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Characterisation of the differentially regulated trout protein 1 gene (DRTP1) in rainbow trout *(Oncorhynchus mykiss)*

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Abstract

Increased levels of differentially regulated trout protein 1 (DRTP1) mRNA transcripts have been reported in fish after activation of the acute phase response. While the function of the DRTP1 protein still remains to be elucidated, this study focused on the genomic organisation of the gene, the quantification of the DRTP1 transcript in various tissues, and the isolation and analysis the 5' regulatory region of the DRTP1 gene in rainbow trout (*Oncorhynchus mykiss*). Analysis of the DRTP1 genomic and cDNA sequences showed the gene to consist of four exons separated by three introns. Tissue localisation of the DRTP1 gene was performed by Northern analysis and validated by quantitative real-time PCR (qPCR). Six tissues (liver, intestine, spleen, brain, pituitary, and hypothalamus) were analysed. The tissues with the most abundant transcripts were the liver and the pituitary, with lesser amounts detected in the intestine, hypothalamus, brain and spleen. Genome walking allowed the isolation of a 934 bp sequence of the 5' regulatory region of the gene which was cloned, sequenced and in which potential

transcription factor binding were sites identified. Promoter fragments of decreasing size were generated and transiently transfected into the human heptatoma cell line (HepG2). Inducibility of the promoter was determined by stimulation of the HepG2 cells containing the constructs with dexamethasone, polyinosinic:polycytidylic acid (poly I:C) and lipopolysaccharide (LPS) and tumor necrosis factor alpha (TNF-α). One construct, containing two potential C-EBP/β sites and two NF-κB sites, exhibited the highest promoter induction (6.34 fold ± SEM 0.5) when stimulated with human TNFα. A slightly shorter fragment containing one C-EBP/β site and one NF-κB site did not show any significant inducibility when treated with TNFα. The loss of the C-EBP/β and NF-κB in the shorter construct suggests that these sites either individually or in combination are critical for the induction of the DRTP1 promoter by TNFα.

1.   Introduction

Genomic techniques for studying gene expression at the mRNA level include the more traditional methods of Northern blotting, RNase protection and Differential Display-polymerase chain reaction (DD-PCR) with, more recent techniques including Suppression Subtractive Hybridisation (SSH), microarray technology and quantitative real-time PCR (qRT- PCR). Using these techniques a newly identified and as yet uncharacterised gene, DRTP1, was found to be differentially regulated in a variety of fish tissues after the induction of the acute phase response.

DRTP1 was first identified in Brook trout (*Salvelinus fontanalis*) (GenBank accession no. **AF004521**) as a differentially expresssed gene upon stimulation with phorbol

myristate acetate (PMA), which is known to effect several ovarian processes in fish, including ovulation, prostaglandin synthesis and steroidogenesis. The result of DD-PCR carried out on ovarian follicles reported that DRTP1 mRNA was suppressed after treatment with PMA [1]. DRTP1 was also identified and cloned in rainbow trout liver (GenBank accession no. **AF281355**) SSH libraries when livers from unstimulated trout were compared with livers from trout challenged with *Vibrio anguillarum.* DRTP1 was identified as the most abundant clone in the resulting liver SSH library [2]. Through the use of an oligo nucleotide cDNA microarray combined with quantitative real-time PCR (qPCR) these authors further validated the increase in DRTP1 gene expression [3]. Using the SSH technique, DRTP1 was also identified in Atlantic salmon where it was reported to be up-regulated in the liver, spleen and head kidney after challenge with the pathogen *Aeromonas salmonicida* [4], which was confirmed in a further study by cDNA microarray technology [5]. A more recent study reported DRTP1 to be upregulated in the liver and gill of Atlantic salmon, again after challenge with *Aeromonas salmonicida* [6].

DRTP1 is classified as a member of the Ly-6/uPAR protein family. The Ly-6/uPAR superfamily of receptor and secreted proteins contains a carboxy-terminal consensus sequence motif of CCXXXXCN and one or several repeats of the Ly-6/uPAR domain, characterised by a distinct disulfide bonding pattern between of 8 or 10 cysteine residues [7, 8]. The urokinase-type plasminogen activator receptor (uPAR) contains three copies of the Ly-6/uPAR domain whereas other members of this protein family, including CD59, snake neurotoxins, frog cytotoxins, and DRTP1 contain a single Ly-6/uPAR domain. The Ly-6/uPAR family can be classified into two subfamilies based on the presence or absence of a glycosylphosphatidylinositol (GPI)-anchoring sequence [9]. The first subfamily contains 10 cysteine residues and includes CD59, uPAR and

Ly-6. The second subfamily is characterised by the lack of a GPI-signalling sequence and the presence of at least 8 of the 10 cysteine residues. Members of the second subfamily include the single-domain snake and frog cytotoxins, and the recently identified secreted mammalian Ly-6/uPAR-related proteins (SLURP) [10,11,12]. Analysis of the DRTP1 protein would suggest that it belongs to the second Ly-6/uPAR subfamily. The objective of the current study was to investigate the regulation of the DRTP1 gene as a step towards the elucidation of a possible function for the DRTP1 protein.

2.   Materials and Methods

2.1   *Establishing the confinement*

The confinement stress experiments were carried out at the National Environmental Research Council (NERC) Centre for Ecology and Hydrology facility at Lake Windermere, Cumbria in May 2004. Female rainbow trout (Borrowdale strain) were sourced locally and allowed to acclimatise in 1200 L outdoor circular tanks, supplied with 35 litres/min lake water, for three weeks prior to the start of the experiment. The fish were 1-2 years in age, 250-350g in weight and 28-32 cm in size. Prior to the start of the confinement the fish were fed once daily with commercial trout pellets (Skretting Standard Expanded 40) at a rate of 1-2 % body weight per day. Fish were not fed for the duration of the confinement experiment. Water temperature during the course of the confinement ranged from of 14-16 ^{o}C. Water flow in the control circular tanks was 25 L/min and in the confined rectangular 50 L tanks was 5-10 L/min. The photoperiod was natural. Confinement conditions of 65 g/L were established in 50 L rectangular tanks while the control fish were maintained in the 1200 L tanks at a density of 10 g/L.

2.2 Tissue Sampling

Fish were removed from the tank by the use of a small hand-held net before being placed in a solution of water and anaesthetic 2-phenoxy-ethanol (1:2000 dilution). Liver, brain, pituitary, hypothalamus, intestine and spleen tissues were extracted, placed in RNA*later*® (Sigma) and frozen at -80 ^{o}C.

2.3 DNA and RNA extractions

Genomic DNA was isolated using a published protocol [13]. RNA was extracted from tissue using the RNeasy® Maxi Kit for Total RNA Isolation (Qiagen Ltd) as detailed in the manufacturer's instructions. The quantity and quality of nucleic acids were determined using the Bioanalyser 2100 (Agilent). RNA integrity numbers (RIN) of 8 and higher were taken as a measure of RNA quality.

2.4 Generation of DRTP1 cDNA and genomic DNA templates

cDNA synthesis was carried out using the primer set (DRTP1Full_S/ DRTP1Full_AS) which spans the region from + 9 to + 417 of the 455 nucleotide rainbow trout (*O. mykiss*) DRTP1 cDNA sequence (GenBank accession no. **AF281355**). The cDNA and genomic PCR products were cloned into the pCR®2.1 vector (Invitrogen) and transformed into One Shot Top10™ chemically competent cells (Invitrogen). DRTP1-positive colonies were identified by colony PCR. Plasmids were isolated from potential positives and confirmed by sequencing (MWG Biotech, Germany). The genomic and cDNA sequences were manually aligned in Vector NTI and the intron/exon organisation determined. The NetGene2 program for splice site prediction was also used for additional confirmation (http://www.cbbs.dtu.dk/services/NetGene2/).

