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Negative control of *Xenopus* GATA-2 by activin and noggin with eventual expression in precursors of the ventral blood islands

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SUMMARY

To increase our understanding of haematopoiesis during early vertebrate development, we have studied the expression pattern of the transcription factor GATA-2 in *Xenopus* embryos, and asked how this is regulated. We show that the blood island precursors of the ventral mesoderm express GATA-2 RNA at neural tube stages, some 5 hours before globin RNA is detected in their derivatives. Prior to this however, GATA-2 is expressed much more widely within the embryo. Maternal transcripts are uniformly distributed, and zygotic transcription is activated during gastrulation throughout ventral and lateral regions of the embryo, with expression highest in the sensorial ectoderm and only weak in the ventral mesoderm. The domain of GATA-2 expression in neurulae outlines the region of the neural plate and suggests a possible wider role in dorsoventral patterning.

To identify the signals involved in regulating this pattern of expression, we performed experiments with embryo explants. GATA-2 is activated autonomously in isolated animal caps and this activation is suppressed by the mesoderm-inducing factor activin, but not by FGF. Thus, the down-regulation of GATA-2 observed in the region of the Spemann organiser may be a response to an activin-like signal emanating from the dorsal-vegetal region or Nieuwkoop centre. GATA-2 activation in animal caps and ventral marginal zones was suppressed by co-culturing with dorsal marginal zones, suggesting that a signal from the Spemann organiser is involved in suppression of GATA-2 in the dorsal region of the embryo. Expression of a candidate for this signal, noggin, had the same effect. Taken together, the observations presented here suggest that GATA-2 activation occurs by default in the absence of signals, that the restriction of its expression within the early embryo is controlled by negative signals emanating from the Nieuwkoop centre and the organiser, and that noggin and activin-like molecules play a role in these signalling pathways.

Key words: GATA-2, *Xenopus* embryo, activin, noggin, haematopoiesis, ventral mesoderm, animal cap, in situ hybridization

INTRODUCTION

Despite the progress which has been made in understanding the control of haematopoiesis in adult animals, the mechanisms by which haematopoietic cells are first generated in the embryo are largely unknown. The blood cells of vertebrates are derived from pluripotential haematopoietic stem cells, which give rise to progenitors that proliferate and differentiate into the various blood lineages (Moore, 1991). However, the embryonic sources of both the adult stem cells, and those that give rise to the embryonic blood system, are still controversial (Moore and Metcalf, 1970; Medvinsky et al., 1993).

In *Xenopus*, embryonic blood is thought to derive from ventral mesoderm, with adult haematopoietic cells deriving primarily from dorsal lateral plate mesoderm (Kau and Turpen, 1983; Maeno et al., 1985; Dale and Slack, 1987). These conclusions have relied on fate mapping at the 32 cell stage and on embryo transplants at neurula and later stages of development. In between these stages, in the blastula embryo, the mesoderm is induced through an inductive interaction in which vegetal pole blastomeres act on overlying equatorial cells (reviewed by Smith, 1989; Slack, 1994), with the growth factors bFGF and activin emerging as strong candidates for the inducing molecules (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992). In order to relate these early signalling events to blood formation, we need firstly to locate the earliest sites of haematopoietic commitment using probes for genes expressed in blood cell progenitors, and then to study how the early signals affect the expression of these genes. These studies of the specification of ventral and dorsolateral mesodermal tissues may provide insight into more general mechanisms of dorsoventral patterning in the early embryo.

Numerous genes expressed in blood have been shown to contain binding sites for members of the GATA family of zinc

finger transcription factors (Orkin 1992). GATA-1 is the most abundant family member in mature erythroid cells and has been shown to be necessary for the production of these cells by gene targeting experiments in mice (Pevny et al., 1991; Simon et al., 1992). However, differentiation to the proerythroblast stage proceeds apparently normally in the absence of GATA-1 (Pevny et al., 1991), suggesting a non-critical role prior to this stage, which could explain its low levels in haematopoietic progenitors (Mouthon et al., 1993). Furthermore, in early *Xenopus* embryos, the levels of GATA-1 RNA remain low until the tail bud stage of development when globin genes are activated (Zon et al., 1991; Zhang and Evans, 1994). A better candidate probe for blood cell progenitors is GATA-2, which is more abundant both in early *Xenopus* embryos (Zon et al., 1991) and in haematopoietic progenitors (Breigel et al., 1993; Mouthon et al., 1993).

Using in situ hybridisation on *Xenopus* embryos, we show that GATA-2 is expressed in the blood precursor cells of the ventral blood islands. Prior to this, in gastrulae and neurulae, expression occurs throughout ventral and lateral regions of the embryo. The expression in neurulae appears as a 'tennis ball' pattern outlining the neural plate. Using embryo explants and purified signalling molecules, we show that activation of the zygotic gene is autonomous in ventral regions outside the negative influence of dorsalising signals and neural inducers, such as activin and noggin. These data suggest a wider role for GATA-2 in dorsoventral patterning in early embryos.

MATERIALS AND METHODS

Embryos and dissections

Xenopus embryos were obtained by artificial fertilisation as described by Smith and Slack (1983). They were dejellied in 2% cysteine (pH 7.8) and staged according to Nieuwkoop and Faber (1967). Dissections were carried out in MBS (Gurdon and Wickens, 1983) in Petri dishes coated with 1% agarose. For experiments in which explants were treated with activin or bFGF the medium also included 0.1% bovine serum albumin.

Signalling molecules

XTC-MIF (*Xenopus* activin A) was purified from XTC cell-conditioned medium as described by Smith et al. (1990) and used at a concentration of 50 units/ml (see Cooke et al., 1987, for definition of a unit of mesoderm-inducing activity). Recombinant activin A was used at a concentration of 2 ng/ml. Recombinant *Xenopus* basic fibroblast growth factor (bFGF) was used at a concentration of 80 ng/ml. Noggin was expressed in gastrulae by injection of the plasmid pCSKANOG into single cell embryos (Smith et al., 1993, see legend to Fig. 7 for details).

RNA isolation

Embryos or explants were snap frozen in liquid nitrogen in a minimum volume of medium and stored at –70°C. RNA was extracted as described by Sambrook et al. (1989), except that the DNaseI step was omitted. Samples were dissolved in 80% formamide, 0.4 M NaCl, 0.04 M Pipes (pH 6.4), 1 mM EDTA and kept at –70°C until analysis.

