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Original Article

T3-dependent physiological regulation of transcription in the *Xenopus* tadpole brain studied by polyethylenimine based *in vivo* gene transfer

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ABSTRACT The formulation of cationic polymers of polyethylenimine (PEI) with plasmid DNA has been optimized to deliver genes into the *Xenopus* tadpole brain *in vivo*. Using intraventricular microinjections of 1 μl (containing 0.5 to 1 μg DNA) we show that the linear, low molecular weight polymer, 22 kDa PEI was significantly more efficient than a branched 25 kDa polymer. Complexes bearing a slightly positive net charge (formed with a ratio of 6 PEI amines per DNA phosphate) provided the best levels of transfection. Transgene expression was DNA-dose dependent and was maintained over 6 days, the time course of the experiment. Spatial distribution was examined using a β-galactosidase construct and neurones expressing this transgene were found spread throughout the brain. The possibility of using this technique to evaluate physiological regulations was approached by examining the effects of tri-iodothyronine (T_3), on transcription from the mammalian *TRH* and *Krox-24* promoter sequences. Adding physiological concentrations of T_3 to the aquarium water significantly reduced transcription from the rat *TRH* promoter whilst the same treatment increased transcription from a mouse *Krox-24* -luciferase construct. Thus, PEI-DNA transfection provides a versatile and easily applied method for following physiological regulations at the transcriptional level in the tadpole brain.

KEY WORDS: *polyethylenimine, in vivo gene transfer, thyroid hormone, transcriptional regulation, central nervous system*

Introduction

Amphibian metamorphosis provides a versatile experimental model for analyzing regulation of morphogenesis and complex regulatory functions in an integrated context. Indeed, amphibian metamorphosis is one of the best studied hormone-regulated developmental processes (Tata, 1993). In metamorphosing tadpoles most of the tissues undergo transformation as the aquatic-living, herbivorous tadpole prepares for a terrestrial, carnivorous life. The striking morphological changes include eye migration, limb development and tail regression; all of which are induced by the same signal, thyroid hormone (3, 5, 3'-triiodo-L-thyronine or T_3).

The experimental advantages of the model are numerous. First, the free-living tadpole can be easily manipulated and second, the process of metamorphosis can be induced (or blocked) by modifying the thyroid status of the tadpoles. Third, *Xenopus* has recently become amenable to germinal transgenesis (Kroll and Amaya, 1996). This exciting new technical development adds to the spectrum of gene modulation techniques applicable in this species. Indeed, for decades RNA injections have been used to analyze the precocious stages of embryo development (Rebagliati *et al.*, 1987; Giebelhaus *et al.*, 1988; Harland and Misher, 1988; Pierandrei-Amaldi *et al.*, 1988) and over the last few years somatic gene transfer techniques have been developed and applied to study regulations in different organs. On the one hand, a non-viral technique based on naked DNA transfer into the tail muscle tissue has been optimized (de Luze *et al.*, 1993) and used to study the function of thyroid hormone receptors (Ulisse *et al.*, 1995), myosin

Abbreviations used in this paper: CMV, cytomegalovirus; CNS, central nervous system; PEI, polyethylenimine; T_3, 3,5,3'-triiodothyronine; *TRH*, thyrotropin releasing hormone.

