

Zebrafish Enhancer Detection (ZED) Vector: A New Tool to Facilitate Transgenesis and the Functional Analysis of *cis*-Regulatory Regions in Zebrafish

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The identification and characterization of the regulatory activity of genomic sequences is crucial for understanding how the information contained in genomes is translated into cellular function. The *cis*-regulatory sequences control when, where, and how much genes are transcribed and can activate (enhancers) or repress (silencers) gene expression. Here, we describe a novel Tol2 transposon-based vector for assessing enhancer activity in the zebrafish (*Danio rerio*). This Zebrafish Enhancer Detector (ZED) vector harbors several key improvements, among them a sensitive and specific minimal promoter chosen for optimal enhancer activity detection, insulator sequences to shield the minimal promoter from position effects, and a positive control for transgenesis. Additionally, we demonstrate that highly conserved noncoding sequences homologous between humans and zebrafish largely with enhancer activity largely retain their tissue-specific enhancer activity during vertebrate evolution. More strikingly, insulator sequences from mouse and chicken, but not conserved in zebrafish, maintain their insulator capacity when tested in this model. *Developmental Dynamics* 238:2409–2417, 2009. © 2009 Wiley-Liss, Inc.

Key words: enhancer detection; gene regulation; insulators; zebrafish; transgenesis

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INTRODUCTION

At present, considerable efforts are being devoted to understanding transcriptional gene regulation. Although several methods have been developed to identify *cis*-regulatory sequences in vertebrates (Allende et al., 2006; Penacchio et al., 2006), their *in vivo* testing and validation remains a signifi-

cant challenge. To date, most discovery/validation protocols have been carried out in mouse (Visel et al., 2007). However, mouse as a model organism is best suited for small/medium-scale screens. Some of its limitations are overcome by using zebrafish, which allows for rapid, large-scale genomic screening, and has been shown to be a good model for efficient detection of enhancer activity in

vivo (de la Calle-Mustienes et al., 2005; Allende et al., 2006; Navratilova et al., 2009). However, the genetic tools used in zebrafish for enhancer detection are far from optimized.

The commonly used transgenesis vectors consist of a shuttle vector, a minimal promoter and an *in vivo* reporter gene (Nobrega et al., 2003; Penacchio et al., 2006; Woolfe et al.,

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2007; Visel et al., 2008). Yet, this simple design faces two major practical problems. First, the random integration of the shuttle vector in the genome often causes it to be exposed to the enhancer activity present in the surrounding genomic regions, resulting in reporter gene expression that does not result from the DNA sequenced cloned in the vector. This phenomenon is commonly known as “position effect” (Chung et al., 1993). Second, the lack of a positive control of transgenesis makes it difficult to determine the efficiency of the integration events in F0 injected embryos and if a nonexpressing F1 results from the fish carrying an inactive region or no transgene at all.

Here, we have developed a new tool for enhancer detection in zebrafish that avoids these problems: the Zebrafish Enhancer Detection (ZED) vector. ZED is based on the Tol2 Transposon (Kawakami et al., 2000) and introduces several improvements. First, to drive the expression of the reporter gene encoding the enhanced green fluorescent protein (EGFP), we have used a *gata2a* minimal promoter (Ellingsen et al., 2005), which has been selected from a test set of several TATA-containing and TATA-less promoters because of its high efficiency of enhancer activity detection. Second, we have placed a Gateway entry site 5' of the *gata2a* minimal promoter to facilitate the insertion of candidate enhancers in the reporter construct (Hartley et al., 2000; Cheo et al., 2004). Third, the enhancer reporter cassette in ZED is flanked by insulator sequences that we show decrease position effects, thereby increasing the specificity of the signal. We have used two different insulators, the 5'HS4 insulator from the chicken beta-globin gene (Chung et al., 1993; Recillas-Targa et al., 2002) and the GAB insulator element from the mouse tyrosinase gene (Montoliu et al., 1996), which we show here to maintain their insulator function in zebrafish. Also, ZED contains a cardiac actin promoter driving the expression of a red fluorescent protein (DsRed) that serves as control for transgenesis efficiency in vivo in the F0 and the F1 embryos.

Furthermore, ZED contains two excision cassettes, one targeted by Cre-recombinase (loxP sites) and the other

by Flip-recombinase (FRT sites). We show here that these cassettes are functional and allow the genetic deletion, from stable transgenic lines, of both the positive control of transgenesis and the putative enhancer under study. The latter deletion allows one to confirm that the enhancer activity in stable lines depends on the element being assayed. Finally, as a collateral product of this work, we have also generated an efficient vector for rapid evaluation (in F0 injected embryos) of insulator activity in genomic DNA.

RESULTS AND DISCUSSION

Screening for an Optimal Promoter to Test Enhancer Activity

To identify an optimal minimal promoter to detect cis-regulatory activity, we compared the sensitivity to enhancers of different promoters isolated from the zebrafish *gata2a*, *irx3a*, and *rhodopsin* genes, and the human β -globin gene (named herein as: *pgata2*, *pirx3*, *prhodopsin*, and *p β -globin*, respectively). An ideal promoter should be sensitive (i.e., capable of responding efficiently to different enhancers) and specific (i.e., not able to drive any pattern of expression on its own). As test enhancers, we isolated four different sequences from the zebrafish genome that are highly conserved among vertebrates and whose homologous regions have been shown to promote distinct expression patterns in mice (Visel et al., 2007). These sequences are E187, E200, E261, and E298 (<http://enhancer.lbl.gov/>; Visel et al., 2007). Each of these sequences was cloned upstream of the four promoters. EGFP was used as a reporter gene. These constructs were assembled in a Tol2 transposon and injected in 0–1 hours postfertilization (hpf) embryos. More than 100 injected (or F0) embryos for each construct were observed at 24 hpf (Supp. Fig. S1A, which is available online) and embryos with a reproducible EGFP expression pattern were scored. In this assay, we found that, with the four different enhancers, *pgata2* and *pirx3* allowed GFP detection on an average frequency of 35% and 47% of the injected embryos, respectively (Supp. Fig. S1B). These results are consider-

