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Exogenous retinoic acid rapidly induces anterior ectopic expression of murine *Hox-2* genes in vivo

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Summary

Exogenous retinoic acid (RA) has teratogenic effects on vertebrate embryos and alters *Hox-C* gene expression in vivo and in vitro. We wish to examine whether RA has a role in the normal regulation of *Hox-C* genes, and whether altered *Hox-C* gene expression in response to RA leads to abnormal morphology. The expression of 3′ *Hox-2* genes (*Hox-2.9*, *Hox-2.8*, *Hox-2.6* and *Hox-2.1*) and a 5′ gene (*Hox-2.5*) were examined by whole-mount in situ hybridization on embryos 4 hours after maternal administration of teratogenic doses of RA on embryonic day 7 to 9. The expression of the 3′ *Hox-2* genes was found to be ectopically induced in anterior regions in a stage-specific manner. The *Hox-2.9* and *Hox-2.8* genes were induced anteriorly in the neurectoderm in response to RA on day 7 but not at later stages. Expression of *Hox-2.6* and *Hox-2.1* was ectopically induced anteriorly in neurectoderm in response to RA on day 8. *Hox-2.1* remained responsive on day 9, whereas *Hox-2.6* was no longer responsive at this stage. The expression of the 5′ gene *Hox-2.5* was not detectably altered at any of these stages by RA treatments. We also examined the response of other genes whose expression is spatially regulated in early embryos. The expression of *En-2* and *Wnt-7b* was not detectably altered by RA, whereas RARβ expression was induced anteriorly by RA on day 7 and 8. *Krox-20* expression was reduced in a stage- and region-specific manner by RA. The ectopic anterior expression of *Hox-2.8* and *Hox-2.9* induced by RA on day 7 was persistent to day 8, as was the altered expression of *Krox-20*. The altered pattern of expression of these genes in response to RA treatment on day 7 may be indicative of a transformation of anterior hindbrain to posterior hindbrain, specifically, a transformation of rhombomeres 1 to 3 towards rhombomere 4 identity with an anterior expansion of rhombomere 5. The ectopic expression of the 3′ *Hox-2* genes in response to RA is consistent with a role for these genes in mediating the teratogenic effects of RA; the rapid response of the *Hox-C* genes to RA is consistent with a role for endogenous RA in refining 3′ *Hox-C* gene expression boundaries early in development.

Key words: retinoic acid, Hox genes, mouse embryos.

Introduction

Retinoids have long been recognized as teratogens for vertebrate embryos with effects on a wide variety of structures at a number of stages of development (Hale, 1933; Cohlan, 1953; Shenefelt, 1972). The responding organ or structure typically has a period of sensitivity to exogenous retinoids that corresponds approximately to the time of establishment of the anlage for that organ or structure (Shenefelt, 1972). The morphology of some of the retinoid-induced abnormalities has been interpreted as a transformation of anterior cell fates to more posterior cell fates. The most striking morphological transformations are seen in the chick limb bud where local application of retinoids at the anterior margin of the limb bud results in mirror-image duplications of posterior limb structures (reviewed by Tickle, 1991; Tabin, 1991). Recently, retinoid-induced morphological transformations have been documented for structures other than the limb. The otic vesicle is positioned more anteriorly in embryos treated with retinoic acid (RA) in mouse, *Xenopus* and zebrafish (Sulik et al., 1988; Ruiz i Altaba and Jessell, 1991b; Holder and Hill, 1991), and the extent of the hindbrain and spinal cord may be increased at the expense of the midbrain and forebrain in *Xenopus* embryos treated with high doses of RA (Durston et al., 1989). Application of exogenous RA to mouse embryos causes somites to form at levels more anterior than normal (Morriss-Kay et al., 1991) as well as causing alterations in the axial skeleton suggestive of anterior-to-posterior transformations (Kessel and Gruss, 1991). The transformation of cell fates implies that important developmental processes, particularly specification along the anterior-posterior (AP) axis, can be disrupted by exogenous retinoids. It is possible that exogenous RA causes anterior-to-posterior transformations by disrupting an endogenous RA-based signalling system which specifies position along the AP axis of the embryo.

Vertebrate embryos, including the chick, the mouse and *Xenopus*, all contain endogenous RA (Thaller and Eichele,

1987; Satre and Kochhar, 1989; Durston et al., 1989). Vertebrate embryos also contain ligand-activated receptors, the retinoic acid and retinoid X receptors (Ellinger-Ziegelbauer and Dreyer, 1991; Rowe et al., 1991; Ruberte et al., 1991; Smith and Eichele, 1991; Mangelsdorf et al., 1992), as well as retinoic acid- and retinoid-binding proteins of undefined function (Dencker et al., 1990; Maden et al., 1991; Ruberte et al., 1991), all of which could cooperate to transduce a RA signal. The distributions of the retinoic acid receptors and binding proteins during embryogenesis are complex and dynamic, and the distribution of RA itself is unknown; however, the domains of RA action almost certainly depend not just on RA distribution, but on the integration of receptor and binding protein distribution, and, possibly, interactions with other transcription factors (Husmann et al., 1991; Schüle et al., 1991; Yu et al., 1991; Kliewer et al., 1992; Leid et al., 1992; Zhang et al., 1992). Despite this apparent complexity, a RA-responsive transgene (*RAREhsplacZ*) designed to reveal RA-mediated transcriptional activity in the mouse embryo generates a domain of β-galactosidase staining with a sharp anterior boundary across all germ layers at the early neural plate stage of development (Rossant et al., 1991). The expression of the *RAREhsplacZ* transgene presumably represents an integrated response to the RA signal, and supports a role for RA in patterning along the AP axis.

The target genes of the RA-transducing system in the early embryo are not known, but it has been suggested that members of the *Hox-C* gene clusters might normally be spatially regulated by RA in the undisturbed embryo and that ectopic expression of these genes induced by exogenous RA leads to morphological transformations (Krumlauf et al., 1991). Mice have four clusters of *Hox-C* genes related by sequence, organization and expression to the homeotic complexes (HOM-C) of *Drosophila* (Duboule and Dollé, 1989; Graham et al., 1989; McGinnis and Krumlauf, 1992). Genetic manipulation and phenotypic analysis has suggested that the vertebrate *Hox-C* genes, like the *Drosophila* HOM-C genes, specify regional identity along the body axis (McGinnis and Krumlauf, 1992). Mutational analysis has been performed on only a few of the approximately 40 members of the murine *Hox-C* family; however, the elimination or ectopic expression of *Hox-C* genes result in regionally restricted defects and homeotic transformations, as one would predict if *Hox-C* genes specify regional identity (Balling et al., 1989; Kessel et al., 1990; Chisaka and Capecchi, 1991; Lufkin et al., 1991; Chisaka et al., 1992). Also, the morphological defects caused by ectopic expression of the *Hox-1.1* gene are similar to some of the defects caused by exogenous retinoic acid (Balling et al., 1989).

If the normal boundaries of *Hox-C* gene expression are set by an RA-based signalling system, then one might predict that *Hox-C* genes should respond to additional, exogenous RA by shifts in their boundaries of expression. The *Xenopus* labial-class homeobox genes *Xhox.lab1* and *Xhox.lab2*, the sex combs reduced class gene *XlHbox4* and the Abdominal-B class gene *Xlhbox6* are induced to higher levels of expression in *Xenopus* embryos treated with RA (Cho and De Robertis, 1990; Sive et al., 1990; Papalopulu et al., 1991b; Sive and Cheng, 1991). In addition, morphological transformations caused by exogenous RA have been shown to be accompanied by ectopic anterior expression of *Hox-C* genes in the mouse embryo (Morriss-Kay et al., 1991; Kessel and Gruss, 1991). However, in these studies, the expression of *Hox-C* genes was not assayed until well after morphological abnormalities were evident, leaving it unclear whether the alteration in *Hox-C* gene expression was a primary response to RA or was secondary to the morphological changes.

