A binding site for Gli proteins is essential for *HNF-3β* floor plate enhancer activity in transgenics and can respond to Shh in vitro

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SUMMARY

The floor plate plays important roles in ventral pattern formation and axonal guidance within the neural tube of vertebrate embryos. A critical event for floor plate development is the induction of a winged helix transcription factor, Hepatocyte Nuclear Factor-3β (HNF-3β). The enhancer for floor plate expression of *HNF-3β* is located 3′ of the transcription unit and consists of multiple elements. *HNF-3β* induction depends on the notochord-derived signal, Sonic hedgehog (Shh). Genetic analysis in *Drosophila* has led to the identification of genes involved in the Hh signalling pathway, and *cubitus interruptus* (*ci*), encoding a protein with five zinc finger motifs, was placed downstream. In the present work, we test the involvement of Gli proteins, the mouse homologues of Ci, in activation of the floor plate enhancer of *HNF-3β*. Transgenic analysis shows that a Gli-binding site is required for the activity of the minimal floor plate enhancer of *HNF-3β* in vivo. Three *Gli* genes are differentially expressed in the developing neural tube. *Gli* expression is restricted to the ventral part, while *Gli2* and *Gli3* are expressed throughout the neural tube and dorsally, respectively. Strong *Gli* and *Gli2*, and weak *Gli3* expressions transiently overlap with *HNF-3β* at the time of its induction. Consistent with ventrally localized expression, *Gli* expression can be up-regulated by Shh in a cell line. Finally, the Gli-binding site acts as a Shh responsive element, and human GLI, but not GLI3, can activate this binding site in tissue culture. Taken together, these findings suggest that Gli, and probably also Gli2, are good candidates for transcriptional activators of the *HNF-3β* floor plate enhancer, and the binding site for Gli proteins is a key element for response to Shh signalling. These results also support the idea that Gli/Ci are evolutionary conserved transcription factors in the Hedgehog signalling pathway.

Key words: Gli, HNF-3β, floor plate, Sonic hedgehog, enhancer, pattern formation, *Drosophila*

INTRODUCTION

The floor plate is a group of specialized cells located in the ventral midline of the vertebrate neural tube. It plays important roles in neural tube development as a source of signalling molecules for dorsoventral patterning and axonal guidance (for reviews, see Jessell and Dodd, 1992; Dodd and Jessell, 1993). A floor plate-derived signalling molecule, Sonic hedgehog (Shh), which is a vertebrate homologue of *Drosophila* Hedgehog (Hh), induces differentiation of ventral type neurons (Ericson et al., 1995; Marti et al., 1995; Roelink et al., 1995). Another floor plate-derived signalling molecule, netrin-1, works as both a chemoattractant and a chemorepellent of axons, depending on neuronal cell types (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995).

Differentiation of the floor plate is induced by a signal derived from the notochord or by Shh. High concentrations of Shh-N peptide or contact with the notochord are required for floor plate induction in neural explants (Marti et al., 1995; Roelink et al., 1995). A critical event of floor plate development is the expression of a winged-helix transcription factor, HNF-3β (Ang et al., 1993; Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993, 1994). Ectopic expression of *HNF-3β* in the neural tube induces ectopic expression of series of floor plate marker genes and prevents dorsal/lateral maker expression, indicating that *HNF-3β* acts as a regulator of floor plate development (Sasaki and Hogan, 1994; Ruiz i Altaba et al., 1995a). The *HNF-3β* expression in the floor plate is induced by a signal derived from the notochord or by Shh, and its induction does not require de novo protein synthesis (Roelink et al., 1995; Ruiz i Altaba et al., 1995b; Chiang et al., 1996). This makes *HNF-3β* a candidate for a direct target of Shh signalling.

Previously, we have identified cis-regulatory regions of the mouse *HNF-3β* gene in transgenic mouse embryos. Enhancers for floor plate expression and node/notochord expression were

Fig. 1. Schematic presentation of the previous knowledge of mouse *HNF-3β* floor plate enhancer. The transcription unit and its direction are indicated by boxes and an arrow, respectively. Minimal floor plate enhancer activity requires combination of a 1.5 kb 'A fragment' and a 400 bp *Hae*III fragment. Analysis using internal deletion series within the 400 bp fragment identified multiple regions involved in regulation. The regions del-3 and del-4 are required for anterior floor plate gene expression, while regions del-5 and del-6 are essential for the enhancer activity (Sasaki and Hogan, 1996).
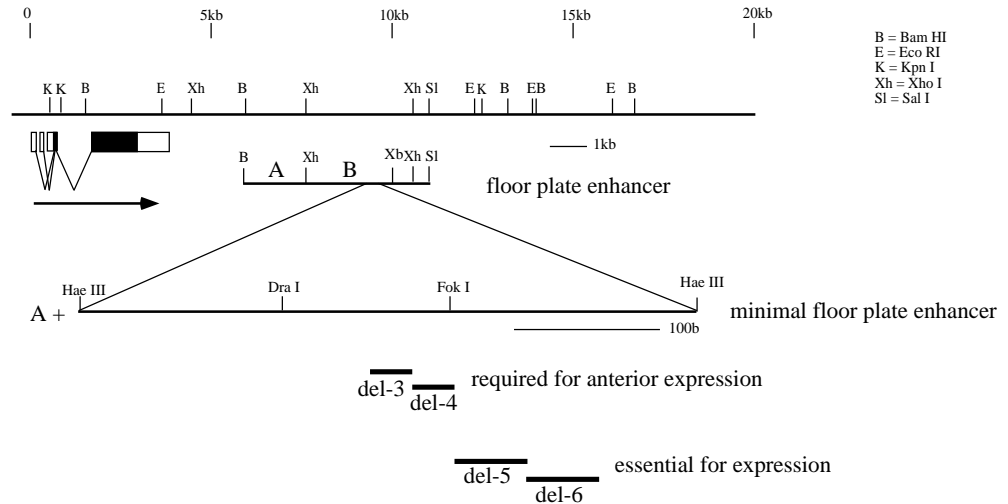
identified in separate regions 3′ and 5′ of the transcription unit, respectively. Deletion analysis of the minimal floor plate enhancer identified multiple elements required for its activity (Fig. 1) (Sasaki and Hogan, 1996). The present study was initiated by finding a Gli-protein-binding site within one of these essential elements.

GLI is a gene that was originally identified as an amplified gene in a human glioblastoma (Kinzler et al., 1987). It encodes a nuclear protein, containing five zinc finger motifs, which binds to DNA in a sequence-specific manner (Kinzler et al., 1988; Kinzler and Vogelstein, 1990). In humans, there are three *GLI* genes (*GLI, GLI2, GLI3*) (Ruppert et al., 1988), which have closely related zinc fingers, and their mouse counterparts have also been identified (*Gli, Gli2, Gli3*) (Walterhouse et al., 1993; Hui et al., 1994).

