Cloning and expression pattern of EPAS1 in the chicken embryo

Colocalization with tyrosine hydroxylase

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Abstract EPAS1 is a hypoxia-inducible transcription factor, highly expressed in vasculature and recently shown to be necessary for catecholamine production during embryogenesis. We report here the cloning and detailed expression pattern of this factor in the chicken embryo. We show that chicken EPAS1 presents an overall identity of 76% with the human sequence and that it is strongly expressed in the blood vessel wall, mostly in endothelial cells, but also in vascular smooth muscle cells. Moreover, we report non-vascular expression sites: liver, kidney, and, quite interestingly, cells of the sympathetic nervous system where EPAS1 is coexpressed with one of its putative target genes, the tyrosine hydroxylase. EPAS1 could therefore represent the link between the vascular system and the sympathetic nervous system, both sensitive to hypoxia.

Key words: EPAS1; Hypoxia-inducible factor 2α; Hypoxia; Tyrosine hydroxylase; Angiogenesis; *Gallus gallus*

1. Introduction

The family of proteins harboring a basic helix-loop-helix PAS (bHLH-PAS) domain (for review see [1]) constitutes a class of transcription factors encompassing a broad range of functions, mostly in differentiation and metabolic processes. Thus, they regulate genes involved in neurogenesis [Single-minded (Sim), NPAS], xenobiotic metabolism [aryl hydrocarbon receptor (AHR)], control of circadian rhythm [Period (Per), Clock, Timeless], tracheal formation [trachealess (trh)] and response to low oxygen tension [hypoxia-inducible factor 1α (HIF1α)]. All family members comprise a bHLH domain implicated in DNA binding and dimerization, located in the amino-terminus of a PAS domain. The PAS (Per-Arnt-Sim) region which displays two hydrophobic repeat motifs (A and B) functions as a dimerization interface.

Endothelial PAS protein 1 (EPAS1) is a member of the bHLH-PAS family recently identified in human [2], mouse [2] and bovine [3] species. It has also been reported as HIF1α-like factor (HLF) [4], HIF1α-related factor (HRF) [5], member of PAS superfamily 2 (MOP2) [6] and HIF2α [7]. The primary amino acid sequence of EPAS1 is 48% identical to that of HIF1α. Like HIF1α, EPAS1 can form active heterodimers with ARNT (aryl hydrocarbon receptor nuclear translocator) [2] and ARNT3 [8], and transactivate the expression of a reporter gene containing HREs (hypoxia responsive elements) in the promoter region. In transfection assays, this activity is enhanced 2–4-fold in hypoxic conditions (1% O_2) [2], mediated by an accumulation of EPAS1 protein – but not mRNA – [9], as previously shown for HIF1α [10,11]. Although these two transcription factors seem to share common functional mechanisms in vitro, their patterns of expression studied in the mouse suggest that they play different and perhaps complementary roles during embryogenesis. In the mouse fetus, the expression of HIF1α is ubiquitous and does not seem to be specific to any cell or tissue type [12], unlike EPAS1 which is particularly expressed in endothelial cells (ECs) [2,5,12]. HIF1α has been previously shown to be necessary for both embryonic and tumoral angiogenesis [13–15]. The high homology of EPAS1 with HIF1α together with its increased activity under hypoxic conditions and its vascular expression pattern suggested an important role in regulation of angiogenesis. Moreover, EPAS1 displays activity on the promoters of three genes known to be essential for angiogenesis, i.e. the vascular endothelial growth factor (VEGF) [4,9], its receptor Flk-1 [16] and the endothelial receptor Tie2 [2]. These observations still further point to a central role of EPAS1 in blood vessel formation.

Unexpectedly, the recent results of EPAS1 gene inactivation in the mouse [17] revealed a crucial role of this factor, not in vascular formation, but in catecholamine production. Indeed, EPAS1−/− mice die of heart failure between day 12 and 16 of embryonic development, a probable consequence of a profoundly reduced level of norepinephrine. These observations pointed toward other potential target genes to be regulated by EPAS1 such as tyrosine hydroxylase (TH) and dopamine β-hydroxylase, two key enzymes implicated in catecholamine release and known to be overexpressed during hypoxia [18,19].

