Development 128, 2199-2208 (2001)
Printed in Great Britain © The Company of Biologists Limited 2001
DEV2719

Hindbrain patterning involves graded responses to retinoic acid signalling

Valérie Dupé and Andrew Lumsden*

MRC Centre for Developmental Neurobiology, King's College London, Guy's Campus, London SE1 1UL, UK
*Author for correspondence (e-mail: andrew.lumsden@kcl.ac.uk)

Accepted 9 April 2001

SUMMARY

Several recent studies have shown that retinoic acid signalling is required for correct patterning of the hindbrain. However, the data from these studies are disparate and the precise role of retinoic acid signalling in patterning the anteroposterior axis of the neural tube remains uncertain. To help clarify this issue, we have cultured a staged series of chick embryos in the presence of an antagonist to the all three retinoic acid receptors. Our data indicate that retinoic acid is the transforming signal involved in the expansion of posterior hindbrain structures. We find that the hindbrain region of the neural tube down to the level of the sixth somite acquires the identity of rhombomere 4 when retinoic acid signalling is blocked. Specification of future rhombomere boundaries has a retinoic acid dependency between stage 5 and stage 10$^+$ that is lost progressively in an anterior-to-posterior sequence. Furthermore, the application of various concentrations of antagonist shows that successively more posterior rhombomere boundaries require progressively higher concentration of endogenous retinoic acid for their correct positioning, a result that strengthens the hypothesis that a complex retinoid gradient acts to pattern the posterior hindbrain. Our dissection of early retinoic acid functions allows us to re-interpret the wide disparity of hindbrain phenotypes previously observed in various models of retinoic acid deficiency.

Key words: Anteroposterior patterning, Hindbrain, Segmentation, Retinoic acid, Morphogen, Chick

INTRODUCTION

The effects of retinoids on hindbrain development, which include posteriorization of the anterior hindbrain, have long been indicated by the actions of exogenous retinoids (Durston et al., 1989; Conlon, 1995; Marshall et al., 1992). Direct evidence that endogenous retinoids are required for hindbrain patterning has been slower to emerge, and has come first from embryos produced by quail hens subjected to complete dietary vitamin A (retinol) deficiency (VAD). In these embryos, the caudal hindbrain region is mis-specified such that rhombomeres (r) 4-7 apparently fail to develop (Maden et al., 1996; Maden et al., 1997; Maden et al., 1998b; Gale et al., 1999).

The spatial and temporal nature of retinoid influence on gene expression depends on a large number of parameters. These include the local availability of retinoic acid (RA) receptors (RARα, β, γ and RXRα, β, γ) which transduce the retinoid signal, enzymes necessary for RA synthesis and/or catabolism, and intra- or intercellular carrier proteins (Chambon, 1996; Duester et al., 1998). The distribution of RA in the early embryo correlates with the opposing action of the two main RA metabolic enzymes: Raldh2 (Maden et al., 1998a; Niederreither et al., 1997; Berggren et al., 1999), which converts retinaldehyde into RA, and Cyp26, a cytochrome P450 that oxidatively inactivates RA (Swindell et al., 1999; de Roos et al., 1999; Fujii et al., 1997). *Raldh2* is strongly expressed in the somites adjacent to the caudal hindbrain and cervical spinal cord (Swindell et al., 1999; Berggren et al., 1999), whereas *Cyp26* is strongly expressed in the fore- and midbrain region of the neural plate (Swindell et al., 1999). It has yet to be shown that a posterior-to-anterior gradient of RA exists during hindbrain patterning, but the expression of *Raldh2* and *Cyp26* is suggestive of a source and sink that would be required to set up such a gradient.

Recent genetic analysis in the mouse has revealed that *Raldh2* (*Aldh1a2* – Mouse Genome Informatics) is crucial for regulating patterning events in the posterior hindbrain. However, the phenotype of *Raldh2* knockout mice differs from that described for the VAD quail, as r4 molecular markers are still expressed in these embryos (Niederreither et al., 1999; Niederreither et al., 2000). In female rats, complete VAD leads to reproductive failure, whereas a partial deficiency leads to variable hindbrain defects in the progeny (White et al., 1998; White et al., 2000). Targeted disruption of murine RAR genes also provides information on the roles of RA in anteroposterior patterning. Interestingly, the RARα:RARβ compound mutant embryos have normal anterior rhombomeres (r1-r4), but posterior hindbrain markers expand in a posterior direction (Dupé et al., 1999). Similarly, overexpression of dominant negative retinoic acid receptors partially anteriorises the posterior rhombomeres of *Xenopus* embryos (Blumberg et al., 1997; Kolm et al., 1997; Van der Wees et al., 1998). Targeted disruption of *Hoxa1* and *Hoxb1* RA response elements (RAREs) reveal further levels of complexity in the actions of RA in anteroposterior

patterning (Dupé et al., 1997; Studer et al., 1998). Collectively, these results are not easy to interpret, given the wide disparity of phenotype. In the case of both knockout and dominant negative receptor experiments, uncertainty arises on account of the incomplete inactivation of RA signalling. For both of these approaches, as for the VAD embryos, uncertainty also surrounds the timing of an RA requirement for different aspects of patterning, as RA signalling is depleted throughout development. An approach that could potentially resolve both uncertainties would be to block RA signalling completely or partially at successive, defined stages of development.

Thus, in order to investigate the diverse roles of RA signalling during anteroposterior nervous system patterning, and to explore the exact timing of RA actions, we have used a culture system in which a staged series of chick embryos are exposed to various concentrations of an antagonist that blocks activation of all three RAR isotypes (Wendling et al., 2000). We find that the entire posterior hindbrain develops as a default r4 in absence of RA. Consequently, RA is the transforming signal involved in the specification of posterior hindbrain structures from stage 5 to stage 10^+. These actions take place through the regulation of hindbrain segmentation genes in an environment of a retinoid activity gradient. More generally, we have made a thorough analysis of the early RA functions during chick hindbrain patterning.

MATERIALS AND METHODS

Roller-tube culture and antagonist treatments

Fertile hens eggs were incubated in a humidified 38°C room to the desired stages. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). From stage 10, the transitory first somite is not counted. Embryos were collected at various times between stage 4 and stage 12 (16 somite stage) and cultured as described (Connolly et al., 1995). Briefly, egg yolks complete with blastoderms were transferred to a dish of Pannet and Compton saline, and, having been freed gently from the vitelline membrane with fine forceps and washed from the yolk with a pipette, each blastoderm was placed hypoblast side up. Using a pair of forceps, one edge is gently grasped and the blastoderm folded along the longitudinal axis of the embryo to form a 'pitta bread' configuration with hypoblast/endoderm on the inside and epiblast/neural plate outside. The free edges were sealed by cutting with iridectomy scissors along a line passing just within the area opaca. Folded and sealed embryos were transferred to 5ml plastic bijou bottles containing 500 μl of Liebovitz tissue-culture medium. The lightly capped bottles were placed on a roller apparatus rotating at 30 revs/minute, inclined at a angle of about 10°, in a 38°C incubator. Development then proceeded normally for 24-30 hours.

The pan-RAR synthetic retinoid antagonist BMS493 (Bristol-Myers-Squibb, Princeton, NJ, USA), diluted in ethanol, was added to the Liebovitz medium at various concentration (10^{-5}M to 10^{-8}M). In control embryo cultures, vehicle (ethanol) was added at the same final dilution.

Molecular analyses

Whole-mount in situ hybridisation with digoxigenin-labelled riboprobes was performed as described by Wilkinson (Wilkinson, 1992), using probes from template plasmids produced in our laboratory (for Hoxb1, follistatin, Cepu-1 and Hoxa1; Guthrie et al., 1992; Graham and Lumsden, 1996; Jungbluth et al., 2001) or kindly provided by D. Wilkinson (Krox-20; Wilkinson et al., 1989), J. Dodd (Wnt-8c; Hume and Dodd, 1993) and I. McKay (MafB/kr).