RNase protection and in situ hybridisation probes

A stage 17 *Xenopus* embryo λgt10 cDNA library (Kintner and Melton, 1987) was screened at low stringency with a full length *Xenopus* GATA-1 cDNA (kind gift of Len Zon). Positive clones were rescreened with a probe from outside the highly conserved zinc finger region in order to exclude GATA-1. GATA-2 clones were identified by comparison with the published GATA-2 sequence (Zon et al., 1991). To make an RNase protection probe, a 378 bp *Eco*RI fragment covering the zinc finger region of GATA-2 was cloned into the *Eco*RI site of pGEM7. This plasmid was cut with *Pvu*II and *Xba*I and an antisense probe was prepared by transcription of an isolated 680 bp fragment with SP6 RNA polymerase. This produces a probe of 482 nucleotides and a protected fragment of 378 nucleotides. In later experiments (Fig. 7), an improved probe for GATA-2 was used (T. Sykes, unpublished data). A 136bp *Ban*I/*Eco*RI fragment from the 3′ end of the zinc finger region was blunt ended and subcloned into the *Sma*I site of pGEM7. SP6 polymerase transcription yields an antisense probe of 222 nucleotides, which generates a protected fragment of 136 nucleotides using RNase A and T1. For NCAM, an *Eco*RI/*Pvu*II subclone of N1 cDNA (Kintner and Melton, 1987), as used by Dixon and Kintner (1989), was transcribed with SP6 polymerase and a partial product of length 215 nucleotides was excised from a polyacrylamide gel for use as probe. Digestion with RNase T1 yields a doublet of protected fragments of size 208 and 190 nucleotides. Probes detecting EF-1α (see Sargent and Bennett, 1990) and muscle-specific actin (Mohun et al., 1988) were as described. For αT4 globin, a 260 bp *Cla*I/*Bgl*II fragment from the cDNA (Banville and Williams, 1985) was subcloned into the *Cla*I and *Bam*HI sites of pGEM7. An antisense probe of 310 nucleotides length was prepared using SP6 polymerase. Digestion with RNase T1 yields protected fragments of 260 and 230 nucleotides. Signals were quantitated by scanning densitometry and normalised to EF-1α to control for RNA loading.

For in situ hybridisation, the construct used in the RNase protections was used to probe for αT4 globin. For GATA-2, two *Eco*RI fragments, of sizes 1.3 kb and 750 bp, representing the complete coding region of GATA-2 cDNA, were subcloned into pGEM7 and used for in situ hybridisation interchangeably. For whole-mount in situ hybridisation, both probes were used together. Probes were transcribed in both the sense and the antisense directions essentially as described by Harland (1991).

In situ hybridisation

Transverse sections of *Xenopus* embryos at stages 15-26 were mounted on slides coated with 2% 3-aminopropyl triethoxysilane as described by O'Keefe et al (1991). *In situ* hybridisation of sections was carried out using a modification of the whole-mount protocol of Harland (1991). Briefly, paraffin sections were dewaxed in xylene and rehydrated through graded alcohols. They were then fixed in 4% paraformaldehyde in PBS for 20 minutes at room temperature, rinsed in 2× SSPE and digested with 3 μg/ml Proteinase K in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA for 30 minutes at 37°C. Sections were then rinsed again in 2× SSPE, incubated in 0.2 M HCl for 15 minutes, rinsed in 2× SSPE and acetylated in 0.1 M triethanolamine pH 8.0, 0.2% acetic acid, 0.5% acetic anhydride for 10 minutes at room temperature. They were then rinsed in 2× SSPE, dipped in H_2O and prehybridised in hybridisation buffer for 2 hours at 60°C. This was then replaced with hybridisation buffer containing labelled probe at 3 μg/ml. Hybridization conditions were as described by Harland (1991).

Posthybridisation washes, before RNase treatment, were as described by Harland (1991) except that 2× SSPE was used instead of 2× SSC. Slides were then incubated in 20 μg/ml RNase A in 4× SSPE for 30 minutes at 37°C, washed in 50% formamide/2× SSPE at 50°C for 1 hour followed by two 10-minute washes in 2× SSPE/0.3% Chaps at room temperature. Labelled probe was then identified using a detection kit for digoxigenin-labelled nucleic acids (Boehringer Mannheim) essentially according to the manufacturer's instructions. Stained specimens were then fixed in MEMFA for 45 minutes at room temperature, washed in PBS/0.1% Tween-20 and mounted in 30% PBS/70% glycerol.

Whole-mount in situ hybridisations were performed essentially as described by Harland (1991). Albino and non-albino embryos were used.

RESULTS

xGATA-2 is expressed early in the ventral blood islands

The blood islands of *Xenopus* have been identified in late tail bud embryos by morphology, ultrastructure, benzidine staining and in situ hybridisation using a globin probe (Nieuwkoop and Faber, 1967; Mangia et al., 1970; Hemmati-Brivanlou et al., 1990). Detection in both late (stage 35) and mid (stage 24-26) tail bud embryos by whole-mount in situ hybridisation using a probe for αT4 globin is presented in Fig. 1A,B. We have been unable to detect cells containing globin RNA prior to stage 23, even on sectioned material (data not shown), which contrasts with its detection at neurula stages by northern analysis (Zon et al., 1991; Sive and Cheng, 1991). These workers were using a probe for the α larval IA gene (Widmer et al., 1981) which

