



Published in final edited form as:

Dev Dyn. 2012 June ; 241(6): 1125–1132. doi:10.1002/dvdy.23794.

A Gal4/UAS system for conditional transgene expression in rhombomere 4 of the zebrafish hindbrain

Seong-Kyu Choe^{*,#}, Mako Nakamura^{*,&}, Franck Ladam, Letitia Etheridge, and Charles G. Sagerström[^]

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation St Worcester MA 01605

Abstract

Background—The zebrafish is well established as a model organism for the study of vertebrate embryogenesis, but transgenic lines enabling restricted gene expression are still lacking for many tissues.

Results—We first generated the *hoxb1a(β-globin):eGFP^{um8}* line that expresses eGFP in hindbrain rhombomere 4 (r4), as well as in facial motor neurons migrating caudally from r4. Second, we generated the *hoxb1a(β-globin):Gal4VP16^{um60}* line to express the exogenous Gal4VP16 transcription factor in r4. Lastly, we prepared the *UAS(β-actin):hoxa3a^{um61}* line where the *hoxa3a* gene, which is normally expressed in r5 and r6, is under control of Gal4-regulated UAS elements. Crossing the *hoxb1a(β-globin):Gal4VP16^{um60}* line to the *UAS(β-actin):hoxa3a^{um61}* line drives robust *hoxa3a* expression in r4. We find that transgenic expression of *hoxa3a* in r4 does not affect *hoxb1a* expression, but has variable effects on migration of facial motoneurons and formation of Mauthner neurons. While cases of somatic transgene silencing have been reported in zebrafish, we have not observed such silencing to date – possibly because of our efforts to minimize repetitive sequences in the transgenic constructs.

Conclusion—We have generated three transgenic lines that will be useful for future studies by permitting the labeling of r4-derived cells, as well as by enabling r4-specific expression of various transgenes.

Introduction

The zebrafish has become well established as a model system due to its small size, rapid external development of optically transparent embryos and genetic tractability. Much work in zebrafish embryos has relied on the microinjection of mRNA or antisense oligonucleotides to modulate gene function, but it was rapidly realized that the transient nature of such approaches has limited utility for some applications and that methods for direct manipulation of the zebrafish germline are also needed. While the development of such tools initially lagged behind technology development in other areas, it has recently seen significant advances. In particular, the introduction of transgenes, which initially suffered from low rates of integration and high rates of mosaicism, has been significantly improved by the development of transposon-based (Tol2) techniques (Kawakami et al., 2000; Kawakami et al., 2004). Indeed, with the aid of the Tol2 system, it is now feasible to

[^]Author for correspondence: Phone: 508.856.8006, Fax: 508.856.8007, charles.sagerstrom@umassmed.edu.

^{*}The first two authors contributed equally to this work

[#]Current address: Wonkwang University School of Medicine, Center for Metabolic Function Regulation, 460 Iksandae-Ro, Iksan, Jeonbuk, 570-749, South Korea

[&]Current address: Faculty of Agriculture, Kyushu University, Rm.113, Agr. Bldg.2, 6-10-1 Hakozaki, Higashi-Ku, Fukuoka 812-8581 JAPAN

generate transgenic lines to address a multitude of biological questions. Initially, much of this effort was directed at deriving zebrafish reporter lines expressing markers such as GFP in various tissues, but more recently transgenesis efforts have been focused on generating lines permitting conditional gene expression. Conditional gene expression is achieved by the use of promoters that can be activated in response to specific external stimuli (e.g. heat shock), or that are active only in specific tissues. Both of these approaches have been applied successfully in zebrafish (e.g. (Higashijima et al., 1997; Scheer et al., 2002)). Conditional transgene expression is further improved by bi-partite systems (e.g. Cre/Lox and Gal4/UAS) where a tissue specific ‘driver line’ is used to control expression of genes in ‘reporter lines’. These systems permit both temporal and spatial control of gene expression and also enable multiple uses of lines such that one driver line can be used to control several different reporter lines (and *vice versa*).

The Gal4/UAS system makes use of the yeast Gal4 transcription factor, which activates transcription of genes under control of UAS (upstream activator sequence) promoter elements. The Gal4/UAS system was first implemented in *Drosophila* (Fischer et al., 1988; Brand and Perrimon, 1993), but is also being used in the mouse (e.g. (Ornitz et al., 1991; Matsumoto et al., 2004; Iyer et al., 2005; Ovchinnikov et al., 2008)). By selective use of promoters, transgenic lines can be generated with Gal4 expressed in tissue-specific patterns. These tissue-specific Gal4 lines are then crossed to transgenic lines where genes of interest have been placed under UAS control. While this approach requires an initial investment in the generation of multiple lines, it has the advantage of subsequently allowing ‘mixing and matching’, such that any Gal4 line can be crossed to any UAS line and the various lines become useful to the entire research community. The first application of the Gal4/UAS system to zebrafish preceded the development of Tol2-based transgenesis and resulted in relatively inefficient expression, as well as infrequent integration events (Scheer and Campos-Ortega, 1999). The efficiency of expression was subsequently improved upon by using Gal4VP16 – a fusion between the Gal4 protein and the strong transcription activation domain from herpes simplex viral protein 16 – as well as by using a UAS construct that contains 14 repeats of the UAS element. While these modifications improved expression in transient assays (Koster and Fraser, 2001), stable lines prepared using Gal4VP16/14xUAS revealed variegated expression, possibly as a result of the incorporation of multiple transgene copies that were subsequently subjected to silencing (e.g. (Sagasti et al., 2005)). In an effort to minimize variegation effects, the Gal4/UAS technology has recently been integrated with the Tol2 transposon system, which increases the frequency of single insertions and may therefore minimize silencing effects. While Tol2-mediated Gal4/UAS transgenesis has now been used successfully by several groups for gene- and enhancer-trapping, as well as for the generation of tissue specific Gal4 driver lines (Davison et al., 2007; Scott et al., 2007; Asakawa et al., 2008; Distel et al., 2009), there have been reports of variegation effects and transgene silencing with this system as well (Goll et al., 2009; Akitake et al., 2011). Nevertheless, we reasoned that sufficient information can be derived from previous efforts at Gal4/UAS transgenesis in zebrafish that it is now feasible to establish Gal4/UAS tools to probe the development of specific tissues in the zebrafish embryo.

