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Metabolic inactivation of retinoic acid by a novel P450 differentially expressed in developing mouse embryos

Hideta Fujii[1,2], Takashi Sato[3], Sinya Kaneko[1], Osamu Gotoh[4], Yoshiaki Fujii-Kuriyama[5], Kanju Osawa[6], Shigeaki Kato[3,7] and Hiroshi Hamada[1,2,8]

[1]Tokyo Metropolitan Institute of Medical Science, 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113, [2]Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamadaoka, Suita 565, [3]Department of Agricultural Chemistry and [6]Nodai Research Institute, Tokyo University of Agriculture, Setagaya-ku, Tokyo 156, [4]Department of Biochemistry, Saitama Cancer Research Institute, Inamachi, Saitama 362, [5]Department of Chemistry, Faculty of Science, Tohoku University, Sendai 980-77 and [7]Institute of Molecular and Cellular Bioscience, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

[2]Present address: Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565, Japan

[8]Corresponding author
e-mail: hamada@imcb.osaka-u.ac.jp

Retinoic acid (RA) is a physiological agent that has a wide range of biological activity and appears to regulate developmental programs of vertebrates. However, little is known about the molecular basis of its metabolism. Here we have identified a novel cytochrome P450 ($P450_{RA}$) that specifically metabolizes RA. *In vitro*, $P450_{RA}$ converts all-*trans* RA into 5,8-epoxy all-*trans* RA. $P450_{RA}$ metabolizes other biologically active RAs such as 9-*cis* RA and 13-*cis* RA, but fails to metabolize their precursors, retinol and retinal. Overexpression of $P450_{RA}$ in cell culture renders the cells hyposensitive to all-*trans* RA. These functional tests *in vitro* and *in vivo* indicate that $P450_{RA}$ inactivates RA. The $P450_{RA}$ gene is not expressed uniformly but in a stage- and region-specific fashion during mouse development. The major expression domains in developing embryos include the posterior neural plate and neural crest cells for cranial ganglia. The expression of $P450_{RA}$, however, is not necessarily inducible by excess RA. These results suggest that $P450_{RA}$ regulates the intracellular level of RA and may be involved in setting up the uneven distribution of active RA in mammalian embryos.

Keywords: cytochrome P450/embryogenesis/metabolism/retinoic acid

Introduction

Retinoic acid (RA) is a vitamin A derivative that has a remarkably wide range of biological activity, such as in cell growth, differentiation and morphogenesis (for reviews, see Chambon, 1994; Mangelsdorf *et al.*, 1994). RA exists endogenously in vertebrate embryos and adults. Multiple forms of RA have been found *in vivo*, including all-*trans* RA (Thaller and Eichele, 1987; Durston *et al.*, 1989), 9-*cis* RA (Heyman *et al.*, 1992; Levin *et al.*, 1992; Kraft *et al.*, 1994) and 4-oxo all-*trans* RA (Pijnappel, *et al.*, 1993), all of which are known to be biologically potent. These active forms of RA exert their effects by serving as a ligand for nuclear receptors called RARs (Giguere *et al.*, 1987; Petkovich *et al.*, 1987) and RXRs (Mangelsdorf *et al.*, 1992). In the presence of RA, an RAR–RXR homodimer or heterodimer can regulate expression of a set of genes.

RA has been implicated in many aspects of embryogenesis. Severe vitamin A deficiency during pregnancy often results in congenital abnormalities (known as the fetal vitamin A-deficiency syndrome). Also, experimental administration of excess RA to vertebrate embryos leads to abnormal development of many organs. Furthermore, RA appears to be distributed unevenly in developing embryos. Several previous studies have shown that some cells (or some organs) contain a higher concentration of RA than others. For instance, a graded distribution of RA has been reported in developmentally important regions such as the ZPA area of the limb bud (Thaller and Eichele, 1987) and Hensen's node (Chen *et al.*, 1992). Recently, the role of RA has been examined more directly by manipulating the *RAR* and *RXR* genes. These studies have clearly demonstrated the importance of these receptors in development. As expected, mice deficient in *RAR* or *RXR* show a variety of developmental defects (Kastner *et al.*, 1994; Lohnes *et al.*, 1994), which overlap with the phenotype of the fetal vitamin A-deficiency syndrome.

In spite of extensive studies on RAR and RXR, surprisingly little is known about the molecular basis of retinoid metabolism. The major sources of retinoids in the diet are provitamin A carotenoids and retinyl esters. Retinyl esters are enzymatically converted to retinol in the intestinal lumen before absorption, and carotenoids are converted to retinol in enterocytes (Blomhoff *et al.*, 1990). RA may be synthesized by oxidizing retinol or directly from β-carotene, but the metabolic pathway of RA synthesis, isomerization and degradation has not been established yet. In order to understand the physiological roles of RA, it is crucial to know how RA is metabolized in the human body. Such knowledge is also clinically important because RA has been used in the treatment of certain human diseases such as acute promyelocytic leukemia. Unfortunately, however, only a few of the enzymes involved in retinoid metabolism have been molecularly identified.

In this study, we have identified an RA-catabolizing enzyme, a novel cytochrome P450 which we refer to as $P450_{RA}$. Functional analysis *in vivo* and *in vitro* clearly demonstrated that $P450_{RA}$ encodes an enzyme that specifically metabolizes RA to an inactive form. Interestingly, $P450_{RA}$ is not expressed uniformly in embryos or adult organs. Rather, $P450_{RA}$ expression is found in restricted regions of developing embryos which are known to be sensitive to excess RA. We propose that $P450_{RA}$

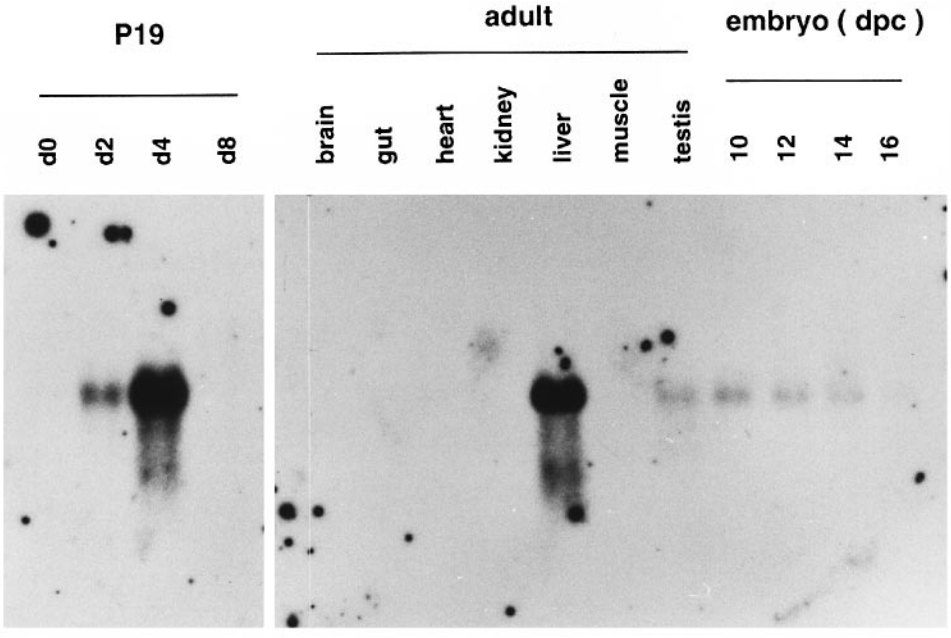

Fig. 1. Expression of $P450_{RA}$ in P19 cells, adult mouse organs and developing embryos. Poly(A)$^+$ RNA (5–10 μg) from the indicated cells, adult organs or developing embryos was subjected to Northern blot analysis. The *Eco*RI–*Xho*I insert of a partial cDNA clone 10245 was used as a probe. d0, undifferentiated P19 cells; d2, d4 and d8, P19 cells 2, 4 and 8 days after RA induction, respectively. The integrity of each poly(A)$^+$ RNA had been confirmed with a β-actin probe (data not shown).

may be a determinant that sets up the uneven distribution of RA during embryogenesis. The presence of such an RA-inactivating enzyme further strengthens, from a new point of view, the physiological importance of RA in establishing body plan as well as in homeostasis.

