The zebrafish neckless mutation reveals a requirement for raldh2 in mesodermal signals that pattern the hindbrain

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SUMMARY

We describe a new zebrafish mutation, neckless, and present evidence that it inactivates retinaldehyde dehydrogenase type 2, an enzyme involved in retinoic acid biosynthesis. neckless embryos are characterised by a truncation of the anteroposterior axis anterior to the somites, defects in midline mesendodermal tissues and absence of pectoral fins. At a similar anteroposterior level within the nervous system, expression of the retinoic acid receptor α and hoxb4 genes is delayed and significantly reduced. Consistent with a primary defect in retinoic acid signalling, some of these defects in neckless mutants can be rescued by application of exogenous retinoic acid. We use mosaic analysis to show that the reduction in hoxb4 expression in the nervous system is a non-cell autonomous effect, reflecting a requirement for retinoic acid signalling from adjacent paraxial mesoderm. Together, our results demonstrate a conserved role for retinaldehyde dehydrogenase type 2 in patterning the posterior cranial mesoderm of the vertebrate embryo and provide definitive evidence for an involvement of endogenous retinoic acid in signalling between the paraxial mesoderm and neural tube.

Key words: Zebrafish, Anteroposterior patterning, Vitamin A deficiency, Retinoic acid, Retinoic acid receptor, Craniofacial development, Neural crest, raldh2, hoxb4

INTRODUCTION

In all vertebrates, inductive cellular interactions result in stable differences in cell states between head, trunk and tail derivatives. In particular, anteroposterior (AP) patterning of the neural tube is regulated by signals from organiser-derived tissues, such as the notochord and prechordal plate, and from paraxial mesoderm (reviewed by Jessell, 2000; Stern and Foley, 1998). Somites clearly influence posterior identities of cells in the neural tube. In heterotopic grafts of hindbrain or somites, neural cells can be respecified to more posterior identities when juxtaposed to posterior mesoderm (Grapin-Botton et al., 1995; Itasaki et al., 1996; Stern et al., 1991). In the posterior head region, putative posteriorising factors have been implicated in patterning both the identities of hindbrain rhombomeres and of neural crest-derived pharyngeal arches (Gould et al., 1998; Muhr et al., 1999; Wendling et al., 2000).

Retinoids are prime candidates for such posteriorising factors since they can have a wide range of effects on AP-patterning in the developing central nervous system (CNS), limbs and neural crest. Exposure of embryos to an excess of retinoic acid (RA) inhibits anterior development in the neural tube and craniofacial mesenchyme through the suppression of fore- and midbrain-specific gene expression and the expansion of the expression domains of more caudally restricted genes (reviewed by Durston et al., 1998; Gavalas and Krumlauf, 2000). These effects correlate well with the distribution of endogenous RA: both in chick and mouse embryo, RA is detected only after gastrulation with a sharp anterior boundary at the level of the first somite, and at high concentrations caudal to this boundary (Mendelsohn et al., 1991; Rossant et al., 1991; Colbert et al., 1995; Horton and Maden, 1995; Maden et al., 1998). Similarly, in zebrafish the anterior trunk contains high levels of RA (Marsh-Armstrong et al., 1995).

Depriving embryos of RA causes a variety of developmental defects, among them neural crest cell death, the absence of limb buds and posterior branchial arches, small somites, and hindbrain segmentation defects, which collectively are known as vitamin A-deficient (VAD) syndrome (Morriss-Kay and Sokolova, 1996; Maden et al., 1996; Dickman et al., 1997; Maden et al., 2000). In the hindbrain, embryonic RA depletion leads to graded phenotypic effects: with decreasing amounts of RA, expression of genes normally restricted anteriorly progressively extends posteriorly until finally, in the absence of RA signalling, embryos lack rhombomeric and gene expression boundaries posterior to rhombomere 3 (Blumberg
et al., 1997; Dickman et al., 1997; Dupe et al., 1999; Kolm et al., 1997; van der Wees et al., 1998; White et al., 1998; White et al., 2000).

The effects of RA and other retinoids are mediated through nuclear receptors of the RAR and RXR families which act as ligand-activated transcriptional regulators (reviewed by Mangelsdorff et al., 1995). Inactivation of single receptors in mice has revealed extensive receptor redundancy, while compound mutations in some receptors recapitulate the phenotypic defects observed in VAD, including the disruption of AP patterning in the cranial neural crest and hindbrain (Dupe et al., 1999; Kastner et al., 1994; Kastner et al., 1997). These complex phenotypes are not surprising, given the widespread distribution of RA and expression of its receptors. For example, zebralfish RARα and RARγ (rara and rarg – Zebrafish Information Network) expression show little overlap; RARα is expressed in paraxial mesoderm, posterior hindbrain and spinal cord, whereas RARγ is expressed more anteriorly in head mesenchyme and in the brain (Joore et al., 1994).

AP patterning of the CNS is mediated through the regulated expression of Hox genes, which are expressed with discrete AP expression boundaries within the developing neural tube and adjacent mesoderm. Binding sites for RA receptors have been characterised in the regulatory regions of hoxa1, hoxb1, hoxb4 and hoxd4, and shown to confer RA-mediated gene activation in vivo and in vitro, suggesting that RA directly regulates Hox gene transcription (Marshall et al., 1994; Morrison et al., 1996; Dupe et al., 1997; Gould et al., 1998; Studer et al., 1998). Thus, the spatial distribution of RA and its receptors are all thought to be critical for regulating Hox gene expression in the neural tube.

The biosynthesis of RA involves the sequential conversion of vitamin A into retinaldehyde, which is then oxidised to RA. At least two cytosolic alcohol dehydrogenases (ADH), or microsomal retinol dehydrogenases, catalyse the first step, while the second step requires cytosolic retinal dehydrogenases, members of the aldehyde dehydrogenase (ALDH) family (reviewed by Duester, 2000). Two aldehyde dehydrogenases, ALDH1 and ALDH6V1, are predominantly expressed in spatially restricted domains of the head and retina and are unlikely to contribute to the high levels of RA posteriorly (Haselbeck et al., 1999; Maden et al., 1998). In contrast, retinaldehyde dehydrogenase 2 (RALDH2), a nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase, is expressed posteriorly in a pattern that correlates with RA-mediated gene activation (Wang et al., 1996; Zhao et al., 1996; Niederreither et al., 1997; Berggren et al., 1999; Swindell et al., 1999). In mouse, loss-of-function mutations in Raldh2 mimic the most severe phenotypes associated with VAD, implicating Raldh2 as the main source of RA in the vertebrate embryo (Niederreither et al., 1999; Niederreither et al., 2000).

