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Expression of the acatalytic carbonic anhydrase VIII gene, *Car8*, during mouse embryonic development

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Summary

The carbonic anhydrase (CA)-like protein, CA VIII, lacks the typical carbon dioxide hydrase activity of the CA isozymes. However, the high degree of amino acid sequence similarity between the products of the mouse and the human CA VIII genes suggests an important biological function. We have attempted to investigate the function of this gene in mammalian development by conducting an *in situ* hybridization study on sagittal sections of mouse embryos at gestation days of 9.5–16.5 using a ^{35}S-labelled riboprobe. Results indicate that this gene (called *Car8* in mice) is expressed as early as day 9.5 in a variety of organs including liver, branchial arches, neuroepithelium and developing myocardium. Between days 10.5 and 12.5, it showed a widespread distribution of mRNA expression that became more restricted as development progressed. The level of expression of *Car8* mRNA was relatively high in the brain, liver, lung, heart, gut, thymus and epithelium covering the head and the oronasal cavity.

Introduction

The Æ-carbonic anhydrases (Æ-CAs) in reptiles, birds and mammals (amniotes) have been designated CA I–X and are under the control of ten autosomal genes (see Hewett-Emmett & Tashian, 1996). Eight of these CAs (CA I–VII and CA IX) catalyse the reversible hydration of CO_2, with turnover numbers ranging from about 1 3 10^4 s^{-1} in the CA III isozyme to extremely high levels of approximately 1 3 10^6 s^{-1} in the CA II isozymes. However, two of these, CA VIII and CA X, do not appear to have any CO_2 hydrase activity owing to lack of critical active site residues that are essential for this activity (Hewett-Emmett & Tashian, 1996; N. Bergenhem, unpublished).

CA VIII (originally termed CA-related polypeptide, CARP) was first identified from the adult mouse brain cDNA library (Kato, 1990). Its derived amino acid sequence revealed the substitution of an Arg for the critical Zn-liganded His-94 residue at the active site, thereby abolishing its CO_2 hydrase activity. In spite of this, its amino acid sequence remained highly conserved between human and mouse (percentage identity of about 98%) during 150 million years or so of mammalian evolutionary radiation. Initially, CA VIII was believed to be expressed exclusively in the Purkinje cells of the mouse cerebellum (Kato, 1990); since then, its expression has been reported from human testis, salivary glands, placenta and rat lung (Skaggs *et al*., 1993; Ling *et al*., 1994).

In an attempt to understand the biological role of this acatalytic CA-like protein, we have initiated studies on its mRNA expression in embryonic and adult mice. Here we show that the mRNA for the CA VIII gene (called *Car8* in mice) is expressed in a variety of tissues during mouse embryonic development.

Materials and methods

EMBRYO ISOLATION AND SECTIONING

Mouse embryos (gestation days 9.5–16.5) were isolated from pregnant female CD1 mice (Charles River Breeding Laboratories), frozen immediately at −308C and stored

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frozen at −808C. Just before sectioning, they were equilibrated to cryostat temperature (−188C), mounted in cold optimal cutting temperature embedment (OCT; Lab-Tek Division, Miles) and allowed to freeze as solid blocks. The blocks containing the embryos were cryosectioned at 14-ì m thickness and mounted on polylysine-coated microscope slides.

RIBOPROBE PREPARATION

A cDNA probe was made by polymerase chain reaction (PCR) amplification of cDNA prepared by reverse transcription (RT–PCR) of mRNA isolated from the mouse brain using *Car8*-specific primers (2584D and 2585D). These primers amplified the *Car8* gene cDNA between nucleotides 120 and 619 of the coding sequence producing a fragment of about 500 bp in size. This DNA was then closed in pAMP1 vector (Life Technologies) flanked by two transcriptional controls. The antisense transcript was under the control of T7 promoter, whereas the sense transcript was under SP6 promoter control. Transcription was carried out by standard procedures (Melton *et al.*, 1984) using either T7 or SP6 polymerase in the presence of [^{35}S]UTP. All probes were subjected to partial alkaline hydrolysis to produce fragments of about 100–150 nucleotides long (Cox *et al.*, 1984; Wilkinson & Nieto, 1993).