ably higher than the average frequency of expression obtained with the other two promoters tested (Supp. Fig. S1B). In F1s stable lines, *pgata2* transgenic embryos showed very specific expression patterns. This contrasted with the expression patterns observed in *pirx3*-derived stable lines, which consistently drove some expression in the somites and eye (Fig. 1). These results were confirmed, for each construct, with two or more stable lines derived from independent insertion events. Therefore, the highest sensitivity and the good specificity of *pgata2* prompted us to use it as the minimal promoter in the further assembling of the ZED vector. In addition, these experiments also demonstrated that the conserved zebrafish sequences maintain their function as enhancers and they are able to drive expression, to a large extent, in similar anatomical regions than their human counterparts in mouse (Visel et al., 2007; Supp. Table S1).

Implementation of Insulator Sequences to Minimize Position Effects

An important source of noise in enhancer detection assays is the cis-regulatory activity from genomic regions surrounding the transposon insertion site, also known as position effect (Chung et al., 1993). One way to reduce this position effect is to flank the integration cassette with insulators (Montoliu et al., 1996, 2009; Allen and Weeks, 2005). To do this, we have first determined the insulator potential of known sequences in zebrafish. Two insulator sequences were used, the 5'HS4 insulator from the chicken β -globin gene (Chung et al., 1993) and the GAB insulator element from the mouse tyrosinase gene (Montoliu et al., 1996). Although the 5'HS4 insulator has been previously tested in zebrafish (Zhu et al., 2007), no information in this model was available for the GAB insulator element. For that reason, we have developed a new vector to test insulator activity in zebrafish. This vector is Tol2 transposon-based, and contains the zebrafish *irx* Z48 enhancer (also known as Z54390), which promotes strong expression in the midbrain (de la Calle-

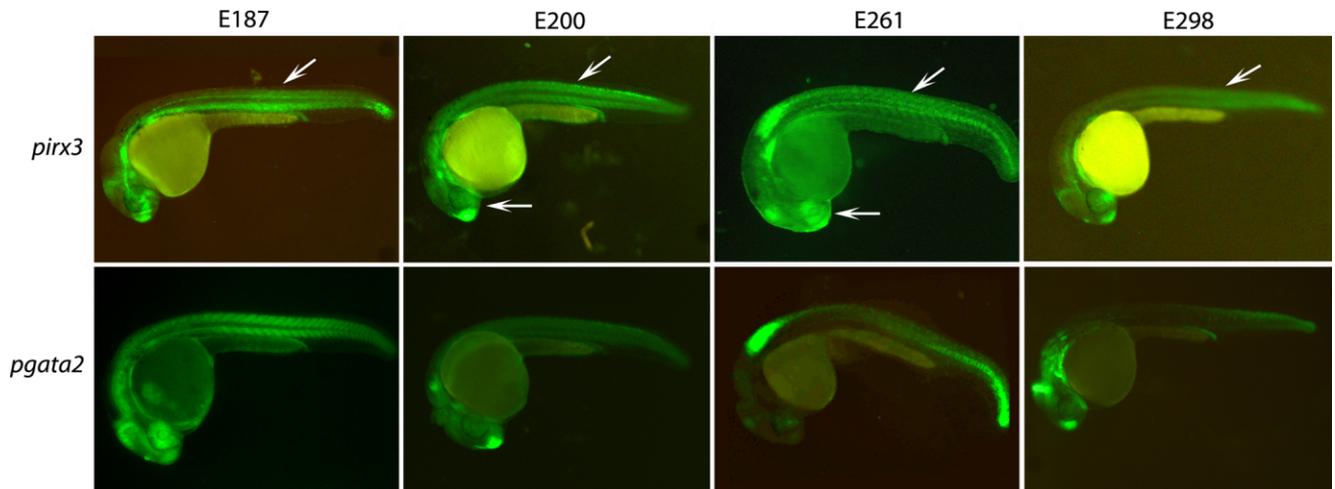


Fig. 1. Capacity of *irx3* and *gata2* promoters to respond to different enhancers. Two or more independent F1 stable transgenic lines were generated with the four tested enhancers (E187, E200, E261, and E298, from left to right columns) positioned 5' of either the *irx3* (upper row) or the *gata2* (lower row) minimal promoters, both driving green fluorescent protein (GFP) expression. Both *irx3* and *gata2* minimal promoters respond efficiently to all four enhancers. However, the *irx3* promoter shows some promoter-specific expression in somites and eye (arrows).