Discrepancies between the importance of EPAS1 in angiogenic processes as suggested by both in vitro and in situ studies and the results obtained in vivo remain to be clarified. To address these questions, we have undertaken a study of this transcription factor using an experimental model well established for the study of development and particularly angiogenesis, the chicken embryo. We have cloned the avian homologue of EPAS1 and studied its expression during chicken development. We report here that both the sequence and the expression pattern reveal a high conservation of EPAS1 between mammals and avian species. Moreover, our observations also show a precise colocalization of this transcription factor with TH in cells of the sympathetic system. The coexpression of EPAS1 and TH provides a simple explanation for the lethality due to insufficient levels of catecholamine observed in EPAS1−/− mice and strongly suggests a direct regulation of TH by EPAS1 under normal or hypoxic conditions.

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2. Materials and methods

2.1. Cloning of cEPAS1

2.1.1. RT-PCR. Total RNAs were extracted from chicken lung according to the method of Chomczynski and Sacchi [20]. One μg of total RNA was reverse transcribed in a 20 μl reaction volume for 1 h at 37°C, using the MMLV reverse transcriptase (Gibco-BRL) as described by the manufacturer. Primers used to amplify the chicken EPAS1 cDNA (A: 5′-AGCAAGACCTTCCTGAGCC-GC-3′; B: 5′-GGTGACTCTTGGTCATGTTCTC-3′) were designed from the human sequence and corresponded to the highly conserved domain PAS-B. The PCR reaction was performed as follows, on a Perkin Elmer Cetus 480 thermal cycler: the cDNAs were denatured (94°C for 5 min), subjected to 30 cycles of amplification (94°C for 1 min, 54°C for 1 min and 72°C for 1 min) and to a final elongation (72°C for 10 min). After purification on a QIAquick PCR Purification column (Qiagen), the PCR products were sequenced, using the Big-Dye terminator Cycle Sequencing Ready Reaction on an Applied Biosystem 377 DNA Sequencer.

2.1.2. Rapid amplification of cDNA ends (RACE). Poly(A)$^+$ RNAs were purified from total chicken lung RNAs with the mRNA Separator Kit (Clontech). First and second strand cDNA synthesis, adapter ligation and 5′-RACE or 3′-RACE were performed using the Marathon cDNA Amplification kit (Clontech) and the Advantage cDNA Polymerase Mix (Clontech), as described by the manufacturer. For both 5′ and 3′ elongations, two successive reactions were performed, with a first and a second nested primer, respectively. These primers were: cEP5-1: 5′-CGGCCCAGCAACTCTTCTGGGTGG-3′ and cEP5-2: 5′-GCGGCTCAGGAAGGTCTTGCT-3′ for the 5′-RACE; and cEP3-1: 5′-CCACCCAGAAGAGTTGCTGGGCCG-3′ and cEP3-2: 5′-GAGAACATGACCAAGAGTCACC-3′ for the 3′-RACE. The first amplification reactions consisted of 30 cycles (94°C for 30 s and 68°C for 4 min), whereas the nested amplifications were carried out with only 18 cycles. After sequencing of the RACE products, the cEPAS1 cDNA was finally generated using Pfu polymerase (Promega) with the following primers: cEP-ATG: 5′-GGGGTACC-GCCGCGTGACAGCTGACAAGG-3′ and cEP-TGA: 5′-GTCTA-GAATATTGCAGGATTGCTCAGGTTGCC-3′ and cloned in a pcDNA3.1zeo+ vector (Invitrogen) between the *Kpn*I and *Xba*I restriction sites.

