# Long-chain polyunsaturated fatty acid synthesis in fish: Comparative analysis of Atlantic salmon (*Salmo salar* L.) and Atlantic cod (*Gadus morhua* L.) Δ6 fatty acyl desaturase gene promoters

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ABSTRACT

Fish vary in ability to biosynthesise n-3 long-chain polyunsaturated fatty acids (LC-PUFA), with marine fish such as cod being inefficient in comparison to freshwater and salmonid fish. We investigated differences in the gene promoters of Δ6 fatty acyl desaturase (Δ6 FAD), a critical enzyme in LC-PUFA biosynthesis, in cod and salmon. Progressive deletions and targeted mutations of the promoters were tested for activity in a transfected fish cell line under low or high LC-PUFA treatment, and regions sufficient to direct transcription were identified. Comparison of these regions with sequences of corresponding regions of Δ6 FAD genes from mammals, amphibians and fish indicated a remarkable conservation of binding sites for SREBPs and NF-Y. In addition to these sites, a site was identified in salmon with similarity to that recognised by Sp1 transcription factor, and which was required for full expression of the salmon Δ6 FAD gene. The cod promoter was less active and lacked the Sp1 site. Eicosapentaenoic acid suppressed LC-PUFA synthesis in AS cells and also suppressed activity of the salmon Δ6 FAD promoter although this activity was likely mediated through sites other than Sp1, possibly similar to those recognised by NF-Y and SREBP transcription factors.

*Keywords*:
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## 1. Introduction

In vertebrates, biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic (EPA; 20:5n-3) and arachidonic (20:4n-6) acids from 18:3n-3 and 18:2n-6, respectively, requires the sequential activity of a Δ6 fatty acyl desaturase (FAD), an elongase and Δ5 FAD (Cook, 1996). Synthesis of docosahexaenoic acid (DHA; 22:6n-3) requires two further elongation steps, a second Δ6 desaturation and peroxisomal chain shortening (Sprecher, 2000), However, the production of LC-PUFA from C18 fatty acids is inefficient in humans (Salem et al., 1999; Brenna, 2002; Burdge and Calder, 2005). This fact underpins the well-known phenomenon that increasing our dietary intake of EPA and DHA has beneficial effects in a number of inflammatory and pathological conditions, including cardiovascular and neurological diseases (Brouwer et al., 2006; Eilander et al., 2007; Ruxton et al., 2007), and the much greater efficacy of fish oil containing these LC-PUFA than linseed/flax oil, rich in 18:3n-3, in intervention studies (Torrejon et al., 2008; Brenna et al., 2009).

Fish are unique sources of the omega-3 or n-3 LC-PUFA, EPA and DHA, in the human diet (Givens and Gibbs, 2006), and an increasing proportion of fish are now farmed (FAO, 2009). However, sustainable development of aquaculture to produce the fish required for the burgeoning human population requires n-3 LC-PUFA-rich fish oil in the feed to be replaced by vegetable oils that are devoid of LC-PUFA, with consequences for fish lipid metabolism and human health (Sargent et al., 2002). The ability of fish to convert 18:3n-3 to EPA and DHA is also rather inefficient and our overarching hypothesis is that understanding the molecular basis of LC-PUFA biosynthesis and its regulation in fish will allow us to manipulate and optimise the activity of the pathway to enable efficient and effective use of vegetable oils in aquaculture while maintaining the nutritional quality of farmed fish.

Our understanding of the biochemical and molecular mechanisms of LC-PUFA biosynthesis in fish has advanced considerably in recent years (Tocher, 2003; Tocher et al., 2003a). Several FADs have been characterised in various fish species, and all are Δ6 FADs (Zheng et al., 2004, 2005a, 2009; Tocher et al., 2006), except for a zebrafish bifunctional Δ6/Δ5 FAD (Hastings et al., 2001) and an Atlantic salmon (*Salmo salar*) Δ5 FAD (Hastings et al., 2005). To date no Δ5 FAD has been isolated from a marine fish species and, supported by biochemical studies in fish cell lines (Ghioni et al., 1999; Tocher and Ghioni, 1999), this has led to the hypothesis that some groups of fish may be unable to biosynthesise LC-PUFA because they lack specific genes in the pathway (Leaver et al., 2008a). However, Δ6 FADs cDNAs have been cloned from all fish species studied so far, and all showed significant

activity in heterologous yeast expression systems. In contrast, Δ6 desaturation activity and expression of Δ6 FAD are very low in Atlantic cod (*Gadus morhua*) tissues compared to the activity and expression of Δ6 FAD in salmon tissues (Tocher et al., 2006). Furthermore, Δ6 FAD expression and activity are under nutritional regulation in freshwater and salmonid fish. The activity of the LC-PUFA pathway in carp cells was increased by essential fatty acid (EFA)-deficiency (Tocher and Dick, 1999), and modulated by different C18 PUFA (Tocher and Dick, 2000). *In vivo* dietary trials have shown that activity of the LC-PUFA biosynthetic pathway is increased in freshwater and salmonid fish fed vegetable oils rich in C18 PUFA compared to fish fed fish oil, rich in EPA and DHA (Tocher et al., 1997, 2002, 2003b). Expression of Δ6 FAD mRNA was reduced in salmon fed diets containing fish oil, rich in EPA and DHA, in comparison to fish fed diets containing vegetable oils lacking LC-PUFA (Zheng et al., 2005a,b; Leaver et al., 2008b; Taggart et al., 2008). Again, in contrast, there was little difference in Δ6 FAD expression and activity in cod fed diets containing either vegetable or fish oil (Tocher et al., 2006). Thus cod may be deficient, both in critical LC-PUFA biosynthetic genes and in the expression levels and regulation of Δ6 FAD compared to salmon.

In the present study, we have compared the sequences of the putative promoter regions for the Δ6 FAD genes of Atlantic salmon and Atlantic cod, and tested these regions for their ability to drive reporter gene expression in a transfected fish cell line. This enabled delineation of the regions of the promoters that drive both basal expression and confer responsiveness to LC-PUFA. Comparison of these regions with the sequences of corresponding regions of Δ6 FAD genes from other fish, an amphibian and human indicated remarkably high conservation of sequence elements implicated in regulating Δ6 FAD in humans. Atlantic salmon, in addition, possess a site with similarity to that recognised by Sp1 transcription factor that was responsible for higher expression of the salmon Δ6 FAD gene compared to cod, which lacked the Sp1 site.

## 2. Materials and methods

### *2.1. Genomic organization and promoters of salmon and cod FAD genes*

Atlantic salmon and cod genomic DNA libraries were constructed in lambda FIX II (Stratagene, La Jolla, CA, USA). The respective DNA libraries were probed with full-length

salmon (GenBank accession no. AY458652) and cod Δ6 FAD cDNAs (Accession no. DQ054840). Inserts of positive recombinant phages were isolated and subcloned into the pBluescript II KS vector for sequencing (Stratagene, La Jolla, CA, USA). The full desaturase genomic nucleotide sequences were assembled using BioEdit version 5.0.6 (Tom Hall, Department of Microbiology, North Carolina State University, USA). The gene structures and the upstream sequences were identified and confirmed by comparing the genomic sequences with the relevant FAD cDNA sequences reported previously (Zheng et al. 2005a; Tocher et al. 2006).

