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# Effects of retinoic acid excess on expression of *Hox-2.9* and *Krox-20* and on morphological segmentation in the hindbrain of mouse embryos

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**Mouse embryos were exposed to maternally administered RA on day 8.0 or day 7¾ of development, i.e. at or just before the differentiation of the cranial neural plate, and before the start of segmentation. On day 9.0, the RA-treated embryos had a shorter preotic hindbrain than the controls and clear rhombomeric segmentation was absent. These morphological effects were correlated with alterations in the spatiotemporal distribution patterns of two genes, *Hox-2.9* and *Krox-20*, which are expressed in the otic and preotic hindbrain and in specific neural crest cell populations. *Hox-2.9* was expressed throughout the preotic hindbrain region, instead of being confined to rhombomere 4. *Krox-20* was not expressed rostral to the *Hox-2.9* domain, i.e. its normal rhombomere 3 domain was absent. The *Hox-2.9/Krox-20* boundary was ill-defined, with patches of alternating expression of the two genes. In migrating neural crest cells, *Hox-2.9* expression was both abnormally extensive and abnormally prolonged. Neural crest cells expressing *Krox-20* remained close to the neural tube. Embryos exposed to RA on day 8¼ appeared to be morphologically normal. We suggest that early events leading to rhombomeric segmentation and rhombomere-specific gene expression are specifically vulnerable to raised RA levels, and may require RA levels lower than those in the region of somitic segmentation.**

*Key words: Hox-2.9/Krox-20*/retinoic acid/rhombomere/segmentation

## Introduction

It has long been known that vitamin A (retinol) and its metabolites play essential roles in the development of a variety of systems: vitamin A deficiency causes a wide spectrum of congenital abnormalities (Warkany *et al.*, 1948), and there is good evidence that retinoic acid is involved in pattern formation in the chick limb bud (Wanek *et al.*, 1991; Noji *et al.*, 1991, and references therein). Retinoic acid (RA) is thought to exert its effects by modifying the transcriptional activity of specific genes through its binding to the three nuclear retinoic acid receptors (RAR) RAR-α, RAR-β and RAR-γ (Petkovich *et al.*, 1987; Giguère *et al.*, 1987; Brand *et al.*, 1988; Green and Chambon, 1988; Krust *et al.*, 1989; Zelent *et al.*, 1989). The genes which are activated by RA in embryos are unknown, but in human embryonal carcinoma cells they include the nine genes of the *Hox-2* cluster (Simeone *et al.*, 1990).

The hindbrain and the neural crest cells derived from it are of particular interest in relation to the developmental functions of RA because they are abnormal in rodent embryos exposed to a retinoid excess during or shortly before early neurulation stages of development (Morriss, 1972; Morriss and Thorogood, 1978; Webster *et al.*, 1986). Human infants exposed to a retinoid excess *in utero* at early developmental stages likewise show abnormalities of the brain and of structures to which cranial neural crest cells contribute (Lammer *et al.*, 1985). Retinoid-induced abnormalities of hindbrain morphology in rodent embryos include shortening of the preotic region in relation to other head structures, so that the otocyst lies level with the first pharyngeal arch instead of the second (Morriss, 1972; Morriss and Thorogood, 1978).

In normal rodent and human embryos, the hindbrain is organized as a series of rhombomeric segments, composed of alternating sulci and gyri, by the time neural tube closure is complete (Adelmann, 1925; Bartelmez and Evans, 1926; Bartelmez and Dekaban, 1962; Tuckett *et al.*, 1985; Wilkinson *et al.*, 1989b). The morphological structure of these segments is maintained by apical microfilament bundles in the sulci and fan-shaped arrays of microtubules in the gyri (Tuckett and Morriss-Kay, 1985). In mouse embryos, the otocyst lies level with rhombomere 5 and the upper part of rhombomere 6 (Wilkinson *et al.*, 1989b). It originates as the otic placode, which is thought to be induced by the adjacent neural epithelium (Van de Water and Ruben, 1976), possibly involving segment-specific expression of the FGF-like proto-oncogene *int-2* in rhombomeres 5 and 6 (Wilkinson *et al.*, 1988; Wilkinson, 1990). A rostral shift in the position of the otocyst therefore suggests that the normal pattern of development of rhombomeres 1–4 is altered as a result of exposure to retinoid excess.

Segment-specific gene expression in the mouse hindbrain at the stage of clear rhombomeric segmentation includes *Krox-20*, which is expressed in rhombomeres 3 and 5, and *Hox-2.9*, which is expressed only in rhombomere 4 (Wilkinson *et al.*, 1989a,b; Murphy *et al.*, 1989; Murphy and Hill, 1991). These genes are also expressed in early migrating neural crest cells adjacent to rhombomere 4 (*Hox-2.9*) and rhombomeres 5/6 (*Krox-20*). Both are putative transcription factors, and *Hox-2.9* is related to homeotic genes involved in segmentation and in the determination of segment identity in *Drosophila*.

In this study we have examined the relationship between the morphogenesis of the early mouse brain and the expression of *Hox-2.9* and *Krox-20* by investigating the effects of RA excess on both processes. We have used scanning electron microscopy and histology in order to observe the morphological abnormalities of the affected embryos, and *in situ* hybridization in order to reveal the sites

of expression of the two genes in normal and RA-treated embryos. The results demonstrate that the effects of RA on morphogenesis of the brain and cranial neural crest correlate closely with its effects on the expression of the two genes and suggest that RA may play an important role in pattern formation along the rostrocaudal axis of the hindbrain and its derived neural crest.

## Results

### *Appearance of live embryos*

For each time of RA administration, there were no detectable morphological differences between embryos from dams given 10 mg/kg RA and 12 mg/kg RA. The majority of embryos from dams given RA on day 8 + 6 h appeared to be morphologically normal and were within the same range of somite number as equivalent control embryos; three embryos in each of two litters showed a mild version of the abnormalities seen in all embryos exposed to RA on day 8.0 (these embryos were not processed further).

Embryos from dams given RA on day 8.0 had 8–14 somite pairs on day 9.0 compared with 17–20 in equivalent controls. This 6 to 9 somite number difference was maintained at later stages. Control embryos showed a strict correlation between the stage of cranial neurulation and somite number as previously observed in rat embryos (Morriss and Solursh, 1978; Morriss-Kay and Tuckett, 1987). Cranial neural fold development was retarded for somite number in RA-treated embryos. The position of the otocyst or otic pit was abnormal, being level with the first pharyngeal arch instead of the second pouch/second arch as in controls; the length of the preotic hindbrain was correspondingly reduced. The forebrain was also slightly reduced in size. In embryos from dams given RA on day 7 + 18 h, and dissected on day 10.0, the otocyst was level with the maxillary region and the forebrain was markedly reduced in size. In all day 7 + 18 h-treated embryos, and in some day 8.0-treated embryos, somites extended as far rostrally as the rostrally-positioned otocyst/otic pit, so that the first somite was level with the second pharyngeal arch. (Control

