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Induction of a *RARβ2-lacZ* transgene by retinoic acid reflects the neuromeric organization of the central nervous system

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Summary

The hormone retinoic acid (RA) has been implicated in the organization of the anteroposterior (AP) body axis. In this paper, we describe the effects of RA on the activity of the RA-inducible retinoic acid receptor-β2 (*RARβ2*) promoter. When transgenic embryos carrying a *RARβ2-lacZ* reporter gene were exposed to a single dose of RA between gestational days 8.5 to 10.5, *lacZ* expression was induced in the anterior central nervous system (CNS). Strikingly, the transgene was expressed in a segmented pattern reminiscent of that of *Drosophila* 'pair-rule' genes. RA treatment of midgastrulation embryos at day 7.5 disturbed the segmentation and produced severe craniofacial defects. We discuss the possibility that the entire anterior CNS is segmented and that this segmentation is reflected by the *RARβ2-lacZ* induction pattern.

Key words: transgenic mice, segmentation, retinoic acid, *lacZ*, gene expression.

Introduction

Vertebrate development involves the spatial organization of embryonic tissues into repetitive metameric units or segments. These segments become most conspicuous as the paraxial mesoderm condenses into somites, epithelial structures that will eventually give rise to dermis, axial muscles and the axial skeleton. It is primarily the axial skeleton that elicits the segmented pattern in the adult organism.

The segmented organization of the ectoderm was first described more than 160 years ago (Baer, 1828) as transiently formed metameric bulges of the neural tube (termed neuromeres; Orr, 1887). However, the functional significance of neuromeres has only recently come to light. Tissue transplantation and gene expression studies, as well as cell tracing experiments demonstrated clearly that neuromeres (rhombomeres), the overlying ectoderm, and the neural crest of the branchial region are domains for cellular differentiation, gene expression, and cell lineage restriction (Noden, 1983; Lumsden and Keynes, 1989; Murphy et al., 1989; Couly and Le Douarin, 1990; Fraser et al., 1990; Sundin and Eichele, 1990; Hunt et al., 1991). The status of the neural tube rostral to the hindbrain is still unclear. It is often considered to be unsegmented (Kandel et al., 1991), although neuromeres have been morphologically identified (Baer, 1828; Bartelmez, 1923; Adelmann, 1925; reviewed by Vaage, 1969).

The molecular mechanisms underlying segmentation in mammals are poorly understood. Several lines of evidence, however, suggest a role for the hormone retinoic acid. Exposure of gastrulating amphibian embryos to low RA concentrations results in CNS transformation (Dursten et al., 1989; Sive et al., 1990; Ruiz i Altaba and Jessell, 1991). Congenital CNS malformations and axial defects have also been reported after maternal RA administration in rodents (Kochhar, 1967; Morriss, 1972; Shenefelt, 1972) and humans (Lammer et al., 1985). It has recently been shown that RA can disturb the segmented organization of the hindbrain (Holder and Hill, 1991; Morriss-Kay et al., 1991; Papalopulu et al., 1991) and the axial skeleton (Kessel and Gruss, 1991; Kessel, 1992).

RA modulates the transcriptional activity of its target cells via nuclear retinoic acid receptors. These receptors belong to the steroid/thyroid hormone receptor superfamily of transcription factors (Benbrook et al., 1988; Brand et al., 1988; Giguére et al., 1987; Krust et al., 1989; Petkovich et al., 1987; Zelent et al., 1989). Two subfamilies (termed *RAR*s and *RXR*s) have been distinguished based on sequence-homology and ligand-affinity (Mangelsdorf et al., 1990). Each sub-family is composed of at least three unlinked genes (-α, -β, and -γ) which in turn seem to encode several receptor-isoforms as a result of differential splicing and/or promoter usage (Giguére et al., 1990; Kastner et al., 1990; Zelent et al., 1991).

