Molecular analysis and growth evaluation of northern pike (*Esox lucius*) microinjected with growth hormone genes

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ABSTRACT


Bovine (bGH) or chinook salmon (Oncorhynchus tshawytscha) growth hormone (csGH) cDNA genes were transferred by microinjection into newly fertilized northern pike (*Esox lucius*) eggs. Non-lethal screening of fin tissue showed genomic integration of transgenes in 88 of 1398 putative transgenic fish. Expression of bGH transgenes under transcriptional control of the Rous sarcoma virus long terminal repeat was detected in 36 of 1218 putative transgenic fish examined by radioimmunoassay of blood serum. Bovine growth hormone was also detected in mesodermal tissue of fins from microinjected fish using thin slice immunohistochemistry. Southern hybridizations of six tissues from a sample of 40 microinjected individuals revealed a high degree of mosaicism, with 30% of the fish containing detectable transgenic DNA in one or more tissues and only 41% of these containing detectable transgenes in fins.

Growth of microinjected fish was quantitatively evaluated in three experiments. Average weight of microinjected fish was greater than that of controls of the same sex in four out of six groups. Significant growth enhancement (*P<0.05*) was detected only for microinjected males in one experiment. Comparisons among molecular assays and individual fish growth in the founder generation indicated that the high degree of mosaicism prevented non-lethal identification of all transgenic individuals and influences detection of growth enhancement.

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*Authors contributed equally; order was determined by a coin toss.
INTRODUCTION

The creation of growth-enhanced mice using gene transfer (Palmiter et al., 1982) has sparked interest in applying these techniques to improve traits in fish. While the classical method of improving traits is to develop a genetic selection program, the application of gene transfer has the potential to further enhance traits such as growth, disease resistance, and feed efficiency. Gene transfer experiments in fish have concentrated on growth enhancement using growth hormone genes (Guise et al., 1990; Kapuscinski and Hallerman, 1990). In some mammals containing growth hormone transgenes, increased growth hormone levels have caused deleterious perturbations in their endocrine balance resulting in serious side effects. For example, uncontrolled expression of growth hormone in pigs produced developmental problems that ranged from diabetes and infertility to lethality (Pursel et al., 1989). Fish, however, may have the greatest promise for the action of transferred growth hormone genes since their rate of growth has been shown to be highly responsive to injections of crude, purified, or recombinant-derived growth hormone protein (Adelman, 1977; Agellon and Chen, 1986; Weatherly and Gill, 1987a,b).

Landings from the world's fisheries are rapidly approaching the predicted maximum sustainable yield for natural waters. This trend has contributed to the increasing importance of aquaculture production (Stickney, 1990). Because many aquaculture operations utilize wild or recently domesticated stocks, genetic improvements could potentially be financially beneficial. Several research groups have successfully introduced growth hormone genes into fish (Zhu et al., 1985; Chourrout et al., 1986; Dunham et al., 1987; Rokkones et al., 1989) with a few noting increased growth in the treated group (Zhu et al., 1986; Zhang et al., 1990). The latter reports, however, presented limited information about rearing conditions and statistical analyses, making it difficult to quantitatively evaluate conclusions about the observed growth enhancement. In conjunction with molecular analyses, we therefore initiated growth studies under carefully controlled rearing conditions to quantitatively evaluate the performance of transgenic fish containing extra growth hormone genes.

MATERIALS AND METHODS

DNA constructs

A fish expression vector (FV), FV-2/csGH, was constructed by inserting the fragment containing chinook salmon (Oncorhynchus tshawytscha) growth hormone cDNA (Hew et al., 1989) into the EcoRI site of pHIN/Nco (Liu et al., 1990a). Thus, FV-2/csGH contains the proximal promoter and enhancer regulatory elements of the common carp (Cyprinus carpio) β-actin gene. To
Fig. 1. Construction of FV-2/csGH. Restriction sites are as indicated.
Isolation of 330bp Hinf I fragment containing one copy of the LTR

