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An activated form of type I serine/threonine kinase receptor TARAM-A reveals a specific signalling pathway involved in fish head organiser formation

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SUMMARY

The role of Transforming Growth Factor β (TGF-β)-related molecules in axis formation and mesoderm patterning in vertebrates has been extensively documented, but the identity and mechanisms of action of the endogenous molecules remained uncertain. In this study, we isolate a novel serine/threonine kinase type I receptor, TARAM-A, expressed during early zebrafish embryogenesis first ubiquitously and then restricted to dorsal mesoderm during gastrulation. A constitutive form of the receptor is able to induce the most anterior dorsal mesoderm rapidly and to confer an anterior organizing activity. By contrast, the wild-type form is only able to induce a local expansion of the dorsal mesoderm. Thus an activated form of TARAM-A is sufficient to induce dorsoanterior structures and TARAM-A may be activated by dorsally localized signals. Our data suggest the existence in fish of a specific TGF-β-related pathway for anterior dorsal mesoderm induction, possibly mediated by TARAM-A and activated at the late blastula stage by localized dorsal determinant.

Key words: zebrafish, dorsal mesoderm, induction, organizer, serine-threonine kinase receptor, transforming growth factor β, TARAM-A

INTRODUCTION

Early vertebrate development involves cell-cell interactions, called inductions, changing the fate and/or behaviour of a population of cells under the influence of signals emitted by neighbouring cells. Mesoderm formation and patterning in frogs is one of the best studied example of inductive process. According to models developed during recent years, early signals provided by the prospective endoderm both induce and pattern mesoderm along the dorsoventral (D/V) axis. Ventral mesoderm is induced by ventral endoderm, whereas the most dorsal mesoderm, known as Spemann organizer, is induced by a group of dorsal endoderm-fated cells called the Nieuwkoop center (NC), (Smith, 1993; Kessler and Melton, 1994). Further mesoderm patterning is achieved around gastrulation by both ventralizing signals, provided by ventral mesoderm and dorsalizing signals provided by the organizer. The exact molecular nature of NC-provided signals is not yet clear but may include mesoderm inducers related to FGF and TGF-β as well as competence modifiers such as *wnt* which would dorsalize mesoderm (Smith, 1993; Kessler and Melton, 1994). Within the TGF-β class, three candidates inducers have been implicated. In the relevant in vitro assays, *Xenopus* activins (Smith, 1993; Thomsen et al., 1990), processed Vg-1 (Thomsen and Melton, 1993) and nodal-related gene products Xnr-1 and Xnr-2 (Jones et al., 1995), can induce dorsal mesoderm formation. A full range of dorsoanterior structures including heads can be rescued in UV-ventralized embryos lacking a Spemann organizer, by NC cells grafts or by expression of either activins, processed Vg1 or nodal-related factors, suggesting that these signals may indeed be produced by NC (Thomsen and Melton, 1993; Jones et al., 1995). However, as expected for an activity generated by the NC, only activins and Vg-1 are present as maternal components (Asashima et al., 1991; Thomsen and Melton, 1993). On the contrary, Xnr-1 and Xnr-2 are expressed as zygotic transcripts, after the MBT, initially in the prospective endoderm and mesoderm (Jones et al., 1995).

During fish embryogenesis, a fate map can be drawn just before gastrulation (Kimmel et al., 1990) in which mesoderm originates from the margin of the gastrula blastoderm while cells from the animal hemisphere primarily generate ectodermal derivatives. This map shows a topology very similar to the *Xenopus* map (Dale and Slack, 1987). In parallel, genes involved in formation and differentiation of the mesoderm like *ntl* (homologue of *Xenopus brachyury* and mouse *T* genes, Schulte-Merker et al., 1994), *axial* (homologue of mouse *HNF3β*, Strähle et al., 1993), *goosecoïd* (Stachel et al., 1993; Thisse et al., 1994) and *lim-1* (Toyama et al., 1995) show a similar pattern of expression in *Xenopus* and zebrafish embryos strongly suggesting that mechanisms of vertebrate mesoderm patterning have been conserved during evolution (Beddington and Smith, 1993).

In fish, there is experimental evidence that cell interactions control the formation of dorsal mesoderm. Particularly, blastoderm rotation experiments in trout have shown that signals emanating from an extraembryonic layer, the yolk syncitial layer (YSL), can respecify the position of the dorsal mesoderm (Long, 1983). Molecular mechanisms controlling fish mesoderm patterning are very poorly understood, but the conservation and the implication of TGF-β-related molecules in mesoderm formation and/or patterning in fish, frogs and mice (activins, Vg-1, nodal-related) suggest that they may represent

common cues for mesoderm patterning in different vertebrates. Zebrafish activins and mouse nodal induce posterior axis duplications, including trunk and tail structures, but no heads (Wittbrodt and Rosa, 1994; Toyama et al., 1995). A Vg1-related maternal transcript has been isolated in zebrafish and its corresponding processed protein is also able to induce dorsal mesoderm in zebrafish (Dohrmann et al., 1996).

Thus, in frogs and fish, the identity and the mechanisms of action of endogenous inducer(s) for dorsal mesoderm remain elusive. One way to address this question is to characterize the corresponding receptors. Specific intracellular signalling by TGF-β-related molecules is achieved by formation of a complex between a ligand and a serine/threonine kinase type II receptor followed by the recruitment of a type I receptor with a similar kinase domain (Wrana et al., 1994). In turn, the type II receptor transphosphorylates a particular domain of the type I receptor, the GS domain, which is believed to trigger the activation of intracellular signalling by the type I receptor. Activation of a defined type I receptor can depend on the formation of different ligand/type II receptor complexes (Yamashita et al., 1995). Specificity of the response relies on the activity of the type I receptor (Carcamo et al., 1994).

We characterized a zebrafish cDNA encoding a type I receptor, TARAM-A. First present as maternal ubiquitous transcripts in the zebrafish embryo, *TARAM-A* expression is progressively restricted to the presumptive mesoderm and the migrating axial mesoderm. Ectopic expression in zebrafish embryo of a gain-of-function mutant protein shows that TARAM-A activity is sufficient to induce the most anterior axial mesoderm and to confer an anterior organizing activity. The wild-type protein is only able to induce dorsal mesoderm markers on the dorsal margin of the embryo, indicating that only a local activation of TARAM-A may occur in the embryo. Our data sustain the view of a signalling pathway activated by a TGF-β-related factor able to support anterior dorsal mesoderm induction in fish.

