

EVALUATION OF A FISH GENE TRANSFER SYSTEM:
EXPRESSION, FATE, AND GERMLINE TRANSMISSION OF CAT RECOMBINANT PLASMID
AND PHAGE SEQUENCES MICROINJECTED INTO NEWLY FERTILIZED EGGS OF THE
JAPANESE MEDAKA, Oryzias latipes (TEMMINCK & SCHLEGEL)

By

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ABSTRACT

The creation of 'transgenic' animals has provided insights into mechanisms of gene regulation, as well as opened up a new avenue for genetic improvement of livestock, including fish.

In this thesis, the suitability of the Japanese ricefield fish or 'medaka' (*Oryzias latipes*) as a gene expression system was evaluated. The procaryotic chloramphenicol acetyltransferase (CAT) gene regulated by a double eucaryotic promoter-enhancer region was chosen as a reporter. This reporter was introduced as either a supercoiled or linear recombinant plasmid (pUSVCAT), as a phage, or as purified phage DNA. DNA or phage was microinjected into the cytoplasm of newly fertilized medaka eggs at the 1-2 cell stage. Expression and fate of the injected DNA or phage were monitored by harvesting medaka at various developmental stages and performing CAT enzyme assays and Southern blot analyses, respectively. Several injected eggs were allowed to develop to sexual maturity, and their offspring were pooled and tested by CAT enzyme assay for inheritance of the CAT sequences.

The patterns of expression of injected supercoiled and linear pUSVCAT DNA were very similar, indicating that DNA conformation does not affect the efficiency of expression. CAT enzyme activity was detectible from the early high blastula stage (4 hr post-injection), was strongest at the late gastrula/early neurula stage (1 day post-injection), and was sustained but slightly weaker in the one-week old embryo. Expression was significantly reduced in hatchlings (2 weeks post-injection), varying noticeably among the individuals analysed. CAT expression was still detectible in free-swimming fish (4 weeks post-injection). Recombinant CAT phage particles or purified CAT phage DNA were also able to express the CAT gene up to the free-swimming fish stage. However, in these treatments, the strongest CAT expression was

seen in the one-week old embryo instead of in the gastrula/neurula, raising the possibility of a role played by different vector sequences on gene expression.

Studies on the fate of injected supercoiled and linear pUSVCAT revealed conversion of the input forms to high molecular weight head-to-tail and randomly oriented concatemers respectively. Total plasmid DNA increased rapidly during cleavage and gastrulation, indicative of plasmid replication, whereas degradation of plasmid sequences was observed by the early high blastula stage. In the gastrula/neurula derived from injection of supercoiled pUSVCAT, total plasmid DNA increased ten-fold, whereas injection of linear pUSVCAT resulted in a 12-fold increase at the same stage. In both cases, most of the observed increase was contributed by the high molecular weight concatemers. The amount of plasmid DNA decreased after the gastrula/neurula stage, and this DNA was exclusively of the high molecular weight form at hatching and could persist to the free-swimming stage.

Neither the DNA from injected CAT phage particles nor the injected purified CAT phage DNA appeared to be concatenated during early embryogenesis. In both cases, however, the phage DNA appeared as higher molecular weight DNA by the one-week old embryonic stage, probably formed by covalent end-to-end ligations. DNA of CAT phage particles did not increase until after the early high blastula stage, but by the flat blastula stage (10 hr post-injection) a three-fold increase over the input amount was observed. There was no significant increase at the gastrula/neurula stage, nor was there an immediate decrease thereafter. Injected purified CAT phage DNA increased through the stages of cleavage and gastrulation, the gastrula/neurula having seven-fold more CAT phage DNA than that injected, and decreased thereafter. Both DNA of injected phage particles and injected phage DNA could persist to the free-swimming stage.

CAT gene expression was detected in a number of pooled offspring from several DNA and phage-treated fish, indicating inheritance of the input sequences. The data in this study suggest that the germline-positive parents are probably mosaic for the presence of the CAT sequences, and that germline transmission is possible with plasmid DNA of both conformations, DNA-carrying phage particles, or purified phage DNA.

The above results, coupled with the ease of handling and manipulation of the medaka embryo, strongly favour the use of the medaka as a transient expression and transgenic animal model.

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INTRODUCTION

The Xenopus oocyte and fertilized egg have been successfully employed as transient expression systems in the analysis of the temporal and spatial regulation of gene expression during early development. For example, the oocyte has been used to decipher the transcriptional regulation of Xenopus genes coding for 4S tRNA and 5S rRNA, and of sea urchin histone genes (reviewed in Etkin, 1982); the fertilized egg has been used to analyse the temporal regulation of a Xenopus gastrula-specific gene (Krieg and Melton, 1985) and the tissue-specific regulation of a Xenopus actin gene (Wilson *et al.*, 1986). Also commonly used in transient expression analyses is the fertilized sea urchin egg, in which ontogenic regulation of sea urchin genes coding for cytoskeletal actin (Davidson *et al.*, 1985; Flytzanis *et al.*, 1987; Franks *et al.*, 1988; Katula *et al.*, 1987), and early and late histones (Colin *et al.*, 1988; Vitelli *et al.*, 1988), has been demonstrated. Spatially correct expression of a cytoskeletal actin gene was also observed in this system (Hough-Evans *et al.*, 1987, 1988).

Two major characteristics of transient expression systems, mosaicism and gradual loss of the introduced gene over time, limit the scope of investigations to which they can be applied. Hough-Evans *et al.* (1988) point out, however, that mosaicism may in certain circumstances be advantageous since the presence of incorporated DNA only in a percentage of cells impinges less on the viability of an animal because competitive depression of endogenous genes is alleviated. Nevertheless, only animals which stably retain at least one copy of the gene in every cell are amenable, for example, to studies of cell lineage or phenotypic effects of insertional mutagenesis, among others (reviewed in Jaenisch, 1988). Integration of introduced genes and inheritance by progeny, *i.e.*, the creation of stable transgenic lines has been reported for several organisms, for example mice (reviewed in Palmiter

and Brinster, 1986, and Jaenisch, 1988), Drosophila (Rubin and Spradling, 1982; Spradling and Rubin, 1982, 1983), and Caenorhabditis (Fire, 1986), and have proven invaluable in such studies.

Transgenic technology has also been applied to the genetic manipulation of commercial livestock. Cloned growth hormone genes have been introduced into cattle (McEvoy *et al.*, 1987; King and Wall, 1988), sheep (Hammer *et al.*, 1985; Nancarrow *et al.*, 1987), pigs and rabbits (Hammer *et al.*, 1985), and fish (Zhu *et al.*, 1985, 1986; Chourrout *et al.*, 1986; Dunham *et al.*, 1987; Guyomard *et al.*, 1988). The transfer of a disease resistance gene into poultry (Crittenden and Salter, 1985) and of a cold temperature tolerance gene into fish (Hew *et al.*, 1987) have also been reported. In many instances, successful integration and or expression of the transferred genes has been demonstrated.

The recent reports of gene transfer into fish (reviewed in Maclean *et al.*, 1987) have been concerned with improvement of certain traits in commercially important species, such as enhancement of growth and tolerance of low temperatures. The aim of the present study was to evaluate the feasibility of using the Japanese medaka Oryzias latipes as a transient, and also as a stable, expression system for testing developmentally important fish genes, or any other genes with possible applications to the genetic engineering of commercial fish species. The medaka appears to be an excellent model for such studies since its biology, including embryonic development and physiology, has been extensively studied, hundreds of eggs may be obtained daily (Yamamoto, 1967, 1975), and a transparent chorion permits easy observation of embryonic development. In one of the earlier gene transfer attempts into fish, Ozato *et al.* (1986) microinjected the chicken δ -crystallin gene into the germinal vesicle of medaka oocytes because of the difficulty in locating the nucleus in fertilized eggs. However, the female had to be sacrificed to obtain the oocytes, and several other manipulations before and

after microinjection were required. In the present study, a simplified procedure of microinjection into the cytoplasm of fertilized medaka eggs prior to or immediately after first cleavage (one to two cell stage embryo) was adopted. The chloramphenicol acetyltransferase (CAT) gene was chosen as a reporter gene because simple and rapid tests are available for testing CAT activity, and because 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 virus (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, 1988).

A number of reports have compared the expression of exogenous genes when applied in various conformations *in vivo*. Etkin and Balcells (1985) found that supercoiled DNA injected into Xenopus embryos exhibited higher expression than did linear DNA, but conflicting observations were made by Wilson *et al.* (1986). To test whether DNA topology has any effect on efficiency of gene expression in the medaka embryo, the CAT transcriptional unit encompassed in recombinant plasmid was injected in either supercoiled or linear conformation, and CAT expression monitored during medaka development. The expression of a recombinant CAT phage, the genome of which contains three CAT transcriptional units, was also evaluated since 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 than did DNA-mediated gene transfer, presumably because the phage coat protects the exogenous DNA from degradation by DNases. Additionally, recombinant phage technology allows larger genes (15 to 20 kb) to be cloned, and phage are commonly used in cloning genomic libraries. Successful expression of the DNA carried by the injected phage particles not only would allow long stretches of genomic DNA to be tested for the effects of various introns and of distant

regulatory regions on gene transcription and translation (Bendig and Williams, 1983), but also would obviate the need to use purified phage DNA. CAT phage DNA purified from the recombinant CAT phage was also tested for expression in medaka embryos since Wilson *et al.* (1986) reported poor transcription for recombinant phage DNA, possibly due to interference by the arms of the phage vector.

Several other studies have concentrated on the fate of exogenous DNA injected into the fertilized egg, since correct temporal or spatial expression depends, in part, on the survival and persistence of the DNA for a sufficient period of time for the gene to be regulated. It has been observed that linear plasmid molecules, when injected into the cytoplasm of fertilized Xenopus eggs, persist for a longer period than do supercoiled molecules (Etkin *et al.*, 1984; Wilson *et al.*, 1986; Marini *et al.*, 1988). In addition, Wilson *et al.* (1986) observed that supercoiled DNA remained unchanged whereas linear plasmid DNA was processed into high molecular weight concatenes. Marini *et al.* (1988), however, reported that both supercoiled and linear plasmid molecules were converted to high molecular weight species, the supercoiled DNA-derived species consisting of head-to-tail tandem arrays and the linear plasmid-derived species consisting of either head-to-tail or random arrays. In sea urchins, McMahon *et al.* (1985) observed that only linear, but not supercoiled, DNA was processed when injected into the cytoplasm of fertilized eggs. Wilson *et al.* (1986) also reported that a λ clone carrying a Xenopus cardiac actin gene persisted poorly in the Xenopus embryo unless the insert was separated from the vector arms prior to injection. To determine if DNA conformation, phage particle packaging or vector sequences have an effect on DNA viability and processing in the medaka embryo, supercoiled or linear pUSVCAT DNA, recombinant CAT phage particles, or purified CAT phage DNA were injected into fertilized medaka eggs and their fates monitored during development.

Aside from transient expression studies, another major objective in gene transfer experiments is the production of stable transgenic animals, i.e. animals in which injected exogenous DNA is incorporated into the host genome and is transmitted through the germline to offspring. In mice, it has been shown that only microinjections into the pronuclei of the fertilized egg resulted in high frequencies of DNA integration, and nuclear injections resulted in more efficient integration when linear DNA molecules rather than supercoiled molecules were used (see Brinster *et al.*, 1985). Also, Costantini and Lacy (1981) demonstrated that pronuclear microinjection of a λ DNA clone resulted in high frequency of integration into mouse tissues and subsequent germline transmission. In contrast to the almost absolute necessity for nuclear injections in mice, genomic integration of a cytoplasmically injected linear plasmid DNA has been shown in the sea urchin (Flytzanis *et al.*, 1985), and Etkin and Pearman (1987) detected germline transmission of a supercoiled plasmid to offspring by a Xenopus adult male derived from cytoplasmic injection of the exogenous DNA. Recently, Guyomard *et al.* (1988) and Stuart *et al.* (1988) were able to demonstrate that linear plasmid DNA injected into the cytoplasm of fertilized trout or zebrafish eggs persisted in adult fish and was inherited by a certain percentage of offspring. Vielkind *et al.* (1988) also reported that supercoiled pUSVCAT DNA cytoplasmically injected into fertilized zebrafish eggs resulted in stable transformants whose offspring not only inherited the foreign sequences but also expressed the CAT gene. To determine if cytoplasmic injections of supercoiled or linear pUSVCAT DNA, recombinant CAT phage, or purified CAT phage DNA would result in stable germline-positive transformants, offspring from DNA or phage-treated parents were analysed by CAT enzyme assay for inherited expression of the CAT gene.

MATERIALS AND METHODS

Egg collection and embryo culture

Clusters of fertilized medaka eggs attached to the females were collected 1 to 2 hr after the start of the light cycle and were transferred to and maintained in Ringer's solution (0.75% NaCl, 0.02% KCl, 0.02% CaCl₂, pH 7.3; Yamamoto, 1961) for up to 2 hr at 12 °C prior to injection to slow down the cleavage process. Injected embryos were reared in medium consisting of 1% NaCl, 0.03% KCl, 0.04% CaCl₂·2H₂O, 0.163% MgSO₄·7H₂O, 0.001% methylene blue (Kirchen and West, 1976). Staging of medaka embryos was according to Matsui (1949). Embryos that hatched were transferred to and maintained in normal tank water until analysed.

