Int. J. Dev. Biol. 35: 481-484 (1991)

Short Contribution

Gene transfer into intact vertebrate embryos

B.A. DEMENEIX[1]*, G. FREDRIKSSON[4]*, F.LEZOUAL'CH[1], N. DAUGERAS-BERNARD[1], J.P. BEHR[2] and J.P. LOEFFLER[3]

[1]*Laboratoire de Physiologie Générale et Comparée, URA 90 CNRS, Muséum National d'Histoire Naturelle, Paris,* [2]*Laboratoire de Chimie Génétique, URA 1386 CNRS, Faculté de Pharmacie, Strasbourg and* [3]*Laboratoire de Physiologie Générale, URA 1446 CNRS, Université Louis Pasteur, Strasbourg, France*

ABSTRACT Intact chick embryos at 40 h incubation were transfected *in vivo* with chimeric vectors expressing chloramphenicol acetyl transferase (CAT) under different promoter sequences. The cationic lipid, dioctadecylamidoglycyl spermine (DOGS) used as the transfecting agent had no noticeable toxic effects on embryonic development. CAT activity was monitored 48 h post-transfection on homogenates of embryos dissected free of all annexes. Of the various constructs tested, those containing the AP-1 response element linked to CAT (TRE-tk-CAT) gave high expression and consistent enzyme responses within groups. Co-transfection experiments in which embryos were exposed simultaneously to a CAT vector containing the cAMP response element and to a vector expressing the catalytic subunit of protein kinase A showed that the promoters of the introduced genes can be regulated by their respective transacting factors. This method may therefore represent a general tool for introducing genes into intact vertebrate embryos at precise developmental times.

KEY WORDS: *gene transfer, cationic lipid, chick embryos*

Analysis of the roles specific genes play during development is currently hampered by the lack of experimental tools that can be applied *in vivo*. We are faced with the paradoxical situation where we have on the one hand a number of cloned, developmentally regulated genes and, on the other hand, no efficient means of modulating the expression of these genes in spatially delimited areas of embryos at defined times.

At the moment micro-injection of DNA or RNA is most commonly used for introducing foreign genes into fertilized eggs or early embryos. This approach can be used to generate transgenic animals (Palmiter and Brinster, 1986) or used for transient expression in early embryogenesis (see for example Kintner, 1988; Ruis Altaba and Melton, 1989). When RNA is injected into cells, levels of translation products rise and fall rapidly so microinjection is useful but time-consuming and tedious, necessitating injections into individual cells of an embryonic area under study.

Retroviruses can be used to circumvent this latter problem, and to introduce nucleic acids *in vivo*. This approach has already been applied successfully to cell lineage studies in the CNS of birds (Sanes *et al.*, 1986) and mammals (Price *et al.*, 1987). However, difficulties encountered include achieving high enough titers to infect significant numbers of cells *in vivo*, obtaining retroviral constructs that contain the gene under study and maintain their efficiency and, finally, the limitations arising from cell-specific infection. Thus a method based on receptor-independent mechanisms is sought. However, the most commonly used methods, such as calcium phosphate/DNA co-precipitation and use of high molecular weight polycations (*e.g.*, dextran) are not suitable for use *in vivo*.

Recently, Holt and collaborators (Holt *et al.*, 1990) achieved lipid-based transfection in *Xenopus* embryos by placing either parts of the embryonic brain or embryos dissected free of the epidermis into solutions containing lipofectin and a vector expressing luciferase cDNA. Alternatively, the DNA/lipofectin mixture was injected into the eye vesicle from whence it entered the neutral tube (Holt *et al.*, 1990). This latter method however produces lower total luciferase activity in the brain than the methods involving dissection of embryos.

To transfect intact chick embryos we chose to use dioctadecylamidoglycyl spermine (DOGS= Transfectam™, I.B.F.,

Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; CRE, cAMP responsive element; CREBP, cAMP responsive element binding protein; DOGS, dioctadecylamidoglycyl spermine; PKA, protein Kinase A; RSV, Roux sarcoma virus; tk, thymidine kinase; TRE, 12-O-tetradecanoylphorbol-13-acetate-responsive element.

