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Polymerase chain reaction and DNA sequence of rainbow trout tumour suppressor gene *p53* exons 5, 6 and 7 to 9

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Abstract

A protocol for the extraction of genomic DNA from teleost fish was developed and produced high molecular weight DNA from English sole, white sucker and rainbow trout. A protocol for polymerase chain reaction (PCR) of tumour suppressor gene *p53* from rainbow trout genomic DNA was developed. Several primers were chosen inside exon 5 and 9 and exon 6 and 7. A successful PCR method yielded the conserved exon 5 to 9 region of genomic rainbow trout *p53* in two fragments: fragment 1 comprising exons 5 and 6 and fragment 2 spanning the region of exons 7 to 9. The apparent molecular weight of fragments 1 and 2 were 510 and 760 base pairs respectively. The sequences of both fragments including the introns were determined and gave 515 and 793 bp for the two PCR fragments. These sequences will facilitate examination of tumour suppressor gene *p53* conserved exons from genomic DNA e.g. for study of *p53* mutations in fish.

Keywords: *Oncorhynchus mykiss*, polymerase chain reaction, tumour suppressor gene, DNA.

Réaction de polymération en chaîne et séquence d'ADN du gène suppresseur de tumeur p53, exons 5, 6 et 7 à 9, chez la truite arc-en-ciel.

Résumé

Un protocole pour l'extraction de l'ADN du génome de poissons téléostéens a été développé et produit de l'ADN de haut poids moléculaire de la plie (*Pleuronectes vetulus*), du meunier (*Castostomus commersoni*), et de la truite arc-en-ciel (*Oncorhynchus mykiss*). Un protocole pour la réaction de polymérisation en chaîne (PCR) sur le gène suppresseur de tumeur *p53* a été développé chez la truite arc-en-ciel. Plusieurs primers ont été choisis à l'intérieur des exons 5 et 9 et des exons 6 et 7. Une technique de PCR a permis de mettre en évidence 2 fragments conservés dans la région 5 à 9: le fragment 1 comprenant les exons 5 et 6 et le fragment 2 comprenant la région des exons 7 à 9. Le poids moléculaire apparent des fragments 1 et 2 étaient respectivement de 510 et 750 paires de bases.

Mots-clés : ADN, gène, génome, mutation, tumeur, poisson téléostéen, truite.

INTRODUCTION

An area of cancer research which is currently receiving considerable attention is genetic alteration of oncogenes and tumour suppressor genes by carcinogens. Perhaps the most common target of genetic change is the *p53* tumour suppressor gene. Mutations in the *p53* gene have been implicated in a virtually all major human malignancies (reviewed by Levine *et al.*, 1991 and Hollstein *et al.*, 1991).

To date, cDNA clones coding for human, murine, rat, chicken, frog, and rainbow trout *p53* proteins have been characterized (reviewed in Soussi *et al.*, 1990; Caron de Fromentel *et al.*, 1992). Five highly conserved domains have been identified among the *p53* sequences from these divergent species, suggesting that these regions are important for biological function.

A variety of molecular techniques have been employed to detect and characterize cancer-related

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mutations in the *p53* gene. For instance, polymerase chain reaction (PCR) has been used to amplify regions of the *p53* gene in which mutations associated with malignancies tend to occur. Recently, a sensitive two-stage PCR was developed for amplification of the individual exons 5, 6, 7, 8, and 9 (Kusser *et al.*, 1993). In he first round of PCR, a "touchdown" protocol was used to amplify a 1.84 kb fragment spanning exons 5-9 from DNA extracted from human tumours. The first-round PCR product was then used as a template for amplification of individual exons in a second PCR.