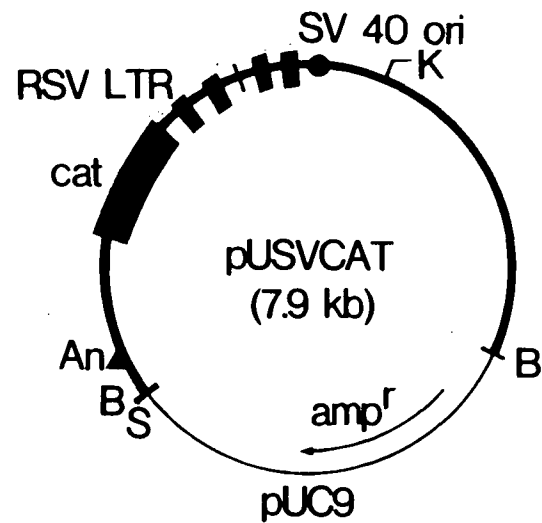
Recombinant CAT gene constructs

Figure 1 illustrates the CAT gene constructs used in this study. The transcriptional unit consists of the bacterial CAT coding sequence under the regulation of the Rous sarcoma virus (RSV) LTR and Simian virus 40 (SV 40) early region double promoter-enhancer regions. This CAT transcription unit was tested as a recombinant plasmid pUSVCAT (Karlsson *et al.*, 1985) in supercoiled form and also as a linear molecule after digestion with SalI. A recombinant CAT phage particle containing three tandem copies of the CAT transcription unit was also used in this study (Vielkind and Vogel, 1988), as was the purified DNA from this recombinant phage.

pUSVCAT DNA was extracted by using a modified Brij detergent method (Clewell and Helinski, 1969) and was purified by two successive CsCl-ethidium bromide equilibrium density centrifugations. Recombinant CAT phage were purified from plate lysates by CsCl step gradient centrifugation and were dialysed against SM buffer (100 mM NaCl, 8 mM MgSO₄·7H₂O, 50 mM Tris pH 7.5,

Figure 1 (a) Plasmid map of pUSVCAT showing CAT coding region, promoter-enhancer regions (■), and relevant restriction sites. (b) pUSVCAT linearized at the Sall site. (c) Map of recombinant CAT phage DNA. The insert consists of three tandem copies of a 5200 bp BamHI fragment of pUSVCAT containing the CAT transcription unit. A_n, polyadenylation site; amp^r, ampicillin resistance; K, KpnI restriction site; S, Sall restriction site; B, BamHI restriction site.

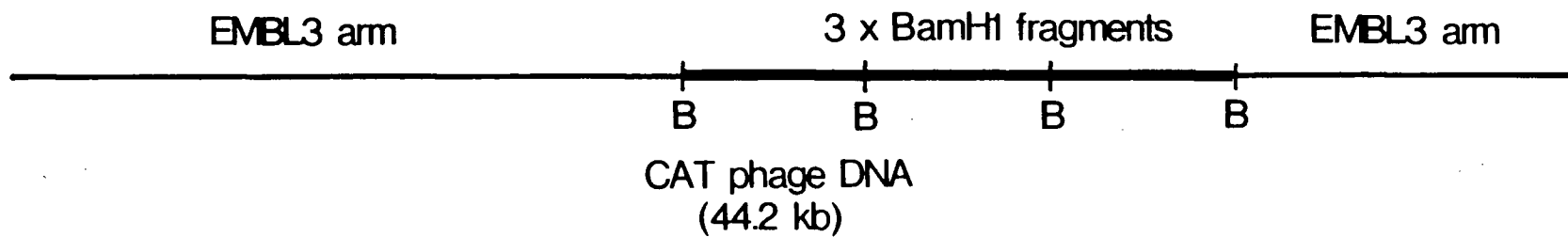
a



b



c



0.01% gelatin). CAT phage DNA was extracted from some of the phage as described in Maniatis *et al.* (1982).

Microinjection

Supercoiled or linear pUSVCAT DNA, recombinant CAT phage particles, or purified CAT phage DNA were microinjected into the cytoplasm of the medaka zygote prior to, or immediately after, first cleavage (1-2 cell stage embryo), by using a borosilicate glass capillary needle (3 to 5 μm diameter) mounted on a micromanipulator. Injections were done under a binocular microscope (Zeiss) with a magnification range of 8x to 50x. Concentrations of 50 $\mu\text{g}/\text{ml}$ plasmid DNA, 8×10^{10} phage particles/ml (equivalent to 5 $\mu\text{g}/\text{ml}$ phage DNA), and 5 $\mu\text{g}/\text{ml}$ or 20 $\mu\text{g}/\text{ml}$ purified phage DNA were used. Phenol red had been added to the DNA/phage solutions to a final concentration of 0.25% to aid in estimation of injection volume (ca. 500 pl).

CAT assay

CAT assays were performed essentially as described by Gorman *et al.* (1982). Individual embryos, hatchlings, and free-swimming fish or pools of three or five embryos or hatchlings were homogenized in 100 μl 250 mM Tris pH 8.0, and then subjected to three 5 min cycles of freeze-thawing; extracts were obtained after centrifugation (Eppendorf, 5 min, 4 $^{\circ}\text{C}$). To 100 μl extract, 20 μl dH_2O , 2 μl ^{14}C -chloramphenicol (NEN DuPont, 60 mCi/mmol, 100 $\mu\text{Ci}/\text{ml}$), and 20 μl 4 mM acetyl coenzyme A (Boehringer Mannheim) were added, and the mixture was incubated for 1 hr at 37 $^{\circ}\text{C}$. The ^{14}C -chloramphenicol and its acetylated forms were extracted with 1 ml ethyl acetate, dried under vacuum, resuspended in 30 μl ethyl acetate, and then spotted and separated on silica gel chromatography plates (J.T. Baker Co.) for 50 min in chloroform:methanol (95:5). After the plates were air-dried, autoradiograms were produced by

exposure of X-ray film (Kodak XAR-5) to these plates for one or seven days in the presence of an intensifying screen (DuPont).

DNA extraction

Individual embryos, hatchlings, and free-swimming fish, as well as pools of ten embryos, were homogenized in 200 μ l of 1x SET (100 mM NaCl, 20 mM EDTA, 50 mM Tris pH 7.8), 0.5% SDS, 0.5 mg/ml proteinase K and incubated for 2 to 4 hr at 37 °C. The samples were then extracted with one volume of phenol:chloroform:isoamyl alcohol (25:24:1), re-extracted with an equal volume of butanol:isopropanol (7:3), precipitated with 2 volumes of 95% ethanol for at least 2 hr at -20 °C and redissolved overnight in TE (10 mM Tris pH 8.0, 1 mM EDTA).

Determination of embryo DNA content

The genomic DNA content of medaka embryos at stages up to the early high blastula stage was calculated by multiplying the amount of DNA present per diploid medaka cell (see Uwa and Iwata, 1981) to the estimated number of cells present at the embryonic stage. For later developmental stages, DNA was extracted from embryos and measured fluorometrically using the bis-benzimidazole (Hoechst 33258, Hoefer Scientific Instruments) method as specified by the manufacturer.

Southern blots

Total DNA from individual embryos, hatchlings, or free-swimming fish, or aliquots of DNA from pooled samples (equivalent to single embryos of the various stages), either non-digested or completely digested by restriction enzymes as specified by the manufacturer, were subjected to electrophoresis in 0.8% agarose (Bio-Rad) gels. Gels were soaked once in 250 mM HCl for 10 min to partially hydrolyse DNA, twice in 1.5 M NaCl, 0.5 M NaOH for 15 min to

denature DNA strands, and twice in 1.5 M NaCl, 0.5 M Tris pH 7.5 for 15 min to neutralize the pH of the gel. After capillary transfer of DNA onto nylon filters (Schleicher & Schuell Nytran) in 20x SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7.0), the filters were dried *in vacuo* for 2 hr at 80 °C.

Hybridizations

Filters were prehybridized for 15 min at 60 °C with a solution containing 3x SSC, 10 mM Tris pH 7.6, 10 mM EDTA, 0.5% SDS, 1x Denhardt's (0.02% BSA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), and 0.1 mg/ml yeast RNA. The filters were then hybridized overnight at 60 °C in a similar solution containing pUSVCAT DNA, which had been labelled by random hexamer priming (Feinberg and Vogelstein, 1983/84) with ^{32}P -dCTP to a specific activity of $>5 \times 10^8$ cpm/ μg . The filters were washed twice in 2x SSC, 0.5% SDS for 30 min at room temperature (low stringency), and twice in 0.1x SSC, 0.5% SDS for 30 min at 60°C (high stringency). Autoradiograms of the filters were then obtained by exposure of the dried filters to X-ray film in the presence of intensifying screens.

Quantitation of DNA hybridization signals

Hybridization bands of each filter lane were excised and placed in 7 ml plastic scintillation vials; the remainder of each lane were placed in separate vials to be counted. 5 ml of a toluene (BDH chemicals) scintillation cocktail containing 0.4% w/v PPO (2,5-diphenyloxazole; BDH chemicals) and 0.01% w/v dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene; Packard) was added to each vial, and the vials counted in a Packard scintillation counter for 10 min each. Hybridization bands containing known amounts of DNA were also counted in the same manner and used as a reference to convert cpm (counts per minute) values to picogram DNA amounts.

RESULTS

1. Studies of CAT reporter gene expression during medaka development

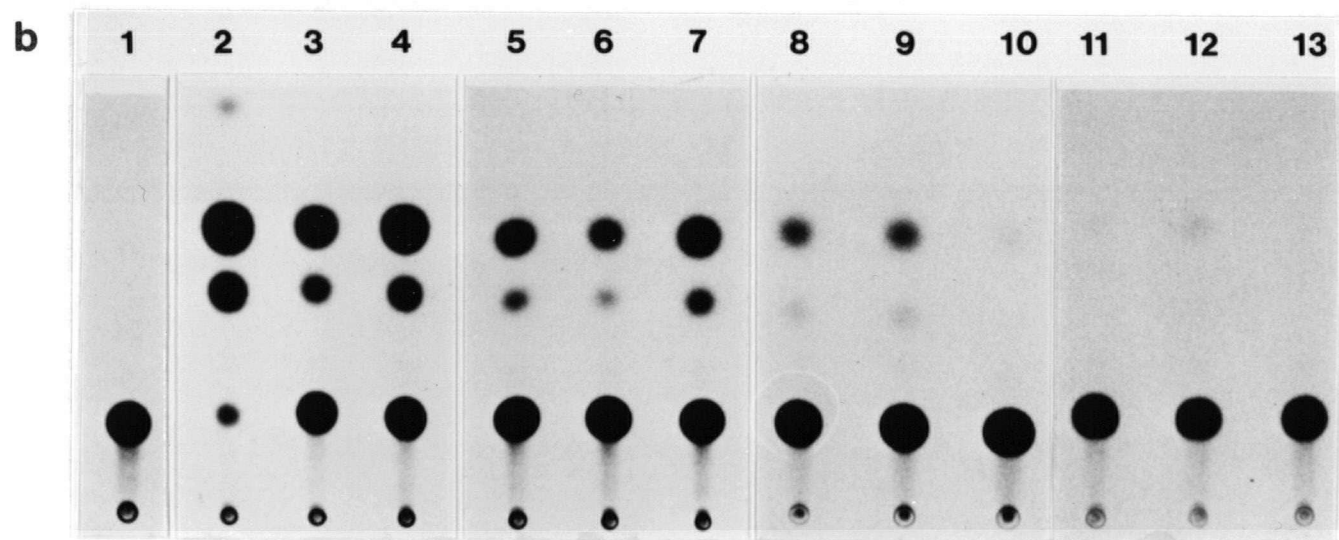
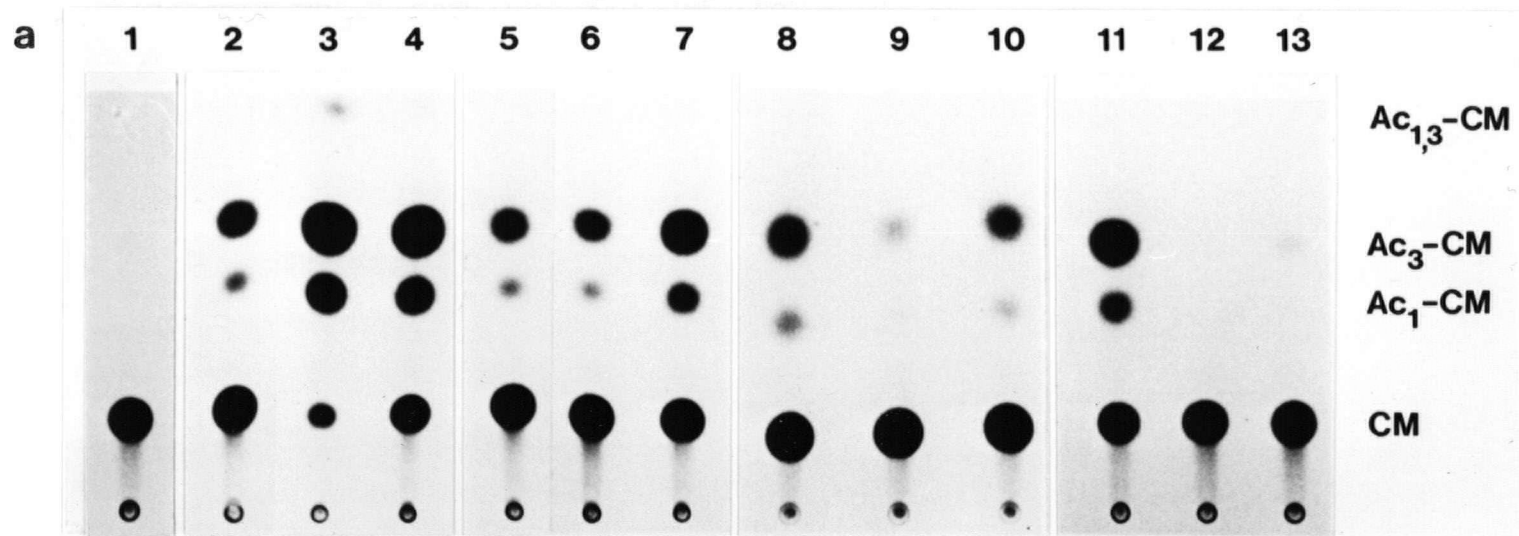
CAT expression gene during medaka development was monitored by performing CAT enzyme assays on individuals or pooled batches at various stages from the early cleavages of the egg up to the free-swimming fish stage.

