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Retinoic acid receptors and cellular retinoid binding proteins

I. A systematic study of their differential pattern of transcription during mouse organogenesis

PASCAL DOLLÉ[1,*], ESTHER RUBERTE[1], PIERRE LEROY[1], GILLIAN MORRISS-KAY[2] and PIERRE CHAMBON[1,†]

[1]*Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg-Cédex, France*
[2]*Department of Human Anatomy, South Parks Road, Oxford OX1 3QX, UK*

* Present address: European Molecular Biology Laboratory, Meyerhofstrasse 1, Postfach 10.2209, 6900 Heidelberg 1, FRG
† Author for correspondence

Summary

We report here the gene expression patterns, as revealed by *in situ* hybridisation, of the retinoic acid receptors alpha, beta and gamma (RAR-α, -β and -γ), and the cellular binding proteins for retinol and retinoic acid (CRBP, CRABP) in non-neural tissues of mouse embryos during the period of organogenesis. At all stages, RAR-α transcripts were almost ubiquitous, whereas the distribution of transcripts of the other four genes was distinctive in all systems. At early stages in the formation of an organ, the expression patterns were different in the epithelium, the adjacent mesenchyme, and in mesenchyme more distant from the epithelium, suggesting a role for RA and RA receptors in epithelial–mesenchymal tissue interactions. In the developing face, limb bud and genital tubercle, where large expanses of mesenchyme are present, differential patterns of expression were established before the onset of overt tissue differentiation, suggesting some significance for pattern formation in these regions. The distribution of RAR-β transcripts in tracheobronchial, intestinal and genital tract epithelia is consistent with the possibility that RAR-β plays a role in mediating retinoid effects on the differentiated stage of these epithelia. Possible developmental roles of RARs in relation to the expression patterns of other genes are discussed. CRBP expression domains showed a high degree of overlap with RAR-β and RAR-γ, and a mutual exclusivity with CRABP expression domains. Correlation of these expression patterns with the morphogenetic effects of vitamin A deficiency and retinoid excess lead us to propose that the function of CRBP is to store and release retinol where high levels of RA are required for specific morphogenetic processes, while CRABP serves to sequester RA in regions where normal developmental functions require RA levels to be low. Where both binding protein genes are expressed in a non-overlapping pattern within a large area of mesenchyme, a gradient of free RA may be created between them by release of retinol-derived RA from CRBP-expressing cells, with binding to CRABP enhancing the steepness of the decline in concentration distant to the source.

Key words: RAR-α, β and γ, mouse embryo, CRABP, CRBP.

Introduction

Retinoic acid (RA), the most biologically active natural metabolite of vitamin A (retinol), appears to play a key role in vertebrate development. Vitamin A deficiency or excess have long been known to be teratogenic, inducing a wide spectrum of embryonic malformations in several mammalian species (Kalter and Warkany, 1959; Morriss, 1972; Shenefelt, 1972; Kochhar, 1961, 1973, 1977 and, 1985; Marin-Padilla, 1966; Geelen, 1979; Sulik *et al.* 1988; and refs therein), including human (Rosa, 1984; Rosa *et al.* 1986; Lammer *et al.* 1985). Studies on chick limb development led to the suggestion that RA may act as a morphogen (Tickle *et al.* 1982; Eichele *et al.* 1985; Tickle *et al.* 1985). This proposition has been supported by the finding of a graded anteroposterior distribution of RA across the wing bud, with maximal levels in the posterior margin which contains the zone of polarizing activity (ZPA) (Slack, 1987; Thaller and Eichele, 1987; Smith *et al.* 1989; Tickle *et al.* 1989; Summerbell and Maden, 1990; and refs therein). RA has spectacular effects on limb regeneration and can induce extra limbs in amphibians (Maden, 1982; Brockes, 1989; Eichele, 1989; Summer-

bell and Maden, 1990; and refs therein). RA is also known to affect mammalian limb development (Kochhar, 1973 and, 1977; Satre and Kochhar, 1989; Sulik and Dehart, 1988; Alles and Sulik, 1989). Furthermore retinoids have been observed to affect the development of the nervous system in amphibians (Durston *et al.* 1989) and mammals (Langman and Welch, 1967; Morriss, 1972; Lammer *et al.* 1985). In animal models, craniofacial and axial skeletal anomalies are also produced by exposure to RA (Morriss and Thorogood, 1978; Webster *et al.* 1986; Wedden, 1987; Wedden *et al.* 1987 and, 1988; Langille *et al.* 1989; Alles and Sulik, 1990; and refs therein). Finally, retinoids have marked effects on differentiation and maintenance of epithelial cells *in vivo* and *in vitro*, skin being a major target for retinoids both in its normal (Roberts and Sporn, 1984; Brown *et al.* 1985; Shapiro, 1985; Asselineau *et al.* 1989; Kopan and Fuchs, 1989; and refs therein) and pathological (Peck, 1984) states.

How can RA exert such a wide spectrum of biological activities? It has been recognized for some time that RA-induced differentiation of embryonal carcinoma cells *in vitro* is accompanied by a change in gene expression (Roberts and Sporn, 1984; Mavilio *et al.* 1988; Vasios *et al.* 1989; Simeone *et al.* 1990; and refs therein). The discovery of RA receptors (RARs) belonging to the steroid/thyroid hormone family of nuclear receptors was a major step towards the elucidation of the molecular mechanisms linking RA signals to the control of gene expression. Indeed, nuclear receptors act as ligand-inducible transcriptional enhancer factors (reviewed in Green and Chambon, 1988; Evans, 1988). Three different mammalian RAR genes have been identified. The corresponding receptors, RAR-α (Petkovich *et al.* 1987; Giguère *et al.* 1987), RAR-β (Brand *et al.* 1988; Benbrook *et al.* 1988) and RAR-γ (Krust *et al.* 1989; Zelent *et al.* 1989) can potentially differ in their affinity for retinoids and in their recognition specificity for the RA-responsive elements of the target genes. A fourth receptor, termed RAR-δ, has been found in the newt (Ragsdale *et al.* 1989). In addition, recent data indicate that, for each mammalian RAR gene receptor, isoforms can be generated by alternative splicing (Giguère *et al.* 1990; Kastner *et al.* 1990; and our unpublished results). The existence of several nuclear RARs, whose expression could be spatio-temporally regulated during development, may thus account for the diversity of RA effects. Indeed, differential expression of the three RAR genes has been demonstrated in adult mouse tissues (Krust *et al.* 1989; Zelent *et al.* 1989; Kastner *et al.* 1990), and during mouse embryogenesis (Dollé *et al.* 1989; Ruberte *et al.* 1990; Kastner *et al.* 1990). Our previous study of the localisation of RAR-γ transcripts during mouse embryogenesis revealed that this receptor is a candidate for mediating the effects of RA during early morphogenesis and differentiation of cartilage and cornified squamous epithelia (Dollé *et al.* 1989; Ruberte *et al.* 1990). Noji *et al.* (1989*a*,*b*) have also reported a specific distribution of RAR transcripts in developing finger bones and in the skin.

The natural source of retinoids for embryonic tissues is maternal retinol. In view of its highly pleiotropic effects, it is likely that RA is synthesized from retinol in discrete areas of the embryo, close to its sites of action. Cellular retinoid binding proteins have been characterized, that specifically bind retinol (cellular retinol binding protein – CRBP) and RA (cellular RA binding protein – CRABP) with high affinity (Chytil and Ong, 1984; and refs therein). The function of these cytoplasmic proteins, which have no counterparts in the mechanism of action of steroid/thyroid hormones, is not clear. They may be involved in the metabolism of retinoids; it has also been proposed that they may define populations of cells that are RA responsive (Maden *et al.* 1989*b*) and may be responsible for regulating the intracellular concentration of free retinoids, e.g. to generate concentration gradients of free RA (Robertson *et al.* 1987; Maden *et al.* 1988; Smith *et al.* 1989). Several recent studies have indicated that CRABP and CRBP transcript or protein are tissue-specifically expressed in vertebrate embryos (Maden *et al.* 1989*a*,*b*; Vaessen *et al.* 1989; Dollé *et al.* 1989; Perez-Castro *et al.* 1989), but no systematic study of their distribution during mammalian embryogenesis has yet been carried out. Moreover it appears that there are two types of CRBP (I and II) and CRABP (I and II). Rat CRBP II (Li *et al.* 1986; Demmer *et al.* 1987) differs from rat CRBP I (Sundelin *et al.* 1985*b*) by 58 out of 133 amino acid residues, and the CRBP I sequence appears highly conserved during evolution (Nilson *et al.* 1988). Thus a CRBP I nucleic acid probe cannot hybridize with CRBP II sequences under stringent hybridization conditions. CRABP I cDNA has been cloned from bovine (Shubeita *et al.* 1987; Wei *et al.* 1987) and murine (Stoner and Gudas, 1989) sources and the sequence of bovine CRABP I has been determined (Sundelin *et al.* 1985*a*). CRABP I appears highly conserved in evolution at least between chick and bovine (Eriksson *et al.* 1981; Kitamoto *et al.* 1988). CRABP II, which has been purified from rat (Bailey and Siu, 1988) and chick (Kitamoto *et al.* 1988) sources, is also conserved in evolution, but to a lesser extent than CRABP I. Moreover, a comparison of the first 25 amino acids of chick CRABP I and II shows 3 non-conservative amino acid changes, whereas a similar comparison of rat CRABP I and II reveals 7 non-conservative changes (Bailey and Siu, 1988; Kitamoto *et al.* 1988). Accordingly, antibodies against rat CRABP I did not cross-react with rat CRABP II (Bailey and Siu, 1988), and antibodies against chick CRABP II did not recognize chick CRABP I (Kitamoto *et al.* 1989), although curiously two antibody preparations directed against mammalian CRABP I appear to cross-react with both chick CRABP I and II (Kitamoto *et al.* 1989; Maden *et al.* 1990). The murine CRABP II cDNA has not yet been cloned and therefore it is not known whether the mouse CRABP I probe used in the present study is specific for CRABP II transcripts.

As an approach to clarifying the function and the relationships between CRBP, CRABP and RARs, we have analysed and compared the distribution of their

respective transcripts during mouse embryogenesis. Using the *in situ* hybridization technique, we have systematically compared the distribution of the transcripts of the genes encoding murine RAR-α, -β and -γ, CRABP I and CRBP I (designated as CRABP and CRBP thereafter) through various developmental stages from gastrulation to late organogenesis. Data concerning the early period of mouse morphogenesis and the development of the nervous system will be published separately. In the present study, the distribution of RARs, CRABP and CRBP transcripts is analyzed in non-neural tissues during the period of organogenesis, i.e. in fetuses aged from 10.5 to 14.5 days *post-coitum* (*p.c.*).