While considerable attention has focused on establishing a relationship between alterations in the *p53* gene and exposure to mutagenic or carcinogenic agents in human cancer, few studies have examined the molecular basis of carcinogenesis in fish. Neoplasms have been found in nearly all of the major organs of many fish species and are often similar histologically to those occurring in humans (Van Beneden *et al.*, 1990). Moreover, the occurrence of tumours in fish has been correlated with exposure to chemical carcinogens, both in the laboratory and in the environment (reviewed by Hinton, 1989). For instance, an extremely high incidence of liver tumours was reported in Atlantic tomcod (*Microgadus tomcod*) collected from the heavily polluted Hudson River compared to a low incidence of tumours in fish from unpolluted control sites (Wirgin *et al.*, 1989). We believe that fish species could prove valuable in assessing the effects of potential carcinogens at the *p53* loci. Moreover, such studies could be extended to identify carcinogenic hazards in aquatic environments.

So far, only the human, mouse and *Xenopus p53* genes have been fully characterized. Here we report a DNA extraction procedure affording high molecular weight DNA, PCR of exons 5 and 6 and 7 to 9 from rainbow trout liver genomic DNA and the sequence of the fragments.

MATERIALS AND METHODS

Samples and DNA extraction

Livers were collected from three fish species: rainbow trout from the Pacific Northwest (*Oncorhynchus mykiss*), white sucker (*Castostomus commersoni*), and English sole (*Pleuronectes vetulus*). Genomic DNA was extracted from the livers by proteinase K digestion and phenol chloroform extraction. The tissue was finely minced with scalpel blades and suspended in TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) containing 0.2 mg/ml RNAse and 1% SDS. The tissue suspension was further homogenized in a 7 ml Dounce tissue grinder (Wheaton) then incubated at 37 °C for 1 hour. Proteinase K (0.8 mg/ml) was added, the homogenate was mixed and incubated at 50 °C overnight. The DNA was extracted three times using 1 volume of phenol chloroform (3 parts phenol: 2 parts 500 mM Tris/20 mM EDTA pH = 8.0: 4 parts chloroform). The DNA was precipitated and isolated by spooling onto a glass rod. To spool the DNA, 1/10 volume of 3M sodium acetate (pH 5.2) was added to the aqueous phase and two volumes of 100% ethanol (cooled to –20 °C) were gently layered on top of the mixture. The two layers were gently mixed with a glass rod causing the DNA to "spool" onto the rod as a thread-like precipitate. The DNA material collected was rinsed with cold 70% ethanol and dissolved in TE buffer (pH 7.0), precipitated with 2 volumes of 70% ethanol and centrifuged for 15 min at 10 000 xg. The supernatant was pipetted off, and the pellet was air-dried. Isolated DNA was rehydrated in TE buffer (pH 7.0). DNA concentrations were determined with a Hoefer TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco), and working solutions of 10 ng/ml were prepared.

Polymerase chain reaction

Primers of 20 mers were synthesized by Dalton Chemical Laboratories Ltd. (North York, Ontario). The oligonucleotide sequences were made to regions of the rainbow trout *p53* cDNA adjacent to sites considered most likely to be splice junctions. Oligo software was used to determine optimal primer-pair annealing temperatures and to check for primer-primer interaction, self-annealing and primer-dimer extension. *Table* 1 provides the sequences of all primers used.

In attempting to amplify the region spanning exons 5-9 of the rainbow trout *p53* gene, a number of reaction conditions were manipulated. The PCR reactants were combined in Micro-Amp tubes (Perkin-Elmer) to give a final volume of 32.5 µl and final concentrations of 15.3 mM Tris/HCl (pH = 8.5), 30-150 mM KCl, 1.5-4.6 mM $MgCl_2$, 0.76 mM of each dNTP (Pharmacia), 5 pmols of each primer (sense and antisense), 20 ng of genomic DNA, and 2.5 units of Taq polymerase (Perkin Elmer). PCR was carried out in a Perkin-Elmer DNA Thermal Cycler (Cetus 9600). Each assay was preheated for 5 min at 95 °C to denature the template

Table 1. – Oligonucleotide sequences used as primers for amplification and sequencing of *p53* fragments from teleost fish liver genomic DNA. Primers were named for the exon to which they were made (*i.e.* 5, 6, 7, 8 or 9) and the DNA strand for which they were directed against (*i.e.* sense = F and antisense = R). The position refers to the nucleotide number in rainbow trout cDNA (De Fromentel *et al.*, 1992).

