*Molecular Marine Biology and Biotechnology* (1992) 1(4/5), 326–337

# Analysis of heterologous and homologous promoters and enhancers *in vitro* and *in vivo* by gene transfer into Japanese Medaka (*Oryzias latipes*) and *Xiphophorus*

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## Abstract

Efficient expression systems are required for analysis of gene regulation and function in teleost fish. To develop such systems, a number of inducible or constitutive promoter and enhancer sequences of fish or higher vertebrate origin were tested for activity in a variety of fish cell lines and in embryos of the Japanese medaka fish (*Oryzias latipes*) and *Xiphophorus*. The activity of the different promoter-enhancer combinations were quantitated. Considerable differences were found for some constructs if tested *in vitro* or *in vivo*. From the data obtained, a set of expression vectors for basic research as well as for aquaculture purposes were established.

## Introduction

Regulation of gene expression in fish has gained increasing interest (Powers, 1989; Chen and Powers, 1990). On the one hand, teleost fish are useful model systems for studies (e.g., on vertebrate development or cancer biology). On the other hand, modern-day aquaculture centers on genetic modification of fish as a major resource for protein. A steadily growing number of various important fish genes have been cloned and even more may be expected in the near future. *In vitro* and *in vivo* model systems are required for studies on regulation of expression of a gene of interest and the functional significance of its encoded protein. Besides initial characterization in tissue culture, the most significant data for application to transgenic animals may be obtained after gene transfer into a suitable laboratory fish. Such a fish should meet the following requirements: easy breeding, daily production of large amounts of eggs and embryos for microinjection, resistance of eggs and embryos against micromanipulation and exogeneously applied DNA, easy conditions for rearing injected embryos to adulthood, short generation time, and availability of genetic markers.

In addition to the well-known zebrafish, the Japanese medaka has been successfully used for gene transfer studies in several laboratories (Ozato et al., 1986; Chong and Vielkind, 1989; Inoue et al., 1989; Tamiya et al., 1990; Winkler et al., 1991). However, in all cases where stable transgenic lines have been attempted in the medaka, as well as in zebrafish, salmon, trout, or others, the ultimate goal of stable integration, as well as transmission and correct expression of the transgene have not been reached. The most problematic part seems to be correct expression of the foreign gene integrated into the host genome; however, integration and transmission have been readily established in several laboratories (Stuart et al., 1988; Guyomard et al., 1989).

In transgenic fish, like in higher vertebrates, the suitability of the promoters used determines whether a transgene is expressed, rather than the artificial situation of combining donor DNA sequences with the host genome. Using the Japanese medaka, we have established a system to rapidly screen various heterologous and homologous enhancers and promoters for their activity in fish *in vivo* (Winkler et al., 1991) and to assay for gene function (unpublished data). We report on a comparative analysis of 9 different promoters that are of potential use for expression of a foreign gene in transgenic fish for aquaculture as well as for basic research purposes. In addition to the more recent experimental systems mentioned, which use eggs or very early embryos for

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DNA injection, it has been reported that injection of native DNA directly into tissues of late embryos of the livebearing fish *Xiphophorus* also leads to expression of a foreign gene (Schwab et al., 1976; Vielkind et al., 1982). Because those findings are reminiscent of reports in higher vertebrates (Wolff et al., 1990; Acsadi et al., 1991), where DNA was injected into adult muscle tissues and found to be expressed, we used those promoter-enhancer constructs found to be most potent in the medaka to evaluate the *Xiphophorus* system in more detail.

## Results

*Analysis of promoters in different fish cells* in vitro

X47 is a metallothioneine (MT) promoter-like sequence isolated from a platyfish (*Xiphophorus maculatus*) genomic library using the human MTIIA 5′ flanking region as a probe. It is characterized by the presence of one metal responsive element (MRE) consensus sequence, a CAAT box, and a SP1 binding site (Friedenreich and Schartl, 1990). To see if this promoter is also metal-inducible like authentic MT promoters, transient expression was studied in various cell lines following fusion to the CAT gene. The promoter activity was previously demonstrated in the A2 cell line (Friedenreich and Schartl, 1990). In this cell line, X47 was constitutively active, significantly more active than the thymidine kinase (Tk) promoter, and only slightly lower than the herpes simplex virus Tk promoter in combination with two copies of the SV40 virus enhancer.

Authentic MT promoters analyzed so far contain from 2 MREs (the rainbow trout MTa and MTb promoters) (Zafarullah et al., 1988; Hong Y. unpublished observations) to multiple MREs (mammalian MTs) (Stuart et al., 1985). To investigate whether metal induction of X47 can be obtained in other cell types or whether the single MRE of X47 in general cannot render metal responsiveness, two cell lines of liver origin were used, because expression of MT genes occurs mainly in the liver. In human hepatoblastoma cells (HepG2; Knowles et al., 1984), X47 showed an extremely low basal expression barely above that of pBL-CAT3, a promoterless CAT-containing plasmid. Treatments with $Zn^{++}$, however, clearly enhanced CAT conversion by approximately two-fold. This expression level was approximately 50% of the Tk promoter activity in the same cell line, suggesting a weak metal inducibility of the X47 promoter. When a fish hepatoma cell line, namely the rainbow trout hepatoma (RTH-149; Fryer et al., 1980) cell line) was used, no CAT activity above background was detected, even after exposure of the cells to zinc and cadmium (Table 1). This finding is surprising because in this cell line, the human MTI I A and the rainbow trout MTb promoter direct the highest CAT expression (Hong et al., 1992), as well as another fish MT promoter, the rainbow trout MTa promoter (Hong Y. unpublished observations).

**Table 1.** CAT assays for the *Xiphophorus* MT-like promoter sequence (X47) in three cell lines.

| | A2 | HepG2 | RTH |
|---|---|---|---|
| pBL-CAT3 | 0.5[a] | 0.13 | 0.37 |
| pBL-CAT2 | 1 | 1 | 1 |
| ptk-CAT2E | 96.31 | 100.08 | 31.89 |
| pX47-CAT | 45.55 | 0.19 | 0.21 |
| Plus zinc | 43.71 (0.85) | 0.44 (2.32) | 0.32 (1.52) |
| Plus cadmium | 44.42 (0.9) | 0.11 | 0.26 (1.24) |
| ptMTb-CAT | 0.71 | 0.1 | 0.45 |
| Plus zinc | 4.48 (6.16) | 0.33 (3.3) | 12.47 (27.68) |
| Plus cadmium | 11.23 (15.82) | 0.11 (1.1) | 2.29 (5.01) |

[a] Conversion; number in brackets: fold induction. The conversions were normalized to that of pBL-CAT2[2], whose actual conversion was defined as 1. Promoterless plasmid pBL-CAT3 was used as a negative control, whose conversion was seen as background. Induction value was calculated by dividing the metal-induced conversion rate by the basal value (no metal treatment). The data represent the mean of three independent parallels.

