Differential expression of cold- and diet-specific genes encoding two carp liver Δ9-acyl-CoA desaturase isoforms


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Polley, S. D., P. E. Tiku, R. T. Trueman, M. X. Caddick, I. Y. Morozov, and A. R. Cossins. Differential expression of cold- and diet-specific genes encoding two carp liver Δ9-acyl-CoA desaturase isoforms. Am J Physiol Regul Integr Comp Physiol 284: R41–R50, 2003. First published September 12, 2002; 10.1152/ajpregu.00263.2002.—Carp respond to cold by the upregulated expression of Δ9-acyl-CoA desaturase. Here we report the cloning and characterization of Cds2, a second Δ9-acyl-CoA desaturase expressed in carp liver. Both Cds1 and Cds2 complemented the ole1 mutation in Saccharomyces cerevisiae, permitting the synthesis of Δ9-monounsaturates, confirming their identity as Δ9-desaturases. We demonstrate that under a standard feeding regime it is the Cds2, and not Cds1, transcript that is transiently upregulated during the first few days of cooling from 30°C to 10°C, the period when cold-induced membrane restructuring occurs. Cds2 exists as two differentially spliced transcripts, differing by a small segment from the 3′-untranslated region, the ratio of which varies with temperature. Feeding a diet enriched in saturated fats produced a fourfold increase in Cds1 transcript levels, which was blocked by cooling to 15°C. Cds2 transcript levels, however, showed no substantial response to the saturated diet. Thus carp liver uniquely expresses two isoforms of Δ9-acyl CoA desaturase, possibly formed by a recent duplication event, that are differentially regulated by cooling and dietary treatment.

temperature adaptation; lipid adaptation; membrane adaptation; homeoviscous adaptation

The physical properties of the phospholipid membranes are heavily dependent on the saturation of their constituent fatty acids (11). Maintaining an appropriate balance between saturated and unsaturated fatty acids, in the face of a variable dietary supply, is therefore an essential compositional requirement for all living organisms. This situation is further complicated by changes in cell temperature. This is because membrane physical properties are highly temperature dependent, and fluctuations in cellular temperature may disturb the normal function of membrane systems. Organisms that regularly experience variations in body temperature (i.e., poikilotherms), and therefore cell temperature, mitigate these effects by activating a series of corrective mechanisms to preserve function over the normal range of temperatures and to prevent breakdown at thermal extremes (7). In the case of cellular membranes, this is evident as a cold-induced increase in fatty acid unsaturation that provides a disordering influence to offset the direct ordering effect of cooling. Warm acclimation induces the reverse response. The resulting homeostatic regulation of membrane physical structure is termed homeoviscous (28) or homeophase adaptation (14) and is a highly conserved process observed widely in microorganisms, plants, and animals.

Recent progress using molecular genetic techniques in a wide range of organisms has identified a central role of acyl-desaturases in this environmental response (19). For example, in the cyanobacterium Synechocystis, cold causes the rapid transcriptional upregulation of the acyl-CoA Δ9-desaturase (18), and a similar response has been recorded in higher plants (22). This enzyme inserts the first double bond typically at the 9–10 position of a saturated carbon chain, a position that maximizes the change in physical properties (3).

In the common carp Cyprinus carpio, a hepatic desaturase is transiently upregulated in the few days after a slow progressive cooling treatment (27, 36), and this correlates particularly with an increase in monoenic fatty acids in the sn-1 position of ethanolamine phosphoglycerides (32). We have previously cloned a carp homolog of a rat stearoyl-CoA Δ9-desaturase (SCD1) and have shown that transcript amounts increase 8- to 10-fold in the few days after cold treatment, due at least in part to enhanced transcription (32). The induction of desaturase activity was also brought about by the activation of preexisting but latent desaturase protein, perhaps posttranslationally. The transcriptional response occurs with more extreme cooling treatments and with a slower time course than the activation response, the two offering a graded response of desaturase activity to the magnitude and speed of the change in temperature (33). In mammals the expression of the hepatic Δ9-desaturase is subject to dietary control (29), although little is known about dietary influences on the cold-induced carp Δ9-desaturase.

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We now report the cloning and characterization of a second carp desaturase, termed Cds2, which is also expressed mainly in the liver. We have developed probes to distinguish between the two coexpressed desaturase genes. We have developed transcripts and demonstrate that expression of Cds2 is upregulated by cooling from 30°C to 15°C, instead of Cds1 as previously reported (32). We demonstrate that Cds1 is strongly induced by feeding a saturated diet, indicating a quite different physiological regulation compared with Cds2. This situation appears to have arisen by promoter divergence of duplicated carp desaturase genes after a genome duplication event.