Our specific goal is to generate Gal4/UAS tools for the analysis of hindbrain development in zebrafish and here we report transgenic lines specifically for the analysis of rhombomere 4 (r4) development. We first identified a 1.0 kb fragment from the zebrafish *hoxb1a* promoter and demonstrate that it is sufficient to drive expression of GFP in r4 of stable transgenic *Tg(hoxb1a-βglobin:eGFP)^{um8}* embryos, suggesting that this promoter fragment contains all required regulatory elements. We next established a transgenic line where this 1.0 kb fragment drives expression of the Gal4VP16 transcription factor *Tg(hoxb1a-βglobin:Gal4VP16)^{um60}*. We find that Gal4VP16 expression is restricted to r4 in

Tg(hoxb1a-βglobin:Gal4VP16)^{um60} embryos and we further demonstrate that this r4-restricted Gal4VP16 can transactivate GFP expression from a transiently introduced UAS:GFP plasmid. Lastly, we report the derivation of a UAS:Hoxa3a transgenic line *Tg(UAS:hoxa3a)^{um61}* and we demonstrate that *Tg(hoxb1a-βglobin:Gal4VP16)/Tg(UAS:hoxa3a)^{um61}* embryos display robust expression of *hoxa3a* in r4. This ectopic expression disrupts Mauthner neuron formation in r4 and interferes with facial motoneuron migration from r4, but does not affect *hoxb1a* expression in r4. We have not yet observed any variegation or silencing of these transgenes, in spite of the *Tg(hoxb1a-βglobin:eGFP)^{um8}* line having been bred for 6 generations. We speculate that the lack of silencing may stem from our use of a bipartite system where the Gal4VP16 driver and the UAS reporter are established as separate lines, as opposed to co-integrated as has previously been the norm in zebrafish, as well as to our use of fewer UAS elements in the UAS line.

Methods

Generation of transgenic lines

A genomic fragment of the *hoxb1a* enhancer encompassing position +1 (*hoxb1a* translation start site as defined by Zv9 Ensembl release) to position -1158 was PCR amplified (using forward oligo 5'-GGACTAGTTTTTGGCTTGCCAATTCAA-3' and reverse oligo 5'-CGGGATCCTCTGGAAGTGTCCATACG-3') and subcloned into the pCR2-TA vector (Invitrogen). The *hoxb1a* promoter fragment was excised from the pCR2-TA vector by digestion with SpeI (an SpeI site was included in the forward primer used for PCR amplification) and EcoRI (cuts in pCR2-TA polylinker) and cloned into a pBS vector (via SpeI/EcoRI sites in the pBS polylinker) that already contained the eGFP gene in the EcoRV site. The resulting *pBS-hoxb1a:eGFP* construct was digested with NarI (cuts at an internal site at position -174 of the *hoxb1a* promoter) and EcoRI (cuts in pBS polylinker), thereby eliminating the *hoxb1a* translation start site. In place of this sequence was ligated an adapter (generated by hybridization of two oligos: 5'-CGCCGGGCTGGGCATAAAAGTCAGGGCAGAGCCATCTATTGCTTACATTTGCTTCTGG-3' and 5'-AATTCAGAAGCAAATGTAAGCAATAGATGGCTCTGCCCTGACTTTTTATGCCCA GCCCGG-3') that contains 53bp from the human basal β-globin promoter. The resulting *pBS-hoxb1a(β-globin):eGFP* construct was modified by insertion of an oligo containing an XhoI site into the NotI site of the pBS polylinker. The *hoxb1a(β-globin):eGFP* construct was then released as an XhoI fragment (XhoI now cuts both at the modified NotI site upstream of the *hoxb1a* promoter and downstream of *eGFP* in the pBS polylinker) and cloned into the XhoI site of the pTOL2 vector to generate *hoxb1a(β-globin):eGFP*.

To generate *hoxb1a(β-globin):Gal4VP16*, Gal4VP16 was amplified from *pBSGal4VP16* (using primers 5'-GGAATTCGCCACCATGGATTATAAGGATGATGACGACAAAAAGCTACTGTCTTCTATCGAA-3' and 5'-CCGAAGCTTTTAAAAAACCTCCCACACCTCCCC-3'), followed by digestion with EcoRI (cuts site in 5' primer) and HindIII (cuts site in 3' primer). The *eGFP* gene was released from *pBS-hoxb1a(β-globin):eGFP* by digestion with EcoRI and HindIII and the Gal4VP16-containing EcoRI/HindIII fragment was cloned in its place to generate *pBS-hoxb1a(β-globin):Gal4VP16*. A NotI adapter was inserted into the HindIII site of *pBS-hoxb1a(β-globin):Gal4VP16* and the *hoxb1a(β-globin):Gal4VP16* fragment was released as a NotI fragment followed by cloning into the Pspom1 site of pTol2 to generate *hoxb1a(β-globin):Gal4VP16*.

