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The bifunctional protein DCoH/PCD, a transcription factor with a cytoplasmic enzymatic activity, is a maternal factor in the rat egg and expressed tissue specifically during embryogenesis

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ABSTRACT The bifunctional protein DCoH/PCD is both a cytoplasmatic enzyme (PCD) involved in the tetrahydrobiopterin regeneration and a transcription coactivator (DCoH). Originally detected in liver cell nuclei, it forms a 2:2 heterotetrameric complex with the nuclear transcription factors HNF1α and the variant form HNF1β and enhances their transcriptional potential. To address the role of DCoH in tissue specific and developmental gene regulation we analyzed its spatial and temporal expression pattern in the rat. DCoH might have a function in tissue specific gene expression mediated by HNF1 in the adults and in the developing embryo as it is found in the kidney and the liver, organs known to contain HNF1. In addition DCoH is a maternal factor in the rat egg lacking HNF1 transcription factors. The maternal protein enters the cell nuclei at the 8-cell stage suggesting a role in early embryonic gene regulation and excluding a cytoplasmatic enzymatic function. Evidence for a HNF1 independent function of DCoH is also given by the fact that DCoH is present in the eyes (pigmented epithelium) and the brain (ependym cells) of the rat embryos, cell types lacking HNF1 proteins. The tightly regulated expression pattern of DCoH in distinct cell types originating from endo- meso- and ectoderm is conserved between the rat and the frog indicating a fundamental role for DCoH in early gene regulation among the vertebrates.

KEY WORDS: *coactivator, enzyme, DCoH/PCD, embryogenesis, rat*

Introduction

Temporally and spatially defined gene expression is mediated by the interaction of transcription factors with DNA elements present in the promoters and enhancers of the genes. This process is fundamental for the development of a multicellular organism and relies on the localized activities of maternal determinants. Most convincingly the role of maternal factors involved in a regulatory network has been shown for the early development of *Drosophila* (St. Johnston and Nüsslein-Volhard, 1992; Coury and Huang, 1995). Also in *Xenopus* there is clear evidence for the activity of transcriptional cascades responsible for the very early differentiation processes in development (e.g. Brewer *et al.*, 1995; Holewa *et al.*, 1995; Horb and Thomsen, 1997; Partington *et al.*, 1997). However, data concerning maternal components of the mammalian egg involved in gene regulation are limited.

In the *Xenopus* egg and early developmental stages we have recently identified the transcriptional regulator DCoH as a maternal factor (Pogge v. Strandmann and Ryffel, 1995). DCoH is a bifunctional protein with combined catalytic and regulatory properties (for review see Hansen and Crabtree, 1993; Suck and Ficner, 1996). One of the functions of DCoH also called PCD (**p**terin-4α-**c**arbinolamine-**d**ehydratase) concerns its carbinolamine dehydratase activity, that is involved in the phenylalanine hydroxylase enzyme complex as well as in the function of other mono-oxygenases (Citron *et al.*, 1992). There are human diseases linked to a deficiency of this enzymatic activity like a mild form of hyperphenylalaninemia and the depigmentation disorder vitiligo. The other regulatory role of DCoH is linked to the function of the homeodomain transcription factors HNF1α and HNF1β (Mendel *et al.*, 1991b). The transcriptional potential of both factors, that bind as homo- and heterodimers to a common DNA recognition sequence (Mendel *et al.*, 1991a) is enhanced by DCoH, which stabilizes their dimerization

Abbreviations used in this paper: DTT, dithiothreitol; E22, embryonic day 22; EDTA, ethylendiaminietetraacetic acid; LDH, lactate dehydrogenase; LHRH, luteinizing hormone releasing hormone; P0, P2, etc., postnatal days 0, 2, etc; pGlu-, pyroglutamyl-; TRH, thyrotropin releasing hormone.

