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HNF1β is required for mesoderm induction in the *Xenopus* embryo

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SUMMARY

***XHNF1β* is a homeobox-containing gene initially expressed at the blastula stage in the vegetal part of the *Xenopus* embryo. We investigated its early role by functional ablation, through mRNA injection of an XHNF1β/engrailed repressor fusion construct (*XHNF1β/EngR*). Dorsal injections of *XHNF1β/EngR* mRNA abolish dorsal mesoderm formation, leading to axial deficiencies; ventral injections disrupt ventral mesoderm formation without affecting axial development. *XHNF1β/EngR* phenotypic effects specifically depend on the DNA-binding activity of its homeodomain and are fully rescued by coinjection of *XHNF1β* mRNA. Vegetal injection of *XHNF1β/EngR* mRNA blocks the mesoderm-inducing ability of vegetal explants. Both B-Vg1 and VegT maternal determinants trigger *XHNF1β* expression in animal caps. *XHNF1β/EngR* mRNA blocks *B-Vg1*-mediated, but not by *eFGF*-mediated, mesoderm induction in animals caps. However, wild-type *XHNF1β* mRNA does not trigger *Xbra* expression in animal caps. We conclude that *XHNF1β* function is essential, though not sufficient, for mesoderm induction in the *Xenopus* embryo.**

Key words: *HNF1β*, Mesoderm induction, *XHNF1β/EngR*, Organizer, *Xenopus*

INTRODUCTION

Mesoderm induction was first demonstrated by Pieter Nieuwkoop in 1969. By recombining animal and vegetal explants, which would in isolation originate ectoderm and endoderm, respectively, mesoderm formed from the animal cap cells (Nieuwkoop, 1969). Successive research has identified candidate mesoderm inducers within the TGFβ and FGF families of molecules (reviewed in Harland and Gerhart, 1997).

Several TGFβ factors are involved in mesoderm induction and patterning. Activin, Vg1 (in its activated form, B-Vg1), Nodal-related factors and Derrière can promote development of mesoderm with different dorsoventral values in animal cap induction assays (Green and Smith, 1990; Thomsen and Melton, 1993; Jones et al., 1995; Sun et al., 1999). BMP factors (a subgroup of TGFβ-like molecules) are involved in ventralization of mesoderm, rather than in its initial induction (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994). Involvement of TGFβ-like molecules in mesoderm induction and patterning has been proved by impairing their signalling pathways. Overexpression of dominant negative activin receptors completely abolishes mesoderm development, while selective inhibition of Vg1, Xnr1 or Xnr2 signalling also disrupts or suppresses mesoderm formation (Hemmati-Brivanlou and Melton, 1992; Chang et al., 1997; Joseph and Melton, 1998; Osada and Wright, 1999; Piccolo et al., 1999). In contrast, impairment of BMP signalling causes dorsalization of ventral mesoderm (Graff et al., 1994).

FGF signals are also crucial for mesoderm induction. Overexpression of a dominant negative FGF receptor, either by mRNA injection or in transgenic embryos, demonstrated that FGF signalling is required for mesoderm development and maintenance (Amaya et al., 1991; Kroll and Amaya, 1996). Although able to induce ventral mesoderm in animal caps (Kimelman and Kirschner, 1987; Slack et al., 1987), FGF may work as a competence factor for TGFβ-like-mediated signalling (Cornell et al., 1995).

Initial work suggested that mesoderm-inducing signals are produced in the vegetal part of the amphibian embryo very early in development (Jones and Woodland, 1987), well before zygotic gene activation. However, recent observations on heterochronic animal/vegetal conjugates demonstrated that only a low level of mesoderm-inducing signals is produced from vegetal explants before mid-blastula transition (MBT), suggesting that vegetal cells may become fully efficient in releasing inducing signals only after the onset of zygotic transcription (Wylie et al., 1996). The T-box transcription factor VegT/Xombi/Antipodean/Brat (Zhang and King, 1996; Zhang et al., 1998 and references therein) plays a crucial role in this: depletion of its maternal, vegetally localized, mRNA, completely abolishes mesoderm and endoderm formation and impairs the ability of vegetal explants to induce mesoderm from animal cap cells in recombination experiments (Zhang et al., 1998; Kofron et al., 1999). These observations have suggested that mesoderm induction may thus be biphasic, with a first pre-MBT step of weak induction, and a second post-MBT step of strong induction (Wylie et al., 1996; Zhang et al., 1998; reviewed by Kimelman and Griffin, 1998). Zygotic gene activation within the vegetal cells may therefore set up the biochemical conditions required for their full signalling activity.

HNF1β/vHNF1/LFB3 (De Simone et al., 1991; Mendel et al., 1991; and references therein) is a transcriptional activator, with a strongly divergent homeodomain containing 21 extra amino acids (aa) between the 2nd and the 3rd α-helix regions. HNF1β and its close relative HNF1α (Frain et al., 1989) are among the best-characterized transcriptional regulators. Several reports have demonstrated that they bind and transactivate DNA target sequences required for the expression of different liver-specific genes, both in vitro and in vivo (Rey-Campos et al., 1991; De Simone et al., 1991; and references therein). Within the N-terminal part of the protein, three regions are essential for DNA binding; specific deletions or changes within these domains completely impair DNA binding (Nicosia et al., 1990). The A region (aa 1-32) is required for homodimerization and heterodimerization of HNF1 proteins (Nicosia et al., 1990). Region B (aa 100-184) and region C (aa 198-281) are involved in DNA sequence recognition. Finally, the C-terminal part of the proteins contains the transactivation domain (Nicosia et al., 1990; De Simone et al., 1991; Mendel et al., 1991). We previously cloned the *Xenopus* homologue *XHNF1β* (also named *XLFB3*) and studied its developmental expression (Demartis et al., 1994 and present work). We here address the question of *XHNF1β* early function. *XHNF1β* function was impaired by overexpression of a fusion construct (*XHNF1β/EngR*), in which the *XHNF1β* activation domain was replaced by the *Drosophila engrailed* repressor domain (*EngR*), following the approach introduced by Conlon et al. (1996). We also report on *XHNF1β* overexpression experiments. Our results show that *XHNF1β* is expressed in the vegetal part of the *Xenopus* embryo at MBT and activated by B-Vg1 and VegT maternal determinants, and interference with its function causes mesoderm suppression.

MATERIALS AND METHODS

XHNF1β constructs

XHNF1β full-length cDNA was excised from the recombinant λ phage described in Demartis et al. (1994) and cloned into the *Eco*RI site of pGEM7Z(–) (Promega Biotec), to give plasmid pXLFB3, used for in situ hybridization. The *XHNF1β* coding region plus 123 bp of 5′UTR and 41 bp of 3′UTR were cloned into the *Bam*HI-*Xba*I site of pCS2 (Rupp et al., 1994), to give plasmid pCS2B3 used for overexpression (Fig. 1). The dominant negative plasmid pB3EngR (Fig. 1) was constructed by in-frame fusion of the 5′ filled-in *Eco*RI fragment of pXLFB3 to the engrailed repressor domain of pENG-N (kind gift of D. Kessler). Mutagenized versions of pB3EngR were produced using Stratagene Quick Change Kit; the A residue in position 9 of pB3EngR recognition helix was changed either to E (never present in this position in known homeodomains) or to Q (typical of the Antennapedia type of homeodomains; Gehring et al., 1994), giving mutant plasmids pX3 and pM1, respectively (Fig. 1). The identity of all plasmids was confirmed by sequencing.