2.5   Generation of radioactive probes

Radioactive probes were generated from a purified PCR product generated using pCR®2.1 incorporating the + 9 to + 417 sequence of the DRTP1 cDNA as template. PCR reactions were made up to 50 µl volume containing 5 µl NH_4 10X buffer, 1.5 µl 50 mM $MgCl_2$, 1 µl 10 mM dNTPs, 1 µl of 10 µM DRTP1Full_S/DRTP1Full_AS primers and 1 µl (5 units) BIOTAQ™ DNA polymerase (Bioline). PCR conditions were 94 ^{o}C for 2 min, followed by 35 cycles of 94 ^{o}C for 30 s, annealing temperature of 55-65 ^{o}C (depending on the primer pair) for 30 s, 72 ^{o}C for 45 s followed by 72 ^{o}C for 5 min. PCR reactions were carried out on a Peltier Thermal Cycler PTC-225 (MJ Research). PCR products were gel purified using the QIAquick™ Gel Extraction Kit (Qiagen). For the generation of radioactive probes PCR product (25ng) in a final volume of 11 µl was denatured at 95 ^{o}C for 10 min, before 4 µl of High Prime (Roche Diagnostics) and 5 µl (50 µCi) [α^{32}P] dCTP (6000 Ci / mmol) was added, incubated for 1 h at 37 ^{o}C before inactivation by heating at 65 ^{o}C for 10 min. The probes were purified using the ProbeQuant™ G-50 Micro Columns (Amersham). Rainbow trout 18S ribosomal RNA (GenBank accession no. **AF308735**) was used as a housekeeping gene for Northern analysis of the various trout tissues.

2.6   Northern analysis and hybridisation

Total RNA from control, non-confined rainbow trout tissue was separated on denaturing formaldehyde gels containing 1% agarose in 1X MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA) and 2.2 M formaldehyde. RNA samples (5 µg RNA / lane) were denatured for 10 min at 65 ^{o}C in 0.5X MOPS, 2 M formaldehyde, 50% formamide containing 10 ng ethidium bromide. One tenth volume of 10X loading dye (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue) was added to each sample prior

to loading on the gel. Electrophoresis was carried out at 65 volts for 4 to 5 h and RNA

was transferred to nylon membranes (Amersham) by capillary action according to the

procedure set out in Molecular Cloning [14]. Pre-hybridisation (4 h) and hybridisation

(16 to18 h) were carried out in ULTRAhyb™ (Ambion) at 65 ^{o}C. Filters were washed at

65 ^{o}C for 10 min in 2X SSC/0.5% SDS, 15 min in 2X SSC/0.1% SDS, and 30 min in

0.1% SSC/0.5% SDS. Following washing, blots were sealed and exposed to X-ray film

at -80 ^{o}C.

2.7 *Primer design*

Primers were designed using Vector NTI Suite 6 (Invitrogen) and commercially

synthesised (MWG Biotech, Germany). Primers used in this study are listed in Table 1.

2.8 *Quantitative PCR using a cRNA standard curve*

A primer pair was designed to incorporate a T7 promoter upstream of the DRTP1

fragment (see Table 1) (T7 DRTP1-S and T7 DRTP1-AS). The PCR product was

amplified from the pCR$^{®}$2.1 clone incorporating the $+ 9$ to $+ 417$ sequence of the trout

DRTP1 cDNA. PCR reactions were made up to 50 μl volume containing 5 μl NH$_4$ 10X

buffer, 1.5 μl 50 mM MgCl$_2$, 1 μl 10 mM dNTPs, 1 μl 10 μM of each primer and 1 μl

(5 units) BIOTAQ™ DNA polymerase. The PCR product was gel purified using the

QIAquick™ Gel Extraction Kit (Qiagen) and added as template for cRNA synthesis

using the MEGAscript High$^{®}$Yield Transcription Kit (Ambion). cRNA transcription

was carried out in a volume of 20 μl containing 2 μl reaction buffer, 2 μl of 75 mM

ATP, CTP, GTP, and UTP, 1 μg of DNA template and 2 μl enzyme mix. The reaction

was incubated at 37 ^{o}C for 3 h, followed by DNase digestion for 15 min. The cRNA was

purified using NucAway™ spin columns (Ambion), quantified using a Bioanalyser

2100 (Agilent) and the number of DRTP1 cRNA molecules/µl was calculated. A cRNA

standard curve (4.0×10^2 to 4.0×10^8 copies) for absolute quantification was generated.

Quantitative PCR assays were performed in the Mx3000P™ Real-Time PCR System

(Stratagene, La Jolla, CA, USA) using the RT-SYBR Green Kit (Qiagen). Reverse

transcription and PCR reactions were carried out in a 20 µl total reaction volume

consisting of 10 µl of 2X QuantiTect® SYBR Green RT-PCR master mix, 0.2 µl

QuantiTect® RT mix, 1 µl of 10 µM sense and anti-sense gene specific primers, 2.8 µl

dH_2O and 5 µl of total RNA template (10 ng/µl). The thermal profile of the Mx3000P™

system (Stratagene) was set up to include a reverse transcription step of 50 ^{o}C for 30

min, 95 ^{o}C for 15 min, followed by 40 cycles of 94 ^{o}C for 15 s and 60 ^{o}C for 30 s and

72 ^{o}C for 30 s. In order to detect the presence of non-specific amplification, control

reactions containing all the reaction components except for the template were included

for each primer set. At the end of each amplification cycle DNA melt curve analysis

was performed using a ramping rate of 1°C/min over a temperature range of 60 to 95 ^{o}C.

Specificity of the PCR reaction was confirmed by the detection of a single peak.

Quantification of the number of molecules of DRTP1 was automatically calculated from

the standard curve by inputting the average Ct value obtained for each tissue into the

'Initial template quantity calculator' on the standard curve screen of the MX3000P™

system (Stratagene).

2.9   Genome Walking

The Universal GenomeWalker™ Kit (Clontech) was used to obtain the 5' flanking

sequence of the DRTP1 gene from rainbow trout liver total genomic DNA. Briefly,

blunt-end fragments of the genomic DNA were created by individual digestion with

Dra I, *Eco* RV, *Pvu* II, and *Stu* I enzymes followed by a phenol:chloroform purification.

Ligation of adapters to the blunt-ended fragments was carried in a final volume of 8 μl

with 1.9 μl of 25 μM GenomeWalker™ adapter, 1.6 μl of 10X ligation buffer, 0.5 μl of

T4 DNA ligase (6 units/ μl). Ligation reactions were incubated at 16 °C overnight.

Primary PCR was carried out in 50 μl using 2 μl of the adapter-ligated genomic DNA as

template, 5 μl 10X *Tth* PCR reaction buffer, 1 μl of 10 mM dNTP, 2.2 μl 25mM

Mg(OAc)$_2$, 1 μl of the AP1 primer and 1 μl of the gene-specific primer (GWAS)

(10μM), and 2 μl 50X Advantage® Genomic Polymerase Mix (See Table 1 for primer

sequences). PCR reactions were carried out in a Peltier Thermal Cycler PTC-225 (MJ

Research). Primary PCR conditions were 94 °C for 5 min, followed by 7 cycles of 94 °C

for 20 s and 70 °C for 3 min, and 32 cycles of 94 °C for 20 s and 65 °C for 3 min,

followed by 65 °C for 7 min. Secondary PCR was carried out as for the primary PCR

except using 2 μl of a 1:50 dilution of the primary PCR product as template, and 1 μl of

the AP2 primer and 1 μl of the nested gene-specific primer (GWASN) (10 μM).

