Highly Unsaturated Fatty Acid Synthesis in Vertebrates: New Insights with the Cloning and Characterization of a Δ6 Desaturase of Atlantic Salmon

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ABSTRACT: Fish are an important source of the n-3 highly unsaturated fatty acids (HUFA), eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids that are crucial to the health of higher vertebrates. The synthesis of HUFA involves enzyme-mediated desaturation, and a Δ5 fatty acyl desaturase cDNA has been cloned from Atlantic salmon (*Salmo salar*) and functionally characterized previously. Here we report cloning and functional characterization of a Δ6 fatty acyl desaturase of Atlantic salmon and describe its genomic structure, tissue expression, and nutritional regulation. A salmon genomic library was screened with a salmon Δ5 desaturase cDNA and positive recombinant phage isolated and subcloned. The full-length cDNA for the putative fatty acyl desaturase was shown to comprise 2106 bp containing an open reading frame of 1365 bp specifying a protein of 454 amino acids (GenBank accession no. AY458652). The protein sequence included three histidine boxes, two transmembrane regions, and an N-terminal cytochrome b_5 domain containing the heme-binding motif HPGG, all of which are characteristic of microsomal fatty acid desaturases. Functional expression showed that this gene possessed predominantly Δ6 desaturase activity. Screening and sequence analysis of the genomic DNA of a single fish revealed that the Δ6 desaturase gene constituted 13 exons in 7965 bp of genomic DNA. Quantitative real-time PCR assay of gene expression in Atlantic salmon showed that both Δ6 and Δ5 fatty acyl desaturase genes, and a fatty acyl elongase gene, were highly expressed in intestine, liver, and brain, and less so in kidney, heart, gill, adipose tissue, muscle, and spleen. Furthermore, expression of both Δ6 and Δ5 fatty acyl desaturase genes in intestine, liver, red muscle, and adipose tissue was higher in salmon fed a diet containing vegetable oil than in fish fed a diet containing fish oil.

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Highly unsaturated fatty acids (HUFA), including arachidonate (20:4n-6), EPA (20:5n-3), and DHA (22:6n-3), are crucial to the health and normal development of higher vertebrates (1–3). Fish are the most important source of n-3 HUFA for humans, but with fisheries in decline, an increasing proportion of fish are being provided by rapidly expanding aquaculture (4). Paradoxically, aquaculture is itself dependent on fisheries for the provision of fish meals and oils traditionally used in the feed formulations (5). Their use ensures the high nutritional quality of farmed fish through the high levels of n-3 HUFA that fish oil (FO) and fish meal provide. However, feed-grade fisheries have reached sustainable limits. Along with concern over organic contaminants in FO, this has dictated that alternatives to FO must be found if aquaculture is to continue to expand and supply more of the global demand for fish (6).

The only practical, sustainable alternative to FO is vegetable oils (VO), which are rich in C_{18} PUFA but devoid of the n-3 HUFA abundant in FO (7). Consequently, tissue FA compositions in fish fed VO are characterized by increased levels of C_{18} PUFA and decreased levels of n-3 HUFA, which may reduce their nutritional value to the human consumer (8). The extent to which fish can convert C_{18} PUFA to HUFA varies, associated with their complement of FA desaturase enzymes. Although Atlantic salmon (*Salmo salar* L.) are capable of producing DHA from 18:3n-3, and so express the necessary desaturase activities, the production is insufficient to maintain n-3 HUFA in fish fed VO at levels found in fish fed FO (9–11). Our primary hypothesis is that understanding the molecular basis of HUFA biosynthesis and its regulation in fish will enable us to optimize the activity of the pathway to ensure efficient and effective use of VO in aquaculture while maintaining the nutritional quality of farmed fish for the consumer.

Δ5 and Δ6 fatty acyl desaturases and elongases are critical enzymes in the pathways for the biosynthesis of HUFA. In recent years, significant progress has been made in characterizing FA desaturases involved in HUFA synthesis (12). Full-length cDNAs for Δ6 desaturases have been isolated from the filamentous fungus *Mortierella alpina* (13), the nematode *Caenorhabditis elegans* (14), rat (15), mouse and human (16). FA Δ5 desaturase genes have been isolated from *M. alpina* (17), *C. elegans* (18,19), and humans (20,21). Moreover, we have reported isolation of a cDNA of zebrafish (*Danio rerio*, GenBank accession no. AF309556) that has high similarity to mammalian Δ6 desaturase genes. Functional analysis by heterologous expression in the yeast *Saccharomyces cerevisiae* indicated that the zebrafish gene was unique in that the cDNA encoded an enzyme having both Δ6 and Δ5 desaturase activities (22). Putative FA desaturase cDNAs have now also been

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Abbreviations: FO, fish oil; HUFA, highly unsaturated fatty acids (carbon chain length ≥20 with ≥3 double bonds); MMLV, Moloney murine leukemia virus; ORF, open reading frame; qrtPCR, quantitative (real-time) polymerase chain reaction; RACE, rapid amplification of cDNA ends; SCMM, *Saccharomyces cerevisiae* minimal medium; UTR, untranslated region; VO, vegetable oil.

isolated and cloned from rainbow trout (*Oncorhynchus mykiss*, GenBank accession no. AF301910) (23) and gilthead seabream (*Sparus aurata*, GenBank accession no. AY055749) (24). Functional analysis showed that these two desaturase genes, along with cDNAs recently cloned from common carp (*Cyprinus carpio*, GenBank accession no. AF309557) and turbot (*Psetta maximus*, GenBank accession no. AF301910) encoded basically unifunctional Δ6 FA desaturase enzymes responsible for the first and possibly rate-limiting step in the biosynthesis of HUFA from 18:3n-3 and 18:2n-6 (25). Recently, a full-length cDNA for a desaturase containing 1365 bp encoding 454 amino acid residues has been cloned from Atlantic salmon (GenBank accession no. AF478472). Functional analysis showed that this gene was primarily a Δ5 desaturase with virtually no Δ6 activity (26). Therefore, it was presumed that other FA desaturase genes should be present in Atlantic salmon.

The objectives of the study described here were, first, to clone and functionally characterize a Δ6 desaturase gene of Atlantic salmon; second, to describe its genomic structure; and third, to place it in evolutionary and physiological contexts. Therefore, we detail the exon/intron organization of a salmon Δ6 desaturase gene, describe the expression profile of both Δ6 and Δ5 fatty acyl desaturase and fatty acyl elongase genes in various tissues, and demonstrate nutritional regulation of the fatty acyl desaturase genes.

MATERIALS AND METHODS

Putative desaturase cloning and its genomic organization. An Atlantic salmon genomic DNA library constructed previously with the lambda FIX II/Xho I partial fill-in vector kit (Stratagene, La Jolla, CA) was probed with a full-length salmon Δ5 fatty acyl desaturase cDNA (GenBank accession no. AF478472). Inserts of positive recombinant phages were isolated and subcloned into the pBluescript KS II vector for sequencing (Stratagene). The full putative desaturase genomic nucleotide sequence was assembled using BioEdit, version 5.0.6 (Tom Hall, Department of Microbiology, North Carolina State University, Raleigh, NC).

