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Regulation of expression of transgenes in developing fish

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The transcriptional regulatory elements of the β-actin gene of carp (*Cyprinus carpio*) have been examined in zebrafish and goldfish harbouring transgenes. The high sequence conservation of the putative regulatory elements in the β-actin genes of animals suggested that their function would be conserved, so that transgenic constructs with the same transcriptional control elements would promote similar levels of transgene expression in different species of transgenic animals. To test this assumption, we analysed the temporal expression of a reporter gene under the control of transcriptional control sequences from the carp β-actin gene in zebrafish (*Brachydanio rerio*) and goldfish (*Carrasius auratus*). Our results indicated that, contrary to expectations, combinations of different transcriptional control elements affected the level, duration, and onset of gene expression differently in developing zebrafish and goldfish. The major differences in expression of β-actin/CAT (chloramphenicol acetyltransferase) constructs in zebrafish and goldfish were: (1) overall expression was almost 100–fold higher in goldfish than in zebrafish embryos, (2) the first intron had an enchancing effect on gene expression in zebrafish but not in goldfish, and (3) the serum-responsive/CArG-containing regulatory element in the proximal promoter was not always required for maximal CAT activity in goldfish, but was required in zebrafish. These results suggest that in the zebrafish, but not in the goldfish, there may be interactions between motifs in the proximal promoter and the first intron which appear to be required for maximal enhancement of transcription.

Keywords: β-actin gene; goldfish; promoter; transcription; zebrafish

Introduction

Complex spatial and temporal regulation of gene expression in multicellular organisms are required for both proper development and homeostasis. This is achieved primarily at the level of transcription, involving activators, repressors and squelchers of RNA synthesis (Ptashne, 1988). Tissue-cultured cells are insufficient for identifying and characterizing the transcriptional regulatory sequences responsible for differential gene expression that occurs in multiple differentiated tissues of developing and adult organisms. To circumvent this problem, transgenic organisms can be used to characterize tissue- and developmental-specific transcriptional control elements. For many reasons, fish are being recognized as an excellent model system for investigations of developmental genetics (Kimmel, 1989; Powers, 1989; Rossant and Hopkins, 1992) and cancer genetics (Schwab, 1987; Schartl *et al.*, 1990). Firstly, most fish embryos develop outside the mother, allowing easy inspection and access to the developing embryo. Secondly, fish eggs are easy to obtain in large quantities without any trauma or injury to the female and, in some species, such as the zebrafish, may be produced daily. Thirdly, the embryos are fairly large and hardy, simplifying their handling. Several groups have microinjected genes into fish zygotes for a variety of

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purposes including development of growth-enhanced fish (reviewed by Fletcher and Davies, 1991; Hackett, 1992). Early experiments employed promoter elements from mammals, birds and viruses. More recently, promoter elements isolated from piscine species have been tested (Liu *et al.*, 1990a,b,c; Friedenreich and Schartl, 1990; Gong *et al.*, 1991; Shears *et al.*, 1991; Winkler *et al.*, 1991; Moav *et al.*, 1992a,b; Du *et al.*, 1992; Xiong *et al.*, 1992). All of these studies demonstrated that transcriptional control sequences from mammals, birds and fish are able to direct RNA synthesis in vertebrate cells.

We (Liu *et al.*, 1990c; Moav *et al.*, 1992a,b) have constructed expression vectors that employ the regulatory elements of the well-characterized carp β-actin gene (Liu *et al.*, 1990a,b, 1991; Moav *et al.*, 1992b). These studies showed that the organization of the β-actin gene of carp is similar to that found in land-vertebrate genomes, in which the first exon is non-coding and transcriptional control elements reside in the first intron. Like β-actin genes in other higher eukaryotes, fish β-actin genes have a serum-responsive element, the $CC(A/T)_6GG$ (CArG) box, which is evolutionarily conserved in actin and other serum-responsive genes (Minty and Kedes, 1986; Boxer *et al.*, 1989; Orita *et al.*, 1989; Subramaniam *et al.*, 1989; Walsh, 1989; Liu *et al.*, 1990a).