1.1 CAT gene expression due to cytoplasmic injection of recombinant plasmid in supercoiled and linear conformation.

The results of the CAT enzyme assay of individual medaka of various stages derived from fertilized eggs injected with supercoiled pUSVCAT DNA is shown in Figure 2a. Altogether, ten individuals at each stage of development were assayed, of which representative results are shown. CAT enzyme activity was not detectible in the 32-64 cell stage embryo (lane 1, 2 hr post-injection). However, CAT enzyme activity in gastrula/neurula stage embryos was very prominent (lane 2-4, 1 day post-injection), and was sustained but slightly weaker in the one-week old embryo (lane 5-7), a stage when the eye and most major organs are formed. Reduced CAT activity was observed at the time of hatching and varied noticeably among the hatchlings assayed (lane 8-10, 2 weeks post-injection). By the free-swimming fish stage, CAT activity was still detectible in a few of the fish assayed, one of which had a moderately strong signal (lane 11-13, 4 weeks post-injection).

No CAT activity was detected in untreated medaka at similar developmental stages (data not shown), arguing that the CAT enzyme activity in treated embryos is not due to expression of endogenous genes. Sleight (1986) and Crabb and Dixon (1987) have reported the presence of substances in cell extracts that interfere with CAT enzyme activity. As control, CAT assays were performed on homogenates of uninjected embryos at different stages to which equal amounts of commercially available CAT enzyme had been added. No

Figure 2 CAT gene expression after injection of (a) supercoiled, and (b) linear, pUSVCAT DNA into fertilized medaka eggs. Eggs were injected with 25 pg of either plasmid form and allowed to develop until harvested. Individuals at various developmental stages were harvested and assayed for CAT enzyme activity, of which representative autoradiograms are shown. CM, ^{14}C -chloramphenicol; Ac₁- and Ac₃-CM, monoacetylated forms of CM; Ac_{1,3}-CM, diacetylated CM.



Stages: 32-64 cells late gastrula/early neurula one-week old embryo hatchling free-swimming fish

detectible differences in CAT activity signal were observed among these control groups (data not shown), indicating that the observed pattern of CAT signals obtained by injecting supercoiled pUSVCAT DNA is not a result of different inhibitory capacities of the embryonic stages, but represents the CAT expression pattern of the introduced gene.

For embryos injected with Sall-linearized pUSVCAT (Fig 2b), an expression pattern similar to that for supercoiled pUSVCAT injected embryos was observed, but with marginally stronger signals at the gastrula/neurula stage and slightly weaker signals at the hatchling stage. Very weak CAT expression was detected in a few free-swimming stage fish (lane 11-13).

1.2 CAT gene expression due to cytoplasmic injection of CAT recombinant phage particles and purified CAT phage DNA.

The results of the CAT expression experiments using CAT phage particles is shown in Figure 3a. No CAT expression was detectible in embryos assayed at the 32-64 cell stage (lane 1), but weak CAT expression was observed in several late gastrula/early neurula stage embryos (lane 2-4). A slightly stronger CAT expression was consistently observed in one-week old embryos (lane 5-7), and CAT expression was either weak or not detected at the hatchling stage (lane 8-10). However, weak CAT expression was still detectible in a few free-swimming stage fish (lane 11-13).

Injections with purified CAT phage DNA resulted in a pattern of CAT gene expression similar to that obtained with CAT phage particles (Fig 3b). However, generally stronger signals were observed, presumably resulting from the higher CAT phage DNA concentration used, which was the equivalent of five times the DNA administered through phage particle injections. For example, a moderately strong CAT activity was consistently detected in the late gastrula/early neurula (lane 2-4), whilst one-week old embryos displayed a significantly stronger CAT activity (lane 5-7). CAT signals were generally

Figure 3 CAT gene expression after injection of (a) CAT phage particles, and (b) CAT phage DNA, into fertilized medaka eggs. Eggs were injected with 4×10^4 recombinant phage particles (equivalent to 2 pg CAT phage DNA) or 10 pg purified phage DNA and allowed to develop until harvested. Individuals at various developmental stages were harvested and assayed for CAT enzyme activity, of which representative autoradiograms are shown.

weaker and varied widely in individual hatchlings (lane 8-10), and were detectible only in a few of the free-swimming fish tested (lane 11-13).

1.3 Onset of CAT gene expression in the early medaka embryo

The very strong CAT expression observed in gastrula/neurula stage embryos derived from treatment with either supercoiled or linear pUSVCAT DNA suggest that CAT expression began at an earlier developmental stage. CAT gene expression was therefore examined between the 32-64 cell stage and the late gastrula/early neurula stage on pooled batches of 3 embryos (Fig 4). Expression appeared to begin from the early high blastula stage (lanes 2, 4 hr post-injection), a clear signal being apparent by the flat blastula stage (lanes 3, 10 hr post-injection), and a very strong CAT activity was observed in the gastrula/neurula (lanes 4, 1 day post-injection). No differences were observed between the supercoiled and linear DNA-treated groups.

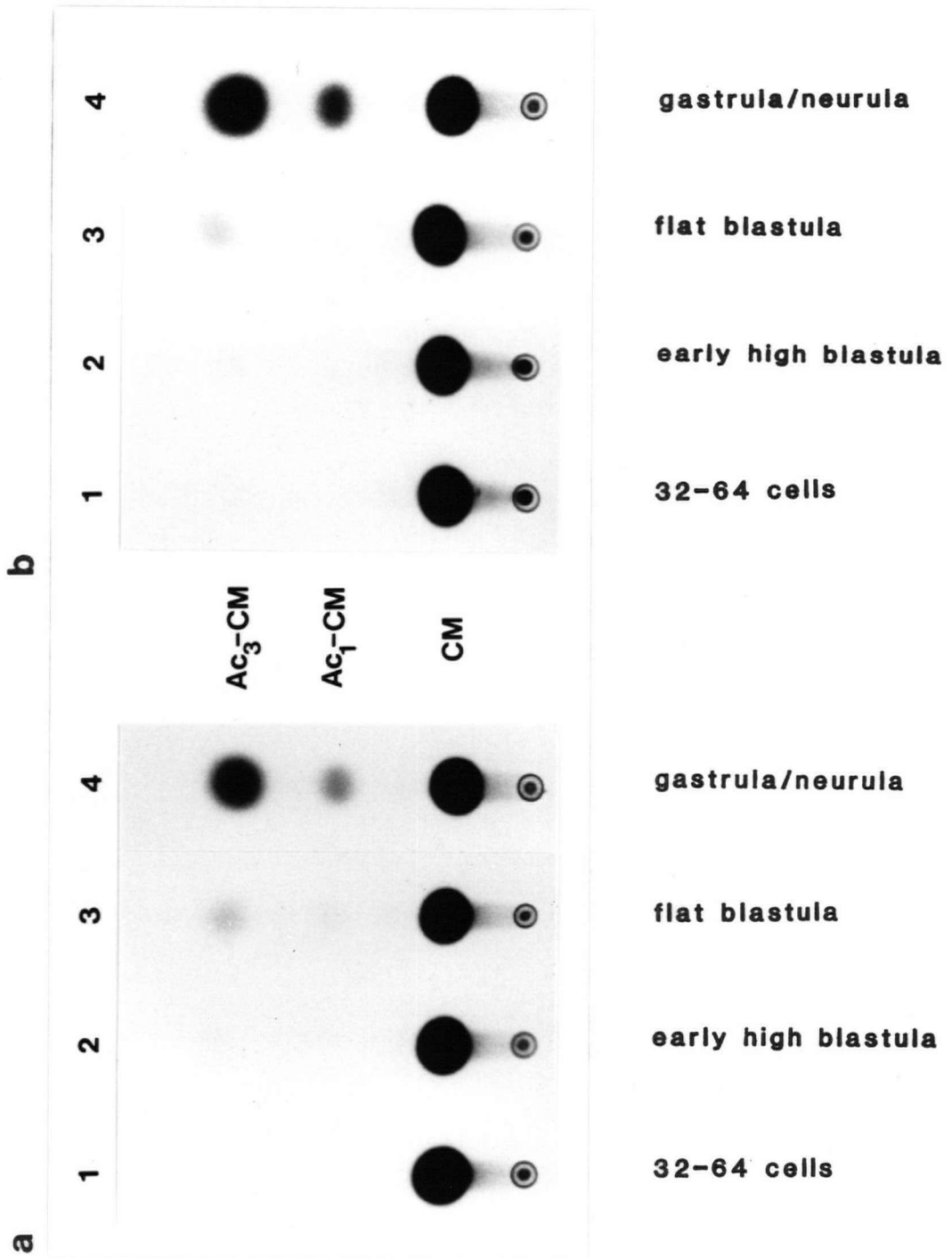
2. Studies on the fate of introduced CAT DNA sequences during medaka development

The fate of the introduced CAT DNA sequences during medaka development was monitored by performing Southern blot analysis of DNA samples obtained from individuals or pools at various stages from the 1-2 cell embryonic stage to the free-swimming fish stage.

2.1 Conformational changes of injected supercoiled and linear plasmid DNA

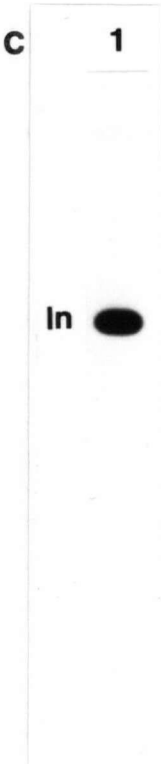
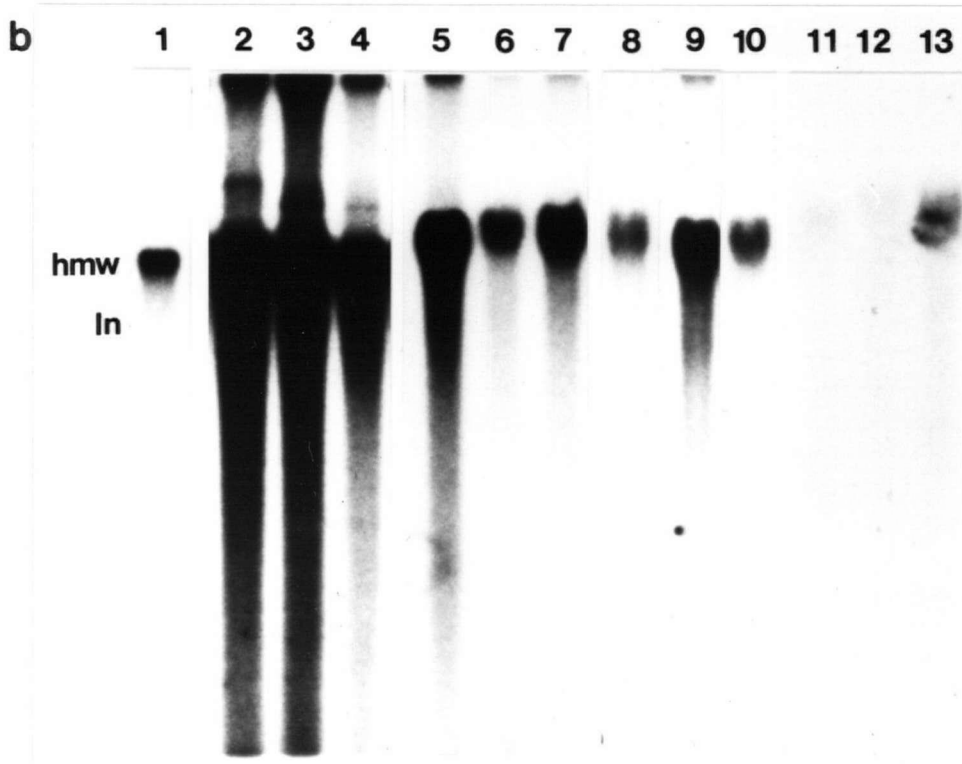
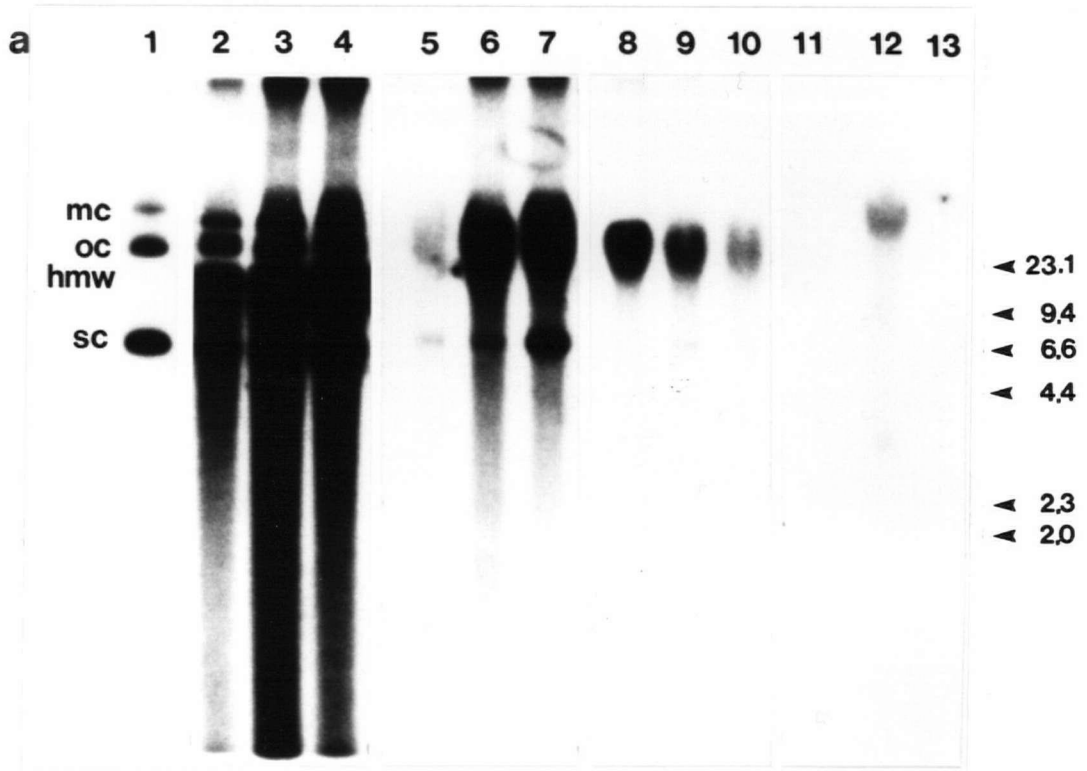
The results of the Southern blot analyses of individual medaka DNA, arising from experiments using supercoiled pUSVCAT DNA, is shown in Figure 5a. At the 32-64 cell stage (lane 1), all three forms which are present in pUSVCAT plasmid DNA preparations (mostly supercoils, and some open circles and multimeric circles; not shown) were detected. By the late gastrula/early neurula stage an additional, high molecular weight form of greater than 23.1