Clone into Hind III site of pUC119

Clone the complete copy of bGH cDNA (829bp) into pUC119

Clone the isolated Hind III fragment of LTR (343bp) into the Hind III site of pUCbGH

Figure Legend:
B, BamH I; E, EcoR I; H, Hind III; Hf, Hinf I; K, Kpn I; P, Pst I; S, Sst I; Sa, Sal I; Sm, Sma I; Sp, Sph I; X, Xba I

bGH LTR of RSV vector sequences

Fig. 2. Construction of pRSV/bGH. Restriction sites are as indicated.
insert the cDNA fragment into the EcoRI site of pHin/Nco, the clone that harbors the cDNA for csGH was partially digested with HindIII, filled-in with Klenow fragment DNA polymerase I, ligated to EcoRI linkers and digested with EcoRI endonuclease (Fig. 1).

To construct pRSV/bGH, the 0.83 Kb cDNA of bovine growth hormone was cloned into the PstI site of pUC119 by partial digestion of pBH27 (provided by Hank George, Molecular Genetics Inc., Edina, MN) with PstI to produce pUCbGH. The Rous Sarcoma virus (RSV) promoter contained within a 0.33 Kb HindIII fragment (Yoon et al., 1990) was blunted with the Klenow fragment of polymerase I. HindIII linkers were added and this fragment was cloned into the HindIII site of pUCbGH to yield pRSV/bGH (Fig. 2).

Gamete collection, fertilization, and microinjection

Northern pike (Esox lucius) gametes were collected from wild individuals and transported at 4°C to the lab. Eggs from 2–3 females were mixed and fertilized with sperm from 2–3 males. A portion of the fertilized eggs was randomly allocated for controls. Twenty minutes after fertilization, a smoothly tapered borosilicate needle with an inner tip diameter of approximately 2 μm (Yoon et al., 1990) was used to microinject approximately 50 picoliters of linearized DNA solution into the germinal disc directly through the chorion and vitelline membranes. The plasmid pRSV/bGH was linearized with the restriction endonuclease KpnI, extracted with phenol/chloroform, precipitated in ethanol, and re-dissolved in 88 mM NaCl, 10 mM Tris·HCl, pH 7.6 to a final concentration of 25 ng/μl. The plasmid FV-2/csGH was microinjected in the superhelical form at the same final concentration. Microinjection proceeded under a stereo microscope with the borosilicate needle attached to a Brinkman MM33 micromanipulator and the amount of plasmid solution injected was controlled by a Medical Systems Corporation pico-injector (Model PLI-100).

DNA analysis

The physical state of the transgene DNA was characterized by Southern blot hybridization analysis, as described by Hallerman et al. (1990). DNA was isolated from individual 6-day-old embryos or juvenile fish fin tissues by sodium dodecyl sulfate lysis (SDS) and proteinase K digestion. DNA (7 μg) was digested with excess BamHI restriction endonuclease, electrophoresed through a 0.8% agarose gel, transferred onto nylon membranes by Southern blotting and then probed for the presence of transgenic DNA. The entire plasmid, either pRSV/bGH or FV-2/csGH, was labeled with 32P by the random priming method.

For two-dimensional gel electrophoresis, uncut and cellular DNA was electrophoresed first in 0.4% agarose and then at right angles in 1.2% agarose,
transferred to nylon membranes, and hybridized to labeled pRSV/bGH DNA (Manias et al., 1989). This method separates open circular and superhelical extra-chromosomal transgene DNA, both unit length and multimers, from linear cellular and integrated transgene DNA (Manias et al., 1989).

