Transient expression directed by homologous and heterologous promoter and enhancer sequences in fish cells*

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ABSTRACT

In order to construct fish specific expression vectors for studies on gene regulation in vitro and in vivo a variety of heterologous enhancers and promoters from mammals and from viruses of higher vertebrate cells were tested for expression of the bacterial chloramphenicol acetyl transferase reporter gene in three teleost fish cell lines. Several viral enhancers were found to be constitutively active at high levels. The human metallothionein promoter showed inducible expression in the presence of heavy metal ions. A fish sequence was isolated that can be used as a homologous constitutively active promoter for expression of foreign genes. Using the human growth hormone gene with an active promoter in fish cells for transient expression insufficient splicing and lack of translation were observed, pointing to limitations in the use of heterologous genes in gene transfer experiments. On the contrary, some heterologous promoters and enhancers functioned in fish cells as well as in their cell type of origin, indicating that corresponding transcription factors are sufficiently conserved between fish and human over a period of 900 million years of independent evolution.

INTRODUCTION

Studies on the regulation of gene expression in fish have gained considerable importance (1). Reasons for this are on the one hand that teleost fish lend themselves to basic science as useful model systems e.g. for studies on vertebrate development (2) and cancer biology (3, 4). On the other hand modern-day aquaculture centers around the improvement of fish as a major protein resource, present and future. Several genes which are of particular interest to the various disciplines have been isolated so far, e.g. the homeobox-containing genes of salmonids and zebrafish (5, 6), proto-oncogenes and/or oncogenes from *Xiphophorus* (7, 8, 9, 10), goldfish (10), and trout (4), metallothionein gene from rainbow trout (11), growth hormone genes e.g. of chum salmon (12), coho salmon (13) and rainbow trout (14), the alpha-globin gene (15) and β-crystallin gene (16) of carp, the anti-freeze protein of wolf-fish (17) and a much larger and steadily increasing number is currently attempted.

After the structural characterization, studies on the regulation of expression of the respective gene and on the functional significance of this expression are desired. Putative regulatory sequences are studied by linking them to a reporter gene whose transcription is readily and reliably assayed in the experimental system used. The functional significance can be studied by attaching the respective gene to formerly characterized manipulable regulatory sequences. Both types of experiments can be performed in tissue culture cells or in transgenic animals. While such systems are available in mammals and have been successfully used over the past years to study the regulation and function of numerous mammalian genes, in fish the situation is much more insufficient. Several attempts have been made to produce transgenic fish by introducing avian or mammalian genes into fish via microinjection into the oocyte nucleus (18), the egg cytoplasm (19); or into early (20, 21, 22) or late embryos (23). Although the DNA was present for a prolonged period in the recipient, the production of bona fide transgenic fish (with integration of the transgene into the germline) has been shown so far only in two cases (21, 24). However, in no case expression of an integrated transgene was observed. Considering *in vitro* systems, the situation is just as unsatisfactory. There exists a single report on transfection and stable expression of the mammalian gene for xanthine guanine phosphoribosyltransferase associated with the promoter of Simian virus 40 (SV40) in a cell line of the goldfish (25). So far only one fish sequence that would direct expression of foreign genes is known, namely the trout metallothionein promoter which has been reported to direct transient expression of the bacterial gene for chloramphenicol acetyl transferase (CAT) (26). However, a systematic study on expression and regulation of exogenous DNA in fish cells is lacking. There exists no information on the activity of known promoters and enhancers from higher vertebrates or from avian and mammalian viruses in fish cells.

The aim of this work was therefore to obtain expression systems suitable for teleost cells for studies *in-vitro* as well as for the production of transgenic fish. For this purpose, we have studied the activity of different heterologous promoters and

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enhancers on the expression of the CAT gene used as reporter gene after transfection into established cell lines of the poeciliid teleost fish *Xiphophorus* and of the common carp. In addition we report the cloning and characterization of a fish sequence that can be used as a constitutive promoter for foreign genes.

MATERIAL AND METHODS

Cell lines

A2 cells, derived from *Xiphophorus xiphidium* embryos (27) and PSM cells, derived from spontaneous hereditary melanoma of *Xiphophorus* hybrids (28) were cultured in F12 nutrient medium (Gibco, Biochrom), supplemented with 1.25 g $NaHCO_3$/l, 10% fetal calf serum (Gibco), 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cells were grown at 28°C under 5% CO_2. For characterization of the cell lines see ref 29. EPC cells, derived from carp (30) were cultured at room temperature in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 25 mM Hepes pH 7.4, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine.

