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Cooperation of *sonic hedgehog* enhancers in midline expression

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Abstract

In zebrafish, as in other vertebrates, the secreted signalling molecule Sonic hedgehog (Shh) is expressed in organiser regions such as the embryonic midline and the zona limitans intrathalamica (zli). To investigate the regulatory mechanisms underlying the pattern of *shh* expression, we carried out a systematic analysis of the intronic regulatory sequences of zebrafish *shh* using stable transgenesis. Deletion analysis identified the modules responsible for expression in the embryonic shield, the hypothalamus and the zli and confirmed the activities of previously identified notochord and floor plate enhancers. We detected a strong synergism between regulatory regions. The degree of synergy varied over time in the hypothalamus suggesting different mechanisms for initiation and maintenance of expression. Our data show that the pattern of *shh* expression in the embryonic central nervous system involves an intricate crosstalk of at least 4 different regulatory regions. When compared to the enhancer activities of the mouse *Shh* gene, we observed a remarkable divergence of function of structurally conserved enhancer sequences. The activating region *ar-C* (61% identical to *SFPE2* in mouse *Shh*), for example, mediates floor plate expression in the mouse embryo while it directs expression in the forebrain and the notochord and only weakly in the floor plate in the zebrafish embryo. This raises doubts on the predictive power of phylogenetic footprinting and indicates a stunning divergence of function of structurally conserved regulatory modules during vertebrate evolution.

Keywords: Embryonic shield; Notochord; Floor plate; Zona limitans intrathalamica; Hypothalamus; Zebrafish; Sonic hedgehog; Transgene; Enhancer

Introduction

The secreted signalling molecule Sonic hedgehog (Shh) controls a multitude of different differentiation processes during vertebrate embryogenesis. In the neural tube, it acts as a morphogen that drives the differentiation of specific neurons in a concentration-dependent manner and is important for neurogenesis in the neonate and the adult (Ingham and McMahon, 2001; Jessell, 2000; Stecca and Ruiz i Altaba, 2005). Other functions include patterning of the endoderm, the somites and the paired appendages and Shh was also implicated in hair and tooth development (Ingham and McMahon, 2001; Jessell, 2000). Moreover, misregulation of the *shh* signalling pathway can cause a variety of tumours in humans including basal cell carcinoma and medulloblastoma (Bale and Yu, 2001; Ingham, 1998; Marino, 2005; Stecca and Ruiz i Altaba, 2005).

In zebrafish as in higher vertebrates, expression of *shh* is highly restricted to regions with organiser activity (Krauss et al., 1993; Scholpp et al., 2006; Strahle et al., 1996). In the zebrafish embryo, *shh* is expressed initially in the embryonic shield, and subsequently in the notochord, prechordal plate and the floor plate. In the brain, *shh* expression is detected in the ventral midbrain, the hypothalamus, the zona limitans intrathalamica (zli) and in a small patch of cells in the telencephalon (Krauss et al., 1993). In the 2-day-old embryo, strong expression of *shh* is also found in the endoderm and its derivatives (Strahle et al., 1996).

Misexpression experiments indicated that the correct spatial and temporal pattern of *shh* expression is critical for the normal

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development of the embryo (Krauss et al., 1993; Roelink et al., 1994; Strahle et al., 1997). Moreover, the absolute levels of *shh* expression are crucial to trigger the correct differentiation programs (Ingham and McMahon, 2001; Jessell, 2000). To investigate the molecular mechanisms that control *shh* expression in the notochord and floor plate, we had previously carried out a screen for enhancer sequences in the zebrafish *shh* gene by transient co-injection experiments (Muller et al., 1999). Activating regions (*ar-A*, *B*, *C*, *D*, see Fig. 7E for overview) that direct floor plate and notochord expression were detected upstream and in two intronic regions of the *shh* locus. The promoter (including sequences up to −2.4 kb) drove expression in the floor plate of the midbrain, hindbrain and anterior spinal cord (Muller et al., 1999). Enhancers *ar-A* and *-B*, which are located in the first intron, mediated expression in the notochord and the floor plate, respectively. A fourth region, *ar-C*, in the second intron, directed expression in the notochord and weakly in the posterior floor plate in these transient experiments (Muller et al., 1999). The sequences of *ar-A* and *ar-C* are highly conserved in the mouse and human *Shh* genes (Jeong and Epstein, 2003; Muller et al., 2002). Both *ar-A* and *ar-C* fall into the class of highly conserved non-coding sequences that are frequently associated with developmental regulators and transcription factors (Dickmeis et al., 2004; Woolfe et al., 2005; Plessy et al., 2005).

The previous co-injection experiments in the 24 hpf embryo (Muller et al., 1999) were limited by mosaicism of the transient expression analysis thereby preventing a detailed analysis of the identified regulatory regions in early stages or in small domains of expression such as particular brain regions or in the embryonic shield. In addition, it was not clear whether the overlapping activities of the enhancers indicate redundancies or specific, as yet undetected activities. Thus there was a need to re-assess the activities of these previously identified enhancers by a stable transgenic approach. Here, we identified the modules responsible for expression in the embryonic shield, the zona limitans intrathalamica and the hypothalamus. We found a remarkable cooperation of the enhancers in the floor plate, notochord and hypothalamus indicating non-redundant functions of these modules. When compared with the function of homologous regions of the mouse *Shh* locus, the function of the structurally conserved enhancers has diverged during vertebrate evolution raising concerns on the functional relevance of conserved sequences. Our work demonstrates a regulatory complexity of the *shh* locus that was not anticipated from the previous transient analysis of the modules and suggests that the enhancer regions do not act redundantly but rather in a fine-tuned concert to control the correct temporal and spatial expression of *shh* in the zebrafish embryo.

Material and methods

Fish stocks

The wild-type line is an intercross between the AB line (University of Oregon, Eugene) and the wtOX line (Goldfish Bowl, Oxford, UK) has been bred for several years in our laboratory as described (Westerfield, 1993).

Plasmid construction

All cloning was done following standard procedures (Sambrook, 2001). The *−2.4shh:gfp* plasmid was constructed by inserting the *−2.4shh* promoter (Chang et al., 1997; Muller et al., 1999) as a *Sal*1, *Xho*1 fragment in the *pCS2:gfp* vector (Blader and Strähle, unpublished data). Note that the previous name *−2.2shh:gfp* (Muller et al., 1999) was changed to *−2.4shh:gfp* as it was found upon sequencing that the promoter construct contains sequence from −2432 to +221 relative to the proximal transcription start site (Chang et al., 1997; Ertzer, unpublished data). Plasmids *−2.4shh:gfpA*, *−2.4shh:gfpB* and *−2.4shh:gfpC* were constructed by inserting PCR-amplified *Not*I/*Kpn*I fragments corresponding to positions +549 to +2381, +2382 to +3592, +3593 to +5366, respectively. Amplification primers contained in addition *Spe*1 and *Sfi*1 restriction sites. *−2.4shh:gfpAC* and *−2.4shh:gfpAB* plasmids were created by inserting *ar-A* as *Not*I/*Spe*I PCR fragment into *−2.4shh:gfpC* and *−2.4shh:gfpB*. The *−2.4shh:gfpABC* plasmid harbours the *ar-C* fragment in the *Sfi*1/*Kpn*1 sites of *−2.4shh:gfpAB*. During the course of the work the *I-Sce*I meganuclease protocol became available (Thermes et al., 2002). The efficiency of obtaining stable transgenics is higher with this approach. Thus the *I-Sce*1 *−2.4shh:gfpABC* plasmid was constructed by inserting double-stranded oligonucleotides containing an *I-Sce*1 restriction site into the *Sal*1/*Kpn*1 restriction sites of *−2.4shh:gfpABC*. The other *I-Sce*1-containing deletion plasmids were constructed by selectively removing *ar-A*, *ar-B* or *ar-C* fragments from this plasmid. The sequences of the oligonucleotides and further details on the construction of the plasmids are available upon request.

