Multiple microarray platforms utilized for hepatic gene expression profiling of GH transgenic coho salmon with and without ration restriction

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Abstract

The objectives of this study are to examine hepatic gene expression changes caused by GH transgenesis and enhanced growth. This is the first use of cDNA microarrays to study the influence of GH transgenesis on liver gene expression in a non-mammalian vertebrate, and the first such study using sexually immature animals. Three groups of coho salmon were examined: GH transgenic on full ration (T), GH transgenic on restricted ration (R), and control non-transgenic (C). Specific growth rates for weight in T were approximately eightfold higher than in C, and fourfold higher than in R. Differential gene expression in T, R, and C samples was determined using ~3500 and 16 000 gene microarrays, and R and C samples were compared on a different ~4000 gene microarray. The use of multiple microarray platforms increased the overall proportion of the hepatic transcriptome considered in these studies. Cross-platform comparisons identified genes behaving similarly between studies. For example, genes encoding a precerebellin-like protein and complement component C3 were downregulated in R relative to C ($R < C$) in two microarray studies, and hemoglobins α and β were $R > C$ in all three studies. Comparisons of informative gene lists within and between studies inferred causes of altered gene expression. For example, ten genes, including 78 kDa glucose-regulated protein, glycerol-3-phosphate dehydrogenase, hemoglobins α and β , and a C-type lectin, were likely induced by GH transgenesis due to their presence in both $T > C$ and $R > C$ gene lists. Eleven genes, including hepcidin, nuclear protein p8, precerebellin-like, transketolase, and fatty acid-binding protein, were present in both $T < C$ and $R < C$ gene lists and were, therefore, likely suppressed by GH transgenesis. A large number of salmonid genes identified in these studies are involved in iron homeostasis, mitochondrial function, carbohydrate metabolism, cellular proliferation, and innate immunity. Pentose phosphate pathway genes phosphogluconate dehydrogenase, transaldolase, and transketolase, were dysregulated in GH transgenic samples relative to control samples. Changes in the expression of genes involved in maintaining hemoglobin levels (heme oxygenase, hemoglobins α and β , Kruppel-like globin gene activator, hepcidin) in R and T fish indicate a need for additional hemoglobin in the transgenic fish, perhaps due to higher metabolic rate required for enhanced growth.

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Introduction

Growth hormone (GH), synthesized largely by the pituitary gland, regulates longitudinal growth in vertebrates. GH-mediated signaling occurs in many tissues, and impacts cellular metabolism, proliferation, and differentiation (Thompson *et al.* 2000). In the liver, an important GH-target organ, GH regulates carbohydrate and lipid metabolism (Davidson 1987). In the last two decades, much research on GH and mammalian

growth has involved the study of dwarfism (caused by chronic underproduction of GH), acromegaly (chronic overproduction of GH), hypophysectomized (Hx) animals (surgically removed pituitary glands, modeling severe hormone deficiency), chronic and acute administration of exogenous GH, and GH transgenic animals. Until recently, studies of the effects of GH on tissue gene expression have been hampered by the lack of genomic tools and methods for global gene expression profiling. The advent of DNA microarrays has allowed detailed

analyses of the impacts of dwarfism (Tsuchiya *et al.* 2004), hypophysectomy (Flores-Morales *et al.* 2001), continuous GH treatment (Ahluwalia *et al.* 2004), and GH transgenesis (Olsson *et al.* 2003) on mammalian hepatic gene expression. The present study is the first to use cDNA microarrays to study the influence of GH transgenesis on liver gene expression in a non-mammalian vertebrate, and the first such study using sexually immature animals.

GH transgenic salmonids experience extraordinary rates of growth in their 1st year, well before the onset of sexual maturation (Devlin *et al.* 1994, 2001, 2004a). Feed availability has a profound influence on growth rates of sexually naïve GH transgenic coho salmon (*Oncorhynchus kisutch*). Our objectives are to study the consequences of growth, GH transgenesis, and ration level, through comparisons of global hepatic gene expression data from three groups of sexually immature coho salmon: GH transgenic on a full ration (group T), GH transgenic on a restricted ration (group R), and control non-transgenic (group C). We have conducted three experiments, each in a different laboratory and using different cDNA microarray platforms containing primarily Atlantic salmon (*Salmo salar*) sequences. The microarray platforms used in this research were a 3554 gene (3.5K) microarray developed by the Genomic Research on Atlantic Salmon Project (GRASP; Rise *et al.* 2004b), a 16 006 gene (16K) GRASP microarray (von Schalburg *et al.* 2005b), and a 4104 gene (4K) microarray developed by the Institute for Marine Biosciences (IMB; Ewart *et al.* 2005). Heterologous hybridizations indicate that Atlantic salmon cDNA microarrays are effective tools for global gene expression studies involving any salmonid species (Rise *et al.* 2004b, von Schalburg *et al.* 2005b). The use of multiple microarray platforms increased the number of different genes, and therefore the proportion of the liver transcriptome, considered in the present study. Cross-platform comparisons confirmed results by identifying genes present in similar informative gene lists between microarray studies, and comparisons within and between studies inferred biological causes of altered gene expression. The functional annotations of informative genes, and the pertinent literature, point to hepatic molecular pathways and biological functions altered by GH transgenesis, growth, and/or ration restriction.

Materials and methods

Aquaculture, sampling, and growth assessment

Coho salmon examined in this study were derived from the Chehalis River, which is a main tributary river of the

Fraser River in southwestern British Columbia, Canada. All fish culture was conducted at Fisheries and Oceans Canada's West Vancouver Laboratory, which is a non-commercial contained research facility specially designed to prevent the escape of genetically modified fish to the natural environment. GH transgenic coho salmon were initially produced by microinjecting a GH-gene construct (OnMTGH1) into the eggs from wild parents from the Chehalis River (Devlin *et al.* 1994). The strain used in the present study (M77) is stable and shows highly enhanced growth rates relative to wild-type sibling controls (Devlin *et al.* 2004a).

The objectives of the present study are to examine gene expression changes caused by GH transgenesis and enhanced growth. It is possible that there are both direct effects of GH transgenesis and indirect effects, which arise as a consequence of greatly accelerated growth. To separate these effects, three types of fish were examined in the present study: group T, transgenic fish reared on a full ration (which fully satiates their voluntary intake of food) for 9 months; group C, control non-transgenic coho salmon reared on a full ration for 21 months; and group R, transgenic salmon reared for 21 months on a restricted ration equivalent (per kilogram body weight) to that consumed by control salmon on a full ration. R salmon were pair fed in parallel with a group of C salmon of the same biomass, and were daily provided with the same amount of food consumed by the C group. Thus, group T grew at the very rapid rate typical of this GH transgenic strain, whereas groups C and R grew at similar rates typical of non-transgenic salmon. Use of fish of differing ages of T and C fish was necessary to ensure that fish were at the same developmental stages at the time of analysis. Comparisons of age-matched groups growing at their maximum growth rates are inappropriate due to the very large difference in size, which rapidly occurs between transgenic and non-transgenic individuals resulting in fish of different developmental stages (i.e. after some 4 months of growth, non-transgenic fish are still fry and are adapted for fresh water growth, whereas GH transgenic salmon are smolts, which are adapted for marine conditions). Age-matched samples at the same developmental stage have been generated by restricting the feeding of transgenic fish (group R), but these fish will differ due to effects from ration limitation. Thus, elucidation of the consequences of growth, GH transgenesis, and ration level requires assessment of all three groups (T, C, and R). Comparison of groups T and C salmon allows examination of effects of both growth and GH transgenesis, comparison of groups T and R salmon reveals effects arising indirectly from growth rate and/or ration restriction, and comparison of groups C and R allows identification of effects of GH transgenesis and/or ration restriction.

Group T transgenic salmon used in this study were F₅ generation progeny (year 2001 brood) produced by crossing a father homozygous for the transgene insert with five wild females derived from the Chehalis River. These crosses stably produce 100% transgenic hemizygous progeny (Devlin *et al.* 2004a). Group C were non-transgenic salmon (year 2000 brood) produced by crossing wild salmon sires with five wild females. Group R transgenic salmon were F₄ progeny (year 2000 brood) derived as for group T, except using the same females as in group C, but homozygous fathers from a previous year class. Group R salmon were reared on a restricted, control level, ration throughout the pre-experiment period. The variance associated with intrafamily growth rates is very small relative to the difference in growth observed between transgenic and non-transgenic salmon. Nevertheless, transgenic strain M77 has been maintained in an otherwise wild genetic background by continuous backcrossing to wild fish. Thus, the transgenic and non-transgenic strains should on average differ genetically only at the transgenic locus. All fish groups were initially incubated in Heath trays during embryonic development followed by culture under parallel conditions in oxygen-saturated fresh well

water at 10 °C with a flow rate in excess of 1 l/min/kg and a fish density <5 kg/m³. Stage-specific feeds were obtained from Skretting Canada (Vancouver, BC, Canada).

During the pre-sample period (August 21, 2002), 11 control non-transgenic (C), 11 ration-fed GH transgenic (R), and 11 full ration GH transgenic (T) post-smolt coho salmon were identified with unique passive integrated transponder tags implanted in their i.p. space, and placed in separate 200 l tanks and fed twice daily. C fish were fed to satiation, R fish were fed the same amount of food as C salmon (one R fish died and ration was adjusted accordingly), and T fish were fed to satiation. Length-specific growth rates (SGR_L) and weight-specific growth rates (SGR_W) were calculated using the equation, $SGR = [\ln(x_2/x_1)/(t_2 - t_1)] \times 100$, with x_1 as length or weight at time t_1 , and x_2 as length or weight at time t_2 (Fig. 1; see Table S1 in Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2/>). One-way ANOVA was used to assess differences in SGR_L and SGR_W. Tukey *post-hoc* tests were used for pairwise comparisons of growth rates for the C, R, and T treatment groups, and a significance level of $P=0.05$ was utilized.

Figure 1 Growth data for control non-transgenic (C), ration-fed GH transgenic (R), and full ration GH transgenic (T) year class 1 fish at times t_1 (August 21, 2002) and t_2 (September 13, 2002). (A) Average C, R, and T weights (g) at t_1 and t_2 . (B) Average C, R, and T lengths (cm) at t_1 and t_2 . (C) Average C, R, and T weight-specific growth rates. (D) Average C, R, and T length-specific growth rates. Error bars=S.E.M. Within each graph, identical letters (upper case for t_1 data, lower case for t_2 data) indicate no significant difference ($P > 0.05$) between the C, R, and T treatment groups.

Following anesthetization in tricaine–methanesulphonate (100 mg/l with 100 mg/l sodium bicarbonate), fish were sampled for weight and length, and weight-matched fish from each group were euthanized by a sharp blow to the head, and tissues rapidly removed by team dissection. Tissues were flash frozen in liquid nitrogen and stored at -75°C until RNA extraction.

