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Misexpression of *Cwnt8C* in the mouse induces an ectopic embryonic axis and causes a truncation of the anterior neuroectoderm

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SUMMARY

Transgenic embryos expressing *Cwnt8C* under the control of the human *β-actin* promoter exhibit duplicated axes or a severely dorsalised phenotype. Although the transgene was introduced into fertilised eggs all duplications occurred within a single amnion and, therefore, arose from the production of more than one primitive streak at the time of gastrulation. Morphological examination and the expression of diagnostic markers in transgenic embryos suggested that ectopic *Cwnt8C* expression produced only incomplete axis duplication: axes were always fused anteriorly, there was a reduction in tissue rostral to the anterior limit of the notochord, and no duplicated expression domain of the forebrain marker *Hesx1* was observed. Anterior truncations were evident in dorsalised transgenic embryos containing a single axis. These results are discussed in the light of the effects of ectopic *Xwnt8* in *Xenopus* embryos, where its early expression leads to complete axis duplication but expression after the mid-blastula transition causes anterior truncation. It is proposed that while ectopic *Cwnt8C* in the mouse embryo can duplicate the primitive streak and node this only produces incomplete axis duplication because specification of the anterior aspect of the axis, as opposed to maintenance of anterior character, is established by interaction with anterior primitive endoderm rather than primitive streak derivatives.

Key words: mouse, axis formation, wnt, forebrain, *Cwnt8C*

INTRODUCTION

Since the initial transplantation experiments by Mangold and Spemann (Spemann and Mangold, 1924), amphibians, and especially *Xenopus*, have served as model systems for the analysis and manipulation of axis formation in the vertebrate embryo. The embryonic axis emerges during gastrulation and its elaboration depends on the activity of the dorsal blastopore lip, or organiser. While the organiser initiates gastrulation movements and itself gives rise predominantly to axial mesoderm, classified as the most dorsal type of mesoderm in the embryo, it also has the ability to convert ventral mesoderm to a more dorsal character and to turn prospective ectoderm into neural tissue. Organisers have subsequently been identified in all vertebrate embryos, being located at the anterior aspect of the primitive streak in avian and mammalian embryos (Beddington, 1994; Waddington, 1932, 1933). Cells constituting the organisers of different vertebrates share similar fates, exhibit similar gene expression patterns and, by definition, when heterotopically grafted all vertebrate organisers can induce a secondary axis (reviewed in Beddington and Smith, 1993; Conlon and Beddington, 1995).

The mechanisms responsible for establishing the dorsal lip organiser in *Xenopus* have been studied intensively (reviewed in Kessler and Melton, 1994; Sive, 1993), and it is now clear that the future position of dorsal blastopore lip is dictated by events associated with cortical rotation in the fertilised egg, which redistributes and probably causes activation of maternal 'determinants'. As a result, a signalling centre, the Nieuwkoop centre, is established on the future dorsal side of the embryo opposite the original site of sperm entry. The activity of the Nieuwkoop centre, seen as its ability to induce dorsal mesoderm, is apparent about the time that zygotic transcription commences (the mid-blastula transition) and thus precedes gastrulation (Moon et al., 1993; Wylie et al., 1996). The dorsal mesoderm induced by the Nieuwkoop centre constitutes a second signalling centre: the Spemann organiser of the gastrulating embryo. A number of secreted protein factors, such as certain Wnt family members (Du et al., 1995; Hume and Dodd, 1993; McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991), noggin (Smith and Harland, 1992), activin (Thomsen et al., 1990) and Vg1 (Thomsen and Melton, 1993) are capable of inducing a second axis in *Xenopus* embryos. While activin and Vg1 can induce mesoderm in animal cap assays (Smith et al., 1989; Sokol and Melton, 1991; Thomsen and Melton, 1993) Wnts and noggin cannot (Christian et al., 1992; Lamb et al., 1993; Smith and Harland, 1992). Wnts have therefore been implicated in establishing the Nieuwkoop centre

while the members of the TGF-β family and possibly noggin may serve to induce the organiser. The strongest evidence that members of the Wnt1 class of proteins act to establish the Nieuwkoop centre, rather than serving as the Nieuwkoop centre signal, comes from the non-cell autonomous axis induction effects seen when intracellular downstream components of the Wnt receptor (such as inactive GSK3β (He et al., 1995; Pierce and Kimelman, 1995), β-catenin (Funayama et al., 1995; Gruger and Gumbiner, 1995; Wylie et al., 1996) or Lef1 (Behrens et al., 1996; Molenaar et al., 1996)) are ectopically expressed in vegetal cells. Furthermore, maternal β-catenin is asymmetrically distributed and only localised to nuclei of dorsal cells as befits a determinant of dorsal polarity (Larabell et al., 1997) and reduction in β-catenin compromises dorsal mesoderm formation (Heasman et al., 1994). Since no Wnts have yet been found that show the requisite expression profile for an inducer of the Nieuwkoop centre, it is becoming increasingly likely that this signaling centre is established by ligand-independent activation of intracellular signal transduction components.

Interestingly, ectopic expression of genes of the *Wnt1* class (Du et al., 1995) later in *Xenopus* development produces a quite different response (Christian and Moon, 1993; Moon et al., 1993). Misexpression of *Xwnt8* dorsally after mid-blastula transition results in a dramatic truncation of anterior structures. In particular, the forebrain, eyes and cement gland are either absent or grossly reduced, and there is a less well characterised effect on notochord formation. It was thought that the perturbation of anterior development stems from the ventralising influence of Xwnt8 on anterior mesoderm, thus compromising its ability to induce forebrain (Christian and Moon, 1993). However, excess levels of Xwnt8 dorsally may also interfere with induction or maintenance of *cerberus* expression, a gene whose injected RNA can induce anterior head structures including eyes and cement gland and whose normal expression appears to depend on signals from Spemann's organiser (Bouwmeester et al., 1996).

