Molecular cloning, gene organization and expression of rainbow trout (*Oncorhynchus mykiss*) inducible nitric oxide synthase (iNOS) gene

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A full-length inducible nitric oxide synthase (*iNOS*) gene has been sequenced for the first time outside the mammals, and the gene organization compared with that already determined for human *iNOS*. While there are some differences from the human gene, overall the exons show remarkable conservation in sequence and organization. As in human, the trout *iNOS* gene has 27 exons, with 18 of the trout exons being identical in size with the equivalent human exons. The cofactor-binding domains are found in the same exons and in some cases are absolutely conserved. Differences include the start of the ORF in exon 3 instead of exon 2, resulting in a deletion at the 5' end of the trout iNOS protein. Exon 27 also shows a large difference in size and although the trout exon is larger this is due to the length of the 3'-UTR. Several non-mammalian features are notable, and include a conserved potential glycosylation site in chicken and fish, and an insertion at the boundary of exons 20 and 21 in fish. The intron sizes in trout were generally much smaller than in

human iNOS, making the trout *iNOS* gene approximately half the size of the human gene. Analysis of RNA secondary structure revealed two regions with complementarity, which could interfere with reverse transcription. Using a trout fibroblast cell line (RTG-2 cells), it was shown by reverse transcriptase (RT)-PCR that virus infection was a good inducer of iNOS expression. However, when using a combination of Superscript™ II for reverse transcription and primers at the 5' end of the gene only very weak products were amplified, in contrast with the situation when primers at the 3' end of the gene were used, or ThermoScript™-derived cDNA was used. The impact of such results on RT-PCR analysis of iNOS expression in trout is discussed.

Key words: exon organization, iNOS cDNA, iNOS expression, phylogenetic analysis, RNA secondary structure.

INTRODUCTION

We have previously reported on the partial cloning and sequencing of rainbow trout (*Oncorhynchus mykiss*; formerly *Salmo gairdneri*) [1] and goldfish (*Carassius auratus*) [2] inducible nitric oxide synthase (iNOS) cDNA, as the first fish (and poikilotherm) sequences reported. Attempts to obtain the full-length rainbow trout iNOS cDNA, by PCR and cDNA library screening approaches, were unsuccessful at that time. Since then a full-length carp (*Cyprinus carpio*) iNOS cDNA has been cloned [3], which has 57% amino acid identity and 60% nucleotide identity with the human and chicken *iNOS* genes. The inability to clone the rainbow trout iNOS transcript may relate to differences in expression level in comparison with other teleost fish groups. Thus in the superorder Ostariophysi, including goldfish [4,5], carp [3] and catfish [6,7], and in the superorder Acanthopterygii, including turbot [8,9] and seabream [10], leucocytes can be readily stimulated to produce nitric oxide. This is not the case with trout (superorder Protacanthopterygii), where detection of the iNOS transcript has required nested PCR following stimulation with equivalent stimuli such as lipopolysaccharide or live bacteria [1,11]. As an alternative approach to obtaining the full-length rainbow trout *iNOS* gene, we have undertaken genomic library screening, and have obtained sequence from two clones that have allowed the full rainbow trout *iNOS* gene to be sequenced, the first outside mammals. This sequence has been used to obtain the full-length rainbow trout cDNA to confirm the exon boundaries. In addition, a method to

elicit strong induction of the rainbow trout iNOS transcript has been found and used to identify one factor that contributes to the difficulty in measuring the expression of this gene in rainbow trout.

EXPERIMENTAL

Cloning and sequencing of the full-length genomic DNA

Nested PCR was used to amplify a rainbow trout iNOS probe for screening of a rainbow trout genomic library. A bacterially stimulated rainbow trout gill cDNA library [12] was used as template, since it has been shown previously that the gill is a site of high iNOS expression in rainbow trout [11]. iNOS-specific primers F6 and R6 were used for the first round of PCR, followed by primers F7 and R7 (see Table 1). A ≈ 1.2 kb fragment was amplified and cloned into the TOPO-TA cloning vector. Sequencing results confirmed that the cloned cDNA was identical with the previously reported rainbow trout partial iNOS sequence [1].

