

## Suppression of GATA factor activity causes axis duplication in *Xenopus*

T. G. Sykes<sup>‡</sup>, A. R. F. Rodaway, M. E. Walmsley and R. K. Patient<sup>\*</sup>

Developmental Biology Research Centre, The Randall Institute, King's College London, 26-29 Drury Lane, London WC2B 5RL, UK

<sup>‡</sup>Present address: INSERM U.382, Developmental Biology Institute of Marseille (IBDM), CNRS-INSERM Université de la Méditerranée, Campus de Luminy, Case 907, 13288 Marseille Cedex 09, France

<sup>\*</sup>Author for correspondence (e-mail: roger.patient@kcl.ac.uk)

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### SUMMARY

In *Xenopus*, the dorsoventral axis is patterned by the interplay between active signalling in ventral territories, and secreted antagonists from Spemann's organiser. Two signals are important in ventral cells, bone morphogenetic protein-4 (BMP-4) and Wnt-8. BMP-4 plays a conserved role in patterning the vertebrate dorsoventral axis, whilst the precise role of Wnt-8 and its relationship with BMP-4, are still unclear. Here we have investigated the role played by the GATA family of transcription factors, which are expressed in ventral mesendoderm during gastrulation and are required for the differentiation of blood and endodermal tissues. Injection ventrally of a dominant-interfering GATA factor (called G2en) induced the formation of secondary axes that phenocopy those induced by the dominant-negative BMP receptor. However, unlike inhibiting BMP signalling, inhibiting GATA activity in the ectoderm does not lead to neuralisation. In addition,

analysis of gene expression in G2en injected embryos reveals that at least one known target gene for BMP-4, the homeobox gene *Vent-2*, is unaffected. In contrast, the expression of *Wnt-8* and the homeobox gene *Vent-1* is suppressed by G2en, whilst the organiser-secreted BMP antagonist chordin becomes ectopically expressed. These data therefore suggest that GATA activity is essential for ventral cell fate and that subsets of ventralising and dorsalising genes require GATA activity for their expression and suppression, respectively. Finally, using G2en, we show that suppression of *Wnt-8* expression, in conjunction with blocked BMP signalling, does not lead to head formation, suggesting that the head-suppressing Wnt signal may not be Wnt-8.

Key words: Dorsoventral patterning, *Xenopus*, BMP-4, Wnt-8, GATA factors

### INTRODUCTION

Patterning of the dorsoventral axis in *Xenopus* has been intensively studied (reviewed by Lemaire and Kodjabachian, 1996). A central role in this process is played by the Spemann organiser, a field of cells on the dorsal midline that has the ability to organise a secondary axis when transplanted ventrally. Recently, however, it has become clear that signalling by ventral cells also plays a crucial role in dorsoventral patterning of the embryonic axis. Two such signals in the ventral mesoderm are known to be important: *BMP-4* (Dale, 1992; Jones et al., 1992) and *Wnt-8* (Christian et al., 1991). The emerging picture is that the primary signals to which cells respond come from ventral mesoderm whilst the organiser secretes antagonists to these signals: *follistatin*, *noggin* and *chordin* in the case of *BMP-4* (Piccolo et al., 1996; Sasai et al., 1995; Zimmerman et al., 1996), and *Frzb*, *cerberus* and *dickkopf-1* in the case of *Wnt-8* (Glinka et al., 1997, 1998; Leyns et al., 1997; Wang et al., 1997). One of the keys to understanding the patterning of the dorsoventral axis therefore rests with an understanding of the events leading to the production and interpretation of ventral signals.

There is now strong evidence that *BMP-4* plays a major role

in patterning the dorsoventral axis, both in the mesoderm and the ectoderm (Dosch et al., 1997; Neave et al., 1997; Wilson and Hemmati-Brivanlou, 1995). Antagonists secreted by the organiser act to set up a gradient of BMP-4 activity which is interpreted by recipient cells to produce a range of fates along the dorsoventral axis. *Wnt-8* meanwhile, which is expressed in ventral and lateral mesendoderm, appears to act to restrict the organiser field to the dorsal midline (Christian and Moon, 1993). This is of particular significance for head induction, an early function of the organiser, which is to some extent separable from the trunk organiser. The two secreted proteins which have head inducing activity, *Cerberus* and *Dickkopf-1*, are both able to antagonise *Wnt-8* signalling (Glinka et al., 1997, 1998).

In addition to these secreted signals, transcription factors with ventralising properties have also been isolated. These include the related homeobox transcription factors *Vent-1* (Gawantka et al., 1995) and *Vent-2* (Onichtchouk et al., 1996, also known as *Xom*, *Xbr-1* and *Vox*; Ladher et al., 1996; Papalopulu and Kintner, 1996; Schmidt et al., 1996). Both these genes are regulated by BMP-4 in the early embryo and appear to mediate the effects of this secreted factor. In addition, although the expression patterns of *Vent-1* and *Vent-2* are

overlapping, they show distinct dorsal boundaries, both in ectoderm and in mesoderm. These boundaries are regulated by the level of *BMP-4* activity (Dosch et al., 1997). The relationship between these *Vent* genes and the *Wnt-8* pathway is still unclear. Overexpression of *Vent-1* in dorsal marginal zones upregulated *Wnt-8* but did not upregulate *BMP-4*. This gave rise to the suggestion that *Vent-1* may represent crosstalk between the *BMP-4* and *Wnt-8* pathways (Gawantka et al., 1995; Lemaire, 1996). However the demonstration that *Vent-1* acts as a transcriptional repressor implies that this link is not mediated through a direct effect of *Vent-1* on the *Wnt-8* promoter (Onichtchouk et al., 1998). A full understanding of dorsoventral patterning requires that the relationships between these two pathways and the *Vent* genes be clarified further.

We and others have been studying the GATA family of zinc finger transcription factors within the context of early development. The GATA family comprises six members in vertebrates, with diverse roles in the formation of blood, heart, ectoderm and endoderm-derived tissues (Laverriere et al., 1994; Read et al., 1998; Yamamoto et al., 1990). The family can be divided on the basis of both sequence homology and expression profile into two subgroups. *GATA-1*, *GATA-2* and *GATA-3* are all expressed in blood lineages and are required at different stages during the ontogeny of the blood system (Pandolfi et al., 1995; Pevny et al., 1991; Tsai et al., 1994). *GATA-2* and *GATA-3* are also expressed in the ectoderm (Bertwistle et al., 1996; Neave et al., 1995; Read et al., 1998). *GATA-4*, *GATA-5* and *GATA-6* are involved in the formation of the heart and endodermal derivatives (Laverriere et al., 1994). All are able to bind to a consensus sequence (A/T)GATA(A/G), the so-called GATA motif, that is found in a range of promoters active in the tissues listed above. In *Xenopus*, all six members of this family are expressed by the onset of gastrulation (Bertwistle et al., 1996; Gove et al., 1997; Jiang and Evans, 1996; Kelley et al., 1993, 1994; Walmsley et al., 1994; Read et al., 1998; Zon et al., 1991). The expression of these factors in all three germ layers of *Xenopus* gastrulae suggests that they may play fundamental roles in early development.

