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Intronic enhancers control expression of zebrafish *sonic hedgehog* in floor plate and notochord

F. Müller, B.-E. Chang, S. Albert, N. Fischer, L. Tora and U. Strähle*

Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP, BP 163, 67404 Illkirch Cedex, CU de Strasbourg, France
*Author for correspondence (e-mail: uwe@titus.u-strasbg.fr)

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SUMMARY

The signalling molecule Sonic hedgehog (Shh) controls a wide range of differentiation processes during vertebrate development. Numerous studies have suggested that the absolute levels as well as correct spatial and temporal expression of *shh* are critical for its function. To investigate the regulation of *shh* expression, we have studied the mechanism controlling its spatial expression in the zebrafish. We employed an enhancer screening strategy in zebrafish embryos based on co-injection of putative enhancer sequences with a reporter construct and analysis of mosaic expression in accumulated expression maps. Enhancers were identified in intron 1 and 2 that mediate floor plate and notochord expression. These enhancers also drive notochord and floor plate expression in the mouse embryo strongly suggesting that the mechanisms controlling *shh* expression in the midline are conserved between zebrafish and mouse.

Functional analysis in the zebrafish embryo revealed that the intronic enhancers have a complex organisation. Two activator regions, ar-A and ar-C, were identified in intron 1 and 2, respectively, which mediate mostly notochord and floor plate expression. In contrast, another activating region, ar-B, in intron 1 drives expression in the floor plate. Deletion fine mapping of ar-C delineated three regions of 40 bp to be essential for activity. These regions do not contain binding sites for HNF3β, the winged helix transcription factor previously implicated in the regulation of *shh* expression, indicating the presence of novel regulatory mechanisms. A T-box transcription factor-binding site was found in a functionally important region that forms specific complexes with protein extracts from wild-type but not from notochord-deficient mutant embryos.

Key words: floor plate, notochord, sonic hedgehog, HNF3β, enhancer, gene regulation, zebrafish, mouse transgenic, co-injection, T-box

INTRODUCTION

Cell-cell signalling is crucial to shape the vertebrate body. Signals from the axial mesoderm pattern the ventral neural tube (Placzek et al., 1993). Sonic hedgehog (Shh), a member of the hedgehog family (Hh) of signalling molecules induces floor plate and motor neurons, the ventral-most structures in the vertebrate neural tube (Chiang et al., 1996). In comparison to the phenotype of $shh^{-/-}$ mice (Chiang et al., 1996), lack of *shh* (named *sonic-you* in zebrafish) causes much milder defects in the zebrafish ventral neural tube. In particular, the floor plate forms in mutants homozygous for loss-of function alleles (Schauerte et al., 1998). Two other, members of the *hedgehog (hh)* gene family are expressed in domains in the zebrafish body axis overlapping with that of *shh* (Ekker et al., 1995; Currie and Ingham, 1996). Loss of *shh* function may be compensated for by these closely related members of the *hh* family.

Numerous experiments suggest that the correct spatial and temporal expression of Shh signal is crucial for patterning (Echelard et al., 1993; Krauss et al., 1993; Johnson et al., 1994; Barth and Wilson, 1995). During gastrulation, *shh* is expressed in the midline mesoderm comprising notochord and prechordal plate precursors and also in the directly overlying neuroectoderm giving rise to the floor plate. Other regions of expression are the posterior limb bud and the endoderm (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Strähle et al., 1996). In addition, there is evidence that the absolute levels of secreted protein determine which cell type is induced. At high concentrations, Shh induces floor plate while at lower concentrations motor neurons and interneurons can be induced in neural tube explants in vitro (Marti et al., 1995; Roelink et al., 1995; Ericson et al., 1997).

Shh binds to Patched, a multipass transmembrane protein that controls a signalling cascade in target cells (Goodrich et al., 1996; Marigo et al., 1996; Stone et al., 1996; Alcedo and Noll, 1997). Shh signalling leads to activation of Gli zinc finger transcription factors (Alexandre et al., 1996; Ruiz i Altaba, 1997; Ding et al., 1998; Matise et al., 1998), which induce expression of specific genes including that of the winged-helix

transcription factor HNF3β (Sasaki et al., 1997). There is, however, also evidence that Shh controls target gene expression by transcription factors other than Glis as in the case of *COUP-TFII* (Krishnan et al., 1997a,b).

Members of the winged helix class of proteins have been implicated in the control of *shh* expression. Ectopic expression of *HNF3β* (named *Axial* in the zebrafish) causes ectopic activation of *shh* expression (Ruiz i Altaba et al., 1995a; Sasaki and Hogan, 1994; Chang et al., 1997). Furthermore, targeted mutation of *HNF3β* in the mouse results in loss of *shh* expression in the body axis (Ang and Rossant, 1994; Weinstein et al., 1994). In agreement, the zebrafish *shh* promoter has been shown to contain two HNF3β (Axial)-binding sites, which are required for high HNF3β (Axial)-dependent promoter activity in a cell culture system (Chang et al., 1997). Mouse embryos homozygous for targeted mutations in the *shh* gene fail to form the floor plate (Chiang et al., 1996). Therefore, *shh* controls not only floor plate differentiation but possibly also its own expression in the floor plate, in a process that has been proposed to be mediated by HNF3β (Ruiz i Altaba et al., 1995b; Chang et al., 1997). Thus, HNF3β seems to act both downstream and upstream of *shh* in the developmental pathway specifying floor plate.

In zebrafish, mutations identified several other genes to be required for notochord and/or floor plate development and *shh* expression in these tissues. Mutations in *floating head* (*flh*; zebrafish homologue of *Xenopus XNot1*) cause impaired development of the notochord and the floor plate (Talbot et al., 1995). Embryos carrying mutations in either *cyclops* or *squint*, which encode closely related members of the Nodal subfamily of TGFβ signalling proteins, disrupt development of the ventral neural tube and *shh* expression (Feldman et al., 1998; Krauss et al., 1993; Rebagliati et al., 1998; Sampath et al., 1998). Hence, signalling by Nodal-related molecules may be required for specification of the ventral neural tube and *shh* expression. Mutations in *one-eyed pinhead*, which encodes a secreted protein containing an EGF and a CFC domain (Zhang et al., 1998), indicate yet another signalling pathway required for *shh* expression and floor plate development in zebrafish (Schier et al., 1997; Strähle et al., 1997b).

shh expression in axial mesoderm and the ventral neural tube appears to be the result of multiple converging, signalling pathways. In order to better understand the mechanism underlying expression of *shh* in the notochord and floor plate, we have investigated the *cis*-regulatory elements directing *shh* expression in the zebrafish embryo. We employed a co-injection approach in zebrafish embryos. This involves the detection of activating sequences by co-injection of gene fragments with a reporter construct (Müller et al., 1997). This technique exploits the rapid concatamerisation of injected DNA in fish embryos (Stuart et al., 1988; Winkler et al., 1991) and by-passes the need to generate multiple expression constructs. We identified enhancers in intron 1 and 2 that control notochord and floor plate expression. These enhancers direct midline expression in both mouse and zebrafish embryos indicating that the mechanisms of *shh* regulation in these tissues are conserved. Detailed analysis of the introns revealed multiple modules, two of which control predominantly notochord expression while another module controls expression in the floor plate. Detailed deletion analysis of one of the modules (activating region C, ar-C), which mediates predominantly notochord expression revealed that three subregions are essential for activity. One subregion, which contains a potential T-box transcription factor-binding site, forms specific complexes with protein extracts from zebrafish embryos. Protein binding is increased in extracts from dorsalised embryos in which the *shh* expression domain is expanded, and decreased in extracts from *flh* mutants lacking the notochord.

MATERIALS AND METHODS

Fish stocks, embryo production

The wild-type zebrafish line wtOX is derived from fish purchased from the Goldfish Bowl, Oxford, UK and has been bred for several years in the laboratory. Fish were bred and raised as described (Westerfield, 1993). The origin of the flh^{P3B} allele has been described (Strähle et al., 1996).

Plasmid construction

All cloning was performed following standard procedures (Sambrook et al., 1989). Briefly, plasmids *–563shh::lacZ* and *–2.2shh::lacZ* containing the zebrafish *shh* promoter from –563 to +221 and approximately –2200 to +221, respectively, (numbers are relative to the proximal transcription start site) are derivatives of *–563shhCAT* and *–2200shhCAT* described previously (Chang et al., 1997). The CAT coding region in these plasmids was replaced by the *Kpn*I-*Sal*I fragment of *pHMGFNFlacZ* (kindly provided by J. Pearce), which encodes a *lacZ* gene with a nuclear localisation signal.

