

Expression of HGF/SF, HGFI/MSP, and c-met Suggests New Functions During Early Chick Development

CLOTILDE THÉRY, MELANIE J. SHARPE, SARAH J. BATLEY, CLAUDIO D. STERN,
AND ERMANNO GHERARDI

Department of Genetics and Development, College of Physicians and Surgeons, Columbia University, New York, New York (C.T., C.D.S.); ICRF Cell Interactions Laboratory, Cambridge University Medical School, Cambridge, United Kingdom (M.J.S., S.J.B., E.G.)

ABSTRACT We report the cloning of full-length cDNAs for a plasminogen-related growth factor, hepatocyte growth factor/scatter factor (HGF/SF), its tyrosine kinase receptor, c-met, and a close member of the same family, hepatocyte growth factor-like/macrophage stimulating protein (HGFI/MSP), from the chick. We have used these cDNAs to provide the first report of the expression of this family of growth factors and the c-met receptor at early stages of vertebrate development. RNAase protection and wholemount in situ hybridization were used on chick embryos between formation of the primitive streak and early organogenesis. We find patterns of expression for HGF/SF and its receptor c-met consistent with their known roles in epithelial-mesenchymal transformation and angiogenesis. In addition, these genes and HGFI/MSP are expressed in discrete locations within developing somites, suggesting a role in paraxial mesodermal development. Very strong and early expression of HGF/SF in the elevating limb buds suggests its involvement in limb outgrowth. HGFI/MSP is expressed in the notochord and then in the prospective floor plate region and could play a role in development of the neural tube. Interestingly, c-met is often more closely associated with HGFI/MSP than with its known ligand, HGF/SF, raising the possibility that c-met expression may be induced by HGFI/MSP.

© 1995 Wiley-Liss, Inc.

Key words: Hepatocyte growth factor, scatter factor, HGF/SF, hepatocyte growth factor-like, macrophage stimulating protein, HGFI/MSP, c-met, chick embryo

Weidner *et al.*, 1991]. Unlike plasminogen, however, HGF/SF is devoid of any protease activity and has pleiotropic effects on target cells in vitro [for review, see Gherardi *et al.*, 1993]. Although this factor has been proposed as an effector of various epithelial-mesenchymal interactions occurring during organogenesis [Sonnenberg *et al.*, 1993] and as an angiogenic factor [Grant *et al.*, 1993], it may have more roles, particularly during early embryonic development.

Its closest relative and one that also lacks protease activity, is hepatocyte growth factor-like/macrophage stimulating protein (HGFI/MSP) [Degen *et al.*, 1991; Han *et al.*, 1991; Yoshimura *et al.*, 1993]. Apart from the known effects of this factor on the motility and responsiveness of macrophages in vitro [Skeel *et al.*, 1991], its roles in vivo are still poorly understood.

The receptor for HGF/SF has been identified as the transmembrane tyrosine kinase encoded by the c-met protooncogene [Bottaro *et al.*, 1991; Naldini *et al.*, 1991]. Unique extracellular domain features of the Met protein define a new subfamily of tyrosine kinase receptors comprising two other members: the chick Sea [Huff *et al.*, 1993] and the human Ron [Ronsin *et al.*, 1993] and its putative mouse homologue STK [Iwama *et al.*, 1994]. Recently, Ron has been identified as a receptor for human HGFI/MSP [Gaudino *et al.*, 1994; Wang *et al.*, 1994].

Despite a few studies on HGF/SF and c-met at relatively late stages of mammalian development [De Frances *et al.*, 1992; Sonnenberg *et al.*, 1993] and a recent Northern analysis of expression of HGF/SF in amphibian embryos [Nakamura *et al.*, 1995], we know little about the expression of these molecules at early

INTRODUCTION

Hepatocyte growth factor/scatter factor (HGF/SF) is a fibroblast-derived growth factor related to the blood serine protease plasminogen [Nakamura *et al.*, 1989;

Received for publication December 30, 1994; accepted March 8, 1995.

Address reprint requests to C.D. Stern, Dept. of Genetics and Development, College of Physicians and Surgeons, Columbia University, 701 W. 168th St., New York, NY 10032.

stages in any vertebrate embryo, and there is no information about the spatial pattern of expression of HGF1/MSP at any stage in any species. To learn more about the roles of these molecules during early embryonic development, we opted to use the chick embryo. The chick is an ideal system because of its transparency and because of the large amount of information already available concerning the events of early embryogenesis. Here, we report the cloning of full-length cDNAs of chick homologues of all three genes, HGF/SF, HGF1/MSP, and c-met, and their expression patterns during the first 4 days of chick embryonic development.

As expected, HGF/SF is found in locations consistent with its roles in angiogenesis and cell migration or dissociation. In addition, all three molecules are expressed transiently in regions of the embryo where cell interactions important for patterning the embryonic axis take place. For example, HGF/SF is first expressed in Hensen's node at the primitive streak stage. Later, it is expressed very strongly in the limb buds as soon as they start to elevate. During development of the neural tube, HGF1/MSP is expressed transiently in the notochord, and shortly afterward expression shifts to the future floor plate region. All three molecules are expressed in specific regions of the developing somites, suggesting roles in cell interactions during development of the paraxial mesoderm. Surprisingly, we often find c-met expressed in distant locations from its known ligand, HGF/SF, in closer association with cells expressing HGF1/MSP.

MATERIALS AND METHODS

RNA Isolation and Preparation of cDNA

RNA was isolated from frozen newborn chick tissues or chick embryos according to Chomczynski and Sacchi [1987]. Oligo (dT) primed first strand cDNA was prepared from 10 µg of newborn chick liver RNA in a reverse transcription reaction containing 140 mM KCl, 8 mM MgCl₂, 50 mM Tris-HCl pH8.3, and 40 units of reverse transcriptase (HT Biotechnology) for 1 hr at 42°C. One-tenth of the reaction was subsequently used in the PCR reactions described below.

PCR Cloning of a Fragment of Chick c-met

A 1.4 kb partial clone of the chick c-met was obtained using the PCR technique in two successive steps. Since in mouse and human, the liver is a major site of expression of c-met [Di Renzo *et al.*, 1991; Prat *et al.*, 1991], we used chick liver cDNA to clone fragments of the chick c-met.

Because c-met is a member of the receptor tyrosine kinase family [Park *et al.*, 1987], we first used two highly degenerate primers designed on extremely conserved regions of the tyrosine kinase genes [Wilks, 1989; Lai and Lemke, 1991]: 5' primer: 5'-CGTACAY-CGNGAYYTNGCNGCNCG-3' (VHRDLAA sequence in domain VI), 3' primer: 5'-CCGGAYNCCRWAR-

WCCANACRTC-3' (DVWSFGV sequence in domain IX).

PCR amplification of newborn chick liver cDNA was carried out for 40 cycles of 95°C (1 min), 37°C (2 min), and 63°C (3 min). The 220 bp product generated was cloned into the EcoRV site of pBluescript KS- (Stratagene). In order to distinguish c-met from other tyrosine kinases amplified by PCR, bacterial colonies containing the plasmid were transferred to Hybond-N filters (Amersham) and hybridized with a 3.9 kb probe encompassing most of the human c-met coding sequence (kindly provided by P. Comoglio, Turin, Italy). Hybridization was performed at 65°C in 0.34 M Na Phosphate pH7, 9.7 mM EDTA, 9.7% dextran sulfate, 5% SDS, 250 µg/ml salmon sperm DNA. The final wash was to stringency 0.5 × SSC at 42°C. Three clones were selected and sequenced, and one of them exhibited a strong homology with the human and mouse c-met sequences.

Two 3' primers were then designed from the sequence of the 220 bp fragment (external primer, 5'-GACTTTGTCGGTGAACCTTCTG-3'; nested primer, 5'-CACTGGCAGCTTAGCTCC-3'). A degenerate 5' primer (5'-GARATHATHGTGYAC-3') was designed based on the conserved amino acid sequence EIICCT in the extracellular domain of human and mouse c-met, 136 aminoacids upstream the transmembrane domain.