MATERIALS AND METHODS

Cloning of a *TARAM-A* cDNA and generation of *TARAM-A-D*

A 180 bp *TARAM-A* cDNA fragment was obtained by RT-PCR from RNAs prepared at the shield stage. Three degenerated oligonucleotides covering the conserved kinases subdomains were used, VI 5′gccggatcCAC/TCGNGAC/TATAAAA/GTCNAAA/GAA3′ and IX 5′ ccggaattCCC/GAA/TG/AG/CA/T/CA/GTAG/CACTA/GTC3′ (first round) and VI and VIII 5′gccgaattcNAGNAC/TC/TTCNGGNGCCATA/GTA3′ (second round) in order to favor specific amplification. This fragment was used to clone a partial cDNA, FL, lacking the 5′ part of the ORF, from a gastrula-stage cDNA library (gift of R. Riggleman and K. Helde). Another cDNA, NT, containing the 5′ part of the ORF was obtained from a gastrula-stage cDNA library (gift of M. Rebagliati and I. Dawid), with a 5′ probe obtained from a RACE reaction. *TARAM-A-WT* cDNA was reconstructed by fusion of FL and NT at the unique *Bam*HI site. In order to generate *TARAM-A-D*, a PCR fragment carrying the mutation changing a threonine into aspartic acid (underlined) was amplified from the *TARAM-A-WT* plasmid with the oligonucleotides 5′GGATGTTGAGGATCCATCCTGTGAT3′ and 5′tcgcggatcctctagatcgtggtaccggctcggatgcccgggCTGCAGTACAT<u>GTC</u>CCTGGCCAG3′ and inserted into the *TARAM-A-WT* plasmid between the uniques *Bam*HI and *Pst*I sites.

Preparation of capped RNA and injection

cDNAs to be transcribed were inserted into the expression vector psP64T. Plasmids were linearized and in vitro transcribed by using the SP6 Ambion Megascript in vitro transcription kit. Injections were performed as described by Wittbrodt and Rosa (1994). Below a 5 μg/ml concentration of RNA to be tested, β-galactosidase RNA carrier was added at a final concentration of 40 μg/ml. When mentioned, rhodamine coupled to 2 MDa dextran was included at 50 mg/ml in the injection mix. This tracer has been shown not to diffuse to neighbouring cells (Strehlow et al., 1994).

Whole-mount in situ hybridisation and immunohistochemistry

Digoxigenin- and fluorescein-labeled antisense probes were prepared with a commercially available nucleotide mix (Boehringer). *TARAM-A* probe was transcribed with T7 polymerase from a cDNA covering the 3′ untranslated part or the ORF of the transcript. *ntl*, *gsc*, *axial*, *lim* and *eve-1* probes were prepared as described respectively in Schulte-Merker et al. (1994) Stachel et al. (1993), Strähle et al. (1993), Toyama et al. (1995) and Joly et al. (1993). Whole-mount in situ hybridisation were carried out as described by Hauptman and Gerster (1994). Staining of embryos incubated with NTL antibody was performed with PAP as described by Westerfield (1993). β-galactosidase staining was performed as described by Vize et al. (1991).

RESULTS

Cloning of TARAM-A a novel serine/threonine kinase type I receptor related to TGF-β and activin receptors

We cloned from a late zebrafish blastula library a cDNA containing an ORF that encodes a typical type I serine/threonine kinase receptor of 506 aminoacids, TARAM-A (Figs 1, 3). TARAM-A is mainly characterized by a conserved spacing of cysteines in the extracellular domain, the existence between the transmembrane and the kinase domains of a TSGSGSGLP motif (GS domain) absent from type II receptors. Comparison of TARAM-A sequence with other type I receptors showed that it was most closely related to mouse and human activin type I receptor m/hActR-IB (Carcamo et al., 1994), to the human TGF-β type I receptor TβR-I (Franzen et al., 1993) and the *Xenopus* TGF-β-related receptor XTrR-I (Mahony et al., 1995). TARAM-A intracellular kinase domain is highly conserved with m/hActR-IB (94%), TGFβR (88%) and the XTrR-I (88%). By contrast, the extracellular domain (ending at the consensus sequence GPVE, Fig. 1) is poorly related to ActR-IB (43%), TGFβR (25%) and XTrR-I (23%). This comparison defines this receptor as a new member of this group. Because of its expression during gastrulation and its effects on axial mesoderm formation, the receptor was named TARAM-A (TGF-β-Activin-related Receptor expressed in Axial Mesoderm). RT-PCR analysis confirmed that *TARAM-A* is expressed during early embryonic stages first as a maternal transcript(s) in the egg and maintained at a constant level until gastrulation (data not shown).

TARAM-A displays a spatial restriction during zebrafish early embryogenesis

Whole-mount in situ hybridisations have been performed with digoxygenin-labeled antisense RNA probes encompassing the *TARAM-A* ORF or its 3′ UTR and gave the same results. Sense probe, used as a control, did not reveal any staining. *TARAM-A* RNA is expressed maternally (Fig. 2A) and homogeneously