**Growth hormone radioimmunoassay**

Blood serum samples collected from dorsal aortae of 2-month-old fish were analyzed for the presence of bGH protein by radioimmunoassay. Because the pRSV/bGH construct included the signal peptide sequence, detection of bGH protein was expected. Radioiodinated bGH was prepared by adding four iodo-Beads (Pierce Chemical Co., Rockford, IL) to a vial containing 1.5 mCi$^{125}$I and 0.2 ml buffer (0.25 $M$ Na$_3$PO$_4$ pH 7.5). After 5 min, 15 $\mu$g bovine growth hormone (USDA) solubilized in 0.01 $M$ NaHCO$_3$ diluted with 20 $\mu$l of buffer was added and incubated for 15 min. The mixture was purified by column chromatography using Bio-gel P-30 (Bio-Rad) equilibrated and eluted with 0.1% bovine serum albumin (BSA)–barbitol buffer pH (Sigma) as three peaks. The middle peak was diluted in 1% BSA–0.01 $M$ phosphate-buffered saline (PBS) pH 7.5. Anti-serum to ovine GH (NIAMDD-anti-oGH) was diluted1:5000 in 1% normal rabbit serum–0.05 $M$ EDTA-PBS pH 7.0, and 0.1 ml dispensed into assay tubes containing 0.3 ml 1% egg albumin–PBS pH 7.0 and 0.3 ml test plasma. After 24 h, 0.1 ml tracer (20 000 cpm) was added, followed 24 h later by the addition of 0.1 ml precipitating antiserum (sheep anti-rabbit gamma globulin) and incubation for 48 h. Samples were centrifuged at 15 000 $\times$ g for 20 min and counted in a Beckman gamma counter. Assay sensitivity was approximately 1 ng/tube, equivalent to 1.1 ng/ml (Wheaton et al., 1986).

**Immunohistochemical detection of tissues expressing bGH**

Fin tissue collected from individual fish in the microinjected groups was fixed, sectioned, and mounted on slides using standard clinical techniques. Slides were deparafinized by a 30 min room temperature dip in xylol, followed by two 5 min dips in 100%, 90%, and 70% ethanol. Slides were washed three times in PBS pH 7.6 for 10 min, dipped in a 0.3% H$_2$O$_2$ in PBS for 30 min, and rewashed three times in PBS for 5 min. Blocking was by incubation in a 1 $\times$ dilution of normal sheep serum for 30 min at 37°C, followed by a gentle wiping. Slides were incubated in polyclonal rabbit anti-bGH diluted 1:100 in normal sheep serum overnight at 4°C, washed three times in PBS for 10 min, and incubated with goat anti-rabbit Ig diluted 1:2000 in normal sheep serum for 30 min at room temperature. Slides were washed three times in PBS for 10 min, and incubated with the DAB (diaminobenzidine tetrahydrochloride) substrate (5 mg DAB in 20 ml 0.03% H$_2$O$_2$ in PBS) for 10 min at room temperature in the dark. Slides were washed three times in PBS, counterstained in 0.15% methylene blue for 10 min, washed twice in tap water,
dehydrated by a series of dips in 70%, 95%, and 100% ethanol, dipped in xylol twice, and mounted with Permount.

Fish culture

While optimum values for most culture variables have not been determined for northern pike, our culture methods were based on known requirements of the species and empirical data (Westers, 1986; Coolwater Fish Culture Workshop Minutes, 1988, 1989, 1990). We set conservative values for maximum density index, minimum dissolved oxygen tension and maximum ammonia concentrations by using recommendations for rainbow trout (*Oncorhynchus mykiss*), a species requiring relatively high water quality (Meade, 1985; Piper et al., 1982; Soderberg, 1982).

Incubation and hatching. Microinjected and control embryos were separately incubated in divided compartments of a flow-through vertical tray incubator. Embryos were incubated at 10°C until hatching (approximately 9 days). Immediately before hatching, embryos were placed in plastic pans in which the water was allowed to warm to room temperature, thereby reducing variability in fry size by synchronizing hatching of all embryos within an hour.

Fry rearing. Microinjected and control fry were separately reared in 210-l circular tanks at similar densities, ranging from 10 fry/l at hatching to less than 1 fry/l (or 15 g/l) at 2 months of age. Water temperature and flows were kept constant at 21°C and one exchange per hour, respectively (Westers, 1986). After fry swim-up (approximately 3 days), automatic feeders delivered brine shrimp at 5-min intervals during 24 h of light. After 10 days, fry were converted to BioDiet pelleted feed (size #2; BioProducts Inc.), which was delivered by automatic Louden trough feeders (North Star Co.) at frequent intervals during light hours. Dual feeding of brine shrimp and BioDiet usually lasted 3 days.