**EXPERIMENTAL PROCEDURES**

### Carp maintenance and cooling treatment.

Carp (Cyprinus carpio L., 0.2–0.5 kg) were obtained from a local fish farm (Clearwater, Fiddlers Ferry Power Station, Widnes, UK) and held for at least 2 mo at 30 ± 0.5°C in large 2,000-liter tanks provided with recirculation filters. The carp were routinely fed twice daily on trout pellets (Trouw UK, Preston, UK) containing 21% (wt/vol) crude oils and 49% (wt/vol) crude protein. For cooling treatment, fish were transferred to 1,000-liter tanks and cooled at 1°C/h to a maximum of 7°C/day, reaching a temperature of 10°C on day 3 of the cooling (27) at which temperature they were held for up to 69 days.

**Dietary treatment.** Fish were transferred to 1,000-liter tanks and fed at 0.5% of their body weight twice daily, two groups being fed the trout pellet diet and another two groups a specially formulated and pelleted diet containing elevated proportions of saturated fats (see Table 1). The saturated fat diet contained (in %dry weight) 38.8% total saturates, 30.2% total polyunsaturates, and 16.0% unsaturation index. Yeast strain Aw3a was grown on synthetic complete drop-out (SCDO) medium, and all physical manipulations, including protein extraction and transformation, were performed as described by Adams (1). Aw3a were grown on SCDO containing 0.5 mM palmitoleic acid and 0.5 mM oleic acid plus 1% (vol/vol) tergitol type NP-40 (Sigma Chemicals). Heterologous expression of Cds1 and Cds2 as fusion proteins was achieved using the S. cerevisiae expression vector pXY213 (R&D Systems, Abingdon, UK). Oligonucleotide primers (Csd1 BamHI, 5′-GGGATCCTGACAGGACATCAAAATCTCCA-3′; Csd2 BamHI, 5′-GGGATCCGACACGGAAAATCAAATCTCC-3′) were used to introduce a Cds1 and Cds2 reading frames by PCR. PCR was carried out using the Accuraseq Taq Polymerase (Biogene) according to the manufacturer’s recommendations, and the resulting products were cloned into pGEM-TEasy (Promega) and sequenced to confirm sequence fidelity. The BamHI sites were then used to excise the Cds coding regions. This allowed the Cds1 and Cds2 fragments to be ligated into the pXY213 expression vector in frame with the translation initiation site to create pXY::Cds1 and pXY::Cds2, respectively. The yeast oel1 mutant strain Aw3a was transformed with either pXY::Cds1, pXY::Cds2, or the empty expression vector pXY213:MBV and transformants selected on SCDO plus glucose plus oleic acid and palmitoleic fatty acids. Mutants expressing a functional ∆9-desaturase were selected by their growth on SCDO plus galactose as sole carbon source in the absence of fatty acid supplementation.

### Fatty acid composition.

**Ura** cells were isolated and grown for 120 h on SCDO medium plus galactose as sole carbon source in the presence of 0, 0.1, and 1 mM linoleic acid. Yeast cells were washed into 100 mM phosphate-buffered saline by repeated centrifugation. Total lipid fraction was extracted from the resulting pellet as described previously (4). Fatty acids were saponified, methylated, identified, and quantified by capillary gas liquid chromatography as described (17).

### General molecular genetic techniques.

Standard molecular techniques were performed as described (26). Southern and Northern transfers and hybridization were performed using Zeta Probe GT membrane (Bio-Rad) according to manufacturer’s instructions. For low-stringency probing, posthybridization washes were conducted at 50°C. **SCDI** homologs were isolated by screening a commercial carp liver cDNA library (Stratagene) as described previously (32). RNA was isolated from carp liver as described by Chomczynski and Sacchi (6). Northern blots were quantified using the STORM 840 and ImageQuant software (Molecular Dynamics).

Genomic DNA was isolated from carp erythrocytes by a modified extraction protocol (M. Hughes, personal communication). Washed erythrocytes from 0.5 ml blood were hypo-translationally lysed with 0.1% SDS containing 5 mM MgCl2, 50 mM Tris·HCl, pH 8.0 in a sterile Oakridge test tube. One-half milliliter of a solution containing 5% SDS (wt/vol) and 4 mg/ml proteinase K was added, and the mixture was

### Table 1. Fatty acid composition of diets fed to carp

<table>
<thead>
<tr>
<th>Fatty Acid Species</th>
<th>Normal Treat</th>
<th>Saturated Pellet</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>14:0</td>
<td>8.6</td>
<td>±0.5</td>
</tr>
<tr>
<td>16:0</td>
<td>27.1</td>
<td>±1.2</td>
</tr>
<tr>
<td>18:0</td>
<td>3.12</td>
<td>±0.2</td>
</tr>
<tr>
<td>18:1</td>
<td>15.5</td>
<td>±0.6</td>
</tr>
<tr>
<td>18:2</td>
<td>10.5</td>
<td>±0.5</td>
</tr>
<tr>
<td>20:1</td>
<td>4.9</td>
<td>±0.5</td>
</tr>
<tr>
<td>20:5</td>
<td>7.9</td>
<td>±0.7</td>
</tr>
<tr>
<td>22:6</td>
<td>10.4</td>
<td>±0.28</td>
</tr>
<tr>
<td>Total saturates</td>
<td>38.8</td>
<td>±1.11</td>
</tr>
<tr>
<td>Total monounsaturates</td>
<td>31.0</td>
<td>±0.4</td>
</tr>
<tr>
<td>Total polyunsaturates</td>
<td>30.2</td>
<td>±0.7</td>
</tr>
<tr>
<td>Unsaturation index</td>
<td>160.0</td>
<td>±4.2</td>
</tr>
</tbody>
</table>

