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All-fish gene constructs for growth hormone gene transfer in fish

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Résumé

Afin de développer des vecteurs d'expression de poisson, entièrement homologues, destinés aux microinjections dans des oeufs fertilisés, les constructions suivantes ont été préparées: promoteurs de la metallothionine, a ou b, de truite arc-en-ciel d'une part, et promoteur de l'actine β de carpe d'autre part, associés à l'ADNc de l'hormone de croissance de daurade royale (ptMTa-gsbGH cDNA, ptMTb-gsbGH cDNA, et pcAβ-gsbGH cDNA).

Les promoteurs de la metallothionine ont été clonés en utilisant la technique de la RCP. La tMTa comprend 430 pb, tandis que la tMTb en comprend 260 (Hong *et al.* 1992). Ces deux promoteurs ont été insérés dans pGEM-3Z qui contenait l'ADNc de GH de *Sparus aurata*, pour former, respectivement, ptMTa-gsbGH et ptMTb-gsbGH. Le gène de l'actine cytoplasmique β de carpe a été choisi comme source d'isolement de séquences régulatrices fortement constitutives. Une de ces séquences régulatrices a été liguée à l'ADNc de GH de *S. aurata* dans pUC118, pour réaliser la construction pcAβ-gsbGH cDNA.

L'expression des constructions contenant les promoteurs de la metallothionine a été tentée dans des cultures de cellules de poisson, où elle a été effectivement induite par le zinc. La construction ptMTa-gsbGH cDNA a été microinjectée dans des oeufs fertilisés de carpe. Son intégration dans le génome de carpe a pu être détectée dans l'ADN isolé à partir de nageoires d'animaux agés de 2 mois.

Abstract

In order to develop all-fish expression vectors for microinjection into fertilized fish eggs, we have prepared the following constructs: rainbow trout metallothionein a/b and the gilthead seabream growth hormone cDNA (ptMTa-gbsGHcDNA, ptMTb-gsbGHcDNA), carp β-actin gilthead seabream GH cDNA (pcAβ-gsbGHcDNA). The inducible metallothionein promoters *a* and *b* were cloned from rainbow trout, and the constitutive promoter β-actin was isolated from carp.

The metallothionein promoters were cloned by using the PCR technique. The tMTa contains 430 bp, while the tMTb contains 260 bp (Hong *et al.* 1992). These two promoters were introduced to pGEM-3Z containing the GH cDNA of *Sparus aurata* to form ptMTa-gsbGH and ptMTb-gsbGH, respectively. The carp cytoplasmic β-actin gene was chosen as a source for isolating strong constitutive regulatory sequences. One of these

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regulatory sequences in pUC118 was ligated to GH cDNA of *S. aurata* to form the pcAβ-gsbGHcDNA.

Expression of the constructs containing the metallothionein promoters was tested in fish cell culture and was found to be induced effectively by zinc. The ptMTa gsb-GH cDNA construct was microinjected into fertilized carp eggs, and integration in the genome of carp was detected in the DNA isolated from fins at the age of two months.

Introduction

Current transgenic fish research is being directed mainly toward growth promotion, because of the economic importance of increasing the growth rate of commercial fish. For reviews of transgenic fish studies, see Ozato *et al.* (1989) and Chen and Powers (1990).

It has been shown in many cases that the foreign gene was integrated into the fish genome, and also inherited. Expression of the GH gene in the growing transgenic fish was, however, observed in few cases. This includes Atlantic salmon (Rokkones *et al.* 1989; Du *et al.* 1992), carp and loach (Chen *et al.* 1990; Zhang *et al.* 1990).

In most of the studies of transgenic fish, the human GH gene was used. Exceptions are rainbow trout GH cDNA (Chen *et al.* 1990; Zhang *et al.* 1990), and salmon GH (Du *et al.* 1992).

The regulatory sequences mostly used are the gene promoters of mouse metallothionein and the Rous sarcoma virus (RVS). There are several studies with promoters of piscine origin. Liu *et al.* (1990) prepared several constructs containing the carp β-actin promoter and the CAT reporter gene for studying their expression efficiency. Du *et al.* (1992) used the anti freeze protein promoter from the ocean pout.

The purpose of the production of transgenic fish with the GH gene is to produce a new commercial line of "super fish" which grow larger and faster. It is therefore important that the DNA constructs used for gene transfer will be of fish origin, and not from human or viral sources.

