A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway

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A functional assay to clone mouse mesoderm inducers has identified the mouse gene Mad related 2 (Madr2). Madr2 induces dorsal mesoderm from Xenopus ectoderm and can mimic the organizer in recruiting neighboring cells into a second axis. By analyzing the expression of a lacZ/Madr2 fusion protein, we find Madr2 confined to the nucleus in the deep, anterior cells of the second axis, whereas in epidermal and more posterior cells the protein is cytoplasmically localized. This context-dependent nuclear localization suggests that in certain regions of the embryo, Madr2 responds to a localized signal and amplifies this signal to form the second axis. Furthermore, although Madr2 remains unlocalized in ectodermal explants, addition of activin enhances the concentration of Madr2 in the nucleus. Significantly, a functional lacZ fusion to a carboxy-terminal portion of Madr2 is nuclear localized even in the absence of activin. This indicates that Madr2 contains a domain that can activate downstream components and a repressive domain that anchors the protein in the cytoplasm. Nuclear localization of Madr2 in response to activin, and the activin-like phenotypes induced by overexpression of Madr2, indicate that Madr2 is a signal transduction component that mediates the activity of activin.

[Key Words: Madr2, activin, mesoderm; nuclear localization]

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The crucial first step in patterning of the vertebrate embryo is the induction of mesoderm. In Xenopus, disruption of peptide growth factor signaling leads to loss of the mesoderm, supporting the idea that cell–cell communication, or induction, is responsible for mesoderm formation (Amaya et al. 1991; Hemmati-Brivanlou and Melton 1992). Several members of the transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) families have been shown to have mesoderm-inducing activity (Kessler and Melton 1994; Slack 1994). However, to date, mutations in specific FGFs or activins have not implicated any of these molecules in mesoderm induction in the mouse (Deng et al. 1994; Vassalli et al. 1994; Yamaguchi et al. 1994; Feldman et al. 1995). One interpretation of these experiments is that additional activities are required in the mouse for mesoderm formation.

The formation of mesoderm in the mouse is mechanistically different from that in amphibians. In the cup-shaped epiblast of the mouse embryo, mesoderm formation initiates in a population of ingressing cells adjacent to the embryonic/extraembryonic junction. This population forms the primitive streak, which initiates at one point on the rim of epiblast. Cells intercalate between the ectoderm and primitive endoderm of the streak, emerging as mesoderm (Hogan et al. 1994). In the mouse, the origin of mesoderm-inducing signals is unknown. These signals could emanate from the embryonic endoderm, the embryonic ectoderm, or even the extraembryonic ectoderm. Identifying molecules important in mammalian mesoderm formation has been difficult in part because of the small size and inaccessibility of the mouse embryo. However, analysis of naturally occurring or induced genetic mutations has been successful in identifying genes essential to mesoderm formation, such as nodal and brachyury. Embryos lacking nodal contain little or no mesoderm and fail to form and maintain the primitive streak [Conlon et al. 1991, 1994; Zhou et al. 1993]. Brachyury mutant embryos do not generate enough mesoderm and have severe defects in the development of the notochord [Herrmann et al. 1990]. Even though these approaches have yielded important molecules, the effort involved in generating mutations and subsequently isolating the molecules responsible for the defects is extensive.

To identify mouse genes involved in mesoderm formation, we have used a functional screen for mammalian molecules capable of inducing mesoderm in Xenopus ectodermal explants. Precedent for such a screen comes from observations that mammalian proteins can act on Xenopus tissues, even when the mammalian sequence has diverged considerably from the nearest Xenopus homolog [e.g., nodal and the Xenopus nodal-related genes, Jones et al. 1995]. In some cases, a mouse gene is more potent than the frog homolog, perhaps because regulatory mechanisms that suppress activity of the protein in

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normal development are not conserved (Rupp et al. 1994).