Microarray analysis

Three different Atlantic salmon microarray platforms were utilized in this study: a 3·5K GRASP microarray, 16K GRASP microarray, and 4K IMB microarray. Differential gene expression in T, R, and C liver samples was determined using the 3·5K and 16K GRASP microarrays, and R and C samples were compared on the IMB microarray. Construction and initial testing of the GRASP microarrays described previously (Rise *et al.* 2004*a,b*, von Schalburg *et al.* 2005*a,b*). Briefly, clones were stringently selected from an expressed sequence tag (EST) database containing approximately 80 000 sequences derived from over 30 high-complexity salmonid cDNA libraries at the time the 3·5K microarray was designed and built, and over 300 000 sequences from over 175 high-complexity salmonid cDNA libraries at the time the 16K microarray was constructed. Inserts were amplified by PCR, cleaned, and printed as double, side-by-side spots on ez-rays aminosilane slides (Matrix) in $3\times$ SSC. *Arabidopsis thaliana* cDNAs were spotted on each microarray (Rise *et al.* 2004*b*, von Schalburg *et al.* 2005*b*) and used for thresholding (see Supplemental data at <http://jme.endocrinology-journals.org/content/vol37/issue2/>).

Construction of the IMB microarray has been described previously (Ewart *et al.* 2005). Briefly, 4104 clones were selected from four non-normalized salmonid cDNA libraries from liver, head kidney, macrophage, and spleen, as well as cDNA libraries constructed by suppression subtraction hybridization from the same tissues infected by the pathogen *Aeromonas salmonicida*. cDNAs were amplified by PCR, purified, resuspended in dH_2O , and spotted in duplicate on Gap II glass slides (Corning, Corning, NY, USA) in 50% DMSO. The entire array of 8208 spots was reprinted on the lower half of the slide. Twofold dilutions of a plasmid encoding chlorophyll synthetase G4 from *A. thaliana* were included at the bottom of each sub-array to serve as internal controls.

RNA isolation, and microarray hybridization and analysis

Microarray experiments were designed to comply with Minimum Information About a Microarray Experiment (MIAME) guidelines (Brazma *et al.* 2001). For GRASP microarrays, all scanned microarray TIF images and

quantified raw data files are available as online supplemental data at <http://web.uvic.ca/cbr/grasp>. Corresponding data for IMB microarrays are available as online supplemental data at http://www.imb.nrc.gc.ca/projects/salmonlivergeneexp/index_e.php. In addition, microarray TIF images and raw data (ImaGene or QuantArray files) have been deposited in gene expression omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>). For the 3·5K GRASP microarray experiment, the GEO platform accession number is GPL966; the GEO sample accession numbers are GSM44847 (slide DD035·038), GSM44848 (DD035·040), GSM44849 (DD035·041), GSM44988 (DD035·042), GSM44989 (DD035·043), GSM44990 (DD035·044), GSM44991 (DD035·046), GSM44992 (DD035·047), and GSM44993 (DD035·048); the GEO series accession number is GSE2388. For the 16K GRASP microarray experiment, the GEO platform accession number is GPL2989; the GEO sample accession numbers are GSM78638 (slide EB017·061), GSM78641 (EB017·062), GSM78664 (EB017·063), GSM78665 (EB017·064), GSM78666 (EB017·065), and GSM78667 (EB017·066); the GEO series accession number is GSE3477. For the 4K IMB microarray experiment, the GEO platform accession number is GPL2844; the GEO sample accession numbers are GSM78628 (slide 12605187-L-n-bottom), GSM78629 (slide 12605533-L-n-top), GSM78630 (slide 12605532-L-n-bottom), GSM78631 (slide 12605532-L-n-top), GSM78632 (slide 12841776-L-n-bottom), GSM78633 (slide 12841776-L-n-top), GSM78634 (slide 12841777-L-n-bottom), and GSM78635 (slide 12841777-L-n-top); the GEO series accession number is GSE3475.

GRASP microarrays

GRASP microarray studies were conducted using liver samples from fish of year class 1 (Fig. 2A and B). To minimize technical variability within a study, all targets were synthesized in one round, and all hybridizations were conducted simultaneously on slides from a single batch (DD035 for 3·5K study and EB017 for 16K study). Total RNA was individually prepared from flash-frozen adult liver tissues using TRIzol reagent and methods (Invitrogen). RNA from each fish was quantified and quality-checked by spectrophotometer and agarose gel respectively. The microarray experiments used pooled RNA templates (pooled C, pooled R, and pooled T; Fig. 2A and B), and each individual fish contributed an equal quantity of high-quality total RNA to a pool. Since the microarray studies involved pooled RNA samples, they did not provide information on biological variability of expression of informative genes. However, individual fish total RNA samples contributing to pools were archived at -80°C , and used as templates in the quantitative reverse transcription-PCR (QPCR) validation of a selection of microarray-identified genes

Overview of microarray experimental designs and results

Figure 2 Overview of microarray experimental designs and results (results compiled from Tables S3–S16 in Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2/>).

(A) 3.5K GRASP microarray study. (B) 16K GRASP microarray study. (C) 4K IMB microarray study. Arrows between samples symbolize microarrays with the base of each arrow on the Cy3-labeled target and the arrowhead on the Cy5-labeled target. Numbers of replicates are indicated next to arrows. Microarray features having identical top (most negative *E*-value) BLASTX hits are considered to be the same transcript. Summary data are shown adjacent to T versus C, R versus C, and T versus R sample comparisons. C, control non-transgenic; R, ration-fed GH transgenic; T, full ration GH transgenic.

(Fig. 3). The QPCR data, therefore, reveal biological variability of expression levels for genes validated in this manner. Microarray experimental design (Fig. 2A and B) involved three comparisons (C versus R, C versus T, and R versus T), each run in triplicate (two replicates and one dye flip) for the 3.5K study, or duplicate (one microarray and a dye flip) for the 16K study. The microarray experiments included only technical replicates (i.e. with averaged biological variation contained within technical replicates), while QPCR experiments included both technical and biological replicates. Microarray hybridizations were performed using the 3DNA Array 50 Expression Array Detection Kit and instructions (Genisphere, Inc., Hatfield, PA, USA). The Array 50 instruction manual, which includes an explanation of the chemistry involved in the Array 50 labeling system, is available online at

http://www.genisphere.com/pdf/array50v2_10_19_04.pdf. Briefly, 20 µg total RNA (pooled C, pooled R, or pooled T) were reverse transcribed using oligo d(T) primers with unique 5-prime sequence overhangs for the Cy3 or Cy5 labeling reactions. Microarrays were prepared for hybridization by washing 2×5 min in 0.1% SDS, washing 5×1 min in MilliQ H₂O, immersing 3 min in 95 °C MilliQ H₂O, and drying by centrifugation (5 min, 2000 r.p.m. in 50 ml conical tube). Microarray hybridizations were run in the dark under HybriSlips hybridization covers (Grace Biolabs, Bend, OR, USA) in slide hybridization chambers (Corning, Corning, NY, USA) submerged in a 48 °C water bath. The coho salmon liver cDNAs were hybridized to the salmonid cDNA microarray in a formamide-based buffer (25% formamide, 4× SSC, 0.5% SDS, 2× Denhardt's solution) for 16 h at 48 °C.

Coverslips were floated off at 48 °C in 2× SSC, 0.1% SDS buffer, and arrays were washed once for 10 min in 2× SSC, 0.1% SDS at 48 °C, twice for 5 min in 2× SSC, 0.1% SDS at room temperature (RT), twice for 5 min in 1× SSC at RT, and twice for 5 min in 0.1× SSC at RT, and dried by centrifugation as before. The Cy3 and Cy5 three-dimensional fluorescent molecules (3DNA capture reagent, Genisphere) were hybridized to the bound cDNA on the microarray in a formamide-based buffer (25% formamide, 4× SSC, 0.5% SDS, 2× Denhardt's solution) for 3 h at 48 °C, and washed and dried as before.

Fluorescent images of hybridized arrays were acquired immediately at 10 µm resolution using ScanArray Express (PerkinElmer, Wellesley, MA, USA). The same laser power (90%) and photomultiplier tube (PMT) settings were used for all slides in each study (3.5K study Cy3 PMT 75, Cy5 PMT 65–67; 16K study Cy3 PMT 78–80, Cy5 PMT 72–76). Fluorescent intensity data were extracted from TIF images using ImaGene (BioDiscovery, El Segundo, CA, USA; 3.5K study) or QuantArray software (PerkinElmer; 16K study). Quality statistics were compiled in Excel (see Table S2 in Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2/>), and data transformation (background correction, and setting background corrected values <0.01 to 0.01), normalization (Lowess), and analysis (formation and comparison of fold change gene lists) were performed in GeneSpring (Silicon Genetics Agilent Technologies, Palo Alto, CA, USA). In the GRASP microarray studies, transcripts with greater than twofold difference in expression between RNA samples of a given comparison (i.e. pooled T versus pooled C) in all slides of a study are reported in Supplementary data, Tables S3–S14 (see <http://jme.endocrinology-journals.org/content/vol37/issue2/>). These relatively strict criteria for identifying reproducibly informative transcripts were utilized to minimize false positives occurring due to dye bias and technical variability.

IMB microarrays

The IMB microarray study was conducted using liver samples from fish of year class 2 (Fig. 2C). mRNA, prepared from adult liver tissues preserved in RNALater (Ambion, Austin, TX, USA) using the FastTrack kit (Invitrogen), was quantified and quality-checked by spectrophotometer and agarose gel. Microarray target

synthesis used 100 ng pooled C or pooled R mRNA template, and each pool contained approximately equal quantities from five individual fish. All experiments were conducted in quadruplicate (two replicates and two dye flips). The top array from each slide was scanned and analyzed separately from the bottom array, yielding eight data sets and 16 possible spots for each gene. Preparation of cDNA using the SuperSmart PCR cDNA Synthesis Kit (BD Biosciences, San Jose, CA, USA), amplification using the Advantage 2 PCR Kit (BD Biosciences), labeling of cDNA with Cy3- and Cy5-dCTP (Amersham), hybridization, and scanning were performed as previously described (Ewart *et al.* 2005). Normalization and data analysis were carried out using the GeneTraffic software (Tobion Informatics, La Jolla, CA, USA). Background fluorescence was subtracted from the spot intensities and spots were flagged and removed from the data set, if the signal to background ratio was <1, the signal to average background ratio was <1, or the signal intensity was <1000. Data were normalized on a sub-grid basis using both locally weighted non-linear regression (Lowess) and intensity-based methods. Differentially regulated genes were identified using the following filter: normalized log 2 ratios of spots >0.9 or <−0.9 in the arrays using RNAs from R versus C fish, eight or more (out of 16) unflagged spots contributing to this assessment, and an overall coefficient of variation for that spot of <0.3. Since biologically significant changes in gene expression may not be greater than twofold or <0.5-fold, the non-parametric significance analysis of microarrays (SAM; Tusher *et al.* 2001) method was carried out on the normalized data to identify differentially regulated genes based on their changes in expression in relation to the s.d. for multiple measurements. The false discovery rate was set at 1%, meaning that, on average, 1% of the genes would be classified incorrectly using this method. Genes were identified as up- or downregulated only if they were identified using both the filter and SAM methods.