RESULTS

Antagonist treatment selectively impairs hindbrain development in a stage-dependant manner

To characterise the time at which RA-dependant hindbrain patterning occurs, chick embryos were treated with an RA antagonist that blocks activity of the three RARs (Bristol Myers Squibb compound 493). The antagonist was applied to embryos at various stages in roller-tube culture at a concentration of 5×10^{-6} M. The molecular identity of rhombomeres 3, 4 and 5 was evaluated by looking at *Krox-20* and *Hoxb1* levels. *Krox-20* provides a particularly useful marker for analysis of hindbrain segmentation as its expression precedes the appearance of rhombomere boundaries. In control embryos, *Krox-20* is expressed in r3 as early as stage 8^+ and in r5 by stage 9^+ (Wilkinson et al., 1989). To assess r6 and r7 molecular identities, we monitored the expression of *follistatin* and *MafB/kr*. In the chick CNS at stage 11, *follistatin* is expressed at high levels in r2, r4, r5 and r6, more weakly in r7, and absent from more posterior regions of the neural tube (Graham and Lumsden, 1996). *MafB/kr* is expressed in r5 and r6 as early as stage 8 (Eichmann et al., 1997).

Exposure of stage 4 (full-length primitive streak) and stage 5 (head process) embryos to a high concentration (5×10^{-6} M) of antagonist resulted in all embryos developing a shorter anteroposterior axis with smaller and poorly defined somites compared with controls. Treated embryos examined at stage 11 show expanded *Krox-20* expression in the r3 domain, with a posterior limit that is not well defined, and lack the characteristic stripe of expression in r5 as well as the neural crest cells (NCC) migrating from r5/r6 (Fig. 1a). Furthermore, the characteristic stripe of *Hoxb1* expression in r4 disappears, with *Hoxb1* being continuously expressed from a region just rostral to the first somite down to the posterior neuropore (Fig. 1k,l). Corresponding with the posterior limit of *Krox-20* expression, the rostralmost region of *Hoxb1* expression lacks a sharp border and contains patches of unlabelled cells. The characteristic expression of *MafB/kr* in r5 and r6 is absent (compare Fig. 1i with 1j). Identical results were obtained using a higher concentration of antagonist (10^{-5} M).

Embryos treated at stage 6 (headfold) appear to have normal r3 *Krox-20* expression, but lack the r5 stripe (Fig. 1b), suggesting that establishment of the r3/r4 boundary is no longer sensitive to RA deficiency at this stage. As before, *MafB/kr* is not expressed and *Hoxb1* is continuously expressed from r3/r4 down to the posterior neuropore (Fig. 1m).

When treated with the antagonist at stage 6^+, embryos have normal r3 *Krox-20* expression but display patches of *Krox-20*-positive cells in the r5 region, although these patches extend posteriorly beyond the expected r5 territory (Fig. 1c,d). This patchy expression of *Krox-20* may represent the appearance of r5-like cells. Indeed, in embryos treated at stage 6^+, we observed patches of *Hoxb1*-negative cells at the expected axial level of the r5 region (compare Fig. 1n with Fig. 1c,d). *Hoxb1* expression domains such as this are not seen when embryos are treated slightly earlier (stage 6). *MafB/kr* expression also appears in scattered patches, but the staining does not appear

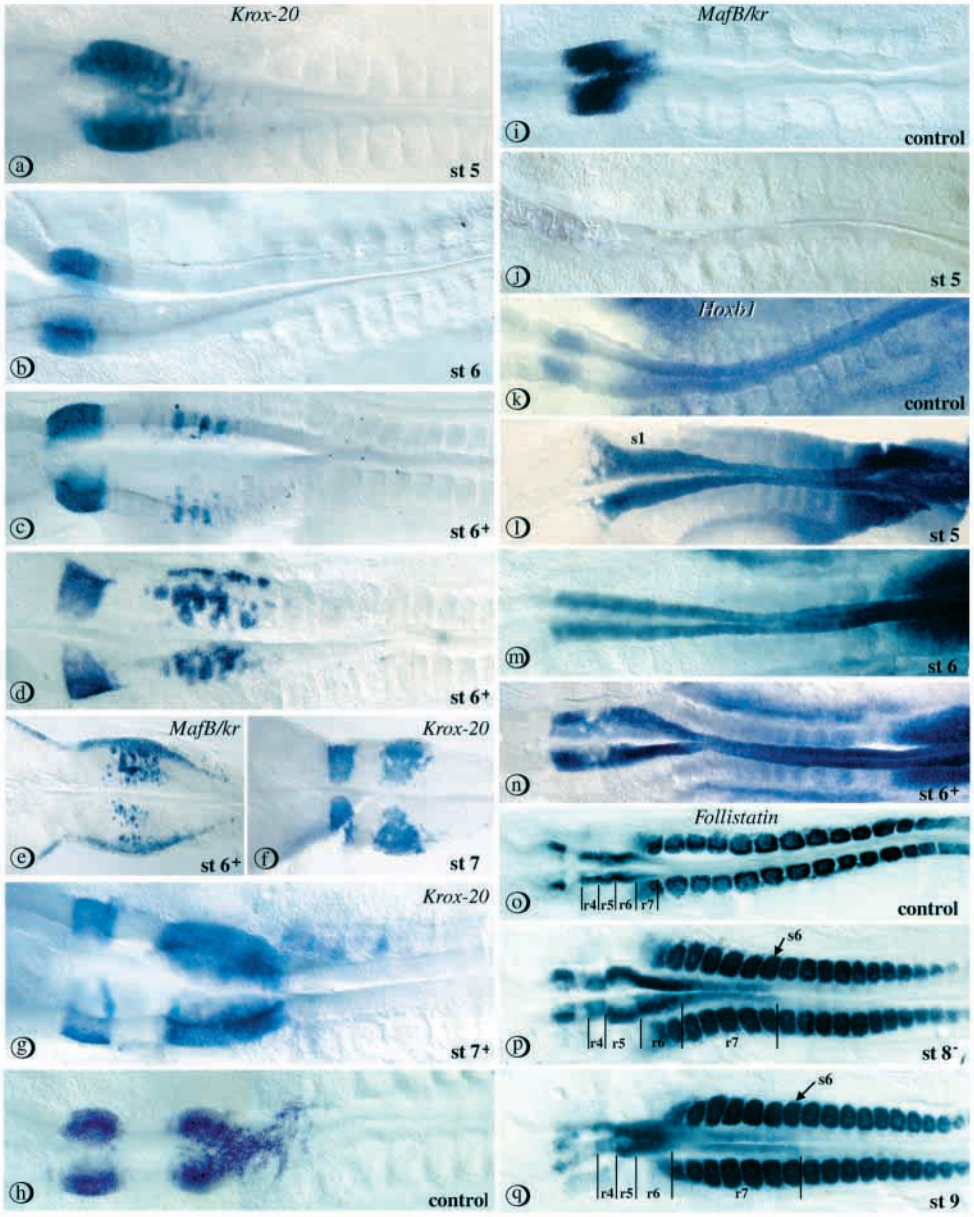


Fig. 1. BMS493, a retinoid antagonist, treatment impairs hindbrain development in a stage-dependant manner. Dorsal view of whole-mounts (a-c,g-k,m-q) or flat-mounted hindbrains (d-f,l) of control (h,I,k,n) and antagonist-treated (5×10^{-6} M) chick embryos at stage 5 (a,j,k), stage 6 (b,m), stage 6^+ (c-e,n), stage 7 (f), stage 7^+ (g), stage 8^- (p) and stage 9 (q). The embryos were collected after approximately 24 hours of culture and the expression of *Krox-20* (a-d,f-h, stage 11), *MafB/kr* (e,i,j, stage 9), *Hoxb1* (k-n, stage 11) and *follistatin* (o-q, stage 12) was analysed. Stages indicated at the bottom-right of the pictures correspond to the stage of treatment. s1, first somite; s6, sixth somite; r4-r7, rhombomeres 4 to 7.

more extended than that of *Krox-20*, indicating that, at this stage of treatment, r6-like identity is absent (Fig. 1e).

Embryos treated at stage 7 (one-somite stage) display a normal r3 and the number of *Krox-20*-positive cells in the most posterior domain is increased; their anterior limit corresponds to a normal anterior r5 boundary, whereas their posterior limit extends beyond the expected location of r5/r6 boundary (Fig. 1f). At this stage of treatment, *Hoxb1* expression in r4 is normal (data not shown).

Treatment of stage 7^+ and 9^- embryos (two- to six-somite stages; n=12) does not affect the size of r4, but r5 is markedly enlarged, as shown by the expression of *Krox-20* (Fig. 1g). *follistatin* expression at stage 13 in embryos treated between stage 7^+ and 9^- confirms the expansion of the morphologically recognisable r5, and shows abnormal caudal expansion of putative r6 and r7 (Fig. 1p). However, *follistatin* expression is not detected in the neural tube posterior to the sixth somite. *MafB/kr* expression is also extends beyond the expected location of r6/r7 boundary (data not shown).