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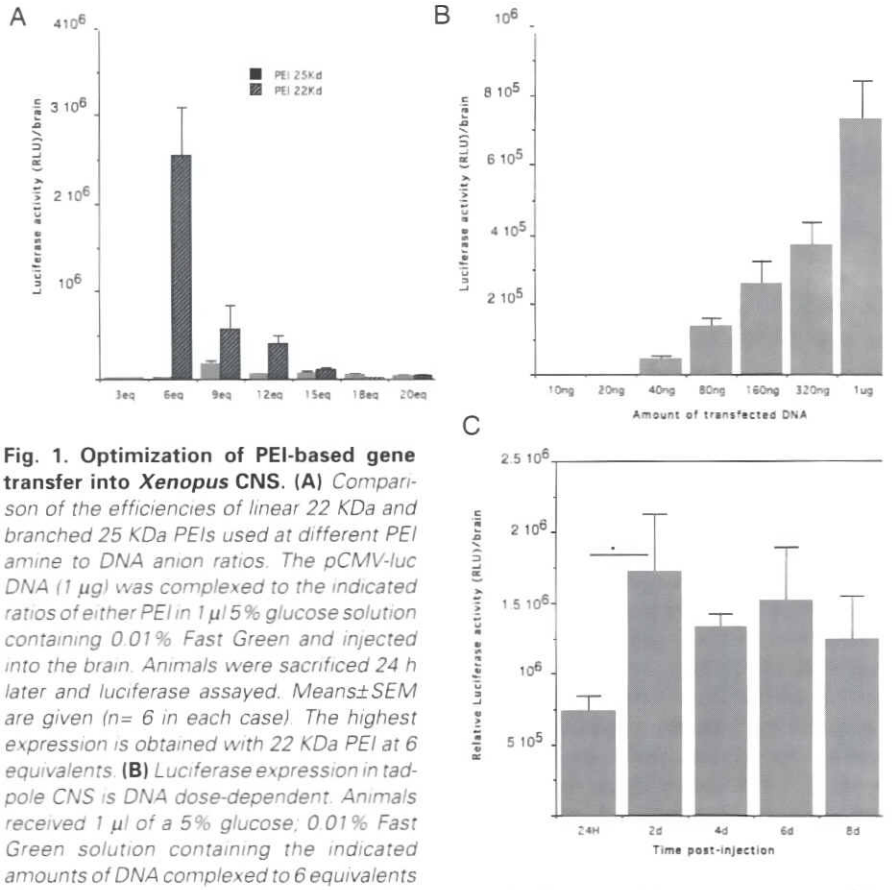

Fig. 1. Optimization of PEI-based gene transfer into *Xenopus* CNS. (A) *Comparison of the efficiencies of linear 22 KDa and branched 25 KDa PEIs used at different PEI amine to DNA anion ratios. The pCMV-luc DNA (1 μg) was complexed to the indicated ratios of either PEI in 1 μl 5% glucose solution containing 0.01% Fast Green and injected into the brain. Animals were sacrificed 24 h later and luciferase assayed. Means±SEM are given (n= 6 in each case). The highest expression is obtained with 22 KDa PEI at 6 equivalents.* **(B)** *Luciferase expression in tadpole CNS is DNA dose-dependent. Animals received 1 μl of a 5% glucose; 0.01% Fast Green solution containing the indicated amounts of DNA complexed to 6 equivalents of 22 KDa PEI. Tadpoles were sacrificed 24 h later and luciferase activity assayed. Means±SEM are given (n= 6 in each case).* **(C)** *Time course of expression of pCMV-luc in the CNS. Animals were injected with 1 μl of a 5% glucose; 0.01% Fast Green solution containing 1 μg of pCMV-luc complexed to 6 equivalents of 22 KDa PEI. Animals were sacrificed at the times indicated post-injection. Means±SEM are given (n= 6 in each case).*

gene regulation (Sachs *et al.*, 1996) and apoptosis (Sachs *et al.*, 1997a,b). On the other hand, vaccinia virus has been used to introduce genes in the tadpole central nervous system (Wu *et al.*, 1995) and exploited to analyze maturation of glutamatergic synapses (Wu *et al.*, 1998).

