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Expression and fate of CAT reporter gene microinjected into fertilized medaka (*Oryzias latipes*) eggs in the form of plasmid DNA, recombinant phage particles and its DNA

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Summary. Fertilized medaka (*Oryzias latipes*) eggs were cytoplasmically injected with the chloramphenicol acetyltransferase (CAT) gene encompassed in supercoiled and linear plasmid DNA, as well as in intact recombinant phage particles and DNA isolated from the phage. Expression for the CAT plasmid DNA was highest at the gastrula/neurula stage, while for the DNA of the phage, it peaked in the 1-week old embryo; then expression declined but was still detectable in early adulthood (4 weeks post injection). Following the fate of exogenous DNA, an extensive replication was observed in early embryogenesis, and DNA was still found 4 weeks after injection, suggesting a possibility of integration. The system is useful as a transient expression system for the analysis of early developmental genes in particular, but also as a test system for the analysis of cloned genes of interest for the farming of economically important fish species. The fact that DNA transferred in intact phage particles or its DNA is functionally active opens the possibility to introduce larger DNA pieces (20 kb), e.g., for the functional test of larger and more distant control regions.

Key words: Fish – Cytoplasmic injection – Recombinant phage – Gene transfer – Gene expression

Introduction

The analysis of the expression of exogenously introduced genes in oocytes, fertilized eggs and developing embryos has greatly contributed to an understanding of the requirements for controlled temporal and spatial gene expression. This approach has made use of transient expression systems such as developing *Xenopus* (Etkin 1982; Wilson et al. 1986) and sea urchins (Katula et al. 1987), and also of stably transformed lines, mostly of the mouse (for reviews, see Palmiter and Brinster 1986; Jaenisch 1988) and *Drosophila* (Rubin and Spradling 1982; Spradling and Rubin 1982, 1983), and recently also of *Caenorhabditis* (Fire 1986). Gene transfer also offers the potential of altering commercially important traits such as growth rate, immunity to specific diseases or tolerance of temperature extremes in livestock (Hammer et al. 1985).

Fish have not been exploited widely in gene transfer studies (Maclean et al. 1987). Most of the studies were centered on fish species of commerical interest, which may not be optimal systems for gene transfer studies because of the large yolk mass and thick, opaque chorion of the eggs, which make locating and microinjecting the nucleus difficult. In addition, many of these species have a long generation time, hampering the analysis of transgenes. The Japanese medaka (*Oryzias latipes*), however, appears to be an excellent model for such studies since its biology, including embryonic development and physiology, has been extensively studied. It has a short generation time, hundreds of eggs may be obtained daily (Yamamoto 1967) and the relatively large egg size and thin, transparent chorion make microinjection easy and permit easy observation of embryonic development. In this report, we wish to evaluate the potential of cytoplasmic injection of DNA into the fertilized eggs of the medaka prior to or immediately after first cleavage (1–2 cell stage embryo), with regard to its use as a model transient expression system for testing developmentally important genes, or genes with potential applications to the genetic engineering of commercial fish species. The chloramphenicol

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acetyltransferase (CAT) gene was chosen as a reporter gene because simple and rapid tests are available for the CAT gene product and no similar enzyme has been found in eucaryotic systems. A double viral promoter-enhancer consisting of the Simian virus 40 (SV 40) early region and the long terminal repeat (LTR) of the Rous sarcoma viurs (RSV) was chosen to regulate transcription of the CAT gene (Karlsson et al. 1985), because of its high CAT expression in many cell lines as compared to other CAT constructs (Vielkind and Vogel 1989; J. R. Vielkind, unpublished results).

There are a number of reports correlating the expression patterns of exogenous genes with the various conformations and the fate of the transferred DNA (Etkin and Balcells 1985; Wilson et al. 1986). We therefore applied the CAT transcriptional unit encompassed in recombinant plasmid of supercoiled and linear conformations and monitored CAT expression and the fate of the two DNA forms in the developing medaka embryo. Cell transfection studies by Ishiura et al. (1982) and Okayama and Berg (1985) have shown that phage particle-mediated gene transfer resulted in higher transformation rates in mouse cell lines compared to DNA-mediated gene transfer, presumably because of the phage coat protecting the exogenous DNA from degradation by nucleases. Additionally, phage allow larger sized genes of 15–20 kb to be cloned and are commonly used in the construction of genomic libraries. Successful expression of the DNA carried by the injected phage particles would not only allow long stretches of genomic DNA to be tested for effects of various introns and distant regulatory regions on gene transcription and translation, but would also obviate the need to use purified DNA. We therefore studied CAT expression and DNA fate after injection of recombinant phage particles, the genome of which contains three CAT transcriptional units. For comparison, we also studied expression and fate of microinjected purified CAT phage DNA.

Materials and methods

Egg collection and embryo culture. Clusters of fertilized eggs attached to medaka females were collected 1–2 h after the onset of light and maintained in Ringer solution (0.75% NaCl, 0.02% KCl, 0.02% $CaCl_2$, pH 7.3; Yamamoto 1961) at 12 °C prior to injection, to slow down the cleavage process and hardening of the chorion. Injected embryos were reared in medium of 1% NaCl, 0.03% KCl, 0.04% $CACl_2 \cdot 2H_2O$, 0,163% $MgSO_4 \cdot 7H_2O$, 0.001% methylene blue (Kirchen and West 1976) and transferred to tank water after hatching. Staging of medaka embryos followed Matsui (1949).

Recombinant CAT plasmid and phage. Figure 1 illustrates the various constructs encompassing the CAT gene used in this study. The transcriptional unit consists of the bacterial CAT coding sequence driven by the Rous sarcoma virus LTR and early SV 40 promoter-enhancer regions. This CAT transcription unit was subcloned into pUC9, yielding the recombinant plasmid pUSVCAT (details in Karlsson et al. 1985); digestion with SalI results in a linear molecule, also shown in the Figure. Three tandem copies of the 5.2-kb BamHI fragment of the pUSVCAT were subcloned into the EMBL3 phage (details in Vielkind and Vogel 1989), yielding the recombinant CAT phage.

DNA extraction and phage preparation. Plasmid DNA was extracted using a modified Brij detergent method (Clewell and Helinski 1969) and purified by two successive CsCl-ethidium bromide equilibrium density centrifugations. CAT phage was purified from plate lysates by CsCl step gradient centrifugation and dialysed against SM buffer (0.1 *M* NaCl, 0.01 *M* $MgSO_4 \cdot 7H_2O$, 0.05 *M* TRIS pH7.5, 0.01% gelatin). CAT phage DNA was extracted as described in Maniatis et al. (1982).