A rainbow trout genomic library in the λGEM-11 vector (Promega) was screened with the ³²P-labelled 1.2 kb probe (Figure 1A). Two independent clones were isolated. One of the clones was digested with *SacI* (Figure 1A) and subcloned into the pGEM 3zf(+) vector (Promega). The full-length sequences of the subclones were obtained using vector- and gene-specific primers, although in some cases the Erase-A-Base system (Promega) was used to produce serial deletions to allow sequencing of the larger (4–5 kb) subclones. To eliminate the

Abbreviations used: iNOS, inducible nitric oxide synthase; VHSV, viral haemorrhagic septicaemia virus; RT, reverse transcriptase; FCS, foetal calf serum; UTR, untranslated region; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession numbers AJ295231 and AJ300555.

Table 1 Primers used for cloning and expression

Name	Gene	Position in the cDNA sequences	Sequence (5' to 3')
INOS F1	iNOS	1–23	TTCCC,GCTCT,CTTGT,CTTCC,TTT
INOS F2	iNOS	282–304	GTGCG,TAACG,TGAAG,GATGG,CTC
INOS F3	iNOS	1475–1497	CTTCT,TCTAC,TACCA,GCCTG,ACC
INOS F4	iNOS	2394–2417	CATAC,GCCCC,CAACA,AACCA,GTGC
INOS F5	iNOS	4098–4419	CGAAT,GGAGC,TATCG,TCAGA,CC
INOS F6	iNOS	1126–1146	GTTGG,TACAT,GGGCA,CTGAG,A
INOS F7	iNOS	1273–1293	TTGCA,GTCA,ATACA,GTTC,C
INOS R1	iNOS	4331–4308	CGGGA,ACGTT,GTGGT,CATAA,TACC
INOS R2	iNOS	2063–2040	CTCCT,TATAA,GCATC,CTTGA,GAGC
INOS R3	iNOS	750–727	CGGTA,ATCGC,AGACC,TTAGG,TTTC
INOS R4	iNOS	515–482	CTCCA,ATCTG,CGGAG,GTGGT,GTTC
INOS R5	iNOS	3139–3116	CCTCG,CCCTC,TCATC,TCCAG,TGTC
INOS R6	iNOS	2533–2513	GTAAT,GTCCA,GGAAG,TAGGT,G
INOS R7	iNOS	2454–2434	TCATT,CGGAA,GGTGT,CAGAA,C
GAPDH F	GAPDH	45–64	ATGTC,AGACC,TCTGT,GTGG
GAPDH R	GAPDH	559–539	TCCTC,GATGC,CGAAG,TTGTC,G
Adapter DT	Kit for RACE-PCR		GCTGT,CAACG,ATACG,CTACG,TAACG,GCATG,ACAGT,G(T) ₁₈
GR3P	Kit for RACE-PCR		GCTGT,CAACG,ATACG,CTACG,TAACG
LGEMF	Vector		CCTCA,CTGGC,CTAAT,ACGAC,TCAC
LGEMR	Vector		CCATT,TAGGT,GACAC,TATAG,AAGAG,C

A: Cloning of Rainbow trout iNOS gene **B: Cloning of Rainbow trout iNOS cDNA** **Figure 1** Schematic diagram showing the cloning strategy used to obtain the rainbow trout iNOS genomic (A) and cDNA (B) sequences

Vertical arrows indicate the *SacI* sites used for subcloning. Horizontal arrows show the relative positions of forward and reverse primers used for PCR (as detailed in Table 1). According to the predicted transcription start site from the genomic sequence, the primers used to obtain the cDNA 5' untranslated region (UTR) begin 43 bp downstream (i.e. the predicted 5' UTR is 228 bp). Shading indicates sequenced regions.

possibility that sequence gaps could exist between the sequenced subclones, and to locate the subclones in the genomic sequence, PCR was performed (see below for conditions) with gene-specific

primers between all subclones using the lambda DNA as template, and the products were sequenced directly with an ABI 377 Automated Sequencer (Applied Biosystems).

Sequence analysis of the lambda clone revealed that the 5' end of the coding region of the trout *iNOS* gene was potentially missing although the main part of the gene, including part of the 3'-flanking region, was cloned (Figure 1A). To isolate the 5' end of the gene, two primers were designed to the 5' end of the known sequence obtained above (F2 and R4, Table 1) and were used to screen the genomic library for further clones containing the *iNOS* gene using a PCR-based strategy [13] (Figure 1A). Briefly, sub-libraries prepared from 4000 plaque-forming units were amplified with primers F2 and R4. Positive sub-libraries were amplified with R4 and one of the two vector primers to determine whether more sequence was present at the 5' end compared with the clone isolated above. A positive sub-library was identified with about 4 kb more sequence at the 5' end, which was used to isolate a single clone. In total, 6595 bp at the 5' end of this clone was sequenced with gene-specific primers to allow overlap with the 5' end of the original lambda clone isolated.

