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Comparative expression of the plasmids pCMV-CAT and pCMVTtk-lacZ in embryos and larvae of the seabream

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SUMMARY - DNA transfer to fish embryos allows the creation of genetically manipulated strains in commercial species and the *in vivo* study of gene expression by means of transient expression systems. Medaka and zebrafish have been widely utilized as model fish species, however accurate expression patterns from other different species can only be obtained when self specific systems are used. Due to high commercial value of the gilthead seabream species, a successful DNA transfer technique has been developed, to analyse the behaviour and the expression patterns of "standard" plasmids in different conformations, with quantifiable (pCMV-CAT) and spatially (pCMVTtk-lacZ) detectable gene products. This step is previous to perform the screening of gilthead seabream regulatory elements involved in genes of productive traits. For that purpose embryos in 1 or 2 cell stage, were microinjected with both the circular and linear forms of the plasmids. Their respective temporal expression patterns were analysed in embryos and larvae of up to 4 days development for lacZ and up to 13 days for CAT. The results revealed that the initiation of expression in either plasmid forms, occurs at mid-late blastula stage, thus marking the activation time of endogenous genes. Once the expression has been initiated, the CAT activity appeared at different levels in all the screened times, while no b galactosidase activity was detected after 72 hours. When the expression levels with the two forms of the respective plasmids were compared, slightly higher levels were shown by the linear form, probably due to a differential persistence of the exogenous DNA. In this work it has been demonstrated that the gilthead seabream is a suitable system to test regulatory sequences from the own species. Additionally CAT and lacZ markers have proved to be respectively useful for quantification and spatial expression studies.

Key words: Seabream, gene transfer, transient expression, DNA fate.

RESUME *- "Expression comparative des plasmides pCMV-CAT et pCMVtk-lacZ chez des embryons et des larves de daurade". Le transfert d'ADN permet l'obtention de souches transformées chez les poissons d'intérêt commercial, ainsi que l'analyse in vivo de l'expression de gènes au moyen de systèmes d'expression transitoire. Le medaka et le poisson-zèbre sont des modèles largement utilisés dans les études de biologie du développement des Vertébrés; cependant, l'analyse précise de patrons d'expression chez d'autres espèces requiert l'utisation de systèmes qui leur sont propres. A cause de la grande importance commerciale de la daurade, nous avons développé chez cette espèce une méthode efficace de transfert d'ADN qui permet l'analyse du comportement et de l'expression de plasmides "standard" de différentes conformations; les produits d'expression en sont localisables et quantifiables, permettant la recherche d'éléments régulateurs chez la daurade. Dans des embryons au stade une ou deux cellules, nous avons micro-injecté les formes linéaire et circulaire des plasmides CMV-CAT et pCMVtk-lacZ. Leur patrons d'expression respectifs ont été analysés au cours du temps, chez des stades embryonnaires et larvaires jusqu'à quatre jours de développement pour lacZ et jusqu'à 13 jours pour CAT. L'analyse des résultats a montré que l'expression de ces plasmides (dans les deux conformations) débute à la transition mi-blastuléenne, et marque donc l'activation de gènes endogènes. Après ce démarrage, l'activité CAT persiste à des niveaux variables à toutes les étapes étudiées, alors qu'aucune activité galactosidase n'est plus détectable après 72 heures. En comparant les niveaux d'expression des deux formes de chaque plasmide, nous avons observé une expression légèrement plus intense des formes linéaires, ce qui correspond probablement à une différence de persistance de l'ADN exogène. Ce travail démontre que la daurade peut être considérée comme un modèle valable pour tester des séquences régulatrices intervenant*

dans la différenciation cellulaire, puisque plusieurs types cellulaires sont présents aux stades larvaires. De plus, nous avons montré que CAT et lacZ sont utiles pour l'analyse quantitative et spatiale, respectivement, de l'expression génétique dans ce système.

Mots-clés : *Daurade, transfert d'ADN, expression transitoire.*

Introduction

The transfer of exogenous DNA in fish as well as in other animal groups, has two main objectives consisting on the study of the regulation of gene expression *in vivo* and on the creation of genetic manipulated strains more productive in aquaculture. For the former, short-term expression assays have been preferentially used, due to the high difficulties in obtaining suitable long-term expression strains. Furthermore when the genes under study are related to productive traits, the information obtained can be applied to the second objective.

