

Growth/Differentiation Factor-10: A New Member of the Transforming Growth Factor- β Superfamily Related to Bone Morphogenetic Protein-3

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We have identified a new member of the transforming growth factor- β (TGF- β) superfamily, growth/differentiation factor-10 (GDF-10), which is highly related to bone morphogenetic protein-3 (BMP-3). The nucleotide sequence of GDF-10 encodes a predicted protein of 476 amino acids with a molecular weight of approximately 52,000. The GDF-10 polypeptide contains a potential signal sequence for secretion, a putative RXXR proteolytic processing site, and a carboxy-terminal domain with considerable homology to other known members of the TGF- β superfamily. In the mature carboxy-terminal domain GDF-10 is more homologous to BMP-3 (83% amino acid sequence identity) than to any other previously identified TGF- β family member. GDF-10 also shows significant homology to BMP-3 (approximately 30% amino acid sequence identity) in the pro- region of the molecule. Based on these sequence comparisons, GDF-10 and BMP-3 define a new subgroup within the larger TGF- β superfamily. By Northern analysis, GDF-10 mRNA was detected primarily in murine uterus, adipose tissue, and brain and to a lesser extent in liver and spleen. In addition, GDF-10 mRNA was present in both neonatal and adult bone samples, with higher levels being detected in calvaria than in long bone. These results suggest that GDF10 may play multiple roles in regulating cell differentiation events, including those involved in skeletal morphogenesis. Gdf10 was mapped to the proximal region of mouse chromosome 14 close to a region known to contain a spontaneous recessive mutation that is associated with a craniofacial defect.

KEYWORDS: growth/differentiation factor, bone morphogenetic protein-3

INTRODUCTION

The bone morphogenetic proteins (BMPs) constitute a family of proteins that are capable of inducing the aggregation, proliferation, and differentiation of mesenchymal cells when implanted subcutaneously or intramuscularly in rats (for review, see Wozney, 1992; Kingsley, 1994; Reddi, 1994). This process leads to a series of histological transformations that closely resemble those seen in normal embryonic bone formation and ultimately result in *de novo*

cartilage and bone formation at the site of implantation. Based on their biological activities, their expression patterns during embryonic development, and the phenotypes of mice carrying mutations in these genes, it seems clear that the BMPs are key regulators of skeletal development *in vivo*.

The BMPs represent a subset of the larger TGF- β superfamily of growth and differentiation factors. In mammals this superfamily includes Mullerian inhibiting substance (Cate et al., 1986), the activins and inhibins (Mason et al., 1985; Forage et al., 1986; Mayo et al., 1986), nodal (Zhou et al., 1993), GDNF (Lin et al., 1993), GDF-1 (Lee, 1990), GDF-3/Vgr-2 (Jones et al., 1992; McPherron and Lee, 1993), GDF-9 (McPherron and Lee, 1993), and the TGF- β s

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(Derynck *et al.*, 1985, 1988; de Martin *et al.*, 1987; ten Dijke *et al.*, 1988). Each member of the TGF- β superfamily is synthesized as part of a larger precursor protein containing a hydrophobic signal sequence, a long, poorly conserved amino-terminal pro-region, and a shorter, more highly conserved carboxy-terminal domain. The biologically-active C-terminal region is generated by proteolytic cleavage of the precursor protein at an RXXR site located approximately 110–140 amino acids from the carboxy terminus. It is the mature region that contains the seven positionally conserved cysteine residues characteristic of most TGF- β family members. For most family members, the biologically active molecule appears to be a homodimer of C-terminal fragments; however, certain family members, such as the inhibins (Ling *et al.*, 1986) and the TGF- β 's (Cheifetz *et al.*, 1987), can also exist as heterodimers, and these heterodimers appear to have different biological properties than the respective homodimers.

Using degenerate oligonucleotides based on the sequence of BMP-3 as primers for polymerase chain reactions, we have identified a novel member of the TGF- β superfamily that we have designated growth/differentiation factor-10 (GDF-10). GDF-10 shows significant homology to BMP-3 in the C-terminal domain (83% amino acid sequence identity) as well as in the pro-region of the molecule (30% amino acid sequence identity). Based on these sequence comparisons GDF-10 and BMP-3 define a new subgroup within the TGF- β superfamily.

METHODS

Analysis of Polymerase Chain Reaction (PCR) Products

GDF-10 was identified from a mixture of PCR products obtained with primers NSC1 (5'-CGGAATTCAA(A/G)GT(G/A/T/C)GA(T/C)TT (T/C)GC-(G/A/T/C)GA(T/C) AT(T/C/A)GG(G/A/T/C)TGG-3'), which encodes the amino acid sequence KVDFAIDGW, and NSC2 (5'-CGGAATTCA(A/G)CA(G/A/T/C)GC(A/G)CA(A/G)CT(C/T)TC(G/A/T/C)AC (G/A/T/C)GTCAT-3') and NSC3 (5'-CCGGAATTC (A/G)CA(G/A/T/C)GC(A/G)CA-(G/A/T/C)GA(C/T)TC(G/A/T/C)AC(G/A/T/C)GTCAT-3'), the reverse complements of which encode the amino acid sequence MTVESAC. PCR using primers NSC1 with NSC2 or NSC1 with

NSC3 was carried out with cDNA prepared from 0.25 μ g of lung or brain mRNA at 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min for 35 cycles. PCR products of ~300 base pairs were gel-purified, digested with *EcoRI*, gel purified again, and cloned in the Bluescript vector (Stratagene). Of 11 clones that were sequenced, 9 corresponded to murine BMP-3, and two represented a novel sequence, which was designated GDF-10.