Cloning and sequencing of the full-length cDNA

To clone the full-length rainbow trout *iNOS* cDNA, primers were designed against the known genomic sequence and used for PCR with cDNA produced from RTG-2 cells (a trout fibroblast cell line) [14] after infection with viral haemorrhagic septicaemia virus (VHSV) for 1 day (see expression studies below). Extracted total RNA was reverse-transcribed with ThermoScript[™] reverse transcriptase (RT; Gibco BRL) and primed by an adapter-DT primer (Table 1) ready for PCR. PCR was performed using 2 µl of cDNA template, 5 µl of 10× PCR buffer, 2 µl of 50 mM MgCl₂, 2 µl of 2.5 mM dNTP, 2 µl of each primer (10 µM), 2.5 units of *Taq* polymerase, 0.05 unit of *Pfu* DNA polymerase and the volume made up to 50 µl with water. The cycling protocol was 1 cycle at 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 62 °C for 1 min and 72 °C for 2 min and 1 cycle at 72 °C for 5 min. PCR was performed with primer pairs F1/R2, F3/R1 and F4/GR3P (Figure 1B), and the three overlapping products obtained were cloned into the pGEM-T EASY vector (Promega). At least three clones per product were sequenced with vector primers, with sequence-specific primers being designed to allow further sequencing of the internal sequence of each clone.

Sequence analysis

All sequences generated were analysed with programmes within the Wisconsin Genetics Computer Group (GCG) Sequence Analysis Software Package (version 10, 1999) and the Human Genome Mapping Project Resource Centre (<http://www.hgmp.mrc.ac.uk>). Initial comparison with sequences in the GenBank and EMBL databases were performed using FASTA [15] and BLAST [16], to search for similarity. Direct comparison between two sequences was performed using the GAP program [17], and multiple sequence alignments generated using CLUSTALW (version 1.7) [18]. Patterns defined by the *PROSITE* Dictionary of Protein Sites and Patterns (<http://www.expasy.org/tools/scnpsite.html>) were searched for using the MOTIFS program. Phylogenetic trees were created by the neighbour-joining method [19] within the CLUSTAL package using NOS amino acid sequences, and was bootstrapped 1000 times. Analysis of RNA secondary structure was performed with GeneBee [20].

Expression studies

To study further factors able to induce *iNOS* expression in rainbow trout, viral stimulation was used, since previous studies

with turbot leucocytes have shown that this is an efficient means of eliciting nitric oxide *in vitro* [8]. VHSV was obtained from the Marine Laboratory, Aberdeen, U.K., and passaged in CHSE-214 cells cultured in L15 medium (Gibco BRL) plus 1% foetal calf serum (FCS, Gibco BRL) at a low multiplicity of infection [0.01 TCID₅₀ (tissue-culture infective dose 50)/cell]. After 5 days incubation at 22 °C, the virus was harvested and separated from cell debris by centrifugation at 6000 g for 30 min. The VHSV was titrated in CHSE-214 cells, aliquoted and stored at -70 °C ready for use. For *iNOS* induction, RTG-2 cells in L15 medium, 10% FCS, 100 units/ml penicillin and 100 µg·ml⁻¹ streptomycin, at 80% confluence, were infected with VHSV at a high multiplicity of infection (100 TCID₅₀/cell). After 2 h the cells were washed to remove unattached virus, and cultured for a further 6, 22 or 46 h in L15 medium plus 1% FCS prior to RNA extraction.

Total RNA was prepared from cell cultures for RT-PCR by homogenizing 10⁷ cells with 1.8 ml of RNazol (Biogenesis). Chloroform (0.18 ml) was then added and the homogenate shaken vigorously for 15 s. After settling on ice for 5 min, the aqueous phase was separated by centrifugation (12000 g, 15 min) and the RNA precipitated by addition of an equal volume of isopropanol. The RNA was washed with 75% ethanol and dissolved in 0.1 ml of nuclease-free water.

Reverse transcription was performed with ThermoScript[™] RT, allowing relatively high temperatures to be used. Total RNA (5 µg), 50 pmol of oligo(dT)₂₀, 4 µl of 5× cDNA synthesis buffer, 1 µl of 0.1 M dithiothreitol, 40 units of RNase OUT, 2 µl of 10 mM dNTP and 15 units of ThermoScript[™] RT were combined and the volume adjusted to 20 µl with water. The mixture was then incubated at 60 °C for 20 min followed by 65 °C for 50 min, and reverse transcription terminated by incubation at 85 °C for 5 min. The cDNA was treated with 2 units of RNase H at 37 °C for 20 min prior to PCR.