Microinjection. Supercoiled and linear pUSVCAT plasmid DNA, phage particles, or phage DNA were microinjected in approximately 500 pl into the cytoplasm of the medaka zygote

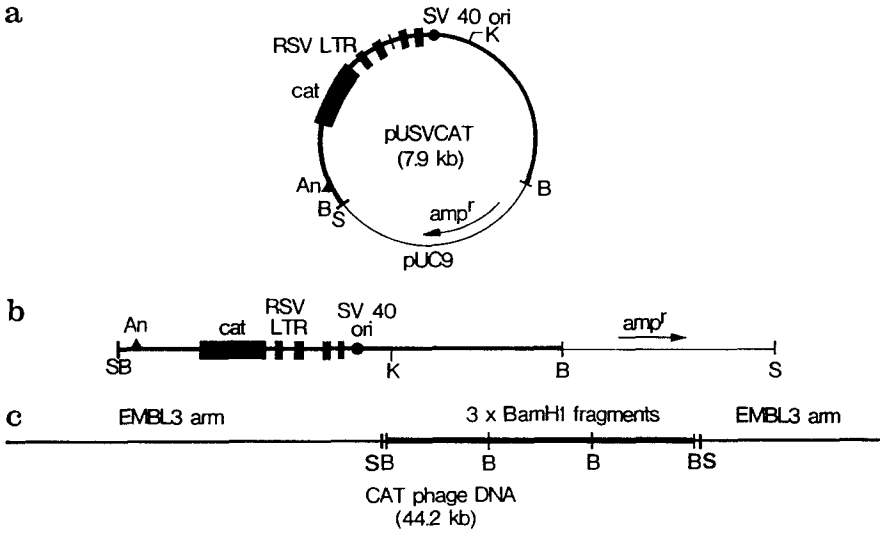

Fig. 1 a–c. Diagram of **a** supercoiled and **b** SalI-linearized pUSVCAT, and of **c** recombinant CAT phage. Illustrated are the CAT transcription unit (CAT coding sequence, LTR, and SV40 regions that contain enhancer-promoter regions, An: poly adenylation site) and the 5.2 kb-BamHI fragment containing the CAT transcription unit of pUSVCAT ligated to the EMBL3 phage arms. B, K, and S: BamHI, KpnI and SalI restriction sites

prior to or immediately after first cleavage (1–2 cell stage embryo), using a borosilicate glass capillary needle (3–5 µm diameter) mounted on a micromanipulator. Injections were done under a stereomicroscope (Zeiss) with a magnification range of 8–50×. Concentrations of 50 µg/ml plasmid DNA, 8×10^{10} particles per ml CAT phage (equivalent to 5 µg/ml CAT phage DNA), and 20 µg/ml CAT phage DNA were used. Phenol red was added to the DNA/phage solutions to a final concentration of 0.25% to aid in estimation of injection volume.

CAT assay. CAT assays were performed in principle as described by Gorman et al. (1982). Batches of three embryos, or individual embryos, hatchlings and free-swimming fish were homogenized in 100 µl 250 m*M* TRIS pH 8.0, subjected to three 5-min cycles of freeze-thawing and extracts were obtained after centrifugation (Eppendorf, 5 min, 4 °C). To 100 µl extract, 20 µl dH_2O, 2 µl ^{14}C-chloramphenicol (NEN DuPont, 60 mCi/mmol, 100 µCi/ml), and 20 µl 4 m*M* acetyl coenzyme A (Boehringer Mannheim) were added and the mixture was incubated for 1 h at 37 °C. The ^{14}C-chloramphenicol and its acetylated forms were extracted with 1 ml ethyl acetate, dried down under vacuum, resuspended in 30 µl ethyl acetate, then spotted and separated on silica gel chromatography plates (J.T. Baker) for 50 min in chloroform:methanol (95:5). The plates were air-dried and autoradiograms were produced by exposure to X-ray film (Kodak XAR-5) for 7 days in the presence of an intensifying screen.

DNA extraction from medaka. Batches of 10 embryos, as well as individual embryos and hatchlings were homogenized in 200 µl of 1 × SET (100 m*M* NaCl, 20 m*M* EDTA, 50 m*M* TRIS pH 7.8), 0.5% SDS, 0.5 mg/ml proteinase K (Boehringer Mannheim) and incubated for 2–4 h at 37 °C. The samples were then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by extraction with butanol:isopropanol (7:3). The DNA was precipitated with 2 vol of 95% ethanol for at least 2 h at −20 °C and redissolved overnight in TE (10 m*M* TRIS pH 8.0, 1 m*M* EDTA).

Southern blots. Aliquots of DNA (equivalent to single embryos) from pooled samples or DNA from individual embryos, hatchlings and free-swimming fish, either undigested or after complete digestion with restriction enzymes as specified by the manufacturer, were electrophoresed on 0.8% agarose (Bio-Rad) gels. Gels were soaked in 250 m*M* HCl for 10 min to partially hydrolyse DNA, two times in 1.5 *M* NaCl, 0.5 *M* NaOH for 15 min each to denature DNA strands, and two times in 1.5 *M* NaCl, 0.5 *M* TRIS pH 7.5 for 15 min each to neutralize gel pH; extensive washing followed the acid and alkaline steps. After capillary transfer of DNA onto nylon filters (Nytran, Schleicher and Schuell) in 20 × SSC buffer (1 × SSC is 150 m*M* NaCl, 15 m*M* Na-citrate, pH 7.0) for 2 h, the filters were dried under vacuum for 1 h at 80 °C.

Hybridizaitions. Filters were prehybridized for 15 min at 60 °C with a solution containing 3 × SSC, 10 m*M* TRIS pH 7.6; 10 m*M* EDTA, 0.5% SDS, 1 × Denhardt's (Maniatis et al. 1982) and 0.1 mg/ml yeast RNA. The filters were then hybridized in the same buffer overnight at 60 °C to pUSVCAT DNA labelled by random hexamer priming (Feinberg and Vogelstein 1983, 1984) with ^{32}P-dCTP to a specific activity of $> 5 \times 10^8$ cpm/µg. High stringency washes were done twice in 0.1 × SSC, 0.5% SDS for 30 min each at 60 °C, before autoradiography on X-ray film in the presence of intensifying screens.

Quantitation of DNA hybridization signals. Radioactive bands of hybridized medaka Southern blots were excised and radioactivity was counted in toluol cocktail in a scintillation counter; the remainder of each lane was also counted. Radioactive counts of bands resulting from Southern blots of known amounts of plasmid or phage DNA were used as references to convert the cpm values of medaka blots to picogram DNA amounts.

