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The cap 'n' collar family member NF-E2-related factor 3 (Nrf3) is expressed in mesodermal derivatives of the avian embryo

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ABSTRACT NF-E2-related factor 3 (Nrf3) is a recently identified member of a family of transcription factors homologous to the *Drosophila* «cap 'n' collar» or CNC protein. The *cnc* gene is located immediately 3' to the *Drosophila* homeobox gene cluster and has been shown to regulate at least one of those genes, *deformed*. Likewise, human and mouse CNC homologues are located immediately 3' to each of the four Hox complexes, although no genetic interactions have yet been demonstrated in vertebrates. Work presented here demonstrates that *Nrf3*, adjacent to the Hox A cluster, is expressed during early development of the chicken embryo. Expression begins in the presumptive heart myocardium from the time of cardiac tube fusion through the looping process. *Nrf3* transcripts then disappear from the heart and are next observed in the myotomal compartment of maturing somites, restricted to the medial portion along the rostrocaudal axis and fading after muscle precursors migrate. Central nervous system expression appears gradually and persists at low levels in ventricular neuroepithelial cells until at least embryonic day 6. Strong expression is observed in the early epiphysis, in the collecting ducts of the developing kidney and in individual cells of the yolk sac, underlying blood islands. This is the first description using *in situ* hybridization of the expression of a CNC family member and its dynamics through the course of early development.

KEY WORDS: *cap 'n' collar, maf, Hox, FGFR2b*

Introduction

In bilaterians, segmental identity along the rostrocaudal axis is conferred through the action of Hox transcription factors, of which the DNA-binding domains have been highly conserved over the course of evolution. *Hox* genes are organized in chromosomal clusters, where the order of representation in the 3' to 5' direction is reproduced in nested expression domains along the length of the body. The transcription factor *cap 'n' collar* is located immediately 3' to the unique *Drosophila* homeobox gene cluster (Mohler *et al.*, 1991, 1995). Four CNC homologues are found just 3' to each of the four *Hox* gene clusters of humans and mice: *NF-E2/p45* (Andrews *et al.*, 1993) near the *Hox C* complex, *Nrf1* (Chan *et al.*, 1996) also known as *LCR-F1* (Caterina *et al.*, 1994) near the *Hox B* complex, *Nrf2* (Moi *et al.*, 1994); also known as *ECH* (Itoh *et al.*, 1995) near the *Hox D* complex and *Nrf3* (Kobayashi *et al.*, 1999; Genbank NM_004289 annotated as *NFE2L3*), near the *Hox A* complex.

The CNC factors are basic region-leucine zipper proteins and interact with structurally related factors such as c-fos, small Maf (F, G, K) or Jun proteins, for DNA binding activity. The resultant heterodimers appear to behave as transcriptional activators (reviewed in Veraksa *et al.*, 2000), with the exception of the divergent Bach proteins which show repressor activity (Muto *et al.*, 1998; Oyake *et al.*, 1996). *BACH1* and *BACH2* map to distinct chromosomes (6q15 and 21q22.1 in humans) and contain additional regulatory domains for protein-protein interactions (Blouin *et al.*, 1998; Ohira *et al.*, 1998; Sasaki *et al.*, 2000). In the absence of a large CNC-type subunit, the small Maf protein partners inhibit promiscuous activation of a consensus binding motif on target DNA sequences, which otherwise can be induced by AP-1-like transcription factors.

The originally identified cap 'n' collar protein exists as three isoforms through differential splicing. One of these, CncB, is expressed in the pharyngeal endoderm and has been shown to suppress the transcription of Deformed in the *Drosophila* mandible, thereby maintaining mandibular identity (McGinnis *et al.*, 1998). *cncA* and *cncC* transcripts are ubiquitously expressed. Deformed is a homeobox-containing transcription factor, located at the 3'

Abbreviations used in this paper: CNC, cap 'n' collar protein; Nrf3, NF-E2-related factor 3.