Microinjection and expression analysis

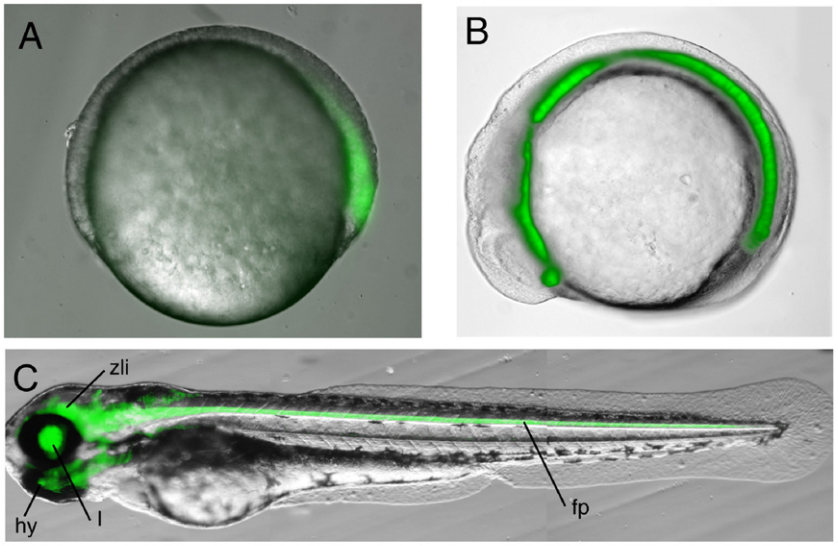
Transgenes were excised from plasmids and separated by agarose gel electrophoresis followed by purification with the Qiaquick Kit (QIAGEN). Eggs were dechorionated using Pronase E as described (Westerfield, 1993). Dechorionated eggs were transferred to agar-coated plastic dishes containing 10% Hank's solution (Westerfield, 1993). Before injection, phenol red was added to 0.1% final concentration. DNA fragments were injected into the yolk of 1- to 2-cell stage zebrafish embryos at a concentration between 50 and 100 ng/μl. Injections of *I-Sce*1-modified plasmids were performed as described (Thermes et al., 2002), with some modifications. Embryos were placed in 10% Hank's solution and injected at room temperature. DNA was injected through the chorion into the cytoplasm of one cell stage embryos. The injection solution contained 10 ng/μl plasmid DNA, *I-Sce*1 meganuclease buffer 0.5× (New England Biolabs), 1 μg/μl *I-Sce*1 meganuclease (New England Biolabs) and 0.1% phenol red. *gfp*-expressing embryos were raised to adulthood and transgenic carriers were identified by crossing with wild-type fish.

GFP expression was analysed with a Leica DMIRBE inverted microscope. *In situ* hybridisation was performed as described (Oxtoby and Jowett, 1993).

Results

A shh transgene drives faithful reporter expression in the embryonic midline

We generated stable transgenic lines (referred as *−2.4shh:gfpABC* or wild-type transgene) with *shh* sequence from −2432 to +221 relative to the *shh* transcription start site inserted upstream of the *green fluorescent protein* (*gfp*) reporter gene and *shh* sequences from +549 to +5366 inserted downstream of *gfp* (Chang et al., 1997). Expression of this transgene, which contains all three intronic enhancer sequences *ar-A*, *-B* and *-C*, is first detected in the embryonic shield in a pattern similar to that of the endogenous *shh* gene (Figs. 1A and 2A) (Krauss et al., 1993). At the 8-somite stage, the transgene is expressed in the notochord, the floor plate and in the anlage of the ventral forebrain as described for the



D

Constructs	Abbr.	lines
-2.4 gfp A B C	-2.4shh:gfpABC	(ABC)	3
-2.4 gfp	-2.4shh:gfp	(-)	2
-2.4 gfp A	-2.4shh:gfpA	(A)	4
-2.4 gfp B	-2.4shh:gfpB	(B)	4
-2.4 gfp C	-2.4shh:gfpC	(C)	5
-2.4 gfp A B	-2.4shh:gfpAB	(AB)	2
-2.4 gfp A C	-2.4shh:gfpAC	(AC)	2
-2.4 gfp B C	-2.4shh:gfpBC	(BC)	3

Fig. 1. The −*2.4shh:gfpABC* transgene recapitulates the expression of the endogenous *shh* gene. GFP expression was detected in the embryonic shield (A, 70% epiboly), the notochord and neural plate (B, 8-somite stage) and in the ventral neural tube (including the zona limitans intrathalamica, zli) (C, 48 hpf, for further details of the brain expression see also Fig. 6). The *shh* minilocus is composed of a 2.4 kb promoter and 5.8 kb downstream including the previously described activating region A (*ar-A*) and B (*ar-B*) in the first intron and the activating region C (*ar-C*) in the second intron. A series of deletion constructs was generated to test the activating regions either alone or in the three possible combinations. The −*2.4shh:gfp* construct lacking the intronic activating regions served as a promoter control. Several stable transgenic lines were generated from each construct to assess integration site effects (number of lines is indicated). Orientation of embryos: anterior is up, dorsal is right (A) and anterior is left and dorsal is up (B, C). Abbreviations: fp, floor plate; hy, hypothalamus; l, lens; zli, zona limitans intrathalamica.

endogenous *shh* gene (Fig. 1B) (Krauss et al., 1993). Expression in the 48 hpf brain includes the zona limitans intrathalamica (zli), the tegmentum and the hypothalamus (Fig. 1C and data not shown). GFP expression was also detectable in the entire medial floor plate at this stage and was still detectable at three weeks after fertilisation in the brain and the ventral midline of the spinal cord (data not shown). With the exception of ectopic expression in the anal region, the transgene was not expressed in the endoderm, indicating that regulatory regions crucial for endoderm expression are missing in the transgene (Krauss et al., 1993; Strahle et al., 1996). Three independent lines carrying the −*2.4shh:gfpABC* transgene gave identical results in the midline (Fig. 1). The transgenes are also expressed in the pectoral fins. This expression differs, however, among lines. Moreover, the fin expression does not recapitulate the pattern of *shh*, suggesting integration site effects and lack of fin regulatory modules (data not shown).

In summary, our results indicate that the re-constructed *shh* mini-locus recapitulates expression of the endogenous *shh* gene in the neural tube and in the notochord. These results confirm and extend the conclusion drawn from our previous transient analysis at 24 hpf (Muller et al., 1999).

ar-A and ar-C control expression in the embryonic shield

With the onset of gastrulation, *shh* starts to be expressed in the embryonic shield and then in the extending midline mesoderm, comprising notochord and prechordal plate (Krauss et al., 1993) (Fig. 2A). The wild-type transgene −*2.4shh:*

