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Inhibition of retinoic acid receptor-mediated signalling alters positional identity in the developing hindbrain

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SUMMARY

Retinoids regulate gene expression via nuclear retinoic acid receptors, the RARs and RXRs. To investigate the functions of retinoid receptors during early neural development, we expressed a dominant negative RARβ in early *Xenopus* embryos. We obtained evidence that dominant negative RARβ specifically inhibits RAR/RXR heterodimer-mediated, but not RXR homodimer-mediated, transactivation. Both all-trans- and 9-cis-RA-induced teratogenesis were, however, efficiently opposed by ectopic expression of dominant negative RARβ, indicating that only RAR/RXR transactivation is required for retinoid teratogenesis by each of these ligands. Experiments with two RXR-selective ligands confirmed that activation of RXR homodimers does not cause retinoid teratogenesis. Dominant negative RARβ thus specifically interferes with the retinoid signalling pathway that is responsible for retinoid teratogenesis. Dominant negative RARβ-expressing embryos had a specific developmental phenotype leading to disorganization of the hindbrain. Mauthner cell multiplications in the posterior hindbrain, and (both anteriorly and posteriorly) expanded *Krox-20* expression domains indicated (partial) transformation of a large part of the hindbrain into (at least partial) rhombomere 3, 4 and/or 5 identity. In contrast, the fore- and midbrain and spinal cord appeared to be less affected. These data indicate that RARs play a role in patterning the hindbrain.

Key words: *Xenopus*, Retinoic acid, All-trans-RA, 9-cis-RA, Dominant negative RAR, RAR/RXR heterodimer, RXR homodimer, SR11246, HX600, Hindbrain, Mauthner, *Krox-20*, *Hoxb-3*

INTRODUCTION

Neural induction in amphibia has been proposed to consist of (at least) two steps: a neural activation step, when anterior neural tissue is specified, and a transformation step, via which newly induced anterior neural tissue is transformed from an anterior into a posterior character (hindbrain and spinal cord) (reviewed by Nieuwkoop and Albers, 1990). Several secreted proteins with the capacity to induce anterior neural tissue, viz. noggin (Lamb et al., 1993), follistatin (Hemmati-Brivanlou et al., 1994), chordin (Sasai et al., 1995), cerberus (Bouwmeester et al., 1996), and Xnr-3 (Hansen et al., 1997), have recently been identified. Retinoids are among factors which have been proposed as candidates for neural transformation (or posteriorising) signals: when added to *Xenopus* embryos during gastrulation, all-trans-retinoic acid (ATRA) disturbs proper development of the anteroposterior axis, with loss of anterior neural and mesodermal structures, and shortening of the tail (Durston et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991a, b). Depending on the concentration used, the volume of the hindbrain increases (Durston et al., 1989; Simeone et al., 1995), suggesting transforming activity of retinoids. The enlargement of the hindbrain is accompanied by increased expression of posterior neural genes, including *Hox* genes (Conlon and Rossant, 1992; Dekker et al., 1992; Kessel, 1993), at the expense of the expression of fore- and midbrain markers like *Otx-2* (Pannese et al., 1995; Simeone et al., 1995) and Engrailed-2 (Sive et al., 1990). Similarly, ATRA treatment of noggin-induced neurectoderm explants induces expression of posterior instead of anterior markers (Papalopulu and Kintner, 1996; Godsave et al., personal communication). Explanted anterior neurectoderm (presumptive forebrain) cultured in a medium containing ATRA is also induced to express *Hox* genes (Dekker et al., 1992). These data indicate that ATRA causes an anterior-to-posterior transformation of neural tissue, whereas further observations (below) also emphasize the importance of endogenous retinoids for hindbrain patterning.

Retinoids activate transcription by activating retinoid receptors, which are members of the superfamily of ligand-

inducible transcription factors. Two classes of retinoid receptors have hitherto been identified: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), each comprising a family of different isoforms (reviewed in Mangelsdorf et al., 1994). RARs need RXRs as heterodimerisation partners for effective transcriptional activation (Kliewer et al., 1992; Leid et al., 1992; Zhang et al., 1992a) via retinoic acid response elements (RAREs) present in promoters and/or enhancers of target genes. RXRs can form active homodimers (Levin et al., 1992; Zhang et al., 1992b) and transactivate via RXREs. ATRA can only activate RAR/RXR heterodimers, whereas 9-cis-RA, a different stereoisomer, can bind to both RARs and RXRs, and activate transcription both via RAR/RXR heterodimers and via RXR homodimers (Heyman et al., 1992; Zhang et al., 1992b).

The relevance of retinoid signalling for patterning the posterior central nervous system (CNS) is indicated by a number of observations. A number of retinoid receptors show localized expression in the developing vertebrate CNS (Ruberte et al., 1991; Sharpe, 1992), and these expression patterns are consistent with a function for RARs in regulating *Hox* gene expression and hindbrain patterning. Functional RAREs have actually now been identified in the regulatory regions of three *Hox* genes that are expressed in the hindbrain: *Hoxa-1* (Langston and Gudas, 1992; Frasch et al., 1995; Dupé et al., 1997; Langston et al., 1997), *Hoxb-1* (Marshall et al., 1994; Studer et al., 1994; Ogura and Evans, 1995a; b; Langston et al., 1997), and *Hoxd-4* (Pöpperl and Featherstone, 1993; Moroni et al., 1993; Morrison et al., 1996). At least some of these RAREs are involved in posterior neural patterning (reviewed by Marshall et al., 1996; Maconochie et al., 1996). Loss-of-function studies have also revealed certain possible functions for RARs and retinoid signals in early CNS development. For example, compound *RARαγ* mutants can have hindbrain defects, including agenesis of the motor nucleus of the abducens nerve, which is localized in rhombomere (r) 5 and r6 (Lohnes et al., 1994). Another recent study revealed that vitamin A deficiency leads to absence of the posterior hindbrain (r4-r8) in the quail embryo (Maden et al., 1996). It is now almost certain that retinoid signalling is indispensable for correct patterning of the hindbrain (see also Morriss-Kay et al., 1991; Papalopulu et al., 1991; Holder and Hill, 1991; Sundin and Eichele, 1992). The mechanisms involved are clearly complex and they may include the action of a retinoid as a neural transformation signal.

Here, we have investigated the role of RARs in neural patterning during early *Xenopus* development, by overexpression of a dominant negative (DN) RAR that lacks the ligand-dependent transactivation domain. Previous studies using similar approaches (Smith et al., 1994; Schuh et al., 1993; Kolm and Sive, 1995; Blumberg et al., 1997; Sharpe and Goldstone, 1997) did not deal with effects on hindbrain patterning in particular. In the present study, we show that ectopic expression of DN RARβ interferes with hindbrain development. We find further that two RXR-selective ligands are not teratogenic during *Xenopus* embryogenesis, indicating that retinoid-induced teratogenesis is mediated exclusively by RAR/RXR heterodimers, not by RXR homodimers. From the defects resulting from injection of DN RARβ, which include Mauthner cell multiplications in the posterior hindbrain and altered expression patterns of hindbrain markers, we conclude that RAR-mediated signalling is necessary for correct hindbrain patterning.