Plasmids

Promoter-CAT hybrid plasmids

pBL-CAT2 (31) is a pUC 18 derivative containing the chloramphenicol acetyl transferase (CAT) gene fused to a simian virus 40 (SV40) polyadenylation signal and the thymidine kinase (tk) promoter derived from herpes simplex virus in front of the gene. 5′ and 3′ beside the tkCAT unit convenient polylinker sequences are located.

pBL-CAT3 (Luckow and Schütz, 1987) is a derivative of pBL-CAT2 in which the tk-promoter was removed.

phMTIIA-CAT was constructed by inserting the 835 bp human metallothionein IIA (hMTIIA) HindIII / NcoI promoter fragment of p84H (32) in front of the CAT gene of pBL-CAT3 into the HindIII / XhoI sites.

pLTR-CAT was generated by insertion of the 0.6 kb Rous sarcoma virus long terminal repeat (RSV LTR) NdeI/HindIII fragment into pBL-CAT-3 in front of the CAT gene.

pMMTV-CAT (33) contains the mouse mammary tumor virus LTR sequences (−631/+125) in front of the CAT gene of pBL-CAT3 (34)

pX47-CAT; pX26-CAT contain 830 bp or 790 bp, respectively, of a *Xiphophorus* sequence in front of the CAT gene. The additional sequence in pX47-CAT contains a short GC rich region which shows considerable similarity to the consensus sequence for the transcription factor SP1 (35). Plasmids were constructed by cloning the 830 bp and 790 bp Eco RI / HindIII fragments of Exo III deletion clones of pXP1 after partial HindIII digestion into the NdeI / HindIII sites of pBL-CAT3.

Enhancer-tkCAT hybrid plasmids

ptkCAT-2E (Altschmied, Schulz and Renkawitz, unpublished) contains the 72 bp repeats of the SV40 enhancer as a double insertion downstream of the CAT gene of pBL-CAT2.

phMTIIA-tkCAT (Altschmied, Schulz and Renkawitz, unpublished) contains the enhancer region of the human metallothionein IIA (hMTIIA) gene from nucleotide −39 to −366 cloned into pBL-CAT2 in front of the tkCAT unit.

pmMTI-tkCAT (Altschmied, Schulz and Renkawitz, (unpublished) contains the enhancer region of the mouse metallothionein I (mTI) gene from nucleotide −95 to −600 in reverse orientation in front of the tkCAT unit of pBL-CAT2.

pCMV-tkCAT (Altschmied, Schulz and Renkawitz, unpublished). A 700 bp Alu I fragment containing the human cytomegalovirus enhancer (CMV) (−16 / −667) was cloned into pBL-CAT2 in front of the tkCAT unit.

pIg-tkCAT (Altschmied, Schulz and Renkawitz, unpublished) contains the mouse immunoglobulin heavy chain enhancer (Ig; 992 bp XbaI fragment) in front of the tkCAT unit of pBL-CAT2.

pPy-tkCAT (Altschmied, Schulz and Renkawitz, unpublished) contains the polyoma virus enhancer (Py; 1.2 kb HindIII / BamHI fragment) in front of the tkCAT unit of pBL-CAT2.

Human growth hormone expression vector

phMTIIA-GH was constructed by cloning the 835 bp HindIII / BamHI fragment of the hMTIIA promoter from pHSI (36) into p0GH (37), which contains the 2.2 kb human growth hormone gene.

Human glucocorticoid receptor expression vector

pRShGRα contains the RSV-LTR in front of the human glucocorticoid receptor α cDNA fused to the SV 40 polyadenylation signal (38).

Transfection of fish cells

Plasmids used for DNA transfection were prepared essentially as described (37). All transfection experiments were carried out in triplicate and repeated at least twice. All cell lines were transfected by a modification of the calcium-phosphate method (39). Briefly, cells were seeded 4−7 days prior to transfection on 10 cm dishes at a density of 1×10^6 cells/plate (PSM), 2×10^6 cells/plate (A2), or 4×10^6 cells/plate (EPC). 4−7 hrs before adding the DNA to the cells the medium was changed (DMEM containing Hepes, pH 7.4). Per dish 3.5 pmol of superhelical plasmid DNA was added. After 18 hrs the medium was removed, the cells were washed and shocked for 4 min. with 3 ml of 15% glycerol in 1×HBS. Fresh medium (F12 (PSM, A2) or DMEM (EPC)) was added which in case of induction experiments was supplemented with 20μM C dCl_2, 150 μM $ZnCl_2$, 100μM Cu SO_4, 10^{-6}M dexamethasone or 10^{-6}M cortisone. 2 to 7 days later, cells were harvested and CAT activity was determined. For steroid induction of pX47-CAT and pX26-CAT the cells were grown in media supplemented with charcoal treated fetal calf serum. For coexpression with the human glucocorticoid receptor 0.6 pmol/dish of pRShGRα were cotransfected with 1.4 pmol/dish of pMMTV-CAT into A2 cells.