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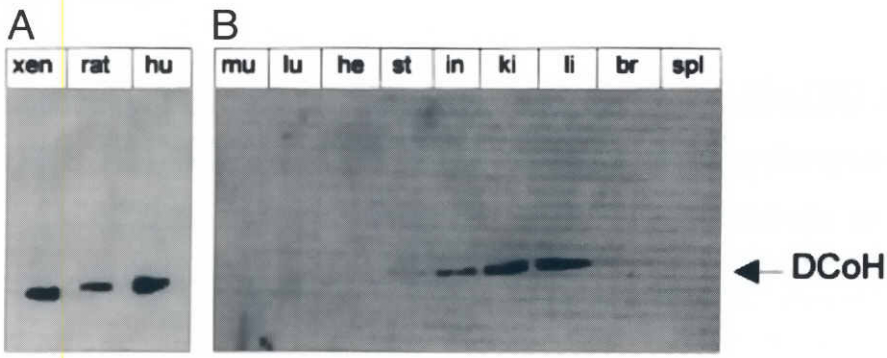

Fig. 1. Specificity of the DCoH antiserum and tissue-specific expression of DCoH. *Western blotting: Aliquots of liver extracts from Xenopus (xen), rat (rat) and human (hu)* **(A)** *and aliquots of rat extracts derived from various tissues* **(B)** *were separated on a 15% SDS gel, blotted and the membrane was incubated with the DCoH specific rabbit-antiserum as the first antibody. Signals were visualized with anti-rabbit antibodies coupled to the m peroxidase and ECL reaction. mu, muscle; lu, lung; he, heart; st, stomach; in, intestine; ki, kidney; li, liver; br, brain; spl, spleen.*

(Mendel *et al.*, 1991b; Rhee *et al.*, 1997). HNF1α and β were originally described as regulators for the establishment and maintenance of the hepatocyte phenotype. However, the distribution of these factors is not restricted to the liver as one of them or both are in addition detectable in kidney, pancreas, lung and the intestinal tract (Blumenfeld *et al.*, 1991; Ott *et al.*, 1991; Cereghini *et al.*, 1992). Thus a cofactor with a characteristic tissue specific expression pattern might be involved in the regulation of selective gene activation by HNF1 in different tissues. Indeed in *Xenopus* it has been shown that DCoH is colocalized with HNF1α in liver and kidney but the protein is absent or detectable only in trace amounts in stomach and intestine (Pogge v. Strandmann and Ryffel, 1995), organs containing HNF1α levels comparable to the liver and kidney (Bartkowski *et al.*, 1993).

Investigating the expression of DCoH during *Xenopus* development implied a new role for DCoH independent of the HNF1 proteins, because it is detectable in cell types lacking HNF1. Thus DCoH is, in contrast to HNF1, a maternal component of the egg and nuclear and ubiquitously expressed in the early development. It is, in addition, found in the embryonic amphibian eye in the absence of HNF1 (Pogge v. Strandmann and Ryffel, 1995). The localization of the protein in the cell nuclei implies a regulatory function rather than a cytoplasmatic enzymatic activity of DCoH/PCD in early embryogenesis and in the eye.

The three dimensional structure of DCoH (Endrizzi *et al.*, 1995; Ficner *et al.*, 1995a,b; Cronk *et al.*, 1996; for reviews see Kim and Burley, 1995 and Suck and Ficner, 1996) supports the idea of a multifunctional protein with the ability to bind as yet unidentified macromolecules. The structure shows that DCoH exists as a stable homotetramer in the absence of HNF1 and reveals a new protein motif reminiscent of the molecular saddle seen in the TATA box binding protein TBP (Nikolov *et al.*, 1992). This saddle is typical for a structure binding nucleic acids or proteins. However no DCoH affinity to DNA or RNA has been observed and the binding domain of HNF1, that forms a 2:2 heterotetrameric complex with DCoH (Mendel *et al.*, 1991b) is not known. Recently, the stabilization of HNF1/DNA complexes by DCoH resulting in a promoted interaction with suboptimal DNA target sequences and, interestingly, a suppression of HNF1 binding to RNA has been shown, proving a role for DCoH in regulating the binding of HNF1 to nucleic acids (Rhee *et al.*, 1997).

