Development 126, 2751-2758 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV2412

Specification of distinct motor neuron identities by the singular activities of individual *Hox* genes

Stefan Jungbluth*, Esther Bell* and Andrew Lumsden‡

Department of Developmental Neurobiology, King's College London, Hodgkin Building, Guy's Campus, London SE1 9RT, UK

*These authors contributed equally to this study

‡Author for correspondence (e-mail: a.lumsden@umds.ac.uk)

Accepted 15 April; published on WWW 19 May 1999

SUMMARY

Hox genes have been implicated in specifying positional values along the anteroposterior axis of the caudal central nervous system, but their nested and overlapping expression has complicated the understanding of how they confer specific neural identity. We have employed a direct gain-of-function approach using retroviral vectors to misexpress _Hoxa2_ and _Hoxb1_ outside of the normal _Hox_ expression domains, thereby avoiding complications resulting from possible interactions with endogenous _Hox_ genes. Misexpression of either _Hoxa2_ or _Hoxb1_ in the anteriormost hindbrain (rhombomere1, r1) leads to the generation of motor neurons in this territory, even though it is normally devoid of this cell type. These ectopic neurons have the specific identity of branchiomotor neurons and, in the case of _Hoxb1_-induced cells, their axons leave the hindbrain either by fasciculating with the resident cranial motor axons at isthmic (trochlear) or r2 (trigeminal) levels of the axis or via novel ectopic exit points in r1. Next, we have attempted to identify the precise branchiomotor subtypes that are generated after misexpression and our results suggest that the ectopic motor neurons generated following _Hoxa2_ misexpression are trigeminal-like, while those generated following _Hoxb1_ misexpression are facial-like. Our data demonstrate, therefore, that at least to a certain extent and for certain cell types, the singular activities of individual _Hox_ genes (compared to a combinatorial mode of action, for example) are sufficient to impose on neuronal precursor cells the competence to generate distinctly specified cell types. Moreover, as these particular motor neuron subtypes are normally generated in the most anterior domains of _Hoxa2_ and _Hoxb1_ expression, respectively, our data support the idea that the main site of individual _Hox_ gene action is in the anteriormost subdomain of their expression, consistent with the phenomenon of posterior dominance.

Key words: *Hox* gene, Rhombomere, Hindbrain, Development, Motor neuron

INTRODUCTION

The motor neurons of the hindbrain are segmentally organised and they display subtype variation along the anteroposterior (AP) axis. They represent an attractive system in which to study patterning along the anteroposterior neural axis (Lumsden and Krumlauf, 1996). Initially each of the hindbrain segments, or rhombomeres, contains a similar basic composition of neural precursors (Clarke and Lumsden, 1993), but as development progresses, segment-specific neuronal diversification becomes evident. It is thought that this is achieved by rhombomere-specific activities of candidate genes such as the *Hox* genes (Lumsden and Krumlauf, 1996). In order to test for *Hox* gene functions, gain-of-function and loss-of-function studies have been performed in mouse, chick and zebrafish embryos. These studies have implicated *Hox* genes in both the initial establishment of rhombomeres as well as the maintenance of specific rhombomeric identities (Lufkin et al., 1991; Chisaka et al., 1992; Carpenter et al., 1993; Dollé et al., 1993; Mark et al., 1993; Zhang et al., 1994; Alexandre et al., 1996; Goddard et al., 1996; Studer et al., 1996; E. Bell, R. Wingate and A. Lumsden, unpublished). It remains unclear, however, how *Hox* genes confer positional values on neural tissue. Their overlapping expression patterns suggest that positional identity could be conferred by a cooperative action of different *Hox* genes ('The *Hox* code'), or by the sum of individual singular *Hox* gene activities present at a given axial level, or by a combination of both (see McGinnis and Krumlauf, 1992). However, with the exception of the targeted disruption of *Hoxa2* (Gavalas et al., 1997), which is the only *Hox* gene expressed in rhombomere (r)2, functional studies have focussed on areas of the developing neural tube that express other *Hox* genes in addition to the particular *Hox* gene under investigation, thereby complicating the distinction between a combinatorial and singular mode of *Hox* gene action. *Hoxb1*, for example, is initially expressed up to the r3/r4 boundary and later is maintained at high levels in r4 and, at lower levels, caudal to r6. It is coexpressed with *Hoxa1*, *Hoxa2* and *Hoxb2* in r4, which gives rise to, amongst other cells, branchiomotor neurons of the facial motor nucleus

(Lumsden and Krumlauf, 1996). Gene targeting studies have demonstrated that despite the absence of *Hoxb1,* motor neurons differentiate in r4, suggesting that *Hoxb1* might not be required to initiate motor neuron generation (Goddard et al., 1996; Studer et al., 1996). Alternatively, loss of *Hoxb1* activity might be partially compensated by the remaining *Hox* genes, thereby giving rise to the differentiation of motor neurons in r4 with an atypical migration behaviour as described for *Hoxb1*−/− embryos (Studer et al., 1996; Goddard et al., 1996; Gavalas et al., 1998). However, motor neurons are still generated in the r2 of *Hoxa2*−/− mutants despite the fact that this is the only *Hox* gene expressed in that rhombomere (Gavalas et al., 1997). Although the particular subtype of motor neurons generated in the absence of *Hoxa2* has not been determined, this result does suggest that *Hox* genes are not required for motor neuron induction. Furthermore, at more anterior levels of the axis, somatomotor neurons of the trochlear (isthmus) and oculomotor (midbrain) nerves form in the normal absence of *Hox* gene expression.

We were interested in finding out whether the singular activities of individual *Hox* genes might be sufficient to impart distinct developmental fates to neuronal precursor cells. Using a direct gain-of-function approach, and avoiding possible complications resulting from the presence of other *Hox* genes, we analysed the effects of misexpressing *Hoxb1* and *Hoxa2* in vivo in r1, i.e. anterior to the normal *Hox* expression domain. Our results show that ectopic *Hoxb1* and *Hoxa2* activity leads to the generation, respectively, of ectopic facial and trigeminal branchiomotor neurons in an area of the neural tube never normally giving rise to any motor neurons, demonstrating that *Hox* genes can operate through their individual singular activities to specify the development of distinct neuronal subtypes.

