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Retinoic acid receptors and cellular retinoid binding proteins

III. Their differential transcript distribution during mouse nervous system development

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SUMMARY

We have studied the transcript distribution of the retinoic acid receptors (RARs) and the cytoplasmic retinoid binding proteins during embryonic development of the mouse nervous system. Of the three retinoic acid receptors, only RAR-γ was not expressed in developing neural structures. RAR-β and RAR-α both showed rostral limits of expression in the medulla oblongata equivalent to their patterns of expression in the neuroepithelium of the early hindbrain neural tube. Within their expression domains in the spinal cord and brain, RAR-α was ubiquitously expressed, whereas RAR-β transcripts showed very specific patterns of expression, suggesting that this receptor is involved in mediating retinoic acid-induced gene expression in relation to the development of specific neural structures or pathways.

The cytoplasmic binding proteins, cellular retinoic acid binding proteins type I and II (CRABP I and CRABP II) and cellular retinol binding protein type I (CRBP I), were widely distributed in developing neural structures. Their differential spatiotemporal patterns of expression suggest that fine regional control of availability of retinoic acid (RA) to the nuclear receptors plays an important role in organization and differentiation of the nervous system. For instance, expression of CRABP I in the migrating cells that give rise to the olivary and pontine nuclei, which develop abnormally in conditions of retinoid excess, is consistent with observations from a variety of other systems indicating that CRABP I limits the access of RA to the nuclear receptors in normal physiological conditions. Similarly, expression of CRBP I in the choroid plexuses, which develop abnormally in conditions of vitamin A deficiency, is consistent with observations indicating that this binding protein mediates the synthesis of RA in tissues requiring high levels of RA for their normal developmental programme. RAR-β and CRABP II, which are both RA-inducible, were coexpressed with CRBP I in the choroid plexus and in many other sites, perhaps reflecting the fact that all three genes are RA-inducible. The function of CRABP II is not well understood; its domains of expression showed overlaps with both CRABP I and CRBP I.

Key words: RARs, retinoid binding proteins, mouse nervous system development, in situ hybridization, mRNA distribution, mouse

INTRODUCTION

Vitamin A (retinol), and its active metabolite retinoic acid (RA), play important roles in vertebrate neural development. Retinoids (vitamin A and related compounds) are potent teratogenic agents in both excess and deficiency (Lammer et al., 1985; Morriss, 1972; Rosa et al., 1986; Shenefelt, 1972; Warkany et al., 1943; Wilson et al., 1953). Exposure to high levels of retinoids during the early stages of development interferes with the normal migration of the neural crest cells leading to craniofacial skeletal abnormalities (Morriss, 1972; Morriss and Thorogood, 1978; Webster et al., 1986). Retinoid excess also affects the normal proliferation and differentiation of the neural epithelium leading to brain and spinal cord deformities (Langman et al., 1966; Langman and Welch, 1967; Lammer and Armstrong, 1992). Experimental vitamin A deficiency in animals has been associated with degeneration of the cranial and peripheral nerves, and of the grey and white matter of the spinal cord (Wolbach and Bessey, 1941; Mellanby, 1944). Raised intracranial pressure has been described in the offspring of both vitamin A deficient mothers and mothers treated with excess retinoids.

RA exerts its effects by binding to nuclear receptors, retinoic acid receptors and retinoid X receptors (RARs and RXRs respectively) that cooperate to modulate the transcriptional activity of specific target genes (Benbrook et al., 1988; Brand et al., 1988; Giguère et al., 1987; Petkovich

et al., 1987; Zelent et al., 1989; Krust et al., 1989; Leid et al., 1992; Mangelsdorf et al., 1990, 1992). In addition to the nuclear receptors there exists a family of low molecular mass cytoplasmic proteins that bind retinol and RA with high affinity, cellular retinol binding protein (CRBP) and cellular retinoic acid binding protein (CRABP) respectively. Two CRBPs have been described, cellular retinol binding protein I and II (CRBP I and II; Blomhoff et al., 1990; Giguère et al., 1990; Stoner et al., 1989). They are involved in the conversion of retinol to retinaldehyde and then to RA (Napoli et al., 1991). Expression of CRBP may therefore indicate cells in which RA is synthesized. Only CRBP I is found in embryos. Similarly there exist at least two CRABPs, cellular retinoic acid binding protein I and II (CRABP I and II). There is good evidence that CRABP I binds and retains RA in the cytoplasm (Boylan and Gudas, 1991), and also facilitates its catabolism to polar metabolites (Napoli et al., 1991), so that little or no RA is available to bind to the nuclear receptors in CRABP I-containing cells. The function of CRABP II is less well understood. Mouse CRABP I and CRABP II have a sequence identity of 73% (Giguère et al., 1990). CRABP II has a lower affinity for RA than that of CRABP I, with a 15 times higher dissociation constant (Ong and Chytil, 1978; Bailey and Siu, 1988). In cells containing both binding proteins, RA does not appear to bind to CRABP II until the binding capacity of CRABP I is saturated (Bailey and Siu, 1988). Unlike CRABP I, CRABP II is inducible by RA (Giguère et al., 1990). Expression of CRABP II may therefore indicate cells in which RA is available for binding to the nuclear receptors, even where CRABP I is also present.

We have previously described the transcript distribution of the RARs and retinoid binding proteins during early stages of development (Ruberte et al., 1991, 1992a), during organogenesis (Dollé et al., 1990; Ruberte et al., 1992a) and limb development (Dollé et al., 1989; Ruberte et al., 1992 a,b) of the mouse.

We describe here their trancript distribution in the developing central nervous system from day 11.5 of gestation. The results are discussed in relation to the teratogenic effects of retinoids on the development of the nervous structures.

MATERIALS AND METHODS

^{35}S-labelled RNA probes were synthesized using T7-polymerase (according to the suppliers directions, Promega Biotec) from full-length cDNAs coding for RAR-α, RAR-β, RAR-γ, CRBP I, CRABP I and CRABP II cloned in an antisense orientation. The mouse RAR-α, β and γ, CRBP I, and CRABP I plasmids have been described in Dollé et al. (1990). The murine CRABP II plasmid has been described in Ruberte et al. (1992a). Probe length was reduced to an average of 150 nucleotides by limited alkaline hydrolysis, as described in Ruberte et al. (1990). In situ hybridization was performed on sections of day-10.5 to day-14.5 mouse embryos. Brains from 18.5 day foetuses and newborn mice were dissected out from skulls. 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). The time of autoradiographic exposure was 15 days for the nuclear receptors and CRBP I probes, 7 days for the CRABP I probe and 10-12 days for the CRABP II probe.

RESULTS

We have analyzed the transcript distribution of the RARs and retinoid binding proteins (CRABP I and II, and CRBP I) during development of the nervous system of the mouse. At every stage analyzed consecutive sections have been hybridized with at least two different probes to allow comparison of their transcript distribution. Only RAR-γ was not expressed in the differentiating nervous structures at the stages analyzed here (see Ruberte et al., 1990 and Fig. 5); it is therefore excluded from the description of the results.

Spinal cord

According to a general craniocaudal gradient of differentiation of the embryonic structures, the cranial regions of the spinal cord are more advanced in development than the caudal regions. These spatiotemporal differences are also reflected in the transcript distribution, so that at any one stage the most caudal regions of the neural tube show the transcript distribution pattern observed in more cranial regions at an earlier stage. From day 10.5 of development RAR-α transcripts were more abundant in the neuroepithelium (ventricular zone) of the spinal cord than in the differentiating mantle layer (intermediate zone; Fig. 1).

RAR-β transcripts were observed in the neuroepithelium of the spinal cord at 10.5 and 11.5 days of gestation (Fig. 1A,B). At 11.5 days transcripts were also detected in the mantle layer the most cranial regions of the spinal cord (Fig. 1B). At 12.5 days RAR-β transcripts were still found in the neuroepithelium, which has at this stage become thinner ventrally (adjacent to the basal plate). In the mantle layer of the basal plate, the signal has become progressively restricted to a group of presumptive motor neurons (Fig. 1C). No RAR-β expression was detected in the floor plate. At 14.5 days, when most of the neurons are in their final positions, RAR-β transcripts were found in a single (bilateral) group of motor neurons and in the dorsal horns and medial regions of the spinal cord (Fig. 1D). RAR-β expression was also detected in the ependymal layer (Fig. 1D).

Labelling with the CRABP I probe at 10.5 days was detected in the periphery of the neural tube, where some cells showed higher levels of transcripts (Fig. 2A, see also Fig. 1A). Bright-field examination of the sections revealed that the labelling was more intense in the cells bordering the neuroepithelium of the alar plate and in the most dorsal and lateral cells of the basal plate. At 11.5 days of gestation the signal in the neuroepithelial layer was more intense in the regions dorsal and lateral to the mantle layer. CRABP I transcripts were not observed in the ependymal layer, nor in the roof and floor plates (Fig. 1). The most caudal regions showed the pattern of expression observed more cranially at the previous stage, e.g.: the hindlimb level at 11.5 days (Fig. 1A) resembles the more cranial level at 10.5 days (Fig. 2A).

