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Somatic transgenesis in Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

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The tilapia fish is one of the most extensively farmed fish in aquaculture second only to carp. It is now found in over 100 countries, though it originated from the African continent. The application of biotechnology to aquaculture has focused on many areas to improve commercial stocks, but in particular, on increasing output and protecting stocks from disease. Successes in the field of germline transgenesis have fuelled interest in whether similar successes may be achieved through somatic gene transfer. The presentation of DNA into somatic cells may potentially result in the conferral of the gene product, without permanent change to the genome and subsequent germline transgenesis.

There were two main objectives for this project. The first was to investigate the fate and persistence of transgene DNA after direct injection into the somatic cells of tilapia. This was achieved using the bacterial reporter gene lacZ. The difference in expression levels gained using tilapia β-actin/lacZ and carp β-actin/lacZ constructs were also investigated, as well as the effect of co-injection of these constructs with the linear tilapia β-actin enhancer element, isolated from intron I of the tilapia β-actin promoter. The second of the objectives was to investigate the effect on growth of juvenile tilapia after somatic gene transfer of an 'all-tilapia' growth hormone plasmid construct (tiβAP/tiGH) as well as the opAFP/csGH construct into the muscle tissue of tilapia fish.

The results suggest that the tilapia promoter sequence gives rise to higher gene expression probably owing to the greater homology of this promoter when compared to that from the other species. Co-injection of the tilapiine enhancer element from intron I significantly boosted levels of gene expression. It was found that the gene product was mobile around the body, unlike the DNA itself which remained, for the most part, in the myofibres around the site of injection. On injection of either of the growth hormone plasmids, there was a putative endocrine controlled down-regulation of endogenous GH, making GH-injected fish show reduced growth as compared with controls. A putative contributing limiting factor may be attributed to the quantity of plasmid DNA which can become nuclear, since the active nuclear uptake mechanism can become saturated.

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ABBREVIATIONS

AFP	anti-freeze promoter
β-gal	beta-galactosidase
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyl tranferase
cβAP/lacZ	plasmid carp β-actin promoter spliced to lacZ reporter gene construct
CI	confidence interval
CoA	coenzyme A
csGH	Chinook salmon growth hormone
CMV	cytomegalo virus
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DPE	down stream promoter elements
Enh	enhancer element from intron I in the tilapia β-actin promoter
EtOH	ethanol
FAO	United Nations Food and Agricultural Organisation
FCR	food conversion ratio
GFP	green fluorescent protein
GH	growth hormone
GHRH	growth hormone releasing hormone
GMO	genetically modified organism
GnRH	gonadotropin releasing hormone
HCL	hydrochloric acid
IGF	insulin like growth factor
i.m	intramuscular injection
i.p	intraperitoneal injection
kb	kilobase
LUC	luciferase
mRNA	messenger ribonucleic acid
MT	metallothionein
MUG	methylumbelliferyl-β-D-galactoside

MyCH	myosin heavy chain
MyLC	myosin light chain
NaCl	sodium chloride
NEO	neomycin phophtrasnferase
NP-40	nonidet P-40
OD	optical density
opAFP/csGH	plasmid ocean pout anti-freeze promoter spliced to chinook salmon growth hormone gene construct
PCR	polymerase chain reaction
2-PE	2-phenoxyethanol
pg	picogram
RSV	rous sarcoma virus
SDS	sodium dodecyl sulphate
SDW	sterile distilled water
SE	standard error
SGR	specific growth rate
SRIF	somatostatin
SV40	simian virus 40
tiβAP/lacZ	plasmid tilapia β-actin promoter spliced to lacZ reporter gene construct
tiβAP/tiGH	plasmid tilapia β-actin promoter spliced to tilapia growth hormone gene
tiGH	Tilapia growth hormone
X-gal	5-bromo 4-chloro 3-indolyl β-D-galactoside

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Chapter one:

Introduction and literature review

1.0 General introduction

1.1 General introduction and overview of study

Tilapia *Oreochromis niloticus* (L.) a species of freshwater fish originating from the African continent (de la Fuente *et al.*, 1999), is one of the world's most important species used in aquaculture, second only to carp in terms of human food resources (Maclean *et al.*, 2002). The suitability of tilapia for aquaculture stems from their ease of breeding, high reproductive capacity, rapid growth rate and their omnivorous feeding habits allowing them to convert organic and domestic wastes into high quality protein. They show great capacity for survival in waters of poor quality with low oxygen concentrations, while demonstrating good disease resistance (Suresh 1999). Tilapia can also tolerate rapid fluctuations of these environmental parameters, important when stocking in the high densities seen in aquaculture.

In order to optimise growth of the fish in conditions where overcrowding results in the harvest of smaller fish, and to facilitate multiple harvests annually, genetic manipulation has been used to enhance growth and induce sterility. Sterile tilapia can show greater growth potential than those allowed to mature sexually, (Razak *et al.*, 1999, Maclean *et al.*, 2002). Seminal work performed on transgenic fish involved the microinjection of a mouse metallothionein promoter spliced to human growth hormone gene (Zhu 1986), inspired by the work of Palmiter and colleagues (1982). Palmiter *et al.*, (1982) microinjected copies of the rat growth hormone (rGH) gene, driven by a mouse metallothionein-l (mMT-I) promoter, into the pronuclei of fertilised mouse eggs. The result of this work showed that, of the mice carrying the construct, most grew significantly larger than the littermates that did not. This growth enhancement was thought to be a result of the production of growth hormone in liver, as dictated by the metallothionein promoter, rather than in the pituitary tissue as in the non transgenics. Since the pioneering work of these groups, the focus of transgenic research has widened and the number of species included has grown appreciably, encompassing many genes of interest.

Genetically modified organisms (GMO's) have been subject to increasing public scrutiny, with controversy regarding the ethics of such applications, safety for human

consumption and risk posed to the environment (FAO 2002). Despite this there has been substantial interest in genetic engineering owing to the considerable benefits to be had, both commercially and scientifically. The application of biotechnology to aquaculture, in order to increase output and protect stocks from disease, has been the subject of much diverse research. Commercially advantageous traits that are being engineered into fish include improved growth (Rahman *et al.*, 2001; Rahman and Maclean 1999, Rahman *et al.*, 1998), enhanced cold tolerance (Wu *et al.*, 1998; Wang and Zhang 1995), reversible sterility (Razak *et al.*, 1999; Iyengar *et al.*, 1996), improvement of carbohydrate breakdown for reduced dependence on fish meal feeds (Krasnov *et al.*, 1999; Pitkanen 1999), and the enhanced/increased immunity of fish to various significant diseases (Lorenzen *et al.*, 2002a, b; Ferandez-Alonso *et al.*, 2001).

Concern over the depletion of wild fish stocks across the world coupled with the growing world population, must result in the development of sustainable means to augment current demand, and potentially alleviate pressure on wild fish stocks. According to global statistics compiled by the United Nations Food and Agriculture Organization (FAO), the number of wild fish stocks that are being overexploited is high, and rising (FAO 2004). World supply in 2001 from fisheries (capture and aquaculture) totalled approximately 128 million tonnes and of this over 37 million was supplied from aquaculture (FAO 2002). The total catch, according to the United Nations must increase seven-fold by 2020 to supply the world's growing population, a daunting prediction given that the current quota achieved from capture fisheries has reached a plateau, with most stocks fully exploited. Catch reports for 2000-2002 gathered from all major fishing countries indicate that there is a marked decrease in global capture production (FAO 2004). It is thought that continuation of capture fisheries at such levels will lead to a severe decline in stocks owing to current fishing practices being substantially unsustainable. Conversely to the declining capture fisheries, output from aquaculture has been increasing annually and has already been augmenting catches from wild stocks. All continents showed increases in production during 2000–2002, except Europe, where production remained relatively unchanged (FAO 2004).

In the Southampton laboratory three separate lines of transgenic fish have been developed to attempt to provide enhanced growth (Rahman and Maclean 1999; Rahman *et al.*, 1998), with one line showing on average 2.5 fold increase in size over non-transgenic siblings (Rahman *et al.*, 2001). The commercialisation of these transgenic lines of tilapia for use in aquaculture raises some important concerns, since such fish could potentially impact native populations, through introgression of transgene DNA, should accidental escape occur. Taken together it is these factors which prompted research into whether growth enhancement could be achieved with the methods used for somatic transgenesis, the direct introduction of DNA into the somatic cells of tilapia. This introduction will provide an outline of the history of transgenic research, briefly reviewing germline transgenesis, and the production of transgenic fish. It will set out to define somatic transgenesis, to outline the current methods and uses for this type of genetic manipulation, and put into context the area of research in which this study has been undertaken.

1.2 Technology of germline transgenesis in fish

1.2.1 History and development of transgenic fish

Palmiter and colleagues in 1982 worked on the production of transgenic mice and succeeded in creating the 'super mouse', which grew approximately twice the size of its littermates. It was this work that inspired the initial research into the production of transgenic fish. The preliminary work performed on transgenic fish used a mouse metallothionein promoter to drive human growth hormone (Zhu *et al.*, 1986). Since that time the number of fish species that have been included in transgenic research has grown, resulting in the number of genetically engineered fish species surpassing all other GM vertebrates, and are subject to research in laboratories across the world (Pinkert and Murray 1999). These include species such as tilapia (*Oreochromis niloticus*, e.g., Caelers *et al.*, 2005; Rahman *et al.* 1999), carp (*Cyprinus carpio*, e.g., Tran and Nguyen 1993), rainbow trout (*Oncorhynchus mykiss*, e.g., Tymchuk and Devlin 2005; Anderson *et al.* 1996a, b), Atlantic salmon (*Salmo salar*, e.g. Devlin *et al.* 1994), coho salmon (*Oncorhynchus kisutch* Devlin *et al.*, 2004,1994b) loach (*Misgurnus fossilis*, e.g., Kozlov *et al.* 1988), mud loach (*Misgurnus mizoler*, e.g., Noh

et al., 2003; Nam *et al.*, 2001), channel catfish (*Ictalurus punctatus*, e.g., Dunham *et al.*, 1992), African catfish (*Clarias gariepinus*, Muller *et al.*, 1992), Indian catfish (*Heteropnustes fossilis*, e.g. Anathy *et al.*, 2001 and Sheela *et al.*, 1999). Also the use of zebra fish (*Danio rerio*, e.g., Udvadia and Linney 2003), medaka (*Oryzias latipes*, e.g., Matsumoto *et al.*, 1992) and goldfish (*Carassius auratus*, Wang and Zhang 1995), is noteworthy, since these species are commonly used as models for genetic research.

Several species of fish have shown integration of copies of novel genes into their genome (Rahman *et al.*, 1997; Devlin *et al.*, 1995a,b), although sometimes problems arise when trying to induce germline transmission and long-term stable integration, owing to mosaicism of the transgene throughout the cells (Iyengar and Maclean 1995). The initial experiments of Zhu and colleagues (1986) used the mouse metallothionein promoter to drive expression of a human growth hormone gene, growth hormone being highly conserved between species (Marins *et al.*, 2002). Since this original research many fish species, such as Atlantic salmon (e.g. Hew and Fletcher 2001; Saunders *et al.*, 1998; Choy *et al.*, 1996; Du *et al.*, 1992), tilapia (e.g. Rahman *et al.*, 1999; Martinez et al., 1999; de la Fuente *et al.*, 1998) Coho and Pacific salmon (e.g. Devlin *et al.*, 1995a,b) common carp (e.g. Wu *et al.*, 2003; Hinits and Moav 1999; Fu *et al.*, 1998; Chatachondi *et al.*, 1995), to list some of the more important commercial species, have shown enhanced growth from many different sources of growth hormone and a wide variety of promoters isolated from various fish species, as well as promoters from both viral and mammalian origin.

1.2.2 The promoter and regulatory sequences

To facilitate transgene expression it is important to select a suitable regulatory sequence with which to drive gene expression. Regulatory elements may be tissue specific or ubiquitous in their expression, although they may show more expression in certain tissues when compared with others. The metallothionein promoters were among the first eukaryotic promoters used in fish (Zahfarullah *et al.*, 1989; Zhu *et al.* 1986). Metallothioneins are proteins that bind heavy metals in cells, in particular zinc, cadmium, copper and mercury (Maclean and Penman 1990). The MT promoter is inducible, and their synthesis up-regulated in the presence of heavy metals such as zinc and copper, as well as corticosteroid hormone (Wu *et al.*, 2002). Kinoshita *et al.*,

(1994) spliced the 0.5 kb regulatory sequence isolated from rainbow trout MT-A, to the CAT reporter gene to assess transient expression studies in medaka fry *in vivo*. These authors show the activation of the CAT gene in a dose dependent response on addition of $ZnCl_2$. Devlin *et al.*, (1994) however, showed that the Sockeye salmon MT promoter spliced to a sockeye salmon GH gene induced dramatic growth enhancement but without heavy metal induction. The metallothionein gene promoter, like the β-actin promoter and the ocean pout antifreeze protein promoter, is active in most tissues particularly the liver (Caelers *et al.*, 2005; Maclean and Penman 1990). Viral promoters have been found to yield high levels of transgene expression and have been used extensively in many fish species. These include the promoters from the human cytomegalovirus (CMV; e.g., Venugopal *et al.*, 2004; Martinez *et al.*, 1999), simian virus (SV40), the enhancer/promoter from Rous sarcoma virus long terminal repeat (RSVLTR; Zhang *et al.* 1990) and pseudotyped retroviral vectors such as the vesicular stomatitis virus (VSV; Lin *et al.*, 1994). However there is evidence to suggest that regulatory sequences from viral or mammalian origin are frequently less efficient in transgenic fish than are those sequences isolated from fish origin (Alam *et al.*, 1996; Maclean and Rahman 1994; Penman *et al.*, 1990). The use of viral or murine regulatory sequences in transgenic fish intended for human consumption is seen as morally and/or socially unacceptable, resulting in a move towards developing 'all fish' constructs, entirely piscine in origin (Hackett 1993; Du *et al.*, 1992a). Two other promoters have shown greater promise for use in fish transgenics and these are the antifreeze protein promoter and β-actin promoter. .

1.2.2.1 AFP

Fish regulatory sequences from antifreeze protein (AFP) genes have been widely and successfully used *in vivo* and *in vitro* (Rahman *et al.*, 1997; Du *et al.*, 1992a; Gong *et al.*, 1991). AFP is synthesized in the liver of a number of polar fish, it acts by absorbing ice crystals forming in the serum thus preventing the freezing of the blood and tissues by stopping continued ice crystal growth and have been classed into types I, II and III. Type I has been found to be seasonally regulated, whereas types II and III show little seasonal difference (Davies *et al.*, 1989). Gong *et al.*, (1991) identified both positive and negative regulatory elements isolated from type II and type III AFP genes after splicing these putative control regions to the CAT reporter gene and transfecting

them into a number of cell lines. Du *et al.*, (1992b) used an ocean pout antifreeze promoter (opAFP) to drive a chinook salmon GH gene in the production of transgenic salmon and reported growth enhancement of between two to six fold over controls. This construct was successfully used by Rahman *et al.*, (1997) in the creation of transgenic tilapia expressing exogenous GH. Later Rahman and Maclean (1999) demonstrated transmission of the opAFP/csGH construct from the G_0 to G_3[1] generations and obtained enhanced growth of approximately three times in transgenic tilapia as compared with non-transgenic siblings.

1.2.2.2 β-actin

Actin is an abundant cytoskeletal protein found in all cells and can be divided into two types; muscular and non-muscular (or cytoplasmic actin) types of which there are six isoforms. Distribution of these is tissue specific and expression of these result in the change in composition of muscle tissue throughout development (Johnston 1999). The β-actin isoform is of the non-muscular type, which is down-regulated during myogenesis - the differentiation of myoblasts to form myotubes. Expression patterns of the different actin isoforms change, the cytoplasmic actins, beta and gamma, are down-regulated and the muscle specific isoforms are up-regulated (Lloyd and Gunning 2002). The region responsible for the down-regulation of the β-actin gene is located in the 3' end of the gene (Erba *et al.* 1988). The β-actin promoter, without the 3' sequence, results in constitutive expression in all cell types including muscle. Liu *et al.*, (1990a) isolated the putative β-actin proximal promoter of carp and found it to be transcriptionally active in both mammalian and piscine cells. Liu *et al.*, (1990b) went on to analyse the functional elements affecting expression of the β-actin gene in carp by studying the putative control regions when spliced to the CAT reporter gene. These authors found that the basal promoter directed a high level of expression, with a distal upstream negative element which regulated this expression and, within the first intron a strong enhancer element was identified, which interestingly, acted in a position and orientation dependent fashion. Since this work, carp β-actin regulatory sequences have

[1] In the production of transgenic fish the founder generation, those individuals raised from eggs with novel DNA introduced, are defined as G0 rather than the conventional F0. The subsequent generations are known as G1, G2 and so on, resulting from crosses of transgenic and wild type individuals, generation denoted conventionally by number. F is used between crosses of the same generation and not the wild type.

been shown to drive high levels of reporter gene expression in a variety of fish species (Moav *et al.*, 1993, 1992b). Alam *et al.*, (1996) in a comparative study of β-actin regulatory sequences from mammalian and piscine origin, reported on the level of expression from the reporter gene, lacZ, when driven by rat or carp β-actin in tilapia and rainbow trout embryos. These authors were able to demonstrate that the carp construct expressed at substantially higher levels than the rat construct in the fish embryos. Moav *et al.*, (1995) showed successful transmission of a carp β-actin/csGH transgene from the F_0 to F_1 generation. Noh *et al.*, (2003) reported on the usefulness of the regulatory regions from the mud loach β-actin gene, showing that it exhibited strong transcriptional activity which may prove useful in autogenic mud loach transgenesis. Hwang *et al.*, (2003) went on to isolate and characterise the tilapia β-actin promoter using primers designed from the conserved sequences of carp β-actin (Liu *et al.*, 1990a) and medaka β-actin (Takagi *et al.*, 1994). The activity of the tilapia β-actin promoter (including untranslated exon 1 and intron 1) was tested in comparison with the 1.5 kb and 4.7kb carp β-actin promoters. These authors described higher reporter gene expression when using the tiβA promoter in tilapia embryos and in tilapia somatic tissue after intramuscular injection of the tiβA construct,, with up to three times the expression after transfecting blue gill cells, with the same construct. These data demonstrated the potential of tilapia β-actin as a promoter especially for use in autotransgenics.

1.2.3 The use and increased expression associated with enhancer elements

Enhancer elements, generally speaking, are able to stimulate transcription of a cognate promoter, independent of orientation and distance from the promoter. They act by binding sequence specific transcription factors which may then act in a positive or negative manner on the promoter. After the rat myosin light chain enhancer was shown to increase reporter expression in mice (Rosenthal *et al.*, 1989), Muller *et al.*, (1997) co-injected the rat foetal myosin light chain enhancer and enhancer elements isolated from the first intron from the carp β-actin regulatory sequence (Lui *et al.*, 1990b) into zebrafish embryos. Using either the carp (0.9 kb) myosin heavy chain promoter spliced to a lacZ gene (MyHCP/lacZ) or carp β-actin promoter spliced to a lacZ gene (cβAP/lacZ). The two reporter constructs were microinjected into zebrafish embryos, either covalently linked by splicing the enhancer after the promoter and gene sequence,

or co-injected in a separate linear form in a 1:3 ratio. The subsequent expression was observed and mapped. The authors recorded location and type of cells expressing lacZ, as well as the level of expression obtained. As much as a 13-fold increase in the proportion of cells positive for lacZ was recorded when co-injected with an enhancer element, and higher expression was achieved when the enhancer was co-injected separately in linear form as compared with the covalently linked promoter/gene/enhancer sequences. An increase in expression was mapped after microinjection of the MyHC/lacZ sequence and MyLC enhancer sequence, as compared to that injected with the MyHC/lacZ construct alone. However, the largest increases were observed when the regulatory elements from the carp β-actin first intron were co-injected with the cβAP/lacZ construct, perhaps owing to the higher homology of the fish derived sequences.

1.3 Production of transgenic fish

1.3.1 Producing transgenic fish

The method of producing transgenic fish, while not directly involved with this present project is pertinent, provides a well established benchmark to which the methods and results from somatic transgenesis may be compared with. Germline transgenesis has been achieved in the Southampton laboratory with success, by the method of microinjection, and although this by no means is the only way to produce transgenic fish will be the focus of this brief review. More in-depth reviews are available in Moav (2000); Hackett and Alverez (2000); Maclean and Rahman (1994), Fletcher and Davies (1991) Maclean and Penman (1990), and Powers and Kirby (1990).

1.3.2 Egg microinjection to create transgenic fish

Microinjection procedures are the most frequently used of the techniques to create transgenic fish and introduce DNA into fertilized eggs, as well as oocytes (Maclean and Laight 2000). Introduction of multiple copies of the transgene (approximately 10^6 copies into fertilized eggs) raises the probability of integration into chromosomal DNA, though the copy number, integration frequency and position of transgene within the recipient genome occurs randomly (Maclean and Laight 2000; Rahman *et al.*, 2000,

1997; Iyengar *et al.*, 1996). Matsumoto *et al.*, (1992) carried out research on the injection of DNA transgenes into medaka oocytes. This method is unusual and depends on the introduction of DNA into the large tetraploid nucleus of the egg before fertilization occurs. By introducing transgene DNA before the first cell division, in theory all developing cells are expected to carry a copy of the transgene, including the future germ cells, should integration take place. The injection of material at the oocyte stage does however have its difficulties, and is why this type of procedure is uncommon compared with cytoplasmic injection. Oocytes are only readily obtained from certain species of fish, and the procedure may damage the micropyle, the site in which sperm can access the egg.

Injection into the cytoplasm of the egg may be accomplished by using a microneedle to penetrate through the chorion. This tough, protective shell is impermeable to sperm except through the micropyle. In tilapia and Atlantic salmon, egg microinjection can be carried out through the micropyle (Shears *et al.*, 1992; Brem *et al.*, 1988) and in other species such as mud loach or common carp, the chorion is soft enough for microinjection (Nam *et al.*, 2001; Moav *et al.*, 1992). The chorion also may be chemically treated with agents such as glutathione to soften it after fertilisation; medaka and trout eggs have been treated this way to facilitate penetration of the microneedle (Kinoshita *et al.*, 1996; Yoshizaki *et al.*, 1991). Injection is carried out as soon as possible after fertilisation, the timing of which is controlled by stripping milt and eggs from the fish and combining them *in vitro*. The DNA is released from the microneedle in the area of the perinuclear cytoplasm, since the fertilised pronuclei can not be visualised with a light microscope, and so large numbers of the transgene are used. It has been reported that the introduction of considerable quantities of transgene DNA can prove fatal to the developing embryo (Fletcher and Davis 1991; Penman *et al.*, 1990), and so an optimum concentration between providing enough DNA into the perinuclear cytoplasm to ensure a chance at integration without harm to the embryo must be found.

The low rate of integration of the transgene into the germ cells is a product of mosaicism observed in the founder generation. Only a small percentage of the founder

generation transmit copies of the injected DNA to their progeny (Rahman *et al.*, 1997; Alam *et al.*, 1996; Du *et al.*, 1992). The integration of copies of injected DNA occurs after the first division of the egg, and often into eggs that have developed past the one cell stage into early multicellular embryos. The result is of a random distribution of the injected copies of DNA throughout the adult cells. Only between one and five percent of injected eggs result in adults stably expressing the transgene from integrated copies (Maclean *et al.*, 2002; Moav *et al.*, 1995; Stuart *et al.*, 1990). Transient expression occurs in most embryos from the transcription and subsequent translation of unintegrated copies. These expression patterns relate to the extrachromosomal persistence of the transgene and are found to reduce, fol lowing cell division and transgene degradation (Iyengar *et al.*, 1996; Stuart *et al.*, 1988).

Linear transgene sequences in fish, (as also in *Xenopus*) often rapidly establish long concatemers after introduction to the egg, and this phenomenon is attributed to the activity of the stored ligases present in the egg (Marini *et al.*, 1988). The conformation of these concatemers alters, formed from randomly ligated copies of the linear sequence. Iyengar and Maclean (1995) report the effects of transgene concatemerisation in rainbow trout after injection of both linear and circular DNA, finding that expression was higher and more persistent with linear DNA. It was postulated that linear DNA was more efficient at concatemerisation, resulting in subsequent elevated expression. It is hoped that, of the integrated transgene DNA, some copies will be present, at least in some individuals, in the germ cells of the G_0 founders and so will be transmitted to subsequent generations. Transfer of the transgene from G_0 to their progeny in less than the expected Mendelian ratio indicates that the transgenic fish are showing germline mosaicism (Shears *et al.*, 1991; Penman *et al.* 1990). However, by selecting progeny positive for the transgene, the resultant transmission between G_1 and G_2 generations means a proportion, according to mendelian laws of inheritance, should be hemizygous for the transgene (reviews: Chen *et al.*, 1992; Chen and Powers 1990).

1.4 Reasons for producing transgenic fish

Why produce transgenic fish? There are two main targets for transgenesis in fish

1) For studies on gene regulation
2) To improve fish stocks by manipulating commercially important traits

1.4.1. For studies on gene regulation

1.4.1.1 Reporter genes

Reporter genes are used in order to study gene expression and regulation, isolated from exogenous origin, they encode sequences for enzymatic activity not normally present in the host cells, producing a product with distinguishable features. The product of the gene must be easily detected with the level of expression readily quantified, enabling cells and tissues that express the gene to be easily distinguished from non-expressing ones. By splicing various reporter genes to a promoter, its temporal and spatial expression can be assayed and the activity of the promoter investigated.

The bacterial chloramphenicol acetyltransferase (CAT) gene is one such reporter. After incubation of cell or tissue extracts, with unlabeled chloramphenicol and ^{14}C – labelled acetyl CoA, CAT activity may be detected and quantified by the amount of cell or tissue lysates that will acetylate labelled chloramphenicol. The amount of chloramphenicol converted to the acetylated form can then be measured in a liquid scintillation counter (Gorman *et al.*, 1982). It has been widely and successfully used in transgenic fish and also for direct injection into fish skeletal muscle (Tan and Chan 1997; Rahman and Maclean 1992; Hansen *et al.*, 1991).

Luciferase, also widely used in fish transgenics, was first isolated from the firefly *Photinus pyralis*, and provides a simple, effective reporter gene system. The enzymatic activity of tissue homogenates may be detected, after starting the reaction by adding luciferin substrate to the homogenate, in a reaction containing acetyl CoA and ATP, and subsequently measured by determining photo emission using a scintillation counter (de Wet *et al.*, 1987). Alestrom *et al.*, 1991, showed that the reporter gene may be

detected in living medaka embryos, using a photon counting acquisition system. The living embryos were immersed in a solution of luciferin, which allowed the penetration of the substrate into the tissues. Then the photons are subsequently counted in a scintillation counter or by placing the living embryos on a sensitive X-ray film (Gibbs *et al.*, 1994).

Neomycin phosphotransferase (NEO) is an antibiotic resistance gene that confers resistance of cells or organisms to the antibiotic neomycin. Its use in fish transgenics has been limited since the mosaic nature of G0 transgenic fish leads to only a subset of cells producing NEO expression. These individuals subsequently do not survive exposure to the antibiotic or its analogue G418 (Yoon *et al.*, 1990). Guise *et al.*, (1992) reported successful microinjection of NEO into goldfish, however the survival rate ranged between 10-50% compared to a 90% survival rate in controls.

Green fluorescent protein (GFP) originating from jellyfish *Aequorea victoris*, differs from other reporter gene systems in that it does not require the addition of other substrates in order to activate any measurable properties of the gene. Rather it is intrinsically fluorescent and may be visualised in expressing cells when exposed to UV light, where upon it fluoresces (Chalfie *et al.*, 1994). Like luciferase it may be used *in vivo* without harm to the organism and has been developed to express different emission spectra making several artificial fluorescent colours available (Gong *et al.*, 2003; Gibbs and Schmale 2000).

1.4.1.2 LacZ reporter gene

The reporter gene used in this study, lacZ, may be used in two assays which allows the enzymatic activity of the gene to be easily detected. The product of the lacZ gene is betagalactosidase (β-gal), its activity can be visualised after histochemical assay; the staining of fixed embryos or tissues, or can be detected in the 4-methylumbelliferyl-β-D-galactoside (MUG) assay, which quantitatively measures the hydrolysis of a β-galactosidase substrate linked to a fluorogen. The substrate, 4-methylumbelliferyl-β-D-galactoside, is cleaved yielding 4-methylumbelliferone, a fluorescent molecule, the increase of which, is directly proportional to the amount of β-galactosidase, and so the level of β-galactosidase expressed in the tissue tested can be ascertained. It is sensitive

enough to enable picogram quantities of β-gal to be detected. The disadvantage of this method is that it requires tissue homogenates and therefore can only provide an average of the product present in the whole to be measured.

LacZ has been widely used in fish transgenesis as well as to survey uptake in skeletal muscle after histochemical staining on direct injection into trout skeletal muscle (Gomez-Chiarri *et al.*, 1996) and zebrafish skeletal muscle by Tan and Chan (1997). The lacZ reporter gene was selected to use in this study owing to the wide applications for its use. It was used here, both to quantify expression levels and to look at the pattern of uptake, as well as migration of β-galactosidase from within the myofibres of the injected skeletal muscle by histochemical staining of sectioned muscle blocks, taken from the site of injection. LacZ has been shown to be successful in the creation of fusion proteins, where two genes are spliced together over the stop codon, so that one long transcript is produced, resulting in as fusion protein. An excellent example of this was demonstrated by Coen *et al.*, (1997) who in a murine system, successfully introduced by direct injection in muscle tissue, a hybrid protein fusing TCC, a fragment of tetanus toxin, which has the ability to bind to nerve cells and be retrogradely transported through a synapse with a lacZ gene. They showed that the fusion protein retained the biological function of both genes, and after intramuscular injection (into mouse tongue) β-gal activity could be detected along motorneurons. The creation of fusion proteins provides a method of visualising the movement of proteins from genes which otherwise do not have the attributes of reporter genes.

1.4.2 To improve fish stocks by manipulating commercially important traits

This following section will cover the three main areas, about which the majority of transgenic fish research is carried out, these include growth enhancement of stock, increased resistance of fish to cold temperatures and the application of DNA vaccines to protect stocks in aquaculture against disease.

1.4.2.1 Growth enhancement

Growth hormone was proven to have an effect on fish growth as early as 1948 when Pickford and Thomson administered mammalian GH to killifish (*Fundulus heteroclitus*) which resulted in a significant growth increase. Since this time, during

the 1970's, the administration of exogenous bovine GH to salmonids was investigated, resulting in the increased growth, enhanced appetite and feed conversion efficiency (Guise *et al.*, 1992). Bovine GH while showing the potential to enhance growth in salmonids, resulted in varying outcomes with other fish species. This and the commercial nature of such research, especially with fish intended eventually for human consumption, led to a move away from the use of mammalian GH to that of GH of piscine origin (Funkenstein 2000). The purification of GH from the pituitary, particularly from fish, makes this impractical on the scale needed for aquaculture, unless other commercially viable ways to create GH are established. Guillen *et al.*, (1998) injected tilapia (*Oreochromis hornorum*), intraperitonially with *E.coli* derived homologous GH, and recorded a significant increase in both body length and weight when receiving 0.5 μg tiGH g^{-1} body weight, which corresponded with an increase in IGF mRNA levels in the liver. Li *et al.*, (2003) transformed a strain of yeast (*Pichia pastoris*) GS115 to express a transgene consisting of common carp (*Cyprinus carpio*) GH, extracted and cloned from the pituitary, spliced to the promoter from the alchohol oxidase gene, with an α-factor signal peptide. After induction of the transgene using methanol, the recombinant carp GH was expressed in the culture medium at a yield of 300-400 mg l^{-1} and when introduced via intraperitoneal injection of 0.10 μg and 1 μg g^{-1} body weight $week^{-1}$, resulted in significant growth of juvenile tilapia over controls.

1.4.2.2 Growth enhancement by germline transgenesis

The 'all-fish' construct splicing the ocean pout anti freeze promoter sequence to chinook salmon GH (opAFPGHc) has been transferred into Atlantic, coho and chinook salmon as well as rainbow and cutthroat trout, yielding results after *ca.* one year of growth enhancement of between three and ten fold (Devlin 1995a,b; Du *et al.*, 1992b). Germline transmission of this construct was reported by Rahman *et al.* (1997) with three transgenic individuals transmitting the GH transgene to their progeny. Devlin (1994) reported that microinjection of an 'all-salmon' construct (pOnMTGH1) using a concept similar to that used in the production of transgenic mice in which the transgene was driven by a metallothionein - B promoter, resulted in transgenic individuals that were 11-fold larger than their siblings with a range from zero difference to one fish that was 37 times larger than controls. Interestingly the fish precociously developed a silver body colour, which is indicative of salmon undergoing smoltification enabling the fish

to migrate in the spring from fresh water to a marine environment. Winter levels of GH in the body of transgenic fish were found to be 40 times higher than controls. Levels of serum GH are generally very low until spring when they are needed for migration. The increase suggests unregulated over-expression of GH, hence the premature colour change of the transgenic fish.