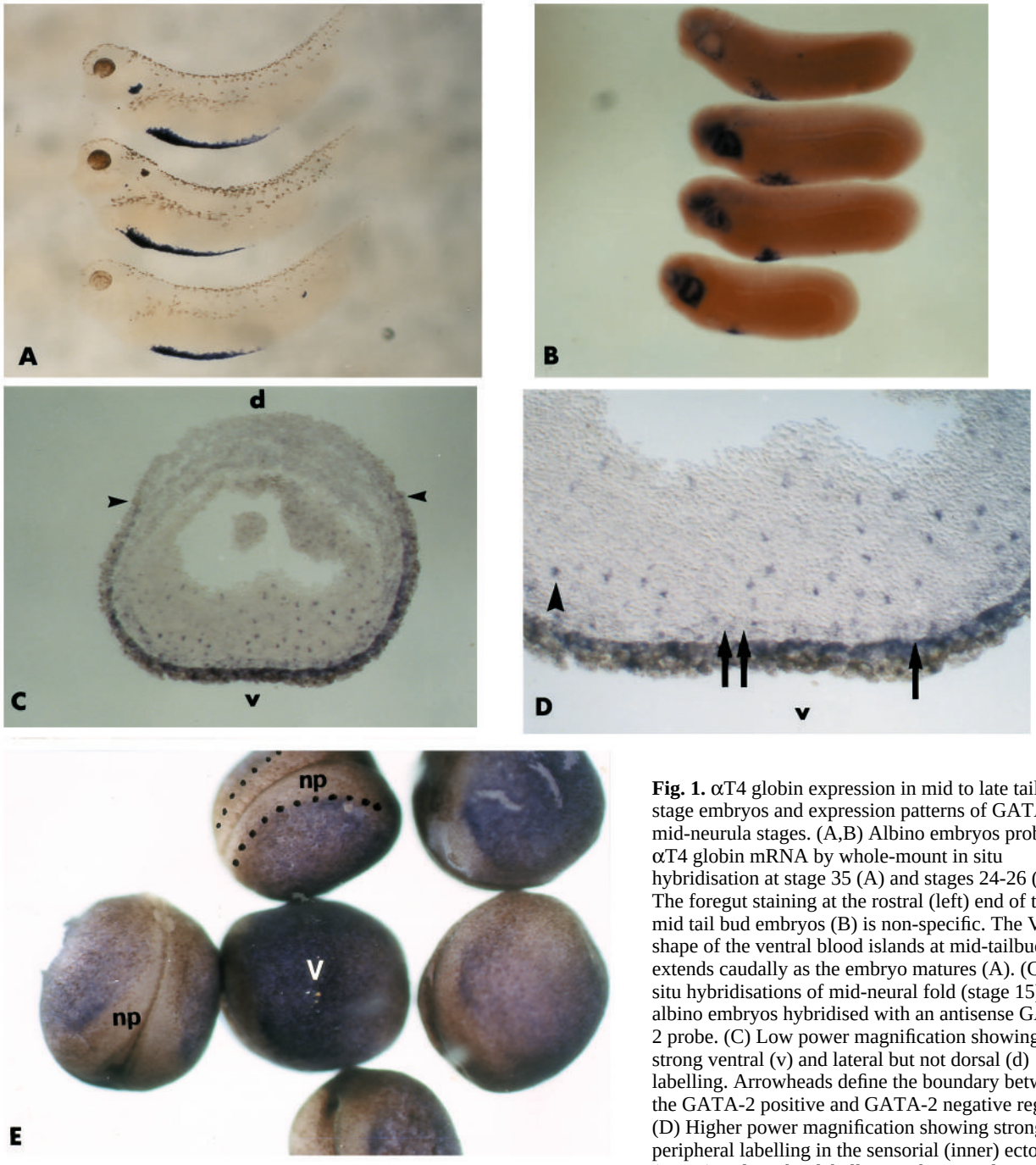

Fig. 1. αT4 globin expression in mid to late tailbud stage embryos and expression patterns of GATA-2 in mid-neurula stages. (A,B) Albino embryos probed for αT4 globin mRNA by whole-mount in situ hybridisation at stage 35 (A) and stages 24-26 (B). The foregut staining at the rostral (left) end of the mid tail bud embryos (B) is non-specific. The V-shape of the ventral blood islands at mid-tailbud (B) extends caudally as the embryo matures (A). (C-E) In situ hybridisations of mid-neural fold (stage 15) non-albino embryos hybridised with an antisense GATA-2 probe. (C) Low power magnification showing strong ventral (v) and lateral but not dorsal (d) labelling. Arrowheads define the boundary between the GATA-2 positive and GATA-2 negative regions. (D) Higher power magnification showing strong peripheral labelling in the sensorial (inner) ectoderm (arrow) and weaker labelling in the mesoderm (double arrows) and endodermal cells (arrowhead). (E) Stage 15 non-albino embryos probed for GATA-2 RNA (purple) by whole-mount in situ hybridisation, before clearing. Staining is apparent throughout the ventral surface up to the edges (dashed line) of the developing neural plate (np, brown due to pigmentation) giving rise to a 'tennis ball' pattern. The embryo in the centre (V) is viewed from the ventral aspect.

in the alternative terminology is the αT3 gene (Banville and Williams, 1985). The αT4 probe used here has 98% nucleotide identity to the αT3 probe used in the northern analyses and therefore the differing results cannot be explained by probe differences. The simplest explanation is that in neurulae the globin RNA is present at a very low background level throughout the embryo, which is below the limits of detection by in situ hybridization.

In order to determine if the cells of the ventral blood islands are GATA-2 positive, mid tail bud embryos (stage 25/26) were sectioned and alternate sections were hybridised with the αT4 globin probe or with a GATA-2 probe (Fig. 2). The strong

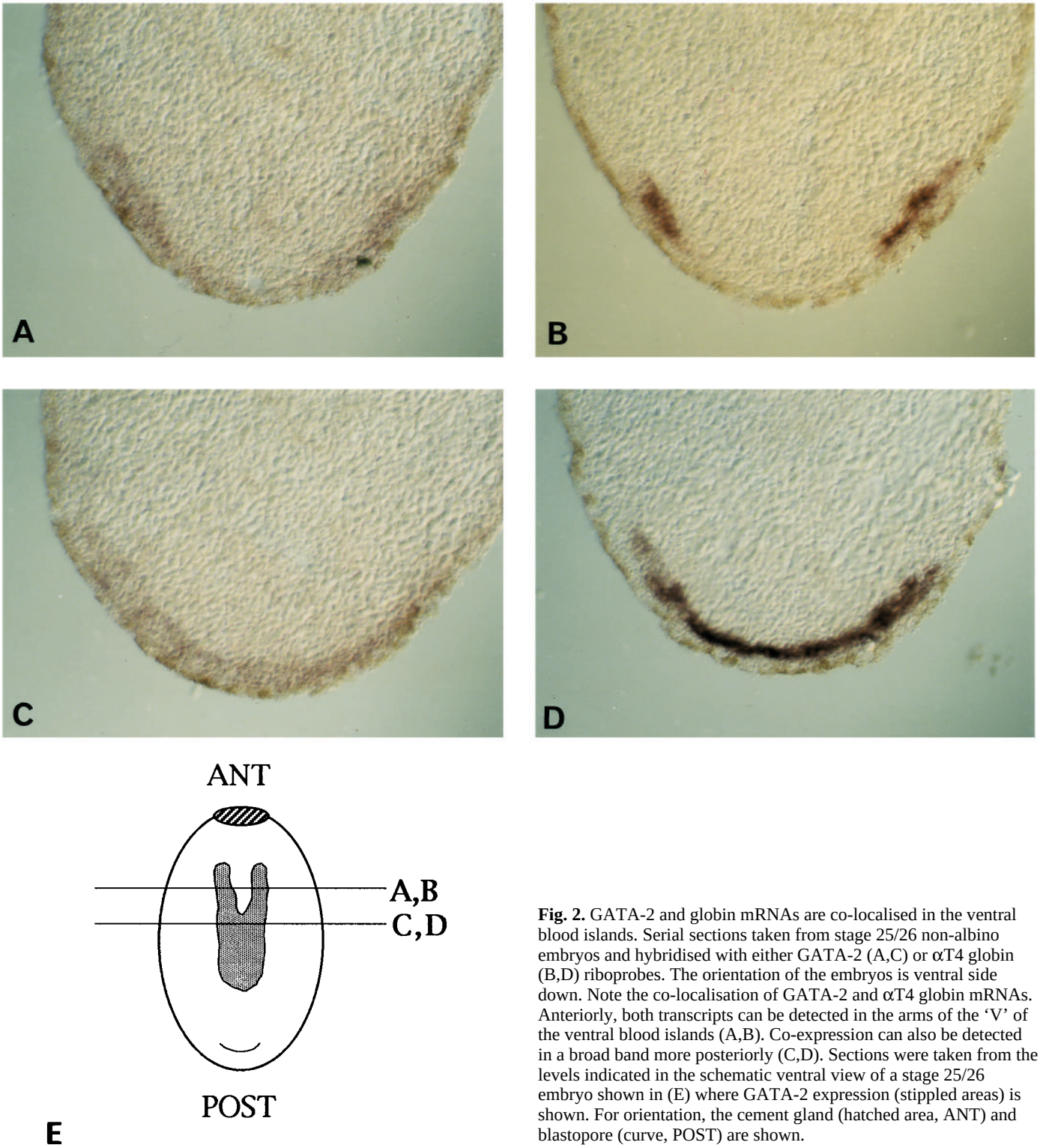

Fig. 2. GATA-2 and globin mRNAs are co-localised in the ventral blood islands. Serial sections taken from stage 25/26 non-albino embryos and hybridised with either GATA-2 (A,C) or αT4 globin (B,D) riboprobes. The orientation of the embryos is ventral side down. Note the co-localisation of GATA-2 and αT4 globin mRNAs. Anteriorly, both transcripts can be detected in the arms of the 'V' of the ventral blood islands (A,B). Co-expression can also be detected in a broad band more posteriorly (C,D). Sections were taken from the levels indicated in the schematic ventral view of a stage 25/26 embryo shown in (E) where GATA-2 expression (stippled areas) is shown. For orientation, the cement gland (hatched area, ANT) and blastopore (curve, POST) are shown.

globin signal in the ventral mesoderm (Fig. 2B,D) overlaps the weaker GATA-2 signal (Fig. 2A,C) in a characteristic 'V' shape (Fig. 2E and Mangia et al., 1970), suggesting that the majority of the cells in this region contain both RNAs. Thus, GATA-2 is indeed expressed in the ventral blood island cells.

