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Mechanisms of Development 49 (1995) 37–48

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A homeobox gene involved in node, notochord and neural plate formation of chick embryos

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Received 5 August 1994; accepted 7 September 1994

Abstract

We have isolated a chicken cDNA clone, *Cnot*, resembling in sequence and expression pattern the *Xenopus* homeobox gene *Xnot*. The major, early transcription domains of *Cnot* are the node, the notochord and prenodal and postnodal neural plate caudal from the prospective hindbrain level. All these cell populations appear to be descendants of the *Cnot*-expressing cells of the node, suggesting a cell lineage relationship. After the onset of somitogenesis, a second, independent expression domain appears in the neural folds at the prospective mid- and forebrain levels, and further transcripts are found in the epiphysis, the ventral diencephalon, the preoral gut and the limb buds. Transplantation of nodes from extended streak embryos leads to the formation of ectopic notochords, which express *Cnot* in the typical, cranially decreasing gradient. Transplantation of young nodes to young hosts has previously been described to induce secondary embryos. We observed that secondary chick embryos express *Cnot* in node derived, notochord-like structures and in the anterior neural plate, similar to the domains seen in primary embryos. However, expression was absent from the posterior neural plate, which in the induction experiments is excluded from the node lineage. This finding corroborates our initial conclusion about a cell lineage relationship between node, notochord, and neural plate defined by *Cnot* expression. The midline mesoderm of vertebrate embryos consists of two tissues, the prechordal mesoderm and the notochord. The anterior notochord, the head process, may represent an intermediate form. The transition from prechordal to chordal mesoderm can be followed by the expression of the two marker homeobox genes *goosecoid* and *Cnot*, first in the primitive streak, and then in the head process. We suggest that expression of *goosecoid* or *Cnot* is involved in the specification of a prechordal or notochordal identity, respectively. A transition from *goosecoid* to *Cnot* expression may proceed, while cells are still in the epiblast, but not after becoming mesodermal. A molecular coding of axial positions in the midline mesoderm may occur by specific homeobox genes, similar to the situation in the neural tube and the somitic mesoderm.

Keywords: Homeobox; Gastrulation; Node; Primitive streak; Notochord

1. Introduction

The basic patterning of a vertebrate embryo occurs during gastrulation. Omni- or pluripotency of cells observed in pregastrulation embryos is from now on gradually or stepwise lost with the generation of definitive endoderm and mesoderm. A rostrocaudal body axis with a pronounced dorsoventral pattern becomes defined. While cell ingression occurs through a pore in amphibia, amniotes (reptiles, birds, and mammalia) form a longitudinally extended 'streak' (Bellairs, 1986; Schoenwolf, 1991). The extension of the primitive streak in chick embryos is preceded by an initial spreading of extraembryonal endoderm, the secondary hypoblast, from the posterior to the anterior pole of the embryo (Vakaet, 1970; Harrisson et al., 1988). Signals from the hypoblast must pattern the formation of the streak, as shown by the classical hypoblast rotation experiments performed in chick (Waddington, 1933; Azar and Eyal-Giladi, 1981). Although not formally proven, it seems likely that the growth factor activin is involved in the signalling from endodermal hypoblast (Mitrani and Shimoni, 1990; Mitrani et al., 1990). While streak formation indicates already the location of the prospective body axis, the initially ingressing cells do not contribute to the material of the body axis, as shown by extensive fate mapp-

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0925-4773/95/$09.50
SSDI 0925-4773(94)00300-C

ing studies in the chick (Spratt, 1952; Selleck and Stern, 1991). Three preaxial phases can be distinguished, before axial mesoderm indicates the beginning of axis formation: (a) Initial streak; definitive endoderm ingresses through the tip of the streak, (b) Intermediate streak; while endoderm continues to ingress anteriorly, extraembryonal mesoderm migrates through the posterior streak and (c) Definitive streak; endoderm still ingresses through the tip, paraxial head mesoderm through the anterior streak and heart mesoderm around the middle of the streak. Extraembryonic mesoderm continues to leave the posterior portion.

Formation of the actual body axis begins after the streak has reached its definitive length. Generation of the endoderm layer is then finished and medial (or axial) mesoderm starts to leave the tip of the streak, the node. Three different forms of medial or axial mesoderm can be discerned along the rostrocaudal extension of the body axis. Initially, the midline mesoderm stays mesenchymal and forms the prechordal mesoderm at the rostral end of the embryo (Adelman, 1922; Seifert et al., 1993). It is followed by the anterior notochord, the head process, which can be structurally distinguished from the notochord proper. The head process then turns into the notochord, a rod-like structure eventually surrounded by a fibrous sheath. Approximately, the prechordal mesoderm underlies the midline of the prospective forebrain (prosencephalon), the head process the prospective midbrain (mesencephalon), and the notochord the hindbrain (rhombencephalon) and spinal cord. Cells ingressing through streak levels posterior to the node, but still in the anterior half of the streak, will obtain positions located increasingly further lateral from the midline, e.g. the medial part of a somite, the lateral part of a somite, intermediate mesoderm and lateral mesoderm (Selleck and Stern, 1991; Schoenwolf et al., 1992).

The node of higher vertebrates is functionally equivalent to the dorsal blastopore lip of amphibia, both groups of cells with 'organizer' activity (Dias and Schoenwolf, 1990; Izpisúa-Belmonte et al., 1993 and references therein). Transplantation of an organizer to competent regions of a host embryo leads to the formation of a secondary embryo, which is partly derived from grafted cells by self differentiation, and partly from host cells by inductive processes (Spemann and Mangold, 1924).

Homeoboxes are DNA sequences encoding highly conserved parts of proteins, the homeodomains, and were found in the genomes of all metazoa (Kappen et al., 1993). Homeobox genes seem to play a role in the specification of cells during embryogenesis, as indicated by expression analyses (Kessel and Gruss, 1990), ectopic expression experiments (e.g. Kessel et al., 1990), targeted inactivations (e.g. Chisaka and Capecchi, 1991; Lufkin et al., 1991), and in vivo modulations by the developmental signalling molecule retinoic acid (e.g. Kessel and Gruss, 1991; Kessel, 1992; Kessel, 1993). Overlapping, nonidentical expression domains of a subfamily of homeobox genes, the *Hox* genes, characterise the trunk region caudal from the otic region (Kessel and Gruss, 1990). Similarly, nested homeobox gene expression patterns are found in fore- and midbrain regions, where *Otx* and the *Emx* genes are transcribed (Simeone et al., 1992). The Xenopus homeobox gene *goosecoid* is expressed in the blastopore lip apparently as a prime target of the growth factor activin, is causally related to anterior cell migration and discussed as a key gene of the head organizer (Cho et al., 1991; Niehrs et al., 1993). The chicken *goosecoid* gene is transcribed before streak formation in a few cells of the posterior marginal zone, and then prominently in the anterior portion of the elongating primitive streak, including the node (Izpisúa-Belmonte et al., 1993). After the streak reaches its full extension it is expressed in the cells of the prechordal mesoderm. In the head process, expression quickly fades to nondetectable levels.

