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Generation of stable *Xenopus laevis* transgenic lines expressing a transgene controlled by weak promoters

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Abstract

Combining two existing protocols of transgenesis, namely the REMI and the *I-SceI* meganuclease methods, we generated *Xenopus laevis* expressing a transgene under the control of a promoter that presented a restricted pattern of activity and a low level of expression. This was realized by co-incubating sperm nuclei, the I-SceI enzyme and the transgene prior to transplantation into unfertilized eggs. The addition of the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) in our constructs further enhanced the expression of the transgene without affecting the tissue-specificity of the promoter activity. Using this combination of methods we produced high rates of fully transgenic animals that stably transmitted the transgene to the next generations with a transmission rate of 50% indicating a single integration event.

Keywords: *Xenopus laevis*; transgenesis; I-SceI Meganuclease; Posttranscriptional regulatory element (WPRE)

Introduction

The amphibian *Xenopus laevis* has played a key role in the study of vertebrate development and the establishment of important concepts in developmental biology (for review see Stern 2005; Heasman 2006). The large size of eggs and embryos permit microsurgical manipulation. It is also possible to over-express specific gene products in particular regions of the embryo by micro-injection of synthetic mRNA or to inhibit zygotic gene function by the use of dominant negative approach (Amaya et al. 1991) or antisense morpholinos (Heasman et al. 2000).

The ability to generate transgenic *Xenopus* has reinforced the interest in this model. This was first achieved in the late 90's when Kroll and Amaya (1996) described a method based on the Restriction Enzyme Mediated Integration (REMI) developed in *Dictyostelium discoideum* (Kuspa and Loomis 1992). According to these authors, the transgene was supposed to integrate in the chromatin-decondensed sperm nuclei before transplantation in unfertilized eggs. This method was further simplified by omitting egg extracts and restriction enzyme to avoid the weakening of decondensed sperm nuclei (Sparrow et al. 2000). It enables the production of non mosaic and stable transgenic animals. However, one of the major drawbacks is that chromosomal integration occurs as concatemers (2 to 6 copies) at 4-8 sites of the genome.

In 2002, a new approach based on the use of a yeast transposase, the meganuclease *I-SceI*, was proposed to generate transgenic fishes (Thermes et al. 2002). The co-injection in the cytoplasm of one-cell stage embryos of the enzyme and a plasmid carrying the transgene flanked by two restriction sites for *I-SceI* allowed the rapid integration of a functional insert. Moreover, in the fish species, the observed germline transmission rates were about 50% suggesting a single integration of the transgene into one-cell stage embryos. This was confirmed by Southern blot analysis since the reporter was found integrated as a single copy or few copies in tandem into mostly single sites within the genome (Thermes et al. 2002).

This transgenesis method using meganuclease has been successfully used in other fish species

(zebrafish *Danio rerio*: Grabher et al. 2004) and the ascidian *Ciona savignyi* (Deschet et al. 2003). More recently, it has been adapted to different amphibian species such as axolotl (Sobkow et al. 2006) or the anurans *Xenopus laevis* (Pan et al. 2006) and *Xenopus tropicalis* (Ogino et al. 2006). Contrary to what happens in fishes, transgenic amphibians produced with this method were mainly mosaic and only 10 to 15% of developing embryos showed a non-mosaic expression of the transgene. The majority of the F0 founders transmitted the transgene to less than 50 % of their progeny. Interestingly, the main advantage of this method, namely the few transgene copy number, was preserved in *Xenopus*.

Other methods, based on the use of different transposases, were also proposed. However, these protocols need the co-injection of the plasmid carrying the transgene and the mRNA encoding the transposase. The delay between the injection of mRNA and the production in the cells of the active enzyme led to the production of mosaic animals that were of limited use for promoter analysis in the F0 founder generation (Allen and Weeks 2005; Hamlet et al. 2006; Sinzelle et al. 2006).

From all these studies, it seems quite difficult to obtain stable F1 generation which will be useful for the amphibian community. Moreover, all the experiments conducted so far have been realised with a little number of promoters in combination with GFP. Despite the obvious advantage of GFP to monitor expression, the threshold level required for detection makes it inappropriate to assess low level of gene expression.

Using a combination of REMI and *I-SceI* meganuclease methods we produced high rates of fully transgenic *Xenopus* bearing a few number of transgene copies. We used the promoter of the pan neural gene *neuro-βtubulin* (NβTub) which has been previously used (Ryffel and Lingott 2000) and the promoter of the bHLH neurogenic differentiation factor NeuroD encoding gene which has a low and restricted transcriptional activity (Lee et al. 1995). However, this method was not sufficient to obtain convincing expression of GFP under the control of the *neuroD* promoter. This could be achieved either by boosting transcription or by acting on post-transcriptional events, which would limit the potential alteration of the promoter tissue-specificity. The ability of a post-transcriptional

regulatory element from woodchuck hepatitis virus (WPRE) to enhance the expression of a transgene in retrovirus (Zufferey et al. 1999) or adenovirus (Glover et al. 2002; Xu et al. 2003) vectors without affecting the tissue specificity (Glover et al. 2002) has already been demonstrated. However, to date, the efficiency of cis-acting elements such as WPRE has only been assessed in mammals or mammalian cell lines and little is known about its ability to enhance the *in vivo* expression of a transgene in a non-mammalian species. Thus, in this work, we also performed transgenesis in *Xenopus laevis* with constructs containing the *neuroD* promoter, which drives the expression of the enhanced green fluorescent protein (EGFP) and the WPRE element, localized in the 3'UTR of the EGFP sequence.

