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Differential Activation of the Clustered Homeobox Genes *CNOT2* and *CNOT1* during Notogenesis in the Chick

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***CNOT2*, a newly identified homeobox gene, is physically linked to the *CNOT1* gene in the chicken genome. The two chicken genes represent two different subgroups of the *Not* gene family, the first including *CNOT1* and the *Xenopus* genes *XNot1* and *XNot2*, and the second *CNOT2* and the zebrafish *floating head* gene. The overall expression pattern of *CNOT2* in Hensen's node, notochord, neural plate, tailbud, and epiphysis resembled the *CNOT1* pattern. However, several significant differences occurred: *CNOT2* expression was much stronger and more widespread in the pregastrulation embryo, it showed an additional, transient domain on the anterior intestinal portal, and lacked expression on the early anterior neural folds and the anterodistal limb bud. We studied CNOT expression by transplanting parts of the primitive streak into growing embryos or by explanting them into tissue culture. *CNOT* gene expression from young nodes was maintained *in vivo*, but required *in vitro* the addition of retinoic acid. The generation of differentiated notochord structures could only be obtained, if either older node grafts were used *in vitro* or young node grafts were transplanted close to the primary axis *in vivo*. We conclude that *CNOT* expression in the anterior streak is not enough for notochord differentiation, but further influences are necessary. A *Not*-related gene has previously been isolated from *Drosophila melanogaster* and its expression was detected in the posterior brain and the neuroblasts (Dessain and McGinnis, 1993. *Adv. Dev. Biochem.* 2, 1–55). The correspondence between *Not* gene-expressing cells in the nervous system of *Drosophila* and the early neuroectoderm in the chick and its implication for a phylogenetic relationship between neuroectoderm and the notochord is discussed.**

INTRODUCTION

The axial mesoderm of vertebrates lies dorsal to the gut and ventral to the central nervous system. It consists of the rostrally located prechordal mesoderm, at the level of the forebrain, and the notochord, extending from the hindbrain all the way to the tail. At the transition between these two tissues lies a notochord-like structure (the head process), which demarcates the level of the midbrain. With regard to its cell biology, inductive properties, and gene expression, the head process possesses some unique properties. It lacks a notochordal sheath and induces a unique type of ventral central nervous system (Hynes *et al.*, 1995). The common origin of the axial mesoderm during embryogenesis is the tip of the primitive streak, the node (Bellairs, 1986). In the chick, the node begins generating prechordal mesoderm once the streak has reached its definitive length (Hamburger–Hamilton stage 4, HH st.4 ; Hamburger and Hamilton, 1951) and subsequently it produces notochord during its regression toward the caudal pole of the embryo from HH st.6 onward (Jurand, 1962; Meier, 1981; Rosenquist, 1983; Sausedo and Schoenwolf, 1993; Seifert *et al.*, 1993; Selleck and Stern, 1991). Finally, the node and the streak melt into a common structure, the tailbud, where the ''chordoneural hinge'' becomes the site of notochord generation (Catala *et al.*, 1995).

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. X98049.

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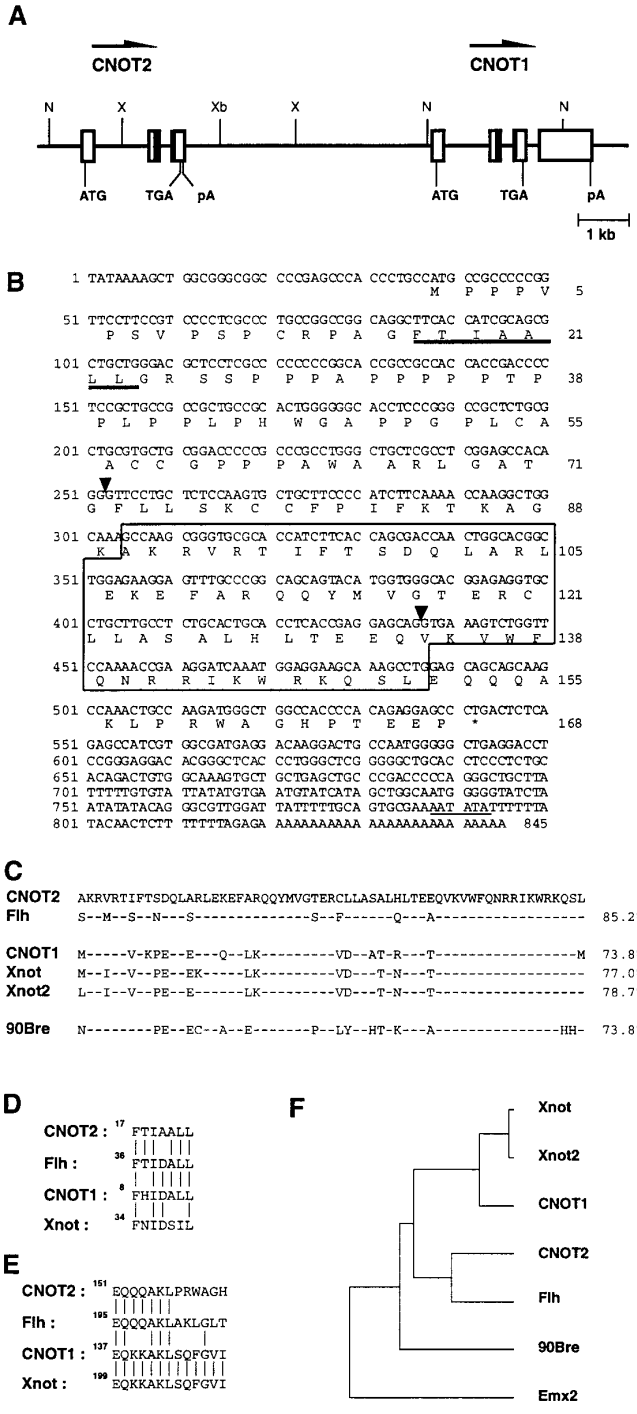


FIG. 1. (A) Genomic structure of the chicken *CNOT* cluster. Exons are indicated by boxes and filled boxes depict the two homeodomains. Note the similar organization of the first three exons of *CNOT1* and *CNOT2*, respectively. N, *Nco*I; X, *Xho*I; Xb, *Xba*I; ATG, start codon; TGA, stop codon; pA, polyadenylation site. (B) Nucleotide and deduced amino acid sequence of the *CNOT2* cDNA. The homeodomain is boxed and the conserved heptapeptide and the polyadenylation signal underlined (bold or thin line, respectively). Triangles indicate the positions of the introns. The first 82 nucleotides are derived from the genomic sequence. (C) Comparison of the amino acid sequence of the CNOT2 homeodomain to the other members of the *Not* family (for references see text). Dashes represent residues that are identical to the CNOT2 sequence. (D, E) Comparison of short amino acid sequence similarities in the region of the heptapeptide (D) and the amino acids following the homeodomain (E). Numbers indicate the position of the first shown amino acid in the respective protein. Outside the domains described in C, D, and E CNOT2 shows no significant similarities to other Not proteins. (F) Subdivision within the family of Not homeodomains. The dendrogram was constructed from the homeodomains shown in C by using the PILEUP program of the Wisconsin GCG Sequence Analysis Package (Devereux *et al.*, 1984) and also includes the homeodomain of the closest relative of the *Not* genes, *Emx2* (Simeone *et al.*, 1992). Note the grouping of *CNOT1*/*Xnot*/*Xnot2* and *CNOT2*/*Not*, respectively.

Homeobox genes seem to play critical roles in the specification of cells by defining the identity of tissues, organs, regions, or positions. More than 170 different homeobox genes have been cloned from the genomes of vertebrates (Stein *et al.*, 1996), representing a significant percentage (>0.1%) of the total number of genes in a higher vertebrate. A candidate gene for the specification of the prechordal mesoderm is *goosecoid*, which is in addition expressed in the node, when prechordal mesoderm cells migrate out, and with decreasing levels in the head process (Izpisúa-Belmonte *et al.*, 1993). Other homeobox genes with related patterns of expression in the prechordal area of mice or chicken are *Lim1*, *Otx2*, and *Rpx* (Bally-Cuif *et al.*, 1995; Hermesz *et al.*, 1996; Shawlot and Behringer, 1995). The availability of murine null mutants has shed further light on the specification of the prechordal region. *Otx2* and *Lim1* mutants are severely affected in the rostral part of the head, suggesting the major influence of these genes (Acampora *et al.*, 1995; Matsuo *et al.*, 1995). *goosecoid* mutants, however, did not show dramatic abnormalities in the head (Rivera-Peréz *et al.*, 1995; Yamada *et al.*, 1995). In these studies the authors postulated the presence of a second, *goosecoid*-related gene, which could substitute for *goosecoid* during gastrulation and prechordal mesoderm specification of the mutant mice.