We have characterised the neckless (nls) mutation in zebralfish, which recapitulates many aspects of VAD. We link nls to a missense mutation in raldh2, structural analysis of which predicts a non-functional protein. Consistent with the molecular nature of nls, we show that exogenous application of RA rescues the fin and mesodermal defects in nls mutants. We also show that zebralfish require raldh2 for formation of posterior head mesoderm and notochord, as well as for cell specification in the anterior spinal cord. Finally, we show that the lack of expression of hoxb4 in the CNS is due to defects in RA signalling from the paraxial mesoderm. Our findings suggest a model in which RA directs AP patterning directly in the mesoderm, and that these cells, in turn, indirectly pattern the neural tube.

**MATERIALS AND METHODS**

**Zebrafish husbandry**

London wild-type and WIK strains of zebrafish were reared and staged at 28.5°C (Kimmel et al., 1995).

**Mutant screening**

Diploid F2 progeny of male fish mutagenised with ethyl-N-nitrosourea (ENU) (Mullins et al., 1994; Solnica-Krezel et al., 1994) from a London wild-type background (Currie et al., 1999) were fixed at 24 hours postfertilisation (hpf) and hybridised with probes for knox20 (Oxtoby and Jawett, 1993), pax2 (Krauss et al., 1992), shh (Krauss et al., 1993) and myoD (Weinberg et al., 1996). In situ hybridisation was performed essentially as previously described (Begemann and Ingham, 2000), using 24-well plates. For double in situ hybridisations, strongly expressed transcripts were labelled with fluorescein and detected with p-iodo nitrotetrazolium violet (INT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP), and weakly expressed ones were labelled with NBT/BCIP (Roche Diagnostics).

**Mapping and linkage testing**

nls28 was outcrossed to the WIK strain and the pooled DNA from F2 homozygous mutants and siblings was analysed using SSLPs. An EST (GenBank Accession Numbers, AI476832 and AI477235) that mapped between z11119 and z8693 on the LS54 radiation hybrid panel (Hukriede et al., 1999), was shown to encode raldh2 by sequence similarity to other vertebrate Raldh2 genes. Linkage was determined by RFLP analysis of pooled cDNAs, from 40 ‘London wild type’ and nls/nls embryos (oligonucleotides: 5¢-AACCTGCC-AGGAGAGGTGAAGAACGAC-3¢ and 5¢-ACGGCCAATTGGG-ACATTTTGAATC-3¢). Psrl restriction of the amplificates generated a restriction fragment length polymorphism (RFLP) of 0.6 and 0.77 kb in nls/nls, and of 1.46 kb in wild type, owing due to a missense mutation in nls28.

**Cloning of raldh2**

Degenerate primers against the peptide sequences IIPWNPF (5¢-ATA/C/CT ATA/C/T CCI TGG AAC/T TTC/T CC-3¢) and PFGFKM (5¢-CAT C/TTT A/GAA ICC ICC A/GAA IGG-3¢) were used to amplify a 0.9 kb raldh2 fragment by RT-PCR from 30 hours hpf wild-type embryos. Fragments were subcloned into the pCR2.1-vector using the TOPO kit (Invitrogen) and sequenced, revealing one with similarity to vertebrate Raldh2. The fragment was screened against a zebralfish late somitogenesis stage cDNA library (Max-Planck-Institute for Molecular Genetics, Berlin) under stringent conditions to obtain a full-length clone of raldh2 (ICRFp524L2053Q8)(GenBank Accession Number, AF339837). Several cDNAs from different homozygous nls mutant embryos and London wild-type embryos were obtained by RT-PCR and sequenced using raldh2-specific primers.

**Retinoic acid treatments**

Batches of 60-80 embryos from wild-type or nls heterozygous parents were incubated in the dark from late blastula stage onwards in varying dilutions (in embryo medium) of a10-4 M all-trans RA (Sigma)/10% ethanol solution (from a 10-2 M stock solution in DMSO). As controls, siblings were treated with equivalent concentrations of ethanol/DMSO alone. Mild teratogenic effects (e.g. disrupted heart development and smaller eyes) were observed at higher concentrations.
mRNA rescue experiments

Full-length RALDH2 cDNA was cloned as a SpeI/NorI fragment into the XhoI and NorI sites of pSP64TXTB (Tada and Smith, 2000). The resulting plasmid, pSP64T-RALDH2 was linearised with XhoI and transcribed using the ‘SP6 mMessage mMachine’ kit (Ambion). 3 nl of in vitro synthesised mRNA was injected into embryos at the one- to four-cell stage.

Morpholino injections

Two partially overlapping morpholinos against raldh2 (5'-gtt caa ctt cac tgg agg tca tc-3' and 5'-gca gtt caa ctt cac tgg agg tca t-3') were obtained from GeneTools, LLC, and solubilised in water at a stock concentration of 1 mM (8.5 mg/ml). 4-5 nl of 1:2, 1:4 and 1:10 dilutions in water, respectively (approximately 4, 2 and 0.85 mg/ml) were injected into one-cell stage embryos. The injected dilutions resulted in strong (1:2) to weak (1:10) phenotypes of the nls phenotype.

