Coho salmon (*Oncorhynchus kisutch*) transgenic for a growth hormone gene construct exhibit increased rates of muscle hyperplasia and detectable levels of differential gene expression

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Abstract: Transgenic coho salmon (*Oncorhynchus kisutch*) containing a growth hormone gene construct were compared with nontransgenic coho salmon in terms of gross anatomy, muscle cellularity, muscle enzyme activity, and differential gene expression. Transgenic fish were found to have significantly higher numbers of small-diameter muscle fibres in both the dorsal and lateral region of the somitic muscle, suggesting that they grow by greater rates of hyperplasia relative to slower growing nontransgenic fish. Higher levels of activity were found for phosphofructokinase and cytochrome oxidase in white muscle of the transgenic fish. This difference indicates a higher glycolytic and aerobic requirement in the muscle of transgenic fish. Subtractive hybridisation of muscle RNA of transgenic fish from control fish provided a library of cDNAs whose expression is upregulated in the transgenic fish. This library contains genes that may be involved in, or related to, both high growth rates and muscle hyperplasia. We have sequenced a number of fragments and have found a preponderance of myosin light chain 2 mRNAs, consistent with a putative high level of expression in the early stages of muscle fibre construction.

Résumé : On a comparé des saumons cohos (*Oncorhynchus kisutch*) transgéniques contenant un gène chimère d'hormone de croissance à des saumons cohos non transgéniques aux chapitres de l'anatomie générale, de la cellularité musculaire, de l'activité d'enzymes des muscles et de l'expression génique différentielle. On a observé que les poissons transgéniques renfermaient des quantités significativement supérieures de fibres musculaires de faible diamètre dans les régions dorsale et latérales du muscle somitique, ce qui indique que leur croissance est tributaire de taux d'hyperplasie plus élevés que chez les poissons non transgéniques à croissance plus lente. Les activités de la phosphofructokinase et de la cytochrome oxydase étaient plus élevées dans le muscle blanc des poissons transgéniques. Cette différence indique que le muscle des poissons transgéniques exige une glycolyse et une consommation d'oxygène plus intenses. Grâce à l'hybridation soustractive d'ARN musculaire des poissons transgéniques à partir du matériel de poissons témoins, on a obtenu une banque d'ADNc dont l'expression est régulée en amont chez les poissons transgéniques. Cette banque contient des gènes déterminant directement ou indirectement les taux de croissance élevés et l'hyperplasie musculaire. Nous avons séquencé certains fragments et observé une prépondérance d'ARNm de chaînes légères de myosine de type 2, ce qui va dans le sens de notre supposition selon laquelle il y aurait une forte expression dans les premiers stades de la construction des fibres musculaires.

[Traduit par la Rédaction]

Introduction

Muscle development and growth in vertebrates occurs by two fundamental growth processes: recruitment of new muscle fibres (hyperplasia) and increase in size of existing fibres (hypertrophy) (Goldspink 1972). In higher vertebrates, hyperplasia ceases shortly after birth (Goldspink 1972; Campion 1984) and postnatal growth continues by hypertrophy of already existing fibres (Goldspink 1972), making the number of fibres formed during embryosis one of the main factors determining postnatal growth dynamics of muscle in mammals. In teleosts, muscle hyperplasia may continue beyond embryogenesis (Greer-Walker 1970; Stickland 1983; Weatherley et al. 1988). This allows growth potential of muscle in fish to be affected postnatally, making growth dynamics of skeletal muscle of adult fish remarkably plastic compared with that of mammals and birds. Growth strategy (hypertrophy versus hyperplasia) has been shown to be influenced by both exogenous and endogenous factors (Carpene and Veggetti 1981; Weatherley and Gill 1982; Higgins and Thorpe 1990). There are also marked species differences in the contribution of hyperplastic and hypertrophic processes to muscle growth in fish. Weatherley et al. (1988) examined 10 different species of teleost fish and

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found that the largest and fastest growing species showed sustained recruitment of fibres to a large body size, in contrast with the slowest growing species, where cessation of recruitment led to slow growth and small size. Large variations in muscle fibre growth within populations of fish also occur in many fish species (Higgins and Thorpe 1990; Rowlerson et al. 1995). Weatherley and Gill (1982) demonstrated that fast-growing rainbow trout (*Oncorhynchus mykiss*) achieved a greater proportion of their growth by recruiting new fibres than did slow-growing fish. It therefore seems likely that high growth rates are linked to hyperplasia in postlarval muscle growth of fish.

Kiessling et al. (1991*a*) presented contradicting results in that the disappearance and appearance of small and large fibres in white muscle of rainbow trout (growth by hyperplasia) was related to body length independent of growth rate. However, in the study of Weatherley and Gill (1982), a much smaller size and growth range of the experimental fish and a shorter time period were used than in the study of Kiessling et al. (1991*a*). It is therefore possible that the importance of hyperplastic versus hypertrophic growth is different in growth spurts of more limited time periods compared with muscle growth over longer time periods.

Supplementation of growth hormone (GH) has been shown to promote hyerplastic growth in rainbow trout (Weatherley and Gill 1982) and common carp (*Cyprinis carpio*) (Fauconneau et al. 1996). However, the genetic control of fish muscle growth is poorly understood. For example, although muscle regulatory factors are known to transcribe contractile proteins (for a review, see Buckingham 1994), little is known about genes (or cellular factors) that influence the choice of muscle stem cells to form new fibres or to fuse with existing fibres.

White muscle in fish is composed of a mixture of small diameter fibres dispersed between large diameter fibres, giving the muscle a mosaic appearance (Greene 1913). The presence of small diameter fibres has been related to hyperplasia, and has been used as a method to identify continuing hyperplastic growth (Greer-Walker 1970; Weatherley et al. 1980; Stickland 1983). Strong indications exist suggesting that fibre size composition of muscle in fish is an important factor in flesh quality in terms of texture. In addition, other influences of accelerated growth on muscle, for example lipid deposition, enzyme titres and differential expression of structural protein isoforms may result in significant quality changes of farmed products (Johansson et al. 1995).

Transgenic coho salmon (*Oncorhynchus kisutch*) have been produced using the all-salmon GH/metallothionen (MT) gene construct (OnMTGH1) (Devlin et al. 1994). These fish are extremely fast growing relative to controls, averaging a size more than 11-fold larger than that of controls by the age of 15 months (Devlin et al. 1994). In swimming stamina tests (U_{crit} swimming test in a standard Brett-type swimming tunnel), transgenic fish showed a significantly lower performance compared with control fish (Farrell et al. 1997), suggesting that there may be a number of important biochemical and structural differences in the transgenic fish muscle. The extent to which transgenic fish provide a model for the study of fast growth has not been established.

In this paper, we present a study of muscle cellularity analysis of growth-enhanced transgenic coho salmon relative to older controls of the same size. The aim was to establish whether there was an increase in hyperplasia under the influence of the transgene. A number of other comparative techniques were also used to examine other factors that might be altered. These included enzyme substrate assays and differential gene transcription through production of a subtracted library of cDNAs. In order to produce such a library, we have employed the technique of subtractive hybridisation. This technique reveals the difference in expression between transgenic versus nontransgenic fish of all activated genes. The advantage of such an approach is that unexpected differences are revealed, giving much better coverage of the true effect of the insertion of a gene complex that leads to major differences in growth rate. This differs markedly from earlier work where only expected effects have been monitored, as the technique to monitor gene or protein expression by an untargeted approach has been lacking. This study therefore provides a unique comparison with earlier studies of fish in which high growth rate was stimulated through other factors, e.g., environmental or physiological. The data presented in this paper will also provide a starting point for the use of transgenic coho salmon as an experimental model in studies of fish growth and development and the effects of rapid growth on farmed fish products.

