Effect of exogenous DNA microinjection on early development response of the seabream (*Sparus aurata*)

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Abstract

DNA transfer techniques allow genetic manipulation of commercial fish. However, marine species have received little attention because of their difficult zootechnical requirements. The seabream (*Sparus aurata*) has become one of the most important species in the aquaculture of Mediterranean countries, and the development of suitable DNA transfer procedures represents a main step in its genetic improvement. To assess the response of the seabream to exogenous DNA, naturally fertilized eggs were injected with the plasmids pCMV-CAT, pCMVTKlacZ, and pEGFP-N1, in supercoiled and linearized forms. Embryo and larval survival, DNA fate, and reporter gene expression were analyzed during early development. The survival results indicate that microinjection is an effective transfer method in spite of the unfavorable conditions. Linearized plasmids were more efficiently polymerized than supercoiled ones; however, no significant differences were detected either in their persistence or in their expression levels. Reporter gene expression was initiated after mid-blastula transition. The duration of transient expression varied between the promoter-gene combinations, and no integration of transgenes into fish chromosomes was detected. Results suggest that the main factor affecting the persistence and expression of DNA seems to be related to developmental processes. Among the markers used, CAT proved to be the most sensitive, but GFP had obvious methodologic advantages over the spatial marker lacZ. The usefulness of GFP for diagnosis of transgenesis is enhanced by the transparency of embryos and larvae in *S. aurata*.

Introduction

The transfer of exogenous DNA to fish and to other animal embryos has two main objectives, the study of the function of regulatory sequences during development and the genetic manipulation of commercial fish species to produce transgenic lines with higher commercial value. For the former, short-term or transient expression assays have been preferred (Chong and Vielkind, 1989; Gong and Hew, 1993; Reinhard et al., 1994) owing to the difficulties in obtaining suitable long-term expression strains. So far, foreign genes have been introduced mostly by microinjecting newly fertilized eggs with DNA constructs, though other innovative procedures to improve efficiency have been attempted with variable success (reviewed in Iyengar et al., 1996).

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 because they are easier to maintain in the laboratory and partly because of their tradition in aquaculture. In contrast, marine species have received much less attention despite their increasing importance in aquaculture (Gordin, 1989).

The marine fish *S. aurata* has emerged in the last few years as one of the most important species in the aquaculture of the European Mediterranean countries. It can be considered as a model in the domestication process of various other sparid fish. Consequently, there is considerable interest in the isolation and characterization of genes involved in the productive traits of this species for possible genetic improvement.

Because evolutionary diversity among fish is greater than in all other vertebrate classes together, the use of species-specific regulatory elements for
transgene expression is recommended when possible (Devlin et al., 1994; Takagi et al., 1994). This work is a step toward using S. aurata as an in vivo system for studying the function of autologous regulatory sequences, mostly related with genes involved in productive traits. Previous to these studies it was convenient to assess the response of S. aurata to DNA transfer processes. For this purpose the plasmids pCMV-CAT, pCMVTklacZ, and pEGFP-N1 were injected. The embryo and larval survival rates and the respective DNA fate and expression during the early development were evaluated. The relative utility of the three markers was emphasized, especially the advantages of the GFP over the lacZ marker.

Results

The seabream response to the transfer of pCMV-CAT, pCMVTklacZ, and pEGFP-N1 plasmids (Figure 1) was assessed by analyzing embryo and larval survival, DNA persistence, and transgene expression, in early development.

Survival analysis

In order to minimize the negative effect of the injected DNA on embryo viability (Fletcher and Davies, 1991), different DNA concentrations were evaluated, (results not shown). Results from these experiments indicated that the dose of 10^8 plasmid copies (100 ng/µl) per embryo produced the highest levels of expression without adversely affecting survival. The effect of the microinjection technique on mortality was evaluated by comparing the survival values of embryos microinjected with the plasmid pCMV-CAT and an un.injected control group at different times (Figure 2). The survival rates, expressed as the means of the percentages of living embryos, showed a general decreasing tendency with respect to the increasing development time. The survival values of the two groups were compared by the statistical test for differences between two regression coefficients (Sokal and Rohlf, 1996) and found to be significantly different ($F = 4.58$, $p < .01$). The estimated average reduction in viability in the transgenics relative to the controls was around 23%.