We (Reynolds et al., 1991), and others (Mendelsohn et al., 1991) recently utilized *RARβ2* promoter sequences to drive the expression of a *lacZ* reporter gene in transgenic mice. We found that 250 bp of *RARβ2* promoter sequences were sufficient to direct expression of the *lacZ* reporter gene in a pattern that closely resembled that of the endogenous *RARβ2* (Reynolds et al., 1991; Ruberte et al., 1991).

Interestingly, *RARβ2* expression can be induced by RA treatment in vitro in tissue culture cells (de The et al., 1989) and also in vivo in chicken limb buds (Noji et al., 1991). This induction is dependent on a retinoic acid responsive element (RAREβ) in the *RARβ2* promoter. As RARβ can bind to this element, an autoregulatory mechanism for *RARβ2* expression has been suggested (de The et al., 1990; Sucov et al., 1990). Maternal administration of RA may also influence *RARβ2* expression in mammalian embryos, thus contributing to its teratogenic effects.

We describe here the expression pattern of *RARβ2-lacZ* reporter genes in transgenic mouse embryos after maternal RA administration. Exposure of day-7.5 embryos to low RA concentrations was highly teratogenic. The same concentration had no obvious effects when administered at day 8.5. *RARβ2-lacZ* induction was found in the migrating cranial mesoderm of day-7.5 embryos and in a segmented pattern in the anterior CNS after RA treatment at day 8.5 or 9.5.

Materials and methods

Animals

Inbred FVB mice (obtained from Taconic, Germantown, NY) were used in this study. Females were adapted to a dark-light cycle (light cycle from 6 a.m. to 6 p.m.) for at least one week prior to matings. Transgenic males were housed together with three non-transgenic females. Mice housed under these conditions will usually mate around midnight. Successful matings were evaluated every morning on the presence of vaginal plugs. The morning of this day was counted as day 0.25, the noon as day 0.5 and the evening at day 0.75.

Retinoic acid treatment and RARβ2-lacZ *expression analysis*

All-*trans* RA (Sigma) was dissolved in methylsulfoxide to a concentration of 25 mg/ml. This stock-solution was aliquoted and stored in the dark at −20ºC. It was suspended prior to use in sesame oil (Sigma) to a final concentration of 1 mg/ml. Pregnant mice were fed by gavage a single dose of 10 mg RA per kg body weight (0.2-0.25 ml). Times of RA treatment and the isolation of embryos is summarized in Table 1. Embryos were isolated, stained and sectioned as described previously (Reynolds et al., 1991). Briefly, embryos were dissected in phosphate-buffered saline, fixed in 4% formaldehyde, 0.5% glutaraldehyde and stained for β-gal activity in staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$). Cryosections were cut from some of the embryos at 25 µm and counterstained with 0.1% neutral red.

Results

The data presented in this paper were obtained with two independent *RARβ2-lacZ* transgenic lines, TG3 and TG16. These lines carry the reporter-constructs ΔRLZ79 (TG16) or RLZ79 (TG3). These constructs differ in the 5′-*RARβ2* promoter-sequences utilized to drive the expression of the *lacZ* gene (ΔRLZ79 : −250 to +625; RLZ79: −620 to +625; Reynolds et al., 1991). We have previously analyzed the expression pattern of the *lacZ* reporter gene during embryonic development in these lines (Reynolds et al., 1991): no expression was detected in day-7.5 embryos. The onset of expression was between day 8.5 (4 somites) and day 8.75 (10 somites). The expression pattern of both lines very closely resembled that of the endogenous gene (Reynolds et al., 1991). Expression in the central nervous system was restricted to the spinal cord, with an anterior boundary at the level of rhombomere 7 (rh7, Figs 1D, 2G).

In addition to the comprehensive analysis of RA treatment on *RARβ2-lacZ* expression in transgenic lines TG3 and TG16, we have also analyzed two other RLZ79 and ΔRLZ79 lines at day 9.5 and 10.5. The expression pattern after RA treatment was identical at all times in all transgenic lines. Hence, the unique expression pattern described below was independent of the integration site of the transgene.

