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Exposure to retinoic acid before or after the onset of somitogenesis reveals separate effects on rhombomeric segmentation and 3′ *HoxB* gene expression domains

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SUMMARY

We have compared the relationship between the patterns of altered morphogenesis and of altered gene expression in mouse embryos exposed to excess retinoic acid (RA) (a) just before and (b) just after the onset of somitic segmentation (day 7.75 to day 8.25). Exposure to RA prior to the onset of somitic segmentation results in suppression of rhombomeric (but not somitic) segmentation, and conversion of the genetic identity of the whole preotic hindbrain to that of rhombomere 4. In contrast, exposure to RA at early somite stages results in near-normal rhombomeric segmentation; rhombomeric gene expression domains indicate that only rhombomere 2 has changed its genetic identity to that of rhombomere 4, the other preotic segments showing normal expression patterns for *HoxB* genes and *Krox-20*. The results indicate that RA has separable effects (1) on the genes mediating the process of rhombomeric segmentation per se, such as *Krox-20*, and (2) on the genes that influence the nature of the structures that subsequently develop from the individual rhombomeres, such as the *Hox* genes.

Key words: retinoic acid, mouse embryo, *HoxB*, *Krox-20*, segmentation, rhombomeres

INTRODUCTION

Mammalian embryos exposed to an excess of retinoic acid (RA), or any of its precursors, exhibit a range of morphological abnormalities, the nature of which is related to the stage of development at the time of exposure (Shenefelt, 1972; Morriss, 1973). Treatment around the time of neural induction produces characteristic craniofacial malformations, an early feature of which is shortening of the preotic hindbrain (Morriss, 1972; Morriss and Thorogood, 1978).

By the time cranial neurulation is complete (day 9.0 in the mouse), the normal hindbrain consists of seven segments or rhombomeres and an unsegmented occipital region. A number of genes expressed during the period of cranial neurulation show rostral boundaries of expression that coincide with rhombomeric boundaries (Wilkinson et al., 1989b; Hunt et al., 1991). These include the homeobox-containing genes of the *HoxB* (formerly *Hox-2* (Scott, 1992)) complex (Fig. 1), which are thought to influence the nature of the structures that derive from individual rhombomeres (Wilkinson et al., 1993, and references therein). Between 8.5 and 9.5 days of gestation, transcripts of the zinc-finger gene *Krox-20* are found in two domains that correspond to rhombomeres 3 and 5 (Wilkinson et al., 1989a, and Fig. 1). The two domains are distinct before rhombomeres form, suggesting that this gene is involved in the segmentation process (Wilkinson and Krumlauf, 1990; Nieto et al., 1991). Expression domains for two RA receptors, RAR-α and RAR-β, also show a rhombomere-specific pattern at day 9, but detectable levels of RAR transcripts were not observed in rhombomeres 1-3 (Ruberte et al., 1991). Two RA binding proteins, CRABP I and CRABP II, show changing patterns of expression in the hindbrain neuroepithelium during neurulation (Ruberte et al., 1991, 1992).

In embryos exposed to raised RA levels on day 7.5 or 7.75, two different patterns of altered expression of *Hoxb-1* and *Krox-20* have been described. Morriss-Kay et al. (1991) and Conlan and Rossant (1992) found that *Hoxb-1* was expressed in an enlarged domain which occupied most of the preotic hindbrain in the affected embryos; *Krox-20* was expressed only in a small band thought to correspond to the normal rhombomere 5 domain. Conlan and Rossant (1992) also showed rostral displacement of the *Hoxb-2* expression domain. The authors of both of these studies considered that the first four rhombomeres were transformed to a single enlarged rhombomere 4. In contrast, Marshall et al. (1992), using the same RA administration timing and dosage as Conlan and Rossant (1992), but using transgenic mice containing *lacZ* reporter genes to reveal gene expression patterns, observed embryos with normal hindbrain segmentation, showing ectopic expression of *Hoxb-1* and *Hoxb-2* in rhombomere 2 only, in addition to embryos showing similar patterns of expression to those observed in the two previous studies. These results were described as the transformation of rhombomeres 2/3 into a rhombomere 4/5 identity, and RA-treated embryos with

unsegmented hindbrains were considered to be an early developmental stage that was followed by hindbrain segmentation.