RNA Isolation and Northern Analysis

All murine tissue samples were obtained from the strain CD-1 (Charles River). Human uteri were obtained from the International Institute for the Advancement of Medicine (Exton, PA). RNA isolation and poly A selections were carried out as previously described (Lee, 1990). Two and a half micrograms of poly (A)⁺ RNA were electrophoresed on formaldehyde gels, transferred to Gene Screen Plus (Dupont), and hybridized with the 300 base pair (bp) GDF-10 PCR product probe essentially as described (Lee, 1990), except that the hybridization was done in 5X SSPE (50 mM sodium phosphate, pH 7.0, 5 mM Na₂EDTA, 0.9 M NaCl), 10% dextran sulfate, 50% formamide, 1% SDS, 200 μ g/ml salmon sperm DNA, and 0.1% each of bovine serum albumin, ficoll, and polyvinylpyrrolidone. For the bone Northern blots, 12 μ g total RNA were electrophoresed on formaldehyde gels, transferred to Gene Screen Plus, and hybridized and washed as above except that the probe was the full length GDF-10 cDNA.

Isolation and Sequencing of cDNA Clones

Oligo (dT)-primed cDNA libraries were prepared from 3 μ g of murine or human uterine poly (A)⁺ RNA in the lambda ZAP II vector according to the instructions provided by Stratagene. The libraries were screened without amplification. Filters were hybridized as described (Lee, 1991). DNA sequencing of both strands was carried out using the dideoxy chain termination method (Sanger *et al.*, 1977) and a combination of the S1 nuclease exonuclease III strategy (Henikoff, 1984) and synthetic oligonucleotide primers.

Interspecific Backcross Mapping

Interspecific backcross progeny were generated by mating (C57BL/6) \times *Mus spretus*) F₁ females and

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C57BL/6J males as described (Copeland and Jenkins, 1991). A total of 205 N₂-mice were used to map the *Gdf10* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed essentially as described (Jenkins et al., 1982). All blots were prepared with Zetabind nylon membrane (AMF-Cuno). The probe, an ~500 bp *EcoRI/BamHI* fragment of mouse GDF-10 cDNA, was labelled with [α^{32} P]dCTP using a nick translation labelling kit (Boehringer Mannheim); washing was done to a final stringency of $0.5 \times \text{SSC}$, 0.1% SDS, 65°C. A fragment of 5.2 kilobase pairs (kb) was detected in *SphI* digested C57BL/6J DNA, and a fragment of 9.8 kb was detected in *SphI* digested *M. spretus* DNA. The presence or absence of the 9.8 kb *M. spretus*-specific *SphI* fragment was followed in backcross mice. A description of the probes and restriction fragment length polymorphisms (RFLPs) for the loci linked to *Gdf10*, including plasminogen activator, urokinase (*Plau*), and SP-A pulmonary surfactant protein (*Sftp1*), has been reported previously (Moore et al., 1992). One locus not previously reported is retinol binding protein interstitial (*Rbp3*). The probe was an ~2.2 kb *EcoRI* fragment of human cDNA that detected major *SacI* fragments of 4.4, 1.9, and 1.6 kb (C57BL/6J) and 5.4 kb (*M. spretus*). Recombination distances were calculated as described (Green, 1981) using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of recombination events required to explain the allele distribution patterns.

RESULTS

Identification and Expression of GDF-10

In order to isolate a cDNA clone encoding murine BMP-3, we designed degenerate oligonucleotides corresponding to the known human BMP-3 amino acid sequence and used these oligonucleotides for polymerase chain reactions on cDNA prepared from murine brain and lung mRNA. Nucleotide sequence analysis of individual cloned PCR products showed that in addition to BMP-3, a sequence related to BMP-3 but distinct from other known members of the TGF- β superfamily had been amplified in these reactions. This novel sequence was designated GDF-10.

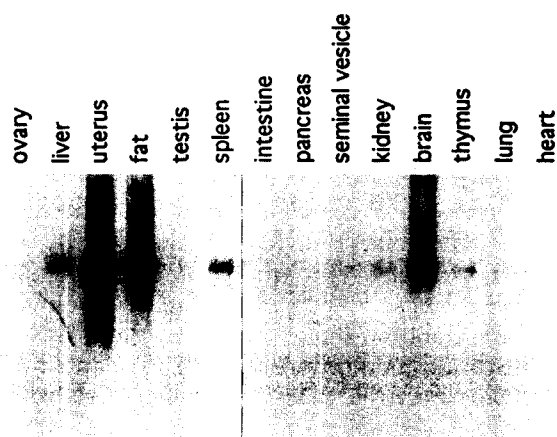
To determine the pattern of expression of GDF-10, Northern analysis was carried out using poly (A)⁺ RNA prepared from a number of adult mouse tissues. As shown in Fig. 1a, the GDF-10 probe detected an approximately 2.7 kilobase transcript expressed at highest levels in uterus, adipose tissue, and brain and at lower levels in liver and spleen. An identical blot probed a murine BMP-3 probe revealed a completely different pattern of bands (data not shown) consistent with the previously-reported pattern of BMP-3 expression (Ozkaynak et al., 1992); hence, under these hybridization and washing conditions, there was no cross-hybridization between the GDF-10 and BMP-3 probes. Because GDF-10 is highly related to BMP-3 (see below), we also examined the expression of GDF-10 mRNA in bone. As shown in Fig. 1b, GDF-10 mRNA was detected in various bone samples with higher levels being detected in calvaria than in femur, both in neonatal and in adult animals.

Sequence of Murine GDF-10

In order to isolate a full-length cDNA clone encoding GDF-10, a uterine cDNA library consisting of 3 million independent recombinant phage was constructed in the lambda ZAP II vector. From this library seven phage were isolated that hybridized to a probe derived from the original GDF-10 PCR product. The entire nucleotide sequence of the longest of these cDNA clones is shown in Fig. 2. The 2322 base pair sequence contains a single long open reading frame beginning with a methionine codon at nucleotide 126. The encoded polypeptide is 476 amino acids in length with a predicted molecular weight of approximately 52,500, as determined by nucleotide sequence analysis. The predicted GDF-10 amino acid sequence contains a core of hydrophobic amino acids near the N-terminus suggestive of a signal sequence for secretion. GDF-10 contains four potential N-linked glycosylation sites at asparagine residues 114, 152, 277 and 467.