*Comparative analysis of different homologous and heterologous promoters in Medaka embryos* in vivo

To evaluate a series of expression vectors for production of transgenic fish, various constructs containing different enhancer/promoter combinations were injected into the cytoplasm of two-cell stage medaka embryos. In this and previous studies (Winkler et al., 1991), we observed that the level of expression of a reporter gene mediated by one and the same promoter varies unavoidable from one set of injection experiments to another. This variation is probably due to inconsistency of injected DNA volume, egg quality, among others. For an exact quantitation of promoter strength *in vivo*, it was therefore necessary to inject the different constructs in a short time span in one series of injections performed by only one person to guarantee constant conditions for every vector injected. A well-characterized vector (pCMVTkCAT), containing the Tk promoter in combination with the cytomegalovirus (CMV) enhancer, was used as a standard in every set of injections to allow comparison of different experiments to each other.

In previous studies, we found that the expression of the injected constructs is highest 48 hours after injection, independent of the promoter used. To compare the different promoters at their level of highest activity, we analyzed the CAT expression at this time point in extracts from single embryos. After injection of pBLCAT3, which contains only the CAT gene but no additional eukaryotic promoter or enhancer, we found CAT expression values significantly above background, as determined in noninjected embryos or embryos injected with buffer alone (Figure 1). This observation has also been made in different cell lines *in vitro* and illustrates "promoter-like" sequences existing in the vector. No activity was found in embryos injected with buffer and phenol red alone, excluding the existence of endogeneous CAT-like enzymes. The Tk promoter (pBLCAT2) revealed only a slightly higher CAT activity. This activity was only detected in some embryos. The activity of this promoter was even lower or at best equivalent to pBLCAT3. Injection of constructs containing the fish promoter X47 or the human metallothioneine promoter IIA (phMTCAT) led to high values of CAT activity, comparable to pCMVTkCAT (see Figure 1A). We found large variation in the CAT expression values, ranging from 1.8% with pX47CAT in one embryo to 95.5% CAT conversion in another. The same level of variation (up to approximately 100-fold) was also found in embryos injected with phMTCAT, whereas with the standard pCMVTkCAT, the variation between single embryos was less (only approximately eight-fold). In this set of injections, the highest values obtained with pX47CAT and phMTCAT were even higher than those with pCMVTkCAT.

When the CMV enhancer with its own promoter was compared to the CMVTk combination, it showed weaker activity (see Figure 1B). This observation is in contrast to results obtained with mammalian cells *in vitro* (data not shown). The highest activity in medaka embryos found was obtained for the SV40 enhancer in combination with the Tk promoter (see Figure 1C). Under the conditions used (protein extract diluted 1:10), nearly complete CAT conversion (>75%) in 80% of injected embryos was obtained. Because CAT conversion values above 70% are not linear with respect to enzyme activity, it is not possible to quantitate pSVTkCAT exactly in comparison with standard pCMVTkCAT. It can be estimated from our data, however, that the promoter strength of SVTk is at least ten-fold higher than that of CMVTk.

Further, we tested whether there is a position effect of the SV40 enhancer on the activation of the Tk promoter (see Figure 1C). In our studies, no dramatic difference in activation was found, regardless of whether the enhancer was put in front or behind the Tk promoter/CAT gene unit. Because of the very high conversion rates, no detailed quantitative comparison was possible. Removing the Tk promoter from the construct pTKCATE (pCATE) resulted in a drastic reduction of expression to levels barely above background.

The trout metallothioneine B promoter (tMTb) was also tested for its activity in medaka embryos. The analysis of embryos at different time points after injection (Figure 2) revealed high CAT activity only in a single embryo (2 days after injection) out of 6 tested; treatment with $Zn^{++}$ resulted in no significant induction of the promoter (data not shown).

*Effect of DNA conformation on the expression of foreign DNA*

To compare the level and the temporal pattern of expression after injection of supercoiled and linearized DNA molecules, the construct pSVTkCAT was used (Figure 3). To guarantee that constant amounts of DNA were injected, the number of linearized versus supercoiled pSVTkCAT molecules was increased by the factor 2.4, because prokaryotic plasmid sequences had been removed as well.

The expression pattern with supercoiled pSVTkCAT plasmid was found to be very similar to the pattern seen with other constructs described earlier