Results

$P450_{RA}$ is a new member of the P450 superfamily

P19 is a pluripotent embryonic carcinoma (EC) cell line that can differentiate to neural cell types when induced with all-*trans* RA (Rudnicki and McBurney, 1987). By applying a sensitive subtraction procedure (Saijoh *et al.*, 1996) to the P19 cell system, we have isolated a number of genes whose expression is induced 2 days after RA induction (our unpublished results). One such clone (clone No. 10245; referred to as $P450_{RA}$ below) was used in this study. Northern blot analysis confirmed that $P450_{RA}$ is not expressed in undifferentiated P19 cells, but is highly expressed 2 days after RA treatment (Figure 1). The expression is transient: it reaches its maximum level 4 days after the RA induction, but disappears on the eighth day.

$P450_{RA}$ mRNA encodes an open reading frame of 497 amino acid residues (Figure 2). The encoded amino acid sequence showed significant homology to a number of P450 members, with the highest (25% identical overall) homology to the CYP4 family. The carboxy-terminal region was more conserved than the amino-terminal region and includes a highly conserved cysteine residue (at amino acid residue 442), which is a heme-binding site. The amino-terminal region rich in hydrophobic residues is probably a transmembrane domain, a domain commonly found in microsome-associated P450s. In all, $P450_{RA}$ is probably a diverged member of the P450 superfamily.

$P450_{RA}$ metabolizes RA in vitro

For several reasons, we speculated that $P450_{RA}$ might encode an enzyme which metabolized RA. First, a number of P450 genes are known to be inducible by their substrate (Nebert and Gonzalez, 1987; Gotoh and Fujii-Kuriyama, 1988). In P19 cells, $P450_{RA}$ appeared to be inducible by RA (as described below, however, the $P450_{RA}$ gene is not necessarily inducible by RA). Secondly, $P450_{RA}$ was closest to the CYP4 family, some members of which are known to metabolize linear molecules such as lipoic acid, prostaglandin and leukotriene (Kikuta *et al.*, 1993; Roman *et al.*, 1993). All-*trans* RA also contains a linear structure. Finally, the involvement of cytochrome P450 in RA metabolism has been suggested by previous pharmacological studies (Martini and Murray, 1993).

We first tested whether $P450_{RA}$ could metabolize all-*trans* RA *in vitro*. 293T cells were transfected with a $P450_{RA}$ expression vector. Since $P450_{RA}$ possesses a transmembrane domain typical of microsome-associated P450s, microsome fractions were prepared from transfected and untransfected cells. Microsome fractions were incubated with various retinoids and the reaction products were analyzed by HPLC. The microsomes from untransfected 293T cells did not metabolize all-*trans* RA (data not shown). In contrast, the microsomes from the transfected 293T cells could convert all-*trans* RA into a new form (peak X, Figure 3A). The substrate specificity of $P450_{RA}$ was examined and it was found that $P450_{RA}$ could also metabolize 9-*cis* RA (Figure 3B). This enzyme also metabolized other retinoic acids such as 13-*cis* and 9,13-di-*cis* RAs (data not shown). However, it failed to metabolize precursors of these RAs such as retinal, retinol and β-carotene (Figure 3C and D and data not shown), which are not (or much less) potent with respect to biological activity when compared with all-*trans* and 9-*cis*

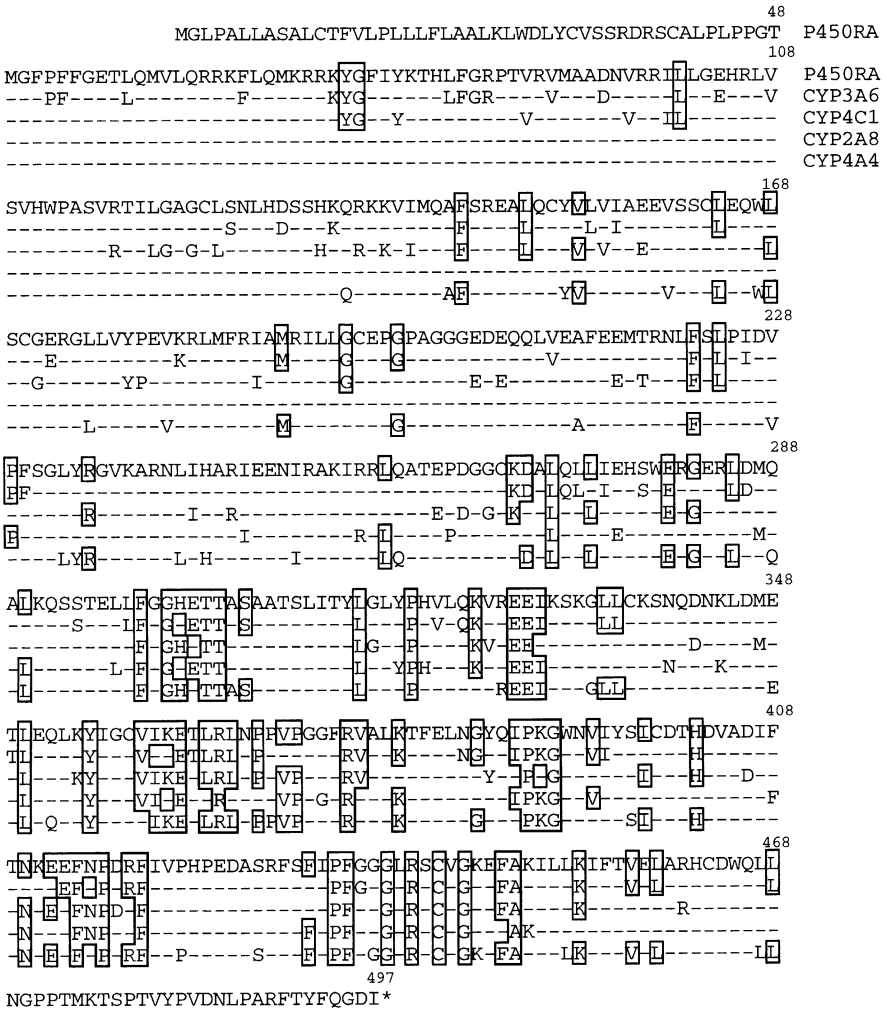

Fig. 2. P450$_{RA}$ is a new member of the P450 superfamily. The amino acid sequence encoded by *P450$_{RA}$* mRNA is compared with four other P450s. Identical amino acid residues are indicated by upper case letters. Amino acid residues conserved among more than three of the P450 members are boxed.