Here we report the identification of a mouse transcript, Madr2, which causes mesoderm to form in Xenopus animal caps. Madr2 is similar to the Drosophila gene, mothers against dpp (Mad) (Sekelsky et al. 1995) as well as the human tumor suppressor gene DPC4 (Hahn et al. 1996). The sequence is very similar to Xmadr2, which was recently isolated by sequence similarity to Drosophila Mad (Graft et al. 1996). We address the mode of action of Madr2 using animal cap and whole embryo assays. The clone isolated in the screen is a partial cDNA and encodes a truncated protein. This truncation functions similarly to the full length transcript, which was subsequently isolated. Using lacZ fusion proteins we show that nuclear localization of Madr2 can be induced by activin. In addition, the truncated form of Madr2, is localized exclusively in the nucleus with or without activin treatment. This suggests that the Mad proteins contain a domain that is sufficient to activate downstream components, and a regulatory domain, which anchors the protein in the cytoplasm.

Results

Expression cloning of Madr2

We have developed a screen for mouse molecules which can change the fate of Xenopus ectoderm to mesoderm. As a source for potential inducers, we made a cDNA library from mouse embryonic stem (ES) cells which had begun to differentiate into mesoderm. When cultured in the presence of leukemia inhibitory factor (LIF), ES cells can be induced into mouse embryos and can contribute to all three germ layers (Beddington and Robertson 1989). However, in the absence of LIF, these cells can be cultured in vitro to differentiate into mesodermal derivatives (Robertson 1987). During differentiation, ES cells turn on genes such as nodal and brachyury (Johansson and Wiles 1995), suggesting that they may also turn on other genes in the normal mesoderm induction pathway. Poly(A)+ RNA was isolated from CCE ES cells after being grown without LIF for 24 hr and a plasmid cDNA library constructed from this material.

The library of cDNAs was divided into 97 pools of 1000 clones each, these were used to synthesize capped mRNA that was injected into one-cell Xenopus embryos. At the blastula stage (8–9), the ectoderm [animal cap] was explanted and grown in culture until gastrula stage (10.5). To examine whether the animal cap has become mesoderm in response to the injected RNA, the aged caps were analyzed for the expression of the mesodermal marker, Xbra [Fig. 2A, below]. If the injected pool of synthetic RNAs contains a message which can instruct the ectoderm to become mesoderm, then the animal cap will express mesodermal markers. If no inducing molecule is present, or if present at low concentrations, then the animal cap will develop as epidermis.

Of the first 60 pools screened, 4 pools had potential mesoderm-inducing activity. One pool was split into subpools of 200 colonies and reassayed. Subsequently active pools were split into smaller pools and reassayed until a single active clone was identified. This clone was used to isolate a full-length cDNA from the original ES cell library.

Sequence analysis (GenBank accession no. U60530) showed the isolated clone is 65% identical at the amino acid level to Drosophila Mad (Sekelsky et al. 1995). In addition, Madr2 is 52% and 45% identical to the Caenorhabditis elegans Sm2 and Sm3 proteins, respectively (Savage et al. 1996) and is 46% identical to the human tumor suppresser DPC4 (Hahn et al. 1996). Drosophila Mad and C. elegans sma genes have been shown by genetic analysis to be downstream effectors of the BMP family of TGFβ-like molecules. Xenopus Mad2 (Xmad2) is 99% identical to mouse Madr2 at the amino acid level (Graft et al. 1996). Interestingly the cDNA we initially cloned encodes a truncated form of Madr2, which lacks the first 183 of 467 amino acids. We assume that this form of Madr2, which we call Madr2(C), initiates at a downstream methionine residue [amino acid 241] that is in a favorable context for initiation.

To determine the temporal and spatial expression of Madr2, in situ hybridization was performed on embryonic day 6.5 (e6.5)–10.5 mouse embryos. With the possible exception of the heart and tail bud, Madr2 is expressed widely at these stages [Fig. 1]. This evidence, along with sequence data showing no signal sequence or transmembrane domains, is consistent with a role for Madr2 as a signal transduction component.