Growth enhancement in tilapia using tilapia growth hormone (tiGH) and an enhancer/promoter from the human CMV, resulted in ectopic expression within muscle and gonad tissues in a percentage of the F0 generation but stable transmission to F1 and F2 generations with expected Mendelian ratios (Martinez *et al.*, 1996). Transgenic tilapia also have higher feed conversion ratios as compared with their non-transgenic siblings or controls, more than 20% greater while still showing enhanced growth of up to four times the body size (Rahman *et al.*, 2001). Transgenic fish appear to be more efficient at utilising protein and energy (Rahman *et al.*, 2001; Fu *et al.*, 1998). Thus transgenic carp when studied for body composition and nutritional status appear to have improved levels of protein and lower lipid content (Chatakondi *et al.*, 1995). Methods of improving integration of novel genes have been explored by the research of Rahman *et al.*, (1997) by co-injecting carp β-actin/lacZ with an integration rate of about 14% together with GH spliced to the ocean pout antifreeze promoter (opAFPcsGH the 'all fish gene' construct from Du *et al.*, 1992a with integration rates lower than 2%. By injecting both these constructs simultaneously, the integration rate was improved to *ca.* 5%, thought to be owing to the greater efficiency of cβAP/lacZ construct at forming concatemers and, therefore, its persistence and expression resulted in the increased integration and germ-line transmission of the commercially important GH gene. There can however be adverse effects to the levels of GH in the body. Devlin *et al.*, (1995b) noted deformities in those individuals that grew most rapidly, such morphological changes in the cranium can be compared to acromegaly in mammals. If in severe enough form, acromegaly can affect feeding, respiration and growth rate. Growth is affected in farmed fish by a number of factors including gas super-saturation, high stocking densities and inappropriate light regimes which all contribute to creating stress, thus acting as a suppressant to growth by appetite reduction and/or stimulated catabolism (Pickering 1998). Implying that good husbandry combined with genetically engineered growth enhancement and the

introduction of DNA at optimum conditions, to induce greater body mass without incurring acromegaly, may lead to substantial increases in output from aquaculture.

1.4.2.3 The regulation of growth hormone in teleosts

Growth hormone (GH) is one of a cascade of hormones that stimulate somatic growth. It is a polypeptide of about 22 kDa and produced in the anterior pituitary gland by somatotrophs (Funkenstein 2000). Other hormones in this group include the insulin like growth factors one and two (IGF-I and IGF-II), which are believed to regulate GH. Endocrine regulation in fish is reviewed with greater detail in Blazquez *et al.*, (1998), Melamed *et al.*, (1998 and 1995), briefly however endocrine regulation with reference to the regulation of growth hormone is discussed here.

In mammals, hormones originating in the hypothalamus regulate the release of GH from somatotrophs located in the anterior pituitary. These primarily are the stimulator, the growth hormone releasing hormone (GHRH) and the inhibitor, somatostatin (SRIF) (Blazquez *et al.*, 1998). GHRH belongs to a large family of regulatory neuropeptides and migrates from the hypothalamus to the pituitary through axons of hypothalamic neurons, via the hypothalamo-hypophyseal portal blood system. After binding to growth hormone receptor, a transmembrane protein found on the somatotrophs, there is stimulation of GH production and release (Montero *et al.*, 2000). With teleosts the anterior pituitary is directly innervated by neurons synthesizing regulatory neuropeptides and neurotransmitters, rather than the hypothalamo-hypophyseal portal blood system, which is non functional (Blazquez *et al.*, 1998). Many factors have been implicated in the endocrine control of growth in fish including sex steroids, which act on the somatotrophs, influencing their receptiveness, neuropeptide Y and IGF-I, which down-regulates GH (Shepherd *et al.*, 1997). Recent reviews include Blazquez *et al.*, (1998), Melamed *et al.*, (1998), although the main regulatory neuropeptides acting in tilapia are discussed below.

In fish a GHRH-like peptide is implicated in elevating levels of cAMP, the elevation of which results in the potent stimulation of GH secretion in teleosts, however the GH releasing activity of GHRH in fish is low (Blazquez *et al.*, 1998). In tilapia GH secretion has been found to be potently stimulated by gonadotropin releasing hormone

(GnRH) and acts both *in vivo* and *in vitro* in a dose dependent manner resulting in a 6-fold increase in plasma GH as compared with controls (Melamed *et al.*, 1995). It is mediated through activation of protein kinase C (PKC) as well as the involvement of the cAMP-protein kinase A (PKA) pathway, although the endogenous hormone involved in elevating cAMP has yet to be determined. Dopamine has also been implicated in GH regulation. In goldfish the binding of dopamine to D1-type receptors, located on somatotrophs leads to an increase in cAMP levels, although dopamine does not potently stimulate GH secretion in tilapia (Melamed *et al.*, 1998; Peter and Marchant 1995). As with mammals, the hypothalamic regulation of GH secretion is regulated by SRIF. SRIF has been found to decrease levels of cAMP in tilapia pituitary and then potently inhibit GH release, it is thought by modulation of translation, since surprisingly steady state GH mRNA levels remain constant, despite the reduction in circulating GH levels (Melamed *et al.*, 1998).

GH is necessary for normal metabolism of proteins, carbohydrates, lipids and minerals. GH acts directly on GHR to enhance the break down of stored triglyceride, prevents the uptake of circulating lipid and so enhances the metabolic rate, decreasing lipid content and increasing lean body mass. Circulating GH also up-regulates the synthesis and secretion of insulin like growth factors (IGF-I, IGF-II) which mediate the functional effects of GH. IGF acts on soft tissue, by increasing the number of cells, stimulating cell division and preventing apoptosis. Other activities include increasing cell size by stimulating synthesis of proteins, inhibiting cell degradation and promotes amino acid uptake. In bone tissues, IGF stimulates cell division and maturation of cartilage cells in the epiphyseal plate as well as stimulating bone forming cell activity at the epiphyseal plate (Kopchick *et al.*, 1999; Peter and Marchant 1995).

1.4.2.4 Increased resistance of fish to cold temperatures

Fish are generally sensitive to changes in temperature and temperate climates pose a greater risk to aquacultural fish stocks; the risk of a particularly cold winter may result in extremely high fish mortality. Cold is a considerable stressor to fish, causing suppression of the immune system and dysfunction of the membrane bilayer at reduced temperatures (Melamed *et al.*, 2002; Tort *et al.*, 1998). It is thought that by increasing farmed fishes resistance to cold temperatures, there may be the possibility of

geographical range extension as well as the conferral of a better cold tolerance[2] to aid in the protection of stocks against severe winter temperatures. Some marine teleosts found in coldwater conditions have evolved high levels of serum antifreeze proteins (AFP) or glycoproteins (AFGP) between 10-25mg/ml, which protect the fish from freezing temperatures by halting the growth of the ice crystals in the serum. Four classes of AFP are found and one class of AFGP, the isoforms of AFP are formed in the skin, gills, scales and fins, with seasonal variation, which in some are negatively controlled by growth hormone (Harding *et al.*, 2003; Fletcher *et al.*, 2001).

A great deal of interest has been focused on the winter flounder (*Pseudopleuronetes americanus*) that can survive arctic conditions, which vary between –1.4 to –1.9°C and produce at least seven of the AFPs during the winter season (Jiang 1993). Wang and Zhang (1995) introduced ocean pout AFP genes to the oocyte of goldfish (*Carassius auratus*) eggs and reported that the surviving adults were significantly more cold tolerant than controls. The survival of tilapia (*Oreochromis massambicus Peters*) and milkfish (*Chanos chanos Forsskal*) in cold-tolerance tests, after feeding or anal injection of AFP, resulted in a 60% mortality rate in the control group and 41.9% in the AFP group, suggesting that AFP is able to enhance cold tolerance to some degree in these fish (Wu *et al.*, 1998). The commercially important Atlantic salmon responded to the gene coding for liver AFP, which integrated into the germ line and was then passed down to F3 generation where it was expressed specifically in the liver ranging between 200-400μg/ml. This conferred about 70% of the potential antifreeze activity as seen expressed in winter flounder (Hew *et al.*, 1999). It would seem that results are promising for the development of cold tolerance in commercial species of fish but to date full conferral and therefore protection of AFP has not yet been achieved.

1.4.2.5 Application of DNA vaccines

Recombinant DNA technology can be used to attenuate pathogens to produce live, safe vaccines by replacing or deleting genes used for survival and virulence *in vivo*. Genetic immunization uses introduced plasmid vectors to synthesize the immunizing

[2] It should be noted that cold tolerance and freeze resistance are different in that freeze resistance enables fish, such as the ocean pout to live in polar conditions, whereas the conferral of cold tolerance may allow commercial fish species to survive unusually cold winter conditions.

protein/proteins directly in the host, commonly by intramuscular injection or gene gun particle bombardment. Unlike live-attenuated vaccines, DNA vaccines use selected coding sections of the pathogen as opposed to the whole pathogen to elicit a long-lived antibody response with no risk of infection, and can provoke both humoral and cellular immune responses. Reviews covering the development and uses of DNA vaccines are those of Gurunathan *et al.*, (2000), Davis and McCluskie (1999) and Robinson and Torres (1997), as well as their application to aquaculture, Heppell and Davis (2000), Kanellos *et al.*, (1999) and Gudding *et al.*, (1999). By using DNA vaccines the reliance on antibiotics from which bacteria may develop a resistance is reduced and the fish acquire an innate disease resistance so reducing the costs and difficulties of the practices in preventing and curing disease.

1.4.2.6 Immunisation against viral infection

DNA vaccines can be used to treat bacterial, viral and parasitic infections and early achievements with mammals have prepared the way for vaccine development in fish. Success has been achieved in channel catfish against herpes virus (IHV-1; Nusbaum *et al.*, 2002) rainbow trout against the rabdoviruses, viral hemorrhagic septicaemia virus (VHSV; Lorenzen *et al.*, 2002a, b; Lorenzen *et al.*, 2000) and infectious hematopoietic necrosis virus (IHNV; LaPatra *et al.*, 2001; Anderson and Mourich 1996). IHNV also affects Atlantic salmon, and vaccination with a form of the virus isolated from rainbow trout was successful (Traxler *et al.*, 1999). Gomez-Chiarri and Chiaverini (1999) evaluate the efficacy of eukaryotic promoters for piscine vaccines and found levels of expression similar to those gained when using viral promoters. The development of non-viral promoter sequences would allay concerns expressed over the viral origins of current promoters (Liu and Huang 2002). DNA vaccination by immersion and subsequent short pulses of ultrasound treatment, which facilitates the transfer of plasmids into rainbow trout, resulted in preliminary data indicating the immunization of trout fingerlings for the viral hemorrhagic septicaemia virus. This could provide a viable way to vaccinate small fish (Navot *et al.*, 2005; Ferandez-Alonso *et al.*, 2001). Research into the area of DNA vaccines, though still perhaps in its development stage, seems to provide adequate proof of the effectiveness of DNA vaccines and the disease resistance they elicit.

1.5 Somatic transgenesis

1.5.1 Genetic manipulation and somatic transgenesis

Somatic transgenesis allows the introduction of novel DNA constructs into the somatic tissues of animals, in such a way that the properties of the genes may be transiently expressed *in vivo*, with little evidence to suggest permanent change to the genome or integration to the germline (Kanellos *et al.*, 1999; Wolff *et al.*, 1992). Direct injection of plasmid DNA has been widely investigated in small animals such as mice and rats, and to a lesser extent in fish. The application of this technology creates a simple method to characterise gene expression, tests the efficacy of promoter and enhancer sequences, and has heralded an era of research into genetic immunisation and gene therapy. The introduction of novel DNA into the somatic cells of a host species can be achieved either by using exogenous DNA, derived from one species and donated into another, or by using sequences of DNA which have originated from that same species, a method known as autotransgenesis.

Somatic transgenesis differs from other applications of transgenesis in the way the DNA is introduced, as well as the site and method of introduction. It may be regarded as a form of gene therapy, since both requires the isolation of appropriate genes and regulatory regions, the development of safe and efficient vectors for packaging of the novel genes and introduction of the recombinant genes into appropriate target cells and tissues.

Typically using juveniles, the DNA may be administered by direct injection, with the use of a gene gun, orally using micro encapsulation, transferred by immersion or with the use of viral vectors. Conversely germline transgenesis involves novel DNA introduced into a fertilized egg by microinjection or other methods. This, unlike somatic transgenesis, leads to the production of species with a genetically altered genotype which, should the transgene be present in the germ cells of the G0 generation, is passed on to the progeny which inherit the altered genotype and therefore the commercially beneficial phenotype.

1.5.2 Definition of a transgenic organism in view of somatic transgenesis

The definition of what is classed as a transgenic organism has created some confusion since the development of this type of molecular biology. Reviewed by Beardmore (1997), the various differing applications of the use of the word transgenic have been explored, culminating in the definition "transgenic should be used to denote an individual, or a cell with a DNA sequence, which has been transferred and integrated, into that individual or cell, by techniques of genetic engineering". Somatic transgenesis by this definition could therefore create a transgenic individual, since individuals are injected with DNA sequences, and the DNA may become integrated at random, although evidence points to this being unlikely with this type of genetic engineering (Anderson *et al.*, 1996; Zhu *et al.*, 1993; Wolff *et al.*, 1992, 1990). The Commission Directive 97/35/EC states that a genetically modified organism (GMO) is one in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination. Germline transgenesis produces individuals that are in the true sense of the word genetically modified. The novel genes can be introduced and integrated into the genome, which may then result in the new genetic information being passed onto the next generation through germline transmission. Somatic transgenesis allows the introduction of novel DNA into an individual with little evidence to suggest that this either becomes integrated into the host genome or subsequently gets passed to their progeny (Anderson *et al.*, 1996; Zhu *et al.*, 1993; Wolff *et al.*, 1992). Some forms of medical treatment arising from gene therapy involve the transfection of novel DNA into the patient's cells *ex vivo*. This method allows the new genetic material to become incorporated into the chromosomes with the hope that on reintroduction to the patient, it provides long-term correction of the original genetic defect.

Gene therapy is a potentially low risk application of genetic engineering; somatic transgenesis does not permanently alter the genotype of the target species. Aside from the ethical issues raised by the genetic manipulation and production of GMO's, a major concern is the risk of altered genes being passed to the wild type of that species, with consequent irrevocable changes to their genotypes and phenotypes. In natural conditions, this may result in the species being placed under different selection pressures, with either positive or negative net effects making the ultimate survival of

the species and the ecosystem, of which is it an integral part, questionable (Muir and Howard 2002). These factors must be considered carefully with the application of genetic engineering from which the resulting products may be produced commercially and on a large scale.

1.5.3.1 Treatment using gene therapy

Introduction of novel genes into the somatic tissues of a juvenile or adult organism and the subsequent expression of the recombinant genes give great prospects for the treatment of many inherited and acquired human diseases. As defined by Verma and Weitzman (2004), "the concept of gene therapy involves the transfer of genetic material into a cell, tissue, or whole organ, with the goal of curing a disease or at least improving the clinical status of a patient". Gene therapy works by changing or modifying the expression of genes, and has promise for future therapeutic needs within the field of medicine. Gene therapy allows the introduction of DNA into cells of a patient *in vivo*, which often results in the novel DNA becoming degraded over a period of time and so eroding the long-term desired effect of this DNA, or cells may be removed and transfected *ex vivo* to allow integration into the chromosomes, and therefore facilitate longer term treatment after reintroduction of the cells to the patient (Onodera and Sakiyama 2000). In ethical terms gene transfer into somatic cells rather than germ cells should not raise such problems as treatment which may result in germline transmission of the novel DNA, and gene therapy may be regarded as a type of pharmacotherapy (Morishita *et al.*, 2005).

Attenuated viral vectors are often used to deliver the novel DNA; these are highly efficient at integration owing to the ontogeny of the viral agents, which have evolved effective ways of targeting and delivering their genes into the host where they are replicated. Other non-viral vectors include complexes of DNA, proteins or lipids, which are constructed as particles capable of efficiently transferring genes (Pfeifer and Verma 2001). Non-viral approaches satisfying the various requirements for effective *in vivo* gene therapy are reported in Nishikawa and Hashida (2002). Problems that remain include integration of novel genes into non dividing cells such as liver, muscle and neurons, as well as the avoidance of immune response, since often the vector can elicit

an antibody response or provoke inflammation (review Gardlik *et al.*, 2005; Verma and Weitzman 2004).

1.5.3.2 Trials conducted in gene therapy

The first trials began in 1990 using an *ex vivo* strategy with two children suffering from severe combined immunodeficiency characterised by depletion of T lymphocytes, resulting in low levels of the enzyme adenosine deaminase (ADA). Somatic cells were removed, the gene encoding normal ADA introduced into the cells *in vitro*, and the modified cells reintroduced into the bodies of the children. This treatment was repeated during a two-year trial, with results showing the production of nearly normal T lymphocytes (Onodera and Sakiyama 2000). A gene therapy model that treated cystic fibrosis (*in vivo*) used an attenuated version of an adenovirus as a vector, which has a natural tropism for the lungs. This delivers a cell membrane glycoprotein - the transmembrane conductance regulator protein (TCRP) to the tissues of the lungs. However success is limited by the amount of tissues in contact with the novel DNA inhaled into the system and the persistence of the novel DNA (Griesenbach *et al.*, 2004 and 2003; Davies *et al.*, 1998). In terms of gene therapy, the *ex vivo* strategy allows the long-term persistence of therapeutic novel DNA, which is one of the main limiting factors with *in vivo* approaches. However, it is hoped that gene therapy may be used to treat conditions such as cardiovascular disease, Huntington's, Parkinson's disease, AIDS, as well as cancer (review Le and Frim 2002). Cancer is the most common disease targeted by gene therapy and accounts for up to 67% of all present trials; of these 31% use *in vitro* and 32% use *in vivo* immunotherapies (Stephens 2003; Anderson 2002).

1.5.4 Somatic transgenesis with a gene gun

The introduction of DNA to somatic cells has been achieved by the use of a hand held helium propelled gene gun. This employs particle mediated transfer of DNA to the cells usually by DNA coated gold beads, a few microns across, and bombarding the area into which the DNA is to be introduced. The force of the particle bombardment is thought to propel the beads through the plasma membrane so that any cells in the path of the particles are transfected with the transgene DNA. With the Helios® hand held gene gun no vacuum is necessary so any cell, tissue or organ that can be made

accessible may be treated. The introduction of foreign genes by particle bombardment first described by Sanford *et al.*, (1987) has been explored both *in vivo* and *in vitro* in mammals and fish. Yang *et al.*, (1990) showed transient expression of chimeric CAT and β-gal marker genes in rat and mouse tissues recovered from liver, skin and muscle, when bombarded with DNA covered gold particles *in vivo*. Transfer and expression was also achieved *in vitro* with rat and human tissues, the eight cell lines of human derivation showed expression in cells of epithelial, endothelial, fibroblast and lymphocyte origin with two of these cell lines showing stable integration. Williams *et al.*, (1991) used particle bombardment of the firefly luciferase reporter gene using an adaptation of the gun to reduce the exposed area, to show expression in liver and skin cells of living mice.

This early work outlined the uses of particle bombardment with a gene gun and this approach in mammals has been used to explore the possibilities of DNA vaccination, already shown to elicit a response that is protective in other models. Vaccination via particle bombardment uses non-infectious, non-replicating DNA that encodes only the protein of interest and this can be made inexpensively with high levels of purity. Plasmid DNA promotes a long lived, cell-mediated and humoral immunity unlike recombinant viral vaccines that contain a heterologous protein component often associated with a host response, which can reduce the effectiveness of a booster vaccination. Lodmell *et al.*, (1998) reports on the immunity conferred by vaccination using particle bombardment against rabies virus infection in mice with results that strongly suggest 100% immunity amongst those that received the vaccine and were subsequently exposed to a rabies virus challenge. Tan *et al.*, (1999), and Smooker *et al.*, (1999) review DNA vaccination using a gene gun as been reported in and the current applications of the gene gun have been outlined in Lin *et al.*, (2000) reviewing cutaneous gene therapy.

Lee *et al.*, (2000) reported on expression of foreign genes delivered by particle bombardment into the muscle of rainbow trout via the use of a gene gun. Two CAT reporter gene constructs within plasmids were used, the cytomegalovirus immediate promoter (pCMV-CAT) and the simian virus 40 early promoter (pSV2-CAT), resulting in CAT expression in the muscle tissue of all fish bombarded during a 90 day trial.

Reporter genes have also been introduced by somatic gene transfer, in Zebrafish with expression of luciferase and lacZ (Torgersen *et al.*, 2000) and also with GFP (Sudha *et al.*, 2000). Tucker *et al.*, (2001) assesses the potential use of particle bombardment for the application of DNA vaccination using GFP and CAT (pCMV-CAT and pSV2-CAT of Lee *et al.*, 2000) in juvenile Japanese flounder (*Paralichthys olivceus*). The results of this study suggest long term and stable expression with the potential for vaccination of juvenile Japanese flounder a viable option. The stable and long term expression of plasmid DNA in muscle makes this tissue an attractive target for the introduction of DNA with reference to both growth enhancement and DNA vaccination.

1.5.5 Direct injection

Direct injection is an alternative method to the gene gun for the introduction of DNA to the somatic cells, and also results in the relatively immediate biological effects observed with this type of gene transfer. The technique used by Wolff *et al.*, (1990) used direct injection of pure DNA or RNA administered into skeletal muscle of mice *in vivo* and Lin *et al.*, (1990) showed expression of reporter genes in rat myocardium following direct injection *in vivo*. Its use in gene therapy is outlined in Raz *et al.*, (1993). This technique also has been applied to various fish species; the initial experiments conducted using the common carp by Hansen *et al.*, (1991). Plasmids were constructed using the SV40 early promoter, rabbit β-cardiac myosin heavy chain promoter, human MxA promoter or an artificial promoter to drive expression of the reporter genes CAT or LacZ and were injected *in vivo*. The results of histochemical analysis after staining sections of muscle showed expression of lacZ in muscle fibres distributed along the path made by the injection needle and the CAT assay detected high levels of expression, yielding about 90% chloramphenicol conversion. Direct injection of linear transgene DNA into muscle of tilapia fish resulted in expression of the CAT reporter gene reaching maximum counts 48 hours after first injection. The decline after this time was thought to be due to destruction of the transgene by nuclease activity (Rahman and Maclean 1992).

Rainbow trout showed gene expression after direct injection of plasmid DNA containing the firefly luciferase reporter gene controlled with either viral and fish promoters. This occurred in muscle cells anterior to, as well as, along the site of

injection with maximal activity five to seven days after injection, the highest activity resulting from the viral cytomegalovirus (Anderson *et al.*, 1996). Gomez-Chiarri *et al.*, (1996) reported expression in rainbow trout tissues after both injection and particle bombardment *in vivo*, direct injection of plasmid DNA with the reporter gene luciferase yielded expression levels that persisted for 60 days after injection. Particle bombardment using DNA coated beads of the same construct luciferase showed expression that was significantly lower than was obtained by direct injection (*t*-test >0.02). Tan and Chan (1997) use Zebrafish to show pCAT expression driven by human cytomegalovirus immediate early promoter (CMV-IE), resulted in expression levels that correlated to the amount of plasmid DNA injected with high expression levels lasting up to 1 year. It was reported that the site of uptake of the transgene was in the myofibres along the site of injection concurrent with similar findings from Anderson *et al.*, (1996) and Hansen *et al.*, (1991). Direct injection of plasmid DNA is commonly used in the development of DNA vaccines (Heppell and David 2000). It is hoped that these DNA vaccines may be used to induce immunity against common pathogens which threaten commercial fish stocks, and are of interest given that the methodology used to induce immunity by the introduction of DNA to the somatic cells is interchangeable with those used in this study.

1.5.6 The case for using GH for somatic transgenesis

In vivo approaches show promise in the field of human gene therapy (Nishikawa and Hashida 2002). There has been success in conferring protective immunity after the use of DNA vaccines introduced somatically in both murine (Lodmell *et al.*, 1998) and fish (Lorenzen *et al.*, 2002 a,b; Naubaum *et al.*, 1998) and the stable long-term expression of plasmid DNA in muscle tissue (Wolff *et al.*, 1992), suggests that exogenous DNA exists long enough to usefully confer the properties of interest derived from these novel genes.

The case for growth enhancement of tilapia, as discussed, prompted the question of the feasibility of enhancing the growth of juvenile tilapia by somatically injecting the 'all tilapia' growth hormone construct (tiβA/tiGH), kindly provided by Dr G. Hwang and the opAFP/csGH construct, kindly provided by Professor C. Hew. It was hoped that the direct injection of DNA would produce a growth response in injected batches

without permanent integration into the genome as occurs with egg micro-injection. This type of manipulation has potential environmental as well as marketing benefits, since the resultant product would not contain transgenes associated with GM technology.

1.6 Objectives of this project

The first aim of this project was to investigate the longevity of plasmid DNA once injected into the skeletal muscle of tilapia, and to determine the persistence of expression gained after somatic injection of plasmid transgenes. A comparison of promoter activity was made looking at expression levels after introducing either of the reporter constructs; the tilapia β-actin promoter spliced to the bacterial β-galactosidase gene, as well as carp β-actin promoter spliced to the lacZ gene to study the effectiveness of these promoters *in vivo*.

An investigation was made into the mobility of the transgene DNA once injected into the muscle tissues from around the site of injection and from the organs after intramuscular injection. Histological staining was carried out after injection of the tilapia β-actin promoter spliced to the lacZ reporter gene, plasmid construct, to ascertain the behaviour of the protein product once in the myofibres of the injected tilapia and to find whether the exogenous protein product stayed at the site targeted by the injection procedure or was able to migrate to other sites.

The second aim of this project was to evaluate the possibility of inducing a positive growth response in juvenile tilapia after somatic gene transfer of growth hormone plasmid constructs using two different plasmid constructs; tilapia β-actin promoter spliced to tilapia growth hormone gene, and the ocean pout antifreeze promoter spliced to chinook salmon growth hormone. The tilapia β-actin enhancer element isolated from intron I was also isolated and co-injected with these constructs as well as a reporter construct to ascertain the effect this putative enhancer sequence might have on transcription of the plasmid transgene in the myofibres.

Chapter two:

Materials and methodology

2.1 Gel preparation

2.1.1 Preparation of agarose gels for analysis of nucleic acids

Migration and subsequent separation of nucleic acid bands through an agarose gel allows identification of target fragments. Selection of agarose concentration depends on fragment size. Typically 0.8 % agarose concentration was used for a DNA fragment range of between 0.8-12 kb and a 1.2 % gel to separate a fragment size between 0.4-7 kb.

Preparation of a large gel

1) 1.32 g of agarose was added to 110 ml of 1x TEB buffer for 1.2 % gel and heated in a microwave on medium until the solution clears (for 0.8 % gel, 0.88 g of agarose was added)

2) The solution was left to cool for 20 minutes after which 2 µl of EtBr was added and gently swirled to mix.

3) The agarose mix was poured into a large gel tray and combs inserted, ensuring no bubbles were trapped under the comb teeth.

4) The gel was allowed to set for 40 minutes before use.

Medium gel @ 1.2 %

65 ml of 1 x TEB buffer + 0.78 g of agarose; 15 minutes cooling + 1.5 µl of EtBr.

Small gel @ 1.2 %

45 ml of 1x TEB buffer + 0.54 g of agarose; 10 minutes cooling + 1 µl EtBr.

Preparation of TEB buffer (10x) per 100 ml

- Tris (88mM) 10.8 g
- Boric acid (88mM) 5.5 g
- EDTA (2.5 mM) 0.93 g

Adjust pH to 8.3 with Tris or boric acid

2.2 Preparation of plasmid DNA

2.2.1 Transformation of E.coli (adapted from Sambrook et al., 1989)

For transformation

1) Two 15 ml polypropylene round-bottom Falcon tubes (2059) were placed on ice to prechill.

2) Competent cells were thawed quickly on ice (XL1-blue Stratagene) and mixed carefully using a sterile pipette tip.

3) 20 µl aliquots of mixed cells were placed in each of the 15 ml Falcon tubes.

4) 0.4 µl of β-mercaptoethanol was added to each tube and swirled gently, then incubated on ice for 10 minutes. Swirling was repeated every two minutes.

5) An aliquot of plasmid DNA between 0.1-50 ng, was added to one Falcon tube. The second tube received 20 µl of TE buffer for use as a negative control. Both were mixed carefully by gentle swirling.

6) The cells were incubated on ice for a further 30 minutes.

7) The cells were heat-shocked by placing the tubes in a preheated water bath at 42°C for 45 seconds, timed heat exposure is critical for obtaining high plasmid uptake efficiencies.

8) The cells were then transferred onto ice and incubated for a further 2 minutes.

9) 0.9 ml of preheated (42°C) SOC medium (life-technologies) was added and incubated at 37°C for 1 hour while shaking 225-250 rpm.

Incubate on agar plate

Four agar plates with an ampicillin concentration of 50 µg/ml were allowed to reach room temperature in fume cupboard to avoid contamination. Aliquots were made with two separate volumes, between 10-50 µl of cells onto each plate. The cells were spread over agar until the surface of agar appeared dry, using disposable, sterile spreader.

The inoculated plates were incubated overnight until suitable colonies had developed.

Preparation of LB medium per litre

- NaCl 10 g
- trypton 10 g
- yeast extract 5 g
- SDW to final volume of 1 litre

adjust to pH 7 with 5 M NaOH and autoclave

Preparation of LB agar per litre

- NaCl 10 g
- trypton 10 g
- yeast extract 5 g
- agar 20 g
- SDW to final volume of 1 litre

Adjust to pH 7 with 5 M NaOH and autoclave, pour into 250 ml bottles for storage

Preparation of LB ampicillin agar

- prepare 1 litre of LB agar
- autoclave
- allow to cool to 55 °C
- add 1 ml of stock ampicillin (stock solution 50 mg/ml)

Pour into Petri dishes and allow to cool

2.2.2 Amplification of plasmid (After Qiagen Maxi and Qiafilter handbooks)

1) From an overnight culture of transformants, suitable colonies were picked from the agar plate using a sterile, disposable loop and transferred individually to a second agar plate (amp 50 μg/ml), by dabbing the loop onto the agar lightly. Colonies were separated using a numbered grid system marked on base of the petri dish, then each colony was placed into the centre of the formed squares.

2) The same loop was used to inoculate 3 ml of LB medium (amp 50 μg/ml) after careful retouching of the original colony, into a correspondingly labelled Falcon tube (2059).
3) The starter cultures were incubated at 37°C for 6-8 hours, while shaking vigorously at ~ 300 rpm. The plated colonies were also incubated for this time.
4) 100 ml of LB medium amp (50 μg/ml) was inoculated with 100 μl of starter culture, in a 500 ml conical flask. This was incubated overnight at 37°C with vigorous shaking for 12-16 hours until late logarithmic phase was reached and a spectrophotometer reading OD_{600} = 0.6 was obtained.
5) Cells were harvested by centrifugation at 6000 rpm for 15 minutes at 4°C.
6) The pellets were either used as needed or stored at -20°C. Pellets can be stored for several weeks at –20°C.

2.2.3 Long-term storage of E.coli - Glycerol stocks

1) A stock solution of glycerol was sterilized for storage at room temperature.
2) 250 μl of sterile glycerol was added to 750 μl of logarithmic phase *E.coli* cells removed from the starter culture and mixed thoroughly using a pipette to ensure an even suspension of cells in the glycerol.
3) The suspension was frozen in liquid nitrogen and stored at -80°C.

2.2.3.1 Recovery of transformed colonies from glycerol stocks

1) The glycerol stock was thawed on ice.
2) Then using a sterile, disposable loop, an inoculum was streaked across LB agar containing ampicillin (50 μg/ml).
3) The inoculum was spread using same loop with three more streaks running the second streak through the first and so on to ensure proper spreading of the E.coli.
4) The agar plate was left to incubate overnight at 37°C overnight until suitable colonies appeared.

5) Colonies were picked off the plate with a sterile disposable loop and used to inoculate a 3 ml starter culture.

2.2.4 Extraction of E.coli cultures (after Qiafilter Plasmid Purification Handbook)

Composition of buffers listed in Qiafilter plasmid purification handbook.

1) After harvesting bacterial cells by centrifugation (II.2 step 5), the cells were resuspended in 10 ml of buffer P1.
2) Lysis buffer P2 was then added and the tubes gently mixed, to avoid shearing the genomic DNA by inverting between 4–6 times and allowed to stand for less than 5 minutes, allowing maximal release of plasmid DNA without release of chromosomal DNA.
3) Chilled neutralisation buffer P3 was added to the lysate and mixed gently by inversion of tube 4-6 times.
4) The lysate was then poured into a Qiafilter Cartridge and left to incubate at room temperature for 10 minutes, in which time the white precipitate floats to the top containing proteins, genomic DNA and detergent.
5) During the incubation of the lysate a Qiagen-tip 500 was allowed to equilibrate by the application of 10 ml of buffer QBT.
6) After incubation the lysate was syringed using the supplied plunger from the cartridge to the Qiagen-tip and allowed to clear through by gravity flow. The through flow was collected and poured back through the tip once more to ensure maximum yields of plasmids.
7) The tip was then washed twice, each wash with 30 ml of buffer QC, allowing to clear by gravity flow.
8) The DNA was then eluted with two 10 ml applications of buffer QF, leaving one minute between treatments.
9) DNA was precipitated by 14 ml of isopropanol and spun in a Sorvall RC-5B centrifuge, with Sorvall SLA-600m rotor at 9000rpm for 30 minutes 4°C.
10) The formed pellet of DNA was washed with 5 ml of room temperature 70% EtOH and then spun again as before for 10 minutes.