The predicted GDF-10 sequence contains two potential proteolytic processing sites, one at amino acid residues 283–286 (RVRR) and another at amino acid residues 331–334 (RTAR). Cleavage of the GDF-10 precursor at the first site would generate a mature GDF-10 protein 190 amino acids in length with a predicted nonglycosylated molecular weight

(A)



(B)

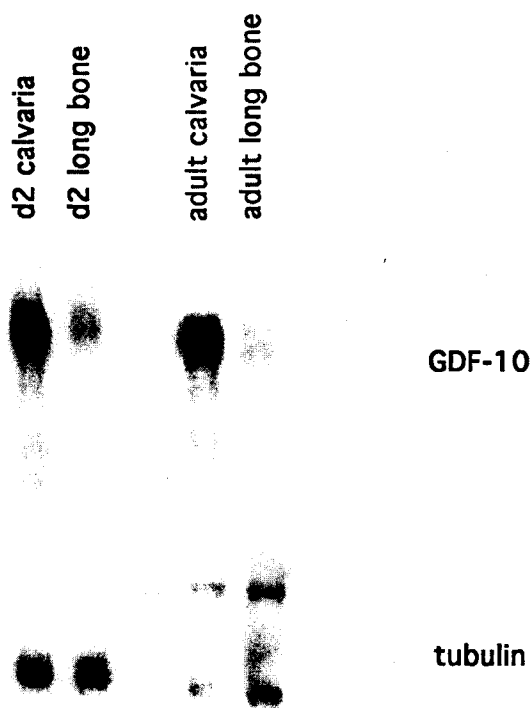


FIGURE 1. Expression of GDF-10 mRNA. (A) 2.5 micrograms of poly (A)⁺ RNA prepared from each adult tissue were electrophoresed on a formaldehyde gel, transferred to Genescreen, and probed with GDF-10 cDNA. (B) Twelve μ g total RNA prepared from calvaria or long bone of neonatal day 2 or adult animals were electrophoresed on a formaldehyde gel, transferred to Genescreen, and probed with GDF-10 cDNA. Following autoradiography, this blot was stripped and probed with a tubulin cDNA to demonstrate equivalent loading.

of ~22,000; cleavage at the more distal site would generate a mature GDF-10 protein 142 amino acids in length with a predicted nonglycosylated molecular weight of ~16,000. Verification of which processing site is actually utilized will have to await N-terminal sequencing of mature GDF-10; however, by analogy with BMP-3 (see below), we presume that the more distal site is the one that is used *in vivo*. Several other TGF- β family members have been shown to contain multiple RXXR sites in the pro-region (Ozkaynak *et al.*, 1992).

The C-terminal region of GDF-10 following the putative proteolytic processing site shows significant homology to the known members of the TGF- β superfamily (Fig. 3). The predicted GDF-10 sequence contains most of the highly conserved amino acids present in the other family members, including the seven cysteine residues with their characteristic spacing. A tabulation of the sequence comparisons between GDF-10 and the other family members in the C-terminal region is shown in Fig. 4. Among the known mammalian TGF- β family members, GDF-10 is most homologous to BMP-3 (83% amino acid sequence identity beginning with first conserved cysteine residue). GDF-10 also shows significant homology to BMP-3 (~30% amino acid sequence identity) in the pro-region of the molecule. Based on these sequence comparisons, GDF-10 and BMP-3 define a new subfamily within the larger superfamily.

Comparison of Human and Murine GDF-10

Members of the TGF- β superfamily typically show extensive conservation across species. To isolate human GDF-10, a human uterine cDNA library consisting of 16.2 million independent recombinant phage was constructed in lambda ZAP II and screened with the full length murine GDF-10 probe. From this library, 20 hybridizing clones were isolated. Partial nucleotide sequence analysis of the longest clone showed that human and murine GDF-10 are highly homologous; the predicted amino acid sequences are 97% identical, beginning with the first conserved cysteine residue following the predicted cleavage site (Fig. 3). This high degree of conservation of GDF-10 across species supports the notion that GDF-10 may play an essential regulatory role in embryos and/or in adult animals.

