

# Identification of a Novel Member (GDF-1) of the Transforming Growth Factor- $\beta$ Superfamily

Se-Jin Lee

Carnegie Institution of Washington  
Department of Embryology  
Baltimore, Maryland 21210

A cDNA clone encoding a new member (designated GDF-1) of the transforming growth factor- $\beta$  (TGF $\beta$ ) superfamily was isolated from a library prepared from day 8.5 mouse embryos. The nucleotide sequence of GDF-1 predicts a protein of 357 amino acids with a mol wt of 38,600. The sequence contains a pair of arginine residues at positions 236–237, which is likely to represent a site for proteolytic processing. The C-terminus following the presumed dibasic cleavage site shows significant homology with the known members of the TGF $\beta$  superfamily, matching the other family members at all of the invariant positions, including the seven cysteine residues with their characteristic spacing. GDF-1 is most homologous to *Xenopus* Vg-1 (52%), but is not likely to be the murine homolog of Vg-1. *In vitro* translation experiments were consistent with GDF-1 being a secreted glycoprotein. Genomic Southern analysis indicated that GDF-1 may be highly conserved across species. These results suggest that GDF-1 is most likely an extracellular factor mediating cell differentiation events during embryonic development. (Molecular Endocrinology 4: 1034–1040, 1990)

## INTRODUCTION

A growing number of polypeptide factors playing critical roles in regulating differentiation processes during embryogenesis have been found to be structurally homologous to transforming growth factor- $\beta$  (TGF $\beta$ ). Among these are Mullerian inhibiting substance (MIS) (1), which causes regression of the Mullerian duct during male sex differentiation; the bone morphogenetic proteins (BMPs) (2), which can induce *de novo* cartilage and bone formation; the inhibins and activins (3–6), which

regulate FSH secretion by pituitary cells and which (in the case of the activins) can affect erythroid differentiation; the *Drosophila* decapentaplegic gene product (7), which influences dorsal-ventral specification as well as morphogenesis of the imaginal disks; the *Xenopus* Vg-1 gene product (8), which localizes to the vegetal pole of eggs; and Vgr-1 (9), a gene identified on the basis of its homology to Vg-1 and shown to be expressed during mouse embryogenesis. In addition, one of the most potent mesoderm-inducing factors, XTC-MIF, also appears to be structurally related to TGF $\beta$  (10, 11). The TGF $\beta$ s themselves are capable of influencing a wide variety of differentiation processes, including adipogenesis, myogenesis, chondrogenesis, hematopoiesis, and epithelial cell differentiation (for review, see Ref. 12), and at least one TGF $\beta$ , namely TGF $\beta$ 2, is capable of inducing mesoderm formation in frog embryos (10).

Here I report the characterization of a new member of the TGF $\beta$  superfamily isolated from an 8.5-day-old mouse embryo cDNA library. This gene, which I have designated GDF-1, is most homologous to Vg-1, but is not likely to be the murine homolog of Vg-1. The structural homology between GDF-1 and the other members of this family as well as its expression in early mouse embryos suggest that GDF-1 may play an important role in mediating developmental decisions related to cell differentiation.

## RESULTS AND DISCUSSION

### Cloning and Nucleotide Sequence of GDF-1

To identify new members of the TGF $\beta$  superfamily that may be important for mouse embryogenesis, a cDNA library was constructed in  $\lambda$ ZAP II using poly(A)-selected RNA from whole embryos isolated on day 8.5 postcoitum (pc). The library was screened with oligonucleotides selected on the basis of the predicted amino acid sequences of conserved regions among members of the superfamily. Among 600,000 recombinant phage

screened were three clones that hybridized with the single nondegenerate 27-mer, 5'-GCAGCCA-CACTCCTCCACCACCATGTT-3', the complement of which corresponds to the amino acid sequence NMVVEECG. Partial sequence analysis revealed that the three cDNA clones contained overlapping sequences and were, therefore, likely to represent mRNAs derived from the same gene, which was designated GDF-1 (growth/differentiation factor-1). Northern analysis of day 8.5 embryonic RNA (which had been used to prepare the cDNA library) using the GDF-1 probe detected a single predominant mRNA species approximately 1.4 kilobases (kb) in length (Fig. 1). Because the original three cDNA isolates were all smaller than 1.4 kb, portions of the longest clone were used to rescreen the cDNA library to isolate a full-length clone. Hybridizing recombinant phage were seen at a frequency of approximately 1 per 200,000.