The high degree of conservation between DCoH from *Xenopus* and rat, that are 85% identical on the amino acid level and functionally interchangeable (Pogge v. Strandmann and Ryffel, 1995), suggests a common and essential function for the protein among vertebrates. We have now analyzed the expression of DCoH in the rat to investigate a potential role in early regulatory processes in mammalian embryogenesis.

Results

DCoH is expressed in a tissue specific manner

An essential prerequisite for the analysis of the temporal and spatial expression pattern of DCoH in the rat is the availability of specific antibodies detecting the mammalian protein. In this study we used a polyclonal rabbit antiserum raised against recombinant DCoH from *Xenopus* (XDCoH). This antiserum was monospecific as it has been purified by an affinity matrix coupled to DCoH (Pogge v. Strandmann and Ryffel, 1995). To test the specificity of the antibodies for mammalian DCoH we performed a Western blot with crude extracts from *Xenopus*, rat and human liver, an organ known to express the protein (Fig. 1A). In all these extracts there is a single immunoreactive protein of 12 kDa, the expected size of DCoH. Although DCoH from the various species are identical in size (104 amino acids each), the mammalian proteins reveal a slightly retarded mobility in the gel. This is probably due to the 15% sequence differences of DCoH from *Xenopus* versus rat and human (Pogge v. Strandmann and Ryffel, 1995). The proteins from rat and human have a single amino acid change (Mendel *et al.*, 1991b) and therefore show the same electrophoretic mobility. Having proven the specificity and sensitivity of the antiserum we analyzed the tissue distribution of DCoH with extracts derived from various tissues of adult rats (Fig. 1B). The DCoH protein is detected predominantly in kidney and liver, in smaller amounts in intestine and in trace amounts in stomach. The other organs tested lack the protein. These results are in a good agreement with the DCoH mRNA expression, that is predominantly found in the protein containing tissues (Mendel *et al.*, 1991b).

To analyze the subcellular distribution of DCoH in the adult liver and kidney we performed immunostainings (Fig. 2). Comparing the immunofluorescence (A, right panel) and the phase contrast (A, left panel) of the same liver section the DCoH specific fluorescence is visible in the hepatocytes and in the kidney the cells forming the nephric tubules show the DCoH specific fluorescence (Fig. 2B).

The DCoH protein in the hepatocytes and the kidney cells is sequestered in the nucleus, but especially in the hepatocytes significant amounts are also detectable in the cytoplasm (Fig. 2).

We assume that this localization reflects the bifunctional character of DCoH. It is well documented that in addition to its role in transcriptional regulation in the nucleus DCoH acts as an enzyme involved in the cytoplasmic regeneration of tetrahydrobiopterin (Citron *et al.*, 1992).

These results demonstrate that DCoH is expressed tissue specifically in the adult rat and may contribute to the HNF1 mediated gene regulation. In addition the protein is correctly localized to fulfil its second function as a cytoplasmatic enzyme.

Embryonic expression of DCoH

To evaluate the significance of DCoH during rat embryogenesis we examined its developmental expression pattern. Using Western blots DCoH is detectable in extracts derived from rat eggs and it comigrates with recombinant DCoH and with DCoH of rat liver (Fig. 3 lane 1, 2 and 12). DCoH is therefore in contrast to the HNF1 transcription factors (Blumenfeld *et al.*, 1991; Ott *et al.*, 1991; Cereghini *et al.*, 1992; Pontoglio *et al.*, 1996) and the phenylalanine hydroxylase (Sanchez-Urretia *et al.*, 1978) a maternal factor stored in the egg. The protein is also detectable in total extracts of gastrula stages and day 12 p.c. embryos (lane 3 and 4). In later embryonic stages we observed the tissue restriction of DCoH, that correlates with the presence of DCoH in adult tissues. The protein is detectable in the embryonic kidney and liver with increasing amounts from day 16 (lane 5 and 6) to day 19 p.c. (lane 8 and 9), whereas DCoH is not found in the extracts from heart and brain. Interestingly, DCoH is expressed in the eye of a 16 day p.c. embryo (lane 7) lacking HNF1 and the activity of the phenylalanine hydroxylase dehydratase (Sanchez-Urretia *et al.*, 1978; Blumenfeld *et al.*, 1991; Ott *et al.*, 1991; Cereghini *et al.*, 1992; Pontoglio *et al.*, 1996). Therefore, DCoH may play a role distinct from the HNF1 proteins and its dehydratase function in these cells.

