Development 126, 281-292 (1999)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV4093

Regionalization of *Sonic hedgehog* transcription along the anteroposterior axis of the mouse central nervous system is regulated by Hnf3-dependent and -independent mechanisms

Douglas J. Epstein[1,*], Andrew P. McMahon[2] and Alexandra L. Joyner[1,3,‡]

[1]Developmental Genetics Program and Howard Hughes Medical Institute, Skirball Institute of Biomolecular Medicine, and
[2]Department of Cellular, Molecular and Developmental Biology, Harvard University, 1600 Divinity Avenue, Cambridge MA, USA
[3]Departments of Cell Biology and Physiology and Neuroscience, NYU School of Medicine, 540 First Avenue, New York, NY 10016, USA

*Present address: Department of Genetics, University of Pennsylvania Medical School, Philadelphia, PA 19104-6145, USA (epsteind@mail.med.upenn.edu)
‡Author for correspondence (e-mail: joyner@saturn.med.nyu.edu)

Accepted 20 October; published on WWW 14 December 1998

SUMMARY

The axial midline mesoderm and the ventral midline of the neural tube, the floor plate, share the property of being a source of the secreted protein, Sonic hedgehog (Shh), which has the capacity to induce a variety of ventral cell types along the length of the mouse CNS. To gain insight into the mechanisms by which *Shh* transcription is initiated in these tissues, we set out to identify the *cis*-acting sequences regulating *Shh* gene expression. As an approach, we have tested genomic clones encompassing 35 kb of the *Shh* locus for their ability to direct a *lacZ* reporter gene to the temporally and spatially restricted confines of the *Shh* expression domains in transgenic mice. Three enhancers were identified that directed *lacZ* expression to distinct regions along the anteroposterior axis including the ventral midline of the spinal cord, hindbrain, rostral midbrain and caudal diencephalon, suggesting that multiple transcriptional regulators are required to initiate *Shh* gene expression within the CNS. In addition, regulatory sequences were also identified that directed reporter expression to the notochord, albeit, under limited circumstances. Sequence analysis of the genomic clones responsible for enhancer activity from a variety of organisms, including mouse, chicken and human, have identified highly conserved binding sites for the hepatocyte nuclear factor 3 (Hnf3) family of transcriptional regulators in some, but not all, of the enhancers. Moreover, the generation of mutations in the Hnf3-binding sites showed their requirement in certain, but not all, aspects of *Shh* reporter expression. Taken together, our results support the existence of Hnf3-dependent and -independent mechanisms in the direct activation of *Shh* transcription within the CNS and axial mesoderm.

Key words: Shh, Gene regulation, Central nervous system, Mouse, Hnf3

INTRODUCTION

The establishment of regional identity along the two major axes of the vertebrate nervous system involves the coordinated expression of signaling molecules localized in time and space to discrete organizing centers (Tanabe and Jessell, 1996; Lumsden and Krumlauf, 1996). Two of such embryonic territories, the axial mesoderm (prechordal plate and notochord) underlying the neural plate and the ventral midline of the spinal cord (floor plate) have been defined as organizing centers based on their ability to promote ventral CNS differentiation in tissue recombination assays (reviewed in Tanabe and Jessell, 1996).

The cloning of the secreted factor, *Sonic hedgehog* (*Shh*) (Echelard et al., 1993; Riddle et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Chang et al., 1994) and the finding that it is expressed within organizing centers such as the notochord and floor plate prompted experiments to test its ability to function as an inductive signal during nervous system development. Gain- and loss-of-function experiments have revealed a crucial role for Shh in patterning the ventral CNS along the length of the anteroposterior neuraxis (Tanabe and Jessell, 1996). At spinal cord levels, Shh has been shown to be a sufficient and required signal for the induction of motor neuron and floor plate differentiation (Roelink et al., 1995; Marti et al., 1995a; Chiang et al., 1996). At more rostral axial levels, Shh has been shown to be dependent on the cooperation of additional factors, including members of the BMP and Fgf superfamilies, for the induction of distinct neuronal cell types (Dale et al., 1997; Ye et al., 1998).

Considerable attention has focussed on the roles of mediators in Shh signal transduction (reviewed in Hammerschmidt et al., 1997; Ruiz i Altaba, 1997; Kalderon, 1997). Stemming largely from studies of hh signaling in

Drosophila, the framework of a pathway is now emerging to explain how on a molecular level these conserved transducers of the Shh signal participate in the patterning of the ventral CNS. Shh, secreted from the notochord, is thought to be bound by its co-receptors Ptc and Smo in the overlying medial neural plate (Stone et al., 1996; Marigo et al., 1996a), presumably leading to the downregulation of PKA activity (Hammerschmidt et al., 1997) and activation of members of a family of transcriptional regulators, the *Gli* genes (Hui et al., 1994; Lee et al., 1997; Platt et al., 1997). Gli2 has been shown to be required for proper floor plate development (Ding et al., 1998; Matise et al., 1998) and Gli1 can positively regulate the transcription of two target genes in the ventral CNS, the winged helix transcriptional activator *Hnf3β* and *Ptc* (Sasaki et al., 1997; Hynes et al., 1997; Lee et al., 1997). Hnf3β, in turn, has been shown sufficient to activate *Shh* expression in the CNS (Echelard et al., 1993; Hynes et al., 1995a; Ruiz i Altaba et al., 1995) hence, completing a molecular cascade that culminates in the initiation and maintenance of floor plate gene expression. Subsequent to floor plate induction, the maintenance of floor plate gene expression is no longer dependent on Gli activity, as expression of all family members is downregulated from the floor plate (Lee et al., 1997; Platt et al., 1997).

While progress has been made in deciphering the pathway downstream of Shh, little is known about the mechanisms involved in activating *Shh* gene expression within the aforementioned organizing centers. Regulation of the *Shh* gene is critical for proper growth and development, as loss of a single copy can result in the human condition holoprosencephaly (Belloni et al., 1996; Roessler et al., 1996). Furthermore, inappropriate activation of *Shh* (Echelard et al., 1993; Fan et al., 1995; Oro et al., 1997) or components of the pathway (Epstein et al., 1996; Johnson, 1996; Dahmane et al., 1997; Xie et al., 1998) has been implicated in over-proliferation phenotypes including the formation of tumors, thus suggesting that the *Shh* pathway requires a tightly balanced level of regulation.

Hnf3β has been proposed to be an upstream activator of *Shh* based initially on the observation that it is expressed prior to and in the same cells as *Shh* in the axial mesoderm and floor plate (Sasaki and Hogan, 1993; Ang et al., 1993; Monaghan et al., 1993; Echelard et al., 1993; Marti et al., 1995b). That ectopic expression of *Hnf3β*, in a variety of developmental systems, can lead to the induction of floor plate gene expression including *Shh*, suggests that Hnf3β may be involved in initiating *Shh* transcription, however, it has yet to be determined whether this activation is direct (Echelard et al., 1993; Sasaki and Hogan, 1994; Ruiz i Altaba et al., 1995). As mice carrying loss-of-function alleles of *Hnf3β* result in the loss of a functional node, notochord and floor plate, they have proved uninformative with respect to addressing whether Hnf3β is necessary for the activation of *Shh* expression (Ang and Rossant, 1994; Weinstein et al., 1994).

Interestingly, another transcriptional component of the Shh pathway, Gli3, has been shown to negatively regulate the expression of *Shh* (Marigo et al., 1996b). The spontaneously arising *extra-toes* mouse mutant, which harbors a deletion of *Gli3* (Vortkamp et al., 1992; Hui and Joyner, 1993), shows ectopic expression of *Shh* in both the anterior limb bud (Masuya et al., 1995; Buscher et al., 1997) and dorsal CNS (Ruiz i Altaba, 1998), two sites where *Shh* is not normally found.

In an attempt to further characterize the transcriptional regulators that act upstream of mouse *Shh* in polarizing centers such as the floor plate and notochord, we initiated an in vivo reporter assay to identify the *cis*-acting sequences that regulate *Shh* gene expression. Several enhancers were detected that can direct the expression of a *lacZ* reporter gene to discrete regions of the ventral CNS in transgenic mice, suggesting that activation of *Shh* transcription is regionalized along the anteroposterior axis and that not one, but multiple, regulators are responsible for initiating *Shh* expression in the CNS. Consensus binding sites for Hnf3 class transcriptional regulators were identified in some, but not all, enhancers supporting the view that Hnf3-dependent and -independent mechanisms exist to activate *Shh* gene expression in the floor plate.

MATERIALS AND METHODS

Generation of Reporter Constructs

Genomic clones overlapping the *Shh* locus were isolated from a λ Dash II library (Stratagene) as described in Echelard et al. (1993). Reporter constructs were generated by subcloning DNA restriction fragments from the purified λ clones into a reporter cassette modified from that previously described (Echelard et al., 1994). The reporter vector contained either a *Shh* (1.1 kb *Eco*RI-*Sac*II) or *hsp68* (Kothary et al., 1989) promoter, a *lacZ* cDNA followed by SV40 polyadenylation sequences, and a multiple cloning site polylinker located either upstream or downstream of the *lacZ* cDNA. As the 1.1 kb *Eco*RI-*Sac*II fragment proved to be toxic to bacteria when propagated in a high replication copy plasmid, the low copy pBR322 vector was used in the cloning of all reporter constructs. Genomic fragments were blunt ended with T4 DNA polymerase (NEB) and cloned into the *Sna*BI restriction site of the reporter vector, situated either upstream or downstream of the promoter depending on its native orientation. The restriction sites listed on reporter constructs 1 through 19 (Figs 1, 3, 5) correspond to those used in subcloning.