2.2. In situ hybridization (ISH)

In situ hybridization was performed as described previously [21]. Briefly, embryos were fixed in freshly prepared 4% paraformaldehyde in phosphate buffered saline (PBS), dehydrated in a graded series of ethanol solutions and embedded in Paraplast. Sections of 5–7 μm thick were mounted on silanized slides. For hybridization, the sections were deparaffinized and rehydrated, subjected to a microwave heating treatment for signal enhancement [21], digested with proteinase K (20 μg/ml), postfixed in 4% paraformaldehyde, and dehydrated in ethanol. Single strands sense and antisense [^{35}S]RNA probes were generated by in vitro transcription of a partial cEPAS1 clone (nucleotides 251–1438) in pCRII (Invitrogen) using [^{35}S]UTP and SP6 or T7 polymerase. Hybridization was performed overnight at 50°C. Slides were washed in increasing stringency solutions and non-hybridized probes were hydrolyzed by RNase A (20 μg/ml). After dehydration, slides were dipped in NTB2 emulsion (Kodak), exposed in the dark for 2 weeks, photographically developed and counterstained with toluidine blue.

2.3. Immunohistochemistry (IHC)

Sections were deparaffinized, rehydrated, washed in PBS and non-specific binding was blocked with 3% normal goat serum in PBS. An anti-tyrosine hydroxylase antibody (Institut Jacques Boye, Reims, France) was used at a 1/1500 dilution in 1% normal goat serum (1.5 h at room temperature), followed by a secondary antibody (goat biotinylated anti-rabbit IgG 1/200, 45 min) and by amplification with the avidin-biotin complex (ABC Elite, Vector, Burlingame, CA, USA) for 45 min. The peroxidase reaction was then developed with 0.5 mg/ml diaminobenzidine tetrahydrochloride (DAB) in Tris (pH 7.6, 50 mM), and 0.03% H_2O_2 for 10–15 min. The slides were washed in Tris (pH 7.6, 50 mM) for 10 min, dehydrated and mounted in Eukitt.

2.4. Double labeling (ISH+IHC)

The ISH protocol was performed as described above, but interrupted in NaCl-TE, before the final wash in 0.1 SSC which was