1	TGGGGTCATCCGGGCTGTCCGAGTCCCACAGGGACAACTCCAGCCGCGGACGAGGTGCAC	60
61	AGCCAACACTGAGCCCTCCTTGTCTGTCTCCTGGGCTCAGACCCTTCACCACCGTTACT	120
121	CAGCCATGGCTCCAGGTCCTGCTCGGATCAGCTTGGGGTCCCAGCTGCTGCCCATGGTGC	180
	M A P G P A R I S L G S Q L L P M V P	
181	CGTGCTCCTGTGCTGCGGGGCGCAGGCTGCGGCCACAGGGGCCCTCATGGTCTCAT	240
	L L L L L R G A G C G H R G P S W S S L	
241	TGCCCTCGGCAGCTGCCGGTCTGCAGGGGACAGGACTCCCAGCAGTCACCCGGGGACG	300
	P S A A A G L Q G D R D S Q Q S P G D A	
301	CAGCAGCCGCTCTGGGCCCAGGCGCCAGGACATGGTCGCTATCCACATGCTCAGGCTCT	360
	A A A L G P G A Q D M V A I H M L R L Y	
361	ATGAGAAGTACAACCGAAGAGGTGCTCCACCGGGAGGAGGCAACACCGTCCGAAGCTTCC	420
	E K Y N R R G A P P G G G N T V R S F R	
421	GTGCCCCGCTGGAATGATCGACCAAAAGCCTGTGTATTTCTTCAACTTGACTTCCATGC	480
	A R L E M I D Q K P V Y F F N L T S M Q	
481	AAGACTCGAAAATGATCTCACAGCCGCTTCCACTTCTACTCAGAACCTCCACGGTGGC	540
	D S E M I L T A A F H F Y S E P P R W P	
541	CCCGGGCTGGTGAAGTATTCTGCAAGCCCCGAGCTAAGAACGCATCTGCGCCCTCTGA	600
	R A G E V F C K P R A K N A S C R L L T	
601	CCCCAGGCTGCTGCAAGCTTGCACCTAATCTTCCGAGTCTTTCCAGAACACCGCCA	660
	P G L P A R L H L I F R S L S Q N T A T	
661	CTCAGGGCTGCTCCGCGGGCCATGGCCCTGACGCCTCCACCACGTGGCCTGTGGCAGG	720
	Q G L L R G A M A L T P P P R G L W Q A	
721	CCAAGGACATCTCCTCAATCATCAAGGCTGCCGGAAGGGATGGAGAGCTGCTTCTCTG	780
	K D I S S I I K A A R R D G E L L L S A	
781	CTCAGCTGGATACTGGGAGAAGGACCCCGAGTGCCACGGCCAGTCCACATGCCCT	840
	Q L D T G E K D P G V P R P S S H M P Y	
841	ATATCCTTGTCTACGCCAATGACCTGGCCATCTCCGAACCCAAACAGTGTAGCAGTGTGCG	900
	I L V Y A N D L A I S E P N S V A V S L	
901	TACAGAGATACGACCCATTTCAGCTGGAGACTTTGAGCCTGGAGCAGCCCCAACAGCT	960
	Q R Y D P P P A G D F E P G A A P N S S	
961	CAGCTGATCCCCGCTGCGCAGGGCGGCTCAGGTGTCAAAACCCCTGCAAGACAATGAAC	1020
	A D P R V R R A A Q V S K P L Q D N E L	
1021	TGCCGGGGCTGGATGAAAGACCAGCGCCTGCCCTGCATGCCCAGAATTTCCACAAGCAGC	1080
	P G L D E R P A P A L H A Q N F H K H E	
1081	AGTTCTGGTCCAGTCTTTCCGGGCACTGAAACCCCGCAGCGCGCAAGACCCGAAGA	1140
	F W S S P F R A L K P R T A R K D R K K	
1141	AGAAGGACCAAGACACATTACCGCGCCTCCTCTCAGGTGCTGGACTTTGACGAGAAGA	1200
	K D Q D T F T A A S S Q V L D F D E K T	
1201	CGATGCAGAAAGCCAGGAGCGGCAAGTGGGATGAGCCCCGGGTCTGCTCCAGGAGGTACC	1260
	M Q K A R R R Q W D E P R V C S R R Y L	
1261	TGAAGGTGGATTTTGCAGACATCGGGTGAATGAATGGATCATCTCTCCAAATCCTTTG	1320
	K V D F A D I G W N E W I I S P K S F D	
1321	ACGCCTACTACTGTGCTGGGGCCTGCGAGTTCCCATGCCCAAGATTGTCCGCCCATCCA	1380
	A Y Y C A G A C E F P M P K I V R P S N	
1381	ACCATGCCACCATCAGAGCATCGTCAGAGCTGTGGGCATTGTCCCTGGCATCCAGAGC	1440
	H A T I Q S I V R A V G I V P G I P E P	
1441	CATGCTGTGTTCCAGACAAGATGAATCCCTTGGAGTCTTTTCTGGATGAAAATCGGA	1500
	C C V P D K M N S L G V L F L D E N R N	
1501	ATGCGGTTCTGAAGGTGTACCCCAATATGTCCTAGAGACCTGTGCCTGTGCGTAAGATG	1560
	A V L K V Y P N M S V E T C A C R *	
1561	GCTTCAAGATAGAAGACAGACCTGCTTCATCCCTGCCCTGCAGAGTGGCAATCTTGGAGC	1620
1621	CAGGGACTTGACTCGGGGAGGTTCCAGGTGCTAGACAGAGCTTACAGGCAGCCCTGCTGG	1680
1681	GACCAAGAAAGATCTGCCACACATCGCAATTCTTCAGTTCTTCCGTGCTGGTGGTAGC	1740
1741	TCTGTAAAGACGTTGTGAGTTCTCTGGAAGAAATCTGGAATTAATGTGGTCTGCAATTG	1800
1801	CCCATCATCCCTGCCACACTTTTCAAGGCCTAGAAATAACGTGTGTCTCAAATGTCAA	1860
1861	CTCCAGGCAATTTGCTCTCAAACCTAGAAAGACTATGCAAAATCTTGGGGTACTCCGCC	1920
1921	CCCCCATGGCAGTTAAATGCTGTTTTAAACCCCTCAGGCTGCATTCTAGAAACAGGGCC	1980
1981	TAACCCATGGCAGGAGTGAATTTTCTTACGTTTCACTACAGTCTTTTATACATG	2040
2041	CAGTATGCACATGTAATCACGGTTGATTCTTTTAAATATATGTAATTTCTATTTCAA	2100
2101	GCAAAACGGAGAGAGTCGATCCCATCCCTGCAGAGGTAATTAATGCAAGTTAGGTGTTGG	2160
2161	TTGCTAAGCATGTGTATGGAATAATACATACAGTAATATGCTGGAATACAAAAAAGT	2220
2221	AACCAAGATTTTATATTTTGTAAATTAATCTTTGTATCTGTAGATTGTGAGTGTCTG	2280
2281	TGTTTTATGGAAGCTAATAAATTAAGGTGCGGAGGTATC	2322