So far, the introduction of foreign genes has been mostly carried out by microinjecting newly fertilized eggs with DNA constructs, though other innovative procedures to improve its efficiency, have been attempted with variable success (reviewed in Iyengar *et al.*, 1996).

Successful transgenic fish from model and commercial species have been produced in the last decade, although most experiments have been restricted so far to freshwater teleosts (reviewed in Maclean and Rahman, 1994), partly for being easier to be maintained in the laboratory and partly because of their tradition in aquaculture. Marine species on the contrary have received much less attention, despite their increasing importance in aquaculture (Gordin, 1989).

The marine fish *Sparus aurata* has emerged in the last years as one of the most important species in the Aquaculture of the European Mediterranean countries and it can be considered as a "model" in the domestication process of other various sparid fish, so that there is a considerable interest in the isolation and characterization of genes of economical importance.

Though preliminary results of microinjecting seabream eggs have been reported (Cavari *et al.*, 1993; Knibb *et al.*, 1994), no routine gene transfer procedures have been developed in this species, due to complicated zootechnical requirements and to the characteristics of the spawned eggs (Knibb *et al.*, 1994). The highly successful manipulation conditions developed by our group (García-Pozo *et al.*, submitted), prompted us to analyse the expression of quantifiable (CMV-CAT) and spatial (pCMVTklacZ) reporter genes, with the eventual goal to characterize functionaly gilt-head seabream regulatory gene elements, related with productive traits.

Material and methods

Egg manipulation

The cytoplasm of naturally fertilized seabream embryos in 1- or 2-cell stage, were

cytoplasmic microinjected with a red coloured solution containing approximately 10^8 copies of either supercoiled or linear forms of the plasmids pCMV-CAT and pCMVTtk-lacZ. Microinjection was performed at room temperature with a mechanical micromanipulator, using an air-pressure-controlled injector and borosilicate needles. Subsequent to injection, the treated and the control embryos were incubated in slow-flowing water tanks at 18°C until they were collected for further analysis. The sample size was greater than 80 specimens for each screened development time.

The gene constructs

The cloramphenicol acetyl transferase (CAT) gene has been widely used as reporter in transgenic fish, since its expression can be detected by simple and sensitive assays that also allow its quantification. The plasmid CMV-CAT (5 kb) contains the enhancer and promoter of the immediate early gene of human cytomegalovirus, fused to the CAT gene, along with the early SV40 splice and polyadenylation signal (Fig. 1), (Foecking and Hofstetter, 1986). This plasmid was previously tested for fidelity of expression in cell cultures and in trout eggs. To obtain the linearized form for microinjection, the plasmid was digested with XbaI.

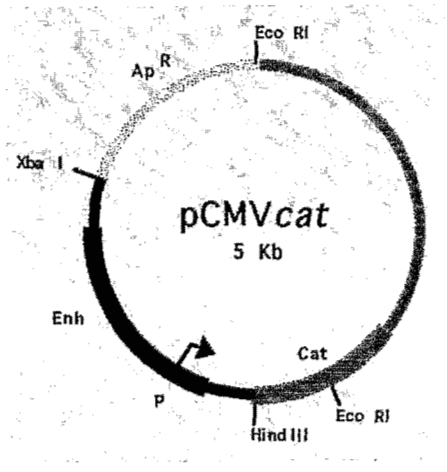

Fig. 1. The pCMV-CAT gene construct containing the human cytomegalovirus promoter spliced to the CAT gene.

The lacZ reporter gene is most useful for determining the site of expression in a tissue or a whole animal, since the activity of its product, the bacterial b galactosidase (b gal), can be detected using histochemical staining thus allowing *in situ* detection. The construct pCMVTk-lacZ of 8.05 kb (Fig. 2), contains the enhancer of the immediate early gene of human cytomegalovirus and the timidine kinase promoter along with the E. coli lacZ gene and the polyadenylation signal of the Simian Virus.