To try to ascertain whether exogenous RA could result in primary changes in gene expression in vivo, we investigated the patterns of expression of several members of the *Hox-2* gene cluster, in addition to other spatially regulated genes (*Krox-20*, *En-2*, *Wnt-7b* and RARβ), shortly after treatment with RA. To expedite the comparison of many different patterns of gene expression, we developed a whole-mount in situ hybridization procedure for postimplantation mouse embryos. Exogenous RA induced ectopic anterior expression of the RARβ and *Hox-2.8*, *Hox-2.9*, *Hox-2.6* and *Hox-2.1* genes within 4 hours of treatment, before morphological abnormalities were evident. This rapid response supports a primary role for RA in AP patterning through the establishment of anterior expression boundaries of the anterior *Hox-C* genes. Further analysis suggested that RA-mediated positional signalling has spatial and temporal limits within the embryo, and that other factors are involved in the stabilization of the boundaries of expression established through the action of RA.

Materials and methods

Administration of RA and ethanol

Pregnant CD-1 female mice were administered RA essentially as described by Rossant et al. (1991). A stock solution of 25 mg/ml all-*trans* retinoic acid (Sigma) in DMSO was diluted one in ten in vegetable oil just before use, and 0.2 ml delivered by gavage for a final dose of approximately 20 mg/kg of maternal body weight. Control mice were administered the same mixture without RA. For treatments of 4 hours, mothers were treated at 8 am, 12 pm or 4 pm on embryonic day 7, and 8 am on days 8 and 9. The day of plug observation was counted as day 0. For examination of long-term effects, mothers were treated at 12 pm on embryonic day 7 and the embryos were recovered at 8 am on the following day. In our mouse colony, embryos were typically preheadfold at 8 am, headfold stage at 12 pm and possessed foregut pockets by 6 pm on day 7. At 8 am on day 8, most embryos had four to six somites.

Ethanol was injected intraperitoneally as 0.75 ml of 25% ethanol in 0.1 M NaCl. This dose of ethanol has been shown to be teratogenic (Sulik et al., 1981). Control mice received 0.75 ml of 0.1 M NaCl. The administration of carrier solutions alone had no effect on the expression of the genes studied as assayed by in situ hybridization (data not shown).

Histological detection of β-galactosidase

Embryos hemizygous for the *RAREhsplacZ* transgene were obtained by mating outbred CD-1 females to hemizygous or homozygous males of the Tg12 line (Rossant et al., 1991). The embryos were stained for β-galactosidase as described therein. They were subsequently cleared in 50 and 80% glycerol.

Preparation of embryo acetone powder

The procedure for preparation of acetone powder for preabsorption of the antidigoxigenin antibody is that of Harlow and Lane (1988). Briefly, 13.5 day embryos were homogenized in a minimum volume of calcium- magnesium-free PBS on ice. Four volumes of cold acetone were added and mixed vigorously. This was incubated at 0°C for 30 minutes with occasional vigorous mixing. The precipitate was collected by centrifugation at 10,000 ***g*** for 10 minutes. The pellet was resuspended in cold acetone and kept on ice for 10 minutes. The precipitate was collected by centrifugation again, and the pellet air-dried.

Synthesis of hybridization probes

Single-stranded RNA probes containing digoxigenin were synthesized from linearized template DNA exactly as directed by the manufacturer (Boehringer Mannheim Biochemicals). The amount of RNA synthesized was determined by spotting aliquots of the probe on a nylon filter, as well as digoxigenin-labelled DNA standards, and detecting with an anti-digoxigenin antibody as described by the manufacturer.

Hox-2 plasmids were generously provided by R. Krumlauf, the *Krox-20* plasmid by D. Wilkinson, the RARβ plasmid by V. Giguère, and the *Wnt-7b* plasmid by A. McMahon. For the synthesis of hybridization probes, plasmid DNA was linearized with the appropriate restriction enzyme and transcribed in vitro. The plasmid 866 contains an 800 bp *Hox-2.9* cDNA fragment in pBluescript KS. The plasmid 865 is a 3 kb *Hox-2.8* fragment in pBluescript KS. Probes for *Hox-2.6* were transcribed from linearized 650, a 1.3 kb *Hox-2.6* fragment in pBluescript KS. The plasmid 267 is a 800 bp *Hox-2.1* cDNA fragment in pGEM-1. The plasmid 755 is a 400 bp fragment of *Hox-2.5* cloned in pBluescript KS. The plasmid pBSEn-2 is a 260 bp *En-2* cDNA fragment cloned in pBluescribe M13+. This plasmid was constructed using the *Eco*RI/*Hin*dIII insert of pC5-BS12 (Davis et al., 1988). The plasmid pBSRARβ is a 450 bp fragment of the RARβ ligand-binding domain cloned in pBluescribe M13+. This was generated with the *Eco*RI/*Hin*dIII insert of pGmRARβ/RV-RI. The plasmid mKrox 20 *Pst*/*Apa* is a 800 bp fragment of *Krox-20* in pBluescript KS. Probes for *Wnt-7b* were synthesized from linearized plasmid consisting of 3066 bp of 3′ untranslated *Wnt-7b* mRNA cloned into pGem 3Zf(+).

Whole-mount in situ hybridization

Whole-mount in situ hybridizations were performed in a manner similar to procedures for embryos of other organisms (Tautz and Pfeifle, 1989; Hemmati-Brivanlou et al., 1990). Embryos were dissected free of extraembryonic membranes in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS for 2 hours at 4°C. Embryos were rocked gently throughout the procedure on a mechanical rocking platform unless otherwise indicated. The embryos were washed three times with PBS containing 0.1% Tween 20 (PBT), dehydrated into 100% methanol and stored at −20°C. Fixed embryos can be stored for several months in this manner. To resume, the embryos were treated with 5:1 methanol/30% hydrogen peroxide for 3 to 5 hours at room temperature. After several washes with methanol, the embryos were rehydrated through a methanol-PBT series and washed three times in PBT. Embryos were treated with 20 μg/ml proteinase K in PBT for 5 minutes at room temperature, followed by two washes for 5 minutes each with PBT containing 2 mg/ml glycine, and then two washes with PBT. The embryos were refixed in 0.2% glutaraldehyde and 4% paraformaldehyde in PBS for 20 minutes at room temperature and then washed three times with PBT. The embryos were treated with freshly prepared 0.1% sodium borohydride in PBT for 20 minutes in upright tubes with the caps loosened to allow evolved gas to escape, followed by three washes with PBT. Embryos were prehybridized for 1 hour at 63°C in hybridization buffer (50% formamide, 0.75 M NaCl, 10 mM Pipes pH 6.8, 1 mM EDTA, 100 μg/ml tRNA, 0.05% heparin, 0.1% BSA, 1% SDS). The hybridization buffer was replaced, and single-stranded RNA probes labelled with digoxigenin were added to 2 μg/ml, and the embryos were hybridized overnight at 63°C. The embryos were washed through three changes of Wash 1 (0.3 M NaCl, 10 mM Pipes pH 6.8, 1 mM EDTA, 1% SDS), followed by two washes for 30 minutes each at 63°C in Wash 1. The embryos were washed twice with Wash 1.5 (50 mM NaCl, 10 mM Pipes pH 6.8, 1 mM EDTA, 0.1% SDS), then once with the same for 30 minutes at 50°C. The wash buffer was replaced by two changes of RNAase buffer (0.5 M NaCl, 10 mM Pipes pH 7.2, 0.1% Tween 20). The embryos were incubated with 100 μg/ml RNAase A and 100 U/ml RNAase T1 in RNAase buffer twice for 30 minutes at 37°C. The embryos were washed twice with RNAase buffer, twice with Wash 2 (50% formamide, 300 mM NaCl, 10 mM Pipes pH 6.8, 1 mM EDTA, 1% SDS), then once at 50°C for 30 minutes in Wash 2. This was followed by two changes through Wash 3 (50% formamide, 150 mM NaCl, 10 mM Pipes pH 6.8, 1 mM EDTA, 0.1% Tween 20) after which the embryos were washed at 50°C for 30 minutes in Wash 3. The embryos were washed twice with Wash 4 (500 mM NaCl, 10 mM Pipes pH 6.8, 1 mM EDTA, 0.1% Tween 20), then placed in a heating block at 70°C for 20 minutes to inactivate endogenous alkaline phosphatases.

