INTRODUCTION

Formation of the central nervous system (CNS) in vertebrates such as *Xenopus* is initiated during gastrulation and largely depends on the inductive interaction between the ectoderm and adjacent dorsal mesoderm (Spemann organizer). The CNS is characterized by distinct anteroposterior (AP) and dorsoventral patterning (review by Hamburger, 1988; Doniach, 1993). The predominant concept of how AP patterning is established was suggested by Nieuwkoop (Nieuwkoop, 1952). In this two-step model, the initial neural inducing signal is thought to specify anterior neuroectodermal structures, such as cement gland and forebrain; this first step is referred to as ‘activation’. The second caudalizing step is called ‘transformation.’ During this second step, anterior neural tissue is respecified to more posterior fates, such as midbrain, hindbrain and spinal cord. Several molecules have been identified which participate in the ‘activation’ and ‘transformation’ processes. Non-neural ectoderm is induced to anterior-neural tissue by inhibition of bone morphogenetic protein (BMP) activity (reviewed by Harland and Gerhart, 1997). Secreted BMP antagonist molecules bind the BMP molecule and inhibit its receptor binding activity (Zimmerman et al., 1996; Piccolo et al., 1996; Fainsod et al., 1997; Hsu et al., 1998). BMP antagonists are expressed in Spemann’s organizer during gastrulation and induce anterior neural tissue in adjacent ectoderm (Lamb et al., 1994; Hemmati-Brivanlou and Melton, 1994; Sasai et al., 1994).

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Three secreted ‘transformation’ factors have been shown to caudalize neural tissue in whole embryos or explants: retinoic acid (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991; Sharpe, 1991; Kolm and Sive, 1995; Papalopulu, and Kintner, 1996; Papalopulu, and Kintner, 1996; Kolm and Sive, 1995; Papalopulu, and Kintner, 1996; Papalopulu, and Kintner, 1996) basic fibroblast growth factor (bFGF; Kenkgaku and Okamato, 1995; Lamb and Harland, 1995; Cox and Hemmati-Brivanlou, 1995) and Xwnt3a (McGrew et al., 1995; McGrew et al., 1997). These factors and/or their receptors are expressed in the neural plate in a temporal and regional manner, supporting their roles as caudalizers of the nervous system. All three of these molecules caudalize in non-equivalent manners and it is still not clear how they interact to specify proper AP pattern in the CNS (Kolm et al., 1997; rev. in Gamse and Sive, 2000).

In *Xenopus* embryos and explants, Meis family homeobox proteins have been shown to regulate cell fate specification in vertebrate and invertebrate embryos. Ectopic expression of RNA encoding the *Xenopus* Meis3 (XMeis3) protein caused anterior neural truncations with a concomitant expansion of hindbrain and spinal cord markers in *Xenopus* embryos. In naïve animal cap explants, *XMeis3* activated expression of posterior neural markers in the absence of pan-neural markers. Supporting its role as a neural caudalizer, *XMeis3* is expressed in the hindbrain and spinal cord. We show that XMeis3 acts like a transcriptional activator, and its caudalizing effects can be mimicked by injecting RNA encoding a VP16-XMeis3 fusion protein. To address the role of endogenous XMeis3 protein in neural patterning, XMeis3 activity was antagonized by injecting RNA encoding an Engrailed-XMeis3 antimorph fusion protein or XMeis3 antisense morpholino oligonucleotides. In these embryos, anterior neural structures were expanded and posterior neural tissues from the midbrain-hindbrain junction through the hindbrain were perturbed. In neuralized animal cap explants, XMeis3-antimorph protein modified caudalization by basic fibroblast growth factor and Wnt3a. XMeis3-antimorph protein did not inhibit caudalization per se, but re-directed posterior neural marker expression to more anterior levels; it reduced expression of spinal cord and hindbrain markers, yet increased expression of the more rostral En2 marker. These results provide evidence that XMeis3 protein in the hindbrain is required to modify anterior neural-inducing activity, thus, enabling the transformation of these cells to posterior fates.

Key words: *Xenopus laevis*, XMeis3, Antimorph, Antisense morpholino oligonucleotides, Caudalization, Hindbrain
expressed in the hindbrain from rhombomere 2 (r2) to rhombomere 4 (r4), and in the anterior spinal cord (Salzberg et al., 1999). Ectopic XMeis3 expression in embryos causes anterior truncations, with a loss of anterior neural tissues from the cement gland/forebrain until the midbrain-hindbrain junction. In parallel, hindbrain and spinal cord cell types are expanded in embryos that overexpress XMeis3. Expression of pan-neural markers is unaltered by ectopic XMeis3 expression.

In neuralized animal cap explants, ectopic XMeis3 expression inhibits anterior neural induction by BMP antagonists such as noggin or the BMP2/4 dominant-negative (DN) receptor; however, XMeis3 does not inhibit the ability of these BMP antagonists to induce pan-neural markers (Salzberg et al., 1999). Strikingly, in naïve animal cap ectoderm, ectopic XMeis3 expression induces transcriptional activation of hindbrain and spinal cord neural markers, albeit in the absence of pan-neural marker expression (Salzberg et al., 1999). This effect is ectoderm-specific, as XMeis3 does not activate transcription of mesodermal markers in injected animal cap explants (Salzberg et al., 1999). Thus, the XMeis3 protein ‘uncouples’ neural caudalization from neural induction.