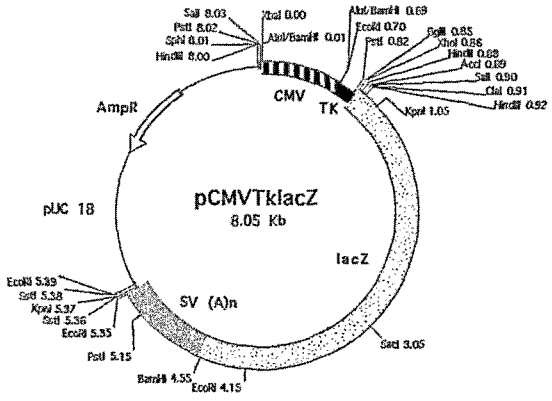

Fig. 2. The pCMVTk-LacZ gene construct, containing the timidina kinase promoter, spliced to the lacZ gene and the SV40 polyadenilation sequence.

Preparation of samples

For CAT expression and DNA fate analysis, groups of five seabream embryos or larvae were homogenized in 55 µl of 250 mM Tris-HCl, pH 7.5 and subjected to three cycles of freezing-thawing (by freezing in liquid nitrogen and thawing at 37°C for 5 minutes each) to lyse the cells. After centrifugation for 20 min at 4°C, the supernatant to be used for the CAT assay was heated at 55°C for 10 min and the pellets were kept at -80°C for DNA analysis.

Embryos microinjected with the lac-Z marker, were frozen for DNA analysis. For LacZ expression, undechorionated embryos were fixed for 30min at 4ºC in a fixative composed by: 2% paraformaldehyde, 0.2% glutaraldehyde and 0.02 % NP-40, in PBS.

Extraction and analysis of DNA

For Southern analysis the pooled DNA was homogenated. The DNA extraction was carried out by incubating the homogenate with 150 µl of the extraction buffer (10 mM Tris, pH 8, 100 mM EDTA, 0.5% sodium dodecyl sulfate (SDS) and 200 µg/ml of proteinase K). Upon phenol and chloroform extraction, the DNA was precipitated by addition of 5 M NaCl and 2X volume of ethanol, washed in 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). Subsequently half of the DNA was digested with the appropriate enzyme to be linearized and the other half remained undigested.

DNA fragments were separated by electrophoresis on 0.8% agarose gels, stained with ethidium bromide and denatured and neutralized after been treated for 10 min with 0.25 M HCl. DNA was transferred to a nylon membrane (Dupont, Boeringher Mannheim). The complete plasmid was used as probe and ^{32}P labelled with Klenow polymerase by random primer (Amersham Megaprime Kit). Filters were prehybridized in 25 ml of hybridization buffer (0.5 M phosphate buffer, pH 7.2 , 7% SDS and 1 mM EDTA) at 65°C for 5 hours. Hybridization occurred in the same

solution after adding the probe at 65°C overnight (18 hours). Filters were washed 2 times in 2% SDS/2 x SSC at room temperature and 2 more times in 0.1% SDS/0.1 x SSC at 65°C and then they were exposed to radiographic film (X-omat Kodak) in a cassette at -80°C for 1 to several days.

CAT assay

For the CAT assay the protein extract was incubated for 1.5 hours at 37°C with 8 µl ^{14}C-labelled chloramphenicol (Amersham; 25 µCi/ml) and 20 µl of 4 mM aqueous solution of acetyl coenzyme A (Sigma). The mix was completed up to 150 µl with 250 mM Tris-HCl, pH 7.5. After extraction with ethylacetate, the reaction products were dried down in a speed-vac, redisolved in ethylacetate, spotted on silica gel thin layer plate and run with chloroform-methanol mixture (95:5) for 50 min. The plates were air-dried and the autoradiogram was developed after 4-5 days (Gorman *et al.*, 1982).

LacZ staining

The embryos were stained for at least 16 hours at 30ºC, in a solution containing a mix of 974 ml of the solution A and 26 ml of solution B. Solution A: 0.2M Na phosphate buffer ph 7.3, 5M NaCl, 1M $MgCl_2$, 0.1M $K_4(Fe_3(CN)_6)$ and 0.1M $K_3(Fe_2(CN)_6)$. Solution B: 8% X-gal (5-bromo-4chloro-indolyl-b-D galactosidase) in DMF. Stained embryos were kept in 1XPBS.

Results

CAT- expression

The results of expression of the CMV-CAT construct, are summarized in the histogram of Fig. 3. The overall expression values are expressed as the percentage of positive samples (one sample represents a pool of five individuals) at a specific time. With both forms, the CAT expression initiates at 7 hours after fertilization and has been maintained at different levels in all stages screened. Embryos of up to three days old were available for the linear plasmid and of up to 13 days, for the circular one.

