Resource

A method for using direct injection of plasmid DNA to study cis-regulatory element activity in F₀ Xenopus embryos and tadpoles

Chen Wang, Ben G. Szaro

Department of Biological Sciences and the Center for Neuroscience Research, University at Albany, State University of New York, 1400 Washington Avenue, Albany, NY 12222, USA

Abstract

The ability to express exogenous reporter genes in intact, externally developing embryos, such as Xenopus, is a powerful tool for characterizing the activity of cis-regulatory gene elements during development. Although methods exist for generating transgenic Xenopus lines, more simplified methods for use with F₀ animals would significantly speed the characterization of these elements. We discovered that injecting 2-cell stage embryos with a plasmid bearing a dQC31 integrase-targeted attB element and two dual β-globin HS4 insulators flanking a reporter transgene in opposite orientations relative to each other yielded persistent expression with sufficiently high penetrance for characterizing the activity of the promoter without having to coinject integrase RNA. Expression began appropriately during development and persisted into swimming tadpole stages without perturbing the expression of the cognate endogenous gene. Coinjected plasmids having the same elements but expressing different reporter proteins were reliably coexpressed within the same cells, providing a useful control for variations in injections between animals. To overcome the high propensity of these plasmids to undergo recombination, we developed a method for generating them using conventional cloning methods and DH5α cells for propagation. We conclude that this method offers a convenient and reliable way to evaluate the activity of cis-regulatory gene elements in the intact F₀ embryo.

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Introduction

Embryonic development requires coordinating expression of genes whose products direct the fate and define the phenotype of differentiating cells. A crucial component of defining such transcriptional networks is identifying and characterizing the activities of the cis-regulatory elements that bind the transcription factors controlling this expression (Howard and Davidson, 2004). This is best done within the intact embryo, which maintains both the lineage of and intercellular interactions among cells as they progress through development. Thus, characterizing the activity of cis-elements controlling gene expression during development requires introducing synthetic reporter constructs into embryos. Because of the inherent difficulties associated with testing synthetic constructs in mammalian embryos, which develop internally, serious efforts have been made to develop methods for studying cis-regulatory activity in embryos of externally developing vertebrates such as Xenopus. The earliest method used injection of plasmid DNAs directly into fertilized embryos. Although simple to perform, this method yielded mosaic, largely ectopic expression that was short-lived, due to loss of the DNA within the first few days of development (Rusconi and Schaffner, 1981; Andres et al., 1984; Etkin et al., 1984; Bendig and Williams, 1984; Etkin and Pearman, 1987; Mayor et al., 1993). To circumvent these shortcomings, a number of methods have since been developed for achieving stable transgenesis in Xenopus, including but not limited to, Restriction Enzyme-Mediated Integration (REMI) of DNA (Kroll and Amaya, 1996; Amaya and Kroll, 1999; Sparrow et al., 2000), transposon-mediated transgenesis (Hamlet et al., 2006; Sinzelle et al., 2006; Vergeau et al., 2009), and I-SceI meganuclease-mediated transgenesis (Ogino et al., 2006; Pan et al., 2006; Ishibashi et al., 2012). Although more involved than simply injecting plasmids into embryos, these efforts have proved enormously successful for studying cis-regulatory elements, as well as targeting expression of altered genes to specific cell types, especially in F₁ animals.

The desire to assess cis-element activities more rapidly and to avoid maintaining transgenic lines has continued to fuel efforts to develop simpler methods for use with F₀ animals [e.g., Bacterial Artificial Chromosome (BAC) injection (Fish et al., 2012)]. Because stable transgenesis is unnecessary for addressing many experimental questions, the goal with these methods is rather to obtain expression that reveals the activity of cis-elements with greater fidelity and for longer periods in development than is achievable.
with simple plasmid injections. One variation is to introduce into the injected plasmid elements that promote integration into the genome and protect the transgene’s own regulatory elements from endogenous enhancers acting at the insertion site. One such method coinjects into fertilized eggs a plasmid containing a prokaryotic attB site together with RNA encoding phage φC31 integrase, which leads to the plasmid’s incorporation into pseudo-attP sites within the genome (Allen and Weeks, 2005). Due to the widespread existence of such sites in multiple organisms, this technique has also worked well in cells and embryos ranging from mammals (Tsaygarjan et al., 2001; Olivares et al., 2002; Hollis et al., 2003) to fruit flies (Bateman et al., 2006). Flanking the transgene with dual β-globin Hypersensitive Site 4 (HS4) insulator elements from chicken effectively minimizes the influence of endogenous regulatory elements (Chung et al., 1993; Pikaart et al., 1998; West et al., 2002; Allen and Weeks, 2005; Love et al., 2011). However, the tendency of the flanking dual insulators to stimulate recombination during cloning (Allen and Weeks, 2006) has proved challenging when multiple constructs need to be generated. Since inverting the orientation of one of the insulators relative to the other further exacerbates this tendency (Sekkali et al., 2008), they are typically oriented in the same direction (i.e., 5’ to 3’). Yet, because insulators have direction-dependent as well as –independent effects (Steinhaerder and Lieber, 2000; West et al., 2002; Yannaki et al., 2002), orienting them differently than in earlier studies, in which both were pointed in the same direction as in the chicken genome (Allen and Weeks, 2005; Sekkali et al., 2008), might be expected to exhibit novel behaviors in injected embryos.

Initially motivated by trying to overcome recombination while generating our own plasmids with attB and HS4 elements to study cis-elements of the Xenopus medium neurofilament (NF-M) gene, we discovered that injection of a plasmid having its flanking dual insulators both pointing inward toward the transgene yielded robust reporter expression that was characteristic of the promoter (i.e., “promoter-typical”) and persisted well into early feeding tadpole stages, even when no integrase RNA was injected. To overcome the problem of recombination, we developed a two-step cloning strategy that enabled us to more reliably engineer multiple such plasmids using standard cloning methods and a cloning strategy, which enabled us to more reliably engineer.