Two successive rounds of PCR (95°C 1 min, 50°C 1 min, 72°C 1 min) were performed on newborn liver cDNA, using the 5' primer and successively the two nested 3' primers. The resulting 1.35 kb product was cloned into the EcoRV site of pBluescript KS- (Stratagene). Five clones were sequenced and two of them exhibited homology with the c-met gene. The longest clone was sequenced fully on both strands after generating a set of nested exonuclease III deletions (Henikoff, 1984) from each end of the clone.

Cloning of Full-length Genes: Library Screening

Cloning of full-length chick HGF/SF. Cloning of a 1.4 kb fragment of the chick HGF/SF has been reported elsewhere [Streit *et al.*, 1995]. This fragment has been used as a probe to screen a stage 12–14 chick whole embryo cDNA library made in λZAP (kindly provided by A. Nieto and D. Wilkinson, NIMR, Mill Hill, UK). Approximately 7.5×10^5 phages were transferred to Hybond N filters. Hybridization was performed overnight at 65°C in 0.5 M NaHPO₄ pH7, 7% SDS, 1 mM EDTA; washes were performed at high stringency (final wash: 0.5 × SSC, 0.1% SDS, 65°C). Three positive clones were obtained. The longest was fully sequenced.

Cloning of full-length chick c-met. The 1.35 kb chick c-met fragment was used as a probe to screen the same chick embryo cDNA library. Six positive clones corresponding to the chick c-met were selected. Smaller subclones of the longest clone were generated and the entire coding sequence of the chick c-met was determined.

Cloning of chick HGF-like gene. An adult liver chick cDNA library made in λ gt10 (Clontech) was transferred to Hybond N filters and screened with a 3kb cDNA fragment containing the full coding sequence of mouse HGF/SF (M. J. Sharpe, unpub. results). Hybridization conditions were $6\times$ SSC, $5\times$ Denhardt's solution (0.1% Ficoll, 0.1% poly(vinyl)pyrrolidone, 0.1% BSA), 0.1% SDS, and 100 μ g/ml salmon sperm DNA. Filters were washed to a stringency of $3\times$ SSC at 65°C. Approximately 7.5×10^5 phages were screened and six positive plaques were isolated. The clone containing the longest insert was subcloned into pBluescript SK+ (Stratagene). Its full sequence was determined by sequencing a set of nested exonuclease III deletions (Henikoff, 1984) generated from each end of the clone.

DNA Sequencing and Sequence Analysis

Sequencing was performed either on an ABI 373A automated sequencer using the Taq dye dideoxy terminator method (ABI) according to manufacturer's instructions, or manually by the dideoxy method using a Sequenase II kit (USB). Sequences were assembled using Staden software and database searches performed using the program BLAST [Higgins and Sharp 1988; Karlin and Altschul 1990].

RNAase Protection Mapping

RNAase protection analysis was performed according to Krieg and Melton [1987]. Briefly, 40 μ g of total RNA from either newborn tissues or embryo stages was hybridized overnight at 45°C with 32 P-labeled RNA probes in the presence of 80% formamide. The probes, generated from templates in pBluescript, were designed to give protected fragment sizes as follows. Chick cytoskeletal beta actin as an internal loading control (E. Gherardi unpub. results): a 110 bp Eco0109I linearised PCR fragment; chick HGF/SF: a 140 bp HpaII fragment of the Pollo 23 clone [Streit *et al.*, 1995]; chick HGF-like gene: a 240 bp BglII-AvaII fragment; chick c-met: a 270 bp fragment obtained by Henikoff deletion of the 1.35kb clone. In one experiment, probes for c-met, HGF/SF, and actin were hybridized simultaneously and in the second experiment probes for HGF-like and actin were hybridized simultaneously. Digestion was then performed for 1 hr at 37°C with both RNAase A and RNAase T1. Samples were run on a 8% (HGF/SF and c-met) or a 5% (HGF-like) polyacrylamide gel.

Wholemout In Situ Hybridization

In situ hybridization on chick embryos was performed as follows [modification of Wilkinson, 1993; D. Ish-Horowicz, pers. comm.]. Briefly, chick embryos were dissected in PBS containing 2 mM EGTA, staged according to Hamburger and Hamilton [1951], and fixed overnight in 4% formaldehyde in PBS-EGTA.

They were dehydrated in methanol and kept at -20°C before processing.

The probes were obtained by transcription in the presence of DIG-UTP (Boehringer) of the 1.4 kb clone for HGF/SF, of the 1.35 kb clone for c-met, or of the full-length clone for HGF-like (2.2 kb).

Embryos were progressively rehydrated in PBS-0.1% Tween, treated with proteinase K (10 μ g/ml at room temperature for a number of minutes approximately equal to their H&H stage), postfixed in 4% formaldehyde 0.1% glutaraldehyde, and washed in PBS-Tween before prehybridization. Hybridization was carried out at 70°C overnight, in 50% formamide, $1.3\times$ SSC pH4.5, 100 μ g/ml heparin, 50 μ g/ml yeast tRNA, 5 mM EDTA, 0.2% Tween-20, 0.5% CHAPS, with a probe concentration of 0.1–0.5 μ g/ml. Washes were performed in the hybridization solution at 70°C. Antibody incubation (1:3,000, overnight 4°C) and washes were performed in TBS containing 1% Tween. The alkaline phosphatase reaction was carried out in the presence of NBT and BCIP for 3–5 hr at room temperature and if necessary overnight at 4°C. Embryos were refixed overnight in 4% formaldehyde in PBS before being photographed.

RESULTS

Chick HGF/SF and HGF-like Genes

The predicted protein sequences of chick HGF/SF and HGF1/MSP and comparisons with their mammalian counterparts are shown in Figure 1 and Table 1. Chick HGF/SF and HGF1/MSP have all the features described for the mammalian homologs: (1) an N-terminal domain containing four cysteine residues forming a hairpin loop, (2) four kringle domains, with additional conserved cysteine residues in both kringles 2 and 3, and (3) the presence of an inactive serine protease domain.

Conversely, the following features distinguish chick (as well as mouse and human) HGF/SF from HGF1/MSP: (1) the length of the inner loop in the N-terminal domain, (2) the length of the linker peptides between kringles 2 and 3 and between kringles 3 and 4, (3) the length of the activation domain, and (4) the number of cysteine residues in the serine protease domain.

Chick c-met Gene

Figure 2 shows an alignment of the protein sequences of the chick, mouse and human c-met receptors and the chick c-sea receptor. Sequence identities between these proteins are given in Table 1. A putative leader peptide of 24 residues in length is conserved in chick c-met as well as a furin cleavage site in the extracellular domain (Fig. 2) suggesting that the mature form of chick c-met is a heterodimer of two disulfide-linked subunits as are human c-met (Giordano *et al.*, 1989) and Ron (Gaudino *et al.*, 1994; Wang *et al.*, 1994).