MATERIALS AND METHODS

Construction of recombinant retroviruses and infection procedure

The full coding region of chick *Hoxa2* (Prince and Lumsden, 1994) was cloned into the RCAS(BP)B retroviral vector (Hughes et al., 1987) using the adaptor plasmid Slax12 (Morgan and Fekete, 1996). The adaptor plasmid sequence was altered from the ATG of the *Nco*I site to add the start of the *Hoxa2* coding sequence up to the *Sfu*I site by PCR. An *sfu*I/*Aat*II *Hoxa2* fragment was subcloned into Slax12 digested with *Sfu*I/*Hin*dIII. Sequences were verified to ensure no PCR errors had occurred. The Slax12/*Hoxa2* was cut with *Cla*I and cloned into the RCAS vector cut with *Cla*I. The construction of the RCAS/*Hoxb1* and RCAN/*Hoxb1* vectors will be described elsewhere (E. Bell, R. Wingate and A. Lumsden, unpublished).

Collection of virus and infections into the neural tube or on top of the primitive streak were done according to established protocols (Morgan and Fekete, 1996). Virus titers were between 10^8 and 10^9 particles/ml. For each probe or antibody between 5 and 30 infected embryos were stained and analysed.

In situ hybridisation, immunostaining, and nerve tracing

Whole-mount in situ hybridisation with digoxigenin- and fluorescein isothiocyanate-labelled riboprobes was performed as described (Wilkinson, 1992). For whole-mount antibody stains, embryos were left after blocking in primary antibodies for 3 days (in PBS/10% serum/1% Triton X-100), 4°C, followed after washing by an overnight incubation in secondary antibodies (in PBS/5% serum/1% Triton X-100), 4°C. After washing, embryos were fixed (for fluorescence-coupled secondary antibodies) or developed in DAB (for peroxidase-coupled secondary antibodies). Probes and antibodies were: chick *Hoxa2* (Prince and Lumsden, 1994); chick *Hoxb1* (V. Prince); mouse *Hoxb1* (R. Krumlauf); chick *Hoxb2* (A. Kuroiwa); chick *Isl2* (Tsuchida et al., 1994); chick *BEN* (O. Pourquie); anti-ISL1/2 (Thor et al., 1991); anti-BEN (Pourquie et al., 1990; Developmental Studies Hybridoma Bank); anti-mouseHoxb1 (N. Manley and M. Capecchi); anti-neurofilament antibody clone RMO-270 (Zymed). The hindbrains of stained embryos were dissected out, flat-mounted and analysed by bright field and Nomarski microscopy.

Anterograde and retrograde nerve tracing was performed by injecting DiI (6 mg/ml in dimethyl formamide; Molecular Probes) into the ventral neural tube or into the branchial arches of formaldehyde-fixed embryos. After 3-7 days in fix, embryos were analysed by confocal microscopy.

RESULTS

Generation of ectopic motor neurons in the anterior hindbrain following retroviral misexpression of *Hoxb1*

To test whether the activity of a single *Hox* gene is sufficient to induce the generation of ectopic motor neurons, chick embryos were infected between stages HH5 and HH8 (according to Hamburger and Hamilton, 1951) with an RCAS(BP)B retrovirus expressing mouse (m) *Hoxb1*. Subsequently, r1 was analysed for the presence of ectopic motor neurons using an anti-ISL-1/2 antiserum. r1 never normally expresses any *Hox* genes and the only endogenous motor neurons present in r1 are those of the trochlear nucleus, which lie in the most anterior part of r1, in the isthmus (Fig. 1A). Misexpression of *Hoxb1* led to the presence of ectopic motor neurons in infected areas of r1 when analysed 2 days after infection (HH17-19), which at these stages were found only in the ventral aspect of r1, immediately lateral to the floor plate (Fig. 1B). 4 days after infection (approximately HH25), the ectopic motor neurons had migrated from their place of birth in ventral r1 to a more lateral position within r1 (Fig. 1C,D), thereby manifesting a migration behaviour characteristic for hindbrain branchiomotor neurons (Fig. 1C) (Heaton and Moody, 1980; Covell and Noden, 1989; Simon et al., 1994) (see below). Ectopic motor neurons in r1 were only generated in infected areas of the neural tube as assessed by simultaneous detection of ISL-1/2 and m*Hoxb1* mRNA or mHoxb1 protein (see Fig. 2B and not shown). To control for possible non-specific viral effects, infection was performed with a control retrovirus RCAN/*Hoxb1*, which generates unspliced viral transcripts preventing translation of m*Hoxb1*. Following injection of RCAN/*Hoxb1,* ectopic motor neurons did not appear and no abnormalities of development were detected (not shown).

Axon pathways of ectopic motor neurons generated in r1 following *Hoxb1* misexpression

To study the axonal projections of the ectopic motor neurons in r1, infected embryos were collected at HH17-19 and stained with an anti-BEN antibody (Pourquié et al., 1990). With the exception of trigeminal motor neurons, which show only a very low level of BEN expression at both the mRNA and protein levels, BEN is strongly expressed on the cell surface of young

motor neurons (Guthrie and Lumsden, 1992; Simon et al., 1994). Ectopic motor neurons generated after misexpression of *Hoxb1* show a high level of BEN expression (compare left and right r1 in Fig. 2A,B; see below). Three main axonal projection pathways were taken by the ectopic motor neurons located in r1. (1) Axons of ectopic motor neurons grow laterally within r1 and then join the axons of endogenous trochlear motor neurons. The latter are located immediately anterior to the ectopic motor neurons in the ventral isthmus, project dorsally around the circumference of the isthmus and, after decussation, exit from the dorsal aspect of the brain (Fig. 2C,D). (2) Ectopic motor axons join the pathway of trigeminal motor neurons. Trigeminal cell bodies are situated caudal to the ectopic motor neurons, in r2 and r3, and exit the hindbrain via an exit point in lateral r2 (Fig. 2A,C,D). (3) Ectopic projections of fasciculated motor axons project dorsally within r1 without joining either of the flanking endogenous pathways, sometimes forming ectopic nerve exit points in the dorsolateral aspect of r1 (Fig. 2D).