Recently a second, midline expressed homeobox sequence was isolated from *Xenopus* laevis gastrula cDNA (von Dassow et al., 1993). Transcripts of the *Xnot* gene,

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Cnot     MKRVRTVFKPEQLERLEQEFLKQQYMVGTERVDLAATLRLTETQVKVWFQNRRIKWRKQSM

Xnot     ...I....T.....K..K.................S..N.....................L     88.5 %
Xnot2    L..I....T.....K..K.................S..N.....................L     88.5 %

Emx2     P..I..A.S.S..L...HA.E.NH.V..A..KQ..HS.S.............T.FKR.KL     60.7 %
Emx1     P..I..A.S.S..L...RA.E.NH.V..A..KQ..GS.S.S...........T.YKR.KL     59.0 %
e5       P.....A.S.T..LK..HA.EGNH.V..A..KA..QG.S.............T.HKRMQ      57.4 %
ems      P..I..A.S.S..LK..HA.ESN..V..A..KA..QN.N.S...........T.HKRMQQ     55.7 %
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Fig. 1. Comparison of the deduced chicken *Cnot* homeodomain with the homeodomains encoded by the *Xenopus Xnot* (von Dassow et al., 1993) and *Xnot2* (Gont et al., 1993) genes; by the murine *Emx1* and *Emx2* genes (Simeone et al., 1992); and by the *e5* and *ems* genes of *Drosophila* (Dalton et al., 1989). Dots indicate amino acid identity with *Cnot*.

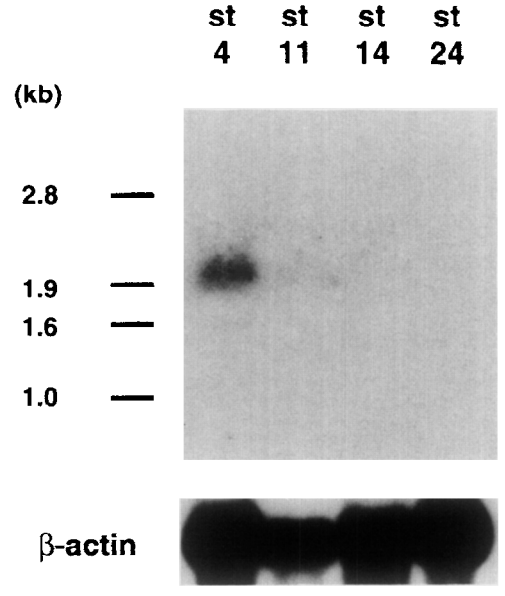


Fig. 2. Northern blot analysis of *Cnot* expression. Total RNA of the indicated stages was analyzed as described in the methods section. Note the high expression of a 2 kilobase *Cnot* RNA in HH st.4 embryos.

or the almost identical *Xnot-2* gene (Gont et al., 1993), were found adjacent to the dorsal lip, the notochord and, in later stages, the epiphysis and the tailbud. Here, we report the cloning and analysis of a chicken homolog of *Xnot*. We studied in detail its expression during chick embryogenesis, in particular during the development of endogenous and transplanted notochord cells. Our data indicate that expression of this chicken gene defines a cell population in the node, notochord and neural plate, suggesting a cell lineage relationship.

2. Results

2.1. Isolation of chicken Cnot cDNA

Two degenerate primers were designed based on the conserved first and third helix of the *Xnot* homeobox (von Dassow et al., 1993). They were used to amplify by PCR a 120 bp fragment from cDNA produced from HH st.4 chicken embryos. The cloned PCR fragment was used to screen a cDNA library under low stringency conditions. A 880 bp cDNA clone showed 88.5% identity to the *Xnot* genes of *Xenopus* at the deduced amino acid level in the homeodomain (Fig. 1), and was therefore designated *Cnot* in analogy. Genomic blots revealed only a single band under high stringency conditions probing with a 600 bp cDNA fragment (not shown). Besides the *Xnot* homeodomains, sequence similarities were found to the *empty spiracles* homeobox gene subfamily, where two members both from *Drosophila* (*e5* and *ems*; Dalton et al., 1989) and mouse (*Emx1* and *Emx2*; Simeone et al., 1992) are known. However, homology between *Cnot* and the *empty spiracles* homeodomains lies only between 55 and 60% (Fig. 1).

2.2. Expression of Cnot during chick development

A 2.0 kb transcript was detected in HH st.4 embryos by Northern blot analysis. In later stages the relative expression level of *Cnot* mRNA is greatly decreased, and is no longer detectable in HH st.24 embryos by northern blot analysis (Fig. 2). Spatio-temporal expression of the *Cnot* gene was analyzed in chick embryos during gastrulation and neurulation by means of digoxygenin-labelled riboprobes. Several expression domains are characteristic for the *Cnot* gene and can be followed from precursor structures through development. Below, the dynamics of expression are described chronologically, following the stages of chick development as defined by Hamburger and Hamilton (Hamburger and Hamilton, 1951). The major features of expression are schematically represented in Fig. 8.

Intermediate and definitive streak (HH st.3$^+$ and HH st.4). The first transcripts are detected in intermediate streak embryos (HH st.3$^+$), where weak staining occurs in the tip of the still elongating primitive streak (data not shown). Strong expression is seen in definitive streak embryos (HH st.4), which possess a clearly delineated node (Fig. 3A). *Cnot* RNA is detected in the node, with more intensive staining in its dorsal portion (Figs. 4A, 4C). Significant *Cnot* staining is present in the epiblast directly anterior of the node, whereas the underlying mesoderm or endoderm are negative (Fig. 4B). At the posterior boundary the signal was detected also in the most anterior streak and its flanking epiblast.

Head process (HH st.5) to head fold (HH st.6). The formation of the head process/notochord as an elongated, finally rod-like structure could be followed in the *Cnot* staining pattern, typically with stronger staining in younger as compared to older notochord regions (Figs. 3B–F; Fig. 4D–G). Histological analysis revealed, however, that *Cnot*-expressing cells were always preceded by non-expressing, still more anterior midline mesoderm, belonging to the prechordal mesoderm (not shown). The strong staining of notochord continued into the node, which becomes less and less precisely delineated with further development. A distinct prenodal, as well as a paired postnodal region of the epiblast express *Cnot* in continuation with the node signal (Figs. 3B,C; Figs. 4D,F,G). These *Cnot* transcribing cells indicate the extent of the neural plate in these stages. DiI labelling determined the fate of the postnodal cells in HH st.6 embryos as spinal cord neuroectoderm (Fig. 4D, 4H) and tailbud epiblast (not shown), as predicted also from fate maps established for HH st.5 embryos by Spratt (Spratt, 1952). Noteworthy, the neural plate overlying the head process in HH st.5 and 6, fated to become mesencephalon (Spratt, 1952), does not express *Cnot* during HH st.5/6 (Fig. 4E).