The *Drosophila* homologue of *Gli* genes is *cubitus interruptus (ci)*, which is involved in the Hedgehog (Hh) signalling pathway (Orenic et al., 1990). According to genetic analysis, the Hh pathway also includes transmembrane Hh receptor Patched (Ptc), a seven-transmembrane protein Smoothened (Smo), a serine-threonine kinase Fused (Fu), cAMP-dependent protein kinase A (PKA) and a gene *costal-2 (cos-2)* (Alcedo et al., 1996; Chen and Struhl, 1996; van den Heuvel and Ingham, 1996; for reviews, see Forbes et al., 1993; Perrimon, 1995). Among them, Ci was proposed to be a transcription factor that works at the most downstream point of the pathway (Forbes et al., 1993; Domínguez et al., 1996). In fact, recent experiments showed that Ci acts as a transcriptional activator and that it activates one of the target genes, *ptc*, through GLI-binding sites (Alexandre et al., 1996).

At least a part of the Hh signalling pathway seems to be conserved between *Drosophila* and vertebrates, while *hh* homologues appear to form a multigene family in vertebrates (Echelard et al., 1993; Ekker et al., 1995a,b; Currie and Ingham, 1996). For example, it has recently been shown that vertebrate Ptc (vPtc) directly binds Shh and Dhh, suggesting its involvement as a receptor (Marigo et al., 1996a; Stone et al., 1996), and that vertebrate Smo (vSmo) makes a complex with vPtc, suggesting involvement of vSmo in the Hh signalling pathway (Stone et al., 1996). In addition, injection of RNAs encoding Shh, Indian hedgehog (Ihh) or PKI, a dominant-negative regulatory subunit of PKA, into zebrafish embryos results in equivalent phenotypes, indicating that PKA acts as a negative regulator of Hh signalling in vertebrates (Hammerschmidt et al., 1996). By extension of such similarities, the three Gli proteins are good candidates for downstream transcription factors of the Hh pathway in vertebrates (Hui et al., 1994).

In this study, the involvement of Gli proteins in activation of the *HNF-3β* floor plate enhancer was tested using transgenic embryos. A Gli-binding site within an essential region of the minimal enhancer was found to be essential for the enhancer activity. Three *Gli* genes are differentially expressed in the developing neural tube, and transiently overlap with *HNF-3β*. In addition, Shh and human GLI, but not GLI3, can activate this Gli-binding site in tissue culture. The results suggest that some of the Gli proteins are involved in induction of *HNF-3β* by Shh as transcription activators of the floor plate enhancer, and that downstream transcription factors of the Hh signalling pathway are conserved between the mouse and the fly.

MATERIALS AND METHODS

Production of GST fusion proteins

Glutathione S-transferase (GST) fusion proteins were produced in *E. coli* using pGEX expression vectors. The pGEX-KG (Guan and Dixon, 1991) was used for synthesis of GST. The plasmid pGEX-HNF-3β (a gift from Richard O'Brien, Vanderbilt University, USA), which produces a fusion protein of GST with full length HNF-3β protein, was used for GST-HNF-3β. The plasmid pGEX-GLI, which was constructed by cloning *Hinc*II-*Xba*I fragment of human GLI cDNA (pGLI•K12) (Kinzler et al., 1988) into *Sma*I and *Xba*I sites of pGEX-KG, was used for GST-GLI. For protein production, bacterial culture was induced with 0.1 mM IPTG. After 3 hours of induction, bacteria were harvested by centrifugation and disrupted by sonication with a Branson sonifier in PBS containing 1% Tween-20. The sonicate was cleared by centrifugation at 12,000 *g* for 10 minutes and cleared lysate was used as GST fusion protein.

Gel mobility shift assay

The binding reaction was set up as follows: 1 μl of cleared *E. coli* lysate, 0.01 pmoles of ^{32}P-labeled probe, 1 μg poly(dI-dC) with or without 0.5 pmoles of non-radioactive competitor were combined in 10 μl of 1× binding buffer (10 mM Tris HCl, pH 7.5, 50 mM NaCl,

5 mM MgCl$_2$, 0.5 mM DTT, 4% glycerol) on ice. After incubation for 30 minutes on ice, protein-DNA complexes were separated on a 6% acrylamide gel (acrylamide:bis-acrylamide = 60:1) containing 0.25×TBE (22 mM Tris-borate pH 8.3, 0.5 mM EDTA).

Immunoblot analysis

0.5 μl of a cleared *E. coli* lysate was electrophoresed in a 12% SDS polyacrylamide gel. After transfer of separated proteins onto a nitro-cellulose membrane, GST fusion proteins were detected by a combination of anti-GST antibody (Z-5) (Santa Cruz Biotechnology), protein A-HRP (Amersham) and ECL (Amersham), following the manufacturers' protocols.

Production of transgenic mouse embryos

Mutations were introduced in the 400 bp *Hae*III fragment of the enhancer by a PCR-based in vitro mutagenesis method using Pfu DNA polymerase (Stratagene). The sequence was verified using a ABI 373 DNA sequencer. Transgenes were constructed by introducing enhancer fragments upstream of the hsp68 promoter-lacZ-poly(A) cassette (ASShsplacZpA vector) (Sasaki and Hogan, 1996). Transgenic embryo production, genotyping and lacZ staining of embryos were performed as described (Sasaki and Hogan, 1996).

Transfection assay

To construct reporter plasmids, eight directly repeated copies of 3′Gli-BS or m3′Gli-BS were cloned into the *Bam*HI site of the plasmid pδ51*Luc*II (Kamachi and Kondoh, 1993). pJT4/Shh, which expresses chick *Shh* cDNA under the control of the CMV promoter, was a gift from Dr Sumihare Noji (Tokushima University). pJT4 was created by removing *Shh* cDNA from pJT4/shh. pSRα-GLI and pSRα-GLI3, which express human GLI and GLI3 cDNAs under the control of the SRα promoter (Takebe et al., 1988), respectively, were gifts from Dr Shun-suke Ishii (RIKEN). pSRα was a gift from Dr Ryohei Sekido (Osaka University). A rat neural stem cell line MNS70 was maintained as described (Nakafuku and Nakamura, 1995; Nakagawa et al., 1996). For transfection, MNS70 cells were plated into 6 cm Primaria dish (Falcon) at a density of 6×10^5 cells/plate 1 day before. Medium was changed 1 hour before DNA addition. The CaPO$_4$/DNA mixture, containing effector (3 μg), reporter (3 μg) and reference (SV-β-gal, 1 μg) plasmids, was added to the MNS70 cells and incubated for 10 hours. In the experiments shown in Fig. 5C, the CaPO$_4$/DNA mixture contains effector 1 (2 μg), effector 2 (2 μg), reporter (2 μg) and reference (1 μg). Cells were washed twice with PBS and re-fed with fresh medium. Cells were harvested 2 days after DNA addition. Preparation of lysates, luciferase and β-galactosidase assays were as described (Fiering et al., 1991; Kamachi and Kondoh, 1993). Luciferase activities were normalized by β-galactosidase activities. All transfection experiments were independently repeated at least twice and the results were reproducible.