Initially, PCR was performed with primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1), which were used as a positive control for RT-PCR since the gene is expressed constitutively. The resulting PCR products obtained using 22 cycles of amplification, which was within the linear phase of signal amplification for GAPDH, allowed titration of the amount of template subsequently used for PCR to generate consistent amounts of product with these primers between samples. PCR with *iNOS*-specific primers F2 and R3 (product size 469 bp) was then carried out, and products visualized on a 1.6% agarose gel containing ethidium bromide (0.5 µg·ml⁻¹). PCR conditions were as above except that the elongation time was reduced to 30 s.

Having established a reproducible method for induction of *iNOS* expression in RTG cells, this system was used to study whether factors such as predicted RNA secondary structure may influence the ability to detect expression of this gene in rainbow trout. Using primers F2 and R3 at the 5' end of the trout *iNOS* gene, primers F5 and R1 at the 3' end of the gene (product size 234 bp) or primers F4 and R5 between these two primer pairs (product size 746 bp), PCR was undertaken with cDNA prepared as above or prepared with Superscript[™] II (Gibco BRL) for reverse transcription, using DNase-treated RNA (since primers F5 and R1 do not cross an intron) from challenged (VHSV for 24 h) or unchallenged RTG-2 cells. For reverse transcription with Superscript[™] II, 1 µl of oligo(dT)₁₂₋₁₈ (500 µg·ml⁻¹) and 5 µg of total RNA in 12 µl of water were incubated at 70 °C for 10 min and then chilled on ice. Next, 4 µl of 5× cDNA synthesis buffer, 2 µl of 0.1 M dithiothreitol and 1 µl of 10 mM dNTP mix were added and the mixture was incubated at 42 °C for 2 min. Superscript[™] II (1 µl; 200 units) was then added and the mix

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incubated at 42 °C for a further 50 min. Reverse transcription was halted by incubation at 75 °C for 15 min. Following PCR with GAPDH primers, the amount of template to be used was again titrated and then used for PCR with the iNOS primers. As above, products were visualized on agarose gels containing ethidium bromide. Preliminary PCR studies using random hexamer-primed cDNA prepared with Superscript[®] II showed that in general this was a less efficient method of producing the cDNA compared with using oligo(dT), with all primer combinations tested, and was not used further.

RESULTS

Following digestion of the isolated lambda clone with *SacI*, seven subclones were prepared and sequenced, giving 18602 bp of sequence. In case some cleavage sites gave rise to small products that were not cloned, PCR was performed between all subclones, and in three cases extra sequence was found between clones, of 38, 227 and 228 bp. The compiled contiguous sequence

of the lambda clone measured 19095 bp and was found to contain nine *SacI* sites, as predicted from the above results (Figure 1A). This sequence contained 24 putative exons, according to the homology with other *iNOS* genes, with two potential polyadenylation sites within the last predicted exon. Unfortunately it did not contain the putative 5' end of the trout *iNOS* gene, and thus a further lambda clone containing *iNOS* sequence was identified using a PCR screening strategy with primers to the 5' end of the above sequence. The second lambda clone was ≈ 20 kb in length, and 6595 bp at the 5' end was sequenced, giving 2455 bp overlap (with complete identity) with the first lambda clone isolated and a contiguous sequence of 23235 bp (Figure 1A). The second lambda clone had a predicted TATA box at 3973 bp and a predicted transcription start at 4007 bp, with the compiled full-length *iNOS* gene measuring 18157 bp (GenBank accession no. AJ295231).

The full-length trout iNOS cDNA, prepared from VHSV-infected RTG-2 cells, was sequenced to allow identification of the exon/intron boundaries of the above genomic sequence.

Figure 3 Multiple alignment of the predicted trout iNOS translation with other known iNOS sequences (human sequence represents a typical mammalian sequence)

Asterisks indicate gaps in the alignment and dashes indicate amino acids common to the trout sequence. Conserved cofactor-binding sites are boxed in grey and a conserved, non-mammalian, potential glycosylation site is shown in bold and underlined.

Figure 4 Unrooted phylogenetic tree showing the relationship between the trout iNOS amino acid sequence and other published NOS isoforms

The tree was created by the neighbour-joining method and bootstrapped 1000 times. Bootstrap values of the branches are given inside the circles. eNOS, endothelial NOS.