Values represent means ± S.D for 3 determinations. Unsaturation index has been calculated as the sum of the %weight for each fatty acid multiplied by the number of unsaturation bond.

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incubated overnight at 50°C. This was mixed with an equal volume of saturated phenol (pH 8.0), overlaid with 2 ml of L phase lock gel (5 Prime > 3 Prime, Boulder, CO), and the tube was centrifuged at 13,000 g for 5 min. The resulting supernatant was removed and extracted against equal volumes of phenol-chloroform and chloroform-isooamyl alcohol, again using phase lock gel. DNA was precipitated from this solution with 0.1 vol of 3 M sodium acetate (pH 5.2) and 0.7 vol isopropanol before washing with 70% ethanol followed by resuspension in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8).

Plasmid DNA was prepared using Wizard Miniprep kits (Promega) according to the manufacturer’s instructions. cDNAs and DNA inserts were sequenced using the ABI 373A sequencer, and the resulting sequences were compiled and analyzed using DNASTAR (LaserGene). Computer analysis of predicted protein sequences was performed using the PROSITE program, while homology searches were performed using the TFAST and BLITZ programs. All homology programs were accessed via the EBI website (http://www.ebi.ac.uk/).

RESULTS

Identification of a second carp desaturase, Cds2. A putative Δ9-desaturase gene had previously been identified by screening a commercial carp hepatic cDNA library with the rat SCD1 (32). This gene has now been designated Cds1 (carp desaturase 1, GenBank NC01864). The original cDNA clone isolated, pcDsL-7, was used to reprobe the same cDNA library under conditions of low stringency to determine if any additional homologs were present. One hundred sixty eight cDNAs were isolated, and their DNA was dot blotted as a sublibrary onto charged nylon membrane. This sublibrary was probed sequentially, at high stringency, with the coding region, the 3′-UTR, and the 5′-UTR of pcDsL-7.

Seven clones were identified that cross-hybridized to the coding region of pcDsL-7 but not the 3′-UTR of this clone. All seven clones were sequenced. The longest clone contained a single long ORF encoding a putative protein of 325 amino acids (Fig. 1). This protein shows 62% identity to rat SCD1 and 93% identity to the putative product of Cds1 (32). The gene encoding this second putative carp Δ9-desaturase has been named Cds2 (EMBL AJ249259). A second Cds2 sequence was found with an identical ORF but with the addition of a 269-nt sequence in the 3′-UTR, this extra sequence being present in the genomic copy of Cds2 (data not

Fig. 1. Amino acid alignment of the predicted protein products of Cds1 (CDS1, U31864) and Cds2 (CDS2, AJ249259) compared with the grass carp (G29) homolog (AJ243835) and the rat homologs SCD1 (AB032243) and SCD2 (AF509569). Residues conserved with the CDS1 sequence are boxed in black.
shown). The genomic sequence excised from the shorter cDNA was flanked by splice sites. The two cDNAs are therefore likely to represent alternatively spliced transcripts. This is consistent with a Northern analysis that revealed two alternative Cds2 transcripts (Fig. 2).

Forty-five of the remaining 161 clones cross-hybridized with the coding region and the 3'-UTR of pcDsL-7. Sequencing of the largest of these clones revealed the presence of a 152-nt sequence that was absent in pcDsL-7. This sequence was also present in the genomic sequence of Cds1 (data not shown). PCR on all 45 of the Cds1-derived cDNA clones using primers flanking this region showed that it was absent only in clone pcDsL-7. This clone may therefore represent a very rare or incorrectly spliced transcript or alternatively a deletion within this specific clone. The revised sequence extends the putative product of Cds1 by 39 amino acids at the COOH terminus of the predicted sequence (Fig. 1) (32). pcDsL-7 was also found to be carrying a sequence fused onto the 5'-end of Cds1 that was not present on the genomic sequence. The sequence has subsequently been isolated from the cDNA library as an independent transcript, confirmed by Northern analysis, which shows high identity to the yeast transcription initiation factor SUI1 (S. D. Polley, unpublished observation). Primer extension was used to identify the transcription initiation start sites for Cds1 and Cds2 (data not shown). Under this regime Cds1 was expressed in fish at 30°C, before cooling, but was repressed on cooling to 15°C (Fig. 2). A separate cooling experiment has shown that even a modest temperature reduction to 23°C is sufficient to repress its expression (data not shown). By contrast, the Cds2 probe revealed two transcripts that were shown by Northern analysis to be present in only one of three control fish acclimated to 30°C and then only at a very low level.