Materials and methods

^{35}S-labelled antisense RNA probes were synthesized using a T7 polymerase *in vitro* transcription reaction, from full-length cDNAs coding for murine RAR-α, -β and -γ, CRABP and CRBP subcloned in an antisense orientation in the pSG5 vector (Green *et al.* 1988). The mouse RAR-α, -β and -γ and CRABP plasmids have been described in Dollé *et al.* (1989). The type I CRBP cDNA was cloned from a mouse testis cDNA library (P. Leroy, unpublished results). The CRABP and CRBP cDNAs used here correspond to type I CRABP and CRBP, since their deduced amino acid sequences are highly homologous to those of CRABP I (Sundelin *et al.* 1985*a*; Shubeita *et al.* 1987; Stoner and Gudas, 1989; Wei *et al.* 1987; Kitamoto *et al.* 1988) and CRBP I (Sundelin *et al.* 1985*b*; Li *et al.* 1986; Nilsson *et al.* 1988), which have been previously purified, sequenced and cloned. Probe length was reduced to an average of 150 nucleotides by limited alkaline hydrolysis, as described in Ruberte *et al.* (1990).

Recovery and paraffin embedding of mouse embryos, *in situ* hybridization, emulsion autoradiography and staining of histological sections were performed as previously described (Ruberte *et al.* 1990).

In situ hybridization analysis was performed on mouse embryo sections using ^{35}S-labelled antisense RNA probes transcribed from full-length cDNAs of murine RAR-α, -β and -γ, CRABP I and CRBP I (called hereafter CRABP and CRBP). To compare the spatial distribution of the corresponding mRNA species, series of 5 adjacent sections, 6 μm thick, were hybridized to these probes in a repeated manner so that the entire embryo or fetus was analyzed. Thus, the spatial distribution of the 5 mRNA species was compared within 30 μm thick sectioning planes.

Results

Each of the 5 probes hybridized specifically to its corresponding mRNA, as shown clearly by the distinct labelling patterns that were obtained (e.g. Fig. 1). At all stages RAR-α transcripts were almost ubiquitously expressed; thus their distribution is not systematically displayed in this study. Local differences of RAR-α signal grain intensity generally reflect variations in cell density, except in areas of the central nervous system (CNS) and in the liver where regions with significant lower transcript abundance could be detected (see Fig. 1). The distribution of RAR-α transcripts in the CNS will be described elsewhere (Ruberte *et al.* unpublished data). In contrast RAR-β, RAR-γ, CRABP and CRBP transcripts exhibited restricted and distinct domains of expression. The intensity of specific labelling with RAR-β and -γ probes was in general weaker than with CRBP and CRABP probes, indicating a higher abundance of transcripts for the binding proteins than for the nuclear receptors. The sections hybridized with the CRABP probe were systematically exposed for half as long as the others.

Facial and pharyngeal derivatives

RAR-β, RAR-γ, CRABP and CRBP transcripts were not uniformly distributed in craniofacial structures prior to cytodifferentiation. By day 10.5 *p.c.* (Fig. 2A), RAR-β transcripts were most abundant in anterior facial mesenchyme [e.g. around the eye (e) and in the frontonasal mesenchyme, (fn)], but were absent from the maxillary process (mp) and from the rostral half of the mandibular arch (I). There was a weak labelling in the caudal half of this arch, but little or none in arches 2 to 6. As described previously (Ruberte *et al.* 1990), RAR-γ transcripts were more homogeneously distributed in the facial and pharyngeal arch mesenchyme (Fig. 2A). Of the 5 gene transcripts studied here, RAR-α and RAR-γ were the only ones that showed expression throughout the maxillary and mandibular components of the first arch. CRABP transcripts were most abundant in the rostral part of the frontonasal mesenchyme and in the distal part of the mandibular arch mesenchyme, but labelling was weak or absent elsewhere (Fig. 2A). CRBP transcripts exhibited a somewhat complimentary distribution to that of CRABP, being abundant in central regions of the facial mesenchyme, in the caudal half of the first arch and in more caudal pharyngeal arches (Fig. 2A). The maxillary region was negative or very weakly labelled by both binding protein probes.

Differential expression of the RAR-β, RAR-γ, CRABP and CRBP genes in facial structures was even more accentuated as development proceeds. By day 12.5 *p.c.*, craniofacial tissues are still poorly differentiated (Fig. 2B). RAR-β transcripts were regionally restricted to very central frontonasal mesenchymal cells located close to the brain (b), and to discrete regions at the base of the tongue (t) and within the mandible (m) (see also Figs 1 and 4A). The signal for RAR-γ transcripts was more diffuse. It was, however, stronger in several areas not expressing RAR-β transcripts (Fig. 2B, see also Fig. 1) and corresponding to precartilaginous mesenchymal condensations (see Ruberte *et al.* 1990). The distribution of CRABP transcripts was clearly distinct from those of RAR-β and RAR-γ transcripts. They were most abundant in superficial frontonasal mesenchyme and absent in central regions; they were also preferentially expressed in the distal half of the tongue, and exhibited a complex distribution in the mandible (see also Fig. 1). CRBP transcripts were homogeneously distributed in the tongue (note however, their absence in precartilaginous areas at the base of the tongue in the precise region that strongly

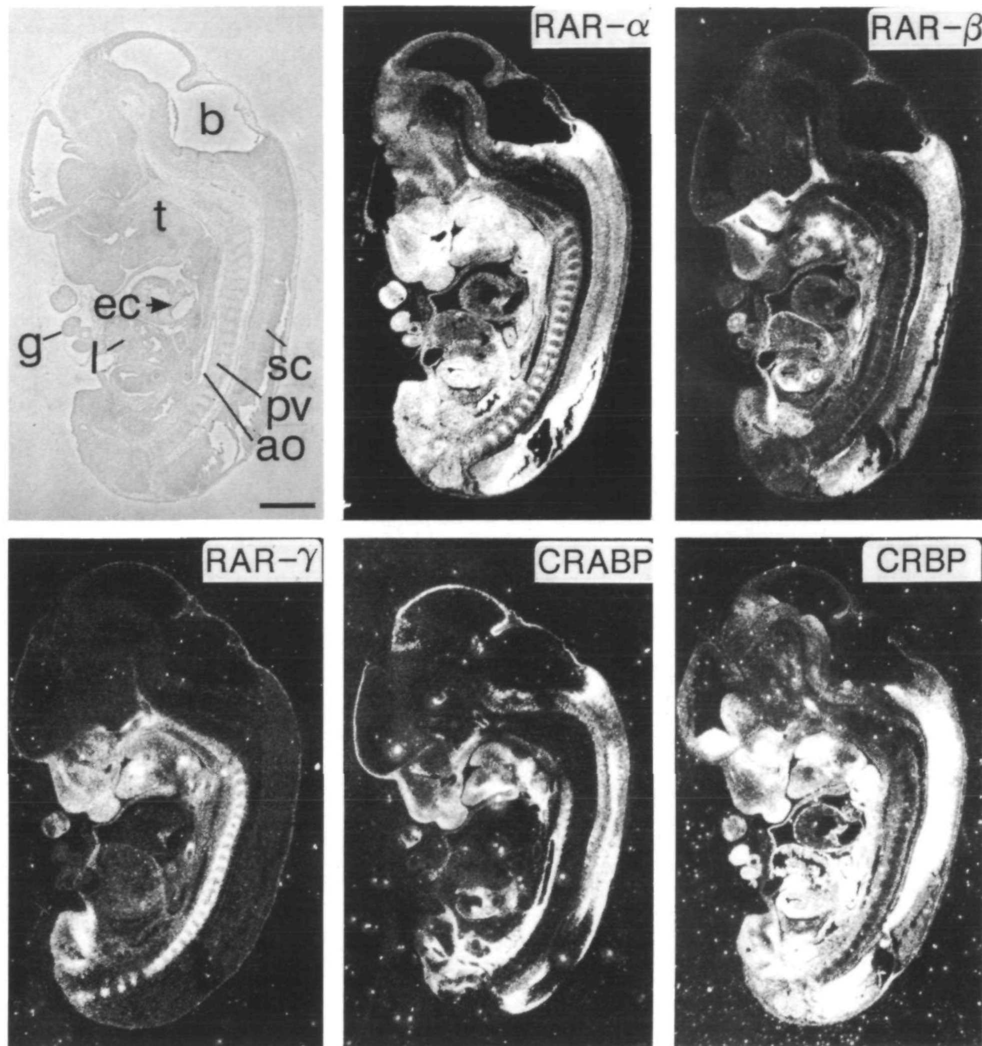

Fig. 1. Tissue-specific distribution of RARs, CRABP and CRBP in the mouse fetus. 5 adjacent midsagittal sections of a 12.5 day old fetus, hybridized as indicated to RNA probes specific for RAR-α, -β and -γ, CRABP and CRBP transcripts, photographed under dark-field illumination so that the autoradiography signal grain appears white. A bright-field photograph of the section is shown for histological identification. In this and subsequent figures, bright-field photographs are arbitrarily taken from sections hybridized to the RAR-β probe. Bar: 625 μm. b=brain; t=tongue; ec=endocardial cushion; g=gut; l=liver; pv=prevertebral column; ao=aorta; sc=spinal cord.

expressed RAR-γ) and were not expressed or at a very low level in mandible and frontonasal areas where CRABP was highly expressed (Fig. 2A, see also Figs 1 and 4A).

At later stages in development (day 13.5 and 14.5 *p.c.*), the distribution of RAR-β and CRBP transcripts became very similar and was highly restricted to cells located close to the base of the skull and surrounding the nasal cavities (data not shown and see below). At the same stages, RAR-γ transcripts were associated with cartilage, and CRABP transcripts were generally (but not always, see e.g. Fig. 7B and C) found in areas surrounding cartilaginous elements, and in the dermal layer of the skin (Fig. 8B and data not shown). Thus, the late distribution of CRABP transcripts in the regions surrounding bone models, in the face and more generally in the whole body, was similar to that previously described in the limbs (Dollé *et al.* 1989).

In the developing submandibular salivary gland, which develops from the oral epithelium and adjacent mesenchyme, RAR-β transcripts were found exclusively in the mesenchymal cells (Fig. 2C). CRBP exhibited a similar expression pattern (not shown), whereas a complimentary but weak RAR-γ labelling was specifically seen in the glandular epithelium (Fig. 2C). Only RAR-α and CRBP transcripts were detected in the thyroid and thymus glands (data not shown).

By day 14.5 *p.c.*, the tooth germs are 'bell-shaped' structures (Fig. 3) composed of an oral ectoderm-derived dental lamina (l) with the ameloblast layer (a), invaginated by a neural crest cell-derived mesenchymal dental papilla (p) from which the odontoblasts (o) will differentiate. At this stage, RAR-β transcripts were detected in the superficial region of the dental lamina, but not in its deeper invagination (i.e. not in the ameloblast layer, Fig. 3). RAR-β transcripts were also found in the mesenchymal dental sac (s) that surrounds the tooth germ. The RAR-γ transcript signal, which was clearly seen in the dental sac mesenchyme and in the base of the dental papilla, was very weak or absent in the dental lamina. Both CRABP and CRBP transcripts were found only in the mesenchyme, where their distribution was complimentary. CRBP transcripts were present in the superficial regions of the dental sac and in the dental papilla, whereas CRABP transcripts were found in the deeper regions of the dental sac and in cells close to the jaw cartilage.

