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Molecular Cloning of the *Xenopus* c-met/Hepatocyte Growth Factor Receptor and Its Regional Expression during Early Development[1]

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Hepatocyte growth factor (HGF), a ligand for c-Met receptor tyrosine kinase, regulates the cell growth, migration and morphogenesis of a wide variety of cells, and plays important roles in organogenesis in embryos as well as regeneration of organs. HGF is structurally conserved in *Xenopus laevis*, and the *Xenopus* HGF gene is expressed in neurula and tailbud embryos. In the present work, we cloned complementary DNA for the *Xenopus* Met/HGF receptor and analyzed its expression during embryogenesis. The open reading frame of *Xenopus c-met* is 4,125 base pairs long and encodes a putative polypeptide of 1,375 amino acids. The deduced amino acid sequences of the *Xenopus* and human Met proteins are 63% identical, the tyrosine kinase domains in the intracellular regions showing particular homology, over 90% identity in the amino acid sequences between *Xenopus* and human. During *Xenopus* embryogenesis, *c-met* mRNA was expressed at a high level from the gastrula to the neurula stage, while HGF mRNA expression was seen from the early neurula stage. Whole mount *in situ* hybridization showed that *c-met* mRNA was specifically localized in the foregut region, mesenchymal tissue of the tailbud, and neural tissues in neurula embryos. Thus, c-Met is a highly conserved molecule in a wide range of species, as is its ligand, HGF. The HGF-Met system may be involved in early multiple organogenesis in *Xenopus* embryos.

Key words: c-met, embryogenesis, epithelial-mesenchymal interaction, hepatocyte growth factor, *Xenopus laevis*.

Hepatocyte growth factor (HGF), originally identified and purified as a potent mitogen for mature hepatocytes (*1-5*), is composed of a four kringle-containing α-chain and a pseudo serine protease-like β-chain (*6, 7*). Further extensive studies focusing on the biological functions of HGF revealed multi-potent characteristics for a wide variety of cells. HGF exhibits mitogenic activity toward various types of epithelial cells (*8-10*), and also exhibits motogenic activities toward certain types of epithelial cells, promoting cell scattering and motility (*11, 12*). HGF also functions as an epithelial morphogen, inducing branching tubule formation (*13-15*). These biological activities of HGF are mediated by the high affinity cell surface receptor, Met membrane-spanning tyrosine kinase (*16-18*). Binding of HGF to the extracellular domain of Met leads to autophosphorylation, which elicits further activation of an intracellular tyrosine kinase and the following activation of specific downstream signal-transduction pathways through its unique docking sites (phosphorylated tyrosine residues) that interact with intracellular molecules containing the Src homology 2 (SH-2) domain (*19*).

Together with the unique multipotent characteristics of HGF, the preferential expression patterns of the HGF and *c-met* genes in embryogenesis of vertebrates means that HGF is likely to be a mesenchymal-derived paracrine mediator for cell-cell interactions between epithelial and mesenchymal tissues during embryonic organogenesis (*9, 13, 20*). The epithelial histogenesis of liver and placental tissues in HGF gene knock-out mouse embryos are specifically disrupted (*21, 22*), and the same disruption of epithelial histogenesis was observed in mice lacking the HGF receptor/c-met gene (*23*). Likewise, disruption of the coupling between HGF and Met inhibited organogenesis of kidney and tooth tissues in organ culture systems (*24, 25*).

The *Xenopus* embryo is an appropriate system for studies on the molecular mechanisms of embryonic events, *e.g.*, mesoderm induction and neural induction. The conventional transgene techniques of microinjection of synthetic RNAs and numerous molecular markers for cellular differentiation all facilitate investigation of early embryogenesis. Activation and inhibition experiments on *Xenopus* embryos successfully revealed the function of various molecules including growth factors, receptors, transcrip-

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Abbreviations: HGF, hepatocyte growth factor; HLP, HGF-like protein; ODC, ornithine decarboxylase.

tional factors, *etc.* To better understand the roles of signals mediated by HGF and HGF receptor/c-Met in embryogenesis, particularly in the process of multiple organogenesis, cDNA encoding *Xenopus* HGF was cloned and the pattern of gene expression during embryogenesis was analyzed (*26*). It appears that HGF may regulate early embryogenesis in *Xenopus laevis*, being a highly conserved molecule from amphibians to mammalians. In the present study, we isolated cDNA encoding a *Xenopus* homologue of the c-Met/HGF receptor gene, and analyzed the embryonic expression of the *c-met* gene during embryogenesis. The expression of the *Xenopus* c-met gene was detectable from the gastrula to the neurula stage, and was localized in the foregut region, mesenchymal tissue of the posterior region, and neural tissues. These findings suggest that the HGF-Met system may be involved in early processes of multiple organogenesis.