The entire nucleotide sequence of the longest cDNA clone obtained encoding GDF-1 is shown in Fig. 2. The 1387-basepair (bp) sequence contains a single long open reading frame beginning with an initiating ATG at nucleotide 217 and potentially encoding a protein of 357 amino acids with a mol wt of 38,600. Up-stream of the putative initiating ATG are two in-frame stop codons and no additional ATGs. Nucleotides 1259-1285 show a 25/27 match with the complement of the oligonucleotide selected for the original screening. The 3' end of the clone does not contain the canonical AAUAAA

1.4 kb—

Fig. 1. Northern Analysis of Embryonic RNA

Two micrograms of twice poly(A)-selected mRNA isolated from day 8.5 pc mouse embryos were electrophoresed on formaldehyde gels, transferred to nitrocellulose, and probed with GDF-1 cDNA. The assignment of the size of the major band was based on the mobilities of RNA standards transcribed *in vitro*.

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1  CCCTTCCAGGACTCTGGCTGCCAGCAGCTCCGCTTTCAGATCAATTCGACCAAC 60
61  CACCTTGGGACTGCCGCCAGTCTGCTGCTGATCAGTGGGTCAGACAGCCCTCT 120
121  CCAGGAGCTCAAGACACCCCGACCTAAGGTACACAGCCCACTGGCCCAAGACGATGG 180
181  GCTCCGCTGACTCTCTTGGACACCTCTGGGAGGAAATGCTCCCTGCTGCACTGTT 240
      H L P V C H R F
241  TTGGACCACTCTCTCTCTCTCTCTCTGCTGCTGCTGCCCTGACGACCTTGGCCCGGCGAGC 300
      C D H L L L L L L L L L P S T T L A P A P A
301  ATCCATGGGCGCGCTGCCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 360
      S H G P A A A L L O V L G L P E A P R S
361  CGTCCCAACACAGGAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 420
      V P T H R P V P P V H M R L F R R R D F
421  CCAGGAGGCGAGAGTGGGACGCGCTCTGCGGCTATGCCACGTGGAGGAATAGGGGTCGC 480
      Q E A R V G R P L R P C H V E E L G V A
481  CGGAACATTTGTCGCGACATCCCGACAGCGGTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 540
      G N I V R H I P D S G L S S R P A Q P A
541  CAGGAGCTCGGGGCTGTGCGCGAGTGGACAGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 600
      R T S G L C P E W T V V F D L S N V E P
601  CACAGAGCGCCCAACACGCGCGCTTAGAGTTCGGGCTGGAGGCTGAGTGTGAAGATAC 660
      T E R P T R A R L E L R L E A E C D T
661  AGGAGGGTGGGAGCTAAGCGTGGCACTGTGGCGGACGAGCAGCATCCAGGCGCTGAGCT 720
      G G M E L S V A L W A D A E H P G P E L
721  GCTGCGGCTGCCGCGCACAGGGGTGCTGCTGCGGCGAGCAGCTACTGGGAGTCACT 780
      L R V P A P P G V L L R A D L L G T A V
781  AGCGCCCAACGACAGTCACTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 840
      A A N A S V P C T V R L A L S L N P G A
841  CACTGCACTGTGGGCGCTGCTGAGGCTCCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 900
      T A A C G R L A E A S L L V T L D P R
901  CCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 960
      L C P L P R L R R H T E P R V E V G P V
961  GGGCACTGTGCTACCGGAGGTTGATGTGAGTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1020
      G T C R T R L H V S T F R E V G M H R W
1021  GGTGATGCGCGCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1080
      V I A P R G F L A N F C Q G T C A L P E
1081  AACGCTGAGGGAGCGCGCGCGCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1140
      T L R G P G G P P A L N H A V L R A L M
1141  GCACGCACTGCTCCACCGCGGGTGCAGGCTCCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1200
      H A A A P T P G A G S P C V F E R L S
1201  ACCATCTCGCTGCTCTTCTGACAATAGTGACACGCTGGTCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1260
      P I S V L F F O N S D N V L R N Y E D
1261  CATGGTGTGATGAGTGTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1320
      M V V D E C G C R
1321  CCACGCCAAGCAGGAGCTGTTTTCATGTTTATTGGTGACAAAAGCTTAAACAAA 1380
1381  TTTGACT 1387