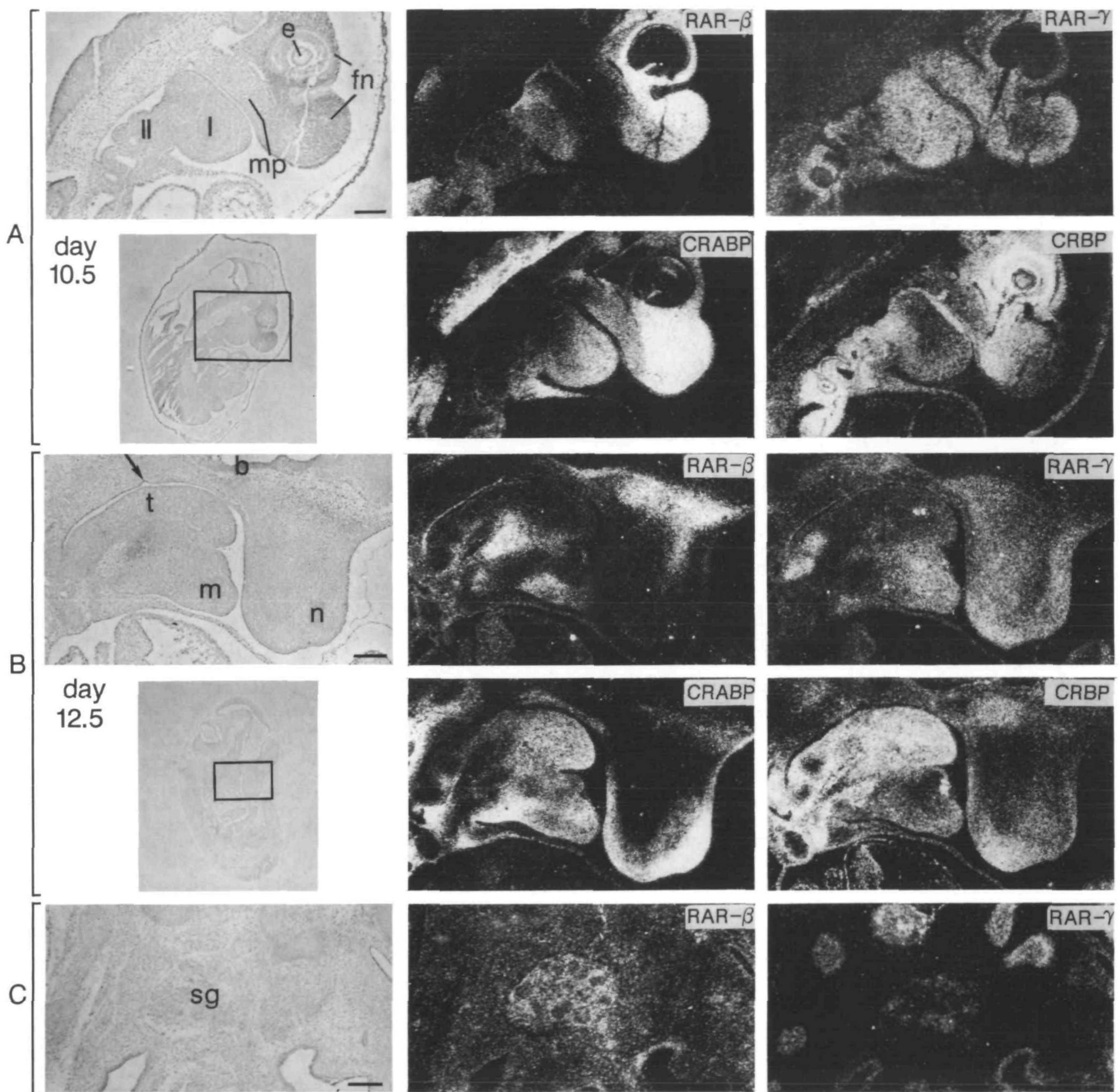

Fig. 2. RARs, CRABP and CRBP transcript domains in craniofacial and pharyngeal derivatives. (A) Sagittal sections of a 10.5 day old fetus, enlargement of the facial and pharyngeal region (boxed in the whole-fetus view). Bar: 160 μm. (B) Midsagittal sections of a 12.5 days *p.c.* fetus, enlargement of the facial region. Bar: 200 μm. The arrow points to the limit between ectoderm- and endoderm-derived epithelium. (C) Sections through the submandibular gland anlage of a 14.5 day old fetus. Bar: 160 μm. e=eye; mp=maxillary process; fn=frontonasal mesenchyme; I=mandibular arch (first pharyngeal arch); II=hyoid arch (second pharyngeal arch); b=brain; n=nasal mass; t=tongue; m=mandible; sg=submandibular gland.

Respiratory system

The endodermal expression of the RAR-β and CRBP genes starts early in development (Ruberte *et al.* 1991) and persists at later stages in endodermal components of the gut and respiratory tract. In the oral cavity of 12.5 day embryos, RAR-β transcripts were detected in the endoderm-derived epithelium located caudally to the pituitary anlage, while the more rostral ectoderm-derived epithelium was negative for RAR-β transcripts (Fig. 2B and Fig. 4A, in which the arrows indicate the limit of expression in the oral ectoderm).

The RAR-β gene was strongly expressed in the endoderm-derived tracheal epithelium and in the adjacent mesenchyme (Fig. 4A, tr). CRBP was also strongly expressed in the tracheal mesenchyme and in the mesenchyme located between the trachea and the

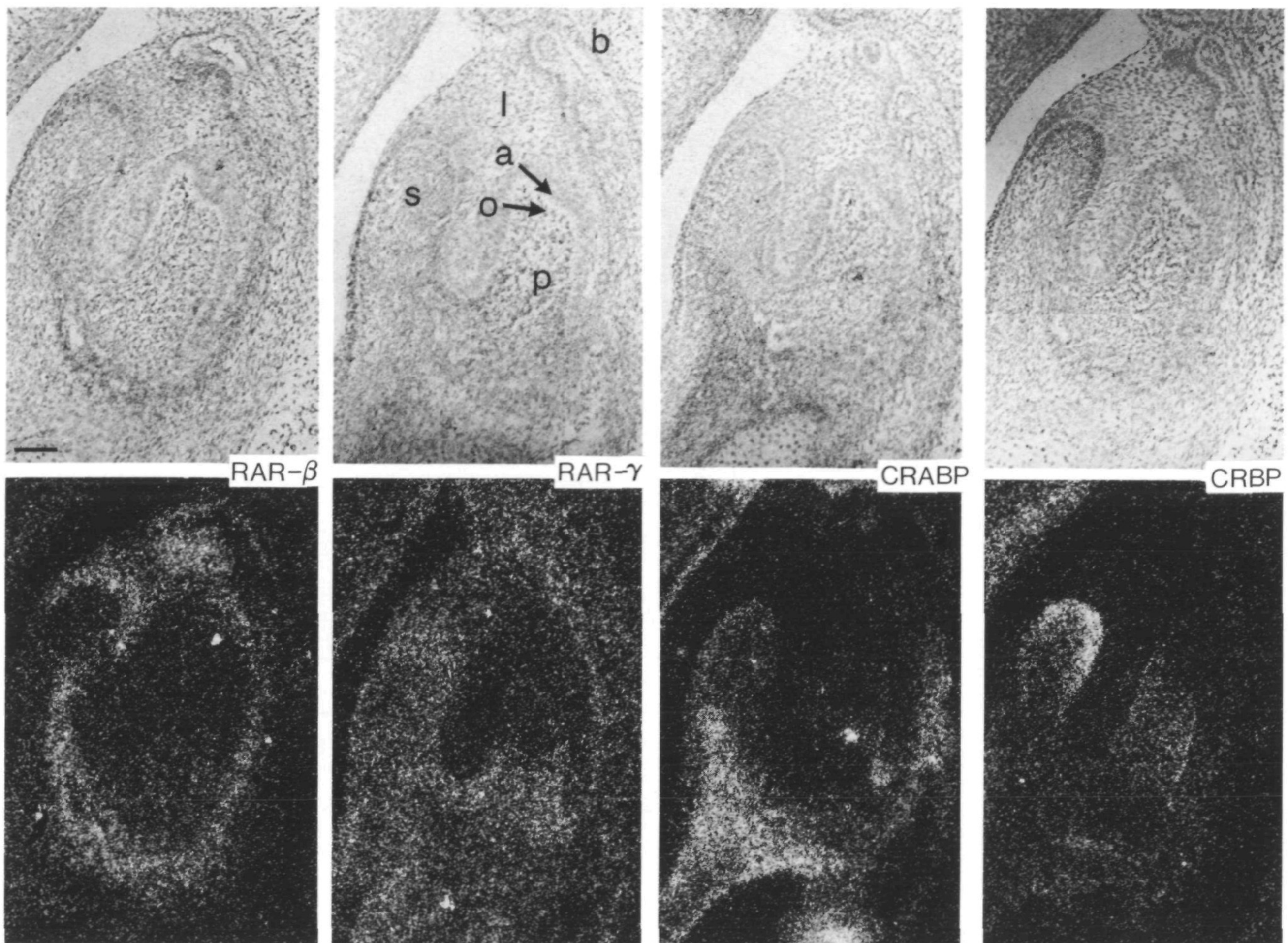

Fig. 3. RARs, CRABP and CRBP transcript domains in the developing tooth germ. Adjacent sections of a molar tooth germ by day 14.5 *p.c.* Bar: 100 μm. l=dental lamina; s=dental sac; p=dental papilla; a=ameloblast layer; o=odontoblast layer; b=bud of the permanent tooth.

heart (h), as well as in the tracheal epithelium, where its expression was essentially apical, in marked contrast with the homogeneous RAR-β transcript distribution. RAR-γ expression in the trachea was strictly mesenchymal, while CRABP was expressed in cells located between the trachea and the heart, but not in the trachea (Fig. 4A).

By day 12.5 *p.c.*, several branchings of the bronchial buds have already occurred. RAR-β transcripts were found only in the epithelium of proximal bronchi, while smaller distal bronchi and the mesodermal components of the lungs were not labelled (not illustrated). Two days later (day 14.5 *p.c.*), RAR-β expression was detected in the epithelium of the intrapulmonary segmental bronchi (br), whereas the lung parenchyma, the bronchioles and alveolar ducts were not labelled (Fig. 4B). Thus, RAR-β expression in the bronchial epithelium was related to its differentiation status. At day 14.5 *p.c.*, RAR-γ expression was weak and rather homogeneous in the lungs. CRBP transcripts could be detected in the 12.5 day bronchial epithelium and lung mesenchyme (not shown), but two days later, CRBP transcripts appeared to be restricted to mesenchymal cells of the lungs (Fig. 4B). No CRABP transcripts could be detected in the lungs at any of these developmental stages (not shown).