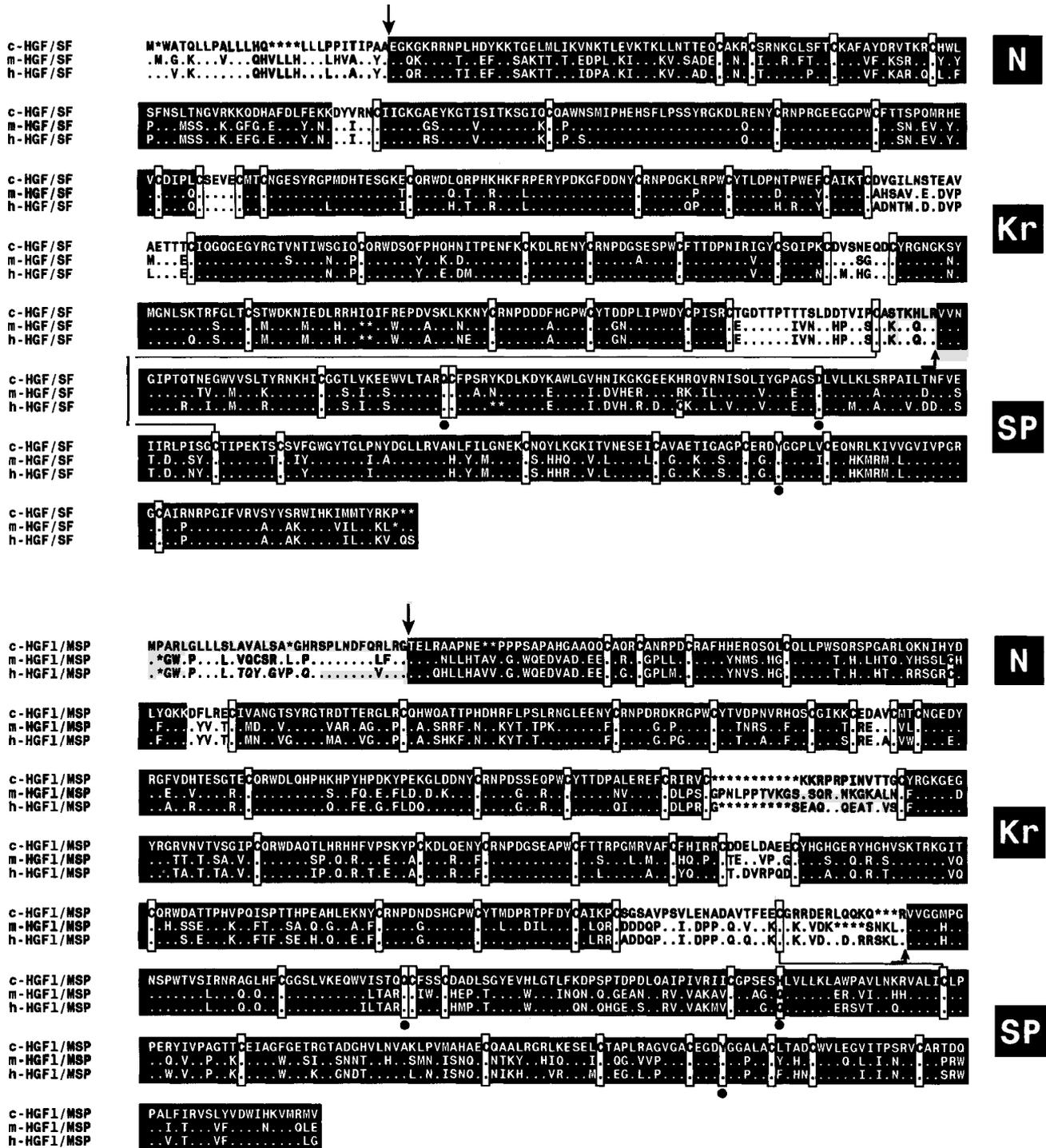


Fig. 1. Alignment of the protein sequences of chick, mouse, and human HGF/SF (top) and chick, mouse, and human HGF1/MSP (bottom). The amino-terminal domain (N), four kringle domains (Kr), and the serine protease domain (SP) are shown as white characters on black background. Cysteine residues are shown as white boxes. The predicted cleavage sites for the signal peptide and between the A and B chains are shown with arrows above and below the sequences respectively. The black line illustrates the single, predicted disulfide bond between the A and B chains. The residues corresponding to the catalytic triad of functional serine protease domains are identified with a black circle below the sequence. Dots indicate residues in the mouse and human sequences identical to those of the chick sequence

(top lines). Asterisks are padding characters inserted for optimal alignment of the three sequences [Higgins and Sharp, 1988]. The sequences used for alignment are the following: chick HGF/SF (EMBL X84045) and HGF1/MSP (EMBL X84043), mouse HGF/SF (translated from EMBL accession MMHGF1), human HGF/SF (SwissProt accession HGF_HUMAN), mouse HGF1/MSP (SwissProt accession HGFL_MOUSE) and human HGF1/MSP (SwissProt HGFL_HUMAN). A second clone of chick HGF/SF (not shown) carries a 5-residue deletion in kringle 1 (aminoacids FLPS) as previously observed in human [Rubin *et al.*, 1991; Seki *et al.*, 1991; Weidner *et al.*, 1991] and mouse [Sasaki *et al.*, 1994] HGF/SF.

TABLE 1. Percent Sequence Identity Between Chick Genes and Mammalian Counterparts

	Chick HGF/SF	Chick HGF1/MSP
Chick HGF/SF		41
Mouse HGF/SF	74	
Human HGF/SF	74	
Mouse HGF1/MSP		58
Human HGF1/MSP		62
		Chick c-met ^a
	Total	TK
Mouse c-met	72	94
Human c-met	72	94
Chick c-sea	35	75

^ac-met identities in the whole molecule (total) and in the tyrosine kinase domain (TK) are shown.

Expression of Growth Factors and Receptor During Embryonic Development

The time course and pattern of expression of the two related growth factors, HGF/SF and HGF1/MSP, and of the HGF/SF receptor, c-met, were analyzed during chick development by in situ hybridization and RNAase protection. The results are shown in Figures 3 (RNAase protection) and 4–6 (in situ hybridization).

Early Development: Gastrulation to neural plate formation. RNAase protection performed on whole embryos showed no expression of HGF/SF from gastrulation up to the 7-somite stage (stage 9) (Fig. 3). However, in situ hybridization reveals that at stages 3 to 4+, HGF/SF transcripts are present and confined to Hensen's node [Streit *et al.*, 1995]. After stage 5, no expression is detected until stage 10–11. c-met was detected from gastrulation onward by RNAase protection, although at a low level (Fig. 3 shows the result of an experiment performed with 40 µg of RNA and exposed for 8 days). It was not possible to visualize any c-met transcripts by in situ hybridization until stage 7 (see below). Unlike HGF/SF and c-met, HGF1/MSP is expressed at a fairly high level from stage 3 onward (Fig. 3). By in situ hybridization, the entire area pellucida is seen to express HGF1/MSP at stage 4 (Fig. 4A), and this pattern persists up to stage 7.

After the start of neural plate formation, expression of all three genes becomes stronger and confined to a few sites. The salient features are considered below.

Expression in axial structures: Neural tube and notochord. The only one of the three genes expressed in the axial structures is HGF1/MSP, until regional differentiation of the spinal cord begins. From stage 8, the forming neural tube strongly expresses HGF1/MSP (Fig. 4B). As closure of the neural tube proceeds, by stages 10–11, expression disappears from the more anterior part (Fig. 4C), but remains in the more posterior part of the neural tube, which is still open.

At stage 9, the notochord begins to show expression of HGF1/MSP (Fig. 4C). However, this excludes the most anterior (head process) and the most posterior regions and is stronger in the region where the somites

have formed. At stage 11, expression in the notochord starts around the middle of the segmental plate and often ends anteriorly at the level of the fourth youngest somite, but there is some variability in this anterior limit. From stage 10, at levels anterior to the fourth youngest somite, the floor plate region expresses the mRNA, and this is maintained up to stage 19–21 (Fig. 4D, E), after which it disappears. Interestingly, the floor plate of the hindbrain never seems to express HGF1/MSP.

As the nervous system develops further, c-met starts to be expressed there (Fig. 5D). A low level of transcripts is seen in the spinal cord, including the lateral motor columns at the brachial level at stage 23 [see also Sonnenberg *et al.*, 1993], and in components of the developing peripheral nervous system: dorsal root ganglia and motor and sensory roots.

Expression in the somites. Striking patterns of HGF/SF, c-met, and HGF1/MSP expression can be seen at different stages of somite development. At stage 7, c-met appears in the first forming somite (Fig. 5A); in some embryos at stages 7–8, this expression covers the first three or four somites. After this localised and transient expression, c-met is not detectable by in situ hybridization until stage 11. At that time, a narrow band of expression appears at the dorsomedial edge of each somite (Fig. 5B). This band, in transverse sections, corresponds to the dorsal edge of the dermomyotome (green arrows in Fig. 5D), but is seen even in somites that are still epithelial, including the most recently formed one (Fig. 5B). This pattern persists up to the oldest stages studied (stage 23). From stage 11 onward, we could also see weak expression of HGF/SF in the sclerotome. Finally, HGF1/MSP becomes very strongly expressed in the myotome of stage 19 somites, and remains highly expressed up to the latest stage analysed (stage 21–23) (Fig. 4E–G).