Plasmids *I1+I2/–2.2shh::lacZ* and *I2+I1/–2.2shh::lacZ* were constructed by inserting the *Xho*I-*Sal*I fragment (Chang et al., 1997) comprising *shh* downstream sequences from +221 to +5995 into the *Sal*I site in both orientations upstream of the *shh* promoter in *–2.2shh::lacZ*. The *I1+I2/–2.2shh::lacZ* hybrid gene with the *shh* introns 1 and 2 were isolated by cleavage with *Not*I and *Kpn*I. The linear fragment *I1/–2.2shh::lacZ* was produced by isolating the *Msc*I-*Kpn*I fragment from *I2+I1/–2.2shh::lacZ* and the linear fragment *I2/shh::lacZ* was generated by isolating the *Msc*I-*Kpn*I fragment from *I1+I2/–2.2shh::lacZ*. Plasmid *arC/HSP68::lacZ* was constructed by insertion of a PCR generated *Xho*I-*Xma*I-cleaved fragment containing the region +5168/+5631 and inserted into the *phsp68PTlacZ* plasmid (Kothary et al., 1989) into *Xho*I-*Xma*I sites upstream of the *HSP68* promoter. The sequence of the zebrafish *shh* genomic gene between the transcriptional start site and the translational stop has been submitted to GenBank (Accession Number AF124382).

Microinjection in zebrafish embryos

–2.2shh::lacZ or *HSP68::lacZ* reporter genes and derivatives thereof were excised and the vector fragments were removed by agarose gel electrophoresis. Likewise, fragments for co-injection were either cut out from plasmids (intron 1 and 2) or amplified by PCR (fine mapping of ar-A, ar-B and ar-C; oligonucleotide sequences used for PCR amplification of *shh* intronic fragments are available on request) and then purified by agarose gel electrophoresis (Sambrook et al., 1989). DNA fragments were isolated with a Quiex DNA purification kit (Quiagen), ethanol-precipitated and redissolved in water. For injection, phenol red was added to a final concentration of 0.1%. The *–2.2shh::lacZ* or *HSP68::lacZ* hybrid gene fragments were injected at a concentration of 100 μg/ml unless otherwise stated. Co-injected fragments were provided in a 3-fold (fragments >80 bp) to 5-fold (80 bp fragments) molar excess over the reporter gene fragments.

Fish 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). and were injected with a gas-driven microinjector (Eppendorf) at the 1- to 8-cell stage.

Transgenic mouse embryos

Vector sequences were removed from *I1+I2/–2.2shh::lacZ, arC/HSP68::lacZ* and *–563shh::lacZ* by restriction digestion and preparative gel electrophoresis. DNA, after electroelution from agarose blocks, was further purified by sucrose gradient centrifugation (5-20% sucrose in 0.02 M Tris-HCl, 0.2 M NaCl, 2 mM EDTA, pH7.4, 0.005 mg/ml ethidium bromide) (Sambrook et al., 1989). Purified DNA was then extracted with phenol-chloroform, precipitated and redissolved in TE (10 mM Tris-HCl pH7, 0.1 mM EDTA). Fragments diluted to 4 ng/μl in TE were injected into the pronucleus of fertilized mouse eggs.

Embryos were either collected at 8.5, 9.5 and 11.5 days postcoitum (d.p.c.) or grown to adulthood to establish transgenic lines. Transgenic embryos and mice were identified by PCR using DNA from proteinase-K-digested yolk sacs or mouse tails and primers specific to the zebrafish *shh* promoter.

In situ analysis

For β-gal staining, fish or mouse embryos were fixed at 4°C, 1 hour in BT-fixative (4% (w/v) sucrose, 0.12 mM $CaCl_2$, 23 mM NaH_2PO_4, 77 mM Na2HPO4, 4% (w/v) paraformaldehyde) and 0.2% (w/v) glutaraldehyde. After three washes in PBS, the embryos were rinsed and incubated in staining buffer (15.4 mM Na_2HPO_4, 4.6 mM NaH_2PO_4, 150 mM NaCl, 3 mM $K_4Fe_3(CN_6)_6$, 3 mM $K_3Fe_4(CN_6)_6$) and X-gal was added to a final concentration of 0.04% (w/v). Embryos were incubated at 37°C for several hours to overnight. The pattern of expressing cells was scored by using differential interference contrast optics.

The restaining of β-gal-labelled embryos with the monoclonal antibody Zn12 (Metcalfe et al., 1990; Trevarrow et al., 1990) was carried out as described previously (Strähle et al., 1993, 1997a).

EMSA analysis

Whole-cell extracts were prepared on ice from 1-day-old zebrafish embryos by rupturing the embryos in 20 mM Tris-HCl pH 7.6, 2 mM DTT, 20% glycerol, followed by extraction by freeze thawing in 20 mM Tris-HCl pH 7.6, 2 mM DTT, 400 mM KCl, 20% glycerol. The homogenate was centrifuged (10000 revs/minute, 10 minutes, 4°C) and protein concentration in supernatants was determined. 20 μg protein were incubated together with 40 bp long ^{32}P-labelled double-stranded oligonucleotides at room temperature for 40 minutes in 10 mM Tris-HCl pH 7.6, 0.5 mM EDTA, 500 ng poly(dI-dC), 4% Ficoll and 100 mM KCl. In competition experiments, protein extracts were incubated first with cold double-stranded oligonucleotides for 10 minutes prior to the addition of the labelled probe. Complexes were separated by 6% PAGE prior to autoradiography. LiCl treatment of embryos was carried out as described (Stachel et al., 1993).

RESULTS

The *–563shh* promoter is not sufficient for notochord and floor plate expression

To test whether the zebrafish *shh* promoter previously characterised in a mammalian cell system (Chang et al., 1997) is sufficient to drive expression in the floor plate and notochord, we constructed *lacZ* reporter genes containing the *shh* promoter from position –563 to +221 relative to the proximal transcription start site. The purified fragment *–563shh::lacZ* was injected into early cleavage stage zebrafish embryos and *lacZ* expression was monitored at 24 hours of development (*n*=60 embryos). Only weak expression in a random pattern was observed (Table 1, data not shown) suggesting that the promoter region from –563 to +221 is not sufficient to direct faithful expression in the embryo.

This type of analysis in the zebrafish is characterised by significant mosaicism of transgene expression (Westerfield et al., 1992). We also injected the *–563shh::lacZ* construct into mouse embryos. At 11.5 days of embryonic development, 3 out of the 22 transgenic mice had β-gal staining in the posterior portion of the limb bud (Fig. 1). The intensity of *lacZ* expression as well as the size of the *lacZ* expression domain varied between embryos. Also ectopic expression outside of the normal *shh* expression domain was noted (Fig. 1B) indicating influences of the transgene integration site. These data suggest that the promoter region (–563 to +221) contains elements that control at least partially expression in the posterior region of the limb bud in mouse embryos. However, none of these transgenic mice showed expression in the notochord and floor plate.

Downstream sequences of the zebrafish *shh* gene control floor plate and notochord expression in both zebrafish and mouse embryos

To search for regulatory elements required for *shh* expression in notochord and neural tube, we employed a co-injection

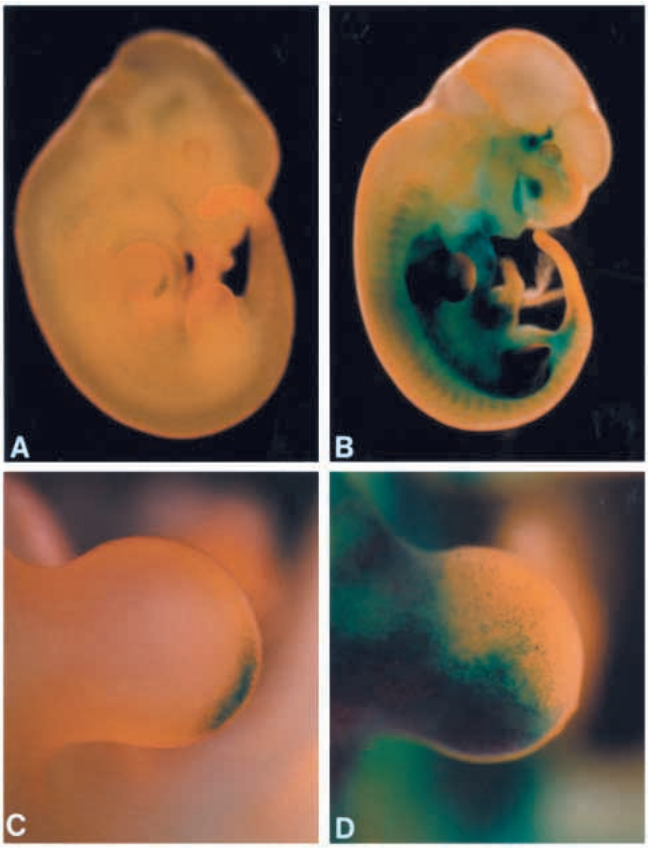

Fig. 1. The zebrafish *shh* promoter from –563 to +221 directs at low frequency *lacZ* expression in the posterior limb bud but not in floor plate and notochord in transgenic mouse embryos. (A,B) Two examples of transient transgenic mouse embryos expressing *lacZ* from *–563shh::lacZ.* (C,D) Magnification of the forelimb buds of embryos shown in A and B. The posterior region of the forelimb buds expresses *lacZ.* Ectopic expression was also evident in the ventral prosencephalon, diencephalon, heart, ventral somites and the limb bud. (B,D). Embryos are oriented anterior up and dorsal to the left.