Secondary PCR conditions were 94 °C for 5 min, followed by 7 cycles of 94 °C for 20 s

and 70 °C for 3 min, and 20 cycles of 94 °C for 20 s and 65 °C for 3 min, followed by

65 °C for 7 min. The PCR products were cloned into pCR®2.1 and transformed into One

Shot Top10™ chemically competent cells (Invitrogen). Plasmid DNA was prepared

using a GenElute™ Plasmid Miniprep Kit (Sigma) and sequenced (MWG Biotech,

Germany).

2.10   Directional cloning of DRTP1 5' regulatory region into pGL3-Basic.

The 5' regulatory fragment of DRTP1 insert was subcloned into the pGL3-Basic

reporter vector (Promega) containing the firefly Luciferase gene using the *Kpn* I and

Xho I restriction sites on either side of the DRTP1 in the multiple cloning site of the

pCR$^{®}$2.1 plasmid. The pGL3-Basic/DRTP1 construct was again verified by sequencing (MWG Biotech, Germany).

2.11   Generation of DRTP1 promoter constructs

Promoter constructs were generated using the Erase-a-Base$^{®}$ System (Promega) as detailed in the manufacturer's instructions. The Erase-a-Base® System uses exonuclease III (*Exo* III) to specifically digest insert DNA from a 5' protruding or blunt-end restriction site. The adjacent site on the plasmid is protected from digestion by a 4-base 3' overhang restriction site, which is *Exo* III resistant. *Kpn* I was used to generate the 4-base 3' exonuclease-resistant site and *Mlu* I was used to generate 5' overhangs at the position where *Exo* III nuclease digestion initiates. *Exo* III digestion was carried out at 25 ^{o}C at an assumed digestion rate of 90 nucleotides per minute. The 12 plasmids of decreasing size which were generated were religated before transformation into One Shot Top10™ cells.

2.12   Screening of promoter constructs in pGL3-Basic

Colony PCR was carried out to screen for clones containing suitably sized inserts. Clones were picked into 50 µl of dH$_2$0 of which 25 µl was used as template for colony PCR in a final reaction volume of 50 µl. Colony PCR reactions were carried out in 50 µl volumes containing 25 µl template, 5 µl 10X Advantage 2 buffer (Clontech), 2 µl of 10 µM pGL3_For and pGL3_Rev, and 1 µl 50X Advantage$^{®}$ 2 Polymerase mix (Clontech). Colony PCR conditions were 94 ^{o}C for 5 min, 30 cycles of 94 ^{o}C for 60 s, 55 ^{o}C for 60 s, 72 ^{o}C for 60 s, followed by 72 ^{o}C for 5 min. The colony PCR products were separated on a 1.4% agarose/SYBR safe™ gel. Inserts were verified by sequencing (MWG Biotech, Germany).

2.13   Identification of potential transcription factor binding sites

Potential transcription factor binding sites were identified using MatInspector V6.2 (Genomatix) [15]. Settings for identification of potential transcription factor binding sites were 1.0 for core similarity and >0.85 for core matrix similarity.

2.14   Analysis of DRTP1 promoter basal activity

Human heptatoma (HepG2) cells were obtained from the American Tissue Culture Collection (ATCC). Cells were maintained at 37 ^{o}C/5% CO_2 in T75 cm^2 flasks in Williams E medium supplemented with 2 mM L-glutamine, 10% fetal calf serum (FCS) and 1X penicillin/streptomycin. HepG2 cells were seeded at a density of 2 x 10^4 cells / well in 24-well plates (Nunc). The following day promoter constructs and control plamsids (0.6 μg/well) were transfected into the HepG2 cells using Lipofectamine™ 2000 (Invitrogen) as per manufacturer's instructions. Transfection controls included pGL3-Basic (negative control), pGL3-SV40 promoter (positive control) and non-transfected cells. The pRL-SV40 vector containing the *Renilla* luciferase was co-transfected as a control for transfection efficiency at a ratio of firefly luciferase to *Renilla* luciferase of 10:1. The activities of firefly luciferase and *Renilla* luciferase were assayed 2 days after transfection using the Dual-Luciferase$^®$ Reporter Assay (DLR) system (Promega) and read on a microtiter plate luminometer (Wallac).

2.15   Analysis of DRTP1 promoter inducibility

Promoter inducibility was tested using the synthetic glucocorticoid dexamethasone, polyinosinic:polycytidylic acid (poly I:C) - a synthetic double-stranded RNA that models viral infections *in vivo,* lipopolysaccharide (LPS) - a glycolipid found in the outer membrane of the gram-negative bacterial cell wall and with the cytokine tumor

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necrosis factor-alpha (TNFα). Human heptatoma liver cells (HepG2) were co-transfected with the pGL3-Basic reporter vector and the pRL-SV40 vector for 48 h after which the cells were stimulated for a further 6 h with either dexamethasone (100 nM), poly I:C (25 µg/ml), LPS (5 µg/ml) or TNFα (100 ng/ml). Growth medium was removed from the HepG2 cells and the cells washed in 1X PBS (0.5 ml) before 100 µl of 1X Passive Lysis Buffer (PLB) was added per well of the 24-well plate for 15 min and collected into 1.5 ml microfuge tubes. The supernatant was centrifuged at 10,000 rpm for 1 min to remove the cell debris before the supernatant was transferred to a fresh 96-well plate for analysis. LAR II reagent (100 µl) was added to the cell lysate (20 µl), mixed by pipetting and read. Stop and Glo reagent (100 µl) was added and samples read again.

2.16   *Transfection data analysis*

A basal reading was taken first to get the background luminescence of the plate, followed by the firefly and then the *Renilla* luciferase reading. After the background was subtracted the luminescence for each sample was calculated by the following formula - firefly luminescence/*Renilla* luminescence X *Renilla* pGL3-Basic. In analysing the basal promoter activity in each construct the data was reported as a percentage of the pGL3-SV40 positive control construct designated as 100%. For the promoter inducibility studies the data was reported as the fold induction of the promoter activity relative to the same construct without induction.

2.17   *Statistics*

The results of the HepG2 transfection experiments were represented as the luciferase activity mean (± SEM) of at least 3 independent transfections. Significance differences

between DRTP1 promoter constructs was determined using One-way Anova with the Tukey post test for multiple comparisons using GraphPad InStat. *P* values less than 0.05 were accepted as significant.

3. Results

3.1 Tissue distribution of DRTP1 transcripts in rainbow trout

Identification of the DRTP1 mRNA transcript in fish tissue was performed by Northern analysis and quantified by absolute real-time PCR. Six tissues (liver, intestine, spleen, brain, pituitary and hypothalamus) from healthy, non-confined fish were analysed. The tissues with the most abundant transcripts were the liver and the pituitary, with lesser amounts detected in the intestine, hypothalamus, brain and spleen. Northern analysis identified a strong 0.7 kb transcript in both the liver and pituitary with much weaker transcripts in hypothalamus and intestine and no evidence of expression in the spleen or brain. To confirm the tissue expression pattern, quantitative real-time PCR (qPCR) was also performed. DRTP1 mRNA present in each tissue was quantified by means of an RNA standard curve. Using this method DRTP1 transcripts in the liver were determined to be 3 times greater than in the pituitary (4.21×10^4 copies/ng of total RNA and 1.32×10^4 copies/ng of total RNA, respectively) and over 20 times greater than in the intestine (1.87×10^3 copies/ng of total RNA). DRTP1 transcripts were also detected in the hypothalamus (1.11×10^3 copies/ng of total RNA), brain (7.65×10^2 copies/ng of total RNA) and spleen (4.06×10^2 copies/ng of total RNA) by qPCR (Fig. 1).