**Construction of initial plasmids with Xenopus NF-M promoters**

To generate pSPORT1[attB/Ins1](1.5 kb) NF-M promoter/glGFP/β-globin 3’-UTR/Ins2, a fragment spanning the 1.5 kb Xenopus NF-M promoter (gene symbol nefm), Green Lantern FLP (glGFP) extracted from pGreenlantern-1, GigoBCL(R), and a rabbit β-globin 3’-UTR (originally from Richard Harland, UC Berkeley) was first cut from pNF-M(2)URS-GFP (Roosa et al., 2000) with KpnI[5’ end; New England Biolabs (NEB) and EcoRV (3’ end; NEB) and directionally cloned into the corresponding sites of pSPORT1 (Invitrogen). Next, single HS4 insulator core (250 bp) elements (Chung et al., 1999, 1997) were isolated by two separate polymerase chain reactions (PCR; GoTaq Green, Promega) from chicken genomic DNA prepared from commercially available muscle tissue (Davis et al., 1994) using the following primer pairs [Integrated DNA Technologies (IDT); see Supplementary Table 1 for primer sequences]: 1) Snabl-5’HS4-forward, XbaI-3’HS4-reverse; 2) XbaI-5’HS4-forward, SpeI-3’HS4-reverse. A dual insulator (Ins1 comprising two 250 bp HS4 core elements linked in tandem through the intervening XbaI site was created via sequential directional cloning of the PCR product between the Snabl/XbaI and XbaI/SpeI sites within the pSPORTI sequences of the plasmid. This inserted dual insulator was then re-amplified with BstEII-5’HS4-forward and ApaI-3’HS4-reverse primers (Supplementary Table 1) for insertion into the BstEII and ApaI sites of the pSPORT1 vector, downstream of the β-globin 3’-UTR element, to yield a second, flanking dual insulator (Ins2) of opposite orientation relative to Ins1 and pointing inward toward the transgene. Finally, the attB element was generated by annealing two single-stranded oligonucleotides (Supplementary Table 1) to produce a double-stranded attB element with AarI-compatible overhangs, which was subsequently ligated into the corresponding pSPORT1 site.

pSPORT1[attB/Ins1](1.5 kb) NF-M promoter/DSRed2/[β-globin 3’-UTR/Ins2] was generated from the previous plasmid by replacing glGFP (flanked by Notl sites) with DSRed2, obtained by PCR (GoTaq Green, Promega) from DSRed2-C1 (Clontech; see Supplementary Table 1 for primer sequences). To generate pSPORT1[attB/Ins1/(0.4 kb) NF-M promoter/DSRed2/[β-globin 3’-UTR/Ins2], KpnI was first used to release the 1.5 kb NF-M promoter along with DSRed2 from the previous plasmid. PCR was then used to amplify from this fragment the sequence extending from 0.4 kb upstream of the transcription initiation site through the end of DSRed2 (see Supplementary Table 1 for primer sequences). After digestion with KpnI to create compatible overhangs, the PCR product was ligated back into the original KpnI-digested, calf intestinal alkaline phosphatase-treated vector.

**Two-step cloning strategy for plasmids bearing the NfT and CarA promoters**

Because of the difficulty encountered in engineering plasmids bearing dual flanking, inverted HS4 insulators, subsequent plasmids were constructed using a two-step cloning strategy (Fig. 1). Briefly, an intermediate cloning vector (pBluescript[attB/Ins1]) was used to place the promoter of interest downstream of the attB and Ins1 elements. This intermediate vector was created by directionally cloning a fragment spanning a small piece of pSPORT1, along with the downstream attB and Ins1 elements, into the ClaI and SpeI sites of pBlueScript II SK (+) (Agilent Technologies). (See Supplementary Table 1 for the primers used in PCR to isolate the attB and Ins1 elements from pSPORT1[attB/Ins1/(1.5 kb) NF-M promoter/glGFP/β-globin 3’-UTR/Ins2].) The new promoter of interest was then directionally cloned into the appropriate restriction sites within the multiple cloning site of...
this intermediate vector (e.g., SpeI and NotI, in the case of the CarA and NβT promoters described below). A second host vector (pSPORT1[Nhel/gGFP/β-globin 3′-UTR/Ins2]) was used for inserting the elements from the new promoter-bearing plasmid to create the final construct used for injection. (See Supplementary Table 1 for the primers used to introduce a Nhel site into pSPORT1[attB/Ins1/New Promoter] or pBluescript[attB/Ins1/New Promoter] (in this case NβT or CarA); see Supplementary Table 1 for primer sequences) from their parent plasmids [gifts of Enrique Amaya, University of Manchester; sequences derived from those of pTransgenesis plasmids available from the European Xenopus Resource Centre, University of Portsmouth, UK (Love et al., 2011)] and then directionally cloned into the SpeI and NotI sites of the intermediate vector. Fragments spanning the attB site, Ins1, and promoter of interest were then isolated by XL-PCR (Applied Biosystems) from the resulting plasmid (pBluescript[attB/Ins1/New Promoter] (in this case NβT or CarA); see Supplementary Table 1 for primer sequences) to add Nhel sites for subsequent cloning into the host vector. After final propagation in DH5α cells, all plasmids used for injection were sequenced (GENEWIZ) to confirm their fidelity.

Immunohistochemistry

For immunohistochemistry, MS222-anesthetized embryos and tadpoles were fixed overnight in 4% paraformaldehyde/2% sucrose in 0.1 M sodium phosphate buffer (pH 7.4; PB). For whole mount immunostaining, embryos were post-fixed in Dent’s fixative and processed as described (Dent et al., 1989; Szaro et al., 1991). For staining of sections on slides, tissues were first cryoprotected in 30% sucrose/PB, then embedded and frozen in Tissue Freezing Medium (Triangle Biomedical Sciences) for cutting of 20 µm transverse sections and immunostaining as described (Hutchins and Szaro, 2013). Primary antibodies were directed against: 1) Green Lantern GFP [Anti-GFP (Goat), Rockland; used at 1:500]; 2) DsRed2 [Living Colors DsRed Polyclonal Antibody (Rabbit), Clontech; used at 1:500]; 3) a Xenopus neuronal class I β-tubulin (NβT) isotype [clone JDR. 38B, Sigma-Aldrich (Moody et al., 1996); used at 1:100]; and 4) Xenopus NF-M [clone RM0270 (Szaro et al., 1989; Wetzel et al., 1989; Liu et al., 2008); used at 2 µg/ml]. Species-appropriate Alexa Fluor 488 or 546 antibodies (Life Technologies, used at 1:1000) were used as secondary antibodies, as noted.