Table 1. Downstream enhancers of *sonic hedgehog* control expression in floor plate and notochord

Construct	% embryos expressing lacZ* (number of embryos scored)	% β-gal-positive cells in notochord and floor plate (number of cells)‡
–563shh::lacZ	13.3 (60)	0 (16)
–2.2shh::lacZ (100 μg/ml)	8.3 (60)	0 (34)
–2.2shh::lacZ (500 μg/ml)	26.6 (105)	13 (110)
–2.2shh::lacZ+ (I1+I2) (100+300 μg/ml)	76 (50)	72 (76)
I1+I2/–2.2shh::lacZ (200 μg/ml)	12.7 (102)	96 (54)
I2+I1/–2.2shh::lacZ (200 μg/ml)	28.7 (101)	93 (69)
I1/–2.2shh::lacZ (200 μg/ml)	45 (122)	55 (153)
I2/–2.2shh::lacZ (200 μg/ml)	42 (88)	78 (146)

*Expression in the yolk syncytial layer was excluded from the calculations.
‡Cells mean total number of cells showing β-gal activity excluding the yolk syncytial layer.

protocol in which genomic fragments were injected together with a linearised minimal promoter driving the *lacZ* reporter gene (Müller et al., 1997). Since transient transgene expression results in strong mosaicism in the zebrafish embryo, expression maps were plotted in which positions of *lacZ*-expressing cells in embryos were scored and superimposed (Westerfield et al., 1992). A larger promoter/*lacZ* construct (*–2.2shh::lacZ*) containing *shh* 5′ sequences up to approximately –2.2kb was used in these experiments. When this reporter construct was injected at 100 μg/ml alone, a weak and random pattern of β-gal activity was observed similar to that seen upon injection of the shorter promoter construct *–563shh::lacZ* (Table 1; Fig. 2A). When *–2.2shh::lacZ* was injected at higher concentrations (500 μg/ml) expression in the ventral brain anterior to the otic vesicle, but not in the spinal cord or notochord, was frequently observed (Fig. 3A,B) suggesting that the –2.2kb promoter contains elements that weakly direct expression in the ventral brain. When a fragment comprising sequences from +221 to +5995 including intron 1 and 2 was co-injected together with *–2.2shh::lacZ*, (at 100 μg/ml), we found increased β-gal staining that was predominantly located in cells of the notochord and floor plate (Figs 2B, 3C). Strong expression was found in the yolk syncytial layer (YSL), irrespective of co-injected fragments (data not shown). High YSL expression was also often observed when other reporter genes were injected and is thus unlikely to reflect a specific regulatory property of the *shh* promoter but points rather to non-specific transgene activity of the polyploid YSL nuclei (Williams et al., 1996).

To verify that the results obtained by the co-injection approach reflect regulatory activity of the downstream sequences, we constructed an expression plasmid in which the 5.8 kb fragment was cloned upstream of the *shh* promoter in

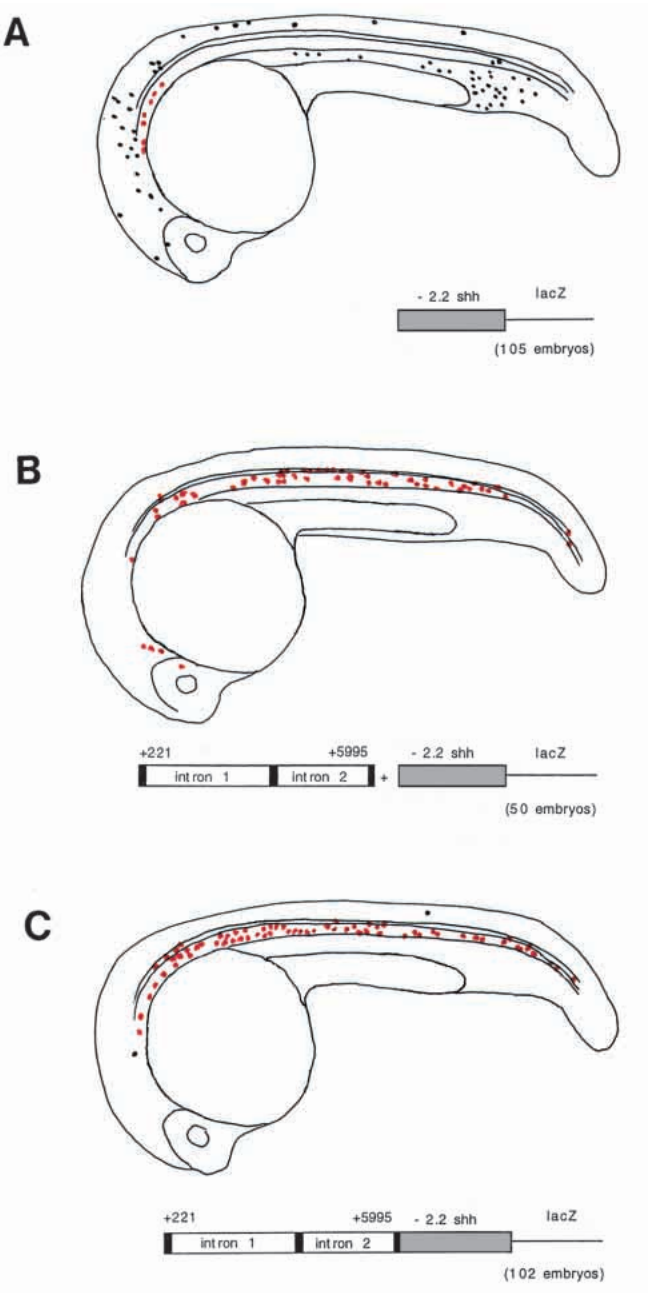

Fig. 2. Downstream sequences containing intron 1 and 2 of the *shh* gene drive expression in floor plate and notochord of zebrafish embryos. Embryos were injected with linearised and vector deleted *–2.2shh::lacZ* alone (A) or together with a 5.8 kb fragment comprising *shh* intron 1 and 2, parts of exon 1 and 3 and the entire exon 2. Embryos in C were injected with *I1+I2/–2.2shh::lacZ* in which the 5.8 kb fragment was cloned upstream of the *–2.2shh* promoter. Embryos were injected at early cleavage stages and grown until 24 hours before fixing and β-gal staining. Expressing cells were scored in injected embryos (numbers of embryos are indicated) and position of expressing cells in individual embryos were superimposed. Co-injection of reporter and enhancer fragments (B) gave the same results as injection of construct *I1+I2/–2.2shh::lacZ* (C) in which the enhancer fragment had been cloned upstream of the *–2.2shh::lacZ* reporter gene prior to injection. Red dots indicate cells that fall into the expression domain of endogenous *shh*. Black dots indicate ectopic expression. Ectopic expression in the yolk syncytial layer is not shown.

–2.2shh::lacZ. Injection of this construct (*I1+I2/–*2.2*shh::lacZ*) into zebrafish embryos directed the same pattern of β-gal expression as obtained when the intron-containing fragment was co-injected (Fig. 2C); 96% *lacZ*-expressing cells were located in the notochord and floor plate (Table 1; Fig. 2C). The same restriction of expression to notochord and floor plate was obtained when *I2+I1/–2.2shh::lacZ* was injected, in which the 5.8 kb fragment was inserted in the opposite orientation (Table 1 and data not shown).

Transgenic mouse embryos were produced containing the fragment *I1+I2/–2.2shh::lacZ* to investigate whether the intronic regions would also drive expression in the notochord and floor plate of mouse embryos. One transient transgenic mouse was identified by PCR and was shown to express the transgene. Furthermore, three stable transgenic lines were established, out of which two lines expressed the transgene. Between 8 and 11.5 days, all expressing transgenic mice showed β-gal activity specifically in the notochord and floor plate albeit with a mosaic pattern (Fig. 4), while expression in the gut endoderm was restricted to a very low number of cells. When compared with previously reported expression of *shh* in the mouse brain (Echelard et al., 1993), the zebrafish gene does not appear to faithfully reproduce the pattern in the anterior brain, expression in the ventral diencephalon is missing.

Taken together these results demonstrate first that *cis*-regulatory elements, which are able to drive expression in the floor plate and notochord are located in the 5.8 kb fragment that includes intron 1 and 2. Also, the regulatory mechanisms underlying *shh* expression in the notochord and floor plate are likely to be conserved between zebrafish and mouse. Finally, our results show that co-injection technique and transient expression analysis in zebrafish embryos provides correct spatial data, and that, together with the sampling technique, a realistic picture of the expression activity of the injected constructs can be obtained.

Multiple elements control floor plate and notochord expression

In subsequent experiments, we have focused on the regulatory elements responsible for controlling *shh* expression in the floor plate and notochord. To delineate the position of the regulatory element(s) in the 5.8 kb fragment more precisely, either intron 1 or intron 2 was deleted from the 5.8 kb fragment upstream of the *shh* promoter in *–2.2shh::lacZ*. The construct *I1/–2.2shh::lacZ* contains *shh* sequence from position +221 to +3898 comprising intron 1 and part of exon 1 and 2 (Fig. 5A,B) while construct *I2/–2.2shh::lacZ* harbours intron 2 and the flanking coding sequences from position +3898 to +5995 (Fig. 5A,G).