In this paper we demonstrate a crucial role for GATA factors in the development of the ventral mesendoderm. Repression of GATA activity by injection of a dominant-interfering GATA mutant leads to dorsalisation, suggesting that GATA factors are required for ventral cell fate. Molecular analysis demonstrates that *Vent-1* and *Wnt-8* are dependent on GATA activity for their expression, whilst other ventralising genes such as *Vent-2* and *BMP-4* are GATA factor-independent. Suppression of these genes in the ventral mesoderm results in the ectopic expression of *chordin* but not *noggin* or *gooseoid*.

## MATERIALS AND METHODS

### Construction of expression constructs

A full length *Vent-1* cDNA fragment (977 bp) was removed from pXVent-1 (Gawantka et al., 1995) by digestion with *XhoI* and *NotI* (blunted) and subcloned (by M. Law) into the *XhoI/HindIII* sites of the expression plasmid p $\beta$ -UT2 (see below) to produce a template for the synthesis of *Vent-1* RNA which is more stable. The region comprising the zinc finger domain of *GATA-2* (amino acids 263-380 in xGATA-2; Zon et al., 1991) was amplified by PCR, and subcloned into the expression plasmids p $\beta$ -UT2en and p $\beta$ -UT2myc. All these plasmids (Adam Rodaway, maps available on request) contain the *Xenopus*  $\beta$ -

globin 5' and 3' untranslated regions (p $\beta$ -UT2), and either the engrailed domain and a myc epitope (p $\beta$ -UT2en) or just a myc epitope (p $\beta$ -UT2myc). The engrailed domain used extends from amino acids 2 to 298 (Badiani et al., 1994, kind gift from K. Weston).

### Embryo and oocyte manipulation

Culture and microinjection of embryos have been described previously (Walmsley et al., 1994). UV treatment of embryos was performed by placing them in a quartz petri dish, and irradiating the vegetal poles for 2-3 minutes with a UV lamp suspended 7 cm below. Embryos were injected at the 4-cell stage into two adjacent blastomeres. Oocytes were injected with 2-10 ng of mRNA into the cytoplasm, and cultured overnight at 18°C. Oocytes were then injected with 5 ng of reporter DNA, targeting the injection to the germinal vesicle. Since non-specific initiation from cryptic sites is reduced with elevated levels of DNA (Walmsley and Patient, 1987), 20 ng of plasmid DNA (pGEM7) was coinjected in each case. After 24 hours, luciferase assays were performed on the oocyte extracts according to the manufacturer's instructions (Promega). Luciferase levels were normalised for injection efficiency by comparison to supercoiled plasmid levels, representing the level of DNA in the germinal vesicle (Walmsley and Patient, 1987). This was assayed by Southern blotting.

### In situ hybridisation

Whole-mount in situ hybridisation was performed as previously described (Bertwistle et al., 1996), using BM Purple (Boehringer) as the alkaline phosphatase substrate. All probes have been previously described: *Vent-1* (Gawantka et al., 1995), *Vent-2* (Ladher et al., 1996), *chordin* (Sasai et al., 1994), *Wnt-8* (Christian et al., 1991), *noggin* (Smith and Harland, 1992), *cardiac actin* and *NCAM* (Walmsley et al., 1994). *Gooseoid* was probed using a 367 bp fragment from the 5' end of the cDNA inserted into the *EcoRI* and *Apal* sites of pBSKS (kind gift from J. Smith) and transcribed using T3 RNA polymerase.

### Ribonuclease protection and RT-PCR

Preparation of RNA, and RNase protection assays were as described by Walmsley et al. (1994). Probes for *cardiac actin*, *NCAM*, and *EF1 $\alpha$*  are also described by Walmsley et al. (1994). Other probes have also been described: *BMP-4* (Dale et al., 1992), *Wnt-8* (Christian et al., 1991), *chordin* (Howell et al., 1997), *Xhox3* (Ruiz i Altaba and Melton, 1989). For RT-PCR, the method of Wilson and Melton (1994) was broadly followed. RNA was extracted using the method of Jonas et al. (1989) omitting the LiCl step. Approximately 5  $\mu$ g of total RNA was reverse transcribed using an oligo[dT]-V primer (where V=GAC) and Superscript II reverse transcriptase (GIBCO). For the amplification, all primers were tested on a dilution series of whole embryo RNA to show that the product was a linear function of the input cDNA. PCR reactions were performed in a standard 30  $\mu$ l PCR reaction with Platinum Taq (GIBCO). Reaction parameters were 95°C for 5 minutes, followed by cycles of 95°C for 20 seconds, 60°C for 20 seconds, 72°C for 30 seconds. A final hold of 72°C for 5 minutes was performed. Conditions were the same for all primer pairs. Primers were: *BMP-4* forward GCTTATATGCGCGACCTGTA, reverse GTGATGGGTTTCATAACTTC, giving a product of 322 bp; *noggin* forward CCCTTGACTGCGATGAGAG, reverse TCTGCCTAA-GAAGGAGATCC, giving a product of 448 bp; *Vent-1* forward GGAACAGGCATTCAACAAGC, reverse GAGCTCATTCTAAC-ATGGTG, giving a product of 320 bp; *Vent-2* forward TACAGCACTAGCACTGACTC, reverse GGTGGTATGAGTCTG-GTCTG, giving a product of 333 bp. All other primers have been described previously (*chordin*: Darras et al., 1997; *FGFR1*, *gsc*, *Wnt-8*: Lemaire and Gurdon, 1994).

### Histology

Embryos were fixed, dehydrated in ethanol and xylene, and embedded in paraffin wax. Sections were cut at 20  $\mu$ m. Sections were stained with haematoxylin diluted three-fold in 70% ethanol. Excess stain was washed out with tap water, before mounting in 70% glycerol.