The purpose of the present study is to investigate whether the two RA-induced patterns of hindbrain gene expression and morphology are merely sequential developmental stages, or are the result of RA effects on different stages of embryogenesis. In order to distinguish between rhombomeres 3 and 5, we have monitored *Hoxb-3* expression in addition to the genes studied previously.

MATERIALS AND METHODS

Animal mating and retinoic acid administration

Virgin female C57Bl/6 mice were exposed to males either overnight or from 8-11 am and were examined at 11 am for vaginal plugs. Embryonic stage at day 8 and day 9 of gestation was not affected by the timing of exposure to males but was altered by changing the timing of the light/dark cycle in the animal room. In order to obtain animals at the stages required for maternal RA administration and dissection for these experiments, the lighting was on from 8 am to 10 pm GMT.

A solution was made up containing 5 mg of all-*trans* RA (Tretinoin) (kindly donated by Hoffmann-La Roche) in 0.8 ml of absolute alcohol and 9.2 ml of arachis oil. This was gassed with argon and stored in the dark at 4°C for up to 2 days. Fifty pregnant C57Bl/6 females were dosed with 12 mg/kg of RA (0.6 ml of stock solution) by oral gavage at day 8.0 post coitum. 10 pregnant dams were given RA at day 7.75 in order to reproduce the embryonic stage studied in detail previously (Morriss-Kay et al., 1991), and 20 were dosed at day 8.25. Control mice were dosed with 0.6 ml of vehicle only. Embryos were recovered 1 hour after day 7.75, day 8.0 and day 8.25 and dissected for observation of the morphology at the time of RA exposure. The morphological differences of exposure to RA at presomite and early somite stages were clearly distinguishable by day 9.0, so for the in situ hybridization studies, treatment on day 8.0 was used to obtain both types of embryo. 24 hours after maternal RA administration, the embryos were dissected free of their membranes and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2-3 hours or overnight, then dehydrated and embedded in paraffin wax; 7 μm sections were cut and mounted onto microscope slides coated with 3-aminopropyltriethoxysilane (TESPA); the slides were baked overnight at 60°C and stored at room temperature in an airtight box containing silica gel.

In situ hybridization

The *HoxB* probes used in these experiments were kindly provided by Robb Krumlauf and the *Krox-20* probe by David Wilkinson. The in situ hybridization protocol used in these experiments was based on that described by Wilkinson and Green (1990). ^{35}S-labelled RNA probes were prepared using linearized template DNA. The exposure time of the dipped slides depended on the individual probe; the *Hoxb-3* slides were exposed for 3-4 weeks, *Hoxb-2* for 2-3 weeks, *Hoxb-1* for 2-3 weeks and *Krox-20* for 3 weeks. The sections were counterstained with haematoxylin.

RESULTS

Morphology of living and sectioned embryos

Live embryos were examined 1 hour after the RA treatment, since maternally administered RA is significantly raised in the embryos within this time (Creech Kraft et al., 1989). All day-7.75 embryos were at presomite stages, and all day-8.25 embryos were at early somite stages (2-6 pairs of somites). Day-8.0 litters consisted of a mixture of the two. Fig. 2 shows two representative embryos taken from the same litter at day 8+1 hour of development. The embryo shown in Fig. 2A is at the presomitic stage and has a flat neural plate, while that shown in Fig. 2B is at the 3-somite stage and the cranial neural folds are well developed.

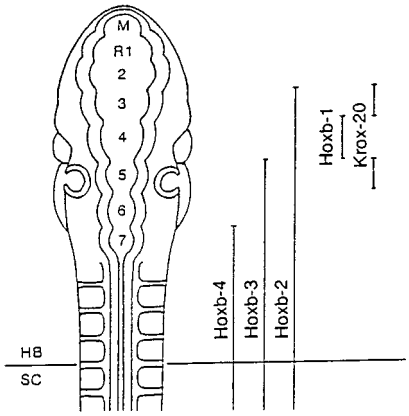

Fig. 1. Patterns of expression of the genes of the *HoxB* complex and of *Krox-20* in the neuroepithelium of the mouse hindbrain at day 9.0 of gestation. M, midbrain; R1-7, rhombomeres; HB/SC, hindbrain/spinal cord junction.