Effects of RA exposure at day 7.5

Gastrulating rodent embryos are particularly sensitive to low doses of RA. Administration of a single dose of all-

Table 1. *Schedule of RA treatment and isolation of embryos*

Group	RA treatment†	Isolation of embryos†	No. experiments	No. embryos total / *lacZ*+	*lacZ* induction pattern
A	7.25	7.75	2	24 / 9	mesoderm, primitive streak
B	7.25	8.25	1	5 / 3	unsegmented hindbrain*
C	7.75	8.25-8.75	5	52 / 30	unsegmented hindbrain*
D	7.75	9.5	2	20 / 18	none*
E	7.75	11.5	2	23 / 17	none*
F	8.25-8.5	8.75	4	37 / 19	segmented brain neural crest
G	8.75	9.25-9.5	3	31 / 15	segmented brain
H	8.75	10.5-13.5	9	97 / 71	none
I	9.25	9.5-9.75	2	18 / 10	segmented brain
K	9.75	10.25	3	35 /16	segmented brain
L	10.75	11.25	3	31 / 19	segmented brain
M	11.75	12.25	2	19 / 9	segmented brain
N	7.75 + 8.25	8.5-8.75	4	36 /17	unsegmented brain*
O	7.75 + 9.25	9.5	2	20 / 13	different categories*,‡

Embryos were staged as described in experimental methods. Embryos of group E, F, and P were developmentally retarded as a result of RA treatment. †Gestation days. *Embryos with obvious CNS defects. ‡See text for details.

trans-RA (10 mg/kg) by gavage at day 6.75 produced severe teratogenic effects in 50% of all litters, while RA treatment at day 7.25 and day 7.75 resulted in a delay in neural tube closure and microcephaly in 100% of all litters (group B-E in Table 1; Fig. 1F). Exposure to the same dose of RA at day 8.5 caused brain defects with only very low incidence (1 out of 9 litter; group H in Table 1). Hence, the period of strongest RA sensitivity starts at day 7 and ends at day 8.

To determine if *RARβ2-lacZ* expression could be prematurely induced by RA in gastrulating embryos, day-7.75 embryos were stained for β-gal activity 6 hours after RA treatment (group A in Table 1). We found strong *lacZ* expression in a characteristic, wing-shaped pattern with the origin in the primitive streak and the tips at the head process (Fig. 1A). This pattern is indicative of migrating cranial mesoderm cells (Lawson et al., 1991). No staining was detected in the neuroectoderm (Figs 1A, 3A). Hence, the *RARβ2* promoter present in our constructs is selectively activated in the cranial mesoderm. This suggests that mesoderm transformation may play a role in the teratogenic effects of RA treatment at this stage.

When embryos were isolated 12 to 16 hours after RA administration (at day 7.75), we found that *RARβ2-lacZ* was induced in the midbrain and hindbrain. Expression levels were high in the caudal hindbrain and decreased progressively towards the midbrain (Fig. 1B,C). Expression in the trunk region was similar to untreated embryos (Fig. 1D), but the expression level in somites was slightly elevated. *RARβ2-lacZ* induction was still detectable after 24 hours (day 8.5; group C in Table 1; Fig. 1E), but embryos isolated at day 9.5 (group D in Table 1; Fig. 1F) or 11.5 (group E in Table 1) had the same anterior and posterior expression boundaries as untreated embryos (compare Figs 1F, 2G).

RA exposure at later stages induces segmented RARβ2-lacZ *expression*

To test if *RARβ2-lacZ* could be induced after the RA sensitive period, we treated them with the same dose between day 8 and day 12 (Table 1). When embryos were stained for β-gal activity 6-16 hours later, we found strong *lacZ* expression in a segment-like pattern in the CNS (Fig. 2).