MATERIALS AND METHODS

Preparation of Xenopus Embryos—*X. laevis* were purchased from Hamamatsu Seibutsu Kyouzai (Hamamatsu). The embryos were prepared by standard methods. The jelly coat of the embryos was removed by treatment with 3% cysteine-hydrochloride/MMR (pH 7.3-7.4). The *Xenopus* embryonic development stage was determined according to the normal stage table presented by Niewkoop and Faber (*27*).

Isolation of Xenopus c-met cDNA—PCR comprised three step reactions. A set of degenerate oligonucleotide primers was designed in tyrosine kinase domains, based on the amino acid sequences of mouse and human *c-met* (*16, 28*). The primers used for the first PCR were 5′-AGAAAG-CTTTT(TC)GG(ATGC)TG(TC)CT(ATGC)TA(TC)CA(TC)-GG-3′, and 5′-CGTTCTAGAACT(TC)TC(ATGC)A(AG)-(ATGC)GCCATCCA-3′. The primers for the second PCR were 5′-AGAAAGCTTTT(TC)GG(ATGC)TG(TC)CT-(ATGC)TA(TC)CA(TC)GG-3′, and 5′-TGTTCTAGATA-(TC)TC(TC)TT(AG)TC(AG)TACAT(GA)TC-3′. The primers for the third PCR were 5′-AATAAGCTTAA(AG)-AA(AG)AT(ATC)CA(TC)TG(TC)GC-3′, and 5′-TGTTC-TAGATA(TC)TC(TC)TT(AG)TC(AG)TACAT(GA)TC-3′. The expected 400 bp PCR products in the agarose gel were purified and then subcloned into pBluescript II SK(−). One of the plasmid clones was tentatively named pXM1.

Approximately 1.0×10^6 recombinant phases in a *Xenopus* liver library were screened by plaque hybridization, using the [α-^{32}P]dCTP labeled 400 bp cDNA fragment of pXM1 as a probe. Consequently, five independent positive clones were identified and tentatively named pXMA, pXMB, pXMC, pXMD, and pXME, respectively. The deleted clones, pXMC and pXME, were sequenced, and the entire nucleotide sequence of the open reading frame was determined by the dideoxynucleotide chain termination method using a Dye Deoxy™ cycle sequencing kit with an ABI 373A automated DNA sequencer (Perkin-Elmer).

RNA Isolation and RT-PCR Analysis—Total RNA was purified from various *Xenopus* organs and embryos by the acid guanidium thiocyanate/phenol-chloroform method (*29*). RT-PCR were performed as described in the literature (*30*). Complementary DNA for RNA, used for PCR, was synthesized with MoMLV RTase (Gibco BRL). The oligos used for PCR in this assay were as follows. *Xenopus c-met*; sense primer: 5′-AGGTGAAGTTCTGAAAGTT-GG-3′, and antisense primer: 5′-GACTAATGCTTCTGGC-ACTCA-3′. The *Xenopus* HGF sense primer: 5′-TCAAGA-TGTGAAGGCGATAC-3′, and antisense primer: 5′-TCC-AACAATATGCAGAGTACC-3′ (*26*). The *Xenopus* ornithine decarboxylase (ODC) sense primer: 5′-AATGGATTT-CAGAGACCA-3′, and antisense primer: 5′-CCAAGGCT-AAAGTTGCAG-3′ (*31*). The histone H4 sense primer: 5′-GACAACATCCAGGGCATCACC-3′, and antisense primer: 5′-GAGAGCGTACACCACATCCAT-3′ (*32*). The conditions used for PCR were as follows: denaturation at 94°C for 30 s, followed by annealing at 60°C (48°C for ODC) for 1 min and extension at 72°C for 1.5 min. Twenty-five reaction cycles were performed in the presence of [α-^{32}P]-dCTP. The PCR products were separated in 4.8% polyacrylamide gels, and the gels were dried for autoradiography.

Whole Mount In Situ Hybridization—Whole mount *in situ* hybridization was performed as described previously (*33*), except that BM purple AP substrate (Boeringer) was used as a substrate for alkaline phosphatase. A 4.0 kbp *Xenopus* c-Met cDNA clone was used as a template for the synthesis of cRNA probes. The anti-sense and sense probes were synthesized using T3 RNA polymerase (Gibco/BRL) and T7 RNA polymerase (Gibco/BRL), respectively.