In the mouse, an unequivocal anteroposterior axis can only be identified once the primitive streak forms at the onset of gastrulation. What determines where the primitive streak will form, a decisive event in establishing anteroposterior polarity of the egg cylinder, remains unknown. Recently, the axis of bilateral symmetry in the preimplantation mouse embryo has been shown to coincide with the animal vegetal axis of the fertilised egg, suggesting that information present in the oocyte could influence the subsequent definition of axes in the embryo (Gardner, 1997). However, there is no compelling evidence yet to suggest a causative link between the axis of bilateral symmetry in the blastocyst and the future site of primitive streak formation. By whatever mechanism mammalian axes are determined, once gastrulation is underway the patterning of the embryo and its reliance on inductive interactions emanating from the organiser is thought to be very similar to that documented in *Xenopus*.

Here we report the effects of ectopic expression of chicken *Wnt8C* on axis formation and anterior patterning of the mouse embryo. Ectopic expression of *Cwnt8C*, under the control of the human *β-actin* promoter, caused axis duplication and truncation of the anterior neurectoderm in transgenic mouse embryos. The similarities between the *Wnt* misexpression phenotypes in mouse and *Xenopus* embryos suggest that, in spite of their very different developmental histories as they approach gastrulation, they may employ the same molecular mechanisms to establish the critical organising centres required for initiating and executing gastrulation. However, the results also suggest that a distinct strategy, independent of the classical organiser, may be employed to establish rostral identity in the mouse.

MATERIALS AND METHODS

Generation of transgenic mice

Plasmid *pβ-actinCwnt8C* was constructed in *pBluescript KS*$^+$ and contains a 4.3 kb human *β-actin* promoter with intron 1 in the 5′ untranslated region (Ng et al., 1985) and an 0.3 kb SV40 polyadenylation signal, both subcloned from plasmid *pLβ-actin(IVS)lacZ* (gift from Jeff Mann (Mann and McMahon, 1993)). The *Cwnt8C* coding region, contained within a 1.29 kb *Eco*RI-*Xmn*I fragment of plasmid *pGEMwnt8.1R1* which originated from clone *pMT23-HNWnt8C* (Hume and Dodd, 1993), was inserted by blunt ligation into a unique *Hin*dIII site between the *β-actin* promoter and polyadenylation signal.

Transgenic mice were generated by microinjection of linear, vector-free DNA fragments at 1 ng/μl into a pronucleus of zygotes. Fertilised eggs were obtained from crosses between F_1 hybrids (CBA×C57) or from crosses between F_1 (CBA×C57) females with homozygous males of a transgenic line for construct 1 (Pöpperl et al., 1995) generated in this hybrid background. This line carries the *lacZ* gene under control of the *Hoxb1 rhombomere 4 enhancer*. Founder embryos transgenic for *β-actin-Cwnt8C* were identified using PCR on yolk sac DNA or DNA from extraembryonic tissue with primer pairs specific for the transgene (*Cwnt8C/SV40pA*: 5′-GGCGTTCCTCGTGCAGTAGTCGG-3′; 5′-GATGAGTTTGGACAAACCAC-3′) and primers to *myogenin* as an internal control (5′-CCAAGTTGGTGTCAAAAGCC-3′; 5′-CTCTCTGCTTTAAGGAGTCAG-3′).

Whole-mount in situ hybridisation

Embryos analysed by whole-mount in situ hybridisation were dissected in PBS, immediately fixed in 4% paraformaldehyde in PBS for about 24 hours at 4°C, then dehydrated through a methanol series and processed as described by Sasaki and Hogan (1993). No RNAse step was performed in hybridisations using the heterologous rat *vhh1* probe. Digoxigenin-labelled antisense riboprobes used to detect the expression of *T*, *Cwnt8C*, *Hesx*1, *Shh* or *Wnt1* were generated from linearised plasmids (see below) with the appropriate RNA polymerase according to manufacturer's instructions (Boehringer Mannheim).

Digoxigenin-labelled antisense riboprobes for in situ hybridisation were generated using the following plasmids:

Brachyury (*T*) probe: A plasmid containing a 2.0 kb full-length cDNA encoding mouse *T* (gift from Dr B. Herrmann; Wilkinson et al., 1990).

Cwnt8C probe: Plasmid *pGEMwnt8* (Hume and Dodd, 1993) containing a 1.7 kb *Eco*RI fragment of a cDNA clone spanning the entire coding region.

Hesx1 probe: The riboprobe was transcribed from a 394 bp *Alu*I fragment (Thomas and Beddington, 1996).

Shh probe: A plasmid containing an *Xho*I fragment of a rat *vhh1* cDNA (from Tom Jessell; Roelink et al., 1994).

Wnt1 probe: Plasmid *dnWnt-1 CS2*$^+$ (from R. T. Moon; Hoppler et al., 1996), containing 915 bp of the mouse *Wnt1* cDNA.