Detection of hybridization

The embryos were incubated for 1 hour at room temperature with 10% normal goat serum (heat-inactivated just before use at 70°C for 30 minutes) and 2 mM levamisole (fresh) in TBST (137 mM NaCl, 25 mM Tris-HCl pH 7.6, 3 mM KCl, 0.1% Tween 20). Sufficient embryo acetone powder was heat-inactivated just before use in a small volume of TBST at 70°C for 30 minutes. Anti-digoxigenin antibody coupled to alkaline phosphatase (BMB) at a 1/5,000 dilution was preabsorbed for 30 minutes at 4°C with 1% (w/v) embryo acetone powder in TBST containing 1% heat-inactivated normal goat serum and 2 mM levamisole. Debris was removed from the antibody by centrifugation at 10,000 ***g***. Embryos were incubated with the preabsorbed antibody overnight at 4°C. The embryos were washed three times at room temperature with TBST containing 2 mM levamisole, followed by five to seven washes for 1 hour each at room temperature. The last wash was followed by three changes of NTMT (100 mM NaCl, 100 mM Tris-HCl pH 9.5, 50 mM $MgCl_2$, 0.1% Tween 20) containing 2 mM levamisole. The colour reaction was initiated by washing the embryos into NTMT containing 2 mM levamisole, 4.5 μl/ml NBT (75 mg/ml nitroblue tetrazolium salt in 70% dimethylformamide) and 3.5 μl/ml BCIP (50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidine salt in 100% dimethylformamide). Staining was allowed to proceed overnight in the dark at 4°C for moderately prevalent messages, or at room temperature for rare messages. When staining was satisfactory, the embryos were washed three times with TBST, then dehydrated through 30, 50, 70 and 100% methanol to intensify the reaction product. The embryos were rehydrated and cleared in 50 and 80% glycerol.

We have found that the sensitivity of this whole-mount in situ hybridization procedure is comparable to standard procedures for mouse embryos utilizing hybridization of radiolabelled probes to sections. We have been able to visualize the expression pattern of RARβ transcripts, which are of low prevalence, and we detected expression of this gene in head mesenchyme that was not reported in sections (see below).

Results

We wished to examine the early effects of exogenous RA on the spatial patterns of expression of a number of genes thought to have roles in specifying region-specific morphology along the AP axis.

A preliminary experiment was conducted to determine conditions of RA treatment for which responses in gene expression might be expected. The mothers of embryos transgenic for *RAREhsplacZ*, a RA-responsive transgene (Rossant et al., 1991), were fed teratogenic doses of RA on embryonic day 7 when the majority of the embryos were at the early headfold stage, and the embryos were harvested at 2 hour intervals after treatment and examined histologically for β-galactosidase activity. The *RAREhsplacZ* transgene directs β-galactosidase expression in the posterior half of headfold-stage embryos (Rossant et al., 1991). RA treatment results in a spread of transgene-directed β-galactosidase expression into domains that do not normally express the transgene. We designated maximal induction as the state where β-galactosidase staining was equally intense throughout the embryo. With less than maximal induction, β-galactosidase expression was induced anteriorly in the neural folds, and this staining spread to the more medial portions of the anterior neural plate with increased length of treatment or dose of RA (not shown). The minimal time and dose for maximal induction was 4 hours and 20 mg/kg of maternal body weight (not shown). There were no morphological abnormalities apparent in embryos 4 hours after treatment with RA, but this dose regime produced a syndrome of defects by 20 hours after treatment at the headfold stage (see below). Subsequent experiments to determine the immediate response of genes to RA used this dose and interval.

The responses to RA reported below were consistently observed in almost all embryos. For reasons that are obscure, some embryos within litters, accounting for less than ten percent of the total, did not show the induced changes in gene expression or morphology. In addition, there was some slight variability in the strength of the responses to treatment. The responses reported here were representative of the majority of embryos in each case (out of approximately 10 to 50 embryos for each treatment).

Immediate effects of RA on gene expression on day 7

We examined responses to exogenous RA of five different genes (*Hox-2.9*, *Hox-2.8*, *Hox-2.5*, *Krox-20* and RARβ) on embryonic day 7. Treatments were initiated at times when the majority of embryos could be expected to be at preheadfold, early headfold and late headfold stages (see Materials and methods). We found three different modes of response. Three genes (*Hox-2.9*, *Hox-2.8* and RARβ) responded to RA by an anterior shift in their boundaries of expression within 4 hours, one gene (*Krox-20*) responded by a decrease in expression, whereas one gene (*Hox-2.5*) did not respond at all, as assayed by whole-mount in situ hybridization.

(A) Hox-2 *gene family*

Throughout this study, we examined the response to RA of several members of the *Hox-2* gene family, ranging from the 3′, most anteriorly expressed genes *Hox-2.8* and *Hox-2.9*, through *Hox-2.6* and *Hox-2.1*, which show boundaries of expression around the developing hindbrain-spinal cord junction, to *Hox-2.5* which is the most posterior, and 5′-most member of the *Hox-2* gene family identified to date (Hunt et al., 1991b). On day 7, only *Hox-2.8*, *Hox-2.9* and *Hox-2.5* are expressed, and the most 3′, *Hox-2.8* and *Hox-2.9*, responded to RA with an anterior shift in gene expression.

The normal pattern of expression of *Hox-2.9* in primitive streak and early neural plate stages of development has been well defined (Murphy et al., 1989; Frohman et al., 1990; Hunt et al., 1991a,c; Murphy and Hill, 1991). *Hox-2.9* is expressed in embryonic ectoderm and mesoderm in the posterior half of the embryo by late primitive streak stages. By the headfold stage, *Hox-2.9* expression extends slightly anterior to the node in the neural epithelium and the underlying mesoderm. Shortly before formation of the first somite, *Hox-2.9* expression in the neurectoderm extends to the posterior edge of the preotic sulcus. About the time of the formation of the first somite, the most anterior neural staining at the preotic sulcus becomes separated from the staining in the posterior of the embryo by a region of lower expression, and the mesodermal expression has retracted towards the posterior to about the level of the node. The anterior limit of neural expression of *Hox-2.9* in late headfold embryos is thought to be maintained during closure of the anterior neural tube, and to represent the anterior boundary of the later expression domain in rhombomere 4 (Frohman et al., 1990; Murphy and Hill, 1991). RA treatment resulted in an expansion of the domain of *Hox-2.9* expression in an anterior direction relative to carrier-treated control embryos both prior to and after the formation of headfolds (Fig. 1A, and not shown). Ectopic anterior expression of *Hox-2.9* was induced both laterally and in the midline but extended more anterior along the neural folds of headfold embryos, and in the lateral margins of the presumptive neural plate in preheadfold embryos (Fig. 1A, and not shown). Careful examination and dissection of stained embryos revealed that induced ectopic expression of *Hox-2.9* RNA was present in both ectodermal and mesodermal cell layers.

To ascertain if the expansion of the domain of *Hox-2.9* expression upon treatment with exogenous RA was simply a response to insult, we administered teratogenic doses of ethanol to the mothers of headfold embryos and examined the embryos 4 hours later. No change in the spatial pattern of *Hox-2.9* expression was detected relative to carrier-treated controls (not shown).

Hox-2.8 expression was first detected in the posterior of primitive streak stage embryos, and in headfold embryos in an apparently graded distribution at the posterior of the embryo in both ectoderm and mesoderm. Unlike *Hox-2.9* expression, which showed a sharp anterior boundary in headfold embryos, the expression of *Hox-2.8* gradually decreased anteriorly, ending slightly anterior to the node. By the time of the formation of the first somite, *Hox-2.8* expression in the neurectoderm extends anteriorly to the level of the preotic sulcus (Wilkinson et al., 1989b; Hunt et al., 1991a). 4 hours after application of RA to headfold embryos, the expression of *Hox-2.8* was induced more ante-

riorly in both ectoderm and mesoderm, although the response was not as dramatic as that seen with *Hox-2.9*. In headfold embryos, the most anterior induced expression was seen in the neural folds (Fig. 1B).