Cds2 was expressed on cooling to 17°C caused a significant increase in amounts of both Cds2 transcripts, which reached a maximum on day 3, by which time the fish had been cooled to 10°C. After day 3 Cds2 transcript levels partially decreased, reaching approximately one-half their maximum level on day 6. The relative abundance of the Cds1 and Cds2 transcripts over the time course of cold induction was quantified using the coding region of Cds1 as a probe. This probe binds Cds1 and Cds2 with equal intensity. In warm-acclimated animals, Cds1 accounted for >90% of Cds1-like transcripts, a situation that was reversed in 10°C carp. Similarly, the relative levels of the two Cds2 transcripts changed over the time course of cold treatment, with the smaller transcript responding most strongly to cold induction, such that its abundance increased from 0.5 times (day 0) to over two times that of the larger species (day 3). From these data, it is clear that expression of Cds2 and not Cds1 is induced during cold acclimation and that Cds2 is subject to temperature-dependent differential splicing.

**Dietary regulation of Cds1 and Cds2.** We have explored the differential regulation of Cds1 and Cds2 in response to combined dietary and thermal manipulation. Groups of 16 carp sampled randomly from a common preacclimated stock were placed in each of

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**Fig. 2.** Northern analysis of total hepatic RNA extracts from carp subjected to chronic cooling. Carp were previously acclimated for >60 days at 30°C and subjected to a standard cooling regime as described in EXPERIMENTAL PROCEDURES. RNA was probed after Northern blotting with probes specific to either Cds1, Cds2, or 18S rRNA transcripts. Each lane represents a single fish.

**Fig. 3.** Southern analysis of desaturase isoforms in carp genomic DNA. Each lane contains DNA digested with the indicated restriction enzyme with the exception of the DNA ladders (HP). The DNA in the left and right panels was probed with the 3'-untranslated region (UTR) probes for Cds1 and Cds2, respectively. Sizes of the resulting bands are indicated.
four identical 1,000-liter tanks at 30°C. Carp in two of the four tanks were fed a normal trout pellet while the remaining animals were fed a pelleted diet enriched in saturated fats at the expense of polyunsaturated fats. Fish were killed and sampled for transcript analysis at 0 and 14 days. At day 14, a subsample of animals from both dietary regimes was cooled to 15°C and sampled, together with control fish maintained at 30°C, at 4 days (day 18) and 10 days (day 24) after cooling. Figure 4, A and B, shows the transcript levels of Cds1 and Cds2 in replicate carp at each of the sampling times while Fig. 4, C–J, plots their amounts relative to 18S rRNA.

By comparing animals sampled from the same time point and dietary regime but subjected to different temperature profiles, we can evaluate the effect that temperature has on Cds1 and Cds2 transcript levels. When we considered only those fish fed the normal unsaturated diet, at 30°C a low but somewhat variable expression of both isoforms was seen (as had been observed previously) (Fig. 4, A and B). These carp express both Cds1 and Cds2 transcripts simultaneously, with the ratio between the two isoforms being similar from one carp to another. After progressive cooling of animals over days 14 and 15, down from 30°C to 15°C, the Cds1 transcript (Fig. 4C) showed no significant cold-induced increase in amounts at day 18 in cold-acclimated animals (15°C) compared with warm-acclimated (30°C) controls (P = 0.686). A significant difference was observed on day 24 (P = 0.029), but overall transcript amounts were very low. For Cds2 transcript amounts (Fig. 4D), a consistent and substantial increase was seen in cooled fish (15°C) compared with warm-acclimated (30°C) controls, which is significant at day 18 (P = 0.029) and tending toward significance on day 24 (P = 0.057). Similar results were obtained for those animals transferred to a saturated diet on day 0; Cds1 amounts (Fig. 4E) were reduced by cooling at day 18 or day 24, and Cds2 amounts (Fig. 4F) showed substantial increases compared with warm-acclimated (30°C) controls at day 18 (P = 0.029). These results confirm previous results that it was Cds2 and not Cds1 that was cold induced, and this was unaffected by dietary treatment.