Mutations in Hnf3-binding sites 4 and 5 (RC18 and RC19) were generated using a multistep PCR-based approach. For Hnf3-binding site 4, two sets of primers (J23-5′ GCCAGAGCCCCGGGTCG-CATTCC 3′, J24-5′ TTTTAAGCTTCCAGAAAGAATCTTTCTGC 3′, and J25-5′ TTTTAAGCTTGCCAAAAGCATGATCC 3′ J26-5′ TTTTCTCGAGAAGGGTATTTGAATTTAAAATGGC 3′) were used to amplify sequences on either side of the Hnf3 site, that were subsequently digested with *Sma*I-*Hin*dIII and *Hin*dIII-*Xho*I, respectively, and cloned into the corresponding sites within the pBSKII (Stratagene) vector. This strategy effectively replaced the Hnf3 site 4 with a *Hin*dIII restriction site. The 350 *Sma*I-*Xho*I fragment was then cloned into a reporter vector containing the 150 bp (*Eco*RI-*Sma*I) fragment (RC18). Two point mutations that abolish the ability of Hnf3 site 5 to bind to DNA were also generated during the aforementioned mutagenesis of Hnf3-binding site 4 through the introduction of mismatches within the J26 primer (underlined bases). To generate RC19, RC18 was digested with *Bam*HI/*Xho*I and the 500 bp fragment containing the mutated HNF3 sites 4 and 5 was cloned into the corresponding sites of RC17.

The sequencing of all PCR-generated mutations, as well as the genomic fragments contained within RC1 (GenBank Accession Number AF098925), RC6 (GenBank Accession Number AF098926), RC14 (Genbank Accession Number AF098927) and RC15 was performed by dye terminator cycle sequencing (PE Applied Biosystems) using an ABI Prism 377 DNA sequencer.

Primer extension analysis

The start site of *Shh* transcription was mapped by primer extension. For this, an oligo complementary to nucleotides –211 to –230 (5′ CGACGCTACCGTCCGACGGC 3′) of the *Shh* cDNA was end labeled with T4 polynucleotide kinase (NEB) and annealed overnight (4 M NaCl, 100 mM Pipes pH 6.4) at 56°C to 3 μg of poly(A)+ RNA isolated from the lungs of 18.5 dpc embryos. As negative controls, both tRNA and poly(A)+ E18.5 liver RNA were used as primer extension templates. The annealed primer was extended using Superscript II reverse transcriptase (GIBCO BRL) under conditions recommended by the supplier. After phenol/chloroform extractions and subsequent precipitation, the primer extension products were electrophoresed on an 8% sequencing gel along side a sequencing reaction generated with the identical primer and a genomic clone overlapping the region. The gel was dried and exposed to autoradiography film (Kodak) for 7 days at –80°C using an intensifying screen.

Production and genotyping of transgenic mice

Outbred Swiss webster mice (Taconic) were used to produce transgenic embryos and mouse lines essentially as described (Hogan et al., 1994). Transgenes were prepared for microinjection as described (Epstein et al., 1996). The genotyping of transgenic embryos and mice was carried out by PCR using proteinase K-digested yolk sacs or tail biopsies as DNA templates. Upstream primers directed against either the *Shh* (5′ GACAGCGCGGGGACAGCTCAC 3′) or *hsp68* (5′ GACGAACCTTCCCAGGAGCATC 3′) promoters and a downstream primer directed against *lacZ* (5′ AAGGGCGATCGGTGCGGGCC 3′), generating DNA fragments of (200 bp) and (220 bp), respectively, were used under the following PCR conditions: 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, for 30 rounds followed by a final extension at 72°C for 10 minutes. For staging embryos, the day of vaginal plug detection corresponded to 0.5 days post coitus (dpc).

Whole-mount β-galactosidase and in situ hybridization

β-galactosidase activity was detected in whole-mount embryos by using X-gal (Sigma) or Salmon-gal (Biosynth) as substrates according to Echelard et al. (1994). The embryos were stained from 20 minutes to overnight depending on the strength of transgene expression. Whole-mount RNA in situ hybridization of embryos was performed essentially as described (Matise et al., 1998) using an *Shh* riboprobe (Echelard et al., 1993).

Histological analysis

After whole-mount staining some embryos were sectioned at 50-75 μm on a vibrotome and counterstained with Nuclear Fast Red (Poly Sciences).

RESULTS

As an impetus towards the identification of the critical *cis*-acting sequences responsible for the regulation of *Shh* gene expression, three overlapping λ phage clones encompassing 35 kb of the *Shh* locus were isolated and a high resolution restriction map constructed (Fig. 1). Based on this map, genomic fragments surrounding the *Shh* gene were cloned into a reporter vector comprising of a minimal promoter, the *bacterial β-galactosidase* gene and an SV40 polyadenylation signal. All genomic fragments were initially cloned in the orientation found within the endogenous *Shh* locus, thus preserving its normal position with respect to the *Shh* promoter. The reporter constructs were then injected into fertilized mouse eggs and assayed for X-gal staining at either 9.5 or 10.5 dpc. These stages of development were chosen because *Shh* expression in tissues with polarizing activity is well developed.

The pattern of *Shh* expression during mid-embryogenesis in the mouse has been documented (Echelard et al., 1993). Briefly, at 9.5 dpc, *Shh* mRNA can be detected throughout much of the ventral neuraxis including the floor plate of the spinal cord, hindbrain and midbrain. In the ventral midline of the midbrain and caudal diencephalon *Shh* expression is broader than in other regions whereas in the rostral diencephalon *Shh* is absent from the midline but found in two stripes of lateral expression that merge in the floor of the telencephalon (Echelard et al., 1993) and Fig. 2A). Expression of *Shh* can also be observed in tissues of non-ectodermal origin including the notochord, gut and pharyngeal endoderm and, at 10.5 dpc, the posterior region of the limb bud (Echelard et al., 1993). Although the analysis focussed at identifying regulatory sequences mediating *Shh*-like expression at these stages, relevant reporter expression was also assessed in stable mouse lines at earlier and later time points.

As gene regulation can be conferred by promoter-specific interactions with tissue-specific enhancers (Hansen and Tjian, 1995; Merli et al., 1996), all reporter constructs were initially tested utilizing a *Shh* promoter. The *Shh* transcriptional start site was mapped by primer extension analysis to a position 352 bp upstream of the initiator methionine where a consensus TATA box (TATAAT)-binding site was identified (data not shown). A *Shh* promoter comprising 1 kb of upstream sequence was cloned into a *lacZ* reporter construct [Fig. 1, Reporter Construct 1 (RC1)] and tested for its ability to recapitulate the pattern of *Shh* gene expression in transgenic mouse embryos. Although four of eight embryos carrying the RC1 transgene expressed *lacZ* at 10.5 dpc, suggesting that the promoter was functional, none of them displayed staining in a pattern consistent with that of *Shh.* Given this finding, the relevance of the previous report that maintenance of *Shh* expression in cultured limb bud cells is dependent on multiple consensus binding sites for *Hoxd-12* situated in proximity of the *Shh* promoter is questionable (Knezevic et al., 1997). However, the possibility remains that binding sites for Hoxd-12 located elsewhere are still pertinent for *Shh* regulation.

An upstream *Shh* enhancer directs expression to the floor plate

Extending the enhancer search to include an additional 13 kb of genomic DNA upstream of the start of *Shh* transcription (Fig. 1, RC2) revealed *lacZ* expression exclusively in the ventral midline of the neural tube in transgenic embryos (Fig. 2C). In contrast to the pattern of *Shh* expression normally seen at 9.5 dpc, embryos carrying the RC2 transgene showed X-gal staining in a limited pattern along the anteroposterior axis (compare Fig. 1A and C). The rostral expression boundary of the reporter gene was detected at the mid-hindbrain junction and the caudal limit extended only to the hindlimbs. Vibrotome sections cut in the transverse plane through several of these embryos also demonstrated that expression was restricted to the floor plate of the spinal cord and hindbrain and absent from the notochord and gut (compare Fig. 2A with C and Fig. 2B with D). Founder lines of mice were generated with the RC2 transgene and, other than the floor plate, no additional sites of reporter activity were revealed in embryos between 8.5 and 13.5 dpc.