Histology

Cartilage staining was performed as described (Schilling et al., 1996). TUNEL staining for apoptotic cells was performed as described previously (Williams et al., 2000). Labelled DNA was detected with alkaline phosphatase-coupled anti-fluorescein (Roche), followed by a NBT/BCIP (Roche) colour reaction. For live labelling of apoptosis, dechorionated embryos were incubated in 5mg/ml Acridine Orange fluorescent tyramide substrate (Renaissance TSA kit; Dupont) detected with diaminobenzidine (for brightfield microscopy) or a fluorescent tyramide substrate (Renaissance TSA kit; Dupont) detected with diaminobenzidine (for brightfield microscopy) or a fluorescent tyramide substrate (Renaissance TSA kit; Dupont)

Mosaic analysis

Donor embryos were injected at the one-cell stage with 2.5% lysine fixable tetramethyl-rhodamin-dextran and 3.0% lysine fixable biotin-dextran (Mf 100,000)(Molecular Probes) dissolved in 0.2 M KCl. Transplants were done blindly, and donor genotypes determined at 24 hpf. At late blastula stages, groups of 10-30 donor cells were transplanted into unlabelled host embryos of the same stage and placed either along the margins of the blastoderm, which gives rise to the mesendoderm, or further away from the margin in regions that give rise to neural ectoderm (Kimmel et al., 1990). Transplanted cells were labelled using a peroxidase-coupled avidin (Vector Labs) and detected with diaminobenzidine (for brightfield microscopy) or a fluorescent tyramide substrate (Renaissance TSA kit; Dupont Biotechnology Systems), and examined for fluorescence.

RESULTS

Mutation of the neckless gene disrupts posterior head mesoderm and pectoral fin development

The neckless (nls) mutation was isolated in an in situ hybridisation screen of ENU-mutagenised zebrafish through its effects on gene expression along the AP axis (Fig. 1; Currie et al., 1999). Simultaneous detection of krox20 expression in the hindbrain and myoD expression in somite precursors reveals a reduction in the spacing between rhombomere 5 (r5) and the first somite as early as the tailbud stage in 25% of the progeny of nls heterozygotes (Fig. 1G-J). The nls<sup>26</sup> allele is inherited in a Mendelian fashion as a recessive lethal trait and homozygotes die between 4-6 days postfertilisation. At 18 hpf, the posterior head in mutants is thickened just anterior to the first somite (Fig. 1A,B), and the distance between the otic vesicle and the first somite is reduced compared with wild type (Fig. 1C,D). At 30 hpf mutants have weak heartbeats, swollen pericardial cavities and lack apical folds of the developing pectoral fin buds. By 4 days, mutant larvae lack pectoral fins (Fig. 1E,F). Alcian staining of cartilage showed that homozgyotes lacked both a pectoral girdle and endoskeletal elements of the pectoral fins (see Fig. 6J). Body shape and fin defects were 100% penetrant in nls mutants either in AB or London genetic backgrounds, whereas pericardial swelling had a lower penetrance (data not shown).

To investigate its mesodermal defects further, we compared markers of different mediolateral regions (paraxial, lateral plate and axial). Analysis of myoD and her1 expression revealed no differences in the length of the somitic plate, or number of somites formed, in nls homozygotes (Fig. 1G-J). The nephric tubules, which derive from lateral plate mesoderm and express pax2, are invariably located lateral to somite 3 in nls, as in wild type (Fig. 1K,M). By contrast, nls mutants have fewer notochord cells, visualised using an anti-No tail antibody in the posterior head (Fig. 1G-J; see Table 1). At 12 hpf the number of No tail-positive cells between r5 and somite 1 is reduced by approximately 50% in nls (Table 1). The number of developing anterior somites is unchanged, as expression of hoxc6 is detected up to the boundary between the fourth and fifth somites in nls as in wild type (Molven et al., 1990; Prince et al., 1998a). Thus, nls mutants exhibit early defects in both paraxial and axial mesoderm in the region that will form the posterior head and pectoral fins.

A mutation in raldh2 co-segregates with nls

Using bulked segregant analysis we mapped nls between SSLPs z11119 and z8693 on LG7 (Fig. 2B,C). This location coincides with that of an EST predicted to encode a close relative of mammalian Raldh2. As the nls phenotype shows some similarities to that of VAD quail and Raldh2 mutant mouse embryos, Raldh2 seemed a good candidate for the nls gene. We sequenced a full-length cDNA encoding zebrafish raldh2 (Fig. 2A) and six independent isolates of RALDH2 cDNAs from homozygous nls embryos that all contain a point mutation (Gly204Arg) (Fig. 2D). This creates a fortuitous PstI restriction site with which we confirmed linkage to nls by RFLP analysis (Fig. 2E). Glycine 204 is one of 23 residues that are invariantly located lateral to somite 3 in nls, as in wild type (Fig. 1K,M). By contrast, nls mutants have fewer notochord cells, visualised using an anti-No tail antibody in the posterior head (Fig. 1G-J; see Table 1). At 12 hpf the number of No tail-positive cells between r5 and somite 1 is reduced by approximately 50% in nls (Table 1). The number of developing anterior somites is unchanged, as expression of hoxc6 is detected up to the boundary between the fourth and fifth somites in nls as in wild type (Molven et al., 1990; Prince et al., 1998a). Thus, nls mutants exhibit early defects in both paraxial and axial mesoderm in the region that will form the posterior head and pectoral fins.

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<tr>
<th></th>
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<th>Average number of No tail-expressing cells</th>
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<tr>
<td></td>
<td>Sample size (n)</td>
<td>Rostral to r3</td>
</tr>
<tr>
<td><strong>Wild type</strong></td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td><strong>nls</strong></td>
<td>9</td>
<td>11</td>
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Cell numbers expressing the nuclear No tail protein in 10-somite-old embryos, counterstained with krox20 and myoD.
suggest that this mutation prevents a secondary structure that allows interaction of the protein with the co-enzyme NAD (data not shown). Owing to the tight spacing of glycine 204 within its surroundings, replacing this residue with arginine appears to be sterically prohibitive and would create a protein of reduced or no activity.