To prepare *UAS(β-actin):hoxa3a*, HA-tagged *hoxa3a* was released from the pCS2+ vector with BamHI and NotI, the NotI site was blunted and the resulting fragment was cloned into the BamHI/BssHIII site of the pUAS-L vector. A fragment containing 5 UAS elements

followed by the carp β -actin promoter and the HA-tagged *hoxa3a* gene was PCR amplified from the resulting plasmid (using primers 5'-GAAGATCTGTGGATCAGCTTGCATGCCTG-3' AND 5'-CAAGATCTACCCACACCTCCCCCTGAACC-3') and cloned into the BglII site of pTol2 via BglII sites included in the primers.

Microinjections

Linearized DNA constructs were injected at the early 1 cell stage together with *Tol2 transposase* mRNA. Injected embryos were raised to adulthood and genotyped by PCR using oligos 5'-CCGAAGCTTTTAAAAAACCTCCCACACCTCCCC-3' and 5'-GGAATTCGCCACCATGGATTATAAGGATGATGACGACAAAAAGCTACTGTCTTC TATCGAA-3' for *hoxb1a*(β -globin):*Gal4VP16^{um60}* and oligos 5'-ATTGCCGAGCCGTCGCAGTA-3' and 5'-GCTGCAAATAGCAGGAAACG-3' for *UAS*(β -actin):*hoxa3a^{um61}*, or by assaying the offspring for GFP expression for *hoxb1a*(β -globin):*eGFP^{um8}*. The lines used herein have been outbred to wild type fish (TL and/or Ekwill) for 6 generations (*hoxb1a*(β -globin):*eGFP^{um8}*) or 3 generations (*hoxb1a*(β -globin):*Gal4VP16^{um60}* and *UAS*(β -actin):*hoxa3a^{um61}*). *In situ hybridizations and immunohistochemistry.* In situ hybridizations and immunohistochemistry were carried out as reported previously (Vlachakis et al., 2001; Choe et al., 2002).

Results

A 1.0kbp *hoxb1a* promoter fragment is sufficient to drive GFP expression in r4 of the hindbrain

The zebrafish *hoxb1a* gene is the earliest *hox* gene to be expressed in a rhombomere-restricted manner. Previous work has identified a series of binding sites for paralog group 1 (PG1) Hox proteins and their Meis and Pbx cofactors as being necessary and sufficient to drive *hoxb1* expression in r4 of the murine hindbrain (Popperl et al., 1995; Jacobs et al., 1999). In zebrafish, these elements are located in a region approximately between position -285 and -210 upstream of the *hoxb1a* translation start site (as defined in the Zv9 Ensembl release). To obtain a genomic region capable of driving gene expression in zebrafish rhombomere 4, we isolated a 1.0kbp fragment from the zebrafish *hoxb1a* locus by PCR (see methods section for details of cloning) that includes sequence from position -1158 to position -174 upstream of the *hoxb1a* translation start site. This fragment lacks the *hoxb1a* translation start site and is also likely to lack the *hoxb1a* transcription start site (TSS), although the location of the *hoxb1a* TSS is less well defined. The resulting 1.0kbp fragment and the location of the Hox/Meis/Pbx binding elements are shown in Fig. 1A.

The 1.0kbp fragment was placed upstream of a 53bp sequence encoding the basal promoter of human β -globin followed by the eGFP gene and used to generate stable transgenic lines (Fig. 1B, see methods section for details). The existence of this line has been reported previously (Choe et al., 2009), but it has not been described in detail. Analysis of the *hoxb1a*(β -globin):*eGFP^{um8}* transgenic line demonstrates detectable eGFP mRNA in the zebrafish hindbrain at 10hpf (Fig. 2D). eGFP mRNA is robustly detected by 12hpf and persists at least until 22hpf (Fig. 2E, F). This time course of eGFP mRNA transcription appears somewhat delayed relative to the expression of *hoxb1a* mRNA, which is detectable by 8hpf and robust by 10hpf (Prince et al., 1998), Fig 2A, B). We also note that eGFP mRNA is detected primarily in the hindbrain, while endogenous *hoxb1a* mRNA is detected both in the hindbrain and in adjacent mesoderm (asterisk in Fig. 2B), suggesting that the 1.0kbp fragment contains regulatory elements required for hindbrain expression, but may lack elements required for mesoderm expression.

eGFP protein is readily detectable by 13hpf (Fig. 2G) and remains highly expressed until 24hpf (Fig. 2H, I). While we occasionally observe the onset of eGFP expression in a salt-and-pepper like pattern, this pattern appears to be transient and resolves into a uniform eGFP expression domain in r4. By 24hpf, both *eGFP* mRNA and eGFP protein is detectable in a well-defined r4 expression domain as well as in nVII facial motoneurons migrating caudally from r4 (arrows in Figs. 2F, H, I). *hoxb1a* expression in nVII neurons has been reported previously and *hoxb1a* is required for proper nVII migration (Cooper et al., 2003).