By comparing carp sampled from the same time points and thermal regime but fed either a saturated or unsaturated diet (Fig. 4, G–J), we can determine the effects of diet on Cds1 and Cds2 transcript amounts. Feeding the saturated diet to carp maintained at 30°C led to a progressive and significant increase in the transcript levels of Cds1 (Fig. 4G) on days 14 (P = 0.029) and 18 (P = 0.029) compared with unsaturated diet controls, but not on day 24 (P = 0.057). Cooling of carp to 15°C (Fig. 4I) abolished this effect (P = 0.342 on day 18 and 0.685 on day 24). Regarding Cds2, we see a modest but statistically significant increase in transcript amounts in fish held at 30°C (Fig. 4H) and fed a saturated diet on days 14 (P = 0.029) and 18 (P = 0.028), but not on day 24 (P = 0.342), compared with unsaturated controls. This dietary induced increase in Cds2 transcript levels was maintained in fish cooled to 15°C (Fig. 4J, P = 0.029, day 18 and P = 0.342, day 24). We conclude, first, that Cds1 is substantially elevated and Cds2 is slightly elevated in response to feeding a saturated diet, and, second, for Cds1 this effect can be prevented by cooling.

Tissue-specific expression of desaturase isoforms. The tissue specificity of Cds1 and Cds2 expression has been examined in response to cooling. Replicate warm-acclimated carp were killed on day 0 and day 2 of the standard cooling regime, and total RNA extracts from a range of tissues were prepared and pooled for each time point. Figure 5 shows Northern blots probed with the Cds1 ORF, which under the conditions used will hybridize to both the Cds1 and Cds2 transcripts. Cds1 homologous transcripts were evident in carp cooled to 17°C but not in control fish held throughout at 30°C. High levels of transcript were only observed in the liver, although faint bands were seen in several other tissues, including brain and spleen. These data suggest that the liver is the principal tissue for the expression of both desaturase isoforms.

Complementation of the S. cerevisiae ole1 mutation by both Cds1 and Cds2. The suggested functions of Cds1 and Cds2 were based on their similarity to the rat Δ9-desaturase structural gene SCD1. We have sought to confirm this by testing the ability of both Cds1 and Cds2 to complement the ole1 mutation in the yeast S. cerevisiae. This mutation disrupts the endogenous yeast Δ9-desaturase making the growth of mutant strains dependent on provision of unsaturated fatty acids in the culture medium (30). Functional complementation of this mutation by a heterologous gene has previously been used to demonstrate that SCD1 encodes a Δ9-desaturase (31). We have developed an inducible construct containing either Cds1 or Cds2 to allow the activity of the enzyme to be directly tested.

Both Cds1 and Cds2 were introduced into a galactose-inducible, glucose-suppressible yeast expression vector. pXY::Cds1 and pXY::Cds2 were engineered to express the carp Cds1 and Cds2 transcripts, respectively. Both expression constructs and the empty expression vector pXY213::MV were transformed separately into the ole1 strain Aw3a. Transformants were selected on the basis of uracil prototrophy in the presence of oleic and palmitoleic acid. These transformants were then transferred to uracil-deficient plates either in the presence or absence of monounsaturated fatty acid supplementation and using either glucose or galactose as sole carbon source (Fig. 6).

pXY::Cds1 and pXY::Cds2 transformed cells showed definite growth in the absence of oleic and palmitoleic acids in the presence of galactose, as sole carbon source, but failed to grow in the presence of glucose on a medium lacking in these fatty acids (Fig. 6). Only on the addition of oleic and palmitoleic acid to the medium were these transformants able to grow in presence of glucose. pXY213::MV transformed cells failed to show any growth in the absence of monounsaturated fatty acid supplementation using either galactose or glucose as sole carbon source.
Fig. 4. Expression of \textit{Cds1} and \textit{Cds2} in carp subjected to dietary and chronic cooling treatments. \textit{A} and \textit{B}: total hepatic RNA extracts from individual carp fed an unsaturated and saturated diet, respectively, and analyzed for \textit{Cds1}, \textit{Cds2}, and 18S rRNA transcript levels. For each diet, a random sample of fish was cooled to 10°C on day 14 as described in EXPERIMENTAL PROCEDURES and sampled on days 18 and 24. \textit{C}–\textit{J} quantify the transcript amounts of both \textit{Cds1} and \textit{Cds2} relative to the 18S rRNA, and the results are plotted against time. \textit{C} and \textit{D} illustrate results for carp fed the normal unsaturated diet (Unsat) from day 0, while \textit{E} and \textit{F} relate to carp fed a saturated diet (Sat) throughout. For these 4 plots, open squares indicate fish held at 30°C and closed squares fish transferred on day 14 to 15°C and held at that temperature to the end of the experiment. \textit{G}–\textit{J} compare results for carp fed saturated and unsaturated diets for a given temperature regime, 30°C (\textit{G} and \textit{H}) and 15°C (\textit{I} and \textit{J}). For \textit{G}–\textit{J}, closed circles indicate a saturated diet while open circles indicate an unsaturated diet. For \textit{I} and \textit{J}, only the last 2 time points occur in fish held at 15°C; earlier time points (30°C) are included for continuity and indicated by a gray triangle. *Significant differences between animals experiencing 30°C and 15°C or saturated and unsaturated diets (in relevant panels) as calculated by a Wilcoxon's signed rank test.
Differential expression of desaturase isoforms

