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GATA-1 Inhibits the Formation of Notochord and Neural Tissue in *Xenopus* Embryo

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The expression of *GATA-1*, which encodes for a hemopoietic transcription factor, initiates at gastrula stage in the *Xenopus* embryo (1). In order to examine a possible function of *GATA-1* in dorso-ventral patterning of mesoderm and ectoderm derivatives, the synthesized RNA of *GATA-1* was overexpressed in embryonic cells to assess its biological effects. In the embryos injected with *GATA-1* RNA in the dorsal marginal zone at 4-cell stage, dorsal epidermis did not cover the vegetal cells so that the gastrulation was not completed. The same dose of *GATA-1* RNA injected into ventral marginal zone did not influence the development, and *GATA-2* RNA transcribed from the same vector had little effect, suggesting that this phenomenon is physiologically important. The morphological and immunohistochemical studies revealed that notochord and neural tissue were mostly eliminated in the embryos or the dorsal marginal zone explants after injection of *GATA-1* RNA. *GATA-1* also inhibited neurogenesis in animal cap explants, which was induced by the injection with *noggin* RNA. Northern blot analysis using dorsal marginal zone explants showed, however, that only a slight amount of *α-globin* message was induced, and cardiac *α-actin* message was retained. Therefore, *GATA-1* did not convert completely the dorsal phenotype to the ventral one. Furthermore, the injection of *GATA-1* RNA didnot alter the expression of early dorsal and ventral markers at the onset of gastrulation. These results suggest that GATA-1 is an potential inhibitor of the dorsalization and the neurogenesis, but it affects on the specification of dorsal tissues in relatively later steps.

The *Xenopus* embryo is an excellent model system to study the specification of lineages from multi-potential embryonic cells. The recent extensive studies have shown that dorso-ventral specification is regulated by the members of the TGF-β super family, such as activin, BVg-1, and BMP-4. The activin/BVg-1 induces dorsal types of mesoderm in animal cap assay, thus this signal has a dorsalizing function and induces the expression of Spemann's organizer-specific genes, such as *goosecoid* (2), *Xlim* (3), *noggin* (4), *follistatin* (5) and *chordin* (6). On the other hand, BMP-4 is a key factor in the formation of ventral mesoderm (7, 8, 9). The recent functional studies utilizing dominant negative receptor (10, 11, 12) showed that BMP-4 prevents the prospective ventral mesoderm from differentiating into the dorsal axial structures (13, 14).

Many investigators have made efforts to elucidate downstream factors of BMP-4 signaling, and isolated several homeobox-containing transcription factors which possess the ventralizing activity (15, 16, 17, 18, 19, 20). Among these factors, mix-1 could be a key target of BMP-4 signaling for blood cell development, since the overexpression of mix-1 caused differentiation of functional erythrocytes in dorsal marginal zone explants (20). Furthermore, as a cellular common component of TGF-β signaling molecules, mad-1 (Smad-1) was shown to have an activity to ventralize explants and induce erythrocyte differentiation (21, 22).

In mammals, a group of Zn-finger motif has an important role in determining erythroid and myeloid cell lineages (23). Both *GATA-1* and *GATA-2* genes are expressed in erythroid progenitor cells, but *GATA-2* is expressed at earlier stages and in a broader distribution (24, 1). As the expression of *GATA-1* arises, the expression of *GATA-2* is down-regulated during terminal differentiation (14). Knockout experiments of these factors in mice showed that both factors are unequivocal for red blood cell program, but loss of *GATA-2* causes more general defects in whole hemopoietic lineages (25, 26).

In *Xenopus*, *GATA-1* and *GATA-2* were expressed in the prospective ventral blood island mesoderm. In gastrula to neurula stages, however, *GATA-2* is expressed in both prospective epidermis and ventral areas (24, 1), whose expression pattern is similar to that of *BMP-4*. In the previous study, we showed that *BMP-4* regu-

