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Cwnt-8C: a novel *Wnt* gene with a potential role in primitive streak formation and hindbrain organization

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SUMMARY

To begin to examine the possibility that Wnt proteins act as cell signalling molecules during chick embryogenesis, PCR was used to identify *Wnt* genes expressed in Hensen's node. We have identified a novel member of the *Wnt* gene family, *Cwnt-8C*, which is expressed prior to gastrulation in the posterior marginal zone, the primitive streak and Hensen's node. Injection of Cwnt-8C mRNA into *Xenopus* embryos caused axis duplication and dorsalization of mesodermal tissues. During neurulation, *Cwnt-8C* is expressed transiently in a restricted domain of the prospective hindbrain neurectoderm that will give rise to rhombomere 4. This domain is defined prior to the formation of rhombomere boundaries and also precedes the up-regulation and restriction of expression of Hox B1 in the same region. Thus, *Cwnt-8C* is potentially involved in the regulation of axis formation and hindbrain patterning.

Key words: *Wnt*, *Cwnt-8C*, Hox B1, gsc, chick, rat, posterior marginal zone, primitive streak, rhombomere

INTRODUCTION

Axis formation in the chick blastula is initiated by interactions between cells of the two layers of the marginal zone, the hypoblast and the epiblast (Waddington, 1933; Azar and Eyal-Giladi, 1981; reviewed in Stern, 1991). Cells in the epiblast of the posterior marginal zone specify the location at which the future primitive streak will form (Stern, 1990). Epiblast cells of the area pellucida migrate towards the posterior marginal zone and then move anteriorly forming the primitive streak (Rudnick, 1935; Pasteels, 1940; Waddington, 1956; Stern, 1990; Eyal-Giladi et al., 1992). During gastrulation, prospective endoderm and mesoderm cells in the epiblast ingress through the primitive streak and migrate laterally and rostrally (Stern and Canning, 1990). Neural differentiation of epiblast cells is induced by signals that appear to derive from cells of the anterior-most region of the primitive streak and Hensen's node, and from axial mesoderm (Dias and Schoenwolf, 1990; Storey et al., 1992). Grafting of Hensen's node next to unspecified chick ectoderm results in the development of a second, host-derived, axis (Waddington, 1933; Vakaet, 1965; Gallera, 1971; Dias and Schoenwolf, 1990 ; Storey et al., 1992), suggesting that cells of Hensen's node have functions equivalent to those of the dorsal lip of the blastopore in amphibians (Hara, 1978). In support of this, Hensen's node from avian or mammalian embryos also induces patterned neural properties in *Xenopus laevis* ectoderm (Kintner and Dodd, 1991; Blum et al., 1992). Many of the events leading to axis formation and the regionalization of mesoderm and neural ectoderm may therefore be regulated by similar signals in chick and frog.

Several peptide growth factors have been proposed to mediate the axis-inducing and patterning properties of the dorsal lip of the blastopore in frog embryos (McMahon and Moon, 1989; Thomsen et al., 1990; Smith and Harland, 1991, 1992; Sokol et al., 1991; Ku and Melton, 1993). These include members of the Wnt family of proteins that have been implicated in regulation of early development and neural differentiation in a variety of vertebrate species (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; and reviewed in Nusse and Varmus, 1992). In *Xenopus*, injection of Wnt mRNAs causes axis duplication and rescues UV-irradiated, axis-deficient embryos (McMahon and Moon, 1989; Cho et al., 1991; Sokol et al., 1991; Chakrabarti et al., 1992; Smith and Harland, 1992; Ku and Melton, 1993). Furthermore, *Wnt* gene expression has been shown to dorsalize the ventral mesoderm induced by FGF in *Xenopus* animal cap explants (Otte and Moon, 1990; Sokol and Melton, 1992; Christian et al., 1992). Together, these findings have led to the suggestion that a Wnt family member may be responsible for some of the inductive activities of the dorsal lip of the blastopore. The localization of *Xwnt-11* (Ku and Melton, 1993) suggests that it may be involved in dorsalization in frog embryos but the spatial and temporal distributions of all other identified *Wnt* genes are not consistent with roles in dorsal axis induction.

To begin to examine the possibility that *Wnt* genes might be involved in axis formation and in neural development in chick embryos, we used PCR to clone *Wnt* genes that are

expressed in Hensen's node. We show that Hensen's node expresses a novel *Wnt* gene, termed *Cwnt-8C*, which is related to but distinct from *Xwnt-8. Cwnt-8C* is expressed prior to gastrulation in the posterior marginal zone and causes axis duplication when injected into *Xenopus* embryos. During neurulation, *Cwnt-8C* is expressed transiently in a restricted domain of the prospective hindbrain neuroectoderm that will give rise to rhombomere 4. Together, these results suggest an involvement of Cwnt-8C in both axis formation and neural patterning.

MATERIALS AND METHODS

Embryos

Fertile White Leghorn chicken eggs were obtained from SPAFAS Inc (Norwich, CT) and incubated for up to three days at 38°C. Staging of embryos prior to primitive streak formation was based on the extent of hypoblast advancement towards the anterior margin of the area pellucida as described by Eyal-Giladi and Kochav (1976) [stages X-XIV]. After the appearance of the primitive streak, embryos were staged according to Hamburger and Hamilton (1951) [stages 2-45]. For early embryonic stages (X-XIII), fresh, unincubated eggs were used. Embryos were explanted into L15 medium at 4°C. For RNA preparation, Hensen's nodes (approximately 0.2×0.2 mm) were dissected from stage 4-5 embryos, immediately frozen on dry ice and stored at −80°C. Embryos for immunohistochemistry and in situ analysis were trimmed to leave only a small border of area opaca, rinsed in ice-cold L15 and then fixed for 2 hours with MEMFA (3.7% formaldehyde, 100 mM MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$) at room temperature (Harland, 1991). Fixed embryos were dehydrated and stored at −20°C in 100% methanol.

Xenopus laevis eggs and embryos were cultured under standard conditions. Embryos were staged according to Nieuwkoop and Faber (1967). RNA injections were performed essentially as described by Moon and Christian (1989).

Hensen's node cDNA library construction

Poly(A)$^+$ RNA was prepared from 750 stage 4-5 Hensen's nodes. A directional plasmid cDNA library was constructed using the SuperScript kit (Gibco/BRL) with minor modifications to permit cloning of the final double-stranded cDNA into the COS cell expression vector, pMT23 (a derivative of pMT21, provided by G. Wong; Wong et al., 1985; Wong, 1990). Approximately 2×10^7 independent clones were obtained after electroporation into the recombination-deficient bacteria, SURE (Stratagene, La Jolla CA). To facilitate PCR-based screening of the cDNA library, plasmid DNA was prepared from 10^6 clones amplified by growth in 0.3% semi-solid agarose (Sea Prep, FMC). This method was used to minimize disproportionate amplification in plasmid cDNA libraries (Kriegler, 1990). General procedures for library construction and screening were as described in Maniatis et al. (1982).

PCR amplification of *Wnt*-related genes

Degenerate primers for amplification of *Wnt*-related genes from Hensen's node were synthesized based on two stretches of amino acids that are highly conserved between known *Wnt* genes (Gavin et al., 1990; Christian et al., 1991a) (see Fig 1):

Wnt-5′: QECKCH:
5′-GGGGAATTCCA(A/G)GA(A/G)TG(C/T)AA(A/G)TG(C/T)CAT-3′
Wnt-3′: FHWCC:
5′-AAAATCTAGA(A/G)CACCA(A/G)TG(A/G)AA-3′

1 μg of plasmid DNA from the amplified library was used as a template in a PCR reaction ((1) 3 cycles: 95°C, 1 minute; 37°C, 1 minute; 72°C, 2 minutes; (2) 32 cycles: 95°C, 40 seconds; 50°C, 1 minute; 72°C, 1 minute; (3) 10 minutes at 72°C). The amplified products were run on a 3% NuSieve/1% agarose gel and bands of the expected sizes (350-500 bp) were picked with a Pasteur pipette. After reamplification using the same primers, the fragments were digested with *Eco*RI and *Xba*I and subcloned in pGEM-1. Inserts were sequenced by the dideoxy termination method using the Sequenase Kit (USB) and vector primers. Amino acid sequences were aligned with those of known *Wnt* genes using Geneworks (Intelligenetics).

Isolation of full-length Cwnt-8C cDNA clones

500,000 clones of the amplified stage 4-5 Hensen's node cDNA library were screened under high stringency with a partial length chick Cwnt-8C cDNA clone. The three longest clones isolated were approximately 2.1 kb. The 5′ 1735 bp *Eco*RI fragment of pMT23-HNWnt8C contains all of the predicted coding sequence, 5′ untranslated region and 576 bp of 3′ untranslated region. This fragment was subcloned into pGEM-1 and sequenced on both strands using primers complementary to the vector and multiple internal sites. Ambiguous regions were resolved by comparing independent cDNA clones and by substituting dITP for dGTP in sequencing reactions. The nucleotide sequence of *Cwnt-8C* has the GenBank accession number U02097.

Isolation of a Cwnt-8C-related cDNA from rat

To isolate cDNA clones for Wnt-8C from rat, PCR primers (W8-1 and W8-2) were designed based on stretches of amino acids both of which are conserved between Cwnt-8C and Xwnt-8 (Fig. 1). W8-2 is also present in Xwnt-8B, but neither sequence is conserved in other *Wnt* gene family members.

W8-1: WSVNNF: 5′ CGGAATTCTGGTCIGTIAA(T/C)AA(T/C)TT 3′
W8-2: EDSPDY: 5′ AAATCTAGATA(A/G)CTIGGIGA(A/G)TC 3′

These primers were used to amplify a 680 nucleotide *Cwnt-8C*-related sequence from rat E9 primitive streak cDNA. Antisense digoxigenin-labelled RNA probes were used in in situ hybridization analyses of E9-E11 rat embryos essentially as described below for chick.

Isolation of chicken Goosecoid cDNA

A cDNA clone encoding chicken *Goosecoid (Gsc)* was isolated by screening the stage 4 Hensen's node library with a *Xenopus* Gsc cDNA probe (provided by Dr A. Ruiz i Altaba). Sequence analysis revealed that the clone shared 97% amino acid identity with mouse *Gsc* over the homeobox region.

Isolation of chicken Hox B1 cDNA

A cDNA clone encoding amino acids 148-282 of chicken Hox B1 was isolated by PCR from the stage 4 Hensen's node library.