The high sequence conservation of the putative regulatory elements in the β-actin genes of animals suggests that their function is also highly conserved. We assumed that transgenic constructs with similar transcriptional control elements would promote similar levels of transgene expression, allowing the elements to be used in the construction of expression vectors for transgenic animal studies. To test this assumption, we analysed the temporal expression of a reporter gene under the control of transcriptional control sequences from the carp (*Cyprinus carpio*) β-actin gene in two species of fish used as model systems; the zebrafish (*Brachydanio rerio*) and the goldfish (*Carrasius auratus*). Our results indicated that, contrary to expectations, some combinations of transcriptional control elements affected the level of duration of gene expression differently in developing zebrafish and goldfish.

Materials and methods

Recombinant constructs

The constructs used have been described previously (Liu *et al.*, 1990b, 1991). Essentially, all the tested constructs contain portions of the carp β-actin gene ligated to the bacterial chloramphenicol acetyltransferase (CAT) gene to which either the 3′-end of the SV40 early region with its intron and poly(A) sequence (Gorman *et al.*, 1982) or the 3′-end of the chinook salmon growth hormone gene (Hew *et al.*, 1989) was juxtaposed as described previously (Liu *et al.*, 1990c).

Collection of fish eggs

Zebrafish (*Brachydanio rerio*) eggs were harvested within 1 h after fertilization. Essentially, two females and one male were kept per breeding box in an aquarium with a controlled photoperiod. The procedures recommended in *The Zebrafish Book* (Westerfield, 1989) were used to induce and enhance mating. To obtain newly fertilized eggs, a funnel with tygon tubing at the bottom was placed underneath the breeding box to allow easy and gentle collection by siphoning aquarium water with eggs into a beaker. Zygotes were washed with 10% Hank's saline (13.7 mM NaCl, 0.54 mM KCl, 0.13 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.025 mM Na_2HPO_4, 0.42 mM $NaHCO_3$) and placed in Petri dishes with a 2 mm layer of 1.5% agarose. The eggs were conveniently transferred with large-mouth Pasteur pipettes. Most of the solution was removed to leave the zygotes barely covered; this procedure prevents drying and enhances the adhesion of the zygotes to the plate during the microinjection. Collection of goldfish (*Carrasius auratus*) sperm and eggs from males and females was done by standard stripping procedures; sperm and eggs were kept at 4° C until use. One hundred eggs were mixed with sperm and activated by well water on a Petri dish. Water and excess sperm were removed after 1 min and the eggs that adhered to the plastic were washed several times with well water at room temperature and placed in Holtfreter's solution (3.5 g NaCl, 0.05 g KCl, 0.1 g $CaCl_2$, 0.2 g $NaHCO_3$, in 1 L) (Holtfreter, 1931) for microinjection.

Microinjection of the zygotes

Microinjection was performed at room temperature with a mechanical micromanipulator, using an air-pressure-controlled PLI-100 Pico-Injector apparatus (Medical Systems Co., Greenvale, NY, USA) and microcapillaries with 1-5μm tip diameters. Appoximately 4–20 nl (50–250 pg) of DNA, in solution stained with a drop of food dye (Schilling), was microinjected into each egg. After microinjection (about 10 eggs per min) the eggs were washed in Hank's solution and placed in 250-ml glass beakers (50–100 eggs per beaker) and kept at 28° C in a water bath. Five hours after microinjection, the embryos were treated for 15 min with 0.1% formaldehyde in Hank's solution to prevent fungal infection.

Culture of zebrafish and goldfish zygotes

Following fertilization and microinjection, the zebrafish embryos were washed several times a day until hatching. After hatching, the young zebrafish were fed with live infusoria (*Little Fry Formula*, Jungle Labs Co., Cibolo, TX, USA) for 2 weeks and then transferred to regular aquaria at 28° C for further growth. After 10 h in the Holtfreter's solution following formaldehyde treatment, the goldfish embryos were placed in 0.5× Holtfreter's solution for 12 h and then washed and kept in well water

until hatching (about 4–5 days at 20°–22° C). Fry were reared in net cages submerged in an aquarium at 20° C.