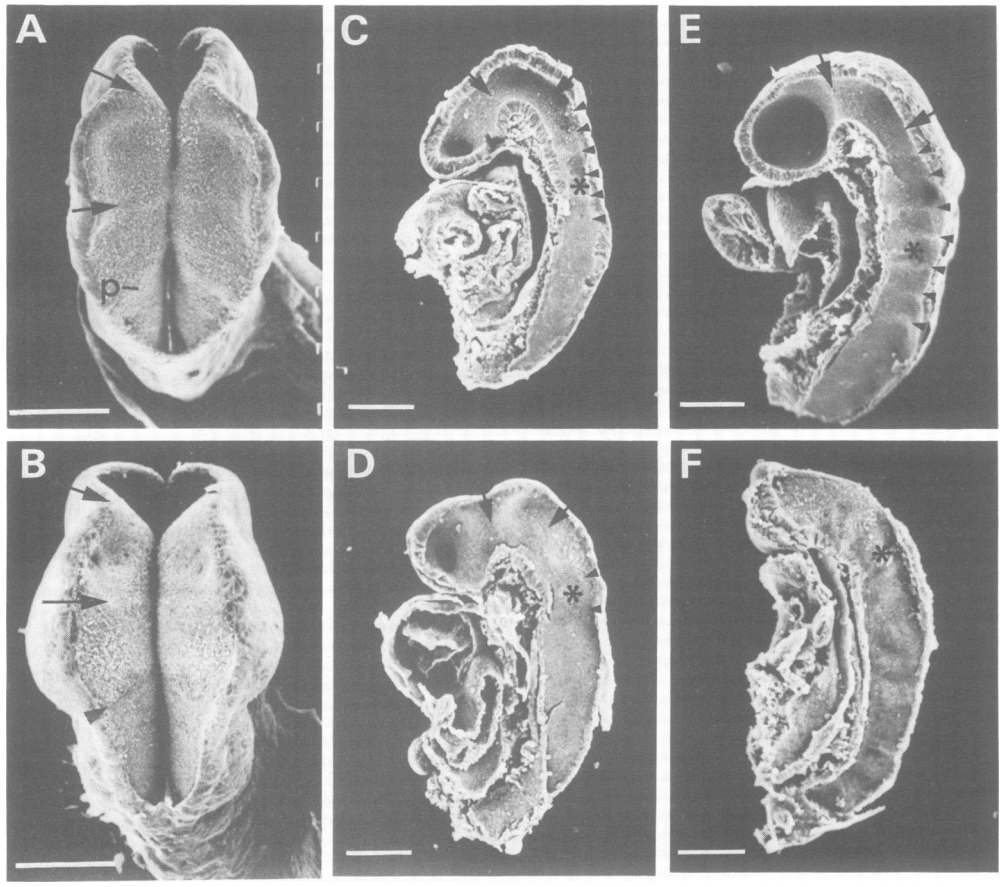


**Fig. 1.** Scanning electron micrographs of control (A, C, E) and RA-treated (B, D, F) mouse embryos. **A**: Dorsal view of a 9-somite stage control embryo (day 9.0). The forebrain/midbrain and midbrain/hindbrain boundaries are indicated by arrows in this and subsequent illustrations in this figure; the lowest transverse line is the preotic sulcus (p). **B**: An 11-somite stage embryo exposed to RA on day 8.0, showing the similarity of neural fold morphology to that of a 9-somite stage control embryo. The area between the midbrain/hindbrain boundary (lower arrow) and the arrowhead corresponds to the *Hox-2.9*-expressing domain shown in Figures 2E, K. **C**: interior view of a sagittally-halved 11-somite stage control embryo. The hindbrain is subdivided into six rhombomeric subdivisions at this stage; arrowheads indicate the inter-rhombomeric gyri. **D**: Equivalent view of a 12-somite stage day 8.0-RA-treated embryo. Only two rather gently convex gyrus-like structures are present (arrowheads). **E**: 16-somite stage control embryo (day 9.5): seven rhombomeric sulci are now present, with the unsegmented occipital region of the hindbrain caudal to them. **F**: 16-somite stage (day 10.0) embryo exposed to RA on day 7 + 18 h. The neuroepithelial surface shows irregular undulations, not rhombomeres. In C–F, asterisks indicate the position of the otic pit, as observed in external views of the other halves of the same specimens. Bar lines represent 100 μm.

embryos have a gap between the otocyst and the first somite, which lies level with the fourth pharyngeal arch.) The trunk region of all embryos appeared to be morphologically normal.

### *Scanning electron microscopy (SEM)*

Cranial neurulation was retarded in RA-treated embryos, so that the cranial neural folds of embryos with 11 or 12 somite pairs showed a superficial resemblance to 9-somite stage