Assays for transient expression

CAT assays were performed according to ref 40 with the following modifications: cells were lysed by three freeze/thaw cycles. After centrifugation the protein concentration of the supernatant was measured by the BioRad assay and identical protein amounts per sample were used for the enzymatic reaction. Expression of the GH constructs was determined using a radioimmuno assay (Laboserv, Giessen, F.R.G.) or immunoradiometric assay (IRE-Medgenix, Fleurus, Belgium).

Cloning of a fish sequence that directs transcription of heterologous reporter genes

An EMBL4 genomic library of *Xiphophorus maculatus* (provided by S.M. Robertson) was screened by plaque filter hybridization with a nick-translated ^{32}P labelled human metallothionein IIA

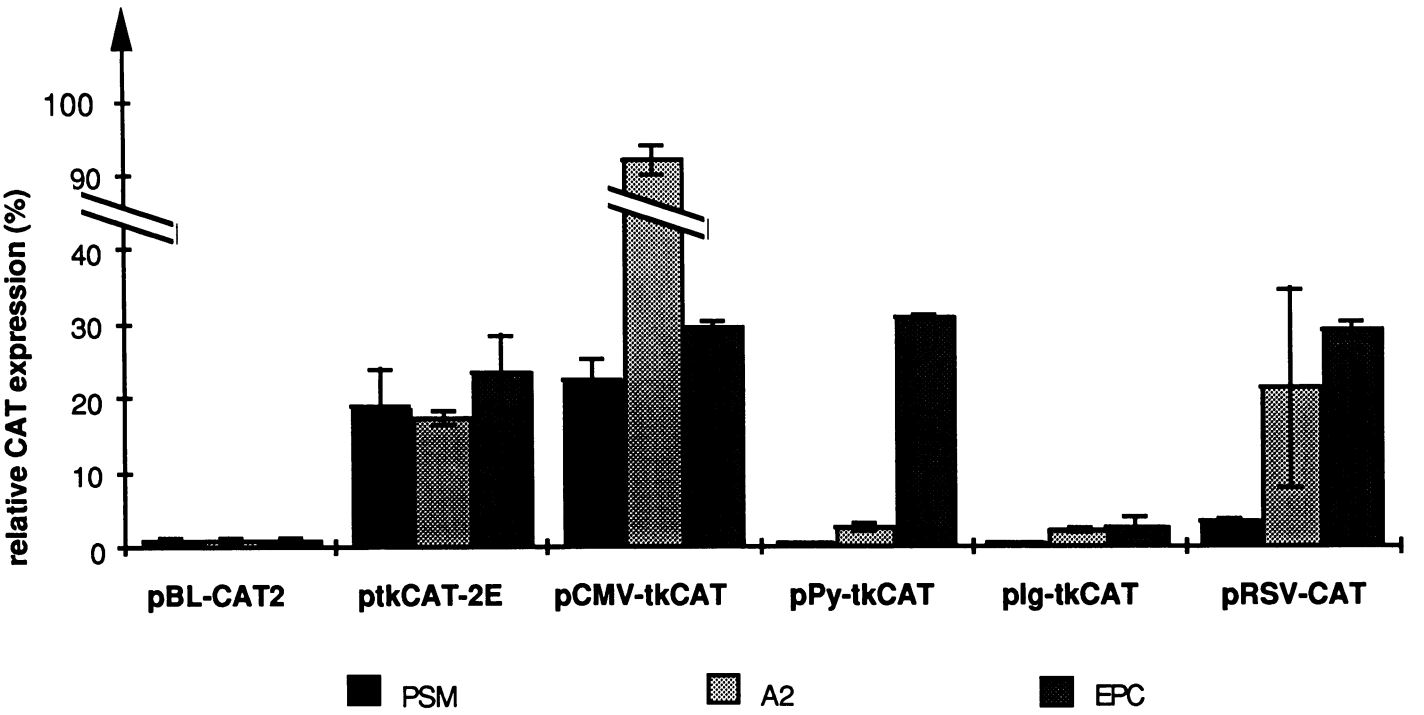

Figure 1. Expression of CAT-gene with constitutive heterologous promoter and enhancer sequences in PSM, A_2 and EPC cells