DNA persistence

The fates of both linear and supercoiled forms of the three plasmids, pCMV-CAT, pCMVTklacZ, and pEGFP-N1 (Figure 1), were followed by Southern blotting. The results from the respective supercoiled forms are exemplified by the pattern obtained from the pEGFP-N1 plasmid (Figure 3). The signal intensities, representative of the amounts of pEGFP-N1, sharply decreased after 12 hours of development, while pCMVTklacZ and pCMV-CAT decreased after 24 hours (data not shown). In lanes corresponding to samples with undigested DNA (odd lanes from 5 to 15), bands of different mobilities are shown, indicating that the plasmid is present in various configurations, such as supercoiled (z), linearized (y), open circular (x), and higher molecular weight fractions (w and v). The interpretation of the monomeric conformations was based on a Southern blot using DNA under the same condi-

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**Figure 1.** DNA constructs used in the microinjection process after linearization with the corresponding restriction enzyme (see text): (a) the pCMV-CAT; (b) the pC-MVTklacZ; and (c) the pEGFP-N1 plasmids. Bacterial plasmid regions are represented with striped boxes, open boxes are the regulatory regions, closed boxes are the coding sequences, and shaded boxes are the polyadenylation sequences. The relevant restriction sites are indicated.

**Figure 2.** Survival rates of seabream embryos and larvae over the first 3 days of development. Open circles represent controls; closed circles, microinjected embryos. The standard error is represented by the T bars.
The bands from the odd lanes indicate that the DNA was present in the transgenic embryos in either monomeric or multimeric forms. In the even lanes (4–14), where the DNA was linearized with BamHI (Figure 1c), a strong band of 4.7 kb was present. The conversion of the high molecular weight bands (w and v) into unit size bands (y) after digestion (even lanes) is indicative of either head-to-tail multimers or concatenates. The patterns obtained from the samples with the pCMVTklacZ and pCMV-CAT plasmids (data not presented) were similar to those described for pEGFP-N1 (Figure 3) in terms of the presence of monomeric and multimeric configurations of the transgenic DNA.

The results from the linearized forms of the three plasmids are exemplified by the Southern blot pattern obtained from the pCMVTklacZ plasmid (Figure 4). Upon the injection of its XbaI linearized form, the Southern blotting showed in lanes with undigested DNA (5, 7, 9, 11, and 13), bands of approximately 16 kb (u), as well as faint bands of higher molecular weight (t). In lanes 4, 6, 8, 10, and 12, in which the transgene was digested with KpnI (Figure 1b), the presence of two bands of approximately 4.2 kb (x) and 3.8 kb (y), indicates that bands from odd lanes correspond to head-to-tail multimeric molecules. Two other bands of about 2 kb (z) and 5.6 kb (w), in lanes 4 and 6, suggest the presence of a smaller amount of polymeric plasmids in either head-to-head or tail-to-tail conformations.

The patterns obtained from the linearized forms of the pCMV-CAT and pEGFP-N1 plasmids (Figures 1a and 1c) were very similar in terms of signal intensity decreasing after 12 to 24 hours and the presence of only multimeric configurations (data not presented).

**Reportor gene expression**

The expression efficiencies of the three constructs were assessed through the proportions of positive individuals from various developmental stages, after being assayed for the corresponding reporter gene. The CAT activity shown by transgenic embryos is documented in Figure 5, in which all groups screened in the plate were positive. The expression frequencies are summarized in the histogram of Figure 6a. Exogenous CAT expression initiated at 7 hours after fertilization with linearized and supercoiled plasmids and was maintained through 4 days of development. To evaluate statis-
tically the influence of plasmid conformation and time of development on the expression levels, a two-way analysis of variance (ANOVA) was performed, revealing a significant effect as a function of the plasmid forms ($F = 3.40, p < .005$) as well as the time of development ($F = 8.02, p < .005$).