To further examine the role of XMeis3 protein in Xenopus neural development, two distinct strategies have been used to inhibit endogenous XMeis3 protein activity. In the first strategy, fusions of a XMeis3 open reading frame to either the Engrailed transcriptional repressor domain or the VP16 transcriptional activation domain were compared in embryos and explants. We found that the Eng-XMeis3 fusion protein acted as an antimorph, blocking the effects of wild-type XMeis3-encoding RNA in Xenopus embryos and explants, while the VP16-XMeis3 fusion protein acted as a transcriptional activator to caudalize embryos and explants. In embryos, ectopic XMeis3-antimorph (XMeis3-AM) protein expression caused a loss of hindbrain marker expression, with a concomitant posterior expansion of anterior neural markers into the hindbrain region. Spinal cord and pan-neural marker expression was unaltered by the XMeis3-AM protein. In a second experimental approach, inhibition of XMeis3 mRNA translation by injection of XMeis3 antisense morpholino oligonucleotides (AMOs) also disrupted Xenopus hindbrain formation.

In animal cap explants caudalized by bFGF or Wnt3a, antagonism of XMeis3 protein activity did not specifically inhibit caudalizer activity, but it did rostralize the AP extent of posterior neural marker expression. XMeis3 activity is probably required for cells to overcome anterior neural signaling, thus enabling proper hindbrain cell fate identity in the developing Xenopus CNS.

**MATERIALS AND METHODS**

**Construction of XMeis3-antimorph constructs**

Two sets of Eng-XMeis3 and VP-XMeis3 fusion proteins were constructed. In the first set, PFU DNA polymerase (Promega) generated full-length fragments (amino acids 1-1385) that were cloned in frame 3’ to the VP16 or Engrailed domains in the pcS2 vector (Kessler, 1995). In the second set of constructs, the region spanning amino acids 1-333 was cloned in the same manner. The latter two constructs contain the Xenus homology-box and homeodomain regions, but lack all of the region C-terminal to the homeodomain. All four of these constructs were subcloned into the pSP64T+globin vector. Both sets of Eng-XMeis3 constructs acted as antimorphs; the shorter version was more effective and was used in the shown experiments. Both sets of VP-XMeis3 constructs acted as caudalizers; the longer version was more effective and was used in the shown experiments.

**Xenopus embryos, explants and inducing factors**

Ovalution, in vitro fertilization, embryo culture and dissections were carried out as described by Re’em-Kalma et al. (Re’em-Kalma et al., 1995). Embryos were staged according to Nieuwoop and Faber (Nieuwoop and Faber, 1967). Xenopus bFGF (XbFGF) treated (50 ng/ml) animal cap explants were cultured as described by Lamb and Harland (Lamb and Harland, 1995).

**RNA injections**

Different concentrations of capped sense in vitro transcribed full-length XMeis3 (Salzberg et al., 1999), Eng-XMeis3 and VP16-XMeis3 (0.1-1.8 ng in a volume of 5-10 nl) were injected into the animal hemisphere of embryos at the one or two-cell stages. Capped in vitro transcribed Xenopus noggin RNA (200 pg) and mouse Wnt3a RNA (100 pg) were injected into the animal hemisphere of embryos at the one-cell stage (Smith and Harland, 1992; Baker et al., 1999).

**Injection of antisense morpholino oligonucleotides (AMOs)**

Antisense morpholino oligonucleotides (AMOs) complementing the 5’ region of the XMeis3 mRNA were designed by and purchased from Gene Tools, LLC; Corvallis, OR (www@gene-tools.com; Heasman et al., 2000; Nasevicius and Ekker, 2000). The XMeis3 AMO sequence is 5’-ATACCTTTGTGCCATTCCGAGTTGG-3’. A standard control morpholino oligonucleotide (CMO) was also used in each experiment (Gene Tools). AMOs and CMOs were dissolved at 2 mg/ml in sterile water. One-cell embryos were routinely injected in the 10-20 ng range in 5-10 nl volumes. In two-cell stage embryos, one blastomere was injected with 7.5 ng in a 5 nl volume. The AMO was toxic at levels above 30 ng/embryo and experiments were performed at significantly lower concentrations.

**In situ hybridization**

Whole-mount in situ hybridization was carried out with digoxigenin-labeled probes, as described previously (Hemmati-BriVanlou et al., 1990; Harland, 1991; Knecht et al., 1995). Double in situ hybridization experiments were performed with probes generated from fluorescein and digoxigenin RNA-labeling mixes (Roche). Embryos were stained with BM purple and Fast Red substrates (Roche; Hollemann et al., 1998). In some cases, both probes were stained with BM purple. Two-cell stage albino embryos were injected unilaterally into the animal hemisphere of one-cell with 50-100 pg of RNA encoding the XMeis3-AM protein or 6-7.5 ng of the AMO. Embryos were cultured until late neurula stages and subsequently fixed for in situ hybridization. The uninjected side served as an internal control in all experiments. For lineage tracing analysis, 50 pg of RNA encoding the β-galactosidase protein (β-gal; Smith and Harland, 1991) and RNA encoding the XMeis3-AM protein were co-injected unilaterally at the two-cell stage. Embryos were stained in red for β-gal activity and fixed for whole-mount in situ hybridization as described previously (Bonstein et al., 1998). The perturbations seen in the embryos were always seen on the red stained XAG1 primers and XMeis3-AM or AMO injected side (data not shown).