Mustienes et al., 2005), and the cardiac actin promoter isolated from *Xenopus laevis* driving EGFP. This promoter drives strong expression in somites and heart (Mohun et al., 1986; Ryffel et al., 2003). To facilitate the transference of putative insulator sequences to the reporter vector, we introduced a Gateway entry site between the promoter and the enhancer (Hartley et al., 2000; Cheo et al., 2004; Fig. 2). With this vector design, active insulator sequences should decrease the ability of the midbrain enhancer to activate the reporter gene. The GFP expression in the somites, driven by the Cardiac Actin promoter, serves two purposes: (1) as an internal control for transformation efficiency (the more GFP-positive somites, the higher the integration efficiency) and (2) as an internal control for GFP normalization. Thus, if compared with muscle expression in midbrain expression, the GFP activity in midbrain should be reduced in the presence of an insulator (Fig. 2). Using this vector, with both 5'HS4 and GAB cases we detect a decrease (45–65% of the embryos with decreased midbrain enhancer activity, $n > 100$ F0 injected embryos), although not a complete loss, in the levels of midbrain-specific GFP when compared the control, which carries no insulator sequence (Fig. 2). This demonstrates that both insulators function in zebrafish. Interestingly, these sequences are not conserved in the ze-

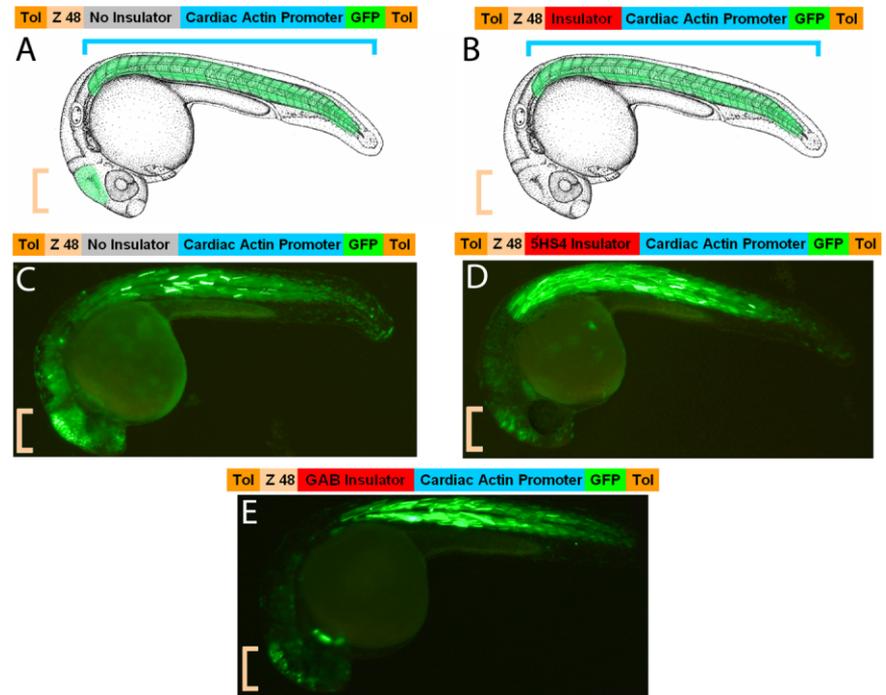


Fig. 2. The mouse “GAB” and the chicken “5’HS4” insulators are functional in zebrafish. We have generated a new Tol2 transposon-based vector to test the insulator potential of candidate sequences. This vector contains a strong midbrain-specific enhancer (Z48) 5' of the Cardiac Actin promoter. **A:** These two components drive GFP expression in the midbrain (Pale orange bracket), due to activity of the Z48 enhancer, and in the somites (pale blue open bracket), due to an endogenous activity of the Cardiac Actin promoter. **B:** If a strong insulator is placed between the enhancer and the promoter, the midbrain expression should be reduced. **C–E:** When we placed either the mouse GAB or the chicken 5’HS4 insulators in this vector, we observe that the ratio between midbrain and muscle expression is decreased (D,E) compared with that observed in controls bearing the Gateway entry sequence of 1.7 kb, which has no insulator activity (C). For each insulator, the reduction of the midbrain enhancer activity was observed in 45–65% of the injected embryos ($n > 100$).

brafish genome. This suggests that, despite of lack of sequence conserva-

tion, the molecular machinery that operates through these insulators is present and functional in this organism. To test the ability of these insu-

lators to reduce the position effects, we injected embryos with a reporter construct consisting in the *pgata2* promoter driving EGFP alone or flanked by 5'HS4 and GAB in the in the 5' and 3' region, respectively. Because these vectors do not harbor any enhancer sequence, we assume that any GFP expression we might observe in the injected embryos is only due to position effects. As expected, when the reporter vector is flanked by insulators, we detect a dramatic decrease in both the number of embryos showing GFP-expressing cells and in the number of cells in each embryo with reporter gene expression (Fig. 3). Using an internal control of transgenesis described below, we demonstrated that this loss of expression is not due to a decrease in the efficiency of integration events (not shown).

Implementing the Cardiac Actin Promoter RFP Cassette as an Internal Control for Transgenesis

Next, to estimate transgenesis efficiency, we introduced the *Xenopus* Cardiac Actin promoter driving the expression of the gene encoding for DsRed (a red fluorescence protein) "outside" of the insulated enhancer reported cassette. The Cardiac Actin promoter activates the expression of DsRed in heart and, very conspicuously, in muscles (Mohun et al., 1986; Ryffel et al., 2003). There are several benefits of this DsRed-expressing internal control. First, it allows selecting among injected embryos those where the transgenesis has occurred efficiently, which correspond to those with broad expression of DsRed in muscles (Fig. 4). Second, this control allows us to identify unambiguously transgenic F1 individuals by observing red fluorescence in heart and muscles, independently of whether the tested sequence is capable of induce EGFP expression or not. Of interest, in both F0 and F1 embryos, strong DsRed expression was only detected at 72 hpf, facilitating the detection of EGFP at earlier stages (not shown). A diagram of the final configuration of the ZED vector is shown in Figure 5A. To validate the ZED vector, the E187, E200, E261, and E298 enhancers as well as Z176 (previously denoted

Z54102 in de la Calle-Mustienes et al., 2005) and Z48 were cloned in the ZED vector, and embryos were injected (Figs. 5B, 6). The F0 embryos showed EGFP expression (30–50% of the injected embryos, $n > 100$) in the expected domains (Fig. 6) and also muscle RFP expression (Fig. 5B). As found with the empty vector, the injected embryos showed reduced background as compared to those injected with the noninsulated *pgata2* constructs (Supp. Fig. S1). We also generate several independent stable lines for the Z176 and Z48 constructs. These lines express EGFP in territories that showed reporter gene activity in the F0 injected embryos (Fig. 6). In addition, in these lines, RFP is detected at 48–72 hpf (insets in Fig. 6).