Rearing after 2 months of age. Water temperatures, flow rates, and delivery of pelleted feed were as described for fry rearing. Densities ranged from 15 g/l when average fish length was 12 cm to 95 g/l when average fish length was 30 cm. Photoperiod was 14 h light: 10 h dark and feed type Glencoe Trout Grower pellets (International Multifoods). When individual weights averaged 15 g, microinjected and control fish of an experiment were tagged intraperitoneally with passive integrated transponders (Biosonics Corp.; Prentice et al., 1987) which permit individual identification. All microinjected and control individuals in a given growth experiment were then transferred to a common rearing tank.
TABLE I

Percent hatch and numbers of fish retained for growth experiments

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Construct</th>
<th>Percent hatch</th>
<th></th>
<th>No. males analyzed</th>
<th></th>
<th>No. females analyzed</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Injected</td>
<td>Control</td>
<td></td>
<td>Injected</td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>pRSV/bGH</td>
<td>62</td>
<td>58</td>
<td>22</td>
<td>7</td>
<td>24</td>
<td>9</td>
</tr>
<tr>
<td>B</td>
<td>pRSV/bGH</td>
<td>63</td>
<td>78</td>
<td>38</td>
<td>17</td>
<td>98</td>
<td>26</td>
</tr>
<tr>
<td>C</td>
<td>FV-2/csGH</td>
<td>66</td>
<td>75</td>
<td>61</td>
<td>7</td>
<td>67</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Totals</td>
<td>64*</td>
<td>70*</td>
<td>121</td>
<td>31</td>
<td>189</td>
<td>59</td>
</tr>
</tbody>
</table>

*Average value.

Growth analysis

A randomly chosen subset of the molecularly analyzed fish was compared to controls in three growth experiments (Table 1). Individual weights (to the nearest 0.1 g) and lengths (to the nearest 1.0 mm) were recorded on a monthly basis. As fish gained weight and space became limiting, numbers of fish in each experiment were reduced to maintain optimum rearing conditions. To cull fish, a computer-generated random number was assigned to each individual within the control or microinjected group in a common tank, the numbers were sorted and the appropriate numbers of fish were eliminated from the top of the list for the group.

Because females are usually larger than males and skewed sex ratios are known to exist in populations of northern pike (Hassler, 1969; Scott and Crossman, 1985), growth data were analyzed by sex. At 1 year of age, fish were sexed based on the shape of the urogenital opening (Demchenko, 1963; Casselman, 1974). Our ability to determine sex in this manner was verified by dissection of 100 fish. Weight differences between treatments of the same sex were analyzed by t-tests at approximately 6, 9, and 12 months of age. F-statistics were calculated to test the equality of variances and determine whether pooled or separate variance t-tests applied (Zar, 1984).

RESULTS

By microinjection into the germinal disc of one-cell northern pike embryos, we obtained successful transfer and genomic integration of cDNA for either: bovine growth hormone linked to the Rous sarcoma virus long terminal repeat (pRSV/bGH; Fig. 2); or chinook salmon growth hormone linked to the carp β-actin promoter (FV-2/csGH; Fig. 1). For some fish microinjected with pRSV/bGH, we detected expression of bGH in blood serum and growth enhancement under controlled growth trials. In earlier studies (Liu et al., 1990a),
we demonstrated expression of transgenes in fish under the transcriptional control of the carp $\beta$-actin promoter.

\textit{Production of transgenic northern pike}

For the three growth experiments (Table 1), a total of 14,000 one-cell northern pike embryos was microinjected. Hatching rates for both control (58-78\%) and microinjected (62-66\%) groups were normal for artificial incubation of this species (Westers, 1986). Of the surviving microinjected fish, 440 were randomly chosen for growth analyses (Table 2). In addition to these growth experiments, another 10,000 embryos were microinjected, of which 958 juvenile fish were molecularly analyzed (Table 2).