During the prehatch (less than 48 hours), the overall expression was higher with the linear than with the circular form (excepting at 7 and 8 hours), while between 48-72 hours, 100% of the samples showed expression with both forms. The expression in larvae containing the circular plasmid (from 4 to 13 days old), is still significant, though lower than in the precedent stages.

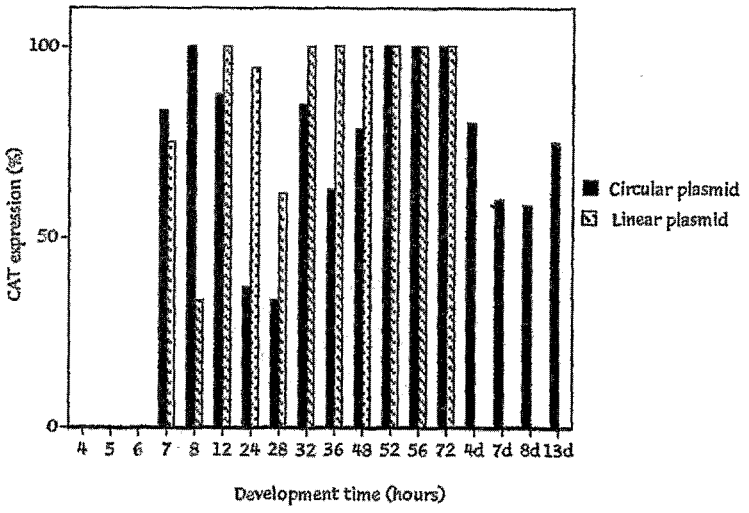

Fig. 3. Temporal CAT expression pattern of the seabream embryos and larvae from different stages. Development times are represented in the abscissa and the percentage of positive samples in the ordinate. The two plasmid forms are indicated in the panel.

Lac-Z expression

The results of the temporal expression of the construct pCMVTk-lacZ are presented in Fig 4. The percentage values of expression correspond to positive single individuals showing b galactosidase activity. The expression initiates at 7 hours and gradually decreases, showing the lowest values at 72 hours. No expression has been detected beyond this time.

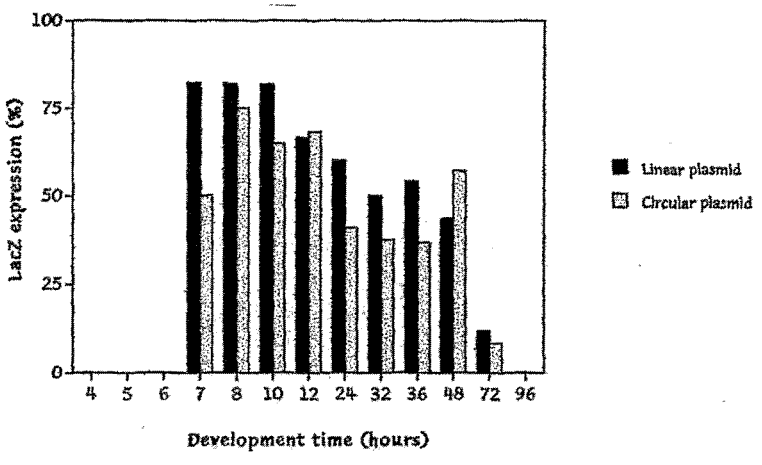

Fig. 4. Temporal LacZ expression pattern of the seabream embryos and larvae from different stages. Development times are represented in the abscissa and the percentage of positive samples in the ordinate. The two plasmid forms are indicated in the panel.

The spatial expression pattern indicated that all positive embryos and larvae from all stages investigated, were mosaic and showed a large variation in the extent of

the stained areas. Few of them displayed large stained areas, never surpassing 50% of the whole embryo, while most of them exhibited small stain patches, both in the embryonic as well as in the extraembryonic tissues. In any case non tissue specific pattern was shown, according to the ubiquity of the promoter used.