Xenopus embryos and in situ hybridizations

Embryos were obtained as previously described (Demartis et al., 1994) and staged according to Nieuwkoop and Faber (1967). For in situ hybridization, embryos were fixed in MEMFA (Harland, 1991); for RNA extraction, they were frozen in liquid nitrogen. Whole-mount in situ hybridization was performed as previously described (Harland, 1991). Proteinase K treatment was omitted for hybridization of explants and conjugates. Embryos were bleached following Mayor et al. (1995) and clarified in 2:1 BB:BA (Harland, 1991). In situ hybridization probes were previously described: cardiac actin (Mohun et al., 1984),

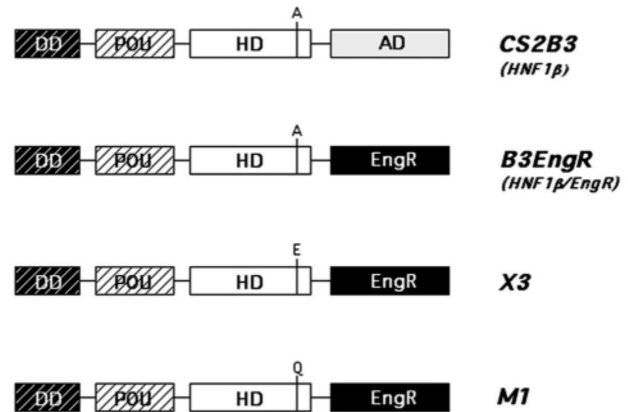


Fig. 1. *XHNF1β*-derived constructs used in this study. DD, dimerization domain; POU, POU-like box; HD, homeodomain; AD, activation domain; EngR, engrailed repressor domain. A, E and Q indicate the aminoacid encoded at position 9 of the homeodomain recognition helix.

goosecoid (Cho et al., 1991), *Xbra* (Smith et al., 1991), *Xpo* (Sato and Sargent, 1991), *Xwnt8* (Smith and Harland, 1991), *Xnot2* (Gont et al., 1993), *chordin* (Sasai et al., 1994), *nrp-1* (Knecht et al., 1995), *Xnr3* (Smith et al., 1995), and *Xvent2* (Onichtchouck et al., 1996).

Explants and conjugates

Dissections were performed in 50 μg/ml gentamicin-supplemented medium. For conjugate experiments, animal and vegetal explants were dissected out of uninjected or control stage 8 embryos and recombined in 1× MBS; after healing, conjugates were cultured in 0.5× MBS until stage 11. Separate animal and vegetal explants were similarly cultured and processed to verify correct dissection. In animal cap experiments, injected and control caps were processed in the same way.

RNA methods and microinjections

Capped RNAs were prepared as described (Krieg and Melton, 1984). *XHNF1β* and *XHNF1β/EngR* mRNAs were transcribed from pCS2B3 and pB3EngR templates, respectively; mutant forms of *XHNF1β/EngR* mRNA were transcribed from pX3 and pM1 plasmids (Fig. 1). *B-Vg1* (Thomsen and Melton, 1993), *VegT* (Zhang and King, 1996) and *eFGF* (Isaacs et al., 1992) mRNA were transcribed from pSP64TBVg1, pCS2-VegT and CS2-eFGF, respectively. Embryos were injected in 0.1× MMR, 4% Ficoll, grown overnight and subsequently transferred in 0.1× MMR, along with sibling controls.

Embryo RNA was extracted with Qiagen RNeasy kit and treated with DNase I prior to RT-PCR, performed as previously described (Gawantka et al., 1995; Henry and Melton, 1998). PCR cycling conditions were: 95°C, 30 seconds; annealing temperature, 30 seconds; 72°C, 1 minute. Primers were as follows (5′ to 3′):

XHNF1β: CACCACACCATACTCAACCAAG (forward) and ACTTTGTGCCTGAGAGGTGTTTA (reverse);
siamois: GGAGACAGACATGACCTATGAG (forward) and CTTCATGTTTTTGCTGACCTGAG (reverse);
Xnr1: TAAAAGCAAAGGAAAGGGCAGAG (forward) and GATGCTTCCTATTGATAAGTGATG (reverse);
Xnr2: TTGTTCTTCGTCATTGCTTCCCT (forward) and CCTTGATGGAGATAATACTGGAG (reverse);
Xnr4: TTTACTGTCTCATCCTCCTATTTG (forward) and CACTAATACTTTGGCACGATGAG (reverse);
chordin: CCTCCAATCCAAGACTCCAGC (forward) and GGAGGAGGAGGAGCTTTGGG (reverse).

Annealing temperature was 3°C below T_m (according to A/T=2°C; G/C=4°C); 28 cycles were used for all these genes. Other primers and

Table 1. *XHNF1β/EngR* mRNA microinjection results

2-cell-stage bilateral injections

	n	D.A.I. 0-2	D.A.I. 3	D.A.I. 4	D.A.I. 5	Posterior bulges
250 pg/bl	40	5 (12%)	4 (10%)	5 (12%)	17 (42%)	9 (22%)
400 pg/bl	87	14 (16%)	28 (32%)	14 (16%)	22 (25%)	9 (10%)
500 pg/bl	172	76 (44%)	10 (6%)	–	8 (5%)	78 (45%)
Uninjected	131	–	–	–	131 (100%)	–

4-cell-stage dorsal bilateral injections

	n	D.A.I. 0	D.A.I. 1	D.A.I. 2	D.A.I. 3	D.A.I. 4	D.A.I. 5
500 pg/bl	74	57 (77%)	11 (15%)	–	1 (1%)	4 (6%)	1 (1%)
500 pg/bl	35	22 (63%)	9 (26%)	1 (3%)	–	3 (8%)	–
500 pg/bl	31	–	7 (23%)	20 (65%)	3 (9%)	–	1 (3%)
Uninjected	130	–	–	–	–	–	130 (100%)

4- to 8-cell-stage injections (500 pg/bl)

Stage and site of injection	*n*	D.A.I. 0-1	D.A.I. 2	D.A.I. 3-4	D.A.I. 5
4C/2D	28	16 (57%)	5 (18%)	7 (24%)	–
4C/2V	27	–	–	–	27 (100%)
8C/2D-Vg	30	12 (40%)	1 (3%)	15 (50%)	2 (6%)
8C/2D-An	24	–	4 (16%)	14 (58%)	6 (25%)
Uninjected	17	–	–	–	17 (100%)

n, number of embryos; D.A.I., dorso-anterior index (Kao and Elinson, 1988); amount of injected RNA is indicated in picograms/blastomere (pg/bl). 4C/2D, 4-cell stage dorsal bilateral injection; 4C/2V, 4-cell stage ventral bilateral injection; 8C/2D-Vg, 8-cell stage dorsovegetal bilateral injection; 8C/2D-An, 8-cell stage dorsoanimal bilateral injection.