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end of the homeobox complex. In vertebrates, the *Hoxa4, b4, c4* and *d4* orthologues of Deformed are expressed within and caudal to the hindbrain in the CNS, in somites and in the esophageal and posterior gut during embryonic life (reviewed in Couly *et al.*, 1996). Expression of cnc family members in vertebrates has been restricted to Northern blot analysis. *NF-E2* is only seen in hematopoietic cell lines and tissues such as mouse fetal liver and human adult spleen and bone marrow (Peters *et al.*, 1993). *Nrf1* has a reportedly ubiquitous expression (Chan *et al.*, 1993). *Nrf2* transcripts are found in mouse red blood cells, kidney and intestine and at lower levels in brain, liver, skeletal muscle and heart (Itoh *et al.*, 1995). *Nrf3* is expressed at high levels in the placenta and lower levels in adult human heart, brain, lung, kidney and pancreas (Kobayashi *et al.*, 1999).

In the context of recent data from the Institut d'Embryologie in Nogent-sur-Marne, demonstrating the influence of pharyngeal endoderm and *Hox* gene expression on the morphogenesis of cephalic neural crest derivatives (Couly *et al.*, 2002; Creuzet *et al.*, 2002), I examined the developing chicken embryo for the possible presence and localization of *CNC* gene transcripts. One of these, the putative chicken homologue of Nrf3, showed a surprising and dynamic expression pattern in non-cephalic mesodermal derivatives during embryogenesis, notably in the heart, somites, yolk sac and kidney. This pattern was unexpected given the ubiquitous expression described for the CNC genes to date and implies an earlier, more specific role in target gene regulation by Nrf3.

Results

Nrf3 is first transcribed at detectable levels at Hamburger-Hamilton stage (HH) 10 in the wall of the fused cardiac tube (not shown). This expression becomes increasingly intense until stage 18 (Figure 1A) and disappears from the myocardium by HH20 (Figure 1G). Expression appears stronger in the inflow and outflow tracts than in the ventricular wall (Figure 1D).

Somitic expression begins at HH15 in the rostralmost somites and progresses in a caudal direction (Figure 1C). *Nrf3* expression occurs in the median portion of the myotomal compartment along the rostrocaudal axis (Figure 1A, C, E). As the myotome differentiates, the strong expression levels are downregulated but not entirely abrogated; migrating hypaxial muscle precursors appear to maintain some *Nrf3* expression (Figure 1A and 1F, arrows) and muscle masses in the embryonic day 5 limb continue to faintly express *Nrf3* (Figure 1J, arrow). However, expression disappears from the somites by HH20, which precedes terminal muscle differentiation from the myotome; therefore, it is concluded that not all muscle precursors express *Nrf3*.

The pharyngeal endoderm only transcribes *Nrf3* transiently and at low levels during branchial arch formation. Expression is observed within the endodermal outpocketings separating branchial arches 2-3 and 3-4 at HH18 (Figure 1A, open arrows) and is already absent from this area at HH20. Uniformly faint levels of *Nrf3* mRNA are detected throughout the neuroepithelium at both HH18 and HH20, with the exception of the strongly expressing epiphyseal placode at these stages (Figure 1G, 1H, arrows). The Wolffian ducts do not express *Nrf3* at any stage preceding HH18, when faint expression is observed (Figure 1A, arrowhead).

Between HH26-28, *Nrf3* transcription increases dramatically in the mesonephric tubules, resembling collecting ducts (Figure 1I-K). Not all tubules are positive, but mesonephric glomeruli are essentially negative with the exception of scattered cells (Figure1L, arrow). Expression is also present in the rostral end of the regressing mesonephric duct (Figure 1M).

Concordant with studies showing the importance of CNC type genes in erythroid and platelet differentiation (Andrews *et al.*, 1993; Caterina *et al.*, 1994), *Nrf3* is found in a subpopulation of cells within the yolk sac at HH26 (Figure 1N). These cells are located abutting the blood islands forming in the mesoderm on their endodermal face, a location consistent with hematopoietic precursors that have not yet matured into blood islands (Figure 1N; Manaia *et al.*, 2000).

Discussion

Nrf3 is a member of the cap 'n' collar gene family that has been largely conserved during evolution between the insect and the vertebrate subphyla. In this work, the expression pattern of *Nrf3* has been examined at chosen stages of development in the avian embryo. The dynamic and specific activation of Nrf3 in tissues of mesodermal origin shown here is the first demonstration of its potential role in vertebrate embryogenesis.