MATERIALS AND METHODS

Plasmids

DN RARβ: the sticky ends of a 1.5 kb *Sst*I-*Bam*HI fragment of construct βΔ384 from Shen et al. (1993) were blunted and ligated in *Eco*RV-linearized T7Ts, a plasmid that adds 5′- and 3′-untranslated *Xenopus globin* sequences to inserted DNA (Krieg and Melton, 1984). A 1.8 kb *Eco*RI *hRARα* fragment (Petkovich et al., 1987) and a 1.9 kb *Eco*RI *hRXRα* fragment (Mangelsdorf et al., 1990) were similarly ligated in T7Ts. A 3.7 kb *Bgl*II-*Bam*HI *lacZ* fragment was ligated in *Bgl*II-linearized T7Ts. Gal4-xRXRβ(DE) was constructed by ligating a 970 bp *Rsa*I fragment encoding most of the D and the complete E domain of xRXRβ (Marklew et al., 1994) into *Sma*I-digested pSG424 (Sadowski and Ptashne, 1989). The reporters −1470+156Luc, containing a 1.6 kb fragment of the human $RAR\beta_2$ promoter (which contains a DR5 RARE) fused to the *luciferase* gene, and Gal-Luc have been described before (Folkers and van der Saag, 1995).

Embryo culture

Xenopus embryos were obtained by in vitro fertilization and staged according to Nieuwkoop and Faber (1967). Embryos were cultured in 10% MMR (Newport and Kirschner, 1982) containing 50 μg/μl gentamicin, and retinoids where indicated, at 16-20°C. Retinoid treatments were done in 24-well plates (10 embryos/ml/well).

RNA transcription and microinjection

RNA was synthesized from *DN RARβ*, *RARα*, *RXRα*, or *lacZ* in T7Ts linearized with *Xba*I, or from *xGR* in T7Ts, linearized with *Sal*I (Gao et al., 1994), using the T7 mMessage mMachine (Ambion), and injected as a single dose into one- or two-cell-stage wild-type or albino *Xenopus* embryos. Embryos were injected in 3% ficoll, 25% MMR, and after at least 4 hours they were transferred to 10% MMR. For reporter experiments, −1470+156Luc or Gal-Luc DNA was diluted in microinjection buffer (88 mM NaCl, 10 mM Tris-HCl, pH 8). Reporter DNA was mixed with *DN RARβ* RNA, with or without added *RAR* or *RXR* RNA or Gal4-xRXRβ(DE) DNA respectively, and injected into the zygote. Groups of 5-10 embryos were homogenized in 100 μl reporter lysis buffer (Promega) and mixed with 300 μl assay buffer (0.1 M potassium phosphate buffer pH 7.8 (KPi) containing 1 mM DTT, 3 mM ATP, and 15 mM $MgSO_4$). The luciferase reaction was started by addition of 0.4 mM luciferine, 1 mM DTT in 100 μl 0.1 M KPi. Relative light units were measured during 10 seconds in a luminometer (Bicounter, Lumac).

In situ hybridization

Digoxigenin-labeled RNA probes were transcribed from linearized plasmids according to the manufacturer's (Boehringer Mannheim) instructions. The *Otx-2* probe was as described by Blitz and Cho (1995). The *Krox-20* probe was synthesized according to Bradley et al. (1992). An *Engrailed-2* (*En-2*) probe was generated as described by Eizema et al. (1994). *Hoxb-1*, *b-3*, *b-7*, and *b-9* probes were generated as described by Godsave et al. (1994). Embryos in which *lacZ* RNA was co-injected with the *DN RARβ* or *GR* RNA were fixed for 40 minutes at room temperature in 4% paraformaldehyde in 0.1 M KPi containing 4% sucrose and 0.15 M $CaCl_2$. They were washed 3× 15 minutes in PBS and stained for 45 minutes at 37°C in 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 1 mg/ml X-Gal, 0.1% Triton X-100 in PBS. Stained embryos were refixed for 1 hour in MEMFA (Harland, 1991) and stored in methanol at –20°C before being used for in situ hybridization, which was as described by Harland (1991) with slight modifications as described by van der Wees et al. (1996).

Immunofluorescence and confocal microscopy

For immunostaining of the CNS, embryos were fixed overnight at 4°C in methanol. The pigment was bleached in 80% methanol, 6% H_2O_2, 25 mM NaOH, for approximately 3 hours. After bleaching, the

embryos were washed extensively in PBS containing 0.2% Tween, and blocked for 30 minutes with PBT (0.2% Tween, 3% bovine serum albumin in PBS). Incubation with anti-neural antibodies 2G9 (Jones and Woodland, 1989) and Xen-1 (Ruiz i Altaba, 1992) at 1:1 2G9 and 1:5 Xen-1 in PBT was overnight at room temperature. The embryos were washed with PBT and incubated overnight with the secondary antibody conjugated to the Cy-5 far-red fluorophore (Jackson Research Labs, Inc.). Specimens were cleared in 1:2 benzyl alcohol: benzyl benzoate and viewed using confocal scanning laser microscopy (CSLM). Approximately 20 optical sections, covering the complete CNS, of each embryo were recorded on a BioRad MRC600 confocal microscope and reconstructed into (three-dimensional) images.

Retrograde tracing of reticulospinal neurons

MS222 anaesthetized stage-48 embryos were placed on moist tissue. Their spinal cords were incised using iridectomy scissors at the level of the posterior end of the gut. Immediately after incision, a crystal of fluorescein-labeled dextran (M_r 10×10^3; lysine fixable; Molecular Probes) was placed in the wound. After 2 minutes, embryos were transferred to 25% MMR and incubated for 7 hours, after which they were killed by an overdose of MS222 and fixed overnight in 4% paraformaldehyde in PBS. After a few rinses with PBS, CNSs were dissected, incised at the dorsal midline and placed, dorsal side facing downwards, on an object glass. The superfluous fluid was soaked away and, by gentle pushing, the CNS was pressed to the object glass. A cover glass with 30 µl of Elvanol (the supernatant of a solution consisting of 10 g Moviol 4-88 (Hoechst), 40 ml 0.1 M phosphate buffer, and 20 ml glycerin) was used to cover the CNS. Neurons and axons of the mid- and hindbrain were viewed using a fluorescence microscope (Zeiss).

RESULTS

DN RARβ inhibits ATRA induced activation of a natural DR5 RARE and reduces retinoid teratogenesis in *Xenopus* embryos

The DN RAR construct used in this study was made by C-terminal exchange between human (h) RARβ$_2$ and the truncated RARα from an ATRA-resistant embryonal carcinoma cell line (RAC65; Kruyt et al., 1992) (construct βΔ384; from Shen et al., 1993). This mutation deletes the ligand-dependent transactivation domain (see Durand et al., 1994), and because the inactive receptor can still dimerise with endogenous RXRs and bind to DNA, it inhibits transcriptional activation by endogenous RARs via DR5 RAREs in embryonal carcinoma (EC) cells (Shen et al., 1993). To assess the dominant negative activity of DN RARβ in *Xenopus* embryos, *DN RARβ* mRNA was co-injected with a luciferase reporter construct driven by a h*RARβ*$_2$ promoter element, which contains a DR5 RARE in its natural context. This reporter construct was almost silent in *Xenopus* embryos, and was inducible by addition of ATRA to the culture medium (Fig. 1, 'no RNA'). Co-injection of *DN RARβ* mRNA abolished ATRA-induced reporter activation (Fig. 1, 'DN RARβ'). Thus, DN RARβ expression can prevent ATRA-induced transactivation of a DR5 RARE in *Xenopus*.

To investigate whether expression of DN RARβ was sufficient to prevent ATRA-induced teratogenesis, *Xenopus* embryos that had been injected before the first cleavage with *DN RARβ* mRNA were treated with different concentrations of ATRA during gastrulation (14-hour treatment), and their phenotypes were scored at tadpole stages. Injected (non-ATRA treated) embryos showed a specific phenotype (Fig. 2A, top embryo; for further details see below). DN RARβ rather efficiently rescued both the anterior and posterior defects which result from ATRA treatment (compare Fig. 2B with 2C). The rescue of forebrain-related eye development is particularly obvious, as discussed below. An example of maximal rescue from treatment with 10^{-6} M ATRA by DN RARβ expression is also shown in Fig. 2C (bottom). We investigated further whether ectopic expression of DN RARβ could restore the expression of *Otx-2*, a marker for the fore- and midbrain (Blitz and Cho, 1995; Pannese et al., 1995), which disappears after ATRA treatment (Pannese et al., 1995, and Fig. 2E). In the majority of cases, expression of DN RARβ prevented ATRA-induced loss of expression of *Otx-2* (Fig. 2F), and of *En-2*, a marker of the mid-hindbrain border (Hemmati-Brivanlou et al., 1991), which is also not expressed in RA-treated embryos (Sive et al., 1990) (not shown). These results indicated that expression of DN RARβ also diminishes ATRA teratogenesis.