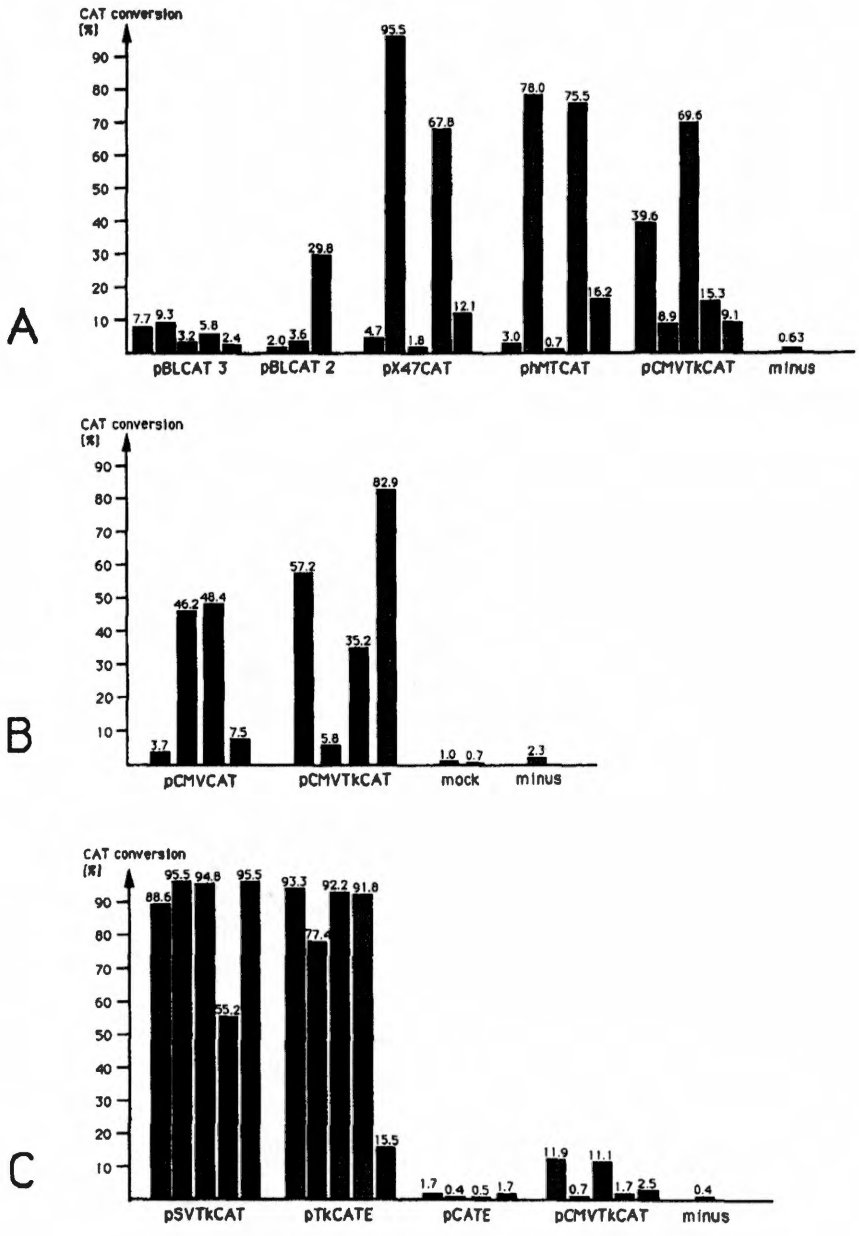


**Figure 1.** CAT expression in 2-day-old medaka embryos injected with various promoter-enhancer constructs in supercoiled conformation at the two-cell stage in 3 independent injection series (A, B, C). Each bar represents the activity of CAT enzyme (in terms of substrate conversion) as assayed in 10% of protein extracts from single embryos. Mock-activity in a 2-day-old embryo injected with buffer and phenol red alone; minus-activity in a 2-day-old noninjected embryo.

(Winkler et al., 1991). Six hours after injection, when the embryo is in the early blastula stage, no activity of CAT enzyme was found, followed by high activity with up to 95% CAT conversion after 24 and 48 hours. Thereafter, a decrease in activity to approximately 16% of conversion is observed 8 days after injection.

A few differences in expression pattern were found with linearized pSVTkCAT as compared with the pattern described. As with supercoiled plasmids, no activity was seen 6 hours after injection. Maximum expression started at 24 hours. However, 48 hours after injection, expression decreased to approximately 20% and stayed at this level until 8 days after injection.

No significant change in the level of expression was found after 24 hours in supercoiled compared with linearized plasmid. At 48 hours, however, CAT activity was clearly lower with linearized plasmids, whereas 8 days after injection it appeared to be higher, as in embryos injected with supercoiled pSVTkCAT. The variations obtained in one-stage embryos injected with linearized plasmids (e.g., at 48 hr: 2.8-fold) appeared not as high as with circular DNA (14.6-fold). A larger number of embryos need to be analyzed to confirm these initial observations.

*Onset of reporter gene expression*

To exactly determine the onset of expression of reporter genes, pCMVTkCAT-injected embryos were

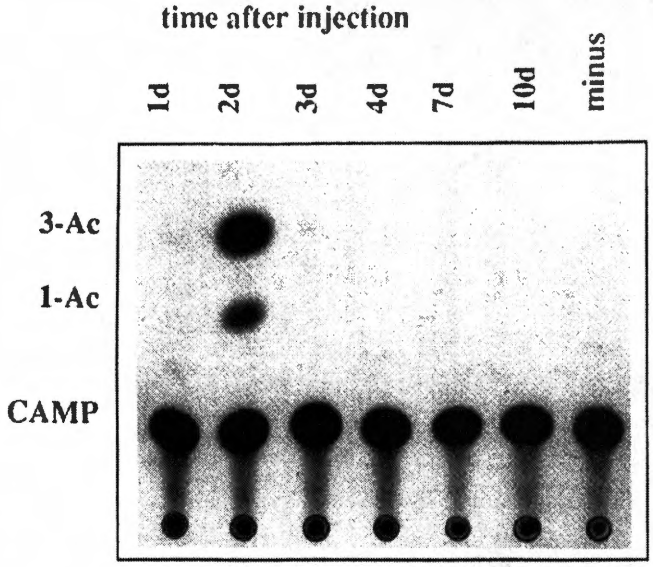


**Figure 2.** CAT expression in medaka embryos at different time points of embryonic development injected with a CAT reporter gene construct containing the trout metallothioneine-B promoter (ptMTbCAT). Total protein extracts from single embryos were used for each time point. Minus-activity in a 2-day-old noninjected embryo.

analyzed for CAT enzyme activity from 5 to 48 hours after injection (Figure 4). The first CAT activity was found around stage 13 (14 hours after injection), when the embryo is in the dorsal lip gastrula stage, approximately 3 to 4 hours after midblastula stage. To see whether onset of expression is influenced by the amount of injected DNA, the concentration of the injected DNA was increased by the factor of 2, whereas the volume of injected solution was kept constant. The first CAT activity was again found around stage 13. However, the level of expression was increased by 1.4-fold. No negative effect on the survival rate of injected embryos was observed as long as the injection volume was kept constant.

pSVTkCAT, which has been shown to yield approximately 10 times higher activity than pCMVTkCAT, was injected to see whether a strong enhancer/promoter can mediate expression earlier than the midblastula stage (stage 10). At this stage, CAT activity in pSVTkCAT-injected embryos was found to be only barely above background levels, whereas at stage 16 (20 hr), CAT conversion was at a maximum (approximately 98%) in every embryo tested.