RT-PCR

For the *Gli* induction study, 7 μg of effector plasmid were transfected into MNS70 cells by the CaPO$_4$ method, as described above. RNA was prepared 2 days after DNA addition using Ultraspec RNA (Biotex). One fifth of the RNA from each plate was subjected to reverse transcription using Ready To Go You-Prime First-Strand Beads (Pharmacia) and oligo dT primer (Pharmacia). 1 μl of the reverse transcript was used for each PCR reaction. Primers for mouse *Gli* (5′-CGCGAATTCATGTGTGAGCAAGAAGGTTGC-3′, 5′-GCCTCTAGAAGTCGAGGACACTGGCTATAGG-3′) (Walterhouse et al., 1993) were used for amplification, and authenticity of the products was verified by their size and Southern hybridization with a mouse *Gli* cDNA fragment. The conditions of the PCR were 95°C for 45 seconds, 58°C for 30 seconds, 72°C for 1.5 minutes for 30 cycles, followed by 72°C for 10 minutes. The radioactivity of each band was quantitated by BAS2000 (Fuji Film). *β-actin* primers were as described (Nakagawa et al., 1996).

In situ hybridization

A partial mouse *Gli* cDNA was cloned by RT-PCR from a mixture of total RNAs of 8.5- and 12.5-day-old mouse embryos using primers, as described above. A partial mouse *Gli3* cDNA was obtained by screening a mouse 8.5-day post coitum (d.p.c.) cDNA library (a gift from Kathy Mahon, Baylor College of Medicine, USA) with human *GLI3* cDNA (Ruppert et al., 1990) as a probe. Mouse *Gli2*, *HNF-3β* cDNAs were as described (Sasaki and Hogan, 1993; Hui et al., 1994). A *Shh* cDNA was a gift from Dr Hiroshi Hamada (Osaka University). In situ hybridizations using digoxigenin-labeled RNA probes on sections were performed as described (Sasaki and Hogan, 1994). Two or three embryos were analyzed for gene expression at each stage, and similar results were obtained reproducibly.

RESULTS

Identification of a Gli-binding site within an essential region of the minimal *HNF-3β* floor plate enhancer

Previously, we identified a floor plate-specific enhancer of *HNF-3β* downstream of the transcription unit. The genetic elements responsible for the enhancer activity were refined to a combination of a 1.5 kb 'A' fragment and a 0.4 kb *Hae*III fragment (Fig. 1) (Sasaki and Hogan, 1996). By making an internal deletion series within the 0.4 kb fragment, two regions (del-5 and del-6) were found to be essential (Sasaki and Hogan, 1996). Therefore, it is possible that one of these regions contains an element responsive to the Shh signal from the notochord.

Within one of the essential regions, del-5, a sequence motif (3′Gli-BS: 5′-GA<u>A</u>CACCCA-3′) that resembles a known consensus sequence of human GLI-binding sites (hGLI-BS: 5′-GA<u>C</u>CACCCA-3′; different base underlined) was found (Fig. 2A) (Kinzler and Vogelstein, 1990). To test if the 3′Gli-BS motif is actually bound by Gli, a gel mobility shift DNA-binding assay was performed (Fig. 2B). Bacterial lysate containing a fusion protein of glutathione-S-transferase and human GLI (GST-GLI) was used as a GLI protein. Bacterial lysates containing either GST or GST-HNF-3β were used as negative controls. Each lysate contains approximately equal amounts of GST fusion proteins, as shown by immunoblotting (Fig. 2C). Only GST-GLI bound to the 3′Gli-BS probe as well as the hGLI-BS probe in a specific manner (Fig. 2B lanes 3, 4, 7, 8, 10). Sequence alteration within the 3′Gli-BS motif (m3′Gli-BS: 5′-GAA<u>GTGGG</u>A-3′: modified bases are underlined) abolished GST-GLI binding in a competition assay, confirming that the fusion protein bound to this motif within the probe (Fig. 2B, lane 9). The apparent binding affinity of GST-GLI to the 3′Gli-BS is slightly lower than that of the hGLI-BS, probably because 3′Gli-BS deviates from the consensus sequence (hGLI-BS) (Fig. 2B and data not shown). These results suggest that Gli protein can bind to 3′Gli-BS in a specific manner. Considering that GLI and GLI3 have similar DNA-binding activities (Vortkamp et al., 1995) and that all three Gli proteins have very similar DNA-binding domains (Hui et al., 1994), it is likely that other Gli proteins can also bind to the 3′Gli-BS. Because of this, the phrase 'Gli-binding site' will be used with the meaning of 'generic Gli-binding site' throughout this paper. Since *Gli* genes are mouse homologues of *Drosophila ci*, and since Ci is a downstream transcription factor of the Hh signalling pathway, it is possible that this Gli-binding site mediates the responsiveness of *HNF-3β* to a Shh signal derived from the notochord.
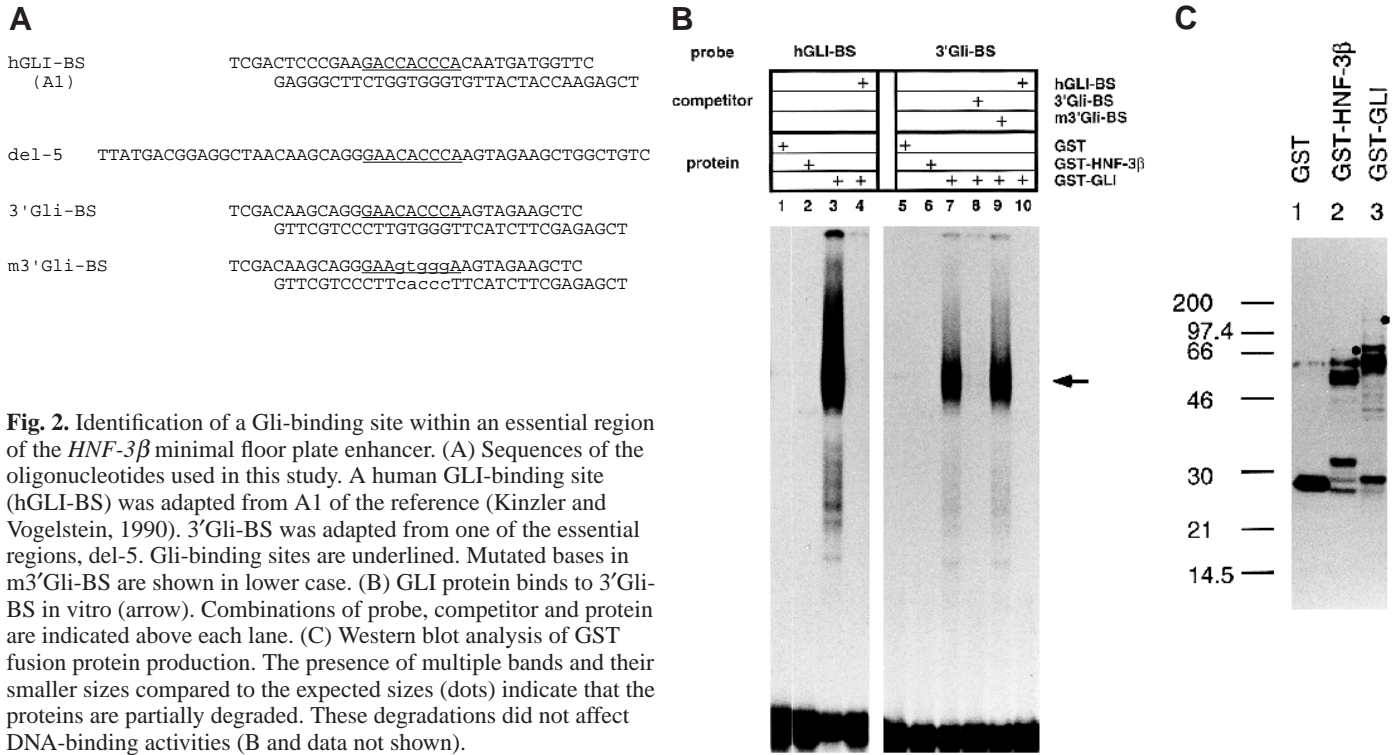
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hGLI-BS      TCGACTCCCGAAGACCACCCACAATGATGGTTC
  (A1)         GAGGGCTTCTGGTGGGTGTTACTACCAAGAGCT