Flipase- and Cre-Mediated Excision Cassettes

Foreseeing the need to erase the internal control of transgenesis (Cardiac Actin Promoter-DsRed) or to revert possible unpredictable or mutagenic effects generated by the GAB insulator from stable transgenic lines, we have designed a Flipase-mediated recombination cassette by cloning two FRT sequences (Harrison and Perrimon, 1993; Werdien et al., 2001) flanking the Cardiac Actin promoter-DsRed-GAB insulator cassette (Fig. 5A). In the presence of Flipase, a recombination event should result in the deletion of this cassette (Harrison and Perrimon, 1993; Werdien et al., 2001). To test the functionality of these sites, we injected an F1 transgenic stable line with 200 pg of Flipase mRNA. In these injected embryos, the homogeneous muscle-specific expression is transformed into a mosaic DsRed pattern, which confirms that the Flipase/FRT-mediated excision of the cassette can be induced in vivo (Fig. 7A). At the other end of the vector, we have also designed another excision cassette that includes the 5'HS4 insulator and the cloning site for the sequence to test. For that, we flanked this region by Cre recombinase target loxP sites (Fig. 5A). The purpose of this removable cassette is to remove the insulator or to test if the pattern of EGFP expression in stable transgenic lines can be only attributed to the cloned sequence (enhancer), or whether it is

contaminated by position effects. If the GFP pattern were exclusively due to the cloned sequence, Cre-mediated excision should transform a homogeneous tissue-specific EGFP expression into a mosaic pattern. To test this cassette, we injected 100 pg of Cre mRNA in F1 stable transgenic embryos containing the Z48 enhancer-EGFP insertion (Fig. 7B). At 24 hpf, most of the Cre-injected embryos showed, instead of a homogeneous EGFP expression, a mosaic distribution of the fluorescent protein in the domain where this enhancer promotes expression. These results demonstrate that the Cre-mediated excision in stable transgenic lines harboring the ZED vector is very effective and that, as in this particular case, it can be used to determine if the pattern of EGFP expression is indeed driven by the cloned enhancer (Fig. 7B). Because in some cases the simultaneous removal of the insulator could make it difficult to discriminate if the resulting EGFP expression depends on the excision of the enhancer or on the position effect becoming now evident after elimination of the insulator, Cre-mediated enhancer removal should be done in more than one independent stable line. It is very unlikely that two independent genomic landscapes promote reporter expression in the same domain as the enhancer under evaluation.

In this study, we have discussed the need to improve the transgenic vectors for enhancer assays in zebrafish. This need arises from the fact that current standard methods are not able to cope with common problems associated with this kind of assay, such as the noise generated by position effect and the ambiguity in the detection of the transgenic events. Here, we have presented an improved vector for functional enhancer assays in transgenic experiments in zebrafish, the ZED vector. Several features make the ZED vector an optimal tool for assessing enhancer activity of candidate sequences: (1) it detects enhancer activity efficiently; (2) it shows reduced sensitivity to the position effect due to the presence of insulators flanking the EGFP expression cassette; (3) it also allows investigators to easily evaluate the efficiency of transgenesis by direct in vivo visualization of a DsRed-positive control cassette in F0 injected em-

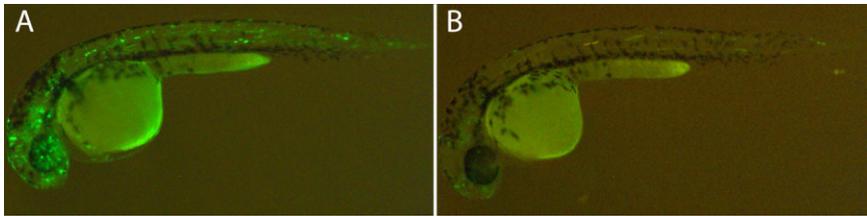


Fig. 3. GAB and 5'HS4 insulators decrease the position effect. **A:** F0 embryos injected with a Tol2 transposon containing the *gata2* minimal promoter driving green fluorescent protein (GFP). The GFP background expression is due to position effect in independent random insertions and was observed in a large number of cells in 40% of the injected embryos ($n > 100$). **B:** Most of this background is lost in embryos injected with similar construct flanked GAB and 5'HS4 insulators, as we observed GFP expression in only 16% of the injected embryos ($n > 100$) and in a much reduced number of cells.