## RESULTS

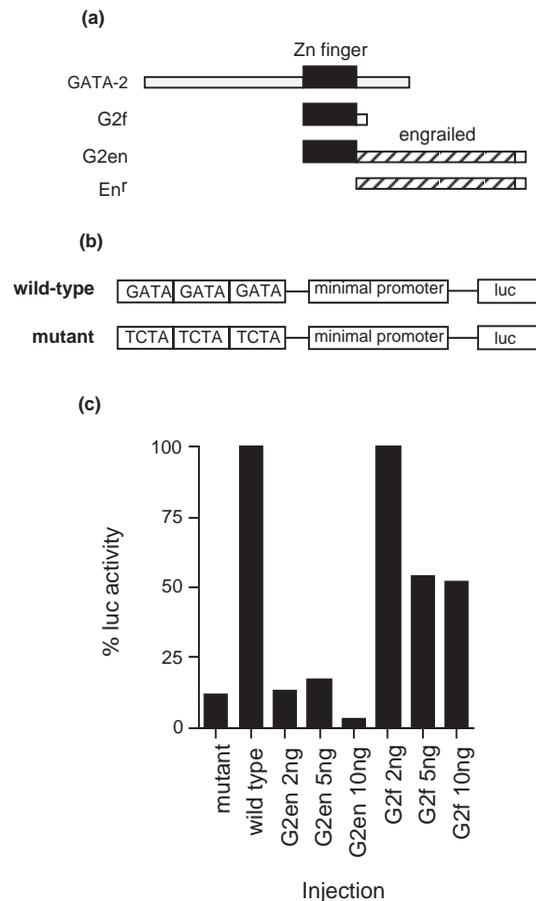
### Injection of a dominant interfering GATA factor results in secondary axis formation

The repressor domain from the *Drosophila* engrailed protein can be fused to a transcription factor of interest to convert an activator of transcription into a site-specific repressor (Badiani et al., 1994; Conlon et al., 1996; Han and Manley, 1993; Jaynes and O'Farrell, 1991). The construction of such a dominant-interfering form of GATA-2 (G2en) is shown schematically in Fig. 1a, along with two control constructs containing either the engrailed repressor domain (En<sup>r</sup>) or the zinc finger DNA binding domain of GATA-2 (G2f) alone. We tested the ability of G2en and G2f to inhibit the activity of maternal GATA-2 in *Xenopus* oocytes (Partington et al., 1997), using a GATA-dependent reporter gene shown in Fig. 1b. The activity of maternal GATA-2 is apparent from the expression of the reporter containing consensus GATA sites (wild type) compared to a control promoter with these sites mutated (Fig. 1c). Strong repression of the wild-type reporter construct by G2en was observed, reducing transcription to basal levels at the lowest dose tested, whereas, even at the highest dose tested, G2f repressed transcription by only 50%. Therefore G2en is able to inhibit GATA-2 activity *in vivo*.

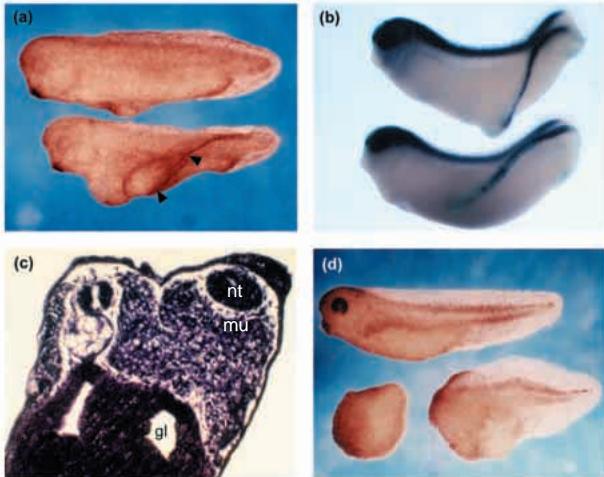
To test the effects of G2en on early ventral development, *Xenopus* embryos were injected with RNA into the marginal zone of both ventral blastomeres at the 4-cell stage, and cultured to tail-bud stages. The amount of RNA injected (50 pg) represents a level equivalent to levels of endogenous GATA-2 mRNA (as assayed by RNase protection, data not shown). Injection of G2en induced formation of secondary axes (Fig. 2a), that were incomplete, in that they lacked head and notochord structures, and yet contained a neural tube (Fig. 2b), ectopic muscle and an ectopic gut lumen (Fig. 2c). This effect was specific for G2en in that injection of either the GATA-2 fingers alone (G2f) or the engrailed domain alone (En<sup>r</sup>) did not induce secondary axes. The dose response for this phenotype (Table 1) showed that the number of embryos containing secondary axes (up to 61%) was proportional to the amount of RNA injected over the range tested (10–50 pg per blastomere). As a second assay for axis inducing activity, the ability of G2en to rescue UV-ventralisation was tested (Fig. 2d). Embryos were UV irradiated at the 1-cell stage, and injected with 50 pg of G2en RNA at the 4-cell stage. The extent of dorsoventral axis formation was quantified according to the Dorso-Anterior Index (DAI; Kao and Elinson, 1988). Uninjected UV-treated embryos lacked all axial structures (DAI=0.7±0.1; mean±s.e.m., n=94). G2en was able to rescue trunk and tail axial development, but did not result in head formation (DAI=2.1±0.1, n=46). Once again, a neural tube and muscle were observed, but no notochord (data not shown). These experiments suggest that GATA-2 is an important regulator of ventral cell fate.

To address the question of which GATA factor may represent the critical activity inhibited by G2en, we tested the ability of several GATA factors to rescue the axis inducing activity of G2en. All GATA factors have been shown to bind to a consensus GATA site via their highly conserved zinc finger domain, and in some cases, overlapping functions for the GATA factors have been reported (Blobel et al., 1995; Kuo et al., 1997; Tsai et al., 1998; Visvader et al., 1992; Visvader and

Adams, 1993; Weiss et al., 1994, 1997). Table 1 shows that coinjection of 50 pg of *GATA-1*, *GATA-2* or *GATA-6* mRNA, along with 50 pg G2en mRNA, led to substantial rescue of the G2en phenotype. These rescue experiments suggest firstly that G2en is indeed interfering with normal GATA factor function, and secondly that the targets of G2en may include those of GATA factors other than GATA-2.



**Fig. 1.** Construction and testing of G2en. (a) The structure of GATA-2, G2f, G2en and En<sup>r</sup>. Both G2en and G2f contain sequences around the zinc finger DNA binding domain (black box) that are conserved in all vertebrate GATA factors. In addition, G2f has a myc epitope fused at the 3' terminus (open box), G2en has a repressor domain from the *Drosophila* engrailed protein, and a myc epitope, both at the 3' end. En<sup>r</sup> contains only the engrailed repressor domain fused to a myc epitope (kind gift from J. Smith). (b) Reporter constructs used for testing the effect of G2en and G2f on GATA-dependent transcription. The 'wild type' construct contains three GATA sites from the mouse  $\alpha 1$ -globin promoter, upstream of a minimal promoter from the rabbit  $\beta$ -globin gene (wild type pRBG3xM $\alpha$ Gluc, kind gift from M. Yamamoto). The 'mutant' construct contains the three GATA sites mutated to TCTA. (c) The ability of G2en and G2f to inhibit maternal GATA-2 in *Xenopus* oocytes was tested. The activity of maternal GATA-2 is seen from a comparison between the expression of the wild-type reporter and the reporter with mutated GATA sites. Coinjection of G2en mRNA at the doses shown led to substantial repression of GATA-dependent transcription. Injection of G2f meanwhile only resulted in a 2-fold decrease at the highest dose. The experiment was performed twice with similar results. All levels of normalised reporter activity are expressed as percentages, with the wild-type reporter activity representing 100%.