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Fig. 2. Sequence of GDF-1

The entire nucleotide sequence of GDF-1 derived from a single cDNA clone is shown, with the predicted amino acid sequence below. The poly(A) tail is not shown. Numbers indicate nucleotide position relative to the 5' end of the clone.

polyadenylation signal. Sequence analysis at the 3' end of four independent cDNA clones (all differing at their 5' ends) showed that two clones terminated at the same nucleotide, and the other two clones terminated at a site seven nucleotides further down-stream (these clones contained an additional AAAAAATT sequence at the 3' end).

The predicted amino acid sequence identifies GDF-1 as a new member of the TGF $\beta$  superfamily. A comparison of the C-terminal 122 amino acids with those of the other members of this family is shown in Fig. 3A. The predicted GDF-1 sequence contains all of the invariant amino acids present in the other family members, including the seven cysteine residues with their characteristic spacing, as well as many of the other highly conserved amino acids. In addition, like other family members, the C-terminal portion of the predicted GDF-1 polypeptide is preceded by a pair of basic residues (R-R) at positions 236-237, potentially representing a site for proteolytic processing. All of the known members of this family, except MIS, have a cluster of basic residues approximately 120 amino acids from the C-terminus, and, at least in the case of TGF $\beta$ s (13, 14),

# A

GDF-1 ALNHAVLRALMHAAA-PTPGAGSPCCV--PERLSPIVLFF-DNSDNVLRHYEDMVVDECCOR

Vg-1 -SNHAILOTLVHS--IEPEDIPLPCCV--PTKMSPISMLFY--DNNDNVLRHYENMAVDECCOR

Vgr-1 -TNHAIIVOTLVHL--MNPEIYVPKPCA--PTKLNATISVLFE--DDNSNVLLKRYNMVVRACGCH

BMP-2a -TNHAIIVOTLVNS--VNSKIPKACV--PTELSAISMLYL--DEYDKVVLKNYQDMVVEGCGH

BMP-2b -TNHAIIVOTLVNS--VNSSIPKACV--PTELSAISMLYL--DENKNVVLKYPNMVTVESACR

BMP-3 -NHATISIVIRA-VGVVPGPIPECV--PEKMSSISLFF--OSTVVLKNYQDMVTVGCGH

DPP -TNHAVIOTLVNN--MNPCKVPKACV--PTOLDSDAMLYLND--ISAHVKNPMVATESACR

MIS -GNH-VULL--KMGARGAALRPCCV--PTAYAGKLLISLSEER--ISAHVKNPMVATESACR

Inhibin  $\alpha$  -PGAPPT---PAOPYSILPGAOPCOALPGTMRPLHVRTSDGGYSFKYETVPMNLITQELCI

Inhibin  $\beta$ A -SEHSTVINHYRMRGHSPFANKLSCV--PTKLRTMSMLFY--DDGONIKKIDNVNMIVEECCGA

Inhibin  $\beta$ B -SEHSTAVNVOYRMRGLNPGT-VNSCGI--PTKLSTSMMLFY--DEYINIVKRDVPMNMIVEECCGA

TGF- $\beta$ 1 -TOYSKVALALYN--OHNPICASAPCCV--POALEPLPIVYY--VGRKPKI-EQLSNMIVKSSNGS

TGF- $\beta$ 2 -TOHSRVLALYN--TINPEASASPCV--PODLEPLTILYY--VGRTPKV-EQLSNMVVKSNGS

TGF- $\beta$ 3 -TTHSTVLGLYN--TLNPEASASPCV--PODLEPLTILYY--VGRTRPV-EQLSNMVVKSNGS

TGF- $\beta$ 4 -TOYTKVALALYN--OHNPICASAPCCV--PDLDPLPIIYY--VGRNVRV-EQLSNMVVRAKNGS

TGF- $\beta$ 5 -TOYSKVALALYN--OHNPICASAPCCV--PDVLEPLPIIYY--VGRATAKV-EQLSNMVVRSNGS

# B

[illegible]