In the day-9.0 control embryos, the neuroepithelium of the hindbrain consisted of a series of seven evenly spaced segments or rhombomeres, the otocyst being level with rhombomere 5 (for example, Fig. 3A). All embryos exposed to RA on day 8.25, and some embryos exposed to RA on day 8.0, appeared essentially normal when examined live, although on sectioning it was found that the hindbrains of some of the day 8.0-exposed embryos showed minor variations of rhombomeric segmentation, the rhombomeres being slightly irregular in form and having less clearly defined boundaries than normal (Fig. 3B). RA treatment on day 7.75, and in some cases on day 8.0, resulted in a hindbrain morphology characterized by a rostral shift in the position of the otocyst with corresponding shortening of the preotic hindbrain, as observed previously (Morriss-Kay et al., 1991). Cranial neural tube closure was also delayed in these embryos. On sectioning, the preotic hindbrain was found to consist of a single large sulcus that bore some resemblance to a rhombomere, but the post-otic hindbrain was unsegmented (Figs 3C, 4C).

Two distinct phenotypes were therefore distinguishable at day 9.0: an unsegmented hindbrain phenotype, following exposure to RA at day 7.75, and a normal or near-normal rhombomeric segmentation phenotype, following exposure to RA at day 8.25. Exposure to RA on day 8.0 resulted in litters containing embryos with both phenotypes; these litters were used for the in situ hybridization study, in order to minimise the difference in developmental stage between embryos, and so that gene expression patterns in all embryos were analysed 24 hours after maternal RA administration.

HoxB and *Krox-20* expression patterns at day-9.0 post coitum

In the RA-exposed embryos that showed hindbrain segmentation, *Hoxb-1* was expressed in two domains: one correspond-

ing to the usual rhombomere 4 position (the caudal boundary coinciding with the rostral expression boundary of *Hoxb-3*), and the other corresponding to rhombomere 2 (Figs 3B, 4B). *Hoxb-2* was also ectopically expressed in rhombomere 2, its rostral boundary having moved from the rhombomere 2/3 to the rhombomere 1/2 boundary (Fig. 3B). Both *Hoxb-1* and *Hoxb-2* were ectopically expressed in the neural crest migrating from rhombomere 2. Therefore, in rhombomere 2 and its associated neural crest, neither of which normally shows expression of any of the genes of the *HoxB* complex, the combination of genes usually associated with rhombomere 4 was expressed. Rhombomere 3 showed its normal complement of gene expression (*Hoxb-2* and *Krox-20*; Figs 3B, 4B).

In the embryos lacking hindbrain segmentation, the preotic hindbrain consisted of a single large rhombomere-like sulcus (Figs 3C, 4C). *Hoxb-1* was expressed throughout this region, the caudal expression boundary again corresponding to the rostral boundary of *Hoxb-3* expression (Fig. 4C). The rostral boundaries of *Hoxb-1* and *Hoxb-2* expression were less clearly defined than in the controls, but appeared to coincide (Fig. 3C). *Hoxb-1* and *Hoxb-2* were also expressed in the neural crest migrating from this large sulcus.

The expression pattern of *Krox-20* was normal in the embryos with the segmented hindbrain phenotype, being expressed in rhombomeres 3 and 5 (Fig. 4B). Rhombomere 3 was therefore unaffected by the RA treatment with respect to both *HoxB* and *Krox-20* expression. In the embryos with a single large rhombomere in the preotic hindbrain, the rhombomere 3 domain of *Krox-20* expression was missing (Fig. 4C and as described previously; Morriss-Kay et al., 1991). Therefore, in the unsegmented hindbrain phenotype, rhombomere 3 was not represented and what remained of the preotic hindbrain region expressed the combination of genes usually associated with rhombomere 4.

DISCUSSION

The results of this study show that exposure of mouse embryos to a single pulse of increased concentration of RA within a 12-hour period (day 7.75-8.25) during early neurulation results in two different craniofacial phenotypes as observed on day 9.0 p.c. The characteristics of these two phenotypes may be summarised as follows. (1) Exposure to RA on day 7.75, when embryos are at the late presomite stage, results in a phenotype in which the hindbrain is morphologically unsegmented and the shortened preotic hindbrain has the morphology of a single large rhombomere-like structure. The otocyst is abnormally rostral in position and the first pharyngeal (mandibular) arch is partially or completely incorporated into the maxillary region of the face, indicating that the shifted otocyst has maintained its normal position relative to the second pharyngeal arch. (2) Exposure to RA on day 8.25, when embryos are at early somite stages, results in a phenotype that appears to be normal except for a very slightly shortened preotic hindbrain and a correspondingly shortened gap between the mandibular arch and the maxillary region.