CAT assays

CAT assays were done on 10–20 embryos, which were suspended in 1 ml of 0.25 M Tris (pH 7.5) by homogenization with a Polytron Homogenizer for 30 s at room temperature followed by three cycles of freezing and thawing. The homogenate was centrifuged at 15000 × g for 10 min at 4° C to pellet cellular debris. The supernatant was used for assay of CAT activity at 37° C for 1 h with [^{14}C] chloramphenicol (Amersham, Arlington Heights, IL, USA) and acetyl-coenzyme-A (Sigma, St. Louis, MO, USA) (Gorman *et al.*, 1982). In order to get a quantitative measure of transgene activity in early development, we had to take into consideration the non-linearity of CAT conversion percentages. The original data of percentage conversion of [^{14}C]-labelled chloramphenicol to acetylated chloramphenicol was corrected using a standard curve. The standard curve was obtained by adding purified CAT enzyme (Sigma, St. Louis, MO, USA), at concentrations ranging from 1 to 75 units, to our standard assay and measuring the resultant chloramphenicol acetylation. The CAT activity values presented in Figs 2, 3 and 4, and in Table 1 indicate corrected CAT enzyme activity per zygote. Repeated assays of CAT activity in triplicate samples, taken from single pools of embryonic lysates, varied by 5 to 10% (Z. Liu and B. Moav, unpublished).

DNA analysis

DNA was extracted and analysed by dot-blotting, using uniformly [^{32}P]-labelled CAT gene as a probe, according to Sambrook *et al.* (1989). Twenty embryos were taken at 12 h and 15 embryos were pooled at 2, 4, 6, 8 and 10 days after microinjection, and pooled for DNA extraction and analysis.

Results

Regulatory sequences for transcriptional units in vertebrates generally reside in three locations: 1) the proximal promoter within the first 100 nucleotide pairs preceding the transcriptional initiation site; 2) distal sequences, sometimes extending several kilobases, upstream of the proximal promoter; and 3) regulatory regions behind the promoter, often in introns (Mitchell and Tjian, 1989). Accordingly, we analysed the transcriptional regulatory regions of the carp β-actin gene by linking these sequences to the bacterial CAT reporter gene (Gorman *et al.*, 1982; Liu *et al.*, 1990b) and examining resultant CAT activities in zygotes that developed from fish eggs microinjected with the various constructs. The constructs used for this investigation are shown in Fig. 1; all included the first non-coding exon of the β-actin gene. These four constructs were selected from the 28 constructs that have been examined in tissue-cultured mammalian and piscine cells (Liu *et al.*, 1990b, 1991; Moav, 1992a,b). Construct 1 has only the proximal promoter in 204–bp of 5′ flanking sequence. Construct 2 has, in addition to the sequences in construct 1, the remainder of exon-1 plus the complete first intron and the leading portion of exon-2 that contains the 3′-splice site. Constructs 3 and 4 lack the conserved CArG box in their proximal promoters but are otherwise identical to constructs 1 and 2. These constructs were microinjected into either zebrafish or goldfish within 1 h after fertilization and gene expression was examined over the following 7–10 days. The larger goldfish produce many more eggs per female and zygotes hatch about 4 to 5 days after fertilization at 20° C–22° C, compared to zebrafish which hatch at about 3 days post-fertilization at 28° C. Supercoiled constructs were injected into the zygotes to reduce complications due to differential rates of integration, sites of integration, and other modifications of the transgenic DNA that could occur with linearized constructs (Moav *et al.*, 1992a; Liu *et al*, 1991).

Transgene expression in zebrafish and goldfish

The necessity of an intact β-actin proximal promoter was shown in several cell lines (Liu *et al.*, 1990b,c; Moav, 1992a,b). For analysis of transgene expression in zebrafish, batches of several hundred zygotes were microinjected with each construct. From these, 15–20 embryos or hatchlings were randomly selected for each assay of CAT gene activity. The data in Fig. 2 demonstrated that an intact 204-bp proximal promoter consisting of a TATA box, a CArG box, and a CAAT box (Fig. 1) was sufficient to drive expression of the CAT reporter gene in zygotes of both fish species but the level of expression in goldfish zygotes was more than 100-fold higher per embryo than in zebrafish. In both species the onset of CAT activity was around day 4.