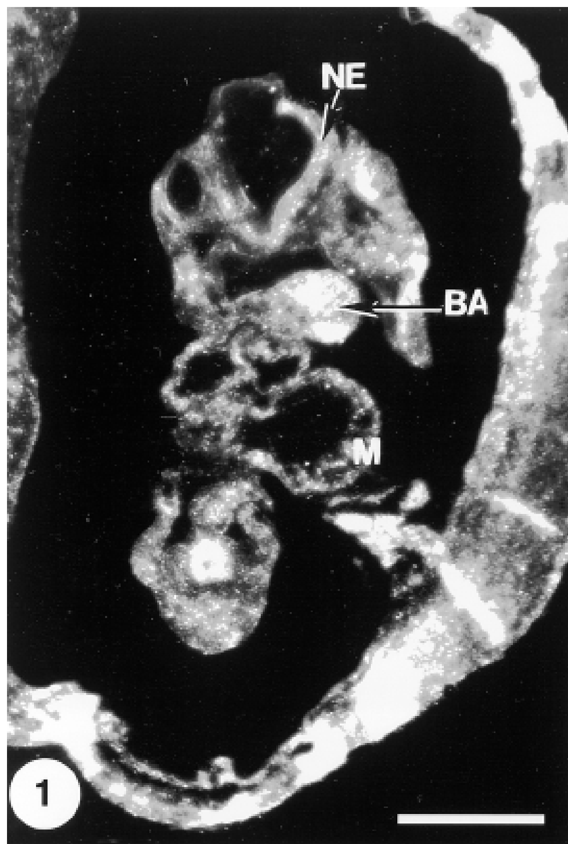
IN SITU HYBRIDIZATION

In situ hybridization was carried out using adaptations of previously described procedures (Hayashi *et al.*, 1986; Sassoon & Rosenthal, 1993; Wilkinson & Nieto, 1993). Embryo sections were first thawed to room temperature and fixed with 4% paraformaldehyde, washed with distilled water and treated with 0.2 N hydrochloric acid (HCl) for 5 min, followed by treatment with proteinase K (1 ì g ml^{-1}) at room temperature for 5 min. These sections were then acetylated with 0.1 M triethanolamine containing 0.25% acetic anhydride and washed with distilled water. Hybridization was performed by applying 50 ì l of Novagen hybridization solution (50% formamide, 0.6 M sodium chloride (NaCl), 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 50 ì g ml^{-1} heparin, 10 mM dithiothreitol, 0.5 mg ml^{-1} tRNA, 10% polyethylene glycol-8000 and 1 3 Denhardt's), containing 1 3 10^{5} cpm ì l^{-1} ^{35}S-labelled probe, directly on the sections and incubating them at 558C overnight. A positive control (mouse cerebellum sections) was included using the same probe. Negative controls of mouse embryo sections were also hybridized under the same conditions but using the opposite sense strand as a probe. The slides were then washed twice with 2 3 saline sodium citrate (SSC) (150 mM NaCl, 15 mM sodium citrate), 50% formamide at 658C for 30 min each, treated with RNAase A (200 ì g ml^{-1}) for 30 min at 378C, followed by a final wash in 0.1 3 SSC at 558C for 30 min. Autoradiography was then performed by dipping the slides in Kodak NTB2 film emulsion (Angerer & Angerer, 1981). Exposure time was about 10 days, after which they were developed and fixed in Kodak developer and fixer respectively. Finally, slides were stained with 5% aqueous Giemsa stain (Sigma) for 2 min, dried and coverslipped. Photography was carried out using Kodak TMAX 100 black and white film using dark-field illumination.

Results

The temporal and spatial pattern of expression of mouse *Car8* was investigated in mid-to-late gestational stages of embryonic development by RNA *in situ* hybridization, using a ^{35}S-labelled probe applied to whole mouse embryo sections at sequential stages of gestation. The study included embryos from gestation days 9.5 to 16.5. The specificity of the probe was tested on a positive control, which was the cerebellum of the adult mouse (data not shown) where it was previously shown to be expressed in the Purkinje cells (Kato, 1990). One set of hybridization experiments was carried out with the sense probe to indicate the non-specific background (negative controls) and another set was hybridized to the antisense probe to indicate the specific hybridization signal that represents mRNA levels and thus gene expression. The resulting signals were examined by dark-field microscopy, photographed under optimal magnification and the specific embryonic structures were identified following Kaufman (1992).