Figure 4 Earliest appearance of CAT gene expression after injection of (a) supercoiled, and (b) linear, pUSVCAT DNA. 25 pg of either plasmid form was injected into each fertilized egg. At various early embryonic stages, 3 samples were pooled together and assayed for the presence of CAT enzyme activity.



Stages:

Figure 5 Southern blot analysis of the fate of injected (a) supercoiled, and (b) linear, pUSVCAT molecules during medaka development. Fertilized eggs were injected with 25 pg of either plasmid form. DNA was extracted from individuals at various developmental stages, subjected to electrophoresis in 0.8% agarose gels, and transferred to nylon membranes. The blots were probed then with pUSVCAT DNA labelled by random hexamer priming, representative autoradiograms of which are shown. (c) linear pUSVCAT DNA standard, equivalent to input amount (25 pg). sc, supercoiled pUSVCAT DNA; oc, open circular pUSVCAT DNA; mc, multimeric circular pUSVCAT DNA; ln, linear pUSVCAT DNA; hmw, high molecular weight pUSVCAT DNA. Lambda HindIII size standards (kb) are shown at right of panel (a).



Stages:

32- 64 cells	late gastrula/ early neurula	one-week old embryo	hatchling	free- swimming fish
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kb was clearly evident (lane 2-4), co-migrating with the high molecular weight fraction of medaka DNA (as seen by ethidium bromide staining of the gel prior to Southern blotting; data not shown). An increase in all the plasmid forms was seen, with the high molecular weight form showing the greatest increase. Strong hybridization smears were also observed, suggesting degradation of a significant percentage of plasmid DNA. In the one-week old embryo, the open circular and multimeric forms could not be detected, some supercoiled plasmid was still evident, and the rest of the plasmid DNA was of the high molecular weight form (lane 5-7); plasmid DNA degradation was still evident. In hatchlings, plasmid DNA was further reduced and only the high molecular weight form remained (lane 8-10). Input plasmid DNA persisted in one of several free-swimming fish analysed (lane 11-13).

When linear pUSVCAT DNA was used, DNA extracted from 32-64 cell stage embryos contained a strongly hybridizing high molecular weight form (Fig 5b, lane 1), suggesting a fairly rapid conversion of the injected linear molecules. A faint smear suggests that plasmid DNA degradation had already begun. In the late gastrula/early neurula, a very strong hybridization in the high molecular weight band was observed, indicating a large increase in plasmid DNA. A strong hybridization smear was also apparent, suggesting substantial degradation of the plasmid DNA. Only the high molecular weight form was present in the one-week old embryo, and plasmid degradation was still evident (lane 5-7). In hatchlings, the amount of plasmid DNA present was further reduced, and its continued degradation was still detected in some samples (lane 8-10). Plasmid DNA was still clearly apparent in one of several free-swimming stage fish (lane 11-13).

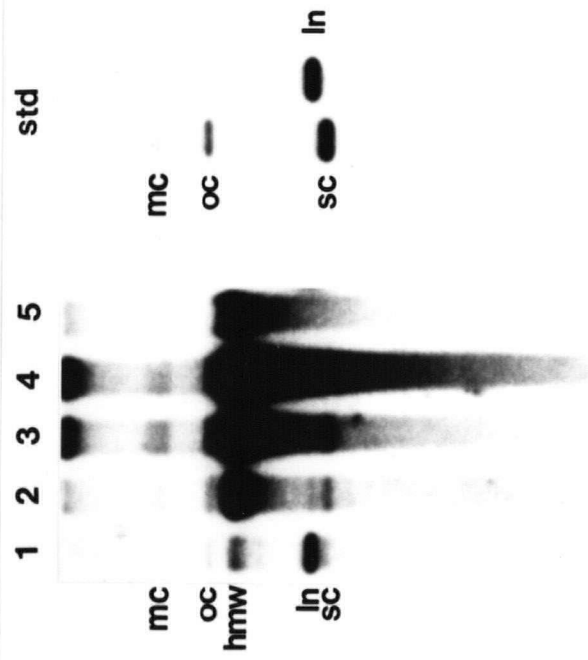
The very strong hybridization signals already seen at the gastrula/neurula stage in both the supercoiled and linear pUSVCAT experiments suggest that processing of the injected plasmids must have begun earlier. In order to have an idea of the rapidity of plasmid conversion, embryos at

earlier stages between the 1-2 cell stage to the 30 somite neurula stage were analysed. Ten embryos at each stage were pooled, and Southern blot analysis was performed on embryo-equivalent DNA aliquots after complete digestion with XhoI, which does not recognize any site on pUSVCAT DNA. Digestion with XhoI reduces high molecular weight medaka DNA to smaller fragments, thus minimizing any possible impedance of pUSVCAT DNA migration during electrophoresis. DNA extracted from 1-2 cell stage embryos within 5 min of injection with supercoiled pUSVCAT DNA contained the expected supercoiled, open circular, and multimeric circular forms (Fig 6a, lane 1). The high molecular weight form, which was not different from that observed before, became apparent at the early high blastula stage, and an overall increase in the other pUSVCAT forms was seen (lane 2, 4 hr post-injection). By the flat blastula stage (lane 3, 10 hr post-injection), the high molecular weight form became more prominent than the other forms, suggesting preferred replication of the high molecular weight form. In addition, a strong smear was observed, signalling the onset of plasmid DNA degradation. The strongest overall DNA hybridization signal was observed in the late gastrula/early neurula, as previously noted (lane 4, 1 day post-injection) but by the 30 somite stage total plasmid DNA had significantly declined (lane 5, 3 days post-injection).

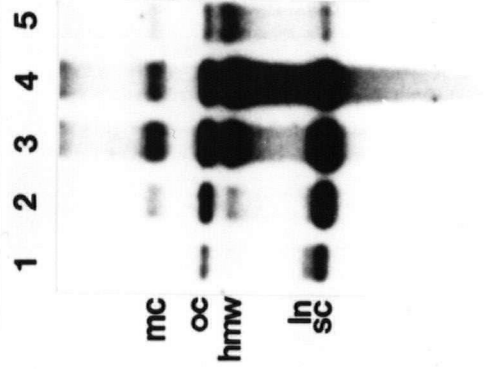
In contrast to the experiment with supercoiled pUSVCAT DNA, much of the linear pUSVCAT DNA injected into fertilized eggs was almost immediately converted to high molecular weight form (Fig 6b, lane 1, 5 min post-injection). In addition, faint bands corresponding to supercoiled and open circular pUSVCAT forms could be made out, indicating some conversion of the linear DNA to these forms too. In the early high blastula stage, the amounts of supercoiled, open circular, and especially high molecular weight pUSVCAT forms increased while that of the linear form diminished (lane 2). Multimeric pUSVCAT circles were clearly apparent in flat blastula stage embryos (lane 3), while total plasmid DNA was further increased. The strongest DNA

Figure 6 Southern blot analysis of the early fate of injected (a) supercoiled, and (b) linear, pUSVCAT molecules. Fertilized eggs injected with either supercoiled or linear pUSVCAT DNA were harvested at various early embryonic stages, and total DNA was obtained from pools of ten embryos. Embryo-equivalent aliquots were subjected to electrophoresis, blotted, hybridized to radio-labelled pUSVCAT probe, and autoradiographed as described in Fig 5. std, standards of the various circular and linear pUSVCAT forms.

b



a



30 somites
gastrula/neurula
flat blastula
early high blastula
1-2 cells

30 somites
gastrula/neurula
flat blastula
early high blastula
1-2 cells

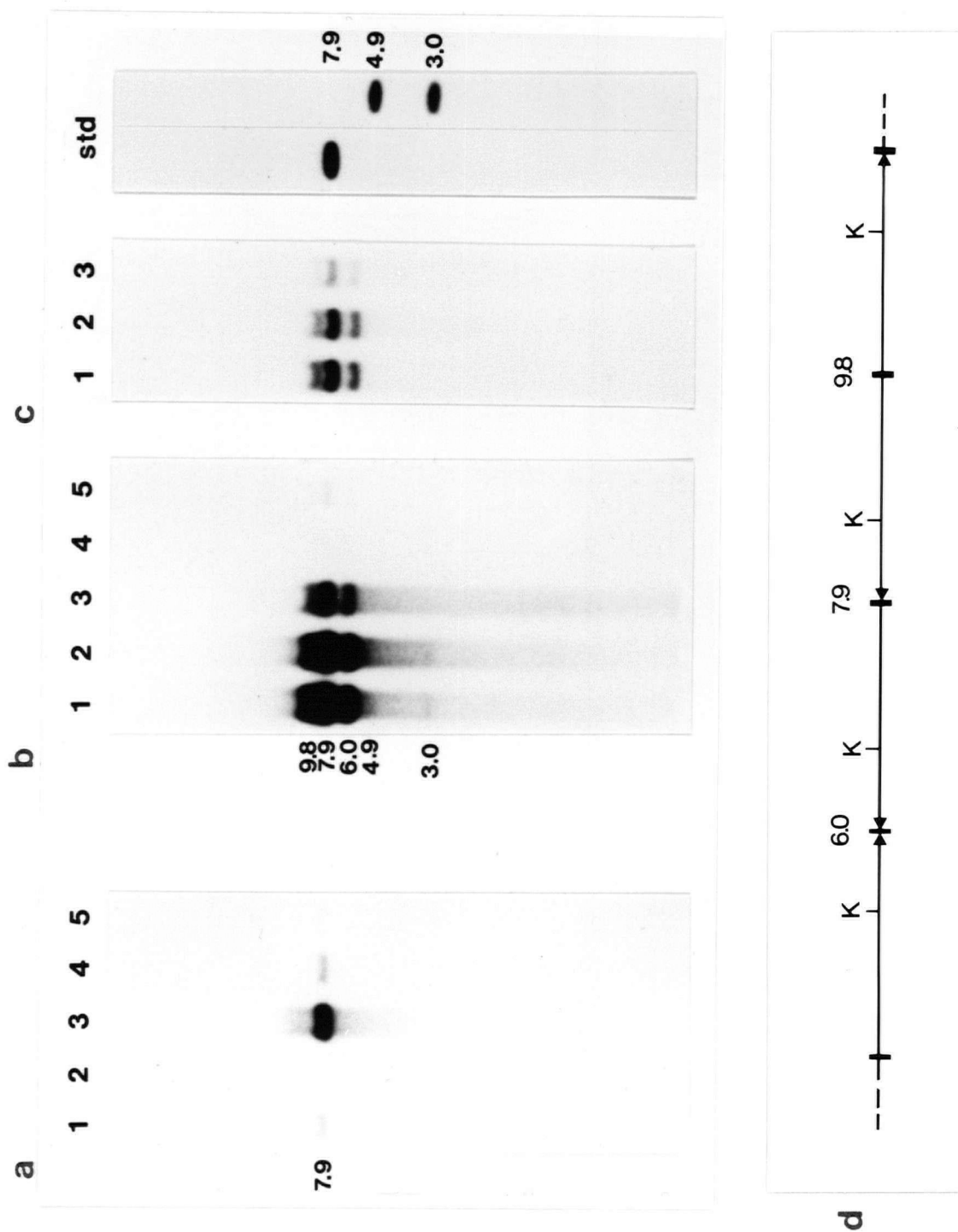
Stages:

hybridization signal and smearing was seen in the late gastrula/early neurula (lane 4). By the 30 somite stage (lane 5), total plasmid DNA markedly decreased and only the high molecular weight form remained.