Figure 1. Madr2 expression in mouse embryos. (A) Whole-mount in situ hybridization of e8.5 mouse embryos with either sense (top) or antisense (bottom) probes. (B, C) e10.5 mouse embryos hybridized with either antisense (B) or sense (C) Madr2. Arrowheads point to the heart where expression is not detected.
Madr2 induces dorsal mesoderm in a dose-dependent fashion

The mesoderm-inducing activity of the full length and truncated forms of Madr2 was further assayed to determine what type of mesoderm was being induced. Animal caps explanted from embryos injected with either Madr2 (full length) or Madr2(C) (truncated) were grown to stage 10.5 and assayed by RT–PCR for the expression of several mesodermal markers (Fig. 2B). A quantitative comparison between Madr2 and Madr2(C) demonstrates that the truncated form Madr2(C) is more active in inducing a variety of markers than the full-length Madr2. Xbra, a general mesodermal marker is induced by Madr2(C) at 50 pg and by Madr2 at 100 pg. The organizer-specific dorsal mesodermal markers noggin and goosecoid were both expressed when treated with 50 pg of Madr2(C).

High doses (500–1000 pg) of Madr2 were needed to express these markers. Xwnt8, a marker of ventral mesoderm, is induced over a restricted range of doses of both Madr2(C) and Madr2-injected caps (50–100 and 100–500 pg, respectively). Thus, we conclude that Madr2 is an inducer of dorsal mesoderm and can induce different mesodermal cell types in a dose-dependent manner. These results are similar to those obtained with members of the TGFβ family, namely nodal and activin (Green et al. 1992; Jones et al. 1995) and to the effects of Xmad2 (Graff et al. 1996).

Morphogenetic movements were observed in Madr2(C)-injected animal caps. Explants recovered from embryos that received 100 pg of Madr2(C) elongated compared to controls (Fig. 3B, C). At higher concentrations of Madr2(C) the caps also turned inside out, possibly mimicking the involution movements of gastrulation (Fig. 3A). Animal caps injected with high concentrations (1 ng) of Madr2 do not undergo the extensive morphological movements seen in caps injected with 1 ng of Madr2(C). However, these animal caps do elongate. The ability of Madr2 to induce dorsal mesoderm and to induce elongation indicate that Madr2 has activin-like activities, not the bone morphogenetic protein (BMP)-like activity shown in invertebrate Mad homologs (Sekelsky et al. 1995; Savage et al. 1996).

Madr2(C) mRNA injection induces a second axis in host embryos

The ability of Madr2(C) to specify dorsal mesoderm in ectodermal explants prompted us to see if Madr2 could induce ventral cells of the embryo to adopt a dorsal fate. In multiple experiments, 100 pg of Madr2(C) was injected into two ventral blastomeres at the four cell stage. Subsequently, the embryos were examined for the formation of a secondary axis. Of embryos injected with 100 pg of Madr2(C) RNA, 82% developed a partial second axis (Table 1). Madr2 also generates a second axis, albeit less reliably than Madr2(C). Second axes generated by injection of Madr2(C) or Madr2 contain neural tissue, muscle and some notochord (Fig. 4 E–G).

Activin also induces a partial second axis in embryos and can induce organizer activity, which recruits neighboring cells into a second axis (Cooke 1989; Thomsen et al. 1990). To address whether Madr2-expressing cells could induce neighboring, nonexpressing cells to form ectopic mesoderm, we traced the fate of Madr2-expressing cells by coinjecting 200 pg of lacZ RNA with 1 ng Madr2(C) RNA or 200 pg of lacZ RNA with 1 ng of Madr2 RNA in two ventral blastomeres of the four-cell embryo. As a control, 200 pg of lacZ RNA was injected in the same manner. The embryos were allowed to grow to the tailbud stage and stained for β-galactosidase to visualize cells receiving high concentrations of injected mRNA. The results of multiple experiments are included in Table 1 and are shown in Figure 4 (A, B). These results show that cells expressing high amounts of Madr2 are localized to the anterior portion of the second
movements of the leading edge of mesendoderm (Symes et al. 1994).

