Development 128, 3485-3495 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV1706

Hedgehog signaling is required for primary motoneuron induction in zebrafish

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Accepted 29 June 2001

SUMMARY

Sonic hedgehog (Shh) is crucial for motoneuron development in chick and mouse. However, zebrafish embryos homozygous for a deletion of the *shh* locus have normal numbers of motoneurons, raising the possibility that zebrafish motoneurons may be specified differently. Unlike other vertebrates, zebrafish express three *hh* genes in the embryonic midline: *shh*, *echidna hedgehog* (*ehh*) and *tiggywinkle hedgehog* (*twhh*). Therefore, it is possible that Twhh and Ehh are sufficient for motoneuron formation in the absence of Shh. To test this hypothesis we have eliminated, or severely reduced, all three Hh signals using mutations that directly or indirectly reduce Hh signaling and antisense morpholinos. Our analysis shows that Hh signals are required for zebrafish motoneuron induction. However, each of the three zebrafish Hhs is individually dispensable for motoneuron development because the other two can compensate for its loss. Our results also suggest that Twhh and Shh are more important for motoneuron development than Ehh.

Key words: Hh, Smoothened, Motoneuron, *syu*, *smu*, *cyc*, *flh*, *shh*, *ehh*, *twhh*, Morpholino, Ventral neural tube, Floor plate, Spinal cord patterning

INTRODUCTION

Motoneurons develop on both sides of the floor plate in the ventral neural tube, and a variety of experimental studies in mouse and chick implicate Shh signals from the embryonic midline in their formation. For example, either the notochord or the floor plate, both of which express *Shh*, can induce ectopic expression of motoneuron markers in chick explant cultures; this activity is mimicked by recombinant Shh protein and blocked by Shh antibodies (Roelink et al., 1994; Marti et al., 1995; Ericson et al., 1996). In addition, mouse loss-of-function *Shh* mutants completely lack expression of the motoneuron marker Islet 1 (Chiang et al., 1996). These, and other experiments (Litingtung and Chiang, 2000a; Eisen, 1999) provide evidence that in chick and mouse Shh is both sufficient and required for motoneuron development.

Zebrafish have two distinct populations of motoneurons: primary motoneurons are born earlier and are larger than secondary motoneurons, and may be specific to anamniote vertebrates such as fish and amphibians (Kimmel and Westerfield, 1991; Kimmel et al., 1994). Prospective motoneurons can be identified by soma position and *islet* expression. *Islet* genes encode members of the LIM homeodomain (LH) family of transcription factors and are expressed by vertebrate motoneurons in all species examined to date. In chick, all spinal motoneurons first express *Islet-1*; later, subsets of motoneurons express other LH family members (Tsuchida et al., 1994). Similarly, in zebrafish, prospective primary motoneurons express *islet1* soon after birth, just after the end of gastrulation, but at mid-somitogenesis stages two identified classes of primary motoneurons, CaPs and VaPs, initiate expression of *islet2* and then downregulate expression of *islet1*. This is in contrast to the other two identified primary motoneuron classes, MiPs and RoPs, which continue to express *islet1* and never express *islet2* (Appel et al., 1995; Inoue et al., 1994; Tokumoto et al., 1995). Later-developing secondary motoneurons also express *islet1* and *islet2*, but because they are born several hours after primary motoneurons they do not express either of these genes until late somitogenesis stages.

In contrast to the *Shh* loss-of-function mouse, zebrafish embryos homozygous for the t4 allele of *sonic-you* (*syu*), which completely deletes the *shh* locus, have normal numbers of both secondary and primary motoneurons (Schauerte et al., 1998), though the axon tracts of many of them are aberrant, probably owing to abnormal muscle patterning in these mutants. Zebrafish *shh* is expressed as in other vertebrates, starting at 60% epiboly in the embryonic shield or organizer region during gastrulation and then in notochord and floor plate (Krauss et al., 1993). However, unlike other vertebrates, zebrafish express two additional *hh* genes in different subsets of the *shh* expression domain during the time when motoneurons are likely to be specified: *tiggywinkle hedgehog* (*twhh*) is expressed in the embryonic shield from 50% epiboly and then in floor plate and ventral brain, and *echidna hedgehog* (*ehh*) is expressed in the notochord from late gastrulation, starting slightly later than the first expression of *shh* (Ekker et al., 1995; Currie and Ingham, 1996). Therefore, it is possible

that Ehh and Twhh, both of which are expressed normally in *syu*^{t4} mutants, are sufficient for motoneuron formation in the absence of Shh (Schauerte et al., 1998). Unfortunately, it is not yet possible to test this hypothesis by analyzing fish that lack the functions of all three *hh* genes, because mutations in *twhh* and *ehh* have yet to be isolated.

In addition to *syu*, several other loci have been implicated in the Hh signaling pathway in zebrafish. Mutants at these loci have been referred to as 'u' mutants because of their characteristic curved (u-shaped) somites. They include *you-too*, the zebrafish homolog of the Hh pathway transcription factor *gli2* (Karlstrom et al., 1999), and *detour*, *chameleon*, *you* and *iguana*, which are as yet unidentified molecularly (van Eeden et al., 1996; Lewis et al., 1999; Odenthal et al., 2000). Normal numbers of spinal cord motoneurons form in embryos mutant for each of these genes, although many of the mutants have disturbed axon tracts reminiscent of *syu* mutants, and cranial motoneurons are missing in *detour* mutants (van Eeden et al., 1996; Brand et al., 1996; Chandrasekhar et al., 1999). This suggests that either zebrafish spinal cord motoneurons form independently of Hh signals or that none of these mutations causes a complete loss of Hh signaling.

More recently, a newly characterized mutation, *slow muscle omitted* (*smu*) (Barresi et al., 2000) has been shown to disrupt the zebrafish homolog of *smoothened* (*smoh*) (Varga et al., 2001). Smoothened is a seven-pass transmembrane protein postulated to form part of the receptor complex for Hh signaling (Ingham, 1998). In zebrafish, only one *smoothened* gene has been identified, which, in combination with the severity of the *smu* mutation, suggests that Smoh is required for signaling from all three Hh proteins (Varga et al., 2001). *smu* mutant embryos have a more severe morphological phenotype than any of the 'u' mutants, including *syu*. In addition, *smu* mutants have a complete loss of secondary motoneurons and a severe reduction of primary motoneurons. This phenotype is reminiscent of *cyclops;floating head* (*cyc;flh*) double mutants, which also lack secondary motoneurons and have fewer primary motoneurons (Beattie et al., 1997). *cyc;flh* mutants also have a severe reduction in Hh signaling: they lack both notochord and floor plate, and consequently have no *ehh* or *twhh* expression, and express *shh* only transiently during gastrulation.

Strikingly, despite severe motoneuron reductions, a substantial, though less than normal, number of primary motoneurons still form in the anterior spinal cord of both *smu* and *cyc;flh* mutants. This suggests that zebrafish anterior primary motoneurons might be a unique population that does not require Hh signaling. This is not without precedent: Hh signaling is insufficient and possibly not required to induce the median subset of zebrafish floor plate (Schauerte et al., 1998; Odenthal et al., 2000; Varga et al., 2001; Muller et al., 2000), which instead seems to require Nodal signals (Sampath et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998a; Rebagliati et al., 1998b). However, there is still residual expression of *shh* during gastrulation in *cyc;flh* mutants (Beattie et al., 1997), and *smu* mutants have maternal *smoh* transcripts (Varga et al., 2001), raising an alternative hypothesis: that sufficient early Hh signaling remains in both of these cases for anterior spinal cord primary motoneurons to form.