Methods

Plasmid constructs

The pEGFP-1 reporter vector (Clontech) was modified by introducing double-strand oligonucleotides containing an *I-SceI* recognition site (Colleaux et al. 1988; Thermes et al. 2002) in the *Bgl* II and *Afl* II restriction sites to generate pSce-EGFP-Sce. For transgenesis, a 3.8 kb *neuro-βtubulin* promoter from *Xenopus* (accession number: EF989124) was inserted in the *Hind* III site of pSce-EGFP-Sce to generate pSce-NBT-EGFP-Sce. A 1.3 kb *neuroD* promoter fragment (accession number: EF591766) was cloned from genomic libraries generated using the Universal Genome Walker kit (Clontech) according to the manufacturer's protocol. Two reporter plasmids were then constructed, containing either the entire cloned 5' flanking region (*1.3neuroD*) or only the 320 bp proximal region (*0.3neuroD*) in the *Sac* I / *Hind* III sites of pGL3 reporter plasmid (Promega) to perform transfection assays. These constructs were then designated as p*0.3neuroD*-Luc and p*1.3neuroD*-Luc. An expression vector, pCS-MT-xNGNR-1a, (provided by Dr. D.J. Anderson, Howard Hughes Medical Institute, California Institute of Technology, Pasadena, California) was used in co-transfection assays to check the ability of the *xneuroD* promoter fragments to be induced. The two fragments of *xneuroD* promoter were also introduced into the *Xho* I / *Bam* HI (*0.3neuroD*) or *Sma* I (*1.3neuroD*) restriction sites of pSce-EGFP-Sce for transgenesis. A WPRE element (Woodchuck Post-transcriptional Regulatory Element, provided by Pr D. Trono, Geneva, Switzerland; accession number: J04514) was inserted in the *Xba* I site of pGL3, p*0.3neuroD*-Luc and p*1.3neuroD*-Luc used in transfection studies or the *Not* I site of p*0.3neuroD*-EGFP and p*1.3neuroD*-EGFP used in transgenesis, between the reporter gene and the polyadenylation signal.

Culture of P19 cells and transfection assays

P19 mouse multipotent embryonic carcinoma cells were grown in DMEM (Dulbecco's Modified Eagle Medium, Invitrogen Gibco BRL) supplemented with 10% foetal calf serum (Invitrogen Gibco BRL) at 37°C in a humidified 5% CO₂ atmosphere. P19 cells were plated at a density of 0.5 x 10⁵

cells per well of 24 well plates (BD Falcon). Cultures were transfected with 400 ng of reporter vector (pGl3, pGl3-WPRE, p0.3*neuroD*-Luc, p0.3*neuroD*-Luc-WPRE, p1.3*neuroD*-Luc or p1.3*neuroD*-Luc-WPRE) with or without 50 ng of expression vector (pCS-MT-xNGNR-1a) using the calcium phosphate transfection method (Wigler et al. 1978). All transfections were achieved with 100 ng of an internal control vector (pCH110, Amersham Pharmacia Biotech). The total amount of DNA was supplemented at 1 µg per transfected well with the addition of pBluescript plasmid. During the transfection step, cells were grown at 37°C in a humidified 2% CO₂ atmosphere. Luciferase activity was assayed 45 hours after transfection using the luciferase assay system according to the manufacturer's protocol (Promega) and normalized with the β-Galactosidase activity (Sambrook et al. 1989). Each experiment was performed at least four times in triplicates.

Embryo manipulation and microinjection of transgenes

The use of the *I-SceI* meganuclease was combined with the method based on the transplantation of purified sperm nuclei (Sparrow et al. 2000). The sperm nuclei were prepared according to the protocol described by Kroll and Amaya (1996). For transgenesis, 500 ng of plasmid vectors were digested for 30 min at room temperature with *I-SceI* meganuclease (Roche Applied Science) in the appropriate buffer and then combined with 500 000 sperm nuclei and incubated for 5 minutes at room temperature. The reaction was diluted in 200 µl of MOH injection buffer (KPO₄ 10 mM, Kgluconate 125 mM, NaCl 5 mM, MgCl₂ 0.5 mM, Sucrose 250 mM, Spermidine 0.25 mM and Spermine 0.125 mM, pH 7.2; Offield et al. 2000) and back filled into glass needles. Unfertilized eggs were dejellied using 1.5% cysteine in 0.1×MMR (Marc's Modified Ringers: Sive et al. 2000), rinsed in 0.1×MMR and loaded into the square well of agarose dishes containing 6% Ficoll in 0.1×MMR at 16°C. The injection was performed using a Harvard 22 syringe pump (Harvard Instruments) with a flow rate of 0.6 µl/min.

A few hours after injection, embryos were transferred into 0.1×MMR without Ficoll and incubated

at 19-20°C. Developing embryos were staged according to Nieuwkoop and Faber (1967).

Genomic PCR and Southern blot hybridization

Genomic DNA was extracted from F1 *0.3neuroD*-EGFP-WPRE positive or negative tadpoles. 20 ng were used for genomic PCR reactions. A part of the transgene was amplified using the following primer set: xNDfor (5' TCCgCAgAAAgCACCACT 3') and EGFPprev (5' TTgTCgggCAgCAgCACgg 3'). The PCR cycle conditions were 4 min at 94°C followed by 35 cycles of 20 sec at 94°C, 20 sec at 58°C, 45 sec at 72°C and a final extension 7 min at 72°C. PCR products were separated on a 0.8% agarose gel.

For the Southern blot analysis, 25 µg of DNA was digested with either *BsrGI* or *BamHI*, separated on a 0.8% agarose gel and transferred to a Hybond-N hybridization membrane (Amersham). Hybridization was performed with a 800 bp EGFP cDNA probe labeled with [α^{32} P]-dCTP .

***In situ* hybridisation on tissue sections**

Larvae were fixed overnight at 4% (v/v) formaldehyde and processed for *in situ* hybridisation as previously described (Coumailleau and Duprez 2009). Antisense RNA probes were labelled with digoxigenin according to manufacturer's instructions (Roche Diagnostics). The probes were detected by an alkaline phosphatase-coupled antibody against digoxigenin using nitroblue tetrazolium/5-bromo-chloro-3-indolyl phosphate (NBT/BCIP) as the chromogenic substrate for alkaline phosphatase. Antisense NeuroD RNA probes were prepared as previously described (Lee et al. 1995). Antisense EGFP was produced using a TOPO/EGFP plasmid generated by subcloning a PCR product corresponding to the entire EGFP into the pCR2.1-TOPO plasmid (Invitrogen).