We have previously pointed out that the homeobox gene *CNOT* (now *CNOT1*) is present at the right time and space expected for a gene involved in the specification of the notochord. *CNOT1* transcripts were found in the node and, with rostrocaudally increasing levels, in the head process, and in the notochord (Stein and Kessel, 1995). Recently, further descriptions of the same chicken gene ("*Gnot1*") were reported by Mackem and colleagues, who also found a second *Not* homeobox in chicken (Ranson *et al.*, 1995). *CNOT1* is a member of the "*Not*" subfamily of homeobox genes together with the *Xenopus* genes *Xnot* and *Xnot2* (Gont *et*

al., 1993; von Dassow *et al.*, 1993), the zebrafish *floating head* (*flh*) gene (Talbot *et al.*, 1995), as well as the *Drosophila* gene *90Bre* (Dessain and McGinnis, 1993). Recently, three zebrafish mutants with point mutations or deletions in the *Not* gene *flh* were described (Halpern *et al.*, 1995; Talbot *et al.*, 1995). The primary defect of *flh* mutants is the nonformation of notochord and the consequential loss of its inductive influences. In a gain-of-function experiment, Gont and colleagues studied the effects of injected *Xnot2* mRNA in *Xenopus* 4-cell embryos, where they observed the generation of greatly expanded notochords extending to the most rostral part of the body axis (Gont *et al.*, 1996). Both loss- and gain-of-function phenotypes strengthened the close correlation between expression of a *Not* gene and generation and maintenance of a notochord. These findings place the *Not* genes toward the top of a regulatory hierarchy establishing a notochord identity. During further development the notochord exerts its function as an embryonic signaling center via secreted factors like sonic hedgehog (for review see (Placzek, 1995) or chordin (Sasai *et al.*, 1995, 1994).

In the present study we demonstrate that a second *Not* gene, *CNOT2*, with similar, but nonidentical sites of expression, is clustered with *CNOT1* in the chick genome. We discuss the relationship between *Not* genes of chicken, frog, zebrafish, and fruit fly and their role in the ontogenetic and phylogenetic development of the notochord.

MATERIALS AND METHODS

Embryos

Fertilized White Leghorn chicken eggs were purchased from Lohmann Tierzucht (Cuxhaven, Germany). Embryos were staged according to Eyal-Giladi and Kochav (1976) for stages preceding the formation of the primitive streak (EK) and according to Hamburger and Hamilton (1951) for later stages (HH). HH st.3 was further subdivided according to Schoenwolf (1992).

cDNA Cloning

A *CNOT2* cDNA clone (569 bp) was isolated from a chicken HH st.10 cDNA library (Charlebois *et al.*, 1990) with a PCR-derived probe of *CNOT1* at low stringency (Stein and Kessel, 1995). Subsequently, a cDNA library prepared from HH st.3^+/4 Hensen's nodes was screened with a 270-bp PCR fragment spanning the region between the homeobox and the poly(A) tail of the original *CNOT2* cDNA. This subclone was also used to isolate two overlapping phage clones (G3 and G4) from a genomic chicken library (EMBL3, SP6/T7, Clontech).

Whole-Mount in Situ Analysis

Antisense digoxigenin-labeled RNA probes for *CNOT2* were synthesized by T7 RNA polymerase (Boehringer Mannheim) from the originally isolated *CNOT2* cDNA clone and from the 270-bp subclone as templates after linearization with *Hin*dIII. For detection of *CNOT1* mRNA a 600-bp subclone was used (see Stein and Kessel, 1995). Sense RNA probes gave no significant staining. Embryo preparation, whole-mount analysis and histology were essentially done as described previously (Stein and Kessel, 1995; Wilkinson, 1992).

RNase Protection Assays

As DNA templates for the *CNOT1* and *CNOT2* probes, cloned fragments of the respective homeoboxes were used. For preparation of the *β-actin* template two complementary 50-bp oligonucleotides designed to the 5′ region of cytoplasmic chicken *β-actin* (Paterson and Eldrigde, 1984) were hybridized to one another and cloned into a Bluescript II vector (Stratagene). All templates were transcribed with T7 RNA polymerase (Boehringer Mannheim) to yield [^{32}P]UTP-labeled antisense riboprobes for *CNOT1* (254 bp), *CNOT2* (244 bp), or *β-actin* (115 bp), which should give rise to protected fragments of 92, 123, or 50 bases, respectively. Total RNA was prepared from single, carefully staged embryos, in order to escape staging artifacts from pooled specimens. By using the Micro RNA Isolation kit (Stratagene) around 4 μg of RNA could be obtained per embryo. It was replenished with 36 μg yeast tRNA and was hybridized for 14 hr at 60°C with the three probes simultaneously, using 5×10^5 cpm of each of the *CNOT* riboprobes, as well as 5×10^4 cpm of the *β-actin* probe. After digestion (50 μg/ml RNase A; 200 U/ml RNase T1) protected fragments were electrophoresed in a 6% denaturing gel and analyzed by autoradiography.

Transplantations and Explantations

Transplantation studies were performed in New-culture (New, 1955) essentially as described earlier (Stein and Kessel, 1995). For explant cultures primitive streaks were dissected from embryos of various stages, cut into a rostral and a caudal half, and placed into standard tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM). All-*trans* retinoic acid (RA) was diluted in DMEM to a final concentration of 10^{-5} *M*. In most cases the explants adhered to the dish after an overnight incubation at 37°C. Only a few rounded up and did not attach. Gene expression was analyzed by whole-mount analysis within the original culture wells.

RESULTS

CNOT2: Genomic Organization, cDNA, and Encoded Protein

Our first indication for the existence of a second *Not* gene in chicken came from a cDNA clone isolated during a screen for *CNOT* cDNAs. We named the new isolate *CNOT2* in order to stay within the logic of our first description of the *CNOT* gene, which we now index as *CNOT1*. Recently, Ranson and colleagues reported the limb expression of the *CNOT1* gene, which they named *Gnot1* (Ranson *et al.*, 1995). They also published a homeodomain sequence identical to CNOT2 under the name Gnot2. We isolated genomic *CNOT2* clones from a chicken phage library using a *CNOT2*-specific probe downstream from the homeobox. The two phages G3 and G4 contained overlapping inserts