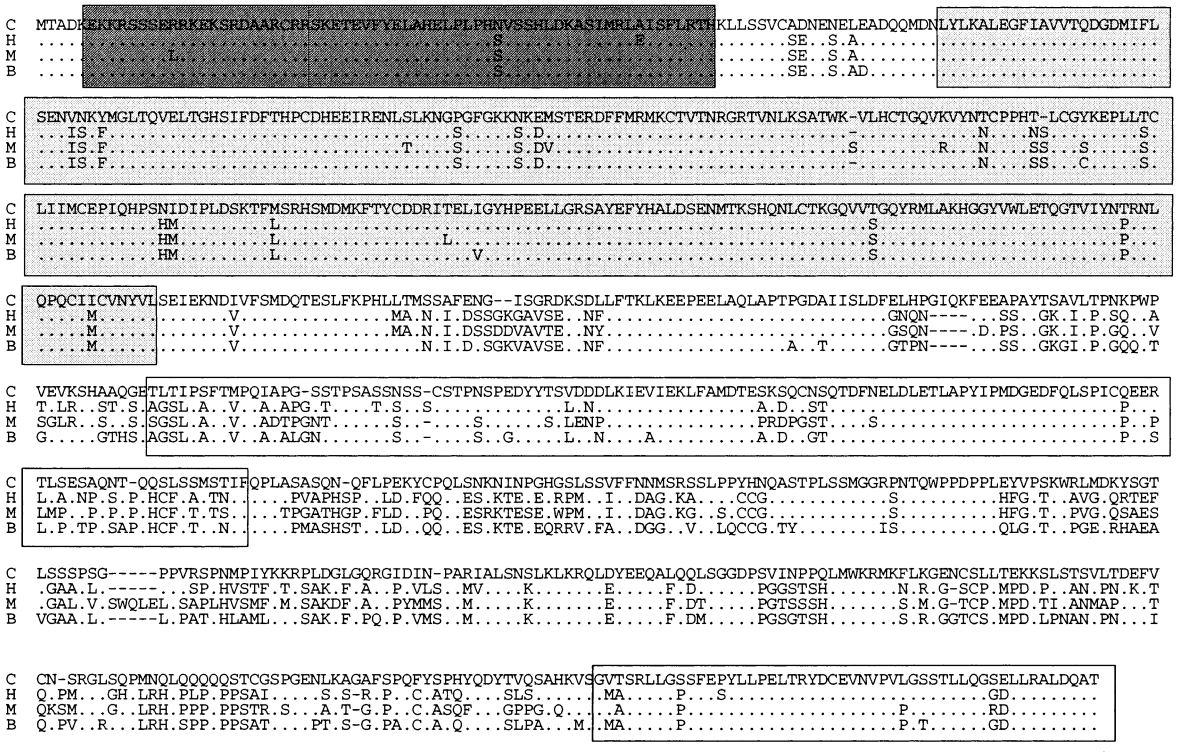

Fig. 1. Sequence alignment of (C) chicken (GenBank accession number AF129813), (H) human, (M) mouse and (B) bovine EPAS1 proteins. Only differences with the chicken EPAS1 are shown. Functional domains are indicated with boxes in dark gray for bHLH, light gray for PAS and plain for activation domains.

replaced by PBS and the IHC protocol was carried out with a final wash in Tris (pH 7.6, 50 mM) overnight. After dehydration, slides were dipped in NTB2 emulsion (Kodak), exposed in the dark for 2 weeks, developed and mounted in Eukitt.

3. Results

3.1. Cloning of chicken EPAS1 cDNA

In order to identify the avian homologue of the bHLH-PAS transcription factor EPAS1, we performed RT-PCR with conserved primers on RNAs extracted from chicken lung. A 160 bp fragment, located into the PAS-B domain was amplified. It presented 88% homology with the human EPAS1 sequence in nucleotides, and 100% identity in amino acids. This sequence was therefore used to design a specific primer, and this first fragment was elongated by 5′- and 3′-RACE. We have sequenced 3112 bp of the chicken EPAS1 (cEPAS1) cDNA, which contains a putative open reading frame of 2604 bp. Translation of this open reading frame into amino acids predicts a 868 residues protein, with high sequence identities to human, bovine and mouse EPAS1 (76%, 74% and 72% overall identity respectively; Fig. 1). As expected, sequence comparison revealed a striking similarity between avian and mammalian EPAS1 in the amino-terminal half, including bHLH (97%) and PAS (93%) motifs. While most of the sequence in the C-terminal half is variable between the different animal species, a high homology stretch is observed in the last 57 amino acids (90%), in a conserved region shown to contain a hypoxia-responsive transcriptional activation domain [22]. Interestingly, a Q-rich activation domain, characteristic of bHLH-PAS proteins, was identified in the chicken sequence whereas it is not present in mammalian EPAS1. A Northern blot experiment revealed a single band of cEPAS1 mRNA at approximately 6.5 kb (data not shown).

3.2. Expression of EPAS1 during chicken embryogenesis

We studied the expression pattern of the transcription factor cEPAS1 by in situ hybridization, between day 1 (E1) and 13 (E13) of chick development.

3.2.1. Vascular system The earliest expression of cEPAS1 was detected in four somite embryos (corresponding to Hamburger and Hamilton's stage 8 [23]) in the extra-embryonic ectoderm, and in the embryo proper at stage 13, in the ECs of the dorsal aorta (Fig. 2A). At later stages, a strong hybridization signal was observed in almost every blood vessel of the embryo, including intersegmental vessels, dorsal aorta (Fig. 2B), sprouting vessels of the neural tube (Fig. 2C), brain capillaries (Fig. 2D), vessels of the choroid plexus epithelium, or capillaries in the lung bud (Fig. 2E). Apart from the noticeable exception of the cardinal veins which lack cEPAS1 in their wall (Fig. 2B), this signal was present in all kinds of vessels, with no restriction of size, type or localization in the embryo. Within the vessel wall, cEPAS1 mRNA was detected predominantly in ECs and, to a lesser degree, in surrounding pericytes or vascular smooth muscle cells (VSMCs). This latter signal was observed in most cases in the inner layer(s) of VSMCs. However, in some large arteries, the labeling could also be localized throughout the whole vessel wall, with a stronger intensity in the outer cell layers (adventitia) (Fig. 2F). Interestingly, we observed a loss of cEPAS1 expression in hematopoietic cells, sprouting from the ventral region of the dorsal aorta at E3 (data not shown).

