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Cell culture systems have provided many insights into eukaryotic gene expression and other biochemical mechanisms. Since the cell represents the smallest living unit of any organism it provides a desirable *in vitro* system, allowing biochemical studies without the complex physiology of an entire animal. However, processes involving intracellular mechanisms, such as development, aging or carcinogenis, eventually require the analysis of the intact organism. Transgenic animals are a very promising tool to approach questions of this magnitude. Fish in general and the zebrafish (Brachydanio rerio) in particular are an excellent model system for transgenic research, mainly due to their extramaternal fertilization and development and their short generation cycle throughout the year. The recent derivation of zebrafish cell lines has opened up possibilities for *in vitro* analysis of this popular model species, and expression of heterologous genes

under the influence of promoter and other regulatory nucleic acid aequences. In contrast to mammalian expression systems, little nucleic acid sequences controlling gene expression in fish are known. Therefore we examined mammalian expression systems in fish cells in order to determine their efficiency quantitatively. Emphasis was given to zebrafish cultures with the goal of eventually injecting in vitro manipulated embryo cells into host embryos and thereby creating transgenic chimera.

Exogenous Gene Expression from Heterologous Promoters in Fish Cell Cultures

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Chapter 1

INTRODUCTION AND LITERATURE REVIEW

Eukaryotic gene expression and regulation

The function of DNA is to carry the genetic message from generation to generation and to allow the expression of that message under appropriate conditions. Multicellular organisms are composed of a variety of cell types, each of which contain an identical copy of the entire genome but yet perform discrete biological functions. There must exist mechanisms which ensure the specific and ordered expression of only those genes characteristic for a given cell type. Besides tissue specific gene expression there must also exist a temporal regulation of gene activity throughout the life of an organism. This may be most apparent during early embryo development, where actively dividing pluripotent stem cells follow a differentiation pattern to become specialized or during growth regulatory processes in adult stages such as hematopoesis, woundhealing, aging or carcinogenisis.

In higher eukaryotes detailed information on the regulation of transcription could not be obtained until the development of techniques for <u>in vitro</u> manipulation of cloned genes. Today we have a detailed, but by no means

complete, understanding of eukaryotic transcription mechanisms (Gluzman Y., 1985; Adams R.L.P.). Central to eukaryotic gene expression are cis-acting elements such as promoters and enhancers. Mammalian genomic promoter/enhancer sequences, such as those of the immunoglobin heavy chain gene, which was the first genomic, non viral, enhancer unit to be isolated, have been identified and cloned (Banerji et al., 1983). Many of the endogenous promoter/enhancer elements are under the influence of spatial or temporal controls, which by themselves are interesing phenomena and currently under investigation (Renkawitz R., 1989). For expression of exogenous DNA, constitutive and possibly tissue independent expression is sometimes desired. For this purpose researchers have taken advantage of viral genomes. The ability of mammalian viruses to utilize mostly host enzymes for transcription of their own genome makes viral promoter/enhancer sequences optimal candidates for exogenous gene expression in mammalian cells (Primrose S.B., 1980). Not only is the cellular transcriptional machinery guaranteed to recognize the foreign promoter/enhancer sequences, but it also can be expected that the viral elements may override any endogenous regulation. Such promoter/enhancer elements have been cloned from mammalian viruses, with primarily broad host ranges. The Rous sarcoma virus long terminal repeat

(RSVLTR) (Xu N.Z., 1983) and the simian virus 40 early promoter (SV40) (Baty et al., 1983) are probably the most commonly used sequences. Other examples are the human cytomegalovirus immediate early promoter/enhancer unit (CMV) (Boshart M. et al., 1985; Thomsen D.R., 1984), the adenovirus 2 major late promoter (AD) (Hearing P. et al., 1983) and the herpes simplex thymidine kinase promoter (HSVTK) (Macknight S. et al., 1985). For fish cells the situation is not so well studied. Cloned fish genes and their 5' regulatory sequences are still few (Friedenreich et al., 1990) and fish viral promoters and enhancers are not available. Therefore researchers have to resort to conventional mammalian systems. This however raises the question of whether these sequences are efficiently recognized and utilized by the transcriptional factors and complexes. This problem is addressed in the work presented in Chapter 2 and the quantitative efficiency of various mammalian promoters is being analyzed in Chapter 3.

Besides cis-acting elements, other gene regulation levels have been observed and are studied (Adams R.L.P., 1986). They involve chromatin structure, DNA methylation, mRNA stability, mRNA secondary structure, differential splicing, gene copy number and translational efficiency. Some if not all of these regulatory mechanisms are even more complicated by the fact that eukaryotic organisms consist of many cell types and therefore temporal and

spacial gene expression as well as cellular interactions add yet another dimension of complexity. Studies of this magnitude are only possible with transgenic animals where exogenous DNA can be introduced into the germ line generating precise animal models as is described in more detail below.

Transgenic animals

The ability to introduce foreign genes into the germ line along with successful expression of the introduced genes within the organism have made transgenics valuable models for biochemical questions. Such questions may involve complex inter cellular mechanisms as are encountered in developmental biology, gene and growth regulation, disease and within the immune system (Hanahan D., 1989; Palmiter R.D., 1986; Gordon J.W., 1985). For example, transgenic animals are indispensible tools to study cell lineage (Sanes J.R., 1986; Turner D.L., 1987) after transplantation of genetically marked embryo- or stem cells. It has been possible to follow such cells along their voyage through developmental tissue pattern formation (Sanes J.R., 1986). Transgenics also provide a system which allows massive gene screening for a systematic mapping of changes in gene expression during development and adult life. By searching for expression of exogenous marker genes randomly integrated into the host genome

(described in more detail below) with no or a weak promoter attached can identify active chromosomal domains (Allen, et al., 1988). The field of transgenic animals is growing rapidly and domestic animals are no longer excluded. They can provide important improvements for the world's food supply and serve for large scale production of genetically improved biological compounds. Approaches to increase growth and disease resistance as well as attempts to produce important peptides in milk have been accomplished in fish, sheep and goats (Church R.B., 1990).

There are several ways of introducing DNA into animals (reviewed by Jaenish R., 1988). The most successful method involves injection of 'naked' DNA directly into either the pronucleus of zygotes (Harbers K., 1981) or into the cytoplasm of early blastula stage zebrafish embryos (Stuart et al., 1988). Microinjection of DNA is tedious and it is for some systems possible to use retroviral infection to transduce foreign DNA into early embryos (up to preimplantation stage) by simply exposing them to infective, but non-lytic viral vectors (Jaenish R., 1977). This method provides an excellent tool for insertional mutagenis screens (Rossant J., 1992). Only single copies, flanked by viral long terminal repeats, rather than multiple copies in concatamerized form, integrate into the host genome. The flanking viral sequences are useful for identification of junction sequences. However, there are

limitations of the size of exogenous DNA that can be incorporated into such vectors and the efficiency of germ cell infection is low (Jaenish R., 1988). Viral vector systems for other species such as the fish are not yet available and therefore make this approach, in general, less useful. A third method involves the production of transgenic chimera (Robertson E.J, 1987). The ability to isolate and grow pluripotential cell lines in tissue culture and returning them to a natural host after in vitro manipulations, such as transfection with exogenous DNA, insertional mutagenesis or deletion or amplification, not only allows preselection for the desired geno- and phenotype, but also provides the researcher with clonal cell lines. The embryo stem cells (ES) are useful for any gene and tissue of interest since they are capable of contributing to all tissues including the germ line (Lord B.I., 1988). Such ES systems have been used successfully in mice (Suemori et al. 1990; Cappechi, 1989) and are presently under investigation for zebrafish (Collodi et al., 1992 b).

Fish as a model system for gene expression and regulation

Fish have provided scientists of all biological disciplines with a tremendous wealth of knowledge for almost a century (Powers, 1989), mainly because they themselves represent the oldest and therefore the largest

and most diverse vertebrate group in the animal kingdom. Furthermore, they are extremely suitable for laboratory experiments: they can be easily kept and bred in captivity and their extra-maternal fertilization and development, along with the vast numbers of offspring, give the researcher unique opportunities to observe and manipulate processes in embryonic development and in the production of transgenic fish.

The zebrafish (Brachydanio rerio) (Staehle, et al., 1992; Laale, 1977) is one of the most favored species for gene manipulation, mostly because of its small size and frequent reproduction cycles providing hundreds of eggs every 3-4 days throughout the year. The development is fast, the embryos transparent and the developmental stages well defined (Warga, 1990; Kimmel, 1990). At 2 hrs post fertilization the embryo has reached a 64 cell stage and the first horizontal cleavage occurs. The next cleavage (128 cells) is referred to as early blastula stage; particular cleavage plans are no longer recognizable and cell division is asynchronous. Mid blastula is reached after 3 hrs (1000 cells) and late blastula after 4 hrs. Gastrulation occurs after 5.2 hrs with 50% epipoly, then somites form and fish hatch after 96 hrs. Sexual maturity is reached after 74 to 75 days. In addition to the fast development it is easy to perform microinjection of 100 or more eggs per day after chorion removal, and survival rates

are high (Stuart et al., 1988). The availability of mutants such as the albino offer additional options for viable chimera detection (Lin S., 1992).

Fish cell lines have greatly expanded the use of fish as model systems for research on viral diseases and toxicological studies on a cellular level (Lannan et al., 1984; Wolf et al., 1980; Collodi et al., 1992 b). They provide a well defined in vitro system, isolated from the complex physiology of entire animals. The derivation of several zebrafish cell lines in our own laboratory (Collodi et al., 1992 a) has expanded the zebrafish model system significantly. The blastula stage derived, near diploid zebrafish embryo lines ZEM1 and ZEM2 (Collodi et al., 1992 a; and Chapter 3) not only allow in vitro studies of biochemical aspects in development or in toxicology but also can be used to optimize gene transfer and expression as is described in Chapter 3. They can be genetically manipulated and selected for desired geno- and pheno-types before transplantation back into a natural host for transgenic chimera production (preliminary data are described in Chapter 4). Cell cultures from various adult tissues, such as fin and gut were also established (Collodi et al., 1992 a). This extension of the in vitro system allows comparison of embryonal- and adult- gene expression and regulation, as well as tissue-specific expression on a cellular, in vitro level.

DNA Transfer

Two systems commonly used are to study expression of exogenous DNA in cell cultures or transgenics (Wynshaw-Boris, et al., 1986): a transient expression system in which transcription is performed from extrachromosomal DNA within the nucleus, and a system in which foreign DNA is stably integrated into the host genome and is passed on to progeny cells. Both methods provide powerful tools to study gene expression. In a transient system gene expression can be monitored immediately after DNA transfer and expression is not influenced by factors related to the integration site within the host genome, such as chromatin structure. Stable integration of any heterologous DNA can be accomplished by cotransfection with a selectable marker and isolation of clonal cell lines of interest. This approach allows insertional mutagenis and gene- promoter- and enhancer-trapping (Rossant, et al., 1992) as well as transgenic chimera production.

Several methods for introduction of exogenous DNA into nuclei of cultured cells include calcium phosphate precipitation (Loyler, et al., 1982; Graham, et al., 1973), lipofection (Felgner, et al., 1989), DEAE-dextran (Maniatis, et al., 1982), electroporation (Potter,H. et al.,1984), and microinjection (Cappecchi,M.R., 1980). The latter two methods can also be applied to developing eggs. In calcium phosphate precipitate-mediated transfections the

DNA is trapped in a tight precipitate which in turn increases the contact between cell surface and DNA thereby facilitating penetration of the negatively charged DNA molecules through the cytoplasmic membrane; lipofection utilizes the ability of cationic liposomes to fuse with the cell membrane allowing the DNA molecules sandwiched between vesicles to be shuttled into the cytoplasm (Chang,A.C.Y. et al, 1988). Anionic vesicles have also been used but with lesser success because they do not generally fuse with the target cell surface, but are taken up by phagocytosis and therefore tend to be deliverd to the lysosomes where the DNA is exposed to digestive enzymes (Mannino, et al., 1988). Calcium phosphate mediated transfection and lipofection are commonly used primarily because they tend to yield higher frequencies of chromosomal integration events and they are easy to perform, although there are differences in success among cell lines.