At 12.5 and 14.5 days the more cranial regions of the spinal cord showed CRABP I transcripts in the periphery

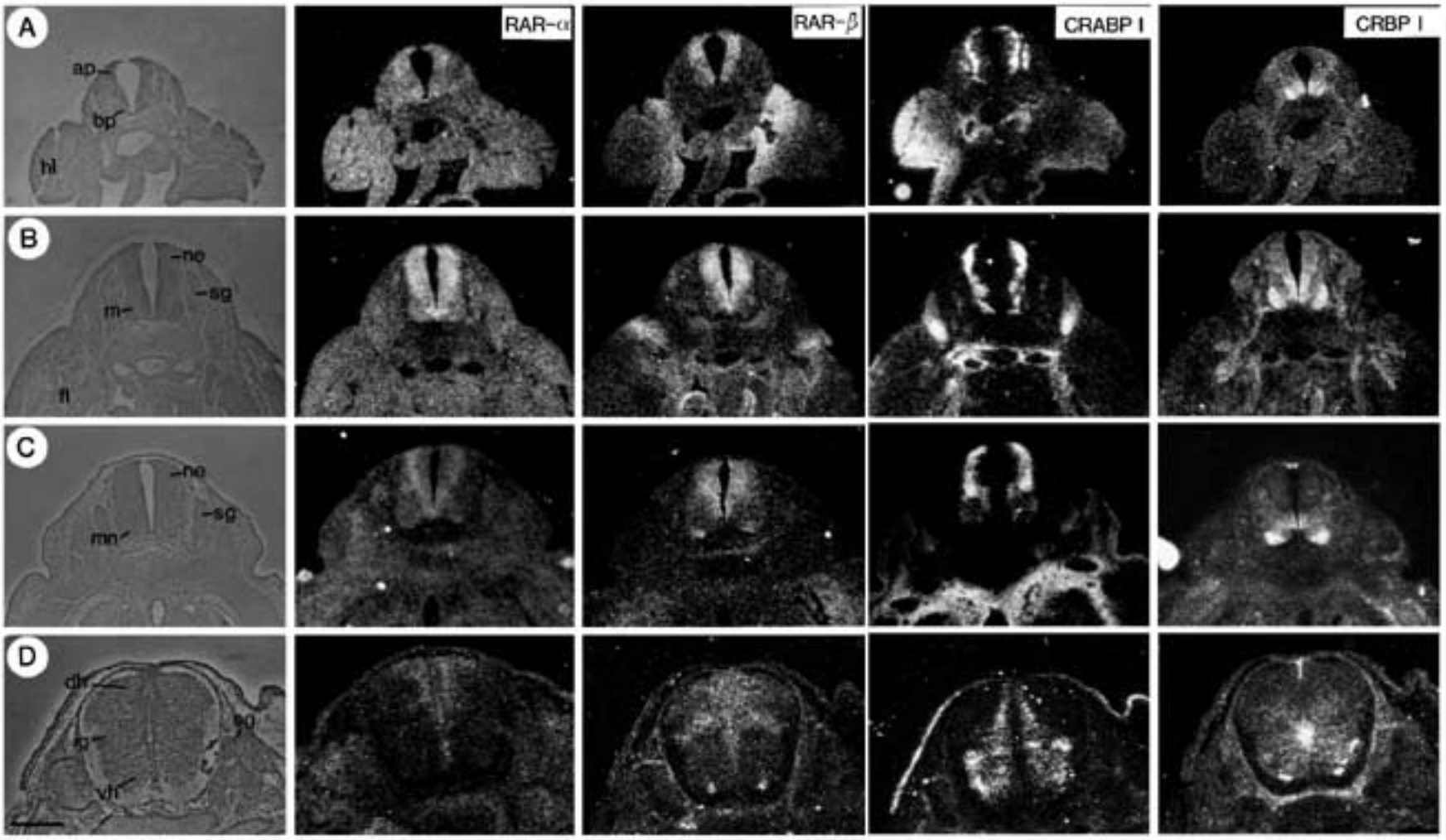

Fig. 1. Transverse sections through the spinal cord at three different developmental stages hybridized with RAR-α, RAR-β, CRABP I and CRBP I probes. (A,B) Transverse sections at the level of the hindlimbs (A) and forelimbs (B) of an 11.5 day embryo to illustrate the spatiotemporal changes occurring in development along the long axis, which are also reflected by the transcript distribution. (C) Transverse sections of a 12.5 day embryo (midtrunk level) when most of the basal neuroepithelium has matured to ependyma. (D) Transverse sections of a 14.5 day embryo (midtrunk level). At this stage most of the neurons are in their final positions. ap, alar plate; bp, basal plate; dh, dorsal horn; fl, forelimb; hl, hindlimb; ig, intermediate gray; m, mantle layer; mn, motoneurons; ne, neuroepithelial layer; sg, spinal ganglia; vh, ventral horn; Bar, 250 μm.

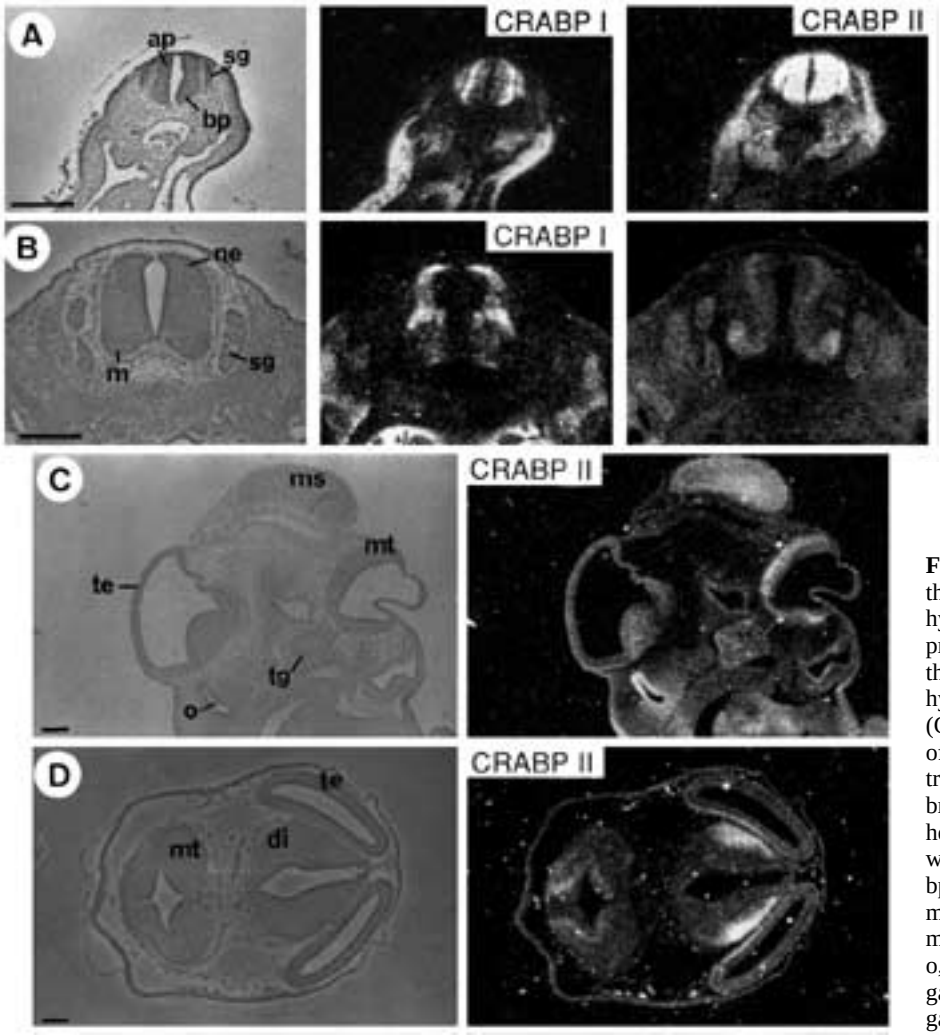

Fig. 2. (A) Transverse sections through the spinal cord of a 10.5 day embryo hybridized with the CRABP I and II probes. (B) Transverse sections through the spinal cord of a 13.5 day embryo hybridized with the CRABP probes. (C) Parasagittal section through the head of a 12.5 day embryo to illustrate the transcript distribution of CRABP II in the brain. (D) Transverse section through the head of a 13.5 day embryo hybridized with the CRABP II probe. ap, alar plate; bp, basal plate; di, diencephalon; m, mantle layer; ms, mesencephalon; mt, metencephalon; ne, neuroepithelial layer; o, olfactory epithelium; sg, spinal ganglia; te, telencephalon; tg, trigeminal ganglion; Bar, 250 μm.

of the neuroepithelium and in the intermediate gray matter as at the previous stage, but the labelling in the mantle layer of the basal plate was more extensive (Figs 1C, 2B). At 14.5 days CRABP I transcripts were detected preferentially in the mantle layer of the basal plate and in the cells close to the ependymal layer of the alar plate (Fig. 1D). At 16.5 and 17.5 days CRABP I transcripts were seen in the ventral horn and intermediate gray matter of the spinal cord. No CRABP I transcripts were detected in the ependymal and marginal layers (Fig. 3C).

At 10.5 days CRABP II transcripts were detected in the neuroepithelium of the spinal cord (Fig. 2A) and a more intense signal was detected in the differentiating motor neurons in the basal plate (not illustrated). At 11.5 and 12.5 days the neuroepithelium still showed CRABP II transcripts and the signal in the basal plate motoneurons was clearer than at previous stages (data not shown). At 13.5 days transcripts were detected in the presumptive motor columns of the basal plate and in the newly formed neuroblasts in the alar plate. Low levels of transcripts were also detected in the intermediate horn of the spinal cord (Fig. 2B). No CRABP II transcripts were detected in the ependymal layer, or in roof and floor plates.

At 10.5 and 11.5 days of development, CRBP I transcripts were observed in the neuroepithelium of the spinal cord, higher levels being detected in the ventral half (Fig. 1A,B). No CRBP I expression was found in the floor plate. CRBP I transcripts were very abundant in the differentiating motor neurons of the basal plate and from day 11.5 labelling with the CRBP I probe was detected in the ventral roots, and in general in all the developing spinal nerves (Figs 1B, 3B). At 12.5 days of gestation transcripts were still seen in the ventral half of the neuroepithelium, although the signal was less intense than in the motor cells of the mantle layer (Fig. 1C). From 14.5 days of gestation CRBP I transcripts were seen in the ventral horn and in the motor columns of the spinal cord, as well as in the ependymal cells (Figs 1D, 3C).

Cranial and dorsal root ganglia

RAR-α transcripts were ubiquitously present in cranial and spinal ganglia at every stage analyzed (Figs 1 and 5). The dorsal root ganglia, which at 11.5 days expressed low levels of RAR-β transcripts (Fig. 4), showed no detectable signal at later developmental stages (Fig. 1B and C).