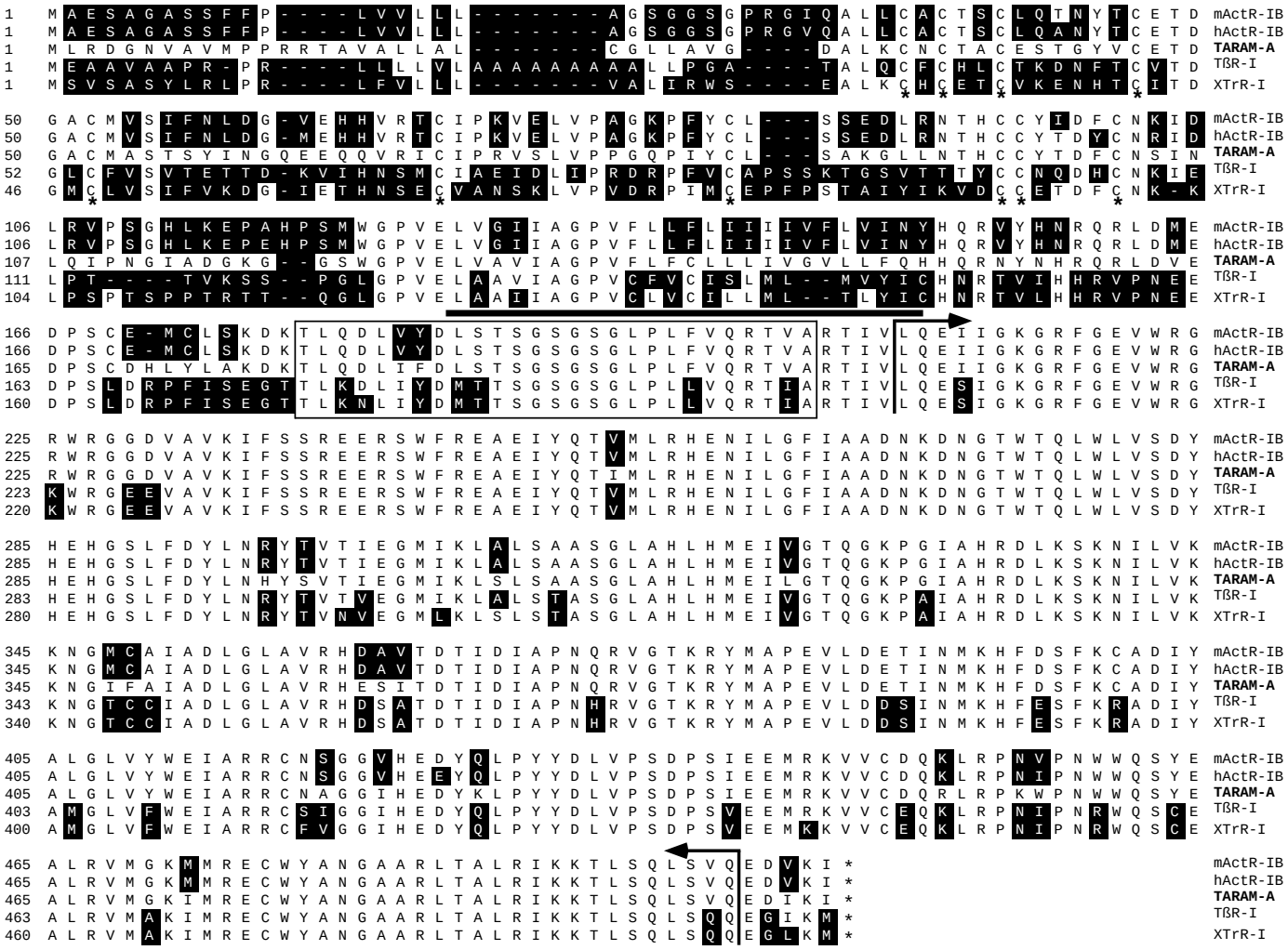

Fig. 1. Comparison of the amino acid sequence of TARAM-A, mouse and human AcTR-IB, human TβR-I and *Xenopus* XTrR-I. Non conserved aminoacids between TARAM-A and the others receptors are black boxed. Conserved cysteine residues in the extracellular domains are indicated by stars. The putative transmembrane domain is underlined, the GS domain is boxed and the borders of the kinase domain are marked by arrows. *TARAM-A* cDNA sequence is available under the accession number X94119.

in all blastomeres until the 250-cell stage. The expression becomes progressively restricted, with exclusion at the 500-cell stage from the prospective yolk syncitial layer (YSL) cells, an extraembryonic layer in immediate contact with the yolk, (Fig. 2B). Further restriction occurs around the dome stage with down-regulation of *TARAM-A* trancripts in animal pole cells (Fig. 2C). From this stage on, *TARAM-A* is expressed only in cells belonging to the marginal zone. At the beginning of epiboly, a new domain of expression appears at the level of the marginal zone and enlarges progressively (Fig. 2D). This domain marks the future dorsal side where the future organizer will form, as shown by colocalization of *gsc*, a dorsal marker (Stachel et al., 1994, data not shown). During epiboly, the level of *TARAM-A* RNA decays progressively in marginal cells except on the dorsal side. As gastrulation begins, the dorsoventral axis becomes morphologically apparent by the formation of a distinct thickening of the cell layers at the dorsal side of the embryo, the embryonic shield. At this stage, the entire deep layer of the shield, corresponding to mesoderm cells that have already involuted (hypoblast) expresses *TARAM-A* (Fig. 2E and data not shown). Some residual expression is also observed in the superficial non-involuted cells and in the rest of the margin. The *TARAM-A* domain then begins to extend towards the animal pole (Fig. 2F); expression is found exclusively in the newly formed hypoblast, the inner cell layer that gives rise to endodermal and mesodermal derivatives (Fig. 2I and data not shown). NTL protein is expressed around the involuting margins of gastrulating embryos and in the axial mesoderm, marking the precursor cells of the future notochord. To map the expression of the *TARAM-A* gene along the developing axis with respect to the *ntl* gene, we performed double-labeling experiments, restaining *TARAM-A* antisense RNA-labeled embryos with the antibody directed against the zebrafish NTL protein, which stains nuclei from NTL-expressing cells (Schulte-Merker et al., 1994). To improve resolution, stained embryos were embedded in wax and sectioned. A sagittal section through the dorsal axis of a mid-gastrula embryo is shown in Fig. 2I. In the posterior half of the hypoblast, cells express both *TARAM-A* RNA and NTL protein, however, *TARAM-A*-positive cells, which are farther up in the hypoblast