Digestive system

In contrast to the expression of the RAR-α gene, which was almost ubiquitous in the digestive tract (e.g. Fig. 6A), expression of the RAR-β gene was spatially restricted along the epithelium of the digestive tract. By day 12.5 *p.c.*, abundant RAR-β transcripts were detected in the epithelium and peripheral mesenchyme of the oesophagus (o, Fig. 4A and C) and the cardiac portion of the stomach, while the pyloric region of the stomach (ps) and the duodenum were not labelled (Fig. 4C). A weak expression of RAR-β was detected again in the epithelium and peripheral mesenchyme of the midgut (e.g. in the herniated gut in Fig. 4C, g; see also Fig. 6A). CRBP was ubiquitously, but not evenly expressed in the gastrointestinal epithelium and mesenchyme (Fig. 4C, and 6A). Within the epithelium of the cardiac portion of the stomach, CRBP expression was highest at the apical surface, while RAR-β was expressed throughout the thickness of the epithelium (see Fig. 8C). CRABP transcripts were detected only in discrete dorsally located regions of the stomach

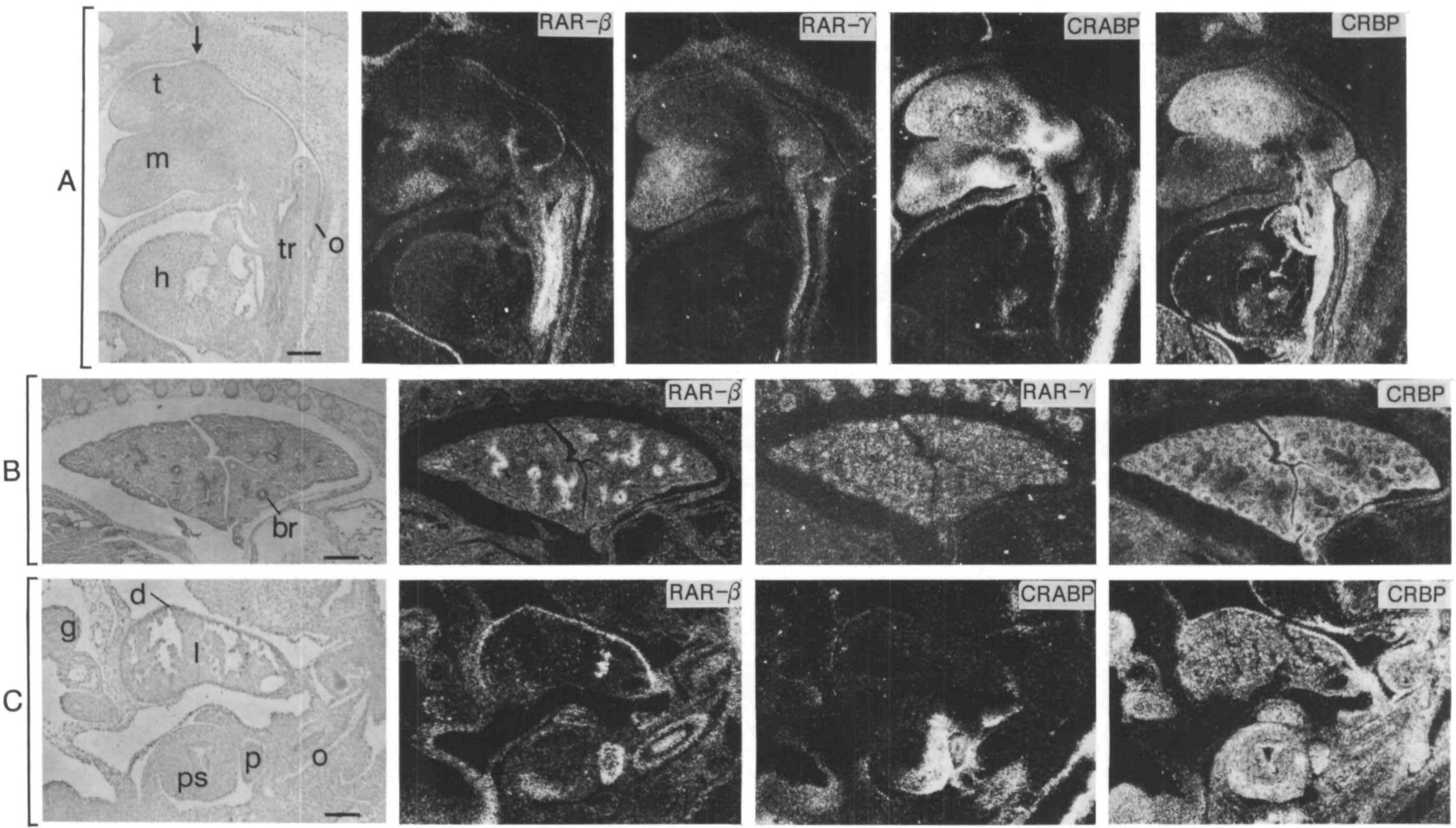

Fig. 4. RARs, CRABP and CRBP transcript domains in respiratory and digestive organs and heart tissue. (A) Sagittal sections across the pharynx, trachea and oesophagus of a 12.5 day old fetus. The arrow marks the boundary between ectoderm-derived and endoderm-derived oral epithelium. Bar: 200 μm. (B) Sections through the lung of a 14.5 day old fetus. Bar: 160 μm. (C) Sagittal sections of a 12.5 day old fetus abdomen, crossing the liver, stomach and pancreas. Bar: 160 μm. t=tongue; m=mandible; tr=trachea; o=oesophagus; h=heart; br=bronchi; g=gut; d=diaphragm; l=liver; ps=pyloric stomach; p=pancreas.

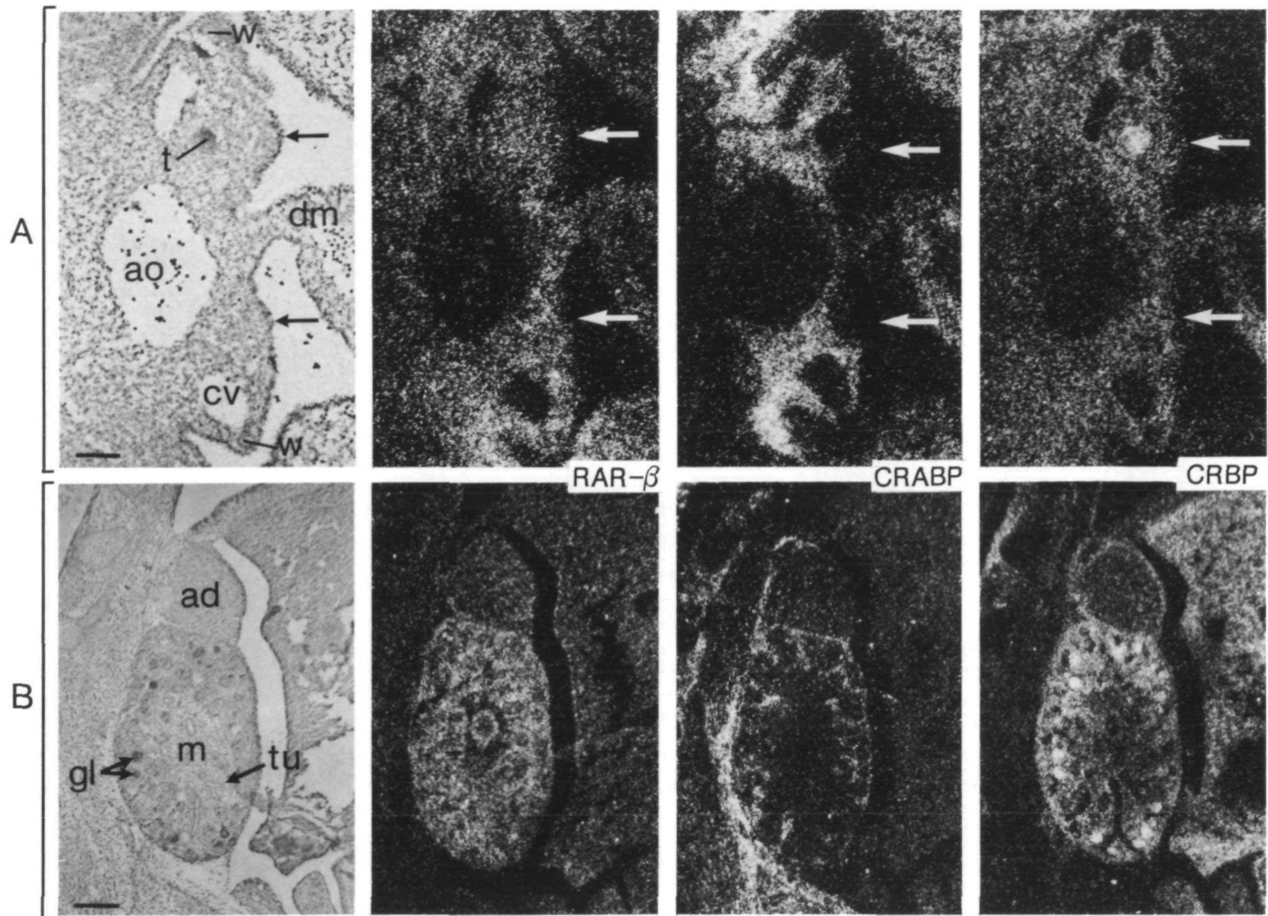

Fig. 5. RAR-β, CRABP and CRBP transcript domains in the mesonephros and definitive kidney. (A) Transverse sections of a 10.5 day old embryo, enlargement of the mesonephros area. The arrows mark the coelomic epithelium. Bar: 100 μm. (B) Sagittal sections through the metanephros (definitive kidney) and adrenal gland of a 14.5 day old fetus. Bar: 160 μm. w=Wolffian duct; t=mesonephric tubule; ao=aorta; cv=posterior cardinal veins; dm=dorsal mesentery; ad=adrenal gland; m=metanephros; tu=metanephric tubule; gl=metanephric glomeruli.

mesenchyme which are close to the dorsal mesentery (Fig. 4C and Fig. 1). RAR-β, CRABP and CRBP expression was readily detected in the dorsal mesentery at earlier developmental stages (Fig. 5A). These expression patterns in the digestive tract became more restricted at later stages of development. By day 14.5 *p.c.*, RAR-β expression in the gut epithelium was confined to the oesophagus, the cardiac region of the stomach (not shown), and to the anal canal (a, Fig. 6B). Interestingly, the RAR-γ gene was also expressed in these epithelia at this gestational stage (Ruberte *et al.* 1990). At the same stage, there was no or weak expression of the CRBP gene in the gut epithelium, but both CRBP and RAR-β were expressed in the gut outer mesenchyme layer (Fig. 6B). The smooth muscle layers of the gut will derive from this layer, which also contains the myenteric nerve plexus.

The liver (l) and diaphragm (d) were homogeneously labelled with the CRBP probe at any developmental stage (Fig. 4C and data not shown). No significant labelling was found with RAR-β, RAR-γ and CRABP probes, with the exception of the RAR-β transcripts expressed in the diaphragm (Fig. 4C and data not shown). The fetal pancreas (p) showed homogeneous CRBP expression, and a more cortical, patchy expression of RAR-β (Fig. 4C), but the resolution was not good enough to assign RAR-β transcripts to a specific cell-type. No CRABP and RAR-γ transcripts were detected in the pancreas.