probe (3.2 kb Hind III fragment from p84H (32). This probe contains the entire coding region of the human metallothionein IIA gene as well as 835 bp of the 5′ untranslated region and the 3′ untranslated region. Hybridizations were performed in batch in a prehybridization / hybridization solution as described for Northern blot hybridizations, except that the final formamide concentration was 40%. Filters were washed at 50°C in 2×SSC, 1% SDS, and the positive clones were characterized by restriction analysis and sequencing. DNA sequence analysis was performed by the dideoxy chain termination method (41, 42) on double-stranded DNA. Nick translations (43) were performed using a kit from Amersham Buchler.

Northern Blot Analysis

Total cellular RNA was extracted by the LiCl/urea method (44) using ultraturrax N8 (Janke & Kunkel, Staufen, FRG) for homogenization. Poly(A)$^+$ RNA was prepared by oligo (dT)-cellulose selection (45). 15 μg of poly (A)$^+$ RNA were denatured with formamide / formaldehyde and electrophoresed in a 1.2% agarose gel containing 2.2 M formaldehyd (46). RNA was electroblotted to GeneScreen membranes according to the protocol of the suppliers (NEN). The filter was stained with methyleneblue (47) to estimate the actual amounts of RNA and to ensure the efficiency of transfer. Hybridization was carried out with a nick-translated (^{32}P) labelled human GH probe (2.2 kb Bam HI / EcoRI fragment from p0GH) or the 1.1 kb Hind III fragment from pXP-1. The filters were hybridized at 42°C for at least 24 hrs in 1 ml of a solution containing 50% Formamid, 10× Denhardt's (0.1g ficoll, 0.1 g polyvinylpyrrolidone 0.1g BSA per 100 ml H_2O), 50 mM Tris-Cl pH7.5, 1 M NaCl, 1% SDS, 250 μg/ml denaturated calf-thymus DNA, and 10^7 dpm ml^{-1} of nick translated probe. The filters were washed at 60°C, 0.1 ×SSC and exposed to Kodak XAR 5 film.

RESULTS

Transfection of fish cells in-vitro

To establish methods for the transfer of exogenous DNA into fish cells supercoiled plasmid DNA of ptkCAT-2E was employed. Determination of CAT activity after transfection revealed the calcium phosphate method (48) to be the most preferable. Transfection utilizing the DEAE-dextran method was rather ineffective due to the high toxicity of the chemicals to the fish cell lines tested. PSM and A2 cells were incubated with the precipitate for 4 or 18h in DMEM. Thereafter cultures were treated for 1, 3 or 5 minutes either with glycerol or DMSO or remained untreated. The cells were then further grown in the appropriate tissue culture media and analyzed for CAT expression after two days. The highest CAT conversion rates were obtained when the precipitate was present for 18h on the cells, followed by a 3–4 min shock with glycerol or DMSO. Because the glycerol shock resulted in a higher viability of the cells as compared to DMSO, only this protocol was used for any further transfection. Addition of 0.1 mM chloroquine during the precipitate incubation had a strong cytotoxic effect and was therefore omitted in further experiments.

Transient expression in fish cells of CAT gene under the control of heterologous promoter and enhancer sequences

To study the activity of heterologous promoter and enhancer sequences in fish cells, a variety of those sequences of mammalian or viral origin were analyzed for their effects on transient expression of a reporter gene (CAT). A construct (pBL-CAT2) containing the thymidine kinase (TK) promoter of herpes simplex virus was used as reference in all transfection experiments, and its relative CAT expression was always taken as 1. For control a plasmid containing only the bacterial CAT gene without any