In situ hybridization

The in situ analysis of the expression of *Cwnt-8C*, chicken *Gsc* and chicken *Hox B1* was performed as described by Harland (1991) but with one minor addition: the sheep anti-digoxigenin antibody (BMB) was preabsorbed for 4 hours at room temperature with chick embryo acetone powder (3 mg/ml). The Cwnt-8C probe used for all examples shown in the figures spanned the entire coding region (1735 bp *Eco*RI fragment). A 200 bp probe consisting entirely of 3′ noncoding sequence gave an identical pattern of hybridization, suggesting that there was no cross-hybridization with other *Wnt* genes. Control hybridization reactions contained Cwnt-8C sense strand probes or antisense probes for other mRNAs. Probes for *Gsc* and *Hox B1* were prepared using standard procedures. The alkaline phosphatase detection reactions were usually developed to saturation by incubating for 4-6 hours at room temperature followed by 12 hours at 4°C. Following the development reaction, embryos were refixed in MEMFA for 4-8 hours, washed

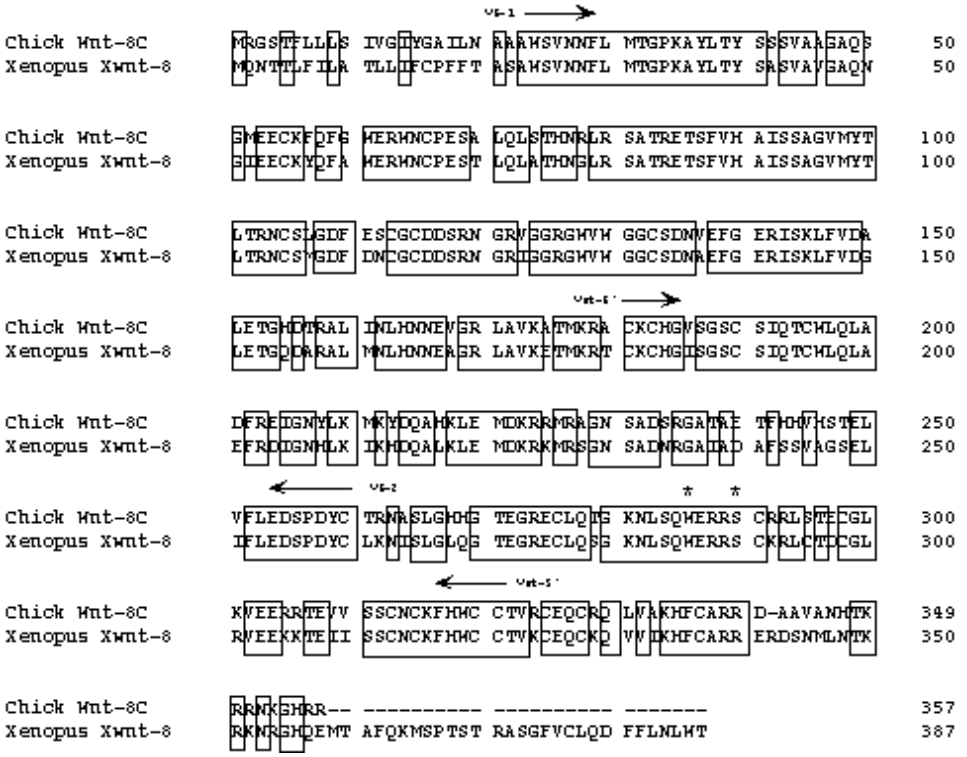

Fig. 1. Predicted amino acid sequence of CWnt-8C and comparison with Xwnt-8. Cysteine residues conserved in known *Wnt* genes are indicated in bold type. Asterisks mark the positions at which two cysteines found in other *Wnt* genes are not conserved in either CWnt-8C or Xwnt-8. Arrows above the amino acid sequence indicate the regions used for making degenerate PCR primers. Wnt-5′ and Wnt-3′ prime all known Wnt genes, while W8-1 and W8-2 are predicted to be relatively specific for genes related to *Cwnt-8C* and *Xwnt-8*.

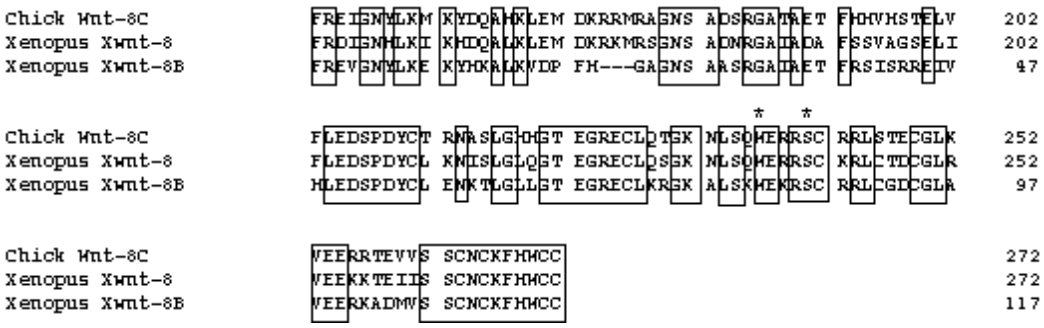

Fig. 2. Comparison of Cwnt-8C with Xwnt-8 and Xwnt-8B over the 115 amino acids of overlapping sequence available for Xwnt-8B.

with PBS and photographed on Kodak EPY-64T using a Zeiss dissecting microscope or Axiophot. For some photographs, embryos were dehydrated in methanol and mounted in Murray's Clearing Solution (benzyl benzoate: benzyl alcohol, 2:1). Embryos were then embedded in Paraplast Tissue: Prep 2 (1:1), sectioned at 8 μm, mounted in Permount and viewed with Nomarski optics.

Immunohistochemistry

Staining for Hox B1 was performed as described by Sundin and Eichele (1990) using an affinity-purified rabbit anti-Ghox 2.9 antibody (a gift of Dr G. Eichele). The secondary biotinylated goat anti-rabbit antibody (1:200, BMB) was detected using a Streptavidin-HRP conjugate (1:400, Chemicon).

In vitro transcription and microinjection of RNAs

Capped mRNA was synthesized in vitro from pMT23-HNWnt-8C using T7 polymerase as described in Krieg and Melton (1984). The Cwnt-8C clone contains a >25-base (poly)A stretch at its 3′ terminus. Control mRNAs consisted of Cwnt-8C-myc and Cwnt-8C antisense. Cwnt-8C-myc contains a human c-myc epitope [(G)EQKLISEEDL] fused to the C terminus of Cwnt-8C. The insertion of this epitope destroys the activity of Cwnt-8C in the *Xenopus* bioassay. Insertion of the myc epitope into Xwnt-1 and Xwnt-8 has been shown to attenuate the activity of these Wnts (McMahon and Moon, 1989; Christian et al., 1991b). Fertilized *Xenopus* eggs were injected with approximately 250 pg of Cwnt-8C mRNA (sense or antisense), Cwnt-8C-myc or Xwnt-1 mRNA at 2- to 32-cell stages. Rhodamine-labelled lysine-dextran (25 mg/ml, Molecular Probes) was mixed with RNAs to localize the descendants of injected blastomeres (Gimlich and Braun, 1985). Injections of control mRNAs caused no phenotype or toxicity.

RESULTS

Cloning of Cwnt-8C from stage 4-5 Hensen's node cDNA

cDNA clones encoding Wnt family members expressed in Hensen's node were isolated using degenerate PCR primers based on stretches of amino acids conserved in known *Wnt* genes. Sequences were amplified from a stage 4-5 Hensen's node cDNA library. Several of the subcloned PCR

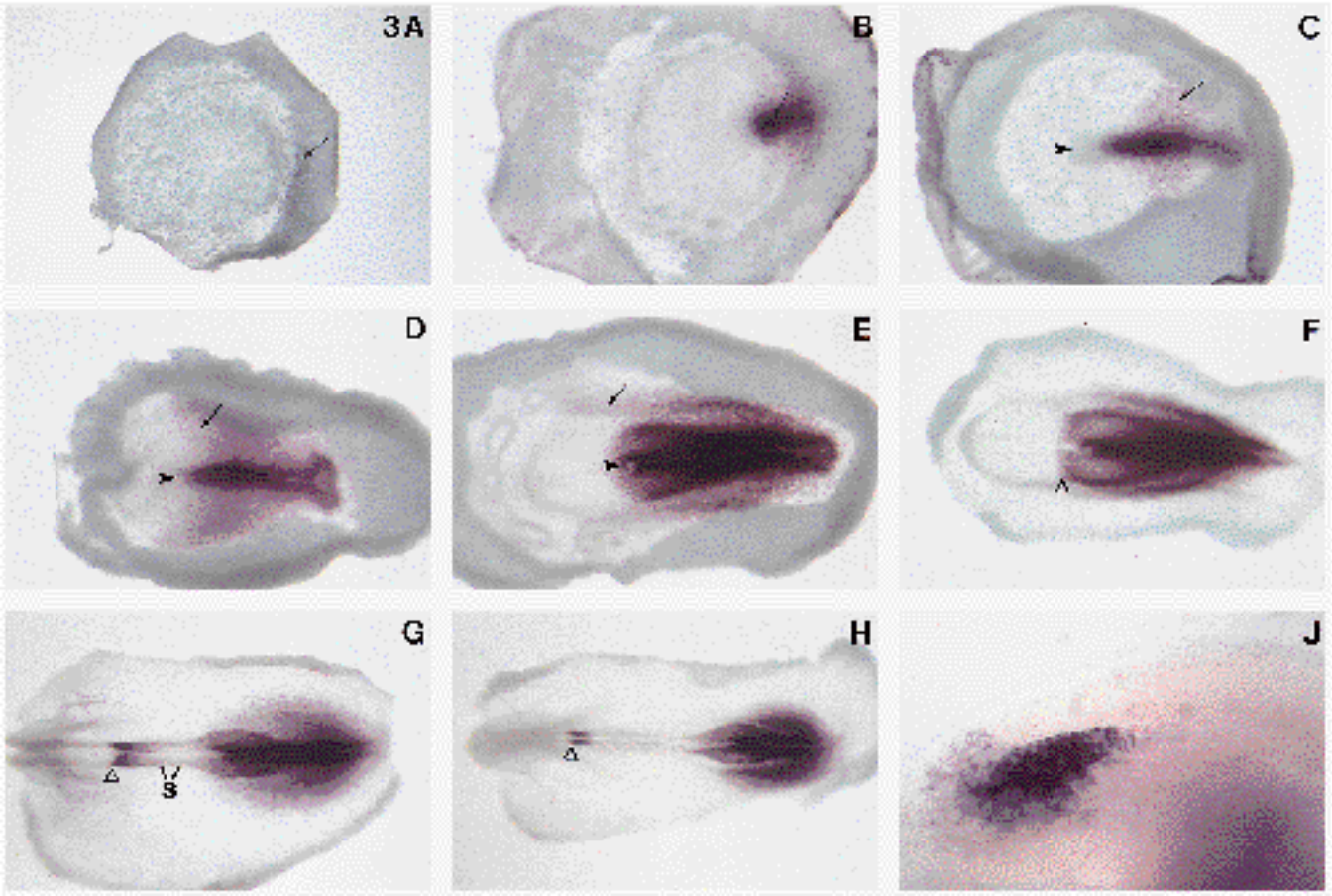

Fig. 3. Expression of *Cwnt-8C* during chick embryogenesis. All panels show whole mounted embryos after in situ hybridization using single-stranded RNA probes labelled with digoxigenin. A-H are oriented dorsal side up with the posterior pole towards the right. J is a lateral view with posterior towards the left. (A) Stage XII, 0 to 2 hours incubation. Labelled cells (arrow) are observed in the posterior marginal zone overlying Koller's sickle. (B) Stage 2, 5 hours. Labelled cells occupy both the posterior marginal zone and the emerging primitive streak (arrow). (C) Stage 3/3^{+}, 12 hours; cells in the posterior primitive streak and those migrating out express Cwnt-8C; arrow shows prospective lateral mesodermal cells migrating out of the streak. Arrowhead indicates anterior tip of streak. (D) Stage 4, 18 hours; entire primitive streak, including Hensen's node, expresses Cwnt-8C. Arrowhead indicates Hensen's node; arrow shows prospective lateral mesoderm. The region of apparent decreased labelling in the midline is an optical illusion due to viewing the embryo in whole mount through a region of folding (see Fig. 4D). (E) Stage 5, 20 hours; arrowhead indicates Hensen's node; arrow indicates lateral mesoderm. (F) Stage 6, 24 hours; open arrowhead indicates rostral boundary of *Cwnt-8C* labelling in neural plate of panels F, G and H. (G) Stage 8, 27 hours, 4 somites; s, somites. With the exception of a band in the prospective hindbrain, Cwnt-8C expression in the neural plate is only transiently expressed. (H) Stage 9, 30 hours, 7 somites. (J) Tail bud region of stage 17 embryo, 3 days. Magnification: A-H, ×25; J, ×50.

fragments encoded a protein (chick Cwnt-8C, see below) with 75% identity to *Xenopus* Xwnt-8 (Christian et al., 1991b). A cDNA corresponding to the chick homologue of *Wnt-4* was also identified.