Examination of several hindbrain genes in *hoxb1a(β-globin):eGFP^{um8}* transgenic embryos did not reveal any expression defects. In particular, *hoxb1a* and *krox20* expression in *hoxb1a(β-globin):eGFP^{um8}* embryos is indistinguishable from that in non-transgenic control embryos (Fig. 2A–C, J–L). We conclude that the 1.0kbp promoter fragment recapitulates *hoxb1a* expression, albeit with a somewhat later onset, and does not affect normal hindbrain gene expression.

The 1.0kbp *hoxb1a* promoter fragment drives Gal4VP16 expression in hindbrain rhombomere 4

We next replaced the eGFP reporter with Gal4VP16 to generate the *hoxb1a(β-globin):GAL4VP16^{um60}* line (Fig. 1C). Gal4VP16 is a fusion protein containing the DNA-binding domain of the yeast Gal4 protein fused in-frame to the activation domain from the viral VP16 protein. The Gal4VP16 protein is a potent transcriptional activator that binds yeast UAS (upstream activator sequence) elements. Since there are no reported UAS elements in the zebrafish genome, Gal4VP16 is not expected to affect zebrafish gene expression or embryogenesis. Accordingly, our analysis reveals that expression of *hoxb1a* (Fig. 3A–C), *valentino* (Fig. 3E), *krox20* (Fig. 3F; detected in red) and *hoxa3a* (Fig. 3G) in the *hoxb1a(β-globin):GAL4VP16^{um60}* line is indistinguishable from control embryos. As expected, in situ hybridization analysis revealed expression of the *Gal4VP16* transcript in r4 of the hindbrain (Fig. 3D, F, H). To confirm that the Gal4VP16 protein is functional, we microinjected a plasmid containing 10 UAS elements driving GFP into 1-cell *hoxb1a(β-globin):GAL4VP16^{um60}* embryos. At 24hpf, GFP expression is readily detected in r4 of the injected embryos (Fig. 3I), confirming that the Gal4VP16 protein is active in zebrafish embryos.

hoxb1a(β-globin):GAL4VP16^{um60} drives expression of a UAS(β-actin):*hoxa3a^{um61}* transgene in r4

To further test the function of the *hoxb1a(β-globin):GAL4VP16^{um60}* line, we generated an additional stable transgenic line where 5 UAS elements are cloned upstream of the carp β-actin minimal promoter driving the zebrafish *hoxa3a* gene (*UAS:hoxa3a^{um61}*; Fig. 1D). This construct should be inactive in zebrafish, which lack endogenous Gal4-like proteins. Accordingly, *UAS(β-actin):hoxa3a^{um61}* embryos were indistinguishable from control non-transgenic embryos with normal *hoxa3a* expression (Fig. 4D), as well as normal expression of *hoxb1a* (Fig. 4A), *valentino* (Fig. 4B) and *krox20* (Fig. 4C).

We next crossed *UAS(β-actin):hoxa3a^{um61}* to the *hoxb1a(β-globin):Gal4VP16^{um60}* fish and examined gene expression in the offspring. We elected to analyze these embryos at 24hpf to ensure that Gal4VP16 had sufficient time to accumulate and drive *hoxa3a* expression. Data from three separate crosses reveal that ~30% of embryos (31/101) express *hoxa3a* ectopically in r4 (arrows in Fig. 5B, E). This is close to the expected 25% and suggests that the Gal4VP16 construct efficiently drives expression from the *UAS:hoxa3* transgene in r4. Notably, *hoxb1a* expression is indistinguishable between *hoxb1a(β-globin):Gal4VP16^{um60}/UAS(β-actin):hoxa3a^{um61}* embryos and control embryos (Fig. 5C), indicating that *hoxa3a* expression does not interfere with *hoxb1a* expression in r4. However, analysis of later stage

hoxb1a(β-globin):Gal4VP16^{um60}/UAS(β-actin):hoxa3a^{um61} embryos revealed disruption of nVII (facial) motorneuron migration from r4 towards the caudal hindbrain (asterisk in Fig. 6C), as well as loss of Mauthner neuron formation in r4 (Fig. 6D), indicating that ectopic expression of *hoxa3a* may affect later stage r4 function. Genotyping of two separate experiments revealed that the majority of embryos with disrupted nVII migration were doubly transgenic (15/17 in Fig. 6E). However, this phenotype is variable and may not be fully penetrant, as indicated by some phenotypically wild type embryos also carrying both transgenes (3/12 in Fig. 6E).