Methods

Fish

Transgenic coho salmon were produced using a strain derived from the Chehalis River in southwestern British Columbia, Canada. The F_2 progeny were generated by mating a single F_1 transgenic male with wild Chehalis River females to yield both control animals and their heterozygous transgenic siblings. The animals utilised in this study contained the GH gene construct OnMTGH1, which we have previously shown results in approximately an 11-fold increase in size in the first year of growth (Devlin et al. 1994). Animals were reared in 10°C freshwater until sampling. Alevins and fry were determined to be transgenic or control using the polymerase chain reaction (PCR), similar to that described in Devlin et al. (1995): DNA was extracted from a small piece of fin tissue and subjected to PCR using primers MT-1 (5′-CTGATTAAGTTTTGTATAGT-3′) and GH-19 (5′-GTTAAATTGTATTAAATGGT-3′).

Transgenic and nontransgenic fish were reared according to Devlin et al. (1995). In brief, this comprised indoor flow-through 200-L glassfibre aquaria supplied with fresh well water (10 ± 1°C) under simulated natural light conditions. Fish were fed ad libatum with a standard commercial feed (Moore-Clarke Ltd., Vancouver, B.C.).

The control fish used in this experiment were offspring of the same family of transgene-carrying fish spawned 1 year earlier. Both groups of fish were sampled on the same day at an approximate fork length of 10 mm. At this length, control fish were aged 14 months post-first-feeding and transgenic fish were aged 2 months post-first-feeding. At present, coho salmon made transgenic with an inactive form of the construct are not available; such fish will, in the future, provide an additional control for comparative experiments analysing transgenic fish, although differences in age will remain an experimental artifact.

Sampling

Fish were sampled by netting and sedated by submersion into water containing 5 ppm Marinil® (metomidate hydrochloride) (Syndel Laboratories, Vancouver, B.C.). The fish were killed by decapitation and two cross sections were immediately cut, one an-

terior to the dorsal fin and one anterior to the adipose fin of the whole fish (Fig. 1). These cross sections were photographed together with a ruler, allowing measurement of whole-body and whole-muscle morphometrics by computerised digitising techniques (Kiessling et al. 1991*a*). Muscle blocks for fibre measurements were cut from the most rostral of these cross sections. Two blocks of tissue were obtained, one just dorsal to the lateral line region containing mediolateral white (MLW) and red muscle (MLR) and the second from the dorsal part of the epaxial muscle denoted dorsolateral white (DLW) (Fig. 1). The muscle block was mounted in Tissue Tek® (Miles Laboratories Inc., Elkhart, Ind.), frozen in isopentane, and cooled to its freezing point in liquid nitrogen. Samples were then packed and stored in liquid nitrogen after a maximum delay of 5 min after decapitation. Concurrently (<1 min after decapitation), muscle samples for measurement of enzymatic activity and purification of total RNA were removed anterior to the rostral chop (Fig. 1). Samples for enzymatic activity were wrapped in aluminium foil and immediately frozen in liquid nitrogen, while samples for RNA were stabilised in guanidine thiocyanate buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol (Chomczynski and Sacchi 1987)) before freezing in liquid nitrogen. All samples were then stored at –80°C until analyses.

Enzymatic and histochemical analysis

The activity of the three enzymes studied were measured as described in Shonk and Boxer (1964) for phosphofructokinase (PFK, EC 2.7.1.11, indicative of glycolysis), in Bass et al. (1969) for 3-hydroxyacyl-CoA dehydrogenase (HAD, EC 1.1.1.35, indicative of fat oxidation), and in Whereat et al. (1969) for cytochrome oxidase (Cytox, EC 1.9.3.1, indicative of aerobic ATP formation). For enzyme activity determinations, muscle samples (100 mg) were homogenised in an ice-cold, all-glass Potter–Elvehjem homogenise, and the crude homogenate was incubated at 15°C as described in Kiessling and Kiessling (1984). The activity, measured on frozen samples, was converted to fresh tissue values by multiplying by the following factors: 1.0 for PFK, 2.9 for HAD, and 0.6 for Cytox (Kiessling et al. 1990).

Muscle samples were sectioned into slices (10 μm) and stained with acid–Schiff reagent for glycogen and NADH dehydrogenase activity (Novikoff et al. 1961). These two stains were chosen because they produce the overall best results in red and white muscle, respectively (Kiessling et al. 1990).

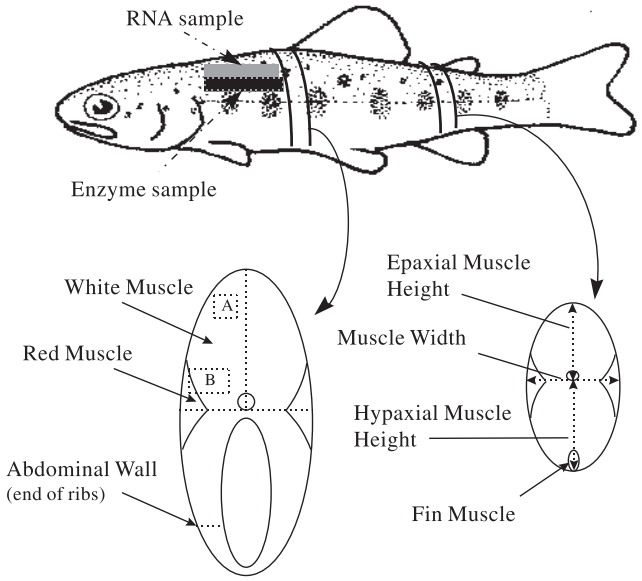
Statistical evaluation

An analysis of variance model was used where anatomical measurements, mean muscle fibre cross-sectional area, theoretical fibre numbers, and enzyme activities were dependent variables and transgenesis was the fixed factor. Extended models (i.e., analyses of covariance models) were carried out with the above-mentioned factors and two continuous variables, body weight and fork length, as covariates. The purpose of these models was to correct the comparisons between factor levels for differences in the continuous variables.

Muscle fibre frequency distribution was compared by paired *t* test (including the following size-classes: DLW, 200 to +4400 μm^2; MLW, 200 to 1300 μm^2; MLW, 1300 to +4400 μm^2; MLR, 500 to +4400 μm^2). This test was used because white muscle fibre size has a skewed distribution, making a comparison by variance doubtful. Further, if the paired *t* test revealed a significant difference, the means of single size-classes were also tested by variance.

The statistical calculations were processed by the statistical program package SAS (version 6.12 for PC-DOS, GLM procedure). All tests were two-tailed. In white muscle, 400 fibres were measured per sample site and fish. In samples of red muscle, 300 fibres were measured. A total of 1100 fibres were counted per fish.

Fig. 1. Graphic of coho salmon sampling sites. "A" represents the dorsolateral sampling area at which white muscle fibres were measured, and "B" represents the mediolateral sampling area at which both red and white muscle fibres were measured. Other samples were taken as indicated by the labels (not to scale).