RAs. Next, we wanted to identify the enzymatic metabolite of all-*trans* RA. As only a small amount of the enzymatic metabolite (peak X in Figure 3A) was available, we chemically oxidized all-*trans* RA and searched for an oxidized compound that would show properties identical to those of peak X. All-*trans* RA was oxidized with *m*-chloroperoxybenzoic acid and the oxidized compounds were separated by reverse phase HPLC (Figure 4A). One of the oxidized forms, peak 2, showed the same retention time as that of the enzymatic metabolite (peak X) on HPLC (Figure 4B). It should be noted that peak 2 was clearly different from 4-oxo all-*trans* RA (Figure 4B). Peak 2 was then analyzed by means of gas chromatography/mass spectroscopy (GC/MS) and was identified as 5,8-epoxy all-*trans* RA (peak 1 was identified as 5,6-epoxy all-*trans* RA). Finally, peak 2 (5,8-epoxy all-*trans* RA) co-migrated with the enzymatic metabolite (peak X) on reverse phase HPLC (Figure 4D) and normal phase HPLC (Figure 4E). These results strongly suggest that peak X, an enzymatic metabolite of all-*trans* RA, is 5,8-epoxy all-*trans* RA.

P450$_{RA}$ inactivates RA in vivo

We next examined whether P450$_{RA}$ could inactivate the biological activity of RA *in vivo*. First, we tested whether constitutive expression of *P450$_{RA}$* would have any effect on RA-induced differentiation of P19 cells. Undifferentiated P19 cells were transformed with a *P450$_{RA}$* expression vector or a control vector resulting in P19R or P19C cells, respectively. Northern blot analysis confirmed a high level of *P450$_{RA}$* expression in P19R cells but not in P19C cells (Figure 5A). Both types of cells were induced to differentiate with various concentrations of all-*trans* RA, and cell differentiation was monitored by morphological observations (Figure 5B) and by measuring the level of Oct-3 (Figure 5C), a transcription factor specific to undifferentiated cells (Okamoto *et al.*, 1990; Okazawa *et al.*, 1991). As previously reported for P19 cells (Rudnicki and McBurney, 1987), P19C cells could respond to 10^{-9}–10^{-6} M all-*trans* RA. In contrast, P19R cells were hyposensitive to all-*trans* RA. They failed to respond to 10^{-9} and 10^{-8} M all-*trans* RA and remained undifferentiated. With 10^{-7} M all-*trans* RA, the majority of the cells differentiated into neural cells, but many colonies of undifferentiated cells still remained (Figure 5B). A concentration of 10^{-6} M all-*trans* RA could induce all the cells to differentiate.

Retinoic acid receptors can act as transcriptional activators in the presence of their ligands. In a second approach,

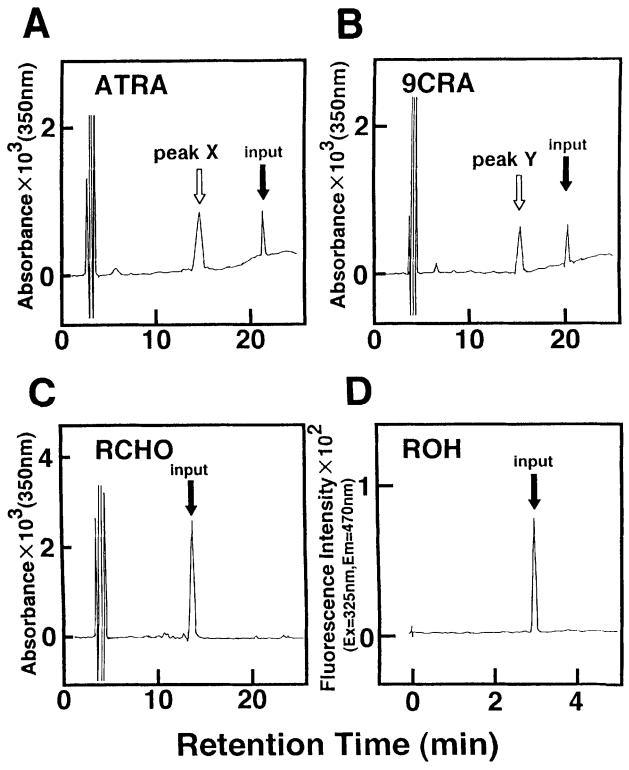

Fig. 3. *P450$_{RA}$* metabolizes RA *in vitro*. The microsome fraction from pBOS*P450$_{RA}$*-transfected 293T cells was incubated with all-*trans* RA (**A**), 9-*cis* RA (**B**), retinal (**C**) or retinol (**D**) and metabolites were analyzed by reverse phase HPLC. Input retinoids are indicated by closed arrows whereas metabolites (peaks X and Y) are indicated by open arrows. The microsome fraction from untransfected 293T cells did not metabolize any of the retinoids used here (data not shown).

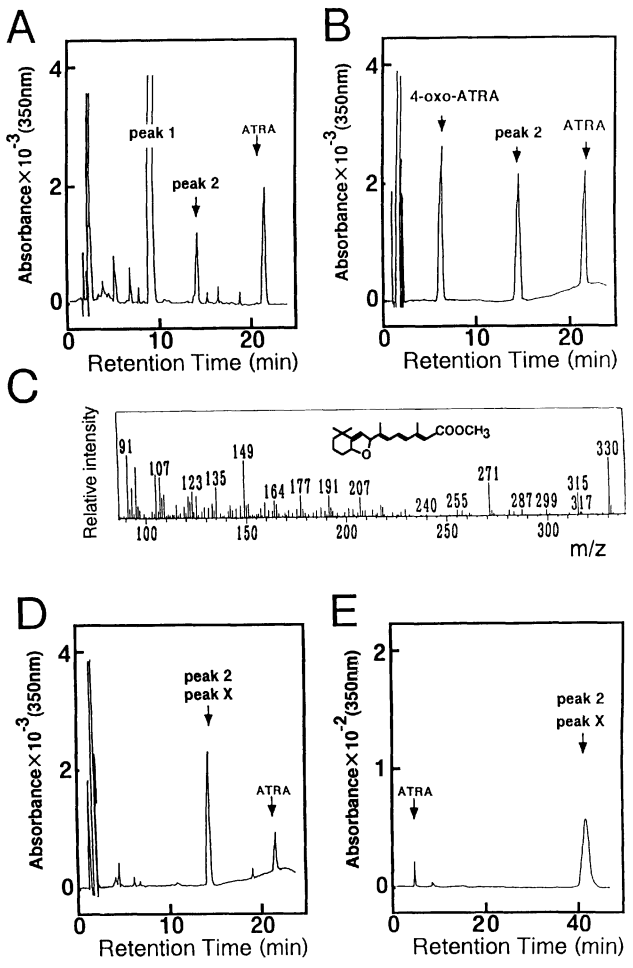

Fig. 4. Identification of the metabolite as 5,8-epoxy all-*trans* RA. (**A**) All-*trans* RA was oxidized with *m*-chloroperoxybenzoic acid and the metabolites were analyzed by reverse phase HPLC. (**B**) One of the oxidized metabolites, peak 2, was subjected to reverse phase HPLC with authentic all-*trans* RA and 4-oxo all-*trans* RA. Note that peak 2 showed an identical retention time to that of the enzymatic metabolite (peak X), but was clearly different from 4-oxo all-*trans* RA. (**C**) Peaks 1 and 2 were methylated with diazomethane and analyzed by GC/MS, identifying peak 2 as 5,8-epoxy all-*trans* RA and peak 1 as 5,6-epoxy all-*trans* RA (data not shown). (D and E) Peak 2 (5,8-epoxy all-*trans* RA) was mixed with the enzymatic metabolite and analyzed by reverse phase (**D**) and normal phase (**E**) HPLC. Note that peak 2 and peak X co-migrated in both cases.

we examined the influence of *P450$_{RA}$* on RA-dependent transactivation by RAR. HeLa cells were transformed with a *P450$_{RA}$* expression vector, and a pool of stable transformants were obtained (referred to as HeLa-R). Northern blot analysis confirmed the expression of *P450$_{RA}$* in HeLa-R cells but not in HeLa cells (Figure 5A). HeLa and HeLa-R cells were co-transfected with an RARβ expression vector and a CAT reporter containing RAR-binding sites in the presence of 10^{-11}–10^{-6} M all-*trans* RA. In HeLa cells, RARβ could activate the reporter gene with 10^{-9} M RA (Figure 6), which was consistent with the findings of a previous report (Brand *et al.*, 1988). In HeLa-R cells, on the other hand, RARβ failed to activate the *cat* gene with 10^{-9} M RA. In HeLa-R cells, an ~100 times higher concentration of all-*trans* RA was required to obtain a comparable level of activation.