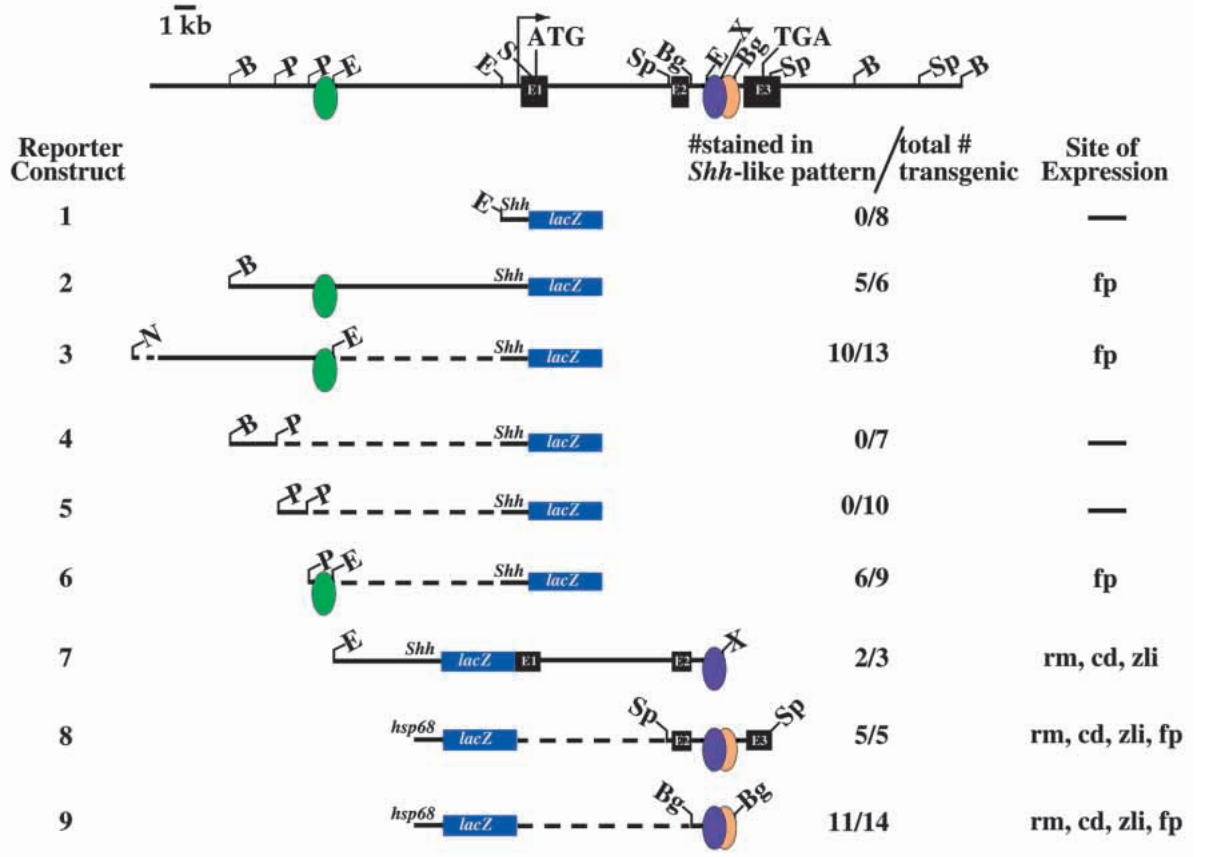

Fig. 1. Characterization of *Shh* regulatory regions in transgenic embryos. Depicted at top is a genomic map surrounding approximately 35 kb of the *Shh* locus, including the position of the *Shh* transcription start site (arrow), coding regions (black boxes) and a partial list of restriction enzyme sites (B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; P, *Pst*I; S, *Sac*II; Sp, *Spe*I; X, *Xho*I) used in the generation of the reporter constructs. The green, purple and peach balloons correspond with the location of the identified enhancers; green, *Shh* floor plate enhancer 1 (SFPE1); purple, *Shh* brain enhancer 1 (SBE1); peach, SFPE2. Listed below the map are reporter constructs 1-9, which were tested for reporter activity in transgenic mice. Where depicted, either the *Shh* or *hsp68* promoters were used in the assay. Solid lines represent genomic fragments present in the reporter constructs, whereas dashed lines correspond to deleted genomic fragments. To the right of the constructs are the results of the transgenic expression analysis indicating the number of transgenic embryos that stained in a *Shh*-like pattern versus the total number of transgenic embryos or lines generated. Sites of expression at 9.5 and/or 10.5 dpc are indicated (fp, floor plate; rm, rostral midbrain; cd, caudal diencephalon; zli, zona limitans intrathalamica).

In addition to assessing spatial control of the RC2 transgene, we also investigated whether temporal regulation was consistent with the onset of *Shh* transcription in the floor plate. *Shh* expression in the CNS is first detected at the 8-somite stage in the presumptive ventral midbrain and then progresses rostrally into the forebrain and, by the 12-somite stage, caudally into the floor plate of the hindbrain and spinal cord (Echelard et al., 1993). Interestingly, the onset of RC2 activity at the 12-somite stage coincided precisely with the initiation of *Shh* expression in the floor plate of the hindbrain and spinal cord (data not shown).

A series of deletion constructs (Fig. 1, RC3-RC6) aided in better defining the location of the *Shh* floor plate enhancer (SFPE1) to within a 1.1 kb genomic fragment approximately 8 kb upstream of the *Shh* transcription start site. This 1.1 kb construct (RC6) displayed similar reporter activity compared to the 14 kb construct (RC2), as assessed by the consistency of expression as well as the minimal time required to initiate staining (20 minutes). Furthermore, the 1.1 kb fragment was found to possess the same activity when directing transcription from a heterologous (*hsp68*) promoter, independent of its orientation, thus, fitting the criteria of a bone fide enhancer (data not shown).

Intronic enhancers direct reporter gene expression to cranial and spinal cord regions

Given that upstream sequences directed *lacZ* to only a portion of the *Shh* expression domain, sequences downstream of the start of *Shh* transcription were also tested for reporter activity. A second enhancer complex was identified when constructs containing intronic sequences were used to generate transgenic embryos (Fig. 1, RC7-9). Once again, X-gal staining was observed in a restricted pattern in comparison to the *Shh* mRNA expression profile. Embryos carrying a transgene that contained all of intron 1 and a portion of intron 2 (RC7) displayed reporter gene activity in the ventral midline of the rostral midbrain and caudal diencephalon at 9.5 dpc (Fig. 2E). The location of this *Shh* brain enhancer (SBE1) was further delineated to within a 2.2 kb segment within the second intron (Fig. 1, RC8, RC9). Interestingly, both RC8 and RC9 displayed

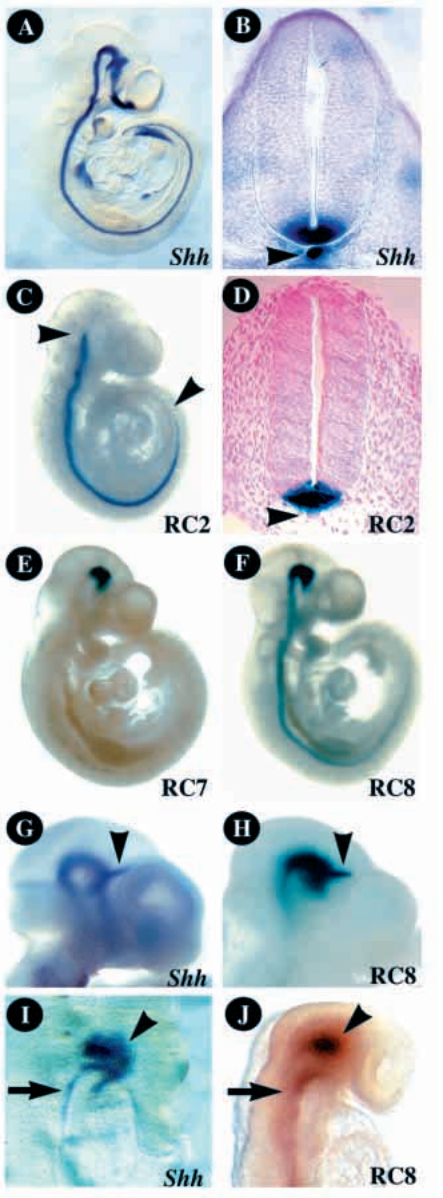

Fig. 2. Comparison of the sites of expression of *Shh* reporter constructs in transgenic embryos to *Shh* mRNA. Whole-mount in situ hybridization of embryos labelled with a *Shh* riboprobe (A,B,G,I) compared to transgenic embryos carrying reporter constructs (from Fig. 1) and stained for β-galactosidase activity (C-F,H,J). (A,B) *Shh* expression at 9.5 dpc. The section in B is taken through a lumbar region of the spinal cord of the embryo in A and shows expression in the floor plate and notochord (arrowhead). (C,D) RC2 expression at 9.5 dpc revealing SFPE1 activity after 30 minutes of staining. Arrowheads in C point to anterior (mid-hindbrain junction) and posterior (hindlimbs) limits of X-gal staining within the CNS. The section in D is taken through a lumbar region of the embryo in C and shows expression exclusively in the floor plate. The arrowhead in D marks the unstained notochord. (E) RC7 expression at 9.5 dpc revealing SBE1 activity in the rostral midbrain and caudal diencephalon after 40 minutes of staining. (F) RC8 expression revealing SBE1 and SFPE2 activity after 40 minutes of staining. (G,H) At 10.5 dpc, both *Shh* and RC8 show expression in the zli (arrowheads). (I,J) At 8.5 dpc (8-somite stage), both *Shh* and RC8 show expression in the presumptive ventral midbrain (arrowhead) as well as the underlying axial mesoderm. No reporter activity was detected in the foregut or pharyngeal endoderm (arrow). The embryo in J is stained with salmon-gal for 15 minutes.

additional expression in the hindbrain and spinal cord, identifying a second *Shh* floor plate enhancer element (SFPE2; Fig. 2F and data not shown).

Stable lines of transgenic mice generated from RC8 showed X-gal staining at 10.5 dpc, within the zona limitans intrathalamica (ZLI), the boundary between the dorsal and ventral thalami, in addition to the midbrain and caudal diencephalon (Fig. 2G,H). 8-somite-stage embryos carrying the RC8 transgene showed staining in the presumptive ventral midbrain, hence marking the initial site of *Shh* expression in the CNS (compare Fig. 2I,J). The RC8-mediated expression progressed into the hindbrain and spinal cord in similar fashion to *Shh* but never advanced further anterior than the caudal diencephalon or more posterior than the hindlimbs. An additional site of expression was also detected, albeit transiently, in the axial mesoderm of embryos at 8.5 dpc (Fig. 2J), however, by 9.5 dpc, there was no noticeable staining in the notochord. The two intronic enhancers, SBE1 and SFPE2, were also found to function in an orientation- and promoter-independent fashion (data not shown).

From this reporter gene analysis, we conclude that activation of *Shh* gene expression along the anteroposterior axis of the mouse CNS is under the transcriptional control of several discrete enhancer elements scattered throughout the *Shh* locus.

SFPE1 activity is regulated independent of Hnf3 function

To further explore the nature of the *cis*-acting sequences within RC6 that mediate floor plate expression, the 1.1 kb genomic fragment was sequenced and analyzed for the presence of consensus binding sites recognized by known transcription factors using the Signal Scan (Advanced Biosciences Computing Center, University of Minnesota) and Best Fit (GCG) algorithms. As expected, a number of general transcription factor binding sites were identified; however, attention was only directed at those suggestive of tissue specificity. Of particular interest was the identification of three 12 nucleotide stretches that matched the recognition site of the hepatocyte nuclear factor 3 (Hnf3) class of winged helix transcriptional regulators (Overdier et al., 1994; see Table 1). Presently, three Hnf3 family members (α, β and γ) have been identified, all of which bind to similar target sequences (reviewed in Kaufmann and Knochel, 1996).