Subtractive hybridisation

Total RNA was isolated from muscle samples from the dorsal proliferative zone of GH-transgenic and nontransgenic coho salmon according to the method of Chomczynski and Sacchi (1987). Samples (approximately 1 g each) from three individual fish were pooled separately for transgenic and nontransgenic (wild type) and homogenised in 400 μL guanidine thiocyanate buffer·g^{-1} followed by the addition of 30 μL of 2 M sodium acetate (pH 4.5). RNA was extracted twice with one volume of water-saturated phenol (pH5.5) – chloroform – isoamyl alcohol (25:24:1) and precipitated with one volume of isopropanol. After centrifugation, the pellet was washed with one volume of 96% ethanol, dried, and redissolved in 50 μL of distilled water. One microlitre of each was run on an agarose gel and the samples were observed to contain approximately the same amount of total RNA at a concentration of about 1 mg·mL^{-1}.

cDNA synthesis was performed on 1 μL (approximately 1 μg) of the total RNA solution for transgenic and wild type using the Clontech SMART™ cDNA Synthesis Kit (Chenchik et al. 1996), which enables proportionate amplification of full-length cDNA during the exponential phase of a PCR. First-strand cDNA was produced in a 10-μL reaction (50 mM Tris–HCl (pH 8.3), 75 mM KCl, 6 mM $MgCl_2$, 1 μM cDNA synthesis primer, 1 μM SMART™ II oligonucleotide, 2 mM DTT, 1 mM dNTP, 20 units of Superscript™ II reverse transcriptase (Gibco BRL)), 1 h, 42°C. This was diluted with 40 μL of 10 mM Tris – 1 mM EDTA buffer (pH 7.6). Two microlitres of each was amplified in a 200-μL reaction volume (40 mM tricine–KOH (pH 9.2), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 75 μg bovine serum albumen·mL^{-1}, 200 μM dNTP, 200 nM PCR primer, 1× Advantage Klentaq Polymerase mix) with the following program: 95°C for 1 min and 21 cycles of 95°C for 15 s, 65°C for 30 s, and 68°C for 6 min. The PCR product was extracted once with Tris (pH 7.6) saturated phenol – chloroform – isoamyl alcohol (25:24:1) and once with chlorofrom – isoamyl alcohol (24:1), precipitated, and redissolved in water. The cDNA was digested overnight with *Rsa*I in the appropriate buffer at 37°C. The digested

cDNA was extracted as before, precipitated, and redissolved in 5.5 μL of distilled water.

The digested transgenic cDNA was divided into two samples (2 μL), TG1 and TG2, and each ligated to a different adapter (adapters 1 and 2, respectively, from the Clontech PCR-select cDNA subtraction kit) in a 10-μL reaction volume (400 units of T4 DNA ligase, 1× standard ligation buffer, appropriate adapter at 1 μM) and incubated at 16°C overnight. The reaction was stopped with 1 μL of 0.2 M EDTA and 1 mg glycogen mix·mL^{-1} and heated to 72°C for 5 min to inactivate the ligase. One microlitre was removed from each reaction for use as an unsubtracted control for PCR.

TG1 and TG2 were separately hybridised to digested wild-type cDNA (denatured at 98°C for 1.5 min, hybridised at 68°C for 8 h in 1× hybridisation buffer supplied with the Clontech kit); the two reactions were then mixed and treated to additional hybridisation in the presence of fresh denatured digested wild-type cDNA and hybridised overnight at 68°C (Diatchenko et al. 1996). The hybridisation reactions were diluted with 200 μL of dilution buffer (20 mM HEPES–HCl (pH 8.3), 50 mM NaCl, 0.2 mM EDTA (pH 8.0)).

Subtracted cDNAs in the hybridisation reactions were then amplified along with the unsubtracted adapter ligated transgenic cDNA with two rounds of PCR. PCR reaction conditions were as before; the first round used a general primer with priming sites present in both adaptors and the following cycling parameters: 75°C for 5 min and 27 cycles of 94°C for 30 s, 66°C for 30 s, and 72°C for 1.5 min. One microlitre from the first PCR was diluted 1:1000 in the second PCR reaction mixture (components as before, except primers) with 400 nM of each of nested primer 1 (priming site built into adapter 1) and nested primer 2 (priming site built into adapter 2). The product of this PCR was analysed on a 2.0% agarose gel in 1× TAE buffer. In addition, a control reaction was run using human skeletal muscle total RNA, half of which was mixed with *Hae*III digested ΦX174 DNA prior to adapter ligation; after the control subtraction, only the *Hae*III-digested bands were present in the sample, indicating a good subtraction. The transgenic subtracted PCR amplification (2 μL) was ligated to a pT-Adv cloning vector (Clontech) in a 10-μL reaction volume (1× standard ligation buffer; 4 units of T4 DNA ligase, 50 ng of pT-Adv vector) overnight at 14°C. Competent cells (TOP10F[1] *Escherichia coli*) were transformed with 5 μL of the ligation reaction in the presence of 20 mM β-mercaptoethanol. The transforming cells were taken through the following temperature regime: ice for 30 min, 42°C for 30 s, and ice for 2 min. SOC (250 μL, 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added and the cells incubated at 37°C for 1 h. Fifty and 200 μL were plated onto LB agar white/blue selection plates (1% tryptone, 0.5% yeast extract, 1.0% NaCl, 1.5% agar, 50 μg ampicillin·mL^{-1}, 0.2 mg X-gal·mL^{-1}, 4 mM IPTG) and incubated overnight. Plate 1 (infected with 50 μL of cells) contained 84 positive colonies and plate 2 (infected with 200 μL of transformed cells) contained >200 positive colonies. Each colony from plate 1 was picked with a sterile cocktail stick and used to infect 2.5 mL of LB broth (1% tryptone, 0.5% yeast extract, 1.0% NaCl) containing 50 μg ampicillin·mL^{-1}, grown overnight at 37°C with shaking (200 rpm), and vector DNA purified by lysing with alkaline and centrifuging at 14 000 rpm in a bench-top microcentrifuge. The supernatant was extracted once with phenol – chloroform – isoamyl alcohol (25:24:1), precipitated in ethanol, air dried, and redissolved in 50 μL of water containing DNase-free RNase.

Dot blots

To test subtraction, 2 μL of each miniprep was denatured in a reaction volume of 250 μL (1 M NaCl, 10 mM EDTA) and was arrayed on a Hybond N+ nylon filter (Amersham). Each blot was rinsed with 0.4 M NaOH, and the filter was rinsed in 2× SSC and air dried. Three such dot blots were tested with probes produced from GH-transgenic muscle total cDNA and nontransgenic muscle total cDNA. Template cDNA (1 μg, transgenic, wild type) was added to 4 μL of DIG High Prime (Boehringer Mannheim) in a 20-μL total reaction volume and incubated overnight at 37°C. Concentration of each probe was determined using DNA standards on test strips (Boehringer Mannheim). The membranes were incubated in hybridisation buffer (5× SSC, 0.1% *N*-laurosarcosinate, 0.02% SDS, 1% blocking reagent (Boehringer Mannheim)) for 30 min at 68°C. The respective DIG-labelled probes were denatured by boiling for 5 min diluted in preheated hybridisation buffer (2 μL of probe in 20 mL of hybridisation buffer). The prehybridisation solution was replaced with hybridisation buffer containing probe, and the filters were incubated overnight at 68°C. The membranes were then washed twice for 5 min in 2× SSC, 0.1% SDS at room temperature and twice for 15 min in 0.5× SSC, 0.1%SDS at 68°C. The filters were washed for 2 min in wash buffer (maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl (pH 7.5), 0.3% Tween 20)), 30 min in blocking solution (1× blocking reagent in maleic acid buffer), 30 min in blocking solution containing 1 : 20 000 anti-DIG-alkaline phosphatase conjugate antibody, twice for 15 min in wash buffer, 2 min in detection buffer (0.1 M Tris–HCl, 0.1 M NaCl (pH 9.5)), and 5 min in CSPD (Boehringer Mannheim), incubated at 37°C for 10 min, and exposed to X-ray film for 10 s. The X-ray film was developed using standard photographic chemicals and rephotographed onto black and white Polaroid film.