**-C**

**C**

	54		133
GDF-1	PVPPVMWRLFRRRDPQEARVGRPLR	PCHVVEELGVAGNIVRHIPD	SLSSRPAQPARTSGLCP
			EWTVVFDLSNVEPTERT
Vg-1	PVPSILWRIFNQRMGSSIQKKKPD	CFVEEFNVPGSVIRVFPDQGRFI	IPYSDDIHPTQCLEKRLFFNISAIEKEERV
	46		124
	219	234	
GDF-1	SLLLVTLDPRLCPLPR		
Vg-1	SLLTVTLNPLRCKRPR		
	228	243	

inhibins (3, 4, 15, 16), and BMP-2a (2, 17), the mature form of the protein is known to be generated by cleavage at these sites. In the case of MIS, it is known that cleavage of the protein can occur at a monobasic site at an analogous position (18).

Figure 3B shows a tabulation of the percentages of identical residues between GDF-1 and the other members of the TGF $\beta$  family in the region starting with the first conserved cysteine and extending to the C-terminus. GDF-1 is most homologous to Vg-1 (52%) and least homologous to inhibin- $\alpha$  (22%) and the TGF $\beta$ s (26–30%). Two lines of reasoning suggest that GDF-1 is not the murine homolog of Vg-1. First, GDF-1 is less homologous to Vg-1 than are Vgr-1 (59%), BMP-2a (59%), and BMP-2b (57%). Second, GDF-1 does not show extensive homology with Vg-1 outside of the C-terminal portion, and it is known that other members of this family are highly conserved across species throughout the entire length of the protein (1, 3, 4, 13, 19, 20). However, GDF-1 and Vg-1 do share two regions of limited homology N-terminal to the presumed dibasic cleavage site, as shown in Fig. 3c. Hence, to my knowledge, this is the first report of the isolation of GDF-1 from any species.

#### *In Vitro* Translation of GDF-1 RNA

The predicted GDF-1 sequence is also noteworthy for the presence of a core of hydrophobic amino acids at the N-terminus, potentially representing a signal sequence, as well as for the presence of a potential N-glycosylation site at amino acid 191. To determine whether these sequences are functional and to confirm that translation initiates as predicted at the first ATG, *in vitro* translation experiments were carried out using a rabbit reticulocyte lysate. As shown in Fig. 4 (lane 2), translation of full-length sense GDF-1 RNA, transcribed and capped *in vitro*, resulted in a major protein species with a mol wt of 39.5K, which agreed well with the predicted mol wt of 38.6K for the translation product initiating at the most up-stream ATG; no such band was seen with translation of antisense GDF-1 RNA (lane 1). Consistent with the interpretation that translation had initiated at the most up-stream ATG was the observation that if the starting DNA template contained a deletion at the 5' end extending past the first ATG codon, the resultant translation product was slightly smaller (lane 4), suggesting that translation in this case had initiated at the next ATG codon (nucleotide 305).