Next, we tested whether DN RARβ expression could also decrease the teratogenic effects resulting from treatment of

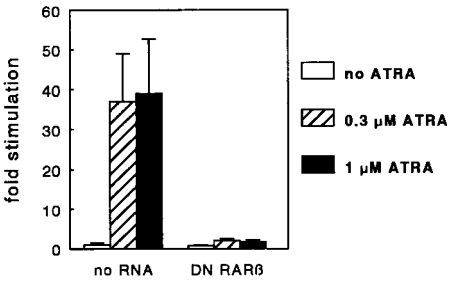

Fig. 1. DN RARβ expression inhibits ATRA-induced transcription of a reporter construct containing a DR5 RARE in its natural context, in *Xenopus* embryos. Embryos were injected with 1.75 ng *DN RARβ* RNA and/or 165 pg reporter (−1470+156Luc; RARβ promoter) DNA ('DN RARβ' or 'no RNA', respectively). At stage 10, embryos were transferred to a medium containing 3×10^{-7}, or 10^{-6} M ATRA, or no additive. After 7-hours incubation, luciferase activity was determined. Each bar represents the mean value of at least 3 pools of 5 embryos ± s.e.m.. Inhibition by DN RARβ of activation of the reporter by ATRA was observed in every reporter experiment performed.

Table 1. DN RARβ expression reduces both ATRA and 9-cis-RA-induced teratogenesis

	Non-injected embryos		*DN RARβ*-injected	
Retinoid treatment	DAI	EYES	DAI	EYES
none	5	2.0 (*n*=52)	5	2.0 (*n*=30)
ATRA (4×10^{-6} M)	2.6	0.2 (*n*=36)	4.5	1.6 (*n*=33)
9-cis RA (1×10^{-6} M)	2.5	0.03 (*n*=36)	4.7	1.8 (*n*=36)

Zygotes were injected with 1.5 ng *DN RARβ* RNA. Groups of 10 early gastrulae (stage 10) were treated with the indicated retinoids (less 9-cis-RA than ATRA was used because 9-cis-RA is more teratogenic) and allowed to develop at 18°C until stage 12. Subsequently, the embryos were washed 4 times and incubated until stage 41, when they were scored for microcephaly, both by using a modified DAI (Sive et al., 1990) and by counting the number of eyes present: 2, two eyes; 1, cyclopic; 0, 0 to 1 (small) eye present. The latter method of measurement was used in addition to the DAI, because it is especially sensitive in revealing differences between highly microcephalic embryos. The number of embryos (*n*) used for scoring is shown in parentheses.

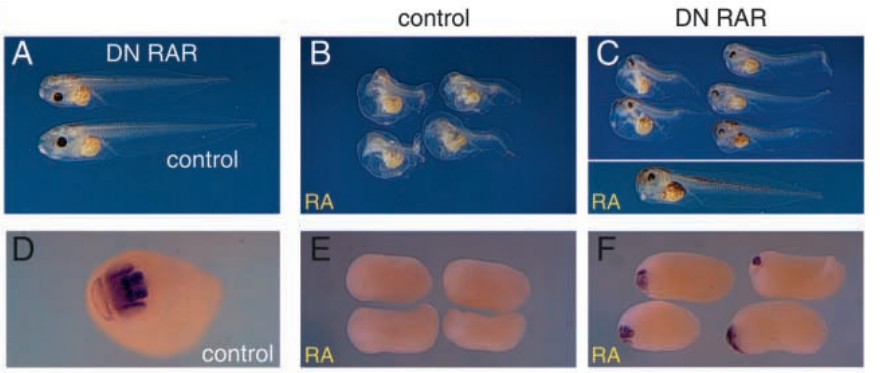

Fig. 2. Effects of DN RARβ expression on normal or ATRA-disturbed *Xenopus* development. DN RARβ reduces ATRA teratogenesis. (A) DN RARβ expression (top embryo) causes no prominent anteroposterior defects compared with control embryos, but experimental embryos are clearly smaller. (B,C) Teratogenic effects of a 14-hour treatment with 10^{-6} M ATRA are much less severe in DN RARβ-expressing embryos (C) than in controls (B). Almost complete rescue from this high dose of ATRA is possible (C, bottom). (D) Wild-type *Otx-2* expression pattern in control stage-21 embryo. (E) Absence of *Otx-2* expression in ATRA-treated *Xenopus* embryos. (F) *Otx-2* is expressed in DN RARβ-expressing ATRA-treated embryos. Anterior is to the left; embryo in D faces upwards. ATRA treatment was from stage 8 until stage 13.

Xenopus embryos with 9-cis-RA. This retinoid binds to and activates both RAR/RXR heterodimers and RXR homodimers and can thus activate all retinoid response elements. To quantify teratogenesis, we used a modified DAI (Sive et al., 1990), but also counted the number of eyes and eye-like structures present. These assays were used to compare control and DN RARβ-expressing embryos treated with incubation medium alone, or with ATRA, or with 9-cis-RA (Table 1). As is clear from the values for uninjected embryos, 9-cis-RA is more potent in inhibiting development of anterior structures (see also Creech Kraft et al., 1994): even at a four-fold lower concentration, 9-cis-RA treatment suppressed development of eye structures more efficiently than ATRA. Ectopic expression of DN RARβ was indeed very effective in reducing the teratogenic effects of 9-cis-RA (Table 1).

The results from the experiments above thus demonstrated that ectopic expression of *DN RARβ* RNA is an efficient tool for inhibiting the activation of endogenous *Xenopus* retinoid receptors, and for reducing teratogenesis induced by the RAR ligand ATRA and by the RAR and RXR ligand 9-cis-RA. This made it likely that the teratogenic effects of 9-cis-RA might be mediated not by RXR homodimers, but only by RAR/RXR heterodimers. To investigate this point further, we performed the experiments described below.

The teratogenic effects of ATRA and 9-cis-RA on *Xenopus* axial patterning are mediated via RAR/RXR heterodimers, not via RXR homodimers

There are at least two conceivable mechanisms that may explain rescue of both ATRA and 9-cis-RA teratogenesis by DN RARβ. The first is specific inhibition of DNA binding to RAR/RXR dimers due to competition by inactive DN RAR/RXR heterodimers for RAREs. The second is squelching of RAR cofactors like RXRs, which might be captured into unproductive DN RAR-cofactor complexes, thus inhibiting the activities of all receptors that need these cofactors. To discriminate between these two possibilities, we investigated whether supplying either additional RAR or additional RXR could counteract the dominant negative effect of DN RARβ on the DR5 response construct. If squelching of RXRs (as an example of a promiscuous cofactor) is the mechanism by which DN RARβ acts, then addition of supplementary RAR would

