

Distribution, expression and germ line transmission of exogenous DNA sequences following microinjection into *Xenopus laevis* eggs

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Summary

We analysed the fate, expression and germ line transmission of exogenous DNA which was microinjected into fertilized eggs of *Xenopus laevis*. DNA was injected into fertilized eggs within 1 h following fertilization. The injected DNA was dispersed around the site of injection and became localized to cleavage nuclei by stage 6. Injected DNA persisted in the tissues of 6- to 8-month-old frogs and exhibited a mosaic pattern of distribution with regard to the presence or absence and copy number between different tissues. We detected the exogenous DNA sequences in 60 % of injected frogs. Restriction digestion analysis of this DNA suggested that it is not rearranged and was organized as head-to-tail multimers. The copy number varied from 2 to 30 copies/cell in various tissues of the

same frog. Plasmid pSV2CAT which contains the prokaryotic gene coding for chloramphenicol acetyl transferase (CAT) enzyme linked to the SV40 early gene promoter was expressed in 50 % of the animals containing the gene. The pattern of expression, however, varied between different animals and could not be correlated with copy number. We also showed that the exogenous DNA sequences were transmitted through the male germ line and that each offspring contained the gene integrated into a different region of the genome.

Key words: transgenic, tissue-specific expression, germ line transmission, gene regulation, transcription, differentiation, development, *Xenopus*, microinjection.

Introduction

Cloned genes have been injected into fertilized eggs to assess the factors involved in regulating their expression during development and cellular differentiation (for reviews see Etkin, 1982; Etkin & DiBerardino, 1983; Gurdon & Melton, 1981). Injected DNA sequences replicate to varying degrees and most are expressed at the midblastula stage of development (Bendig, 1981; Rusconi & Schaffner, 1981; Newport & Kirschner, 1982*a,b*; Etkin, Pearman, Roberts & Bektesh, 1984; Bendig & Williams, 1984; Etkin & Balcells, 1985). During early cleavage injected circular plasmids remain extra-chromosomal and reach maximal amplification at the blastula stage, after which there is a gradual loss of these sequences. There is, however, a fraction of injected DNA that persists in the tissues of young frogs (Rusconi & Schaffner, 1981; Etkin & Roberts, 1983; Etkin *et al.* 1984; Andres, Muellener &

Ryffel, 1984; Bendig & Williams, 1984). The initial transformants appear to be mosaic because some tissues contain the gene while other tissues lack these sequences. In some cases, rearranged episomal DNAs have been detected in tissues of young frogs (Andres *et al.* 1984). No tissue-specific expression of injected sequences has been reported in transformed frogs, though differential expression of adult and tadpole *Xenopus* β globin was observed in embryos (Bendig & Williams, 1984).

In the present study, we injected recombinant plasmids containing the coding sequence for the prokaryotic enzyme chloramphenicol acetyl transferase (CAT) and the *Drosophila* alcohol dehydrogenase gene (psAC-1) into fertilized eggs. Our goals were (1) to determine the nucleocytoplasmic partitioning of the injected DNA during early cleavage stages, (2) to analyse the persistence of the DNA in adult frogs, (3) to analyse the expression of the injected DNA and (4) to test for germ line transmission.

Materials and methods

Plasmids

The recombinant plasmid pSV2CAT (Gorman, Moffat & Howard, 1982) (provided by Dr N. Jones) contains the SV40 early promoter linked to the gene coding for the prokaryotic enzyme chloramphenicol acetyl transferase (CAT). This plasmid lacks the prokaryotic promoter. Plasmid psAC-1 is a recombinant plasmid containing a 4.75 kb insert with the *Drosophila* alcohol dehydrogenase (ADH) gene in pBR322.

Injection of DNA into fertilized eggs

Eggs fertilized by artificial insemination were injected within 1 h with 0.5–1.0 ng of linearized or circular plasmids according to Etkin *et al.* (1984). All injected embryos were raised at 18°C. In most experiments, embryos were kept at 12°C until second cleavage to help reduce leakage after injection.

Extraction of RNA and dot blotting

RNA was extracted from embryos as described previously, utilizing a solution containing phenol (50%), chloroform (48%), isoamyl alcohol (2%) (Etkin & Maxson, 1980; Etkin *et al.* 1984). Contaminating DNA was removed by digestion with RNase-free DNase (BRL Laboratories) at 1 unit/1 µg of DNA for 20 min at 37°C. Digestion of DNA was determined by analysis on agarose gels and staining with ethidium bromide. RNA was denatured using formaldehyde (White & Bancroft, 1982) and it was spotted onto nitrocellulose (Thomas, 1980) using a dot blot apparatus. Filters were baked and hybridized with a nick-translated probe (specific activity 2×10^8 cts min⁻¹ µg⁻¹) prepared from the appropriate DNA. Hybridization was carried out at 65°C in 1 M-NaCl, 1 mM-EDTA, 50 mM-Tris, 4 mM-sarcosyl, 2.5 mM-sodium pyrophosphate, and 10 × Denhardt's solution. The blots were washed with two changes of 2 × standard saline citrate (SSC), 0.1% SDS followed by three washes with 0.1 × SSC, 0.1% SDS at 42°C, and then exposed to X-ray film. Retention of the amount of nucleic acid during DNA dot blotting was monitored by using known standards of plasmid DNA mixed with DNA from noninjected frogs.