**RT-PCR analysis**

RT-PCR was performed as described previously (Wilson and Melton, 1994), except that random hexamers (100 ng/reaction) were used for reverse transcription. Primers for EFlα, En2, Krox20 and HoxB9 have been described elsewhere (Hemmati-BriVanlou and Melton, 1994). The primers for HoxD1 and RARα2 have been described by Kolm et al. (Kolm et al., 1997). The otx2 and XAG1 primers are described...
RESULTS

ENG-XMeis3 protein antagonizes XMeis3 caudalizing activity in contrast to VP16-XMeis3 protein

To inhibit XMeis3 protein activity during early development, we constructed an XMeis3 antimorph protein by fusing two different XMeis3 open reading frames (see Materials and Methods) to either the Engrailed (ENG) transcriptional repressor domain or the VP16 transactivation domain (Fig. 1). In a relatively simple animal cap explant assay, antimorph candidate RNA molecules were screened by coinjection with wild-type XMeis3-encoding RNA (Fig. 2A-B). XMeis3 mRNA activates expression of a panel of posterior neural markers in animal cap ectoderm (Salzberg et al., 1999); thus, any bona fide antimorph-encoding RNA should antagonize XMeis3 caudalizing activity in contrast to VP16-XMeis3.

Co-injection of RNA encoding the ENG-XMeis3 chimera protein inhibited wild type XMeis3 caudalization activity in animal caps (Fig. 2A). As expected, XMeis3 activated Krox20, HoxD1, and HoxB9 expression in animal caps (Fig. 2A, lane 4), whereas injection of XMeis3-AM alone did not transcriptionally activate these markers (Fig. 2A, lane 5). However, in the XMeis3/XMeis3-AM co-injected group, posterior neural marker expression was eliminated (Fig. 2A, compare lane 4 with lane 6). By contrast, injection of RNA encoding the VP16-XMeis3 chimera protein induced posterior neural marker transcription in animal cap explants (Fig. 2B, compare lane 4 with lane 3). Ectopic VP16-XMeis3 expression caused anterior truncations in whole embryos (Fig. 2C); this is the same phenotype observed in embryos ectopically expressing wild-type XMeis3 RNA (Salzberg et al., 1999). These results suggest that XMeis3 protein may act as a transcriptional activator in the embryo to induce posterior neural gene expression.

Ectopic XMeis3-antimorph (XMeis3-AM) expression eliminates the hindbrain region and expands anterior neural tissues

To address the role of endogenous XMeis3 protein in early development, we injected 400-800 pg of in vitro synthesized XMeis3 antimorph (XMeis3-AM) -encoding RNA into the animal hemisphere of one-cell Xenopus embryos. Embryos were scored at tailbud to tadpole stages for phenotypes (Fig. 2D). Overexpression of XMeis3-AM RNA caused anterior expansions in over 80% (n=40/48) of the injected embryos in the shown experiment. In comparison with control embryos, these embryos had enlarged cement glands and a shortened body axis (Fig. 2D).

To further examine the effects of ectopic XMeis3-AM expression on spatial expression of neural markers, whole-mount in situ hybridization was performed on XMeis3-AM-injected late neurula stage embryos. Embryos were unilaterally injected (50-100 pg of XMeis3-AM RNA) into the animal hemisphere of one blastomere at the two-cell stage. Complementing the observation of cement gland expansion (Fig. 2D), in nearly 60% of XMeis3-AM injected embryos (n=33/56), expression of the forebrain/midbrain-specific otx2 (Blitz and Cho, 1995) and forebrain-specific cpl-1 (Knecht et al., 1995) markers was posteriorly expanded (Fig. 3A-D). As seen in the double in situ with Krox20 or En2, otx2 expression was dramatically expanded into the hindbrain region (Fig. 3B-C). In the most extreme phenotypes, otx2 expression extended elsewhere (Knecht et al., 1995). The XE10 primers are described elsewhere (Weinstein et al., 1996). The HoxB3 primers have been described by Hooiveld et al. (Hooiveld et al., 1999).

Western blot analysis

Western blot analysis was performed as described (Henig et al., 1998). For constructing the XMeis3-Myc vector, a full-length XMeis3 PFU generated fragment was subcloned 5' to the Myc fusion site in the pCS2+MT vector. XMeis3-Myc RNA (1.6 ng) was co-injected with 16 ng of XMeis3-AMO or CMO into one-cell stage embryos. Protein was isolated from a pool of ten embryos per group at stage 12.5. A total of 50 µg protein was loaded per sample for electrophoresis. Western blot analysis was performed using the 9E10 Myc antibody. As a positive control, in vitro transcribed/translated Meis3-Myc protein (TNT system; Promega) was loaded for electrophoresis.