2.1.1 Nature of high molecular weight pUSVCAT form

The fact that the migration of the high molecular weight plasmid form was unaffected by digestion of the embryo DNA samples with XhoI, which reduces genomic DNA to smaller fragment sizes but does not cut pUSVCAT DNA, refutes the possibility that it arose from entrapment of supercoiled, open circular, or linear plasmids in the high molecular genomic DNA fraction. To determine the nature of this high molecular weight pUSVCAT form, Southern blot analysis was performed on DNA from hatchlings, a stage in which the remaining pUSVCAT DNA had earlier been observed to be exclusively of the high molecular weight form. DNA from individual hatchlings was digested with KpnI and analysed by Southern blot hybridization using radioactively labelled pUSVCAT DNA as a probe. There is only one KpnI restriction site on pUSVCAT, and on a SalI-linearized molecule, KpnI produces two fragments of 4.9 kb and 3.0 kb. In hatchlings derived from eggs injected with supercoiled pUSVCAT DNA, digestion of the DNA produced a single hybridization band corresponding to a 7.9 kb linear monomer (Fig. 7a). This result suggests that the high molecular weight plasmid form derived from supercoiled plasmid injections probably consisted of tandem arrays of plasmid monomers oriented head-to-tail. However in hatchlings derived from eggs injected with linear pUSVCAT DNA, three strong hybridization bands of approximately 9.8 kb, 7.9 kb, and 6.0 kb were seen (Fig. 7b,c). In addition, two other very weak hybridization bands of approximately 4.9 kb and 3.0 kb could be detected. The restriction pattern obtained indicates that the resident high molecular weight plasmid form consisted of randomly oriented tandem arrays of the linear plasmids. KpnI digestion of such a tandem array would be expected to yield many copies of

Figure 7 Southern blot analysis of high molecular weight pUSVCAT DNA persisting in hatchlings. Hatchlings derived from eggs injected with (a) supercoiled, or (b) linear, pUSVCAT DNA were individually harvested for their DNA. The DNA samples were digested with KpnI, subjected to electrophoresis, blotted, hybridized to radio-labelled pUSVCAT probe, and autoradiographed as described in Fig 5. (c) shorter duration autoradiogram of lanes 1-3 in panel (b). Sizes of the restriction bands are given to the left of panels (a) and (b). std, DNA standards of 7.9kb linear pUSVCAT DNA molecule and the two fragments resulting from KpnI digestion of Sall linearized pUSVCAT. (d) Diagram of a hypothetical multimer of Sall linearized pUSVCAT DNA showing all possible (head-to-tail, head-to-head, and tail-to-tail) ligation products. Unlabelled vertical lines represent Sall ligation junctions. The predicted size (kb) of restriction fragments resulting from KpnI (K) digestion are indicated.



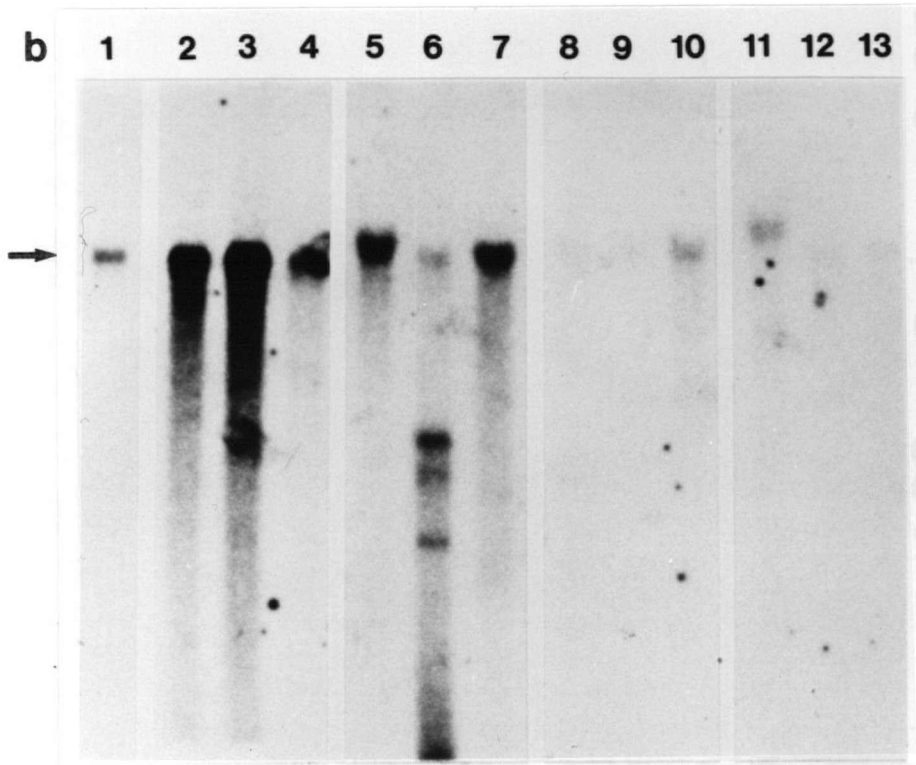
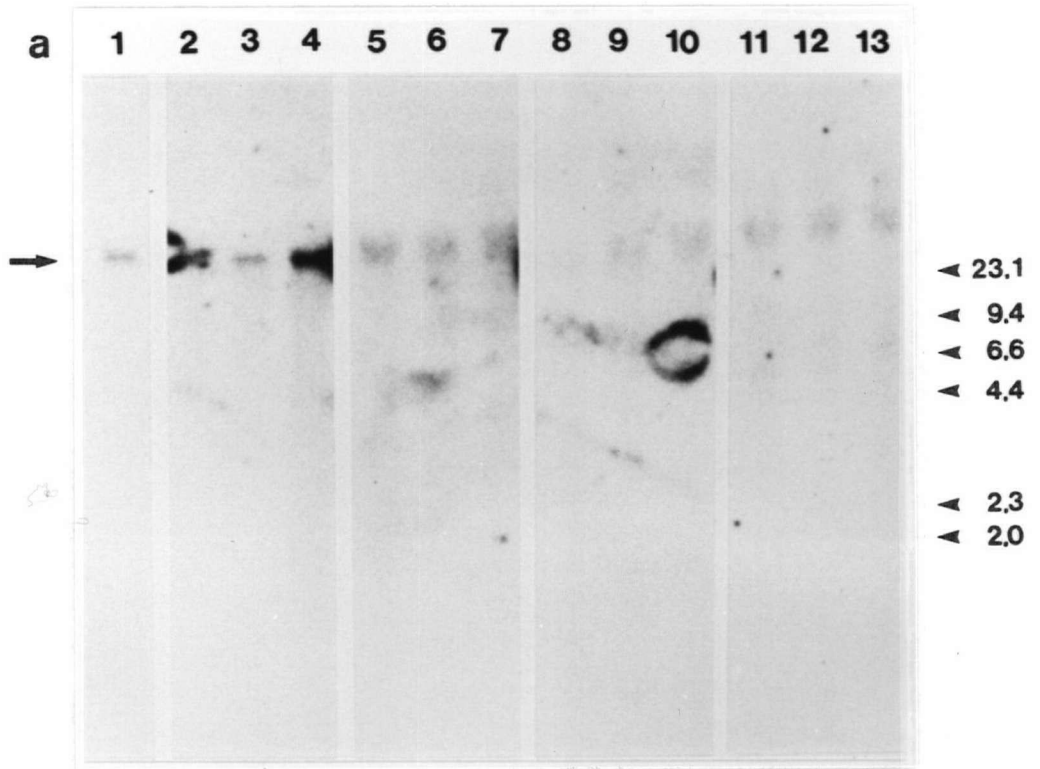
middle fragments with sizes represented by the three strong hybridization bands, and fewer copies of end fragments corresponding to the weak hybridization bands (Fig 7d). The relatively stronger hybridizing signal of the unit length band compared to the other two middle fragment bands implies that a majority of the ligation junctions were of the head-to-tail type.

2.2 Conformational changes of DNA introduced within recombinant phage particles and of purified phage DNA

The results of the Southern blot analyses of individual medaka DNA, arising from experiments using CAT phage particles, is shown in Figure 8a. At the 32-64 cell stage, a band corresponding to CAT phage DNA was observed (lane 1). This band was also present in the late gastrula/early neurula (lane 2-4) but differed from the preceding stage in having a slightly stronger hybridization signal with some very light smearing, indicative of concurrent foreign DNA replication and degradation. This observation therefore suggests that DNA contained within the injected phage particles was released prior to this stage. In the one-week old embryo, the CAT phage DNA appeared to co-migrate with the high molecular weight fraction of medaka DNA (lane 5-7). Overall hybridization signal was only slightly weaker, but smears were more visible. CAT phage DNA was further reduced in hatchlings (lane 8-10), yet appeared to persist through to the free-swimming fish stage (lane 11-13).

Eggs injected with purified CAT phage DNA and analysed at the 32-64 cell stage also showed a band corresponding to unit length CAT phage DNA (Fig 8b, lane 1). By the gastrula/neurula stage, a stronger hybridization band was observed, indicative of CAT phage DNA replication. A prominent smear was also evident, suggesting that CAT phage DNA degradation had also occurred. In the one-week old embryo, there seems to have been a shift toward higher molecular weight in the DNA hybridization band, but CAT phage DNA was reduced and degradation products were still clearly evident (lane 5-7). CAT phage DNA was

Figure 8 Southern blot analysis of the fate of (a) DNA of injected CAT phage particles and (b) injected CAT phage DNA molecules during medaka development. Fertilized eggs were injected with 4×10^4 recombinant phage particles (equivalent to 2 pg CAT phage DNA) or 10 pg purified phage DNA. DNA was extracted from individuals at various developmental stages, subjected to electrophoresis, blotted, hybridized to radio-labelled pUSVCAT probe, and autoradiographed as described in Fig 5. Arrows point to CAT phage DNA monomer. Lambda HindIII size standards (kb) are shown at right of panel (a).



Stages:

32- 64 cells	late gastrula / early neurula	one-week old embryo	hatchling	free- swimming fish
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significantly reduced and no longer detectible in several individuals at the hatchling stage (lane 8-10), but continued to persist to the free-swimming stage in at least one of the fish tested (lane 11-13).

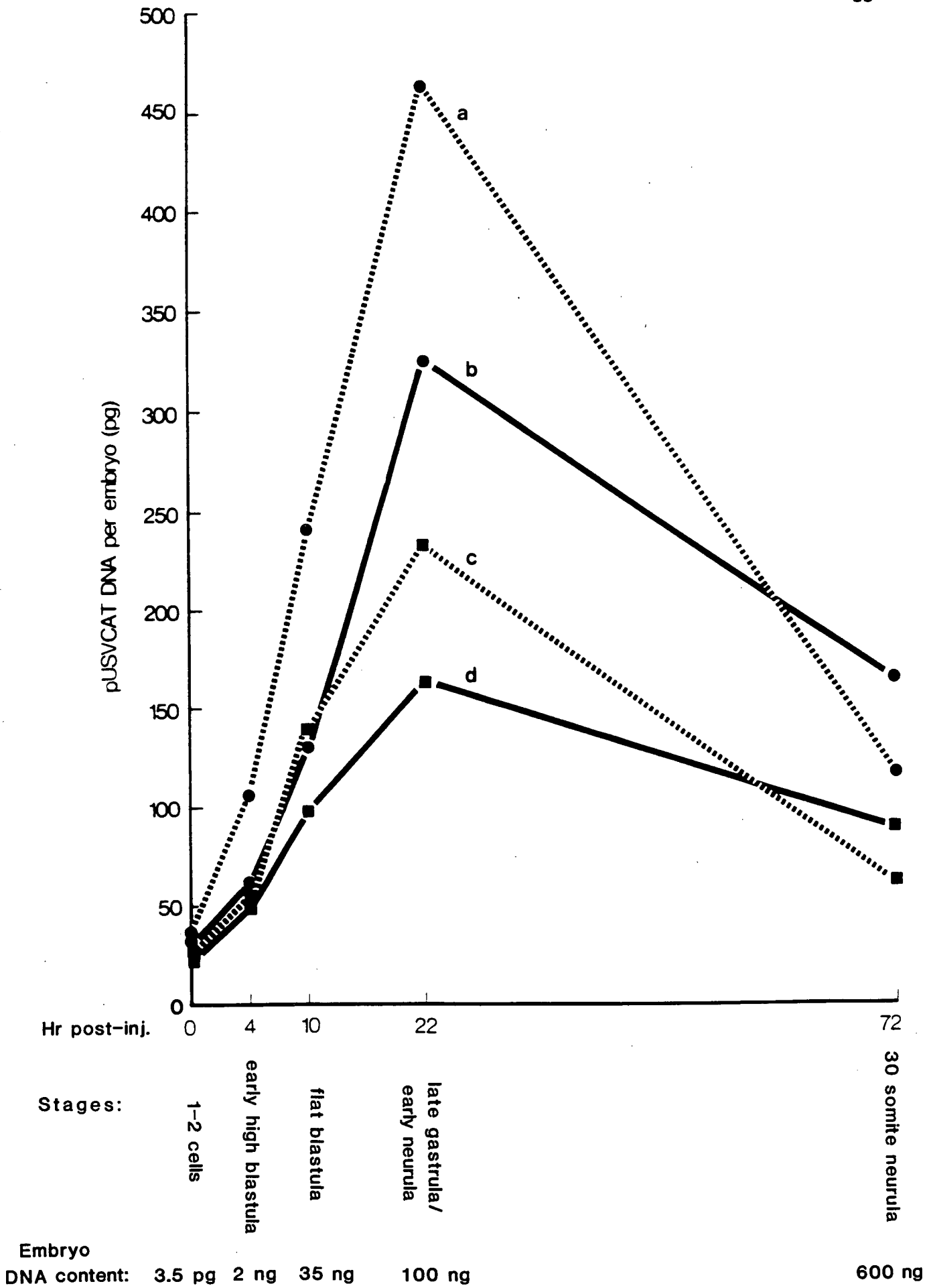
2.3 Replication of injected foreign DNA sequences in early embryos