Hox-2.5 expression was first detected at the headfold stage in the posterior embryonic ectoderm just lateral to the primitive streak, with an anterior limit just posterior to the node (Fig. 1C). Some staining was evident in the base of the allantois, but not in the mesoderm lateral to the streak (not shown). No effect on the pattern of *Hox-2.5* expression was observed 4 hours after RA treatment (Fig. 1C).

(B) Krox-20 *and RAR*β

Expression of *Krox-20*, a zinc finger gene putatively involved in hindbrain segmentation (Wilkinson et al., 1989a,b; Murphy and Hill, 1991), was also found to respond to treatment with exogenous RA. In contrast to the induced ectopic expression of *Hox-2.8* and *Hox-2.9*, *Krox-20* expression was rendered undetectable by RA treatment. *Krox-20* was first expressed in a narrow band of cells across the neural epithelium at the forming preotic sulcus. This domain of *Krox-20* expression is thought to correspond to prospective rhombomere 3 (Wilkinson et al., 1989a; Murphy and Hill, 1991). This band of *Krox-20* expression was undetectable 4 hours after maternal administration of RA late on day 7. (Fig. 1D).

We also examined the spatial patterns of expression of retinoic acid receptor-β (RARβ) on day 7 in response to RA since this gene is known to be directly transcriptionally activated by RA (Sucov et al., 1990; de Thé et al., 1990). RARβ in headfold embryos is normally expressed in the posterior neural folds, in the lateral head mesenchyme and throughout the posterior of the embryo excluding the primitive streak (Ruberte et al., 1991). The expression of RARβ transcripts was induced in the more medial regions of the head mesenchyme, in the anterior neural folds and in the neural plate, 4 hours after administration of RA (Fig. 1E).

Immediate effects of RA on gene expression on days 8 and 9

We also examined the response of a number of genes 4 hours after RA treatment on embryonic day 8. At the beginning of treatment, the majority of embryos were at the 4- to 6-somite stage. Again, some genes were found to respond by induced anterior expression (*Hox-2.9*, *Hox-2.6*, *Hox-2.1* and RARβ), one gene responded by decreased expression in a subdomain of its expression pattern (*Krox-20*), and several genes did not respond at all as monitored by in situ hybridization (*Hox-2.8*, *Hox-2.5*, *Wnt-7b* and *En-2*). The RA-responsiveness of some of these genes changed between embryonic day 7 and 8. *Hox-2.8* expression was responsive on day 7, but no longer responsive on day 8; and the response of *Hox-2.9* and *Krox-20* expression on day 8 was limited to more posterior subdomains of their expression patterns. As on day 7, we observed no differences in morphology between RA- and carrier-treated embryos 4 hours after maternal adminstration of RA.

(A) Hox-2 *gene family*

By embryonic day 8, the patterns of expression of the most anterior *Hox-2* genes, *Hox-2.8* and *Hox-2.9*, have become largely refractory to RA. By early somite stages, the domain of *Hox-2.9* expression has become divided into a discrete band of cells in prospective rhombomere 4 of the hindbrain, and a domain of expression in the posterior neural tube, posterior lateral mesoderm and primitive streak (Frohman et al., 1990; Murphy and Hill, 1991). These domains of expression were not affected 4 hours after RA treatment. However, expression of *Hox-2.9* in the lateral foregut epithelium is initiated on day 8, and extends from the opening of the foregut to the posterior edge of the second branchial arch (Frohman et al., 1990; Murphy and Hill, 1991; and Fig. 2A). 4 hours after maternal administration of RA, *Hox-2.9* expression was present throughout the entire length of the foregut epithelium along the lateral walls (Fig. 2A).

On day 8 *Hox-2.8* is expressed in the neural tube from the level of prospective rhombomere 3 to the posterior of the embryo (Wilkinson et al., 1989b; Hunt et al., 1991a, c). Staining within the neural tube was most intense within prospective rhombomeres 3 to 5 as determined by comparison with normal, untreated embryos stained for *Krox-20* expression (Wilkinson et al., 1989b, and not shown). In addition, there is expression throughout the foregut, in migrating neural crest from prospective rhombomere 4, and in posterior lateral plate mesoderm (Wilkinson et al., 1989b; Hunt et al., 1991c). No change was observed in this pattern of expression in embryos 4 hours after treatment with RA (Fig. 2B).

Hox-2.6, whose anterior boundary of expression is not as clearly established by day 8 as *Hox-2.8* and *Hox-2.9* (Wilkinson et al., 1989b), responded to RA. On day 8, *Hox-2.6* transcripts are expressed in the posterior neural tube, lateral mesoderm and the receding primitive streak (Wilkinson et al., 1989b). The expression within the neural tube does not show a sharp anterior boundary, but rather decreases gradually at more anterior levels (Fig. 2C). 4 hours after treatment with RA, *Hox-2.6* expression in the neural tube displayed a sharp anterior boundary at the level of the junction between prospective rhombomeres 5 and 6, as determined by comparison with normal, untreated embryos stained for *Krox-20* expression. This new, RA-induced *Hox-2.6* boundary is anterior to the normal boundary that *Hox-2.6* expression reaches later on day 9 (between rhombomeres 6 and 7: Wilkinson et al., 1989). In addition, RA treatment induced expression of *Hox-2.6* RNA throughout the foregut, whereas no *Hox-2.6* expression was discernable in the foregut of control embryos (Fig. 2C). By day 9, the expression of *Hox-2.6* in the neural tube had become refractory to induction by RA (not shown).

We first observed expression of *Hox-2.1* at about the 6-somite stage on day 8. Previous studies have characterized the later expression of *Hox-2.1* (Graham et al., 1989; Wilkinson et al., 1989b; Gaunt et al., 1990). The overall expression pattern of *Hox-2.1* was atypical of the *Hox-2* genes examined here in that the expression of *Hox-2.1*, when first detectable, did not extend to the posterior of the embryo. *Hox-2.1* expresssion was first detected in the neural tube, somites and adjacent lateral mesoderm in the trunk of the embryo. The staining in the neural tube displayed a sharp anterior boundary. Expression in all tissue layers

decreased in a graded fashion posteriorly, with very little staining in the tail of the embryo. 4 hours after treatment with RA the anterior boundary of staining in the neural tube had moved more anteriorly (Fig. 2D). *Hox-2.1* expression in the neural tube remained responsive to RA on day 9 (not shown).

As with treatments at earlier stages, the posteriorly expressed *Hox-2.5* did not respond to treatment with RA. *Hox-2.5* expression on day 8 becomes evident in the posterior neural tube, the posterior lateral mesoderm, and, late on day eight, in the posterior somites (Fig. 2E and Bogarad et al., 1989). No change in any aspect of this expression could be detected 4 hours after RA treatment (Fig. 2E). The possibility that this most posterior of the *Hox-2* genes showed an RA response at even later stages was tested by treating mothers on day 9 of pregnancy. On day 9 the pattern of expression of *Hox-2.5* is similar to that seen in late day 8 embryos, with expression in the lateral mesoderm extending up to the posterior margin of the forelimb bud. However, there was no change in the spatial pattern of expression of *Hox-2.5* in response to RA on day 9 (not shown).

(B) Krox-20, En-2, Wnt-7b *and RAR*β

As on day 7, *Krox-20* responded to RA by a decrease in expression. On day 8 the expression of *Krox-20* is restricted to a block of cells just anterior to *Hox-2.9* in the hindbrain, and a new domain of expression appears in a block just posterior to *Hox-2.9* expression, thus marking prospective rhombomeres 3 and 5 (Wilkinson et al., 1989a, b; Murphy and Hill, 1991). Individual *Krox-20*-expressing cells, thought to be neural crest cells, are found just posterior to the posterior block of *Krox-20* expression along the neural folds and in the head mesenchyme ventral to the neural folds. 4 hours after maternal administration of RA, *Krox-20* expression was obliterated in the presumptive neural crest, and the intensity of staining of the posterior block of neural expression (prospective rhombomere 5) was reduced (Fig. 2F).