The fate and stability of injected DNA has been studied in oocytes of Xenopus, (reviewed by Gurdon,J.B. et al., 1981). It was shown that all DNA molecules transferred into the cytoplasm, whether they are supercoiled, circular or linear to begin with, undergo gradual degradation. Even though few molecules find their way into the nucleus, they are stable once separated from the cytoplasmic enzymes by the nuclear membrane. High levels of ligase were detected within the nucleus but

linear DNA molecules were degraded (Willie et al., 1977). Within few hours of injection, nearly all circular DNA deposited in an oocyte nucleus had formed a minichromosomal structure, and displayed similarities with normal chromosomal material, in DNA-protein content, nuclease digestion patterns, electron microscopy images, and sedimentation in sucrose (Willie et al., 1978). Electron micrographs immmages revealed that most injected DNA molecules were packed with nucleosomes at the typical 200 bp spacings but carried no transcripts. Only few molecules were associated with polymerase complexes and transcripts (Trendelenburg,M.F. et al., 1978). Furthermore, it could be demonstrated that in fertilized or unfertilized Xenopus eggs exogenous DNA (single- or double-stranded) was replicating extra chromosomaly (Bending,M.M., 1981).

Selectable and histochemically detectable exogenous cell markers

Exogenous genes which code for unique enzymatic activities or are otherwise easily distinguishable from intra- or extra-cellular proteins provide a straight forward indication of expression after transfection. Several markers of this nature are reviewed (Alam et al, 1990). Exogenous cell markers, if integrated into the host genome, provide means of selection for desired geno- and pheno-types if their gene product results in drug

resistance. In this case cotransfection of selectable markers with any gene of interest is a powerful tool for derivation of clonal cell lines of altered genomes. The genes themselves or gene expression can be detected by PCR, Southern and Northern blotting, in situ hybridization, immunohistochemistry, or by straight forward chemical reaction if the gene product carries out an enzymatic color reaction. Markers which allow in situ detection of cells within a gemisch of unmarked cells or entire tissues are referred to as histochemical markers.

For histochemical markers a very desirable property would be a viable assay rather than one involving fixation and killing of the cells. So far only fluorescent dyes are available to fulfill this goal. They are an extremely useful tool for short term experiments (2 days) and have been used to map the early developmental stages of the zebrafish embryo (Kimmel, et al., 1990; Warga, et al, 1990), but are subject to problems resulting from diffusion between cells (Nolan, et al., 1988).

In the following studies two types of exogenous cell markers have been used and are discussed in more detail: one is E.coli neo gene, a commonly used selectable marker which codes for an aminoglycoside 3' phosphotransferase (Davies, et al., 1980; Southern, et al., 1982). There is absolutely no known eucaryotic homolog, eliminating any anticipated background problems. This enzyme is capable of

inactivating the aminoglycoside antibiotic G418 (neomycin), which has a wide spectrum of activity against bacteria, yeasts, fungi, algae, plant and animal cells. This drug interferes with 80S ribosomes and consequently blocks protein syntesis. For a histochemical marker we chose the E.coli lacZ gene. It codes for the enzyme beta-galactosidase a tetramer with a subunit size of 1023 amino acids. One very nice feature of this gene is that several substrates for different assay types are available. They include antibodies for immunoprecipitation, ONPG (o-nitrophenyl-beta-D-galactopyranoside) for cell free extracts (Maniatis, et al., 1982), FDG (fluorescein di-beta-galactopyranoside) a fluorescent dye used for live staining and flowcytometry (Nolan, et al., 1988) and X-gal (5-chloro-4-bromo-3-indolyl beta-D-galactopyranoside) for histochemical staining of cultures or tissues (Sanes, et al., 1986; MacGregor, et al., 1989). The latter assay was primarily used in the studies presented in Chapter 3. The assay is based on cleavage of the substrate X-gal, which is hydrolyzed to an indolyl and then oxidixes to an indoxyl which in turn dimerizes to form an indigo blue derivative (Pearson et al., 1963). The catalyst for this reaction, a mixture of ferricyanide/ferrocyanide and $MgCl_2$ is required as cofactor for the enzyme beta galactosidase. The pH of the buffer solution used is of critical importance since mammalian cells possess endogenous, lysosomal galactosidase

(Conchie, et al., 1959). However the pH optima for activity of the two enzymes differ substantialy. While the lysosomal enzyme is active in a pH range below 4.5 the bacterial enzyme activity is best at pH 7.5.

Introduction to manuscripts

Fish in general and zebrafish in particular have become popular model systems for studies of development, toxicology, cancer and aquaculture. Transgenic animals provide a valuable tool for all these disciplines and since fish have many technical advantages over mammalian transgenics they are a preferred model system for basic research.

In this study fish cell cultures have been transfected with selectable and histochemical cell markers, under various mammalian viral promoters, to study the expression of those exogenous genes in chromosomaly integrated and transient states. Even though gene manipulations on fish, including the zebrafish, have been undertaken (Sreisinger et.al.,1981; Maclean and Pennman, 1990 reviev), little fish molecular genetics has been done thus far resulting in few isolated fish genes (Friedenreich et.al., 1990) and a poor understanding of gene regulation and expression in fish. The derivation of zebrafish cell culture systems allowed us to address <u>in vitro</u> several questions: 1. Can fish cells be transfected by conventional methods? 2. Can exogenous

DNA be integrated and expressed under heterologous promoters and colony formation be achieved? 3. Which mammalian viral promoters are optimal and are they under any developmental control, (i.e. embryo-derived versus adult-derived cultures)? 4. Can the transfected embryo cells contribute to a normally developing embryo after injection to produce transgenic chimera? Questions 1-3 have been addressed by using a selective marker (neo) and a hystochemical marker (lacZ) in several fish cell cultures and in mouse embryo cultures for comparison. We have applied and optimized mammalian transfection techniques to fish cell cultures with good hope of reaching the goal of question 4, which is presently under investigation. Preliminary data are discussed in Chapter 4 for fish and mouse.

Chapter 2

TRANSFECTION OF CULTURED FISH CELLS WITH EXOGENOUS DNA

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Preface

Coauthor Contribution

George Bailey provided direction in fish physiology and molecular biology and David Barnes directed and financially supported this research.

Abstract

We have applied to fish cell cultures the techniques used routinely to introduce exogenous genes into cultured mammalian cells. Using calcium phosphate-mediated transfection, a plasmid containing bacterial aminoglycoside phosphotransferase under the influence of the simian virus 40 early promoter and polyadenylation signal was introduced into several fish cell lines. The plasmid was expressed in these cells in a stable manner, with transfection occurring at a frequency comparable to that seen with mammalian cells. These results suggest that plasmid constructed for use in mammalian cells may be used efficiently in fish systems without further modification and indicate that the advantages of transfection procedures utilized in mammalian systems can also be applied to fish.

Introduction

Introduction of exogenous DNA to cultured cells through calcium phosphate-mediated transfection is a commonly used technique in the study of regulation of expression of mammalian genes, as well as useful approach for for the engineering of mammalian cells for large scale production of molecules of biological interest (Graham and van der Eb, 1973; Wigler et al., 1980). In this procedure, mammalian cells in culture are presented with DNA trapped in particulate calcium phosphate formed by precipitation

upon the addition of phophate-containing solution at an appropriate concentration to a solution of DNA in calcium chloride (Corsaro and Pearson, 1981).

The cells are capable of internalizing the DNA-containing precipitate and, with low frequency, the internalized DNA is integrated into the cellular genome and expressed. Plasmids containing genes of interest flanked by regulatory signals (Mulligan, Howard, and Berg, 1979; Southern and Berg, 1982; Gorman et al., 1982) are frequently used in such studies to improve the efficiency of expression of the integrated DNA. Mammalian cells genetically altered in this way are not only useful for genetic and biochemical analysis with regard to selected genes, but also in some circumstances can be transplanted into early embryos of the appropriate organism, creating genetically altered individuals (Gordon and Ruddle, 1985).

We have investigated the notion that fish cells in culture might be manipulated in a manner similar to mammalian cells. Here we report that a plasmid containing bacterial aminoglycoside phosphotransferase, APH(3')II, flanked by the simian virus 40 early promoter and polyadenylation signal, which was constructed for the expression of the gene in mammalian cells (Southern and Berg, 1982), is integrated into the genome and expressed by several lines of fish cells with a frequency similar to to that observed for mammalian cells when transfected by the

calcium phosphate-mediated technique. These results suggest that the advantages of these procedures that have been utilized in mammalian systems can also be applied to fish.

Materials and Methods

Cell culture: Cell lines were obtained from Dr. John L. Fryer, Department of Microbiology, Oregon State University. The CHSE-114 line was derived from pooled chinook salmon (Oncorhynchus tshawytscha) embryos (Lannan, Winton and Fryer, 1984). The RTH line was derived from an aflatoxin-induced hepatoma in an adult rainbow trout (Salmo gairdneri) (Fryer, McCain and Leong, 1981). Cells were maintained at 18-20°C in a basal medium consisting of three parts Ham's F12 and one part Dulbecco-modified Eagle's medium (Barnes and Sato, 1980) supplemented with 4.5 g/l glucose, 200 U/ml penicillin, 25 ug/ml ampicillin, 200 ug/ml streptomycin, 10% fetal bovine serum, 30 mM 4-(2-hydroxy-ethyl) 1-piperazine-ethanesulfonic acid (HEPES), pH 7.4 and o.3 g/l sodium bicarbonate. Trypsinization for passage of cultures was accomplished with a solotion containing o.1% crude trypsin and 1 mM ethylenediamine tetraacetate (EDTA) in phosphate-buffered saline.

Cells were maintained in 25 cm^2 plastic tissue culture flasks with 5 ml medium per flask. Medium was changed at 3 or 4 day intervals.

Transfection: The pSV2-neo plasmid (Southern and Berg, 1982) in HB101 was obtained from the American Type Culture Collection (ATCC 37149). The plasmid (5.6 kilobases) was approximately 90% supercoiled and 10% nicked, circular at the time of the transfection. The size of G418-resistance gene insert is 1.4 kilobases. For transfection (Corsaro and Pearson, 1981; Graham and van der Eb, 1973; Wigler et al., 1980), 25 ug of plasmid DNA in o.5 ml o.25 M calcium chloride was added dropwise with constant mixing to o.5 ml HEPES-buffered saline (250 mM NaCl, 50 mM HEPES, pH 7.0) containing 1.8 mM sodium phosphate. The resulting precipitate-containing suspension was incubated 30 minutes at room temperature and the suspension sheared twice through a 25 gauge needle. The suspension was then added directly to a flask containing approximately 10^6 cells that had undergone a medium change approximately four hours previously.

Medium was changed to remove the precipitate six hours after its addition. Cells were not passaged after transfection, and were allowed to grow for 7 to 10 days before addition of the antibiotic G418 (Genetecin, Grand Island, New York) at a concentration of 1 mg/ml. This procedure may lead to overestimation of colony frequency to

some extend due to formation of satellite colonies. The selective componenet was added to the medium at each medium change until most cells of the culture were dead and obvious colonies of resistant cells were observed microscopically. Selection was maintained approximately 10 weeks for all cell lines.

Visualization of G418 resistan colonies was achieved by removal of culture medium from the flask, washing once with phosphate-buffered saline (PBS), followed by the addition of 2 ml of 3.7% formalin in PBS for 10 minutes. The fixative was then removed and the flask washed once with PBS followed by the addition of 2 ml of 1% crystal violet in PBS for 5 minutes. The staining solution was then removed and the flask washed once with PBS. Under these circumstances, colonies resulting from cells actively proliferating in the presence of G418 stained dark blue.

Isolation of DNA: For isolation of DNA from resistant cells (MAnniatis, Fritsch and Sambrook, 1982), G418-resistant colonies were pooled and the cultures expanded to 3 100 mm-diameter tissue culture dishes. Cells were scraped from the plates into PBS and the cells centrifuged from suspension. The cell pellet was lysed by the addition of three ml of Tris-buffered saline, (20 mM EDTA, 25 mM Tris, 136 mM NaCl, pH 7.4), 0.5% sodium dodecyl sulfate (SDS) and 0.2 mg/ml proteinase K (Boehringer-Mannheim) and incubation for 2 to 3 hours at 55°C.