Table 1. Ectopic XMeis3-antimorph action on neural marker expression

<table>
<thead>
<tr>
<th>Gene analyzed</th>
<th>Number of embryos with modified gene expression on injected side</th>
<th>Number of embryos with unchanged gene expression on injected side</th>
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<tbody>
<tr>
<td>En2</td>
<td>48/67 (76%)</td>
<td>19/67 (24%)</td>
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<tr>
<td>Krox20</td>
<td>38/50 (76%)</td>
<td>12/50 (24%)</td>
</tr>
<tr>
<td>XE10</td>
<td>22/22 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>HoxB1</td>
<td>7/12 (58%)</td>
<td>5/12 (42%)</td>
</tr>
<tr>
<td>HoxB3</td>
<td>28/38 (74%)</td>
<td>10/38 (26%)</td>
</tr>
<tr>
<td>HoxB9</td>
<td>1/22 (5%)</td>
<td>21/22 (95%)</td>
</tr>
<tr>
<td>n-tubulin</td>
<td>13/13 (100%)</td>
<td>0</td>
</tr>
<tr>
<td>Nrp1</td>
<td>0</td>
<td>13/13 (100%)</td>
</tr>
</tbody>
</table>
Fig. 2. Eng-XMeis3 RNA encodes an antimorph protein; VP16-XMeis3 RNA encodes a caudalizing protein. (A) One-cell stage embryos were injected in the animal hemisphere with 1.0 ng of XMeis3 RNA (lane 4), 1.6 ng of Eng-XMeis3 RNA (XMeis3-AM; lane 5) or both (lane 6). Eighteen animal cap explants were removed from uninjected (lane 3) and injected groups (lanes 4-6) of blastula embryos (stage 8-9). Explants from each group were grown to stage 20 and total RNA was isolated. RT-PCR analysis was performed with the markers: Krox20, HoxD1 and HoxB9. EF1α served as a control for quantifying RNA levels in the different samples. For controls, RT-PCR (lane 2) and –RT-PCR (lane 1) were performed on total RNA isolated from normal embryos. (B) One-cell stage embryos were injected in the animal hemisphere with 1.6 ng of VP16-XMeis3 RNA (lane 4). Eighteen animal cap explants were removed from uninjected (lane 3) and injected groups (lane 4) of blastula embryos (stage 8-9). Explants from each group were grown to stage 20 and total RNA was isolated. RT-PCR analysis was performed with the markers: Krox20, HoxB3, HoxD1 and RARo2.2. EF1α served as a control for quantifying RNA levels in the different samples. For controls, RT-PCR (lane 2) and –RT-PCR (lane 1) were performed on total RNA isolated from normal embryos. (C) Embryos at the one-cell stage were injected with 1.3 ng of in vitro transcribed VP16-XMeis3. The upper embryo serves as an uninjected control. Injected embryos had anterior truncations and highly reduced cement gland formation (lower panel). The dorsal anterior index (DAI, Kao and Elinson, 1988) was 2.5 (n=39); over 20% of the embryos completely lacked cement glands and another 80% had extreme posterior truncations, with partial cement gland formation (lower panel). Embryos are oriented posterior to anterior: left to right. Embryos were fixed for photography at stages 35/36. (D) Embryos at the one-cell stage were injected with 1.0 ng of XMeis3-AM antimorph RNA. The top embryo serves as an uninjected control. In this representative experiment, over 80% of the XMeis3-AM injected displayed phenotypes. Embryos are oriented posterior to anterior: left to right. Embryos were fixed for photography at stages 30/31.

posteriorly into the r4/r5 boundary (Fig. 3B). cpl-1 expression was also shifted into the hindbrain region, as shown by the double in situ with En2 (Fig. 3D). In the cement gland, XAG1 and XA1 (not shown) expression (Sive et al., 1989) spread posteriorly in over 60% of the embryos (n=25/40). XAG1 expression appeared to extend into both spinal cord and lateral epidermal regions (Fig. 3E).

Hindbrain marker expression was severely inhibited in XMeis3-AM-injected embryos. Krox20 (Bradley et al., 1992) expression in r3/r5 was reduced in over 75% of the injected embryos (Fig. 3G,B,K-M; Table 1). In the mildest of phenotypes, r3/r5 expression is pushed posteriorly into r5/r7 (Fig. 3K). Rhombomere 4-specific markers, such as XE10 (Weinstein et al., 1996) and HoxB1 (Godsave et al., 1998) were also highly reduced in XMeis3-AM-injected embryos (Fig. 3H-I; Table 1). Interestingly, HoxB3 (Godsave et al., 1998) expression in r5/r6 was also eliminated, yet XMeis3 is not expressed at high levels in these rhombomeres (Fig. 3J; Table 1).

En2 (Hemmati-Brivanlou and Harland, 1989) expression was altered in the mid-hindbrain junction in over 75% of the XMeis3-AM-injected embryos (Table 1). However, the perturbed expression of En2 was of a more subtle nature in comparison with hindbrain markers. In embryos displaying the most moderate XMeis3-AM phenotypes, we did not observe major reductions in En2 expression, but expression expanded as far as r3/r4 (Fig. 3C,K). In intermediate phenotypes, we observed a posterior spreading of En2 to r2/r3 with a concomitant loss of Krox20 expression (Fig. 3L). In more extreme phenotypes, En2 expression was lost, together with Krox20 (Fig. 3M,D).

Expression of the pan-neural nrp1 marker (Fig. 3F; Table 1; Richter et al., 1988) and the spinal cord-specific HoxB9 marker (Fig. 3G; Table 1; Wright et al., 1990) was unaltered by ectopic XMeis3-AM expression, despite overlapping XMeis3 mRNA expression in the hindbrain and anterior spinal cord (Salzberg et al., 1999). Interestingly, expression of the neuron specific n-tubulin marker (Hollemann et al., 1998) was highly inhibited (Fig. 3N; Table 1) by ectopic XMeis3-AM activity. In strong phenotypes, both the r2-derived trigeminal neuron as well as the more posterior neural expression was eliminated. In more moderate phenotypes, the trigeminal was still missing but posterior expression was less inhibited (not shown). Thus, XMeis3 may have a role in early neuron specification.