By day 8, we could also examine the response to RA of another homeobox gene, *En-2*, that is expressed at the prospective midbrain-hindbrain junction, anterior to the expression of all *Hox-C* genes (Davis et al., 1988; Davis and Joyner, 1988). The first expression of *En-2* is detected early on day 8, at about the time of formation of the third somite, in delta-shaped domains in the neural plate anterior to the preotic sulcus at the presumptive midbrain-hindbrain junction. This pattern was unchanged in embryos treated with RA (Fig. 2G).

We also examined the behaviour in response to RA of *Wnt-7b*, a gene expressed more anteriorly than the *Hox-C* genes and *En-2*. *Wnt-7b* encodes a member of the Wnt family of growth factors (Gavin et al., 1990), and is first detected at about the eight somite stage in the forebrain anterior to the eye, along the neural folds (B. Parr, G. Vassileva and A. McMahon, personal communication). No change in the pattern of *Wnt-7b* expression was detected by hybridization four hours after RA administration (Fig. 2H).

On day 8, RARβ, like some of the *Hox-2* genes, responded with increased anterior expression. On day 8 RARβ expression is more restricted than earlier: expression is seen in the posterior neural tube from the boundary between prospective rhombomeres 5 and 6 to the level of the posterior neuropore, within the lateral plate mesoderm, and throughout the foregut (Ruberte et al., 1991). The expression of RARβ appears to be graded within the neural tube with highest levels of expression at the anterior. 4 hours after treatment with RA, the expression of RARβ was found throughout all tissues of the anterior of the embryo. The posterior end of the embryo remained negative for RARβ expression (Fig. 2I).

Persistent changes in gene expression in response to RA

We wished to ascertain if the changes in gene expression observed shortly after treatment with RA on day 7 resulted in altered domains of expression on day 8, and if they correlated with morphological alterations. We analyzed RARβ, *Hox-2.9*, *Hox-2.8*, *Krox-20*, *Hox-2.5* and *En-2* gene expression in early somite embryos, 20 hours after maternal administration of RA on day 7 at the headfold stage. We found that some of the genes which responded 4 hours after treatment on day 7 (*Hox-2.9*, *Hox-2.8* and *Krox-20*) showed persistent alterations in their patterns of expression. However, one gene which responded on day 7 did not show persistent altered expression (RARβ). *En-2*, which was not expressed at the time of treatment, did not show an alteration in its later pattern of expression.

In contrast to embryos observed 4 hours after RA application, treatment on day 7 followed by observation 20 hours later revealed a characteristic syndrome of abnormal morphology. The anterior neural tube of RA-treated embryos lacked many of the sulci of untreated and carrier-treated embryos. In particular the prorhombomeric sulci of the hindbrain were apparently missing, resulting in a smooth surface to the open neural folds. The prosencephalic region was reduced in extent, the foregut was cylindrical without the usual diverticula, the branchial arches were reduced or absent, the tail bud was smaller, and the heart was often disorganized in RA-treated embryos (Fig. 3). These changes were not seen in carrier-treated controls. This syndrome of defects is typical for vertebrate embryos treated at this time (Sulik et al., 1988; Holder and Hill, 1991; Papalopulu et al., 1991a)

Treatment of embryos with RA on day 7 resulted in persistent changes in the distribution of *Hox-2.9* and *Hox-2.8* transcripts. The expression of *Hox-2.9* on day 8 in embryos treated with RA 20 hours earlier was similar to that of carrier-treated controls in the foregut, and posterior of the embryo. However, the position of the staining in the anterior neural tube was shifted anteriorly and the length of the anterior *Hox-2.9*-positive domain was greatly expanded relative to carrier-treated controls (Fig. 3A). Since prorhombomeric sulci were abolished by overnight RA treatment, exact correlation of this domain with neural tube landmarks is difficult. However, careful comparison of control and treated embryos, and of domains of expression of different genes suggested that the posterior boundary of the induced *Hox-2.9* domain was beyond the level of its normal anterior boundary in prospective rhombomere 4 in the neural tube (Fig. 3B). In contrast to the sharp boundaries of *Hox-2.9* expression in the hindbrain seen in control embryos, the

boundaries in the RA-treated embryos are irregular (Fig. 3B). These results are similar to those reported by Morriss-Kay et al., (1991) for embryos treated at the same stage but examined on day 9. The anterior boundary of the displaced *Hox-2.9* expression apparently overlapped the domain of expression of *En-2* (Fig. 3A). The pattern of expression of *Hox-2.8* 20 hours after treatment with RA on day 7 was very similar to that of *Hox-2.9* under the same conditions. Induced *Hox-2.8* expression was present in a broad domain anterior to its normal domain of expression within the neural tube (Fig. 3C). As with *Hox-2.9*, the expression boundaries of the ectopic neural *Hox-2.8* expression were irregular (Fig. 3C, and not shown). Without double-labelling experiments on the same embryo, it was impossible to determine whether the induced *Hox-2.8* and *2.9* domains were completely coincident.

The other gene whose expression was affected on day 7, *Krox-20*, also exhibited an abnormal pattern of expression 20 hours after RA treatment. Instead of two bands of *Krox-20* expression across the neural epithelium in prospective rhombomeres 3 and 5, only one broad band of expression was observed (Fig. 3D). The exact position of the altered *Krox-20* domain of expression was difficult to determine given the altered morphology of the treated embryos, but it appears to lie at the level of prospective rhombomeres 4 and 5 in untreated embryos. Similar alterations in the pattern of *Krox-20* expression were reported for treatment at the same time but with analysis on day 9 in the mouse (Morriss-Kay et al., 1991), and for analogous treatments in *Xenopus* (Papalopulu et al., 1991a). Since RA treatment at the headfold stage resulted in the disappearance of the anterior band of *Krox-20* expression 4 hours later, we feel that the band of staining seen 20 hours after treatment is likely to be the posterior band of expression (prospective rhombomere 5).

Despite the fact that the distribution of RARβ transcripts was responsive to RA in embryos from day 7 to 8 (see above), the expression of RARβ transcripts closely resembled the pattern of expression in carrier-treated controls 20 hours after treatment with RA on day 7, (Fig. 3E). It appears that the RARβ gene responded by ectopic expression at the time of treatment, but that by the time the embryos were examined the expression pattern had returned to essentially normal. That the embryos had been exposed to RA was clear from the fact that the embryos displayed the syndrome of RA-induced abnormalities.

Finally, the patterns of expression of genes that were either not expressed or not affected on day 7, showed no alterations 20 hours after RA treatment. No change in the position along the axis, the pattern or intensity of staining were observed in RA-treated embryos relative to carrier-treated controls, for *En-2* and *Hox-2.5* (Fig. 3F, G). Again, since the embryos displayed the syndrome of morphological defects typical of RA treatment, it may be surmised that the embryos were exposed to RA.

Discussion

Exogenous RA, Hox-C *genes and morphological alterations in the head*

Administration of a teratogenic dose of RA to headfold mouse embryos results in a syndrome of defects in the morphology of the head, including a reduction in the size of the forebrain and midbrain, a loss of hindbrain segmentation, an anterior displacement of the otic vesicle, and an anterior displacement of the somites (Morriss-Kay et al., 1991). We have shown that RA treatment at this time results in the rapid anterior displacement of some of the *Hox-2* gene expression boundaries within the prospective hindbrain. We observed an anterior displacement of *Hox-2.9* and *Hox-2.8* expression, and the loss of *Krox-20* expression within 4 hours of RA application (Fig. 4). In addition, the induced changes in expression of these genes was maintained 20 hours later (Fig. 5). The induced changes in *Hox-2.9*, *Hox-2.8* and *Krox-20* expression precede the morphological changes, which suggests that these changes in gene expression are a primary response to retinoic acid rather than the sequelae of the disruption of normal development. Our results also suggest that the ectopic expression of *Hox-C* genes may be responsible at least in part for the morphological defects and transformations seen in RA-treated vertebrate embryos.