Madr2 responds to activin signaling

Because Madr2 appears to be widely expressed in the mouse embryo, contains no signal sequence and is homologous to genes presumed to be involved in the TGFβ signal transduction pathway, it seems likely that Madr2 is a cytoplasmic protein. To determine the intracellular localization of the Madr2 protein, we made fusion proteins between lacZ and Madr2 and also lacZ and Madr2(C). Both fusion proteins were tested for function in two ways. First, animal caps injected with 1 ng of either lacZ/Madr2 or lacZ/Madr2(C) effectively induced the same markers as Madr2 and Madr2(C). Second, 0.5 ng of lacZ/Madr2 and lacZ/Madr2(C) RNA were capable of inducing second axes in 80% and 74%, respectively, of embryos injected [data not shown]. In either assay lacZ/Madr2(C) was more potent than Madr2(C): lacZ/Madr2 reproduced induced Xbra in animal caps at 10 pg and frequently induced head structures (Fig. 4E). Therefore these two criteria show the lacZ fusion proteins retain their biological activity and provide confidence that the lacZ activity will reflect the subcellular location of the active protein.

The embryos generated by ventral injections of the lacZ/Madr2 mRNAs were stained with X-gal to assay for the presence of β-galactosidase [Fig. 5]. The results were similar to those obtained with lacZ and Madr2 RNA injections in that expression was predominantly in the anterior cells of the second axis. Interestingly when injected with lacZ/Madr2, the stain in the anterior cells of the second axis was exclusively confined to the nucleus, suggesting that Madr2 is a nuclear protein [Fig. 5A]. However, the fusion proteins were not nuclear in all cell

| Injection | n | Number of second axis induced | Expressing cells in anterior of second axis |
|-----------|---|------------------------------|----------------------------------|
| none      | 116 | 0                           | N.A.                              |
| Madr2(C) | 92  | 76                          | N.A.                              |
| Madr2     | 74  | 21                          | N.A.                              |
| none      | 60  | 0                           | N.A.                              |
| Madr2(C) and lacZ | 56 | 53                          | 40                                |
| none      | 15  | 0                           | 0                                |
| Madr2 and lacZ | 20 | 18                          | 15                                |

Embryos were injected with 100 pg of Madr2(C), with 1 ng of Madr2, and with 0.2 ng of lacZ and 1 ng of either Madr2(C) or Madr2 into two ventral blastomeres at the four-cell stage. They were allowed to develop and then assayed for the presence of a second axis. If injected with lacZ, the embryos were subsequently stained for β-gal. Data are summarized from several experiments. The effectiveness of Madr2 in secondary axis induction varies, as illustrated by the difference between Madr2 and the Madr2 + lacZ mixture.
Figure 4. Lineage tracing of Madr2(C)-expressing cells in either dorsal or ventrally injected embryos. We injected dorsal or ventral blastomeres of the four-cell embryo with either lacZ alone (B,D) or coinjected with Madr2(C) and lacZ RNA (A,C). Embryos were allowed to develop to stage 35 and were stained with X-gal. (A) Embryos coinjected into ventral blastomeres with 1 ng Madr2(C) and 200 pg lacZ developed a partial second axis and show Madr2(C) expressing cells to be localized to the anterior portion of the second axis. (B) Control embryos injected ventrally with 200 pg of lacZ. (C) Embryos injected dorsally with 1 ng of Madr2(C) and 200 pg of lacZ, showing the expressing cells in the anterior endoderm. (D) Control embryos injected dorsally with 200 pg of lacZ. Comparison of Madr2(C)-injected embryos (A,C) with lacZ controls (B,D) demonstrate the ability of Madr2 to change the prospective fate of ventral and dorsal cell types. Immunostaining of axes generated by injecting 500 pg of lacZ/Madr2(C) (E), 500 pg of Madr2 (F), and 500 pg of lacZ/Madr2 (G) into ventral blastomeres. Embryos were stained with 6F11 (E), a neural specific mAb; Tor70 (F), a notochord specific mAb; and 12101 (G), a muscle specific mAb. Higher magnification shows that the notochord cells are not well organized, and the Tor70 antigen is deposited around individual cells rather than in an organized notochord sheath. Tor70 also stains the otic vesicle, which can be seen rostral to the notochord in the second axis. In each panel arrowheads indicate the induced axis. The neural staining in E shows that eyes are induced in the second axis by lacZ/Madr2(C). The eyes in the second axis contained pigmented retina that was bleached after immunostaining.
Madr2 responds to activin signaling