We report here the production of all-fish gene constructs which consist of the carp β-actin, or the rainbow trout metallothionein (tMT) promoters and the gilthead seabream GH (gsbGH) cDNA. These constructs were checked for expression in the carp cell line, and some were microinjected into carp fertilized eggs.

Materials and methods

Construction of plasmids

All recombinant plasmids were constructed by standard molecular techniques. All constructs were verified either by restriction endonuclease mapping or by dideoxy sequencing.

Cell culture and transfection

Carp epithelial cell line (EPC) was grown at 28°C under 5% CO_2 in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine, 100 U/ml of penicillin G and 100 μg/ml of streptomycin. Plasmid DNA for transfection was prepared by the alkaline lysis method (Maniatis *et al.* 1982). Cell transfection was performed by the calcium phosphate coprecipitation method of Gorman *et al.* (1982) modified for fish cells (Friedenreich and Schartl 1990; Hong *et al.* 1992). Briefly, approximately 10^6 cells were plated on 10 cm tissue culture dishes and allowed to grow for 1–3 days to nearly half-confluence. The $CaPO_4$-DNA precipitates were applied onto the cell surface on dishes. Each dish received 3.5 pM (in a total volume of 10 ml) of the proper circular plasmid DNA. After 18h of incubation with the $CaPO_4$-DNA precipitates, the cells were shocked with 15% glycerol for 3 min. The cells were allowed to recover for 18h and then exposed to metal induction treatment with $ZnCl_2$ at a final concentration of 150 μM for 72h before harvesting.

RNA isolation

RNA isolation from cell cultures was performed according to Chomczynski and Sacchi (1987) with some modifications. About 10^7 cells in a 10 cm

dish were washed in phosphate buffered saline. Cells were scraped using a rubber policeman in 1 ml of TEN buffer (40 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, and 150 mM NaCl) and transferred into a 2 ml Eppendorf tube. Following centrifugation for 5 min (12,000 rpm) at 4°C, the cell pellet was loosened by a brief vortexing, lysed in 0.8 ml of denaturing solution (4M guanidine thiocyanate, 25 mM Na-citrate pH 7.0, 0.5% sarcosyl, and 0.1M 2-mercaptoethanol), and mixed by inversion for 5 min. Then 80 μl of 2M sodium acetate (pH 4.0), 0.8 ml of phenol (TE-saturated) and 160 μl of chloroform-isoamyl alcohol mixture (49:1) were sequentially added to the sample, with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 1 min and cooled on ice for 15 min. After centrifugation (12,000 rpm, 20 min at 4°C), RNA was present in the aqueous phase whereas DNA and proteins were present in the interface and phenol phase. The aqueous phase was transferred to a fresh tube, mixed with 1 volume of isopropanol, and placed at -20°C for at least 1h to precipitate RNA. Following spinning again as above, the resultant RNA pellet was dissolved in 0.3 ml of denaturating solution, and precipitated with 1 volume of isopropanol at -20°C for 1h. After centrifugation for 10 min at 4°C, the RNA pellet was washed with 75% ethanol, precipitated, dried and dissolved in 50 μl of either 0.5% SDS (in DEPC-treated water) or 10 mM Tris-HCl, 1 mM EDTA (pH 8.0, in DEPC-treated water) at 65°C for 10 min. Typically, a 10 cm dish of the EPC cells can yield 50–100 μg of total cytoplasmic RNA.

Northern blot analysis

Total cellular RNA (about 20 μg for each sample) was heat-denatured in formamide/formaldehyde, separated by electrophoresis on 1.5% agarose-2.2M formaldehyde gels, and transferred to a GeneScreen nylon membrane by the capillary blotting method according to the supplier's protocols (New England). RNA standards (RNA ladder, BRL) were run in parallel for size determination. After baking for 2h with Gel dryer (model 583, BioRad), the membranes were stained with methylene blue for quantification of the RNA amounts. Filters were prehybridized overnight at 42°C in10 ml of hybridization mixture containing 50% deionized formamide, 5 × SSC (1 × SSC is 0.15M NaCl plus 0.015M sodium citrate), 50 mM Tris-HCl (pH 7.5), 1% SDS, 5 × Denhardt's solution, 0.1% NaH_2PO_4 and 0.1 mg/ml sonicated calf thymus DNA. Hybridization was carried out at 42°C for at least 24h in the same solution containing gbsGHcDNA probe (about 1 × 10^6 cpm per ml) labeled by the random-priming method (Feinberg and Vogelstein 1983) with [^{32}P]dCTP. Following washing twice in 2 × SSC – 1% SSC for 30 min each at room temperature, once in 1 × SSC – 1% SDS for 30 min at 65°C, and up to three times in 0.1 × SSC – 1% SDS for 30 min each at 65°C, the filters were exposed to X-ray film (Kodak X-Omat AR film or Agfa film) for 1–15 days at -80°C.