Thus the two broad groups of techniques that can be applied for somatic gene transfer, viral or non-viral, have been successfully applied respectively to the nervous system and skeletal muscle of the *Xenopus* tadpole. Both systems have their advantages, that of the viral approaches is their high efficiency. However, non-viral, plasmid-based methodologies have numerous advantages over viral systems, even if in general they are of lower efficiency. Besides their lesser toxicity and the lower immune responses, no integration into the genome occurs thus reducing risks of tumorigenesis. Their mechanisms of interactions with the cell membrane are not receptor mediated and therefore are not limited by cell or species specific mechanisms. What is more, there is no limitation to the size of genes to be introduced. Whereas, most viral constructions are limited to 6-8 kb, plasmid constructions can accommodate 2 to 3 times that. This allows one to envisage the preparation and introduction of a complete gene and/or complex constructions coding for more than one protein under the control of different regulatory sequences. Moreover, plasmid constructs designed to be delivered by different non-viral vectors, are much easier to engineer, verify and to produce in large quantities. Finally, using somatic gene transfer allows one to follow physiological regulations despite the fact that the DNA is not integrated into chromatin. This has been seen in the *Xenopus* tail muscle where parallel regulations were found between mammalian promoter constructs (assayed by following naked DNA gene transfer) and endogenous genes expression measured by RNAse protection assay (Sachs *et al.*, 1996). It is also the case for TRH transcription in the mouse hypothalamus (Guissouma *et al.*, in press).

Given these numerous benefits we chose to develop a non-viral method for analyzing gene regulation in the brain of *Xenopus* tadpoles. A major recent advance in these techniques came from the development of polyethylenimine (PEI) and its application to the mammalian CNS in our laboratory (Boussif *et al.*, 1995; Abdallah *et al.*, 1996). We thus optimized this technique for delivering genes into the developing *Xenopus* brain and show its versatility by using it to examine regulation of neuronal genes subjected to positive and negative regulation by T_3.

Results

Twenty-two KDa is more efficient than 25 KDa PEI for gene transfer in the tadpole CNS

The relative transfection efficiencies of two low molecular weight PEIs were compared at different PEI amine/DNA anion ratios. As shown in Figure 1A, both the linear 22 KDa polymer and the branched 25 KDa polymer provided measurable levels of luciferase expression following transfection with 1 μg of the pCMV-luc construct. However, the 22 KDa polymer provided significantly higher levels of transfection at PEI/DNA anion ratios of 6, 9 and 12. Forming complexes at 3 eq gave levels of transfection only just above background and increasing charge ratios to over 12 eq significantly reduced transfection levels with both PEIs. The most efficient combination tested was that of 22 KDa used at 6 eq per DNA anion, the level of transfection obtained ($2.5x10^6 \pm 5x10^5$ RLU/brain) was five times that obtained at 9 eq with the same PEI.

PEI-based gene transfer is dose-dependent and stable over one week post-injection

We next examined expression as a function of the amount of DNA transfected using pCMV-luc complexed with 6 eq PEI 22 KDa. As seen in Figure 1B, expression was dose-dependent, being maximal at 1 μg DNA. Higher doses were not tested as complexing DNA at concentrations over 1 μg/μl tends to precipitate it. The time course of expression was followed by using optimal amounts of DNA and PEI/DNA ratios: i.e., 1 μg pCMV-luc/μl at 6 eq 22 KDa PEI. Expression was $7.3x10^5 \pm 1.1x10^5$ RLU/brain at 24 h and

increased significantly ($p<0.05$) to $1.7x10^6 \pm 4.1x10^5$ RLU/brain at 48 h (Fig. 1C). Expression was maintained over the time course of the experiment, values not being statistically different at days 2 and 8.

PEI-based DNA transfection provides wide distribution of transgene expression

Spatial expression of the transgene was analyzed using a pCMV-β-gal construct (Fig. 2B-F). Carrying out revelation of β-galactosidase expression on whole brains and then clarifying the tissues by delipidation showed numerous neuronal like cells (Fig. 2B-F) to express the transgene throughout the brain in a dose dependent manner. Expression was found around the ventricles but also in the hypothalamus (Fig. 2B) and the hindbrain. Neither abnormal morphology nor toxic effects were observed. No staining was seen with an irrelevant plasmid (CMV-luc).