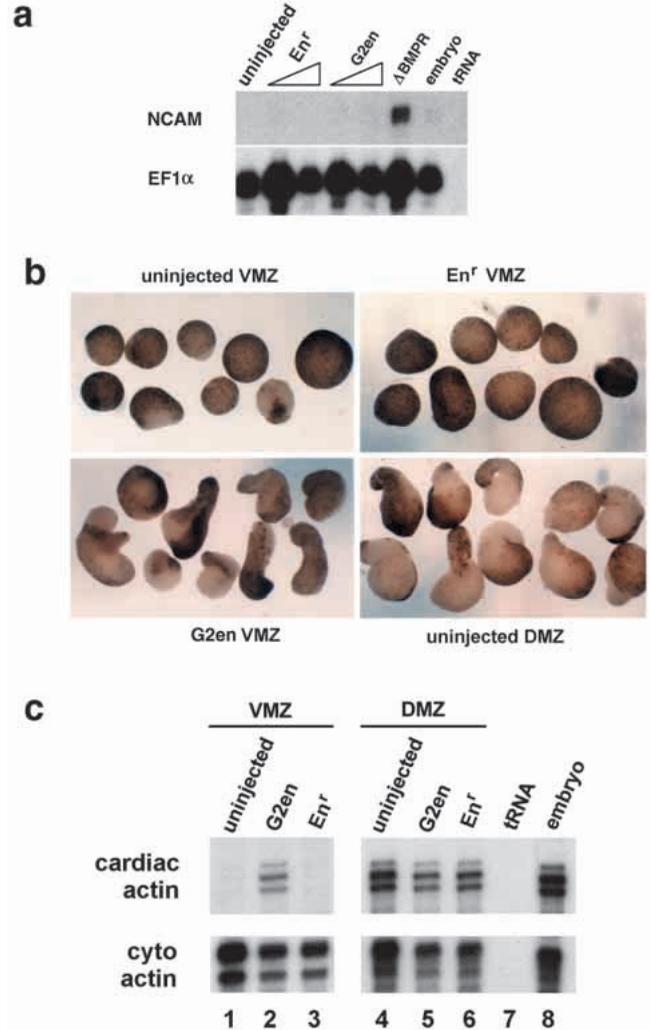


**Fig. 2.** Phenotype of G2en injected embryos. (a) Stage 34 embryo injected into both ventral blastomeres at the 4-cell stage with 50 pg G2en (bottom) develops a secondary axis (black arrows), whereas G2f has no axis inducing activity (top), but occasionally produces vesicles in ventral tissues that are typical of RNA injection. (b) St 26 G2en-injected embryos have secondary neural tubes as shown by *NCAM* in situ hybridisation. (c) Transverse section through a G2en-injected embryo, showing lack of notochord formation in the secondary (right-hand) axis. Secondary axes contain ectopic gut lumen (gl), muscle (mu) and neural tube (nt). (d) G2en can partially rescue axis formation in embryos ventralised with UV treatment. Top is a stage 38 control embryo, left is an uninjected UV-treated embryo lacking all axial structures, right is a UV-treated G2en injected embryo showing partial axis rescue.

### The effect of G2en is restricted to the mesendoderm

*GATA-2* and *GATA-3* are expressed in the presumptive epidermis along with *BMP-4* (Bertwistle et al., 1996; Fainsod et al., 1994; Read et al., 1998; Walmsley et al., 1994). Since injection of G2en induces ectopic neural tissue in whole embryos, we tested whether inhibition of GATA activity, like inhibition of BMP-4 activity (Wilson and Hemmati-Brivanlou, 1995), could cause direct neural induction in ectoderm. Presumptive epidermis (animal caps) was taken from embryos injected with the amount of RNA required to induce a second neural axis, and probed for the expression of the pan-neural marker *NCAM* (Kintner and Melton, 1987). Fig. 3a shows that neither *Enf* nor G2en induced *NCAM* expression, unlike injection of a dominant-negative BMP receptor ( $\Delta$ BMPR, Schmidt et al., 1995). Since higher doses of G2en and *Enf* were toxic, it is unlikely that this negative result represents a difference in the level of RNA required. This suggests that the induction of *NCAM* expression in whole embryos injected with G2en is not a direct consequence of inhibiting GATA activity in the ectoderm.

To test whether G2en works by dorsalising ventral mesendoderm, ventral marginal zone (VMZ) explants were taken from G2en injected embryos. An isolated VMZ explant normally forms a spherical structure, whereas an explant from the dorsal side (DMZ) elongates as a result of the convergent extension movements associated with dorsal development. Injection of G2en, but not *Enf*, led to elongation of isolated VMZ explants, implying that dorsalisation had occurred (Fig. 3b). This was confirmed by monitoring the expression of the



**Fig. 3.** G2en dorsalises mesoderm but not ectoderm. (a) G2en (50–100 pg) and *Enf* (200–400 pg) did not induce neural tissue in ectoderm explants (animal caps) cultured to Stage 23, unlike injection of  $\Delta$ BMPR (500 pg), as judged by the upregulation of the pan-neural marker *NCAM*, assayed by RNase protection. The low signal in the whole embryo lane reflects dilution by non-neural tissues compared to the ectoderm-only explants. (b) VMZ explants from embryos injected with the same dose of G2en as above (50 pg) became elongated (lower left, 20/22 explants) in a similar manner to dorsal marginal zone explants (DMZ, lower right, 21/22 elongated). VMZ explants from uninjected embryos remained spherical (top left, 0/22 elongated) as did VMZ explants from embryos injected with *Enf* (top right, 0/22 elongated). Explants are stage 23 equivalent. (c) The explants in b were probed for *cardiac actin* expression by RNase protection, using cytoskeletal actin as a loading control. G2en but not *Enf* induced *cardiac actin* expression in VMZ explants, to levels equivalent to those seen in DMZ explants, confirming dorsalisation.

dorsolateral muscle marker *cardiac actin*. This gene is not expressed in ventral tissues, but was upregulated by injection of G2en and not by *Enf* (Fig. 3c). These experiments therefore suggest that the dorsalising activity of G2en is confined to the mesendoderm, and that the second neural tube formed in G2en injected embryos is a result of secondary induction by the underlying dorsalised mesoderm.