In this paper we address the question of whether zebrafish anterior spinal cord primary motoneurons require Hh signaling. To determine if there really is a distinct, Hh-independent class of motoneurons in zebrafish, we used a variety of different 'knockdown' and mutational approaches to severely reduce, or eliminate Hh signaling. We also investigated whether Hh signaling is required for primary motoneuron induction or maintenance and whether individual Hedgehog genes contribute differently to motoneuron development. Our results provide evidence that zebrafish motoneurons require Hh signals. Shh, Twhh and Ehh can all act redundantly to specify motoneurons, but our results suggest that Ehh has a lesser role than Twhh or Shh. In addition, we show that Hh signals are required for initial motoneuron expression of *islet1*, suggesting that Hh signaling induces the motoneuron fate.

MATERIALS AND METHODS

Propagation and identification of zebrafish wild-type and mutant embryos

Zebrafish (*Danio rerio*) embryos were obtained from natural spawnings of a wild-type colony (AB) or crosses of identified carriers, heterozygous for specific mutations. Fish were maintained in the University of Oregon Zebrafish Facility on a 14 hour light/10 hour dark cycle at 28.5°C and embryos staged according to Kimmel et al. (Kimmel et al., 1995) by number of somites or hours post fertilization at 28.5°C (h). To produce parental fish heterozygous for mutations at two different loci, fish heterozygous for one of the mutations were mated with fish heterozygous for the other mutation, and the progeny identified as carrying one or both mutations by single pair matings and examination of embryo morphological phenotypes. Triple mutant carrier fish, heterozygous for the *cyc*, *flh* and *syu* mutations, were generated by mating fish heterozygous for both *cyc* and *flh* mutations with fish heterozygous for the *syu* mutation. Mutant embryos were identified on the basis of morphology or expression of *islet* genes (Table 1). In all cases the expected Mendelian ratios of mutant to wild-type progeny were observed.

The strongest available mutant alleles of each locus were used: *syu*^{t4} (Schauerte et al., 1998); *smu*^{b641} (Varga et al., 2001); *cyc*^{b16} (Hatta,

Table 1. Main criteria used to identify mutant embryos

Mutant	Morphological phenotype	Motoneuron (*islet*) phenotype
syu	U-shaped somites	Normal
smu	U-shaped somites	Loss of posterior pmns
cyc	Cyclopia	Very slight reduction in pmns
flh	No notochord and blocky somites	Slight reduction in pmns
cyc;flh	Cyclopia, no notochord and blocky somites	Loss of posterior pmns
cyc;syu	Cyclopia and u-shaped somites	Reduced pmns
flh;syu	No notochord and blocky somites	Reduced pmns
cyc;flh;syu	Cyclopia, no notochord and blocky somites	Five or fewer pmns

pmns, primary motoneurons.

1992); *flh* ^{n1} (Talbot et al., 1995). *syu*^{t4} is a deletion that encompasses the *shh* gene (Schauerte et al., 1998). *cyc*^{b16} is a deletion that encompasses the Nodal-related gene *ndr2* (Rebagliati et al., 1998b; Talbot et al., 1998). *flh*^{n1} is a 2 bp deletion in the *xnot* gene that causes the protein to be truncated upstream of the homeodomain and is therefore a putative null allele (Talbot et al., 1995). *smu*^{b641} is a point mutation that changes a glycine into an arginine in the predicted second transmembrane domain of Smoh, and is also a putative null allele (Varga et al., 2001). The *cyc*, *flh*, *syu* and *smoh* genes are all unlinked: *cyc* maps to linkage group 12, *flh* to linkage group 13, *syu* to linkage group 7 and *smoh* to linkage group 4 (Postlethwait et al., 1994; Geisler et al., 1999; Varga et al., 2001).

In situ RNA hybridization

In situ RNA hybridization was performed as previously described (Concordet et al., 1996): *islet1* and *islet2* probes were synthesized as in Appel et al. (Appel et al., 1995); *α-collagen type II* as in Yan et al. (Yan et al., 1995).

Specimens were analyzed using a Zeiss Axioplan microscope and photographed with Kodak Ektachrome 64T or 164T film. Images were scanned on a Nikon LS-1000 35 mm film scanner and processed using Adobe Photoshop software.

Motoneuron counts are presented for individual embryos, or as mean±s.e.m.

Morpholino injections

Morpholino oligonucleotides were obtained from Gene Tools. Morpholinos are antisense oligonucleotides that block translational initiation through an RNase-H independent process (Summerton, 1999) and have recently been demonstrated to specifically 'knock down' function of a number of zebrafish genes, including *twhh* (Nasevicius and Ekker, 2000). Two different *twhh* morpholinos were used and gave similar results, so the data from these experiments were pooled. Morpholinos were designed against the following 5′ UTR or 5′ coding sequence, depending on publicly available sequence and the manufacturers' recommendations. Control morpholinos have four mismatches spread throughout the oligonucleotide. (ATG start codons are shown in bold, mismatches in lower case. *smu*-MO-3 is upstream of the ATG but overlaps with *smu*-MO-2.)

twhh-MO-1: **ATG**GACGTAAGGCTGCATCTGAAGCAATT

twhh-MO-2*:* AAGAGATAATTCAAACGTC**ATG**G (also used in Nasevicius and Ekker (Nasevicius and Ekker, 2000)

ehh-MO: **ATG**AGACTCTCCACGGCGGCGGCGCTCCTC

twhh-MO-control: **ATg**GACgTAAGGCTGCAtCTGaAGCAATT

ehh-MO-control: **ATg**AGAcTCTCCACGGcGGCgGCGCTCCTC

smu-MO-1: TTGG**ATG**CTTTGGATCTGGACAGCT

smu-MO-2: ATTGTTGGAAGCTTTTGG**ATG**CTTT

smu-MO-3: CGCCCCTGCTCCATTGTTGG

Morpholinos were dissolved in 1×Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM Ca $(NO_3)_2$, 5 mM Hepes, pH 7.6), and then further diluted with distilled water and a 1% solution of Phenol Red. Morpholinos were injected into the yolk at the one- to four-cell stage at an approximate volume of 10 nl. *hh* morpholinos were injected at a final concentration of 1 or 2 mg/ml. These concentrations produced embryos with normal morphology but with the specific phenotypes described below. Consistent with earlier findings (Nasevicius and Ekker, 2000), higher concentrations generated some embryos with short anteroposterior axes or other morphological defects, and hence these experiments were not analyzed. Control morpholinos were injected at comparable concentrations: *twhh*-MO-control alone (2 mg/ml, *n*=92); *twhh*-MO-control and *ehh*-MO-control (2 mg/ml each, *n*=57; 1 mg/ml each, *n*=72). *smoh* morpholinos were injected at final concentrations of 2-10 mg/ml. At 10mg/ml, some embryos showed nonspecific morphological defects so higher concentrations were not used.