*2.2. LC-PUFA synthesis in Atlantic salmon cell line (AS)*

The activity of the LC-PUFA synthesis pathway was determined in the Atlantic salmon cell line (AS) as described in detail previously (Ghioni et al., 1999). Briefly, AS cells were seeded into 75 $cm^2$ flasks in medium containing 10 % delipidated (charcoal/dextran-treated) fetal bovine serum (dFBS; HyClone, Logan, Utah, USA) and grown to 80% confluence. Cultures were supplemented with 50 μM EPA or DHA, with control cultures receiving the same volume of ethanol carrier alone and, 24 h later, [1-$^{14}$C]18:3n-3 was added as a bovine serum albumin (BSA) complex (0.25 μCi/flask). After a further 24 h, medium was aspirated, cultures washed with 5 ml phosphate buffered saline (PBS), cells dissociated with 0.05 % trypsin/0.02 % EDTA, harvested in 5 ml PBS and centrifuged at 300 g for 5 min at 4 °C. Cells were washed with 5 ml PBS containing 1 % fatty acid free-BSA, prior to extraction with 5 ml ice-cold chloroform/methanol (2:1, v/v) containing 0.01 % butylated hydroxytoluene as antioxidant, according to Folch et al. (1957). Fatty acid methyl esters were prepared by acid-catalysed transmethylation and separated by argentation chromatography on TLC plates impregnated with 2 g silver nitrate in 20 ml acetonitrile and activated at 110 °C for 30 min as described previously (Ghioni et al., 1999). Plates were developed with toluene/acetonitrile (95:5, v/v) to separate PUFA and autoradiography performed using Kodak BioMax MR film for 6 days at room temperature. Silica corresponding to individual fatty acids, identified in comparison with known standards, was scraped into scintillation vials containing 2.5 ml Ecoscint A and radioactivity determined in a scintillation counter (TRI-CARB 2000CA, United Technologies Packard, Pangbourne, U.K.). Data were corrected for counting efficiency and quenching of $^{14}$C under these conditions.

*2.3. Construction of deletion mutants of FAD gene upstream sequences*

The salmon and cod FAD genomic DNA fragments, which included the upstream promoter regions, were used as templates for PCR cloning. Forward primers, augmented with a 5' Hind III site, and anti-sense primers containing an Nco I site (SD6REV1 and CD6REV1) encompassing the ATG translation start site of each gene, were used to generate promoter DNA fragments (Table 1). The promoter fragments were amplified using high-fidelity PfuTurbo® DNA Polymerase (Stratagene, La Jolla, CA, USA). PCR products were digested with Hind III and Nco I, and inserted into pGL4.10 [luc2] vector (Promega Corporation, Madison, WI, USA) digested with the same restriction enzymes. Recombinant plasmids were isolated using NucleoBond PC 100 plasmid DNA purification kit (ABgene), and the constructs verified for accuracy by sequencing using GenomeLab™ DTCS Quick Start Kit and the CEQ™ 8800 Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

*2.4. Cell culture, transfection and dual luciferase assay*

AS cells were seeded on 96-well cell culture plates in Dulbecco's Modified Eagle Medium with 10 % dFBS and grown for 16–24 h to 80 % confluence. AS cells were transfected with 125 ng of each reporter firefly luciferase construct using Superfect transfection regent (Qiagen Ltd, West Sussex, UK). Cells were co-transfected with 125 ng of pGL4.75, a vector expressing Renilla luciferase under the control of a CMV promoter (Promega Corporation, Madison, WI, USA) as an internal control vector to normalize variations in transfection efficiency. The promoterless pGL4.10 basic vector was used as negative control for each transfection assay. Each plasmid was transfected in triplicate in three independent experiments. Fresh culture medium with 50 μM EPA or with the same volume of ethanol carrier alone was replaced at 24 h post-transfection. Luciferase assays were performed at 48 h post-transfection using the Dual-Glo$^{TM}$ luciferase assay system (Promega Corporation, Madison, WI, USA), with duplicate readings obtained using a Wallac 1420 multilable counter (PerkinElmer Life Sciences, Wallac Oy, Turku, Finland). The promoter activity was calculated from the ratio of firefly:Renilla luciferase for each construct, and then normalized to the activity of pGL4.10 with no insert (no insert control; NIC) luciferase in the presence or absence of EPA.

*2.5. Site-directed mutagenesis*

Mutations were performed using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol with mutations confirmed by sequence analysis. For salmon FAD, the construct SD2 was used as wild type and for cod FAD, the construct CD34 was used, and five mutants were produced from each (Table 2). The selection of sites for mutation was based on an alignment of the salmon and cod sequences across the region under study, which indicated areas of sequence conservation, and also by the use of an *in silico* online Patch™ tool with the TRANSFAC® Professional database to predict potential transcription factor binding sites.

### *2.6. Electrophoretic mobility shift assay (EMSA)*

Complementary sense and anti-sense oligonucleotides (Table 1, single strand only is shown) were labelled separately with Biotin 3′ End DNA Labeling Kit (Pierce, Rockford, IL, USA). They were then annealed to produce the double strand DNA probe. Oligos SDB1 and SDA1 included salmon putative binding sites 2 and 5 (bold letters), respectively. Oligos CDC1, CDB1 and CDA1 included cod putative binding sites 3, 4 and 5 (bold letters), respectively. Mutant sites were tested using oligonucleotides as shown in Table 2. Nuclear extract was prepared from AS cells using the Active Motif Nuclear Extract Kit (Carlsbad, CA, USA) and protein content determined by the Quick Start Bradford Protein Assay Kit supplied by Bio-Rad Laboratories (Hercules, CA, USA). The EMSA reaction was performed with LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. The binding reaction was subjected to a 5 % non-denaturing polyacrylamide gel and transferred to a nylon membrane. The biotin end-labeled DNA was detected using a streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate. The signal was then detected by autoradiography with CL-XPosure x-ray film (Pierce, Rockford, IL, USA)**.**

## 3. Results

### *3.1. Organisation of salmon, cod and other vertebrate Δ6 FAD genes*

Previously, we reported the full-length cDNAs for salmon and cod Δ6 FADs, which showed they contained open reading frames of 1365 and 1344 bp, coding proteins of 454 and 447 aa, respectively (Zheng et al., 2005a; Tocher et al., 2006). The putative transcription start

sites for these two genes were identified according to the published 5'UTR sequences, which were obtained by 5' SMART RACE PCR. The salmon and cod Δ6 FAD genes both consisted of 13 exons that spanned 12090 bp and 5106 bp of genomic DNA (GenBank accession numbers AY736067 and FJ859898), respectively (Fig.1). The translation start sites (ATG) were located in exons 2 and stop codons in exons 13. Thus, the tested promoter regions, in addition to upstream untranscribed sequence, contained transcriptional start sites, upstream non-coding exon and an ATG initiation codon for each gene. A total of 5.2 Kbp and 7.2 Kbp of 5'sequence upstream of the ATG were sequenced for salmon and cod Δ6 FADs, respectively. Of these, 4226 bp and 2962 bp upstream of the ATG, of salmon and cod respectively were investigated for promoter activity. Searches of the ENSEMBL genome sequences of zebrafish (*Danio rerio*), medaka (*Oryzias latipes*) and stickleback (*Gasterosteus aculeatus*) for Δ6 FAD genes identified a homologous annotated gene in each of these species. In each case these genes were organised similarly to the cod and salmon genes, with an initial non-coding exon followed by 12 coding exons, encoding a complete Δ6 FAD homologue. In the case of the amphibian *Xenopus tropicalis* an annotated Δ6 desaturase was not clearly recognisable, however a search using the salmon cDNA and TBLASTX identified 12 coding exons for a Δ6 FAD in this species. It was not possible to identify any 5' non-coding exons.