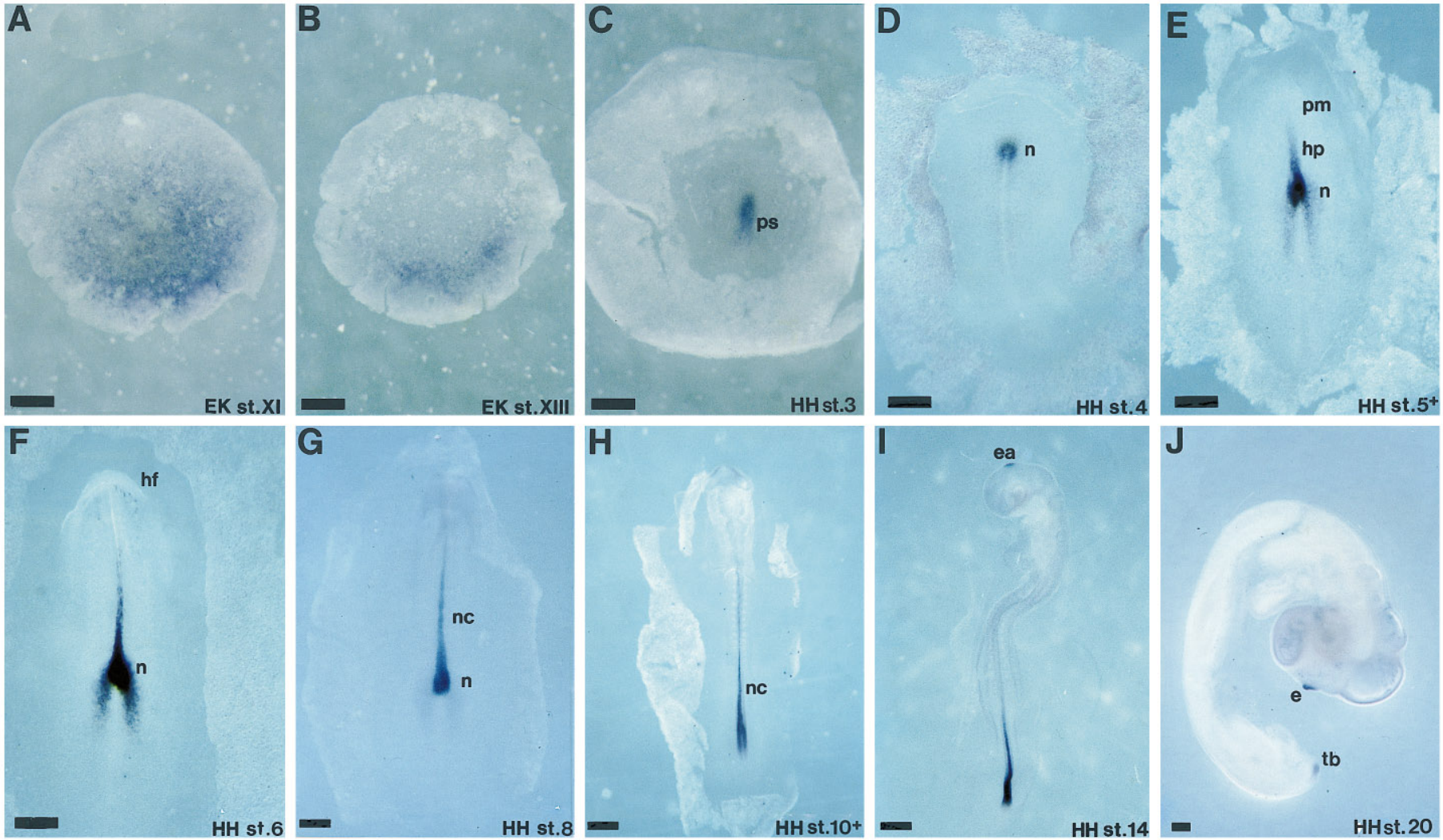


FIG. 2. Expression pattern of *CNOT2* during chick development. Embryos were subjected to whole-mount *in situ* hybridization. Stages are given in the lower right corner; the magnification bar represents 500 μm. Indicated are the primitive streak (ps), Hensen's node (n), the prechordal mesoderm (pm), the head process (hp), the headfold (hf), the notochord (nc), the epiphysis anlage (ea), the epiphysis (e), and the tailbud (tb). For a detailed description of the expression see text.

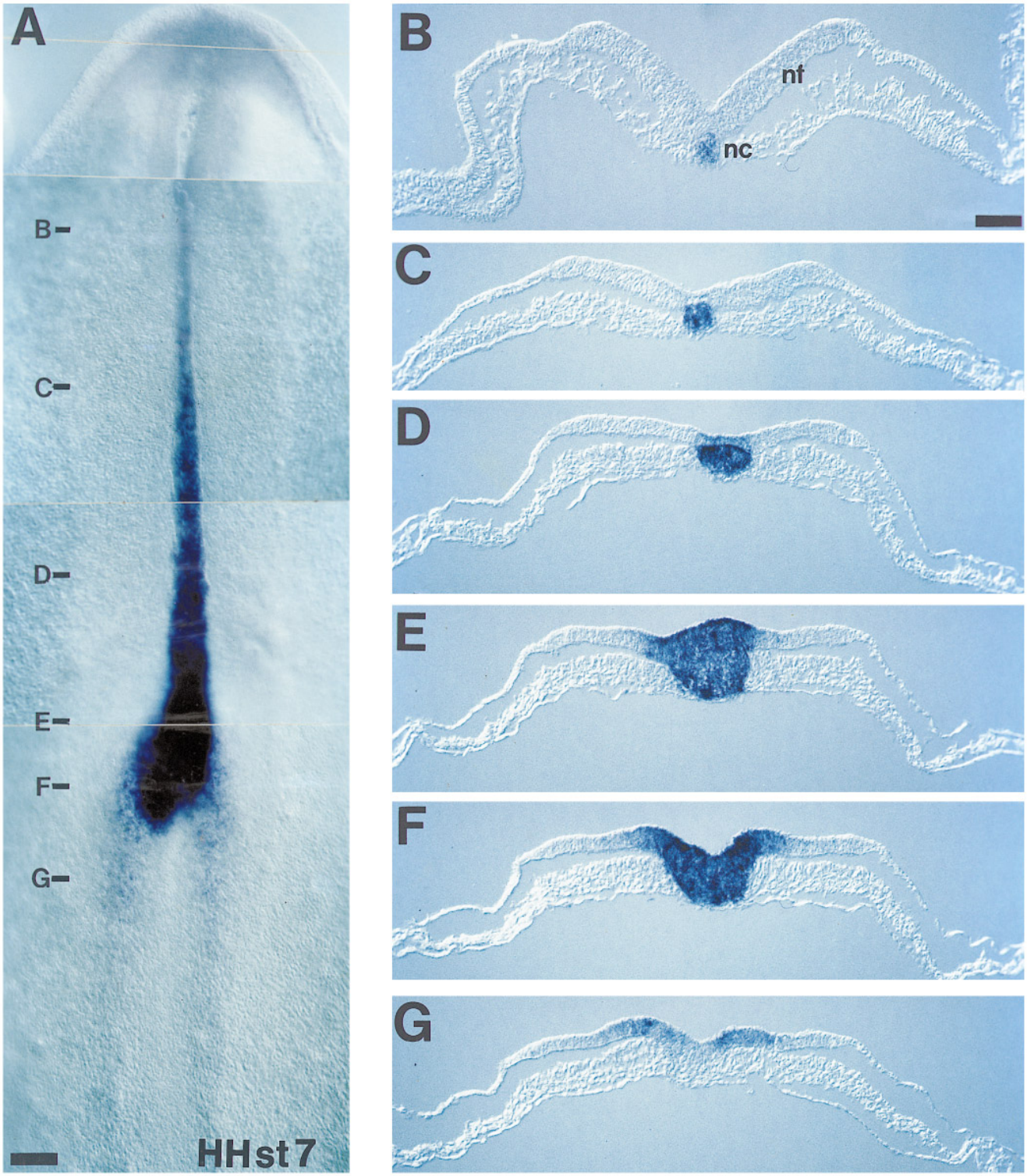


FIG. 4. *CNOT2* expression in a one-somite chick embryo (HH st.7). The whole-mount (A) and the paraffin sections are viewed by Nomarski optics. The levels of the sections (B–G) are marked in (A); the magnification bar represents 50 μm. Note the absence of expression from the neural plate (prospective midbrain) overlying the head process/anterior notochord (B and C). Strong expression is seen in the neural plate around the node (E and F). The expression in the postnodal neural plate flanking the primitive streak is tightly restricted to a narrow region (G), whereas *CNOT1* in a comparable embryo would label a slightly wider area of the postnodal neural plate.