3.2 Genomic organisation of the DRTP1 gene

A PCR amplification product was generated from cDNA using a DRTP1-specific primer pair (DRTP1Full_S/DRTP1Full_AS) which spanned the region from + 9 to +

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417 of the 455 nucleotide sequence. The resulting PCR products were ligated into pCR®2.1, positive clones identified, and sequenced in both directions. The cDNA and genomic sequences were aligned in Vector NTI and introns identified. The NetGene2 prediction program was also used to confirm the intron/exon junctions. In addition to confirming the intron/exon junctions identified by the Vector NTI alignments, the NetGene2 program predicted that additional splice sites in the region of the cDNA which did not get amplified (8 nucleotides at the 5' end of the cDNA and 38 nucleotides at the '3 end) were improbable. Therefore we conclude that the DRTP1 gene contains 4 exons separated by 3 introns. The sequence and genomic organisation of the DRTP1 gene are illustrated in figure 2.

3.3 Analysis of the 5' regulatory sequence of DRTP1

The 934 nucleotide 5' regulatory fragment of DRTP1 obtained by genome walking was subcloned into the pGL3-Basic reporter vector. The transcription start site (TSS +1) was identified using the neural network promoter prediction (NNPP) program (http://searchlauncher.bcm.tmc.edu/). Potential transcription binding sites were identified using Matinspector V6.2. The transcription factor binding sites identified included AP1 (4), c-MYB (2), CAAT (2), C-EBP/β (4) CREB, CREB2 (2), GFI1 (2), GATA1, GATA2 (3), GRE (2), HSF2, IRF1, NF-κB (2), NF-AT, OCT1 (2) and YY1. The 5' region of the DRTP1 gene was cloned into pGL3-Basic and verified by sequencing. Using the Erase-a-Base® System (Promega) the pGL3-Basic/DRTP1 construct was digested at an approximate rate of 90 nucleotides per min at 25 $^{\circ}$C. (fig. 3) The start of the constructs, relative to the TSS, were determined to be D1 at -934, D2 at -786, D3 at -726, D4 at -610, D5 at -536, D6 at -420, D7 at -278, D8 at -188, D9 at -57, D10 at +55, and D11 and 12 at position +149. The 5' regulatory region of the

DRTP1 gene illustrating the different DRTP1 constructs and potential transcription factor binding sites is represented in figure 4.

3.4   Analysis of the DRTP1 promoter constructs

The basal promoter activity of the DRTP1 constructs D1 to D6 ranged between 20% and 26% of the pGL3-SV40 positive control (mean 23.85% ± SEM 1.41) (Fig. 4.) Subsequent decreases in the size of the promoter construct lead to decreasing basal activity of the promoter. Construct D7 (-278) had 8.7% promoter activity while construct D8 (-188) had 0.5%. The promoter activities of construct D9 (-57), D10 (+55), and D11/12 (+149) were similar to that of D8 and were therefore excluded from future transfection experiments.

3.5   Analysis of DRTP1 promoter construct inducibility

HepG2 cells were co-transfected with both the pGL3 promoter constructs expressing the firely luciferase gene and the pRL vectors constitutively expressing *Renilla* luciferase. After 48 h the cells were stimulated for 6 h with either dexamethasone, poly I:C, LPS, or TNFα and the luciferase activities measured. Two potential glucocorticoid regulatory elements (GRE) sites were located at -581 and -210 in the 5' regulatory region of the DRTP1 gene. DRTP1 promoter stimulation with dexamethasone resulted in an approximate 2 fold induction (of DRTP1 promoter constructs D1 to D7), with construct D8 exhibiting no inducibility.

Treatment with poly I:C resulted in a small induction in constructs D1-D7, approximately 1.5 fold, with D8 showing no induction. DRTP1 promoter stimulated with LPS did not lead to significant activation in any of the D1 to D7 DRTP1 promoter constructs, with D8 showing a slight reduction in promoter activity relative to the D8

untreated control. In the absence of induction constructs D1-D6 had a mean of 23.8% promoter activity, construct D7 had a lower promoter activity (9.1%) with D8 showing minimal activity (0.53%). Upon stimulation with TNFα the induction of constructs D6 to D1 ranged from 3.45- 4.1 fold relative to the untreated construct. Construct D7 proved to be the most inducible increasing 6.34 fold upon stimulation. Construct D8 showed the least induction, increasing 1.81 fold, relative to the other constructs being analysed (Fig. 5).

3.6   DRTP1 and the Ly-6/uPAR protein family.

DRTP1, based on the patterns of conserved cysteine residues involved in disulphide bridge formation, was characterised as a member of the Ly-6/uPAR family of proteins. The Ly-6/uPAR family can be classified into two subfamilies based on the presence or absence of a glycosylphosphatidylinositol (GPI)-anchoring sequence [9]. Examples of Ly-6/uPAR GPI-linked cell surface glycoproteins include the urokinase plasminogen activator receptor (uPAR), the mouse family of Ly-6 T-cell antigens and CD-59, an inhibitor of the complement membrane attack complex. Included in the non-GPI linked, secreted proteins Ly-6/uPAR subfamily are snake neurotoxins, cytotoxins and the recently identified mammalian secreted Ly-6/uPAR family of proteins (SLURPs). DRTP1 was originally hypothesised to be a CD59-like protein [1] exhibiting 30% homology to CD59 [2]. Using the GPI predictions programs, big-PI Predictor (http://www.expasy.org/) [16] and DGPI (http://129.194.185.165/dgpi/) [17] the C-terminal hydrophobic region of the DRTP1 protein, identified as the last 3 amino acids, was predicted to be too short to function as a GPI-anchoring signal sequence. The presence of an N-terminal signal sequence and the lack of a C-terminal GPI-anchoring

signal sequence would place DRTP1 in the non-GPI linked secreted Ly-6/uPAR subfamily of proteins.

4.   Discussion

In the current study the expression of DRTP1 in six tissues from rainbow trout was examined, namely liver, intestine, spleen, brain, pituitary and hypothalamus. By Northern analysis abundant transcripts were identified in the liver and pituitary with much weaker transcripts in hypothalamus and intestine, with no evidence of DRTP1 expression in the spleen and brain. Using the more sensitive technique of qPCR, DRTP1 transcripts were detectable in all tissues including the brain and spleen. This is the first study where the DRTP1 transcript has been investigated in the brain, hypothalamus and pituitary. One interesting result of the current study was the high level of expression of the DRTP1 transcript in the pituitary. We have been able to show that conditions which lead to the induction of DRTP1 in the liver do not lead to the induction of the gene in the pituitary, a tissue where the gene is abundant under normal conditions (unpublished results).

The potential transcription factor binding sites identified in the 5' regulatory region of the DRTP1 gene included AP1, c-MYB, CAAT, C-EBP/β, CREB, CREB2, GFI1, GATA1, GATA2, GRE, HSF2, IRF1, NF-κB, NF-AT, OCT1 and YY1. The two potential NF-κB transcription sites were identified at positions -204 and -158. In excess of 150 different stimuli, including bacterial lipopolysaccharides (LPS), proinflammatory cytokines (tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1)), hormones and mitogenic agents are able to promote NF-κB activation [19]. NF-κB family members are transcriptional factors that regulate the expression of a large number of target genes

involved with physiological processes such as immune response, programmed cell death (apoptosis), inflammation, and progression of the cell cycle in different organisms [20, 21, 22, 23]. STAT3 is a second transcription factor with core binding site identical to the NF-κB binding site and therefore STAT3 could also be binding at these two sites. The acute phase response element/signal transducer and activator of transcription (APRE/STAT) is a motif common to almost all acute phase protein (APP) genes [24]. Four potential binding sites for CCAAT/enhancer binding protein beta (C/EBPβ) were identified (-389, -357, -232, -140) in the 5' regulatory region. C/EBPβ is activated in liver cells following exposure to interleukin-6 and other cytokines. During inflammation when both NF-κB and the C/EBP family of proteins are increased and activated, they form a heteromeric complex and act synergistically [25]. C/EBPβ and NF-κB are known to be important regulatory factors contributing to tissue specificity and to the high rate of transcription in the major acute phase gene serum amyloid A (SAA) in response to inflammatory mediators in the liver [26, 27].