*Fate of DNA injected into the neural tube of* Xiphophorus

The plasmid pCMVTkCAT was injected in linear and in supercoiled conformation into the neural crest region of early organogenesis embryos, either complexed with lipofectin or in fish Ringer's solution (Figure 5). The survival rates of the injected embryos did not differ from the uninjected control group (data not shown). Numbers of survivors injected with lipofectin-complexed DNA showed no striking difference as compared with the control group injected with noncomplexed DNA. Also, conformation (linearized versus supercoiled) of the DNA had no influence on the survival rates of the embryos (data not shown). The embryos tolerated high amounts (2 ng) of injected DNA (approximately 100 times the amount injected into the medaka blastomeres) without any decrease in viability.

To follow the fate of the injected constructs, total DNA was prepared from injected embryos at different time points after injection. The DNA was analyzed by Southern hybridization, which showed that the amount of injected linear DNA decreased rapidly during embryonic development. The amount of plasmid DNA injected in supercoiled conformation also decreased, but exhibited a prolonged stability in comparison to the linear DNA. No conformational change, as observed for DNA injected into medaka two-cell stage embryos (Winkler et al., 1991), was noticed.

*Expression analysis after injection of CAT reporter genes into* Xiphophorus embryos

To address the question of whether the injected DNA was expressed before becoming degraded, the construct pCMVTkCAT was injected. Two days after injection, CAT activity was found exclusively in those embryos injected with lipofectin-complexed DNA, and CAT activity was observed up to 10 days after injection (Figure 6). CAT activity, however, was very low, suggesting that the DNA was expressed only in a small number of cells. To directly correlate the CAT activity with the amount of expression construct present in the embryo, protein and DNA were isolated from the same embryos in parallel. CAT assay and Southern blot analysis with a homologous CMVTk probe were performed (Figure 7). There is no obvious correlation between the CAT activity and the amount of plasmid DNA present in the embryos, demonstrating that the majority of the foreign DNA is not expressed.

## Discussion

Gene transfer has become a powerful tool for studies of gene function and regulation. To ensure the effective expression of transferred genes, it is necessary that proper regulatory sequences be established

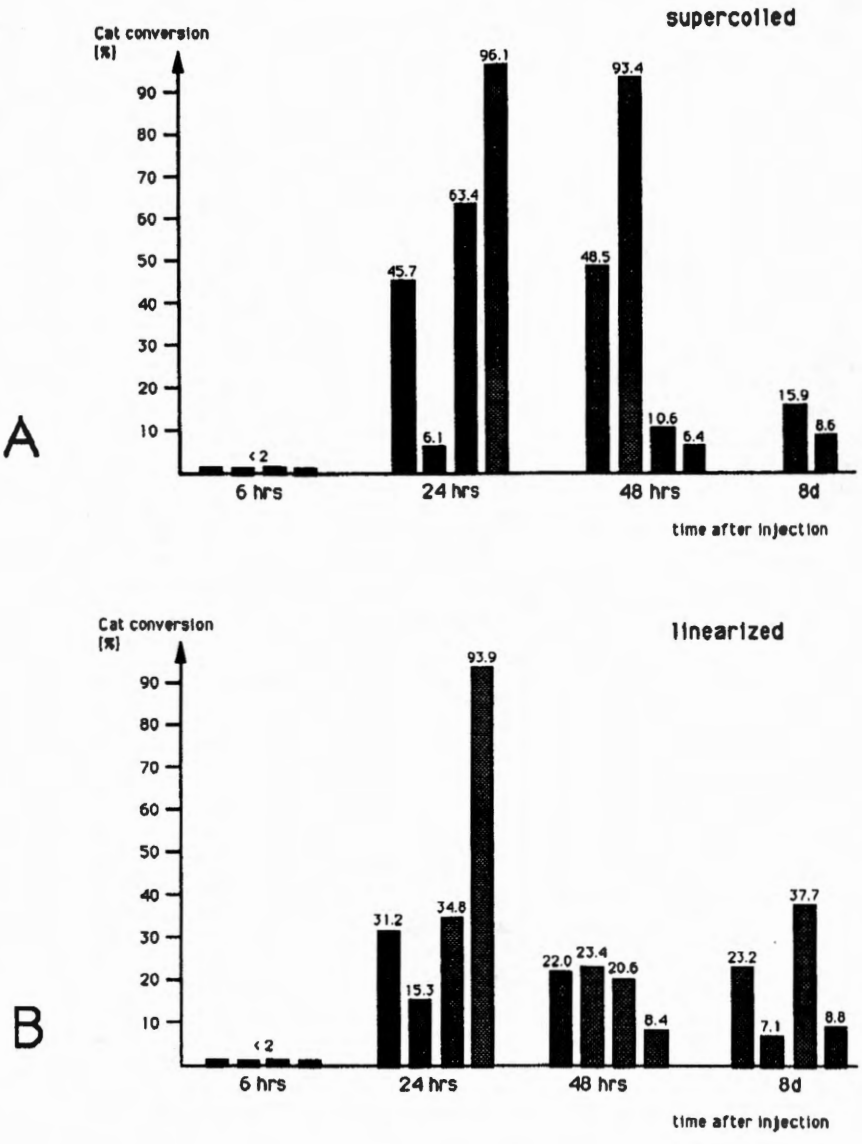


**Figure 3.** CAT expression pattern during embryonic development of medaka embryos injected with (A) "supercoiled" and (B) linearized pSVTkCAT construct at the two-cell stage. Each bar represents the activity as measured in 10% of protein extracts from single embryos. Incubation of extracts with substrates in the CAT assay procedure was only 1 hour compared with 2.5 hours in Figures 1 and 4, which explains the relatively low levels of expression at 24 hours after injection as compared with the levels in Figures 1 and 4.

and analyzed. Fish are especially ideal for production of transgenics due to the large number of eggs produced and external development, but the lack of fish promoters has been a limiting factor and has led to use of heterologous promoters. In most transgenic fish experiments, the novel genes were not effectively expressed (Chen and Powers, 1990). This finding may be largely due to the heterologous origins of promoters used, which are perhaps not as effective in fish as in a homologous host. Indications for use come from previous experiments in tissue culture (Friedenreich and Schartl, 1990) (e.g., from inactivity of the mouse MT promoter in fish cells). The rainbow trout MTa and MTb promoters, in contrast, have a much higher activity in fish cells than in heterologous cells (Hong et al., 1992; Hong Y., unpublished observations).