Examining the subcellular localization of DCoH may help to distinguish the cytoplasmic enzyme activity from the nuclear gene regulating function. Therefore the expression of DCoH protein was investigated in rat preimplantation stage embryos by a whole-mount immunohistochemical approach (Fig. 4). The protein is not restricted to specific areas of the egg, but is ubiquitously distributed. This expression pattern is maintained in the 4-cell stage, whereas it changes at the 8-cell stage. Comparison with the phase contrast reveals the translocation of DCoH into the nuclei at this developmental stage and the nuclear localization persists in the morula and in the blastocyst. It is visible that each cell contains DCoH, as the inner cell mass forming the embryo and the cells of the trophoectoderm building the extraembryonal structures show DCoH specific fluorescence.

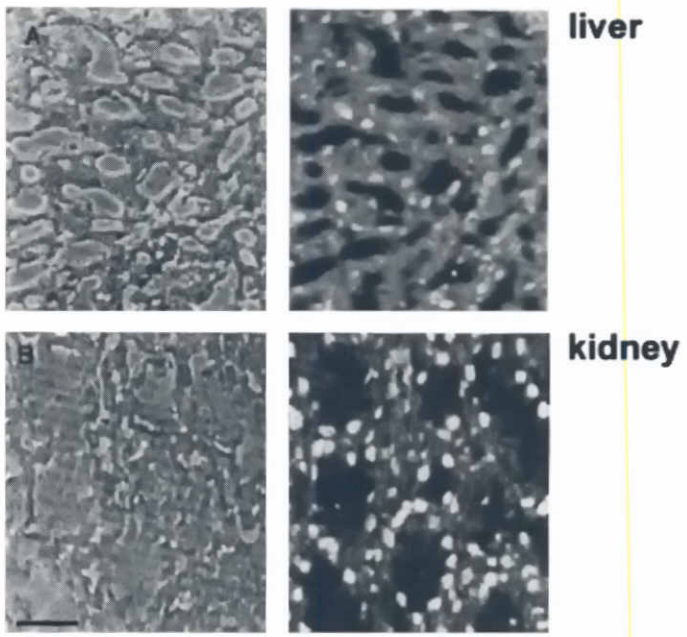

Fig. 2. Immunocytochemistry to detect DCoH in the liver and the kidney of adult rats. *Cryostat sections were incubated with the DCoH specific antiserum as the first antibody and a second fluorescence labeled antiserum (right panel). The left panel shows the corresponding phase contrast image. Control stainings using the DCoH antiserum, that was depleted by incubation with an excess of recombinant DCoH gave no signal. Bar, 50 μm.*

At later stages we analyzed the tissue distribution of DCoH in the embryo more precisely using cryosections of a day 16 p.c. embryo (Fig. 5). In the liver there are about 50% of the hepatocytes stained and we find DCoH predominantly in the nuclei. The embryonic kidney reveals poor morphological differentiation in the phase contrast image. But the staining of the DCoH containing cells reveals that some of the kidney cells are arranged in circles to form the characteristic tubules. The staining of the eyes is found in its outer cell layer known as the pigmented epithelium in later developmental stages. DCoH is additionally found in the brain restricted to the highly specialized ependym cells of the brain