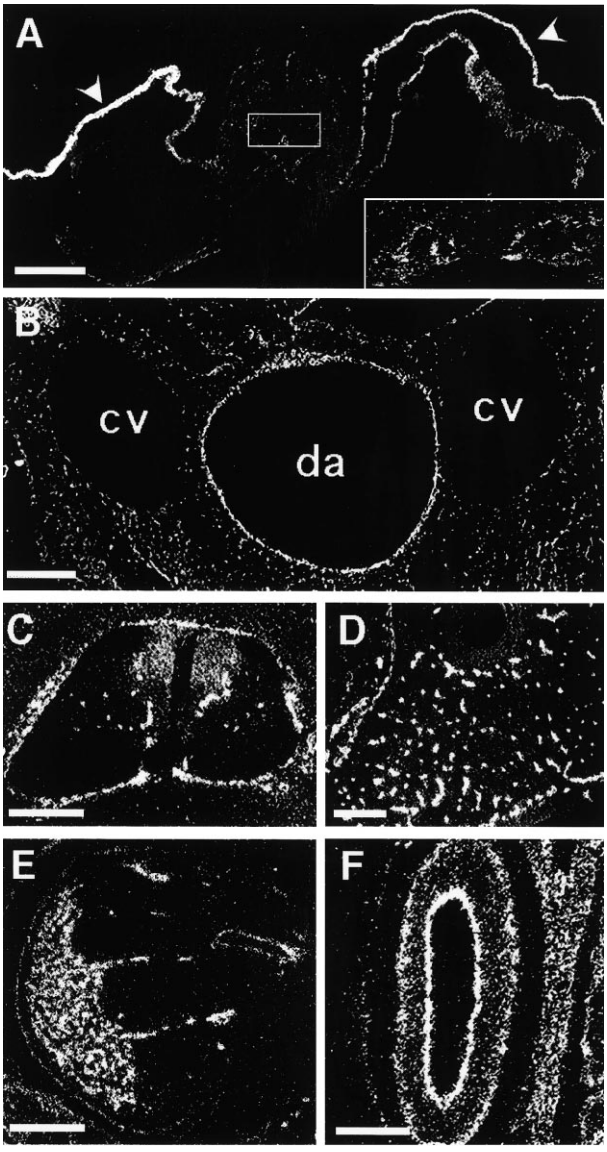

Fig. 2. Vascular expression of cEPAS1 in the chicken embryo. In situ hybridization signal is observed under dark field illumination. Note the expression of cEPAS1 at stage 13 (A) in the ectoderm of the extra-embryonic area (arrowheads) and in ECs of dorsal aorta (insert). At stage 24 (B), cEPAS1 is present in dorsal aorta (da), but not in cardinal veins (cv). During development, the vascular expression of cEPAS1 remains very intense in all blood vessels and capillaries as in neural tube (C) and brain (D) at day 6 or lung bud (E) at day 8. Note that although cEPAS1 is always expressed in ECs, it can also be present in VSMCs (F). Bar: 200 μm (A, C, D, E); 100 μm (B, F).