Photomicroscopy

Images of living transgenic animals were obtained using either a Leica MZF LIII stereomicroscope and a Leica DC300F digital camera or an inverted Leica TCS SP2 laser scanning confocal

microscope (PIXEL platform of GIS Europa, Rennes). Confocal imaging was performed using a HC PL APO 10x (NA=0.40) objective together with a S23 (NA=0.53) condenser. Excitation was provided by the 488 nm laser line of Ar laser source.

Image and statistical analysis

After collection, data were analyzed with the open source software ImageJ (<http://rsb.info.nih.gov/ij/>). The stacks were then projected along the z-axis to give the best overview of the structure. The data obtained from transfected P19 cells were analyzed using the Student's t-test.

Results

Generation of transgenic *Xenopus* using a new combination of transgenesis procedures

Our aim was to obtain transgenic animals carrying a few number of transgene copies. In this purpose, we chose a method combining the simplified version of the REMI protocol (Sparrow et al. 2000) and the Meganuclease-based method (Thermes et al. 2002; Pan et al. 2006). First attempts were performed with the construct pSce-N β Tub-EGFP-Sce based on the promoter of a class II- β Tubuline (Neuro- β Tubuline) that is specifically and strongly expressed in the neurons of *Xenopus* embryos (Moody et al. 1996). Using this construct, the EGFP fluorescence was detected in the neural tube of embryos from stage 17-18 (**Fig. 1A**). At tailbud stage, the spinal cord and developing brain were fluorescent (**Fig. 1B**). On later stage, EGFP expression was localized in the nervous system. The fluorescence was easily detected in the brain, spinal cord, retina, optic and olfactory nerves and olfactory epithelia (**Fig. 1C**). At stage 55, it was still detectable in the nerves of head, tail myotomes and limbs (**Fig. 1D,E,F**).

Of 5588 injected eggs, 142 (2.5%) developed normally to stage 40. Among these, 41 showed a strong and uniform expression in the central nervous system (CNS). 37 embryos expressed EGFP in the CNS as well as in various other tissues and 10 others expressed EGFP only outside of the CNS. This ectopic expression varied between the embryos in term of localization or intensity. One embryo uniformly expressed EGFP in only one half of his CNS reflecting a possible integration of the transgene after the first cellular division (**Table 1**).

Seven N β Tub-EGFP founders, presenting a specific expression in CNS, were raised to sexual maturity and mated to wild-type animals and germline transmission rates were estimated by scoring EGFP expression (**Table 2**). One of these founders transmitted the transgene to 71% of its progeny, probably reflecting the existence of two integration sites. For another one, the transgene was not transmitted to the offspring suggesting either the absence of transgene integration or an integration that occurred after the differentiation of the germ cell precursors. However, five of these founders

produced offspring with 48 to 58% of F1 animals expressing EGFP in the correct tissues. These rates are close to the Mendelian ratio for the transmission of a single locus to the offspring. This single integration event was confirmed on one of these lines by the sequence analysis of the integration site (data not shown). F2 embryos were also generated from this transgenic line. The transmission rate of the transgene was still about 50% in these animals indicating that the transgene is stably transmitted in the successive generations (**Table 2**).

Improvement of reporter gene expression from the *xneuroD* promoter by the addition of WPRE element

The use of weak promoter is often limited in transgenesis by the fact that their activity is hardly detectable. Thus we tried to improve our transgenesis method by integrating a viral element in our construct. As a weak promoter, we choose that of the *Xenopus* neurogenic differentiation factor *neuroD* gene which shows low level and restricted patterns of expression (Lee et al. 1995).

Using PCR on a *Xenopus laevis* genomic library, we generated a 2.4 kb DNA fragment containing a part of the *xneuroD* gene (accession number: EF591766). Determination of the transcription start site by RACE PCR and comparison with human (Miyachi et al. 1999) and mouse (Xu and Murphy 1998) *neuroD* genes showed that this fragment comprises about 1.3 kb of the 5' flanking region of this gene (data not shown).

The transcriptional activity of this *neuroD* promoter was assayed in transiently transfected mouse P19 cells. Luciferase reporter plasmids containing two fragments of the *neuroD* promoter region, termed *0.3neuroD* and *1.3neuroD*, were transiently transfected in mouse P19 stem cells to assess their transcriptional activities. These two fragments contained the three E-boxes involved in *neuroD* transcription activation (Miyachi et al. 1999; Huang et al. 2000). As shown in **Fig. 2 A**, the relative luciferase activities of p*0.3neuroD*-Luc and p*1.3neuroD*-Luc were found to be 5 to 10-fold higher than that of pGL3. The basal activity of p*0.3neuroD*-Luc was 1.8 to 2-fold above p*1.3neuroD*-Luc activity (p<0.01). In the P19 cells, it is known that the basal activity of *neuroD* promoter is very

low (Itoh et al. 1997; Kim et al. 2004) but can be strongly induced by another bHLH factor, Neurogenin (Ngn) (Kim et al. 2004). Thus, in order to determine the ability of Ngn-1 to enhance the activity of the *neuroD* promoter fragments, P19 cells were co-transfected with a *Xenopus* Neurogenin-related-1 expression vector, pCS2-X-Ngnr-1a. This resulted in a strong increase of both *0.3neuroD* and *1.3neuroD* activity (9 and 11.6-fold respectively, $p < 0.001$) (**Fig. 2A**).

To increase reporter gene expression both *in vitro* and *in vivo*, WPRE was inserted in our plasmid constructs downstream of the open reading frame of the reporter gene. The efficiency of WPRE was then assessed after transient transfection of two constructs, p0.3*neuroD*-Luc-WPRE and p1.3*neuroD*-Luc-WPRE, in P19 cells. Adding WPRE induced a 1.75 and 1.9-fold ($n=18$, $p < 0.002$) increase of the luciferase activity when used with the *0.3neuroD* or *1.3neuroD* promoter fragments respectively, when compared with the same constructs lacking WPRE (**Fig. 2B**).