We previously found that the first intron of the carp β-actin gene enhanced downstream gene expression five-fold in mouse cells (Liu *et al.*, 1990b), presumably owing to interactions between factors binding to sequences in the proximal promoter and in the first intron (Kawamoto *et al.*, 1988; Ng *et al.*, 1989; Liu *et al.*, 1991). The effects of the intron on CAT gene expression were tested in microinjected zebrafish and goldfish eggs. The addition of intro-1 to the promoter resulted in a 5-fold increase in CAT activity in zebrafish (Fig. 3A) but only a modest increase in the goldfish zygotes (Fig. 3B). In these experiments, as well as those that follow, we did not examine mRNA levels directly; we have previously shown that CAT expression from these β-actin/CAT constructs was proportional to steady-state message levels and that neither splicing nor stability was noticeably affected by the juxtapositioning of the different elements in our constructs (Liu *et al.*, 1990b). As shown in Figs. 2B and 3B,

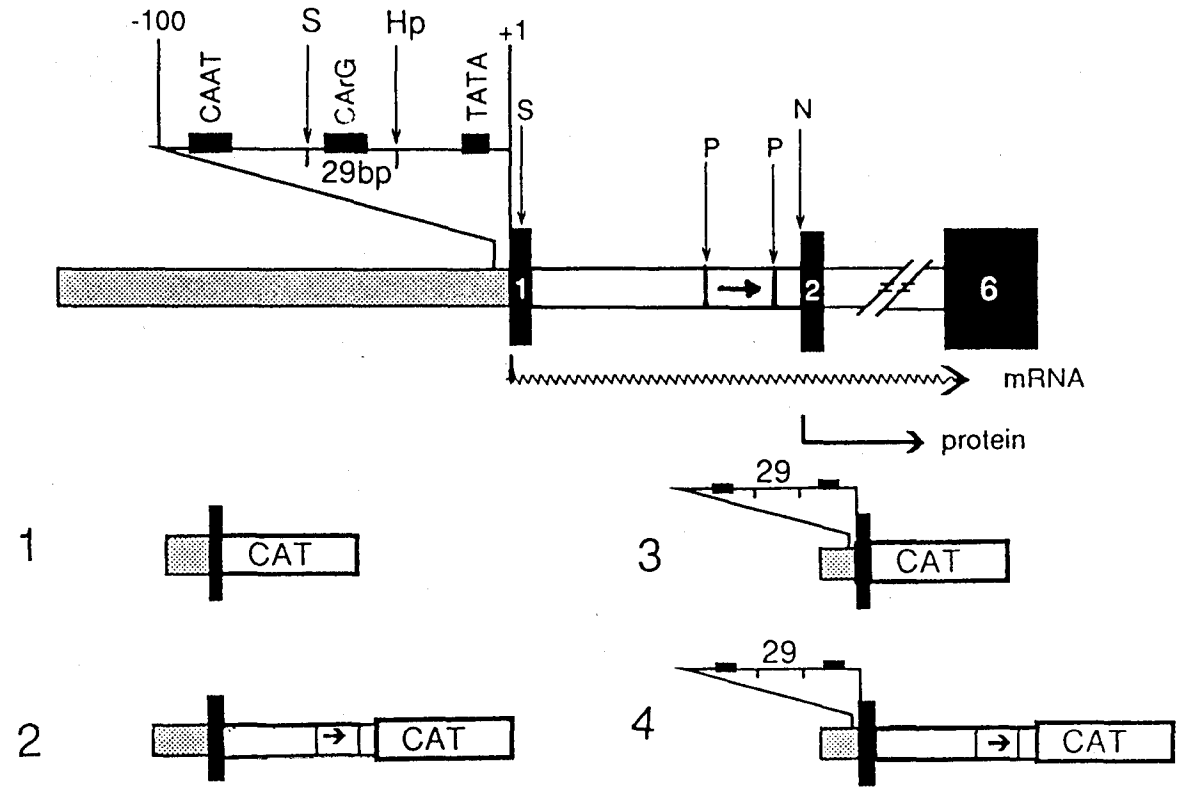

Fig. 1. Schematic diagram of constructs used in this study. Top: schematic of the carp β-actin gene with 5′-flanking sequences (shaded region), expanded 100-bp sequence in the proximal promoter with the CAAT, CArG and TATA boxes shown, exons 1, 2, and 6 (blackened regions), and introns (unshaded) showing regions of intron-1 that contain conserved motifs (arrows designate orientation). The transcriptional (+1) and translational initiation sites are indicated. S = *Sst* I; Hp = *Hpa* II; P = *Pst* I; N = *Nco* I; restriction endonuclease sites. Bottom: the constructs are shown with 5′-flanking sequences which are not to scale; constructs 1–4 have the 204-bp proximal promoter. In constructs 3 and 4, a 29-bp sequence containing the CArG box in the proximal promoter was replaced by a 29-bp linker. The region in the intron flanked by *Pst* I sites contains a copy of the CArG motif. Behind the CAT gene were either the SV40 3′ sequences from pSV40 or the salmon growth hormone gene 3′-end (not shown in the diagrams, see Liu *et al.*, 1990c).