As already observed in the Southern analyses of DNA from embryos injected with supercoiled and linear pUSVCAT DNA, CAT phage DNA and CAT phage particles, the total amount of plasmid DNA was greatest at the gastrula/neurula stage, decreasing significantly thereafter. In the experiments using supercoiled or linear plasmid DNA, the increasing intensities of DNA hybridization signals from the 1-2 cell stage to the gastrula/neurula stage suggest that the injected sequences were replicated during the cleavage and gastrulation stages of medaka embryogenesis. At the same time, the hybridization intensity of the smears indicate that a fraction of the hybridization signal consisted of degraded foreign DNA.

In an attempt to determine the degree of foreign DNA replication during early embryogenesis, a Southern blot hybridization assay of Sall digested embryo-equivalent DNA was performed. This method was chosen over the simpler 'dot blot' assay in order to separate undegraded DNA from degraded DNA. For pUSVCAT injected eggs a Sall band migrating as a 7.9 kb molecule corresponds to intact (*i.e.* transcriptionally functional) pUSVCAT monomers, whereas for eggs injected with CAT phage DNA, a Sall band migrating as a 15.6 kb molecule corresponds to intact CAT insert (*i.e.* 3 tandemly arranged CAT transcription units).

In experiments using supercoiled pUSVCAT DNA, total pUSVCAT DNA increased approximately ten-fold by the late gastrula/early neurula stage (22 hr post-injection), but the amount of intact pUSVCAT molecules at this stage represented only a six-fold increase (Fig 9b & d). Of the total pUSVCAT sequences present at this stage, 50% were recovered as unit length molecules.

Figure 9 Rapid increase and subsequent decrease of input pUSVCAT DNA during early embryogenesis, as measured by Southern blot hybridization assay. Embryo-equivalent aliquots of the DNA samples generated in the experiments in Fig 6 were digested with SallI, subjected to electrophoresis, blotted, hybridized to radio-labelled pUSVCAT probe, and autoradiographed as described in Fig 5. Hybridization bands on each filter lane corresponding to unit intact linear pUSVCAT molecules were cut out and quantified by scintillation counting. The remainder of each lane corresponding to plasmid smears were counted separately; total pUSVCAT DNA present in each embryo was calculated from the counts obtained from the entire lane. (a) and (b) Total pUSVCAT DNA per embryo after injection of fertilized eggs with linear and supercoiled pUSVCAT DNA, respectively. (c) and (d) intact pUSVCAT DNA per embryo after injection with linear and supercoiled pUSVCAT DNA, respectively. The time of harvest, embryonic stage, and genomic DNA content of each stage are indicated on the abscissa.



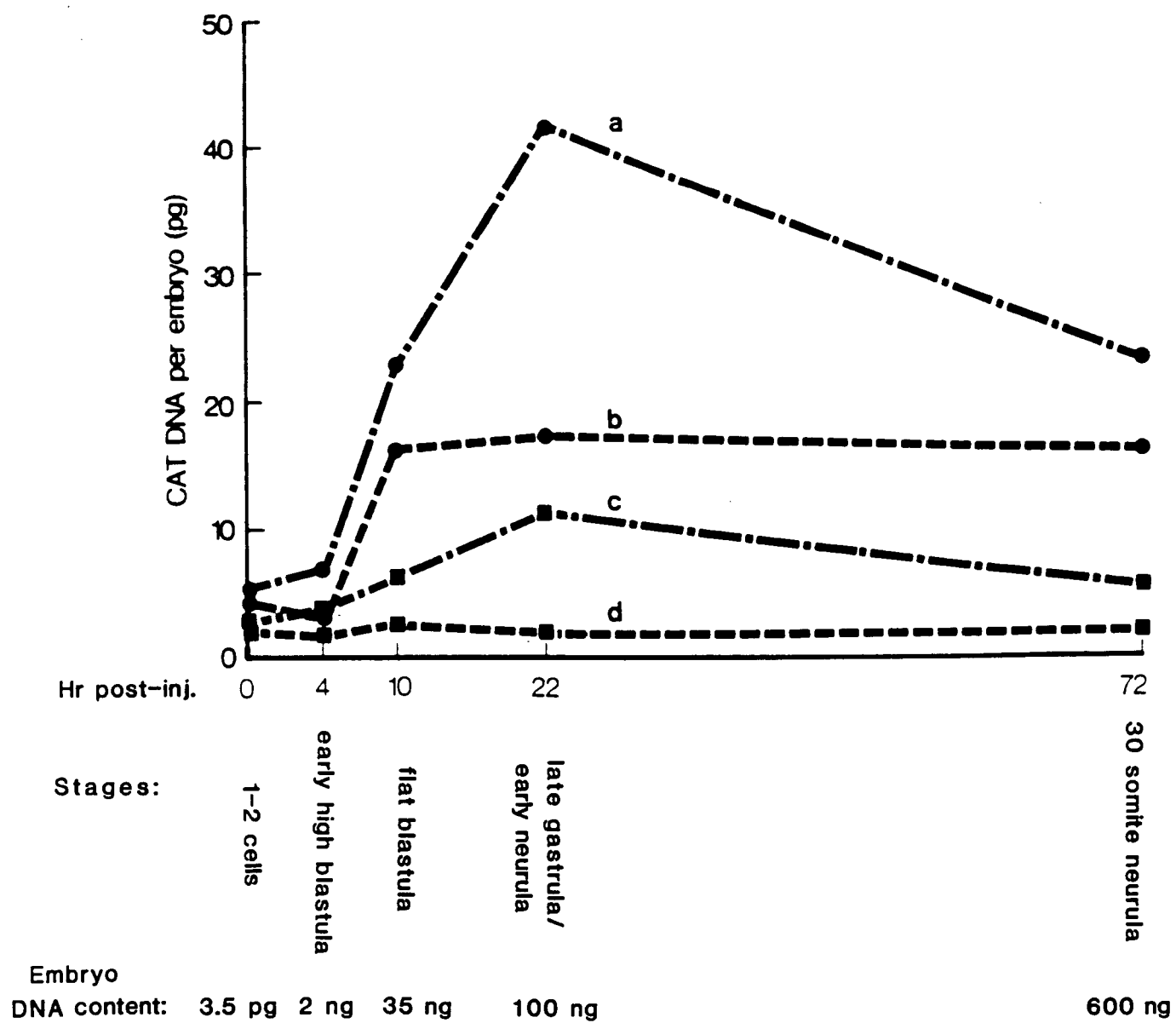
The greatest degree of increase was recorded with linear pUSVCAT DNA; total pUSVCAT DNA increased 12-fold by the gastrula/neurula stage, while the intact molecules were approximately nine-fold greater than that at the time of injection (Fig 9a & c). Intact pUSVCAT units also constituted 50% of the total pUSVCAT DNA sequences present at the gastrula/neurula stage. When CAT phage particles were used, total CAT DNA increased three-fold, but intact CAT insert increased less than one-fold, by the flat blastula stage (Fig 10b & d). With purified CAT phage DNA, total CAT DNA sequences increased approximately seven-fold by the gastrula/neurula stage; intact CAT insert increased only three-fold and accounted for roughly 30% of the total CAT DNA sequences at the gastrula/neurula stage (Fig 10a & c).

By the 30 somite stage of embryonic development (72 hr post-injection), all three DNA-injected groups (supercoiled pUSVCAT, linear pUSVCAT, and CAT phage DNA), but not the CAT phage particle-injected group, had significantly reduced total and intact CAT DNA. These results reflect a change in replication of the foreign sequences relative to their degradation, suggesting a slowdown or complete halt in CAT DNA replication after the gastrula/neurula stage, while the CAT DNA that were still present continued to be degraded. Throughout the period of embryonic development analysed (1-2 cell stage to 30 somite stage), the genomic DNA content of embryos continues to increase, although at diminishing rates of increase with progressing development.

3. Inherited expression of CAT reporter gene in offspring

One of the objectives in this study was to determine if the injected DNA or phage would result in genomic integration of the foreign DNA as well as in the inheritance and expression of these sequences by progeny fish. In order to identify positive founder fish, several treated fish were out-crossed with untreated medaka. One-week old F₁ embryos or F₁ hatchlings obtained from each out-cross were harvested and pooled in batches of five and analysed for

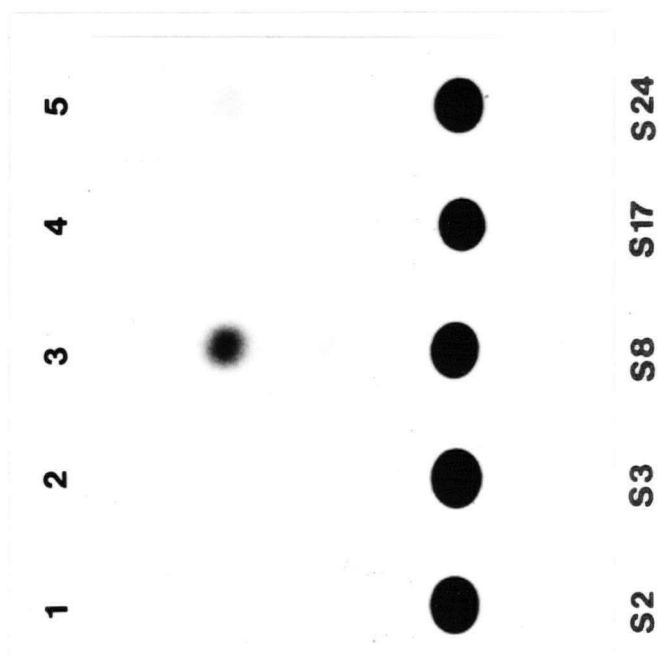
Figure 10 Increase and subsequent decrease of input CAT phage DNA during early embryogenesis. Fertilized eggs were injected with 4×10^4 recombinant phage particles (equivalent to 2 pg CAT phage DNA) or 2 pg purified CAT phage DNA. Total DNA was extracted from pools of ten embryos at various early embryonic stages. Embryo-equivalent aliquots were digested with Sall, subjected to electrophoresis, blotted, hybridized to radio-labelled pUSVCAT probe and autoradiographed as described in Fig 5. Hybridization bands in each filter lane corresponding to intact CAT DNA inserts, and the remainder of each lane (smears), were separately quantified by scintillation counting as described in Fig 9. Total CAT DNA present in each embryo was calculated on a similar basis as in Fig 9. (a) and (b) Total CAT DNA per embryo after injection with purified CAT phage DNA and recombinant CAT phage particles, respectively. (c) and (d) intact CAT DNA inserts per embryo after injection with CAT phage DNA and CAT phage particles, respectively.



inherited CAT gene expression. Representative CAT assays of pooled F_1 batches from several parents derived from eggs cytoplasmically injected with supercoiled and linear pUSVCAT, recombinant CAT phage, and purified CAT phage DNA are shown in Figures 11 and 12. Altogether, three to four batches of F_1 , representing 15 and 20 offspring respectively, were obtained from each out-cross, and analysed for CAT expression, the results of which are listed in Tables 1 through 4. CAT-positive offspring from parents representing all four injection groups (supercoiled pUSVCAT, linear pUSVCAT, recombinant CAT phage, and CAT phage DNA) were detected. CAT expression signals varied in strength, and are arbitrarily denoted by '+' to '+++' symbols. The observation in many instances that some F_1 batches were positive while other batches from the same parent were negative indicate that the percentage of CAT-positive offspring was low, and that the germline-positive parents were probably mosaic for the CAT sequences.