Microscopy

DsRed2 and GFP expressions viewed directly, without immunostaining, were imaged in live embryos and tadpoles through either a Leica MZ16 FA fluorescence stereo microscope with a DFC 340 FX camera (monochrome) or an Olympus SZX12 fluorescence stereo microscope with a Nikon DSR1 camera (color) running NISElements D software version 3.10, as specified. Fixed, immunostained, and cleared (benzyl alcohol: benzyl benzoate, 1:2) embryos and tadpoles in whole mount were imaged through either the Olympus SZX12 fluorescence stereo (above) or a Zeiss LSM 510 confocal laser scanning microscope (CLSM; 10 × FLuar, N.A., 0.5), as specified. Immunostained sections of stages (st.) 43–46 tadpoles were imaged on a Zeiss LSM 510 CLSM (20 × Plan-APOCHROMAT, N.A., 0.75), and sections of st. 55–56 tadpoles were imaged on a Leitz Laborlux S compound microscope (25 × PL FLUOTAR; N.A., 0.6) equipped with the Nikon DS-R1 camera.

Analysis of colocalization of reporter proteins in “reference” and “test” plasmid coinjections

To calculate the number of cells expressing both the “reference” and “test” coinjected plasmids (identical except for containing gGFP and DsRed2, respectively), we used MosaicSuite for ImageJ (Rizk et al., 2014) to compute the percentages of overlapping expression in sections (3 for each structure) averaged over 3 tadpoles, immunostained for GFP and DsRed2.
**Determination of expression levels of endogenous mRNAs**

The levels of endogenous NF-M, NfT, and CarA mRNAs relative to that of gapdh (ΔCt) were determined by reverse transcription followed by quantitative real time PCR (qRT-PCR) from st. 43–46 tadpoles (Nieuwkoop and Faber, 1956). For each sample, 10 tadpoles were anesthetized by immersion in 0.1% ethyl 3-aminobenzoate methanesulfonate (MS222, Sigma-Aldrich), then homogenized (Polytron 3000, Kinematica AG) and processed for extraction of total RNA (RNasea Plus Kit, Qiagen). Oligo dT-primed (2 pmol, Invitrogen) reverse transcription was performed on 1 μg of total RNA (SuperScript III, Invitrogen), and 5% (1 μl of 20 μl) of the resulting cDNA was used for qRT-PCR with SYBR green (Applied Biosystems; see Supplementary Table 1 for primer sequences). Results were averaged across 3 to 6 biological replicates as noted (10 embryos for each replicate; see legend, Fig. S2) and compared between uninjected and plasmid-injected embryos by two-tailed t-test, with p < 0.05 considered statistically significant. To verify that the number of replicates was sufficient to yield statistically valid conclusions, power analysis (P) was performed with G*Power 3.1 (Faul et al., 2007) and considered adequate when P > 0.8.

**Results**

**Direct injection of modified plasmid DNA generated robust promoter-typic reporter protein expression in swimming tadpoles**

While testing plasmids constructed for studying control elements of the X. laevis NF-M gene with the flC31 integrase method, we discovered that injecting plasmid (75–150 pg) containing two inwardly pointing, dual HS4 insulator elements flanking the transgene (pSPORT1 [attB/Ins1]/1.5 kb/NF-M promoter/gsGFP/β-globin 3′–UTR[Ins2]) yielded moderately to highly penetrant, promoter-typic expression at st. 43–46, even without the integrase RNA. To quantify variability in promoter activity among animals, 75 pg of the plasmid was unilaterally injected into over two hundred 2-cell stage embryos (Table 1). Consistent with what was concluded for the same promoter from Roosa et al. (2000), expression was largely mosaic, with only 17% of the surviving (115 of 125 injected) tadpoles exhibiting some degree of promoter-typic expression (categories 1 and 2, combined). Notably, although most tadpoles from this second group exhibited some reporter expression earlier in development (not shown), expression largely faded to undetectable levels by st. 43–46 (38%, category 4), whereas only 7% of group injected with plasmid having all three elements had no expression at this stage. Statistically, the distributions of the expression patterns among animals injected with these two plasmids were significantly different ($\chi^2$ test, p < 0.001), confirming that injection of the construct bearing an attB and inwardly pointing, flanking dual HS4 insulator elements promoted robust promoter-typic expression. In being confined largely to the nervous system but with some muscle expression, this promoter-typic expression was consistent with what was concluded for the same promoter from earlier REMI experiments (Roosa et al., 2000).

To determine whether this penetrant, promoter-typic expression occurred with other promoters as well, we tested two additional promoters: NfT, which drives expression that is both stronger and more neuron-specific than that of the 1.5 kb NF-M promoter (Oschwald et al., 1991; Kroll and Amaya, 1996; Roosa et al., 2000), and CarA, which drives expression in muscle and is inactive in neurons (Mohun et al., 1986; Kroll and Amaya, 1996; Latinkic et al., 2002; Love et al., 2011). The NfT injected animals were scored using

| Table 1 | Phenotype scoring for promoter-only and elements-included groups at stages 43–46. |
|-----------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|-----------------------------|
| 1. NfT promoter          |                                  |                                  |                                  |                                  |                                  | 2 test $^{b}$                  |
| Promoter only (N=102) $^{a}$ | Neural                       | 19%                            | Neural+ weak muscle               | 24%                            | Ectopic                        | 52%                           | 6%                           |
| Elements included (N=200) |                                | 33%                            |                                  | 45%                            |                                  | 21%                           | 3%                           |
| 2. NF-M promoter         |                                  |                                  |                                  |                                  |                                  | 2 test $^{b}$                  |
| Promoter only (N=115)     | Neural                       | 4%                             | Neural+ weak muscle               | 13%                            | Ectopic                        | 45%                           | 38%                          |
| Elements included (N=245) |                                | 23%                            |                                  | 48%                            |                                  | 22%                           | 7%                           |
| 3. CarA promoter          |                                  |                                  |                                  |                                  |                                  | 2 test $^{b}$                  |
| Promoter only (N=136)     | High penetrance              | 20%                            | Medium penetrance                 | 22%                            | Low penetrance                  | 58%                           | 2%                           |
| Elements included (N=236) |                                | 34%                            |                                  | 25%                            |                                  | 41%                           | 1%                           |

$^{a}$ Embryos were injected at the 2-cell stage and scored for reporter protein expression at st. 43–46. See the text for descriptions of the promoters, injected plasmids, and scoring criteria.  
$^{b}$ Results of a $2 \times 2 \chi^2$ test determining whether the two distributions differed significantly.

