Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine involved in both acquired and innate immunity. MIF also has many functions outside the immune system, such as isomerase and oxidoreductase activities and control of cell proliferation. Considering the involvement of MIF in various intracellular and extracellular events, we expected that MIF might also be important in vertebrate development. To elucidate the possible role of MIF in developmental processes, we knocked down MIF in embryos of the African clawed frog *Xenopus laevis*, using MIF-specific morpholino oligomers (MOs). For the synthesis of the MOs, we cloned a cDNA for a *Xenopus* homolog of MIF. Sequence analysis, determination of the isomerase activity, and x-ray crystallographic analysis revealed that the protein encoded by the cDNA was the ortholog of mammalian MIF. We carried out whole mount *in situ* hybridization of MIF mRNA and found that MIF was expressed at high levels in the neural tissues of normal embryos. Although early embryogenesis of MO-injected embryos proceeded normally until the gastrula stage, their neuralization was completely inhibited. At the tailbud stage, the MO-injected embryos lacked neural and mesodermal tissues, and also showed severe defects in their head and tail structures. Thus, MIF was found to be essential for axis formation and neural development of *Xenopus* embryos.

Macrophage migration inhibitory factor (MIF) was originally discovered as a lymphokine derived from activated T cells that inhibited the random migration of macrophages (1, 2). Later studies have revealed that this protein has various functions. For example, MIF is released from the anterior pituitary gland of lipopolysaccharide-challenged mice and potentiates lethal endotoxemia (3). MIF was also found to be essential for T cell activation (4). In addition, MIF has isomerase activity that catalyzes the tautomerization of D-dopachrome and phenylpyruvate (5, 6). Oxidoreductase activity has been implicated in the regulation of oxidative cell stress (7, 8). MIF is involved in the control of cell proliferation (9, 10), including the suppression of p53-mediated growth arrest (11) and the regulation of cell growth mediated by binding to c-Jun activation domain-binding protein 1 (Jab1) (12). Moreover, MIF regulates innate immunity through the modulation of Toll-like receptor 4 (13).

The involvement of MIF in a variety of intracellular and extracellular events has led us to speculate that MIF might also have important functions in the development of mammals and other vertebrates. Little is known about the involvement of MIF in vertebrate development, except that the expression of chicken MIF in the developing eye lens is correlated with cell differentiation (14). To investigate the possible functions of MIF in developmental processes, we carried out loss-of-function experiments in embryos of the African clawed frog *Xenopus laevis*. For this purpose, we cloned a cDNA for *Xenopus* MIF (XMIF), and synthesized XMIF-specific antisense morpholino oligomers (MOs). MOs are gaining wide use in developmental biology for blocking the translation of mRNA because of their high efficacy and specificity (15–17). In this study, the MO-mediated knockdown of MIF caused a severely altered phenotype, which demonstrated that MIF was an essential factor in *Xenopus* embryogenesis, and which, furthermore, suggested the importance of mammalian MIF in the development of mammals.

**EXPERIMENTAL PROCEDURES**

Cloning of XMIF cDNA—We prepared total RNA from *X. laevis* liver and carried out rapid amplification of cDNA ends (RACE) reactions using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA). The primers used for 5′- and 3′-RACE were 5′-GCCCAGCGCA-CAAGAATCAGTTGA-3′ and 5′-CTGATCTTTTGTGCCCGTGCGTCTGC-TG-3′, respectively. The 5′ and 3′ primers used for the amplification of the full-length cDNA were 5′-AGTTGTCGACCCGGTCTCATCCTTT-3′ and 5′-AATTGCTCTACTTTGTTTACTGAGAAGATG-3′, respectively. The product of this end-to-end PCR was cloned into pBluescript KS+. We sequenced four clones and found that all of them were identical.

Preparation of Recombinant MIF Proteins—*Escherichia coli* BL21(DE3)pLysS, transformed with pET-3a containing *Xenopus* or rat MIF cDNA, was cultured, and MIF expression was induced by isopropyl-β-D-1-thiogalactopyranoside. The cells were disrupted with a French pressure cell disrupter. From the clarified homogenate, MIF was purified by S-ethylglutathione affinity column chromatography.