Figure 11 Inherited CAT gene expression in pooled offspring of pUSVCAT DNA-treated parents. Fertilized eggs that had been injected with (a) supercoiled, and (b) linear, pUSVCAT DNA were reared to maturity and outcrossed with untreated fish. Offspring from each outcross were pooled into batches of 5 and assayed for the presence of CAT enzyme activity. Autoradiograms show representative CAT assays of pooled F₁ from 5 different parents for each treatment group. Alpha-numerals at the bottom of each lane identify the different parents. See Table 1 and 2 for detailed results of F₁ CAT assays.

a



b

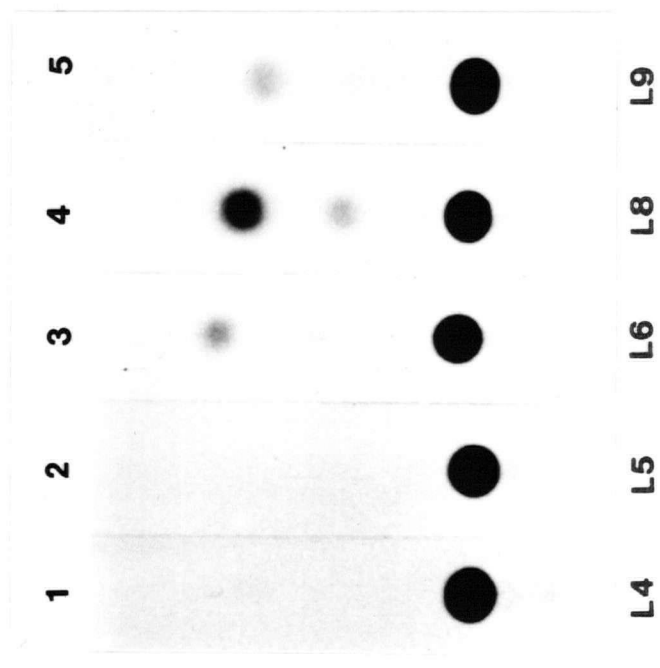


Figure 12 Inherited CAT gene expression in pooled offspring of CAT phage particle and CAT phage DNA treated parents. Fertilized eggs that had been injected with (a) recombinant CAT phage particles, and (b) purified CAT phage DNA, were reared to maturity and outcrossed with untreated fish. Offspring were pooled and assayed for CAT activity, and autoradiograms of representative CAT assays shown, as described in Fig 11. See Table 3 and 4 for detailed results of F₁ CAT assays.

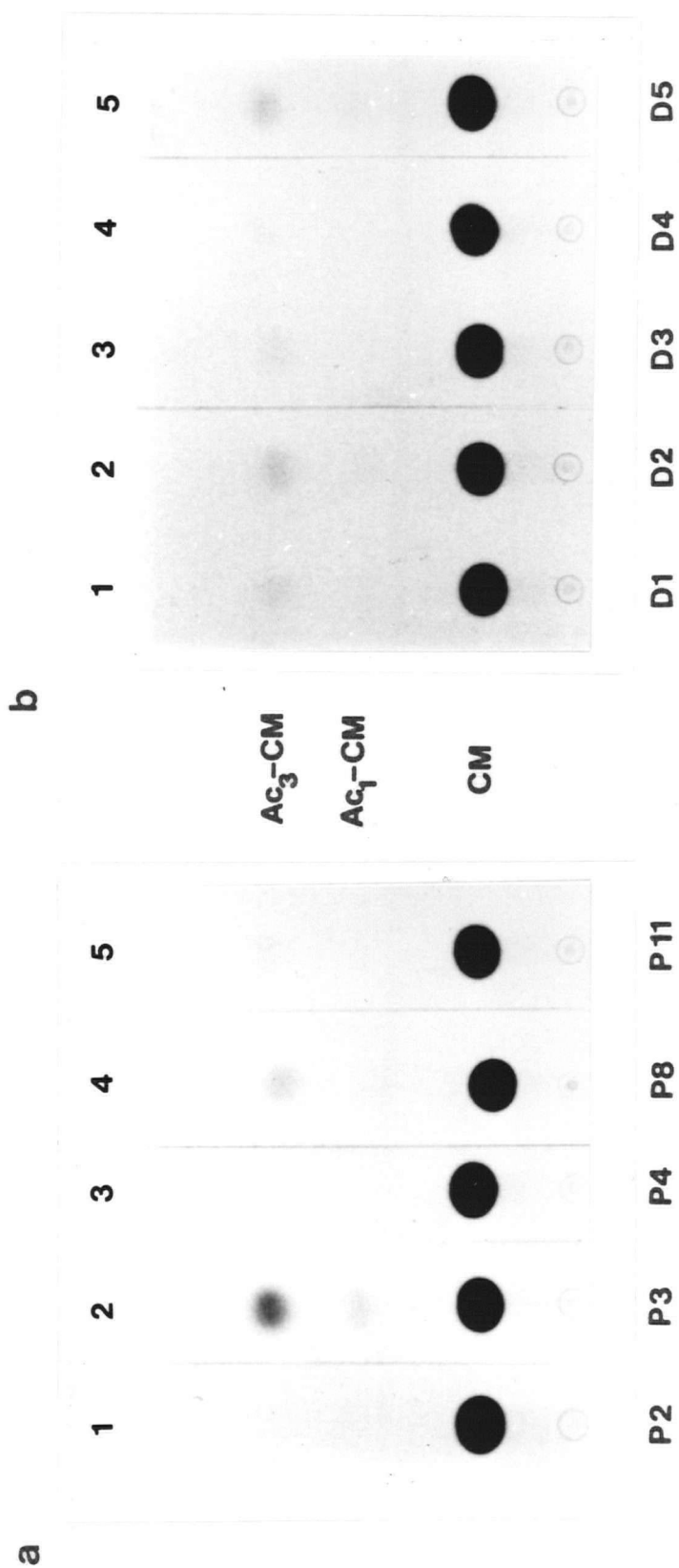


Table 1. Inherited expression of supercoiled pUSVCAT DNA in offspring. Medaka eggs injected with supercoiled pUSVCAT DNA were reared to adulthood and outcrossed with untreated fish. Offspring were pooled into batches of 5 and assayed for CAT enzyme activity. m, male; f, female; -, no detectible CAT activity; + to +++, relative CAT activity strengths; nd, not done.

<u>F₀</u> Sex	<u>F₁ batch no.</u>		
	<u>1</u>	<u>2</u>	<u>3</u>
S1 m	+	+	nd
S2 m	-	-	-
S3 m	-	-	-
S4 m	-	+	-
S5 f	+	+	-
S6 f	+	+	+
S7 f	+	+	-
S8 f	-	+++	+
S9 f	+	+	-
S10 f	+	+	+
S11 m	-	-	nd
S12 f	-	-	nd
S13 m	+	+	nd
S14 f	+	+	-
S15 f	+	+	+
S16 m	-	+	-
S17 m	+	+	+
S18 f	-	-	+
S19 f	+	+	+
S20 f	-	-	-
S21 f	-	-	-
S22 f	-	-	-
S23 f	++	-	nd
S24 f	++	++	++

Table 2. Inherited expression of linear pUSVCAT DNA in offspring. Parents were derived from eggs injected with linear pUSVCAT DNA. Procedure for screening of offspring as described in Table 1.

<u>F₀</u>	<u>Sex</u>	<u>F₁ batch no.</u>			
		<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
L1	m	-	+	++	nd
L2	m	-	-	-	nd
L3	m	-	-	+	-
L4	m	-	-	-	-
L5	f	-	-	-	-
L6	f	++	-	+++	-
L7	f	-	-	+	-
L8	m	-	+	+	+++
L9	f	+	++	+	nd

Table 3. Inherited CAT expression in offspring of parents derived from fertilized eggs injected with recombinant CAT phage particles. Procedure for screening of offspring as described in Table 1.

<u>F₀</u>	<u>Sex</u>	<u>F₁ batch no.</u>		
		<u>1</u>	<u>2</u>	<u>3</u>
P1	f	-	-	nd
P2	f	-	-	-
P3	m	+	+++	-
P4	m	-	-	-
P5	m	-	+	-
P6	f	+++	+	-
P7	m	-	-	+
P8	f	++	++	++
P9	m	-	-	-
P10	f	-	-	+
P11	f	+	++	+

Table 4. Inherited CAT expression in offspring of parents derived from fertilized eggs injected with purified CAT phage DNA. Procedure for screening of offspring as described in Table 1.

<u>F₀</u>	<u>Sex</u>	<u>F₁ batch no.</u>			
		<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
D1	f	+	+	+	+
D2	f	++	++	+	+
D3	f	+	++	-	-
D4	f	+	+	+	+
D5	f	++	++	++	nd

DISCUSSION

The expression and fate of a CAT reporter gene after microinjection into the cytoplasm of fertilized medaka eggs (1-2 cell stage embryos) were monitored during embryonic development and up to the free-swimming fish stage. This gene was applied as supercoiled or linear plasmid DNA, recombinant phage particles, or purified phage DNA.

The injection of supercoiled or linear pUSVCAT DNA resulted in peak CAT gene expression at the gastrula/neurula stage (1 day post-injection), followed by a sustained though slightly weaker expression in the one-week old embryo. Expression was significantly reduced, and varied noticeably, in the hatchlings sampled (2 weeks post-injection). By the free-swimming fish stage (4 weeks post-injection), expression was detected in only a few of the sampled fish. The relatively similar expression patterns obtained with supercoiled and linear plasmid DNA indicate that physical conformation of input DNA has no significant effect on its ability to be expressed. These results contrast with two conflicting observations in the Xenopus embryo; Etkin and Balcells (1985) reported a higher expression for supercoiled pSV2CAT DNA and lower CAT activity if the plasmid was first linearized, whereas Wilson *et al.* (1986) observed that a cloned actin gene and an actin-globin fusion gene were efficiently transcribed only if the circular plasmid containing either gene was first linearized.

The CAT expression pattern obtained when medaka eggs were injected with CAT phage DNA differed significantly from that obtained with pUSVCAT DNA; peak CAT gene expression did not occur until the one-week old embryonic stage. Expression characteristics at the hatchling and free-swimming fish stages, however, were similar to that observed with pUSVCAT injections. The increase in expression from the gastrula/neurula stage to the one-week old embryo stage is surprising since the amount of CAT phage DNA is maximal at the

gastrula/neurula stage and is significantly reduced thereafter. With CAT phage particles, a similar pattern of increasing CAT activity up to the one-week old embryo stage and subsequent decline was observed. These observations suggest a possible role of the phage vector arms in post-gastrula/neurula enhancement of gene expression. In an experiment involving the microinjection of a lambda DNA clone into fertilized Xenopus eggs, correctly localized transcription was achieved when a mixture of the actin gene insert and the vector arms was injected, but not when the intact clone was used (Wilson *et al.*, 1986). This suggests that the lambda sequences may have a cis-inhibitory effect on transcription of the actin gene in the Xenopus embryo.

Analysis of embryos at earlier stages revealed that the injected supercoiled or linear pUSVCAT DNA was not expressed prior to the early high blastula stage (4 hr post-injection) although approximately 25 pg DNA, corresponding to 3×10^6 copies of the CAT gene, was injected into each 1-2 cell stage embryo. The onset of CAT expression in medaka embryos only after the early high blastula stage appears to parallel a phenomenon observed in Xenopus embryos by Newport and Kirschner (1982a,b). These authors showed that transcription of endogenous or injected DNA in Xenopus embryos begins at stage termed the 'mid-blastula transition' (Gerhart, 1980), when a critical ratio between nucleus and cytoplasm is reached. They also showed that transcriptional suppression of a yeast leucine tRNA gene which was injected at pre-mid-blastula stages could be reversed by injecting an amount of pBR322 DNA equivalent to the total genomic DNA that is present in a mid-blastula stage embryo, thereby titrating out presumed suppressor components. Etkin and Balcells (1985), using the pSV2CAT plasmid DNA, were also unable to detect expression of the CAT gene prior to the mid-blastula transition. They argued against the possibility that the appearance of CAT activity only at the mid-blastula transition 8 hr after injection was simply a consequence of increase in pSV2CAT DNA copy number after several rounds of replication, since

injection of an equal amount into non-replicating oocytes elicited CAT expression as early as 2.5 hr after injection. Furthermore, injection of a different CAT plasmid construct together with a trans-activating enhancing factor did not induce earlier expression, but did induce a much stronger expression at the mid-blastula transition. In the present study, injection of higher DNA amounts equivalent to the genomic DNA content of the medaka mid-blastula was not attempted because exogenous DNA doses above 250 pg have been shown to be lethal to the medaka embryo (Vielkind *et al.*, 1988). Although CAT phage DNA injected eggs were not analysed at the stages earlier than the gastrula/neurula stage, the moderately strong signal seen at this stage implies that the expression of the input CAT phage DNA also began an earlier embryonic stage. In marked contrast, a transcription initiation stage similar to the Xenopus mid-blastula transition is not present during sea urchin embryogenesis. For example, sea urchin early histone genes are expressed during early cleavage (reviewed in Davidson, 1976) since the limited histones present in the comparatively smaller egg are sufficient only for a few cleavages (Poccia *et al.*, 1981). Injected early histone H2A (Vitelli *et al.*, 1988) and early histone H2B (Colin *et al.*, 1988) genes showed peak transcription together with endogenous genes during the early blastula stage.