Total RNA was extracted from liver tissue of Atlantic salmon fed a standard extruded diet based on fish meal and FO using TRIzol® reagent (GibcoBRL, Grand Island, NY). 3′-RACE (rapid amplification of cDNA ends) cDNA was synthesized using MMLV (Moloney murine leukemia virus) reverse transcriptase (Promega, Madison, WI) primed by the oligonucleotide, T7PolyT, 5′-TACGACTCATATAGGGCGTGCAGTTTT TTTTTTTT-3′. The specific sense primer, D6P31, 5′-CAGGGGTGGGCCCGGTGGAGGGCTA-3′ was designed for 3′-RACE PCR based on the genomic sequence described above. This was used in conjunction with T7PolyT primer for the RACE PCR isolation of the salmon desaturase cDNA fragment predicted to contain the 3′ UTR (untranslated region). PCR amplification was performed using the Hotstar Taq master kit (Qiagen, Crowley, West Sussex, United Kingdom) and involved an initial denaturation step at 95°C for 15 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 3 min. The final extension at 72°C was for 10 min. 5′-RACE-cDNA was synthesized using the SMART™ RACE cDNA amplification kit (Clontech, Palo Alto, CA). The primer, SD6PPR3, 5′-GTCGCATTCCATCCCAATCC-3′ was designed according to the 3′-RACE PCR fragment sequence. This was used in conjunction with universal primer mix: long 5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTACAACGGAGT-3′ and short 5′-CTAATACGACTCACTATAGGGC-3′ to perform 5′-RACE PCR using high-fidelity DNA polymerase (Roche Diagnostics Ltd., Lewes, East Sussex, United Kingdom). Amplification involved an initial step at 95°C for 1 min and 70°C for 3 min, and four cycles of denaturation at 95°C for 15 s, annealing at 62°C for 1 min, and extension at 72°C for 1 min 30 s, followed by 27 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 30 s and extension at 72°C for 1 min 30 s. The final extension at 72°C was for 10 min.

All RACE PCR products were cloned into the pBluescript KS II$^+$ vector for sequencing. The 3′ and 5′ RACE PCR fragment sequences were aligned to assemble the full nucleotide sequence of the putative desaturase cDNA using BioEdit, version 5.0.6. The assembled putative fatty acyl desaturase cDNA sequence and its genomic DNA sequence were aligned to assign consensus donor and acceptor splice recognition sequences.

Heterologous expression of desaturase open reading frames (ORF) in S. cerevisiae. PCR amplification was carried out to clone the salmon putative desaturase cDNA ORF. Sense primer, D6RF2, 5′-ATGGGGGGCGGAGGCCAGCAGAATGATTCAG-3′, and antisense primer, D6RR1, 5′-ATGCGATGGATTAAATCCCG –3′ (located in the 3′ UTR) were designed for first-round PCR after comparing nucleotide sequences of this putative cDNA and the Δ5 desaturase cDNA. Expression primers were designed for a second round of PCR. The sense primer, SalpYESFOR, 5′-CCCAAGCTTACTATGGGGGGCGGAGGCC-3′ contains a *HindIII* site (underlined) and antisense primer, SalPYESREV2, 5′- CCGCTCGAGTCATTTATGGAGATATGCAT-3′ contains an *XhoI* site (underlined). PCR was performed using high-fidelity DNA polymerase (Roche Diagnostics Ltd.) following the manufacturer's instructions. Amplification involved an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min and 30 s followed by a final extension at 72°C for 10 min.

Following PCR, the DNA fragments were restricted with the appropriate enzymes, *HindIII* and *XhoI*, and ligated into the similarly digested yeast expression vector pYES2 (Invitrogen Ltd., Paisley, United Kingdom). Ligation products were then used to transform Top10F′ *Escherichia coli* competent cells (Invitrogen Ltd.), which were screened for the presence of recombinants. Transformation of the yeast *S. cerevisiae* (strain InvSc1) with the recombinant plasmids was carried out using the S.c.EasyComp Transformation Kit (Invitrogen Ltd.). Selection of yeast containing the desaturase/pYES2

constructs was on *S. cerevisiae* minimal medium (SCMM) minus uracil. Culture of the recombinant yeast was carried out in $SCMM^{-uracil}$ broth as described previously (22), using galactose induction of gene expression. Each culture was supplemented with one of the following PUFA substrates: α-linolenic acid (18:3n-3), linoleic acid (18:2n-6), eicosatetraenoic acid (20:4n-3), dihomo-γ-linoleic acid (20:3n-6), docosapentaenoic acid (22:5n-3), or docosatetraenoic acid (22:4n-6). PUFA were added to the yeast cultures at concentrations of 0.5 mM (C_{18}), 0.75 mM (C_{20}), and 1 mM (C_{22}), as uptake efficiency decreases with increasing chain length. Yeast cells were harvested, washed, and dried, and lipid was extracted by homogenization in chloroform/methanol (2:1, vol/vol) containing 0.01% BHT as antioxidant as described previously (22). FAME were prepared, extracted, purified by TLC, and analyzed by GC, all as described previously (22). The proportion of substrate FA converted to the longer-chain FA product was calculated from the gas chromatograms as 100 × [product area/(product area + substrate area)]. Unequivocal confirmation of FA products was obtained by GC–MS of the picolinyl derivatives as described in detail previously (22).

Salmon tissue RNA extraction and quantitative real-time PCR (qrtPCR). Tissue expression profiles and effects of diet were investigated in Atlantic salmon that had been fed one of two diets from the first feeding. The diets consisted of a control in which FO was the only added oil and an experimental diet in which 75% of the FO was replaced by a VO blend containing rapeseed, palm, and linseed oils in a 3.7:2:1 ratio. Both diets were fishmeal-based and contained 48% protein, 26% lipid, 7% moisture, and 8% ash as determined by proximate analyses. The FA compositions of the diets (6 mm pellet) are given in Table 1. The diets were prepared by the Nutreco Aquaculture Research Centre (Stavanger, Norway) and formulated to satisfy the nutritional requirements of salmonid fish (27).

Fish were sampled in November 2003, 6 mon after seawater transfer, following 18 mon on the diets, at which point the weights of the fish fed the FO and VO diets were 1250.0 ± 84.9 and 1280.0 ± 79.4 g, respectively. Eight fish per dietary treatment were sampled, and liver, brain, heart, kidney, gill, intestine (pyloric caeca), spleen, white and red muscle, and adipose tissue were collected, frozen immediately in liquid nitrogen, and subsequently stored at –80°C before extraction. Total RNA extraction was performed as described above. Five micrograms of total RNA was reverse-transcribed into cDNA using an MMLV reverse transcriptase first strand cDNA synthesis kit (Promega UK, Southampton, United Kingdom). Gene expression of the fatty acyl Δ6 and Δ5 desaturase, and fatty acyl elongase genes in tissue from individual salmon fed the different diets were studied by quantitative real-time PCR (qrt-PCR). β-Actin was used for normalization of mRNA levels. The PCR primers were designed according to Δ6 desaturase (accession no. AY458652), and the published Δ5 desaturase (accession no. AF478472), elongase (accession no. AY170327), and β-actin (accession no. AF012125) cDNA sequences. For the Δ6 desaturase, the forward primer was 5′-CCCCAGACGTTTGTGTCAG-3′, and the reverse primer was 5′-CCTGGATTGTTGCTTTGGAT-3′. For the Δ5 desaturase, the forward primer was 5′-GTGAATGGGGATCCATAGCA-3′, and the reverse primer was 5′-AAACGAACGGACAACCAGA-3′. For the elongase, the forward and reverse primers were 5′-TGATTTGTGTTCCAAATGGC-3′ and 5′-CTCATGACGGGAACCT CAAT-3′, respectively. For β-actin, 5′-ACATCAAGGAGAAGCTGTGC-3′ and 5′-GACAACGGAACCTCTCGTTA-3′ were the forward and reverse primers, respectively. PCR product sizes were 181, 192, 219, and 141 bp, respectively. The linearized plasmid DNA containing the target sequence for each gene was quantified to generate a standard curve of known copy number. Amplification of cDNA samples and DNA standards was carried out using a SYBR Green PCR kit (Qiagen) under the following conditions: 15 min denaturation at 95°C, 45 cycles of 15 s at 94°C, 15 s at 55°C, and 30 s at 72°C. This was followed by product melt to confirm single PCR products. Thermal cycling and fluorescence detection were conducted in a Rotor-Gene 3000 system (Corbett Research, Cambridge, United Kingdom). The copy numbers of the specific genes in the sample, normalized to total RNA, were used to compare expression levels between different tissues, and the ratios of copy numbers between the target genes