Sequencing

Each clone was sequenced by dye terminator cycle sequencing (Lee et al. 1992; Prober et al. 1992) using the Thermo Sequenase (Amersham) premix, 2 μL (0.4 μg) of the appropriate miniprep, and nested primer 1 (0.5 μM) from adapter 1 of the subtractive hybridisation kit in a 20-μL reaction volume. Thermal cycling parameters were as follows: 96°C for 1 min followed by 28 cycles of 96°C for 30 s, 45°C for 15 s, and 60°C for 4 min. The reaction was then precipitated in 70% ethanol in the presence of 2 M ammonium acetate, redissolved in loading dye, and run on an ABI Prism 377 sequencer according to the manufacturer's instructions.

Results

General characterisation of transgenic coho salmon

Controls (1994 nontransgenic fish) and the transgenic fish (of the 1995 year-class) were not significantly different in size when comparing fork length, body weight, condition factor, or slaughter yield (carcass weight percentage) (Table 1). However, significant differences were found in the body conformation variables abdominal wall thickness, area of total chop, muscle width, and muscle height (shown indirectly by the quota of width/height) (Table 1).

Muscle frequency distribution

Muscle fibre average cross-sectional area differed significantly between transgenic and nontransgenic fish in the two white muscle sample sites (Table 2). No difference in mean fibre size was evident in red muscle, even though an increase in variation of fibre sizes (occurrence of larger fibres in transgenic fish, $p < 0.0027$) (Fig. 2) was observed. This difference was abolished by including body weight in the model. In white muscle, body size, described by body weight (BW) and fork length (FL), asserted no significant effect on either average cross-sectional area or fibre size distribution (DLW: BW, $p < 0.26$; FL, $p < 0.28$; MLW: BW, $p <$

Table 1. Anatomical measurements of transgenic and nontransgenic control coho salmon.

	Transgenic group	Control group	*p*
Body weight (BW, g)	8.9±0.6	9.6±0.5	<0.447
Carcass weight (% of BW)	79±1.0	88.0±0.6	<0.060
Fork length (FL, mm)	9.5±0.2	9.8±0.2	<0.395
Condition factor	1.02±0.04	1.03±0.04	<0.5
Abdominal wall (AW, mm)	0.57±0.04	1.13±0.04	<0.0001*
AW/FL (%)	0.60±0.05	1.16±0.05	<0.0001*
Chop anterior (mm^2)	76.8±3.9	101.5±5.2	<0.0001*
Chop posterior (mm^2)	36.8±2.1	43.6±1.4	<0.01*
Height epaxial anterior (mm)	6.4±0.40	7.08±0.20	<0.14
Height epaxial posterior (mm)	4.9±0.16	5.04±0.20	<0.57
Width/FL anterior (%)	9.3±0.28	10.2±0.34	<0.0001*
Width/FL posterior (%)	5.3±0.27	5.49±0.14	<0.45
Width/height anterior (%)	1.42±0.10	1.40±0.01	<0.83
Width/height posterior (%)	1.02±0.05	1.07±0.04	<0.51
Red muscle anterior (%)	1.61±0.16	0.96±0.16	<0.007*
Red muscle posterior (%)	1.98±0.20	0.97±0.22	<0.002*

Note: The majority of anatomical measurements were not significantly different between transgenic and nontransgenic control fish. The fish from each group were of similar weight and length (top section, whole-body measurements). Among the anatomical measurements taken (lower section, see Fig. 1), abdominal wall thickness, cross-sectional area, and the percentage of red muscle showed significant differences (*$p < 0.05$, errors are standard errors).

Table 2. Mean muscle fibre cross-sectional area in transgenic and nontransgenic control coho salmon.

	Transgenic group	Control group	*p*
DLW (μm^2)	735±61	1645±5 ($n = 5$)	<0.0001*
MLW (μm^2)	1267±112	1941±155 ($n = 5$)	<0.006*
MLR (μm^2)	771±115	953±142 ($n = 3$)	<0.364

Note: In white fibre, but not in red fibre, transgenic fish have significantly smaller fibres (*$p < 0.05$, errors are standard errors).

0.60; FL, $p < 0.94$). On the contrary, a strong effect of body weight was observed for both average red muscle fibre cross-sectional area and fibre size distribution (MLR: BW, $p < 0.001$; FL, $p < 0.45$).

Comparing fibre size distribution (Fig. 2) with a paired *t* test indicated a significant difference in sizes-classes of DLW (size classes >200 μm^2, $p < 0.04$) and of MLW (size-class <1.300 μm^2, $p < 0.048$; size-class >1.300 μm^2, $p < 0.036$) but not of MLR (size-class >500 μm^2, $p < 0.17$). Comparing variance in individual size-classes indicated a significant difference between DLW (size-class 0–200 μm^2, $p < 0.0025$ with 42.1 ± 4.9 and 10.9 ± 2.4 of transgenic and nontransgenic fish, respectively) and MLW (size-class 0–500 μm^2, $p < 0.0071$ with 68.0 ± 3.7 and 45 ± 4.0% of transgenic and nontransgenic fish, respectively). A corresponding comparison between size-class 0–500 μm^2 in MLR yielded no significant difference ($p < 0.97$) with an average of 36.8 ± 10.3 and 36.0 ± 11.5% of transgenic and nontransgenic fish, respectively. Body size did not seem to influence this variable (BW, $p < 0.91$; FL, $p < 0.68$).

Comparison of average fibre size in the smallest size class (0–200 μm^2) yielded a significant difference in this class of DLW (transgenic fish, 126 ± 2.3 μm^2; nontransgenic fish, 186 ± 4.0 μm^2 ($p < 0.0001$)), while no such differences were observed in any other sample site (MLW: transgenic fish, 170 ± 4 μm^2; nontransgenic fish, 176 ± 8 μm^2 ($p < 0.83$); MLR: transgenic fish, 167 ± 6 μm^2; nontransgenic fish, 170 ± 9 μm^2 ($p < 0.80$)).