3.2.2. Sympathetic nervous system Considering the gene inactivation of EPAS1 which results in a decrease of catecholamine levels, the expression of cEPAS1 in sympathetic neurons and ganglia was studied in detail. We compared the expression of this transcription factor, detected by ISH, to that of TH, a putative target of EPAS1 in this system, revealed by IHC. Starting at E4, expression of cEPAS1 was observed in the para-aortic ganglia forming the primary sympathetic chain, visible at that early stage as two rows of cells ventral to the somites and immediately lateral to the aorta. TH protein was also detected in the same cells (shown at E5 in Fig. 3A). Then, this coexpression continued at E5–E6 at least in

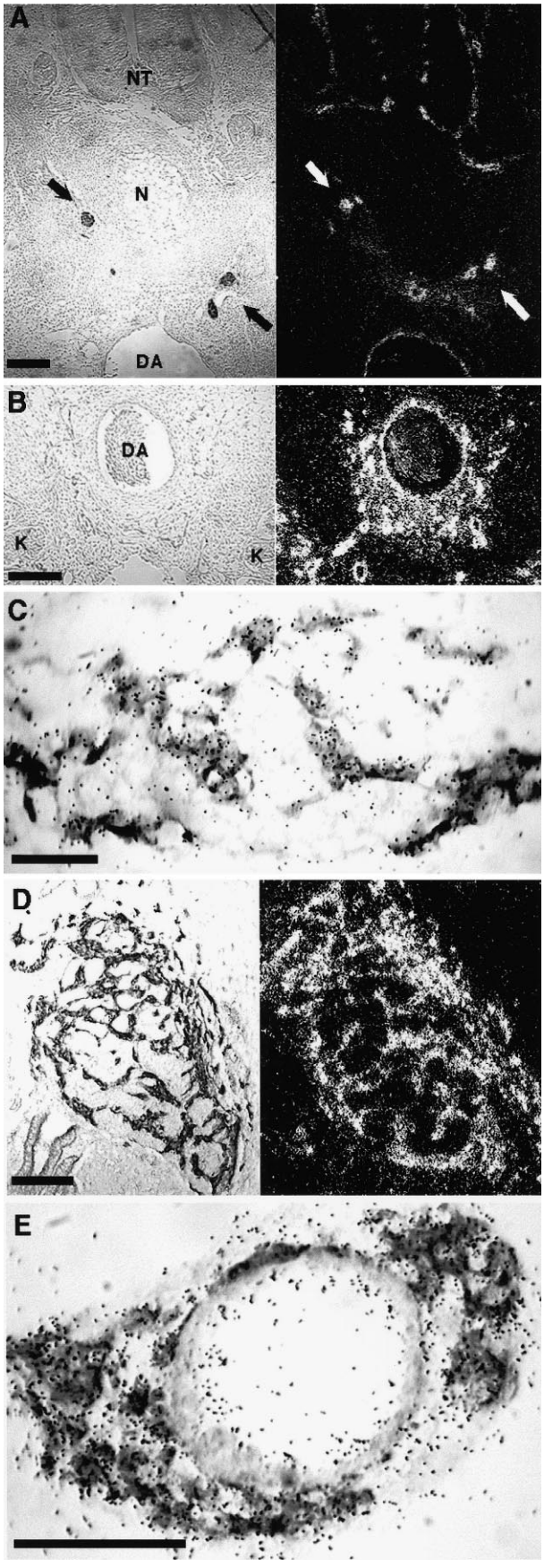

Fig. 3. Expression of cEPAS1 and tyrosine hydroxylase in the sympathetic nervous system. At E5, EPAS1 and TH are coexpressed in para-aortic ganglia (arrows) (A). At this stage, cEPAS1 is also present in mesenchymal cells in between. In the migrating sympathetic chain, cEPAS1 expression can be observed at the caudal level, earlier than that of TH, as shown at day 5 (B). Double labeling experiments confirm that cEPAS1 and TH are expressed in the very same cells of the sympathetic chain at E6 (C). In the adrenal gland, both cEPAS1 and TH are present in chromaffin cells at E8 (D) and are also coexpressed in neurons innervating a mesenteric artery at E13 (E). A, B and D: Observation of TH labeling (left column) and cEPAS1 hybridization signal by dark field illumination (right column). C and E: Double labeling cEPAS1-TH observed in bright field. NT: neural tube; N: notochord; DA: dorsal aorta; K: kidney. Bar: 100 μm (A, B, D); 25 μm (C, E).
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the secondary chain migrating to form the medullary plexus of the adrenal gland and a double labeling experiment confirmed that cEPAS1 and TH were indeed expressed in the very same cells (Fig. 3C). However, at the caudal level, a large condensation of cEPAS1-positive cells, ventral to the aorta, appeared TH-negative (Fig. 3B). At E8, a cEPAS1 hybridization signal was detected together with the TH immunoreactivity in the chromaffin tissues within the adrenal gland (Fig. 3D). Finally, at E9 and E13, the expression of cEPAS1 mRNA was observed in all TH-positive ganglia and in sympathetic neurons innervating mesenteric arteries (Fig. 3E).