The results from the two approaches described above indicate that P450$_{RA}$ metabolizes all-*trans* RA into a biologically inactive form.

P450$_{RA}$ is expressed differentially in developing embryos

The expression profile of *P450$_{RA}$* during mouse development was examined by whole mount *in situ* hybridization. *P450$_{RA}$* seems to be expressed in a stage-/region-specific fashion. At 6.0 days post-coitum (d.p.c.), *P450$_{RA}$* is expressed in the extra-embryonic and embryonic endoderm but not in the ectoderm (data not shown). At 7.0 d.p.c. (Figure 7A and E), the extra-embryonic and embryonic endoderm continue to express *P450$_{RA}$*. In addition, the embryonic (but not the extra-embryonic) mesoderm is beginning to express the gene. The extra-embryonic ectoderm is positive for expression while the embryonic ectoderm is negative. A similar expression pattern can be seen at 7.25 d.p.c., but expression in mesoderm and the primitive streak (excluding the node) becomes more evident (Figure 7B and F). There is a postero-anterior gradient in the mesodermal expression (Figure 7F). The *P450$_{RA}$* expression pattern changes dramatically in a short period between 7.25 and 8.5 d.p.c. The expression seen in the posterior mesoderm and primitive streak (at 7.25 d.p.c.) has become weaker by 7.5 d.p.c. At 7.5 d.p.c., *P450$_{RA}$* expression is found in different domains: the anterior region of all three embryonic germ layers (Figure 7C and G). In the extra-embryonic region, only the endoderm is positive. This expression pattern disap-

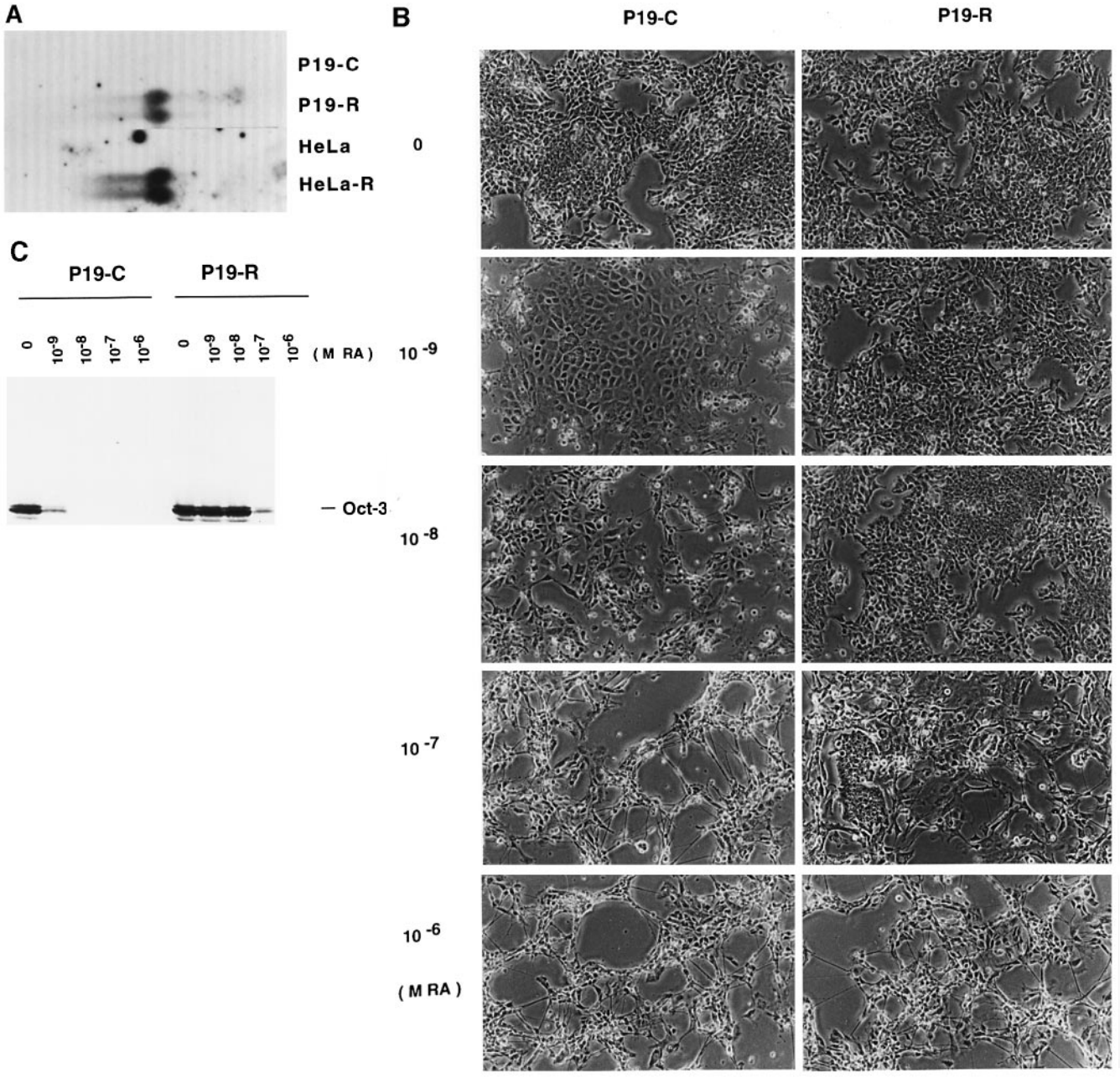

Fig. 5. *$P450_{RA}$* renders P19 cells hyposensitive to RA. (**A**) Northern blot analysis. Total RNA (20 μg) from P19C, P19R, HeLa or HeLa-R cells was run on a formamide gel, blotted and hybridized to a *$P450_{RA}$* probe. Note that *$P450_{RA}$* is not expressed in P19C or HeLa cells but is highly expressed in P19R and HeLa-R cells. (**B**) P19C and P19R cells were induced to differentiate with various concentrations of all-*trans* RA. Phase contrast photographs of the cells were taken 6 or 7 days after induction. (**C**) RA-induced cells were harvested at 6 or 7 days after induction. The level of the Oct-3 protein in each cell population was determined by Western blot analysis using an anti-Oct-3 antibody.

pears by 8.0 d.p.c. At 8.5 d.p.c. (7–8 somite stage), *$P450_{RA}$* is highly expressed in a posterior region, including the caudal neural plate, tailbud mesoderm and hindgut endoderm (Figure 7D and data not shown). It is also expressed in an anterior region which includes the prospective rhombomere 2, foregut epithelium and the first branchial arch epithelium (Figure 7D and H). *$P450_{RA}$*-expressing cells located in the ventrolateral region of the rhombomere can first be detected at the 5–6 somite stage (data not shown). The timing of their appearance and their location suggest that these cells are pre-migratory cranial crest cells. *$P450_{RA}$*-expressing cells are also found at 8.5 d.p.c. in mesenchyme between the first branchial arch and the hindbrain (Figure 7H), which are probably migrating neural crest cells. Between 9.5 and 10.5 d.p.c., *$P450_{RA}$* is expressed mainly in two distinct regions: a posterior region (Region 1), including the caudal neural plate, hindgut and tailbud mesoderm, and an anterior region (Region 2), including neural crest cells for cranial ganglia. At 9.5 d.p.c. (Figures 7I and 8A), the anterior margin of Region 1 has regressed toward the posterior end. Expression in Region 1 can be found in the caudal neural plate as well as in the hindgut and tailbud mesoderm (Figure 8G). Transverse sections of Region 2 indicate that it is clusters of neural crest cells for cranial ganglia V, VII/VIII and IX/X that express *$P450_{RA}$* (Figure 8C, E, I, K and M). It

