dextran/cycloheximide, 5 h.

A.




B.

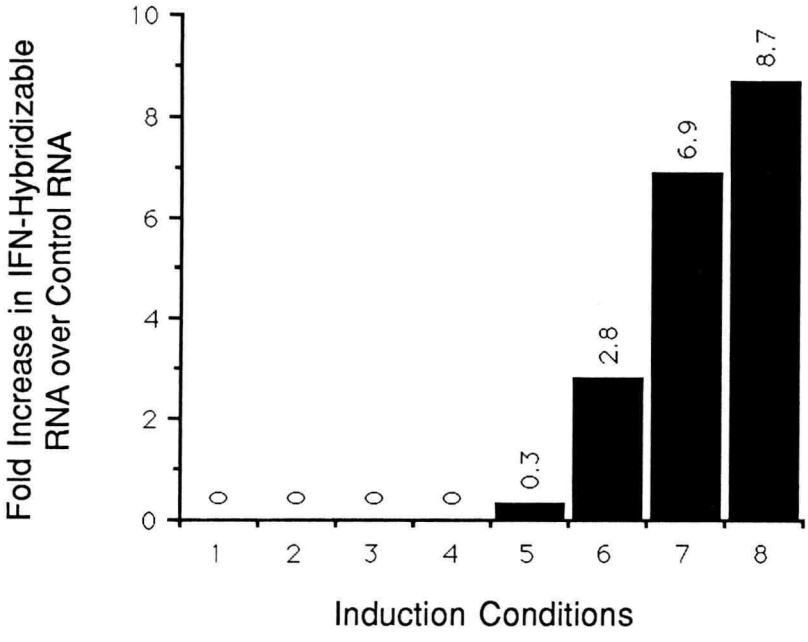



Figure III.3.

Figure III.4. Interferon probe-hybridizable RNA is present at 3 h post-induction and continues to be present at 5 h post-induction. The presence of fish IFN-like RNA at these times post-induction is similar to what has been demonstrated in the mammalian system.

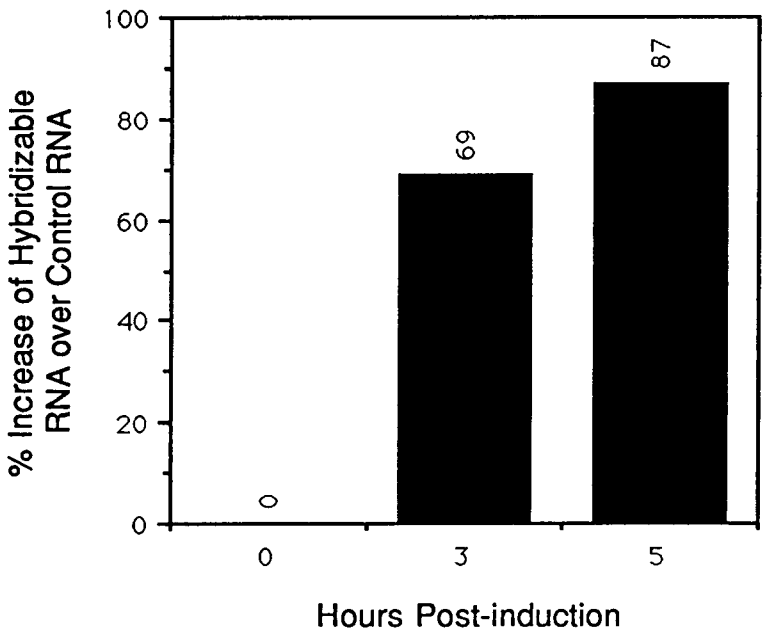

% Increase of Hybridizable RNA over Control RNA
100
80
60
40
20
0
0
69
87
0
3
5
Hours Post-induction

Figure III.4.

In Vivo RNA Production

Northern analysis of the in vivo induced RNA revealed that the 15 g fish-derived RNA hybridized to the human $\beta_1$-IFN probe. Long exposures of the film were necessary to yield an adequate signal from in vivo derived IFN-like RNA. Figure III.5 demonstrates the inducible IFN-like RNA species. Fish of approximately 15 g preferentially produce a 1.4 knt RNA species and sparingly produce a smaller 0.9 knt RNA species. It appears that the more mature 600 g fish preferentially produced the smaller 0.9 knt species and a smaller amount of the larger 1.4 knt species.

PCR

Figure III.6 illustrates codon preference determined by pooling codon usages from the rainbow trout protamine gene (Dixon et al.,1981), rainbow trout growth hormone gene (Agellon and Chen 1986), and the rainbow trout Mx gene (Staeheli et al.,1989). The PCR primers designed acording to codon preference are shown in Figure III.7. Figure III.8 illustrates the PCR product sequence. Similarities were found when the PCR product was aligned with the hu-$\beta_1$-IFN sequence shown in Figure III.8. A 36% similarity between the putative rt-PCR-IFN and hu-$\beta$-IFN was found. The predicted amino acid sequence of the PCR product is shown in Figure III.9. Alignment of the putative amino acid sequence with both human type 1 IFNs demonstrated 4 exact amino acid matches between all three IFN species. These 4 highly conserved amino acids are: Leu, Ser, Trp, and Val (Figure III.9).

Figure III.5. Induced and uninduced IFN-like RNA from rainbow trout liver was fractionated through a formaldehyde, 1% agarose gel. RNA was transferred to a nylon membrane and probed for sequences similar to human β-IFN. The hybridization pattern is shown here. Lane 1-4 contain 20 μg of total cell RNA while lanes 5-8 contain 40 μg of total cell RNA and represent a repeat of lanes 1-4. Lane designations include 1.) 15 g fish; control, 2.) 15 g fish; poly I:C, 3.) 600 g fish; control, 4.) 600 g fish; poly I:C. The arrowheads indicate bands unique to the induced lanes.

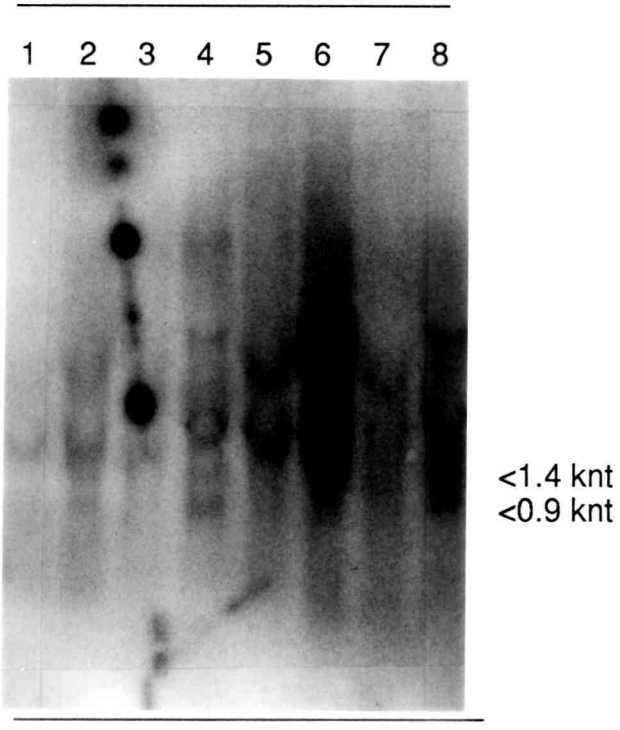



Figure III.5.

Figure III.6. Codon preference from published rainbow trout sequences is shown here as the % of usage in the sequences examined.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| ALA | GCC | 42% | PHE | UUU | 81% | LEU | CUG | 53% |
| ILE | AUC | 60% | VAL | GUG | 55% | SER | UCC | 39% |
| PRO | CCC | 53% | THR | ACC | 38% | TYR | UAC | 100% |
| HIS | CAU | 54% | GLN | CAG | 74% | ASN | AAC | 78% |
| LYS | AAG | 73% | ASP | GAC | 71% | GLU | GAG | 68% |
| CYS | UGC | 67% | ARG | CGC | 31% | GLY | GGA | 36% |

Figure III.6.

Figure III.7. The nucleotide sequence of PCR primers #66 and #67 are shown with degenerate positions listed below the sequence line. The primer #66 begins with amino acid number 33 and primer #67 begins with amino acid number 98. These amino acid positions designate the corresponding protein borders from the human β-IFN gene sequence.