Determination of Isomerase Activity—The rates of the keto-enol tautomerization of p-hydroxyphenylpyruvate (HPP) catalyzed by MIF were measured by high-performance liquid chromatography.
Table I

<table>
<thead>
<tr>
<th>Resolution (Å)</th>
<th>30–2.5 (2.59–2.5)</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>Unique reflections</td>
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</tr>
<tr>
<td>Completeness (%)</td>
<td>97.0 (97.0)</td>
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<tr>
<td>Averaged redundancy (%)</td>
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<tr>
<td>Averaged I/σ(I)</td>
<td>5.1 (1.7)</td>
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<tr>
<td>Rmerge (%)</td>
<td>8.7 (26.3)</td>
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<tr>
<td>Total number of non-hydrogen atoms</td>
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</tr>
<tr>
<td>Protein</td>
<td>52</td>
</tr>
<tr>
<td>Solvent</td>
<td>40.6</td>
</tr>
</tbody>
</table>

Rmerge = \( \sum \frac{I_n - I_C}{\sigma(I_n)} \), where \( I_C \) is the ith used observation and \( I_n \) is the mean intensity for unique reflections. The Rmerge for protein and solvent are calculated and are 8.7 and 40.6, respectively.

For the synthesis of XMIF mRNA, including its 5' untranslated region (UTR), the pBlueScript plasmid containing the full-length XMIF cDNA was linearized with HindIII. From this template, capped mRNA was synthesized using the mMessage mMachine T7 Kit (Ambion, Austin, TX). Xenopus EF-1α RNA was synthesized from the control template using the same kit. For the synthesis of XMIF RNA without its 5' UTR, the coding sequence was amplified and subcloned. The primers used were 5'-CCGGTCGACGCGCCACCATGCTGTCCTCAGCAT-3' and 5'-GCTTCTAGGATGCAAGCGGATC-3'. A 9-nucleotide Kozak consensus sequence for translation initiation (underlined) was included in the 5' primer. The PCR product was inserted into the XhoI-HindIII site of pcBS2+. The plasmid was linearized with NotI, and capped RNA was synthesized using the mMessage Machine SP6 Kit.

In Vitro Translation—XMIF or EF-1α protein was synthesized using nuclease-treated rabbit reticulocyte lysate (Promega, Madison, WI). The translation reaction mixture (20 μl) contained 14 μl of reticulocyte lysate, 20 μM methionine-free amino acid mixture, 5 μCi of [35S]methionine (Amersham Biosciences), and 0.5 μg of capped RNA. Translation was carried out at 30 °C for 90 min, followed by addition of RNase A to a concentration of 0.2 mg/ml and incubation at room temperature for 5 min. Translated products were analyzed by 15% Tricine-SDS-PAGE and visualized by autoradiography.

Phylogenetic Analysis of MIF Sequences—The phylogenetic tree was constructed with the aligned amino acid sequences of MIF homologs by the neighbor-joining method (23) using the expanded ClustalW program. Positions with gaps were excluded from the analysis. The degree of support for internal branches of the tree was assessed by 1000 bootstrap replicates (24).

RESULTS
cDNA Cloning of XMIF—Although the cloning of full-length cDNA for MIF homologs from Xenopus has not been previously described, an expressed sequence tag (EST) that is similar to mammalian MIF cDNA has been isolated from Xenopus embryos (GenBank™ number BE681403). We assumed that this EST was derived from a gene encoding a protein that was the Xenopus homologue of mammalian MIF. Thus, we refer to the protein of the gene as XMIF (we also refer to it simply as MIF when there is no need to distinguish it from mammalian MIF), and we cloned its full-length cDNA from Xenopus liver by the RACE method. The primers used for the RACE reactions were selected from the EST. Sequencing of 17 clones obtained from 5' RACE identified two major transcrip-
tion start sites at −59 and −47, relative to the start codon. Using the terminal sequences of the 5'- and 3'-RACE products, we amplified and cloned a full-length cDNA for XMIF. The resultant 527-bp cDNA started at 59, and contained an open reading frame, the length of which was identical to that of mammalian MIF. The sequence of this cDNA has been deposited in the GenBank™/EMBL/DDBJ database under accession number AB111063.