*3.2. AS cells as a model system for LC-PUFA biosynthesis*

AS cells have been previously shown to have a fully functional LC-PUFA synthesis pathway (Ghioni et al., 1999) indicating that the Δ6 FAD gene should be expressed in this cell line. In order to determine whether LC-PUFA biosynthesis in AS cells is regulated by fatty acids, the pathway was assayed by determining the desaturation of [1-$^{14}$C]18:3n-3 in the presence or absence of EPA and DHA. Addition of EPA or DHA to AS cells cultured in dFBS showed that LC-PUFA synthetic activity was significantly reduced by the presence of the LC-PUFA (Fig.2). Thus, EPA was chosen as a potential modulator of FAD expression in AS cells growing in lipid-deficient media.

*3.3. Promoter deletion analysis*

To determine the minimal FAD gene sequences that are sufficient to direct transcription, the DNA fragment encompassing upstream, untranscribed exon and initiation codon

sequence, and deletions thereof, was fused to a promoterless luciferase reporter gene and examined for the ability to mediate basal transcription. Transfection of AS cells in dFBS with a series of salmon promoter deletion constructs showed that maximal promoter activity was evident when up to 546 nucleotides upstream of the transcription start site (TSS) were included in the construct (SD2) (Fig.1). A deletion with 321 nucleotides upstream of the TSS (SD21) exhibited much reduced activity, although still significantly higher than all other deletions, which ranged from 321 nucleotides upstream, to 726 downstream of the TSS. The deletion constructs were also tested in parallel for response to LC-PUFA, with EPA treatment causing a significant reduction in promoter activity of both the SD2 and SD21 constructs (36 and 26% reduction, respectively). Deletions beyond 321 nucleotides upstream of the TSS showed general reductions of around 15% after EPA treatment, although these were not statistically significant when compared to the same constructs in the absence of EPA. A similar experiment conducted with deletion constructs from Atlantic cod showed that deletion of more than 167 nucleotides upstream of the TSS greatly reduced activity (Fig.1). No further decreases in activity were observed in further deletions up to 983 nucleotides downstream of the TSS. Although EPA treatment significantly reduced activity in some of the cod promoter deletions, the reductions were more variable and did not show a clear pattern as in the salmon constructs.

*3.4. Promoter mutagenesis*

The sequences of the salmon and cod Δ6 FAD fragments identified by promoter deletion were subjected to further *in silico* analysis that showed it was possible to align an area with similarity across all species, with the areas of identity across all genes corresponding to possible nuclear factor Y (NF-Y) and sterol regulatory element binding protein (SREBP) sites (Fig.3). Candidate transcriptional regulatory sequences were selected based primarily on the effects of promoter deletion, with focus on sequences that varied between salmon and cod Δ6 FADs, and promoter activity investigated in AS cells transfected with mutants targeting these sequences (Fig. 4). For salmon Δ6 FAD, mutations at site 2 and site 5 caused significant reductions of promoter activity of 38% and 73% of the wild type activity, respectively (Fig.5). For cod Δ6 FAD, mutations in sites 3, 4 and 5 caused significant reductions of promoter activity of 48 %, 60 % and 65 % of the wild type activity, respectively (Fig. 6). Mutation of various other sites had no significant affects on promoter activity.

*3.5 EMSA*

In order to show that regulatory sites in the salmon and cod promoters were capable of binding factors present in AS cell nuclei, EMSAs were performed using synthetic oligonucleotides corresponding to the sequences of the putative binding sites and AS cell nuclear extracts. The putative binding sites 2 and 5 for salmon, and sites 3, 4 and 5 for cod were investigated based on the results of the mutagenesis experiment. However, no shifts were observed using salmon site 2 and cod site 4 and site 5 probes (data not shown), but DNA-protein complexes were observed with salmon site 5 (Fig.7, lane 2) and with cod site 3 (Fig.8, lane 2). Salmon site 5 was the potential Sp1 site and cod site 3 was a possible CCAAT/enhancer binding protein (C/EBP) alpha site. A 200-fold molar excess of competitor DNA, unlabeled consensus oligonucleotides (lanes 3) completely inhibited the complex formations in both cases. The complexes were not observed when mutated oligonucleotide probes were used (lanes 4, 5 and 6). Similar results were obtained when the nuclear extract was prepared using cells cultured in dFBS without or with EPA (lanes 7 and 8), or more nuclear extract was used (lane 9, Fig.7).

**4. Discussion**

The LC-PUFA EPA was chosen as a know modulator of FAD expression in salmon, and the AS cell line was chosen as the cell system in which to study the salmon and cod promoters, based on results of several previous studies. The activity of the LC-PUFA biosynthesis pathway was significantly lower in liver and intestine from salmon being fed EPA and DHA in the form of fish oil compared to fish fed vegetable oils that are devoid of LC-PUFA (Tocher et al., 1997, 2001, 2003c). There is also a significant correlation between the activity of the LC-PUFA biosynthetic pathway and dietary n-3 LC-PUFA levels (Tocher et al., 2003b). Moreover several studies have demonstrated that the expression of Δ6 FAD mRNA was lower in livers of salmon fed fish oil compared to fish fed vegetable oils (Zheng et al., 2005a,b; Leaver et al., 2008b; Taggart et al., 2008). Therefore, there is strong evidence that the effects of dietary EPA and DHA in salmon are mediated through effects on the transcription of Δ6 FAD. Since the LC-PUFA biosynthesis pathway in AS cells has been previously investigated in some detail (Tocher and Sargent, 1990; Ghioni et al., 1999), and considering the evidence for regulation of this pathway by PUFA *in vivo*, the effect of potential lipid mediators was tested in this cell line. The results clearly showed that both EPA

and DHA suppress the LC-PUFA pathway in AS cells growing in lipid-deficient media, indicating that this cell line would be a suitable model for the investigating the mechanisms of transcriptional regulation of salmon FAD genes. Unfortunately no suitable cell lines exist for cod but, given the overall similarity in biochemical mechanisms of fatty acyl desaturation in vertebrates (Tocher et al., 1998; Behrouzian and Buist, 2002; Wallis et al., 2002; Nakamura and Nara, 2004), AS cells should have all the necessary ancillary enzyme machinery for testing both salmon and cod promoters.