The Cwnt-8C PCR fragment was used as a probe to isolate full-length clones from the stage 4-5 Hensen's node cDNA library. Six cDNAs were characterized, which differed only in the length of 5′ sequence. The 3′ end of each clone contained a consensus polyadenylation signal followed by a poly(A) tail. The three longest clones were each 2.1 kb and terminated at the 5′ end within ten bases of each other, suggesting that they represented nearly full-length messages. The predicted open reading frame of 357 amino acids is shown in Fig. 1. As in *Xwnt-8*, there was no in-frame termination codon preceding the predicted AUG at position 87, leaving open the possibility that translation

Fig. 5. Expression of *goosecoid* during chick embryogenesis. (A,D-F) Dorsal views of embryos subjected to whole-mount in situ hybridization using an antisense Gsc probe. Position of the AP axis in A is shown by an arrow in B-F; the posterior pole is to the right. (A) Stage XII, 0 to 2 hours of incubation. Gsc is expressed in the posterior marginal zone and extends anteriorly up to the margin of the secondary hypoblast. (B,C) Mid-sagittal sections through stage XII embryos labelled by whole mountin situ hybridization with Gsc (B) and Cwnt-8C (C) probes. Gsc expression is restricted to the hypoblast, including Koller's sickle (k) while Cwnt-8C is expressed in the epiblast overlying Koller's sickle. (D) Stage 2+, 6 hours. Gsc is expressed in the anterior end of the primitive streak (arrow). (E) Stage 4, 18 hours. Gsc is expressed primarily in Hensen's node (arrowhead) and in cells of the prechordal plate mesoderm (arrow) (F) Stage 6, 24 hours. Magnification: A,D-F, ×25; B,C, ×200. k, Koller's sickle.

initiates further upstream. In vitro translation of synthetic mRNA for *Cwnt-8C* generated a protein with the predicted relative molecular mass of 39×10^3 when unglycosylated, and a range of $40\text{-}45\times10^3$ M_r in the presence of pancreatic microsomes (data not shown). Cwnt-8C contains 21 of the 23 cysteine residues within the predicted mature protein that are conserved in other *Wnt* family members (Gavin et al., 1990; Christian et al., 1991b; Sidow, 1992). The two

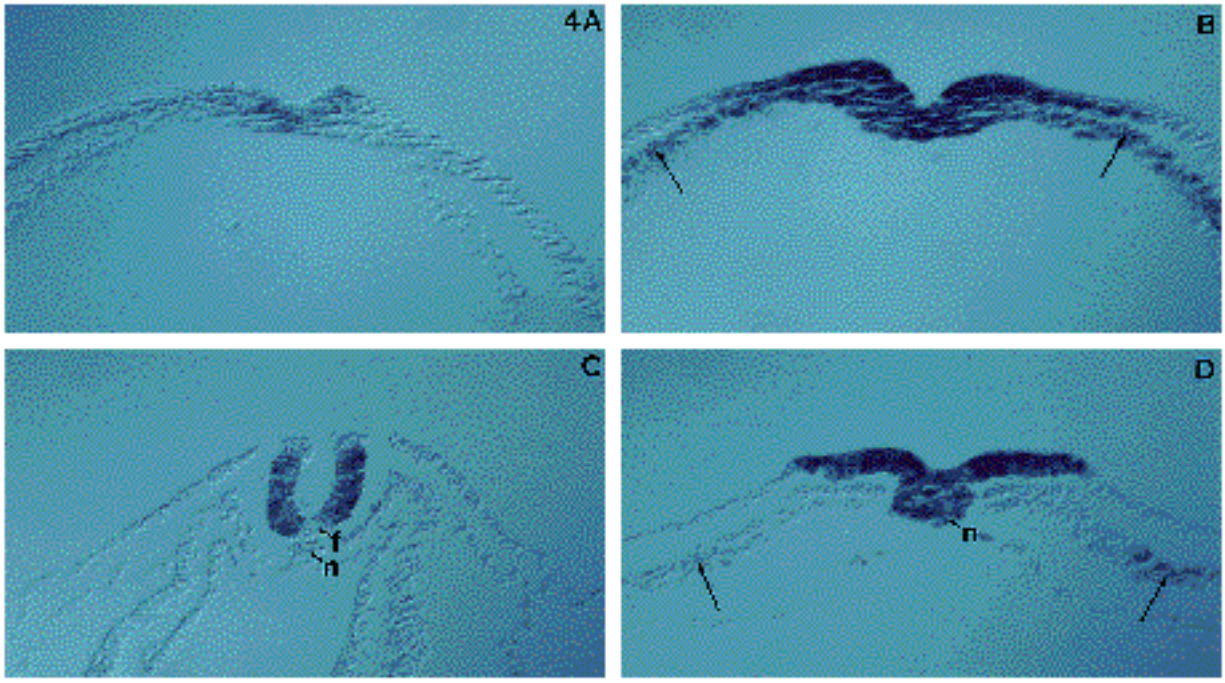

Fig. 4. Expression of *Cwnt-8C* during gastrulation and neurulation. Sections through chick embryos labelled by whole mount in situ hybridization. (A,B) Transverse sections through stage 4 embryo (Fig. 3A) at the level of Hensen's node (A) and midway between Hensen's node and the posterior pole (B). Arrows indicate labelled prospective lateral mesoderm. (C,D) Transverse sections through stage 9+ embryo (Fig. 3H) at the level of prospective rhombomere 4 in the hindbrain (C) and immediately anterior to Hensen's node (D). Paraffin sections were cut at 8 μm. Magnification: ×200. f, floor plate; n, notochord.

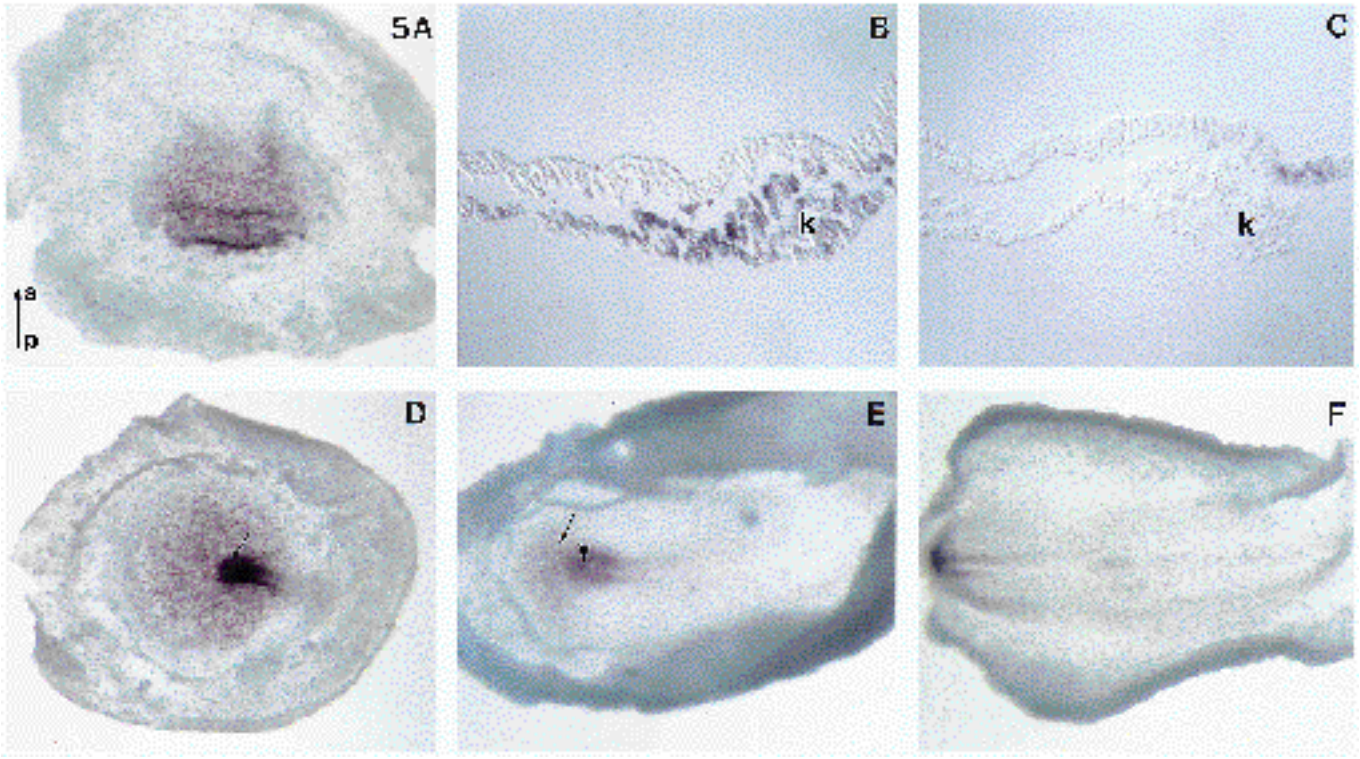

Table 1. Results of injecting Cwnt-8C mRNA into 4- to 8-cell *Xenopus* embryos

	Percentage of injected embryos (*n*)				
	Hyperdorsalized	Anterior duplication	Normal	Dead	Total number
Cwnt-8C+	41(65)	53(83)	5(8)	1(2)	158 (10 expts)
Cwnt-8C− no RNA	0	0	99(84)	1(1)	85 (10 expts)
Xwnt-1		82(42)	18(9)	0	51 (2 expts)