**Figure 5.** Nuclear localization of the Madr2 and Madr2(C) proteins. (A, B) 0.5 ng of lacZ/Madr2 RNA was injected into the two ventral blastomeres at the four-cell stage. Embryos were allowed to develop until stage 35 and were subsequently stained with X-gal. Madr2 protein present in the anterior of the second axis was localized to the nucleus (A), whereas Madr2 protein present in the epidermis was cytoplasmically localized (B). 0.5 ng of the full-length fusion lacZ/Madr2 RNA, 0.5 ng of the truncated fusion lacZ/Madr2(C) RNA, or 0.5 ng of the full-length fusion lacZ/Madr2 and 0.1 ng of activin βB was injected into the animal pole of the one-cell embryo. At the blastula stage (8–9) animal caps were explanted, grown to gastrula stage (10.5), and stained with X-gal to assay for the presence of β-galactosidase (C, E, G) and bisbenzamide to accurately identify nuclei (D, F, H). LacZ/Madr2 full-length protein was expressed ubiquitously throughout the cells (C, D). When exposed to activin βB, lacZ/Madr2 was predominantly nuclear localized (E, F). Interestingly, the truncated fusion protein lacZ/Madr2(C) was localized only in the nucleus (G, H) with or without the addition of activin. Scale bars, 100 μM (A, B), 50 μM (C–G).

In most cells, the addition of activin was not enough to ensure complete nuclear localization. This cannot be attributed to a weak activin signal, because this dose of activin is 100-fold higher than the optimal dose for Xbra induction (Graff et al. 1994; D. Hsu, pers. comm.) and causes death in embryos because of excessive gastrulation movements.

To determine whether the truncated Madr2(C) can also respond to activin signaling, we subsequently assayed the subcellular location of a lacZ/Madr2(C) fusion after expression in animal caps. Interestingly, lacZ/Madr2(C) is localized to the nucleus in every expressing cell, even in the absence of activin (Fig. 5G, H). This suggests that the amino-terminal domain of the full-length protein is required for regulating entry into the nucleus. We suggest that upon the addition of activin, the signal transduction pathway is activated and either stimulates removal of a cytoplasmic retention activity, or causes refolding of the protein to expose a cryptic nuclear localization signal. Thus, the amino-terminus of the protein is likely to be regulatory, and the carboxy-terminus acts in the nucleus to activate downstream signals.

The activin-induced nuclear localization coupled with the activin-like induction of mesodermal gene expression strongly suggest that Madr2 is a member of the activin or activin-like signal transduction pathway.

**Discussion**

**Mad proteins can be Bmp or activin-like signaling molecules**

The TGFβ family of signaling molecules has been shown to be important in a wide range of developmental phenomena. During embryogenesis in vertebrates, members are needed to set up the body plan, to control the formation of limbs, cartilage and bone and to induce the formation of mesoderm (Lyons et al. 1991; Kingsley 1994). In addition, changes in the activities of these molecules have been implicated in the onset of several diseases, including fibrosis and cancer (Roberts and Sporn 1993).

The signaling effects of the TGFβ family are mediated by transmembrane serine threonine kinase receptors, which are represented in two classes, type I and type II (Kingsley 1994). The type II receptors appear to be constitutively active. Upon ligand binding, a heteromeric complex forms and the type II receptor activates the type I receptor by phosphorylation (Wrana et al. 1994). The events triggered by the activation of the type I recep-
tor are beginning to be elucidated (Massagué 1996). Genetic screens in Drosophila and C. elegans have uncovered molecules that seem to play important roles in the signal transduction pathway of the BMP subclass of the TGFβ superfamily. A screen looking for dominant enhancers of a imaginal disk phenotype caused by lack of dpp, the Drosophila homolog of Bmp2/4, revealed the gene Mad (Sekelsky et al. 1995). Similar screens in C. elegans for genes acting downstream of daf-4, a BMP-like receptor, revealed three genes, sma-2, sma-3, and sma-4, all of which are homologous to Drosophila Mad (Savage et al. 1996). It was shown that sma-2 and daf-4 are required in the same cells and, in addition, expression of daf-4 cannot rescue sma-2 mutations. This suggests that the C. elegans sma genes are involved in a BMP-like signal transduction pathway.