TABLE 1
FA Composition (percentage of total FA) of Diets[a]

	FO	VO
14:0	6.1	2.4
16:0	14.7	16.0
18:0	2.8	3.3
Total saturated[b]	24.3	21.9
16:1n-7[c]	5.0	2.0
18:1n-9	13.5	35.2
18:1n-7	2.5	2.3
20:1n-9[d]	10.4	3.6
22:1n-11[e]	14.9	4.8
24:1n-9	0.7	0.3
Total monoenes	47.0	48.2
18:2n-6	4.0	11.8
20:4n-6	0.5	0.2
Total n-6 PUFA[f]	5.1	12.2
18:3n-3	1.1	8.5
18:4n-3	2.4	0.8
20:4n-3	0.7	0.2
20:5n-3	6.7	2.8
22:5n-3	1.1	0.4
22:6n-3	10.4	4.5
Total n-3 PUFA[g]	22.4	17.3
Total PUFA[h]	28.7	29.9

[a]Data are the means of two samples. FO, fish oil; VO, vegetable oil blend.
[b]Totals contain 15:0 present at up to 0.5%.
[c]Contains 16:1n-9.
[d]Contains 20:1n-11 and 20:1n-7.
[e]Contains 22:1n-9.
[f]Totals contain 18:3n-6, 20:2n-6, 20:3n-6, and 22:5n-6 present at up to 0.2%.
[g]Totals contain 20:3n-3 present at up to 0.1%.
[h]Totals contain C_{16} PUFA.

and β-actin were calculated and used to compare the gene expression levels in fish fed the two diets.

Sequence analysis. Nucleotide sequences were determined by standard dye terminator chemistry using a PerkinElmer ABI-377 DNA sequencer following the manufacturer's protocols (PerkinElmer, Applied Biosystems). Deduced amino acid sequences of desaturases from various species were aligned using ClustalX, and sequence phylogenies were predicted using the Neighbour Joining method (28). Confidence in the resulting phylogenetic tree branch topology was measured by bootstrapping through 1000 iterations.

Materials. Eicosatetraenoic (20:4n-3), docosapentaenoic (22:5n-3), and docosatetraenoic (22:4n-6) acids (all >98–99% pure) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Linoleic (18:2n-6), α-linolenic (18:3n-3), and eicosatrienoic (20:3n-6) acids (all >99% pure), BHT, 1,1′-carbonyldiimidazole, 2,2-dimethoxypropane, FA-free BSA, galactose, 3-(hydroxymethyl) pyridine, HBSS, nitrogen base, raffinose, tergitol NP-40, and uracil dropout medium were obtained from Sigma Chemical Co. Ltd. (Dorset, United Kingdom). TLC (20 cm × 20 cm × 0.25 mm) plates precoated with silica gel 60 (without fluorescent indicator) were purchased from Merck (Darmstadt, Germany). All solvents were HPLC grade and were from Fisher Scientific (Loughborough, United Kingdom).

RESULTS

Sequence analyses. The full length of the putative salmon desaturase cDNA (mRNA), as determined by 5′- and 3′-RACE PCR, was shown to be 2106 bp, which included a 5′-UTR of 284 bp and a 3′-UTR of 457 bp. Sequencing revealed that the cDNA included an ORF of 1365 bp, which specified a protein of 454 amino acids (GenBank accession no. AY458652). The protein sequence included all the characteristic features of microsomal FA desaturases, including three histidine boxes and an N-terminal cytochrome b_5 domain containing the heme-binding motif, HPGG (Fig. 1). The protein sequence also contained two transmembrane regions. These features are similar to those of other FA desaturase genes including salmon Δ5 desaturase, the zebrafish Δ6/Δ5 desaturase, and the human Δ5 (GenBank accession no. AF126799) and Δ6 (GenBank accession no. AF199596) desaturases. However, the new salmon desaturase, like the salmon Δ5 desaturase and the rainbow trout Δ6 desaturase sequences, had an insertion of 10 amino acid residues at the N-terminal end.

A pairwise comparison was made between fish and human desaturase sequences. The amino acid sequence predicted by the salmon putative (Δ6) desaturase ORF shows 91% identity to the salmon Δ5 desaturase, and 94% identity to the trout Δ6 desaturase. The salmon cDNA shows 65% identity to that of the zebrafish Δ6/Δ5 desaturase, and 65 and 58% identity to the human Δ6 and Δ5 cDNAs, respectively.

A phylogenetic tree was constructed on the basis of the amino acid sequence alignments between the salmon fatty acyl desaturases and 15 other desaturases of fish and mammals (Fig. 2). The phylogenetic analysis clustered the new Atlantic salmon putative desaturase sequence with the Atlantic salmon Δ5 desaturase, rainbow trout Δ6 desaturase and other, as yet uncharacterized, masou (cherry) salmon (*Oncorhynchus masou*) desaturase genes, but closest to the trout Δ6 desaturase. The salmonid desaturases clustered more closely with turbot, sea bream, and tilapia (*Oreochromis nilotica*) desaturases than with carp Δ6 desaturase and zebrafish Δ5/Δ6 desaturase. All of the fish desaturase genes clustered together, and closer to the mammalian (mouse and human) Δ6 desaturases than to the mammalian Δ5 desaturases.