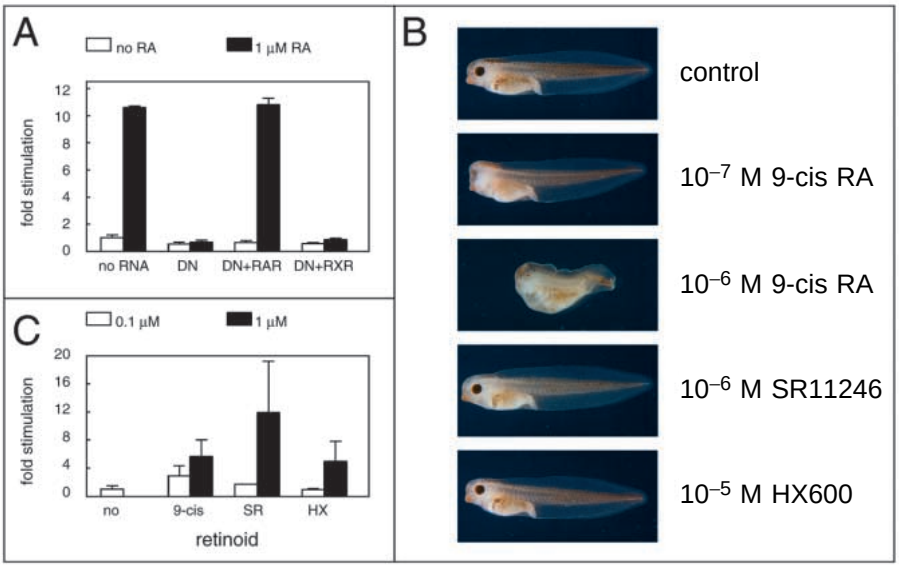

Fig. 3. (A) DN RARβ-inhibited transactivation of a DR5 reporter construct can be alleviated by co-injection of *RAR* RNA, not *RXR* RNA, indicating that DN RARβ specifically inhibits activities of endogenous RARs and that it does not squelch RXRs (or other cofactors). The reporter DNA (165 pg of −1470+156Luc; RARβ promoter) was co-injected with 2 ng h*RARα* RNA (DN + RAR), or 2 ng h*RXRα* RNA (DN + RXR), and/or 0.5 ng *DN RARβ* RNA. (B) Two synthetic RXR-selective ligands, SR11246 and HX600, are not teratogenic for *Xenopus* embryos, in contrast to 9-cis-RA. Treatment with retinoid ligands was from stage 10 till stage 12. (C) 9-cis-RA, SR11246, and HX600 penetrate *Xenopus* embryos and activate the ligand-binding domain of xRXRβ. Zygotes were injected with a mixture of 55 pg Gal4-xRXR(DE) and 165 pg Gal-Luc, treated from stage 10 with the indicated retinoids for 7 hours, and assayed for luciferase activity. Bars represent means ± s.e.m. of 2 pools of 10 embryos. no, no ligand (value set to 1); 9-cis, 9-cis-RA; SR, SR11246; HX, HX600. Similar results were obtained in three independent experiments.

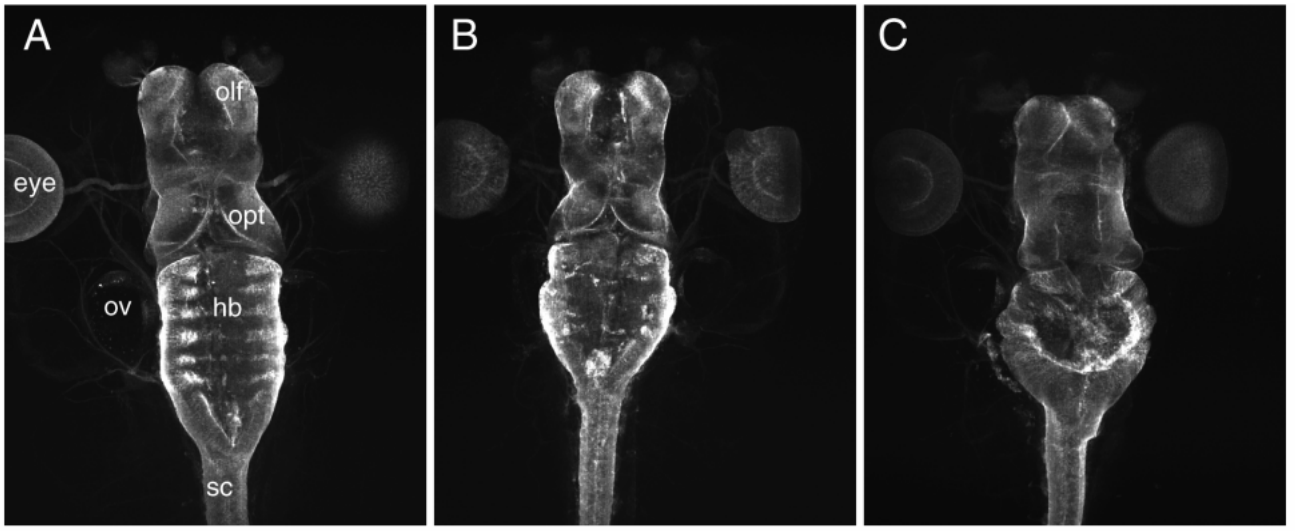

Fig. 4. DN RARβ expression causes morphological changes in the hindbrain of *Xenopus* embryos. CSLM images of fluorescent CNSs of *Xenopus* stage 47-tadpoles labeled with anti-neural antibodies 2G9 and Xen-1. (A) CNS of control (*GR* injected) embryo. Clearly visible are the forebrain (pronounced telencephalic olfactory bulbs (olf) connecting to the olfactory placodes), the optic lobes (opt) of the midbrain, and the individual rhombomeres of the hindbrain (hb) (r1-6 are separated, whereas r7 and r8 form one unit). (B,C) CNS of tadpoles injected with 1.75 ng *DN RARβ* RNA before the first cleavage. A relatively normal fore- and midbrain are accompanied by a severely malformed hindbrain. ov, otic vesicle; sc, spinal cord.

have no effect on reporter activity (because the amount of available cofactors would still be limiting), whereas addition of RXR would make RXRs available for dimerisation, and would thus restore transactivation (see also Shen et al., 1993). Therefore we co-injected *RARα* or *RXRα* RNA with the DR5 reporter construct and *DN RARβ* RNA. As is shown in Fig. 3A, co-injection of *RARα* RNA restored DN RARβ-inhibited ATRA-induced transactivation, whereas co-injection of *RXRα* RNA did not significantly induce reporter activity. The fact that addition of extra RAR could counteract the effects of DN RARβ indicates that RARs (but not RXRs or other cofactors) were limiting, and thus that expression of DN RARβ in *Xenopus* does not cause squelching of available RXRs or other cofactors. Thus, 9-cis-RA teratogenesis, which is alleviated by DN RARβ expression, is mediated only by RAR/RXR heterodimers, and not by RXR homodimers. The rescue by RARα supplementation of DN RARβ-blocked expression of the reporter indicates that inhibition of transcription of the reporter by DN RARβ is not specific for RARβ.