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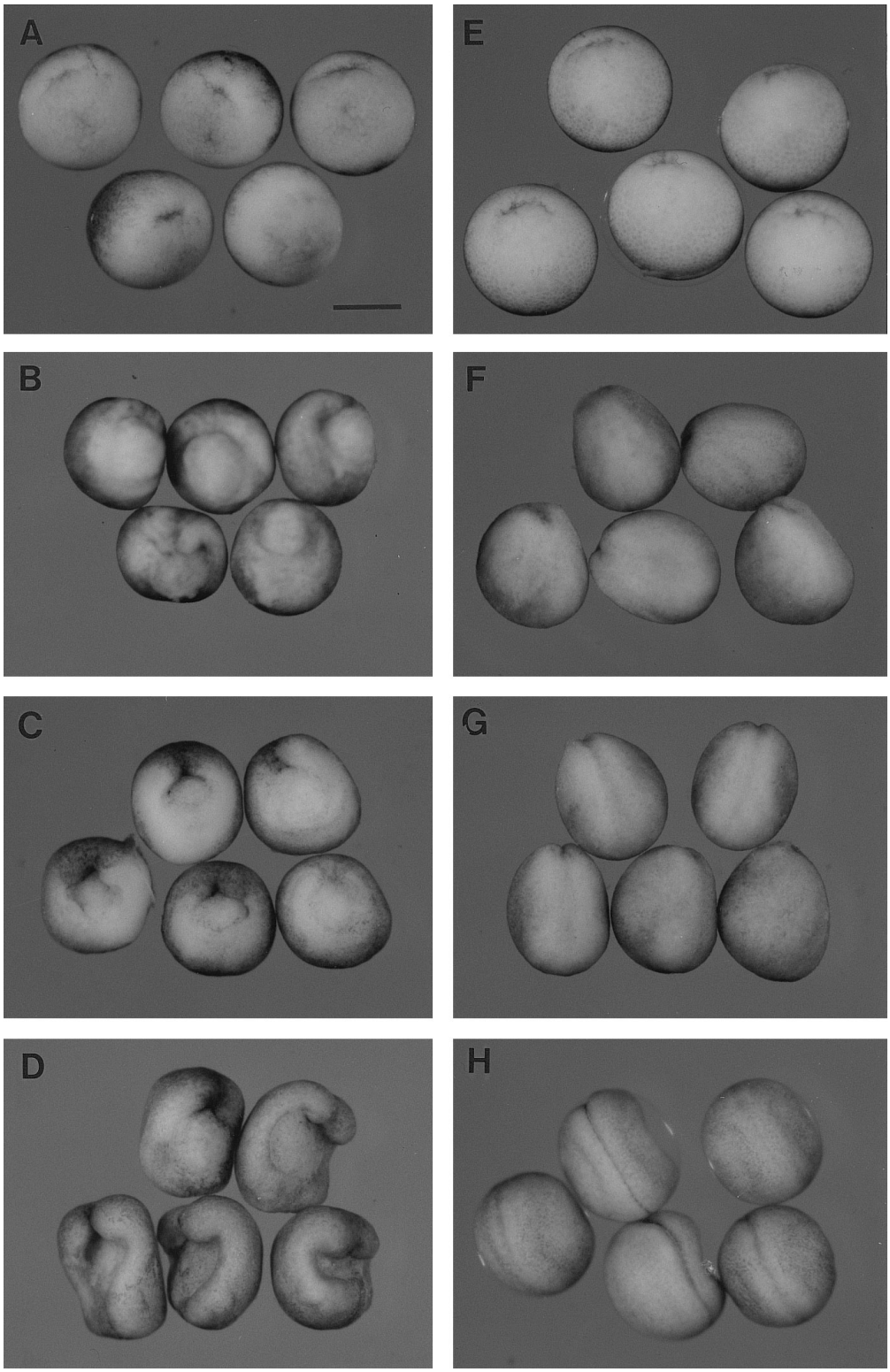


FIG. 1. Morphological views of *GATA-1* RNA-injected embryos. Dorsal two blastomeres at 4-cell stage were injected with *GATA-1* RNA (1ng/embryo), and the injected (A-D) and uninjected (E-H) embryos were fixed at early gastrula (stage 10+, A and E) (38), late gastrula (stage 13, B and F), neurula (stage 15, C and G), and late neurula (stage 20, D and H). *GATA-1*-injected embryos appear normal at the onset of gastrulation (A), but the delay of invagination process is obvious during gastrulation stages (B and C). Finally as they start to elongate at late neurula stage, the endoderm cells are exposed to outside without any structures of neural tissue differentiation (D). A, E; vegetal view. B-D; dorsal-vegetal view. F-H; dorsal view. Bar in A indicates 0.5 mm.

lates the expression of *GATA-2* in ventral mesoderm and animal pole tissue, and that it functions as a stimulator of epidermis-dependent erythropoietic differentiation (27). More recently, GATA-1 and SCL, a homeobox transcription factor that also regulates erythropoietic differentiation, are activated depending on BMP-4 signaling (14). Thus all the hemopoietic transcription factors need the BMP-4 signaling for initial activation.

The present study was intended to examine whether GATA-1, one of differentiation factors specifically expressed in erythroid lineage, has an additional activity to ventralize or modify the mesodermal patterning during early development of *Xenopus* embryos. We observed that the embryos injected with *GATA-1* RNA had a severe defect in the dorsal region of embryo. In those embryos, dorsal mesodermal cells invaginated abnormally at mid-gastrulation stage, and the development of notochord was totally abortive. The expressional analysis showed, however, that the dorsal and ventral markers at early gastrula were not altered by the injection of *GATA-1* RNA. We speculate that *GATA-1* affects on the specification of dorsal tissues after early gastrula stage.