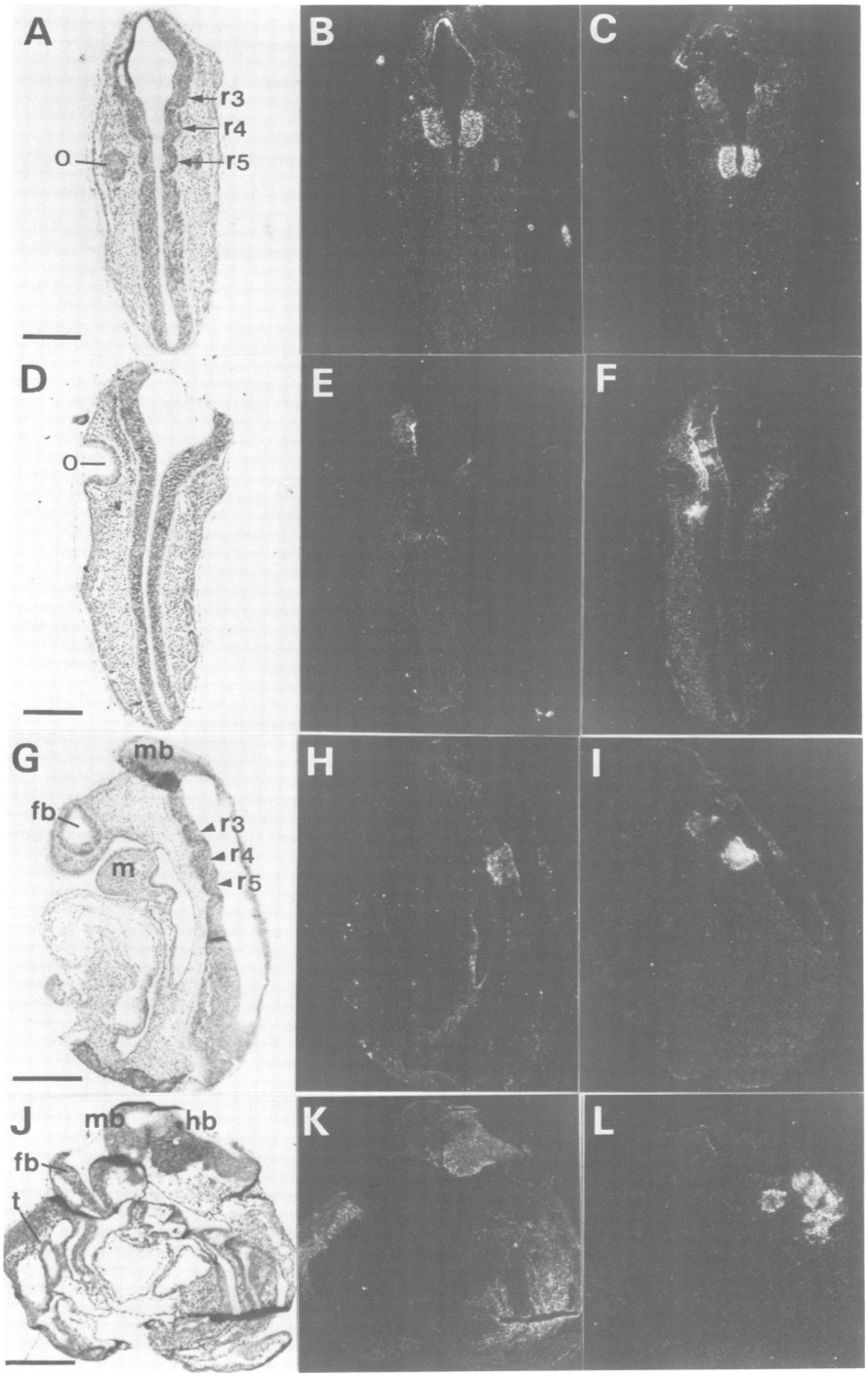


**Fig. 2.** Day 9.0 embryos cut coronally (A, D) or near-sagittally (G, J); control (**A**–**C** and **G**–**I**) or day 8.0 RA-treated (**D**–**F** and **J**–**L**); bright-field views (left column), dark-field views of sections hybridized with *Hox-2.9* (middle column) or *Krox-20* (right column) probes. In the control specimens the cranial neural tube is closed, while in the RA-treated specimens the forebrain and rostral hindbrain are still open. The expression domain of *Hox-2.9* is restricted to rhombomere 4 at this stage in control embryos (B, H), but in RA-treated embryos it extends throughout a larger territory immediately caudal to the midbrain boundary (E, K). Foregut-associated mesenchyme also shows more extensive *Hox-2.9* expression in RA-treated embryos than in controls. *Krox-20* is expressed in rhombomeres 3 and 5 in control embryos (C, I); in RA-treated embryos (F, L) there is a single but broken *Krox-20* domain which alternates with patches of cells expressing *Hox-2.9*. Neural crest cells expressing *Krox-20* can be seen immediately adjacent to the neural epithelium in RA-treated embryos (F, L), whereas in control specimens they have already dispersed. fb, forebrain, h, heart; m, mandibular arch; mb, midbrain; o, otocyst/otic pit; r, rhombomere; t, tail bud mesenchyme. Bar lines represent 100 $\mu$m.

control embryos except that the forebrain was partially apposed (Figure 1A, B). 11- and 12-somite stage control embryos had a completely apposed forebrain and a narrow spindle-shaped midbrain/rostral hindbrain neuropore, within which rhombomeric sulci could be seen; in sagittally halved 11- or 12-somite stage control embryos, up to seven rhombomeric sulci of approximately equal size could be distinguished (Figure 1C). The first rhombomere of 12-somite RA-treated embryos was larger than that of 12-somite stage controls; there was another small rhombomere caudal to it, but no others could be distinguished at this stage (Figure 1B) or later (Figure 1D). The forebrain and midbrain regions of RA-treated embryos were slightly smaller than those of controls in day 8.0-treated embryos (Figure 1C, D); the apical neuroepithelial surface of the midbrain was pitted with up to six small but relatively deep depressions in all 11 specimens examined (e.g. Figure 1B). The surface of the midbrain of control embryos was unpitted. The significance of this observation is obscure, and does not correlate with any other feature of the RA effects.

In sagittally halved 16- to 20-somite stage control embryos (day 9.5), the characteristic eight rhombomeric sulci were clearly visible (Figure 1E). Day 8.0 RA-treated embryos of 16- to 20-somite stages (day 10.0) still showed no clear rhombomeres. Day 7 + 18 h-treated embryos showed a large number of irregular sulci and gyri extending from the trunk (spinal cord) region to the midbrain/forebrain junction (Figure 1F); these structures were asymmetrical, and had the appearance of undulations rather than rhombomeres, so were not segmental structures. The forebrain region was

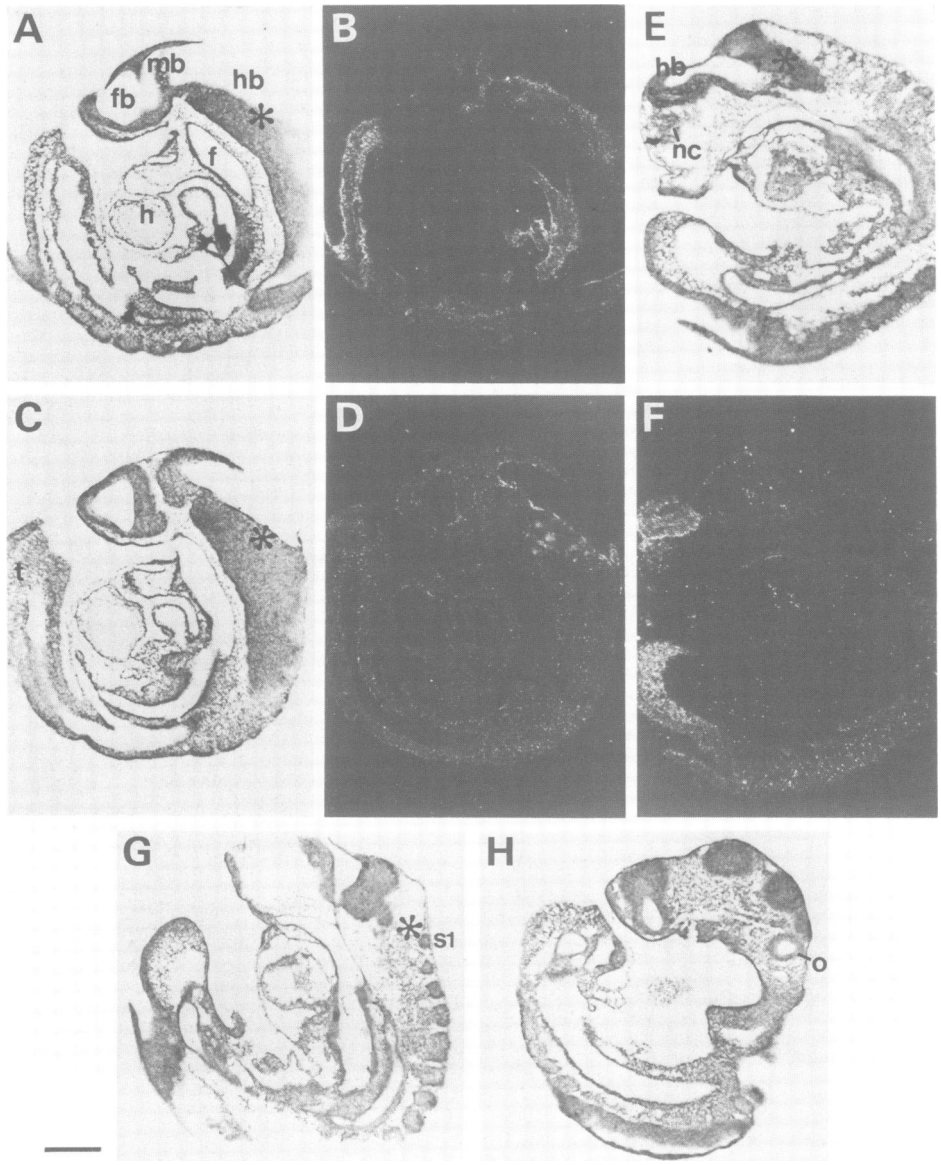


**Fig. 3.** Parasigittal sections of a single day 7+18 h RA-treated embryo fixed on day 10.0. **A** and **B**, **C** and **D**, **E** and **F** are brightfield/darkfield pairs; **G** and **H** are shown in brightfield only. The position of the otocyst on parallel sections (see H) is indicated by an asterisk. B shows a long domain of expression of *Hox-2.9*; D shows that this domain is punctuated by a number of small patches of cells expressing *Krox-20*. F shows more clearly that the *Hox-2.9* domain extends to the midbrain/hindbrain boundary, and that neural crest cells expressing this gene are associated with the most rostral part of the hindbrain. Comparison of G and H shows that there is no unsegmented mesenchyme between the otocyst (o) and the first somite (s1). f, foregut; nc, neural crest; other symbols as Figure 2. Bar line represents 100 $\mu$m.

much smaller than that of the control embryos, and the cranial flexure was poorly developed.