There are aspects of the response of *Hox-2* genes to exogenous RA other than the promptness of the response that support the idea that the misexpression of *Hox-C* genes in RA-treated embryos is at least partly responsible for the induced morphological abnormalities. The direction of induced ectopic *Hox-C* gene expression within RA-treated embryos correlates with the direction of morphological transformations, where anterior cells adopt more posterior fates. In addition, there is a spatial correlation between the anterior expression boundaries of the responsive *Hox-C* genes, and the regions of the embryo that are affected. For example, genes at the 3′ end of the *Hox-2* cluster like *Hox-2.8* and *Hox-2.9* normally have anterior expression boundaries in the posterior hindbrain, and are induced in anterior hindbrain and possibly posterior midbrain regions by exogenous RA. The hindbrain region appears to be most sensitive to RA treatment at this stage in the mouse and other organisms (Holder and Hill, 1991; Morriss-Kay et al., 1991; Papalopulu et al., 1991a). *Hox-2.5*, a gene at the 5′ end of the *Hox-2* cluster, has an anterior expression boundary in the prospective trunk of the embryo, and was not ectopically induced by RA at these early stages. The morphology of the posterior region of the embryo is relatively unaffected by RA treatments at this stage (Shenefelt, 1972; Morriss-Kay et al., 1991; Kessel and Gruss, 1991). Finally there is a temporal correlation between the periods of sensitivity to RA of both head morphology and anterior *Hox-C* gene expression within the neural tube. The expression of *Hox-2.8* and *Hox-2.9* was responsive to exogenous RA in headfold embryos, but, by the early somite stages, expression within the neural tube was no longer responsive to exogenous RA. Similarly the syndrome of head defects generated by RA are largely limited to treatment of headfold embryos (Kessel and Gruss, 1991; Morriss-Kay et al., 1991).

On the basis of the later, altered domains of expression of *Krox-20* and *Hox-2.9* after RA-treatment, it has been suggested that treatment with RA at the headfold stage of mouse embryogenesis may lead to the anterior hindbrain

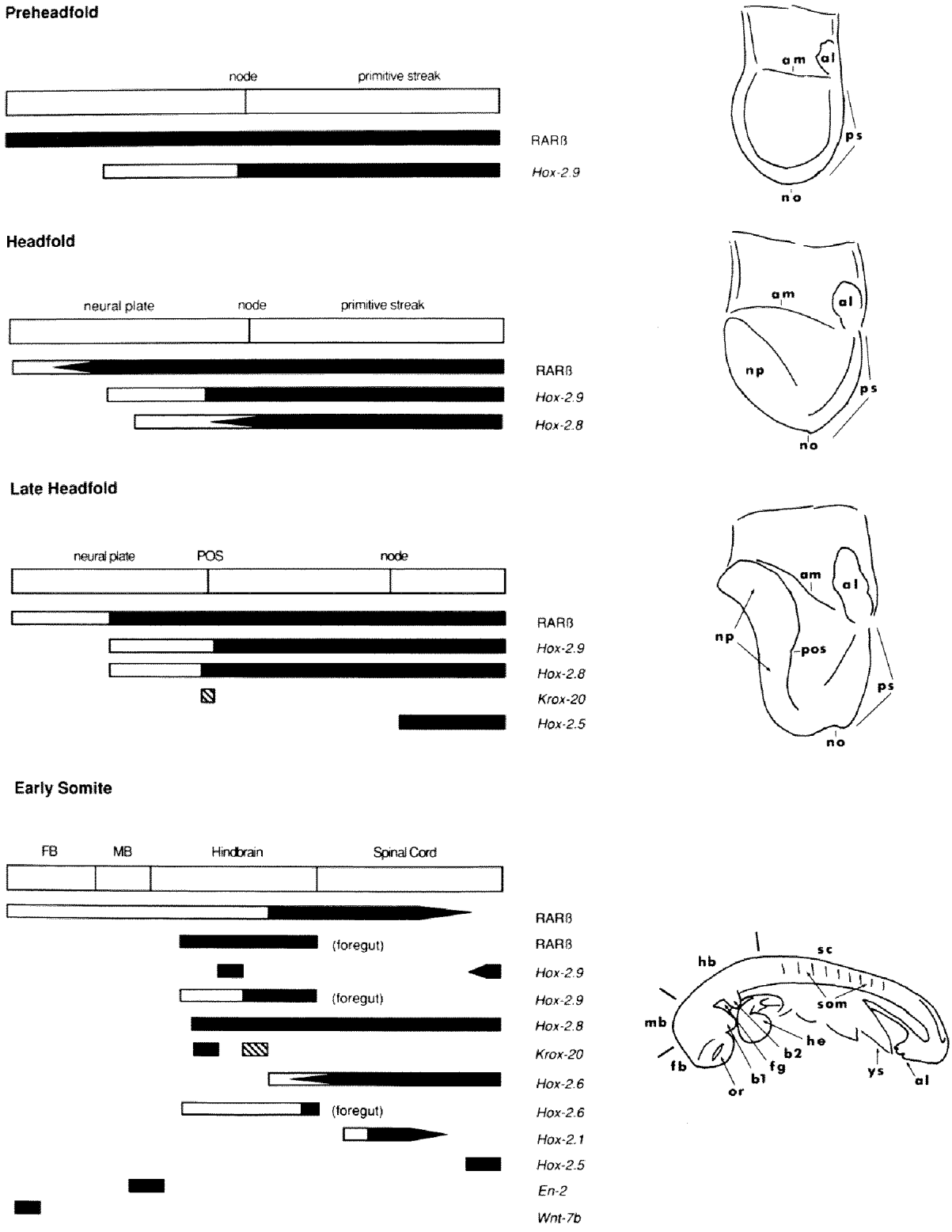

Fig. 4. Summary of the alterations in gene expression 4 hours after treatment with RA. The large open box represents the AP axis of the embryo for each stage with some morphological landmarks indicated. At the right, cartoons diagram these morphological landmarks, in addition to others referred to in the text. Filled and open boxes represent the approximate limits of expression of the given genes, where filled boxes represent expression in untreated and carrier-treated embryos, and open boxes indicate expression induced 4 hours after RA treatment. A hatched box is used to denote domains of expression that are repressed by RA-treatment. (al, allantois; am, amnion; b1, 1st branchial arch; b2, 2nd branchial arch; fb, forebrain; fg, foregut; hb, hindbrain; he, heart; mb, midbrain; no, node; np, neural plate; or, optic rudiment; pos, preotic sulcus; ps, primitive streak; sc, spinal cord; som, somites; ys, yolk sac)

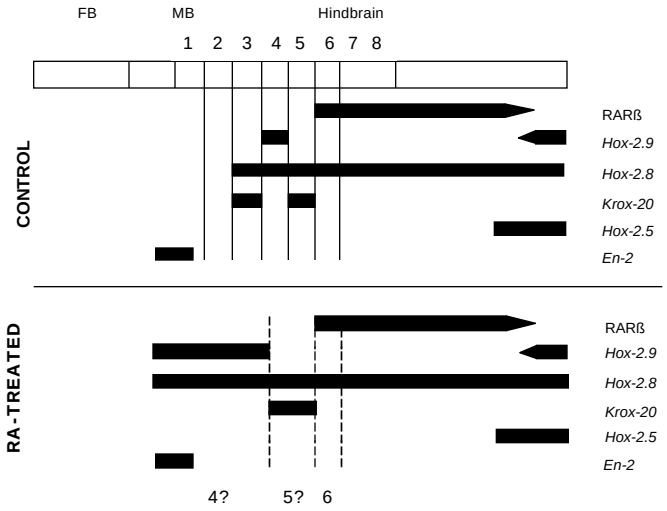

Fig. 5. Summary of the persistent changes in gene expression 20 hours after administration of RA at the headfold stage. The large open box at the top indicates the AP axis of the embryo. The approximate domains of gene expression are indicated by filled boxes for both control and RA-treated embryos. In the diagram for carrier-treated control embryos, the boundaries of prospective rhombomeres 1 to 8 in the hindbrain are indicated by solid vertical lines. In the RA-treated embryos, the proposed prospective rhombomere boundaries are indicated by dashed lines. Abbreviations are as in Fig. 4.