***Address for reprints**: Laboratoire de Physiologie Générale et Comparée, URA 90 CNRS, Muséum National d'Histoire Naturelle, F-75231 Paris cedex 5, France. FAX: 33-1-40.79.34.84

[4]**Present address:** Dept. of Ultrastructure Research, Stockholm University, S-10691 Stockholm, Sweden. FAX: 46-81 15 98 71

0214-6282/91/$03.00

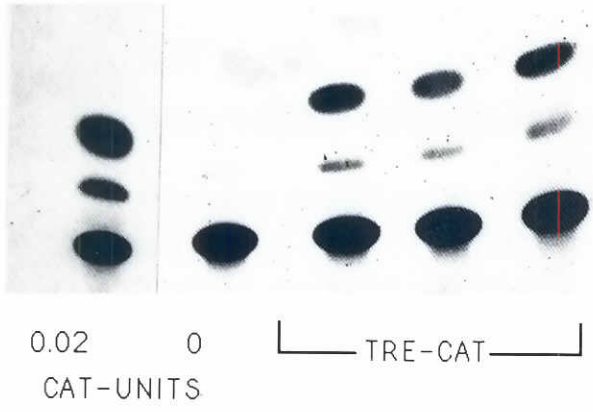

Fig. 1. Cationic lipid-based transfection in chick embryos gives reproducible enzyme activities. *Three successive embryos were transfected at 40 h incubation (at 38°C±5°C, stage 11 according to Hamburger and Hamilton, 1951) with 10 µl of 8.6‰ NaCl containing 2 µg TRE-tk-CAT plasmid mixed with 2 µl of a 4 mM ethanolic solution of Transfectam. This DNA/lipid preparation was placed in a water bath and sonicated (1 min, 20 kHz) to evenly disperse the complexes and avoid clumping. Forty-eight hours later embryos were removed, and CAT assays performed as described in the Methods. A CAT enzyme standard (0.02 units) is shown on the extreme left, next to a blank.*

France). This cationic lipid can be used innocuously to mediate efficient transfection in endocrine and neuronal cells maintained in primary culture (Behr *et al.*, 1989; Demeneix *et al.*, 1990; Loeffler *et al.*, 1990). In this study, we chose to work on embryos incubated for 36-40 h at 37.5°C±0.5°C, *i.e.*, at embryonic stages 10 to 12 (Hamburger and Hamilton, 1951). This corresponds to the time at which the embryo is most accessible and not yet covered by the amniotic membrane.

Preliminary experiments showed that at least 10 µl of a 2 mM Transfectam solution in 8.6% NaCl could be applied to embryos at this stage with no deleterious effects on subsequent development. Indeed, the transfection procedure did not result in increased mortality over the 48 h post-transfection, as compared to shams treated with 2 µl 100% ethanol in 10 µl 8.6‰ NaCl, or 2 µl of a 4 mM ethanolic solution of Transfectam in 10 µl 8.6‰ NaCl. In general mortality 48 h post-transfection in all groups varied between 10-15%.

Fig. 1 shows that when 2 µl of a 4 mM ethanolic solution of Transfectam is mixed with 2 µg TRE-tk-CAT in 8.6‰ NaCl (total volume 10 µl) and applied to a 40 h old embryo, high CAT activity is measurable in the embryo 48 h later. The levels of enzyme activity resulting from transfection of a given amount of DNA are remarkably constant. Fig. 1 shows the CAT activity resulting from transfection of three successive embryos with 2 µg of TRE-tk-CAT complexed with 2 µl of a 4 mM ethanolic solution of Transfectam in a total volume of 10 µl. The individual responses were fairly equivalent (Fig. 1, lanes 1-3) and about half that given by 0.02 units of CAT (Fig. 1, lane 5), included systematically in all assays as an internal standard.

In contrast, the same amount of TRE-tk-CAT (2 µg), applied alone in NaCl in the absence of lipid gave no measurable response (Fig. 2A, lane 2). The second plasmid tested, CRE-tk-CAT, when applied at the same concentration (2 µg/10 µl final volume) as the TRE-tk-CAT and with the same amount of Transfectam, produced no detectable CAT activity (Fig. 2A, lane 3; Fig. 3, left lanes). However, when 1 µg CRE-tk-CAT was co-transfected with 1 µg of a vector, Mt-cα (McKnight *et al.*, 1988) expressing the catalytic subunit of protein kinase A (PKA), measurable CAT activity was found at 48 h post-transfection (Fig. 3).