Administration of RA on day 8.0 resulted in litters that at day 9.0 had embryos of both phenotypes. In contrast to litters exposed to RA on day 7.75 or day 8.25, the range of stages in these litters at the time of RA exposure includes the stage of onset of somitic segmentation. The only sign of intermediate forms was the rare observation of embryos of the segmented hindbrain phenotype, in which the rhombomeres were slightly irregularly spaced or had less clearly defined boundaries than normal. The precise timing for induction of the segmented and unsegmented hindbrain phenotypes varies between different laboratories and has shifted over a period of time in our own stock; it is not related to RA dosage (different phenotypes were produced by administration of 20 mg/kg in the Rossant and Krumlauf laboratories).

These observations reveal a direct correlation between exposure to RA at presomite stages and failure of rhombomeric segmentation; in contrast, RA is unable to prevent rhombomere formation in embryos of early somite stages, suggesting that a commitment to undergo hindbrain segmentation is made close

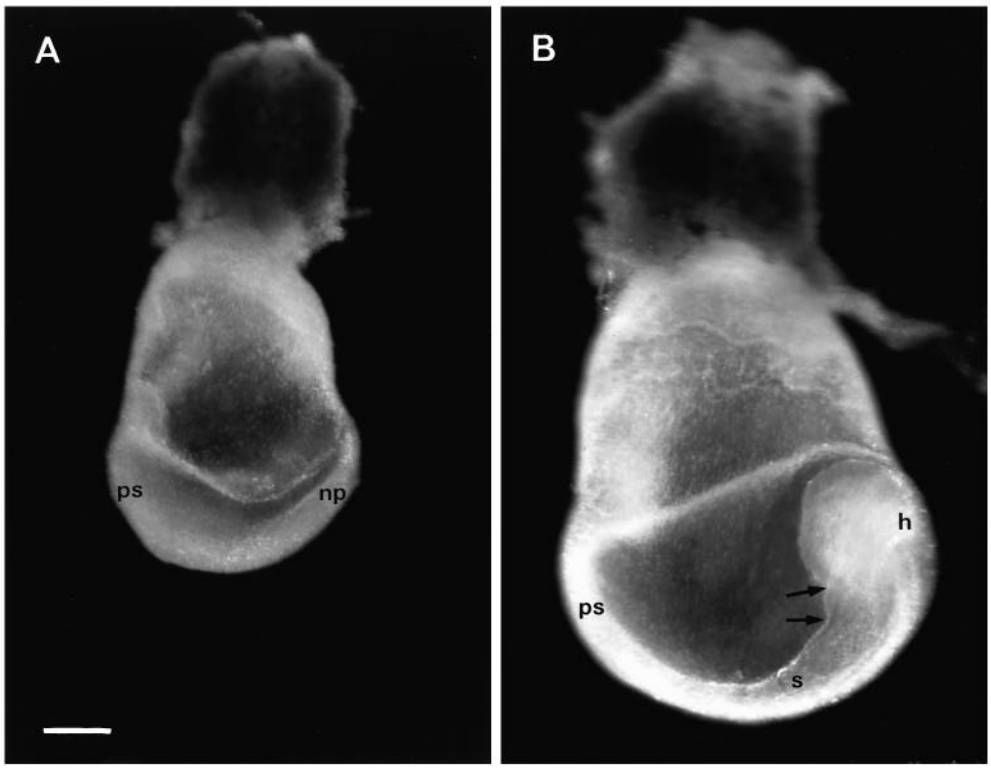

Fig. 2. Two embryos taken from a control litter at day 8+1 hour of development. (A) Presomite stage (as also observed for all embryos explanted at day 7.75), with flat neural plate (np) and primitive streak (ps). (B) Early somite stage (all embryos explanted at day 8.25 were at or beyond this stage): the cranial neural folds are well advanced and the preotic and otic sulci have formed (arrows); the heart (h) and foregut (beneath it) are developing and there are three pairs of occipital somites (s, first somite). Bar, 100 μm.