Analysis of the promoter constructs (D1 to D6) in HepG2 cells showed the basal promoter activity to be quite high relative to the SV40 positive control (30%). With a reduction in promoter size (D6 to D7), which resulted in the loss of 2 C/EBPβ sites (-389, -357) and 1 AP-1 (-244) site, the basal level of promoter activity was reduced by approximately 60%. The basal promoter activity was effectively lost in going from promoter construct D7 to D8 with the further loss of C/EBPβ (-232), GRE (-210) and NF-κB (-204) sites. The addition of stimuli, including dexamethasone, poly I:C, LPS and TNFα, to transfected heptatoma cells was used to test the inducibility of the DRTP1 promoter. Promoter induction using dexamethasone resulted in a maximal increase of 2 fold activity, irrespective of whether the promoter construct contained one or two GRE sites. The promoter induction with poly I:C was minimal, approximately 1.5 fold, while

no change in promoter activity was evident with LPS. TNFα was the only stimulus capable of inducing substantial promoter activity, ranging from 3.45 fold to 6.34 fold depending on the construct. Construct D7, containing two C/EBPβ sites (-232, -140) and two NF-κB sites (-204 and -158), exhibited the highest induction (6.34 fold ± SD 0.49) when stimulated for 6 h with human TNFα (100ng/ml). The 90 nucleotide shorter construct D8 containing one C/EBPβ site (-140) and one NF-κB site (-158) showed both a reduced promoter basal activity and a reduction in inducibility. The loss of the C/EBPβ and the NF-κB site in the D8 construct suggests that these sites, either individually or in combination, are critical for the induction of the DRTP1 promoter by TNFα.

DRTP1 was initially identified as a possible CD59 homologue [1, 2] despite the apparent absence of a C-terminal GPI-signalling sequence. CD59, which protects cells by inhibiting the formation of the membrane attack complex during activation of the complement system [28, 29], is an 18-20 kDa membrane protein. Recently however, two CD59-like proteins CD59-like 1 and CD59-like 2, (GenBank accession nos. **AAT94063** and **CAI54280**) have been identified in *O. mykiss*. In contrast to DRTP1, these CD59-like proteins are of comparable size to their human orthologs (120 and 119 amino acids respectively) and are predicted to be GPI anchored [31].

The DRTP1 gene (O. mykiss) has 4 exons (~20, 111, 112 and 225 nucleotides, respectively) and 3 introns (158, 198 and 222 nucleotides, respectively). Intron 1 is in the 5'UTR of the gene and also seems to be present in Atlantic salmon, but in at least one Atlantic salmon EST (GenBank accession no **AM049603**), it is not spliced out. It is also not spliced out in the 'long' form of the brook trout transcript (GenBank accession nos **AF004739**). The second intron is before the first conserved Cys (towards the end of the signal peptide sequence) and the third intron is between the 5th and 6th conserved

Cys residues. CD59 also has four exons separated by three introns (5.4 kb, 5 kb and 7 kb respectively) [30]. The position of coding sequence introns seems to be consistent across the DRTP1, CD59 and SLURP sub-families, with the only inconsistency being the presence or absence of the intron within the 5'UTR. Although most SLURP1s and DRTP1s (not O.mykiss) are reported to have 3 exons and 2 introns, and most CD59s to have 4 exons and 3 introns, O.mykiss DRTP1 has 4 exons and we believe that many of the other DRTP1-like genes could also have 4 exons (unreported because the first exon is so short).

Snake neurotoxins and cytotoxins act by binding to nicotinic acetylcholine receptors (nAChRs) and prevent the binding of acetylcholine, thereby blocking the excitation of muscles [32]. The nAChRs are ionotrophic receptors that form ion channels in the cell plasma membrane. Bungarotoxin (BTX), like DRTP1, is a member of the Ly-6/uPAR family of proteins. Two nACh antagonists (αBTX and *n*-BTX), have been isolated from the venom of *Bungarus multicinctus,* the Formosan banded krait [33].

Until recently the only recognised secreted mammalian three-fingered proteins (TFP) containing a Ly-6/uPAR domain were SLURP-1 (secreted mammalian Ly-6/uPAR related protein) [34] (Adermann *et al*., 1999) and SLURP-2 (Tsuji *et al*., 2003). Consistent with the putative ligand function of some secreted TPF/Ly-6/uPAR proteins, the SLURP proteins function by binding to nicotinic acetylcholine receptors (nAChRs). SLURP-1 activates the alpha7 subtype of keratinocyte nicotinic acetylcholine receptors (nAChRs) and facilitates keratinization and programmed cell death [36]. SLURP-2 competes with acetylcholine predominantly at the alpha3 nAChR, and that receptor ligation with SLURP-2 delays keratinocyte differentiation and prevents apoptosis [37]. The newest secreted mammalian members of the TPF/Ly-6/uPAR protein family, the

PATE proteins (Prostate And Testes Expression) [38] also have demonstrated the ability to modulate the activity of nicotinic acetylcholine receptors (nAChRs).

Amino acid alignment alone would not have suggested that the SLURPs or PATEs, would bind to acetylcholine receptors as do the toxins (fig.7a). The amino acid residues that were originally deemed important in binding of the neurotoxins to the receptors are clearly not conserved in the PATEs or SLURPs. Phylogenetic analysis of these sub-families suggests that these proteins belong to distinct Ly-6/uPAR subfamilies (fig. 7b). Analysis of the protein sequence of DRTP1 would suggest that it is also a secreted, non-GPI-anchored member of the Ly-6/uPAR subfamily and therefore the possibility exists that it too might function as a modulator of the nicotinic acetylcholine receptor. However, further work either through the isolation of the native DRTP1 protein or through the generation of a recombinant protein, will be required before the function of this protein can be tested and this hypothesis validated.

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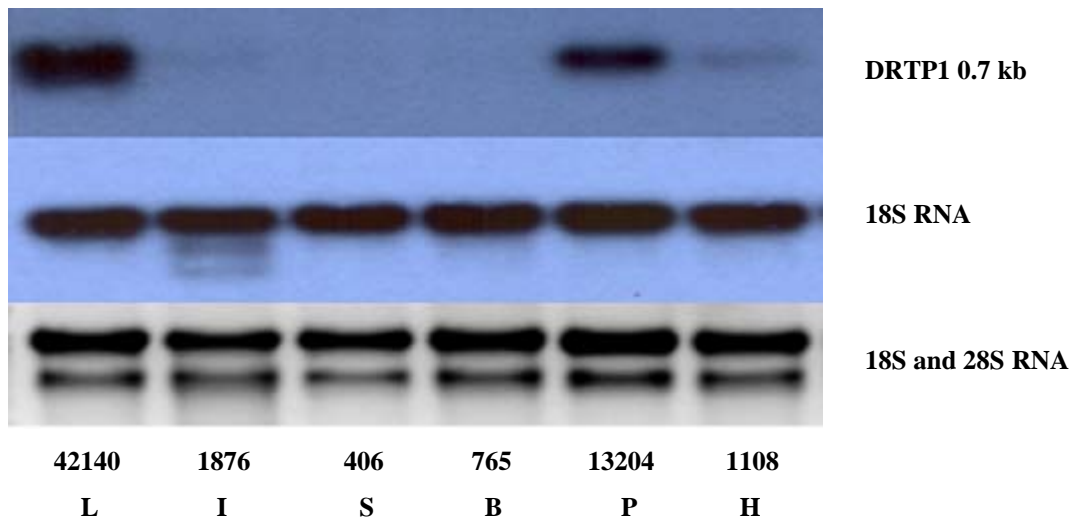
Fig. 1   Tissue distribution and quantification of DRTP1