\textit{DNA analysis}

As shown in Fig. 3A, uncut genomic DNA from 6-day-old pRSV/bGH microinjected embryos appeared to be in two forms: linear copies (3.9 Kb) and high molecular weight DNA. The latter form, which represents the majority of the DNA, migrated as two separate bands: a band ($\sim$23 Kb) representing large concatamers of the injected DNA; and a band larger than 40 Kb that migrated with cellular DNA and represents integrated transgenic DNA. In the lane for embryonic DNA digested with \textit{BamHI} several prominent bands exist: (1) the 3.9 Kb band represents linear copies of pRSV/bGH derived from digestion of both integrated and concatamer forms as well as the free linear copies present in the uncut lane; (2) the two faint high molecular weight bands most likely represent trimers and larger concatamers that have lost the \textit{BamHI} site during concatamerization; (3) the unexplainable 2.2 Kb band was found in all 6-day-old microinjected embryos analyzed but not in control embryos (data not shown). The exact nature of the band is unknown at this point, but probably represents a common deletion product of the microinjected DNA.

\begin{table}
\centering
\caption{Numbers of presumptive juvenile transgenic fish molecularly analyzed and numbers in which transgene DNA or protein product (bGH) was detected}
\begin{tabular}{lcccc}
\hline
Expt.$^a$ & Fish analyzed & DNA fin positive & Serum bGH \\
\hline
A & 52 & 6 (11\%) & nd \\
B & 260 & 29 (11\%) & 7 (3\%) \\
C & 128 & 9 (7\%) & nd \\
D$^b$ & 958 & 44 (5\%) & 29 (3\%) \\
\hline
Total & 1398 & 88 (6\%) & 36 (3\%)$^c$ \\
\hline
\end{tabular}
\end{table}

$^a$DNA constructs used are described in Table 1.
$^b$These fish were microinjected with pRSV/bGH but were not part of a growth experiment.
$^c$Represents 3\% of total for experiments B and D (36/1218).
\textit{nd} = not done.
Fig. 3. Southern blot hybridization analysis of DNA from selected northern pike transgenic for pRSV/bGH. (A) 10 μg of either BamHI digested or uncleaved DNA from embryos was electrophoresed and transferred to nylon membranes, then hybridized to a [32P]-labelled probe made from a BamHI/HindIII (0.83 Kb) fragment of pRSV/bGH containing the coding region of bGH. 20 pg of linearized pRSV/bGH cleaved with BamHI is included as a control. Lambda DNA cleaved with HindIII was used for size markers (fragment sizes given in Kb). (B) 5 μg of DNA from juvenile (Juv) northern pike fins were cleaved with EcoRI, electrophoresed, transferred, then hybridized to a [32P] labeled probe made from the entire pRSV/bGH plasmid. Expected band sizes corresponding to EcoRI cleaved pRSV/bGH are 0.96 and 2.9 Kb. HindIII cleaved lambda phage DNA served as size markers (fragment sizes given in Kb). The bands at 2.5 and 2.1 Kb represent vector sequences from a co-microinjected pRSV/CAT plasmid (Hallerman et al., 1990) included in some Juv fish. (C) Two-dimensional gel electrophoresis analysis of uncut genomic DNA from Juv17. Arrow indicates the expected location of episomal and concatamerized forms of DNA if they were present.
Two-month-old juvenile fish were sufficiently large to allow removal of fin tissue without affecting survival. Therefore, to determine which of the surviving microinjected fish were transgenic, DNA was extracted from fin clips and screened for the presence of transgenic DNA by Southern blot hybridization. As shown in Table 2, 5–11% of microinjected fish in experiments A–D and 6% (88) of all surviving juvenile fish contained detectable levels of transgenic DNA within their fin tissue. Unlike embryonic DNA, however, the transgenic DNA in juvenile fish appeared to be only in an integrated form. For example, in Fig. 3A, the pRSV/bGH transgenic sequences in the uncut Juv77 and Juv78 lanes only migrated as high molecular weight DNA. Furthermore, two-dimensional Southern blot hybridization analysis indicated that the transgenic DNA had probably been integrated into the genomes of juvenile fish (Fig. 3C). Transgenic DNA in the form of episomes or large supercoiled concatamers have been observed by other researchers; however, such forms would migrate above (see arrow Fig. 3C) the main bulk of the DNA. The possibility remains that some transgenic DNA is in a very long linear form, although such DNA generally does not have a long half-life in developing organisms. Additionally, dominant bands from 70% of pRSV/bGH transgenic fish DNA’s, when analyzed by Southern hybridization, migrated at the expected band sizes: BamHI linearizes pRSV/bGH and produces a 3.9 Kb fragment; whereas EcoRI digestion gives bands of 0.96 and 2.9 Kb. For example, DNA of Juv78 digested with BamHI produced a dominant band at 3.9 Kb representing linear pRSV/bGH as well as many putative junction fragments (Fig. 3A). In Fig. 3B, all juvenile fish shown were digested with EcoRI and yielded the expected bands at 0.96 and 2.9 Kb. Putative junction fragments are also evident. However, other samples, including Juv77 (Fig. 3A) gave bands upon restriction digestion that were of unexpected sizes, suggesting transgene rearrangement. Transgene copy number per fish ranged from less than one to hundreds of copies.