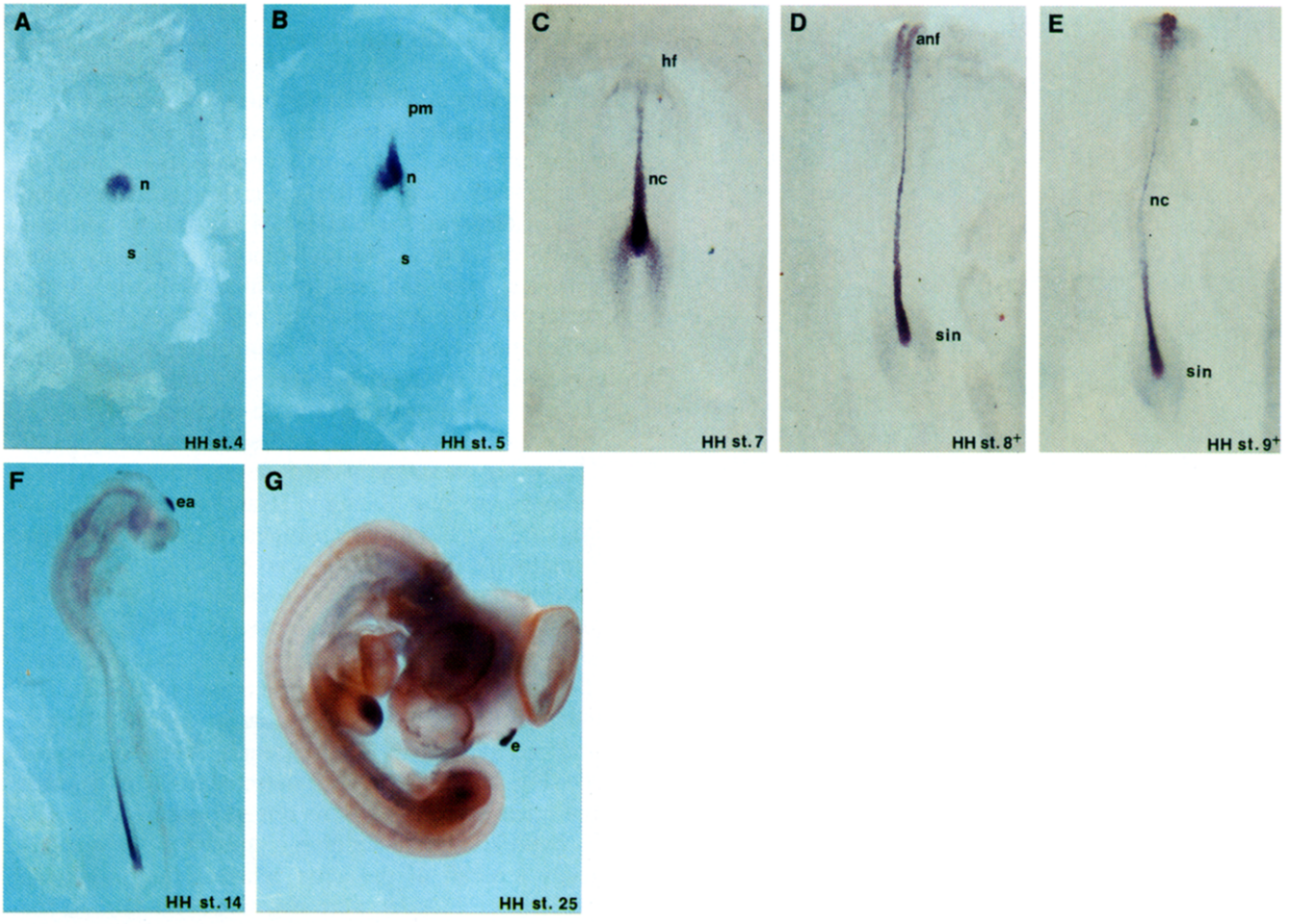
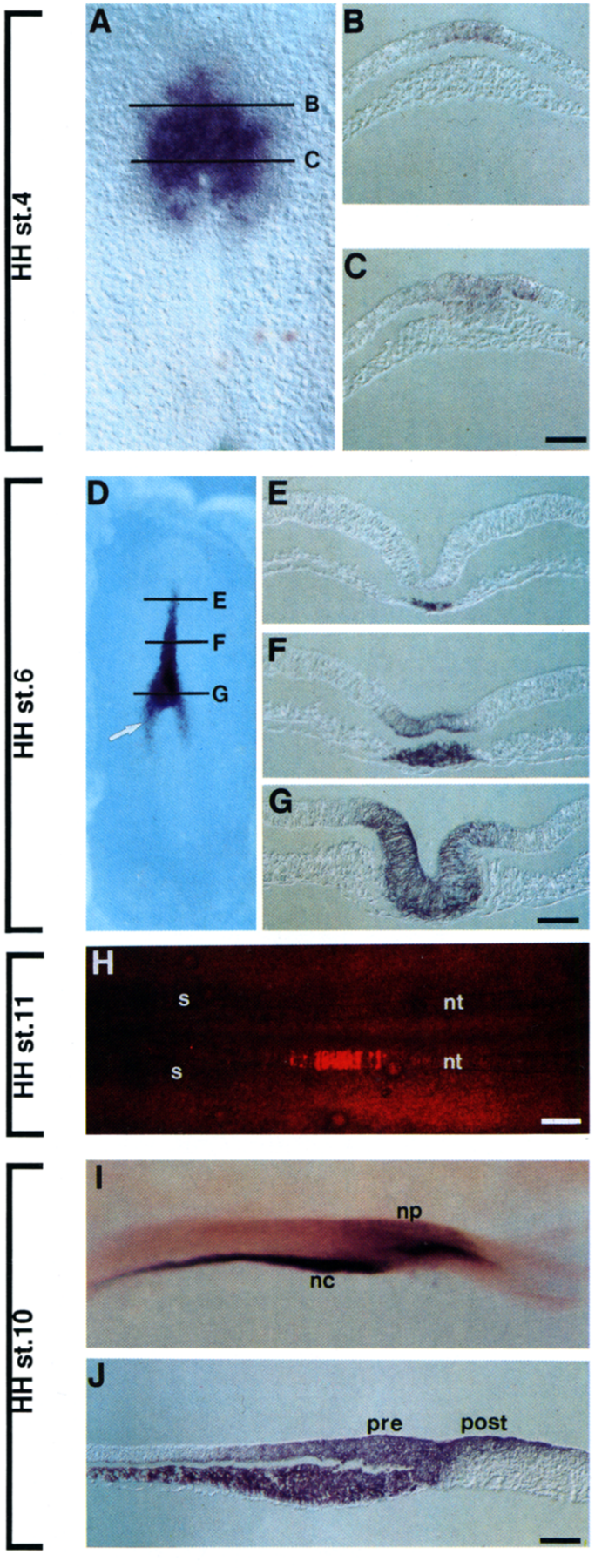


Fig. 3. Expression of the *Cnot* gene in chick embryos. Embryos hybridized with a *Cnot* probe as whole mount preparations are displayed, photographed under a dissecting microscope using a semidarkfield (A,B,F,G) or brightfield (C,D,E) illumination. A–E represent the same magnification (original 32×), F (25×) and G (12×). Indicated are the node (n), the primitive streak (s), the prechordal mesoderm (pm), the headfold (hf), the notochord (nc), the anterior neural plate (an), the sinus rhomboidalis (sin) consisting of pre- and postnodal neural plate, the epiphysis anlage (ea), and the epiphysis (e). Stages according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) are given in the right corner. The expression patterns are explained in the results section.