Effects of WPRE on transgene expression *in vivo*

In order to obtain transgenic embryos, *0.3neuroD* and *1.3neuroD* promoter fragments were bound to EGFP, with or without WPRE, and flanked by an *I-SceI* recognition site at both extremities.

At stage 40, 18% of the animals ($n=34$) expressed EGFP in the eye, brain and spinal cord when placed under the control of *0.3neuroD* promoter. For 9% of these embryos, the EGFP detection in the future nervous system was already possible at stages 18/20. The addition of WPRE to the same construct gave 63% of EGFP-expressing animals at stage 40 ($n=51$), and fluorescence detection began earlier at stage 15 for 19% of embryos. At stages 18/20, 35% of neurula embryos were labelled in the future nervous system (**Table 3**). With *1.3neuroD* promoter fragment, no expression in the nervous system was obtained. However, the addition of WPRE allowed EGFP detection in the CNS from stage 33 with p1.3*neuroD*-EGFP-WPRE construct. At stage 40, EGFP expression was localised in the brain and eyes for two of the eight surviving animals (**Table 3**). No expression was observed in the spinal cord contrary to observations with *0.3neuroD* promoter fragment.

The preservation of tissue and cellular specificity was checked for the *0.3neuroD* promoter

construct carrying the WPRE using both confocal microscopy on living embryos and *in situ* hybridisation on fixed tissues. At stage 40, confocal microscopy imaging performed on living embryos showed EGFP fluorescence in the five encephalic vesicles (telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon) as well as in the retina (**Fig. 3A**) and the spinal cord (not shown). At stage 48, the fluorescence level remained elevated in the pallia, the cerebellum and the rhombencephalon (**Fig. 3B**). In order to precisely check the activity of the *0.3neuroD* promoter in transgenic embryos, we performed *in situ* hybridisation to detect both EGFP and endogenous NeuroD transcripts. We showed that EGFP expression was clearly restricted to the brain area that expressed the endogenous neuroD gene since both signals are superimposed in the cerebellum (**Fig.4A,B**) and rhombencephalon (**Fig. 4C,D**).

NeuroD, also referred as BETA2, is expressed in the pancreas (Naya et al. 1995; Kelly and Melton 2000). Spectral analysis in confocal microscopy showed that EGFP was expressed in the pancreas of transgenic animals (data not shown). However, this fluorescence was quite difficult to observe in classical fluorescent microscopy because of the high auto-fluorescence of this tissue.

Some embryos expressing EGFP under the control of *0.3neuroD* promoter from a construct containing WPRE element were raised to sexual maturity and used to estimate the transmission rate to the F1 offspring. As previously obtained with pSce-N β Tub-EGFP-Sce, a majority of the founders (5 out of 6) transmitted the transgene to about 50% of their progeny (**Table 4**). Transgene integration was analyzed in one of these lines using genomic PCR and Southern blot. Both methods showed that EGFP fragments can be detected only in EGFP-positive animals indicating that the absence of expression in EGFP-negative animals is not due to transgene silencing (**Fig. 5 A,C**). Moreover, the patterns observed in the Southern blot experiment are consistent with the hypothesis of a single integration of the transgene in the host genome. The presence of a weak band of about 2 kb indicated that the transgene might be inserted as a tandem repeat (**Fig 5B,C**). Transmission rate to the F2 generation was also assessed for one of these transgenic lines and was found to be stable between the different generations (**Table 4**).

Discussion

In this study, we showed that the combination of two previously described protocols, namely the REMI (Kroll and Amaya 1996) and *I-SceI* meganuclease (Thermes et al. 2002) methods, allowed the obtention of a high rate of fully transgenic animals in the F0 generation with a limited number of transgene integration events. This was assessed with different constructs based on the use of promoters of two genes, N β Tubuline and NeuroD, that have different transcriptional activities. N β Tubuline is a class-II β tubulin that is specifically and strongly expressed in differentiated neurons (Moody et al. 1996) whereas NeuroD is a transcription factor that belongs to the bHLH superfamily and promotes the differentiation of neuronal precursor cells in neurons (Lee et al. 1995). Moreover, this latter gene presents low level and restricted pattern of expression.

The limitation of integration events was shown by the transmission rates close to 50% obtained from a majority of F0 founders and genomic DNA analysis, both indicating an integration in a single site of the host genome. In this case, all the siblings obtained from a single founder exhibited the same pattern of expression and similar level of fluorescence. This is in contrast with the results published by Marsh-Armstrong et al. (1999) reporting that most of the founders produced by the REMI method presented 2 to more than 4 integration events which resulted in siblings presenting various fluorescence intensities. F2 embryos expressing EGFP were also produced from some F1 adults mated with wild type animals showing that the transgene is stably integrated in germline cells. Moreover, the transgene transmission rate is the same between F1 and F2 generations as between F0 and F1.

Combining the protocols was found to be far more efficient than using the *I-SceI* meganuclease alone in term of transgene transmission between F0 and F1. Indeed, with *I-SceI* alone, the transmission rate rarely reaches 50% whatever the animal species indicating that, in this case, the integration into the host genome occurs mainly after the first cellular division (Thermes et al. 2002; Deschet et al. 2003; Grabher et al. 2004; Ogino et al. 2006; Pan et al. 2006; Sobkow et al. 2006). It

is interesting to note that in our hands this method gave almost the same results (one transgenic line with a transmission rate close to 50% on 6 tested, unpublished data).

The mechanisms involved in the limitation of integration events when *I-SceI* meganuclease is used together with sperm nuclei remain largely unknown. However, it is clear that the incubation of the transgene with sperm nuclei promotes its rapid integration in the genome reducing the number of embryos presenting a mosaic expression of this transgene. Although this cannot be totally ruled out, it is very unlikely that *I-SceI* facilitates transgene integration by generating double strand breaks (DSB) in *Xenopus* genomic DNA since it has a 18-bp long recognition site and therefore a low probability of cutting in the host genome. Another possibility is that the integration happens after the introduction of sperm nuclei into the oocyte. In this case, sperm nuclei could only be vectors that carry the transgene into the cell and the integration could occur at the time of pronuclei fusion. As previously demonstrated, exogenous DNA incubated with sperm cells or purified nuclei is rapidly internalized (Francolini et al. 1993) and mature sperm cells can even be used as vectors to introduce DNA into eggs and generate transgenic animals (Lavitrano et al. 1989). Whatever the precise mechanism involved in the integration, *I-SceI* could intervene in DSB repair as it apparently proceeds in yeast where it promotes intron homing (for review, see Dujon 1989).