MATERIALS AND METHODS

RNA injection and explants. *Xenopus GATA-1* and *GATA-2* plasmids were generous gift from Dr. L. I. Zon (Children's hospital, Boston). The *GATA-1* and *GATA-2* vectors (constructed in pGEM-HE) were linearized with NheI, and capped RNA was synthesized using T7 RNA polymerase according to manufacture's protocol (Ambion). The two ventral or dorsal blastomeres of dejellied embryos at 4-cell stage were injected with these RNAs (18.4 nl/embryo) in 3% ficoll/Steinberg's solution. The embryos were developed in Steinberg's solution up to designated stages. In some of experiments, dorsal marginal zone (DMZ), or ventral marginal zone (VMZ) tissue was excised at stage 10+, and further cultured in sterilized Steinberg's solution with 30 μg/ml kanamycin.

Northern blot analysis and RT-PCR. Total RNA from explants injected with *GATA-1* RNA was extracted by AGPC method (RNA isolation kit , Stratagene), loaded in denatured 1% agarose gel, and transferred to N-bond membrane (Amersham). Probes used in this study were as follow; *α-actin,* 1.2 kb BamHI/HindIII fragment (28); *α-globin,* 0.8 kb PstI fragment (29); *EF-1α,* 0.4 kb PstI/SacI fragment (30). Hybridization was performed in Hybrisol I (Oncor), and membrane was exposed to RX film (Fuji) after washing. The same blot was sequentially hybridized with the different probes to detect each message in the same explant samples. RT-PCR was performed according to the previous study (31). Primers used for RT-PCR in this study are as follows: *chordin,* forward 5′-tta-gag-agg-aga-gca-act-cgg-gca-at-3′, reverse 5′-gtg-ctc-ctg-ttg-cga-aac-tct-aca-ga-3′ (27 cycles); *goosecoid,* forward 5′-aca-act-gga-agc-act-gga-3′, reverse 5′-tct-tat-tcc-aga-gga-acc-3′ (27 cycles); *Xwnt-8,* forward 5′aga-tga-cgg-cat-tcc-aga-3′, reverse 5′-tct-ccc-gat-atc-tca-gca-3′ (20 cycles); *EF1α,* forward 5′-cct-gaa-tca-ccc-agg-cca-gat-tgg-tg-3′, reverse 5′-gag-ggt-agt-ctg-aga-agc-tct-cca-cg-3′ (21 cycles).

Immunostaining and whole-mount in situ hybridization. Whole mount immunostaining was performed as described (32) using monoclonal antibodies against N-CAM (4d, from Developmental Studies Hybridoma Bank) and keratan sulphate (MZ-15, a gift from Dr. F. M. Watt) to stain neural tissues and notochord, respectively. Embryos and explants were fixed in Dent solution (20% dimethyl sulfoxide in methanol), incubated in diluted first antibodies, and followed by 1:400 diluted peroxidase-conjugated secondary antibodies. Staining was visualized with diaminobenzidine and H_2O_2. For immunostaining of sections, embryos were fixed in 2% paraformaldehyde/50% MMR solution, dehydrated, embedded in paraffin, and sectioned at 7 μm. Hydrated sections were incubated with first antibodies as described above, and followed by 1:100 diluted rhodamine-conjugated secondary antibodies. Whole-mount in situ hybridization was performed as described before (33). The antisense probe used for the detection of *goosecoid* was derived from a PCR fragment (434 bases from nucleotide 362 to 795) kindly provided by Drs. A. Suzuki and N. Ueno.

TABLE 1

The Dorsal Defect Induced by Heterotopic Injection of *GATA-1* RNA

Exp. No.	Site of injection	RNA[a]	No. Embryos with dorsal defect[b]/ No. of embryos survived	(%)
1	Dorsal	*GATA-1*	153/192	(80)
	Dorsal	*GATA-2*	20/123	(16)
	Dorsal	*β-gal*	0/139	(0)
	Dorsal	H_2O	0/74	(0)
2	Dorsal	*GATA-1*	107/137	(78)
	Ventral	*GATA-1*	5/115	(4)

[a] One ng of *GATA-1*, *GATA-2* or *β-gal* RNA was injected into dorsal or ventral two blastomeres of 4-cell stage embryo.