Three overlapping PCR products were prepared using gene-specific primers, from the 5' UTR (43 bp from the predicted transcription start) of the *iNOS* gene to the polyadenylation tail, the latter requiring the use of adaptor primers in 3' rapid amplification of cDNA ends (RACE)-PCR (Figure 1B). The contiguous sequence was 4381 bp, containing 185 bp of 5' UTR, a 3252 bp ORF (coding for 1083 amino acids) and 924 bp of 3' UTR upstream of a 20 bp polyadenylation tail (Figure 2; GenBank accession no. AJ300555). The 3' UTR contained a potential polyadenylation signal 27 bp after the stop codon, but no clones were found with a downstream polyadenylation tail. In contrast, the polyadenylation signal at 4334 bp was 22 bp upstream of the polyadenylation tail. Five mRNA instability motifs (ATTTA) were also present within the 3' UTR. The cDNA sequence showed 62–64% nucleotide identity with known mammalian and chicken iNOS sequences, and 70% identity with the carp sequence, with lower identities with other NOS isoforms. The predicted amino acid sequence had 58–60% identity with homeotherm iNOS sequences and 69% identity with the carp sequence, and a predicted molecular mass of 123 kDa. Multiple alignment of the predicted trout iNOS amino acid sequence with other known sequences showed good homology throughout the sequence, with clearly identifiable cofactor-binding domains (the haem and NADPH domains being absolutely conserved; Figure 3). However, the 5' end of the trout iNOS cDNA was relatively short compared with all other known iNOS sequences. One potential glycosylation site was present within the trout iNOS sequence, in common with the other non-mammalian iNOS

sequences. Phylogenetic analysis of the amino acid sequences of all known *NOS* genes showed the trout *iNOS* gene branched with carp *iNOS* and grouped with the mammalian and chicken *iNOS* sequences (Figure 4). Lastly, analysis of the mRNA secondary structure with GeneBee revealed two 13 bp regions with complementarity (Figure 2), where a melting point of > 42 °C would be expected.

Using the cDNA sequence it was possible to determine that the trout *iNOS* gene contained 27 exons, in agreement with the known mammalian gene (Table 2). All the intron/exon boundaries in the trout sequence conformed strictly to the known GT/AG donor/acceptor site rule. Remarkably, the intron phase was identical between the human and trout *iNOS* genes with the exception of intron 2, and the cofactor-binding domains were located within identical exons (Table 2). The main difference was the beginning of the open reading frame, which started within exon 2 of the human *iNOS* gene but at the beginning of exon 3 in trout. In many cases exon sizes were also identical, with the trout exons 1–5 being somewhat smaller than in human, and trout exons 20–22 and 27 being somewhat larger. In contrast, the trout intron sizes were generally much smaller than in the human *iNOS* gene, with only introns 7, 8, 10, 13 and 16 being larger in trout. This made the trout *iNOS* gene considerably smaller ($\approx 50\%$) than the human gene, ≈ 18 kb compared with 38 kb for human *iNOS*.

Infection of RTG-2 cells with VHSV was shown to be a good inducer of trout iNOS expression, where expression was detectable at 24 h and 48 h post-infection (Figure 5). Using 24 h

Table 2 Comparison of the intron/exon sizes and intron phase of the *iNOS* gene between human and rainbow trout

Exon sizes in bold indicate differences between the human and trout genes.

Exon no.	Exon size (bp) and features				Intron phase			Intron size (bp)	
	Human		Trout		No.	Human	Trout	Human	Trout
1	192	5' UTR	122*	5' UTR	1	II	II	1500	1021
2	183	5' UTR/Start	106	5' UTR	2	II	0	1000	273
3	85		57	Start	3	0	0	700	96
4	123		99		4	0	0	1000	188
5	149	Zn binding	140	Zn binding	5	II	II	6000	443
6	163	Haem	163	Haem	6	0	0	800	170
7	92		92		7	II	II	900	1852
8	142		142		8	0	0	129	153
9	140		140		9	II	II	1800	809
10	175		175		10	0	0	90	223
11	102		102		11	0	0	4000	2883
12	195	Tetrahydrobiopterin	195	Tetrahydrobiopterin	12	0	0	900	122
13	83	Calmodulin	83	Calmodulin	13	II	II	600	1904
14	145	Calmodulin	145	Calmodulin	14	0	0	1200	334
15	105		105		15	0	0	1500	108
16	50		50		16	II	II	400	1612
17	175	FMN binding	175	FMN binding	17	0	0	1200	247
18	133		133		18	I	I	1600	124
19	79		79		19	II	II	800	106
20	182	FAD (ADP)	191	FAD (ADP)	20	I	I	1500	128
21	164		170		21	0	0	1000	206
22	208	FAD (flavin)	211	FAD (flavin)	22	I	I	1700	187
23	88		88		23	II	II	400	148
24	122	NADP (ribose)	122	NADP (ribose)	24	I	I	600	107
25	149		149		25	0	0	1000	165
26	195	NADP (ADP)	195	NADP (ADP)	26	0	0	1400	134
27	606	Stop/3' UTR	975	Stop/3' UTR					