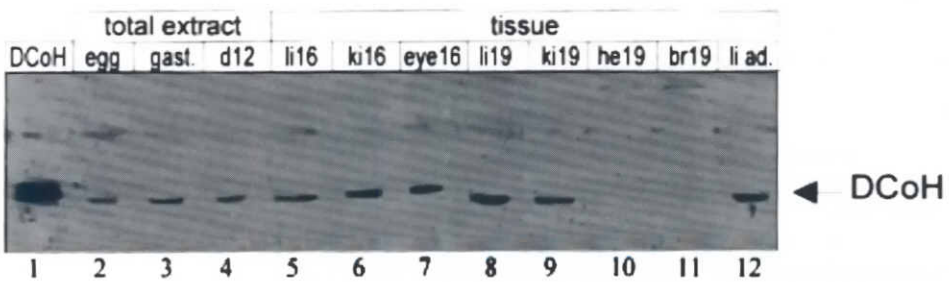

Fig. 3. Expression of DCoH during rat embryogenesis. *Total extracts from rat embryos (5-10 μg protein) of different developmental stages (egg; gast., gastrula, d12, day12 p.c.) and from embryonic tissues of day 16 (li, liver; ki, kidney; eye, eye) and of day 19 p.c. (li, liver; ki, kidney; he, heart; br, brain) were analyzed by Western blotting using the DCoH specific antiserum. Lane 1 was loaded with 20ng recombinant Xenopus DCoH protein and lane 12 was loaded with liver extract derived from an adult rat.*

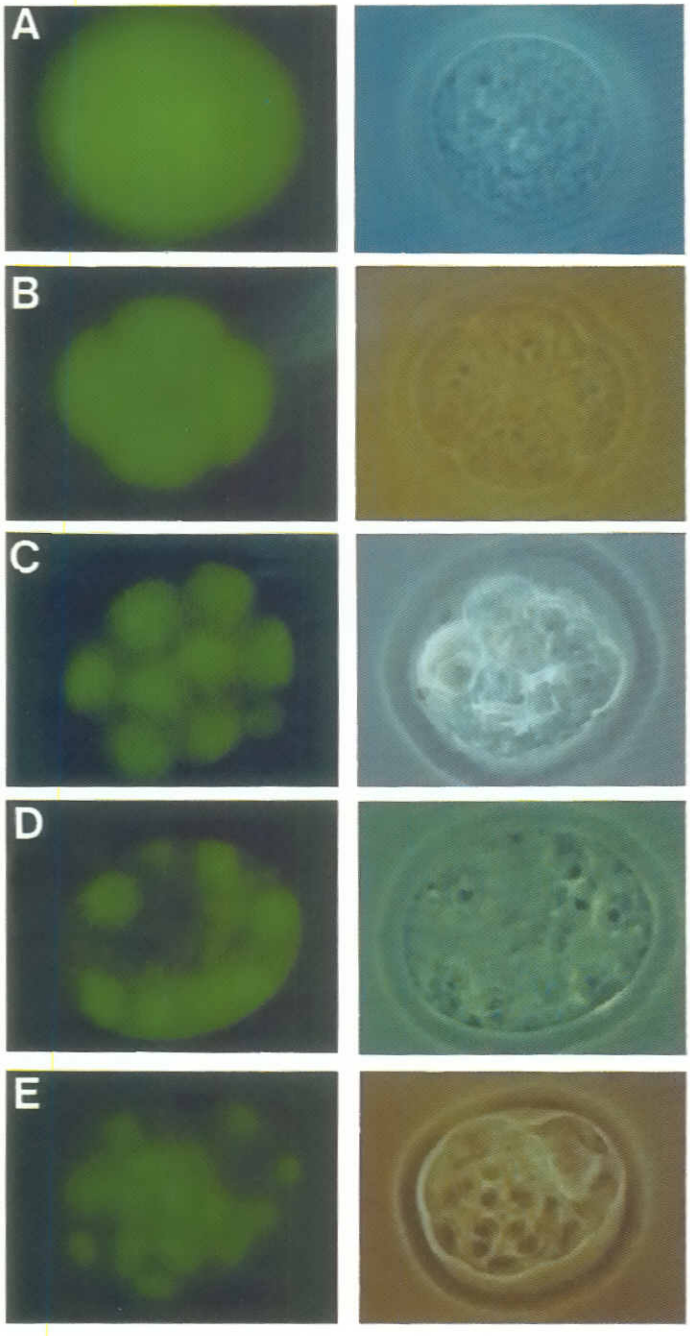

Fig. 4. Distribution of DCoH in early rat embryos. *Whole-mount immunostaining: The rat embryos were incubated with the DCoH specific antibodies from rabbit and a fluorescence labeled anti-rabbit antiserum. The left panel shows the phase contrast and the right panel the corresponding fluorescence images.* **(A)** *egg;* **(B)** *4-cell stage;* **(C)** *8-cell stage;* **(D)** *morula;* **(E)** *blastocyst.*

ventricles. We suppose that the lack of DCoH in Western blots of brain extracts (Fig. 3, lane 11) is due to the fact that the ependym cells constitute only a small fraction of the brain cells.