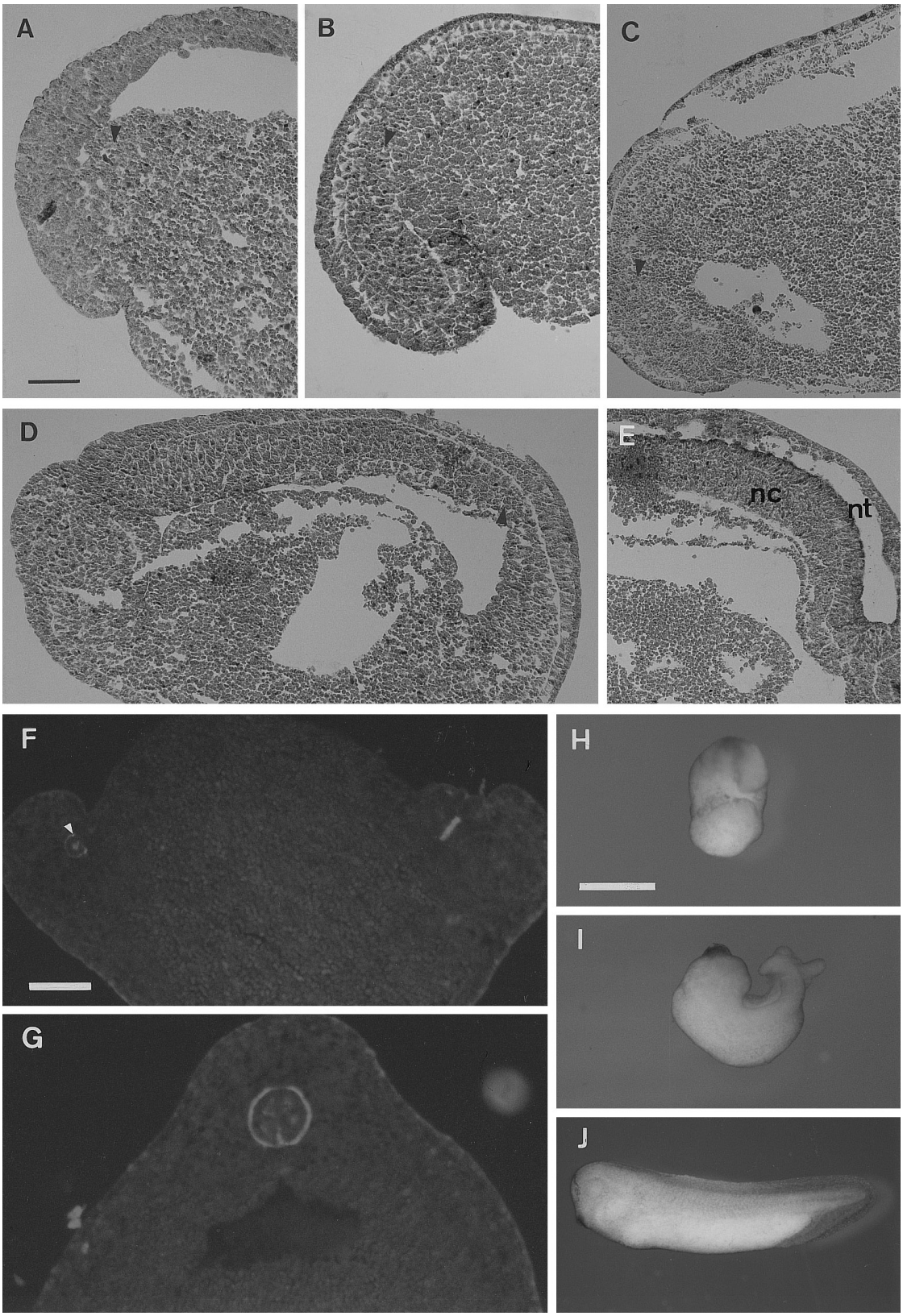
[b] The injected embryos were cultured for overnight and the number of embryos with the dorsal defect was scored at st. 20. The results were summarized from 7 independent experiments. The number of embryos with non-specific damages was excluded from the score. Typical morphology of the dorsal defect was shown in Fig. 1.

Western blot analysis. Western blot analysis was performed using monoclonal antibody to N-CAM (4d, as described above). Ten to tweny animal cap explants, which were previously injected with RNAs, were harvested, and the lysates from 3 explants were separated on a 7.5% SDS-PAGE. After the proteins were transferred to Immobilon-P (Millipore), the membrane was blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20, and incubated with 4d antibody (1:10 diluted culture supernatant), followed by 1:10,000 diluted peroxidase-conjugated secondary antibodies against mouse IgG. Staining was visualized with ECL assay system (Amersham).

Histology. The embryos at different stages were fixed in 2% paraformaldehyde/50% MMR solution, dehydrated, embedded in paraffin, sectioned at 7 μm, and stained with aniline blue and orange G.