Functional characterization. The salmon desaturase cDNA was functionally characterized by determining the FA profiles of transformed *S. cerevisiae* containing either the pYES vector alone or the vector with the salmon desaturase cDNA insert, grown in the presence of a variety of potential FA substrates, including Δ6 substrates (18:2n-6 and 18:3n-3), Δ5 substrates (20:3n-6 and 20:4n-3), and Δ4 substrates (22:4n-6 and 22:5n-3). The FA composition of the yeast transformed with the vector alone showed the four main FA normally found in *S. cerevisiae*, namely 16:0, 16:1n-7, 18:0, and 18:1n-9, together with the exogenously derived FA. This is consistent with *S. cerevisiae* not possessing Δ5 or Δ6 FA desaturase activities (Figs. 3 and 4). The most prominent additional peaks were observed in the profiles of transformed yeast grown in the presence of the Δ6 desaturase substrates, 18:3n-3 and 18:2n-6 (Fig. 3). Based on GC retention time and confirmed by GC–MS, the additional peaks associated with the presence of the salmon desaturase cDNA were identified as 18:4n-3 (Fig. 3B) and 18:3n-6 (Fig. 3D), corresponding to the Δ6 desaturation products of 18:3n-3 and 18:2n-6, respectively. Approximately, 60.1% of 18:3n-3 was converted to 18:4n-3 and 14.4% of 18:2n-6 was converted to 18:3n-6 in yeast transformed with the salmon desaturase (Table 2). However, a very small additional peak representing desaturated FA product, as confirmed by GC–MS, was observed in the lipids of *S. cerevisiae* transformed with the desaturase cDNA when the transformed yeast was incubated with 20:4n-3 (Figs. 4A and 4B). About 2.3% of 20:4n-3 (n-3 Δ5 activity) was desaturated by the salmon clone, but no product of desaturation of the 20:3n-6 substrate was detected, indicating no significant n-6 Δ5 desaturase activity. The desaturase cDNA did not express any Δ4 desaturase activity as evidenced by the lack of any observable additional peaks representing desaturated products of 22:5n-3 or 22:4n-6 (data not shown). Overall, therefore, the results showed that the salmon desaturase cDNA-encoded enzyme was essentially a Δ6 fatty acyl desaturase, with only a very low level of Δ5 desaturase activity, and no Δ4 desaturase activity.

Genomic structure. The alignment of the Δ6 fatty acyl desaturase cDNA and the genomic sequences revealed 13 exons spanning 7965 bp of genomic DNA as illustrated in Table 3.

FA desaturase and elongase gene expression in salmon tissues. To identify which tissues were likely to contribute to HUFA synthesis in the Atlantic salmon, reverse transcription qrtPCR was used to examine the tissue distribution of Δ6 and

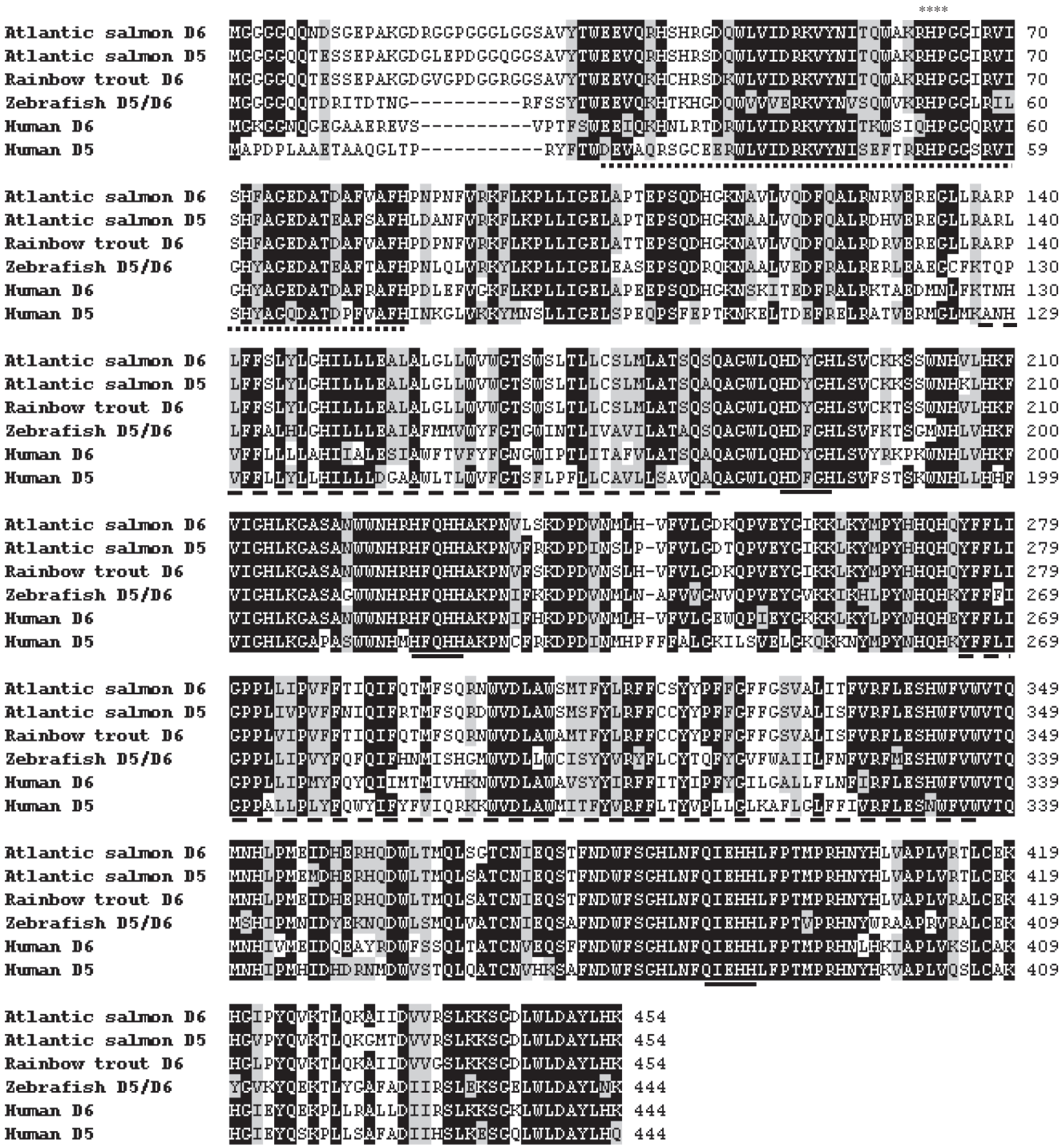

FIG. 1. Comparison of the deduced amino acid sequence of Δ6 and Δ5 polyunsaturated fatty acyl desaturases from Atlantic salmon with that of desaturases from trout, zebrafish, and humans. Identical residues are shaded black, and similar residues are shaded grey. Identity/similarity shading was based on the BLOSUM62 matrix, and the cutoff for shading was 75%. The cytochrome b_5-like domain is dot-underlined, the two transmembrane regions are dash-underlined, the three histidine-rich domains are solid underlined, and asterisks on the top mark the heme-binding motif, HPGG.

Δ5 fatty acyl desaturase and fatty acyl elongase mRNAs. The results showed that the three genes were expressed in all tissues examined, with the highest expression in terms of the absolute copy numbers (mean ± SD, $n = 8$) in intestine, followed by liver and brain (Fig. 5). In comparison with the Δ5 desaturase, the transcript copy abundance for the Δ6 desaturase was higher in these tissues with higher expression, but lower in tissues with lower expression, other than kidney. The transcript copy abundance for fatty acyl elongase was much lower than that for the Δ6 and Δ5 desaturases in all tissues.