DNA extraction, Southern blotting and restriction enzyme digestion

DNA was extracted from embryos, larvae and tissues by homogenizing embryos in 0.5–1.0 ml of homogenization buffer [300 mM-NaCl, 100 mM-Tris (pH 7.5), 10 mM-EDTA, 2% SDS, with proteinase K (1 mg ml⁻¹) (Beckman Incorporated)]. Homogenates were incubated at room temperature for 30 min and extracted one or two times with an equal volume of phenol/chloroform/isoamyl alcohol and once with chloroform. DNA was precipitated with two volumes of absolute ethanol at -20°C in 2.5 M-ammonium acetate. RNA was removed with RNase treatment. Electrophoresis was performed on 0.7% agarose gels using TAE buffer (40 mM-Tris, 5 mM-sodium acetate and 1 mM-EDTA, pH 7.5) and the DNA was transferred to nitrocellulose filters. Filters were hybridized with a nick-translated probe from pSV2CAT or psAC-1. Restriction

enzyme (New England Biolabs or BRL) digestion of DNA was carried out according to manufacturers' specifications.

CAT enzyme assay

CAT enzyme assays were performed according to the method of Gorman *et al.* (1982), with slight modifications (Etkin & Balcells, 1985). Tissue samples were homogenized in 100–300 µl of 0.25 M-Tris (pH 8.0) depending on size of the tissue sample. Homogenates were centrifuged in a microfuge for 15 min at 4°C. Supernatants were removed and added to Eppendorf tubes containing 1.5 µCi of [¹⁴C]chloramphenicol (Amersham Corp.). The reaction was started by adding 3 µl of acetyl CoA (3.0 mg ml⁻¹, P&L Biochemicals). The mixture was incubated at 37°C for 1–2 h. Chloramphenicol was extracted with cold ethyl acetate, and the ethyl acetate removed by drying. The residue was dissolved in 20 µl of ethyl acetate and spotted onto silica gels; it was then chromatographed for 30 min in 95% chloroform/5% methyl alcohol. The gels were air dried and autoradiographed. Conversion of [¹⁴C]chloramphenicol to the acetylated forms was quantified by cutting out the areas on silica gels and determining the radioactive counts in a scintillation counter. The linearity of the assay was confirmed by controls in which known amounts of purified enzyme were analysed and by time course analysis of extracts.

Labelling of plasmid DNA and histology of injected embryos

An M13 subclone of the 1.2 kb *Bam*H1–*Sal*I fragment of psAC-1, which contains the *Drosophila* alcohol dehydrogenase gene, was labelled to 2×10^6 cts min⁻¹ µg⁻¹ by synthesis of the complementary strand using the M13 primer, Klenow fragment of *E. coli* polymerase I, and ³H-TTP and ³H-ATP. The labelled DNA was isolated from the unincorporated nucleotides by 2.5 M-ammonium acetate-ethanol precipitation. 1 ng of the labelled DNA was injected into fertilized *Xenopus laevis* eggs. Embryos were fixed at appropriate stages in Bouin's fixative for 15 min; rinsed in 70% ethanol; dehydrated with 80%, 90% and 95% ethanol; cleared with amyl acetate and embedded in paraffin. The embryos were sectioned at 12 µm, fixed to slides, dehydrated in a 30–95% ethanol series and dipped in Kodak NTB-2 emulsion (diluted 1:1 with dH₂O). The slides were exposed at 4°C. The autoradiographs were developed for 2 min in a 1:1 dilution of Dektol (Kodak), rinsed in distilled water, fixed 5 min in Kodak fixer and washed 5 min in distilled water. The sections were stained with 0.5 µg ml⁻¹ Hoechst dye in distilled water for 10 min and rinsed in distilled water. The slides were flooded with 0.42 M-citric acid, 0.8 M-Na₂HPO₄ and a coverslip was applied and sealed with rubber cement.

Results

Injected DNA is localized in cleavage nuclei by stage 6

We followed the fate of the injected DNA by labelling a recombinant M13 vector containing the *Drosophila* ADH gene with [³H]thymidine, injecting