11) The pellet was left upside down to air dry and then resuspended in 1ml TE buffer.

2.2.5 Spectrophotometric quantification of DNA

Allow the SP6-550 UV-VIS spectrophotometer to warm up before use for at least 30 minutes to ensure optimal working conditions. For quantification, readings should be taken at 260 nm for DNA and for RNA 280 nm. An optical density (OD) of 1 is equal to ~ 50 μg / ml for double stranded DNA and ~ 40 μg / ml for single stranded DNA and RNA. Measuring the ratio between OD_{260}/OD_{280} gives an approximation of the purity of samples. Pure samples of DNA result in a value of 1.8 and RNA 2.0, contamination lowers these values.

1) The wavelength of the spectrophotometer was set to 260 nm, ensuring that the light filters were positioned correctly.
2) The counter was set to zero using 1 ml of buffer, the same as was used to suspend the DNA sample in and placed in a crystal cuvette.
3) 10 μl of buffer was removed and replaced with 10 μl of sample DNA. The OD_{260} and OD_{280} were taken.
4) The amount of DNA in sample (μg / ml) is calculated using the following equation;

$$\text{Sample } \mu g / ml = OD_{260} \times \text{dilution factor} \times 50$$

2.2.6 Gel quantification of DNA

1) Samples and λ DNA marker were run through a gel with electrophoresis.
2) A Polaroid photograph was taken of the gel after visualisation on a transilluminator.
3) The amount of fluorescence given off by the DNA is proportional to the total mass of the DNA and so an estimation of the quantity of DNA in samples was made by comparison to the nearest band from the λ DNA marker

2.3 DNA amplification by Polymerase Chain Reaction (PCR)

In these experiments the length of time the plasmid persists was assessed using PCR on muscle tissue taken from the site of injection. This study also investigated whether the DNA migrated around the body, away from the area targeted, and in particular whether it was transported to any other organs. In order to assess this, organ tissue was collected from the liver, spleen, gonads and brain of individuals; the DNA extracted and amplified using PCR. These key questions provide knowledge about the behaviour of DNA once somatically introduced and the length of time the constructs can survive in the host cell.

The amplification of a segment of DNA can be achieved using PCR resulting in multiple copies of the target sequence. This occurs in a three-stage cycle of denaturation, annealing, and extension of primers.

The following conditions were used to amplify genomic DNA in order to investigate whether the exogenous DNA was present in the organs of DNA injected individuals and also to assess the length of time the plasmid DNA remained at the site of injection.

2.3.1 Amplification of tiβAP/LacZ plasmid using PCR

tiβAP TBA forward primer 5′-AGT GGT TCT GTC GGC TAG

LacZ reverse primer 5′-GCC ATT CAG GCT GCG CAA C

The Hybade PCR machine was programmed to run the following temperatures and times; initial denaturation of DNA duplex, genomic, 95 °C for 5 minutes, followed 30 cycles of denaturation 95 °C for 30 seconds, an annealing temperature of 52 °C for 50 seconds and 72 °C for extension with a final extension time of 5 minutes at 72 °C, when the samples were removed and stored at 4 °C until needed. The PCR product generated using the TBAF primer and LacZR primer was 439 bp in length.

2.3.2 Amplification of cβAP/LacZ

cβA forward primer 5′-CAG TGT GCA GCC CTT CAG TC

LacZ reverse as used in 2.3.1

Initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of 95 °C for 50 seconds, to 52 °C for 50 seconds with the extension time set at 50 seconds at 72 °C. Final extension 72 °C for 5 minutes, resulting in a 845 bp product.

2.3.3 Amplification of tiβA/tiGH

tiβA forward primer as used in 2.3.1

TIGH reverse 5'-CTG CTG CAG GTT ACA A

Initial denaturation at 95 °C for 5 minutes, followed by 95 °C for 30 seconds, annealing temperature of 66 °C for 30 seconds, extension 72 °C for 50 seconds all over 25 cycles, before a final extension time of 5 minutes at 72 °C, resulting in a 560 bp product.

2.4 Preparation and injection technique

1) Following purification of plasmids and spectrophotometric determination of DNA concentration, varying concentrations of plasmid DNA were prepared.
2) Using, either a Hamilton 50 µl syringe or 1 ml insulin syringe, a given dosage was correctly measured and loaded into syringe
3) 2-Phenoxyethanol (2-PE; Sigma) was used to anaesthetise the fish at a suitable concentration (~3% v/v) for the size of fish used in each experiment and mixed into a receptacle of tank water (to minimise discomfort and reduce shock) at a volume that enabled total immersion fish.
4) The anaesthetised fish were placed on a damp clean cloth on a platform, head facing to the left each time, to enable easy location of the injection site from tissue recovery.
5) The fish were then blotted dry at the injection site using tissue and injected, the needle inserted in between the dorsal and anal fin, slightly above the lateral line.
6) After depression of the syringe, the needle was left in the tissue for approximately 30 seconds, then withdrawn while pressing down on the injection site with tissue to prevent excess loss of injected fluid.

7) The site was then sealed with a small amount of Vaseline, and the fish allowed to recover in a well-aerated tank.

2.5 Fish maintenance

The sibling batches of control tilapia were reared in large tanks until needed. They were then transferred into experimental tanks after injection. Water temperature was maintained at 28.0 ± 1°C. An automatic switch controlled the 12 hour dark and light cycle. The fish were fed twice a day on commercial trout feed pellet.

Chapter three:

Determination of the fate, persistence and uptake of plasmid transgene DNA in tilapia muscle

3.1 Introduction

In the field of fish transgenics, prevenient research often utilised mammalian sequences for introduction into a wide variety of fish species, resulting in varying degrees of success, prompting a move to using sequences of piscine origin (Alam *et al.*, 1996; Penman *et al.*, 1991). These early experiments demonstrated that both mammalian and fish promoters were effective in providing gene expression in fish. The same was found to be true after the direct injection of DNA into muscle tissue *in vivo*. This method was first used successfully by Wolff *et al.*, (1990). These authors directly injected plasmid DNA into mouse skeletal muscle, reporting on the rapid internalisation of the DNA and its subsequent expression, and later went on to detail gene expression in rat myocardium following direct injection of a reporter construct (Wolff *et al.*, 1992). Strong expression of the CAT and LacZ reporter genes driven by mammalian regulatory sequences was reported by Hansen *et al.*, (1991) after directly injecting DNA into the muscle tissue of juvenile common carp. They described high CAT activity in juveniles after injection of 50 μg of DNA, while the doubling of this dose resulted in a retardation of CAT activity. Rahman and Maclean (1992) used the pFV1 and pFV2 constructs developed by Liu *et al.*, (1990c) for direct injection into the muscle of tilapia fish. Both constructs resulted in CAT activity, although increased concentrations of the DNA did not yield an obvious increase in expression. They reported that expression was, on the most part, detected 48 hours post injection, whilst at 24 hours and 7 days there was reduced CAT activity, probably owing to the lag time in which the DNA is taken up into the nuclei of cells and from transgene destruction by nuclease activity respectively.

Intramuscular injection can induce strong and long lasting expression of reporter genes, although different times of peak activity and total duration of expression have been reported (Kanellos *et al.*, 1999; Heppell *et al.*, 1998; Anderson *et al.*, 1996; Rahman and Maclean 1992). Anderson *et al.*, (1996) using promoters of mammalian, fish and viral origins spliced to the luciferase (LUC) reporter gene, found that expression persisted in the tissues for up to 115 days with maximal activity up to 7

days post injection into rainbow trout skeletal muscle. Gomez-Chiarri *et al.*, (1996) obtained results which suggested expression in rainbow trout muscle increased for up to 60 days and reported significantly higher luciferase expression when injecting 50 μg as compared with 25 μg or 100 μg. These authors used expression vector with a viral regulatory region; cytomegalovirus (CMV) enhancer and herpes simplex thymidine kinase (tk) promoter (pCMVtkluc) after Winkler *et al.*, (1991). Tan and Chan (1997) introduced a CMV/CAT reporter construct into zebrafish muscle, which at a concentration of 5 μg of CMV/CAT was shown to increase up to 7 days post injection with high levels of expression lasting for up to a year.

The production of transgenic animals through gene transfer is well established in fish primarily for the improvement of broodstock for use in aquaculture. Transgenic technology has been applied to many farmed fish species such as tilapia (de la Fuente *et al.*, 1999; Rahman *et al.*, 1999), carp (Tran and Nguyen 1993), rainbow trout (Anderson *et al.*, 1996), and Atlantic and coho salmon (Devlin *et al.*, 2004, 1994). Much of the developmental research is applied to species commonly used as models for genetic research and include the zebrafish (Udvadia and Linney 2003), medaka (Matsumoto *et al.*, 1992) and goldfish (Wang *et al.*, 1995). These species are often used for functional analyses of *cis*-acting regulatory elements owing to the availability of mutants and fast, cheap transgenic technologies (Muller *et al.*, 2002). The identification of *cis*-acting regulatory elements depends on these types of transient expression studies where temporary extrachromosomal transcription of exogenous genes occurs.

Gong *et al.*, (1991) used transgenic medaka as a transient expression system to analyse the fish promoter regions of AFP genes from several species of fish. Winkler *et al.*, (1992, 1991) used transgenic medaka and zebrafish to test a number of inducible or constitutive promoters and enhancer elements isolated from both piscine and higher vertebrate origins. Moav *et al.*, (1993) studied the function of elements from the regulatory region of the carp β-actin gene in both goldfish and zebrafish embryos. The rapid development of these small fish is ideal for these types of functional analyses to

study early events of transgene expression. However, although model species can provide detailed genetic information in developmental research, they are not always a good substitute for the target species which may respond differently owing to genetic heterogeneity (Moav *et al.*, 1993, 1992).

Great variation in expression levels are obtained when introducing novel DNA by direct injection into different vertebrate species (Hengge *et al.*, 1996). Many early experiments in the field of fish transgenics used mammalian, viral or avian promoters, but these often resulted in poor expression (Alam *et al.*, 1996; Penman *et al.*, 1991; Guyomard *et al.*, 1989). There was, therefore, a move to using regulatory sequences of piscine origin (Cavari *et al.*, 1993; Du *et al.*, 1992; Moav *et al.*, 1992a, b; Gong *et al.*, 1991; Shears *et al.*, 1991; Liu *et al.*, 1990a, b, c). Du *et al.*, (1992) developed the opAFP/csGH construct which proved to yield transgenic coho salmon with enhanced growth. This construct was also introduced into Atlantic salmon, again with success in positively altering the growth of transgenic individuals, as compared with their non transgenic siblings (Devlin *et al.*, 1994). Other promoter regions from genes of piscine origin include the rainbow trout metallothionein A and B gene (Olsson *et al.*, 1995; Zararullah *et al.*, 1989), winter flounder AFP genes (Gong *et al.*, 1995), the zebrafish myosin heavy chain gene (Muller *et al.*, 1997) as well as regulatory sequences isolated from β-actin genes of various fish origins.

There has been a long history of using β-actin promoters to drive gene expression in fish. Liu *et al.*, (1991, 1990a, 1989) made a detailed functional analysis of the transcriptional control elements in the carp β-actin gene. Studying the transcriptional activity of the various cβAP/CAT constructs resulted in the characterisation of four transcriptional control regions; a negative element upstream of the transcription start site, the proximal promoter (in the first 100 bp upstream) and in intron I; a negative control element close to the 5′ end, and an enhancer located near the 3′ end of intron I. Within the proximal promoter, a serum-response element, the $CC(A/T)_6GG$ unit, known as the CArG motif, was identified along with the CAAT box, and TATA motif (Liu *et al.*, 1990b). These transcriptional elements are similarly found in the control

regions of many other genes within the genome and are evolutionarily conserved across all classes of vertebrates, all containing a non-coding first exon and transcriptional control elements in intron I (Liu *et al.*, 1990a; Boxer *et al.*, 1989; Minty and Kedes 1986; Orita *et al.*, 1989; Subramanium *et al.*, 1989; Walsh *et al.*, 1989).

The conservation of β-actin regulatory elements in vertebrates suggested that their function was also conserved, and so these regulatory elements may drive similar levels of expression in different transgenic vertebrate species. Great variation in levels of expression, however, were recorded in heterogeneous piscine tissues and across different species when examining CAT reporter gene activity transfected into carp epithelial cells, rainbow trout hepatoma cells and rainbow trout fibroblasts, as well as, goldfish and zebrafish embryos (Moav *et al.*, 1993, 1992). When comparing zebrafish and goldfish embryos, the intron I containing the putative enhancer region was found to have an enhancing effect on expression in zebrafish embryos and the CArG motif was required for maximal activity but this was not the case in goldfish embryos, where expression from the construct was overall approximately 100-fold higher. Since fish are an evolutionary diverse group, with teleosts being the most diverse class of vertebrates with approximately 25,000 species, it is preferable to use a specific promoter isolated from the same species of fish, ensuring higher homology than might otherwise be achieved when using regulatory regions from other unrelated fish (Devlin *et al* 1994; Rahman *et al.*, 1998; Nam *et al.*, 2001).

It is important to be able to assess the length of time the plasmid itself remains in the tissues post injection. This may indicate how lasting expression from the DNA would be, as well as how long the exogenous DNA survives post injection, which is significant when considering the potential commercial aspects of introducing DNA in this way to ensure that they persist long enough to confer the properties of the genes. Anderson *et al.*, (1996) noted the plasmid luciferase reporter construct once injected into rainbow trout muscle was very stable, with little plasmid degradation over the period of study (> 115 days). Gomez-Chiarri *et al.*, (1996) reported on luciferase expression lasting for 60 days, but did not note the length of time the injected plasmid

DNA persisted for. Kanellos *et al.*, (1999) injected plasmid DNA, containing the lacZ reporter gene into goldfish muscle, reporting that the DNA lasted for up to 70 days in the tissues without migrating elsewhere or becoming integrated. Expression peaked at 21 days and degraded slowly after which time.

As documented, there is much variation in the results obtained after somatic gene transfer into various fish species. This relates both to studies investigating gene regulation as well as the development and application of DNA vaccines. The commercial importance of tilapia and the novel nature of this type of gene transfer in tilapia fish results in the need to investigate what occurs to transgene DNA once injected into tilapia directly, rather than extrapolating from models based on other fish species.

The experiments in this chapter were designed to investigate the following objectives;

1) The determine the optimum DNA concentration and volume of buffer required to facilitate uptake in muscle tissue, and to ascertain the behaviour of exogenous DNA immediately after somatic injection into tilapia muscle tissue using a tilapia beta actin/lacZ reporter construct.
2) To compare levels of expression of beta-galactosidase under the control of two different promoter elements isolated from piscine origin, after somatic injection of these plasmid transgenes.
3) To detect the persistence of exogenous plasmid DNA once introduced, to acertain whether it will persist long enough to usefully confer the associated properties of the transgene.
4) To investigate the length of time the expression from the injected DNA may be detected for in muscle tissue samples from the site of injection.
5) To investigate the fate of the transgene, to determine whether the transgene DNA can migrate from the site of injection and into the organs.
6) To ascertain whether on injection of the lacZ reporter gene into the muscle tissue, the subsequent beta-gal expression from this transgene could be detected in any organs, suggesting movement of the protein away from the target site, and if so which organs.
7) To visualise the spread of beta-galactosidase through the muscle cells using histochemical staining after intramuscular injection of a reporter construct.

3.2 Materials and Methods

3.2.1 The MUG assay for quantification of the expression of β-galactosidase protein

The protein and β-galactosidase (MUG) assay were used in this study to determine the expression of the lacZ reporter gene after injection into muscle tissues of sibling juvenile tilapia. The protein assay ensures that there is a uniform level of protein in each sample across the sample group and the subsequent MUG assay quantitatively measures the hydrolysis of a β-galactosidase substrate linked to a fluorogen. The substrate 4-methylumbelliferyl-β-D-galactoside is cleaved yielding 4-methylumbelliferone (7-hydrosy-4-methylcoumarin, MU) a fluorescent molecule when ionised above pH 8 after excitation at 365nm. The increase of fluorescence is directly proportional to the amount of β-galactosidase, and so the level of β-galactosidase expressed in the injected tissue tested may be ascertained.

3.2.1.1Preparation of samples

Fish that were subject to injection of plasmid DNA of either the carp β-actin/lacZ reporter construct, the tilapia β-actin/lacZ reporter construct, or control fish, injected with buffer alone were allowed to recover and held in tanks in accordance with fish injection technique and fish care techniques in chapter two. The fish were overdosed with 2-PE and decerebrated in accordance with the Animals (Scientific Procedures) Act 1986. Tissue samples were harvested from individuals, weighing 0.29 g +/- 0.01. These were removed from the site of injection, after descaling and detaching the skin using a scalpel. The samples were then finely chopped into small pieces using a scalpel and placed into 1.5 ml eppendorf tubes. 200 µl of homogenisation buffer were added to each tube and immediately frozen in liquid nitrogen for storage at -80 °C.

The organs harvested for the assay included the liver, weighing 0.12 g +/- 0.01, the spleen weighing 0.05 g +/- 0.01 and blood samples using a BD-1 ml insulin syringe. The syringe was thoroughly rinsed using sodium citrate solution in order to prevent the blood from clotting. 1 ml of sodium citrate solution was placed into a falcon tube (2059) ready to receive 9 ml of the sampled blood following the advised 1:9 ratio

(Sigma). The blood was collected from the caudal vein, decanted into the falcon tubes mixed carefully by inversion and stored on ice until ready for use. The samples were spun at 3000 rpm in the TKO mini-centrifuge for 10 minutes, the supernatant was drawn off and stored at -80 °C with the red blood cells for later use in the protein and MUG assay.

3.2.1.2 Materials for Protein assay

Homogenisation buffer

- 1M Tris pH 7.5 100 µl
- 20 µl 5M NaCl 20 µl
- Triton (for 0.1 %) 10 µl
- SDW (to make up to 10 ml)

Bovine serum albumin (BSA) standard (25 µg/ml)

- stock BSA (Promega) 2.5 µl
- SDW 997.5 µl

3.2.1.3 Materials for MUG assay

Reaction cocktail

Prepare without 4-methylumbelliferyl-β-D-galactoside, at room temperature, then add as last step.

- 1M Tris-HCl, pH 7.5 250 µl
- 250 µl 5M NaCl 250 µl
- 20 µl 1M $MgCl_2$ 20 µl
- 2-mercaptoethanol 8.3 µl
- SDW (to make 10 ml) 9.422 ml
- (60 mM stock) 4-methylumbelliferyl-β-D-galactoside 50 µl

4-methylumbelliferyl-β-D-galactoside (60 mM stock)

Dissolve 10 mg of 4-MU-β-D-Gal., FW 338.3, into 0.5 ml EtOH. Store at – 20 °C.

Trichloroacetic acid solution

Add 25 % TCA to 75% SDW (v/v) for 25% TCA stock solution.

Glycine-carbonate reagent (GC) 200 ml

- Glycine FW 75.07 1.997 g
- 1.760 g Na2CO3 FW 106.0 1.760 g
- SDW 200 ml

pH adjusted to 10.7 with 5M NaOH and filter through 0.45 μm filter

Methylumbelliferone Standard MU stock solution.

First make concentrated stock solution; 2 mM MU: 4-methylumbelliferone, sodium salt (FW 198.2)

- MU 39.6 mg
- SDW 100 ml

Working stock solution; 1 μM MU

- 10 μl of concentrated stock MU solution
- 10 ml SDW

3.2.1.4 Methods; sample preparation for protein assay

(adapted from Bradford, M.M. 1976)

1) The samples were thawed on ice then homogenised.
2) Then centrifuged at full speed for 5 minutes and the supernatant drawn off and placed into new 1.5 ml eppendorf tubes.
3) Then the samples were placed in water bath at 37 °C for 10 minutes
4) The samples were then spun down again for a few minutes.
5) 5 μl was drawn off and added to 0.5 ml tubes with 95 μl SDW, the rest of the supernatant was stored at –20 °C for the MUG assay.

3.2.1.5 Protein assay

To make the standard curve, each standard concentration was mixed and the absorbency read of the spectrophotometer after 5 minutes as shown in table 3.1.

Conc (µg/ml)	0	1.25	2.5	3.75	5	7.5
BSA (25ug/ml)	0	50 µl	100 µl	150 µl	200 µl	300 µl
DW	800 µl	750 µl	700 µl	650 µl	600 µl	500 µl
Dye reagent	200 µl	200 µl	200 µl	200 µl	200 µl	200 µl

Table 3.1 Table showing each concentration needed for standard curve.

Dye reagent supplied by Biorad.

1) After all readings were taken for the standard curve, 5 µl of homogenate from 3.2.1.4, step 5, were added to 795 µl DW and then mixed in a 1 ml cuvette.
2) 200 µl of dye was added and the sample was placed in the spectrophotometer.
3) Absorbency was read after 5 minutes.

3.2.1.6 MUG assay

1) The samples were thawed on ice and centrifuged.
2) Following the result of the protein assay, shown in table 3.1, the correct volume of homogenisation buffer was added to each sample to result in identical protein concentrations in a total volume of 40 µl.
3) 160 µl of reaction cocktail was added to each sample and incubated at 37 °C for 30 minutes. During this time the standard curve was made.

3.2.1.7 Standard curve

The TKO 100 mini-fluorometer was turned on 15 minutes before use (as instructed in manufacturers instructions) and calibrated as follows.

1) 1.9 ml GC reagent + 100 µl DW was put into a crystal cuvette, mixed well and the machine zeroed by adjusting the 'zero' knob on the fluorometer.

2) The cuvette was cleaned using fresh GC reagent, then 1.9 ml of GC reagent was added with 100 µl of the 1 µM MU working solution, mixed well and the machine was adjusted to read 500 using the 'Scale' knob.
3) The standard curve was then made using the following dilutions, shown in table 3.2

	GC reagent	1 uM MU stock
25 nM	1.95 ml	50 µl
75 nM	1.85 ml	150 µl
100 nM	1.8 ml	200 µl
150 nM	1.7 ml	300µl
200 nM	1.6 ml	400 µl

Table 3.2 Shows the concentrations of standards (MU) for the standard curve.

3.2.1.8 β-galactosidase assay

1) After incubation the samples were removed and the reaction stopped with the addition of 50 µl of 25 % TCA and cooling on ice.
2) The samples were then spun down at full speed to clear the solution.
3) Each sample was then loaded into a 2 ml crystal cuvette with GC reagent at appropriate volumes in order to obtain a reading from the TKO, i.e. 100 µl of sample with 1.9 ml GC; 10 µl of sample with 1.99 ml GC etc.

Readings were converted into nM concentration = MU unit/µg protein.

3.2.2 Removal of organs and extraction of genomic DNA

For detection of the transgene the polymerase chain reaction was used in order to locate any transgene DNA which may be present in samples. PCR conditions are set out in chapter two, the general materials and methods section but extraction of genomic DNA is set out here. After injection of sibling juvenile tilapia with transgene DNA, the following DNA extraction technique was used.

1) Fish were overdosed using 2-PE and decerebrated. The liver, spleen and gonads were removed from the peritoneal cavity and placed into eppendorf tubes.
2) The brain was removed from the cranium, all organ samples were frozen immediately in liquid nitrogen and stored at – 80 °C for later use.

3.2.2.1 Extraction of genomic DNA

1) The tissue samples were removed from storage at – 80 °C and thawed on ice.
2) 300 µl of TEN (1.5 % SDS) were added to each sample
   - 18.5 ml TEN
   - 1.5 ml 20 % SDS
3) 100 µl of proteinase K was then added to each sample, mixed well by pipetting up and down.
4) The samples were then placed in an incubator for 2 hours at 60 °C, shaking samples to mix after 1 hour.
5) After incubation, samples were chilled on ice and 400 µl of phenol was added in a fume cupboard after taking appropriate precautionary safety measures.
6) The samples were loaded onto a spinning tray and allowed to mix for 40 minutes, to separate fats and proteins.
7) The samples were centrifuged for 5 minutes at full speed, then the bottom organic layer was removed using a P 1000 pipette, leaving the top aqueous layer.
8) 400 µl of phenol, chloroform, isoamyl alcohol (IAA) (25:24:1 v/v/v) was then added to each tube in a fume cupboard and allowed to mix for 30 minutes.
9) The emulsion was then spun down at full speed in a bench top centrifuge.
10) Step 7 was repeated
11) Finally chloroform and IAA (24:1 v/v) was added and mixed for a further 30 minutes.
12) The tubes were then spun at full speed and the top, clear, aqueous layer removed and placed into a clean 1.5 ml eppendorfs.

To prepare TEN buffer

- Tris (hydroxymethyl) aminomethane (100mM) 1.211 g
- EDTA (Ethylenediaminetetraacetic acid) (10mM) 0.372g
- NaCl (250mM) 1.461 g

Adjust to pH 8.0 with HCL add 1 % SDS (w/v) for solid tissues

3.2.2.2 To precipitate DNA

1) 0.6 x volume of homogenate of EtOH was added to samples and mixed well. Precipitate appeared like cotton wool.

2) The samples were centrifuged at full speed for 3 minutes and the supernatant carefully aspirated so as not to dislodge the DNA pellet. This step removes salts, phenol, chloroform and alcohol.

3) The pellets were washed in 70 % EtOH, centrifuged, then supernatant removed.

4) The eppendorf tubes were placed upside down and the pellets allowed to air dry.

5) Once dry they were resuspended in either 200 µl or 400 µl of SDW, depending on the size of the DNA pellet obtained and the quantity of genomic DNA obtained as determined by gel electrophoresis, then stored at – 20 °C, until needed.

3.2.3 Histology

The muscle tissue was sampled from the site of injection after the intramuscular injection of 25 µg 100 μl^{-1} of plasmid tiβAP/lacZ and was harvested 11 days after initial injection to ensure high levels of β-galactosidase expression. The tissue was first stained with X-gal, sectioned and fixed to slides for observation with a light microscope. Images were produced using an overhead Nikon microscope camera. The photographs were taken under different magnifying resolutions (x 10, x 422 and x 844) in order to examine the staining pattern obtained more closely, as well as from lower resolutions, to allow larger sections of tissue fibres for observation.

3.2.3.1 Materials needed for sectioning and histology

20 ml of working fixative

(2 % Paraformaldehyde / 0.2 % Glutaraldehyde) was prepared as follows;

- (4 %) paraformaldehyde 10 ml
- 0.1M Sodium phosphate pH 7.6 9.84 ml
- (66 mM $Na_2H(PO_4)$ + 33 mM NaH_2 (PO4))
- 25 % glutaraldehyde 0.16 ml
- Nonidet P-40 (0.02%) 4 µl

4 % Paraformaldehyde

This was made in fume cupboard, and was prepared as follows;

- 8 g Paraformaldehyde powder was dissolved in 150 ml 0.1M Sodium phosphate pH 7.6, while heated to 60°C and stirred continuously.
- 10 M NaOH was added to clear the solution, one drop per minute.

The total volume was brought to 200 ml using 0.1M Sodium phosphate pH 7.6 and was subsequently filtered using Whatman filter paper. The solution was stored in 4°C until needed.

X-gal stain 20 ml

- 0.1 M Sodium phosphate pH 7.6 17.2 ml
- 2 M $MgCl_2$ 13 µl
- 50 mM $K_3Fe(CN)_6$ 1.2 ml
- 50 mM $K_4Fe(CN)_6$ 1.2 ml
- 400 µl of 1 mg/ml X-Gal 400 µl

When combined, the stain was then filtered through 0.45 µm disposable filtration unit. Stocks were made and stain was freshly made, all staining was conducted at 4°C in the dark.

PBS (phosphate buffered saline) 500 ml of solution, pH 7.4

- NaCl 40 g
- KCl 1 g
- $Na_2H(PO_4)$ 7.2 g
- KH_2PO_4 1.2 g

These components were dissolved in 450 ml of SDW, then topped up to make 500 ml and pH adjusted with 4 M HCl.

3.2.3.2 Methods for tissue sectioning

1) Samples of tissue were taken from the injection site (11 days post injection) and immediately immersed in working fixative overnight at room temperature.
2) The samples were then washed three times for 15 minutes each in PBS and placed in freshly made X-gal stain for 3 days at 4°C.
3) The samples once removed from the X-gal were dehydrated in ascending concentrations of ethanol for 1 hour at each concentration (30 %, 50 %, 70 %, 80 %, 90 % and 100 % ethanol)
4) The ethanol was removed by immersion in 50:50 (v/v) 100 % ethanol:butan-1-ol, left overnight, then 25:75 (v/v) 100 % ethanol:butan-1-ol, left overnight and finally three changes of 100 % butan-1-ol over 24 hours.
5) The butan-1-ol was replaced with paraffin wax (Sigma; paraplast). The following steps were all completed at 58°C when paraffin wax is molten. Firstly washed in 50:50 butan-1-ol:paraffin wax overnight, the samples were placed in 25:75 butan-1-ol:paraffin wax, again overnight, and finally placed in 100 % paraffin wax, replacing the wax for new three times, for 48 hours each wax replacement.
6) The samples were then moulded into a block, allowed to set and cut into a trapezium shape then mounted onto wooden blocks.

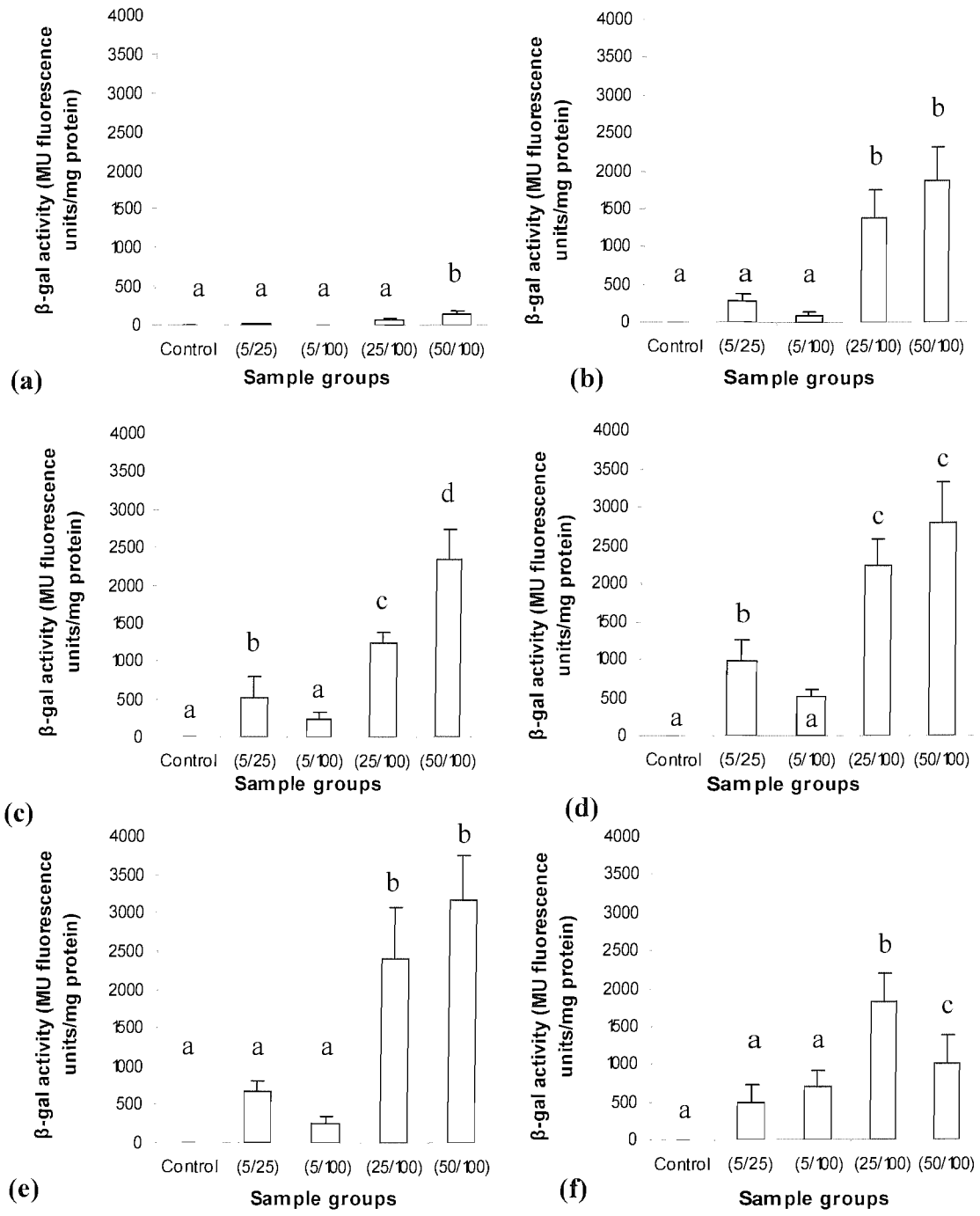
7) Disposable knives (R.A. Lamb) were used to section the tissue to a thickness of 10 μm on a rotary microtome, collected and placed into a water bath set at 40°C using a brush.
8) After complete expansion and smoothing of the tissue sections, a silane coated slide (Sigma) was used to pick the sections up and left to dry overnight at room temperature.
9) Once dried the sections were treated in Histoclear (Sigma) by immersion for 30 minutes twice and allowed to dry once more overnight.
10) The sections were then mounted using DPX and a coverslipped for later analysis under a light microscope.