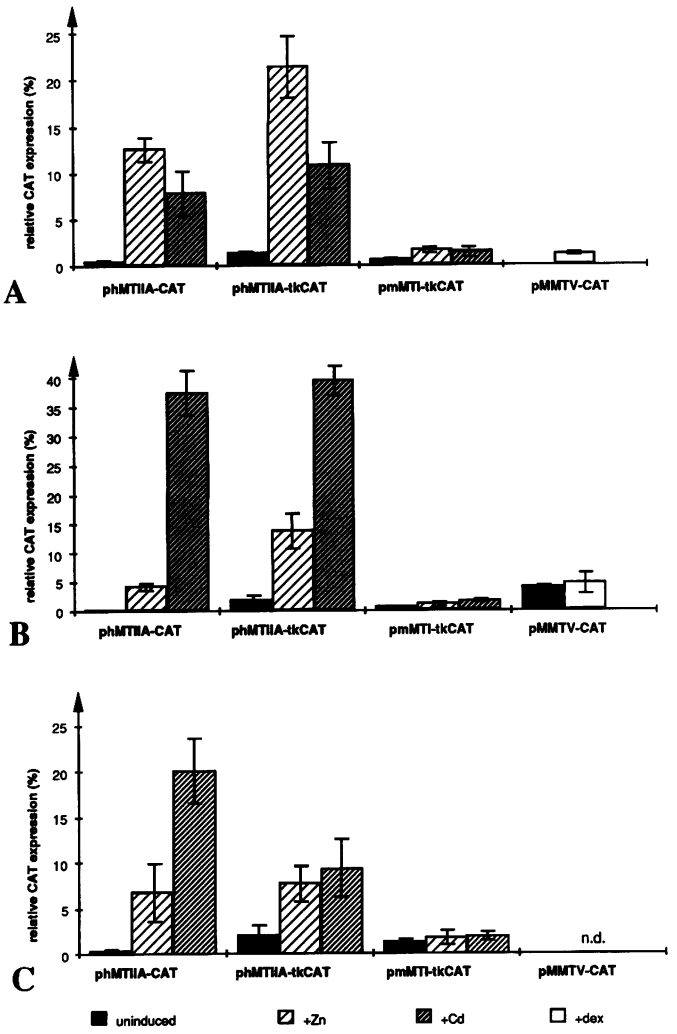

Figure 2. Expression of CAT gene with inducible heterologous promoter and enhancer sequences in a) PSM, b) A_2 and c) EPC cells

promoter sequence was tested in all cell lines used and found only to render CAT conversion barely above background. All constructs used have previously been shown to be active in homologous systems (HD 11, T47-D, MCF-7 and HeLa cells; Altschmied, Schulz and Renkawitz, unpublished and data not shown).

Using the A2 embryonal epithelial cell line the RSV promoter on its own and the SV40 enhancer as well as the cytomegalovirus (CMV) enhancer in combination with the TK promoter led to a considerable enhancement of CAT expression as compared to pBL-CAT2 (20–100 fold)(Fig. 1). A similar expression pattern was found in the two other cell lines; the relative CAT conversion rates, however, were generally higher in the EPC cells (with the exception of the CMV promoter) and lower in PSM (exception SV40 enhancer). The polyoma virus enhancer (Py) was found to be active only in the carp EPC cells (Fig. 1). No enhancement was found with the mouse immunoglobulin (Ig) enhancer in any cell line. Constructs containing the metal-inducible human metallothionein IIA (hMTIIA) enhancer in reverse orientation in front of the TK promoter (phMTIIA-tkCAT) or containing the entire hMTIIA promoter on its own (phMTIIA-CAT) were

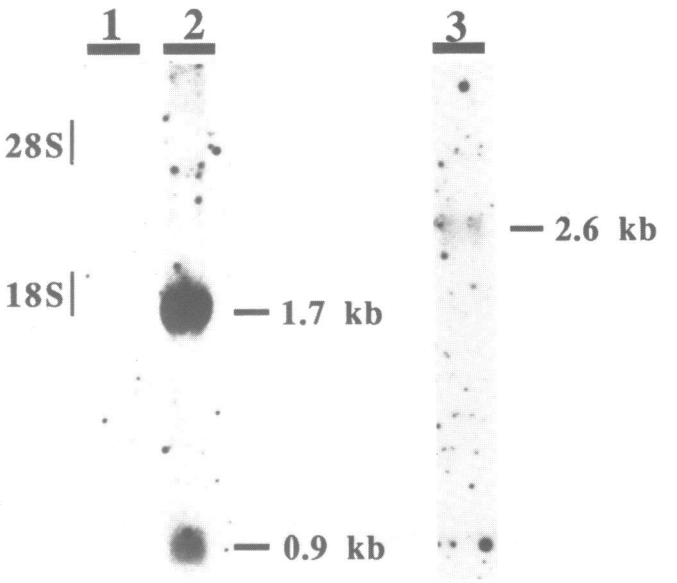

Figure 3. Northern blot analysis of polyA$^+$ RNA from A_2 cells. (1) Control. Probe: 2.2 kb Bam HI/Eco RI fragment from phMTIIA-GH. (2) Cells transfected with the phMTIIA-GH construct induced with 20 mM cadmium, probe as for (1). (3) Detection of the XP-1 expression. Probe: 1.1 kb Hind III fragment from pXP-1 (see Fig. 5a).