Heart and circulatory system

At 12.5 day *p.c.* RAR-α transcripts were present in the heart, but no significant expression of RAR-β was seen (Figs 1 and 4A). RAR-γ transcripts were present only in the endocardial cushion tissue and in the vicinity of large blood vessels (Fig. 1 and data not shown). CRBP transcripts were specifically detected in the outer or epicardial layer, in the endocardial cushion tissue and in the wall of large blood vessels close to their junction with cardiac cavities (Fig. 4A). In contrast, CRABP transcripts were strongly expressed in the tissue adjacent to the endocardial cushions and in the walls of large arterial trunks (e.g. aorta, carotids, brachial and iliac arteries: see Fig. 1 and data not shown); then were absent from the walls of the vascular trunks in the vicinity of the heart, in the precise regions that contain

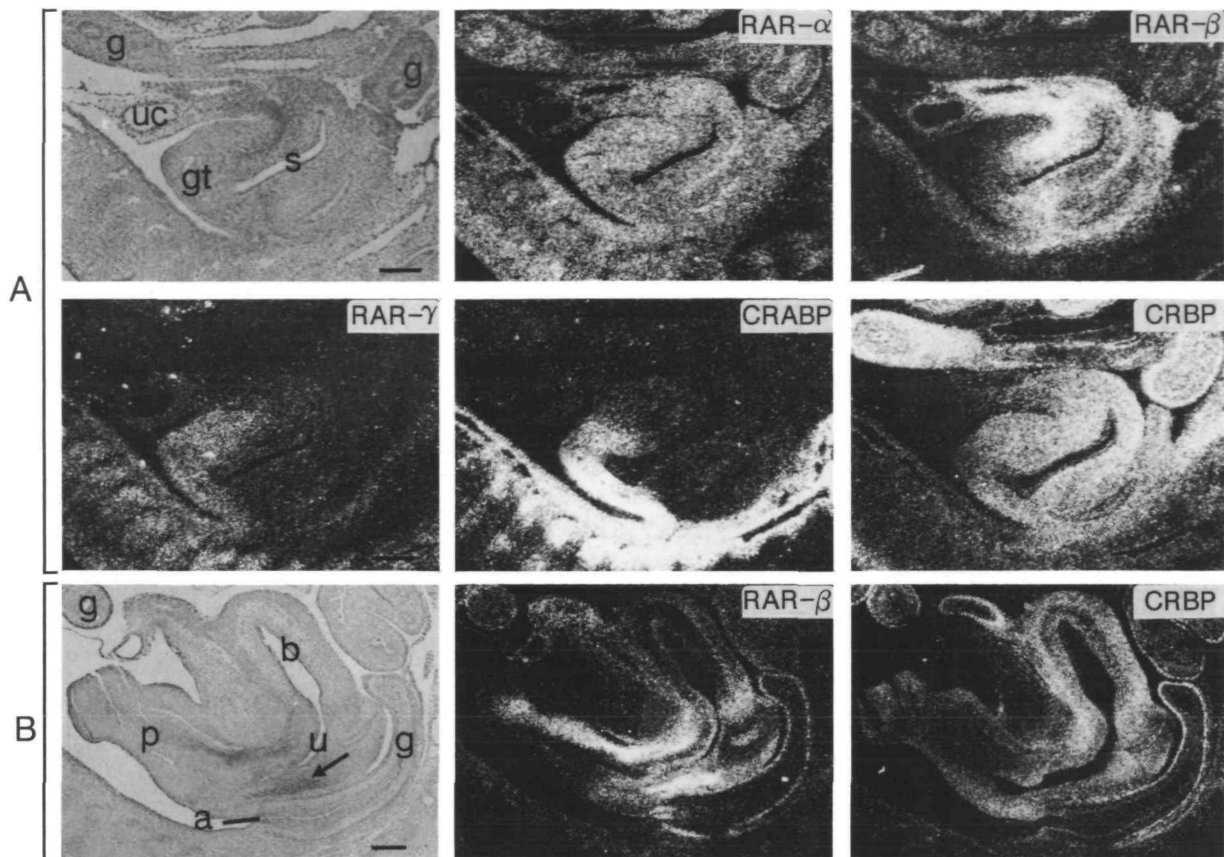

Fig. 6. RARs, CRABP and CRBP transcript domains in the genital eminence and posterior gut. (A) Midsagittal sections across the genital tubercle of a 12.5 day old fetus. Bar: 160 μm. (B) Midsagittal sections across the urinary bladder and phallus of a 14.5 day old male fetus. The arrow points to the developing prostate area. Bar: 250 μm. g=gut; uc=umbilical cord; gt=genital tubercle; s=urogenital sinus; b=urinary bladder; u=urethra; p=phallus; a=anal canal.

CRBP transcripts (Fig. 4A and data not shown). Similar transcript distributions were observed at 14.5 days *p.c.*, except that CRABP was no longer expressed in the heart.

Urogenital system

A differential expression of RARs, CRABP and CRBP transcripts was observed in the mesonephroi, which are transitory paired segmented kidneys, derived from the intermediate mesoderm. RAR-α transcripts were ubiquitously found, whereas no significant RAR-γ expression was detected (data not shown). Diffuse RAR-β transcripts were detected in the mesonephros by day 10.5 *p.c.*, but not in the cells of the genital ridge ventral to it (Fig. 5A, arrows). CRBP transcripts exhibited a similar distribution, but higher amounts were found in the mesonephric tubules (t). CRABP transcripts were more restricted to the mesenchyme in the vicinity of the aorta (ao) and posterior cardinal veins (cv) (Fig. 5A). In subsequent developmental stages, no RAR-β, -γ, CRABP or CRBP transcripts were found in the genital ridge and fetal gonad (not shown). In the developing metanephros (definitive kidney anlage) at 14.5 days, labelling with the RAR-β probe was seen among the stromal cells, with no obvious labelling of the metanephric tubules (tu) or glomeruli (gl) (Fig. 5B). CRBP labelling was also detected in the metanephros, and was particularly strong in the glomeruli. CRABP labelling was restricted to the cortical (nephrogenic) mesenchymal cells and to the metanephric capsule (Fig. 5B). RAR-β, RAR-γ, CRABP and CRBP transcripts could not be detected in the adrenal glands (Fig. 5B and data not shown).

The genital tubercle (gt) is the sexually undifferentiated precursor of the male and female external genital organs. Only RAR-α transcripts were ubiquitously distributed in this region at 12.5 days *p.c.* (Fig. 6A). RAR-β transcripts were restricted to the proximal mesenchyme of the genital tubercle, close to the urogenital sinus (s), and to the allantoic mesenchyme within the umbilical cord (uc). The RAR-γ expression pattern was almost complimentary to that of RAR-β, with RAR-γ transcripts absent from the central core, but present in the distal tip. CRABP transcripts were even more restricted to the distal mesenchyme, whereas CRBP transcripts were abundant in the mesenchyme around the urogenital sinus, decreasing towards the distal tip of the genital tubercle. Such regionally restricted expression persists in later stages. For instance, RAR-β transcripts were still restricted to the central core of the phallus (p) of the 14.5-day-old fetus, where CRBP transcripts were very poorly expressed

(Fig. 6B). In contrast, at the same stage, RAR-γ transcripts were uniformly expressed in the distal tip of the phallus, whereas they were excluded from the central core in which RAR-β transcripts were abundant (data not shown). RAR-β transcripts were also detected in the urethral epithelium (u) and in the prostatic mesenchyme at the base of the urinary bladder (b) (arrow in Fig. 6B), but were only weakly expressed in the bladder epithelium (Fig. 6B).

Sense organs

Distinct domains of transcription of RARs, CRABP and CRBP were observed in three developing sensory structures: the eye, the inner ear and the olfactory system. Within these three sense organs, RAR-α transcripts were homogeneously expressed in all cell types, and RAR-γ transcripts were restricted to surrounding precartilaginous and cartilaginous areas (data not shown; Ruberte *et al.* 1990). At 10.5 day *p.c.*, no RAR-β transcripts could be detected in the eyes. The CRBP gene was strongly expressed throughout retina and lens, whereas a weak expression of CRABP could be seen in the neural retina (Fig. 2A). Within the developing eye at 12.5 days, RAR-β transcripts were located in the pigmented retina (p), the vitreous body (v), and in the condensed mesenchyme around the eye cup which will later form the choroid layer (ch) (Fig. 7A). CRABP transcripts were seen in the central area of the neural retina (n), and in the innermost layer of the prechoroidal mesenchyme. CRBP transcripts were present in the neural and pigmented layers of the retina, with particularly strong signals in the superficial layer of the neural retina, which at this stage contains some newly post-mitotic retinal ganglion cells (Dräger, 1985). The pigment layer at this stage is already synthesizing melanin (Silver and Sapiro, 1981). Only CRBP transcripts were detected in the lens (l), where they were located in the superficial cell layer.

Within the developing inner ear, RAR-β transcripts were strongly expressed in the mesenchyme (m), as well as in the thickened parts of the vestibular epithelium (e), which will acquire a sensory function (Fig. 7B). CRABP transcripts were generally not detected in the inner ear, except in a very limited area of the cochlea epithelium (not shown), which may be the presumptive organ of Corti. In contrast, CRBP transcripts were abundant in the inner ear mesenchyme and in most of the epithelium (Fig. 7B).

The nasal cavities develop from the olfactory pits, which are invaginations of an ectodermal placode, and are composed of two functionally distinct regions. The roof of each nasal cavity is the olfactory portion and contains the sensory receptor cells, while the remainder of the nasal cavities, the respiratory portion, is lined by a non-sensory epithelium. By day 10.5 *p.c.*, transcripts of RAR-β, RAR-γ and CRABP were undetectable in the ectodermal component of the olfactory pits (data not shown), although the three transcript species were expressed in the fronto-nasal mesenchyme (cf. see Fig. 2A). Labelling with the CRABP probe was particularly intense in the mesenchyme closely surrounding the olfactory pits (not shown). In contrast, CRBP transcripts were uniformly expressed in the epithelial and mesenchymal layers of the olfactory pits (not shown). By 13.5 days *p.c.*, the RAR-β gene was not expressed in respiratory epithelium and mesenchyme (data not shown). In contrast, RAR-β transcripts were present in the olfactory mesenchyme (m) and in a limited area of the olfactory epithelium (e) (Fig. 7C). CRABP transcripts were abundant in the olfactory epithelium and respiratory mesenchyme, but absent from the respiratory epithelium and olfactory mesenchyme (Fig. 7C and data not shown). Examination of the developing olfactory epithelium at higher magnification revealed a distinct distribution of RAR-β and CRABP transcripts. RAR-β transcripts were detected in the apical layer of the epithelium, whereas the strong CRABP labelling was more basal (Fig. 8A). This differential labelling may reflect cell-type-specific expression, since the nuclei of the developing olfactory receptor cells are situated at this stage in the middle zone of the epithelium, while supporting cells differentiate from the apical layer (Cuschieri and Bannister, 1975). CRBP transcripts, which were strongly expressed in the mesenchyme surrounding the nasal cavities (Figs 7C and 8A), were restricted to the respiratory portion of the epithelium (not shown).

Skin and whisker follicles

The distribution of RAR and CRABP transcripts in the whole fetal skin was identical to that reported previously in the skin of the fetal limb (Dollé *et al.* 1989). RAR-β transcripts could not be detected in any of the skin layers, whereas RAR-α and RAR-γ transcripts were expressed during late gestation in both epidermis and dermis, and CRABP transcripts were specific to the dermal cells. CRBP transcripts could not be detected in the differentiating skin (not shown). The hair and whisker follicles initially develop from epidermal placodes and invaginations, upon local induction from the underlying dermis. Interestingly, strong CRABP labelling was detected in dermal cells that lie in the close vicinity of the developing whisker follicles, whereas the epidermal component was not labelled (Fig. 8B). In contrast, the RAR-γ labelling, which was weaker, was homogeneously distributed in both cell types, and RAR-β transcripts could not be detected.