The ratios of copy numbers between the target genes and β-actin were determined (means ± SD, $n = 4$), and the fold

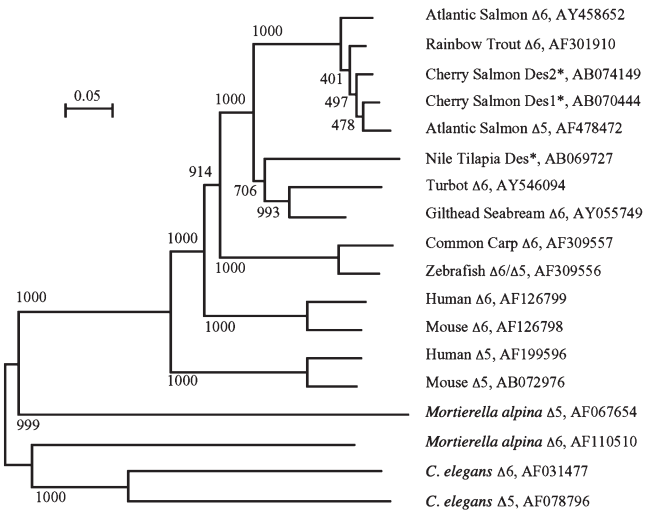

FIG. 2. Phylogenetic tree of Δ6 and Δ5 desaturases from salmon, and desaturases from other fish species (zebrafish, cherry salmon, rainbow trout, seabream, common carp, turbot, and tilapia), mammals (mouse and human), fungus (*Mortierella alpina*), and nematode (*Caenorhabditis elegans*). The tree was constructed using the Neighbour Joining method using *ClustalX* and *NJPLOT*. The horizontal branch length is proportional to the amino acid substitution rate per site. The numbers represent the frequencies with which the tree topology presented here was replicated after 1000 bootstrap iterations. Sequences marked with an asterisk are not functionally characterized.

difference between the mean value of target gene expression in the tissue of fish fed VO was calculated relative to the expression in tissues of fish fed FO (Fig. 6). The results revealed that Δ6 and Δ5 fatty acyl desaturase gene expression in liver and red muscle of fish fed VO was significantly increased compared with fish fed the FO diet, whereas the expression of both desaturases in heart and spleen, and Δ5 in gill and kidney was decreased in fish fed VO (Fig. 6). Expression of both desaturases in intestine and adipose tissue was also higher in fish fed VO, although with the high variation these effects were below the level of statistical significance. However, feeding VO decreased the expression of the fatty acyl elongase gene in most tissues, significantly so in heart, gill, brain, adipose, spleen, and kidney (Fig. 6).

DISCUSSION

Several fish desaturases have been cloned and functionally characterized in recent years. These are the bifunctional zebrafish enzyme showing both Δ6 and Δ5 desaturase activity (22), an Atlantic salmon desaturase that was shown to be predominantly an n-3 Δ5 desaturase (26), and common carp, rainbow trout, gilthead seabream, and turbot desaturases that were all shown to be predominantly Δ6 desaturases (25). The bifunctional nature of the Δ6/Δ5 desaturase of zebrafish suggested that it may be a prototypic or ancestral progenitor desaturase (22,29). But the subsequent characterization of several essentially unifunctional Δ6 fish desaturases and the salmon Δ5 desaturase indicates that the zebrafish enzyme might be atypical.

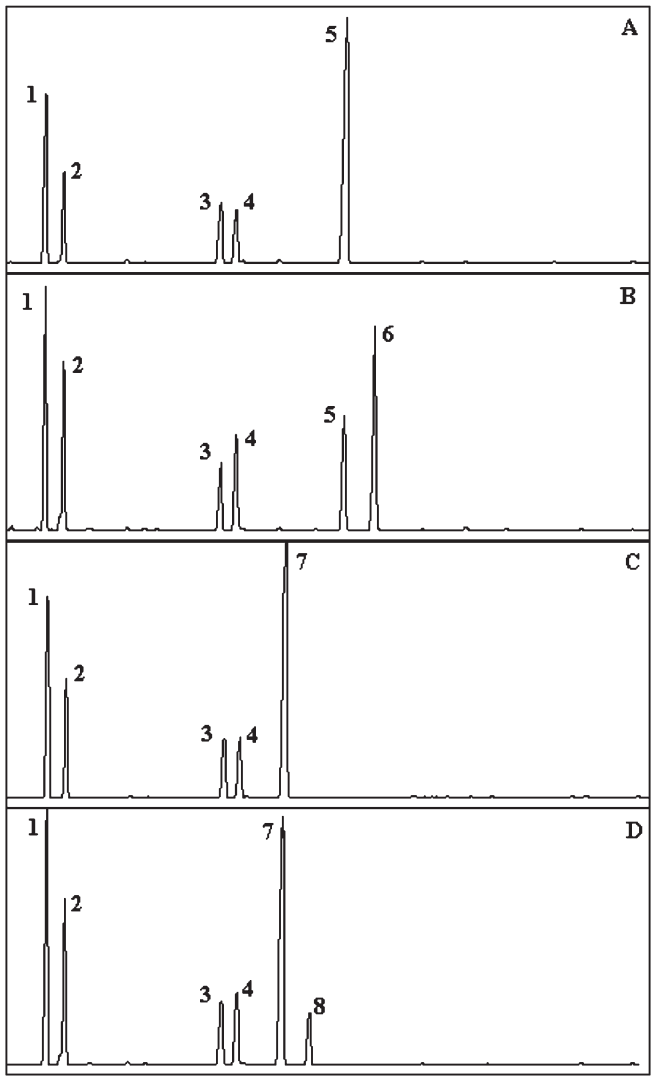

FIG. 3. Functional expression of the Atlantic salmon putative fatty acyl desaturase in transgenic yeast (*Saccharomyces cerevisiae*) grown in the presence of Δ6 substrates, 18:3n-3 and 18:2n-6. FA were extracted from yeast transformed with pYES vector without insert (A and C) or containing the putative FA desaturase cDNA insert (B and D). The first four peaks in panels A–D are the main endogenous FA of *S. cerevisiae*, namely, 16:0 (1), 16:1n-7 (2), 18:0 (3), and 18:1n-9 (with 18:1n-7 as shoulder) (4). Peak 5 in panels A and B, and peak 7 in panels C and D are the exogenously added substrate FA, 18:3n-3 and 18:2n-6, respectively. Peaks 6 and 8 in panels B and D were identified as the resultant desaturated products, namely, 18:4n-3 and 18:3n-6, respectively. Vertical axis, FID response; horizontal axis, retention time.

The study described here has further increased our knowledge of PUFA desaturases in fish. The cloning and functional characterization of a predominantly Δ6 desaturase gene makes the Atlantic salmon the first fish species to be shown to have separate and distinct genes for Δ6 and Δ5 desaturases, as reported previously for *C. elegans* (14,18,19) and humans (16,20,21). The salmon Δ6 desaturase clone also showed measurable, but very low, levels of Δ5 activity and thus was similar to other fish Δ6 desaturases of carp, trout, seabream,