Previously, the only detailed analysis of a vertebrate desaturase gene involved in LC-PUFA biosynthesis was that for the human FADS2 (Tang et al., 2003; Nara et al., 2002), and so the present paper is the first report comparing FAD gene structures of fish and other vertebrate species. Comparison of the salmon and cod Δ6 FAD genes with those identified in the ENSEMBL genome sequences of zebrafish, medaka and stickleback, showed that all the fish FADs consisted of 13 exons, the first of which was non-coding followed by 12 coding exons. In contrast, human Δ5 and Δ6 desaturases (FADS1 and FADS2, respectively) consisted of 12 coding exons and no 5' non-coding exons (Marquardt et al., 2000). The structure of *Xenopus* Δ6 FAD is less certain, but based on available EST sequences, it may also lack the 5' non-coding exon found in the fish species. Despite this difference, comparison of the sequences immediately 5' of the first exon for all these genes, showed an area with similarity across all species that included NF-Y and SREBP sites (Nara et al., 2002). However, the specific aim of the present study was to determine if there were differences in the promoters of salmon and cod Δ6 FADs that could account for, or contribute to, the differences in gene expression and enzymatic activity observed between these species (Tocher et al., 2006), and therefore studies were targeted to identify promoter sequences that were not conserved between salmon and cod.

The promoter deletion studies clearly defined regions that had transcriptional activity for both salmon and cod Δ6 FADs. The salmon promoter contained two sites, sites 2 and 5, which when absent from the deletion construct, either following deletion or by mutation, resulted in significantly lower activity. Sites 2 and 5 contain consensus sequences for a nuclear receptor half site and for the ubiquitous general transcription factor Sp1, respectively, with sites for the latter also demonstrated in human Δ6 FADS2 gene promoter (Tang et al., 2003). Although deletion of these sites considerably reduced transcriptional activity, they did not abolish it, nor did they have a significant effect on the relative reduction in activity caused by EPA. This suggests that Sp1 particularly may be important for constitutive expression of salmon Δ6

FAD, but is not responsible for the suppressive effect of EPA. Therefore, the sequence(s) driving the effect of EPA must reside in a region less than 315 nucleotides upstream of the salmon Δ6 FAD TSS.

In comparison to salmon, the cod promoter showed less activity but a region with significant promoter activity could still be defined. It was noteworthy that this region did not contain an obvious Sp1 binding site. Consistent with the low activity of the cod gene promoter observed here, Δ6 FAD is expressed at considerably lower levels in cod and other marine fish compared to salmon (Tocher et al., 2003a, 2006), and Δ6 desaturation activity is much lower in tissues of cod compared to salmon (Tocher et al., 2006). Therefore, based on the results presented here, it is possible to speculate that this difference in constitutive or basal expression of Δ6 FAD between salmon and cod may be due the presence of an Sp1-like site in the salmon promoter, which is not present in the corresponding region of the cod. The effects of EPA on the activity of the cod Δ6 FAD promoter were not consistent, and there was no substantial evidence to suggest that EPA affected promoter activity, although this may be a consequence of a lack of assay sensitivity, given the lower overall activity of this promoter compared to salmon. Similar inconclusive results were obtained *in vivo* for the effects of LC-PUFA on Δ6 FAD gene expression in cod. Although, the expression of Δ6 FAD in cod appeared lower in fish fed EPA- and DHA-rich fish oil compared to cod fed vegetable oil, this was not statistically significant (Tocher et al., 2006). For sure, only very low levels of fatty acyl desaturation activity were apparent in cod hepatocytes and enterocytes, irrespective of diet (Tocher et al., 2006).

The effects of LC-PUFA such as EPA, on fatty acid desaturation pathways have also been studied in mammalian systems. The levels of hepatic mRNA for Δ6 and Δ5 FADs in rats fed menhaden oil (EPA and DHA) were only 25 % of those in rats fed a fat-free diet or a diet containing triolein (18:1n-9) (Cho et al., 1999). Furthermore, a region of the human FADS2 gene promoter that is responsible for mediating transcriptional suppression by LC-PUFA has been described (Nara et al., 2002). This region contained a sterol regulatory element (SRE) adjacent to elements that are known to bind NF-Y transcriptions factors. These sites and the SREPB transcription factor appeared to mediate the suppressive effect of LC-PUFA (Nara et al., 2002). In an attempt to define regions of the salmon and cod desaturase genes that may also mediate the effects of LC-PUFA, the sequences were compared with that of the human FADS2 promoter, and also with putative promoter sequences for other fish and amphibian Δ6 FADs extracted from the ENSEMBL genome databases (Fig. 3). Interestingly, a region

bearing striking resemblance to the human FADS2 NF-Y and SREBP binding sites, in a similar position relative to the putative TSS, was present in all of the fish and amphibian desaturase genes. These results indicate a high level of sequence conservation of the Δ6 FAD promoter in diverse fish, amphibians and mammals, and suggest some evolutionary conserved mechanisms of regulation.

Studies on mammalian genes of the cholesterogenic and lipogenic pathways have shown that regulation by SREBP through binding to sterol response elements (SREs) is frequently dependent on the presence of adjacent NF-Y and/or Sp1 sites (Amemiya-Kudo et al., 2002; Teran-Garcia et al., 2007) and furthermore binding of SREBP to SREs induced the recruitment of NF-Y transcription factor to adjacent sites (Bennett and Osborne, 2000). Although to date SREBPs have not been characterised in fish species, their involvement in desaturase expression in Atlantic salmon has been previously suggested based on the increase in expression of both desaturase mRNA and an mRNA for an SREBP-like sequence following feeding of salmon diets low in LC-PUFA (Leaver et al., 2008b), and thus the involvement of SREBP in LC-PUFA regulation of Δ6 FAD genes in salmonids and/or freshwater fish is possible (Seiliez et al., 2001; Zheng et al., 2005a,b). Testing this possibility will be dependent on the identification and isolation of piscine SREBPs and other transcription factors. In addition however, as described above, there are differences between salmon and cod in the apparent regulation of Δ6 FAD and fatty acid desaturation by LC-PUFA. Thus, the clear correlations between dietary LC-PUFA, Δ6 desaturase expression and desaturation activity that have been observed in salmon, with reduced expression and activity in fish fed EPA- and DHA-rich fish oil compared to fish fed vegetable oils lacking LC-PUFA (Tocher et al., 2003b; Zheng et al., 2005a,b) have not been observed in cod (Tocher et al., 2006). Therefore, if SREBPs are involved, the molecular mechanism(s) underpinning the difference in LC-PUFA sensitivity between salmon and cod are unclear as both show conserved SREs in the Δ6 FAD gene promoters. This further highlights the importance of the identification and isolation of the relevant piscine transcription factors that will be required to elucidate the effects of LC-PUFA on salmon and cod FAD promoters. Finally, the EMSA results were positive in as much as the AS cell nuclear extract was shown to contain proteins that did bind to promoter regions, sites 3 (a possible C/EBPα site) and 5 (Sp1) in cod and salmon, respectively, that were shown to possess significant activity. However, purified piscine factors will also greatly enhance the insight that EMSA could provide to elucidating the complex mechanisms of gene regulation in fish.