External views of 11- to 16-somite stage embryos showed the normal position of the otic pit in control embryos and the abnormally rostral position of the otic pit indicating shortening of the preotic hindbrain in RA-treated embryos. Otic pit position, as revealed by these SEM observations, is indicated by an asterisk on the interior views (Figure 1C–F).

### *Morphology of sectioned embryos*

Histological examination of embryos prepared for *in situ* hybridization was carried out using brightfield light microscopy (Figures 2–6). At 9 days, coronal and sagittal sections of control embryos clearly showed the characteristic rhombomeric structure of the hindbrain. RA-treated embryos showed the enlarged rhombomere-like structure seen with SEM, while the remainder of the hindbrain lacked sulci and gyri. In day 8.0-treated embryos this smooth appearance was maintained at 10 days (Figure 6), while in day 7 + 18 h-treated embryos of that age of hindbrain and spinal cord showed irregular undulations (Figure 5C). Cranial neural crest cells were not easily distinguishable from primary mesenchyme in control embryos, except where they formed preganglionic condensations. In treated embryos, groups of neural crest cells were located close to the rostral hindbrain. Their relation to other structures will be discussed in the next section. The reduced forebrain size, enlarged first rhombomere and otherwise smooth hindbrain neuroepithelium of day 8.0-treated embryos at 10½ days are illustrated in Figure 6. The abnormally rostral extension of somitic segmentation observed in whole, live embryos (see above) is illustrated in Figure 3G and H.

### *Expression of Hox-2.9 and Krox-20 in the hindbrain*

The expression patterns of *Hox-2.9* and *Krox-20* in control embryos were as previously described (Murphy *et al.*, 1989; Wilkinson *et al.*, 1989a,b; Murphy and Hill, 1991): in the hindbrain on day 9, *Hox-2.9* was expressed in rhombomere 4 and *Krox-20* in rhombomeres 3 and 5 (Figure 2B, C, H and I). The concentration of *Krox-20* transcripts in rhombomere 3 fell below detectable levels by day 9.5. In day 8.0-treated embryos at 9 days, *Hox-2.9* was expressed in a domain which coincides with a large rhombomere-like structure and corresponds to the enlarged first rhombomere seen with SEM (Figure 2E, K). This domain extended abnormally far rostrally, being immediately adjacent to the midbrain and level with the maxillary region as well as the first pharyngeal arch; however, it extended caudally to approximately the normal position of rhombomere 4 in relation to the otic pit or otocyst. In adjacent sections of the same embryos, *Krox-20* was expressed in patches in a single domain of the hindbrain (Figure 2F, L). In relation to the otocyst, this domain was located slightly rostral to the normal

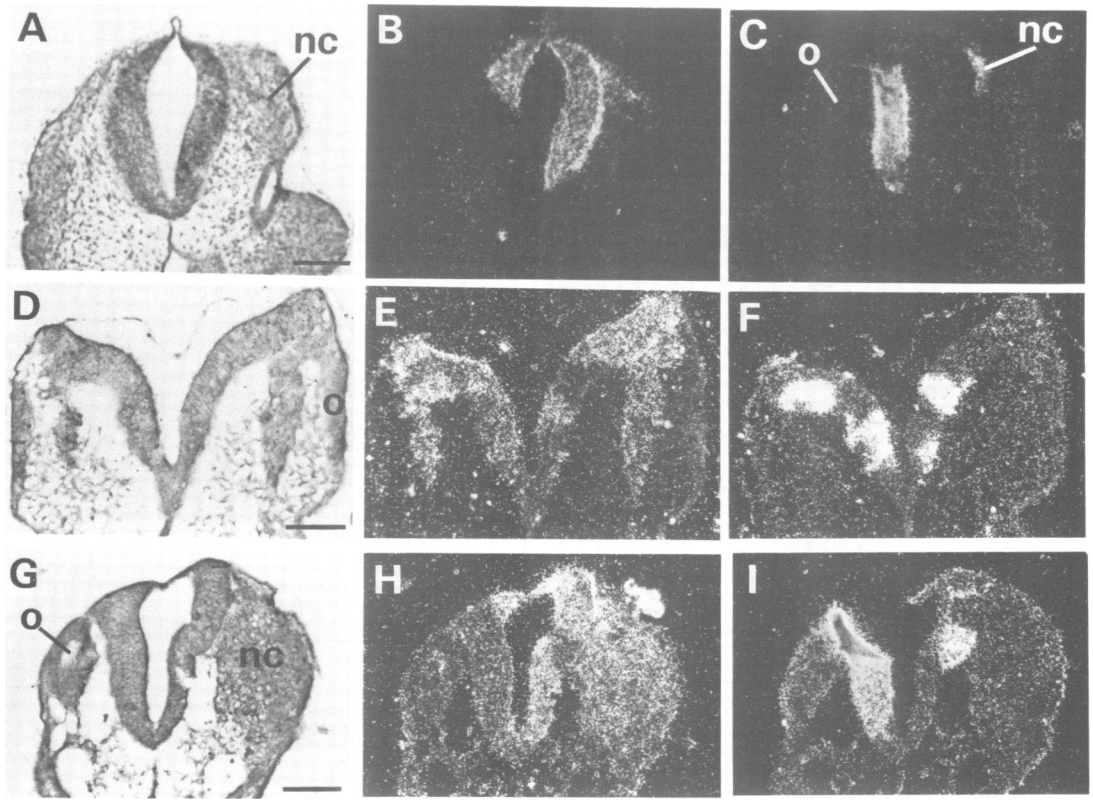


**Fig. 4.** Transverse sections rostral to or through the otic regions of a day 9.0 control embryo (**A–C**), a day 9.0 (**D–F**) and a day 10.0 (**G–I**) RA–(day 8.0)–treated embryos. In A–C and G–I the sections are oblique, with the right side being rostral to the left. A, bright-field view of B; C is a slightly more caudal section, passing through the otocyst on the left side. Both B and C pass through rhombomere 4 dorsally and on the right, and rhombomere 5 on the left, as shown by the distribution of *Hox-2.9* (B) and *Krox-20* (C) transcripts. Some neural crest cells adjacent to the more caudal part of rhombomere 4 appear to contain *Krox-20* transcripts; *Hox-2.9* transcripts are only present in neural crest cells close to the neural tube. The sections from RA-treated embryos show the characteristic complimentary patches of *Hox-2.9* (E, H) and *Krox-20* (F, I) transcripts. *Hox-2.9* transcripts are present in neural crest cells at a much greater distance from the neural tube than in control embryo sections. o, otic placode (D) or otocyst (A, G); nc, neural crest cells. Bar lines represent 100 μm.