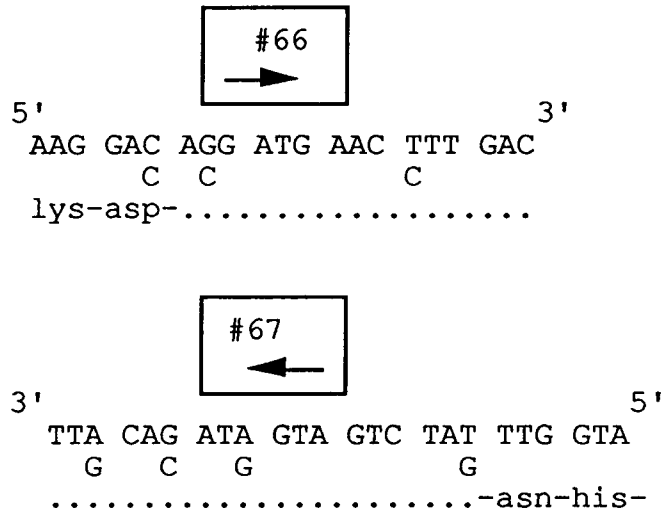

#66
5'
3'
AAG GAC AGG ATG AAC TTT GAC
C C C
lys-asp-.....
#67
3'
5'
TTA CAG ATA GTA GTC TAT TTG GTA
G C G G
.....-asn-his-

Figure III.7.

Figure III.8. Human $\beta_1$-IFN nucleotide sequence is shown in alignment with the fish PCR IFN-like product sequence. The human $\beta_1$-IFN sequence begins at nucleotide 354. The predicted amino acid sequence of the fish product is shown. The asterisks indicate shared homology at the nucleotide level. A.) human β-IFN, B.) fish PCR product

A. ser ser leu his leu lys arg tyr tyr gly arg ile leu his tyr
agc agt ctg cac ctg aaa aga tat tat ggg agg att ctg cat tac
* * * ** * * ** * * * ** * * * *
B. aac aca ctt gct cag caa ata ccc ttg cca tac atg tta cgt ttt
asn thr leu ala gln gln ile pro leu pro tyr met leu arg phe

A. leu lys ala lys glu tyr ser his cys ala trp thr ile val arg
ctg aag gcc aag gag tac agt cac tgt gcc tgg acc ata gtc aga
* * ** ** * * *** * * ** *
B. ttt tct caa ttg cgt aca agc cat ttt tgg atc tgt gtt gaa
phe ser gln leu arg thr ser his phe trp ile cys val glu

A. val glu ile leu arg asn phe tyr phe ile asn arg leu thr
gtg gaa atc cta agg aac ttt tac ttc att aac aga ctt aca g
** * * * * *** * * * * * * * ** **
B. atg tat cta cag cag aac aat gtg tac cat cag ata aac cca t
met tyr leu gln gln asn asn val tyr his gln ile asn thr

Figure III.8.

# DISCUSSION

In 1983, Wilson et al. surveyed a wide variety of vertebrate and non-vertebrate species for the presence of human α- and β-IFN hybridizing sequences. Human α-IFN DNA hybridized to many mammalian and non-mammalian DNA genomes but did not hybridize to perch (Perca fluviatilis), clawed frog (Xenopus tropicalis), or invertebrate DNA. β-IFN sequences could be detected in many nonmammalian species ranging from "birds to bony fish". The fish species examined for β-IFN sequences included perch (Perca fluviatilis), minnow (Phoxinus phoxinus), dace (Leuciscus leuciscus), and stone loach (Noemacheilus barbatulus). In all cases, faint bands were detectable upon Southern blot hybridization ranging in size from 3.8 kbp to approximately 8.0 kbp.

Leung et al. (1984) surveyed mammalian genomes from human, bovine, feline, murine, porcine, and equine sources, for the ability to hybridize to human and bovine β-IFN gene probes. Bovine DNA was found to possess a β-IFN multigene family. Murine, feline and human DNA were determined to contain only a single β-IFN gene or a very small multigene family in their respective genomes by hybridization. Equine and porcine DNA was found to contain multiple copies of β-IFN, as was found in the ungulates.

Zoo-blots were carried out here to examine the distribution of IFN-like sequences among Oncorhynchus species. The Southern blot contained bovine DNA as a control as well as rainbow trout, coho salmon, and chinook salmon genomic DNA. The probe, human $\beta_1$-IFN from Genentech, hybridized to a major band of 3.5 kbp in bovine DNA digested with Hind III, as well as two minor bands at 2.0 and 2.4 kbp. Rainbow trout genomic DNA, digested with

Figure III.9. Human α-IFN, beginning at amino acid 107, and human β-IFN, beginning at amino acid 110, were aligned with the predicted amino acid sequence of the fish PCR product. Note #1-#4 are highly conserved amino acid positions between all three IFNs. Sequences shared by the PCR product and either the α– or β-IFN sequence are boxed. A.) human α-IFN, B.) rainbow trout sequence, and C.) human β-IFN.

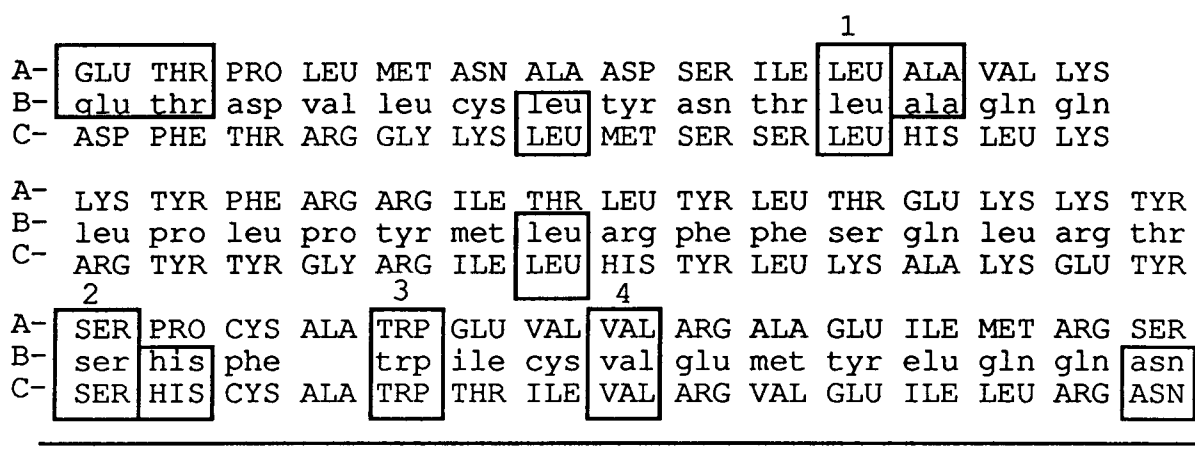

1
A- GLU THR PRO LEU MET ASN ALA ASP SER ILE LEU ALA VAL LYS
B- glu thr asp val leu cys leu tyr asn thr leu ala gln gln
C- ASP PHE THR ARG GLY LYS LEU MET SER SER LEU HIS LEU LYS
A- LYS TYR PHE ARG ARG ILE THR LEU TYR LEU THR GLU LYS LYS TYR
B- leu pro leu pro tyr met leu arg phe phe ser gln leu arg thr
C- ARG TYR TYR GLY ARG ILE LEU HIS TYR LEU LYS ALA LYS GLU TYR
2
3
4
A- SER PRO CYS ALA TRP GLU VAL VAL ARG ALA GLU ILE MET ARG SER
B- ser his phe trp ile cys val glu met tyr elu gln gln asn
C- SER HIS CYS ALA TRP THR ILE VAL ARG VAL GLU ILE LEU ARG ASN

Figure III.9.

BamH1, yielded three bands of 1.0, 2.0, and 4.2 kbp. The same DNA digested with Hind III demonstrated two bands at 4.0 and 4.3 kbp upon hybridization. Chinook DNA was found to possess four hybridizing bands of 0.6, 1.65,1.8, and 4.1 kbp when digested with BamH1. Hind III digestion of chinook DNA yielded 2.2, 3.8, and 4.1 kbp fragments. Coho DNA hybridized at 1.2, 2.4 and 3.8 kbp from BamH1 fragmentation while the Hind III digestion led to a major band at 2.3 kbp and a minor band at 1.7 kbp. It is well documented that ungulates possess a multigene β-IFN family. The multiplicity of hybridizing bands found within the salmonid genomes, under conditions of moderate stringency, indicated that fish may also contain a multigene family of at least three β-IFN-like genes or pseudogenes.

β-interferon is an inducible gene product which is under tight control and which itself regulates the gene expression of many other gene products. Interferon has been well documented as inducible in the presence of mitogens, polyribonucleotides, such as poly I:C, and viruses. Experiments using Newcastle's Disease Virus (NDV) appear to induce both α- and β-IFN from L-cells (Hiscott et al., 1984), while poly I:C is believed to preferentially induce fibroblast (β)-IFN over leukocyte (α)-IFN. Therefore, poly I:C was preferentially utilized over viral inducers as an IFN inducer in these experiments.