Morpholino-mediated translational inhibition of RALDH2 phenocopies nls

To investigate whether loss of RALDH2 activity could account for the nls phenotype, we injected raldh2-specific morpholino antisense oligonucleotides into wild-type embryos and assayed the ensuing effects by in situ hybridisation with appropriate marker probes. Injection of either 8.5 or 17 ng of the raldh2-morpholino resulted in a strong reduction in the space between the somite 4/5 boundary in nls (Fig. 3A), a phenotype indistinguishable from that caused by the morpholino. To investigate whether loss of RALDH2 activity could account for the nls phenotype, we injected in vitro transcribed raldh2 mRNA into one- to four-cell nls embryos and assayed for the rescue of tbx5.1 expression in the pectoral fin buds, as well as for development of an apical fin fold. The concentration of injected raldh2 mRNA was progressively reduced until overexpression phenotypes, similar to those observed in RA-treated embryos, were no longer observed. This concentration (approx. 500 pg per embryo) was used to assay phenotypic rescue in batches of embryos derived from a cross between nls-heterozygotes (Table 3). Partial or complete restoration of tbx5.1 expression was seen in 83% of mutants (30/36 expected nls embryos), indicating that wild-type raldh2 is sufficient to rescue nls embryos.

Exogenous RA or RALDH2 activity rescues aspects of the nls mutant phenotype

As RALDH2 catalyses the last step in the synthesis of all-trans-RA, the main constituent of retinoids in zebrafish embryos (Costaridis et al., 1996), we investigated whether or not exogenous RA can rescue the mesodermal and fin defects caused by nls. Two early fin markers, tbx5.1, which labels the entire pectoral fin field (Begemann and Ingham, 2000), as well as shh, a marker of posterior fin mesenchyme (Krauss et al., 1993), are undetectable in the presumptive fin mesenchyme of nls homozygotes (Fig. 3C,E,G-I). Exposure to all-trans-RA rescued caudal head mesoderm development at 12 and 17 hpf (Fig. 3B; Table 2), as well as the pectoral fin expression of tbx5.1 at 36 hpf (Fig. 3J,L), consistent with the nls mutation causing a reduction or loss of RA signalling.

To confirm that the molecular lesion in nls/raldh2 is responsible for the nls mutant phenotype, we injected in vitro transcribed raldh2 mRNA into one- to four-cell nls embryos and assayed for the rescue of tbx5.1 expression in the pectoral fin buds, as well as for development of an apical fin fold. The concentration of injected raldh2 mRNA was progressively reduced until overexpression phenotypes, similar to those observed in RA-treated embryos, were no longer observed. This concentration (approx. 500 pg per embryo) was used to assay phenotypic rescue in batches of embryos derived from a cross between nls-homozygotes (Table 3). Partial or complete restoration of tbx5.1 expression was seen in 83% of mutants (30/36 expected nls embryos), indicating that wild-type raldh2 is sufficient to rescue nls embryos.

nls/raldh2 is expressed in early trunk paraxial mesoderm

To investigate the spatial and temporal patterns of nls/raldh2 expression during embryogenesis, we used whole-mount in situ hybridisation. raldh2 mRNA is first detectable at 30%
Fig. 2. Cloning and mapping of zebrafish nls/raldh2. (A) Comparison of the predicted RALDH2 amino acid sequence of human (Accession Number, O94788), mouse (Q62148), chick (O93344) and zebrafish proteins. Zebrafish RALDH2 protein has 79% amino acid identity and 91% similarity to the human protein. Sequence conservation within the first 20 amino acids suggests that RALDH2 proteins may be translated from the first methionine, rather than methionine 20; the 19 N-terminal amino acids for the mouse and chick proteins were derived from their cDNA sequences. Underlined sequences correspond to primers used for RT-PCR cloning. Alignment was performed using PILEUP. (B) Schematic of part of linkage group 7 (LG7), showing the nls/raldh2 map position in relation to SSLP markers. Data were combined with those from the meiotic and LN54 radiation hybrid panels to determine the position of the raldh2 EST and nls. (C) Linkage analysis with SSLP primers on genomic DNA from nls, outcrossed to the WIK strain. In parental strains, primer pair z11119 amplified a single band of 205 base pairs (bp) in the heterozygous nls carriers and two bands of 195 bp and 130 bp, in WIK. In F2 progeny, these primers amplified the 205 bp fragment in only homozygous nls/nls embryos, demonstrating linkage to this marker. Likewise, SSLP marker z4999 amplifies a fragment of 210 bp in heterozygous nls/+ parents, and a fragment of 200 bp in WIK (a fragment of 180 bp is amplified in both strains). These primers amplified only the 210 bp fragment in pools of homozygous F2 nls embryos, whereas sibling embryos contained both fragments. Thus, nls is closely linked to SSLP markers z11119 and z4999, and to z11894 and z8693 (not shown, see Materials and Methods). (D) A single point mutation in all of six independently subcloned cDNAs within the nls open reading frame, generates a missense mutation of Gly204 to Arg204 (asterisk), located in a loop structure. (E) PstI restriction of PCR-fragments, amplified using raldh2-specific primers, of cDNA pools of 40 London wild-type (+/+) and nls/nls embryos, respectively, generates RFLPs of 0.6 and 0.77 kb in nls/nls, and of 1.46 kb in wild type. M, molecular weight marker.

Table 2. Pharmacological rescue of nls embryos by RA treatment

<table>
<thead>
<tr>
<th>A. Rescue of pectoral fin development*</th>
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<tr>
<td>Concentration (M) and sample size</td>
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<tr>
<td>Wild type</td>
</tr>
<tr>
<td>10⁻⁷ (n=122)</td>
</tr>
<tr>
<td>10⁻⁸ (n=126)</td>
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<tr>
<td>10⁻⁹ (n=115)</td>
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<td>10⁻⁶ (n=56)</td>
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<th>B. Rescue of caudal head mesoderm‡</th>
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<tr>
<td>Concentration (M)</td>
</tr>
<tr>
<td>Wild type</td>
</tr>
<tr>
<td>10⁻⁶ (n=56)</td>
</tr>
<tr>
<td>5x10⁻⁷ (n=55)</td>
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*Treated embryos were classified at 36 hpf for the presence (wild type + rescued nls) or absence (nls) of pectoral fin buds. Embryos from a wild type cross treated under identical conditions did not show alterations to pectoral fins upon RA treatment.