Analysis of muscle structure

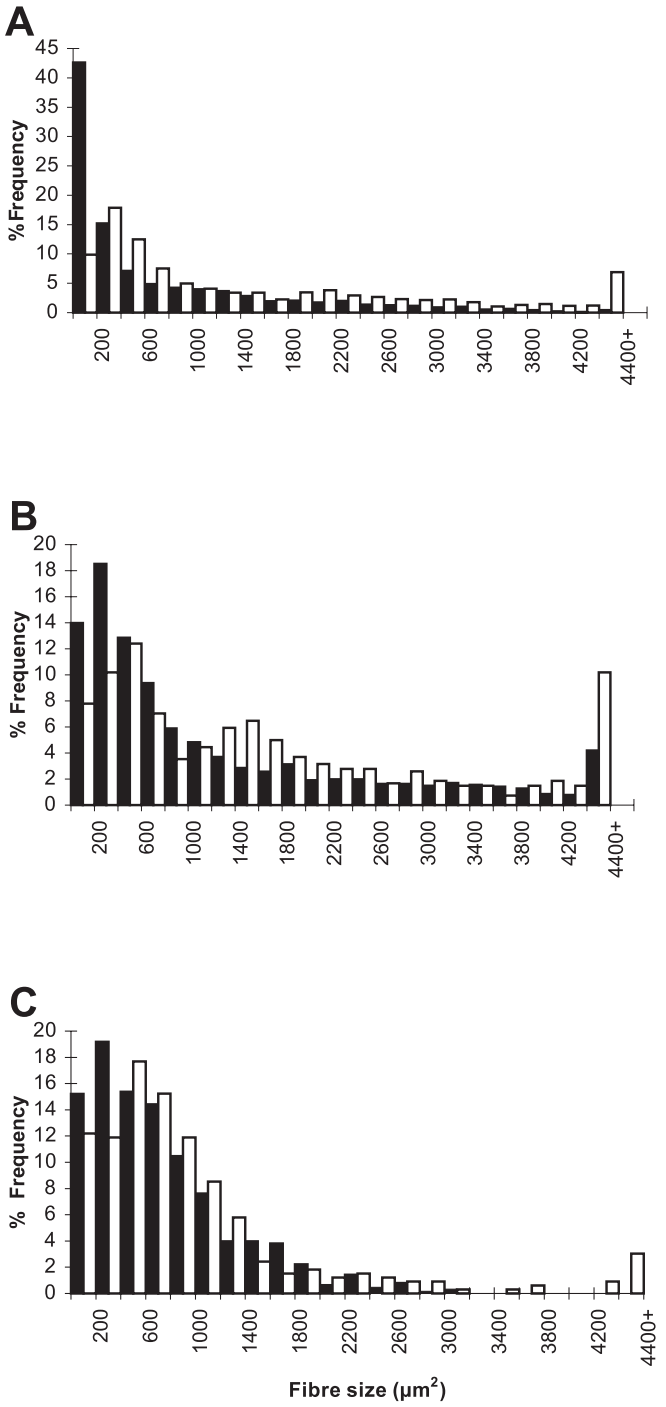
Comparing the relative composition of white versus red muscle in the total cross section of the fish revealed highly significant differences. Transgenic fish had more red muscle than the nontransgenic controls (Table 1). This difference in total amount of red muscle was most prominent in the caudal region.

The anatomical positions of muscle blocks taken for fibre area measurements within the white muscle (Fig. 1) represent zones of prolific growth in growing adult fish, established by traditional histological techniques in a number of species (Kiessling et al. 1990, 1991*a*, 1993). DLW and MLW fibres were both significantly smaller than controls on the basis of mean fibre area (Table 2); however, in an analysis of the difference in the distribution curve of fibre size, only the fibre frequency pattern from DLW fibres was significantly different (Figs. 2 and 3). This is consistent with previous observations that the distribution curve of fibre size in the mediolateral muscle reflects a more prominent hypertrophic growth (Kiessling et al. 1990, 1991*a*), indicating lower recruitment of small fibres and an increase in fibres in larger size groups. Average fibre size and size distribution of the MLR did not show any significant difference between transgenic fish and controls.

Enzyme analysis

Of the three enzymes analysed during the study, PFK and Cytox were significantly higher in activity in the transgenic fish (Table 3). PFK levels were approximately fourfold higher, while Cytox levels were increased by approximately 20%. No difference was found in activity of the third investi-

Fig. 2. Distribution of fibre cross-sectional area transgenic coho salmon (solid bars) and nontransgenic control coho salmon (open bars). (A) DLW muscle in transgenic fish has a greater number of small fibres than that in control fish (paired *t* test comparison of distribution, $p < 0.0064$ by variation in standard error). (B) MLW muscle also has a higher number of small fibres in transgenic fish than in the control group ($p < 0.0009$ by variation in standard error). (C) MLR fibres from transgenic fish show more similarity in distribution but are significantly different by variation in standard error ($p < 0.0027$).



gated enzyme, HAD, between transgenic and nontransgenic fish.

Differential expression of myosin light chain 2 (MLC2) in GH-transgenic coho salmon

Differences in gene expression were detectable between the transgenic fish and the controls using subtractive hybridisation. A number of cDNA clones isolated from the subtracted cDNA (Fig. 4A) library are differentially expressed when tested with probes generated from total cDNA from nontransgenic (Fig. 4B) or transgenic (Fig. 4C) coho salmon. This analysis was repeated several times with different concentrations of probe and different exposures to X-ray sensitive film. Results were consistent throughout, and all clones (with the exception of the negative control) were found to hybridise to both probes (results not shown). All of the differences in expression indicated higher expression in transgenic fish than in nontransgenic fish. No differences were observed when the two probes were hybridised against cDNA pools of transgenic and nontransgenic coho salmon. A negative control of a miniprep containing no insert was negative to both probes.

A number of these clones were sequenced, and several cDNAs with high homology to vertebrate MLC2 were identified along with a number of others (summarised in Table 4). Two different-sized fragments were identified that resulted from the *Rsa*I digestion of the initial cDNA pool, and we were able to identify the ends of each fragment, allowing us to construct a single continuous cDNA fragment. The combined cDNA fragment of 683 base pairs aligns with zebrafish (*Danio rerio*) MLC2 (Xu et al. 1998) mRNA with a homology of 88% for a region of 528 base pairs, which includes the entire coding region (Fig. 5A), and a homology of 89% at the amino acid level (Fig. 5B). The only major difference between the two proteins appears to be the insertion of a glycine at position 16 on the chain.

Discussion

In this experiment, we tested the effect of growth rate on muscle tissue by measuring muscle architecture and the titres of three important muscle enzymes. We have also isolated a number of genes by subtractive hybridisation that are more highly expressed in the transgenic fish. There are a number of points that must be remembered during discussion of these results. Firstly, we have no evidence that the muscle architecture observed in transgenic fish is causal to fast growth rates or growth efficiency but only that fibre distribution changes fast-growing transgenic fish. Secondly, we have no way to assess whether the differences seen between slow-growing nontransgenic fish and fast-growing transgenic fish are a result of growth rate or are a direct result of high levels or ectopic expression of the transgene. Also, this paper addresses changes in muscle under the influence of the GH transgene; no attempt has been made to address the effects on other tissues that are also an important component of whole-body growth.

Observations in rainbow trout suggest that submaximal growth is not expressed as more large and fewer small fibres when compared with maximally growing fish. In fact, an identical fibre distribution was found in fish of the same

Fig. 3. Cross-sectional view of muscle fibres from the DLW sampling site in (A) nontransgenic control coho salmon clearly showing that muscle fibres are larger than those in (B) transgenic coho salmon. M indicates a muscle fibre and the fibre boundary is indicated by arrowheads. Magnification 1075×.