del-5    TTATGACGGAGGCTAACAAGCAGGGAACACCCAAGTAGAAGCTGGCTGTC


3'Gli-BS     TCGACAAGCAGGGAACACCCAAGTAGAAGCTC
               GTTCGTCCCTTGTGGGTTCATCTTCGAGAGCT


m3'Gli-BS    TCGACAAGCAGGGAAgtgggAAGTAGAAGCTC
               GTTCGTCCCTTcacccTTCATCTTCGAGAGCT
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Fig. 2. Identification of a Gli-binding site within an essential region of the *HNF-3β* minimal floor plate enhancer. (A) Sequences of the oligonucleotides used in this study. A human GLI-binding site (hGLI-BS) was adapted from A1 of the reference (Kinzler and Vogelstein, 1990). 3′Gli-BS was adapted from one of the essential regions, del-5. Gli-binding sites are underlined. Mutated bases in m3′Gli-BS are shown in lower case. (B) GLI protein binds to 3′Gli-BS in vitro (arrow). Combinations of probe, competitor and protein are indicated above each lane. (C) Western blot analysis of GST fusion protein production. The presence of multiple bands and their smaller sizes compared to the expected sizes (dots) indicate that the proteins are partially degraded. These degradations did not affect DNA-binding activities (B and data not shown).
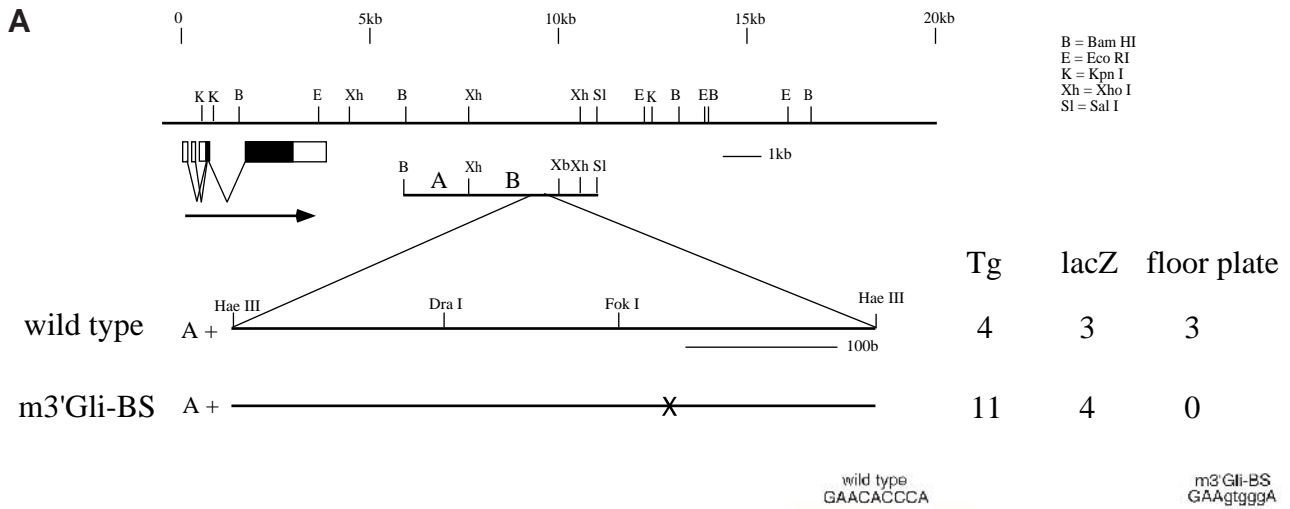
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	Tg	lacZ	floor plate
wild type A +	4	3	3
m3'Gli-BS A +	11	4	0

Fig. 3. Gli-binding site is required for minimal floor plate enhancer activity. (A) Summary of a transgenic mouse experiment. Tg, number of transgenic embryos identified by PCR of yolk sac DNAs; lacZ, number of embryos with lacZ staining; floor plate, number of embryos with lacZ staining in the floor plate. (B,C) Transgenic embryos with the wild-type minimal enhancer express β-galactosidase in the floor plate with some lateral ectopic expression (B,C and data not shown). (D) On the other hand, transgenic embryos with mutant enhancer having base alterations of the 3′Gli-BS into m3′Gli-BS show either no β-galactosidase expression (D and data not shown) or unrelated expression, probably caused by positional effects (data not shown).

The Gli-binding site is essential for the floor plate enhancer activity

The significance of the 3′Gli-BS in *HNF-3β* regulation in vivo was investigated with transgenic mouse embryos. The sequence of the 3′Gli-BS in the minimal enhancer was changed into the m3′Gli-BS so as to abolish binding of Gli proteins (Fig. 3A), and transgenic embryos were produced using the mutated enhancer. With the wild-type enhancer, three out of four transgenic embryos showed gene expression in the floor plate (Fig. 3A-C). On the contrary, with the mutant enhancer, none out of 11 transgenic embryos showed β-galactosidase expression in the floor plate (Fig. 3A,D). Transgenic embryos showed either no β-galactosidase expression (Fig. 3D and data not shown) or expression in unrelated sites, probably caused by integration site position effects (data not shown). The gene expression patterns observed with the mutant enhancer were different among the transgenic embryos. The results indicate that the 3′Gli-BS is essential for the minimal floor plate enhancer activity.