Segmentation of the hindbrain involves successive subdivisions of three pro-rhombomeres (RhA, RhB and RhC) into 8 final rhombomeres (rh1 to rh8; for review see Vaage, 1969; Lumsden, 1990). The central rhombomeres 4 and 5 are the first to establish clear boundaries. We found strong *lacZ* expression in day-8.75 embryos that had not yet turned (6-8 somites) 6 hours after RA exposure (Group F in Table 1) in rh4 and caudal to rh5. Rh5 and rh3 became resistant to *RARβ2-lacZ* induction. A dorsoventral band of expressing cells spanned from rh4 to the second brachial arch (Fig. 2B). The analysis of serial horizontal sections revealed that these cells are migrating neural crest cells (Fig. 3B). We also found *RARβ2-lacZ* induction in one domain rostral to rh3. This domain corresponds to pro-rhombomere A, which subsequently divides into rh1 and rh2. *RARβ2-lacZ* induction was again transient as embryos isolated between day 10.5 and 13.5 (Group H in Table 1) had the same expression pattern as untreated embryos.

Rhombomeres are fully developed at day 9.5 (Tuckett et al., 1985) and *RARβ2-lacZ* was markedly induced in rh1, rh4, and caudal of rh5 6 hours after treatment. Expression was also weakly induced in rh2, rh3 and rh5 (Fig. 2E, Group I in Table 1). These differential expression levels were most distinct in day-9.5 embryos that were treated at day 8.75 (group G in Table 1; Fig. 2D). No expression was detected in the midline region and at inter-rhombomeric junctions.

RARβ2-lacZ induction was also detected in the mesencephalon and prosencephalon at day 8.75 (Fig. 2A,C). The expression domains were initially rather diffuse, but subsequently formed clearly demarcated bands dividing the anteroposterior (A-P) axis of the brain into alternating expressing and non-expressing regions (Figs 2D,F,H,I, 4). We classified these regions consecutively, into two telencephalic (t1 and t2), three diencephalic (d1, d2, and d3) and two mesencephalic (m1 and m2) domains (Figs 2F, 4B, 6).

The anterior limit of the expressing mesencephalic domain m1 was at the mes-diencephalic boundary (Fig. 2D,F,H,I). This was particularly evident in parasagittal sections through the ventral mesencephalon, where β-gal staining was just rostral to the tuberculum posterius (Fig. 4A,B). In the diencephalon, we found *RARβ2-lacZ* induction in the lateral hypothalamus rostral to the optic stalk (d2; Figs 2F,H, 4A,B). The most anterior region of the diencephalon (d1) did not express the transgene. In the telencephalon, we found an expression domain (t2) in the caudal region of the lateral vesicles (Figs 2C,D,F,H, 4A). The posterior boundary of this expression domain delineated the di-telencephalic boundary.

High levels of induction were still detected in the midbrain and caudal hindbrain of day-11.25 embryos (group L in Table 1), while induction in the forebrain was weak (data not shown). In day-12.25 embryos (group M in Table 1), *RARβ2-lacZ* expression levels were still high in the caudal hindbrain, and low in the midbrain. No induction was seen in the forebrain. *lacZ* expression in the spinal cord of these embryos was uninterrupted, while untreated embryos had two expression domains at this stage (data not shown).

RA exposure at day 7.5 disrupts the segment-like induction pattern

The hindbrain neuroepithelium of embryos treated with RA at day 7.75 appeared very smooth (Fig. 1E), in contrast to the rhombomeric bulges seen in untreated embryos (Fig. 2A,B). This finding supports the observation that RA exposure during gastrulation can lead to morphological alterations of the hindbrain (Holder and Hill, 1991; Morriss-Kay et al., 1991; Papalopulu et al., 1991).

To analyze the effects of RA on the organization of the neuromeres more closely, we re-examined the *RARβ2-lacZ* induction pattern in embryos that were treated twice (group N and O in Table 1). The first exposure at day 7.75, causes teratogenic effects and induces *RARβ2-lacZ* transiently. This transient induction does not last much longer than 24 hours (see Fig. 1). The *RARβ2-lacZ* expression pattern induced by a second RA treatment at day 8.25 or day 9.25 should therefore reveal alterations in the CNS segmentation caused by the first treatment. Embryos were isolated and stained for β-gal activity 4 hours after the second treatment. The results are shown in Fig. 5.