As noted for all four treatment groups, for each of the stages up to the one-week old embryo stage, CAT expression signals were highly consistent among the samples analysed. At the hatchling stage, signal strengths among the samples varied noticeably, and by the free-swimming fish stage CAT expression could not be detected in a significantly large fraction of the samples. These observations are reflected in the fate of the introduced plasmid and phage DNA sequences whereby, at embryonic stages up to the one-week old embryo stage, the amount of foreign DNA sequences present at each stage was fairly consistent among the sampled embryos. Amounts of exogenous DNA increased during cleavage and gastrulation and were significantly reduced by the one-

week old embryo stage. Thereafter, DNA amounts fluctuated among the sampled hatchlings, and was no longer detectible in most of the sampled free-swimming fish. Upon injection, supercoiled pUSVCAT DNA was gradually converted to a high molecular weight concatemer oriented head-to-tail. This high molecular weight form was observed to increase greatly during cleavage and gastrulation, suggesting that it is the preferred replicative structure. When linear pUSVCAT DNA was injected, a very rapid conversion to high molecular weight concatemers occurred, restriction analysis of which revealed a random orientation of the ligated molecules. Some conversion of the linear molecules to supercoils, open circles, and multimeric circles was also evident and the high molecular weight form was also rapidly replicated during cleavage and gastrulation. It is highly improbable that the 'appearance' of the three circular forms resulted from replication of trace amounts already present in the stock DNA solution; some supercoils and open circles were already detectible in embryos analysed within 5 min of their injection with linear pUSVCAT (see Fig 6b, lane 1), and Southern blot hybridization of linear pUSVCAT standards containing the equivalent of one to four times the injected amount showed no evidence of these circular forms (data not shown). The results obtained with supercoiled and linear plasmid DNA are virtually identical to the observations by Marini *et al.* (1988) in the Xenopus embryo but differ somewhat from the processes occurring in the sea urchin embryo. In the sea urchin embryo, supercoiled plasmids neither ligate nor replicate and only linear molecules are rapidly and efficiently assembled into end-to-end concatenates (McMahon *et al.*, 1985). In addition, linear molecules injected into sea urchin embryos are not able to reform supercoils or circular molecules (McMahon *et al.*, 1985). Marini *et al.* (1988) noted that the high molecular weight concatemer formed in Xenopus embryos from linear plasmid molecules may exist as a random concatemer or may exhibit a preference for head-to-tail orientation. A similar mechanism may exist in the medaka embryo,

which could explain why restriction analysis of the linear plasmid-derived concatemers yielded more of the fragment expected from both unidirectional and random ligations and less of the fragments only expected from random ligations. The formation of random concatemers generated by linear molecules is presumably due to random ligation of linear termini. A Xenopus ovary specific protein that promotes concatenation *in vitro* of linear DNA (Bayne *et al.*, 1984) and a sea urchin early embryo specific DNA ligase that acts on linear DNA with cohesive or blunt ends (Prigent *et al.*, 1987) have been isolated. Presumably a similar activity is present in medaka embryos to assemble long concatemers from unit length linear DNA. The exclusively head-to-tail orientation of concatemers generated by supercoiled DNA indicate that, apart from random ligation, another mechanism related to homologous recombination possibly exists in the medaka embryo, as postulated for Xenopus embryos (Marini *et al.*, 1988). Folger *et al.* (1982) have shown in mammalian cells that injected linear or supercoiled plasmids are organized into tandem head-to-tail arrays through homologous recombination between plasmid molecules. The presence of high molecular weight forms after injection of linear plasmid DNA has been reported in embryos of other fish species (Zhu *et al.*, 1986; Dunham *et al.*, 1987; Stuart *et al.*, 1988).

No rapid conversions of the sort observed with plasmid DNA injections occurred when medaka eggs were injected with either CAT phage particles or CAT phage DNA. Instead, an upward shift consistent with a change into higher molecular weight structures was obvious only from the one-week old embryonic stage onwards. Heating of the embryo DNA extracts at 65 °C followed by rapid cooling prior to gel electrophoresis and Southern blot analysis did not produce a band corresponding to CAT phage DNA monomers. It is possible that the linear phage molecules were covalently ligated into concatemers either with or without modification of their cohesive ends. Covalent end-to-end

ligation of a recombinant λ clone has been observed after pronuclear injection into fertilized mouse eggs (Costantini and Lacy, 1981).

A comparison of the extent of replication between supercoiled and linear plasmid DNA injected embryos points to a slightly greater degree of plasmid DNA replication when the linear molecule is used. This might be expected considering the much quicker conversion of input linear molecules into high molecular weight concatemers, which appear to be the preferred replicative form. For eggs injected with CAT phage particles, total CAT DNA was relatively unchanged up to the early high blastula (4 hr post-injection), possibly suggesting that most of the phage DNA has yet to be released from the phage coats. The increased total CAT DNA at the flat blastula stage (10 hr post-injection) might be explained by a subsequent release of DNA from the phage coats and ensuing DNA replication. There appears to be no further increases in DNA after this stage, amounts remaining unchanged even at the 30 somite neurula stage (72 hr post-injection). The lack of a protective phage coat around the injected purified CAT phage DNA probably accounts for the significantly greater degree of DNA replication and degradation as compared to CAT phage particle-injected embryos. The input CAT phage DNA was, however, not as extensively replicated as input plasmid DNA of supercoiled or linear conformation. By the 30 somite neurula stage, embryos derived from plasmid and phage DNA injections contained significantly reduced exogenous DNA. Similar observations of replication up to the gastrula/neurula stage and subsequent loss of exogenous supercoiled and linear plasmid DNA has been observed in Xenopus (Rusconi and Schaffner, 1981). In sea urchins (McMahon *et al.*, 1985) and fish (Zhu *et al.*, 1986), a similar stage specific increase and decrease of injected linear plasmid DNA has been reported.

Since the majority of input DNA sequences that were replicated were continually being degraded and subsequently lost, it is likely that they remained extra-chromosomal throughout embryogenesis. Presumably, extra-

chromosomally replicated foreign DNA is unequally segregated among the daughter cells of a rapidly dividing and growing embryo, resulting in some of the cells not having the exogenous sequences at all. In addition, exogenous DNA segregated into actively dividing cells of rapidly growing tissues would continue to be replicated, whilst those ending up in non-dividing or slowly dividing cells of differentiated tissues would be quickly degraded and lost. The net result would be an animal mosaic for the presence of the foreign DNA. Mosaicism has been demonstrated in Xenopus where pSV2CAT 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 direct *in situ* DNA hybridization that exogenous CAT DNA sequences injected into fertilized sea urchin eggs are mosaically distributed to most or all cell types or lineages of the embryo. This unequal distribution and resultant mosaicism of non-chromosomal exogenous DNA may explain the greater CAT expression variations among the medaka hatchlings and loss of CAT enzyme activity in many free-swimming fish.

Despite the fact that most of the input and replicated DNA remain extra-chromosomal, the possibility exists that genomic integration of some of the exogenous sequences occurred. In this present study, integration of the injected CAT plasmid and phage sequences into the medaka genome is supported by the persistence of CAT DNA sequences and of CAT gene expression in a few free-swimming fish, and by the demonstration of inherited CAT gene expression in pooled offspring of several DNA and phage-treated fish. Since germline-positive parents were identified from all four treatment groups, it would appear that neither the initial DNA conformation, nor different vector sequences adversely affect the ability of a gene to be inherited by offspring. Neither does packaging of DNA into phage particles appear to be a barrier to germline transmission of the enclosed gene. The observation that some F₁ pools were positive, while others from the same parent were not, suggests that

the germline-positive parents were mosaic for the integrated sequences. This would be expected if the assumption is made that the incorporation of the foreign sequences did not occur immediately after injection, and that the exogenous DNA was incorporated into only one or a few of several primordial germ cells. In the medaka, Gamo (1961) determined that no primordial germ cells could be distinguished prior to gastrulation, but that up to 20 such cells could be determined at the early gastrula stage (15 hr post-fertilization, the equivalent of 13 hr post-injection). Genomic integration at an early cleavage stage would theoretically result in a higher probability that all the primordial germ cells contain the integrated foreign sequence. The observation that some of the tested fish were not germline positive does not exclude the possibility that they may harbour exogenous sequences in a fraction of their cells in other tissues. Genomic integration of exogenous DNA sequences after injection into the cytoplasm of fertilized sea urchin egg has been demonstrated by Flytzanis *et al.* (1985). In addition, germline transmission of cloned genes after microinjection into the egg cytoplasm has recently been reported in Xenopus (Etkin and Pearman, 1987) and in fish (Guyomard *et al.*, 1988; Stuart *et al.*, 1988; Vielkind *et al.*, 1988). In all the above cases, the animals were mosaic for the exogenous sequences, and mosaicism of the founder animals was inferred from the observation that only a percentage of the offspring from each founder were positive for the presence of the exogenous sequences. It should be noted that germline transmission *per se* does not prove stable host genome integration, as was shown in the nematode in which an episomal structure was passed to the offspring (Stinchcomb *et al.*, 1985). Thus, genomic integration has to be further substantiated by analysis of Mendelian inheritance of the transgene. Stuart *et al.* (1988) were able to demonstrate stable integration of a linear plasmid in the zebrafish genome, showing that 50% of F₂ progeny issued from a foreign DNA positive F₁ outcrossed to an untreated fish carried the foreign sequence.

The F_1 CAT assay procedure adopted in this study was devised as a quick screen to enable a large number of parents to be rapidly tested for germline transmission of functional CAT sequences. Dot blot or slot blot analysis has been employed in zebrafish (Stuart *et al.*, 1988) and Southern blot analysis used in trout (Guyomard *et al.*, 1988) to test offspring for the presence of foreign DNA sequences. However, DNA loading constraints for dot/slot blots dictate the need to load equal amounts of DNA within a narrow range. A minimum amount of total DNA is required in order for single copy genes to be detected. Offspring cannot be pooled since excess total DNA may compete out any weak hybridization signal or conversely result in high background signals. The DNA extraction and hybridization procedures are also comparatively elaborate. Southern blot analysis allows more DNA to be loaded and thus could allow pooling of offspring from a single parent, but is comparatively slower than either dot/slot blot analysis or CAT enzyme assay. Most importantly, a positive signal from DNA analysis provides no information on whether the transmitted CAT gene is functional. Using the simpler and quicker CAT assay protocol, tests with pooled embryos or hatchlings from non-treated parents were negative for CAT activity, and no inhibitory effect could be detected when a commercial CAT enzyme was incubated in pooled untreated embryo/hatchling extracts. Thus, not only could F_1 embryos and hatchlings from the same DNA/phage treated parent be pooled, but a positive CAT signal indicated germline transmission of fully functional CAT sequences.

Taken together, the results of this study indicate that cloned DNA and recombinant phage particles, cytoplasmically injected at the 1-2 cell stage of medaka embryos, persist and are expressed during embryogenesis. No significant advantage with regard to expression and persistence was observed when either linear or supercoiled plasmid molecules were used, but an apparently more efficient expression was observed with the plasmid DNA clone than with the phage DNA clone. Nonetheless, the successful expression and

replication of input phage DNA clone opens the possibility of testing larger genes, especially genomic sequences containing long stretches of introns or distant regulatory regions, which cannot be cloned into plasmids. In addition, providing sufficiently high phage particle titres are used, genes cloned into recombinant phages can be tested by direct injection of phage particles without having to first extract the DNA.

The very consistent CAT expression signals in the early embryonic stages of the medaka strongly favour its use as a transient expression system in the analysis of 'early' gene regulatory regions, as has been done, e.g. in sea urchins (Flytzanis, *et al.*, 1987). In addition, the medaka embryo can be used in the functional testing of genes intended to be used in the genetic engineering of economically important fishes. For example, genes coding for the winter flounder antifreeze proteins (Lin and Gross, 1981; Davies *et al.*, 1982; Gourlie *et al.*, 1984), salmon beta-gonadotropin (Trinh *et al.*, 1986), and rainbow trout growth hormone (Agellon *et al.*, 1988) have been cloned. Introduction of such genes into the genome of fish could potentially alter their cold water tolerance, fertility, and growth rate, respectively. The ability of the exogenous DNA sequences to persist in some free-swimming stage medaka, and the positive F₁ CAT assay results, also open the possibility of creating transgenic lines of medaka for the study of cell lineages and 'late' gene regulation, among others.

The embryo is well suited for these purposes since the relatively large embryo size (ca 1.5 mm diameter) makes manipulation under low magnifications (8x to 50x) possible, the two cells derived from first cleavage are very well defined, and sharp injection needles can easily penetrate the chorion. Although not employed in these studies, the chorion can be made more penetrable by mild digestion with proteinase K or pronase without having any effect on the viability of the embryos. Thus, harsh treatments such as trypsin-urea used to dissolve the chorion of goldfish eggs (Yamaha *et al.*,

1986) or manipulations such as boring tiny holes into the chorion of trout eggs prior to injection (Rokkones *et al.*, 1985) are unnecessary. This technique also obviates the more difficult task of injecting into the germinal vesicle of the medaka oocyte, a procedure adopted by Ozato *et al.* (1986), since injections into the cytoplasm are sufficient to elicit gene expression and germline transmission of the foreign sequences.

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