Discussion

The zebrafish represents a useful model organism that provides experimentally accessible transparent embryos for the study of vertebrate embryogenesis. Recent advances in zebrafish transgenesis now allow for relative straightforward production of transgenic lines, but the number of such lines available for the analysis of any specific tissue is still limited. Here we report the generation of three transgenic lines for analysis of the zebrafish hindbrain. The *hoxb1a(β-globin):eGFP^{um8}* line drives expression of eGFP in rhombomere 4 (r4) of the zebrafish hindbrain, as well as in facial motorneurons migrating from r4 into the caudal hindbrain. eGFP mRNA is first detectable in r4 at 10hpf (Fig. 2G) and eGFP fluorescence is observed by 13hpf (Fig. 2J). This onset of expression lags behind that of *hoxb1a* expression from the endogenous promoter by approximately 2 hours. The *hoxb1a(β-globin):Gal4VP16^{um60}* line drives expression of the exogenous Gal4VP16 transcription factor in r4 with approximately the same kinetics as observed for eGFP (Fig. 3D). Lastly, the *UAS(β-actin):hoxa3a^{um61}* transgene is not expressed in zebrafish embryos until crossed to the *hoxb1a(β-globin):Gal4VP16^{um60}* line, in which case *hoxa3a* becomes ectopically expressed in r4. These lines will be useful by allowing the marking of r4 cells using the *hoxb1a(β-globin):eGFP^{um8}* line and by enabling forced r4-expression of any UAS-controlled transgene by crossing to the *hoxb1a(β-globin):Gal4VP16^{um60}*. In addition, crossing of the *UAS(β-actin):hoxa3a^{um61}* line to other Gal4 lines will drive *hoxa3a* expression in any number of tissues. We note that a line labeling portions of the murine hindbrain was recently reported (Makki and Capecchi, 2010), but this line uses a different *hox* promoter (*hoxa1*, equivalent to zebrafish *hoxb1b*) and labeling is therefore not restricted to r4. Notably, labeling in the murine line also shows a delay relative to expression of the endogenous promoter, suggesting that this may be a common feature of transgenic reporter lines.

We find that *hoxb1a(β-globin):Gal4VP16^{um60}/UAS(β-actin):hoxa3a^{um61}* embryos express *hoxa3a* ectopically in r4 as expected. Notably, this ectopic expression does not affect *hoxb1a* expression, but has variable effects on Mauthner neuron formation and nVII facial motorneuron migration. This may be the result of partial re-specification of r4 upon expression of *hoxa3a*, but this hypothesis would need to be tested by additional experimentation. Our result is distinct from a recent report that drove *hoxb3* expression in murine r4 by making use of the r4-specific enhancer from the *hoxb2* gene (Wong et al., 2011) and that observed loss of *hoxb1* expression, as well as abnormal neurogenesis in r4. While it is possible that the difference between these studies are due to different activities of *hoxa3a* versus *hoxb3*, we consider it more likely that differences in the time of onset of transgene expression account for the differing effects on *hoxb1a* expression. In particular, since our system relies on initial expression of Gal4VP16 that then transactivates *hoxa3a*, ectopic expression of *hoxa3a* is not initiated in r4 until after *hoxb1a* is already transcribed.

It has been reported that Gal4/UAS transgenesis in zebrafish suffers from somatic silencing, apparently mediated by DNA methylation of the transgenic locus (Goll et al., 2009). This silencing manifests itself as loss of transgene expression in subsequent generations and as

variable expression in individual embryos. While we have observed ‘salt and pepper’ expression of the *hoxb1a(β-globin):eGFP^{um8}* transgene, this appears restricted to the earliest stages of development and is subsequently ‘filled in’ such that consistent expression is observed throughout the eGFP expression domain. We interpret this to represent stochastic onset of eGFP expression rather than silencing and conclude that we do not observe silencing in any of our lines. While we cannot exclude the possibility that silencing will become apparent in subsequent generations, it also is possible that some features of our transgenic constructs make them less susceptible to silencing. In particular, we took several approaches to minimize repetitive sequences in our transgenic lines. First, we made use of the Tol2 system that is more likely to produce single-copy insertions than non-Tol2 approaches, which can lead to insertion of multiple concatamerized transgenes at a single locus. Second, while previous investigators frequently made use of ‘self-reporting’ Gal4/UAS systems where the Gal4VP16-driver and the UAS-reporter are present on the same plasmid, we have separated the two onto different plasmids and used them to raise separate driver and reporter lines. Third, while UAS constructs with 10 or 14 repeated UAS elements have been used previously, we used a UAS reporter containing 5 UAS elements. Similar conclusions were recently drawn by Goll and coworkers in a more systematic study (Akitake et al., 2011). Indeed, by directly comparing silencing of reporter lines containing 4 or 14 UAS elements, these investigators found that fewer UAS elements correlates with lower levels of silencing and lower levels of DNA methylation at the transgenic locus. This supports the general concept that minimization of repetitive elements is important in order to avoid somatic silencing of transgenes in zebrafish.

Acknowledgments

We are grateful to Matthew Gorgoglione for help with fish care and in situ analysis and Nathan Lawson for the gift of pUAS-L vector. This work was supported by NIH grants NS038183 and HD065081 to CGS.

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Bullet points

- A *hoxb1a*(β -*globin*):*eGFP* transgene drives GFP expression in hindbrain rhombomere 4
- A *hoxb1a*(β -*globin*):*Gal4VP16* transgene drives expression of *hoxa3a* from *UAS*(β -actin):*hoxa3a* in r4
- Ectopic expression of *hoxa3a* in r4 disrupts neurogenesis and neuronal migration