From first somites to closing of the anterior neuropore (HH st.7–10). Expression in the notochord continues in these stages with a cranially decreasing gradient (Figs. 3D–F). Behind the last formed somite, the neural folds diverge and merge with the ectoderm lateral to the streak. *Cnot* expression is seen over this large, ovoid area, the 'sinus rhomboidalis' (Hamilton, 1965), marking the extent of the neural plate (Fig. 3E). In a dorsal view, the relatively sharp beginning of the strongly stained notochord marks the middle of the rhomboid area (Fig. 3E). From a lateral view (Fig. 4I) or in sagittal sections (Fig. 4J), the notochord begins at the ventral point of the shallow, cup shaped neural plate. The generation of this pattern can be followed originating from the described node signal in HH st.4 embryos, and continuing in the epiblast signal of HH st.5/6 embryos. As in the early stages, the midline of the primitive streak, piercing into the sinus rhomboidalis area from posterior, does not express *Cnot*.

From HH st.8 onwards, a new *Cnot* expression domain is detected in the still open, anterior neural plate (Figs. 3D, 5A). It extends initially along the folds at the level of the prospective anterior surface ectoderm, prosencephalon and mesencephalon (Fig. 5A; Couly and Le Douarain, 1987). This anterior, ectodermal expression is maintained when brain formation proceeds and the signal then becomes limited to the neuro- and surface ectoderm adjacent to the anterior neuropore (HH st.9/10; Figs. 5B, 5C). A weak signal becomes evident at HH st.10 also in the ventral thalamus (Fig. 5B).

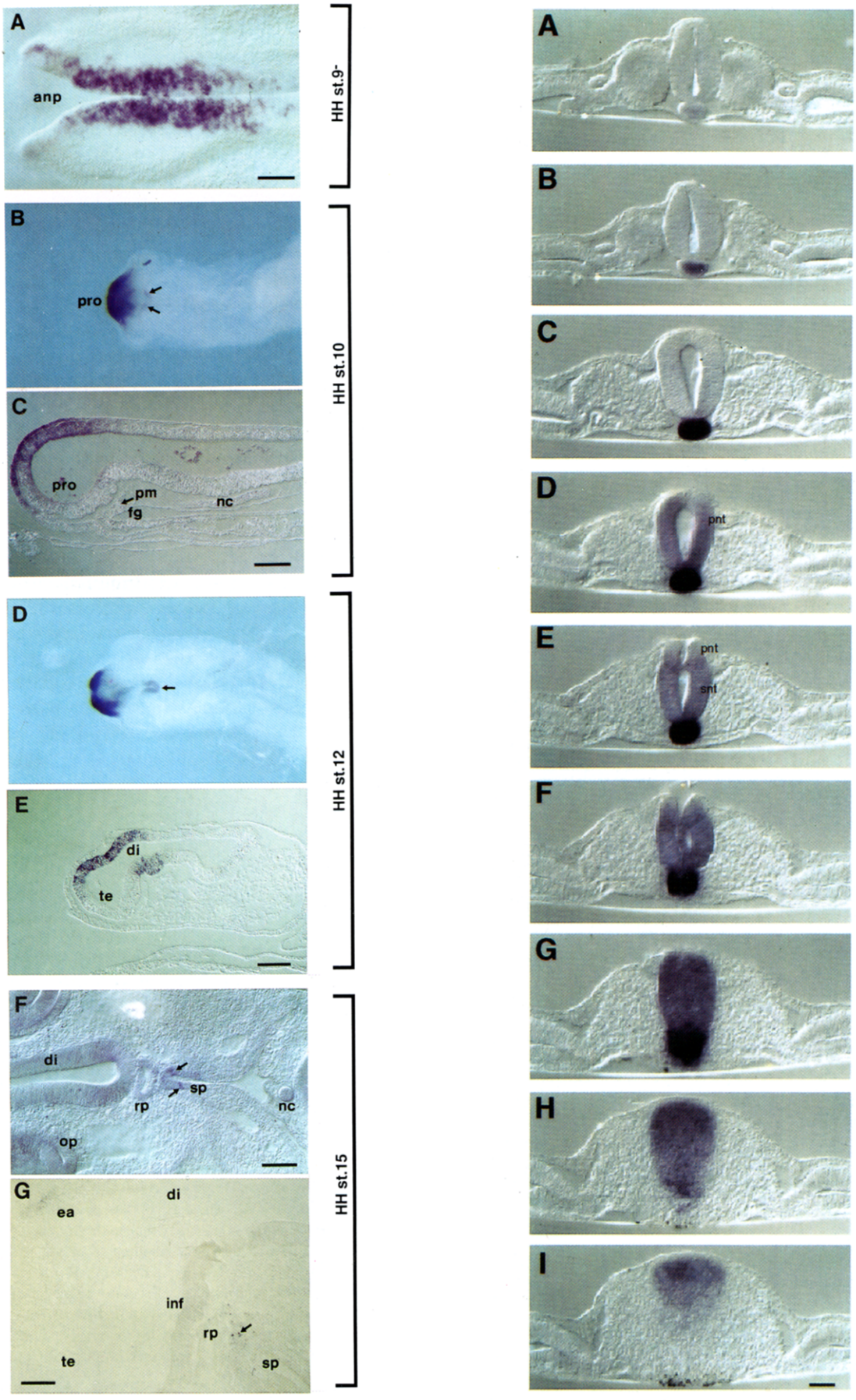
From posterior neuropore to the tailbud (HH st.11–15). From HH st.11 onwards, an extracellular, fibrillar



perichordal sheath forms progressively around the notochord in a craniocaudal direction. Concomitantly, *Cnot* expression in the anterior notochord begins to disappear and staining is only observed parallel and caudal to the last formed somites. The intensity of staining near the notochord origin is however not decreased. By HH st.11, the sinus rhomboidalis becomes converted to an elongate cleft, and subsequently the streak regresses until it fuses with the node to form a mesenchymal structure, the tailbud, by HH st.15. From now on neurulation proceeds no longer by folding and fusion of the neuroectoderm, but by cavitation of the tailbud mesenchyme (Schoenwolf et al., 1985). Fig. 6 shows cross sections of the posterior region from a tailbud stage (HH st.15) embryo. *Cnot* is expressed before generation of the secondary, neuroectodermal epithelium (Figs. 6H, 6I). Then expression is seen in the secondary neural tube (Fig. 6G). Since in the chick primary and secondary neurulation overlap for a short period (Schoenwolf et al., 1985), cross sections show a still folding primary neural tube underlain by a secondary tube becoming cavitated by several, still independent lumina (Figs. 6E, 6F). The neuroectodermal expression fades down quickly (Fig. 6D) and strong expression remains only in the notochord (Fig. 6C), before it decreases (Fig. 6B), and disappears at the level of the posterior somites (Fig. 6A).