This method therefore provides a means of transfecting and co-transfecting foreign genes directly into chick embryos in early development. Small amounts of DNA (1.5 to 2 µg) can be innocuously introduced into whole vertebrate embryos by employing the cationic lipid Transfectam. Usually, we employed 1 µl of a 4 mM Transfectam solution (in 100% ethanol) to transfect 1 µg of DNA.Thus the procedure works satisfactorily when the proportion of lipid to DNA gives a 4-fold charge excess (1 µl 4 mM Transfectam per 1 µg DNA) and is 1.3 times that used for primary cultures (Behr *et al.*, 1989). As responses are obtained with such small amounts

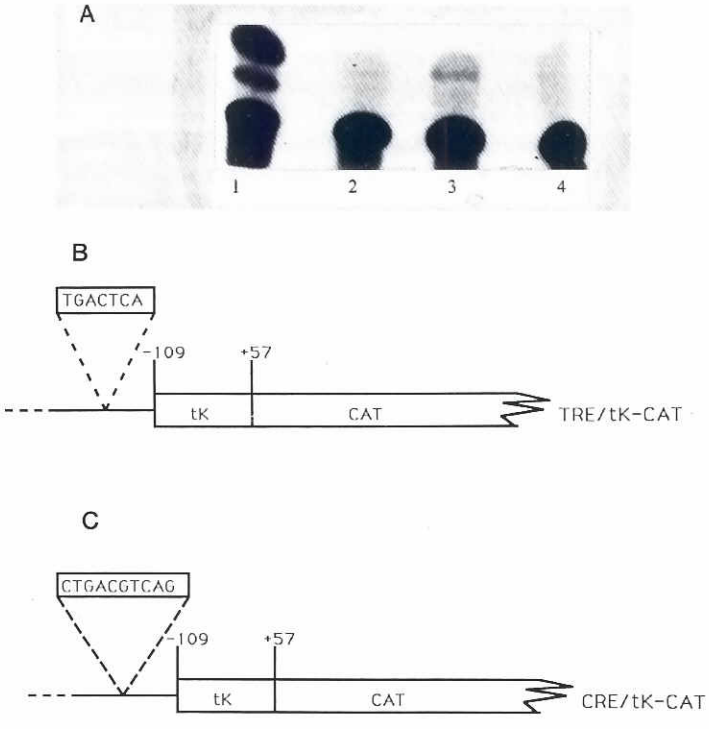

Fig. 2. TRE-tk-CAT but not CRE-tk-CAT is transcribed in stage-11 chick embryos. (A) *Chick embryos at 40 h of incubation were treated with 1) 1.7 µg TRE-tk-CAT (see below) and 1.7 µl of a 4 mM ethanolic solution of Transfectam diluted to 10 µl in NaCl (8.6‰); 2) 1.7 µg TRE-tk-CAT and 1.7 µl ethanol in 10 µl NaCl (8.6‰); 3) 1.7 µg CRE-tk-CAT (see below) and 1.7 µl of a 4 mM ethanolic solution of Transfectam diluted to 10 µl in NaCl (8.6‰); 4) 1.7 µl of a 4 mM ethanolic solution of Transfectam diluted to 10 µl in NaCl 8.6‰. Each DNA/lipid preparation was placed in a water bath and sonicated (1 min, 20 kHz) to evenly disperse the complexes and avoid clumping. Forty-eight hours later the embryos were removed, and CAT assays performed as described in the Methods.* **(B)** *The TRE-tk-CAT plasmid contains a 18 bp oligonucleotide having the TGACTCA core sequence originally from the human methallothionein II gene promoter (Angel* et al., *1987) linked upstream to the thymidine kinase promoter.* **(C)** *The CRE-tk-CAT plasmid contains the somatostatin (som) cyclic AMP response element (CRE) oligonucleotide (core sequence CTGACGTCAG), which covers the -32/-61 promoter region and is linked to the tK promoter (Montminy* et al., *1986).*

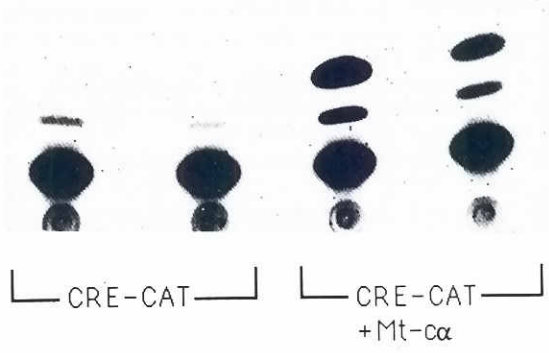

Fig. 3. Co-transfection of CRE-tk-CAT and the catalytic subunit protein kinase A in chick embryos. *Embryos (at 40 h of incubation) were treated with either 1 μg CRE-tk-CAT (1 μg/μl) and 1 μl 4 mM Transfectam in NaCl at a final concentration of 8.6‰, total volume 10 μl (left two lanes) or 1 μg CRE-tk-CAT and 1 μg Mt-cα mixed with 2 μl 4 mM Transfectam in 8.6‰ NaCl, total volume 10 μl (right hand lanes). Each DNA/Transfectam preparation was placed in a water bath and sonicated (1 min, 20 kHz) to evenly disperse the complexes and avoid clumping. Forty-eight hours later the embryos were removed, and CAT assays performed as described in the Methods.*

of DNA, the method would seem to be more efficient than that used by Holt and collaborators (Holt *et al.*, 1990). In their experiments, using vectors expressing luciferase under the RSV long terminal repeat promoter, maximal activity was found when dissected brains of embryonic *Xenopus* were placed in solutions containing 50 to 90 μg DNA, with a DNA/lipofectin ratio of 1:2. However, using isolated heads and the optimal DNA/lipofectin ratio of 1:3, significant luciferase activity was found with 10 to 15 μg plasmid.