Egg microinjection

Experimental carp fish were spawned artificially by induction with GnRH. Eggs were stripped from a mature female into a dry petri dish. Water and milt were added to the eggs with gentle stirring to enhance fertilization and dispersal. Eggs were water-hardened for 5 min and then rinsed. Eggs were covered with Holtfreter's solution and then microinjected. Borosilicate glass microinjection needles with an inner tip diameter of approximately 5μM were prepared by use of vertical micropipette puller (Narishige PW-6). The needles were filled with supercoiled or linearized ptMTa-gsbGHcDNA plasmid dissolved in TE. About 2nl containing approximately 1 × 10^6 copies of the gene were delivered into the germinal disc by penetrating the chorion and the plasma membrane prior to first cleavage. Picoinjector (Medical System Corp., PLI 100) was used together with Narishige MN-100 micromanipulator. Microinjected eggs were allowed to develop in static water at 20–24°C.

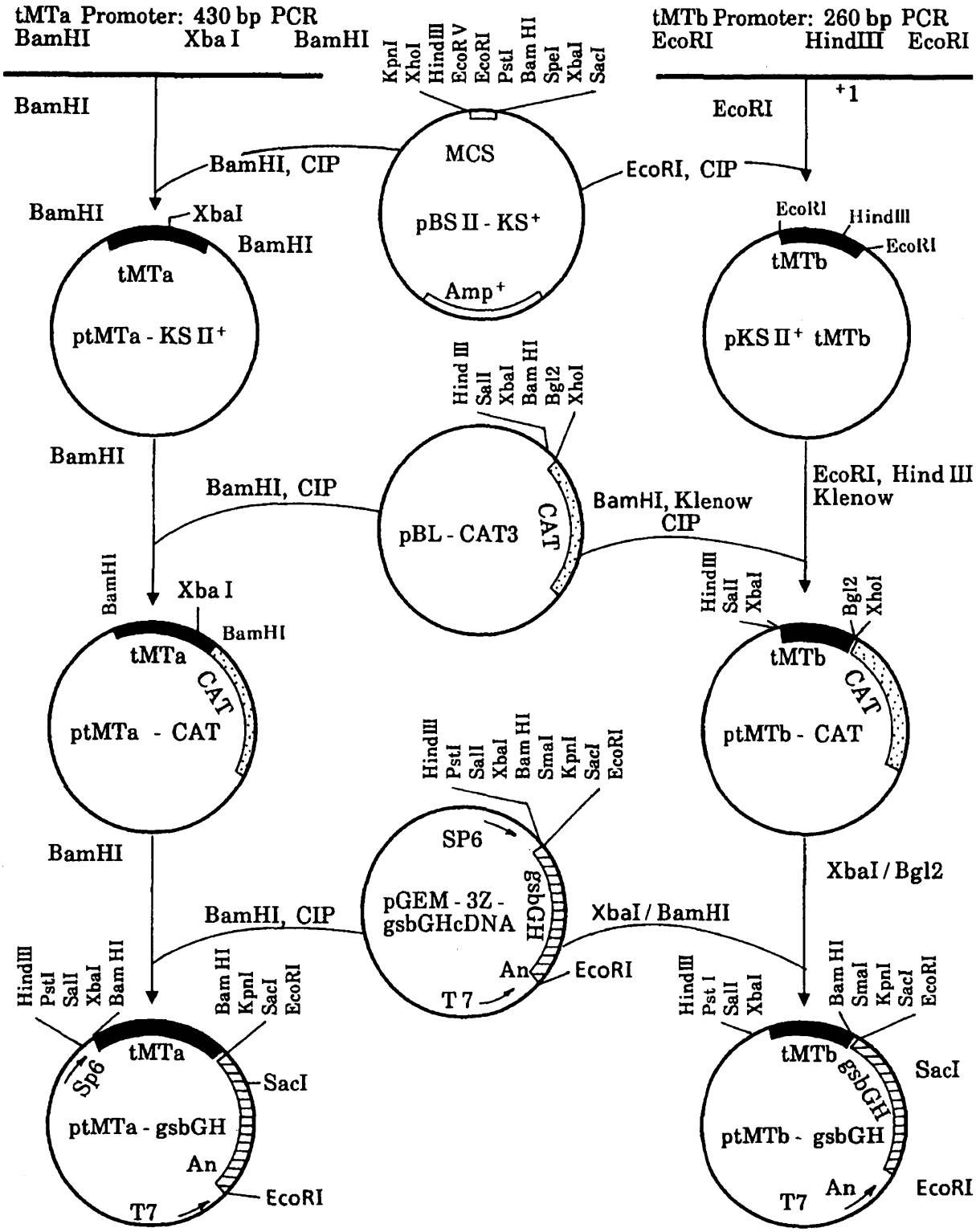

Fig. 1. The construction of plasmids with the MTa and MTb promoters and with the CAT reporter gene and the gilthead sea bream GH cDNA (see details in the text).

Genomic DNA extraction

High molecular weight DNA was extracted from pectoral fin clips of two months old fish by the following method: fins were digested with proteinase K (500 μg/ml) at 50°C for 30–40 min in a buffer containing 50 mM Tris (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM NaCl, and 1% SDS. After repeated extraction with phenol and chloroform, the DNA was precipitated with isopropanol at room temperature.

Southern blot analysis