Real-time quantitative reverse transcription (RT)-PCR (QPCR)

Validating QPCRs were run using RNA templates from the livers of individual fish in year class 1. Total RNA was prepared from flash-frozen C, R, and T coho livers using TRIzol reagent and methods (Invitrogen). For each individual, 250 ng RNA were reverse transcribed using 31.25 ng oligo d(T)₁₆ primer. Reverse transcription

Figure 3 Real-time quantitative RT-PCR (QPCR) validation of 3.5K GRASP microarray results for four transcripts of interest ((A) iron–sulfur cluster assembly U2; (B) hepcidin antibacterial peptide; (C) transaldolase 1; (D) phosphogluconate dehydrogenase) normalized to ubiquitin. Mean ratios (fold changes) are shown. Numbers of replicates: microarrays, $n=3$ slides (two identical replicates and one dye flip) per comparison (e.g. pooled T versus pooled C; since these are technical replicates with pooled samples, rather than biological replicates, microarray data error bars are not included); QPCR, $n=2$ reactions per individual fish, 11 individual C and T fish and ten individual R fish examined. Error bars = s.e.m. The identities of the QPCR amplification products were confirmed by sequencing. C, control non-transgenic; R, ration-fed GH transgenic; T, full ration GH transgenic.

reactions included 5 U RNase inhibitor, 500 µM dNTPs, 5.5 mM MgCl₂, and 15.625 U MultiScribe Reverse Transcriptase with 1× TaqMan RT buffer (TaqMan Reverse Transcription Reagents Kit, Applied Biosystems, Foster City, CA, USA). Thermal cycling parameters for reverse transcription reactions were as follows: 25 °C for 10 min, 48 °C for 30 min, 95 °C for 5 min. First-strand cDNAs were diluted 1:3 and used as templates for QPCR analysis.

QPCR used two PCR primers per gene and SYBR Green I dye. Transcripts of interest and a normalizer gene were selected from the results of microarray experiments. For both the 3.5K and 16K GRASP studies, five different microarray features with ESTs identified as ubiquitin (top BLASTX hit: *Oncorhynchus mykiss* ubiquitin, accession number BAA88568) had normalized Cy5/Cy3 ratios between 0.5 and 2.0 on all slides. Ubiquitin was not dysregulated by GH transgenesis and/or ration restriction, and was therefore an appropriate normalizer gene for QPCR validation of microarray results. Primer pairs for transcripts of interest and ubiquitin were designed from alignments of the transcripts of interest and other available homologous sequences in GenBank using Primer Express software (Applied Biosystems) and the following guidelines: product size 150–250 bp, T_m 60 ± 1 °C, and at least two of the 3' terminal six bases G/C. For each gene, sequences of the forward and reverse QPCR primers respectively, are as follows: ubiquitin, 5'-CAA-CAGCGTCTGATCTTCGC-3', 5'-TTTGTACACAGTTG-TACTTCTGGGC-3'; iron-sulfur cluster assembly U2 (ISCU2), 5'-AGCTTCATCACATCTCCACAAGC-3', 5'-CAAGAAGGTAGTGGACCACTATGAGAA-3'; hepcidin antibacterial peptide, 5'-GTTGGAAGCTTTGA CAGTCCAGTT-3', 5'-AGAAGCCACAGCCAATGTTGT-3'; phosphogluconate dehydrogenase, 5'-CACCGTGG-CCTGTCCAGT-3', 5'-ACCACCGCCTTGTCTTCTTA-3'; and transaldolase 1, 5'-GCCGTCAGAGAGCTTCTCCA-3', 5'-AGAGCTGAGCAAAGACCACAGC-3'. Reactions (20 µl total volume) containing 4 µl diluted template, 300 nM each primer (with the exception of transaldolase at 50 nM), and 1× SYBR Green PCR Master Mix (Applied Biosystems), were run in duplicate using an ABI Prism 7000 Sequence Detection System (Applied Biosystems) and the following cycling parameters: 95 °C for 10 min, then 40 cycles of (95 °C for 15 s, 60 °C for 1 min). Controls (no template) were run for all primer pairs. Standard curves were generated by template dilution, with > 98.95% efficiency, for all primer pairs for relative quantitation by the ABI Prism 7000 Sequence Detection System.

Duplicate data (threshold cycle, or C_t , values) for each transcript of interest and for each individual were first normalized to ubiquitin (individual C_t value minus average ubiquitin C_t value for the same template). Data (normalized C_t values) from control non-transgenic, ration-fed transgenic, and full ration transgenic

templates were compared (converted to fold differences) in Excel using the relative quantification method (Pfaffl 2001) and assuming 100% efficiencies. All C_t values and calculations are available online (Supplementary data, Tables S18–S22; <http://jme.endocrinology-journals.org/content/vol37/issue2/>). Melting curves for the QPCR products for all transcripts of interest and ubiquitin showed single peaks. End-point analysis on agarose gels showed a strong, single band of the expected size for the iron-sulfur cluster assembly U2 primers, but the primers for ubiquitin and the remaining transcripts of interest showed two bands of the appropriate size, as may be anticipated for transcripts derived from a tetraploid genome such as salmon. The two bands were extracted from the gel and sequenced. The resulting sequences matched one another and the EST sequence used for primer design.

Results

Growth characteristics of C, R, and T fish

The following growth data pertain to the year class analyzed using the 3.5K and 16K GRASP microarrays (Figs 1 and 2; See Supplementary data, Tables S1 and S2 at <http://jme.endocrinology-journals.org/content/vol37/issue2/>). At the start of the pre-experiment period (t_1 = August 21, 2002), for both mean weights and length, C and R salmon did not differ but both were larger than T salmon (Fig. 1A and B; See Supplementary data, Table S1). The salmon in all treatment groups were re-measured for length and weight (Fig. 1A and B; See Supplementary data, Table S1) on September 13, 2002 (t_2), at which time T salmon had grown to the same weight as C and R salmon and the same length as C salmon, but were slightly shorter than R salmon (Fig. 1A and B; See Supplementary data, Table S1). Specific growth rates over the period for both weight (SGR_W) and length (SGR_L) were significantly different among all groups, with SGR_W in T salmon greater than eightfold higher than in C salmon and fourfold higher than R salmon (Fig. 1C and D; See Supplementary data, Table S1). Samples for the IMB microarray analysis were from a 2nd year class of fish, which were treated and grew in a similar fashion; however, growth rates were not specifically calculated.

Effects of GH transgenesis on global liver gene expression with and without rationing

Coho salmon transcripts responding reproducibly (as defined in Materials and methods) to GH transgenesis with and without feed rationing are reported in Tables S3–S16 (See Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2/>).

Minimum microarray feature signal requirements (see Supplementary data, Table footnotes) were imposed to prevent inclusion of false positives in candidate informative gene lists due to random variation close to background levels. Changes in transcription levels, and degrees of similarity between salmonid cDNA ESTs and their top (most negative *E*-value) BLASTX or BLASTN hits against GenBank nr. or nt databases, are shown to identify candidate informative transcripts (See Supplementary data, Tables S3–S16). Informative unknown transcripts (cDNAs with no significant BLAST hits) are also reported. Informative transcripts in Tables S3–S14 (See Supplementary data) are listed by descending signal values in the appropriate channel (e.g. T for upregulated in T relative to C, or C for downregulated in T relative to C) of one representative slide in the study. This allows rapid assessment of relative expression level, with highly abundant transcripts towards the top of the list and rare transcripts towards the bottom. For the purposes of intra- and interplatform comparisons, informative salmonid features (or spots) having significant BLAST hits with the same gene name are called by that name. These transcripts may represent identical salmon genes, orthologous sequences (for GRASP microarrays, which are predominantly Atlantic salmon, but contain some rainbow trout sequences), or paralogous sequences.

Many of the microarray-identified hepatic transcripts responsive to GH transgenesis with and without rationing are involved in iron homeostasis, energy metabolism, mitochondrial function, cellular proliferation and stress, and innate immunity. We, therefore, present cross-platform microarray data relevant to these biological processes in Tables 1–5. In cross-platform comparisons, reproducibly informative (see Materials and methods) gene names and accession numbers from individual microarray studies were used to query the normalized data from other microarray studies (See Supplementary data, Tables S3–S17). In Tables 1–5, the reproducibly informative feature data are shown in regular font, and the cross-platform data are shown in italicized font. When cross-platform scans identified multiple same-named microarray features, data were taken from features with signal intensities above threshold and regulation (average fold change between samples and direction of change) most closely resembling that observed in the reproducibly informative platform. With a few exceptions, there is a good level of cross-platform confirmation of informative genes in Tables 1–5. For example, four genes in Table 1 (hepcidin, heme oxygenase, and hemoglobins α and β) receive cross-platform confirmation, while one gene (GABARAP) does not. In Table 2, data from four genes (phosphoglucanate dehydrogenase, glycerol-3-phosphate dehydrogenase, prostaglandin D-synthase, and δ -6 fatty acyl desaturase) were

confirmed by cross-platform comparison. In some instances, features assigned the same gene name between microarray platforms (or even within a platform) may represent distinct but related genes. This could explain apparent disagreements between platforms (i.e. GABARAP in Table 1, and barrier-to-autointegration factor in Table 5).

Reproducibly informative genes identified in these microarray studies were confirmed in the following additional ways. Within each microarray study, several genes received ‘internal confirmation’ by appearing multiple times in a given informative gene list. For example, the 16K GRASP T<C transcript list contains five separate microarray features identified as nuclear protein p8 (See Supplementary data, Table S9). Cross-platform comparisons of informative transcript lists identify genes or closely related genes behaving in a similar fashion between studies (i.e. microarray features identified as 20 β -hydroxysteroid dehydrogenase B are found in the T>C transcript lists from both the 3.5K GRASP and the 16K GRASP studies; Fig. 4A). Comparisons of informative gene lists within and between microarray studies allow identification of suites of genes with the following inferred causes of dysregulation (followed by criteria for inclusion in the suites): genes induced by transgenesis (gene name must appear in at least one T>C list and at least one R>C list, so T and R>C); genes suppressed by transgenesis (gene name must appear in at least one T<C list and at least one R<C list, so T and R<C); genes indirectly induced by growth (gene name must appear in at least one T>C list and at least one R<T list, so T>C and R); genes indirectly suppressed by growth (gene name must appear in at least one T<C list and at least one R>T list, so T<C and R); genes induced by ration restriction (gene name must appear in at least one R>C list and at least one R>T list, so R>C and T); and genes suppressed by ration restriction (gene name must appear in at least one R<C list and at least one R<T list, so R<C and T; Fig. 4B).

The data from reproducibly informative transcripts identified by each microarray study are compiled in Tables S3–S16 (See Supplementary data), and the overlap between these gene lists is shown in Fig. 4. It is important to note that the mean fold change and S.E.M. values presented in Tables S3–S16 (See Supplementary data) pertain to technical replicates (i.e. data from replicate microarrays comparing gene expression in pooled samples), while statistics associated with QPCR experiments (See Supplementary data, Tables S18–S23) pertain to data from biological replicates.