FIGURE 2. Nucleotide and predicted amino acid sequence of GDF-10. The entire nucleotide sequence of GDF-10 derived from a single cDNA clone is shown with the predicted amino acid sequence below. The poly (A) tail is not shown. Consensus N-glycosylation signals are denoted by open boxes. The putative RXXR processing sites are denoted by shaded boxes. Numbers indicate nucleotide position relative to the 5' end of the clone.

mGDF-10	KTMQKARRROWDEPRVCSRRYLKVDF-ADIGWNEWIISPKSFDAYYCAGACEFPMPKIVRP----
hGDF-10	KTMQKARRKQWDEPRVCSRRYLKVDF-ADIGWNEWIISPKSFDAYYCAGACEFPMPKIVRP----
BMP-3/osteogenin	QTLKKARRKQWIEPRNCARRYLKVDF-ADIGWSEWIIISPKSFDAYYCAGACEFPMPKSLKP----
BMP-2	EKRQAKHKQKRLKSSCKRHPLYVDF-SDVGWNDWIVAPPGYHAFYCHGEOPFPLADHLNS----
BMP-4	RSPKHHSQARKKKNKRRHSLYVDF-SDVGWNDWIVAPPGYHAFYCHGEOPFPLADHLNS----
BMP-5	RMSSVGDYNTSEQKQACKKHELYVSF-RDLGWQDWIIAPEGYAAYYCEGECFPLNAHMNA----
Vgr-1 (BMP-6)	RGSGSSDYNGSELKTA CKKHELYVSF-QDLGWQDWIIAPKGYAANYCEGECFPLNAHMNA----
OP-1 (BMP-7)	RMANVAENSSSDQRA CKKHELYVSF-RDLGWQDWIIAPEGYAAYYCEGECFPLNSYMNA----
OP-2 (BMP-8)	LPGIFDDVHGSHGROVRRHLYVSF-QDLGWLDWVIAPOGYSAYYCEGECFPLDSCMNA----
GDF-5 (CDMP-1)	SATROGKRPSKNLKARCSRKALHVNFKDMGWDDWIIAPLEYEAFHCEGLCEFPPLRSHLEP----
GDF-6 (CDMP-2)	FASRHGKRHGKKSRLRCKKPLHVNFKELGWDDWIIAPLEYEAFHCEGLCEFPPLRSHLEP----
GDF-7	GGGGAGRGHGRGRSRCSRKSLHVDF-KELGWDDWIIAPLDYEAHYCEGLCEFPPLRSHLEP----
GDF-1	PRRDAEPVLGGGPGGACRRRLYVSF-REVGWHRWVIAPRGFLANYCQGQCALPVALSGSGGPP----
GDF-3/Vgr-2	KRRRAISVPKGFRCRNFCHRHQLFINF-QDLGWHWVIA PKGFMANYCHGEOPFSMTTYLNS----
GDF-9	FNLSEYFRQFLLPQNECELHDFRLSF-SQLKWDNWIVAPHRYNPRYCKGDCPRAGRHRYS----
MIS	PGRAQSAGATAADGPGALRELSVDL----RAERSVLIPETYQANNQGVGWGWSDRNPRY----
inhibin α	LRLQLRPEEPAAHANCHRVALNISF-QELGWERWIVYPPSFIFHYCHGCGGLHIPNLSLPV----
inhibin β A	RRRGLCECDGKV--NIECKKQFFVSF-KDIGWNDWIIAPSGYHANYCEGECPSHIAGTSGSSL----
inhibin β B	IRKRGLECDGRT--NLCCRQOFFIDF-KDIGWNDWIIAPSGYHANYCEGECPSHIAGTSGSSL----
nodal	WGRRQRHHLPRDSQLCRRVKFOVDF-NLIGWGSWIIYPKQYNAYRCEGECPNPVGEEFHP----
GDNF	NSRGKGRGQRGKNGCVLTAIHLNV--TDLGLGYETKEELIFRYSGSGSDAAETTYDKI----
TGF- β 1	RALDTNYCFSSSTE-KNCCVRQLYIDFRKDLGWK-WIHEPKGYHANFCLGFCPIYIWSLD-----
TGF- β 2	RALDAAYCFRNVQ-DNCCLRPLYIDFRKDLGWK-WIHEPKGYANFCLGFCPIYIWSLD-----
TGF- β 3	RALDTNYCFRNLN-ENCCVRPLYIDFRQDLGWK-WVHEPKGYANFCLGFCPIYIWSLD-----
mGDF-10	-SNHATIQSIVRA-VGIVPGIPEPCCV--PDKMNSLGVLF-DENRNVVLKVYPNMSVETCAACR
hGDF-10	-SNHATIQSIVRA-VGIIPGIPEPCCV--PDKMNSLGVLF-DENRNVVLKVYPNMSVETCAACR
BMP-3/osteogenin	-SNHATIQSIVRA-VGVVPGIPEPCCV--PEKMSSLSILFF-DENKNNVLKVYPNMTVECAACR
BMP-2	-TNHAIQTLVNS---VNSKIPKACCV--PTELSAISMLYL-DENEKVVLKKNYQDMVVECGGCR
BMP-4	-TNHAIQTLVNS---VNSSIPKACCV--PTELSAISMLYL-DEYDKVVLKKNYQDMVVECGGCR
BMP-5	-TNHAIQTLVHL--MFPDHPVPKPCA--PTKLNAISVLYF-DDSNVILKKYRNMVVRACGCH
Vgr-1 (BMP-6)	-TNHAIQTLVHL--MNPEYVVPKPCA--PTKLNAISVLYF-DDSNVILKKYRNMVVRACGCH
OP-1 (BMP-7)	-TNHAIQTLVHL--INPETVPKPCA--PTQLNAISVLYF-DDSNVILKKYRNMVVRACGCH
OP-2 (BMP-8)	-TNHAIQTLVHL--MFPDHPVPKPCA--PTKLNAISVLYF-DDSNVILKKYRNMVVRACGCH
GDF-5 (CDMP-1)	-TNHAIQTLVNS--MDPESTPTTCCV--PTRLSPISILFI-DSANNVVKQYEDMVVECGGCR
GDF-6 (CDMP-2)	-TNHAIQTLVNS--MDPGSTPTTCCV--PTKLTPISILYI-DAGNNVYNEYEEMVVECGGCR
GDF-7	-TNHAIQTLVNS--MAPDAAPASCCV--PARLSPISILYI-DAANNVVKQYEDMVVECGGCR
GDF-1	ALNHAVLRALMHA--AAPGAADLPCCV--PARLSPISVLF-DNSDNVVLKQYEDMVVECGGCR
GDF-3/Vgr-2	-SNYAFMQALMHM---ADPKVPKACCV--PTKLSPISMLYQ-DSDKNVILRHVEDMVVECGGCR
GDF-9	-PVHTMVQNIYE--KLDSSVPRPSCV--PAKYSPLSVLTI-EPDGSIAKEYEDMIATRCR
MIS	-GNHVLLLLKMQA--RGAALARPPCCV--PTAYAGKLLISLSEER--ISAHHVPMVATECGGCR
inhibin α	-FGAPPTPAQFYS---LLPGAQPCCAALPGTMRPLHVRTTSDGGYSFKYETVNNLLTQHCACI
inhibin β A	-SFHSTVINHYMRGHSPTFANLKSCCV--PTKLSPISMLYI-DDGQNIKKDIQNMIVEECGCS
inhibin β B	-SFHTAVVNQYMRGLNPGT-VNSCCI--PTKLSTMSMLYF-DDEYNIVKRDVPMNIVEECGCA
nodal	-TNHAIQTLVNS--YQPHRVPSTCCA--PVKTKPLSMLYV--DNGRVLLHHKDMIVEECGCL
GDNF	-LKNLSRNRRLVS---DKVGQACCR--PIAFDDDLISFL--DDNLVYHIL--RKHSARKGCCI
TGF- β 1	-TQYSKVLALYNQ--HNPGASAPCCV--PQALEPLPIVYV-VGRKPKV-EQLSNMIVKSCCKCS
TGF- β 2	-TQHSRVLSLYNT--INPEASAPCCV--SQDLEPLTILYI-IGKTPKI-EQLSNMIVKSCCKCS
TGF- β 3	-TTHSTVLGLYNT--LNPEASAPCCV--PQDLEPLTILYI-VGRTPKV-EQLSNMIVKSCCKCS