These results demonstrate that ectopic XMeis3-AM expression can cause an anterior transformation of the hindbrain by inhibiting caudalization. Thus, functional XMeis3
Xmeis is required for hindbrain patterning

Antisense morpholino oligonucleotides also inhibit XMeis3 caudalizing activity and hindbrain pattern

An additional molecular tool for antagonism of in vivo XMeis3 protein activity is antisense morpholino oligonucleotides (AMOs). AMOs have recently been shown to inhibit mRNA translation in Xenopus and zebrafish embryos (Haseman et al., 2000; Nasevicius and Ekker, 2000). AMOs, complementary to the 5’ UTR and spanning the initial translated codons of the XMeis3 mRNA (see Materials and Methods) were injected at the one-cell stage into the animal hemisphere of embryos. In every experiment, a control morpholino oligonucleotide (CMO) was injected at an identical concentration to the AMO (see Materials and Methods). As described previously for XMeis3-AM, the specific inhibitory effect of AMOs on XMeis3 activity was screened by co-injection with wild-type XMeis3 RNA. Injection of XMeis3 AMOs together with XMeis3 wild-type RNA inhibited caudalizing activity in both animal cap explants and whole embryos (Fig. 4A). In animal cap explants, XMeis3 ectopically activated expression of the Krox20, HoxB9 RNA. Injection of XMeis3-AM RNA inhibited Krox20 expression (Fig. 4A, lane 7), but in explants co-expressing XMeis3 and the AMO, posterior neural marker expression was eliminated (Fig. 4A, lane 9). In whole embryos, ectopic XMeis3 significantly increased Krox20 and HoxB9 expression (Fig. 4A, lane 2); this increase was inhibited by co-expression with the AMO (Fig. 4A, lane 5). Strengthening this observation, expression of the AMO in embryos significantly reduced normal Krox20 and HoxB3 expression levels, in comparison with CMO-injected control embryos (Fig. 4A, compare lanes 2 and 4). HoxB9 expression

Fig. 3. Expression pattern of neural markers in embryos injected with XMeis3-AM RNA. Two-cell albino embryos were injected unilaterally into the animal hemisphere of one blastomere with 50-100 pg of XMeis3-AM RNA. The red arrow delineates the dorsal midline. In all embryos, XMeis3-AM injection is on the left side. In all cases (except Fig. 3M), embryos are viewed dorsally; embryos are oriented anterior (top) to posterior (bottom). (A) In situ hybridization with otx2. Expression is expanded posteriorly on the XMeis3-AM injected side. The red lines delineate the AP extent of otx2 expression on the uninjected versus injected side. (B) In situ hybridization with otx2 and Krox20. otx2 expression is expanded posteriorly and Krox20 expression (blue arrows) is lost on the XMeis3-AM-injected side. The red lines delineate the AP extent of otx2 expression on the uninjected versus injected side. (C) In situ hybridization with otx2 and En2 (red). Expression of otx2 and En2 (blue arrows) is expanded posteriorly. (D) In situ hybridization with cpl-1 and En2 (red). cpl-1 expression is expanded posteriorly and En2 expression (blue arrow/uninjected side) is lost on the XMeis3-AM-injected side. The red lines delineate the AP extent of cpl-1 expression on the uninjected versus injected side. (E) In situ hybridization with XAG1; expression is expanded posteriorly and laterally on the XMeis3-AM injected side. (F) In situ hybridization with nrp1; expression is unchanged on the XMeis3-AM injected side. (G) In situ hybridization with Krox20 and HoxB9; Krox20 expression (blue arrows/uninjected side) is eliminated on the XMeis3-AM-injected side. HoxB9 expression is unchanged on the XMeis3-AM injected side. (H) In situ hybridization with XE10. XE10 expression is eliminated on the XMeis3-AM-injected side. (I) In situ hybridization with HoxB1. HoxB1 expression is eliminated on the XMeis3-AM injected side. (J) In situ hybridization with HoxB3. HoxB3 expression is eliminated on the XMeis3-AM-injected side. (K) In situ hybridization with En2 (red) and Krox20. En2 expression is pushed posteriorly to the r3/r4 boarder and Krox20 expression is pushed posteriorly to the r5/r7 boarder on the XMeis3-AM-injected side. (L) In situ hybridization with En2 and Krox20. En2 expression is pushed posteriorly to r3 on the XMeis3-AM-injected side. The blue arrow delineates the reduced Krox20 expression (fused stripes) on the injected side. (M) In situ hybridization with En2 (red) and Krox20. Expression of En2 and Krox20 (blue arrows/uninjected side) is eliminated on the XMeis3-AM-injected side. An anterior view of the embryo: dorsal (top) to ventral (bottom). (N) In situ hybridization with n-tubulin. n-tubulin expression is eliminated on the XMeis3-AM-injected side. The trigeminal neuron is marked by blue arrows on both sides.
was not disrupted in AMO-injected embryos (Fig. 4A, lanes 2-5).

To demonstrate that the AMO was indeed inhibiting translation of \textit{XMeis3} RNA, the AMO was co-injected with RNA encoding a \textit{XMeis3}-Myc-tagged protein. As determined by Western blot analysis, co-injection of AMO prevented translation of this \textit{XMeis3}-Myc tagged protein in comparison with embryos co-injected with the CMO (Fig. 4B, compare lanes 2 and 3). The effects on \textit{XMeis3}-Myc translational inhibition were specific, as AMO and CMO injections did not inhibit endogenous \textit{Xenopus} proteins; Erk levels were identical in all groups (Fig. 4B, compare lanes 1-3). In contrast to protein levels, the AMO did not alter \textit{XMeis3}-Myc RNA levels, as RNA levels were identical in the AMO- and CMO-injected groups (Fig. 4C, compare lanes 1 and 2).

In \textit{XMeis3}-Myc/CMO-injected embryos, \textit{XMeis3}-Myc protein acts as a caudalizer, increasing \textit{Krox20} expression in these embryos (Fig. 4C, compare lanes 2 and 3). However, when \textit{XMeis3}-Myc/AMO was co-injected, levels of \textit{Krox20} RNA were highly reduced, significantly below levels in control embryos (Fig. 4C, lanes 2-4).