Expression in limb buds. As soon as they form, at stage 16, the limb buds strongly express HGF/SF (arrows in Fig. 6B). Strikingly, the expression of HGF/SF at stages 13–15 is a predictor of the future position of the limb buds, even before these become visible (Fig. 6A, arrows). Expression in the limbs is maintained to the last stage examined, stage 23 (Fig. 6C), where transverse sections show that it is confined to the limb bud mesenchyme, the ectoderm being devoid of expression.

Stage 23 embryos show weaker expression of c-met (Fig. 5D) and HGF1/MSP in the limb mesenchyme.

Expression in the branchial arches. All three genes are expressed in the branchial arches (stage 23), but in different structures within them. HGF/SF is first expressed in the pharyngeal endoderm at stage 19; later, at stage 21, it is expressed in the ectoderm of the 1st and 6th branchial arches (not shown), and the mesenchyme of the 3rd and 4th (Fig. 6C,D). Interestingly, earlier during development (stage 11), the cranial neural crest cells, some of which will colonize the branchial

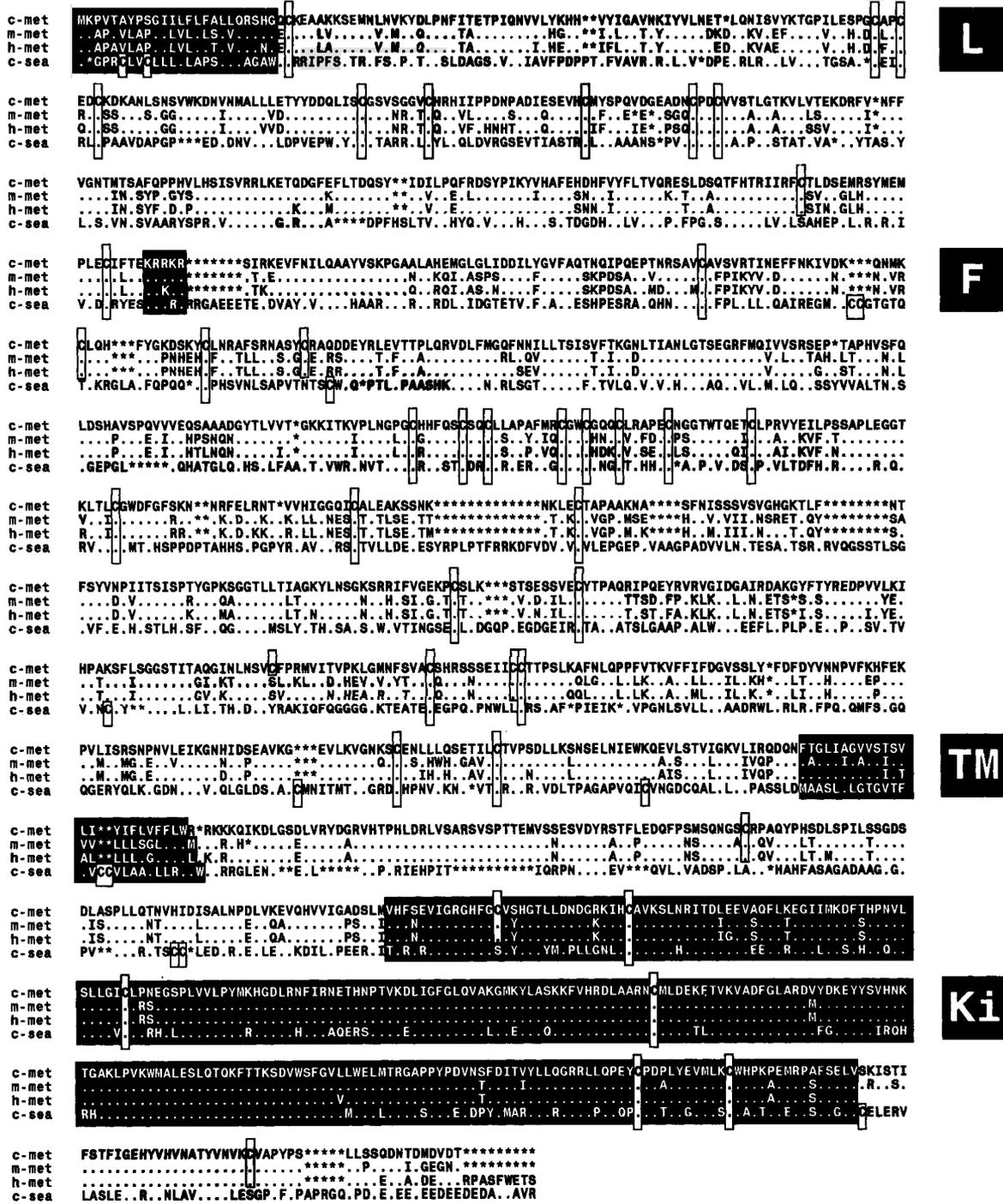


Fig. 2. Alignment of the protein sequences of the chick, mouse, and human c-met receptor and the chick c-sea receptor. The putative signal peptide (L), furin cleavage site (F), transmembrane domain (TM), and kinase domain (Ki) are shown as white characters on black background. Cysteine residues are shown as white boxes and asterisks as in Figure 1. The sequences used for alignment are the following: chick c-met (EMBL accession X84044), mouse c-met (SwissProt accession KMET_MOUSE), human c-met (SwissProt KMET_HUMAN), and chick c-sea (translated from EMBL GGCSEAX).

isks as in Figure 1. The sequences used for alignment are the following: chick c-met (EMBL accession X84044), mouse c-met (SwissProt accession KMET_MOUSE), human c-met (SwissProt KMET_HUMAN), and chick c-sea (translated from EMBL GGCSEAX).

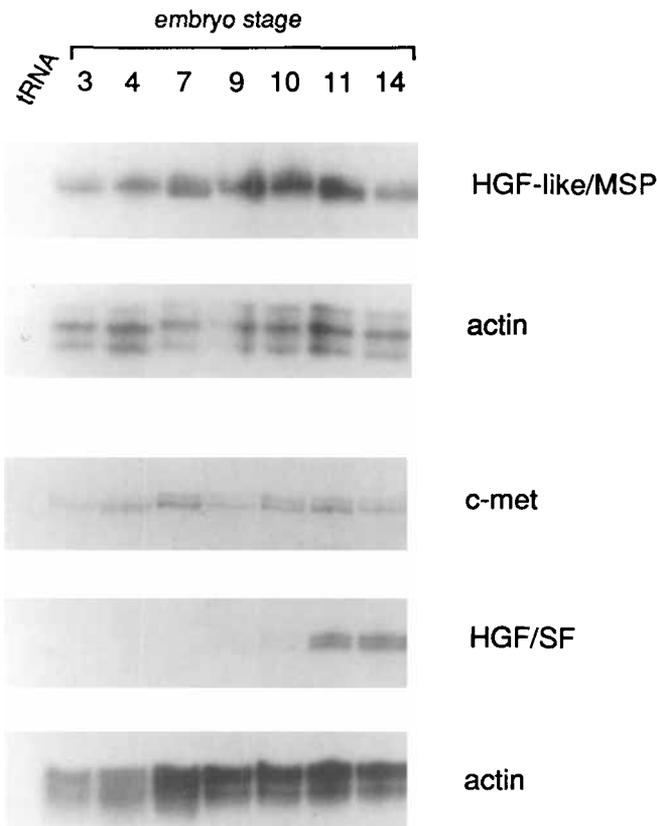


Fig. 3. Time-course of HGF1/MSP, HGF/SF, and c-met expression studied by RNAse protection. Forty micrograms of total RNA from chick embryos at stages 3–14 [Hamburger and Hamilton, 1951] were hybridized with a mixture of HGF1/MSP and actin or HGF/SF, c-met, and actin probes. Actin is used as loading control.

arches, express HGF/SF (Fig. 6E). By contrast, trunk neural crest cells do not express the gene. Simultaneously, c-met is expressed in the endoderm of all the branchial pouches, as well as in the endothelial lining of the aortic arches (Fig. 5C). HGF1/MSP is detectable in the aortic arches (Fig. 4F) and in the endoderm of the branchial pouches.