FIGURE 3. Alignment of the COOH-terminal sequence of GDF-10 with other members of the TGF- β superfamily. The alignment of murine and human GDF-10 with corresponding regions of human BMP-3/osteogenin, BMP-2 and BMP-4 (Wozney *et al.*, 1988), human BMP-5, human Vgr-1 (BMP-6) (Celeste *et al.*, 1990), human OP-1 (BMP-7) (Ozkaynak *et al.*, 1990), human OP-2 (BMP-8) (Ozkaynak *et al.*, 1992), human GDF-5 (CDMP-1), human GDF-6 (CDMP-2) (Chang *et al.*, 1994), murine GDF-7 (Storm *et al.*, 1994), human GDF-1 (Lee, 1991), murine GDF-3/Vgr-2 (Jones *et al.*, 1992; McPherron and Lee, 1993), murine GDF-9 (McPherron and Lee, 1993), human MIS (Cate *et al.*, 1986), human inhibin α , human inhibin β A, human inhibin β B (Mason *et al.*, 1985), murine nodal (Zhou *et al.*, 1993), human GDNF (Lin *et al.*, 1993), human TGF- β 1 (Derynck *et al.*, 1985), human TGF- β 2 (de Martin *et al.*, 1987), and human TGF- β 3 (Derynck *et al.*, 1988; ten Dijke *et al.*, 1988). The conserved cysteine residues are shaded. Dashes denote gaps introduced in order to maximize alignment.

GDF-1
BMP-3
BMP-2
BMP-4
BMP-5
Vgr-1
OP-1
OP-2
GDF-5
GDF-6
GDF-7
GDF-1
GDF-2
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MIS
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	GDF-10	BMP-3/osteogenin	BMP-2	BMP-4	BMP-5	Vgr-1 (BMP-6)	OP-1 (BMP-7)	OP-2 (BMP-8)	GDF-5 (CDMP-1)	GDF-6 (CDMP-2)	GDF-7	GDF-1	GDF-3/Vgr-2	GDF-9	MIS	inhibin α	inhibin β A	inhibin β B	nodal	GDNF	TGF- β 1	TGF- β 2	TGF- β 3
GDF-10	100	83	46	45	42	44	42	40	43	42	42	40	39	28	31	29	35	34	40	19	29	30	29
BMP-3/osteogenin	-	100	48	47	43	44	42	41	47	44	46	42	42	29	30	29	36	37	41	17	32	32	32
BMP-2	-	-	100	92	61	61	60	55	57	54	57	42	53	34	27	22	42	42	42	23	35	34	36
BMP-4	-	-	-	100	59	60	58	55	57	55	57	43	50	35	27	22	41	42	40	22	34	33	35
BMP-5	-	-	-	-	100	91	88	74	52	54	52	46	50	31	24	24	43	37	40	23	34	35	36
Vgr-1 (BMP-6)	-	-	-	-	-	100	87	75	51	53	52	46	53	31	24	25	44	41	43	21	35	37	39
OP-1 (BMP-7)	-	-	-	-	-	-	100	74	51	53	53	47	50	30	27	24	43	42	41	22	34	38	38
OP-2 (BMP-8)	-	-	-	-	-	-	-	100	50	51	53	47	55	27	27	26	42	37	44	24	30	35	38
GDF-5 (CDMP-1)	-	-	-	-	-	-	-	-	100	82	80	46	48	32	27	24	40	37	42	22	33	34	37
GDF-6 (CDMP-2)	-	-	-	-	-	-	-	-	-	100	82	43	46	32	26	25	43	38	43	22	34	35	37
GDF-7	-	-	-	-	-	-	-	-	-	-	100	48	45	33	25	26	41	36	45	24	36	35	38
GDF-1	-	-	-	-	-	-	-	-	-	-	-	100	50	28	34	23	37	35	35	18	33	32	33
GDF-3/Vgr-2	-	-	-	-	-	-	-	-	-	-	-	-	100	32	22	25	42	41	40	24	36	31	32
GDF-9	-	-	-	-	-	-	-	-	-	-	-	-	-	100	21	26	30	30	31	20	22	25	24
MIS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	18	24	25	22	18	28	23	25
inhibin α	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	26	25	23	20	23	22	24
inhibin β A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	63	39	18	41	37	36
inhibin β B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	37	22	35	34	37
nodal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	19	28	32	31
GDNF	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	19	17	21
TGF- β 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	74	78
TGF- β 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	82
TGF- β 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100