**bGH assays**

Because non-lethal removal of blood from 2-month-old fish was also possible, blood serum from these same fish was analyzed for the presence of circulating bovine growth hormone (Table 2). Radioimmunoassays detected bGH hormone in the serum of 3% (36) of the tested fish. The majority of these positive fish had levels below 4 ng/ml of circulating bGH hormone within their serum, although nine fish had levels above 10 ng/ml with the highest being 37 ng/ml. Interestingly, 34 of the 36 fish had no detectable transgenic DNA within their fins, implying a high degree of mosaicism within the transgenic fish.

Lack of any positive results with blood serum from control fish indicated that our bGH radioimmunoassay did not cross-react with native northern pike growth hormone. Additional evidence supporting this conclusion came
Fig. 4. Thin slice immunohistochemical analysis of northern pike fins employing a polyclonal rabbit anti-bGH antibody. Panel A shows fin tissue from a control fish. Panel B shows fin tissue from a fish microinjected with pRSV/bGH whose transgene expression is limited to undifferentiated mesenchymal tissue, appearing as dark areas (especially prevalent within the chondrocytes). Abbreviations for tissue types are: (ch) chondrocytes, (epi) epithelial tissue, (ray) fin ray, and (um) undifferentiated mesenchymal tissue.
TABLE 3

The extent of tissue DNA mosaicism in a random sample of northern pike microinjected with pRSV/bGH

<table>
<thead>
<tr>
<th>Fish no.</th>
<th>Brain</th>
<th>Gonad</th>
<th>Kidney</th>
<th>Liver</th>
<th>Muscle</th>
<th>Fin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
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<tr>
<td>16</td>
<td>+</td>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>19</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
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<td>2/12</td>
<td>10/12</td>
<td>6/1</td>
<td>6/12</td>
<td>5/12</td>
</tr>
</tbody>
</table>

% of positive fish

| % of all analyzed fish | 25 | 16 | 83 | 50 | 50 | 41

#12/40 fish were positive in one or more tissues, or 30% overall.

from tests with a chinook salmon GH assay. This assay, which cross-reacted with northern pike blood, showed no cross-reactivity with purified bGH (Brian McKeown, Dept. of Biological Sciences, Simon Fraser University, Burnaby, B.C., personal communication).

**Immunohistochemical detection of bGH in fins**

Two fish, with bGH in their serum but no detectable transgene in their fins, expressed bGH protein within their fins, as detected by a thin slice immunohistochemistry assay employing a polyclonal rabbit anti-bGH antibody (Fig. 4). Expression was restricted to a thin layer of mesodermal tissue within the fin, and was especially prevalent within the chondrocytes. The identical expression pattern was found in similar thin slices from several fish that were positive for both transgene DNA in fin tissue and for bGH in serum. At this time, we do not know whether transgene DNA is present in a percentage of all the various types of fin cells, or only in those cells with observed expression of the hormone.