All embryos analyzed at day 8.75 expressed the trans-

gene at very high levels in the heart and in the entire CNS except in the rostral tip of the headfolds after the second RA treatment (Fig. 5A,B; Group N in Table 1). We did not find any segmented expression pattern. These results indicate that RA treatment during gastrulation induces molecular transformations so that most tissues become susceptible to *RAR*β*2-lacZ* induction.

The expression pattern after a second treatment at day 9.25 (Group O in Table 1) was more complex. Embryos of this group fall into three categories. The first group is shown in Fig. 5C. In those embryos, expression was strong in the entire hindbrain region and exhibited no signs of hindbrain segmentation. Expression was also found in small patches in midbrain and at a low level in one domain in the forebrain. There was no expression in the anterior CNS of embryos of the second category (Fig. 5D). However, *RAR*β*2-lacZ* expression was induced in the caudal hindbrain (compare Fig. 5D with 1F). Embryos of the third category (Fig. 5E) showed expression in the entire hindbrain except for rh5. There was also expression in small domains in the prosencephalon and mesencephalon. Common among these three different categories was *RAR*β*2-lacZ* induction caudal to the otic vesicle, and a reduction in size of the midbrain and forebrain domains that are susceptible to *RAR*β*2-lacZ* induction. It is important to note that these different categories were equally represented in both litters that were analyzed. We can, therefore, exclude the possibility of simple experimental variations. These results show that the disruption of the morphological segmentation of the hindbrain is reflected by alterations in the *RAR*β*2-lacZ* expression domains. They also suggest that RA disturbs the segmented organization of anterior brain regions.

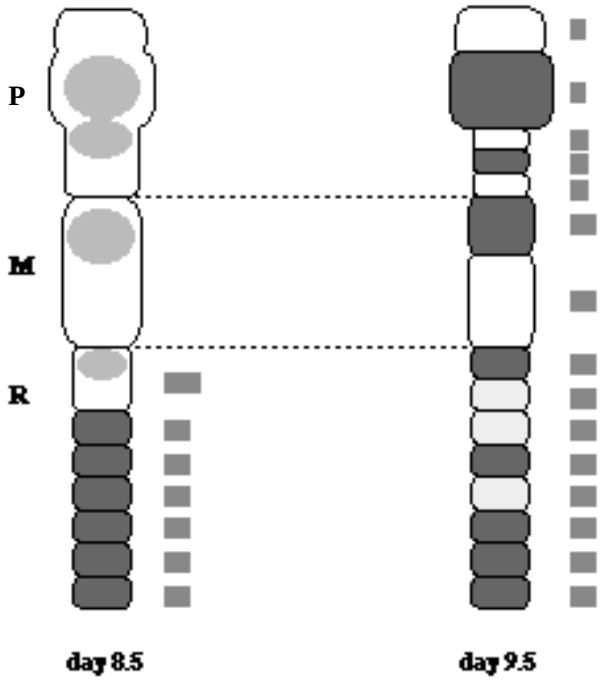

Fig. 6. Summary of the *RAR*β*2-lacZ* induction pattern in the brain at day 8.5 and 9.5. Rhombomeres 3-8 are readily detectable in day-8.5 embryos. However, the most anterior rhombomere RhA has yet to divide into rh1 and rh2. The formation of neuromeres in the mesencephalon (M) is just starting at this developmental stage (Tuckett et al., 1985). *RAR*β*2-lacZ* induction in the prosencephalon (P), mesencephalon, and in RhA was found at lower levels and without clearly demarcated boundaries (indicated by grey areas). Segment boundaries are fully established at day 9.5. Indicated are two telencephalic (t1, t2), three diencephalic (d1 to d3), two mesencephalic (m1, m2), and eight rhombencephalic (rh1 to rh8) domains. Relative *lacZ* expression levels in different brain regions are indicated by differential shading.

Discussion

Morphological and evolutionary considerations argue against a common segmented ancestor of insects and vertebrates. Yet, studies in *Drosophila* and mice established remarkable analogies in the molecular mechanisms of pattern formation in these diverse species. It appears that both organisms have utilized similar segmentation strategies and recruited similar groups of genes to establish the primary body axis (Wilkinson and Krumlauf, 1990; Kessel and Gruss, 1990). The analysis of homeobox containing genes in particular led to the conclusion that these genes have (i) a similar genomic organization in gene clusters; (ii) similar expression patterns in segmented structures along the AP axis and in respect to their genomic position; and (iii) similar functions, i.e. the specification of positional information.