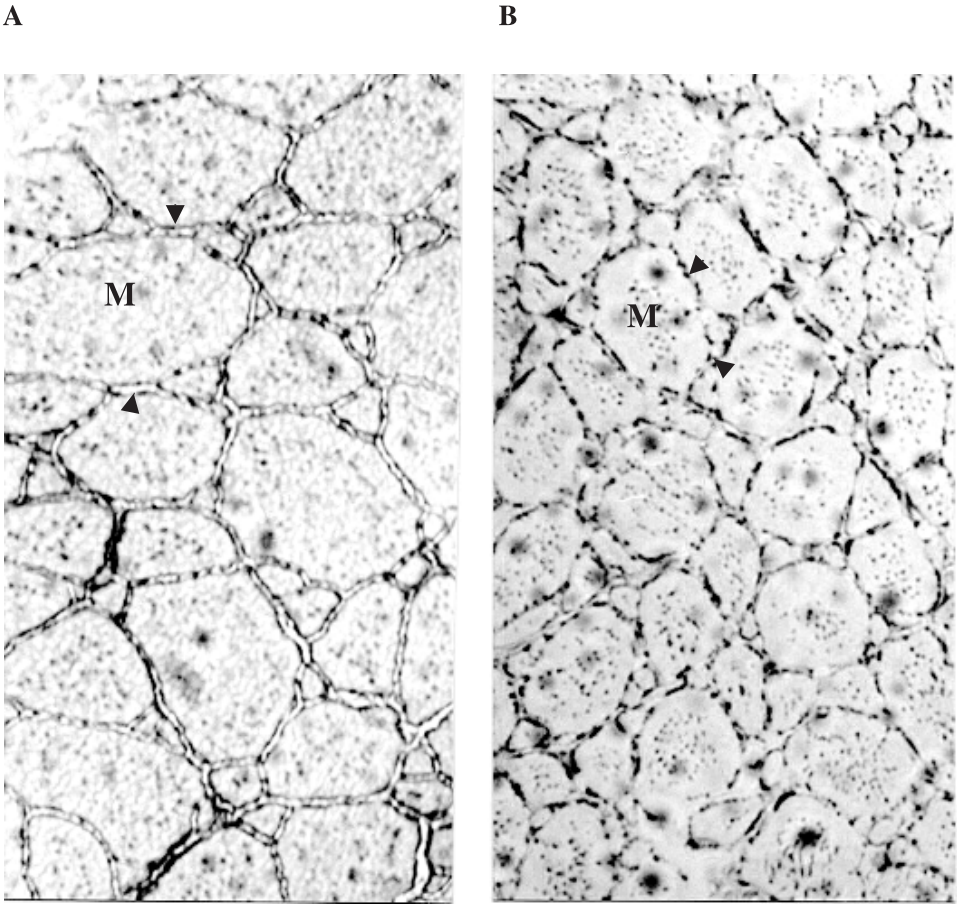


Table 3. Enzyme analysis of transgenic and nontransgenic control coho salmon muscle.

Enzyme ($\mu mol \cdot g^{-1} \cdot min^{-1}$)	Transgenic	Control	p
PFK	9.33 ±2.23	2.49±0.58	<0.014*
HAD	4.08±1.11	4.30±0.85	<0.879
Cytox	7.22±0.55 (n = 5)	5.53±0.49	<0.047*

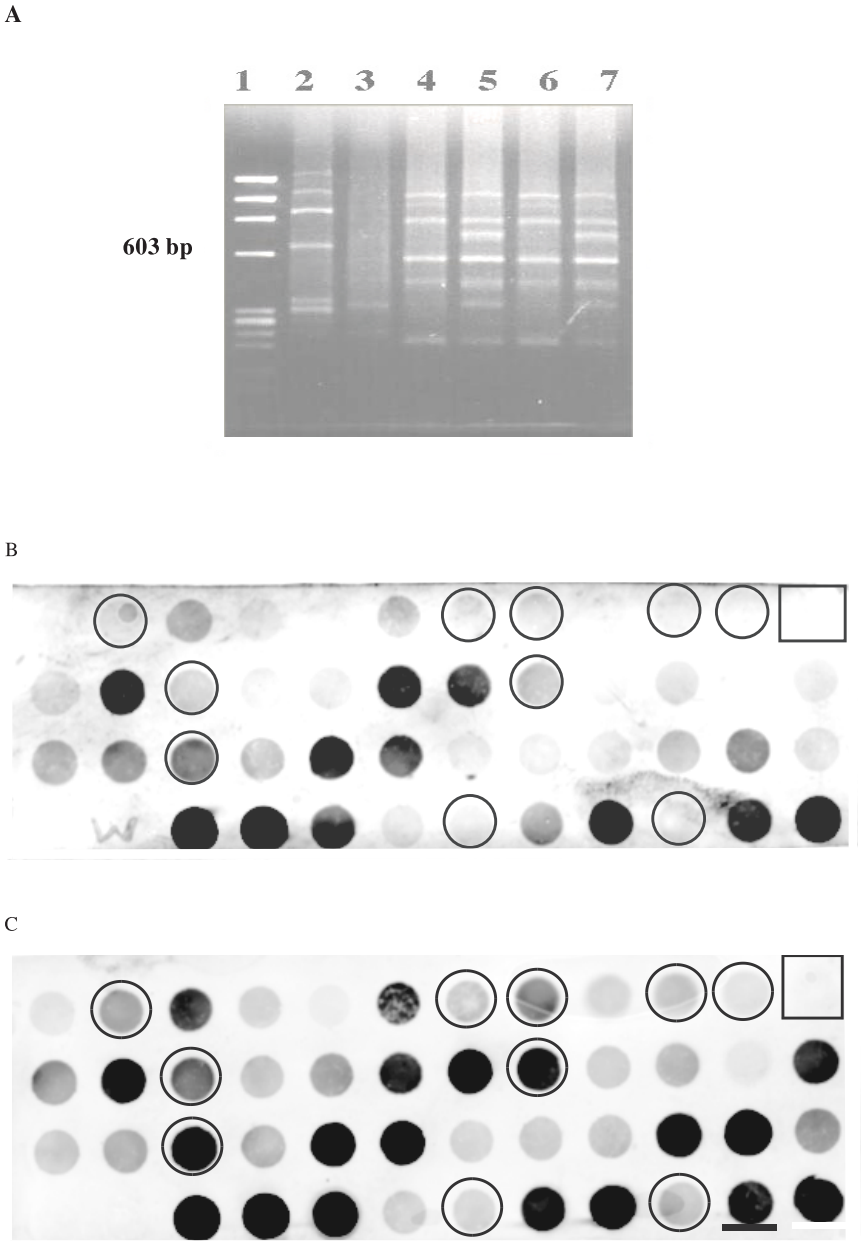
Note: Transgenic fish muscle has significantly higher activity levels of PFK and Cytox (*p < 0.05, errors are standard errors).

weight but of widely different age (Kiessling et al. 1991*a*). However, the latter study was conducted over a much longer time period with larger sampling intervals. A possibility therefore exists that rapid muscle growth supported by hyperplasia is limited in time and its contribution to growth is replaced by hypertrophy when measured over longer time periods. This cyclic growth pattern is supported by our own observations (Kiessling et al. 1991*a*), with a "burst" of small fibre production occurring in large fish but only during the autumn. The same phenomenon with small fibres occurring only during autumn was reported by Carpene et al. (1982) in mullet.