Our results demonstrate a weak metal inducibility for the X47 sequence. Its metal responsiveness, however, was only approximately threefold, which is approximately 10 times lower than those of the authentic fish or human MT promoters (Friedenreich and Schartl., 1990; Hong et al., 1992). This finding may be explained by the single MRE of X47 and, consequently, by the lack of the synergistic interaction between two or more MREs, as has been shown by others (Stuart et al., 1985, Zafarullah et al., 1988).

Despite the consensus sequence of MRE, MT promoters of different origins differ considerably with respect to their expression patterns (Hong et al., 1992). They share a general characteristic, however, namely metal inducibility. Sequences that function both as inducible and constitutive promoters depending on the cell type where the promoter is active appear to be very rare in eukaryotes. In this context, the X47 sequence is peculiar, because it acts as a strong constitutive, as a weak metal-inducible pro-

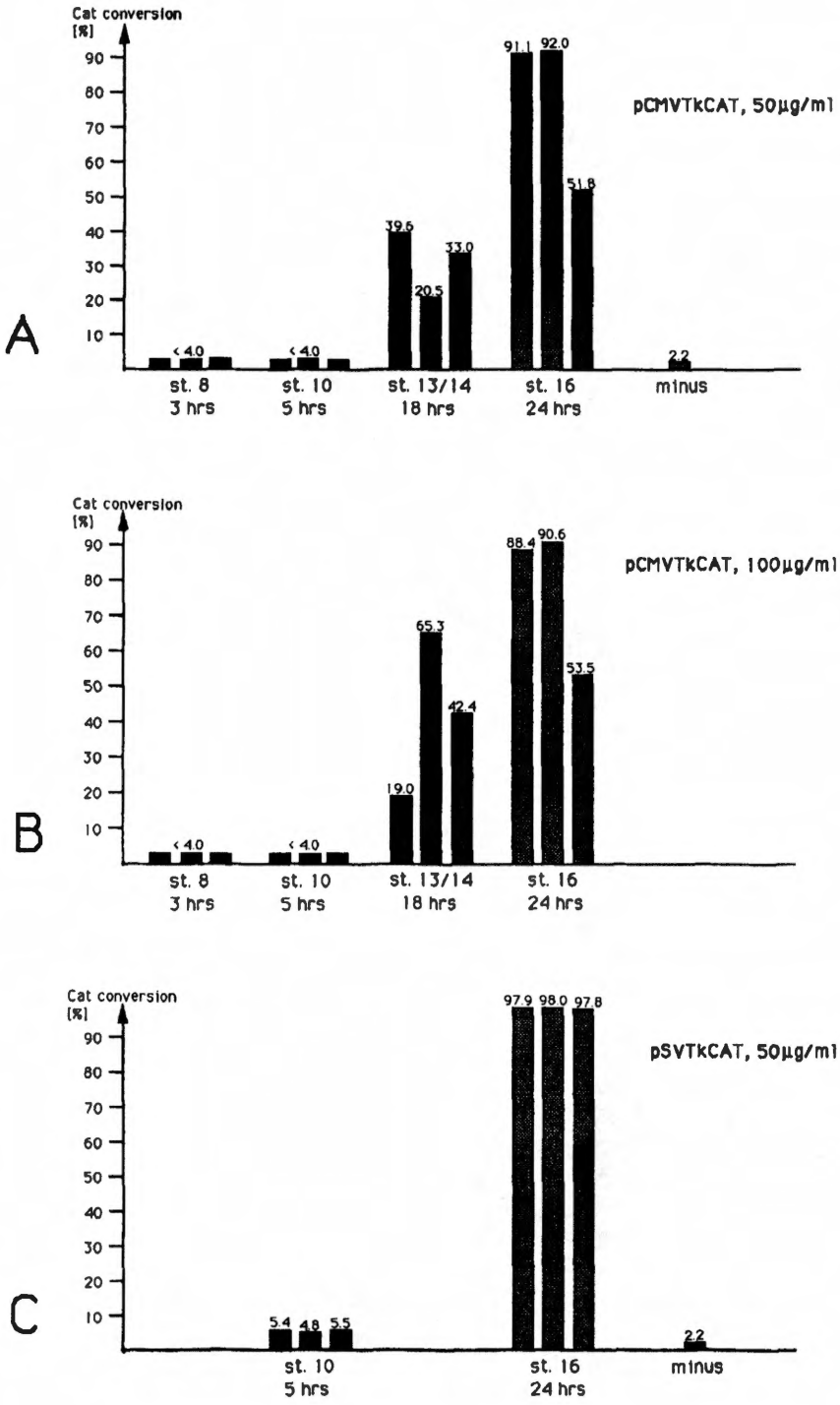


**Figure 4.** CAT expression pattern during early development of medaka embryos injected with "supercoiled" pCMVTkCAT construct at a concentration of (A) 50 µg/mL or (B) 100 µg/mL, or with pSVTkCAT at 50 µg/mL (C). Each bar represents the activity obtained with 50% of protein extracts from single embryos. Minus-activity in 24-hour-old noninjected embryo.

moter, or is completely inactive, depending on the cell lines used. Furthermore, X47 behaves differently compared with the two trout MT promoters with respect to cell type–specific expression patterns. This difference illustrates the conclusion that not only MREs but also other thus far uncharacterized sequences may have an important role in metal regulation of the expression of MT and MT-like promoters. Further analysis of X47 using deletion, mutagenesis, and DNA-protein interaction studies will provide information about the regulation of expression of X47 as well as other inducible (e.g., MT) promoters.