constructs 1′ and 2′, substitution of the Atlantic salmon growth hormone polyadenylation signal for the comparable sequences from SV40 did not appreciably affect expression. The activities of these constructs are shown since they contain only piscine transcriptional regulatory sequences, which is important when these constructs are used as expression vectors (Liu *et al.*, 1990c). CAT gene expression over days 3–8 post-fertilization was calculated and the total activity integrated for each microinjection experiment to facilitate comparison of the different transgenic constructs. Table 1 shows the CAT activity values for the data in Figs 2 to 4 as well as results obtained previously from tissue-cultured mouse cells for comparison.

A single CArG box is sufficient for high-level gene expression in goldfish

The conserved CArG motif was required for expression of actin genes in tissue culture (Chow and Schwartz 1990; Liu *et al.*, 1991). However, requirements for conserved motifs for expression may differ in tissue culture and in transgenic animals (Pinkert *et al.*, 1987; Tronche *et al.*, 1989; Swift *et al.*, 1989). We tested the requirement for the CArG box in the proximal promoter during zebrafish development, using constructs 3 and 4 which lack the CArG motif but maintain the normal spacing between the CAAT and TATA boxes. In constructs with just the proximal promoter, precise replacement of the CArG element in a 29 bp region lowered CAT activity 2-fold in zebrafish and 100-fold in goldfish relative to construct 1 (Fig 4A,B). When constructs which included the first intron, but lacked the CArG box in the promoter, were injected in developing zebrafish zygotes, expression was reduced 20-fold relative to that from construct 2 (Fig. 4A). In distinct contrast, removal of the CArG motif from the proximal promoter did not impair, and possibly may have enhanced, transcription from the intron-containing construct in goldfish (Fig. 4B and Table 1). The reductions in CAT gene expression suggest a positive activation role of the CArG element in the proximal promoter

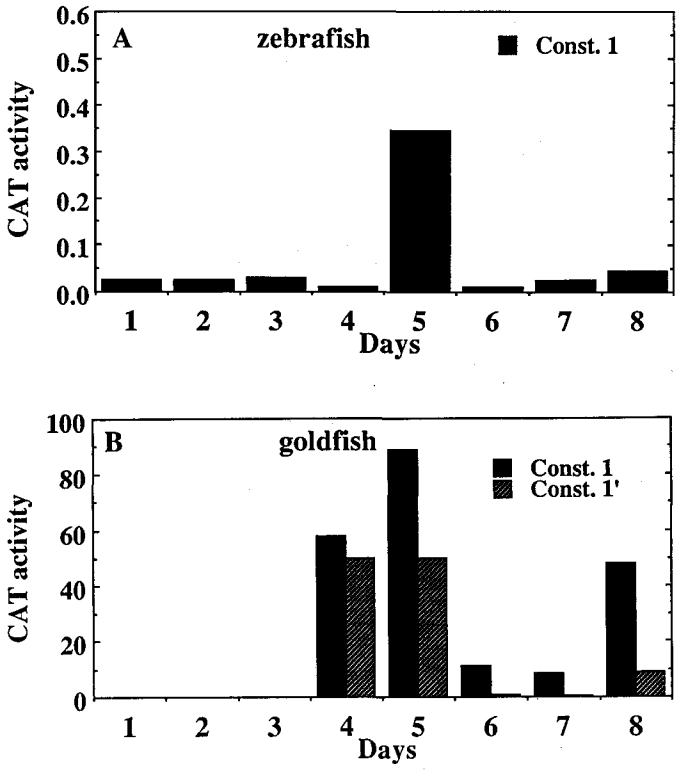

Fig. 2. CAT activity per embryo or hatchling from carp β-actin/CAT constructs in zebrafish and goldfish. Microinjected zygotes were maintained as described (Materials and methods). 10-20 embryos or hatchlings were pooled on various days post-fertilization/microinjection for analysis of CAT activity. The equivalent of one embryo extract (5-10% of the pooled extract) was analysed by thin layer chromotography on silica gels as described (Materials and methods). Construct designations (Fig. 1) are given in the upper right corner of each panel. Two constructs are shown in goldfish; the prime on construct 1′ indicates that the salmon growth hormone 3′-region replaced the SV40 3′-region. Note the difference in scale for levels of CAT activity for constructs in zebrafish and goldfish.