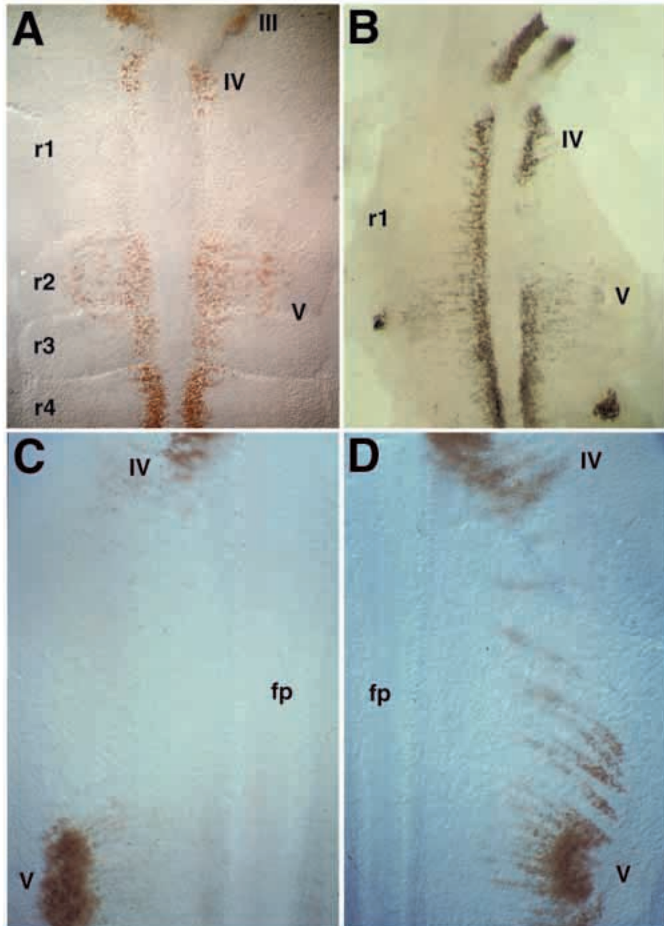

Fig. 1. Generation of ectopic motor neurons in r1 as assessed by detection of ISL1/2 following misexpression of *Hoxb1*. Flat-mount preparations of embryonic hindbrains. (A) Normal pattern of motor neuron generation in an HH18 embryo. Motor neurons are generated lateral to the floor plate and trigeminal branchiomotor neurons (V) already have started migrating laterally. Note that with the exception of trochlear motor neurons (IV) at the very rostral end of r1, r1 does not generate any motor neurons. (B) Misexpression of *Hoxb1* in left r1 leads to the generation of ectopic motor neuron throughout the entire ventral aspect of infected r1, as assessed 48 hours after infection (HH17-19). (C) At HH25, trigeminal branchiomotor neurons (V) in the hindbrain have migrated laterally towards their exit points, whereas trochlear somatomotor neurons (IV) remain close to the ventral midline. (D) 4 days after infection (HH25), ectopic motor neurons in r1 have migrated laterally, similarly to endogenous trigeminal branchiomotor neurons (V). r1-r4, rhombomeres1-4; fp, floor plate; III, IV and V, oculomotor, trochlear and trigeminal motor nuclei.

To analyse further the projection patterns of *Hoxb1*-induced motor neurons, we performed anterograde nerve tracing. Infected embryos were collected at approximately HH25 and the pathways of ectopic neurons present in the ventral aspect of r1 analysed by DiI labelling in the central region of ventral r1. In addition to joining trochlear (Fig. 2E) and trigeminal motor axon pathways (Fig. 2G), as already seen after detection of BEN, ectopic neurons in r1 were found that projected their axons posteriorly as far as r4 and entered the second branchial arch in conjunction with facial axons (Fig. 2F). Ectopic motor neurons in r1 exiting via the trigeminal exit point in r2 were also detected after retrograde tracing from the first branchial arch (Fig. 2H). Moreover, ectopic motor neurons in r1 were found to exit the hindbrain via dorsolateral exit points never normally present in r1 (Fig. 2G). These results therefore demonstrate that in addition to the induction of motor neurons exiting the hindbrain together with other cranial nerves, *Hoxb1* activity is also sufficient to instruct the generation of additional nerve exit points.

Misexpression of *Hoxb1* selectively induces the generation of branchiomotor neurons in r1

Depending on the target tissue they innervate, motor neurons in the hindbrain can be classified into three different subtypes: visceromotor, branchiomotor and somatomotor. We sought to determine whether misexpression of *Hoxb1* leads to the induction of a particular subtype of motor neuron or rather to the induction of a generic type of motor neuron. The migratory behaviour displayed by the ectopic motor neurons in r1 (Fig. 1D) already indicated that they might be of the branchiomotor subtype. To further test this at the molecular level, we made use of the observation that in the hindbrain only somatomotor neurons but not branchiomotor neurons express the LIM-homeobox gene *Isl2* (Varela-Echavarria et al., 1996). When analysed at HH25, somatic trochlear and abducens motor neurons were found to coexpress *Isl1* and *Isl2*, whereas branchiomotor neurons expressed *Isl1* but not *Isl2*, as previously reported (Fig. 3A,B). Ectopic motor neurons in r1 were never found to express *Isl2* in addition to *Isl1* (Fig. 3A,B), consistent with their being of the branchiomotor subtype. This result demonstrated, therefore, that the activity of *Hoxb1* was sufficient to selectively induce the generation of a distinct specified subtype of motor neurons.

Singular activity of *Hoxb1* is responsible for the generation of ectopic branchiomotor neurons in r1

Evidence exists for positive cross-regulatory interactions among *Hox* genes. The effects of misexpressing *Hoxb1* might thus be due to the induction of other *Hox* genes. In the case of *Hoxb1*, both *Hoxb2* and *Hoxb1* itself are known targets (Popperl et al., 1995; Maconochie et al., 1997). However, we never observed any induction of *Hoxb2* in