A positive signal for *Car8* expression was detected in the embryo proper at the earliest time point examined (i.e. day 9.5). At this stage, a strong positive signal was observed in the developing neuroepithelium, the first branchial arch and the myocardium (Fig. 1). By day 10.5, a moderate level of expression signal appeared throughout the embryo (Fig. 2), with higher expression signal in some parts of the developing brain (forebrain, midbrain and hindbrain). This general expression pattern was maintained at day 11.5 (Fig. 3), but expression increased visibly in the liver. At day 12.5 (Fig. 4), the general expression signal was less intense but a strong signal was maintained in the developing liver and corpus striatum mediale (medial aspect of ganglionic eminence) of the forebrain. By days 13.5–14.5 (Figs 5 and 6 respectively), a general signal was still visible but a high level of expression of *Car8* was present in many specialized developing structures, including the forming liver, lung, gut, kidneys and parts of the brain. In addition, a very strong signal appeared in the nasal placode and in the developing cerebellum. By gestational day 15.5 (Fig. 7), a significant change in the pattern of mRNA expression of this gene was observed. The signal became more localized to discrete regions, being abundant in the liver and some parts of the heart. A moderate level of expression was also present in the brain, lungs and other abdominal organs, including the developing gut and kidneys. Under higher magnification, the expression in the kidney appeared to be particularly high in the glomeruli. The same pattern was observed at day 16.5 (Fig. 8) with an additional strong signal in the developing cerebellum and the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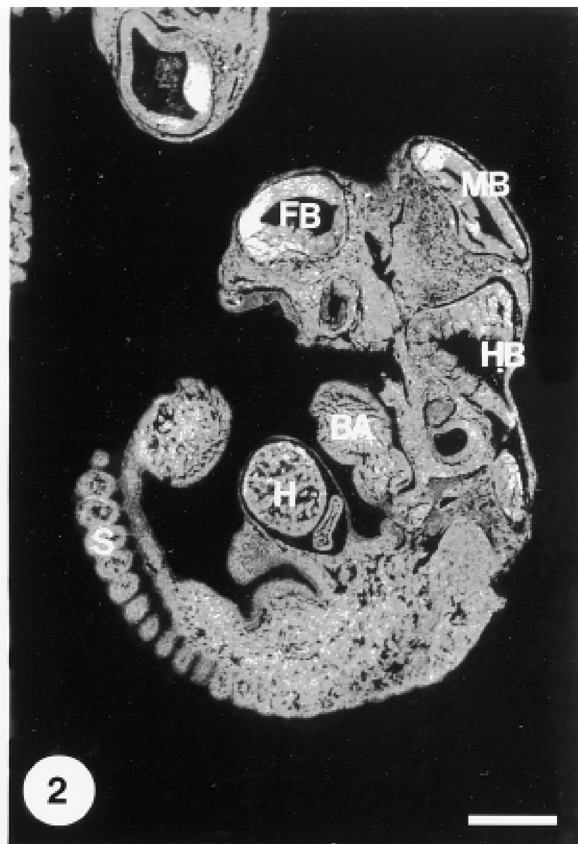
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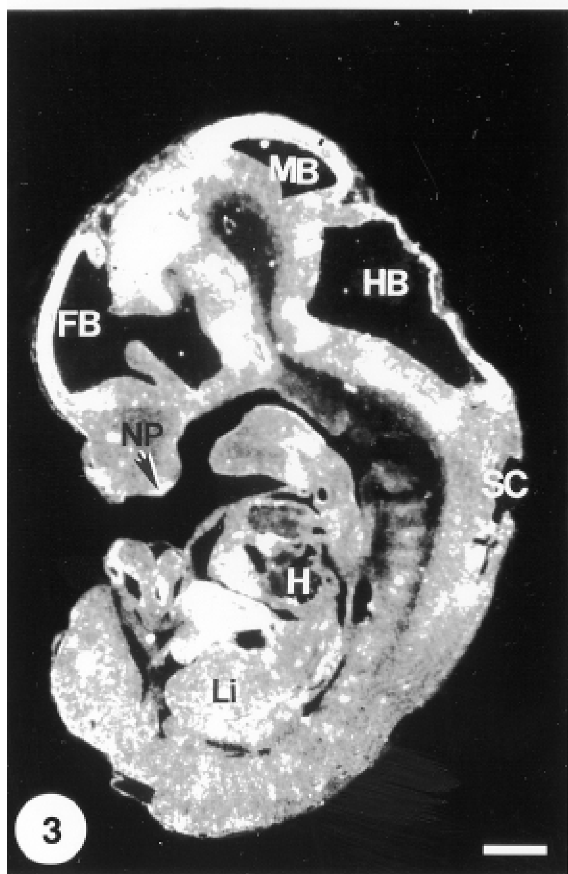
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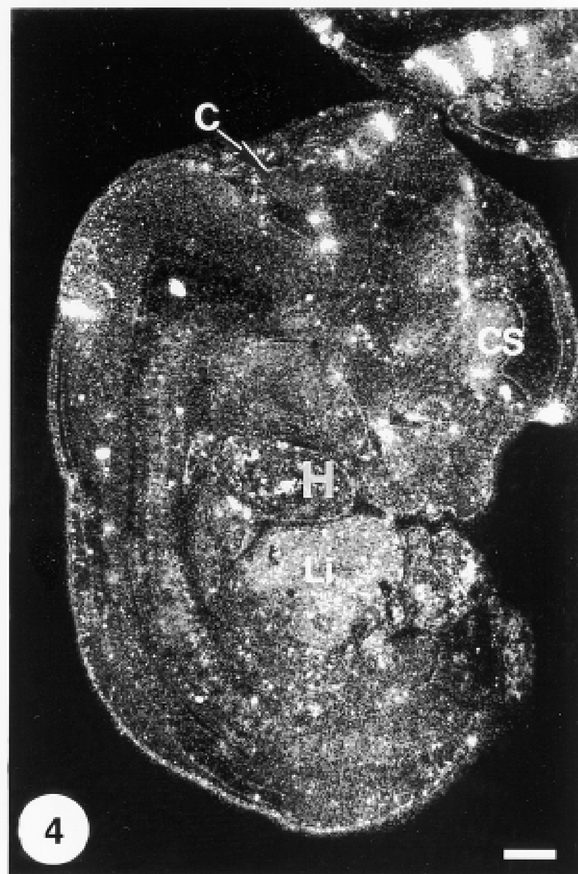