Embryos treated between stage 9 and 9^+ (seven- to eight-somite stages) have normal expression of *Krox-20* in r3 and r5 but, as shown by *follistatin* expression, they have an enlarged r6 and r7 (Fig. 1q). Again, this expanded expression in the neural tube is not found beyond somite 6.

In embryos treated between stage 10^- and 10^+ (nine- to 11-somite stages), only the r7 is expanded, reaching the 3rd somite, as *MafB/kr* expression is normal and the characteristic weak expression domain of *follistatin* in r7 is enlarged (data not shown). From stage 11^-, the presence of antagonist in the culture medium no longer affects patterning of the hindbrain.

These data implicate RA in the specification of rhombomere boundaries from stage 5 to 10^+. Furthermore, this RA dependency is lost progressively from anterior to posterior as development proceeds.

Establishment of hindbrain boundaries is dependent on RA concentration

To test the idea that a graded RA concentration could mediate hindbrain patterning, we applied various concentrations of the antagonist to cultured embryos at stage 4, and used *Krox-20*, *follistatin*, *MafB/kr* and *Hoxb1* to reveal the size of rhombomeres. As a function of the concentration used, we have obtained a range of phenotypes that we have classified in six arbitrary types from very affected embryos, as described previously for a 5×10^{-6} M concentration, to normal embryos (Table 1).

As described above, when embryos are treated with a 5×10^{-6} M (or 10^{-5} M) concentration of the antagonist at stage 4, r3 is enlarged and r5-r6 are absent (Table 1). The same phenotype

Table 1. Hindbrain phenotypes observed after antagonist treatment at various concentrations

	r3 enlarged r5 absent	r3 normal r5 absent	r3 normal r4 and r5 very enlarged	r3 normal r4-r7 slightly enlarged	r3 normal r4 slightly enlarged r5-r7 normal	Normal hindbrain segmentation	Total embryos analysed
5×10^{-6} M	**100%**	0%	0%	0%	0%	0%	33
2.5×10^{-6} M	**53%**	**47%**	0%	0%	0%	0%	17
10^{-6} M	15%	33%	**45%**	7%	0%	0%	33
5×10^{-7} M	0%	17%	35%	**41%**	8%	0%	34
2.5×10^{-7} M	0%	0%	14%	27%	**41%**	18%	49
10^{-7} M	0%	0%	0%	10%	**46%**	**44%**	39
5×10^{-8} M	0%	0%	0%	0%	14%	**86%**	14
10^{-8} M	0%	0%	0%	0%	0%	**100%**	12

A decreasing concentration of RA antagonist allows the progressive establishment of more posterior rhombomeres. Figures are percentage of embryos showing each category of defect.

appears in 53% of the embryos treated with slightly lower antagonist concentration (2.5×10^{-6} M). However, the remaining 47% have a normal r3, while r5-r6 are absent, as indicated by *Krox-20* and *MafB/kr* expression (this phenotype is similar to the one observed when embryos are treated with 5×10^{-6} M antagonist at stage 6; Fig. 1b and Table 1). This result suggests that a major RA signalling deficiency is required to produce an enlarged r3.

At a lower concentration of antagonist (10^{-6} M), we observed new phenotypes, the most significant of which was the appearance of *Krox-20*-expressing cells in ectopic posterior positions. Indeed, of the embryos treated at 10^{-6} M, 45% developed either patches of *Krox-20*-expressing cells or a continuous domain of *Krox-20* expression with poorly defined boundaries (Fig. 2a, Table 1). The anterior limit of this ectopic *Krox-20* expression lies as far posterior as the level of the second somite, whereas the posterior limit can reach the level of the 6th somite. We never observed *Krox-20* expression in the neural tube posterior to somite 6. This ectopic expression of *Krox-20* may represent the appearance of a greatly enlarged r5-like territory in a more caudal region. In this case, the large unstained region of the hindbrain between the r3 caudal border and the level of somite 2 would correspond to r4. Indeed, we found *Hoxb1* to be expressed throughout the expanded *Krox-20*-negative region between r3/r4 and somite 2, and to be absent from the expanded *Krox-20*-positive domain between

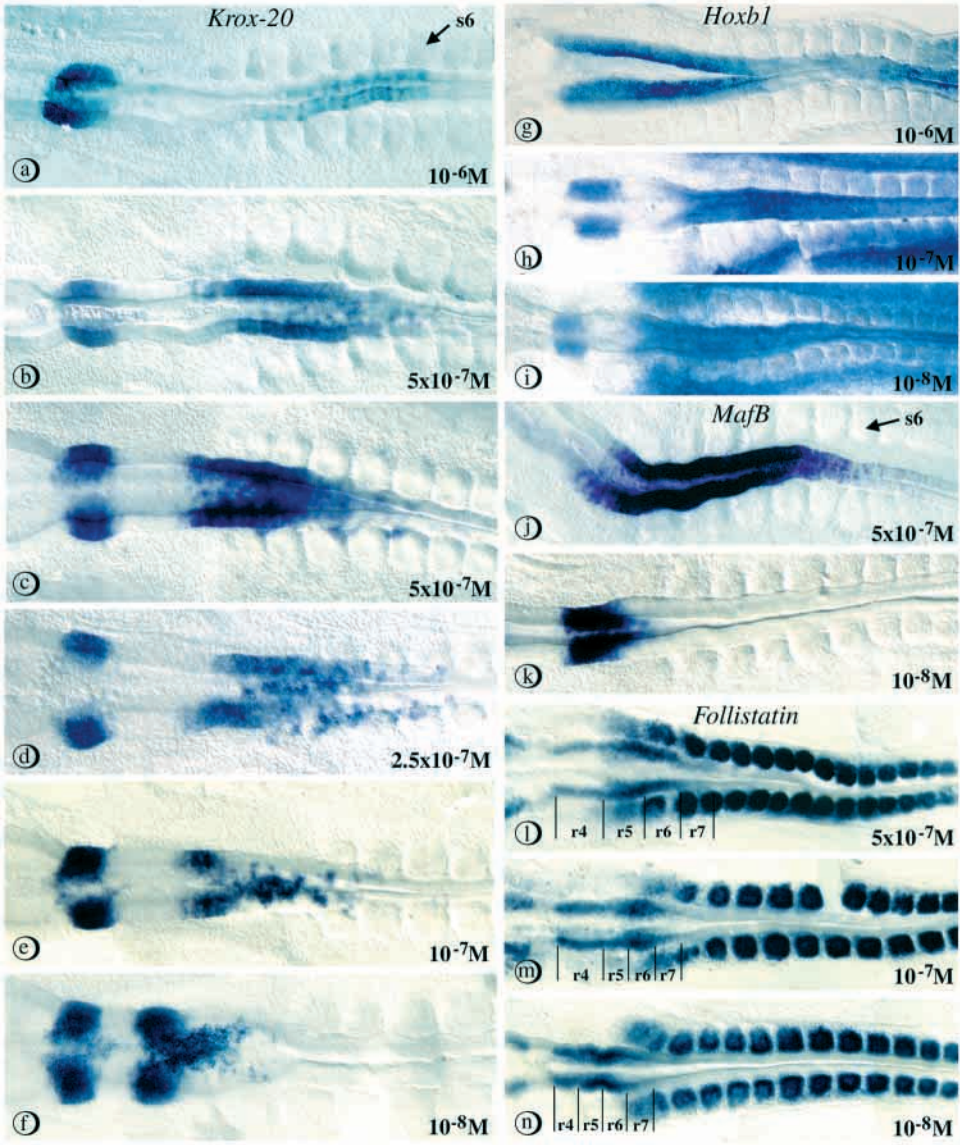


Fig. 2. Establishment of hindbrain boundaries is dependent on retinoic acid concentration. Dorsal view of whole-mounts of BMS493-treated chick embryos at stage 5 at the following concentration: 10^{-6} M (a,g), 5×10^{-7} M (b,c,j,l), 2.5×10^{-7} M (d), 10^{-7} M (e,h,m) and 10^{-8} M (f,i,k,n). Whole-mount in situ hybridization was performed on embryos using digoxigenin-labelled probes for *Krox-20* (a-f, stage 11), *Hoxb1* (g-i, stage 11), *MafB/kr* (j,k, stage 10) and *follistatin* (l-n, stage 12). s6, sixth somite. r4-r7, rhombomeres 4 to 7.

somite 2 and somite 6 (Fig. 2g). Therefore, the elongated anterior stripe of *Hoxb1* expression does correspond molecularly to an enlarged r4.