3.2.3. Miscellaneous. In the heart (Fig. 4A), cEPAS1 was strongly positive in the endocardium, in both the ventricles and the atria. At the tip of the valves, the hybridization signal was particularly intense in endocardial cells and extended beneath, in the mesenchymal cells at the site of putative epithelial-mesenchymal transformation [24,25].

A very strong expression of cEPAS1 was observed in the liver. At E3.5, it was detected in ECs of the sinusoids and it appeared at day 5 in hepatocytes, although less intensely. From E6 to E13, these two cell types presented very high and comparable levels of expression of cEPAS1 transcripts (Fig. 4B).

In the mesonephros as well as in the metanephros, the expression of cEPAS1 was detected, from E3 and E10 onward respectively, within the glomerulus (Fig. 4C). Even though it is difficult to identify the type of the positive cells, it is likely that cEPAS1 transcripts are present in both endothelial and mesangial cells, but not in podocytes where VEGF is strongly expressed (data not shown).

4. Discussion

In this study, we describe the cloning and the expression pattern of the chicken EPAS1 transcription factor. Its deduced amino acid sequence is very close to its mammalian homologues, in particular in the functional motifs bHLH, PAS and activation domains located in the amino-terminus or carboxy-terminus of the variable region [22]. We performed a detailed study of the expression pattern of cEPAS1 mRNA during chicken development and our results reveal, in addition to the expected vascular sites of expression, a close topographical association with the sympathetic nervous system.

cEPAS1 mRNA appears in the extra-embryonic area where it is localized mostly in the ectodermal layer, in cells known to express VEGF [26] which is a putative target gene for EPAS1 [4]. This ectoderm has been shown to inhibit hemangioblast