CRABP I and II transcripts were both detected in cranial ganglia from their formation and they were present throughout all the stages studied. The cranial ganglia were

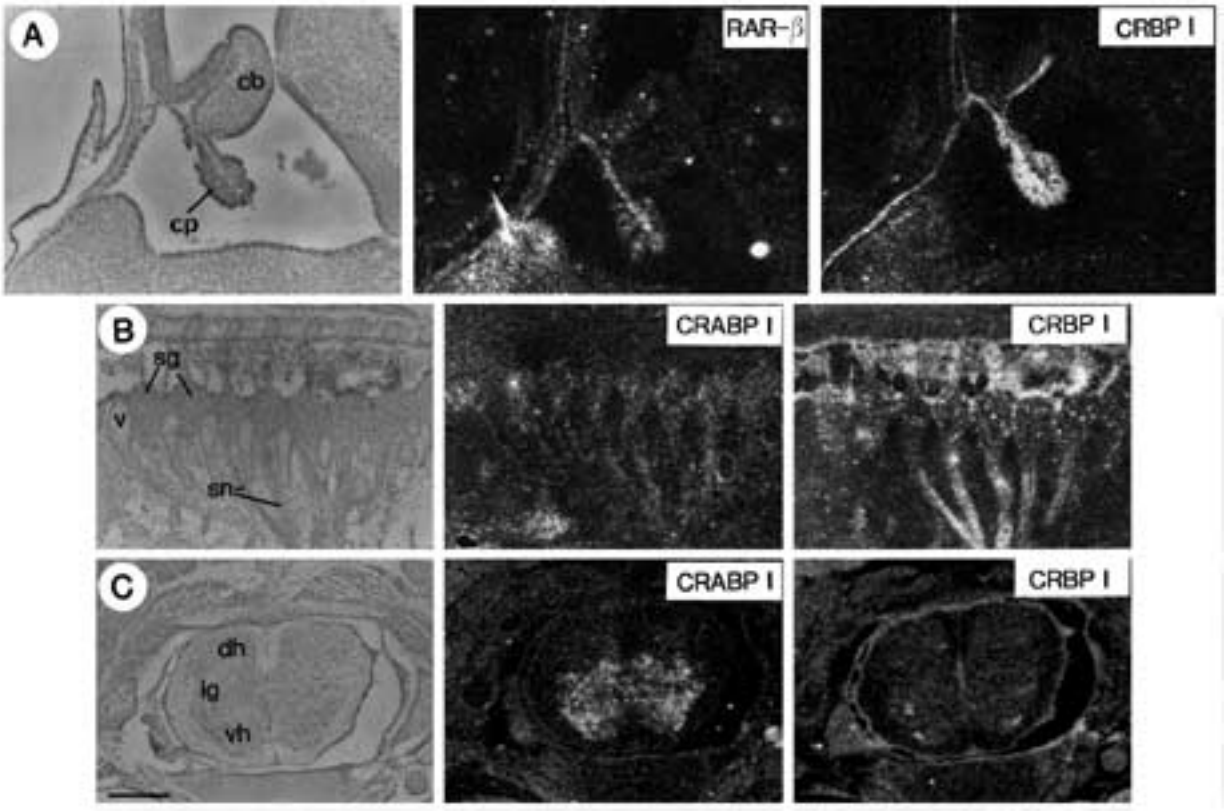

Fig. 3. (A) Sagittal sections through the cerebellum and choroid plexus of a 14.5 day embryo hybridized with the RAR-β and CRBP I probes. (B) Sagittal sections through the cervical plexus of a 14.5 day embryo hybridized with the CRABP I and CRBP I probes. (C) Transverse sections through the spinal cord of a 17.5 day foetus hybridized with the CRABP I and CRBP I probes. cb, cerebellum; cp, choroid plexus; dh, dorsal horn; ig, intermediate gray; sg, spinal ganglia; sn, spinal nerves; v, vertebrae; vh, ventral horn; Bar, 250 μm.

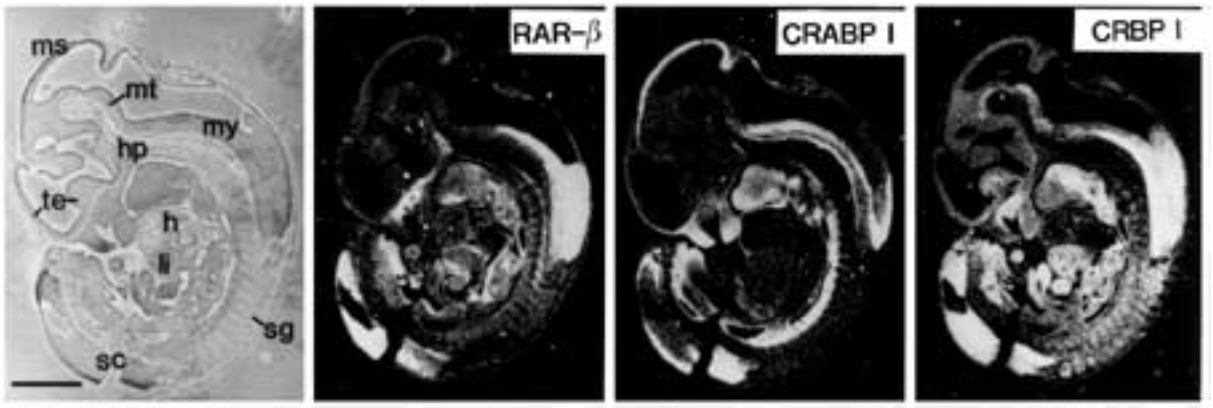

Fig. 4. Parasagittal sections of an 11.5 day embryo hybridized with RAR-β, CRABP I and CRBP I probes. h, heart; hp, hypophysis; li, liver; ms, mesencephalon; mt, metencephalon; my, myelencephalon; sc, spinal cord; sg, spinal ganglia; te, telencephalon; Bar, 250 μm.

at first homogeneously labelled with the CRABP I probe; from day 12.5 they showed a punctate labelling indicating that only a subpopulation of cells maintained expression of CRABP I (Fig. 6C). CRABP I and II transcripts were both detected in the dorsal root ganglia from the very early stages since they were present in the migrating neural crest cells from which they originate. By day 11.5 of gestation, and in a craniocaudal sequence, the dorsal root ganglia showed a decrease in the intensity of labelling with the CRABP I probe and it was very low or completely absent by day 12.5 of gestation (Fig. 1, compare A to B-D and 2A and B). At day 15.5 low levels of CRABP I transcripts were again detected in the dorsal root ganglia and peripheral nerves (Fig. 3B). With the CRABP II probe the labelling in the dorsal root ganglia persisted throughout all the stages analyzed (Fig. 2A,B and data not shown).

CRBP I transcripts were detected in the cranial and dorsal root ganglia from day 11.5 (Figs 1, 4, 3B). The spinal

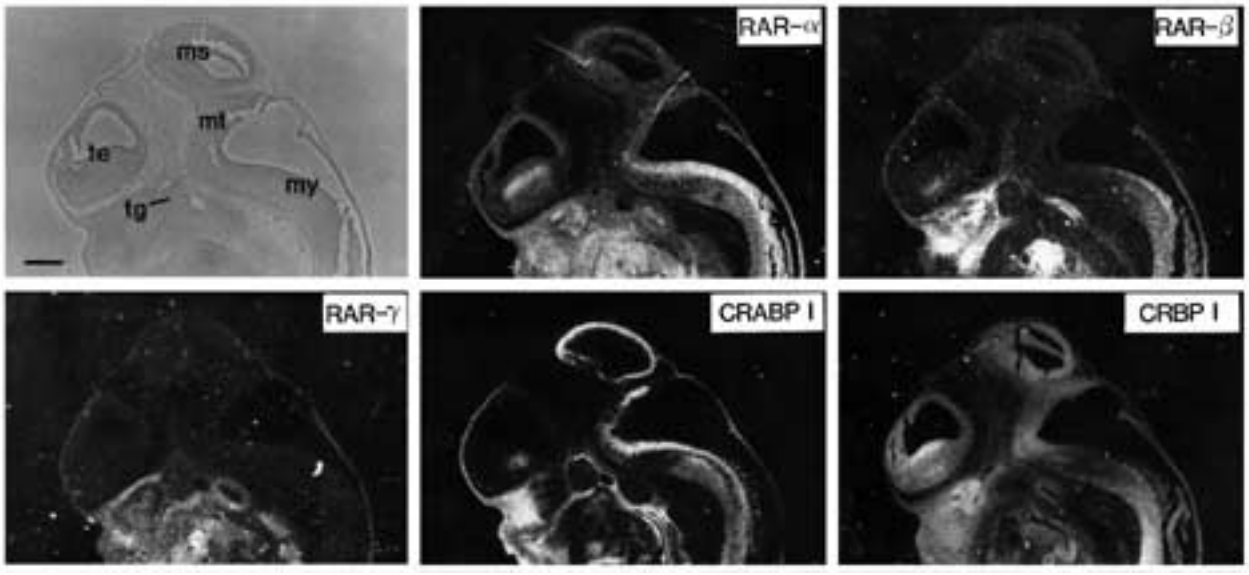

Fig. 5. Parasagittal sections through the head of a 12.5 day embryo to show the transcript distribution of the RARs, CRABP I and CRBP I transcripts in the brain vesicles. ms, mesencephalon; mt, metencephalon; my, myelencephalon; te, telencephalon; tg, trigeminal ganglion; Bar, 250 μm.

nerves also appeared to be labelled with the CRBP I probe (Figs 1B and 3B).

Brain

Up to 12.5 days of development RAR-α transcripts were found in the myelencephalon but were very low or absent in the rest of the brain vesicles (data not shown), as previously observed on day 9.5 (Ruberte et al., 1991). From day 12.5 of gestation RAR-α transcripts were also found in the forebrain, where they were most abundant in the developing corpus striatum (Fig. 5).