Studies on adult haematopoiesis in mammals and the chicken have revealed that GATA-2 is strongly expressed in progenitor cells (Mouthon et al., 1993; Breigel et al., 1993). Therefore, if embryonic haematopoiesis resembles that in adults, we might expect the cells of the ventral blood islands

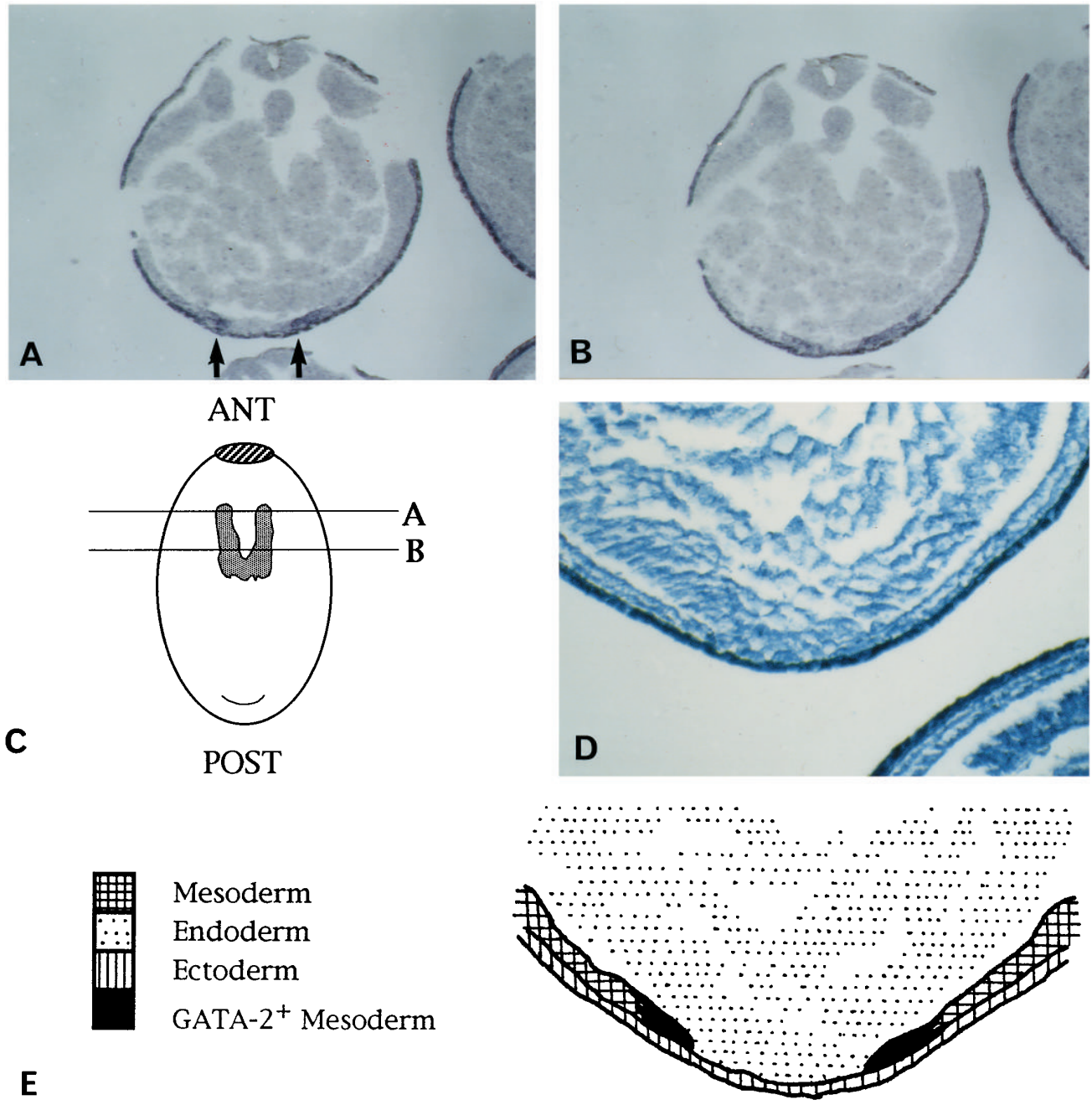

Fig. 3. GATA-2 is localised in presumptive haematopoietic cells before globin expression is established. Late neural tube (stage 21) non-albino embryos were hybridised with an antisense GATA-2 probe. (A,B,D,E) The orientation of the embryos is ventral side down. (A) Transverse section through the anterior end of the presumptive blood islands showing ventral position and two discrete expression domains (arrows). (B) Section taken just posterior to (A) showing the two discrete expression domains closer together. (C) Schematic diagram of the ventral aspect of a stage 21 embryo showing the mesodermal expression of GATA-2 (stippled area) reconstructed from examination of serial sections. Note the 'V' of the future blood islands. For orientation, the cement gland (hatched area, ANT) and blastopore (curve, POST) are shown. (D) Section from the same region as in (A), at higher power, stained with brilliant cresyl blue for histological detail. (E) A schematic illustration of the section shown in (D) to indicate the three primary germ layers. Note that in this position along the anteroposterior axis of the embryo, the mesoderm does not extend to the ventral midline. Areas of GATA-2 expression (shown in black) are restricted to the medial edges of the mesoderm in this region.

to be GATA-2 positive before globin can be detected. To test this, late neural tube embryos (stage 21) were sectioned and hybridised to the GATA-2 probe (Fig. 3). The characteristic 'V' shape was apparent even at this stage using the GATA-2 (but not the αT4 globin) probe (Fig. 3A-C). Thus, the cells detected with GATA-2 at these early stages are very likely to be haematopoietic progenitors.

By comparing the in situ hybridisation data with the histology of an equivalent section (Fig. 3D,E), we can see that the expression domains of GATA-2 correspond to the medial edges of the ventral mesoderm. Thus, the characteristic 'V' shape of the ventral blood islands reflects the arrangement of the mesoderm in this region, which is continuous caudally but discontinuous rostrally. It has been noted previously, using light and electron microscopy, that the consequential 'groove-shaped' endoderm surrounds and makes intimate contacts with the blood island mesoderm (Mangia et al., 1970). Our data are consistent with these earlier observations. The significance of this arrangement for blood island differentiation is discussed below.

xGATA-2 is expressed more broadly at earlier stages of development

With a view to identifying the blood precursors earlier in development, in situ hybridisation was carried out on mid neural fold embryos (stage 15). Surprisingly, GATA-2 RNA was detected throughout the ventral and lateral parts of the embryo in all three germ layers (Fig. 1C-E). Expression was strongest in the sensorial ectoderm and in the nuclei of endodermal cells (arrow and arrowhead in Fig. 1D), with only weak staining visible in the ventral mesoderm from which the blood islands derive (double arrows in Fig. 1D). Nuclear expression, as seen in the endoderm, has been reported previously for a number of other genes soon after their initial activation in *Xenopus* embryos and is thought to reflect rapid synthesis of the primary transcript (Smith and Harland, 1991). When viewed by whole mount, the pattern of expression resembles half a tennis ball where the other half contains the neural plate (Fig. 1E).