Comparison of XMIF and Mammalian MIF—Fig. 1 shows the deduced amino acid sequence of XMIF compared with those of MIF homologs from different craniate (vertebrate and hagfish) species, including a jawed fish (Paralabidochromis chilotes) and two jawless fishes (Petromyzon marinus and Myxine glutinosa) of distant taxa (25). The N-terminal proline residue, which is the catalytic center of the isomerase activity of mammalian MIF (26), is conserved in XMIF. In mammalian MIF, a number of aromatic residues are clustered around this proline (27, 28). These surrounding residues are all conserved in XMIF, suggesting their importance in the function or formation of the structure of MIF. In addition, the invariant lysine residue at position 32, which contributes to the isomerase activity of the protein (29, 30), is conserved in XMIF. Two cysteine residues that form the catalytic center of oxidoreductase activity (7) are also conserved. One of these cysteines (Cys56) is conserved in all known MIF homologs from craniate species, including d-dopachrome tautomerase (DDT), and also in some homologs from nematodes. The other cysteine (Cys59) residue of the catalytic center undergoes cysteinylation in human suppressor T hybridoma cells. This modification is essential for the immunosuppressive activity of MIF as a glycosylation-inhibiting factor (31). Cys59 is conserved in chicken MIF and in P. chilotes MIF as well, but not in those of jawless fishes (25). We carried out a phylogenetic analysis of XMIF and the other MIF homologs listed in Fig. 1, and found that the branching pattern of the resultant phylogenetic tree corresponded essentially with the evolutionary relationships among the species (Fig. 2).

Because crucial amino acid residues for the isomerase activity of mammalian MIF were shared with XMIF, we expected XMIF to have the same activity as mammalian MIF. The determined rate of keto-enol tautomerization of HPP catalyzed by XMIF was comparable with that catalyzed by rat MIF (Fig. 3).

We carried out x-ray crystallographic analysis to compare the structure of XMIF with that of mammalian MIF. In the crystal structure of XMIF (Fig. 4), one trimer and one monomer were contained in an asymmetric unit, in which MIF formed a

Fig. 1. Amino acid sequence alignment of MIF and DDT proteins from craniate species. The numbering system is for mammalian MIF. Hyphens denote gaps, which were inserted to optimize the alignment according to a previous report (25). Residues conserved in all the proteins listed are boxed, including the N-terminal proline that is the catalytic center of the isomerase activity of MIF (26), and those conserved in mammalian MIF and XMIF are marked by a plus sign (+). The asterisks represent the residues clustered around the N-terminal proline (27, 28). The dollar sign ($) denotes the lysine residue, which has also been implicated in the enzymatic activity (29, 30). The number sign (#) indicates the catalytic center of the oxidoreductase activity of MIF (7, 8). The secondary structure elements shown below are those of human MIF (27). Species: Bota, Bos taurus (cattle); Gaga, Gallus gallus (chicken); Hosa, Homo sapiens (human); Meun, Meriones unguiculatus (Mongolian gerbil); Mumu, Mus musculus (mouse); Mygl, M. glutinosa (hagfish); Pach, P. chilotes (cichlid); Pema, P. marinus (lamprey); Rano, Rattus norvegicus (rat); Xela, X. laevis. The GenBank™ accession number for each protein is indicated to the right of the C terminus, except in the case of bovine MIF (SWISS-PROT accession number P60177).
trimer around the crystallographic 3-fold axis. Mammalian MIF proteins also exist as a trimer (27, 33). Each monomer contains two \( \alpha \)-helices and a four-stranded \( \beta \)-sheet. Two additional \( \beta \)-strands interact with the \( \beta \)-sheets of adjacent subunits. The barrel structure, which contains three \( \beta \)-sheets (one from each monomer), forms the solvent-accessible channel positioned at the center of the trimer. These structural features are conserved in mammalian MIF. The root mean square deviation for main chain atoms between XMIF and human MIF and between XMIF and rat MIF are 0.55 and 1.30 Å, respectively.