To determine whether Hnf3 proteins were able to bind to the identified Hnf3 recognition sequences, electromobility shift assays using liver extracts as the source of all three Hnf3 proteins were performed. Each of the three Hnf3 isoforms was shown to be capable of binding to the three identified Hnf3-binding sites (data not shown). Moreover, the three Hnf3-binding sites were also found to be 100% conserved with human sequences when a best fit alignment (GCG) of the 1.1 kb (RC6) mouse sequence was performed with the sequence of a 30 kb cosmid overlapping the human *Shh* locus (Table 1). The preservation of the sequence of the Hnf3-binding sites between mouse and human is suggestive of functional conservation.

In order to determine whether the 5′ segment of the 1.1 kb

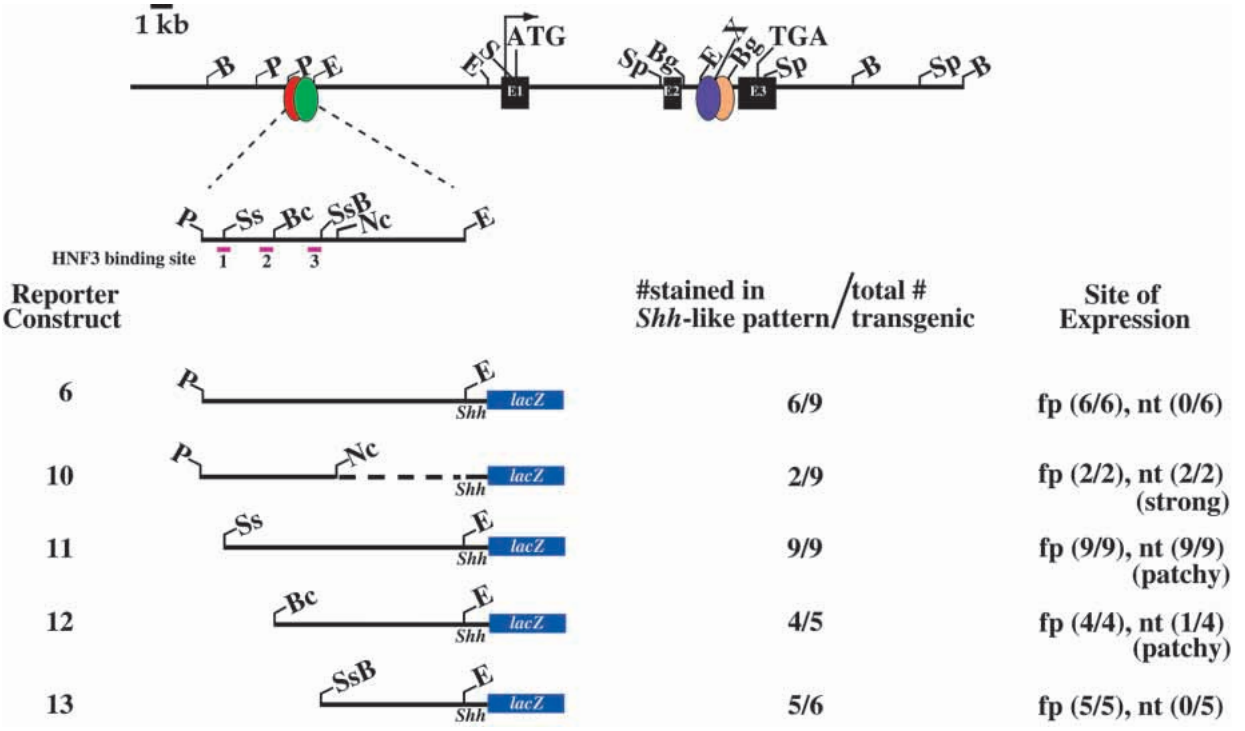

Fig. 3. Dissection of the upstream *Shh* notochord and floor plate enhancers. The schematic map of the *Shh* locus is as described in Fig. 1, with the addition of a red balloon depicting the position of a *Shh* notochord enhancer (SNE). The 1.1 kb *Pst*I-*Eco*RI genomic fragment (RC6) is enhanced to reveal the position of additional restriction enzyme sites (Bc, *Bcl*I; E, *Eco*RI; Nc, *Nco*I; P, *Pst*I; Ss, *Ssp*I; SsB, *Ssp*BI) used to generate RC10-13. The position of Hnf3-binding sites 1-3 (magenta bars) is also shown. RC6 (from Fig. 1) serves as a reference point for comparison with RC 10-13. Sites of expression (fp, floor plate; nt, notochord).

(RC6) fragment, encompassing the cluster of Hnf3 sites, was sufficient for floor plate expression, embryos carrying a transgene containing only the proximal half of RC6 were generated (Fig. 3, RC10). Remarkably, strong X-gal staining was detected in the notochord, whereas floor plate staining was found to be relatively weak (Fig. 4A). Of the nine transgenic embryos carrying RC10, only two showed this pattern with the remainder being devoid of any *Shh*-like expression. This suggests that a *Shh* notochord enhancer (SNE) is present in RC10 and is particularly sensitive to the site of integration.

Given the unexpected finding that the proximal portion of the 1.1 kb fragment (RC10) is capable of directing reporter expression to the notochord, whereas the full fragment (RC6) is sufficient for only floor plate expression, we set out to determine to what degree this dual enhancer activity was dependent on Hnf3 function. For this, a series of constructs containing small deletions resulting in the sequential removal of the Hnf3-binding sites were tested in the reporter assay. The first construct generated contained an 80 bp deletion of RC6, overlapping Hnf3-binding site 1 (Fig. 3, RC11). 10.5 dpc embryos carrying the RC11 transgene displayed strong reporter activity in the floor plate and consistently patchy X-gal staining in the notochord (Fig. 4B). When embryos carrying a construct that removed the first and second Hnf3-binding sites of RC6 were assessed for reporter expression, X-gal staining was observed in the floor plate of all four transgenic animals generated, while patchy notochord expression was only detected in one embryo (Fig. 3, RC12). Embryos carrying a construct that deleted all three Hnf3-binding sites (Fig. 3, RC13) showed strong staining in the floor plate but were completely devoid of reporter activity in the notochord (Fig. 4C).

As no other sequences within the distal portion of RC13 were found to match the consensus for Hnf3-binding proteins as defined by Overdier et al. (1994), we conclude that SFPE1 activity occurs in an Hnf3-independent manner. Moreover, SNE activity may be dependent on Hnf3 function as the loss

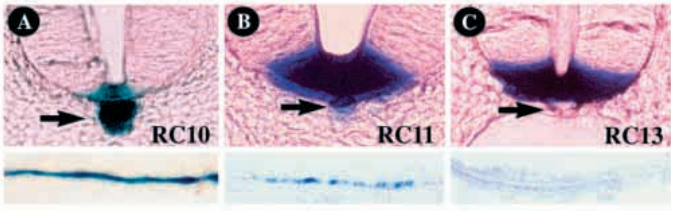

Fig. 4. *Shh* notochord and floor plate enhancers are independently regulated. (A) RC10 expression in the notochord (arrow) showing robust expression compared to the floor plate. The section is from the lumbar region of a 9.5 dpc embryo stained for 60 minutes. After staining, the notochord was dissected away from other tissues to reveal extent of staining along a portion of its length (below). (B) RC11 expression in the notochord (arrow) showing weak and patchy (below) expression along its length compared to the strong floor plate staining. The section is from the thoracic region of a 9.5 dpc embryo stained for 3 hours. (C) RC 13 expression is absent from the notochord (arrow and below) yet persists in the floor plate. The section is from lumbar regions of a 9.5 dpc embryo stained for 3 hours.

Table 1. Sequence of mouse Hnf3-binding sites identified in *Shh* regulatory regions compared to human and chicken

Hnf3 binding site	Mouse sequence	Human sequence	Chicken sequence
1 (RC10)	AAATATTTAGTC	AAATATTTAGTC	NA
2 (RC10)	CCTTATTTGATT	CCTTATTTGATT	NA
3 (RC10)	ACATATTTATAC	ACATATTTATAC	NA
4 (RC16)	CAATCTCTATTT	NA	AGATCTCTGTTT
5 (RC16)	AGATGTGTATTT	NA	AGATGTGTATTT
6 (RC17)	GAATGTTTGTCC	NA	——
7 (RC17)	GGATATTTACTC	NA	——
8 (RC17)	GGTTGTTGGCAT	NA	——
HNF3 consensus binding site:	VAWTRTTKRYTY		

The Hnf3-binding sites were identified according to their sequence matching that of the consensus site for Hnf3β (Overdier et al., 1994). Best-fit alignment (GCG) determined the overlap between mouse and human as well as mouse and chicken sequences. Underlined bases in Hnf3-binding site 4 show differences between the mouse and corresponding chicken sequence yet, still match the consensus. Solid line refers to the fact that no homology was detected between the mouse and chicken sequence at the corresponding positions. NA, sequence not available for comparison. Mismatches between certain bases and the Hnf3 consensus were occasionally detected, however binding to the site persisted (Overdier et al., 1994; data not shown). V: A,C or G; W: A or T; R: G or A; K: G or T; Y: C or T.