At 5×10^{-7} M antagonist, the proportion of embryos with an enlarged r4 associated with an enlarged r5 decreased to 35%, whereas, in 41% of the embryos r4 and r5 were still enlarged but to a lesser extent, both being approximately twice their normal size (Fig. 2b,c). In these embryos, however, the expression domain of *MafB/kr* is dramatically enlarged, with neural tube expression extending from somite 1 to somite 6 (Fig. 2j). Thus, at the molecular level, this lower concentration of antagonist still increases the AP length of r4 and r5 but has a greater effect on enlarging r6 and r7 (Fig. 2b,c,j,e). Again, the affected region extends as far caudal as somite 6 but no further (Fig. 2j).

When the concentration of antagonist was reduced to 2.5×10^{-7} M, all of the embryos had a more clearly defined r5 and the proportion of embryos with slightly enlarged r4, r5, r6 and r7 decreased to 27%. Interestingly, at this concentration, 41% of the embryos had a slightly enlarged r4, whereas r5, r6 and r7 were of normal size (Table 1). This is shown by expression domains of *Krox-20*, *Hoxb1* and *follistatin*. Furthermore, some embryos developed bilaterally asymmetric phenotypes, with an enlarged r4 and r5 on one side of the hindbrain and a less enlarged r4 and almost normal r5 on the other (Fig. 2d).

At 10^{-7} M antagonist, 46% of the embryos had a slightly enlarged r4 (Fig. 2e,h,m), whereas 44% had a normal hindbrain segmentation; at 5×10^{-8} M antagonist, only 14% had a slightly enlarged r4, whereas 86% had a normally segmented hindbrain; at 10^{-8} M antagonist, we saw no effects at all (Table 1).

RA acts as a posteriorising signal

During the formation of rhombomere boundaries, *Hoxb1* is selectively expressed in the developing r4 in rodents, quail and chick (e.g. Sundin and Eichele, 1990; Murphy et al., 1989). In contrast to rodents, where *Hoxb1* disappears from the rest of the neural tube, expression persists in the stage 11 chick and quail embryos all along the neural tube posterior to r7 (Sundin and Eichele, 1990). The expression of *Hoxb1*, *Krox-20* and *MafB/kr* in VAD quail embryos led Maden et al. (Maden et al., 1996) to conclude that r4 was absent in these animals. Conversely, *Hoxb1* expression in VAD rat embryos and *Raldh2* knockout mouse embryos suggests that r4 is still present, but enlarged (White et al., 2000; Niederreither et al., 2000).

In order to determine whether or not r4 had totally disappeared in antagonist-treated chick embryos, we have used additional hindbrain markers. Embryos exposed to highly concentrated antagonist (5×10^{-6} M or 10^{-5} M) at stage 4 and allowed to develop to stage 11, display an extended expression of *follistatin* in r2 (Fig. 3b). Surprisingly, in these embryos, *follistatin* is strongly expressed in the neural tube from the r3 caudal boundary into the spinal cord down to the axial level of somite 6 (Fig. 3b). Furthermore, there is a strong downregulation of *follistatin* expression in the anterior mesencephalon of treated embryos (Fig. 3b).

In normal stage 8 embryos, *Wnt8c* expression is restricted in the presumptive r4 domain and to the region of the posterior neuropore (Fig. 3c). This r4-specific expression disappears by stage 10^{-} (Hume and Dodd, 1993). In antagonist-treated embryos, cultured from stage 4 to 9^{-}, we found that *Wnt8c* was no longer expressed in a stripe at the normal r4 position, but in an extended domain of the neural tube alongside somites 1-6 (Fig. 3d).

CEPU-1 is a cell-adhesion molecule that is specifically expressed in r3 and r4 during early hindbrain segmentation (Fig. 3e) (Jungbluth et al., 2001; Spaltmann and Brummendorf, 1996). Embryos exposed to antagonist (5×10^{-6} M) at stage 4 and allowed to develop to stage 10, display a considerably extended expression domain of *Cepu-1* in the parasomitic neural tube (Fig. 3f). As for Wnt8c, this expanded expression of *Cepu-1* in the neural tube extends posteriorly as far as somite 6, but no further. As the enlarged r3 is morphologically distinguishable at this stage, we conclude that the neural tube

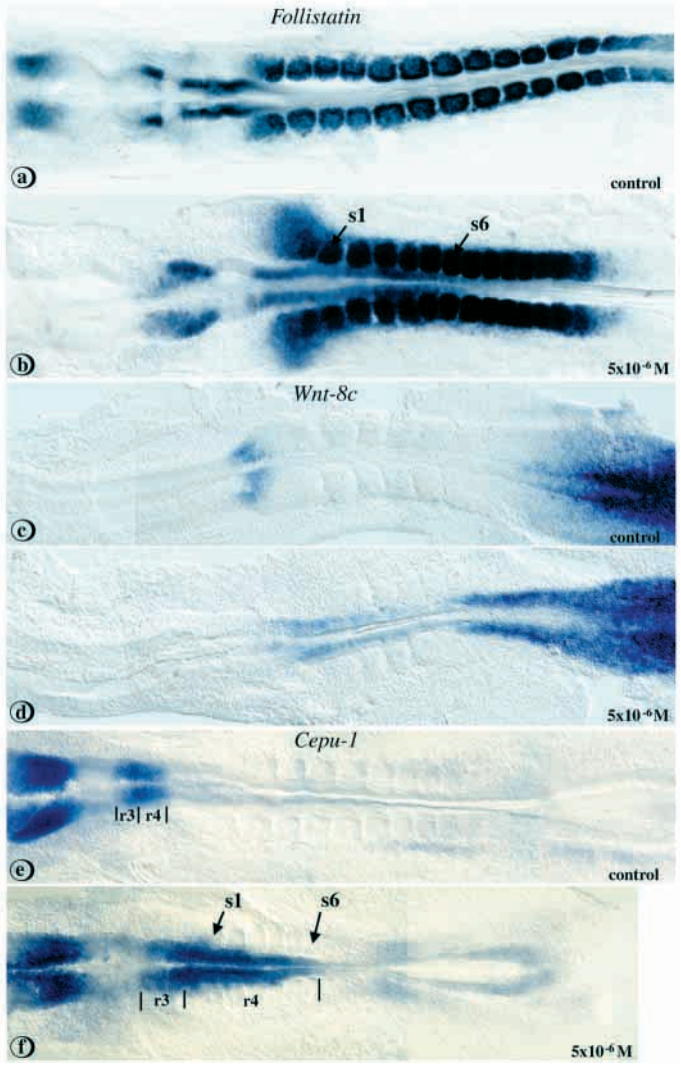


Fig. 3. In retinoic acid-deficient chick embryos, r4 forms a defined domain from the r3/r4 border to the level of the sixth somite. Dorsal view of whole mounts of control embryos (a,c,e) and BMS493-treated (5×10^{-6} M) chick embryos (b,d,f). In treated embryos, *follistatin* expression is ectopically expressed in the neural tube (b, stage11), *Wnt8c* expression is not restricted to the normal position of r4 (compare d with c, stage8^{+}) and *Cepu-1* expression is throughout the posterior hindbrain (f, stage10). r3, r4, rhombomeres 3 and 4. s1, s6, first somite and sixth somite.

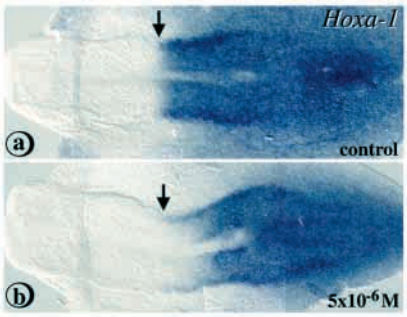


Fig. 4. Downregulation of *Hoxa1* in BMS493-treated embryos. Dorsal view of whole mounts of control (a) and antagonist-treated (5×10^{-6} M) chick embryos at stage 4 (b). Note the downregulation of *Hoxa1* in its anterior domain of expression in b. Arrows indicate the normal rostral limit of *Hoxa1* expression.

posterior to this rhombomere expressing *Cepu-1* has acquired an r4-like identity down to the level of the sixth somite.