RESULTS

The heterotopic expression of GATA-1 caused a defect of dorsal structure. To assess a possible involvement of *GATA-1* on mesoderm patterning during gastrulation and following differentiation steps, we injected *GATA-1* RNA into two dorsal blastomeres at 4-cell stage. These embryos were cultured for 18 hours until control sibling embryos reached stage 20 (late neurula stage). The embryos recieved injection of 1ng *GATA-1* RNA exhibited invagination at early gastrula stage as control embryos (Fig. 1A, E). However, drastic differ-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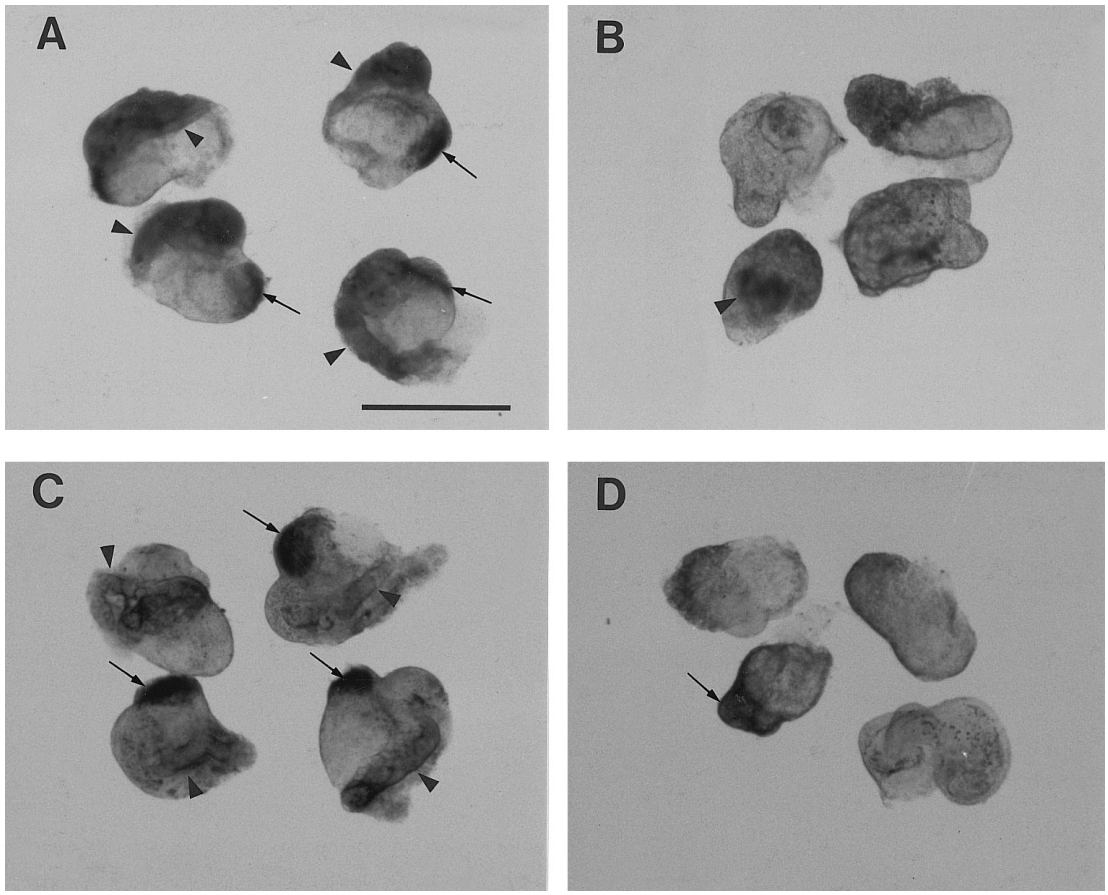


FIG. 3. Injection of *GATA-1* RNA inhibits the differentiation of notochord and neural tissue in dorsal marginal zone (DMZ) explants. Dorsal two blastomeres at 4-cell stage were injected with *GATA-1* RNA (1ng/embryo), and the DMZ explants (B, D) or control uninjected explants (A, C) were allowed to develop to stage 35/36. The explants were immunostained with anti-N-CAM antibody, 4d (A, B) or with anti-notochord antibody, MZ-15 (C, D), showing significant reduction of both markers in injected embryos (B, D). Arrowheads show the positively stained sites. Arrows show pigments of cement gland. Bar in A indicates 0.5 mm.

ence from the control embryos was observed in the mid- to late-gastrulation period. At late gastrula stage (stage 13), at which mesodermal mantle reached the most anterior position, and the yolk plug is covered with ectodermal layer (Fig. 1F), the retardation of gastrulation became obvious (Fig. 1B). Invagination was still not completed in injected embryos at neurula stage (stage 15), at which control embryos formed neural groove (Fig. 1C, G). There seems no neurulation occurring in the predicted dorsal anterior ectoderm. As embryos elongated along antero-posterior axis at late neurula stage (stage 20, Fig. 1H), the endodermal area without overlaying mesoderm expanded toward antero-posterior direction (Fig. 1D). This deficiency in *GATA-1* RNA-injected embryos was observed at high frequency (78–80%, Table 1). The sibling eggs were injected with *GATA-1* RNA either into dorsal or ventral two blastomeres at 4-cell stage, and the effects of injection were compared. As shown in Table 1, the embryos injected with *GATA-1* RNA into dorsal blas-