To the cell lysate an equal volume of 0.1 M Tris-saturated (pH 7.6) redistilled phenol containing 1 mg/ml 8-hydroxy quinolin and 0.1% (v/v) 2-mercaptoethanol was added. The mixture was mixed and centrifuged in a bench-top centrifuge at 3500 rpm. The aqueous phase was removed and extracted again with phenol. The aqueous phase was then mixed with an equal volume of chloroform/isoamyl alcohol (24:1), mixed and centrifuged as described above. The aqueous phase was removed and extracted again with chloroform/isoamyl alcohol. DNA was then precipitated from the aqueous phase by addition of NaCl to give a final concentration of 250 mM and the addition of 2.5 volumes of 95% ethanol. Tubes containing the mixture were stored at -20°C overnight.

Precipitated DNA was recovered by centrifugation and washed one time with 10 ml of 70% ethanol followed by one wsh with 95% ethanol. Pellets were air dried and resuspended in 1 ml of a solution containing 10 mM Tris, pH 7.4, 5 mM NaCl and 0.1 mM EDTA. Suspensions were shaken for 2 hours or more at room temperature to fully solubilize the DNA. DNA preparations contain RNA contamination detectable after agarose gel electrophoresis was treated with RNAse (40 ug/ml) for 30 to 60 minutes at 37°, and reextracted with phenol. DNA concentration was assayed by spectrophotometric analysis of absorbance of the DNA at 260 nm.

Ratio of absorbance at 260 nm to absorbance at 280 nm was about 2. Agarose electrophoresis and comparison to lambda bacteriophage markers indicated that the DNA isolated was greater than 50 kilobases in size.

Restriction endonuclease digestion, electrophoresis and nitrocellulose blotting of isolated DNA: Isolated DNA (10 ug) was reprecipitated with ethanol and lyophilized. The DNA was then redissolved in 10 ul 100 mM Tris-HCl, pH 7.5, 50 mM NaCl and 10 mM $MgCl_2$ and incubated overnight at 37°C with 2 U/ug DNA EcoRI restriction endonuclease (1 U/ml, Bethesda Research Laboratories, Bethesda, Maryland) with buffers and conditions recomended by the supplier. Fragments were separated by loading the mixture after incubation onto 0.8% TBE agarose gels (Maniatis, Fritsch and Sambrook, 1982) and electrophoresis for 4 to 5 hours at 150 Volts (4.5 volts/cm). Gels were stained in ethidium bromide (15 to 20 minutes, 0.5 ug/ml), destained in water for 10 to 20 minutes and visualized under ultraviolet light to verify digestion and separation of fragments.

Prior to nitrocellulose blotting of the electrophoretically separated DNA fragments, the gel was subjected to depurination in 0.25 M HCl for 10 to 15 minutes while shaking, denaturation by incubation with shaking for 1 hour in 1.5 M NaCl containing 0.5 N NaOH and neutralization in 1.5 M NaCl containing 0.121 mg/ml Tris, pH 8.0. Gels were blotted as described by Southern (1975)

on nitrocellulose (Schleicher and Schuell, NC BA-85) for two days with a reservoir containing 175 g/l NaCl and 88.2 g/l sodium citrate, pH 7.0 (20x SSC). Nitrocellulose after blotting was baked under vacuum at 80°C for two hours.

Hybridization analysis: The SV2-neo plasmid (0.5 ug) was labeled with ^{32}P by nick translation (Rigby et al., 1977). Labeled plasmid was separated from unincorporated nucleotide by Sephadex G-50 gel filtration chromatography. Specific activity was 1.9 x 10^8 dpm/ug. Blots were prehybridized and hybriduzed (2 x 10^7 cpm labeled plasmid, 10 ml total volume) in 50% formamide as described (Maniatis, Fritsch, and sambrook, 1982). Hybridization was carried out for 2 days at 43°C.

After hybridization, blots were washed (Peden, Mounts and Hayward, 1982) at 65°C in a shaking water bath with 1 M NaCl, 50 mM Tris, pH 8.5, 2 mM EDTA, 1% SDS (2 times, 30 minutes); 0.5 M NaCl, 50 mM sodium phosphate, pH 6.5, 2 mM EDTA, 0.5% SDS (one time, 60 minutes); 0.5 M NaCl, 50 mM sodium phosphate, pH 8.5, 2 mM EDTA, 0.5% SDS (one time, 60 minutes); and rinsed with 2x SSC (10 minutes, room temperature). Hybridized, washed blots were exposed to x-ray film (Kodak Diagnostic film, X-Omat AR) for 16 hours at -70°C and developed using Kodak developer and fixer.

Results

Isolation of G418-resistant fish cells resulting from calcium phosphate-mediated transfection of an exogenous gene: The antibiotic G418 is toxic for mammalian cells in culture because of interference of the compound with protein synthesis Davies and Jiminez, 1980). Transfection of mammalian cells with the pSV2neo plasmid results in appearance of G418 resistant cells with a frequency of one in 104 to 105, due to the integration and expression in the resistant cells of the enzyme aminoglycoside phosphotransferase which inactivates the antibiotic (Southern and Berg, 1982). Untransfected rainbow trout and chinook salmon cells in culture are also killed by G418 (Fig.2.1), and the compound is similarly toxic for perch and steelhead trout cells (not shown).

Obvious cell death occurred in fish cultures 10 to 20 days after adding G418. This lengthy incubation time in G418, compared to mammalian cells, neccessary to produce a toxic effect may relate to the lower incubation temperature and slower growth rate of fish cells. The concentration of fish cells used in these studies is within the range commonly used for similar selections in mammalian cells (0.2 to 1.0 mg/ml). A more detailed examination of the toxicity range of G418 for the fish cell lines was not attempted.

Although no colonies of resistant cells were detected in untransfected control cultures, obvious colonies of resistant cells were apparent within 30 days after transfection of cultures with the pSV2neo plasmid. These colonies grew to macroscopically visible islands of cells on the flasks by 60 days after transfection (Fig.2.1) and cells of these colonies could be continually propagated in the presence of 1 mg/ml G418. Frequency of appearance of resistant colonies after transfection for the RTH rainbow trout hepatoma cell line and the CHSE-114 chinook salmon cell line was approximately 1 in 10^4 cells, a frequency similar to that observed with some mammalian cells.

G418-resistant colonies were also observed upon transfection of perch and steelhead trout cell lines with pSV2neo and cultivation in G418. The frequency of transfection for perch cells was somewhat higher than that seen with trout and chinook salmon, while the frequency of transfection of steelhead trout was considerably lower (about 1 in 10^6). Variations in frequency of transfection also are obseved among different mammalian cell lines (Southern and Berg, 1982).

Identification of plasmid-related nucleotide sequences in transfected G418-resistant fish cells: In order to confirm that the resistance of cells from transfected cultures was the result of incorporation of genetic information from the pSV2neo plasmid, we examined DNA

isolated from G418-resistant rainbow trout and chinook salmon cells for the presence of plasmid-related sequences. DNA isolated from plasmid-transfected, G418-resistant cells from both cell lines contained sequences hybridizing to radiolabeled plasmid DNA under high stringency, while no plasmid-related sequences were detected under these conditions in DNA isolated from controll cells of either cell line (Fig.2.2). The major fragment containing plasmid sequences in DNA from G418-resistant rainbow trout liver cells was approximately 6.5 kilobases in size.

Discussion

Our results indicate that the pSV2neo plasmid, originally designed for expression in mammalian cells, was incorporated and expressed by rainbow trout and chinook ssalmon cells in culture with a frequency comparable to that seen with mammalian cells (Corsaro and Pearson,1981; Southern and Berg, 1982). This work suggests that other plasmids constructed for use in mammalian cell culture systems may be directly suitable for use in fish cell culture systems without further modification. In a recent abstract, it has been reported that exogenous xanthine-guanine phosphoribosyltransferase under the control of the rous sarcoma virus promoter can be transfected and

expressed in cultured fish cells, although the frequency of successful transfection of cells was not given (Huang, Gourlie and Price, 1987).

It also seems possible that additional genetic manipulations of plasmids originally intended for use in mammalian systems, such as replacement of the SV40 early promoter or other genetic regulatory elements with those derived from the fish genome or fish viruses, might further increase the transfection frequency. As with other organisms, the potential to genetically alter individual fish cells by exposure to exogenous DNA represents apowerful technique for exploration of various aspects of both basic and applied research.

In experiments analogous to those carried out with mammalian systems (Gordon and Ruddle, 1985), the alteration of cells in culture followed by transplantation of these cells to the developing or adult organism, or the direct exposure of embryos to exogenous DNA through microinjection could lead not only to insights in the areas of molecular, cell and developmental biology of fish, but also could lead to the production of strains with greater growth potential, increased resistance to disease, or other commercially useful characterisitics. Introduction of recently cloned salmon growth hormone gene (Sekine et al., 1985) into

embryonic salmonid cells and generation of chimeric organisms expressing increased levels of this hormone represents attractive example of this potential.

Thus far integration of exogenous DNA into fish genomes has been achieved by direct microinjection of embryos, but little data exists to indicate that exogenous genes can be expressed under these circumstances (Zhu et al., 1985; Chorrout, Guyomard, and Houdebine, 1986). An advantage of our approach is the direct selection for expression of the exogenous gene, and the ability to do so easily with a population of 10^7 or greater individual cells, compared with a lack of of selective pressure after microinjection and the limited potential to inject and examine only few individuals.

The advantage of fish model systems, compared to mammalian animal models, as sensitive organisms in toxicology and carcinogenisis studies (Bailey et al., 1984), in the production of large numbers of embryos from single animals, and the ability to produce cloned individuals in several species, including rainbow trout (Steisinger et al., 1981; Parson and Thorgaard, 1985) suggest that the use of techniques to introduce exogenous genes into fish cell cultures may provide alternative approaches to some kinds of questions that cannot be easily addressed with mammalian models.

Examples of situations in which these techniques might be useful include introduction into cultured cells derived from early fish embryos of genes for enzymes involved in genomic repair, detoxication or metabolic activation pathways, oncogenes or other genes involved in growth control and selection for expression at amplified levels, possibly followed by transplantation back into a large number of developing embryos and examination of in vivo phenomena in which statistically significant responses of a large population of individuals can be compared. Other approaches include introduction into cultured embryo cells of genes under the control of inducilbe promoters, followed by transplantation of cells back into developing embryos, or transplantation of nuclei derived from cells genetically manipulated in culture into oocytes in which the endogenous nucleus had been inactivated, followed by procedures inducing development of gynogenetic diploid animals.

Acknowledgements

WE thank M. Solem for initial exploration of the fish cell culture systems, C. Lannan and Dr. J. Fryer for cell lines, Dr. G. Thorgaard for useful discussions and Dr. C.L. Rawson for pSV2neo plasmid DNA preparations and advice. This work was supported by National Cancer Institute Grant

R01CA44317 and National Institute of Environmental Health Sciences Center Grants ES00210 and ES03850. DWB is the recipient of NIHNCI Research Creer Development Award 01226.

Fig. 2.1. **Growth of G418-resistant colonies from cultures transfected with pSV2neo plasmid DNA.** Cultures were transfected as described and maintained for 60 days in the presence of 1 mg/ml G418, followed by fixing of the flasks with formalin and staining with crystal violet. (A), Chinook salmon embryo (CHSE-114) cells, control; (B), Chinook salmon embryo cells, plasmid-transfected; (C), Rainbow trout hepatoma (RTH) cells, control; (D), Rainbow trout hepatoma cells, plasmid-transfected.