3.3 Results

3.3.1 Optimising expression of exogenous plasmid DNA

It was important to ascertain the way in which plasmid DNA behaved once intramuscularly injected into tilapia muscle. It was important to study the effect of changes in buffer volume on expression levels and whether this provided a way to reduce the range about the mean. These types of experiments can result in large variation owing to the variable efficiency of the injection procedure and recovery of tissue from this site (Anderson *et al.*, 1996; Gomez-Chiarri *et al.*, 1996). Batches of fish were selected from sibling brood stock to reduce any differences that may occur when using juvenile fish from different lines. Figure 3.3.1 (a) – (f), shows the mean (±SE) β-gal expression of muscle tissue homogenates after (a) 24 hours, (b) 3 days, (c) 5 days, (d) 7 days, (e) 9 days and (f) 11 days after intramuscular injection of 5 μg 25 μl^{-1} and 5, 25 and 50 μg 100^{-1} of the plasmid tiβAP/lacZ (n = 10). At 11 days post injection, all muscle sample homogenates were tested for the presence of the transgene using PCR, and results proved positive in each sample (results not shown). The ANOVA general linear model was used to look for significance using Tukey pairwise comparison to a 95 % C.I. ($P < 0.05$). Significance is indicated by corresponding letters above mean (±SE) values.

Figure 3.3.1 (a) – (f)



Figures 3.3.1 (a) – (f). Optimisation of β-galactosidase expression. The effect of DNA dose and buffer volume over time after single an initial intramuscular injection of plasmid tiβAP/lacZ (n = 10). Those which are statistically different are marked using different letters (a:b).

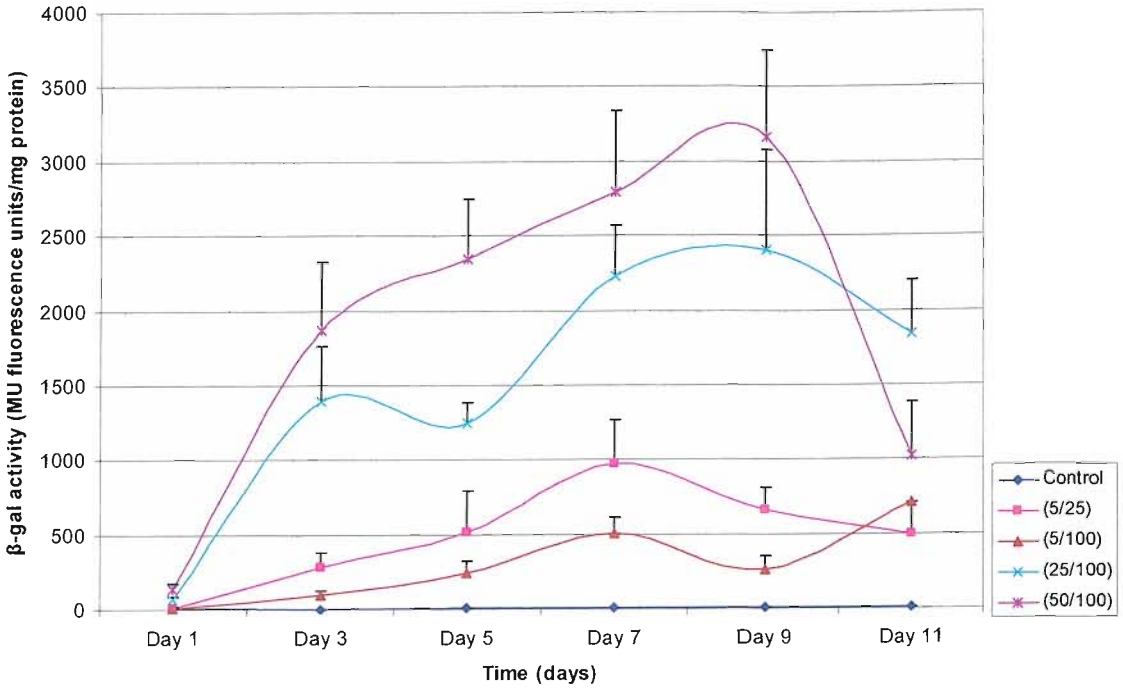


Figure 3.3.1.1 Summary of all treatments over time. This graph shows the collation all of treatments over time with mean (±SE) β-gal expression in muscle tissue homogenates from tilapia sacrificed over 11 days, after intramuscular injection of 5 μg 25 μl^{-1} and 5, 25 and 50 μg 100^{-1} of the plasmid tiβAP/lacZ (n = 10).

The changes in β-galactosidase expression observed after intramuscular injection of plasmid tiβAP/lacZ, were plotted over time to observe the resulting trend lines (figure 3.3.1.1). This data falls into two groupings and can be picked out in figure 3.3.1.1; those resulting in high β-gal expression and those which result in lower β-gal expression. Firstly, the upper set which produces comparatively strong expression levels and includes the sample set injected with 25 μg 100 μl^{-1} and 50 μg 100 μl^{-1} of DNA, and the second grouping, containing the controls injected with buffer alone, the 5 μg 25 μl^{-1} and 5 μg 100 μl^{-1} sample sets, which never reach more than 1000 MU of β-gal activity.

Initially, 24 hours after the introduction of the plasmid transgene, the highest concentration of DNA (50 μg 100 μl^{-1}) proved significantly different from all other volume and concentration parameters (figure 3.3.1 a). From days 3 to 9 (figures b,

Initially, 24 hours after the introduction of the plasmid transgene, the highest concentration of DNA (50 μg 100 μl^{-1}) proved significantly different from all other volume and concentration parameters (figure 3.3.1 a). From days 3 to 9 (figures b, d, e), there was no difference, statistically, when comparing the 50 μg DNA dose with the 25 μg samples, except day 5, where a dip in expression of the 25 μg DNA dose was observed (figure 3.3.1 (c), 3.3.1.1). The 25 μg sample group resulted in significantly higher β-gal expression than the two smaller (5 μg) DNA concentrations and the control group, but for days 5 and 7 when, although the expression obtained by this sample group yielded higher expression than the 5 μg 25 μl^{-1} samples, it was not significant. The smaller volume used in the 5 μg 25 μl^{-1} sample set consistently expressed more β-gal than the 5 μg 100 μl^{-1} sample group. These factors formed the rational for the results set out in this section and provided guidelines with which to progress to injecting GH constructs into tilapia.

3.3.2 Promoter efficiency; a comparison of cβAP/lacZ and tiβAP/lacZ constructs

Prior to injecting any growth hormone constructs into the somatic cells, it was vital to ascertain the length of time the DNA persisted in the tissue of tilapia and important to identify for how long the DNA continued to give rise to detectable expression. It was also necessary to select a promoter and determine its efficiency once injected into the muscle tissue of juvenile tilapia. I chose to compare the tilapia β-actin promoter, useful in autotransgenics, with the other well characterised and frequently used β-actin promoter isolated from carp. Testing promoter efficiency by the method of direct injection is fairly novel (Hansen *et al.*, 1991; Rahman and Maclean 1991). The characterisation of the tilapia β-actin promoter was essential to be able to ascertain its success as a promoter, and whether high levels of expression might be gained by this method of gene transfer.

The results in figure 3.3.2 (a)-(f) show the β-galactosidase expression gained after somatic injection of the plasmid carp β-actin promoter spliced to a lacZ reporter gene (4.7 kb cβAP/lacZ) as described in Rahman *et al.*, (1997) and the plasmid tilapia β-actin spliced to lacZ (1.6 kb tiβAP/lacZ) kindly provided by Dr GL Hwang.

Figure 3.3.2 (a) – (f)

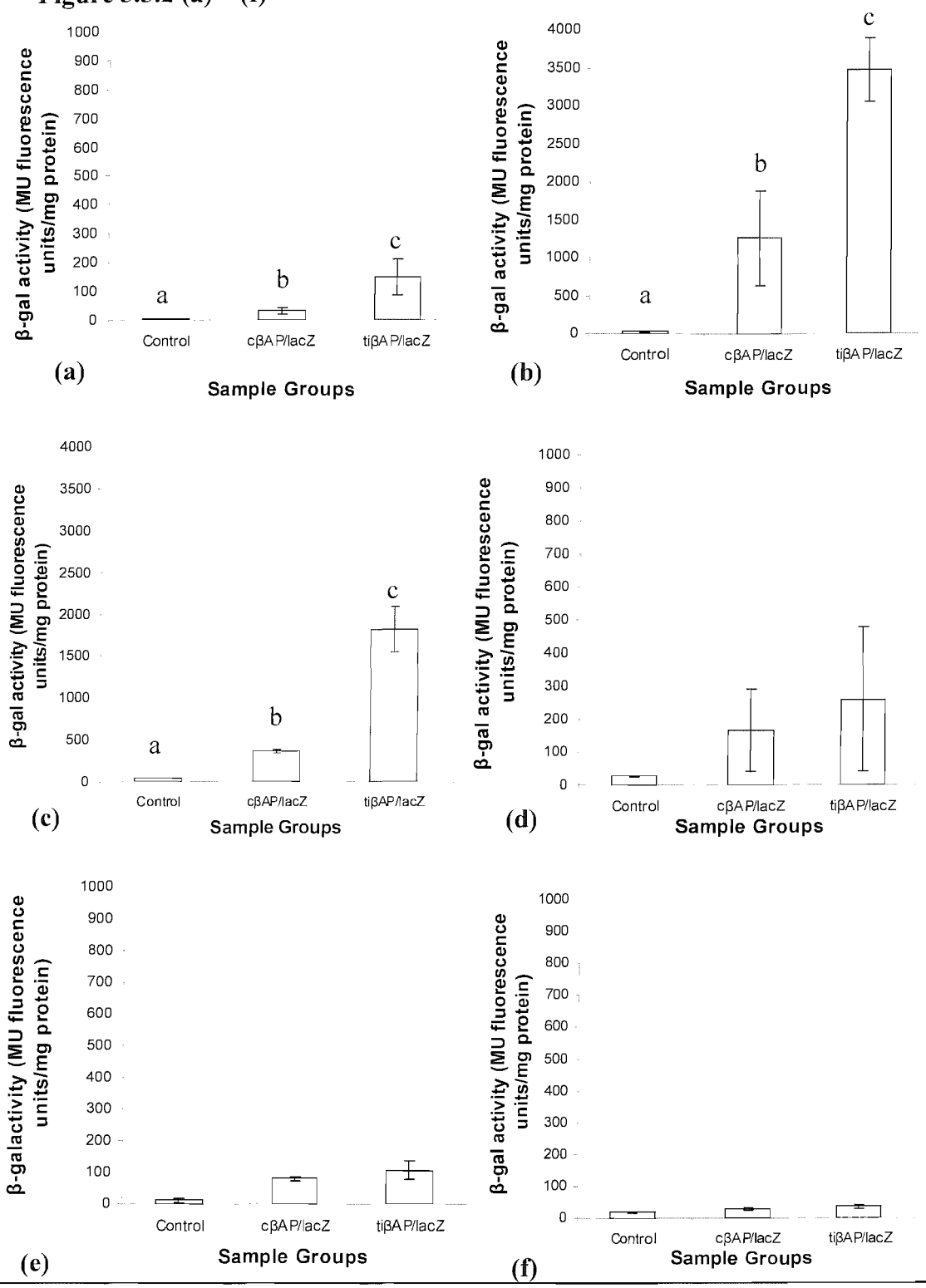


Figure 3.3.2 Comparison of the mean (±SE) β-gal expression levels recorded over time after intramuscular injection of the cβAP/lacZ and tiβAP/lacZ constructs. Significant tested with ANOVA to 95 % CI ($P < 0.05$). Those statistically significant are marked with different letters (a:b). Please note that the scales of the vertical axis differ in (b) and (c) from other figures.

After the introduction of the two lacZ reporter constructs into juvenile tilapia skeletal muscle, the tilapia β-actin promoter produced expression levels that were significantly higher than those obtained with the carp β-actin promoter, by approximately three fold (figure 3.3.2.1). The statistical analysis performed compared all three sample sets, the control group, those injected with tiβAP/lacZ and those injected with cβAP/lacZ, and significance was proved up to 46 days post injection (figures 3.3.2 a – c; 3.3.2.1). The expression obtained from each of the constructs continued to increase over 21 days. At 56 days the expression from both constructs was not statistically different from the control group (figure 3.3.2.1).

Figure 3.3.2.1

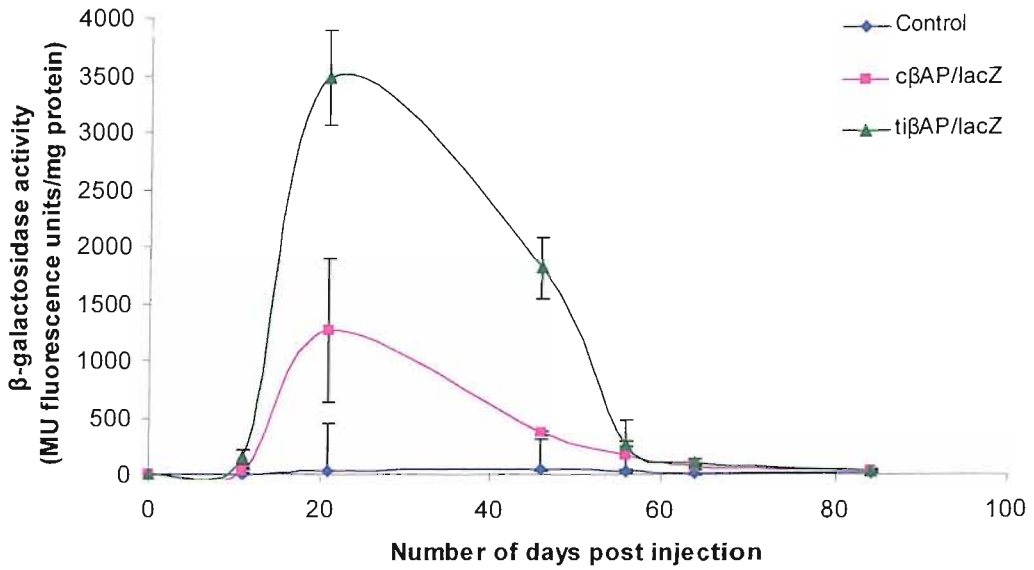


Figure 3.3.2.1 Summary of all data comparing promoters. The graph shows a summary of figures 3.3.2 (a) to (f), plotted on a single axis so that a trend line may be visualised (n = 5).

3.3.3 Fate of the transgene

The length of time the plasmid persists is important to be able to usefully assess the length of time the DNA is able to resist degradation and survive to transmit its product *in vivo*. The PCR assay was used to detect transgene DNA from the site of injection after extraction of genomic DNA in order to determine how long the transgene DNA remained at the site of injection. PCR was also employed to investigate whether the plasmid DNA migrated around the body, away from the area targeted, and in particular whether it was transported to any other organs. In order to assess this, organ tissue was collected from the liver, spleen, gonads and brain of individuals; the DNA extracted and amplified using PCR, since PCR was able to detect picogram quantities of plasmid DNA suspended in 400 µl of buffer during experimental test batches (data not shown). These key questions provide knowledge about the behaviour of DNA once somatically introduced and the length of time the constructs can survive in the host cell.

From a total of 489 organs extracted from 152 individual fish, examined by PCR for the presence of plasmid DNA up to six weeks after intramuscular injection, three were found to have some plasmid transgene present. In each of these cases the plasmid DNA was located in the gonad tissue alone and was never present in the liver, spleen or brain tissues. The cβAP/lacZ plasmid introduced at a concentration of 5 µg 100 μl^{-1} was found in gonad tissue homogenates four weeks post injection (figure 3.3.3 a). The gel shows the amplification of genomic DNA extracted from organ tissues of 2 individuals injected intramuscularly with cβAP/lacZ. Lane 1 shows a positive result for the presence of cβAP/lacZ in the gonad tissue, marked with an asterisk. Lanes 2-4 show negative results for spleen, brain and liver. Lanes 5-8 show negative results for gonad, spleen, brain and liver amplified from second tilapia fish. Lane 9 shows control tissue, lane 10 positive PCR control and lane 11 the negative PCR control.

The tiβA/lacZ plasmid was amplified from homogenates sampled from gonad tissue using PCR, 24 hours after injection of 25 µg 100 μl^{-1} of the tiβAP/lacZ construct

(figure 3.3.3 b). 5 individual fish were harvested 24 hours after injection. Lanes 1-4 show the PCR result from the four organs; liver, spleen, gonads and brain of individual I. Lanes 5-8 show the four organs for individual II. Lanes 9-12 the organs from individual III, lanes 13-16 that of individual IV, and lanes 17-20 from individual V. Lane 21 shows tissue extracted from control tilapia, lane 22 the PCR negative control, lane 23 the 100 bp DNA marker ladder and lane 24 shows the positive PCR control. Lane 11, marked with an asterisk shows putative positive result, evidence of the tiβAP/lacZ construct in the gonad tissue.

The all-tilapia growth hormone plasmid construct tiβAP/tiGH, was found in gonad tissue homogenates two weeks after injection of 25 μg 100 μl^{-1} (figure 3.3.3 c). The gel image shows lanes 1-16 run with genomic D NA amplified from the organs of 4 individual fish. Lane 6 shows putative positive result amplified from gonad tissue, marked with an asterisk (*). Lane 17 shows control tissue, lane 18 positive PCR control and lane 19, a 100 bp DNA marker ladder. These samples were harvested 14 days post intramuscular injection with a DNA concentration of 25 μg in 100 μl. Strong dark areas seen in lanes 11 and 15 are the result of high concentrations of genomic DNA in the PCR reaction mix.

Figure 3.3.3 (a) – (c)

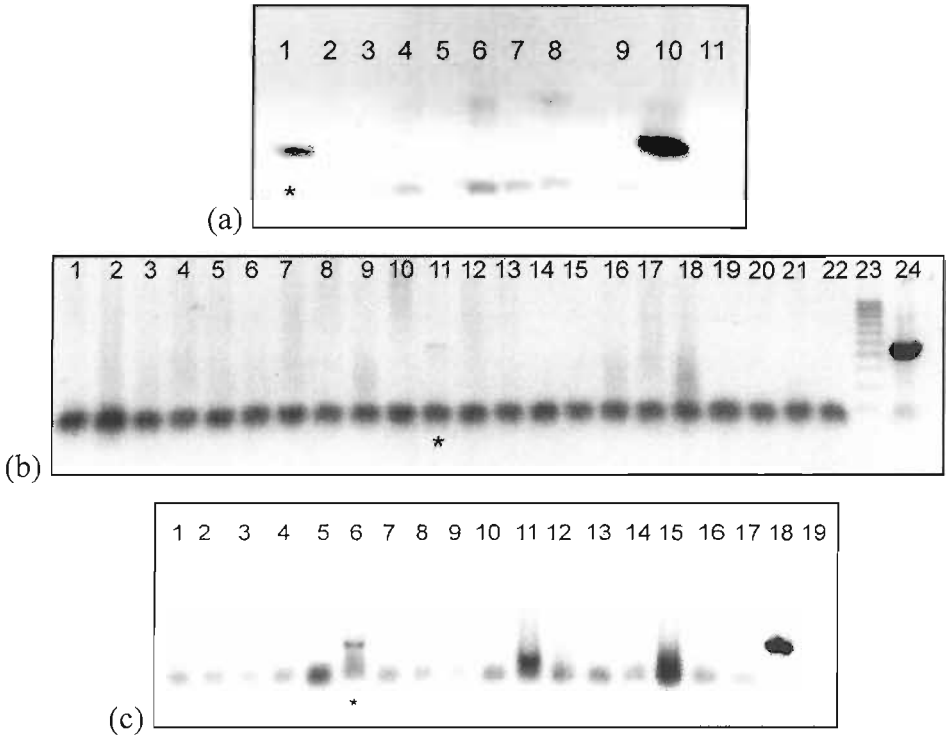


Figure 3.3.3 Detection of plasmid transgene DNA in organ tissue (liver, spleen gonad and brain tissue) homogenates by PCR. Inverted gel image shows PCR product run on agarose gel, samples with putative positive detection of transgene in organs marked with asterisk. (a) PCR result from organ samples taken 4 weeks after intramuscular injection of 5μg $25\mu l^{-1}$ cβA/lacZ construct (b) PCR result from organ samples taken 24 hours after intramuscular injection of 25μg $100\mu l^{-1}$ tiβA/lacZ construct (c) PCR results from organ samples taken 2 weeks after intramuscular injection of 25μg $100\mu l^{-1}$ tiβA/tiGH construct.

3.3.3.1 Persistence of the transgene

The inverted agarose gel images show the PCR product after amplification of extracted genomic DNA, taken from muscle tissue located at the site of injection. The tiβAP/lacZ, cβAP/lacZ and tiβAP/tiGH constructs were used for injection into muscle tissue and were investigated using the PCR for up to 6 months post injection of the transgene DNA into the tissues. The length of time the DNA persists for in the tissues is an important factor in this type of gene transfer. Unlike germline transgenesis, the nature of somatic transgenesis and in part, its attractiveness for commercial application owes to putative DNA degradation reported *in vivo* after its application. This persistence of transgene DNA must be investigated to determine the length of time the

DNA may usefully survive and in what time frame the exogenous transgene is removed from the hosts cells. The inverted gel images are shown with positive and negative PCR controls and a negative tissue control, which was extracted along side the DNA injected batch and underwent the same PCR conditions to avoid false positives from contamination, a problem with this sensitive assay.

Figure 3.3.3.1 (a) – (c)

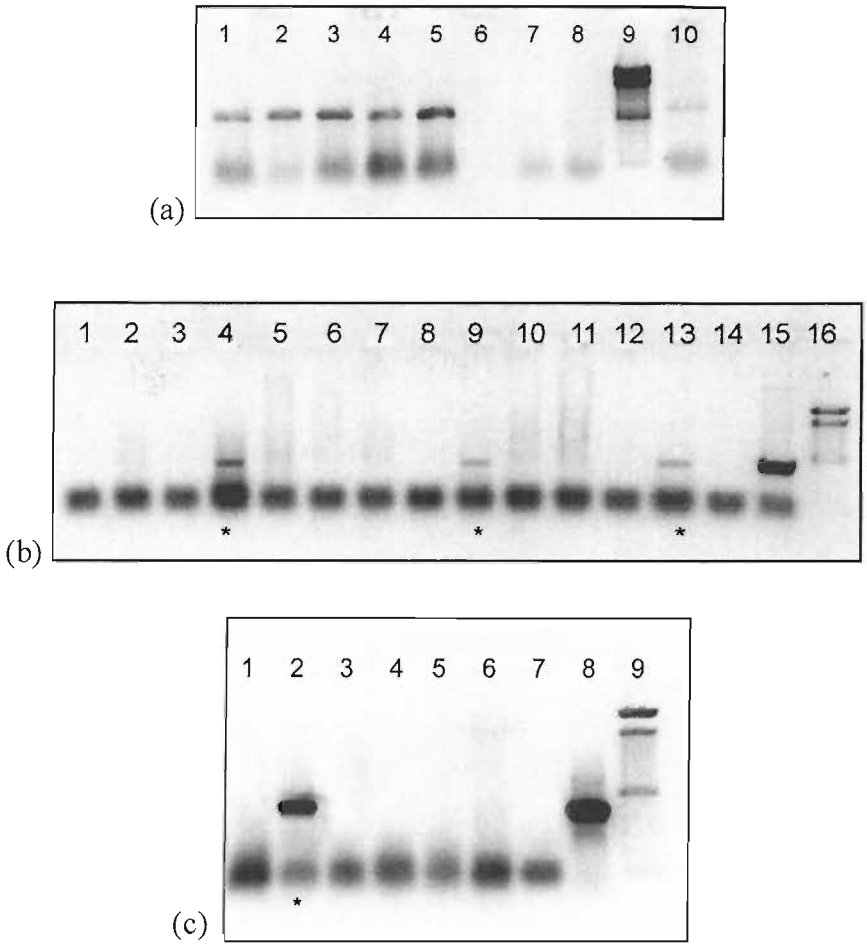


Figure 3.3.3.1 Detection of tiβAP/lacZ plasmid transgene DNA in muscle tissue harvested from the site of injection using PCR of muscle tissue homogenates (a) one week post injection (n=5), (b) lanes 1-5, 6 months post injection (n=5) and lanes 9-13, 3 months post injection (n=5) (c) six months post injection of transgene (n=6). Putative positives marked with an asterisk.

Figure 3.3.3.1 all inverted gel images in this figure show the amplified PCR product from extracted genomic DNA isolated from the muscle tissue of tilapia, intramuscularly injected with 25μg $100\mu l^{-1}$ tiβA/lacZ. Figure 3.3.3.1 (a) shows 5 individuals harvested 1 week post injection, lanes 1-5. Control tissue is shown in lane 7, negative control lane 8, 100 bp DNA marker ladder and positive control lanes 9 and 10 respectively. Figure 3.3.3.1 (b) shows in lanes 9 -13, five individual fish that were harvested three months after a single intramuscular injection, one individual per lane, lane 8 shows control tissue extracted with this batch. Lanes 1 – 5, figure 3.3.3.1 (b), show five fish harvested six months after initial injection, lane 7 shows control tissue extracted with this batch. The PCR negative shown in lane 14, PCR positive shown in lane 15 and 100 bp DNA marker ladder shown in lane 16. Putative positive results shown with asterisks resulting in 2 positives from the 5 tilapia fish tested after 3 months and 1 positive from the 5 tilapia fish tested after 6 months. Figure 3.3.3.1 (c) Lanes 1-5 show the result gained from five tiβAP/lacZ injected fish 6 months after the initial injection, lane 6 shows control tissue, PCR negative and positive controls are shown in lanes 7 and 8 respectively. Putative positive results shown with asterisk (*), resulting in 1 positive out of 5 tilapia fish tested.

Fish were harvested in batches for up to 6 months after injection with the tiβA/lacZ construct, and were subsequently harvested for tissue samples from the site of injection. The gels in fig. 3.3.3.1 are representative of the findings in this investigation. At three months, 9 of the tested fish were found to contain plasmid DNA at the site of injection out of a total of 22 individuals tested. At six months, 3 of the 14 surviving fish were shown to contain the plasmid transgene.

Figure 3.3.3.2 (a) – (c)

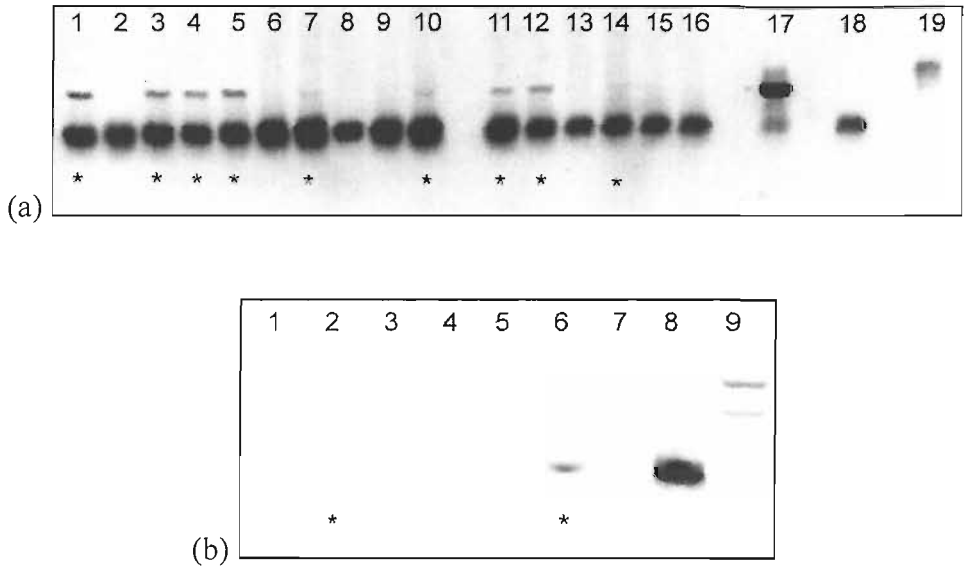


Figure 3.3.3.2 (a)-(b) Detection of (a) tiβAP/tiGH plasmid transgene DNA in muscle tissue harvested from the site of injection using PCR of muscle tissue homogenates, lanes 1-16, 3 months after initial injection of transgene DNA, (b) lanes 1-5, 3 months post injection of the cβAP/lacZ plasmid construct. Putative positives marked with an asterisk.

Figure 3.3.3.2 (a) shows an inverted gel image with genomic DNA amplified from 15 individuals injected with 25μg $100\mu l^{-1}$ tiβAP/tiGH. 1-15 were harvested 3 months after an initial intramuscular injection. Control tissue is shown in lane 16, positive control lane 17, the negative control in lane 18 and a 100bp DNA marker ladder in lane 19. Putative positive samples indicated with asterisks resulting in 9 positive samples in the 15 injected fish. The total number of fish which survived to be sampled at 3 months for this construct was 31. 18 of these fish were shown using PCR to contain this construct in the transgene injected muscle tissue.

Figure 3.3.3.2 (b) shows amplification of extracted genomic DNA using PCR from the muscle tissue of tilapia, intramuscularly injected with 25μg $100\mu l^{-1}$ cβAP/lacZ. Six individual fish were harvested three months after a single intramuscular injection, one individual per lane, lanes 1-6. The PCR negative shown in lane 7, PCR positive shown in lane 8 and 100 bp DNA marker ladder shown in lane 9. Putative positive results

shown with asterisks resulting in 2 positives of the 6 individuals tested. No fish injected with either of these constructs were found to be positive for the injected transgene after 6 months (n = 17), results are not shown. The total number of fish which survived to be sampled at 3 months for the presence of the cβAP/lacZ construct was 12. Four of these fish were shown using PCR to contain this construct in the transgene injected muscle tissue.

3.3.4 MUG assay from spleen, liver and blood serum

Preliminary research suggested that β-galactosidase expression was detected in the liver after approximately two weeks had elapsed (figure 3.3.4.1) and was also detected at this time in spleen tissue (results not shown). It was important to be able to determine whether any expression from the transgene DNA occurred in sites other than that targeted. In the instance of DNA vaccination, the positive finding of expression in other organs may suggest the possibility of conferring greater immunity (Heppel *et al.*, 1998). However regardless of whether using a ubiquitous promoter or one that was tissue specific, the finding of expressed protein elsewhere from the site targeted by the exogenous DNA, is interesting and has wider implications [for example, gene therapy] since the localisation of expression in sites, other than the target may prove to be deleterious for the organism (Kelly 2003).

Controls, fig. 3.3.4.1, were injected with 100 µl SDW, and the tiβAP/lacZ construct was injected intramuscularly at a concentration of 25 µg 100 μl^{-1}. Statistical analysis was completed using a one-way analysis of variance, comparing sample sets to a 95 % confidence interval ($P < 0.05$).