Three *Gli* genes are differentially expressed in the neural tube/plate and transiently overlap with *HNF-3β*

In order to study the possible involvement of *Gli* genes in *HNF-*

$3β$ induction, the expression patterns of *Gli, Gli2* and *Gli3* in the neural tube/plate were re-examined in comparison with *HNF-3β* and *Shh* by in situ hybridization of serial sections of the mouse embryos at 8.5 and 9.5 d.p.c. (Fig. 4).

Three *Gli* genes show specific and dynamic changes of expression patterns along the anteroposterior axis of the neural tube/plate of 8.5 d.p.c. embryos (9-11 somites). *Gli* transcripts were abundant in the ventral region of the neural tube/plate and gradually lost dorsally (Fig. 4C,H). In the posterior part, where *HNF-3β* but not yet *Shh* is transcribed in the ventral midline (Fig. 4A,B), its expression overlaps with *HNF-3β* (Fig. 4C). However, in the anterior part, where both *HNF-3β* and *Shh* are co-expressed in the floor plate, *Gli* expression is excluded from the floor plate (Fig. 4H). *Gli2* transcripts were rather evenly distributed throughout neural tube/plate (Fig. 4D,I). As observed with *Gli*, its expression also overlaps with *HNF-3β* in the posterior floor plate (Fig. 4D), but is excluded from the anterior floor plate (Fig. 4I). On the other hand, *Gli3* seems to be expressed at a very low level throughout the neural tube in the posterior part, since signals above background levels were observed reproducibly (Fig. 4E). In the anterior part, strong *Gli3* expression is widely observed but in an opposite gradient to *Gli*, i.e. it is strong in the dorsal region and weak in the
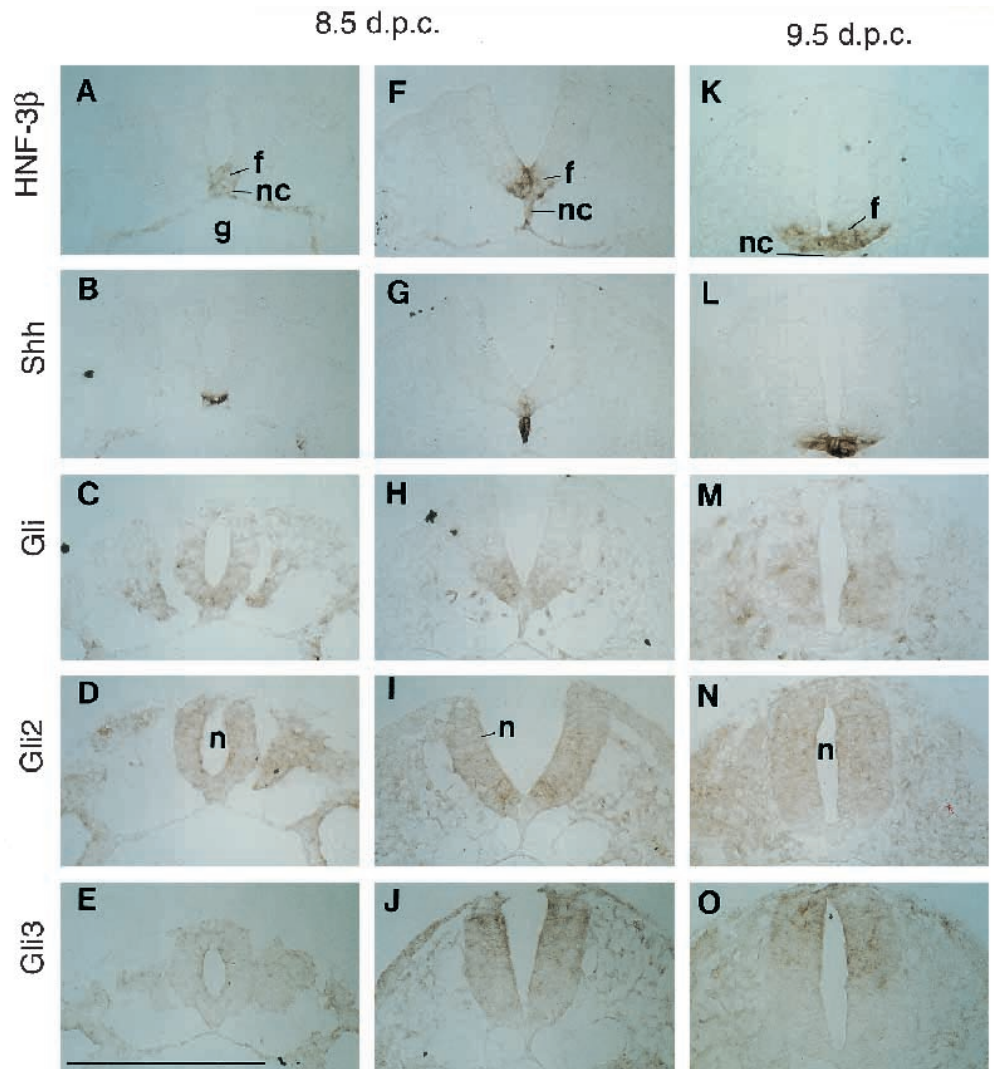

Fig. 4. Comparison of *Gli, Gli-2* and *Gli-3* expressions with *HNF-3β* and *Shh* in developing neural tubes. In situ hybridization of *HNF-3β* (A,F,K), *Shh* (B,G,L), *Gli* (C,H,M), *Gli-2* (D,I,N) and *Gli-3* (E,J,O) on serial cross sections of 8.5 (A-J) and 9.5 d.p.c. (K-O) mouse embryos. Sections through posterior part (A-E) and hindbrain level (F-J) of an 8.5 d.p.c. embryo. Left side somites of D and E are damaged. Sections through posterior forelimb bud level of a 9.5 d.p.c. embryo (K-O). f, floor plate; nc, notochord; n, neural tube/fold; g, gut. Scale bar, 0.2 mm.

ventral (Fig. 4J). Again, no signal is seen in the floor plate. Differential expression of the three *Gli* genes similar to Fig. 4H-J, which are essentially the same as our previous observation (Hui et al., 1994), were also observed in more anterior regions, up to the level of the diencephalon (data not shown). However, in the mid- and forebrains, where *HNF-3β* expression domains are clearly broader than Shh, the ventral boundary of *Gli* expression apparently coincides with the Shh boundary (data not shown).

It is likely that the differential expression of the three *Gli* genes along the anteroposterior axis of the neural tube/plate correlates with the differentiation of the neuroectoderm rather than with its position along the body axis per se. Supporting this idea, sections through posterior forelimb bud level (around 11-13th somites) of a 9.5 d.p.c. embryo (Fig. 4K-O), which is equivalent in position to the section of the posterior level of the 8.5 d.p.c. embryo shown in Fig. 4A-E, now show gene expression patterns similar to those in the anterior of the 8.5 d.p.c. embryo shown in Fig. 4F-J. In addition, the expression patterns of three Gli genes in the brain level of the 7.5 d.p.c. embryo (Hui et al., 1994) are similar to those in the posterior of the 8.5 d.p.c. embryo (Fig. 4C-E).