RESULTS

Isolation and Characterization of Xenopus c-met cDNA—A *Xenopus* homologue of the *c-met* gene was initially isolated from embryonic cDNA by PCR with degenerate oligonucleotide primers designed on the basis of mammalian *c-met* sequences (*16, 28*). Complete cDNA clones were obtained from a *Xenopus* liver library, using the cloned PCR product as a probe. The open reading frame of *Xenopus c-met* is 4,125 base pairs long and encodes a putative polypeptide of 1,375 amino acids (Fig. 1). The deduced amino acid sequences of the Met proteins of human and *Xenopus* are 63% identical (Fig. 2A). Conversely, the identity of the *Xenopus* Met amino acid sequence to those of other members of the mammalian and avian Met families is relatively low. Human Ron, a receptor for HGF-like protein (HLP)/macrophage stimulating protein (MSP) (*34, 35*), and chick c-Sea (*36*) are 31 and 30% identical to *Xenopus* Met, respectively.

The N-terminal hydrophobic stretch of 20 amino acid residues is likely to be a signal sequence, and an intramolecular hydrophobic stretch of 925-949 amino acid residues to be the transmembrane domain. Cleavage of proteins at a cluster of basic amino acid residues (296-300) is one of the posttranslational modifications common to Met family proteins, and the basic amino acid cluster, KRRKR, is conserved in this *Xenopus* homologue of the Met protein. Thus, putative *Xenopus* Met may be a heterodimeric molecule composed of an extracellular α-chain and a membrane spanning β-chain, which contains a cysteine-rich domain in the extracellular region and an intracellular tyrosine kinase domain. These domain structures are consistent with those of human Met (Fig. 3).

Although the sequence homology of the extracellular region between human and *Xenopus* Met is relatively low; 56% in the α-chain and 54% in the extracellular β-chain, the number and locations of extracellular cysteine residues,