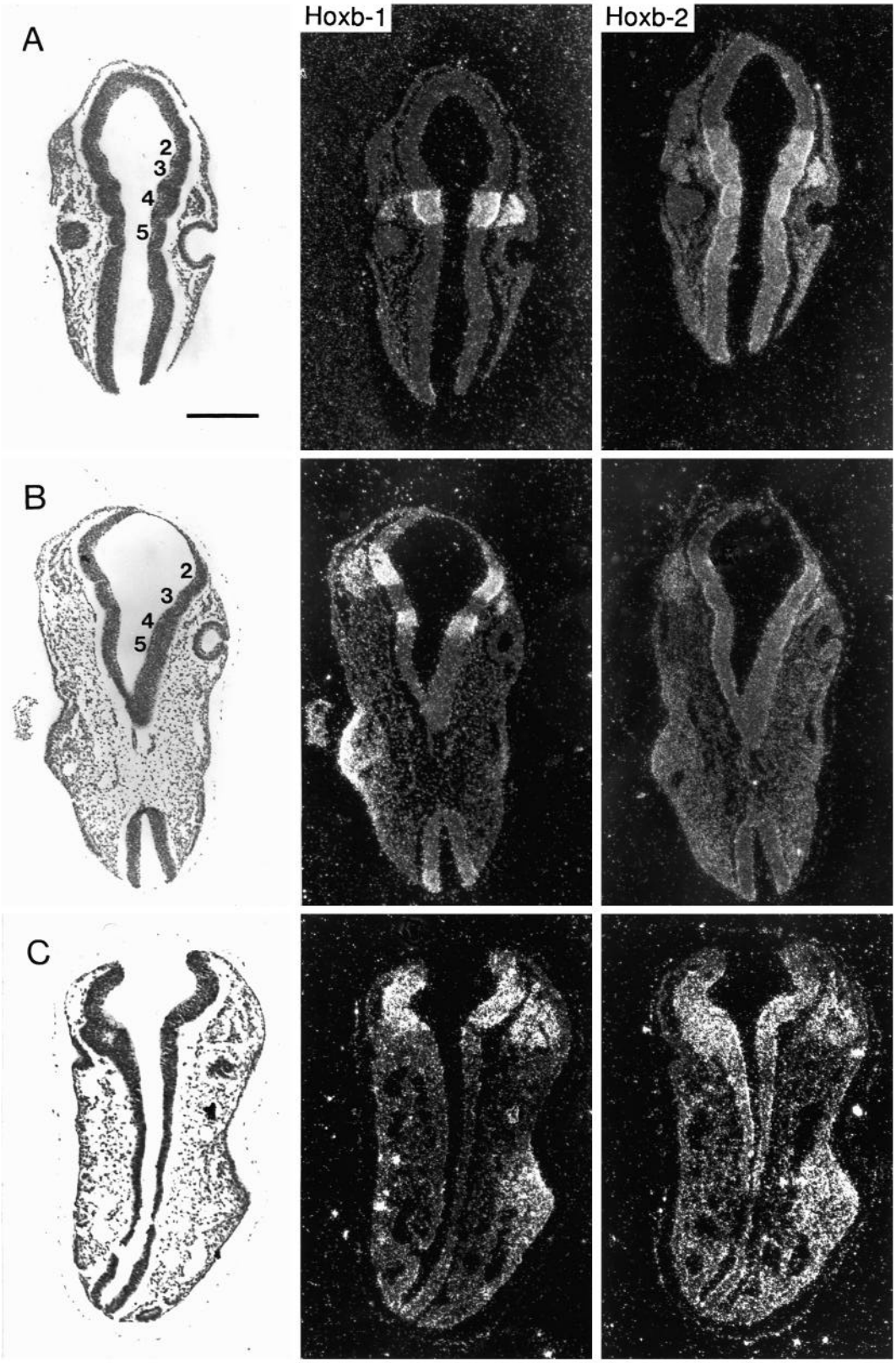

Fig. 3. In situ hybridization on paired adjacent coronal sections of day-9.0 embryos showing expression patterns of *Hoxb-1* and *Hoxb-2*. Rhombomeres, where present, are numbered. (A) Control embryo: *Hoxb-1* transcripts are confined to rhombomere 4 and *Hoxb-2* is expressed up to the rhombomere 2/3 boundary; *Hoxb-1* and *Hoxb-2* transcripts are present in neural crest cells adjacent to rhombomere 4. (B) 24 hours after RA administration on day 8.0; segmented hindbrain phenotype: *Hoxb-1* is expressed ectopically in rhombomere 2 and normally in rhombomere 4; *Hoxb-2* is ectopically expressed in rhombomere 2; neural crest cells adjacent to rhombomere 2 show expression of *Hoxb-1* and *Hoxb-2*. (C) 24 hours after RA administration on day 8.0; unsegmented hindbrain phenotype: *Hoxb-1* and *Hoxb-2* are expressed throughout a large rhombomere-like sulcus in the preotic hindbrain, and in its associated neural crest cells. Bar, 200 μm.

to the time of onset of somitogenesis and that this decision is irreversible by RA. It is interesting to note that a correlation between the onset of overt somitic segmentation and the onset of hindbrain segmentation is shown by the tyrosine kinase gene *Sek*, which is expressed during segmentation in both the hindbrain and the mesodermal segmental plate (Nieto et al., 1992).