Lane 1, liver (L), lane 2, intestine (I), lane 3, spleen (S), lane 4, brain (B), lane 5, pituitary (P), and lane 6 hypothalamus (H). The top section represents a Northern blot probed with DRTP1, the middle is the Northern blot probed with 18S RNA, the bottom section is the formaldehyde/EtBr gel showing ribosomal RNA bands. Transcript copies of DRTP1 per ng of total RNA in each tissue, as determined by qPCR, are listed under each lane.
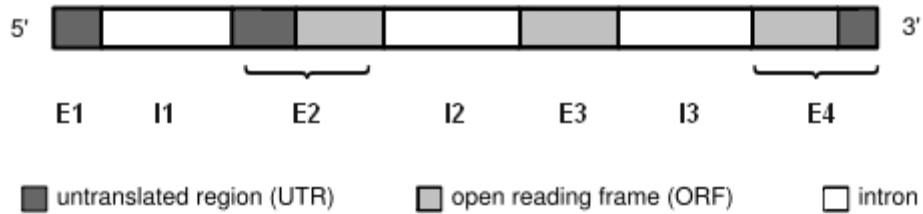
taaaggaagctcaataca**CCTAAAGGAAACACAATCAG**gtgagcaactactctactatctacagcaccta
atactttgtctcgtgatactgtgctactgggcttgtgaaatggttggtttcagaatgttcctgcacatttcaaataaatc
aaaataacatgttgcaattgttgaacattgtataatcccaccacat**AGAGGCATTATTTTCTATTGTTGG**
AACGATCAGATCGAAAGACTCCTGAAGAAAGGAAAGATG**AAAGCTGTATGTTTTTCTCTG**
CTCCTCCTGCTGCTGGCGTGTAGCTTTGgtgagtctgcccaacaaggtcttcatcttatttcatgtctttat
ttgacagggacaatgccaaataaacacatgtaccagattaaagctagattattctcatctccattatttatgctcc
agtctcccagtcaacacattggataaccagacacactccaatacaacagagatgatgctgttcttctgttttctcc
ag**GAGAGGGCCTGAAATGCAACCGTTGTGTTGGCAAAGGCTGCAGGAACACAGTGGAG**
ACCTGCCGTGTTGACCATGACACCTGCGGCACGGTCTTATTCAAGCCCCCACTCCgtacgt
atctattatccccagtttaatctgtatataccccttcactaggcactttactgtgtttaaccctaggatcagtaaccaa
tcgggaaaacacaaaatacctgatccttttgattgacatctaaggaaaacgtttgctttatccaatcaagaaaat
gtgctggtttgttgtcttctgtatctggtgtttatcctgtctcgcttctctctctccacag**CTATCTCATACTTCAAG**
AGATGCATGAAGATGTCAGAATGCTTGCTGCTGGGAAGCAACAAGGACATTGATGCCTA
TTGCTGCACCACCAACCAATGCAACTGAGAGACACATCCTCTGTCTACCATACTGTATAC
TATACAGTCCATTAATTGTATTAATCAATGCAAATGATTGAAACTGCCAAGAAGTAACAG
AAATAAAGTTGTACTTTTTCTCCAAAAAA

Fig. 2   Sequence and genomic organisation of the DRTP1 gene

The genomic organisation of the DRTP1 gene. Exon 1 consists of 38 nucleotides, exon

2 consists of 112 nucleotides of which only the last 52 are translated, exon 3 has 111

nucleotides and exon 4 has 225 nucleotides, of which only the first 104 are translated,

respectively (upper case bold letters) separated by three introns of 158, 198 and 222

nucleotides (lower case), respectively. Lower case bold letters in exon 1 indicate

sequence for which there is not evidence of transcription in any *Oncorhynchus mykiss*
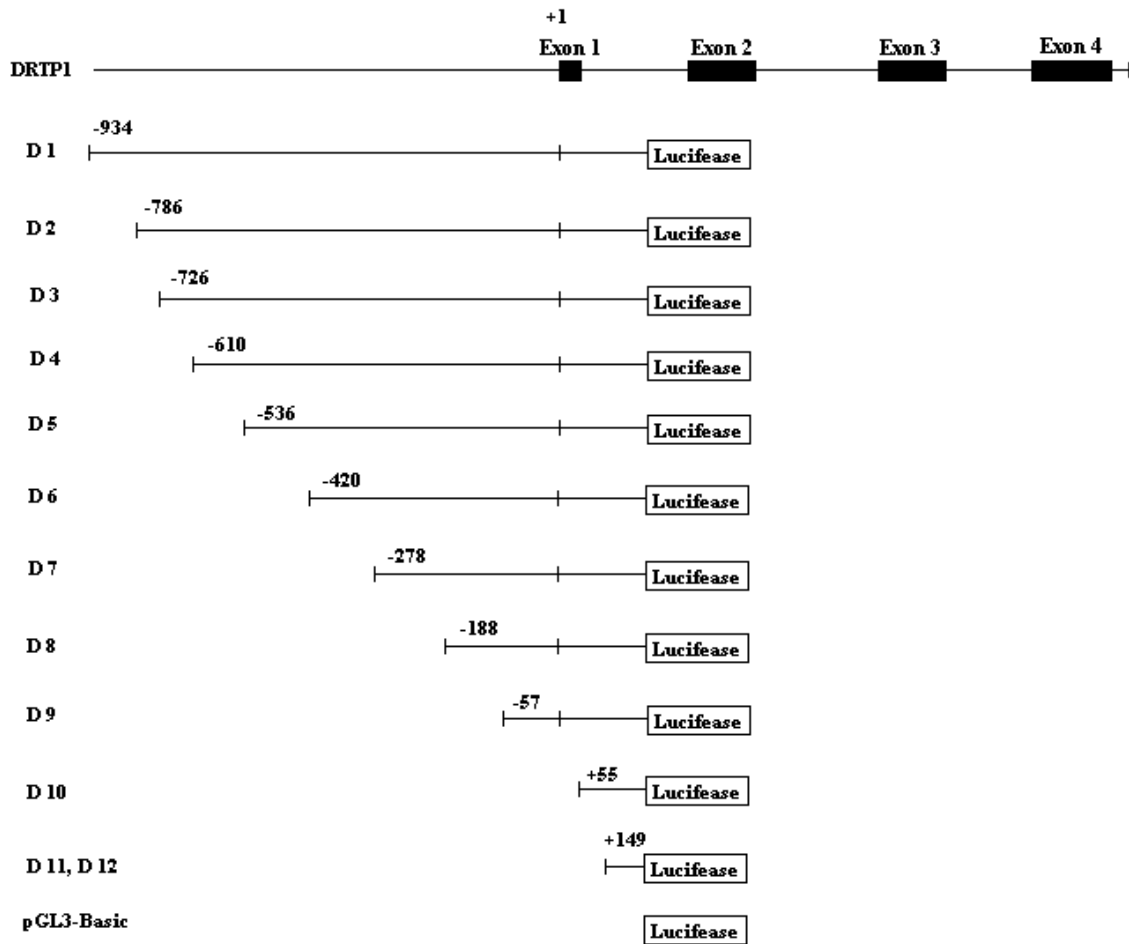
EST.