Interestingly, in our experiments the only construct to produce measurable basal activity within the window of time examined was that containing the AP-1 (activator protein-1) consensus sequence, *i.e.*, plasmid TRE-tk-CAT. In *Drosophila*, the expression of FRA protein (Fos-related AP-1 protein) is developmentally regulated (Perkins *et al.*, 1990). In vertebrates expression of Fos protein (one component, with Jun proteins of AP-1 complexes, Curran and Franza, 1988) is associated with differentiating tissues and during development Fos transcription is observed in a spatially restricted manner (see for instance Mohun *et al.*, 1989). Therefore, given the role of AP-1 proteins in development it is not surprising to find high transcription of CAT from the AP-1 consensus sequence in the TRE-tk-CAT construct.

The co-transfection experiment (Fig. 3) shows that although the chimeric gene CRE-tk-CAT is transcribed at levels below those permitting detection of CAT activity by the methods employed here, the plasmid is effectively transfected into the embryo and transcription can be increased by modulation of transacting factor activity. Indeed, overexpressing the catalytic subunit of PKA by transfection of Mt-cα, could possibly increase phosphorylation of target proteins, such as the transacting factor CREBP, which bind to CRE elements including that in the CRE-tk-CAT vector, thus modulating transcription.

In conclusion, the method described here provides a non-toxic, non-invasive means of introducing specific genes into intact vertebrate embryos at a defined stage of development. We are currently applying immunocytochemistry to investigate whether the genes introduced are expressed in specific tissues and to monitor the introduction of genes into defined, spatially delimited parts of the embryo. Thus, the approach will circumvent one of the main problems arising from the use of transgenic animals, namely that incurred by overexpression of certain genes early in ontogeny, when they might prove lethal or alter subsequent stages of development. The methodology developed here permits investigation of the effects of modifying gene expression within defined time periods without worrying about earlier expression and its sequels.

Experimental procedures

Transfection of embryos

Chick embryos at <40 h of incubation (at 38°C±5°C, stage 11 according to Hamburger and Hamilton, 1951) were treated with varying amounts of DNA mixed with a 4 mM ethanolic solution of Transfectam (1 μl 4 mM Transfectam /1 μg DNA) in a final volume of 10 μl in NaCl 8.6‰. Each preparation was placed in a water bath and sonicated through the water bath (1 min, 20 kHz) to evenly disperse the DNA/lipid complexes and avoid clumping of particles.

To apply the solutions, eggs were opened, the shell membrane over the embryo removed, a small hole made in the vitelline membrane and the solutions deposited evenly over the embryo using a hand-held micropipette, pulled from a siliconized 50 μl glass capillary tube. Eggs were closed with 3M transparent adhesive tape and incubated for a further 48 h. Embryos were then removed, dissected free of all annexes and sonicated in Tris-HCl 200 mM (pH 7.4), containing antiproteases (5 units aprotinin and 16 μg phenylmethylsulfonyl fluoride per ml). The extracts were heated (65°C, 10 min) then centrifuged and the supernatant used to assay CAT activity.

CAT assay

CAT activity was determined by the method of Gorman *et al.* (1982). To 180 μl of the supernatant (see above) 40 μl of Tris-HCL (pH 7.4) containing ^{14}C- labelled chloramphenicol (N.E.N., 0.1 μCi; specific activity 47 μCi/mmol) was added. After 5 min at 37°C the reaction was initiated by adding 40 μl of 4 mM acetyl co-enzyme A. The mixture was incubated at 37°C for 2 h and then extracted with 0.5 ml ethyl acetate. After separation by thin layer chromatography (in chloroform/methanol; 95/5) the acetylated and unreacted forms of chloramphenicol were visualized by autoradiography.

Plasmids

The tK-CAT vector contains the herpes simplex virus thymidine kinase (tK) promoter region between position -109 and +57 linked to the bacterial gene coding for CAT (Montminy *et al.*, 1986). Oligonucleotides containing the original sequences of cyclic AMP (cAMP) - and 12-0-tetradecanoylphorbol-13-acetate (TPA) - responsive elements (CRE and TRE respectively) were linked upstream of the tK promoter.