After closing of the anterior neuropore (HH st.11) two large *Cnot* expression domains, originating from the closing folds, were found symmetrically to the midline in the dorsal diencephalon and overlying surface ectoderm (Fig. 5D). They condense at HH st.14 to a single, ex-

Fig. 4. *Cnot* expression in the node/notochord/neural plate lineage. (A) HH st.4 embryo viewed under Nomarski optics. The level of cross sections shown in B and C are indicated. (B) Note *Cnot* expression in ectoderm, but not in meso- or endoderm directly anterior of the node. (C) Note expression in the dorsal part of the node. (D) HH st.6 embryo, with indication of cross sections shown in E, F, and G. The white arrow indicates the site of DiI injection performed in the experiment documented in panel (H). (E) Note expression in anterior mesoderm (head process), but not in the overlying ectoderm (prospective midbrain level). More anterior sections (not shown) revealed unstained, prechordal mesoderm. (F) Note staining in notochord, as well as corresponding ectoderm (prospective hindbrain). (G) Expression in the node region, with strong signal in epiblast, but not in paraxial mesoderm. (H) Cell fate determination of the *Cnot* expression domain lateral to the streak. A HH st.6 embryo was injected with DiI at the position indicated in D (white arrow), as described under methods. After incubation to HH st.11 the embryo was viewed under epifluorescence. Note unilateral labelling of neural tube adjacent to the segmental plate. Somites (s) and neural tube (nt) are indicated. (I) Dorso-lateral view and (J) sagittal section of posterior neural plate (np) and notochord (nc) of a HH st.10 embryo. Note staining of the notochord with cranially decreasing intensity, and of pre- and postnodal neural plate (pre-, post-). Bars indicate 50 μm, except for panel H (100 μm).


A
anp
HH st.9-
B
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tremely strong domain marking the area of the prospective epiphysis (Fig. 3F). A relatively weak, triangular expression domain of yet unidentified cellular origin was observed in the dorsal roof of the caudal mesencephalon at HH st.14 (Fig. 3F). In HH st.12–13 embryos, strong *Cnot* expression is further observed in the hypothalamus area including the infundibulum (Figs. 5D, 5E), but has faded by HH st.14–15 (Fig. 5F). A few positive cells were detected in HH st.15–17 embryos in and around the roof of Seessel's pocket, a preoral evagination of the foregut close to Rathke's pouch (Figs. 5F, 5G). Rathke's pouch itself remains negative throughout development.

Brain and limb expression in older embryos (from HH st.16 on). *Cnot* expression in the notochord is confined to the tailbud region from HH st.16 onwards, and becomes less and less intensive, until it disappears around HH st.22, when somitogenesis also comes to an end, and formation of the body axis by budding ceases.

The epiphysis forms at HH st.17 as a distinct evagination in the mid dorsal wall of the diencephalon. Here, the originally more diffuse bilateral signal of the open neural folds, (Hemiepiphysis; Couly and Le Douarin, 1987) becomes condensed into one extremely high expressing structure protruding from the dorsal diencephalon (Fig. 3G). No signal was observed in the floor plate at any axial level, although the uniform expression in the posterior neural plate includes the floor plate precursors. No significant staining occurred in the primitive gut.

With the onset of limb bud formation at HH st.17, a prominent *Cnot* expression domain develops, restricted to the distal, anterior region (Fig. 3G). The detailed pattern and dynamics will be described elsewhere.

2.3. Transplantations of node or notochord

The fate of node cells has been extensively mapped during chick development (Selleck and Stern, 1992). Presumptive notochord cells lie in the rostral medial sector of the HH st.4 node and stem cell like characteristics were described in labelling and transplantation experiments. In order to study the dynamics of *Cnot* expression in an ectopic environment the following type of transplantations was performed.

A triangular, rostral node sector was grafted to a variety of sites in somite stage embryos, including segmented and unsegmented paraxial mesoderm in dorsal or ventral locations of the presumptive head ($n = 26$) or trunk ($n = 30$) region. Regardless of the grafting site, the generation of ectopic notochords was observed and indicated by strong *Cnot* staining in whole mount in situ analysis (Figs. 7A–E). Structural and histological analyses revealed that older ectopic notochords, which had differentiated already into a more rod-like structure, quickly stopped expressing *Cnot* in the ectopic sites (Fig. 7D). Occasionally ectopic somites were also formed in our experiments (Fig. 7C). Most probably they indicate self-differentiation of prospective somite cells from the grafted node sector (Hornbruch et al., 1979).

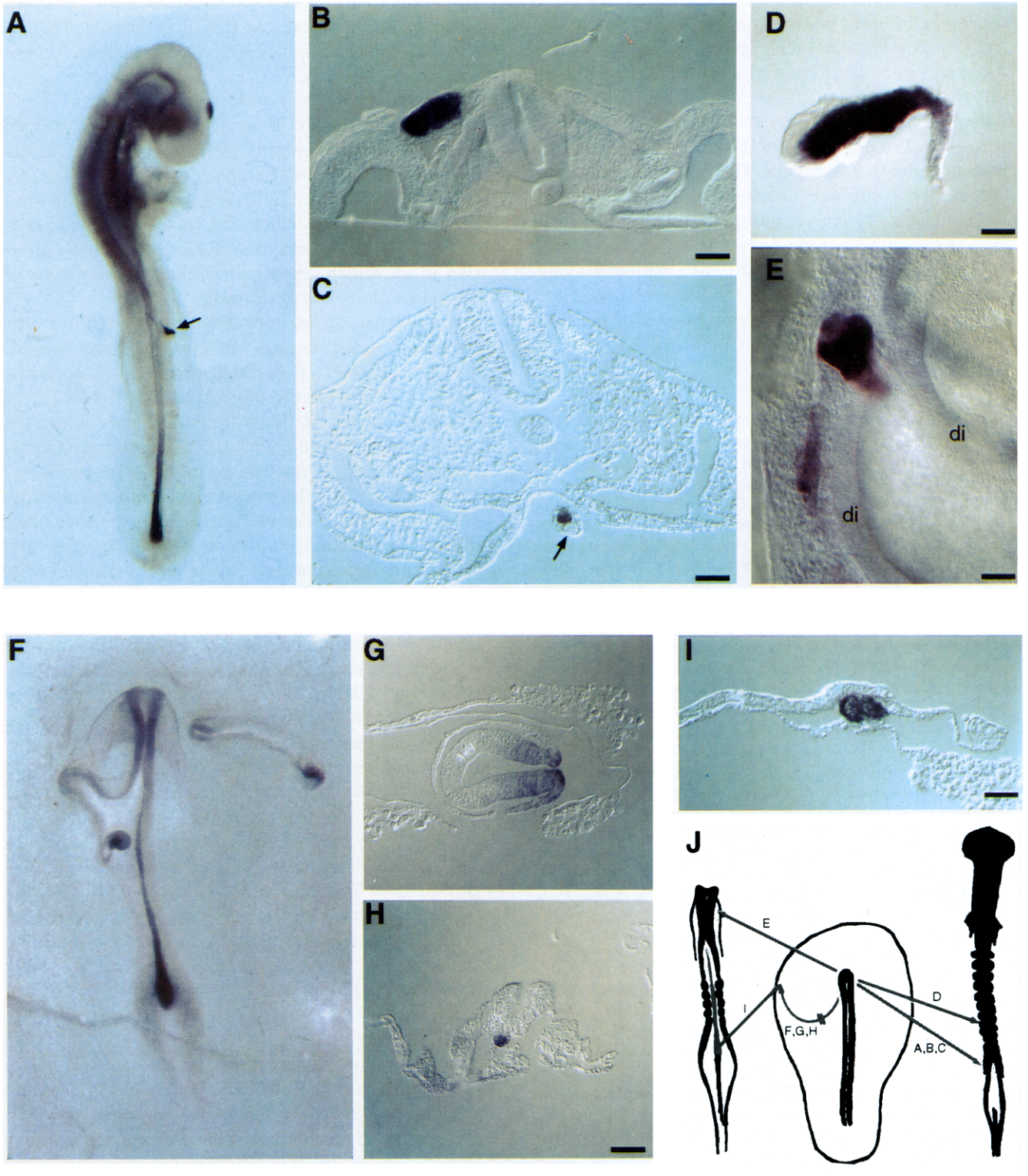
Grafting of HH st.3$^+$ or 4 nodes to lateral epiblast of similar stage embryos is known to lead to the formation of secondary embryos by induction and self-differentiation processes (Dias and Schoenwolf, 1990; Storey et al., 1992). To analyze the requirements for induction of *Cnot*, we followed its expression in such secondary embryos. We chose the boundary between the area opaca and area pellucida of HH st.3$^+$ embryos in culture as grafting sites for nodes of the same stage ($n = 26$). We regularly observed elongated secondary embryos with prominent neuroectodermal structures after around 18 h of incubation. After whole mount staining with *Cnot* probes and paraffin sectioning we clearly detected *Cnot* expression in the secondary embryos (Figs. 7F–H). Most strongly stained were node- or notochord-like structures, while elongated, rod-like notochords rarely formed in accordance with the observations of Dias and Schoenwolf (1990). In addition, a *Cnot* signal appeared on the elevated neural folds of the prospective secondary forebrain (Figs. 7F, 7G), equivalent to the rostral *Cnot*