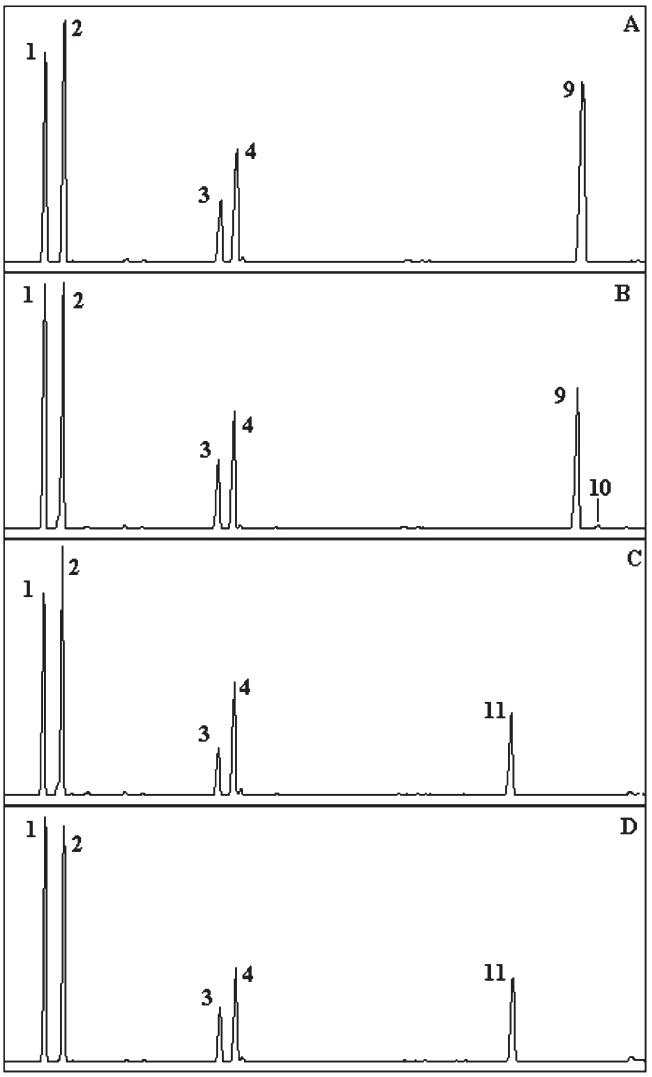

FIG. 4. Functional expression of the Atlantic salmon putative fatty acyl desaturase in transgenic yeast (*Saccharomyces cerevisiae*) grown in the presence of Δ5 substrates, 20:4n-3 and 20:3n-6. FA were extracted from yeast transformed with pYES vector without insert (A and C) or containing the putative FA desaturase cDNA insert (B and D). The first four peaks in panels A–D are as described in the caption to Figure 3. Peak 9 in panels A and B, and peak 11 in panels C and D are the exogenously added substrate FA, 20:4n-3 and 20:3n-6, respectively. Peak 10 in panel B was identified as the resultant desaturated product of 20:4n-3, namely, 20:5n-3. Vertical axis, FID response; horizontal axis, retention time.

TABLE 2
Functional Characterization of Salmon FA Desaturase cDNA Clone in the Yeast *Saccharomyces cerevisiae*

PUFA substrates	Products	Desaturase activity	Conversion rate[a] (%)
α-Linolenic acid (18:3n-3)	18:4n-3	Δ6	60.1
Linoleic acid (18:2n-6)	18:3n-6	Δ6	14.4
Eicosatetraenoic acid (20:4n-3)	20:5n-3	Δ5	2.3
Dihomo-γ-linolenic acid (20:3n-6)	20:4n-6	Δ5	0
Docosapentaenoic acid (22:5n-3)	22:6n-3	Δ4	0
Docosatetraenoic acid (22:4n-6)	22:5n-6	Δ4	0

[a]Conversion rates represent the proportion of substrate FA converted to the longer-chain FA product, calculated from the gas chromatograms as 100 × [product area/(product area + substrate area)].

TABLE 3
Exon and Intron Boundaries of Atlantic Salmon Δ6 Fatty Acyl Desaturase

Exon	Size (bp)	3′ splice acceptor	5′ splice donor	Intron size (bp)
1	25[a]		..AATATTGgtgagtg..	698
2	496[b]	..tttgcagCTGGCCC..	..TGCCACGgtcagta..	1127
3	111	..tttgtagGACGCAT..	..GAAAAATgtgagga..	744
4	198	..catacagGCAGTAC..	..GTCTCAGgtaccat..	228
5	102	..ctctcagTCCCAGG..	..CCTAAAGgtaggct..	345
6	126	..tttccagGGTGCCT..	..TGTAGAGgtagtta..	515
7	61	..attgcagTATGGTA..	..TTCCTCAgtaagtc..	128
8	77	..ctttcagTTGGACC..	..CTGGGTGgtgagat..	303
9	98	..tgtgaagGATCTGG..	..TCGTCAGgtaaagt..	161
10	97	..tatatagGTTTTTG..	..CATGCAGgtaacat..	1011
11	80	..gtcttagTTGAGTG..	..AACACCAgtaagtg..	383
12	126	..ctcccagTCTGTTT..	..TTGTCAGgtaagtg..	216
13	509[c]	..tctccagGTCACTG..		

[a]Exon is a 5′-untranslated region (5′-UTR)
[b]Exon includes a 5′-UTR of 259 bp.
[c]Exon includes a 3′-UTR of 457 bp.

and turbot (25). But, unlike the zebrafish desaturase, which showed very significant Δ5 desaturase activity at around 70% of the Δ6 activity (22), the n-3 Δ5 activity in the salmon cDNA product was only 3.8% of the Δ6 activity. It is likely that the level of Δ5 desaturase activity measured is of limited physiological significance.

The study described here also clearly showed that the salmon Δ6 desaturase has a marked preference for the n-3 substrate 18:3n-3 over the n-6 substrate 18:2n-6. A similar preference for n-3 FA substrates rather than n-6 substrates on heterologous expression in yeast was observed previously with the zebrafish Δ6/Δ5 desaturase, salmon Δ5 desaturase (22,26), and trout, seabream, carp, and turbot Δ6 desaturases (25). These data are consistent with earlier enzymological studies investigating the desaturation of ^{14}C-labeled FA substrates in primary hepatocytes (9), primary brain astrocytes (30), and established cell lines (31). Therefore, it appears that greater activity toward n-3 PUFA may be a characteristic of fish fatty acyl desaturases. In contrast, functional characterization of Δ6 desaturases of other organisms including nematodes, mammals, fungi, mosses, and higher plants failed to show a preference for either 18:3n-3 or 18:2n-6 substrates, although recently Δ6 desaturases have been identified in *Primula* sp. that have a preference for n-3 substrates (32). However, data of these kinds obtained from heterologous expression can be regarded as only semiquantitative, as there are likely to be differences between FA, for example, in their uptake into organisms such as yeasts (33).