Cds1 ORF

Galactose Glucose

+MUF

pXY::Cds1 pXY::Cds2

Wild Type pXY213

Fig. 6. Complementation test of putative carp desaturases in the ole1 mutant of the yeast Saccharomyces cerevisiae. pXY::Cds1 and pXY::Cds2 were transformed into the Aw3a strain of yeast as described in EXPERIMENTAL PROCEDURES. Transformants were grown in the presence of glucose, which acts as a repressor, and galactose, which acts as an inducer of the GAL1 promoter, which is responsible for the transcription of the 2 carp genes. The presence or absence of monounsaturated fatty acid (MUFA) supplementation in the medium was as indicated.

Although S. cerevisiae does not form linoleic acid (18:2 Δ9–12) under normal growth conditions, it is a strong repressor of OLE1 expression and is preferentially incorporated into the membrane lipids of wild-type cells when added into the medium, to replace the 16:1 and 18:1 products of the Δ9-desaturase activity (20). ole1 strains show good growth on media not containing monounsaturated fatty acids but supplemented with linoleic acid (Fig. 6). The pXY::Cds1, pXY::Cds2, and pXY::MBV transformed cells were grown on media supplemented with varying levels of linoleic acid using galactose as sole carbon source and then harvested for analysis of their total fatty acids. The traces showed an abundance of 18:2 fatty acids in the pXY::Cds2 transformed yeast. By contrast, the pXY::Cds1 transformants showed peaks corresponding to the Δ9-desaturation products, 16:1 and 18:1, that increased in relative magnitude as the level of linoleic acid supplementation was reduced.

Fig. 5. The tissue-specific expression of Cds1 and Cds2 in carp. Northern analysis of RNA isolated from various tissues of warm-acclimated and cold-treated carp. The blots were probed with the coding sequence of Cds1, which is unable to discriminate between Cds1 and Cds2. ORF, open reading frame.

DISCUSSION

A revised structure for Cds1. We have previously cloned a homolog of rat SCD1 from a commercial carp liver cDNA library (32). The original clone, designated pcDsL-7, possessed a single long ORF encoding a putative protein of 292 amino acid residues with 55 and 53% identity with rat and mouse SCD1 Δ9-desaturases, respectively. However, the predicted protein product of pcDsL-7 was ~30 residues shorter at the COOH-terminal end than the putative product of SCD1, and the pcDsL-7 transcript possessed an unexpectedly long 5’-UTR of 320 nucleotides. We now show through characterization of other SCD1 homologous clones that pcDsL-7 appears to contain an internal deletion within the ORF and a distinct cDNA sequence erroneously fused to the 5’ end of Cds1. The ORF contained by the other cDNAs encoded a putative protein of 327 amino acid residues and a molecular mass of 37.7 kDa both of which more closely match the COOH terminal sequences of the rat and yeast homologs. This revised carp gene has been redesignated Cds1.

Relationship of Cds1 and Cds2. Rescreening the carp liver cDNA library revealed a group of transcripts with high identity to the coding sequence of Cds1 but not with the corresponding 3’-UTR. Sequencing of these clones revealed a second putative desaturase gene with high sequence similarity to the putative protein products of Cds1 (93%) and mouse SCD1 (62%). This new gene has been designated Cds2. Both isoforms are expressed in liver and not in any other tissue, at least in amounts detectable by our methodology. We have also identified and isolated the genomic sequences encoding these genes and confirmed their identity by sequencing (S. D. Polley, H. Evans, B. Cossins, and P. E. Tiku, unpublished data).

Mouse also expresses two Δ9-desaturase genes that are 89% identical at the amino acid level; one (SCD1) is...
expressed constitutively in adipose tissue and induced in liver by dietary treatment (23) and the other (SCD2) is expressed in brain but not in liver (16). An important question is whether these isoforms are related to the two hepatic isoforms in carp or have arisen independently. Figure 8 shows a dendrogram based on similarity analysis from which it is evident that the two carp isoforms are more similar to each other than either is to the mouse or rat isoforms. Moreover, of the 33 amino acid substitutions between the two mouse homologs, only five coincided with substitutions between the two carp homologs and only two of these involved similar substitutions, indicating no relationship between the respective mouse and carp homologs. Because of this and the different tissue-specific patterns of expression, we conclude that the two carp isoforms have a phylogenetic origin different from the two mouse Δ9-desaturases and are therefore likely to have a different physiological significance compared with those observed in the mammals.