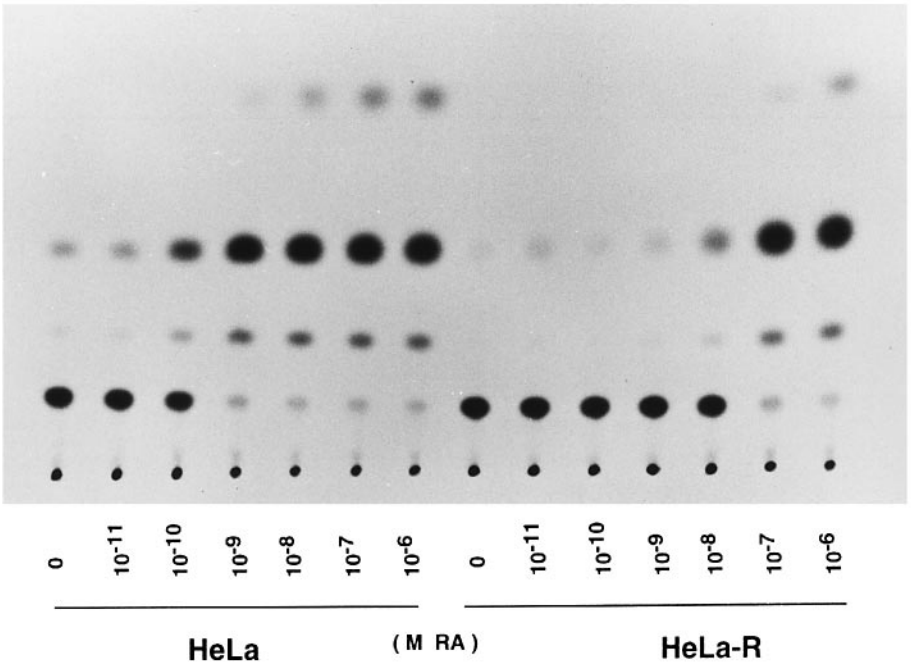

Fig. 6. *P450$_{RA}$* can inhibit RA-dependent transactivation by RAR. HeLa or HeLa-R cells were transfected with 5 μg of pRARE-Tkcat and 1 μg of pSGRARβ (Brand *et al.*, 1988) in the presence of the indicated concentrations of all-*trans* RA. RA-dependent, RARβ-mediated transactivation of the *cat* gene was examined by measuring the CAT activity.

appears that, among cranial crest cells, only those of the cranial ganglial lineage express *P450$_{RA}$*. At 10.5 d.p.c. (Figure 7J), expression in Region 1 is detected only at the tail end, while expression in Region 2 is still evident. Expression in Regions 1 and 2 almost disappears at 11.5 d.p.c. (Figure 7K). Between 9.5 and 11.5 d.p.c., a weaker level of expression is detected in other regions such as the forelimb bud, hindlimb bud, otic vesicle, eye and maxilo-mandibular cleft (Figure 7I–N). In the developing eye, *P450$_{RA}$* is expressed in the dorsoventral boundary region of the neural retina (Figure 7K and L). In the limb buds, a weak level of expression is evenly detected in the whole limb buds at 9.5 and 10.5 d.p.c. (Figure 7I and J). At 11.5 d.p.c., however, the expression is restricted to the interdigital space (Figure 7K, M and N). In addition, *P450$_{RA}$* expression is found in a base region of the forelimb bud as a sharp line (Figure 7K and M) but not in the corresponding region of the hindlimb bud (Figure 7N).

Expression at later developmental stages was examined by Northern blot analysis (Figure 1). A low level of expression was detected in 10, 12, 14 and 16 d.p.c. embryos. In adult mice, *P450$_{RA}$* was expressed in the liver and testis but not in any of the other organs examined.

The P450$_{RA}$ gene can be repressed or induced by all-trans RA in mouse embryos

In P19 cells, expression of *P450$_{RA}$* was induced when these cells differentiated into neural cells in response to RA and cell aggregation. However, it was not certain whether induction of *P450$_{RA}$* was a consequence of RA treatment or of the differentiation into a neural cell lineage. Therefore, we next examined whether *P450$_{RA}$* expression can be induced by RA in mouse embryo.

All-*trans* RA or vehicle alone was administered orally to pregnant mice at 8.5 and 9.0 d.p.c. *P450$_{RA}$* expression in the embryos was examined at 9.5 d.p.c. The results were rather unexpected. The expression in Region 1 (the caudal neural plate, tailbud mesoderm and hindgut) was abolished or greatly reduced in 100% (8/8) of the RA-treated embryos (Figure 8). Transverse sections confirmed the presence of the neural plate and hindgut in the RA-treated embryos (Figure 8H). On the other hand, the expression in Region 2 was up-regulated by exogenous RA; not only did the expression level in the cranial ganglia increase, but the expression domain was extended (Figure 8B, D and F). The expression domain in the otic vesicle was also extended by excess RA. In control 9.5 d.p.c. embryos, *P450$_{RA}$* was expressed in the ventrolateral region of the otic vesicle, which extended halfway from its posterior end (Figure 8I, K, M and O). In RA-treated embryos, the expression domain shifted to the ventral region (compare Figure 8M with N) and extended almost entirely from the posterior end to the anterior end (Figure 8J, L, N and P).

These results suggest that, unlike other P450s that metabolize xenobiotic agents, *P450$_{RA}$* is not necessarily inducible by its substrate. The gene is regulated differentially by all-*trans* RA in Regions 1 and 2.

Discussion

P450$_{RA}$ as an RA-inactivating enzyme

The functional tests *in vitro* (Figures 3 and 4) and *in vivo* (Figures 5 and 6) indicate that P450$_{RA}$ catabolizes RA and possesses substrate specificity. It can metabolize biologically active retinoids such as all-*trans* and 9-*cis* RAs, whereas it fails to recognize their precursors such as β-carotene, all-*trans* retinol and all-*trans* retinal (Figure 3), which are known to be much less active. It appears that several different stereoisomers of RA can be the substrate for this enzyme, and the presence of the carboxy-terminus