Other sites of expression. From stage 12, some expression of HGF/SF is seen in the intermediate mesoderm (which will give rise to the nephric system). The lateral plate mesoderm of stage 23 embryos also expresses c-met.

We also detected HGF/SF and c-met in the circulatory system. HGF/SF is expressed in loose endocardial mesenchymal cells, including the region of the developing endocardial cushions as early as stage 11 (Fig. 6F), as well as in extraembryonic hemopoietic blood islands (Fig. 6G). The receptor, c-met, is expressed in older embryos in most of the arterial blood vessels, including branchial aortic arches (Fig. 5C), intersomitic arteries (Fig. 5E), and major vessels near the heart.

Finally, in the oldest embryos (stage 23), the boundaries between all rhombomeres of the hindbrain ex-

press HGF/SF, c-met and HGF1/MSP (Fig. 6H). However, no such expression is seen at the boundaries between adjacent neuromeres in the diencephalon or elsewhere in the brain.

DISCUSSION

The purpose of this study was to compare the patterns of expression of a plasminogen-related growth factor, HGF/SF, reported to be an effector of epithelial-mesenchymal interactions, and its receptor, c-met, at early stages of development in the chick embryo. Since preliminary observations had shown some discrepancies in the sites of expression of the ligand and its receptor, we also analyzed the expression of another related growth factor, HGF1/MSP, whose functions *in vivo* are still unknown. Our observations suggest that these molecules may be involved in multiple cell-cell interactions during early organogenesis and patterning of the embryonic axis. In the following discussion, we consider some of the possible processes in which the gene products could play a role.

Chick Homologs of HGF/SF, HGF1/MSP, and c-met

Analysis of the protein translation of the chick clones suggest that they encode functional proteins. One clone—the cDNA encoding chick HGF/SF—has been expressed and found to be biologically active in the sensitive scattering assay on MDCK cells (M. J. Sharpe, unpub. results).

Chick HGF1/MSP shows higher sequence divergence from the mammalian homologs (58–62%) than does HGF/SF (73%), as expected from previous comparisons of the mouse and human clones. Thus, although the GC content and intron size of the HGF/SF [Seki *et al.*, 1991] and HGF1/MSP genes [Han *et al.*, 1991] suggest that the latter is more closely related to the ancestral precursor gene, it seems likely that the HGF1/MSP has undergone a greater evolutionary drift.

The structural features shared by HGF/SF and HGF1/MSP define a new subfamily of plasminogen-related growth factors. The existence of these two proteins in chick was expected, since sequence comparison of the serine protease domains of the proteins containing also one or more kringle domains suggests that the genes for HGF/SF and HGF1/MSP have diverged in excess of 500 million years ago [Donate *et al.*, 1994], well before the separation of birds from the other vertebrate classes. HGF/SF [Nakamura *et al.*, 1995] as well as another related growth factor (A. Ruiz i Altaba, pers. comm.) have been found in *Xenopus*, suggesting that this subfamily exists in all vertebrates.

HGF/SF in Cell Migration, Cell Dissociation, and Angiogenesis

HGF/SF has several interesting properties on target epithelial cells: dissociation and migration [Stoker *et*

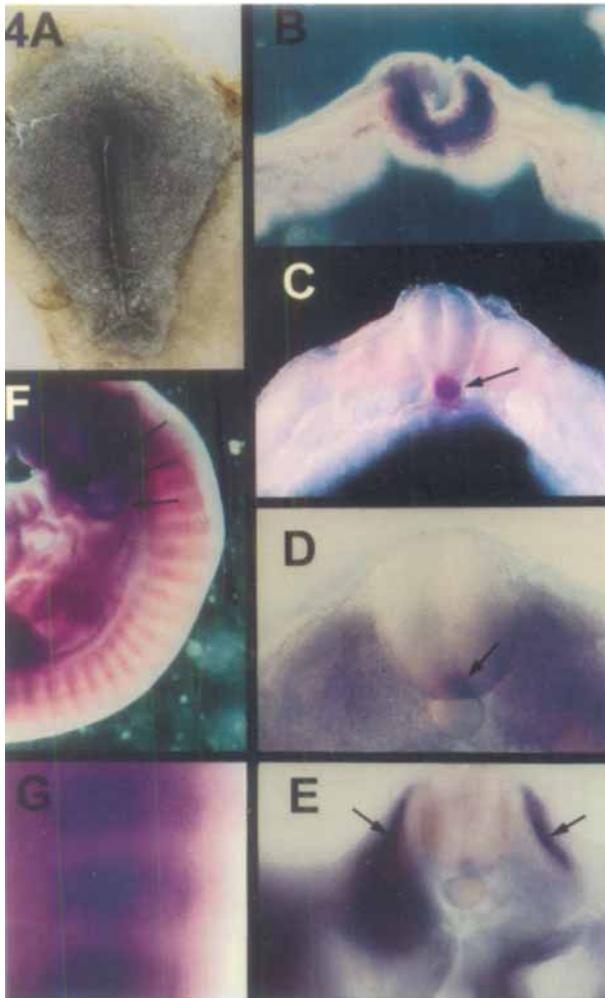
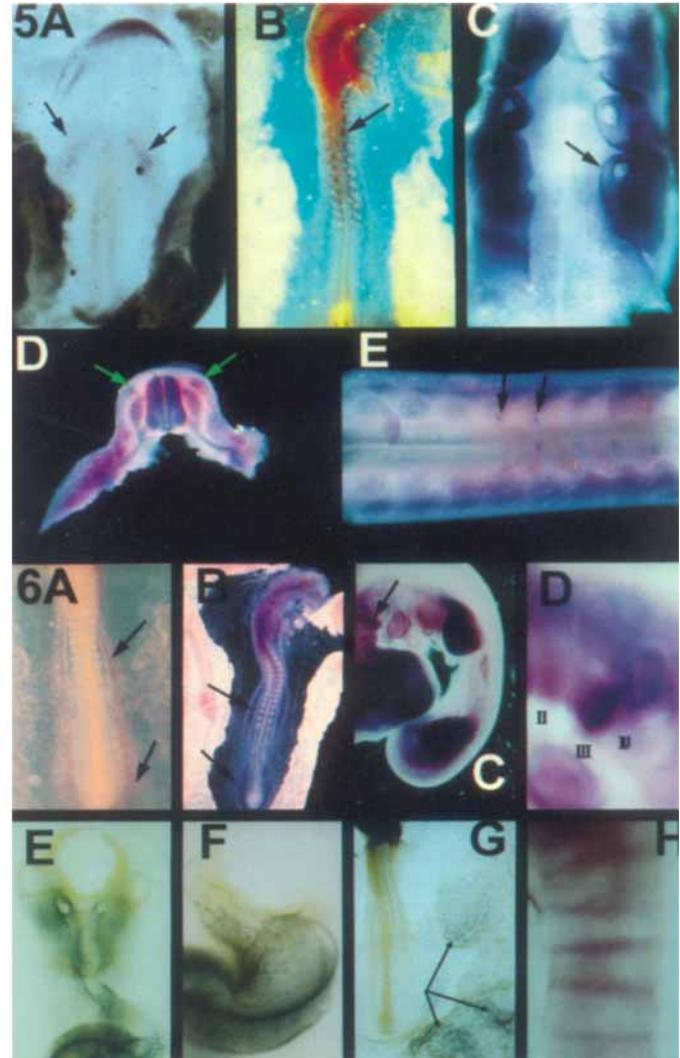


Fig. 4. Expression of HGF1/MSP visualized by wholemount in situ hybridization. **A.** Stage 4 embryo showing overall expression of HGF1/MSP in the area pellucida. **B.** Transverse section of a stage 9 embryo, showing expression in the closing neural tube and in the notochord. **C.** Transverse section of a stage 11 embryo: the closed neural tube is now negative, whereas the notochord (arrow) is strongly positive. **D.** Transverse section of a stage 19 embryo, showing expression in the floor plate (arrow); the notochord underlying it is negative. The floor plate expression persists up to stage 23 (**E**). **E.** Transverse section of stage 23 embryo, showing strong expression in the myotomal portion of the somite (arrows). This expression in the myotome is also seen in wholemounts of embryos at this stage viewed at low (**F**) and high (**G**) magnification. Strong expression is also associated with the aortic arches (**F**, arrows).