Both positive and negative transcriptional regulations can be analyzed in vivo by PEI-based DNA transfection

To evaluate whether PEI-based gene transfer could be used to study physiological regulation in the tadpole CNS we used the *pKrox-24*-luc and the p*TRH*-luc constructs. In the mammalian brain, the *Krox-24* gene has been shown to be up-regulated by T_3 (Pipaon *et al.*, 1992; Mellström *et al.*, 1994; Ghorbel *et al.*, in press), whilst the *TRH* gene is a negatively regulated T_3 target gene (Lezoualc'h *et al.*, 1992; Guissouma *et al.*, in press). A first step was to verify the time course of expression from a physiologically relevant promoter. Thus we followed expression from the *TRH*-luc construction over 6 days, a period that amply covers the experimental period used for studying physiological regulation. Expression was 1659±527 RLU/brain at 48 h and increased significantly ($p<0.05$) to 36962±16858 RLU/brain at 96h (Fig. 3A). At day 6, expression was maintained and reached a maximum $2,5x10^6 \pm 9x10^5$ RLU/brain, values being significantly different at days 2 and 6. We next examined T_3-dependent regulation of this gene in the tadpole brain during the first 24 h post-injection. As shown in Figure 3B, transcription from the p*TRH*-luc construct was significantly ($p<0.01$) down-regulated by physiological amounts of T_3 (10 nM). In contrast, expression from the *Krox-24* construct was significantly ($p<0.01$) up-regulated by T_3 in a dose dependent manner (Fig. 4). The physiological specificity of these regulations is underlined by the fact that there was no difference in the constitutive luciferase expression driven by CMV promoter (500 ng) between control and T3-treated (10nM) tadpoles, the treatment beginning immediately after gene injection. In these two groups, luciferase expression in whole brain homogenates were respectively in control animals (24 h: $2.6x10^6 \pm 4x10^5$ RLU, n= 10; 96 h: $1.0x10^6 \pm 4.2x10^5$ RLU, n =5) and in T3-treated animals (24 h: $2,8x10^6 \pm 4.0x10^5$ RLU, n =10; 96 h: $0.8x10^6 \pm 1.3x10^6$ RLU, n =5).

Discussion

The data presented here show that a cationic polymer, PEI, provides a simple and convenient method for introducing genes into the *Xenopus* tadpole brain. Two previous methods for gene transfer into the amphibian CNS have been reported. First, in 1990, Holt *et al.* showed that a monocationic lipid (Lipofectin™) could be used to obtain low levels of transfection in this tissue, but no reports of the use of the method to study regulation have appeared.