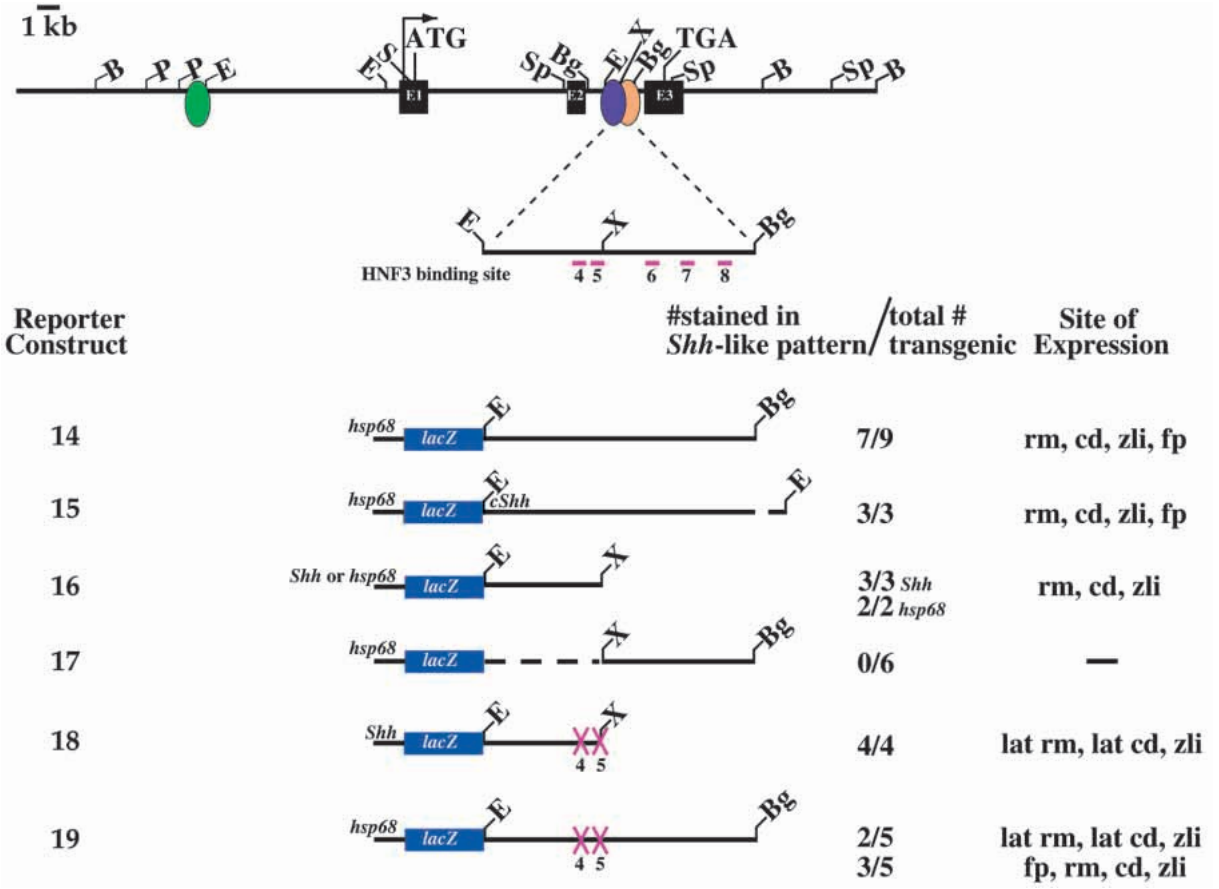

Fig. 5. Dissection of the SBE1 and SFPE2 intronic enhancers. The schematic map of the *Shh* locus is as described in Fig. 1. The 1.3 kb *Eco*RI-*Bgl*II genomic fragment is enhanced and shows the position of Hnf3-binding sites 4-8 as well as the restriction enzymes used in the generation of RC14-19. RC 15 contains chicken genomic DNA isolated from a similar region of intron 2 compared to RC 14. Hnf3-binding sites 4 and 5 are mutated in RC 18 and 19 (magenta X). RC18 expression is absent from the ventral midline of the CNS in transgenic embryos but shows expression in lateral regions of the rostral midbrain and caudal diencephalon (lat rm, lat cd). Similarly, 2/5 embryos transgenic for RC19 showed absence of ventral midline expression in the CNS (lat rm, lat cd). In contrast, 3/5 embryos transgenic for RC19 also showed either very weak expression (2/5) or normal expression (1/5) in the ventral midline of the CNS (fp, rm, cd, zli).

of binding sites correlated with the loss of reporter activity in the notochord. This conclusion is not definitive as the possibility remains that, in addition to the Hnf3-binding sites, we have removed additional sequences required for SNE activity.

SBE1 and SFPE2 rely on Hnf3 function for midline but not lateral *Shh* expression

In an effort to delineate the region containing the critical sequences for SBE1 and SFPE2 activity, a 1.3 kb genomic fragment (RC14) was cloned downstream of the hsp68 *lacZ* reporter cassette and injected into fertilized zygotes (Fig. 5). At 9.5 dpc, transgenic embryos were found to stain in the same pattern as those carrying the RC8 transgene including the ventral midline of the spinal cord, midbrain and caudal diencephalon (Fig. 6A).

Shh has been cloned from most experimental organisms and shows a pattern of expression highly conserved across phyla (Hammerschmidt et al., 1997). The pattern of *Shh* expression within the CNS and notochord in the chick is similar to that in the mouse. To benefit from this high degree of conservation of expression, we generated a reporter construct containing DNA overlapping a similar region of intron 2 from the chicken *Shh* locus (Fig. 5, RC15). Interestingly, embryos carrying the chicken DNA construct showed X-gal staining in a similar pattern to those carrying the mouse DNA (compare Fig. 6A with B). The one difference in the staining of embryos carrying chicken versus mouse constructs related to the posterior extent of expression within the CNS. Embryos carrying the RC14 mouse DNA construct showed a posterior limit of *lacZ* expression at the level of the hindlimbs whereas embryos carrying the RC15 chicken DNA construct showed staining into the tail bud.

Relying on the premise that conservation of sequence underlies conservation of function, the 1.3 kb (RC14) intron fragment containing the *cis*-acting sequences required for SBE1 and SFPE2 activity was sequenced from mouse and compared to the comparable region of intron 2 from chicken. The overall sequence identity between the mouse and chicken fragments approached 70% including two conserved Hnf3-binding sites (Table 1) that were found capable of binding Hnf3 proteins (data not shown). Three other Hnf3-binding sites matching the consensus were also identified in the mouse DNA fragment but were found not to be conserved in the corresponding chicken DNA (Table 1).

Before determining the requirement of the Hnf3-binding sites for SBE1 and SFPE2 activity, the positions of the two enhancers were better delineated. The *Xho*I restriction site appeared to demarcate the boundary between SBE1 and SFPE2 given the results obtained with RC7 and RC9 (Fig. 1). Two constructs were therefore generated, RC16 and RC17 (Fig. 5), which contained sequences proximal and distal to the *Xho*I restriction site, respectively. Although RC16 conveyed SBE1 activity, RC17 showed no *Shh*-like expression, suggesting that SFPE2 activity depends on sequences both proximal and distal to the *Xho*I site and that on their own, Hnf3-binding sites 6-8 are not sufficient for floor plate expression (see Discussion).

Hnf3-binding sites 4 and 5 were then mutated in the context of the 532 bp fragment that mediates SBE1 expression (Fig. 5, RC18). Transgenic embryos carrying RC18 displayed X-gal staining that was excluded from the ventral midline of all embryos tested (n=4) but was found present in lateral cranial regions (compare Fig. 6C,E with D,F) including, at 10.5 dpc, the zli (not shown).

To determine whether SFPE2 activity was also dependent on Hnf3-binding sites 4 and 5, they were perturbed in the context of the 1.3 kb fragment (Fig. 5, RC19). Two of five embryos carrying the RC19 transgene showed a complete absence of X-gal staining in the ventral midline of the spinal cord, midbrain and caudal diencephalon (compare Fig. 6G with H). The staining observed lateral to the floor plate at spinal cord levels in Fig. 6H is derived from the *hsp68* promoter which has endogenous β-gal activity and thus, serves as a positive control for staining (Song et al., 1996). In contrast, staining in lateral cranial regions, where *Shh* is normally found broader than in more posterior regions, was unaffected by the loss of Hnf3 sites 4 and 5 in these embryos. In the remaining three embryos carrying the RC19 transgene, expression in the midline was weak and patchy (2/5), or unaffected by the loss of the Hnf3 sites (1/5) (data not shown).

Taken together, these results suggest that Hnf3 function is required for consistent floor plate activation by SBE1 and SFPE2 but that an Hnf3-independent pathway exists to activate lateral SBE1 activity.

DISCUSSION

CNS expression of *Shh* is regulated by multiple enhancers

With the intent of identifying the transcriptional regulators that act upstream of *Shh*, we conducted a reporter gene assay in transgenic mice that assessed the ability of 35 kb of genomic DNA surrounding the locus to direct reporter gene expression in a *Shh*-like pattern. From this assay, several enhancers were identified and shown to direct transcription to discrete regions of the *Shh* expression domain. Within the CNS, where *Shh* is expressed along the length of the ventral neuraxis, two independent enhancers, *Shh* floor plate enhancer 1 and 2 (SFPE1 and SFPE2), were shown to direct reporter expression exclusively to the floor plate of the hindbrain and spinal cord, whereas a third, *Shh* brain enhancer 1 (SBE1), directed reporter expression to the ventral midbrain and caudal diencephalon. With respect to the genomic organization of the enhancers, SFPE1 was localized to a 500 bp fragment approximately 8 kb upstream of the *Shh* transcription start site, whereas, SFPE2 and SBE1 were identified on overlapping 1.3 kb fragments situated within intron 2. Transcriptional activity from these three enhancers, as judged by X-gal staining, covered the majority of the anteroposterior neuraxis with the exception of the rostral forebrain and the region caudal to the hindlimbs, which presumably fall under the control of additional enhancers lying outside of the genomic region analyzed.