Evidence of β-galactosidase activity by immunohistochemical assays could be detected in microinjected embryos but never in controls, indicating that the activities were exclusively of exogenous origin. The results of the temporal expression of the pCMV-TklacZ plasmid are shown in Figure 5b. The blue patches started to appear at 7 hours, and the proportion of positive individuals gradually decreased to the lowest values at 72 hours. No expression was detected beyond this time. According to the ANOVA test, the expression activity was influenced by time of development ($F = 3.58, p < .005$), but not by the plasmid conformation ($F = 9.18, p > .005$). The spatial expression patterns (Figures 7a–7d) were mosaic at all stages with a large variation in the extent and distribution of the stained areas. In general the extension of the stained areas never surpassed 50% of the whole embryo, was most extensive at about 12 hours (Figure 7a), and progressively decreased thereafter. No tissue-specific patterns of expression were seen (Figure 7b), as would be expected owing to the ubiquity of the promoter used, so that the expression areas involved both extraembryonic and embryonic tissues (Figure 7b). However, expression in ventral tissues surrounding the resorbing yolk sac was relatively common (Figure 7c).

The temporal expression patterns from the supercoiled pEGFP-N1 plasmid (Figure 6c) revealed fluorescent cells in about 50% of the embryos at around 7 hours after fertilization, shortly after the mid-blastula transition. At 24 hours the level of positive reaction sharply decreased, and no fluorescence was detected after 72 hours. The patterns were very similar to those found with the two forms of the lacZ plasmid. However, with the linearized GFP plasmid, expression was not observed earlier than 12 hours, and the GFP was still visible in 4-day-old embryos and even persisted in later stages (unpublished observations). The ANOVA test revealed a significant effect of developmental time ($F = 4.02, p < .005$), but not a significant influence of plasmid conformation ($F = 9.28, p < .005$), on the expression rates. The spatial expression patterns were similar to those described for lacZ in terms of mosaicism, ubiquity, and lack of tissue specificity (Figures 8a and 8d).

Discussion

Effect of microinjection on embryo viability

Because of the reported technical difficulties in microinjecting seabream embryos (Knibb et al., 1994), the first step of this study consisted in evaluating the damage produced by the microinjection technique itself, without using any method to prevent the chorion from hardening. This was accomplished by comparing the survival rates of the microinjected groups with their respective uninjected controls (Figure 2). The results showed a parallelism between the microinjected groups and the respective controls and a clear tendency of decreasing survival with development time. Previous microinjection attempts in this species had reported survival rates of around 10% at 48 hours (Cavari et al., 1993), or around 34% at 24 hours (Knibb et al., 1994). In the latter study different chemical treatments had to be used to inhibit the chorion from hardening to ease the microinjection process. Their reported survival values are clearly lower than ours, which reach around 50% (Figure 2). Survival data from other fish are available only from freshwater species, with less hard chorions (Fletcher and Davies, 1991). In general these data vary considerably between species and experiments, but the highest values are in all cases very close to ours. Both kinds of comparisons, within the same species and among other species, indicate that in spite of the unfavorable natural conditions of seabream eggs for microinjection, the procedure adopted in
this work without using any chorion treatment proved relatively successful in terms of viability.

**DNA persistence**

The strategy to inject linearized or supercoiled plasmids was influenced by the discrepant results in other fish species in terms of persistence and expression. The comparison of the Southern blot patterns obtained from both forms of the plasmids (Figures 3 and 4) allowed us to evaluate the fates of each form of DNA. A common observation from all of the plasmids in the supercoiled and linear configurations was a decrease in the signal intensity in samples after 24 to 48 hours, which can be interpreted as an overall reduction in the total amount of the foreign DNA remaining in the embryos. Similar patterns of degradation have been reported in other fish species (Winkler et al., 1991; Volckaert et al., 1994), presumably owing to random nuclease action, occurring at a higher rate than the replication process. This is similar to what happens in *Xenopus* (Marini et al., 1988). Although the signals are very faint or nonexistent beyond 3 days of development, DNA persistence after that time has

![Figure 6](image_url). Temporal expression pattern of the seabream embryos and larvae from different stages, microinjected with the pCMV-CAT plasmid (a), pCMVTKlacZ plasmid (b), and pEGFP-N1 plasmid (c). The number of individuals per sample ranged from 52 to 71, from 33 to 36, and from 110 to 128, respectively. Development times are represented along the abscissa and the percentage of positive samples on the ordinate.
been implied from expression activity detected at 13 days (data not shown). To delimit the actual duration of the extrachromosomal plasmid persistence, further developmental stages must be screened.