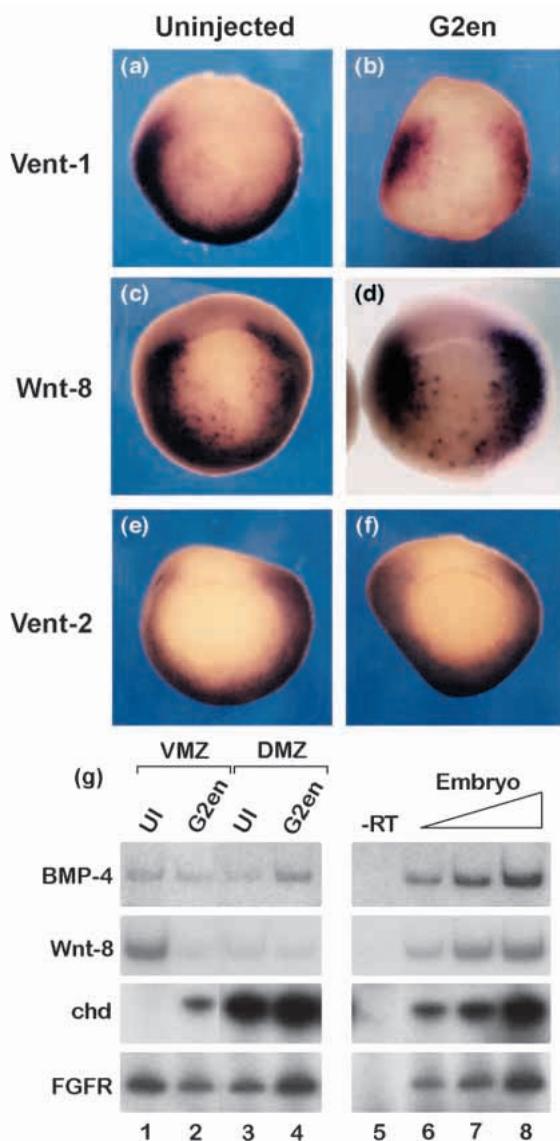
**Table 1. G2en induced axis duplication and rescue by heterologous GATA factors**

Injection	Experiments <i>n</i>	Total <i>n</i>	Survived <i>n</i>	Normal (%)	Secondary Axes (%)	Gastrulation defects (%)
G2f (200 pg)	3	82	69	80	1	19
G2en (10 pg)	3	67	54	76	14	10
G2en (25 pg)	4	87	63	46	43	11
G2en (50 pg)	6	209	178	25	61	14
G2en+GATA-1	2	61	47	83	13	4
G2en+GATA-2	2	40	35	77	3	20
G2en+GATA-6	2	35	31	65	0	35

Embryos were injected with the amount shown, into each ventral blastomere at the 4-cell stage. Rescue of twin axis formation induced with 50 pg G2en mRNA was achieved with 50 pg of GATA-1, GATA-2 or GATA-6 mRNAs.

### G2en affects a subset of ventrally and dorsally expressed genes

To investigate the mechanism by which G2en dorsalises ventral mesoderm, we analysed expression of four genes expressed in this region that are known to have ventralising properties: the related homeobox transcription factors *Vent-1* and *Vent-2*, and the secreted factors *Wnt-8* and *BMP-4*.



The normal expression patterns of these genes have been described (Fainsod et al., 1994; Ladher et al., 1996; Lemaire and Gurdon, 1994; Onichtchouk et al., 1996; Papalopulu and Kintner, 1996; Schmidt et al., 1996) and are shown here for three of the genes at the onset of gastrulation (Fig. 4a,c,e). Injection of 50 pg of G2en into the ventral marginal zone at the 4-cell stage resulted in the repression of *Vent-1* expression (Fig. 4b, 16/20 embryos), but did not affect the expression of *Vent-2* (Fig. 4f, 0/13 embryos). In addition, a clear suppression of *Wnt-8* transcription was seen (Fig 4d, 14/23 embryos). No effect of G2en on *BMP-4* expression could be detected by in situ hybridisation (data not shown), however expression levels of *BMP-4* at stage 10.5 are low and detection by this method is difficult (Dale et al., 1992; Jones et al., 1992). We therefore carried out RT-PCR analysis of VMZs from G2en injected embryos (Fig. 4g), to quantitate the effect of G2en on *BMP-4* and the results were confirmed by RNase protection (not shown). G2en reproducibly had no effect on *BMP-4* expression levels at this stage, while *Wnt-8* levels were again seen to be reduced. Thus, expression of *Wnt-8* and *Vent-1* is suppressed by G2en while that of *BMP-4* and *Vent-2* is unaffected in the early gastrula.

**Fig. 4.** Expression of ventralising genes in G2en-injected embryos at stage 10.5. Panels are in pairs, with uninjected embryos on the left; all embryos are viewed from the vegetal pole, with dorsal at the top. (a) *Vent-1* expression is seen in ventral and lateral mesoderm and in endoderm. (b) G2en injection represses expression of *Vent-1* (16/20 embryos affected), in both mesoderm and endoderm. (c) *Wnt-8* expression is seen in ventral and lateral mesoderm and in endoderm. (d) Injection of G2en represses expression of *Wnt-8* in both mesoderm and endoderm (14/23 embryos affected). (e) *Vent-2* expression is seen in ventral and lateral mesoderm but not endoderm. The probe used was from the *Xom* cDNA (Ladher et al., 1996), which is 100% identical to *Vent-2*. (f) *Vent-2* expression is unaffected by G2en (0/13 embryos affected). (g) Quantitation of levels of gene expression in G2en-injected VMZ explants at stage 10.5 by RT-PCR. G2en injection results in the suppression of *Wnt-8* in VMZ explants, whereas the expression of *BMP-4* was unaffected (lanes 1 and 2). In addition, G2en induced the expression of *chordin* (*chd*). Note that in G2en-injected VMZ explants (lane 2) that the amplification products are slightly underloaded, as judged by levels of FGFR1 expression. All products were amplified over 27 cycles, except *chordin* (*chd*) which was amplified for 26. No non-specific products were seen in any reactions, and no products from genomic DNA were generated, as judged by lack of amplification in the -RT lane (lane 5). All amplifications were performed in the linear range of the reaction, as shown by two-fold increases in product from stage 10.5 whole embryo cDNA (lanes 6-8).

The repression of ventralising gene expression and the whole embryo phenotype suggests that inhibiting GATA activity results in the formation of a partial ectopic organiser. This was investigated directly by examining the expression of genes normally expressed in the dorsal mesoderm. Fig. 5a,c,e show the normal expression patterns of the three genes examined: the homeobox transcription factor *gooseoid* (Cho et al., 1991), and the secreted BMP antagonists *noggin* (Smith and Harland, 1992) and *chordin* (Sasai et al., 1994). In embryos injected with G2en, ectopic expression of *chordin* was seen in the ventral mesendoderm (Fig. 5f, 12/19 embryos, also assayed by RT-PCR, Fig. 4g, and confirmed by RNase protection). In contrast, there was no induction of *gooseoid* expression (Fig. 5b, 0/25 embryos), nor of *noggin* (Fig. 5d, 0/22 embryos).