The two RA-induced phenotypes observed on day 9.0 were

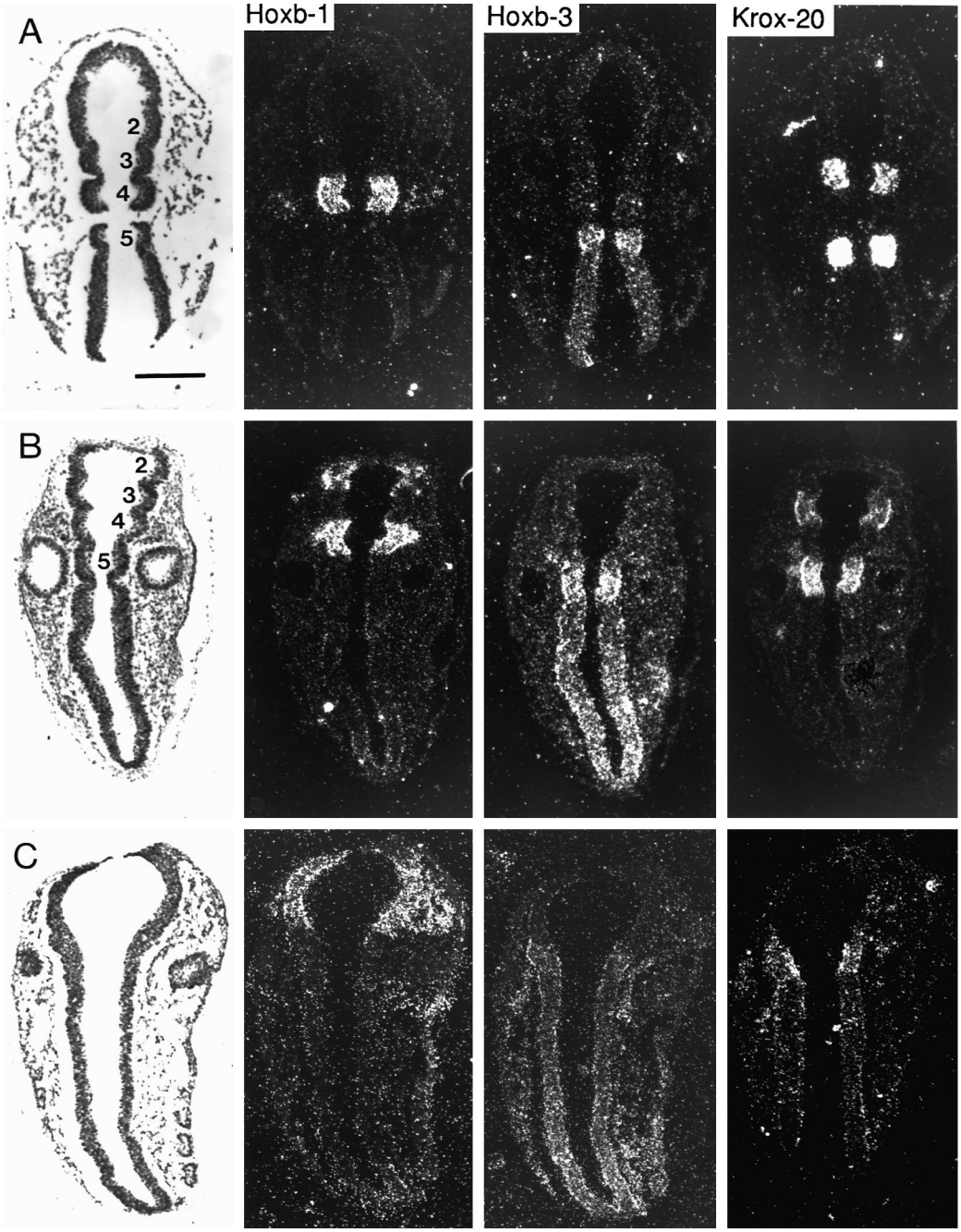

Fig. 4. In situ hybridization on adjacent coronal sections of day-9.0 embryos showing expression patterns of *Hoxb-1*, *Hoxb-3* and *Krox-20*. (A) Control embryo: *Hoxb-1* is expressed in rhombomere 4, *Hoxb-3* is expressed up to the rhombomere 4/5 boundary and *Krox-20* is expressed in rhombomeres 3 and 5. (B) 24 hours after RA administration on day 8.0; segmented hindbrain phenotype: *Hoxb-1* is expressed in rhombomere 4, and is also ectopically expressed in rhombomere 2; the expression patterns of *Hoxb-3* and *Krox-20* are unchanged. (C) 24 hours after RA administration on day 8.0; unsegmented hindbrain phenotype: *Hoxb-1* is expressed in a single enlarged domain in the preotic hindbrain, and in its associated neural crest; *Hoxb-3* is expressed up to the caudal expression boundary of *Hoxb-1* and in neural crest cells between the neuroepithelium and otic pit; *Krox-20* is expressed in a single domain adjacent to the otic pit (and in adjacent neural crest cells, not clearly shown here), but its rhombomere 3 expression domain is absent. Bar, 200 μm.

associated with different patterns of gene expression (summarised in Fig. 5). In the unsegmented hindbrain phenotype, the entire preotic hindbrain and its associated neural crest expressed the combination of *HoxB* genes normally characteristic