The abnormal expression patterns of *follistatin*, *Wnt8c* and *Cepu-1* in RA-deficient chick embryos collectively indicate that cells with r4 molecular expression characteristics form a defined domain from the r3/r4 border to the region of the neural tube at the axial level of the sixth somite. Thus, in the absence of RA signalling the posterior hindbrain (including the posteriormost, parasomitic region) is anteriorised and has an r4-like identity.

Hoxa1 is downregulated in antagonist-treated embryos

During early gastrulation, *Hoxa1* mRNA expression extends from the posterior end of the embryo along both the neurectodermal and mesodermal layers up to the presumptive r3/r4 boundary (Fig. 4a). At early somite stages, *Hoxa1* expression begins to regress caudally, later becoming localised to the tailbud (Sundin et al., 1990). The product of *Hoxa1* is essential during hindbrain segmentation. *Hoxa1*-knockout mice have an enlarged r3 and it has been shown that expression of *Hoxa1* posterior to the prospective r3 is regulated through RAREs during the presomite stage (Dupé et al., 1997; Studer et al., 1998). In order to determine if the enlarged r3 observed in antagonist-treated embryos is due to downregulation of *Hoxa1*, we exposed stage 4 embryos to 5×10^{-6} M antagonist and allowed them to develop to stage 7. This treatment downregulates *Hoxa1* expression anteriorly, with the anterior limit of high level expression retreating posteriorly from the expected r3/r4 boundary (Fig. 4b). This abnormal *Hoxa1* expression is not observed in embryos treated with a 5×10^{-7} M and 10^{-7} M of the antagonist.

DISCUSSION

Treatment of chick embryos in culture with a pan-RAR antagonist abrogates RA signalling. The same hindbrain phenotypes are observed at 5×10^{-6} M and 10^{-5} M BMS493, suggesting that the former concentration is saturating. This phenotype is rescued by the simultaneous addition of 10^{-5} M RA to the culture medium (data not shown). Furthermore, hindbrain phenotypes that are near identical to those observed have been noted for VAD quail and rat embryos (Maden et al., 1996; White et al., 2000), and embryos lacking RARs (Dupé et al., 1999). Therefore, BMS493 specifically blocks retinoid signalling and may do so completely. This approach has enabled us to dissect the action of RA on hindbrain development thoroughly. In the light of this work, we are able to re-interpret the wide disparity of hindbrain phenotypes previously observed in various models of retinoic acid deficiency.

Abrogation of RA signalling results in the enlargement of r4

Treating chick embryos at stage 4 with the 5×10^{-6} M antagonist, produces a hindbrain phenotype that is identical, in terms of morphology and molecular marker expression, to that described for VAD quail embryos that have a complete absence of endogenous RA (Maden et al., 1996; Maden et al., 1998b). Embryos develop a shorter AP axis with smaller and poorly defined somites (Fig. 3b,f). Furthermore, patterning of the r2-r7 region is profoundly altered, with enlarged r2 and r3, absence of the r5 *Krox-20* expression, loss of *MafB/kr* expression, and with *Hoxb1* expressed continuously throughout the posterior hindbrain and spinal cord (Figs 1, 3). Interestingly, the few rat embryos obtained under conditions of absolute retinoid deficiency exhibit similar *Hoxb1* expression (White et al., 2000).

However, the phenotype of the VAD quail and that resulting from the blockade of RA signalling by the BMS493 antagonist are interpreted differently. In their studies on VAD quail embryos, Maden et al. concluded that r4 is absent (Maden et al., 1996; Maden et al., 1997; Maden et al., 1998b). This was on the basis of *Hoxb1* expression, which, in the neural tube of VAD quail embryos, extends caudally from just anterior to the first somite in these embryos; the distinctive 'r4' stripe of *Hoxb1* expression is absent but the continuous posterior domain looks similar to the cervical spinal cord domain of *Hoxb1* in normal embryos (see Fig. 3 in Maden, 1996). In the absence of other r4 markers, the reasonable conclusion by Maden et al. (Maden et al., 1996) was that r4 is lost, and that r3 abuts spinal cord. We see an identical pattern of *Hoxb1* expression in the antagonist-treated embryos but, by contrast, we offer a different interpretation of the upper reaches of the parasomitic domain, arguing that it is not spinal cord but hindbrain. Specifically, it is r4. Thus, we have shown that in antagonist-treated embryos, *follistatin* is expressed posterior to the enlarged r3 down to the level of the 6th somite, indicating that this region has a hindbrain identity. Furthermore, expression of the r4 marker *Wnt8c* in antagonist-treated embryos shows that as early as stage 8, presumptive r4 cells are not restricted to a stripe, as in control embryos, but are found in parasomitic neural tube down to the level of somite 6. Finally, *Cepu-1*, another r4 marker, is also expressed down to the level of somite 6. We conclude, therefore, that the blockade of RA signalling in chick does not result in the loss of r4; rather, it results in the formation of an enlarged r4. In the light of this observation, the hindbrain phenotypes observed in RA-deficient chick and VAD quail and rat are actually similar to the one observed in *Raldh2*-knockout mouse, where *Hoxb1*- and *Wnt8*-expressing cells expand posteriorly into the 'spinal cord' region (Niederreither et al., 1999; Niederreither et al., 2000).

On the basis of the interpretation by Maden et al. of the VAD quail (Maden et al., 1996), where RA signalling is completely absent, it has recently been suggested that the *Raldh2* mutants may have residual RA signalling (Gavalas and Krumlauf, 2000). Our analysis, however, suggests that there is a complete deficiency of RA in the *Raldh2* mutant hindbrain. It is therefore reasonable to assume that the RA implicated in hindbrain patterning is produced exclusively by Raldh2 activity.

As early as stage 4, *Raldh2* is expressed lateral to the primitive streak with a sharp anterior boundary. At stage 7, transcripts are abundant in the paraxial mesoderm and display an anterior boundary at the level of the first somite (Berggren et al., 1999; Swindell et al., 1999), corresponding with the future location of the r6/r7 boundary. A similar anterior boundary of RA production has been shown by Maden et al. in the chick using a RA reporter cell assay (Maden et al., 1998a). It has been proposed that RA synthesised at high levels in the first and subsequent somites diffuses anteriorly through the hindbrain at early stages and contributes to establishment of *Hox* gene expression patterns (Swindell et al., 1999; Berggren et al., 1999; Maden, 1999; Grapin-Botton et al., 1998). As *Raldh2* is detectable in cervical somites but not in the more rostral unsegmented paraxial mesoderm, and because our results are consistent with *Raldh2* being the sole source of RA in hindbrain patterning, the RA effects we and others have described must be long range.

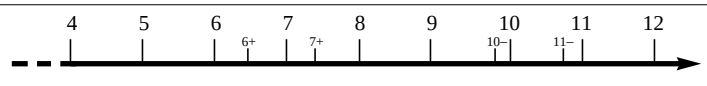
The posteriorising effect of RA is restricted to the hindbrain

Our results show that proper specification of the post-otic neural tube also requires RA. Interestingly, only the region of neural tube down to level of the sixth somite is anteriorised by abrogating RA signalling. Indeed, we never saw expression of hindbrain markers posterior to the sixth somite. What might be distinct between the neural tube regions anterior and posterior to the sixth somite? There is no obvious correlation with weaker expression of *Raldh2* in the somites, as those posterior to somite 6 also express the gene (Swindell et al., 1999; Berggren et al., 1999). However, there is known to be a fundamental change of neurectodermal cell fate and potential at this juncture in the neural tube, with particular respect to the neural crest. Rostral to somite 7, the neurogenic crest is fated to form enteric ganglia whereas caudal to somite 5 it is fated to give rise to sympathetic neurons (Le Douarin and Kalcheim, 1999). That the cells rostral to this point have the broad ectomesenchymal potentials of cephalic crest, which are lacking from more caudal crest (Nakamura and Ayer-le Lievre, 1982), is perhaps an even more significant difference. Fate-mapping studies have shown that the avian skull is derived from neural crest ectomesenchyme, together with paraxial mesoderm, down to the level of the fifth somite (Couly et al., 1993), the scelerotome of the upper cervical somites being incorporated into the occipital region of the skull. Although the neural tube of this region acquires a dorsoventral cell pattern that is characteristic of spinal cord, it is contained within the skull; it is thus properly designated as hindbrain rather than spinal cord, thus corresponding to the 'rhombomere 8' described by Lumsden and Keynes (Lumsden and Keynes, 1989; see also Cambronero and Puelles, 2000). In summary, it appears that dependency on RA for correct anteroposterior patterning is restricted to the hindbrain, by this strict anatomical definition, and that in embryos that completely lack RA signalling, the posterior hindbrain (r5-r8), takes on the identity of a large r4 region by default.