Although it probably needs to be confirmed in various promoter contexts, we showed in this paper that WPRE could be useful to enhance transgene expression in *Xenopus* without affecting its tissue-specificity. WPRE is a post-transcriptional regulatory element (Donello et al. 1998) that does not interfere with the promoter activity and its efficiency was previously demonstrated in different tissues using adenovirus vectors (Xu et al. 2003). It probably functions by modifying the RNA polyadenylation, export and/or translation (Zufferey et al. 1999; Mastroyiannopoulos et al. 2005), so that the transgene product accumulates more quickly and is detectable earlier on. The RNA export function of WPRE involves in a cellular factor CRM-1, an export receptor for leucine-rich nuclear export signals (Fornerod et al. 1997; Popa et al. 2002). A deficiency in this factor may explain the negative effects of WPRE observed in some cases (Werner et al. 2004; Klein et al. 2006). In

Xenopus, the CRM-1 factor is present during the early stage of embryogenesis and becomes functional before the neurulation (Callanan et al. 2000).

The efficiency of the association between the *xneuroD* promoter and WPRE was first assessed by transfection studies in the pluripotent P19 embryonal carcinoma cells that can differentiate in neuronal cells upon a retinoic acid treatment. The difference in the activities observed with the two promoter fragments used can be explained by the presence of a potential binding site for the transcriptional repressor IA-1 in the *1.3neuroD* fragment (Breslin et al. 2002). The addition of WPRE significantly increased the reporter gene expression with both promoter fragments. Our results are close to the 1.5-fold induction in RNA or protein levels obtain in a plasmid vector context when this vector is stably integrated in the host cells (Johansen et al. 2003). Using adenoviral vector, Glover et al. (2002) also reported a similar elevation with the SYN1 promoter which drives a neuron-specific expression. Other studies reported higher elevations. However, these works were based on the use of retroviral or lentiviral vectors and strong promoters such as CMV (cytomegalovirus) or MMTV (mouse mammary tumour virus) promoters (Zufferey et al. 1999; Klein et al. 2006).

The ability of WPRE to promote gene expression was then evaluated *in vivo* in transgenic *Xenopus* embryos. Addition of this element in the plasmid vector used for transgenesis increased the reporter gene expression driven by the *0.3neuroD* promoter and allowed an earlier detection of EGFP in more embryos (63%) without modifying the localization in the nervous system. This improvement in the detection of transgene expression was notably observed with the *1.3neuroD* promoter fragment. The enhancement of reporter expression, due to the improvement of the transgenic technique and the addition of the WPRE element, permitted an early detection (stage 15) of EGFP controlled by *Xenopus neuroD* promoter, which occurs slightly later than the appearance of *neuroD* mRNA at stage 13.5/14 (Lee et al. 1995; Ma et al. 1996). Transgene expression with p*0.3neuroD*-EGFP-WPRE was found to be specifically localized in the developing nervous system, in structures in which *neuroD* mRNA was detected (Lee et al. 1995; Ma et al. 1996; Huang et al. 2000; Schlosser

and Northcutt 2000; Schlosser et al. 2002). This concordance was maintained at later stage. Indeed, at stage 48, confocal microscopy imaging as well as *in situ* hybridisation studies allowed the detection of EGFP in limited areas corresponding to neuroD-expressing brain areas during neurogenesis in *Xenopus* (Wullimann et al. 2005).

Thus, in this study, we describe a transgenesis method that allows the production of fully transgenic F0 animals with a limited number of insertion events. Moreover, we show that regulatory elements such as WRPE can be successfully used to improve transgene expression in *Xenopus* making possible the use of a promoter with low activity to label a group of cells or a particular tissue.

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Figure legends

Fig. 1 *In vivo* Expression of the N β Tub-EGFP transgene co-injected with *I-SceI* meganuclease and sperm nuclei into *X. laevis* oocytes

Fluorescence can be detected in the neural tube at stage 18 (A), the brain and spinal cord at stage 34 (B) or 46 (C), the brain, retina and cranial nerves such as optic or olfactory nerves at stage 55 (D). The innervation of the tail myotomes (E) and hind limbs (F) is also easily detectable (NT: neural tube; B: brain; SC: spinal cord; Olf: olfactory nerve; Opt: optic nerve). Scale bar: (A) 500 μ m; (B,C) 1 mm; (D,E,F) 2 mm.

Fig. 2 Activation of *XneuroD* promoter fragments in P19 cells

(A) P19 cells were transfected with pGL3, p0.3*neuroD*-Luc or p1.3*neuroD*-Luc constructs. *NeuroD* promoter fragments activities were assayed with pCS2-X-*Ngfr-1a* (+NGN) cotransfection. Activities were normalized to that of pGL3. (B) Effect of the WPRE element addition in the reporter constructs was assessed. Data represent relative luciferase activity obtained 45 hours after transfection and normalized to β -Galactosidase activity, expressed from a cotransfected control plasmid. Each activity is the mean \pm SEM of at least four independent experiments with triplicate transfections (A and B).