PCR conditions were previously described as follows: *goosecoid* and *Xbra* (Gawantka et al., 1995), *ODC* (Bouwmeester et al., 1996), *Xsox17α* (Hudson et al., 1997), *Milk* (Ecochard et al., 1998), *Mixer* and *Mix.1* (Henry and Melton, 1998), and *derrière* (Sun et al., 1999).

RESULTS

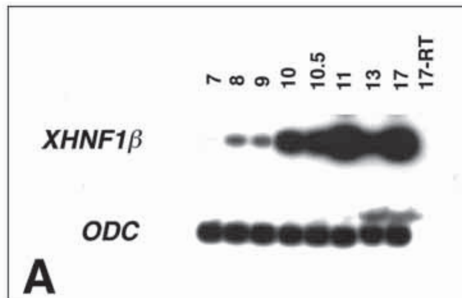
XHNF1β expression in the early *Xenopus* embryo

XHNF1β transcripts are initially detected by RT-PCR at stage 8, becoming more abundant from stage 10 onwards (Fig. 2A). Transcripts were detected in the vegetal part of blastula embryos by in situ hybridization (stage 8.5 and 9), but not in their animal or marginal regions (Fig. 2B,C). At the gastrula stage, the *XHNF1β* expression maps to the presumptive endoderm of the vegetal pole (Fig. 2D). Comparison of *XHNF1β* expression to *chordin* and *Xbra* expression in BB:BA cleared embryos (Fig. 2D-F) shows that *XHNF1β* is excluded from the marginal zone. *XHNF1β* is expressed in prospective endodermal cells throughout development, in the trunk neuroectoderm transiently during neurulation, and in the developing excretory system from stage 16 onwards (Fig. 2G,H). Our present data confirm and extend those of Demartis et al. (1994).

Phenotypic effects of *XHNF1β/EngR*

To suppress *XHNF1β* function, we replaced the *XHNF1β* activation domain with the *Drosophila engrailed* repressor domain (see Materials and Methods; Fig. 1) and injected the corresponding mRNA into *Xenopus* embryos.

Bilateral injection of 250-500 pg *XHNF1β/EngR* mRNA/blastomere at the 2-cell stage mainly results in axial deficiencies, scored according to the dorsoanterior index (D.A.I.; Kao and Elinson, 1988; Table 1). Reduction of dorsal tissues was confirmed by in situ hybridization of injected embryos with probes for cardiac actin, a marker of somitic muscle (Mohun et al., 1984), and for *nrp-1*, a pan-neural marker (Knecht et al., 1995; Fig. 3). A significant number of embryos also showed bulges in their ventroposterior region (Table 1; data not shown).



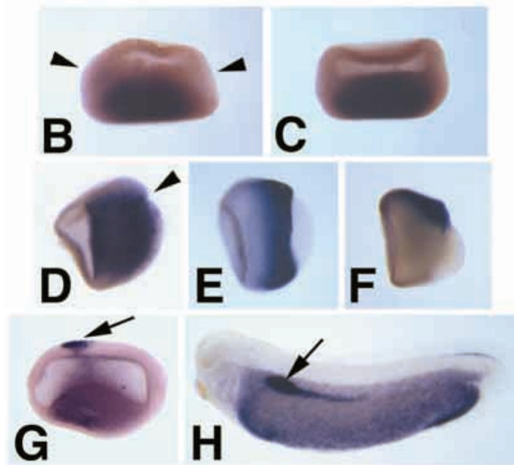


Fig. 2. Expression of *XHNF1β*. (A) Developmental RT-PCR analysis of *XHNF1β* expression. *Xenopus* embryonal stages are indicated in the upper part of the panel. Ornithine decarboxylase (ODC) was used as an internal control. (B,C,D,G,H) *XHNF1β* expression as detected by whole-mount in situ hybridization at blastula stage 8.5 (B) and stage 9 (C), gastrula stage 10.25 (D), neurula stage 14 (G) and tailbud stage 29 (H); expression of *Xbra* (E) and *chordin* (F) at gastrula stage are shown for comparison. Arrowheads indicate the embryo marginal zone (B), or the dorsal blastopore lip (D); arrows in G and H point towards *XHNF1β* expression in neuroectoderm and pronephric system, respectively.

At the 4- and 8-cell stages it was possible to map the blastomeres as the phenotypic effects of the chimeric mRNA are stronger. At the 4-cell stage, injection of 500 pg mRNA/blastomere in both dorsal blastomeres results in a high frequency of axially deficient embryos (Fig. 4A; Tables 1, 2); on the contrary, similar ventral injections result in embryos with normal axial development, but with bulges in their ventroposterior parts (Fig. 4B; Table 1). 8-cell-stage injections show that the axis-suppressing effect of *XHNF1β/EngR* mRNA is maximal in the dorsovegetal quadrant (Table 1): when 500 pg mRNA/blastomere are injected into dorsovegetal blastomeres, embryo axial development is severely impaired (Fig. 4D), but embryos appear only partly disturbed by dorsoanimal injections (Fig. 4E).

The effects of *XHNF1β/EngR* mRNA depend on the DNA-binding specificity of its encoded homeodomain and are rescued by coinjection of *XHNF1β* mRNA

To demonstrate that the effect of the chimeric construct is specific and due to suppression of *XHNF1β* function (dominant negative effect), we adopted two experimental strategies: in the first, we changed the DNA-binding specificity of the

Table 2. Specificity of *XHNF1β/EngR* mRNA effect

		n	D.A.I. 0	D.A.I. 1	D.A.I. 2	D.A.I 3	D.A.I. 4	D.A.I. 5
(A) Injection of mutant construct mRNA compared to *XHNF1β/EngR* mRNA								
	XHNF1β/EngR	53	16 (30%)	12 (23%)	12 (23%)	6 (11%)	–	7 (13%)
	X3	56	–	–	–	–	–	56 (100%)
	M1	93	–	–	–	–	3 (3%)	90 (97%)
	Uninjected	64	–	–	–	–		64 (100%)
(B) Rescue of *XHNF1β/EngR* axis-suppressing activity by *XHNF1β*								
I	*XHNF1β/EngR*	35	1 (3%)	2 (6%)	12 (34%)	4 (11%)	12 (34%)	4 (11%)
	XHNF1β/EngR+ XHNF1β 1:4	55	–	–	–	–	5 (10%)	50 (90%)
	Uninjected	53	–	–	–	–	–	53 (100%)
II	*XHNF1β/EngR*	64	4 (6%)	15 (23%)	40 (62%)	2 (3%)	1 (1%)	2 (3%)
	XHNF1β/EngR+ XHNF1β 1:4	97	2 (2%)	1 (1%)	3 (3%)	3 (3%)	22 (22%)	66 (66%)
	XHNF1β/EngR+ XHNF1β 1:2	54	–	2 (4%)	8 (15%)	8 (15%)	21 (39%)	15 (28%)
	Uninjected	93	–	–	–	–	–	93 (100%)

Embryos were injected in both dorsal blastomeres with: (A) 500 picograms/blastomere (pg/bl) of mRNA derived from each construct; (B) 150 pg/bl of *XHNF1β/EngR* mRNA *(XHNF1β /EngR)*, or with 150 pg/bl *XHNF1β/EngR*+600 pg/bl *XHNF1β* mRNA *(XHNF1β/EngR+XHNF1β 1:4),* or with 150 pg/bl *XHNF1β/EngR*+300 pg/bl *XHNF1β* mRNA *(XHNF1β/EngR+XHNF1β 1:2). n*, number of embryos; *D.A.I.,* dorso-anterior index (Kao and Elinson, 1988).