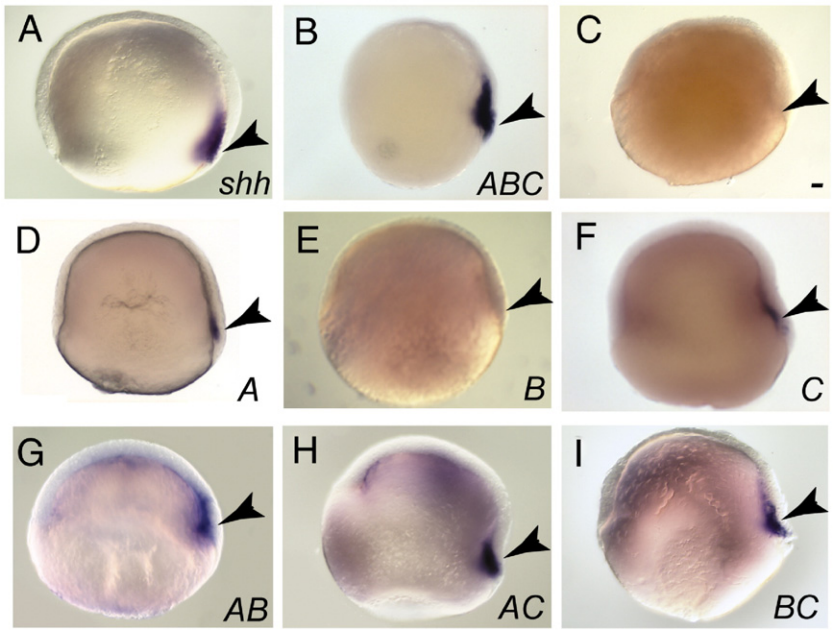


Fig. 2. *ar-A* and *ar-C* direct expression in the shield. Shield stage embryos were hybridised to a *shh* (A) or a *gfp* (B–I) antisense probe. At 50% epiboly, *shh* expression is restricted to the shield (A, arrowhead). The full-length construct *−2.4shh:gfpABC* mimics this expression (B) while a transgenes deleted for the intronic sequences (C) or containing *ar-B* alone (E) show no expression in the embryonic shield. All constructs containing *ar-A* alone (D), *ar-C* alone (F) or in combinations – *ar-A* and *ar-B* (G), *ar-A* and *ar-C* (H), *ar-B* and *ar-C* (I) – direct expression in the shield (arrowhead). Lateral views, animal pole up and dorsal right. The transgenic constructs are indicated in the bottom right corner. See Fig. 1 for the structure of the transgenes.

gfpABC recapitulates the expression of the endogenous *shh* gene in the embryonic shield (Figs. 1A and 2B).

To delineate the enhancer regions responsible for expression in the embryonic shield, deletion derivatives were analysed in the gastrulating embryo. The wild-type transgene had been designed in such a way that the intronic regulatory regions (Muller et al., 1999) could be tested alone or in combination (Fig. 1D). Previously, we narrowed down the three enhancer elements *ar-A*, *-B* and *-C* to 250 to 500 bp in introns 1 and 2 (Muller et al., 1999). For the purpose of this study, we decided, however, to leave the spacing and sequences between the three elements as intact as possible, just introducing unique restriction sites to delete individual elements.

The expression activity of the promoter construct *−2.4shh:gfp*, that lacks the elements *ar-A*, *-B*, *-C*, in the anterior floor plate at 24 hpf served as a control for transgene integration and expression (Fig. 1D). Transgenes were stably introduced into the germ line. Each transgene was analysed in at least two independently established transgenic lines (Fig. 1D). Expression of the reporter gene was monitored by *in situ* hybridisation with an antisense probe complementary to *gfp* mRNA (Fig. 2).

The promoter construct *−2.4shh:gfp* does not mediate *gfp* mRNA expression in the embryonic shield (Fig. 2C) indicating that the promoter is not sufficient. Transgenes containing either *ar-A* (*−2.4.shh:gfpA*) or *ar-C* (*−2.4shh:gfpC*) are expressed in the embryonic shield (Figs. 2D, F) suggesting that both regulatory regions direct expression to the embryonic shield. Transgene *−2.4shh:gfpB* is inactive at this stage (Fig. 2E) indicating that *ar-B* is not sufficient to drive expression in the embryonic shield (0/2 lines analysed). *gfp* expression is, however, detectable in these lines (4/4 lines) at later stages (see Fig. 5E for example) indicating that these transgenes were integrated and can be expressed. Transgenes containing pairwise combinations of either *ar-A* or *ar-C* with *ar-B* are also expressed in the embryonic shield (Figs. 2G, H, I).

Distinct regulatory regions co-operate to drive expression in the floor plate and notochord at early somitogenesis stages

At the 3-somite stage, the emergence of the notochord, somitic mesoderm and neural plate highlight the dorsoventral subdivision of the embryo. Also the pattern of expression of *shh* has significantly been modified at this stage anticipating the later expression in the body axis. At the 3-somite stage, *shh* is expressed strongly in the midline of the anterior neural plate, giving rise to the ventral forebrain. In addition, expression is detectable in the notochord and in the midline of the posterior neural plate, the nascent floor plate of the future midbrain, hindbrain and spinal cord (Krauss et al., 1993) (Fig. 3A).

The transgenes *−2.4shh:gfpABC* harbouring all three activating regions drive expression in the midline of the 3-somite stage embryo, in a pattern similar to that of the endogenous *shh* gene (Fig. 3B). In the absence of the three activating regions, expression was missing with the exception of a restricted region in the midline of the anterior neural plate representing the anlage of the anterior floor plate (Fig. 3C).

The transgenic lines harbouring one of the three activating regions alone (Figs. 3D to F) or in pairwise combinations (Figs. 3G to I) reproduce the pattern of the wild-type *−2.4shh:gfpABC*

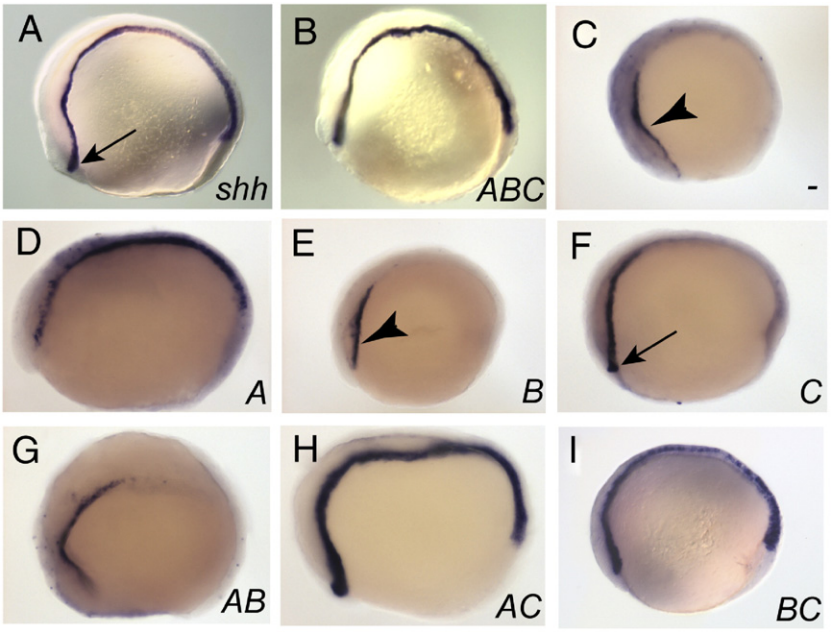


Fig. 3. The shield enhancers *ar-A* and *ar-C* mediate strong expression in the notochord in early somitogenesis stage embryos. Embryos at the 3-somite stage were hybridised to the *shh* (A) or the *gfp* (B–I) antisense probe. The full-length transgene *−2.4shh:gfpABC* (B) drives expression in the embryonic midline in a similar manner to the endogenous *shh* gene (A) including the floor plate (see Fig. 4 for higher magnification). Lack of the intronic sequences in the *−2.4shh:gfp* transgene restricts expression to the midline of the presumptive ventral midbrain and hindbrain (C, arrowhead). Note that the expression in the more rostral part is strongly reduced or missing compared to that of the endogenous *shh* gene or the *−2.4shh:gfpABC* transgene. The presence of the *ar-A* fragment restores expression in the notochord (D) whereas addition of the *ar-B* fragment (E, arrowhead) does not significantly change the pattern of *gfp* mRNA expression in comparison to *−2.4shh:gfp* (C, arrowhead) expression. The presence of *ar-C* leads, however, to partial rescue of expression in the posterior notochord and increases expression in the midline of the rostral neural plate (F, arrow) in a manner similar to the *shh* gene (A, arrow). Fragments *ar-A* and *ar-B* in combination do not restore the expression in the notochord contrary to *ar-A* alone (G) (suggesting that *ar-B* can repress the activity of *ar-A*). *ar-A* and *ar-C* combined give a strong expression in the notochord and anterior neural plate (H and Fig. 4D). The combination of *ar-B* and *ar-C* drive expression in the notochord and in the midline of the neural plate including posterior regions forming the floor plate (I). Lateral views, anterior is left and dorsal is up. The transgenic constructs are indicated in the bottom right corner. See Fig. 1 for the structure of the transgenes.

lines only partially. This suggests that expression in the midline of the 3-somite stage embryo requires the cooperation of all three modules. *ar-A* or *-C* alone drives weak expression in the notochord. However, a combination of the two modules results in a much stronger expression in the notochord in both independently isolated transgenic lines (compare Figs. 3D, F with H).