* Determined from the predicated transcription start site from the genomic sequence.

Figure 5 RT-PCR analysis of *iNOS* expression in cDNA samples produced with ThermoScript™, from RTG-2 cells infected with VHSV for various timesPCR was performed with *iNOS* primers F2 and R3 (product size 469 bp) for 35 cycles or with GAPDH primers (515 bp) for 22 cycles. C, control uninfected cells; M, 100 bp DNA ladder.

infected samples, the impact of the predicted secondary structure was studied by comparing cDNA produced using ThermoScript™ or Superscript™ II for reverse transcription. The latter reverse-transcribes at 42 °C, below the predicted melting point of the hairpin identified. Using primers F5 and R1 at the 3' end of the gene (before the secondary structure, Figure 6A), clear *iNOS* products were seen in both the uninfected and infected samples, with the latter showing evidence of induction by infection, whether using ThermoScript™ or Superscript™ (Figure 6B). However, to be at the 3' end of the gene these primers were located in the final exon and hence there was a risk that, although DNase treatment of the RNA was carried out, genomic DNA

contamination could have affected the result. Thus a further primer pair (F4 and R5) was used that crossed introns (20–24) but that was located between the two regions of secondary structure (Figure 6A). Using this primer pair very similar results were obtained, with clear products being detectable whether using cDNA reverse transcribed with ThermoScript™ or Superscript™ (Figure 6C). In contrast, when primers F2 and R3 were used, located upstream of the secondary structure, a marked difference between the ThermoScript™- or Superscript™-produced cDNA was apparent (Figure 6D). In this instance only the samples reverse transcribed with ThermoScript™ gave a strong product after infection with VHSV.

DISCUSSION

A full-length *iNOS* gene has been sequenced for the first time outside the mammals, and the gene organization compared with that already determined for human *iNOS*. Whereas there are some differences from the human gene, overall the exons show remarkable conservation in sequence and organization. As in human, the trout *iNOS* gene has 27 exons, with 18 of the trout exons being identical in size with the equivalent human exons [21]. The cofactor-binding domains are found in the same exons and in some cases, as with the haem and NADPH domains, are absolutely conserved. Differences at the 5' end of the gene, including the start of the ORF in exon 3 instead of exon 2, result in a deletion at the 5' end of the trout *iNOS* protein, as seen in the multiple alignment. This is not a non-mammalian or fish phenomenon, since both the chicken [22] and carp [3] cDNA sequences are full length. However, in the carp there are three

Figure 6 RT-PCR analysis of iNOS expression in cDNA samples produced with ThermoScript[™] or SuperScript[™], from RTG-2 cells infected with VHSV for 24 h

(A) PCR was performed with three pairs of iNOS primers in different positions relative to the predicted RNA secondary structure. Products obtained with primers F5 and R1 (product size 234 bp), primers F4 and R5 (746 bp) or primers F2 and R3 (469 bp) for 35 cycles, or with GAPDH primers (515 bp) for 22 cycles, are shown in (B) to (E) respectively. C, control uninfected cells; I, VHSV-infected cells; NC, no template control; M, 100 bp DNA ladder.

repeats in this region, of TPN^{TQ}/_MCENNNVIL, which if deleted would give a molecule similar in length to that in the trout. Exon 27 also shows a large difference in size between trout and human, and although the trout exon is larger this is due to the 3' UTR, since the trout iNOS protein is also short at the 3' end relative to mammals (51 bp of coding sequence in trout exon 27 compared with 108 bp in human), in common with carp. A couple of other non-mammalian features are also present, notably the conserved potential glycosylation site in chicken, carp and trout, and an insertion of several amino acids (CRDD in trout) at the boundary of exons 20 and 21 in both fish species.