Another approach to test how DN RARβ interferes with retinoid signalling, and which receptors are important for teratogenesis, was to investigate whether DN RARβ could protect *Xenopus* embryos against teratogenic effects caused by RXR-selective ligands (see Minucci et al., 1996). To this end, *Xenopus* embryos were treated either with the RXR-selective ligand SR11246 (Dawson et al., 1995b) or with HX600, a synthetic retinoid (Umemiya et al., 1995) which also appears to be RXR-selective in HeLa cells (Umemiya et al., 1997). Strikingly, neither RXR-selective ligand had any apparent effect on anteroposterior development in *Xenopus* embryos, not even when these embryos were treated with 10^{-5} M HX600 during gastrulation (the most sensitive period for retinoid teratogenicity) (Fig. 3B). To confirm that both ligands could activate *Xenopus* (x) RXRβ, the prominent RXR isoform present during *Xenopus* gastrulation (Marklew et al., 1994; Sharpe and Goldstone, 1997), we co-injected an expression construct encoding the xRXRβ ligand-binding domain coupled to the DNA-binding domain of the yeast transcriptional activator Gal4 (Gal4-xRXRβ(DE)), with a luciferase reporter containing 5 Gal4 responsive elements (Gal-Luc) into *Xenopus* zygotes. Injected embryos were treated during gastrulation with either 10^{-7} or 10^{-6} M 9-cis-RA, SR11246, or HX600, and luciferase activity was determined after 7 hours of incubation (Fig. 3C). This experiment showed that both SR11246 and HX600 are able to penetrate the *Xenopus* embryo and to activate xRXRβ. SR11246 treatment resulted in a stronger activation of Gal4-xRXRβ(DE) than 9-cis-RA treatment (Fig. 3C). Besides transactivation via this chimaeric receptor, we also observed synergistic effects of both RXR-selective ligands on teratogenesis evoked by the RARα-selective ligand Am580 (WWM Pijnappel, personal communication; see also Minucci et al., 1997), again demonstrating the ability of both RXR ligands to enter the embryo and to activate RXRs. Together, these data confirm the hypothesis that the effects of retinoids on anteroposterior patterning of *Xenopus* are not mediated by RXR homodimers.

Ectopic expression of DN RARβ disturbs patterning of the hindbrain

The results described above demonstrate that DN RARβ is an excellent experimental tool for specifically decreasing RAR-mediated signalling during *Xenopus* embryogenesis. We investigated the effect of this treatment on embryonic development. As was shown in Fig. 2A, expression of DN RARβ (top embryo) caused no gross morphological defects. Injected embryos were slightly but significantly shorter than embryos injected with a control RNA that does not affect *Xenopus* development (*GR* mRNA, encoding the *Xenopus* glucocorticoid receptor; Gao et al., 1994): the mean length of stage-39 *DN RARβ* injected tadpoles was 9.2±0.3 mm,

compared to the mean length of *GR*-injected tadpoles which was 9.9±0.1 mm; P=0.04, unpaired two-tailed *t*-test.

To examine the brains of embryos expressing DN RARβ, we used a combination of anti-neural antibodies 2G9 (Jones and Woodland, 1989) and Xen-1 (Ruiz i Altaba, 1992). In control (*GR*-injected) embryos, the organization of the developing *Xenopus* CNS is clearly visible (Fig. 4A). The most notable defect in DN RARβ-expressing embryos was that their hindbrain was clearly reduced in length. Usually this shorter hindbrain was thicker, and sometimes very obviously disorganized: in the more extreme cases rhombomere boundaries were no longer visible (Fig. 4B,C).

To examine the organization of the hindbrain in more detail, we performed retrograde tracing of reticulospinal neurons in unilaterally DN RARβ- or GR-expressing embryos. The Mauthner cell, a reticulospinal neuron, which is specified during gastrulation (Vargas-Lizardi and Lyser, 1974) and which is easily recognizable as a single pair of large, contralaterally projecting neurons, is located in prospective r4. Ectopic expression of *GR* RNA had no effect on Mauthner cell development: 14/14 embryos had two Mauthner cells (Table 2 and Fig. 5A). In contrast, multiple Mauthner neurons were found in 76% of the DN RARβ-expressing embryos (16/21) (Table 2 and see Fig. 5B,C). Supernumerary Mauthner neurons were always located posteriorly from the 'original' Mauthner neuron, in r5 and/or r6, and sometimes also in r4 (Fig. 5C), but they were never observed in r1-3 or r7 or r8. This finding suggests a (partial) change of identity of r5 and r6 into (partial) r4 identity.

To investigate whether other changes had also taken place, we used a number of molecular markers to examine specific parts of the brains of DN RARβ- or (as a control) GR-expressing embryos in detail. Embryos were injected unilaterally at the two-cell stage (resulting in an internal control side).

Krox-20 marks r3 and r5 and the neural crest derived from r5 (Bradley et al., 1992, and Fig. 6A). DN RARβ expression affected *Krox-20* expression in several ways. The expression in r3 was rarely expanded anteriorly to r1 and r2 (3 cases out of 86 DNRAR injected embryos) (Fig. 6B,E), suggesting an occasional posterior transformation of these anterior rhombomeres. Alterations were much more frequently observed in the *Krox-20* expression in r5 (54 cases out of 86 injected embryos), such that expression could be expanded posteriorly to r6 (Fig. 6C,F,H,I), also to r7 (Fig. 6J), and sometimes even to r8 (Fig. 6D,E), suggesting an anterior transformation of the posterior part of the hindbrain. Ectopic expression of *Krox-20* was also observed in r4 (Fig. 6D,E,H,I). Ectopic *Krox-20*-expression was also seen outside the CNS, possibly representing ectopic *Krox-20* expression in neural crest cells (Fig. 6C). Of 43 control (GR-injected) embryos examined, none showed any aberration in *Krox-20* expression.

In separate experiments, DN RARβ-expressing embryos were simultaneously hybridized to *Krox-20* and *En-2* probes (Fig. 6G-J). *En-2* marks the mid-hindbrain border and is thus partially expressed in r1. Whereas DN RARβ-expressing embryos had ectopic *Krox-20* expression in r4 and r6 (Fig. 6H,I) or in r7 (Fig. 6J), aberrant *En-2* expression was never observed in these embryos (Fig. 6H-J), indicating a normal development of the mid-hindbrain border area. Similarly, we usually detected no changes in the expression pattern of *Otx-2* (a marker of fore- and midbrain; Pannese et al., 1995) (Fig. 6N,O), although in 1 out of 25 embryos the *Otx-2* expression domain in the midbrain was slightly elongated into a more posterior brain region (not shown). The expression of *Hoxb-1*, which is expressed in r4 (Godsave et al., 1994), was not changed (not shown).

Table 2. DN RARβ-expressing embryos have multiple Mauthner neurons

No. of Mauthner cells at injected side	Injected RNA	
	GR (*n*=14)	*DN RARβ* (*n*=21)
1	14	5
2	0	8
3	0	6
4	0	2

Two-cell-stage embryos were unilaterally injected with 1.5 ng *GR* or *DN RARβ RNA* and cultured until stage 48. Reticulospinal neurons were retrogradely labeled with fluorescein-labeled dextran, and the numbers of Mauthner neurons present in successfully labeled tadpoles were counted.

The expression of *Hoxb-3*, which is most abundant in r5 and r6, and in the neural crest derived from r5 (Godsave et al., 1994, and Fig. 6K), was repressed and/or posteriorised in some DN RARβ expressing embryos (Fig. 6L), but ectopic expression of this gene was also sometimes observed in a region posterior to the neural crest cells that normally express *Hoxb-3* (Fig. 6M), suggesting anteriorisation of posterior neural crest cells.

The posterior-most markers used in this study (*Hoxb-7* (not shown) and *Hoxb-9*), which are expressed in the spinal cord, seemed to be relatively unaffected; however, *Hoxb-9* expression in the spinal cord sometimes extended somewhat further laterally in DN RARβ-expressing embryos (Fig. 6P,Q). This effect was associated with slightly delayed closure of the neural tube and was clearly aspecific since it was always bilateral, also in unilaterally injected embryos.