Results

CAT gene expression during medaka development: experiments with supercoiled and linear pUSVCAT

CAT enzyme assays of ten individual medaka of various stages derived from fertilized eggs injected with supercoiled pUSVCAT DNA were performed; representative results of this analysis are shown in Fig. 2a, indicating the range of expression and the number of positive animals. CAT enzyme activity was not detectable in the 32–64 cell stage embryo (2 h post-injection) but was very prominent in the gastrula/neurula stage (1 day post-injection), decreasing only slightly in the 1-week-old embryo (a stage when the eye and most major organs are formed). CAT activity decreased further at the time of hatching (2 weeks post-injection) and varied noticeably among the hatchlings assayed; it was still detectable in tow medaka of free-swimming fish stage (4 weeks post-injection), one of which yielded a strong signal.

Since in preliminary experiments we neither detected endogenous enzyme activities similar to the CAT enzyme nor inhibitory factors (Sleigh 1986; and Crabb and Dixon 1987) that interfere with CAT enzyme activity, the observed CAT activity patterns must represent the CAT expression pattern of the introduced CAT gene.

To analyse whether gene expression is affected by the conformation of the introduced DNA, the pUSVCAT was injected in linear form after digestion with SalI (Fig. 1). Representative results of these experiments are shown in Fig. 2b. The expression pattern is similar to that observed for supercoiled pUSVCAT injected embryos, but with possibly slightly stronger signals at the gastrula/neurula stage and marginally weaker signals at the hatchling stage. Very weak CAT expression was observed in two free-swimming stage fish.

CAT gene expression during medaka development: experiments with recombinant CAT phage particles and purified CAT phage DNA

Results of CAT expression in various stages as a consequence of injection of CAT phage particles into the fertilized egg are shown in Fig. 3a. No CAT gene expression was detectable in embryos assayed at the 32–64 cell stage, but by the gastrula/neurula stage weak expression was apparent. CAT expression was consistently found in the 1-week-old embryos, but subsequently decreased in hatchlings; weak expression was detectable in free-swimming stage fish.

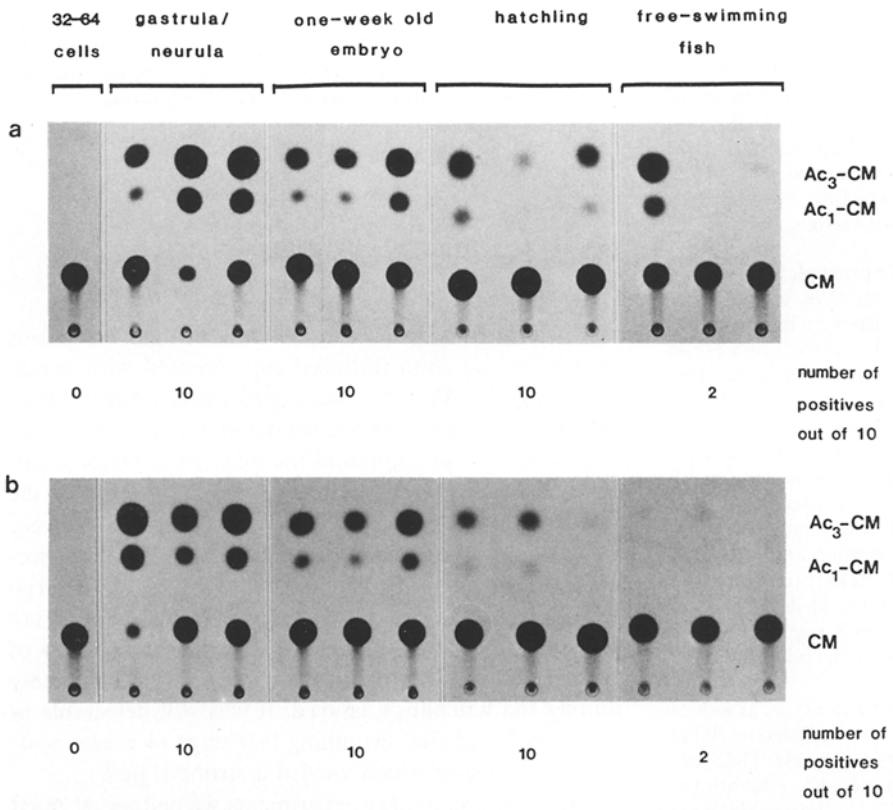

Fig. 2 a and b. CAT gene expression in individuals of various developmental stages derived from fertilized eggs injected with **a** supercoiled and **b** linear pUSVCAT. Injection volume was approx. 500 pl = 25 pg of 50 µg/ml DNA; CM = ^{14}C-chloramphenicol, Ac_1- and Ac_3-CM = acetylated forms of CM

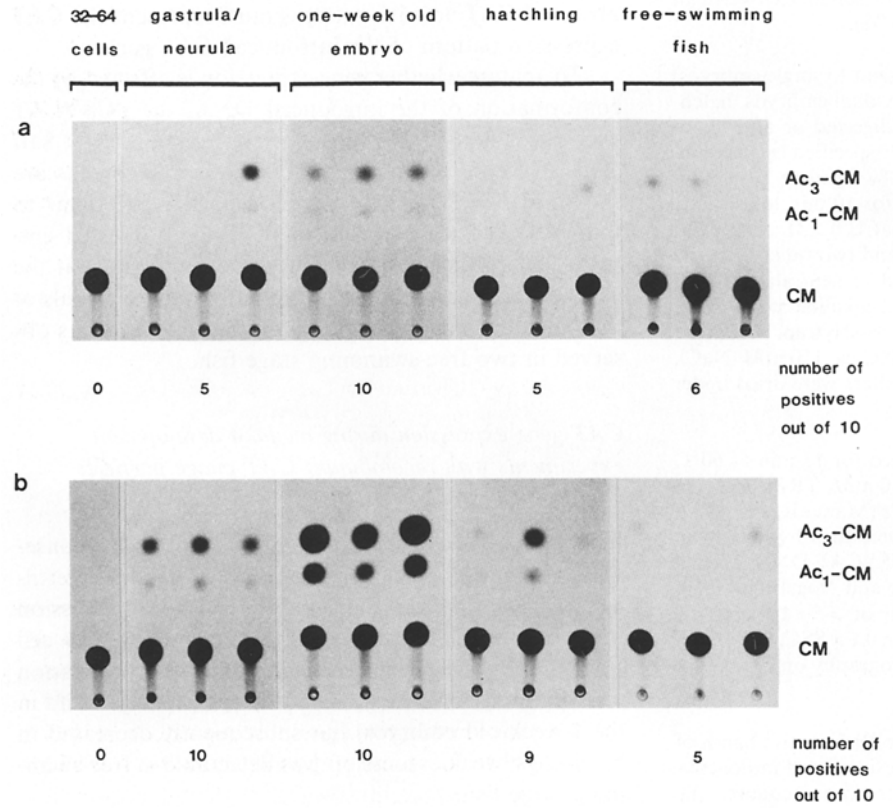

Fig. 3 a and b. CAT gene expression in individuals of various developmental stages derived from fertilized eggs injected with **a** recombinant CAT phage particles and **b** purified recombinant CAT phage DNA. Injection volume was approximately 500 pl = 4×10^4 phage particles of 8×10^{10} ml/phage and 10 pg of 20 µg/ml CAT phage DNA. For further details, see Fig. 2