Expression of XMIF mRNA—RNAs from Xenopus embryos and those from organs of an adult frog were subjected to RT-PCR followed by Southern blot analysis to determine the expression levels of MIF mRNA. Fig. 5A shows the expression of MIF mRNA in embryos at different stages of development. Early embryos contained a substantial amount of maternally expressed MIF mRNA. The mRNA level decreased at the gastrula and neurula stages (stages 11 and 15, respectively), and increased again at the tailbud stage (stage 20). B, MIF was ubiquitously expressed in various organs in the adult frog. Histone 4 (H4) and EF-1α served as the RNA loading controls.

FIG. 3. HPP tautomerase activities of rat MIF and XMIF. Plotted values of absorbance at 330 nm (\( A_{330} \)) represent the MIF-catalyzed increase in end HPP. •, rat MIF; □, XMIF.

FIG. 4. Structural comparison of XMIF and human MIF. The main chains of XMIF (red) and human MIF (blue) are superimposed. The atomic coordinates for XMIF protein have been deposited in the Protein Data Bank under code 1UIZ.

FIG. 5. Expression of XMIF in developing embryos and organs of an adult frog. The RT-PCR/Southern blot analysis was performed as described under “Experimental Procedures.” A, MIF was expressed maternally; levels decreased at the gastrula and neurula stages (stages 11 and 15, respectively), and increased again at the tailbud stage (stage 20). B, MIF was ubiquitously expressed in various organs in the adult frog. Histone 4 (H4) and EF-1α served as the RNA loading controls.
MIF Is Essential for Xenopus Development

Embryos hybridized with antisense (bar) and sense (LH and posterior) larvae shown to have no effect on the translation of that RNA (Fig. 7, lanes 7 to 24), was derived. We used two MOs for targeting MIF. MO1, which was designed for inhibition of the translation of endogenous MIF RNA of \( \text{MIF} \), MO2 was as effective as the standard control MO (Fig. 7, lanes 6 and 8). MO2 also inhibited the translation of the same RNA, with reduced efficiency (lanes 4 and 5). MO2 did not inhibit the translation of MIF RNA lacking an MIF-derived 5' leader sequence (lanes 7 and 8). MO1 and MO2 had no effect on the translation of an RNA unrelated to MIF (lanes 9–11).

Knockdown of XMIF Inhibits Neurulation—Because MIF mRNA was detected at high levels in neural tissues, we expected that MIF might play important roles in neural development. Thus, we injected antisense MOs for MIF into two dorsal blastomeres of 4-cell stage embryos, from which neural tissues are derived. We used two MOs for targeting MIF. MO1, which hybridizes to the area including the start codon (~1 to 24), was predicted by the manufacturer to be optimal for blocking the translation of MIF. MO2, which was complementary to a portion of the 5' UTR (~28 to ~4), was used for a rescue experiment, because it hybridizes only to endogenous MIF mRNA, not to co-injected MIF RNA lacking an MIF-derived 5' leader sequence.

We examined the effect of the MIF MOs in an \textit{in vitro} translation system (Fig. 7). As expected, both MO1 and MO2 inhibited the translation of the full-length MIF RNA. MO1 almost completely blocked the translation at a concentration of 1 \( \mu \text{M} \) (Fig. 7, lane 2). In this system, MO2 had a lower targeting efficiency than MO1. With 1 \( \mu \text{M} \) MO2, an appreciable amount of MIF protein was synthesized. At 10 \( \mu \text{M} \), MO2 was as effective as 1 \( \mu \text{M} \) MO1 (Fig. 7, lanes 4 and 5). Thus these MOs were likely to inhibit the translation of endogenous MIF mRNA in injected embryos, at different efficiencies. MO2, which was designed for co-injection with MIF RNA without its 5' leader sequence, was shown to have no effect on the translation of that RNA (Fig. 7, lanes 7 and 8). In addition, MO1 and MO2 had no detectable effect on the translation of an RNA unrelated to MIF (EF-1a) (Fig. 7, lanes 9–11).