3.5K GRASP T<C

In the 3.5K GRASP T versus C study on fish from year class 1 (Fig. 2A), 28 microarray features representing 26 different transcripts were reproducibly greater than

Table 1 Informative genes with functions related to iron homeostasis

Gene name ^a	Function ^b	Regulation	Mean fold change in study ^c		
			3·5K GRASP	16K GRASP	4K IMB
Hepcidin antibacterial peptide	Signaling molecule, iron homeostasis	T < C R < C	4·63 2·34	2·15 2·03	NA LQ
GABA _A receptor associated protein (GABARAP)	Binds transferrin receptor, which plays key role in iron metabolism	T > C R < C R < T	2·34 2·23 4·18	0·80 1·11 0·91	NA LQ NA
Hemoglobin α chain	Oxygen transport	T > C R > C	1·59 2·64	2·28 2·20	NA 1·91
Hemoglobin β chain	Oxygen transport	T > C R > C	2·14 3·23	2·36 1·65	NA 1·88
Kruppel-like globin gene activator	Transcription factor that activates globin promoters	T > C	NP	2·33	NA
Heme oxygenase	Heme oxygenase (decyclizing) activity	R < C	NP	1·31	1·82
Iron–sulfur cluster assembly U2 (ISCU2)	Binds iron, and participates in iron–sulfur cluster biosynthesis	T < C	3·21	LQ	NA

C, control non-transgenic; T, full-ratio GH transgenic; R, ration-fed GH transgenic.

^aFor expressed sequence tags (ESTs) corresponding to informative microarray features, the gene names of the most significant (lowest *E*-value) BLASTX hits are shown.^bFunction associated with the EST's top BLAST hit or a functionally annotated putative ortholog, from Swiss-Prot (<http://us.expasy.org/sprot/>) or references discussed in this report.^cNumber of replicate microarrays in 3·5K GRASP study: 3; in 16K GRASP study: 2; in 4K IMB study: 4. 'T < C'-fold change values are presented as C/T ratios, 'T > C' as T/C ratios, 'R < C' as C/R ratios, 'R > C' as R/C ratios, 'R < T' as T/R ratios, and 'R > T' as R/T ratios. Entries in regular font were reproducibly informative (see Materials and methods). Complete lists of reproducibly informative features, including EST accession numbers, BLAST results (accession numbers, species affiliations, and *E*-values of top BLAST hits), Gene Ontology (GO) and other functional annotations, and fold change values, are available online in Supplementary data (<http://jme.endocrinology-journals.org/content/vol37/issue2>) Tables S3–S8 (3·5K GRASP study), Tables S9–S14 (16K GRASP study), and Tables S15–S16 (4K IMB study). Entries in bold font correspond to genes appearing multiple times in an informative gene list; data from a single feature are shown, and additional feature data are available online in Supplementary data. Entries in italics were not present in reproducibly informative gene lists; selection criteria for these entries are presented in Results, and supporting data are available online in Supplementary data Table S17. NP, not present on microarray; NA, not applicable; as only the R versus C comparison was made using the IMB microarray (see Fig. 2). LQ, low quality signal data (signal intensities fell below threshold; see Supplementary data Table S17).**Table 2** Informative genes with functions related to energy metabolism

Gene name	Function	Regulation	Mean fold change	
			3·5K GRASP	16K GRASP
Glutamic-pyruvic transaminase	Cellular nitrogen metabolism, gluconeogenesis	T < C	2·23	LQ
Transaldolase	Carbohydrate metabolism, pentose phosphate pathway (PPP)	R < C	2·14	LQ
Phosphogluconate dehydrogenase	Pentose-phosphate shunt, oxidative branch	R < T	4·49	2·38
Transketolase	Regulation of growth, PPP	T < C R < C	LQ LQ	3·39 3·54
3-Phosphoglycerate dehydrogenase	Utilizes substrate from glycolysis to synthesize serine and glycine	R > C R > T	8·22 10·37	LQ LQ
Glycerol-3-phosphate dehydrogenase	Carbohydrate metabolism, gluconeogenesis	T > C R > C	2·72 2·50	2·39 1·87
Prostaglandin D-synthase	Prostaglandin metabolism, transporter activity	T > C R > C	4·13 3·17	3·83 1·52
δ -6 fatty acyl desaturase	Involved in bioconversion of linolenic acid to eicosapentaenoic acid (EPA)	T > C R < T	2·58 3·64	1·44 1·90
Fatty acid amide hydrolase	Terminates the signaling functions of bioactive fatty acid amides	T < C	4·04	LQ
Fructose-1,6-bis phosphatase	Carbohydrate metabolism, gluconeogenesis	T < C	NP	2·41

All column constructions and abbreviations are as described in Table 1 footnotes. Entries in regular, bold, or italicized font: see Table 1 footnotes.

For supporting information, see Tables S3–S17 in Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2/>.

Table 3 Informative genes involved in the mitochondrial electron transport chain

Gene name	Function	Regulation	Mean fold change	
			3·5K GRASP	16K GRASP
Cytochrome <i>c</i> oxidase polypeptide VIII (COX8A)	Nuclear-coded polypeptide chain of cytochrome oxidase (terminal oxidase in mitochondrial electron transport)	T>C	2·97	NP
Cytochrome <i>c</i> oxidase subunit VIb isoform 1 (COX6B)	Connects the two COX monomers into the physiological dimeric form	T>C	3·79	NP
Cytochrome <i>c</i>	Electron carrier protein in the mitochondrial matrix	T>C R>C	4·48 3·60	LQ LQ
Ubiquinol-cytochrome <i>c</i> reductase complex 11 kDa protein	Part of mitochondrial respiratory chain; may mediate formation of complex between cytochrome <i>c</i> and <i>c</i> 1	T>C	2·11	1·57
Ubiquinol-cytochrome <i>c</i> reductase complex core protein 2	Part of mitochondrial respiratory chain; required for assembly of the cytochrome <i>b-c</i> 1 complex	T>C	3·40	1·69

All column constructions and abbreviations are as described in Table 1 footnotes. Entries in regular, bold, or italicized font: see Table 1 footnotes. For supporting information, see Supplementary data, Tables S3–S17 (<http://jme.endocrinology-journals.org/content/vol37/issue2>).

twofold downregulated in T liver samples relative to C liver samples (See Supplementary data, Tables S3a and S3b; 'a' tables show mean fold change values for a non-redundant set of informative transcripts as well as the presence of same-named genes in other informative gene lists, and 'b' tables show data for each reproducibly informative feature from each contributing microarray). The most abundant downregulated transcript in T samples relative to C samples was alcohol dehydrogenase. Different microarray features with identical top BLAST hits in an informative gene list represent single genes or closely related paralogs.

For example, there are two CCAAT/enhancer-binding protein delta microarray features and two iron–sulfur cluster assembly U2 (ISCU2) microarray features in Table S3 (see Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>). The presence of multiple entries of genes in an informative gene list provides internal validation of microarray results. From 3·5K GRASP microarray data, transcripts most highly suppressed (>4·5-fold) in T relative to C liver were alcohol dehydrogenase (8·2-fold), metalloproteinase inhibitor 2 (8·2-fold), differentially regulated trout protein 1 (6·1-fold), and hepcidin

Table 4 Informative genes with functions related to cellular proliferation and stress

Gene name	Function	Regulation	Mean fold change in study		
			3·5K GRASP	16K GRASP	4K IMB
H2A histone family member Z	Chromosome organization and biogenesis; nucleosome assembly	T>C R>C	4·33 3·26	2·36 1·53	NA –
Histone H5	Chromatin condensation	R>C	NP	1·57	2·13
Non-histone chromosome protein 2-like 1	Regulation of progression through cell cycle; RNA binding	T>C R>C	2·25 3·11	1·87 1·63	NA –
Proliferating cell nuclear antigen	Various roles in DNA replication, DNA repair, cell-cycle control	T>C	2·84	LQ	NA
Glucose-regulated protein 78 (GRP78)	Chaperone that plays a central role in the ER stress response	T>C R>C	3·33 4·08	LQ LQ	NA –
Nuclear protein p8 (candidate of metastasis 1)	Induction of apoptosis; may promote cell growth to help tissue counteract diverse injuries	T<C R<C R>T	NP NP NP	7·32 3·22 2·97	NA – NA
Second mitochondria-derived activator of caspase	Induction of apoptosis by oxidative stress; promotes apoptosis by activating caspases	T<C R>T	3·59 1·97	3·70 2·61	NA NA

All column constructions and abbreviations are as described in Table 1 footnotes. Entries in regular, bold, or italicized font: see Table 1 footnotes. Dashes indicate genes were not confirmed due to low intensities, non-significance using SAM (see Materials and methods), or absence of the spot. For supporting information, see Supplementary data, Tables S3–S17 (<http://jme.endocrinology-journals.org/content/vol37/issue2>).

Table 5 Immune-relevant informative genes

Gene name	Function	Regulation	Mean fold change in study		
			3·5K GRASP	16K GRASP	4K IMB
C-type lectin	Agglutinates microorganisms <i>in vivo</i> ; sugar binding	T > C R > C R < T NP ^a	NP ^a NP ^a NP ^a NP	8·93 2·36 6·20 NP	NA 2·60 NA 1·89
Tachylectin	Innate immunity; cell adhesion	R < C	NP	NP	1·89
Complement component C3-3	Complement activation; inflammatory response	R < C	NP ^b	2·20	1·80
Complement C3-4	Endopeptidase inhibitor activity	R < C	NP	NP	1·82
Complement component C4	Complement activation; inflammatory response	T < C	LQ	2·74	NA
Complement component 7	Response to pathogenic bacteria	T < C R < T	3·33 2·69	LQ LQ	NA NA
Precerebellin-like protein	Acute phase response (Gerwick <i>et al.</i> 2000); immune-relevant gene (Bayne <i>et al.</i> 2001)	T < C R < C R > T	1·73 1·33 0·80	5·27 2·12 6·82	NA 2·26 NA
Differentially regulated trout protein 1	Immune/acute phase gene (Bayne <i>et al.</i> 2001)	T < C R < C	6·14 4·61	3·38 2·66	NA —*
CCAAT/enhancer-binding protein δ	Regulation of genes involved in immune/inflammatory responses	T < C	2·53	LQ	NA
Immunoglobulin H chain variable region	Antibody component (humoral immunity)	R > T	4·35	LQ	NA
Thymus-specific serine peptidase	Protease that may play a role in T-cell development	R > T	2·49	LQ	NA
Serum albumin-related protein	Immune/acute phase gene (Bayne <i>et al.</i> 2001)	T < C	NP	2·45	NA
Liver expressed antimicrobial peptide 2 isoform B	Defense response to bacteria	R > C	NP	NP	2·97
Barrier-to-autointegration factor	Response to virus	R > C	1·02	LQ	2·58

All column constructions and abbreviations are as described in Table 1 footnotes. Entries in regular, bold, or italicized font, and dashes: see Table 1 footnotes. For supporting data, see Tables S3–S17 (see Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>).
^aThe C-type lectin genes informative in the 16K GRASP and 4K IMB microarray experiments are most similar to newt (*Pleurodeles waltli*) and nematode (*Caenorhabditis elegans*) lectin genes respectively see Tables S10, S12, S13, and S16 (see Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>), and are not found on the 3·5K GRASP microarray. The C-type lectin genes on the 3·5K GRASP microarray are most similar to rainbow trout (*Oncorhynchus mykiss*) C-type lectin 2-1 and C-type lectin 2-2.
^bThe complement C3-like genes informative in the 16K GRASP and 4K IMB experiments are most similar to *O. mykiss* C3-3 and C3-4, and are not found on the 3·5K GRASP microarray. The C3-like genes on the 3·5K GRASP microarray are most similar to *O. mykiss* complement component C3-1. Asterisk: the IMB microarray study found a gene identified as differentially regulated trout protein 1 to be significantly R > C.

antibacterial peptide (4·6-fold). QPCR validation of microarray results using tissue samples from individual fish of year class 1 showed a 2·3-fold suppression of ISCU2 (s.e.m. 0·2) and a 3·2-fold suppression of hepcidin antibacterial peptide (s.e.m. 0·5) in T relative to C liver samples (Fig. 3A and B; See Supplementary data, Tables S18–S20 and S23). QPCR with fish from year class 1 also showed that ISCU2 was 2·2-fold suppressed (s.e.m. 0·2) in R relative to C samples (Fig. 3A; See Supplementary data, Tables S19 and S23).