RESULTS

Strategy

In this paper we present a series of complementary experiments in which we reduced Hh signaling in different ways and analyzed the resulting motoneuron phenotypes. We were unable to remove unambiguously all zebrafish Hh signaling because mutations in *ehh* or *twhh* have not been isolated and *smu* mutants have maternal *smoh* transcripts (Varga et al., 2001) and thus residual Hh signaling (shown below) and fragments of other *hh* genes have been reported, but their patterns of spatial and temporal expression are unknown (Krauss et al., 1993; Ekker et al., 1995). Given these provisos, we describe experiments in which we reduced or eliminated one or more of the three characterized Hh signals and zygotic Smoh function.

In all our experiments, we visualized primary motoneurons by in situ hybridization for *islet1* and/or *islet2*, and we counted motoneurons on both sides of the embryo along the complete length of the spinal cord, starting at the first somite and continuing to the end of the tail. We primarily analyzed *islet2* expression, as this is a marker of more differentiated motoneurons and is easier to use at later somitogenesis stages when some interneurons have also started to express *islet1*. However, to analyze whether Hh signals are required for induction or maintenance of motoneurons, we visualized *islet1*

Table 2. The number of motoneurons is proportional to Hh levels

Mutant/experiment	*islet 2* (18-24 h)	*islet1* + *islet2* (18-24 h)	*islet1* (11-12 h)
Wild type	53.2±3.0 (*n*=5) [48]	94.5±2.8 (*n*=10) [79]	57.3±1.3 (*n*=25) [46]
smu	18.1±1.0 (*n*=14) [14]	28.4±1.2 (*n*=18) [20]	25.3±1.0 (*n*=21) [17]
smu;cyc	6.2±0.4 (*n*=20) [3]	10.5±0.6 (*n*=21) [6]	n.d.
smu;syu	8.4±0.6 (*n*=12) [4]	n.d.	n.d.
cyc;flh	14.1±0.4 (*n*=7) [12]	31.5±0.9 (*n*=8) [28]	12.5±0.7 (*n*=10) [9]
syu;flh	n.d.	35.0±0.5 (*n*=27) [30]	n.d.
syu;cyc	n.d.	17.1±1.0 (*n*=23) [6]	n.d.
cyc;flh;syu	2.3±0.7 (*n*=8) [0]	2.3±0.6 (*n*=4) [1]	2.9±0.6 (*n*=7) [1]
twhh morpholino injected into *syu*	[6]	n.d.	n.d.
twhh + *ehh* morpholinos injected into *syu*	[0]	n.d.	n.d.
twhh + *ehh* morpholinos injected into *smu;syu*	[0]	n.d.	n.d.

Results are average of *n* embryos±s.e.m. Numbers in square brackets indicate the lowest number of motoneurons seen in any embryo. n.d., not done.

Motoneurons were counted along the complete length of both sides of the spinal cord. Counts are not given for *syu* mutants or for single morpholino injections into wild-type embryos, as in all of these cases a normal number of primary motoneurons formed. Averages were not calculated for morpholino experiments because there was a large range of phenotypes, probably owing to variability in the amount injected or its distribution.

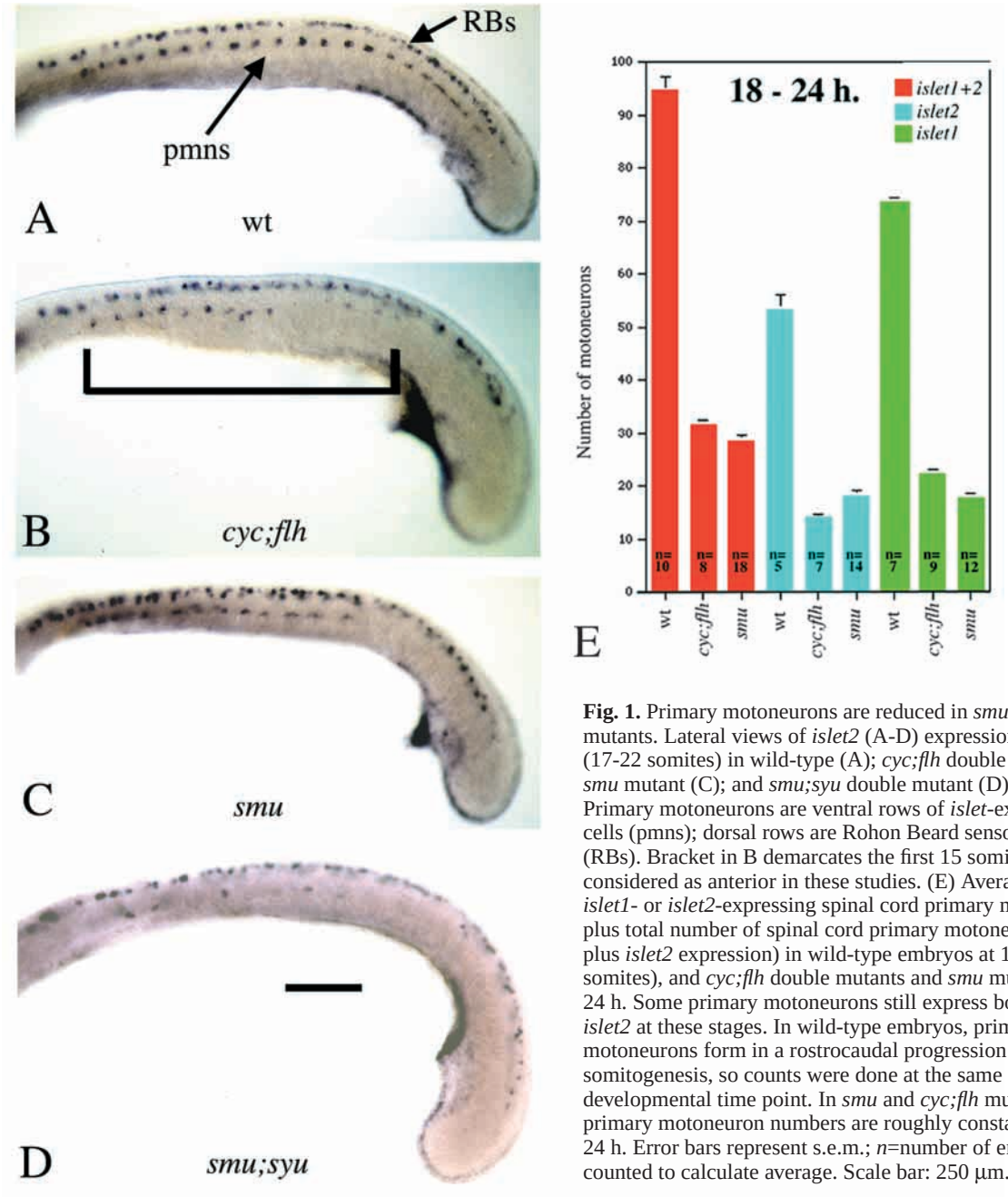

Fig. 1. Primary motoneurons are reduced in *smu* and *cyc;flh* mutants. Lateral views of *islet2* (A-D) expression at 17.5-20 h (17-22 somites) in wild-type (A); *cyc;flh* double mutant (B); *smu* mutant (C); and *smu;syu* double mutant (D) embryos. Primary motoneurons are ventral rows of *islet*-expressing cells (pmns); dorsal rows are Rohon Beard sensory neurons (RBs). Bracket in B demarcates the first 15 somites, considered as anterior in these studies. (E) Average number of *islet1*- or *islet2*-expressing spinal cord primary motoneurons plus total number of spinal cord primary motoneurons (*islet1* plus *islet2* expression) in wild-type embryos at 17-18 h (16-18 somites), and *cyc;flh* double mutants and *smu* mutants at 17-24 h. Some primary motoneurons still express both *islet1* and *islet2* at these stages. In wild-type embryos, primary motoneurons form in a rostrocaudal progression throughout somitogenesis, so counts were done at the same developmental time point. In *smu* and *cyc;flh* mutants, primary motoneuron numbers are roughly constant from 17-24 h. Error bars represent s.e.m.; *n*=number of embryos counted to calculate average. Scale bar: 250 μm.