In summary DCoH is expressed in several distinct but not related cell types suggesting that the protein interacts with different cellular partners.

Discussion

Analyzing tissues of adult rats with DCoH specific antibodies in Western blots we localized DCoH in liver and kidney and in smaller amounts in stomach and intestine (Fig. 1). DCoH is therefore together with HNF1α and HNF1β found in liver and kidney, organs known to express these transcription factors (for a review see Tronche and Yaniv, 1992). In these organs we find DCoH at least partially in the cell nuclei (Fig. 2), probably involved in HNF1 dependent gene regulation. However, DCoH is only found in small amounts in the intestine and hardly detectable in the stomach, although the HNF1α level in these organs compared to liver and kidney is invariant (Blumenfeld *et al.*, 1991). The lung, which along with the liver and kidney is known to contain high levels of HNF1β (Cereghini *et al.*, 1992), does not contain any detectable DCoH protein. The DCoH protein data correspond to the results of a biochemical assay for the PCD enzyme (Davis *et al.*, 1992), which is identical to DCoH (Citron *et al.*, 1992). The enzyme activity is most abundant in liver and kidney and also detectable in certain brain compartments as pineal, pituitary and hypothalamus (Davis *et al.*, 1992).

The differential expression of HNF1 and DCoH supports the hypothesis that the presence of DCoH may modulate HNF1 activity in a tissue specific way. It is an attractive idea that the cofactor may contribute for example to the different regulatory functions of HNF1α in the liver and the stomach, two tissues that contain HNF1α, but express different target genes of the same transcription factor. In this context it is noteworthy that Rhee *et al.* (1997) have recently described an altered DNA binding affinity for HNF1/DCoH complexes compared to HNF1 homodimers using non optimal HNF1 binding sites.

A discrepancy between the expression of a transcription factor and its target genes has been noted also for Oct1, a ubiquitous factor paradoxically mediating B-cell specific gene expression. The cell specificity of the Oct1 regulatory potential is achieved by the activity of the coactivator Bob1, necessary for the Oct1 function and strictly restricted to B-cells and other lymphatic derivatives (Gstaiger *et al.*, 1995; Strubin *et al.*, 1995).

In the embryo we detected DCoH as a maternal factor in the egg (Figs. 3, lane2 and 4A). At day 16 p.c. the protein is in addition to liver and kidney expressed in the pigmented epithelium of the eye and the ependym cells of the brain (Fig. 5). Obviously, the DCoH activity in all these cell types is independent of HNF1 since HNF1α and HNF1β are neither maternally expressed nor found in neuroectodermal derivatives (Blumenfeld *et al.*, 1991; Ott *et al.*, 1991; Cereghini *et al.*, 1992; Miura *et al.*, 1993; Pontoglio *et al.*, 1996). As DCoH enters the cell nuclei very early in development at the 8-cell stage (Fig. 4) it is likely that the factor plays a role for the gene regulation rather than an enzymatic function in the embryo.

The expression pattern of DCoH in the rat and in *Xenopus* (Pogge v. Strandmann and Ryffel, 1995) is highly conserved and this adds to the high degree of homology in structure and function. Such a similarity most convincingly seen in the translocation of the maternal DCoH into the cell nuclei very early in rat and *Xenopus* development suggests a common role for the protein among the vertebrates.