FIG. 2. Histological analyses of *GATA-1* RNA-injected embryos. Dorsal two blastomeres at 4-cell stage were injected with 1ng *GATA-1* RNA. A-E, Sagittal views of injected (A, stage 10+; B, stage 15; C, stage 20) and uninjected control embryos (D, stage 15; E, stage 20). Note that *GATA-1* RNA injection arrests of the invagination of mesoderm, and disturbs the differentiation of notochord. Arrowheads in A-D indicate the edge of involuting cells. Dorsal to the left of figures in A-C. Anterior to the left of figures in D and E. Bar in A indicates 100 μm. nc, notochord; nt, neural tube. F-G, Transverse sections of injected (F) and uninjected (G) embryos at a trunk level. The sections were stained by anti-notochord antibody, MZ-15, indicating that the injected embryos lack the differentiated notochord. A trace of positive staining was observed in an embryo exceptionally (white arrowhead in F). Bar in F indicates 100 μm. H-J, Effect of increased doses of injected *GATA-1* RNA. Dorsal two blastomeres at 4-cell stage were injected with 5ng (H) or 1ng (I) *GATA-1* RNA, and the embryos were cultured until stage 32 (2 days old). High dose of RNA injection causes loss of eye capsules and cement gland, suggesting that neural tissues are completely eliminated in these embryos. J shows uninjected control embryo. Bar in H indicates 1 mm.

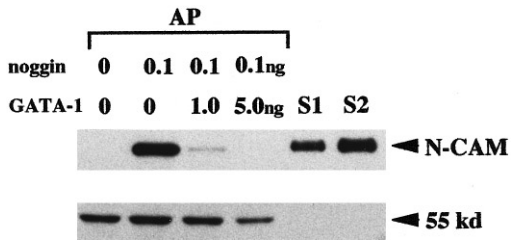


FIG. 4. Injection of *GATA-1* RNA inhibits the differentiation of neural tissue in animal cap explants. Two balstomeres at 2-cell stage were injected with *noggin* RNA, or coinjected with *noggin* and *GATA-1* RNAs, and the animal cap (AP) explants were cultured for 2 days. The explants were harvested, and the lysate from 3 explants was separated by 7.5% SDS-PAGE. The expression of N-CAM antigen was detected by Western blot analysis as described in Materials and Methods. The 55 kd major band, which is detected nonspecifically but in all the embryonic tissues is shown for a protein loading control. In lanes S1 and S2, lysates from 25 and 12.5 μg of adult brain were run.

tomeres showed a typical dorsal defect at high frequency (78%), whereas those injected into ventral blastomeres did not exhibit such abnormality (4%). Since both *GATA-1* and *GATA-2* are shown to be essential for erythrocyte differentiation and may share common DNA binding motif (25, 26), we compared the effect of injection of these two RNAs. In contrast to the results obtained by the injection of *GATA-1* RNA, *GATA-2* RNA did not affect much on dorsal morphogenesis (16%). Therefore, we concluded that the defect observed in dorsal region of embryo by *GATA-1* RNA was not due to general toxic effect of RNA injection.

Injected embryos lacked notochord and neural tissues. Although the injected embryos exhibited abnormal invagination, the onset of gastrulation started normally. The histological observation indicated that mesodermal layer did not reach animal roof even at neurula stage (Fig. 2A, B). When the control embryos reached late neurula stage, no further movement of invaginated mesoderm layer was observed in *GATA-1* RNA-injected embryos (Fig. 2C). In order to detect molecular marker expressed in notochord, we performed immunostaining using monoclonal antibodies to keratan sulfate. As shown in the transverse sections in Fig. 2F and 2G, the*GATA-1* RNA-injected embryos apparently lacked notochord.

An increased dose of *GATA-1* RNA did not affect on the morphology at neurula stage, but caused loss or shrinkage of the neurulation-dependent tissues, such as cement gland and eye capsule, at later stages. At stage 32, 100% (27/27) and 74% (23/27) of embryos injected with 1 ng *GATA-1* RNA had eye capsules and cement gland, respectively, while only 35% (8/27) and 13% (8/23) of embryos injected with 5 ng RNA had these tissues (Fig. 2H-J). This data suggested that injection of higher dose of *GATA-1* RNA inhibited the neurulation completely.

Effects of GATA-1 on tissue pattern in dorsal maginal zone and animal cap explants. To analyze tissue pattern in embryo injected with *GATA-1* RNA, the dorsal marginal zone (DMZ) was excised from stage 10 embryos which were previously injected with 5 ng *GATA-1* RNA, and the explants were cultured for 2 days. These explants and control explants were immunostained with anti-N-CAM antibody and anti-keratan sulfate antibody. As shown in Fig. 3, all the explants from control embryos (11/11, 100%) had neural tissue as stained by anti-N-CAM antibody. In contrast, *GATA-1*-injected explants exhibited the differentiation of neural tissue in 3/12 (25%). The notochord was observed in 8/11 (73%) of normal DMZ explants, and in 0/12 (0%) of *GATA-1*-injected explants. We also examined the effect of *GATA-1* on the neurogenesis. Animal caps, which were previously injected with *noggin* RNA, were explanted at blastula stage, and cultured for 2 days. These explants expressed N-CAM as revealed by Western blot analysis. Coinjection of *noggin* and *GATA-1* RNAs, however, clearly inhibited the expression of N-CAM in the animal cap explants, indicating that *GATA-1* can inhibit neurogenesis directly (Fig. 4).