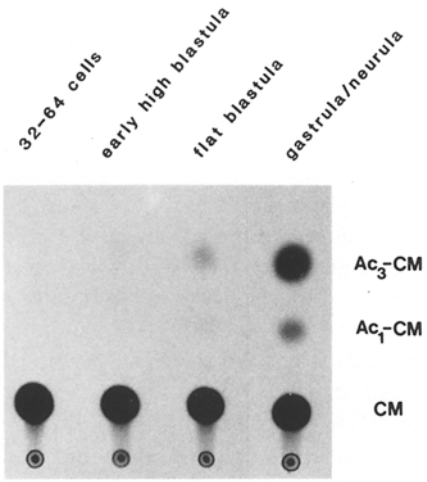

Fig. 4. Onset of CAT expression in early embryogenesis after injection of supercoiled pUSVCAT. Signals are from three pooled embryos, for further details, see Fig. 2

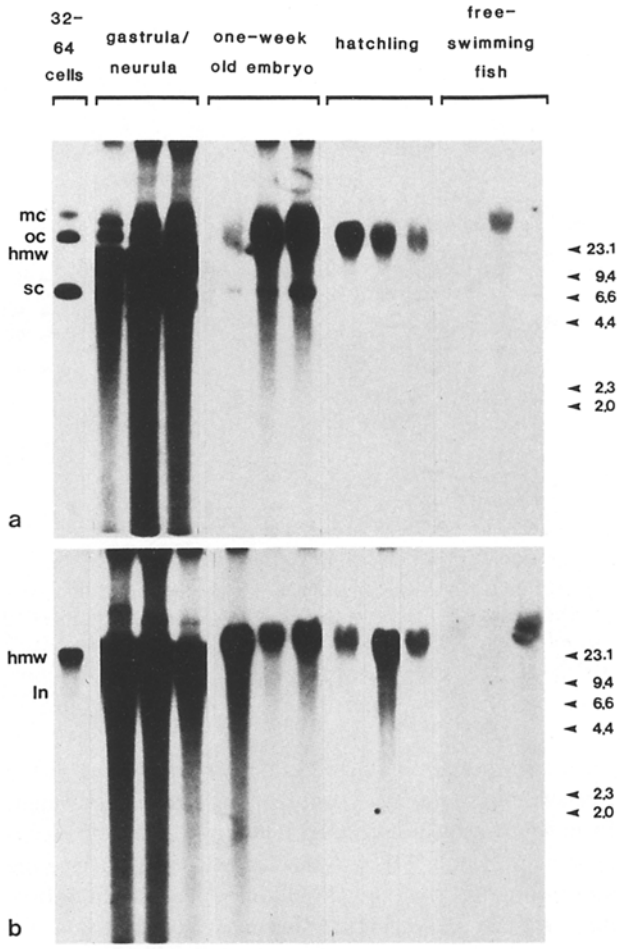

Fig. 5a and b. Fate of injected **a** supercoiled and **b** linear pUSVCAT in various developmental stages. DNA from individual medaka was electrophoretically separated in 0.8% agarose gels, transferred to nylon membranes and probed with ^{32}P radioactively labelled pUSVCAT (spec.act. $> 5 \times 10^8$ cmp/μg). Injection details as in Fig. 2; sc – supercoiled, oc – open circular, mc – multimeric circular, ln – linear, and hmw – high molecular weight form; 23.1 – 2.0 are in kb representing the standard fragments of a HindIII digest of phage lambda DNA

Injection with CAT phage DNA resulted in a pattern of CAT expression similar to that observed with CAT phage particle-injected embryos, but signals were generally stronger (Fig. 3b). Again, no CAT activity was seen at the 32–64 cell stage, while moderately strong signals were consistently detected at the gastrula/neurula stage and even stronger expression was observed in the 1-week-old embryos. Expression decreased sharply in the hatchling stage and varied widely in individual hatchlings, but CAT expression was still detectable in free-swimming stage fish.

Onset of CAT expression in early embryogenesis

The CAT gene was already strongly expressed by the gastrula/neurula stage. To investigate the expression pattern more closely and to investigate whether or not a similar situation might exist in medaka as for *Xenopus* in which nuclear genes are turned on at the mid-blastula stage (Newport and Kirschner 1982a, b) plasmid-injected embryos were harvested at earlier embryonic stages, i.e. between the 32–64 cell stage and the late gastrula/early neurula stage, in batches of three, and assayed for CAT cativity. The results (Fig. 4) show that CAT expression was clearly apparent at the flat blastula stage (10 h post-injection) and that it sharply increased at the gastrula/neurula stage (22 h post-injection). No differences in pattern of expression were observed when linear DNA was injected (data not shown).

Fate of supercoiled and linear plasmid DNA during medaka development

The fate of the introduced DNA was followed by Southern blot analysis. Representative results of analysed DNA from individual medaka derived from fertilized eggs injected with supercoiled pUSVCAT DNA are shown in Fig. 5a; with the exception of the Southern blot for the 32–64 cell stage, three examples out of six for each stage are shown. At the 32–64 cell stage, the extracted DNA contained supercoiled pUSVCAT and, in addition, open circular and multimeric circular forms, forms which we always found in pUSVCAT plasmid DNA preparations. By the gastrula/neurula stage an additional, high molecular weight, form migrating as a broad band of 20–30 kb, which is seen more clearly in Fig. 6a (see also below), could be detected. In the 1-week-old embryos, some of the plasmid DNA was still in supercoiled form, but most was of high molecular weight