Because the effects of DN RARβ on *Krox-20* expression were variable, we co-injected *lacZ* mRNA with the *DN RARβ* RNA as a control for distribution of the RNA. An example of this is shown in Fig. 6J. Whereas the whole length of the left (injected) part of the neural tube stains positive for β-galactosidase (and thus presumably also expresses DN RARβ), ectopic *Krox-20* expression is only observed in r7, and *En-2* expression is not influenced. 16/16 lineage labelled embryos showed similar patterns, with rather general *lacZ* expression on the injected side of the embryo and very localised disturbances to *Krox-20* expression, within the β-gal labelled domain. Thus, mosaic distribution of *DN RARβ* RNA is probably not the reason for the variability of the effects of DN RARβ on *Krox-20* expression. In conclusion, ectopic DN RARβ expression severely disturbed hindbrain development, but not fore- or midbrain or spinal cord development. Changes in the organization of the hindbrain can be summarized as follows (Fig. 7): from the multiplied Mauthner cells, it appears that r5 and r6 become (partially) r4-like; and similarly, the posteriorly expanded r5 expression of *Krox-20* points to a (partial) change of the identities of r6-8 into an r5 like identity. The rare ectopic *Krox-20* expression in r1 seems to point to an occasional (partial) transformation of r1 into an r3-like identity. Thus, decreased retinoid signalling results in anteriorisation of the

posterior hindbrain and occasional posteriorisation of the anterior hindbrain. This loss of strictly regulated rhombomere-specific localisation of gene expression may also reflect a partial disturbance of segmentation.

DISCUSSION

DN RARβ is a powerful tool for investigating the functions of RARs

We used ectopic expression of *DN RARβ* mRNA in *Xenopus* embryos to evaluate the role of RARs in anteroposterior patterning. First, we tested whether this mutant receptor was indeed dominant negative in *Xenopus* embryos, by co-injecting a RARβ promoter reporter construct containing a DR5 RARE into the zygote. Without addition of retinoids, the activity of this reporter was too low to reliably determine whether DN RARβ could block signalling by endogenous retinoids. Treatment of *Xenopus* embryos with ATRA induced reporter activity 10- to 40-fold. Expression of DN RARβ was clearly adequate to completely block RA-induced activation of the reporter. DN RARβ partially prevented ATRA-induced teratogenesis during *Xenopus* embryogenesis. Retinoid-induced teratogenesis thus appears to be opposed less efficiently than retinoid-induced reporter activity. Possible reasons for this are discussed below.

We have not completely addressed the issue of the specificity of inhibition of retinoid signalling by DN RARβ, although we have some indications that DN RARβ does not specifically inhibit RARβ-mediated retinoid signalling: in Fig. 3A we show that RARα is capable of counteracting the inhibitory effects of DN RARβ on RA-induced reporter activity. Furthermore we have observed that DN RARβ reduced the teratogenic effects caused by the RARα-selective ligand Am580 (Jetten et al., 1987) and the RARγ-selective ligand SR11254 (Dawson et al., 1995a) as efficiently as it reduced ATRA teratogenicity (not shown). Thus DN RARβ represses a considerable part of RAR-mediated signalling. However, there are also indications that DN RARβ has specific effects (see below).

Phenotypic penetrance and specificity of DN RARβ

Several observations suggested that DN RARβ expression failed to give a complete block of retinoid signalling. These observations included incomplete rescue of retinoid teratogenesis (although not of a co-injected reporter construct) and very localized effects on *Krox-20* expression. We note also that the expression of a gene (*Hoxb-1*) which is known to be regulated by retinoid response elements was not altered in this experiment. Incomplete penetrance might be caused by mosaic expression of the injected RNA. Co-injected reporter DNA will be repressed efficiently by DN RARβ, even if the injected *DN RARβ* RNA is expressed mosaically, because each cell that contains reporter DNA also contains *DN RARβ* RNA (DNA spreads less efficiently through the cytoplasm than RNA). However, it is possible that *DN RARβ* RNA is distributed unevenly in the developing embryo, and that low- or non-expressing cells remain susceptible to retinoids. We generally injected into the animal pole so as to target presumptive ectodermal and neural tissue. Although mosaicism is potentially a valid explanation, for example, for incomplete rescue of retinoid-induced teratogenesis, our control experiments using a lineage tracer showed however that wide-spread marker RNA distribution can be observed in parallel with very localized effects on *Krox-20* expression, arguing against mosaicism being the major cause of the observed phenotypical variation. Furthermore, the effects of DN RARβ on the development of Mauthner neurons were very consistent: 16/21 embryos had one or more additional posterior Mauthner neurons. Because Mauthner neurons are specified during gastrulation, this observation implies that at that time the

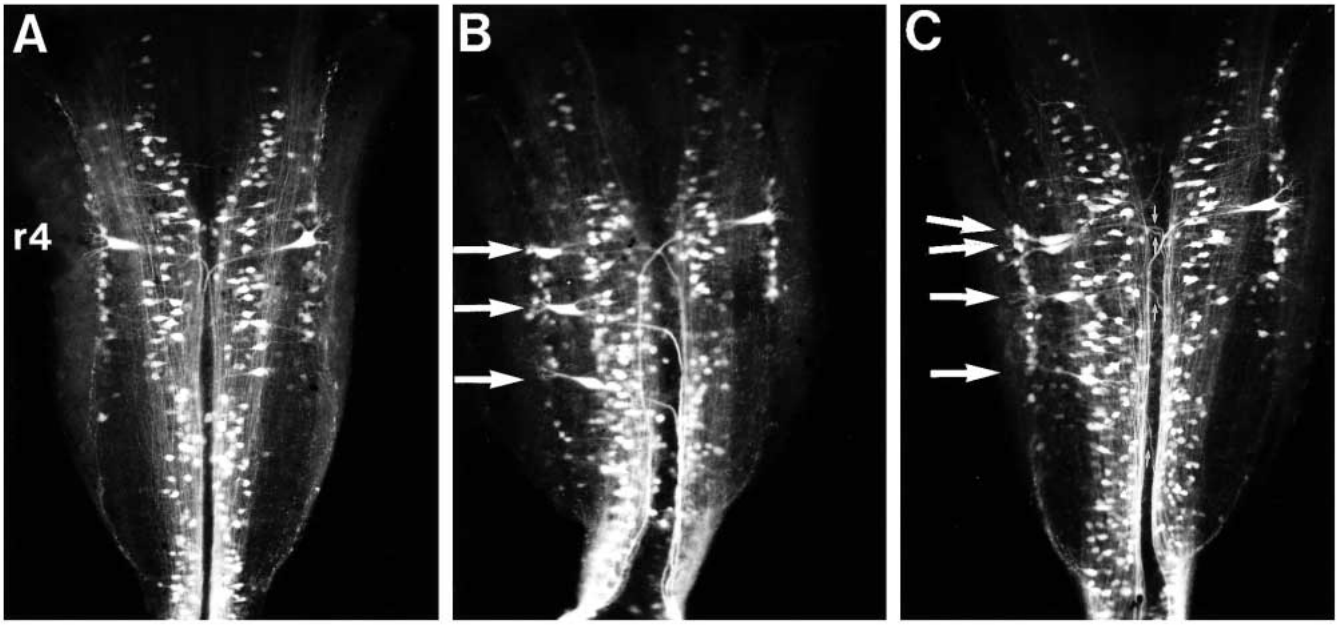

Fig. 5. DN RARβ anteriorises rhombomeres 5 and 6. Flat-mounted hindbrains of GR- (A) or DN RARβ-expressing embryos (B,C) in which the reticulospinal neurons have been retrogradely labeled with fluorescein-labeled dextran. Ectopic Mauthner neurons are found in r5 and r6 (large arrows in B,C) and sometimes also in r4 (C). Small white arrows in C point to Mauthner axons crossing the midline.

distribution of the *DN RARβ* RNA is uniform enough to anteriorise part of the prospective posterior hindbrain in 76% of the injected embryos. This is thus consistent with our direct observations that injected RNA is distributed uniformly enough to influence *Krox-20* expression consistently (in 54/86 cases). Another potential explanation for incomplete penetrance might be instability of the injected RNA, but this is presumably also not the case, because at stage $12\frac{1}{2}$, DN RARβ was present in sufficiently large amounts to repress reporter activity totally (see Fig. 1). DN RARβ will thus be active during gastrulation, the period when the anteroposterior pattern of the CNS is first established (Gerhart et al., 1989).