We have analyzed the activity of the RA inducible *RAR*β*2* promoter in transgenic *RAR*β*2-lacZ* mouse embryos after maternal RA administration. Strikingly, *RAR*β*2-lacZ* was strongly induced in a segment-like pattern in alternating rhombomeres and in the anterior CNS. This pattern is very reminiscent of the expression pattern of *Drosophila* 'pair-rule' genes and suggests that segmentation is a feature of the whole brain.

The segmented organization of the neural tube is easily discernible by morphological means only in the hindbrain region during a brief developmental period. Segmentation of the hindbrain is, however, supported by cell tracing experiments which demonstrated that rhombomeres are units of cell lineage restriction (Fraser et al., 1990). Moreover, the expression patterns of *Krox-20* and many *Hox* genes correlates well with segment boundaries (Wilkinson et al., 1989; Murphy et al., 1989; Sundin and Eichele, 1990).

The *RAR*β*2-lacZ* induction domains at day 8.5 and day 9.5 are summarized in Fig. 6. At day 9.5, *RAR*β*2-lacZ* induction was strong in rh4 and rh6 and low in rh3 and rh5. We also found an alternating pattern in the mesencephalon and prosencephalon. The 'pair-rule' type pattern was disturbed only in the anterior rhombomeres. Here, *RAR*β*2-lacZ* was strongly induced in rh1, and very weakly in rh2 and rh3. However, day-8.5 embryos (prior to the differentiation of rhombomeres 1 to 3) showed expression in only one domain in the rostral hindbrain, in RhA, and thus showed a perfectly alternating expression pattern (Fig. 2C). Rh1 and rh2 are the latest to differentiate (Lumsden, 1990). Hence, it is possible that the subdivision of rh1 and rh2 is a secondary event to an underlying perfectly alternating two-segment-repeated molecular patterning in CNS development.

Evidence for functional diversity of alternating rhombomeres came from the analysis of neurogenesis in the chick hindbrain (Lumsden and Keynes, 1989). Motor neurons develop first in rhombomeres 2, 4, and 6 and subsequently in rhombomeres 3 and 5. Furthermore, motor neuron pathways are different in even-numbered and odd-numbered rhombomeres.

Several morphological studies suggested that the neural tube rostral to the hindbrain has a neuromeric organization (Baer, 1828; Bartelmez, 1923; Adelmann, 1925; Vaage, 1969; Tuckett et al., 1985). But, the number of potential neuromeres and their developmental appearence remained controversial due to the lack of supportive experimental cellular and molecular evidence (reviewed by Vaage, 1969). A relatively good consensus concerning the number of neuromeres in anterior brain regions exists only for the mesencephalon, where most authors found two neuromeres or mesomeres (Bartelmez, 1923; Adelmann, 1925; Bartelmez and Evans, 1926; Bergquist, 1952; Tuckett et al., 1985; reviewed by Bergquist and Källén, 1954; Vaage, 1969). The neuromeric organization of the mesencephalon becomes evident at the beginning of day 8.5 with the subdivision of an initially single large mesomeric sulcus (Tuckett et al., 1985). The rostral neuromere subsequently enlarges and forms the optic tectum later. *RAR*β*2-lacZ* induction in the mesencephalon was first detected in the rostral region (m1) of day-8.5 embryos. Thus, *RAR*β*2-lacZ* induction correlates well with the morphological onset of mesencephalon segmentation.