These results suggest that GATA factor activity is an important regulator of a subset of ventral gene expression, and predict that full length GATA-2 RNA injected dorsally would cause some degree of ventralisation. Injection of GATA-2 at the 100-200 pg level resulted in reduction or loss of anterior structures, whilst higher levels resulted in abnormal gastrulation or toxicity (data not shown). To investigate the effect of GATA-2 at the molecular level, DMZ explants were taken from embryos injected with sub-toxic amounts of GATA-2 (100-200 pg), and were analysed for ventral and dorsal gene expression by RT-PCR at stage 10.5 (Fig. 6). Consistent with the G2en data, a subset of the markers was affected with increases detectable for *Vent-1* and possibly *Wnt-8*, decreases for *chordin* and possibly *gooseoid*, and minimal effects on the other markers. Therefore, the observed phenotype is likely to result from changes in dorsal and ventral gene expression. The weaker effects of the full length protein compared to G2en may reflect the inability to achieve sufficient levels of GATA-2 without toxicity but may also reflect inadequate levels of a critical cofactor in dorsal cells. A large number of partners for GATA factors have now been identified, some of which are essential for transactivation (Durocher et al., 1997; Evans, 1997; Molkenkin et al., 1994, 1998; Tsang et al., 1997; Wadman et al., 1997).

Taken together, the molecular data for G2en and GATA-2 injections implicate GATA activity in the expression of *Vent-1* and *Wnt-8*, and in the suppression of *chordin*.

### Rescue of G2en-induced dorsalisation

In order to determine if these changes in gene expression in G2en injected embryos were responsible for the phenotype observed, we carried out rescue experiments. Rescue was recorded as the normal rounded morphology of G2en-injected VMZ explants, backed up by either the formation of a single axis in whole embryos, or molecular markers.

Coinjection of *Vent-1* with G2en in VMZ explants dramatically reversed the G2en phenotype. Rescue was 100%, as judged by explant morphology (Table 2), which was confirmed by analysis of *cardiac actin* and the ventroposterior marker *Xhox3* (Fig. 7). In comparison to *Vent-1*, rescue by *Wnt-8* was incomplete. Similar results were observed whether measured in whole embryos or in VMZ explants (Table 2). However in these experiments, *Wnt-8* was expressed from a DNA template to avoid activation of the maternal Wnt-like pathway (Sokol et al., 1991). It therefore remains possible that even in the limited confines of a VMZ explant, this partial rescue represents differential diffusion rates of injected DNA and RNA.

G2en injection induces the ectopic expression of *chordin*, a BMP antagonist, suggesting that the activity of BMP-4 becomes compromised during gastrulation. We therefore attempted to rescue G2en-injected embryos by overactivating the BMP pathway. Injection of either BMP-4, or a cell-autonomous activator of BMP signalling – a constitutively active BMP receptor (CA-BR; Candia et al., 1997), led to complete rescue in both cases (Table 3). Whilst these rescues may simply be explained by upregulation of *Vent-1*, a BMP target gene in the ventral mesoderm, it confirms nevertheless the importance of the upregulation of *chordin* to the G2en phenotype. Overall, these rescue experiments indicate that the molecular changes we have recorded are responsible for the dorsalised phenotype we observe.

**Table 2. Rescue of G2en by injection of pCSKA-Wnt-8 plasmid DNA or Vent-1 RNA**

Injection	Total <i>n</i>	Survived <i>n</i>	Rounded/Normal (%)	Elongated (%)	Secondary axes (%)
<b>G2en+Vent-1 in VMZs</b>					
Uninjected	32	32	100	0	–
G2en (50 pg)	32	32	25	75	–
G2en+Vent-1 (100 pg)	32	32	72	28	–
G2en+Vent-1 (200 pg)	33	33	100	0	–
<b>G2en+Wnt-8 in VMZs</b>					
Uninjected	18	18	100	0	–
G2en (50 pg)	16	15	33	67	–
G2en+Wnt-8 (100 pg)	22	22	46	54	–
G2en+Wnt-8 (150 pg)	15	13	54	46	–
<b>G2en+Wnt-8 in whole embryos</b>					
Uninjected	168	150	100	–	0
G2en (50 pg)	30	29	38	–	62
G2en+Wnt-8 (100 pg)	47	40	65	–	35
G2en+Wnt-8 (150 pg)	42	25	76	–	24

Three types of experiment are shown, the last being assayed in whole embryos at stage 34, the other two being assayed in VMZ explants at stage 23 equivalent. Rescue was taken as the formation of a single axis after coinjections with G2en or as the formation of a VMZ with normal spherical morphology. RNAs or DNA were injected, at the amounts shown, into ventral blastomeres at the 4-cell stage.

**Table 3. Rescue of G2en by BMP-4 or CA-BR**

Injection	Total <i>n</i>	Survived <i>n</i>	Rounded/Normal (%)	Elongated (%)	Secondary axes (%)
<b>G2en+BMP-4 in VMZs</b>					
Uninjected	11	11	100	0	–
G2en (50 pg)	14	14	7	93	–
G2en+BMP-4 (250 pg)	15	15	100	0	–
<b>G2en+Ca-BR in whole embryos</b>					
G2en (50 pg)	47	37	40	–	60
G2en+CA-BR (50 pg)	36	22	95	–	5

Two experiments are shown, the first being assayed in VMZs at stage 23 equivalent and the second being assayed in whole embryos at stage 34. Criteria were as described in Table 2. RNAs (amounts as stated) were injected into ventral blastomeres at the 4-cell stage.

### Inhibition of Wnt-8 synthesis does not phenocopy dominant-negative Wnt in coinjections with the dominant-negative BMP receptor

An apparent anomaly in these experiments exists between the whole embryo phenotype and the observed effects on signalling. G2en injection results in repression of both the BMP pathway, via the upregulation of *chordin*, and the Wnt pathway, via an effect on *Wnt-8* transcription. According to the model of Glinka et al. (1997), whilst repression of the BMP pathway alone results in trunk dorsal mesoderm formation, simultaneous repression of BMP and *Wnt* pathways results in the additional formation of head structures. Therefore the phenotype resulting from G2en injection should be a secondary axis that includes head structures. This phenotype was never observed in G2en-injected embryos. One possible explanation for this is that the induced *chordin* expression accumulates too late to inhibit BMP signalling at the time of head mesoderm specification. This view is supported by the normal expression of the *BMP-4* target gene, *Vent-2*, at the onset of gastrulation (see Fig. 5e). We therefore asked what would be the phenotype resulting from G2en injection in embryos in which BMP signalling was inhibited early. To examine this we coinjected either G2en or, as a control, a dominant negative Wnt (dnXwnt-8; Hoppler et al., 1996), with a dominant negative BMP receptor ( $\Delta$ BMPR). Coinjection of dnXwnt-8 with  $\Delta$ BMPR produced embryos with secondary axes containing head structures at high efficiency (Table 4), as previously described (Glinka et al., 1997). Surprisingly however, coinjection of G2en with  $\Delta$ BMPR produced no embryos with ectopic head structures, despite the high proportion of embryos with secondary axes (83%, Table 4). Given the repression of *Wnt-8* transcription by G2en (Fig. 4d and g), this result suggests that *Wnt-8* signalling may not represent the crucial activity suppressed by dnXwnt-8 in head induction. Blockage

by dnXwnt-8 of signalling by Wnts other than *Wnt-8* was also indicated by its effects on animal cap tissue, despite the lack of expression of *Wnt-8* in this region (Glinka et al., 1997).