Figure 3.3.4.1 (a) – (d)

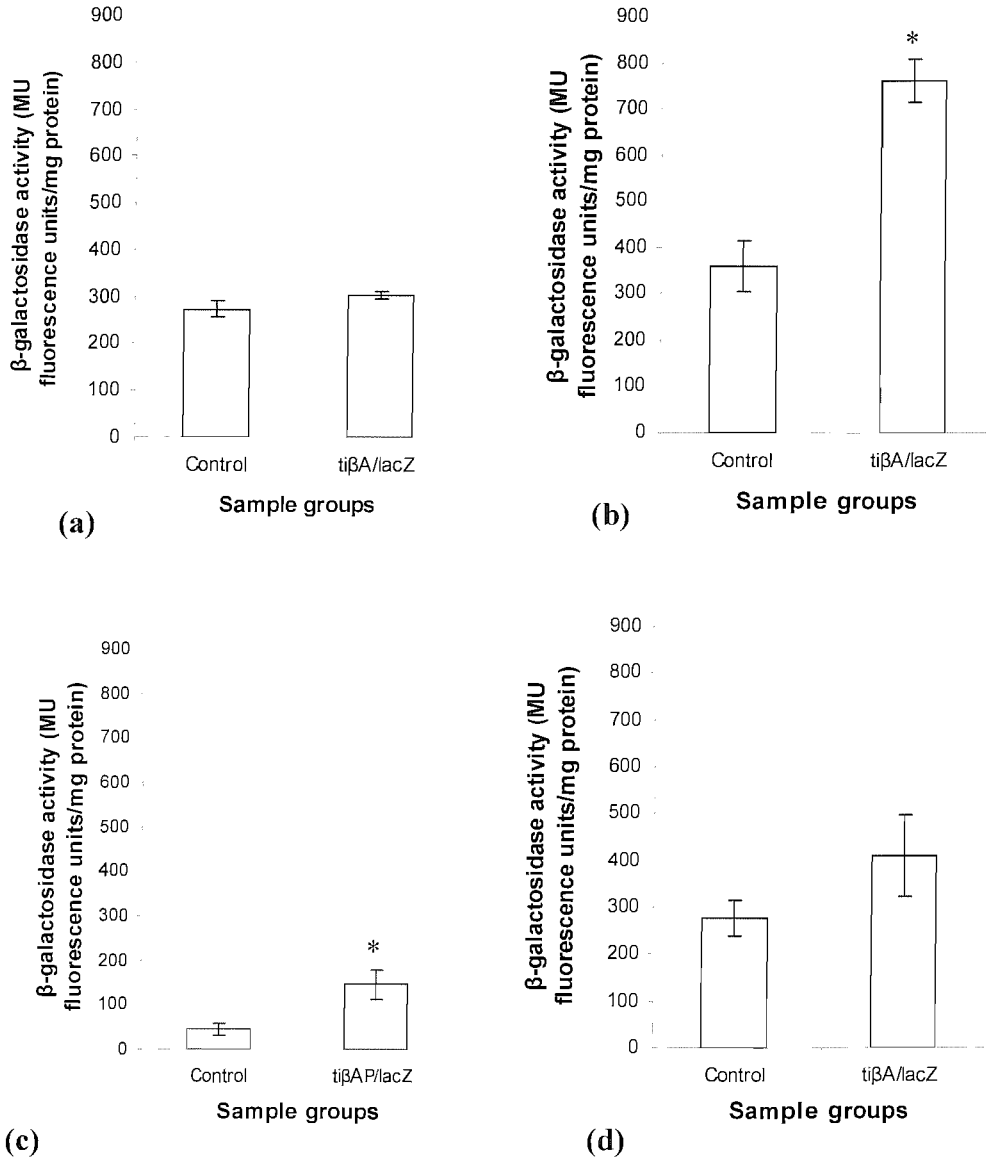


Figure 3.3.4.1 (a) – (d) Mean (±SE) β-galactosidase expression found in liver tissue homogenates sampled (a) 1 week, (b) 2 weeks, (c) 3 weeks and (d) 4 weeks after a single intramuscular injection of 25 μg 100 μl^{-1} of plasmid tiβAP/lacZ (n = 5). Significance was tested using a one-way ANOVA assuming a 95 % CI, ($P < 0.05$). Those significantly different to control are denoted with an asterisk (*).

The following experiments were designed to detect any β-galactosidase expression which may have occurred in the liver, spleen and red blood cells of juvenile tilapia after intramuscular injection over a 5 week period (figure 3.3.4.3). Anderson *et al.*, (1996) and Heppel *et al.*, (1998) reported luciferase expression in the organs, the highest detected in the kidney and spleen after intramuscular injection, although the maximal levels of expression obtained were from the site of injection. Since expression peaks at 21 days after injection in muscle tissue (figure 3.3.2.1), it was reasonable to assume it may take some time for the β-gal to appear in the organs of the DNA injected fish. At the conclusion of each trial, the tissue homogenates were kept for PCR analysis, no exogenous transgene DNA was detected in the tested organ tissues (results not shown), but was found in one blood sample taken from a single individual (n = 30) figure 3.3.4.2. Any expression which may have occurred in the organs could reasonably have been assumed to have come from the site of injection, in muscle tissue, given the absence of detectable DNA in the organ tissue. Controls were injected with 100 µl SDW, and the tiβAP/lacZ construct was injected at a concentration of 25 µg 100 μl^{-1}. Statistical analysis was completed using a one-way analysis of variance, comparing sample sets to a 95 % confidence interval ($P < 0.05$).

Figure 3.3.4.2

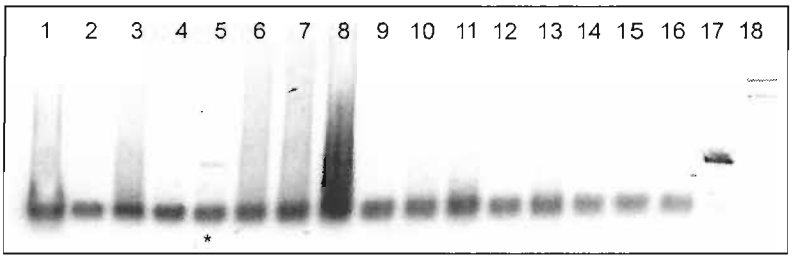
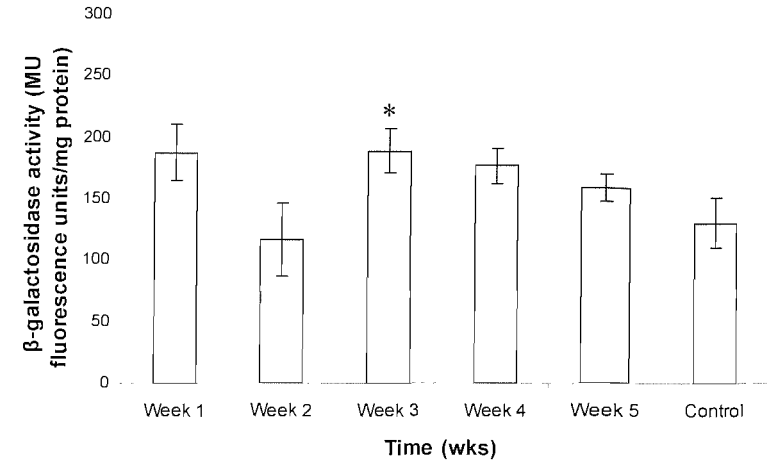
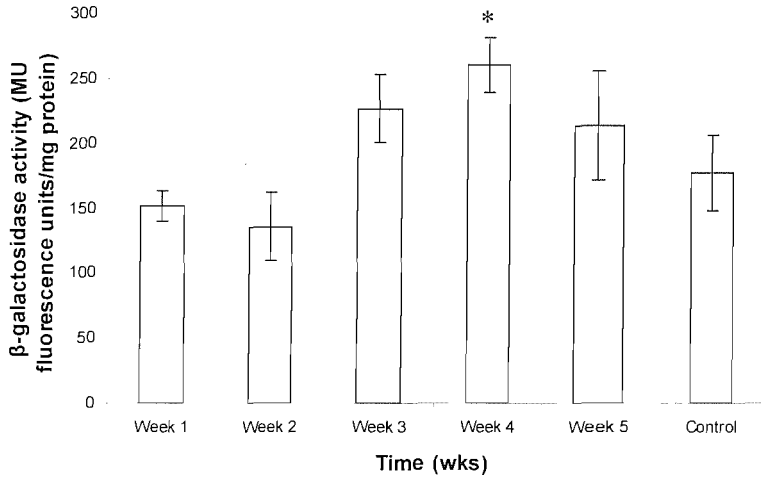


Figure 3.3.4.2 Shows PCR detection of tiβAP/lacZ in blood samples in DNA injected fish. Lanes 1-5; five individual fish harvested 7 days post injection. Lanes 6-10; five injected fish 3 days post injection and 11-15 show control individuals. PCR negative and positive controls are shown in lanes 16 and 17 respectively. Putative positive results shown with asterisks resulting in 1 positive out of 5 tilapia fish tested for the presence of the lacZ transgene after 1 week.

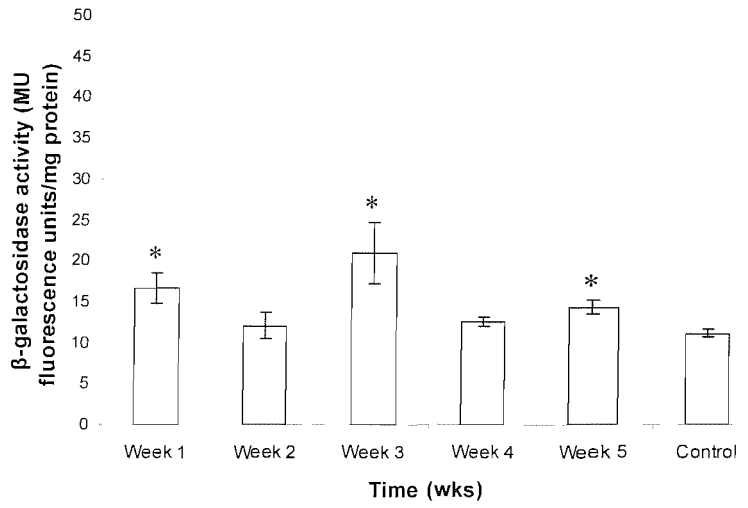
Figure 3.3.4.3 (a) – (c)



(a)



(b)



(c)

Figure 3.3.4.3 (a) - (c) Mean (± SE) β-gal expression in samples over 5 weeks in (a) liver (b) spleen and (c) red blood cells (n = 5). Samples were injected with a single injection of 25 μg 100 μl^{-1} tiβAP/lacZ. Statistical analysis; one-way analysis of variance, comparing sample sets to a 95% C.I ($P < 0.05$). Those marked with an asterisk (*) are significant from controls.

3.3.5 X-gal stained sectioned tilapia tissue injected with tiβAP/lacZ

LacZ expression may be visualised after X-gal staining of tissue sections. By examining the pattern of X-gal staining, the uptake and possible movement of the plasmid transgene may be ascertained. Expression of lacZ in fish muscle has been found to be present in cells distributed along the path of the injection needle (Romoren *et al.*, 2003; Tan and Chan 1997; Gomez-Chiarri *et al.*, 1996; Hansen et al., 1991). Skeletal muscle fibres lie in register, each fibre consisting of bundles of myofibrils, between 1-2 μ across, contained within a sarcoplasmic matrix, which contains the organelles. Each multinucleated fibre is bound by sarcolemma, in which the nuclei are embedded, with the endoplasmic reticulum and Golgi bodies situated around each of the nuclei. The fibres have blunt ends and are bordered by the sarcolemma. The structural bands around the circumference of the cell, known as desmin filaments, create the easily visualised Z bands. Using a concentration of 25 μg 100 μl^{-1} of the tiβAP/lacZ construct, the fate of the transgene and the protein product, once injected, was investigated by sectioning muscle tissue 11 days post injection in juvenile tilapia of about 4 months of age (figures 3.3.5.1-2).

3.3.5.1 Results

Figures 3.3.5.1 (a) and (b) show photographs of sectioned tilapia muscle. The tissue was sampled from the site of injection after the introduction of 25 μg 100 μl^{-1} of plasmid tiβAP/lacZ, harvested 11 days after initial injection. Figure 3.3.5.2 (a) shows a section of control tilapia muscle tissue stained in X-gal and magnified by x 10. The photographs were taken under different magnifying resolutions (x 10, x 422 and x 844) in order to examine the staining pattern obtained more closely, as well as from lower resolutions, to allow larger sections of tissue fibres for observation.

Figure 3.3.5.1 (a) – (b)

(a)

(b)

Figure 3.3.5.1 (a) - (b) Tissue stained with X-gal after intramuscular injection with tβA/lacZ. Putative nuclei (N) are stained heavily and are indicated with arrows. The white connective (C) tissue is marked with arrows and the Z bands, lying across the fibre are also clearly stained and are pointed out in two areas with (Z). (a) magnification x 422 (b) magnification x 844.

Figure 3.3.5.2 (a) – (b)

(a)

(b)

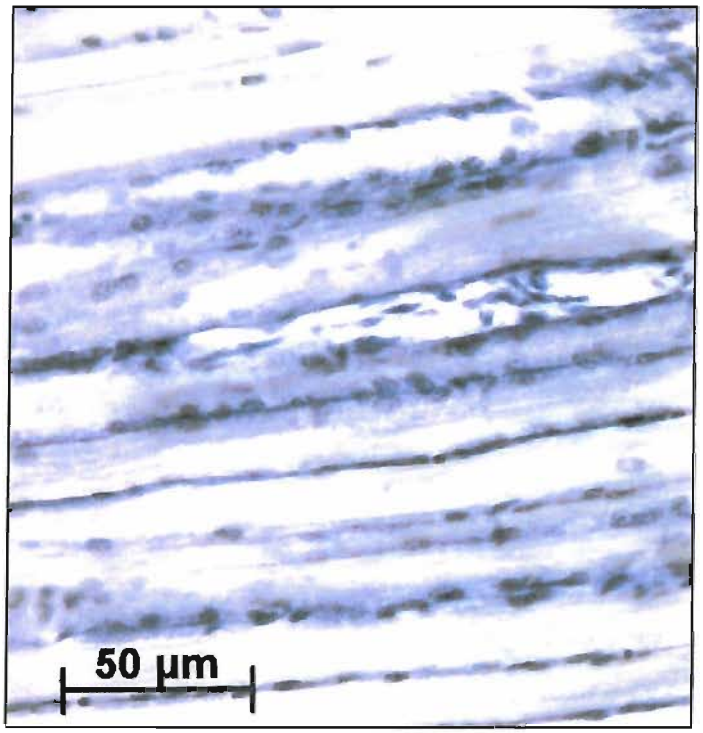


Figure 3.3.5.2 (a) - (b) These images show (a) X-gal staining of control tissue (x 10), there is no visible staining pattern in this tissue. (b) Zebrafish muscle section after nuclear staining. A longitudinal section kindly supplied by Professor Ian Johnson, St. Andrews University.

It should be noted that X-gal is not a nuclear stain. The putative results suggest the areas showing concentrated blue staining fig. 3.3.5.1, are located around the nuclear region and not the actual nucleus itself. The inclusion of zebrafish muscle stained with nuclear stain, kindly supplied by Professor Ian Johnson (figure 3.3.5.2 b), was to provide a reference with which to compare the location and situation of the nuclei in multinucleated muscle fibres. This shows the tissue in longitudinal section, after nuclear staining, each nuclei clearly visible as dark spots on the external surface of the fibre.

3.4 Discussion of results

3.4.1 Short term analysis of β-galactosidase expression in muscle tissue.

In order to optimise the delivery of exogenous transgene DNA to the muscle tissue *in vivo*, various parameters were investigated over time. These included variation of buffer volume, in which the DNA was delivered, and variation in the concentration of the administered DNA. Skeletal muscle has long been proved to be efficient in plasmid DNA uptake and subsequent expression (Rahman and Maclean 1992; Ascardi *et al.*, 1990; Wolff *et al.*, 1991; 1990). It would appear that initially, the highest concentration dose of 50 μg resulted immediately in elevated expression levels. However, this margin did not remain significantly higher after 24 hours, the reduced dose of 25 μg of DNA, yielded statistically similar expression levels. Dowty *et al.*, (1995) reported that plasmid DNA gained access to postmitotic nuclei of primary rat myotubes, probably via active transport through nuclear pores. This active transport mechanism may become saturated and finally inhibit nuclear transport (Schulte *et al.*, 1998; Dowty *et al.*, 1995). It is therefore possible that a threshold point may be reached, where significant increases in DNA concentration are not reflected in the observed expression levels. These authors report that substantial increase in DNA concentration may not affect expression levels since a high level of DNA remains at the site of injection, with less than 1% of cells expressing cytoplasmically injected plasmid DNA in murine tissues. It would appear that both these factors may be considered limiting to expression levels after somatic injection of plasmid DNA.

In terms of studying the effect of gene expression once injected somatically into the tissues, there may be no significant advantage in injecting a larger dose than 25 μg 100 μl^{-1}. This appears to be the optimum volume / DNA concentration in order to achieve high expression. As shown doubling the DNA concentration does not result in significantly increased expression, and has been noted in other research (Heppell *et al.*, 1998; Shulte *et al.*, 1998; Anderson *et al.*, 1996; Rahman and Maclean 1992; Hansen *et al.*, 1991). DNA introduced in high concentrations is also likely to be toxic to cells (Fletcher and Davis 1991; Penman *et al.*, 1990).

Damage to the muscle tissue allows the plasmid DNA to infiltrate the muscle fibres. Regenerating muscle tissue, after localised damage, has been shown to significantly improve plasmid DNA uptake and expression in murine skeletal tissues (Wells *et al.*, 1997; 1993). The larger volume of buffer (100 µl) presumably allows wider coverage of tissue area and therefore greater damage to the muscle fibres, enhancing uptake of DNA. The smaller volume used in the 5 µg 25 μl^{-1} sample set consistently expressed more β-gal than the 5 µg 100 μl^{-1} sample group. While a larger volume of buffer must result in greater damage to muscle fibres and therefore enhance DNA uptake, with such a small DNA concentration the comparative advantage may be lost.

Variation within each sample group was observed. Each sample set represents a batch of 10 individual fish from a sibling group, between these there may be variation in the efficiency of gene transfer among individual injections as well as between batches taken at each time point. This variation has been related to differences in plasmid distribution within muscle tissues between injected individuals (Davis *et al.*, 1993). The variation within each sample group may be attributed to a number of factors, including differences in plasmid uptake, distribution within muscle tissues (Davis *et al.*, 1993), and distance of the plasmid from each nucleus, since cytoplasmic sequestration of plasmid DNA, which dramatically reduces lacZ expression, occurs with increased distance from the nuclei (Dowty *et al.*, 1995). Variation may also result from DNA release after injection, since re-submersed fish sometimes lose the Vaseline plug which covers the injection site as well as when harvesting the tissue. This may occur when the mark left by the injection has long healed and is why extreme care was taken when injecting each fish to ensure that the site of injection was readily identifiable in each case.

3.4.2 Comparison of cβAP/lacZ and tiβAP/lacZ constructs

As expected, after the introduction of the two lacZ reporter constructs into juvenile tilapia skeletal muscle, the tilapia β-actin promoter produced expression levels that were significantly higher than those obtained with the carp β-actin promoter, by

approximately three fold. The transcriptional activity from the well characterised carp β-actin promoter has been found to be regulated by four transcriptional control regions including the serum response element, termed the CArG motif and the enhancer region located near the 3′ end of the first intron (Liu *et al.*, 1991, 1990a). These have been shown to result in high levels of variation in transgenic fish species and also in cultured piscine cells (Moav *et al.*, 1993, 1992). The 1.6 kb tilapia β-actin promoter used in these experiments contained the CAAT and TATA box in the proximal promoter as well as the CArG motif and also an enhancer found in intron I (Hwang *et al.*, 2003). Hwang *et al.*, (2003) reported that expression obtained after micro-injection of the 1.6 kb tilapia β-actin construct resulted in higher expression than either the 4.7 kb or 1.5 kb carp β-actin constructs in both tilapia embryos and in transfected blue gill cells. The increased homology of the native tilapia sequence resulted in significantly higher expression after intramuscular injection into juvenile tilapia muscle.

Plasmid DNA is a comparatively large molecule with an overall negative charge, and it must be quickly taken up into the nucleus to avoid degradation (Kelly 2003). The mechanism for plasmid uptake into the nucleus *in vivo* is as yet unknown (Bremner *et al.*, 2001; Pouton and Seymour 2001; Hagstrom *et al.*, 1996). Plasmid DNA has been shown to be taken up though nuclear pores, (Dowty *et al.*, 1995) and has been related to *cis*-acting elements known as nuclear localisation signals (NLS's) which may signal and enhance uptake of plasmid DNA by active transport through the nuclear pores (Marcello *et al.*, 2002; Wilson *et al.*, 1999). In these studies the plasmid DNA contained sequences isolated from the SV40 virus, known to attract cellular transcription factors containing NLS. The viral DNA 'coats' itself in these transcription factors which aid in nuclear uptake.

The metallothionein (MT) promoter is known to be up-regulated by the presence of metals or glucocorticoid steroid hormone. The promoter contains response elements known as the metal response element (MRE) and the glucocorticoid response element (GRE). In the presence of intracellular glucocorticoid steroid hormone, the cytosolic glucocorticoid receptor (GE) forms a dimer with another receptor unit, on dimerization

the complex is translocated to the nucleus (Latchman 1998). On characterising the GR receptor protein, two motifs which act as NLS's were isolated (Yamamoto 1987). It may well be possible that in the presence of the MT promoter in the cytosol (used in transgenic constructs, which allows the delivery the transgene to the cytosol) the GR dimer binds to the GRE unit located upstream of the transcription start site on the promoter, thus effectively coating it with nuclear localisation signals. This was similarly observed with the SV40 viral DNA, and enables the complex to be translocated to the nucleus from the cytosol (Marcello *et al.*, 2002; Dean 1997). The β-actin promoter contains a serum response element (SRE), also termed the CArG motif, this may well function on introduction into the cytosol, rather like the proposed model for uptake of the MT promoter construct into the nucleus, this discussion is purely speculative however.

Once copies of the transgene become nuclear, subsequent expression may persist over long periods of time; this persistence is attributed to the stability of the differentiated, post-mitotic muscle fibres and their nuclei (Heppell *et al.*, 1998; Wells *et al.*, 1997). It may also be that following the damage inflicted by muscle injection, some cellular infiltration occurs and nuclear mitosis with concomitant dissolution of the nuclear envelope may occur. This would facilitate uptake of plasmid DNA into post mitotic nuclei (Wells 1993).

On uptake of the plasmid DNA, persistence of expression has been shown to vary widely. Differences have been attributed to the various promoters used, volume of buffer used to suspend the DNA, as well as the species and age of the fish. Age has been implicated in altering expression levels, with younger, fast growing animals showing higher expression than those that are older, in both murine and piscine systems (Wells and Goldspink 1992; Hansen *et al.*, 1991). Kannellos *et al.*, (1999a) reported on expression gained from a CMV/lacZ construct lasting for up to 70 days in approximately half the injected goldfish. Heppell *et al.*, (1998) noted that CMV/LUC activity lasted for about 80 days in rainbow trout but lasted at least 112 days in zebrafish, different results than were obtained by Andersen *et al.*, (1996) after injecting

the same construct into the muscle tissue of rainbow trout. This group reported that luciferase activity peaked at 7 days post injection, the difference in findings being thought to arise from the change to buffer volume used in Heppell's research as compared with Anderson's; the latter group injected plasmid DNA suspended in larger volumes, shown to reduce expression levels of the luciferase gene (Heppell *et al.*, 1998).

In the present work the expression levels gradually reduce, falling to background levels 84 days after the initial injection of DNA. There is evidence of strong 5′ - 3′ exonuclease activity in the nucleus (Marcello *et al.*, 2002), although plasmid DNA is believed to be less susceptible to nuclease activity at lower temperatures as associated with piscine rather than murine systems, and is resistant to 5′ - 3′ exonuclease attack (Kanellos *et al.*, 1999b). The finding that the plasmid DNA was totally absent in a number of fish, from about half the sample group 10 weeks after injection, suggests that other factors assist in the removal of transgene DNA from the cells. The PCR is a highly sensitive assay and can detect picogram quantities of DNA. The primers designed for amplification of the plasmid result in an amplified fragment of between 400-600 bp, dependant on which primer pair is used. The absence of any detectable plasmid DNA by this technique suggests other processes used for the removal of the exogenous DNA from the muscle fibres, other than nuclease activity alone. This is especially true given the finding that plasmid DNA is highly stable once in the nucleus (Marcello *et al.*, 2002; Tan and Chan 1997). Wells *et al.*, (1997) reported that a loss of muscle fibres positive for β-gal expression resulting from an immune response against the exogenous protein. These authors reported that β-gal was a strong immunogen, eliciting an immune response to the gene product after the introduction of lacZ into muscle tissue and its subsequent transcription/translation. This research suggested that muscle fibres transfected with exogenous DNA may elicit a strong immune response which ultimately is responsible for the clearing of these cells and therefore the associated decline in expression as observed in this research.

3.4.3 Fate of the transgene DNA

Muscle tissue, including cardiac muscle, is purported to be unique in its ability to uptake naked plasmid DNA, as demonstrated in both mammalian and piscine tissues (Tan and Chan 1997; Gomez-Chiarri *et al.*, 1996; Wells 1993). It was important to discover whether plasmid DNA was able to migrate from the site of injection, since it was significant whether the exogenous DNA can transfer to other parts of the body that had not been targeted by the gene treatment. The finding of the three different constructs in sites other than the muscle tissue, suggests that they behave in much the same way once introduced into tilapia muscle tissue. Previous studies have noted expression from exogenous DNA apparent in the organs of the fish, although the plasmid DNA itself has not been found present at these sites. Romoren *et al.*, (2003) reported finding luciferase expression after intramuscular injection of plasmid DNA in kidney, liver and spleen tissues, though no evidence of the plasmid itself being present in any of the organs tested. Similarly Anderson *et al.*, (1996) reported on luciferase expression, but not plasmid DNA, in the organs of rainbow trout.

The appearance of the DNA in gonad tissue indicates that it must migrate from the site of injection to the gonad tissue. Evidence was obtained which suggested that plasmid DNA was occasionally present in blood samples of fish injected with the tiβA/lacZ construct. This occurred in only one sample 7 days after injection and was found on only one occasion, although other attempts were made to detect transgene presence in blood samples from DNA injected fish at different time points after injection. This coupled with the evidence that in almost 500 samples, plasmid DNA appeared in organ tissues only three times. This suggests that such migration is rare and most probably occurs immediately after injection when the plasmid DNA has yet to be taken up into the muscle fibres. The transgene DNA may access the capillaries supplying the tissues at the site of injection. In fish, a single circulatory system pumps blood from the atrium, having been collected from the body, and is pumped into the ventricle as the heart relaxes. From here it moves across the gills where gas exchange occurs and then through the body via a network of capillaries. It is possible that the plasmid moved from the site of injection through the capillaries before becoming lodged in the

gonad tissue. These putative migration events to the gonad tissue occur at such a low frequency that it may be concluded that the plasmid DNA appears to be most frequently restricted to the locality of injection site.

3.4.4 Persistence of the transgene

The transgene DNA was shown to be present in muscle tissue for up to 6 months. The exogenous DNA degrades slowly over time, the tiβAP/lacZ construct expressing in 100% of individuals tested after 1 week this figure falling to 20 % of individuals after 6 months. The carp construct and all-tilapia GH construct was detected by PCR after 3 months but not 6 months after the initial injection. Egg microinjection suggested that larger linear constructs were more persistent than smaller inserts (Rahman *et al.*, 1997), though this was attributed to concatemerisation, a phenomenon more likely to occur in eggs rather than somatic cells, owing to the abundance of stored ligase present in the egg (Marini *et al.*, 1988). The clearance of muscle fibres containing the lacZ constructs may result from the fact the β-galactosidase protein has been found to be highly immunogenic and elicits an immune response. This occurs primarily via the activation of cytotoxic $CD8^+$ cells, which have been shown to attack the fibres expressing the endogenous antigen, β-galactosidase (Wells *et al.*, 1997). Endogenous antigens are thought to be broken down within the cell into peptide fragments, which are then bound to MHC class I molecules produced by all nucleated cells. This complex is then transported to the cell membrane where cytotoxic $CD8^+$ T cells (T_c) recognise the antigen associated with class I MHC molecules where they attack and kill the cell (Goldsby *et al.*, 2000). By this mechanism muscle fibres expressing β-galactosidase are removed, resulting in a clearance of those fibres positive for the transgene.

Kanellos *et al.*, (1999) reported on plasmid DNA being present in the muscle tissue of trout at least 4 weeks after injection and in goldfish 10 weeks post injection, although no plasmid DNA was detected in trout for this time. Anderson *et al*, (1996) noted intact plasmid DNA present up to 2 months after injection into rainbow trout muscle tissues, and suggested that the DNA was either degraded away or simply became

'diluted' from the growth of the fish and the subsequent increase in the number of muscle fibres at the site of injection. Although there may be a 'diluting' effect, the total absence of transgene DNA, detectable by PCR at the site of injection would suggest both removal of transfected muscle fibres, governed by an immune response to the presence of the exogenous protein, as well as degradation of the plasmid DNA itself by nuclease activity. The tiβAP/tiGH construct is unlikely to elicit an immune response given that the protein product is tilapia growth hormone, the degradation of this plasmid may be attributed to nuclease activity or the presence of CpG motifs known to be found in plasmid DNA.

CpG motifs present in the plasmid backbone have been implicated in reducing levels of transcription via methylation of the plasmid DNA. Methylation of CpG replete plasmid DNA resulted in a 95 % decrease in plasmid expression in murine tissues 1 day after i.m injection, and CpG-reduced plasmids resulted in significant but more modest reduction in protein levels (Hodges *et al.*, 2004). These authors suggest that the difference in protein levels and the time dependant decline in transgene expression results from the number of CpG dinucleotides and the extent to which they are methylated. The introduction of non-methylated CpG DNA into Atlantic salmon has been shown to induce the production of antiviral cytokines, since in eukaryotes unmethylated CpG motifs have been found to elicit an immune response (Jorgensen *et al.*, 2001). These factors must be considered since the plasmid vectors are bacterial and unmethylated CpG motifs are common in bacterial DNA but are less frequent as well as methylated in vertebrate DNA (Bird 1987). This may contribute to the reduction observed in expression levels over time, as well as being implicated in the immune response which endogenous antigen (in this case the protein product from plasmid DNA) has been found to elicit (Wells *et al.*, 1997).

3.4.5 MUG assay from spleen, liver and serum

Significant β-galactosidase expression was detected in a number of samples taken from the liver, spleen and red blood cells of individuals up to five weeks after a single injection of tiβAP/lacZ. Similarly Andersen *et al.*, (1996) reported finding luciferase

expression in organs of small fish after direct injection in the muscle. Romoren *et al.*, (2003) reported expression of luciferase after introduction of naked plasmid DNA injected intramuscularly in the spleen, head kidney and liver. The finding of significant expression of lacZ in the liver between 2 and 3 weeks post injection of the plasmid DNA suggested that time may be a factor in the arrival of the protein into the organs. Accordingly over a 5 week time frame, significant expression was detected in the liver and spleen, three and four weeks respectively, after an initial injection of tiβAP/lacZ.

The images resulting from histological staining of tissues injected with tiβAP/lacZ and stained with X-gal show movement of β-galactosidase protein through the muscle fibres after the construct became nuclear. The finding of significant levels of β-gal protein in the red blood cells might explain the discovery of β-gal expression in the liver and spleen when the presence of plasmid DNA was, at no time, detected in these organs. The protein was shown to move through the muscle fibres and staining was found in other cells such as the connective tissue. It is possible that it enters the blood via the capillaries in the muscle tissue, where it is conveyed to the spleen and liver by cells of the immune system. Heppell and Davis (1997) noted on the finding of reporter gene expression in the epithelial cells of the capillaries, in fish intramuscularly injected with plasmid DNA. The spleen receives blood which is filtered and monitored by B-lymphocytes and T cells, the blood is then passed on into the liver. Romoren *et al.*, (2003) suggested that the presence of luciferase in the organs, but absence of any plasmid DNA resulted from the more rapid degradation of the plasmid DNA than its associated protein product. However, in the results presented here, plasmid DNA was never detected in the liver or spleen at any time point after injection, although the protein product from the DNA was detected at significant levels. By inference, blood moving through the capillaries provides a feasible mechanism by which the protein product is moved from the site of injection to the organs, which are known to filter the blood of any immunogenic substance.

3.4.6 X-gal stained sectioned muscle tissue

After staining with X-gal, lacZ expression is clearly visible in the injected muscle, with varying degrees of staining intensity, probably related to copy number of the transgene within each muscle fibre. Hansen *et al.*, (1991) reported on β-gal expression occurring along the path of injection into fish muscle tissue. In this study, the staining pattern was seen to spread from the needle track; it is likely that the resulting damage to the cell membranes allowed the introduced plasmid DNA access to the sarcoplasm. The dark blue staining concentrated in small areas along the muscle fibre corresponds with the positioning of nuclei as are observed in the zebra fish muscle tissue sections. The high level of staining shown over the nuclei would perhaps have resulted from the increased presence of the β-galactosidase protein under going translation in the associated organelles outside the nuclear envelope, where ribosomes and golgi body are found to be situated in muscle cells. Since β-gal is translated in the nucleus and subsequently processed in the transcriptional machinery located outside the nucleus, this could result in the high concentrations of β-gal before it diffuses along the length of the fibre.

Without access to the nucleus, plasmid DNA cannot be transcribed, and gene expression is dependant on the ability of the gene to become nuclear to function, regardless of how the DNA is introduced to the cell. During mitosis the migration of cytoplasmic DNA occurs during the removal of the nuclear envelope, when no barrier exists between cytoplasm and nucleus; a factor perhaps in explaining why improved levels of transgene expression have been reported after prolonged periods of muscle regeneration (Wells 1993). Dowty *et al.*, (1995) and Dean (1997) report on nuclear uptake of plasmid DNA and suggest this may be a function of the nuclear pore complex, since uptake occurred in both post-mitotic nuclei of primary rat myotubes, although the exact uptake mechanism is largely unknown.