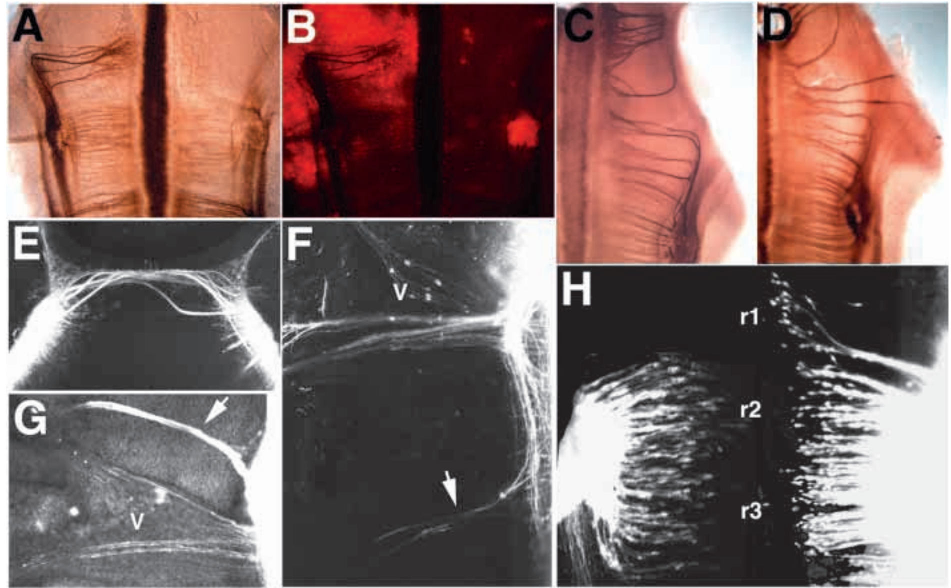

Fig. 2. Axonal pathways taken by ectopic motor neurons generated in r1 after misexpression of *Hoxb1*. Flat-mount preparations of embryonic hindbrains. (A-D) Detection of BEN; (B) Detection of mHoxb1; (E-G) Anterograde DiI labelling; H: Retrograde DiI tracing. (A) Ectopic motor neurons in r1 initially project dorsally within r1 and then turn posterior to join trigeminal motor neurons. (B) Same specimen as in A. Detection of mHoxb1 (red) demonstrates that ectopic motor neurons are only generated in infected areas of r1 misexpressing Hoxb1 (compare to A). (C) In this particular embryo, ectopic motor neurons in r1 join both trochlear and trigeminal motor neurons to exit the hindbrain. (D) In addition to joining the flanking trochlear and trigeminal motor neurons, ectopic motor neurons continue projecting dorsally within r1 to form structures reminiscent of ectopic nerve exit points. (E) After anterograde labelling from r1 of an HH25 embryo, ectopic neurons can be detected in r1 exiting in conjunction with trochlear motor neurons. Dorsal view onto the isthmic nerve crossing. (F) Ectopic motor neurons in r1 project along the hindbrain down until r4 and project into second branchial arch territory (arrow). Note that some trigeminal sensory neurons have been labelled (see labelled cell bodies in trigeminal ganglion). Anterograde labelling from r1 in an HH25 embryo. (G) Ectopic motor neurons in r1 exit the hindbrain via an ectopic exit point in dorsal r1 (arrow), as detected after anterograde labelling in an HH25 embryo. (H) After retrograde labelling from the first branchial arch, ectopic motor neurons can be detected in r1 exiting in conjunction with trigeminal motor neurons located in r2/r3 (right half of the figure). The left half of the figure shows a retrograde tracing of trigeminal motor neurons from the first branchial arch of a control embryo for comparison. No motor neurons can be detected in r1. r1-r3, rhombomeres1-3; V, trigeminal ganglion.

Hoxb1-infected embryos when assessed 2 days after infection (Fig. 4A,B). Moreover, using a chick-specific probe, we were also unable to detect any induction of chick *Hoxb1* by the retrovirally expressed *mHoxb1* (Fig. 4C,D). In addition, the expression of *Hoxa2* (which is not a target of *Hoxb1*) was unaffected by the misexpression of *Hoxb1* except for a few scattered cells in the lateral aspect of r1 observed in a few embryos which, however, never colocalised with the ectopic motor neurons (Fig. 4E,F). In addition, we did not observe any transient induction of *Hoxa2*, *Hoxb1* or *Hoxb2*, as assessed 24 hours after infection (data not shown). Therefore, the ectopic branchiomotor neurons found in r1 appear to be generated in response to the singular activity of *Hoxb1* itself rather than to the singular or combinatorial activities of other *Hox* genes.

Induction of ectopic branchiomotor neurons in r1 following misexpression of *Hoxa2*

To find out whether *Hox* genes other than *Hoxb1* might be sufficient to induce the generation of motor neurons in r1, we constructed a recombinant RCAS(BP)B retrovirus driving the expression of chick *Hoxa2*, which is the *Hox* gene expressed most anteriorly in the neural tube up to a rostral limit at the r1/r2 boundary. After infection of HH5-8 embryos and collection of the embryos 2 days later (HH17-19), we examined r1 for the presence of ectopic motor neurons by staining for ISL1/2. As in the case of *Hoxb1* misexpression, *Hoxa2* expression was also sufficient to confer on precursor cells in r1 the competence to form motor neurons, which were initially found in ventral r1 lateral to the floor plate (Fig. 5A).

Axonal projections of ectopic motor neurons in r1 following *Hoxa2* misexpression

Embryos infected with RCAS/*Hoxa2* were harvested 48 and 72 hours after infection and axonal pathways analysed by BEN immunostaining. The majority of ectopic motor neurons in r1 appeared to project dorsally within r1. In a few cases, we found ectopic motor axons joining the trochlear nerve, as observed after misexpression of *Hoxb1*. We were unable, however, to detect ectopic motor axons joining any of the other cranial nerves (Fig. 5B).

The projections of ectopic motor neurons generated after misexpression of *Hoxa2* were further investigated by retrograde and anterograde tracing from the first branchial arch and r1, respectively. We were unable to find any ectopic motor neurons in r1 exiting the hindbrain in conjunction with trigeminal motor neurons after retrograde labeling (not shown). Moreover, the anterograde labeling did not reveal any axonal projections from r1 exiting the hindbrain along with other cranial nerves nor via ectopic exit points. Occasionally, a few ectopic axons appeared to join the trochlear nerve (not shown).

Analysis of *LIM* and *Hox* expression patterns following misexpression of *Hoxa2*

To determine the subclass of motor neurons generated, we analysed the expression profiles of *Isl1* and *Isl2* and found that the ectopic motor neurons generated following *Hoxa2* misexpression expressed *Isl1* but not *Isl2*. (Fig. 5C,D). Therefore, as with *Hoxb1*, *Hoxa2* misexpression also resulted in the generation of motor neurons of branchiomotor subtype identity, as shown by their lateral migration behaviour and by their *Isl* gene expression patterns.

In order to see whether misexpression of *Hoxa2* would lead to the induction of other *Hox* genes, expression patterns of *Hoxb1* and *Hoxb2* were analysed 48 hours after infection. We did not find any changes in the expression patterns of the aforementioned genes (not shown).