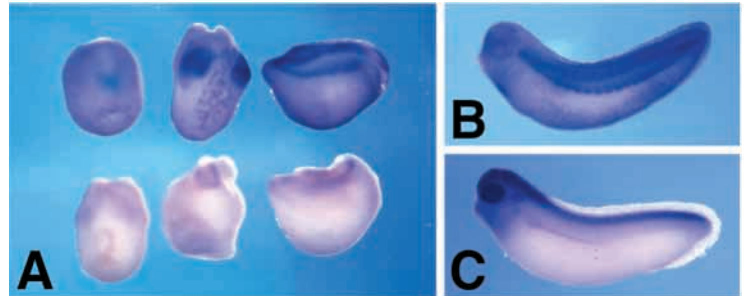


Fig. 3. Results of *XHNF1β/EngR* mRNA bilateral injections in 2-cell-stage embryos. (A) Injected stage 28 embryos were processed by whole-mount in situ hybridization either with a cardiac actin probe (top row) or with *nrp-1* (bottom row). (B) Control embryo probed with cardiac actin. (C) Control embryo probed with *nrp-1*.

XHNF1β/EngR construct by mutating the homeodomain recognition helix at residue 9, crucial for DNA-binding affinity (reviewed by Gehring et al., 1994); in the second, we performed rescue experiments by coinjecting both *XHNF1β/EngR* and wild-type *XHNF1β* mRNAs into the dorsal quadrant of the embryo.

In the first set of experiments, bilateral dorsal injection of 500 pg/blastomere of either pX3 or pM1 mutant mRNA (see Material and Methods) at the 4-cell stage did not produce any phenotypic effect (Fig. 4H,I) in contrast to the strong effect of *XHNF1β/EngR* mRNA (Fig. 4G, Table 2).

In the second set of experiments, the effects of 150 pg *XHNF1β/EngR* mRNA/blastomere (Fig. 4M, Table 2) on axial development were efficiently rescued by coinjection of 600 pg of wild-type *XHNF1β* mRNA/blastomere (1:4 molar ratio) (Fig. 4O; Table 2). Coinjection of 150 pg *XHNF1β/EngR* mRNA and 300 pg wild-type mRNA (1:2 molar ratio) consistently resulted in a lower rescue success (Fig. 4N; Table 2). It is also important to note that bilateral injection of 600 pg *XHNF1β* mRNA/blastomere does not have any phenotypic effect on embryos, either in ventral or in dorsal injections at the 4-cell stage: the resulting embryos always had a normal appearance and a D.A.I. value of 5 (Fig. 4J-L; data not shown).