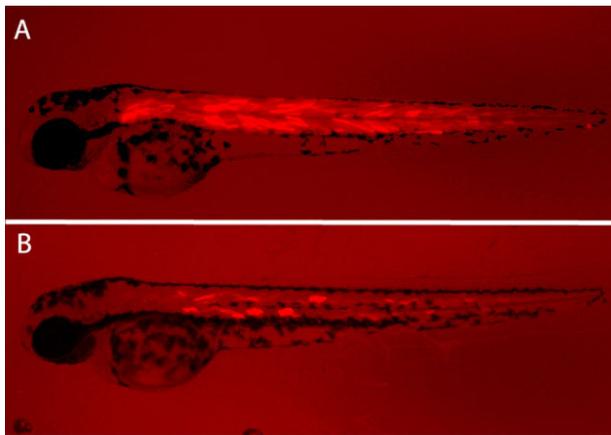


Fig. 4. The Cardiac Actin-RFP cassette is effective as an internal control of transgenesis. The Cardiac Actin-RFP cassette drives expression of RFP in the somites. **A:** The broad RFP expression in the muscles of this embryo indicates high efficiency of integration. **B:** In this embryo, the transgenesis procedure was less effective. Thus, the possibility of germ line integrations is reduced.

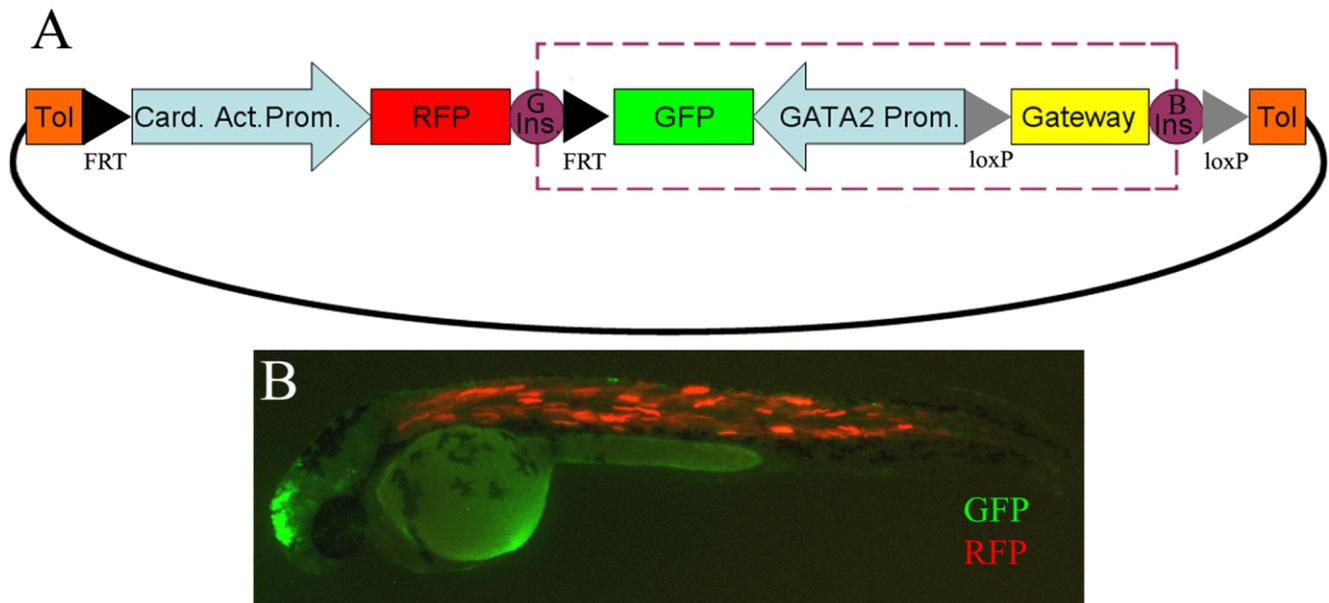
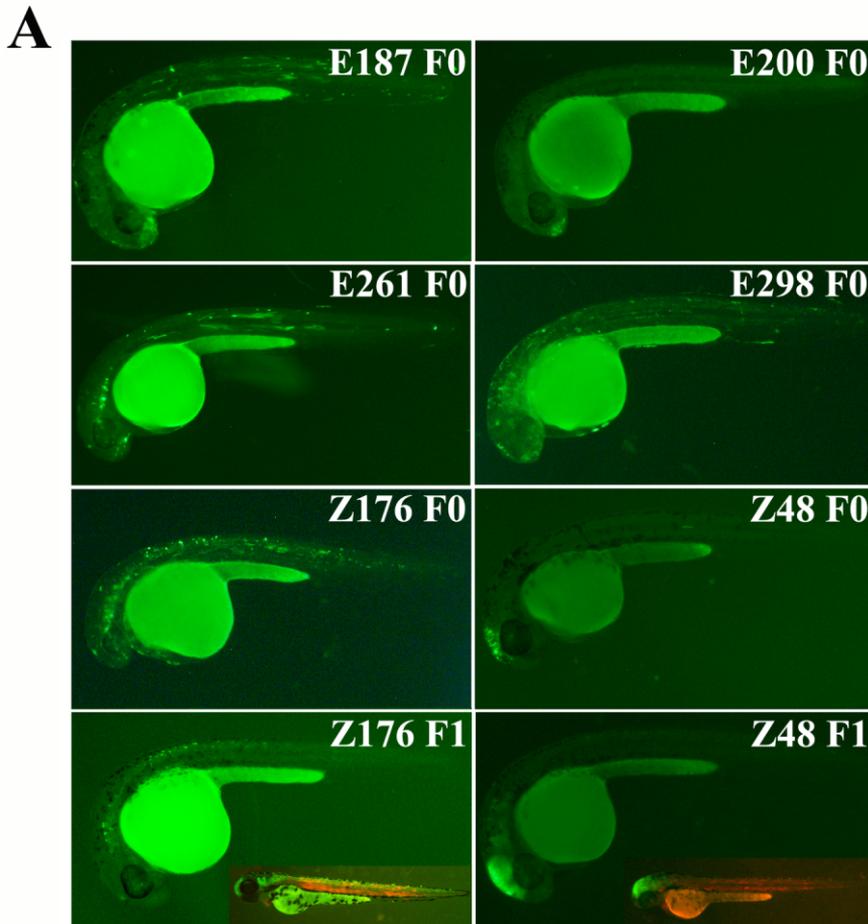