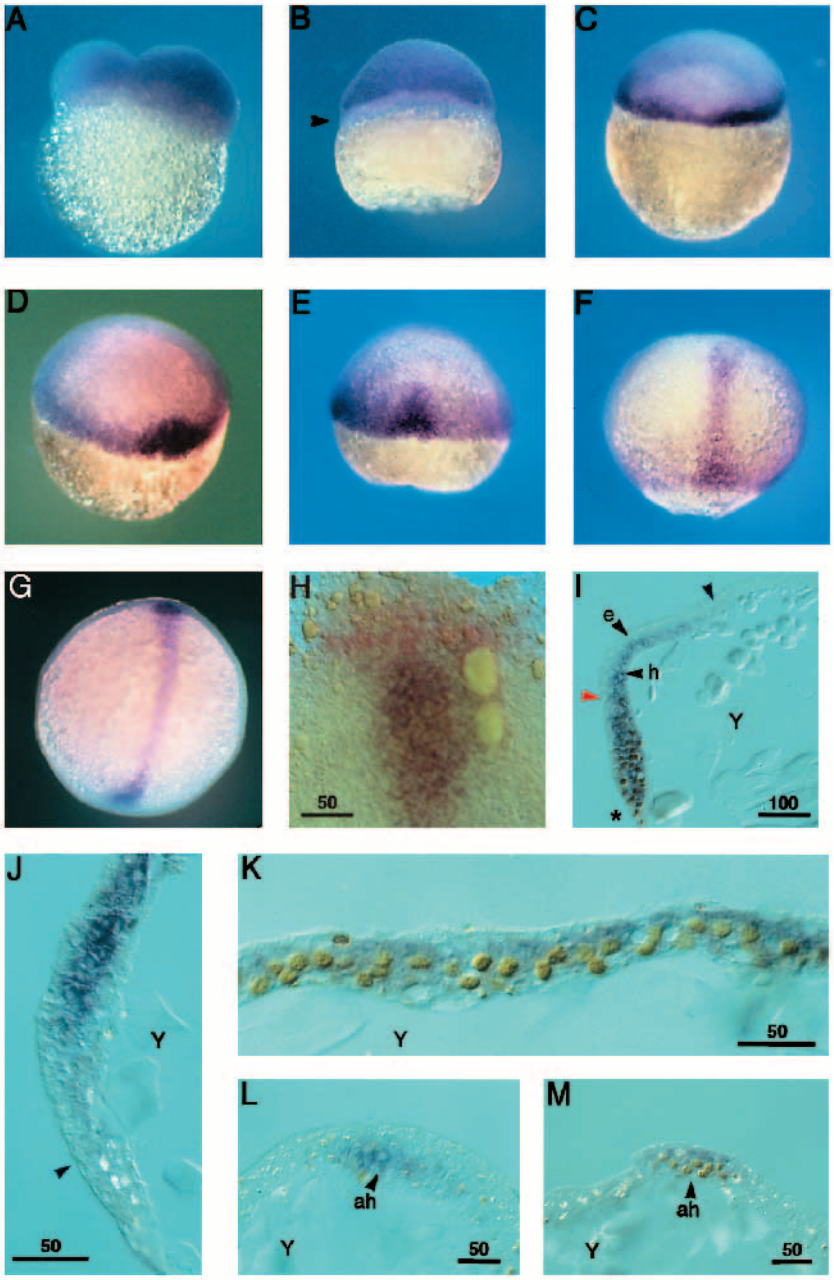

Fig. 2. (A-G) *TARAM-A* RNA expression in early zebrafish embryos stages. Whole-mount view from lateral (A-C), or dorsal (D-G) side; (A-F) animal pole is to the top; (G,H) anterior is to the top. (A) 2-cell-stage. (B) 500-cell stage. *TARAM-A* expression is fading from the marginal cells corresponding to the prospective yolk syncitial layer cells (YSL) (arrowhead). (C) Dome stage. *TARAM-A* expression is no longer detected in animal pole cells and is restricted to the marginal zone. (D) 40% epiboly stage. An additional domain of *TARAM-A* appears on the future dorsal side. (E) Shield stage. The expression in the marginal zone is fading and the *TARAM-A* territory expression occupies the shield area. (F) 80% epiboly stage and (G,H) 100% epiboly stage; labelling is restricted to the embryonic axis. (H) Flat-mount view of head of an embryo at the end of gastrulation labelled with *gsc* (red) and *TARAM-A* (purple). The anterior limit of *TARAM-A* expression is bordered by the anterior, crescent-shaped *gsc* domain. (I-M) Sections of embryos labelled with a *TARAM-A* probe (blue) and an anti-NTL antibody (nucleus in yellowish brown). (I-K) Sagittal section; (L,M) cross sections. Y indicates the position of the yolk, the black arrowheads indicate the positions of the epiblast (e) and the hypoblast (h) or axial hypoblast (ah). (I) Sagittal section through a 70% epiboly stage embryo; the asterisk indicates the blastoderm margin, the red arrowhead indicates the anterior boundary of NTL protein distribution and the blue arrowhead the anterior boundary of *TARAM-A* expression in the hypoblast (dorsal to the left). Brachet's cleft, delimiting the hypoblast-epiblast boundary, is visible in the anterior part of the embryo (Kimmel et al., 1995). (J,K) Sagittal section through the head (J), or the trunk (K), of a 95% epiboly stage embryo, (J, anterior down). The head can be divided into three regions. Anterior to the blue arrowhead, where the crescent-shaped expression of *gsc* is detected (see H), no *TARAM-A* expression is detected. Posterior to the blue arrowhead, both hypoblast and epiblast weakly express *TARAM-A*. More posteriorly, strong *TARAM-A* expression is graded, with peak levels around hypoblast-epiblast boundary. Periderm is not labelled. (K; anterior left) The axial hypoblast corresponding to the future notochord expresses specifically *ntl* and is labelled by *TARAM-A*. In addition to the hypoblast the epiblast is labelled. (L,M) Transverse sections through the head (L) and the trunk (M) of a 95% epiboly stage embryo. Staining is axial in both cases. Values for scale bars are indicated in μm.

at its most anterior tip, having involuted slightly earlier, do not express the NTL protein. At the end of gastrulation, *TARAM-A* is expressed in axial structures in formation (Fig. 2F,G). The anterior limit of *TARAM-A* expression at 100% epiboly is posterior crescent-shaped anterior expression of *gsc* RNA (Fig. 2H), thereby partially overlapping the pillow region (prospective hatching gland). Transverse section analysis of embryos stained for *TARAM-A* RNA and NTL protein confirm the axial expression of *TARAM-A* (Fig. 2J-M). Analysis of sagittal and transverse sections shows that *TARAM-A* RNA is briefly expressed in both trunk axial hypoblast and epiblast (prospective neural tube) at the end of gastrulation (Fig. 2K,M). Expression in the head appears graded with highest levels

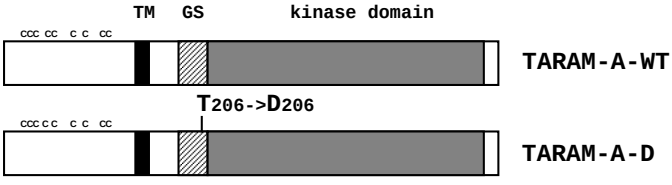

Fig. 3. Schematic representation of the structure of wild-type TARAM-A (TARAM-A-WT) and mutant TARAM-A-D receptors. C indicates the nine cyteine residues of the extracellular domain, the black box represents the transmembrane domain (TM), the hatched box the GS domain and the grey box the kinase domain. In TARAM-A-D the threonine 206 (T) has been replaced by an aspartic acid (D).

around the hypoblast-epiblast boundary (Fig. 2J). Soon after the end of gastrulation, *TARAM-A* expression fades quickly from trunk structures first and then progressively later from the anterior territory and from the tailbud (data not shown).