Histochemical and Immunohistochemical staining

β-galactosidase activity in whole embryos was assayed by histochemical staining with X-gal as previously described (Whiting et al., 1991). Immunohistochemical staining using an anti-T antibody (gift of Dr B. Herrmann) was performed on whole embryos either directly or after in situ hybridisation (Kispert and Herrmann, 1994). For wax

histology, embryos were dehydrated and embedded according to Beddington (1994) and 7 μm serial sections were cut (Bright 6030 Microtome). Once dewaxed, sections were mounted in DPX mountant (BDH, Ltd.).

RESULTS

Gross assessment of the phenotypes caused by ectopic *Cwnt8C* expression

To examine the early influences of ectopic expression, founder embryos transgenic for a *β-actin-Cwnt8C* construct were analysed between 6.5 and 9.5 days post coitum (dpc). Transgenesis was confirmed either by PCR analysis of extraembryonic tissues or by whole-mount in situ hybridisation using a *Cwnt8C* probe. The transgene can be expressed in all tissues of the embryo at 6.5-7.5 dpc, although this expression is usually mosaic and variable from embryo to embryo. The highest level of transcripts invariably appears to be in visceral endoderm cells (Fig. 1A,B). Variable expression is most likely caused by position effects at the independent integration sites in the genome and compounded by mosaicism resulting from the stage of transgene integration.

The penetrance of the phenotypes was high in that about 80% of the *Cwnt8C* transgenic embryos displayed an abnormal morphology. The severity of defects was variable in accordance with differences in transgene expression (Table 1; Fig. 1C). Nonetheless, embryos could be readily classified into distinct categories. Approximately a quarter of transgenic embryos at the egg cylinder stage exhibited overt axis duplication but, in all cases, only a single amnion was present. The embryonic region of a further 54.1% was consistently deformed such that, in late streak stage embryos, the rostral half of the cylinder appeared relatively enlarged and often gave the embryo an uncharacteristic 'boat-shaped' appearance (Fig. 3B). A profound constriction at the embryonic-extraembryonic junction was evident in most transgenic embryos (64.9%). In older embryos (from headfold to 25-somite stage; Fig. 1C), axis duplication was apparent at about the same frequency (22.1%) and again all 'twins' occurred within the same amnion. However, the predominant phenotype at these stages was overt truncation of anterior structures (56%; Table 1). None of these phenotypes were observed in founder embryos containing other *β-actin* expression constructs or unrelated transgenes (Pöpperl et al., 1995 and data not shown).

Axis duplication in *Cwnt8C* transgenic embryos

Embryos exhibited two different forms of axis duplication: either the axes were in opposing orientation giving a head-to-head duplication (Fig. 2A,B) or they were parallel and fused caudally (Fig. 2C,D). Embryos with axial duplications appeared somewhat developmentally retarded and never showed signs of turning. In addition, no complete axial duplication which included the formation of two approximately normal heads was ever observed.

In all cases of head-to-head duplications, the two axes were fused rostrally (Fig. 2A,B) and the secondary axes did not contain patent somites. The presence of a notochord in each of the axes was demonstrated by whole-mount in situ hybridisation to *Shh* transcripts (Fig. 2B). Where the *Cwnt8C* transgene had been introduced into oocytes from a transgenic marker line of mice containing the *Hoxb1 rhomobomere (r) 4 enhancer* driving a *lacZ* reporter, it was clear that the secondary axis at 9.5 dpc also contained a fourth rhombomere and thus duplication extended at least as far rostrally as the hindbrain (Fig. 2A). Although not always straightforward to interpret, some 8.5 dpc embryos showed evidence of two opposing primitive streaks with the presumed headfolds and anterior aspect of each embryonic axis displaced to the distal aspect of the conceptus (Fig. 2F). That such displacement of prospective anterior tissue can occur in *Cwnt8C* transgenic embryos is supported by the presence of *Hesx1* transcripts in a distal domain of some 7.5 dpc transgenic embryos, when normally this gene is expressed in an anterior domain corresponding to prospective forebrain and adjacent endoderm (Fig. 3B). Furthermore, *Brachyury* (*T*) expression was sometimes observed as a discontinuous circumferential ring abutting the extraembryonic region of 7.5 dpc embryos (Fig. 3A), indicating that two primitive streaks may be able to form on opposite sides of the proximal epiblast.

In the second group of embryos showing axis duplication, the two axes are oriented parallel to one another and are invariably fused caudally. Thus sections of 9.5 dpc embryos exhibiting this phenotype reveal two notochords which merge in the posterior trunk (data not shown). Exteriorly, axis duplication in these embryos is manifested in patterning defects in the caudal brain and in the trunk. In one embryo, an additional,

Table 1. Summary of the phenotypes observed in *Cwnt-8C* transgenic embryos recovered between 6.5 and 9.5 dpc

	No. transgenic	No. resorbing	No. normal	No. single streak but abnormal morphology[ø]	No. overt duplication	No. junctional constriction
Egg cylinder§ 6.5-7.5 dpc	37	1 (0.3%)	7 (18.9%)	20 (54.1%)	9* (24.3%)	24 (64.9%)
	No. transgenic	**No. normal**	**No. mild anterior truncation**	**No. severe anterior truncation**	**No. duplication**	**No. uninterpretable**
Headfold-25 somite 7.5-9.5 dpc	68	14 (20.6%)	18 (26.5%)	20 (29.4%)	15† (22.1%)	1 (1.5%)

§Only embryos subjected to diagnostic in situ hybridisation are classified as having duplicated axes.
[ø]The embryos tend to be 'boat-shaped': the diameter of the embryonic portion of the cylinder is greater than its length and the neural plate appears broader.
*Two embryos appeared to have multiple axes.
†One embryo appeared to have multiple axes.