DNA samples (20 μg) were digested with Hind III and electrophoresed on 0.8% agarose gel. The gel was denaturated and neutralized, DNA samples in the gel were transferred to N-Hybond membrane, and the DNA fixed by exposure to UV light for 4 min. Hybridization was performed in a solution containing 50% formamide at 42°C, and the probe used was the 430 bp Bam HI fragment of the MTa promoter.

Results and discussion

Plasmid constructs

The basic plasmids were pBluescript II KS + (Stratagene) and pGEM-3Z (Promega). The pgsbGHcDNA is a derivative of pGEM-3Z which contains the GH cDNA sequence from the gilthead sea bream (*Sparus aurata*) (Funkenstein *et al.* 1991). The vectors ptMTaCAT, ptMTbCAT (Hong *et al.* 1992) were the sources for the tMT promoters. The ptMTaCAT and ptMTbCAT were the derivatives of the promoterless pBL-CAT3 (Luckow and Schütz 1987), into which the 430 bp tMTa and 260 bp tMTb promoters were separately inserted 5′ to the chloramphenicol acetytransferase (CAT) gene.

The 430 bp BamHI fragment of the tMTa promoter was released from the ptMTaCAT and inserted in the BamHI site upstream of the gsbGH cDNA, resulting in the ptMTa-gsbGH cDNA. For the construction of ptMTb-gsbGH cDNA, the 260 bp XbaI-BglII tMTb promoter excised from the ptMTbCAT was directionally introduced 5′ to the gsbGH cDNA in pGEM-gsbGH cDNA which had been cut with XbaI-BamHI. The construction of ptMTaCAT, ptMTa-gsbGH cDNA, ptMTbCAT, and ptMTb-gsbGH cDNA is shown in Fig. 1.

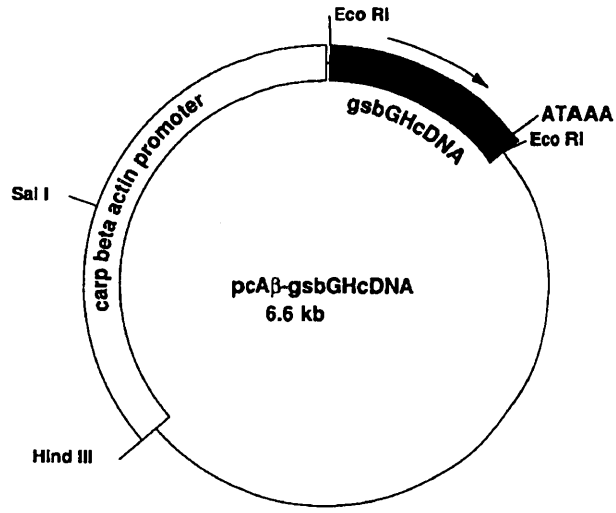

Fig. 2. Structure of the gsbGHcDNA expression vector pcAβ-gsbGHcDNA. The construction strategy was described in Results and discussion. The carp β-actin promoter is shown in the open box, the gsbGH cDNA is in the black box with the arrow indicating the direction for open reading frames.