Regarding expression in other tissues, the proximal half of RC6 was shown to occasionally direct expression to the notochord and RC8 transiently directed expression to the axial mesoderm at early somite stages. Due to the general inconsistency in the reporter gene activity at these sites of expression, it is likely that additional enhancers must exist that control the full extent of *Shh* expression in the axial mesoderm. Further support for this view emanates from our finding that a 100 kb BAC clone, overlapping the 35 kb of DNA tested in our

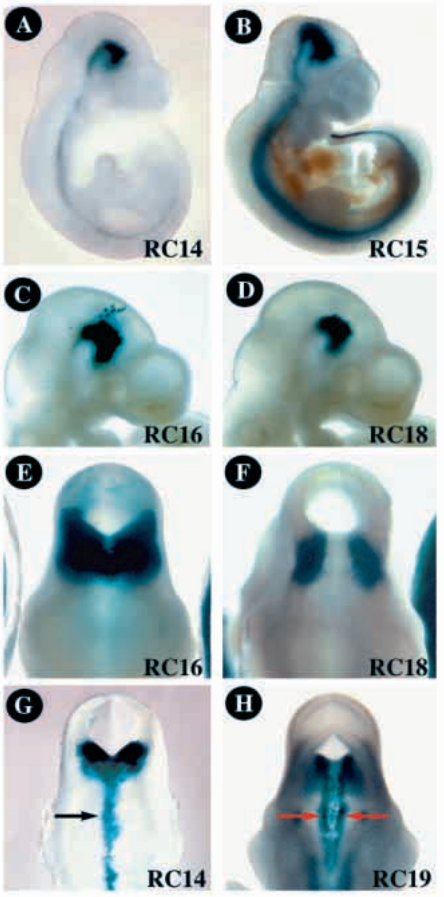

Fig. 6. Dependence on Hnf3-binding sites for proper reporter gene expression. RC14 (A) and RC15 (B) both reveal SBE1 and SFPE2 activity at 9.5 dpc showing the conservation of *Shh* regulatory elements between mouse and chicken. (C) Lateral and (E) frontal views of RC16 showing normal SBE1 activity at 9.5 dpc. Mutations in Hnf3-binding sites 4 and 5 (RC18) show little effect on lateral SBE1 activity (D), but abolish expression from the ventral midline as seen in F. (G) Dorsal view of RC14 expression at 9.5 dpc showing SFPE2 (arrow) and SBE1 activity. (H) In the presence of Hnf3-binding site mutations, reporter expression (RC19) is cleared from the ventral midline of the CNS. Lateral expression adjacent to the floor plate is derived from the *hsp68* promoter (red arrows). Embryos were stained for either 40 minutes (C,E,G) or overnight (D,F,H).

study, is unable to rescue the notochord-derived induction of *Shh* expression in the floor plate in mice carrying a targeted disruption at the *Shh* locus (D. J. E. and A. L. J., unpublished observations).

It was surprising that reporter activity was detected in the notochord only when various deletions of RC6 were generated. One possibility is that negative regulatory sequences restrict *Shh* notochord expression, since removal of either the proximal 80 bp or distal 500 bp of RC6 unmasked *Shh* notochord enhancer (SNE) activity. To test whether such repression could be general to other notochord enhancers, a construct was generated containing proximal sequences of RC6 attached to the notochord enhancer of the *Hnf3β* gene (Sasaki and Hogan, 1996) and assayed for whether these sequences could actively repress notochord-specific reporter activity in transgenic animals. The proximal sequences were found incapable of repressing the *Hnf3β* notochord enhancer, suggesting that the mechanism that silences SNE activity is specific for *Shh* (data not shown).

Given the general distribution of upstream components of the Shh pathway, namely the *Gli* genes and *Hnf3β*, along the rostrocaudal neuraxis, it was rather unexpected to find that the regulation of *Shh* expression is regionalized within discrete domains along this axis. This suggests that the transcriptional control of *Shh* gene expression is far more complicated than was previously expected and brings to question why such a manner of regulation has evolved.

Shh has previously been shown to induce distinct cell types in a graded fashion along the dorsoventral axis (Roelink et al., 1995; Ericson et al., 1996). Moreover, at different anteroposterior levels of the neuraxis, Shh has been shown to induce diverse neuronal populations (Hynes et al., 1995b; Ericson et al., 1995, 1996). Differences in anteroposterior restrictions of cell types induced by Shh can be explained, in part, by signals that cooperate with Shh along the axis. For instance, Fgf8 and Shh are required together for the induction of midbrain dopaminergic neurons (Ye et al., 1998), and BMP7 and Shh are both required for the induction of rostral diencephalic cell fates (Dale et al., 1997). Given the extent of regionalization of *Shh* transcription observed in our reporter assay, it would be rather intriguing if the level of transcriptional activation from the *Shh* enhancers varied along the anteroposterior axis and this correlated with the ability of Shh to induce specific cell fates at different axial levels. This speculation stipulates that the level of Shh required for the induction of a particular cell type differs along the anteroposterior axis. Interestingly, the character of ventral midline, whether rostral diencephalic or spinal cord, was shown to be dependent on exposure to differing concentrations of Shh (Dale et al., 1997). High concentrations of Shh were capable of inducing rostral ventral midline character by activating BMP7, while lower concentrations of Shh supported the induction of spinal cord markers in the absence of BMP7 (Dale et al., 1997). Whether differences in transcriptional activation of *Shh* could evoke a similar outcome remains to be determined.

Aspects of *Shh* transcription are dependent on Hnf3 and cooperating factors

A common feature underlying the regulation of SBE1, SNE and SFPE2 activity is the requirement of Hnf3-binding sites contained within their critical *cis*-acting sequences. Perturbation of the Hnf3 sites in each of these three enhancer constructs alters reporter gene activity, thus confirming that Hnf3 transcriptional regulators are required for aspects of *Shh* gene expression in the floor plate and notochord. Of the three *Hnf3* family members, *Hnf3α* and *Hnf3β* have been implicated in Shh signaling based on their overlapping expression with *Shh*, as well as the ability of Hnf3β to positively regulate *Shh* transcription in ectopic expression assays (Echelard et al., 1993; Sasaki and Hogan, 1994; Hynes et al., 1995a; Ruiz i Altaba et al., 1995). Relying on expression data alone has been difficult in assessing whether Hnf3α and/or Hnf3β is the activator of *Shh* transcription in the CNS. Interestingly, *Hnf3β* precedes *Shh* expression in the floor plate by approximately 12 hours (Echelard et al., 1993), while *Hnf3α* is expressed at approximately the same time as *Shh* in both the floor plate of

the ventral midbrain and spinal cord (Ang et al., 1993); (D. J. E. and A. L. J., unpublished observations). Why such a delay exists between the onset of *Hnf3β* expression and activation of *Shh* transcription could be explained by the presence of an inhibitory signal in the floor plate (Arkell and Beddington, 1997). Ectopic expression of BMP family members in the ventral midline of the CNS inhibits transcription of *Shh* even in the presence of Hnf3β (Arkell and Beddington, 1997; McMahon et al., 1998). Interestingly, *BMP7* expression is normally found in the floor plate at cranial levels, at a time prior to the initiation of *Shh* transcription and its downregulation corresponds with the time when *Shh* is first detected (Arkell and Beddington, 1997). Whether this inhibition by BMP7 acts directly on Hnf3β, a cooperating transcriptional partner required for *Shh* activation (see below) or through the activation of an inhibitor is unclear.

Genetic data, on the other hand, do not support a required role for Hnf3α in the transcription of *Shh*, as mice carrying a targeted disruption of the *Hnf3α* gene still possess a floor plate and maintain *Shh* expression (Duncan et al., 1998) (Klaus Kaestner, personal communication). Moreover, Hnf3α appears to be a less potent transcriptional activator than Hnf3β, at least for targets of endodermal origin (Duncan et al., 1998). The targeted disruption of *Hnf3β* in mice results in the lack of an organized node and notochord, and the mice do not possess a floor plate (Ang and Rossant, 1994; Weinstein et al., 1994). As a result, these mice have not proved informative with respect to whether Hnf3β is required for *Shh* expression, because the tissues in which it is expressed are not specified. This reasoning holds true for two other genes, *nodal* and *goosecoid*, which have been shown to genetically interact with *HNF3β* (Varlet et al., 1997; Filosa et al., 1997). Mice homozygous for a mutation in *goosecoid* and heterozygous for a mutation in *Hnf3β*, as well as mice doubly heterozygous for mutations in *nodal* and *Hnf3β*, each show varying degrees of loss of the ventral midline CNS which result, in part, from a primary defect in the functioning of the node and, as a secondary consequence, the loss of cell types that express *Shh*. Only upon the generation of either a conditional *Hnf3β* mutant or chimeric mice that are wild type for *Hnf3β* expression in the node and notochord and mutant in the floor plate, will the requirement of Hnf3β for floor plate expression of *Shh* be fully determined.

Hnf3 family members have been shown to promote the expression of endoderm-derived genes either by activating transcription directly (Kaufmann and Knochel, 1996) and/or by positioning nucleosomes into transcriptionally stable states (Shim et al., 1998). Although we have not determined by which of these mechanisms Hnf3 is mediating SBE1 and SFPE2 activity, it is important to note that the presence of the Hnf3 sites alone is not sufficient for the transcription of the reporter transgenes in all tissues expressing *Hnf3* family members. For instance, reporter constructs containing SBE1 and SFPE2 activity never displayed expression in the gut, where *Shh* and *Hnf3β* overlap (Echelard et al., 1993), even though Hnf3-binding sites were identified in these constructs. Although it is conceivable that Hnf3 family members are not involved in the regulation of *Shh* gut expression, another possible explanation for this observation is that Hnf3 requires cooperation with additional factors to regulate *Shh* expression and, therefore, only when DNA-binding sites for all the obligatory components are present is *Shh* transcribed in a particular tissue. This postulation is further supported by the finding that RC16, which possesses SBE1 activity, does not activate reporter expression in the floor plate of the spinal cord or elsewhere in the embryo, even though it contains two required Hnf3-binding sites. Only when more 3′ sequences were added (RC14) was floor plate expression in the spinal cord detected. Although these distal sequences also contained Hnf3 sites, when tested on their own (RC17), transgenic embryos did not reveal floor plate expression (Fig. 5). From this, we conclude that Hnf3-binding sites alone are not sufficient for floor plate expression in the spinal cord.