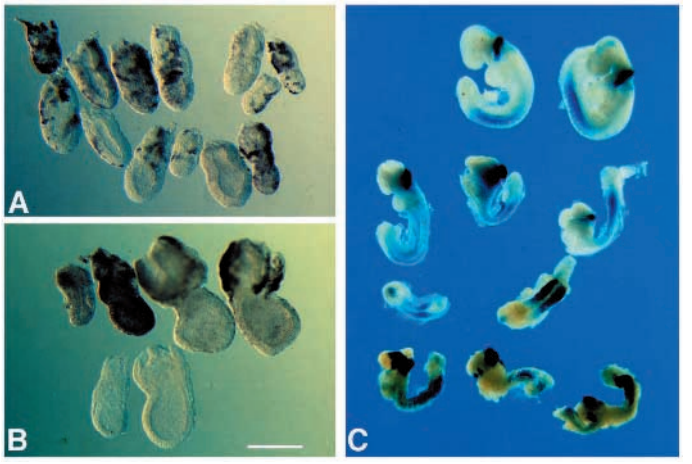

Fig. 1. Variable transgene expression and range of morphological abnormalities in *β-actin-Cwnt8C* transgenic founder embryos. (A) Expression of the *Cwnt8C* transgene in 6.5 dpc embryos visualised by whole-mount in situ hybridisation. Single cells or groups of cells expressing high levels of the transgene predominate in the visceral endoderm. (B) *Cwnt8C* expression in transgenic embryos displaying abnormal morphologies at 7.5 dpc. Transgenic embryos expressing different levels of the transgene are shown on the top. In the two embryos on the right, the highest level of *Cwnt8C* transcripts is apparent in the visceral endoderm of the extraembryonic region. The extraembryonic-embryonic junctions show a mild constriction and the embryonic regions are abnormally shaped. On the bottom are two non-transgenic control embryos. Bar, 500 μm. (C) Range of phenotypes generated in the background of a *Hoxb1 r4 enhancer* line. The two embryos on the top show no obvious defects. β-galactosidase activity is evident in r4 of the hindbrain and the second branchial arch as well as more caudally in somitic mesoderm. The three embryos in the second row exhibit mild anterior truncations as judged by the shortened relative distance between r4 and the rostral limit of the head. In two embryos with severe anterior truncations (3rd row), a narrow r4 region is located almost at the anterior limit of the embryos. Embryos on the bottom show various degrees of axis duplication. The irregularly shaped r4 of the embryo on the left indicates an anterior axial defect. The other two embryos have duplicated axes and both contain a second r4 domain (see also Fig. 2A). Bar, 1 mm.