acquiring the character of more posterior hindbrain (Morriss-Kay et al., 1991). We have confirmed the pattern of response of these two genes, shown that the changes are an early response to RA, and extended the results to include the response of *Hox-2.8*. If *Hox-C* genes specify regional identity through combinations of *Hox-C* gene expression (Kessel and Gruss, 1991), these results now allow a more specific prediction to be made: Firstly, that rhombomeres 1 to 3 may be transformed towards rhombomere 4, and, secondly, that rhombomere 5 may be expanded anteriorly. In embryos treated with RA at the headfold stage, *Hox-2.8* and *Hox-2.9* are expressed in large overlapping domains from approximately the anterior limit of the hindbrain to the level of the boundary between prospective rhombomeres 3 and 4 (ie. in prospective rhombomeres 1 to 3). In the undisturbed embryo, *Hox-2.8* and *Hox-2.9* neural expression overlaps in prospective rhombomere 4. If this combination of *Hox-C* gene expression has a role in specifying rhombomere 4 identity, then one might predict that, in embryos treated at the headfold stage with RA, rhombomeres 1 to 3 are transformed towards rhombomere 4 (Fig. 5). It has been suggested that *Hox-C* gene expression in the hindbrain may also serve to specify the fates of hindbrain-derived neural crest (Hunt et al., 1991b). In support of this is the fact that loss-of-function mutations in hindbrain *Hox-C* genes lead to defects in structures derived from hindbrain neural crest (Chisaka and Capecchi, 1991; Lufkin et al., 1991). Thus one might expect the ectopic expression of *Hox-2.8* and *Hox-2.9* in RA-treated embryos to lead to alterations in the fates of neural crest cells. Disruption of the development of crest-derived cranial ganglia V and VII have been reported for *Xenopus* and zebrafish embryos treated with moderate doses of RA (Holder and Hill, 1991; Papalopulu et al., 1991a). The resolution of the identity of disrupted structures requires careful analysis using markers specific for structures derived from different axial levels of the hindbrain.

As well as a possible transformation to rhombomere 4 identity, expansion of the rhombomere 5 domain is also indicated by the broadened *Krox-20* domain of expression seen in RA-treated embryos (Fig. 5). The forward displacement of the otic vesicle in embryos treated with RA at the headfold stage is also consistent with this (Sulik et al., 1988; Morriss-Kay et al., 1991). The otic vesicle is thought to be induced from the surface ectoderm by the action of the *Int-2* growth factor in the neural epithelium (Represa et al., 1991). The expression of *Int-2* becomes restricted to rhombomeres 5 and 6 in the hindbrain, beneath the site at which the otic vesicle forms (Wilkinson et al., 1988). An anterior expansion of rhombomere 5 would thus move the domain that induces otic vesicle formation more anterior.

In addition to the putative anterior to posterior transformations caused by RA-treatment on day 7, the sulci marking rhombomeric boundaries within the hindbrain are obliterated (Morriss-Kay et al., 1991). The overt segmentation of the rhombencephalon is established by day 9 of embryonic development, after many of the *Hox-C* genes have apparently stabilized their anterior expression boundaries within this region. The normal expression of the *Hox-C* genes is highly ordered and regular, whereas after treatment with RA the expression boundaries of *Hox-2.8, Hox-2.9* and *Krox-20* are much less regular, and the normal combinations of expressed genes are disrupted. Either the lack of regularity in the boundaries of *Hox-C* gene expression, or uncoordinated *Hox-C* gene expression caused by RA treatment, may be responsible for the lack of regular segmentation of the hindbrain.

The reduction in extent of the forebrain and midbrain region and the anterior displacement of somites seen in embryos treated with RA is more difficult to explain in terms of the altered *Hox-C* gene expression that we observed. Since RA treatment did cause ectopic anterior expression of RARβ in prospective forebrain regions, it is possible that the presence of both receptors and excess ligand serve to repress the expression of genes required for anterior head development. Consistent with this, there are instances of RARs acting as transcriptional repressors (Husmann et al., 1991; Mangelsdorf et al., 1991; Schüle et al., 1991; Kliewer et al., 1992).

The response of Hox-C *genes to exogenous RA*

Our evidence for a rapid response of certain of the *Hox-2* genes to RA in vivo is consistent with studies showing upregulation of *Hox-C* gene expression in response to RA in *Xenopus* embryos, and in human and mouse teratocarcinoma cells in vitro (Cho and De Robertis, 1990; Simeone et al., 1990, 1991; Sive et al., 1990; Papalopulu et al., 1991b; Sharpe, 1991; Sive and Cheng, 1991; Ura and Hirose, 1991). The general relationship between responsiveness and position in the cluster was also conserved, with *Hox-C* genes at the 3′ end of the *Hox-2* cluster being

responsive to RA whereas those at the 5′ end were relatively insensitive. However, it is difficult to relate the spatial response that we observe in vivo with the quantitative responses seen in vitro. The difference between quantitative and spatial responses is exemplified by the case of *Hox-2.5*. The presumed homologue of *Hox-2.5* in *Xenopus*, *XlHbox6*, has been shown to be induced to higher levels of expression by RA in isolated RNA from whole embryos and various tissue explants (Cho and De Robertis, 1990; Sive et al., 1990; Sharpe, 1991), whereas *Hox-2.5* was refractory to alterations in the extent of its expression domain at all stages of mouse development that we examined. The in situ hybridization methodology used did not allow us to address directly quantitative differences. However, if there was an increase in *Hox-2.5* expression in our embryos, it must have occurred within the normal boundaries of *Hox-2.5* expression.

The mechanism of the response of *Hox-C* genes to exogenous RA is not clear. Both transcriptional and post-transcriptional mechanisms have been invoked for the response of *Hox-C* genes in vitro on the basis of nuclear run-on experiments (for example, Papalopulu et al., 1991b; Simeone et al., 1991; Ura and Hirose, 1991). Our results do not provide direct evidence for either mechanism in vivo, but it is tempting to think that there is a transcriptional component involved since the response of *Hox-C* genes is rapid, and occurs within the same interval and in similar regions as the transcriptional activation of the retinoic acid-responsive transgene, *RAREhsplacZ* (Rossant et al., 1991).

Endogenous RA and AP patterning

From the studies described here and elsewhere, there is now clear evidence that RA can induce ectopic expression of *Hox-C* genes and other genes putatively involved in patterning the embryo in vivo. What remains to be ascertained is the importance of the endogenous RA-signalling pathway in the normal spatial regulation of these genes and hence in AP patterning of the embryo. The evidence for a role for endogenous RA in specifying the normal anterior expression boundaries of the 3′ *Hox-C* genes is still largely circumstantial.

If RA does have a role in specifying the anterior boundaries of expression of *Hox-C* genes, we suggest that this role is limited to only certain axial levels, probably to the level of the hindbrain, at least at early neural fold stages. This suggestion is based on the fact that only those *Hox-2* genes with anterior boundaries in the hindbrain or anterior spinal cord responded to RA by anterior ectopic expression. The hindbrain *Hox-2* genes whose expression we investigated were sequentially expressed in the embryo in a manner that corresponded to their order 3′ to 5′ in the *Hox-2* cluster: the initial detectable expression occurred in the order *Hox-2.9*, *Hox-2.8*, *Hox-2.6* and *Hox-2.1* (Fig. 4). Similarily, the order of cessation of sensitivity to exogenous RA of neural expression was the same (Fig. 4). The development of vertebrate embryos proceeds from anterior to posterior in the sense that anterior structures are specified and differentiate earlier than more posterior structures. It is possible that RA is involved in the specification of boundaries of the 5′ *Hox-C* genes at stages later than we have examined. Consistent with this idea is the generally posterior movement of the teratogenic defects induced by exogenous RA with developmental age (Shenefelt, 1972; Kessel and Gruss, 1991). However, the expression of the 5′ *Hox-2.5* gene remained insensitive to exogenous RA as late as embryonic day 9, and the 5′ *Hox-2* genes are largely insensitive to RA in vitro (Papalopulu et al., 1991b).