This conclusion is further supported by experiments that deleted selected Hnf3-binding sites. In the absence of Hnf3-binding sites 4 and 5 (in the context of RC19), *lacZ* expression in the floor plate of the spinal cord was dramatically reduced. In some transgenic embryos however, floor plate expression persisted, perhaps suggesting that, in the absence of functional Hnf3-binding sites, the site of transgene integration was important for consistent reporter activity. That RC19 expression was dependent on its site of integration could be indicative of its sensitivity to chromatin structure, with expression being revealed only when the transgene integrates within a transcriptionally permissive chromatin environment. We would predict then, that the role of Hnf3 in mediating SFPE2 activity is to ensure a transcriptionally suitable chromatin organization, permitting the binding and activation of *Shh* transcription by cooperating factors. In support of this, the crystal structure of Hnf3 has been shown to share considerable similarity with histone H5, which plays a fundamental role in chromatin organization (Clark et al., 1993). Moreover, similar conclusions have been proposed with respect to the regulation of albumin gene expression by Hnf3 (Shim et al., 1998).

The zebrafish *Shh* promoter has recently been characterized in co-transfection experiments using HeLa cells (Chang et al., 1997). Although the relevance of HeLa cells to the various tissues that express *Shh* is not clear, results from these assays suggested that axial, a closely related Hnf3β homologue in zebrafish and frogs, and retinoic acid receptors are direct regulators of *Shh* (Chang et al., 1997). In comparing the sequence surrounding the zebrafish and mouse *Shh* promoters, we identified a stretch of 73 nucleotides that show a sequence identity of 73%, including a perfectly conserved TATA box. Of particular interest was the identification of at least one Hnf3-binding site showing 100% sequence identity to that of mouse. Although this particular binding site was shown to be required for reporter activity in HeLa cells, the equivalent segment of DNA in mouse yielded no activity in vivo (Fig. 1, RC1). This finding suggests that not all Hnf3-binding sites are equivalent, and/or that there are different requirements for the activation of *Shh* in vitro as compared to in vivo.

Shh can be activated by Hnf3-independent mechanisms

We have shown that Hnf3 is necessary for consistent SFPE2 activity and the ventral most activity of SBE1, but that ventral-lateral expression found in the rostral midbrain, caudal diencephalon and ZLI, where *Shh* expression is broader than the ventral midline, is independent of Hnf3 function. In addition, SFPE1 activity within RC13 also appeared to be independent of Hnf3 function, as no binding sites matching the

Hnf3 consensus were identified within the 500 bp sequence. From this, it is apparent that additional DNA-binding sites within the various enhancers are also required for activating *Shh* gene expression. A powerful strategy used for the identification of critical regulatory sequences relies upon the comparison of sequences regulating gene expression in divergent organisms (Aparicio et al., 1995; Manzanares et al., 1997). To this end, we have compared the sequence of mouse RC16 that contains SBE1 activity, with a similar fragment from the chicken *Shh* locus, as well as the mouse RC13 sequence (SFPE1) with the comparable human sequences. In both instances, a high degree of sequence conservation was identified, 70% and 75%, respectively. With respect to RC16, several perfectly conserved binding sites matching the consensus of homeodomain and octamer binding proteins were identified. Comparison of RC13 sequences showed several conserved E-box-binding sites that serve as targets for bHLH transcriptional regulators. Future studies will be required to address the significance of these and other potentially critical *cis*-acting regulatory sequences directing *Shh* gene expression.

We would like to thank Randy Johnson for providing us with the chicken *Shh* genomic clone and Stephen Duncan for providing us with liver nuclear extracts. The assistance of the staff at the Skirball Institute Transgenic Core Facility was much appreciated. We are grateful to Brian Bai and Alex Schier for critically reading the manuscript. D. J. E. was supported by a Centennial Fellowship from the Medical Research Council of Canada. A. L. J. is an investigator of the Howard Hughes Medical Institute. Work in A. P. M.'s laboratory was supported by a grant (NS 33642) from the NINDS. Work in A. L. J.'s laboratory was supported by a grant (R01 HD35768) from the NIH.

REFERENCES

Ang, S.-L. and Rossant, J. (1994). HNF-3β is essential for node and notochord formation in mouse development. *Cell* **78**, 561-574.

Ang, S.-L., Wiereda, A., Wong, D., Stevens, K. A., Cascio, S., Rossant, J. and Zaret, K. S. (1993). The formation and maintenance of the definitive endoderm lineage in the mouse: involvement of HNF3/*forkhead* proteins. *Development* **119**, 1301-1315.

Aparicio, S., Morrison, A., Gould, A., Gilthorpe, J., Chaudhuri, C., Rigby, P., Krumlauf, R. and Brenner, S. (1995). Detecting conserved regulatory elements with the model genome of the Japanese puffer fish. Fugu rubripes. *Proc. Natl. Acad. Sci.* **92**, 1684-1688.

Arkell, R. and Beddington, R. S. P. (1997). BMP-7 influences pattern and growth of the developing hindbrain of mouse embryos. *Development* **124**, 1-12.

Belloni, E., Muenke, M., Roessler, E., Traverso, G., Siegel-Bartelt, J., Frumkin, A., Mitchell, H. F., Donis-Keller, H., Helms, C., Hing, A. V., Heng, H. H. Q., Koop, B., Martindale, D., Rommens, J. M., Tsui, L.-C. and Scherer, S. W. (1996). Identification of *Sonic hedgehog* as a candidate gene responsible for holoprosencephaly. *Nature Genetics* **14**, 353-356.

Buscher, D., Bosse, B., Heymer, J. and Ruther, U. (1997). Evidence for genetic control of *Sonic hedgehog* by Gli3 in mouse limb development. *Mechanisms of Development* **62**, 175-182.

Chang, B.-E., Blader, P., Fischer, N., Ingham, P. W. and Strahle, U. (1997). Axial (HNF3β) and retinoic acid receptors are regulators of the zebrafish *sonic hedgehog* promoter. *EMBO J.* **16**, 3955-3964.

Chang, D. T., Lopez, A., von Kessler, D. P., Chiang, C., Simandl, B. K., Zhao, R., Seldin, M. F., Fallon, J. F. and Beach, P. A. (1994). Products, genetic linkage, and limb patterning activity of a mouse *hedgehog* gene. *Development* **120**, 3339-3353.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking *Sonic hedgehog* gene function. *Nature* **383**, 407-413.

Clark, K. L., Halay, E. D., Lai, E. and Burley, S. K. (1993). Co-crystal structure of the HNF-3/fork head DNA recognition motif resembles histone H5. *Nature* **364**, 412-420.

Dahmane, N., Lee, J., Robins, P., Heller, P. and Ruiz i Altaba, A. (1997). Activation of the transcription factor Gli1 and the Sonic hedgehog signalling pathway in skin tumours. *Nature* **389**, 876-881.

Dale, J. K., Vesque, C., Lints, T. J., Sampath, T. K., Furley, A., Dodd, J. and Placzek, M. (1997). Cooperation of BMP7 and SHH in the induction of forebrain ventral midline cells by prechordal plate. *Cell* **90**, 257-269.

Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J. and Hui, C.-c. (1998). Diminished Sonic Hedgehog signaling and lack of floor plate differentiation in *Gli2* mutant mice. *Development* **125**, 2533-2543.

Duncan, S. A., Angeles Navas, M., Dufort, D., Rossant, J. and Stoffel, M. (1998). Regulation of a Transcription Factor Network Required for Differentiation and Metabolism. *Science* **281**, 692-695.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signaling molecules is implicated in the regulation of CNS and limb polarity. *Cell* **75**, 1417-1430.

Echelard, Y., Vassileva, G. and McMahon, A. P. (1994). *Cis*-acting regulatory sequences governing *Wnt-1* expression in the developing mouse CNS. *Development* **120**, 2213-2224.

Epstein, D. J., Marti, E., Scott, M. P. and McMahon, A. P. (1996). Antagonizing cAMP-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway. *Development* **122**, 2885-2894.

Ericson, J., Muhr, J., Placzek, M., Lints, T., Jessell, T. M. and Edlund, T. (1995). Sonic hedgehog induces the differentiation of ventral forebrain neurons: a common signal for ventral patterning within the neural tube. *Cell* **81**, 747-756.

Ericson, J., Morton, S., Kawakami, A., Roelink, H. and Jessell, T. M. (1996). Two critical periods of sonic hedgehog signaling required for the specification of motor neuron identity. *Cell* **87**, 661-673.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heynigen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-180.

Fan, C.-M., Porter, J. A., Chiang, C., Beachy, P. A. and Tessier-Lavigne, M. (1995). Long range sclerotome induction by Sonic hedgehog: direct role of the amino terminal cleavage product and modulation by the cyclic AMP signaling pathway. *Cell* **81**, 457-465.

Filosa, S., Rivera-Perez, J. A., Gomez, A. P., Gansmuller, A., Sasaki, H., Behringer, R. R. and Ang, S.-L. (1997). *goosecoid* and *HNF-3β* genetically interact to regulate neural tube patterning during mouse embryogenesis. *Development* **124**, 2843-2854.

Gualdi, R., Bossard, P., Zheng, M., Hamada, Y., Coleman, J. R. and Zaret, K. S. (1996). Hepatic specification of the gut endoderm in vitro: cell signaling and transcriptional control. *Genes Dev.* **10**, 1670-1682.

Hammerschmidt, M., Brook, A. and McMahon, A. P. (1997). The world according to hedgehog. *Trends in Genetics* **13**, 14-21.