found to be active in fish cells, however, with a differential transient expression pattern depending on the cell line, the construct and the heavy metal used for induction. (Fig. 2). While in the carp epithelial cell line (EPC) and in *Xiphophorus* embryonal epithelial cells (A2), cadmium was more efficient for induction than zinc, in the *Xiphophorus* melanoma cells (PSM) zinc revealed higher expression. Unlike with mammalian cells, where maximum levels are reached within 6 to 24 hrs of induction, the relative CAT expression increased at least as long as 7 days after transfection as assayed for phMTIIA-CAT (data not shown). Due to the low level of basal expression of phMTIIA-CAT the induction factor for this construct was always higher than for phMTIIA-tkCAT (up to 100–150 fold as compared to 20–50 fold) although the relative CAT expression levels were comparable. Surprisingly the mouse metallothionein I enhancer of pmMTI-tkCATwas not inducible with zinc or cadmium in all three cell lines. Although all metallothionein constructs contain a glucocorticoid responsive element (GRE), we found no enhancement of expression using dexamethasone or cortisone for induction. In line with this result is the failure to induce expression with these steroids of the mouse mammary tumor virus promoter MMTV-CAT construct, although a low level of constitutive expression was observed (Fig. 2). However, after cotransfection with the human glucocorticoid expression vector marked induction of the MMTV-promoter was observed.

Transfection of the human growth hormone gene (GH) into fish cells

In our attempts to express the human growth hormone gene (GH) a plasmid was constructed containing the human MTIIa promoter in front of the GH gene. This construct was tested for expression in mouse L-cells. Upon induction with heavy metal ions secretion

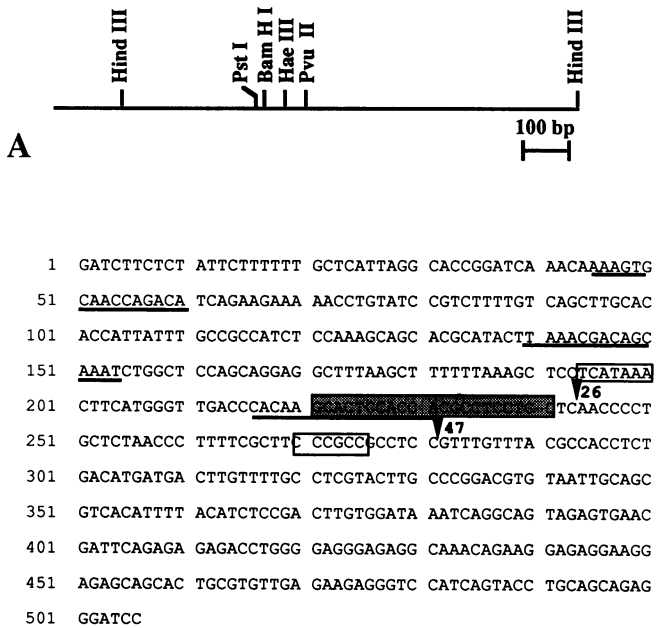

Figure 4. a) Restriction map of pXP-1. b) pXP-1 nucleotide sequence upstream of the Bam HI site. Sequence motifs with similarity to MREs are underlined, TATA- and SP.1-like sequences are boxed, the 21 bp indentity to the human MTIIA gene is shadowed. Arrowheads with 26 and 47 indicate the extent of the sequence used to construct the expression plasmid pX26-CAT or pX47-CAT, respectively.

