AQUACULTURE CRSP 21ST ANNUAL TECHNICAL REPORT

IGF AS A GROWTH RATE INDICATOR IN OREOCHROMIS NILOTICUS

Tenth Work Plan, Reproduction Control Research 3 (10RCR3)
Final Report

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ABSTRACT

Insulin-like growth factor-I (IGF-I) is considered to be the primary proximal cue for the peripheral signalling of growth reponses in tilapia as in other vertebrates. It is therefore possible that the detection of IGF-1 may be a precise and practical means of determining the growth status of a fish. Our research thus far lays the groundwork for establishment of a method of detecting IGF-1 in the Nile tilapia as a means of assessing growth status, that offers the potential of more precision than other indicators (growth hormone levels, RNA/DNA ratios, etc) and which is potentially far more efficient and cost-effective than measuring fish following an extended period of growout.

We undertook a series of studies to clone the cDNA encoding a portion of the IGF-I gene for Nile tilapia (Oreochromis niloticus) and to examine whether liver IGF-I gene (mRNA) expression differs in fish grown at normal and slow rates. We also examined tissues from tilapia on intensive growth and maintenance diets. This work is the first part of a doctoral program being carried out by Mr. Emmanual Vera Cruz from Central Luzon State University, who is approaching the end of his first year of study. Plans are to complete this work under the coming work plan or work plans.

INTRODUCTION

The proposed program of doctoral research consisted of a focused collaborative effort to clone the Insulin-like Growth Factor (IGF-I) gene for Nile tilapia (*Oreochromis niloticus*), to use this gene to generate one or more probes that can be used in the detection of the gene product, and to conduct a series of preliminary examinations in order to determine the efficacy of this approach as a means of determining instantaneous growth rate in the tilapia.

Studies carried out under the support of the Aquaculture CRSP, including our own studies under Work Plan 10 (10PDVR2), involve the growout of tens of thousands of fish under controlled (on-station) or semi-controlled (farm) conditions. Typically, this approach is used to optimize production parameters such as feeds, fertilization, and other controllable variables. To date, we have carried out a series of on-station and on-farm trials that have led us to conclusions about the cost-effectiveness of feeding strategies (for example, see Bolivar et al., 2001, 2003; Brown et al., 2000). Surprisingly, we have found in the course of these studies that reducing rations by as much as 50% do not impact or barely impact growth and yield. The cost of such studies is considerable. A total of nine farms each grew fish over a period of more than four months, and throughout these studies, tens of thousands of fish must be weighed in order to ascertain the effect of a particular feeding strategy or method on growth. These are exceedingly simple experiments, in which hundreds of man-hours are required to carry out a statistically valid comparison of a single pair of feeding techniques, and we have carried out four such studies over the past two biennia, or an average of one growout study per year.

It is theoretically possible to ascertain growth rate without going through a large-scale, long duration growout process. The proposed approach is to develop a molecular method for detection and quantification of IGF-I, which is activated by the presence of growth hormone and which in turn signals body musculature to grow. Because of its close relationship with growth, IGF-I is gaining increasing acceptance as an indicator of growth rate (Fruchtman et al., 1997). Rapid detection of IGF-I could enable experimenters to screen combinations of variables (temperature, feed content and delivery method, and dissolved oxygen content of the water, for example) that would be exceedingly difficult or practically impossible on a farm-trial scale, since growth rate could be measured without the extensive commitment of resources needed for replicate trials on a large scale. Our efforts to develop, validate, and begin to apply an effective means of detecting IGF-I are closer to the outset than they are to completion, but we have passed some important milestones.

METHODS AND MATERIALS

The first objective was to clone the tilapia (*O. niloticus*) IGF-I

gene. Liver was collected from freshwater *O. niloticus* grown at Florida International University, frozen immediately in liquid nitrogen, and stored at -80° C. The frozen tissues were transported to North Carolina State University (NCSU) for processing in Dr. R. Borski's laboratory, with the participation of personnel from both laboratories.

Total RNA was isolated using the guanidium thiocyanate procedure as described elsewhere. Liver total RNA was further extracted with lithium chloride to remove excess glycogen and other potentially interfering compounds. Oligonucleotide primers based on *O. mossambicus* IGF-I were used to clone the gene in O. niloticus. The outer primers span from within the 5′ promoter region (forward: 5′-TTCTCCAAAACGAGCCTGCG-3′) into the E domain (reverse: 5′-TCTGCTACTAACCTTGGGTGC-3′). First strand cDNA was synthesized from 1 μg total RNA using hot start Taq polymerase commercial reagents (Omniscript, Qiagen) in a total reaction volume of 20 μl. Extension was performed for 60 min at 37ºC. Termination of the reaction occurred by incubation at 94ºC for 5 min.

The minute quantity of cDNA obtained by means of the process outlined above was amplified using the Polymerase Chain Reaction (PCR). A 3 μl sample of the reverse transcription reaction was combined with IGF-I specific primers. The PCR was performed for 30 cycles, each consisting of denaturation at 94ºC for 1 min, annealing at 56ºC for 1 min, and extension at 72ºC for 90 sec, followed by a final extension at 72ºC for 15 min. PCR products were purified (Qiagen, Valencia, CA) and ligated and cloned into pCRII vector (Invitrogen, San Diego, CA). The final product for each gene was sequenced in the forward and reverse directions (NCSU DNA Sequencing & Mapping Facility and University of Chicago Cancer Research Center DNA Sequencing Facility) and compared with known IGF-I sequences using BLAST (Basic Local Alignment Search Tool; National Center for Biotechnology Information).