To further determine the role of endogenous \textit{XMeis3} protein in the embryo, we used the AMO to inhibit endogenous \textit{XMeis3} mRNA translation during early development. We injected 12.5-20 ng of the AMO into the animal hemisphere of one-cell stage embryos; these embryos were scored at tailbud to tadpole stages for phenotypes (Fig. 5). Like ectopic \textit{XMeis3-AM} expression, the AMO (17.5-20 ng) caused anterior expansions and cement gland enlargement in over 80% (\textit{n}=41/49) of the injected embryos (Fig. 5A, lower panel), in comparison with control CMO-injected embryos (Fig. 5A, upper panel). As in the case of the \textit{XMeis3}-AM phenotypes, these embryos also had a much shorter body axis; body length was reduced by approximately 25-33% in AMO phenotypic embryos. At lower AMO concentrations (12.5-15 ng), body length was still altered in over 75% of the embryos (\textit{n}=37/54), and anterior expansion phenotypes were weaker (Fig. 5A, middle panel). Thus, like the \textit{XMeis3}-AM protein, injection of the AMO caused a prominent dose-dependent anteriorized phenotype in embryos.

By RT-PCR analysis, neural marker expression was examined over the 12.5-20 ng AMO concentration range (Fig. 5B). \textit{Krox20} expression is the most sensitive to loss of \textit{XMeis3}...
**Figure 5.** *XMeis3* antisense morpholino oligonucleotide causes posterior truncations and anterior expansions in embryos. (A, top) Embryos at the one-cell stage were injected with 20 ng of the CMO. These embryos resembled un.injected controls. All embryos were fixed for photography at stage 38. (A, middle) Embryos at the one-cell stage were injected with 12.5 – 15 ng of the AMO. (A, bottom) Embryos at the one-cell stage were injected with 17.5-20 ng of the AMO. (B) Embryos from the experiment in A were grown to late neurula stages. Total RNA was also isolated from pools of seven embryos from each of the injected groups (lanes 1-6). RT-PCR analysis was performed with the markers: En2, Krox20 and HoxB9. EF1α served as a control for quantifying RNA levels in the different samples. For controls, RT-PCR (lane 2) and −RT-PCR (lane 1) were performed on total RNA isolated from control CMO injected embryos (lane 2). (C) Embryos at the one-cell stage were injected with 15 ng of the CMO (top) or the AMO (bottom). CMO- and AMO-injected embryos were co-injected with either 1.6 ng of *XMeis3* RNA (middle) or 1.6 ng of *hth* RNA (right). White arrows mark the cement glands. All embryos were fixed for photography at stage 27/28.

*Xmeis* is required for hindbrain patterning activity, being highly reduced at all concentrations examined (Fig. 5B). *HoxB3* expression was also inhibited (not shown). *En2* expression is lost, but in a graded manner and only at higher AMO concentrations (Fig. 5B). *HoxB9* expression was not reduced, being slightly increased in AMO-injected embryos (Fig. 5B). Expression levels of the ectodermal and mesodermal markers, *epidermal cytokeratin* and *muscle actin* were not significantly altered by the AMOs (not shown).

To demonstrate AMO specificity, *XMeis3* and *Drosophila hth*-encoding RNAs were separately co-injected into embryos together with the AMO. We have previously shown that ectopic *hth* expression can caudalize *Xenopus* embryos and animal cap explants, in the same way as *XMeis3* (Salzberg et al., 1999). As the *hth* gene lacks the *XMeis3* 5′ region encoded by the AMO, we expect that its caudalizing activity should not be affected by the AMO. Indeed, both RNAs caudalized embryos...
Fig. 6. XMeis3 antisense morpholino oligonucleotide eliminates hindbrain marker expression. Two-cell albino embryos were injected unilaterally into the animal hemisphere of one blastomere with 6-7.5 ng of the XMeis3 AMO. In situ hybridization was performed in late neurula stage embryos. In all cases, embryos are viewed dorsally; embryos are oriented anterior (top) to posterior (bottom). The red arrow delineates the dorsal midline. Embryos were injected on the right side. (A) In situ hybridization with Krox20 and En2 (red); expression of both markers is eliminated on the AMO-injected side. (B) In situ hybridization with XE10; expression is unchanged on the AMO-injected side. (C) In situ hybridization with XMeis3; expression is eliminated on the AMO-injected side. (D) In situ hybridization with XMeis3 and En2 (red); expression of both markers is posteriorized on the AMO-injected side. (E) In situ hybridization with XMeis3; expression is inhibited on the AMO-injected side. The XMeis3 expression in r2 is indicated by arrows on both sides.

in a similar manner: 85% of the XMeis3/CMO injected embryos (n=8) and 75% of the hth/CMO (n=16) -injected embryos had small cement glands (Fig. 5C, upper panel). XMeis3/AMO-injected embryos had rescued caudalized phenotypes: nearly 80% of the embryos (n=18) had normal or expanded cement glands (Fig. 5C, lower panel). In sharp contrast, 70% (n=20) of the hth/AMO-injected embryos had small cement glands (Fig. 5C, lower panel), like the XMeis3/CMO-injected group (Fig. 5C, upper panel). In the AMO-injected control group, 75% (n=16) of the embryos had expanded cement glands (lower left panel), in comparison with the CMO-injected (upper left panel) group (n=10). Similar results were also seen in animal cap explants; expression of Krox20 was reduced in XMeis3/AMO versus XMeis3/CMO-injected explants, but levels of Krox20 expression were identical in hth/AMO- and hth/CMO-expressing explants (not shown). These results show that the AMO cannot inhibit hth caudalizing activity, thus, the AMO is indeed specific to the XMeis3 gene.