**Complementation of yeast ole1 mutation by Cds1 and Cds2.** Although both the carp putative desaturases have a high sequence identity with the rat SCD1, it was important to determine whether either or both sequences code for a functional Δ9-desaturase. We have tested both genes by complementation of a yeast strain (ole1) that is deficient in its endogenous Δ9-desaturase activity and is auxotrophic for monounsaturated fatty acids (30). We have shown that both genes restored growth to yeast cultures when their expression is induced by growth with galactose as sole carbon source but not when it is repressed by glucose (Fig. 6). Moreover, cultures complemented with either Cds1 or Cds2 produced monounsaturated fatty acids demonstrating unequivocally that these genes code for Δ9-desaturases.

**Temporal and spatial regulation of the two isoforms during chronic cooling.** Previous work has shown that the enzymatic activity of the hepatic desaturase was low in 30°C-acclimated carp but increased 8- to 10-fold in the 4–5 days after cooling to 10°C (32). This was associated with a transient increase in amounts of Cds transcript levels, caused at least in part by an increased rate of transcription. We now show that the transcript evident in 30°C-acclimated carp is mainly that encoded by Cds1 (Fig. 2). Cooling of fish down to 10°C over 3 days led to a substantial increase in the level of Cds2 transcripts while the level of Cds1 was reduced. Thus only Cds2 is cold inducible while Cds1 expression is transiently repressed by cold.

Although both the carp putative desaturases have a high sequence identity with the rat SCD1, it was important to determine whether either or both sequences code for a functional Δ9-desaturase. We have tested both genes by complementation of a yeast strain (ole1) that is deficient in its endogenous Δ9-desaturase activity and is auxotrophic for monounsaturated fatty acids (30). We have shown that both genes restored growth to yeast cultures when their expression is induced by growth with galactose as sole carbon source but not when it is repressed by glucose (Fig. 6). Moreover, cultures complemented with either Cds1 or Cds2 produced monounsaturated fatty acids demonstrating unequivocally that these genes code for Δ9-desaturases.

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Despite the low levels of Cds1 transcript in the liver of 30°C-acclimated carp, we have previously shown by Western immunoassay that these animals possess significant amounts of largely inactive desaturase protein whose enzymatic activity increases two- to fourfold during the first 2 days of cooling (32). The prevalence of Cds1 transcript in these animals suggests that this protein is largely, if not entirely, composed of CDS1, and that this isoform is subjected to activation. Because cooling leads specifically to increased levels of the Cds2 transcript, it follows that the subsequent increase in desaturase protein abundance observed in Western immunoassays is solely due to CDS2. Thus during the early period of chronic cooling the population of desaturase proteins comprises a mixture of the two isoforms with CDS2 possibly becoming predominant after prolonged cooling. At present we are unable to confirm this scenario first because the polyclonal antibody we have is unable to discriminate between the two isoforms and second because little is known about the degradation of desaturase proteins in fish. It is known, however, that the degradation of desaturases in the bacterium *Escherichia coli* is markedly temperature dependent (10).

**Dietary regulation of hepatic desaturases.** The presence of two desaturase isoforms in carp liver may permit the differentiated regulation of desaturase activity to different stimuli, perhaps as part of different response systems. It is well known that mammalian hepatic Δ9-desaturases, most notably in rat and mouse, are greatly induced by a dietary regime of starvation followed by refeeding with a fat-free diet (23, 29). More significantly in the present context, Wodtke and Cossins (37) have demonstrated a long-lasting increase in hepatic desaturase activity in carp fed a commercial diet containing elevated proportions of saturated fatty acids.

We have therefore tested the effects of manipulation of dietary lipid saturation on desaturase expression by

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*Fig. 8. An unrooted additive tree illustrating the amino acid identity between the 2 carp desaturase proteins CDS1 (accession number U31864) and CDS2 (AJ249259), the desaturases for the grass carp (*Clenopharyngodon idella*, AJ249835), the Antarctic *Chionodraco hamatus* (AJ249579), the milk fish (*Chanos chanos* AY082003), and the mammalian homologs, mouse SCD1 (AF509567), mouse SCD2 (M26270), rat SCD1 (AB032243), and rat SCD2 (AF509569), together with an insect homolog as an outgroup (*Epiphyas postvittana*, AY061988). The tree was generated by the least-squares method program Fitch, using the Dayhoff PAM matrix to calculate pairwise distances. Bootstrap analysis with 100 replicates gave values of 99% or above for all branches. Fitch is part of Felsenstein's Phylib program (25).*
feeding carp a pelleted diet containing elevated levels of coconut oil and reduced levels of fish oil. Feeding this diet to carp held at 30°C over a 2-wk period led to a statistically significant increase in the level of Cds1 transcript (10-fold over that observed with an unsaturated diet) with a much smaller absolute effect on Cds2. Cooling from 30 to 15°C abolished the diet-induced increase in Cds1 transcript observed at 30°C but instead caused a substantial induction of Cds2, a response to cooling that was more marked in animals fed the saturated diet.