expression, as this is the earliest marker available for motoneuron fates. When it was important to determine total numbers of motoneurons at later stages, we examined expression of both *islet1* and *islet2*.

cyc;flh and *smu* mutants have very similar motoneuron phenotypes

In both *smu* and *cyc;flh* mutants, anterior primary motoneurons form but posterior motoneurons are absent. However, previous analysis of the *smu* mutant examined *islet1* expression, whereas analysis of *cyc;flh* primarily examined *islet2* expression (Beattie et al., 1997; Varga et al., 2001), so it was unclear how similar these mutant phenotypes were. *cyc;flh* mutants lack both notochord and floor plate, hence, in addition to reduced Hh signaling, they presumably lack other signaling molecules expressed by these structures, some of which might also be involved in motoneuron development. Therefore we directly compared the patterns of *islet1* and *islet2* expression in these mutants to determine the similarity of the phenotypes.

We observed very comparable phenotypes in *cyc;flh* and *smu* mutants: primary motoneurons were absent from spinal cord posterior to about somite 15, but about 30 primary motoneurons persisted more anteriorly. *islet1*-expressing and *islet2*-expressing primary motoneurons were reduced in a similar manner both within and between mutant classes, with the exception that *cyc;flh* mutants had a more severe reduction of *islet2*-expressing than of *islet1*-expressing motoneurons.

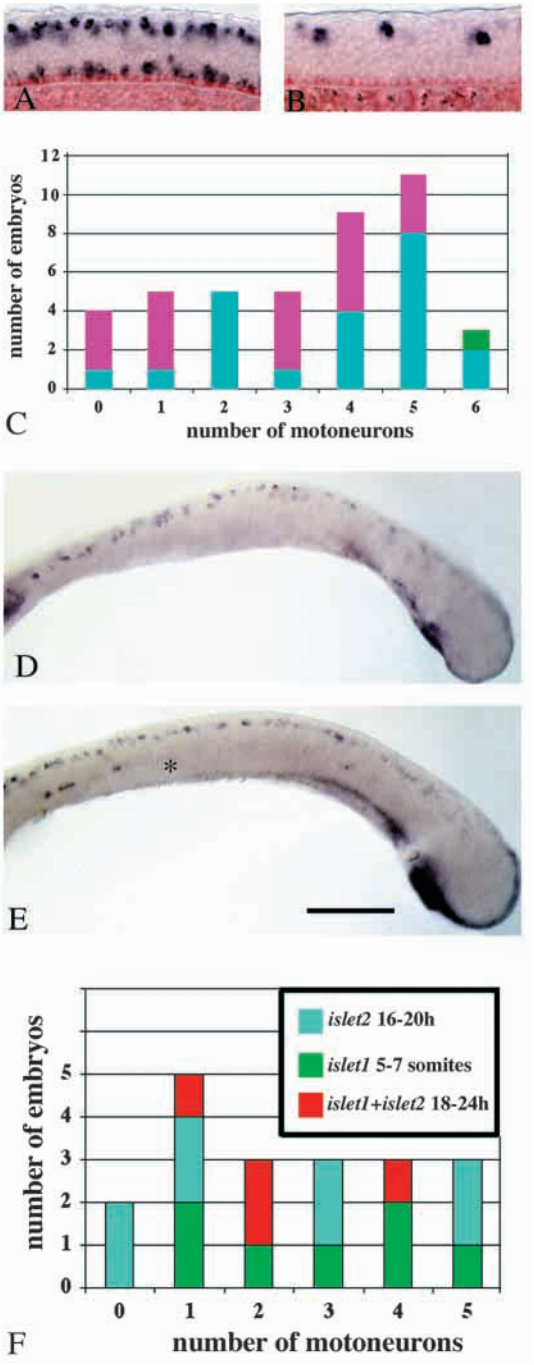

Fig. 2. The less Hh signal, the fewer primary motoneurons, but all embryos still form median floor plate. Lateral views of *islet2* expression in blue (A,B,D,E) and *α-collagen type II* expression in red (A,B) at 18-21 h (18-22 somites) in wild-type uninjected embryo (A); MO-injected *syu* mutant (B); and embryos from a triple mutant cross (D,E). (B) entirely lacked primary motoneurons but still formed median floor plate. (D) has no *islet2*-expressing primary motoneurons and is presumably a $cyc^{-/-};flh^{-/-};syu^{-/-}$ triple mutant. (E) has only six *islet2*-expressing primary motoneurons but has a notochord (*) and is probably $cyc^{-/-};flh^{+/-};syu^{-/-}$ or $cyc^{-/-};flh^{+/+};syu^{-/-}$. (C) shows total number of embryos from all the morpholino injection experiments that formed six or fewer *islet2*-expressing primary motoneurons. These are broken down into embryos from $syu^{+/-}$ parents (blue) or $syu^{+/-};smu^{+/-}$ parents (pink) injected with *twhh*-MO + *ehh*-MO and the one *syu* mutant injected with *twhh*-MO that formed six primary motoneurons (green). (F) The number of motoneurons in individual triple mutants. These were embryos from $cyc^{+/-};flh^{+/-};syu^{+/-}$ parents that had no notochord and five or fewer primary motoneurons. *islet2* experiment, n=609: expect about 9.5 triple mutants; observed eight embryos with five or fewer motoneurons. *islet1* experiment, n=549: expect about 8.5 triple mutants; observed seven embryos with five or fewer motoneurons. *islet1* + *islet2* experiment, n=367: expect about 5.7 triple mutants; observed four embryos with four or fewer motoneurons. Scale bar: 100 μm (A,B) and 250 μm (D,E).

However, this was only a slight difference and may reflect a slight developmental delay in these embryos (Fig. 1; Table 2).

These results suggest that the motoneuron phenotype of *cyc;flh* mutants can be explained solely by a reduction in Hh signaling. In addition, they confirm that both *islet1*- and *islet2*-expressing posterior primary motoneurons require Hh signals, but suggest that either sufficient Hh signaling remains in both *cyc;flh* and *smu* mutants for anterior primary motoneurons to form, or that some anterior primary motoneurons form independently of Hh signals.

smu;syu and *smu;cyc* mutants have a more severe phenotype than *smu* mutants

To determine whether maternal *smoh* is responsible for formation of the remaining motoneurons in *smu* mutants, we attempted to reduce maternal Smoh activity with *smoh* morpholinos. We injected three different morpholinos at a variety of concentrations into wild-type embryos (n=24); progeny from $syu^{+/-}$ parents (n=143) and progeny from $smu^{+/-}$ parents (n=252). However, in the first two cases, all of the injected embryos formed normal numbers of motoneurons, and in the later case embryos either had wild-type numbers of motoneurons or the normal zygotic *smu* mutant phenotype (61/252). This suggests that none of these morpholinos 'knocks down' zygotic or maternal Smoh function, at least as assayed by motoneuron phenotypes. There are three possible reasons for this, one is that the morpholinos are ineffective, as found for some other genes by Nasevicius and Ekker (Nasevicius and Ekker, 2000); alternatively the morpholinos may not act early enough, or efficiently enough to affect motoneuron development.