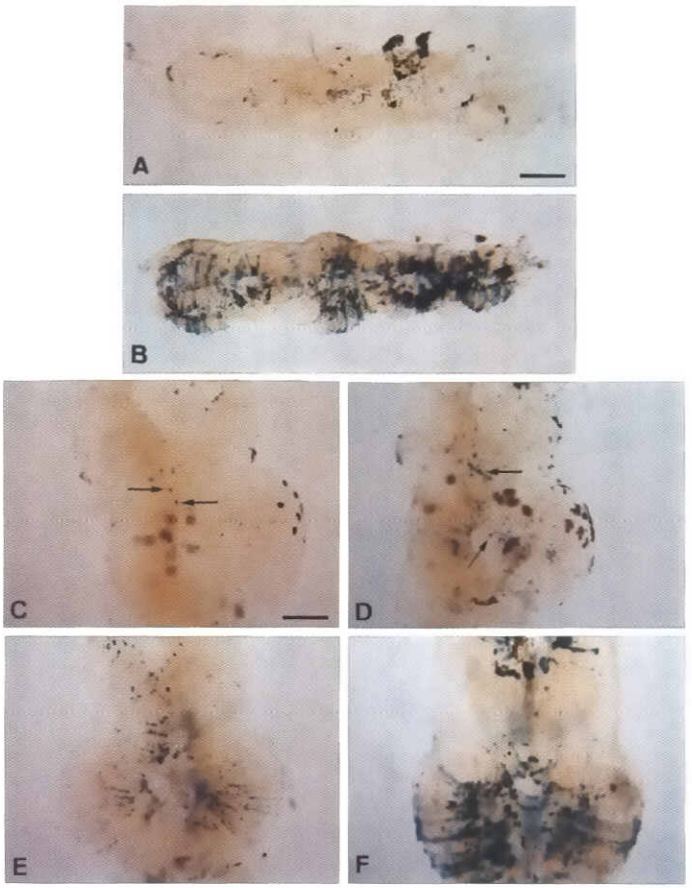

Fig. 2. Spatial expression of transgenes. *Whole brain localization of β-gal expression was followed after introducing* **(A)** *control 500 ng pCMV-luc and* **(B)** *500 ng pCMV-β-gal. Expression in the hypothalamic region of graded concentrations of pCMV-β-gal was dose dependent* **(C)** *50 ng,* **(D)** *100 ng,* **(E)** *250 ng,* **(F)** *500 ng. Injected plasmids were complexed to 6eq 22 KDab PEI in 1 μl 5% glucose solution containing 0.01% Fast Green per brain. Animals were sacrificed 48 h post-injection, brains dissected and β-gal revealed overnight at room temperature. Brains were photographed after delipidation in 2/1 benzyl benzoate/benzyl alcohol. (Magnifications: A and B x20, bar, 0.5 mm; C,D,E and F x40, bar, 0.25 mm).*

Second, more recently, the vaccinia virus has been successfully used to study neuronal maturation, as its very high levels of infection produce massive levels of transgene expression (Wu *et al.*, 1995,1998). Despite the high efficiency of viral vectors in general, there are a number of reasons for developing non-viral, synthetic vectors (Behr and Demeneix, 1998). Some of the most convincing reasons are their practicality, with ease of production of gene constructs and application. It is with this versatility in mind that we chose to optimize the use of a non-viral vector for gene transfer in the tadpole brain.

We now have a couple of decades of perspective on the performances of cationic non-viral vectors *in vitro* and nearly a decade for the *in vivo* context. During this period, polycationic vectors have been shown to be generally more efficient than their monocationic counterparts (Behr *et al.*, 1989; Remy *et al.*, 1994; Behr and Demeneix, 1998; Remy *et al.*, 1998), the density of the cation being a major player in the transfection processes. The gene

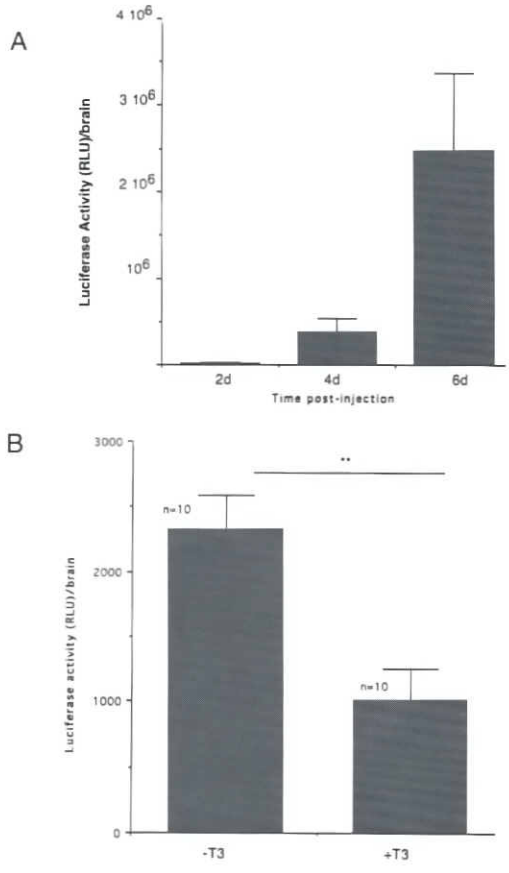

Fig. 3. Rat TRH-Mc expression and regulation. (A) *Rat TRH promoter driven-time course of luciferase expression in the CNS. Animals received 1 µl of a 5% glucose 0.01% Fast Green solution containing 500 ng of pTRH-luciferase plasmid condensed to 22 KDa PEI at 6 equivalents. Tadpoles were sacrificed at the times indicated post-injection. Means±SEM are given (n= 6 in each case).* **(B)** *Rat TRH promoter activity is down-regulated by T_3 in the CNS. Tadpoles were injected with 1 µl of a 5% glucose 0.01% Fast Green solution containing 500 ng of pTRH-luciferase plasmid complexed to 22 KDa PEI at 6 equivalents. Tadpoles were immediately treated with 10 nM T_3 and sacrificed 24 h later. Means±SEM are given (n= 6 in each case).*