of rhombomere 4, and rhombomere 3 was not represented. In embryos with the segmented hindbrain phenotype, only rhombomere 2 and its neural crest showed an abnormal pattern of gene expression, having the *HoxB* characteristics of rhombomere 4. Marshall et al. (1992), using mice containing *lacZ* reporter genes that reproduced the expression patterns of *Hoxb-1*, *Hoxb-2* and *Krox- 20*, described the effects of RA exposure as the transformation of rhombomeres 2 and 3 to a rhombomere 4 and 5 identity, giving a rhombomeric sequence of 1,4,5,4,5 in the preotic hindbrain. This interpretation is inconsistent with our results, which showed that rhombomere 3, where present, expressed the normal combination of genes (*Hoxb-2* and *Krox-20*), without ectopic *Hoxb-3* expression, indicating that the RA-induced rhombomeric sequence was 1,4,3,4,5.

The *Krox-20* gene has recently been disrupted (Schneider-Manoury et al., 1993), and the resulting phenotype provides some insight into our results. *Krox-20*$^{-/-}$ embryos have an unsegmented hindbrain that lacks *Krox-20* promoter activity in the rhombomere 3, but not the rhombomere 5, position; *Hoxb-2*, which has previously been suggested to be under the control of *Krox-20* (Sham et al., 1993) and is normally expressed in rhombomeres 3, 4 and 5, is expressed in a single domain thought to correspond to rhombomere 4, together with *Hoxb-*