Transgenic fish have a greater percentage population of small white fibres compared with large white fibres (fibre area <200 mm^2 = 42% of the total fibres compared with 22% for the controls) (Fig. 2), and the anatomical region with the greatest difference is the dorsolateral area (Fig. 3). This was most prominent, not only as a larger difference in mean average cross-sectional area, but also as a more marked difference in number of small fibres in the smallest size-class (Figs. 2 and 3) and the mean average size of the fibres within this smallest size-class. Not only are many more small fibres found in DLW of transgenic fish, but the average size of these fibres is significantly smaller. This has been shown to be the major area of new fibre production in a number of teleost species (see Kiessling et al. 1990, 1991*a*, 1993). These observations are consistent with those of (Weatherley and Gill (1982) and Fauconneau et al. (1996), which show that exogenous GH induces hyperplasia in fish muscles. It is therefore possible that the constant and elevated level of GH in the transgenic fish replaces a cyclic shift between which growth type, hypertrophy or hyperplasia, dominates. In transgenic fish, hyperplastic growth may dominate constantly.

We observed no effect of body size on either white muscle mean cross-sectional area or fibre distribution within the size range used in this study. This tallies well with earlier published data on rainbow trout (Kiessling et al. 1991*a*). Further, in the same study, red muscle fibre cross-sectional area was found to relate more to growth rate (measured as feed level) than to attained body size. Our data agree with this

Fig. 4. (A) A 1.2% agarose gel electrophoresis of the result of the subtractive hybridisation. Lane 1, ΦX174 *Hae*III digest DNA marker (603 base pair band indicated); lane 2, subtracted control human skeletal muscle cDNA showing amplification of ΦX174 *Hae*III digest DNA bands, which are slightly longer than the marker lane due to the ligation of adapters during the experiment; lane 3, unsubtracted control human skeletal muscle cDNA with little amplification of specific bands; lanes 4 and 6, independent duplicate subtracted coho salmon muscle cDNA; amplified bands are differentially expressed cDNA in the transgenic fish; lanes 5 and 7, independent duplicated unsubtracted cDNA from coho salmon muscle showing additional bands that are subtracted out by the experiment. (B) Dot blot of 44 minipreps from the subtracted library probed with wild-type cDNA probe. Minipreps containing MLC2 identified by sequencing are circled. Also shown are the negative control (within a square), transgenic cDNA (underlined in black), and wild-type cDNA (underlined in white). (C) Dot blot identical to that in Fig. 4B probed with transgenic cDNA probe. Note that the majority of the minipreps are differentially expressed in the two cDNA populations and that this is true for all of the MLC2 clones.



observation in that only in red muscle was any significant relationship found between muscle fibres and body size, indicating that growth of red muscle fibres by hyperplasia or hypertrophy is more plastic than in white muscle, at least during earlier life stages.

The most prominent difference in body conformation between transgenic and nontransgenic fish is a leaner body in relation to fork length of the transgenic fish. Also the abdominal wall of the transgenic fish is much thinner, again indicating a less stout body conformation. Whether this difference is a result of altered growth rates between muscle and other tissues such as skeleton or whether it is caused by a shift in muscle growth between hyperplasia and hypertrophy cannot be deduced from these data. What is apparent is

Table 4. Identified genes from the subtracted library.

cDNA fragment similar to:	Homology	NCBI database accession No.	Comment
MLC2	Significant	AF251130	High representation, two fragments
Parvalbumin-β	Significant	—	High representation, two fragments
Skeletal α-actin	Significant	—	
Myosin heavy chain	Significant	—	
Ribosomal protein S17	Significant	—	
Nucleolar protein B23	Significant	—	
tRNA	Significant	—	
Plasmodium gene		—	Possible parasite?
Hs myoblast KIAA0005		—	Similar to human myoblast gene

Note: Genes were assessed for significant homology using online BLAST database homology searching (see Altschul et al. 1990).

that a much higher total number of fibres in the anterior region (Table 1) still results in a reduced total cross section of the muscle.

Comparing the enzyme activities found in this study with those in rainbow trout of equivalent size (approximately 40 $\mu mol \cdot g^{-1} \cdot min^{-1}$, Kiessling et al. 1991*b*) indicates that the activity of PFK is much lower. The level of PFK measured for transgenic fish resembles that of rainbow trout exposed to severe underfeeding (Kiessling et al. 1989), and that of the controls is well below any value reported earlier for salmonids (based on crude homogenate). However, distinct species and age differences exist in activity of this enzyme, indicative of muscle glycolytic capacity. In rainbow trout, a marked increase in activity of this enzyme was reported in white muscle during the first winter, increasing from 40 $\mu mol \cdot g^{-1} \cdot min^{-1}$ to a level close to 90 $\mu mol \cdot g^{-1} \cdot min^{-1}$ as the fish reached the size of saltwater tolerance (Kiessling et al. 1991*c*). This is the same activity level found in white muscle of sea-running sockeye salmon (*Oncorhynchus nerka*) (Kiessling et al. 1994*b*). An activity level of 10 $\mu mol \cdot g^{-1} \cdot min^{-1}$ was measured in the white muscle of adult seawater-adapted chinook salmon (*Oncorhynchus tshawytscha*), much higher than the activity level (2.49 $\mu mol \cdot g^{-1} \cdot min^{-1}$) measured in the control fish (Kiessling et al. 1994*a*). From this, it is clear that widely different levels of glycolytic activity exist in white muscle between different species of Pacific salmon.

The higher activity level of PFK found in the transgenic fish, relative to controls, and the less marked increase in the mitochondrial-bound enzyme Cytox (indicative of aerobic potential) may indicate that transgenic fish have a higher basal metabolic level than controls. This difference is intriguing, considering the observation that transgenic fish have a lower sustained swimming capacity than non-transgenic fish (Farrell et al. 1997), in spite of a white muscle metabolic profile normally associated with a higher capacity for sustained and burst activity. The superior sustained swimming capacity of the control fish is even more puzzling, considering the difference in muscle type profile (amount of red versus white muscle) between the transgenic and control fish. The muscle type profile of the control fish would, if found in humans, be indicative of a sprinter, while that of the transgenic fish would be indicative of a long-distance runner. This may be explained by physical limitations outside the muscle, perhaps differential growth rates between muscle and other organ systems such as circulation or skeletal development, although small white muscle fibres have been shown, by semiquantitative histochemical staining techniques, to contain higher levels of glycogen and a higher density of mitochondria (Kiessling et al. 1990).