Fig. 5. Expression of c-met visualized by wholemount in situ hybridization. **A.** Stage 7 embryo showing c-met expression in the first somite (arrows). **B.** Stage 11 embryo; note the very restricted expression of c-met at the dorso-medial edge of each somite (arrow). **C,D,E.** Stage 23: thick coronal section through the pharyngeal region, viewed from its ventral aspect, showing stronger expression in the endoderm (arrow) and endothelial lining of the aortic arches (**C**); transverse section at the level of the forelimb bud, showing c-met expression in



the dorsomedial edge of the dermomyotome (green arrows), the dorsal root ganglia, the spinal cord, peripheral nerves, and limb bud mesenchyme (**D**); thick coronal section through the trunk viewed from the ventral side, showing c-met expression in the intersomitic blood vessels; two of these are highlighted by arrows (**E**).

Fig. 6. Expression of HGF/SF visualized by wholemount in situ hybridization. **A,B,C:** Expression in the developing limb bud: HGF/SF transcripts are first seen in the region where the limb buds will appear, as early as stage 13 (arrows) (**A**); by stage 15, this expression has become both stronger and more localized (arrows) (**B**), and persists up to stages 21–23 (**C**). At this stage, HGF/SF is also expressed in the branchial arches (arrow). A close-up of this region is shown in **D**, where the mesenchymal expression in arches III and IV can be seen. **E.** Expression of HGF/SF in cranial neural crest at stage 11. **F.** Expression in the loose mesenchyme of the heart, including prospective endocardial cushions, at stage 11. **G.** At stage 12, transcripts are seen in the developing blood islands in the area opaca (arrows). **H.** Thick sagittal section along the hindbrain showing HGF/SF expression localized to the boundaries between rhombomeres (dorsal to the right, anterior to the top). HGF1/MSP, and c-met show the same pattern of expression in this region (not shown).

al., 1987], growth [Miyazawa *et al.*, 1989; Nakamura *et al.*, 1989], angiogenesis [Bussolino *et al.*, 1992; Grant *et al.*, 1993], and kidney tubule formation [Montesano *et al.*, 1991; Santos *et al.*, 1994].

During organogenesis, HGF/SF and c-met are often expressed in adjacent epithelial (c-met) and mesenchymal (HGF/SF) cells [Sonnenberg *et al.*, 1993]. At earlier stages of development, we have observed expression of HGF/SF during stages at which epithelial-mesenchymal transitions and extensive cell migration take place, such as cranial neural crest and endocardial cushions. We also find strong expression in the extraembryonic blood islands, where blood vessel formation is occurring. However, unlike the later stages (with the sole exception of the endothelial lining of the aortic arches), the c-met receptor could not be detected either in the same, or in adjacent cells.

HGF/SF is thought to be sequestered *in vivo* in the extracellular matrix [Naldini *et al.*, 1991], where it is proteolytically processed to form the biologically active peptide. Its production by migrating cells could therefore result in the presence of active factor at a distance from the producing cell, and possibly closer to target cells expressing the receptor. This could account for the apparent discrepancy in the sites of expression of the ligand and its receptor.

Multiple interactions between related growth factors and receptors have been observed in other families, e.g., FGFs and their receptors [Dionne *et al.*, 1990; Keegan *et al.*, 1991; Partanen *et al.*, 1991]. Thus, another hypothesis would be that a ligand for c-met other than HGF/SF exists *in vivo*. We therefore analyzed the expression of HGF1/MSP. The results obtained suggest that, in addition to the roles of HGF/SF in epithelial morphogenesis and angiogenesis, this factor, as well as HGF1/MSP and the c-met receptor, fulfill other roles during development.

HGF/SF, HGF1/MSP, and c-met During Neural Induction

As we have shown previously, HGF/SF transcripts are present in a very restricted area of the stage 3-4 + embryo: Hensen's node [Streit *et al.*, 1995]. Expression at these stages is both very localized and at a low level, which accounts for our inability to detect HGF/SF by RNAase protection from whole stage 3 embryos. In contrast, c-met expression is seen between stages 3 and 5 at a low level by RNAase protection, but not at all by *in situ* hybridization. This suggests that c-met is probably expressed in a large region of the embryo, but at a level below the sensitivity of the *in situ* technique. Altogether, our results show that c-met and HGF/SF are expressed simultaneously, consistent with the hypothesis that HGF/SF plays a role during the early steps of neural induction [Stern *et al.*, 1990; Stern and Ireland 1993; Streit *et al.*, 1995].

The L5 epitope [Streit *et al.*, 1990] has been sug-

gested to be a marker for cells that are competent to respond to neural induction [Roberts *et al.*, 1991; Streit *et al.*, 1995]. Before stage 5, the epitope is expressed in a broad region of the embryo, including areas that lie quite distant from Hensen's node, in a pattern reminiscent of that of HGF1/MSP mRNA. This raises the possibility that HGF1/MSP is responsible for the initial expression of L5 and that this is maintained and enhanced by HGF/SF as L5 expression gradually becomes restricted to the prospective neural plate region between stages 3 and 5 [Streit *et al.*, 1995].

HGF1/MSP and Neural Tube Development

During closure of the neural tube, which progresses in approximately rostral-to-caudal sequence between stages 8-11, the ventral midline of the neuraxis undergoes some important changes. The axial mesoderm (notochord) is responsible for induction of the floor plate from the adjacent median hinge point cells, or notoplate [Van Straaten *et al.*, 1985; Jessell *et al.*, 1989; Schoenwolf and Smith, 1990; Van Straaten and Hekking, 1991; Yamada *et al.*, 1991]. This interaction takes place in caudal regions of the neural tube, while this is still open [Yamada *et al.*, 1991]. The expression of HGF1/MSP correlates closely with this process. It is expressed in a short portion of the notochord that underlies the closing neural plate. In more anterior (older) regions, expression appears instead in the floor plate itself. Interestingly, in those regions of the neural tube where the floor plate does not express HGF1/MSP (e.g., the hindbrain), the notochord/head process also never expresses.

The floor plate is also known to attract commissural axons, a candidate effector of this process being the recently described netrins [Kennedy *et al.*, 1994]. Netrin-1 is expressed, like HGF1/MSP, in the notochord, the floor plate, and in boundaries between adjacent rhombomeres in the hindbrain [Kennedy *et al.*, 1994]. A possible hypothesis is therefore that HGF1/MSP also might be involved in axon guidance. If so, it is also conceivable that it may be involved in the guidance of motor axons to the myotome, which strongly expresses HGF1/MSP, as well as to more distant targets such as the limb bud.

HGF/SF in Limb Bud Development

One of the strongest sites of HGF/SF expression is the developing limb bud, an observation consistent with a recent report by Myokai *et al.* [1995]. In addition, we show that this localization begins even before the limb buds become morphologically recognizable in the flank. These patterns of expression are more consistent with a role in initiation of limb bud outgrowth than in polarizing activity, as previously hypothesized by Yonei *et al.* [1993] and Myokai *et al.* [1995].

HGF/SF, HGF1/MSP, and c-met in Somite Development

Between the second and fourth day of development, the paraxial mesoderm undergoes some dramatic changes [for review, see Keynes and Stern, 1988; Tam and Trainor, 1994]. First, the loosely arranged mesenchyme of the segmental plate becomes condensed into epithelial spheres (the somites) in rostral-to-caudal sequence. The ventromedial portion of the somites dissociates again into a mesenchyme to become the sclerotome and the dorsolateral part (the dermomyotome) remains epithelial. Later still, the dorsomedial edge of the dermomyotome involutes to give rise to the myotome. The initial formation of somites from the segmental plate appears to be cell autonomous, but subsequent changes require interactions with neighboring tissues including the neural tube, notochord, surface ectoderm, and endoderm [Keynes and Stern, 1988; Tam and Trainor, 1994].