In a novel functional screen, we have cloned a vertebrate Mad homolog, Madr2. Madr2 is 65% and 52% identical to Drosophila Mad and C. elegans Sma-2, respectively. Because these proteins are well conserved, it would be a logical assumption that Madr2 would also function as a BMP signal transduction molecule. However, the Madr2 overexpression phenotypes are not similar to those of BMP4, which is a ventral mesoderm inducer (for review, see Harland 1994). Instead the activities of Madr2 are strikingly similar to another TGFβ molecule, activin, which is a dorsal mesoderm inducer (Green and Smith 1990, Thomsen et al. 1990). Recently other vertebrate homologs of Drosophila Mad and C. elegans sma-2 and -3 have been cloned based upon their sequence similarities (Graff et al. 1996, Hoodless et al. 1996, Thomsen 1996). Xenopus mad1 and mad2 (Xmad1, Xmad2), which are 62% identical, produce distinctly different responses in embryos: Xmad1 induces ventral mesoderm, whereas Xmad2 induces dorsal mesoderm (Graff et al. 1996; Thomsen 1996). In this study we have provided evidence that the addition of activin stimulates the transport of full-length Madr2 protein into the nucleus, demonstrating that Madr2 responds to the activin signaling. Similar results have been obtained for BMP-dependent nuclear translocation of Mad1 (Hoodless et al. 1996, Liu et al. 1996). Therefore, there may be a Mad protein or set of proteins for each TGFβ pathway, whether BMP- or activin-like. Interestingly many of the TGFβ superfamily ligands bind the same receptors, and yet result in different responses. It seems likely that the biological response to members of the superfamily will result not only from the affinity of the ligand for different receptor combinations, but may also result from integration of the signal at the level of the transducing Mad-related proteins.

Nuclear localization of Madr2 and Madr2(C) in response to activin

We have shown that Madr2 is localized to the nucleus in vivo in response to endogenous signals located at the anterior portion of a second axis. We infer that this signal is an activin-like molecule from experiments where we analyze ectoderm expressing a lacZ/Madr2 fusion or ectoderm expressing the fusion and activin. In the absence of activin lacZ/Madr2 is predominantly cytoplasmic whereas with the addition of activin, expression is mostly nuclear. Even with high quantities of activin, Madr2 is not always nuclear. The residual cytoplasmic protein in these explants can be explained in a variety of ways. The activin signaling pathway may not be sufficiently effective by itself to modify or release all the cytoplasmic fusion protein. Alternatively the cytoplasmic protein may be the result of cleavage of the fusion protein, Western blotting indicates some LacZ reactive material that is smaller than the full-length fusion [data not shown]. Even without activin signaling, the full-length Madr2 molecule can function to induce mesoderm in Xenopus ectoderm. When the localization of full-length LacZ/Madr2 was examined, a large amount of mRNA was injected [500 pg]. Under these conditions, the protein is evenly distributed throughout the cell. Currently, we do not know whether physiological levels of Madr2 are excluded from the nucleus. Thus, the activity of the full-length Madr2 in injection experiments could be attributed to overwhelming the cytoplasmic retention machinery, eventually leading to the induction of mesoderm.

All Mad homologs discovered thus far contain conserved amino-terminal and carboxy-terminal domains with a variable proline-rich intervening region. Madr2(C) gives us a glimpse at the importance of these two conserved regions. It appears that the amino-terminal region is not required for signaling, but may regulate activity by acting as a cytoplasmic tether. A precedent for this type of mechanism has been studied extensively in the transcription factor dorsal, which is required to direct the development of ventral structures in the fly (Morisato and Anderson 1995), and NF-κB, which is involved in the activation of κ chain gene transcription in B lymphocytes (Verma et al. 1995). Before activation, dorsal is held in the cytoplasm through an association with cactus. When dorsal receives a signal from the receptor toll, dorsal is freed from the cactus protein and enters the nucleus. NF-κB is similarly anchored in the cytoplasm by the protein I-κB. In the case of Mad proteins, a cytoplasmic tether could be binding to the conserved amino-terminal domain of Madr2, anchoring the protein in the cytoplasm. When the amino-terminal region is deleted [as in Madr2(C)] the cytoplasmic protein would no longer sequester Madr2 and allow it to be freely transported to the nucleus. The observation that Madr2(C) is confined to the nucleus as well as the finding that Madr2(C) is more active than Madr2 are consistent with this model. Fusion to lacZ increases the activity further, perhaps by providing stability, or more efficient translation. In the case of full-length Madr2 the activated receptor could disrupt the Mad/tether complex and allow the full-length molecule to accumulate in the nucleus.