Transgenic lines containing *ar-C* (Figs. 3F, H, I) show expression in the midline of the anterior neural plate like the endogenous *shh* or the wild-type transgene (Figs. 3A, B). This suggests a regulatory function for *ar-C* in the anlage of the hypothalamus.

At the 3-somite stage, *−2.4shh:gfpA*, *−2.4shh:gfpB*, *−2.4shh:gfpC*, *−2.4shh:gfpAB* and *−2.4shh:gfpAC* are not expressed in the midline of the posterior neural plate, the anlage of the floor plate of the spinal cord (Figs. 3D–H and 4D). Expression in the midline of the posterior neural plate was obtained, when *ar-B* was combined with *ar-C* (Figs. 3I and 4E) suggesting that *ar-C* interacts with *ar-B* in the floor plate. Transgenes *−2.4shh:gfpAC* without *ar-B* has strong notochord expression but lacks floor plate expression (Figs. 4B, D). Taken together, enhancer *ar-C* appears to play a role as a synergising enhancer that increases the activity of *ar-A* and *ar-B* in the 3-somite stage embryo. At the 6- to 10-somite stage, *−2.4shh:gfpB* transgenes start to be expressed in the posterior midline, extending midline in a progressive fashion more posteriorly (data not shown). This suggests that *ar-B* can drive floor plate expression independent from *ar-C* at later stages (4/4 lines).

Floor plate and notochord expression in the 1-day-old embryo require multiple modules

At 24 hpf, *shh* expression in the trunk and the tail is restricted to the medial floor plate, the ventral brain and the posterior part of the notochord (Krauss et al., 1993) (Fig. 5A). These *shh* expression territories are reproduced by the wild-type transgene harbouring all three modules (Fig. 5B). The deletion of the three downstream modules in transgene *−2.4shh:gfp* restricts expression to the anterior floor plate (Fig. 5C). The location of *ar-D* mediating anterior floor plate expression was mapped by transient co-injection experiments to the regions between −1413 and −913. This region harbours a 220-bp stretch with homology to the SFPE1 of the mouse *shh* gene (data not shown).

When *ar-B* was placed downstream of the *−2.4shh:gfp* reporter construct (*−2.4shh:gfpB*, Fig. 5E), expression was restored in the posterior floor plate, indicating that *ar-B* can mediate expression in the posterior floor plate in the 1-day-old embryo. The floor plate expression is the result of the

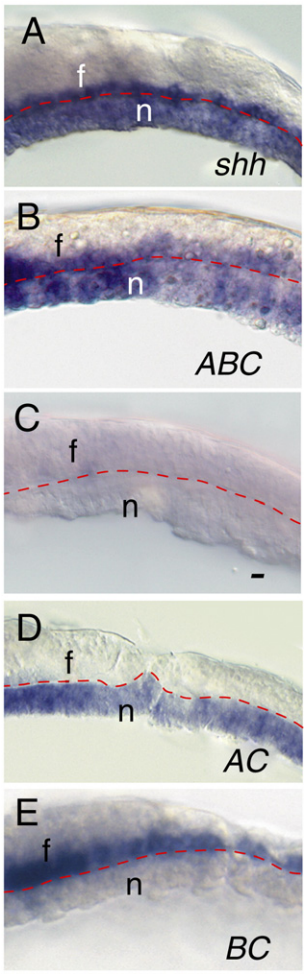


Fig. 4. The combined action of *ar-B* and *ar-C* is required for expression in the floor plate at the 3-somite stage. 3-somite stage embryos were hybridised to a *shh* (A) or a *gfp* antisense probe (B–E). The endogenous *shh* gene is expressed in the floor plate and notochord (A) like the full-length transgene *−2.4shh:gfpABC* (B). Deletion of *ar-A*, *ar-B and ar-C* abolishes the expression in the floor plate (C). The combination of *ar-A* and *ar-C* mediates notochord expression (D). The combination of *ar-B* with *ar-C* mediates expression in the floor plate (E). Lateral view, anterior is left and dorsal is up. f: floor plate, n: notochord. The dashed line highlights the border between the notochord and the floor plate. The transgene analysed is indicated in the bottom right corner of each panel. The yolk was removed and the embryonic midline dissected with needles.

composite activities of *ar-B* and *ar-D* (Figs. 5C, E). Similarly, the other *ar-B* containing transgenes also show expression in the posterior medial floor plate (Figs. 5B, G, I). However, *ar-C* is dispensable for floor plate expression at this stage in contrast to the 3-somite stage (Fig. 5F). None of the *−2.2shh:gfpC* lines (5/5 lines) shows expression of *gfp* mRNA in the medial floor plate in the 1-day-old embryo. When we analysed however the more sensitive and stable expression of the green fluorescent protein (GFP), 1 out of the 5 *−2.4shh:gfpC* lines showed weak GFP expression in posterior regions of the spinal cord**.** Moreover, transient mosaic *gfp* expression was noted in the posterior floor plate when a transgene containing only the 240 bp conserved core of *ar-C* was tested in a stably integrated state (1 out of 2 lines, data not shown) or in transient expression experiments (Muller et al., 1999). This suggests that *ar-C* can have weak and transient floor plate enhancer activity. Irrespective of this activity of *ar-C*, our results show that timely establishment of early expression in the floor plate requires the interaction of *ar-B* and *ar-C*. However, at later stages, *ar-B* is sufficient to drive expression in the floor plate on its own.

Normally, at 24 hpf, only the posterior tip of the notochord expresses *shh* mRNA and this expression is reproduced by the wild-type transgene (Figs. 5A, B). Similarly, when *ar-A* and *ar-C* are present (Figs. 5B, H) the posterior end of the notochord shows prominent expression of *gfp* mRNA (two out of two *−2.4shh:gfpAC* lines, three out of three *−2.4shh:gfpABC* lines) while transgenes containing *ar-A* or *ar-C* alone have weak or no expression in the notochord (except one out of five *−2.4shh:gfpC* lines). This cooperation is also reflected in the results from transient expression experiments: *−2.4shh:gfpAC* injected embryos expressed more frequently in the notochord (83%, $n=50$), than embryos injected with *−2.4shh:gfpA* (15%, $n=33$) or *−2.4shh:gfpC* (30%, $n=42$) alone. Thus, the combination of *ar-A* and *ar-C* appears to mediate stronger notochord expression not only in the 3-somite stage but also at 24 hpf. This contrasts with the cooperation between *ar-B* and *ar-C* for floor plate expression, which is no longer evident at 24 hpf (Figs. 5E, I).

The presence of enhancer *ar-A* leads to expression in the anus (Figs. 5B, D, G, H). However, in comparison with the endogenous gene at the same stage (Fig. 5A), the transgene appears to be expressed ectopically in the anal region. Variable ectopic expression was noted in the hindbrain region of several lines (e.g. Figs. 5E, G). However, the patterns were not reproducible and could not be correlated with the presence of a particular regulatory region suggesting integration site effects.