$^{c}$ N represents the total number of injected tadpoles that survived to st. 43–46 and had a normal appearance.
the same categories as for those injected with the NF-M promoter. Among 224 injected embryos, 200 survived to st. 43–46. Of these surviving tadpoles, 33% exhibited robust GFP expression restricted to CNS (Fig. 2A1 and category 1, Table 1). Such expression at equivalent developmental stages has been observed by others working with this promoter using transgenesis procedures such as REMI and the I-SceI meganuclease methods (Ryffel and Lingott, 2000; L’hostis-Guidet et al., 2009); 45% showed some weak expression in tail muscle in addition to strong expression in CNS (Fig. 2A2; category 2, Table 1); 21% showed high degrees of ectopic expression outside of CNS (Fig. 2A3; category 3, Table 1). Notably, the distribution of these expression patterns was significantly more promoter-typic than that derived from a promoter-only plasmid having neither attB nor insulator elements ($\chi^2, p < 0.001$). With the CarA promoter, we never observed expression outside of muscle. Therefore, we used a scoring criteria adapted from previous reports categorizing muscle expression patterns according to their penetrance when expression was driven by the CarA promoter [REMI (Kroll and Amaya, 1996); I-SceI meganuclease transgenesis in fish (Thermes et al., 2002)], as well as by other promoters [Tol2 and piggyBAC transposon methods and REMI (Loots et al., 2013); Tol2 transposon (Lane et al., 2013)]. These categories were 1) “high penetrance”, representing smooth, consistent muscle expression (e.g.,Fig. 2C1), which resembled that observed by others using transgenesis procedures [REMI (Kroll and Amaya, 1996; Ryffel and Lingott, 2000; Sekkali et al., 2008); I-SceI meganuclease (Ogino et al., 2006)]; 2) “medium penetrance”, representing consistent expression in muscle cells, but with lower intensity (e.g.,Fig. 2C2); 3) “low penetrance”, representing sporadic, varying levels of expression scattered throughout the myotome, and with a contiguous gap of non-expressing cells located between the dorsal and ventral myotome (e.g.,Fig. 2C3); 4) “no expression” (e.g.,Fig. 2C4). Of embryos surviving to st. 43–46 (236 of 247 injected), 34% had highly penetrant expression (category 1), 25% had medium penetrant expression (category 2), 41% had sporadic expression (category 3), and 1% had no detectable expression (category 4). Within categories 1 and 2, 82% (e.g.,Fig. 3D1, inset) and 11% exhibited expression in the heart, respectively, whereas animals within the remaining two categories typically showed no heart expression. Consistent with what was observed from the neuronal promoter groups, CarA driven expression was significantly more promoter-typic when the attB element and insulator sequences were included in the injected plasmid (Table 1), with a decrease in low penetrant, and an increase in high and medium penetrant expression compared to the promoter only plasmid ($\chi^2, p < 0.01$). In general, the degree of
highly promoter-typic expression driven by the elements-included construct was comparable to transgenesis methods that inject non-modified, promoter-bearing plasmids with enzymatic co-factors (Sparrow et al., 2000; Ogino et al., 2006). Interestingly, another study that used insulator elements (different from our construct) with REMI transgenesis also reported that adding insulator sequences promoted higher frequency of tissue specific expression compared to the uninsulated group (Sekkali et al., 2008).

To characterize further reporter expression driven by the three promoters in more detail, we selected st. 43–46 tadpoles that exhibited highly promoter-typic expression from groups injected with constructs bearing the attB element and both insulators, cut cryosections through the eye, brain, and spinal cord, and immunostained the sections for GFP (Fig. 3). Expression characteristic of all promoters at st. 43–46 was confirmed in these sectioned tissues. For the NβT and NF-M groups, expression on the injected side was readily visible in dorsal retina layers as well as in neurons of the head and spinal cord. On the uninjected side, it was seen in the decussated (Jacobson and Hirose, 1978; Hirose and Jacobson, 1979) ventral retina (not shown) and axons of brain and spinal cord (Fig. 3B2–B4 and C2–C4). CarA-driven expression was restricted to muscle (Fig. 3D1–D4).

Expression emerged appropriately at earlier developmental stages

We next examined embryos at earlier stages to assess when expression first appeared, using co-reared embryos from the same spawning to make side by side comparisons between promoters. Consistent with the known time course of neuronal and muscle cell differentiation and what has been reported using these promoters with other methods (Kroll and Amaya, 1996; Roosa et al., 2000; Latinkic et al., 2002; Della Gaspera et al., 2012), expression from all three promoters first emerged between late neural fold and early neural tube stages (Fig. 4B1–D1; Table 2). At this earliest stage (st. 18–20), fewer embryos exhibited the most specific (NβT, 9%; NF-M, 13%) or highly penetrant (CarA, 10%)
expression patterns (category 1) compared with what was seen at st. 43–46 (from Table 1: 33, 23, 34% for NβT, NF-M, and CarA, respectively), with more exhibiting either ectopic or no detectable expression. By st. 22–24, expression became more readily visible, with the numbers of embryos exhibiting no detectable expression declining to levels comparable to what was seen at st. 43–46. Expression became more visible throughout brain and spinal cord in the two groups of neuronal promoters (Fig. 4B2 and C2 and Table 2), and the vast majority of embryos injected with the CarA plasmid (490%) began to exhibit visible expression in muscle (Fig. 4D2, Table 2). By st. 32–33/34, patterns of expression that were characteristic of each of the two neuronal promoters (categories 1 and 2) became evident in the majority of embryos (NβT, 60%; NF-M, 59%), and these numbers improved through st. 39–40 (Table 2) and st. 43–46 (Table 1), when expression in live animals became easier to observe as they became more transparent. The expression patterns and steadily growing frequency of promoter-typic expression that we observed here at these earlier stages were similar to what has been reported for these same promoters in F0 animals with transgenesis methods (Kroll and Amaya, 1996; Roosa et al., 2000; Sparrow et al., 2000; Ryffel and Lingott, 2000; Ogino et al., 2006; Sekkali et al., 2008; L’hôstis-Guidet et al., 2009).