An alternate mechanism for nuclear localization is exemplified by Signal Transducer and Activator of Transcription (STATs). Upon activation of cytokine receptors, the STATs are phosphorylated, dimerize, and a
cryptic nuclear localization signal is exposed, allowing translocation and activity (Karin and Hunter 1995).

What Madr2 does once it enters the nucleus is still unknown. The Mad homolog Smad1/Mad1 does have transcriptional activation activity, which is dependent upon BMP signaling. This transcriptional activating activity resides in the carboxy-terminal domain, consistent with the activity observed for Madr2(C) (Liu et al. 1996).

Our screen for mouse mesoderm inducers has explored the relatively easy assays that can be carried out in *Xenopus*. It remains to be determined to what extent Madr2 plays a role in the development of mesoderm in the mouse. Several TGFβ family members have been shown to be important in early gastrulation in the mouse, including Bmp4, Bmpr1A, and nodal. Mice lacking Bmpr1A do not form mesoderm, whereas mice lacking Bmp4, which can signal through Bmpr1A, have a variable phenotype (Mishina et al. 1995; Winnier et al. 1995). Although many Bmp4 homozygous embryos do not survive past gastrula stages, some can develop to the headfold stage. In addition, mutations are being studied that have defects in mesoderm formation. The *eed* mutant fails to gastrulate normally and generates little mesoderm whereas embryos mutant for the *msd* locus do not produce mesoderm. The molecular lesion in the *eed* or the *msd* genes have not been reported (Holdener et al. 1994; Faust et al. 1995).

Previous functional screens have assayed for axis formation, either in ventralized embryos or on the ventral side of normal embryos (Smith and Harland 1991, 1992; Lemaire et al. 1995; Smith et al. 1995). Such assays have previously revealed molecules that pattern the mesoderm, but have no intrinsic mesoderm-inducing ability. The current screen was more specific, in that it assayed for *Xbra* induction and therefore for mesoderm induction. However, the animal cap assay can be adapted to screen for components of the wnt signaling pathway, which activate *Xnr-3* expression (Smith et al. 1995), or for activities that antagonize BMP signals, which result in neural induction and activation of neural cell adhesion molecule [NCAM] and *npr-1* (Lamb et al. 1993; Knecht et al. 1995; Sasai et al. 1995; Wilson and Hemmati-Brivanlou 1995). Signal transduction components are usually less active than ligands in such assays, but if small pools of cDNAs are screened, even weak activities should be identifiable. We therefore expect that further screens will allow us to identify additional secreted signaling molecules and intracellular signal transduction components.

### Materials and methods

#### Growth of ES cell and isolation of RNA

CCE ES cells were grown on LIF-transfected STO feeder layers (Robertson 1987) in Dulbecco's modified Eagle medium containing 10% fetal calf serum, 10% newborn calf serum, and 0.1 mM β-mercaptoethanol at 37°C in 5% CO2. To induce differentiation, the STO feeder layers were removed by washing 80% confluent plates in 1× PBS, trypsinized, and then transferred in medium to tissue culture plates, that had been treated with 0.1% gelatin. The STO cells were allowed to adhere to the gelatinized plates for 30 min at 37°C. Subsequently, the medium, containing mostly ES cells, was transferred to new gelatinized plates and the procedure repeated to ensure removal of all STO cells. The density of ES cells in the medium was analyzed with a hemacytometer and cells were plated at a density of 9×10³ cells per 10-cm gelatinized plate. These cells were cultured for 24 hr at 37°C. At this time, twelve 80% confluent plates were harvested by the acid phenol extraction method (Chomczynski and Sacchi 1987). Poly(A)⁺ RNA was isolated by the PolyTract isolation system [GIBCO-BRL].