At 10.5 and 11.5 days of gestation RAR-β transcripts in the brain were restricted to the caudal myelencephalon, consistent with their earlier pattern of expression (Fig. 4). From 12.5 days RAR-β transcripts were also found in the corpus striatum and the olfactory tubercle (Figs 5, 6D, 7A). In the caudal myelencephalon, transcripts were more abundant in the neuroepithelial layer than in the mantle layer and the signal was particularly intense in the general somatic efferent group of the basal plate (Fig. 6A). At 13.5 days of development, RAR-β transcripts were detected in the general somatic and visceral motor nuclei of the medulla oblongata (nucleus hypoglossus, nucleus dorsalis of vagus; Fig. 7C). At 18.5 days of gestation RAR-β transcripts could only be detected in the corpus striatum (caudate-putamen), in the accumbens nucleus and in the tuberculum olfactivum (Fig. 6D).

CRABP I transcripts at 10.5 and 11.5 days were seen in the outer cells of the dorsal mesencephalon and in the thin roof plate of the metencephalon, and were more generally distributed in the myelencephalon (Fig. 4). At this stage, labelling with the CRABP I probe was also detected in the diencephalon-derived optic stalk (data not shown). At 12.5 days of development CRABP I transcripts appeared in the corpus striatum (Fig. 5). In the dorsal mesencephalon CRABP I transcripts were found, as at the previous stage, in the outer cells (Fig. 6B). In the metencephalon CRABP I transcripts were detected in the mantle layer and they showed a rather patchy distribution (Fig. 6B). Labelling with the CRABP I probe was also seen in the intermediate layer of the pontine region and upper medulla, leaving a stripe of tissue in which no signal was detected (Fig. 6B, arrowheads). Transcripts were also detected in a group of cells in the mantle layer of the basal plate. In the caudal part of the medulla oblongata, CRABP I transcripts were present in both the alar and basal plates, being most abundant in the lateral region of the alar plates (Fig. 6A). Cells from the alar plate of the rhombencephalon migrate ventrally to contribute to the olivary and pontine nuclei; CRABP I transcripts in this region may be associated with these migratory cells.

At 13.5 days of gestation CRABP I transcripts were still detected in the corpus striatum (Fig. 7A) and in the mesencephalon, where the pattern was the same as at earlier stages, although the intensity of the signal had decreased (data not shown). In the rostral medulla, abundant CRABP I expression was detected in the vestibular nuclei and in the sensory nucleus of the fifth cranial nerve (Fig. 7B). Labelling with the CRABP I probe was also detected in the prepositus of the hypoglossal nucleus (Fig. 7B). Transcripts were also detected in cell groups located ventrally on both sides of the midline. A very intense signal was also observed in a group of densely packed cells located at the outer margins of the lower pons/rostral medulla region lateral to the trigeminal nerve root. This signal extended caudally to cell groups bordering the alar plates of the rostral medulla, just below the choroid plexuses (Fig. 7B, arrow) and was continuous with a more ventral signal in the lower medulla (Fig. 7C, arrow). These labelled cells constitute the pontine migration and will contribute to the development of the olivary nuclei. At 15.5 days of development CRABP I transcripts were also detected in the cerebellum (data not shown). In 18.5 day fetal brain and in the newborn brain, the transcript distribution of CRABP I was

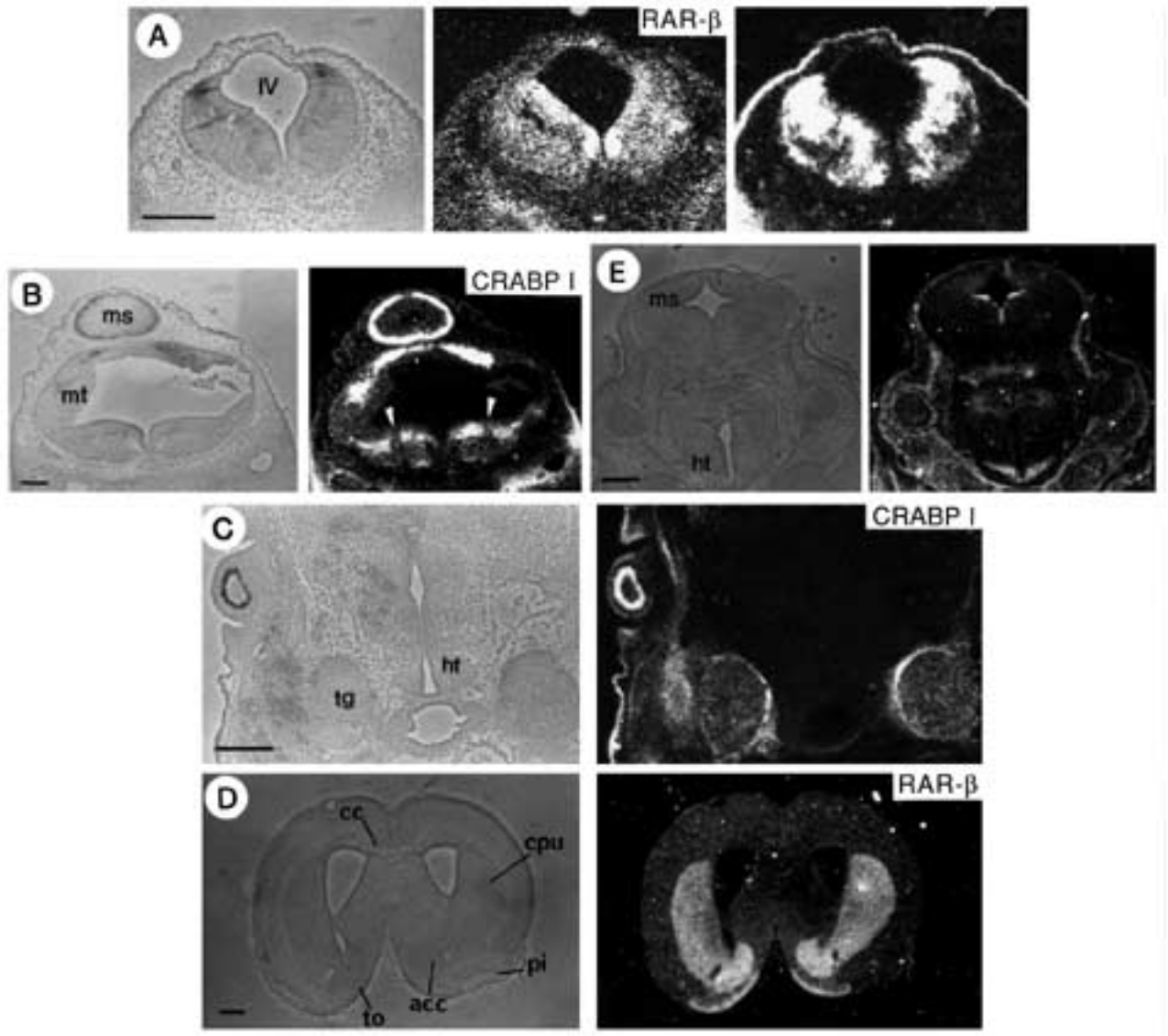

Fig. 6. (A) Frontal sections through the lower medulla oblongata of a 12.5 day embryo hybridized with the RAR-β and CRABP I probes. (B) Frontal section through the head of a 12.5 day embryo hybridized with the CRABP I probe. The arrowheads point to the stripe of tissue lacking CRABP I transcripts (C) Transverse section across the trigeminal ganglion of a 14.5 day embryo to show CRABP I transcript distribution. (D) Coronal section of an 18.5 day foetus brain hybridized with the RAR-β probe. (E) Frontal section across the mesencephalon and hypothalamus of a 13.5 day embryo hybridized with the CRBP I probe. acc, nucleus accumbens septi; cc, corpus callosum; cpu, caudate putamen; ht, hypothalamus; ms, mesencephalon; mt, metencephalon; pi, piriform cortex; tg, trigeminal ganglion; to, tuberculum olfactivum; IV, fourth ventricle; Bar, 250 μm.

essentially the same (Figs 8, 9). Transcripts were found in the corpus striatum (caudate-putamen) and in the tuberculum olfactivum (Figs 8A,B, 9D). In the newborn brain transcripts were also detected in the amygdala (Fig. 9D), in the cerebellum (data not shown) and in the hippocampus, where they were restricted to the CA3 sector of the cornu ammonis, and to the dentate gyrus (Figs 8B, 9E). Labelling was detected in the mesencephalic nucleus of the fifth cranial nerve (Figs 8D, 9B). CRABP I transcripts were detected in the thalamus, in a thin band of cells located dorsal to the medial lemniscus (Figs 8C, 9A). The shape and localization of these labelled cells suggest that they could correspond to the subparafascicular thalamic nucleus parvocellularis.