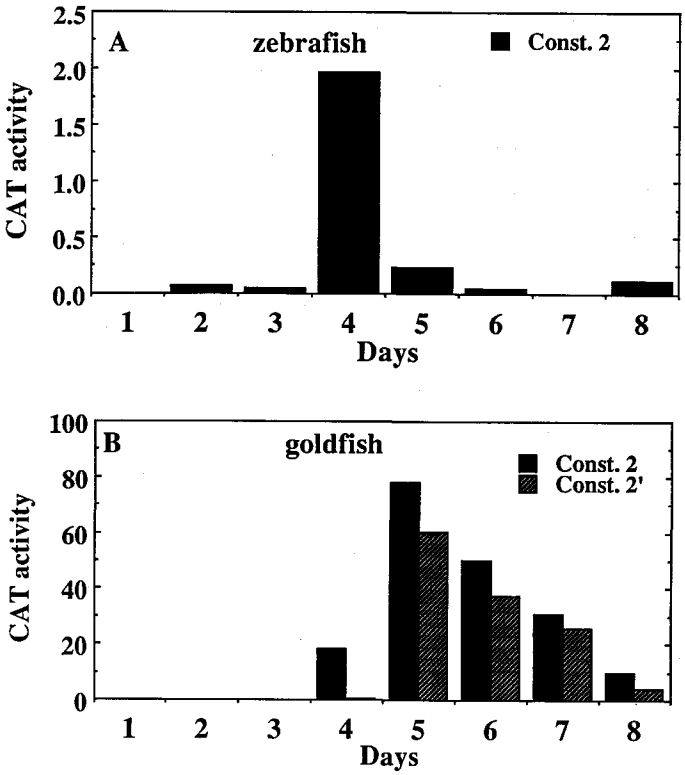

Fig. 3. CAT activity per embryo or hatchling from constructs with intron elements microinjected into embryos as described in Fig. 2. Construct designations (Fig. 1) are given in the upper right corner of each panel. Two constructs are shown in goldfish; the prime on construct 2′ indicates that the salmon growth hormone 3′-region replaced the SV40 3′-region.

during zebrafish development, whereas only a single CArG box in either the proximal promoter or the first intron is required during goldfish development.

Rates of transgene loss after microinjection

In every experiment, expression of the different β-actin promoter/CAT constructs decreased after peaking between days 4 and 7. In several experiments where expression was followed for 14 or more days, CAT activities in the embryos dropped to levels near to background although in some cases at later times the CAT activities rose a little (Moav *et al.*, 1992a and unpublished results). The decrease in CAT activity may have been due to a reduction in transcription of the transgenes, disappearance of the constructs, or both. Accordingly, we examined the persistence of the transgenes over the first ten days of zebrafish development by microinjecting approximately 5×10^5 copies of different constructs into embryos. Over the four days following microinjection, the zygotes lost about 50–70% of these constructs. A further 50–90% reduction in the number of constructs/embryo occurred during the next four days (Fig. 5 and Table 2). The persistence of five different constructs was tested in this way; all showed approximately the equivalent stability (L. Caldovic, unpublished). These results suggest that a major contributor to the decrease in CAT gene expression in the developing zygotes was degradation of template, rather than repression of transcription.

Discussion

Our results indicate that expression vectors previously tested in tissue-cultured mouse cells behaved differently during development in the zebrafish and goldfish model systems (Table 1). The major differences in expression of the β-actin/CAT constructs in zebrafish and goldfish were: (1) overall expression was about 100-fold higher in embryos and hatchlings of goldfish than zebrafish; (2) the

Table 1. Expression of transgenic constructs in fish

Construct	*Zebrafish*[a]	*Goldfish*	*Mouse cells*[b]
1	0.4 (100)	215[c] (100)	100
2	2.3 (600)	187[c] (87)	500
3	0.2 (50)	0.5 (0.3)	7[d]
4	0.1 (25)	158 (73)	80[d]

[a]Values in parentheses are normalized to construct 1.
[b]Data taken from Liu *et al.*, 1990c except where noted.
[c]Constructs have the 3'-end of the salmon growth hormone gene replacing the SV40 3'-poly(A) region.
[d]Data taken from Liu *et al.*, 1991 and normalized to Liu *et al.*, 1990c.