Regulatory region ar-C mediates expression in the zona limitans intrathalamica

In the brain of 24 hpf embryos, *shh* expression is detectable in the mid-diencephalic boundary forming the zli, in the hypothalamus, in a small area in the telencephalon and in the floor plate of the midbrain and hindbrain (Barth and Wilson, 1995; Krauss et al., 1993; Macdonald et al., 1995; Strahle et al., 1996; Wilson and Rubenstein, 2000) (Fig. 5A). The zli is an important signalling centre in the diencephalon and Shh plays a crucial function in its activity (Scholpp et al., 2006). As the zli becomes more prominent at later stages, we analysed transgene expression in the brain of the 32 hpf stages in detail.

The full-length transgene recapitulates diencephalic expression of the endogenous *shh* gene (Figs. 6A, B). Deletion of the activating sequences *ar-A*, *-B* and *-C* restricts the reporter expression to the floor plate of the midbrain (Fig. 6C). Insertion of enhancer *ar-A* or *ar-B* downstream of *−2.4shh:gfp* leads to expression in the tegmentum (Figs. 6D, E). The presence of region *ar-C* restores prominent expression in the zli in addition to expression in the tegmentum (Fig. 6F). All the other transgenes carrying enhancer *ar-C* show expression in the zli, strongly indicating that *ar-C* is crucial for driving expression in the zli. Unexpectedly, the combination of *ar-A* with *ar-B* (Fig.

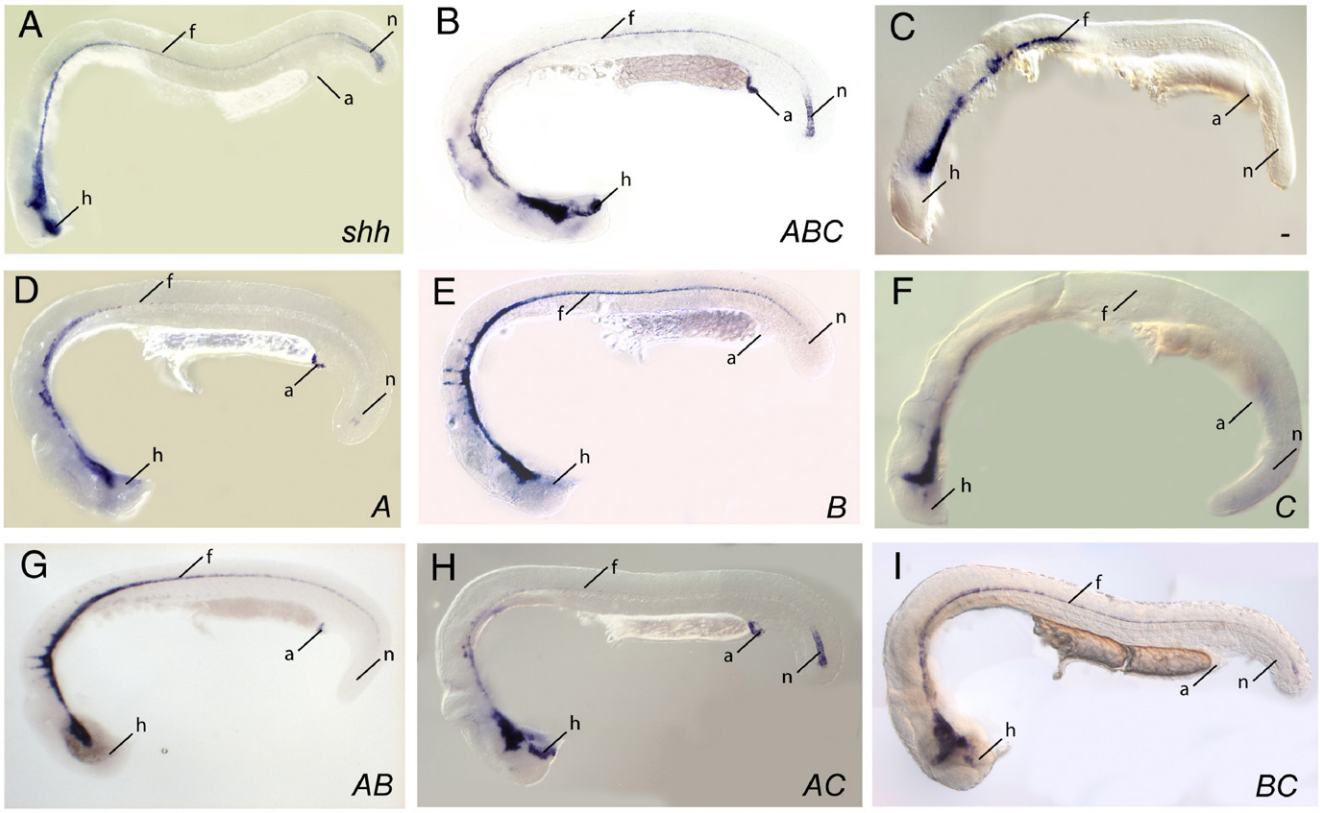


Fig. 5. The expression in the midline of the 1-day-old embryo depends on the combination of several activating regions. One-day-old embryos were hybridised to a *shh* (A) or a *gfp* (B–I) antisense probe. At 24 h, *shh* is expressed in the hypothalamus, tegmentum, floor plate and the posterior part of the notochord (A). *shh* is also expressed in the zona limitans intrathalamica (zli) but, due to its small size, the zli is not always clearly distinguishable at this stage. The full-length transgene *−2.4shh:gfpABC* recapitulates *shh* expression in the midline. It also shows an ectopic expression in the anus (B). Deletion of the downstream-activating sequences restricts the transgene expression to the cephalic floor plate (C). Addition of the *ar-A* gives a very weak expression in the posterior notochord but also an ectopic expression in the anus (D). Addition of the *ar-B* restores the expression in the trunk and tail floor plate (E) whereas addition of the *ar-C* restores the expression in the zli (F) and rarely in the hypothalamus (1 out of 5 lines). The combinations *ar-A* plus *ar-B* (G) and *ar-B* plus *ar-C* (I) show an additive effect. The combination *ar-A* plus *ar-C* leads to the correct expression in the hypothalamus and the posterior notochord (H). Yolks were removed and embryos oriented with anterior is left and dorsal is up. a: anus, f: floor plate, h: hypothalamus, n: notochord.

6G) abolishes the expression in the tegmentum in 2 out of 2 stable lines. Whether this reflects an artefact caused by the integration site or whether this is a genuine repressive effect of the *ar-A/B* combination remains to be determined.

Forebrain expression depends on all three activating regions

The hypothalamus originates from the midline of the anterior neural plate (Woo and Fraser, 1995). At the 3-somite stage, transgenic lines carrying enhancer *ar-C* drive expression in the midline of the anterior neural plate in a pattern similar to that of the endogenous *shh* gene and the wild-type transgene (Figs. 3B, F, H, I). However, hypothalamic expression at 24 hpf was not detected in all transgenic lines harbouring *ar-C* (Figs. 5B, F, H, I). Only 1 out of the 5 transgenic lines carrying *−2.4shh:gfpC* had *gfp* mRNA expression in the hypothalamus suggesting that *ar-C* is not sufficient to maintain *gfp* mRNA expression at 24 hpf. We detected, however, in all the lines (5/5) the rather stable GFP protein in the hypothalamus at this stage (data not shown) representing an echo of the expression of the *−2.4shh:gfpC* transgene in the anlage of the hypothalamus earlier. Lines containing either all three enhancers (Fig. 5B) or the combination of *ar-A* with *ar-C* (Fig. 5H) had significant *gfp* mRNA expression in the hypothalamus at 24 hpf. Thus, the combination of *ar-A* with *ar-C* appears to be necessary for efficient hypothalamic expression at 24 hpf.