Supporting an activity of DCoH in fundamental processes of gene regulation and amino acid metabolism a bacterial homolog has been cloned (Zhao *et al.*, 1994). This gene product (*phhB*) is

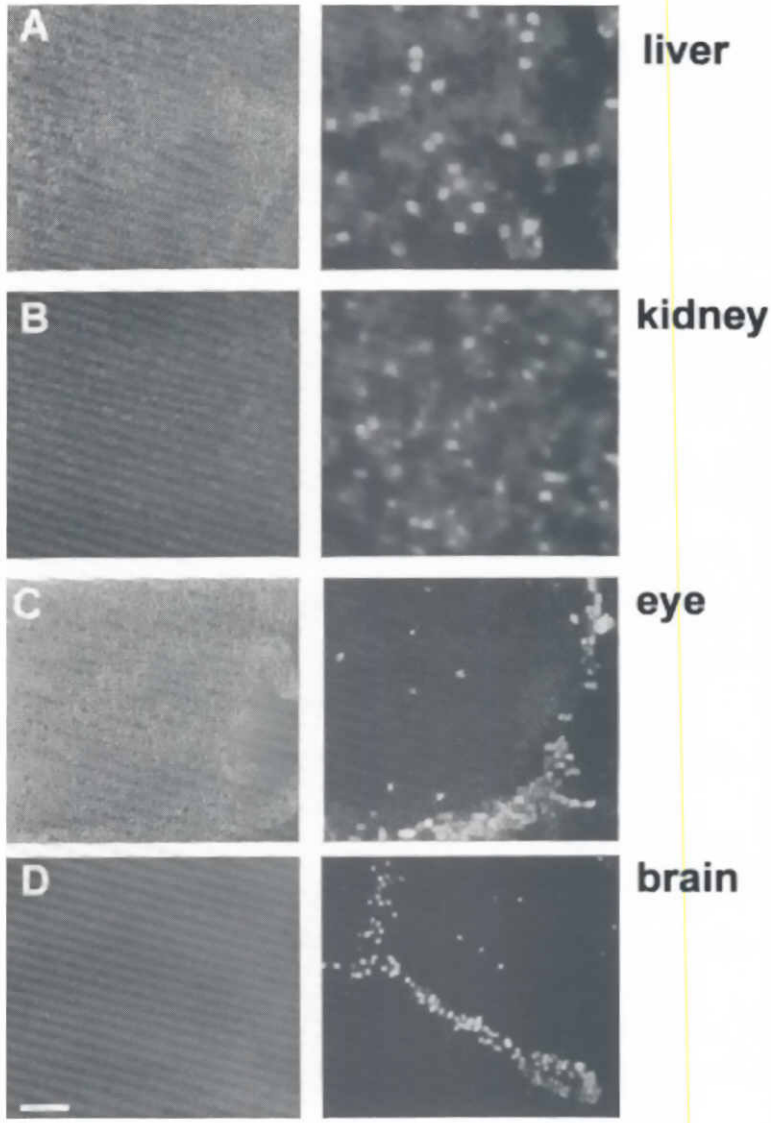

Fig. 5. Immunocytochemistry to detect DCoH in the day 16 p.c. embryo. *Cryosections were incubated with the DCoH specific antiserum and fluorescence labeled secondary antibodies. The left panel represents the phase contrast, the right panel the fluorescence images. Control experiments were performed for each section which demonstrated that preincubation of the antibodies with recombinant Xenopus DCoH abolishes staining.* **(A)** *liver;* **(B)** *kidney;* **(C)** *eye;* **(D)** *brain.*

essential for the transcription of the operon containing the enzymes of the phenylalanine-hydroxylase complex including the *phhB* gene itself and it possesses in addition the enzymatic dehydratase activity (Zhao *et al.*, 1994).