RNase protection assays of dissected neurula and tail bud embryos are consistent with the patterns of expression described above (Fig. 4B, lanes 8-17). The signal detected in the anterior region of tail bud embryos (stage 22, lane 16) has

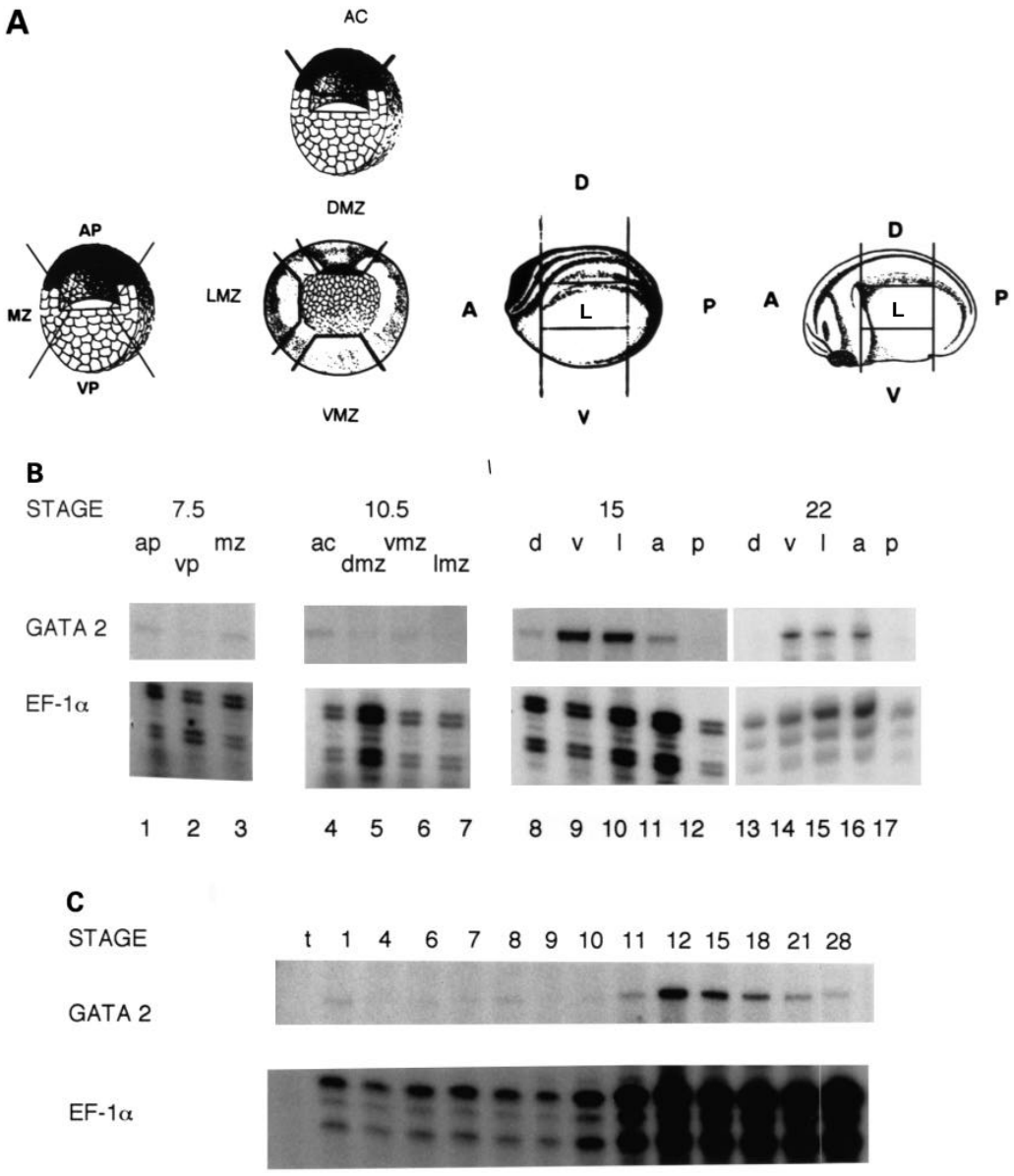

Fig. 4. Spatial and temporal distribution of GATA-2 during early development up to stage 28. (A) Embryo dissections. Embryos were dissected into animal pole (AP), vegetal pole (VP) and marginal zone (MZ) at stage 7.5 (lanes 1-3), into animal cap (AC), dorsal marginal zone (DMZ), ventral marginal zone (VMZ) and lateral marginal zone (LMZ) at stage 10.5 (lanes 4-7), or into dorsal (D), ventral (V), lateral (L), anterior (A) and posterior (P) regions at stages 15 and 22 (lanes 8-17). (B) RNase protection analysis. RNA was extracted from 5 explants in each case and analysed using antisense probes for GATA-2 and EF1-α as a loading control. Results were quantitated by scanning densitometry. (C) RNase protection analysis showing expression of GATA-2 from the fertilised egg (stage 1) through to tail bud (stage 28). RNA from 10 embryos at each developmental stage was analysed using antisense probes for GATA-2 and EF1-α as a loading control. tRNA (t) acted as a negative control.

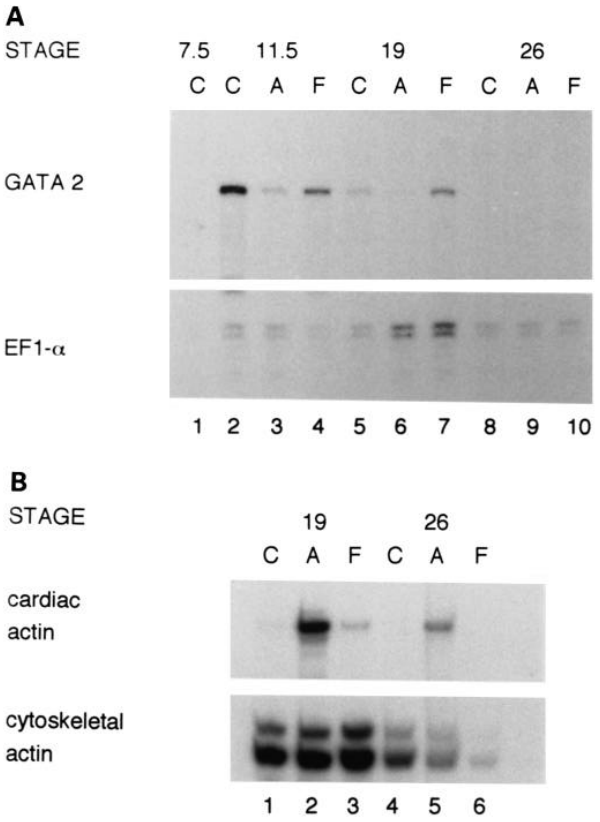

Fig. 5. Response of GATA-2 to mesoderm induction factors in vitro. (A) Animal caps were dissected at stage 7.5. Some were snap frozen immediately (lane 1), others were cultured for various lengths of time either alone (lanes 2, 5 and 8), or in the presence of activin (lanes 3, 6 and 9) or bFGF (lanes 4, 7 and 10). RNA extracted from 5 animal caps was analysed by RNase protection for GATA-2 and EFlα. Results were quantitated by scanning densitometry and relative GATA-2 levels were determined after normalisation to EF1α. (B) The same RNA samples at stages 19 (lanes 1-3) and 26 (lanes 4-6) were analysed by RNase protection for cardiac actin (cytoskeletal actin detected by this same probe is used as a loading control). C, control animal caps cultured alone; A, animal caps cultured in the presence of 50 units/ml activin; F, animal caps cultured in the presence of 80 ng/ml bFGF.