In conclusion, deletion analysis of Δ6 FAD promoters in Atlantic salmon and Atlantic cod identified specific regions of 5' sequence that had promoter activity in a transfected salmon cell line. These areas were also identified as the areas associated with EPA-suppression of expression and are highly conserved across diverse vertebrates, indicating potentially common regulatory mechanisms. In addition though, a site was identified in salmon with similarity to that recognised by Sp1 transcription factor, and which was required for full expression of the salmon Δ6 FAD gene. In contrast, the cod promoter was less active and lacked the Sp1 site. The study represents the first report of functional promoter analysis of FAD genes in fish, and a further step in elucidating the molecular mechanisms of transcriptional regulation of LC-PUFA biosynthesis towards our goal of designing nutritional strategies to enhance and/or optimise the activity of the pathway in fish.

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Legends to Figures

**Fig. 1.** Structure and deletion analysis of salmon and cod Δ6 FAD gene promoters. Deletion constructs are represented on the left. Non-coding exon is indicated with open boxes, luciferase coding by closed boxes. Sequence is numbered relative to the first base of the transcription start site, assumed to be the first base of the 5'non-coding exon. Promoter activity of constructs is represented on the right and is values represent normalised activity (firefly luciferase: Renilla luciferase) relative to a construct containing no insert. Asterisks indicate that the effect of eicosapentaenoic acid (EPA) is significant compared to ethanol on the same construct (T-test; $p < 0.05$). The results are representative of three independent experiments. The gene structure of the cod and salmon desaturase genes is represented below. Numbers refer to exon (boxes) and intron (lines) sizes in base pairs. Salmon intron/exon size is indicated above the diagram and cod below. The dashed line indicates the point at which the promoter containing 5' region, untranslated exon sequence, the first intron and ATG start codon was ligated to replace the ATG of the luciferase gene.

**Fig. 2.** Effect of EPA and DHA on the activity of the LC-PUFA synthesis pathway in AS cells. Results are means ± S.D. (n = 4) and represent the rate of conversion ($pmol.h^{-1}.mg\ protein^{-1}$) of [1-$^{14}$C]18:3n-3 to all desaturated products (sum of radioactivity recovered as 18:4n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3) in AS cells. Significant differences between means as determined by ANOVA are indicated by different letters (Zar, 1984).

**Fig. 3.** Alignment of the salmon and cod promoter regions with the corresponding regions in other fish, amphibian and human FADS2. Salmon and cod sequences are as described herein. Stickleback (stick), zebrafish (zfish),medaka and *Xenopus tropicalis* sequences were extracted from regions upstream of the FADS2 homologues predicted in ENSEMBL genome databases. The numbers indicate the sequence position relative to possible transcriptional start sites (based on cDNA and EST sequence information). Shading indicates that the majority of nucleotides at that position are identical. NF-Y, nuclear factor Y binding site; SRE, sterol regulatory element.

**Fig. 4.** The nucleotide sequences of the 5' flanking region of the salmon and cod Δ6 FAD genes. Numbers are given relative to the first base of the transcription start site (TSS). Putative promoter regions are showed in bold letters (-546 to -321 for salmon, and -167 to -62

for cod). Potential transcription binding motifs investigated by mutational analysis are indicated by SITE 1-5 for salmon and SITE 1-5 for cod, with nucleotide mutations indicated by lower-case letters above the wild-type sequence.

**Fig. 5.** Effect of mutation on salmon Δ6 FAD gene promoter. Various mutations of the salmon promoter deletion SD2 (-546 to ATG, Fig. 1) were generated according to Fig. 4 and the resulting reporter gene activity expressed as a percentage of the corresponding non-mutated (wild type) promoter. Relative positions of Sp1 (●), NF-Y (▲) and SRE (◆) sites, as described in text and in Fig. 3 are indicated. Open boxes represent untranslated exon forming the wild type FAD gene, closed box represents luciferase reporter gene. Values represent the mean ± SEM of four different experiments in triplicate. *P < 0.01 by one-way ANOVA followed by Scheffe's test (Zar, 1984).

**Fig. 6.** Effect of mutation on cod Δ6 FAD gene promoter. Various mutations of the cod promoter deletion CD34 (-167 to ATG, Fig.1) were generated according to Fig. 4 and the resulting reporter gene activity expressed as a percentage of the corresponding non-mutated (wild type) promoter. Relative positions of Sp1 (●), NF-Y (▲) and SRE (◆) sites, as described in text and in Fig. 4 are indicated. Open boxes represent untranslated exon forming the wild type FAD gene, closed box represents luciferase reporter gene. Values represent the mean ± SEM of three different experiments in triplicate. *P < 0.01 by one-way ANOVA followed by Scheffe's test (Zar, 1984).

**Fig. 7.** Electrophoretic mobility shift assay (EMSA) of salmon Δ6 desaturase site 5 using AS cell nuclear extract. Ten μg of nuclear extract prepared from AS cells treated with normal medium (lane 2, 3, 5 and 6), lipid-free medium (lane 7) and EPA supplemented medium (lane 8), and 20 μg of nuclear extract prepared from AS cells treated with normal medium (Lane 9) were incubated with biotin labelled probe for site 5 (Sp1) (lanes 2, 3, 7, 8 and 9) or biotin labelled mutated site 5 probe (lanes 5 and 6). Unlabelled self probes were used as competitor DNA. Band A was the shifted DNA-protein complexes. Band B was the free probe.

**Fig. 8.** Electrophoretic mobility shift assay (EMSA) of cod Δ6 desaturase site 3 using AS cell nuclear extract. Twenty μg of nuclear extract prepared from AS cells treated with normal medium (lanes 2, 3, 5 and 6) and 15 μg from AS cells treated with lipid-free medium (lane 7)

and EPA supplemented medium (lane 8) were incubated with biotin labelled site 3 probe (lanes 2, 3, 7 and 8) or biotin labelled mutated site 3 probe (lanes 5 and 6). Unlabelled self probes were used as competitor DNA. Band A was the shifted DNA-protein complexes. Band B was the free probe.