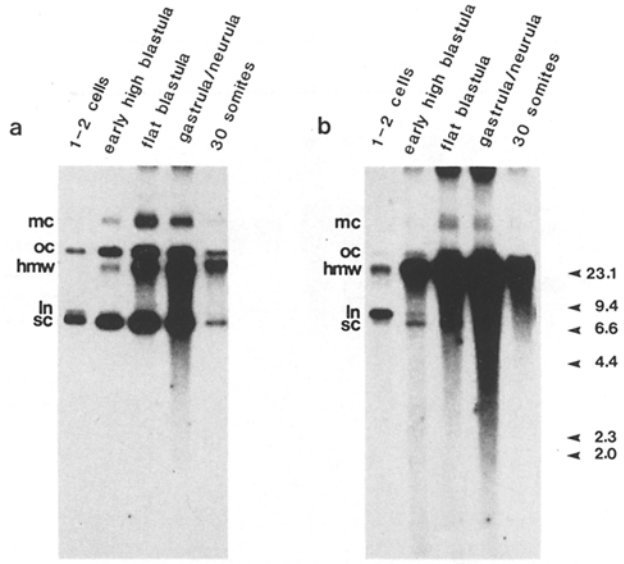

Fig. 6 a and b. Fate of injected **a** supercoiled and **b** linear pUSVCAT in early embryogenesis. DNA embryo aliquots of 10 pooled embryos were analysed. For further details, see Figs. 2 and 5

form larger than 30 kb. In hatchlings and in one of the free-swimming stage fish, only the high molecular weight form was detected; it co-migrated with the high molecular weight medaka DNA as determined by comparing the autoradiogram with the UV-photo of the ethidium bromide stained gels prior to Southern blotting (data not shown). Judging by the stronger hybridization signal present in the DNA of the gastrula/neurula stage, a sharp increase in amount of plasmid DNA seemed to have occurred, probably as a result of plasmid DNA replication (discussed in greater detail below). The hybridization signal then decreased continuously with progression of development. This decrease is apparently due to degradation of the plasmid DNA, as can be inferred from the hybridization smears most prominently observed in the DNA from gastrula/neurula stage embryos, but also seen in that of the 1-week-old embryos.

Southern blot analyses of experiments with linear pUSVCAT DNA are shown in Fig. 5b. DNA extracted from 32–64 cell stage embryos contained plasmid DNA in high molecular weight form; no linear molecules were seen. In the gastrula/neurula, additional bands which may represent multimeric circular pUSVCAT could be seen; further details are discussed below (see also Fig. 6b). In the other developmental stages, only the high molecular weight form that migrated at a higher position than in the stages before was observed; it co-migrated with high molecular weight recipient DNA (data not shown). A faint smear indicates that plasmid DNA degradation has already begun. There was also a marked increase in the amount of plasmid DNA in the gastrula/neurula stage and a decrease due to degradation in the later stages.

Fate of plasmid DNA during early embryogenesis

As described above, a significant amount of high molecular weight plasmid DNA was already present in the gastrula/neurula stage, implying that conversion of the injected forms to this high molecular weight form must have occurred at an earlier stage. Southern blot analysis was, therefore, performed on embryo-equivalent aliquots of DNA from ten pooled embryos of earlier stages, ranging from the 1–2 cell stage to the 30-somite neurula stage. The extracted DNA was digested with XhoI prior to Southern analysis. This enzyme digests high molecular medaka DNA but not pUSVCAT, thus reducing any possible influence of the larger size medaka DNA on the plasmid DNA separation pattern (Rusconi and Schaffner 1981).

The results of these experiments are shown in Fig. 6. DNA extracted from 1–2 cell stage embryos within 5 min of injection with supercoiled pUSVCAT DNA (Fig. 6a) contained the expected supercoiled, open circular and multimeric circular forms. The high molecular weight form appeared in the early high blastula stage (4 h post-injection); all forms were observed in the later stages. The amount of the pUSVCAT forms began to increase by the early high blastula stage and increased further in the flat blastula and gastrula/neurula stages, before decreasing in the 30-somite neurula. A smear was apparent in the DNA from the flat blastula, indicative of the onset of plasmid degradation which was more prominent in the gastrula/neurula stage.

DNA extracted from 1–2 cell stage embryos within 5 min of injection with linear pUSVCAT DNA (Fig. 6b) contained a high molecular weight form in addition to the linear form. A faint band corresponding to the open circular pUSVCAT form could also be observed. In the later stages the supercoiled as well as the multimeric circular forms could be observed. A similar pattern of increase and decrease in plasmid DNA was observed as described above for the fate of supercoiled injected DNA.

Nature of plasmid DNA sequences of high molecular weight

As described above, the input plasmid is exclusively in high molecular weight form by the hatchling stage. In order to determine the nature of this plasmid form, DNA was obtained from hatchlings derived from eggs injected with supercoiled and linear pUSVCAT DNA, digested with the single cutter KpnI (for details, see Fig. 1) and analysed by Southern blotting. The results are shown in Fig. 7a–c; in Fig. 7d the results of a blot of KpnI-digest-

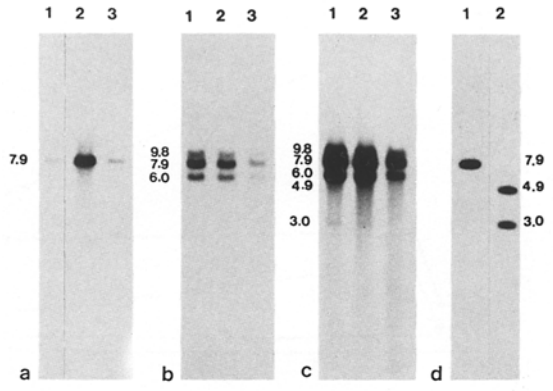

Fig. 7 a–d. Nature of high molecular weight pUSVCAT in three individual hatchings each derived from fertilized eggs injected with **a** supercoiled and **b** linear pUSVCAT (Fig. 2). DNA from individuals was digested with KpnI and further analysed (Fig. 5); **c** represents an overexposure of blot shown in **b**; **d** separation of KpnI digest of supercoiled (*lane 1*) and SalI-linearized pUSVCAT *lane 2*; **e** diagram of hypothetical multimer derived from linear head-to-head, head-to-tail and tail-to-tail junctions

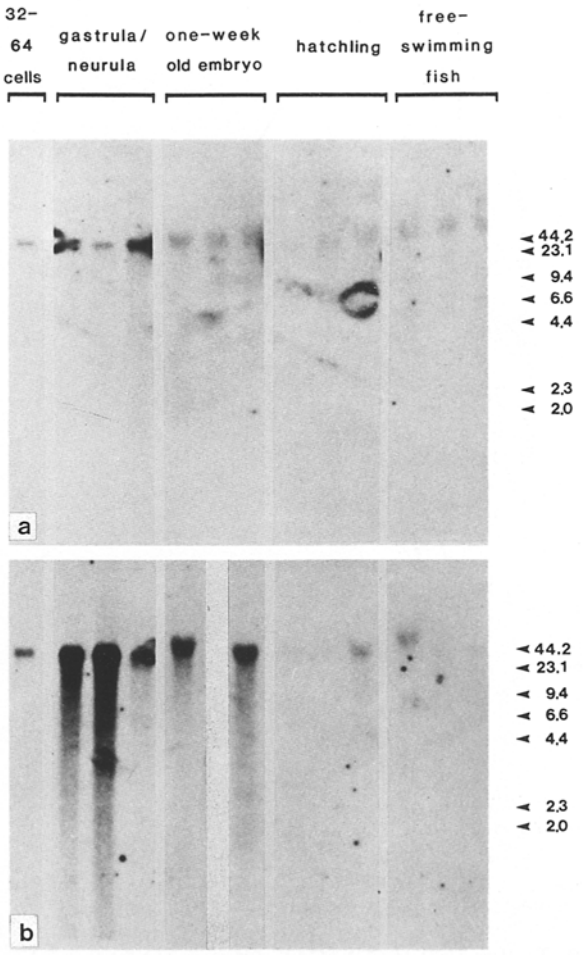

Fig. 8 a and b. Fate of DNA injected as **a** CAT phage particles and **b** purified CAT phage DNA. 44.2 (in kb) marks the position of one genome length of the recombinant phage DNA. For further details, see Figs. 3 and 5