16K GRASP T < C

In the 16K GRASP T versus C study on fish from year class 1 (Fig. 2B), 28 microarray features representing 20 different transcripts were reproducibly greater than twofold downregulated in T liver relative to C liver (See Supplementary data, Table S9a and S9b). The most

abundant downregulated transcript in T samples relative to C samples was an unknown EST (GenBank nt accession number CA038313). There are five nuclear protein p8, three differentially regulated trout protein 1, and three fatty acid-binding protein microarray features in Table S9 (See Supplementary data). From 16K GRASP microarray data, transcripts most highly suppressed (> 4·4-fold) in T relative to C liver were nuclear protein p8 (7·3-fold), precerebellin-like protein (5·3-fold), alcohol dehydrogenase (4·7-fold), and fatty acid-binding protein (4·5-fold).

Overlap of T < C from studies 1 and 2 (Fig. 2A and B)

Four genes (alcohol dehydrogenase, differentially regulated trout protein 1, second mitochondria-derived activator of caspase protein, and hepcidin antibacterial peptide) were greater than twofold suppressed in

A Cross-platform confirmation of informative genes

		Microarray platform: 3-5K GRASP								16K GRASP				IMB			
		T < C	T > C	R < C	R > C	R < T	R > T	T < C	T > C	R < C	R > C	R < T	R > T	R < C	R > C	R < T	R > T
In Supplemental Table S-		3	4	5	6	7	8	9	10	11	12	13	14	15	16		
R < C	Antifreeze protein LS-12																
	Differentially regulated trout protein 1																
	Precerebellin-like protein																
	Complement component C3-3																
R > C	Hemoglobin alpha chain																
	Hemoglobin beta chain																
	C-type lectin																
T < C	Alcohol dehydrogenase																
	Differentially regulated trout protein 1																
	SMAC protein; Diablo																
	Putative hepcidin antibacterial peptide																
T > C	Prostaglandin D synthase																
	20beta-hydroxysteroid dehydrogenase B																
	Glycerol-3-phosphate dehydrogenase																
R < T	Phosphogluconate dehydrogenase																
R > T	Alcohol dehydrogenase																
	RNA terminal phosphate cyclase-like 1																

B Summary of inferred causes of altered gene expression

In Supplemental Table S-		3	4	5	6	7	8	9	10	11	12	13	14	15	16		
Genes induced by transgenesis (T & R > C) • must be in at least one T > C table (S4, S10), and at least one R > C table (S6, S12, S16)	Prostaglandin D synthase																
	78 KD glucose-regulated protein																
	Glycerol-3-phosphate dehydrogenase																
	Non-histone chromosome protein 2-like 1																
	Cytochrome c																
	Single stranded D box binding factor 2																
	H2A histone family, member Z																
	Hemoglobin alpha chain																
	Hemoglobin beta chain																
	C-type lectin																
Genes suppressed by transgen. (T & R < C) • must be in at least one T < C table (S3, S9) and at least one R < C table (S5, S11, S15)	Differentially regulated trout protein 1																
	Putative hepcidin antibacterial peptide																
	Metalloproteinase inhibitor 2; TIMP-2																
	Guanylate binding protein 5																
	Nuclear protein p8																
	Similar to other reported ESTs (BG934783)																
	Precerebellin-like protein																
	Transketolase																
	Similar to other reported EST (BI468158)																
	Fatty acid binding protein																
	Similar to zebrafish DNA sequence (AL606751)																
Indirectly induced by growth (T > C & R)	Delta-6 fatty acyl desaturase																
	GABA(A) receptor associated protein																
	Unknown cDNA from normalized liver library																
	C-type lectin																
	Unknown cDNA from normalized gut library																
Indirectly suppressed by growth (T < C & R)	Alcohol dehydrogenase																
	SMAC protein; Diablo																
	Nuclear protein p8																
	Precerebellin-like protein																
	Similar to other reported EST (BG935059)																
Induced by ration restriction (R > C & T)	Translation initiation factor SUI1																
	3-phosphoglycerate dehydrogenase																
Suppressed by ration restriction (R < C & T)	GABA(A) receptor associated protein																
	Thyroid hormone-inducible hepatic protein																
	Unknown cDNA from norm. mixed tissue library																
	TSC domain family protein 4																

T relative to C in both the 3·5K and 16K GRASP microarray studies (Fig. 4A).

3·5K GRASP $T > C$

In the 3·5K GRASP T versus C study on fish from year class 1 (Fig. 2A), 36 microarray features representing 27 different transcripts were reproducibly greater than twofold upregulated in T liver relative to C liver (See Supplementary data, Tables S4a and S4b). The most abundant upregulated transcript in T relative to C liver was retinoid-binding protein 7. There are five prostaglandin D-synthase, three cytochrome *c*, three proliferating cell nuclear antigen (PCNA), and two H2A histone family member Z microarray features in Table S4 (See Supplementary data). From 3·5K GRASP microarray data, transcripts most highly induced ($> 4\cdot1$ -fold) in T relative to C liver were cytochrome *c* (4·5-fold), replication factor C 4 (4·5-fold), H2A histone family member Z (4·3-fold), and prostaglandin D-synthase (4·2-fold). Since there was a high level of redundancy (providing internal validation) in this informative gene list, transcripts of interest for QPCR validation were selected from informative gene lists with less redundancy.

16K GRASP $T > C$

In the 16K GRASP T versus C study on fish from year class 1 (Fig. 2B), 31 microarray features representing 18 different transcripts were reproducibly greater than twofold upregulated in T liver relative to C liver (See Supplementary data, Tables S10a and S10b). The most abundant upregulated transcript in T samples relative to C samples was ubiquitin carboxy-terminal hydrolase L1. There are two C-type lectin, seven β hemoglobin, two α hemoglobin, and six prostaglandin D-synthase microarray features in Table S10 (See Supplementary data). From 16K GRASP microarray data, transcripts most highly induced ($> 3\cdot7$ -fold) in T relative to C liver were C-type lectin (8·9-fold) and prostaglandin D-synthase (3·8-fold).

Overlap of $T > C$ from studies 1 and 2 (Fig. 2A and B)

Three genes (prostaglandin D-synthase, 20 β -hydroxysteroid dehydrogenase B, and glycerol-3-phosphate dehydrogenase) were greater than twofold induced in

T relative to C in both the 3·5K and 16K GRASP studies (Fig. 4A).

3·5K GRASP $R < C$

In the 3·5K GRASP R versus C study on fish from year class 1 (Fig. 2A), ten microarray features representing ten different transcripts were reproducibly greater than twofold downregulated in R liver relative to C liver (See Supplementary data, Tables S5a and S5b). The most abundant downregulated transcript in R relative to C liver was related to antifreeze protein LS-12 and is probably an apolipoprotein. From 3·5K GRASP microarray data the transcripts most highly suppressed ($> 3\cdot2$ -fold) in R relative to C liver were differentially regulated trout protein 1 (4·6-fold), cysteine protease CP14 (3·6-fold), guanylate-binding protein 5 (3·4-fold), and antifreeze protein LS-12 (3·3-fold). From this informative transcript list, hepcidin antibacterial peptide and transaldolase 1 were selected for QPCR validation, because their functions relate to immunity and carbohydrate metabolism respectively. QPCR validation of microarray results using tissue samples from individual fish of year class 1 showed a 2·8-fold suppression of hepcidin antibacterial peptide (S.E.M. 0·2) and a 2·3-fold suppression of transaldolase 1 (S.E.M. 0·2) in R relative to C liver samples (Fig. 3B and C; See Supplementary data, Tables S18, S20, S21, and S23). QPCR with year class 1 liver template samples and transaldolase 1 primers also showed 1·5-fold higher expression in T relative to C samples (S.E.M. 0·2), and 3·1-fold higher expression in T relative to R samples (S.E.M. 0·3; Fig. 3C; See Supplementary data, Tables S21 and S23).

16K GRASP $R < C$

In the 16K GRASP R versus C study on fish from year class 1 (Fig. 2B), 20 microarray features representing 16 different transcripts were reproducibly greater than twofold downregulated in R liver relative to C liver (See Supplementary data, Tables S11a and S11b). The most abundant downregulated transcript in R samples relative to C samples was thyroid hormone-inducible hepatic protein. There are three antifreeze protein LS-12, two nuclear protein p8, and two fatty acid-binding protein microarray features in Table S11 (See Supplementary data online). From 16K GRASP microarray data, transcripts most highly suppressed ($> 3\cdot1$ -fold) in R relative to C liver were antifreeze protein LS-12

Figure 4 Intra- and interplatform comparisons of reproducibly informative genes from microarray studies, compiled from Tables S3–S16 (see Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>). (A) Cross-platform comparisons of informative transcript lists. To be included, a gene name had to be present in a particular informative transcript list (e.g. upregulated in R relative to C, or $R > C$) in studies using at least two different microarray platforms. Black boxes indicate the presence of gene names in tables in this report. (B) Overlap of informative gene lists within and between microarray studies infers causes of altered gene expression. C, control non-transgenic; R, ration-fed GH transgenic; T, full ration GH transgenic.

(3·9-fold), thyroid hormone-inducible hepatic protein (3·7-fold), transketolase (3·5-fold), and nuclear protein p8 (3·2-fold).

4K IMB $R < C$

In the IMB R versus C study on fish from year class 2 (Fig. 2C), the reproducibly downregulated transcripts in R liver relative to C liver encoded precerebellin-like protein, tachylectin, heme oxygenase, two complement C3 components, and four unknown products (See Supplementary data, Tables S15a and S15b).

Overlap of $R < C$ between microarray studies

Two genes (antifreeze protein LS-12 and differentially regulated trout protein 1) were greater than twofold suppressed in R relative to C in both the 3·5K and 16K GRASP microarray studies (Fig. 4A). Two other genes (precerebellin-like protein and complement C3-3) were present in the $R < C$ gene lists of both the 16K GRASP and the 4K IMB studies (Fig. 4A). Interestingly, the IMB microarray study found that differentially regulated trout protein 1 was upregulated in R relative to C and this was found to be significant using SAM analysis (see Materials and methods). None of the remainder of the downregulated transcripts uncovered using the 3·5K GRASP microarray was confirmed using the IMB microarray, either because the intensities fell below threshold (e.g. hepcidin, GABA_A receptor associated protein), because SAM analysis did not find them to be statistically significant (transaldolase, amoebocyte aggregation factor, antifreeze protein LS-12), or because they were not present on the IMB microarray (metalloproteinase inhibitor 2, retinoid X receptor α , guanylate-binding protein 5, cysteine protease CP14). Using the IMB microarray with year class 2 fish samples, antifreeze protein LS-12 homolog was significantly downregulated in R relative to C head kidney, but not in liver (data not shown).