transfer activity of these molecules is related to their capacity for condensing DNA, interacting with anionic proteoglycans of the cell membrane (Labat-Moleur *et al.*, 1996; Mislick and Baldeschwieler, 1996) and inducing endosome swelling and rupture. This latter function is dependent on the protonation of certain cationic groups (Remy *et al.*, 1998). PEI is a commercially available polymer in which one in every third atom is an amino nitrogen that can be protonated. In fact, PEI is the cationic polymer having the highest charge density potential and the overall protonation level of PEI increases from 20 to 45% between pH 7 and 5 (Suh *et al.*, 1994).

We have already shown that branched polymers of PEI can be used for vectorising DNA into the new-born (Boussif *et al.*, 1995) and adult (Abdallah *et al.*, 1996) mouse brain. The work in the adult mouse brain showed that the most efficient of the branched polymers was that with the lowest molecular weight (25 KDa). Since that work appeared a linear form of PEI with an even lower molecular weight (22 KDa) that has been synthesized and commercialized (Euromedex, Souffleweyersheim). We compared the performances of these two low molecular weight PEIs in the tadpole brain and found the linear form to be significantly better than the branched 25 KDa form. With the 22 KDa we find over 10^6 light units/µg DNA injected. This corresponds to nmolar concentrations of luciferase (about 10 ng luciferase), a result equal to that obtained on similar cell types (embryonic chick neurones) in the less stringent *in vitro* culture conditions (Boussif *et al.*, 1995).

It must be noted that the optimal result was obtained with an amine/phosphate ratio of 6. This produces complexes bearing a slight positive charge (Remy *et al.*, 1998). This overall cationic charge is necessary for interaction with the cell membrane but formulating complexes at higher ratios (and therefore higher net charge) decreased transfection efficiency, probably due to complexes sticking too rapidly to the extracellular matrix around the site of injection.

Indeed, examining the spatial distribution of transgene expressing cells in the transfected brain following transfection into the ventricle, shows a DNA dose dependent increase in number of transgene expressing cells, as well a wide spread pattern of expression all along the ventricle. Such expression is equivalent in extension to that obtained with ventricular injection of vaccinia virus expressing the same gene. Moreover, the time of expression is at least equal to the six days seen with vaccinia virus (Wu *et al.*, 1995). The difference between the results obtained by the two methods lies in the number of cells transduced, vaccinia virus being far more efficient.

However, for studying physiological regulation *in vivo* it is not necessary to have all the neurones in a given region transfected. Moreover, in our studies in mice CNS (Guissouma and Ghorbel, unpublished data), we have found that even with plasmid based somatic gene transfer, one will disrupt neuronal function if proteins are expressed from certain strong viral promoters. Here we show that the levels of transfection obtained with PEI are sufficient and

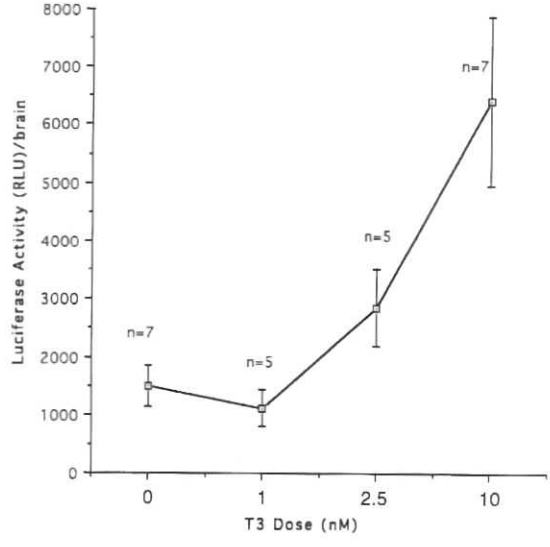

Fig. 4. Dose dependency of the T_3 effect on *Krox-24* promoter driven-luciferase expression. *The pKrox-24-luciferase plasmid (1 µg) was condensed to 22 KDa PEI at 6 equivalents in 1 µl of a 5% glucose 0.01% Fast Green solution and injected per animal. Tadpoles were treated by T_3 48 h post-injection and sacrificed two days later. Means±SEM are given (n indicates the number of individuals in each experiment).*