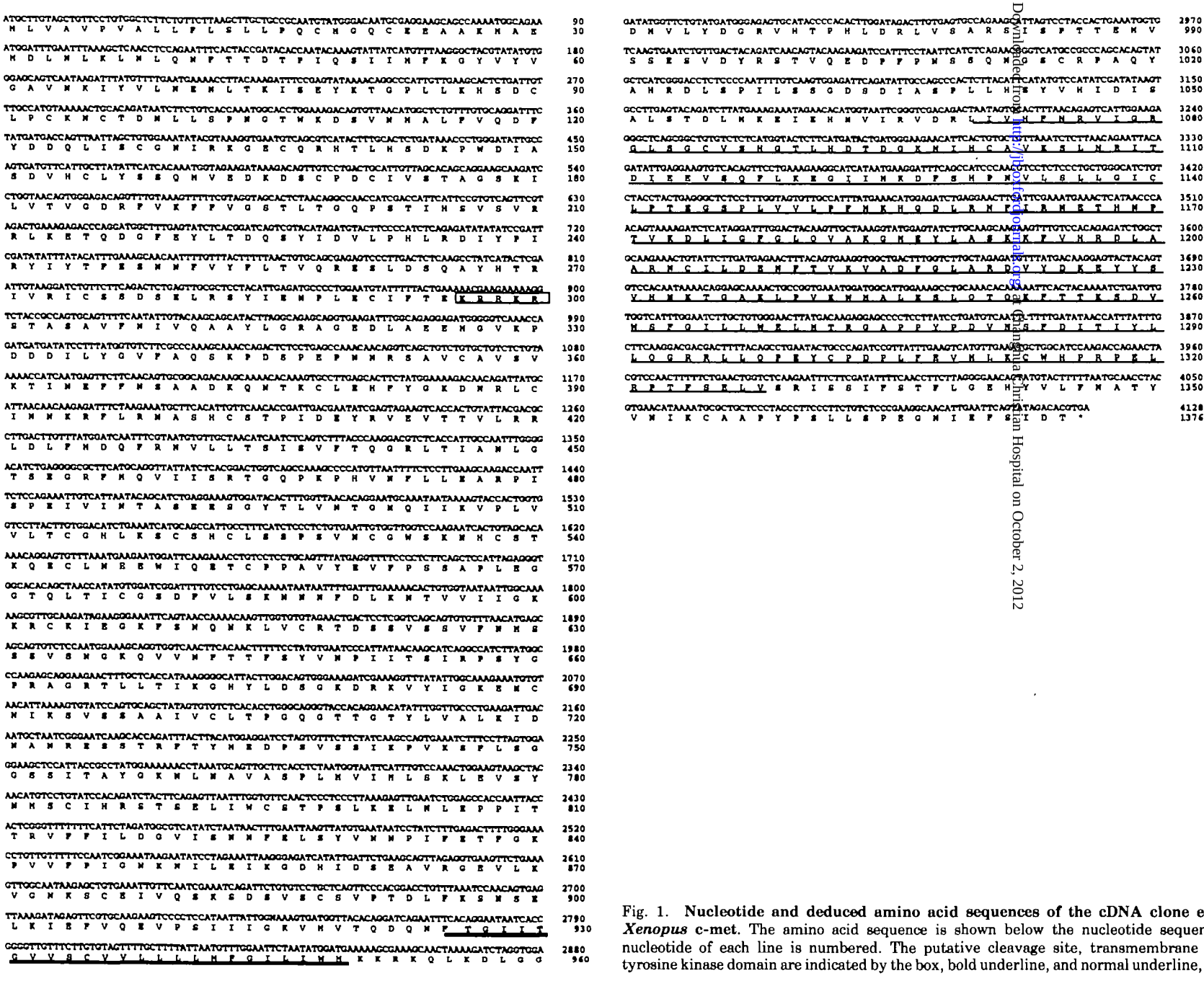

Fig. 1. **Nucleotide and deduced amino acid sequences of the cDNA clone encoding for *Xenopus* c-met.** The amino acid sequence is shown below the nucleotide sequence. The last nucleotide of each line is numbered. The putative cleavage site, transmembrane domain, and tyrosine kinase domain are indicated by the box, bold underline, and normal underline, respectively.