The construct 6 of Liu *et al.* (1990) was the source for the carp β-actin (cAβ) promoter. The construct was sequentially cut with Kpn I (the site is located between the cAβ promoter and the CAT gene), blunt-ended by removing the protruding 3′ termini with T4 DNA polymerase, and digested with Eco RI to remove the CAT gene. The 1 kb gsGH cDNA was prepared by cutting the pgsbGHcDNA with Bgl II (the site is situated 5′ most of the gsbGH cDNA insert), filling-in with Klenow fragment, and re-cutting with Eco RI. The fragment was recovered following gel electrophoresis and ligated to the linearized vector. The resultant plasmid was termed pcAβ-gsbGHcDNA; its structure is illustrated in Fig. 2.

Transfection and Northern blot analysis

Previous studies by transient expression of a reporter gene indicated that the carp epithelial cell line

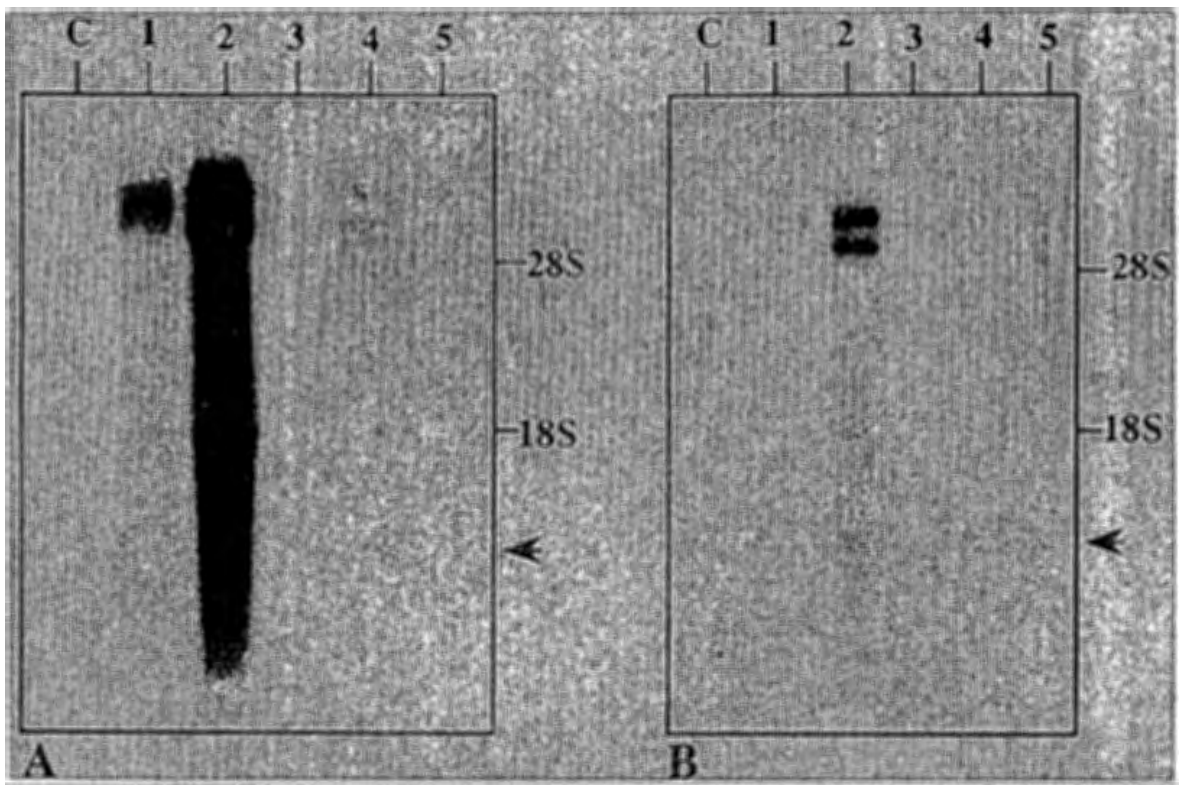

Fig. 3. A representative Northern blot analysis. Total RNA (approx. 20 μg per lane) from the EPC cells transfected with different gsbGHcDNA-containing plasmids were hybridized with the 1-kb gsbGHGcDNA probe. The transfected cells were treated with zinc (lanes C, 2 and 4) or untreated (lanes, 1, 3 and 5) as described in Materials and methods. Lane C: mock-transfected control sample. Lanes 1 and 2: ptMTa-gsbGHcDNA-transfected samples. Lanes 3 and 4: ptMTb-gsbGHcDNA-transfected samples. Lane 5: pcAβ-gsbGHcDNA-transfected sample. The positions of 28S and 18S rRNAs are shown. The arrow indicates the position for the gsb mRNA expected. A: the filter was washed for up to 1h in 0.2 × SSC – 1% SDS at 60°C and exposed for 3 days at −80°C with intensifying screen. B: the same filter was further washed for 1h in 0.1 × SSC – 1% SDS at 65°C and exposed for 15 days under the same conditions.