Fig. 8 shows the results of the injection of MO1. In the embryos injected with MO1, early embryogenesis up to gastrulation proceeded normally, but neurulation was completely inhibited, i.e., embryos that had received the injection did not form any trace of a neural plate. These embryos also had severe defects in the head and tail structures, and histological examination showed that they were devoid of neural tissues, resulting in the absence of the brain, spinal cord, eye capsules, and otic vesicles. Although a mass of cells in the anterior dorsal region showed staining similar to that of neural cells (Fig. 8F), they did not form any structure found in normally developed neural tissues. More surprisingly, mesodermal tissues such as the notochord and somites did not form. The disappearance of these structures indicated that both anteroposterior and dorsosventral axis formation were entirely disordered in these embryos. We further confirmed the absence of neural tissues by \textit{in situ} hybridization of two pan-neural markers, zic-2 and nrm-1. As shown in Fig. 8N, no expression of zic-2 was observed in the MO-injected embryos. Similarly, nrm-1 was not detected (data not shown). These results indicated that the injection of MIF MO inhibited the differentiation of neural precursor cells; in addition, it was found that even the anterior dorsal cells showing neural cell-like staining had not differentiated normally.

To examine the specificity of the effect of MIF MO on embryogenesis, we used MO2 and MIF RNA in a rescue experiment. When 9.2 pmol of MO2 (equal to the amount of MO1 used in the...
experiment described above) was injected into the embryos, only a slight morphological effect was observed (data not shown). We hypothesized that the reduced effect was because of the lower targeting efficiency of MO2 than that of MO1. However, injecting the embryos with 36.8 pmol of MO2 caused essentially the same phenotypic changes observed in MO1-injected embryos, although the effects were slightly less prominent (Fig. 9, B, E, and G). When MIF RNA (2 ng per embryo)
was co-injected with MO2, we observed a partial phenotype recovery (Fig. 9, C, F, and H). However, no specific change of phenotype was observed by injecting MIF RNA alone (data not shown), suggesting that the constitutively expressed MIF level might be sufficient to maintain physiological functions, and, moreover, that extra expressed MIF minimally affected the phenotypes. The average ± S.D. of the body length of the control embryos (n = 20) was 4.52 ± 0.13 mm and that of MO2-injected embryos (n = 17) was 2.11 ± 0.30 mm, whereas that of co-injected embryos (n = 16) was 3.00 ± 0.70 mm. In addition to the recovery of body length, the head and tail structures were morphologically restored in many of the co-injected embryos (Fig. 9, E and F). A higher dose of MIF RNA (4 ng per embryo) did not help to improve the restoration of the phenotype, suggesting the saturation of MIF RNA.

Histological observation revealed that, in the rescued embryos, the neural tube at the trunk level had formed (Fig. 9H). However, the anterior neural structures, including the brain, eyes, and ears were still disorganized in the co-injected embryos. Table II summarizes the results of the rescue experiments. When the embryos were injected with MO2, we found severely defective head structures in all 17 viable embryos, and no eye capsules had formed (Grade 2). On the other hand, when MIF RNA was co-injected with MO2, 12 of 16 viable embryos showed only moderate defects (Grade 1), i.e. the eye capsules were partially formed and the head structure was less disordered than in the previous case. These data suggested that the disappearance of neural tissues and defective head and tail structures in MIF MO-injected embryos were specific effects of the knockdown of MIF. Thus, we concluded that MIF was essential for axis formation and neural development in Xenopus embryogenesis.

**DISCUSSION**

MIF was initially identified as a lymphokine, and today it is regarded as an important immunoregulatory protein and as a key component of the cytokine network that plays a number of roles in both acquired and innate immunity (3, 4, 13, 35, 36). The significance of MIF in the immune system is being recognized to an increasing extent. Clinically, MIF is emerging as a prospective target of therapy for human diseases primarily affecting immunity (35, 37–40). On the other hand, a number of findings have revealed many functions of MIF that are not restricted to those carried out as part of the immune system (9–12, 39, 41–44). Considering the various functions of MIF, it seemed possible that MIF might have essential roles in vertebrate development.

The amino acid sequence of XMIF is 67–71% identical to that of mammalian MIF, and the crucial residues required for some MIF functions are conserved in XMIF. Of all the MIF homologs with known amino acid sequences, the most similar to XMIF is chicken MIF, the amino acid sequence of which is 77% identical to that of XMIF. In addition to the EST that was used to clone the XMIF cDNA, an EST that is similar to mammalian DDT has also been isolated from Xenopus (GenBank™ accession number BC043871). This EST contains an entire open reading frame for a possible product of 117 amino acids, which is 66% identical to human DDT and only 27% identical to XMIF. The presence of the open reading frame is supporting evidence for our initial assumption that the functions of XMIF correspond to those of mammalian MIF.