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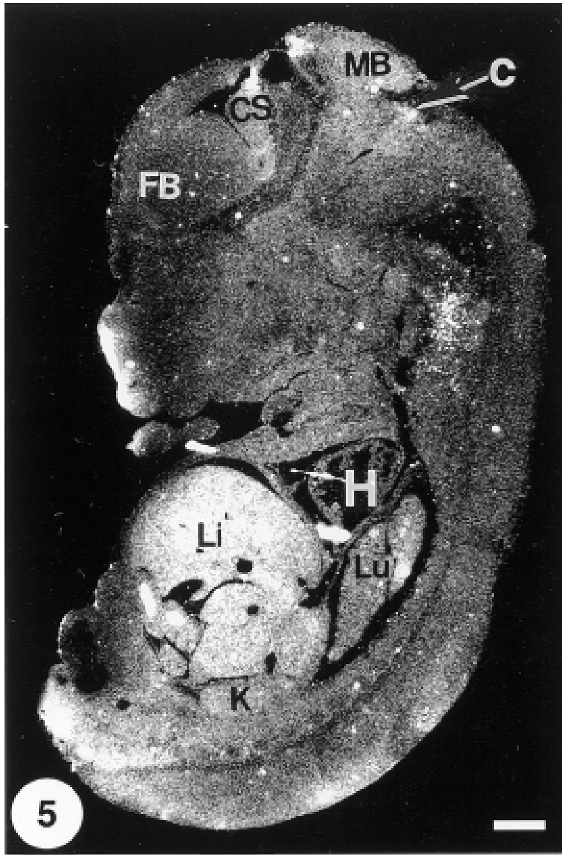
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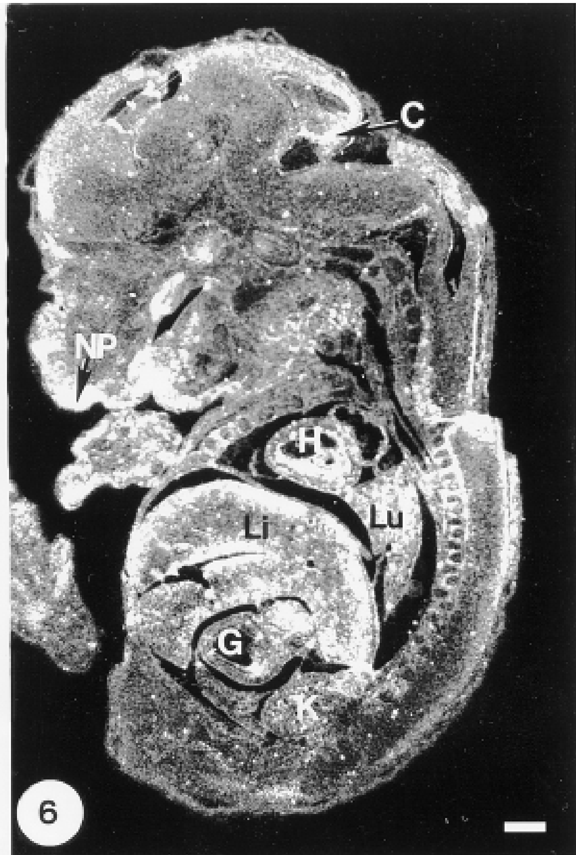
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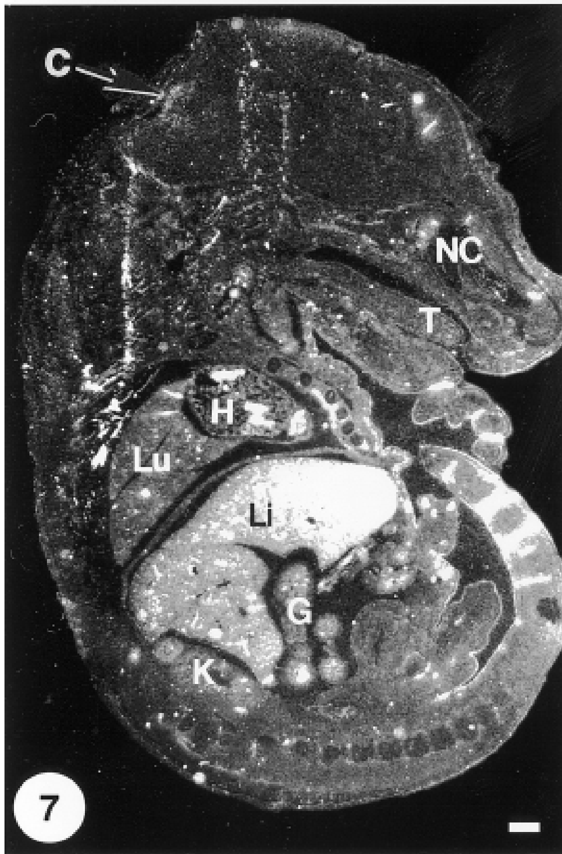
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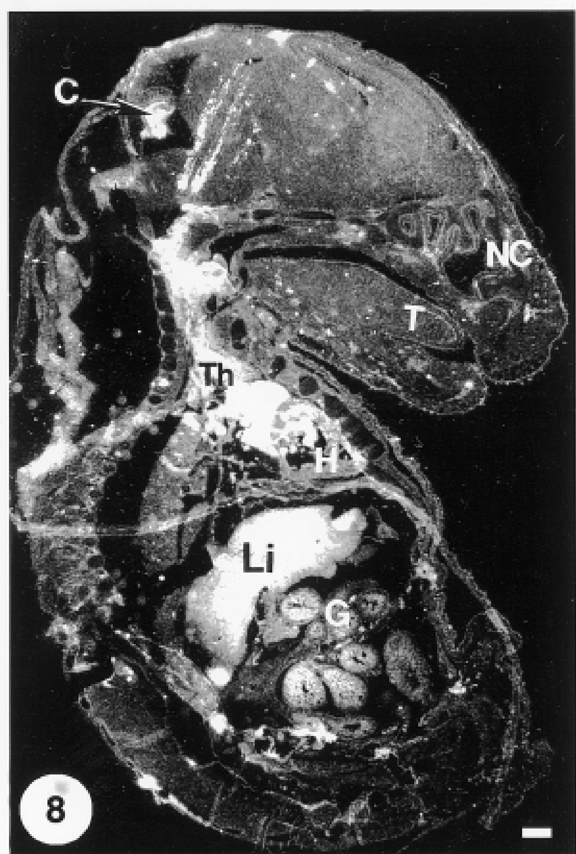
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thymus. In both later stages, a strong signal was also present in the regions underlying the surface epithelium covering the head, particularly at the base of vibrissae, the tongue, the nasal cavity and the eye. This unique signal could be an indication of a possible involvement in the neural wiring, including the sensory epithelia of the taste buds and the olfactory epithelium. In addition, a signal of positive expression was observed in the perichondrium of the forming bones throughout all the stages studied.