Therefore, to establish if there is residual Hh signaling in *smu* mutants and if this is responsible for the remaining motoneurons, we examined *smu;syu* and *smu;cyc* double mutants to see if they had a more severe phenotype than *smu* single mutants.

We found that both *smu;syu* and *smu;cyc* double mutants had only half the number of motoneurons present in *smu* single mutants (Fig. 1D; Table 2). In addition, crosses between $smu^{+/-};syu^{+/-}$ parents produced a class of progeny with motoneuron numbers intermediate between *smu* single mutants and *smu;syu* double mutants, suggesting that $smu^{-/-};syu^{+/-}$ or $smu^{+/-};syu^{-/-}$ embryos may also form fewer motoneurons than *smu* single mutants.

Our double mutant results demonstrate that removing either Shh or Twhh from *smu* mutants exacerbates the motoneuron phenotype, suggesting that there is still some Hh signaling present in *smu* mutants. However, about six to eight primary motoneurons still form in both of the double mutants. These could be due to remaining Hh signaling, from *ehh* and/or *twhh*

in *smu;syu* mutants and from *ehh* and/or *shh* in *smu;cyc* mutants, or these motoneurons might form independently of Hh signaling. To distinguish between these possibilities we used two different methods to severely reduce or eliminate all three Hh signals.

'Knockdown' of all three Hedgehogs prevents primary motoneuron formation

There are no identified mutations in *ehh* or *twhh*, so we used morpholinos against these genes, in combination with *syu* and *smu* mutations, to reduce or eliminate the function of all three Hh proteins in vivo. We injected morpholinos against *twhh* (*twhh*-MO) and *ehh* (*ehh*-MO) separately and in combination, and analyzed primary motoneuron development by in situ hybridization with *islet2* at 18-24 h. The degree to which motoneurons were reduced varied considerably within each experiment, presumably because of variation in the amount of morpholino injected or its distribution in individual embryos. Therefore, as we were primarily interested in whether reducing Hh signals could reduce the number of primary motoneurons below that seen in *smu;syu* and *smu;cyc* double mutants, we counted the number of motoneurons in the most severely affected embryos in each experiment.

Our most severe results were obtained by injecting a combination of *ehh*-MO and *twhh*-MO into *smu;syu*, *smu* and *syu* mutants. When we injected progeny from $smu^{+/-};syu^{+/-}$ parents, 49% of embryos had a reduction in primary motoneurons and 24.7% of these had six or fewer motoneurons (n=157). When we injected into progeny from *syu* heterozygous parents, 28.7% of the embryos had a reduction in the number of motoneurons and 18% of these had six or fewer motoneurons (n=425). These results suggest that the *ehh* and *twhh* morpholinos reduced only the number of motoneurons in embryos that also lacked Shh and/or Smoh function. In both cases, we observed embryos that lacked all motoneurons (Fig. 2; Table 2). In addition, even embryos with fewer than six motoneurons appeared to form a morphologically normal median floor plate. To examine this further, we visualized expression of *islet2* and *α-collagen type II*, a marker for median floor plate (Yan et al., 1995) in embryos from *syu* heterozygous parents injected with *twhh*-MO + *ehh*-MO. Embryos with a severely reduced number of motoneurons (0-14; n=16) were examined in more detail: all appeared to form median floor plate along the whole length of the embryo (Fig. 2B).

We also observed a reduced number of motoneurons when we injected *twhh*-MO alone into *syu* mutants, but in this case the most severe reduction was to six motoneurons (Fig. 2; Table 2). Again, only a quarter (24.3%, n=189) of the progeny from *syu* heterozygous parents had fewer motoneurons. By contrast, progeny from *syu* heterozygous parents injected with *ehh*-MO alone formed normal numbers of motoneurons (n=70; data not shown). Control morpholinos had no effect on motoneuron formation. We injected progeny from *syu* heterozygous parents with *twhh*-MO-control alone (n=92, data not shown) and with a combination of *twhh*-MO-control and *ehh*-MO-control (n=139, data not shown).

These results suggest that Hh signals are required for primary motoneuron formation: less Hh signal results in fewer motoneurons. In addition, they suggest that complete loss of primary motoneurons may require inactivation of all three *hh* genes. Loss of just one Hh appears to have no effect on motoneuron formation, neither does loss of Ehh plus Shh or Ehh plus Twhh. Loss of Twhh and Shh can produce substantial reductions in motoneuron numbers, but reductions are more severe when all three Hhs are removed. This suggests that all of the morpholinos have at least some activity, and it is also consistent with previous observations that *twhh*-MO had no independent effect on somite and head development, but acted redundantly in combination with a *shh* morpholino (Nasevicius and Ekker, 2000). However, even when all three Hh signals were removed by mutation or morpholinos, in most embryos at least one primary motoneuron still formed.

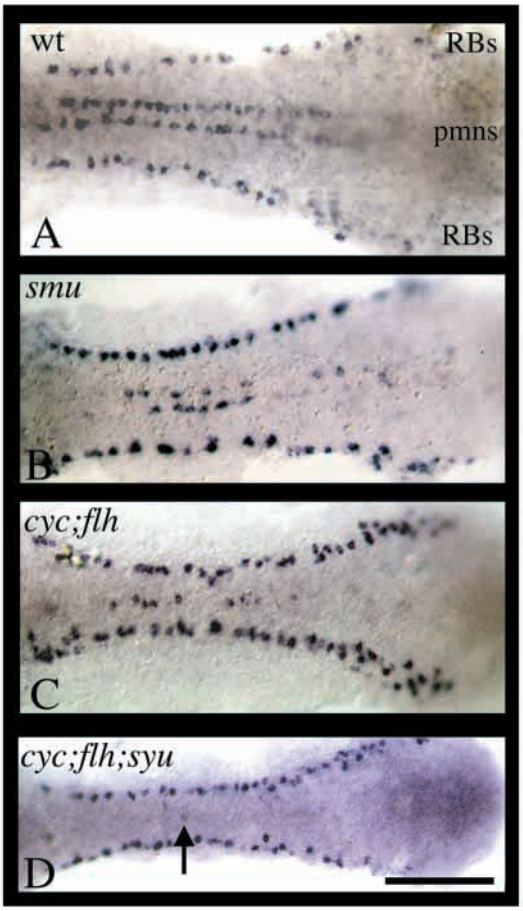

Fig. 3. Hh signals are required for primary motoneuron induction. Dorsal views of flat mounted preparations of *islet1* expression in wild-type (A); *smu* mutant (B); *cyc;flh* double mutant (C) and *cyc;flh;syu* triple mutant (D) embryos at 11-12 h (five to six somites). At this stage, primary motoneurons (pmns) normally form two rows in the median neural plate and Rohon Beard (RB) sensory neurons are visible at the lateral edges of the neural plate. The arrow demarcates a single primary motoneuron in the triple mutant embryo in D. Scale bar: 250 μm.

cyc;flh;syu triple mutants lack primary motoneurons

cyc;flh;syu triple mutants lack notochord, floor plate and the *shh* locus, and hence lack expression of *shh*, *twhh* and *ehh* (Beattie et al., 1997; Schauerte et al., 1998). These mutants therefore provide an alternative, although partially indirect

assay of the effects of removing all Hh signaling, as well as allowing us to address the specific question of whether the anterior primary motoneurons that form in *cyc;flh* mutants require Shh signals.