Whereas the gene organization and exon sizes are similar, the trout introns are typically smaller than in the human gene, resulting in a gene that is $\approx 50\%$ smaller (18 kb versus 38 kb). All of the intron/exon boundaries conform strictly to the GT/AG donor/acceptor site rule, as seen in the human gene [21], and only one trout intron has a different intron phase. Indeed, the

intron phase is generally conserved across all three NOS isoforms [21], with many exons containing critical cofactor-binding domains having an intron splice type O with the potential to allow insertion or deletion events without disrupting the reading frame.

In these studies virus was found to be a good inducer of iNOS expression, as predicted from studies in other fish species where nitric oxide production had been measured [8]. Transcript was readily detectable 24–48 h after infection of the RTG-2 cells, similar to the kinetics of virus-induced iNOS expression in mammals [23]. This relatively late induction implies that this is an indirect effect, possibly dependent upon earlier cytokine (interferon?) [24] release. In carp, transcript can be detected as early as 4 h post-stimulation with lipopolysaccharide, with maximum transcription apparent between 4 and 12 h post-stimulation [3]. Stimulation of carp phagocytes with the blood flagellate *Trypanoplasma borelli* has a similar effect, although the peak

in transcription is somewhat later (12–24 h). That the induced transcript results in the appearance of a functional enzyme capable of generating nitric oxide has been implicated from previous *in vivo* studies on rainbow trout [11], and supported by studies in the goldfish, where a direct correlation between induced transcript and detectable nitric oxide was shown using a goldfish macrophage cell line [2,4]. Whereas virus-induced iNOS expression may be beneficial to host defences [25,26], there are many examples where nitric oxide production contributes to the immunopathological consequences of virus infection [27]. Clearly the scenario in fish remains to be determined.

The virus-induced expression of iNOS in trout RTG-2 cells was used to study further the apparent difficulty in detecting expression in this species. From sequencing of the iNOS cDNA it was apparent that a region of secondary structure was predicted in the RNA that could interfere with reverse transcription at low (42 °C) temperatures. To examine this possibility, reverse transcription was performed with ThermoScript[™] or Superscript[™] enzymes, and the resulting cDNA tested in PCR with primers before, between or after the regions of predicted secondary structure. This experiment clearly showed that when using Superscript[™] and primers at the 5' end of the gene a very weak product was amplified in the virus-stimulated samples. In contrast, primers at the 3' end gave products comparable with those seen using ThermoScript[™]-derived cDNA. No such difference was seen if primers were located at the 3' end of the gene, but care about genomic DNA contamination of samples is needed. Thus whereas differences may well exist between fish species, with respect to the ease of induction of iNOS expression, it is also apparent that secondary structure may contribute to the apparent low-level expression seen previously in trout, where nested PCR was needed to obtain detectable products [11]. Nevertheless, detection of nitric oxide in supernatants from trout cells with detectable iNOS transcript is very low, and perhaps the mRNA instability motifs in the 3' UTR contribute to a rapid degradation of the produced mRNA. To study this further, we are currently cloning the trout iNOS 5'-flanking region into a reporter gene construct to examine in detail factors influencing the expression of iNOS in trout cells.