When full-length GDF-1 RNA was translated in the presence of dog pancreatic microsomes, some of the translated product migrated slower than the full-length product (lane 3).<sup>1</sup> This slower migrating species (41K) could be converted to a 38K form by treatment with endoglycosidase-H (lane 7), consistent with the 41K and 38K species representing the glycosylated and deglycosylated forms, respectively, of the GDF-1 protein lacking a signal peptide. Furthermore, the 41K species (unlike the unprocessed 39.5K species) was resistant to treatment with trypsin in the absence (lane 9), but not in the presence (lane 13), of detergent, suggesting that the 41K species was protected from cleavage by its presence within the microsomes. In contrast, parallel experiments carried out with protein translated from a deletion template lacking the signal sequence showed no shift to a high mol wt species in the presence of microsomes (lane 5) and no protection from cleavage by trypsin (lane 11). Taken together, these data suggest that GDF-1 may be a secreted glycoprotein like many of the other members of this superfamily.

#### Southern Blot Analysis

To determine whether GDF-1 is likely to be a single copy gene, Southern blot analysis was carried out using mouse genomic DNA. As shown in Fig. 5, the GDF-1 probe detected a single predominant band in three different digests of mouse DNA. However, even at high stringency, additional weakly hybridizing bands were detected. These minor bands are not likely to represent the products of partial digestion, because many of these bands were smaller than the predominant band, and the intensities of these minor bands relative to that of the major band could be enhanced by reducing the stringency of the washing conditions (data not shown). Whether these bands represent other known members of this superfamily or genes more highly homologous to GDF-1 remains to be determined.

Southern analysis was also extended to DNA isolated

<sup>1</sup> The band migrating at 39.5K in lane 3 (Fig. 4) is presumed to represent the unprocessed protein resulting from the relative inefficiency of processing by the dog pancreatic microsomes. Consistent with this is the resistance of the 39.5K species to endoglycosidase-H (lane 7) and the susceptibility of the 39.5K species to trypsin (lane 9). In addition, the relative ratio of the intensities of the 41K and 39.5K species in lane 3 varied depending on the amount of added microsomes (data not shown).

**Fig. 3.** Comparison of the Predicted GDF-1 Amino Acid Sequence with the Previously Described Members of the TGF $\beta$  Superfamily. A, Alignment of the C-terminal amino acid sequence of GDF-1 (beginning at amino acid 236) with the corresponding regions of *Xenopus* Vg-1 (8); murine Vgr-1 (9); human BMP-2a, 2b, and 3 (2); *Drosophila* DPP (7); human MIS (1); human inhibin- $\alpha$ , - $\beta$ A, and - $\beta$ B (19); human TGF $\beta$ 1 (13); human TGF $\beta$ 2 (32); human TGF $\beta$ 3 (33, 34); chicken TGF $\beta$ 4 (35); and *Xenopus* TGF $\beta$ 5 (36). The seven invariant cysteines are shaded. Dashes denote gaps introduced in order to maximize the alignment. B, Amino acid homologies among the different members of the superfamily. Numbers represent percent identities between each pair calculated from the first conserved cysteine to the C-terminus. C, Homology between GDF-1 and Vg-1 up-stream of the presumed dibasic cleavage site. Two different regions are shown. A single gap of one amino acid has been introduced into the Vg-1 sequence in order to maximize the alignment. Numbers indicate amino acid positions in the respective proteins.