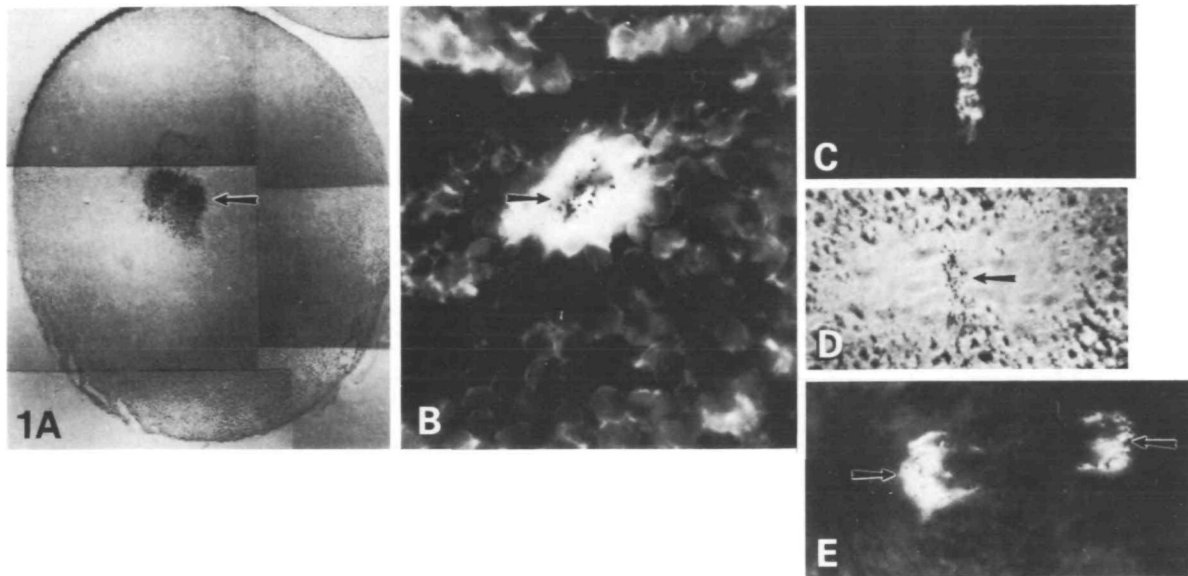


Fig. 1. Localization of radiolabelled plasmids injected into fertilized eggs. 1 ng of radiolabelled psAC1 (specific activity 2×10^6 cts $\text{min}^{-1} \mu\text{g}^{-1}$) was injected into fertilized eggs. Embryos were fixed and sectioned at various stages of development and processed for autoradiography (see Materials and Methods). The slides were stained with Hoechst dye and examined by either fluorescent or light microscopy. (A) Embryo at stage 1 immediately following injection of labelled plasmid. The arrow points to the localization of silver grains. (B) Section showing localization of silver grains over a stage 6 nucleus viewed under fluorescent light to show presence of DNA. (C) Stage 6 metaphase plate under fluorescent light. (D) The same section as in C viewed under light microscopy to show silver grains. (E) Stage 6.5 anaphase viewed under fluorescent light, showing grains localized over the chromosomes.

Table 1. Number of grains over nuclei in autoradiographs of stage 6.5 embryos

	Number of grains			
	0-5	6-10	11-25	26 or more
Number of nuclei	15 (14 %)	9 (8 %)	25 (23 %)	61 (55 %)

Number of nuclei counted = 110.

this into fertilized eggs and performing autoradiography on histological sections at various developmental stages. The sectioned embryos were stained with Hoescht dye to stain nuclei and serial sections were examined by fluorescent microscopy.

We injected 1 ng of psAC-1, which contains the *Drosophila* ADH gene, into fertilized eggs within 1 h following fertilization. Immediately following injection the plasmid was localized to an area surrounding the injection site (Fig. 1A). By stage 6 (midcleavage) the injected DNA was observed localized in many of the cleavage nuclei (Fig. 1B). More interestingly this DNA appeared to be sticking to the chromosomes during mitosis and segregating in a random fashion (Fig. 1C-E).

Analysis of serial sections showed that grains were detectable in 60-85 % of the nuclei, indicating that the injected plasmids were distributed in a mosaic pattern (Table 1). It is unlikely that the results

obtained were due to the degradation and reincorporation of the labelled nucleotides into the genomic DNA because the label was not detected in all nuclei.

It is probable that the majority of these sequences were extrachromosomal circular plasmids which will be degraded following the blastula stage of development. This was tested by analysing the persistence of injected labelled templates by extracting the DNA, separating it by electrophoresis on agarose gels and blotting it to nitrocellulose. The blot was coated with scintillant and exposed to X-ray film. The fluorograph showed that the plasmids persisted as circular molecules and there was no detectable loss or increase of the ^3H -labelled plasmids through the early blastula stages, but there was a loss of circular plasmid by the late gastrula stage (data not shown).

Injected DNA is detected in various tissues of young frogs

Between 0.5 and 1.0 ng (10^7 copies) of the *Bam*H1 linearized plasmid pSV2CAT was injected into each egg. Linearized plasmids were injected since results from previous studies suggested that persistence of exogenous DNA was more efficient if the plasmids were injected as linear rather than circular molecules (Etkin *et al.* 1984). The embryos were reared until postmetamorphosis stages, at which time tissues were removed and analysed.