Alternatively, some of the specific effects we observed following ectopic expression of DN RARβ may reflect specificity of the DN RARβ treatment. Several receptor-subtype-specific retinoid- or retinoid-like inputs may be involved in the signalling pathways which can be stimulated by retinoid treatment. Specificity might explain the different effects of DN RARβ expression on two retinoid-responsive markers for r4: expression of *Hoxb-1* (not changed) versus development of Mauthner neurons (DN RARβ induces posterior multiplications). These observations indicate that DN RARβ blocks only a subset of RAREs. This might also explain the differences between alterations in gene expression observed in this study (expression of DN RARβ) and the study of Blumberg et al. (1997), who ectopically expressed a DN RARα (see below).

Although ectopic expression of *DN RARβ* RNA may not be fully effective in inhibiting the complete retinoid signalling repertoire in the *Xenopus* embryo, the effects of this treatment on embryonic development are substantial and interesting. Investigating them will help to resolve the role of retinoid receptors during development.

Teratogenic effects of retinoids are apparently mediated by RAR/RXR heterodimers, not by RXR homodimers

Our investigations showed that DN RARβ could prevent disturbed development just as efficiently in more severely affected 9-cis-RA treated embryos as in less affected ATRA-treated embryos. These findings suggest that 9-cis-RA teratogenesis is mediated exclusively by RAR/RXR heterodimers. We investigated this idea further by treating embryos with two RXR-selective ligands. To our surprise, these retinoids did not affect anteroposterior patterning at all, although both retinoids did enter the embryo and efficiently transactivated xRXRβ. These results conflict with the report by Minucci et al. (1996) that the RXR-selective ligands SR11237 and Ro25-6603, when used at 10^{-5} M, induced teratogenicity in *Xenopus* embryos. The observed difference probably resides in the loss of receptor-selectivity at the high ligand concentration used by Minucci et al. (1996). At this concentration, the retinoids used by them were able to induce

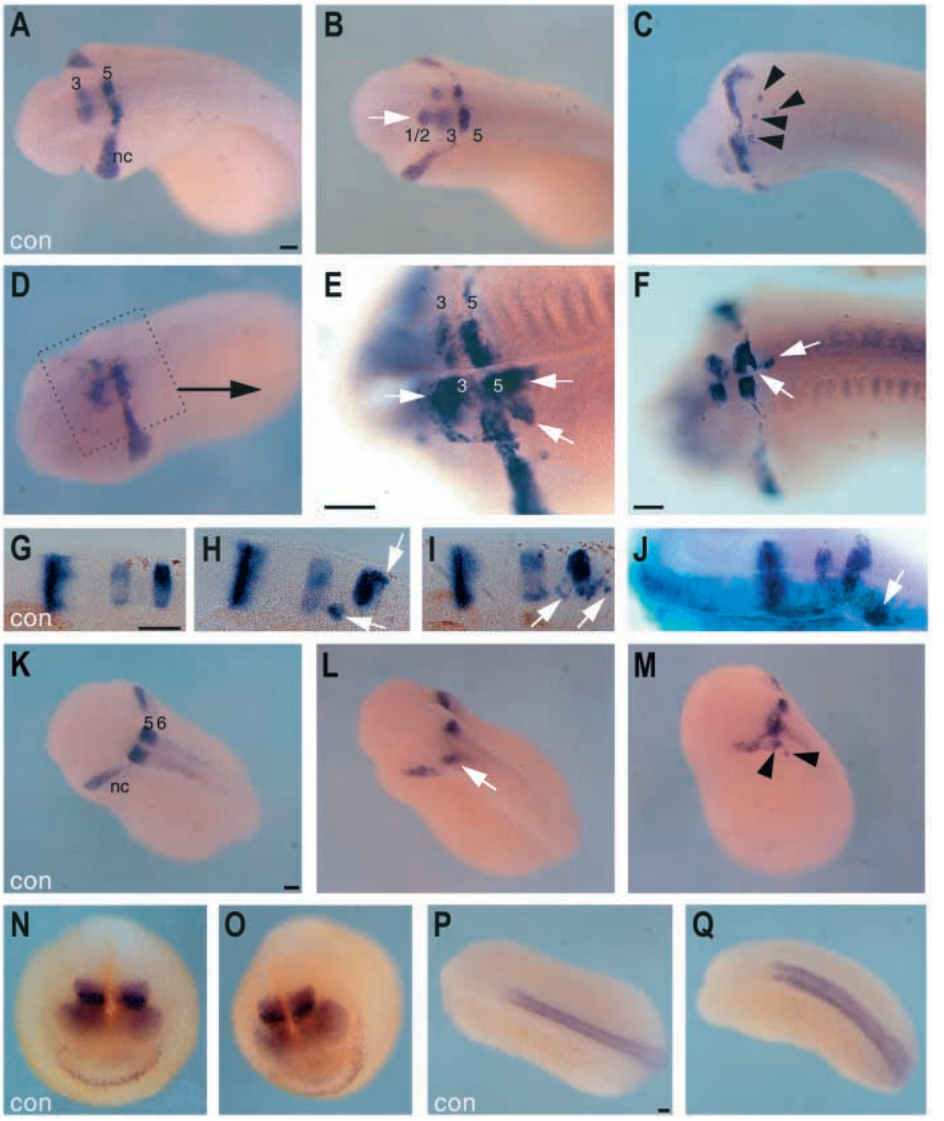

Fig. 6. Expression of *Krox-20* (A-F), *Krox-20* and *En-2* (G-J), *Hoxb-3* (K-M), and *Hoxb-9* (P,Q) in stage-25 control (i.e. unilaterally *GR* RNA injected) embryos (A,G,K,P) and unilaterally DN RARβ-expressing embryos (B-F,H-J,L-M, Q); anterior is to the left. (N) Expression of *Otx-2* in a stage-19 *GR* RNA-injected control embryo and (O)in a DN RARβ-expressing embryo; anterior is towards the reader. White arrows indicate ectopic expression in the CNS. Black arrowheads point to cells ectopically expressing *Krox-20* (C) or *Hoxb-3* (I) outside the CNS. Embryos in E-J were cleared with 1:2 benzyl alcohol/benzyl benzoate. Scale bars (in A for A-D; in G for G-J; in K for K-O; in P for P-Q) 100 μm. The embryo in J was stained for β-gal to reveal the expression of co-injected *lacZ* RNA.

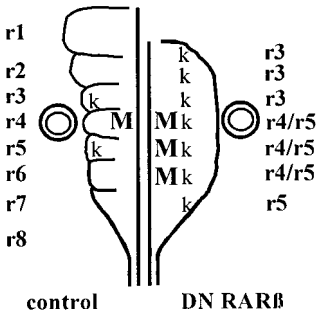

Fig. 7. Summary of the major results of expression of DN RARβ. The left part depicts the control situation for the *Xenopus* hindbrain: a segmented structure marked by restricted *Krox-20* (k) expression domains in r3 and r5, and one Mauthner neuron (M) in r4. DN RARβ expression can probably disturb segmentation of the hindbrain and can result in a hindbrain with preferential r3 (k), r4 (M), and r5 (k) identity.

a βRARE reporter 1.5- to 2-fold, respectively. This level of induction is non-negligible because ATRA at the same concentration gives only a 5-fold induction (Minucci et al., 1996). Moreover, RXR-selective ligands have been found to be non-teratogenic in mouse (Jiang et al., 1995; Kochhar et al., 1996) and zebrafish (Minucci et al., 1996, 1997). From the above and the finding that co-injection of *RAR* RNA rather than *RXR* RNA can counteract the dominant-negative effect of DN RARβ on the RARE reporter, we conclude that retinoid teratogenesis occurs via RAR/RXR heterodimers only, probably both via ligand activation of the RAR partner and synergistic activity of the RXR partner. Further experiments need to be performed to address this issue, but in the light of recent findings (e.g. Roy et al., 1995; Horn et al., 1996; Minucci et al., 1997; Lu et al., 1997), synergism of RAR and RXR ligands seems a likely mechanism involved in teratogenesis.