In 18.5 day fetal brains, intense signal was observed in the outermost cells of the pons (Fig. 8D). In the medulla oblongata CRABP I transcripts were widely distributed at this stage. Labelling was detected in the vestibular nuclei, in the prepositus hypoglossal nuclei and in the spinal motor nucleus of the fifth nerve (Fig. 9F-H and data not shown). Intense labelling was also detected in the inferior olivary nuclei, particularly in the dorsal accessory and principal nuclei. Moreover, CRABP I transcripts were observed in a rather spotty distribution in the paragigantocellularis and gigantocellularis reticular nuclei, and in the reticular paramedian nuclei (Fig. 9F-H). At 18.5 days transcripts were seen in the region of the nucleus solitarius and in the dorsal efferent nucleus of the vagus nerve (Fig. 9G-H), and in the

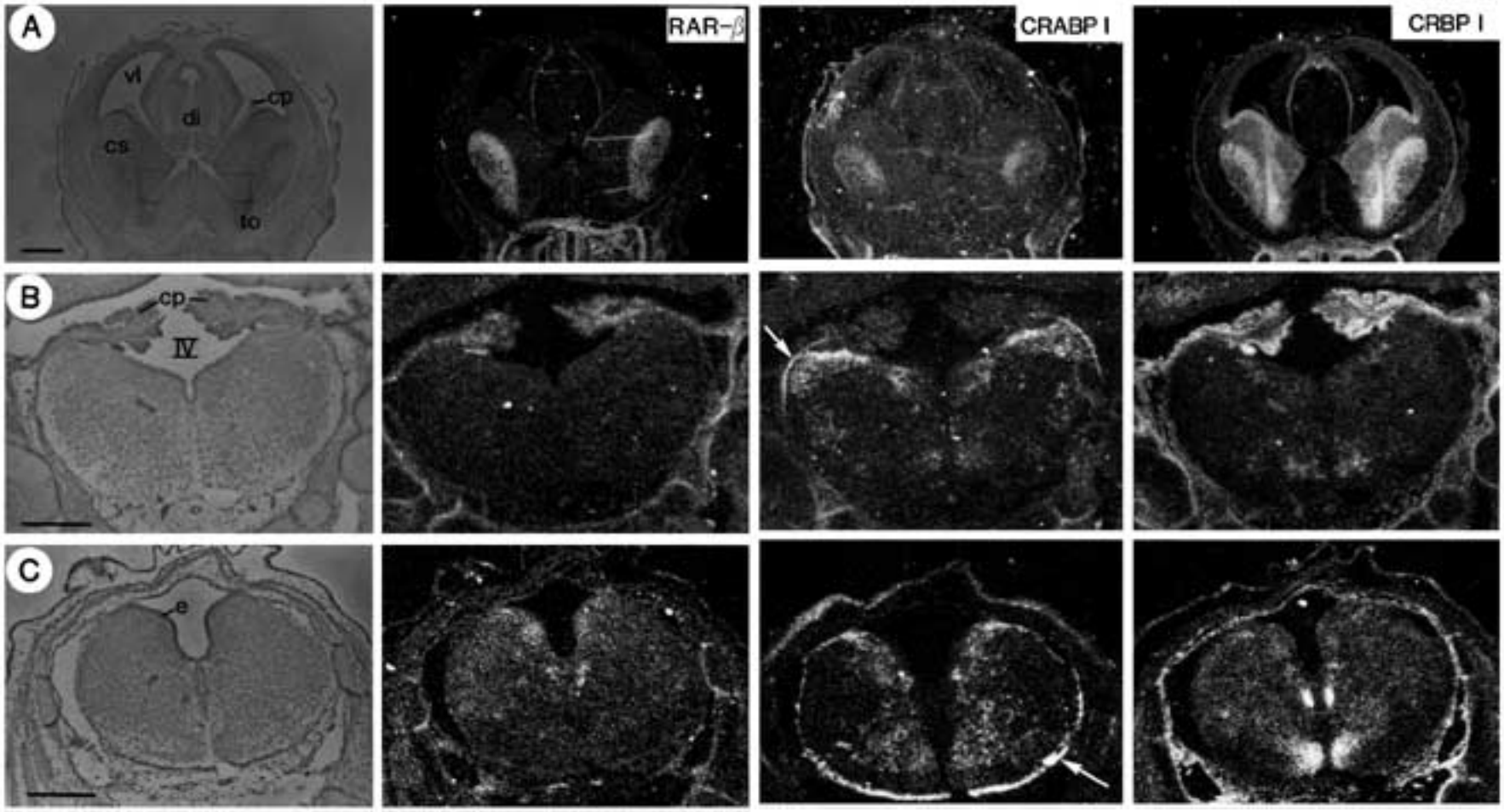

Fig. 7. Serial frontal sections of a 13.5 day embryo hybridized with RAR-β, CRABP I and CRBP I probes. (A) Consecutive sections across the corpus striatum and diencephalon. (B) Consecutive sections across the upper medulla oblongata. (C) Consecutive sections across the low medulla oblongata. cp, choroid plexus; cs, corpus striatum; di, diencephalon; e, neuroepithelial layer; to, tuberculum olfactivum; vl, lateral ventricles; IV, fourth ventricle; arrow, pontine migration; Bar, 250 μm.

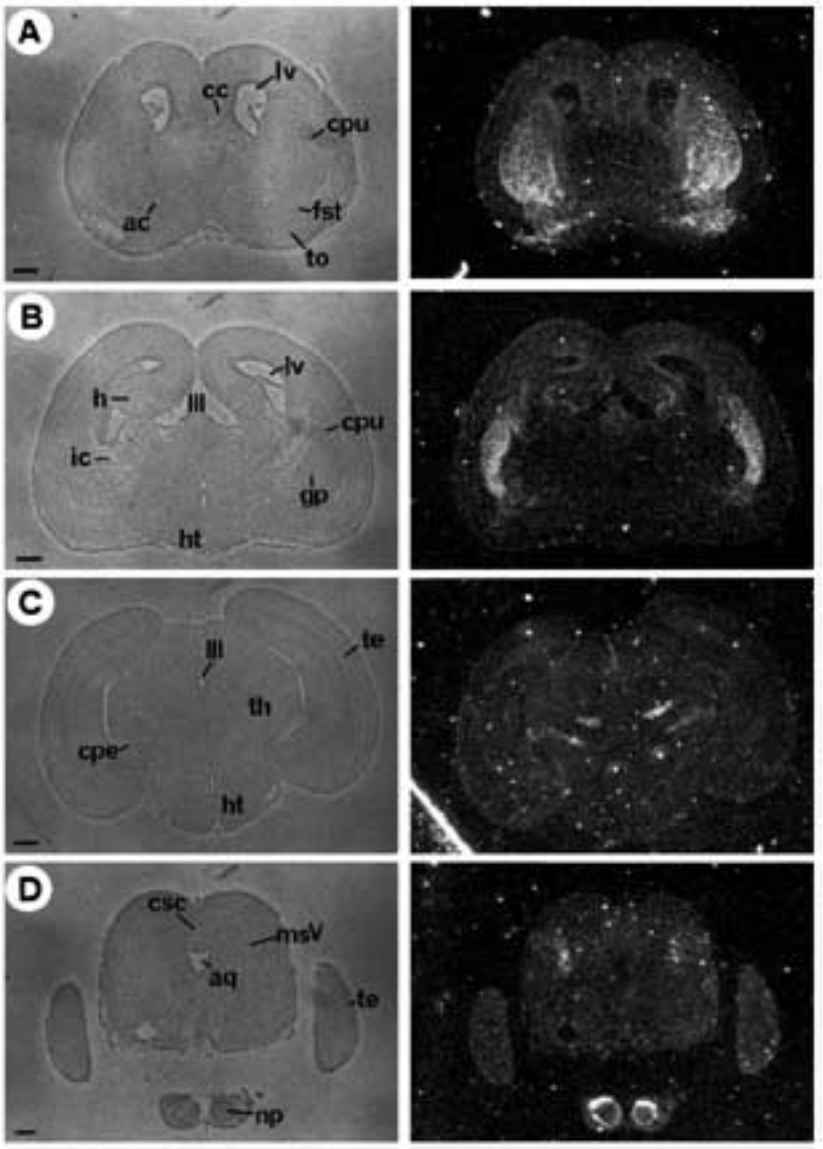

Fig. 8. Frontal sections through the brain of an 18.5 day foetus hybridized with the CRABP I probe. (A) Section across the caudate putamen and tuberculum olfactivum. (B) Section across the caudate putamen and globus pallidus. (C) Section through the thalamic region and hypothalamus. (D) Section across the colliculus and pons. ac, anterior commisure; aq, aqueduct (Sylvius); cc, corpus callosum; cpe, cerebral peduncle; cpu, caudate putamen; csc, commisure of superior colliculus; fst, fundus striatum; gp, globus pallidus; h, hippocampus; ht, hypothalamus; ic, internal capsule; lv, lateral ventricles; msV, mesencephalic nucleus of trigeminal (V) nerve; np, nucleus pontis; te, telencephalon; th, thalamus; to, tuberculum olfactivum; III, third ventricle; Bar, 250 μm.

region of the area postrema (data not shown). In the newborn brain the area postrema was no longer labelled (Fig. 9H).

At 11.5 days of gestation CRABP II transcripts were widely distributed among the brain vesicles and were particularly abundant in the mesencephalon, metencephalon and myelencephalon (data not shown). At day 12.5 CRABP II transcripts were still found in the telencephalic regions (Fig. 2C). In the diencephalon CRABP II transcripts were found in the anterior lateral walls and in the derived optic stalk. The mesencephalon was homogeneously labelled, whereas in the metencephalon and myelencephalon, the mantle layer was preferentially labelled (Fig. 2C). At 13.5 days the corpus striatum and the cerebral hemispheres showed only a diffuse labelling. CRABP II transcripts were also detected in the lateral walls of the diencephalon (Fig. 2D) and abundant expression was detected in the posterior region of the mesencephalon (data not shown). At this stage labelling was also detected in the pontine nuclei and a diffuse labelling was observed in the cerebellum (data not shown). A column of motor neurons in the myelencephalon was also labelled. The pattern of distribution of CRABP II transcripts was very similar in the brain tissue from gestational day 15.5 until birth. Transcripts were found in the ventral posterolateral and posteromedian thalamic nucleus and in the geniculate body (Fig. 10A). In the medulla oblongata transcripts were detected in the motor nucleus of the fifth and seventh cranial nerves (Fig. 10B,C) and in the dorsal efferent nucleus of the vagus nerve (Fig. 10D).