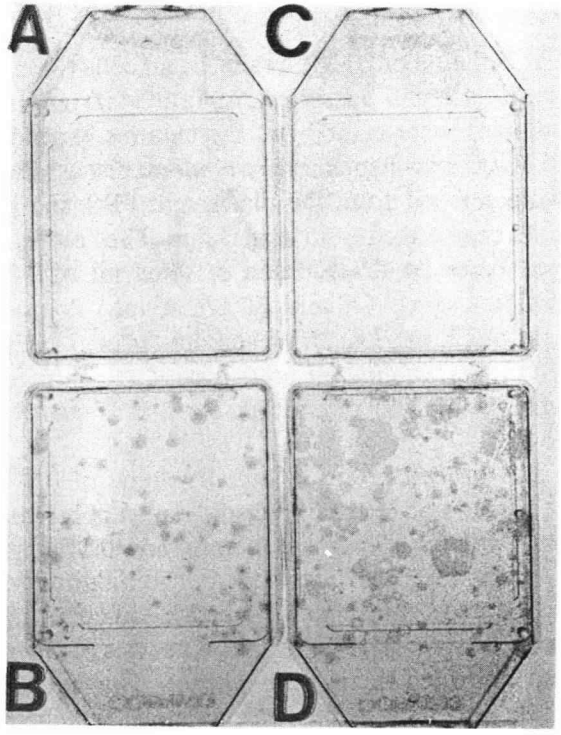
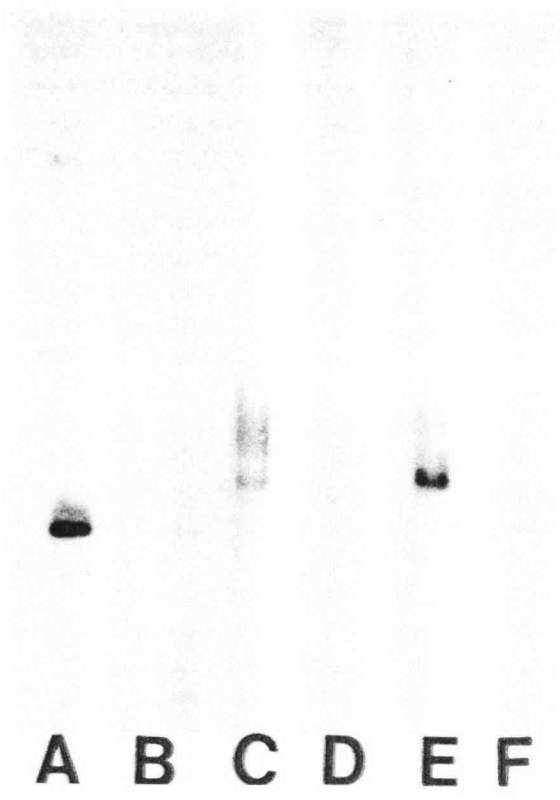


Fig. 2.2. **Hybridization analysis of pSV2neo plasmid sequences in DNA of G418-resistant cells derived from plasmid-transfected cultures.** DNA isolation and hybridization was carried out as described. (A), pSV2neo plasmid DNA (1 ng); (B), DNA isolated from control chinook salmon embryo cells; (C), DNA isolated from G418-resistant chinook salmon embryo cells; (D), Dna isolated from control rainbow trout hepatoma cells; (E), DNA isolated from G418-resistant rainbow trout hepatoma cells; (F), trout liver DNA (control).



Chapter 3

COMPARISON OF ACTIVITIES OF MAMMALIAN VIRAL PROMOTERS DIRECTING GENE EXPRESSION IN VITRO IN ZEBRAFISH AND OTHER FISH CELL LINES

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and David Barnes

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Preface

Coauthor Contribution

Kazuo Nishiyama worked out and optimized conditions for the lipofection experiments. Paul Collodi assisted with valuable advice on fish cultures and stimulating discussions. And David Barnes directed this research project and provided scientific and financial support.

Summary

Zebrafish are an important model species for several disciplines of basic biology and recently have become a model for development of transgenic organisms. A prerequisite for transgenic animal production is successful expression of the introduced gene under the control of a strong promoter. Recently we developed cell lines derived from zebrafish embryo and adult tissues, allowing quantitative evaluation of promoter activity in an *in vitro* system. We tested five commonly used strong mammalian viral promoters using zebrafish cell lines as well as lines derived from carp epithelioma (EPC) and chinook salmon embryo (CHSE-214). All cell lines were transfected with the bacterial *lacZ* gene as a histochemical marker. Of the promoters tested the human cytomegalovirus immediate early enhancer/promoter unit gave best resylts.

Introduction

The production of transgenic fish is an important tool for studies of developmental biology, aquaculture and toxicology. One of the most popular fish models for genetic manipulations is the zebrafish (*Brachydanio rerio*) (Streisinger et al., 1981). Small size and relatively short generation time along with transparency of the embryos throughout development make the zebrafish a favorable species for transgenic studies. Because

straightforward interpretation of transgenics requires expression of the introduced genes with a strong constitutive or inducible promoter, most researchers have resorted to well characterized mammalian promoters. Few fish promoters have been isolated and promoter-directed expression patterns in various tissues in vivo are not well studied (Liu et al., 1990; Gong et al., 1991; Fu et al., 1991). In addition, promoters from fish viruses are not readily available.

Construction of transgenic fish with conventional promoters adopted from mammalian systems has been achieved, integration of the exogenous DNA into the host genome verified by Southern blotting and germ-line transmission confirmed by subsequent breeding experiments (Powers, 1989). However, only a few investigators have been successful in obtaining protein expression with heterologous transgenic promoter systems. Successes include bacterial chloramphenicol acetyl transferase under control of a Rous sarcoma virus (RSV)-simian virus 40 (SV40) chimeric promoter in transgenic zebrafish (Stuart et al., 1990), rainbow trout growth hormone linked to RSV promoter and human growth hormone linked to the mouse metallothionein promoter in carp and loach (Chen et al., 1990).

Exogenous genes with 5′ flanking sequences have also been expressed: a sequence from winter flounder

(Pseudopleuronectes americanus) coding for antifreeze protein was detected in transgenic salmon (Davies, et al.,1990), and the chicken delta-crystallin gene was expressed in medaka (Oryzias latipes) (Inoue et al., 1989). However the relative activities of various promoters cannot be easily evaluated from experiments with intact organisms, because tests in vivo provide primarily qualitative information and are influenced by undefined factors affecting frequency of integration and tissue-specific or developmental stage-dependent expression.

Reports of systematic in vitro studies of promoter-directed exogenous gene expression in fish cells are rare and have been limited to a few established fish cell lines (Friedenreich et al., 1990; Inoue et al., 1990). Despite the usefulness of zebrafish for transgenics, no zebrafish cell cultures have been available and quantitative in vitro studies of promoter efficiencies were not possible. Recently we derived several zebrafish cell lines from blastula stage embryos and adult tissues (Collodi et al., 1992). Here we report a quantitative study on the expression in zebrafish cells of the bacterial lacZ gene under the influence of various heterologous mammalian promoters. We also present a comparison with other fish cell lines and with a mammalian cell line routinely used in transfection studies.

Results and Discussion

Time course of transient expression after calcium phosphate mediated transfection with a plasmid containing lacZ under the control of the SV40 early promoter was determined for five cell lines. Cell lines tested were two embryo-derived zebrafish lines (ZEM1 and ZEM2, Collodi et al., 1992), a salmon embryo-derived line (CHSE214), a commonly used carp line (EPC) (Wolf et al., 1980) and, as a control, a commonly used mouse embryo-derived cell line (NIH3T3)(Loo et al., 1989). At the indicated times after transfection, lacZ expression was assayed histochemically and the number of positive (blue) cells/10^6 transfected cells was determined (Fig. 3.1).

Time course of transient expression followed the same general pattern in all cases; expression frequencies increased during the first few days, reached a maximum and then returned to near zero. Maximal expression varied with each cell line and frequency of expression was in general much greater with mouse and carp cells than with zebrafish or salmon embryo cells (Fig. 3.1 A,B). Maximal expression of transfected EPC cells was seen earlier with cells cultured at 26°C, when compared with cells cultured at 18°C, but maximal expression frequency was about the same at both temperatures. Although frequencies of expression varied with time and cell line, the intensity after histochemical

staining for lacZ expression was similar in all cases.

The time-dependent increase in lacZ expression is presumably representative of accumulation of gene product within the cell and the subsequent decline in positive cells is presumably due to nuclease degradation of the plasmids or dilution of the plasmid copy number as cells divide. The time-dependent increase in positive cells we observed is not due simply to replication of cells that initially received multiple plasmid copies and redistribution of these plasmids to progeny cells, because positives were single, isolated cells in the culture population, rather than groups of two or more stained cells deriving from a common progenitor. A time-dependent increase in gene dosage due to autonomous replication of transfected plasmids has been reported in developing medaka and xenopus after injection of exogenous DNA (Marini et al., 1988; Chong et al., 1989; Winkler et al., 1991), even though the introduced plasmids did not contain specific sequences known to initiate replication in eucaryotes. The significant delay in appearance of positive cells in our in vitro system suggests that delayed transient expression of transgenic lacZ often observed in experiments in which plasmids are injected directly into developing embryos may not necessarily indicate developmental regulation of plasmid expression. To further explore the transient transfection response we characterized the dose response

relationship between amount of plasmid exposed to cells and frequency of lacZ expression (Fig.3.2). Maximum expression was obtained with 35 to 50 ug plasmid DNA per transfection. Higher DNA concentrations generally are not used in calcium phosphate mediated transfections for technical reasons. Because expression from calcium phosphate-mediated transfection was relatively poor with the zebrafish cultures, we also examined lipofection as an alternative transfection method with ZEM2 cells. Transient expression frequency with lipofection was similar to that obtained with calcium phosphate-mediated DNA transfection (Fig. 3.2). Although transient expression was not improved by lipofection, the technique may be useful to achieve stable integration.

For a comparison of promoter activities five different promoters were tested for ability to direct transient expression of lacZ on zebrafish and salmon embryo, carp and mouse embryo cell lines. In some cases we also tested an adult zebrafish fin-derived line (ZPF, Collodi et al., 1992). Promoters tested were: SV40 early promoter, cytomegalovirus (CMV) immediate early promoter, adenovirus 2 (AD) late promoter, herpes simplex virus thymidine kinase promoter (TK) and RSV long terminal repeat (LTR).

CMV and SV40 early promoters were in general best for transient expression (Fig. 3.3 A,B), while AD, TK and RSVLTR showed relatively low expression frequencies (Fig. 3.3 C,D,E). Expression of most plasmids in EPC cells was

strikingly better than in the other fish cell lines, and approached levels of expression seen in the control mouse cell line. The CMV promoter was best for all cell lines except EPC, which showed highest transient expression rates from Ad and SV40 promoters. These results suggest that data derived with EPC cells as a model for testing plasmid constructions for transgenic purposes may not be directly applicable to other fish systems.

CMV and SV40 expression frequencies were similar in zebrafish embryo-derived (ZEM1 and ZEM2) and in zebrafish adult fin-derived cells (ZPF) (Fig. 3.3 A,B), suggesting that low expression in embryo-derived cells may not be the result of developmental regulation of the introduced lacZ gene. However, temporal and spatial regulation of introduced viral promoters, such as SV40 and RSV LTR in in vivo systems have been reported in transiently expressing medaka, as well as in stable transgenic mice (Overbeek et.al.,1986; Brinster et.al., 1984; Chong et al., 1989; Winkler et.al., 1990).

We and others have shown that several standard fish cell lines can be transfected with and stably express common selectable markers such as the procaryotic aminoglycoside 3' phosphotransferase gene (neo) or the E. coli xanthine-guanine phosphoribosyl transferase gene under the control of the SV40 early promoter (Isa et al.,1987, Helmrich et al., 1988). The resulting colony frequencies

are comparable to those found in mammalian systems for stable transfection, but are influenced by relative frequencies of stable genomic integration and replication in addition to successful promoter-directed expression. To quantify the mammalian virus promoter-directed expression of an exogenous gene in single cells under conditions in which genomic integration is not a factor, we transfected zebrafish embryo and other cell lines with bacterial lacZ and assayed for transient expression rates.

Although the CHSE214 and all zebrafish cell lines showed relatively low transient expression frequencies, these low frequencies can be overcome experimentally by cotransfecting with a selectable marker such as neo and clonal isolation of stable transformants (Helmrich et al., 1988). SV40 and RSV-derived promoters have been used in the production of transgenic zebrafish, although expression of the transgene is not uniformly observed (Stuart et al., 1990, Culp et al., 1991). We are presently testing stable transformants of blastula-derived zebrafish cells utilizing several of the promoters examined in this report for the ability to contribute to embryonic development after injection into host blastulas. Such cultures could provide a means to produce transgenic zebrafish chimeras.