The attB element and both dual insulators were required for persistent expression

To test the relative contributions of the attB and two insulator elements to the fidelity, penetrance and persistence of NF-M promoter-driven expression, which was the weakest promoter of the three tested, we injected at the 2-cell stage, five constructs with these three elements in various combinations. We then assayed expression in live tadpoles at st. 43–46 (Fig. 5) and then again, in a smaller number of animals, approximately 2 months later at st. 55–56 (Fig. S1). Consistent with an earlier study (Roosa et al., 2000), expression at st. 43–46 derived from plasmid lacking all three elements was barely detectable and largely ectopic (Fig. 5A, showing a tadpole whose expression fell into the category with the highest frequency in this group, 45% ectopic of 115 survivors scored; same data as used in Table 1). Adding back just the attB element increased the intensity of expression at st. 43–46 and the frequency of promoter-typic expression (N=117, 22% neural only and 15% neural + weak muscle), but ectopic expression remained widespread, especially in epidermis and anterior trunk muscle (Fig. 5B, arrows, 43% ectopic), consistent with earlier reports on the necessity of having two insulators (Allen and Weeks, 2005). In animals injected with plasmid having all three elements, expression was predominantly promoter-typic (categories 1 and 2) at this stage, and ectopic expression was less frequent than with all the other groups (Fig. 5E, same data as used in Table 1 and Fig. 2B). Deleting the attB element but retaining both flanking dual insulators decreased the level of expression dramatically (Fig. 5D, showing 44% weak expression, see below), indicating that the attB element was required. Notably, the little
Table 2
Phenotype scoring for early developmental stage embryos injected with plasmids bearing all elements.

<table>
<thead>
<tr>
<th>Stages 18–20</th>
<th>Neural</th>
<th>Neural + weak muscle</th>
<th>Ectopic</th>
<th>No expression</th>
</tr>
</thead>
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<tr>
<td>N/T (N = 118)</td>
<td>9%</td>
<td>36%</td>
<td>47%</td>
<td>9%</td>
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<tr>
<td>NF-M (N = 119)</td>
<td>13%</td>
<td>21%</td>
<td>51%</td>
<td>15%</td>
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<tr>
<td>CarA (N = 139)</td>
<td>10%</td>
<td>21%</td>
<td>22%</td>
<td>48%</td>
</tr>
<tr>
<td>High penetrance</td>
<td>Medium penetrance</td>
<td>Low penetrance</td>
<td>No expression</td>
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<table>
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<tr>
<th>Stages 22–24</th>
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<th>Neural + weak muscle</th>
<th>Ectopic</th>
<th>No expression</th>
</tr>
</thead>
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<td>42%</td>
<td>40%</td>
<td>4%</td>
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<tr>
<td>NF-M (N = 114)</td>
<td>18%</td>
<td>31%</td>
<td>44%</td>
<td>7%</td>
</tr>
<tr>
<td>CarA (N = 137)</td>
<td>23%</td>
<td>23%</td>
<td>47%</td>
<td>7%</td>
</tr>
<tr>
<td>High penetrance</td>
<td>Medium penetrance</td>
<td>Low penetrance</td>
<td>No expression</td>
<td></td>
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<th>Neural + weak muscle</th>
<th>Ectopic</th>
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<td>37%</td>
<td>3%</td>
</tr>
<tr>
<td>NF-M (N = 110)</td>
<td>20%</td>
<td>39%</td>
<td>34%</td>
<td>7%</td>
</tr>
<tr>
<td>CarA (N = 137)</td>
<td>31%</td>
<td>21%</td>
<td>45%</td>
<td>4%</td>
</tr>
<tr>
<td>High penetrance</td>
<td>Medium penetrance</td>
<td>Low penetrance</td>
<td>No expression</td>
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<table>
<thead>
<tr>
<th>Stages 39–40</th>
<th>Neural</th>
<th>Neural + weak muscle</th>
<th>Ectopic</th>
<th>No expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/T (N = 104)</td>
<td>25%</td>
<td>44%</td>
<td>28%</td>
<td>3%</td>
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<tr>
<td>NF-M (N = 104)</td>
<td>22%</td>
<td>44%</td>
<td>28%</td>
<td>6%</td>
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<tr>
<td>CarA (N = 134)</td>
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</tr>
<tr>
<td>High penetrance</td>
<td>Medium penetrance</td>
<td>Low penetrance</td>
<td>No expression</td>
<td></td>
</tr>
</tbody>
</table>

| N/T (N = 14) | 13%     | 15%                  | 19%     | 3%           |
| NF-M (N = 114) | 20%     | 39%                  | 34%     | 7%           |
| CarA (N = 137) | 31%     | 21%                  | 45%     | 4%           |
| High penetrance | Medium penetrance | Low penetrance | No expression |

<sup>a</sup> Embryos were injected at the 2-cell stage with plasmids containing the indicated promoter and all elements, and then scored at the indicated stages using criteria as in Table 1.

<sup>b</sup> N represents the total number of surviving tadpoles with normal appearance at the corresponding stage.

Fig. 5. Persistent, promoter-typic reporter protein expression required injection of a plasmid containing an attB site plus two dual insulators. Plasmid maps for the corresponding groups are shown above each column, with the presence or absence of an attB site and one or two dual insulators, as indicated by the symbols (+ or −, respectively). Total numbers of surviving animals scored for each group at st. 43–46 are indicated (N). Table at the bottom shows percentages of tadpoles bearing different expression patterns as characterized in Tables 1 and 2. (A–E) Dorsolateral views of the heads and trunks of live st. 43–46 tadpoles, taken with a Leica DFC 340 FX monochrome camera through a fluorescence stereo microscope, illustrate representative examples from the category with the highest frequency of expression pattern from each group. Most tadpoles injected with plasmid lacking all three elements (A) exhibited only very weak, barely detectable expression that was mostly ectopic. All three groups injected with plasmids containing the attB site yielded robust expression in the CNS (B,C,E), but among these, those lacking either one (C) or two (B) dual insulators exhibited significantly more ectopic expression in muscle and epidermis (arrows) than did those possessing all three elements (E). Groups injected with plasmid lacking the attB element but having both insulators (D) exhibited only very weak to undetectable expression at these stages. Dotted lines outline the brain and spinal cord; autofluorescence from the gut is labeled on all panels.
expression that remained when the attB was deleted was nevertheless restricted to the nervous system, indicating that the two flanking insulators indeed improved the fidelity of the promoter. Thus, all three elements appeared necessary for achieving robust, promoter-typical expression at st. 43–46.