In situ hybridization for *islet2* on the progeny of $cyc^{+/-};flh^{+/-};syu^{+/-}$ parents at 18-24 h revealed a number of embryos with a much more severe loss of primary motoneurons than *cyc;flh* double mutants. Surprisingly these fell into two groups: those with a notochord, that were presumably $syu^{-/-};cyc^{-/-};flh^{+/-}$ or $syu^{-/-};cyc^{-/-};flh^{+/+}$ (Fig. 2E); and those lacking a notochord, the most severe of which were presumably triple mutants (Fig. 2D). We expected to see about 9.5 triple mutants and we observed eight embryos without a notochord that had severely reduced motoneuron numbers. We counted the motoneurons in these presumptive triple mutants and observed similar results to our *twhh*-MO + *ehh*-MO injections into *syu* mutants. The triple mutants formed an average of only two motoneurons and two of them had no motoneurons (observed in whole mounts and confirmed with transverse sections; Fig. 2; Table 2). However, we also saw embryos with notochords but as few as three or four motoneurons (Fig. 2E).

It remains possible that embryos lacking *islet2*-positive motoneurons still had *islet1*-positive motoneurons, so we also analyzed embryos by in situ hybridization with a combination of *islet1* and *islet2* probes. The disturbed morphology of the mutants and the expression of *islet1* in some interneurons at these stages, made it difficult to count the number of motoneurons in whole mounts, so the numbers of motoneurons in embryos with severe reductions were confirmed with transverse sections. Again, we observed embryos with no notochords and severe reductions in primary motoneurons at the expected frequency for triple mutants. These presumably triple mutants still had an average of only two motoneurons but all of them had at least one (Fig. 2; Table 2).

The loss of primary motoneurons in *cyc;flh;syu* triple mutants was consistent with our morpholino results, demonstrating that with the possible exception of an occasional cell, the anterior motoneurons in zebrafish require Hh signals, and the motoneurons that form in *cyc;flh* mutants require Shh.

Early requirement for Hh signaling suggests a role in induction

To determine whether Hh signaling is required for motoneuron induction or maintenance, we examined *islet1* expression in *smu*, *cyc;flh* and *cyc;flh;syu* mutants at the time when it is first expressed by primary motoneurons (11-12 h; four to five somites). In all cases, the motoneuron phenotype resembled that seen at later stages. Both *smu* and *cyc;flh* mutants had fewer motoneurons even at this early stage, but, in agreement with later stages, the reduction was predominantly in posterior segments, and anterior spinal cord motoneurons still formed (Fig. 3; Table 2). Similar to later stages, crosses from fish heterozygous for *cyc*, *flh* and *syu* generated embryos with no notochords and an average of only three motoneurons at the expected frequency for triple mutants (Fig. 2F; Fig. 3; Table 2). In addition, some embryos from these crosses had very few motoneurons but still formed a notochord (data not shown). These results suggest that Hh signaling is required to induce primary motoneuron fates.

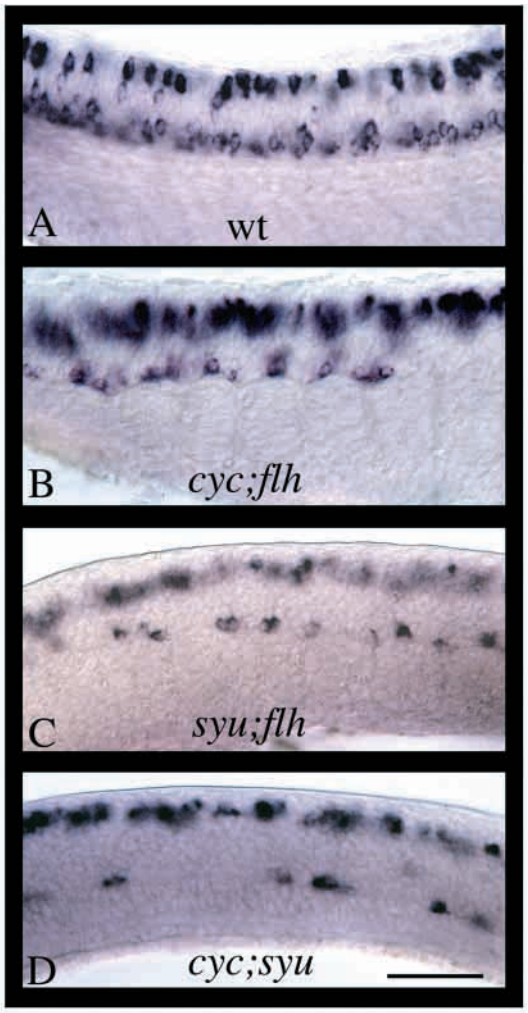

Fig. 4. Ehh is less effective than Shh and Twhh for primary motoneuron formation. Lateral views of *islet1* + *islet2* expression (both in blue) in wild-type embryo at 24 h (A); *cyc;flh* double mutant at 24 h (B); *flh;syu* double mutant at 18-19 h (C); and *cyc;syu* double mutant at 18-19 h (D). Scale bar: 50 μm.

Twhh and Shh are more effective at inducing motoneurons than Ehh

Our morpholino injections into *syu* mutants suggested that removing Ehh and Shh, or Ehh and Twhh, has less effect on motoneuron development than removing Twhh and Shh. Our triple mutant analysis, in particular the embryos that formed a notochord but very few motoneurons, also suggested that Ehh plays a minimal role in motoneuron development. To investigate the roles of different Hhs in motoneuron development more precisely, we compared the expression of *islet1* and *islet2* in different double mutants. As described above, *cyc;flh* mutants form an average of 31.5 motoneurons, despite their lack of *twhh* and *ehh* expression, and only transient expression of *shh*. Similarly *syu;flh* mutants, in which only *twhh* is expressed, form an average of 35 motoneurons. By contrast, *syu;cyc* mutants, in which only *ehh* is expressed, form an average of 17 motoneurons (Fig. 4; Table 2). This suggests that Ehh is less efficient at inducing motoneurons than Shh or Twhh.

DISCUSSION

Formation of zebrafish primary motoneurons requires Hh signaling

We have demonstrated, using both mutational and antisense techniques, that formation of at least the vast majority of zebrafish primary motoneurons, like formation of zebrafish secondary motoneurons (Beattie et al., 1997), requires Hh signaling. This is consistent with findings for motoneurons in other vertebrates, despite the idea that zebrafish primary motoneurons are less similar to other vertebrate motoneurons than are zebrafish secondary motoneurons. Our results also suggest that the difference between the motoneuron phenotypes of zebrafish and mouse *shh* mutants is due to the ability of *ehh* and *twhh* to compensate for loss of *shh* function in zebrafish.