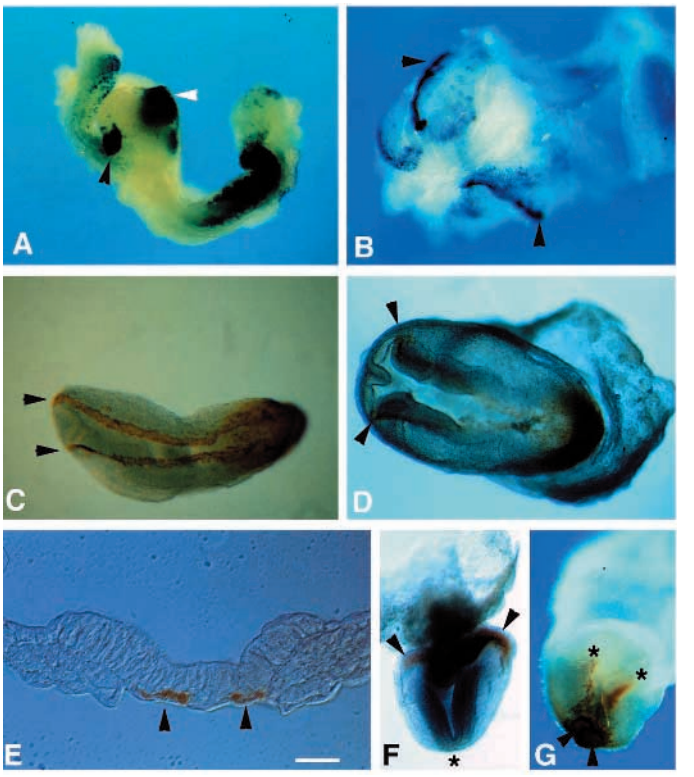

Fig. 2. Axial duplication in *β-actin-Cwnt8C* transgenic embryos. (A,B,F) Head-to-head duplication of the embryonic axis. (C,D,E,G) Parallel, caudally convergent axes. (A) Lateral view of a *Cwnt8C* transgenic embryo at 9.5 dpc generated in the background of a *Hoxb1 r4 enhancer* line (see Fig. 1C). The embryo is oriented such that the anterior end of the primary body axis is on the left. β-galactosidase activity marks r4 in the hindbrain (white arrowhead). The secondary axis, which is developmentally retarded relative to the primary axis, is fused with the primary axis in a head-to-head orientation and includes a second r4 domain (black arrowhead). The posterior end of the secondary axis is on the left and pointing up. Bar, 200 μm. (B) Frontolateral view of a 9.5dpc transgenic embryo with duplicated axes. The ventral aspect of each axis is visible and each axis contains a notochord marked by expression of *Shh*. (arrowheads). The posterior ends of the embryo are to the right and the axes are fused rostrally. Bar, 100 μm. (C,D) Ventral views of 8.5 dpc embryos with duplicated notochords and primitive streaks stained with anti-T antibody. Anterior is to the left. The two notochords (arrowheads) are parallel and the anterior extent of the duplicated axes coincides with the anterior limits of the two notochords. Bar, 150 μm. (E) Transverse section of an embryo similar to those shown in C,D showing two notochords stained with T antibody (arrowheads) underlying an expanded neural plate. Bar, 30 μm. (F) Lateral view of an 8.5 dpc embryo, stained with anti-T antibody and subjected to *Hesx1* whole-mount in situ hybridisation. T expression reveals two opposed primitive streaks (arrowheads) arising from a severely constricted extraembryonic-embryonic junction. The headfolds of each axis meet at the distal aspect of the egg cylinder (asterisk; see also Fig. 3B) but no *Hesx1* transcripts are detectable indicating anterior truncation. Bar, 200 μm. (G) Frontal view of a 8.5 dpc embryo with two irregular notochords (asterisks) and two morphologically distinct nodes (arrowheads). Bar, 150 μm.

prominent, fold of neurectoderm was present in the hindbrain region and, as found in the trunk region of another embryo, a widened column of somites. In this case, the enlarged somites occurred in a region containing two notochords although one notochord was displaced ventrally and no longer in contact with the neural tube indicating a fusion between the two adjacent somite files. These embryos also showed signs of a foreshortened preotic brain region (see below). Immunohistochemical staining for Brachyury (T) protein at 8.5 dpc clearly demonstrates the presence of two notochords which converge caudally and the primitive streak is broader than normal (Fig. 2C-E; n=4). In some embryos at this stage two distinct nodes can still be identified (Fig. 2G). Interestingly, there is no evidence for axial duplication rostral to the most anterior limit of the duplicated notochords. Instead the two axes appear to be joined rostrally by a single epithelial fold (Fig. 2C,D). The lack of extreme rostral duplication, and the possible fusion observed where the prospective forebrain should be, is consistent with the observation that no *Hesx1* transcripts could be detected in 2 embryos at the late streak stage, which had duplicated axes stained with T antibody.

These data suggest that two primitive streaks forming on

opposite sites of the epiblast could generate two opposing axes, while the formation of two streaks in closer proximity would lead to the production of two approximately parallel axes, which subsequently merge posteriorly.

Anterior truncations in *Cwnt8C* transgenic embryos

The development of anterior structures was examined in embryos that showed features of dorsalisation but which did not show overt axial duplication. Apart from being the most common phenotype of *β-actin-Cwnt8C* transgenic embryos, this class of embryo was a better subject for studying anterior development since duplications and fusions did not complicate the analysis.

At 9.5 dpc, *Cwnt8C* transgenic embryos, produced in the background of the *lacZ* reporter line for the *Hoxb1 r4 enhancer*, showed different degrees of anterior defects (Fig. 1C). A restricted, albeit sometimes enlarged, domain of β-galactosidase activity in the hindbrain demonstrated that r4 and, therefore, at least the posterior hindbrain was present and patterned. However, more anterior cranial regions were clearly reduced (Fig. 1C). Transgenic embryos between 8.0 and 9.0 dpc exhibited a range of altered head morphologies. Consistent with a deletion of anterior structures, the notochord extends closer to the anterior limit of the embryo (Fig. 4A-C). Midline mesoderm that underlies the prospective forebrain is composed of prechordal mesoderm which does not express *T* (Herrmann, 1991).

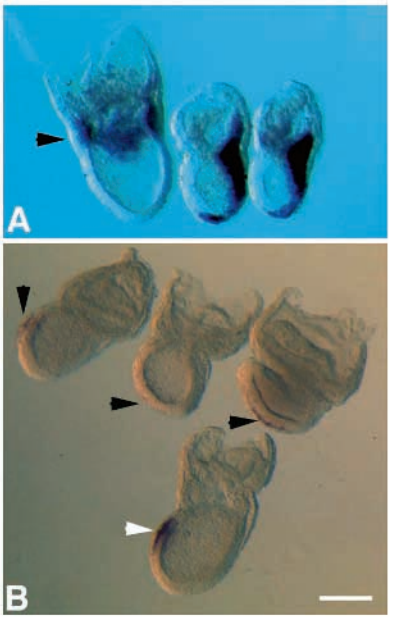

Fig. 3. Altered expression of early anterior and posterior markers in 7.0-7.5 dpc *Cwnt8C* transgenic embryos. (A) Patchy, circumferential *T* expression in a transgenic embryo (left; arrowhead) indicative of ectopic primtive streak formation. Normally, T expression marks the primitive streak and the head process as shown in two control embryos on the right. (B) Distally displaced and reduced *Hesx1* expression in the primitive endoderm of three *Cwnt8C* transgenic embryos (top; arrowheads) compared to normal *Hesx1* expression in anterior primitive endoderm of a control embryo (bottom; white arrowhead). One of the transgenic embryos (top; right) also exhibits a severe 'boat-shaped' morphology often seen in *Cwnt8C* transgenic embryos where the width of the embryonic region is greater than its length and the distal tip of the egg cylinder is blunt. (A,B) Bar, 300 μm.