A subset of these animals (6–10 from each group) were raised for an additional 2 months to prometamorphic stages (st. 55–56; Fig. S1) to determine the extent to which expression seen at st. 43–46 persisted. Only those animals injected with plasmid bearing all three elements still exhibited visible expression at this late stage in live tadpoles (Fig. S1E1), and more sensitive immunostaining of sectioned tissues for GFP further confirmed that reporter expression persisted in only this group (Fig. S1E2). This expression at st. 55–56 remained restricted to the nervous system (e.g., retinal ganglion cells, Fig. S1E2; hindbrain neurons, Fig. S1E3; spinal cord neurons, Fig. S1E4), although it was less intense than that seen earlier. Expression even persisted in the two to three animals receiving the NF-T and NF-M plasmids that were raised to froglet stage (not shown), but this expression was even more diminished than what had been seen at st. 55–56, being restricted to only a few populations of neurons. This diminishing penetrance over time convinced us that our method is much better for rapid characterization of gene regulatory elements in F0 tadpoles rather than for generating transgenic lines, and thus, we did not follow up these observations with a systematic analysis of older animals.

Coinjection of plasmids bearing different reporter genes facilitated studies of cis-regulatory elements

Although the penetrance and fidelity of expression with our plasmid was higher in F0 animals than with other plasmids, the variability in expression among animals injected with the same promoter is nonetheless apt to introduce uncertainty into the interpretation of patterns seen with novel, previously uncharacterized cis-regulatory elements. Ideally, one would like to identify and analyze those animals with expression patterns that are the most characteristic of the promoter. Here we present a strategy to identify such animals, and thereby better control for these variations, by coinjecting a plasmid expressing GFP to serve as a reference, together with a second plasmid expressing DsRed2 and the elements to be tested. We first tested whether two plasmids that were identical except for their reporter gene would exhibit the same expression pattern when coinjected. Even with the weakest of the three promoters (i.e., NF-M), coinjection of equal amounts (75 pg) of two such plasmids yielded extensively overlapping expression. Coinjected tadpoles (N = 65) were raised to st. 43–46, encoded, and then scored live, first for GFP and then for DsRed2 patterns of expression. Of those displaying the most promotor-typical pattern of expression for this promoter with GFP (i.e., neural and neural with weak muscle, 40 animals), 80% displayed the same pattern for DsRed2, with the remainder yielding either ectopic (15%) or no expression (5%). Of those expressing GFP ectopically (21 animals), 86% also expressed DsRed2 ectopically, and no embryos that failed to express GFP (4 animals) expressed DsRed2. At the cellular level, in animals yielding promoter-typical expression (N = 3), which were sectioned and immunostained for both GFP and DsRed2 (Fig. 6), 90 ± 2% (SD), 84 ± 2%, and 84 ± 3% of cells in hindbrain, spinal cord, and eye, respectively, expressed both markers. Examination of tadpoles expressing each reporter gene separately confirmed that the extensive overlap was not due to crosstalk between fluorescence channels (not shown). Thus, we concluded that by pre-screening animals for expression of a coinjected, GFP-expressing reference plasmid, the activity of a test plasmid expressing DsRed2 can be reliably determined. With a previously uncharacterized test plasmid, we typically coinject 50 to 100 embryos to account for the occasional outlier.

To demonstrate this idea in practice, we injected a DsRed2 “test” plasmid under the control of a shorter, 0.4 kb NF-M promoter together with a GFP “reference” plasmid using the longer, 1.5 kb NF-M promoter described above (Fig. 7). Previous studies with the shorter, 0.4 kb promoter indicated that although it contained minimal elements necessary for neural expression, it generally yielded expression that was considerably more widespread among non-neural tissues than that seen with the longer 1.5 kb promoter (Roosa et al., 2000). Consistent with this earlier study, at st. 43–46, embryos injected with
this test plasmid exhibited two expression patterns with comparable frequency (Fig. 7B and C; 54% and 46%, respectively; total \( N = 106 \)), both of which were more widespread among non-neural tissues than what was observed with the longer, 1.5 kb promoter (Fig. 7A). Although both 0.4 kb NF-M promoter test groups exhibited expression in the nervous system, one group exhibited muscle expression that was stronger and extended further rostrally (Fig. 7B) than with the longer, 1.5 kb promoter. DsRed2 expression in the second group was strikingly more ubiquitous with little to no preference for tissue type (Fig. 7C). Neither pattern resembled what was observed with the 1.5 kb NF-M promoter (Fig. 7A). To determine whether this more widespread expression extended to the cellular level, expression in coinjected animals was compared between GFP (Reference) and DsRed2 (Test) within the same sections of embryos exhibiting the ubiquitous DsRed2 expression pattern [i.e., as in Fig. 7C; representative examples of sections shown in Fig. 7D–F for DsRed2 (Test) and 7D’,E’,F’ for GFP (Reference)]. These embryos all exhibited promoter-typic (i.e., categories 1 and 2) GFP expression. This comparison confirmed that the shorter, 0.4 kb promoter had indeed yielded expression that was more widespread in non-neural tissues (e.g., Fig. 7D and E, muscle; Fig. 7F, gut) and therefore lacked upstream elements required for the more neuron-specific expression of the longer, 1.5 kb promoter. Thus, this strategy provides a stronger basis for making comparisons between the activities of two different promoters by observing their simultaneous expression within the same individual.

**Plasmid injection had no influence on expression of the corresponding endogenous genes**

One remaining concern with using methods that involve injecting large amounts of plasmid DNA into embryos is that this DNA will compete with endogenous genes for trans-factors needed for proper transcription and translation, thereby altering either normal expression of endogenous genes and the animal’s development or the activity of the elements being tested due to inadequate availability of the necessary trans-factors. If this is the case, one would reasonably expect to see alterations in the