From the data presented herein, it is obvious that early medaka embryos provide a useful and efficient system for comparative screening of promoter and enhancer sequences to be used in gene transfer experiments. In stable transgenic animals, expression of the transgene often depends on the site of integration with regard to the state of activity of

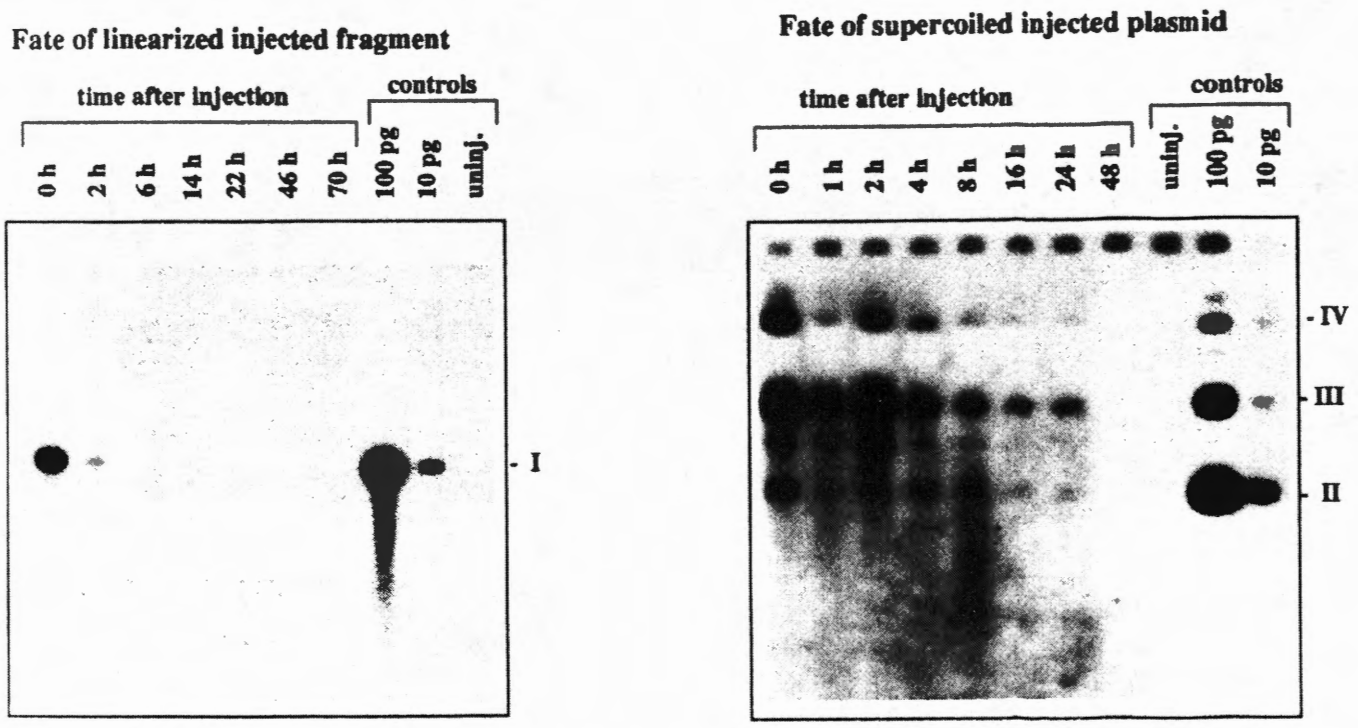


**Figure 5.** Southern blot analysis of DNA extracted from *Xiphophorus* embryos at different stages after injection of linearized or "supercoiled" pCMVTkCAT constructs into the neural crest region. DNA from single embryos at different time points after injection, as well as DNA from noninjected embryos and 100 and 10 pg undigested linear fragment or "supercoiled" plasmid mixed with DNA from untreated embryos, were electrophoresed and hybridized after blotting with a $^{32}$P-labeled 0.85-kb fragment containing the CMVTk enhancer-promoter region. (I) Plasmid as linearized fragment; (II) plasmid in supercoiled conformation (RF I); (III) nicked open circular (RF II); and (IV) concatenates.

chromosomal domains, state of methylation, among others. In a transient system where the injected DNA persists extrachromosomally, expression is dependent solely on the promoter or enhancer used. In such transient systems, however, the injected DNA is distributed unequally to the daughter cells during mitotic division, resulting in mosaics (Stuart et al., 1988; Chong and Vielkind, 1989). Different levels of expression may be obtained depending on the contribution of the different tissues in which the DNA persists. This major drawback can be compensated for by analyzing larger numbers of embryos. In our studies, in which we analyzed at least 5 embryos per construct and per time point, the SV40 enhancer in combination with the Tk promoter proved to be the most powerful regulatory sequence. This finding is in contrast to studies in tissue culture; in two of three cell lines tested, the CMVTk construct gave higher expression than SVTk by an order of magnitude, and in a third cell line was of equal activity. Although it is difficult to compare the activity of a given promoter quantitatively in tissue culture and in embryos, in general CAT conversion values obtained were approximately 100-fold higher in extracts from a single early embryo versus a 10-cm dish of cells. The mosaic distribution can also give some hints for qualitative promoter studies. Given a tissue-specific promoter, one would expect a higher variability in expression levels between parallel samples due to the uneven distribution of the DNA to the tissue, which gives the correct expression for constitutive promoters. This clearly was the case for the human metallothioneine promoter, the X47, and the trout metallothioneine promoter.

In *Xiphophorus* embryos, measurable CAT gene expression was only obtained using the highly effective lipofection transfection procedure in combination with a highly potent expression construct. In addition, in contrast to the medaka system, only a minor fraction of the foreign DNA was expressed. It is tempting to speculate that this DNA may have ended up in muscle tissue, which at this time is already differentiated. Such explanation would be in agreement with the findings in mammals (Wolff et al., 1990; Acsadi et al., 1991). The *Xiphophorus* system seemed to be a very ineffective system for gene transfer. In light of this notion, it is difficult to explain the results where injection of native linear