Strong expression was also be detected in the Z lines, formed by desmin filaments, at the borders of sarcomeres. This pattern of staining was also detected in embryos after egg microinjection of the linear form of the reporter gene (Hwang *et al.*, 2003). The

overall pattern of staining suggests that the DNA migrates along the muscle fibre from the point of access to the cell and is not just diffusion of β-gal protein. Tan and Chan (1997) note expression of lacZ was found only in the myofibres and no other cells types were positive around the site of injection. They reported that other cells which exhibit staining next to cells which show heavily staining, probably resulted from diffusion of β-gal at the time of cryosectioning. Here the connective tissue was also found to show light staining in areas, and it is unclear however, whether this pattern of staining has resulted from diffusion of the stain from the muscle fibres, or whether the connective tissue is actually expressing lacZ after uptake of the injected transgene. Heppell and Davis (2000) note that reporter gene expression was found in fish myocytes, as well as in cells infiltrating the muscle tissue and epithelial cells of the capillaries. It would appear that both the transgene and the protein product are fairly mobile within each fibre and that there is evidence which may suggest diffusion of lacZ from cell to cell (Heppell and Davis 2000). The finding of β-galactosidase in liver and spleen tissue a number of weeks after the initial injection may be attributed to the length of time between the optimal expression levels in the muscle tissue itself, suggested in this study to be 3 weeks and the subsequent arrival of the exogenous protein in the organs, which are well supplied with blood, and are known to filter immunogenic substances from the body.

Chapter four:

Somatic transgenesis using the tilapia β-actin enhancer element and 'all-fish' growth hormone genes to promote growth enhancement

4.1 Introduction

4.1.1 The potential for growth enhancement by transgenesis

Growth enhancement by germline transgenesis has been tried with varying degrees of success in many fish species such as tilapia (e.g. Rahman *et al.*, 1999; Martinez et al., 1999; de la Fuente *et al.*, 1998), Atlantic salmon (e.g. Hew and Fletcher 2001; Saunders *et al.*, 1998; Choy *et al.*, 1996; Du *et al.*, 1992), Coho and Pacific salmon (e.g. Devlin *et al.*, 1995a; Devlin *et al.*, 1995b) and common carp (e.g. Hinits and Moav 1999; Fu *et al.*, 1998; Chatachondi *et al.*, 1995; Wu *et al*, 1994). Nam *et al.*, 2001 reported on exceptional growth enhancement after autotransgenesis of the mud loach, a commercially important species in Asia. This group used the mud loach β-actin promoter to drive the mud loach growth hormone gene, creating three lines showing incorporation into the germline. The growth achieved was between 2 and 35 times greater than the non-transgenic controls, and significantly reduced the time required to reach market size.

Devlin *et al.*, (1995) incorporated the opAFP/csGH construct into coho salmon which resulted in fish achieving enhanced growth of an average of 11 fold, as compared with controls. Later Devlin *et al.*, (2001) reported that the potential for growth enhancement is strongly dependent on genetic background. These authors compared growth rates of a strain of slow growing wild rainbow trout with a fast growing strain of domesticated rainbow trout after introducing the rtMTP/csGH construct by egg microinjection. The transgenic slow growing wild rainbow trout responded well to the exogenous GH, growing on average 17 times larger than the non transgenic controls. The transgenic domesticated rainbow trout showed no significant growth enhancement over non-transgenic controls. Tilapia however have shown to be responsive to exogenous growth hormone after introduction of the opAFP/csGH construct resulted in transgenic tilapia becoming three times, on average, larger than their non-transgenic siblings (Rahman and Maclean 1999; Rahman *et al*,. 1998). The mud loach appears to exhibit great phenotypic plasticity, showing that growth enhancement by autotransgenesis can have strongly positive results. This group were the first to report

on enhanced growth by the method of somatic transgenesis, the DNA injected fish achieving an increase of growth of 102 %, as compared with controls reaching between 35-42 % of their original size in four weeks (Nam *et al.*, 2001).

4.1.2 The isolation and action of the beta-actin enhancer element

The carp β-actin enhancer element, first isolated by Liu *et al.*, (1990a) was found to be located near the 3′ end of intron I. Its presence was confirmed after the development of two constructs for use in transgenic research, pFV1 and pFV2, of which pFV1 contained the carp β-actin proximal promoter and pFV2 contained the untranslated first exon, the first intron and part of the second exon. Both constructs, used in a number of transgenic fish studies, resulted in significantly better expression from the pFV2 construct which contained the putative enhancer element (Moav *et al.*, 1993; Maclean *et al.*, 1992; Rahman and Maclean 1992b; Liu *et al.*, 1990c). The enhancer contains a CArG motif: a serum response element, this motif is also commonly found in DNA sequences of other higher eukaryotes (Minty and Kedes 1989; Orita *et al.*, 1989). The CArG motif binds serum response factor (SRF) which activates expression of several genes in response to growth factors present in the serum. The motif, found in the carp β-actin enhancer element, was shown to act in a position and orientation dependant manner in carp epithelial cells, rainbow trout hepatoma cells and fibroblasts when spliced to a CAT reporter gene, although there was great variation in gene expression attributed to the range of different *trans*-acting factors available in each cell type (Moav *et al.*, 1992).

Muller *et al.*, (1997) reported that β-galactosidase expression in zebrafish embryos was similarly increased when the lacZ gene, spliced to the 4.7 kb carp β-actin regulatory element, containing the full length upstream regulatory regions, was either co-ligated or co-injected (in separate linear form) with a rat-derived foetal myosin light chain enhancer (MyLC) element. This work points to the orientation and position independent action of the enhancer element. Since enhancers are known to act over very long distances and on multiple promoter sites along the chromosome (Nobrega *et al.*, 2003; Bengten *et al.*, 2000) and have been shown to act in a position/orientation

independent manner, it seemed a logical step to isolate the putative tilapia enhancer element from intron I of the tilapia β-actin promoter (tiEnh) and co-inject it in linear form with a reporter construct to ascertain whether any affect on expression might occur. I also linearised the lacZ construct using SpeI, leaving a 4.6 kb linear construct. It was interesting to see whether the linear reporter construct would give higher expression after co-injection with the enhancer element than when injected together with the construct in plasmid form.

The following experimental technique was applied in order to assess the feasibility of enhancing the growth of juvenile tilapia by somatically injecting the 'all tilapia' growth hormone construct (tiβAP/tiGH), kindly provided by Dr G. Hwang and also introducing the opAFP/csGH construct, kindly provided by Professor C. Hew. The objectives of this research were;

1) To investigate the effect of co-injecting a reporter gene with the 100 bp tilapia beta actin enhancer element isolated from intron I of the tilapia beta actin promoter.
2) To investigate the effect of injecting a linear reporter construct into the somatic tissues of juvenile tilapia, and to see whether the co-injected enhancer element boosted expression of the linearized gene sequence.
3) To attempt to positively influence the growth of juvenile tilapia by the method of somatic transgenesis using the 'all-tilapia' autotransgenic GH plasmid construct.
4) To inject the ocean pout anti freeze promoter / Chinook salmon GH plasmid construct, known to produce a strong growth response in transgenic tilapia, to promote growth.
5) To co-inject both of the 'all-fish' GH plasmid constructs with the linear enhancer element to investigate whether this provoked an enhanced growth response in juvenile tilapia fish.

4.2 Materials and Methods

4.2.1 Information on fish behaviour in aquarium conditions

Tilapia were selected from sibling batches to minimise any variation as observed when injecting transgene DNA into the somatic tissues as observed in chapter 3. The juveniles were weighed to ensure they all had the same weight to begin each trial with. It should be noted that tilapia, when not kept in high enough densities, will form hierarchies. Those which are dominant become extremely aggressive to subordinate fish, which may result in the death of these fish from harassment and fighting. However, the density of fish must be such that they survive the duration of each trial, are not subject to stressful tank conditions with low oxygen concentrations that can occur with heavy stocking since these can lead to low condition factor. The numbers used in the following experiments reflect the optimum tank conditions depending on which tanks were used and on which aquarium system, all in accordance with the Animals (Scientific Procedures) Act 1986.

4.2.2 Preparation of DNA and injection into juvenile tilapia

1) Following purification of plasmids and spectrophotometric determination of DNA concentration, varying concentrations of plasmid DNA were prepared.
2) Using, either a Hamilton 50 µl syringe or 1 ml insulin syringe, a given dosage was correctly measured and loaded into syringe (either 25 µl or 100 µl respectively).
3) 2-Phenoxyethanol (2-PE; Sigma) was used to anaesthetise the fish at a suitable concentration (~3% v/v) for the size of fish used in each experiment and mixed into a receptacle of tank water (to minimise discomfort and reduce shock) at a volume that enabled total immersion fish.
4) Control juvenile tilapia were carefully weighed by placing anaesthetised fish into a water bath, set at tare, on weighing scales and measured from tip of mouth to the end of caudal peduncle where it meets the caudal fin using callipers.

5) Fish were distributed between groups (control and GH injected) so that the average was approximately the same, to give an equal starting point for trial.
6) Once measurements were recorded, the anaesthetised fish were placed on a damp clean cloth on a platform, head facing to the left each time, to enable easy location of the injection site after GH trials.
7) The fish were then blotted dry at the injection site using tissue and injected, the needle inserted in between the dorsal and anal fin, slightly above the lateral line.
8) After depression of the syringe, the needle was left in the tissue for approximately 30 seconds, and then withdrawn while pressing down on the injection site with tissue to prevent excess loss of injected fluid.
9) The site was then sealed with a small amount of Vaseline, and the fish allowed to recover in a well-aerated tank.
10) Amount of feed was calculated initially at 8% then as required to 6% of the total body mass held in each of the tanks and measured into 7 separate trays for the week. Feed was administered twice daily, half in the morning and half in the late afternoon.
11) After each week the fish were caught, anaesthetised and reweighed in order to assess growth rate and recalculate feed levels.

4.2.3 PCR amplification of 100 bp enhancer element

Enh forward primer 5′-GTA GCC GCA AAG CTG CTC AA

Enh reverse primer 5′-GTG CCG CAC ATT CCG AGT AA

Initial denaturation at 95 °C for 5 minutes, followed by 95 °C for 30 seconds, annealing temperature of 64 °C for 30 seconds, extension 72 °C for 30 seconds all over 30 cycles, before a final extension time of 5 minutes at 72 °C, resulting in a 100 bp product.

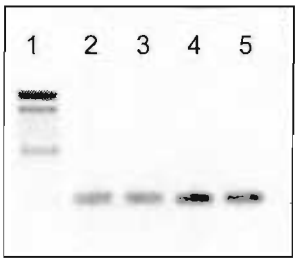


Figure 4.2.3 Tilapia enhancer element amplified from the tilapia β-actin promoter, using PCR. Lane 1 shows the 100 bp DNA marker ladder, lanes 2 – 5 show the 100 bp tiEnh PCR product.

4.2.4 PCR Purification of gel product

Adapted from Qiaquick spin protocol, composition of buffers listed in Qiaquick handbook.

1) The enhancer element obtained using PCR, was run on a 1.2 % agarose gel and visualised on a UV tray. The 100 bp product was excised from the gel using a clean, sharp scalpel, cutting as close to the DNA band as possible.
2) The gel fragments were placed in a 1.5 ml eppendorf tube and weighed.
3) 3 volumes of buffer GQ were added for each 1 volume of gel (100 mg ~ 100 µl), i.e. 300 µl of buffer to each 100 mg of gel.
4) The eppendorf tubes were incubated at 50 °C for up to 10 minutes, or until each of the gel slices were completely dissolved.
5) The colour of the solution was checked after the gel had dissolved to ensure it had maintained the desired yellow colour (if the dissolved gel had turned a violet in colour, the addition of 3 M sodium acetate pH 5, would have restored the yellow colouring).
6) 1 gel volume of isopropanol was added to the sample and mixed well.
7) The Qiaquick column was placed in a collection tube and spun in the centrifuge for 1 minute at 2.5 rpm, allowing the DNA to bind to the column. This step was repeated twice to ensure a high yield of product, after which the through flow was discarded

8) 0.5 ml of buffer QC was used to wash the column, and moved through the column using the centrifuge at 4 rpm. The through flow was discarded.
9) 0.75 ml of buffer PE was used to wash the column at 4 rpm, and the through flow was discarded.
10) After the removal of any collected buffer PE the tubes were spun again to ensure all of the buffer was moved through the column
11) The eppendorf tubes were placed in the drying oven at 50 °C for 5 minutes to ensure all the ethanol has evaporated.
12) To elute the DNA, 50 µl of buffer EB was placed in the top of the column and the entire column was placed in a clean 1.5 ml eppendorf tube. After allowing to rest for 1 minute, the tube was spun for 1 minute at 10 rpm. The elute was stored at -20 °C until required for use.

There is approximately a 75 % recovery rate for DNA purified in this way.

To calculate the 3:1 ratio used for co-injection technique injection, the following equation was used;

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio} \frac{\text{insert}}{\text{vector}} = \text{ng of insert}$$

4.2.5 Linearization of the tiβA/lacZ plasmid transgene

After quantification of DNA concentration, aliquots were prepared containing a total of 10 µl;

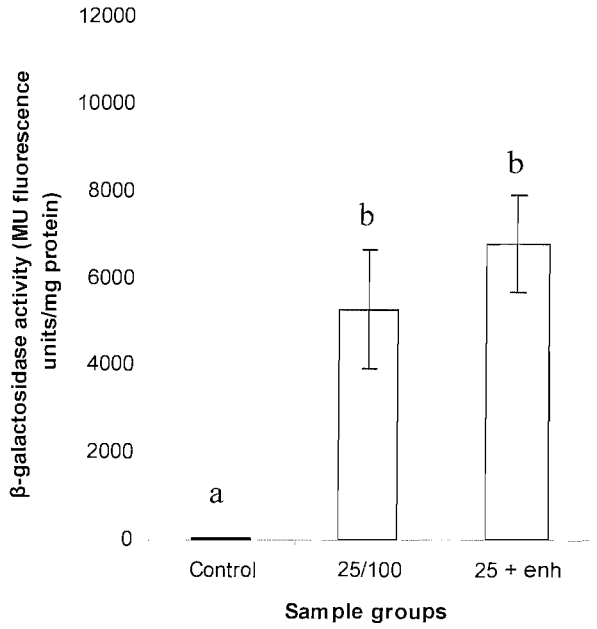
- 0.5 µl of tiβA/lacZ (= 2.4 µg plasmid DNA)
- 3 µl of 10 unit SpeI restriction nuclease
- 1 µl of 10 X buffer to dilute to 1 X
- 5.5 µl with SDW

General rule; 1 unit of restriction enzyme, cuts 1 µg of target DNA in 1 hour. The reaction was allowed to take place overnight at 50 °C, stored at – 20 °C until needed. After restriction the 7.2 kb plasmid was cut into a linear gene sequence of 4.6 kb and

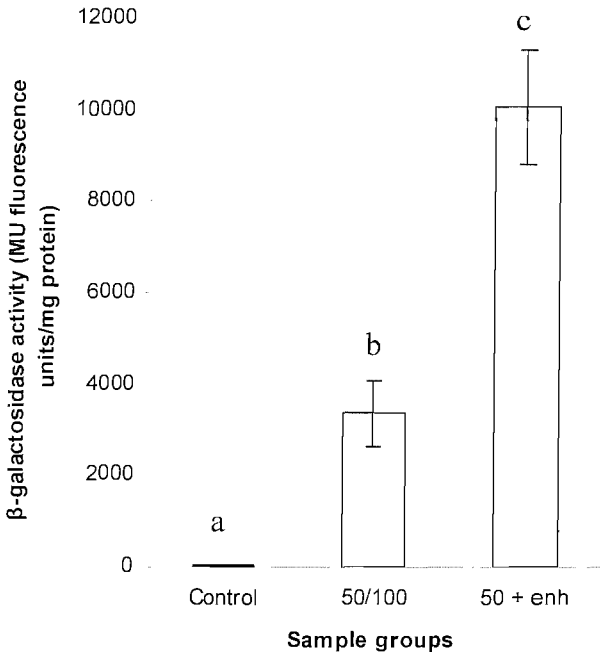
the remaining plasmid was 2.6 kb. Each reaction tube contained, after restriction, 64 % linear gene sequence and 36 % plasmid DNA. Each sample was allowed to run on an agarose gel to separate the two bands and the larger 4.6 kb band excised for purification of DNA as shown in step 4.2.4. Approximately 80 μg of linear DNA was made from 40 tubes (in above reaction), allowing for the reduction during gel extraction. The linear reporter gene sequence was collected and reduced by evaporation until 250 μg of linear plasmid was ready for injection into tilapia.

4.3 Results

Figure 4.3.1 (a) shows mean (± SE) β-galactosidase expression in muscle tissue homogenates from tilapia (n = 10). The controls injected with 100µl of SDW, the 25/100 were injected with 25 µg 100 μl^{-1} of plasmid tiβA/lacZ and the 25 + enh sample group were co-injected with the tilapia β-actin enhancer element suspended in 25 µg 100 μl^{-1} plasmid tiβA/lacZ, at a 3:1 ratio (there was three times the amount of linear enhancer element than plasmid DNA, as determined in micrograms of DNA). There was no significant difference between all treatments at the conclusion of the trial as tested with ANOVA (P = 0.345), but there was a significant difference between control and 25 µg 100 μl^{-1} tiβA/lacZ, (P = 0.000) as well as control and tiβA/lacZ + tiEnh (P = 0.000), significance tested at a 95 % confidence interval. Same letter indicates statistical significance (P < 0.05).



(a)



(b)

Figure 4.3.1 (a) – (b) Mean (± SE) β-galactosidase expression in muscle tissue homogenates from tilapia (n = 10). The graph displays the difference in expression levels of β-gal, taken from tissue homogenates from the following sample groups harvested 8 days post injection of plasmid tiβA/lacZ or co-injected with linear enhancer element (a) with 25 μg of plasmid DNA and (b) with 50 μg of plasmid DNA. Those indicated with the same letter i.e. a:a are statistically different ($P < 0.05$)

Figure 4.3.1(a) shows mean (± SE) β-galactosidase expression in muscle tissue homogenates from tilapia (n = 10). The co-injection of the enhancer element with plasmid tiβAP/lacZ at a 3:1 ratio can be seen to increase expression levels of the construct. There was an increase in expression, (though not statistically significant), when co-injecting 25 μg of tiβAP/lacZ with the enhancer element, as compared with injection of the reporter construct alone, although both prove statistically different from the control group, injected with SDW. Figure 4.3.1 (b) shows a 50/100 sample group which received an injection of 50 μg 100 μl^{-1} of plasmid tiβA/lacZ and the 50 + enh were co-injected with the tilapia β-actin enhancer element suspended in 50 μg 100 μl^{-1} plasmid tiβA/lacZ at a 3:1 ratio. The greater concentration of tiβAP/lacZ, 50 μg 100 μl^{-1}, resulted in an approximate 3-fold increase in expression when co-injected with the linear enhancer element ($P < 0.05$).

Figure 4.3.1.1

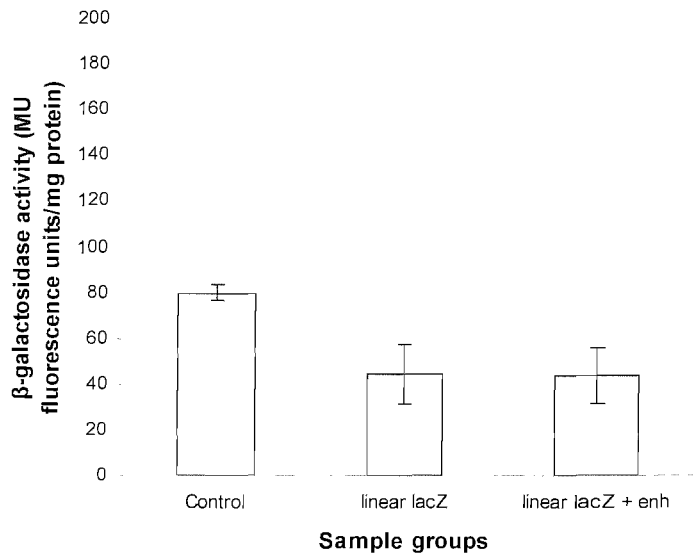


Figure 4.3.1.1 Mean (± SE) β-galactosidase expression in muscle tissue homogenates from tilapia (n = 5). The graph displays the difference in β-gal expression, taken from tissue homogenates from the following sample groups harvested 8 days post injection of linearised tiβA/lacZ.

Figure 4.3.1.1 shows the mean (± SE) β-galactosidase expression in muscle tissue homogenates from tilapia (n = 5). Controls were injected with 100µl of SDW, the 25/100 sample group were injected with 25 µg 100 μl^{-1} linear tiβA/lacZ, and the 25 + enh sample group were co-injected with the tilapia β-actin enhancer element suspended 25 µg 100 μl^{-1} linear tiβA/lacZ, at a 3:1 ratio. There was no significant difference between treatments at the conclusion of the trial as tested with ANOVA (P = 0.071). Linear sequences did not result in expression of the transgene, even when co-injected with the enhancer element. The expression level obtained on injection of 25 µg 100 μl^{-1} of linear tiβAP/lacZ transgene was not statistically different from the control group or when co-injected with the enhancer element.

It should be noted that the reduced number of fish in this experiment as compared with the other enhancer trials reflects the difficultly of producing large amounts of linear plasmid transgene. This had to be cut with the SpeI restriction nuclease, separated and purified on an agarose gel and quantified in a spectrophotometer until the correct concentration was achieved. This was a time consuming and costly experiment, therefore n = 5 for each of the sample groups.

Growth trials were conducted over time with variation in the concentration of DNA, the frequency of injection, and the volume of buffer the DNA was suspended in, to ascertain whether any growth response could be induced by somatically introducing the tiβAP/tiGH plasmid construct into the muscle tissue of juvenile tilapia. Figures 4.3.2 – 4.3.14 show the development of different trials, conducted with varied total DNA concentration injected, as well as, investigating frequency of injection and its effect on growth. This technique was applied in conjunction with other sections of this project, namely the finding of expression levels and persistence over time, as discussed in chapter III. This was in order to best utilise the maximum expression levels post injection and minimise trauma to the fish which can cause an adverse growth reaction through stress and low condition factor. Significance was tested for at the conclusion of each of the trials using a t-test (two-sample assuming equal variances).

Tables 4.3.2 – 4.3.14 summarise the initial average weight of each sample groups, their final weight, percentage survival, as well as, the food conversion ratio (FCR) and specific growth rate (SGR) achieved for each group. FCR and SGR were calculated according to the following equations; FCR = $W_{dt} / (W_2 - W_1)$; SGR = $(\ln W_2 - \ln W_1 \times 100) / t$, where W_1 and W_2 are the initial and final weights of the sample groups, W_{dt} is the dry weight of food and t is time in days. Feed conversion ratio (FCR) is calculated from the number of kilos of feed that are used to produce one kilo of whole fish. A reduced FCR shows better utilisation of feed. SGR indicates the growth response over time; a higher SGR reflects better growth of the fish.

Figure 4.3.2

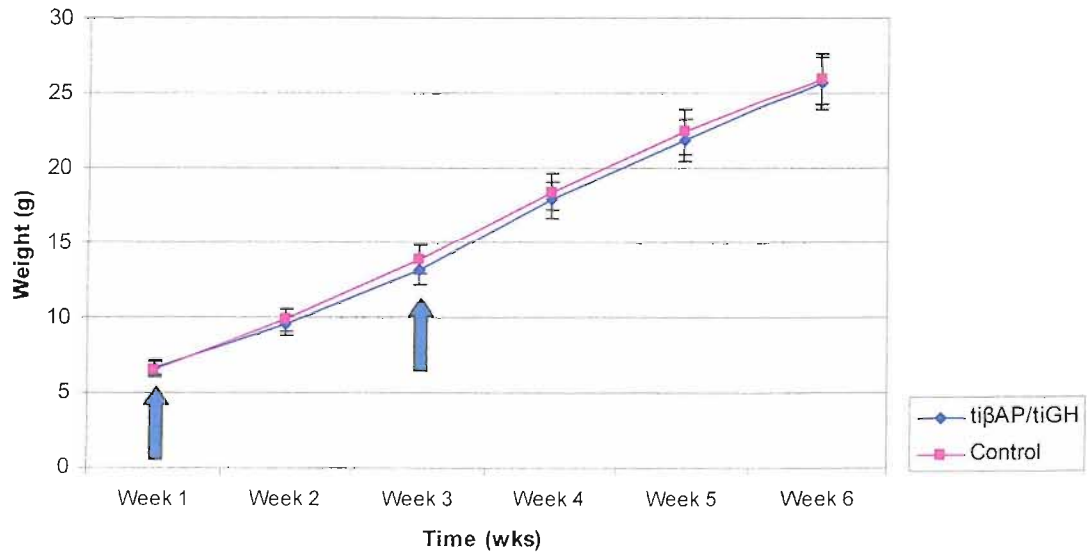


Figure 4.3.2 Graph depicting the mean (±SE) rate of increase in growth over time. DNA injected fish received a concentration of 7 μg g^{-1} $fish^{-1}$ (calculated from the average weight of the sample group) of plasmid tiβAP/tiGH intramuscularly injected. Controls receive SDW (n = 30).

Figure 4.3.2 shows two batches of juvenile tilapia initially weighing an average of 6.2 g. The tiβAP/tiGH group each intramuscularly received 7 μg g^{-1} $fish^{-1}$ (calculated from the average weight of the sample group) of plasmid tiβAP/tiGH. The control group were injected with an identical amount of SDW (n = 30). The arrows indicate the weeks when the sample groups were treated with this dose, allowing a week free

from injection in between doses to reduce stress to the fish. Significance was tested for at the conclusion of the trial, there was found to be no significant difference (P = 0.45).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	6.24	187.25	26.11	783.31	2.9	0.54	100
tiGH (7ug/g of fish)	6.19	185.72	26.02	780.67	2.9	0.52	100

Table 4.3.2 Feed conversion ratio and specific growth rate (7 μg g^{-1} $fish^{-1}$), shown with average and total weight gain of fish during the trial period, shown in figure 4.3.2.

Table 4.3.2 shows the change in weight of each of the sample groups at the beginning of the growth trial and again at the conclusion. The specific growth rate and food conversion ratio were calculated and indicate that there was no difference between sample groups at utilisation of available food.

Figure 4.3.3

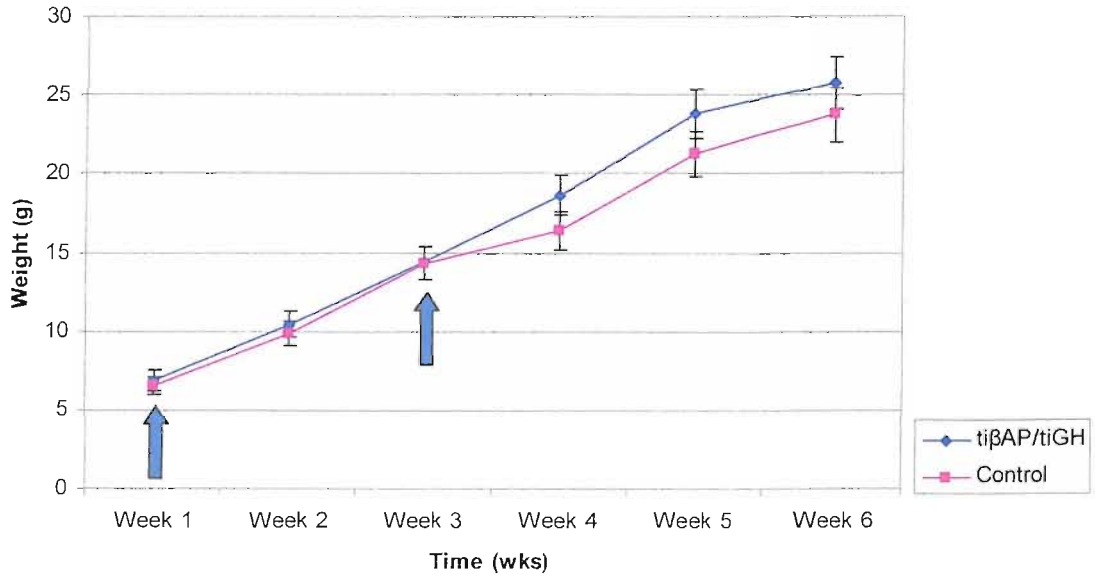


Figure 4.3.3 Graph depicting the mean (±SE) rate of increase in growth over time. GH sample group received a concentration of 14 μg g^{-1} $fish^{-1}$ (calculated from the average weight of the sample group) and controls received a sham injection of SDW.

Figure 4.3.3 shows the tiβAP/tiGH groups growth performance after intramuscular injection of the GH construct. Controls were injected with an identical amount of

SDW (n = 30). Here the fish were injected on two separate occasions to allow for fish recovery and peak expression of plasmid transgene. The GH injected group exhibiting a better growth response and grew marginally larger than the control group. Significance was tested for at the conclusion of the trial, there was found to be no significant difference (P = 0.19).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	6.89	206.72	24.02	720.62	2.55	0.61	100
tiGH (14ug/g of fish)	6.93	207.98	25.81	748.4	2.61	0.61	96.7

Table 4.3.3 Feed conversion ratio and specific growth rate (14 μg g^{-1} $fish^{-1}$) shown with average and total weight gain of fish during the trial period, as shown in figure 4.3.3.

Table 4.3.3 shows the change in weight of each of the sample groups at the beginning of the growth trial and again at the conclusion. The specific growth rate and food conversion ratio were calculated resulting in the GH injected fish having a better growth rate than the controls, as shown in figure 4.3.3, although the FCR was the same for both fish groups.

Figure 4.3.4

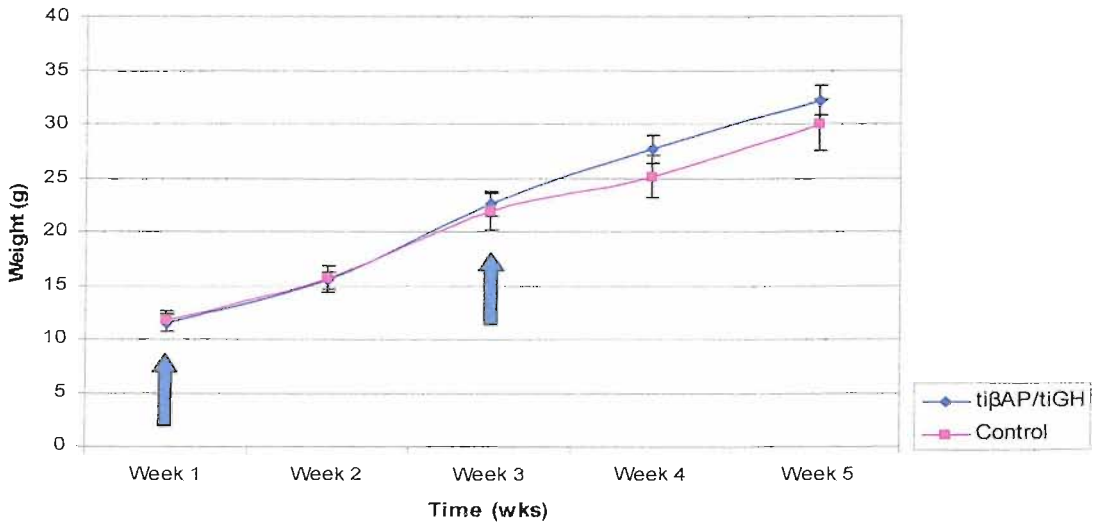


Figure 4.3.4 Graph depicting the mean (±SE) rate of increase in growth over time. GH sample group received two intramuscular injections at a concentration of 40μg 30$μl^{-1}$ of plasmid tiβAP/tiGH intramuscularly injected at weeks 1 and 3 as indicated with arrows. Controls were injected with an identical amount of SDW (n = 15) on the same injection dates

Figure 4.3.4 shows experimental batches injected with a high concentration of DNA in a small buffer volume (40μg $30\mu l^{-1}$).The fish injected with plasmid tiβAP/tiGH showed marginally better growth over time than the sham injected controls (n = 15). However, significance was tested for at the conclusion of the trial, there was found to be no significant difference (P = 0.34).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	11.71	175.71	30.09	451.35	2.7	0.82	100
tiGH (2 x 40 ug)	11.52	172.74	32.59	488.85	2.97	0.74	100

Table 4.3.4 Feed conversion ratio and specific growth rate (40μg $30\mu l^{-1}$) shown with average and total weight gain of fish during the trial period, as shown in figure 4.3.4.

Table 4.3.4 shows the change in weight of each of the sample groups at the beginning of the growth trial to the conclusion. The specific growth rate and food conversion ratio were calculated, resulting in a marginally better utilisation of available food exhibited by the GH injected group in comparison to the control group as well as a better growth rate.