Rainbow trout gonad-2 fibroblast cells (RTG-2), maintained in tissue culture, were analyzed for their ability to produce β-IFN-specific RNA under a variety of inducing conditions. These conditions have been well documented for mammalian species previously examined. These conditions include early harvest of RNA, induction with the polyribonucleotide poly I:C, and utilization

of both the protein synthesis inhibitor cycloheximide and DEAE-dextran which enhances the permeability of cell membranes.

Rainbow trout fibroblast IFN-like RNA was examined at early times post- induction. The rational behind this time choice was made according to research done by Cavalieri et al. (1977). Cavalieri analyzed human fibroblast IFN RNA from 0-10 h post-poly I:C induction. Their research was based on the capacity of the induced IFN RNA to program the biosynthesis of biologically active IFN protein in Xenopus laevis oocytes. Cavalieri et al. concluded that poly I:C-induced IFN accumulated for 1-2 h post-induction but was then rapidly degraded with a half-life of 18 min.

Interferon mRNA production has been postulated to be regulated by negative control mechanisms at both the transcriptional and translational level. When cells are treated with the transcription inhibitor Actinomycin D, β-IFN mRNA continues to accumulate in poly I:C treated cells (Raj and Pitha, 1983). Tan and Berthold (1977) postulated the existence of a rapidly turned-over repressor and demonstrated that inhibitors of protein synthesis could induce IFN synthesis presumably by reducing the effective level of this repressor. Cycloheximide prolongs the synthesis of IFN RNA by this phenomenon known as superinduction (Vilcek, 1970). Greater than a 100-fold increase in message levels has been described in the case of human cells by superinduction. Treatment of cells with cycloheximide elongated the time of accumulation of intact IFN RNA, in concert with poly I:C induction, as long as 3 h post-induction. In the presence of cycloheximide, the RNA half-life increased from 18 to 49 min (Cavalieri et al., 1977).

Interferon production is also known to be enhanced by DEAE-dextran. Rosztoczy (1971), demonstrated that DEAE-dextran alone was incapable of

both virus titre inhibition and induction of any IFN activity. However, DEAE-dextran amplified the level of IFN mRNA by presumably increasing permeability of the cell membrane to the potent IFN inducer poly I:C. Together DEAE-dextran and poly I:C accentuated the production of IFN over poly I:C treatment alone. DEAE-dextran is also thought to diminish the possible effects of RNases on poly I:C, thus effectively increasing the presence and induction capabilities of the polymer poly I:C (Stewart II et al., 1972).

According to this information, rainbow trout RNA was induced in a manner which would optimize both the production and accumulation of RNA and decrease the rate of transcript degradation. As described in the Materials and Methods section, IFN RNA was induced at either 3 or 5 h post-induction. It was determined that by 5 h post-induction, in the presence of both DEAE-dextran and cycloheximide, IFN-like RNA was easily detectable. There was a 87% increase over uninduced cells by this method. It is unclear how long the level of IFN mRNA accumulation continues in fish cells following induction, but it is clear that by 5 h post-induction the levels are well above control, uninduced levels.

These inducible messages were subsequently evaluated for their length and compared to previously described β-IFN messages. Following IFN induction in the presence of poly I:C and cycloheximide, Sehgal and Sager (1980) isolated the polyadenylated population of FS-4 cell cytoplasmic RNA and found that two populations of message were produced. By microinjecting fractionated RNA species into Xenopus laevis oocytes for in vitro translation and IFN activity analysis, the investigators were able to resolve two translationally active RNA populations. There was an abundant 1.3 knt species and a less abundant 0.9 knt species. Hybridization to fractionated

rainbow trout RNA by northern blot analysis using the human β-IFN Pst1-Bgl II fragment demonstrated predominantly the 0.9 knt RNA species. A 1.3 knt β-IFN "long" transcript was found to be inducible but not greatly affected by prior priming with β-IFN protein (Nir et al., 1985). The investigators were able to verify that the transcripts all originated at primarily the main β-IFN start point by nuclease S1 mapping. A minor internal start site was also found. Mapping of the 3' end mRNAs concluded that the transcripts were colinear with the main 0.9 knt transcript.

Poly I:C inducible in vivo IFN RNA production was analyzed from two size/age groups of rainbow trout, Oncorhynchus mykiss. Fish RNA was probed with the Pst 1-Bgl II fragment of human β-IFN containing most of the coding sequence. This probe was able to detect both a 1.4 and 0.9 knt IFN RNA species from fish liver RNA. It has been demonstrated that it is very difficult to visualize any RNA produced by poly I:C induction alone so a two week exposure was necessary to visualize the RNA species induced (Tovey et al., 1987), thus explaining the high background.

Developmentally, it appears that the small 15 g fish utilize the larger 1.4 knt IFN mRNA preferentially while at later times in life, at an as yet undetermined time but before they reach 600 g, they switch production of IFN RNA to primarily the smaller species.

In the fish, the two RNA species found by northern analysis could have arisen from a number of potential sources. As fish diverged from mammals 200 to 400 million years ago, it is possible that the fish IFN may not have diverged as much in fish as they have within humans. Alternatively, they represent two different RNA species arising from varying processing events

ocurring from the same DNA template which would be representative of colinear, poly I:C inducible genes containing some or all of the 3' 2/3 of the β-IFN gene and/or some of the 3' flanking downstream sequences. More analysis needs to occur before these questions will be satisfactorily addressed.

In vitro amplification of specific DNA sequences corresponding to rainbow trout β-IFN was carried out. The polymerase chain reaction (PCR), as originally described by Saiki et al. (1985) specifically amplified a DNA segment as defined by flanking oligonucleotides. The sequence utilized as the master template for these flanking oligonucleotides was published by Derynck et al. (1980) and corresponded to the human β-IFN gene. The level of homology is apparent, although not striking, at 40% when compared to human $\beta_1$-IFN.

In conclusion, the Oncorhynchus species examined here appear to contain a multigene β-IFN-like family of genes. Representatives of this family demonstrate classic inducible transcription patterns described for more well documented mammalian IFN genes.

Due to the inherent stress factors involved in hatchery rearing and the ubiquitous nature of viral pathogens, it is necessary to implement the most inclusive, low cost methods of prophylaxis. Ideally, classic viral vaccines in conjunction with a non-vaccine antiviral stimulant such as the cytokine, IFN, would be the best approach. Using the expensive bulk polyribonucleotide, poly I:C, as an IFN inducer would be a simple way to approach hatchery management. However, despite the fact that these products have been used in clinical antiviral and cancer trials, they elicit undesirable side effects. Clinically, poly I:C has been responsible for pyrogenic episodes, leukopenia,

and hypotension (Stringfellow, 1980). Another undesirable condition is known as hyporesponsiveness which occurs after repeated doses. Hyporesponsiveness is described as the temporary loss of the ability to respond fully to the applied inducer effectively eliminating its role as an antiviral agent (Stringfellow et al.,1987). In lieu of the concepts of toxicity and hyporesponsiveness it is undesirable as well as financially prohibitive to consider nucleoside polymers as viral prophylaxis in large scale fishery management protocols. Therefore, it is imperative to produce a low cost product which would boost the non-immune first strike mechanisms against fish pathogens.

# CHAPTER FOUR

## Retroposon-like Elements Found Within the Genome of *Oncorhynchus mykiss*: Fish Repetitive Elements

Leslie A. Tengelsen and Jo-Ann C. Leong

Department of Microbiology

Oregon State University

Corvallis, OR 97331-3804

# ABSTRACT

Most Oncorhynchus species (Kido et al., 1991) examined to date have been shown to contain repetitive elements possessing signals for polyadenylation and polyadenylate tracts. Winkfein et al. (1988) have reported the presence of repetitive sequences in the genome of the rainbow trout known as RSg-1 elements. These elements possess poly A tracts at the 3' end of the element, a concensus polyadenylation signal, and redundant terminal sequences characteristic of retroposons and similar to the protamine pseudogene p101, another retroposon-like element found in the rainbow trout genome. We report two sequences found in the genome of rainbow trout (Oncorhynchus mykiss). These genes share similarities with the RSg-1 and p101 elements at the extreme 3' end of the element in that they contain poly A tracts and polyadenylation signal sequences. However, one of the elements reported here diverges from the RSg-1 elements by the presence of reverse transcriptase-like sequences which have not been found previously within the genome of O. mykiss. We report the finding that the L1 retroposon elements possessing pol (ORF2) sequences described extensively within mammalian genomes is also represented within the genome of rainbow trout.