Fig. 3 Confocal microscopy images of EGFP expression in live transgenic F1 embryos obtained with p0.3*neuroD*-EGFP-WPRE construct

(A) EGFP fluorescence was found in the retina and the five embryonic brain vesicles of stage 40-41 (Tel: Telencephalon, Di: Diencephalon, Mes: Mesencephalon, Met: Metencephalon, Myel: Myelencephalon, Ret: Retina). (B) *In vivo* spatial expression of EGFP fluorescence in the whole brain of a stage 48 embryo. (Pa: pallium, OT: Optic Tectum, C: Cerebellum, Rho: Rhombencephale); Scale bar: 100 μ m

Fig. 4 *In situ* hybridisation analysis of x*NeuroD* and EGFP expression in transgenic 0.3*NeuroD*-EGFP-WPRE larva (stage 48)

Consecutive transverse sections at the level of cerebellum (A,B) and rhombencephalon (C,D) were hybridized with the x*NeuroD* (A,C) and EGFP (B,D) probes. Dorsal is to the top. Arrow indicates the position of a ganglion of the cranial nerve IX. Scale bar: 100 μ m

Fig. 5 PCR and Southern blot analysis on genomic DNA from *0.3neuroD*-EGFP-WPRE animals

(A) Genomic PCR of F1 siblings from a *0.3neuroD*-EGFP-WPRE founder. Lanes labeled (-) or (+) represent genomic DNA from individual EGFP-negative or EGFP-positive tadpoles respectively. Wt is a control performed on wild-type genomic DNA. M: DNA ladder (B) Schematic representation of the transgene with the relative position of the probe and restriction sites. Below, products expected after transgene integration according to the different possibilities of concatemerization. (C) Southern blot analysis of F1 progeny. DNA from EGFP-positive tadpoles was digested by either *BsrG* I (lane1) or *Bam* HI (lane 2). DNA from EGFP-negative tadpoles was digested by *BsrG* I (lane3).