Conserved Hox partners link Hox and FGF activity

Nrf2 and Nrf3 have recently been demonstrated to be upregulated through the action of FGF7, or keratinocyte growth factor (KGF), in healing skin wounds (Braun *et al.*, 2002). The embryonic expression pattern of *FGF7* significantly overlaps with that of Nrf3 as shown here. Like chicken *Nrf3, FGF7* is first detected in the developing heart of the mouse and at higher levels in the atrial than the ventricular end of the cardiac tube, disappearing after further differentiation (Mason *et al.*, 1994). These authors also showed that *FGF7* is not detected in epithelial somites but, once the myotome differentiates, is expressed in a rostrocaudal temporal gradient within each somitic myotome and disappears from the somites with the dispersion of the myotome. While this early pattern greatly resembles that of *Nrf3* (cf. Figure 1), in contrast, *FGF7* continues to be strongly expressed thereafter within all skeletal muscles at levels not equivalent to the low *Nrf3* expression visible in muscle masses of the chicken embryo. Other differences include strong localized expression of *FGF7* within the telencephalic ventricle, foregut subepithelial mucosa, perichondral mesenchyme and the dermis (Mason *et al.*, 1994), not seen for *Nrf3*. Finch and colleagues (1995) extended the work of Mason *et al.* (1994) to compare the expression of *FGF7* and its receptor KGFR or FGFR2b (Miki *et al.*, 1992). Examination of the mouse urogenital system revealed strong *KGFR* expression in the collecting duct epithelia of the kidney, analogous to *Nrf3* expression. However, *FGF7* itself was expressed in the surrounding mesenchyme of the collecting ducts; neither ligand nor receptor was found in the vicinity of the nephrons (Finch *et al.*, 1995). Given these expression domains, it is possible that Nrf3 is a target of FGF7 activity through FGFR2b, but that its transcription is also regulated by other factors.

Other fibroblast growth factors acting through FGFR2b include FGF3, FGF10 and FGF15. These FGFs have been shown to be modulated by the Pbx transcription factors. Like the vertebrate