appropriate for studying transcriptional regulation in the tadpole CNS. To do this, we chose the promoters of two neuronal genes that are known to be regulated by T_3 in mammals. We chose *Krox-24* (otherwise known as *NGF1-A*) as previous studies have shown its mRNA to be regulated in the rat brain by thyroid status (Pipaon *et al.*, 1992; Mellström *et al.*, 1994). Moreover, we have shown that this positive T_3-dependent regulation is played out at the transcriptional level in mice (Ghorbel *et al.*, in press). To examine whether negative regulations could be followed by this technique, we used the rat *TRH* promoter as transcription from this sequence is significantly inhibited by physiological concentrations of T_3 in avian and mammalian hypothalamic neurones (Lezoulac'h *et al.*, 1992; Guissouma *et al.*, in press). We found similar, significant, transcriptional regulations following introduction of these heterologous promoters into the tadpole brain. Transcription from the *TRH* promoter was halved by treating the tadpoles during 24 h with T_3 whilst, conversely, the same treatment significantly raised transcription from the *Krox-24* promoter. However, when we treated animals for 48 h with T_3 before measuring TRH-luc, we saw no differences in transcription (data not shown). This may be related to luciferase protein half-life and/or widespread, non-hypothalamic TRH expression. Indeed, in mammals only the cells of the paraventricular hypothalamic nucleus expressing TRH are sensitive to T3-feedback (Jackson, 1982).

A number of experimental possibilities are raised by this demonstration. First, the technique will be useful *per se* for studying how various inputs impinge on promoter function in integrated, physiologically relevant contexts. Second, one can analyze how the functions of different transcription factors (such as different isoforms of T_3 receptors) affect transcription from given promoters. A third application could be the labor-saving testing of promoters prior to their use in germinal transgenesis (of either *Xenopus* or mice). Finally, somatic gene transfer can be applied to tadpoles or adult *Xenopus* in which the genetic background has been modified by germinal transgenesis (Kroll and Amaya, 1996) with the aims of either rescuing certain phenotypes or better defining the properties of transcription factors in these altered environments.

In conclusion, the use of linear, low molecular weight PEI provides a manageable, versatile and non-toxic method for introducing genes into the *Xenopus* tadpole brain. The transgenes introduced are regulated in a physiologically appropriate manner, opening up more experimental possibilities for this invaluable model of development.

Materials and Methods

Plasmid constructs

Super-coiled DNA plasmids were prepared using commercial columns (Jetsorb, Bioprobe Systems, Paris), suspended in Tris-HCl 10 mM, EDTA 1 mM pH 8 and stocked as aliquots at -20°C.

The pCMV-β-galactosidase expression vector was from Clonetech (Paris).

The pCMV-luciferase plasmid was a generous gift from Vical Inc. (San Diego, USA); it contains the viral CMV promoter upstream of a modified firefly luciferase gene. The p*TRH*-luciferase plasmid was a generous gift from Pr S.L. Lee (Boston). The construct contains a Pst I-Hind III fragment extending from + 84 to -600bp of The 5'UTR region of the rat *TRH* gene, upstream of the luciferase gene.

The p*Krox-24*-luciferase expression vector was constructed by subcloning a 1716bp EcoRI/PstI fragment from the mouse *Krox-24* promoter region (a generous gift from Dr. P. Charnay, ENS, Paris) into the EcoRI/PstI sites of the pBluescriptII SK Vector (Stratagene, La Jolla, CA, USA). Then the fragment was cut with EcoRV/SacI and reinserted in SmaI/SacI sites of the pGL2-basic vector (Promega, Madison, WI, USA), upstream of the luciferase coding sequence (Ghorbel *et al.*, in press).