CRBP I transcripts appeared to be rather ubiquitously distributed in the developing brain but higher levels of transcripts were found in the lateral regions of the telencephalon and in the developing diencephalon from day 10.5 of gestation (Figs 4, 5). In motor areas of the metencephalon and myelencephalon transcripts were as abundant as in the motor areas of the spinal cord (data not shown). At day 13.5 CRBP I transcripts were found in the corpus striatum, in both the epithelial and mantle layer, and in the olfacory tubercle (Fig. 7A). Labelling with the CRBP I

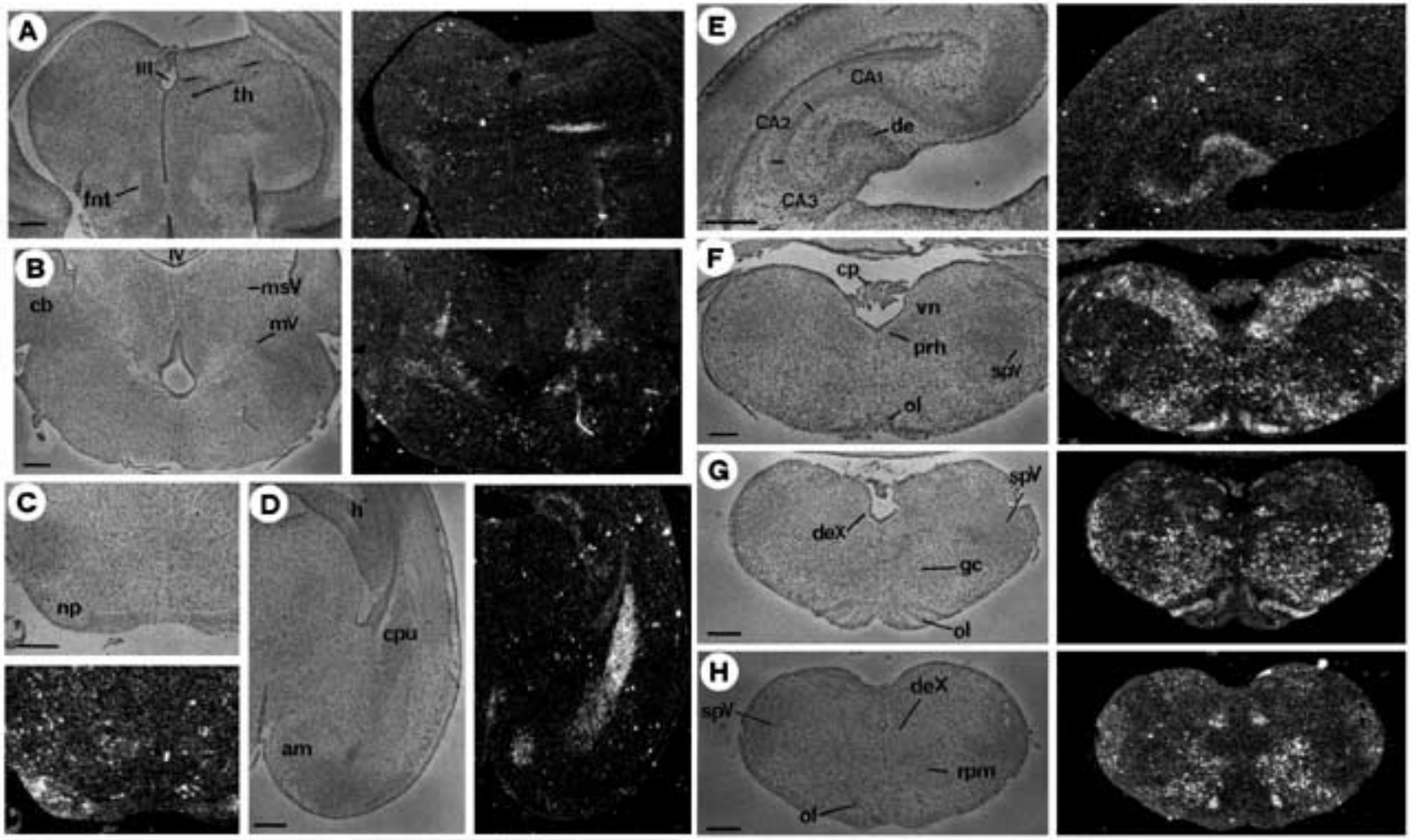

Fig. 9. Frontal section across the brain of a newborn mouse hybridized with the CRABP I probe. (A) Section across the thalamic region. (B) Section across the mesencephalon and pons. (C) Section across the pons. (D) Section through the caudate putamen and amygdala. (E) Section through the hippocampus. (E-H) Serial sections across the medulla oblongata. am, amygdala; CA1-3, cornu Ammonis 1-3; cb, cerebellum; cp, choroid plexus; cpu, caudate putamen; de, dentate gyrus; deX, dorsal efferent nucleus of vagus (X) nerve; fnt, fasciculus mammillothalamicus; gc, nucleus gigantocellularis; h, hippocampus; msV, mesencephalic nucleus of trigeminal (V) nerve; mV, motor nucleus of trigeminal (V) nerve; np, nucleus pontis; ol, olive; prh, prepositus hypoglossi; rpm, nucleus reticularis paramedianus; spV, spinal nucleus of trigeminal (V) nerve; th, thalamus; vn, vestibular nucleus; III, third ventricle; IV, fourth ventricle; Bar, 250 μm.

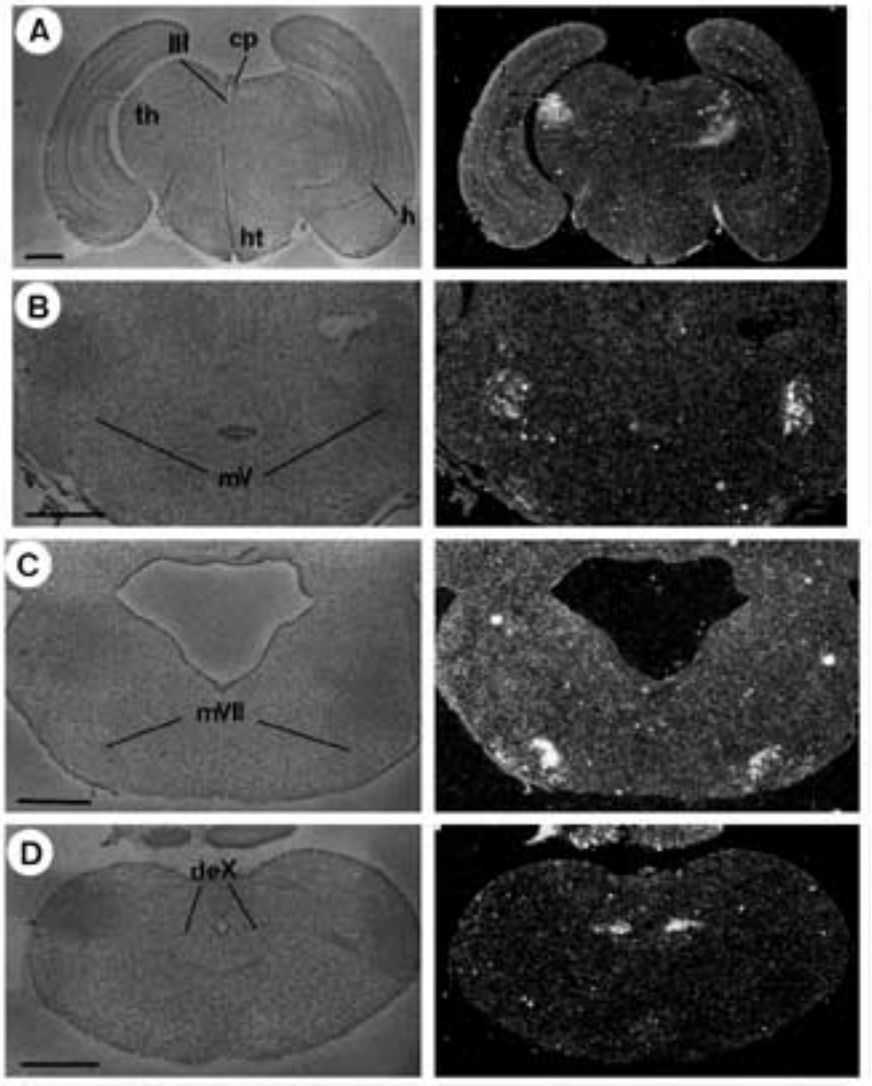

Fig. 10. Frontal sections across a newborn mouse brain hybridized with the CRABP II probe. (A) Section across the thalamic region. (B) Section across the middle pons. (C) Section across the lower pons. (D) Section across the medulla oblongata. deX, dorsal efferent nucleus of vagus (X) nerve; h, hippocampus; ht, hypothalamus; mV, motor nucleus of trigeminal (V) nerve; mVII, motor nucleus of facial (VII) nerve; cp, posterior commisure; th, thalamus; III, third ventricle; Bar, 500 μm.

probe was also detected in the hypothalamus (Fig. 6E). In the mesencephalon intense signal was observed in the ependymal cells of the basal plate (Fig. 6E) and in the mesencephalic nucleus of the trigeminal nerve (data not shown). CRBP I transcripts were also detected in the germinal layer of the cerebellum (data not shown). In the upper medulla, transcripts were seen in a group of neuroepithelial cells on both sides of the midline between the vestibular and the prepositus of the hypoglossal nuclei which corresponds to the group of cells that was less intensely labelled with the CRABP I probe (Fig. 7B). Transcripts were also detected in the dorsal and ventral cochlear nuclei (data not shown). Ventrally, CRBP I transcripts were expressed in a group of cells whose shape and position suggested that they were the migrating motor nucleus of the facial nerve (data not shown). Transcripts were also detected in the developing olivary nuclei (Fig. 7B). In the lower medulla high CRBP I expression was detected in the hypoglossal nuclei and in the inferior olive (Fig. 7C). In the brain of 18.5 day fetuses, CRBP I was expressed in the corpus striatum (Fig. 11A), in the olfactory area, in the ventromedial nucleus of the hypothalamus (Fig. 11B) and in the paraventricular nucleus of the thalamus. CRBP I transcripts were also detected in the mesencephalic nucleus of the fifth cranial nerve (data not shown) and in the Purkinje cell layer of the cerebellum (Fig. 11E). In the upper medulla oblongata CRBP I expression was detected in the motor nuclei of the seventh cranial nerve and in the inferior olivary nuclei (Fig. 11D-F). A low signal was also detected in the dorsal cochlear nucleus (data not shown). CRBP I transcripts were also detected in the region of the area postrema (Fig. 11F). In the newborn brain, transcripts were detected in the mesencephalic nucleus of the fifth cranial nerve and mesencephalic tract (Fig. 12B), in the paraventricular nucleus of the thalamus (Fig. 12A), in the molecular and Purkinje cell layer of the cerebellum (data not shown), and in the most dorsal cells of the motor nucleus of the facial nerve (Fig. 12C), as at the previous stage. The signal in the inferior olive had decreased considerably by this stage and the most intense labelling was seen in the inferior olive cap kooy medial nucleus and in the accessory nucleus (Fig. 11D). At this stage the labelling in the region of the area postrema had almost completely disappeared (data not shown).