To further examine the role of the AMO in neural patterning, whole embryos at the two-cell stage were injected unilaterally into one blastomere with 6-7.5 ng of AMO, and whole-mount in situ hybridization was performed. We saw a dramatic reduction in hindbrain marker expression on the injected side: Krox20 (Fig. 6A), XE10 (Fig. 6B) and HoxB3 (Fig. 6C). HoxB9 expression in the spinal cord was not decreased in AMO-injected embryos (Fig. 6A,C). In AMO-injected embryos, XE10 expression was exclusively inhibited in the hindbrain (where XMeis3 expression overlaps), but not in ectoderm regions found lateral to the neural tube, where XMeis3 was not expressed (Fig. 6B). We also examined how AMO injection altered endogenous XMeis3 expression in r2-r4 and the anterior spinal cord. In moderate phenotypes (Fig. 6D), we detected a shift of the XMeis3 rhombomeric expression from r2-r4 to r5-r7 with a fusion of the expression domain to the spinal cord. In the same embryo, En2 expression is pushed to the approximate r2/r3 border (Fig. 6D). In more extreme phenotypes (Fig. 6E), the XMeis3 expression pattern is again shifted posteriorly, but XMeis3 mRNA levels are also highly reduced. These data strongly corroborate the results obtained with the XMeis3-AM protein (Fig. 3), providing substantial proof that XMeis3 protein activity is obligatory for proper cell fate determination in the hindbrain.

Caudalized animal caps: XMeis3-AM protein rostralizes AP coordinates

To elucidate specific XMeis3 interactions with caudalizing pathways, XMeis3-AM was ectopically expressed in animal caps caudalized by either Xenopus bFGF (XbFGF) or mouse Wnt3a proteins. At the one-cell stage, XMeis3-AM, noggin and/or mouse Wnt3a RNAs were injected in the animal hemisphere; at blastula stages animal cap explants were removed. In the experiments with XbFGF, animal caps were aged until stage 10.25, when XbFGF treatment was initiated (Lamb and Harland, 1995). Total RNA was isolated for RT-PCR analysis at late neurula stages.

In noggin-neutralized animal cap explants, XMeis3-AM protein modified caudalization by XbFGF and mouse Wnt3a. In these caps, the perturbation of XMeis3 activity did not inhibit caudalization per se, but did bias neural marker expression in a more anterior manner. In both noggin/XbFGF- and XbFGF-treated animal caps explants, XMeis3-AM protein decreased expression of spinal cord-specific HoxB9 marker, yet increased expression of the more anterior En2 marker (Fig. 7A). This effect was dependent on the initial AP coordinates of the explant. When caps are treated with XbFGF, only HoxB9 is induced (Fig. 7A, lane 3), yet in the presence of the XMeis3-AM protein, both HoxB9 (reduced levels) and En2 are expressed (Fig. 7A, lane 4). In XbFGF/noggin-treated caps, both En2 and HoxB9 are expressed (Fig. 7A, lane 5); however, in the presence of XMeis3-AM protein, En2 is exclusively expressed and at increased levels (Fig. 7A, lane 6). Thus, the final extent of anteriorization in the XMeis3-AM injected explants appears determined by the initial AP patterning coordinates in the explant that were pre-determined by FGF±noggin. The presence of the XMeis3-AM protein shifted neural marker expression in favor of the more anterior mid-hindbrain.
We have used two experimental approaches to inhibit endogenous XMeis3 protein activity in developing Xenopus embryos. In the first approach, we constructed an antimorph protein, by fusing XMeis3 open reading frames to the engrailed repressor or VP16 activator transcriptional domains. In the second approach, we injected an antisense morpholino oligonucleotide (AMO) homologous to the 5' end of the XMeis3 mRNA. In a relatively simple bioassay in animal cap explants, we determined that the Eng-XMeis3 fusion protein inhibited caudalizing activity when co-injected with wild-type XMeis3 RNA. In the same animal cap assay, the VP16-XMeis3 fusion protein strongly activated expression of posterior neural markers. We thus concluded that the Eng-XMeis3 fusion protein acted as a bonafide antimorph protein.

Further supporting this observation, studies in transgenic flies that express either Xenopus Eng-XMeis3 or Drosophila Eng-HTH chimera proteins demonstrated hth loss-of-function like phenotypes (Inbal et al., 2001). In a complementary manner, transgenic flies expressing VP16-HTH chimera protein displayed hth gain-of-function like phenotypes; VP16-HTH also rescued phenotypes in hth mutant embryos (Inbal et al., 2001). Our previous studies demonstrated that ectopic expression of either wild-type HTH or XMeis3 proteins caudalized Xenopus embryos and animal cap explants (Salzberg et al., 1999). These results suggest that the Meis family transcriptional activator function has been conserved for nervous system development in such diverse organisms as flies and frogs.

Like the XMeis3-AM protein, injection of AMOs also inhibited wild-type XMeis3 caudalizing activity in embryos and animal cap explants. XMeis3-Myc protein levels were eliminated by the AMO. These results show that the AMO also acts as a potent inhibitor of XMeis3 activity, by preventing mRNA translation.

To address the role of XMeis3 during Xenopus CNS development, one-cell embryos were injected with the ENG-Xmeis3 (XMeis3-AM) RNA, VP16-XMeis3 RNA or AMOs. Ectopic expression of VP16-XMeis3 RNA caudalized Xenopus embryos in a manner similar to the wild-type XMeis3-encoding RNA. By contrast, both XMeis3-AM and AMOs had distinct posterior-truncation/anterior-expansion phenotypes. In these embryos, the cement gland was expanded and the body axis was shortened.