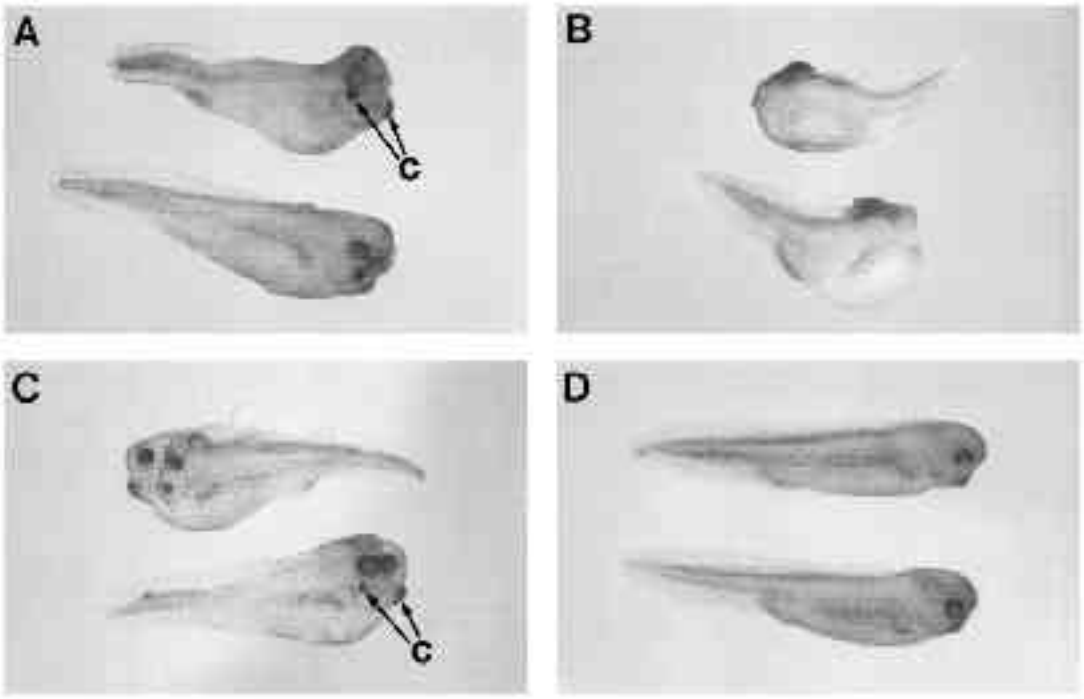

Fig. 6. *Xenopus* embryos injected with Cwnt-8C RNA. Embryos were injected with 250 pg of either Cwnt-8C sense mRNA (A-C) or control, inactive, Cwnt-8C-myc (D) RNA. Cwnt-8C sense mRNA was injected at the 8-cell stage into ventral (A) or dorsal (B) animal blastomeres or at the 32-cell stage into a ventral marginal blastomere (tier c) (C). Embryos shown represent the most common phenotypes observed at stage 38. Injections of sense mRNA into ventral blastomeres resulted in double axes including duplication of cement glands (c in A and C) and eyes. Injection into a dorsal blastomere caused partial duplication without extra cement glands or eyes (B).

cysteine residues that are absent in Cwnt-8C (indicated by asterisks at positions 286 and 290 in Figs 1, 2) are also absent in Xwnt-8 (Christian et al., 1991b) and Xwnt-8B (Wolda and Moon, 1992).

Within the family of known *Wnt* genes, *Cwnt-8C* appears to be most similar to *Xwnt-8*. At the amino acid level, Cwnt-8C exhibits 76% identity to the Xwnt-8 protein, with the most extensive divergence in sequence at the putative signal peptide region and at the carboxyl terminus (Fig. 1). As shown in Fig. 2, Cwnt-8C is 63% identical to Xwnt-8B, compared over the 115 amino acids of available sequence (Wolda and Moon, 1992). Over this same region Cwnt-8C is 73% identical to Xwnt-8. Although sequences for other chick *Wnt* genes have not been reported, the level of identity between orthologous *Xenopus* and mouse *Wnt* genes ranges from 69 to 90% (Noordermeer et al., 1989). Wnt-8 and Wnt-8B have been identified thus far only in *Xenopus* and because distinct *Wnt* gene family members within a species have been shown to have conservation as high as 90% (Gavin et al., 1990; Roelink and Nusse, 1991; Sidow, 1992), the degree of identity between chick *Cwnt-8C* and *Xwnt-8* does not indicate whether these genes are orthologues or members of a closely related subfamily of *Wnt* genes. As described below, the distribution of *Cwnt-8C* differs strikingly from that of *Xwnt-8* and the developmental expression patterns of *Cwnt-8C* and *Xwnt-8B* also differ, suggesting that the three genes are distinct. We therefore refer to this chick *Wnt-8*-related gene as *Cwnt-8C*, following the terminology for other paralogous *Wnt* genes (Sidow, 1992)

The expression of *Cwnt-8C* during embryogenesis

The distribution and timing of *Cwnt-8C* expression during the blastula, gastrula and neurula stages of chick embryonic development was examined using whole-mount in situ hybridization with subsequent histological analysis of sections.

(1) Pre-streak, blastula stages (X-XIV) and axis formation

Cwnt-8C was first detected in the chick blastula, at stage X. At this stage it was expressed in a small number of cells scattered in a ring of peripheral epiblast in the area opaca. In most stage X embryos, *Cwnt-8C* was also expressed in the epiblast of the posterior marginal zone. At stages XI-XII, expression of *Cwnt-8C* became restricted to the patch of epiblast cells within the posterior marginal zone overlying Koller's sickle (Figs 3A, 5C). Isolated cells expressing *Cwnt-8C* were occasionally observed in the secondary hypoblast underlying the region of labelled epiblast (not shown). Fate-mapping studies suggest that cells in the epiblast converge medially towards the posterior end of the embryo from lateral regions of the marginal zone during gastrulation (Rudnick, 1935; Pasteels, 1940; Waddington, 1956; Stern, 1990). The consolidation of *Cwnt-8C* expression in the posterior marginal zone could result from the movement of cells within the epiblast or from the down-regulation of *Cwnt-8C* expression in other parts of the marginal zone.

As the primitive streak began to form (stages XIII-XIV), *Cwnt-8C*$^+$ cells remained within the epiblast (not shown) of both the primitive streak and the posterior marginal zone. Both the number of *Cwnt-8C*$^+$ cells in the posterior marginal zone and the apparent levels of expression in each cell increased (Fig. 3B).

(2) Gastrulation and neural induction (stages 2-4)

During early primitive streak formation (stages 2-3+), *Cwnt-8C* was expressed in the posterior three quarters of the primitive streak excluding the anterior tip (Fig. 3C). Cells expressing *Cwnt-8C* were found in the superficial and middle layers of the primitive streak (not shown). The deep hypoblast layer of the streak did not express detectable *Cwnt-8C*. Mesodermal cells expressing *Cwnt-8C* were first detected at stage 3$^+$, as they were beginning to emigrate laterally from the middle layer of the primitive streak (arrow in Fig. 3C).

At stage 4, *Cwnt-8C* was expressed throughout the length of the streak, including Hensen's node (Figs 3D, 4A). Within the streak, *Cwnt-8C* was found in cells in the superficial and middle layers (Fig. 4B) but expression in Hensen's node itself was restricted to superficial cells (Fig. 4A). Cells in this region contribute to the notochord and floor plate (Rosenquist, 1966; Selleck and Stern, 1991; Schoenwolf et al., 1989; Schoenwolf and Sheard, 1990). Presumptive mesodermal cells lateral to the primitive streak at stage 4 also expressed *Cwnt-8C* (arrows in Figs 3D and 4B).

Cwnt-8C and *Gsc* are expressed in different layers of the posterior marginal zone

Several genes have been described that are expressed in the dorsal lip of the blastopore in *Xenopus* (eg. *Gsc, Xlim-1, XFKH-1/pintallavis, noggin* and *Xnot*) (Cho et al., 1991; Dirksen and Jamrich, 1992; Ruiz i Altaba and Jessell, 1992; Smith and Harland, 1992; Taira et al., 1992; von Dassow et al., 1993). On the basis of this expression pattern, they have been postulated to be involved in the early specification of mesoderm and to mediate the functions of the dorsal lip of the blastopore (the organizer). Indeed, Gsc and noggin have been shown to mimic some of the actions of the organizer (Cho et al., 1991; Smith and Harland, 1992). To examine whether *Gsc* and *Cwnt-8C* are expressed by the same population of cells during primitive streak formation, we isolated a chick *Gsc* cDNA (see Methods) and compared directly the expression of the two genes in chick.

In contrast to *Cwnt-8C*, *Gsc* was not detected in the epiblast of the blastula (compare Fig. 5B and E). *Gsc* expression in stage X-XII chick embryos was restricted to the hypoblast. It was first observed posteriorly in Koller's sickle (not shown) and in older embryos extended anteriorly up to the margin of the secondary hypoblast (Fig. 5A,B). Although double-labelling experiments were not performed, comparison of stage-matched embryos revealed that the cells expressing *Cwnt-8C* in the blastoderm were localized to the region of epiblast immediately dorsal to the posterior margin of *Gsc* expression in the hypoblast (Fig. 5B,C).

During primitive streak extension and gastrulation, *Gsc* was expressed in the hypoblast and became concentrated in the anterior end of the primitive streak, in deep and middle cells: a pattern that was complementary to the distribution of *Cwnt-8C* (Fig. 5D,E and not shown). At stage 4$^-$, expression in the hypoblast had decreased and *Gsc*$^+$ cells were found primarily in the rostral and deep portions of Hensen's node. Cells in this location contribute to endoderm and prechordal plate mesoderm (Selleck and Stern, 1991). At stages 4-6, *Gsc* was expressed in cells of the prechordal plate mesoderm, anterior to Hensen's node (Fig. 5E,F), suggesting that these cells arose in Hensen's node. Thus, *Cwnt-8C* and *Gsc* are expressed in distinct populations of cells in the chick blastula and during gastrulation. From stages 10-17, some *Gsc* expression persisted in the ventral diencephalon and in pituitary (not shown).

Cwnt-8C has dorsalizing, axis duplicating activity in *Xenopus*

The localization of *Cwnt-8C* in the posterior marginal zone and Hensen's node, suggests that it may be involved in the regulation of axis formation. In the absence of a convenient assay to test the activity of poorly diffusible growth factors, such as Wnts, in chick embryos, a well-characterized assay for dorsal axis induction in *Xenopus* embryos was used. This assay has previously been used to show that mRNAs for several members of the *Wnt* gene family (*Wnt-1, Wnt-3A, Xwnt-8, Xwnt-11, wg*), for *noggin* and for *Gsc* are able to induce the formation of a second axis following injection into *Xenopus* embryos (McMahon and Moon, 1989; Cho et al., 1991; Sokol et al., 1991; Chakrabarti et al., 1992; Smith and Harland, 1992; Ku and Melton, 1993; Wolda et al., 1993), while other *Wnts* (eg *Xwnt-5A*) have different effects in this assay (Wolda et al., 1993).

In vitro transcribed Cwnt-8C mRNA was injected into one cell of 4- to 8-cell *Xenopus* embryos. 94% of the embryos injected with 250 pg of synthetic Cwnt-8C mRNA had either a partial axial duplication (duplicated anterior notochord and neural plate) or a hyperdorsalized phenotype (double heads with duplicated cement glands and eyes) (Table 1). In a second set of experiments the effect of varying the site of injection was examined. Cwnt-8C mRNA was injected into a dorsal animal blastomere, a ventral

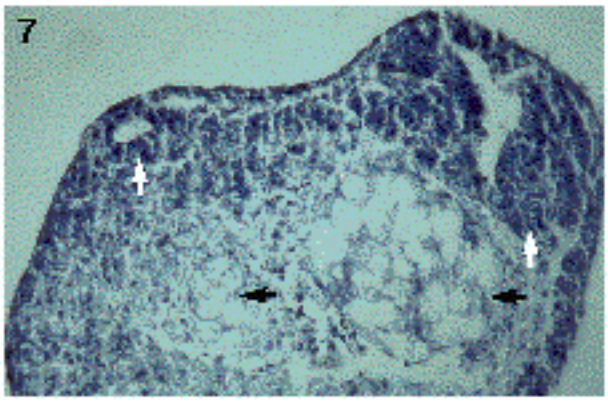

Fig. 7. Injection of Cwnt-8C mRNA causes duplication of the notochord. 8 μm paraffin section through a stage 28 *Xenopus* embryo injected with Cwnt-8C mRNA into a ventral animal blastomere at the 8-cell stage. The section passes through the region of the rostral spinal cord/hindbrain and shows a duplicated notochord (black horizontal arrows) and neural tube (white vertical arrows). Stained with Giemsa. Magnification, ×200.