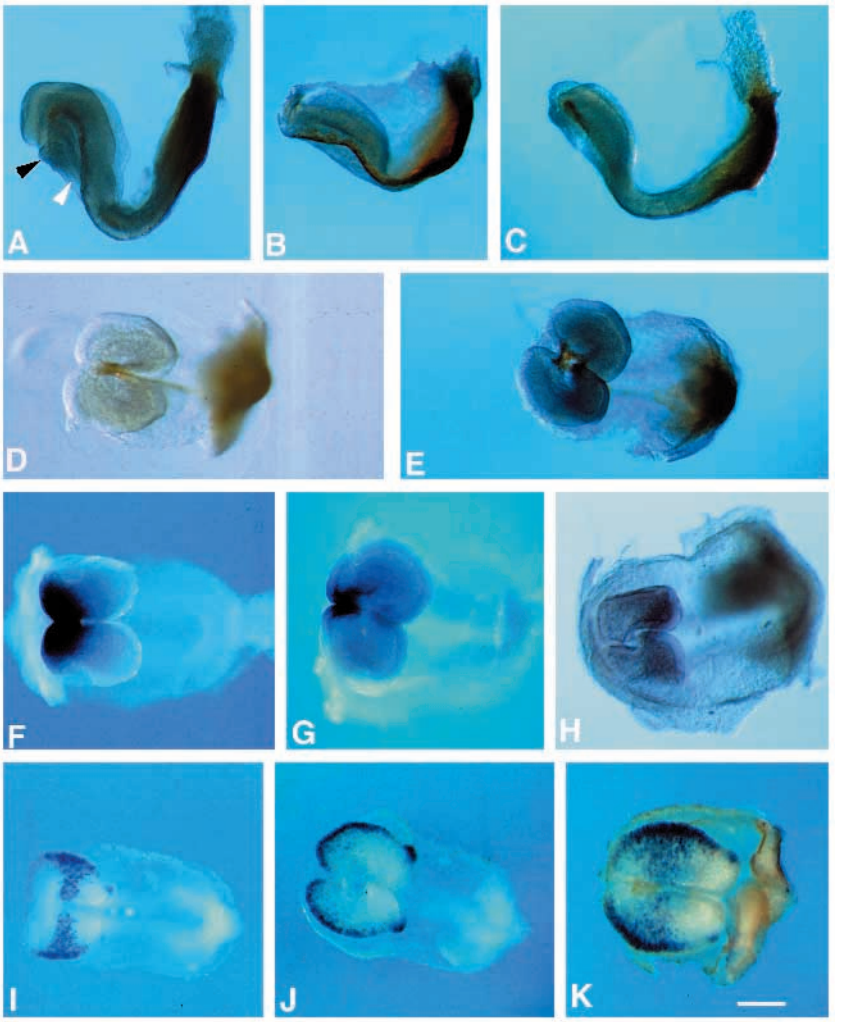

Fig. 4. Anterior truncations in *Cwnt8C* transgenic embryos at 8.5 dpc. (A-E) Immunohistochemical staining of notochord and primitive streak with anti-T antibody. Anterior is to the left. Lateral views of normal headfold and heart morphology in a control embryo (A) and abnormal headfold morphology in transgenic embryos (B,C). The foregut pocket (white arrowhead) and heart (black arrowhead) are absent or reduced, and the notochord extends almost to the anterior limit of the transgenic embryos. (D) Dorsal view of a control embryo and a transgenic embryo (E) showing bifurcation of the rostral notochord. (F-K) Whole-mount in situ hybridisation with forebrain and midbrain markers (dorsal views; anterior to the left). (F) *Hesx1* expression marks the prospective forebrain in a non-transgenic control. Reduction (G) or absence (H) of *Hesx1* expression in transgenic embryos. (I) *Wnt1* expression in the prospective midbrain of a control embryo. The *Wnt1* expression domain is expanded and extends more rostrally in *Cwnt8C* transgenic embryos (J, K) although the most rostral medial domain remains devoid of *Wnt1* transcripts. Bar, 150 μm.

Therefore the extension of T expression more rostrally indicates that the axial mesoderm anterior to the notochord is reduced. Often, the rostral extremity of the notochord is irregularly shaped or bifurcated (Fig. 4E), possibly symptomatic of early and very limited axial duplication. Certainly most embryos in this group show some signs of dorsalisation in that T and *HNF3β* expression indicate an enlarged notochord and, in some embryos, T antibody staining showed the primitive streak to be abnormally broad (data not shown).

Patterning in the cranial neurectoderm was assessed using *Hesx1*, as a molecular marker of the prospective forebrain (Hermesz et al., 1996; Thomas and Beddington, 1996), and *Wnt1* as a marker that is excluded from the forebrain but present in the midbrain and dorsal aspect of the hindbrain (McMahon et al., 1992; Parr et al., 1993). Affected *Cwnt8C* transgenic embryos showed either a reduction in the size of the *Hesx1* expression domain (1 embryo; Fig. 4G) or complete loss of *Hesx1* expression (1 embryo; Fig. 4H). In contrast, the *Wnt1* expression domain was enlarged and extended almost to the anterior limit of the neuroectoderm (Fig. 4J,K; *n*=2). However, *Wnt1* transcripts were never seen in the most rostral medial domain. These altered expression patterns confirm that the anterior cranial folds are truncated and that, in particular, the prospective forebrain is severely reduced while tissue of midbrain and hindbrain character may be expanded.