Expression in marginal cells, fated in part to mesoderm then to axial mesoderm suggests that the TARAM-A receptor could be involved in mesoderm induction or patterning as well as in organizer formation. To test this hypothesis, we studied the effects of ectopic expression of TARAM-A and a gain-of-function variant on early embrogenesis.

Ectopic expression of wild-type TARAM-A protein in embryos enlarges the expression domains of organizer-specific genes

Ectopic expression of TARAM-A-WT (Fig. 3) was carried out by microinjection of synthetic RNA (200 μg/ml) into zebrafish embryos at the 1-cell stage. Injected embryos did not differ markedly from control embryos after 24 hours development. We analysed the expression of early dorsal mesodermal markers in injected embryos. Slightly before the onset of gastrulation (50% epiboly) in control embryos, *gsc* is only expressed in the deep layer of the shield (hypoblast) corresponding to the anterior mesoderm involuting upward (Stachel et al., 1993; Thisse et al., 1994). Microinjection of *TARAM-A-WT* RNA led reproducibly (70%, *n*=85) to an expansion of the *gsc* expression domain and in some cases to the formation of two neighbouring *gsc* expression domains within the margin of the embryo (Fig. 4A). Similar enlargments were observed with *axial*, another shield-specific marker (60% *n*=72), (Fig. 4B). Two others markers, *lim-1* and *ntl*, label the margin and a part of the embryonic shield at the same stage. Similarly, ectopic expression of TARAM-A-WT led to the expansion of *lim-1* dorsal domain (50%, *n*=60) although less marked than *gsc* and *axial*, (Fig. 4C). On the contrary, *ntl* expression domains appeared unchanged (*n*=54, not shown). *eve-1*, a specific marker of the future ventral and posterior cells that is expressed at the level