‡Embryos were classified by in situ hybridisation at 12 hpf (10⁻⁶ M) and 17 hpf (5x10⁻⁷ M) for distance between krox20 and myoD expression. In all experiments, teratogenic effects were observed when treated with 10⁻⁶ M RA.
region and in the pronephric anlage (Fig. 4E). Somite expression persists throughout segmentation (Fig. 4F,G,I,J) becoming progressively restricted to the somite periphery. By 32 hpf, nls/raldh2 is expressed in subsets of the pharyngeal arch mesenchyme adjacent to the otic vesicle (Fig. 4H,K) and in the posterior mesenchyme of the forming pectoral fins (Fig. 4N). Other sites of expression are the endoderm (not shown), cells in somites 1-3 adjacent to the notochord and spinal cord (Fig. 4L), the dorsal retina and choroid fissure (Fig. 4O), and motoneurones that innervate the pectoral fins (Fig. 4M and data not shown). Surprisingly, we found that in nls embryos the expression of nls/raldh2 is upregulated in somites and in the cervical mesoderm that flanks the posterior hindbrain (Fig. 4P-S), whereas expression is absent in structures that are reduced in the mutant (see below).

Craniofacial skeletal and muscle defects in nls
To determine the later consequences of the embryonic patterning defects in nls for larval development, we examined skeletal and muscle anatomy. In all vertebrates, cells of the cranial mesoderm give rise to the pharyngeal and limb musculature (Noden, 1983; Schilling and Kimmel, 1994), which express the myogenic marker myosin heavy chain (Fig. 5A-D). In zebrafish larvae at 72 hpf, pharyngeal muscles can be identified by their segmental attachments and positions along the dorsoventral axis within each pharyngeal arch (Schilling and Kimmel, 1997). nls mutants develop normal patterns of muscles in the first two arches, the mandibular and hyoid, as well as extraocular muscles, while muscles of the five branchial arches (i.e. dorsal pharyngeal wall muscles, rectus ventralis, transversus ventralis), which derive from posterior head mesoderm, are absent (Fig. 5B,D). Consistent with the molecular data indicating that the identities of anterior somites are unaffected in nls, we found that the sternohyal muscles, which are thought to originate from myoblast populations within the somites 1-3 (Schilling and Kimmel, 1997), are present (Fig. 5D).

Defects in the branchial musculature in nls mutants correlate with defects in the neural crest-derived head skeleton, which can also be identified by their segmental locations (Fig. 5E,F). Alcian Blue staining showed that cartilages of the mandibular and hyoid arches are present in nls, though reduced in size compared with wild type. In contrast, skeletal elements in the branchial arches are reduced or absent (from dorsal to ventral these include the ceratobranchials and hyobranchials in arches

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Total number of embryos</th>
<th>Wild type (% of total)</th>
<th>Weak expression (% of total)</th>
<th>No expression (% of total)</th>
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<tr>
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<td>40</td>
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<td>C</td>
<td>45</td>
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<td>D</td>
<td>40</td>
<td>32</td>
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<td>1</td>
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<tr>
<td>Sum</td>
<td>146 (100%)</td>
<td>112 (77%)</td>
<td>27 (18%)</td>
<td>6 (4%)</td>
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Table 3. Injection of RALDH2 mRNA rescues tbx5.1 expression in nls

Microinjection of RALDH2 mRNA into the progeny of nls/WIK heterozygous parents. Selected embryos with missing or partial expression of tbx5.1 in the pectoral fin buds was assayed at 24 hpf. Putatively rescued animals were genotyped and confirmed as being nls homozygotes using SSLP markers. Expression in pectoral fins was assayed as ‘weak’ when less strong than retinal expression in the same specimen. Percentages rounded to the nearest 1%. Uninjected batches were kept as controls.
Fig. 4. Expression of raldh2 in wild-type and neckless embryos. (A-O) In situ hybridisation to detect raldh2 mRNA in wild types. (A) Expression in marginal cells at 30% epiboly. (B) Dorsal view showing expression in the germ ring of the gastrula at 70% epiboly and absence of dorsal expression. (C) Lateral view at 85% epiboly, showing expression in deep, involuted cells of the hypoblast. (D) Dorsal view at tail bud stage showing expression in the presomitic mesoderm. (E) Dorsal view at the 12-somite stage showing expression in somites and lateral plate mesoderm (arrows). (F) Lateral view at 17 hpf showing expression in the anterior of each somite (arrows denote levels of sections in I,J). (G) Lateral view at 32 hpf, showing expression at somite boundaries, dorsal and ventral somite extremities, and pronephric mesoderm (pmn). (H) Dorsal view at 32 hpf, showing expression in the eyes and in mesenchyme flanking the otic vesicle (m1 and m2), pectoral fin buds (pec) and somites (arrows denote levels of sections in K,L). (I-M) Transverse sections at 17 hpf (I,J), 32 hpf (K,L) and 60 hpf (M), showing expression in the distal myotome but not adaxial cells (I, somite 14-level) (n, notochord), in the periphery of mature somites (J, somite 7 level), adjacent to the otic vesicle (ov) (K), and in pectoral fin and somitic mesoderm adjacent to the spinal cord (sc) (L, somite 3 level). (M) Expression in ventral motoneurones (mn) and dorsal spinal cord neurones at pectoral fin level. (N) Dorsolateral view at 30 hpf, showing expression in posterior pectoral fin buds (blue), double-stained for tbx5.1 (orange). (O) Lateral view at 33 hpf showing expression in the dorsal retina and in the periphery of mature somites (J, somite 7 level), adjacent to the otic vesicle (ov) (K), and in pectoral fin and somitic mesoderm adjacent to the spinal cord (sc) (L, somite 3 level). (P-S) Co-localisation of krox20 and raldh2 at 19 hpf in wild types (P,Q) and in nls (R,S). (P,R) Lateral views showing upregulation of somitic expression in nls embryos, (Q,S) Dorsal views of same embryos showing upregulated raldh2 expression in lateral plate mesoderm in nls mutants (arrows); broadened krox20 expression in the hindbrain distinguishes nls from wild-type embryos.

4-7, and an axial row of basibranchials in arches 4 and 5) whereas these elements are still present in branchial arch 1. The expressivity of this phenotype is dependent on the genetic background, so that in individual outcrossed lines of nls ® all five branchial arches may be deleted. All cartilage elements of the pectoral skeleton are also consistently absent. The defects in branchial arch morphogenesis in nls are mirrored by the lack of formation of endodermally derived pharyngeal pouches that form the prospective gill slits (Fig. 4G,H). Thus, the early defects at the head/trunk boundary during somitogenesis in nls correlate with later defects in the formation of tissues derived from all three germ layers in the pharyngeal segments as well as the pectoral fins that form in this location.