Taken together, strong expression of *Gli* and *Gli2* and very weak *Gli3* expression, transiently overlap with *HNF-3β* at the time of its induction. These expression patterns of the three *Gli* genes are consistent with the model of Gli proteins being activators of the floor plate enhancer of *HNF-3β*, although the contribution of Gli3 may be minimal. The transient nature of the expression of *Gli* genes suggests involvement of Gli proteins in induction of *HNF-3β* rather than in the maintenance of its expression.

Similar differential expression of the three *Gli* genes was also observed in paraxial mesoderm, including presomitic mesoderm, somites and head mesenchyme (Fig. 4 and data not shown). The expression patterns of *Gli, Gli2* and *Gli3* in the somites are ventromedial, throughout the somite, and dorsal, respectively. Such differential expression of the three *Gli* genes in paraxial mesoderm also suggests the involvement of *Gli* genes in dorsoventral patterning of the mesoderm.

Gli expression can be up-regulated by Shh

The restricted expression of *Gli* in tissues surrounding Shh-expressing tissues such as the notochord and the floor plate (Fig. 4) suggests that the expression of *Gli* itself is controlled by Shh signalling. Therefore, this possibility was tested using a multipotential rat neuronal cell, MNS70 (Nakagawa et al., 1996). This cell line is derived from the embryonic forebrain and expresses various ventral neuroepithelium-specific genes upon induction by Shh (Nakagawa et al., 1996). Either a *Shh* expression plasmid or a control plasmid was transfected into MNS70 cells and the endogenous *Gli* RNA level was monitored by RT-PCR. *Gli* RNA was up-regulated by transfection of the *Shh* expression plasmid (Fig. 5). Although *Gli2* and *Gli3* were also expressed in this cell line, no obvious change was observed for these transcript levels by transfection of a Shh expression plasmid (data not shown). This result, together with the *Gli* expression patterns in developing mouse embryos (Fig. 4), suggest that it is likely that the expression of *Gli* is controlled by Shh signalling in vivo. It is possible that a similar *Gli* induction by Shh also occurs in the ventral midline of the neural plate, and that this is a part
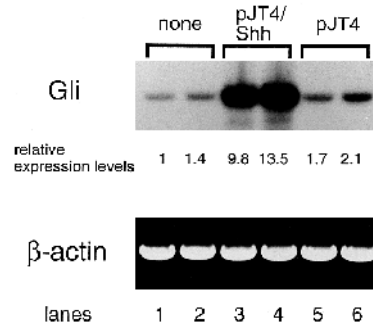

Fig. 5. Expression of *Gli* is controlled by Shh signalling. MNS70 cells were transfected with pJT4/Shh (lanes 3 and 4) or pJT4 (lanes 5 and6), and then analyzed for *Gli* expression by RT-PCR. Lanes 1 and 2 are mock transfected. Expression of *Gli* was increased by transfection of *Shh* expression plasmid. *β-actin* was used as a control. Duplicates are shown for each plasmid. No obvious induction of *Isl-1* by Shh was observed under these conditions (data not shown).

of the scenario responsible for *HNF-3β* induction in the floor plate.

The Gli-binding site is a Shh responsive element

Since floor plate expression of *HNF-3β* is induced by Shh, and since a *Gli* homologue, *ci*, is involved in Hh signalling pathway in *Drosophila*, involvement of the 3′Gli-BS motif of the floor plate enhancer within the Shh signalling pathway was tested by transient transfection assay in MNS70 cells. Although it does not express *HNF-3β* in response to Shh treatment, the basic mechanism of Hh-induced gene expression appears to be common in various tissues, at least in part (see Introduction). Therefore, MNS70 was used to characterize the isolated 3′Gli-BS element in the Shh pathway. A luciferase reporter plasmid having eight copies of either the 3′Gli-BS or the m3′Gli-BS motif was co-transfected with either a Shh expression plasmid or a control expression plasmid into MNS70 cells, expecting autocrine and/or paracrine effects of Shh (Fig. 6A). The expression of the reporter with the 3′Gli-BS motif was increased by co-transfection of the Shh expression vector (Fig. 6B). On the other contrary, the expression of the reporter with the m3′Gli-BS motif was not changed, confirming that the response to Shh was mediated by the Gli-binding site (Fig. 6B). Therefore, the Gli-binding site acts as a Shh responsive element. This is the first demonstration of a Shh responsive element in vitro.

GLI, but not GLI3, can activate the Gli-binding site

Since Shh activates the 3′Gli-BS, we tested whether any Gli proteins can activate the same reporter. Human GLI and GLI3 were used instead of mouse Gli and Gli3, respectively. Gli2 was not tested because of the present unavailability of full-length cDNA. The expression of the reporter with the 3′Gli-BS motif was increased by co-transfection of the GLI expression vector (Fig. 7A,B). The expression of the reporter with the m3′Gli-BS motif was not changed, confirming that the GLI activates reporter gene expression through the Gli-binding site (Fig. 7B). On the other hand, co-transfection of the GLI3 expression vector did not activate, but rather repressed, reporter expression in the 3′Gli-BS-dependent manner (Fig. 7B and

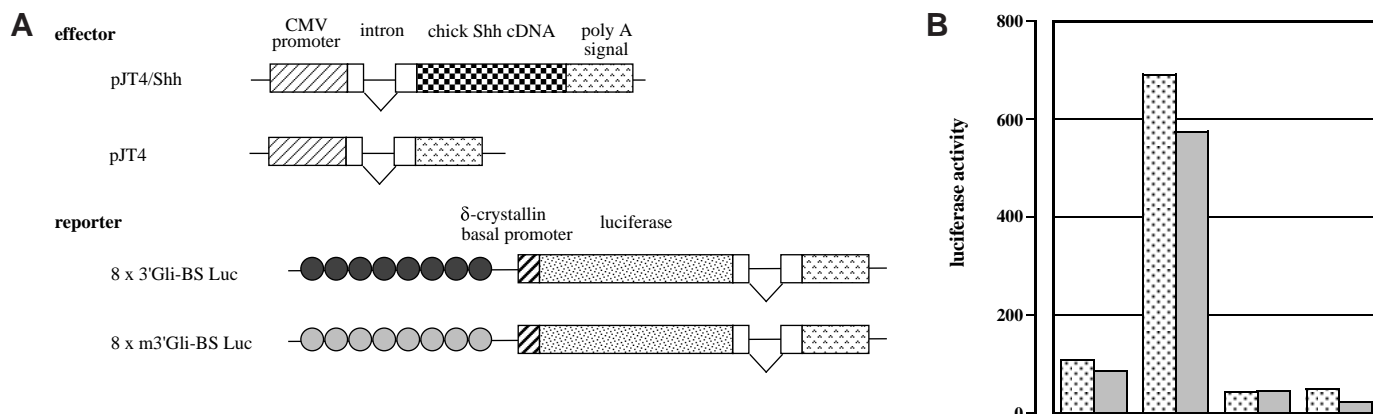

Fig. 6. Gli-binding site is a Shh responsive element. (A) Diagram of effector and reporter plasmids. Each dark and light circle of the reporters indicates 3′Gli-BS and m3′Gli-BS sequences, respectively, listed in Fig. 2A. (B) Stimulation of the transcription of 8×3′Gli-BS by Shh. Gene expression of 8×3′Gli-BS Luc was up-regulated by co-transfection with pJT4/Shh, while no response was observed with 8×m3′Gli-BS Luc. Each combination is shown in duplicate.