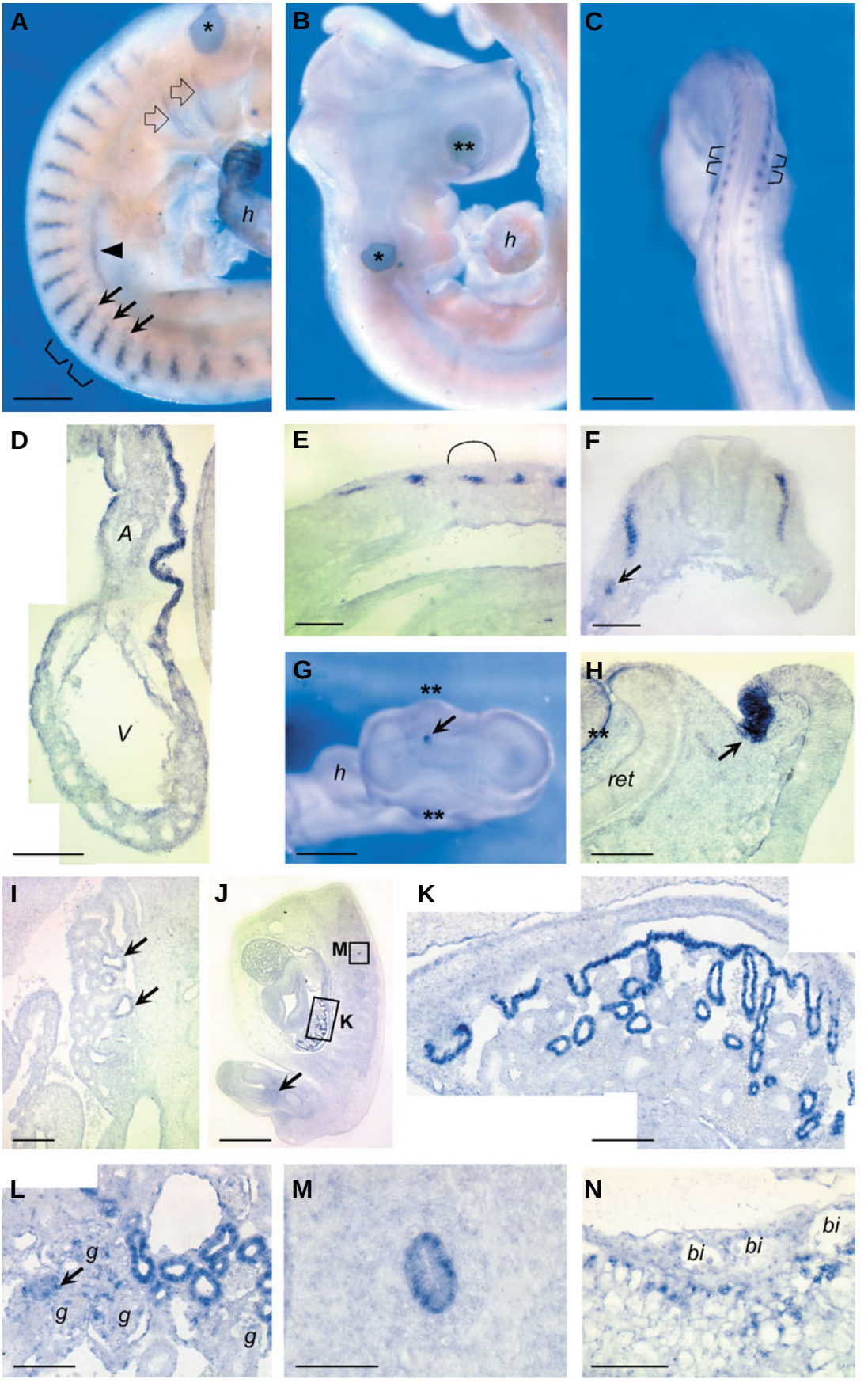

Fig. 1. Expression of *Nrf3* in the embryonic chicken. (A) In situ hybridization with an antisense probe against Nrf3 mRNA in a HH18 embryo. Expression is seen in a medial stripe within each somite and in cells migrating ventrally from these stripes (arrows), in the heart (h) and faintly in the Wolffian duct (arrowhead) and the clefts between branchial arches 2/3 and 3/4 (open arrows). Brackets indicate two examples of somitic boundaries. Asterisk, non-specific signal. **(B)** Hybridization to a sense probe in a HH18 embryo shows non-specific signal in the optic (**) and otic (*) vesicles. **(C)** Somitic Nrf3 expression increases gradually as epithelial somites mature; at HH17, caudal somites do not yet have detectable transcripts. Brackets demonstrate somitic boundaries. Rostral to top. **(D)** Parasagittal section through heart of HH18 embryo showing more intense Nrf3 expression in the atrial (A) than the ventricular (V) portion. **(E)** Parasagittal section through somites showing Nrf3 transcripts restricted to the medial third of myotomes along the rostrocaudal axis (bracketed). **(F)** Myotomal Nrf3 expression at HH18 is evident in this transverse section at thoracic level. Note transcripts in myoblasts migrating in the hypaxial pathway (arrow). **(G)** HH17 epiphysis (arrow) expresses Nrf3 locally, seen from top. Double asterisk, eyes; h, heart. **(H)** Epiphyseal Nrf3 expression is strong at HH18 (arrow). The brain had been opened along the dorsal midline for hybridization, dorsal to right. Ret, retina. **(I)** Nrf3 transcripts are first seen in the developing kidney at HH26 (arrows). **(J)** Oblique parasagittal section of the body at HH28, rostral to top, facing left. Areas enlarged in (K,M) indicated. Note faint expression in muscle masses of proximal hindlimb, arrow. **(K)** Nrf3+ collecting ducts of the HH28 kidney. **(L)** Scattered cells, e.g. arrow, express Nrf3 in some glomeruli (g). **(M)** Rostral end of the receding pronephros also expresses Nrf3. **(N)** Yolk sac Nrf3 transcripts are seen in cells at the interface between the mesodermal blood islands (bi) and the endoderm. Bars: A,B,J, 1 mm; C,G, 2 mm; D,E,F,H,I,K,L,N, 150 μm; M,100 μm.