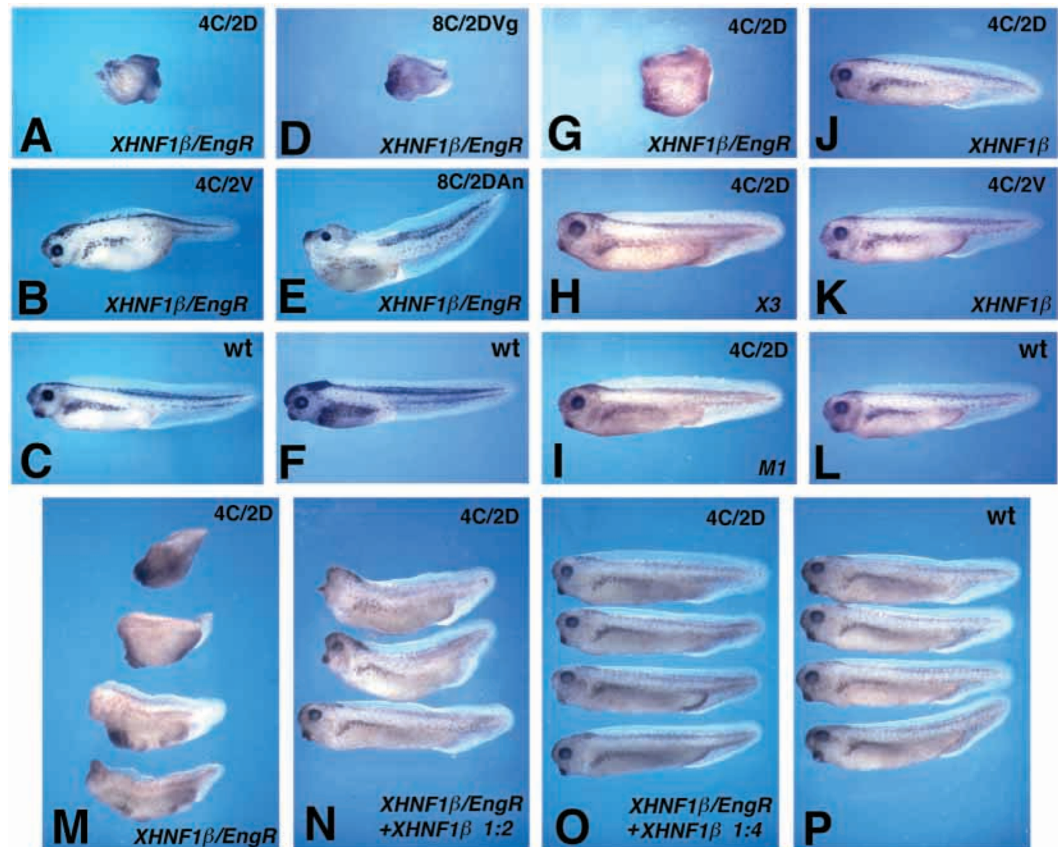


Fig. 4. Microinjection experiments with *XHNF1β*-derived construct mRNAs. Embryos were injected with *XHNF1β/EngR, X3*, *M1,* *XHNF1β* mRNAs, or coinjected with *XHNF1β/EngR+XHNF1β* (1:2 and 1:4 molar ratio) mRNAs, as indicated; control uninjected embryos are shown (wt). Embryos were injected at 4-cell stage in both dorsal (4C/2D) or ventral blastomeres (4C/2V); or at 8-cell stage in both dorsoanimal (8C/DAn) or dorsovegetal blastomeres (8C/DVg). (A-C) 4-cell-stage bilateral injections of *XHNF1β/EngR:* compared to control tadpole embryos (C), dorsal injection leads to strong axial deficiencies (A), while ventral injection results in embryos with ventroposterior bulges (B). (D-F) 8-cell-stage bilateral injections of *XHNF1β/EngR*: compared to uninjected controls (F), dorsovegetal injections severely impair axial development (D), while dorsoanimal injections lead to partly disturbed embryos (E). (G-I) Effects of *XHNF1β/EngR* mRNA depend on the homeodomain DNA-binding specificity; the axis-suppressing effect of *XHNF1β/EngR* mRNA (G) is compared with phenotypically normal embryos similarly injected with *X3* (H) or *M1* (I) mRNA. (J-L) Overexpression of *XHNF1β* at 4-cell stage does not produce any overt external phenotype: (J) dorsally injected embryo; (K) ventrally injected embryo; (L) control embryo. (M-P) Rescue of *XHNF1β/EngR* effect by wild-type *XHNF1β:* (M) dorsal injection of *XHNF1β/EngR* mRNA at the 4-cell stage induce axial deficiencies; (N) partial rescue by coinjection of *XHNF1β/EngR* and wild-type *XHNF1β* mRNA (1:2 molar ratio); (O) complete rescue by coinjection of *XHNF1β/EngR* and wild-type *XHNF1β* mRNA (1:4 molar ratio); (P) control embryos.

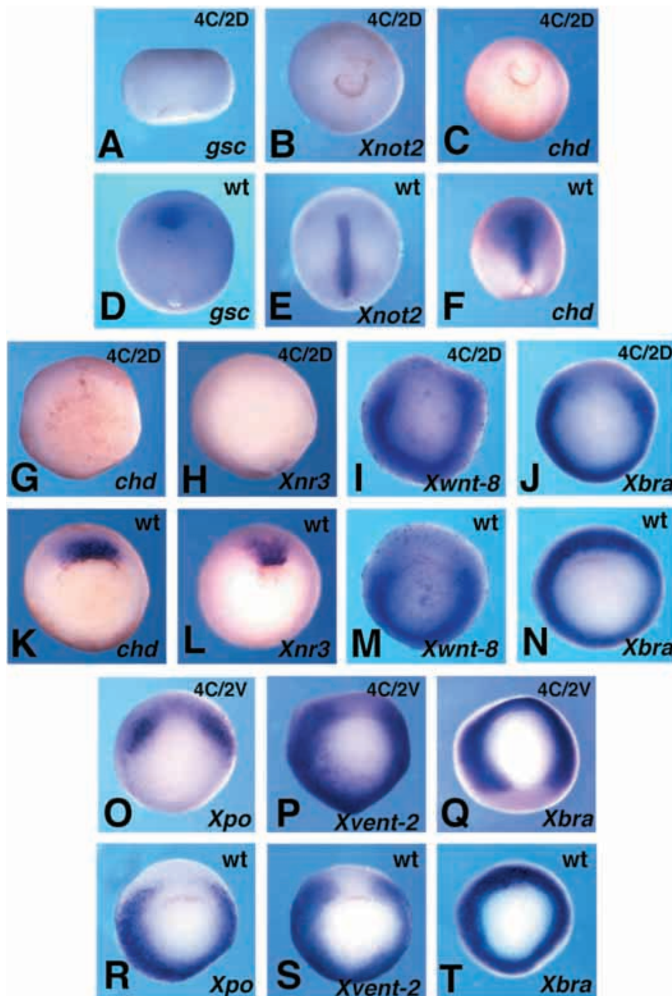


Fig. 5. Molecular marker analysis of *XHNF1β/EngR* mRNA-injected embryos. As indicated, embryos were bilaterally injected at 4-cell stage either dorsally (4C/2D), or ventrally (4C/2V), and processed for in situ hybridization with probes for *goosecoid* (*gsc*), *chordin* (*chd*), *Xnot2, Xnr3, Xwnt-8, Xbra, Xpo* and *Xvent-2*,. Analysis was performed either at early neurula stage 12.5 (A-F; A,D-F, dorsal views; B,C, posterior views), or at gastrula stage 10.25-10.5 (G-T; vegetal view). Control embryos (wt) are also shown. Note absence of dorsal blastopore lip in B and C.

Microinjection of *XHNF1β/EngR* mRNA inhibits expression of several mesodermal markers

500 pg of *XHNF1β/EngR* mRNA were injected into each dorsal blastomere of 4-cell-stage embryos and the expression of various organizer genes was analysed. At stage 12.5, embryos were processed for in situ hybridization with probes for *goosecoid* (in controls at this stage expressed in the prechordal mesoderm; Cho et al., 1991), *Xnot2* (expressed in the control presumptive notochord; Gont et al., 1993), and *chordin* (expressed in both these components in controls; Sasai et al., 1994). Transcription of these genes is almost or completely suppressed in injected embryos (19/19 embryos for *Xnot2*; 11/17 embryos for *goosecoid*; 13/15 embryos for *chordin*; Fig. 5A-F).

Phenotypic analysis of dorsally injected embryos at the gastrula stage revealed absence of the dorsal blastopore lip; eventually only a pigmented rim of bottle cells may appear, but

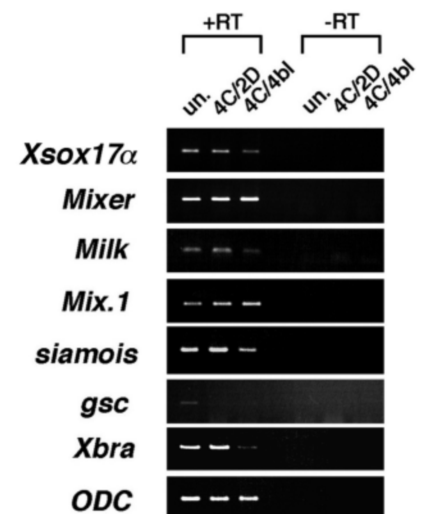


Fig. 6. Expression levels of vegetal molecular markers, as indicated, are compared by RT-PCR at gastrula stage (stage 10.25) in uninjected embryos (un.), *XHNF1β/EngR* dorsally injected embryos (4C/2D) and *XHNF1β/EngR* radially injected embryos (4C/4bl).

a deeper lip fails to form (data not shown, but see absence of dorsal lip at stage 12.5 in Fig. 5B,C). Injected embryos, analysed at stage 10.25-10.5, showed absent or almost no expression of several organizer genes, such as *chordin* (41 out of 57 embryos; 3 experiments), *Xnr3* (17/26; 2 experiments; Smith et al., 1995), *goosecoid* (15/20; 1 experiment; Fig. 5G,H,K,L; data not shown). Suppression of organizer gene expression in these embryos is not due to transformation of the organizer into ventral mesoderm. In fact, dorsally injected embryos do not show any dorsal expansion of *Xwnt8* (Smith and Harland, 1991) domain, which appears to be normal (26/39 embryos; 2 experiments; Fig. 5I,M). Rather, dorsal suppression of the pan-mesodermal marker *Xbra* (Smith et al., 1991; 43/52, 2 experiments) suggests that no mesoderm is present dorsally (Fig. 5J,N).