In addition to truncated neural folds, formation of the foregut and heart development is disrupted in some embryos. Several older embryos (9.5 dpc) clearly lack a morphologically recognisable foregut or heart (*n*=3) and younger embryos at the early somite stage show no sign of heart tube formation (Fig. 4B,C; *n*=4). Since the heart and foregut are also derived from very anterior tissue during gastrulation, these deficiencies may also reflect a form of anterior truncation. Alternatively, their absence could be due to a more indirect effect that inhibits the complex morphogenesis required for the repositioning of the heart, such that, instead of being rostral to the prospective brain, it comes to lie ventral to the cranial neural folds.

DISCUSSION

These experiments provide the first demonstration that ectopic expression of a naturally occurring protein, a member of the Wnt1 class of secreted factors, can induce axis duplication in the mouse embryo. In all cases, this duplication occurred within a single amnion and, therefore, must involve the production of more than one primitive streak within a single egg cylinder rather than an earlier division of the embryo or inner cell mass at preimplantation stages (Kaufman, 1992). In addition, ectopic expression of *Cwnt8C* also caused anterior truncations. These results are reminiscent of those obtained by ectopic expression of *Xwnt8* in *Xenopus* (Christian et al., 1991; Christian and Moon, 1993; Smith and Harland, 1991; Sokol et al., 1991) although there are some important differences, in particular the failure of *Cwnt8C* to induce an entire secondary axis complete with forebrain.

Expression of endogenous vertebrate *Wnt8* genes during gastrulation and the existence of a Nieuwkoop centre in the mouse

Available molecular and functional evidence suggests that *Zwnt8* (Kelly et al., 1995), *Xwnt8* (Christian and Moon, 1993), *Cwnt8C* (Hume and Dodd, 1993) and *Mwnt8* (Bouillet et al., 1996) are true orthologues. Apart from sequence homology they also show many similarities in their expression pattern during gastrulation. All are expressed in the primitive streak, or its equivalent in *Xenopus* and zebrafish, once gastrulation commences. They are not expressed in the population of cells expressing *goosecoid* (De Robertis et al., 1992; Hume and Dodd, 1993; Stachel et al., 1993). Since these cells are destined to form prechordal plate and thus are thought to play a role in forebrain development, the normal exclusion of *Wnt8* transcripts from this population may explain why ectopic dorsal expression of *Wnt8* causes anterior truncations. Only in the chick embryo have localised *Wnt8* transcripts been detected prior to overt gastrulation when they are found in the epiblast of the posterior marginal zone overlying Koller's sickle (Hume and Dodd, 1993), a location and timing of expression that could be consistent with a role in axis induction. However, such precocious localised expression has not been detected in other vertebrates. Therefore, in the mouse, like in *Xenopus*, it is unlikely that Wnt8 is the natural inducer of the primary signalling centre responsible for axis formation.

All the ectopic expression studies in *Xenopus*, the demonstration that Xwnt8 can rescue UV ventralised embryos (Smith and Harland, 1991; Sokol et al., 1991) and the misexpression experiments reported here strongly implicate a Wnt signalling pathway in the early events leading to induction of the organiser. If the molecular mechanism for inducing an organiser is the same in mouse as in *Xenopus* then this would imply that a signalling centre similar to the Nieuwkoop centre may exist in mammals. If so, it is still not clear in which tissue such a signalling centre would reside, although the consistently high levels of *Cwnt8C* transcripts found in the extraembryonic visceral endoderm of gastrulating transgenic embryos (Fig. 1A,B) indicates that this tissue, or the immediately adjacent extraembryonic ectoderm, would be likely sites. The subcellular localisation of β-catenin might serve as a marker for a mouse Nieuwkoop centre (Larabell et al., 1997) but immunohistochemical analysis of pre- and early gastrulating mouse embryos has failed to reveal any sites where β-catenin is concentrated in the nucleus rather than associated with the plasma membrane (F. Conlon and R. Beddington, unpublished observations).

Complete axis duplication in the mouse requires more than a duplicated organiser

Monoamniotic twins are extremely rare in mammals and account for only 1-3% of naturally occurring monozygotic twins in humans. They have been induced experimentally in mice by injection of vincristine sulphate during the early stages of gastrulation (6.5-7.5 dpc; Kaufman, 1992). This microtubule inhibitor generated complete twins in a head-to-head orientation and in only 1 out of 5 of the cases reported were the embryos conjoined in the rostral region. This teratogenic effect of vincristine demonstrates that the gastrulating mouse embryo can physically form two complete axes.

Axis duplication reminiscent to that produced in *Cwnt8C* transgenics is also observed in embryos homozygous either for mutations at the *Fused* locus (Gluecksohn-Schoenheimer, 1949; Perry et al., 1995) or for a targeted mutation in *Lim1* (Shawlot and Behringer, 1995). In both mutants, axis duplica-

tion is restricted to the hindbrain and trunk regions and the embryos exhibit anterior truncations. These similarities between presumed loss-of-function mutations and overexpression of *Wnt8* suggest that *Lim1* and *Fused* may normally serve as direct or indirect antagonists of Wnt signalling. Experimentally axis duplication can also be induced in the mouse by grafting the organiser or node to a posterolateral position in gastrulating embryos, but again no forebrain duplications have ever been observed (Beddington, 1994) even when early organisers are grafted (P. Tam, personal communication). Thus, with the exception of vincristine, which may operate by mechanically splitting the egg cylinder, all molecular perturbations that result in patent duplication of the streak and/or node only lead to duplications of that part of the axis underlain by notochord.