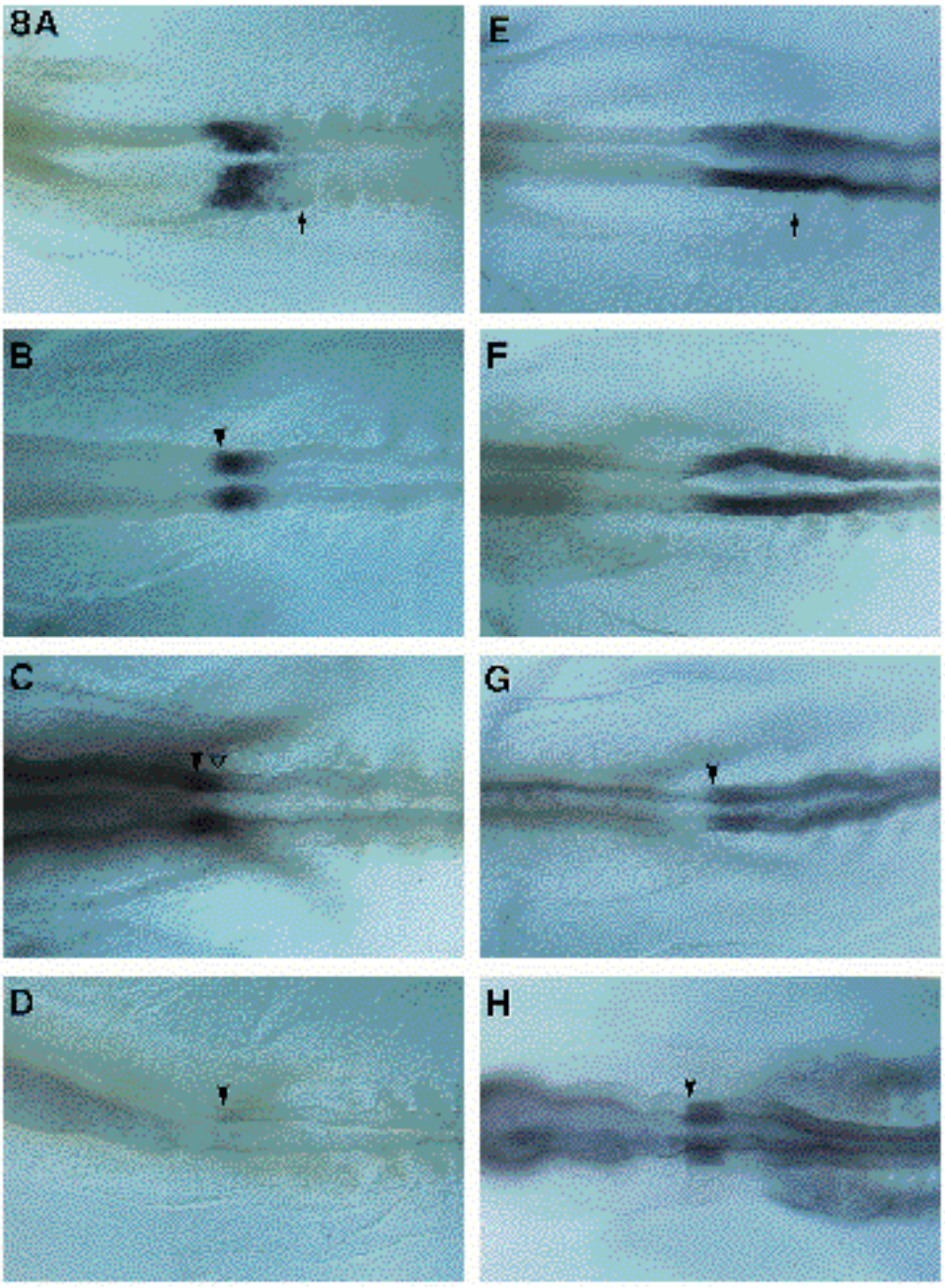

Fig. 8. Expression of *Cwnt-8C* and Hox B1 in the developing hindbrain. Embryos were analysed by whole-mount in situ hybridization for expression of *Cwnt-8C* (A-D) and Hox B1 (E-H). Hindbrain and rostral spinal cord are shown in each panel. (A,E) Stage 8, 4 somites. The restriction of *Cwnt-8C* mRNA to a band in the hindbrain is already apparent. Hox B1 mRNA remains homogeneously expressed in hindbrain and spinal cord. Arrows in A and E indicate the position of the middle of the first somite. (B,F). (C,G) Stage 10^{-}, 9 somites. *Cwnt-8C* expression can be seen to extend throughout rhombomere 4 as the posterior boundary of rhombomere 4 becomes detectable (open arrowhead in C). Hox B1 expression appears upregulated in rhombomere 4 but remains high in posterior hindbrain and spinal cord. In order to reveal the extent of *Cwnt-8C* expression in rhombomere 4 at this stage, the embryo shown in C was incubated for an extended period, resulting in greatly increased background labelling in anterior regions. (D) Stage 10, 10 somites. *Cwnt-8C* expression has declined to almost undetectable levels. (H) Stage 10^{+}, 11 somites. Up-regulation of expression of Hox B1 in rhombomere 4 and down-regulation posteriorly results in an isolated band of expression in rhombomere 4. Black arrowheads in B-H indicate the rostral boundary of rhombomere 4. Magnification, ×100.

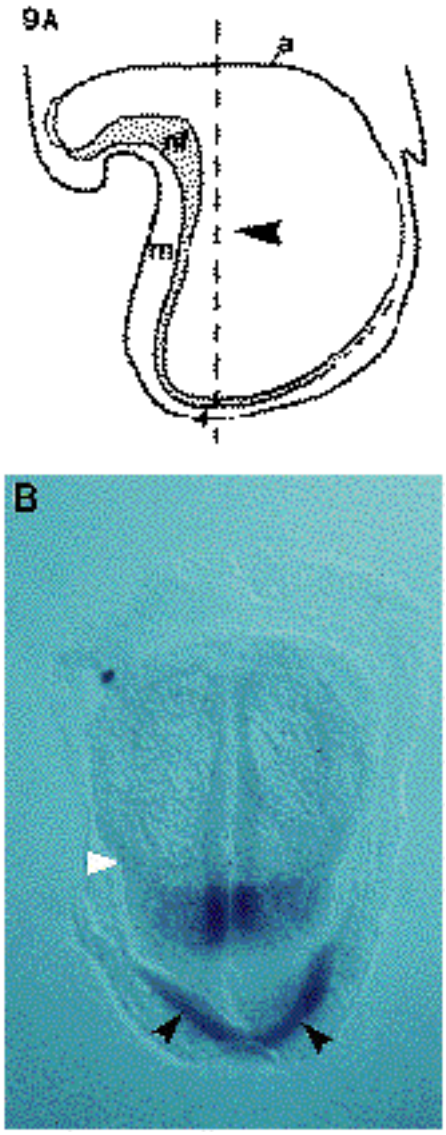

Fig. 9. Expression of a *Cwnt-8C*-related gene, *Rwnt-8C*, in the rat hindbrain. E9 rat embryos were analyzed by whole-mount in situ hybridization using the Rwnt-8C probe. (A) Schematic sagittal section of E9 rat embryo. Because of the shape of the egg cylinder, the embryo was cut as shown by the dotted line and the posterior part removed to permit visualization of the dorsal surface of the neural folds (nf) and the posterior neural plate in cross section (small black arrowhead). Large black arrowhead indicates point of view in B; a, amnion. (B) Labelled E9 rat embryo viewed as indicated in A. *Rwnt-8C* is expressed in a stripe posterior to the preotic sulcus (position indicated by white arrowhead). *Rwnt-8C* is also expressed in the posterior neural plate (black arrowheads) shown here in transverse section. This developmental stage in rat is approximately equivalent to stage 7 in chick and would fall between those depicted in panels F and G of Fig. 3.

animal blastomere or a ventral vegetal blastomere at the eight-cell stage. As noted by others (Sokol et al., 1991; Chakrabarti et al., 1992), the extent of axis duplication was dependent on the site of mRNA injection. Injection of Cwnt-8C mRNA into a ventral animal blastomere induced an anterior bifurcation of the axis and duplication of the cement gland [70% of embryos (26/37)] (Figs 6A,7). Several of these embryos also developed additional eyes. Injections into ventral vegetal blastomeres produced the same phenotype [74% of embryos (20/27)] (Fig. 6C). Dorsal animal injections resulted in partial duplications [74% of embryos (20/27)] (Fig. 6B) including a grossly enlarged or duplicated anterior notochord and neural tube. The majority of these embryos did not develop eyes. Injection of Cwnt-8C mRNA into ventral marginal blastomeres of 32-cell embryos also induced a bifurcation of the axis with duplicated eyes and cement glands (not shown). These phenotypes were indistinguishable from those of embryos injected in parallel with Xwnt-1 mRNA. The results of injections into ventral blastomeres were similar to those described for embryos injected with other Wnt gene mRNAs (Sokol et al., 1991; Chakrabarti et al., 1992) and the reduced phenotype observed with dorsal injections of Cwnt-8C also resembles that previously observed with *Drosophila wg* (Chakrabati et al., 1992). Control embryos, injected with either antisense Cwnt-8C mRNA or Cwnt-8C-myc mRNA, at the 8- or 32-cell stages were indistinguishable from embryos that were not injected with mRNA [100% (38/38)] (Fig. 6D).

Thus, *Cwnt-8C* has a pattern of expression in the chick blastula and a biological activity that together are consistent with an involvement in the regulation of axis formation.

(3) Neurulation and neural patterning (stages 5-10)

Beginning at late stage 5 and continuing thereafter, the expression of *Cwnt-8C* within the epiblast was no longer restricted to the primitive streak and presumptive mesoderm but was also expressed in the neural plate posterior to the middle of the prospective hindbrain.

Expression in rostral neural plate

The rostral boundary of *Cwnt-8C* expression in the lateral neural plate (Fig. 3E,F) coincided with the position that Hensen's node occupied at stage 4^+, the predicted approximate position of the rostral boundary of the prospective hindbrain (Spratt, 1952). As the node regressed, the anterior boundary of expression in neural plate remained constant within the prospective hindbrain (open arrowheads in Fig. 3F-H). By stage 6, *Cwnt-8C* was absent from the midline of the developing hindbrain neural plate and from the underlying notochord (Figs 3F [stage 6] and 4C [stage 9]). The mesoderm immediately lateral to the anterior primitive streak did not express *Cwnt-8C*. However, wing-like regions of *Cwnt-8C*$^+$ prospective lateral plate mesoderm extended from the caudal region of the streak both laterally and rostrally, anterior to the level of the node (arrows in Figs 3E and 4D).

Throughout neurulation, Hensen's node and the neural plate immediately anterior to it expressed *Cwnt-8C*. However, with the exception of the neural plate formed prior to stage 7, the newly formed neural plate expressed *Cwnt-8C* transiently. This resulted in the generation of an isolated transverse band of maintained *Cwnt-8C* expression across the neuroectoderm in a region of the prospective hindbrain (Fig. 3G,H; see also Fig. 8A-D). This anterior band of *Cwnt-8C* expression was maintained until approximately stage 10 when it rapidly declined to undetectable levels (see Fig. 8D).