Experimental Procedures

Plasmids. The pCH110 vector (Clontech Laboratories, Palo Alto, CA) provides an SV40 early promoter for the lacZ gene. Plasmids pADlacZ, pTKlacZ and pCMVlacZ (Clonetech, Palo Alto, CA) have identical backbones and differ only in the promoters (AD, adenovirus 2 major late promoter; TK, herpes simplex virus thymidine kinase promoter; CMV, human cytomegalo virus immediate early enhancer/promoter unit) (Mac Gregor et al., 1989). To produce a plasmid with the lacZ gene under the direction of the RSV LTR, we cloned the 3.5 kb lacZ fragment from a Not I digestion of pCMVlacZ into the unique Not I site of pRc/RSV (Invitrogen, San Diego, CA). Orientation was determined by restriction pattern analysis. Large scale plasmid purifications were performed as described (Maniatis, et al., 1982). Restriction enzymes were obtained from Stratagene (La Jolla, CA),

Cell lines. The EPC cell line was derived from a carp (Cyprinos carpio) epithelioma; the CHSE-214 line was derived from pooled Chinook salmon (Oncorhynchus tshawytscha) embryos (Wolf et al., 1980). ZEM1 and ZPF zebrafish cell lines were derived as described (Collodi et al., 1992). The ZEM2 cell line was derived in the same manner as ZEM1 (designated ZEM in Collodi et al., 1992), except that 30% conditioned medium from Buffalo Rat Liver (BRL) cells was also added. BRL and NIH3T3 lines were

obtained from the American Type Culture Collection (Rockville, Maryland).

Culture conditions. All fish cell lines were maintained in LDF basal nutrient medium as described (Collodi et al., 1992). EPC cells also received 5% calf serum (Hyclone, Logan, Utah). CHSE cells received 10% calf serum. Zebrafish lines were supplemented with 1% fetal bovine serum (FBS) (Gibco/BRL, Grand Island, NY), 0.4% trout serum, 40 ug/ml crude trout embryo extract (Collodi et al., 1990) and 10 ug/ml insulin (Sigma, St Louis, MO). ZEM2 cells received, additionally 30% BRL conditioned medium. To obtain conditioned medium BRL cells were plated (70% confluent) in LDF medium supplemented with 10% FBS and the medium changed the following day to LDF with 1% FBS. Medium was harvested after 3-4 days, sterile filtered and stored at 4°C.

Transfection and histochemical assay. Calcium phosphate-mediated transfection was carried out as described (Collodi et al., 1992; Helmrich et al., 1988). Lipofection was achieved by incubating cells with 50 ul of Lipofectin™ reagent (BRL, Grand Island, NY) in the absence of serum or embryo extract for 1 hr followed by the addition of plasmid DNA. After 6-8 hrs the flasks were changed to complete medium (Felgner et al., 1989). Staining of lacZ expressing cells was achieved as described (Sanes et al, 1986). Variation in percentage of

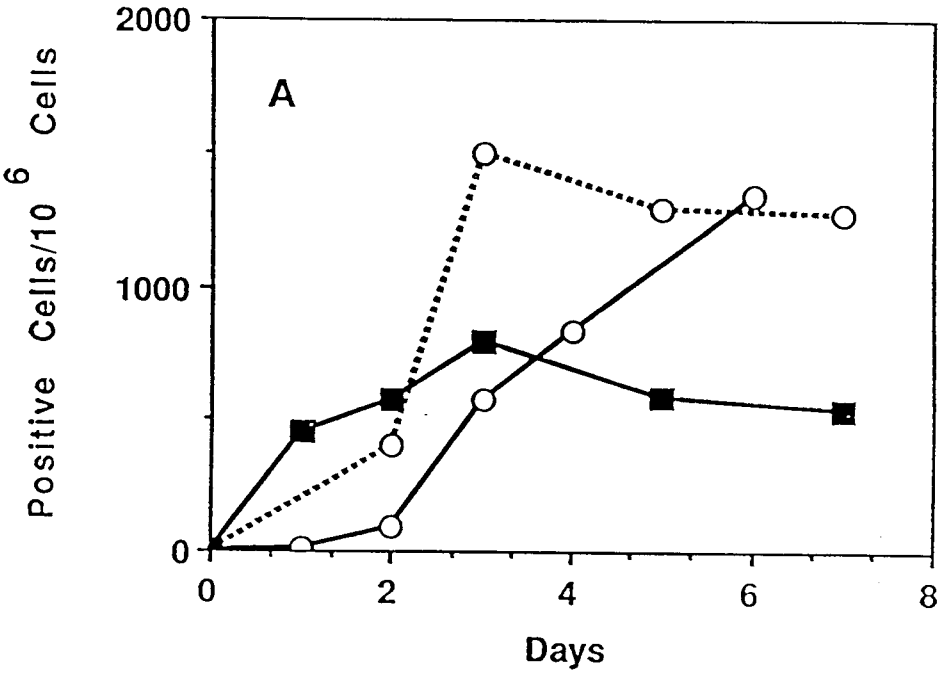
positive cells among replicate transfections for NIH3T3 and EPC cells was about 15% from the average. Variation among replicate transfections for the other cell lines showing reduced level of expression was about six-fold greater due to the 50-fold lower positive cell counts in these experiments. Transfections in which no positive cells were identified were scored as 1 positive/10^6 cells, which was taken as the lower limit of detection in the assay.

Acknowledgments

We thank Dr. G. Merrill, Oregon State University, Dr. D. Grunwald, University of Utah and Dr. S. Shirahata, Kyushu University, for plasmids. We also thank Dr. J. Fryer, Oregon State University, for the EPC and CHSE-214 cell lines and K. Lindburg for assistance in plasmid purifications. This work was supported by US Army Medical Research and Development Contract DAMD17-9121043. Some facilities and materials were provided through center grants ES00210 and ES03580 from the National Institute of Environmental Health Sciences. D.W.B. is the recipient of Research Career Development Award NIHNCI 01226.

Figure 3.1. **Time course of transient SV40lacZ expression in five cell lines.** Cells were plated (10^7/75cm^2 flask), transfected the following day with 50 ug pCH110 DNA/flask, split equally into 5 25 cm^2 flasks after 6-8 hrs and stained histochemically at 5 time points.

A: (-■—■-), NIH3T3 mouse embryo, 37°C; (-O··O-), EPC carp epithelioma, 18°C; (-O—O-), EPC carp epithelioma, 26°C; **B:** (-□—□-), CHSE chinook salmon embryo, 18°C; (-▲—▲-), ZEM1 zebrafish embryo, 26°C; (-△—△-), ZEM2 zebrafish embryo, 26°C.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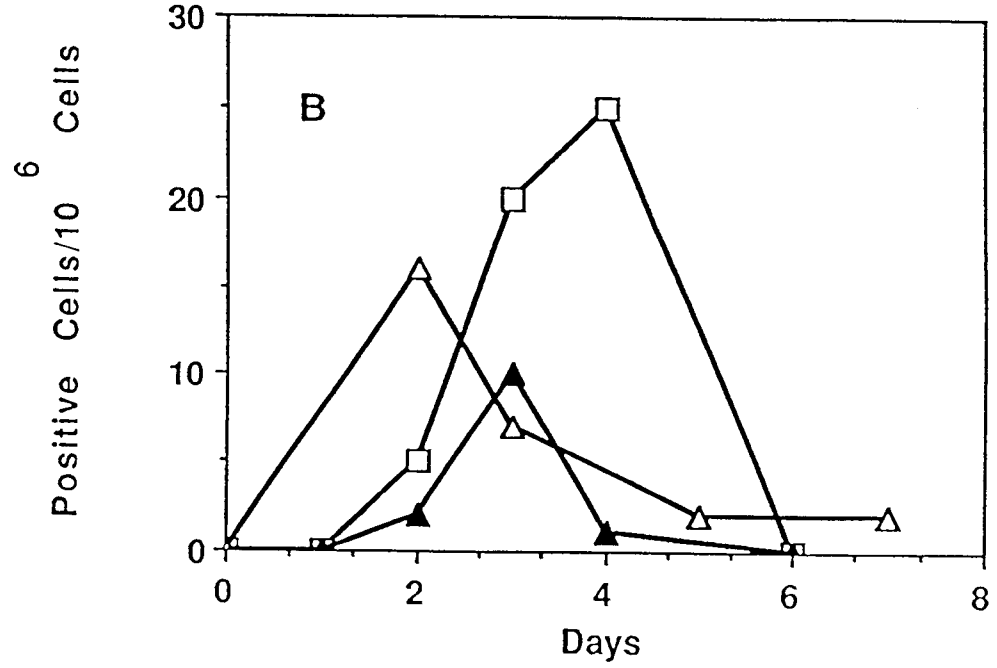
30
20
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Positive Cells/10 6 Cells
0
2
4
6
8
Days

Figure 3.2: **Dose response of SV40lacZ (pCH110) transient expression.** Cells were plated ($5x10^6/75cm^2$ flask), transfected the following day with varying amounts of pCH110 plasmid DNA and stained for lacZ on the appropriate day as determined from the time course. (—O—O—), calcium phosphate-mediated transfection of EPC carp epithelioma, stained on day 5; (—Δ—Δ—), calcium phosphate-mediated transfection of ZEM2 zebrafish embryo cells, stained on day 2; (- Δ - Δ -), lipofection of ZEM2 cells, day 2.