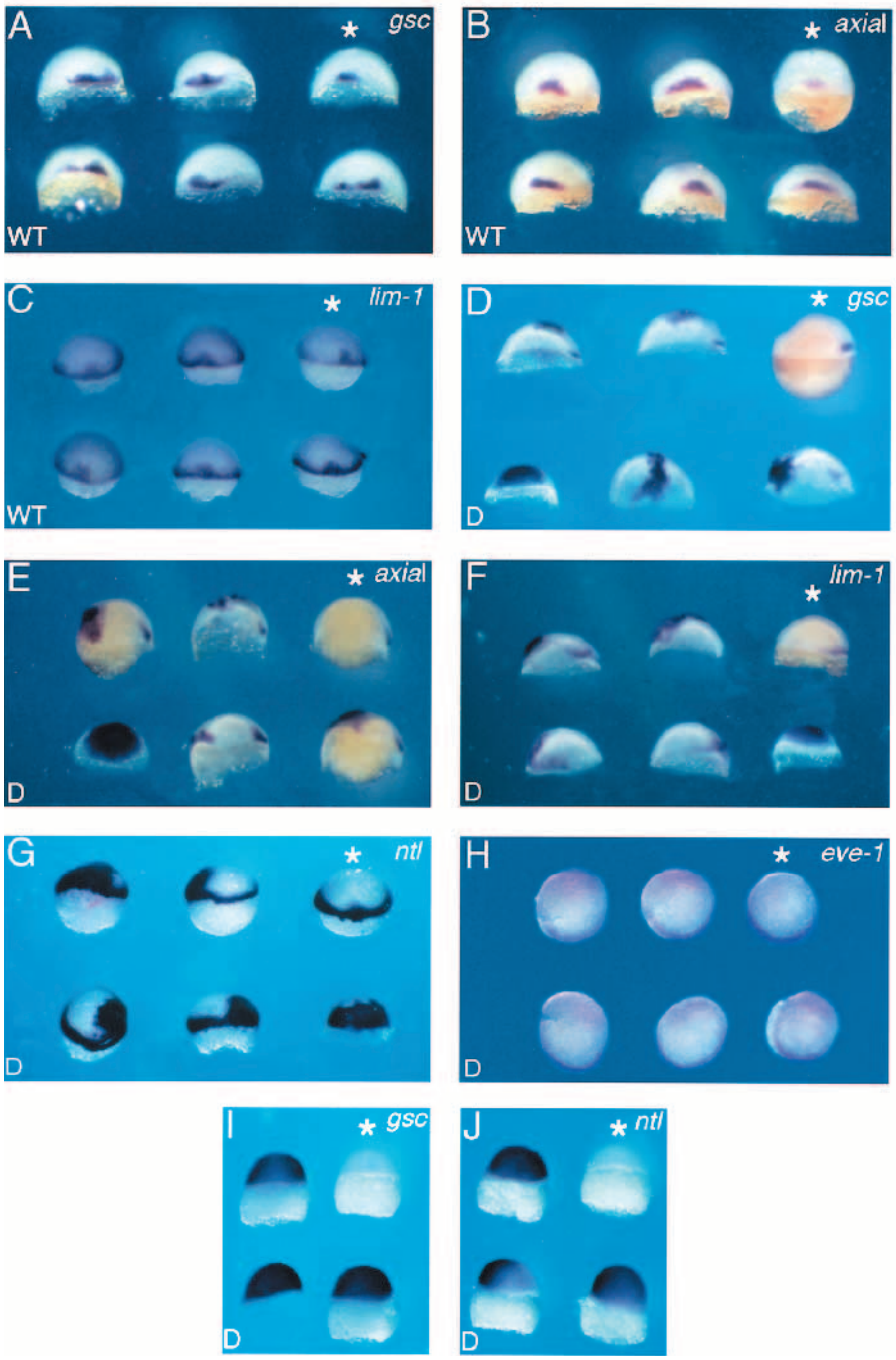

Fig. 4. In vivo analysis of TARAM-A and TARAM-A-D activities; expression of mesodermal markers (indicated in the upper right corner of each picture) in zebrafish late blastula (50% epiboly) (A-H), or high-blastula stage (3.5 h) (I,J). Embryos were injected at the 1- or 2-cell stage into the animal pole region with *TARAM-A-WT* (200 μg/ml), or with *TARAM-A-D* at 0.1 or 2 μg/ml. The asterisk indicates non-injected control embryo. (A-C) Expression of *gsc*, *axial* and *lim-1* respectively upon TARAM-A-WT overexpression (dorsal view). TARAM-A-WT induces an enlargment or a local duplication of the *gsc*, *axial* and *lim-1* domains in the shield area. (D-G) Expression of *TARAM-A-D* RNA (0.1 or 2 μg/ml) induces in varied locations large and thickened ectopic dorsal mesoderm (side view, dorsal to the left). In each case, one embryo injected with 2 μg/ml RNA is included and exhibits expression of the marker in the entire blastoderm. Expression of *TARAM-A-D* RNA (0.1 or 2 μg/ml) does not induce ectopic expression of a ventral marker *eve*-1 (H), (animal pole view, dorsal to the top). At 50% epiboly, *eve-1* follows an arch of 180° on the marginal zone opposite the dorsal side. This arc is slightly reduced in injected embryos (the pink staining corresponds to the lineage tracer coinjected with the RNA). (I,J) Early onset of *gsc* and *ntl* expression in high blastula following injection of *TARAM-A-D* RNA. Induced expression is visible before the endogenous expression can be detected particularly for *ntl* RNA.

of the margin and excluded from the dorsal side, is not affected (*n*=62, data not shown). As a control, we microinjected a BMP type I receptor RNA (*mTFR11*, Suzuki et al., 1994) or β-galactosidase RNA at 200 μg/ml. In these conditions, the *gsc*, *axial* and *lim-1* expression domain were not affected (*n*=80, *n*=71, *n*=68 repectively, data not shown).

Ectopic expression of TARAM-A-D, a gain-of-function mutant induces anterior structure duplications

The local effect of TARAM-A on genes involved in dorsal mesoderm formation suggests that TARAM-A could be involved in their activation and that competence to respond to TARAM-A signalling could be spatially restricted to the region neighbouring the organizer. To test this hypothesis, a gain-of-function mutant corresponding to a constitutively active receptor, TARAM-A-D was created by changing the threonine 206 located in the end of the conserved GS domain to an aspartic acid (Fig. 3). Different type I receptors mutated in this location like TβR-I (Wieser et al., 1995), ActR-IB (Attisano et al., 1996), ALK3 and ALK6 (Hoodless et al., 1996) display specific activities independant of their ligands and the associated type II receptors.

TARAM-A-D RNA was microinjected at very low doses into the animal pole of 1-cell-stage zebrafish embryos and corresponding late blastulae/early gastrulae were analysed by whole-mount in situ hybridization. At an RNA concentration of 0.1 μg/ml, in 100% of the injected embryos, specific markers for dorsal mesoderm *gsc*, *lim-1* and *axial* (*n*=100, *n*=87, *n*=65 respectively) are ectopically expressed in large and thickened domains marked by convergence and involution movements (Fig. 4D-F). At this stage, *ntl* RNA is not yet specific for dorsal mesoderm but very soon will label prospective dorsal mesodermal cells fated to notochord injection of *TARAM-A*-D induces its ectopic expression as well (Fig. 4G). By contrast no ectopic expression of *eve-1*, a marker of the future ventral mesoderm, was detected in injected embryos (*n*=45), and, in some cases, the domain of expression in the margin was slightly reduced (Fig. 4H). A 4-fold lower RNA concentration (0.025 μg/ml) did not affect the expression of the same markers and higher concentrations (2 μg/ml and above) led to an homogenous expression of the dorsal markers in the entire blastoderm. We then determined when ectopic expression of dorsal marker could first be detected by carrying in situ hybridization on injected embryos (2 μg/ml) harvested soon after MBT, the beginning of zygotic transcription. One hour after MBT (high blastula stage, Fig. 4I,J), ectopic expression can be detected for *gsc* (note the endogenous expression on the dorsal side of control embryo) and for *ntl* (endogenous *ntl* is not detectable yet in controls) suggesting that mesoderm-specific markers can be activated as soon as transcription starts.

After 24 hours, injected embryos display partial and very disorganized multiple axes, including clearly recognizable ectopic hatching glands, derived from the most anterior axial mesoderm as expected from the formation of ectopic shields (data not shown). To clarify the nature of these induced structures, injection into one cell of a 16-cell-stage embryo was carried out. At the 16-cell stage, blastomeres are organized as a square over the yolk with twelve marginal cells and four central cells with ill-defined fates. Injection of *TARAM-A-D* RNA into one marginal cell led to the formation of well-formed embryos, but in 25% of the cases (*n*=80) we observed embryos with clearly recognizable duplicated anterior structures ranging from two complete heads on opposite sides (Fig. 5A) or juxtaposed (Fig. 5B), to the formation of one or two additionnal eyes (Fig. 5C). These three groups appear equally represented. Duplication of posterior structures was never observed.

TARAM-A-D activity is thus sufficient to specifically induce anterior dorsal mesodermal structures and to promote an anterior organizing activity. In addition, since the ectopic expression of dorsal mesoderm markers could be achieved in other positions than the endogenous shield, capacity to respond to TARAM-A-D does not appear spatially restricted. These results suggest that activation of TARAM-A-WT signalling is dorsally restricted.

Cells expressing TARAM-A-D are fated to anterior mesoderm

In order to determine the fate of cells expressing TARAM-A-D, we coinjected *TARAM-A-D* RNA (0.5-2 μg/ml) together with rhodamine coupled to 2 MDa dextran, a lineage tracer, into one marginal cell of a 16-cell-stage embryo and analyzed the sites of fluorescence at gastrulation, 24 and 48 hours of development.

At the beginning of the gastrulation, 80% of the *TARAM-A-D*-RNA-injected embryos (*n*=120) possess, in addition to the endogenous shield which appears not to be fluorescent, another shield composed of fluorescent cells deriving from the *TARAM-A-D*-RNA-injected blastomere and positioned randomly with regard to the endogenous one (Helde et al., 1994). In the 20% remaining embryos, a single slightly larger fluorescent shield was observed (data not shown). In both groups, at 24 and 48 hours, the major site of fluorescence was consistently (100%, *n*=120) detected in the derivatives of the most anterior mesoderm including the hatching gland (Fig. 5D). Fluorescence was also observed in the head, just above the yolk and may correspond to pharyngeal endoderm as suggested by analysis of older embryos. Labelling was observed to a lesser extent (less than 10% of the labelled cells) in a few periderm cells and in cells within the tailbud, apparently unable to participate to a given tissue. Other tissues, including notochord cells, were very rarely labelled (less than 5% of the embryos). These data show that any of the marginal cells at the 16-cell stage expressing TARAM-A-D will give rise to the most anterior axial mesoderm.