ed supercoiled (lane 1) and SalI-linearized pUSVCAT DNA (lane 2) are also shown. The DNA from the supercoiled group yielded one band of 7.9 kb, corresponding to the unit length of the pUSVCAT plasmid (Fig. 7a). This suggests that the majority of the high molecular form consists of multimeric circles or of a large tandem array of identical linear pUSVCAT monomers linked head-to-tail, since no end fragments were observed (see below). The DNA from the hatchlings of the linear injected group showed a different pattern; one prominent band of 7.9 kb and two weaker bands of 9.8 and 6.0 kb (Fig. 7b) were observed. A longer exposure of the Southern blot shows, in addition, a very weak band of 3.0 kb and seemingly also a weak band of 4.9 kb, obscured by the smear of the overexposed film (Fig. 7c, lanes 1 and 2). This pattern suggests that most of the high molecular form derived from linear pUSVCAT consists of head-to-tail arrays yielding the most prominent 7.9-kb fragment. The additional 9.8- and 6.0-kb fragments can be explained by assuming that a head-to-head and tail-to-tail ligation has also occurred; such an array is illustrated in Fig. 7e. The very weak 4.9- and 3.0-kb fragments appear in either case of junctional arrangement and represent the end fragments. Their low concentration compared to the other fragments suggests a relatively large size of the concatemers.

Fate of DNA introduced within recombinant phage particles and of phage DNA during medaka development

The results of the Southen blot analysis of the fate of CAT DNA sequences introduced within phage are shown in Fig. 8a. At the 32–64 cell stage, a band corresponding to CAT phage DNA was observed. The phage DNA was also present in the gastrula/neurula and in the later stages. It increased slightly in the gastrula/neurula stage, then decreased in the 1-week-old embryo but stayed the same in the later stages, concurrently showing a very light smear indicative of degradation of the CAT phage DNA.

Embryos injected with CAT phage DNA and analysed at the 32–64 cell stage showed a DNA band corresponding to unit length CAT phage DNA (Fig. 8b). This DNA was also observed in the later stages but seemed to have a wider size distribution. There was an increase in the gastrula/neurula stage with a subsequent

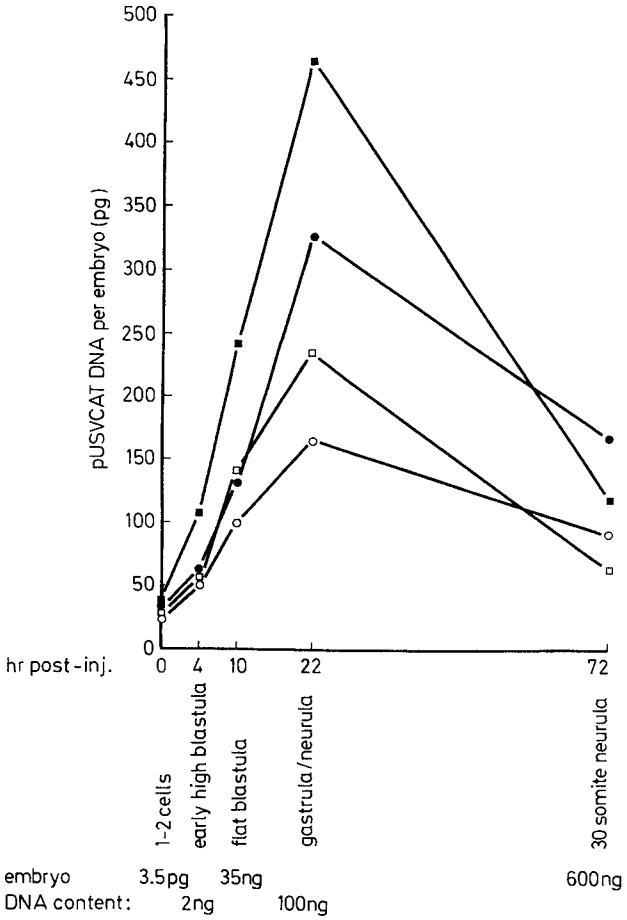

Fig. 9. Total content of pUSVCAT and content of intact CAT transcription units in early embryonic stages derived from eggs injected with supercoiled or linear pUSVCAT (Fig. 2). DNA embryo aliquots of ten pooled embryos were digested with SalI and further analysed (see Fig. 5 and text). ●——● and ○——○ total exogenous pUSVCAT DNA and intact CAT transcription units after injection with supercoiled, and ■——■ and □——□ total DNA and intact CAT transcription units after injection with linear DNA

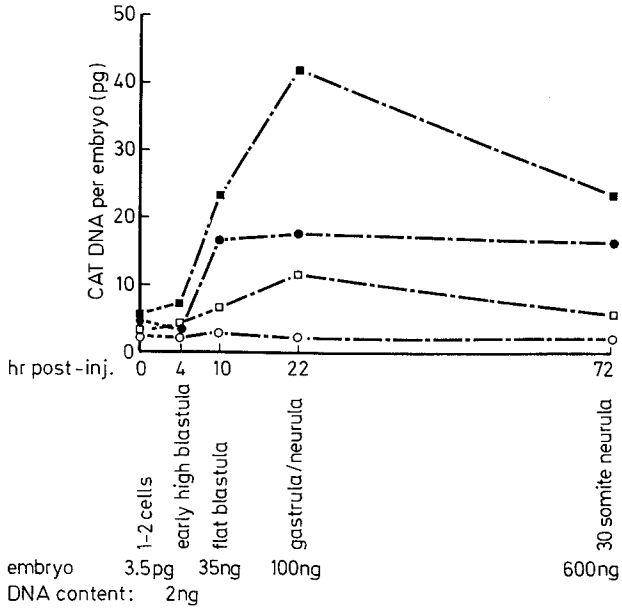

Fig. 10. Total content of phage DNA and content of intact CAT transcription units in early embryonic stages derived from eggs injected with CAT phage particles or CAT phage DNA (Fig. 3, but injection with CAT phage DNA was done with 5 µg/ml DNA). DNA aliquots of ten pooled embryos were digested with SalI and further analysed (see Fig. 5 and text). ●–·–● and ○–·–○ total phage DNA and intact transcription units after injection with phage particles; ■–·–■ and □–·–□ total phage DNA and intact transcription units after injection with phage DNA

decrease in the following stages, presumably due to degradation already obvious by the smear at the gastrula/neurula stage.

