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; Mélè, 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 (Català et al., 1995).
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-Pérez 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., 1995) 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) Nucleotide and deduced amino acid sequence of the CNOT2 Emx2 (Simeone et al., 1992). Note the grouping of CNOT1/Xnot/Xnot2 and CNOT2/Not, respectively. 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). N 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.

*FIG. 1.* (A) Genomic structure of the chicken CNOT cluster. Exons are indicated by boxes and filled boxes depict the two homeodomains. N, Ncol; X, Xhol; XbaI, 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). N 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.
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/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 HindIII. 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 Eldridge, 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 [32P]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 stage-specific artifacts from pooled specimens. By using the Micro RNA Isolation kit (Stratagene) around 4 μg of RNA could be obtained from each embryo. It was replenished with 36 μg yeast RNA and was hybridized for 14 hr at 60°C with the three probes simultaneously, using 5 × 106 cpm of each of the CNOT riboprobes, as well as 5 × 106 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 Explanations**

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 of 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.
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 bases (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; Izpisua-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 epiblast 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 major expression domain (Figs. 2E-2I and 4D) 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. 2I).

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.

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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-
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

### Table 1

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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.

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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 bilatera. 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 slowness 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<sup>n1</sup>, flh<sup>k241</sup>, and flh<sup>m229</sup> 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<sup>m277</sup>, 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...
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 separate 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.

ACKNOWLEDGMENTS

We thank T. Böttger, L. Lemaire, G. Oliver, and E. Pera for discussions and critical reading of the manuscript, R. Grainger for the HH st.10 cDNA library, P. Gruss for comments and support, and W. Behrens for her excellent technical contribution. This work was supported by the Max-Planck Gesellschaft and DFG Grants Ke 513 and SFB 271.

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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 Not1 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 arrows. 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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Received for publication July 15, 1996
Accepted August 20, 1996