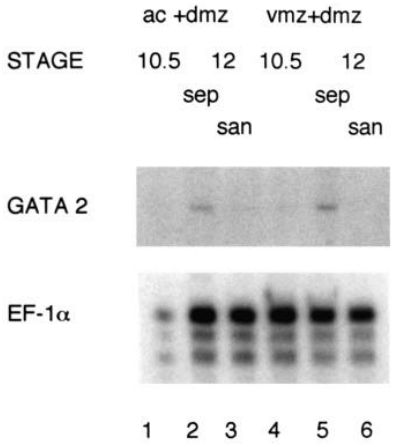

Fig. 6. Inhibition of GATA-2 expression by dorsal marginal zone cells in cultured explants. Embryos at stage 10.5 were dissected into animal cap (ac), dorsal marginal zone (dmz) and ventral marginal zone (vmz) explants (see Fig. 4A). Some were snap frozen immediately as ac/dmz pools (lane 1) or as vmz/dmz pools (lane 4). Others were cultured either separately (sep, lanes 2 and 5) or as ac/dmz conjugates (san, lane 3) and vmz/dmz conjugates (san, lane 6) until stage 12. RNA was extracted from the equivalent of 2.5 embryos and analysed by RNase protection for GATA-2 and EFlα as a loading control.

also been detected by in situ hybridisation and represents expression in the future anterior pituitary and midbrain (data not shown).

Zygotic GATA-2 expression commences at the beginning of gastrulation (Fig. 4C and Zon et al., 1991). To determine if the ventral and lateral expression pattern of GATA-2 observed in neurulae reflects the domains of initial expression, we carried out RNase protections on dissections of early gastrulae (stage 10.5). GATA-2 expression was detectable throughout the embryo but at significantly lower levels in the dorsal marginal zone, suggesting that activation of the zygotic gene occurs predominantly ventrally and laterally (Fig. 4B, lanes 4-7). Prior to this, in blastulae (stage 7.5), a low level of maternal GATA-2 message appears (when RNA loadings are taken into account) to be evenly distributed (Fig. 4B, lanes 1-3).

Zygotic xGATA-2 is activated autonomously and suppressed as part of dorsalisation and neural induction

During early *Xenopus* development, signals are thought to emanate from the vegetal cells of the blastula and commit the overlying animal cap cells to a mesodermal fate (reviewed by Smith, 1989 and Slack, 1994). Two strong candidates for these signals are activin and FGF (Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992). A signal from the dorsal vegetal region, which may be related to activin, is thought to induce the Spemann organiser. This in turn dorsalises the ventral mesoderm of the kind formed in response to FGF. A very strong candidate for the organiser-derived signal is noggin, which has both dorsalising and neural inducing activities (Smith and Harland, 1992; Lamb et al., 1993; Smith et al., 1993). In order to understand how the early pattern of GATA-2 expression relates to these signalling events, we conducted experiments using embryo explants and the purified signalling molecules described above.

(i) Autonomous activation of xGATA-2 and suppression by activin

Animal caps were dissected from *Xenopus* embryos at stage 7.5 (early to mid blastula) and cultured in the presence or absence of levels of activin or bFGF known to induce mesoderm (Cook et al., 1987). GATA-2 expression was measured when sibling embryos had developed to stages 11.5, 19 or 26 (Fig. 5). At stage 11.5, strong expression of GATA-2 was detected in animal caps cultured in the absence of added factors (Fig. 5A, lane 2). A similar result was observed in the presence of bFGF when RNA loading differences are taken into account (Fig. 5A, lane 4). In contrast, a significant suppression of GATA-2 expression was caused by activin (Fig. 5A, lane 3). At stage 19,

levels of GATA-2 expression were lower, but the same pattern was observed. The effect of activin in suppressing GATA-2 expression is unlikely to be due to toxicity, because high levels of cardiac actin were induced in these animal caps (Fig. 5B, lane 2). Similarly the failure of bFGF to affect GATA-2 expression was not due to lack of induction because explant elongation was observed (Howard and Smith, 1993) along with a characteristically weak signal for cardiac actin (Fig. 5B, lane 3). By stage 26, GATA-2 expression levels had declined in a manner similar to that in the intact embryo (Fig. 4C). Thus activation of zygotic GATA-2 expression in animal caps occurs autonomously, and can be down-regulated by activin, an inducer of dorsal mesoderm. The lack of effect observed for bFGF, at least under the conditions used, is consistent with the presence of GATA-2 transcripts in ventral mesoderm (Fig. 1C,D).

(ii) Signals from the dorsal marginal zone down-regulate GATA-2 expression

The suppression of GATA-2 expression by activin suggests that reduced levels of GATA-2 transcripts in the dorsal marginal zone (Fig. 4B, lane 5) may be due to the earlier receipt of activin-like inducing factors from the dorsal-vegetal region of the embryo or Nieuwkoop centre. An additional role may be played by the 'dorsalising' signals derived from the dorsal marginal zone or organiser region (Lettice and Slack, 1993). To investigate this, tissue dissected from the ventral marginal zone, or from the animal cap at stage 10.5, was cultured in contact with dorsal marginal zones until stage 12, when GATA-2 expression is maximal in the intact embryo. As controls, the explants were cultured separately. Fig. 6 shows that by comparison with explants cultured separately (lanes 2 and 5), co-culture with the dorsal marginal zone is sufficient to suppress GATA-2 mRNA accumulation in both the ventral marginal zone (lane 6) and the animal cap (lane 3). Thus, the DMZ appears to emit a signal capable of suppressing GATA-2 expression in cells of both the animal cap and the VMZ, which suggests that down-regulation of GATA-2 may occur as a response to a dorsalising signal.

(iii) Noggin mimics the effects of the dorsal marginal zone on GATA-2 expression in animal caps and ventral marginal zone explants.