CNC proteins, Pbx1 is involved in blood formation; human mutations in *PBX1* are associated with childhood leukemias (Kamps *et al.*, 1990; Nourse *et al.*, 1990). In *Drosophila* , the repressive influence of Nrf3's orthologue, cnc, on Deformed is counteracted by the action of the Pbx orthologue, extradenticle (Veraksa *et al.*, 2000). The feedback binding of Deformed to an element of its own promoter is enhanced by the addition of extradenticle to the reaction (Pinsonneault *et al.*, 1997).

Pbx1 binds DNA cooperatively with some Hox proteins including those of the first four paralogue groups, targeting and modulating Hox activity (Zakany and Duboule, 1999; Selleri *et al.*, 2001; Waskiewicz *et al.*, 2002). In the zebrafish, Pbx proteins (somewhat redundant in their functions as Hox co-factors) are necessary for the early transcription of FGF3 and FGF8 in a central rhombomere, r4. The FGF signals then exert patterning effects on the flanking rhombomeres r3 and r5. For example, FGF3 from r4, acting through FGFR2b (Ornitz *et al.*, 1996), activates an indirect cascade which results in the differential transcription of *Hox* genes of paralogue groups 2 and 3 (Waskiewicz *et al.*, 2002).

The Pbx1 protein also directly activates *FGF15* transcription, presumably in concert with a Hox protein under normal circumstances (McWhirter *et al.*, 1997). The *FGF15* expression domain within the central nervous system is complementary to that of other early-expressed FGFs, including FGF3 and it is also transcribed specifically and transiently within the branchial arch endodermal pouches (McWhirter *et al.*, 1997). Recent evidence implicates FGFR2 and excludes FGFR4 as a possible transducer of the FGF15 signal in the developing mouse brain (Ishibashi and McMahon, 2002), despite an in vitro demonstration that its putative human homologue, FGF19, acts exclusively through FGFR4 (Xie *et al.*, 1999). It would be interesting to compare the *FGF15* expression domain with that of members of *Hox* paralogue group 4, or to see if *FGF15* transcription is repressed by Nrf3 in the branchial arches.

To summarize, FGF signaling through the FGFR2b isoform appears to affect *Hox* transcriptional activity in a feedback loop, perhaps through altering the balance between Hox repressors of the CNC family such as Nrf3 and Hox activators of the Pbx family. Given its expression pattern, *Nrf3* does not appear to be a mediator of hindbrain *Hox* gene regulation, but it may be involved in determination of mesodermal segments, notably the somites or the embryonic mesonephric collecting ducts. Potential roles in the specification of the pineal gland and in hematopoiesis also remain to be explored.

Materials and Methods

The chick EST database maintained by the University of Delaware EST project (http://www.chickest.udel.edu/) was screened for the presence of sequences homologous to cnc. One clone, pgp1n.pk002.o10, isolated from a chicken pituitary/hypothalamic/pineal cDNA library, displayed 46% nucleotide homology to human NRF3 cDNA (Kobayashi *et al.*, 1999) using ALIGN v 2.0 (Myers and Miller, 1989) and was chosen for further analysis. The vector was linearized using Sal I; SP6 RNA polymerase was used to transcribe antisense digoxygenin-labelled RNA probes (Not I and T7 for sense) for *in situ* hybridizations. Paraffin sections at 7 μm or whole embryos were hybridized as described (Henrique *et al.*, 1995; Etchevers *et al.*, 2001); the latter were cut at 10 μm on a Leica microtome after embedding in 15% gelatin/30% sucrose/PBS that had been cross-linked with 2% gluteraldehyde. Hamburger-Hamilton stages 7-13, 15, 17, 18, 24, 26 and 28 were examined.

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Note added in press:
Chenais *et al.* (2004) have recently demonstrated that Nrf3 and its small Maf partner, MafG, are a transcriptional activator in human placental chorionic villus cytotrophoblasts.

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