RA is implicated in the specification of hindbrain borders in a stage-dependant manner

We have shown that RA is required for proper hindbrain segmentation from stage 5 to stage 10^+. Furthermore, the specification of gene expression borders, and presumably future rhombomere boundaries, has an RA dependency that is lost progressively in an anterior-to-posterior sequence as development proceeds. The results of producing a deficiency of RA signalling at successive stages of early hindbrain development are summarized in Table 2. The first action of RA appears to be at stage 5, as we observed the same hindbrain phenotype when embryos are treated (5×10^{-6} M antagonist) at stage 4 or 5. In embryos treated at stage 6, *Krox-20* expression is normal in r3, suggesting that RA is no longer required for maintaining the r3/r4 border after stage 6. Our results also indicate that RA is no longer required for maintaining the r4/r5 border after stage 7^+, the r5/r6 border after stage 9, the r6/r7 border after stage 10^- and the r7/r8 border after stage 10^+ (Table 2). However, Gale et al. were able to rescue the hindbrain phenotype of VAD quails by treating the embryos with RA as late as stage 8 (Gale et al., 1999), showing that the

Table 2. A decreasing concentration of RA antagonist allows the progressive establishment of more posterior rhombomeres



	r3	r4	r5	r6	r7
	enlarged	enlarged	absent	absent	absent
	normal	enlarged	absent	absent	absent
	normal	enlarged	dots	absent	absent
	normal	normal	enlarged	enlarged	enlarged
	normal	normal	normal	enlarged	enlarged
	normal	normal	normal	normal	enlarged
	normal	normal	normal	normal	normal

Thick arrow represent the diverse stages of treatment. The start of the thin arrow represents the stage when treatment commenced.

hindbrain has enough plasticity to respond to RA until this stage.

Interestingly, when embryos are treated at stage 6^+, we observed *Krox-20*-expressing cells in r5 associated with cells not expressing *Hoxb1*. Does downregulation of *Hoxb1* allow the expression of *Krox-20*, or does upregulation of *Krox-20* lead to the downregulation of *Hoxb1*? The latter hypothesis is the more likely, as *Krox-20* misexpression experiments in chick have shown that *Krox-20* can repress *Hoxb1* (Giudicelly et al., 2001). In turn this would suggest an involvement of RA in upregulating *Krox-20* expression in r5, rather than downregulating *Hoxb1* posteriorly to r4. *Hoxb1* is normally downregulated in the chick r5/r6 after stage 9 (Sundin and Eichele, 1990). Here, we show that when the antagonist is applied between stages 7 and 9 we still observed a restriction of *Hoxb1* expression in r4, suggesting that RA does not regulate directly the downregulation of *Hoxb1* in r5. Together, these results suggest that RA would be required to restrict *Hoxb1* expression in r4 through the upregulation of *Krox-20* in r5.

Antagonist treatment can mimic the RARα:RARβ mutant phenotype

Embryos treated at stage 8^- have a similar phenotype to that of the RARα:RARβ compound mutant mouse embryos, which have normal anterior rhombomeres (r1-r4), but enlarged r5 and r6 (Dupé et al., 1999). This suggests that RARβ and RARα are implicated in later aspects of RA signalling in patterning the posterior hindbrain, whereas RARγ may be implicated in earlier aspects, for example, in controlling *Hoxa1* expression (see below). This is underscored by the fact that RARβ has an anterior limit of expression in the neural tube corresponding to the r5/6 boundary (Smith and Eichele, 1991), and RARβ is downregulated in the presence of antagonist (Wendling et al., 2000; data not shown).

RA signalling plays an essential role in the specification of the r3/r4 border by regulating early *Hoxa1* expression

Several anteriorly expressed *Hox* genes harbour RAREs in their regulatory sequences and require RA for establishment of their normal expression domains in the hindbrain (Gould et al., 1998; Marshall et al., 1994; Studer et al., 1994; Langston and Gudas, 1992; Pöpperl and Featherstone, 1993). Antagonist-treated chick embryos and *Raldh2* mutant mouse embryos display altered *Hoxa1* expression, similar to the effect of a targeted disruption of the *Hoxa1* RARE. These mouse mutants later develop an enlarged r3, similar to that noted for our antagonist-treated embryos (Niederreither et al., 1999; Dupé et al., 1997). Activation of *Krox-20* at the level of r3 is dependent on signals that must be propagated from r4, and these signals are downstream from *Hoxa1* and *Hoxb1*. Furthermore, *Hoxa1* is required for the expression of *Hoxb1* in the anterior region of r4 (Helmbacher et al., 1998; Studer et al., 1998; Rossel and Capecchi, 1999; Barrow et al., 2000; Giudicelli et al., 2001). In the present work, the fact that the anterior limit of *Hoxa1* expression is found at a more posterior level in treated embryos explains the enlargement of r3, as the signal is produced at a more posterior level and may not reach its normal limit. Thus, enlargement of r3 in antagonist-treated embryos is directly due to the downregulation of *Hoxa1*. By extension, it indicates that RA is essential for establishing the anterior limit of *Hoxa1* expression at stage 5. Moreover, our culture experiments, using various concentrations of antagonist, suggest that the *Hoxa1* RARE is capable, in vivo, of responding to low concentration of RA, as only a very high concentration of antagonist can lead to an enlarged r3. This is also suggested by the effect of supplementing VAD rat embryos, as a 0.5 μg/g RA diet is sufficient to shift the rostral limit of *Hoxb1* expression from the level of the first somite (in the case of complete retinoid deficiency) to the region of the r3/r4 border (White et al., 2000).

A gradient based on RA responses operates in the posterior hindbrain

Krox-20 and *MafB/kr* are among the earliest known genes to be expressed with an anteroposteriorly restricted pattern within the hindbrain. The ectopic expression (i.e. *Krox-20* in anterior r4) or downregulation (i.e. *Krox-20* in r5 and *MafB/kr* in r5-r6) of these genes in our 5×10^{-6} M antagonist treated embryos, support the idea that RA signalling acts at the head of the genetic hierarchy involved in the control of the hindbrain segmentation.

Our results indicate that the normal non-expression of *Krox-20* posterior to r5 and *MafB/kr* posterior to r6 is due to the presence of a high concentration of RA in this region. Indeed, we have seen from our experiments that a weak RA deficiency leads to upregulation of *Krox-20* and *MafB/kr* in the parasomitic (postotic) hindbrain, and Maden et al. have shown the presence of a high level of RA in the neural tube posterior to r6 (Maden et al., 1998a). Retinoids may cooperate with fibroblast growth factors (FGFs) in this process, as it has recently been shown that FGF signalling pathways are likely to participate in the determination of the posterior limit of expression of *Krox-20* and *MafB/kr* in the hindbrain (Marin and Charnay, 2000).

Furthermore, we have shown that RA signalling controls *Krox-20* and *MafB/kr* in a dose-dependant way in the postotic hindbrain. Indeed, with complete RA deficiency, we lose *Krox-20* and *MafB/kr* expression in r5 and r5/r6, respectively, and a progressive increase of RA availability results in activation of their expression in the neural tube anterior to the level of the sixth somite. Eventually, when RA signalling is unaffected, expression is suppressed in the posterior end of this domain. This indicates that only a low concentration of RA can activate *Krox-20*, *MafB/kr* and maybe other segmental genes in presumptive r7 and r8, whereas a higher concentration is necessary to obtain the normal sharpening expression of these genes (as discussed above). Interestingly, similar ectopic expression of *Krox-20* was previously obtained using VAD rat embryos supplemented insufficiently with RA (White et al., 2000).

Grapin-Botton et al. have postulated the existence of a morphogen in a posterior-to-anterior decreasing gradient (Grapin-Botton et al., 1998). *MafB/kr* would be expressed in a window of morphogen concentration in this gradient. Using various concentration of antagonist, we saw a posterior shift of *MafB/kr* expression, consistent with the posterior displacement of a window of RA signalling strength. When the concentration of RA antagonist is increased, the r4/r5 region produces an enlarged r4, and the r6/r7 region has ectopic expression of *MafB/kr*. This is strong support for the existence of an

endogenous gradient of RA activity, and that this is indeed the morphogen postulated by Grapin-Botton et al., Regulation of *Krox-20* in r5 may well depend on a smaller window in the same gradient.