Hansen, S. K. and Tjian, R. (1995). TAFs and TFIIA mediate differential utilization of the tandem Adh promoters. *Cell* **82**, 565-575.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994). *Manipulating the Mouse Embryo*. Second Edition. Cold Spring Harbor Laboratory Press.

Hui, C.-c. and Joyner, A. L. (1993). A mouse model of Greig cephalopolysyndactyly syndrome: the *extra-toesJ* mutation contains an intragenic deletion of the *Gli3* gene. *Nature Genetics* **3**, 241-246.

Hui, C.-C., Slusarski, D., Platt, K. A., Holmgren, R. and Joyner, A. L. (1994). Expression of three mouse homologs of the Drosophila segment polarity gene cubitus interruptus, Gli, Gli-2, and Gli-3, in ectoderm and mesoderm-derived tissues suggests multiple roles during postimplantation development. *Developmental Biology* **162**, 402-413.

Hynes, M., Poulsen, K., Tessier-Lavigne, M. and Rosenthal, A. (1995a). Control of neuronal diversity by the floor plate: contact-mediated induction of midbrain dopaminergic neurons. *Cell* **80**, 95-101.

Hynes, M., Porter, J. A., Chiang, C., Chang, D., Tessier-Lavigne, M., Beachy, P. A. and Rosenthal, A. (1995b). Induction of midbrain dopaminergic neurons by Sonic Hedgehog. *Neuron* **15**, 35-44.

Hynes, M., Stone, D. M., Dowd, M., Pitts-Meek, S., Goddard, A., Gurney, A. and Rosenthal, A. (1997). Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli1. *Neuron* **19**, 15-26.

Johnson, R. L. (1996). Human homologue of *patched*, a candidate gene for the basal cell nevus syndrome. *Science* **272**, 1668-1671.

Kalderon, D. (1997). Hedgehog signalling: Ci complex cuts and clasps. *Current Biology* **7**, R759-R762.

Kaufmann, E. and Knochel, W. (1996). Five years on the wings of fork head. *Mech. Dev.* **57**, 3-20.

Knezevic, V., De Santo, R., Schughart, K., Huffstadt, U., Chiang, C., Mahon, K. A. and Mackem, S. (1997). Hoxd-12 differentially affects preaxial and postaxial chondrogenic branches in the limb and regulates *Sonic Hedgehog* in a positive feedback loop. *Development* **124**, 4523-4536.

Kothary, R., Clapoff, S., Darling, S., Perry, M. D., Moran, L. A. and Rossant, J. (1989). Inducible expression of an *hsp68-lacZ* hybrid gene in transgenic mice. *Development* **105**, 707-714.

Krauss, S., Concordet, J.-P. and Ingham, P. W. (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.

Lee, J., Platt, K. A., Censullo, P. and Ruiz i Altaba, A. (1997). *Gli1* is a target of Sonic hedgehog that induces ventral neural tube development. *Development* **124**, 2537-2552.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1114.

Manzanares, M., Cordes, S., Kwan, C. T., Sham, M. H., Barsh, G. S. and Krumlauf, R. (1997). Segmental regulation of *Hoxb-3* by kreisler. *Nature* **387**, 191-195.

Marigo, V., Davey, R. A., Zuo, Y., Cunningham, J. M. and Tabin, C. J. (1996a). Biochemical evidence that Patched is the Hedgehog receptor. *Nature* **384**, 176-179.

Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996b). Sonic hedgehog differentially regulates the expression of *Gli* and *Gli3* during limb development. *Dev. Biol.* **180**, 272-283.

Marti, E., Bumcrot, D. A., Takada, R. and McMahon, A. P. (1995a). Requirement of 19K forms of Sonic hedgehog for induction of distinct ventral cell types in CNS explants. *Nature* **375**, 322-325.

Marti, E., Takada, R., Bumcroft, D. A., Sasaki, H. and McMahon, A. P. (1995b). Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. *Development* **121**, 2537-2547.

Masuya, H., Sagai, T., Wakana, S., Moriwaki, K. and Shiroishi, T. (1995). A duplicated zone of polarizing activity in polydactylous mouse mutants. *Genes Dev.* **9**, 1645-1653.

Matise, M. P., Epstein, D. J., Park, H. L., Platt, K. P. and Joyner, A. L. (1998). *Gli2* is required for induction of floor plate but not most ventral neurons in the mouse central nervous system. *Development* **125**, 2759-2770.

McMahon, J. A., Takada, S., Zimmerman, L. B., Fan, C.-M., Harland, R. M. and McMahon, A. P. (1998). Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. *Genes Dev.* **12**, 1438-1452.

Merli, C., Bergstrom, D. E., Cygan, J. A. and Blackman, R. K. (1996). Promoter specificity mediates the independent regulation of neighboring genes. *Genes Dev.* **10**, 1260-1270.

Monaghan, A. P., Kaestner, K. H., Grau, E. and Schutz, G. (1993). Postimplantation expression patterns indicate a role for the mouse forkhead/HNF-3 α, β and γ genes in determination of the definitive endoderm, chordamesoderm and neuroectoderm. *Development* **119**, 567-578.

Oro, A. E., Higgins, K. M., Hu, Z., Bonifas, J. M., Epstein, E. H. J. and Scott, M. P. (1997). Basal Cell Carcinomas in Mice Over expressing *Sonic Hedgehog*. *Nature* **276**, 817-821.

Overdier, D. G., Porcella, A. and Costa, R. (1994). The DNA-binding specificity of the Hepatocyte Nuclear Factor 3/forkhead domain is influenced by amino acid residues adjacent to the recognition helix. *Molec. Cell. Biol.* **14**, 2755-2766.

Platt, K. A., Michaud, J. and Joyner, A. L. (1997). Expression of the mouse *Gli* and *Ptc* genes is adjacent to embryonic sources of hedgehog signals suggesting a conservation of pathways between flies and mice. *Mech. Dev.* **62**, 121-135.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). *Sonic hedgehog* mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. and Dodd, J. (1994). Floor plate and motor neuron induction by vhh-1, a vertebrate homolog of hedgehog expressed by the notochord. *Cell* **76**, 761-775.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A. and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. *Cell* **81**, 445-455.

Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S. W., Tsui, L.-C. and Muenke, M. (1996). Mutations in the human *Sonic Hedgehog* gene cause holoprosencephaly. *Nature Genetics* **14**, 357-360.

Ruiz i Altaba, A., Jessell, T. M. and Roelink, H. (1995). Restrictions to floor plate induction by hedgehog and winged helix genes in the neural tube of frog embryos. *Mol. Cell. Neurosci.* **6**, 106-121.

Ruiz i Altaba, A. (1997). Catching a Gli-mpse of Hedgehog. *Cell* **90**, 193-196.

Ruiz i Altaba, A. (1998). Combinatorial Gli gene function in floor plate and neuronal inductions by Sonic hedgehog. *Development* **125**, 2203-2212.

Sasaki, H. and Hogan, B. L. M. (1993). Differential expression of multiple fork head related genes during gastrulation and axial pattern formation in the mouse embryo. *Development* **118**, 47-59.

Sasaki, H. and Hogan, B. L. M. (1994). HNF-3β as a regulator of floor plate development. *Cell* **76**, 103-115.

Sasaki, H. and Hogan, B. L. M. (1996). Enhancer analysis of the mouse HNF3β gene: regulatory elements for the node/notochord and floor plate are independent and consist of multiple sub-elements. *Genes to Cells* **1**, 59-72.

Sasaki, H., Hui, C.-c., Nakafuku, M. and Kondoh, H. (1997). A binding site for Gli proteins is essential for *HNF-3β* floor plate enhancer activity in transgenics and can respond to Shh in vitro. *Development* **124**, 1313-1322.

Shim, E. Y., Woodcock, C. and Zaret, K. S. (1998). Nucleosome positioning by the winged helix transcription factor HNF3. *Genes Dev.* **12**, 5-10.

Song, D.-L., Chalepakis, G., Gruss, P. and Joyner, A. L. (1996). Two Pax-binding sites are required for early embryonic brain expression of an *Engrailed-2* transgene. *Development* **122**, 627-635.

Stone, D. M., Hynes, M., Armanini, M., Swanson, T. A., Gu, Q., Johnson, R. L., Scott, M. P., Pennica, D., Goddard, A., Phillips, H., Noll, M., Hooper, J. E., de Sauvage, F. and Rosenthal, A. (1996). The tumour-suppressor gene *patched* encodes a candidate receptor for Sonic hedgehog. *Nature* **384**, 129-134.

Tanabe, Y. and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. *Science* **274**, 1115-1123.

Varlet, I., Collignon, J., Norris, D. P. and Robertson, E. J. (1997). Nodal signaling and axis formation in the mouse. *Cold Spring Harbor Symposia on Quantitative Biology* **LXII**, 105-113.

Vortkamp, A., Franz, T., Gessler, M. and Grzeschik, K. H. (1992). Deletion of *GLI3* supports the homology of the human Greig cephalopolysyndactyly syndrome (GCPS) and the mouse mutant extra toes (*Xt*). *Mammalian Genome* **3**, 461-463.

Weinstein, D. C., Ruiz i Altaba, A., Chen, W. S., Hoodless, P., Prezioso, V. R., Jessell, T. M. and Darnell, J. E. (1994). The winged-helix transcription factor HNF-3β is required for notochord development in the mouse embryo. *Cell* **78**, 575-588.

Xie, J., Murone, M., Luoh, S.-M., Ryan, A., Qimin, G., Zhang, C., Bonifas, J. M., Lam, C.-W., Hynes, M., Goddard, A., Rosenthal, A., Epstein, E. H. J. and de Sauvage, F. J. (1998). Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* **391**, 90-92.

Ye, W., Shimamura, K., Rubenstein, J. L. R., Hynes, M. A. and Rosenthal, A. (1998). FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. *Cell* **93**, 755-766.