The TRE/tK-CAT plasmid (TRE-tk-CAT in the text) contains a 18 bp oligonucleotide having the TGACTCA core sequence originally from the human methallothionein II gene promoter (Angel *et al.*, 1987) linked upstream to the thymidine kinase promoter.

The CRE/tK-CAT plasmid (CRE-tk-CAT in the text) contains the somatostatin (som) cyclic AMP response element (CRE) oligonucleotide (core sequence CTGACGTCAG), which covers the -32/-61 promoter region and is linked to the tK promoter (Montminy *et al.*, 1986).

Acknowledgments

We thank Corinne Coitoux and Isabelle Seugnet for expert technical aid. Drs. P. Sassone-Corsi (Strasbourg) and S. McKnight (Seattle) kindly provided recombinant material. This work was supported by the Association de la Recherche pour le Cancer (ARC), and the Swedish Natural Sciences Council, grant B-BU 1809-300.

References

ANGEL P., IMAGAWE, M., CHIN, R., IMBRA, H., RAHMSDORF, C., JONAT, P. and KARIN, M. (1987). Phorbol ester-inducible genes contain a common *cis* element recognised by a TPA-modulated transacting factor. *Cell 49:* 729-739.

BEHR, J.P., DEMENEIX, B.A., LOEFFLER, J.P. and PEREZ-MUTUL, J. (1989). Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proc. Natl. Acad. Sci. USA 86:* 6982-6986.

CURRAN, T. and FRANZA, B.F. (1988). Fos and Jun: the AP-1 connection.*Cell 55:* 395-397.

DEMENEIX, B.A., KLEY, N.A. and LOEFFLER, J.P. (1990). Differentiation to a neuronal phenotype in bovine chromaffin cells is repressed by protein kinase C and is not dependent on c-fos oncoproteins. *DNA Cell Biol. 9:* 335-345.

GORMAN, C.M., MOFFAT, L.M. and HOWARD, B.H. (1982). Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. *Mol. Cell. Biol. 2:* 1044-1051.

HAMBURGER, V. and HAMILTON, H.L. (1951). A series of experimental stages of the development of the chick embryo. *J. Morphol. 88:* 49-92.

HOLT, C.E., GARLICK, N. and CORNEL, E. (1990). Lipofection of cDNAs in the embryonic vertebrate nervous system. *Neuron 4:* 203-214.

KINTNER, C. (1988). Effects of altered expression of the neural cell adhesion molecule N-CAM on early neural development in *Xenopus* embryos. *Neuron 1:* 545-555.

LOEFFLER, J.P., BARTHEL, F., FELTZ, P., BEHR, J.P., SASSONE-CORSI, P. and FELTZ, A. (1990). Lipopolyamine-mediated transfection allows gene expression studies in primary neuronal cells. *J. Neurochem.. 54:* 1812-1815.

McKNIGHT, G.S., GADD, G.G., CLEGG, C.H., OTTEN, A.D. and CORRELL, L.A. (1988). expression of wild-type and mutant subunits of the cAMP-dependent protein kinase. *Cold Spring Harbor Symp. Quant. Biol. 53:* 111-120.

MOHUN, T.J., GARRET, N. and TAYLOR, M.V. (1989). Temporal and tissue specific expression of the proto-oncogene c-fos during development in *Xenopus laevis*. *Development 107:* 835 -846.

MONTMINY, M.R., SEVARINO, K.A., WAGNER, J.A., MANDEL, G. and GOODMAN, R.H. (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA 83:* 6682-6686.

PALMITER, R.D. and BRINSTER, R.L. (1986). Germ line transformation of mice. *Annu. Rev. Genet.. 20:* 465-499.

PERKINS, K.K., ADMON, A., PATEL, N. and TIJAN, R. (1990). The *Drosophila* fos-related AP-1 protein is a developmentally regulated transcription factor *Genes Dev. 4:* 822-834.

PRICE, J., TURNER, D. and CEPKO, C. (1987). Lineage analysis in the vertebrate nervous system by retrovirus-mediated gene transfer. *Proc. Natl. Acad. Sci. USA 84:* 156-160.

RUIZ I ALTABA, A. and MELTON, D.A. (1989). Involvement of the *Xenopus* homeobox gene XHox3 in pattern formation along the anterior -posterior axis. *Cell 57:* 317-326.

SANES, J.R., RUBENSTEIN, J.L. and NICOLAS J-F. (1986). Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. *EMBO J. 5:* 3133-3142.

Accepted for publication: November 1991