This complex experiment supports the idea that the two desaturase isoforms respond quite differently to physiological stimuli, and there is comparatively little cross-talk between them. Cds1 responds to the modified diet, which is substantially more saturated than the trout diet. The main difference in these two diets is the proportion of C14:0 myristate, which could account for the effect we have seen in isolation or in conjunction with the other saturated fatty acids. Although the level of Cds1 transcript was elevated after 10 days of cooling, this response was relatively small and occurred well after the cold-induced changes in fatty acid composition of microsomal phospholipids (32, 33). These compositional changes, representing the acute phase of membrane lipid restructuring, can therefore be attributed exclusively to elevated expression of the CDS2 isoform. Whether the elevation of CDS1 over the longer term has any impact on membrane lipid composition or on the composition of other fatty acid pools is not clear. However, the original report of cold-induced desaturation activity by Schünke and Wodtke (27) claimed a biphasic response to cold with peak enzymatic activities occurring at 5 and 10 days. This may represent the quite separate inductions of Cds2 and Cds1, respectively. Finally, the ~10-fold greater representation of Cds1 clones in the commercial cDNA library compared with Cds2 clones is consistent with the animals used for library construction having experienced warm conditions and a relatively saturated diet. This together with the use of a coding sequence probe probably accounts for the fact that our initial screen identified only Cds1.

Temperature-specific desaturase isoforms? Although we demonstrate that both Cds1 and Cds2 code for Δ9-desaturases, it is much less certain that the resulting proteins are functionally identical. Functionally important changes to proteins can result from very few amino acid substitutions, so the 21 substitutions between CDS1 and CDS2 may well have a functional significance. One possibility is that the two isoforms might be catalytically most effective over a different range of temperatures such that their temperature-specific expression is temperature adaptive as well as being associated with two different response systems. This phenomenon is well documented for the regulation of skeletal muscle contractile activity in summer- and winter-acclimated carp by the differential expression of functionally distinct forms of the myosin heavy chain molecule (11, 15, 34). However, expressing both isoforms in ole1-deficient yeast allowed growth at 30°C, and there was no noticeable difference in the growth characteristics of the two complemented yeast strains.

Recent evidence from the analysis of homeobox genes has indicated that teleost fish experienced a genomic duplication event followed by subsequent selective reduction in the repertoire of expressed genes (2). Furthermore, some groups of fish, including some cyprinid fish, have undergone additional, more recent duplications, and having double the number of chromosomes are thus regarded as tetraploid (8, 25). Figure 8 shows a dendrogram containing the known teleost Δ9-desaturases, including the closely related grass carp, for which there is only one gene (5), and the Antarctic Chionodraco. The dendrogram uses an insect desaturase as an outgroup. This shows that the two carp isoforms are more similar to each other than either is to the Δ9-desaturases of other species, including grass carp, indicating that gene duplication and divergence occurred more recently than the evolutionary divergence of the grass and common carp. By contrast, rat SCD1 shows higher identity to the orthologous protein in mouse (SCD1) than it does to the other rat desaturase (SCD2), indicating that gene duplication and divergence occurred before the divergence of two species. High bootstrap values indicate a robust phylogeny. Even though the existence of a second desaturase cannot be discounted in grass carp by sequencing of cDNAs, Southern analysis with grass carp genomic DNA supports the existence of only one (H. Evans, personal communication). A BLAST search of the genome of the Japanese pufferfish Fugu rubripes (http://Fugu.jgi-psf.org) reveals the existence of two SCD1 homologs in scaffolds 64 and 5415. It is likely however that these two genes result from a very ancient duplication because they show a much higher level of divergence than the two carp desaturases at both the synonymous and nonsynonymous levels, the latter evident as a 70.5% level of identity between the putative protein products of two fugu paralogs. The role of these two SCD1 homologs is unknown at present.

In many tetraploid species the nonallelic gene copies are functional but appear to be fully redundant (25). Divergence in the regulatory sequences might, however, alter the spatial or temporal pattern of expression, giving rise to novel expression characteristics. This has been observed within a developmental context in mice where the homologs Hoxa3 and Hoxd3 encode proteins with an identical biological activity but with different expression patterns within the embryo (13). Within an environmental context, the additional complement of genes might provide a more plastic physiology, capable of tolerating wider environmental conditions than other related groups of fish, giving rise to the flexible genome concept (35). On the other hand, duplicated genes can be fixed by the partitioning of ancestral functions rather than the evolution of new functions per se (9). Although the divergence of the regulatory regions of Cds1 and Cds2 causes them to exhibit differentiated responses to cooling and dietary manipulation, it is not clear whether the ancestral desaturase responded to both stimuli. Distinguishing
between these two contrasting models requires analysis of outgroup species corresponding more closely to the ancestral unduplicated gene, including perhaps the grass carp, *Ctenopharyngodon idella*. This might indicate whether possession of two desaturase isoforms and the partitioning of responsiveness to different stimuli offer any selective advantage with respect to environmental stress.

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