Primer	Position	Sequence (5′-3′)
5F	547	TAC TCG CCA GAC CTG AAC AA
5F	548	ACT CGC CAG ACC TGA ACA AG
6R	827	CTG AGG AGG CTC ATA GGG GA
7F	847	GTG GGA TCA GAG TGT ACC AC
9R	1181	CTG AAG AGT GTA GAT CTC AT
9R	1168	ATC TCA TCG TCA CTC ACA GC
8F	(Sequencing only)	CAA CCT GAA GAA GCA GCA GG
8R	(Sequencing only)	CTC CTC TGT CTT CCT GTC TC

DNA then cycled 30 times using either a touchdown or single-temperature amplification program. Each cycle included a denaturing step at 94 °C for 10-30 seconds (sec.), an annealing step at 53-60 °C for 30-60 sec., and an extension step at 72 °C for 30-40 sec. PCR products were analyzed on a 1 % agarose gel stained with ethidium bromide.

Following a first round of PCR as above, products potentially containing large *p53* fragments were used directly as template for secondary amplification of smaller *p53* fragments. Attempts to amplify smaller *p53* fragments, specifically exons 5-6 and exons 7-9, also involved manipulation of several PCR parameters. Reaction volumes were typically 25 µl, containing 1-4 µl of first round PCR product and final concentrations of 10 mM Tris/HCl (pH 8.5), 20 mM KCl, 1.5-4.6 mM $MgCl_2$, 0.5 mM of each dNTP, 2.5 pmol of each primer, and 1.25 units of Taq polymerase. Each amplification reaction consisted of 30 cycles of 94 °C for 20 sec., 53-56 °C for 40 sec., and 72 °C for 20 sec. PCR products were analyzed by electrophoresis on a 1 % agarose gel stained with ethidium bromide.

Sequencing

Double stranded PCR products were purified by isopropanol precipitation and sequenced with Vent exo DNA polymerase (New England Biolabs) in a cycle sequencing protocol with ^{32}P-endlabeled primers according to manufacturer's instructions.

RESULTS

As a first step to perform PCR of *p53* from fish species we developed an extraction procedure for isolation of pure high molecular-weight DNA from fish liver samples. The extraction procedure uses a proteinase K-phenol/chloroform method developed for human tumour samples (Goelz *et al.*, 1985) combined with the "spooling out" method first described by Marmur (1961). The method was employed to DNA from white sucker, English sole and rainbow trout and yielded high molecular weight DNA as judged by aragose gel electrophoresis and O.D. 260/280 measurements (results shown in *figure* 1).

Amplification of exon regions 5-6 and 7-9 directly from the rainbow trout genomic template was achieved using primer pairs 5F (548)/6R (827) and 7F (847)/9R (1168). The final concentrations of reactants in the PCR assays were: 10 mM Tris, 20 mM KCl, 3 mM $MgCl_2$, 0.5 mM each dNTP, 2.5 pmols each of forward and reverse primer, 10 ng of template, and 1.25 units of Taq polymerase (Perkin Elmer) in a total reaction volume of 25 µl. The assays were first preheated for 5 min. at 95 °C to melt the template DNA, then 30 cycles proceeded for 20 sec. at 94 °C, 40 sec. at 56 °C, and 72 °C for 20 sec. Specific and consistently reproducible bands of 510 and 760 base pairs were