#### Construction of cDNA library

The ES cDNA library was constructed in a modified pCS2 vector (Turner and Weintraub 1994). pCS105 (gift of D. Hsu) was modified to allow directional cloning using the Superscript plasmid system [GIBCO-BRL]. The ligated cDNAs were used to transform DH10B cells [GIBCO-BRL]. The resulting library contained 225,000 clones. Subsequently 100,000 of these clones were plated at a density of 1000 colonies per 97-mm plate. Of 20 randomly chosen plasmids, all had inserts which ranged from 0.6 to 3.0 kb. The average insert size was 1.6 kb. Each plate was harvested by adding 3 ml of Lβ and scraping the cells. An amount of 0.5 ml of this culture was frozen by adding glycerol to 30%. Plasmid DNA was isolated by the alkaline lysis method.

#### In vitro transcription and RT-PCR

Pooled plasmid DNA was linearized with *AciI*. Capped mRNA was made from the pooled plasmid DNA with SP6 RNA polymerase using message machine [Ambion]. By injecting 10 nl of a 1 mg/ml dilution of RNA, 10 ng was introduced into the animal pole of the one cell *Xenopus* embryo. Animal caps were explanted from these injected embryos at blastula stage (8–9), and grown to gastrula stage (10.5). RNA from the caps was either harvested as described [Condie and Harland 1987] and subsequently digested with DNase to remove genomic DNA [Wilson and Melton 1994] or harvested using Trizol [GIBCO-BRL], RT–PCR was performed as described (Wilson and Melton 1994). Primer sets for analysis of Xbra, *Efa1*, *goosecoid*, *noggin*, and *muscle actin* are described by Wilson and Melton (1994). Xnot primers were designed by F. Mariani [University of California, Berkeley]: Upstream [U]-5’-ATCTATGCCCTACCGCTA-3’ (nucleotides 671–688) and Downstream [D]-5’-CA-GTGG-GAGAATGCGAAT-3’ [nucleotides 845–827].

#### LacZ/Madr2 fusion proteins

Primers were made to introduce *EcoRI* sites at the ends of either Madr2 or Madr2(C). The upstream Madr2 primer begins with *EcoRI* and BglII restriction sites, has a few base pairs to maintain a frame and then concludes with Madr2 sequence beginning 7 bp upstream of the ATG. The upstream Madr2(C) primer is arranged in the same way, but concludes with Madr2 sequence beginning 6 bp from the ATG from which it is presumed to initiate. The downstream primer was the same for both Madr2 and Madr2(C). The downstream primer has an *EcoRI* site on the 5’ end, the final 9 amino acids and the stop codon of the Madr2 protein. Madr2 U-5’-[CGAATTTCAGATCTAAGAAA-TGTCGTCCATCTTGCACTCTCCG-3’], Madr2(C) U-[5’-CGAATTTCAGATCTCAAGAGATTTGAGAATGCAAGGCTCTCCGGCTGAA-3’]-D-[5’-CGGAAATTCTTACGACATGCT-TGAGCATCGCACTGAAGG-3’]. The Madr2 fragments were amplified with *Taq* using PCR (1 min at 94°C, 1 min at 60°C and...
1.5 min at 72°C for 20 cycles), digested with EcoRI and cloned into the EcoRI site downstream of the lacZ gene in pC55-ctyβ-gal, constructed by D. Turner, R. Rupp, J. Lee, and H. Weintraub (Fred Hutchinson Cancer Research Center, Seattle, WA). The fusion proteins resulting from the expression of these constructs should initiate at the AUG present in β-galactosidase and terminate at the Madr2 stop codon. [β-Galactosidase protein was visualized using X-gal (Sanes et al. 1986).

In situ hybridization and immunostaining

Whole-mount in situ hybridization was performed according to the protocol described by D. Wilkinson (1992). In three separate experiments sense and antisense probes were generated from Madr2(C), and a probe for an antisense Brachury (Hermann et al. 1990) was used as a positive control. The presence of neural tissue, muscle and notochord in whole Xenopus embryos was determined by immunostaining with the antibodies 6F11 (Lamb et al. 1993), 12101 (Kintner and Brockes 1984), and Tor70 (Bolce et al. 1992), respectively.

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