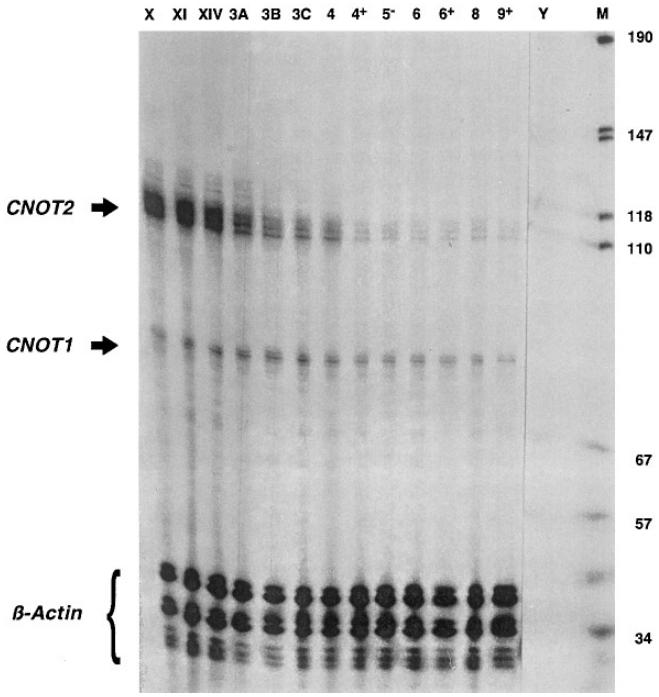


FIG. 3. Determination of relative *CNOT1* and *CNOT2* RNA concentrations in early chick embryos by RNase protection analysis. Each lane represents the analysis of the complete RNA isolated from a single embryo. EK stages are indicated in Roman numbers (X, XI, XIV); the substaging of HH st.3 according to Schoenwolf (1992) is indicated by 3A, 3B, or 3C, respectively, and further HH stages are given in arabic numbers. ''Y'' indicates the negative control lane done with yeast RNA. ''M'' indicates the DNA marker, whose sizes are given on the right margin of the gel. The protected bands from the three probes are indicated on the left margin; their calculated sizes are 123 bases (*CNOT2*), 92 bases (*CNOT1*), or 50 bases (*β-actin*). For details see Materials and Methods.

of 18.5 or 14 kb, respectively, spanning a genomic region of 29 kb. By combining restriction mappings, Southern blotting, sequencing, and comparisons between *CNOT2* and *CNOT1* genomic and cDNA sequences we established a physical map of the genomic locus (Fig. 1A). Phage G3 contains the *CNOT2* and phage G4 the *CNOT1* gene, thus proving a very close clustering of the two genes. The *CNOT2* homeobox lies about 6.4 kb upstream of the *CNOT1* box, and both are transcribed in the same direction. The overall organisation of the two *CNOT* genes is strikingly similar. The reading frames are each separated by two introns of conserved length and position. The first intron interrupts upstream of the homeobox and extends for 1070 (*CNOT2*) or 1064 bases (*CNOT1*). The second interrupts the homeobox with 364 and 352 bases, respectively. For the *CNOT1* gene at least three transcript variants are known since a third, differentially spliced intron was found downstream of the coding sequence. Its removal leads to a long trailer sequence, while the nonspliced RNA can also be terminated by using a more closely located polyadenylation signal (Knezevic *et al.*, 1995; Stein and Kessel, 1995). We have no indication for the occurrence of differently spliced transcripts in the case of *CNOT2*. Additional, overlapping *CNOT2* cDNA clones were isolated from a HH st.4 node cDNA library, and from these we could accumulate an open reading frame including the CNOT2 homeodomain (Fig. 1B). In order to obtain a full open reading frame, 82 bases were substituted from genomic sequences. The predicted CNOT2 protein consists of 149 amino acids (Fig. 1B). This compares to 171 amino acids determined for the CNOT1 protein (Ranson *et al.*, 1995) and 241 for the zebrafish floating head protein (Talbot *et al.*, 1995). Assignment of the start codon was based on the consensus of the translational initiation sequence (Kozak, 1987) as well as on a sequence conservation at the beginning of the CNOT1 and CNOT2 proteins (M-P-P-P). In addition a conserved heptapeptide (F-T-I-A-A-L-L) was found in close vicinity to the N-terminus. A similar heptapeptide is common not only to the otherwise very proline-rich prehomeodomain part of all known Not proteins (Fig. 1D), but is also present more or less conserved in the N-terminal part of many proteins encoded by homeobox genes, such as *goosecoid, engrailed, Nkx2,* and *paired* related genes (Blum *et al.*, 1992; Izpisúa-Belmonte *et al.*, 1993; Joyner and Martin, 1987; Lints *et al.*, 1993; Noll, 1993). This heptapeptide, the homeodomain (Figs. 1C and 1F) as well as a short carboxy-terminal extension of the homeodomain (Fig. 1E) are the only regions where the CNOT2 protein shows significant sequence similarities to the other known Not homeoproteins.

Early Expression Phase of the CNOT Genes

Expression of *CNOT2* is readily detected in a large area of the epiblast of unincubated chick embryos (Fig. 2A). With incubation and thus formation of the hypoblast, expression becomes restricted to more caudal parts of the embryo (Fig. 2B), where *CNOT2* transcripts are found in both the epiblast and the hypoblast layers. Primitive streak formation leads to accumulation of *CNOT2* transcripts in its anterior part, still surrounded by a halo of expressing cells in the epiblast (Fig. 2C). By the definitive streak stage (HH st.4) expression becomes confined to the node (Fig. 2D). Recently, Knezevic and colleagues have demonstrated that *CNOT1* is weakly expressed in slightly caudally concentrated domains of epi- and hypoblast in early, prestreak embryos (Knezevic *et al.*, 1995). In a direct comparison the most striking difference between the two genes is the early onset of *CNOT2* transcription, resulting in strongly positive pregastrulation embryos (EK st.XI), compared to the very low levels of *CNOT1* expression (Figs. 5A and 5B).

In order to compare the activation of the two genes quantitatively we analyzed early embryonic stages by a sensitive RNase protection assay (see Materials and Methods). RNA preparations from single embryos were hybridized simulta-

neously with probes for *CNOT1, CNOT2,* and *β-actin* (Fig. 3). *β-actin* levels per lane proved to be more or less identical, indicating the loading of similar amounts of RNA per lane. *CNOT1* expression was clearly detected before gastrulation, and the detected RNA levels remained almost constant up to HH st.9$^+$. Only a minor increase occurred from the freshly laid egg (EK st.X) through stages EK st.XI, EK st.XIV, HH st.3A, 3B, and 3C to the definitive streak stage HH st.4. After HH st.20 *CNOT1* transcripts were no longer detectable (not shown). Much more dramatic changes were observed for the *CNOT2* gene. The highest level of RNA was found in EK st.X, and it decreased slightly up to HH st.4, when the amount of *CNOT2* RNA was still significantly higher than the *CNOT1* RNA level. Then, between HH st.4 and HH st.4$^+$, *CNOT2* RNA dropped to levels lower than those observed for *CNOT1* and remained constant. This finding corroborated our results from parallel whole-mount analyses, which had indicated that in these stages *CNOT2* expression was weaker than *CNOT1*. In conclusion, the quantitative analysis of *CNOT* expression profiles indicated different phases for the two genes, with *CNOT2* being the predominant gene during the pregastrulation phase.

Expression of CNOT2 during Gastrulation and Neurulation

Localized *CNOT2* expression can be clearly detected in the anterior primitive streak at intermediate length (HH st.3$^+$) and becomes strongly positive in the complete node at the definitive primitive streak stage, HH st.4 (Figs. 2C and 2D). From then onward, *CNOT2* expression remains in the node and as a short extension in the central neural plate directly anterior to the node (Figs. 2D, 2E, 4D, and 4E). The newly generated notochord becomes the main expression domain (Figs. 2E–2I and 4) during the following stages. Connected to the node/notochord/prenodal expression domain are two postnodal areas flanking the primitive streak (Fig. 4G), fated to become neural tube and tailbud (Schoenwolf, 1992; Spratt, 1952; Stein and Kessel, 1995). By HH st.10, the complete sinus rhomboidalis, comprising pre-, para-, and postnodal neural plate (Schoenwolf, 1992), expresses the *CNOT2* gene as does the tailbud later on as the successor of the node (Figs. 2H–2J). By HH st.23, when generation of the notochord from the chordoneural hinge ceases, the expression of *CNOT2* in this posterior region of the embryo also fades out.