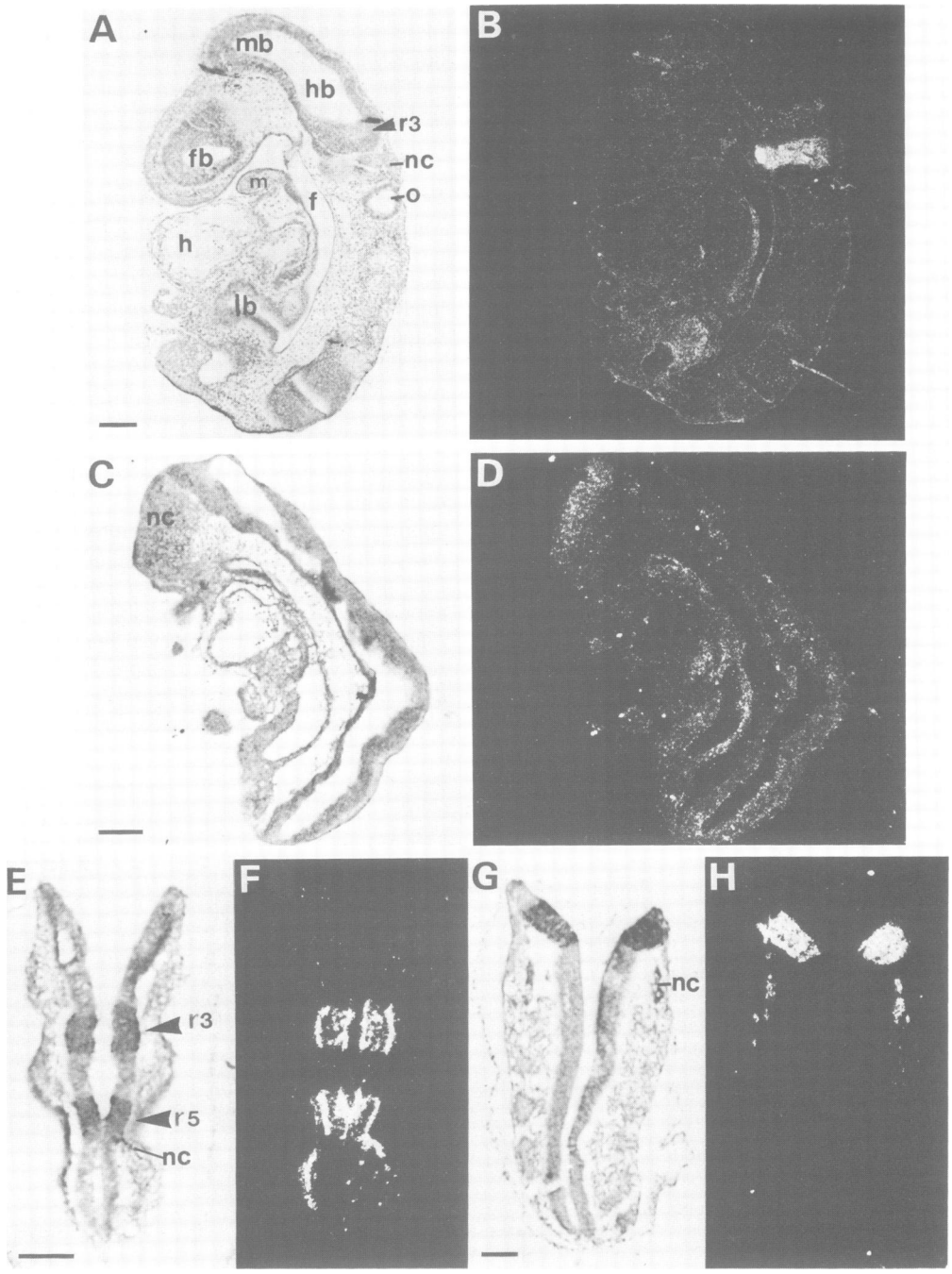


**Fig. 5.** *Hox-2.9* and *Krox-20* transcripts in neural crest cells. **A**, **B**: oblique/sagittal section of a control embryo showing *Hox-2.9*-expressing neural crest cells (nc) lateral to rhombomere 4, exactly level with the mandibular arch (m). **C**, **D**: oblique/sagittal section of a day 7 + 18 h-RA-treated embryo, passing lateral to the forebrain and midbrain and directly through the hindbrain and trunk regions of the neural tube. Neural crest cells (nc) containing *Hox-2.9* transcripts are spread between the most rostral part of the hindbrain and the side of the face. **E**, **F**: coronal section through the hindbrain of a late day 8 control embryo showing neural crest cell (nc) containing *Krox-20* transcripts apparently emanating from the caudal part of the rhombomere 5 *Krox-20* domain of the hindbrain, while the rhombomere 3 domain is not associated with any mesenchymal *Krox-20* expression. **G**, **H**: equivalent coronal section of a day 9.0 embryo exposed to RA on day 8.0. f, foregut; fb, forebrain; h, heart; hb, hindbrain; lb, liver bud, mb, midbrain; o, otocyst; r, rhombomere. Bar lines represent 100 $\mu$m.

rhombomere 5 *Krox-20* domain. Therefore, the hindbrain expression domains of *Hox-2.9* and *Krox-20* were shifted rostrally with respect to other cranial structures, including, though to a lesser extent, the otic vesicle. There was no expression of *Krox-20* rostral to the *Hox-2.9* domain, i.e. no representation of the normal rhombomere 3 domain.

During normal development, *Hox-2.9* is not, at first, expressed in a sharply defined neuroepithelial domain (Murphy and Hill, 1991). Only when rhombomeres are clearly visible (~8¾ days) does *Hox-2.9* expression become defined within sharp planar boundaries at the cellular level. In control embryos, both anterior and posterior boundaries of *Hox-2.9* expression coincide with equally well-defined boundaries of *Krox-20* expression (Figure 2B, C; H, I). In RA-treated embryos only the posterior boundary of the *Hox-2.9* domain adjoined cells expressing *Krox-20* (Figure