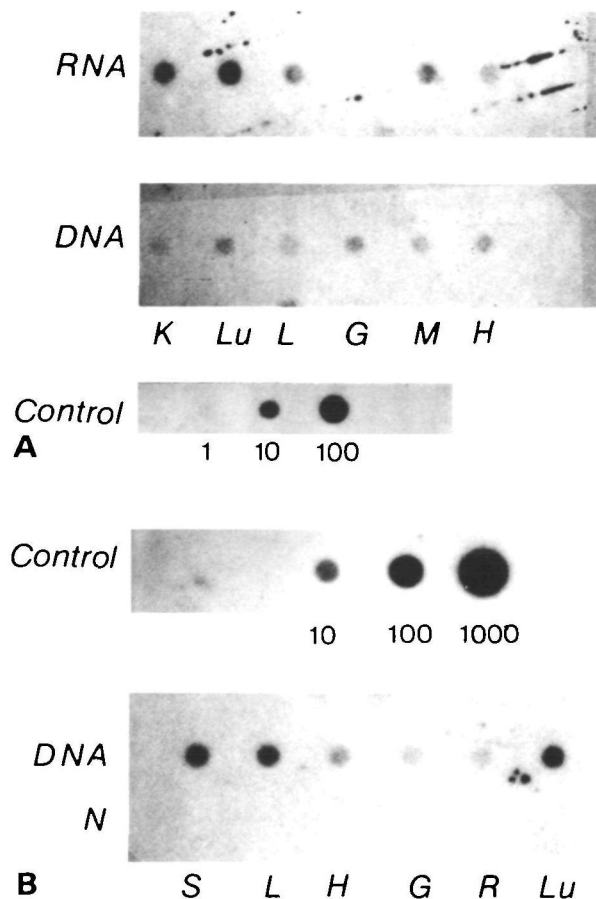


Fig. 2. (A) Dot blots of both RNA and DNA from tissues of a 6-month-old frog. DNA and RNA were extracted from individual tissues as described in Materials and Methods. Approximately $5\mu\text{g}$ of DNA or RNA from each tissue were spotted onto nitrocellulose. The filter was hybridized with a nick-translated probe (2×10^8 cts $\text{min}^{-1} \mu\text{g}^{-1}$), washed and placed in contact with X-ray film. K, kidney; Lu, lung; L, liver; G, gut; M, skeletal muscle; H, heart muscle; Control, standards are purified pSV2CAT plasmid spotted onto nitrocellulose which represent 1 copy, 10 copies or 100 copies of the gene/genome equivalent. (B) Dot blot of DNA from tissues of an 8-month-old frog. Control, standards representing 10, 100 or 1000 copies of the gene/genome equivalent; N, DNA of tissues from a noninjected frog; S, stomach; L, liver; H, heart; G, gut; R, reproductive organs; Lu, lung.

Fig. 2A is a dot blot of DNA extracted from various tissues of a 6-month-old frog demonstrating the presence of pSV2CAT sequences in six tissues (no. 10 in Table 2). In each sample, approximately $5\mu\text{g}$ of DNA was spotted onto nitrocellulose filters and hybridized with a nick-translated probe (2×10^8 cts $\text{min}^{-1} \mu\text{g}^{-1}$) from pSV2CAT. There was a variation in copy number in the different tissues of approximately 2–10 copies/genome as determined by comparison to known DNA standards. Fig. 2B is a dot blot of DNA from tissues of a second frog (animal no. 13, Table 2).

This animal also shows variation in pSV2CAT copy number with approximately 10 copies/genome in stomach, liver and lung and 3–5 copies/genome in heart, gut and reproductive organs. Table 2 summarizes the data from tissues of transformed animals demonstrating both copy number mosaicism and the absence of detectable pSV2CAT DNA in various tissues. Animal number 3 contained 30 copies/genome in lung and 8 copies/genome in muscle, but pSV2CAT sequences were not detected in other tissues. Animal number 8 contained approximately 3 copies/genome in all the tissues examined. Thus, the initial transformants were mosaic in copy number as well as in presence or absence of the gene in various tissues.

We believe that these differences in copy number are not due to artifactual retention on the dot blots since we have observed similar variation on Southern blots and have analysed the variation on dot blots by performing controls in which known amounts of pSV2CAT were mixed with *Xenopus* DNA and dot blotted onto nitrocellulose (data not shown).

CAT gene is expressed in tissues of frogs

We analysed the expression of pSV2CAT in the tissues of individual frogs by utilizing both the functional assay for CAT enzyme activity and RNA dot blots for the presence of transcripts. Fig. 3A,B shows an analysis of CAT enzyme activity in tissues from three frogs. In Fig. 3A (animal no. 5, Table 2), CAT activity was observed in the muscle, jaw cartilage, gut, liver, reproductive organs, heart and brain. Conversion of [^{14}C]chloramphenicol to the acetylated form varied between 1 and 10% in different tissues. In Fig. 3B, tissues from two frogs were examined and activity was detected in the gut from one of the animals (animal no. 1, Table 2). CAT activity was not detected in tissues from animals that were not injected with pSV2CAT. Dot blot analysis of the RNA from tissues of a 6-month-old frog (animal no. 10, Table 2) showed the presence of transcripts from the injected plasmid in five of the six tissues examined (Fig. 2A). The RNA transcripts were most abundant in the kidney and lung though these tissues did not show the highest DNA copy number.