We therefore asked whether *XHNF1β/EngR* mRNA could inhibit mesodermal markers when injected ventrally. Embryos injected with *XHNF1β/EngR* in ventral blastomeres at the 4-cell stage (500 pg/blastomere) do not show ventral expression of either *Xbra* (29/33, 2 experiments; Fig. 5Q,T) or *Xpo* (Sato and Sargent, 1991), a ventrolateral mesodermal marker (18/20, 1 experiment; Fig. 5O,R), and fail to form a ventral blastopore lip (not shown). Interestingly, expression of *Xvent2,* a ventrolateral patterning gene of ectodermal and mesodermal layers, is not affected in injected embryos (20/20 embryos were undisturbed, 1 experiment; Fig. 5P,S). Since *Xvent2* gene responds to BMP4 (Onichtchouk et al., 1996), this result suggests that *XHNF1β/EngR* mRNA may only affect mesoderm specification not ventral patterning. Thus, ablation of *XHNF1β* function in the early embryo is able to abolish mesoderm formation both dorsally and ventrally.

We next investigated whether *XHNF1β/EngR* mRNA could perturb genetic activities within the embryo vegetal region. We injected 500 pg of *XHNF1β/EngR* mRNA into both dorsal or all four blastomeres of 4-cell-stage embryos and tested for the expression of *siamois* (activated in the dorsovegetal quadrant by the Wnt/GSK3/β-catenin dorsalizing pathway; Carnac et al., 1996), *Mix.1* and *Milk* (expressed in both marginal and vegetal cells; Rosa, 1989; Ecochard et al., 1998), and *Xsox17α* and *Mixer* (exclusively expressed in the presumptive endodermal cells; Hudson et al., 1997; Henry and Melton, 1998). While *goosecoid* transcription is suppressed and *Xbra* is strongly downregulated in stage 10.25 injected embryos,

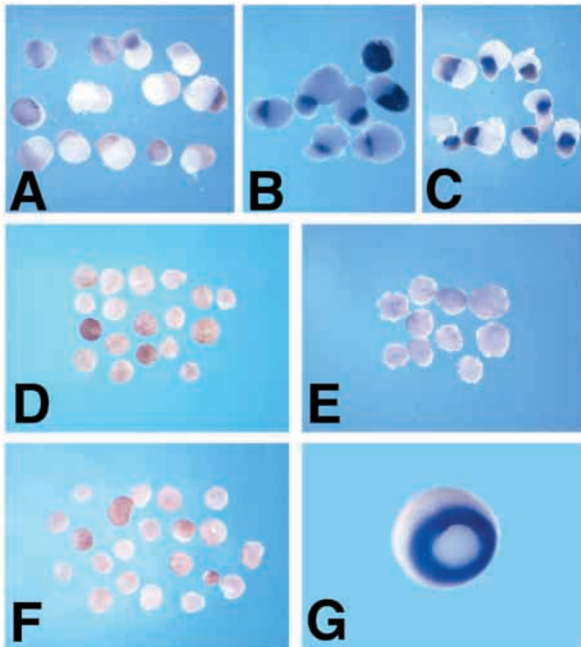


Fig. 7. Mesoderm induction assays on animal/vegetal conjugates and animal caps. (A) Conjugates between *XHNF1β/EngR*-injected vegetal explants and uninjected animal caps; (B) conjugates between *XHNF1β/EngR*-injected animal caps and uninjected vegetal bases; (C) control conjugates of uninjected animal and vegetal explants; (D) control animal explants; (E) control vegetal explants; (F) *XHNF1β* mRNA-injected animal caps; (G) stage 11 control embryo. Explants and embryos were all assayed for *Xbra* expression.

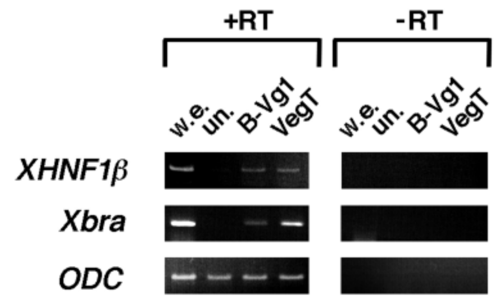


Fig. 8. B-Vg1 and VegT are able to turn on *XHNF1β* expression in animal caps. RT-PCR analysis of RNAs extracted from stage 10 whole embryo (w.e.), uninjected caps (un.), *B-Vg*1-injected caps (B-Vg1), *VegT*-injected caps (VegT).

expression of these vegetal markers is maintained (Fig. 6), suggesting that early endodermal specification may not be affected and that the Wnt/GSK3/β-catenin pathway is still active after *XHNF1β* functional ablation.

XHNF1β function is required in vegetal cells, but is not sufficient in animal caps, for mesoderm induction

The experiments described above indicate that *XHNF1β* functional ablation suppresses mesoderm formation and, because of *XHNF1β*'s early pattern of expression, suggest that activity may be required in the vegetal region for mesoderm induction to occur. To test this, we performed animal/vegetal conjugate assays. 500 pg of *XHNF1β/EngR* mRNA were injected into the four vegetal blastomeres of 8-cell-stage embryos. At stage 8, vegetal explants were dissected from injected embryos and conjugated with animal caps from stage 8 uninjected embryos. Control conjugates were made by recombining uninjected caps and vegetal explants. We also injected 500 pg of *XHNF1β/EngR* into the four animal blastomeres at 8-cell stage and tested the ability of animal caps to respond to vegetal signals in conjugates. Experimental and control conjugates were cultured up to stage 11 and assayed for *Xbra* expression by in situ hybridization. *Xbra* expression was very low or absent in 34/51 experimental conjugates (2 experiments) of injected vegetal explants and uninjected animal caps (Fig. 7A). On the contrary, mesoderm was induced in almost all control conjugates (27/33 positive conjugates, 2 experiments; Fig. 7C), and in conjugates made with uninjected vegetal bases and injected caps (17/21 positives, 2 experiments; Fig. 7B). To test the efficiency of dissections, *Xbra* expression was absent in isolated animal and vegetal control explants (Fig. 7D,E). Therefore, injection of *XHNF1β/EngR* mRNA suppresses the ability of vegetal explants to induce mesoderm in animal caps, but it does not prevent animal caps from responding to mesoderm-inducing signals. Therefore, *XHNF1β* is required in the vegetal part of the embryo for mesoderm to be induced in the marginal zone.