Northern blot analysis indicated that injection of *GATA-1* RNA induced *α-globin* expression. However, the amount of message was obviously less than that induced by 5 ng *BMP-4* RNA, which is a known ventralizing agent (Fig. 5). The expression of cardiac *α-actin* mRNA was not altered by the injection of *GATA-1* RNA. Thus *GATA-1*'s effect was not a typical ventralization as shown in the injection of *BMP-4* RNA. Early molecular markers were also examined. Whole mount *in situ* hybridization analysis showed

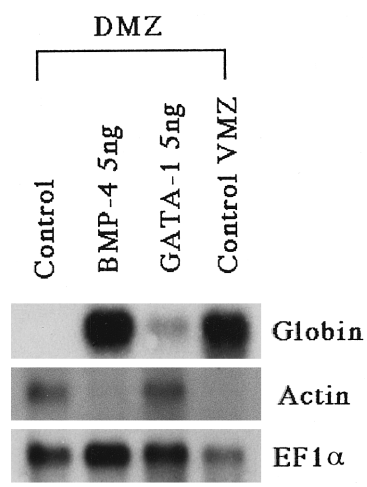
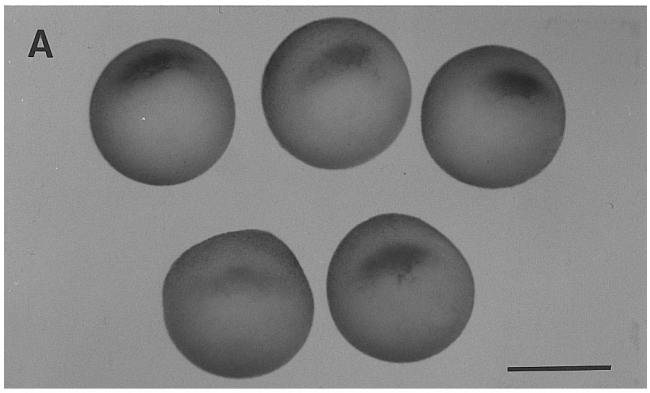
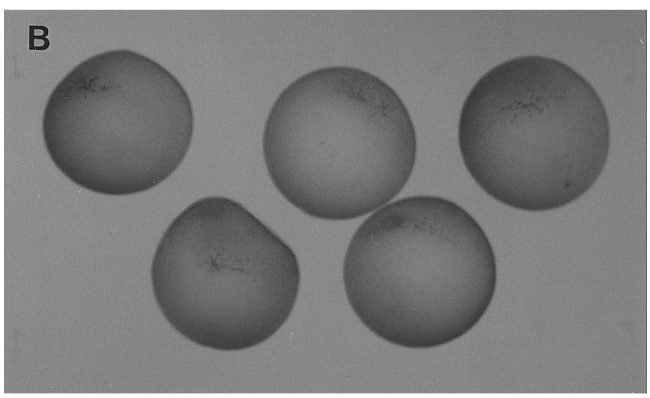
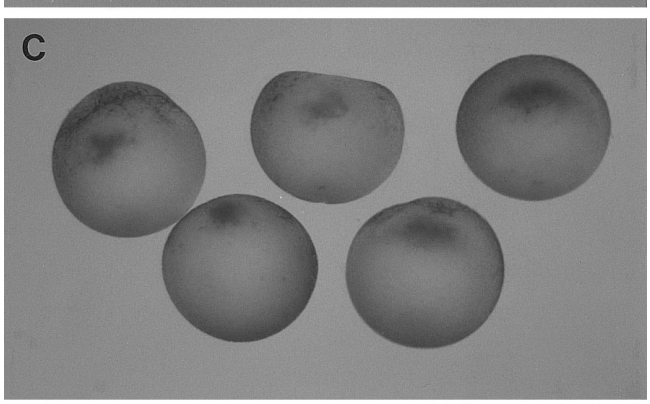
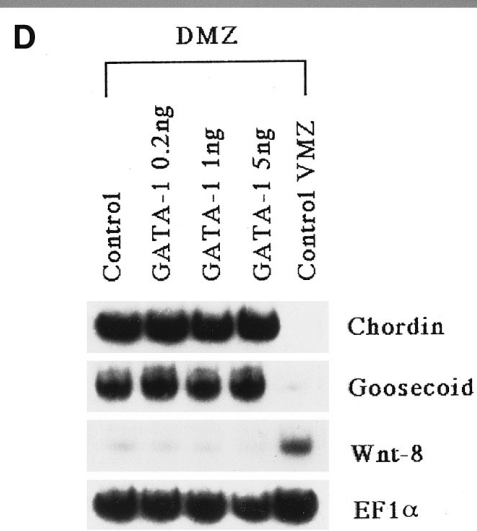