Fig. 5. *Cnot* expression in structures of the anterior ectoderm. (A) Expression in the open and appositioned folds of the anterior neural plate in a HH st.9$^-$ embryo around the wide open anterior neuropore (anp). The axial level of the expression domain corresponds to the pros- and mesencephalon (Couly and Le Douarin, 1987). (B) Ventral view and (C) sagittal section of stained HH st.10 embryos. Note the focusing of the *Cnot* signal adjacent to the closing anterior neuropore in the neuroectoderm of the prosencephalon (pro), and also in the overlying surface ectoderm. Arrows in (B) indicate the first sign of a bilateral expression in the ventral diencephalon. Further indicated in (C) are the prechordal mesoderm (pm), the foregut (fg), and the notochord (nc). (D) and (E) show comparable views to (B, C) for older embryos (HH st.12). Expression has disappeared from the anterior surface ectoderm and is still widespread in neuroectoderm of diencephalon (di) and telencephalon (te). The hypothalamus is now also positive in addition to the ventral thalamus. (F) shows a coronar section through the head of a HH st.15 embryo. The diencephalon (di), Rathke's pouch (rp), Seessel's pocket (sp) and the notochord (nc) are indicated. Note a few positive cells in Seessel's pocket (preoral gut). (G) shows the same region as (F) in a sagittal section. The epiphysis anlage (ea) and the infundibulum (inf) are indicated. *Cnot* positive cells are found in the prospective epiphysis and in the mesenchyme around Seessel's pocket. Note that the *Cnot* expression domains are located in one plane, here visible as a straight line from the epiphysis, the hypothalamus and Seessel's pocket. Bars indicate 50 μm.

Fig. 6. *Cnot* expression in the tailbud and caudal body region. A HH st.15 embryo was cross sectioned with a vibratome starting from its caudal end, the 30 μm sections were numbered. Displayed are section #3 (I), #4 (H), #5 (G), #6 (F), #7 (E), #8 (D), #10 (C), #21 (B), #24 (A). Note the expression in the tailbud before structural manifestation, i.e. epithelialisation, of the neural tube (G–I), then expression in the primary, still open neural tube (pnt) simultaneously with expression in the secondary neural tube (snt; E,F). Note three lumina of the secondary spinal cord, which are generated by cavitation and not by folding, in (F), and decrease of the notochord signal beginning from its outer zone in (B). Bar indicates 50 μm.

signal observed on the forebrain/midbrain neural plate from HH st.8 onwards. We did not, however, detect neuroectodermal *Cnot* expression at the posterior end of the secondary embryos, which could have been expected in analogy to the posterior neural plate/tailbud expression seen in primary embryos. In one case only weak staining was observed in neuroectoderm at the posterior end. Here, the irregular structure suggested that it was derived by self differentiation from grafted epiblast. Also in short incubations (6–10h), where the neurulation inducing effect was not or weakly obvious, no transmission or induction of *Cnot* expression in the overlying epiblast was detected (n = 6; data not shown).

In order to study effects of *Cnot*-expressing trans-



plants in the absence of organizer activity, notochord fragments from HH st.8 embryos were tested in the HH st.3$^+$ embryo culture system ($n = 6$). Notochord pieces isolated from the vicinity of the node, maintained strong *Cnot* expression after 4 h of incubation (Fig. 7I). However the adjacent epiblast cells were not induced to transcribe the *Cnot* gene. After 16 h the *Cnot* signal in the graft had decreased, while relatively little growth and no inductive processes were observable. Notochord fragments taken from further rostral levels did not maintain structural integrity well and expression was not detected after longer (18 h) incubation.

3. Discussion

3.1. The Not gene family

The chicken *Cnot* clone described here was isolated based on structural homology to the *Xenopus* gene *Xnot*. The similarity between the frog and the chicken homeobox is 88.5%, a score not necessarily proving homology. A comparison of the expression patterns, however, suggests that we isolated a close relative of the *Xnot/Xnot-2* genes (Gont et al., 1993; von Dassow et al., 1993), since key features are identical in chick and *Xenopus*. Thus, the node expression of chicken *Cnot* is equivalent to the blastopore expression of *Xnot*, both tissues representing the organizer in the respective gastrulae. Both species show strong expression in the notochord, and, in later embryonic stages, expression in a restricted rostral domain (mainly the epiphysis) and a caudal domain, the tailbud. The *Not* genes seem to represent a unique family of homeobox genes, since their closest relatives, the *empty spiracles* genes of *Drosophila* (*ems*) or vertebrates (*Emx*), are still only distantly similar (Fig. 1). It remains to be investigated, if more than one gene exists per species, especially in light of the relatively weak homology between the chicken and *Xenopus* homeodomains known by now.