first intron had an enhancing effect on gene expression in zebrafish and in some tissue-cultured cell lines but not in goldfish; and (3) the CArG element in the proximal promoter was required for maximal expression of the CAT gene in constructs with just the proximal promoter in both zebrafish and goldfish but, in constructs also containing the first intron, the CArG element was

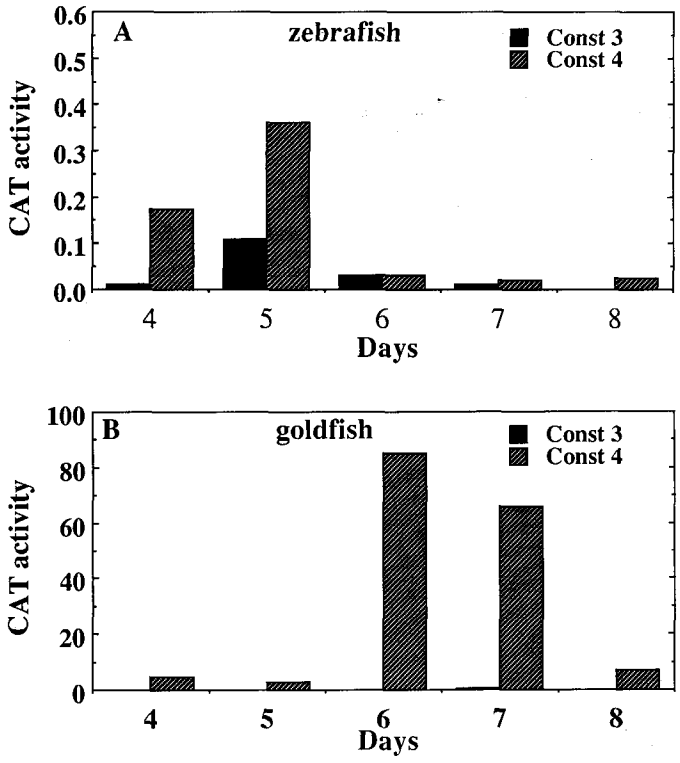

Fig. 4. CAT activity per embryo or hatchling from constructs lacking the CArG box region in the proximal promoter microinjected into zygotes as described in Fig. 2. Construct designations (Fig. 1) are given in the upper right corner of each panel. Expression from constructs 3 and 4 are presented in each panel.

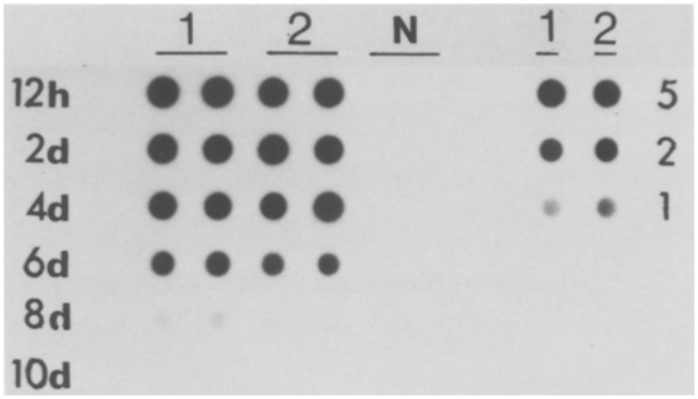

Fig. 5. Persistence of transgenic constructs in zebrafish zygotes. Approximately 5×10^5 copies of either constructs 1, 1a, and 2 were microinjected into fertilized eggs as described in Fig. 2. At 12 h 20 embryos and at 2, 4, 6, 8 and 10 days post-fertilization 15 hatchlings were taken and their DNA was extracted, pooled and split into two fractions for duplicate analysis by dot-blotting, using [^{32}P]-labelled CAT gene as a probe. In the left portion of the autoradiogram, duplicate samples of DNA from zygotes injected with either constructs 1 or 2 are shown; N indicates DNA samples taken from uninjected zygotes. In the right third of the figure, dilution series of constructs were included for a blotting standard; the amounts of DNA applied were 1, 2 and 5×10^6 molecules of each construct.

dispensable for high CAT activity in goldfish but not in zebrafish. This suggests that in the zebrafish there may be interactions between transcriptional elements in the proximal promoter and the first intron (Liu *et al.*, 1991), but not in some cultured rainbow trout cells (Moav *et al.*, 1992b) or goldfish, where a single CArG element sufficed for maximal transcription.