Fig. 5. **A:** Diagram of the Zebrafish Enhancer Detection (ZED) vector. Orange boxes are the Tol2 transposase recognition sequences. This vector is composed of two different cassettes. The transgenesis internal control cassette is composed of the Cardiac Actin promoter (pale blue arrow), and red fluorescent protein (RFP; red block). The enhancer detection cassette contains a Gateway entry site, represented by a yellow box, the *gata2* minimal promoter, shown in pale blue arrow, and the enhanced green fluorescent protein (EGFP) reporter gene, marked with a Green box. This enhancer detection cassette is flanked by two Insulator sequences represented by Violet circles that protect the enhancer detection cassette (dashed purple box) from position effects. The left insulator corresponds to the GAB insulator (G Ins) from the mouse tyrosinase gene and the right to the 5'HS4 insulator (B Ins) from the chicken β -*globin* gene. Additionally, two excision cassettes are also present. One is mediated by Flipase (black triangles), and the other by Cre recombinase (gray triangles). **B:** F0 injected embryos with the ZED vector containing the Z48 enhancer at 48 hours postfertilization (hpf) show both GFP expression in the midbrain (Green) and RFP expression in the somites (Red) in 70% of the injected embryos ($n > 150$).

bryos and F1 stable transgenic lines; (4) the implementation of recombination cassettes mediated by Flip and Cre recombinases allow, if desired, the deletion of this positive control of transgenesis and/or the candidate enhancer under evaluation. This latter deletion permits to validate its enhancer activity by direct injecting Cre mRNA in stable transgenic lines and evaluating its effects in a matter of days. Despite the fact that it has been designed for a very specific application, we propose that its basic architecture, comprising the use of insulators to reduce the position effect and a positive control for transgenesis, can be broadly applied as an improved method of transgenesis in zebrafish. Finally, as an important byproduct of this work, we have also generated a new vector for easy detection of insulator sequences whose application will be described in more detail elsewhere. These two tools will facilitate the identification of positive as well as negative *cis*-regulatory elements essential for gene regulation and genome organization.



B

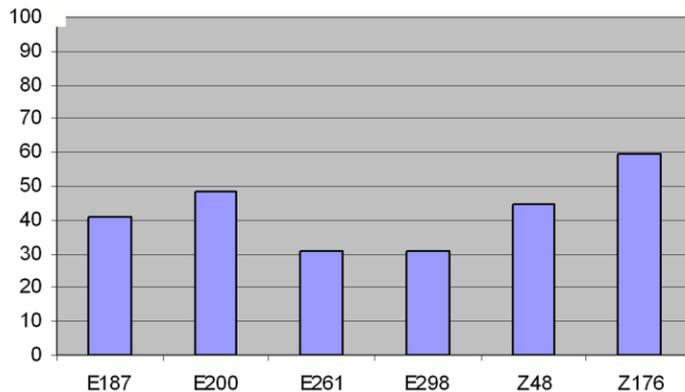


Fig. 6. The Zebrafish Enhancer Detection (ZED) vector effectively detects enhancer activity. For each construct, the number of injected embryos was higher than 100. **A:** Green fluorescent protein (GFP) expression in 24 hours postfertilization (hpf) mosaic F0 embryos injected with the ZED vector harboring the E187, E200, E261, E298, Z176 and Z48 enhancers and F1 embryos derived from the Z176 and Z48 constructs. Insets show GFP and red fluorescent protein (RFP) expression in these two last F1 stable lines at 48 hpf. Note the reduced background in these injected embryos as compared to those injected with the noninsulated *pgata2* constructs shown in Supp. Fig. S1. For the F1 lines, we obtained more than two independent insertions that showed the same pattern. **B:** Graphic representation of the percentage of embryos showing GFP expression in the expected territories. The reduced background observed with the ZED constructs did not compromise the enhancer detection potential of this vector as the GFP activity was detected in 30–50% of the injected embryos ($n > 100$). These percentages are similar to that observed in embryos injected with the noninsulated *pgata2* constructs containing the same enhancers (Supp. Fig. S1).

EXPERIMENTAL PROCEDURES

pgata2, *pirx3*, *prhodopsin*, and *p β -globin* Vectors

The vectors containing the different promoters were built cloning the specific minimal promoter into the *Xho*I and *Bam*HI sites 5' of EGFP in the T2KHG Tol2 vector (Kawakami et al., 2004). In each vector, a Gateway cassette (Invitrogen, Cassette C1 from Gateway Vector Conversion System) was cloned in blunt in the *Xho*I site. The *pgata2* minimal promoter (Meng et al., 1997; Perz-Edwards et al., 2001) was directly excised with *Xho*I and *Bam*HI from the vector pCLGY (Ellingsen et al., 2005). The *pirx3* minimal promoter (de la Calle-Mustienes et al., 2005) was PCR amplified from zebrafish genomic DNA using the primers 5'-CTGCAGAGGACCTCGACTGGC-3' and 5'-GGATCCGCCCTCTTGTTGC-3', which contain a *Pst*I and a *Bam*HI site, respectively (underlined). This PCR fragment was first cloned in pBluescript and then extracted with *Xho*I and *Bam*HI and transferred to the Tol2 vector. *prhodopsin* was PCR amplified from zebrafish genomic DNA using the primers 5'-CTCGAGAAAGTTCTTATTATCTACCTGCTGCTCAG-3' and 5'-GGATCCGGTTGGATGTGGCGCTC-3' that contain a *Xho*I and a *Bam*HI site, respectively (underlined). *p β -globin* was directly excised with *Xho*I and *Bam*HI from the p1230 vector (Manzanares et al., 2000). The genomic coordinates of these promoters and their distance from the first methionine of the transcript (A from the first ATG is considered position 0) are: *pgata2* (-1031 to -1 from ATG), chr11: 2,551,069-2,552,099 (danRer5); *pirx3* (-746 to -41 from ATG), chr7: 27,485,244-27,485,945 (danRer5); *prhodopsin* (-287 to -5 from ATG) chr7: 2,535,681-2,535,962 (danRer5); *p β -globin* (-90 to -38 from ATG), chr11: 5,204,866-5,204,917 (hg18).