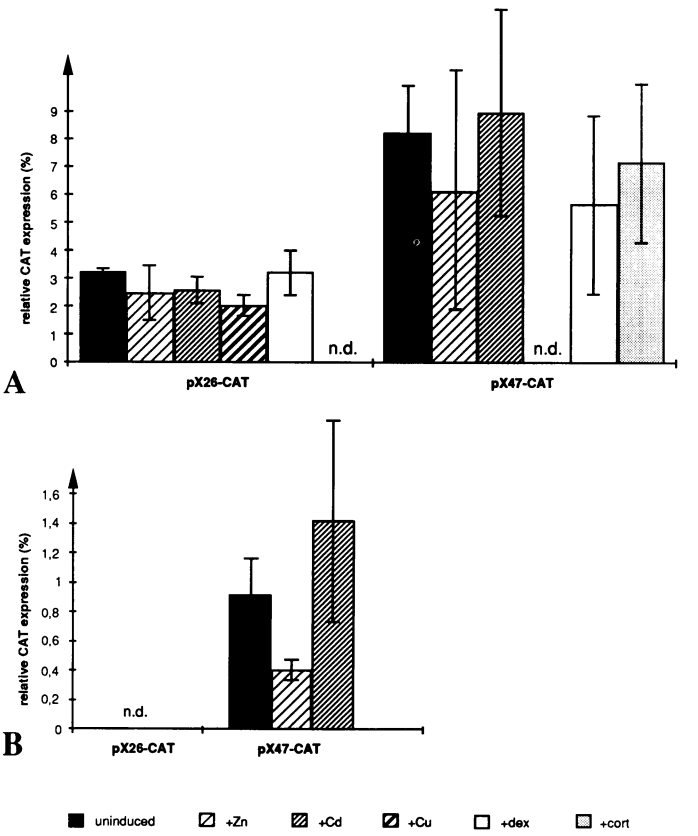

Figure 5. Expression of CAT gene with *Xiphophorus* sequences in a) A_2 and b) EPC cells.

of intact GH into the medium was readily detected (data not shown). The construct was transfected into PSM, A2 and EPC-cells and the promoter was induced with heavy metals. In none of the three fish cell lines GH was detected in the tissue culture medium, using zinc or cadmium for induction, as assayed up to 7 days after transfection. Also cell extracts of transfected cultures showed no immuno-reactivity to anti-GH antibodies. However, RNA of transfected cells gave hybridization bands in Northern blot analysis with the human GH probe of 1.7 kb and 0.9 kb which were not obtained with RNA from non-transfected cells (Fig. 3, lanes 1,2). The bands are approximately of the size that are expected for the unspliced and the correctly spliced GH mRNA, indicating that the construct is expressed on the transcript level, but no immunologically reactive protein product is translated from this message.

Isolation and characterization of a fish sequence that directs transcription of heterologous genes

To obtain a fish sequence that acts as a promoter in transient expression experiments a genomic library of *Xiphophorus maculatus* was screened with a probe containing the 5′ untranslated region of the human MTIIA gene. The rationale for this was that this fragment contains several highly conserved sequence motifs, which are shared by many promoter sequences, especially of the metallothionein gene family from *Drosophila* to man (49). With this probe we isolated a sequence (Fig. 4) from *Xiphophorus*, which upon sequence analysis showed the following features: The sequence contains a stretch of 21 bp which are identical to those around the transcription start site of the human MTIIA gene. Surrounding this sequence several motifs are located which have strong similarity to TATA and CAAT boxes, SP1 binding site and metal responsive elements (MRE). A short open reading frame starting with ATG is found 'downstream' of these motifs, however, the predicted amino acid sequence from it had no similarity with any MT-sequence. If a fragment 'downstream' of the 21 bp identity region is used for Northern blot analysis, a corresponding mRNA of 2.6 kb is detected in polyA$^+$ selected RNA from A2 cells (Fig. 3, lane 3). In PSM cells no such transcript was detected. To test if this isolated fragment can be used as a promoter, we made fusions to the CAT reporter gene. The fusions were made upstream (X26) or downstream (X47) of the presumed SP1 motif. The constructs were transfected into the three cell-lines and show in A2 cells expression values clearly above the level obtained with the herpes simplex virus TK promoter. Only background levels are observed in PSM cells while the EPC cells exhibit values similar to the TK promoter. In A2 cells the X47 constructs containing the putative SP1 site gave 2−3 fold enhanced CAT expression as compared to X26 lacking this sequence (Fig. 5). Our data show that the fragment isolated from the *Xiphophorus* genome indeed can be used as a homologous promoter. The transient expression was found to be constitutive and was not inducible neither by heavy metals (Zn, Cd, Cu) nor glucocorticosteroids (Fig. 5). The promoter appears of intermediate strength as compared with the heterologous promoters. Following microinjection into fish embryos it shows a comparable transient expression profile as

the hMTIIA promoter (Winkler et al., unpublished). When the X47-CAT construct was transfected into mouse L-cells, expression was about 10 fold higher as compared to the TK promoter (data not shown).