Table 2 summarizes the data on the expression of pSV2CAT from eight different frogs. There was no detectable expression of either CAT enzyme or RNA in four of the eight frogs examined, though tissues of three of these frogs contained from 2 to 13 copies of the gene/cell (Table 2). Frog number 3 (Table 2) had 8 copies of the gene/cell in the muscle tissue while there was no detectable expression; frog number 8 had approximately 3 copies of the gene/cell in seven different tissues with a high level of expression in gut and brain, slightly less in heart and reproductive

Table 2. Presence and expression of DNA in different tissues in transformed frogs

Animal no.		Tissue									
		Heart	Cartilage	Lung	Muscle	Gut	Brain	Kidney	Reproductive organs	Liver	Stomach
3	DNA	ND*	—	+(30)	+(8)	—	—	ND	—	—	ND
	Exp†	ND	ND	ND	—	—	—	ND	ND	—	ND
6	DNA	+(1)‡	ND	+(3)	ND	—	ND	ND	—	+(3)	ND
	Exp†	—	ND	—	—	—	ND	ND	—	—	ND
8	DNA	+(3)	ND	+(3)	+(3)	+(3)	+(3)	ND	+(3)	+(3)	ND
	Exp§	++	ND	—	—	+++	+++	ND	++	—	ND
9	DNA	ND	ND	+(5)	+(5)	+(5)	+(10)	+(5)	+(5)	+(5)	ND
	Exp§	ND	ND	—	—	—	+	—	—	—	ND
10	DNA	+(10)	ND	+(10)	+(5)	+(10)	ND	+(2)	ND	+(5)	ND
	Exp§	+	ND	+++	+	—	ND	++	ND	+	ND
11	DNA	—	ND	—	+(4)	—	+(13)	—	—	—	ND
	Exp§	—	—	—	—	—	—	—	—	—	ND
12¶	DNA	—	ND	—	+(5)	—	—	—	—	+(15)	ND
13¶	DNA	+(5)	ND	+(10)	ND	+(5)	ND	ND	+(3)	+(10)	+(10)
1	Exp†	—	ND	ND	—	+	—	ND	—	—	ND
5	Exp†	+	+++	ND	++	+	++	ND	++	+	ND

* ND, no data; +, presence of detectable sequences; —, no sequence detectable.

† CAT assay performed

+++ 11–15 % conversion of nonacetylated to acetylated chloramphenicol,

++ 6–10 % conversion of nonacetylated to acetylated chloramphenicol,

+ 1–5 % conversion of nonacetylated to acetylated chloramphenicol,

— no detectable activity.

‡ Number in parentheses represents copy number/genome equivalent as determined by comparison to standards of known amounts of pSV2CAT DNA.

§ RNA dot blot

+++ hybridization signal 15-fold above background,

++ hybridization signal 10-fold above background,

+ hybridization signal 5-fold above background,

— no detectable signal.

¶ No data for expression.

|| No data for presence of pSV2CAT DNA.

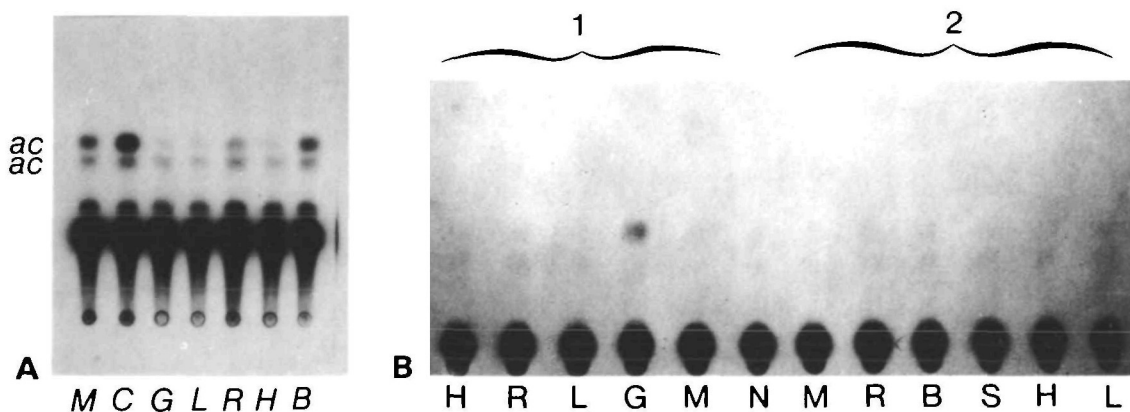


Fig. 3. (A) CAT assay of tissues from a 3-month-old frog. Individual tissues were homogenized in buffer and equivalent amounts analysed for CAT activity using the assay as described in Materials and Methods. The signals range from 1–10 % conversion of [14 C]chloramphenicol to the acetylated form (ac). M, skeletal muscle; C, jaw cartilage; G, gut; L, liver; R, reproductive organs; H, heart; B, brain. No CAT activity was ever detected in extracts from tissues of noninjected frogs. (B) CAT enzyme activity from 2 frogs 5 months of age. H, heart, animal no. 1; R, reproductive organs, no. 1; L, liver, no. 1; G, gut, no. 1; M, skeletal muscle, no. 1; N, muscle from noninjected frog; M, muscle, no. 2; R, reproductive organ, no. 2; B, brain, no. 2; S, stomach, no. 2; H, heart, no. 2; L, liver, no. 2.

organs, and no detectable expression in lung, muscle and liver; animal number 10 had 10 copies/cell in both gut and lung, but expression only in the lung. The pattern reveals that, while there was expression in various tissues in different animals, there was no correlation between copy number and level of expression.