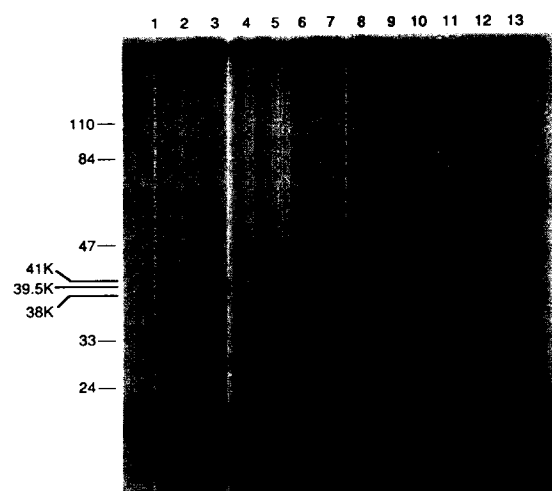


Fig. 4. *In Vitro* Translation of GDF-1

Antisense (lane 1) or sense (lanes 2–13) RNA, transcribed and capped *in vitro*, was translated with a rabbit reticulocyte lysate in the presence of [<sup>35</sup>S]methionine with (lanes 3, 5, 7, 9, 11, and 13) or without (lanes 1, 2, 4, 6, 8, 10, and 12) added dog pancreatic microsomes. Lanes 2 and 3, translation products from a full-length GDF-1 template; lanes 4 and 5, translation products from a deletion template lacking the putative signal sequence; lanes 6 and 7, endoglycosidase-H-treated translation products from a full-length GDF-1 template; lanes 8 and 9, trypsin-treated translation products from a full-length GDF-1 template; lanes 10 and 11, trypsin-treated translation products from a deletion template lacking the putative signal sequence; lanes 12 and 13, translation products from a full-length GDF-1 template treated with trypsin in the presence of Triton X-100. Samples were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel under reducing conditions, followed by fluorography. Equal amounts of products prepared in a single translation reaction were used for lanes 2, 6, 8, and 12, for lanes 3, 7, 9, and 13, for lanes 4 and 10, and for lanes 5 and 11. Numbers at the left indicate sizes of mol wt standards. The 41K, 39.5K, and 38K positions were calculated relative to the mobilities of these standards.

from other species. Even at high stringency, the GDF-1 probe detected a single predominant band in both hamster and human DNA (Fig. 5), suggesting that GDF-1 is conserved across species. Moreover, as was seen with mouse DNA, additional minor bands could be detected in both human and hamster DNA at relative high stringency.

The TGF $\beta$  superfamily encompasses a group of proteins affecting a wide range of differentiation processes. Many of these proteins are likely to play key roles in regulating embryonic development. In addition, some of these proteins appear to perform important endocrine functions in adult animals as well. The predicted sequence of GDF-1 clearly identifies it as a new member of this superfamily. By analogy with the other members of this superfamily, it seems reasonable to hypothesize that GDF-1 is an extracellular factor involved in mediating developmental decisions related to cell differentiation. The expression of GDF-1 in early mouse embryos

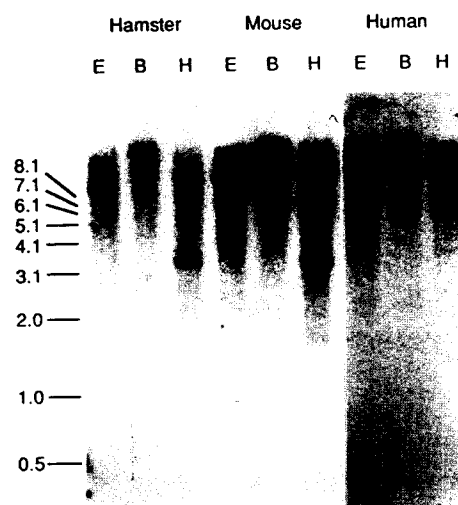


Fig. 5. Genomic Southern Analysis of GDF-1

Ten micrograms of genomic DNA isolated from Chinese hamster ovary cells (hamster), BNL cells (mouse), or BeWo cells (human) were digested with *EcoRI* (E), *BamHI* (B), or *HindIII* (H); electrophoresed on a 1% agarose gel; transferred to nitrocellulose; and probed with GDF-1. Numbers at the left indicate sizes (kilobases) of standards. The lanes containing human DNA were exposed twice as long as the lanes containing hamster and mouse DNA.

and the *in vitro* translation experiments showing that GDF-1 contains a functional signal sequence support this hypothesis. Also, by analogy with the known members of this superfamily, it seems likely that the C-terminal portion is cleaved from the full-length precursor and that GDF-1 is active as a dimer. It will be important to determine whether such cleavage and dimerization occur and whether GDF-1 is capable of forming heterodimers with other related proteins. The high degree of conservation of GDF-1 across species supports the notion that GDF-1 may play an essential regulatory role in the embryo and/or the adult. An elucidation of the specific role(s) played by GDF-1 during embryogenesis and/or in adult animals awaits characterization of the temporal and spatial patterns of GDF-1 mRNA expression and the functional activities of GDF-1 protein both *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Construction and Screening of an 8.5-Day-Old Embryonic cDNA Library