DISCUSSION

In order to obtain expression systems suitable for teleost cells we have studied a variety of mammalian and viral promoter and enhancer sequences as well as a promoter isolated from the teleost *Xiphophorus* in three fish cell lines and observed a specific expression pattern depending on the construct and the cell line used. In the case of the heterologous promoter and enhancer sequences, expression directed by the human MTIIA promoter, the RSV-LTR, the TK promoter and the CMV, SV40 and Py enhancer show that all the factors necessary for transcriptional control by these sequences are sufficiently conserved in teleosts and higher vertebrates which evolutionary diverged as early as 450 million years ago. This is also true for the factor(s) involved in heavy metal induction of the metal responsive elements (MRE) of the MTIIA promoter. In line with findings is that the MRE nucleotide sequence of metallothionein genes is highly conserved even between such distant organisms as the fruit fly and man (49). The SV40 enhancer and the RSV-LTR were found to be active in all three cell lines. This is reminiscent of the situation in higher vertebrates. The RSV-LTR has been found to be active e.g. in monkey kidney cells, chicken embryo fibroblasts, chinese hamster ovary cells, Hela cells or mouse NIH/3T3 cells (50). The SV40 enhancer has been found to be constitutively expressed in most cells of higher vertebrates (51). The Py enhancer in our study varies considerably in its activity between different cell types. It was only active in the carp epithelial cells but not in the embryo and the transformed pigment cell line. In mammals this sequence is inactive in undifferentiated mouse F9 embryonal carcinoma cells, and becomes active after their differentiation, it is active in fibroblasts, whereas it is inactive in myeloma cells (52).

The failure to obtain expression following steroid induction of the human MTIIA promoter as well as the MMTV promoter is obviously due to the absence of a functional corresponding receptor in the cell lines used. If such a receptor was coexpressed, the MMTV promoter was readily inducible. In addition, a low point conservation of transcription factors instrumental in this process might complicate the situation. The latter point is supported by the fact that the trout MT promoter was not inducible with dexamethasone in a human hepatoblastoma cell line while the human MT promoter was readily induced with this steroid (26). The Ig enhancer used has been shown in mammals to be active only in lymphoid cells (53) so the zero effect in the fish cell lines used is within the expectation. We do, however, have no explanation why in contrast to the specific expression of the human metallothionein enhancer the corresponding mouse construct was silent.

In all experiments in which the bacterial CAT gene was used as reporter gene, obtaining the ultimate gene product, namely the active enzyme, presented no problem. Moreover CAT activity was found stably for a prolonged period as compared to avian or mammalian cells, obviously a peculiarity of the cells from a cold-blooded vertebrate. However, when we transfected the human GH gene under control of an active promoter we could not detect with a very sensitive radioimmuno assay any GH protein, neither within the cell nor—as would have been expected—secreted into the medium. On the other hand a reasonable amount of GH mRNA was detected pointing to a problem during the translation process of this message. In addition the large amount of unspliced precursor RNA in this experiment points to an additional problem with splicing of the foreign gene. Our attempts to express the human GH gene in fish cells possibly shows the general limitations for the use of heterologous genes in gene expression studies *in-vitro* or for the production of transgenic fish. Several groups have used constructs with the mouse metallothionein promoter and the mammalian GH genes to produce transgenic fish for the purpose of increasing growth rates. In no case expression of GH was observed (24) which is consistent with our finding on transient expression *in-vitro* . Use of definitely active promoters together with cloned homologous fish genes will help to overcome these problems.

To obtain a homologous fish promoter, we have isolated a sequence with striking structural similarities to the human metallothionein promoter. Although our sequence is obviously a promoter in embryonal fish cells—because it acts as a constitutive promoter in CAT constructs *in-vitro* and also following injection into early embryos (54)—it was not subjected to metal regulation in our experiments. We detected sequences which fit the MRE consensus (49), however, a suggested explanation is that further 5′ sequences, which were not included in our constructs, are needed for this type of regulation. Concerning cell type specificity of expression for the two cell lines the constructs gave the same expression pattern as observed for the promoter *in situ* (no expression in PSM, expression in A2). On the other hand it might be possible that metal regulation of this promoter can only be achieved in distinct cell types. The limited number of established fish cell lines representing distinct differentiated cell types complicates answering this issue. However, further experiments are needed for clarification. The absence of typical metallothionein sequences following the isolated promoter sequence does not rule out the possibility that this promoter might be subject under certain conditions to metal regulation because it is known that teleost fish contain metal inducible genes different from metallothionein (55).

On the basis of the experiments presented here it will be possible to construct a whole variety of different fish specific expression vectors for constitutive and inducible expression either transient or from stably integrated foreign sequences.

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