Fig. 1

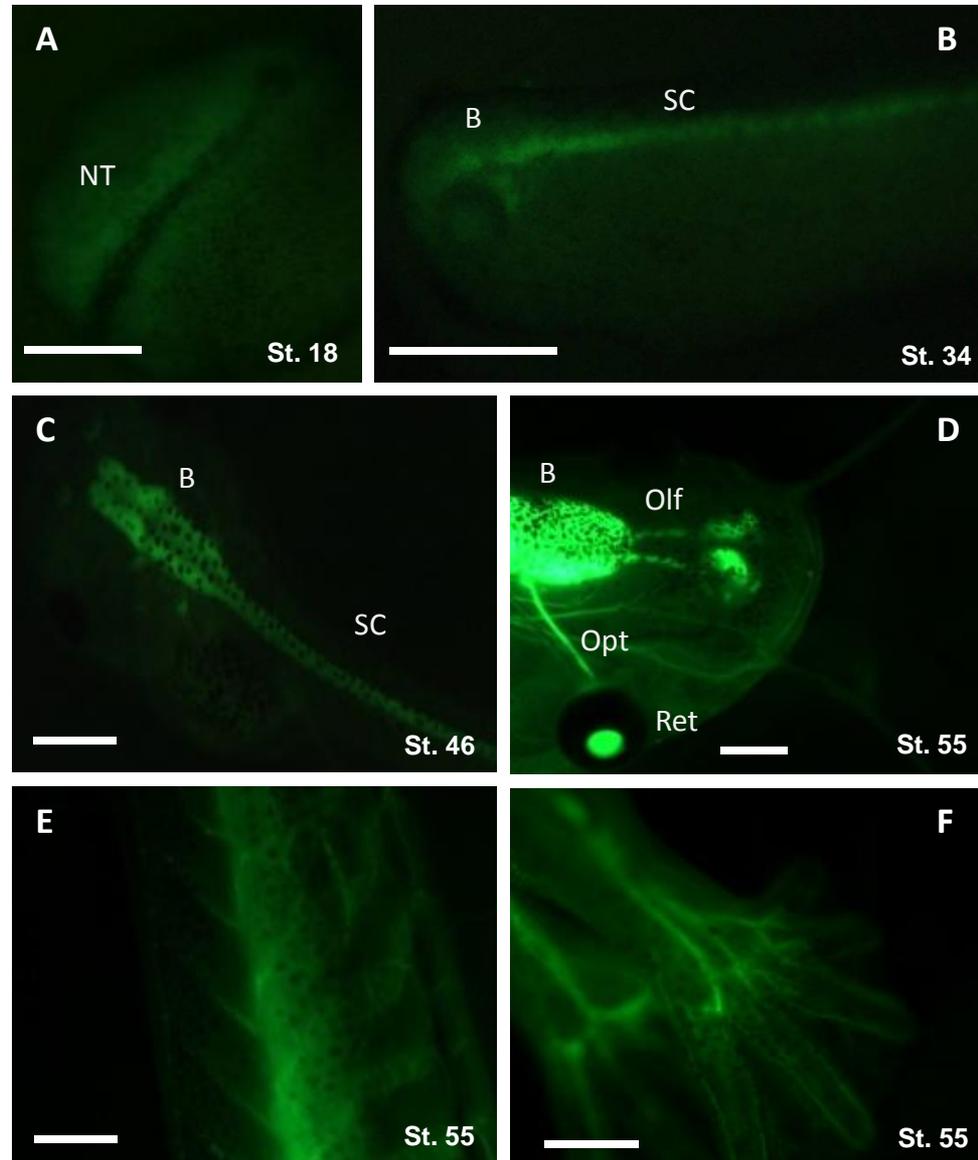


Fig. 2

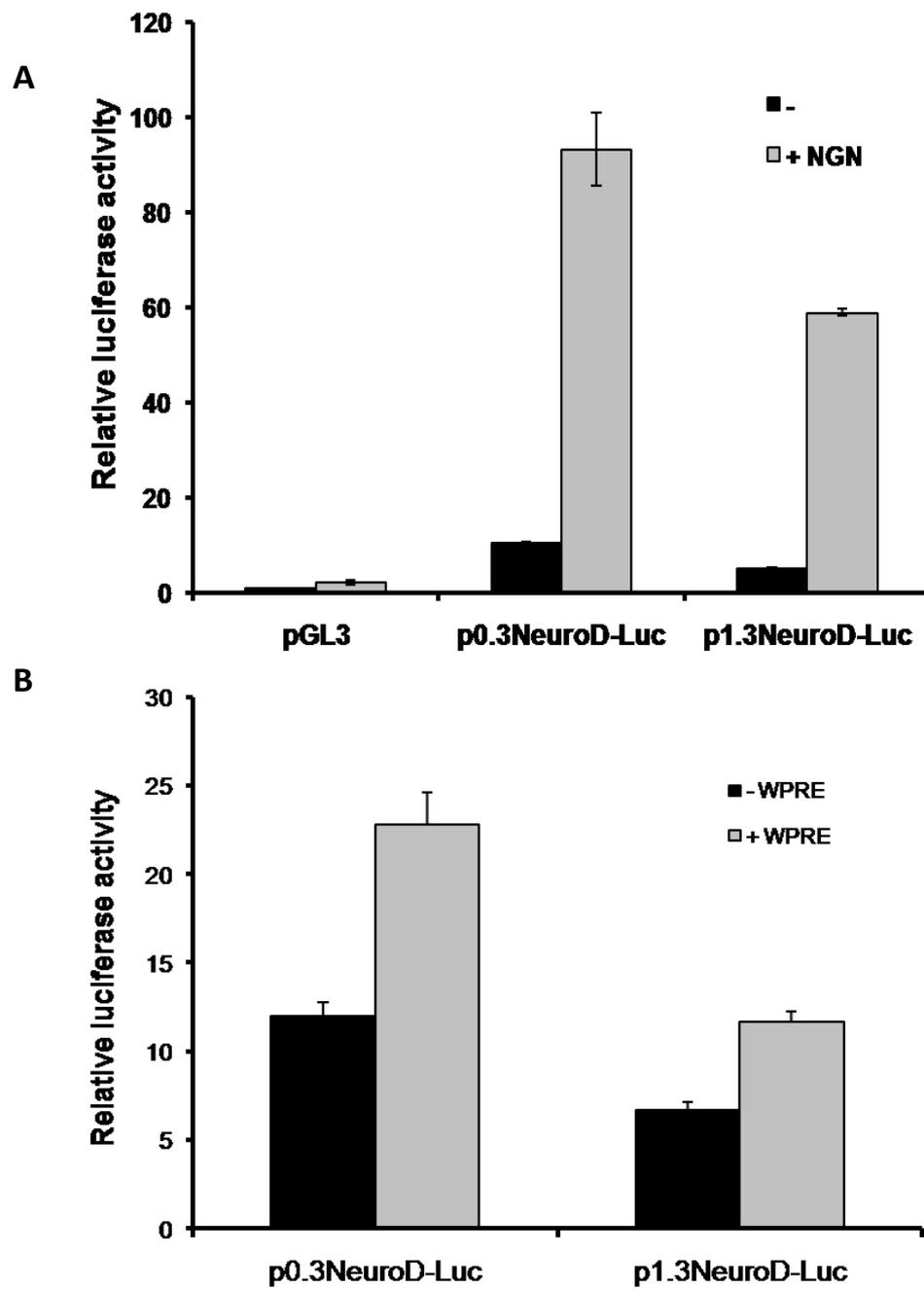


Fig. 3

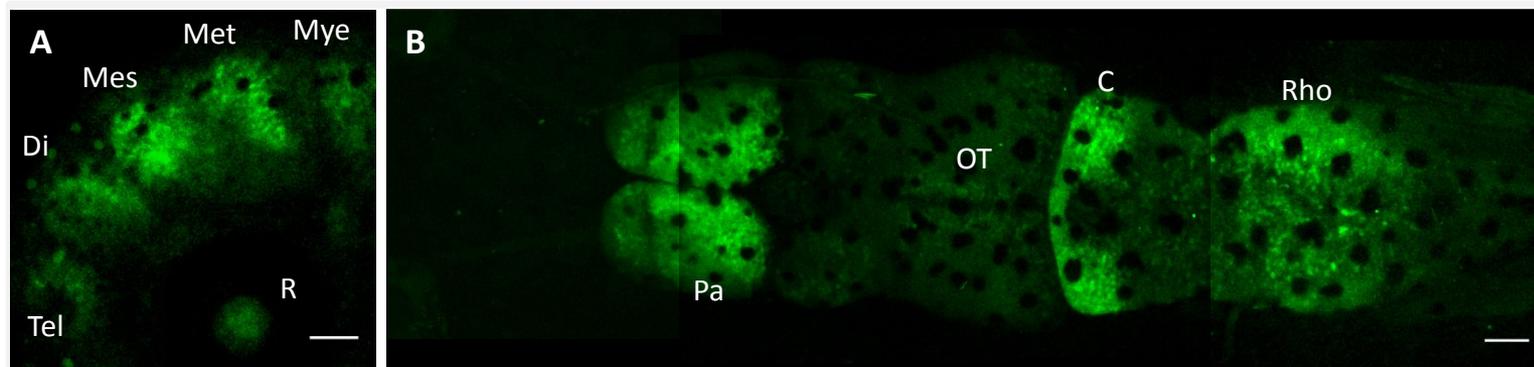


Fig. 4

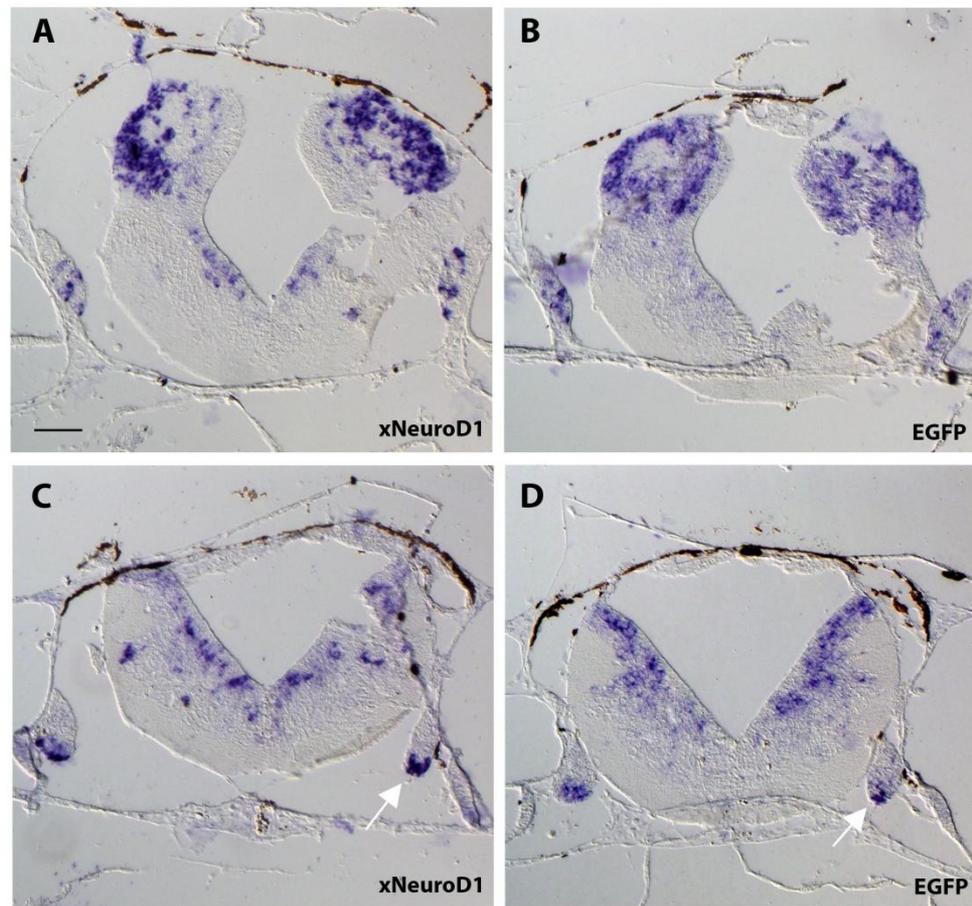
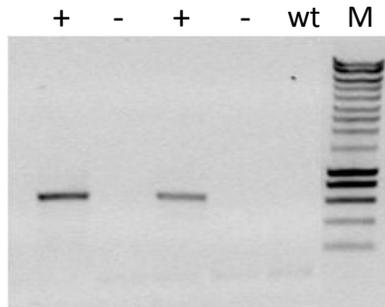


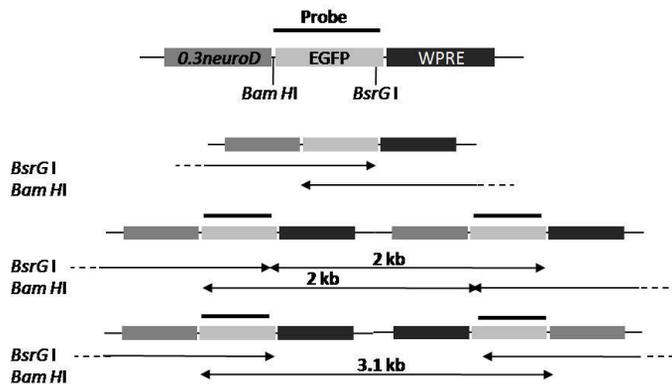
Fig.5

A

0.3neuroD-EGFP-WPRE



B



C

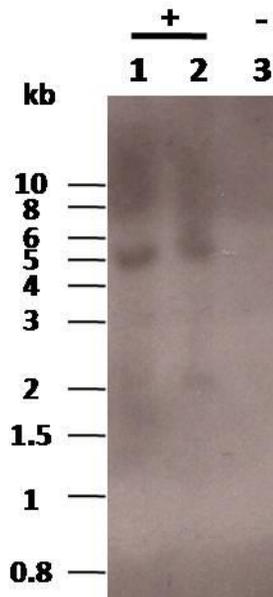


Table 1 : Transgenesis efficiency for the N β Tub-EGFP construct

Plasmid	Injected eggs	surviving embryos at stage 40	% of EGFP expressing embryos at stage 40				
			SNC specific	Hemi body	SNC and other tissues	Ectopic	None
pSce-NbTub-EGFP- Sce	5588	142	29	0.7	26	7	37

Table 2 : Germline transmission of the N β Tub -EGFP transgene

Fo founder	EGFP+ embryos / total F1 embryos	Transmission rate (%)	EGFP+ embryos / total F2 embryos	Transmission rate (%)
pN β Tub-EGFP #1	0/59	0		
pN β Tub-EGFP #2	585/1167	48	255/534	47.53
pN β Tub-EGFP #3	270/529	51		
pN β Tub-EGFP #4	213/365	58		
pN β Tub-EGFP #5	52/98	53		