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REFERENCES

- Laing, K. J., Hardie, L. J., Aartsen, W., Grabowski, P. S. and Secombes, C. J. (1999) Expression of an inducible nitric oxide synthase gene in rainbow trout *Oncorhynchus mykiss*. *Dev. Comp. Immunol.* **23**, 71–85.
- Laing, K. J., Grabowski, P. S., Belosevic, M. and Secombes, C. J. (1996) A partial sequence for nitric oxide synthase from a goldfish (*Carassius auratus*) macrophage cell line. *Immunol. Cell Biol.* **74**, 374–379.
- Saeij, J. P. J., Stet, R. J. M., Groeneveld, A., Verburg van Kemenade, L. B. M., van Muiswinkel, W. B. and Wiergertjes, G. F. (2000) Molecular and functional characterization of a fish inducible-type nitric oxide synthase. *Immunogenetics* **51**, 339–346.
- Neumann, N. F., Fagan, D. and Belosevic, M. (1995) Macrophage activating factor(s) secreted by mitogen stimulated goldfish kidney leukocytes synergize with bacterial lipopolysaccharide to induce nitric oxide production in teleost macrophages. *Dev. Comp. Immunol.* **19**, 473–482.
- Neumann, N. F. and Belosevic, M. (1996) Deactivation of primed respiratory burst response of goldfish macrophages by leukocyte-derived macrophage activating factor(s). *Dev. Comp. Immunol.* **20**, 427–439.
- Schoor, W. P. and Plumb, J. A. (1994) Induction of nitric oxide synthase in channel catfish *Ictalurus punctatus* by *Edwardsiella ictaluri*. *Dis. Aquat. Organisms* **19**, 153–155.
- Yin, Z., Lam, T. J. and Sin, Y. M. (1997) Cytokine-mediated antimicrobial immune responses of catfish, *Clarias gariepinus*, as a defence against *Aeromonas hydrophila*. *Fish Shellfish Immunol.* **7**, 93–104.
- Tafalla, C., Figueras, A. and Novoa, B. (1999) Role of nitric oxide on the replication of viral haemorrhagic septicemia virus (VHSV), a fish rhabdovirus. *Vet. Immunol. Immunopathol.* **72**, 249–256.
- Tafalla, C. and Novoa, B. (2000) Requirements for nitric oxide production by turbot (*Scophthalmus maximus*) head kidney macrophages. *Dev. Comp. Immunol.* **24**, 623–631.
- Mulero, V. and Meseguer, J. (1998) Functional characterisation of a macrophage-activating factor produced by leucocytes of gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol.* **8**, 143–156.
- Campos-Perez, J. J., Ward, M., Grabowski, P. S., Ellis, A. E. and Secombes, C. J. (2000) The gills are an important site of iNOS expression in rainbow trout *Oncorhynchus mykiss* after challenge with the Gram-positive pathogen *Renibacterium salmoninarum*. *Immunology* **99**, 153–161.
- Laing, K. J. (1996) Detection of nitric oxide synthase gene expression in rainbow trout. M.Sc. thesis, University of Aberdeen.
- Bigby, T. D., Hodulick, C. R., Arden, K. C. and Fu, L. (1996) Molecular cloning of the human leukotriene C4 synthase gene and assignment to chromosome 5q35. *Mol. Med.* **2**, 637–646.
- Wolf, K. and Quimby, M. C. (1962) Established eurythermic line of fish cells *in vitro*. *Science* **135**, 1065–1066.
- Pearson, W. R. and Lipman, D. I. (1988) Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2444–2448.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. and Lipman, D. J. (1990) Best local alignment search tool. *J. Mol. Biol.* **215**, 403–410.
- Needleman, S. B. and Wunsch, C. D. (1970) A general method applicable to the search for similarities in the amino acid sequence of two proteins. *J. Mol. Biol.* **48**, 443–453.
- Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680.
- Saitou, N. and Nei, M. (1987) The neighbor-joining method – a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**, 406–425.
- Brodsky, L. I., Ivanov, V. V., Kalaidzidis, Y. L., Leontovich, A. M., Nikolaev, V. K., Feranchuk, S. I. and Drachev, V. A. (1995) GeneBee-NET: internet-based server for analyzing biopolymers structure. *Biochemistry (Moscow)* **60**, 923–928.
- Xu, W., Charles, I. G., Liu, L., Moncada, S. and Emson, P. (1996) Molecular cloning and structural organization of the human inducible nitric oxide synthase gene (NOS2). *Biochem. Biophys. Res. Commun.* **219**, 784–788.
- Lin, A. W., Chang, C. C. and McCormick, C. C. (1996) Molecular cloning and expression of an avian macrophage nitric-oxide synthase cDNA and the analysis of the genomic 5'-flanking region. *J. Biol. Chem.* **271**, 11911–11919.
- Gupta, P. K., Saini, M., Gupta, L. K. and Garg, S. K. (1999) Induction of nitric oxide in bovine peripheral blood mononuclear cells by bovine herpesvirus 1. *Acta Virologica* **43**, 321–324.
- Esteban, M. and Patino, C. (2000) Identification by electron microscopy of the maturation steps in vaccinia virus morphogenesis inhibited by the interferon-induced enzymes, protein kinase (PKR), 2–5A synthetase, and nitric oxide synthase (iNOS). *J. Int. Cytokine Res.* **20**, 867–877.
- Guidotti, L. G., McClary, H., Loudis, J. M. and Chisari, F. V. (2000) Nitric oxide inhibits hepatitis B virus replication in the livers of transgenic mice. *J. Exp. Med.* **191**, 1247–1252.
- Xing, Z. and Schat, K. A. (2000) Inhibitory effects of nitric oxide and gamma interferon on *in vitro* and *in vivo* replication of Marek's disease virus. *J. Virol.* **74**, 3605–3612.
- Akaike, T. and Maeda, H. (2000) Nitric oxide and virus infection. *Immunology* **101**, 300–308.