# INTRODUCTION

Genomic DNA is comprised of not only discrete genetic elements but also highly or moderately repetitive DNA sequences. Historically, repetitive elements have been considered garbage or stuffer DNA; however, their presence may provide clues concerning species evolution. These repetitive elements are dispersed within mammalian genomes and found to occur at a frequency of $10^4$ or more (Singer, 1985; Jelinek et al., 1982). In some cases they represent the majority of the genome as in the case of the rainbow trout, whose genome consists of 84% repetitive elements (Schmidke et al., 1979). Repetitive elements are found to be as simple as dyad repeats and as complex as pseudogenes and retroposons (Winkfein et al., 1988). Retroposons possess structural elements which suggest their genesis by reverse transcription, i.e. sequence bounded by a repetitive (A)-rich tail at the 3' side, and direct terminal repeats of flanking sequences at the 5' and 3' ends. Retroposons are found in both the viral superfamily as retroviruses and the nonviral superfamily comprised of processed pseudogenes, RNA polymerase II transcipts including LINE elements (Singer, 1985) and RNA polymerase III transcipts including heterologous tRNA composites and SINES (Weiner et al., 1986). The nonviral retroposons generate target site duplications, and contain poly adenylation signals and A-rich 3' regions (Rogers, 1985).

We report the isolation of two rainbow trout retroposons. They both contain polyadenylation signals, extensive polyadenylate tracts and a 84 bp 3' element homologous to other fish retroposon sequences (Winkfein et al., 1988; Koishi and Okada, 1991). However, they do not share any homology with tRNA sequences found in fish SINE elements (Matsumoto et

al., 1986) and do not contain any sequences similar to the 5' region of the salmonid Hpa-1 elements (Koishi and Okada, 1991). One sequence, #17 reported here, contains unique sequences homologous to retroposon polymerase sequences, which places this rainbow trout element within the LINE classification of repetitive elements. The "pol" gene-like sequence has never been described before and appears to be restricted to rainbow trout. It is not highly represented within coho sequences and not found in bovine sequences.

## MATERIALS AND METHODS

### Screening a Lambda Genomic Library

A Lambda-Dash II™ (Stratagene) vector-based rainbow trout genomic library (Figure IV.1) was obtained from Dr. Tom Chen (University of Maryland, Baltimore). An initial titration of the library was performed to determine the volume of bacteriophage necessary to yield 5 x $10^5$ phage plaques for screening. The phage preparation was diluted 1:100 in TMG buffer (Maniatis et al., 1982). It was determined that 5 µl of this phage dilution were sufficient to yield approximately 8000 phage plaques per 150 $cm^2$ plate.

### Preparation for Phage Screening

Luria-Bertani (LB) plates (50x 150 $cm^{2)}$ were prepared and allowed to dry for three days so that the plates did not contain any excess water. The *E. coli* cell line necessary for phage attachment and replication, LE392, was prepared as follows. The bacterial cells, strain LE392, were streaked on an LB plate and grown at 37°C overnight. An isolated colony was transferred from the fresh plate into 100 ml of LB containing 2 ml of sterile 20% maltose in a 500 ml Erlenmeyer flask. The flask was shaken at 37°C until the $O.D._{600}$ reached approximately 1.0. Cells were then concentrated by centrifugation in

Figure IV.1. Schematic representation of the parent Lambda-Dash II™ (Stratagene) phage cloning vector. Rainbow trout genomic fragments were engineered into the BamH1 site by Dr. T. Chen at University of Maryland.

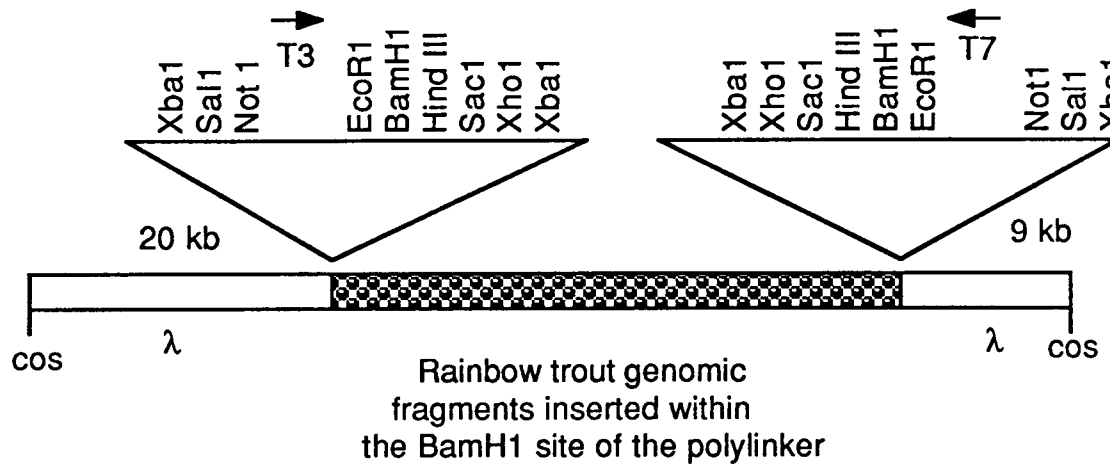

T3
Xba1
Sal1
Not 1
EcoR1
BamH1
Hind III
Sac1
Xho1
Xba1
T7
Xba1
Xho1
Sac1
Hind III
BamH1
EcoR1
Not1
Sal1
Xba1
20 kb
9 kb
cos
λ
λ
cos
Rainbow trout genomic
fragments inserted within
the BamH1 site of the polylinker

Figure IV.1.

50 ml conical tubes at 2500 x g for 10 min and resuspended in 25 ml of cold sterile 10 mM $MgSO_4$ on ice. This yielded approximately 2 x $10^9$ cells per ml. An aliquot of 200 µl of cells was combined with 5 µl of a 1:100 dilution of phage described above. The bacteriophage were allowed to adsorb for 20 min at room temperature. The infected cells were then plated out in 7.5 ml of LB top agar containing 10 mM $MgSO_4$, onto the LB plates. Cell lawn growth was observed for 4 h at 37°C for the emergence of plaques. Following plaque formation, the plates were placed at 4°C for 30 min.

For plaque lifts, each plate was numbered along with a circular nitrocellulose disc (Schleicher and Schuell). Filters were gently applied to the soft agar overlay until completely wet, approximately 5 min. Orientation holes were poked through the nitrocellulose filters and correspondingly marked with a pen on the plate. Nitrocellulose filters were then lifted and allowed to air dry, cell side up, for 30 min. The filters were then consecutively laid on Whatman 3 mm paper soaked in 1) 0.2 M NaOH, 1.5 M NaCL, 2x 5 min.; 2) 2x SSC and 0.4 M Tris, pH 7.4, 2x 5 min; and finally 3) 2x SSC 1x 5 min. The filters were then air-dried for 1 h and baked at 80°C for 2 h under vacuum.

The filters were then probed with a DNA sequence containing the 3' end of the human β-IFN gene and its poly A tract (David Leung, Genentech) (Figure IV.2). Filters were pre-wetted with 2x SSC and then treated with the prehybridization mixture; 5x SSC, 0.2% SDS, 0.1% N-lauryl sarcosine, 40% formamide and 4% Boehringer-Mannheim blocker, for 4 h at 42°C. The Pst1-Pst1 fragment described in Figure IV.2 was labeled to 1 x $10^8$ cpm/ug using the Boehringer-Mannheim random-labeling kit. The labeled probe was boiled for 3 min and 200 ng of the probe was added to the prehybridization mix and allowed to hybridize at 42°C overnight to the Southern blot. Blots were then

washed twice in 2x SSC, 0.2% SDS at room temperature for 15 min each , twice in 1x SSC, 0.1% SDS at room temperature for 15 min each, and once in 0.1x SSC, 0.1% SDS at 42°C for 15 min. Blots were exposed to XAR-X-ray film and positive plaques were located. Of 5 x $10^5$ phage particles, 20 positive plaques were isolated. The twenty plaques were then plaque purified three times.

The DNA from 20 bacteriophage lambda recombinants was purified by the following protocol adopted from Methods in Molecular Biology, 1986.

The bacterial cell line of Escherichia coli, LE392, was prepared as described above for phage infection. One hundred µl of LE392 were added to 10 ml of Luria-Bertani (LB) broth containing $MgSO_4$ to a final concentration of 10 mM in a 17 X 100 mm polypropylene snap cap tube. An agar plug containing a well-isolated single plaque was removed with a sterile Pasteur pipette from a master plate and placed in the LB mixture. The cell/bacteriophage mixture was placed in a 37°C shaker for 12 h, until lysis was apparent; cell debris was seen on the bottom of the tube while the supernatant appeared clearer than a cells-only control tube. Following lysis, 100 µl of chloroform were added to each of the 20 10 ml tubes and shaken for 2 h at 37°C. The tubes were then centrifuged at room temperature for 10 h at 8,000 rpm to remove bacterial debris. The aqueous phase was then removed to a new 50 ml sterile tube. The DNA contained within the 10 ml lysate was then further purified by the following method:

To the 10 ml lysate, 2 ml of a 25% PEG-6000 2.5 M NaCl solution were added. The mixture was swirled and placed on ice for 15 min. The phage particles were then precipitated by centrifugation at 10,000 rpm for 15 min. The supernatant was discarded and the tubes were spun for 5 min more at

Figure IV.2. Pst1 to Pst1 fragment of the human β-IFN gene used as the phage library probe. Note long poly A tract which hybridized to retroposon poly A sequences.