At 32 hpf, hypothalamic expression has faded in lines containing the combination of *ar-A* with *ar-C* (2/2 *−2.4shh:gfpAC* lines) (compare Figs. 5H and 6H). All three activating regions are necessary to maintain expression in the hypothalamus of a 32 hpf embryo (2/2 lines analysed) (Fig. 6B). The fact that *ar-C*-containing lines display *gfp* expression in the anlage of the hypothalamus at the 3-somite stage (Figs. 3B, F, H and I) suggests that distinct regulatory mechanisms underlie establishment and maintenance of hypothalamic expression.

Discussion

We characterised here regulatory modules in the *shh* locus that control expression in the embryonic shield, notochord, floor plate, zli, and hypothalamus. The analysed regulatory regions have both unique and overlapping functions. Moreover, in several instances, regulatory modules cooperate to establish and/or maintain expression in particular structures, suggesting

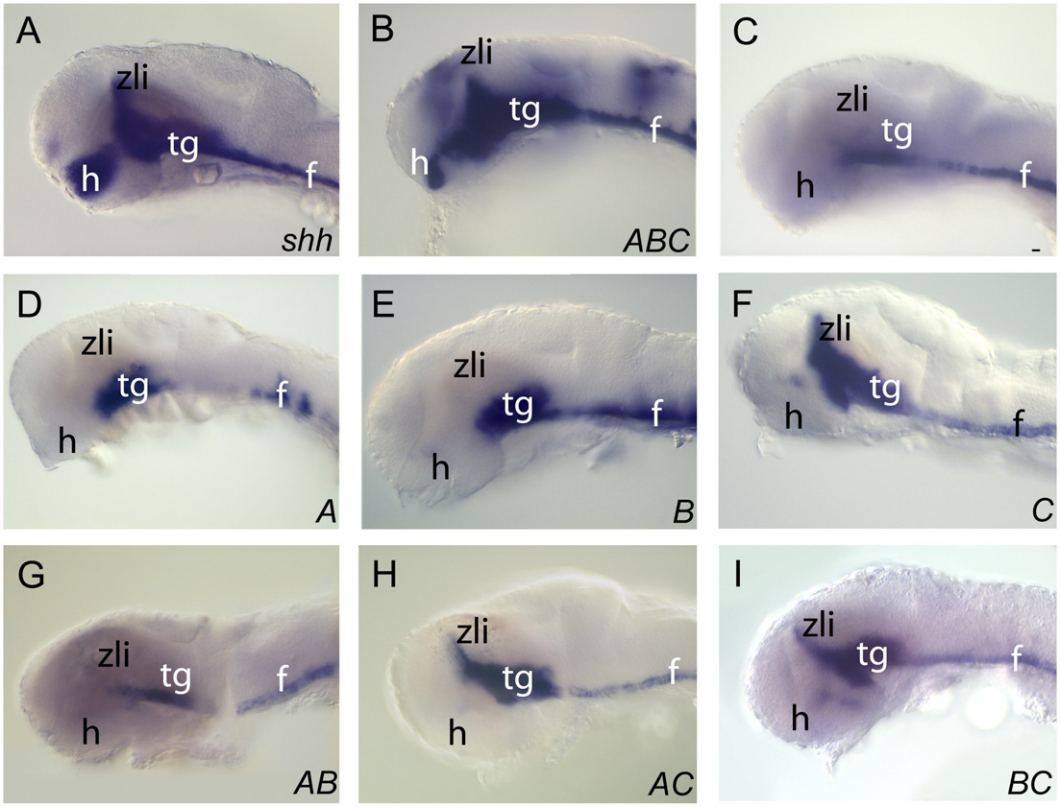


Fig. 6. Strong expression in the hypothalamus of the 32 hpf embryo requires all three activating regions. Embryos were hybridised to a *shh* (A) or a *gfp* (B–I) antisense probes. In the brain of the 32 hpf embryo, *shh* is strongly expressed in the dorsoanterior region of the hypothalamus (h), the zona limitans intrathalamica (zli) the tegmentum (tg) and the floor plate (f) (A). The *−2.4shh:gfpABC* (B) mimics this pattern of expression while the expression of *−2.4shh:gfp* is restricted to the cephalic floor plate (C). The presence of *ar-A* alone does not add to the pattern of expression (D, note that the *−2.4shh:gfpA* line shows mosaic expression in the cephalic floor plate). Similarly, *ar-B* (E) does not add to the overall pattern of *gfp* mRNA expression in the anterior brain although these transgenes show more robust expression in the floor plate. The presence of *ar-C* causes additional expression in the zona limitans intrathalamica. The *−2.4shh:gfpAB* lines (G) express in the same pattern as the *−2.4shh:gfp* line (C). The expression in the tegmentum appears slightly reduced in these lines, consistent with a repressive activity of *ar-B*, which was noted also in the context of the notochord expression at the 3-somite stage (Figs. 3D, G). The *−2.4shh:gfpAC* lines (H) and the *−2.4shh:gfpBC* (I) lines drive expression in a similar pattern as the *−2.4shh:gfpC* lines at this stage. However, in contrast to earlier stages, expression was not detected in the hypothalamus, supporting the notion that the combination of *ar-A* and *ar-C* is not sufficient to mediate expression in the hypothalamus at the 32 hpf stage. Expression in the hypothalamus at this stage requires rather the presence of all three activating regions (B). f: floor plate, h: hypothalamus, tg: tegmentum, zli: zona limitans intrathalamica. Yolks were removed. Embryos are oriented rostral left, dorsal up. The constructs are indicated in the bottom right corner.

that the expression of the *shh* locus is controlled by the orchestrated interaction of several enhancers (Fig. 7 for summary).

Floor plate and notochord expression

It was demonstrated that notochord and floor plate are derived from a common cell pool in the organiser of the chicken embryo (Catala et al., 1996; Le Douarin and Halpern, 2000), leading to the notion that the floor plate is not induced by the notochord as previously suggested (Catala et al., 1996; Le Douarin and Halpern, 2000; Placzek et al., 2000). Rather, floor plate appears to be the result of the distribution of cells from a pool of pre-specified precursors in the organiser. A similar scenario was put forward for the floor plate and notochord precursors in the zebrafish embryonic shield (Le Douarin and Halpern, 2000). The arguments were supported by the early shield expression of the floor plate-specific *tiggy winkle hedgehog* (*twhh*) (Ekker et al., 1995; Etheridge et al., 2001). Moreover, analysis of a *delta-A* mutant also suggested that the embryonic shield contains a common pool of precursor cells for notochord and floor plate (Appel et al., 1999).

The regulatory regions *ar-A* and *-C*, that drive expression in the embryonic shield, direct expression in the notochord. We did not detect *gfp* RNA or GFP protein expression in the midline of the neural plate in *−2.4shh:gfpA* and *−2.4shh:gfpAC* transgenic embryos and only rarely in *−2.4shh:gfpC* transgenics at later somitogenesis stages. This argues that notochord precursors form a distinct population already at the beginning of *shh* expression at the early gastrula stage. The situation in zebrafish appears to be similar to that in the mouse where distinct regulatory modules also control *shh* expression in the floor plate and notochord (Jeong and Epstein, 2003).