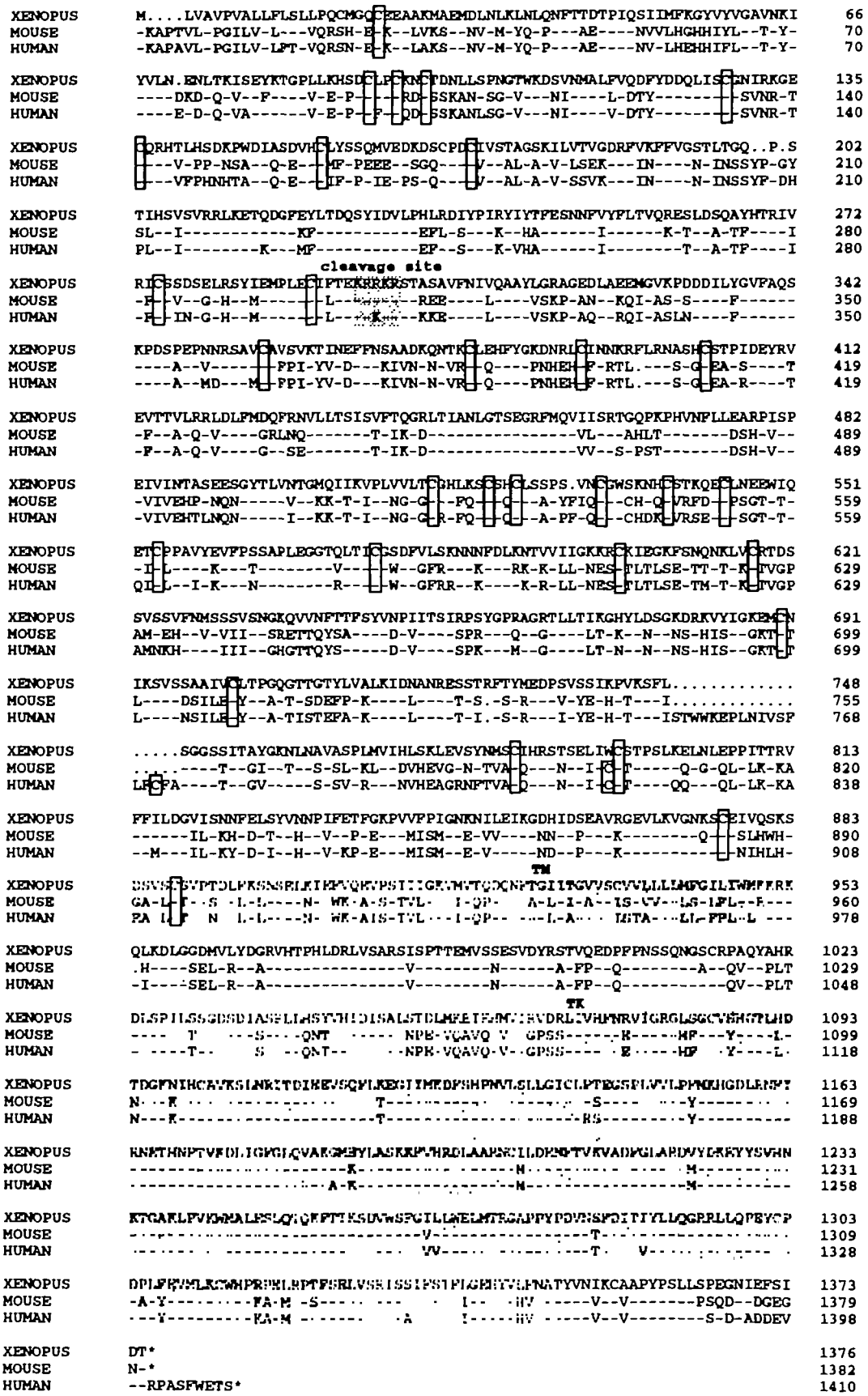

Fig. 2. **Alignment of the amino acid sequences of *Xenopus*, mouse, and human Met.** Amino acid numbers are given on the right. Identical amino acids are indicated by dashes in the sequences of mouse and human Met. The transmembrane domain, the putative cleavage site and a cytoplasmic tyrosine kinase domain are indicated by shaded boxes.

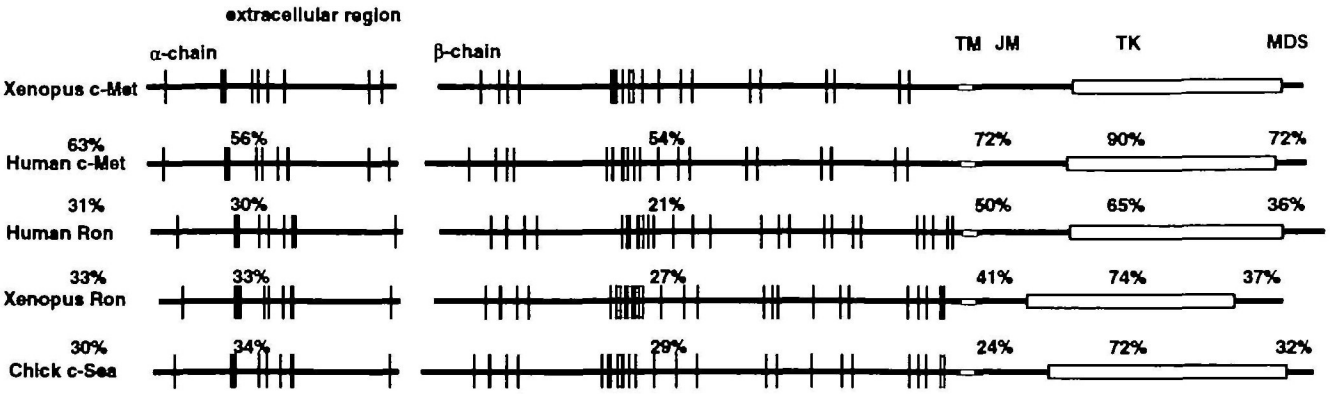

Fig. 3. **Schematic representation of the structures of *Xenopus* Met, human Met, human Ron, *Xenopus* Ron, and chick Sea.** The cysteine residues in extracellular regions are represented by bars. The boxes indicate transmembrane and tyrosine kinase domains. TM, transmembrane domain; JM, juxta-membrane domain; TK, tyrosine kinase domain; MDS, multiple-docking site for SH-2 molecules.