Expression in the caudal neural plate

The pattern of *Cwnt-8C* expression in the newly formed neural plate and notochord of caudal regions of the embryo differed from that in the hindbrain. The caudal neural plate (the future spinal cord) immediately anterior to the node expressed *Cwnt-8C* across its entire breadth, including the midline, and *Cwnt-8C* was expressed transiently in condensing notochord (Fig. 4D). This observation suggests that the development of neural plate and notochord may be slower in spinal cord regions than in hindbrain and is consistent with other studies that have analysed the role of notochord in floor plate induction (Placzek et al., 1993). As Hensen's node regressed and became incorporated into the tail bud, *Cwnt-8C* expression was also detected in the notochord and in the region of the neural tube formed by secondary neurulation (Fig. 3J).

Hindbrain expression of *Cwnt-8C* and rhombomere formation

The restricted expression of *Cwnt-8C* within the presumptive hindbrain preceded the formation of morphological rhombomere boundaries by several hours. This raises the possibility that *Cwnt-8C* expression plays a role in rhombomere specification. Although *Cwnt-8C* expression declined rapidly between stages 9 and 10, the time at which inter-rhombomere boundaries become discernible, several stage 9-10 chicks had sufficiently distinct rhombomere boundaries and levels of *Cwnt-8C* expression that it was possible to determine which rhombomere(s) contained cells that descended from the *Cwnt-8C*-expressing domain. The anterior limit of *Cwnt-8C* expression was sharp and coincided with the rhombomere 3/4 boundary (Fig. 8B-D). The posterior limit was more diffuse but was approximately at the rhombomere 4/5 boundary (Fig. 8C). These results suggest that *Cwnt-8C* is expressed early in most cells that will form rhombomere 4.

Cwnt-8C expression immediately precedes localization of Hox B1 to rhombomere 4 in the hindbrain

The distribution of *Cwnt-8C* within the neural plate and hindbrain is similar to that described previously for Hox B1 (Sundin and Eichele, 1990; Sundin et al., 1990; Frohman et al., 1990; Murphy and Hill, 1991). To begin to determine the relationship of *Cwnt-8C* to other early indicators of hindbrain regional identity, we therefore examined whether Hox B1 and *Cwnt-8C* are expressed in the same population of cells. Whole-mount in situ hybridization was performed on matched-stage embryos for localization of expression of the two genes.

The onset and early distribution of Hox B1 expression in the neural plate was similar to that of *Cwnt-8C*. At stage 5, the rostral-most boundary of expression for both genes was already established as a transverse line immediately anterior to Hensen's node (see Fig. 3E for *Cwnt-8C*; Sundin and Eichele, 1992, Fig. 2 for Hox B1). As the node regressed, both *Cwnt-8C* and Hox B1 were expressed in all cells of the nascent neural plate, excluding the cells of the midline in the prospective hindbrain. In spinal cord regions, both genes were expressed in the midline as well as the lateral neural plate.

Although the early expression of the two genes appeared similar, the timing of restriction of *Cwnt-8C* and Hox B1 to rhombomere 4 differed. The posterior boundary of *Cwnt-8C* expression in the hindbrain was first evident at stage 7, approximately 6 hours before the formation of the rhombomere 3/4 boundary at stage 9 and 10 hours before the appearance of the 4/5 boundary at stage 10^{-}. Thus, *Cwnt-8C* was down-regulated in the neural tube caudal to prospective rhombomere 4 by stage 7 (Fig. 3G). In contrast, Hox B1 was expressed uniformly from spinal cord levels up to the rhombomere 3/4 boundary until stage 10^{-} (Fig. 8E,F). As *Cwnt-8C* expression declined in rhombomere 4, Hox B1 expression was up-regulated (Fig. 8G). The coincident down-regulation of Hox B1 expression in rhombomeres 5 and 6 (Fig. 8H) led to an isolated domain of strong Hox B1 expression in rhombomere 4 by stage $11^{+}/12$. As described in mouse (Frohman et al., 1990), the time course of expression of Hox B1 protein in the chick (not shown) was similar to that observed for the mRNA: both become restricted only after the formation of rhombomere boundaries 3/4 and 4/5 (Frohman et al., 1990). Thus, the restriction of *Cwnt-8C* expression to prospective rhombomere 4 appears to precede both the formation of rhombomere boundaries and the consolidation of Hox B1 expression in the same region.

Wnt-8C expression in other vertebrates

To test the possibility that *Wnt-8C* is expressed with a similar distribution in other vertebrates, a partial cDNA clone of Wnt-8C (*Rwnt-8C*) was isolated from rat E9 primitive streak DNA (see Methods). The predicted amino acid sequence of this fragment (220 amino acids) shares 76% identity with Cwnt-8C and 68% identity with Xwnt-8 (not shown). Comparison with the 52 aa of overlapping sequence available for Xwnt-8B (Wolda and Moon, 1992) revealed 44% identity with Xwnt-8B.

The expression of *Rwnt-8C*, examined by whole-mount in situ hybridization, was found to be similar to that observed for *Cwnt-8C* in chick embryos over a range of comparable stages. Fig. 9 shows an E9 rat embryo. A transverse band of expression is located at the predicted site of the future rhombomere 4, just posterior to the preotic sulcus (Sakai, 1987). Further posterior, expression is found in the nascent neural plate (black arrowheads in Fig. 9) and primitive streak (not shown). By E10, expression had declined to non-detectable levels. The conservation of the expression pattern of *Wnt-8C* in chick and rat suggests that the gene may have a conserved function in vertebrate development.

DISCUSSION

We have identified a novel member of the *Wnt* gene family, *Cwnt-8C*, which is expressed in a spatially and temporally restricted pattern during chick embryogenesis. Prior to gastrulation, *Cwnt-8C* is found in the posterior marginal zone. Later, the domain of *Cwnt-8C* expression expands to include the superficial and middle layers of the primitive streak and Hensen's node. These cells give rise to neural plate and mesodermal tissues such as notochord, somites and lateral plate. The pattern of expression of *Cwnt-8C* during gastru-

lation is different from that of *Gsc*, suggesting that cells expressing these two genes have different roles in the early embryo.

During neurulation, *Cwnt-8C* is expressed transiently in the neural plate extending from the middle of the prospective hindbrain caudally. As Hensen's node regresses, *Cwnt-8C* expression is progressively lost from more anterior regions of the neural plate. Expression is maintained, however, in a band in neurectoderm that will eventually become rhombomere 4 of the hindbrain. Unlike the homeobox gene, Hox B1, the expression of which also becomes restricted to rhombomere 4, the restriction of *Cwnt-8C* expression to prospective rhombomere 4 precedes the formation of morphological boundaries within the hindbrain. Below, we discuss the possibility that *Cwnt-8C* is involved in the regulation of two processes in early embryogenesis, axis formation and hindbrain patterning.

Cwnt-8C is a novel *Wnt* gene

As described above, the degree of identity between *Cwnt-8C* and *Xwnt-8* does not determine whether they are different genes. If *Xwnt-8* and *Cwnt-8C* were orthologous, it would be expected that the proteins would be expressed with the same tissue distribution and perform similar roles during development. Indeed, *Wnt-1*, *Wnt-3A* and *Wnt-4* each have conserved distributions in several species (Wilkinson et al., 1987; Krauss et al., 1992; McGrew et al., 1992; Wolda et al., 1993; C. R. H. and J. D., unpublished observations).

Several studies have analysed the expression of *Xwnt-8* during *Xenopus* embryogenesis (Christian et al., 1991a,b; Smith and Harland, 1991). *Xwnt-8* expression is first detectable in the late blastula (stages 9-10) in a band of cells around the marginal zone, excluding the most dorsal region, the organizer. During gastrulation and neurulation, expression becomes restricted to posterior ventral mesoderm and endoderm. In contrast to *Cwnt-8C*, *Xwnt-8* is never detected in dorsal mesodermal structures such as notochord and somites or their progenitors. Thus, as noted previously (Christian et al., 1991b; Smith and Harland, 1991; Sokol et al., 1991; Sokol and Melton, 1992), *Xwnt-8* is unlikely to be involved in vivo in axis formation or dorsal mesoderm induction because it is not expressed in the dorsal lip of the blastopore. Furthermore, although a few cells expressing *Xwnt-8* were noted in the forebrain (Smith and Harland, 1991), no cells were observed in the developing hindbrain or neural plate. These differences suggest that *Cwnt-8C* and *Xwnt-8* are distinct genes. Similarly, the temporal expression pattern of *Xwnt-8B* (Wolda and Moon, 1992) appears to be distinct from that of *Cwnt-8C*. Finally, the finding that a rat gene that resembles chick *Cwnt-8C* is also expressed in primitive streak, neural plate and hindbrain suggests, moreover, that *Cwnt-8C* is expressed with a similar distribution in several species.

Does *Cwnt-8C* have a role in primitive streak formation?

The pattern of expression of *Cwnt-8C* in the chick blastula and its biological activity in *Xenopus* suggest that it could regulate some aspects of primitive streak formation. *Cwnt-8C* is expressed by a population of epiblast cells in the posterior marginal zone. Epiblast cells in this region are thought to control the site of primitive streak formation by recruiting cells from more lateral regions of the epiblast (Stern, 1990). In common with a subset of other *Wnt* genes, in vitro transcribed Cwnt-8C RNA is able to induce an embryonic axis when injected into early *Xenopus* embryos. *Cwnt-8C* therefore potentially has the biological activity necessary for primitive streak formation in chick. *Cwnt-8C* first appears in the posterior marginal zone epiblast at stage X but is restricted to and concentrated in this region only at stages XI-XII. Thus the time course of expression of *Cwnt-8C* in the posterior marginal zone does not appear to coincide precisely with the peak of axis-forming ability (stage X) of the endogenous posterior marginal zone (Khaner and Eyal-Giladi 1986, 1989; Eyal-Giladi and Khaner, 1989; Eyal-Giladi et al., 1992). It remains possible that other factors mediate the subsequent decrease in activity of the posterior marginal zone.

It is possible that *Cwnt-8C* is responsible for other, later properties of the posterior marginal zone, such as the modulation of the fate of cells that coalesce in the primitive streak. This is suggested by observations of *Wnt* gene action in *Xenopus*. When animal caps are treated with FGF, they form predominantly ventral types of mesoderm and some muscle, but only rarely form dorsal mesoderm such as notochord or neural tissue. Animal caps from embryos injected at early stages with Wnt RNA form only small amounts of ventral mesodermal tissue. In contrast, when the two treatments are combined, muscle, notochord and neural tissues are formed (Otte and Moon, 1990; Sokol and Melton, 1990; Christian et al., 1992). This supports a model in which Wnt proteins either modify the response of the ectoderm to a mesoderm-inducing signal or alter the type of mesoderm after its induction. Thus, endogenous *Cwnt-8C* would modulate the spectrum of mesodermal tissues that derives from the streak and Hensen's node by its interaction with mesoderm-inducing factors. FGFs have, in fact, been shown to be expressed in the primitive streak of mouse by in situ hybridization (Wilkinson et al., 1988; Niswander and Martin, 1992).