The present study shows unequivocally that distinct Δ6 and Δ5 desaturase genes exist in Atlantic salmon, as is the case in humans, and possibly in mammals in general. However, the two salmon cDNAs are very similar in that the predicted amino acid sequence encoded by the Δ6 cDNA is 91%

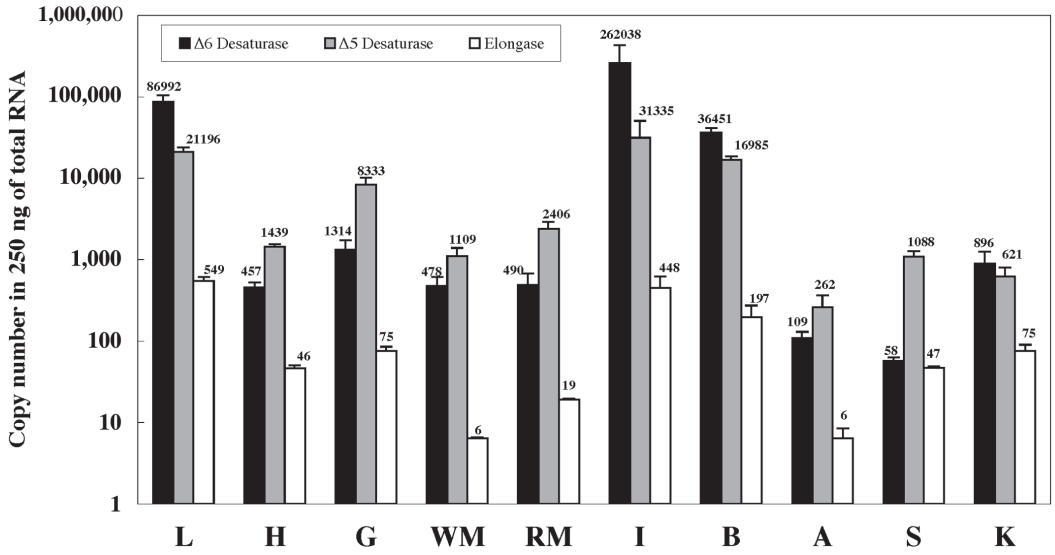

FIG. 5. Tissue distribution of FA Δ6 and Δ5 desaturase and elongase genes in Atlantic salmon. Transcript (mRNA) copy number was determined by quantitative real-time PCR (qrtPCR) as described in the Materials and Methods section. Results are expressed as the copy numbers in 250 ng of total RNA and are means ± SEM (n = 4). L, liver; H, heart; G, gill; WM, white muscle; RM, red muscle; I, intestine; B, brain; A, adipose; S, spleen; K, kidney.

identical with that encoded by the Δ5 desaturase cDNA. In contrast, in humans and *C. elegans*, the two functional Δ6 and Δ5 desaturases share an amino acid identity of only 62 (20) and 45% (19), respectively. Whether or not distinct Δ6 and Δ5 desaturase genes evolved from a common ancestral desaturase progenitor, these data suggest that the process occurred or began more recently in the evolution of Atlantic salmon than in the evolution of humans and *C. elegans*. In this regard, it is pertinent to note that the Atlantic salmon is partially tetraploid, with the tetraploidization event thought to have occurred 25–100 million years ago (34). However, evolution of desaturases in Atlantic salmon and in fish in general remains a subject for speculation. Study of further FA desaturase genes of fish are indicated, and certainly other desaturases are likely to be identified in fish species such as carp and trout, which have the ability to produce DHA from 18:3n-3 (35). But in marine species such as sea bream and turbot, the search for Δ5 desaturases will be particularly intriguing as these species lack the ability to produce EPA and DHA from 18:3n-3. This is attributed to deficiencies in Δ5 desaturation in sea bream, but to $C_{18–20}$ elongation in turbot (36,37).

The salmon Δ6 desaturase showed no Δ4 desaturase activity, perhaps as expected based on the functional characterization of all fish and mammalian Δ6 and Δ5 desaturases reported to date (22,25,26,38). This is consistent with the hypothesis that the synthesis of DHA from EPA in both mammals and fish proceeds *via* elongation to 24:5n-3 followed by a Δ6 desaturation rather than *via* Δ4 desaturation of 22:5n-3 (35,39). Heterologous expression studies of human and rat Δ6 desaturases showed that the same enzymes are active on C_{18} and C_{24} FA (33,40) and that the bifunctional zebrafish desaturase was also capable of desaturating C_{24} FA (41). It will be interesting to determine the activities of all animal Δ6 desaturases toward C_{24} FA substrates. In contrast to higher animals, production of DHA *via* a pathway including Δ4 desaturation appears to operate in some lower organisms such as *Thraustochytrium* sp. (42), and the algae *Euglena gracilis* (43) and *Pavlova lutheri* (44).

Genomic characterization showed that the salmon Δ6 desaturase comprised 13 exons, which is one more than that reported for the human Δ6 desaturase (45). The additional exon in the salmon gene is a small 25 bp exon at the extreme 5′ end. The remaining exons are homologous to the 12 exons in the human Δ6 desaturase, except that exon 2 of the salmon gene is 30 bp longer than exon 1 in the human gene, corresponding to the additional 10 amino acids found in most salmonid desaturases. However, the remaining exons are exactly the same size as their equivalents in the human gene, and splice and acceptor sites are interrupted at similar nucleotide positions, even though the lengths of the introns are quite different. In humans, there is evidence that the desaturase gene cluster has arisen by gene duplication. This is on the basis that the exon organization is nearly identical in the three family members, with each gene consisting of 12 exons and splice and acceptor sites interrupted at identical nucleotide positions within highly conserved codons (45). Further work on the genomic organization of fish desaturases may help to clarify the significance of the additional exon in salmon and the possible evolutionary history of desaturases, as sequence alignments alone are not conclusive (46).

The phylogenetic sequence analyses grouped the fish desaturases largely as expected based on classical phylogeny with the carp and zebrafish (Ostariophysi; cyprinids), trout and salmon (Salmoniformes; salmonidae), and tilapia, sea bream, and turbot (Acanthopterygia; cichlids, perciformes, and pleuronectiformes) appearing in three distinct clusters