Fig. 3   DRTP1 promoter constructs

Constructs in the pGL3-Basic reporter vector were names according to length with D1

the longest and D11/ D12 the shortest. Construct numbering was based on the

transcription start site (TSS +1). The empty pGL3-basic vector was used as a negative

control in all transfection experiments.

```
-934
ACTCAGTCGAAAGTCATTACCCGCTGGACAAAAACTGGTTGAGTCAATGTTGTTCCCATGTCATTTTAACCCCACAAGAATCTATGTGATGA
D1 →                                      AP1 →              OCT1 →
CATTGAATCAACATAGAAAACTGATTGGGTTGGAAAAAGGTCATCAACGTCAGGGCATTTTCTCTTTTTATCACCCAGCTTTGAACCTAAATC
             ← CAAT                          ← CREB     D2 → ← IRF1
CAATGACACGGTGACAGTTCTTGTTGATTTCGTGTTGAATTCACGTAAGTTGACAACTCAACCAAATGTAAATCAAAACTAGATGTTGAACT
CAAT →              D3 →            CREB2 →                    GFI1 →
GATGTCTGTGCCCTGTGGGTATCGTCATCATCATGTCTCTCCCCCCTCTCTATCTTTCTCTCTCTATTTCTCCAGAGAACACCTTCCACAAGTC
          ← CREB2                    D4 →                      ← GRE
CTGCTTCAAGATGATGGAGTGCCTGAAGCTTGGTGTGACCGAGGGTCTGAGAGTCACCTGCTGCAACTGGAACAACTGCAACTTATAAGACC
              D5 →                                            C-MYC →
CATAGCCTCTACTCACCTTCTGTATCATTGGTATGCGTGTGGCAGGAGCCCAGTTCAAATAGCAATGAAATACATTCAGTATTGCAAAATGA
                                    D6 →                        ← C/EBPβ
TATTTTAACCTTTTGTTCCTGATTTCAACATATGTCTAAATGTCATAATATGAACTTGTTTACATGTGATATGAATGATTCATCATCCAACACC
         ← C/EBPβ                                    GATA2 →    AP1 →
ACCTCTTAGATAAACTTCGCACACCAGTCAGTTGTTTTGTTGCGCTTTAATATTTCGAAATTCTCCTAACCCAATGTCCTGGAATGTCCCAGTA
     D7,GATA2 →          ← C-MYB              C/EBPβ →           GRE → ← NF-κB
TGGAACACGACACTGCAAACCTTTGTCTCATGAAATCCGGGAATTTCCATTCTTTTACAGAGAGACAAAGGGCACTGCTTGTGCTAAACCTG
    D8 →                          ← NF-κB
AGTAAATCCATTGCCCAAAATCTCACACAAGTGACTCATAAGATAGCGCTTTATTAGCATACTGTGCTGTATAAAAGGAGAAGGAGAAGGA
AP1 →   ← C/EBPβ              AP1 → D9,GATA2 →                  TATA →
GAGGAGGTAAAGGAAGCTCAATACACCTAAAGGAAACACAATCAGGTGAGCAACTACTCTACTATCTACAGCACCTAATACTTTGTCTCGT
     TSS +1 →              NF-AT →                D10 → ← GATA1
GATACTGTGCTACTGGGCTTGTGAAATGGTTGGTTTCAGAATGTTCCTGCACATTTCAAATAAATCAAAATAACATGTTGCAATTGTTGAACA
          ← YY1              HSF2 →            D10/D11,GFI1 → ← OCT1
TTGTATAATCCCACCACATAGAGGCATTATTTTCTATTGTTGGAACGATCAGATCGAAAGACTCCTGAAGAAAGGAAAGATGAAAGCTGTAT
                                        ← GWASN primer       cDNA start codon
GTTTTTCTCTGCTCCTCCTGCTGCTGGCGTGTGAAGCTTTGGAGGGCCTGAAATGCAACCGTTGTGTTGGCAAAGGCTGCAGGAACACAGTG
                              ← GWAS primer
GAGACCTGCCGTGTTGACCATGACACCTGCGGCACGGTCTTATTCAAGCCCCCACTCCCTATCTCATACTTCAAGAGATGCATGAAGATGTC

AGAATGCTTGCTGCTGGGAAGCAACAAGGACATTGATGCCTATTGCTGCACCACCAACCAATGCAACTGAGAGACACATCCTCTGTCTACCA
                                                              cDNA stop codon
TACTGTATACTATACAGTCCATTAATTGTATTAATCAATGCAAATGATTGAAACTGCCAAGAAGTAACAGAAATAAAGTTGTACTTTTTCTCC

AAAAAA
```

Fig. 4　The 5' regulatory region of the DRTP1 gene

Potential transcription factor binding sites and the transcription start site (TSS) are underlined, with arrows used to indicate direction. The DRTP1 construct fragments are indicated as D1 toD12. The primers used in genome walking (GWASN, GWAS) are also underlined (GWASN, GWAS). The DRTP1 cDNA sequence was obtained from NCBI (GenBank accession no. **AF281355**).
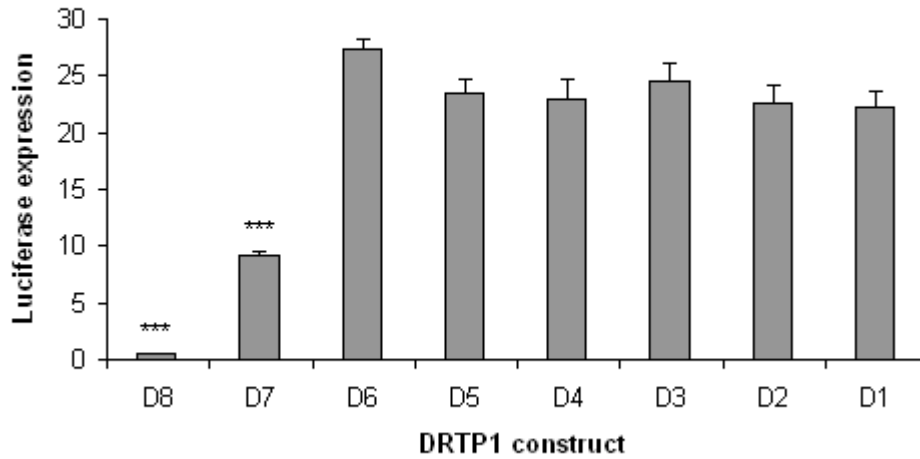
Fig. 5   DRTP1 promoter activity in HepG2 cells

Activity of DRTP1 promoter constructs in HepG2 cells 48 h after transient transfection
with DRTP1 constructs. Results are presented as luciferase expression (mean ± SEM of
4 independent transfections) relative to the positive control (pGL3-SV40). One-way
Anova with the Tukey post test for multiple comparisons was used to determine
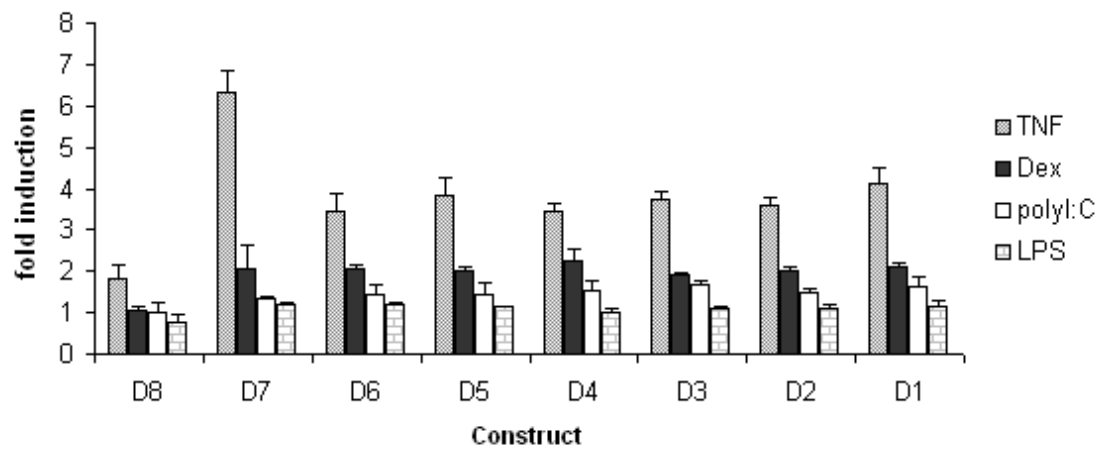significant differences between the DRTP1 promoter constructs (p=<0.05).