The enhancers used to test these vectors were PCR amplified from zebrafish genomic DNA, cloned in TOPO vector (Invitrogen, pCR $\text{\textcircled{R}}$ 8/GW/TOPO $\text{\textcircled{R}}$ TA cloning KIT) and then recombined to each vector by the Gateway in vitro recombination system (Invitrogen, Gateway LR clonase II Enzyme mix).

We used the following primers to amplify these enhancers: E187Forward: 5'-TTTGCAAAACATTTTTGACTG-3'; E187Reverse: 5'-GATCAGCCATTGT-CACCTCCCAG-3'; E200Forward: 5'-TCTGTGGCATTCTCTTCGGCC-3'; E200Reverse: 5'-TGGCATCTCGCA-CACAAAAGC-3'; E261Forward: 5'-GCAATTTTGCCAATGGTTTCC-3'; E261Reverse: 5'-CCAAAGCCT-TCAAATGCCTCGC-3'; E298Forward: 5'-GCGTGGTTCGCATTAAGCAGT-3'; E298Reverse: 5'-CGCTGTGTACAATC-CGAGCCG-3'. The genomic coordinates of these enhancers in the zebrafish genome (danRer5) are as follows: E187: chr6:30,560,951-30,562,277; E200: chr8:13,856,596-13,858,411; E261: chr16:15,190,519-15,192,608; E298: chr19:38,244,824-38,246,847; E48: chr7:27,681,739-27,682,324; E176: chr7:27,497,995-27,501,393.

We have selected EGFP as the reporter gene for enhancer detection in our vectors because, in our hands, it gives stronger and earlier signal than DsRed.

Insulator Vectors

This vector was generated by first cloning a fragment containing the Cardiac Actin promoter driving GFP from the pCARGFP vector (Kroll and Amaya, 1996) into the *XbaI/NotI* restriction sites of the pminiTol2/MCS vector (Balciunas et al., 2006) to generate the pminiTol2-CARGFP. The Z48 enhancer was then PCR amplified from zebrafish DNA using the primers 5'-GGTCTAGAGCTCTCGCAGTTGTGGGC-3' and 5'-CCTCTAGAGGTACCCCCCTGCTTAAGACACAG-3', which contain a *XbaI* restriction sites in the forward primer and a *XbaI* and *KpnI* restriction sites in the reverse one (underlined). This PCR amplified DNA was cloned in the *XbaI* restriction site of pminiTol2-CARGFP to generate pminiTol2-Z48-CARGFP. Clones were selected having the *KpnI* site immediately near 5' the Cardiac Actin GFP insert. The gateway cassette (Invitrogen, Cassette B from Gateway Vector Conversion System) was cloned in blunt into the *KpnI* restriction site generating the insulator vector pminiTol2-