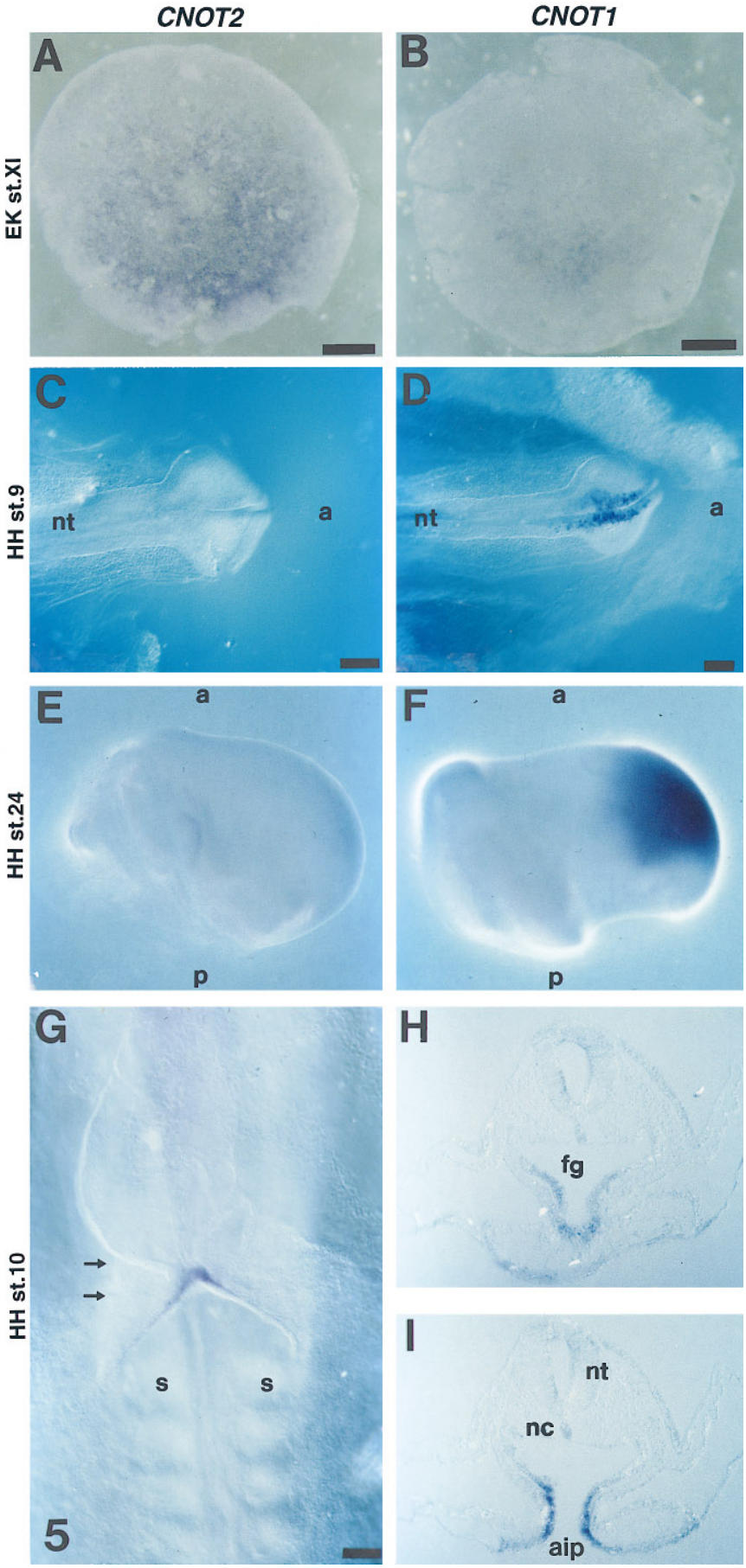
A new *CNOT2* domain arises apparently independently from the described node–notochord–neural plate domain after closure of the brain at HH st.14 (Fig. 2I). It demarcates the anlage of the epiphysis at the dorsal diencephalon, and the epiphysis remains the last strong *CNOT2*-expressing structure beyond HH st.23 (Fig. 2J).

The described expression pattern of the *CNOT2* gene closely resembles the pattern of the *CNOT1* gene, which has been reported in detail previously (Knezevic *et al.*, 1995; Stein and Kessel, 1995). Given this close similarity, the differences are of particular interest. Five significant differences were detected. First, the expression dynamics in pregastrulation embryos identify *CNOT2* as the earlier gene (Figs. 5A and 5B). A second, less obvious, difference is concerning the bilateral expression domains in the postnodal neural plate. During all stages these appear to be more narrow and closer to the streak for *CNOT2,* compared to *CNOT1* (Figs. 2F, 4, and 6). Third, a weak domain on the ventral side of the anterior intestinal portal (HH st.10) is specific for *CNOT2* (Figs. 5G, 5H, and 5I). Fourth, the localized *CNOT1* domain in the limb buds is not observed for *CNOT2* (Figs. 5E and 5F). Finally, the *CNOT2* expression in the anlage of the epiphysis starts much later (HH st.14) and at a lower level than *CNOT1,* which is already quite strong at HH st.8 on the yet unfused neural folds of the prospective prosencephalon (Figs. 5C and 5D). In conclusion we observed highly similar, but not identical, expression for both *CNOT* genes in the pregastrulation epiblast, node, notochord, and neural plate.

Maintenance of CNOT Expression in Primitive Streak Explants

In order to pinpoint differences in maintenance and expression dynamics of *CNOT* genes we used a tissue culture system and analyzed explants of primitive streaks from various stages (see Materials and Methods, Table 1, Fig. 6). Explants from early (HH st.2), intermediate (HH st.3), or definitive (HH st.4) primitive streaks grew readily in overnight micromass cultures. After *in situ* analysis of streaks grown in culture three types of signals could be clearly differentiated: strong blue staining of notochord structures, with the rest of the explant culture unstained (+++, Fig. 6A); clearly positive, partially localized staining, but no notochord formation (++, Figs. 6E and 6F); background staining only (−/+, Figs. 6B, 6C, and 6D).

FIG. 5. Differences between *CNOT1* and *CNOT2* expression. Indicated are neural tube (nt), anterior (a), posterior (p), first somite (s), foregut (fg), notochord (nc), and anterior intestinal portal (aip). (A and B) In prestreak embryos of EK st.XI the expression of *CNOT2* is significantly stronger and more widespread than that observed for *CNOT1.* The tendency to become localized in the posterior part of the embryonic disc is evident for both genes. (C and D) Note the absence of *CNOT2* staining from the anterior neural folds (prospective forebrain) at HH st.9. *CNOT2* expression in the epiphysis anlage appears only at HH st.14. (E and F) No localized *CNOT2* expression is observed in the limb bud (HH st.24), in contrast to the striking anterior-distal domain of *CNOT1.* (G–I) Note endodermal *CNOT2* staining at the anterior intestinal portal (aip) and in the closed and unclosed foregut. Scale bar, 500 μm in A–D and 50 μm in G–I.