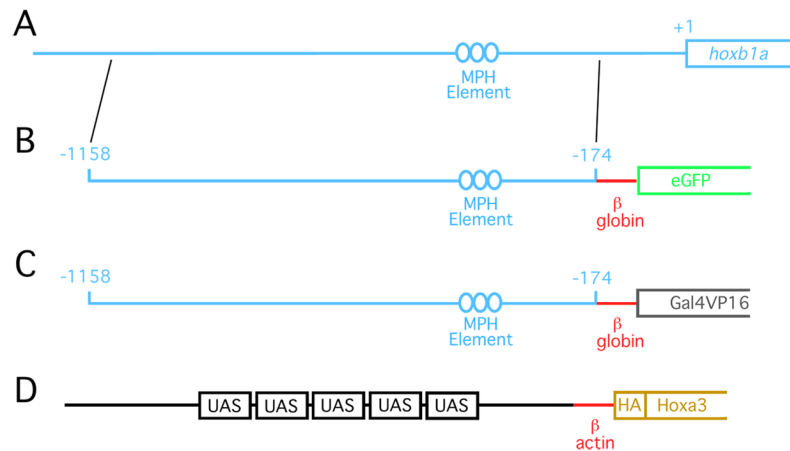
**Figure 1.**

Diagram of transgenic constructs. (A) Genomic DNA between positions -1158 and -174 (relative to translation start site) was isolated from the zebrafish *hoxb1a* locus. This sequence was cloned upstream of the murine β -globin promoter and used to drive eGFP (B) or Gal4VP16 (C). (D) Five UAS elements were cloned upstream of the carp β -actin promoter and used to drive HA-tagged *hoxa3a*. See methods section for specific details of transgene construction. ‘MPH element’ indicates the position of previously defined Meis/Pbx/Hox binding sites required for r4-restricted expression of the *hoxb1a* gene.

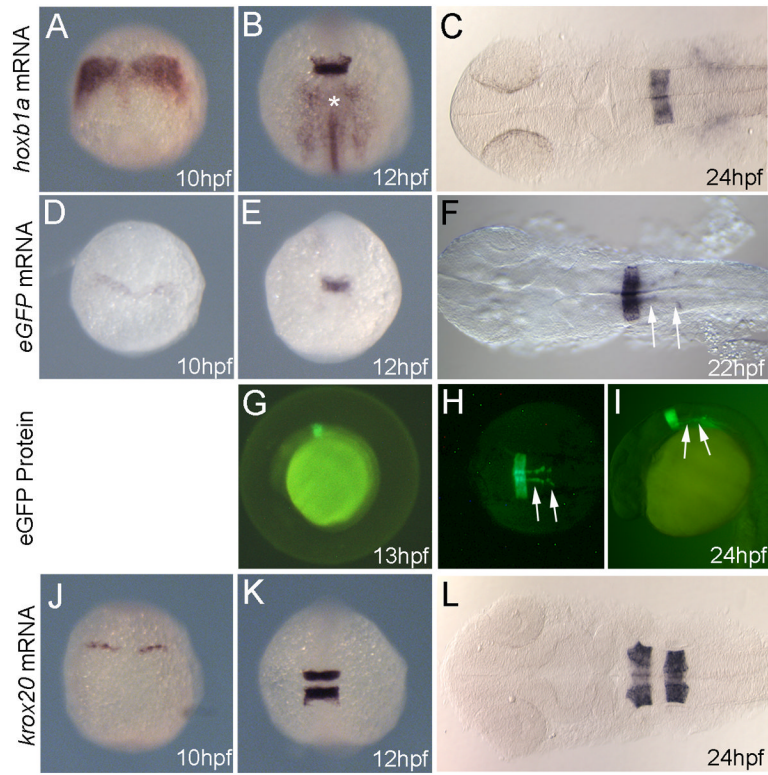


Figure 2. *hoxb1a*(β -globin):*eGFP^{um8}* transgenic embryos express GFP in r4, but are otherwise indistinguishable from non-transgenic siblings. Embryos were collected at the indicated stages and assayed for expression of *hoxb1a* (A–C), *eGFP* (D–F) or *krox20* (J–L) by in situ hybridization as well as for eGFP fluorescence (G–I). All embryos are from an outcross of a heterozygous *hoxb1a*(β -globin):*eGFP^{um8}* carrier. Embryos in D–I are heterozygous for the *hoxb1a*(β -globin):*eGFP^{um8}* transgene. Embryos in A–C and J–L may be either heterozygous or non-transgenic, but there is no difference in expression of *hoxb1a* and *krox20* within the clutch of embryos. Asterisk in B indicates *hoxb1a* expression in mesoderm. Arrows in F, H, I indicate eGFP expression in migrating nVII facial motor neurons. All embryos are in dorsal view except G and I that are lateral views. Anterior is to the top (A, B, D, E, J, K) or to the left (C, F, G, H, I, L).

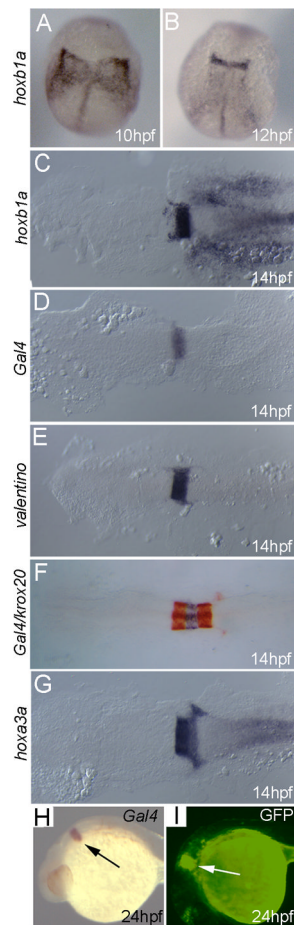


Figure 3.