5'- Pst1
I

gaagaaaaactggagaaagaagattttaccaggggaaaactcatgagcagtctgcacctgaa
aagatattatgggaggattctgcattacctgaaggagtacagtcactgtgcctggaccatagtcagagtgga
aatcctaaggaacttttacttcattaacagacttacaggttacctccgaaactgaagatcttcctagcctgtccct
ctgggactggacaattgcttcaagcattcttcaaccagcagatgctgtttaagtgactgatggctaatgtactgc
aaatgaaaggacactagaagattttgaaatttttattaaattatgagttatttttttatttatttaaattttatttttggaaaat
aaattatttttttggtgcaaagtcAAAAAAAAAAAAAAAAAAAAAAAAAAA.....Pst1

Figure IV.2.

5,000 rpm to bring down any remaining liquid on the side of the tube. The pellet was resuspended in 400 µl of TM buffer (50 mM Tris, pH 7.4, 10 mM $MgSO_4$) and placed in a sterile 1.5 ml microfuge tube. RNase A (2 µl of 10 mg/ml) and DNase I (2 µl of 1 mg/ml in 2.5 mM HCl/50% glycerol) were added, gently mixed and set at room temperature for 30 min. Following incubation, the solution was then extracted with phenol (pH 8.0) one time, followed by a chloroform extraction. The aqueous phase was then precipitated with 1/10 volume of 3 M NaOAc (pH 4.8) and 2 volumes of 95% ethanol for 20 min at -20°C . The chilled solution was then centrifuged in a Beckman microcentrifuge for 5 min and the subsequent pellet was rinsed with 150 µl of 80% EtOH and finally dried under vacuum. The final pellet was resuspended in 50 µl TE.

Phage Southern

Phage DNA was digested to completion with either EcoR1 or Xba1 and separated on a 0.8% agarose gel in the presence of 1x TAE. DNA was transferred to a nylon membrane (Schleicher and Schuell) by capillary action in the presence of 20x SSC as described by Maniatis et al. (1982). Following transfer, the membrane was rinsed with 5x SSC to remove any gel fragments and UV cross-linked. The membrane was then prehybridized and hybridized as described above. Fragments were probed for the presence of polyadenosine sequences, as described above.

Sequencing

Positively hybridizing fragments were subcloned and sequenced for content by the manufacturers' instructions for Sequenase™ (Pharmacia) using $^{35}$S-dCTP or $^{32}$P-dCTP (Amersham) and the Sanger dideoxy-sequencing methodology. Sequences were fractionated on 6.6% polyacrylamide gel

matrices, dried on a BioRad gel dryer, exposed to XAR-X-ray film and read. Sequences were compared with the GenBank and EMBL sequence databases for homology and alignment properties. The BESTFIT and GAP programs (IntelliGenetics, Inc.) were utilized.

Genomic Dot Blot

Bovine, coho, and rainbow trout liver genomic DNA were prepared as described by Maniatis et al. (1982) and probed by dot blot analysis for the presence of polymerase-like sequences present in the representative genomes. The oligonucleotide used for probing purposes was oligonucleotide H-12, constructed from the sequence TTG CAT TGT AGT ATT AAC ATG TTT TAA TAT TTG TGC, which corresponds to the unique polymerase-like sequences found in the rainbow trout genome. H-12 was constructed in the antisense orientation. The oligonucleotide is marked in Figure IV.6 as the #2 arrow. Bovine, coho, and rainbow trout genomic DNA were immobilized onto a Nytran™ nylon membrane using a dot manifold suction device (Schleicher and Schuell). Dilutions of DNA containing 10, 5, and/or 2.5 µg carried in 100 µl of 5x SSC, held at 65°C for 10 min, and chilled on ice for an additional 5 min. The DNA was applied to the manifold device and drawn onto the membrane until dry. An additional 100 µl of 10x SSC were passed through the wells and suction was applied for an additional 5 min. The device was then disassembled and the blot removed for further treatment. The membrane was treated for 5 min with 0.4 M NaOH followed by neutralization with 1 M Tris, pH 7.4. The DNA was subsequently fixed to the membrane by UV cross-linking. Prehybridization was carried out as described above. Hybridization was carried out with 1 x $10^9$ cpm $^{32}$P-$\gamma$ ATP-labeled oligo H-12 at 55°C for 18 h. The blot was subsequently rinsed 4x for 5

min each in 2x SSC, 0.1% SDS at room temperature and exposed to XAR-X-ray film.

RNA Dot Blot

Two-fold dilutions of total cellular RNA were applied to a nylon membrane using a dot blot manifold (Schleicher and Schuell) beginning with 2.5 µg of RNA. The RNA samples were derived from RTG-2 cells (rainbow trout gonad cells) or Vero cells (African green monkey kidney cells). The RTG-2 cells had been treated with either cycloheximide, poly I:C, or DEAE-dextran alone or in combination before RNA was extracted from these cells. These combinations included poly I:C + DEAE-dextran, poly I:C + cycloheximide, or poly I:C + DEAE-dextran + cycloheximide. The RNA samples were prepared by the RNAzol method. Samples are diluted to a final concentration of 50% formamide, 17% formaldehyde and 1x SSC before heating to 68°C for 15 min and then chilling in ice for 2 min. Samples were then diluted with 2 volumes of 20x SSC. The manifold was assembled with blotting paper and a nylon membrane presoaked in 10x SSC. Once the apparatus was assembled, 1 ml of 10x SSC was applied to each well before each sample was applied. Finally, 1 ml of 10x SSC was applied to wash each sample. After an additional 5 min of suction was applied, the apparatus was disassembled. The membrane was air-dried and subsequently UV cross-linked with a Stratagene UV Stratalinker™ 1800. Blots were hybridized with the oligonucleotide probe, H-12, described above in the presence of 6xSSC, 0.5% SDS and 5x Denhardt's (50x stock: 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA) at 65°C for 6 h. Blots were then washed with 2x SSC, 0.5% SDS at room temperature for 4x at 5 min for each wash and exposed to film for 24 h.

# RESULTS

## Phage Screening

The rainbow trout liver genomic library was screened with a radiolabeled probe from the human β-IFN gene (Figure IV.2). The initial screen of 5 x $10^5$ phage plaques yielded ten positive phage clones after three plaque purification steps (Figure IV.3). The DNA from each phage clone was prepared and analyzed by restriction endonuclease digestion. Subsequent Southern blot hybridization of the restricted DNA identified the phage DNA fragments containing the hybridizing sequence (Figure IV.4). The phage clones were numbered 1, 3, 5, 6, 10, 15, 17, 18, 19, and 20. EcoR1 restriction analysis yielded positive fragments in phage # 1, 5, 6, 10, 17 and 20. Phage # 1, 5, 17, and 20 were chosen for further analysis and the hybridizing fragment for each clone was 8.0 kbp (#1), 5.8 kbp (#5), 5.0 kbp(#17), and 3.8 kbp (#20). Phage #5 and #6 appeared to possess the same restriction fragment and were assumed to be the same clone. Subclone analysis (not shown) yielded smaller fragments which were subsequently cloned into pUC18 and sequenced. This produced a 2.5 kbp Sph1 fragment for phage clone #5 and a 4.3 kbp Xba1 fragment for phage clone #17.

## Sequence Analysis

The Sph1 fragment for #5 and the Xba1 fragment for #17 were sequenced and found to resemble retroposon-like elements. The Sph1 (#5) gene fragment (Figure IV.5) and the Xba1 (#17) gene fragment (Figure IV.6) both possess long polyadenylated 3' sequences directly downstream from AAATAAA polyadenylation signals. The sequences also possess 3' terminal direct repeats. The Xba1 (#17) fragment was found to contain, in addition, a "pol" gene sequence in open reading frame 2. The "pol" gene sequence

Figure IV.3. Circles A-D represent the stages in obtaining pure phage clones. A. Initial screening showing hybridization to a single positive plaque (arrow head). B. Replating of phage from original plaque and screening of the plaque by hybridization with radiolabeled probe. C. Third plating of phage from a positive plaque taken from plate B. D. Negative control. Hybridization was to a human β-IFN probe containing a poly A tract. Large outer dots are orientation markers which were added with India ink.