Expression in the floor plate is under the main influence of two distinct regulatory regions, *ar-B* and the −2.4 kb promoter containing the promoter–distal module *ar-D* (Muller et al., 1999, 2000). The activity of these two regulatory regions differs along the AP axis. While the −2.4 kb promoter is mostly active

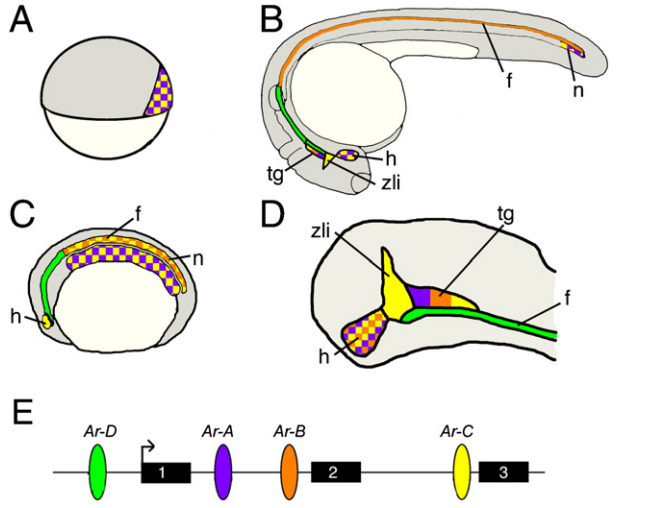


Fig. 7. Summary of the enhancer activities of the zebrafish *shh* locus in (A) shield stage embryos, (B) 3-somite stage, (C) 24 hpf embryo and (D) 32 hpf brain. The activating regions *ar-A*, *ar-B* and *ar-C* and the promoters are colour-coded. Enhancer activities are indicated in panels A to D by colour combinations. The white area in panel D represents the tegmentum in which expression is mediated by *ar-A*, *-B* or *-C* alone. (Note that the combination of *ar-A* and *-B* does not drive the expression in this region suggesting a repressive effect of this combination.) The expression in the dorsoventral hypothalamus at 32 hpf requires the combined action of all three activating regions (D) while *ar-C* and the combination of *ar-A* with *ar-C* at the 3-somite (B) and 24 hpf (D), respectively, are sufficient. f: floor plate, h: hypothalamus, tg: tegmentum, zli: zona limitans intrathalamica.

in the floor plate of the midbrain, hindbrain and anterior trunk, *ar-B* directs expression also in the posterior floor plate. The *ar-D* in the extended promoter shares homology with the enhancer *SFPE1* of the mouse *Shh* gene (Epstein et al., 1999; Muller et al., 1999, 2000) (Hadzhiev, unpublished data), which also mediates floor plate expression (Epstein et al., 1999).

The activity patterns of enhancers *ar-A* and *-C* in the notochord and the modules *ar-B* and *-D* in the floor plate reflect the mechanistic differences between notochord and floor plate expression. Our data are consistent with an induction of *shh* expression in the floor plate that progresses from anterior to posterior at post-gastrulation stages. The timing of the expression of *−2.4shh:gfpB* transgenes in the posterior floor plate coincides with the postulated activity of Midkines that were recently shown in zebrafish to be required for floor plate differentiation (Schafer et al., 2005). *ar-B* may thus be a target of Midkine signalling. It is clear that the activity of both *ar-B* and the *−2.4* promoter is dependent also on Cyclops (Cyc) signals: *cyc* mutants lack expression of the transgenes in the ventral neural tube during somitogenesis and ectopic activation of the Cyc pathway turns on expression of the transgenes in both zebrafish and chicken embryos (Albert et al., 2003; Muller et al., 2000) (and data not shown). Although Shh appears to play no role in medial floor plate induction in zebrafish (Etheridge et al., 2001; Strahle et al., 1997, 2004), there is evidence that Shh is necessary for late differentiation of medial floor plate in *cyc* mutants and maintenance of the medial floor plate in older stages (Albert et al., 2003; Chen et al., 2001; Varga et al., 2001). Preliminary data suggest that this late auto-regulatory input of Shh is mediated by *ar-B* (Müller and Strähle, unpublished data). Thus multiple regulatory pathways appear to converge onto the *ar-B* and *-D* enhancers, although it remains to be determined whether this is direct.

The intronic enhancer regions act synergistically

Our data suggest that the overall pattern of *shh* expression requires the activity of the three intronic activating regions in addition to the promoter. In some locations, the interactions appear to be additive in their effects as in the case of the floor plate expression mediated by the promoter and *ar-B*. In other instances, however, the combinations of enhancers show clear synergies that affect temporal, spatial and quantitative aspects of expression.

The establishment of floor plate expression is occurring in time in lines containing *ar-C* in addition to *ar-B*. Thus, although *ar-C* is not able to drive floor plate expression on its own very efficiently, it is able to cooperate with *ar-B*. Moreover, the *gfp* mRNA levels in the notochord appear to be more than additive when one compares the *gfp* expression in the notochord of *−2.4shh:gfpAC* with that of *−2.4shh:gfpA* and *−2.4shh:gfpC* transgenics (Figs. 3H and 5H). These changes in expression are unlikely to be due to integration site effects or transgene copy number as similar levels of *gfp* mRNA expression were noted in the ventral midbrain anlage of *−2.4shh:gfp*, *−2.4shh:gfpA*, *−2.4shh:gfpB*, *−2.4shh:gfpC* and *−2.4shh:gfpAC*.

A strong combinatorial requirement of enhancer sequences was also noted in the hypothalamus at 32 hpf where the presence of all three intronic enhancer regions is necessary for expression of the reporter. Strikingly, the requirement for interactions seems to change during development. While *ar-C* is sufficient to establish reporter gene expression in the anlage of the hypothalamus at neural plate stages (Woo and Fraser, 1995) the interaction with first *ar-A* and then also *ar-B* is required to maintain expression in the hypothalamus in the 24 and 32 hpf old embryo, respectively. This reflects most likely the complex interplay of signalling inputs that are required for development of the anteriodorsal aspect of the hypothalamus. Shh, Nodal and Wnt signals have been implicated in its specification (Kapsimali et al., 2004; Mathieu et al., 2002). Indeed, inhibition of Wnt signalling, as suggested by the analysis of the phenotype of the *axin1* mutant *masterblind*, was implicated in the determination of hypothalamic identity (Kapsimali et al., 2004). In addition, Nodal signals are cell-autonomously required for specification of the posterior ventral hypothalamus and indirectly by specifying axial mesendoderm for differentiation of the anteriodorsal hypothalmus (Mathieu et al., 2002). Finally, Shh signalling itself appears to be involved in the differentiation of the dorsoanterior hypothalamus (Mathieu et al., 2002), which is the main expression domain of Shh at 32 hpf. The synergism of regulatory modules may reflect the integration of the different activating and inhibitory inputs that control the spatial and temporal aspects of *shh* expression in the hypothalamus.

It is obvious that the mosaicism is reduced when all three downstream enhancers are present. For example, transgenes

containing *ar-B* alone showed frequently mosaic expression (2/4 transgenic lines). This mosaicism was strongly reduced or absent, when other modules were combined with *ar-B*. The combination of the enhancers may provide a chromatin environment that makes expression less prone to negative influences from the neighbourhood.

Other examples of such enhancer synergism are scarce in the vertebrate literature, possibly because these modules are usually analysed in isolation. Analysis of the mouse *ikaros* locus encoding a zinc finger transcription factor required for the balanced production of a number of blood and immune cells indicated a similar functional interdependence of regulatory regions: while the DHS-C3 region was active during early T-cell differentiation, the interaction with a downstream second regulatory regions DHS-C6 was necessary to maintain high expression in T-cells during later stages (Kaufmann et al., 2003). Crosstalk between individual modules is a major theme in the *cis*-regulatory system of the *endo16* gene of the invertebrate *Strongylocentrotus purpuratus*. Module A, which controls establishment of *endo16* expression in the vegetal plate during early development, interacts with modules G and B during later stages to drive high level expression in the gut (Yuh et al., 1998). Only one (module A) of the seven mapped *cis*-regulatory modules of the *endo16* gene encodes spatial information. The other modules act by influencing the activity of module A (Yuh et al., 1998). In contrast, the interaction of the regulatory modules of zebrafish *shh* appear to generate novel pattern of expression.