Other studies have addressed the potential role of growth factors in axis formation in chick. In in vitro cultures of isolated chick epiblast, activin induces the formation of axial mesoderm (Mitrani and Shimoni, 1990; Mitrani et al., 1990; Cooke and Wong, 1991). Activin beta-B mRNA has been shown to be expressed in the chick blastula (Mitrani et al., 1990) and in Hensen's node (unpublished observations), although its spatial and temporal localization in the prestreak embryo is not known. In contrast to Wnt RNAs, however, activin RNA is not able to rescue fully the dorsal axis of UV-irradiated *Xenopus* embryos or to induce a complete secondary axis in microinjected normal embryos (Sokol and Melton, 1992).

The differential expression of *Cwnt-8C* and *Gsc* throughout gastrulation suggests that the two genes play different roles in the early embryo. *Gsc* has been suggested to contribute to the formation of the organizer in *Xenopus* and in mouse to mimic some of the actions of the organizer. The expression of *Gsc* in anterior and ventral Hensen's node supports this idea. Fate mapping has not resolved whether some cells of the hypoblast enter the primitive streak and hence contribute to Hensen's node. It is therefore not clear

whether *Gsc*$^+$ cells in Hensen's node originate in the hypoblast or whether *Gsc* is expressed in a novel cell population. The roles of *Gsc* in the hypoblast and in Hensen's node may thus be distinct.

Does *Cwnt-8C* have a role in the hindbrain in rhombomere 4 specification?

In chick, the first evidence of hindbrain segmentation is discernible around stage 9 (Vaage, 1969). Single cell marking experiments have shown that rhombomeres represent regions within which lineally related cells are restricted (Fraser et al., 1990). Rhombomeres are eventually characterized by a distinct set of antigenic and molecular markers and morphological features such as motor nuclei, axonal projections, associated neural crest and cranial nerves (Lumsden and Keynes, 1989; Lumsden et al., 1991; Hunt et al., 1991). Transplantation studies in which late properties of rhombomeres were examined suggest that rhombomere identity is determined coincident with the generation of morphological boundaries (Guthrie et al., 1992; Kuratani and Eichele, 1993). Similar experiments, examining the propensity of prospective rhombomeres to form boundaries, indicate that cells within alternating segments first acquire information about their segmental identity before boundary formation (Guthrie and Lumsden, 1991).

Cwnt-8C is selectively expressed in a prospective hindbrain segment before the generation of morphological boundaries. In chick, by stage 7 (1 somite) *Cwnt-8C* is restricted to cells that appear to give rise to rhombomere 4. *Krox-20*, a zinc finger gene (Wilkinson et al., 1989), and *Sek-1*, a gene encoding a tyrosine kinase receptor (Nieto et al., 1992), are also expressed before the formation of rhombomere boundaries. They are both first expressed at stage 8$^-$ (3 somites) in a single domain in the region of the hindbrain that will form rhombomere 3 (Nieto et al., 1991,1992). By stage 9 (7 somites), *Krox-20* and *Sek-1* are also expressed in prospective rhombomere 5 (Nieto et al., 1991,1992). Thus, *Cwnt-8C*, *Krox-20* and *Sek-1* are expressed in a reciprocal fashion in the regions of the hindbrain that will give rise to rhombomeres 3 to 5. Prior to morphological boundary formation, at least some hindbrain cells have the capacity to contribute to more than one rhombomere (Fraser et al., 1990). Since each of these three genes is expressed in most cells of a restricted domain before the appearence of boundaries, it is possible that expression is regulated by region-specific factors other than these genes or that the three genes themselves interact to maintain their reciprocal distribution. The possibility remains, however, that only a few cells close to the prospective rhombomere boundaries can or do move and that these cells do not express *Cwnt-8C*, *Krox-20* or *Sek-1*.

A striking feature of hindbrain organization is the pattern of Hox gene expression with relation to rhombomere boundaries. The anterior boundaries of individual Hox genes correspond to given rhombomere boundaries and expression extends posteriorly throughout the hindbrain. The anterior limit of early Hox B1 expression coincides with the prospective rhombomere 3/4 boundary, but, unlike the distribution of other Hox genes, after morphological boundaries have formed the expression of Hox B1 in the hindbrain becomes restricted to rhombomere 4. The correlation between the domain of *Cwnt-8C* and Hox B1 up-regulation and restriction in rhombomere 4 raises the possibility that *Cwnt-8C* regulates the expression of Hox B1 and the identity of rhombomere 4.

Members of the *Wnt* gene family have previously been shown to play a role in cellular differentiation in early embryos. *Wnt-1* is required for midbrain development in the mouse (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas et al., 1991). The mechanisms by which *Wnt-1* acts in the midbrain (McMahon et al., 1992) may resemble those described for another member of the *Wnt*-gene family in *Drosophila* embryos, *Dwnt-1*. The extracellular wg signal is required for maintenance of en expression in adjacent cells and hence for their correct fate (DiNardo et al., 1988; Martinez-Arias, 1988). This model may also apply to the potential role of *Cwnt-8C* in the hindbrain. The presence of *Cwnt-8C* may be required for the up-regulation and maintenance of expression of Hox B1 in rhombomere 4. Conversely, the early loss of *Cwnt-8C* from more posterior cells in the hindbrain may contribute to the failure of maintained Hox B1 expression in these segments.

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The sequence of *Cwnt-8C* has been submitted to Gen Bank.

REFERENCES

Azar, Y. and Eyal-Giladi, H. (1981). Interaction of epiblast and hypoblast in the formation of the primitive streak and teh embryonic axis in the chick as revealed by hypoblast rotation experiments. *J. Embryol. Exp. Morph.* **61,** 133-144

Blum, M., Gaunt, S. J., Cho, K. W. Y., Steinbeisser, H., Blumber, B., Bittner, D. and De Robertis, E. (1992). Gastrulation in the mouse: the role of the homeobox gene *goosecoid*. *Cell* **69**, 1097-1106.

Chakrabarti, A., Matthews, G., Colman, A. and Dale, L. (1992). Secretory and inductive properties of *Drosophila* wingless protein in *Xenopus* oocytes and embryos. *Development* **115**, 355-369.

Cho, K.W.Y., Blumberg, B., Steinbeisser, H. and De Robertis, E. M. (1991). Molecular nature of Spemann's organizer: the role of the *Xenopus* homeobox gene *goosecoid*.*Cell* **67**, 1111-1120.

Christian, J. L, Gavin, B. J., McMahon, A. P. and Moon, R. T. (1991a). Isolation of cDNAs partially encoding four *Xenopus wnt-1/int-1* related proteins and characterization of their transient expression during embryonic development. *Dev. Biol.* **143**, 230-234.

Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991b). *Xwnt-8*, a *Xenopus Wnt-1/int-1* related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111,** 1044-1055.

Christian, J. L., Olsen, D. J. and Moon, R. T. (1992). *Xwnt-8* modifies the character of mesoderm induced by bFGF in isolated *Xenopus* ectoderm. *EMBO J.* **11**, 33-41.

Cooke, J. and Wong, A. (1991). Growth factor related proteins that are inducers in early amphibian development may mediate similar steps in amniotes (birds). *Development* **111**, 197-212.

von Dassow, G., Schmidt, J. E. and Kimelman, D. (1993). Induction of the *Xenopus* organizer: expression and regulation of *Xnot*, a novel FGF and activin-regulated homeobox gene. *Genes Dev.* **7,** 355-366

Dias, M. and Schoenwolf, G. C. (1990). Formation of ectopic

neuroepithelium in chick blastoderms:age-related capacities for induction and self-differentiation following transplant of quail Hensen's nodes. *Anat. Embryol.* **229**, 437-448.

DiNardo, S. Sher, E. Heemskerk-Jongens, J, Kassis, J. A. and O'Farrell, P. H. (1988). Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* emrbyogenesis. *Nature* **332**, 604-609.

Dirksen, M. L. and Jamrich, M. (1992). A novel activin-inducible, blastopore lip-specific gene of *Xenopuslaevis* contains a forkhead DNA-binding domain. *Genes Dev.* **6**, 599-608.

Eyal-Giladi, H. and Kochav, S. (1976). From cleavage to primitive streak formation: a complemental normal table and a new look at the first stages of the development of the chick. *Dev. Biol.* **49**, 321-337.

Eyal-Giladi, H. and Khaner, O. (1989). The chick's marginal zone and primitive streak formation. *Dev. Biol.* **134**, 215-221.

Eyal-Giladi, H., Debby, A. and Harel, N. (1992). The posterior section of the chick's area pellucida and its involvement in hypoblast and primitive streak formation. *Development* **116**, 819-830.

Fraser, S., Keynes, R. and Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344,** 431-435.

Frohman, M. A., Boyle, M. and Martin, G. R. (1990). Isolation of the mouse Hox-2.9 gene; analysis of embryonic expression suggests that positional information along the anterior-posterior axis is specified by mesoderm. *Development* **110,** 589-607.

Gallera, J. (1971). Primary induction in birds. *Adv. Morphog.* **9**, 149-180.

Gavin, B. J., McMahon, J. A and McMahon, A. P. (1990). Expression of multiple novel *Wnt-1/int-1*-related genes during fetal and adult mouse development. *Genes Dev.* **4**, 2319-2332.

Gimlich, R. L. and Braun, J. (1985). Improved fluorescent compounds for tracing cell lineage. *Dev. Biol.* **109,** 509-514

Guthrie, S. and Lumsden, A. (1991). Formation and regeneration of rhombomere boundaries in the developing chick hindbrain. *Development* **112**, 221-229.

Guthrie, S., Muchamore, I., Kuroiwa, A., Marshall, H., Krumlauf, R. and Lumsden, A. (1992). Neuroectodermal autonomy of *Hox-2.9* expression revealed by rhombomere transpositions. *Nature* **356**, 157-

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.

Hara, K. (1978). Spemann's organizer in birds. In *Organizer - a Milestone of a Half-Century from Spemann.* (ed. O. Nakamura and S. Toivonen). pp. 221-265. Amsterdam: Elsevier, North Holland.

Harland, R. (1991). In situ hybridization: an improved whole-mount method for *Xenopus* embryos. *Methods in Cell Biol.* **36**, 675-685.

Hunt, P., Whiting, J., Nonchev, S., Sham, M., Marshall, H., Graham, A., Cook, M., Allemann, R., Rigby, P.W.J., Gulisano, M., Faiella, A., Boncinelli, E. and Krumlauf, R. (1991). The branchial Hox code and its implications for gene regulation, patterning of the nervous system and head evolution. *Development* **1991 Supplement**, 63-7

Khaner, O. and Eyal-Giladi, H. (1986). The embryo-forming potency of the posterior marginal zone in stages X through XII of the chick. *Dev. Biol.* **115**, 275-281.

Khaner, O. and Eyal-Giladi, H. (1989). The chick's marginal zone and primitive streak formation. *Dev. Biol.* **134**, 206-214.

Kintner, C. R. and Dodd, J. (1991). Hensen's node induces neural tissue in *Xenopus* ectoderm. Implications for the action of the organizer in neural induction. *Development* **113**, 1495-1505.

Krauss, S., Korzh, V., Fjose, A. and Johansen, T. (1992). Expression of four zebrafish *Wnt*-related genes during embryogenesis. *Development* **116**, 249-259.

Krieg, P.A. and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucl. Acids Res.* **12**, 7057-7070.

Kriegler, M. (1990). *Gene Transfer and Expression. A Laboratory Manual.* NY: Stockton Press.

Ku, M. and Melton, D. A. (1993). *Xwnt-11*: a novel maternally expressed *XenopusWnt* gene. *Development* (in press).