Limbs

The distribution of RARs and CRABP transcripts during limb development has been described previously (Dollé *et al.* 1989; see also Fig. 9). We have searched for CRBP transcripts in the developing limbs, and found no significant signal in day 9.5 and 10.5 *p.c.* limb buds (not shown). These results are consistent with an immunohistochemistry study on chick limb buds, where CRBP could not be detected at an equivalent developmental stage (Maden *et al.* 1989*b*). By days 12.5 to 14.5 *p.c.*, CRBP transcripts were detected only in the mesenchyme of the distal extremities of the limbs, mainly in the interdigital mesenchyme, in a domain which is overlapping, but more extended than that of RAR-β

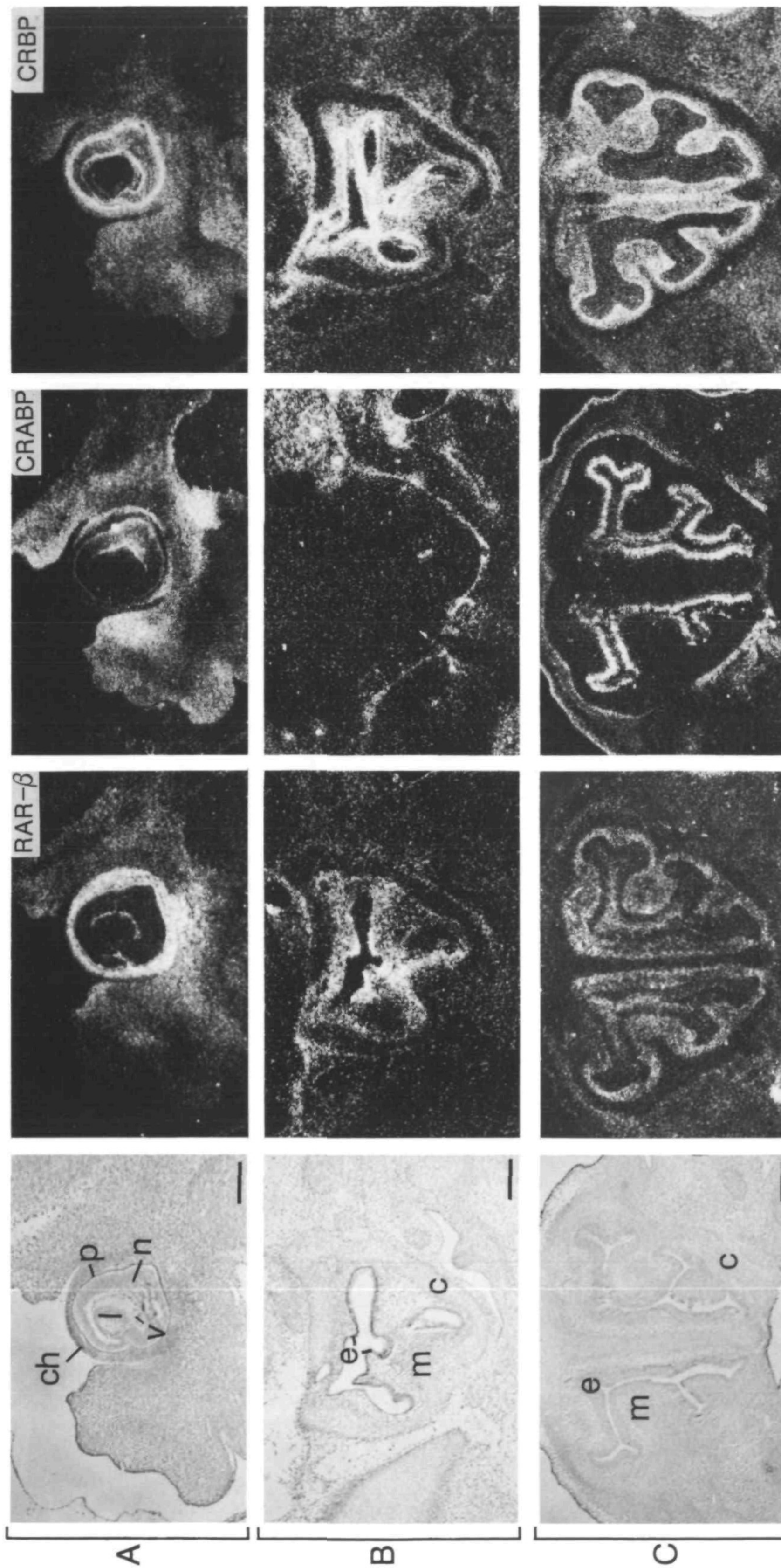

Fig. 7. RAR-β, CRABP and CRBP transcript domains in sensory organs. (A) Sections through the eyeball of a 12.5 day old fetus. Bar: 160 μm. (B) Sections through the inner ear of a 14.5 day old fetus. Bar: 160 μm. (C) Transverse sections through the olfactory portion of the nasal cavities of a 13.5 day old fetus. Bar: 200 μm. v=vitreous body; ch=choroid; p=pigment layer; l=lens; n=neural retina; e=epithelium; m=mesenchyme; c=cartilage.

transcripts (Fig. 9), and distinct from those of CRABP and RAR-γ.

Pituitary gland

The anterior pituitary arises from the epithelium of Rathke's pouch, an invagination of the roof of the oral ectoderm. By day 10.5 *p.c.*, the epithelium of the still open Rathke's pouch was specifically labelled by RAR-α, RAR-β and CRBP probes, but not by those for RAR-γ and CRABP (not shown). By day 12.5 *p.c.* (Fig. 10A), the anterior pituitary anlage (pars distalis, d) is visible as a cell proliferation that originates from the ventral side of the closed Rathke's pouch. RAR-α transcripts were ubiquitously expressed, whereas RAR-γ transcripts were not seen. RAR-β transcripts were detected in cells of the pars distalis anlage and in the adjacent mesenchyme (Fig. 10A); the dorsal aspect of Rathke's pouch epithelium, which will form the intermediate lobe (i) of the pituitary, was very poorly labelled. In contrast, CRBP transcripts were found in both the pars distalis and intermediate lobe. Neither of these two probes labelled the neurohypophysis anlage (n). CRABP transcripts were detected only in some surrounding mesenchymal cells. By day 14.5 *p.c.* (Fig. 10B), RAR-β transcripts were still preferentially located in the pars distalis (in both epithelium and mesenchyme), while CRBP transcripts were observed in mesenchymal, but not epithelial, tissues of the whole pituitary gland.

Discussion

In the present study, we have systematically compared the distribution of transcripts coding for RAR-α, RAR-β, RAR-γ, CRABP and CRBP in differentiating organ systems during the second half of mouse development. Table 1 summarizes their distribution in various fetal organs. We have previously shown (Dollé *et al.* 1989; Ruberte *et al.* 1990) that RAR-γ gene expression is restricted during the second half of mouse development to differentiating cartilage and cornified squamous epithelia, irrespective of their embryological origin. We show here that the expression of the RAR-β gene is also spatio-temporally restricted, and that RAR-β and

Table 1. *Schematic summary of the expression pattern of RAR-α, -β and -γ, CRABP and CRBP genes in various differentiating organs*

	RAR-α	RAR-β	RAR-γ	CRABP	CRBP
SUBMANDIBULAR GLAND	+	* mesenchyme	* epithelium	−	* mesenchyme
THYROID GLAND	+	−	−	−	+
TRACHEA epithelium	+	+	−	−	+
TRACHEA mesenchyme	+	+	+	−	+
LUNG	+	* bronchi	* late	−	* parenchyma
OESOPHAGUS	+	+	* late	−	+
GUT epithelium	+	* spatially restricted	−	−	+
GUT mesenchyme	+	+	−	* dorsal mesentery	+
LIVER	+	−	−	−	+
PANCREAS	+	* mesenchyme?	−	−	+
HEART	+	−	* endocardial cushions	* adj. to end. cushions	* epicard
GONAD	+	−	−	−	−
KIDNEY	+	* stroma	−	* cortex	* glomeruli
ADRENAL GLAND	+	−	−	−	−
EYE	+	* mesenchyme choroid	−	* neural retina	* retina lens
SKIN	+	−	* late	* dermis	−
PITUITARY	+	+	−	−	+
OLFACTORY epithelium	+	+	−	+	−

(+) and (−) indicate the presence or absence, respectively, of the corresponding mRNA. (*) indicates that the expression of the corresponding gene is restricted, either temporally, or to a cell subset of the organ, as indicated.