Discussion

Because transcriptional activity is a major molecular event regulating cell growth and differentiation, the temporal and spatial expression pattern of *Car8* was carried out in correlation with developmental events in order to obtain clues to its function during mouse embryogenesis. Since *Car8* belongs to a gene family, it was important to maintain high-stringency hybridization and washing conditions to avoid cross-hybridization with other members of the carbonic anhydrase gene family. Appropriate negative controls were also run in parallel to eliminate any non-specific signal (Fig. 9).

This study revealed several interesting features regarding *Car8* gene expression during embryogenesis. A first look at the results indicates that this gene is not tissue specific; it is rather widely distributed among developing tissues, especially at the earlier stages of development. However, the level of its expression appears to be somehow regulated in a temporal and tissue-specific manner. In general, at the earlier stages of post-implantation development (until day 11.5), it showed a wide-

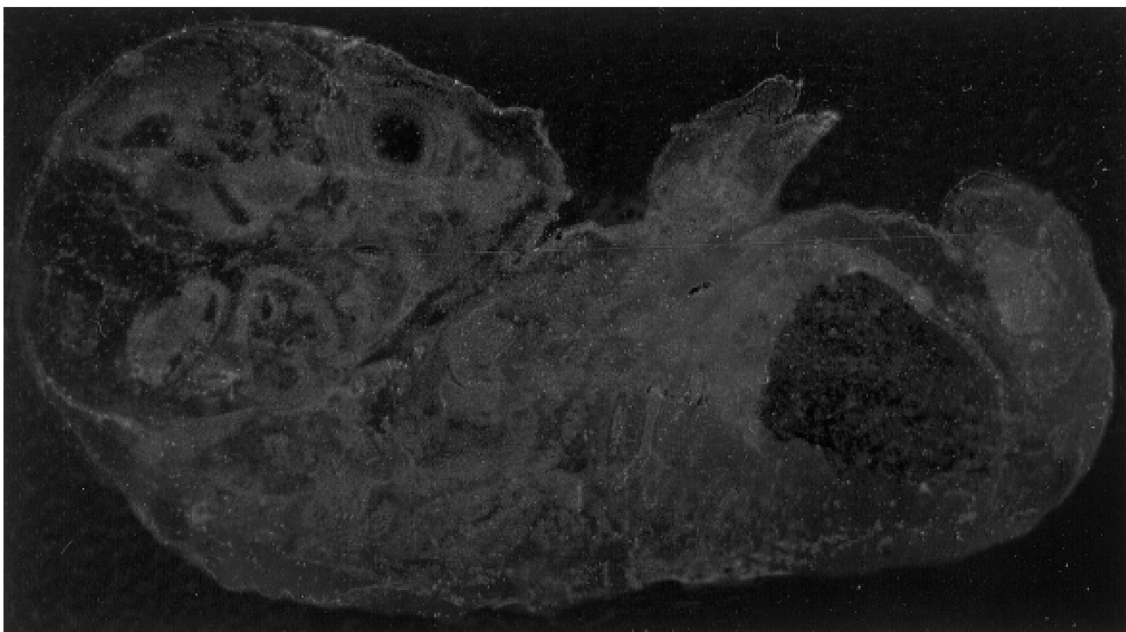

Fig. 9. Dark-field photomicrograph of a negative control, day 14.5 mouse embryo hybridized with the ^{35}S-labelled sense probe, illustrating the non-specific scatter of grains on the tissues.