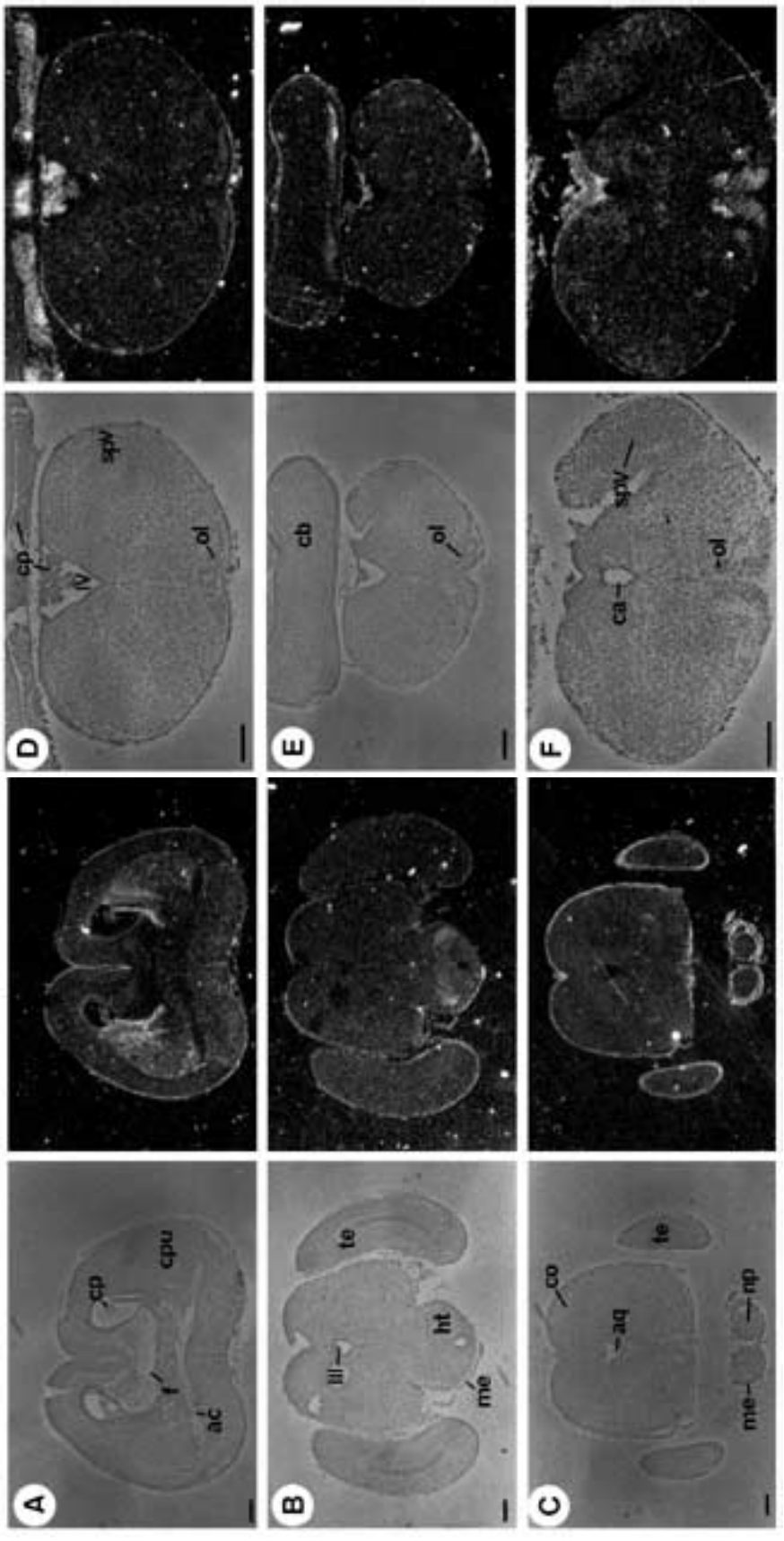

Fig. 11. Frontal sections through the brain of an 18.5 day old foetus hybridized with the CRBP I probe. (A) Section across the caudate putamen. (B) Section across the mesencephalon and hypothalamus. (C) Section across the colliculus and pons. (D-F) Sections across the medulla oblongata. ac, anterior commisure; aq, aqueduct (Sylvius); ca, canalis centralis; cb, cerebellum; co, colliculus; cp, choroid plexus; cpu, caudate putamen; f, fornix; ht, hypothalamus; me, meninges; np, nucleus pontis; ol, olive; spV, spinal nucleus of trigeminal (V) nerve; te, telencephalon; III, third ventricle; IV, fourth ventricle; Bar, 250 µm.

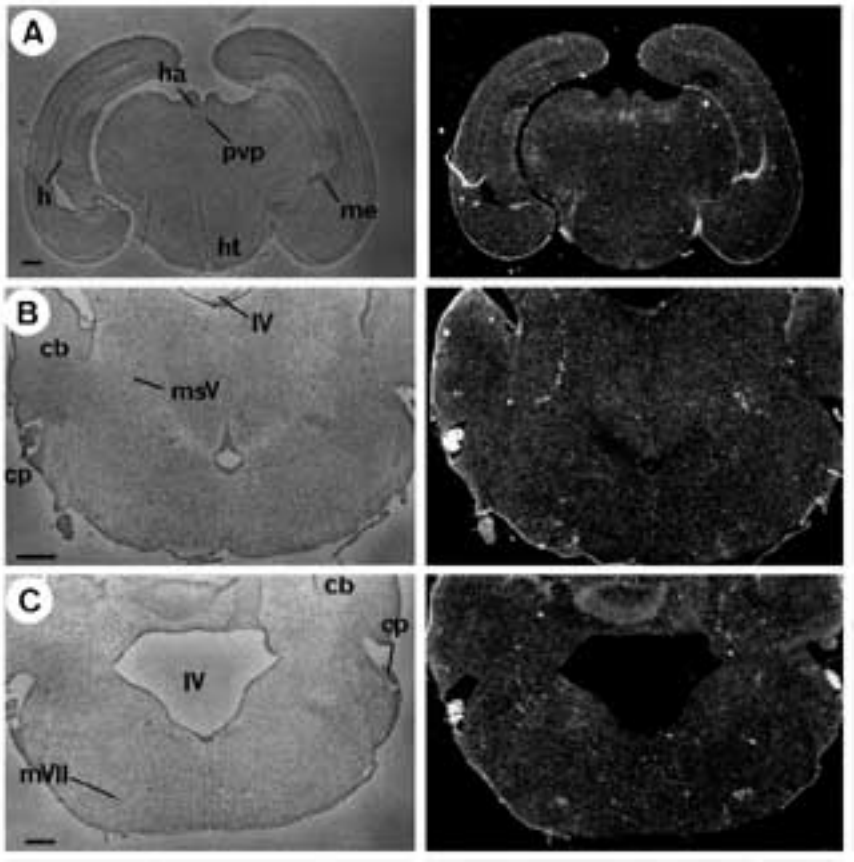

Fig. 12. Frontal sections across the brain of a newborn mouse hybridized with the CRBP I probe. (A) Section across the thalamus and hypothalamus. (B) Section across the mesencephalon and pons. (C) Section across the pons and cerebellum. cb, cerebellum; cp, choroid plexus; h, hippocampus; ha, habenula; ht, hypothalamus; me, meninges; msV, mesencephalic nucleus of trigeminal (V) nerve; mVII, motor nucleus of facialis (VII) nerve; pvp, paraventricular thalamic nucleus, posterior; IV, fourth ventricle; Bar, 250 μm.

Choroid plexus and meninges

At day 14.5 of gestation RAR-β, CRABP I and II, and CRBP I transcripts were detected in the choroid plexus (Fig. 3A and data not shown). Only CRBP I transcripts were expressed in the epithelial component. CRBP I transcripts were still detected at high levels in the newborn choroid plexus (Fig. 12B,C), whereas no detectable signal was observed with other probes at this stage.

At 14.5 days RAR-β, CRABP I and II, and CRBP I transcripts were found in the meninges, and CRBP I was still expressed at high levels in the meninges of the newborn mouse (Figs 3, 11, 12 and data not shown).

DISCUSSION

The RAR and retinoid binding protein transcripts detected by this in situ hybridization study were differentially distributed among the embryonic nervous structures at the developmental stages studied. Only RAR-γ transcripts were absent from the developing nervous system, with the exception of the open trunk neural folds (caudal neuropore) during neurulation (see Ruberte et al., 1990, 1991). The distribution of RAR-α transcripts was confined to spinal cord and myelencephalic levels, but does not otherwise suggest any correlations with developmental processes. In contrast, the distribution of transcripts of RAR-β and the three binding proteins show some significant correlations with specific developmental events, and with the developmental effects of retinoid excess and vitamin A deficiency.

At the stage of neural tube closure, RAR-β transcripts were found in the closed neural tube but not in the open neural folds, and we suggested that RAR-β expression could be related to the maturational state of the neuroepithelium (Ruberte et al., 1991). The patterns of RAR-β expression observed here are consistent with that idea: in the early spinal cord, RAR-β transcripts were found in the proliferating neuroepithelial layer, but with the onset of differentiation of motor neurons, the most intense accumulation of RAR-β transcripts was in the developing motor columns. This pattern of expression is not restricted to the spinal cord but also extends into the columns of general somatic efferent neurons of the low medulla oblongata (represented by the neurons of the hypoglossal nuclei). The rostral limit of RAR-β expression in the general somatic efferent column corresponds to its earlier rostral boundary in the hindbrain rhombomeres (Ruberte et al., 1991; Mendelsohn et al., 1991). Maintenance of this rostral limit from rhombomeric stages until the formation of motor columns suggests a functional association between RAR-β expression and organization of the central nervous system in relation to the innervation of somite derivatives (especially of myotome-derived muscle, including the occipital myotome-derived muscle of the tongue).