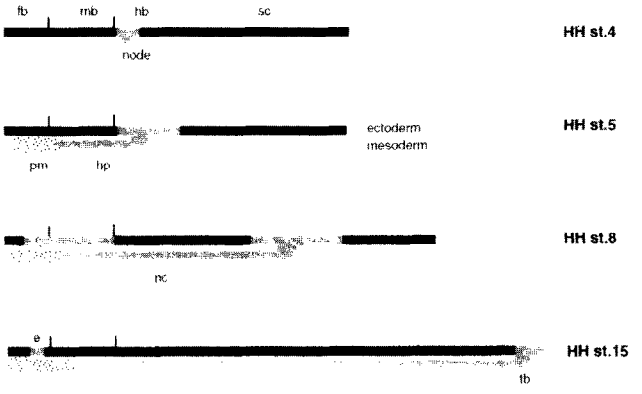


Fig. 8. The relation between axial levels of ectodermal and mesodermal *Cnot* expression domains. For detailed description see results. The schematic representation emphasizes the development of the notochord and neural plate domains (hindbrain/spinal cord levels) from the node. It further demonstrates the apparently independent, later expression domain in anterior ectoderm (fore- and midbrain levels), which persists in the epiphysis. Note that finally all axial levels of neuroectoderm have expressed *Cnot* at one time in development. Indicated are prospective forebrain (fb), midbrain (mb), hindbrain (hb), spinal cord (sc), prechordal mesoderm (pm), the head process (hp), notochord (nc) and tailbud (tb).

3.2. Cnot expression and a node/notochord/neural plate lineage

Genetic probes have been helpful to follow cell lineages, even if gene expression must not be taken as proof for a lineage relationship. For the chicken *Cnot* gene, development could be followed from the node until the end of notochord formation in the tailbud, and also from the node to the neural plate from the hindbrain all the way to the tailbud (for schematic representation see Fig. 8). It appears that the population of *Cnot*-expressing cells in the node becomes separated on the basis of either ingression or non-ingression. If the cells do in-

Fig. 7. Transplantations of notochord and node grafts. A–E: Transplantations of the anterior, triangular node sector (HH st.4) into paraxial mesoderm. (A) The graft was inserted into the segmental plate of a HH st.11 embryo. After further incubation to HH st.15, a whole mount in situ analysis of *Cnot* expression was performed. Note the growth of an ectopic, *Cnot* positive notochord (arrow). A vibratome cross section at the level of the ectopic notochord is displayed in panel (B). (C) From a similar operation as described under (A), a ventrally located ectopic notochord was detected. Note the somite like structure (arrow), which most probably is derived by self differentiation from the graft. (D) Dissected ectopic notochord from a HH st.15 embryo, operated at HH st.10$^+$, by insertion of a graft at the level of the seventh somite. Note the decrease of signal along the length of the notochord. (E) A graft was inserted into the cephalic, paraxial mesoderm at the diencephalon (d) level of a HH st.8$^+$ host. Analysis was performed at HH st.13. F–H: Transplantations of nodes (HH st.3$^+$) into cultured HH st.3$^+$ embryos: (F) Primary embryo (HH st.8) with two secondary embryos resulting from two transplantations. Note on the left side fusion of primary and secondary neural folds, both expressing *Cnot*, and stronger expression in the mostly globular graft derivative. On the right side an independent, secondary embryo has developed, with its anterior neural folds near the primary head. Note anterior (left) and posterior staining. (G) Cross section through anterior neural folds of a secondary embryo, with typical expression in the dorsal part of the neuroectoderm and the adjacent surface ectoderm. Note the absence of mesoderm. (H) Cross section through posterior part of a secondary embryo. Note *Cnot* expression in the notochord-like mesoderm, and absence of staining from the open, posterior neural plate. (I) Lack of *Cnot* expression in epiblast by transplanted, *Cnot*-expressing cells. Newly generated notochord from a HH st.8 embryo was transplanted under neurulation competent ectoderm (HH st.3$^+$) and incubated for 4 h. Note the absence of staining in the epiblast. (J) Schematic representation of the performed operations (see methods section). The left embryo is HH st.8, the right HH st.10/11. The middle symbolizes a HH st.4 embryo in A–E, and HH st.3$^+$ in F–I. Bars indicate 50 μm.

gress, they will remain in the midline and form the mesodermal notochord. If not, they remain in the ectodermal epiblast, and become part of the pre- and postnodal neural plate at the posterior end of the forming neural tube.

The definition of a group of cells in the node on the basis of *Cnot* expression is of interest in view of the phenomenon of planar induction. Evidence is accumulating that not only vertical signals from mesoderm to competent ectoderm, but also planar signals transmitted through the epithelium are involved in neural induction and regionalisation (for review see Ruiz i Altaba, 1993). It is conceivable that in response to an early signal a group of cells becomes defined by expression of *Cnot*. Alternatively, the *Cnot* gene could become activated, when a cell has spent a certain time in a prechordal stage, for example in a phase of *goosecoid* expression (see below). The descendants could then become divided into notochord or posterior neural plate cells by gastrulation mechanisms.

Our transplantation experiments of notochord stem cells to ectopic sites demonstrate that their *Cnot* expression is independent from further signals from the vicinity of the grafting site. The secondary notochords also undergo the typical decrease of signal seen in older, more anterior parts of primary notochords. Transplantation of the node to lateral epiblast is an experiment homologous to the organizer experiment described for amphibia. The type of the chick operation is equivalent to the *Einsteck* procedure (see Hamburger, 1988), and not to the classical transplantation of the blastopore lip as performed originally by Spemann and Mangold (Spemann and Mangold, 1924). While the latter procedure gives a contribution of the graft to the secondary neural ectoderm, this is neither the case in the chick operation, nor the *Einsteck* procedure. Thus, the absence of the posterior neural plate *Cnot* expression domain in secondary embryos corroborates our interpretation of a cell lineage relationship.

3.3. Not-1 expression in the anterior neural plate

The node/notochord/neural plate cell populations characterised by *Cnot* excludes the territory of the prospective fore- and midbrain. From the early somite stages onward a *Cnot* expression domain appears, which seems unrelated to the earlier pattern developing from the node. It covers the anterior neural folds, while they are not yet closely appositioned, at the axial levels of the prospective anterior surface ectoderm, prosencephalon and anterior mesencephalon (Couly and Le Douarin, 1987). Thus the anterior region of the neural plate, which does not express *Cnot* in early stages (HH st.5/6), begins expression in later stages. As a result, the whole length of the neuroectoderm has finally expressed *Cnot* at one point of development (Fig. 8). The bilateral signal becomes more and more focused with closing of the anterior neuropore and finally condenses in one, extremely strong signal in the developing epiphysis at the dorsal diencephalon. Our transplantation experiments demonstrated that the induction of the anterior *Cnot* expression also occurs in the secondary embryos, notably in the absence of any underlying mesoderm. These findings indicate that expression is triggered intrinsically in the secondary neuroepithelium, again pointing to events of planar, rather than vertical induction.

After HH st.10, *Cnot* expression is also detected in more ventral, anterior regions. It begins in the ventral thalamus (HH st.10), is then present in the hypothalamus (HH st.12) and in cells of and around the preoral foregut (HH st.15). The appearance of the ventral domains coincides with the disappearance of *Cnot* expression from the head process. In conjunction with the development of the cephalic flexure, all these anterior expression domains become located in one plane (Fig. 5G). Strikingly, this plane marks quite precisely the most rostral extent of the head process, that is the most rostral site of notochordal *Cnot* expression.