3·5K GRASP $R > C$

In the 3·5K GRASP R versus C study on fish from year class 1 (Fig. 2A), 22 microarray features representing 17 different transcripts were reproducibly greater than twofold upregulated in R liver relative to C liver (See Supplementary data, Tables S6a and S6b). The most abundant and fourth-most abundant upregulated transcripts in R relative to C liver were the α and β chains of hemoglobin respectively. There are three prostaglandin D-synthase, three cytochrome c , and two H2A histone family member Z microarray features in Table S6 (See Supplementary data online). From 3·5K GRASP microarray data, transcripts most highly induced ($>3\cdot5$ -fold) in R relative to C liver were 3-phosphoglycerate

dehydrogenase (8·2-fold), 78 kDa glucose-regulated protein (4·1-fold), nucleolar protein NOP5 (3·8-fold), and cytochrome c (3·6-fold). Due to the high level of internal validation within this informative gene list, transcripts of interest for QPCR validation were selected from informative gene lists with less redundancy.

16K GRASP $R > C$

In the 16K GRASP R versus C study on fish from year class 1 (Fig. 2B), three microarray features representing three different transcripts were reproducibly greater than twofold induced in R liver relative to C liver (See Supplementary data, Tables S12a and S12b). The most abundant upregulated transcript in R samples relative to C samples was hemoglobin α . From 16K GRASP microarray data, transcripts reproducibly induced in R relative to C liver were hemoglobin α (2·2-fold), C-type lectin (2·4-fold), and phospholipid hydroperoxide glutathione peroxidase (2·3-fold).

4K IMB $R > C$

In the IMB R versus C study on fish from year class 2 (Fig. 2C), the reproducibly upregulated transcripts in R relative to C samples encoded liver-expressed antimicrobial peptide 2, C-type lectin, barrier-to-autointegration factor (also called breakpoint cluster protein), histone H5, a novel protein containing a ChaC-like domain, hemoglobins α and β , and two unknown products (See Supplementary data, Tables S16a and S16b). There are two barrier-to-autointegration factor microarray features in Table S16. One additional feature in this informative gene list came from a clone (BG935738) containing two inserts, one for hemoglobin α and the other for monocyte chemoattractant protein-1 (See Supplementary data, Table S16b). Given the positive result from another spot containing hemoglobin α , the signal from BG935738 likely arises from hemoglobin α ; however, there may also be a contribution from monocyte chemoattractant protein. Again, several of the transcripts identified using the 3·5K GRASP microarray were not confirmed using the IMB microarray due to low intensities, non-significance using SAM, or absence of the spot.

Overlap of $R > C$ between microarray studies

Hemoglobin α chain was greater than twofold induced in R relative to C in all three microarray studies (Fig. 4A). Hemoglobin β chain was present in the $R > C$ gene lists of both the 3·5K GRASP and the 4K IMB studies, and C-type lectin was present in the $R > C$ gene lists of both the 16K GRASP and the 4K IMB studies (Fig. 4A).

3·5K GRASP R < T

In the 3·5K GRASP T versus R study on fish from year class 1 (Fig. 2A), 11 microarray features representing 11 different transcripts had greater than twofold lower expression in R relative to T liver in all three slides (See Supplementary data, Tables S7a and S7b). The most abundant transcript in this list was protein disulfide isomerase related. From 3·5K GRASP microarray data, transcripts with the highest T/R fold differences (> 3·4-fold) were an unknown (6·3-fold), phosphogluconate dehydrogenase (4·5-fold), GABA_A receptor associated protein (4·2-fold), kinesin-like protein 2 (3·7-fold), δ -6 fatty acyl desaturase (3·6-fold), and apoptosis inhibitor survivin 1 (3·5-fold). The unknown, GABA_A receptor associated protein, and δ -6 fatty acyl desaturase microarray features in this informative transcript list are also present in Table S4 (See Supplementary data online) (3·5K GRASP T > C; Fig. 4B), suggesting that these genes are induced in T relative to R rather than suppressed in R relative to T. QPCR validation of microarray results, using templates from fish of year class 1 and primers for phosphogluconate dehydrogenase, showed a 4·2-fold T/R difference (S.E.M. 0·2; Fig. 3D; See Supplementary data, Tables S18, S22, and S23). QPCR with year class 1 templates also showed that phosphogluconate dehydrogenase was 1·7-fold induced in T relative to C (S.E.M. 0·1), and 2·8-fold suppressed in R relative to C (S.E.M. 0·3; Fig. 3D; See Supplementary data, Tables S22 and S23).

16K GRASP R < T

In the 16K GRASP T versus R study on fish from year class 1 (Fig. 2B), eight microarray features representing seven different transcripts had greater than twofold lower expression in R liver relative to T liver in both slides (See Supplementary data, Tables S13a and S13b). The most abundant transcript in this list is C-type lectin, and there are two C-type lectin features in Table S13 (See Supplementary data online). From 16K GRASP microarray data, transcripts with the highest T/R fold differences (> 4·0-fold) were C-type lectin (6·2-fold), an unknown (EST accession number CA038900; 5·9-fold), and TSC domain family protein 4 (4·1-fold).

Overlap of R < T from studies 1 and 2 (Fig. 2A and B)

One gene, phosphogluconate dehydrogenase, was greater than twofold induced in T relative to R liver in both the 3·5K and 16K GRASP studies (Fig. 4A).

3·5K GRASP R > T

In the 3·5K GRASP T versus R study on fish from year class 1 (Fig. 2A), nine microarray features representing nine different transcripts had greater than twofold

higher expression in R relative to T liver in all three slides (See Supplementary data, Tables S8a and S8b). The most abundant transcript in this list was alcohol dehydrogenase. From 3·5K GRASP microarray data, transcripts with the highest R/T fold differences (> 3·8-fold) were 3-phosphoglycerate dehydrogenase (10·4-fold), immunoglobulin H chain variable region (4·3-fold), and alcohol dehydrogenase (3·9-fold). Since 3-phosphoglycerate dehydrogenase and translation initiation factor SUI1 are also found in Table S6 (See Supplementary data online) (3·5K GRASP R > C; Fig. 4B), these genes are likely induced in R relative to T rather than suppressed in T relative to R.

16K GRASP R > T

In the 16K GRASP T versus R study on fish from year class 1 (Fig. 2B), 11 microarray features representing ten different transcripts reproducibly had greater than twofold higher expression in R relative to T liver (See Supplementary data, Tables S14a and S14b). The most abundant transcript in this list was precerebellin-like protein. There are two nuclear protein p8 microarray features in Table S14 (See Supplementary data online). From 16K GRASP microarray data, transcripts with the highest R/T fold differences (> 2·8-fold) were precerebellin-like protein (6·8-fold), nuclear protein p8 (3·0-fold), and alcohol dehydrogenase (2·9-fold).

Overlap of R > T from studies 1 and 2 (Fig. 2A and B)

Two genes, RNA terminal phosphate cyclase-like 1 and alcohol dehydrogenase, had greater than twofold higher expression in R relative to T liver in both the 3·5K and the 16K GRASP studies (Fig. 4A). Since alcohol dehydrogenase is also present in the T < C gene lists from both the 3·5K GRASP study (See Supplementary data, Table S3) and the 16K GRASP study (See Supplementary data, Table S9; Fig. 4A and B), this gene is likely suppressed in T relative to R rather than induced in R relative to T.

Discussion

We used three different salmonid cDNA microarray platforms (3·5K GRASP, 16K GRASP, and 4K IMB) to study the impact of GH transgenesis with and without ration restriction on global gene expression in hepatic tissues from pre-reproductive (smolt) coho salmon. DNA microarrays have been used to identify GH-responsive genes in adult mammalian liver (Thompson *et al.* 2000, Flores-Morales *et al.* 2001, Olsson *et al.* 2003, Ahluwalia *et al.* 2004). Here, we report the first use of cDNA microarrays to study the influence of GH transgenesis on liver gene expression in a non-mammalian vertebrate,

and the first such study using sexually immature animals. The use of multiple microarray platforms, each containing a partial salmonid transcriptome, increased the overall proportion of the transcriptome considered in the present studies. Some sequences are lost during the process of cDNA microarray fabrication, largely due to unsuccessful amplification (Rise *et al.* 2004b). An advantage of a multiple platform approach is that clones lost in the fabrication of one platform may be represented in another.

The functional annotations of informative salmonid liver transcripts responding to GH transgenesis with and without food rationing point to molecular pathways altered under these different genotypic and nutritional conditions. A large number of salmonid genes identified in these studies are involved in iron homeostasis, mitochondrial function, carbohydrate metabolism, apoptosis, cellular proliferation, and innate immunity. The discussion is, therefore, focused primarily on these biological processes, with particular attention paid to genes (or gene names) with expression profiles that were confirmed within and/or across platforms, and informative transcripts with growth-relevant functional annotations.

Iron homeostasis and signal transduction

GABARAP, hepcidin, and hepatic transcription factors

In the 3·5K GRASP study, GABA_A receptor associated protein (GABARAP) was upregulated in T relative to C and T relative to R liver, and downregulated in R relative to C liver (Table 1; T>C>R). Thus, in this study, GABARAP was found to be both indirectly induced by growth (T>C and R) and suppressed by ration restriction (R<C and T; Fig. 4B). A transcript identified as GABARAP was not informative in the 16K GRASP study (Table 1), and potential causes of such apparent disagreement between platforms were previously presented. GABARAP is extremely highly conserved in the metazoa, with amino acid identity values ranging from 93 to 98% in such organisms as fruit fly (*Drosophila melanogaster*), amphioxus (*Branchiostoma belcheri tsingtaunense*), and zebrafish (*Danio rerio*). Human GABARAP, first identified by a yeast two-hybrid system using parts of the GABA_A receptor as bait, bears significant amino acid sequence similarity to light chain-3 of microtubule-associated proteins (MAPs) 1A and 1B (Wang *et al.* 1999). GABARAP is ubiquitously expressed, suggesting that it has likely generalized or multiple functions in addition to its putative role in moving, sorting, and clustering GABA_A receptors (Wang *et al.* 1999, Kneussel & Betz 2000, Stangler *et al.* 2002). GABARAP binds transferrin receptor (TfR) and potentially plays a role in trafficking or cycling of TfR (Green *et al.* 2002). Liver TfR plays a key role in iron metabolism,

delivering iron-bound transferrin to the endosomal compartment and stimulating hepatocytes to release hepcidin (Green *et al.* 2002, Sharma *et al.* 2005). In addition to GABARAP, other genes involved in iron homeostasis (e.g. hepcidin and heme oxygenase) were also dysregulated in the GH transgenic coho salmon experiments giving rise to this report.

Microarray (3·5K and 16K GRASP) and QPCR data on tissues from fish of year class 1 (Fig. 2A and B) indicated that a coho salmon transcript encoding a putative polypeptide related to the acute phase protein hepcidin was downregulated in both T and R relative to C liver (Table 1; Figs 3B and 4A and B). Hepcidin was 1 of 11 genes found to be suppressed by GH transgenesis (T and R<C; Fig. 4B). Mammalian hepcidin (or liver-expressed antimicrobial peptide-1) genes are expressed primarily in liver, and hepcidin is believed to be among the most abundant transcripts in adult mammalian liver (Pigeon *et al.* 2001, Courselaud *et al.* 2002). Similarly, coho salmon hepcidin was the fourth-most abundant transcript in the 3·5K GRASP T<C list (See Table S3 at <http://jme.endocrinology-journals.org/content/vol37/issue2>), and the third-most abundant transcript in both the 3·5K GRASP R<C list (See Table S5 in Supplementary data) and the 16K GRASP T<C list (See Supplementary data, Table S9).