**Table 1**

Primers used for creating deletions and EMSA

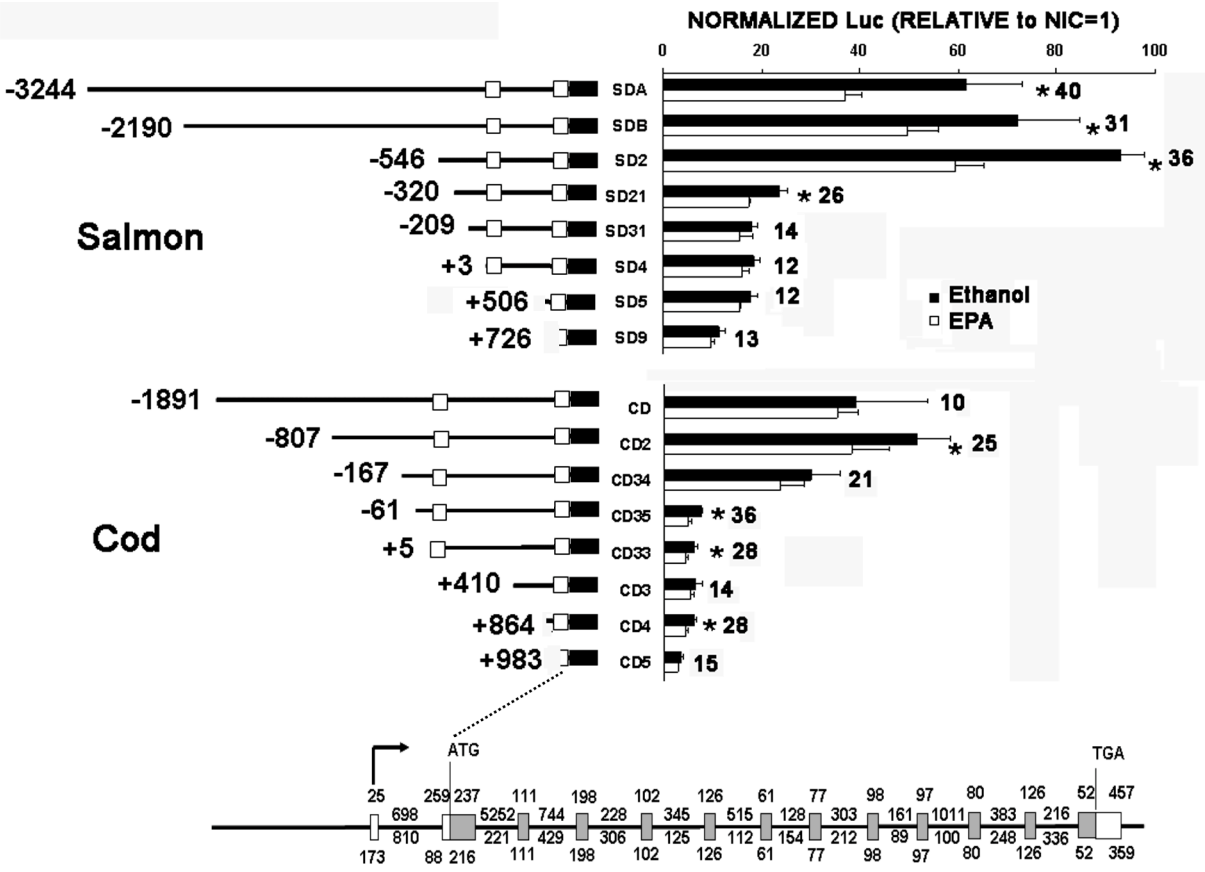
| Construct Name | Primer Name | primer sequences (5'-3') |
|---|---|---|
| Salmon Δ6 desaturase deletions | | |
| SDA | SD6FOR | cctaagcttGAGCTCGGAGCATAAGAATATC |
| SDB | SD6FOR3 | cctaagcttACAGGGAAGAGAAGGAGGTAGG |
| SD2 | SD6FOR5 | cctaagcttGGCCTTTTGGTAGGACATCA |
| SD21 | SD6F5A | cctaagcttGAGTGCGTCCAAGCGTTCTG |
| SD31 | SD6F6A | cctaagcttGGACTAGAGACCCTCGAATG |
| SD4 | SD6FOR7 | cctaagcttCTGGGAATATTGGTGAGTGC |
| SD5 | SD6FOR8 | cctaagcttAGAATCGGAGGTTAGCGATT |
| SD9 | SD6FOR9 | cctaagcttCAGAAATCTCCCAGTTCTGC |
| | SD6REV1 | aatccatggCCTCGGTTCTCTCTGCTCCAC |
| Cod Δ6 desaturase deletions | | |
| CD | CD6FOR | cctaagcttAGGCCTACTAGAATGC |
| CD2 | CD6FOR3 | cctaagcttCACCTCACTTCCAGTTTGCT |
| CD34 | CD6F3B1 | cctaagcttCAACTGTACTGCTGAAACGC |
| CD35 | CD6F3B2 | cctaagcttCGGGATACGCGCGGATTGGC |
| CD33 | CD6FOR3C | cctaagcttGAATGAGTGGGTGAATCTGC |
| CD3 | CD6FOR4 | cctaagcttACGCCAAACCATGTCACTTC |
| CD4 | CD6FOR5 | cctaagcttGGCGGACCTACAGAGACAGA |
| CD5 | CD6FOR6 | cctaagcttGTGGAAAACGTAAGCTCAGC |
| | CD6REV1 | aatccatggCGCCGCTGAGCTGAGTGCTGCC |
| EMSA probes | | |
| Salmon, site 2 | SDB1 | AGTTAAATAA**AGGTCA**AAAT |
| Salmon, site 5 | SDA1 | TCA**GGGGCGGGGCC**CTGGAGTG |
| Cod, site 3 | CDC1 | GATTT**CATTGAGCCAA**TTGCGA |
| Cod, site 4 | CDB1 | ATGCCA**GCGGTCGGGG**ATACGC |
| Cod, site 5 | CDA1 | TACGCGCG**GATTGG**TCCGGGAT |

**Table 2**

Mutated transcriptional binding factors and sequences of salmon and cod desaturase genes

| Genes | Binding site | Sequence | Mutated sequence |
|---|---|---|---|
| Salmon Δ6 | Site 1 | AGGACA | AttAtA |
| | Site 2 | AGGTCA | AttTtA |
| | Site 3 | ATATGTATT | ATAcGTAcc |
| | Site 4 | TGTACA | aaTAcA |
| | Site 5 | GGGGCGGGG | GaaaCaaGG |
| Cod Δ6 | Site 1 | CAACTG | CggCTG |
| | Site 2 | CGCCACACGCAC | CGttACAttCAC |
| | Site 3 | ATTGAGCCAAT | AccGAGaaAAT |
| | Site 4 | GCGGTCGGGG | GCaaTCaaGG |
| | Site 5 | GATTGG | GATTtt |

Fig. 1


NORMALIZED Luc (RELATIVE to NIC=1)
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20
40
60
80
100
Salmon
-3244
-2190
-546
-320
-209
+3
+506
+726
SDA
SDB
SD2
SD21
SD31
SD4
SD5
SD9
* 40
* 31
* 36
* 26
14
12
12
13
Ethanol
EPA
Cod
-1891
-807
-167
-61
+5
+410
+864
+983
CD
CD2
CD34
CD35
CD33
CD3
CD4
CD5
10
* 25
21
* 36
* 28
14
* 28
15
ATG
TGA
25 259 237 111 198 102 126 61 77 98 97 80 126 52 457
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173 88 216 111 198 102 126 61 77 98 97 80 126 52 359