Lack of expression in tissues that contained the gene might have been due to major rearrangements of the coding sequences. We therefore examined restriction enzyme digestion patterns of those tissues not expressing the gene. Fig. 4A shows the results of *Bam*H1 digestion of muscle tissue DNA in which the gene was not expressed. We detected a single 5.0 kb DNA fragment that comigrated with the single 5.0 kb linearized pSV2CAT. Junction fragments were not detected. When DNA from another tissue was digested with *Eco*R1 (which cuts the plasmid two times), we observed the presence of two bands 3.0 kb and 2.0 kb that comigrated with the two DNA fragments produced by *Eco*R1 digestion of control pSV2CAT. We also detected other fragments that represent putative junction fragments (Fig. 4B). These were less intense than the 2.0 and 3.0 kb bands. This finding is consistent with integration of tandem duplicated head-to-tail multimers. The exogenous DNA comigrated with the high molecular weight frog DNA, as we have reported previously (data not shown) (Etkin *et al.* 1984; Etkin & Roberts, 1983). In no case did we detect either circular pSV2CAT or linear 5.0 kb molecules in samples that were not digested with restriction enzymes. These results suggest that the DNA was integrated or persisted as high molecular weight concatemers and was not dramatically rearranged in tissues of transformed animals.

Exogenous DNA sequences are transmitted through the male germ line

Plasmid psAC-1 DNA (1 ng) was injected into *Xenopus laevis* fertilized eggs (parentals) and the embryos were maintained in the laboratory to adulthood. Sperm from a transformed animal was used to inseminate eggs from a 'wild-type' female. The F₁ generation embryos were allowed to develop for 1–3 months. The DNA was extracted from isolated tissues, digested with either *Pst*I or *Bam*H1 restriction endonuclease, separated by agarose gel electrophoresis, blotted onto nitrocellulose and probed with ³²P-labelled psAC-1 DNA.

Six offspring were examined for the presence of psAC-1 sequences. Four of the six animals were positive for the exogenous DNA on dot blots. DNA from two F₁ frogs was analysed by restriction enzyme digestion and Southern blotting. *Bam*H1, which cleaves the injected psAC-1 plasmid at two sites,

produced two fragments of 2.7 and 6.3 kb in frog F1-c similar to the fragments produced in control digests with this enzyme (Fig. 5). Digestion of the DNA with *Pst*I, which cuts plasmid psAC-1 at a single site,

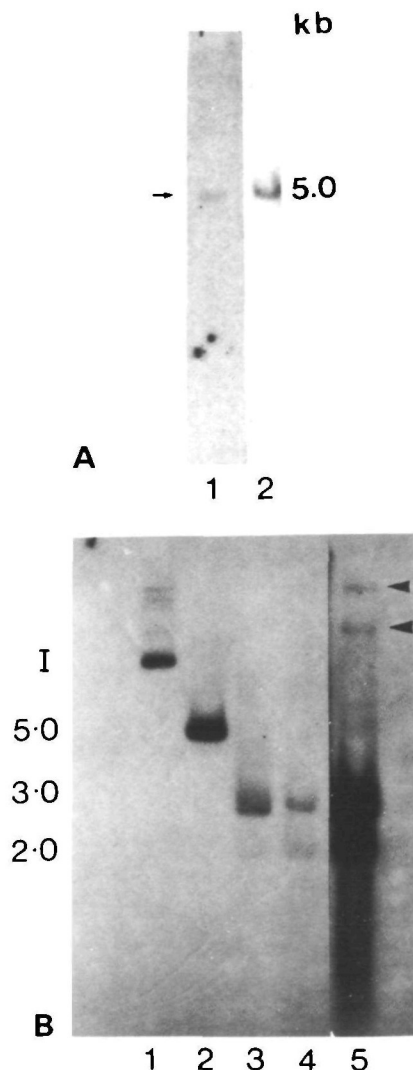


Fig. 4. Southern blots of restriction enzyme digestion of DNA from tissues of frogs transformed with 5.0 kb linear pSV2CAT. 10 µg of DNA were digested with either *Bam*H1 (which cuts pSV2CAT once) or *Eco*R1 (cuts two times), separated on agarose gels and blotted to nitrocellulose. Filters were hybridized with a nick-translated pSV2CAT probe (2×10^8 cts min⁻¹ µg⁻¹) and analysed by autoradiography. (A) Lane 1, *Bam*H1 digestion of DNA from muscle tissue which was negative for CAT activity (lane M, Fig. 3B). Arrow points to 5.0 kb DNA fragment which comigrates with linearized pSV2CAT plasmid. Lane 2, control pSV2CAT DNA digested with *Bam*H1. (B) lane 1, control pSV2CAT undigested (this plasmid is a 15 kb trimer of 5.0 kb pSV2CAT); lane 2, control pSV2CAT digested with *Bam*H1; lane 3, control pSV2CAT digested with *Eco*R1; lane 4, gut tissue DNA from 8-month-old frog digested with *Eco*R1; lane 5, darker exposure of lane 4 showing putative junction fragments (arrows).

produced the predicted 9.0 kb DNA fragment and a putative junction fragment of approximately 2.5 kb in each of the F1-c tissues. This fragment presumably consists of psAC-1 sequences joined to frog DNA, indicating that the psAC-1 DNA was integrated.