The existence of an RA gradient has been proposed previously, based on expression experiments involving the RA-synthetic enzyme Raldh2 and the enzyme responsible for RA inactivation, Cyp26 (Swindell et al., 1999; Berggren et al., 1999). However, in the light of our work, we have to keep in mind that the specification of the rhombomere boundaries by RA (from r3/r4 to r7/r8) is stage dependent. In this context, it is difficult to consider a continuous RA concentration gradient between the posterior and anterior extremities of the hindbrain that would also persist from stage 5^+ to stage 10. Furthermore, a simple source-sink model for such a gradient may be inappropriate, considering that we and others are able to rescue the hindbrain phenotype simply by RA administration to the embryo (Niederreither et al., 2000; Gale et al., 1999; White et al., 2000; data not shown). Together, these features suggest the presence of an active mechanism that would distribute RA along the hindbrain at the appropriate concentration. This mechanism may depend on a large number of parameters, considering that the RARE-containing hindbrain patterning genes have different RAREs that respond differently to RA (Langston and Gudas, 1992; Marshall et al., 1994; Mendelsohn et al., 1991). A further complication is the distribution of CRABPI, which may act as a supplementary local source, or sink, for RA (Ruberte et al., 1991). Furthermore, the results of inactivating Cyp26A1 confirm the role of this enzyme in cleaning RA from specific embryonic areas to protect them against inappropriate RA signalling. In the context of the hindbrain, Cyp26A1 is required to generate an RA-free domain in the rostral hindbrain, defining the position of r3/r4 boundary (Abu-Abed et al., 2001; Sakai et al., 2001). In this respect, other RA-metabolising enzymes may perform additional specific hindbrain functions by modulating RA levels.

In conclusion, our results have enabled us to interpret or re-interpret some of the wide disparity of hindbrain phenotypes previously observed in various models of RA deficiency (Maden et al., 1998b; Niederreither et al., 2000; White et al., 2000; Dupé et al., 1999). Specifically, we have shown that RA signalling is required for correct patterning of the posterior hindbrain, by its wider (anatomical) definition that includes rhombomere 8 (i.e. down to the level of s6) and this entire region acquires an r4-default identity in absence of RA. In terms of RA requirement, anterior rhombomeres are established and independent of RA signalling at an earlier stage than posterior rhombomeres. Stabilisation of rhombomere pattern thus proceeds in a strict rostral-to-caudal procession. It is also clear that successively more posterior rhombomere boundaries are successively more sensitive to a RA deficiency, suggesting that they require progressively higher concentrations of endogenous RA for their correct positioning. Our work strongly supports the hypothesis of a gradient of RA activity in the hindbrain, but this gradient is likely to be a dynamic one, considering the many levels of complexity that accompany RA signalling pathways. Hindbrain development is more likely to be dependent on graded RA responses rather than on a linear RA concentration gradient.

We are grateful to Drs D. Wilkinson, J. Dodd, V. Prince and S. Jungbluth for providing us with cDNA probes. Special thanks to Jamilé Hazan for the chick Hoxa1 probe, to Malcolm Maden for reading our manuscript, and to Professor Pierre Chambon (IGBMC-LGME-U.184-ULPs – Strasbourg) and Bristol Myers Squibb for the gift of BMS 493. The work was supported by the MRC and by a long-term fellowship to V. D. from the HFSP.

REFERENCES

Abu-Abed, S., Dollé, P., Metzger, D., Beckett, B., Chambon, P. and Petkovich, M. (2001). The retinoic acid-metabolizing enzyme, CYP26A1, is essential for normal hindbrain patterning, vertebral identity and development of posterior structures. *Genes Dev*. 15, 226-240.

Barrow, J. R., Stadler, H. S. and Capecchi, M. R. (2000). Roles of *Hoxa1* and *Hoxa2* in patterning the early hindbrain of the mouse. *Development* **127**, 933-944.

Berggren, K., McCaffery, P., Dräger, U. and Forehand, C. J. (1999). Differential distribution of retinoic acid synthesis in the chicken embryo as determined by immunolocalization of the retinoic acid synthetic enzyme, RALDH-2. *Dev. Biol*. **210**, 288-304.

Blumberg, B., Bolado, J., Jr, Moreno, T. A., Kintner, C., Evans, R. M. and Papalopulu, N. (1997). An essential role of retinoic signaling in anteroposterior neural patterning. *Development* **124**, 373-379.

Cambronero, F. and Puelles, L. (2000). Rostrocaudal nuclear relationships in the avian medulla oblongata: A fate map with quail chick chimeras. *J. Comp. Neurol*. **427**, 522-545.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10**, 940-954.

Conlon, R. A. (1995). Retinoic acid and pattern formation in vertebrates. *Trends Genet.* **11**, 314-319.

Connolly, D., McNaughton, L. A., Krumlauf, R. and Cooke, J. (1995). Improved in vitro development of the chick embryo using roller-tube culture. *Trends Genet*. **11**, 259-260.

Couly, G. F., Coltey, P. M. and Le Douarin, N. M. (1993). The triple origin of skull in higher vertebrates: a study in quail-chick chimeras. *Development* **117**, 409-429.

de Roos, K., Sonneveld, E., Compaan, B., ten Berge, D., Durston, A. J. and van der Saag, P. T. (1999). Expression of retinoic acid 4-hydroxylase (CYP26) during mouse and *Xenopus laevis* embryogenesis. *Mech. Dev.* **82**, 205-211.

Duester, G. (1998). Alcohol dehydrogenase as a critical mediator of retinoic acid synthesis from vitamin A in the mouse embryo. *J. Nutr. Suppl.* **128**, 4595-4625.

Dupé, V., Davenne, M., Brocard, J., Dollé, P., Mark, M., Dierich, A., Chambon, P. and Rijli, F. M. (1997). In vivo functional analysis of the *Hoxa-1* 3′ retinoic acid response element (3′ RARE). *Development* **124**, 399-410.

Dupé, V., Ghyselinck, N. B., Wendling, O., Chambon, P. and Mark, M. (1999). Key roles of retinoic acid receptors alpha and beta in the patterning of the caudal hindbrain, pharyngeal arches and otocyst in the mouse. *Development* **126**, 5051-5059.

Durston, A. J., Timmermans, J. P. M., Hage, W. J., Hendricks, H. F. J., de Vries, N. J., Heideveld, M. and Nieuwkoop, P. D. (1989). Retinoic acid causes an anteroposterior transformation in the developing nervous system. *Nature* **340**, 140-144.

Eichmann, A., Grapin-Botton, A., Kelly, L., Graf, T., Le Douarin, N. M. and Sieweke, M. (1997). The expression pattern of the *mafB/Kr* gene in birds and mice reveals that the *kreisler* phenotype does not represent a null mutant. *Mech. Dev.* **65**, 111-122.

Fujii, H., Sato, T., Kaneko, S., Gotoh, O., Fujii-Kuriyama, Y., Osawa, K., Kato, S. and Hamada, H. (1997). Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos. *EMBO J.* **16**, 4163-4173.

Gale, E., Zile, M. and Maden, M. (1999). Hindbrain respecification in the retinoid-deficient quail. *Mech. Dev.* **89**, 43-54.

Gavalas, A. and Krumlauf, R. (2000). Retinoid signalling and hindbrain patterning. *Curr. Opin. Genet. Dev.* **10**, 380-386.

Giudicelli, F., Taillebourg, E., Charnay, P. and Gilardi-Hebenstreit, P. (2001) Krox-20 patterns the hindbrain through both cell-autonomous mechanisms. *Genes Dev.* **15**, 567-580.

Gould, A., Itasaki, N. and Krumlauf, R. (1998) Initiation of rhombomeric

Hoxb4 expression requires induction by somites and a retinoid pathway. *Neuron* **21**, 39-51.

Graham, A. and Lumsden, A. (1996). Interactions between rhombomeres modulate *Krox-20* and *follistatin* expression in the chick embryo hindbrain. *Development* **122**, 473-480.

Grapin-Botton, A., Bonnin, M. A., Sieweke, M. and Le Douarin, N. (1998). Defined concentrations of a posteriorizing signal are critical for *MafB/Kreisler* segmental expression in the hindbrain. *Development* **125**, 1173-1181.