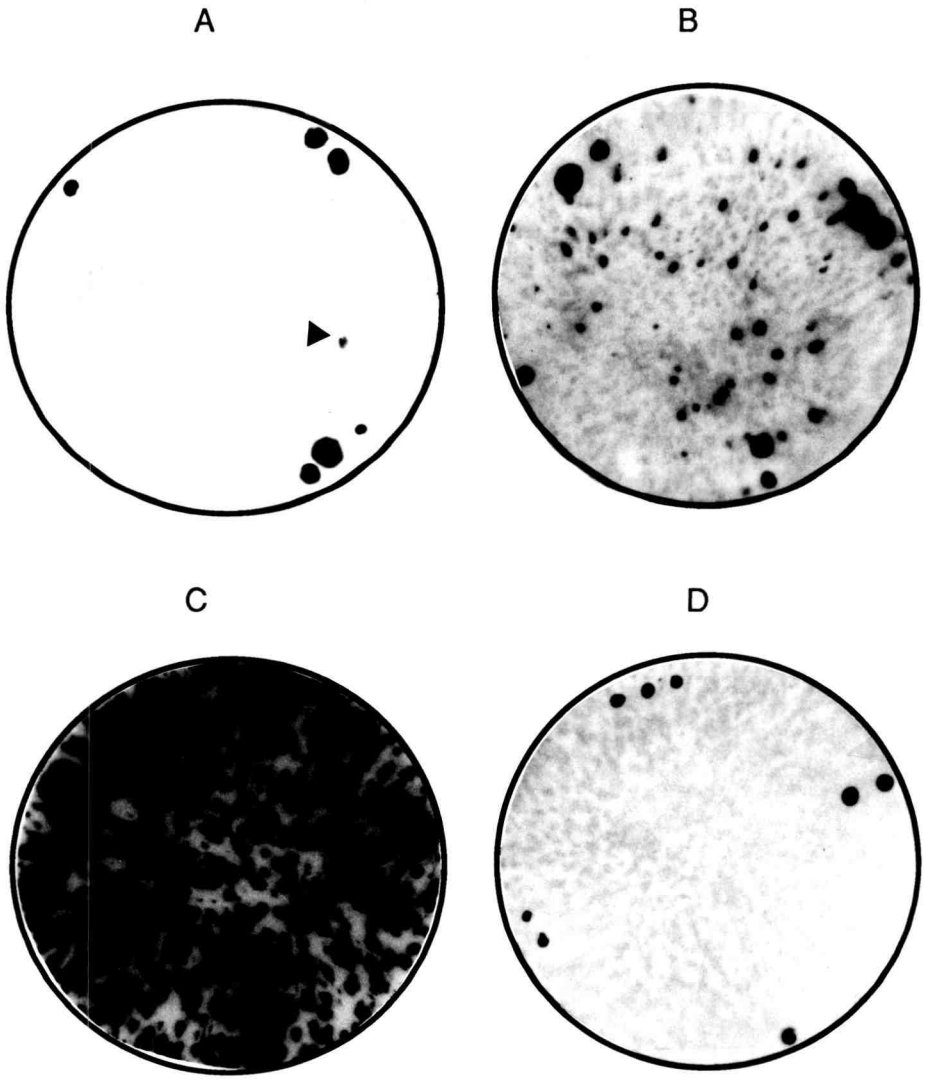



Figure IV.3.

Figure IV.4. Restriction analysis and Southern blot hybridization of cloned phage DNA. The DNA obtained from each purified phage clone preparation was digested with EcoR1 and then separated on a 1% agarose gel. The separated DNA was then transferred to a nylon membrane and probed for the presence of poly A sequences. Letters above the lanes represent the designated number for each phage isolate: a.) #1, b.) #3, c.) #5, d.) #6, e.) #10, f.) #15, g.) #17, h.) #18, i.) #19, and j.) #20.. M; molecular size markers consisting of HindIII digested Lambda DNA fragments with the corresponding size in bp on the right, C; control phage DNA with no insert. A. Ethidium bromide staining of restricted fragments. B. Positive hybridization banding pattern with the corresponding phage clones lettered above.

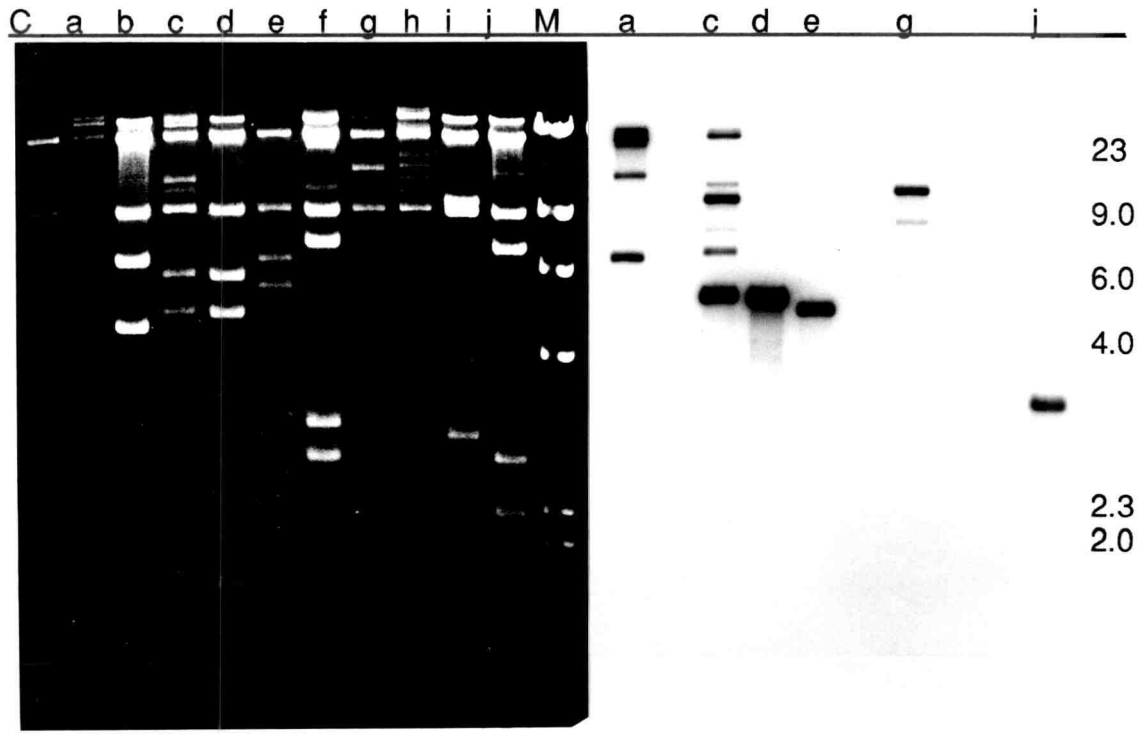



Figure IV.4.

resembles the reverse transcriptase like sequence found in the D. simulans retroposon 297 (Matsuo et al., 1986). There was a 64% similarity between these two sequences at the nucleotide level. Both #17 and #5 sequences possess, at the 3' end, a 84 bp cassette that is highly conserved among this class of retrotransposons. The 84 bp cassette is also found within tRNA-like retroposons and RSg-1 elements which appear to be highly conserved among these sequences. An alignment of the RSg-1 3' cassette with the #17 3' cassette demonstrated an 81% sequence homology between the two elements (Figure IV.7).

DNA Dot Blot

Rainbow trout genomic DNA and, upon long exposure, coho DNA were found to hybridize to polymerase-specific sequences (Figure IV.8). Bovine genomic DNA does not hybridize to the fish derived probe. It is apparent that the sequences are restricted to the fish genomes examined here.

RNA Dot Blot

Dot blot analysis concluded that RNA was not produced in a steady state from the polymerase-like retroposon element, but under the induction conditions where cycloheximide, poly I:C and DEAE-dextran were present, some hybridization was detectable (Figure IV.9).

## DISCUSSION

Repetitive elements are widely dispersed within eukaryotic genomes (Singer, 1985). Their genesis and purpose is unclear; however, many of them possess sequences characteristic of RNA such as polyadenylation signals poly A tracts and the lack of introns. The presence of these elements suggests that these elements arose from a reverse transcription event and/or subsequent transposition (Weiner et al., 1986). A class of common repetitive

Figure IV.5. Retroposon sequence derived from clone #5, a rainbow trout genomic clone. The DR is an element found as a direct repeat in the sequence analysis of clone #17 DNA, shown in figure IV.6. The DR is only represented one time in clone #5. The polyadenylation signal is boxed and the 51-A rich region is underlined. The hatched region indicates homologous sequences between #5 and #17 while the asterisk represents divergence between the two sequences. The sequences shown upstream from the DR element are unique to #5 and not found within #17.