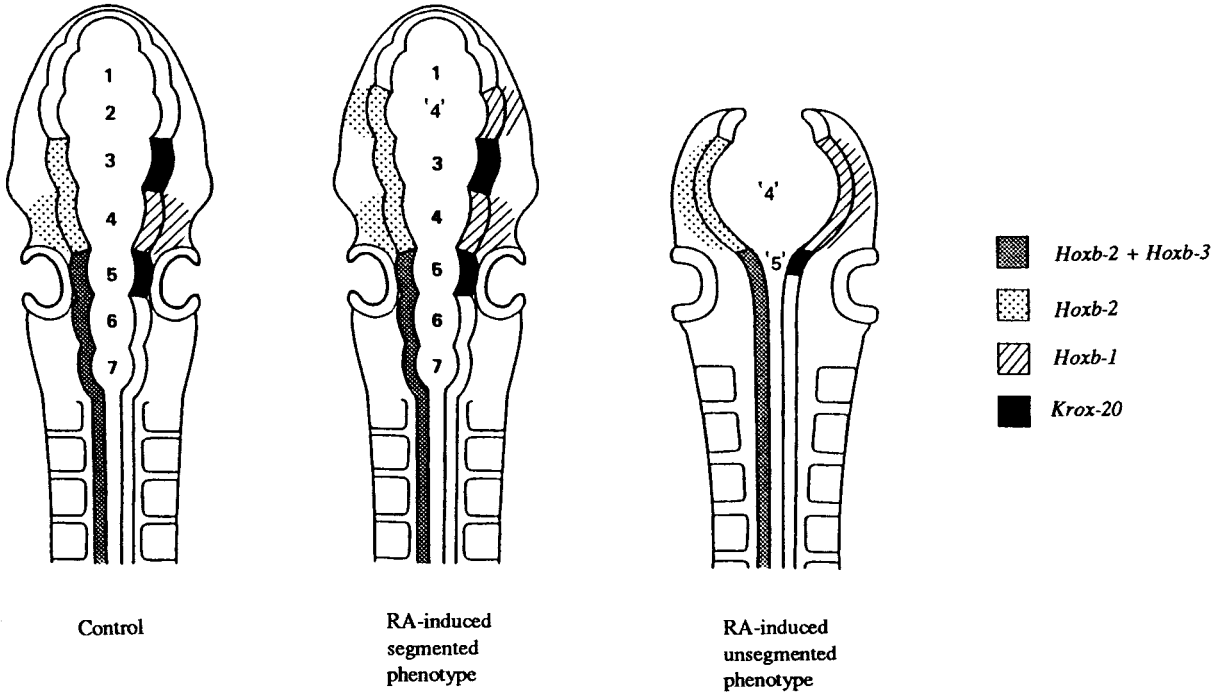

Fig. 5. Domains of expression of the 3′ *HoxB* genes and *Krox-20* in the hindbrain in control embryos (left) and in response to exposure to excess retinoic acid. In embryos exposed to RA after the onset of somitic segmentation (segmented hindbrain phenotype), rhombomere 2 and its associated neural crest show ectopic expression of the *HoxB* gene combination characteristic of rhombomere 4, but rhombomeres 3, 4, and 5 are normal. In embryos exposed to excess RA before the onset of somitic segmentation (unsegmented hindbrain phenotype), the entire preotic hindbrain and its associated neural crest show the *HoxB* gene expression characteristics of rhombomere 4, with *Krox-20* expressed in a single band adjacent to the otic pit, corresponding to the usual rhombomere 5 domain; *Hoxb-3* expression is normal in relation to both the otic pit and to the *Hoxb-1* caudal boundary.

1. Comparison with our results suggests that when presomite-stage embryos are exposed to RA, absence of the rhombomere 3 *Krox-20* domain may be causally correlated with the concomitant failure of segmentation. In contrast, *Hoxb-1* expression is altered in both RA-induced phenotypes but normal in the *Krox-20*$^{-/-}$ embryos, indicating that RA-induced alteration of *Hoxb-1* expression is not mediated by *Krox-20* but is analogous to the RA-induced upregulation of *Hoxb-1* observed in EC cells (Simeone et al., 1990).

Exposure of mouse embryos to RA before and after the hindbrain is committed to undergo segmentation reveals that RA has separable effects on (a) the genes responsible for segmentation and (b) the genes that influence the nature of the structures that subsequently develop from individual rhombomeres. This separate genetic control of segmentation and segment identity is analogous to development in *Drosophila*, in which there are separate sets of genes responsible for setting up the initial segmental pattern (the gap, pair-rule and segment polarity genes) and for specifying the structures that later develop from the individual segments (the homeobox genes) (Doe and Scott, 1988; Ingham, 1988; Graham et al., 1989). Segment formation in *Drosophila* is similar in some respects to rhombomere formation: in both, all the segments are formed within a short time period from a pre-existing epithelium, in contrast to somite formation in which segments are laid down sequentially by mesenchymal condensation.

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