The three-dimensional structure of XMIF (Fig. 4) is very similar to that of mammalian MIF. In addition, the aromatic residues that form the catalytic pocket around the N-terminal proline of MIF are all conserved in XMIF, whereas DDT has a totally different set of residues clustered around the proline (28). Moreover, the HPP tautomerase activity of XMIF was found to be almost equal to that of rat MIF. If we take into account all of these findings, it is clear that XMIF is the ortholog of mammalian MIF. Therefore we consider the results presented here to be useful for understanding the functions of mammalian MIF. We also consider it reasonable to assume that MIF is important in mammalian development.

Targeted disruption of the MIF gene in mice causes no detectable developmental abnormalities (45–47). However, some compensation mechanism that rescues the developing embryos from the loss of MIF may be activated during the generation of knockout mice. By applying the MO-mediated knockdown strategy, we observed an altered phenotype of MIF-targeted Xenopus embryos. In the MO-injected Xenopus embryos, the assumed compensation mechanism may not have been fully

**Fig. 9.** The effect of MIF MO is partially recovered by simultaneous injection of wild-type MIF RNA. Two dorsal blastomeres of the 4-cell stage embryos were injected with MO or MIF RNA, and these injected embryos were cultured until stage 32. Fixed embryos were subjected to morphological and histological analyses. A, un.injected control embryos. B and C, embryos were injected with MO2 (36.8 pmol) (B) or MO2 (36.8 pmol) and MIF RNA (2 ng) together (C). D–F, high magnification views of an uninjected embryo (D), an MO2-injected embryo (E), and an MO2- and MIF RNA-injected embryo (F). G and H, transverse sections at the trunk level of an MO2-injected embryo (G) and an MO2- and MIF-injected embryo (H). Note that MO2 disturbed the differentiation of the neural tube, but MIF RNA was able to rescue neural tube formation. The bars shown in A, D, and G indicate 1 mm, 1 mm, and 100 μm, respectively.
activated. Alternatively, the compensation may be less efficient in *Xenopus* than in mice.

In the RT-PCR analysis, a large amount of MIF mRNA was detected at the blastula stage (stage 9). Because the MIF message was also detected abundantly in early cleaving embryos and the level of expression declined after the blastula stage, the transcript detected at stage 9 was presumed to be of maternal origin. In the whole mount *in situ* hybridization analysis, MIF mRNA was detected in the animal pole area at stage 9, which is likely to reflect the distribution of MIF mRNA in the oocyte. The accumulation of MIF mRNA near the animal pole correlates with the region of the early embryo in which cell division is most accelerated. Thus, MIF might be important for the promotion of cell division in the animal hemisphere. However, the functions of XMIF in very early embryos cannot be examined by MO-mediated targeting because MO injection does not deplete the preformed proteins in the oocyte. The role of XMIF in the early processes of embryogenesis before gastrulation remains to be investigated using a different strategy.

The injection of MIF MO at the 4-cell stage had no detectable effect on subsequent processes up to gastrulation. This may indicate that the MIF protein stored in the oocyte is required for early developmental processes, and that the MIF synthesized from the maternal mRNA after the 4-cell stage has no additional functions. Alternatively, the MIF mRNA in the early embryo may be the source of MIF protein that functions in the gastrula embryo. Although the transcription of MIF mRNA from the zygotic genes may have started at the gastrula stage, the weak MIF signal detected at stage 11 (Fig. 6B) might also have been derived from undegraded maternal mRNA. Even the region in which no MIF signal was detected may have contained MIF protein synthesized from the maternal mRNA, which might have important roles in that region.