pN β Tub-EGFP #6	403/790	51		
pN β Tub-EGFP #7	418/589	71		

Table 3 : EGFP fluorescence driven by *xneuroD* promoter fragments at different stages of development

Plasmids	Injected eggs	% of embryos expressing EGFP in the developing nervous system				% of surviving embryos at stage 40
		stage 15	stages 18/20	stage 33	stage 40	
0.3NeuroD-EGFP	2250	0 (0/69)	9 (6/69)	21 (11/52)	18 (6/34)	1.5
0.3NeuroD-EGFP-WPRE	1357	19 (16/83)	35 (29/83)	48 (30/62)	63 (32/51)	3.75
1.3NeuroD-EGFP	1181	0 (0/18)	0 (0/18)	0 (0/16)	0 (0/2)	0.17
1.3NeuroD-EGFP-WPRE	1647	0 (0/39)	0 (0/39)	7 (2/27)	25 (2/8)	0.5

Table 4 : Germline transmission of the 0.3neuroD-EGFP-WPRE transgene

Fo founder	EGFP+ embryos / total F1 embryos	Transmission rate (%)	EGFP+ embryos / total F2 embryos	Transmission rate (%)
p0.3NeuroD-EGFP-WPRE #1	119/310	46		
p0.3NeuroD-EGFP-WPRE #2	115/125	92		
p0.3NeuroD-EGFP-WPRE #3	604/1137	53	96/189	51
p0.3NeuroD-EGFP-WPRE #4	549/1173	47		
p0.3NeuroD-EGFP-WPRE #5	21/49	43		
p0.3NeuroD-EGFP-WPRE #6	42/104	40		