### DISCUSSION

In this paper we show firstly that inhibition of GATA activity in ventral cells results in dorsalisation, suggesting that GATA activity is an essential requirement for ventral cell fate. Secondly, we show that this effect is not seen in ectoderm and is thus restricted to the ventral mesendoderm. Thirdly, we show that only a subset of ventralising gene activity is affected. The four genes analysed here can therefore be split into GATA-dependent genes (*Vent-1* and *Wnt-8*) and GATA-independent genes (*Vent-2* and *BMP-4*). Finally, we show that suppression of GATA activity leads to an upregulation of *chordin* expression, thereby antagonising BMP signalling.

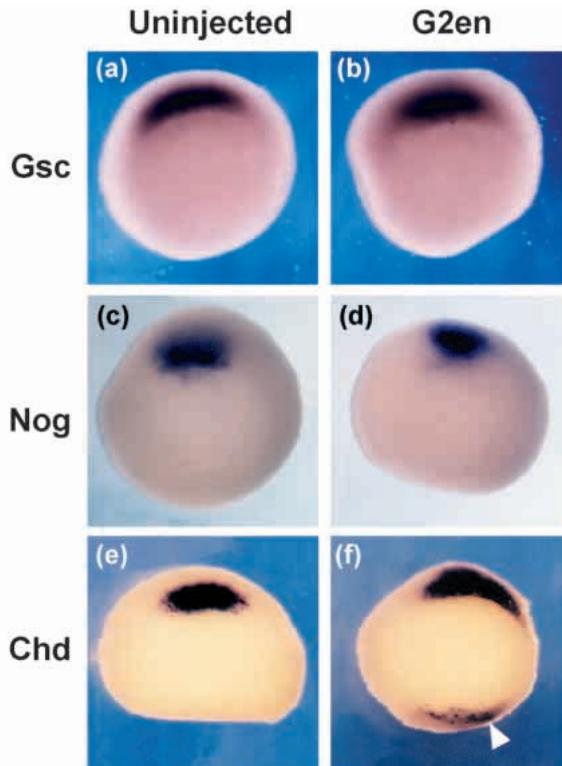
#### The specificity of G2en action

The GATA factor family comprises six members in vertebrates, all of which are able to bind to a consensus GATA site and all of which are able to activate transcription. As for other regulatory gene family members, their overlapping expression patterns are problematic for understanding their specific functions. Whilst specificity of GATA factor function has been demonstrated (Briegel et al., 1993; Gove et al., 1997; Yamagata et al., 1995), considerable evidence exists for overlapping functions of family members, suggesting a degree of redundancy reminiscent of the MyoD family (Rudnicki et al., 1992, 1993). For example, overexpression of *GATA-1*, *GATA-2* or *GATA-3* is sufficient to induce megakaryocytic differentiation in a myeloid cell line (Visvader et al., 1992; Visvader and Adams, 1993), whilst *GATA-1*<sup>-</sup> ES cells,

**Table 4. G2en does not have head inducing activity even when coinjected with  $\Delta$ BMPR**

Injection	Total <i>n</i>	Survived <i>n</i>	Partial secondary axes (%)	Full secondary axes (%)	Normal (%)
Uninjected	115	96	1	0	99
G2en (50 pg)	55	33	36	0	64
G2en+ $\Delta$ BMPR (50 pg)	76	71	83	0	17
dnXwnt-8 50 pg+ $\Delta$ BMPR	39	30	7	67	26

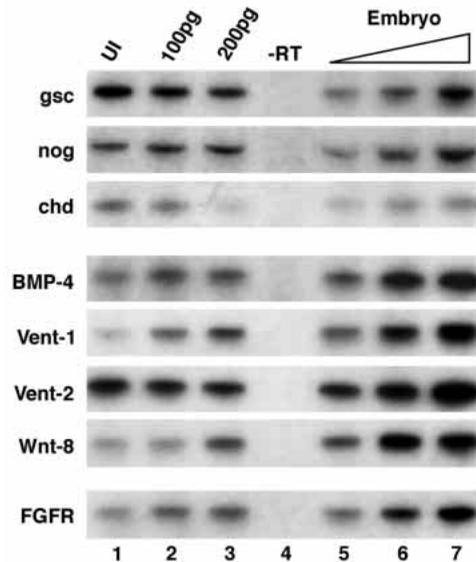
Either G2en or dnXwnt-8 (50 pg) were injected with a concentration of  $\Delta$ BMPR (50 pg) that was optimised for secondary axis formation in previous experiments. Secondary axes were scored as 'full' if they contained head structures with cement glands and eyes, or were classed as 'partial' if head structures were absent.



**Fig. 5.** Expression of dorsalising genes in G2en-injected embryos at stage 10.5. Panels are in pairs, with uninjected embryos on the left; all embryos are viewed from the vegetal pole, with dorsal at the top. (a) *Gooseoid* expression in the organiser. (b) *Gooseoid* is unaffected by G2en injection (0/25 embryos affected). (c) *Noggin* expression. (d) *Noggin* is also unaffected by G2en (0/22 embryos affected) (e) *Chordin* expression. (f) Ectopic *chordin* expression is seen on the ventral side in embryos injected with G2en (arrowhead) (12/19 embryos affected).

differentiated in vitro, express many *GATA-1* responsive genes at wild-type levels possibly as a consequence of elevated *GATA-2* levels (Weiss et al., 1994). Indeed significant rescue of *GATA-1*<sup>-</sup> cells can be effected by *GATA-3*, *GATA-4* or even a fungal *GATA* factor (Blobel et al., 1995; Tsai et al., 1998; Weiss et al., 1997). Similar observations have been made in *GATA-4*<sup>-/-</sup> embryos in which *GATA-6* levels are elevated and heart-specific genes are activated normally (Kuo et al., 1997). In the present study we have shown that *GATA-1*, *GATA-2* and *GATA-6* all rescue the dorsalisation caused by G2en. In addition, ongoing studies with *GATA-5* have revealed that G5en induces secondary axes with similar efficiencies to G2en (H. Weber, C. Symes, A. R. F. R., M. E. W. and R. K. P., unpublished). Whilst confirming that the effects of G2en on the embryo are caused by the antagonism of *GATA* activity, these observations leave open the question of which *GATA* factor is being inhibited.