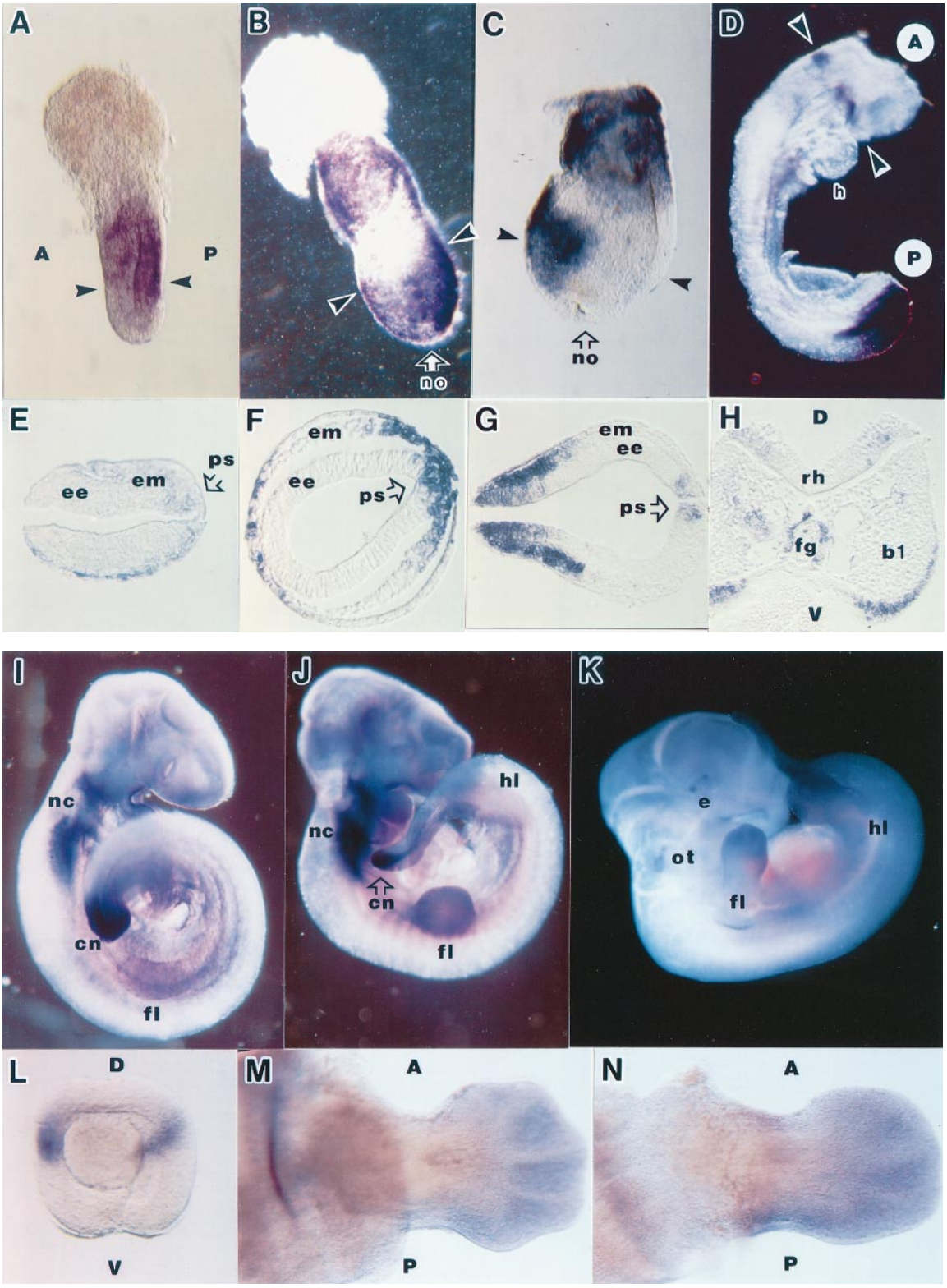

Fig. 7. Restricted expression of *P450$_{RA}$* in developing mouse embryos. Expression of *P450$_{RA}$* in mouse embryos was examined by whole mount *in situ* hybridization. (**A**) 7.0 d.p.c. (early primitive streak stage), (**B**) 7.25 d.p.c. (primitive streak stage), (**C**) 7.5 d.p.c. (advanced primitive streak stage), (**D**) 8.5 d.p.c. (**E–H**) Transverse sections of (A–D), respectively. The level of sectioning is indicated by a pair of closed arrowheads. (**I**) 9.5 d.p.c., (**J**) 10.5 d.p.c., (**K**) 11.5 d.p.c. (**L**) A magnified view of the eye at 11.5 d.p.c. (the eye was excised from the embryo and most of the surrounding tissue has been removed). (**M**) A magnified view of the right forelimb bud at 11.5 d.p.c. (**N**) A magnified view of the right hindlimb bud at 11.5 d.p.c. Abbreviations: A, anterior; P, posterior; D, dorsal; V, ventral; b1, first branchial arch; cn, caudal neural plate; e, eye; ee, embryonic ectoderm; em, embryonic mesoderm; fg, foregut; fl, forelimb bud; h, heart; hl, hindlimb bud; nc, neural crest cells for cranial ganglia; no, node; ot, otic vesicle; ps, primitive streak; rh, future rhombomere 2.