FIGURE 4. Amino acid homologies among different members of the TGF- β superfamily. Numbers represent percent amino acid identities between each pair calculated from the first conserved cysteine to the carboxy-terminus.

Chromosomal Localization of GDF-10

The mouse chromosomal location of *Gdf10* was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J) \times *Mus spretus*] F_1 \times C57BL/6J mice. This interspecific backcross mapping panel has been typed for over 1500 loci that are well distributed among all the autosomes as well as the X chromosome (Copeland and Jenkins, 1991). C57BL/6J and *M. spretus* DNAs were digested with several enzymes and analyzed by Southern blot hybridization for informative restriction fragment length polymorphisms (RFLPs) using a mouse cDNA probe. A 9.8 kb *M. spretus* *SphI* RFLP (see Materials and Methods) was used to follow the segregation of the *Gdf10* locus in backcross mice. The mapping results indicated that *Gdf10* is located in the proximal region of mouse chromosome 14 linked to *Plau*, *Rbp3*, and *Sftp1*.

Although 142 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 5), up to 179 mice were typed for some pairs of markers. Each locus was analyzed in pairwise combinations for recombination frequencies using the additional data. The ratios of the total number of mice exhibiting recombinant chromosomes to the total number of mice analyzed for each pair of loci and the most likely gene order are: centromere-*Plau*-17/179-*Gdf10*-0/154-*Rbp3*-2/154-*Sftp1*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) \pm the standard error] are -*Plau*-9.5 \pm 2.0-[*Gdf10*, *Rbp3*]-1.3 \pm 0.9-*Sftp1*. No recombinants were detected between *Gdf10* and *Rbp3* in 154 animals typed in common suggesting that the two loci are within 1.9 cM of each other (upper 95% confidence limit). The proximal region of mouse chromosome 14 shares a region of homology with human chromosome 10q (summarized in

Fig. 5). In particular, *Rbp3* has been placed on human 10q11.2. The tight linkage between *Rbp3* and *Gdf10* in mouse suggests that *Gdf10* will reside on 10q, as well.

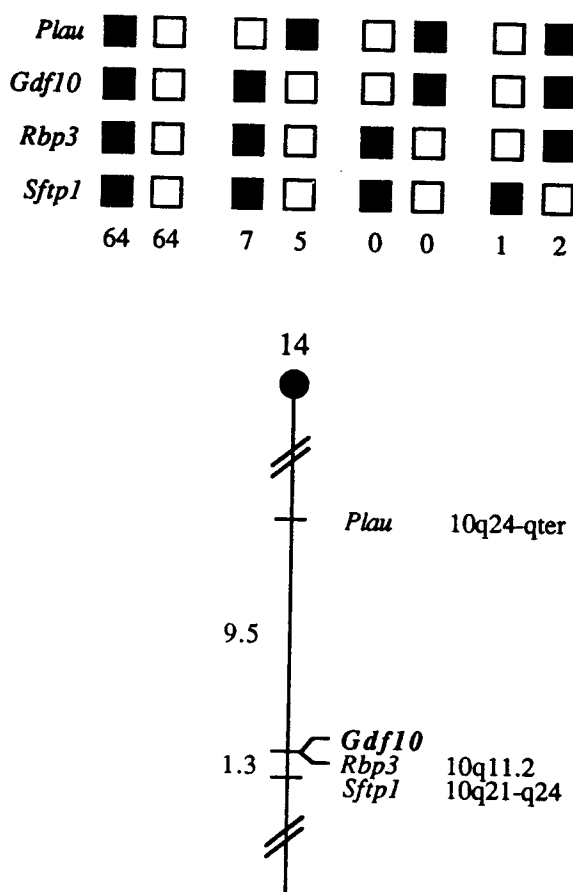


FIGURE 5. *Gdf10* maps in the proximal region of mouse chromosome 14. *Gdf10* was placed on mouse chromosome 14 by interspecific backcross analysis. The segregation patterns of *Gdf10* and flanking genes in 142 backcross animals that were typed for all loci are shown at the top of the figure. For individual pairs of loci, more than 142 animals were typed (see text). Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J) × *M. spretus* F₁ parent. The shaded boxes represent the presence of a C57BL/6J allele, and white boxes represent the presence of an *M. spretus* allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. A partial chromosome 14 linkage map showing the location of *Gdf10* in relation to linked genes is shown at the bottom of the figure. Recombination distances between loci in centimorgans are shown to the left of the chromosome, and the positions of loci in human chromosomes, where known, are shown to the right. References for the human map positions of loci mapped in this study can be obtained from GDB (Genome Data Base), a computerized database of human linkage information maintained by The William H. Welch Medical Library of The Johns Hopkins University (Baltimore, MD).