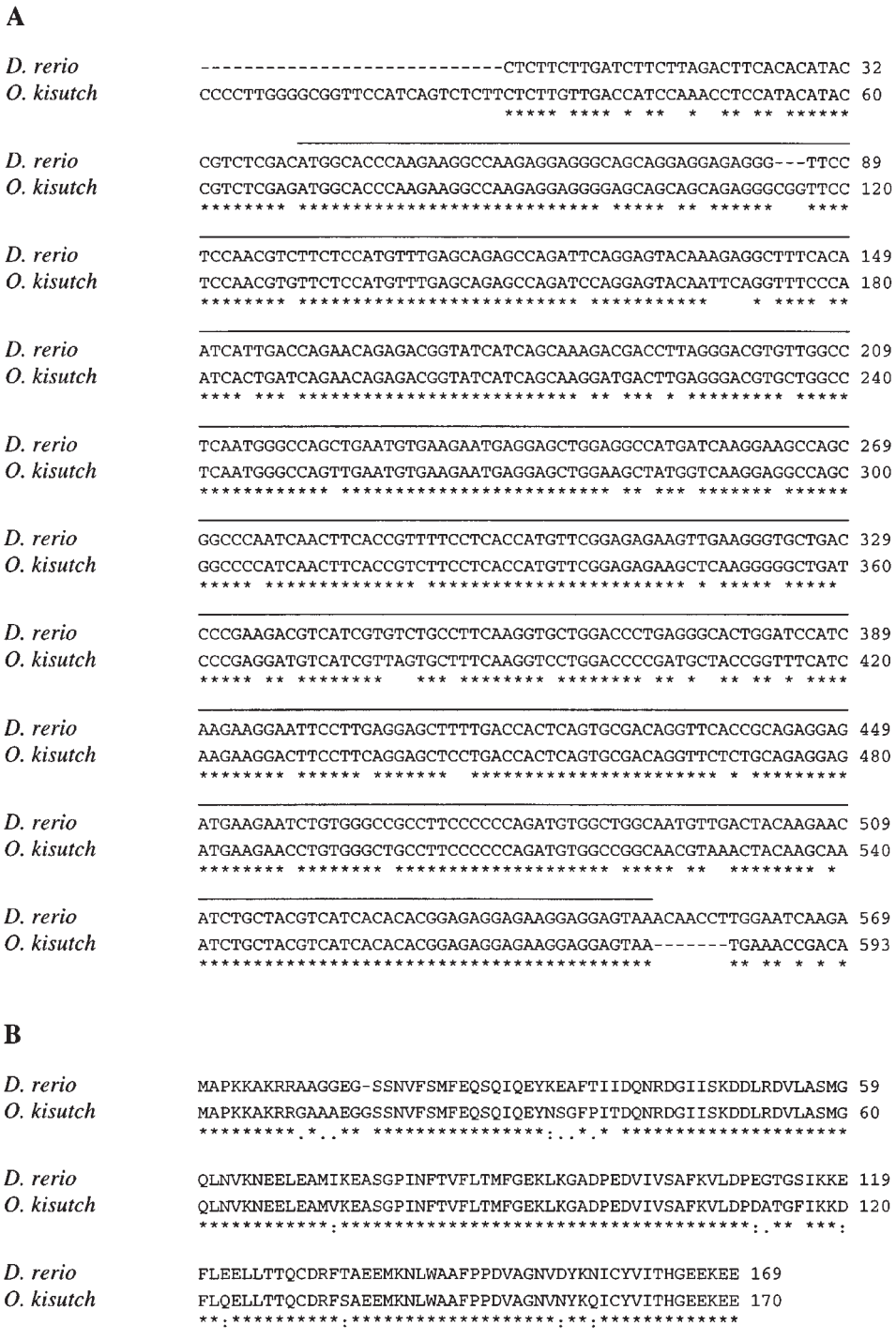
Another explanation is that metabolic performance and swimming performance are affected by ectopic expression of the transgene in muscle cells. Such expression may stimulate genetic responses within the muscle cells, altering the performance profile of the muscle in unexpected ways. With no previous studies available for experimental design, we decided upon an untargeted approach in order to assess whether a broad-scale difference in genetic expression exists between the fast muscle of the transgenic fish and that of the nontransgenic fish.

Subtractive hybridisation is a powerful technique that isolates and identifies mRNA (as cDNA) present at an abundance of 2.5-fold or higher in the tester sample, in this case, fast muscle from the transgenic fish. Analysis of the clones isolated from the subtracted library showed that there was a significant increase in expression of a number of genes in the library. We have yet to isolate a clone in the library that suggests that GH mRNA is present in the library, which is surprising, since the promoter included in the construct is general. The analysis of clones in the library has, however, produced a number of identified cDNAs that are upregulated in transgenic fish fast muscle. The upregulation of some of these genes can be explained by increased growth rate or observed muscle architectural changes, whereas that of others is harder to explain due to the lack of previous studies linking them to growth rate, muscle cellularity, or GH blood titres.

MLC2 has not previously been fully sequenced and characterised in salmonids, and we have included the full sequence of the coding region in this paper. The mRNA has been cloned in zebrafish (Xu et al. 1998); however, there is not yet enough information to indicate how many isoforms of MLC2 are likely to be expressed in fish during development and growth of muscle.

One recent study of Atlantic herring (*Clupea harengus*) development (Johnston et al. 1997) showed by two-dimensional polyacrylamide gel electrophoresis that there are at least two detectable protein isoforms of MLC2, which are expressed in a temporal pattern during development. This indicates that there is at least one MLC2 isoform that is associated with developing muscle fibres and that another (adult) isoform is switched on in existing, growing fibres.

Fig. 5. (A) Nucleic acid sequence analysis and comparison of MLC2 from coho salmon with zebrafish MLC2. The solid line above the sequence indicates the coding region, and an asterisk indicates sequence identity. (B) Deduced amino acid sequence of coho salmon MLC2 compared with zebrafish MLC2. An asterisk indicates identity, a colon indicates fully conserved functionality, and a period indicates partially conserved functionality.



Future studies will establish whether the isoform described in this study is expressed in embryonic muscle or is expressed as a juvenile or adult isoform only. The division of labour of MLC2 isoforms between embryonic and adult muscle has been established in higher vertebrates (Bandman 1992), and the high homology suggests that there may be very little evolution of functional difference. The increased expression of MLC2 in transgenic fish is therefore concordant with the increased emphasis on hyperplastic growth, and this gene may provide a valuable molecular method for monitoring hyperplastic growth in salmonids.

In addition, we identified a number of other clones from the subtracted library (Table 4) that are consistent with elevated levels of transcription (ribosomal proteins and tRNA) and changes in muscle ultrastructure (myosin heavy chain and skeletal α-actin). One interesting finding was that the

Ca^{2+} transport protein parvalbumin-β was subtracted into the library. This gene has been identified as a major human allergen in food fish (Lindstrom et al. 1996), and this finding implies that increased growth rates in salmonids may have unexpected meat quality consequences. This remains to be tested in other experiments on fast growth, for example, temperature or diurnal light cycle studies, since it may be a direct response to the presence of the transgene. However, this result displays the value of the untargeted approach, since parvalbumin has not previously been linked to growth rate changes and can now be monitored in growth rate experiments. A potential risk to consumer health, through increased levels of a strong allergen due to modern farming methods, has been identified.

A number of clones in the library have not yet been identified through homology searches of genetic databases. Cellular analysis of the expression of these genes will provide novel data on genetic expression of muscle cells. For example, one of the clones has some homology to a currently unidentified clone isolated from a human myocyte cell line. We will characterise this cDNA in future studies, since it may prove to be a marker of muscle stem cells. Such a marker has not yet been cloned in salmonids but has great potential for analysis of salmon growth.

In conclusion, increased growth rates, caused by transgenesis, in coho salmon have resulted in changes to muscle architecture consistent with increased rates of hyperplasia. This identifies a meat quality effect of growth rate that can be considered in future studies. Changes in levels of the muscle enzymes PFK and Cytox suggest effects on metabolism that can also lead to changes in meat quality due to postmortality effects. The subtracted library contains genes that are differentially expressed in salmon with high growth rates. These can be used as markers, increasing the analytical tools available for future studies of salmon growth under experimental farming conditions. Our future work will continue to analyse the cDNAs in the library with a view to understanding their relationship to growth processes and to developing diagnostic tests that will enhance growth experiments. In summary, a number of the effects of GH transgene mediated increases in growth rates have been identified. Future experiments will also determine which of these effects can be attributed to growth processes and which, if any, are a direct result of the presence of the transgene.

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