Transgenic embryos from the *hoxb1a*(β -globin):*Gal4VP16^{um60}* line are indistinguishable from their non-transgenic siblings with respect to expression of all hindbrain genes tested. Embryos were collected at the indicated stages and assayed for expression of *hoxb1a* (A–C), *Gal4* (D, F, H), *valentino* (E), *krox20* (F; detected in red) or *hoxa3a* (G) by in situ hybridization. (I). *hoxb1a*(β -globin):*Gal4VP16^{um60}* embryos were injected with a 10X UAS:GFP plasmid and assayed for GFP fluorescence at 24hpf. All embryos are from an outcross of a heterozygous *hoxb1a*(β -globin):*Gal4VP16^{um60}* carrier. Embryos in D, F, H, I are heterozygous for the *hoxb1a*(β -globin):*Gal4VP16^{um60}* transgene while embryos in A–C, E, G may be either heterozygous or non-transgenic, but there is no difference in expression of *hoxb1a*, *valentino*, *hoxa3a* or *krox20* within the clutch of embryos. Arrows in H and I indicate expression in r4. A–G are dorsal views with anterior to the top (A, B) or the left (C–G). H and I are in lateral view with anterior to the left.

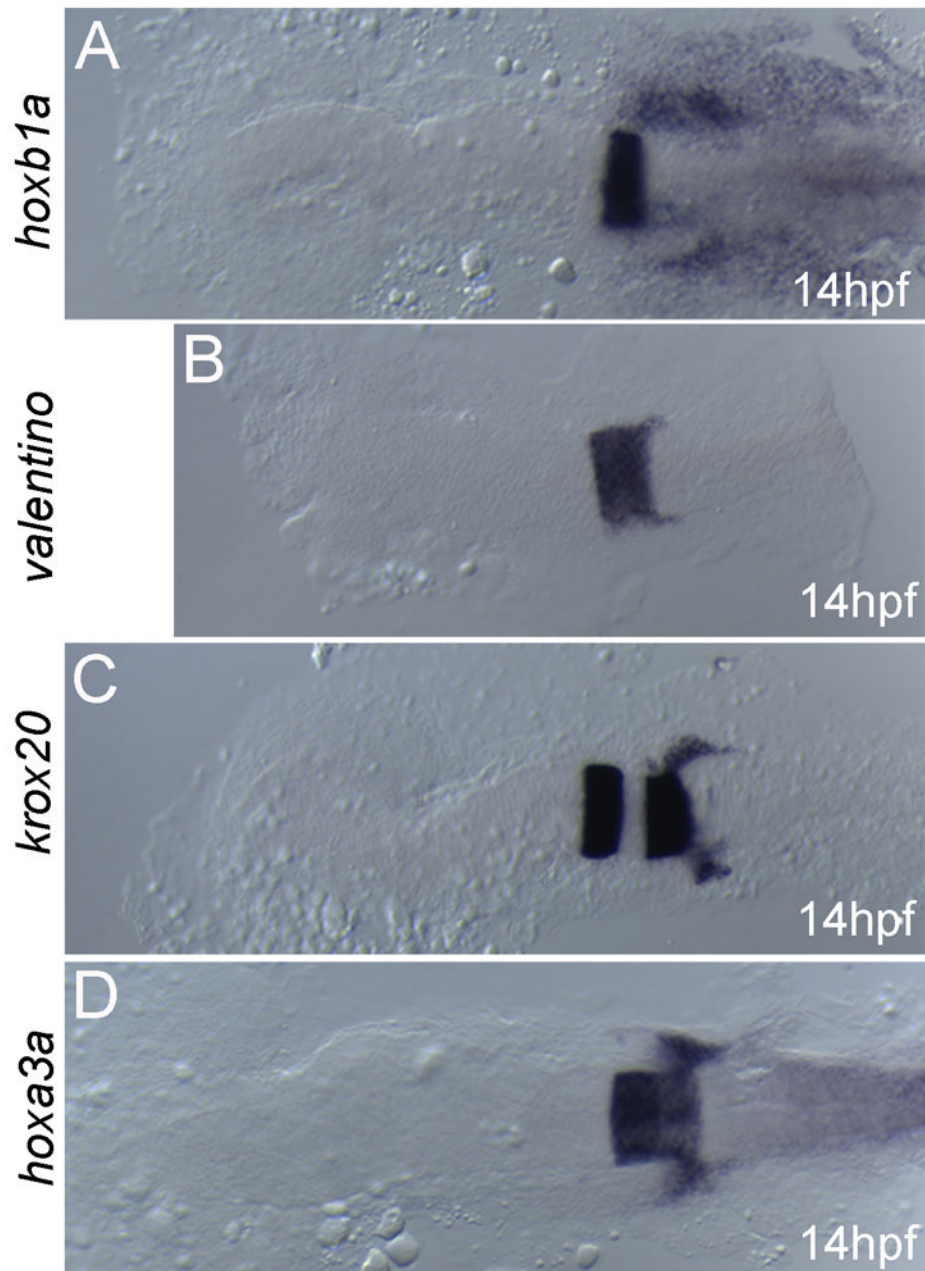


Figure 4.

UAS(β-actin):hoxa3a^{um61} transgenic embryos are indistinguishable from their non-transgenic siblings with respect to expression of all hindbrain genes tested. Embryos were collected at 14hpf and assayed for expression of *hoxb1a* (A), *valentino* (B), *krox20* (C) or *hoxa3a* (D). All embryos are from an outcross of a heterozygous *UAS(β-actin):hoxa3a^{um61}* carrier and may be either heterozygous or wild type, but there is no difference in expression of *hoxb1a*, *valentino*, *hoxa3a* and *krox20* within the clutch of embryos. Embryos are in dorsal view with anterior to the left.