In both treatment groups, a shift of the CAT phage DNA toward higher molecular weight was observed at the hatchling and free-swimming fish stages; this high molecular phage DNA migrated always slightly below the high molecular weight recipient DNA. A dimer of the input phage DNA is almost 100 kb, which would either migrate within or probably more slowly than the medaka DNA. It is, therefore, likely that the detected phage DNA is either a broken phage concatemer or a phage DNA monomer restricted in migration by the high molecular weight medaka DNA.

Degree of foreign DNA replication in early embryos

Regardless in which form the DNA was introduced into the fertilized egg, an increase of input DNA was observed in the gastrula/neurula stage, suggesting replication of the exogenous DNA. In an attempt to quantify the degree of foreign DNA replication in early embryos up to the gastrula/neurula stage, a quantitative Southern blot hybridization assay of SalI-digested aliquots of embryo DNA was performed. SalI digestion of undegraded DNA leads to the 7.9-kb linear pUSVCAT monomer containing a functional CAT transcription unit and to a 15.6-kb fragment of the recombinant phage genome containing the three CAT transciption tandems (Fig. 1). In contrast to the simpler 'dot blot' assay, this method allows the determination of the amount of intact, functional CAT genes, i.e. DNA present in the 7.9- and 15.6-kb fragments, and of total DNA, i.e. intact and degraded DNA present in the fragments and in the smears, respectively.

The results of quantitation of sequences containing intact CAT genes and total input DNA present between 5 min and 3 days after injection are illustrated graphically in Figs. 9 and 10. In experiments using plasmid DNA (Fig. 9), the maximum amount of exogenous DNA was

observed in the gastrula/neurula stage (1 day post-injection) representing a ten-fold increase over the input quantity of supercoiled plasmid DNA; 50% of it was present as intact molecules. For linear input plasmid DNA, a 12-fold increase was seen, of which also 50% represented intact CAT DNA molecules. In experiments with CAT phage particles (Fig. 10), we observed a three-fold increase in total CAT DNA sequences by the gastrula/neurula stage, of which 10% represented intact CAT insert; these amounts were also observed in the 30-somite stage. When purified CAT phage DNA was used (Fig. 10), total CAT DNA sequences increased seven-fold by the gastrula/neurula stage, of which the intact CAT insert accounted for 30%.

The observation that for all injected DNAs a decrease in amount was apparent in the 30-somite stage suggests that this decrease is affected by a decrease in the rate of medaka DNA replication after the gastrula/neurula stage. We therefore determined the DNA content of embryos of the various stages, obtaining values which are also given in the Figures. From these values it can be calculated that, from the 1–2 cell stage to the gastrula/neurula stage, the amount of DNA has increased by a factor of 25,000, while it only increased by a factor of six from the gastrula/neurula stage to the 30-somite stage. Thus, the reduced rate of the host DNA replication presumably also affects replication of input DNA. Since degradation occurs concurrently, this results in continuous decrease of exogenous DNA as observed in later stages (see above).

Discussion

Genes mediating developmental processes can best be studied in the developing organism. Therefore, genes have been transferred into oocytes or fertilized eggs and the gene-specific phenotype examined in the DNA-treated animal or its transgenic offspring. Only a few orgnisms have been extensively studied and used in such types of gene analysis; fish have been rarely used but are of particular interest with regard to improvement of commercially important traits. Whereas in the mouse, pro-nuclear injection is a prerequisite for the survival and functioning of the introduced genetic material (Brinster et al. 1985), in other organisms such as the fruit fly (Spradling and Rubin 1982), nematode (Kimble et al. 1982), sea urchin (McMahon et al. 1985) and frog (Rusconi and Schaffner 1981), cytoplasmic injection has been successfully employed. In most fish species, nuclear injection poses several difficulties (Donaldson 1986); we have, therefore, followed expression and persistence of a marker, the CAT gene, introduced by cytoplasmic injection into the fertilized egg of the medaka, which has been widely used in studies of embryogenesis.

Microinjection of the CAT marker gene yielded CAT expression up to the free-swimming fish stage, but with slightly different expression patterns, depending on whether the marker gene was encompassed in plasmid DNA, or in recombinant phage and in DNA isolated from the phage. For supercoiled and linearized plasmid DNA forms, CAT expression patterns were very similar, if not identical. Expression peaked at the gastrula/neurula stage and, although declining, persisted to the free swimming fish stage. These results indicate that the physical conformation of the applied DNA does not seem to affect its expression. In contrast, Etkin and Balcells (1985) reported a higher expression of the CAT reporter gene in *Xenopus* embryos when administered in supercoiled form, while Wilson et al. (1986) showed that an actin or actin-globin fusion gene was efficiently transcribed only if the supercoiled recombinant plasmid was first lenearized, a result attributed to the poor persistence of the supercoiled form. In the experiments reported here, no great differences in the fate and behavior of either plasmid DNAs were observed. Both forms were replicated considerably during early embryogenesis, with a maximum increase of approximately ten-fold at the gastrula/neurula stage. Replication drastically slowed down coinciding with the decline in early medaka DNA replication. Together with the onset of plasmid degradation at the early high blastula stage, this contributed to the overall decline of injected DNA. Differences were observed with respect to conversion of the input supercoiled and linear plasmid DNA. While the concatemers derived from the supercoiled plasmid DNA seemed to be composed of an ordered array of head-to-tail tandems, the linear-derived concatemers appeared to consist of a more random array of head-to-tail, head-to-head as well as of tail-to-tail tandems; replication and random formation of concatemers were also reported for linear molecules in the zebra fish (Stuart et al. 1988) and for plasmid forms in *Xenopus* (Marini et al. 1988). In sea urchins, however, only linear molecules are efficiently replicated and concatemerized (McMahon et al. 1985).

The expression patterns resulting from injection of CAT phage particles or purified CAT phage DNA were similar to those obtained with plasmid DNA. However, expression peaked in the 1-week-old embryo stage and replication was not as extensive as with plasmid DNA, but also had its maximum increase at the gastrula/neurula stage. Thus, expression does not appear to be correlated with the quantity of DNA present at these stages, but may suggest a possible role of the vector sequences. Unlike the rapid conversions of input plasmid DNA to concatemers, no such prominent changes to input CAT phage DNA could be observed; the phage DNA remained as monomeric DNA molecules. In the mouse,

however, recombinant lambda DNA that was injected into the pronucleus of the fertilized egg was covalently ligated (Costantini and Lacy 1981).