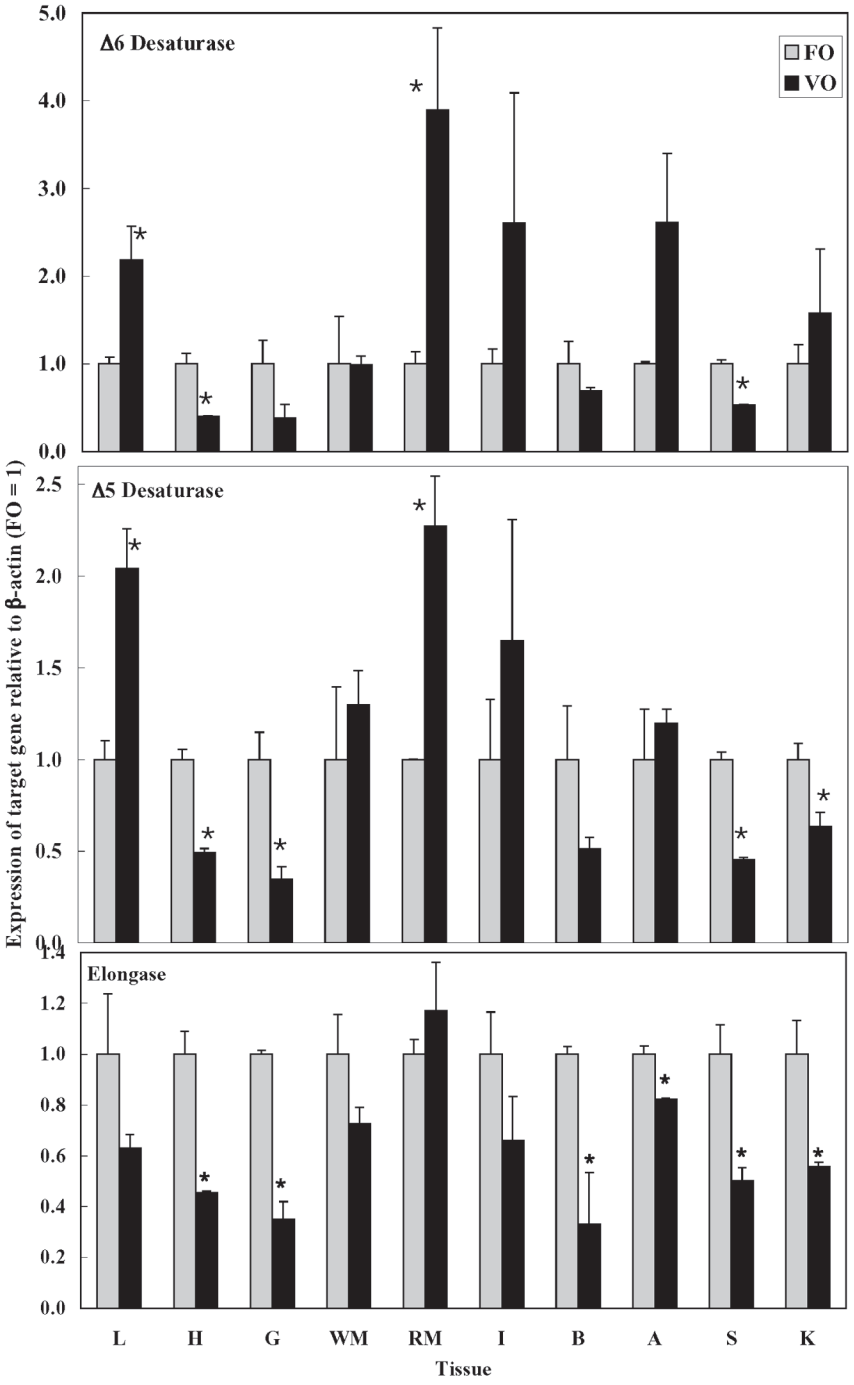

FIG. 6. Effect of dietary vegetable oil (VO) on the expression of FA Δ6 and Δ5 desaturase and elongase genes in tissues from Atlantic salmon. Transcript (mRNA) copy number was determined by qrtPCR as described in the Materials and Methods section. The ratios of copy numbers between the target genes and β-actin were calculated as means ± SEM (n = 4). Results are expressed as the fold differences by comparison of mean values in fish fed the vegetable oil diet compared with those in fish fed the fish oil (FO) diet (FO = 1). For other abbreviations see Figure 5. *Mean values for fish fed VO are significantly different ($P < 0.05$) from those for fish fed FO as determined by the Student t-test.

(47). However, the cloning of Atlantic salmon Δ6 desaturase revealed that both Δ6 and Δ5 desaturases in salmonids contain additional amino acids by comparison with those of other species, having chain lengths of 454 amino acids (or 452 as in cherry salmon Des2) compared with 444 for the cyprinid (carp and zebrafish) and human desaturases (16,20,22,23,26). Furthermore, it has been reported that the desaturase cDNAs encode proteins of 445 amino acids in seabream (24) and turbot

(25), one more residue than in cyprinid and human desaturases. These data support our previous observation that differences in polypeptide length are not in these cases related to function (25).

qrtPCR revealed that the expression of fatty acyl desaturase genes was highest in intestine, liver, and brain and lower in heart, gill, white and red muscle, kidney, spleen, and adipose tissue. Previously, by using RT-PCR, it was shown that Δ6 desaturase of rainbow trout and sea bream was expressed in intestinal tissue (23,24). In the present study, salmon intestinal tissue had levels of Δ6 and Δ5 expression 3- and 1.5-fold greater than liver. Similarly, expression of Δ6 and Δ5 in intestine was 7.2- and 1.9-fold greater than in brain. Therefore, these results suggest that intestine, the first organ to encounter dietary FA, has the capacity to play an important role in the primary processing of dietary FA *via* desaturation. Cho *et al.* (20) reported that human liver expressed 4–5 times more Δ5 desaturase and 12 times more Δ6 desaturase than brain. Our results show that salmon liver contained 2.4 times more Δ6 desaturase mRNA than brain, and the Δ5 desaturase mRNA levels in liver and brain were similar. Regardless of which gene has the higher level of mRNA, the observation that all tissues investigated express detectable levels of Δ6 and Δ5 desaturase and elongase mRNAs is consistent with the important roles that desaturase and elongase enzymes play in maintaining cellular membrane HUFA. That intestine expressed such high levels of both Δ6 and Δ5 desaturase is consistent with data from *in vitro* enzyme assays in isolated enterocytes (48,49) and *in vivo* stable isotope studies (50,51), which have shown enterocytes and intestine to be sites of significant HUFA synthesis in salmonids. The level of Δ6 desaturase mRNA in highly expressing tissues was substantially greater than the amount of Δ5 desaturase mRNA, but the level of Δ6 desaturase mRNA in lower-expressing tissues was lower than the amount of Δ5 desaturase mRNA. In comparison, a study of the relative abundance of Δ6 and Δ5 desaturase mRNA in various human tissues revealed that the level of Δ6 desaturase mRNA in eight different tissues was significantly greater than the amount of Δ5 desaturase mRNA (20). This observation is particularly interesting because Δ6 is often considered the enzyme that catalyzes the rate-limiting step in the synthesis of HUFA (52).

The results of this study show that the expression of Δ6 and Δ5 FA desaturases is under nutritional regulation in Atlantic salmon. Thus, the expression of these genes is higher in liver and red muscle (and possibly intestine and adipose tissue) of salmon fed diets containing C_{18} PUFA-rich VO compared with fish fed diets containing HUFA-rich fish oil. Although Δ6 desaturase is regarded as the main rate-limiting step in the HUFA biosynthesis pathway, Δ5 desaturase is reported also to be under nutritional regulation in mammals (53). In a previous study, the expression and activity of fatty acyl elongase appeared to be nutritionally regulated in Atlantic salmon (54). That study showed that dietary linseed oil increased the expression of both Δ5 FA desaturase and elongase genes in salmon liver (54). Similar effects of dietary linseed oil had been reported previously, with the liver transcript level of Δ6 desaturase being higher in trout fed linseed oil than in trout fed FO (23). However, the present study showed that the expression and activity of the elongase decreased in most tissues of salmon fed diets containing the VO blend compared with fish fed diets containing FO. The precise reason for the different responses in elongase gene expression is unclear but may be related to differences in the FA profiles of the linseed oil and VO-blend diets. In the present trial, the total n-3 HUFA level in the diet in which the VO blend replaced 75% of the FO was over 8%, which compares well with 9% HUFA in the diet in the previous trial in which 25% of the FO was replaced by linseed oil, a level of replacement that did not increase elongase activity (54). Elongase activity was increased only by diets in which 50–100% of FO was replaced with linseed oil, resulting in much lower levels of n-3 HUFA (54).

In conclusion, the study reported here has identified and characterized a Δ6 desaturase gene in Atlantic salmon. It had measurable, but very low, levels of Δ5 desaturase activity. The salmon Δ6 desaturase gene comprises 13 exons, one more than the human Δ6 and Δ5 desaturases. Genes for Δ6 and Δ5 desaturases and elongases were expressed in various tissues of salmon, and highly expressed in liver, intestine, and brain. Both Δ6 and Δ5 desaturase gene expression in intestine, liver, red muscle, and adipose tissue were significantly increased in salmon fed VO compared with fish fed FO.

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