CNOT2
CNOT1
EK st.XI
HH st.9
HH st.24
HH st.10
A
B
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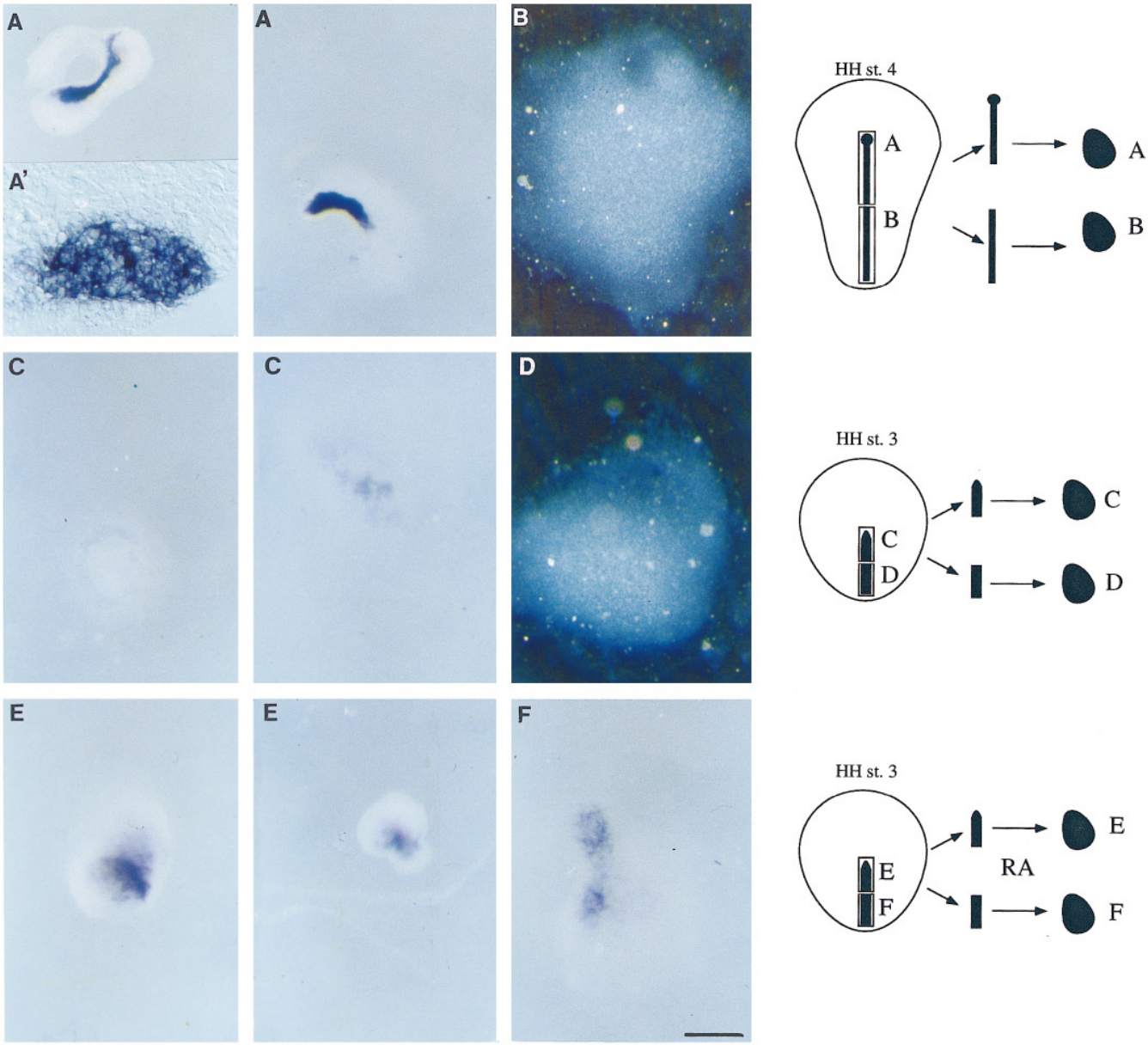


FIG. 6. *CNOT1* expression in cultured explants of the primitive streak. The data are summarized in Table 1. All depicted examples were explanted and analyzed in parallel. The labelings of the panels (A–F) correspond with the explants and culture conditions indicated in the diagrams on the right; A, A′, C, and E are from rostral and B, D, and F from caudal explants. Panels A, A′, and B are derived from HH st.4 streaks, panels C and D from HH st.3 streaks, and panels E and F from HH st.3 streaks, which were cultured in retinoic acid (see schematic drawings). (A, C, E, and F) Photographed in the original dishes under the dissection microscope with illumination through frosted glass; (B and D) with dark-field illumination; A′ is a section viewed by differential interference microscopy. Notochord morphology and a strong *CNOT1* signal is observed in panels A and A′ (symbol +++ in Table 1), definitive, partially localized staining in panels E and F (symbol ++), and completely negative or barely positive cultures are shown in panels B, C, and D (symbol −/+). Scale bar, 250 μm (A–F) or 25 μm (A′).

Exclusively rostral explants from HH st.4 streaks became strongly stained (+++) after hybridization with a *CNOT1* (27/28) or a *CNOT2* (9/10) probe. The signal was restricted to elongated notochord outgrowths with typical histology on a background of nonhybridizing cells (Fig. 6A). Caudal explants of HH st.4 streaks did not express *CNOT1* (29/30; Fig. 6B) or *CNOT2* (12/14) after culture. On the other hand, younger stages behaved differently comparing the mainte-

TABLE 1

CNOT Gene Expression and Notogenesis in Primitive Streak Explants

Analyzed gene	Expression level	Explant of HH st.3		Explant of HH st.4		Culture medium
		Rostral	Caudal	Rostral	Caudal	
CNOT1	−/+	16	20	1	29	DMEM
	++	1	0	0	0	DMEM
	+++	4	0	27	0	DMEM
CNOT2	−/+	5	5	1	12	DMEM
	++	5	6	0	1	DMEM
	+++	4	2	9	1	DMEM
CNOTI	−/+	3	7	nd	nd	DMEM/RA
	++	10	5	nd	nd	DMEM/RA
	+++	5	0	nd	nd	DMEM/RA
CNOT2	−/+	5	14	nd	nd	DMEM/RA
	++	14	17	nd	nd	DMEM/RA
	+++	6	0	nd	nd	DMEM/RA

Note. See text for experimental details and Fig. 7 for schematic drawings and examples for the level of gene expression as detected by *in situ* analysis. Symbols for expression levels are: −/+ (colorless up to light blue); ++ (definitive blue, often partially localized); +++ (strong blue staining of notochord structure, rest of colony unstained). nd, not determined.

nance of *CNOT2* versus *CNOT1* transcripts. Only a few rostral streak explants (5/21) were positive (+++ or ++) for *CNOT1* after culturing, but *CNOT2* signals were observed more often (9/14, Table 1). All caudal explants from younger (HH st. 3) were completely negative for *CNOT1* (20/20), but several of the caudal explants (8/13) scored positive for *CNOT2*. Thus, the *CNOT2* expression in younger primitive streaks appeared not only more significant at the time of explantation (Fig. 2C), but was also maintained better in culture. This difference was no longer observable in older explants. The findings indicated that bona fide notochords only grow in culture, if an *in vivo* maturation of the primitive streak has occurred before. This further extended our previous *in vivo* demonstration of *CNOT1*-positive notochord outgrowths from transplanted HH st.4 nodes regardless of the anteroposterior or dorsoventral level of the transplantation (Stein and Kessel, 1995).

Next we studied the effect of retinoic acid (RA) on notogenesis in HH st.3 explant cultures. In no case was RA able to induce precocious notochord formation *in vitro*. However, *CNOT1* gene expression could be induced by retinoic acid in rostral (15/18) and also some caudal explants (5/12; Figs. 6E and 6F). Since the percentage of *CNOT2*-expressing rostral explants also rose slightly (20/25), the difference between *CNOT1* and *CNOT2* vanished in the presence of RA by elevating the percentage of positive rostral explants for both genes to about 80%.

In summary, we found evidence for a sequential establishment of first *CNOT2* and then *CNOT1* expression in the anterior, intermediate-length streak, involving RA as a potential factor. The step from merely *CNOT* gene-expressing cells to *CNOT* gene expression plus notochord generation, however, could not be obtained *in vitro*.

Maintenance of CNOT Expression in Vivo

Transplantation of Hensen's node to the area opaca-pellucida boundary results in the induction of a ''secondary embryo'' in a reaction comparable to the organizer experiment first performed by Spemann and Mangold in amphibian embryos (1924; Dias and Schoenwolf, 1990, and references therein). We had previously demonstrated that in the secondary embryos *CNOT1* marks the induced, anterior neuroectoderm, the latter being equivalent to the early prosencephalic domain of *CNOT1*. In similar experiments the neuroepithelium remained negative with *CNOT2* probes (Fig. 7A), as predicted from the differential expression of the two genes in normal embryogenesis (Figs. 5C and 5D). Neither gene became induced in the ectoderm adjacent to the graft (Stein and Kessel, 1995).