Hepcidin is thought to play an important role in vertebrate innate immune responses to microbial challenge (Shike *et al.* 2004, Lauth *et al.* 2005). Mammalian hepcidin proteins exhibit antibacterial and antifungal activity (Krause *et al.* 2000, Park *et al.* 2001). Hepcidin expression is upregulated in human (Nemeth *et al.* 2003) and mouse (Pigeon *et al.* 2001) hepatocytes exposed to lipopolysaccharide, and also in Atlantic salmon (Douglas *et al.* 2003), zebrafish (Shike *et al.* 2004), and striped bass (Lauth *et al.* 2005) liver tissues in response to bacterial infections. In addition to its conserved function in innate immunity, hepcidin also serves as a modulator of iron homeostasis (Courselaud *et al.* 2002, Lee *et al.* 2005). Hepcidin expression in mammalian liver is strongly induced by iron excess (Pigeon *et al.* 2001), and strongly suppressed by anemia and hypoxia (Nicolas *et al.* 2002). Therefore, the suppressed hepcidin expression observed in GH transgenic coho salmon liver regardless of nutritional status suggests that these fish should have compromised innate immunity, as well as altered iron homeostasis, specifically increased iron availability for hemoglobin biosynthesis. Challenge studies have indicated that the strain of GH transgenic salmon used in the present study do indeed have reduced resistance to bacterial infections (Jhingan *et al.* 2003). Hemoglobin α and β are two of the ten genes found to be induced by GH transgenesis (T and R>C; Fig. 4B). The induction of these hemoglobin genes in GH transgenic liver was confirmed using all three

microarray platforms (Table 1). Increased expression of hemoglobin genes in R and T relative to C liver tissues indicates a need for additional hemoglobin in the transgenic fish, perhaps due to the higher metabolic rate required for enhanced growth. Increased hemoglobin levels would also result from the increased activation of hemoglobin promoters by upregulated Kruppel-like globin gene activator, and the decreased degradation of existing hemoglobin by downregulated heme oxygenase (Table 1). Furthermore, it is possible that GH transgenic liver may be more vascularized than control liver, resulting in increased numbers of erythrocytes and thus increased levels of hemoglobin transcripts. Analyses of cDNA sequences upstream of mammalian (Pigeon *et al.* 2001) and bass (Shike *et al.* 2002) hepcidin transcriptional start sites identified putative-binding sites for the transcription factors C/EBP β , hepatocyte nuclear factor (HNF), and nuclear factor- κ B. Interestingly, the 3·5K GRASP study found that hepcidin, C/EBP δ , HNF-3 β , and inhibitor of nuclear factor κ B α (I κ B α) were all reproducibly greater than twofold suppressed in T liver relative to C liver (See Supplementary data, Table S3). Using microarray experiments on aortic RNA samples of Hx rats treated with saline or GH, Tivesten *et al.* (2004) found that I κ B α was 54% downregulated by GH. We saw a 3·6-fold downregulation of coho salmon I κ B α in T liver relative to C liver (See Supplementary data, Table S3).

The 3·5K GRASP study found that a coho salmon transcript similar to the Forkhead box/winged helix (Fox) transcription factor HNF-3 β (Foxa2) was suppressed in T relative to C liver (See Supplementary data, Table S3). HNF-3 β has not been identified previously in salmonids, and this EST displays amino acid identity values ranging from 81 to 85% with orthologs from zebrafish (*Danio rerio*), Mozambique tilapia (*Oreochromis mossambicus*), and Japanese medaka (*Oryzias latipes*). HNF-3 β orthologs serve various roles in vertebrate embryogenesis (Nishizaki *et al.* 2001, Suri *et al.* 2004), and mammalian Fox transcription factors expressed in liver play important roles in growth control (Foucher *et al.* 2002). The GH/insulin-like growth factor-I (IGF-I) system plays a key role in mammalian postnatal growth (Lupu *et al.* 2001). GH stimulates the liver to secrete IGF-I, which mediates GH functions (Liu & LeRoith 1999). Transgenic mice overexpressing HNF-3 β in hepatocytes exhibited dramatic postnatal growth retardation, likely due to upregulation of insulin-like growth factor-binding protein (IGFBP)-I, which could sequester functional IGF-I (Rausa *et al.* 2000). Conversely, suppressed HNF-3 β expression in T liver could lower levels of circulating IGFBP, and enhance growth by increasing IGF-I bioavailability.

Iron–sulfur cluster assembly protein

A transcript with 79% amino acid similarity to iron–sulfur cluster assembly U2 (IscU2) from human was suppressed in T and R relative to C liver in fish of year class 1 (Table 1; Fig. 3A). *IscU* genes encode highly conserved proteins that bind iron and participate in iron–sulfur (Fe–S) cluster biosynthesis in prokaryotic and eukaryotic cells (Hwang *et al.* 1996, Zheng *et al.* 1998). Eukaryotic Fe–S proteins (requiring Fe–S clusters for function) are found in the mitochondria, cytosol, and nucleus (Gerber *et al.* 2004), and perform important roles in several cellular processes including electron transport, catalysis, DNA repair, and regulation of gene expression (Ramelot *et al.* 2004). In mammals, most Fe–S proteins are located in the mitochondria (Tong & Rouault 2000). Humans have two *IscU* splice variants: *IscU1* localizes to the cytosol, and *IscU2* localizes to the mitochondria (Tong & Rouault 2000). While it is known that yeast *IscU* mutations cause iron overload in mitochondria (Garland *et al.* 1999, Schilke *et al.* 1999), the precise role played by *IscU* proteins in iron homeostasis is unknown (Tong & Rouault 2000). The downregulation of *IscU2* in GH transgenic coho salmon liver could result in decreased levels of intracellular free iron, potentially leading to the formation of free radicals or increased levels of extracellular free iron that would be available for hemoglobin biosynthesis.

Energy metabolism

GH transgenic coho salmon have enhanced feed intake and specific growth rates (Devlin *et al.* 2004a,b) indicating that their metabolic machinery must be able to handle the increased flux of energy and nutrients through different pathways. Initially, at the organ level, this is achieved at least in part by enhancement of gut surface area (Stevens & Devlin 2000, 2005) to allow increased uptake of nutrients. GH is known to be a hormone that affects metabolism of carbohydrates, lipids, and protein in vertebrates (Higgs *et al.* 1975, Harvey *et al.* 1995, Fauconneau *et al.* 1996, Cook *et al.* 2000a,b), and consequently it is reasonable to anticipate changes in the levels of enzymes involved in these processes in growth-accelerated GH transgenic salmon. Remarkably, relatively few changes in the expression of genes encoding these enzymes have been detected.

Carbohydrate metabolism

The 16K GRASP microarray contains genes for all 11 enzymes involved in glycolysis, five of six genes for enzymes in the TCA cycle, and three of six enzymes involved in glycogen and galactose metabolism. Yet,

among these genes, none was found to change greater than twofold in expression level on any of the three microarray sets nor among any treatment groups, suggesting that if carbohydrate metabolism is being altered (with the exception of the pentose phosphate pathway (PPP) – see below), this is being accomplished primarily by mechanisms other than those that influence transcript abundance.

The PPP

Genes encoding three enzymes in the PPP, transaldolase, phosphogluconate dehydrogenase, and transketolase, were dysregulated in GH transgenic liver samples (Table 2; Figs 3C and D and 4A and B). The PPP consists of two branches: a non-oxidative branch with reversible reactions, and an oxidative branch with irreversible reactions. Transaldolase is thought to be the rate-limiting enzyme in the non-oxidative branch (Puskas *et al.* 2000, Grossmann *et al.* 2004), which contains several reactions catalyzed by transketolase. Phosphogluconate dehydrogenase is in the oxidative branch, which has glucose 6-phosphate dehydrogenase as the rate-limiting enzyme (Puskas *et al.* 2000). The two branches of the PPP are functionally connected, contributing to the generation of NADPH and thereby supplying reducing power needed for cell growth and proliferation (Barroso *et al.* 1998, Puskas *et al.* 2000). NADPH is also required for synthesis of reduced glutathione (GSH), which protects cells from the damaging effects of reactive oxygen intermediates (Banki *et al.* 1996). The PPP is, therefore, an important component of cellular defense against oxidative stress (Grossmann *et al.* 2004). The PPP also plays an important role in the production of D-ribose, a key component required for nucleic acid biosynthesis during growth.

Microarray and QPCR data on tissues from fish of year class 1 showed that both transaldolase and phosphogluconate dehydrogenase were downregulated in R and upregulated in T relative to C (or $T > C > R$, the same gene expression profile as GABARAP; Fig. 3C and D). Phosphogluconate dehydrogenase was in the $R < T$ gene lists of both the 3·5K and the 16K GRASP studies (Table 2; Fig. 4A). Collectively, the data show that transaldolase and phosphogluconate dehydrogenase were indirectly induced by growth ($T > C$ and R) and also suppressed by ration restriction ($R < C$ and T). The relatively low T versus C and R versus C fold differences (Fig. 3C and D), along with the stringent criteria for inclusion in the informative transcript lists arising from GRASP microarray studies (i.e. must be greater than twofold different in all slides of a study) likely contributed to the absence of transaldolase and phosphogluconate dehydrogenase in Fig. 4B. Transketolase is present in the $T < C$ and $R < C$ lists from the 16K GRASP study (Table 2), and it is, therefore, inferred that this gene is suppressed by GH transgenesis (T and $R < C$; Fig. 4B).

Enhanced growth in GH transgenic coho salmon is accompanied by hepatic gene expression profiles indicating increased mitochondrial activity, particularly in T fish (See Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>, Tables S4 and S6), which would result in increased levels of reactive oxygen intermediates. Cultured mammalian lymphocytes respond to treatment with the pro-oxidant dehydroascorbate with increased activities of PPP enzymes (transaldolase, glucose 6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase), increased intracellular GSH, and increased resistance to apoptosis (Puskas *et al.* 2000). In a similar fashion, the induction of transaldolase and phosphogluconate dehydrogenase in T liver could stimulate the PPP to produce the additional NADPH needed to maintain adequate GSH to mitigate cellular damage caused by oxidative stress. However, reductions in transketolase activity as seen in T salmon may be a strategy to ensure that sufficient D-ribose is available for nucleic acid synthesis rather than shifting its conversion to glyceraldehyde-3-phosphate for glycolysis, a pathway which does not appear to be modulated in transgenic salmon (see above). The 3·5K GRASP study identified five different genes involved in mitochondrial electron transport as being induced in T liver relative to C liver compared with one induced mitochondrial gene in R relative to C (Table 3), suggesting that mitochondrial activity and levels of reactive oxygen intermediates would be higher in T than in R or C liver samples. Still, significantly increased growth rates in R relative to C fish (Fig. 1) and elevated cytochrome *c* transcript levels in R relative to C liver (Table 3) suggest that R fish cells may experience increased oxidative stress relative to controls. Both branches of the PPP appear to be suppressed in R liver relative to C and T liver (Fig. 3C and D). Inhibition of the PPP in these fish could result in decreased NADPH and GSH levels, making R tissues more susceptible to oxidative injury. Interestingly, starvation causes decreased 6-phosphogluconate dehydrogenase activity in trout liver (Barroso *et al.* 1998), and decreased transaldolase activity in rat liver (Heinrich *et al.* 1976). We speculate that suppression of PPP genes in R liver may suggest that these fish were in a physiological state approaching starvation. Calorie-restriction in ration-fed GH transgenic coho salmon would likely have physiological consequences such as depressed immune function. The impact of rationing on the global gene expression and health of GH transgenic salmon warrants further investigation.