The presence of the predicted restriction fragments and junction fragments demonstrates that there are no major rearrangements of the injected DNA and that the DNA was probably integrated as tandem head-to-tail concatemers. The presence of similar restriction pattern from different tissues suggests that the psAC-1 DNA is integrated into a single site in F1-c. The intensities of the signals from the predicted fragments and the junction fragments were quantified by scanning densitometry and compared to give an estimate of the copy number of the psAC-1 DNA integrated in the frog F1-c. In each tissue the 9.0 kb fragment was present in a 2:1 relationship with the 2.5 kb junction fragment. Assuming that the junction fragment is present as a single copy per genome then the twofold higher intensity of the 9.0 kb fragment indicates that two complete copies plus a partial copy (as junction fragments) exist per genome equivalent of frog F1-c.

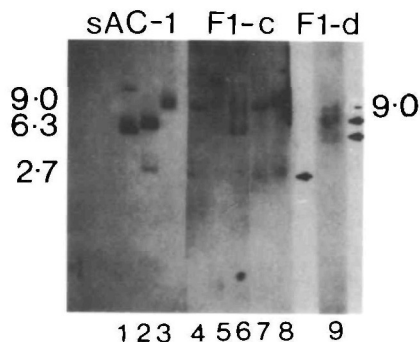


Fig. 5. Germ line transmission of exogenous DNA sequences. Sperm from transformed frogs was used to fertilize 'wild-type' eggs. DNA from tissues of the offspring was extracted as described in Materials and Methods. The DNA was analysed by Southern blotting following digestion with *Bam*H1 or *Pst*I restriction endonucleases. Lanes 1–3 are control psAC1 DNA undigested, digested with *Bam*H1 and digested with *Pst*I, respectively. Lanes 4–8 represent DNA from different organs of F₁ frog F1-c. Lane 4, DNA from liver digested with *Pst*I showing the characteristic 9.0 kb DNA fragment and a putative junction fragment approximately 2.5 kb (arrow). Lane 5, DNA from the gut, undigested. This comigrated with undigested frog genomic DNA as identified on the same gel stained with ethidium bromide prior to blotting. Lane 6, gut DNA digested with *Bam*H1 showing the characteristic 6.3 kb and 2.7 kb restriction fragments. Lane 7, DNA from the brain digested with *Pst*I. Lane 8, muscle DNA digested with *Pst*I. Lane 9, liver DNA from a second F₁ animal F1-d digested with *Pst*I. The arrows indicate the position of putative junction fragments.

Analysis of liver DNA from frog F1-d demonstrated the persistence of psAC-1 DNA in this animal. The psAC-1 DNA comigrated with the high molecular weight cellular DNA when the DNA was analysed without digestion with restriction endonucleases. When the DNA was cleaved with *Pst*I, the characteristic 9.0 kb fragment was observed, as well as a smear of several putative junction fragments (Fig. 5). The size of these junction fragments is significantly different from those in F1-c, indicating that the site of integration was different between these two frogs even though they were from the same mating. This suggests that the male that produced the sperm must have had multiple sites of integration within its germ cells. We detected germ line transmission in one of three initial transformants.

RNA from tissues of several transformed frogs as well as from tissues of F₁ generation frogs was analysed for the presence of *Drosophila* ADH transcripts. No transcripts were detected. Thus, it appears that this gene though present in several copies/genome was not expressed in the adult, or in the offspring. However, we have observed expression of the ADH gene at the midblastula transition of initial transformants (Etkin *et al.* 1984).

Discussion

In the present study we analysed the fate, expression and germ line transmission of several recombinant plasmids in transgenic frogs. Our results show that the injected exogenous DNAs (1) become localized to the cleavage nuclei by stage 6, (2) persist in tissues of adult frogs but exhibit a mosaic pattern of distribution, (3) are expressed in adult tissues, but in a random manner (at least for pSV2CAT) and (4) may be transmitted through the male germ line.

DNA sequences were injected as linear molecules into the cytoplasm of fertilized eggs within 1 h following fertilization. These sequences were detected in the tissues of 60% of the injected frogs. They comigrated with the high molecular weight DNA and did not exhibit gross structural rearrangements as determined by restriction digestion analysis. Data from restriction enzyme digestion of the DNA favour the organization of the injected DNA into tandem head-to-tail multimers. The copy number variation was from 2 to 30 copies/cell in various tissues of different frogs. It is not known, however, whether all cells in a particular tissue possessed the gene or if some cells of the particular tissue contained high copy number.