A strong candidate for the dorsal marginal zone (DMZ) signal that down-regulates

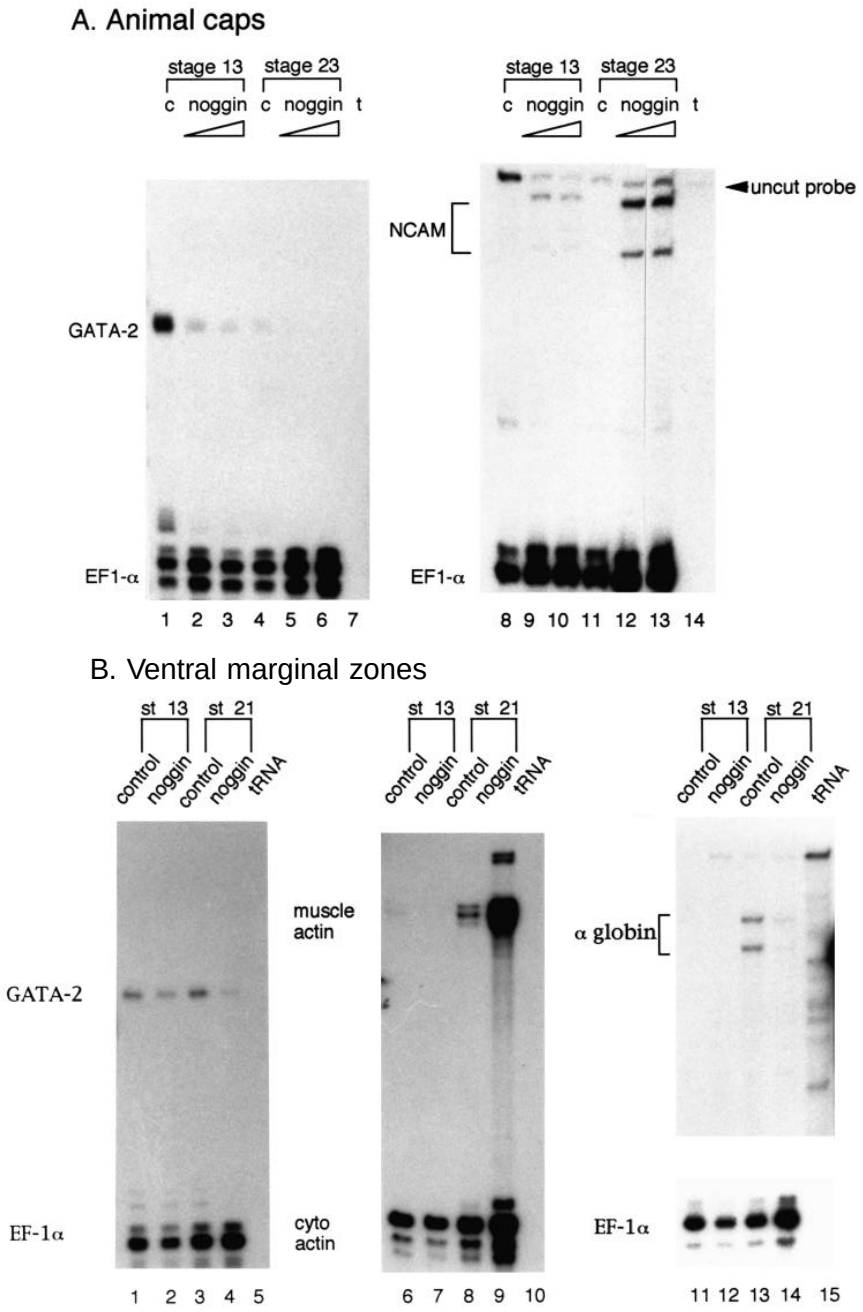

Fig. 7. Noggin mimics the signal from DMZ that down-regulates GATA-2 expression in animal caps and VMZ. (A) Single cell embryos were injected in the animal pole with 200 pg of a control TKβgal plasmid (lanes 1,4,8 and 11) or with 100 pg (lanes 2, 5, 9 and 12) or 200 pg (lanes 3, 6, 10 and 13) of the noggin-expressing plasmid, pCSKANOG. Animal caps were removed at stage 8 and cultured to stage 13 (lanes 1-3 and 8-10) or to stage 23 (lanes 4-6 and 11-13). RNA prepared from injected animal caps was probed by RNase protection for GATA-2 (lanes 1-7) or NCAM (lanes 8-14) and for EF1-α as a loading control. tRNA was used as a negative control (lanes 7 and 14). (B) Single cell embryos were injected into the centre of the embryo with 200 pg of a control TKβgal plasmid (lanes 1, 3, 6 and 8) or with 200 pg of the noggin expressing plasmid, pCSKANOG (lanes 2, 4, 7 and 9). VMZ explants were dissected at stage 10-10.5, removing the animal pole ectoderm lying directly above the VMZ to eliminate the ectodermal GATA-2 signal. VMZs were cultured to stage 13 (lanes 1, 2, 6 and 7) or to stage 21 (lanes 3, 4, 8 and 9). RNA prepared from VMZ explants was probed by RNase protection for GATA-2 (lanes 1-5), muscle actin (lanes 6-10) or αT4 globin (lanes 11-15). EF1-α (lanes 1-5 and 11-15) and cytoskeletal actin (lanes 6-10) acted as loading controls. tRNA was used as a negative control (lanes 5, 10 and 15).

GATA-2 in animal caps and ventral marginal zone (VMZ) explants is noggin. Zygotically expressed noggin has two distinct activities: it induces ectoderm to form neural tissue (Lamb et al. 1993) and dorsalises ventral mesoderm (Smith et al., 1993). To test the effects of noggin on GATA-2 expression in both these germ layers, the noggin plasmid pCSKANOG, which is expressed during gastrula stages (Smith et al., 1993), was injected into single cell embryos. A TKβgal plasmid was injected into control embryos. Animal cap ectoderm was removed from injected embryos at stage 8 and cultured to stage 13 when GATA-2 is optimally expressed, or to stage 23 when the neural marker NCAM is abundantly expressed (Kintner and Melton, 1987). When compared to TKβgal-injected animal caps, noggin-injected caps displayed a down-regulation of GATA-2 expression at stages 13 and 23 (Fig. 7A, lanes 1-6), whilst NCAM was up-regulated by noggin at both stages in the same RNA preparations (Fig. 7A, lanes 8-13). Thus noggin mimics the signal from DMZ that down-regulates GATA-2 expression in animal cap ectoderm.

Ventral mesoderm (VMZ) explants were removed from injected embryos at stage 10-10.5 and cultured to stages 13 and 21. By comparison to TKβgal-injected controls, VMZs from noggin-injected embryos displayed a down-regulation of GATA-2 expression at both stages (Fig. 7B, lanes 1-4). Stimulation of muscle actin (lanes 8, 9) and suppression of globin (lanes 13, 14) demonstrated that the ectopic expression of noggin had sucessfully dorsalised ventral mesoderm. In addition, VMZs from noggin-injected embryos cultured to stage 21 became elongated (data not shown), a characteristic of dorsal development. Thus the ectopic expression of noggin served both to dorsalise VMZ explants and to repress GATA-2 expression.

These experiments are consistent with noggin being a DMZ signal, which down-regulates GATA-2 in animal cap and VMZ explants, and they show that GATA-2 is down-regulated during both dorsalisation and neural induction. Thus the absence of GATA-2 signal in dorsal ectoderm and mesoderm shown by RNase protection and in situ hybridisation of gastrulae and neurulae (Figs 1, 4), may be the result of noggin signalling from the organiser during gastrulation. It is also possible that the activin down-regulation of GATA-2 in animal caps (Fig. 5) is a secondary effect resulting from the production of noggin by the induced dorsal mesoderm.