Both constructs resulted in a significant increase of midline

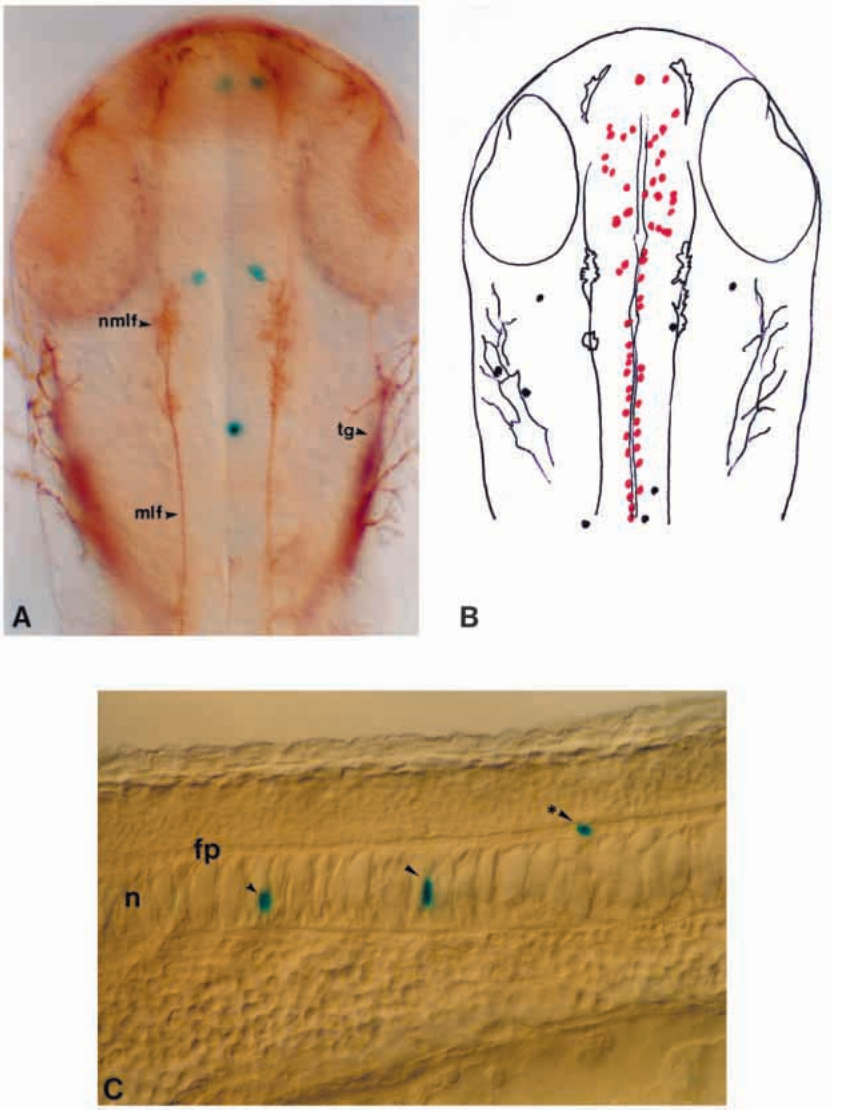

Fig. 3. Mosaic β-gal expression driven by sonic hedgehog genomic sequences. (A,B) β-gal expression in the head of *–2.2shh::lacZ*-injected embryos. (A) Embryos injected with 500 μg/ml *–2.2shh::lacZ* were double labelled by β-gal staining (turquoise) and immunohistochemistry with the anti-HNK1/L2 antibody Zn12 (brown). The ventrally located medial longitudinal fascicles (mlf) and their nuclei (nmlf) served as a reference to assess the position of β-gal-expressing cells with respect to the expression domain of endogenous *shh*. For comparison, see Strähle and Blader (1994). (B) Expression map collected from 23 embryos injected with –2.2*shh::lacZ*. Red dots indicate cells that fall into the expression domain of endogenous *shh*. Black dots indicate ectopic expression. –2.2*shh::lacZ* directs expression predominantly to the ventral midline of the brain where endogenous *shh* is expressed. tg; trigeminal ganglion; nmlf, nucleus of the medial longitudinal fascicle; mlf, medial longitudinal fascicle. The *lacZ* gene driven by the *–2.2shh* promoter contains a nuclear localisation signal sequence. (C) Mosaic expression of nuclear localised β-gal in the trunk of a 24 h old zebrafish embryo co-injected with *–2.2shh::lacZ* and the 5.8 kb downstream fragment containing intron 1 and 2. Embryo is oriented anterior to the left and dorsal up. Arrowhead with asterisk points at a nucleus of a floor plate cell whereas other arrowheads indicate two β-gal-positive nuclei in the notochord. fp, floor plate; n, notochord. Orientation of embryo is dorsal up and anterior to the left.

expression of *lacZ*, when compared to the promoter construct *–2.2shh::lacZ* (Table 1; Fig. 5B,G). However, the fragment comprising intron 2 appeared to be more stringent in its ability to direct expression in the midline (Table 1; Fig. 5G).

To define the elements more accurately, we generated a set of overlapping subfragments spanning both introns (Fig. 5A). The fragments were tested by co-injection with *–2.2shh::lacZ* (100 μg/ml) for their ability to direct β-gal expression in the notochord and floor plate. The fragment spanning the sequence from +2033 to +3098 of intron 1 as well as the subfragment +2033/+2371 were found to direct notochord expression as compared to the basal activity of the *–2.2shh::lacZ* fragment (Fig. 5F,D). The partially overlapping fragment +2351/+2848 showed few expressing cells in the midline not significantly higher than the control promoter construct (data not shown). These findings together with the fact that fragment +553/+2040 and subfragments +1551/+2040 and +2600/+3098 did not have an effect on β-gal activity (Fig. 5A,C) locates activator region A (ar-A) between position +2040 and +2386.

While ar-A directs expression predominantly in notochord, fragment +3099/+4147 of intron 1 resulted in an increase in the number of expressing cells mostly in the floor plate and to a much lesser extent in the notochord. A similar result was obtained when the subfragment +3099/+3597 was injected (Fig. 5E). Neither the flanking fragment +2600/+3098 nor fragment +3598/+3921 showed a detectable effect. Thus activator region B (ar-B) which directs predominantly floor plate expression is located between +3099 and +3597.

In intron 2, two overlapping fragments, +4880/+5374 and +5168/+5631, mediate, like ar-A of intron-1, notochord expression of the *lacZ* reporter gene (Fig. 5A,I). Fragment +4628/+ 5169 and subfragment +4880/+5167 did not lead to specific activation (Fig. 5H). Fragment +5346/+5631 caused only a weak enhancement of notochord and floor plate expression (data not shown). Thus, ar-C appears to be located between +5168 and +5346. In agreement, the subfragment +5168/+5374 mediated strong expression in the notochord (Fig. 7C and data not shown**)**.

In summary, these results show that multiple activator sequences in intron 1 and 2 of *shh* control expression in the floor plate and notochord. While the two activator regions ar-A and ar-C control mostly notochord expression, activator region ar-B appears to direct predominantly floor plate expression.

ar-B and ar-C drive midline expression of a heterologous promoter

To test whether the *shh* promoter is required for the activity of regions ar-B and ar-C, fragments +3099/+3597 and +5168/+5631 comprising ar-B and ar-C, respectively, were co-injected with the mouse heat-shock promoter *HSP68* linked to *lacZ* (Kothary et al., 1989). The *HSP68* promoter was chosen because it directs *lacZ* expression weakly in many tissues (Figs 6D, 7B; Müller et al., 1997).