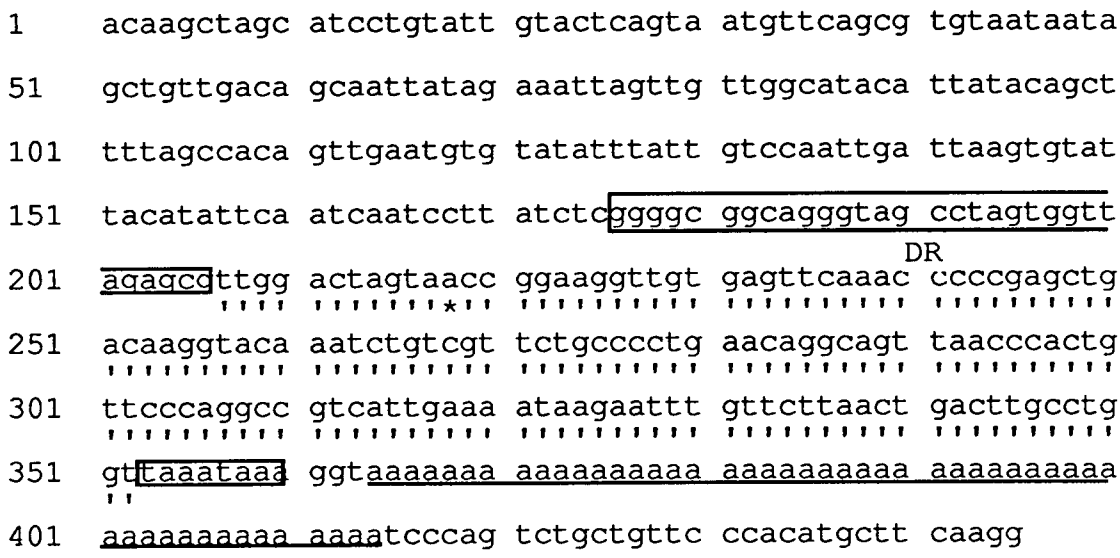

1 acaagctagc atcctgtatt gtactcagta atgttcagcg tgtaataata
51 gctgttgaca gcaattatag aaattagttg ttggcataca ttatacagct
101 tttagccaca gttgaatgtg tatatttatt gtccaattga ttaagtgtat
151 tacatattca atcaatcctt atctcggggc ggcagggtag cctagtggtt
DR
201 agaagcgttgg actagtaacc ggaaggttgt gagttcaaac ccccgagctg
251 acaaggtaca aatctgtcgt tctgcccctg aacaggcagt taacccactg
301 ttcccaggcc gtcattgaaa ataagaattt gttcttaact gacttgcctg
351 gttaaataaa ggtaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa
401 aaaaaaaaaa aaaatcccag tctgctgttc ccacatgctt caagg

Figure IV.5.

Figure IV.6. Retroposon sequence derived from clone #17, a rainbow trout genomic clone. Retroposon-like features are highlighted. 1.) Reverse transcriptase-like sequence is indicated by the asterisks. Each asterisk designates a nucleotides identity with the "pol" gene sequence in retroposon 297 of D. simulans. 2.) Oligonucleotide H-12 used for genomic DNA blotting. 3.) Direct repeats (DR), 4.) polyadenylation signal, and 5.) 54-A rich region. The hatched region of similarity shown in figure IV.5, is located in the same position here between the DR element and the poly adenylation signal.

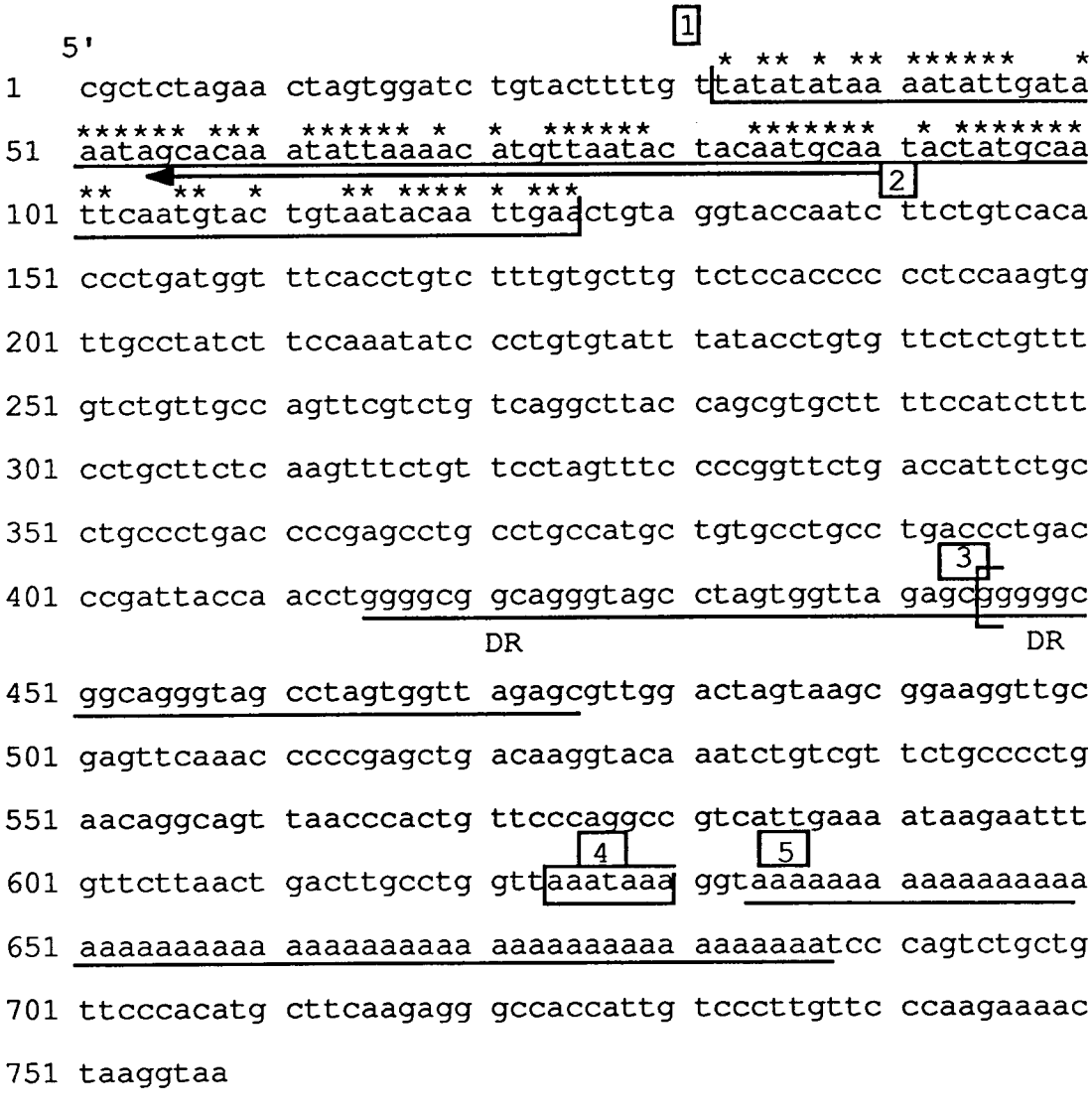

5'
1
2
3
4
5
DR
DR
1 cgctctagaa ctagtggatc tgtacttttg ttatatataa aatattgata
51 aatagcacaa atattaaaac atgttaatac tacaatgcaa tactatgcaa
101 ttcaatgtac tgtaatacaa ttgaactgta ggtaccaatc ttctgtcaca
151 ccctgatggt ttcacctgtc tttgtgcttg tctccacccc cctccaagtg
201 ttgcctatct tccaaatatc cctgtgtatt tatacctgtg ttctctgttt
251 gtctgttgcc agttcgtctg tcaggcttac cagcgtgctt ttccatcttt
301 cctgcttctc aagtttctgt tcctagtttc cccggttctg accattctgc
351 ctgccctgac cccgagcctg cctgccatgc tgtgcctgcc tgaccctgac
401 ccgattacca acctggggcg gcagggtagc ctagtggtta gagcgggggc
451 ggcagggtag cctagtggtt agagcgttgg actagtaagc ggaaggttgc
501 gagttcaaac ccccgagctg acaaggtaca aatctgtcgt tctgcccctg
551 aacaggcagt taacccactg ttcccaggcc gtcattgaaa ataagaattt
601 gttcttaact gacttgcctg gttaaataaa ggtaaaaaaa aaaaaaaaaa
651 aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaatcc cagtctgctg
701 ttcccacatg cttcaagagg gccaccattg tcccttgttc ccaagaaaac
751 taaggtaa

Figure IV.6.