effector	pJT4/Shh		+		+
	pJT4	+		+	
reporter	8 x 3'Gli-BS	+	+		
	8 x m3'Gli-BS			+	+

data not shown). The repression by GLI3 was also tested by co-transfection of both GLI and GLI3 expression vectors. Consistent with the idea of GLI3 being a repressor, GLI3 counteracted the effect of GLI (Fig. 7C). Considering the strong similarities of mouse Gli and Gli3 to human GLI and GLI3, respectively, these results suggest that the Gli, but not Gli3, is a transcriptional activator and can directly activate the 3′Gli-BS.

DISCUSSION

Transcriptional regulation of *HNF-3β* in the floor plate

The initiation of *HNF-3β* expression in the floor plate is induced by a signal, Shh, from the notochord. This study showed that the *HNF-3β* floor plate enhancer contains a binding site for Gli-like proteins (3′Gli-BS) that is essential for floor plate gene expression in transgenic mouse embryos, and that the 3′Gli-BS is activated by Shh and GLI, but not by GLI3, in a cultured cell line. Finally, strong *Gli* and *Gli2,* and very weak *Gli3* expression, transiently overlap with that of *HNF-3β* in the ventral midline of the neural tube/plate at the time of floor plate induction. These results suggest that the Shh signalling from the notochord turns on *HNF-3β* via all or some of the three Gli transcription factors.

Among the three Gli proteins, Gli is a better candidate than Gli3 for activation of the 3′Gli-BS, for the following reasons. Judging from the facts that all three *Gli* genes are co-expressed with *HNF-3β* at the time of floor plate induction (Fig. 4), and that GLI and GLI3 have similar DNA-binding activities in vitro (Vortkamp et al., 1995), it is equally possible for any of the three Gli proteins to be involved in the activation of the 3′Gli-BS. However, the results of the co-transfection assay of GLI and GLI3 (Fig. 7) support the model of 3′Gli-BS activation by Gli but not by Gli3. Although the results of our co-transfection assay do not completely rule out the possibility of transcriptional activation by GLI3 in certain situations, at least co-transfection of the GLI3 and Shh expression vectors did not convert

GLI3 into an activator (H.S., unpublished observation). This model is also consistent with the fact that in the region of overlap with *HNF-3β* the levels of *Gli* and *Gli3* are very high and very low, respectively.

The human GLI cDNA used in the transfection assay was isolated from the glioblastoma cell line, and hence may not be wild type (Kinzler et al., 1987). However, the hypothesis of GLI being a transcriptional activator while GLI3 being a repressor has also been independently proposed by Marigo et al. (1996b), based on analysis with chick limb buds.

Although the role of Gli2 as a transcriptional regulator is not yet known, the phenotype of homozygous *Gli2* null mutant mouse embryos, which have lost *HNF-3β* expression in the floor plate except in the head region (C.-c. H., unpublished observation), suggests that Gli2 functions as an activator. Finally, Gli proteins are involved only in the process of *HNF-3β* induction and not in its maintenance, since none of these three *Gli* genes are expressed in the differentiated floor plate (Fig. 4) (Hui et al., 1994).

Initial Shh signal transduction to Gli proteins probably involves post-translational control, since *HNF-3β* induction by the notochord does not require de novo protein synthesis in the chick neural explant assay (Ruiz i Altaba et al., 1995b). Therefore, the induction or maintenance of a transcriptional activator, Gli, by Shh signalling (Fig. 5) may be a rather late event that may function to back up or stabilize Shh target gene activation by increasing the Gli protein level. Alternatively, the induction or maintenance of *Gli* in cells adjacent to the floor plate may contribute to the later inductive events, such as the induction of oligodendrocyte precursors and motor neuron differentiation (Ericson et al., 1996; Orentas and Miller, 1996; Pringle et al., 1996).

Although the Gli-binding site (3′Gli-BS) is essential for enhancer activity, and can respond to Shh signalling in transfection assays in a cell line, Gli proteins cannot be the only factors involved in floor plate gene expression in vivo. In fact, multiple copies of the 3′Gli-BS alone did not efficiently activate gene expression in transgenic mouse embryos. Only one out of 11 transgenic embryos showed β-galactosidase
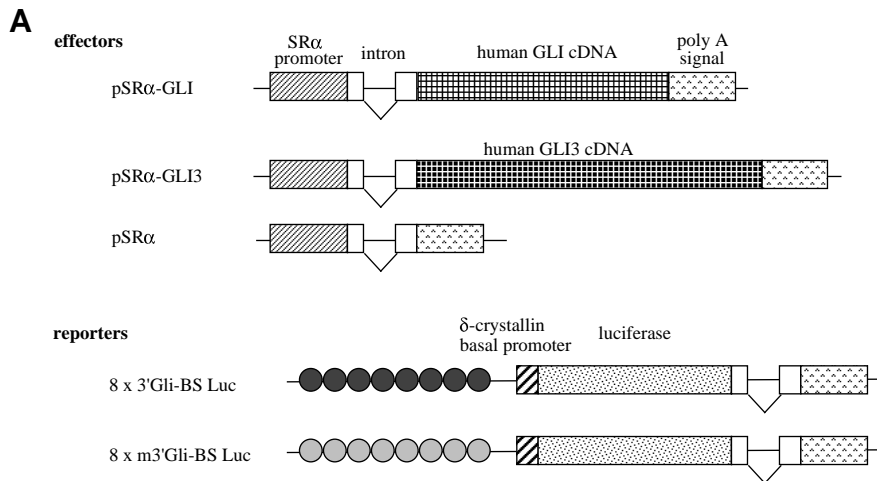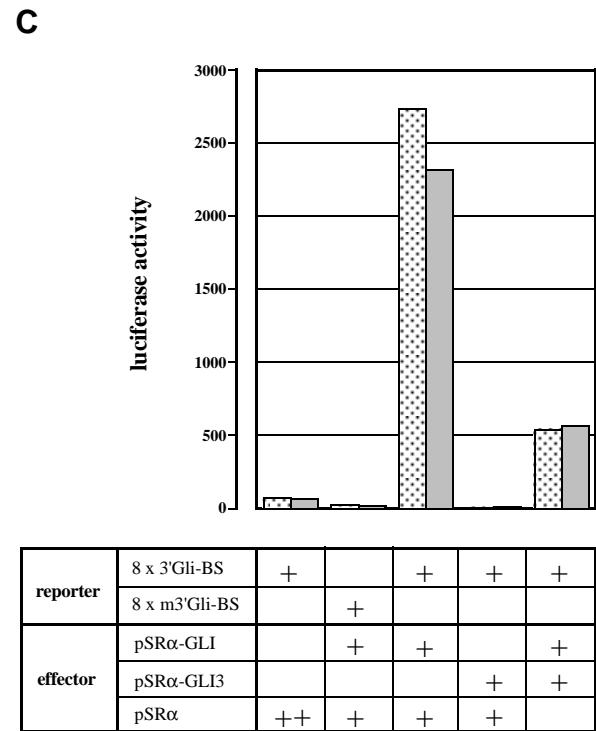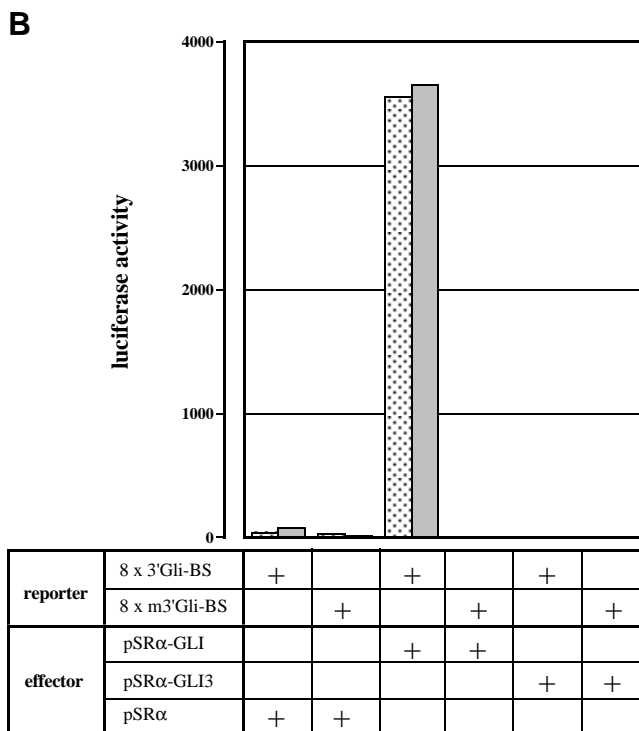
A