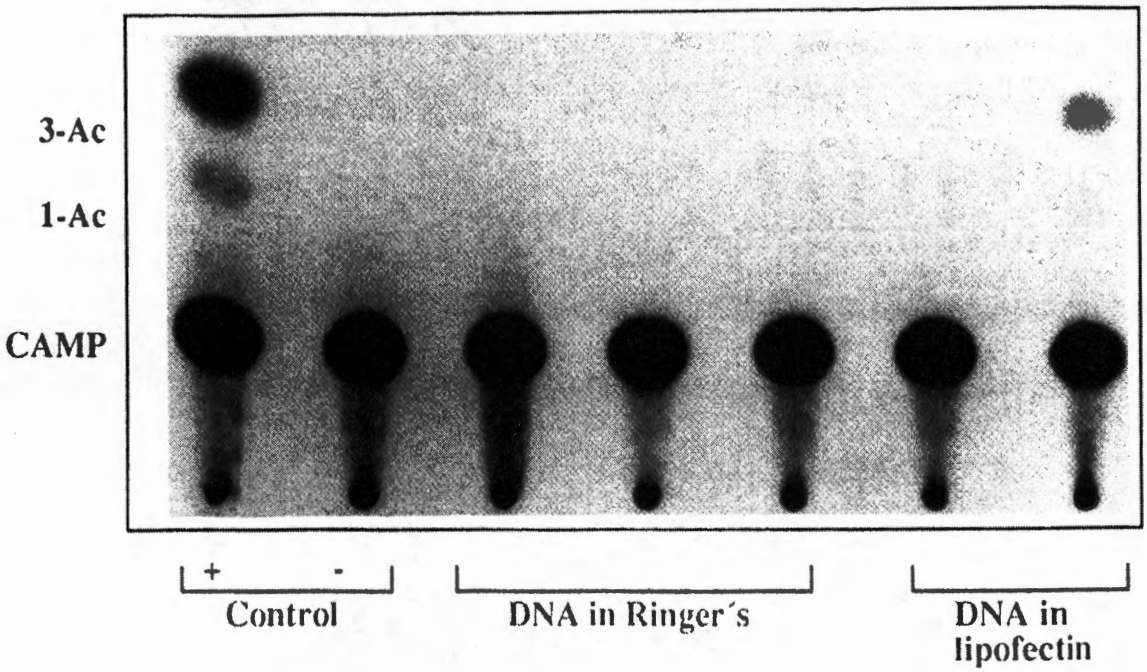


**Figure 6.** CAT expression in *Xiphophorus* embryos injected with pCMVTkCAT in Ringer's solution or complexed with lipofectin. Total protein extracts pooled from 3 embryos were assayed for CAT activity 2 days after injection. Positive control-protein extract from 2-day-old medaka embryo injected with pCMVTkCAT. Negative control-extract from noninjected *Xiphophorus* embryo.

total genomic DNA lead to 1.3% embryos and 7.5% neonate fish that expressed the phenotype encoded by a gene that was presumed to be present only in the donor DNA (Vielkind et al., 1982).

## Experimental Procedures

All methods applied have been described in detail elsewhere (Winkler et al., 1991; Hong et al., 1992). The cell lines used (A2, *Xiphophorus xiphidium* embryonal epithelial cells; HepG2, human hepatoblastoma cells; RTH, rainbow trout hepatoma cells) were maintained as described (Friedenreich and Schartl, 1990; Hong et al., 1992).

### *Transfection of fish cells*

Cells in a 10-cm dish were transfected with 3.5 pmol/L plasmid DNA using the calcium phosphate coprecipitation method. For metal induction, the cells were treated with $ZnCl_2$ (150 μmol/L) or $CdCl_2$ (20 μmol/L) for 48 hours (fish cells) or 24 hours (HepG2 cells) before harvesting.

### *Egg collection and embryo culture*

Medaka fish (*Oryzias latipes;* Teleostei: Cyprinodontidae) were purchased from Carolina Biological Supply Company (Burlington, North Carolina, USA). Adult fish were maintained under standard conditions (Kirchen and West, 1976) with an artificial photoperiod (10 hours of darkness, 14 hours of light) to induce reproductive activities. Clusters of fertilized eggs were collected 1 to 2 hours after the onset of light and kept in Yamamoto Ringer's Saline (0.75% NaCl, 0.02% KCl, 0.02% $CaCl_2$ [pH, 7.3]; Yamamoto, 1961) prior to injection. Injected medaka embryos were reared in a medium containing 0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2 \times 2\ H_2O$, 0.016% $MgSO_4 \times 7\ H_2O$, and 0.0001% methylene blue and transferred to aquarium water immediately after hatching.

To obtain exactly staged embryos of the livebearing fish *Xiphophorus helleri*, pregnant female *X. helleri* were killed by decapitation 10 days after the last spawning. Embryos (stage 9, according to Tavolga, 1949) were carefully removed, washed in PBS adjusted to the osmolarity of *Xiphophorus* embryos (Vielkind et al., 1982) (10 mmol/L NaCl, 1.95 mmol/L KCl, 5.9 mmol/L $Na_2HPO_4$, 1.1 mmol/L $KH_2PO_4$ [pH, 7.3–7.4]; with Pen/Strep, 100 U/mL). After injection, embryos were transferred to single wells of a 24-well dish, each filled with 2 mL embryo medium (Haas-Andela, 1976: 0.8× embryo PBS, 0.67 mmol/L $CaCl_2$, 0.18 mmol/L $MgCl_2$, 100 IE/mL penicillin, 0.1 mg/mL streptomycin, 0.2 chicken embryo extract, 0.25 mg/mL caseinehydrolysate, 0.25 mg/mL glucose; Embryo-PBS: 125 mmol/L