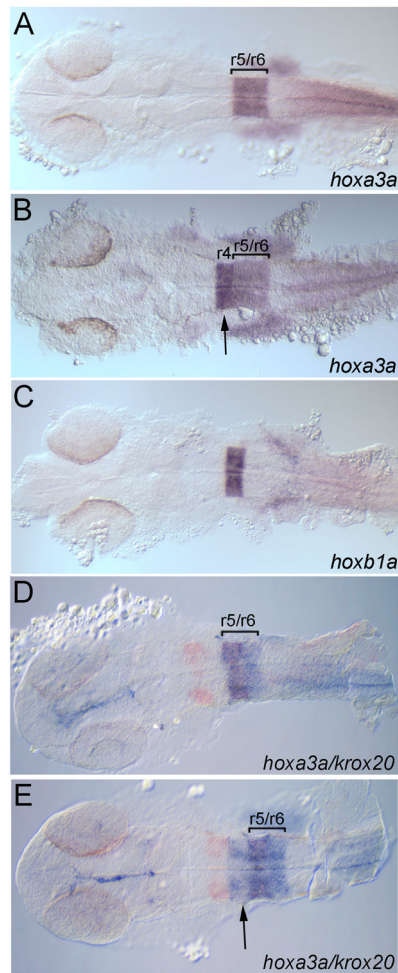


Figure 5. *hoxb1a*(β -globin):*Gal4VP16*^{um60} drives expression of the *UAS*(β -actin):*hoxa3a*^{um61} transgene in rhombomere 4. Embryos were collected at 24 hpf and assayed for expression of *hoxa3a* (A, B, D, E), *hoxb1a* (C) or *krox20* (D, E; detected in red). All embryos are from a *hoxb1a*(β -globin):*Gal4VP16*^{um60} x *UAS*(β -actin):*hoxa3a*^{um61} cross. Embryos in B, E are double transgenic while embryos in A, C, D may be either single transgenic or wild type. Brackets in A, B, D, E delineate r5/r6. Arrows in B, E point to *hoxa3a* expression in r4. All embryos are in dorsal view with anterior to the left.

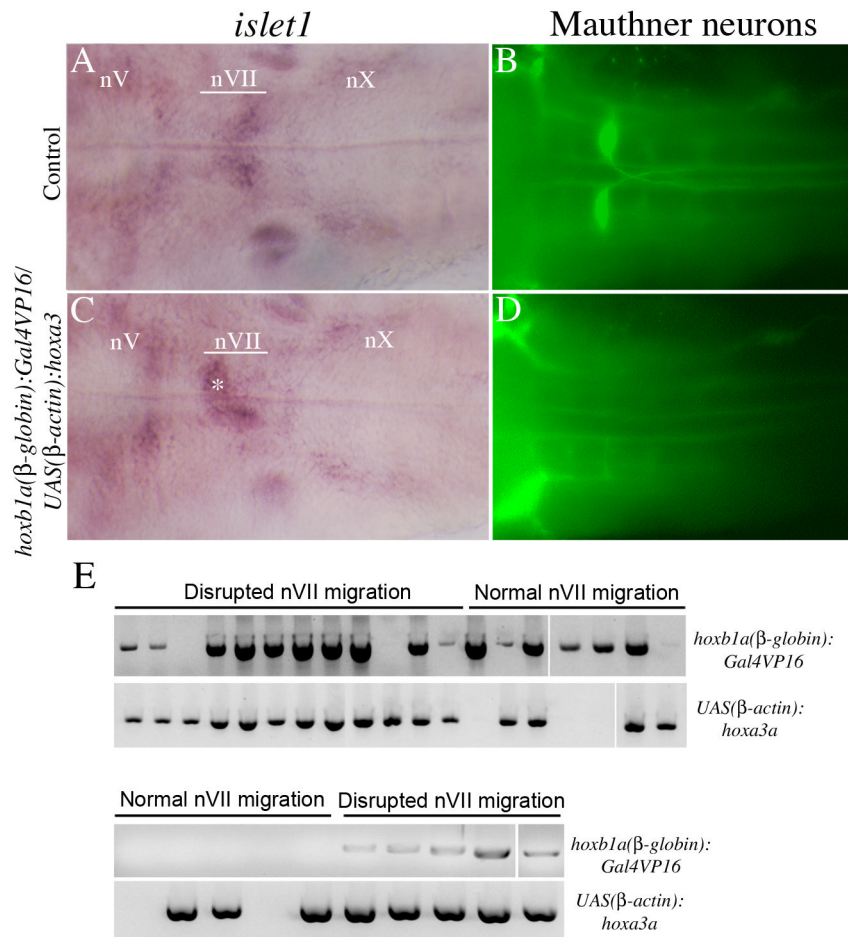


Figure 6. Characterization of embryos from a *hoxb1a(β-globin):Gal4VP16^{UM60}/UAS(β-actin):hoxa3a^{UM61}* cross. Embryos were collected at 48 hpf and assayed for expression of *islet1* to detect motor neurons (A, C) and 3A10 to detect Mauthner neurons (B, D). (E) Embryos with normal or abnormal nVII migration were picked from two representative experiments and genotyped by PCR for presence of the Gal4VP16 and the UAS:Hoxa3a transgene. nV = trigeminal motorneuron, nVII = facial motorneuron, nX = vagal motorneuron. Asterisk in C indicates nVII neurons remaining in r4. All embryos are in dorsal view with anterior to the left.