Overall expression levels in CAT phage particle-treated medaka were weaker than in CAT phage DNA-treated ones, which were weaker than in plasmid treated medaka. The differences in CAT expression strength are likely due to the combined effects of different input amounts of CAT transcription units and of the different degrees of foreign DNA replication and degradation. For exsample, 4×10^4 CAT phage particles injected into each embryo represent only 0.8 pg of CAT transcription unit inserts, while the 10 pg of CAT phage DNA and 25 pg of plasmid DNA represent 3-pg and 16-pg CAT transcription unit inserts, respectively. Nonetheless, this very low amount of DNA introduced within the phage particle still resulted in the successful expression of the CAT gene in the medaka, indicating that genes cloned into recombinant phages can be efficiently tested by direct injection of phage particles without having to first extract the DNA. Phage particles have been used in transfection of cultured cells in which an exceptionally high transfer efficiency has been observed (Vielkind and Vogel 1989). To the best of our knowledge, there is no report on injection of phage particles into eggs or developing embryos.

The onset of CAT expression at the flat blastula stage appears to parallel a phenomenon observed in *Xenopus* embryos, i.e. transcription of endogenous genes begins at a stage termed the mid-blastula transition. At this stage the amount of DNA has accumulated sufficiently to titrate out presumed cytoplasmic suppressor components (Newport and Kirschner 1982a, b); expression at an earlier embryonic stage could be induced by injection of DNA equivalent to the total genomic DNA present in a mid-blastula stage embryo. The amount of DNA we have injected (25 pg) was not sufficient to induce earlier CAT expression; an amount equivalent to the DNA present around the mid-blastula stage (10–20 ng) was not attempted, since exogenous DNA doses above 250 pg have been shown to be lethal to the medaka embryo (Vielkind et al. 1988). Nonetheless, the ability of the CAT gene to be expressed from the flat blastula stage to the free-swimming fish stage allows for the use of the medaka embryo in expression studies on gene regulation during early development.

In all four treatment groups, at each of the stages up to the 1-week-old embryo stage, CAT expression was consistent among the specimens analysed. At the hatchling stage, expression strength varied noticeably among the samples, and by the free-swimming fish stage, CAT expression was no longer detectable in many individuals. These observations are generally reflected in the fate of the introduced DNA sequences. Up to the 1-week-old embryo stage, the amount of foreign DNA sequences present at each stage was fairly consistent. Thereafter, DNA amounts fluctuated among the sampled hatchlings, and could not be detected in many or most of the free-swimming fish. Since input DNA sequences, although being replicated, were continually degraded and subsequently lost, it is likely that most of the DNA remained extra-chromosomal throughout embryogenesis. Presumably, extra-chromosomally replicated foreign DNA segregates unequally to the daughter cells of a rapidly dividing and growing embryo, resulting in some cells not having any exogenous sequences at all. In addition, exogenous DNA segregated into actively dividing cells of rapidly growing tissues would continue to be replicated, while those ending up in non-dividing or slowly dividing cells of differentiated tissues would be quickly degraded and lost. These events would, therefore, result in an animal mosaic for the exogenous DNA, and could explain the observed fluctuations both in expression and in amount of DNA among medaka at the hatchling stage, as well as the loss of activity and absence of detectable foreign DNA in many of the free-swimming fish.

Mosaicism has been demonstrated in *Xenopus,* where DNA injected into fertilized eggs persisted in tissues of adult frogs, but exhibited a mosaic pattern of distribution (Etkin and Pearman 1987). Hough-Evans et al. (1988) have also shown by in situ DNA hybridization that DNA sequences injected into fertilized sea urchin eggs are mosaically distributed to most or all cell types or lineages of the embryo. Yet the facts that some of the free-swimming medaka continued to express the CAT gene and retained detectable amounts of exogenous DNA suggest that genomic integration of some of the exogenous sequences may have occurred. This view is further supported by the observation that the high molecular weight form in hatchlings derived from eggs injected with plasmid DNA co-migrated with the high molecular weight fraction of recipient DNA. The ability of cytoplasmically injected DNA to be integrated into the host genome has been demonstrated in trout (Chourrout et al. 1986) and in zebra fish (Stuart et al. 1988; Vielkind et al. 1988). This technique also obviates the more difficult task of injecting into the germinal vesicle of the medaka oocyte (Ozato et al. 1986), for which expression was followed for only 1 week. It should be noted that germ line transmission itself does not conclusively prove stable host genome integration, since Stinchcomb et al. (1985) have reported that exogenous DNA may be passed on to offspring in the form of episomes. Thus, genomic integration has to be further substantiated by analysis of Mendelian inheritance of the transgene. Stuart et al. (1988) demonstrated stable integration of a linear plasmid in the zebra fish genome using such a strategy, showing that 50% of the F_2 progeny from a foreign DNA positive F_1 outcrossed to an untreated fish carried the foreign sequence.

Taken together, the results of this study indicate that cloned DNA and DNA of recombinant phage particles, cytoplasmically injected at the 1–2 cell stage of medaka embryos, persist and are expressed during embryogenesis and early development. No significant advantage with regard to expression and persistence was observed when either linear or supercoiled plasmid molecules were used. The expression pattern obtained from phage particle and phage DNA injections were weaker than that obtained with plasmid DNA injection but, nonetheless, the successful expression of the phage DNA clone opens the possibility of testing larger genes, especially genomic sequences containing long streches of introns or distant regulatory regions, which cannot be cloned into plasmids. In addition, DNA need not be first extracted from recombinant phages prior to injection, since injection of phage particles alone was sufficient for expression of the enclosed DNA.

The ability of the CAT gene to be expressed throughout embryogenesis thus favors its use as an expression system in the analysis of gene regulation during this period of medaka development, especially of 'early' genes. The relatively large size of the medaka embryo (1.5 mm diameter) allows manipulation under low magnification, and the chorion can be easily penetrated with sharp injection needles. The chorion can also be made penetrable by mild digestion with proteinase K or pronase, without affecting embryo vaibility. Harsh treatments, e.g. trypsin-urea used to dissolve the chorion of goldfish eggs (Yamaha et al. 1986) or manipulations, such as boring holes into the chorion prior to injection of trout eggs (Rokkones et al. 1985), are unnecessary. Thus, in addition, functional testing of genes intended to be used in genetic engineering of economically important fish species can first be performed in the medaka embryo. This would be particularly advantageous if the target species have long generation times, are seasonal breeders, and produce eggs that cannot be easily manipulated.

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