Our experiments show that each of these processes is accompanied by localized and specific expression of each of the three genes: HGF/SF is expressed at a low level in the forming sclerotome, c-met in the region of the dermomyotome from which the myotome starts to be generated, and HGF1/MSP in the whole of the differentiating myotome. Expression of HGF/SF can therefore be correlated with the process of dissociation of the ventral somite to form the sclerotome. However, its known receptor, c-met, does not appear to be expressed in these cells, but rather in the adjacent dorsomedial edge of the dermomyotome. This raises the possibility that the sclerotome induces the de-epithelialization of this region of the dermomyotome, allowing the formation of the myotome. This now has to be investigated by experimental embryological methods.

If correct, however, this proposal requires a mechanism to induce the localized expression of c-met in the dorsomedial edge of the dermomyotome. Although HGF/SF has been shown to enhance the expression of c-met [Boccaccio *et al.*, 1994], its expression in the whole of the sclerotome does not correlate with this role. HGF1/MSP, however, is present in the neural tube at this time, which is immediately adjacent to the c-met expressing region. This raises the possibility that c-met expression may be induced by HGF1/MSP.

Interactions Between HGF1/MSP and c-met?

Although HGF/SF has been shown to be a ligand for c-met [Bottaro *et al.*, 1991; Naldini *et al.*, 1991], we find here that the distribution of the c-met receptor is often more closely correlated with HGF1/MSP than with HGF/SF. However, comparison of the biological effects of the two growth factors on various cell lines have failed to reveal any cross biological activity (K. Lane and E. Gherardi, unpub. obs.). In addition, *in vitro* studies have shown no binding of HGF/SF to Ron (the HGF1/MSP receptor) [Wang *et al.*, 1994], and no acti-

vation of Ron by HGF/SF [Gaudino *et al.*, 1994]. The hypothesis that HGF1/MSP may be a ligand for c-met seems therefore improbable.

Another possible explanation of the correlated expressions of HGF1/MSP and c-met is that the growth factor induces, or enhances, the expression of the receptor. The c-met transcript has a very short half-life [Moghul *et al.*, 1994], and several cytokines and growth factors, including HGF/SF itself, have been shown to increase its abundance in human cell lines [Boccaccio *et al.*, 1994; Moghul *et al.*, 1994]; HGF1/MSP could therefore have the same effect. However, this would mean that before expressing c-met, cells have to express a receptor for HGF1/MSP.

No extensive study has been performed yet to clarify this issue, but in human, the tissue distribution of such a receptor, Ron, is reminiscent of that of c-met [Gaudino *et al.*, 1994], and in one cell line expressing c-met (A549), Ron is also expressed [Boccaccio *et al.*, 1994; Gaudino *et al.*, 1994]. In chick, c-sea is until now the only other known member of the c-met family. Although it does not seem to be the chick homolog of Ron [Ronsin *et al.*, 1993], c-sea is another candidate as a receptor for HGF1/MSP. However, by *in situ* hybridization with a c-sea probe (kindly provided by Dr. J. T. Parsons), we could not reveal expression patterns consistent with this hypothesis (unpub. obs.). It will therefore be interesting in the future to investigate the existence of a chick homolog of Ron and to analyse its expression pattern in the context of the expression patterns of the molecules described here.

ACKNOWLEDGMENTS

We thank Drs. J. Huff and J. T. Parsons for providing the c-sea plasmid, Drs. A. Nieto and D. Wilkinson for the chick embryo library, U. Beauchamp and V. Miljkovic for help with sequencing, Drs. S. Aparicio and A. Streit for helpful discussions, Dr. A. Ruiz i Altaba for sharing results before publication and critical reading of the manuscript, and Dr. D. Ish-Horowicz for sharing his unpublished new protocol for *in situ* hybridization. This work was supported in part by grants from the EEC Human Capital and Mobility Programme, the Wellcome Trust, the Medical Research Council, funds from Columbia University, and the Muscular Dystrophy Association.

REFERENCES

- Boccaccio C, Gaudino G, Gambarotta G, Galimi F, Comoglio PM (1994): Hepatocyte growth factor (HGF) receptor expression is inducible and is part of the delayed-early response to HGF. *J Biol Chem* 269:12846-12851.
- Bottaro DP, Rubin JS, Faletto DL, Chan AM-L, Kmieciak TE, Vande Woude GF, Aaronson SA (1991): Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* 251:802-804.
- Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffer A, Comoglio PM (1992): Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol* 119:629-641.