Transplantation of HH st.3 or HH st.3^{+} nodes resulted in the development of chordoid mesoderm, which did not grow out to form elongated notochords (Fig. 7A; Dias and Schoenwolf, 1990). The graft derived chordoid structures were always strongly positive for either *CNOT* gene. In this respect the *in vivo* grafts behaved similarly to RA-treated *in vitro* explants. The described *in vivo* transplantations and the *in vitro* explantations demonstrated that stable expression of *CNOT* genes is not necessarily coupled to the formation of a morphologically differentiated, elongated notochord. Apparently, the maturation of the tip of the streak occurring *in vivo* between HH st.3 and st.4 is necessary for completion of notogenesis. Experimentally, we could mimic this situation by placing an early node graft near the embryonic axis of the primary embryo so it could develop in parallel and vicinity to the primary node/notochord. Only here, near to the developing midline structures, could we observe the

outgrowth of a long, bona fide notochord from early grafts (Fig. 7B).

In summary, we found *CNOT* expression was not enough to promote notogenesis *in vivo,* but was dependent on further ''maturing'' signals. Once these were received, notogenesis became independent and could proceed also *in vitro* or in non-midline positions *in vivo.*

DISCUSSION

Duplicated Homeobox Genes and Sequential Activation

Many, if not all, homeobox genes were duplicated at least once during evolution of bilateria. Thus many homeobox genes found once in *Drosophila* are present in duplicate copies in vertebrates, where they may stay together in gene clusters or may drift apart to different chromosomal locations (Kappen *et al.,* 1993; Schughart *et al.,* 1989). The best studied homeobox gene clusters are the four *Hox* gene clusters, which together harbor 38 genes in tandem arrays of 9 to 11 genes (Krumlauf, 1994). Further examples for vertebrate homeobox genes occurring in pairs are the murine *Dlx1* and *Dlx2* genes, the *Dlx5* and *Dlx6* genes, as well as the murine *Nkx5.1* and *Nkx5.2* (Bober *et al.,* 1994; Simeone *et al.,* 1994). Thus, it appears not unusual to find the two *CNOT* homeobox genes in close vicinity in the chick genome.

The availability of two initially identical copies of a gene relieves evolutionary pressure and creates the degree of sloppiness necessary in order to extend a genetic function with respect to the biochemical properties of the encoded protein and/or the regulation of gene expression. The primary structures of the predicted CNOT1 and CNOT2 proteins are quite diverged, and it remains to be seen whether this correlates with different functions. However, the maintenance of the clustered genomic organization after the gene duplication event indicates that *CNOT2* and *CNOT1* may share essential regulatory elements of transcription. For several reasons we assume that *CNOT2* represents the original gene and *CNOT1* the duplicated copy, although final proof remains to be furnished. One argument is that it appears more likely to find the original gene with its regulatory elements upstream of the copy. Second, *CNOT2* becomes activated prior to *CNOT1.* Third, the few different expression domains, e.g., in the limb bud and anterior neuroectoderm, may indicate that new regulatory elements were picked up by the duplicated version, *CNOT1.* In conclusion, we favor the idea that an ancestral *Not* gene without its promoter became duplicated during the evolution of bilateria. A single promoter exerted selectional pressure on the maintenance of the clustered chromosomal configuration. In addition, the second gene picked up further promoter elements.

CNOT2 Is Homologous to the Zebrafish Gene floating head

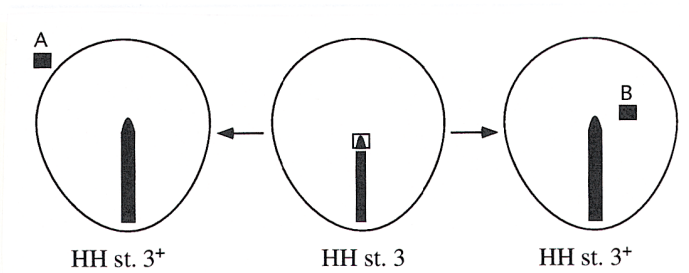
A comparison of the primary sequences from *Xenopus,* zebrafish, chick, and *Drosophila* Not homeodomains reveals that they fall into two significantly different subgroups (Figs. 1C and 1F). The CNOT2 and zebrafish flh homeodomains are 85.2% identical, and a similar value (88.5%) is found when comparing the CNOT1 homeodomain to the *Xenopus* Not alleles. Between the homeodomains of CNOT2 and CNOT1, however, only 73.8% of the amino acids are identical. Sequence similarities extend downstream from the Not homeodomains. Whereas in this region CNOT1 and Xnot share a common peptide sequence of 13 residues, the CNOT2/Flh homeodomains are extended by only seven identical amino acids, where two lysines present in the CNOT1/Xnot subgroup are substituted by glutamines (Fig. 1E). The *Drosophila* gene *90Bre* (Dessain and McGinnis, 1993), on the other hand, is equally distant to both subgroups (73.8% identity in the homeodomain to CNOT1 and CNOT2, respectively; Fig. 1F).

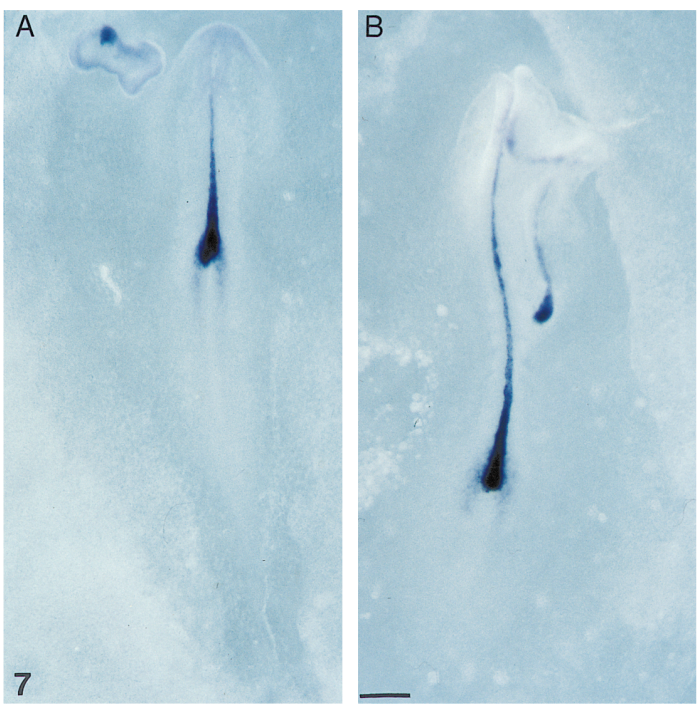
Up to now, two clustered *Not* homeobox genes have only been found in the chick genome. It seems, however, highly likely that two different *Not* genes are characteristic of all vertebrates, meaning a *CNOT1*-type gene remains to be found in fish and a *CNOT2*-type gene in amphibia. Therefore, based on sequence comparisons, the three zebrafish mutants flh^{n1}, flh^{tk241}, and flh^{tm229} appear to be mutants of the zebrafish *CNOT2* homolog and not of the *Xnot*/*CNOT1* gene (Talbot *et al.,* 1995). Extrapolating from chick to zebrafish, we would assume that deletion mutant flh^{b327}, which carries a large chromosomal deficiency in the *floating head* locus, could represent the knockout of a putative zebrafish *Not* gene cluster. The phenotype of the zebrafish *floating head* mutants supports the hypothesis that the chick homolog *CNOT2* is a key gene for notogenesis. It remains to be seen whether *CNOT1* represents a redundant gene of partially conserved function or is essential for notogenesis.