Neural crest defects in nls mutants

To investigate the embryonic basis of defects in the neural crest-derived cartilages of the larva, we analysed markers of both premigratory and migrating neural crest populations. We found no differences in the expression of markers of premigratory crest in nls, such as snail2, which marks most neural crest cells at 12 hpf (data not shown) or krox20, which marks a small group of cells that emigrate from r5 at 13 hpf (see Fig. 1H,J). In contrast, expression of dlx2, which marks all three migrating streams of neural crest and persists in the arch primordia, is disrupted specifically in the most posterior stream that will form the branchial cartilages. Precursors of the mandibular and hyoid arches appeared to migrate normally (Fig. 5I,I’) and dlx2 expression in these arches appeared only slightly reduced by 40 hpf (Fig. 5J,J’). To test the possibility that the branchial neural crest cells undergo apoptotic cell death, we labelled dying cells in nls mutants with whole-mount TUNEL staining or Acridine Orange (Fig. 6C,D). At 24 hpf, we observed increased cell death in nls mutants in the anterior notochord and the third and fourth branchial arches (Fig. 5K,L’), indicating that survival of posterior branchial neural crest cells requires nls.

Hindbrain defects in nls mutants

To investigate whether the mesodermal defects in nls are accompanied by defects in the neurectoderm, we examined the expression of a number of genes that mark specific AP regions of the hindbrain. Expression of krox20, which marks r3 and r5, is initially weaker in r5 at tailbud stage, but subsequently becomes indistinguishable from wild type (Fig. 1G-J). Expression of valentino (Moens et al., 1998), which marks r5/r6, is expanded by 30 μm along the AP axis in nls mutants (Fig. 6A,B). Likewise, eph-b2 expression which marks r7 (Durbin et al., 1998) is slightly expanded in nls as compared to wild type between 14-15 hpf (Fig. 6C,D). Thus, posterior rhombomere territories appear to be established in the appropriate locations in nls mutants, but are slightly enlarged relative to their wild-type counterparts. Consistent with this, we found that hoxb3, which in wild type is strongly expressed in a stripe that includes r5/r6, is expressed in a similar but expanded r5/r6 domain in nls mutants (Fig. 6E-H).
expression, we analysed the distribution of both hoxb4 (Kolm et al., 1997; Gould et al., 1998). To test if the defect in Xenopus, while in the chick, hoxd1 is a direct target of RARα (Kolm et al., 1997; Gould et al., 1998). To test if the defect in hoxb4 expression in nls might be due to a disruption in RAR expression, we analysed the distribution of both RARα and RARY mRNA (Joore et al., 1994). We detected no defects in RARY in nls (not shown); however, RARα expression is downregulated precisely in the region of the neural tube disrupted in mutants. By contrast, RARα expression outside the CNS in the mesoderm appears to be slightly upregulated, and expression in the tailbud is unaffected (Fig. 6M,N). Thus, defects in RARα regulation in nls correlate with the defects in expression of hoxb4.

To examine the consequences of these changes in gene expression for neuronal patterning in the posterior hindbrain we used a combination of neuronal markers and dye labelling techniques. A subset of interneurones express tbx-c (Dheen et al., 1999). In nls, expression in these interneurones is strongly reduced at 36 hpf (Fig. 6P,R). We also retrogradely labelled the large primary reticulospinal interneurones of the hindbrain with rhodamine-dextran by injection into the spinal cord; these are variably disrupted in the caudal hindbrain of nls mutants (data not shown). Likewise, spinal motoneurones that innervate the pectoral fin bud are reduced in nls, as revealed by labelling with the Zn8 antibody (Fig. 6S,T). The loss of hindbrain interneurones, as well as neuronal subpopulations in the rostral spinal cord, correlates with the restricted defects in gene expression at this AP level, such as the failure to initiate hoxb4 expression. Thus, neural defects are milder compared with complete loss of RA signalling, as in the Raldh2−/− mouse, where the caudal hindbrain is absent due to misspecification to a more rostral fate.

RALDH2 is required in the mesoderm for initiation of hoxb4 expression in neural ectoderm

To determine the cellular requirement for nls function, we transplanted cells from embryos labelled with a lineage tracer into unlabeled hosts at the gastrula stage (Fig. 7). First, we asked if nls cells can contribute to tissues disrupted in the nls
mutation, such as the posterior head mesoderm, and axial, paraxial and posterior hindbrain. In an otherwise wild-type embryo, donor derived nls cells were found to be able to spread widely throughout the neural ectoderm of the hindbrain and anterior spinal cord (Fig. 7A,B; Table 4). In other cases, mutant cells readily populated regions of the paraxial (Fig. 7A') and axial (Fig. 7C) mesoderm of the head (Table 4). Likewise, transplants of wild type mesoderm into nls mutants were able to populate the anterior somites (Fig. 7F).

We then used such mesodermal grafts to determine whether the defects in hoxb4 expression in the neural tube of nls mutants (Fig. 7D,E) might reflect defects in a non-autonomous signal from surrounding mesoderm that requires nls/raldh2. With reference to fate maps of head mesoderm (Kimmel et al., 1990), we transplanted mesodermal precursors from biotin-dextran labelled wild-type donors into unlabeled mutant hosts at the gastrula stage. In many cases these transplanted cells spread widely along one side of mutant host embryos and often form muscles in the anterior somites adjacent to the region in which hoxb4 is normally expressed (Fig. 7F-H). In 71% of cases in which wild-type cells populated this region in nls, we observed a partial recovery of early hoxb4 expression several hours before 15 hpf (Table 4). Control transplants of mutant mesoderm into nls hosts had no such effect on hoxb4 expression (Fig. 7G). Thus,
the activity of nls/raldh2 in paraxial mesoderm is necessary for hoxb4 expression in the adjacent neural ectoderm.