Both monomeric and multimeric forms of the three plasmids persisted in all stages screened (Figure 4 and data not shown). Digestion of the multimeric forms suggests that they are concatenates in head-to-tail orientations, as seen in other fish species (e.g., Chong and Vielkind, 1989). However, when linear plasmids were used (Figure 4b), no monomeric forms were observed. Rather, all of the exogenous DNA appeared in high molecular weight conformations in all possible combinations (head-to-tail, head-to-head, and tail-to-tail), presumably as the result of random ligation (reviewed in Iyengar et al., 1996). These results suggest that linear plasmids were rearranged into concatemers at a much higher rate than circular plasmids, which were more often maintained as monomers.

When linearized and supercoiled forms are correlated with the exogenous DNA persistence, our results reveal no significant differences between them, as both forms of the plasmid decrease around the same developmental time (Figures 3 and 4). This suggests that degradation processes equally affect both forms. Our findings are supported by other studies in fish, such as Chong and Vielkind (1989) in medaka and Volckaert et al. (1994) in the African catfish. However, an enhanced persistence of the linear versus the supercoiled form has been found in medaka (Winkler et al., 1991) and rainbow trout (Iyengar and Maclean, 1995). In the latter, the authors proposed that this difference is due to a more efficient concatenation, though no experimental evidence was presented. These observations are supported by the evidence in Xenopus that DNA conformations from linear plasmids are more efficiently replicated and persist longer than those derived from circular molecules (e.g., Endean and Smithies, 1989; Fu et al., 1989). In contrast, we found a higher efficiency for concatenation formation with linear plasmids, but contrary to the experience of others, the persistence was not appreciably different. This lack of agreement could be due to other variables that influence DNA persistence, such as the sequences used in the constructs (Asano and Shiokawa, 1993), or the number and size of the plasmid molecules (Winkler et al., 1991).

In our experiments persistence was not only similar when comparing linear versus circular forms, but also for the three plasmids, which have different sequences and sizes (Figure 1). Accordingly, we suggest that developmental events are the main factor influencing plasmid persistence.

Although the main objective of this work was not to measure the integration of the transgenic constructs, the Southern blot patterns in Figure 3 suggested that all high molecular weight bands were multimers rather than integrants. No integration could be inferred from the blotting data, which is not improbable if one takes into account the low integration rate expected in fish (Rahman et al., 1997) and the limitations of this technique for reliable detection.

**Reporter gene expression**

The temporal patterns of both forms of the three plasmids (Figures 6a–6c) reveal that expression is initiated at about 7 hours after fertilization, which corresponds approximately with the late-blastula stage in seabream (Alessio and Gandolfi, 1975). This event has been associated in zebrafish (Kane and Kimmel, 1993) and in medaka (Tsai et al., 1995) with the activation of endogenous genes, which almost invariably occurs after the mid-blastula transition (MBT), irrespective of the promoter used. The 5-hour delay in initiation of expression from the linearized GFP plasmid relative to the circularized form may be due to differences in time needed to acquire sufficient product for detection of fluorescence (Davis et al., 1995).

Once the exogenous DNA expression was initiated, in the case of the CAT plasmid (Figure 6a) it continued over all the stages surveyed, and the expression levels were higher with the linearized form. However, the two forms of the lacZ (Figure 6b) and the GFP (Figure 6c) plasmids showed progressive declines in their apparently similar expression levels after 10 to 12 hours. This decrease paralleled their respective Southern blot signal intensities (Figures 3 and 4 and data not shown), as well as with the extent of the stained regions during development (Figure 6). The apparent lack of correlation between expression levels (Figure 6a) and persistence in the CAT plasmid (data not shown), as well as the apparently significant differences in expression between the two forms, have to be interpreted cautiously, taking into account that each sample corresponds to five individuals and that statistical tests are not very reliable under these conditions. Accordingly, we conclude that our results do not strongly support a marked influence of the plasmid configuration either in plasmid persistence or in transient expression level, in agreement...