Guthrie, S. C., Muchamore, I., Marshall, H., Kuriowa, A., Krumlauf, R. and Lumsden, A. (1992). Neuroectodermal autonomy of *Hox2-9* expression revealed by rhombomere transposition. *Nature* **356**, 157-159.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Helmbacher, F., Pujades, C., Desmarquet, C., Frain, M., Rijli, F. M., Chambon, P. and Charnay, P. (1998). *Hoxa1* and *Krox-20* synergize to control the development of rhombomere 3. *Development* **125**, 4739-4748.

Hume, C. R. and Dodd, J. (1993). *Cwnt-8C*: a novel *Wnt* gene with a potential role in primitive streak formation and hindbrain organization. *Development* **119**, 1147-1160.

Jungbluth, S., Phelps, C. and Lumsden, A. (2001). *CEPU-1* expression in the early embryonic chick brain. *Mech. Dev.* 101, 195-197.

Kolm, P. J., Apekin, V. and Sive, H. (1997). Xenopus hindbrain patterning requires retinoid signaling. *Dev. Biol.* **192**, 1-16.

Langston, A. W. and Gudas, L. J. (1992). Identification of a retinoic acid responsive enhancer 3′ of the murine homeobox gene Hox1-6. *Mech. Dev.* **38**, 217-228.

Le Douarin, N. M. and Kalcheim, C. (1999). The autonomous nervous system and the endocrine cells of neural crest origin. In *The Neural Crest*, pp. 445. Cambridge: Cambridge University Press.

Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-428.

Maden, M., Gale, E., Kostetskii, I. and Zile, M. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Curr. Biol.* **6**, 417-426.

Maden, M., Graham, A., Gale, E., Rollinson, C. and Zile, M. (1997). Positional apoptosis during vertebrate CNS development in the absence of endogenous retinoids. *Development* **124**, 2799-2805.

Maden, M., Sonneveld, E., van der Saag, P. T. and Gale, E. (1998a). The distribution of endogenous retinoic acid in the chick embryo: Implications for developmental mechanisms. *Development* **125**, 4133-4144.

Maden, M., Gale, E. and Zile, M. (1998b). The role of vitamin A in the development of the central nervous system. *J. Nutr.* **128**, 471-475.

Maden, M. (1999). Heads or tails? Retinoic acid will decide. *BioEssays* **21**, 809-812.

Marin, F. and Charnay, P. (2000). Hindbrain patterning: *FGFs* regulate *Krox20* and *mafB/kr* expression on the otic/preotic region. *Development* **127**, 4925-4935.

Marshall, H., Nonchev, S., Sham, M. H., Muchamore, I., Lumsden, A. and Krumlauf, R. (1992). Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature* **360**, 737-741.

Marshall, H., Studer M., Popperl, H., Aparicio, S., Kuroiwa, A., Brenner, S. and Krumlauf R. (1994). A conserved retinoic acid response element required for early expression of the homeobox gene *Hoxb-1*. *Nature* **370**, 567-571.

Mendelsohn, C., Ruberte, E., LeMeur, M., Morris-Kay, G. and Chambon, P. (1991). Developmental analysis of the retinoic acid-inducible RARβ2 promoter in transgenic animals. *Development* **113**, 723-734.

Murphy, P., Davidson, D. R. and Hill, R. E. (1989). Segment-specific expression of a homeobox-containing gene in the mouse hindbrain. *Nature* **341**, 156-159.

Nakamura, H. and Ayer-le Lièvre, C. S., (1982). Mesectodermal capabilities of the trunk neural crest of birds. *J. Embryol. Exp. Morphol.* **70**, 1-18.

Niederreither, K., McCaffery, P., Drager, U. C., Chambon, P. and Dollé, P. (1997). Restricted expression and retinoic acid-induced downregulation of the retinaldehyde dehydrogenase type 2 (RALDH-2) gene during mouse development. *Mech. Dev.***62**, 67-78.

Niederreither, K., Subbarayan, V., Dollé, P. and Chambon. P. (1999). Embryonic retinoic acid synthesis is essential for early mouse post-implantation development. *Nat. Genet.* **21**, 444-448.

Niederreither, K., Vermot, J., Schuhbaur, B., Chambon, P. and Dollé, P. (2000). Retinoic acid synthesis and hindbrain patterning in the mouse embryo. *Development* **127**, 75-85.

Popperl, H. and Featherstone M. S. (1993). Identification of a retinoic acid response element upstream of the murine *Hox-4.2* gene. *Mol. Cell Biol.* **13**, 257-265.

Rossel, M. and Capecchi, M. R. (1999). Mice mutant for *Hoxa1* and *Hoxb1* show extensive remodeling of the hindbrain and defects in craniofacial development. *Development* **126**, 5027-5040.

Ruberte, E., Dollé, P., Chambon, P. and Morris-Kay, G. (1991). Retinoic acid receptors and cellular retinoid binding proteins. II. Their differential pattern of transcription during early morphogenesis in mouse embryo. *Development* **111**, 45-60.

Sakai, Y., Meno, C., Hideta, F., Nishino, J., Shiratori, H., Saijoh Y., Rossant, J. and Hamada H. (2001). The retinoic acid-signalling enzyme CYP26 is essential for establishing an uneven distribution of retinoic acid along the antero-posterior axis within the mouse embryo. *Genes Dev.* **15**, 213-225.

Smith, S. M. and Eichele, G. (1991). Temporal and regional differences in the expression pattern of distinct retinoic acid receptor-β transcripts in the chick embryo. *Development* **111**, 245-252.

Spaltmann, F. and and Brummendorf, T. (1996). CEPU-1, a novel immunoglobulin superfamily molecule, is expressed by developing cerebellar purkinje cells. *J. Neurosci.* **16**, 1770-1779.

Studer, M., Popperl, H., Marshall, H., Kuroiwa, A. and Krumlauf, R. (1994). Role of a conserved retinoic acid response element in rhombomere restriction of *Hoxb-1*. *Science* **265**, 1728-1732.

Studer, M., Gavalas, A., Marshall, H., Ariza-McNaughton, L., Rijli, F. M., Chambon, P. and Krumlauf, R. (1998). Genetic interaction between *Hoxa1* and *Hoxb1* reveal new roles in regulation of early hindbrain patterning. *Development* **125**, 1025-1036.

Sundin, O. H. and Eichele, G. (1990). A homeo-domain protein reveals the metameric nature of the developing chick hindbrain. *Genes Dev.* **4**, 1267-1276.

Sundin, O. H., Busse, H. G., Rogers, L. J., Gudas, L. J, Eichele, G. (1990). Region specific expression in early chick and mouse embryos of *Ghox-lab* and *Hox1.6* vertebrate homeobox domain containing genes related to Drosophila labial. *Development* **108**, 47-58.

Swindell, E. C., Thaller, C., Sockanathan, S., Petkovich, M., Jessell, T. M. and Eichele, G. (1999). Complementary domains of retinoic acid production and degradation in the early chick embryo. *Dev. Biol.* **216**, 282-96.

Van der Wees, J., Schilthuis, J. G., Koster, C. H., Diesveld-Schipper, H., Folkers, G. E., van der Saag, P. T., Dawson, M. I., Shudo, K., van der Burg, B. and Durston, A. J. (1998). Inhibition of retinoic acid receptor – mediated signalling alters positional identity in the developing hindbrain. *Development* **125**, 545-556.

Wendling, O., Dennefeld, C., Chambon, P. and Mark, M. (2000). Retinoid signaling is essential for patterning the endoderm of the third and fourth pharyngeal arches. *Development* **127**, 1553-1562.

White, J. C., Shankar, V. N., Highland, M., Epstein, M. L., DeLuca, H. F. and Clagett-Dame, M. (1998). Defects in embryonic hindbrain developmental and fetal resorption resulting from vitamin A deficiency in the rat are prevented by feeding pharmacological levels of all-trans-retinoic acid. *Proc. Natl. Acad. Sci. USA* **95**, 13459-13464.

White, J. C., Highland, M., Kaiser, M. and Clagett-Dame, M. (2000). Vitamin A deficiency results in the dose-dependent acquisition of anterior character and shortening of the caudal hindbrain of the rat embryo. *Dev. Biol.* **220**, 263-284.

Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R. and Charnay, P. (1989). Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. *Nature* **337**, 461-464.

Wilkinson, D. G. (1992). Whole-mount in situ hybridisation of vertebrate embryos. In *In Situ Hybridisation, A Practical Approach* (ed. D. G. Wilkinson), pp. 1-83. Oxford: IRL Press.