Our findings argue against there being any major difference other than timing in the requirement of zebrafish anterior and posterior spinal motoneurons for Hh signaling. In particular, we have shown that zebrafish primary motoneurons in both the anterior and posterior spinal cord require Hh signals. Our results suggest that in both *smu* and *cyc;flh* mutants the remaining motoneurons are due to residual Hh signaling. The anterior location of these motoneurons can be explained by motoneurons being induced in a rostrocaudal progression and Hh signaling being present only early in development in both of these mutants.

The mouse *Shh* mutant forms no motoneurons (Chiang et al., 1996; Litingtung and Chiang, 2000b) but even when we attempted to remove all three Hh signals, we often saw at least one remaining motoneuron. One likely explanation for this is that we may not have eliminated all Hh signaling. With respect to our morpholino experiments, we cannot assess the degree to which the morpholinos have interfered with translation without antibodies to the different Hh proteins, and even if antibodies existed, very low levels of protein might remain undetected. With respect to our triple mutant analysis, whereas Beattie and colleagues saw no expression of *twhh* in *cyc* or *cyc;flh* mutants at 90% epiboly and later stages, Ekker and colleagues reported the presence of a few *twhh* expressing cells in *cyc* mutants at 95% epiboly (Beattie et al., 1997; Ekker et al., 1995). This discrepancy may be due to differences in techniques or background differences between *cyc* mutant strains, but it raises the possibility that a small amount of *twhh* message, maybe just at the limit of detection, may remain in our triple mutants. In addition, a fragment of at least one additional *hh* gene has been isolated, although its expression pattern has not yet been reported (Krauss et al., 1993; Ekker et al., 1995); until the zebrafish genome is sequenced, we will not be certain that all of the zebrafish *hh* genes have been identified. Although the severity of the phenotypes we observed suggests that any additional *hh* genes are unlikely to play a major role in motoneuron development, we cannot rule out the possibility that another *hh* gene is responsible for induction of the occasional rogue cell we observed in our experiments. Nevertheless, our results demonstrate that we have reduced Hh signaling to a much greater extent than any previous study in zebrafish. An unambiguous eradication of Hh signals is not possible with existing techniques and mutations. Therefore, it is important that this issue be revisited once the zebrafish genome is sequenced and loss-of-function mutations exist in all of the *hh* genes.

Can motoneurons form independently of Hh signaling?

As discussed above, the simplest explanation for the few remaining motoneurons in our morpholino and triple mutant experiments is that they are induced by residual Hh signals. However, we cannot rule out the possibility that these motoneurons represent a small, Hh-independent population. This alternative hypothesis is reminiscent of observations in other systems. For example, the classic chick in vitro system involves isolating the caudal intermediate neural tube at an early stage and then culturing it with, or without, exposure to potential signaling molecules or embryonic structures. Initial experiments showed that up to five Islet-1-positive, SC1-positive cells still form in these cultures, even in the absence of Hh signals (Yamada et al., 1993). In agreement with this observation, in some notochord ablation experiments in the chick, the floor plate fails to form over a long stretch of the neural tube, but even in the middle of this region a few SC1-positive DM2-positive motoneurons still form (Artinger and Bronner-Fraser, 1993).

Even though Hh signaling is clearly required for the formation of at least the vast majority of motoneurons, it is still unclear exactly how Hh acts. Although it is difficult to explain how a few motoneurons might be independent of Hh signals if Hh directly and solely induces motoneurons, it is less paradoxical if Hh signaling induces motoneurons indirectly by, for example, inhibiting the repression of ventral fates by dorsal signals, or if Hh acts in concert with other signals that have very limited activity on their own to induce motoneurons. There is evidence for both of these scenarios. Litingtung and Chiang (Litingtung and Chiang, 2000b) have recently shown that motoneurons form in *Shh*;*Gli3* double mutant mice, demonstrating that for a substantial number of mouse motoneurons Shh is only required to inhibit Gli3. However these results also demonstrate that differences exist between mouse motoneurons because half of the motoneurons in the lumbar region and most of the motoneurons in the brachial region still require Shh activity, even in the absence of Gli3. This could reflect redundancy between Gli2 and Gli3, or the presence of a second motoneuron-inducing factor in mice that is distributed differentially along the rostrocaudal axis (Litingtung and Chiang, 2000b). Retinoic acid (RA) is a good candidate for a second motoneuron-inducing factor because it has been shown, in vitro, to induce other ventral neuronal fates, specifically V0 and V1 interneurons, in a Shh-independent manner (Pierani et al., 1999) and it can induce motoneurons in chick neural explants (Sockanathan and Jessell, 1998) and embryonic stem cells, although this may be an indirect effect as *Shh* is also induced in these experiments (Renoncourt et al., 1998). However, Shh is also sufficient for induction of V0 and V1 interneurons, and is required for the development of some, but not all, of these neurons, suggesting that RA and Shh may act together to specify the full complement of these neurons. These interactions are still not properly understood, but as they are further elucidated it will be interesting to see whether any parallels can be drawn with motoneuron development.

Other factors may also act in concert with Shh in motoneuron induction. For example, neurotrophin 3 (NT3) acts

synergistically with Shh in vitro to induce motoneurons and a mouse loss-of-function NT3 mutant lacks a subset of spinal motoneurons (Dutton et al., 1999; Kucera et al., 1995; Woolley et al., 1999).

Finally, analysis of other cell fates that are thought to require Hh signals has generated controversy over the precise role of Hh signaling and has raised the possibility that even when the majority of a particular cell type requires Hh signaling, there might be a small population of cells that is independent of this. For example, in the *Shh* loss-of-function mouse, a drastic reduction, but not a complete loss, of *Pax1*-expressing sclerotome precursors and *Myf-5*-expressing median muscle precursors was seen at early stages, suggesting that Hh signaling is required for most, but not all, initial *Pax1* and *Myf5* expression (Chiang et al., 1996). Similar results have also been seen with transplantation and cell culture experiments that have studied myogenic and chondrogenic cell fate determination in the segmental plate mesoderm (George-Weinstein et al., 1998); other studies have also suggested that Shh is a survival and proliferation factor for, rather than a primary inducer of, both epaxial and hypaxial muscles (Kruger et al., 2001; Teillet et al., 1998; Marcelle et al., 1999; Borycki et al., 1999).

Median floor plate still forms in embryos with drastically reduced Hh signals.

Despite an almost complete loss of primary motoneurons, our severely affected morpholino-injected embryos still formed median floor plate. This is consistent with other studies that suggest the formation of zebrafish median floor plate requires Nodal signals but not Hh signals (Schauerte et al., 1998; Odenthal et al., 2000; Muller et al., 2000; Sampath et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998b). The lack of mutations in *ehh* or *twhh* has precluded conclusive analysis of whether these *hh* genes play a role in median floor plate induction in the absence of *shh*. Our morpholino experiments demonstrate that if Ehh and Twhh signaling are reduced in the absence of Shh, a median floor plate still forms. This is in contrast to the mouse *shh* mutant, which does not form a floor plate, and it suggests that not all of the differences between the mouse and zebrafish mutants can be explained by redundancy between different zebrafish Hh proteins.