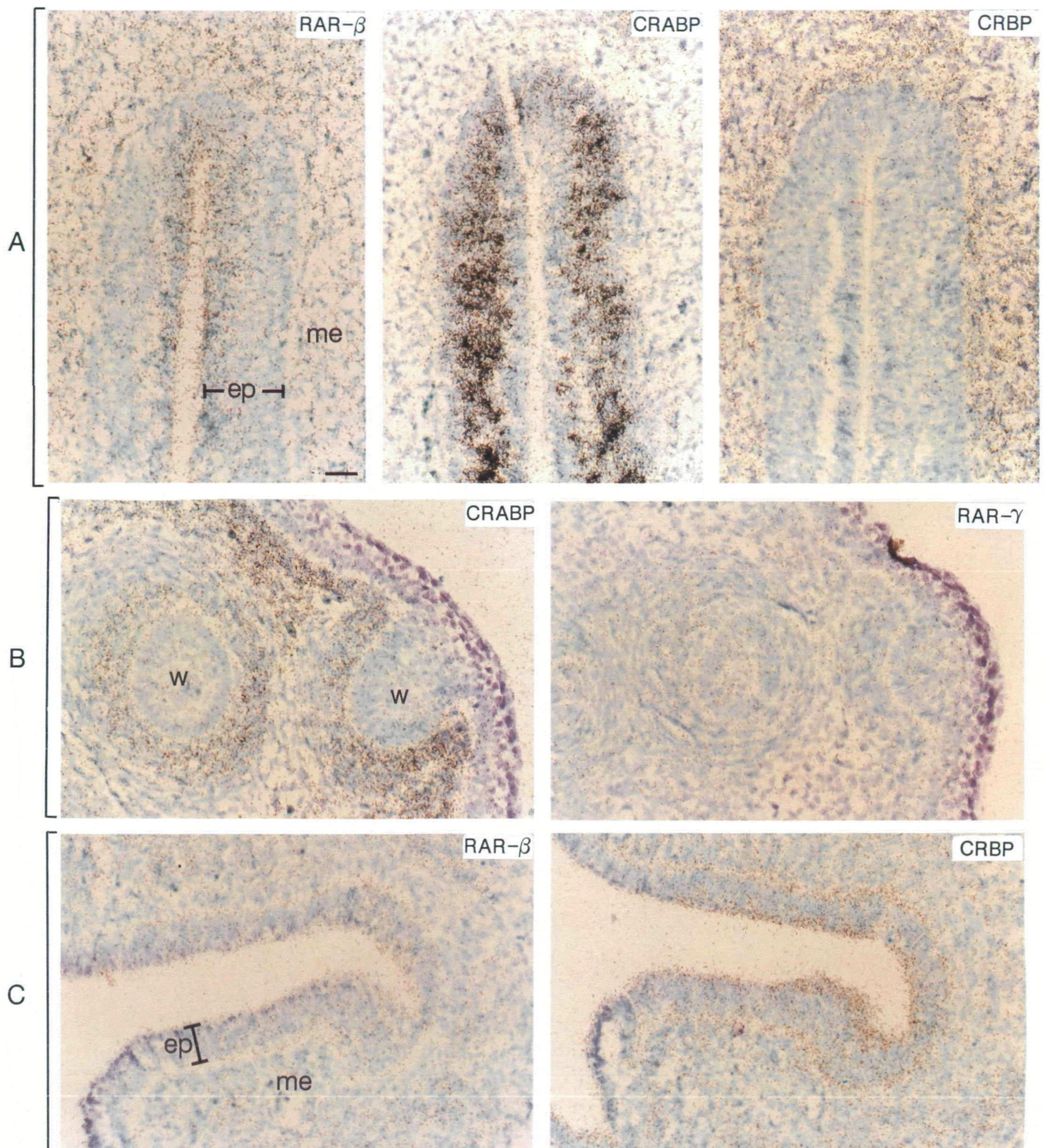

Fig. 8 (A) Differential distribution of RAR-β and CRABP transcripts in the nasal epithelium of a day 14.5 *p.c.* fetus. CRBP transcripts are restricted to the surrounding mesenchyme. Bar: 50 μm. (B) Differential distribution of CRABP and RAR-γ transcripts in whisker follicles of a 14.5 day old fetus. (C) Detection of RAR-β and CRBP transcripts in the stomach epithelium of a 12.5 day old fetus. ep=epithelium; me=mesenchyme; w=whisker follicle.

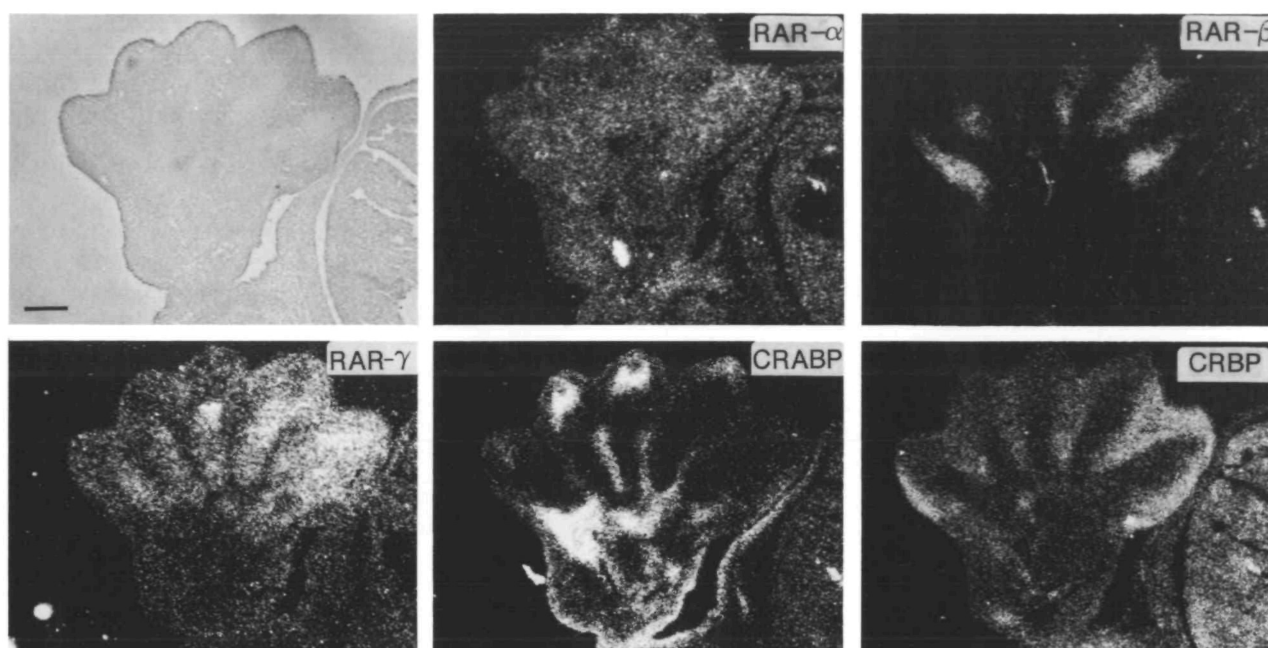

Fig. 9. Transcript domains of the RARs, CRABP and CRBP genes in the hindfoot plate (distal extremity of the hindlimb) of a 13.5 day old fetus. Bar: 160 μm.

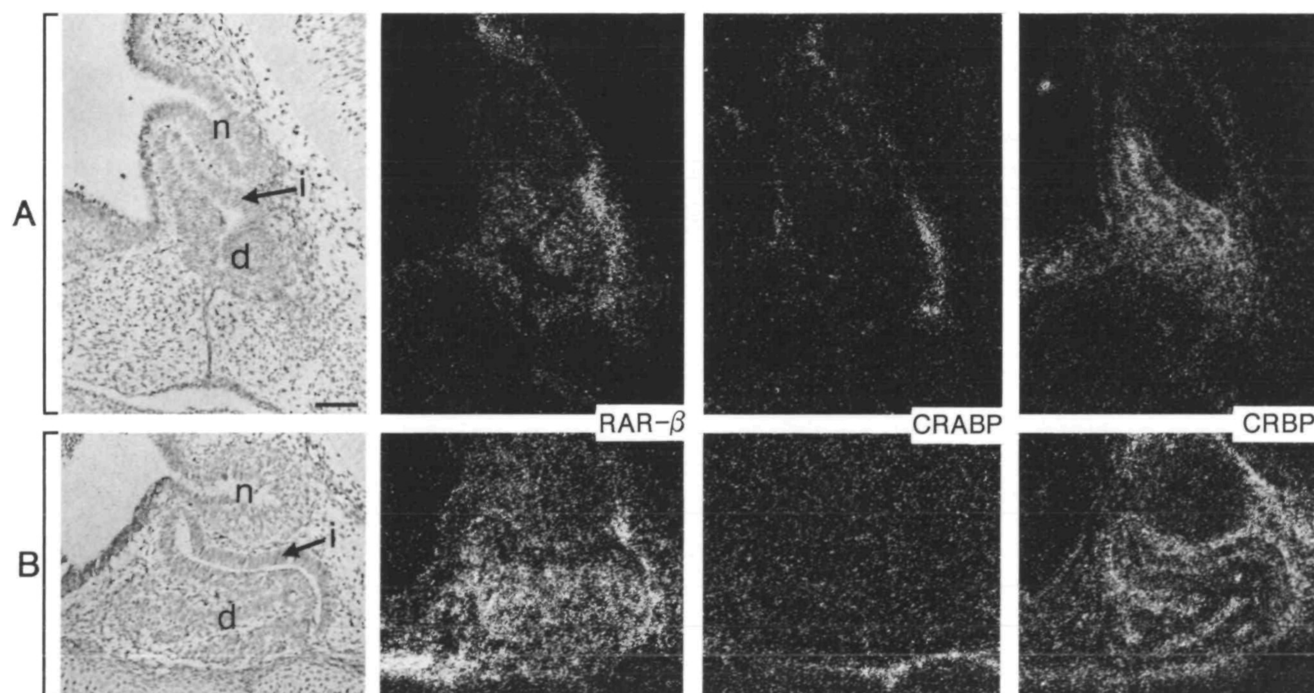

Fig. 10. RAR-β, CRABP and CRBP transcript domains in the developing pituitary gland. (A) Sagittal sections of the pituitary area of a 12.5 day old fetus. Bar: 100 μm. (B) Sagittal section of the pituitary area of a 14.5 day old fetus. n=neurohypophysis; i=pars intermedia; d=pars distalis.

RAR-γ transcript distribution is in general mutually exclusive in late gestational stages. Only RAR-α transcripts appear to be ubiquitously distributed in the mouse fetus. Thus, coexpression of at least two RARs is a common phenomenon in a large number of structures of the mouse fetus. However, several organs, such as the liver, thyroid gland, thymus, gonad or adrenal gland, were found to express only RAR-α transcripts. Altogether, these data indicate that the diversity of RA effects throughout development may be accounted for by differential, tissue-specific expression of the three RAR genes, possibly with the assistance of specific

expression of CRBP and CRABP. In this respect, it is important to note that an additional level of specificity may be provided by the recent finding that multiple isoforms are generated from each of the three RAR genes by differential splicing and multiple promoter usage (Krust *et al.* 1989; Kastner *et al.* 1990; unpublished results of our laboratory). Similarly, two types of CRBP and CRABP, termed I and II, encoded in different genes, have been described (see Introduction). The probes used here do not discriminate between the individual isoforms of the three RARs; the CRABP and CRBP probes correspond to type I binding proteins (see Introduction and below). Further *in situ* hybridization studies will be necessary to investigate whether this additional level of complexity also contributes to the diversity of RA effects.

Possible functions of RAR-β

RAR-β gene expression is topographically restricted within the craniofacial mesenchyme, but its distribution cannot be correlated with a subsequent cell fate. This is contrast with RAR-γ expression, whose distribution in the same region is initially rather general and later becomes specific to precartilaginous cells (Ruberte *et al.* 1990). At 10.5 days, RAR-β is strongly expressed in the frontonasal mesenchyme and only weakly in the caudal part of the first pharyngeal arch; RAR-γ is also expressed in the same regions, but more homogeneously. Morphological and cell lineage studies in rat embryos have indicated that the fronto-nasal mesenchyme is derived from the rostral midbrain neural crest, while the crest cells that populate the maxillary and mandibular mesenchyme are derived respectively from caudal midbrain and rostral hindbrain levels (Tan and Morriss-Kay, 1985 and 1986). Cranial neural cells from different levels of origin have some differences in developmental potential (Noden, 1983; Kirby, 1989); regional differences in RAR-β gene expression may play a role in this aspect of pattern formation.

RAR-β is the only RAR to show a clearcut specificity for endoderm derivatives. Transcripts are detected in the endoderm as early as day 8 *p.c.* (Ruberte *et al.,* unpublished data); in older embryos it is specifically expressed in the tracheobronchial epithelium, in the urogenital sinus epithelium, and in parts of the gut epithelium. As the tracheobronchial apparatus develops, RAR-β expression extends from large proximal bronchi towards the smaller peripheral bronchi. The onset of RAR-β gene expression in bronchial epithelium may therefore be concomitant with a precise differentiation step. The differentiated state of mature tracheal epithelium is modulated by vitamin A; deficiency induces keratinising metaplasia (Wilhelm, 1954; Chopra, 1983), while excess results in an increase in ciliated and mucus-secreting cells (Fell and Rinaldini, 1965; Boren *et al.* 1974). Vitamin A deficiency also decreases the number of goblet cells in the intestinal epithelium (Manville, 1937). Similarly, in the developing urogenital system, vitamin A deficiency is associated with epithelial keratinisation (Wilson and Warkany, 1948). Hence, RAR-β is a prime candidate to mediate retinoid effects on the differentiation status of tracheal, intestinal and genital tract epithelia.