Induction of rhombomere-specific motor neurons by *Hoxb1* and *Hoxa2* activity, respectively

Hox genes have been suggested to exert their principal influence within the anterior regions of their expression domains (see McGinnis and Krumlauf, 1992). In the case of *Hoxa2*, this embraces the area of the hindbrain (r2) giving rise to trigeminal branchiomotor neurons, whereas the anterior domain of *Hoxb1* expression encompasses the region of the hindbrain (r4) generating facial branchiomotor neurons (see Lumsden and Krumlauf, 1996). In order to find out whether *Hoxa2* and *Hoxb1* activities induced the generation of trigeminal-like or facial-like motor neurons, we analysed r1 of infected embryos for the expression of *BEN* mRNA. *BEN* is only very weakly expressed by trigeminal motor neurons, in contrast to the strong expression by other motor neurons (Guthrie and Lumsden, 1992; Simon et al., 1994; see also Fig. 6E). Misexpression of *Hoxa2* resulted in the generation of ectopic motor neurons with no or a very weak induction of *BEN* (Fig. 6A,B) (10/10 embryos), whereas the misexpression of *Hoxb1* led to a strong induction of *BEN* expression in ectopic motor neurons in r1 (Fig. 6C,D) (10/10 embryos). Infection with the control virus RCAN/*Hoxb1* did not result in any change in *BEN* expression (Fig. 6E). This result, therefore, suggests that at least to a certain extent, the individual activities of *Hoxa2* and *Hoxb1* are sufficient to impart on the neural tissue the competence to generate rhombomere-specific branchiomotor neurons of trigeminal or facial type, respectively. This is consistent with the idea of anterior domains of expression as main sites of *Hox* gene activity and suggests a direct role for *Hox* genes in determining the specific AP character of hindbrain motor neurons.

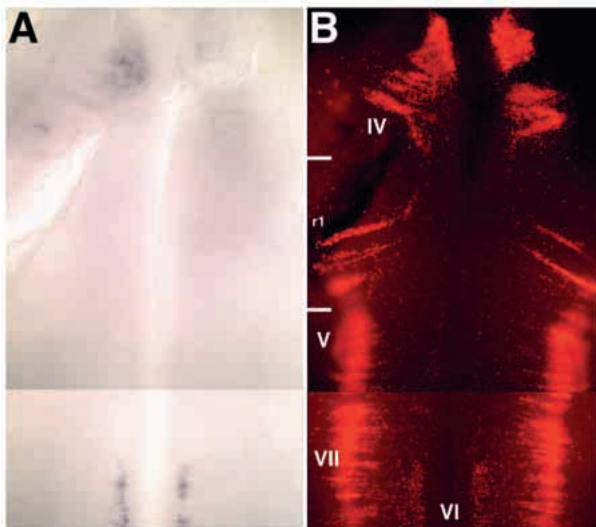

Fig. 3. Ectopic motor neurons in r1 generated after misexpression of *Hoxb1* are of branchiomotor specificity, as assessed by their expression of *Isl1* but not *Isl2*. Flat-mount preparation of HH25 hindbrain. (A) *Isl2* expression by somatomotor trochlear (IV) and abducens (VI) neurons but not by ectopic or branchiomotor neurons (V, VII; compare with B). (B) Same specimen as in A; detection of all motor neurons including ectopic ones in r1 by ISL1/2 immunostaining. IV, V, VI and VII, trochlear, trigeminal, abducens and facial motor neurons.

DISCUSSION

By misexpressing individual *Hox* genes, *Hoxb1* and *Hoxa2*, outside (i.e. anterior to) the endogenous *Hox* expression domain in vivo, we have demonstrated that *Hox* genes are capable of conferring on neural precursor cells the competence to generate distinct cell types that are normally formed in the anterior expression domains of specific *Hox* genes, i.e. trigeminal motor neurons in the case of *Hoxa2*, and facial

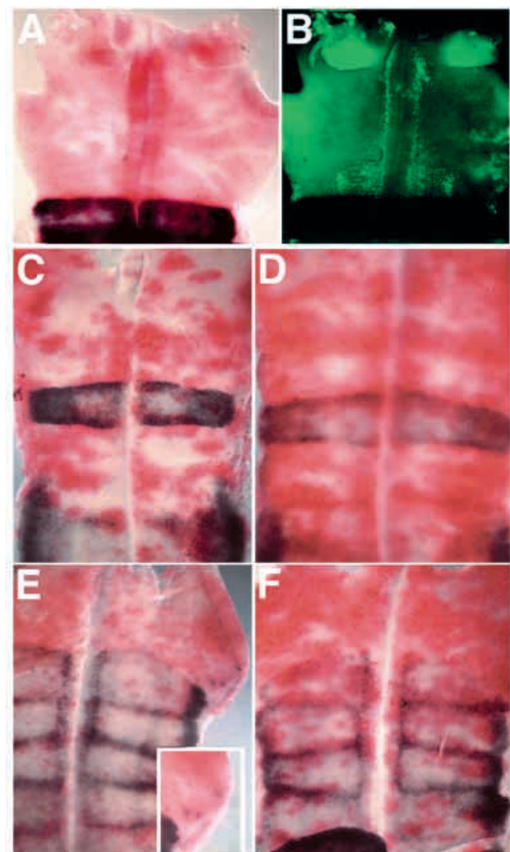

Fig. 4. Lack of cross-regulation of *Hox* gene expression following misexpression of *Hoxb1*. Flat-mount preparations of HH17-19 embryonic hindbrains. (A) Misexpression of *Hoxb1* (red) does not lead to an induction of *Hoxb2* (blue), which has its normal limit of expression at the r2/r3 boundary. (B) Same specimen as in A, stained for motor neurons (ISL1/2, green), demonstrating the generation of ectopic motor neurons in r1 without induction of *Hoxb2* (see A). (C,D) Misexpression of *Hoxb1* (mouse, red) does not lead to an auto-upregulation of endogenous chick *Hoxb1* (blue). Endogenous expression is limited to r4, dorsal r6, and caudal to r6. (E,F) Misexpression of *Hoxb1* (red) does not have any major effect on endogenous *Hoxa2* expression (blue). Only in a very small percentage of infected embryos a few cells in very dorsal r1 could be detected ectopically expressing *Hoxa2* (E, inset). These cells did not overlap with ectopic motor neurons (not shown).