The three markers used in this study can be used in S. aurata if put behind the highly efficient CMV and Tk viral promoters, as has been done in other fish species, both in vitro (Hernandez-Béťancourt et al., 1993) and in vivo (Tewari et al., 1992). Nevertheless, the high percentage of samples showing CAT activity during development times when DNA signals were very faint suggests a higher degree of sensitivity of CAT compared with lacZ and GFP, even though CAT and GFP transcripts were transcribed from the same promoter. The two spatial markers (lacZ and GFP) allowed us to locate the transgene-expressing cells, demonstrated the inherent mosaic patterns of expression, the lack of cell type specificity of the regulating sequences, as well as the high transgene activity in extraembryonic tissues (Figure 7), including the yolk syncitial layer (YSL). This last observation supports the findings of Williams et al. (1996) of higher levels of expression activity in these tissues. According to these authors, this aspect should be taken into consideration in quantifying expression levels in embryos with yolk sac that contain extraembryonic tissues.

While CAT and lacZ have been widely used as markers in fish, the recently described GFP (Chalfie et al., 1994) has been tested only in zebrafish as a convenient live staining marker (Amsterdam et al., 1995; Moss et al., 1996). We found that when the GFP gene was injected into S. aurata embryos, green fluorescence was easily detectable and appeared to have no adverse effects on embryonic development despite widespread expression (data not shown). The utility of the GFP in this species to analyze the regulation of tissue-specific expression is enhanced by the fact that seabream embryos are nearly transparent during the first weeks of development and by day 3 the hatched larvae have almost completed their morphogenesis and rudimentary organs are easily observable. This advantage can also be useful in production of transgenic lines as an indicator of transgene expression in early embryos, thus allowing positive selection with higher efficiency and lower costs. The primary advantage of GFP over lacZ is that gene expression can be continuously followed in living embryos, while fixed embryos are lost to further analysis. Furthermore, the visualization of β-galactosidase activity by the addition of chemical components in living cells can be disturbing. However, the relative degrees of sensitivity of detection of these markers cannot be determined from this study because their promoters were different. However, a superior sensitivity of GFP over lacZ when driven by the same promoters has been demonstrated in mice (Chiocchetti et al., 1997).

In this work we demonstrate the utility of this marine species as a suitable system to be manipulated by DNA transfer and show parameters that affect the persistence and transient expression of transgenes in vivo. This information will be useful for assessing the function of regulatory sequences that have already been cloned from S. aurata.

**Experimental Procedures**

**Maintenance of broodstock and egg manipulation**

The broodstock from a private company, located in the South coast of Spain, has been bred for several generations and is able to spawn through almost the complete year under artificial photoperiods. Naturally fertilized eggs collected from the reproducer tanks were aligned into depressions shaped with 0.9-mm capillaries in a 2% agarose dish stained with methylene blue. Embryos at the one-
to two-cell stage were microinjected in the cytoplasm with 8 nl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8) colored with 0.2% red phenol, containing approximately 10⁸ plasmid copies (100 ng/µl). No specific treatment to weaken or to remove the chorion was applied, and eggs were kept in marine water throughout the process. Microinjection was performed with borosilicate needles and a mechanical micromanipulator. Because the one-cell stage is clearly visible only 15 minutes prior to the first cell division, most microinjections were performed at the two-cell stage, which is visible over a longer duration. To slow down the cleavage rate, the incubating water temperature prior to injection was reduced to 14°C. Subsequently 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 gene constructs

The plasmids used were 5-kb pCMV-CAT (Figure 1a) containing the enhancer and promoter of the immediate early gene of human cytomegalovirus (CMV), fused to the chloramphenicol acetyl transferase (CAT) gene, along with the early SV40 splice and polyadenylation signal (Foecking and Hofstetter, 1986); the 8.05-kb pCMVTklacZ (Figure 1b) containing the CMV enhancer and herpes simplex thymidine kinase (Tk) promoter, fused to the E. coli lacZ gene and SV40 polyadenylation signal (Winkler et al., 1994); the 4.7-kb pEGFP-N1 plasmid from Clontech (Figure 1c) containing the CMV promoter and SV40 polyadenylation signal. To obtain the corresponding linearized forms, the pCMV-CAT and pCMVTklacZ plasmids were digested with XbaI, while the pEGFP-N1 plasmid was digested with ClaI.