An important consideration therefore, in the interpretation of this work, is the patterns of expression of the *GATA* factor family. With the exception of *GATA-1*, all members of this family are strongly expressed during gastrulation (Jiang and Evans, 1996; Zon et al., 1991), although *GATA-3* at this stage is restricted to the non-neural ectoderm (Read et al., 1998). The remaining four (i.e. *GATA-2*, *-4*, *-5* and *-6*) are all expressed in



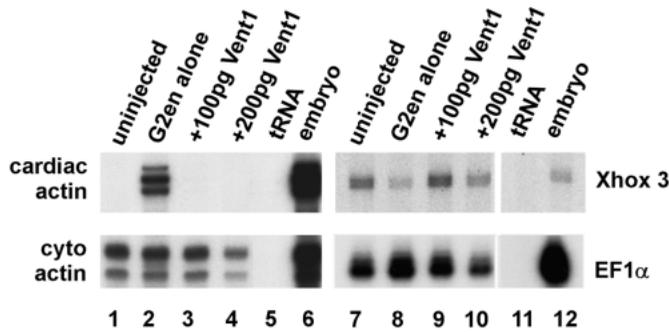
**Fig. 6.** Gene expression in DMZ explants from embryos injected with *GATA-2*. Gene expression was monitored by RT-PCR in stage 10.5 explants. *GATA-2a* RNA, injected either at the 100 pg or 200 pg level (lanes 2 and 3), resulted in down-regulation of the dorsal markers, *chordin* (*chd*) and possibly *gooseoid* (*gsc*), when compared with DMZs from uninjected embryos (UI, lane 1). Analysis of ventral markers showed that *Vent-1* and possibly *Wnt-8* were upregulated, whilst the effect on other ventral genes was negligible. Note that lane 1 is slightly underloaded, as judged by the level of expression of the loading control *FGFR1*. All samples were amplified for 24 cycles, except *chordin* and *gooseoid* which were amplified for 22. No non-specific or genomic products were formed, as shown by the lack of product in samples lacking RT (-RT, lane 4). All amplifications were performed in the linear range of the reaction, as shown by two-fold increases in product from stage 10.5 whole embryo cDNA (lanes 5-7).

ventral mesendoderm (Bertwistle et al., 1996; Walmsley et al., 1994; J. Broadbent, H. Weber and R. K. P., unpublished), whilst *GATA-2* and *GATA-5* are also expressed maternally (Kelley et al., 1993; Partington et al., 1997). These four *GATA* factors are therefore present at the right time and in the right place to be playing the crucial role suggested by these experiments. G2en may act by inhibiting one of these or by repressing target genes common to all of them.

### The role of *GATA* activity in the ventral mesendoderm

Two pathways are important in the specification of ventral mesoderm, represented by *BMP-4* and *Wnt-8*. *BMP* responses appear to be mediated by the *Vent* genes, which are expressed in overlapping domains along the dorsoventral axis. How these genes are related to *Wnt* signalling is still unclear. *Wnt-8* is initially independent of *BMP* signalling, in that it is expressed in a restricted ventral-lateral domain before gastrulation (Christian and Moon, 1993), whilst *BMP-4* acts during gastrulation to pattern the dorsoventral axis (Jones et al., 1996). In addition, the expression of *Wnt-8* occurs normally in dissociated embryos (Lemaire and Gurdon, 1994), suggesting that its early expression does not require *BMP-4*.

Despite the whole embryo phenotype of G2en being



**Fig. 7.** Vent-1 rescues dorsalisation by G2en in VMZ explants. Embryos at the 4-cell stage were injected in the ventral marginal zone with 50 pg of G2en RNA either alone or combined with 100 pg or 200 pg *Vent-1* RNA. VMZ explants were isolated at stage 10 from both uninjected and injected embryos and cultured until stage 18. The RNA prepared from the explants was probed by ribonuclease protection for *cardiac actin* (lanes 1-6) and *Xhox3* (lanes 7-12) using *cytoskeletal actin* and *EF1 $\alpha$*  as loading controls. tRNA (lanes 5 and 11) and RNA extracted from stage 18 whole embryos (lanes 6 and 12) were used as negative and positive controls respectively.

superficially similar to the effect of inhibiting BMP signalling (Graff et al., 1994; Schmidt et al., 1995; Suzuki et al., 1994), there are clear and important differences between G2en and inhibitors of BMPs. Firstly, although one BMP responsive gene (*Vent-1*) is repressed by inhibiting GATA activity, another related gene (*Vent-2*) is unaffected. In addition, the inhibition of GATA activity in ectoderm is unable to induce neural tissue, its effect being restricted to the mesendoderm. Thus, initially at least, G2en inhibits only a subset of BMP responses. Nevertheless the presence of *chordin* in G2en-injected tissue suggests that BMP signalling will become fully compromised during gastrulation, as a consequence of G2en, albeit indirectly. One key therefore to understanding the phenotype of G2en and hence the role of GATA factors in the early embryo is to ask how ectopic *chordin* becomes expressed in the first place, despite BMP-4 being present and active in the early embryo.

In contrast to the BMP pathway, the *Wnt-8* pathway is blocked early by G2en, by virtue of its effect on transcription of the *Wnt-8* gene. This effect on its own is unlikely however to explain the ectopic *chordin* expression, since injection of either *Frzb* or a dominant-negative Wnt does not lead to expression of *chordin* in ventral tissues (Hoppler et al., 1996; Hoppler and Moon, 1998; Leyns et al., 1997). In contrast, the injection of a VP16-Vent-1 fusion construct did lead to an upregulation of *chordin* (Onichtchouk et al., 1998), suggesting that one of the normal functions of *Vent-1* is to repress *chordin* expression in ventral cells. Thus the ectopic *chordin* expression observed in G2en-injected embryos may be a consequence of the repression of *Vent-1*. GATA factor activity is therefore required for two genes which play important roles in restricting organiser gene expression to dorsal cells. This suggests that GATA factors themselves are important regulators of ventral cell fate.

Finally, the observations in this study have allowed us to use G2en as a tool to test the twin repression model for head induction (Glinka et al., 1997). There is strong evidence that a zygotically expressed Wnt is an important regulator of head induction. Two factors (*cerberus* and *dickopf-1*) that can inhibit

overexpressed Wnts can also induce head structures and are expressed in the forming head mesendoderm. Overexpression of these factors, or dnXwnt-8, with a dominant-negative BMP receptor results in the ectopic formation of head structures. Furthermore the expression of *Wnt-8* fits very well with this model, given its ventrally restricted pattern at the time of head formation. If *Wnt-8* represents the crucial factor that is indeed inhibited by the head inducing factors, then G2en in its capacity to inhibit *Wnt-8* production, should phenocopy dnXwnt-8 when coinjected with  $\Delta$ BMPR. With the proviso that it is difficult to compare two different dominant-negative factors that act in different ways, it is nonetheless surprising that G2en and dnXwnt-8 clearly have different effects when coinjected with  $\Delta$ BMPR. The fact that G2en does not, despite its effect on *Wnt-8* transcription, induce head structures implies that inhibition of *Wnt-8* itself may not be the sole requirement for head induction. Other Wnt molecules expressed in the early embryo may therefore be equally important for the process of head formation.

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