While ar-B directed expression mostly in the floor plate

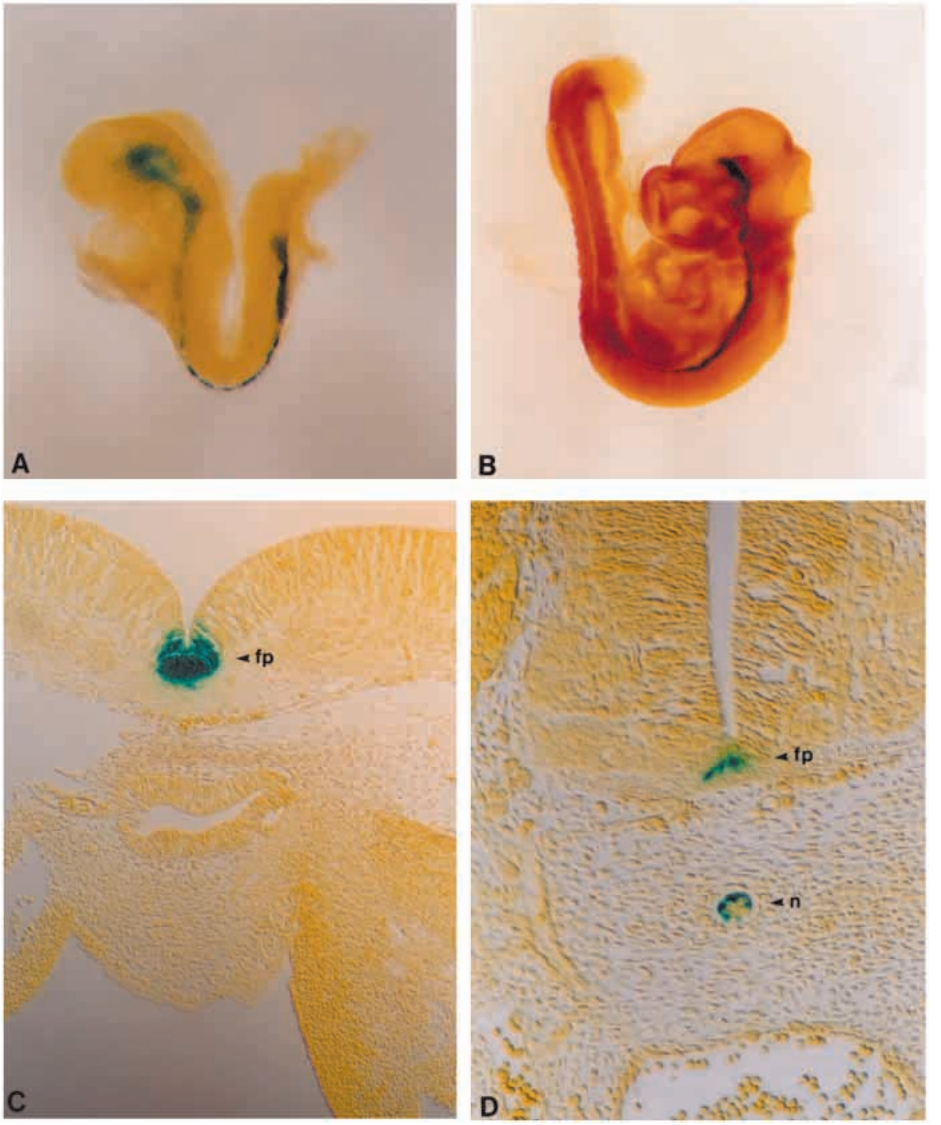

Fig. 4. The downstream 5.8 kb fragment spanning intron 1 and 2 directs nuclear localised β-gal expression in notochord and ventral neural tube in transgenic mouse embryos. Mouse oocytes were injected with *I1+I2/–2.2shh::lacZ* and embryos were either killed at 8.5 days of development for β-gal staining (A) or grown to term to generate a line of stably expressing animals (B). The embryo in B is derived from an outcross of the F_0 at 9.5 days of development. (C,D) Transverse sections through the hindbrain and the trunk of β-gal-stained, 9.5 d.p.c. F_1 transgenic embryo, respectively. β-gal expression is mosaic. Embryos are oriented anterior left (A) and up (B). Dorsal is up in sections (C,D). n, notochord; fp, floor plate.

when co-injected with the *HSP68::lacZ* hybrid gene (Fig. 6B**),** ar-C mediated strong activation of the *HSP68* promoter in the notochord and floor plate (Fig. 6A,C). These data show that both activator regions can work in combination with a heterologous promoter suggesting that elements in the *–2.2shh* promoter are not essential for their activity. However, very few cells showed *HSP68* promoter activity in the ventral brain (data not shown) suggesting that elements present in the *–2.2shh*

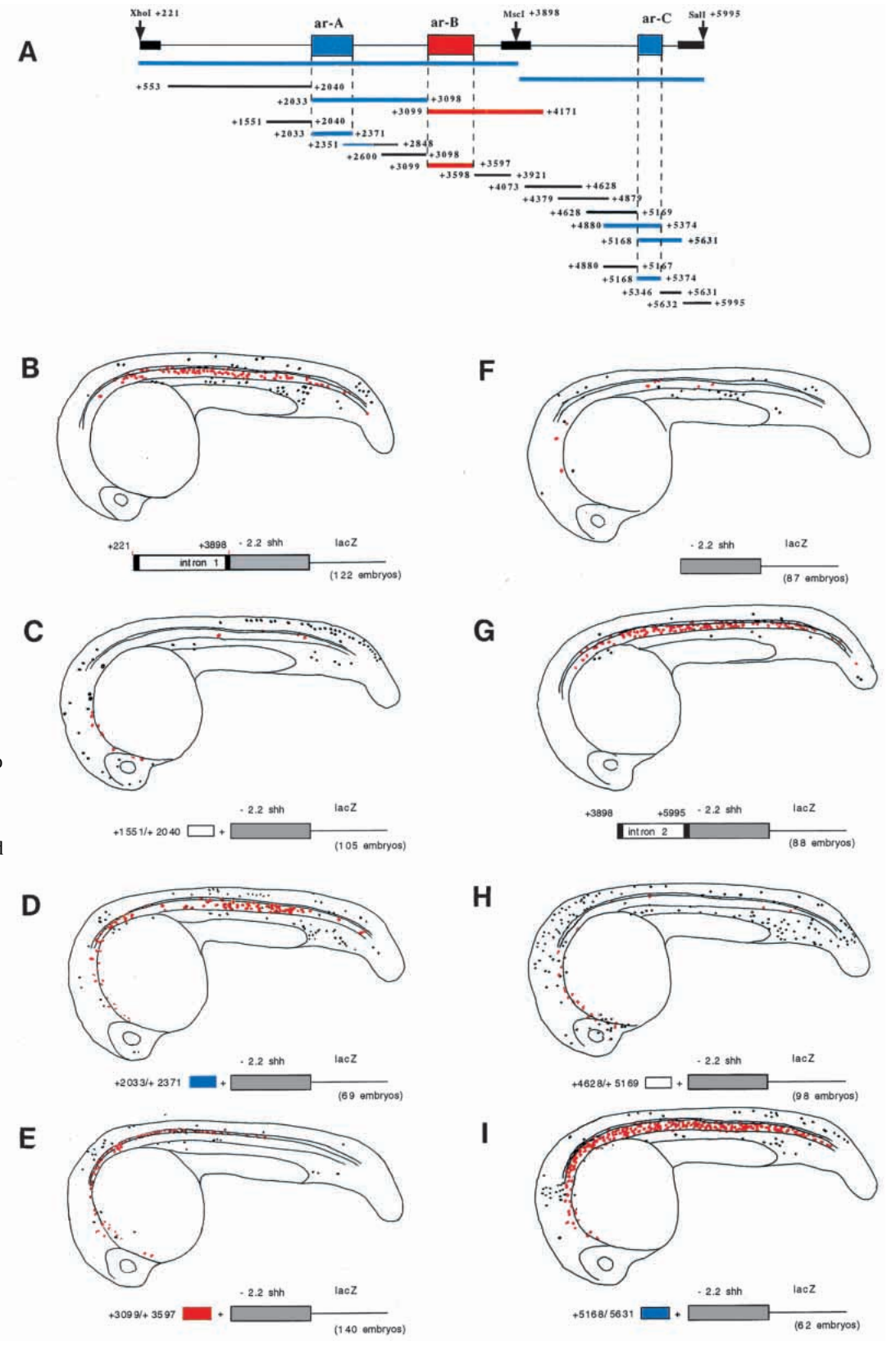

Fig. 5. Mapping of intronic regulatory regions by co-injection into zebrafish embryos. (A) Overview of fragments injected together with *–2.2shh::lacZ*. The position of fragments relative to the transcription start site are indicated. Blue bars indicate regions mediating mostly notochord expression, while red bars indicate fragments that mediate floor plate expression predominantly. Regions outlined by black bars did not show tissue-restricted expression. Examples for expression patterns of fragments with no effect are shown in C and H. The three activating regions identified ar-A, ar-B and ar-C are indicated by coloured boxes. Position of *Xho*I, *Msc*I and *Sal*I restriction enzyme sites used to subclone the intronic regions are shown by arrows. (B-I) Expression maps collected from injected embryos. The DNA injected is outlined below each panel. The number of injected embryos from which data were collected are given in brackets.