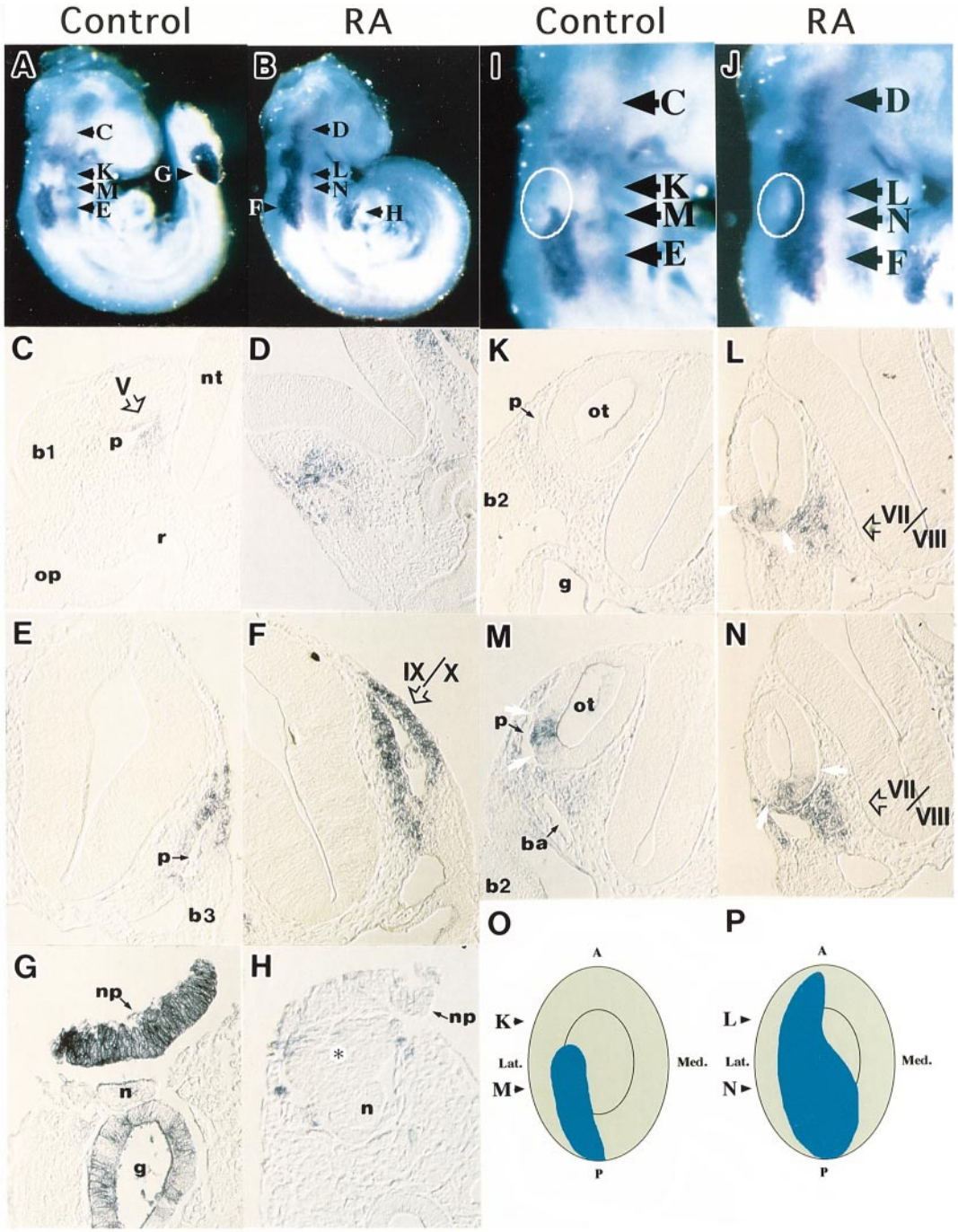

Fig. 8. Expression of *P450$_{RA}$* can be altered by exogenous RA. All-*trans* RA (12.5 mg /kg) (B, D, F, H, J, L, N and P) or vehicle (bean oil) alone (A, C, E, G, I, K, M and O) was administered orally to pregnant mice at 8.5.and 9.0 d.p.c. The embryos were recovered at 9.5 d.p.c. and were subjected to whole mount *in situ* hybridization in parallel. Whole mount staining patterns are shown in (**A**) and (**B**). In order to avoid non-specific accumulation of reagents in the neural tube and to demonstrate clearly the differences in expression pattern between RA-treated and vehicle-alone embryos, the neural tube of these embryos was opened. Administration of vehicle alone had no effect on *P450$_{RA}$* expression (data not shown). (**C–H**) The embryos shown in (A) and (B) were transversely sectioned on comparable planes indicated by the black arrowheads in (A) and (B). (**I** and **J**) Magnified views of the otic vesicle region in (A) and (B), respectively. Open circles show the boundary of the otic vesicle. (**K–N**) Transverse sections at the level of the otic vesicle. Each pair of white arrowheads indicates the boundary of the *P450$_{RA}$*-expressing domain. (**O** and **P**) Schematic representation of *P450$_{RA}$* expression in the otic vesicle viewed from the ventral side. The differences in the expression pattern between control (O) and RA-treated (P) embryos are summarized. Note that the expression domain (shown in blue) is extended in the RA-treated embryo. Abbreviations: A, anterior; P, posterior; Lat., lateral; Med., medial; ba, branchial artery; b1, b2, and b3, first, second and third branchial archs; g, hindgut; n, notochord; np, neural plate; nt, neural tube; op, optic vesicle; ot, otic vesicle; p, primary head vein; r, Rathke's pouch; V, trigeminal neural crest cells; VII/VIII, facio-acoustic neural crest cells; IX/X, glossopharyngeal–vagal neural crest cells.

is essential. It remains to be seen exactly what structure(s) is recognized by $P450_{RA}$.

The chemical analysis (Figure 4) indicated that the enzymatic metabolite of all-*trans* RA (peak X in Figure 3A) was 5,8-epoxy all-*trans* RA. In previous pharmacological studies by others (Barua *et al.*, 1991), all-*trans* RA was injected into adult animals and its *in vivo* metabolites were characterized. The major metabolites of all-*trans* RA included 4-hydroxy all-*trans* RA and 5,6-epoxy all-*trans* RA which are much less active forms (Reynold *et al.*, 1993). However, 5,8-epoxy all-*trans* RA was not the major metabolite detected *in vivo*. There are several explanations for this discrepancy. First, 5,8-epoxy all-*trans* RA may exist *in vivo* as a transient intermediate which is converted rapidly to other forms (such as 5,6-epoxy all-*trans* RA). Secondly, it has been shown that 5,6-epoxy all-*trans* RA can be converted to 5,8-epoxy all-*trans* RA *in vitro* by the acidic conditions used for RA extraction (McCormick *et al.*, 1978). It is possible that the true metabolite of all-*trans* RA produced by $P450_{RA}$ was 5,6-epoxy all-*trans* RA instead of 5,8-epoxy all-*trans* RA. However, the enzymatic metabolite was 5,8-epoxy all-*trans* RA even when the reaction mixture was extracted under non-acidic conditions (data not shown). Finally, $P450_{RA}$ may be the major RA-catabolizer in developing embryos, but may not be the major one in adults. However, expression of $P450_{RA}$ in adult liver (Figure 1) argues against this possibility.

Very recently, White *et al.* (1996) reported a new P450 from zebrafish (referred to as P450RAI) that can metabolize RA. The amino acid sequence of P450RAI shows significant homology to that of our $P450_{RA}$ (~60% of the amino acid residues are identical). However, these two P450s have different activities: P450RAI metabolizes all-*trans* RA to 4-oxo and 4-hydroxy all-*trans* RA whereas $P450_{RA}$ metabolizes all-*trans* RA to 5,8-epoxy all-*trans* RA (Figure 4). For many RA-regulated activities (such as binding to RAR and teratogenic effects on *Xenopus* embryos), 4-oxo all-*trans* RA is as potent as all-*trans* RA (Pijnappel *et al.*, 1993). In this regard, it is most likely that $P450_{RA}$ and P450RAI have a distinct role in RA metabolism: while $P450_{RA}$ catabolizes biologically potent RAs, P450RAI may be involved in the production of active metabolites with functions different from those of all-*trans* RA.

$P450_{RA}$ may regulate the regional concentration of RA in embryos

It has been suggested that biologically active RAs are distributed unevenly in vertebrate embryos. For example, the posterior part of the limb bud and Hensen's node appear to contain more RA than their neighboring regions (Thaller and Eichele, 1987; Chen *et al.*, 1992). It appears that some cells contain more RA than others. If so, what mechanism can account for the uneven distribution of RA in embryos? Formally there are several possibilities; the cellular uptake of RA or the rate of its synthesis/degradation may vary between each cell type. However, there has been no direct evidence supporting any of these possibilities. In this study, we have provided several lines of evidence indicating that $P450_{RA}$ can convert biologically active forms of RA (such as all-*trans* and 9-*cis* RA) into an inactive form(s). Thus, one can envisage that the level of active RA in $P450_{RA}$-expressing cells would be lower than that in non-expressing cells. If this is the case, the region-specific expression of $P450_{RA}$ should result in uneven distribution of active RA in embryos. Our results (Figures 5 and 6) suggest that $P450_{RA}$-expressing P19 and HeLa cells can metabolize 0.01–0.1 μM of all-*trans* RA present in the culture medium. Endogenous all-*trans* RA and 9-*cis* RA are present at ~10^{-8} M in embryos and adult organs (Thaller and Eichele, 1987; Durston *et al.*, 1989; Heyman *et al.*, 1992). Therefore, these $P450_{RA}$-expressing cells are capable of metabolizing physiological levels of RA.

Alternatively, one may argue that expression of $P450_{RA}$ is a protective response to a high concentration of RA, i.e. regional concentration of RA would be determined by some other mechanism not involving $P450_{RA}$, and $P450_{RA}$ is expressed in Regions 1 and 2 simply because the RA concentration in these regions is relatively high. However, our data suggest that this is not the case. The $P450_{RA}$ gene is not necessarily inducible by an increasing concentration of RA; in Region 1, it can in fact be repressed by excess RA (Figure 8B and H). At least the expression in Region 1 should not be a consequence of a high RA concentration in that region. Therefore, we would propose that $P450_{RA}$ may be a determinant of the regional concentration of active RA in developing embryos.