To further address this point, albino embryos were unilaterally injected with XMeis3-AM or AMOs into one blastomere at the two-cell stage. At neurula stages, a wide array of neural markers were examined by whole-mount in situ hybridization. Confirming the phenotypic observations, in XMeis3-AM-injected embryos, we saw an expansion of expression of anterior markers such as XAG1, cpl-1, otx2 and En2 into more posterior regions of the brain. In the most extreme cases, we saw otx2 expression shifted as far back as r4/r5 and En2 expression was eliminated. In more moderate phenotypes, otx2 expression was shifted to r1/r2 and En2 expression was shifted to r3/r4. The posterior spread and loss of En2 and endogenous XMeis3 expression, and the concomitant loss of the r2-derived trigeminal neuron demonstrate that patterning in the most anterior hindbrain r1/r2 regions is greatly disrupted by the loss of endogenous XMeis3.

**Fig. 7.** XMeis3-antimorph protein anteriorizes neural marker expression in animal cap explants caudalized by bFGF or Wnt3a. (A) One-cell stage embryos were injected in the animal hemisphere with 1.6 ng of XMeis3-AM RNA (lane 4) or 0.2 ng of noggin RNA (lane 5) or both (lane 6). Eighteen animal cap explants were removed from uninjected (lanes 2-3) and injected groups of blastula embryos (stage 8-9). Explants from each group were aged until stage 10.25 and XbFGF was added at 50 ng/ml. Explants from each group were grown to late neurula stage and total RNA was isolated. RT-PCR analysis was performed with the markers: En2 and HoxB9. EF1α served as a control for quantifying RNA levels in the different samples. -RT-PCR (lane 1) was performed on total RNA isolated from uninjected embryos. (B) One-cell stage embryos were injected in the animal hemisphere with 1.6 ng of XMeis3-AM RNA (lane 6-9). Eighteen animal cap explants were removed from uninjected (lane 3) and injected groups of blastula embryos (stage 8-9). Explants from each group were grown to late neurula stage and total RNA was isolated. In parallel, total RNA was isolated from uninjected (lanes 2-3) and injected groups of blastula embryos. XMeis3-AM protein acted as a bonafide antimorph protein.

DISCUSSION

Like the XMeis3-AM protein, injection of AMOs also inhibited wild-type XMeis3 caudalizing activity in embryos and animal cap explants. XMeis3-Myc protein levels were eliminated by the AMO. These results show that the AMO also acts as a potent inhibitor of XMeis3 activity, by preventing mRNA translation.

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activity. Rhombomeric expression of the *Krox20, XE10, HoxB1* and *HoxB3* markers was severely reduced by the XMeis3-AM protein or the AMO. In the moderate hindbrain phenotypes, we could detect a two rhombomeric-shift of *Krox20* expression from r3/r5 to r5/r7. By contrast, the spinal cord and pan-neural markers, *HoxB9* and *nrp1* were unaffected by XMeis3-AM or AMO activity.

It also appears that neurogenesis may be affected by the loss of XMeis3 activity. In XMeis3-AM injected embryos, *n-tubulin* expression is always lost in the r2-derived trigeminal neuron, and this could be a reflection of rhombomeric identity loss in this region. In most cases, injection of the XMeis3-AM also inhibited posterior *n-tubulin* expression. Neither XMeis3 nor *noggin* strongly induces *n-tubulin* expression in animal cap explants; however, in the presence of both molecules, we detected high levels of *n-tubulin* expression in animal caps (S. E. and D. F., unpublished). Thus, further experiments need to be performed to determine the exact role for XMeis3 protein in specifying neuron cell fates along the AP axis.

These results show that functional XMeis3 protein maintains a proper AP balance required for hindbrain formation. The spread of expression of anterior neural markers posteriorly into the hindbrain suggests that XMeis3 is essential for actively maintaining a caudalized state in the hindbrain. While XMeis3 does not seem required for neural induction, it seems to fine tune the AP pattern in the forebrain-hindbrain region. The conversion of hindbrain regions to more anterior fates, with concomitant loss of hindbrain markers, *HoxB9* expression did not appear to be required for high hindbrain marker expression by mouse Wnt3a. In whole embryos, *HoxB9* expression is unchanged or even slightly increased in the presence of XMeis3-AM or the AMO. Inhibition of Xwnt-3a activity reduces *HoxB9* expression in embryos and explants (McGrew et al., 1997), so a delicate balance between Wnt and XMeis3 activities may maintain optimal *HoxB9* expression levels in the spinal cord. The implications of these wnt/XMeis3 interactions are still unclear, and further experiments are being carried out to understand how Wnt and XMeis3 pattern posterior neural tissue.

Using two distinct molecular approaches, we have inhibited XMeis3 protein activity in *Xenopus* embryos. In these embryos, a clear perturbation of the posterior CNS is observed, most specifically in the hindbrain region. XMeis3 appears to give distinct spatial identity to hindbrain cells. Without proper XMeis3 activity, anterior neural tissue spreads posteriorly and hindbrain identity is lost. The hindbrain is trapped in a more rostral cell fate. XMeis3 caudalizes the CNS to hindbrain, without inducing neural tissue. When viewing the ‘activation’ and ‘transformation’ model of neural induction, functional XMeis3 activity may be prerequisite for the ‘transformation’ step. XMeis3 probably interpreted spatial information along AP axis in hindbrain cells, thus enabling them to differentiate in a proper manner. Future studies will focus on how XMeis3 functions as a transcriptional activator to caudalize the brain. By identifying genes directly targeted by XMeis3, we intend to determine the genetic hierarchy regulating hindbrain formation in the developing CNS.

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