Figure 4.3.5

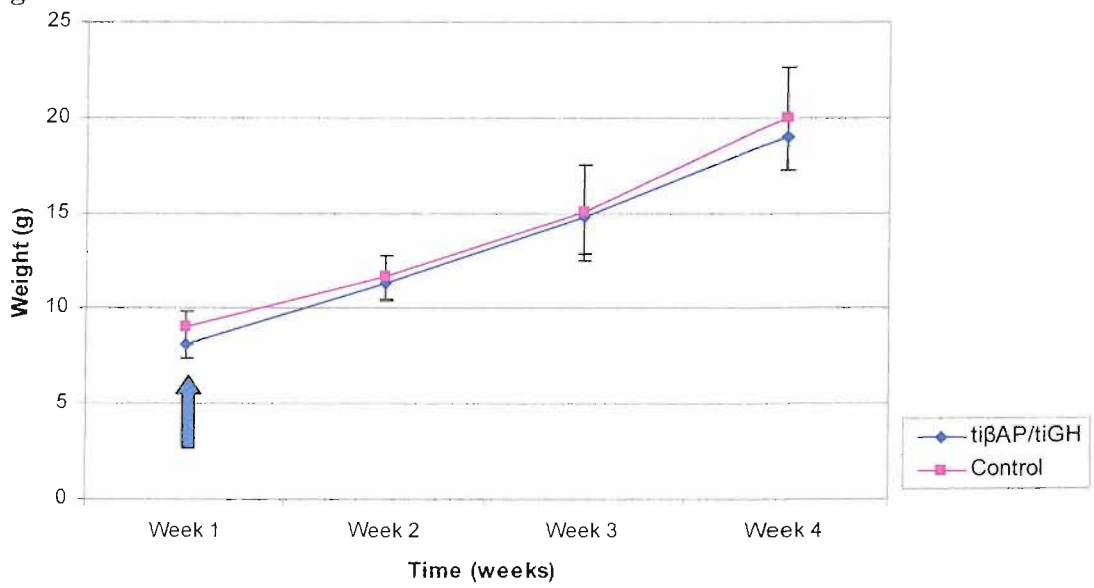


Figure 4.3.5 Graph depicting the mean (±SE) rate of increase in growth over time. GH received 57μg $100\mu l^{-1}$of plasmid tiβAP/tiGH, controls were injected with 100μl of SDW (n = 20)

Figure 4.3.5 shows the increase in growth of two sample groups. The GH sample group received a single intramuscular injection of plasmid tiβAP/tiGH at a concentration of 57μg $100\mu l^{-1}$. Controls were injected with an identical amount of SDW (n = 20). A single injection date was used to test whether by reducing the number of injections and the associated stress response, the fish would better respond to treatment. Significance was tested for at the conclusion of the trial, there was found to be no significant difference (P = 0.34).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	9	180.08	19.94	398.76	2.84	0.42	100
tiGH (57 ug / 100 ul)	8.09	161.79	19.02	380.43	3.05	0.39	100

Table 4.3.5 Feed conversion ratio and specific growth rate (57μg $100\mu l^{-1}$) shown with average and total weight gain of fish during the trial period, as shown in figure 4.3.5.

Table 4.3.5 shows the change in weight of each of the sample groups at the beginning of the growth trial and again at the conclusion. The specific growth rate and food conversion ratio were calculated with the GH injected group proving to have a better SGR and reduced FCR.

Figure 4.3.6

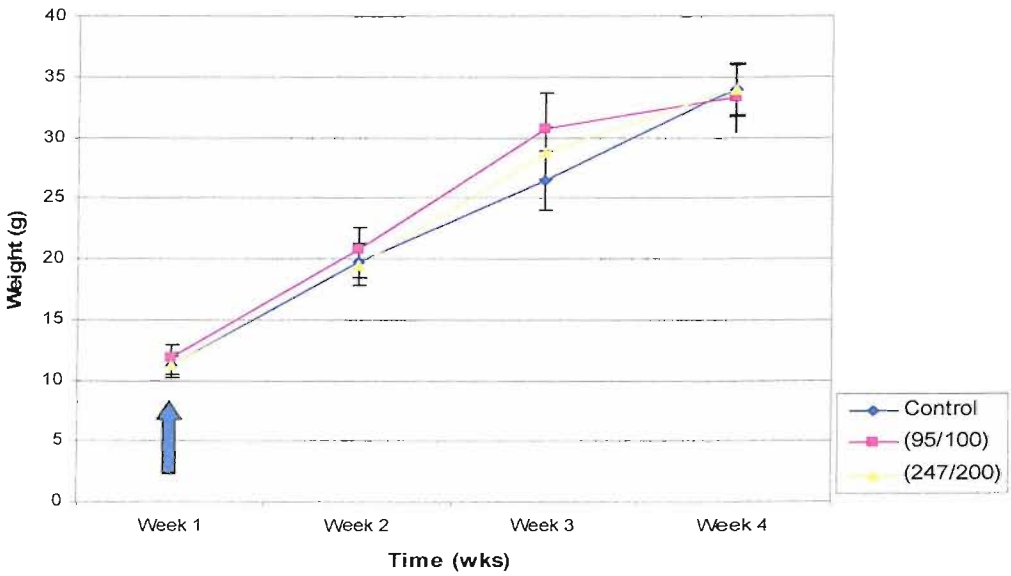


Figure 4.3.6 Graph depicting the mean (±SE) rate of increase in growth over time of the two GH sample groups intramuscularly injected with plasmid tiβAP/tiGH, the first group received a concentration of 95μg $100\mu l^{-1}$ and the second group was given a concentration of 247μg $200\mu l^{-1}$. Controls were injected with 100μl SDW (n = 5).

Figure 4.3.6 shows two batches of GH injected fish both injected with high concentrations of DNA. The lower concentration of 95μg $100\mu l^{-1}$ resulted in a marginally better growth response than either the control group, sham injected with SDW, or the batch that received the highest concentration of DNA (247μg $200\mu l^{-1}$). There was a separation of the mean weights at week three, the advantage gained however, by the 95μg $100\mu l^{-1}$ GH group reduced on reweighing at week 4. There was found to be no significant difference between sample groups (P = 0.975).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Controls	11.24	56.18	33.93	169.64	3.95	0.32	100
tiGH (94ug/100 ul)	11.87	59.34	33.27	166.36	3.68	0.36	100
tiGH (258ug/200 ul)	11.26	56.28	33.93	169.64	3.94	0.33	100

Table 4.3.6 Feed conversion ratio and specific growth rate (95μg $100\mu l^{-1}$; 247μg $200\mu l^{-1}$), shown with average and total weight gain of fish during the trial period, as shown in figure 4.3.6.

Table 4.3.6 shows the change in weight of each of the sample groups at the beginning of the growth trial and again at the conclusion. The specific growth rate and food conversion ratio suggest that there was a similar SGR and FCR for all test batches.

Figure 4.3.7

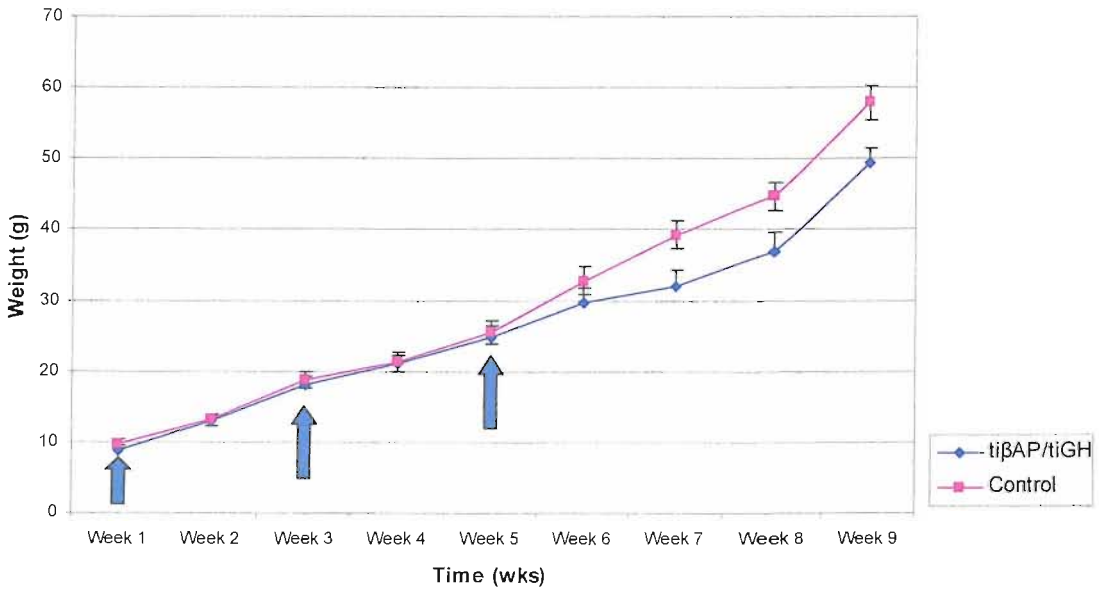


Figure 4.3.7 Graph depicting the mean (±SE) rate of increase in growth over time. GH sample group received a concentration of 50μg $100\mu l^{-1}$ of plasmid tiβAP/tiGH intramuscularly injected, three times over the course of 5 weeks. Controls were injected with 100ul SDW (n = 30).

Figure 4.3.7 shows the increase in mean weight of fish which were subjected to an increased dosing regime. Every other week, for 5 weeks, fish in the GH group received 50µg 100µl^{-1} of plasmid tiβAP/tiGH and the control group received at these time a sham injection consisting of SDW (n = 30). The putative increased amount of DNA made available over time resulted in a significantly reduced growth rate of GH injected fish when compared to the growth rate of controls (P = 0.029).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	9.73	282.13	57.87	1504.69	2.65	0.3	90
tiGH (50ug/100 ul)	8.88	257.53	49.33	1282.66	2.54	0.31	90

Table 4.3.7 Feed conversion ratio and specific growth rate (50µg 100µl^{-1}), shown with average and total weight gain of fish during the trial period, as shown in figure 4.3.7.

Table 4.3.7 shows the change in weight of each of the sample groups at the beginning of the growth trial and again at the conclusion. The specific growth rate and food conversion ratio were calculated and showed that the control group exhibited a better growth rate and food conversion over time than the GH injected group.

Figure 4.3.8

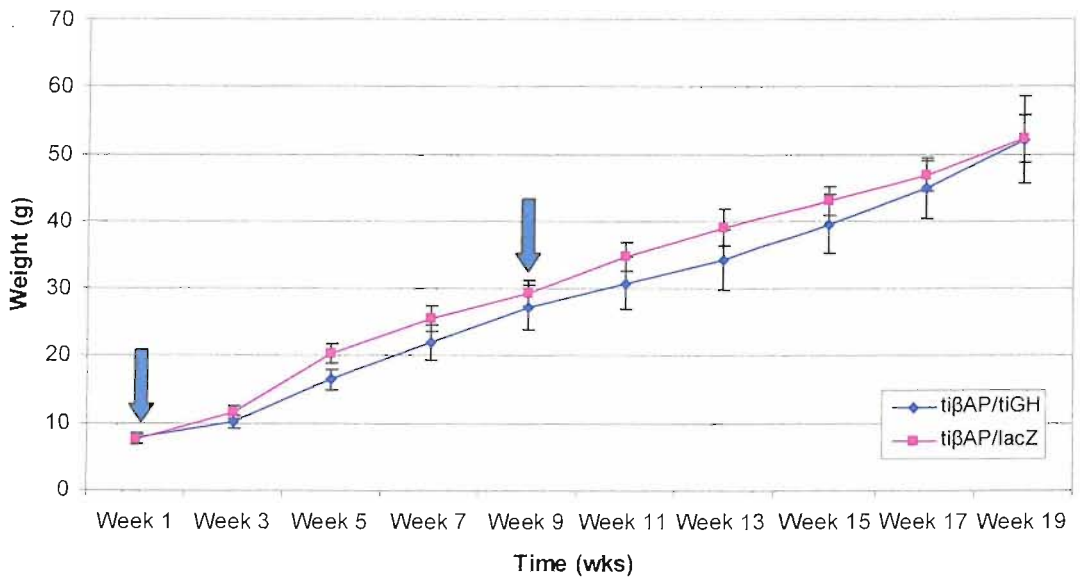


Figure 4.3.8 Graph depicting the mean (±SE) rate of increase in growth over time. GH sample group received a concentration of 50µg 100µl^{-1} plasmid tiβAP/tiGH twice over the course of 19 weeks. Controls were injected with 50µg 100µl^{-1} plasmid tiβAP/lacZ (n = 10).

Figure 4.3.8 shows a 19 week GH trial with a reduced injection regime. This was to allow for fish recovery after injection but to introduce DNA again at a later date to attempt in positively influencing the growth of fish. The GH injected fish twice received a concentration of 50µg 100µl^{-1} of plasmid tiβAP/tiGH (n = 10). Controls were twice injected with a concentration of 50µg 100µl^{-1} of plasmid tiβAP/lacZ. This was to test the hypothesis that the receiving of plasmid DNA into muscle tissues was detrimental to the fish as compared to those receiving only SDW. There was found to be no significant difference between sample groups (P = 0.48).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	7.53	75.32	52.21	522.09	1.46	0.3	100
tiβAP/tiGH	7.71	77.14	52.57	525.69	1.44	0.33	100

Table 4.3.8 Feed conversion ratio and specific growth rate (50µg 100µl^{-1}) of fish injected with the same concentration of plasmid GH construct and plasmid reporter gene construct. Average and total weight gain of fish during the trial period, as shown in figure 4.3.8.

Table 4.3.8 shows the change in weight of each of the sample groups at the beginning of the growth trial and again at the conclusion. The specific growth rate and food conversion ratio were calculated and showed no difference between groups.

Figure 4.3.9

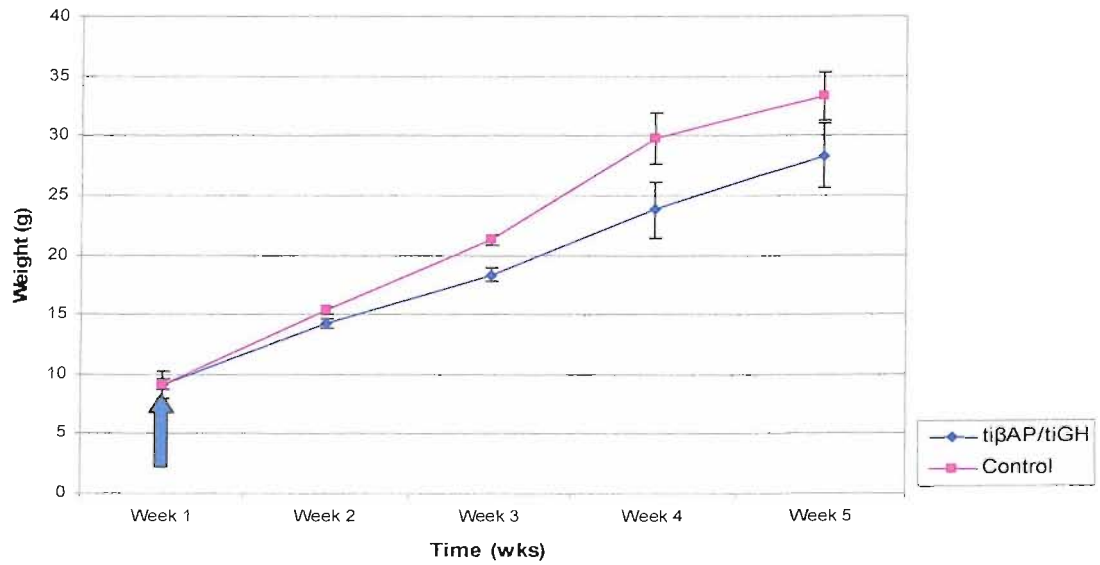


Figure 4.3.9 Graph depicting the mean (±SE) rate of increase in growth over time after intraperitoneal injection of plasmid tiβAP/tiGH. GH sample group received a concentration of 100µg 200µl^{-1} of plasmid tiβAP/tiGH injected into the liver through the peritoneal cavity (n = 5).

Figure 4.3.9 shows the growth response of fish injected once with 100µg 200µl^{-1} of plasmid tiβAP/tiGH into the peritoneal cavity. Controls were injected with 200µl of SDW (n = 5), this high concentration of DNA resulted in the reduced number of fish used in this experiment, it was very costly to produce such high concentrations of DNA. It was hoped that the introduction of the transgene into the liver would enhance the effect from the GH plasmid by supplying GH DNA to an organ known to have a high proportion of GH receptors. Significance was tested for at the conclusion of the trial using a t-test, two-sample assuming equal variances. There was found to be a significant difference between sample groups (P = 0.05) with the control group weighing significantly more than the GH injected.

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	9.13	45.67	33.3	166.48	3.69	0.36	100
tiGH (100ug/200ul)	9.12	45.6	28.32	141.62	3.23	0.39	100

Table 4.3.9 Feed conversion ratio and specific growth rate (100µg 200µl^{-1}) shown with average and total weight gain of fish during the trial period, as shown in figure 4.3.9.

Table 4.3.9 shows the SGR of fish injected intraperitoneally with either the GH construct or SDW. The control group achieved a much higher SGR than the GH injected fish, in line with the increased growth recorded in figure 4.3.9.

Figure 4.3.10

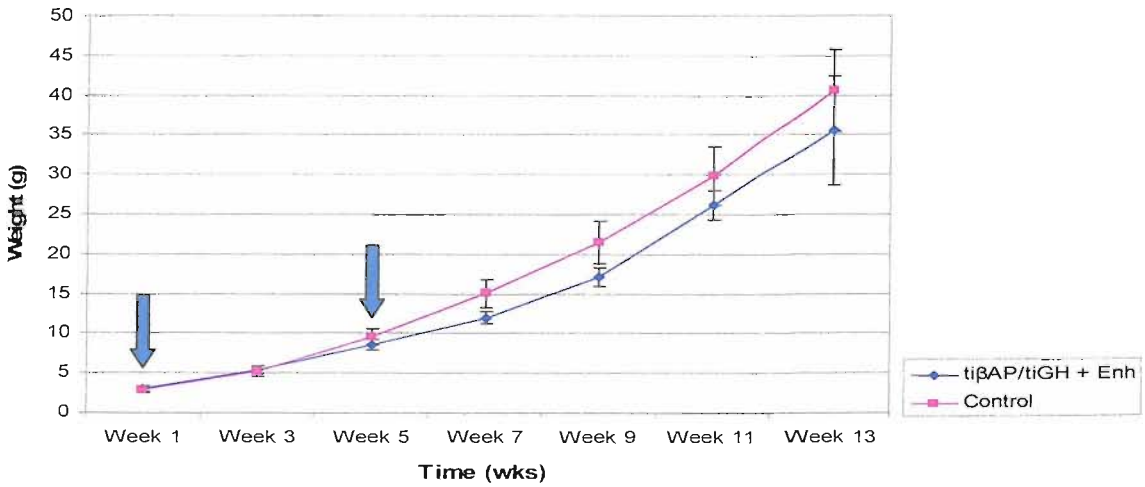


Figure 4.3.10 Graph depicting the mean (±SE) rate of increase in growth over time after intramuscular injection of plasmid tiβAP/tiGH and enhancer element. GH sample group received two intramuscular injections at a concentration of 66µg 100µl^{-1} of plasmid tiβAP/tiGH and linearised tiβA enhancer element at a 3:1 ratio (denoted on key tiβAP/tiGH + Enh). Controls were injected with 100µl of SDW (n = 10).

Figure 4.3.10 shows the growth trial comparing the growth response of control fish against those that received two intramuscular injections of plasmid tiβAP/tiGH co-injected with the 100 bp linear enhancer element isolated from the tilapia beta actin promoter region (n =10). The enhancer element was shown to boost the expression levels of a plasmid reporter gene on co-injection (figure 4.3.1). In this figure (figure 4.3.10) the growth of fish was slightly although not significantly less than the control group (P = 0.284).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	2.89	28.88	40.67	366.02	2.13	0.16	100
tiβAP/tiGH + Enh	3	30.03	35.53	319.75	1.98	0.15	100

Table 4.3.10 Feed conversion ratio and specific growth rate (66μg $100\mu l^{-1}$ of plasmid tiβAP/tiGH and linearised tiβA enhancer element at a 3:1 ratio) shown with average and total weight gain of fish during the trial period, as shown in figure 4.3.10.

Table 4.3.10 shows the change in weight of each of the sample groups at the beginning of the growth trial and again at the conclusion. The specific growth rate and food conversion ratio were calculated and show that the control group resulted in a better utilization of food over time and had a better growth rate than those co-injected with the GH plasmid construct and tilapia enhancer element.

Figure 4.3.11

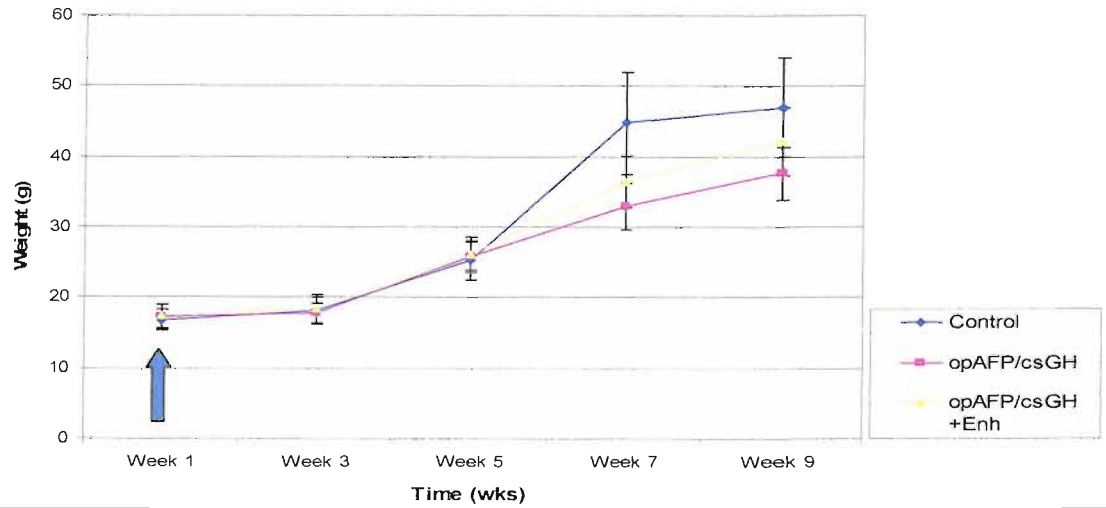


Figure 4.3.11 Graph depicting the mean (±SE) rate of increase in growth over time after intramuscular injection of plasmid opAFP/csGH and enhancer element. Of the two GH sample groups intramuscularly injected with plasmid opAFP/csGH, the first group received a concentration of 80μg $100\mu l^{-1}$ (denoted in the key as opAFP/csGH) and the second group was given a concentration of 80μg $100\mu l^{-1}$ of opAFP/csGH and the tiβA enhancer element (denoted in the key as opAFP/csGH + Enh) at a 3:1 ratio.

The opAFP/csGH construct was used in somatic growth trials, since this construct has been proven to potently stimulate the growth of salmonids as well as tilapia (Rahman and Maclean 1998). The tiβAP/tiGH construct has yet to be rigorously tested in conventional transgenics and so the ocean pout/Chinook salmon construct was injected both alone and co-injected with the tilapia beta actin promoter element. These fish received a single dose of either plasmid opAFP/csGH or plasmid opAFP/csGH and tiENH both at a concentration of 80μg $100\mu l^{-1}$. The control group, similarly to the results obtained when using the 'all-tilapia' construct (figs. 4.3.2 - 4.3.10), reached a larger size at the conclusion of the trial at 9 weeks. There was however, no significant difference between sample groups (P = 0.975).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	16.8	83.99	46.99	234.96	1.63	0.31	100
opAFP/csGH	17.31	86.56	37.62	188.11	1.23	0.42	100
opAFP/csGH + Enh	17.41	87.03	42.15	210.76	1.40	0.36	100

Table 4.3.11 Feed conversion ratio and specific growth rate (80μg $100\mu l^{-1}$ + enh of plasmid pAFP/csGH shown with average and total weight gain of fish during the trial period, as shown in fig 4.3.11.

Table 4.3.11 illustrates that the control group had a better FCR and SGR than the GH injected groups and were better able to utilize available food.

Figure 4.3.12

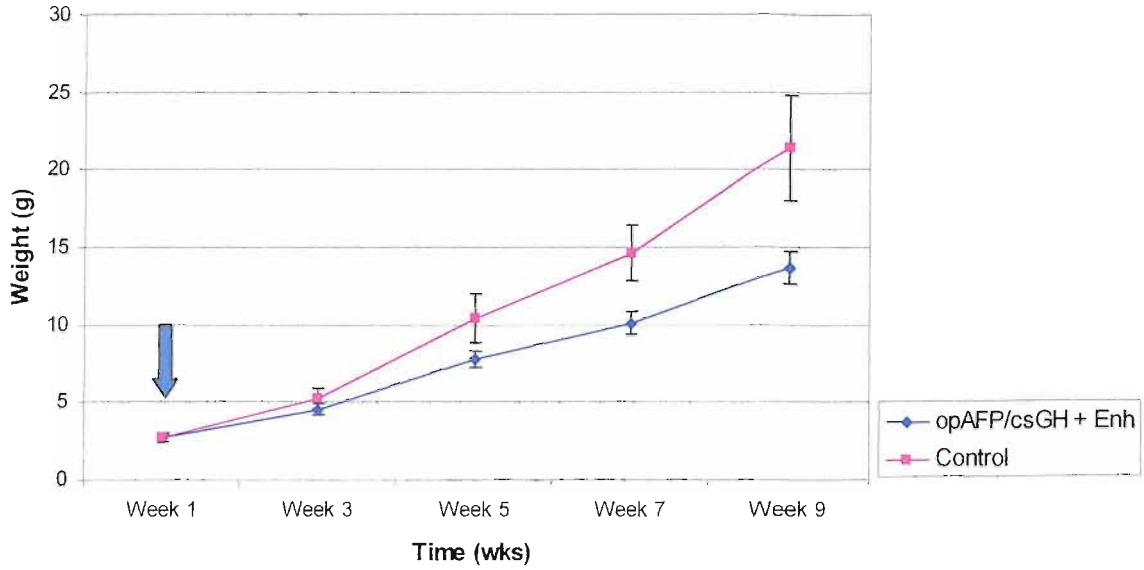


Figure 4.3.12 Graph depicting the mean (±SE) rate of increase in growth over time after intramuscular injection of plasmid opAFP/csGH and enhancer element. GH sample group received a concentration of 60µg 100µl^{-1} of plasmid opAFP/csGH and the tiβA enhancer element (denoted in the key as opAFP/csGH + Enh) at a 3:1 ratio intramuscularly injected. The arrows indicate the week when the sample groups were treated. Controls were injected with 100µl of SDW (n = 10).

Over a 9 week period the growth of fish, co-injected with the opAFP/csGH construct and the tilapia beta actin enhancer element, was significantly reduced in comparison to a control batch injected with SDW alone (P = 0.029). The fish received a single injection at the beginning of the trial to best reduce associated stress responses to the injection procedure (fig. 4.3.12).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	2.72	27.15	21.37	192.29	3.10	0.14	90
opAFP/csGH + Enh	2.68	26.82	13.63	136.26	2.58	0.17	100

Table 4.3.12 Feed conversion ratio and specific growth rate (60µg 100µl^{-1} + Enh of plasmid opAFP/csGH) shown with average and total weight gain of fish during the trial period, as shown in figure 4.3.12.

Table 4.3.12 shows the change in weight of each of the sample groups at the beginning of the growth trial and again at the conclusion. The specific growth rate and food conversion ratio were calculated and show the control group having a higher growth rate than the GH injected as well as being able to better utilize food.

Figure 4.3.13

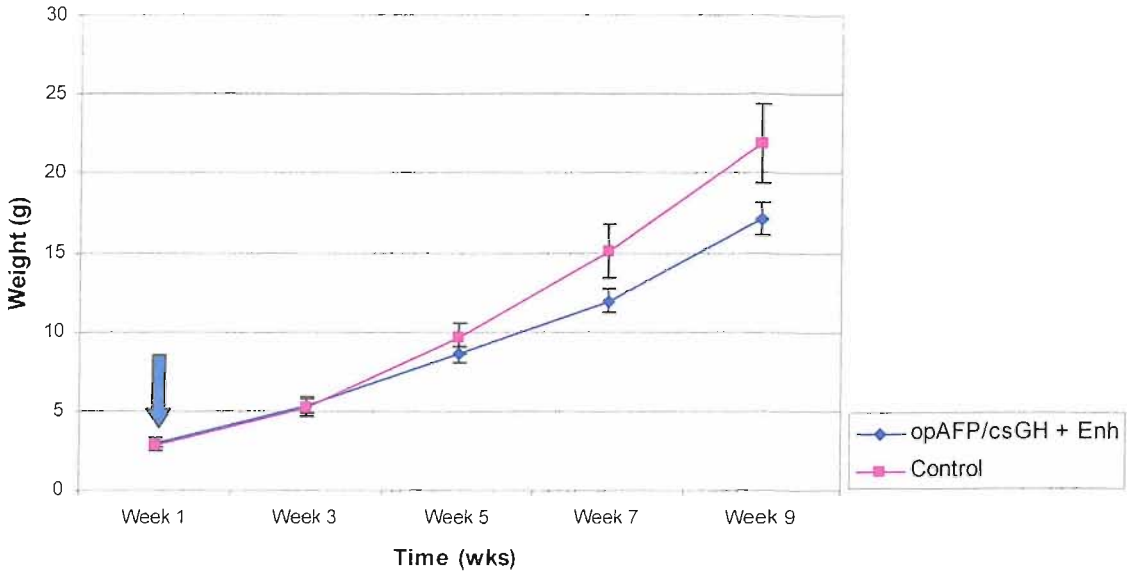


Figure 4.3.13 Graph depicting the mean (±SE) rate of increase in growth over time after intramuscular injection of plasmid opAFP/csGH and enhancer element. GH sample group received a concentration of 66µg $100\mu l^{-1}$ of plasmid opAFP/csGH and the tiβA enhancer element (denoted in the key as opAFP/csGH + Enh) at a 3:1 ratio intramuscularly injected. Controls were injected with 100µl of SDW (n = 10).

Figure 4.3.13 shows the growth response of juvenile tilapia after receiving a single injection of the opAFP/csGH plasmid construct co-injected with the tilapia enhancer element. After 9 weeks the growth of fish injected with the salmon GH construct was significantly reduced when compared with the control group (P = 0.045).

Sample Group	Av initial weight g	Total initial weight g	Av final weight g	Total final weight g	SGR	FCR	% Survival
Control	2.27	27.88	22.28	200.58	3.13	0.16	90
opAFP/csGH + Enh	3	30.03	16.89	152.11	2.57	0.19	90

Table 4.3.13 Feed conversion ratio and specific growth rate (66µg $100\mu l^{-1}$ + Enh of plasmid opAFP/csGH) shown with average and total weight gain of fish during the trial period, as shown in figure 4.3.13.

Table 4.3.13 shows the improved growth rate and food utilization of the control group as compared with the GH and Enh co-injected sample group, reflecting the significant increase in growth of controls as seen in fig. 4.3.13.

4.4 Discussion

4.4.1 The action of the enhancer element

The co-injection of the enhancer element with plasmid tiβAP/lacZ at a 3:1 ratio (there was three times the amount of linear enhancer element than plasmid DNA, as determined in micrograms of DNA) was shown to increase expression levels of the lacZ reporter construct. The higher concentration of tiβAP/lacZ, 50 μ g 100 μ l^{-1}, resulted in an approximate 3-fold increase in expression when co-injected with the linear enhancer element. Muller *et al.*, (1997) similarly reported on the increased expression of a myosin heavy chain promoter / reporter construct after co-injection with a rat-derived foetal myosin light chain enhancer (MyLC) into zebrafish embryos. Interestingly, in this case the expression gained was greater when the enhancer element was co-injected, as compared with covalently linked to the reporter construct.

Linear sequences were shown not to result in expression of the transgene, even when co-injected with the enhancer element. The expression level obtained on injection of linear tiβAP/lacZ transgene was not statistically different from the control group or when co-injected with the enhancer element. This may be attributed to their increased susceptibility to nuclease activity on introduction to the cells. In this regard it is puzzling that co-injection of a circular construct within a plasmid, along with linear copies of the short enhancer sequence results in clear activity of the enhancer sequence. Perhaps the small size of this latter sequence renders it more resistant to endonuclease attack. Also, it may rapidly ligate into some of the plasmids.

Current theory suggesting the interaction between cognate enhancer elements and the associated promoter has been modelled with the 'facilitated tracking model' (Blackwood and Kadonaga 1998) and the 'billboard' model (Arnosti 2003). These describe how enhancer elements are thought to act particularly on specific promoters to yield a temporal or spacial result, when studies in vertebrates indicate that most enhancer/silencers sequences have the ability to activate a wide variety of promoters (Kermekchiev *et al.*, 1991). Butler and Kadonga (2002) showed that the majority of

enhancers activated genes containing either the TATA or DPE motifs in the basal promoter, normally involved in enhancer-promoter specificity, suggesting that many enhancers may interact with disparate promoters.