Hh signaling still occurs in *smu* mutants

Our morpholino injection and *smu;syu* and *smu;cyc* double mutant results demonstrate that some Hh signaling remains in *smu* mutants, at least at early stages. This may be initially surprising, as the *smu* mutation disrupts the *smoothened* gene, which encodes part of the Hh receptor complex and is thought to be essential for Hh signaling. The most likely explanation for this early Hh signaling in *smu* mutants is that maternal expression of Smoh is sufficient for initial signaling by Hh proteins. Alternatively, some Hh signaling may be independent of Smoh, perhaps acting through a second, as yet unidentified, Smoothened protein (but see Varga et al., 2001).

Some of the early Hh signaling in *smu* mutants is due to Shh, as *smu;syu* embryos have a more severe reduction of primary motoneurons than *smu* single mutants. However, some of this early signaling is Shh independent, as *smu;syu* embryos have a less severe phenotype than the most severely affected embryos in the morpholino and triple mutant experiments. Therefore, this early signaling probably requires Twhh and/or Ehh, consistent with the idea that all of the Hh proteins act through Smoh.

Twhh and Shh are more effective than Ehh at inducing motoneurons

Our results demonstrate that the loss of any one Hh protein does not affect motoneuron formation. Thus, any two of the three midline Hh proteins are sufficient for normal motoneuron numbers to form and signaling from any individual Hh protein is sufficient for at least some motoneurons to form. This suggests there is substantial redundancy among the functions of different Hh proteins and argues against motoneuron induction requiring a particular combination or complex of two or more Hhs.

Our results also suggest that Ehh is less efficient at inducing motoneurons than Twhh or Shh. This is consistent with earlier suggestions that cells interpret the overall concentration of Hh signals, because differences in function could reflect the different spatial and temporal expression of the three genes (Lewis et al., 1998). *ehh* is expressed later and possibly at lower levels than *shh* or *twhh* (Currie and Ingham, 1996). In addition, it is expressed exclusively in the notochord and therefore may not be in the same proximity to motoneuron precursors as Shh or Twhh. However, our results are also consistent with Shh and its closest relative Twhh, which resulted from a duplication of the *shh* gene (Zardoya et al., 1996), being inherently more efficient at inducing motoneurons than the zebrafish homolog of Indian Hh, Ehh. Interestingly, Ehh has recently been shown to be less effective than Shh at inducing slow muscle in vitro (Norris et al., 2000), suggesting that these proteins may have different activities. One way to answer this question would be to investigate whether Ehh can rescue *syu* mutants when expressed under the control of the *shh* promoter.

Hh signaling is required for initial motoneuron expression of *islet1*

Our results demonstrate that Hh signaling is required for initial *islet1* expression in motoneurons. Thus, Hh signaling appears to be involved in motoneuron induction. This is consistent with our observation that both the *islet1*- and *islet2*- expressing subsets of primary motoneurons require Hh signals, suggesting that Hh signaling is required before these motoneurons assume their different identities. Although we cannot entirely rule out the possibility that other signals might induce motoneuron fates and Hh might have a very early maintenance role, our experiments show that Hh signals are required from extremely early in motoneuron development. The comparison between *cyc;flh;syu* and *cyc;flh* mutants also suggests that Shh signals are required only during gastrulation for anterior primary motoneuron formation.

We observed that *cyc;flh* mutants had significantly fewer motoneurons at 11-12 h than at 18-24 h, and slightly lower numbers of *islet2*-expressing than *islet1*-expressing motoneurons at 18-24 h. Both of these observations can be explained by *cyc;flh* mutants being developmentally delayed. By contrast, the motoneuron phenotypes of *smu* and *cyc;flh;syu* mutants were very similar at 11-12 h and 18-24 h, suggesting that once motoneurons express *islet1*, Hh signals are no longer required for their maintenance, at least between these stages. However, we did observe a gradual reduction in the number of

motoneurons in *smu* mutants from 18-36 h (K. E. L., unpublished), suggesting that Hh may eventually be required for motoneuron maintenance. This could be an indirect effect: the muscle is severely disturbed when Hh signals are reduced (Lewis et al., 1999; Barresi et al., 2000) and motoneurons may eventually die if they are unable to form synapses with their correct targets.

Motoneurons and slow muscle have different requirements for Hh signaling

Hh signals are crucial for development of slow muscle in zebrafish, but muscle appears to be more sensitive to a partial loss of these signals than primary motoneurons. For example *syu* mutants have a severe reduction in slow muscle formation but no reduction in motoneurons (Schauerte et al., 1998; Lewis et al., 1999) and *smu* mutants almost completely lack slow muscle (Barresi et al., 2000) but still form anterior primary motoneurons (Varga et al., 2001; this paper). We demonstrate here that the number of motoneurons is proportional to the level of Hh signals. However, severe reductions of Hh signals also corresponded to earlier reductions of Hh signals, so our results are consistent with primary motoneurons requiring a lower concentration of Hh signal than slow muscle, or with them losing their requirement for Hh signaling earlier than slow muscle cells.

In conclusion, we have demonstrated that Hh signals are required for development of at least the vast majority of zebrafish primary motoneurons. This is a significant result because primary motoneurons may be distinct from the motoneurons that are most studied in chick and mouse, which have been suggested to more resemble zebrafish and amphibian secondary motoneurons (Kimmel and Westerfield, 1991). We also show that Hh signals are required for initial expression of *islet1* RNA, suggesting that they induce the motoneuron cell fate. In addition, we show that zebrafish Hh proteins act redundantly to specify motoneurons and that all three of the midline zebrafish *hh* genes are individually dispensable for motoneuron development. At least two *hh* genes need to be 'knocked down' to lose any motoneurons, consistent with motoneurons requiring a lower level of Hh signal than some other cell fates such as slow muscle. In addition, our data suggest that Ehh has less of a role in motoneuron formation than Twhh or Shh. However, questions remain unanswered about whether a few cells can acquire aspects of motoneuron identity in the absence of Hh signals. A conclusive answer to this question in zebrafish may require loss-of-function mutations in all of the *hh* genes. Novel methods might be required to find these mutations, because morpholino analysis suggests that their morphological phenotypes as single mutants may be subtle (this paper; Nasevicius and Ekker, 2000). This issue may also be elucidated by studies that further address the mechanism by which Hh signals direct motoneuron fate, and what roles, if any, other motoneuron-inducing factors or co-factors of Hh signaling play in this process.

Note added in proof

Chen et al. (Chen et al., 2001) report a dramatic reduction of primary motoneurons, but normal median floor plate, in *smoothened* mutant embryos treated with cyclopamine and Etheridge et al. (Etheridge et al., 2001) report that median floor plate is normal in *syu* mutants injected with *twhh*-MO.

We are grateful to E. Melançon, E. Hirsinger, M. Westerfield and C. Kimmel for comments on earlier drafts of this manuscript, and to other colleagues at the University of Oregon for discussions about this work. We thank J. Parker and K. Larison for cryostat sectioning and A. Arnett, D. Grant, C. Miller, E. Melançon and the University of Oregon Zebrafish Facility staff for help in maintaining fish stocks. This work was supported through a Wellcome International Prize Travelling Research Fellowship 054975 to K. E. L. and NIH grants NS23915 and HD24498 to J. S. E. Renovation and expansion of the University of Oregon Zebrafish Facility was supported by NIH RR11724, NSF STI-9602828, M. J. Murdock Charitable Trust and W. M. Keck Foundation.

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