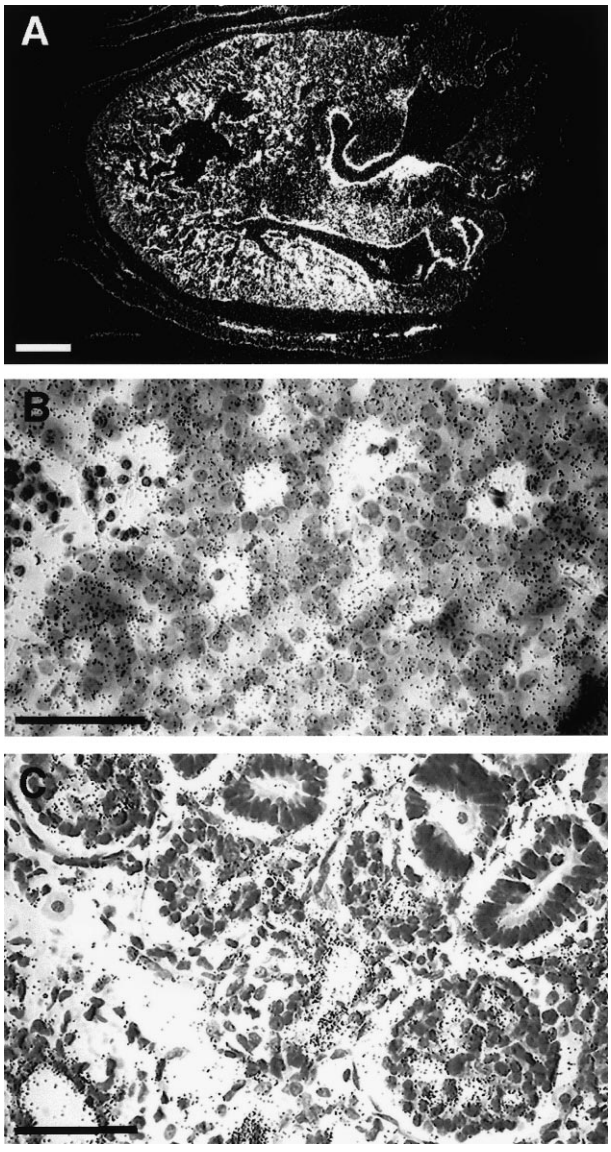

Fig. 4. Expression of EPAS1 in the heart at day 8 (A), the liver at day 6 (B) and the mesonephros at day 5 (C). Visualization of the hybridization signal is by dark (A) or bright field (B, C) illumination. Bar: 200 μm (A); 50 μm (B, C).

induction, and permits the emergence only of angioblasts from the adjacent dorsal (somitic) mesoderm [27]. These observations, together with the loss of cEPAS1 expression in hematopoietic progenitors sprouting from the ventral region of the dorsal aorta at E3, suggest that if cEPAS1 is involved in angiogenesis, it would be related to the angioblastic and not to the hemangioblastic lineage. In the developing embryo, cEPAS1 appears to be expressed in all blood vessels, with the exception of the cardinal veins. As described in the mouse [2,5,12], we could localize this expression, at a very high level, in chick ECs of the vessel wall. However, we show here that in some vessels such as large arteries, cEPAS1 mRNA is also present in pericytes and/or VSMCs, where it may be coexpressed together with VEGF [28]. The presence or absence of cEPAS1 in these cells could not be related to any functional, topographical or morphological characteristics. The vascular expression of cEPAS1 in both ECs and VSMCs supports the results obtained in vitro demonstrating that EPAS1 transactivates genes expressed in both cell types, respectively Tie2 [2] or Flk1 [16] and VEGF [4]. These observations are in keeping with the hypothesis of a major regulatory role for EPAS1 at the interconnection of several pathways of blood vessel formation or remodeling. Although minor defects cannot be excluded, one should then assume that the absence of the vascular phenotype reported in EPAS1−/− mice [17] is the consequence of a compensation by other transcription factors.

Although EPAS1 is most likely involved, at a level which remains to be determined, in angiogenesis, the knock-out results unexpectedly pointed to another essential function of this transcription factor during development. Indeed, it appears that EPAS1 is necessary for catecholamine release and therefore, enzyme(s) involved in catecholamine synthesis may be targets of EPAS1 during (and after) embryogenesis. The colocalization of TH and cEPAS1 mRNA within the same ganglionic cells, as shown here for the first time, provides evidence for a possible direct regulatory role of EPAS1 on catecholamine production. Our results further document the observations made by Tian et al. on the role of EPAS1 in catecholamine production [17], as they reveal that TH is most likely one of the key target genes of EPAS1. Hypoxia is a major inducer of angiogenesis and also strongly and directly affects the sympathetic nervous system. Since both angiogenic factors and TH harbor HREs in their promoter region (for review, see [29]), the colocalization of cEPAS1 and TH in the same cells may represent the missing link that allows the functional connection and interaction between two distinct hypoxia-sensitive systems.

In addition, the remarkable intensity of cEPAS1 expression in ECs and VSMCs raises the question of its function in blood vessel formation, maturation, or stabilization. Other sites of cEPAS1 expression (liver, kidney) also suggest new roles of EPAS1 to be discovered. The chicken embryo model and the cloning of cEPAS1 mRNA offer the opportunity to shed some light on the intricate relationship between angiogenesis, hypoxia and catecholamine production.

5. Note added in proof

During preparation of this manuscript, another avian homologue of EPAS1/HIF2α was cloned in quail [Elvert et al. (1999) Mech Dev. 87, 193–197].

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