effectors

Fig. 7. GLI, but not GLI-3, is a transcriptional activator. (A) Diagram of effector and reporter plasmids. (B) Transcriptional activation of 8×Gli-BS by GLI. Gene expression of 8×3′Gli-BS Luc was up-regulated by co-transfection with pSRα-GLI, while no activation was observed either with 8×m3′Gli-BS Luc reporter or by co-transfection with pSRα-GLI-3 effector. Each combination is shown in duplicate. (C) Transcriptional repression of 8×Gli-BS by GLI-3. Transcriptional activation of 8×3′Gli-BS Luc by co-transfection with pSRα-GLI was partially repressed by addition of pSRα-GLI-3. The amount of each effector was kept constant by addition of same amount of empty effector plasmid (pSRα). Each combination is shown in duplicate.

B

reporter	8 x 3'Gli-BS	+		+		+	
	8 x m3'Gli-BS		+		+		+
effector	pSRα-GLI			+	+		
	pSRα-GLI3					+	+
	pSRα	+	+				

C

reporter	8 x 3'Gli-BS	+		+	+	+
	8 x m3'Gli-BS		+			
effector	pSRα-GLI		+	+		+
	pSRα-GLI3				+	+
	pSRα	++	+	+	+	

expression related to *Shh* (H.S., unpublished observation). Therefore, Gli proteins probably co-operate with other transcription factors that bind to other essential elements of the floor plate enhancer, e.g. the del-6 and 'A fragment' regions, to achieve Shh-inducible and floor plate-restricted gene expression (Fig. 1) (Sasaki and Hogan, 1996).

Possible roles of Gli proteins for dorsoventral patterning of the neural tube and paraxial mesoderm mediated by Shh signalling

Shh plays important roles in the dorsoventral patterning of the neural tube and somites (Johnson et al., 1994; Ericson et al., 1995; Hynes et al., 1995; Roelink et al., 1995; Yang and Niswander, 1995; Chiang et al., 1996). The present study suggests involvement of Gli proteins in the Shh signalling pathway as transcriptional regulators (see below). The present

study also describes the differential expression patterns of the three *Gli* genes in the neural tube/plate and paraxial mesoderm (Fig. 4). Briefly, *Gli* is strongly expressed in the ventral and medial parts of the neural tube/plate and paraxial mesoderm, which are supposed to receive the Shh signal from notochord and/or floor plate. *Gli2* is rather evenly distributed, and the *Gli3* distribution pattern is complementary to *Gli*, i.e. strongly expressed in the dorsal and lateral parts of the neural tube and paraxial mesoderm, which are supposed to barely receive the Shh signal.

These expression patterns, taken together with the results of GLI but not GLI3 being an activator (Fig. 7), and up-regulation of *Gli* by Shh in cultured cell lines (Fig. 5), suggest that ventrally expressed Gli is involved in the activation of Shh target genes, while dorsally expressed Gli3 has the opposite effect. This model is also supported by the facts that *GLI* and *GLI3* are up- and

down-regulated by Shh in chick limb bud, respectively, and that GLI-VP16 can activate another target of Shh, *Ptc* (Marigo et al., 1996b). Although no biochemical information is available at present about the role of Gli2, which is evenly expressed throughout the neural tube, analysis of mutant embryos has shown that *Gli-2* and *Gli-3* have specific and partially redundant functions in skeletal development (Mo et al., 1997).

Possible evolutionary conservation of Hedgehog signalling pathway between vertebrates and *Drosophila*

As described in the Introduction, multiple components of the Hh signalling pathway are conserved between *Drosophila* and vertebrates. Briefly, *Drosophila* Ptc and vPtc are Hh receptors, *Drosophila* Smo and vSmo are receptor-coupled seven-transmembrane proteins, and PKAs are negative regulators of Hh signalling.

The present study suggests the additional conservation of a downstream transcription factor, Ci. In vertebrates, there are three *ci*-related genes, *Gli, Gli2* and *Gli3*, which encode proteins with closely related five-zinc finger motifs (Hui et al., 1994). The present analysis showed that a binding site for Gli proteins is essential for the enhancer activity of a target gene, that Shh can activate a binding site for Gli proteins, and that GLI is a transcriptional activator. These findings are consistent with a role for Gli proteins in the Shh signalling pathway as transcriptional regulators. However, there seem to be some differences, too. In *Drosophila*, there is only one *ci*, and its transcript level is not changed in cells receiving the Hh signal (Domínguez et al., 1996). In mouse, however, there are three *Gli* genes, and expression of *Gli* can be up-regulated by Shh signalling. Therefore, the general scheme of Gli/Ci as downstream transcription factors of the Hh signalling pathways seems to be conserved between vertebrates and *Drosophila*, although the details may be different.

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