Figure IV.7. The RSg-1 consensus sequence from Winkfien et al. (1988) is aligned with the 3' end of the #17 DNA sequence here. The starting base pair positions are indicated. The dotted line indicates identity between the two sequences while nucleotides listed in the RSg-1 sequence line indicate dissimilarity at positions indicated. RSg-1 lacks an extensive poly A tract.

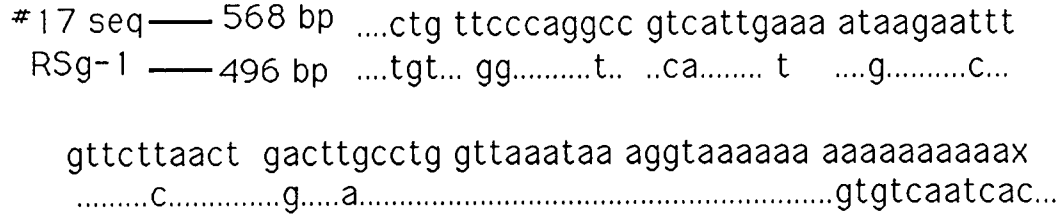

#17 seq —— 568 bp ....ctg ttcccaggcc gtcattgaaa ataagaattt
RSg-1 —— 496 bp ....tgt... gg.....t.. ..ca..... t ....g.....c...
gttcttaact gacttgcctg gttaaataa aggtaaaaaa aaaaaaaaaax
.....c.....g.....a.....gtgtcaatcac.....

Figure IV.7.

Figure IV.8. Dot blot hybridization to bovine, coho, and rainbow trout genomic DNA. Aliquots of 10, 5, and 2.5 μg of DNA were immobilized on a nylon membrane and probed for retroviral polymerase-like sequences using oligonucleotide H-12 (see Figure IV.6).

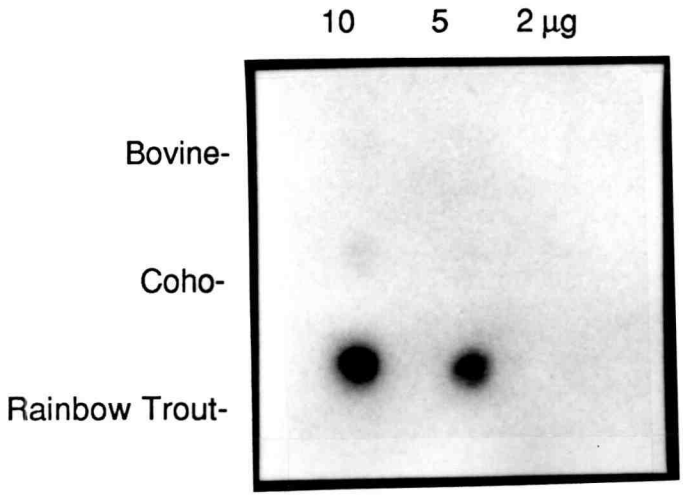



Figure IV.8.

elements sharing these characteristics are called retroposons. Nonviral retroposons can be divided into two main classes: SINES (short interspersed repeats) and LINES (long interspersed repeats) and some smaller, less prevalent subclasses (Weiner et al., 1986). SINES are thought to arise from RNA polymerase III transcripts. The common $tRNA^{lys}$ pseudogene retroposon element is believed to be widely distributed among SINES in the animal kingdom. SINES have been described for O. keta (Matsumoto et al., 1986; Kido et al., 1991) These $tRNA^{lys}$ pseudogene retroposon elements are thought to be the progenitors of the salmon pol III SINES. Kido et al. (1991) reported repetitive element families within salmon species. The highly restricted Sma1 family of repetitive elements only found in chum and pink salmon, the Fok1 repetitive family was restricted to Salvelinus (char), and the ubiquitious Hpa1 family which is found within salmonid species, contains some tRNA sequence similarities. These elements have been found in O. nerka adonis (kokanee) and have been designated elements On-5 and On-8 and in O. masou (cherry salmon) have been designated element Om-2.

LINE (L1 elements) are all thought to be transcribed by RNA pol II. They appear to be truncated at the 5' end and can contain two overlapping ORFs (ORF-1 and ORF-2). ORF-2 possesses some sequence similarities with the pol protein of some retroviruses (Hattori et al., 1986). LINE families can be 6-7 kbp long; however, most are truncated and some contain internal inversions and/or deletions. They also usually contain polyadenylation signals and poly A tracts at their 3' ends and are usually embedded within unrelated sequences. Interspersed, moderately repetitive retroposon-like elements have been decribed for a variety of salmonid species. Rainbow trout (O. mykiss,) were found to contain RNA polymerase II derived structural

Figure IV.9. RNA dot blot representing transcription of nucleic acid in the region of the #17 retroposon element. The oligonucleotide H-12 (see Figure IV.6) was used as a probe. 1.) Herpes simplex type-1 RNA (negative control), 2.) cycloheximide treated RTG-2 cell RNA, 3.) poly I:C treated RTG-2 cell RNA 4.) cycloheximide and poly I:C treated RTG-2 cell RNA, 5.) poly I:C and DEAE-dextran treated RTG-2 cell RNA, 6.) cycloheximide, poly I:C, and DEAE-dextran treated RTG-2 cell RNA, 7.) Vero cell RNA (cell control), 8.) RTG-2 cell RNA.

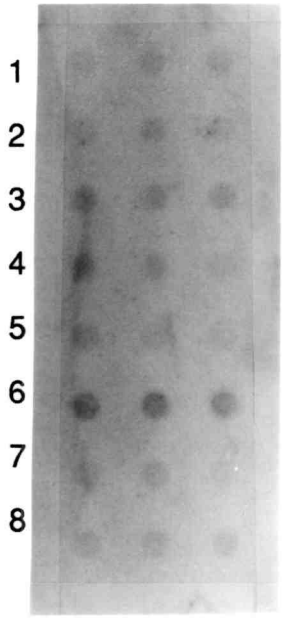



Figure IV.9.

elements which appeared to be related to L1 elements but they exhibited no sequence homology to L1 elements or any other elements described to date. These elements were called RSg-1 elements (Retroposon Salmo gairdneri-1) (Winkfein et al.,1988). RSg-1 elements have been found to be distributed among trout multigene families. These sequences were found to lie upstream of a protamine gene (p101) and upstream of the histone H-4 gene cluster. The RSg-1 elements described contain no ORF-2 and, thus, no sequences that contain any similarities to gag-pol.

The two new sequences reported here are two additional retroposon elements found in the Oncorhynchus genome. These are unique at their 5' ends and share strong identity with the 3' approximately 84 bp sequence directly upstream of the poly A tract in all fish retroposons described to date. They share 81% similarity with the RSg-1 3' cassette; however, the similarity ends there. They are unique in a number of aspects from previously published fish retroposon-like elements. First, they contain much longer poly A tracts than the other fish elements: 50-54 A's versus 6-8 A's for RSg-1. Secondly, #17 contains pol-like sequences with 94 bp sharing 70% homology with D. simulans retroposon 297 ORF-2 pol sequences beginning with base pair 30. The retroposon sequence of #17 also possesses a second direct duplication of the 84 bp 3' element prior to the 54 adenosine residue tract. There is marked variability between the retroposon elements of #5 and #17 at the 5' end; but they also share an extensive poly A tract and an identical 31 bp string following the adenosine tract which may be the target site of duplication. The polymerase-like sequences found within the #17 clone, characterizes this element as a LINE element.

RTG-2 cellular RNA induced in a manner classically used to induce IFN transcription using cycloheximide, poly I:C and DEAE-dextran was examined for the presence of any polymerase-like transcripts corresponding to the #17 clone. It was found that, under the most stringent induction methods using all three reagents together, a small amount of the pol gene transcript was detectable. Some LINES are known to be transcribed; such as the MIF-1/ BamH1 LINE of rodents and the Kpn1 LINE of primates (for review, see Rogers et al., 1985) and some transcripts are thought to be synthesized from fortuitous upstream promoters. Due to the truncated structure of the #17 clone, it is most likely that it is fortuitously transcribed from upstream promoter elements as well.

Bovine, coho, and rainbow trout genomic DNA were probed for polymerase-like sequences using a 36 base oligonucleotide corresponding to the polymerase sequences found within the #17 clone. The results indicated that these particular polymerase-like sequences were strongly represented within the rainbow trout genome and were faintly present in sufficient quantities to be detectable in the coho genome. These sequences were not detectable by this method within the bovine genome. The ubiquitious nature of LINE elements within mammalian and nonmammalian vertebrates has been extended to the fish species. Previously, polymerase-like sequences have not been reported within O. mykiss. The hybridization patterns are indicative of species-specificity which is a hallmark of these retrotransposable elements (Rogers et al., 1985).

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