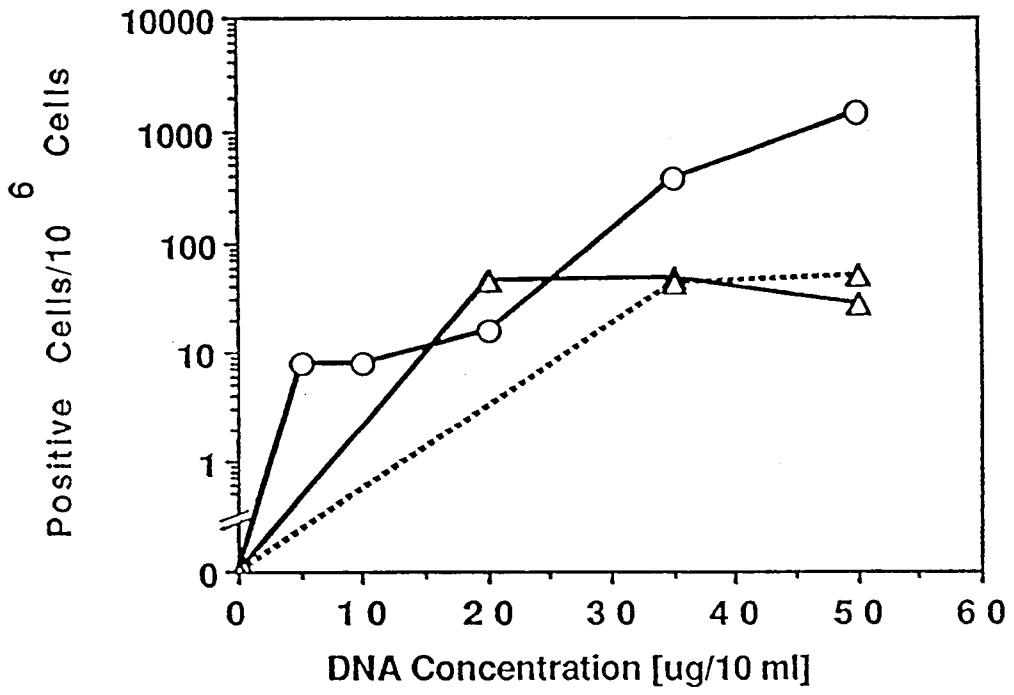
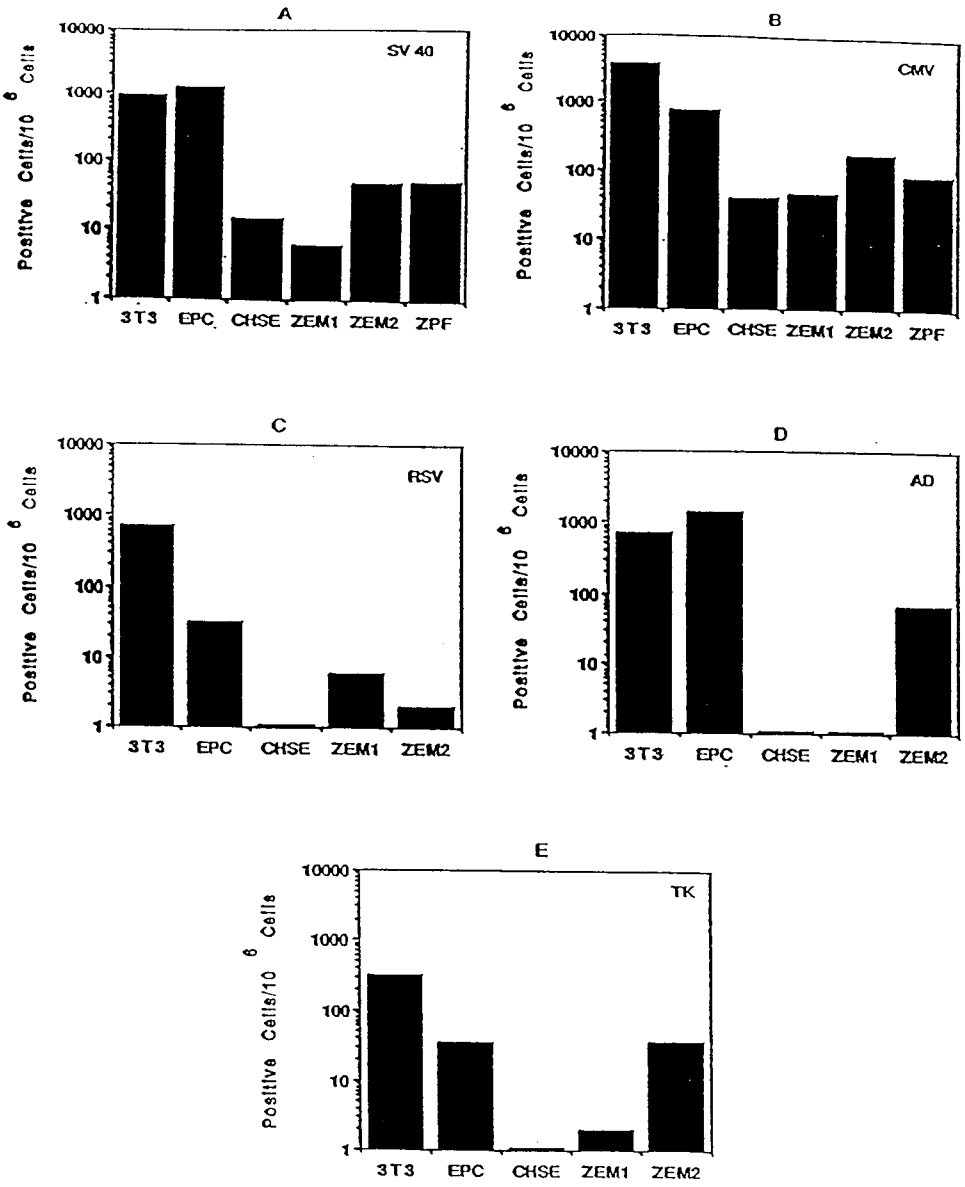


Figure 3.3: **Transient expression with five different promoter sequences directing expression of lacZ in fish cell lines.** Cell were plated (5 x 10^6 cells/75 cm^2 flask), transfected the following day with 50 ug of plasmid DNA and stained histochemically for lacZ expression on the appropriate day as determined from time course (mouse NIH3T3, zebrafish embryo ZEM1 and adult zebrafish fin ZPF, day 3; carp EPC, day 5; salmon embryo CHSE214, day 4; zebrafish embryo ZEM2, day 2). **A**, SV40 early promoter; **B**, CMV immediate early enhancer/promoter; **C**, RSV LTR; **D**, Ad2 major late promoter; **E**, Herpes simplex virus TK promoter.


A
SV 40
B
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C
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D
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Positive Cells/10 6 Cells
10000
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3T3
EPC
CHSE
ZEM1
ZEM2
ZPF

CHAPTER 4

VARIEGATED BETA-GALACTOSIDASE EXPRESSION IN CLONAL CELL LINES AND IN CHIMERA

PRELIMINARY DATA

Preface

Contributors

Katherin Lindburg assisted with SFME cloning and Southern blotting. Paul Collodi injected zebrafish and screened for chimera. David Barnes performed mouse brain injections, directed and finacially supported the research and assisted with helpful discussions.

Abstract

Clonal cell lines transfected with an RSVlacZ-SV40neo plasmid construct have been derived with zebrafish embryo cells (ZEM2) and serum free mouse embryo cells (SFME). Despite the supposedly clonal origin of each cell line, lacZ expression was either absent or mosaic. Only after subcloning was it possible to obtain a 100% expressing SFME clone. We aslo found clones with mixed populations, one 50%-expressing clone and one 1%-expressing clone. None of the ZEM2 clones tested so far expressed lacZ in culture. These clones are used to study the DNA profile of lacZ plasmid within the genome of each clone by Southern blotting. The 100%-expressing (blue) SFME cells are also injected into one day old embryo brains to study their ability to contribute to normal brain development. This has so far not been successful. The inverse case has been observed in zebrafish were we injected non expressing, but supposedly lacZ bearing ZEM2 clones into early blastula stage zebrafish and obtained one chimera out of 80 with lacZ-expressing cells present along the spine. This has since not been reproducible and requires further investigation.

Introduction

Mosaic expression of lacZ in supposedly clonal cell lines and in F2 homozygous transgenic zebrafish has been

observed (MacGregor, et al.,1987 and Stuart, et al., 1990 respectively). These phenomena are poorly understood, and evidence exists supporting several suggested possibilities, some of which are discussed here. In the case of the mosaic lacZ expression in clonal cell lines observed by MacGregor (MacGregor et al., 1987) it was suggested that DNA methylation is responsible for the variegated expression pattern. 5-aza-cytidine was used to block the enzyme methyltransferase resulting in increased RSV promoted lacz expression. Reports have been published supporting this observation (Alterova et al., 1972, Clough et al.,1982); it was shown that in cells infected with RSV and HSV, repectively, viral protein production could be induced upon demethylation by 5-aza-cytidine. DNA methylation is thought to play a role in eykaryotic gene expression, but despite evidence from many experiments, the biochemical mechanisms are not clear (Doerfler, 1984).

Another possible explanation for mosaic expression stems from the discovery of silencers. These are cis acting sequences which can supress the transcriptional activity of a nearby gene. They share similarities with their counter parts, the enhancers, in that they seem to be independent of the integration site and the orientation with respect to the affected gene (Artelt et al., 1991). Endogenous sequences as well as procaryotic and vector sequences have been found to interfere with nearby gene expression in

eucaryotes (Townes, et al., 1985; Kothary, et al., 1989). For example, it was shown that the bacterial neo gene acts as transcriptional silencer in eucaryotic cells (Artelt et al., 1991). An example for an indogenous silencer is one of three GC boxes found 3' to the protamine gene of rainbow trout (Jankowsky et al., 1987).

The presence or absence of introns, as well as their position relative to the promoter can have a profound effect on the expression of exogenous DNA and especially of cDNA in transgenics, but they are not neccessarily important for expression in cell cultures, suggesting that genes are exposed to events during development that are not realized in isolated cultured cells (Palmiter et al., 1991). Introns possibly contain enhancers or other cis-acting elements which bind proteins that influence transcriptional initiation or elongation (Mitchel et al., 1989). Introns may contain sequences that facilitate opening of chromosomal domains for transcription initiation complexes, possibly by altering the chromatin structure (Sveren et al., 1990). Alternatively, the process of mRNA splicing might enhance mRNA stability in the nucleus thereby allowing more accumulation of mature mRNA in the cytoplasm (Dreyfuss et al., 1988). These and many other regulatory mechanisms are encountered when gene manipulations are attempted.

Materials and Methods

Cell culture methods for ZEM2 were as described in Chapter 3. SFME cells are grown as described (Loo, et al., 1989). Transfection for stable integration was performed as described in Chapter 2, followed by colony selection with 100 ug/ml G418 (Gibco/BRL). Colonies were cloned and transfered to 96-well plates after 2-3 weeks for SFME and after 4-6 weeks for ZEM2 cells. Subcloning was initiated by plating a SFME clone with the highest (30%) lacZ-expressing cell population in 10 cm diameter dishes at 10^4 cells/dish.

Injections of SFME cells: 106 -107 cells in 10ul medium were injected into the left hemisphere of one day old Balb/c mouse embryos. Animals were sacrificed after 3-4 weeks, brains dissected out and stained as described (Allen, et al., 1988) with only one variation: the buffer consisted of 50 mM HEPES and 150 mM NaCl, pH7.5. Fish injections were performed as described (Collodi et al., 1992 b).

Results and Discussion

In order to approach our ultimate goal, the production of transgenic chimera via a cell culture stage, we transfected ZEM2 cells with RSVlacZ-SV40neo as well as with CMVlacZ-SV40neo constructs, both in supercoiled and linear forms. After 4 weeks of selection supercoiled plasmid

transfection yielded 11 colonies, while ScaI-lineraized plasmid trtansfection produced 7 colonies. We had hoped to possibly increase the lacZ and neo expression by controlled linearizing of the plasmid at a sequence not involved in the expression of either gene, instead of depending on linearization (neccessary for integration) on random cutting by cellular enzymes. Because the neo and lacZ expression cassettes constituted about 50% of the entire plasmid, we hoped that linearization by ScaI might produce a 50% improvement in lacZ expression within the G418-resistant colony population.

Only a few clones could be successfully expanded, three of which originated from supercoiled and 2 from ScaI linearized plasmid. While this yield may be too low for any significant statistical evaluation, it was surprising to find that none of the clones were expressing lacZ as determined by the histochemical assay described in Chapter 3. Despite this dissappointment we injected clone Lin3C (the first linear DNA-transfected clone to grow sufficiently for experiments) into early blastula stage embryos, hoping that may be these cells would express lacZ after differentiation within the animal. After 10 days of development 80 injected fish were stained histochemically for lacZ and we could indeed identfy one apparently transgenic chimera. The fish showed several layers of blue cells along the spine. None of the 80 uninjected control

fish stained blue except for an ocasional staining of the gut. We think that this background staing may be due to either a low pH in this area or to expression from bacteria colonizing the gut.

The single positive histochemical result is further supported by PCR data obtained from fish injected with neo-transfected ZEM1 (Collodi et al., 1992 b). There we were able to detect 30% transgenic chimeras. Further encouraging news comes from Hopkins laboratory, where chimeric zebrafish have been produced by transplantation of wild type blastula cells into synchonized albino blastulas (Lin, et al., 1992) We also stained 50 fish injected with clone SC6D (the first supercoiled DNA-transfected clone to grow sufficiently), but without any success. Confirmation of intact lacZ integration by Southern blotting or examination of mRNA expression by Northern blotting have not yet been approached for any of the fish clones, due to relativly slow cell growth. While other clones still await testing and more injections are required, these experiments allow some optimism in regard to chimera production with ZEM2.

Parallel to the fish cloning experiments we also applied a similar experimental design to serum free mouse embryo (SFME) cells, because (1) the mammalian viral promoters are more compatible with mouse cells and transfection and cloning protocolls for these cells had been successfuly developed previously (Shirahata, et al.,

1990), and (2) because we have an interest in further characterizing these embryo derived astrocyte precursors and their contribution in the developing and/or adult brain. We generated 20 RSV*lacZ*-SV40*neo* transfected clones and stained for *lacZ*-expression. Two clones showed mosaic expression and none were 100% blue as we had expected. Within one clone about 30% of all cells stained, within the other only about 1% were blue. Only after subcloning was it possible to obtain one 100% expressing clone out of 60 clones tested, however several clones still were mosaic for lacZ. To further investigate this phenomenon we are currently examining DNA from clones with different levels of lacZ expression by Southern blotting to see if the level of expression is related to the number of lacZ copies present.