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**Fig. 7.** Truncation of the 1.5 kb NF-M promoter to 0.4 kb increased expression outside the nervous system. (A) Dorsal view of a representative example of 1.5 kb NF-M: DsRed2 expression in a tadpole at st. 43–46 (map of the injected plasmid is shown on the right). Expression is seen predominantly in the brain and spinal cord (arrowhead) of the injected side; autofluorescence in the gut is labeled. (B,C) Injection at the 2-cell stage of a new DsRed2-expressing construct bearing a truncated, 0.4 kb NF-M promoter (75 pg, plasmid map on the right) yielded two expression patterns with similar frequency in st. 43–46 tadpoles [54% (B) and 46% (C) of 106 scored animals]. Both patterns exhibited higher levels of expression overall and more expression in muscle and surrounding tissues than did the longer, 1.5 kb NF-M promoter. Arrowhead in B labels expression in muscle, at approximately the same rostrocaudal level as the arrowhead pointing to the spinal cord in A. (D–F) This new construct bearing the 0.4 kb promoter (plasmid map shown again in the third row, now designated as “Test”; 75 pg injected) was then coinjected with the GFP-expressing reference plasmid, which used the longer, 1.5 kb promoter (plasmid map shown on the bottom row, designated as “Reference”; 75 pg injected). Animals that expressed GFP predominantly in the nervous system (categories 1 and 2) were selected for analysis and protein expression was assayed by immunostaining transverse histological sections. Representative sections from animals that gave expression similar to that shown in C, immunostained for DsRed2 (D–F) and GFP (D’–F’), are shown. GFP expression driven by the reference plasmid in these animals appeared primarily in the nervous system (e.g., hindbrain (D’) and spinal cord (E’; staining seen in adjacent somites is predominantly in peripheral axons)), but not in gut (F’), whereas DsRed2 expression from the test plasmid appeared not only in neural tissues (e.g., neurons in hindbrain (D) and spinal cord (E)), but also in non-neural tissues (e.g., somitic muscle cells (arrowheads, D,E) and gut (arrowhead, F’)). Scale bars, 50 μm.
expression of the endogenous genes bearing these elements (we already demonstrated that development was normal). Thus, to test whether this was a valid concern, we examined expression of the endogenous genes represented by these three promoters in plasmid-injected (75 pg) animals. For protein expression, we immunostained embryos in whole mount from the NFT-glGFP and NF-M-glGFP groups, as well as un.injected embryos, for the endogenous proteins at three developmental time points (st. 24–25, 31–32, 43–46; Fig. S2A1–D4). (The lack of an antibody specific to cardiac actin prevented us from doing the same with CarA embryos). Results confirmed that endogenous protein expression was spatially and temporally indistinguishable between injected and un injected groups. For mRNA expression, we performed qRT-PCR at st. 43–46. All three groups expressed comparable levels of the corresponding endogenous mRNAs relative to those of un injected animals (ΔC_T relative to gapdh; Fig. S2 E,F,G for NFT, NF-M, and CarA, respectively). Thus, the injected plasmids had no discernible effect on expression of the corresponding endogenous genes and therefore are unlikely to perturb the bioavailability of the trans-factors needed for expression.

Discussion

In constructing plasmids to study cis-regulatory elements of the Xenopus NF-M gene using the attB-ϕC31 integrase technique, we serendipitously discovered that injection of 75–150 pg of plasmid DNA having the second, downstream HS4 dual insulator inverted compared to the orientation within the previously reported plasmids (Allen and Weeks, 2005, 2006) yielded highly penetrant, promoter-typic expression that persisted into feeding tadpole stages without the need for coinjecting exogenous integrase RNA. Injection into 2-cell stage embryos of these reporter plasmids with three separate promoters reliably yielded promoter-typic expression that was consistent with the activity of these promoters when used with bona fide transgenesis methods. Although in some cases expression in the expected cell types exhibited some degree of mosaicism, penetration was nonetheless sufficiently high, and ectopic expression was effectively reduced so that promoter activity was readily deduced from examining multiple embryos. Overall, we saw more than half of the tadpoles exhibiting either highly tissue specific expression or moderate expression with weak mosaicism before early feeding tadpole stages in F0 generation animals. The degree of penetrance and tissue-specificity from injecting these plasmids was consistently higher than what has been reported with other plasmids and with what we observed directly with injecting plasmids that lacked the attB and insulator elements (Table 1). They were also comparable with what has been reported in F0 animals with several bona fide transgenesis methods [e.g., (Sparrow et al., 2000; Ogino et al., 2006; Hamlet et al., 2006; L’hostis-Guidet et al., 2009), and other literature referenced in Results] as well as with injections of bacterial artificial chromosomes [50–60% (Fish et al., 2012)]. The one possible exception was with REMI combined with insulator sequences (Sekkali et al., 2008), where frequencies of animals exhibiting the most highly penetrant expression pattern of the CarA promoter were higher than ours (64% vs. 34%). Nonetheless, the incidence was sufficiently high with our method to allow the activity of the promoters to be assessed accurately. Although characterizing cis-regulatory elements in F1, bona fide transgenics remains the gold standard, consistent with the views of others who have used transgenesis procedures in Xenopus and other organisms (Thermes et al., 2002; Loots et al., 2013; Lane et al., 2013), we believe that examining reporter expression that is either highly penetrant or moderately so can be helpful, and even sufficient, for comparing the activities of cis-regulatory elements.

With that in mind, the higher survival rates of animals and speed with which elements can be characterized with plasmid injections offer distinct advantages over other, more complicated methods. Several factors contribute to the higher survival rates with our method (typically >90% for healthy spawnings). First, because the needles used for injecting plasmids have very small tips (5–6 μm outer diameters) compared with those used to inject either sperm nuclei (REMI) or protein co-factors, there is very little tissue back-flow, and embryos heal very soon after injection. Second, performing injection directly into fertilized 2-cell stage embryos eliminates the possibility of inadvertently injecting unhealthy and unsuccessfully fertilized embryos (Gervasi and Szaro, 2004). Performing unilateral injections also enables one to use the uninjected side as an internal control (Vize et al., 1991), which is useful for characterizing the functionality of injected constructs for mutagenesis and gene knock-down studies (Liu et al., 2008; Hutchins and Szaro, 2013) as well as for verifying that expression of poorly expressed constructs exceeds background, particularly when immunostaining for fluorescent proteins. The one potential drawback of our method, namely variation in expression among animals receiving the same construct, was no worse in F0 animals than with other methods, and could be adequately controlled for by coinjecting a reference plasmid of known expression pattern and a different fluorochrome. Doing so enables one to choose the best animals for analysis, as well as to compare the activities of two different cis-regulatory sequences within the same individual. Thus, we believe this method will prove useful as an adjunct to stable transgenesis methods for rapidly screening and assessing the activities of a variety of cis-regulatory elements during the first few weeks of tadpole development.