3.4. Goosecoid and Cnot: head and trunk organizer, respectively?

The timing and pattern of *Cnot* expression indicates a role during the early organization of the body axis. Several other genes specifically expressed in organizing regions (dorsal blastopore lip or the node area) also maintain high expression levels in the notochord, e.g. the *brachyury* (T) gene (Wilkinson et al., 1990), the forkhead gene *HNF3β* (Sasaki and Hogan, 1993), the homeobox gene *Xlim-1* (Taira et al., 1992) or the growth factor gene *sonic hedgehog* (*shh*; Echelard et al., 1993). A major difference between *HNF3β/shh* and *Cnot* is the pronounced localization of *HNF3β/shh* transcripts in the ventral midline of the neural tube, the floorplate. The likely interaction between these midline genes, including *Cnot*, with regard to the inducing and patterning abilities of the notochord requires further investigation.

A comparison with *goosecoid*, a homeobox gene expressed in the anterior midline mesoderm, is of particular interest. *Goosecoid*-expressing cells can induce the formation of a secondary embryo, with the typical *goosecoid* expression domain in the intermediate, anterior primitive streak (Izpisúa-Belmonte et al., 1993). Such findings have suggested a crucial role for *goosecoid* for the function of the organizer, more specifically the head organizer (see introduction). When *goosecoid* has reached a peak of expression in the anterior streak at HH st.3$^+$, we observed the first sign of *Cnot* expression. In HH st.4 both genes are expressed in the node, where *goosecoid* marks a more ventral position including endoderm, and *Cnot* a more dorsal level. Subsequently, *goosecoid* disappears quickly from the node and marks the prechordal mesoderm and, weakly, the head process. In the head process the expression domains of the two genes overlap, one ending, the other beginning its phase of activity. It will be of interest to see on a single cell

level, if expression of the two genes is mutually exclusive. Since the expression of *Xnot/Cnot* replaces *goosecoid* temporarily and spatially, von Dassow et al. (1993) have, in analogy, discussed *Xnot* as a candidate for a trunk organizer. *Xnot/Cnot* could play a role in the epichordal axis similar to *goosecoid* in the prechordal axis. However, one clear difference between the two putative organizer genes was revealed by transplantation experiments in chick. Direct homeogenetic induction of a gene by expressing graft cells occurs only in the case of *goosecoid* (Izpisúa-Belmonte et al., 1993), but not of *Cnot* (this paper).

A molecular coding mechanism was previously suggested for the function of Hox genes (Kessel and Gruss, 1991). The concept proposed that the combination of expressed Hox genes, the Hox code, determines the axial level of a given cell. It suggested, that cells in the epiblast continue to change Hox expression, while after ingression no changes of the current combination are possible. It is conceivable that the basic concept is applicable in more general terms also for other homeobox genes. In the midline of early embryos a division into pre- and epichordal axis is of relevance. Thus, after a certain time of *goosecoid* expression a cell could make a transition to *Cnot* expression, similar to the transition occuring from one Hox gene to its 5′ neighbour in the cluster. The transition would be prevented by cell ingression, again similar to the situation found for Hox genes. Similar to the function of Hox genes in the periphery, the molecular coding of axial positions in the midline mesoderm could occur by homeobox genes.

4. Materials and methods

4.1. Embryos

Fertile White Leghorn eggs obtained from Lohmann Tierzucht, Cuxhaven, were incubated at 37.8°C in a humidified incubator and the embryos staged according to Hamburger and Hamilton ('HH st.'; Hamburger and Hamilton, 1951).

4.2. PCR cloning

Total RNA from HH st.4 chicken embryos was prepared by the LiCl procedure (Auffray and Rougeon, 1980) and 5 μg were reverse transcribed (Pharmacia). Two degenerate primers were synthesized with the following sequences. A 5′ primer, GGGAGCTC(T/C)TNGA(A/G)AA(A/G)GA(A/G)TT(T/C)(T/C)T and 3′ primer, CGCGGATCC(T/G)NC(T/G)(A/G)TT(T/C)TG(A/G)AACCA. The PCR cycling parameters were 2 initial cycles 94°C for 1 min, 55°C for 3 min, 72°C for 1 min; then 40 cycles of 94°C for 1 min, 60°C for 2 min, 72°C for 1 min; and final extension at 72°C for 10 min. PCR products were ligated into pCRII plasmids (Invitrogen) and identified by dideoxy sequencing. cDNA clones were isolated from a HH st.10 library (Charlebois et al., 1990) by screening with the purified PCR fragment. The sequence of a 880 bp cDNA clone with homology to Xnot is deposited in the EMBL Data Library (EMBL accession number X82575).

4.3. RNA analysis

For Northern blots, 20–30 μg of total RNA (Auffray and Rougeon, 1980) per lane were electrophoresed, blotted on Qiagen nylone plus membranes, and hybridized with a 600 bp *Eco*RI/BstXI cDNA fragment (including the homeobox). Blots were rehybridized with a 2 kb chicken β-actin probe for control. RNA probes for whole mount in situ hybridization were prepared with a DIG labelling mix (Boehringer Mannheim) from the 600 bp *Eco*RI/BstXI subclone of the *Cnot* cDNA. Proteinase K treatment was omitted for embryos up to HH st.5, and the incubation time raised from 1 to 15 min for later stages. Hybridization and washes were essentially done as described (Wilkinson, 1992). However, hybridization was at 55°C, hybridization solution and the first two washing solutions contained 0.1% CHAPS detergent (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate; Sigma; Harland, 1991), and no RNaseA treatment was performed. For paraffin sections (8 μm) stained embryos were dehydrated and embedded in Paraplast plus (Sherwood Medical). 30 μm sections were cut with a vibratome (Pelco 101) after embedding of stained embryos in a gelatin albumen mixture. Embryos were photographed under the dissection microscope or with Nomarski differential contrast optics.

4.4. Manipulation of chick embryos

Grafts were transplanted under the endoderm near the area opaca of HH st.3$^+$/4 hosts, cultured ventral side up in a modified New culture (Stern, 1993), without incision of the epiblast. In many cases two grafts per culture were inserted on the left and right side. Cultures were incubated for 4–10 h (short incubation) or 18 h (long incubation). For grafting in ovo a window was cut into the eggshell and embryos (HH st.8–12) were visualised by injecting black ink (Pelikan India drawing ink, diluted 1:10) into the subblastodermal cavity (Stern, 1993). Eggs were reincubated for 18 h.

For DiI labelling experiments HH st.5 to HH st.7 embryos in ovo were used. A small bolus of dye was applied to the epiblast by using a drawn out glass capillary filled with DiI (1.1′-dioctadecyl-3,3,3′,3′,-tetramethyl indocarbocyanine perchlorate; Molecular Probes) as described previously (Selleck and Stern, 1991). Eggs were reincubated for 14–18 h, embryos were fixed, and photographed under epifluorescence.

Acknowledgements

We thank G. Behrens for excellent technical assistance, M. Gross, M. Hallonet, L. Lemaire, G. Oliver, F.

Pituello, A. Stoykova, A. Voss and R. Wehr for discussions, B. Christ for introduction to chick methodology and P. Gruss for comments and support. The cDNA library was kindly provided by R. Grainger. The project was supported by the Max-Planck Gesellschaft and DFG grants Ke 513 and SFB 271.

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