Extraction and analysis of DNA

For DNA screening, embryos and larvae from the same state 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 minutes at 4°C, the pellets were kept at −80°C. In samples microinjected with the pCMV-CAT, the supernatant was used for CAT assays.

For Southern analysis the DNA from 20 individuals was pooled together. For DNA extraction, the reserved homogenate was incubated 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 2× volume of ethanol, washed in 70% ethanol, and resuspended in TE buffer. Subsequently half of the DNA was restricted with the appropriate enzyme, and the other half remained undigested.

DNA fragments were separated by electrophoresis on 0.8% agarose gels, stained with ethidium bromide, denatured, and neutralized after being treated for 10 minutes with 0.25 M HCl and then transferred to a nylon membrane (Dupont, Boehringer Mannheim). The complete plasmid was used as probe and ³²P-labeled with Klenow polymerase using random primers (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 two times in 2% SDS/2× SSC at room temperature and two more times in 0.1% SDS/0.5× SSC at 65°C. After that they were exposed to radiographic film (X-Omat Kodak) in a cassette at −80°C for 1 day to several days.

CAT assay

Groups of five embryos or hatchlings were pooled in each sample for CAT activity screening. The supernatant obtained from the centrifugation was heated at 55°C for 10 minutes. The protein extract was incubated for 2 hours at 37°C with 8 µl ¹⁴C-labeled chloramphenicol (Amersham; 25 µCi/ml) and 20 µl of 4 mM aqueous solution of acetyl coenzyme A (Sigma). The 150 µl of mix was completed with 250 mM Tris-HCl, pH 7.5. After extraction with ethyl acetate, the reaction products were dried down in a Speed-Vac, redissolved in ethyl acetate, spotted on a silica gel thin layer plate, and run with chloroform-methanol mixture (95:5) for 50 minutes. The plates were air-dried, and the autoradiogram was developed after 4 to 5 days (Gorman et al., 1982).

β-Galactosidase assay

For LacZ expression, undechorionated embryos were fixed for 30 minutes at 4°C in 2% paraformaldehyde, 0.2% glutaraldehyde, and 0.02% nonidet P-40, in phosphate-buffered saline (PBS). The embryos were stained for at least 16 hours at 30°C, in a solution containing a mix of 974 ml of the
solution A and 26 ml of solution B. Solution A: 0.2 M Na-phosphate buffer, pH 7.3, 5.0 M NaCl, 1 M MgCl$_2$, 0.1 M K$_2$Fe$_3$(CN)$_6$, and 0.1 M K$_4$Fe$_6$(CN)$_6$. Solution B: 8% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) in DMF (dimethyl formamide). Stained embryos were kept in 1x PBS.

**GFP observation**

The EGFP protein is a red-shifted mutated variant that is more fluorescent than the wild form. To be visualized, live embryos and larvae were placed on a glass dish with seawater and observed under an Olympus 1 x 50 SBF inverted microscope equipped with an IX-FLA attachment. One inconvenience encountered in observing the GFP was autofluorescence of the GFP. To distinguish the green signal of GFP from the yellow autofluorescence (Figures 8a and 8c), we had to optimize the use of the fluorescein filter set (BP 460-490 and BA 515-550). Owing to the larval mobility, the bright-field and fluorescent micrographs were taken on high-speed film.

**Acknowledgments**

The authors acknowledge the Co. Cupimar, S.A. (San Fernando, Cádiz), for providing the eggs and the facilities. Special thanks to E.R. Bejarano for technical advice.

The plasmids pCMV-CAT and pCMVTklacZ were kindly supplied by D. Chourrout and M. Schartl, respectively. This work has been supported by grant BIO2-CT93-0430 from the European Union.

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