The *RAR*β*2-lacZ* expression pattern suggests the presence of two possible segments in the telencephalon and three in the diencephalon. A subdivision of the diencephalon into three segments has also been suggested on the basis of the pattern of neurogenesis and cell migration (Bergquist and Källén, 1954; Puelles et al., 1987). Close examination of cell tracing experiments in the diencephalon indicates, however, the presence of four segments (Vigdor and Stern, personal communication). It is possible that one of the diencephalic segments that we suggest here, subsequently divides to yield a total of four final segments. This situation would thus be similar to that seen in the rostral hindbrain. Interestingly, thymidine radiographic studies indicated that the fate of hypothalamic and thalamic neurons may be predetermined by the mosaic organization of the diencephalon (Altman and Bayer, 1979). Support for segmentation of the telencephalon was also recently provided by observations of the expression of murine homologs to the *Drosophila* homeobox gene *distalless* in distinct areas of the forebrain (Price et al., 1991; Porteus et al., 1991).

The segmented *RAR*β*2-lacZ* expression pattern was disrupted by RA administration at day 7.5 prior to the onset of neuromere morphogenesis. RA treatment at this stage was also highly teratogenic. These results suggest that endogenous RA plays an important role in the specification of the CNS axis during early stages of neurogenesis. It has previously been shown that exposure of gastrulating mammalian embryos to exogenous RA can result in homeotic transformations of the axial skeleton (Kessel and Gruss, 1991) and in disruption of the segmented hindbrain organization (Morriss-Kay et al., 1991). Our results indicate that RA has a similar effect on the midbrain and forebrain.

What could be the molecular basis for the segmented *RAR*β*2-lacZ* induction by RA in the CNS? When the RAREβ is placed in the context of heterologous promoters, RA-dependent transcriptional activation can be detected in virtually all cells in tissue culture (Sucov et al., 1990) as well as in mouse embryos (Rossant et al., 1991). However, the *RAR*β*2* promoter is itself apparently very tightly regulated. Rossant et al. (1990) used a *RARE*β*-hsp-lacZ* gene to assess RA sensitive tissues during murine development. They found strong expression of the *RARE-hsp-lacZ* expression throughout the CNS after exposure of embryos to RA. It is therefore likely that the segmented *RAR*β*2-lacZ* expression pattern results from the combination of elevated free RA levels and the tissue specific distribution of transacting factors. The absolute concentration of RA may be of little influence on the *RAR*β*2-lacZ* expression as we found the identical pattern when dams were treated on day 9.5 with a 10-fold higher dose of RA (100 mg/kg; A. Z. and A. Z., unpublished observations). This dose was sufficient to induce strong teratogenic effects.

It has recently been shown that RARβ and the TATA box binding factor TFIID act cooperatively to transactivate the *RAR*β*2* promoter. Interestingly, this cooperativity was dependent on the promoter context and was not observed, when the RAREβ was inserted into the TK or SV40 promoters (Berkenstam et al., 1992). Furthermore, the cooperativity was dependent on an E1A-like activity found in embryonic carcinoma (EC) cells (Imperiale et al., 1984). It is conceivable that *RAR*β*2-lacZ* activation is governed by the distribution of such an E1A-like factor in the developing CNS. It will be most challenging to isolate the factors that direct the segmented *RAR*β*2-lacZ* expression, as they may have functional similarity to 'pair-rule' genes and play a central role in the organization of the AP axis of the neural tube.

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Fig. 1. Whole-mount β-gal staining of embryos after maternal RA administration at day 7.25. Embryos were isolated and stained for β-gal activity 6 hours (A), 12 hours (B), 16 hours (C), 24 hours (E), and 48 hours (F) after treatment. Embryos were partially dissected from their membranes (B-F). Day-7.75 embryos (A) revealed the rapid *RAR*β*2-lacZ* induction in a wing-shaped pattern. This pattern suggests that *lacZ* is expressed in mesoderm cells which ingress through the primitive streak (ps) and migrate laterally into the head process (hp). a, anterior; p, posterior. Activation of transgene is transient. No expression in mesoderm derivatives (e.g. the heart) is detected at day 8.25 (B, C) or day 8.5 (E). *RAR*β*2-lacZ* induction is, however, found in the caudal hindbrain region as compared to untreated controls (D). Embryos isolated at day 9.5 (F) exhibit the same expression boundaries as untreated embryos of the same stage (Fig. 2G). Note the delay in neural tube closure after RA exposure (arrows). The asterisk indicates the otic vesicle. All bars, 250 μm.