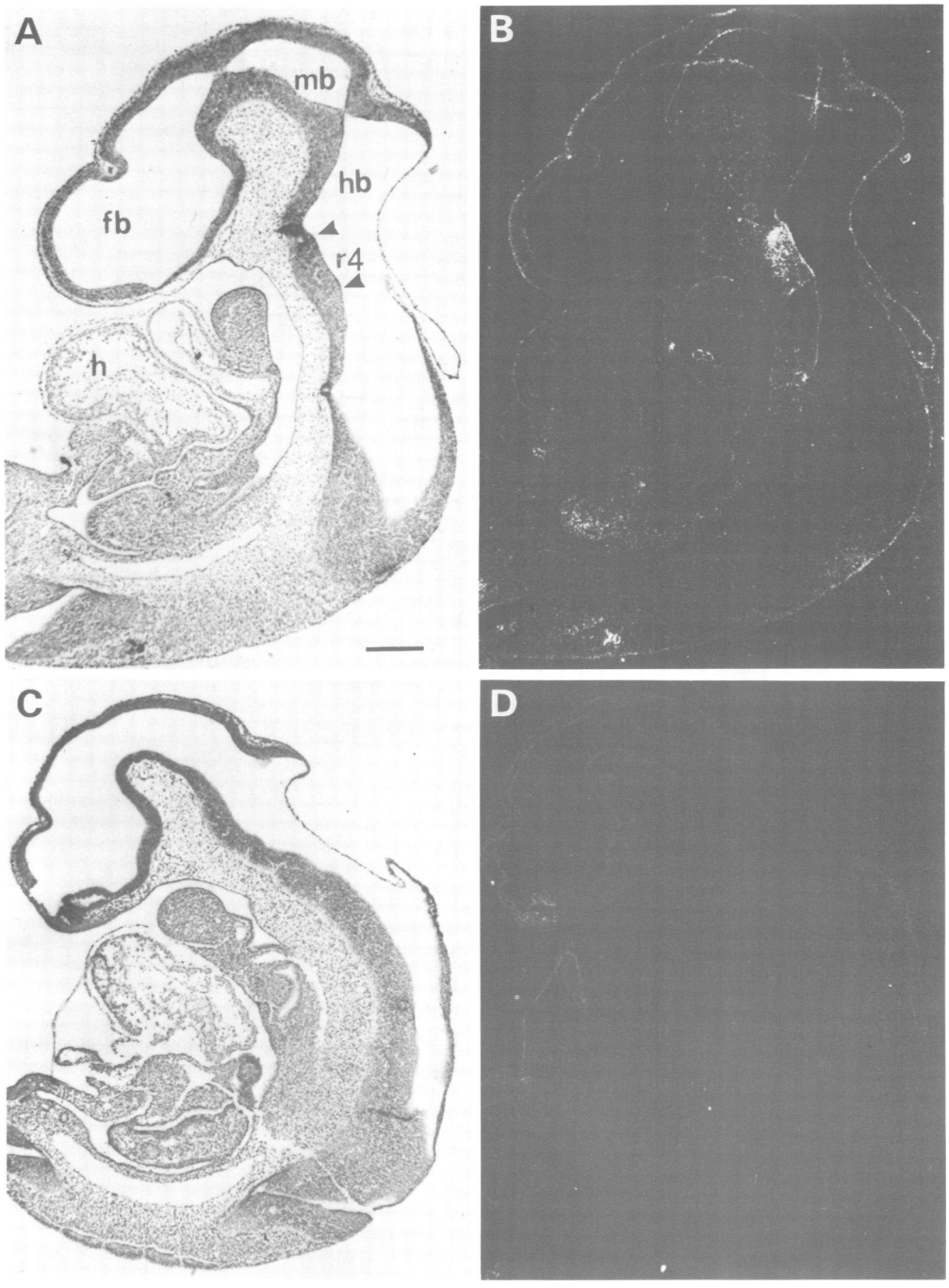


**Fig. 6.** Sagittal sections of control (**A**, **B**) and day-8.0-RA-treated (**C**, **D**) 10.5 day embryos. *Hox-2.9* transcripts are still present in rhombomere 4 and in the gut-associated mesoderm of the control embryo (B), but are absent from the treated embryo (D). Labels as Figure 5. Bar line represents 100 $\mu$m.

2E, F; K, L). Instead of the single planar boundary observed in controls there was an irregular alternation of cells expressing *Hox-2.9* and *Krox-20* resulting in a somewhat mosaic pattern at the interface. This arrangement was also apparent in the transverse plane (Figure 4).

In the day 7 + 18 h-treated embryos, the domain of *Hox-2.9* expression extended from a region showing a high level of expression at the midbrain–hindbrain junction (Figure 3F) to somitic levels of the axis, where expression was less strong (Figure 3B, F). *Krox-20* expression was broken up into small patches of cells within the *Hox-2.9* domain (Figure 3D). As in control embryos and in day 8.0-treated embryos, there did not seem to be intermingling of individual cells expressing the two genes.

Day 8.0-treated embryos were also analysed at 10½ days. *Krox-20* was no longer expressed in the hindbrain of either control or RA-treated embryos. In normal embryos, *Hox-2.9* expression persists within rhombomere 4 until day 11 (Murphy and Hill, 1991); at 10½ days there was no detectable expression of *Hox-2.9* in the hindbrain of RA-treated embryos (Figure 6). Thus, *Hox-2.9* was prematurely down-regulated following exposure to excess RA.

### *Expression of Hox-2.9 and Krox-20 in neural crest cells*

In normal embryos, groups of neural crest cells migrating from specific regions of the hindbrain express *Hox-2.9* and *Krox-20* (Figure 5B, F). Only those which migrate from the region expressing *Hox-2.9* express that gene themselves (Frohman *et al.*, 1990; Murphy and Hill, 1990; our Figure 5B). Neural crest cell expression of *Hox-2.9* is confined to the second wave of emigration, which takes place on day 9; these cells form the acoustico-facial ganglion (Hunt *et al.*, 1991). The first wave of neural crest cell migration from the region (just rostral to the preotic sulcus) which later becomes rhombomere 4 normally migrate into the second pharyngeal arch (Tan and Morriss-Kay, 1985, 1986; Morriss-Kay and Tan, 1987; Nichols, 1987). These cells do not express *Hox-2.9* (Hunt *et al.*, 1991). In the RA-treated embryos, *Hox-2.9*-expressing neural crest cells formed a more extensive group than that of controls (compare Figure 4H with Figure 4B) and were observed in a pathway extending from the *Hox-2.9* domain of the hindbrain across to the side of the face (Figure 5C and D). This pathway is therefore rostral to the normal migration pathway, taking *Hox-2.9*-expressing neural crest cells into an ectopic site; the cells were also abnormal in retaining detectable *Hox-2.9* transcripts at a greater than normal distance from the neural epithelium.

In normal mouse embryos, migrating crest cells just caudal to rhombomere 5 express *Krox-20* (Wilkinson *et al.*, 1989b); it is possible that these neural crest cells originate in rhombomere 5 but immediately take a route caudal to the adjacent otic pit, as suggested by Figure 5F. In RA-treated embryos, *Krox-20*-expressing neural crest cells were situated adjacent to and posterior to the hindbrain domain of *Krox-20*, in a highly condensed group (Figure 2F, L, and Figure 5H). They appeared not to have migrated far from their site of emigration from the neural folds.

### *Expression of Hox-2.9 elsewhere in the embryo*

*Hox-2.9* is normally expressed in several sites at 8½ to 10 days of development including the ectoderm and endoderm of the second and third pharyngeal pouch and cleft, the gut-associated mesoderm, the posterior neural tube and the primary mesenchyme close to the primitive streak (Murphy *et al.*, 1989; Frohman *et al.*, 1990; Murphy and Hill, 1991). *Hox-2.9* transcripts were observed in all of these sites in both control and RA-treated embryos, sometimes more abundantly in the treated embryos (compare Figure 2H and K). In addition, day 7 + 18 h-treated embryos showed *Hox-2.9* expression in the trunk neural tube at 10 days of development (Figures 3F and 5D).

## Discussion

Our results show that the early hindbrain of mouse embryos is specifically vulnerable to raised RA levels at the late presomite stage, and that the effects (summarized diagrammatically in Figure 7) involve both morphological and molecular aspects of development. In this discussion, we consider these two aspects separately and together, and then examine the possibility that they result not only from raised RA levels *per se*, but also from disturbance of normal rostrocaudal differences in free RA levels within the embryonic hindbrain. The abnormalities that we have described derive

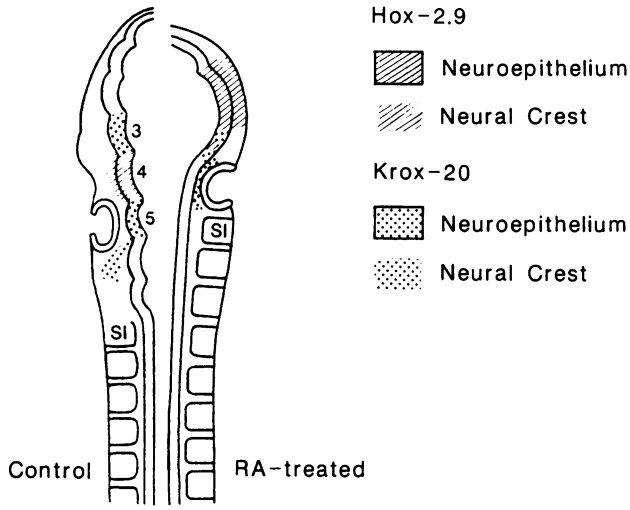


**Fig. 7.** Summary diagram showing a day 9.0 mouse embryo (coronal section). **Left half**: normal morphology and normal pattern of expression of *Hox-2.9* and *Krox-20*. **Right half**: abnormal morphology and gene expression observed in the hindbrain neuroepithelium and neural crest of day 8.0-RA-treated embryos, with the first somite position as observed in day 7¾-RA-treated embryos. The abnormal morphological features include: shortening of the preotic hindbrain; absence of rhombomeric organization; absence of a gap between the otic pit and first somite. The abnormal gene expression pattern includes: expression of *Hox-2.9* in an abnormally large domain beginning at the midbrain–hindbrain junction; caudal to this, alternating patches of *Hox-2.9* and *Krox-20*; absence of a *Krox-20* domain rostral to the *Hox-2.9* domain; more extensive expression of *Hox-2.9* in neural crest cells, which follow a pathway to the side of the face instead of to the acoustico-facial ganglion; less extensive migration of *Krox-20*-expressing neural crest cells.