We then asked whether *XHNF1β* expression alone is sufficient to induce mesoderm in animal caps. A total of either

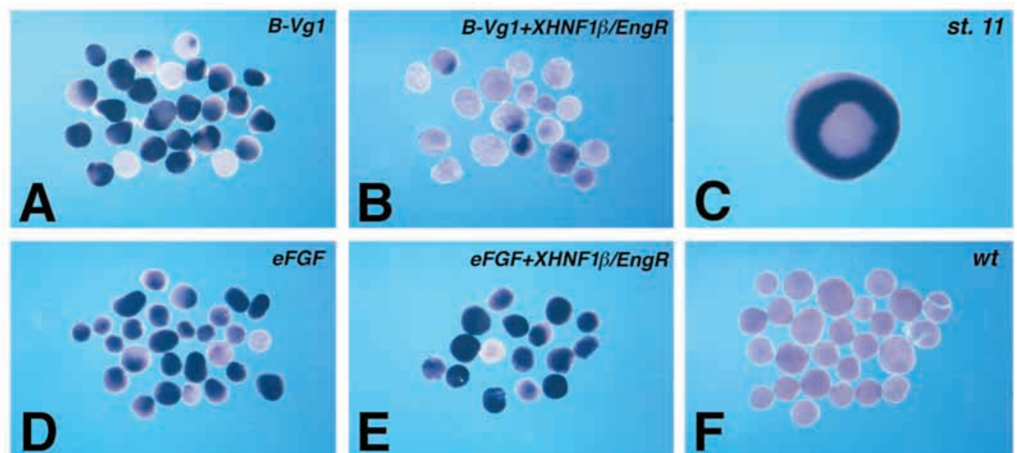


Fig. 9. Injection of *XHNF1β/EngR* mRNA blocks mesoderm induction by *B-Vg1*, but not by *eFGF* mRNA. (A) *B-Vg1* mRNA-injected animal caps; (B) *B-Vg1+XHNF1β/EngR* mRNA-injected animal caps; (C) stage 11 control embryo; (D) *eFGF* mRNA-injected animal caps; (E) *eFGF+XHNF1β/EngR* mRNA-injected animal caps; (F) uninjected animal caps.

1 or 5 ng of *XHNF1β* mRNA was injected in the animal pole of 2-cell-stage embryos; animal caps were dissected at stage 8 and monitored at stage 11 for *Xbra* expression by in situ hybridization. Labeling was not detected in injected caps at either dose (Fig. 7F), or in control caps (Fig. 7D), demonstrating that XHNF1β alone is not sufficient for mesoderm induction.

B-Vg1 and VegT induce *XHNF1β* expression in animal caps

Vg1 and *VegT* mRNA are maternal determinants initially stored in the egg vegetal hemisphere (Weeks and Melton, 1987; Zhang and King, 1996). Vg1 activated form, B-Vg1, and VegT are both able to induce endoderm and mesoderm in animal caps (Thomsen and Melton, 1993; Henry and Melton, 1998; Zhang and King, 1996; Clements et al., 1999). Because of *XHNF1β* vegetal expression, we tested whether B-Vg1 and/or VegT could turn on *XHNF1β* in animal caps. We therefore injected 300 pg *B-Vg1* or 1 ng *VegT* mRNA/blastomere in the animal pole region of 2-cell-stage embryos, dissected out animal caps and assayed them by RT-PCR for *XHNF1β* expression at stage 10. Both *B-Vg1* and *VegT* mRNA induce *XHNF1β* expression in ectodermal explants, suggesting that these maternal determinants may trigger *XHNF1β* transcription in the vegetal part of the embryo (Fig. 8).

XHNF1β/EngR mRNA blocks B-Vg1-mediated, but not eFGF-mediated, mesoderm induction

Because TGFβ- and FGF-like secreted molecules have been demonstrated to trigger mesoderm formation in animal caps, we asked whether *XHNF1β/EngR* mRNA was able to prevent mesoderm induction in animal caps injected with either *B-Vg1* (Thomsen and Melton, 1993) or *eFGF* (Isaacs et al., 1992) mRNA. We coinjected the animal pole region of *Xenopus* embryos with either 1 ng of *XHNF1β/EngR* mRNA+100 pg of *B-Vg1* mRNA/blastomere (2-cell-stage injections), or with 500 pg of *XHNF1β/EngR* mRNA+40 pg *eFGF* mRNA/blastomere (4-cell-stage injections); animal caps were then explanted from these embryos, cultured up to stage 11 and assayed for *Xbra* expression by in situ hybridization. As positive controls, caps were also taken and cultured from embryos similarly injected with either *B-Vg1* mRNA only, or with *eFGF* mRNA only. *XHNF1β/EngR* efficiently blocks *Xbra* activation by B-Vg1 (Fig. 9A,B), but it does not prevent *Xbra* activation by eFGF (Fig. 9D,E).

Effects of *XHNF1β/EngR* mRNA on TGFβ-related gene activities

Xnr1, Xnr2, Xnr4 and Derrière are TGFβ factors involved in late blastula steps of mesoderm induction and initially expressed in the vegetal pole (Jones et al., 1995; Joseph and Melton, 1998; Clements et al., 1999; Sun et al., 1999); we therefore studied the effect of 4-cell-stage radial injection of *XHNF1β/EngR* mRNA (500 pg/blastomere) on transcription of their genes. While *Xbra* and *chordin* were suppressed in stage 9 embryos by *XHNF1β/EngR*, transcription of *Xnr1*, *Xnr2* and *derrière*, initially expressed in the vegetal pole (Jones et al., 1995; Sun et al., 1999), was maintained. On the contrary, *Xnr4* transcription was strongly downregulated (Fig. 10). At stage 10.25, results for injected embryos were slightly different to those at stage 9: *Xnr4*, *chordin* and *Xbra* were strongly downregulated, *Xnr1* (mostly expressed in the dorsal marginal zone in control gastrulae; Jones et al., 1995) was severely reduced, *derrière* (mainly expressed in the dorsal marginal zone; Sun et al., 1999) was consistently reduced, but *Xnr2* expression appeared upregulated (Fig. 10). Overall, these data suggest that *XHNF1β* function may not be required for initial vegetal activation of *Xnr1* and *Xnr2* and *derrière* genes at stage 9, but it may influence *Xnr4* transcription. In addition, it may indirectly influence later expression of all these genes, when they are predominantly transcribed in the marginal zone.

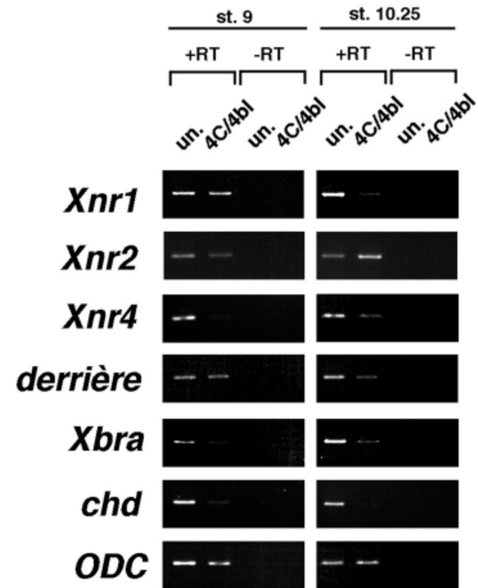


Fig. 10. Expression levels of *nodal*-related and *derrière* genes are compared by RT-PCR at late blastula (stage 9) and gastrula (stage 10.25) in uninjected embryos (un.), and *XHNF1β/EngR* radially injected embryos (4C/4bl).