Injection of the tracer alone or in combination with *TARAM-A-WT* or β-galactosidase RNA led to labelling of cell clusters at random positions along the margin at gastrulation, but, at 24 and 48 hours, progenies from injected cells were found in the different derivatives of the mesoderm as well as in some neural and ectodermal derivatives. In agreement with the relative indeterminacy of early blastomeres, the distribution of the fluorescence is variable between the embryos both in terms of extent of labelling in a given tissue and combination of labelled tissues in one embryo but some correlation was kept between the position of the labelled cells on the margin at the gastrulation and the location of their progenies (Strehlow et al., 1994). In particular, in 80% of the *TARAM-A-WT*-RNA-injected embryos (*n*=115), labelled cells did not populate the embryonic shield and did not later label the hatching gland and axial

mesendoderm. On the contrary, in 20% of the injected embryos, labelled cells ended up in the dorsal domain at gastrulation and later contributed to the axial-derived tissues, notochord, hatching gland and floorplate.

We performed the same experiments using central cells of the 16-cell-stage embryo. The expression of TARAM-A-D in central cells led either to the formation of recognizable ectopic hatching glands, which were fluorescently labelled or to the labelling of hatching glands in otherwise normal embryos. Injections of the tracer alone confirmed previous results showing that central cells do not participate to mesodermal structures (Strehlow et al., 1994, Kimmel and Warga, 1987; data not shown).

In another series of injections, we used β-galactosidase (β-gal) RNA as a tracer (Vize et al., 1991). Injected *TARAM-A-WT/D* and β-gal RNAs are expected to be similarly distributed and the detection of β-gal activity is easy to perform. Results similar to those described for rhodamine-dextran were obtained, confirming the specificity of the fating procedure (Fig. 5E). We conclude from these experiments that activation of the TARAM-A receptor between the 1- and 16-cell stages is sufficient to direct the progenies of individual blastomeres to an anterior mesodermal fate. Both central and marginal blastomeres from the 16-cell embryo appear competent to respond to TARAM-A-D activity.

DISCUSSION

TARAM-A expression is progressively restricted to dorsal mesoderm

TARAM-A expression displays three main phases during early zebrafish embryogenesis. It is first ubiquitously expressed until MBT, then a progressive restriction occurs. *TARAM-A* transcripts are first excluded from the YSL, an extraembryonic layer of cells connected to the giant yolk cell, which represents the first known lineage restriction occurring during early fish development (Kimmel and Law, 1985). At the beginning of epiboly, *TARAM-A* expression is down-regulated from the animal pole cells but is maintained in the marginal zone. Although fate mapping cannot be achieved before the onset of gastrulation (Kimmel et al., 1990), such a differential regulation suggests that genetic programs involved in the specification of the marginal blastomeres have been initiated around the onset of epiboly. During epiboly and early gastrulation, *TARAM-A* expression becomes restricted to axial structures. Thus *TARAM-A*, with *gsc*, is the earliest marker shown to be specific for the dorsal side (Thisse et al., 1994). This may represent one of the first examples of a serine/threonine kinase receptor exhibiting a spatial restriction before gastrulation.

A constitutive form of TARAM-A is able to activate a pathway sufficient to induce anterior axial mesoderm as well as an anterior organizing activity in fish

We analysed the involvment of TARAM-A during early fish embryogenesis by ectopic expression of constitutively active form of TARAM-A. TARAM-A-D was generated by introducing a single aminoacid substitution in the GS domain according to a scheme established for related receptors including TβR-I (Wieser et al., 1995), ActR-IB (Attisano et al., 1996) and BMP type I receptors ALK3 and ALK6 (Hoodless et al., 1996). We provide evidence that TARAM-A or a closely related molecule is directly involved in dorsal mesoderm formation in fish. As expected for a constitutively active receptor, very low levels of *TARAM-A-D* RNA are sufficient to induce anterior dorsal mesoderm formation in fish embryos. TARAM-A-D expression in animal pole or marginal blastomere leads to the formation of ectopic regions of convergence and extension, characterized by the expression of dorsal mesoderm markers *axial, lim-1, gsc* as well as *ntl*. These ectopic sites can be detected by visual inspection on live embryos before the endogenous embryonic shield appears and by in situ analysis showing that dorsal transcripts accumulate soon after MBT. It indicates that once the TARAM-A pathway has been activated, transcriptionally active cells can readily respond to TARAM-A stimulation suggesting that the effect of TARAM-A stimulation on gene activation is direct.

The formation of ectopic anterior mesoderm induced by TARAM-A activation could result from either an anteriorization of existing mesoderm or a de novo induction of ectoderm to axial mesoderm. In the first hypothesis, expression of TARAM-A-D should only affect blastomeres normally fated to mesoderm, e.g. marginal blastomeres (Strehlow et al., 1994). Central blastomeres from the 16-cell-stage embryo, which do not normally contribute to mesoderm but mostly to ectoderm, change their fate to anterior axial mesoderm when expressing TARAM-A-D, making the anteriorization hypothesis unlikely. Rather, these results imply that TARAM-A activation induces a general change of fate from ectoderm to anterior axial mesoderm, suggesting that it may act before normal commitment of blastomeres to a given tissue. Consistent with this idea, we observed that dorsal marginal cells injected with *TARAM-A-D* RNA are no longer found in axial tissues like notochord or floor plate but rather end up in the anterior axial mesoderm.

In addition, injected embryos can exhibit partial or complete duplications of the most anterior structures. In amphibians, it has been demonstrated that dorsal mesoderm possesses an organizing activity regionalized in the anteroposterior axis (the earlier involuting mesoderm will mostly induce heads, whereas the late involuting mesoderm will induce more posterior structures, Hamburger, 1988). Our data strongly suggest that the most anterior mesoderm formed by cells expressing TARAM-D possesses the same anterior organizing activity.