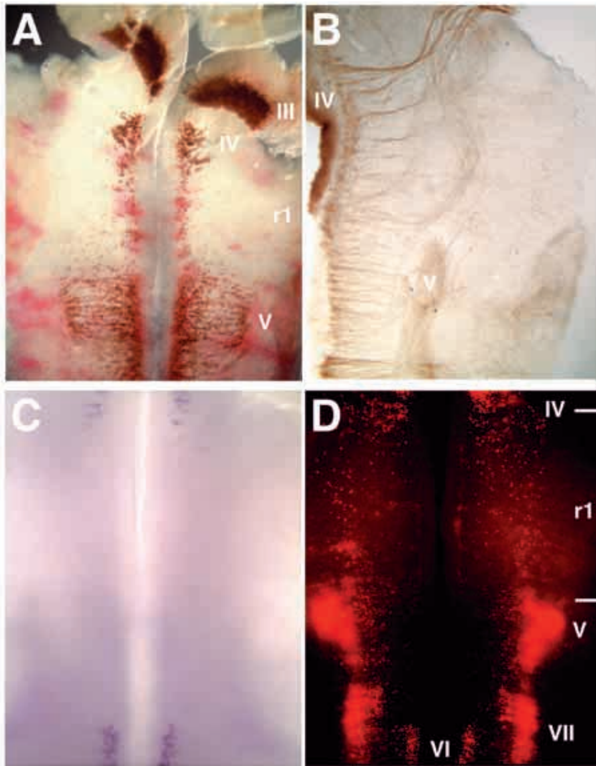

Fig. 5. Generation of ectopic branchiomotor neurons following misexpression of *Hoxa2*. Flat-mount preparation of embryonic hindbrains. (A) Misexpression of *Hoxa2* (red) leads to the generation of ectopic motor neurons in r1 (brown, anti-ISL1/2). Note that ectopic motor neurons are only found in infected areas of r1 (E3 embryo). (B) Immunostaining for BEN 3 days after misexpression of *Hoxa2* (E4 embryo) shows that the majority of ectopic motor neurons in r1 projects dorsally within r1. Some ectopic motor neurons in anterior r1 appear to join the flanking trochlear nerve. Floor plate is to the left, dorsal to the right. (C) Misexpression of *Hoxa2* does not lead to an induction of *Isl2* expression in ectopic motor neurons (compare with D) (E5 embryo). Expression is only found in trochlear and abducens somatomotor neurons (IV, VI). (D) Same specimen as in C, detection of all motor neurons by staining for ISL1/2 (red). Compare with C for *Isl-2* expression. r1, rhombomere1; III, IV, V, VI and VII, oculomotor, trochlear, trigeminal, abducens and facial motor nuclei.

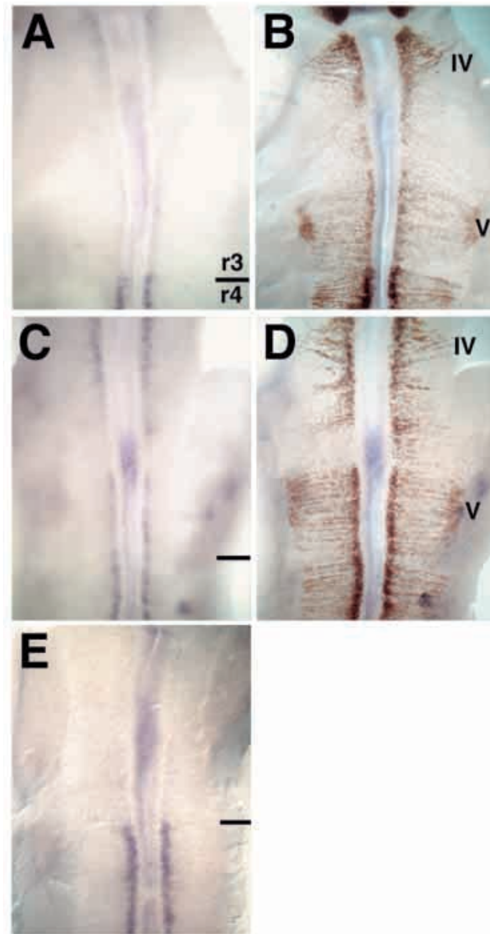

Fig. 6. Ectopic branchiomotor neurons generated following misexpression of *Hoxa2* and *Hoxb1* are trigeminal-like and facial-like, respectively. Flat-mount preparations of embryonic hindbrains. (A,B) Ectopic motor neurons (B, anti-ISL1/2) generated following misexpression of *Hoxa2* only show a very low level of *BEN* expression (A). Strong endogenous expression of *BEN* is only found posterior to r3 (E3 embryo). (C,D) Ectopic motor neurons (D, anti-ISL-1/2) generated following misexpression of *Hoxb1* show a high expression level of *BEN* (C) similar to the endogenous level found posterior to r3 (E3 embryo). (E) Infection of embryos with a control retrovirus RCAN/*Hoxb1* does not lead to any changes in *BEN* expression (E4 embryo). The r3/r4 boundary is labeled by a black bar in A, C and E. Note that in control-infected (E) and *Hoxa2*-infected (A) embryos, *BEN* is strongly expressed only by motor neurons caudal to r3, whereas trigeminal motor neurons in r2/r3 only show a very faint expression of *BEN*. IV and V, trochlear and trigeminal motor nuclei.

motor neurons in the case of *Hoxb1*. This shows that, at least to a certain extent, the activity of a single *Hox* gene is sufficient to determine the positional value of neural tissue and demonstrates that a single *Hox* gene is sufficient to specify the generation of neurons with a distinct subtype. Our data do not, however, rule out the possibility that for cell types other than motor neurons, or under different circumstances, a combinatorial mode of *Hox* gene action, for example, might be operative (see Krumlauf, 1993). Moreover, our data are consistent with the concept of posterior dominance (Duboule, 1991; Duboule and Morata, 1994; see McGinnis and Krumlauf, 1992, for a discussion), whereby the major site of influence of a specific *Hox* gene is in the anterior reaches of its expression domain, implying that the identities of cells at a particular axial level are determined by the activity of the most 5′ *Hox* gene expressed at that axial level.