from the effects of excess RA at the beginning of cranial morphogenesis, at the latest by day 8.0, which in our embryos was before the first pair of somites had formed. Later treatment (day 8¼) had no effect on early brain development. Similarly, in rat embryos *in vitro*, this characteristic pattern of craniofacial dysmorphogenesis has been observed to result from a relatively short exposure of the embryos to RA during the late presomite period of development (Morriss and Steele, 1977); exposure during the early somite stage affects pharyngeal arch size but does not shorten the hindbrain (Ritchie and Webster, 1991). The latest effective time of RA exposure for affecting hindbrain organization is therefore prior to both the onset of *Krox-20* expression and to the stage at which *Hox-2.9* expression first shows a sharp anterior boundary within the hindbrain (Frohman *et al.*, 1990), and well before the morphogenesis of rhombomeres.

### *Differential effects on the brain regions*

In treated embryos, the forebrain, midbrain and preotic hindbrain were reduced in size. These effects varied with the time of RA exposure, reduction of the forebrain and midbrain being more pronounced following exposure at an early stage (day 7¾), whereas the hindbrain effects were consistent following both early and later (day 8.0) exposure. These results differ from those observed in amphibian embryos treated with RA at the stage of neural induction, in which forebrain and midbrain, but not hindbrain structures, were suppressed, an effect described as 'posteriorization' (Durston *et al.*, 1989). In another amphibian study, progressively greater loss of anterior

structures was correlated with increasing RA concentration (Sive *et al.*, 1990), whereas in the present study the response did not depend on the concentration of applied RA within the range examined. These amphibian/mammalian differences may be related to differences in RA receptor expression: Ellinger-Ziegelbauer and Dreyer (1991) have described transcription of an RAR-γ-like receptor in *Xenopus* during gastrulation and early neurulation, transcripts being particularly abundant at the anterior and posterior ends of the neurula. At these stages, RAR-γ in the mouse embryo are not detectable above background, and are first seen at high levels at the posterior end only (Ruberte *et al.*, 1990). In contrast to these differences in the effects of RA on the most anterior structures of amphibian and mammalian embryos, anterior hindbrain effects similar to those described here have now been reported in RA-treated *Xenopus* embryos (Papalopulu,N., Clarke,J.D.W., Wilkinson,D., Krumlauf,R. and Holder,N., submitted for publication).

In accordance with the reduction in size of the preotic hindbrain, the pattern of gene expression in this region appeared to be truncated rostrally. *Krox-20* is normally expressed in rhombomeres 3 and 5 and *Hox-2.9* in rhombomere 4, but RA-treated embryos showed an abnormally large domain of *Hox-2.9* expression at the rostral end of the morphologically defined hindbrain with only the caudal of the two *Krox-20* domains. The presence of adjacent *Krox-20*-expressing neural crest cells suggests that the neuroepithelial *Krox-20* expression represented the normal rhombomere 5 domain only. Taken at face value, these observations would suggest that rhombomeres 1−3 are missing in RA-treated embryos. This interpretation correlates well with the observations of cerebellar abnormalities, including cerebellar hypoplasia and aplasia, in human infants exposed to RA *in utero* (Lammer *et al.*, 1985), since the cerebellum is derived from the most rostral part of the hindbrain (O'Rahilly and Müller, 1987).

### Effects on the neural crest

It is difficult, however, to reconcile the interpretation that the first three rhombomeres are missing in RA-treated embryos with the patterns of migration and development of the cranial neural crest cells. In normal rat and mouse embryos, the midbrain neural crest cells migrate into the frontonasal and maxillary regions, while the hindbrain neural crest rostral to the preotic sulcus migrates into the mandibular part of the first pharyngeal arch (Tan and Morriss-Kay, 1985, 1986; Morriss-Kay and Tan, 1987; Nichols, 1987). The rostral hindbrain neural crest appears to be prespecified to differentiate as the mandibular (Meckel's) cartilage, since in chick embryos this cartilage can also be induced to form in the second arch after appropriate transplantation of rostral hindbrain neural tube and crest (Noden, 1983). In rat embryos exposed to retinoid excess, mandibular arch-like ectopic cartilage forms in the maxillary region (which is normally devoid of cartilage), while the first arch cartilage is reduced or absent (Morriss and Thorogood, 1978; Morriss-Kay, 1991). These observations on cranial neural crest cell pathways and patterning suggest that in the retinoid-treated embryos studied previously (Morriss and Thorogood, 1978), the crest cells which migrate into the maxillary region and differentiate as ectopic cartilage are those which 'belong to' the hindbrain rostral to the preotic sulcus, i.e. to rhombomeres 1−3. Therefore, in terms of their non-neural neural crest derivatives at least, these rhombomeres must be represented within neuroepithelium of RA-treated embryos. The observation of *Hox-2.9*-expressing cells migrating into the side of the face (Figure 5D) suggests that the first three rhombomeres may be morphologically combined with rhombomere 4 to form a single large rhombomere in which *Hox-2.9* is both normally and ectopically expressed. It is also important to note that in normal embryos, *Hox-2.9*-expressing crest cells only contribute to the neural derivative of rhombomere 4 (the acoustico-facial ganglion) and not to the mesenchyme of the second arch, which expresses only *Hox-2.8* (Hunt *et al.*, 1991).

Further information regarding the genetic identity of the region which appears, by morphological criteria, to be midbrain, for example an analysis of the position of expression of the mammalian *engrailed*-like genes (Davis and Joyner, 1988; Davidson *et al.*, 1988; Davis *et al.*, 1988) in RA-treated embryos, is needed to confirm the position of the midbrain−hindbrain boundary in RA-treated embryos. Neither the results of animal studies nor the reported malformations in RA-exposed human infants support the idea that migration of the midbrain-derived fronto-nasal mesenchyme is significantly abnormal.

### Effects on segmentation

In addition to its effect on the size of the major regions of the brain, excess RA inhibited clear rhombomeric segmentation. The single overlarge rhombomere-like structure and the adjacent shallow sulcus that form in RA-treated embryos correspond in position to the abnormal domains of activity of *Hox-2.9* and *Krox-20* respectively. This correspondence might suggest a causal relationship between gene activity and segment morphogenesis, particularly since ill-defined segmental boundaries were a feature of both morphological structure and gene expression domains. The boundaries between the domains of *Hox-2.9* and *Krox-20* normally sharpen as development proceeds, to abut one another at rhombomere boundaries. The absence of cell mixing across rhombomere boundaries has been demonstrated by cell marking experiments (Fraser *et al.*, 1990). In RA-treated embryos, the boundary between *Hox-2.9* and *Krox-20* expression was a mosaic of small patches of cells expressing one or other gene, and the normal sharply-defined gyrus region was represented only by a gentle curve of the epithelium. These results suggest that although RA inhibited normal boundary formation between the domains of expression of the two genes, it had no effect on their ability to intermix individually, or on their mutual exclusivity of expression within individual cells. Morphological rhombomere boundaries are therefore not required for the partitioning of domains of gene expression, but are associated with the normal segmental pattern of these domains.