We injected the 100%-expressing clone into 1 day old Balb/c mouse embryos hoping that these cells would contribute to brain development. Even though the mice developed normally with a 100% survival rate, we could not detect any significantly blue tissue sections. We super transfected the 100% blue clone with pUCEJ6.6, a H-ras containing plasmid vector and are currently selecting for cell growth minus EGF, a property characteristic of ras-transformed SFME cells (Shirahata, et al., 1990). These cells should be tumorigenic upon injection into mice and possibly yield blue-staining tumors. This system should

allow us to determine the technical limitations of the desired experiments, since it is known that cells expressing in culture are not neccessarily expressing in transgenics (Palmiter, et al., 1991).

With both the fish and mouse systems we hope to unravel more of the problems in exogenous gene expression encountered frequently in studies of transgenic animals. The ultimate goal is to succeed in both stable transgenic zebrafish chimera for longterm genetic studies as well as chimeric mouse brains for cell linage studies.

SUMMARY AND CONCLUSIONS

We have analyzed and applied some aspects of mammalian gene expression to fish cell cultures hoping to apply the resulting knowledge eventualy to gene expression in transgenic fish. Since little is known about fish on the molecular level, it seemed neccessary to initially study basic questions of gene expression *in vitro* on a cellular level. In summary, the research presented in this thesis indicates that:

1. Exogenous DNA can be transfected by calcium phosphate precipitation and, as in mammalian cell cuture systems, the DNA is integrated into the host genome.

2. A commonly used bacterial selectable marker (*neo*) is expressed under the controll of the SV40 early promoter with similar transfection frequencies when compared to other mammalian cell culture systems.

3. Transient expression of the *E.coli lacZ* gene as measured by histchemical staining identified single transfected cells in a mixed population - an important property for future histological questions.

4. Quantitative analysis of transient lacZ expression under the control of several mammalian viral promoters revealed that highest expression was obtained with the CMV promoter/enhancer in most fish lines tested, including ZEM2. 5. No differences in *lacZ* expression were observed when ZEM2 were compared to adult ZPF, indicating that

expression is not dependent on cell type or age of the organism from which cell lines were derived, for at least these two cell types. 6. Through stable transfection experiments it was possible to derive a 100%-expressing SFME clone. However, lacZ expression in most clonal SFME lines was either absent or mosaic, and none of the five ZEM2 clones tested so far are positive for lacZ expression. 7. So far no macroscopically obvious contribution of 100%-expressing lacZ-transfected SFME cells could be detected after injection into one day old mouse neonates. However, upon injection of one supposedly lacZ containing but non expressing ZEM2 clone into early blastula stage zebrafish embryos we obtained one chimeric fish with lacZ-expressing cells along the spine.

While more experiments are required to determine the ability of ZEM2 cells to contribute to normal development and possibly to the germline and whether SFME cells expressing lacZ can contribute to normal brain development, there is reason for optimism based on the results summarized above. Zebrafish embryo cells with stem cell-like morpholgy have been isolated (Collodi et al., 1992) and could be used for injection after further in vitro characterization. Such transgenic chimera will be invaluable tools to study cell lineage, aspects of eucaryotic tissue specific and temporal gene expression and regulation, as well as genes already known to play a role

in development or disease. For instance, it could be possible to introduce any growth factor gene under the control of an inducible promoter such as the Drosophila heat shock promoter (Inoue et al., 1989) to study the biochemical mechanisms by which these growth factors are controlled during complex events such as development (Nilsen-Hamilton, 1990). In addition, the fact that haploid zebrafish are viable up to the larval stage (Streisinger et al., 1981) and haploid zebrafish cell lines are available (Collodi et al., 1992) make the zebrafish a primary candidate for large scale genome screens in order to identify genes important in development by insertional mutagenis. Hopkins et al., 199..; Straehle et al., 1992).

Besides the usefulness of transgenics in basic research there is also growing interest in transgenics for commercial use. For instance in aquaculture as well as in agriculture and farming it would be desirable to obtain disease resistant domestic animals, or more productive livestock. Accomplishments in this direction have been reviewed (Church, 1990). While transgenic animals are not a new idea and transgenic mice today are almost taken for granted, there are yet many aspects to be addressed for fish. But once problems are overcome, and present research looks promising, the zebrafish will allow us to approach long persisting questions from a new point of view.

BIBLIOGRAPHY

Adams, R.L.P., Knowler, J.T., and Leader, D.P. (1986). In: The Biochemistry of Nucleic Acids pp. 287-375. Chapman and Hall, London New York.

Alam, J., and Cook, J.L. (1990). Reporter genes: application to the study of mammalian gene transcription. Anal. Biochem. 188:245-254.

Allen, N.D., Cran, D.G., Barton, S.C., Hettle, S., Reik, W., and Surani, A.M. (1988). Transgenes for probes for active chromosomal domains in mouse development. Nature 333:852-855.

Altenerova, V. (1972). Virus production induced by various chemical carcinogens in a virogenic hamster cell line transformed by rous sarcoma virus. J. Natl. Cancer Inst. 49:475-541.

Arelt, P., Grannemann, R., Stocking, C., Friel, J., Bartsch, J., and Hauser, H. (1991). The prokaryotic neomycin-resistance-encoding gene acts as a transcriptional silencer in eukaryotic cells. Gene 99:249-254.

Bailey, G.S., Hendricks, J.D., Nixon, J.E., and Powlowski, N.E. (1984). The sensitivity of rainbow trout and other fish to carcinogens. Drug. Metab. Rev. 15: 725-750.

Banerji, J.L., Olson, L., and Schaffner, W. (1983). A lymphocyte-specific cellular enhancer is located downstream of the joining region in immunoglobulin heavy chain genes. Cell 33:729-740.

Barnes, D.W., and Sato, G.H. (1980). Serum-free cell culture: a unifying approach. Cell 22: 649-655.

Baty, D., Soldana, B.H.A., Everett, R.D., Vigneron, M., and Chambon, P. (1983). Mutational dissection of the 21 bp repeat region of the SV40 early promoter reveals that it contains overlapping elements of the early-early and early-late promoters. Nucleic. Acid Res. 12:915-932.

Bending, M.M. (1981). Persistance and expression of histone genes injected into Xenopus eggs in early development. Nature 292:65-67.

Brinster, R.L., Chen, H.Y., Messing, A., van Dyke, T., Levine, A.J., and Palmiter, R.D. (1984). Transgenic mice harboring SV40 T Antigen genes develop characteristic brain tumors. Cell 37:367-379.

Boshart, M., Weber, F., John, J., Dorsch-Haesler, K., Fleckenstein, B., and Schaffner, W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. Cell 41:521-530.

Capecchi, M.R. (1989). Altering the genome by homologous recombination. Science 244:1288-1292.

Capecchi, M.R. (1980). High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. Cell 22:479-488.

Chang, A.C.Y., and Brenner, D.G. (1988). Cationic liposome-mediated transfection: a new method for the introduction of DNA into mammalian cells. Focus 10:66-69.

Chen, T.T., Lin, C.M., Zhu, Z., Gonzalez-Villasenor, L.I., Dunham, R.A., and Powers, D.A. (1990). Gene transfer, expression and inheritance of rainbow trout and human growth hormone genes in carp and loach. In: Church, R.B. (ed.) Molec. Cell. Biol. New Series 116:127-139.

Chong, S.S.C., and Vielkind, J.R. (1989). Expression and fate of CAT reporter gene microinjected into fertilized medaka (Oryzias latipes) eggs in the form of plasmid DNA, recombinant phage particles and its DNA. Theoret. Appl. Genet. 78:369-380.

Church, R.B., (ed), 1990. In: Transgenic models in medicine and agriculture. UCLA symposia in molecular and cellular biology . New series Vol. 116. Wiley-Liss.

Clough, D.W., Kunkel, L.M., and Davidson, R.L. (1982). 5-azacytidine-induced reactivation of a herpes simplex thymidine kinase gene. Science 216:70-73.

Chourrout, D., Guyomard, R., and Houdebine, L. (19860. High efficiecy gene transfer in rainbow trout by microinjection into egg cytoplasm. Aquaculture 51:143-150.

Collodi, P., and Barnes, D.W. (1990). Mitogenic activity from trout embryos. Proc. Natl. Acad. Sci. USA 87:3498-3502.

Collodi P., Kamei Y., Sharps A., Weber D., and Barnes D.W. (1992 a). Fish embryo cell cultures for derivation of stem cells and transgenic chimera. Molecular Marine Biology and Biotechnology (in press).

Collodi, P., Kamei, Y., Ernst, T., Miranda, C., Buhler, D.R., and Barnes, D.W. (1992 b). Culture of cells from zebra fish (Brachydanio rerio) embryo and adult tissues. Cell Biol. and Toxicol. 8:43-61.

Conchie, J., Findlay, J., and Levvy, G.A. (1959). Mammalian glycosidases distribution in the body. Biochem. J. 71:318-325.

Corsaro, C.M., and Pearson, M.L. (1981). Enhancing the efficiency of DNA-mediated gene transfer in mammalian cells. Somatic Cell Genet. 7:603-616.

Culp, P., Nusslein-Volhard, C., and Hopkins, N. (1991). High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. Proc. Natl. Acad. Sci. USA 88:7953-7957.

Davies, J., and Jimenez, A. (1980). Anew selective agent for eukaryotic cloning vectors. Am. J. Trop. Med. Hyg. 29:1089-1092.

Davies, P.L., Hew, C.L., Shears, M.A., and Fletcher, G.L. (1990). Antifreeze protein expression in transgenic salmon. In: UCLA Symposium on Molecular and Cellular Biology. Church R.B. (ed.) Wiley Liss, Vol. 16 pp. 141-161.

Dunham, R.A., Eash, J., Askins, J., and Townes, T.M. (1987). Transfer of the metallothionein-human growth hormone fusion gene into channel catfish. Trans. Am. Fish. Soc. 116:87-91.

Doerfler, W. (1984). DNA methylation and its functional significance: studies on the adenovirus system. In: Current topics in microbiology and immunology. Haas, W. and Boehmer, H.V. (eds.). Springer-Verlag Berlin Heidelberg.

Dreyfuss, G., Swanson, M.S., and Pinol-Roma, S. (1988). Heterogenous nuclear ribonucleoprotein particles and the pathway of mRNA formation. Trends in Biochem. 13:86-91.

Felgner, P.L. and Holm, M. (1989). Cationic liposome-mediated transfection. Focus 11:21-25.

Friedenreich, H., and Schartl, M. (1990). Transient expression directed by homologous and heterologous promoter and enhancer sequences in fish cells. Nucl. Acids Res. 18:3299-3305.

Fryer, J.L., McCain, B.B, and Leong J.C. (1981). A cell line derived from rainbow trout (Salmo gairdneri) hepatoma. Fish Pathol. 15:193-200.

Fu, L., and Aoki, T. (1991). Expression of CAT gene directed by the 5'-upstream region from yellowtail alpha-globin gene in tissue cultured fish cells. Nip Suisan Gak 57:1689-1696.

Gluzman, Y. (1985). In: Current communications in molecular biology: Eukaryotic transcription, the role of cis- and trans-acting elements in initiation. Cold Spring Harbor Laboratory.

Gluzman, Y., and Schenk, T. (1983). In Current communications in molecular biology: Enhancers and eukaryotic gene expression. Cold Spring Harbor Laboratory.

Gong, Z., Hew, C.L., and Vielkind, J.R. (1991). Functional analysis and temporal expression of promoter regions from fish antifreeze protein genes in transgenic Japanese Medaka embryos. Mol. Mar. Biol. and Biotech. 1:64-72.

Gordon, J.W., and Ruddle, F.H. (1985). DNA-mediated genetic transformation of mouse embryos and bone marrow: a review. Gene 33:121-136.

Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I., and Howard, B.H. (1982). The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Natl. Acad. Sci. USA 79:6777-6781.

Gurdon, J.B., and Melton, D.A. (1981). Gene transfer in amphibian eggs and oocytes. Ann. Rev. Genet. 15:184-218

Graham, F.L. and Van der Eb, A.J. (1973). A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456-467.