A number of maternal transcription factors involved in the differentiation processes and pattern formation of *Drosophila* are well characterized concerning their specific function and target genes (St. Johnston and Nüsslein-Volhard, 1992; Coury and Huang, 1995). In contrast in mammals there are not many maternal regulatory factors described that, based on their embryonic expression pattern, might be involved in early gene regulation. Therefore, our identification of the cofactor DCoH as a maternal component in the rat egg is an interesting novel finding. In mammals the only cell-type specific regulators, that are maternally expressed are *Oct6* and *Oct4*, members of the POU family of transcription factors. While the spatial expression pattern of *Oct6* in the early embryo is not known it is together with *Oct4* found in undifferentiated stem cells (Schöler *et al.*, 1989a,b; Rosner *et al.*, 1990), whereas *Oct4* is specifically found in the cells forming the inner cell mass (Palmieri *et al.*, 1994). Thus the maternal factors *Oct4* and DCoH are differently distributed in the embryo as DCoH is found ubiquitously in the blastocyst. Interestingly the activity of an injected promoter construct containing the octamer motif is restricted to the inner cell mass containing specifically *Oct4* (Schöler *et al.*, 1989a), suggesting that the restricted expression of this transcription factor is one feature of proper gene expression in embryogenesis. Maternal regulatory factors represent therefore good candidates initiating the gene expression in the early mammalian embryo that directs embryogenesis.

Materials and Methods

Preparation of the DCoH antiserum and Western blotting

The polyclonal rabbit antiserum raised against *Xenopus* DCoH (produced by Eurogentec, Pogge v. Strandmann and Ryffel, 1995) was affinity purified using a column with recombinant XDCoH protein covalently coupled to tresyl-activated agarose (Schleicher and Schüll) in 0.1 M $NaCO_3$ pH 8.5. The binding of the antibodies was performed in phosphate buffered saline (PBS) and the elution was undertaken using 0.1 M glycine pH 2.5. The eluted antibodies were dialyzed against PBS.

For extract preparation the embryos and tissues were homogenized in SDS loading buffer (Sambrook *et al.*, 1989), boiled, cleared by centrifugation (10 min, 10°C, 50.000g) and stored at -80°C. Five-ten µg of protein were separated on 15% SDS gels, electrotransferred to nitrocellulose membranes and the blots were incubated with 0.5% blocking reagent (Boehringer). The affinity purified antibodies were used 50-fold diluted in 0.2% blocking reagent and the secondary horse radish peroxidase conjugated mouse anti-rabbit antiserum (Boehringer) was used to visualize bound antibodies with the ECL-system (Amersham).

Tissue fixation and immunofluorescence

The fixation procedure using the periodate-lysine-paraformaldehyde method (McLean and Nakane, 1974) is described previously (Weber *et al.*, 1996). Eight µm cryosections were mounted on BSA coated glass slides, blocked (1 h PBS/10% goat serum), incubated with the purified DCoH specific antiserum (1:20 diluted in PBS/10% goat serum) over night at 4°C and incubated with the secondary fluorescein isothiocyanate-conjugated anti-rabbit IgG antibody from mouse (Boehringer, 500-fold diluted in PBS/10% goat serum) at 20°C for 90 min. To prepare a control serum for antigen specificity, the DCoH-specific antiserum was depleted from DCoH specific antibodies using recombinant XDCoH protein bound to Ni-NTA beads (5mg/ml).

Whole-mount immunostaining

The preimplantation embryos were collected in M2 medium (Quinn *et al.*, 1982), rinsed with PBS/PVP (PBS with 3mg/ml polyvinylpyrolidone) and washed in PHEM (60mM PIPES, 25 mM HEPES, 10 mM EGTA, 1 mM $MgCL_2$, pH 6,9). Fixation was done in 1% paraformaldehyde in PHEM for one hour at room temperature. The fixed embryos were washed 20 min each in PHEM/0.5% Tween and in PBSCMF (PBS without Ca2+ and Mg^{2+}), followed by 30 min incubation in PBSCMF/10% goat serum for

blocking. These steps were done at room temperature. The purified DCoH antibodies and the depleted control serum was used 20-fold diluted in PBSCMF/10% goat serum and incubated with the embryos over night at 4°C. The excess of antibodies was removed by 4 times washing in PBSCMF/0.5% Tween20 for 30 min each. The secondary antiserum (FITC-conjugated swine-anti-rabbit antibodies, Dianova) was used 200 fold diluted in PBSCMF/10% goat serum. The incubation was performed for 1 h at room temperature followed by 4 times washing for 30 min with PBSCMF/0.5% Tween20.

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