Results from this and other studies (Rusconi & Schaffner, 1981; Etkin & Roberts, 1983; Etkin *et al.* 1984) suggest that the injected DNA is integrated into the frog genome. The evidence consists of (1)

persistence of the injected DNA in adult frogs, (2) association of the DNA with the high molecular weight frog DNA and not as discrete circular or linear molecules, (3) generation of putative junction fragments upon digestion of the DNA with restriction enzymes, (4) detection of male germ line transmission and (5) transmission of exogenous DNA sequences by nuclear transplantation. We therefore feel strongly that some of these sequences are integrated into the frog genome. We estimate that integration occurred in approximately 5–10% of the frogs in which the exogenous DNAs persisted. In the other frogs the persisting DNA is probably maintained as extra-chromosomal high molecular weight concatemers. It should be emphasized that this is a crude estimate of the extent of integration and could be an overestimate if there has been some rearrangement of the DNA, and an underestimate if extreme mosaicism results in the lack of detectable junction fragments.

The mosaic pattern of distribution of injected DNA sequences indicates that integration probably occurs during the early cleavage stages and not prior to first cleavage. We speculate that the injected DNA is sequestered in nucleoid-like structures as described by Forbes *et al.* (1983) in unfertilized *Xenopus* eggs. These vesicles probably fuse with nuclei randomly during early cleavage divisions resulting in the mosaic pattern of distribution of the injected DNAs. This is supported by the observation of nucleoid-like structures in embryos injected with lambda DNA which surround the cleavage nuclei (Shiokawa, Sameshima, Tashiro, Miura, Nakakura & Yamana, 1986), and the localization of injected labelled DNA in the nuclei at stage 6 reported in the present study. Localization of injected DNAs in cleavage nuclei was also observed by Gurdon (referred to in Gurdon & Melton, 1981). We routinely inject the DNA into the upper one-third of the animal hemisphere of the fertilized egg. Autoradiography of sectioned material shows that the injected DNA is localized near the site of injection, but by early cleavage stages the labelled plasmid DNA is not distributed to all cells. The pattern of distribution, however, does not appear to correspond strictly to the site of injection, since we detect grains over nuclei of cells in the vegetal and animal hemisphere.

There are several examples of the expression of injected DNAs in *Xenopus* embryos (Bendig, 1981; Rusconi & Schaffner, 1981; Etkin *et al.* 1984; Bendig & Williams, 1984; Etkin & Balcells, 1985). The results of these studies showed that the injected DNAs were all transcribed at the midblastula stage of development except for larval *Xenopus* globin which was expressed at the tailbud stage of development. None of the injected genes were expressed in the proper developmental timeframe. Kreig & Melton

(1985) showed that a cloned *Xenopus* gene, GS17, whose endogenous counterpart is expressed between the midblastula transition and the gastrula stage mimics the same pattern of expression as the endogenous gene when injected into fertilized eggs.

In the present study, the CAT gene was expressed in 50% of the animals that contained pSV2CAT sequences. The pattern of expression, however, was interesting in that the gene was expressed in a variety of tissues in different frogs. There did not appear to be any pattern of expression with regard to tissue type, regional localization or embryological origin of the tissue. By comparing the percent conversion of nonacetylated chloramphenicol to the acetylated form in the tissues to that of commercially available CAT enzyme we estimate that there are approximately 10^3 CAT enzyme molecules/cell in tissues such as cartilage in Fig. 3A. It is unlikely that the lack of detectable expression in various tissues is due to an inhibitor of CAT activity since there are examples of expression in all tissues examined (Table 3).

We observed that there was no correlation between copy number and expression; some tissues with 2–5 copies exhibited relatively high levels of expression, while others with 10 copies or more did not show any detectable expression. We estimate 10–100 copies of RNA/cell in tissues with high levels of expression and 1–10 copies/cell in tissues expressing the gene at low levels. This, of course, represents a steady-state level of RNA and does not take into account RNA stability and turnover. We realize that CAT gene transcription is driven by a viral promoter in this construction, therefore we cannot make any statements regarding the sequence requirements for tissue-specific expression. It is possible that some of the transcripts represent readthrough transcription initiated at an upstream *Xenopus* promoter at the site of integration or even in the plasmid itself. However, some of this RNA is functional mRNA since we observe translational products as determined by assays for CAT enzyme activity. Also, we did not detect transcripts from the *Drosophila* ADH gene in either initial transformants or in their offspring. This may be due to the heterologous nature of this gene. However, we have detected expression of this gene in injected oocytes and in embryos at the midblastula stage of development (Etkin *et al.* 1984).

Restriction enzyme analysis of DNA from tissues in which the CAT gene was not expressed indicate that there were no major rearrangements to the input DNA. Thus, inactivity of the gene in certain tissues is not due to rearrangements as detectable by this analysis.

We detected transmission of exogenous DNA sequences through the male germ line in one of three

transformed animals. Four of the six offspring contained copies of the plasmid psAC-1. Restriction digestion analysis of the DNA from two of these animals showed that the exogenous DNA sequences were probably integrated at the same site in tissues of each animal, but that the sites differed between the two frogs. This result indicates that the two sperm from the original transformed animal contained the gene integrated at a different site. We conclude that the initial transformants were highly mosaic to the point where individual cells from a particular cell type contained the gene integrated into different positions or did not contain the gene at all. Therefore, it may be advantageous to use transformed animals from the F₁ or F₂ generations for future studies examining the tissue-specific expression of injected genes in *Xenopus*.

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