The common boundaries shared by *Hox-C* paralogues expressed in the hindbrain (Hunt et al., 1991a) could be set by similar sensitivities to RA-mediated signalling. A mechanism employing sequential onset of expression of the hindbrain *Hox-C* genes combined with a posterior movement of RA-mediated signalling could generate the initial expression boundaries of the different *Hox-C* gene paralogues expressed in the hindbrain. Consistent with this idea is the fact that the more anterior hindbrain *Hox-C* genes are expressed earlier, and become refractory to exogenous RA earlier than the more posteriorly expressed hindbrain *Hox-C* genes. In addition, expression of a given *Hox-C* gene within different tissues could be set by the same mechanism. For example, the *Hox-2.9* expression boundary in prospective rhombomere 4 has a period of sensitivity to RA that precedes the period of sensitivity of the later, more posterior *Hox-2.9* expression in the foregut. In this case, one gene, *Hox-2.9*, regulated by the same mechanism, could specify regional identity at two different levels along the AP axis in two different germ layers. Most *Hox-C* genes are expressed in the neural tube and at a slightly more posterior level in the mesoderm: both parts of a *Hox-C* gene's expression could be set by this combination of sequential activation in different germ layers and a retreating positioning threshold.

Whether RA acts directly to regulate transcriptionally *Hox-C* genes or not, it is clear that there is a close relationship between the two. However, once the initial boundaries are established through the action of RA-mediated signalling, these boundaries must be stabilized by other factors, perhaps including autoregulation and tissue-specific factors. Stabilization by factors other than RA is apparent in the stably altered domains of expression of the anterior *Hox-2* genes induced by brief RA treatment at day 7. Exogenous RA is known to be rapidly metabolized in the mouse, so that high levels of RA do not persist in the embryo (Satre and Kochhar, 1989). The rapid return to normal expression pattern of the RA-responsive gene RARβ testifies to this short-term effect. However, altered *Hox-2* domains persist, indicating the involvement of other factors, perhaps such as autoregulation, in maintenance of the domains. Presumably similar factors come into play in the untreated embryo, once the period of sensitivity to RA is over.

There must also be antagonistic factors that prevent ubiquitous expression of *Hox-2* genes in response to RA. This is implied by the fact that although *Hox-C* expression spread anteriorly in response to RA, it never spread to the extreme anterior of the embryo. This effect was not mediated by the inability to mount a transcriptional response to RA in the extreme anterior of the embryo since both the retinoic acid receptor β and the *RAREhsplacZ* transgene could both be induced in the extreme anterior of the embryo. It may be significant that when *Hox-C* genes responded to RA, induced expression tended to occur in

those anterior regions where RARβ transcripts are expressed in untreated embryos. Thus, although RARβ was induced by RA treatment, ectopic *Hox-C* gene expression occurred in tissues where there were already high levels of RARβ RNA and, presumably, the receptor itself. This implies an important role for the RARβ receptor in the response of the *Hox-C* genes to exogenous RA.

As yet, direct links between the RA signal transduction system of the embryo and the normal regulation of *Hox-C* genes remain to be demonstrated. The evidence presented here, however, supports the existence of such an interaction. Likewise, it remains to be demonstrated conclusively that the altered *Hox-C* gene expression induced by RA is directly responsible for the teratogenic effects of the same treatment. However, our results support this connection, and enable us to make specific predictions about the nature of the teratogenic alterations.

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Fig. 3. Persistent changes in gene expression after administration of RA at the headfold stage. RNA expression patterns were revealed by whole-mount in situ hybridization 20 hours after treatment with RA, by which time the embryos had reached the early somite stages of development. In all panels except B, the embryos are oriented with anterior to the left, with the carrier-treated embryo above the RA-treated embryo. The morphology of the RA-treated embryos was abnormal after this regime of treatment. The forebrain and midbrain were altered in morphology and reduced in extent, and the heart and foregut were disrupted in the RA-treated embryos. (A) The expression of *Hox-2.9* was induced in a broad domain in the presumptive midbrain and anterior hindbrain by RA. The band of staining in presumptive rhombomere 4 was absent. As a marker for anterior neural structures, the carrier-treated embryos were stained for both *Hox-2.9* and *En-2*: The arrow indicates the domain of expression of *En-2* at the presumptive mindbrain/hindbrain boundary. (B) A dorsal view of embryos similar to those shown in A. The RA-treated embryo is on the left; anterior is up. The arrows indicate the prorhombomeric bulges in prospective rhombomeres 4 and 5 in the hindbrain of the carrier-treated embryo. These morphological specializations are absent in the RA-treated embryo, and the morphology of the hindbrain and more anterior neural regions are greatly altered. (C) *Hox-2.8* expression was present ectopically throughout the hindbrain, and in the midbrain 20 hours after RA-treatment. (D) *Krox-20* expression was present in a single broad domain in the hindbrain in embryos 20 hours after RA-treatment, rather than in the two bands seen in carrier-treated and untreated embryos. (E) The expression of RARβ in embryos treated with RA under this regime closely resembled that of carrier-treated embryos. (F) *Hox-2.5* expression in RA-treated embryos did not appear to differ from that seen in control embryos. (G) *En-2* expression was unaffected by RA-treatment. (The scale bars indicate 200 μm.)

Fig. 1. Changes in gene expression in headfold and late headfold embryos 4 hours after RA adminstration on day 7 visualized by whole-mount in situ hybridization. The embryos are oriented with anterior to the left in all panels except in D and in all panels, the carrier-treated embryo is on the left; the RA-treated embryo on the right. No changes in morphology were evident in RA-treated embryos 4 hours after treatment. For a diagrammatic representation of embryonic anatomy see Fig. 4. (A) *Hox-2.9* expression was induced in anterior neurectoderm and mesoderm by RA in headfold embryos. (B) *Hox-2.8* expression was induced in anterior neurectoderm and mesoderm by RA in headfold embryos. (C) No change in the pattern of *Hox-2.5* expression was seen in RA-treated late headfold embryos. Expression was confined to ectoderm posterior to the node in both RA- and carrier-treated embryos. (D) *Krox-20* expression was rendered undetectable by exogenous RA in late headfold embryos. The dissected anterior neural plates of embryos are shown from the dorsal aspect, with anterior up. (E) RARβ expression was induced by RA in headfold embryos in anterior neurectoderm. (The scale bar indicates 100 μm.)

Fig. 2. Changes in gene expression 4 hours after treatment with exogenous RA in early somite embryos on day 8 as assayed by whole-mount in situ hybridization. Embryos subjected to treatment with carrier are on top in each panel; embryos treated with RA are below. Anterior is to the left in all. No changes in morphology were evident in RA-treated embryos 4 hours after treatment. For a diagramatic representation of embryonic anatomy see Fig. 4. (A) *Hox-2.9* expression was induced in more anterior regions of the foregut in early somite embryos by RA. Staining in other domains of *Hox-2.9* expression appeared to be unaffected. (B) *Hox-2.8* expression in early somite embryos was not detectably altered by treatment with RA. (C) Expression of *Hox-2.6* was induced anteriorly within the neural tube relative to the carrier-treated controls. In addition, expression was induced more anteriorly in the foregut. (D) *Hox-2.1* expression was induced more anteriorly in the neural tube of early somite embryos by RA. (E) The pattern of expression of *Hox-2.5* was unaffected by treatment with RA on day 8. (F) Exogenous RA reduced the amount of staining in the posterior domain of *Krox-20* expression in the neural tube (prospective rhombomere 5). Staining in the presumptive neural crest posterior to prospective rhombomere 5 was also abolished. (G) The expression of *En-2* at the presumptive midbrain/hindbrain boundary was unaffected 4 hours after treatment with RA. (H) *Wnt-7b* expression in the forebrain anterior to the eye ation of RA. (I) Expression of RARβ was induced anteriorly 4 hours after RA treatment in most tissues. There did not appear to be an increase in expression in the posterior of the embryo four hours after RA treatment. (The scale bars indicate 200 μm.)

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