(EPC) is highly suitable for efficient transfection and optimal expression directed by the two fish MT promoters in our conditions. Therefore, this cell line was chosen for this study. Figure 3 represents Northern blot analysis of the expression of the gsbGH containing constructs. There is a band of *ca.* 1 kb which corresponds to the gsbGH mRNA. This band was not visible in the RNA samples from untreated cells. In addition to this, two unexpected bands, for both tMTa- and tMTb-gsbGH, were detected. These bands are approximately 8 and 12 kb, and there is a positive correlation between the occurrence/intensity and Zn-treatment. Moreover, these "extra" bands were much stronger than the "authentic" one. It is possible that the bands resulted from the two or three rounds of run-off transcription of the circular plasmid DNA. Because of the correlation of their appearance with Zn and their absence in the mock-transfected sample (a plasmid containing only the GH gene without any promoter sequence), nonspecific hybridization or DNA contamination is excluded. The authentic band was not clearly visible when the filters were subjected only to low- (1 × SSC – 1% SDS for 1h at 60°C) or moderate-stringency washing (0.2 × SSC – 1% SDS for 1h at 60°C), but after washing in 0.1 × SSC – 1% SDS for 1h at 65°C, the band expected from the correct transcription is clearly observed. These results may be indicative of an inefficiency of the transcriptional terminator of gsbGH plasmid.

Surprisingly, the carp β-actin promoter-containing construct of the gsbGH did not give rise to any detectable signal. The various genes used in the β-actin constructs might be the reason for the differences in the results obtained by us using the

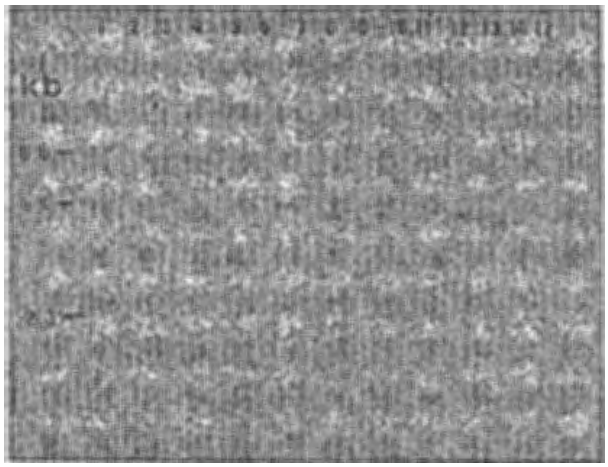

Fig. 4. Southern blot analysis of DNA from fins of microinjected carp. 20 μg of DNA from fins of 2 month old fish were digested with HindIII and electrophoresed on 0.8% agarose gel. After blotting, the filter was hybridized with a 430 bp piece of the MT promoter after random primer labeling. Sample 17 is from a control uninjected fish. Each lane represents a different fish.

GHcDNA, and by others using the CAT reporter gene.

Integration of microinjected construct

It is interesting to determine if the ptMTa-gsb-GHcDNA is correctly transcribed *in vivo*. This construct was therefore microinjected into fertilized eggs of carp. Supercoiled or linearised plasmid were used for injection.

Southern blots of DNA samples extracted from fins of 15 fish are shown in Fig. 4. In 4 of the 14 fish injected with ptMTa-gsbGH, the presence of the injected DNA is demonstrated. Fish # 4 was injected with a BglI 3.1 kb piece containing the promoter and the GH cDNA. HindIII cut this piece to two fragments of similar sizes. In #s 8, 12 and 13, the supercoiled plasmid was injected; # 17 is a control, uninjected fish.

From the results with the 15 microinjected fishes, it appears that in about 25% of the treated fish, the DNA was integrated into the fish genome. It is anticipated that at least some of the integrated DNA will also be expressed in the growing fish, since the cell culture transfected by the same construct used for microinjection into the fish eggs resulted in expression of the gene, as demonstrated by a transcript in the Northern blot.

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