Our plasmids differed from other ones containing insulators, which were used previously with REMI and ϕC31 integrase, chiefly in having the orientation of the second, downstream insulator pointing inwards toward instead of outwards from the transgene. In the absence of adding integrase RNA, expression of plasmids having both insulators oriented in the same direction (i.e., 5’ to 3’) typically fades by st. 46 (Allen and Weeks, 2005), a time when expression with our plasmids remained strong. Although our use of a rabbit β-globin 3′-UTR instead of an SV40 3′-UTR may also have contributed to the enhanced expression, because we have since obtained comparable results with other 3′-UTRs (not shown), we conclude that the novel behavior of these plasmids most likely arose directly from the downstream insulator’s altered orientation.

The 250 bp core element of the HS4 insulator has at least three known, separable activities: a blocking activity that isolates the transgene from enhancers surrounding the site of integration, a barrier activity that prevents incursion of neighboring chromatin modifications, and an enhancer activity that is intrinsic to the insulator (Steinwaerder and Lieber, 2000; Mutskov et al., 2002; Recillas-Targa et al., 2002; Wang et al., 2009). Whereas the first two properties are independent of the orientation of the insulators, the intrinsic enhancer effect is altered significantly, requiring, for example, that an HS4 insulator placed downstream of a reporter gene introduced into baculovirus be pointed towards the transgene (Wang et al., 2009). Notably, this is the same orientation as in our plasmids, but opposite to that used in earlier studies in Xenopus (Allen and Weeks, 2005; Sekkali et al., 2008; Tran and Vlie minckx, 2014), suggesting it contributed to the effect. This intrinsic enhancer activity comes from alterations to DNA topology, with concomitant local effects on histone acetylation and DNA methylation, rather than from direct recruitment of transcription factors (Wang et al., 2009). From our own experience, having two insulator elements in inverted orientations had profound effects on DNAs that appeared to be mediated by alterations in the overall topology of the DNA, even in vitro. For example, precisely the same sequence that was extruded between
the two insulators by recombination during cloning also became deleted during attempts to amplify the sequences between the two insulators by PCR using primers lying outside the insulators. Amplification from the same template, either using primers located within the two insulators or lying on opposite sides of a single insulator invariably yielded the correct product, indicating that the template between the insulators was intact.

Even with the novel properties of the inverted dual insulators flanking the insert, these elements could not completely account for the enhanced expression, since incorporation of an attB site was also required for persistence of expression into later stages (Fig. 5 and Fig. S1). Because we were most interested in developing a tool for use in F0 animals, we were less concerned with the mechanism underlying these effects. Given the effects the insulators had on recombination and PCR amplification, we initially anticipated that they directly facilitated maintenance of the injected plasmids, either by integration into the genome or through the formation of concatemers (Marini et al., 1988), but results of PCR experiments on DNA isolated from these animals indicated that persistence of the exogenous DNA required the attB element (not shown). This need for the attB element to be present together with ϕC31 integrase’s known integration of attB-bearing DNA into pseudo-attP sites further suggests the intriguing possibility that an endogenous recombination activity associated with this element, and perhaps enhanced by the insulators, may be involved. Although determining the molecular mechanism goes beyond the scope of this study, we speculate that alterations in the topology of the DNA induced by the insulators cooperate together with the attB element to produce the enhanced effects these plasmids had on maintaining persistent, promoter-typic expression in vivo.

We suspect that one reason the expression properties of plasmids having their insulators oriented in the same direction as ours had previously gone unnoticed is their high propensity to undergo recombination during construction (Sekkali et al., 2008; Tran and Vleminckx, 2014). By adding each insulator and attB element in succession, we confirmed that the second insulator was indeed the trigger for recombination. We observed that this recombination event occurred only during the initial transformation of newly ligated plasmid into E. coli, and once a plasmid had been successfully constructed, it remained stable through successive transformations. Also, for reasons not entirely understood, inserting a PCR product instead of a restriction fragment recovered from a plasmid into the final ligation product during construction of the injected plasmid significantly reduced the incidence of recombination. These observations led to our developing a successful cloning strategy in which the cis-elements to be tested were inserted first into an intermediate vector containing the upstream insulator and attB element. Recovery of these elements by PCR with subsequent ligation into a second vector bearing the remaining elements to create the final plasmid effectively solved the recombination problem. We now typically obtain desired plasmids by screening fewer than a dozen colonies and have since used this cloning strategy for more than 20 constructs bearing inverted HS4 insulators in DH5α cells successfully.

Overcoming this last technical hurdle has thus enabled us to capitalize on the enhanced expression properties of these plasmids in Xenopus embryos to provide an effective method for rapidly testing the activities of cis-regulatory gene elements in an intact developing vertebrate. We do not envision this method replacing transgenic lines, since the expression obtained with it does not entirely replicate all the features seen in established lines. Rather, because it can be used to test the activity of regulatory elements in dozens of constructs rapidly, we present it as an alternative that people can use to avoid the more cumbersome procedures of generating transgenic lines for every single element being screened. Thus, we see our method as a resource for pre-screening potentially interesting cis-regulatory elements as well as for testing the function of modified genes in specific tissues, either prior to making the transgenic line or in instances where having such a line is not required.

Summary and conclusion

We report here a novel method for studying genetic cis-regulatory element activity in F0 generation X. laevis. While developing a cloning strategy for reliably generating transgenes with inverted, flanking chicken HS4 insulators, we found that injection of plasmid DNA bearing an attB element, two inverted, flanking HS4 insulators, and a rabbit β-globin 3′–UTR, into 2-cell stage Xenopus embryos generated promoter-typic reporter protein expression in early feeding stage tadpoles without the need for coinjecting exogenous integrase RNA. Plasmid injection had no influence on the expression of the cognate endogenous genes, indicating that this method did not titrate out the trans-factors associated with the cis-regulatory elements of the transgene being tested. We further showed that the attB element, acting synergistically with the insulators, was required for the persistence of the exogenous DNA and expression into later developmental stages. Finally, we demonstrated the utility of coinjecting test and reference plasmids expressing two different reporter proteins to control for variations inherent in introducing exogenous genes into F0 animals while testing the functionality of cis-regulatory elements. We conclude that this method provides a convenient and useful adjunct to existing methods for studying cis-regulatory elements during the first few weeks of tadpole development.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.11.010.

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