Figs. 1–8. Dark-field photomicrographs of sagittal sections of mouse embryos illustrating the pattern of *Car8* mRNA expression at the different embryonic stages. (1) E9.5: a positive signal is visible in the neuroepithelium, the first branchial arch and the myocardium; (2) E10.5: a moderate signal appears throughout the embryo with a stronger signal in the brain region (forebrain, midbrain and hindbrain); (3) E11.5: the signal is strong throughout the body, although it is more intense in the brain region; (4) E12.5: a general signal, less intense than the previous stage, is visible throughout the whole body of the embryo with a stronger signal in the liver and the corpus striatum and a moderate signal in the developing cerebellum; (5) E13.5: at this stage, notice the strong signal in the liver, gut, kidney, lung, the nasal placode and the brain region; (6) E14.5: the signal here is similar to that of the previous stage with a new strong signal appearing in the heart; (7) E15.5: expression signal is strong in the liver and moderate in the lung, gut and kidney. In the heart, the signal becomes restricted to discrete spots. A signal also appears in the region underlying the surface epithelium and the base of vibrissae; (8) E16.5: the expression pattern is similar to the previous stage with a new strong signal appearing in the thymus. BA, branchial arch; C, cerebellum; CS, future corpus striatum; FB, forebrain; G, gut; H, heart; HB, hindbrain; K, future kidney; Li, liver; Lu, lung; M, myocardium; MB, midbrain; NC, nasal conchae; NE, neuroepithelium; NP, nasal placode; S, somites; SC, spinal cord; Th, thymus; T, tongue. Each scale bar on the pictures represents 5 ìm of actual embryo section.

spread expression. As development progressed, the transcripts became restricted in developmentally significant domains.

It is noteworthy that the site of higher expression of *Car8* is in the liver, although we have not been able to distinguish the precise cell type. At higher magnification, this expression appeared uniformly strong probably occurring in both the hepatocytes and the haematopoietic cells. This expression pattern was not a dark-field illumination artifact because a similar pattern of expression was observed with digoxigenin-labelled probe (data not shown). The *Car8* mRNA was detected in the liver as early as day 9.5 of gestation and its expression increased to a significant level during later differentiation stages. Another interesting finding of this study is the expression of this gene in the developing nervous system and its derivatives. *Car8* expression in the central nervous system showed interesting regional variations. Beginning at day 14.5 and continuing to day 16.5, *Car8* expression was especially strong in a zone of cells near the site of origin of the Purkinje cells, which is consistent with its adult localization. There was also significant expression in the forming corpus striatum and the sensory epithelia. In addition, it appears that the expression of *Car8* occurs in the regions of mesenchymal interaction with epithelium, particularly lung, forming glomeruli in the kidney and in vibrissae. As development progressed, high levels of *Car8* expression became restricted to the liver, gut and regions of the myocardium, with new high expression in the thymus.

The diversified pattern of mRNA expression observed during early organogenesis stages seems to persist in the adult stage as shown in the various tissues tested (e.g. liver, heart and brain) by reverse transcription–polymerase chain reaction (Fig. 10) with no indication of expression in the lung. It is likely that this gene is not tissue specific and that the lack of detectable signal in some tissues does not exclude the possibility of expression of this gene at low levels in these tissues.

The abundance and relatively widespread distribution of CA VIII mRNA at different developmental stages in the mouse suggest that CA VIII may play a role in development; however, elucidation of this function awaits further studies. Genetic approaches, such as *Car8* knockout studies in mice, are presently under way in our laboratory in an effort to understand the function of this especially interesting acatalytic form of CA.

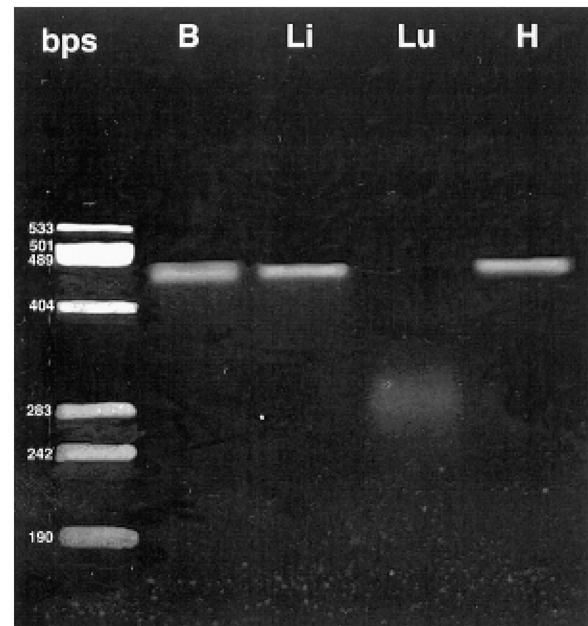

Fig. 10. Reverse transcription–polymerase chain reaction (RT–PCR) amplification of RNA isolated from the brain (B), heart (H), lung (Lu) and liver (Li) of adult mouse, using *Car8*-specific primers, indicating the expression of *Car8* in the brain, liver and heart, but not in the lung of the adult animal.

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