A single experiment was run in order to carry out a preliminary test of the capability of the IGF-I cDNA to be used as a biological indicator of growth rate in tilapia. For this study, tilapia were grown at Florida International University under three feeding rations. In the preliminary study, Northern Blot analyses were used to determine whether the IGF-I gene expression (mRNA) is elevated in the livers of faster versus slower growing animals. To obtain differential growth in a minimal amount of time, fish were held for one month on either a maintenance feed ration, or on a near-satiation feed ration. Liver samples were collected after one month, frozen on dry ice, and stored at -80 ºC until analyzed by the Northern Blot technique.

Total RNA from liver of normal and slow growing *O. niloticus* was extracted with Trizol reagent and quantified by spectrophotometry at 260 nm. Ten micrograms of sample RNA was run on a 1% formaldehyde agarose gel with buffers and protocols provided in the Northern Max kit (Ambion). RNA was blotted onto positively-charged nylon membranes by upward transfer and then fixed to the membrane by UV crosslinking. Blots were prehybridized at 42ºC for two hours prior to hybridizing overnight using a 32P-CTP labeled IGF-I cDNA. After washing, membranes were exposed for 48 hours using a quantitative phosphorimaging method.

In addition, determinations of total RNA, total DNA, and the RNA:DNA ratio were carried out using a spectrophotometer during the course of nucleotide extraction for Northern Blot analysis.

RESULTS

The *O. niloticus* IGF cDNA corresponds to a 539 bp fragment of the prepro IGF-gene that spans from within the 5′ promoter region into the E domain. The deduced amino acid sequence of *O. niloticus* cDNA was compared with known sequences in other vertebrate species. Identities between *O. niloticus* IGF-I and the corresponding region in fish species including *O. mossambicus* ranged from 75–99%, whereas those between *O. niloticus* IGF-I and nonfish species ranged from around 70–80%. The cDNA encoded a mature IGF-I peptide (consisting of B, C, A, and D domains) of 68 amino acids. The mature peptide was 75–100% identical to human and rat, *Xenopus laevis*, carp, *O. mossambicus*, salmon, gilthead seabream, and flounder. These results taken together provide convincing evidence that the IGF-I gene has been successfully cloned and that the peptide has been conserved.

Growth data from Experiment 1 are summarized in Table 1. In this preliminary study, fry with the same initial size and weight were subjected to different diets. The satiation diet supported rapid growth as indiced by increased total length (not shown) and by terminal body weight. Patterns of RNA: DNA ratio were inconsistent—both the highest and lowest ratios seen were in the slow-growing treatment group.

A 32P-IGF cDNA was used as a probe in the Northern Blot. The IGF-I cDNA insert was excised from the plasmid by restriction enzymes and random primed labelled with 32P-CTP. In our initial trial we obtained an incorporation rate of 14% for the IGF-I probe, which was substantially lower than the 30% seen with our B-actin probe, which we use as a control for possible differences in total RNA loading between samples.

Our initial Northern Blots revealed a faint band of around 4 Kb, similar to that observed for *O. mossambicus*, but the amount of trace detected was insufficient for a statistical comparison of the expression of this gene in the two treatments.

DISCUSSION

We have cloned the gene for IGF-I from *O. niloticus* and confirmed its identity through sequence analysis. The first stage of development of a probe for the efficient assessment of the expression of the IGF-I gene was only partially successful, although we find the results encouraging nonetheless. Our initial Northern Blots revealed a faint band of around 4 Kb,

Table 1. Growth, RNA, DNA, and RNA/DNA ratio data, Experiment 1.

Treatment	Tissue	Mean Body Weight (g)	RNA/DNA
Satiation	Muscle	3.37	0.170
Satiation	Liver	3.29	0.170
Maintenance	Muscle	0.88	0.454
Maintenance	Liver	0.88	0.084

similar to that observed for *O. mossambicus*, but the amount of trace detected was insufficient for a statistical comparison of the expression of this gene in the two treatments.

Consequently, we conclude that the IGF-I gene can be detected but that the method of use and detection of the labeled probe must be optimized in order to apply the probe as a molecular indicator of growth. Specifically, we need to improve the efficiency of the probe synthesis reaction beyond the 14% incorporation level to increase the sensitivity of the probe. This should be completed shortly and will be reported in the coming (Work Plan 11) work plan.

We were unable to find an associative pattern of RNA:DNA ratio with growth, although this is sometimes used as a growth indicator. In all likelihood the spectrophotometric method was not sufficiently sensitive, although it has also been argued that these ratios are not sufficiently consistent in their correlation with growth to serve as a reliable indicator of growth.

Conclusions

We conclude that the IGF-I gene has been isolated and cloned and that it can be detected by Northern Blot. We are not done with the refinement and testing of genetic probes using this gene. Our initial attempt to generate a radiolabeled probe for use in the detection of the target gene produced only faint banding and our methods must be refined. That work is presently underway and we believe our goal of using this molecular probe as an indicator of growth will be within reach shortly.

Anticipated Benefits

The tilapia is a crop with established and growing importance worldwide and particularly in Southeast Asia. The generation of new strategies for tilapia culture could have an economic impact throughout the region. Among tilapias, the Nile tilapia (*O. niloticus*) is a worldwide favorite because of both its robustness and its capacity for very rapid growth. Refinement of production techniques and adaptation of technology to local conditions requires extensive testing that could be abbreviated with rapid growth assessment tools.

The proposed development of such tools has passed important milestones—specifically the successful cloning of IGF-I in this species. Our work has not yet delivered the completed product, but we are confident that a genetic probe that will give a quantitative indication of the expression of the IGF-I gene in Nile tilapia will soon be in hand. The testing and resolution of the molecular probe approach will allow almost instantaneous screening of production methods and conditions, which will be of significant value to tilapia farmers and consumers alike.

Acknowledgments

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