**Detection of mosaicism**

Because transgenic fish appeared to be highly mosaic, we analyzed six tissues (brain, gonad, kidney, liver, muscle, and fin) from 40 randomly selected
fish for the presence of transgenic DNA by Southern hybridization (Table 3). Thirty percent of the fish from this sample contained detectable transgenic DNA in at least one or more tissues, with the kidney being the most prevalent (83% of positives). Only one fish (No. 27) was found to have DNA present within all examined tissues. Interestingly, transgene DNA was only detectable within fin tissues of 41% of the positive fish (or 12% of all examined fish), indicating our non-lethal screening procedure did not identify all transgenic individuals.

**Growth analyses**

Average weight of microinjected fish was greater than that of control fish of the same sex in four out of six groups (Fig. 5): females and males of experiment A, males of experiment B, and males of experiment C. At this point in
our ongoing study, however, microinjected males in experiment A are the only experimental group whose weights were significantly different ($P < 0.05$) from those of controls (Fig. 5). Length data (not shown) yielded the same trends as weight data in all experiments. Weights of microinjected and control males in experiment A steadily diverged over time (Fig. 6). A pattern also noted in females of experiment A and males of experiment C (data not shown). Similar patterns of divergence in growth have been observed in studies involving injections of bGH into fish (Weatherly and Gill, 1987a; Schulte et al., 1989). Fish positive for either DNA within their fins or bGH expression within their blood were also compared as a group to controls by $t$-tests. Although they followed the same trends as seen for comparisons involving entire microinjected groups, weight differences from these group comparisons were insignificant (data not shown).

In every experiment the largest individuals of either sex were from the microinjected group, totalling 43 fish for the three experiments. Transgene DNA was detected in fin tissue of only three of these 43 fish. If the remainder of these fish are larger due to proper expression of transgenes, they must bear integrated and expressed transgenes in tissues undetectable by nonlethal methods.

DISCUSSION

Using standard techniques of microinjection into one-cell embryos, we obtained integration rates in juvenile fish (5–11% and 6% overall in fin tissue) comparable to those reported for common carp, *C. carpio* (5.5% in fin tissue; Zhang et al., 1990), tilapia, *Oreochromis niloticus* (6% in whole fish samples; Brem et al., 1988), and zebrafish, *Brachydanio rerio* (5% in fin tissue; Stuart et al., 1988). A higher integration rate (20% in whole fish samples) reported for juvenile channel catfish, *Ictalurus punctatus* (Dunham et al., 1987), was based on a sample size of 2 transgenic fish out of 10 microinjected embryos.

Our detection of circulating bGH in 3% of the analyzed microinjected fish indicated transgene expression but involved small numbers of individuals ($\leq 4$ of each sex) within a growth experiment. Because this reduced statistical power, we did not conduct $t$-test comparisons of their growth to that of controls. We do not know if the minimum effective levels of bGH for growth enhancement are below or above the detection threshold of our assay, which was approximately 1 ng/ml. Individual levels of circulating bGH detected by our radioimmunoassays ranged from 2.0 ng/ml to 37.0 ng/ml. Reports of basal levels of endogenous growth hormone for fish include 7.2–58.4 ng/ml for chum salmon, *Oncorhynchus keta* (Bolton et al., 1986), 10–60 ng/ml for goldfish, *Carassius auratus* (Marchant and Peter, 1986), and 10–70 ng/ml for white sucker, *Catostomus commersoni* (Stacey et al., 1984). We also do not know how levels of hormone expression are developmentally regulated.
Given these unknowns, it is possible to miss detection of physiologically active levels of bGH in transgenic individuals showing growth enhancement.