Two alternative hypotheses can be put forward to explain these results. First, complete axis duplication is induced in all cases but subsequently the most anterior reaches of the axes are posteriorised because the same pathway that leads to induction of a second organiser is also employed to posteriorise the rostral axis. The dual effects of ectopic *Xwnt8* on *Xenopus* development, namely complete axis duplication if expressed early and anterior truncation if expressed late (Moon et al., 1993), lends credence to this hypothesis. The alternative explanation is that duplication of the primitive streak and organiser is not sufficient to generate extreme rostral duplication because the initial specification of the anterior terminal of the axis, as opposed to its maintenance, is not dependent on products of the primitive streak and organiser. Of course, the rostral aspect of the primary axis would still be subject to the later posteriorising influence of ectopic Wnt8. Evidence for this alternative hypothesis remains more circumstantial but the results reported here and previous experiments in the mouse are consistent with such a scenario.

The phenotype of the *Cwnt8C* transgenic embryos shows no evidence of duplication of a specific forebrain marker, *Hesx1*. Although clearly two primitive streaks and nodes can be induced (Fig. 2F,G), and the expression of *Hesx1* may be displaced distally at egg cylinder stages, only a single patch of *Hesx1*-expressing cells is evident (Fig. 3B). Furthermore, the rostral fusion of all duplicated axes in these embryos, even those with parallel axes, argues that the rostral extremity never was duplicated. Finally, the absence of *Wnt1* transcripts from the most rostral medial domain of dorsalised embryos indicates that this region may have a unique specification which cannot be completely corrupted by excess dorsal mesoderm. However, since *Hesx1* transcripts are undetectable in some late streak stage embryos, presumably due to the posteriorising influences of Wnt8, it is difficult without knowing the precise duration and location of *Cwnt8C* expression in each transgenic embryo to be entirely certain of the basis of rostral fusions and absence of apparent forebrain duplication.

That anterior specification may be independent of streak function is supported by other studies on mouse mutants and manipulations of the early gastrulating embryo. Embryos homozygous for a null mutation in *HNF3β*, which is normally expressed in the node, the prechordal plate and notochord (Ang et al., 1993; Sasaki and Hogan, 1993; Weinstein et al., 1994), do not develop a recognisable node (Ang and Rossant, 1994; Weinstein et al., 1994) and yet, despite the absence of axial mesendoderm, these embryos exhibit remarkably normal anterior patterning including expression of forebrain markers such as *Otx2* (Ang and Rossant, 1994). Recently, it has been shown that *Hesx1* is first expressed at the onset of gastrulation in a small patch of endoderm cells at the anterior of the embryo, immediately adjacent to the prospective forebrain region of the epiblast (Hermesz et al., 1996; Thomas and Beddington, 1996). These endoderm cells belong to the primitive endoderm lineage and will not contribute to the embryo itself, but if they are removed forebrain development is compromised. This led to the hypothesis that descendants of the primitive endoderm are responsible for initiating anterior pattern in the embryo (Thomas and Beddington, 1996). Interestingly, other genes that affect prosencephalic development or are later expressed in the node, such as *Otx2* (Acampora et al., 1995; Ang et al., 1994), *nodal* (Varlet et al., 1997), *HNF3β* (Weinstein et al., 1994), and *Lim1* (Shawlot and Behringer, 1995), are expressed first in this primitive endoderm population at the anterior aspect of the egg cylinder. The most compelling evidence that primitive endoderm is involved in anterior patterning of the embryo comes from chimeras containing a mixture of wild-type and *nodal*$^{-}$*/nodal*$^{-}$ cells. Chimeras containing predominantly wild-type cells in the primitive endoderm but a large proportion of mutant cells in the embryo exhibit reasonably normal anterior development. However, if the visceral endoderm is largely mutant and the embryo wild type, severe anterior truncation of the embryonic axis results (Varlet et al., 1997). This may be the reason why some heterozygous *Otx2*$^{+}$*/Otx*$^{-}$ embryos but never *Otx2*$^{+}$*/Otx*$^{-}$↔wild-type chimeras exhibit holoprosencephaly (Matsuo et al., 1995). Thus, the failure to induce a complete second axis by mechanically or genetically manipulating the mouse organiser may be because the anterior end of the embryonic axis is determined independently of the node, by an interaction with visceral endoderm. The role of the prechordal plate would be to sustain bilateral symmetry and to maintain and embellish anterior pattern rather than to initiate it.

Superficially, an organiser-independent mechanism for establishing anterior identity in the mouse conflicts with experiments in amphibians where organiser grafts generate complete secondary axes as does ectopic expression of a variety of genes normally expressed in the organiser. Due to the cylindrical nature of the mouse embryo, the classical organiser associated with the primitive streak and the endoderm now proposed to be responsible for anterior induction happen to be on opposite sides of the conceptus and thus are more easily seen as physically distinct. In *Xenopus* the tissue responsible for inducing anterior structures is the deep endomesoderm of the dorsal half of the embryo which expresses *cerberus* and immediately abuts the dorsal blastopore lip organiser (Bouwmeester et al., 1996). Therefore, as in the mouse, rostral identity appears not to be established by ingressing axial mesendoderm. This difference in topography between the mouse and frog means that early organiser grafts in *Xenopus* will usually include cells responsible for anterior induction whereas mouse grafts will not. Furthermore, manipulations that duplicate the organiser in *Xenopus* may serve to divert the movement of deep endomesoderm rather than to duplicate an anterior signal. Thus, while the effects of ectopic *CWnt8C* in mouse embryos re-emphasise the likely conservation of mechanisms responsible for generating a classical organiser in vertebrates they also point to separate interactions, possibly also conserved amongst all vertebrates, which establish anterior identity.

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