DISCUSSION

We analysed the role of *XHNF1β* in the early *Xenopus* embryo. *XHNF1β* expression begins at around MBT; transcripts are initially detected in the vegetal pole of the late blastula embryo, a crucial region for the production of mesoderm-inducing signals. *XHNF1β* transcription is successively maintained in endodermal cells derived from the vegetal hemisphere and is also activated in other areas of the embryo. *XHNF1β* function was investigated by microinjections of a chimeric engrailed repressor construct mRNA. The rationale for this approach was based on previous experiments on transfected cell lines, which demonstrated that *HNF1β* is a strong transcriptional activator (De Simone et al., 1991); moreover, the possibility that we might interfere with endogenous *XHNF1α* function is ruled out by the observation that XHNF1α protein is not detected until late tailbud stage (Bartkowski et al., 1993). Gain-of-function experiments were performed by injection of wild-type *XHNF1β* mRNA.

XHNF1β/EngR mRNA injection suppresses mesoderm formation either dorsally or ventrally. Dorsal injections result in strong axial deficiencies, while ventral injections produce only minor deficiencies in the form of ventroposterior bulges.

While axial development is severely impaired by dorsal injection of *XHNF1β/EngR*, such effect is not observed upon injections of mutant forms of the chimeric construct, encoding homeodomains with different DNA-binding abilities, thus implying specificity of the effect. Moreover, results of coinjection experiments show that, though not sufficient to produce overt external phenotypes, the wild-type mRNA is able to rescue the effect of *XHNF1β/EngR* mRNA; this strongly suggests that *XHNF1β/EngR* mRNA exerts a dominant negative effect on the endogenous *XHNF1β* gene function.

The axis-suppressing effect of the chimeric mRNA in dorsal injections is clearly the result of the absence of Spemann's organizer, as revealed by morphological and molecular criteria. Absence of dorsal mesoderm is not due to its ventralization (as caused by BMPs; Dosch et al., 1997), but rather to suppression of mesoderm formation on the dorsal side of the embryo. Injection of *XHNF1β/EngR* mRNA impairs mesoderm development also ventrally, as shown by ventral suppression of *Xbra* and *Xpo*. Interestingly, maintenance of *Xvent2* ventral expression suggests that the BMP-4-dependent patterning activity on the ventral side may not be disturbed.

While mesodermal markers are severely affected, expression of vegetal markers, and particularly of the endodermal markers *Xsox17α* (Hudson et al., 1997) and *Mixer* (Henry and Melton, 1998), is unaffected after radial injection of *XHNF1β/EngR* mRNA in 4-cell-stage embryos. Furthermore, expression of *siamois* (Lemaire et al., 1995) is also maintained, indicating that the Wnt/GSK3/β-catenin dorsalizing pathway is not disturbed. The observation that the organizer is not formed in spite of *siamois* being expressed in injected embryos is consistent with the idea that organizer specification, in the whole embryo, requires a synergy between distinct pathways of mesoderm-inducing signals and dorsalizing signals (Darras et al., 1997; Moon and Kimelman, 1998).

Vegetally localized maternal determinants play a crucial role in initiating mesoderm induction by somehow establishing a vegetal signalling centre. We propose that *XHNF1β* is a component of this centre based on the following observations. (1) *XHNF1β* is expressed in the vegetal region of the *Xenopus* embryo, at the right time and place to play a role in the late blastula steps of mesoderm induction. (2) *XHNF1β* is induced in animal caps both by B-Vg1 and by VegT; therefore its vegetal expression may depend on these maternal determinants. (3) Interfering with *XHNF1β* function suppresses mesoderm in whole embryos and impairs the mesoderm-inducing abilities of vegetal explants in animal/vegetal conjugates. All these data strongly suggest that *XHNF1β* is required in the vegetal part of the early *Xenopus* embryo for mesoderm to be induced in the marginal zone. *XHNF1β* may only have a permissive role in mesoderm induction, as it is not sufficient to trigger mesoderm formation in animal caps.

Maternal determinants, such as VegT and possibly early activin-like molecules, may initiate a signalling relay via other TGFβ factors, by activating transcription of *nodal*-related genes and *derrière* (Jones et al., 1995; Clements et al., 1999; Kofron et al., 1999; Yasuo and Lemaire, 1999). With the exception of *Xnr4,* our data suggest that *XHNF1β* functional ablation may have little effect, if any, on transcription of these genes at late blastula, while the effects observed at gastrula, when they are mainly expressed in the marginal zone, may be due to *XHNF1β/EngR* preventing signalling from the vegetal pole. These results, together with the observation that effects of *VegT* mRNA depletion are rescued by vegetal expression of any of *Xnr1*, *Xnr2*, *Xnr4* and *derrière* mRNA (Kofron et al., 1999), suggest that *XHNF1β* may have a function other than mere regulation of their transcription. One possibility is that *XHNF1β* allows the processing or release of inducing molecules from the prospective endoderm of the vegetal region. Interestingly, it has been proposed that mature Vg1 may induce mesoderm after having first induced endoderm (Kessler and Melton, 1995; Joseph and Melton, 1998); indeed, cells producing B-Vg1 are fated to give endoderm both in injected UV-treated embryos and in animal caps (Thomsen and Melton, 1993; Henry and Melton, 1998). The observations that B-Vg1 is able to induce *XHNF1β* in animal caps and that *XHNF1β/EngR* blocks the mesoderm-inducing ability of B-Vg1, but not that of eFGF, are consistent with this view and suggest that *XHNF1β* may work as a permissive factor for vegetal mesoderm induction signalling.

While required for mesoderm induction, *XHNF1β* function may be dispensable for early endodermal specification. This observation is somewhat puzzling, since mesoderm and endoderm appear to result from the same initial induction event, and both can be induced by TGFβ factors (Harland and Gerhart, 1997). However, the recent observation that *Bix4,* a direct target of VegT, rescues endoderm in maternal *VegT*-depleted embryos, while not recovering the mesoderm-inducing ability of vegetal cells (Casey et al., 1999), suggests that *VegT* may specify mesoderm and endoderm through separate genetic cascades.

Recent evidence indicates that mesoderm induction is a zygotic event (Wylie et al., 1996; Kofron et al., 1999; Yasuo and Lemaire, 1999), and emphasize the role of early vegetal transcription factors in conferring full signalling ability to vegetal cells and in initial patterning events. Our data provide further evidence for a primary role of vegetal transcription factors in mesoderm induction. It will be an interesting problem for future research to understand how regulatory proteins may interact to confer full inducing abilities to vegetal pole cells and to lay down the basic body plan of the vertebrate embryo.

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