DISCUSSION

GATA-2 expression and the origins of haematopoietic cells in the embryo

In amphibia, the first location of haematopoietic cells is in the ventral blood islands (which are functionally equivalent to the avian and mammalian yolk-sac blood islands), while later contributions to the blood come from the dorsal lateral plate mesoderm (Kau and Turpen, 1983; Maeno et al., 1985). Previous work, based on morphology, ultrastructure and benzidine staining (Nieuwkoop and Faber, 1967; Mangia et al., 1970), and even in situ hybridisation using a globin probe (Hemmati-Brivanlou et al., 1990), had not visualised formation of the blood islands before stage 31. Here we show that in situ hybridzation, using GATA-2 as a probe, allows detection of the prospective blood islands at stage 21 (late neurula). We have even detected a weak signal in these regions at stage 19 (data not shown). These cells are negative for globin at these stages, although we show that the same regions become globin positive at stage 25/26. This strongly suggests that the GATA-2 positive cells at stages 19-21 represent haematopoietic precursors.

We have not detected GATA-2 in the dorsal lateral plate (DLP) region by in situ hybridisation of embryos up to stage 22. This could mean that GATA-2 plays no role in haematopoiesis deriving from the DLP, although this is unlikely in view of its presence at high levels in precursors of the adult blood in chickens and humans (Breigel et al., 1993; Mouthon et al., 1993). Alternatively, GATA-2 expression may be activated in the DLP later than the stages examined. This interpretation would be consistent with the later contribution of the DLP to blood (Kau and Turpen, 1983; Maeno et al., 1985) and the results of transplantation experiments in which DLP cells from stage 22 embryos grafted into the VBI do not differentiate into blood, suggesting that the cells are not yet committed to haematopoiesis (Maeno et al., 1985). In this scenario, the signal committing the VBI to haematopoiesis would have occurred earlier. Thus, GATA-2 expression may play an early role in haematopoietic commitment.

GATA-2 expression and dorsoventral patterning

GATA-2 is expressed ventrally and laterally from the time of its activation during early gastrulation. Similar domains of expression are observed for zebrafish GATA-3 (Neave et al., unpublished data) and a novel *Xenopus* GATA factor that we have recently isolated (Nijjar et al., unpublished data). Thus, it is possible that this family of transcription factors defines ventral and lateral territory during early development. Wnt-8 is expressed in an overlapping pattern in the mesoderm (Christian and Moon, 1993), while noggin and goosecoid have complementary expression patterns (Smith and Harland, 1992; Cho et al., 1991). Xotch, the *Xenopus* homologue of *Drosophila* notch, also has a complementary expression pattern in the neurectoderm (Coffman et al., 1993), raising the possibility that the GATA factors are involved in defining the boundary of the neural plate. The boundaries of GATA factor expression (the 'seams' of the 'tennis balls') appear to overlap the expression domains of the twist gene and members of the distal-less family (Hopwood, 1989; Papalopulu and Kintner, 1993; Akimenko et al., 1994). It will be interesting to compare the precise boundaries of expression for all these control molecules.

Control of GATA-2 expression by signalling molecules

Activation of zygotic GATA-2 expression is autonomous, in the sense that the gene is turned on in animal cap cells from blastulae in the absence of added inducers. This expression can be prevented by the actions of activin, dorsal marginal zone cells or noggin. DMZ cells and noggin can also suppress expression in ventral marginal zones which contain ventral mesoderm. Thus, it seems that the signals thought to induce dorsal and neural structures in the embryo act to suppress GATA-2 expression in these regions, resulting in the ventral and lateral expression domain observed in early embryos.

Currently favoured models for early signalling in *Xenopus*

involve two mesoderm-inducing signals from presumptive endoderm in blastulae, one of which (the Nieuwkoop signal) induces the organiser. Subsequently, signals emanating from the organiser dorsalise the induced mesoderm (Lettice and Slack, 1993 and references therein). The suppression of GATA-2 expression in animal caps by activin, a candidate for the Nieuwkoop signal, is consistent with the absence of GATA-2 RNA in the region of the organiser in early gastrulae. However, we do not know if this is a direct effect. For example, noggin is expressed in the organiser region as early as stage 9 (Smith and Harland, 1992) and we have shown that noggin suppresses GATA-2 expression. Thus, the absence of GATA-2 in the vicinity of the organiser is likely to reflect noggin activity and the suppression of GATA-2 expression in animal caps by activin may also be a consequence of noggin induction. Noggin has also been shown to have the properties of a neural inducer (Lamb et al., 1993) and we have shown that noggin can suppress GATA-2 expression in animal cap cells. Thus, the absence of GATA-2 RNA in the early neurectoderm may also reflect noggin activity.

An emerging addition to current signalling models is the modulation of the effects of dorsalising signals by 'ventralising' signals. Strong candidate molecules for these signals are Wnt-8 (Christian et al., 1991) and BMP-4 (Dale et al., 1992; Jones et al., 1992). Thus, in addition to the negative signalling by the molecules described above, the ventral expression of GATA-2 may reflect a contribution from positive signalling by Wnt-8 and/or BMP-4. Experiments to test the effects of these molecules are in progress.

The later predominance of GATA-2 expression in the blood island precursors of the ventral mesoderm is likely to result from further signalling events, possibly including the 'ventralising' molecules described above. It is known that presumptive blood island mesoderm cannot completely differentiate in the absence of endoderm (Smith et al., 1989; Keller et al., 1993) or of ectoderm (Maeno et al., 1992), suggesting that ventral endoderm or ectoderm may be the sources of such signals. Alternatively, the unusual arrangement of the three germ layers in the blood island region may have consequences for signalling. Thus, the haematic cords form from the cells at the medial edge of the ventral mesoderm (Fig. 3) and between the arms of the 'V', direct contacts between the endoderm and the ectoderm could generate one of the required signals. A further possibility, in line with the pattern of negative signalling described thus far, is that expression in the ventral region of the embryo outside the medial edges of the mesoderm could be subject to down-regulation. The final predominance in the ventral mesoderm would then represent a default pathway for cells escaping all the negative signalling throughout early development. The increased intensity of the GATA-2 signal over that observed in the mesoderm at earlier stages might then reflect positive autoregulation (Schwartzbauer et al., 1992). It will be important to identify these later signals in order to understand blood cell commitment more fully.

Activin, GATA-2 and erythroid differentiation

It has been known for some time that activin induces erythroid differentiation, both in human bone marrow cultures and in mouse and human erythroid cell lines (Eto et al., 1987; Frigon et al., 1992; Shao et al., 1992; Yu et al., 1987). Furthermore, GATA-2 has been found to be enriched in early haematopoietic cells and to be down-regulated as erythroid differentiation proceeds (Breigel et al., 1993; Mouthon et al., 1993). Indeed, maintenance of GATA-2 expression blocks erythroid differentiation (Breigel et al., 1993). Thus, whilst the patterning of GATA-2 expression in the early embryo may involve activin signalling, it is tempting to suggest that the later suppression of GATA-2 during erythroid differentiation may also be effected by an activin-like molecule.

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