In normal embryos, the caudal part of rhombomere 6 and the whole of rhombomere 7 lie between the level of the otocyst and the first occipital somite, so that the immediately post-otic mesoderm is unsegmented. Somitic segmentation begins where rhombomeric segmentation ends, as if the two are mutually exclusive. In the more severely abnormal RA-treated embryos, somites extended right up to the otocyst (Figure 4). This observation raises the intriguing possibility that in normal embryos, somitic segmentation in this region is actively inhibited, and that the inhibition is absent in

RA-treated embryos, either as a direct response to RA or by the loss of an inhibitory interaction with the hindbrain.

### *Possible roles of RA in organization of pattern in the hindbrain*

Studies in amphibian embryos (Durston *et al.*, 1989; Sive *et al.*, 1990; Cho and DeRobertis, 1991) indicate that RA plays an important role in providing positional information along the long axis of the embryo. Our results are consistent with the idea that RA plays a similar role in the hindbrain of mammalian embryos. This interpretation implies that a mechanism exists for controlling the levels of free RA at specific levels of the rostro-caudal axis. Cellular retinoic acid binding protein I (CRABP I) is a strong candidate for this function. It binds RA with high specificity (Stoner and Gudas, 1989), and is specifically expressed in cells which are vulnerable to high RA levels in chick (Vaessen *et al.*, 1990) and mouse (Ruberte *et al.*, 1991) embryos. Overexpression of CRABP I in F9 teratocarcinoma cells is associated with decreased responsiveness to RA, indicating that less of the exogenous RA reaches the nucleus when CRABP I levels are higher (Boylan and Gudas, 1991). Within the hindbrain, CRABP I is present at particularly high levels in rhombomeres 4 and 6 (Dencker *et al.*, 1990), so nuclear RA levels must be lowest in these segments. In the trunk region, in which low levels of CRABP I transcripts were detected in the cervical region only (Ruberte *et al.*, 1991), effects on the neural tube itself (as opposed to the vertebrae, which are somitic derivatives) required administration of 100 mg/kg RA on day 8.5, i.e. ten times the amount used in our study (M.Kessel, personal communication).

Differential sensitivity to RA is therefore a feature of different axial levels of the early neural epithelium, both within the hindbrain and between the hindbrain and trunk regions. This effect may be mediated by genes of the *Hox-2* cluster. In human embryonal carcinoma cells *in vitro*, genes of the *Hox-2* cluster are induced by RA, those of the 3′ half being affected at the lowest concentration and those at the 5′ end being activated only a higher concentrations (Simeone *et al.*, 1990). This effect reflects the distribution of *Hox-2* domains within the neural tube in terms of vulnerability of different axial levels to RA excess, since the domains of the relatively insensitive 5′ genes are restricted to the trunk region, whereas the domains of the 3′ genes extend into the hindbrain (Graham *et al.*, 1989). *Hox-2.9* lies at the extreme 3′ end of the *Hox-2* cluster (Wilkinson, 1989b). Both the enlarged area of neuroepithelial *Hox-2.9* expression and the prolonged and ectopic expression of this gene in migrating neural crest cells are consistent with the idea that *Hox-2.9* expression has been stimulated by excess RA. The effects of RA on the segment-related expression domains of the other 3′ *Hox-2* genes is of considerable interest.

The different effects of excess RA on the expression of *Krox-20* in its two domains implies significant functional differences between them. RAR-$\alpha$ is expressed in rhombomere 5 but not rhombomere 3 (Ruberte *et al.*, 1991), so it is interesting to note that *Krox-20* binds to a sequence which is present in the upstream promoter region of hRAR-$\alpha$ (Brand *et al.*, 1990).

In general, our observations provide clear evidence that the normal patterns of segmental gene expression and morphological segmentation in the hindbrain and its derived neural crest require free RA to be maintained at very low levels, particularly in the preotic region. Controlled differences in free RA levels within the early hindbrain neuroepithelium may play an important role in the determination of segment-specific patterns of gene expression and morphogenesis.

## Materials and methods

C57Bl/6 mice were housed three to a cage with males at 9 am and checked for plugs at 11 am, 12 noon and 3 pm. The time of observation of a plug was designated the start of day 0 of pregnancy. Retinoic acid was made up as 5 mg RA in 0.8 ml absolute ethanol to which 9.2 ml arachis oil was added (0.5 mg/ml solution). Crystalline RA was kept in the dark under argon at 4°C; the solution was kept in the dark at 4°C for a maximum of 48 h. Mice weighing ~25 g were given 0.5 ml (10 mg/kg) or 0.6 ml (12 mg/kg) of this solution by oral gavage on day 7 + 18 h, day 8.0 or day 8 + 6 h of pregnancy. Control dams of the same stages of pregnancy were given 0.5 ml or 0.6 ml vehicle alone. Orally administered RA reaches near maximal levels in the mouse embryo within 2 h (Creech Kraft *et al.*, 1987) so there is no significant difference between the embryonic stage at the time of maternal dosing and the time of embryonic exposure to RA excess. Mice treated on day 8.0 or day 7 + 18 h were used as the source of embryos for further study. For *in situ* hybridization, embryos were fixed in 4% paraformaldehyde in PBS for ~6 h, dehydrated in alcohols and embedded in paraplast. Sections were cut at 9 $\mu$m and mounted 20–30 to a slide on slides coated with TESPA (3-aminopropyltriethoxysilane, Sigma).

### *Preparation of $^{35}$S-labelled RNA probes for Hox-2.9 and Krox-20*

*Hox-2.9* sense and antisense RNA were synthetically produced using the 3′ *Pst*I–*Eco*RI fragment as a template (Murphy and Hill, 1991). *Krox-20* specific probes were similarly prepared from the 1.5 kb *Apa*I–*Eco*RI fragment (Chavrier *et al.*, 1989). Plasmids containing the insert of interest, and a flanking T7 or T3 promoter sequence, were linearized with appropriate enzymes. Transcription reactions, with T7 or T3 RNA polymerase (Bio-Rad), were carried out in the presence of [$^{35}$S]UTP (NEN). The template was then degraded with RNase-free DNase (Pharmacia), and the labelled RNA was purified by phenol–chloroform extraction and ethanol precipitation. The transcripts were degraded to an average length of 150 bp by random alkaline hydrolysis, to improve access to RNA *in situ*. The probes were dissolved at a working concentration of $1.2 \times 10^5$ disintegrations $min^{-1}$ $\mu l^{-1}$ in hybridization mix (Wilkinson *et al.*, 1987a).

### *In situ hybridization*

The *in situ* hybridization method was largely as described by Wilkinson *et al.* (1987a) with the addition of high stringency washes (Wilkinson *et al.*, 1987b). 40 $\mu$l of the appropriate probe in hybridization mix was added to each slide. Hybridization was carried out overnight at 55°C. The slides were then washed under stringent conditions (65°C, 2 × SSC, 50% formamide) and treated with RNase to remove unhybridized and nonspecifically bound probe. Autoradiography was performed with Ilford K5 emulsion. Exposure times were between 3 and 4 weeks. After developing, sections were stained in 1% methyl green and mounted in DPX. Sections were examined and photographed using a Leitz Orthomat microscope with both dark- and bright-field illumination.

### *Scanning electron microscopy (SEM)*

8- to 20-somite stage embryos were obtained from dams killed 24–42 h after treatment with vehicle only, and 24–48 h after administration of RA. They were dissected free of their membranes and fixed in either 4% paraformaldehyde in PBS or 2.5% cacodylate-buffered glutaraldehyde. According to stage, they were either left whole or sagittally halved after fixation. They were then dehydrated in graded acetones, critical-point dried, gold-coated, and viewed in a Joel T-20 scanning electron microscope.

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