Kuratani, S. C. and Eichele, G. (1993). Rhombomere transplantation repatterns the segmental organization of cranial nerves and reveals cell-autonomous expression of a homeodomain protein. *Development* **117**, 105-117.

Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-428.

Lumsden, A., Sprawson, N. and Graham, A. (1991). Segmental origin and migration of neural crest cells in the hindbrain region of the chick embryo. *Development* **113**, 1281-1291.

Maniatis, T., Fritsch, E. F, and Sambrook, J. (1982). *Molecular Cloning. A Laboratory Manual.* NY: Cold Spring Harbor Laboratory Press.

Martinez-Arias, A., Baker, N. E. and Ingham, P. W. (1988). Role of segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. *Development* **103,** 157-170.

McGrew, L. L., Otte, A. P. and Moon, R. T. (1992). Analysis of *Xnt-4* in embryos of *Xenopuslaevis*: a *Wnt* family member expressed in brain and floor plate. *Development* **115**, 463-473.

McMahon, A. P. and Bradley, A. (1990). The *Wnt-1 (int-1)* proto-oncogene is required for development of a large region of the mouse brain. *Cell* **62**, 1073-1085.

McMahon, A. P., Joyner, A. L., Bradley, A. and McMahon, J. A. (1992). The midbrain-hindbrain phenotype of *Wnt-1*$^{-}$*/Wnt-1*$^{-}$ mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. *Cell* **69**, 581-595.

McMahon, A. P. and Moon, R. T. (1989). Ectopic expression of the proto-oncogene *int-1* in *Xenopus* embryos leads to duplication of the embryonic axis. *Cell* **58**, 1075-1084.

Mitrani, E. and Shimoni, Y (1990). Induction by soluble factors of organized axial structures in chick epiblast. *Science* **247**, 1092-1094.

Mitrani, E., Zif, T., Thomsen, G., Shimoni, Y., Melton, D. A. and Bril, A. (1990). Activin can induce the formation of axial structures and is expressed in the hypoblast of the chick. *Cell* **63**, 495-501.

Moon, R. T. and Christian, J. L. (1989). Microinjection and expression of synthetic mRNAs in *Xenopus* embryos. *Technique* **1**, 76-89.

Morriss-Kay, G. M., Murphy, P., Hill, R. E. and Davidson, D. R. (1991). Effects of retinoic acid excess on expression of *Hox 2.9* and *Krox 20* and on morphological segmentation in the hindbrain of mouse embryos. *EMBO J* **10**, 2985-2995.

Murphy, P. and Hill, R. E. (1991). Expression of the mouse *labial*-like homeobox containing genes, *Hox 2.9* and *Hox 1.6*, during segmentation of the hindbrain. *Development* **111**, 61-74.

Nieto, M. A., Bradley, L. C. and Wilkinson, D. G. (1991). Conserved segmental expression of *Krox-20* in the vertebrate hindbrain and its relationship to lineage restriction. *Development* **1991 Supplement**, 59-62.

Nieto, M. A., Gilandi-Hebenstreit, P., Charnay, P. amd Wilkinson, D. G. (1992). A receptor protein tyrosine kinase implicated in the segmental patterning of hindbrain and mesoderm. *Development* **116**, 1137-1150.

Nieuwkoop, P. D. and Faber, J. (1967). Normal Table of *Xenopuslaevis* (Daudin). Amsterdam: North Holland Publishing Co..

Niswander, L. and Martin, G. (1992). FGF-4 during gastrulation, myogenesis, limb and tooth development in mouse. *Development* **114**, 755-768.

Nordermeer, J., Meijlink, F., Verrijzer, P., Rijsewijk, F. and Destree, O. (1989). Isolation of the *Xenopus* homolog of *int-1/wingless* and expression during neurula stages of early development. *Nucl. Acids Res.* **17**, 11-18.

Nusse, R. and Varmus, H. E. (1992). *Wnt* Genes. *Cell* **69**, 1073-1087.

Otte, A. P. and Moon, R. T. (1992). Ectopic induction of dorsal mesoderm by overexpression of *Xwnt-8* elevates the neural competence of *Xenopus* ectoderm. *Dev. Biol.* **152**, 184-187.

Pasteels, J. (1940). Un apercu comparatif de la gastrulation chez les chordes. *Biol. Rev.* **15**, 59-106.

Placzek, M., Jessell, T. M. and Dodd, J. (1993). Induction of floor plate differentiation by contact-dependent homeogenetic signals. *Development* **117** 205-218.

Roelink, H. and Nusse, R. (1991). Expression of two members of the Wnt family during mouse development-restricted temporal and spatial patterns in the developing neural tube. *Genes Dev.* **5**, 381-388.

Rosenquist, G. C. (1966). A radioautographic study of labeled grafts in the chick blastoderm. *Contrib. Embryol. Carnegie Inst. Wash.* **38**, 71-110.

Rudnick, D. (1935). Regional restriction of potencies in the chick during embryogenesis. *J. Exp. Zool.* **71**, 83-99.

Ruiz i Altaba, A. and Jessell, T. M. (1992). Pintallavis, a gene expressed in the organizer and midline cells of frog embryos: involvement in the development of the neural axis. *Development* **116**, 81-93.

Sakai, Y. (1987). Neurulation in the Mouse 1. The ontogenesis of neural segments and the determination of topographical regions in a central nervous system. *Anat. Rec.* **218**, 450-457.

Schoenwolf, G. C., Bortier, H. and Vakaet, L. (1989). Fate mapping the avian neural plate with quail/chick chimaeras: origin of prospective median wedge cells. *J. Exp. Zool.* **249**, 271-278.

Schoenwolf, G. C. and Sheard, P. (1990). Fate mapping the avian epiblast with focal injections of a fluorescent histochemical marker: ectodermal derivatives. *J. Exp. Zool.* **255**, 323-339.

Selleck, M. A. J. and Stern, C. D. (1991). Fate mapping and cell lineage analysis of Hensen's node in the chick embryo. *Development* **112**, 615-626.

Sidow, A. (1992). Diversification of the Wnt gene family on the ancestral lineage of vertebrates. *Proc. Natl. Acad. Sci.USA* **89**, 5098-5102.

Smith, W. C. and Harland, R. M. (1991). Injected *Xwnt-8* RNA acts early in *Xenopus* embryos to promote formation of a vegetal dorsalizing center. *Cell* **67**, 753-765.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of *noggin*, a new dorsalizing factor localized to the Spemann organizer in *Xenopus* embryos. *Cell* **70**, 829-840.

Sokol, S., Christian, J. L., Moon, R. T. and Melton, D. A. (1991). Injected *Wnt* RNA induces a complete body axis in *Xenopus* embryos. *Cell* **67**, 741-752.

Sokol, S. and Melton, D. A. (1992). Interaction of *Wnt* and Activin in dorsal mesoderm induction in *Xenopus*. *Dev. Biol.* **154**, 348-355.

Spratt, N. T. (1952). Localization of the prospective neural plate in the early chick blastoderm. *J. Exp. Zool.* **120**, 109-130.

Stern, C. D. (1990). The marginal zone and its contribution to the hypoblast and primitive streak of the chick embryo. *Development* **109**, 667-682.

Stern, C. D. (1991). Mesoderm formation in the chick embryo, revisited. In, *Gastrulation, Movements, Patterns and Molecules.* (ed. R. Keller, W. H. Clark and F. Griffin). pp 29-41. New York: Plenum Press.

Stern, C. D. and Canning, D. R. (1990). Origin of cells giving rise to mesoderm and endoderm in chick embryo. *Nature* **343**, 273-275.

Storey, K. G., Crossley, J. M., De Robertis, E. M., Norris, W. E. and Stern, C. D. (1992). Neural induction and regionalization in the chick embryo. *Development* **114**, 729-741.

Sundin, O. H., Busse, H. G., Rogers, M. B., Gudas, L. J. and Eichele, G. (1990). Region-specific expression in early chick and mouse embryos of *Ghox-lab* and *Hox 1.6*, vertebrate homeobox-containing genes related to *Drosophilalabial*. *Development* **108**, 47-58.

Sundin, O. H. and Eichele, G. (1990). A homeo domain protein reveals the metameric nature of the developing chick hindbrain. *Genes Dev.* **4**, 1267-1276.

Sundin, O. H. and Eichele, G. (1992). An early marker of axial pattern in the chick embryo and its respecification by retinoic acid. *Development* **114**, 841-852.

Taira, M., Jamrich, M., Good, P. J. and Dawid, I. B. (1992). The LIM domain-containing homeobox gene *Xlim-1* is expressed specifically in the organizer region of *Xenopus* gastrula embryos. *Genes Dev.* **6**. 356-366.

Thomas, K. R. and Capecchi, M. R. (1990). Targeted disruption of the murines *int-1* proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346**, 847-850.

Thomas, K. R., Musci, T. S., Neumann, P. E. and Capecchi, M. R. (1991). Swaying is a mutant allele of the proto-oncogene *Wnt-1*. *Cell* **67**, 969-976.

Thomsen, G., Woolf, T., Whitman, M., Sokol, S., Vaughan, J., Vale, W. and Melton, D. A. (1990). Activins are expressed early in *Xenopus* emrbyogenesis and can induce axial mesoderm and anterior structures. *Cell* **63**, 485-493.

Vaage, S. (1969). The segmentation of the primitive neural tube in chick embryos (Gallus domesticus). *Ergebnisse Anatomie and Embryologie, Adv. Anat. Embryol. Cell Biol.* **41**, 1-88.

Vakaet, L. (1965). Resultats de la greffe de noeud Hensen d'age diffeent sur le blastoderme de poulet. *C. R. Seanc. Soc. Biol.* **159**, 232-233.

Waddington, C. H. (1933). Induction by the endoderm in birds. *Wilhelm Roux Arch. EntwMech. Org.* **128**, 502-521.

Waddington, C. H. (1956). *Principles of Embryology*. London: Allen and Unwin.

Wilkinson, D. G., Bailes, J. A. and McMahon, A. (1987). Expression of the proto-oncogene *int-1* is restricted to specific neural cells in the developing mouse embryo. *Cell* **50**, 79-88.

Wilkinson, D. G., Bhatt, S., Charrier, P., Bravo, R. and Channay, P. (1989). Segment specific expression of a zinc finger gene in the developing nervous system of the mouse. *Nature* **337**, 461-465.

Wilkinson, D. G., Peters, G., Dickson, C. and McMahon, A. P. (1988). Expression of the FGF-related proto-oncogene int-2 during gastrulation and neurulation in the mouse. *EMBO J.* **71**, 691-695.

Wolda, S. L., Moody C. J. and Moon, R. T. (1993). Overlapping expression of *Xwnt-3A* and *Xwnt-1* in neural tissue of *Xenopus laevis*. *Dev. Biol.* **155**, 46-57.

Wolda, S. L. and Moon, R. T. (1992). Cloning and developmental expression in *Xenopus laevis* of seven additional members of the *Wnt* family. *Oncogene* **7**, 1941-1947.

Wong, G. (1990). Isolation and identification of cDNA genes by their heterologous expression and function. In *Genetic Engineering* **12** (ed. J. K. Skelton). pp. 297-316. New York: Plenum Press.

Wong, G., Witek, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenberg, D. P., Jones, S. S., Brown, E. L., Kay. R.M., Orr, E. C. et al., (1985). Human GM:CSF: molecular cloning of the cDNA and purification of the natural and recombinant proteins. *Science* **228**, 810-815.

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