We confirmed the specificity of the MOs by *in vitro* translation analysis and by co-injection of MO2 and MIF RNA. In the *in vitro* analysis, MO1 and MO2 were both found to inhibit the translation of MIF RNA. Of the two MOs, MO1, which had been predicted to be most effective, inhibited the translation more efficiently than MO2. MO1 and MO2 had different sequences without any overlap and caused essentially the same morphological effect on injected embryos. The lower targetting efficiency of MO2 than of MO1, which was shown in the *in vitro* assay, was consistent with the observation that the MO2-injected embryos were less disordered than the MO1-injected embryos. Moreover, the co-injection of MIF RNA and MO2 partially rescued the altered phenotype caused by the MO2 injection. In addition to the restoration of the head structure (Table II), it was highly noticeable that the co-injected embryos formed the neural tube, which was absent in the embryos injected with MO2 alone. Taken together, these data strongly suggest that the phenotype of the MO-injected embryos, at least except for the disappearance of mesodermal tissues, was caused specifically by the knockdown of MIF.

We initially anticipated that MIF might be required for neural development. Indeed, the MO-mediated knockdown of MIF completely inhibited neurulation. However, it was unexpectedly found that the MIF MO-injected embryos also lacked mesodermal structures such as the notochord and somites. Although the mesodermal derivatives were not restored in the rescue experiment, it is still possible that the disappearance of mesodermal tissues in the MO-injected embryos was specifically caused by the knock-down of MIF, because injection of RNA does not always result in sufficient production of the encoded protein in the cells that require it, or because ectopic production of MIF protein in adjacent cells might have inhibited the normal differentiation of mesodermal cells. Therefore, the essentiality of MIF for developmental processes may not be neural tissue-specific in *Xenopus*. Because the notochord is formed in the dorsal mesoderm and it interacts with the ectoderm to induce neurulation, the initial effect of the MIF MO injection on neural development is thought to be the inhibition of notochord formation. By *in situ* hybridization at the gastrula stage, positive staining of MIF mRNA was hard to identify in the developing notochord. However, it is possible that a very small amount of MIF mRNA (below the limit of detection by *in situ* hybridization) was expressed in the dorsal mesoderm, and that this small amount was critical for the differentiation of the cells in this region to form the notochord. Alternatively, the effect of MO in the formation of the notochord occurred non-specifically. Histological observation in the restoration experiment showed that MO2-injected embryos lost the neural tube and notochord (6 of 6 examined embryos) but the embryos rescued by MIF RNA recovered only the neural tube (5 of 5 examined embryos) (Fig. 9, G and H). This suggests the possibility that MO2 happened to disturb the translation of (an) unknown gene(s) that is required for the development of the notochord but not for the neural induction from the dorsal mesoderm. Although it is difficult to address the exact reason for the incomplete restoration by MIF RNA, the present study at least demonstrates a significant role of MIF in differentiation of neural tissues and in the formation of the anteroposterior axis. In addition, dorsoventral axis formation was shown to be at least partly dependent on MIF, because the neural tube was formed in the embryos co-injected with MO2 and MIF RNA.

At the gastrula stage, MIF mRNA was detected in the deep layer of the dorsal marginal zone (Fig. 6B). Although the MIF signal was weak in the gastrula embryo, an increased signal was detected in the neural plate of the early neurula embryo (Fig. 6D), which indicated that zygotic expression had started. In the RT-PCR analysis (Fig. 5A), the level of MIF expression per whole embryo at the early tailbud stage (stage 20) was
found to be much higher than that at the neurula stage (stage 15). We presume that the observed increase in the amount of MIF mRNA resulted partly or even primarily from proliferation of MIF-producing cells, in addition to elevated expression in those cells. Considering the abundant expression of MIF in the developing neural tissue, it is likely that MIF is required for the differentiation of neural precursor cells in a direct fashion. MIF may also be indirectly involved in neurulation, because notochord formation may require MIF. However, further investigation is necessary to confirm the direct or indirect involvement of MIF in neural development, and to explicate the molecular mechanism underlying the essentiality of MIF in embryogenesis.

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Xenopus laevis Macrophage Migration Inhibitory Factor Is Essential for Axis Formation and Neural Development
Masaki Suzuki, Yumi Takamura, Mitsugu Maéno, Shin Tochinai, Daisuke Iyaguchi, Isao Tanaka, Jun Nishihira and Teruo Ishibashi

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