Amino acid metabolism

More than 20 genes encoding enzymes involved in amino acid metabolism are represented on the 16K GRASP microarray. Glutamate-pyruvate transaminase,

an enzyme involved in metabolism of amino groups collected from amino acids, was found to be moderately reduced in transgenic salmon, possibly to reduce catabolism of amino acids to ensure sufficient supplies are available to support protein synthesis. Another enzyme gene, 3-phosphoglycerate dehydrogenase, was found to be greater than eightfold stimulated in R salmon relative to C or T salmon. This enzyme utilizes a substrate from within the glycolysis pathway to synthesize two non-essential amino acids, serine and glycine. R salmon are provided with insufficient dietary energy relative to T and C salmon, and consequently enhanced synthesis of certain amino acids may be critical to maintain cellular functions.

Lipid, cholesterol, and prostaglandin metabolism

GH is well known to enhance lipid catabolism in many vertebrate systems, including fish (Higgs *et al.* 1975, Harvey *et al.* 1995). The microarray platforms used in the present study contain at least 14 genes involved in lipid and cholesterol metabolism. Among these, glycerol-3-phosphate dehydrogenase is induced in both R and T salmon, consistent with hormonal modulation of lipid metabolism. Prostaglandin D-synthase and δ -6 fatty acyl desaturase were also induced in transgenic salmon, indicating that additional effects of lipid and prostaglandin metabolism are occurring in transgenic salmon. Fatty acid amide synthetase and hydrolase were both suppressed in transgenic salmon, suggesting modification of fatty acid amide signaling molecules. Of interest is the enhancement of the δ -6 fatty acyl desaturase gene in T salmon relative to C and R salmon liver. This encodes activity important for bioconversion of linolenic acid (18:3 n -3) to eicosapentaenoic acid (EPA; 20:5 n -3; Hastings *et al.* 2004). EPA is an important member of the family of omega-3 highly unsaturated fatty acids, which provide significant health benefits to humans consuming fish oil products.

Mitochondrial function

Whereas basal oxygen utilization rates in GH transgenic salmon are not enhanced, metabolic rates following feeding are significantly elevated due to enhanced specific dynamic action (Leggatt *et al.* 2003) suggesting that overall oxygen consumption and energy transport must be enhanced in GH transgenic salmon to meet the demands of accelerated growth. Several genes involved in mitochondrial electron transport (cytochrome *c* oxidase polypeptides VIB and VIII, cytochrome *c*, ubiquinol-cytochrome *c* reductase hinge protein, and ubiquinol-cytochrome *c* reductase core protein 2) are induced in T relative to C liver (Table 3). Cytochrome *c* is also present in the 3·5K GRASP R>C transcript list (See

Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>, Table S6), and is therefore classified as a gene induced by GH transgenesis (T and R>C; Fig. 4B). Flores-Morales *et al.* (2001) found that cytochrome *b5* expression was suppressed in Hx rat livers relative to control rat livers, and induced in GH-treated Hx rat livers relative to untreated Hx rat livers. In addition, several other cytochrome genes were down-regulated in Hx rat livers relative to normal livers (Flores-Morales *et al.* 2001). GH probably influences mitochondrial function in both mammalian and salmonid liver cells due to the increased metabolic demand.

Cellular proliferation and stress response in GH transgenic liver

Several genes dysregulated in GH transgenic coho salmon liver cells are involved in cell proliferation. Multiple microarray features identified as PCNA were greater than twofold upregulated in T relative to C liver (Table 4; See Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>, Table S4). PCNA plays various roles in DNA replication, DNA repair, chromatin remodeling, and cell-cycle control (Maga & Hübscher 2003). Other genes involved in cell-cycle regulation (non-histone chromosome protein 2-like) and chromatin organization (histone H2A family member Z and histone H5) are also upregulated in GH transgenic liver (Table 4). Histone H2A family member Z has various functions in transcriptional regulation and maintenance of genomic stability (Farris *et al.* 2005). Therefore, its induction in growth-enhanced salmon liver may be a part of a mechanism by which GH transgenic cells control genomic fidelity in a hyper-proliferative state.

We observed gene expression changes co-occurring with GH transgenesis which indicate that the transgenic liver is under stress. The 3·5K GRASP study identifies glucose-regulated protein 78 (GRP78) as being upregulated in T and R liver relative to C liver (Table 4). It is, therefore, inferred that GRP78 is induced by GH transgenesis (T and R>C; Fig. 4B). GRPs are endoplasmic reticulum stress-associated proteins that protect cells against damage during changing environmental conditions (Ko *et al.* 2002). GRP genes are induced by various stresses that disrupt endoplasmic reticulum (ER) function (Kaufman 1999). The GRP78 gene is induced in cultured chicken and mammalian cells deprived of glucose (Shiu *et al.* 1977, Lin & Lee 1984) or exposed to calcium ionophores (Wu *et al.* 1981). GRP78 is a chaperone that plays a central role in the ER stress response. In the absence of ER stress, transducers of ER stress signaling (e.g. activating transcription factor 6 (ATF6), protein kinase-like ER kinase (PERK)) are kept inactive through interaction with GRP78 (Lee 2005). Conditions causing ER stress

(e.g. glucose deprivation, pathological conditions, or accumulation of misfolded proteins in the ER) release ER stress transducers from GRP78, activating the apoptosis-inducing ER stress signaling (Lee 2005). GRP78 overexpression decreases the ER stress response, protecting stressed cells from apoptosis (Dorner *et al.* 1992, Kaufman 1999). GRP78 also protects ER-stressed cells from death by interfering with caspase activation (Lee 2005). The upregulation of GRP78 in GH transgenic coho salmon liver tissue suggests that the transgenic liver cells experience ER stress and that GRP78 confers cytoprotection by mitigating this stress.

A salmonid transcript similar to nuclear protein p8, a growth-relevant protein responsive to cellular stress and starvation (Malicet *et al.* 2003), was also found to be dysregulated in GH transgenic salmon liver relative to control samples. In the 16K GRASP study, p8 was downregulated in T relative to C liver and R relative to C liver, and upregulated in R relative to T liver (Table 4; C>R>T). Therefore, p8 is inferred to be both suppressed by GH transgenesis (T and R<C) and indirectly suppressed by growth (T<C and R; Fig. 4B). Nuclear protein p8 is involved in cell-cycle regulation, and has been reported to mediate both the inhibition of growth (Vasseur *et al.* 2002) and the promotion of growth (Vasseur *et al.* 1999) in different cell types. A microarray study identified *Drosophila* p8 as being induced by starvation, prompting speculation that p8 might be activated to stop cell growth when nutrients are scarce (Zinke *et al.* 2002). There may be similarities between fly and fish p8 functions, as we observed an inverse relationship between hepatic p8 expression and salmon growth rate (Fig. 1). A recent study using human pancreatic cancer cell lines demonstrated that activation of growth-promoting intracellular pathways (e.g. Raf→MAPK and ERK kinase (MEK)→extracellular signal-regulated kinase (ERK)) suppressed p8 expression, while activation of growth-inhibiting pathways (e.g. p38) upregulated p8 expression (Malicet *et al.* 2003). These findings point to pathways potentially altered in salmon as a result of GH transgenesis, and possible molecular mechanisms involved in enhanced growth in these fish.

Immune-relevant genes affected by GH transgenesis

In addition to hepcidin and prostaglandin D-synthase, several other immune-relevant salmon liver transcripts (e.g. C-type lectin superfamily members, complement components, and precerebellin-like) were potentially responsive to GH transgenesis with or without ration restriction (Table 5). Salmon transcripts encoding C-type lectins were found in R>C transcript lists from the 16K GRASP study (See Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>,

Table S12) and the IMB study (See Supplementary data, Table S16; Fig. 4A). Since a C-type lectin was also present in the 16K GRASP T>C list (See Supplementary data, Table S10) and R<T list (See Supplementary data, Table S13), the expression of at least one gene in the C-type lectin superfamily appears to be T>R>C. Based on their presence in multiple informative transcript lists across the GRASP and IMB studies, C-type lectins appear to be both induced by transgenesis (T and R>C) and indirectly induced by growth (T>C and R; Fig. 4B). C-type lectin-like transcripts, identified in subtractive cDNA libraries enriched for salmon hepatic genes induced by *A. salmonicida* infection, are thought to play a role in immunity or inflammation (Ewart *et al.* 2005). Other immune-relevant salmonid liver genes potentially responsive to GH transgenesis with or without ration restriction include complement components C7 (T<C and R<T), C4 (T<C), and C3-3 and C3-4 (R<C), as well as tachylectin (R<C), and precerebellin-like (T<C and R<C; Table 5). The upregulation of C-type lectin, and downregulation of several other acute phase reactants in R and T samples, suggest that GH transgenic coho salmon may have altered innate immune function, consistent with studies showing that this strain has reduced resistance to bacterial infections (Jhingan *et al.* 2003).

GH signaling

Several of the components of the signaling pathway by which GH mediates its effects on gene expression (Moutoussamy *et al.* 1998) are present on the GRASP microarrays and provided strong enough signals to yield reliable assessments of expression, although most differed by less than twofold between transgenic and control samples and thus did not meet the criteria used to identify differentially expressed genes as described above. For example, GH receptor was induced in T and R samples (1.15- and 1.73-fold respectively) relative to C, consistent with the known stimulatory effect of GH on its own receptor's transcription in GH transgenic mice (Orion *et al.* 1991). Protein kinase C, which plays a regulatory role in mediating GH effects on cellular processes (Moutoussamy *et al.* 1998), was found to be induced by more than twofold in transgenic salmon on the 3.5K GRASP microarray (see tables S6a and S6b in Supplementary data at <http://jme.endocrinology-journals.org/content/vol37/issue2>). A transcript with similarity to rainbow trout signal transducer and activator of transcription (STAT)-1 appeared to be reduced in both fully fed and ration-restricted transgenic salmon to an average of 0.53 of the level found in controls. Similarly, mitogen-activated protein kinase (MAPK) (which can be activated by the cytoplasmic domain of the GH receptor) is also reduced in GH transgenic salmon relative to controls (to 0.51 in fully fed and to 0.83 in ration-restricted transgenics).

Thus, some effects on signaling pathway components beyond the GH receptor appear to be in directions which would lead to reduction of GH signaling strength, which may indicate the presence of feedback mechanisms, which are operating to attenuate signaling and from chronic overexpression of this hormone (e.g. see Miquet *et al.* 2004). For example, one target of GH signaling, IGF-I, was not found to be induced in any of the transgenic samples examined (range 0.61–1.02-fold). However, in general, effects on signal transduction pathways appear to be quite subtle, indicating that regulation may arise from the cumulative effects of many small changes, rather than large effects on any one regulatory component.

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