Hanahan, D. (1989). Transgenic mice as probes into complex systems. Science 246:1265-1275.

Hearing P., and Schenk, T. (1983). The adenovirus type E1A transcriptional contro region contains a duplicated enhancer element. Cell 33:695-703.

Helmrich, A. Bailey, G.S. and Barnes, D.W. (1988). Transfection of cultured fish cells with exogenous DNA. Cytotech. 1:215-221.

Huang, R.C. Gourlie, B., and Price, J. (1987). Winter flounder antifreeze protein genes: demonstration of a cold-inducible promoter and gene transfer to other species. Federation Proceedings 46:2238 (abst).

Hew, C., Davies, P.L., Shears, M., King, M., and Fletcher, G. (1987). Antifreeze protein gene transfer to atlantic salmon by microinjection. Federation proceedings 46:2039 (abst).

Inoue, K., Akita, N., Yamashita, S., Shiba, T., and Fujita, T. (1990). Constitutive and inducible expression of a transgene directed by heterologous promoters in a trout liver cell line. Biochem. Biophys. Res. Commun. 173:1311-1316.

Inoue, K., Ozato, K., Kondoh, H., Iwamatsu, T., Wakamatsu, Y., Fujita, T., and Okada, T.S. (1989). Stage-dependent expression of the chicken delta-crystallin gene in transgenic fish embryos. Cell Diff. and Devel. 27:57-68.

Isa, K., and Shima, A. (1987). Transfection and stable expression of a dominant selective marker Ecogpt in a cultured cell line of the fish, Crassius auratus. J. Cell Sci. 88:219-224.

Jaenisch, R. (1988). Transgenic animals. Science 240:1468-1474.

Jaenisch, R. (1977). Germ line integration of moloney leukemia virus: Effect of homozygosity at the M-MuLV locus. Cell 12:691-696.

Janowsky, J.M., and Dixon, G.H. (1987). The GC box as a silencer. Bioscience Reports 7:955-963.

Kimmel, C.B., Warga, R.M., and Schilling, T.F. (1990). Origin and organization of the zebrafish fate map. Development 108:581-594.

Kothary, R., Clapoff, S., Darling, S., Perry, M.D., Moran, L.A., and Rossant, J. (1989). Inducible expression of an hsp 68-lacZ hybrid gene in transgenic mice. Development 105: 707-714.

Laale, H.W. (1977). The biology and use of zebrafish, Brachydanyo rerio, in fisheries research. J. Fish Biol. 10:121-173.

Lannan, C.N., Winton, J.R., and Fryer, J.L. (1984). New cell lines: establishment and characterization of nine cell lines from salmonids. In Vitro 20:671-676.

Lin, S., Long, W., Chen, J., and Hopkins, N. (1992). Germ line chimeras in zebrafish by cell transplants from genetically pigmented to albino embryos. Proc. Natl. Acad. Sci. USA in press.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R., and Hackett, P.B. (1990). Functional analysis of elements affecting expression of the beta-actin gene of carp. Mol. Cell. Biol. 10:3432-3440.

Loo, D.T., Rawson, C.L., Ernst, T. Shirahata, S., and Barnes, D.W. (1989a). Serum-free Primary Culture of Mouse Embryo Cells. In: Cell Growth and Cell Division: a Practical Approach, ed. R. Baserga, IRL Press, pp 17-34.

Loo, D., Rawson, C., Helmrich, A., and Barnes, D. (1989b). Serum-free mouse embryo cells: Growth responses in vitro. J. Cell Physiol. 139:484-491.

Lord, B.I., and Dexter, M.D. (1988). In: Stem cells. Journal of cell science supplement 10. The Company of Biologist limited, Cambridge.

Loyter, A., Scangos, G.A., and Ruddle, F.H. (1982). Mechanisms of DNA uptake by mammalian cells: Fate of exogenously added DNA monitored by the use of fluorescent dyes. Proc. Natl. Acad. Sci. USA 79:422-426.

MacGregor, G.R. and Caskey, C.T. (1989). Construction of plasmids that express E.coli beta-galactosidase in mammalian cells. Nucleic Acid Res. 17:2365.

MacKnight, S., Kingsbury, R., Eisenberg, S., Tufaro, F., Weinheimer, S., Triezenberg, S., Johnson, P., and Graves, B. (1985). Studies on the promoter of herpes simplex virus thymidine kinase gene pp 116-122. In Current communications in molecular biology: Eukaryotic transcription. Gluzman Y. (ed.).

MacLean, N., and Pennman, D. (1990). The application of gene manipulation to aquaculture. Aquaculture 85:1-20.

Maniatis,T., Fritsch,E.F., and Sambrook,J. (1982). Molecular cloning: A laboratory manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory.

Mannino, R.J., and Gould-Fogerite, S. (1988). Liposome mediated gene transfer. BioTechniques 6:682-690.

Marini, N.J., Etkin, D.L., and Benbow R.M. (1988). Persistence and replication of plasmid DNA microinjected into early embryos of Xenopus laevis. Dev. Biol. 127:421-434.

Mitchel, P.J., and Tjian, R. (1989). Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science 245:371-378.

Mulligan, R.C., Howard B.H., and Berg, P. (1979). Syntesis of rabbit beta globin in cultured monkey cells following infection with a SV40 beta globin recombinant genome. Nature 277:108-114.

Nilsen-Hamilton, M. (ed.) (1990). In: Current topics in developmental biology Vol. 24: Growth factors and development.

Nolan, G.P., Fiering, S., Nicolas, J., Herzenberg, L.A. (1988). Fluorescence-activated cell analysis and sorting of viable mammalian cells based on beta-D-galactosidase after transduction of E.coli lacZ. Proc. Natl. Acad. Sci. USA 85:2603-2607.

Overbeek, P.A., Lai, S., Van Quili, K.R., and Westphal H. (1986). Tissue-specific expression in transgenic mice of a fused gene containing RSV terminal sequences. Science 231:1574-1577.

Palmiter, R.D., Sandgren, E.P., Avaebock, M.R., Allen, D.D., and Brinster, R.L. (1991). Heterologous introns can enhance expression of transgenes in mice. Proc. Natl. Acad. Sci. USA 88:478-482.

Parson, J.E., and Thorgaard, G.H. (1985). Production of androgenic diploid rainbow trout. J. Heredety 76:177-181.

Pearson, B., Wolf, P.L., and Vazquez, J. (1963). A comparative study of a series of new indolyl compounds to localize beta-galacosidase in tissues. Lab. Invest. 12:1249-1259.

Peden, K., Mounts, P., and Hayward, G.S. (1982). Homology between mammalian cell DNA sequences and human herpes virus genomes detected by hybridization procedure with high-complexity probe. Cell 31:71-80.

Potter, H., Weir, L., Leder, P. (1984). Enhancer-dependent expression of human k-immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc. Natl. Acad. Sci. USA 81:7161-7165.

Powers, D.A. (1989). Fish as model systems. Science 246:352-357.

Primrose, S.B., (ed.) (1980). In: Introduction to modern virology. Blackwell Scientific,Oxford.

Renkawitz R. (ed.) (1989). In: Tissue specific gene expression. VCH New York.

Rigby. P.W.J., Dieckmann, M., Rhodes, C., and Berg, B. (1977). Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J.Mol. Biol. 113:237-252.

Robertson E.J. (ed.) (1987). In: Teratocarcinomas and embryonic stem cells a practical approach. IRL Press Oxford, Washington D.C..

Rossant, J., and Hopkins, N. (1992). Of fin and fur: mutational analysis of vertebrate embryonic development. Genes and Development 6:1-13.

Sanes, J.R., Rubenstein, J.L.R., and Nicolas, J.F. (1986). Use of recombinant retrovirus to study post-implantation cell linage in mouse embryos. EMBO J. 5:3133-3142.

Sekine, S., Mizukami, T., Nishi, T., Kuwana, Y., Saito, A., Sato, M., Itoh, S., and Kawauchi, H. (1985). Cloning and expression of cDNA for salmon growth hormone in E.coli. Proc. Natl. Acad. Sci. USA 82:4306-4310.

Shirahata, S., Rawson, C., Loo, D., Chang, Y., and Barnes, D. (1990). Ras and neu oncogenes reverse serum inhibition and epidermal growth factor dependence of serum free mouse embryo (SFME) cells. J. Cell. Physiol. 144:69-76.

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-523.

Southern, P.J., and Berg, P. (1982). Transformation of mammalian cells to antibiotic resistance with a bacterial gene under the control of the SV40 early region promoter. J. Molec. Appl. Genet. 1:327-341.

Steahle, U., and Ingham, P.W. (1992). Flight of fancy or a major new school? Current Biol. 2:135-138.

Streisinger, G., Walker, C., Dower, N.,Knauber, D. and Singer, F. (1981). Production of clones of homozygous diploid zebrafish (Brachydanio rerio). Nature 291:293-296.

Stuart, G.W., McMurray, J.V., and Westerfield, M. (1988) Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development 103:403-412.

Stuart, G.W., Vielkind, J.R., McMurray, J.V., and Westerfield, M. (1990). Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. Development 109:577-584.

Suemori, H., Kadodawa, Y., Goto, K., Araki, I., Kondoh, H., and Nakatsuji, N. (1990). A mouse embryonic stem cell line showing pluripotency of differentiation in early embryos and ubiquitous beta-galactosidase expression. Cell Diff. Develop. 29:181-186.

Thomsen, D.R., Stenberg, R.M., Goins, W.F., and Stinsky, M.F. (1984). Promoter regulatory region of the major immediate early gene of human cytomegalovirus. Proc. Natl. Acad. Sci. USA 81:659-663.

Townes, T.M., Lingrell, J.B., Chen, H.Y., Brinster, R.L., and Palmiter, R.D. (1985). Erythroid-specific expression of human beta-globin genes in transgenic mice. EMBO J. 4:1715-1723.

Warga, R.M., and Kimmel, C.B. (1990). Cell movements during epiboly and gastrulation in zebrafish. Development 108:569-580.

Trendelenburg, M.F., Zentgraf, H., Franke, W.W., and Gurdon, J.B. (1978). Transcription patterns of amplified Dytiscus genes coding for ribosomal RNA after injection of Xenopus oocyte nuclei. Proc. Natl. Acad. Sci. USA 75: 3791-3795.

Waterfield, M.D. (ed) (1990). In: Growth factors in cell and developmental biology. J. of cell science supplement 13. The Company of Biologists Limited, Cambridge.

Wigler, M., Perucho, M., Kurtz, D., Dana, S., Pellicer, A., Axel, R., and Silverstein, S. (1980). Transformation of mammalian cells with an amplifiable dominant acting gene. Proc. Natl. Acad. Sci. USA 77:3567-3570.

Winkler, C., Vielkind, J.R., and Schartl, M. (1991). Transient expression of foreign DNA during embryonic and larval development of the medaka fish (Oryzias latipes). Mol. Gen. Genet. 226:129-140.

Wolf, K., and Mann, J.A. (1980). Poikilotherm vertebrate cell lines and viruses: a current listing for fishes. In Vitro 16:168-179.

Wyllie, A.H., Gurdon, J.B., and Price, J. (1977). Nuclear localisation of an oocyte component required for the stability of injected DNA. Nature 268:150-152.

Wyllie, A.H., Laskey, R.A., Finch, J., and Gurdon, J.B. (1978). Selective DNA conservation and chromatin assembly after injection of SV40 DNA into Xenopus oocytes. Develop. Biol. 64:178-188.

Wynshaw-Boris, A., Short, J.M., Hanson, R.W. (1986). The determination of sequence requirements for hormonal regulation of gene expression. BioTechniques 4:104-119.

Xu, N.Z., Padmanabhan, R., Gorman, C.M., Howard, B.H. (1983). Comparison of enhancer functions in simian virus 40 and rous sarcoma virus pp. 51-54. In Current communications in molecular biology: Enhancers and eukaryotic gene expression. Gluzman, Y., and *schenk, T. (eds.)

Zhu, A., Li, G., He, L., and Chen, S. (1985). Novel gene transfer into the fertilized eggs of gold fish. Z. Angew. Ichtyol. 1:31-34.