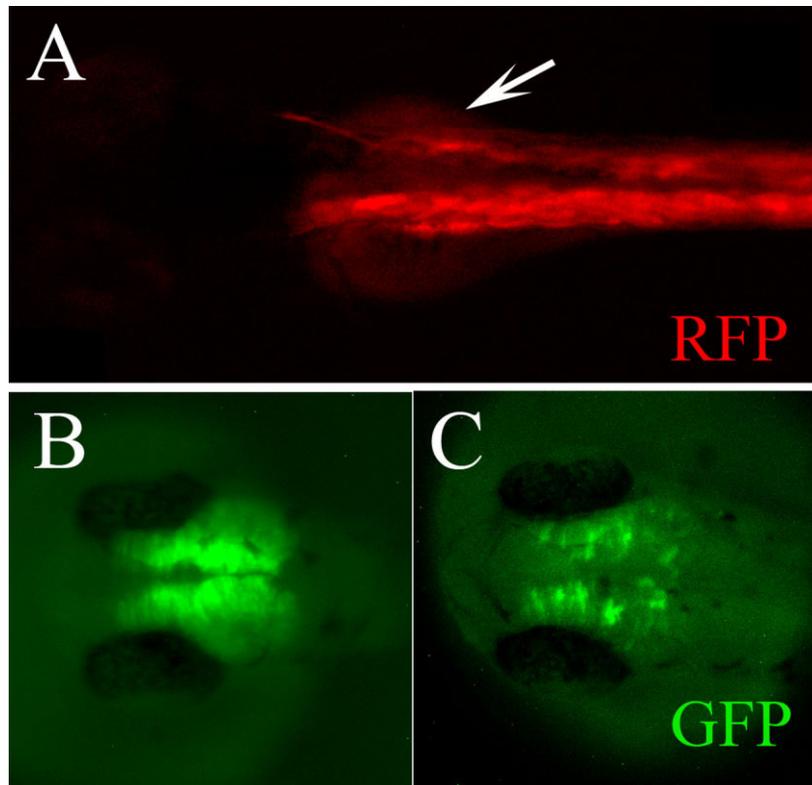


Fig. 7. In vivo excision of the internal control and enhancer detection cassettes of the Zebrafish Enhancer Detection (ZED) vector using Flipase and Cre recombinases. **A:** ZED F1 stable transgenic embryos showing strong red fluorescent protein (RFP) expression in the muscles were injected with Flipase mRNA. This injection caused reduction of RFP expression in some cells of the right side of the embryo (arrow) but not in the left side. RFP reduction upon Flipase mRNA injection was observed in 83% of the injected embryos ($n > 200$). **B:** Embryos from a F1 Z48-ZED stable transgenic line. **C:** Injection of Cre mRNA in these stable transgenic embryos from this line caused mosaic loss of GFP expression. The transition from the homogeneous expression promoted by stable insertion to a mosaic pattern due to Cre-mediated recombination was observed in 85% of the injected embryos harboring the transgene ($n > 200$).

Z48-Gw-CARGFP. Two copies of the β -globin 5' HS4 250-pb insulator (Reccillas-Targa et al., 2002), previously cloned in pBluescript, were amplified with the T3 and T7 primers. The PCR fragment was cloned in TOPO vector (Invitrogen, pCR@8/GW/TOPO® TA cloning KIT) and transferred to the insulator test vector by in vitro recombination (Invitrogen, Gateway LR clonease II Enzyme mix). The GAB insulator element was excised as a 0.57-kb *HindIII/EcoRV* DNA fragment from plasmid pGAB (A. Garcia-Diaz and L. Montoliu, unpublished), carrying a fusion of the G and AB boundary elements previously described within the locus control region (LCR) of the mouse tyrosinase gene (Giraldo et al., 2003). The fragment was cloned blunt into the *KpnI* site of the pminiTol2-Z48-CARGFP plasmid.

The ZED Vector

The 5'HS4 insulator was PCR amplified from the CMV-*gfp* reporter plasmid (Allen and Weeks, 2005) using the primers 5'-ggccagatgggccATA-ACTTCGTATAATGTATGCTATAC-GAAGTTATGAGTTGCGCGCCTGG-GAGC-3' and 5'-ggccagatgggccATA-ACTTCGTATAATGTATGCTATAC-GAAGTTATGAGTTGCGCGCCTGG-GAGC-3'. The forward primer contains an *SfiI* site (underlined and small letters) and *LoxP* recognition site (underlined and capital letters). The reverse primer contains a *NdeI* site (underlined and small letters), a *LoxP* recognition site (underlined and capital letters) and a *XhoI* site (underlined and small letters). This fragment was cloned in the *SfiI/XhoI* sites 5' of the *pgata2*-EGFP-polyA T2KHG plasmid to generate the *LoxP*-5'HS-

LoxP-*pgata2*-EGFP-polyA T2KHG. The Gateway B cassette was cloned blunt into the *NdeI* site. This generated the LoxP-5'HS-Gw-LoxP-*pgata2*-EGFP-polyA T2KHG plasmid. We then introduced a *PacI* and an *SfiI* sites into the pminiTol2/MCS plasmid. To that end, we delete from the LoxP-5'HS-LoxP-*pgata2*-EGFP-polyA T2KHG plasmid a DNA fragment between *NcoI* and *SacI* sites, which includes the LoxP-5'HS-LoxP-*pgata2*-EGFP-polyA cassette, and we religated the remaining vector. From the resulting truncated vector, we excised a 417-pb *HindIII/BglII* fragment that we cloned in the pminiTol2/MCS plasmid. This fragment contains a *PacI* and a *SfiI* sites. We then transferred to these sites the LoxP-5'HS-LoxP-*pgata2*-EGFP-polyA cassette excised with *PacI* and *SfiI* to generate the pminiTol2-LoxP-5'HS-LoxP-*pgata2*-EGFP-polyA plasmid. To introduce the positive control to this vector, we first excised the Cardiac Actin-DsRed-polyA cassette from the ISCarDsR2/Pax6GFP3 vector (gift from H. Ogino and R. Grainger) and cloned into the *XbaI/BamHI* sites of pBluescript KS+ vector. In this plasmid, we then introduced the GAB insulator from the pGAB vector (Giraldo et al., 2003) into the *XhoI/KpnI* sites. We then amplified the GAB insulator-Cardiac Actin-DsRed-polyA fragment with the following primers: 5'ttaattaaTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCCTCACTATAGGGC-GAATTGG-3' and 5'taattaaGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCACGCAATTAACCCTCAC-TAAAGG-3'. These primers contain a *PacI* restriction site (underlined and small letter) and an FRT sequence (underlined and capital letters). This PCR fragment was then transferred to the *PacI* site of pminiTol2-5'HS4-Gw-*pgata2*-EGFP-polyA plasmid to generate the ZED vector.

In Vitro mRNA Synthesis, Microinjection, and Transgenesis

cDNAs were linearized and mRNAs were transcribed as described (Bessa et al., 2008), and 100–200 pg of each mRNA was injected in one-cell stage embryos. Transposase (Kawakami et al., 2004), Cre (Langenau et al., 2005),

and Flipase (Werdien et al., 2001) cDNAs were linearized using *NotI* restriction enzyme, and mRNA was synthesized using Sp6 RNA polymerase.

The Tol2 transposon/transposase method of transgenesis (Kawakami et al., 2004) was used with minor modifications. One nanoliter was injected in the cell of one-cell stage embryos containing 50 ng/μl of transposase mRNA, 40 ng/μl of phenol/chloroform purified DNA, and 0.05% phenol red.

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