**DISCUSSION**

Using a marker-based screening strategy we have identified nls, a new mutation in the zebrafish that disrupts patterning along the AP axis of the embryo. Linkage analysis, together with morpholino phenocopying and phenotypic rescue by RA and raldh2 mRNA injection, strongly suggest that a missense mutation in a conserved glycine residue of the RA metabolic enzyme RALDH2, causing a reduction in RA activity underlies the nls phenotype. Mutant embryos reveal a complex requirement for nls/raldh2 in the formation of both axial and paraxial mesoderm, survival of neural crest cells and specification of cells in the hindbrain at the head/trunk boundary. By generating genetically mosaic embryos we have adduced in vivo evidence for mesodermally derived RA-dependent signals that pattern the CNS. We suggest a model in which RA production in the paraxial mesoderm underlies both short- and long-range effects of RA signalling on the head mesoderm and CNS. The model predicts a direct local role for RARα in hoxb4 regulation in the neural tube in response to RA-signalling from the forming somites. In addition, it postulates a limited influence of RA signalling not only on the neural ectoderm, but also on the head mesoderm, with important secondary consequences for hindbrain and neural crest patterning.

The combination of hindbrain, neural crest and limb defects characteristic of nls mutant embryos is similar to that caused by targeted inactivation of Raldh2 in the mouse (Niederreither et al., 1999), as well as by VAD in the quail (Maden et al., 1996; Gale et al., 1999) and rat (White et al., 2000) embryos. The hindbrain defects in nls embryos are, however, less severe than in these other cases; in both the mutant and VAD embryos, rhombomere-specific characteristics caudal to r4 are disrupted whereas in nls, posterior rhombomeres appear slightly expanded and only neurones near the hindbrain-spinal cord boundary are disrupted. This phenotype is reminiscent of the milder forms of VAD in rat embryos (White et al., 2000) and of the partial rescue of Raldh2-/- mice by maternal application of RA (Niederreither et al., 2000), and suggests that the posterior hindbrain and anterior spinal cord are most sensitive to a reduction in RA levels.

The fact that the nls phenotype is closer to the effects of attenuation, rather than elimination of RA signalling in amniote embryos could be explained if the nls/26 allele behaves as a hypomorph, the mutant protein retaining residual enzymatic activity. Against this, however, structural modelling predicts that the glycine-to-arginine substitution found in nls/26 would result in a complete loss of activity, a view supported by our finding that nls is precisely phenocopied by morpholino-mediated translational inhibition of the raldh2 gene that we have cloned. This raises the possibility that zebrafish may possess a second raldh2 gene that can partially compensate for the loss of nls/raldh2, a possibility consistent with the finding that many teleost genes are duplicated (Amores et al., 1998).

A restricted requirement for nls/raldh2 at the head/trunk boundary

RA has been proposed to act as a graded posteriorising signal throughout the AP axis of the CNS (reviewed by Gavalas and Krumlauf, 2000). Mutation of nls/raldh2 and reduction of RA in zebrafish through pharmacological inhibition of aldehyde dehydrogenases (Perz-Edwards et al., 2001), however, suggest that RA acts in a more localised manner. From tail bud stages onwards, nls/raldh2 expression is confined to trunk and tail mesoderm, yet defects in nls are largely in the posterior head. Thus, nls/raldh2 and, by inference, RA produced in presumptive somites may act at only a short distance and at high concentrations. Perhaps only cells in close proximity to the source of RA are able to respond, while others require different posteriorising signals, such as members of the fibroblast growth factor and Wnt families.

Defects in paraxial mesoderm in nls/raldh2 mutants may secondarily cause its hindbrain defects through loss of local posteriorising induction (Itasaki et al., 1996). nls mutants lack mesoderm between the level of r5 and somite 1 at the beginning of somitogenesis, suggesting that nls/raldh2 activity must be

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**Table 4. Fates of transplanted cells in mosaic embryos**

<table>
<thead>
<tr>
<th>Transplant and sample size (n)</th>
<th>Phenotypes</th>
<th>Fates scored</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Donor</td>
<td>Host</td>
</tr>
<tr>
<td>Paraxial mesoderm (n=51)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=21)</td>
<td>Wild type</td>
<td>Wild type</td>
</tr>
<tr>
<td>(n=14)</td>
<td>Wild type</td>
<td>nls</td>
</tr>
<tr>
<td>(n=10)</td>
<td>nls</td>
<td>Wild type</td>
</tr>
<tr>
<td>(n=6)</td>
<td>nls</td>
<td>nls</td>
</tr>
<tr>
<td>Axial mesoderm (n=21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=11)</td>
<td>Wild type</td>
<td>Wild type</td>
</tr>
<tr>
<td>(n=6)</td>
<td>Wild type</td>
<td>nls</td>
</tr>
<tr>
<td>(n=4)</td>
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<td>nls</td>
</tr>
<tr>
<td>(n=6)</td>
<td>nls</td>
<td>Wild type</td>
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</tbody>
</table>

Transplants were scored at 12–13 hpf for hoxb4 expression by in situ hybridisation and at 24 hpf for contributions to mesodermal or neural derivatives at the head/trunk boundary.
required during gastrulation; this correlates well with the early zygotic expression of nls/raldh2 in the germ ring of gastrulating embryos, which forms the mesendoderm (Kimmel et al., 1990). In VAD quail embryos, a similar defect has been accounted for by apoptosis of mesodermal cells within the first somite during a brief period in early somitogenesis (Maden et al., 1997). Such patterns of apoptosis were not observed, however, during gastrulation stages in nls. Moreover, the mesodermal deficiency in nls/raldh2 occurs in a much broader region anterior to the somites, and includes both axial and paraxial cells. Our molecular analysis rules out the possibility that the posterior head mesoderm is transformed into more caudal tissues, as anterior-most somites in nls develop with their normal identities. RA may instead be involved in maintaining cell proliferation at the head/trunk boundary, though we currently have no direct evidence for such an effect.

Surprisingly, we find defects not only in the paraxial mesoderm of nls, but also in the notochord, a structure that is known to influence patterning of the overlying neural tube. Although the notochord has not been implicated specifically in AP patterning of the hindbrain, recent evidence in zebrafish has shown that some Hox genes are expressed in AP restricted domains near the head/trunk boundary (Prince et al., 1998b). nls/raldh2 is not expressed in the notochord, suggesting that the effects on its development are non-autonomous, which is supported by our mosaic results. Our data suggest that, similar to its restricted influences on the posterior hindbrain, RA signalling from the somites acts locally on axial as well as paraxial mesoderm.