DISCUSSION

The present study describes a novel member (GDF-10) of the transforming growth factor- β superfamily. The nucleotide sequence of GDF-10 encodes a predicted protein of 476 amino acids with molecular weight of approximately 52,500 which has all of the hallmarks of the other family members. The deduced GDF-10 protein has a potential signal sequence for secretion, a putative proteolytic processing site, and the seven invariant cysteine residues found in the C-terminal mature protein of most family members. Tabulation of amino acid sequence homologies with other family members reveals that GDF-10 is most homologous to BMP-3, showing 83% amino acid sequence identity beginning with the first conserved cysteine. GDF-10 also shows significant homology to BMP-3 (approximately 30% amino acid sequence identity) in the pro-region of the molecule.

The BMPs can be divided into subgroups based on the degree of amino acid sequence identity in the mature regions of the proteins. BMP-2 and BMP-4 (Wozney *et al.*, 1988) are 92% identical in the C-terminal region and define one subgroup. BMP-5, Vgr-1 (BMP-6), OP-1 (BMP-7), and OP-2 (BMP-8) (Lyons *et al.*, 1989a; Celeste *et al.*, 1990; Ozkaynak *et al.*, 1990) are 74–91% identical and form another subgroup. GDF-5 (CDMP-1), GDF-6 (CDMP-2), and GDF-7 (Chang *et al.*, 1994; Storm *et al.*, 1994) are 80–82% identical and form a third subgroup. Until now, BMP-3/osteogenin (Wozney *et al.*, 1988; Luyten *et al.*, 1989) was in a fourth subgroup by itself. The identification of GDF-10 suggests that this subgroup also contains multiple members.

Many of the BMPs, as the name suggests, have been demonstrated to have osteogenic potential *in vivo*. Experiments with protein purified from bone extracts or purified recombinant proteins have shown that BMP-2, BMP-3, BMP-4, BMP-5 and OP-1 can induce ectopic cartilage and bone formation (Wozney *et al.*, 1988; Luyten *et al.*, 1989; Celeste *et al.*, 1990; Wang *et al.*, 1990; Cox *et al.*, 1991; D'Alessandro *et al.*, 1991; Hammonds *et al.*, 1991; Sampath *et al.*, 1992). Based on the extensive amino acid sequence homology between GDF-10 and BMP-3 and based on the known ability of BMP-3 to induce bone formation in the rat subcutaneous implant assay (Luyten *et al.*, 1989), it is expected that GDF-10 will also have osteogenic activity. In addition, several of the BMPs have been shown to be involved in the promotion and

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maintenance of cartilage and bone cell phenotypes *in vitro*. For example, BMP-2 (Takuwa et al., 1991) and BMP-3 (Vukicevic et al., 1990) can stimulate alkaline phosphatase activity and collagen synthesis in fetal rat calvarial periosteal cells and osteoblasts. Similarly, in cultures of chick limb bud mesenchymal cells, BMP-3 and BMP-4 can stimulate the differentiation of mesenchymal cells to cells of the chondrocytic lineage as assessed by type II collagen synthesis, proteoglycan profiles, and alcian blue staining (Carrington et al., 1991; Chen et al., 1991). Finally, genetic studies have demonstrated that at least some of these factors play essential roles in regulating skeletal development *in vivo*. In particular, the BMP-5 and GDF-5 genes have been shown to be mutated in *short ear* and *brachypodism* mice, respectively (Kingsley et al., 1992; Storm et al., 1994). Because the specific skeletal abnormalities observed in *short ear* and *brachypodism* mutants differ, it seems likely that specific aspects of skeletal morphogenesis may be regulated by individual members of the BMP family.

Roles other than those involving cartilage and bone formation have been suggested by analysis of the expression patterns of BMPs during mouse embryogenesis. *In situ* hybridization studies have detected BMP-2 transcripts in a variety of embryonic epithelial and mesenchymal tissues, including developing heart, whisker follicles, and tooth buds, as well as in the developing limb bud (Lyons et al., 1989b, 1990; Vainio et al., 1993). BMP-4 mRNA has been detected in developing limb buds, nervous system, heart, and craniofacial tissues (Jones et al., 1991; Vainio et al., 1993). And *Vgr-1* has been shown to be expressed in developing squamous epithelia and nervous system (Lyons et al., 1989b; Jones et al., 1991). The expression of these genes at sites unrelated to bone development suggests that the BMPs have a more wide-spread function *in vivo* than originally appreciated and may play multiple regulatory roles during embryogenesis.

Gdf10 maps to a region of mouse chromosome 14 known to contain a spontaneous recessive mutation termed *pugnose* (*pn*). Mice homozygous for *pugnose* display craniofacial defects that involve abnormalities in the growth and development of skull bones (Kidwell et al., 1961; Green, 1989). Examination of the skeletons from these animals revealed that parietal, frontal, and nasal bones were shorter and wider than normal and slightly misshapen to produce the characteristic facial appearance. Moreover, fertility is markedly reduced in *pugnose* females

(Kidwell et al., 1961); in particular, many of the mutant females either fail to conceive or fail to deliver their young, and mutant females that do deliver at term have a high incidence of uterine prolapse. The chromosomal location of *Gdf10*, the high degree of sequence homology between GDF-10 and a known bone inducing factor, and the high level of GDF-10 expression in the uterus and in calvaria all suggest that GDF-10 may be a reasonable candidate for the gene mutated in *pugnose* mice. The BMP-2/BMP-4 type I receptor gene also maps to this location and is therefore also a possible candidate for *pugnose* (Mishina et al., 1995). Because the *pugnose* mutant strain appears to be extinct, however, definitive proof that either GDF-10 or the BMP-2/BMP-4 receptor is involved in regulating the specific processes that are affected in *pugnose* mice will await the development of knock-out mice in which these genes have been disrupted by homologous recombination.

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