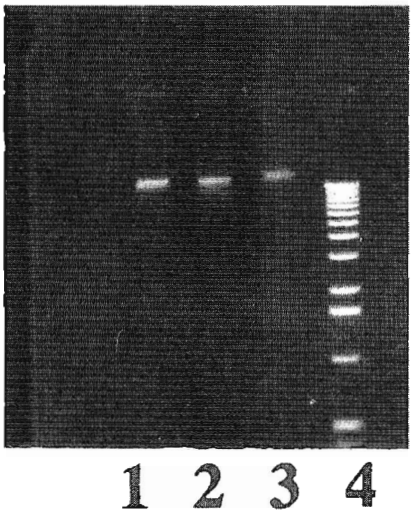

Figure 1. – Photograph of ethidium bromide-stained 1 % agarose gel showing DNA extracted from fish liver samples.
Lane 1: DNA from white sucker
Lane 2: DNA from English sole
Lane 3: DNA from rainbow trout
Lane 4: 1-kb ladder
Twenty nanograms of DNA, or two microlitres of a 1-kb ladder (lane 4), was loaded on each lane and electrophoresed at 90V for 30 min. The gel was photographed under UV light.

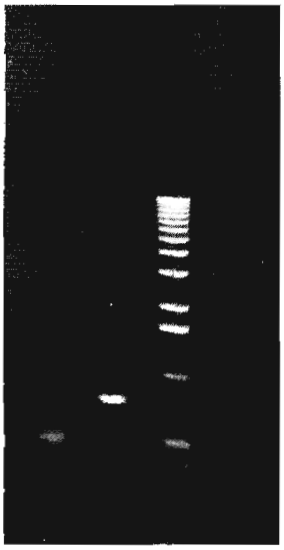

Figure 2. – Photograph of ethidium bromide-stained 1 % agarose gel showing *p53* DNA fragments amplified from rainbow trout genomic DNA. Lanes 1 and 2 show fragments spanning exons 5-6 and exons 7-9, respectively. Twenty nanograms of DNA, or two microlitres of a 1-kb ladder (lane 3), was loaded for each lane and electrophoresis carried out at 90V for 30 min. The gel was photographed under UV light.

amplified (results shown in *figure* 2). For sequencing, the fragments were processed and sequenced using ^{32}P end-labeled PCR primers (*see table* 1 for sequence of primers) in a cycle sequencing protocol as described in Materials and Methods. The fragment spanning exon 5 to 6 had 515 bp, the PCR fragments spanning exon