The RAR-β gene is specifically expressed in cells of the developing adenohypophysis, especially in the pars distalis, as well as in the adjacent mesenchyme, and may thus play some role during the cytodifferentiation process of the anterior pituitary. Interestingly, RAR-β transcripts, but not RAR-γ transcripts, are also found in a number of developing sensory organs (e.g. inner ear, olfactory epithelium), which suggests that RAR-β may play a unique role in their formation.

The relationship between RAR-β and CRBP expression domains is interesting. In most epithelial tissues, such as the digestive tract, there is considerable overlap, although the CRBP domain is more extended. In mesenchymal tissues, there is either an overlapping (footplate, olfactory and inner ear mesenchyme) or a reciprocal pattern of expression domains (tooth bud, genital tubercle at day 14.5 *p.c.*). Reciprocal patterns are also found in sites of epithelial–mesenchymal interactions, such as lung (where RAR-β is epithelial and CRBP mesenchymal) and kidney (where RAR-β is mesenchymal and CRBP epithelial).

CRABP expression domains in relation to RARs

In general, our results are in good agreement with recent immunohistological (Maden *et al.* 1989*a*,*b*; 1990) or *in situ* hybridization (Perez-Castro *et al.* 1989) studies of CRABP I expression in the chicken, mouse and rat embryos. It is, however, noteworthy that the same mammalian CRABP I antibody preparation that was used by Maden *et al.* (1988) to show a distinct pattern of CRABP distribution in the chick limb bud, failed to reveal the presence of CRABP in rat and mouse limb buds, whereas some neural cells were clearly immunoreactive. In contrast our *in situ* hybridization studies (Dollé *et al.* 1989; this study and our unpublished data) and those of Perez-Castro *et al.* (1989) clearly demonstrate the presence of transcripts hybridizing with CRABP I probe in mouse limb buds. Maden *et al.* (1990) have interpreted their results by proposing that their antibody does not distinguish between CRABP I and II in the chick, but does distinguish between mouse CRABP I and II, because they are more dissimilar (see Introduction). According to these authors, CRABP I expression would then be restricted to the nervous system, and CRABP II to the limb. This suggestion is in agreement with the observation of Kitamoto *et al.* (1989) who found, using chick CRABP II antibodies, that CRABP II was expressed in skin, muscles and bones of the chick embryo, but not in the nervous system. It is therefore possible that the transcripts detected in the mouse limb with the present CRABP I probe are in fact CRABP II transcripts. Since the murine CRABP II cDNA has not yet been cloned (see Introduction), we do not know whether murine CRABP I and II can readily cross-hybridize. Further work is clearly required to elucidate this question, and to ascertain whether CRABP I and II have specific

patterns of expression in the mouse embryo, as appears to be the case in the chick embryo.

In our present study, CRABP transcripts are expressed in a highly regulated manner in differentiating mesenchyme. In the craniofacial mesenchyme at 12.5 days, high amounts of CRABP transcripts are detected in superficial regions below the ectoderm, and transcripts decrease and disappear in more central regions. Similar spatial distributions of RAR-β, RAR-γ and CRABP transcripts are found in the mesenchyme of three developing regions: the limb, the craniofacial structures and the genital tubercle. In all of these structures, CRABP transcripts are preferentially superficial, while RAR-β transcripts are limited to central and/or proximal areas. RAR-γ expression is at first homogeneous, and later becomes restricted to precartilaginous cells. Such a similarity in the topographically restricted expression of RARs and CRABP in different developing structures suggests that a common mechanism of response to RA occurs during morphogenesis in these structures, which have distinct embryological origins.

A striking feature during the second half of mouse gestation is the spatial exclusion between CRABP and RAR-β transcript domains. CRABP expression is also exclusive of the late expression domain of RAR-γ, with the exception of the dermal layer of the skin. Such exclusive transcript distributions suggest that CRABP is not required for RAR-β and -γ activity. *In vitro* studies have indicated that CRABP may not be required for RAR activity: some cell lines that do not express CRABP respond to RA (e.g. Dover and Koeffer, 1982); in F9 EC cells, the biological activity of several RA analogs is in keeping with their affinity for RARs, but not necessarily with their affinity for CRABP (Darmon *et al.* 1988). Thus, our results support the idea that CRABP is not necessary for mediating the RA signal in a given cell, but rather that it may compete with the nuclear receptors for free RA. Accordingly, CRABP could modulate RA signalling in embryonic tissues in two important ways. First, a graded distribution of CRABP across a population of cells could create a gradient of 'non-CRABP bound' (i.e. free) RA, so that the amounts of RA available for binding and activating RARs would be maximal in cells lacking CRABP. A CRABP gradient has been found across the anteroposterior axis of the chick wing bud (Maden *et al.* 1988*a*) and a similar gradient of CRABP transcripts has been reported in the developing mouse limb (Perez-Castro *et al.* 1989). Although we have not observed such an anteroposterior CRABP transcript gradient, we found a gradient of CRABP transcripts along the proximodistal axis of mouse limbs (Dollé *et al.* 1989). Interestingly, similar graded distribution of CRABP transcripts were found in the present study in the craniofacial mesenchyme and in the genital tubercle. Second, the presence of CRABP in certain cell-types could serve to prevent RA from activating the RARs in these cells. This could account for the highly restricted late expression of CRABP in arterial walls, perichondrium, dermis, or in the cortical areas of certain organs.

CRBP expression

In agreement with recent immunohistochemical (Maden *et al.* 1990) and *in situ* hybridization (Perez-Castro *et al.* 1989) studies, CRBP I transcripts are widely expressed in the mouse embryo, although not ubiquitously. Restricted expression of CRBP is a possible mechanism for concentrating retinol supplied by the blood circulation in specific areas, where retinol can potentially be converted into RA. In this context, we note that regions of maximal CRBP expression, for instance in craniofacial mesenchyme, are distinct from those of intense CRABP expression. Similarly, in the developing tooth germ, CRBP and CRABP transcripts, which are strictly mesenchymal, have a complimentary distribution. CRBP transcripts are maximal in superficial areas of the dental sac and in the dental papilla, while CRABP transcripts are detected in deeper regions of the dental sac. The mutually exclusive expression of CRABP and CRBP genes is particularly striking in the case of the developing limb. At day 10.5 *p.c.*, there are no detectable CRBP transcripts in the limb bud, while CRABP transcripts form a proximodistal gradient (see Dollé *et al.* 1989). At day 13.5 *p.c.*, the CRBP transcripts are selectively located in the interdigital region, whereas the CRABP transcript distribution is almost complimentary, with maximum expression in the tip of the digits and in the proximal region of the footplate. It is noteworthy that administration of vitamin A is known to affect interdigital necrosis in rat limb bud (Sulik and Dehart, 1988), and that RAR-β is also selectively expressed in the interdigital region of the developing mouse limb (Dollé *et al.* 1989). It is tempting to speculate that the co-expression of CRBP may be necessary to generate the RA required for inducing RAR-β activity.

A similar pattern of mutual exclusivity between CRBP and CRABP, and coincidental or overlapping expression of CRBP and RAR-β, is seen in the genital tubercle and urogenital sinus mesenchyme. The high incidence of lower urogenital tract abnormalities in vitamin A-deficient embryos (Wilson and Warkany, 1948), considered together with our observations, clearly indicates the morphogenetic importance of controlled availability of RA to the cells expressing RARs in this region. In the heart and aortic arches, where vitamin A-deficiency is associated with ventricular septal defects and aortic arch anomalies (Wilson and Warkany, 1949), CRBP is expressed within the cardiac jelly-rich interventricular septum-forming tissues, with CRABP immediately adjacent, while in the aortic arches CRBP, but not CRABP, is expressed. Finally, we note that the CRBP gene is expressed in a variety of epithelia and parenchyma where CRABP transcripts are absent (see Table 1).

These patterns of expression of the two binding protein genes suggest clear differences in their roles. We suggest that the function of CRBP is to concentrate and store retinol in sites where retinoic acid is required in relatively high concentrations, so that retinol can be converted to RA in relation to specific morphogenetic processes. In addition to activating RARs where these

are co-expressed with CRBP, RA may be released from the cells so as to create a concentration gradient, within mesenchymal tissues. Conversely, CRABP may be expressed by cells whose normal developmental function requires low levels of RA. Sequestration of free RA by binding to CRABP would maintain low levels of active RA, and would also serve to steepen the gradient of free RA created by release from CRBP-containing cells.

Relation with other developmental genes

Similarities between the expression domains of RARs and other genes during mouse embryogenesis can provide clues to identify possible target genes of RARs. Although no characterised gene appears to display an identity of expression with that of any of our probes, some interesting comparisons can be made.

TGF-β1 and TGF-β2 are developmentally important molecules which have been localized in many of the structures that express the five genes studied here. However, careful comparison of the expression patterns of TGF-β1 RNA (Lehnert and Akhurst, 1988; Akhurst *et al.* 1990), TGF-β1 protein (Heine *et al.* 1987 and, 1990) and TGF-β2 RNA (Pelton *et al.* 1989) with our results reveals that there is no consistent correspondence of tissue expression between these molecules and any one of our five genes. Nevertheless, since exact comparisons were not always available, the possibility of correlations between the expression of TGF-β and retinoid-binding proteins or receptors should not be ruled out. For instance local similarities, such as that between TGF-β1 protein and two of our probes (RAR-γ and CRBP) in the endocardial cushion mesenchyme, RAR-β and CRBP in the lung, or between CRABP mRNA and TGF-β1 protein in the early vibrissa mesenchyme, may be of developmental significance.

The expression pattern of the homeobox-containing gene Hox 7.1 (Hill *et al.* 1989; Robert *et al.* 1989; Davidson *et al.* in preparation) exhibits some similarity to that of CRBP in the limbs, in the endocardial cushion tissue, and in the craniofacial mesenchyme. Correlations between the expression domains of other homeobox genes and those of our five genes within the central nervous system is discussed in our report of the earlier developmental stages (Ruberte *et al.* 1991).

Finally, a retinoic acid-responsive gene, MK, has been found to be expressed in a spatially restricted manner in the mouse embryo (Kadomatsu *et al.* 1990). Its wide expression domain mostly resembles that of CRBP, and includes areas of RAR-γ (craniofacial and limb bud mesenchyme) and RAR-β (abdominal viscera, pituitary) specific expression.

The importance of these and any other correspondences of gene expression patterns will become clearer when they can be compared on adjacent sections at a series of developmental stages. Coincidences, overlaps and complimentary distributions (e.g. related to epithelial–mesenchymal interactions) may all be developmentally significant. The results presented here show clearly that although the different RARs and the two retinoid binding proteins have distinct patterns of expression, there are many incidences of complimentary or overlapping domains which are consistent with the possibility that gradients within cell populations or differences between adjacent cells are present. Detailed study of specific developing systems such as sites of branching morphogenesis, the sense organs, the heart or the limbs, will further clarify the functional relationships between these molecules and retinoids in normal and abnormal development.

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