DNA fate

Injection of linearized or circularized recombinant DNA in other fish species has suggested differences in terms of persistence and consequently of expression. The fate of both forms of the pCMV-CAT plasmid was followed during embryogenesis and larvae development by Southern blotting. The results with the circular form are presented in Fig. 5, where bands of different mobilities were observed in the undigested DNA lanes, indicating that the plasmid is present in various configurations, such as supercoiled (z), covalently closed circular (x), nicked open circular (w) and high molecular fractions represented by a 10 kb band (v) and a larger than 23 kb band (u). The mobilities of the covalently closed circular conformation (x) and the linearized form (y) are very hard to distinguish from each other. In the lanes in which the DNA was digested with Xbal, (even lanes from 4 to 18), a strong band of 5 kb, corresponding to the plasmid unit size, appeared. This indicates that the bands from undigested DNA represent monomeric or multimeric forms of the plasmid DNA. In the temporal pattern, the decrease in the signal intensity observed in samples above 36 hours can be interpreted as a corresponding overall reduction in the total amount of the exogenous DNA, relative to a progressive degradation.

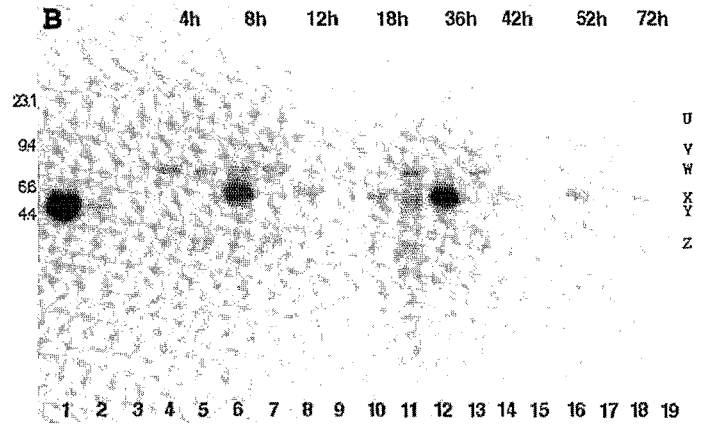

Fig. 5. Southern blot analysis of DNA obtained from various embryonic stages of seabream embryos microinjected with the circular form of the plasmid CMV-CAT. Lanes 1-2 represent positive control samples containing respectively 50 and 5 picograms of the linearized plasmid. Lane 3 negative control sample. Lanes 4 to 19 contain DNA from embryos of times indicated on the top of the figure. There are two lanes for each sample: lanes with even numbers represent digested (Xbal) DNA and lanes with odd numbers represent undigested DNA. The plasmid is present in supercoiled (z), covalently closed circular (x), nicked open circular (w) and high molecular fractions (v and u).

When the plasmid linearized with XbaI was used (Fig. 6), one main band of about 10 kb appeared in the undigested DNA lanes at all times screened. Moreover, some weaker bands of higher molecular weight showing different mobilities were seen in lanes 4, 8, 10 and 15. When the corresponding DNA was digested (even lanes from 4 to 18), one strong band of approximately the same size as the one from the positive control (5 kb), as well as some of the undigested DNA bands were observed. These results suggest that the 10 kb and the higher molecular weight bands, correspond respectively to dimeric and multimeric conformations. The intensities of the signals obtained in the autoradiograph were very low or non-existent from lanes 11 to 16, which in our opinion can be explained by reasons inherent to the Southern blot technique itself and not by a real reduction in the transgene copy number.

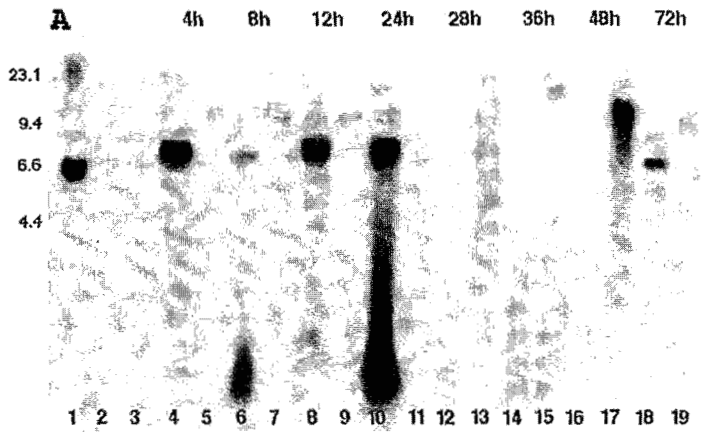

Fig. 6. Southern blot analysis of DNA obtained from various embryonic stages of seabream embryos microinjected with the plasmid CMV-CAT, linearized with XbaI. Lanes 1 and 2 represent positive control samples containing respectively 50 and 5 picograms of the linear plasmid. Lane 3 represents the negative control sample. Lanes from 4 to 19 contain DNA samples from embryos of different times which are indicated on the top. There are two lanes for each sample: lanes with even numbers represent digested (XbaI) DNA and lanes with odd numbers represent undigested DNA. In the digested DNA lanes, monomeric and multimeric plasmid conformations are detected, while in the undigested DNA lanes only bands larger than those of the unit plasmid, are observed.