Following the misexpression of *Hoxa2* and *Hoxb1*, ectopic motor neurons could be detected throughout the entire extent of ventral r1 (Figs 1B, 5A). Cell types in the ventral neural tube have been shown to be induced by the signaling molecule sonic hedgehog, which is initially expressed by the notochord and acts on the overlying neural plate (Tanabe and Jessell, 1996). The particular subtype of ventral neural-tube derived neurons generated in response to sonic hedgehog (e.g. dopaminergic and motor neurons in the midbrain, motor neurons caudal to r1 but not in r1) is dependent on the particular anteroposterior level of the neural axis, whose specification in turn is determined by the patterning genes (e.g. *Hox* genes, *Pax* genes)

expressed at a given level (Simon et al., 1995; Lumsden and Krumlauf, 1996). Thus, our data demonstrate that single *Hox* genes are sufficient to confer on precursor cells in r1 the competence to respond to ventralising signals with the generation of specific motor neuron subtypes not normally found in this region. Although ectopic on the anteroposterior axis, motor neuron progenitors are induced at the correct position on the dorsoventral axis of the neural tube, immediately lateral to the floor plate (Tanabe and Jessell, 1996). Following their birth, the ectopic motor neurons then migrate dorsally within the neural tube (Figs 1D, 5D), a behaviour typical for branchiomotor but not for somatomotor neurons (Heaton and Moody, 1980; Covell and Noden, 1989; Simon et al., 1994). The branchiomotor specificity of the ectopic motor neurons in r1 could further be demonstrated by assessing the expression of the *LIM* genes *Isl-1* and *Isl-2* (Figs 3, 5C,D) whose expression profiles enable the distinction to be made between the different types of motor neurons found in the hindbrain (Varela-Echavarria et al., 1996). In terms of their axonal projections, the ectopic motor neurons generated after misexpression of *Hoxb1* not only exited the neural tube in conjunction with flanking cranial nerves, but also via novel exit points generated in r1. In the case of *Hoxa2* misexpression, the majority of the ectopic motor neurons appeared not to exit the neural tube.

Next, we tried to determine whether misexpression of *Hoxa2* and *Hoxb1* might be sufficient to induce the generation of that particular type of branchiomotor neuron normally found in their most anterior expression domain, trigeminal and facial motor neurons, respectively. Although there are no markers known unequivocally to identify different branchiomotor nuclei, the observed gene expression profiles of *Isl-1*+/*Isl-2*−/*BEN*weak following *Hoxa2* misexpression (Figs 5, 6) and *Isl-1*+/*Isl-2*−/*BEN*strong following *Hoxb1* misexpression (Figs 3, 6) mirror those, respectively, of endogenous trigeminal and facial motor neurons (Guthrie and Lumsden, 1992; Simon et al., 1994; Varela-Echavarria et al., 1996). This therefore suggests that misexpression of *Hoxa2* indeed leads to the generation of trigeminal-like branchiomotor neurons and that of *Hoxb1* to the generation of facial-like branchiomotor neurons. The activities of *Hoxa2* and *Hoxb1*, therefore, are sufficient to assign AP position-specific neuronal subtypes. In the case of misexpressing *Hoxa2*, the axonal projection behaviour displayed by the ectopic motor neurons suggests that additional signals might be required for their further differentiation.

Analysis of *Hoxb1*−/− embryos has shown that even in the absence of *Hoxb1,* motor neurons are generated in r4. These, however, fail to migrate posteriorly to their proper position (within r6) and later die (Goddard et al., 1996; Studer et al., 1996). Using r2-specific reporter lines and gene markers it has been shown that in *Hoxb1*−/− embryos r4 appears to adopt an r2-like identity (Studer et al., 1996). Moreover, misexpression of *Hoxb1* in r2 (normally only expressing *Hoxa2*) has been shown to reassign axonal projections of r2 motor neurons from trigeminal (first arch) specificity to facial (second arch) specificity (E. Bell, R. Wingate and A. Lumsden, unpublished). We now show that the singular activity of *Hoxb1* rather than a cooperative action of several *Hox* genes is sufficient and responsible for the induction of facial-like branchiomotor neurons. Considering the remaining *Hox* genes being expressed in r4 in the absence of *Hoxb1*, *Hoxa1*, *Hoxb2* (at a reduced expression level; Maconochie et al., 1997) and *Hoxa2*, it is most likely that the motor neurons generated in r4 in *Hoxb1*−/− embryos represent trigeminal-like motor neurons rather than facial motor neurons; this would be consistent with the absence of the posterior migration behaviour characteristic for facial branchiomotor neurons (Goddard et al., 1996; Studer et al., 1996; McKay et al., 1997) (see below). As for endogenous trigeminal motor neurons in r2, the motor neurons found in *Hoxb1*−/− embryos in r4 simply migrate laterally within their rhombomere of origin to condense in proximity to their dorsolateral exit point. The subsequent early death of r4 motor neurons in *Hoxb1*−/− embryos may result from the failure of these ectopic trigeminal-like motor neurons to encounter and innervate appropriate target tissues in the second branchial arch rather than first arch derivatives appropriate for trigeminal motor neurons, perhaps due to absence or insufficiency of correct target-derived survival factors.

In the case of *Hoxa2*, it has been reported that despite the absence of *Hoxa2*, trigeminal motor neurons are generated in r2 (no other *Hox* gene present) and r3 (expressing *Hoxb2*), which display defects in pathfinding behaviour, partly exiting the hindbrain from inappropriate exit points as assessed by nerve tracing (Gavalas et al., 1997). These findings might therefore suggest that *Hoxa2* is not required to instruct motor neuron generation. Our data, however, demonstrate that *Hoxa2* is sufficient to induce the generation of trigeminal-like motor neurons.

In conclusion, the specification of trigeminal and facial motor neurons by *Hoxa2* and *Hoxb1* activities, respectively, demonstrates that at least to a certain extent and for certain cell types the singular activities of individual *Hox* genes are sufficient to instruct the generation of distinct cell types.

We thank the following for kindly providing plasmids or antibodies: V. Prince (chick *Hoxb1*); R. Krumlauf (mouse *Hoxb1*); A. Kuroiwa (chick *Hoxb2*); T. Jessell (chick *Isl2*); O. Pourquie (chick *BEN*); T. Edlund (anti-ISL1/2); N. Manley and M. Capecchi (anti-mouse-Hoxb1); C. Cepko and D. Fekete (Slax12); S. Hughes (RCAS(BP)B); J. Gilthorpe (RCAN(BP)B). A. Graham read an early version of this paper. S. Jungbluth was supported by an EC fellowship. The Wellcome Trust and the MRC supported the work.

REFERENCES

Alexandre, D., Clarke, J. D. W., Oxtoby, E., Yan, Y.-L., Jowett, T. and Holder, N. (1996). Ectopic expression of Hoxa-1 in the zebrafish alters the fate of the mandibular arch neural crest and phenocopies a retinoic acid-induced phenotype. *Development* **122**, 735-746.

Carpenter, E. M., Goddard, J. M., Chisaka, O., Manley, N. R. and Capecchi, M. R. (1993). Loss of *Hoxa-1* (*Hox-1.6*) function results in the reorganization of the murine hindbrain. *Development* **118**, 1063-1075.

Chisaka, O., Musci, T. and Capecchi, M. (1992). Developmental defects of the ear, cranial nerves and hindbrain resulting from targeted disruption of the mouse homeobox gene *Hox-1.6*. *Nature* **355**, 516-520.