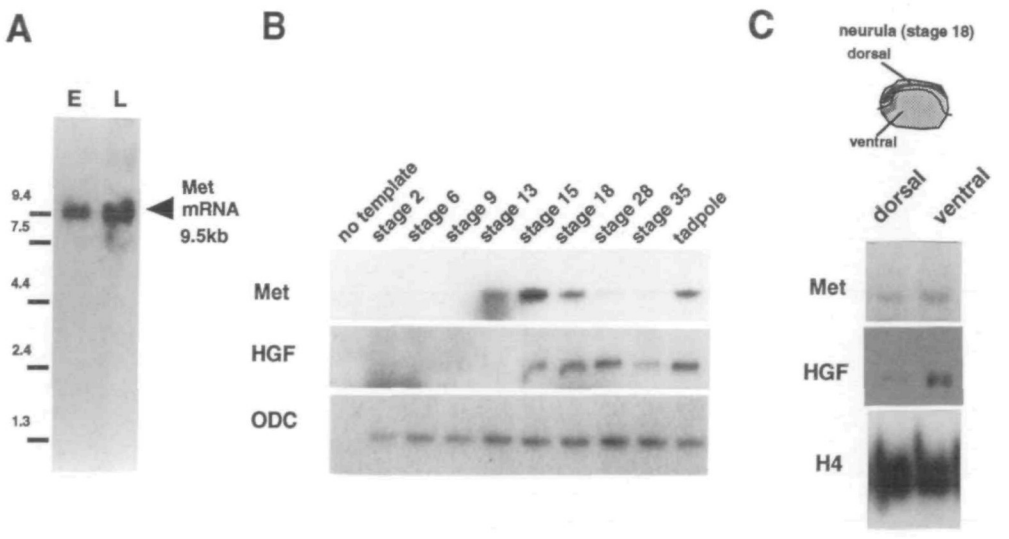

Fig. 4. **Expression of *Xenopus* Met and HGF mRNAs during early development.** (A) Northern analysis of *Xenopus* c-met transcripts in an embryo (tadpole) and liver (from an adult frog). The amount of total RNA for blotting was thirty micrograms. E, embryo; L, liver. (B) Developmental change in the expression of *Xenopus* c-met. The expression of *c-met* and HGF mRNA during embryogenesis was analyzed by RT-PCR. An internal control PCR with *Xenopus* ornithine decarboxylase (ODC) and the same cDNAs was performed. (C) Regional expression of Met and HGF mRNA in *Xenopus* neurula embryos. The expression of *c-met* and HGF mRNA was analyzed by RT-PCR. Neurula embryos (stage 18) were micro-dissected (dorsal and ventral regions). Internal control PCR with *histone H4* and the same cDNAs was performed to confirm the RT reactions.

which restrict the tertiary structure of Met and thus may determine its ligand specificity, are conserved (Fig. 3). In contrast, the amino acid sequence of the tyrosine kinase domain in the intracellular region is well conserved. The homology of Met between mammalians and *Xenopus* in this region is 90%. Moreover, the C-terminal region containing the docking site for multiple SH-2 molecules is also highly conserved (Fig. 3). Likewise, the juxtamembrane region, located between the transmembrane domain and the tyrosine kinase domain, is highly conserved between the human and *Xenopus* Met proteins. Although the biological function of this juxtamembrane domain has yet to be determined, the juxtamembrane region of *Xenopus* Met is 72% identical to that of human Met (Fig. 3).

Temporal and Regional Expression Patterns of Xenopus c-met—We then analyzed, by RT-PCR, the temporal expression pattern of *c-met* during *Xenopus* embryogenesis, and compared it to the pattern of gene expression of its ligand, HGF (Fig. 4). A 9.5 kb transcript of the *Xenopus c-met* gene was expressed in both adult liver and embryos (Fig. 4A). The zygotic expression of the *Xenopus c-met* gene begins at the gastrula stage (stage 13), reaches a plateau at the early neurula stage (stage 15), and then decreases gradually until the tail bud stage (stage 35) (Fig. 4B). When an embryo develops to the tadpole stage, the expression again increases (Fig. 4B). On the other hand, HGF mRNA expression begins at the early neurula stage (stage 15), and becomes maximum during the middle neurula (stage 18) and late neurula (stage 28) stages. It then decreases at the tailbud stage (stage 35), and increases in the tadpole stage (Fig. 4B). Therefore, the zygotic expression of both the HGF and c-met genes is evident following the early neurula stage during the course of *Xenopus* embryonic development.

The regional pattern of expression of HGF and *c-met* in *Xenopus* embryos was also analyzed by RT-PCR using tissues dissected from developing embryos (Fig. 4C). Embryos at the neurula stage were dissected into ventral and dorsal regions (Fig. 4C). The expression of the HGF gene was higher in the ventral than the dorsal region, as previously reported (*26*). The *c-met* gene expression was detectable on both dorsal and ventral sides of neurula

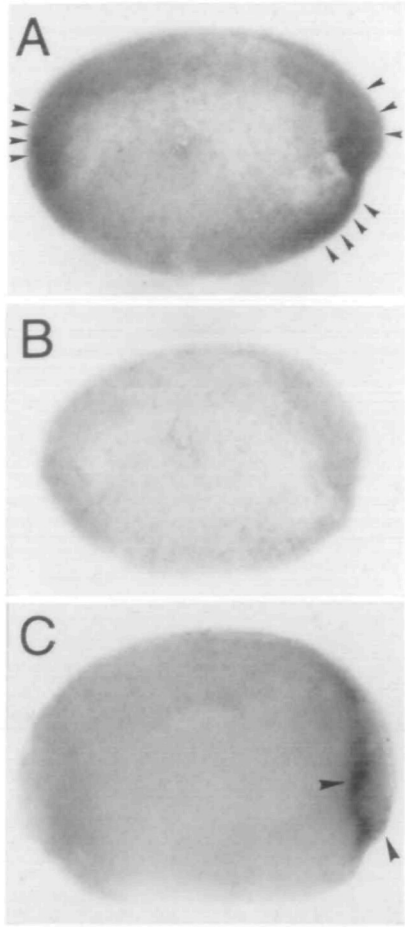

Fig. 5. **Whole mount *in situ* analysis of c-met and ron mRNAs in *Xenopus* embryos.** (A) Hybridization with the antisense c-met probe (stage 18, side view). Arrowheads indicate staining in the ventral foregut, posterior tail mesenchyme and head. (B) Hybridization with the sense *c-met* probe (stage 18, side view). (C) Hybridization with the antisense *Xenopus ron* probe (stage 17, side view). Arrowheads indicate staining in the eye placode and forebrain.