The facilitated tracking mechanism incorporates an enhancer-bound complex containing DNA binding factors and coactivators which 'track' along the chromatin in small steps, until encountering the cognate promoter, whereupon, a stable looped structure is formed. Chromatin structure is thought to be an important component, and coactivators such as CREB binding protein (CBP) or p300, each possessing histone acetyltransferase activity, may recognise and modify chromatin structure, facilitating enhancer-promoter communication and altering repressive chromatin structure (Song *et al.*, 2002). This model allows for the large distances observed between the enhancer sequence and the promoter, and it also allows for the action of boundary elements to shield the promoter from unwanted enhancer action and permits transvection, the action of an enhancer on a cognate promoter on an allele situated on a paired chromosome. The 'billboard' model proposes an information display containing loosely constrained transcription factor binding proteins that interact with the transcriptional machinery in a variety of conformations, with the result that activators send a positive output to promoter, resulting in transcription initiation. In the absence of this signal, co-repressors bind in place of the co-activators, suppressing the weak activity of the widely expressed general activator (Arnosti 2003).

Muller *et al.*, (1997) postulated that there may be two possible actions of the enhancer on the transgene. The first was via DNA looping, as described by the facilitated tracking model (Blackwood and Kadonaga 1998), in which after the formation of extrachromosomal concatemers, there is a resulting *cis* activation of the DNA via looping. Concatamer formation has been shown to readily occur in fish embryos where ligation of linear DNA sequences can occur in any conformation (Iyengar and Maclean 1995; Winkler *et al.*, 1991). Presumably, the random nature of the concatemerisation and the position and orientation independent action of many enhancer elements, would allow the enhancer to boost transcription as noted by Muller

et al., (1997). The second action may be likened to the 'billboard' model (Arnosti 2003), where the non-covalently linked enhancer affects the promoter via *trans*-acting protein binding. Enhancer elements are noted for their ability to activate transcription on an allelic promoter on a pared chromosome known as transvection (Blackwood and Kadonaga 1998). This ability to act in *trans* as well as *cis*, and act over distances that may reach several kilobases in length (Nobrega *et al.*, 2003) may well facilitate the action of the co-injected β-actin enhancer element on the plasmid tiβAP/lacZ construct, resulting in the boosting of expression noted in this study.

However, it remains unclear whether the transgene construct and the enhancer sequence (in various copy numbers) associate by ligation prior to passage into the nucleus, after passage into the nucleus, or whether their cooperative action can occur in the absence of such ligation. As compared with the presumed association of transgene sequences with chromatin during the disassembly of the nuclear envelope during cellular mitosis (following injection of transgene copies in conventional transgenesis), the introduction of transgene copies into muscle tissue presumably requires movement of the transgene sequences into intact nuclei via nuclear pores. Nuclear entry is clearly necessary for transcription, yet muscle cell nuclei probably rarely if ever become involved in mitosis. In this regard somatic transgenesis is very different from conventional egg-injection transgenesis.

4.4.2 The potential of growth enhancement by somatic gene transfer

Despite introducing a wide range of DNA concentrations, from only 7μg g^{-1} per fish to a maximum of 247μg 200μl^{-1}, and varying the frequency of injection, no significant growth response was obtained in these experiments. In the initial experiments, plasmid tiβAP/tiGH was injected twice, the first injection followed by a second in week three, allowing time for expression levels to peak, (according to results obtained in chapter III, section 3.3.2, figure 3.3.2.1) before administering the second dose. After the introduction of 14 μg g^{-1} $fish^{-1}$ there was improved growth of theGH injected batch, but this was not significant and resulted in a similar specific growth rate, as well as, feed conversion ratio.

The specific growth rate and food conversion ratio provides a useful index with which to compare the growth of each sample group, since improved growth performance and/or efficiency in food conversion are desirable traits in farmed fish species. Rahman *et al.*, (1998) showed that in transgenic fish that exhibited growth enhancement after egg injection of the opAFP/csGH construct, the food conversion ratio was significantly improved when compared to controls. The promotion of an increased SGR and reduced FCR indicates increased appetite and overall better utilisation of available food. Guillén (2002) reported that transgenic tilapia showed approximately 3.6 fold less food consumption in relative terms as compared to the control tilapia, demonstrating GH-transgenic tilapia with improved growth also are more efficient in food conversion. It was therefore interesting to see whether somatic injection of either the tiβAP/tiGH or opAFP/csGH constructs altered the specific growth rate and/or feed conversion ratio of the DNA injected tilapia when compared to controls.

It was shown that in the majority of trials, when comparing tiβAP/tiGH injected fish to control fish, the control group demonstrated a marginally better FCR, and therefore that the controls utilised feed more effectively than DNA-injected fish. On average the control group exhibited a better specific growth rate. This suggests that the control fish converted feed to body mass more efficiently than the tiβAP/tiGH injected sample groups in the trials conducted. A similar finding was also made by Guillén *et al.*, (1998) who reported that after intraperitoneal injection of 2.5 μg of tilapia growth hormone per gram of body weight per week, the fish showed a decrease in body weight and length of 1.2 % and 1.5% respectively relative to the control group (although this was not statistically significant). However, direct injection into the liver resulted in the controls being significantly larger than the DNA injected fish, reflected in the SGR and FCR.

In transgenic fish the use of promoters which resulted in low-levels of expressed tiGH, proved to yield growth enhancement in tilapia (Rahman and Maclean 1999; Martínez *et al.*, 1996; de le Fuente *et al.*, 1995) in salmon (Devlin *et al.*, 1995; Devlin *et al.*,

1994; Du *et al.*, 1992b) and carp (Hinits and Moav 1999). It has been documented that high levels of GH may produce an inhibitory effect on growth. Zhang *et al.*, 1990 found that common carp expressing low levels of RSV/rtGH1 cDNA grew faster than those containing higher levels, suggesting GH may produce an inhibitory effect on growth. Hernández *et al.*, (1997) reports that growth acceleration was achieved only after low expression of GH in tilapia, and those fish containing gene inserts with viral promoters (CMV and RSV), known to produce high levels of expression, resulted in similar weights to controls. Mori and Devlin (1999) reported on the effect of increased levels of liver IGF-I mRNA and serum IGF-I in transgenic mammals, which resulted in reduced pituitary GH, owing to suppression of GH gene transcription. They also reported that the expression of the GH transgene resulted in reduced pituitary size and endogenous GH transcription in transgenic coho salmon.

The exogenous GH may be acting to provoke slight endocrine controlled down regulation of endogenous GH, after somatic gene transfer. On injection of the GH constructs into the liver, as well as co-injection with the enhancer element, the negative effect on growth was more pronounced. Perhaps, by making the exogenous DNA more available throughout the body via the bloodstream as was intended by the intraperitoneal injection, or the putative boosting of expression using the enhancer element, down regulation of endogenous growth hormone occurs. Levels of exogenous protein have been shown to increase in muscle tissue for up to 21 days (figure 3.1.4). Perhaps the exogenous GH is produced in sufficient quantities over the first three weeks to create a slight endocrine controlled down regulation of endogenous tilapia GH. After approximately three weeks the signal from the exogenous gene construct has been shown to reduce, it may take some time for endogenous GH levels to recover, after which time the endocrine system may again reach natural homeostasis.

GH stimulates growth promotion by up-regulating the synthesis and secretion of IGF-I and II. In the presence of GH, the GHR forms a dimer. This putative dimerization of the GHR is thought to be essential of proper signal transduction. The binding of GH to the GHR on the extracellular surface allows the sequential binding of 3 forms of JAK's.

On phosphorylation of the third JAK, by the kinase itself, the phosphorylated area becomes a docking site for the STAT transcription factor. This is phosphorylated by the kinase and forms a dimer with another STAT protein, on doing so the dimer is translocated to the nucleus where it binds to the DNA and up-regulates transcription (Kopchick *et al.*, 1999). Guillén *et al.*, (1998) reported that an intraperitoneal injection of 2.5 μg of tilapia growth hormone per gram of body weight per week produced a slight but not significant reduction of growth. These authors postulated that too much growth hormone present in the serum over-loaded the growth hormone receptors which were unable to dimerize and therefore prevented the subsequent signal transduction pathway which would follow normally. This would down-regulate signal transduction capacity of GH and/or IGF receptors.

Since GH acts to stimulate IGF-I and its growth promoting action in an autocrine and paracrine fashion. The localised availability of GH, on intramuscular injection and subsequent processing of the exogenous DNA, may have resulted in an overload of GH acting on local GH receptors which may have prevented the signal transduction of the exogenous and well as endogenous GH. This may be why a growth depression was noted and a significantly so, on co-injection with the enhancer element, and when injected into the liver, an organ with a high incidence of GHR's.

To ensure that the growth repression observed in many of the growth trials was not a product of the plasmid DNA, and that perhaps it was physiologically more stressful to the fish than those in the control group which receive buffer alone, a trial was conducted with the control group receiving plasmid DNA which did not code for GH. Instead the plasmid tiβAP/lacZ construct was injected into the control fish and the GH injected batch received the same concentration of the plasmid tiβAP/tiGH construct. This again, like the other trials, resulted in the slight down regulation of the GH injected batch which may point to the product of the plasmid (i.e. growth hormone) and not the plasmid DNA itself providing the observed effect. In this trial, which was conducted over 19 weeks, the GH-injected group showed slight repression in their growth as compared with the controls, although this was not significant. The DNA-

injected group received two administrations of 50μg $100\mu l^{-1}$ of plasmid tiβAP/tiGH at weeks 1 and 9. The trend shows after each of these administrations there was an increase in the margin between the tiβAP/tiGH injected fish and the control group, that is, the controls gained more weight after each dose of DNA (but not with statistical significance). This margin reduces over time until week 9 where there is little difference between the groups and on re-injection there is a second separation in average fish weight, with the GH injected not growing as fast as the control group. Then seemingly, as the DNA is degraded away, the margin again reduces between the two groups, so that on the conclusion of the trial there is no discernable difference in the final weights of the groups. This may confirm that the disruption of endogenous GH production is short lived, and fish over time regain any losses caused by production of exogenous GH when briefly interrupting the natural growth of the fish. This discussion however is purely speculative, given the very slight decrease in growth noted in these trials.

The intramuscular injection of the opAFP/csGH construct was used for somatic gene transfer since this construct was reported to have been successful in enhancing the growth of transgenic fish (Rahman and Maclean 1999; Rahman *et al*,. 1998; Devlin *et al*., 1995a). This construct as well as the tiβAP/tiGH construct was co-injected with the linear enhancer element, after the putative increase in expression noted when co-injected with the tiβAP/lacZ plasmid reporter construct. This set of trials resulted in the reduced growth of the GH injected batch as compared with the control batch. In the case of the tiβAP/tiGH construct, the difference was not significant, but on co-injection with the opAFP/csGH and enhancer element there was a significant difference; the enhancer element significantly reduced the growth of the DNA + Enh injected. Again this was reflected in the improved FCR and SGR of the controls. Increasing the concentration of DNA, and injecting this increased concentration both with and without the enhancer element, did not in either case result in a significant difference in sample groups at the conclusion of the trial. This finding may reflect the result that somatic gene transfer can reach a threshold point, where the mechanism by

which plasmid DNA is taken up into the nucleus becomes saturated and further increases in DNA concentration are not reflected by increases in expression.

Interestingly, since beginning this project, Noh *et al.*, (2003) reported on the successful growth enhancement of mud loach somatically injected with an autotransgenic plasmid construct containing mud loach β-actin promoter spliced to the mud loach growth hormone gene (mlβAP/mlGH). The method of introduction varied slightly from that used here in that after intramuscular injection, they applied an electrical impulse to the skin within 1 cm of the injection site using electrodes. This resulted in an increase in weight of 102 % for the GH injected group as compared with between 35 – 43 % increase in weight as shown by the control group. This research group used 10 μg of DNA dissolved into buffer and injected into fish weighing approximately 5 g. Perhaps the increased phenotypic plasticity which this fish species exhibits as demonstrated by Nam *et al.*, (2001), in which transgenic mud loach were reported to have reached, in some cases, a 35-fold increase in size as compared with non-transgenic siblings, contributed to making this species a more ready target for this type of gene transfer. However Li *et al.*, (2003) noted that juvenile tilapia exhibited growth enhancement after engineering a strain of yeast to express and secrete carp GH and injecting the purified carp GH into the peritoneal cavity a dose of 1μg g^{-1} $week^{-1}$. This resulted in the fish injected with purified growth hormone growing 53.1 % faster than controls over a 5 week period. They reported that unlike transgenic tilapia, those injected with purified GH showed a slightly higher percentage of lipid, undesirable for aquaculture.

The localised GH expression in the muscle tissues and the absence of widespread availability of this exogenous growth hormone may be a factor in down regulation of endogenous GH. There also may be an immune response to the plasmid DNA which was not observed in the sham injected, certainly cells of the immune system have been shown to recognise endogenous antigens, the expressed proteins from exogenous plasmid DNA. A slight down regulation in growth was still observed in tiβAP/tiGH injected fish, in the trial where the controls received an injection of plasmid tiβAP/lacZ,

even though β-galactosidase is known to be highly immunogenic, which suggests the GH construct effects negatively affects growth although not significantly.

It is clear that improved growth has been attained in transgenic fish, and that growth enhancement has been obtained in mud loach after somatic transgenesis. In tilapia however while there has been success in producing growth enhanced transgenic fish, (Rahman and Maclean 1999; Rahman *et al.*, 1998) it would appear that there is a number of factors which restrict the possibilities for enhanced growth after somatic gene transfer of GH constructs.

Chapter five:

Discussion of results and future work

5.1 Overall discussion and future work

This project had two major objectives, both of which were related to studying the effect of DNA introduced into the muscle cells of tilapia by somatic gene transfer. The first objective was to determine the behaviour of exogenous transgene DNA once injected into the tissues of juvenile tilapia, and to ascertain the length of time the plasmid DNA and its gene product survived *in vivo*. It was necessary initially to determine the effect that DNA concentration and buffer volume had on expression using a reporter gene construct. A larger volume of buffer aided DNA uptake, presumably by increasing the area which is covered and allows greater damage to muscle fibres, the regeneration of which has been proved to enhance plasmid DNA uptake and subsequent expression (Wells *et al.*, 1997; 1993). However, it is important that the concentration of transgene DNA is high enough when suspended in such volumes so that the advantage gained is not lost through dilution effect. It was found that there was an optimum DNA concentration, doubling of the concentration did not yield significantly higher expression in targeted cells. The mechanism used in active plasmid uptake, for transporting the large plasmid vectors from the scarcoplasm to the nucleus of muscle tissues, has a putative saturation point. This is a limiting factor for gene delivery by the method of somatic gene transfer.

The comparison of the two reporter constructs; the tilapia and carp β-actin / lacZ constructs, and their subsequent effect on expression levels, resulted in the finding that the higher homology of the tilapia promoter, as expected, did yield an increase in expression of about 3-fold as compared with the carp construct. This expression was seen to increase over 21 days and reduce after which time, remaining significantly higher than controls for 46 days. PCR analysis at the site of injection found that the plasmid DNA was fairly stable over time but was degraded away slowly, presumably both by nuclease activity within the nucleus and by an immune response mounted against the endogenous antigen. Certainly β-gal is known to be strongly immunogenic and may have contributed to the decline of both the protein and the cell in which the protein was being made, as observed by Wells et al., (1997). Evidence presented by Hodges *et al.*, (2004) and Jorgensen *et al.*, (2001) suggest that CpG motifs, often present in bacterial DNA, result in a decrease in plasmid expression in murine tissues

by methylation of these dinucleotides repeats. The introduction of non-methylated CpG DNA into Atlantic salmon has been shown to elicit an immune response, since in eukaryotes the bacterial CpG motifs are recognised as a signal of infection, resulting in activation of the immune defences (Jorgensen *et al.*, 2001). This evidence should be considered, perhaps as contributing factors, to the observed reduction of expression over time. This would be an interesting subject for further work to ascertain whether the plasmid DNA used in this study was subject to methylation, once introduced into tilapia skeletal muscle by using methylation sensitive isoschizomers. Methylation may be analysed with Southern blots of tilapiine DNA extracted from the site of injection, that have been digested for example, with the methylation-sensitive enzyme *Sma*I (CCCGGG) or its methylation-insensitive isoschizomer *Xma*I and hybridised to a labelled plasmid sequence.

Rare migration of the plasmid DNA was detected. The carp and tilapia β-actin / lacZ constructs and the tiβAP/tiGH construct were found present in gonad tissue in three individual fish from a total of nearly 500 organ samples taken. These putative migration events although rare, are significant. The organ tissues of the injected fish were not targeted with the novel genes, and the finding of the plasmid transgene in gonad tissue suggests that plasmid DNA can move from the target site *in vivo*. The protein product from the injected DNA was proved to be mobile, the finding of β-gal protein in the liver and spleen of muscle injected fish but never the presence of the gene itself suggests that muscle tissue provides a site where, after DNA uptake and expression, the protein product may be distributed systemically. This may prove useful for both DNA vaccine treatment and for genetic applications, such as the introduction of growth hormone genes to enhance growth.

The enhancer element isolated using PCR from intron I of the tilapia β-actin promoter, demonstrated the ability of these short sequences to increase expression of genes in a position and orientation independent manner. It was not proved in these experiments that the enhancer element acted on the tiβAP/tiGH or opAFP/csGH constructs. There was however, an observed significant decrease in growth in those fish co-injected with the tiEnh element and the opAFP/csGH construct. This may be attributed to the action of the enhancer element but has not been proved further than this observation. The

creation of a bicistronic plasmid, in which tilapia growth hormone is spliced to lacZ both under the control of a single promoter would perhaps more fully answer this question. Any increase in expression may be established through a corresponding increase in lacZ expression, fused to growth hormone. It would also enable the study of the movement of the exogenous GH by following the path of its associated reporter protein. This method has been used successfully *in vivo* in a murine system by Coen *et al.*, (1997). They showed that the fusion protein of a neurotoxin, spliced to lacZ, retained the biological function of both genes after intramuscular injection into mouse tongue, since β-gal activity could be detected along motorneurons. Research into the construction of a fusion plasmid was started here in an attempt to directly follow the path of exogenous tiGH once it entered the somatic cells, rather than extrapolating from the lacZ reporter construct alone. The primers designed for the construction of a bicistronic plasmid are listed in appendix I. There was some progress in the production of a fusion plasmid but at the end of this study was not yet completed, and would be an interesting topic for further research.

It would also be useful to investigate the levels of GH protein in the muscle tissue of injected fish as compared with controls using Western blotting since this analysis detects how much protein has accumulated in cells. This would allow the comparison between GH injected and controls and might be used to confirm whether there is increased presence of GH in the tissues of the GH injected fish, as compared with sham injected controls. The protein product from the reporter construct was shown to be highly mobile and was reported at significant levels in the liver, spleen and serum of i.m injected fish. Its mobility was demonstrated after observation of the histology slides, which showed that not only the plasmid DNA itself moved along the fibre and from there into the nuclei, but the protein product once synthesised, gathered in high concentrations around the nuclear region before diffusing along the length of the muscle fibre. This combined with evidence of plasmid DNA in the blood, and significant levels of β-gal in the serum and red blood cells, it is perhaps not surprising that the exogenous protein is later found in the spleen and liver, both organs known to filter immunogenic substances from the blood.

This second of the objectives set out in this project, was to ascertain whether any growth response may be obtained after somatic injection of GH constructs into juvenile tilapia. The commercial importance of the tilapia fish to the developing world means that it is a prime target for genetic manipulation. The improvement in the growth rate of fish may result in more harvests per year and so better annual yields. Success has been achieved with enhancing the growth rate of germline transgenic fish, some species achieving a maximal size not possible in natural conditions. However, the benefit of the introduction of genes by somatic gene transfer, which ultimately degrades away, makes it a highly attractive target by which to achieve growth enhancement. This results in there being no risk of transgene introgression into natural or endemic fish populations and may be more saleable as an item intended for human consumption, since the DNA which affected any changes to the fish would no longer be present at the time of marketing.

The introduction of viral or mammalian gene sequences in fish with the potential to be marketed for human consumption, are not well received by the public. This resulted in the selection of the 'all-fish' GH constructs for researching the possibility of growth enhancement in tilapia. These constructs however, resulted in a slight growth retardation of GH injected tilapia. There appears to be a very small window to target to achieve growth enhancement. De La Fuente *et al.*, (1998) showed that the intraperitoneal injection in tilapia of 0.5 μg/g body weight resulted in a growth acceleration of 6 %, while an injection of 1.5 μg/g body weight, yielded slightly reduced growth of 1 %. The slight down-regulation of growth observed in this research has been attributed to a number of factors, summarized in table 5.1. In light of these results it would be very interesting to inject a GH construct with a strong promoter such as the CMV. The expected high localised expression resulting from a construct of this type may help in understanding exactly what is happening after somatic injection of these constructs by providing greater concentrations of GH expressed in the muscle tissue, any expected results may be exaggerated. While not viral sequences are not suitable in fish for intended for human consumption, the introduction of such constructs would facilitate improved understanding of the processes which occur on introduction to the somatic tissues and could aid ultimately in finding a way to make this method achieve positive results.

Possible explanations	Discussion point
Localised GH expression, as may be expected from i.m injection, may lead to down regulation of endogenous GH in response to the absence of widely available exogenous growth hormone.	In the experiments of Li *et al.*, (2003) and Noh *et al.*, (2003), very small quantities of GH itself, or GH plasmid DNA resulted in growth improvement. Small levels of GH clearly can have a growth promoting effect.
Somatic injection with plasmid DNA is perhaps more physiologically stressful than sham injected controls.	However in growth trial (figure 3.3.2.7) where the control group also received plasmid DNA (tiβAP/lacZ), the tiβAP/tiGH plasmid injected group still exhibited slightly repressed growth by comparison.
There is an immune response to those which receive plasmid DNA not evident in sham injected control fish.	The tiβAP/lacZ plasmid injected controls (figure 3.3.2.7) would have produced the highly immunogenic β-gal protein and yet did not show the (marginally) repressed growth of the GH injected fish.
The exogenous DNA was insufficient to yield dramatic growth enhancement as seen when fish are made germ-line transgenic, which may normally mask the slight negative response seen here.	Is enough exogenous GH produced to yield sufficient levels of growth hormone to induce enhanced growth, given that there seems to be a threshold point for plasmid uptake into the nucleus?
The apparent growth retardation is an artefact of the "all-tilapia" GH construct itself.	Perhaps the expression from this construct binds to GH receptors but does not do so correctly. This "all-tilapia" construct has not yet been rigorously tested in conventional germline transgenics. Its efficacy is as yet not proven.

Table 5.1 Possible explanations and corresponding discussion points for growth response observed in fish injected with either tiβAP/tiGH or opAFP/csGH plasmid constructs.

The research of Noh *et al.*, (2003) demonstrated mud loach fish responded positively to the somatic gene transfer of an 'all-mud loach' construct, similar to the 'all-tilapia' GH construct used in this research. The research of Li *et al.*, (2003) showed that very small amounts of purified carp GH injected intraperitoneally resulted in increasing growth performance of juvenile tilapia over controls. These two groups research suggest that there is a limiting factor which has restricted growth enhancement by somatic gene transfer in tilapia. This could reasonably be attributed to the limitation in the amount of plasmid DNA which becomes nuclear after intramuscular injection, which therefore would limit the amount of growth hormone subsequently produced. The method of injection followed by electroporation employed by Noh's group may well overcome the mechanism of active uptake of plasmid DNA into the nucleus. It is thought that this mechanism may become saturated, and as a result there can be no corresponding increase in expression after substantial increases are made to the injected DNA concentration. As observed in this study, as well as in others (Heppell *et al.*, 1998; Shulte *et al.*, 1998; Anderson *et al.*, 1996; Rahman and Maclean 1992; Hansen *et al.*, 1991). Li's work showed that juvenile tilapia can be induced to grow faster than their control counterparts when injected with comparatively small quantities of growth hormone. It would be very interesting to continue research in this area to find whether increasing the entry of plasmid DNA to the nucleus, perhaps by the methods used by Noh's group, aids in affecting positive growth in juvenile tilapia.

5.2 Summary of key findings

- The expression gained from injected plasmid DNA improves with increased DNA concentration up to a point. Past which, the putative nuclear uptake mechanism used to import plasmid DNA from sarcolemma to nucleus becomes saturated, thus large increases in DNA concentration are not reflected expression levels.
- The plasmid DNA persists in approximately half the sampled tilapia up to 3 months after an initial injection of plasmid DNA into the muscle tissue, and was present in a very few individuals 6 months post injection. The disappearance of cells containing exogenous DNA is thought to be through an immune response. MHC class I molecules with cytotoxic $CD8^+$ T cells remove cells expressing endogenous antigen, resulting in a clearance of those fibres positive for the transgene.

- The expression of the exogenous DNA was found to increase over time taking three weeks to reach the highest expression levels, declining after which time to background levels. The tilapia promoter yielded higher expression levels of the lacZ reporter gene, when directly compared to the carp promoter, presumably from the greater homology of the tilapia sequence.
- Rarely, plasmid DNA was found in gonad tissue, suggesting that DNA intramuscularly injected can infrequently move from the target site, probably via the capillaries.
- The protein product from the exogenous DNA proved mobile, moving freely along muscle fibres after injection, and was detected in the organs more than two weeks post injection.
- The tilapia β-actin enhancer element was proved to significantly boost expression of the plasmid lacZ reporter gene construct when co-injected in linear form.
- The tiβAP/tiGH and opAFP/csGH constructs resulted in a slight repression of growth. On co-injection with the enhancer element this growth repression was significant. It is postulated here that this may be occurring in two ways.
- Firstly, by supplying a localised increase in GH, GH receptors become saturated which prevents them dimerizing correctly. This stops signal transduction of both endogenous and exogenous GH.
- Secondly, the overall increase in GH caused by the addition of the exogenous GH, causes a slight endocrine controlled down-regulation of endogenous GH, which, when the expression from the exogenous DNA decreases over time, results in growth repression of GH injected fish.
- Co-injection with the enhancer element boosts levels of GH and so the effect is more pronounced.

5.3 Summary of suggestions for future work

- To ascertain whether the plasmid DNA used in this study was subject to methylation, by using methylation sensitive isoschizomers. This would identify how long the plasmid DNA remained functional in the cells once injected.
- To create a bicistronic plasmid, in which tilapia growth hormone is spliced to lacZ both under the control of a single promoter. This would enable research

into the putative systemic delivery of the exogenous GH, by assay of the associated reporter protein after injection into muscle tissue.

- To investigate the levels of GH protein in the muscle tissue of injected fish as compared with controls using Western blotting. This analysis would detect how much protein has accumulated in cells and confirm whether there is increased presence of GH in the tissues of the GH injected fish, as compared with controls.
- To inject a GH construct with a strong promoter such as the CMV promoter. The expected high localised expression may exaggerate any effects as observed using the 'all-fish' GH sequences or may provide growth enhancement. This would provide research by which to contrast the results gained here.
- To attempt to enhance growth by somatic injection by providing an electrical pulse directly after injection, to see whether this overcomes the present limitation of getting plasmid DNA into the nucleus of target cells.

Chapter six:

Appendix

Appendix I - Creation of fusion plasmid

Cloning restriction site into tilapia growth hormone gene.

1. The identification of a suitable restriction site was made from tilapia β-actin / tilapia growth hormone construct, which did not occur within the gene sequence. SmaI (CCC/GGG) was identified as a candidate, and was available to buy commercially.

2. A suitable area was identified into which the lacZ gene would be inserted in the tiβA/tiGH construct, upstream of growth hormone gene, in the non-coding untranslated exon 1.

3. The ATG start codon (M), found in the 5′ region of exon I also formed past of NcoI restriction site, which cleaves after the second cytosine (C/CATGG), 1674 bp from beginning of construct sequence.

4. The SmaI restriction site, not present in the GH plasmid sequence, was then cloned into the GH plasmid over 6 bases, 7 - 13 bp upstream from the ATG start codon, with the purpose to insert the lacZ sequence between these two restriction sites (SmaI and NcoI).

5. The selection of a restriction site in the tilapia β-actin promoter sequence which only cuts once was chosen; AfeI (AGC/GCT) was found to be suitable, 1114 bp from beginning of construct sequence.

6. Primers were designed to introduce the new restriction site, 3′ primer spanned the AfeI site (blue) and the 5′ primer spanned the NcoI site (blue) and was inclusive of the new SmaI site (yellow) to be introduced

Forward primer 1) 5′ - CACCAGTCAAGC/GCTTGTGACCG - 3′
Reverse primer 2) 5′ - GTC/CATGGCTGAACCC/GGGAAGAGAAAAAAAA - 3′

PCR amplification of the 576 bp region was carried out using primers 1 and 2 with SmaI site inserted, as shown in yellow, in the reverse primer

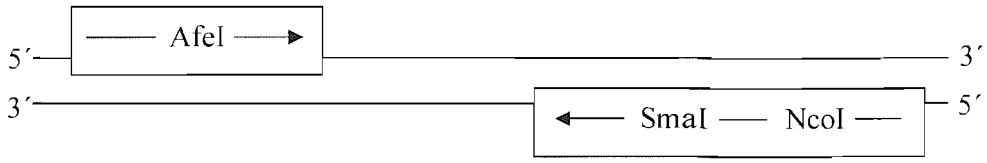


Figure 6.1 Arrangement of restriction sites
The figure shows a diagrammatical representation of the two primers with the intended placement of the new SmaI cloned site on the non-coding strand.

7) The new fragment was amplified using PCR, with the GH plasmid as template and the product was purified with the Qiagen PCR purification kit. The ends were then cut firstly with NcoI and following purification, were cut a second time using AfeI. Refer to figure 6.2 for gel image of the 576 bp linear insert.

8) The growth hormone construct was also separately cut using firstly NcoI, and then AfeI. Both only occur once in sequence. Resulting in a 5299 bp linear tilapia β-actin/tilapia growth hormone sequence. Refer to figure 6.2 for gel image of linear gene sequence.

4) The insert, containing the new SmaI restriction site upstream of the GH start site, was ligated into the linearised growth hormone construct to create a GH construct with new restriction site.

5) The plasmid DNA was transformed into *E.coli*, extracted and the presence of the SmaI site in the plasmid was confirmed by gene scan.

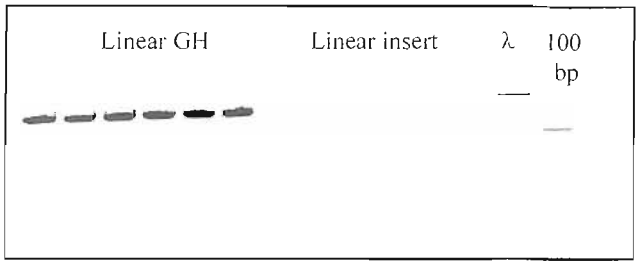


Figure 6.2 Inverted gel image of linear tβAP/tiGH cut with AfeI and NcoI
576 bp insert also cut with AfeI and NcoI, ready for ligation back into plasmid form with new SmaI restriction site added. λ DNA marker and 100 bp DNA marker shown.

Preparation of linear LacZ sequence

1) Primers were designed to cover the stop codon in lacZ gene, which is essential for fusion plasmid, so one long transcript was created spanning both genes. The SmaI site (shown in yellow) was incorporated into the forward primer.

Forward Primer 1) 5′ - ACAGTCCC/GGGATGGCAGATC- 3′
Reverse primer 2) 5′ - AC/CATGGTTTTTGACACCAGACC- 3′

The reverse primer incorporated an NcoI restriction site over stop codon (shown in blue), originally
5′-ATTATT- 3′

2) The 3 kb region of the lacZ gene was amplified using PCR, using carp β-actin/lacZ construct as template DNA.

3) The 3 kb sequence was purified using Qiagen PCR purification kit and then restricted using NcoI and SmaI, ready to insert into tiβAP/tiGH sequence also cut with NcoI and SmaI.

4) The linear lacZ sequence (with NcoI and SmaI ends) was ligated into GH construct and transformed into *E.coli*.

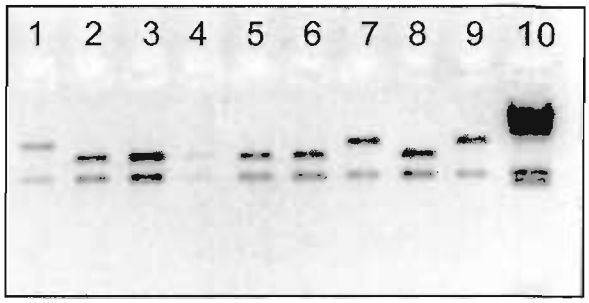


Figure 6.3 Inverted gel image showing transformed colonies. Lanes 1-9 show 9 *E.coli* colonies transformed with fusion plasmid and subsequently restricted with NcoI and SmaI. Lanes 1, 7 and 9 show correct size bands for putative tβAP/tiGH fragment and below the smaller 3 kb lacZ insert. Lane 10 shows λ DNA marker ladder.

These colonies (figure 6.3) did not prove viable and this experiment was not completed by the end of the research conducted for this PhD. This would be a matter of interest for future work, and so I have laid out the framework to these experiments here.

Chapter six:

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