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                EXON 5
TROUT P53       TACTCGCCAGACCTGAACAAGTTGTTCTGCCAGTTGGCGAAGACTTGTCC
HUMAN P53       TACTCCCCTGCCCTCAACAAGATGTTTTGCCAACTGGCCAAGACCTGCCC
                ***** ** * *** ****** **** *****  **** ***** ** **
TROUT P53       AGTTCAGATCGTGGTGGACCACCCTCCTCCTCCTGGGGCAGTGGTACGAG
HUMAN P53       TGTGCAGCTGTGGGTTGATTCCACACCCCCGCCCGGCACCCGCGTCCGCG
                 ** *** *   *** **   * * ** ** ** **  *    ** ** *
TROUT P53       CCCTGGCCATCTATAAGAAGCTGAGTGACGTGGCTGACGTGGTGAGACGC
HUMAN P53       CCATGGCCATCTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGCGC
                ** ********** *** **       ** ** * ** ** ***** ***
                                                      INTRON
TROUT P53       TGCCCTCACCACCAGAGCACCAGCGAGAACAATGAAG   GTACACAACACAG
HUMAN P53       TGCCCCCACCAT--GAGCGCT-GCTCAGATAGCGATG   GTG--------AG
                ***** *****   **** *  **    * *  ** *   **        **
TROUT P53       CGGGAAAGGCTACTCTTACCCATACTAAAACATGTTAGATTGATCTAGAA
HUMAN P53       CAG--------------------CTGGGGC------------------
                * *                     **    *
TROUT P53       TGTGCTTTGATGCAGTATAGAGGGTCCACACTATAAAAGGAGTTTAAACG
HUMAN P53       ----------------TGGAGAG--------ACGACAGGG---------
                              * *** *        *  * ***
TROUT P53       TCATTTATACTTACACTTGTTGCCTATTGAATGAGTAACGCAAATGTTTT
HUMAN P53       --------------CTGGTTGCCCA------GGGT--CCCCAG-GCCT-
                            ** ****** *      * **  * * *  *  *
TROUT P53       GACTGATAGGATATCCTCAGTGTGTCGCTTAATACTCGTGTTTTCTGTGCCTTAG
HUMAN P53       --CTGAT------TCCTCACTG-ATTGCT--------------------CTTAG
                  *****      ****** **  * ***                    *****
                EXON 6
TROUT P53       GTCCTGCCCCGCGAGGTCACCTGGTCAGAGTTGAGGGGAACCAGC
HUMAN P53       GTCTGGCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGC
                ***  ***** *     ** **  ** **** ** ** **   **
TROUT P53       GATCAGAGTATATGGAGGATGGTAACACTCTGAGACACAGTGTGCTCGTC
HUMAN P53       GTGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAGTGTGGTGGTG
                *    ****** **** **  * ****** *  **** ****** * **
TROUT P53       CCCTATGAGCCTCCTCAG
HUMAN P53       CCCTATGAGCCGCCTGAG
                ********** *** **
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Figure 3. – Rainbow trout - Human *p53* exon 5 to 6 sequence alignment. The DNA sequences of trout and human *p53* were aligned using Clustal software. The stars indicate sequence homology.

7 to 9 had 793 bp. The sequences were compared to the equivalent human sequences (EMBL data base accession locator X54156). The sequence and the alignments are shown in *figure* 3 (exon 5 to 6) and *figure* 4 (exon 7 to 9).

DISCUSSION

Several approaches were taken to amplify fragments of the *p53* gene from teleost liver genomic DNA. Specifically, we were interested in amplifying large fragments spanning the conserved exon regions 5-9. The primers used in the PCR assays were non-degenerative oligonucleotide sequences based on the published rainbow trout cDNA sequence (Caron de Fromentel *et al.*, 1992): 5F (547) and 5F (548) were directed at the beginning of exon 5, 6R (827) was directed at the end of exon 6, 7F (847) was directed at the beginning of exon 7, and 9R (1181) and 9R (1168) were towards the end of exon 9. In order to favour the desired products, annealing temperatures and ion concentrations were varied. When unwanted non-specific DNA sequences were amplified, the stringency of the PCR was increased by lowering the magnesium ion concentration in the reaction mixture and/or by increasing the annealing temperature of the PCR. Moreover, to promote selective priming in the first few cycles and avoid accumulation of unwanted products, "touchdown" amplification programs were employed. Alternatively, if the primers did not appear to be amplifying any specific products, the stringency of the PCR was reduced by increasing the potassium ion concentration in the reaction mixture or by reducing the annealing temperature. Successful PCR was performed using primer pairs 5F (548)/6R (827) and 7F (847)/9R (1168). None of the possible primer combinations spanning exons 5 to 9 were successful. The sequence of the two fragments exon 5/6 and 7 to 9 is given in the *figure* 3 and 4. Alignment with the corresponding sequences from human *p53* showed that the exons sequences were highly conserved whereas the intron sequences showed considerable divergence as would be expected. The exon sequences correspond to the sequences published for the *p53* cDNA from rainbow trout (De Fromentel *et al.*, 1992). In addition

Figure 4. – Rainbow trout - Human *p53* exon 7 to 9 sequence alignment. The DNA sequences of trout and human *p53* were aligned using Clustal software. The stars indicate sequence homology.

to studies of mutations in fish species the analysis of sequences in *p53* exons and introns might provide additional clues for the study of molecular evolution in teleost fish.

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