Role of the $P450_{RA}$ gene in development

In post-implantation embryos between 9.5 and 10.5 d.p.c., $P450_{RA}$ is expressed mainly in two regions: Region 1 (the caudal neural plate, hindgut and tailbud mesoderm) and Region 2 (neural crest cells for the cranial ganglia). It may be that the level of RA in these regions needs to be kept low for their normal development. In this regard, it is interesting to note that *RAR*α and *RAR*β are expressed in the closed neural tube but not in the neural plate of the caudal neuropore (Ruberte *et al.*, 1991). Thus, RA-mediated action may be absent in Region 1. Furthermore, it is an interesting coincidence that those $P450_{RA}$-expressing regions are known to be highly sensitive to excess RA. When embryos are challenged by excess all-*trans* RA at 8.5 d.p.c., it alters the migration pattern and identity of neural crest cells for cranial ganglia (Lee *et al.*, 1995). Excess RA also causes underdevelopment of the posterior neural tube, which will very often result in spina bifida at a later stage (Yasuda *et al.*, 1990). The response of the $P450_{RA}$ gene to exogenous RA observed in Region 1 would explain why the posterior neuropore is particularly sensitive to exogenous RA. Since $P450_{RA}$ expression in Region 1 is repressed by exogenous RA, Region 1 (where the RA level is normally kept low) would be the site where the intracellular RA concentration increases most dramatically. The elevated level of RA probably interferes with the normal development of the caudal neural plate.

Recent studies on mice deficient in *RAR/RXR* have clearly demonstrated the importance of these receptors in development (Kastner *et al.*, 1994; Lohnes *et al.*, 1994). However, in a strict sense, it has not been firmly established that all the actions of RA are mediated by the nuclear RA receptors alone: there may be a mechanism not involving RAR/RXR. This is a difficult question to address since it would be impossible to deplete endogenous RA. In this regard, the $P450_{RA}$ gene may serve as a very useful tool

to examine the physiological role of RA. For example, RA has been implicated in limb bud development, but as yet there is no direct evidence for this. One can now introduce ectopic expression of $P450_{RA}$ into the limb bud and examine the role of endogenous RA in limb formation. Recently, we have tested the role of RA in *Xenopus* development by overexpressing $P450_{RA}$ in *Xenopus* embryos. Such ectopic expression of $P450_{RA}$ indeed resulted in malformation of many organs (our unpublished data).

Role of the $P450_{RA}$ gene in the adult

In the adult, $P450_{RA}$ was expressed only in the liver, which resembles the expression pattern of other P450 genes including those involved in the metabolism of xenobiotics. $P450_{RA}$ present in adult liver may be required for the homeostasis of the level of active RA in serum. This would be systemic regulation of the RA level, in contrast to regional regulation in embryos.

All-*trans* RA can induce terminal differentiation of acute promyelocytic leukemia (APL) cells *in vitro* and has been used to treat APL patients. Although treatment with all-*trans* RA leads to remission, most patients later develop RA resistance. As previously proposed (Kizaki *et al.*, 1996), a P450 molecule such as $P450_{RA}$ may be involved in the development of such RA resistance.

Materials and methods

Isolation of the $P450_{RA}$ gene

A cDNA library was constructed from P19 cells at 2 days after RA induction. A number of cDNA clones whose expression was activated 2 days after RA induction were isolated by a sensitive subtraction procedure (Saijoh *et al.*, 1996). One such clone was 10245. The same library was screened with the 5′ end fragment of 10245, and several full-length cDNA clones were obtained. For $P450_{RA}$ expression vectors, a full-length cDNA insert was subcloned in pEFBOS and pEF_{SA}neo (Mizushima and Nagata, 1990), resulting in pBOS$P450_{RA}$ and pEFneo-$P450_{RA}$, respectively.

Whole mount in situ hybridization

Whole mount *in situ* hybridization was performed essentially as described by Wilkinson *et al.* (1992). To generate antisense and sense probes, a 0.5 kb *Eco*RI–*Sac*I fragment from the cDNA clone 10245 was subcloned in Bluescript SK. The antisense and sense probes were produced with T3 RNA polymerase and T7 RNA polymerase, respectively. In most of the experiments, the sense probe was used in parallel. The sense probe did not show any detectable staining with 6.0–11.5 d.p.c. embryos. At least two embryos of the same stage were used in a single experiment to confirm that the staining pattern was reproducible. Developmental stages of mouse embryos were confirmed by their morphology. In the case of embryos later than 8.5 d.p.c., the number of somites was counted.

Culture and differentiation of P19 cells

P19 cells were grown or induced to differentiate essentially as described by Rudnicki and McBurney (1987). To obtain stable transformants, P19 cells were transfected with 2.5 μg of pEFneo-$P450_{RA}$ and 25 μg of pBOS-$P450_{RA}$, and transformants were selected with 200 μg/ml of G418 as described previously (Bhat *et al.*, 1988; Shimazaki *et al.*, 1993). More than 10^3 G418-resistant colonies were pooled, which were referred to as P19R. As a control, P19 cells were also transformed with pEF_{SA}neo alone, and $>10^3$ G418-resistant colonies were pooled (referred to as P19C). P19R and P19C cells were induced to differentiate with various concentrations of all-*trans* RA. Cell aggregates were trypsinized and plated on tissue culture dishes 4 days after induction. The morphology of the cells was examined by phase contrast microscopy (normally, many neurons become visible 6 days after induction). For Western blot analysis, cells were harvested 6–7 days after induction and cell lysates were prepared. The level of Oct-3 in each cell lysate was determined with a polyclonal anti-Oct-3 antibody (Shimazaki *et al.*, 1993).

In vitro RA-metabolizing assay

Ten dishes (10 cm) of 293T cells were transfected with pBOS-$P450_{RA}$. Cells were harvested 48 h after transfection and microsome fractions were prepared from the culture cells. The standard reaction mixture in 1 ml contains 10 μl of microsome fraction (~0.1 mg protein) in a reaction buffer (10 mM NADP, 0.1 M glucose-6-phosphate, 50 mM K_2HPO_4 pH 7.5, 0.2 mM $MgCl_2$, 1 U of glucose-6-phosphate dehydrogenase). After adding 0.1 μM of each retinoid dissolved in 5 μl of dimethylsulfoxide, the mixture was incubated in a light-protected tube at 37°C for 2 h. Retinoids were then extracted from the reaction mixture, concentrated and analyzed by reverse phase HPLC as described previously (Kojima *et al.*, 1994). The retinoids were detected at a wavelength of 350 nm. Standard retinoids were used as described previously (Kojima *et al.*, 1994).

Chemical analysis of RAs

Reverse phase HPLC analysis of RA isomers was performed as previously described (Kojima *et al.*, 1994). Normal phase HPLC analysis was done with a Wakosil-II 5C18AR column (2×25 cm) eluted with methanol–acetonitrile–2% acetate (68:17:15). For GC/MS, all-*trans* RA (30 mg) dissolved in diethyl ether was oxidized with *m*-chloroperoxybenzoic acid and oxidized compounds were first separated by reverse phase HPLC. The oxidized compounds such as peaks 1 and 2 (Figure 4) were methylated further with diazomethane and analyzed by GC/MS (Dadang *et al.*, 1996). The molecular ion (M^+) of methylated peak 2 has a mass/charge ratio of 330, which corresponds to that of 5,8-epoxy all-*trans* retinoic acid methyl ester.

CAT assay

HeLa cells were transfected with 2.5 μg of pEFneo-$P450_{RA}$ and 25 μg of pBOS-$P450_{RA}$ and stable transformants were selected with 400 μg/ml of G418. More than 10^3 G418-resistant colonies were pooled, and were referred to as HeLa-R. For the CAT assay, HeLa and HeLa-R cells were transfected with 5 μg of pRARE-Tkcat, 1 μg of RARβ expression vector (pSGRARβ; Brand *et al.*, 1988) and 3 μg of pEFLacZ as an internal standard. Various concentrations of all-*trans* RA were added to the culture medium after transfection. Cells were harvested 48 h after transfection for CAT assay.

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