Comparisons among data for integrated transgenes in fin DNA, serum bGH assays, and immunohistochemical detection of bGH in fin tissue indicated a high incidence of mosaic individuals in our founder generation of live transgenic fish. This was confirmed by detection of transgene DNA in at least one of six examined tissues for 30% of 40 sacrificed fish. This is consistent with results of a previous study showing the distribution and expression of a marker transgene in goldfish (Hallerman et al., 1990). Stuart et al. (1988) also detected mosaicism in the founder generation of zebrafish based on the incidence of germ-line transmission and on the distribution of transgenes in eight tissues of one female. Patterns of germ-line transmission for the founder generation of common carp also indicated mosaicism (Zhang et al., 1990), although tissue comparisons of transgene integration and expression within individuals were not reported.

In this study we detected bGH expression in mesodermal tissue of fins and, in a previous report, we localized the prevalence of RSV-LTR-driven expression to mesodermal tissues in transgenic goldfish (Hallerman et al., 1990). Both reports parallel the incidence of tumorigenesis in such tissues following RSV virus infection into chickens and mice (Svet-Modalvsky, 1958; Purchase and Burmester, 1978). The molecular basis of this tissue tropism is not completely understood, but is presumed to be due to interactions between tissue specific transcription factors and the RSV-LTR. These same factors may therefore occur within fish, suggesting their high degree of conservation (Liu et al., 1990b), as well as indicating the value of transgenic fish as a model system for elucidating possible mechanisms in transcriptional control.

Our growth results represent a systematic evaluation of performance of founder generation transgenic fish under controlled rearing conditions. While the largest individuals in each experiment had been microinjected, significant growth enhancement ($P < 0.05$) was observed only for microinjected males in one experiment (Figs. 5 and 6). Detection of statistically significant growth enhancement was influenced by three factors: presence of a mixture of genotypes in the founder generation of presumptive transgenic fish; variation in biological activity of the transgene product; and behavioral interactions.

Each microinjected group analyzed for growth performance was handled as one treatment for statistical analysis (F. Martin, Dept. of Applied Statistics, University of Minnesota, St. Paul, personal communication). However, each group actually consisted of several genotypes to which a given live individual could not be accurately assigned. Possible genotypes include: nontransgenic fish (probably > 50% based on results shown in Table 2 and 3); mosaics which lack proper transgene expression, thus performing like nontransgenics; mosaics which express the transgene in relevant tissues (many of which will be undetectable without sacrificing the fish); and completely transgenic individ-
uals which contain transgene DNA within all their cells and express it in a usable manner. The latter is the desired genotype but it probably occurs in only a small fraction of founder individuals using the current technique of microinjection at the one-cell stage of embryos.

Transgene expression leading to proper physiological activity is believed to require secretion of growth hormone into the bloodstream, whereby it is transported to target tissues and binds to cell surface receptors (Weatherly and Gill, 1987b). Therefore, if some founder generation individuals had expression of transgenes in non-target tissues without secretion into the bloodstream, they would not be expected to display growth enhancement. Lack of expression in some transgenic individuals could result from partial enzymatic degradation of the construct prior to incorporation (Maclean et al., 1987) or from rearrangement of the construct. Our Southern hybridization data indicated rearrangement of integrated transgenes in some founder generation individuals.

At this time, we can only speculate about the impact of behavioral interactions within common rearing tanks on observed growth differences in our experiments. Aggressive behavior of a few individuals has been shown to suppress growth of other fish in common tanks (Magnuson, 1962; Weatherly and Gill, 1987b). Additional experiments are needed to test specific hypotheses about the impact of behavioral interactions on growth differences between fish bearing growth promoting transgenes and controls.

As our fish reach sexual maturity, we are breeding them to produce populations of non-mosaic transgenic fish by germ-line transmission and are screening for progeny containing integrated sequences that are not rearranged. Elimination of mosaic genotypes will allow correct identification of transgenic individuals by non-lethal means, thus reducing ambiguities in assays for GH expression and assessment of growth performance. Current plans are to compare growth of transgenic progeny to controls in common and separate rearing tanks. This will permit partitioning of behavioral effects from direct physiological effects of growth hormone expressed by transgenes.

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