Structural but not functional conservation of enhancer sequences

The zli is an organising centre in the vertebrate forebrain that is required for correct patterning of the thalamic and prethalamic areas of the brain (Scholpp et al., 2006). Hedgehog proteins including Shh secreted from the zli are the crucial signals controlling these patterning events in the flanking brain regions (Scholpp et al., 2006). The *ar-C* is required for expression in the zebrafish zli and its regulatory activity appears to be relatively independent of other activating regions. In addition, the *ar-C* region is also responsible for expression in the notochord and is required for expression in the hypothalamus.

The *ar-C* harbours a 240-bp sequence that shares sequence similarity with the *SFPE2* of the mouse *Shh* gene (Muller et al., 1999, 2002). Transgenes harbouring only this 240-bp sequence mediate zli and notochord expression in the zebrafish embryo in the same way as the lines containing the larger *ar-C* sequence (unpublished data) suggesting that the conserved core is sufficient for directing expression in the two tissues.

Mouse *shh* intron 2 sequences can direct expression to the zli (Epstein et al., 1999). However, these sequences named SBE1 lie upstream of ar-C/SFPE2 (see Fig. 8). SBE1 cooperates with SFPE2 to mediate weak and transient notochord expression in the mouse (Epstein et al., 1999, Jeong and Epstein, 2003) while isolated *SFPE2* directs predominantly expression in the floor plate. Although sub-fragments of mouse *SFPE2* appear to have notochord activity (Jeong and Epstein, 2003) as was also noted

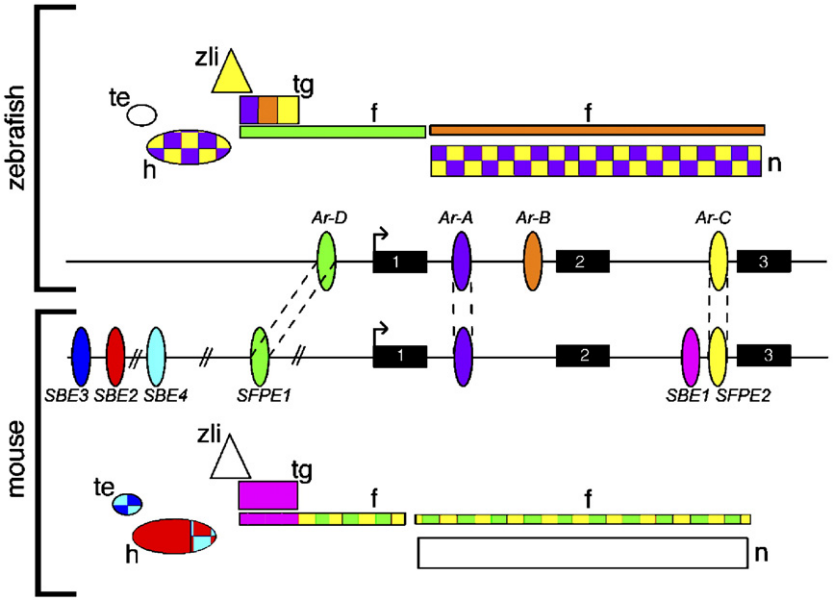


Fig. 8. Comparison of the *shh* enhancer regions driving expression in the central nervous system and the notochord in zebrafish and mouse. Zebrafish *shh* enhancers *ar-A*, *ar-D*, and *ar-C* share sequence similarity with regions in the *Shh* locus of mouse, the latter two being named *SFPE1* and *SFPE2* in the mouse, respectively. While the *SFPE1/ar-D* regions share functional properties by mediating both floor plate expression, the *SFPE2/ar-C* enhancers show differing activities in zebrafish and mouse, giving mostly floor plate in mouse (Epstein et al., 1999) and mostly notochord in zebrafish (Muller et al., 1999). Moreover, the *ar-C* activity in the zli and hypothalamus of the zebrafish embryo is not mediated by *SFPE2* of mouse *Shh*. Rather four distinct sequences (*SBE1* to *-4*) direct reporter gene expression in the diencephalon of the mouse (Epstein et al., 1999, Jeong et al., 2006). While all three enhancer regions *ar-A*, *-B*, *-C* can have activity in the tegmentum of the zebrafish midbrain, a distinct enhancer region in intron 2 (*SBE1*) mediates expression in the mouse midbrain. Moreover, Epstein and co-workers did not detect reporter activity in the mouse *shh* intron 1 despite the fact that *ar-A* is strongly conserved and mediates notochord expression in zebrafish. f: floor plate, h: hypothalamus, n: notochord, tg: tegmentum, zli: zona limitans intrathalamica, te: telencephalon.

for deletion fragments of the other floor plate enhancer *SFPE1* (Epstein et al., 1999), the intact regions mediate floor plate expression in the mouse (Jeong et al., 2006). Thus, *ar-C* of zebrafish *shh* has features that differ from the structurally homologous *SFPE2*. We have no functional evidence that *SBE1* of mouse intron 2 exists in the zebrafish. The activity of *SBE1* appears to reside in *ar-C*.

In contrast to zebrafish *ar-C*, intron 2 of the mouse does not have regulatory activity in the hypothalamus (Jeong et al., 2006). Instead, in the mouse *Shh* locus, regulatory sequences that reside 400 kb upstream of the promoter mediate expression in the hypothalamus (Jeong et al., 2006) (see Fig. 8 for summary).

Moreover, the mouse *Shh* intron 1, despite its striking sequence conservation with *ar-A* (Muller et al., 2002), does not direct notochord expression in the mouse (Jeong et al., 2006). Thus, there can be dramatic changes in the tissue-specific activity of structurally conserved enhancer sequences (Fig. 8). In other instances, such as the regulatory elements of the *ret* gene, function but not structure may be conserved (Fisher et al., 2006).

Modification of regulatory sequences was proposed to be one of the most important driving forces of evolution (Levine and Tjian, 2003). In the case of a conserved regulatory region of the mouse *HoxC8* gene, it was found that the zebrafish and Takifugu homologues of this enhancer drive reporter gene expression at different axial levels in the mouse in comparison to their murine homologue (Anand et al., 2003). This finding was interpreted to reflect the changes in thoracic segments in the different vertebrates (Anand et al., 2003). Furthermore, a diencephalic enhancer of the zebrafish *neurogenin1* gene is active in the lateral telencephalon of the mouse suggesting cooperation of this enhancer in an evolutionary novel structure (Blader et al., 2004). There is no indication, however, that the changes in the function of the *shh* regulatory sequences in mouse and zebrafish would have generated novelty in expression of *shh*. Rather the expression was maintained in homologous structures but by the employment of different regulatory regions (Fig. 8). Zebrafish and mice use different signalling pathways to specify floor plate (for review Placzek and Briscoe, 2005; Strahle et al., 2004). One could speculate therefore that the changes in signalling mechanisms could have enforced the redeployment of enhancers in a different context.

Alternatively, the divergence in the regulatory architecture of the mouse and zebrafish *shh* loci including the change in function of individual enhancers may be a reflection of neutral drift that occurs during evolution of enhancer modules. Regulatory regions with partially redundant functions, such as that of *ar-D* and *ar-B* in the floor plate or *ar-A* and *ar-C* in the notochord, may provide the substrate on which these drifts in function can occur.

A striking feature of *ar-C* is that it controls expression in different structures of the zebrafish and mouse embryo (Jeong et al., 2006) (Fig. 8) despite its structural conservation. Our results suggest that conserved non-coding sequences are useful to detect functionally relevant non-coding sequences. However, one cannot necessarily predict the function of an enhancer by extrapolation of the regulatory activity obtained in one species only.

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