Retinoids as transformation signals

DN RARβ-expressing embryos do not have the phenotype expected of an embryo which has not received any neural transformation signal (a completely anteriorized embryo). This absence of obvious morphological defects was also observed by Smith et al. (1994), who ectopically expressed a DN RARα, and by Sharpe and Goldstone (1997), who showed that a DN RARα could block formation of primary sensory neurons and cause changes in the dorsal/ventral patterning of the spinal cord, but not obviously disturb normal external development. The effects of DN RARα on *Xenopus* development observed by Blumberg et al. (1997) are also quite mild (slight expansion of markers of fore- and midbrain structures, i.c. slightly more posteriorly extending *Otx-2* expression and posteriorized *En-2* expression, and loss of *Hoxb-9* expression, but no loss of tails). These results are different from ours; although we have observed slightly changed *Otx-2* expression in a very small minority of cases, we have never observed posteriorised *En-2* expression. Whereas we observe ectopic *Krox-20* expression along the axis of the hindbrain, Blumberg et al. (1997) observed loss of r5-specific *Krox-20* expression. As discussed above, these differences might be caused by different target gene specificities of our respective DN RAR constructs which were made from RARα (Blumberg et al., 1997) and RARβ (this study) respectively. The results of Blumberg et al. (1997) appear to point in the direction of retinoids as transformation signals. Thus, either retinoids are the transformation signal, and the effects of expression of DN RARs that we and others have observed are not complete, for reasons described above; or retinoids are not the transformation signals, or not the only ones.

Results from RAR loss-of-function experiments (reviewed by Lohnes et al., 1995, and Kastner et al., 1995) and ligand depletion experiments (Maden et al., 1996) have till now also not demonstrated a major role for retinoids in the choice between anterior and posterior neural development; and whereas the RAR loss-of-function studies may suffer from incomplete penetrance because of receptor redundancy, the vitamin A-deficient quail embryo (Maden et al., 1996) is possibly a complete knock-out, which still shows no gross anteroposterior defects.

Although it is still impossible to rule out the possibility that retinoids are the transformation signals, there is also increasing evidence that the transformation process of neural induction is directed by more signals than one. Besides retinoid, FGFs (Doniach, 1995), and (downstream from FGFs) caudal-family transcription factors (Pownall et al., 1996), as well as Wnts (McGrew et al., 1995), are also likely to be involved.

Active RARs are important for correct patterning of the hindbrain

Retinoids have been proposed to have a more specific function in the patterning of posterior neural tissue, namely that they play a role in hindbrain patterning (which might or might not reflect their action as part of a more general transformation process). Such a role is also indicated independently by many findings in the literature (see Introduction). Expression of DN RARβ indeed interfered with hindbrain development, as was clear morphologically, from ectopic Mauthner neurons in the posterior hindbrain, and from alterations in the expression of hindbrain and neural crest markers, notably *Krox-20* and *Hoxb-3*. The effects of expression of DN RARβ on hindbrain development appear to include both anteriorisations and (rare) posteriorisations of the hindbrain. It seems that the effective RAR concentration is critical for correct patterning of the hindbrain, and that changes herein can flatten the patterning system of the hindbrain, resulting in a preferential r3,4,5 identity.

Ectopic expression of DN RARβ very consistently lead to multiple Mauthner neurons, implying (partial) respecification of r5 and r6 into (partial) r4-like identity (anteriorisation of part of the posterior hindbrain), and we also occasionally observed an extra Mauthner cell in r4. Inhibition of retinoid signalling thus induced disturbed development of part of the posterior hindbrain. In zebrafish embryos, the converse treatment (excess ATRA) induces duplication of Mauthner neurons, either in r2 or in r4 (but not in r3, see below), probably acting via ectopic induction of *Hoxa-1* expression (Hill et al., 1995; Alexandre et al., 1996). ATRA can thus apparently at least partly respecify (part of) r2 into an r4-like identity (posterior transformation of part of the anterior hindbrain) (see also Marshall et al., 1994). These results point to the importance of a strictly regulated retinoid availability in the (prospective) hindbrain. Disturbances can lead to a preference for r4. It is

striking that the Mauthner cell in r4 is always present and sometimes duplicated, irrespective of the treatment, and also that the normal two-segment rhombomere periodicity can apparently be lost or disturbed in DN RARβ expressing embryos, as is evident for example from the development of an ectopic Mauthner neuron in r5. This disturbed segmentation was not observed in ATRA-treated zebrafish embryos, where ectopic Mauthner neurons were never observed in r3 (Hill et al., 1995).

The expression of *DN RARβ* could induce ectopic *Krox-20* expression in the hindbrain and in neural crest cells. Instead of a pure change of anteroposterior identity, 'leaking' *Krox-20* expression in neighboring rhombomeres might (additionally) reflect loss of hindbrain segmentation, which allows *Krox-20* expression to spread into more anterior and posterior rhombomeres. A (partial) loss of *Krox-20* from r3 has been reported following ATRA treatment or ectopic *Hoxa-1* expression (Wood et al., 1994; Hill et al., 1995; Alexandre et al., 1996). Gale et al. (1996) showed ectopic *Krox-20* expression in r4 after injection of ATRA into r4. Wood et al. (1994) showed that excess ATRA can lead to loss of anterior (r1-4) hindbrain segmentation. Vitamin A-deficient quail embryos also lose *Krox-20* expression in r3, and in r5 (Maden et al., 1996). Thus, although a clear correlation between activation and inhibition of retinoid signalling and *Krox-20* expression in the hindbrain is lacking, a disturbed retinoid balance results in disturbed *Krox-20* expression.

We were surprised not to observe induced changes in *Hoxb-1* expression by DN RARβ. The transcription of this gene appears to be regulated by retinoid response elements. A 3′ DR2 RARE is responsible for the early expression in neurectoderm, mesoderm, and primitive streak, whereas a 5′ DR2 RARE is involved in suppression of *Hoxb-1* expression in r3 and r5, thereby restricting late *Hoxb-1* expression to r4 (reviewed by Maconochie et al., 1996, and Marshall et al., 1996). *Hoxb-1* expression disappears from r4 when ATRA is injected locally, which was explained by a higher than normal retinoid concentration in r4, thereby allowing binding of active retinoid receptors to the repressor RARE and suppression of *Hoxb-1* expression in r4 (Gale et al., 1996). On the contrary, systemic ATRA treatment can lead to ectopic *Hoxb-1* expression in r2, and not to disappearance of *Hoxb-1* in r4 (Marshall et al., 1994). Vitamin A-deficient quail embryos lose *Hoxb-1* expression in r4 (Maden et al., 1996). Thus, again, the expression of another hindbrain-specific gene appears to be influenced by precisely regulated retinoid concentrations, but we note that our DN RARβ treatment does not eliminate *Hoxb-1* expression in r4. This might indicate that the retinoid response elements regulating *Hoxb-1* expression can not be occupied by DN RARβ, and that development of Mauthner cells and *Hoxb-1* expression are regulated via different pathways.

Although it is not yet clear how, our results demonstrate that retinoid receptors are important for patterning the hindbrain. Further information is needed to determine whether retinoids function during early CNS development as general transformation signals or exclusively as specific signals for patterning the hindbrain, and to determine how retinoids act in patterning the hindbrain.

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