The specificity of the effects observed suggests that the TARAM-A-D pathway is specifically involved in anterior mesoderm formation and acquisition of organizing properties. Interestingly zebrafish early blastomeres exhibit a single threshold in their response to TARAM-A-D. Below a *TARAM-A-D* RNA concentration of 0.025 μg/ml, their fate is not statistically altered as compared to control injections and ectopic dorsal mesoderm is not induced. Above this value and until appearance of toxicity, injected cells systematically contribute only to axial anterior mesoderm, with the exception of some contribution to the periderm (known to originate soon after MBT from the marginal zone and perhaps incompetent to respond to TARAM-A signalling) and some participation of injected cells to the tailbud. Thus TARAMA-D properties contrast sharply with the effects of known mesoderm inducers like activins (Smith, 1993; Wittbrodt and Rosa, 1994), processed Vg1 (Thompsen, 1993) or nodal-related factors like

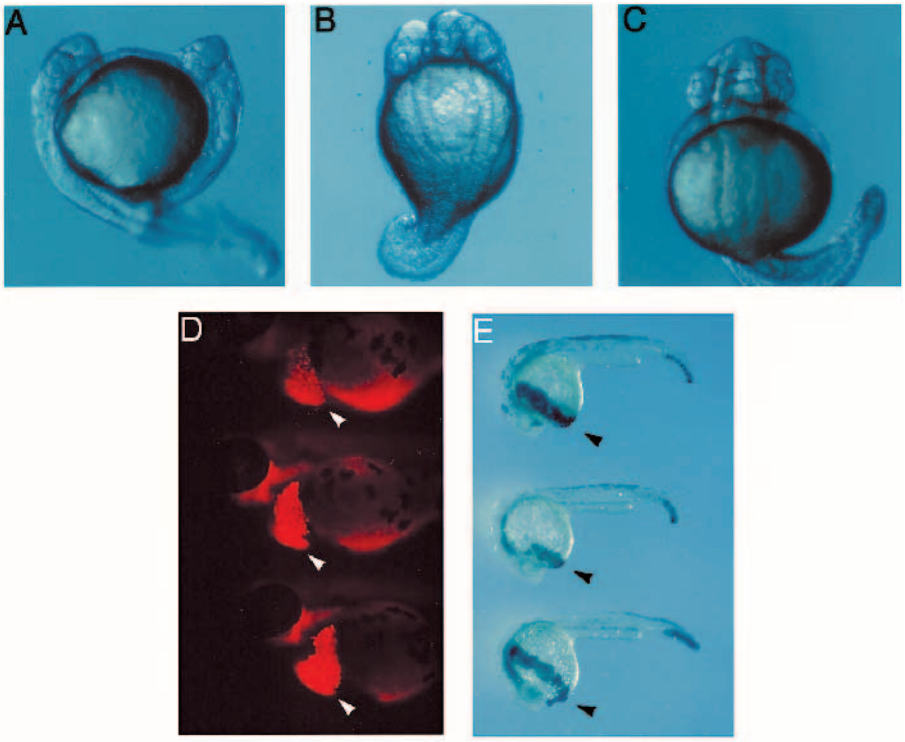

Fig. 5. Duplication of anterior structures in *TARAM-A-D*-injected embryos. (A-C) *TARAM-A-D* RNA was injected at 0.1 μg/ml into a marginal cell from 16-cell-stage embryos. Embryos are observed after 24 hours, three specimens with duplication of anterior structures are shown. (A) A complete duplication of the head is observed, the two heads are facing each other (side view). (B) The two heads are contiguous (dorsal view). (C) One additional eye in a frontal position is observed. (D,E) Fate of TARAM-A-D-expressing cells using coinjection of the rhodamine dextran lineage tracer (D), or the β-gal RNA tracer (40 μg/ml) (E), into one marginal cell of a 16-cell-stage embryo. (D) Left side view from the head and trunk of three 48-hour embryos (anterior to the left). The hatching gland (arrowheads) and head mesendoderm are strongly fluorescent. The apparent labelling of the yolk ball is due to light refraction. (E) Left side view from three 24-hour embryos stained for β-gal activity. Hatching glands (arrowheads) and head mesendoderm display strong β-gal activity. Some scattered periderm cells as well as undifferentiated tailbud cells are also labelled.

Xnrs-1 and Xnr-2 (Toyama et al., 1995; Jones et al., 1995), which can induce all types of mesoderm depending on their concentrations and are not able to generate the most anterior structures. The properties of TARAM-A-D suggest that anterior axial mesoderm induction could rely on an uncharacterized factor binding TARAM-A and that formation of different grades of mesoderm might require different type I receptors.

Activation of the endogenous TARAM-A receptor depends on a signalling component dorsally localized: involvment of TARAM-A in fish head organiser formation

TARAM-A-D can direct animal or marginal blastomere to an anterior dorsal mesoderm fate, thereby showing that early blastomeres are all competent to respond to TARAM-A activation. By contrast, expression of TARAM-A-WT induces dorsal mesoderm markers at ectopic sites only in the immediate vicinity of the endogenous shield. Our results imply that, in situ, only a local activation of TARAM-A may occur. Consistent with this idea, TARAM-A-WT is not able to duplicate anterior structures probably because a regulation may occur when an excess of dorsal mesoderm is formed at the level of the resident shield as already seen in fish embryo (Toyama et al., 1995). Two non-exclusive possibilities can be proposed: either the translation, the processing of TARAM-A or its associated type II receptor is local or other components necessary for TARAM-A activation, including its ligand, are expressed or activated locally. Experimental evidence clearly shows that dorsal mesoderm induction does not rely on factor(s) stored in the blastoderm since neither blastomere ablation nor transplantation reveal any organizing properties (Lin et al., 1992). By contrast, blastoderm transplantation experiments carried out in trout between a mid-blastula and an early gastrula have shown that the embryonic shield position can be specified by signals coming from the gastrula extraembryonic YSL (Long, 1983). The nature and the activity of these signals remains to be determined. At this point, we cannot differentiate the two possibilities mentioned but an interesting possibility is that YSL-provided signals include TARAM-A ligand(s) or regulate its activity. Thus TARAM-A-WT and TARAM-A-D properties suggest strongly that TARAM-A could be responsible for anterior mesoderm specification including organizing activity. One way to answer this question precisely is to block the endogenous TARAM-A-signalling pathway using a dominant negative form of the receptor. However, progenies from one blastomere injected at the 16-cell stage with *TARAM-A-TR* RNA are excluded early from the blastoderm and participate to the yolk syncitial layer (A. R. and F. R., unpublished) thereby impairing the interpretation of the phenotypes obtained by injection at the 1-cell stage. This problem will be alleviated using a conditional dominant negative form of TARAM-A.

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