Animals and T_3 treatment

Xenopus laevis tadpoles (stages 50-54, Nieuwkoop and Faber, 1967) were reared in aerated 15-20l aquariums (>100 tadpoles/ aquarium) until experiments, then grown in 2l aquariums post-injection (<12 tadpoles/ aquarium) until sacrifice.

For T_3 treatment, T_3 (Sigma) was added to the aquarium water either immediately or 48 h post-injection (see figure legends for final concentrations used and timing).

The animals used for the comparison of polyethylenimines (22 and 25 KDa) and for the dose-response experiment were sacrificed 24 h post-injection. Those for the pCMV-luciferase kinetics were sacrificed 1d, 2d, 4d, 6d or 8d post-injection. For gene regulation studies tadpoles were sacrificed at either 24 h (p*TRH*-luc) or 96 h (p*Krox-24*-luc) post-injection (see figure legends).

DNA transfection

All tadpoles were transfected into the third brain ventricle with 60-80 μm (outside diameter) micro-capillaries using a light-weight M-152 manipulator (Narishige, Japan) and an IM-6 micro-injector (Narishige, Japan).

For injection, animals were not anaesthetized, but simply placed in an appropriately sized hollow in a humidified wax support (de Luze *et al.*, 1993).

The injected DNA was complexed to PEI at 4°C in a 5% glucose solution containing 0.01% Methyl Green (Fast Green, Sigma). The required amount of PEI, according to DNA concentration and number of equivalents needed, is calculated by taking into account that 1 μg DNA is 3 nmol of phosphate and that 1μl 0.1M PEI is 100 nmol of amine nitrogen. So, to condense 10 μg DNA (30 nmol phosphate) with 4 eq PEI, one needs 120 nmol PEI (1.2 μl of a 0.1M solution). Two commercially available polyethylenimines (PEI) were compared: a linear polymer with a mean MW of 22 KDa (Euromedex, Souffleweyersheim, France) and a branched polymer with a mean MW of 25 KDa (Sigma). The efficiencies of these PEIs were compared by transfecting tadpoles with 1 μg of DNA complexed to 3, 6, 9, 12, 15, 18 or 20 equivalents of either PEI in 1 μl of a 5% glucose solution containing 0.01% Fast Green. For dose response and physiological regulations, tadpoles received 1 μl of a 5% glucose solution containing either 1 μg/μl DNA (for pCMV-luciferase kinetics and *Krox-24* regulation) or 500 ng/μl DNA (*TRH*-luciferase regulation) or different amounts of DNA (for the dose-response experiment). For all experiments, except that shown in Figure 1A, DNA was complexed with 6 equivalents 22 KDa PEI.

Luciferase activity

Brains from luciferase-transfected tadpoles were dissected and sonicated in 200 ml luciferase lysis buffer (Promega) then centrifuged 10 min at 12000g, 4°C. Twenty μl of the supernatant were mixed with 100 μl luciferase substrate (Promega) and immediately counted for the relative light units during 10s using a single well luminometer (MGM Instruments, Hamden, CT).

Revelation of β-galactosidase activity

β-galactosidase transfected brains were fixed in 2% paraformaldehyde in 0.1 M phosphate buffer saline (PBS) for 2 h at 4°C, rinsed twice in PBS (30 min each) then incubated with X-Gal solution [1 mg/ml X-Gal (Boehringer Mannheim), 3 mM K4Fe(Cn)6, 3 mM K3Fe(Cn)6, 1.3 mM MgCl2, 10 mM Sodium Phosphate, pH 7.4] overnight at room temperature. Brains were dehydrated in successive alcohols, clarified in benzyl benzoate/benzyl alcohol (2/1) and photographed immediately.

Statistical analysis of results

Results are expressed as luciferase activity (relative light units: RLU) per brain. Mean±SEM is given in all figures. Student's t-test was used to

analyze differences between groups. Differences were considered significant when $p<0.05$. Representative results are shown. Each experiment was repeated at least twice producing similar results.

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