Fig. 2

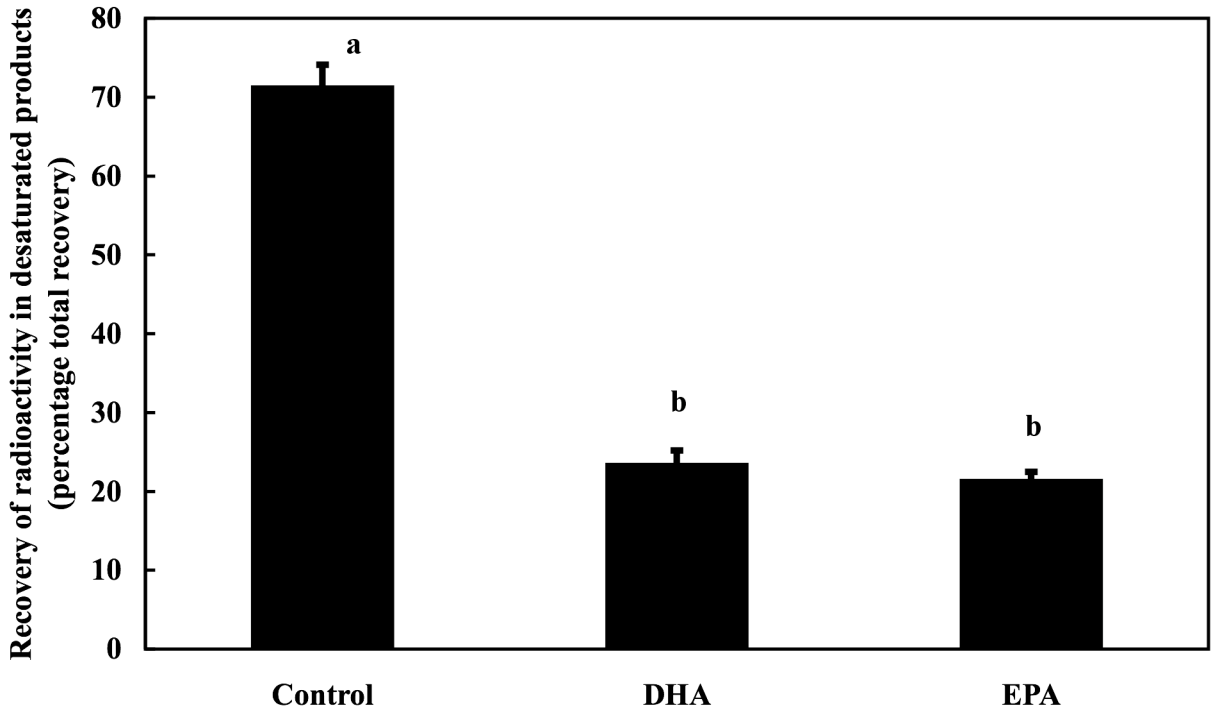
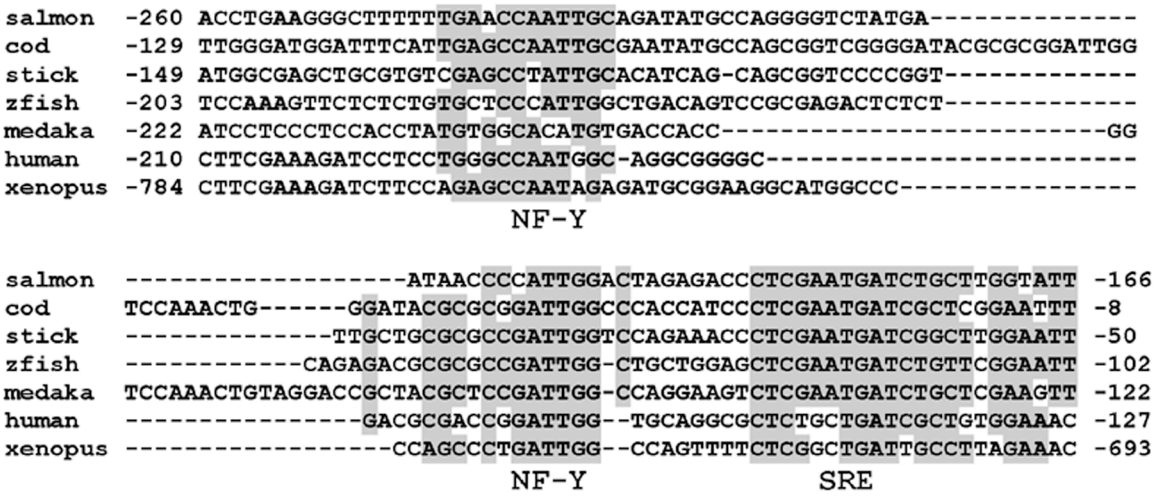

Recovery of radioactivity in desaturated products
(percentage total recovery)
80
70
60
50
40
30
20
10
0
a
b
b
Control
DHA
EPA

Fig. 3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cod -129 TTGGGATGGATTTCATTGAGCCAATTGCGAATATGCCAGCGGTCGGGGATACGCGCGGATTGG
stick -149 ATGGCGAGCTGCGTGTCGAGCCTATTGCACATCAG-CAGCGGTCCCCGGT----------
zfish -203 TCCAAAGTTCTCTCTGTGCTCCCATTGGCTGACAGTCCGCGAGACTCTCT----------
medaka -222 ATCCTCCCTCCACCTATGTGGCACATGTGACCACC----------GG
human -210 CTTCGAAAGATCCTCCTGGGCCAATGGC-AGGCGGGGC----------
xenopus -784 CTTCGAAAGATCTTCCAGAGCCAATAGAGATGCGGAAGGCATGGCCC----------
NF-Y
salmon ----------ATAACCCCATTGGACTAGAGACCCTCGAATGATCTGCTTGGTATT -166
cod TCCAAACTG------GGATACGCGCGGATTGGCCCACCATCCCTCGAATGATCGCTCGGAATTT -8
stick ----------TTGCTGCGCGCCGATTGGTCCAGAAACCCTCGAATGATCGGCTTGGAATT -50
zfish ----------CAGAGACGCGCGCCGATTGG-CTGCTGGAGCTCGAATGATCTGTTCGGAATT -102
medaka TCCAAACTGTAGGACCGCTACGCTCCGATTGG-CCAGGAAGTCTCGAATGATCTGCTCGAAGTT -122
human ----------GACGCGACCGGATTGG--TGCAGGCGCTCTGCTGATCGCTGTGGAAAC -127
xenopus ----------CCAGCCCTGATTGG--CCAGTTTTCTCGGCTGATTGCCTTAGAAAC -693
NF-Y
SRE

Fig. 4

**Salmon**

```
CCTATATTTTTATTTTATTATTTATTTTTAATCCTAACCCTCATACCCACAGGAGGCCTT -541
      tt t
TTGGTAGGACATCATTGTAAATAAGAATTTGTTCTTAACTGACTTGCCTAGTTAAATAAA -481
     SITE 1
tt t      c   cc                   aa
GGTCAAAATATGTATTATGTCATGTTTTATGTTTTGTACAAACCCCAGGAAGACCCCTAA -421
SITE 2   SITE 3                    SITE 4
TAAAATACTACATACTAAAGCATCAAGGGGCTGTCATTTTCTTCTTCTTGAGACAACGTG -361
                          aaa aa
TAGACACGCACTAAGCTGATGTGTCAGGGGCGGGGCCCTGGAGTGCGTCCAAGCGTTCTG -301
                           SITE5
ATTGGTTGAAATATGTGGATGATTGACAGACCTGAAGGGCTTTTTTGAACCAATTGCAGA -241
TATGCCAGGGGTCTATTGAAATAACCCCATTGGACTAGAGACCCTCGAATGATCTGCTTG -181
GTATTGTATAGTGAACGAGCGGGTGAATGACTGATTGAGCTATAGGGCGTCATGGAACGA -121
GAGATAGGCCTACCTTTAGTAGCCGACTGATGATTTGATTGGATAAATGACGAGACTGGT -61
GATCAGAATGAAGTTCATGTATTTTTATGAATAAGAGAAGGAGCGAGAAGTAAAGAACGC -1
```