Clarke, J. D. W. and Lumsden, A. (1993). Segmental repetition of neuronal phenotype sets in the chick embryo hindbrain. *Development* **118**, 151-162.

Covell, D. A. and Noden, D. M. (1989). Embryonic development of the chick primary trigeminal sensory-motor complex. *J. Comp. Neurol.* **286**, 488-503.

Dollé, P., Lufkin, T., Krumlauf, R., Mark, M., Duboule, D. and Chambon, P. (1993). Local alterations of *Krox-20* and Hox gene expression in the hindbrain of *Hoxa-1* (*Hox-1.6*) homozygote null mutant embryos. *Proc. Natl. Acad. Sci. USA* **90**, 7666-7670.

Duboule, D. (1991). Patterning the vertebrate limb. *Curr. Opin. Genet. Dev.* **1**, 211-216.
Duboule, D. and Morata, G. (1994). Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet.* **10**, 358-364.
Gavalas, A., Davenne, M., Lumsden, A., Chambon, P. and Rijli, F. M. (1997). Role of *Hoxa2* in axon pathfinding and rostral hindbrain patterning. *Development* **124**, 3693-3702.
Gavalas, A., Studer, M., Lumsden, A., Rijli, F. M., Krumlauf, R. and Chambon, R. (1998). *Hoxa1* and *Hoxb1* synergize in patterning the hindbrain, cranial nerves and second pharyngeal arch. *Development* **125**, 1123-1136.
Goddard, J. M., Rossel, M., Manley, N. R. and Capecchi, M. R. (1996). Mice with targeted disruption of *Hoxb1* fail to form the motor nucleus of the VIIth nerve. *Development* **122**, 3217-3228.
Guthrie, S. and Lumsden, A. (1992). Motor neuron pathfinding following rhombomere reversal in the chick embryo hindbrain. *Development* **114**, 663-673.
Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. morph.* **88**, 49-92.
Heaton, M. B. and Moody, S. A. (1980). Early development and migration of the trigeminal motor nucleus in the chick embryo. *J. Comp. Neurol.* **189**, 61-99.
Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J. and Sutrave, P. (1987). Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* **61**, 3004-3012.
Krumlauf, R. (1993). *Hox* genes and pattern formation in the branchial region of the vertebrate head. *Trends Genet.* **9**, 106-111.
Lufkin, T., Dierich, A., LeMeur, M., Mark, M. and Chambon, P. (1991). Disruption of the *Hox-1.6* homeobox gene results in defects in a region corresponding to its rostral domain of expression. *Cell* **66**, 1105-1119.
Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-1115.
Maconochie, M. K., Nonchev, S., Studer, M., Chan, S.-K., Popperl, H., Sham, M. H., Mann, R. S. and Krumlauf, R. (1997). Cross-regulation in the mouse *HoxB* complex: the expression of *Hoxb2* in rhombomere 4 is regulated by *Hoxb1*. *Genes Dev.* **11**, 1885-1895.
Mark, M., Lufkin, T., Vonesc, J.-L., Ruberte, E., Olivo, J.-C., Dollé, P., Gorry, P., Lumsden, A. and Chambon, P. (1993). Two rhombomeres are altered in *Hoxa-1* mutant mice. *Development* **119**, 319-338.
McGinnis, W. and Krumlauf, R. (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.
McKay, I., Lewis, J. and Lumsden, A. (1997). Organization and development of facial motor neurons in the *kreisler* mutant mouse. *Eur. J. Neurosci.* **9**, 1499-1506.
Morgan, B. M. and Fekete, D. M. (1996). Manipulating gene expression with replication-competent retroviruses. In *Methods in Avian Embryology* (ed. M. Bronner-Fraser), pp. 185-218. Academic Press Inc., San Diego.
Popperl, H., Blenz, M., Studer, M., Chan, S.-K., Aparicio, S., Brenner, S., Mann, R. S. and Krumlauf, R. (1995). Segmental expression of *Hoxb1* is controlled by a highly conserved autoregulatory loop dependent upon *exd/pbx*. *Cell* **81**, 1031-1042.
Pourquie, O., Coltey, M., Thomas, J. L. and LeDouarin, N. (1990). A widely distributed antigen developmentally regulated in the nervous system. *Development* **109**, 743-752.
Prince, V. and Lumsden, A. (1994). *Hoxa2* expression in normal and transposed rhombomeres: independent regulation in the neural tube and neural crest. *Development* **120**, 911-923.
Simon, H., Guthrie, S. and Lumsden, A. (1994). Regulation of SC1/DM-GRASP during the migration of motor neurons in the chick embryo brain stem. *J. Neurobiol.* **25**, 1129-1143.
Simon, H., Hornbruch, A. and Lumsden, A. (1995). Independent assignment of anteroposterior and dorsoventral positional values in the developing chick hindbrain. *Curr. Biol.* **5**, 205-214.
Studer, M., Lumsden, A., Ariza-McNaughton, Bradley, A. and Krumlauf, R. (1996). Altered segmental identity and abnormal migration of motor neurons in mice lacking *Hoxb1*. *Nature* **384**, 630-634.
Tanabe, Y. and Jessell, T. M. (1996). Diversity and pattern in the developing spinal cord. *Science* **274**, 1115-1123.
Thor, S., Ericson, J., Brannstrom, T. and Edlund, T. (1991). The homeodomain LIM protein Isl-1 is expressed in subsets of neurons and endocrine cells in the adult rat. *Neuron* **7**, 881-889.
Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and Pfaff, S. L. (1994). Topographic organisation of embryonic motor neurons defined by expression of LIM homeobox genes. *Cell* **79**, 957-970.
Varela-Echavarria, A., Pfaff, S. L. and Guthrie, S. (1996). Differential expression of LIM homeobox genes among motor neuron subpopulations in the developing chick brain stem. *Mol. Cell. Neurosci.* **8**, 242-257.
Wilkinson, D. G. (1992). Whole-mount in situ hybridisation of vertebrate embryos. In *In Situ Hybridisation, A Practical Approach* (ed. D. G. Wilkinson), pp. 1-83. Oxford, IRL.
Zhang, M., Kim, H.-J., Marshall, H., Gendron-Maguire, M., Lucas, D. A., Baron, A., Gudas, L. J., Gridley, T., Krumlauf, R. and Grippo, J. F. (1994). Ectopic *Hoxa-1* induces rhombomere transformation in mouse hindbrain. *Development* **120**, 2431-24.