embryos, and it was slightly higher in the ventral than the dorsal region (Fig. 4C).

Localization of Gene Expression of Xenopus c-met in Neurula Embryos—In order to know the localization of c-met mRNA in *Xenopus* embryos in more detail, we carried out whole mount *in situ* hybridization analysis. The specificity of hybridization signals seen with an antisense probe of *Xenopus c-met* was examined by using as the sense probe as a negative control, and also by using a specific probe for *Xenopus ron* (Xron), which is a closely related Met family gene and is a receptor for HGF-like protein (*37*). Because of the background staining in gastrula and early neurula embryos, *Xenopus c-met* mRNA could not be detected unequivocally before the early neurula stage, but we could detect specific signals in late neurula embryos. A lateral side view of a neurula embryo (stage 18) hybridized with the anti-sense probe indicated that *c-met* mRNA was localized in an anterior-ventral region, a posterior region and anterior neural tissues (Fig. 5A). A sense probe for *Xenopus c-met* gave no signals (Fig. 5B). In contrast, ron was expressed in neural tissues, this clearly being distinct from the localization of *c-met* mRNA (Fig. 5C), and these results indicate that the probes for both *c-met* and *ron* mRNAs specifically detect the respective mRNAs without cross-hybridization.

Regions expressing the *c-met* gene in the anterior-ventral region of neurula embryos are likely to be located along the foregut, thus we suspect that the expression corresponds to the region which will develop into multiple endodermal organs, *e.g.* liver, pancreas, and gut. In the posterior tail region of neurula embryos, *c-met* mRNA is expressed in mesenchymal cells and the expression level is the highest in the posterior region. The expression of the *c-met* gene is also detectable in the dorsal region of neurula embryos (Fig. 4C). Likewise, whole mount *in situ* hybridization showed that the dorsal expression of the *c-met* gene is localized in the anterior region of prospective neural tissue (Fig. 5A). In contrast, *ron* mRNA is also detectable in the anterior region of prospective neural tissue, however, the expression in this region is restricted to the forebrain and eye placode (Fig. 5C).

DISCUSSION

Growth factors and related receptors expressed in early embryos play critical roles in the regulation of cell growth and migration, and in inductive processes. Growth factors belonging to the fibroblast growth factor family and to the transforming growth factor superfamily play key roles in the first process, *i.e.*, mesoderm induction. Later studies indicated that secreted proteins such as noggin and chordin are expressed in the organizer region, and are involved in the second inductive process, neural induction. Thus, identification of growth factors and their receptors expressed during embryogenesis, as well as disruption of functional coupling, shed light on molecular mechanisms underlying successive inductive processes and morphogenic processes needed to construct normal tissue structures. In the present study, we cloned the cDNA encoding *Xenopus c-met*, and analyzed the expression of both the HGF and Met genes during early development of *Xenopus* embryos.

The nucleotide and deduced amino acid sequences of *Xenopus* c-Met are conserved along the entire coding region, compared with the mammalian counterparts. In particular, a set of intracellular domains, *i.e.*, the juxtamembrane domain, tyrosine kinase domain and a specific region containing a multiple docking site (binding motif for SH-2 molecules), are highly conserved between *Xenopus* and mammalian Met. The marked structural conservation in intracellular domains of the Met/HGF receptor likely indicates that these domains play critical roles in transduction of the unique intracellular signals of HGF, including mitogenic, motogenic and morphogenic signals, all essential for the construction of normal multicellular structures in embryonic organogenesis, as well as for tissue regeneration. In contrast, the overall sequence homology in the extracellular region between human and *Xenopus* c-Met is relatively low compared to that in the intracellular region. However, it should be emphasized that the locations of the 30 extracellular cysteine residues of *Xenopus* c-Met are completely conserved (Figs. 2 and 3). We recently cloned *Xenopus* homologues of HGF-like protein/macrophage stimulating protein (HLP/MSP) and Ron/HLP receptor tyrosine kinase (*37*). The heterodimeric structure and each