Correlated with these spatially restricted phenotypes in the mesoderm and nervous system, nls mutants later exhibit defects in branchial arches. Again these are confined to the posterior head and recapitulate the branchial hypoplasia in chick embryos treated with pan-RAR antagonists and in Raldh2-deficient mice (Niederreither et al., 1999; Wendling et al., 2000). In this case, however, Raldh2 appears to be required for cell survival in the neural crest-derived skeleton. Crest cells are apoptotic in the pharyngeal primordia in nls, as they are in VAD and Raldh2−/− mice (Maden et al., 1996; Niederreither et al., 1999). The correlation between the loss of posterior head mesoderm and posterior arches in nls mutants further suggests that apoptosis may be a secondary consequence of earlier defects in posterior head mesoderm and/or endoderm. Patterning of cranial neural crest has been shown to respond to both mesenchymal-mesenchymal interactions with mesoderm, as well as epithelial-mesenchymal interactions with surrounding endoderm in the arches (Trainor and Krumlauf, 2000; Tyler and Hall, 1977). Alternatively, crest cells may require nls/raldh2 directly for their survival, as crest cells appear to be particularly sensitive to alterations of RA levels (Ellies et al., 1997). RARα/RARβ double mutant mice have hypoplastic posterior branchial arches similar to those seen in ablations of postotic neural crest in chick embryos (Dupe et al., 1999; Ghyselinck et al., 1997), despite the normal generation and migration of crest. Thus, the developmental deficiencies observed in the pharyngeal region of nls are likely to be caused by local defects in postotic mesoderm and endoderm, rather than by a long-range graded requirement for RA.

Another striking aspect of the nls phenotype is the complete lack of pectoral fin buds. nls/raldh2 is required early during pectoral fin induction locally in the fin field, as one of the earliest markers of the fin field, tbx5.1, is not expressed in the lateral plate mesoderm in nls mutants. This phenotype correlates well with expression of nls/raldh2 in this region of the mesoderm between the 6- and 12-somite stage (12-15 hpf). raldh2 expression precedes (not shown) and is then maintained during outgrowth of the apical fold at the posterior of the pectoral fin bud (Fig. 3R). As we have shown for hoxb4 in the neural tube, nls/raldh2 may also be required for the expression of Hox genes in the prospective fin field, thus being involved in setting up limb position along the AP axis of the lateral plate mesoderm (Cohn et al., 1997). A common model of limb-field determination proposes a function for fibroblast growth factors (FGFs) as limb inducers and locates the source of limb inducing activity in the intermediate mesoderm (reviewed in Martin, 1998). RA induces FGF or generates competence of the flank to respond, and FGFs are capable of inducing ectopic limb buds in the lateral plate mesoderm of the chick flank. raldh2 may thus be required for the local induction of an FGF or another inducing signal.

**The role of the mesoderm in neural patterning**

A large body of evidence has previously implicated RA in mediating signals from the mesoderm to the neural tube. In *Xenopus*, tissue recombination experiments have demonstrated a requirement for RARs in the ectoderm for induction of Hox gene expression through mesoderm-derived signals (Kolm et al., 1997). Conversely, upregulation of RA during somitogenesis has been shown to be required for cultured paraxial mesoderm to induce cells of spinal cord fate, while loss of this inducing capacity by freeze-thawing paraxial cells can be restored through administration of RA (Muhr et al., 1999). In line with these data, transgenic RARβ-lacZ reporter constructs (where the RA-responsive element of the RARβ gene has been fused to the lacZ reporter gene) (Balkan et al., 1992; Mendelsohn et al., 1991; Rossant et al., 1991; Zimmer, 1992), as well as similar reporter constructs in zebrafish (Marsh-Arstrong et al., 1995; Perz-Edwards et al., 2001), are activated in the neural tube. Such activation of neural RA-responsive genes can be explained by diffusion of RA from the paraxial mesoderm. While the lack of detectable nls/raldh2 mRNA in the prospective neural tube during gastrulation and segmentation stages is highly suggestive of a non-autonomous action of RA, the results of our mosaic analyses provide the first conclusive evidence for this mode of action. Transplantation of wild-type cells into the somitic mesoderm of nls embryos restores early hoxb4 expression, whereas that of nls cells in the same position does not. Likewise, nls ectodermal cells intercalate normally into a wild-type CNS in the hoxb4-expressing domain, suggesting that nls/raldh2 is not required for cells to take on the identity of this region of the neural tube.

Our analysis of hoxb4 expression in nls reveals that, as in the amniote embryo (Gould et al., 1998), the zebrafish hoxb4 gene follows a biphasic mode of transcriptional regulation. The first phase establishes neural expression and depends on raldh2 activity, while the second phase is independent of raldh2. These regulatory steps are linked to neural promoter elements that regulate hoxb4 expression in chick and mouse embryos, one of which acts before rhombomere formation and one of which acts later in maintenance of expression. Activation of the early neural enhancer is mediated by RA response elements.
expression. Similarities in the expression of a result from a failure to initiate this first phase of interneurones and neurones in the anterior spinal cord may in the avian neural tube blocks induction of RAR (Gould et al., 1998).

For stimulating discussions and A. Meyer for his generous support.


REFERENCES


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Fig. 8. Model for the role of nsl/raldh2 in patterning the posterior head and neural tube. High local concentrations of RA are required for patterning of posterior axial and paraxial head mesoderm, survival of posterior neural crest cells, and induction of gene expression in the neural tube. Dorsal schematic view of an idealised zebrafish embryo during early segmentation stages with anterior to the left.

Our model of short-range RA-dependent interactions between the mesoderm and neural tube (Fig. 8) is based on our genetic analysis in zebrafish is consistent with earlier results obtained from experimental manipulation of the chick embryo. This model makes testable predictions and thus provides a framework for future experiments, both to explore the exact source and graded nature of the RA signal and the nature of the responses of neural cells to RA during vertebrate development.

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