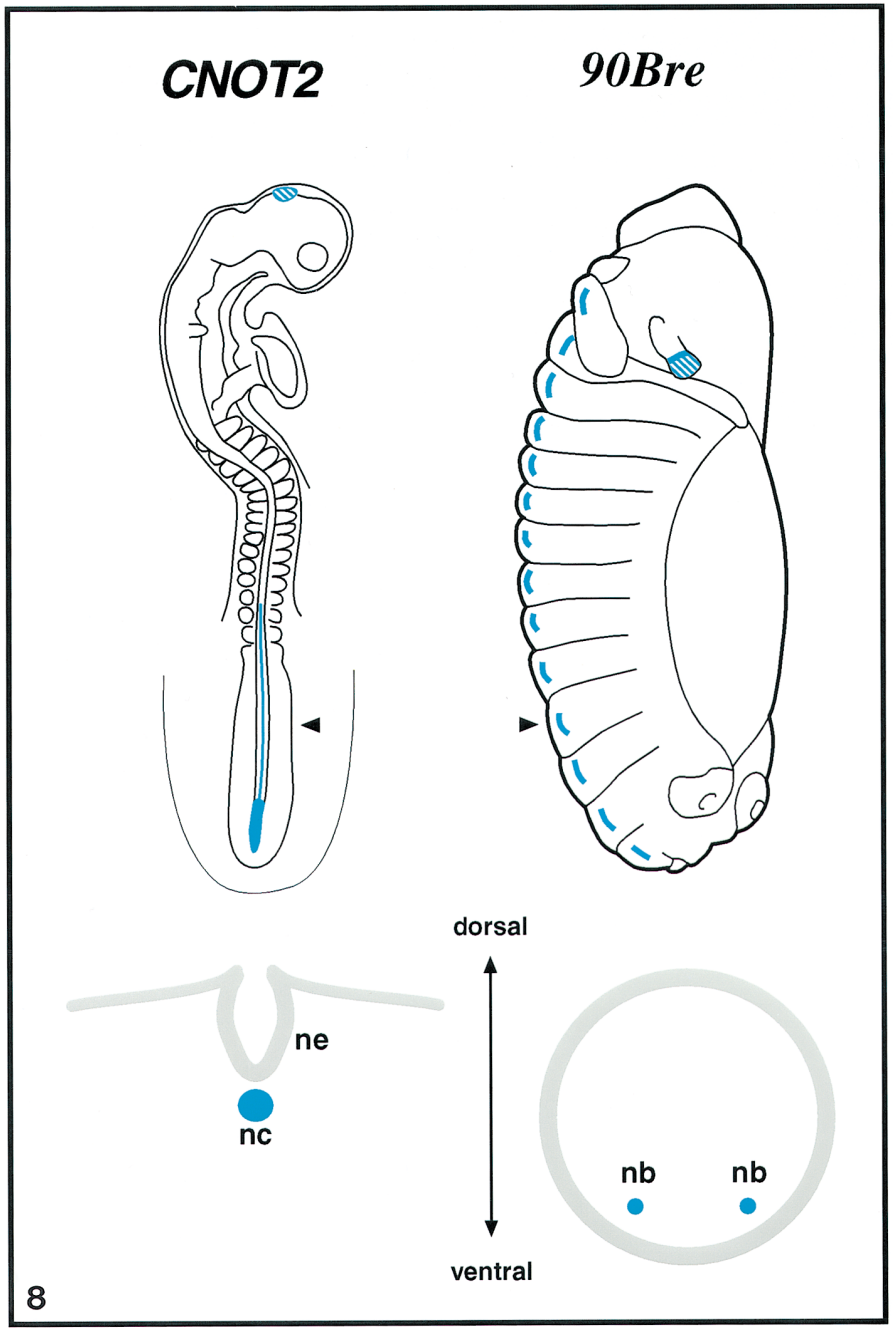
Other examples for a pair of vertebrate homeobox genes with partially redundant functions are the two murine *engrailed* genes, *En1* and *En2.* Only the inactivation of *En1,* the slightly earlier expressed gene, led to a dramatic phenotype in the mid-hindbrain anlage, which is also the major domain of *En2* expression (Wurst *et al.,* 1994). On the other hand, *En2* was able to rescue *En1* mutants (Hanks *et al.,* 1995). These remarkable findings demonstrated that the difference between *En1* and *En2* stems from their divergent expression patterns and not from the difference of their primary structures.

CNOT Expression and Notochord Identity

''Identity'' has become a popular term in modern developmental biology. It is most easily inferred from morphology in relatively far advanced embryos. Thus, being part of a rod-like structure in the central midline of a vertebrate embryo would be enough to recognize the ''notochord identity'' of a certain cell. However, rod formation represents only the endpoint of development and a notochord identity must be established much earlier. How does the acquisition of a notochordal fate occur? Cellular specification is a dynamic







process, being labile at the beginning, potentially even a reversible process. This high degree of plasticity allows cells to follow different decisions, influenced by all kinds of external and internal signals.

One such signal relevant for *CNOT* genes appears to be RA, a substance which has been shown in many other systems to have on the one hand a differentiating and on the other a caudalizing activity (e.g., Kessel, 1992, 1993; Kessel and Gruss, 1991). An influence of RA on *CNOT1* gene expression has previously been demonstrated (Knezevic *et al.*, 1995). We have found in our explant cultures that RA may play a role in the establishment of stable *CNOT1* gene expression and abolishing the slight developmental advance of *CNOT2*.

The early fate maps of pregastrulation embryos of all vertebrates indicate that the prospective notochord and neural plate cells are located in adjacent domains. The boundary between these two separates nongastrulating (neural) from gastrulating (notochordal) cells. The large *CNOT* expression domain in pregastrulation embryos becomes restricted to demarcate mainly the notochord, with only small elements left in the para- and postnodal neural plate. We will discuss below how the expression dynamic of *CNOT* genes in the neural plate and notochord may reflect a phylogenetic link between those two different identities.

Bilaterian Not Genes

The *90Bre* gene is the only member of the *Not* gene family isolated from the *Drosophila* genome (Dessain and McGinnis, 1993). A preliminary investigation by Dessain and McGinnis demonstrated *90Bre* expression in the posterior brain, i.e., the optic lobes, and in the 14 paired neuroblasts, which are derived from the ventral neurogenic ectoderm and delaminated from positions flanking the ventral furrow. Comparison of the expression patterns in fly and chicken reveals some interesting parallels (Fig. 8). These are particularly evident if the hypothesis about an inversion of the dorsoventral axis occurring during the evolution of the common, bilaterian ancestor of the gastroneuralian insects and the notoneuralian chordates is accepted, which has been discussed elsewhere in detail (Arendt and Nübler-Jung, 1994; De Robertis and Sasai, 1996).

Not genes from both fruitflies and chicks possess a small site of expression in an originally bilateral part of the brain related to visual processes, the optic lobes in flies, and the epiphysis in chicken. Both genes are expressed along the rostrocaudal axis in the developing nervous system flanking the site of gastrulation, the ventral furrow, or the primitive streak, respectively. While the neuroectoderm may represent the phylogenetically original site of *Not* gene expression, it corresponds with a primitive, ontogenetically early neuroectoderm in vertebrates. However, while in flies the neuroectodermal expression appears to be the definitive site of expression, in chicken it is only transient and the notochord becomes the major and final domain of expression. Thus, with the evolution of chordates the identity conferred by *Not* genes was transferred to a major new structure, the notochord. The *Not* cells in chordates could interact with surface ectoderm to induce neuroectoderm and to influence the generation of a dorsoventrally patterned, highly complex neural tube.

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FIG. 7. Transplantation of Hensen's node to ectopic sites. Hensen's node from HH st.3 embryos was transplanted to the area opaca margin (A) or to a site within the area pellucida (B) of HH st.3^+ hosts as shown in the schematic drawing. (A) The result of a typical operation outside of the influence of the primary embryo, resulting in an independent secondary nervous system. The graft-derived, chordoid material did not grow out to form an elongated notochord. Note that the *CNOT2* probe used for the depicted whole-mount specimen does not label the anterior neural folds. (B) After transplantation within the area pellucida the graft can end up very near to the axis of the primary embryo. In this case three rows of somites develop and the neuroectoderm of the forebrain level fuses between the primary and the secondary embryo. Note in this specimen staining of the anterior neuroectoderm with the *CNOT1* probe. The grafted node grows out to form a well-differentiated and elongated notochord.

FIG. 8. Comparison of *Not* gene expression in fruit fly and chick embryos. The data for the *D. melanogaster* gene *90Bre* are taken from Dessain and McGinnis (1993). A comparative discussion of the *Not* gene expression patterns in these two branches of bilateria is presented in the text. Note that both genes possess a small anterior domain (hatched) as well as an extended domain along the axis. Indicated are neuroblasts (nb), notochord (nc), and neuroectoderm (ne); *Not* gene expression is symbolized in blue. The lower panels show simplified cross sections at the sites indicated by arrowheads. Note the inversion of the dorsoventral axis between the insect and the chordate. Note that the differentiated Not cells in the chicken notochord are of mesodermal origin, while the *Drosophila* neuroblasts are ectodermal. For discussion see text.

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