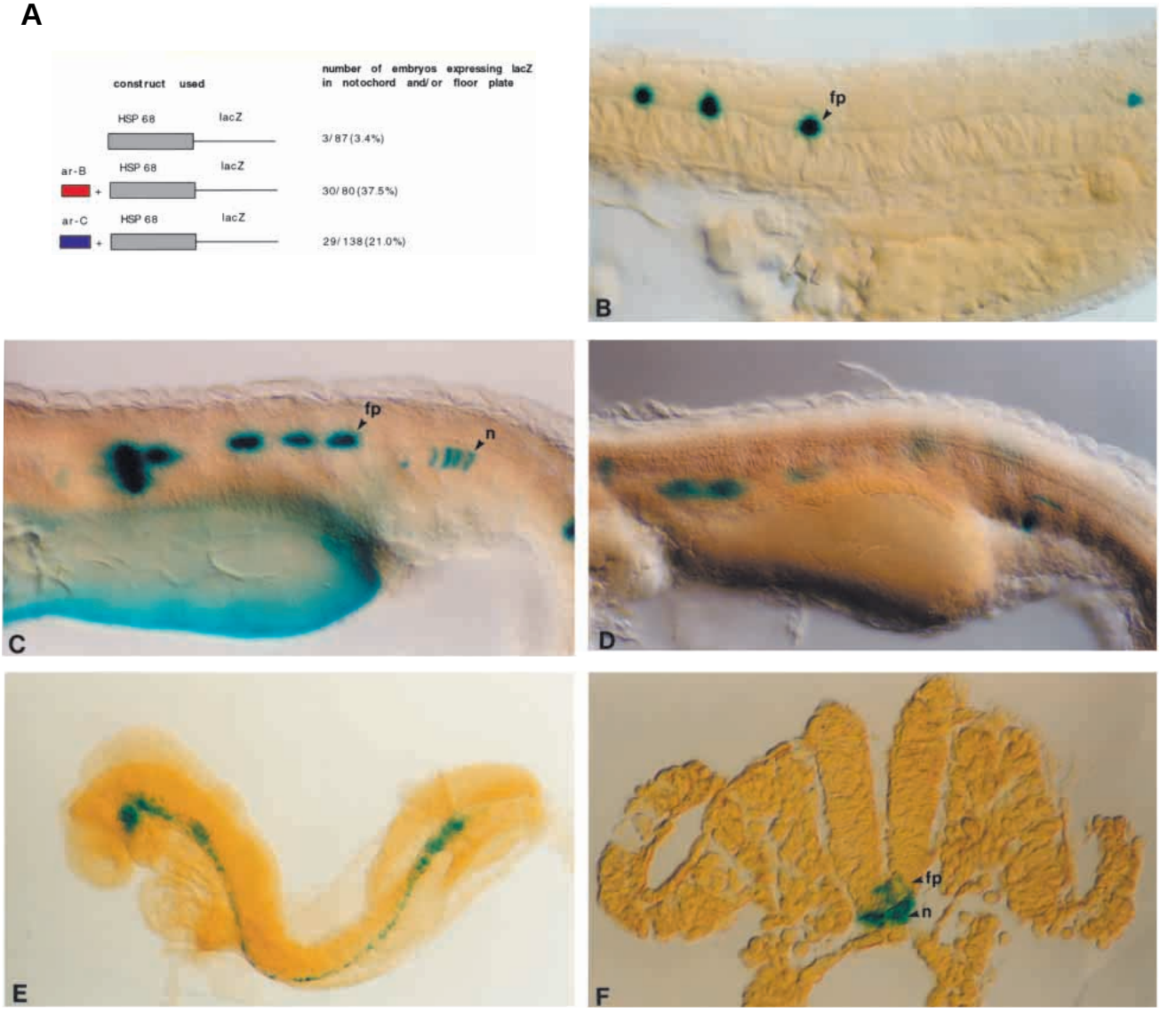

Fig. 6. ar-B and ar-C activate the heterologous *HSP68* promoter in a tissue-restricted manner. (A) Overview of constructs injected and quantitation of results. Numbers give embryos that express in the floor plate (ar-B) or notochord and floor plate (ar-C and *HSP68::lacZ* alone) per total number of injected embryos from one representative experiment. (B) Embryo co-injected with *HSP68::lacZ* and ar-B (fragment +3099/+3597) showing expression of β-gal in the floor plate (fp). (C) Embryo co-injected with *HSP68::lacZ* and ar-C containing fragment +5168/+5631. β-gal positive cells are present in both the floor plate (fp) and the notochord (n). (D) Embryo injected with *HSP68::lacZ* alone. The basal activity of this promoter is higher than that of the *shh* promoter resulting in a higher number of ectopically expressing cells. (E,F) Mouse oocytes were injected with *ar-C/HSP68::lacZ* and recovered at 8.5 days of development for β-gal staining. Whole-mount embryo is oriented anterior to the left (E), and transverse section through the trunk is shown dorsal up (F). n, notochord; fp, floor plate.

promoter are necessary for activation in this region. We also injected *ar-C/HSP68::lacZ* in which the fragment +5168/+5631 was cloned upstream of the *HSP68* promoter. The results were the same as those obtained in the co-injection experiments (data not shown). The zebrafish *shh* promoter contains multiple HNF3β (Axial) sites, two of which were previously shown to be functional in a cell culture transfection assay for mediating HNF3β (Axial) activation (Chang et al., 1997). The activity of ar-C was not altered when co-injected with a *shh* promoter construct containing point mutations in the two HNF3β sites (Chang et al., 1997), suggesting that these two HNF3β (Axial) sites are not required for the activity of ar-C (data not shown).

We also injected *ar-C/HSP68::lacZ* into mouse oocytes. *lacZ* expression was observed in four injected embryos out of 44. Three embryos showed β-gal activity in the notochord and floor plate exclusively confirming functional conservation of the ar-C region (Fig. 6E,F).

The ar-C is composed of multiple elements

Since ar-C of intron2 showed the most dramatic effect in activating notochord expression, we characterised this region further. To narrow down the limits of ar-C**,** we tested a series of overlapping PCR-generated, 80 bp long fragments (Fig. 7A) by co-injection with *–2.2shh::lacZ* (300 μg/ml). The four fragments spanning the region between +5168 to +5367 still

enriched β-gal expression in the midline (28%, 29%, 40% and 20% expressing cells were located in the notochord and floor plate, respectively, compared to 6.5% midline cells when *–2.2shh::lacZ* was injected alone). Fragment +5368/+5447 did not have a specific effect on the promoter activity limiting ar-C between positions +5168 and +5367. Since several non-overlapping 80 bp fragments retained specific activity, it suggests that activator region C is itself composed of multiple elements.

Fragments harbouring 40 bp internal deletions over the +5168 to +5327 region (Fig. 7A) were generated to test the functional relevance of the individual activator elements in the

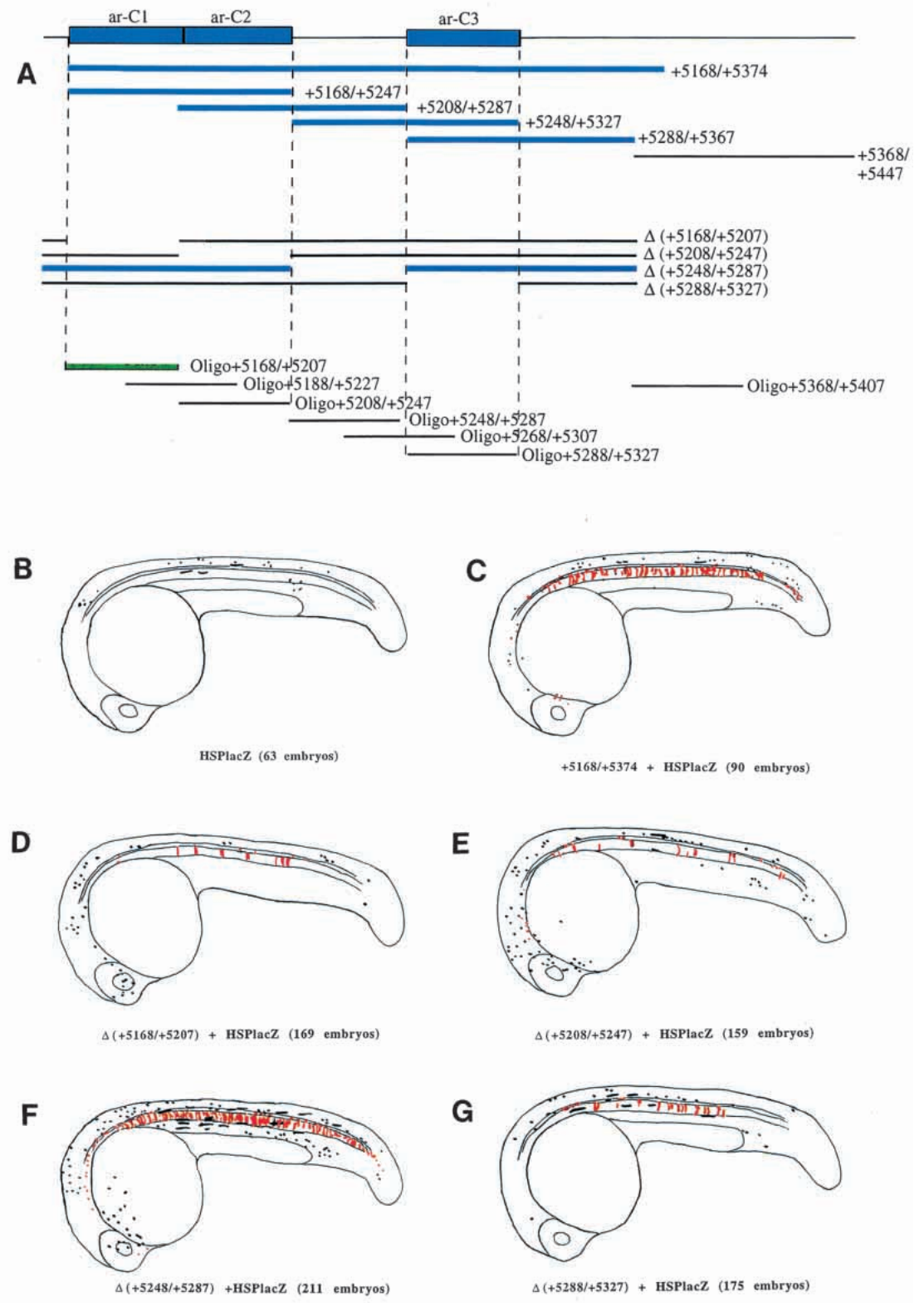

Fig. 7. Fine mapping of activating region ar-C. (A) Overview of fragments injected. Fragments giving rise to expression in the midline are indicated by blue bars. At the bottom, the position of oligonucleotides used in the mobility shift assays (Fig. 9) are outlined. Green bar indicates oligonucleotide to that specific protein complex binding was detected. (B-G) Expression maps collected from embryos injected with *HSP::lacZ* alone (B), or together with fragment +5168/5374 (C) or with internal deletion Δ(+5168/+5207) (D), Δ(+5208/+5247) (E), Δ(+5248/+5287) (F), or Δ(+5288/+5327) (G).