Fig. 2. Whole-mount staining of embryos treated with RA after the onset of CNS segmentation at day 8.25 (A-C), day 8.75 (D), day 9.25 (E, F) or day 9.75 (H, I). Embryos were isolated 6 hours (A, B, C, E, F) or 16 hours (D, H, I) after treatment. (G) *RAR*β*2-lacZ* expression in untreated embryos. The asterisk indicates the otic vesicle at the level of rh5. *RAR*β*2-lacZ* induction in a segmented pattern was first detected in day-8.75 embryos (A, B). At this stage, induction is strong in rh4 and rh6. Individual rhombomeres are identified by numbers. Weak transgene expression was found in pro-rhombomere A (RhA, open arrow), in the midbrain and the forebrain. Neural crest cells derived from rh4 (arrows) also express the transgene. Expression in the mid- and forebrain is markedly enhanced in embryos that have already turned (C). These rather diffuse *RAR*β*2-lacZ* induction domains become subsequently very distinct at day 9.5 (D-F) and delineate alternating expressing and nonexpressing segments (classified as telencephalic domains t1 and t2; diencephalic domains d1, d2, d3; and mesencephalic domains m1 and m2). Note that expression levels are higher in embryos isolated 6 hours after RA treatment (E, F). The *RAR*β*2-lacZ* induction pattern remains basically unchanged in day-10.5 embryos (H, I). rh, rhombencephalon; m, mesencephalon; t, telencephalon. Arrowheads indicate corresponding expression domains in day-9.25 (D) and day-10.25 embryos (H). All bars, 250 μm.

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Legends for Figs 1–5 for colour

Fig. 3. (A) Sagittal section through a day-7.75 embryo as shown in Fig. 1A. Embryos were isolated 6 hours after RA treatment, stained for β-gal expression, sectioned, and counterstained with neutral red. The primitive streak (PS) shows high *lacZ* expression levels, but not the neural fold (NF) region. (B) Transverse section through a day-8.75 embryo 6 hours after RA treatment demonstrates *RAR*β*2-lacZ* induction in migrating neural crest (NC) cells originating from rh4. Picture was taken with Nomarski optics. NG, neural groove; F, foregut. All bars, 100 μm.

Fig. 4. (A) Parasagittal sections through a day-10.25 embryo demonstrates the segmented *RAR*β*2-lacZ* induction in the CNS. Embryos were treated with RA at day 9.75 and isolated approximately 12 hours later. T, lateral ventricle (telencoel); III, third ventricle; IV, fourth ventricle. (B) High magnification of the area indicated in A shows the alternating *RAR*β*2-lacZ* expression domains in the ventrolateral region of the diencephalon (d2), the mesencephalon (m1) and the rostral hindbrain (rh1). Arrows indicate expression boundaries. All bars, 100 μm.

Fig. 5. Whole-mount β-gal staining of embryos that were exposed to RA at day 7.75 and again at day 8.25 (A, B) or day 9.25 (C-E). All embryos were isolated 4 hours after the second treatment. RA exposure during gastrulation made embryos initially highly susceptible to *RAR*β*2-lacZ* induction and the second RA treatment at day 8.25 induced the transgene in virtually all tissues, except the most anterior region of the neuroectoderm (A, B). Subsequently, anterior regions of the embryo became resistant to *RAR*β*2-lacZ* induction and we observed three different lacZ expression patterns after a second treatment at day 9.25. In the first group (C), *RAR*β*2-lacZ* was induced uniformly in the hindbrain, in small cell patches in the midbrain and in one domain in the forebrain. The otic vesicle is indicated by an arrow. All tissues rostral to rh6 were resistant to *RAR*β*2-lacZ* induction in embryos of the second group (D). Embryos of the third group (E) showed expression of the transgene in a large domain rostral to the otic vesicle (arrow) and in very small domains in the midbrain and forebrain. All bars, 250 μm.