The consistent levels of expression in several cases when different constructs were used and the uniformity of the duration of gene expression indicate the relatively high reproducibility of the experiments. By pooling 10–20 embryos or hatchlings for each measurement, we have determined the average activity of the test plasmids following 10–20 separate microinjection into 10–20 individual zygotes. We have not investigated the variation in CAT gene expression between individual microinjected eggs for each of these constructs; however, expression of constructs 1 and 2 has been relatively constant in zebrafish, when normalized per injected zygote, over the course of 10 months when the pool size varied from 4–20 embryos (B. Moav, unpublished).

The single most important finding was the distinct differences in two species of warm water fish in the roles played by the putative transcriptional control elements during early development. We had noted previously variations in transcriptional responses to the intron element in different tissue-cultured cell lines; in mouse L cells and epithelial carp cells (EPC) the intron enhanced

Table 2. Persistence of constructs after microinjection into embryos

Days of development	*Construct (Molecules per embryo × 10^{-5})*	
	1	*2*
0.5	7.5	3.0
2.0	4.6	6.4
4.0	2.2	3.3
6.0	1.0	1.3
8.0	0.2	0.3
10.0	bmd	bmd

bmd: below the minimal level of detection; less than 0.05×10^5 molecules/embryo.
Calculation of the average number of molecules per embryo was done by scanning densitometry of the black dots on the x-ray film in Fig. 5, using for calibration known number of molecules of the corresponding constructs, and dividing by the number of embryo equivalents of DNA per dot.

expression 3- to 5-fold, whereas in rainbow trout hepatoma (RTH149) and gonad cells (RTG2) expression was reduced 80% (Moav *et al.*, 1992b). These effects were assumed to be due to differences in available *trans*-acting factors that bind to sequences in the proximal promoter and intron elements (Liu *et al.*, 1991; Moav *et al.*, 1992b). The results presented here suggest that, contrary to our initial expectations, the relative levels of gene expression in equivalent tissues may differ during development in goldfish and zebrafish. The results shown here and those obtained from tissue-cultured cell lines from different species (Moav *et al.*, 1992b) demonstrate that identical transgenic constructs may behave differently in closely related species. There are precedents for common genes to require species-specific transcriptional factors; for example, the rRNA promotors of mammals have minor variations that demand species-specific transcriptional factors (Bell *et al.*, 1990).

The major question that emerges from these experiments concerns the temporal regulation of the β-actin control elements. The expression from each construct should be due to a combination of the number and geometry of *cis*-acting transcriptional control regions and the *trans*-acting transcriptional factors available in the different cells at different times in the developing fish. The differences in duration of activity between the goldfish and zebrafish may be the result of differences in availability of *trans*-acting transcriptional factors and/or differences in the pace of development in the two species. In northern pike and walleye, which develop more slowly at lower temperatures, transgene activity is maintained for more than three weeks (Moav *et al.*, 1992a). Zebrafish were grown at 28° C and hatched in 3–4 days, goldfish were grown at 20-22° C and hatched in 4–5 days, and northern pike and walleye were grown at 11° C and hatched in 9–14 days, respectively. Thus, perhaps not surprisingly, the transgenic DNA appears to have periods of expression that may be related to stage of development in different species of fish.

Our constructs did not contain all of the known control elements for the β-actin gene (Ng *et al.*, 1989; Seiler-Tuyns *et al.*, 1984; Petropoulos *et al.*, 1989). Sequences more than 2.2 kb upstream, which were previously shown to have minor effects on transcription (Liu *et al.*, 1990b), were not examined and none of our constructs contained the conserved motif at the end of the β-actin gene (DePonti-Zilli *et al.*, 1988; Liu *et al.*, 1990a) which is apparently responsible for suppressing β-actin gene transcription during myogenesis. Omission of this negative element should not have reduced CAT gene transcription in our studies.

Our results demonstrate the value of using fish as a model system for investigation of early gene expression. Hundreds of microinjected zygotes, obtained easily and inexpensively, were used for this investigation. This study is the initial investigation into the use of transcriptional control regions from the carp β-actin gene for studying gene expression in developing fish. The next step is the linkage of another reporter gene such as *lac*Z (e.g., Westerfield *et al.*, 1992) to the various vectors to determine tissue specificity and sites of gene expression *in situ* during early growth and development.

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