Discussion

The expression pattern of exogenous DNA in fish is the final outcome of the interaction of several factors, such as the construct itself, the plasmid configuration, the DNA persistence and the development stages.

The expression of the reporter gene CAT has been highly efficient (Fig 3), such as would be expected from the strong promoter used, which have worked very efficiently in different fish species both *in vivo* (Tewari *et al.*, 1992) and *in vitro* (Hernandez Bétancourt *et al.*, 1993) conditions. The lacZ construction expression (Fig 4) also proved to be quite efficient in this case, in spite of the poor activity shown by the combination of the tk promoter with the CMV enhancer in various fish cell lines (Hernandez Bétancourt *et al.*, 1993).

The temporal expression data of CAT and lacZ activities (Figs. 3 and 4 respectively), indicate that it initiates with the two forms of both plasmids at 7 hours after fertilization, which approximately corresponds to the late-blastula stage in seabream (Alessio and Gandolfi, 1975). It seems that initiation of exogenous DNA expression in other fish species, is coincident with the activation of endogenous genes, which almost invariably occurs after the Mid Blastula Transition stage (MBT), irrespectively of the kind of promoter used (reviewed in Iyengar *et al.* 1996). Therefore the mid-blastula transition event, characterized so far in the embryogenesis of zebrafish (Kane and Kimmel, 1993) and medaka (Tsai *et al.*, 1995), has also been revealed in this marine species.

Once the exogenous DNA expression was initiated, the activity of CAT has been detected at different levels all over the stages surveyed, while the activity in the lacZ has apparently been interrupted in larvae beyond 3 days old. The different duration in both expression patterns can be explained by the differential activity of their respective regulatories sequences as mentioned above. Additionally it seems that the kind of expression in both cases (data from the lacZ plasmid not stated) is transient, since the DNA fate analysis were not able to detect "junction" fragments, that is bands of high molecular weight which size is not multiple of the plasmid unit, which might indicate integration of the exogenous DNA and subsequently permanent expression.

The overall higher expression levels shown by the linear plasmid form in relation to the circular one (Figs 3 and 4), can be interpreted in terms of the different fate of the respective plasmid forms, once they are inside the host embryonic cells. When circularized plasmids were used (Fig 5) both monomeric and multimeric DNA conformations persisted in the development times screened.

However when the linearized plasmid was used (Fig. 6), no monomeric forms were observed as opposed to circular, instead all the exogenous DNA appeared in high molecular conformations, thus indicating that linear plasmids have been concatemerized at a higher rate than circular ones. This observation is coincident with studies in medaka (Winkler *et al.*, 1991) and in rainbow trout (Iyengar and Maclean, 1995). In addition to this, the possibility of a higher vulnerability of the monomeric forms from circular plasmids to degradation, should not be discarded as a reasonable alternative.

In spite of the former findings, not all studies in fish have reported significant differences in the persistence of both forms, such as Chong and Vielkind (1989) in medaka and Volckaert *et al.*, (1994) in the African catfish. These contrasting results may be interpreted by considering other variables influencing the amplification efficiency besides the plasmid conformation, such as the kind of sequences used in

the constructs (Asano and Shiokawa, 1993), or the number and size of the plasmid molecules (Winkler *et al.*, 1991). In any case and according to the previous observations, we propose that the higher overall expression with linear forms, can be due to the higher presence of multimers observed, that in turn results in higher levels of expression (Iyengar and MacLean, 1995).

In this work it has been demonstrated the interest of the seabream as a suitable system to be manipulated by DNA transfer. In short-term expression studies, the CAT and lacZ markers have proved to be very useful for quantification and spatial characterization of autologous sea bream regulatory sequences and subsequent testing of their function. Additionally linearized plasmids seem to be more active in terms of persistence and expression than circularized ones.

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