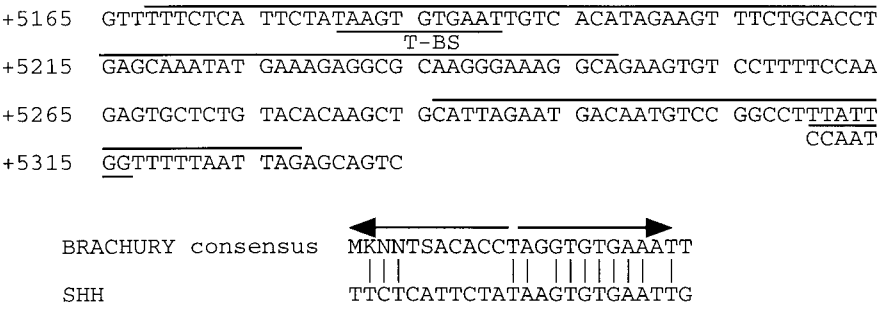

Fig. 8. Sequence of activating region ar-C. Lines above the sequence indicate the extent of deletions that impair function of ar-C. T-BS indicates position of the homology to the Brachyury monomer-binding site (a comparison with the palindromic consensus is shown at the bottom). A homology to a CCAAT-box is present at position +5310 in inverted orientation. (GenBank accession number, AF124382)

context of the whole ar-C. We utilised the *HSP68::lacZ* reporter in these experiments as this reporter gene provides higher activity than the *–2.2shh::lacZ* hybrid gene thereby facilitating the analysis. Deletion of the regions +5168/+5207, +5208/+5247 and +5288/+5327 significantly reduced specific activation in the midline (Fig. 7D,E,G). Removal of sequences between +5248 and +5287 did not affect the activity of the fragment and caused tissue-restricted expression similar to that obtained with the wild-type sequence (Fig. 7, compare F and C). These data support the notion that ar-C is composed of at least three modules that contribute to the activity of the region.

In order to identify possible regulators of the ar-C region, we inspected the sequence for binding sites of transcription factors (Fig. 8). A homology to a monomer-binding site of the T-box transcription factor Brachyury was found at position +5180 in ar-C1 (Casey et al., 1998; Kispert and Herrmann, 1993). A CCAAT-box in inverted orientation is located in ar-C3 (Fig. 8). Neither HNF3β-(Axial) (Kaufmann et al., 1995; Overdier et al., 1994) nor Gli (Kinzler and Vogelstein, 1990; Sasaki et al., 1997)-binding sites were found in the functionally relevant regions.

The ar-C is specifically bound by proteins from zebrafish embryos

We next tested whether proteins from zebrafish embryos bind to the regions identified by deletion mapping. Extracts from 24 h old zebrafish embryos were prepared and electromobility shift analysis (EMSA) was carried out with overlapping 40 bp oligonucleotides covering the region from +5168 to +5327 (Figs 7, 9). When protein extracts were prepared from wild-type embryos and incubated with radioactively labelled double-stranded oligo+5168/+5207, two distinct complexes were detected (uc, lc, Fig. 9, lane 1). The same fragment when added to the reaction in a 100-fold excess as a cold competitor, abolished complex formation (Fig. 9, lane 3). In contrast, another fragment, oligo+5368/+5408, when added as a competitor did not influence formation of the uc and lc complexes (data not shown) suggesting that the extract proteins bind specifically to the sequence from +5168 to +5207. The overlapping oligo+5188/+5227 showed only weak binding (Fig. 9, lane 4), suggesting that the sequences responsible for strong complex formation with oligo+5168/+5207 are confined between +5168 to +5187, which also contains the homology to the Brachyury monomer-binding site. DNA fragments containing sequences between +5208/+5247, +5248/+5287, +5268/5307, +5268/+5327 and +5368/+5407 did not show specific complex formation under the conditions employed (Fig. 9, from lane 7 to 9, and data not shown).

LiCl treatment of cleavage stages dorsalises the zebrafish embryo (Stachel et al., 1993) and expands the domain of *shh* expression (data not shown). We thus reasoned that LiCl treatment would also enrich the factors specifically interacting with the *shh* control region in whole embryo protein extract. When protein extract from LiCl-treated embryos was incubated with the oligo+5168/+5207 probe the upper, slower migrating complex (uc) was increased while the lower complex (lc) remained unaltered in intensity (Fig. 9 compare lane 1 and 2, or 10 and 11). These results suggest that, in the uc complex, a factor is bound to the DNA fragment whose abundance in the extract or binding activity is increased by the LiCl-induced dorsalisation of embryos.

Embryos homozygous for mutations in *floating head* (*flh*), the zebrafish homologue of the *Xenopus* homeodomain transcription factor *XNot1*, fail to form a notochord and have severe defects in floor plate formation and *shh* expression in trunk and tail (Talbot et al., 1995; Strähle et al., 1996). To assess whether mutations in *flh* would affect the pattern of complex formation on oligo+5168/+5207 EMSA assays were carried out with an extract from 24 h homozygous *flh* mutant

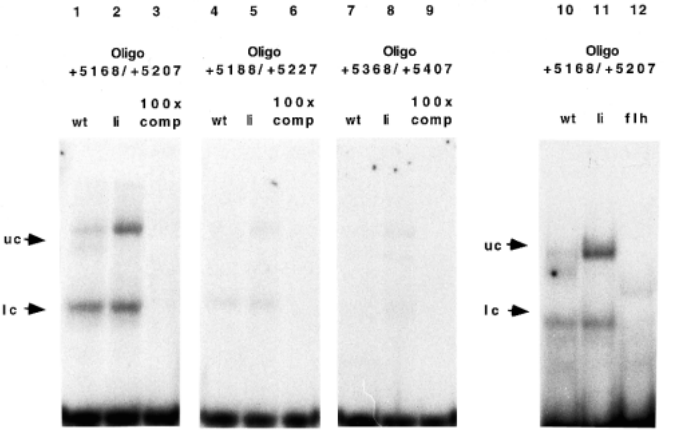

Fig. 9. Electrophoretic mobility shift assays of ar-C. Radioactively labelled, double-stranded oligonucleotides (oligo) covering sequences +5168/+5207 (lanes 1-3), or +5188/+5227 (lanes 4-6) or +5368/5407 (lanes 7-9) were incubated with protein extract prepared either from wild-type embryos (wt) or from embryos treated with LiCl (li and 100× comp) or *floating head* mutants (flh). Addition of a 100-fold excess of cold oligonucleotide to the binding reaction (100× comp) abolished binding in extracts treated with LiCl. Only a weak retarded band is detectable with oligo+5188/+5227 while no specific complex is formed with oligo+5368/5407. Uc, upper complex; lc, lower complex.

embryos. Complexes uc and lc did not form with extract from *flh* mutant embryos (Fig. 9, lane 12). When the same extracts were probed with a DR-5 retinoic acid response element (Chang et al., 1997), extract from *flh* mutant and wild-type embryos formed the same specific complexes (data not shown). These data together with the results obtained by using the LiCl-induced embryo extracts strongly support the functional relevance for the complexes formed in vitro with oligo+5168/+5207.

DISCUSSION

We report here the identification of regulatory elements in the *shh* gene that mediate expression in the notochord and ventral neural tube. The −563*shh* promoter, which contains HNF3β (Axial)-binding sites (Chang et al., 1997), does not efficiently direct expression in these regions of the embryo. Our analysis suggests that elements in both introns of the *shh* gene are required for expression in notochord and floor plate.

The *−563shh* promoter by itself gave rise to *lacZ* expression in the posterior limb bud in transgenic mouse embryos, suggesting that the promoter contains elements that may direct expression in these regions. The retinoic acid response element previously identified in the *shh* promoter could be involved in this regulation (Chang et al., 1997). However, only a fraction of mouse embryos harbouring *−563shh::lacZ* displayed limb expression and furthermore none of the transgenic mice that expressed the −2.2*shh* promoter fused to the intronic enhancers in notochord and ventral neural tube showed expression in the limb buds. Thus, additional elements must be required to mediate efficient expression in the limb buds.

Regulation of expression in the ventral neural tube appears to be complex. The intronic enhancers as well as an element in the *−2.2shh* promoter appear to contribute to expression in the ventral neural tube. While the intronic enhancers mediate expression in the floor plate of the spinal cord, another element in the *−2.2shh* promoter appears to weakly drive expression in the ventral brain. However, the anterior-most expression of *shh* in the ventral diencephalon does not appear to be recapitulated by any of the transgenes suggesting that elements outside of these regions are involved in this aspect of *shh* expression. Moreover, neither the zebrafish nor the mouse embryos showed significant expression in endoderm, another site of *shh* expression (Echelard et al., 1993; Krauss et al., 1993; Strähle et al., 1996). Thus, the regions that we have studied here are able to direct efficient expression in the notochord and floor plate but not in the endoderm, limbs/fins and the anterior neural tube.