- Chomczynski P, Sacchi N (1987): Single-step method of RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
- De Frances MC, Wolf HK, Michalopoulos GK, Zarnegar R (1992): The presence of hepatocyte growth factor in the developing rat. *Development* 116:387–395.
- Degen SJF, Stuart LA, Han S, Jamison CS (1991): Characterization of the mouse cDNA and gene coding for a hepatocyte growth factor-like protein: Expression during development. *Biochemistry* 30:9781–9791.
- Di Renzo MF, Narsimhan RP, Olivero M, Bretti S, Giordano S, Medico E, Gaglia P, Zara P, Comoglio PM (1991): Expression of the Met/HGF receptor in normal and neoplastic human tissues. *Oncogene* 6:1997–2003.
- Dionne CA, Crumley G, Bellot F, Kaplow JM, Searfoss G, Ruta M, Burgess WH, Jay M, Schlessinger J (1990): Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. *EMBO J* 9:2685–2692.
- Donate L, Gherardi E, Srinivasan N, Sowdhamini R, Aparicio S, Blundell T (1994): Molecular evolution and domain structure of plasminogen-related growth factors (HGF/SF and HGF/MSP). *Protein Sci* 3:2378–2394.
- Gaudino G, Follenzi A, Naldini L, Collesi C, Santoro M, Gallo KA, Godowski PJ, Comoglio PM (1994): Ron is a heterodimeric tyrosine kinase receptor activated by the HGF homologue MSP. *EMBO J* 13:3524–3532.
- Gherardi E, Sharpe M, Lane K (1993): Properties and structure-function relationship of HGF-SF. In Goldberg ID, Rosen EM (ed): "Hepatocyte Growth Factor-Scatter Factor (HGF-SF) and the c-Met Receptor." Basel, Switzerland: Birkhäuser Verlag, pp. 31–48.
- Giordano S, Ponzetto C, Di Renzo MF, Cooper CS, Comoglio PM (1989): Tyrosine kinase receptor indistinguishable from the c-met protein. *Nature* 339:155–156.
- Grant DS, Kleinman HK, Goldberg ID, Bhargava MM, Nickoloff BJ, Kinsella JL, Polverini P, Rosen EM (1993): Scatter factor induces blood vessel formation in vivo. *Proc Natl Acad Sci USA* 90:1937–1941.
- Hamburger V, Hamilton H (1951): A series of normal stages in the development of the chick embryo. *J Morphol* 88:49–92.
- Han S, Stuart LA, Degen SJF (1991): Characterization of the DNF15S2 locus on human chromosome 3: identification of a gene coding for four kringle domains with homology to hepatocyte growth factor. *Biochemistry* 30:9768–9780.
- Henikoff S (1984): Ordered deletion for DNA sequencing and in vitro mutagenesis by polymerase extension and endonuclease III gapping of circular templates. *Nucl Ac Res* 18:2961–2966.
- Higgins DG, Sharp PM (1988): CLUSTAL: A package for performing multiple sequence alignments on a microcomputer. *Gene* 73:237–244.
- Huff J, Jelinek MA, Borgman CA, Lansing TJ, Parsons JT (1993): The protooncogene c-sea encodes a transmembrane receptor protein-tyrosine kinase related to the Met/hepatocyte growth factor/scatter factor receptor. *Proc Natl Acad Sci USA* 90:6140–6144.
- Iwama A, Okano K, Sudo T, Matsudo Y, Suda T (1994): Molecular cloning of a novel receptor tyrosine kinase gene, STK, derived from enriched hematopoietic stem cells. *Blood* 83:3160–3169.
- Jessell TM, Bovolenta P, Placzek M, Tessier-Lavigne M, Dodd J (1989): Polarity and patterning in the neural tube: The origin and function of the floor plate of the neural tube. Cellular basis of morphogenesis, CIBA symposium 144:255–280.
- Karlin S, Altschul SF (1990): Methods for assessing the statistical significance of molecular sequence features by using general scoring schemes. *Proc Natl Acad Sci USA* 87:2264–2268.
- Keegan K, Johnson DE, Williams LT, Hayman MJ (1991): Isolation of an additional member of the fibroblast growth factor receptor family, FGFR-3. *Proc Natl Acad Sci USA* 88:1095–1099.
- Kennedy TE, Serafini T, De la Torre JR, Tessier-Lavigne M (1994): Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* 78:425–435.
- Keynes RJ, Stern CD (1988): Mechanisms of vertebrate segmentation. *Development* 103:413–429.
- Krieg PA, Melton DA (1987): In vitro RNA synthesis with SP6 polymerase. *Methods in Enzymology* 155:397–415.
- Lai C, Lemke G (1991): An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. *Neuron* 6:691–704.
- Miyazawa K, Tsubouchi H, Naka D, Takahashi K, Okigaki M, Arakaki N, Nakayama H, Hiron S, Sakiyama O, Takahashi K, Gohda E, Daikuhara Y, Kitamura N (1989): Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. *Biochem Biophys Res Commun* 163:967–973.
- Moghul A, Lin L, Beedle A, Kanbour-Shakir A, DeFrances MC, Liu Y, Zarnegar R (1994): Modulation of c-MET protooncogene (HGF receptor) mRNA abundance by cytokines and hormones: Evidence for rapid decay of the 8 kb c-MET transcript. *Oncogene* 9:2045–2052.
- Montesano R, Matsumoto K, Nakamura T, Orci L (1991): Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 67:901–908.
- Myokai F, Washio N, Asahara Y, Yamai T, Tanda N, Ishikawa T, Aoki S, Kurihara H, Murayama Y, Saito T, Matsumoto K, Nakamura T, Noji S, Nohno T (1995): Expression of the hepatocyte growth factor gene during chick limb development. *Dev. Dynamics* 202:80–90.
- Nakamura H, Tashiro K, Nakamura T, Shiohara K (1995): Molecular cloning of Xenopus HGF cDNA and its expression studies in Xenopus early embryogenesis. *Mech Dev* 49:123–131.
- Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S (1989): Molecular cloning and expression of human hepatocyte growth factor. *Nature* 342:440–443.
- Naldini L, Weidner KM, Vigna E, Gaudino G, Bardelli A, Ponzetto C, Narsimhan RP, Hartman G, Zarnegar R, Michalopoulos GK, Birchmeier W, Comoglio P (1991): Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *EMBO J* 10:2867–2878.
- Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Vande Woude G (1987): Sequence of Met proto-oncogene cDNA has features characteristic of the tyrosine kinase family of growth factor receptors. *Proc Natl Acad Sci USA* 84:6379–6383.
- Partanen J, Mäkelä TP, Eerola E, Korhonen J, Hirvonen H, Claesson-Welsh L, Alitalo K (1991): FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. *EMBO J* 10:1347–1354.
- Prat M, Narsimhan RP, Crepaldi T, Nicotra MR, Natali PG, Comoglio PM (1991): The receptor encoded by the human c-met oncogene is expressed in hepatocytes, epithelial cells and solid tumors. *Int J Cancer* 49:323–328.
- Roberts C, Platt N, Streit A, Schachner M, Stern CD (1991): The L5 epitope: An early marker for neural induction in the chick embryo and its involvement in inductive interactions. *Development* 112:959–970.
- Ronsin C, Muscatelli F, Mattei M-G, Breathnach R (1993): A novel putative receptor protein tyrosine kinase of the met family. *Oncogene* 8:1195–1202.
- Rubin JS, Chan AM-L, Bottaro DP, Burgess WH, Taylor WG, Cech AC, Hirschfield DW, Wong J, Miki T, Finch PW, Aaronson SA (1991): A broad spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. *Proc Natl Acad Sci USA* 88:415–419.
- Santos OFP, Barros EJJ, Yang X-M, Matsumoto K, Nakamura T, Park M, Nigam SK (1994): Involvement of hepatocyte growth factor in kidney development. *Dev Biol* 163:525–529.
- Sasaki M, Nishio M, Sasaki T, Enami J (1994): Identification of mouse fibroblast-derived mammary growth factor as hepatocyte growth factor. *Biochem Biophys Res Commun* 199:772–779.
- Schoenwolf GC, Smith JL (1990): Mechanisms of neurulation: Traditional viewpoint and recent advances. *Development* 109:243–270.
- Seki T, Hagiya M, Shimonishi M, Nakamura T, Shimizu S (1991): Organization of the human hepatocyte growth factor-encoding gene. *Gene* 102:213–219.

- Skeel A, Yoshimura T, Showaltzer SD, Tanaka S, Appella E, Leonard EJ (1991): Macrophage stimulating protein: Purification, partial amino acid sequence, and cellular activity. *J Exp Med* 173:1227-1234.
- Sonnenberg E, Meyer D, Weidner KM, Birchmeier C (1993): Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J Cell Biol* 123:223-235.
- Stern CD, Ireland GW (1993): HGF-SF: a neural inducing molecule in vertebrate embryo? In Goldberg ID, Rosen EM (ed): "Hepatocyte Growth Factor-Scatter Factor (HGF-SF) and the c-met Receptor." Basel, Switzerland: Birkhäuser Verlag, pp 369-380.
- Stern CD, Ireland GW, Herrick SE, Gherardi E, Gray J, Perryman M, Stoker M (1990): Epithelial scatter factor and development of the chick embryonic axis. *Development* 110:1271-1284.
- Stoker M, Gherardi E, Perryman M, Gray J (1987): Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature* 327:239-242.
- Streit A, Faissner A, Gehrig B, Schachner M (1990): Isolation and biochemical characterization of a neural proteoglycan expressing the L5 carbohydrate epitope. *J. Neurochem.* 55:1494-1506.
- Streit A, Stern CD, Théry C, Ireland G, Aparicio S, Sharpe MJ, Gherardi E (1995): A role for HGF/SF in neural induction and its expression in Hensen's node during gastrulation. *Development* 121:813-824.
- Tam PPL, Trainor PA (1994): Specification and segmentation of the paraxial mesoderm. *Anat Embryol* 189:275-305.
- Van Straaten HMW, Hekking JWM (1991): Development of floor plate, neurons and axonal outgrowth pattern in the early spinal cord of notochord-deficient chick embryo. *Anat Embryol* 184:55-63.
- Van Straaten HWM, Hekking JWM, Thors F, Wiertz-Hoessels EL, Drukker J (1985): Induction of an additional floor plate in the neural tube. *Acta Morphol Neerl Scand* 23:91-97.
- Wang M-H, Ronsin C, Gesnel M-C, Coupey L, Skeel A, Leonard ED, Breathnach R (1994): Identification of the Ron gene product as the receptor for the human macrophage stimulating protein. *Science* 266:117-119.
- Weidner KM, Arakaki N, Hartmann G, Vanderkerckhove J, Weingart S, Rieder H, Fonatsch C, Tsubouchi H, Hishida T, Daikuhara Y, Birchmeier W (1991): Evidence for the identity of human scatter factor and human hepatocyte growth factor. *Proc Natl Acad Sci USA* 88:7001-7005.
- Wilkinson DG (1993): In situ hybridization. In Stern CD, Holland PWH (ed): "Essential Developmental Biology: A Practical Approach." Oxford: IRL Press, pp 257-274.
- Wilks AF (1989): Two putative protein-tyrosine kinases identified by application of the polymerase-chain reaction. *Proc Natl Acad Sci USA* 86:1603-1607.
- Yamada T, Placzek M, Tanaka H, Dodd J, Jessell T (1991): Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. *Cell* 73:635-647.
- Yonei S, Tamura K, Koyama E, Nohno T, Noji S, Ide H (1993): MRC-5, human embryonic lung fibroblasts, induce the duplication of the developing chick limb bud. *Dev Biol* 160:246-253.
- Yoshimura T, Yuhki N, Wang M-H, Skeel A, Leonard EJ (1993): Cloning, sequencing and expression of human macrophage stimulating protein (MSP, MST1) confirms MSP as a member of the family of kringle proteins and locates the MSP gene on chromosome 3. *J Biol Chem* 268:15461-15468.