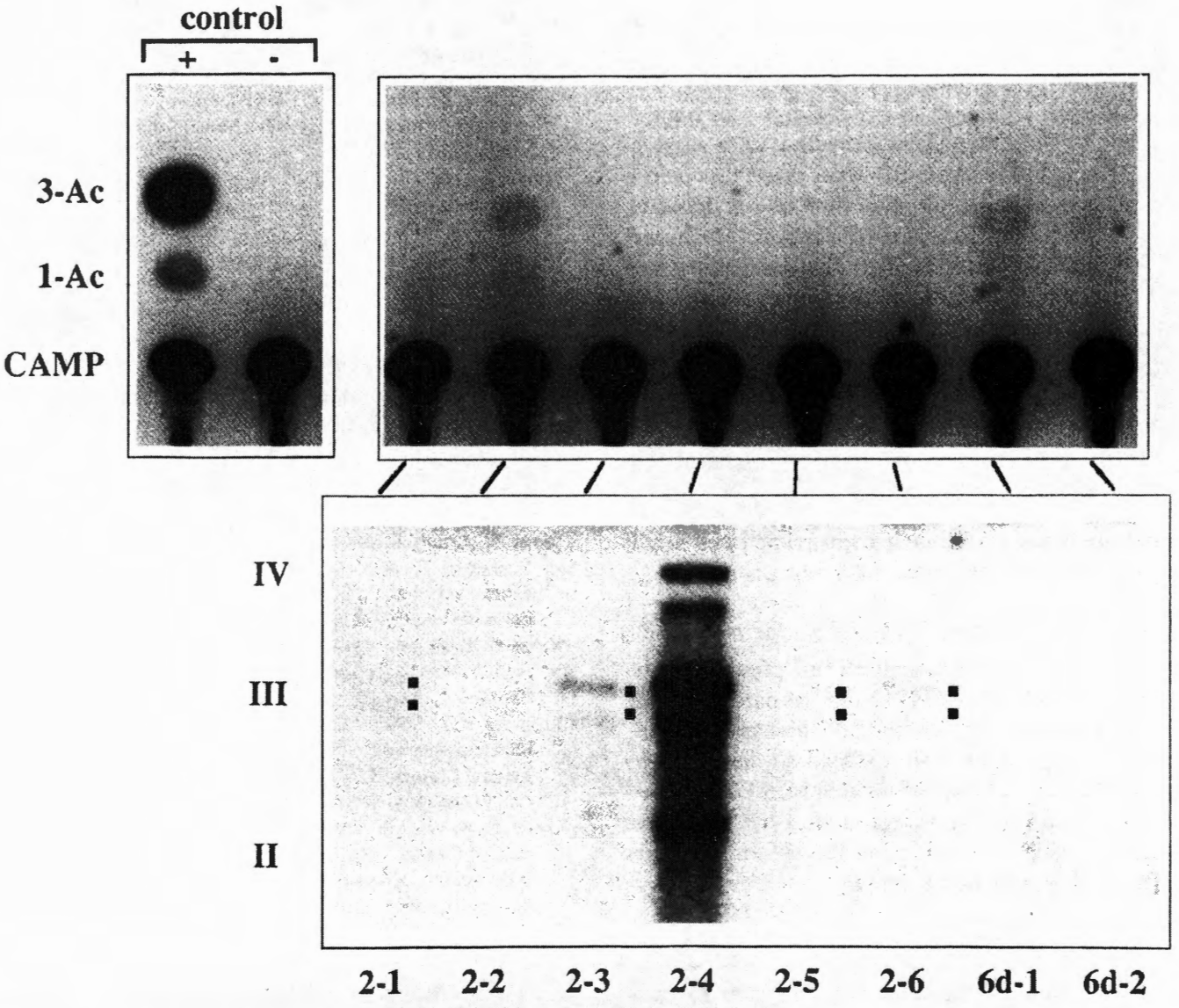


**Figure 7.** CAT activity and persistence of DNA in parallel samples of *Xiphophorus* embryos injected with pCMVTkCAT into the neural crest region. Three embryos each were pooled 2 (2-1 to 2-6) or 6 days after injection. One third of extracts were assayed for CAT activity; one corresponding third was analyzed by Southern blotting. Probe and plasmid conformations as in Figure 5, controls as in Figure 6.

NaCl, 2.4 mmol/L KCl, 9.3 mmol/L $Na_2HPO_4$, 1.3 mmol/L $KH_2PO_4$ [pH, 7.4]) with a sterile platinum loop. Embryos were kept under sterile conditions at 28°C in the dark. All buffers for embryo culture were prepared with sterile filtered aquarium water.

*Microinjection*

Medaka embryos were injected cytoplasmatically into one cell of the two-cell stage embryo as previously described (Winkler et al., 1991). *Xiphophorus* embryos were injected in a 96-well microtiterplate filled with PBS-penicillin/streptomycin with approximately 10 nL ($3 \times 10^8$ copies) DNA solution (0.1% phenol red, 1× fish-Ringer's, 40% lipofectin (Gibco-BRL), 250 ng/μL plasmid DNA) into the neural tube region of the embryos.

*Protein extraction*

Cells of 10-cm plates and embryos were homogenized in 250 mmol/L Tris (pH 7.8) and subjected to three cycles of freeze-thawing (−70°C to 25°C). The protein extract was obtained after centrifugation at 4°C, and the amount of protein was determined using Bio-Rad assay.

*CAT assay*

Diluted or undiluted protein extracts in a total volume of 100 μL were incubated for 2.5 hours (see

Figures 1, 3, 4), 1 hour (see Figure 2), or 6 hours (see Table 1; Figures 6, 7) at 37°C with 1 μL $^{14}$C-labeled chloramphenicol (Amersham; 54 mCi/mmol, 200 μCi/mL) and 20 μL an aqueous acetyl coenzyme A solution (3.4 mg/mL). After extraction with ethylacetate, the reaction products were dried down in a vacuum concentrator, redissolved in ethylacetate, and spotted on TLC plates (Polygram, Macherey-Nagel, Düren). The reaction products were separated by thin-layer chromatography in a chloroform-methanol mixture (95:5) for 50 minutes. The plates were air-dried and scanned in a TLC linear analyzer (raytest, Stratec, Steubenhardt) to determine CAT conversion values. Even with diluted protein extracts, CAT conversion values of up to 95% were obtained in some cases. Due to the known fact that values above 70% are not linear with respect to substrate conversion, such conversion rates might be indicative of even higher CAT expression.

*Preparation of genomic DNA and Southern blotting*

DNA was prepared essentially as previously described (Winkler et al., 1991). For the parallel preparation of protein extracts for CAT assays and DNA, the soluble proteins were isolated as described by freeze-thawing. The pellet obtained after centrifugation was used to prepare genomic DNA. Southern blotting was performed using the alkaline transfer method (Reed and Mann, 1985).

## Acknowledgments

We thank R. Renkawitz, Martinsried, for kindly providing the plasmids pCMVTkCAT, pCMVCAT, pSVTkCAT, pTkCATE, pCATE, pBLCAT 3, and pBLCAT 2; and H. Friedenreich, Martinsried, for phMTIIaCAT and pX47CAT. This work was supported by grants to M.S. supplied by the G.I.F. (Nr. I-18-127.3/87) and the Bundesministerium für Forschung und Technologie through Schwerpunkt "Grundlagen und Anwendungen der Gentechnologie" (Nr.26). Y.H. is a fellow of the DAAD.

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