domain structure, *i.e.*, an extracellular cysteine-rich domain, a juxtamembrane domain and a tyrosine kinase domain, are conserved in both Met and Ron. However, the positions and clustering of cysteine residues in the extracellular region, particularly the presence of two juxtamembrane cysteine residues in Ron tyrosine kinase are likely to specify different structural properties in Met family proteins (Fig. 3). Additionally, the number of extracellular cysteine residues of c-Met differs from those of Ron and c-Sea (*34, 36*). Human Ron, *Xenopus* Ron-like proteins and chick c-Sea have 38, 40, and 36 extracellular cysteine residues, respectively. Both HGF and HLP are composed of a four kringle-containing α-chain and a serine protease-like β-chain, and exhibit a 50% amino acid sequence homology, yet cross-interaction between HGF and Ron, as well as HLP and Met does not occur (*35*). Thus, the distinct positional and clustering features of extracellular cysteine residues between Met and Ron are likely to determine the tertiary structures required for the specific recognition and binding of each ligand. The conserved structures of the extracellular regions of *Xenopus* c-Met also indicate that the ligand-binding specificity of c-Met seems to be highly conserved between *Xenopus* and mammalians.

Members of the growth factor family, several growth factors among the same family, show a common cross reaction with known receptors and hence show overlapping biological activities. Two members of the met family, c-Met and Ron respectively, receive the signals of HGF and HLP/MSP, members of the HGF family, however, no cross reactions occur among these ligands and receptors, at least in cultured cells, hence Met and Ron may have distinct biological functions. In *Xenopus* embryos, c-met is expressed in multiple tissues, the ventral foregut region, mesenchyme of posterior tailbud, and anterior neural tissues. The ventral foregut expressing c-met is likely to give rise to endodermal organs, *e.g.* liver, pancreas, and gut, the posterior mesenchyme will develop into tail structure in the tadpole stage, and the anterior neural tube gives rise to segmental brain structures. Thus, c-met is likely to play important roles in multiple organogenesis in embryos. In contrast, ron is expressed in the restricted region of presumptive neuronal tissues, the eye placode and the forebrain in neurula embryos (Fig. 5C and Ref. *37*), and this expression pattern suggests the specialized biological function of Ron in neuronal development. Therefore, the biological functions of the HGF-Met and HLP-Ron systems are distinct from each other during embryogenesis.

Interaction between the epithelium and mesenchyme, *e.g.*, epithelial-mesenchymal interaction, mediates crucial aspects of normal development, affecting tissue induction, organogenesis, and morphogenesis of specific multicellular constructions (*38-40*). Studies focusing on localization of HGF and Met in the embryo suggested that these molecules are responsible for signal exchange between epithelial and mesenchymal (or stromal) tissues (*20, 41*). The expression of the HGF and c-met genes is evident during murine embryogenesis, and epithelial cells in various developing organs express Met, while neighboring mesenchymal cells express HGF (*20, 41*). In *Xenopus* embryos, both the HGF and c-Met genes are expressed during early embryonic development. The embryonic expression of the HGF gene is localized in the ventral mesoderm surrounding the endodermal epithelium (*26*). Together with the present observations that temporal and regional expression of the *Xenopus* c-met gene is significantly coupled with the expression of the HGF gene on the ventral side of the foregut region in neurula embryos, we speculate that the HGF-Met system may play a role in the endodermal organogenesis in *Xenopus* embryos. The dominant expression of the tyrosine kinase-minus Met/HGF receptor on the ventral side of *Xenopus* embryos blocks epithelial histogenesis of the liver, pronephros, and gut (manuscript in preparation). It is worth noting that certain epithelia of the liver and placenta were not formed in mice lacking the HGF or c-met gene (*21, 23*). The similarity in the loss-of-function phenotypes between *Xenopus* and mouse suggests that essential functions of HGF and Met in epithelial-mesenchymal interactions for organogenesis may be highly conserved over a wide range of species, as are their conserved molecular structures.

HGF was initially considered to be a hepatotrophic factor for liver regenerations (*1-5, 8-10*), and ongoing studies show that this factor has a vital role in the regeneraton of various organs including the liver, kidney and lung (*8, 9*), and is involved in the organogenesis of various developing organs (*21-25*). In light of our present results, the HGF and Met system may also play an important role in the development of *X. laevis*, and specific functions of the HGF-Met system seem to be conserved from amphibians to mammalians. In ongoing studies we hope to determine the biological functions of HGF and its family member, HLP, during the early development of *X. laevis*, using strategies to disrupt functional coupling between these ligands and their receptor kinases.

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