Enhancer screening by co-injection and mosaic analysis

The production of stable lines of transgenic zebrafish and faithful expression of transgenes in transgenic lines has been demonstrated recently (Long et al., 1997; Meng et al., 1997). However, given the lack of efficient integration, the number of transmitting lines obtained remains low per experiment. To overcome this problem, we have employed a transient expression assay that has previously been shown to provide information about the activity of tissue-specific regulatory elements (Reinhard et al., 1994; Westerfield et al., 1992). A major drawback of transient expression in the zebrafish embryo is the mosaicism of transgene distribution. To obtain a spatial pattern of enhancer activity, positions of expressing cells were recorded from a large number of embryos resulting in the generation of expression maps (Westerfield et al., 1992; Müller et al., 1997).

Fast delineation of regulatory elements was possible by exploiting the efficient concatamerisation of microinjected fragments, a phenomenon well characterised in *Xenopus* (Marini et al., 1988) and also described in fish embryos (Stuart et al., 1988; Winkler et al., 1991). It was previously shown that strong muscle and neural-tube-specific enhancers convey tissue-restricted expression to reporter genes when co-injected into zebrafish embryos (Müller et al., 1997). In this report, we show for the first time that this property of transient transgenic fish embryos can be exploited to identify novel regulatory elements in gene fragments. Our results show that this technique is not limited to intact and thus strong enhancer regions but can be utilised to dissect elements to a very high resolution. By co-injecting sequentially shorter fragments, we were able to identify enhancers in large fragments in a significantly shorter period of time than would have been required for establishing stable lines of transgenics.

Multiple modules with overlapping functions are present in the intronic regions

Both notochord and floor plate derive from intermingled cells in the embryonic shield, the zebrafish equivalent of the amphibian organizer (Woo and Fraser, 1995). In avian embryos, notochord and floor plate cells were even shown to be descendants of the same precursor cells in the node (Catala et al., 1996). Although there is no direct evidence for a common precursor in the zebrafish, the notochord and floor plate do share expression of a number of genes (Hatta, 1992; Krauss et al., 1993; Strähle et al., 1993; Yan et al., 1995), suggesting that these two cell types are also very closely related in fish. However, despite shared gene expression in precursor cells and a common descent, floor plate and notochord differentiate into morphologically and functionally distinct structures. In line with these differences, the regulation of the *shh* gene is mediated by distinct elements that control predominantly notochord expression and an element that directs predominantly floor plate expression. However, the activity of both types of elements does not appear to be entirely restricted to one or the other tissue. The notochord elements ar-A and ar-C also mediate to a lesser, but still significant, degree activation in the floor plate while the converse holds true for the floor plate enhancer ar-B. This implies that some regulatory factors are employed in both tissues. This promiscuous activity of the activating regions may be a reflection of the common descent of floor plate and notochord.

Notochord expression appears to be redundantly controlled by two activator regions. However, fragments that contain both intronic enhancer regions appear to direct more specific expression in notochord and floor plate. Moreover, we can not entirely rule out the possibility that certain elements may have unique functions that will only be evident in the context of the intact *shh* gene. The regulation of *shh* expression in the midline is dynamic: it is first expressed in the embryonic shield and then in the maturing notochord and midline of the neural plate. While expression is maintained in the neural tube, expression

is turned off with an anterior-to-posterior progression in the notochord during somitogenesis (Krauss et al., 1993). Therefore, it remains to be established whether these multiple elements are truly redundant or whether they are involved in specific temporal aspects of *shh* regulation.

Which factors are involved?

The presence of a sequence with homology to one half of the palindromic *Brachyury*, (*no tail*, *ntl* in zebrafish, Schulte-Merker et al., 1994) binding site in ar-C1 is intriguing. The *Xenopus eFGF* gene is regulated by *Xbra*, the *Xenopus* homologue of *Brachyury*, through a response element also containing only one half of the palindrome which was previously shown by binding site selection to be the preferred recognition sequence for mouse Brachyury in vitro (Casey et al., 1998; Kispert and Hermann, 1993). It is unlikely, however, that *ntl* is indeed involved in regulation of *shh* expression: onset of *shh* expression in the shield is normal in *ntl* mutants. At later stages, *ntl* mutants have an expansion of floor plate, most likely as a result of a cell fate change of notochord cells to floor plate, and the widened floor plate appears to express *shh* at normal levels (Halpern et al., 1993, 1997; Krauss et al., 1993; Odenthal et al., 1996; Strähle et al., 1996). Other related T-box genes are expressed in the midline, however, and they may have a function in regulating *shh* expression (Dheen et al., 1999).

Many lines of evidence suggest that HNF3β (Axial) is crucial for development of the notochord and the floor plate. Mutant mouse embryos lacking HNF3β activity lack the notochord, floor plate and also *shh* expression in the midline (Ang and Rossant, 1994; Weinstein et al., 1994). Overexpression of HNF3β results in ectopic activation of *shh* expression (Chang et al., 1997; Ruiz i Altaba et al., 1995a; Sasaki and Hogan, 1994). Furthermore, the zebrafish *shh* promoter contains HNF3β-binding sites, which mediate activation by HNF3β (Axial) in a cell system, suggesting that HNF3β (Axial) is a direct regulator of the *shh* gene (Chang et al., 1997). We did not find any sequence with homology to the HNF3β (Axial) consensus recognition site (Kaufmann et al., 1995; Overdier et al., 1994) in the functionally important regions of ar-C, suggesting that the activity of these regions depends on factors other than HNF3β. Given the low resolution of the functional analysis of ar-A and ar-B, it remains unclear whether the HNF3β (Axial)-binding site homologies found in these regions (F. M. and U. S., unpublished) are functionally relevant. The observation that both ar-B and ar-C act on the heterologous *HSP68* promoter shows that the activity of these elements is not restricted to the *shh* promoter. ar-C appears to activate the mutant −563*shh* promoter with mutations in the two HNF3β sites that mediate activation by HNF3β (Axial) in a cell system. Thus, the two HNF3β-binding sites do not appear to be required for activation in our assay system in the embryo. These findings do not, however, rule out a requirement of HNF3β for promoter activity as a number of other HNF3β-binding site homologies are present in the promoter region that could potentially compensate for the mutations. Other promoter elements may substitute for the HNF3β in the more ubiquitously expressed *HSP68* promoter.

Previously, it has been proposed that Shh controls floor plate differentiation and floor-plate-specific gene programs via post-transcriptional activation of Gli-1, which in turn switches on transcription of *HNF3β* (Lee et al., 1997; Sasaki et al., 1997). ar-B, which mediates floor plate expression, harbours a sequence (+3260 TAGCACCCA) with significant homology to a Gli-1-binding site (Kinzler and Vogelstein, 1990; Sasaki et al., 1997). This homology suggests that ar-B may be a target of Gli-1 and that *shh* may regulate its own expression directly via Gli-1.

The regulation of *shh* expression in notochord and floor plate are conserved between mouse and zebrafish

While it is clear that *shh* is crucial for development of the floor plate in the mouse embryo (Chiang et al., 1996), the picture is less clear in zebrafish. Zebrafish embryos carrying loss-of-function alleles in *sonic-you* encoding the zebrafish orthologue of *shh* form floor plate and express floor plate markers (Schauerte et al., 1998). These observations raise the possibility that the regulatory mechanisms underlying floor plate specification in the zebrafish may be different from those in the mouse. Our finding that the zebrafish intronic enhancers also drive expression in the notochord and floor plate of the mouse argues strongly against such an interpretation. Furthermore, the ar-C sequence delineated by deletion mapping shares high sequence similarity with the floor plate enhancer SFPE2, which was recently identified in intron2 of the mouse *shh* gene (Epstein et al., 1999). Highest homology was scored in the subregions ar-C2 and ar-C3 shown by deletion analysis to be required for full activity of ar-C in the co-injection approach in the zebrafish. A homology to the T-box transcription-factor-binding site that resides in ar-C1 of the zebrafish enhancer was found 22 bp downstream of the mouse region homologous to ar-C3 (F. M. and U. S. unpublished). Thus, zebrafish and mouse embryos appear to employ the same regulatory sequences to drive expression of the *shh* gene. The mechanism underlying floor plate development in the two organisms appears to be conserved.

In view of this conservation and the floor plate deficiency and impaired *shh* expression in *cyc* and *oep* mutant zebrafish embryos (Hatta et al., 1991; Schier et al., 1997; Strähle et al., 1997b), it is likely that these genes, which encode Nodal-related and EGF domain-containing signals, respectively (Rebagliati et al., 1998; Sampath et al., 1998; Zhang et al., 1998), are also required for floor plate development in higher vertebrates. Future experiments will have to show whether *oep* and *cyc* act directly on *shh* expression and if so whether they act through the intronic elements.

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