The significance of the patterns of expression of the retinoid binding proteins during development relates to their role in controlling the availability of RA to the nuclear receptors. Activation of nuclear RARs by RA (and of the related RXR receptors by 9-*cis*-RA) modulates the transcription of responsive genes by binding to retinoic acid

response elements (RAREs) in their promoter region, leading to further downstream transcriptional events. Hence, cells in which RA availability to the nucleus is high will have a different programme of development from these in which nuclear RA is low or absent. CRBP I is involved in the synthesis of RA from retinol (Napoli et al., 1991), so RA-induced transcriptional events may be particularly important in cells expressing this gene. In contrast, CRABP I is associated with the degradation of RA to inactive metabolites (Napoli et al., 1991), and little or no RA is available to the nuclear receptors in cells expressing this gene (Boylan and Gudas, 1991).

CRBP I transcripts were observed in the spinal cord, but in contrast to RAR-β, the CRBP I signal was most intense in the ventral half of the neuroepithelium and in the adjacent basal plate, where it was maintained throughout the period of differentiation of the motor neurons. By day 14.5, this signal was colocalised, with that of RAR-β, to the motor columns, and was also strong in the central and most dorsal ependymal cells. Since CRBP I facilitates the synthesis of RA from retinol, its pattern of localization suggests that higher levels of RA are available to the nuclear receptors in the ventral region during the period of regionalization of the neural tube into alar (associative) and basal (motor) plates, and during the further differentiation of the motor column cells. This preferential ventral labelling with the CRBP I probe was already present in the neural tube at the time of its formation (Ruberte et al., 1991). As differentiation of the motor column cells proceeds, CRBP I transcripts become restricted to the differentiated motor neurons as previously reported for CRBP I protein (Maden et al., 1990). CRBP I expression was also found in the general somatic efferent column of the lower medulla oblongata (nucleus hypoglossus) at 12.5 and 13.5 days of development, but this was lost by day 18.5 of development, suggesting that CRBP I, or the RA synthesized by cells expressing this gene, may be involved in the differentiation and maturation of these cells, but not in their mature function.

CRABP II transcripts were also expressed in the motor columns of the spinal cord. As discussed elsewhere (Ruberte et al., 1992a), RAR-β, CRBP I and CRABP II are found coexpressed in a number of embryonic structures. The three genes are RA-inducible and contain a RARE in their promoter region (de Thé et al., 1990; Mendelsohn et al., 1991; Mangelsdorf et al., 1991; Smith et al., 1991; B. Durand and P. Chambon, unpublished results) suggesting that they may be involved in a common morphogenetic mechanism in which retinol converted to RA may activate the transcription of responsive genes through the nuclear receptor RAR-β. However, unlike RAR-β, the expression of CRABP II transcripts in the general somatic efferent column does not extend up into the hypoglossal nucleus but stops at the rostral end of the spinal cord. CRABP II transcripts were found in two other motor nuclei of the brainstem, the nuclei of the facial (VII) and trigeminal (V) nerves.

In contrast, CRABP I transcripts were preferentially found in the sensory nuclei derived from the alar plates of the myelencephalon. Hybridization signal was detected in the vestibular nuclei and in the spinal nucleus of the trigeminal nerve. The signal in the spinal nucleus of the trigeminal nerve decreased in a rostrocaudal sequence during development, suggesting that CRABP I expression and nuclear RA levels are related to the state of differentiation, since a rostrocaudal gradient in the differentiation of these neurons has been observed (Altman et al., 1982). The mesencephalic nucleus of the trigeminal nerve, which in birds is derived from the mesencephalic neural crest (Narayanan et al., 1978; Rogers et al., 1973), showed a rare overlap of expression of the CRBP I and CRABP I genes. It is interesting to note that CRABP I transcripts are found in the migrating neural crest cells and in a number of their derivatives (Dencker et al., 1990; Maden et al., 1990; Vaessen et al., 1990; Ruberte et al., 1991, 1992a).

High levels of CRBP I and CRABP I transcripts were detected in the inferior olivary complex from the earliest stages of its development, although not in the same nuclei. This pattern of labelling is of interest in relation to the abnormal organization of the olivary nuclei induced by exposure to high levels of retinoids (see below).

RARs and retinoid binding proteins in relation to retinoid status

The malformations induced by retinoid excess are dose and stage dependent, indicating that retinoids are able to interact with a great number of events occuring during morphogenesis, and thus lead to a wide spectrum of malformations.

Exposure to an excess of retinoids during pregnancy has been shown to affect the development of nervous structures (Alles et al., 1990; Lammer et al., 1985; Lammer and Armstrong, 1992; Langman and Welch, 1967). However, little attention has been paid to this effect of retinoid excess compared with the studies on the effects of RA on the development of the craniofacial (Morriss and Thorogood, 1978; Webster et al., 1986) and limb skeletal elements (for a review see Tabin, 1991). Adverse effects of excess retinoids on the nervous system have been demonstrated in *Xenopus* (Durston et al., 1989; Krumlauf et al., 1991; Papalopulu et al., 1991a), mouse (Langman and Welch, 1967; Morriss, 1972) and human (Lammer et al., 1985; Lammer and Armstrong, 1992). In mouse and *Xenopus* embryos, RA has been shown to affect rhombomere boundary formation and to alter the segmental patterns of expression of some genes, including homeogenes (Morriss-Kay et al., 1991; Papalopulu et al., 1991b).

In human, the effects of maternal RA excess during pregnancy on the developing nervous system has similarly been shown to involve mainly the hindbrain structures (Lammer et al., 1985; Lammer and Armstrong, 1992). RA excess interferes with the normal development of the cerebellum leading to absence of cerebellar vermis, cerebellar hemisphere heterotopias and dysplasia of the dentate nucleus. Hydrocephaly was frequently observed and was generally thought to result from aqueductal stenosis. In exposed fetuses and infants, RA has also been reported to lead to dysplasia of the olivary nuclei and abnormal development of the pontine nuclei (Lammer et al., 1985; Lammer and Armstrong, 1992). These effects are reported to have resulted from maternal ingestion of 13-*cis*-RA at both early (e.g. 1-32 days of gestation) and late (e.g. 38-66 days of

gestation) stages of brain development (Lammer and Armstrong, 1992). We have shown in previous studies that there is a clear relationship between tissues expressing CRABP I and their vulnerability to excess retinoids (Dollé et al., 1990; Ruberte et al., 1991), suggesting that RA does not normally reach the nucleus in these cells, annd that RA excess may lead to saturation of the binding and metabolic activities of CRABP I, enabling RA to reach the nuclear receptors and induce an abnormal programme of gene expression. In the present study, CRABP I was expressed in the alar plates of the metencephalon and myelencephalon at stages consistent with the observation that RA excess induces abnormal morphogenesis of the cerebellum and related nuclei. Of particular interest are the malformations of the olivary and pontine nuclei in relation to the pattern distribution of CRABP I transcripts, since they were present in the cells migrating from the alar plates of the myelencephalon and contributing to the olive and pons. CRABP I transcripts are also present in migrating primary mesenchyme cells (Ruberte et al., 1991), and neural crest cells. RA excess has been shown to alter the normal migratory behaviour of these two cell populations both, in vivo and in vitro (Morriss, 1972, 1975; Morriss and Thorogood, 1978; Thorogood et al., 1982; Webster et al., 1986). It is possible that CRABP I plays a similar role in relation to the bulbo-pontine migration, so that if RA levels are raised, the migration pathways are altered, resulting in abnormal development of the olivary nuclei. Another possibility is that RA excess alters the normal differentiation of the olivary structures, since CRABP I transcripts are also present at later stages of their development.

Congenital hydrocephalus has been observed in infants and experimental animals exposed to both excess and deficient levels of retinoids. In RA-exposed infants, hydrocephalus was associated with aqueductal stenosis (Lammer and Armstrong, 1992). In contrast, calves born to vitamin A-deficient dams were found to have hydrocephaly without aqueductal stenosis (Okamoto et al., 1962), and in a similar study in mice, hydrocephaly was associated with poorly developed choroid plexuses (Mahmood and Morriss-Kay, unpublished observations). RA-related gene expression in the choroid plexus is complex, with both RA binding proteins, RAR-β and CRBP I transcripts being present. However, only CRBP I was present in the cerebrospinal fluid-secreting epithelial layer from its early stages until the latest stage examined (one day post natally). All of these observations suggest that RA is required for proper control of cerebrospinal fluid secretion, and that when maternal dietary retinol levels are adequate, the presence of CRBP I within the choroid plexus epithelium ensures that RA is synthesized there.

CRBP I transcripts were also detected in the developing peripheral nerves suggesting that RA is synthesized by differentiating Schwann cells. In experimental vitamin A deprivation, degeneration of the spinal and cranial nerves and spinal cord has been reported (Mellanby, 1944; Wolbach and Bessey, 1941). Although this degeneration was interpreted as the result of cessation of bone growth and increase of mechanical pressure by bony structures, an active role in the differentiation and maintenance of these structures cannot be excluded.

The differential patterns of transcription of the three retinoid binding proteins observed in this study suggest that fine regional control of RA availability to the nuclear receptors plays an important role in organization and differentiation in the developing nervous system. These patterns, together with those of RAR-β transcripts, point to an important role for RA-induced gene expression in development of the motor system and in normal function of choroid plexus. In contrast, the normal developmental programme of the hindbrain, particularly in the olivary nuclei and cerebellum, appears to depend on the ability of specific cell populations to restrict the access of RA to the nuclear receptors.

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