Fig. 6   Induction of DRTP1 promoter constructs

HepG2 were transiently transfected with DRTP1 constructs 48 h prior to stimulation with TNFα (100 ng/ml), dexamethasone (100 nM), poly I:C (25 µg/ml), LPS (5 µg/ml) for an additional 6 h before detection of luciferase activity. The results are relative to non-stimulated constructs and are a mean (± SEM) of 3 independent transfection experiments.

Fig.7a Alignments of Ly-6/uPAR sequences (graphic)
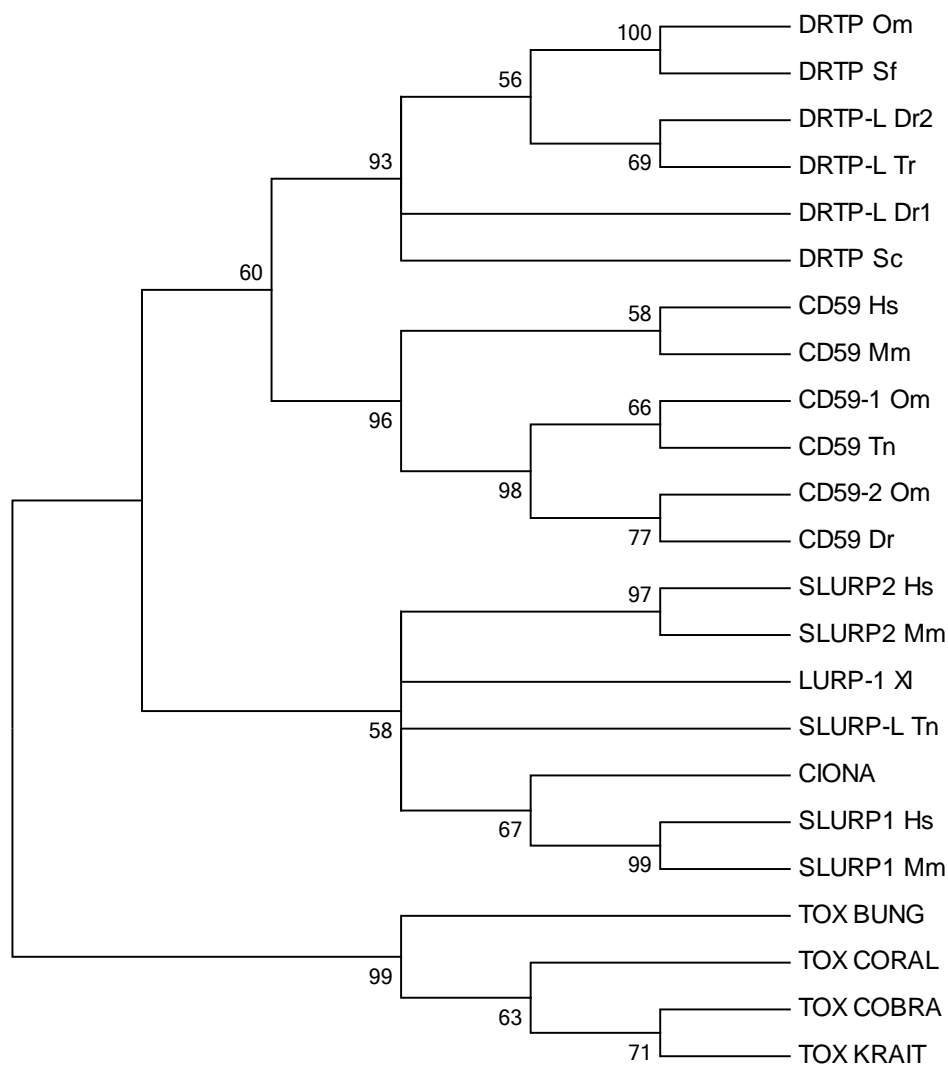
Fig.7b Phylogenetic tree of Ly-6/uPAR sequences

Fig. 7 Aligment and phylogenetic tree analysis of Ly-6/uPAR containing proteins including DRTP1, CD59, SLURP and snake toxins from various species.

DRTP OM (rainbow trout DRTP1, Q9DFD50), DRTP Sf (brook trout DRTP1, O13165), DRTP-L DR2 (zebra fish DRTP1-like EST, EH492308), DRTP1-L Tr (tiger pufferfish DRTP1, AL837306), DRTP1 Sc (Chinese perch DRTP1, Q2KKY5), CD59 Hs (human CD58, P13987), CD59 Mm (mouse CD59, O55186), CD59-1 Om (rainbow trout CD59-1, Q64HX7), CD59-2 Om (rainbow trout CD59-2, Q5F2I9), CD59 Dr (zebrafish CD59-like, XP_001336789), CD59 Tn (spotted green pufferfish EST,

CAG01322), SLURP1 Hs (human SLURP-1, P55000), SLURP1 Mm (mouse SLURP1, Q9Z0K7), SLURP2 Hs (human SLURP-2, Q86SR0), SLURP2 Mm (mouse SLURP2, AB272582), SLURP-L Tn (spotted green pufferfish EST, CAG07813), Ciona (Ciona intestinalis EST, BW387906), LURP-1 Xl, (African clawed frog Ly-6/uPAR-related protein-1, Q90ZP3), TOX BUNG (kappa-6 bungarotoxin, Q9W729), TOX CORAL (coral snake toxin, Q9PUB7), TOX COBRA (King cobra toxin, ABB83639), TOX KRAIT (Many-banded krait toxin, O12963). Sequence alignments and phylogenetic tree analysis was constucted using MEGA 4 (Tamura *et al*., 2007). Branch points were validated by 500 bootstrap replications.

Table 1

Primers used in this study

Name	Sequence (5'- 3')	Application
DRTP1Full_S	AGGCATTATTTTCTATTGTTGGCACG	Gene organisation/ Northern probe
DRTP1Full_AS	CTTGGCAGTTTCAATCATTTGC	Gene organisation/ Northern probe
DRTP1_GWAS	ACACAACGGTTGCATTTCAGGCCCTC	Genome Walking
DRTP1_GWASN	TCCTTTCTTCAGGAGTCTTTCGATCTG	Genome Walking
AP-1	GTAATACGACTCACTATAGGGC	Genome Walking
AP-2	ACTATAGGGCACGCGTGGT	Genome Walking
pGL3_For	CTAGCAAAATAGGCTGTCCC	Colony PCR
pGL3_Rev	TATGTTTTTGGCGTCTTCC	Colony PCR
RT18S_For	CGGAATTAACCAGACAAATCGCTCC	Northern probe (18s RNA)
RT18S_Rev	TCAGATACCGTCGTAGTTCCGACCA	Northern probe (18s RNA)
T7 DRTP1-S	TAATACGACTCACTATAGGGGTAGCT-TTGGAGAGGGCCTGAAATG	cRNA Standard curve
T7 DRTP1-AS	CAATGTCCTTGTTGCTTCCCAGCAGC-AAGCATTCTGACATCTTCA	cRNA Standard curve
DRTP1_RTS	GTAGCTTTGGAGAGGGCCTGAAATG	Real-time PCR
DRTP1_RTAS	CAATGTCCTTGTTGCTTCCCAGC	Real-time PCR