**Cod**

```
TTCATCAGTATTTTTAGACTAATTGTTATGGACTCAACCGATTCTTTCACTATTTGGCTA -241
TATCCTCGTTGTTGAAGTCACTTTGGCCAAATTAGTATGCCAATGCAAATGCAATGTACC -181
              gg                     tt   tt
AGCATTTCCAGTTCAACTGTACTGCTGAAACGCACGCCACACGCACGCCACATTTTGGGA -121
              SITE 1                   SITE 2
          cc   aa                   aa  aa                tt
TGGATTTCATTGAGCCAATTGCGAATATGCCAGCGGTCGGGGATACGCGCGGATTGGTCC -61
           SITE 3                    SITE 4           SITE 5
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Fig. 5

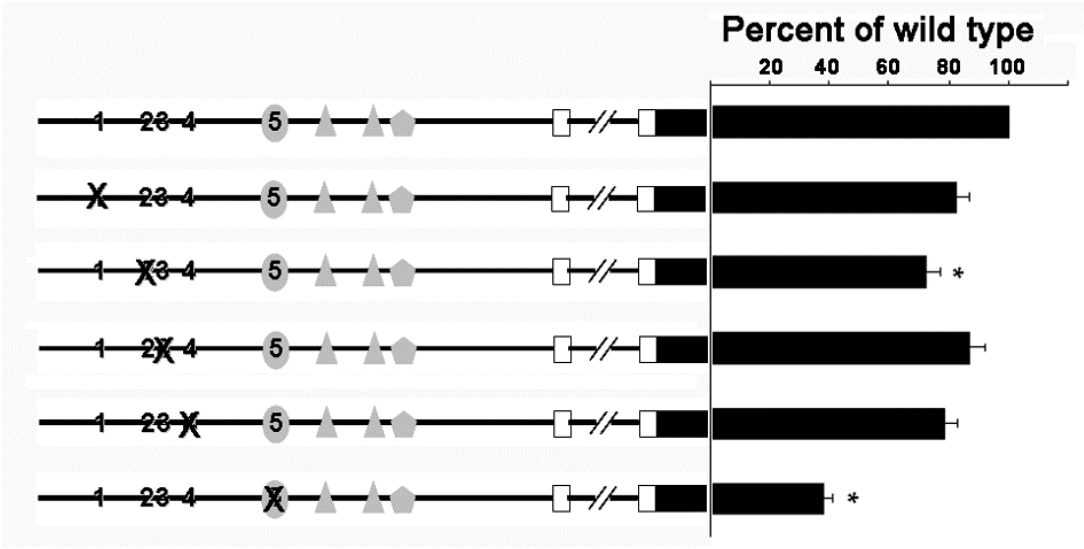
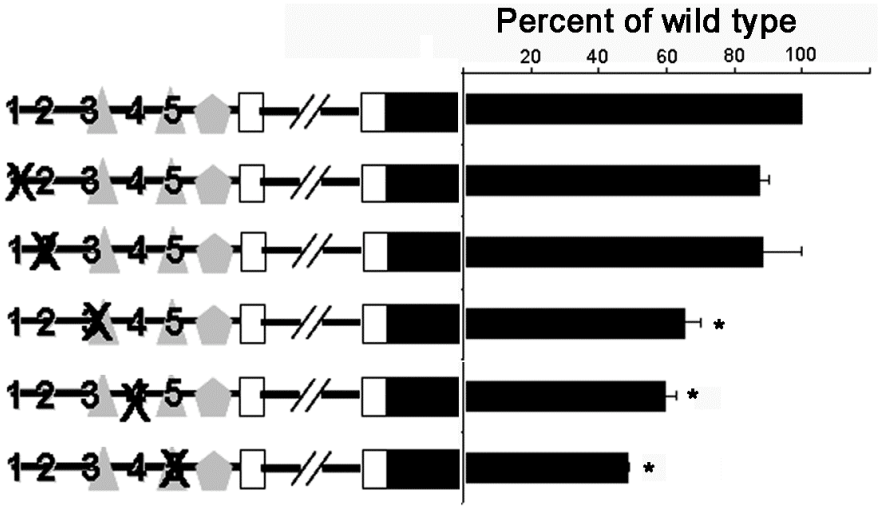


Fig. 6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60
80
100
1-2-3-4-5
2-3-4-5
1-3-4-5
1-2-4-5
1-2-3-5
1-2-3-4
*
*
*

Fig. 7

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|
| Nuclear Extract | - | + | + | - | + | + | + | + | + |
| Site 5 probe | + | + | + | - | - | - | + | + | + |
| Mutated probe | - | - | - | + | + | + | - | - | - |
| Competitor DNA | - | - | + | - | - | + | - | - | - |

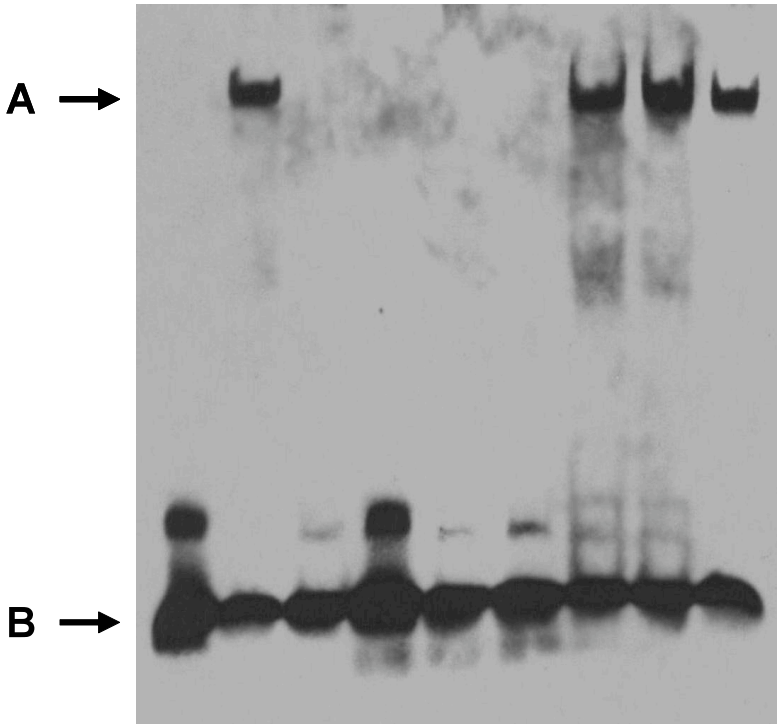

Fig. 8

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Nuclear Extract | - | + | + | - | + | + | + | + |
| Site 3 probe | + | + | + | - | - | - | + | + |
| Mutated probe | - | - | - | + | + | + | - | - |
| Competitor DNA | - | - | + | - | - | + | - | - |