All embryonic materials were obtained from random matings of CD-1 mice (Charles River, Wilmington, MA). Mice were maintained according to the NIH guidelines for care and maintenance of experimental animals. The day on which the vaginal plug was noted was designated day 0.5 pc. Embryos were dissected out from the uterus, freed of all extraembryonic membranes, and frozen rapidly. Total RNA was prepared by homogenization in guanidinium thiocyanate buffer and centrifugation of the lysate through a cesium chloride cushion (21).

Poly(A)-containing RNA was obtained by twice selecting with oligo-dT cellulose (22). A cDNA library was constructed in the  $\lambda$ -ZAP II vector using the RNase-H method (23, 24) according to the instructions provided by Stratagene (La Jolla, CA). 3.2 million recombinant plaques were obtained from 2  $\mu$ g starting RNA. The library was screened with the oligonucleotide 5'-GCAGCCACACTCTCCACCACCATGTT-3', which had been end labeled using polynucleotide kinase. Hybridization was carried out in  $6 \times$  SSC,  $1 \times$  Denhardt's, 0.05% sodium pyrophosphate, and 100  $\mu$ g/ml yeast tRNA at 50 C. Filters were washed in  $6 \times$  SSC-0.05% sodium pyrophosphate at 60 C.

#### DNA Sequencing and Blot Hybridizations

DNA sequencing of both strands was carried out with the dideoxy chain termination method (25), using the exonuclease III/S1 nuclease strategy (26).

For Northern analysis, RNA was electrophoresed on formaldehyde gels (27, 28), transferred to nitrocellulose, and hybridized in 50% formamide,  $5 \times$  SSC,  $4 \times$  Denhardt's, 0.1% sodium dodecyl sulfate (SDS), 0.1% sodium pyrophosphate, and 100  $\mu$ g/ml salmon DNA at 50 C. Filters were washed first in  $2 \times$  SSC, 0.1% SDS, and 0.1% sodium pyrophosphate, then in  $0.1 \times$  SSC-0.1% SDS at 50 C.

For Southern analysis, DNA was electrophoresed on 1% agarose gels, transferred to nitrocellulose, and hybridized in 1 M NaCl, 50 mM sodium phosphate (pH 6.5), 2 mM EDTA, 0.5% SDS, and  $10 \times$  Denhardt's at 65 C. The final wash was carried out in  $2 \times$  SSC at 68 C.

#### In Vitro Translation Experiments

The full-length 1387-bp GDF-1 cDNA or a deletion mutant lacking the first 251 nucleotides was subcloned into the Bluescript vector (Stratagene), and sense or antisense RNA was transcribed *in vitro* from the T3 or T7 promoters (29, 30) in the presence of cap analog, as described by Stratagene. *In vitro* translations were carried out by incubating 0.5  $\mu$ g RNA, 17.5  $\mu$ l rabbit reticulocyte lysate (Promega, Madison, WI), 20  $\mu$ M cold amino acid mixture (Promega), and 20  $\mu$ Ci [ $^{35}$ S]methionine (New England Nuclear, Boston, MA) in the presence or absence of 10 equivalents of dog pancreatic microsomes (Promega) for 60 min at 30 C. Endoglycosidase digestions were carried out by diluting the translation reaction 1:30 with 100 mM sodium acetate (pH 5.5), 0.1% SDS, and 17 mU/ml endoglycosidase-H (Boehringer-Mannheim, St. Louis, MO). Protease digestions were carried out by diluting the translation reaction 1:20 with PBS-1 mg/ml trypsin (Boehringer-Mannheim) in the presence or absence of 0.1% Triton X-100. All digestions were carried out for 3 h at 37 C. Translation products were analyzed by electrophoresis on 10% SDS-polyacrylamide gels (31), followed by fluorography with Enhance (New England Nuclear).

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Address requests for reprints to: Dr. Se-Jin Lee, Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore, Maryland 21210.

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