FIG. 5. Expression of late markers in dorsal marginal zone explants. Two dorsal blastomeres of 4-cell stage embryo were injected with either *BMP-4* RNA (5 ng/embryo) or *GATA-1* RNA (5 ng/embryo), and explanted DMZ tissues were cultured for 2 days. Two μg total RNA from each sample was electrophoresed in each lane for Northern blot analysis to detect a larval *α-globin* and muscle *α-actin*. For RNA loading control, the same blot was rehybridized with *EF-1α* probe.









that the *goosecoid* expression was completely suppressed by *BMP-4* RNA injection in the dorsal marginal zone, whereas the expression was not altered by the introduction of *GATA-1* RNA (Fig. 6A-C). Furthermore, another dorsal marginal zone marker, *chordin*, and a ventral marginal zone marker, *wnt-8*, were not changed by *GATA-1* RNA injection, as revealed by RT-PCR analysis (Fig. 6D). These results strongly suggest that the effect of *GATA-1* on mesodermal tissue pattern is not based on the alteration of regulatory genes at early gastrulation, but of later steps of differentiation after gastrulation.

DISCUSSION

The recent studies have shown that *GATA-1* and *GATA-2* are expressed prior to hemopoietic cell differentiation in *Xenopus* embryo (34). Especially *GATA-2* is abundantly expressed in the ectodermal region of embryo at gastrula and neurula stages. Our previous study showed that *GATA-2* expression can be induced by the *BMP-4* overexpression in animal pole area, and *GATA-2* can activate the epidermis-dependent stimulation on the ventral mesoderm to form blood cells (27). However, as shown in the present study, *GATA-2* was not able to ventralize embryo if its RNA was injected into presumptive dorsal blastomeres. Furthermore, the knockout of BMP-4 signal by a dominant-negative receptor (*DN-TFR11*) did not inhibit completely the *GATA-2* expression in animal pole tissue (27, 14). Thus in spite of the similar expression pattern of *BMP-4* and *GATA-2* (24), *GATA-2* has a distinct role from BMP-4 signaling on dorso-ventral specification and blood formation program. The result in the present study suggests, in addition, that GATA-1's activity is a part of BMP-4 signal, and it may have a function in specification of mesodermal patterning. Since *GATA-1* did not disturb the development of ventral mesoderm derivatives and the inhibitory effect was specific on the dorsal tissues, the physiological role of GATA-1 may be to inactivate dorsalizing and neuralizing factors in ventral and non-neural areas.

FIG. 6. Expression of early markers in *GATA-1*-injected embryos. A–C. The expression of *goosecoid* in dorsal lip region at stage 10+ embryos was examined by whole-mount *in situ* hybridization after injection of *BMP-4* (B)or*GATA-1* (C) RNA (5ng/embryo) into dorsal marginal zone at 4-cell stage. A indicates uninjected control embryos. All figures are vegetal view, and dorsal to up. Dorsal to up. Bar in A indicates 1 mm. D. The RT-PCR anaysis showing the expression of *goosecoid*, *chordin*, *Xwnt8*, and *EF1α* in dorsal marginal zone (DMZ) or control ventral marginal zone (VMZ). The dorsal blastomeres of 4-cell stage embryo were injected with designated amounts of *GATA-1* RNA, and DMZ or VMZ was excised at stage 10+ early gastrula, and 0.5 μg total RNA from these tissues was used for subsequent RT-PCR analysis. Note that no significant change was observed in the expression of early dorsal and ventral marker genes by injection of *GATA-1* RNA.

The *Xenopus GATA-1* has two subtypes, which are expressed distinctly in embryogenesis (35). Recently, a similar effect of *GATA-1* RNA was reported by Xu et al. (36). They described an inhibitory effect of *GATA-1b*, which is a subtype of *GATA-1*, on neurulation and on dorsal mesoderm formation. On the other hand, the *GATA-1* used in this study was *GATA-1a*, which was cloned previously by Zone et al. (37), and its DNA sequence was determined also in our laboratory (data not shown). Although the morphological phenotype was similar with that induced by *GATA-1b*, it is uncertain that the activities caused by these two subtype genes are identical or not. *GATA-1b* in the previous report clearly inhibited the expression of *chordin*, which encodes for an antagonizer of BMP-4 signal (36). In contrast, the present study showed that the ventral and dorsal markers at early gastrula were not altered by the *GATA-1a* overexpression in dorsal region. The difference in results may be due to the place and the stage of RNA injection, and/or the activity of RNAs used for the experiments. Since, as also pointed out previously (36), these two genes are highly homologous each other, it may be proper to predict that both factors possess a similar activity, but*GATA-1b* 's activity is stronger. Therefore, in the present study we could show that, not only *GATA-1b* but also*GATA-1a* affects on the expression of regulatory genes in dorsal mesoderm and neural ectoderm. In future, in order to elucidate the physiological importance of GATA-1 factors on mesoderm patterning and tissue differentiation other than hemopoietic lineages, the other strategies, for instance, the dominant-negative constructs of *GATA-1s*, will be important.

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