# The Bone Morphogenetic Protein 15 Gene Is X-Linked and Expressed in Oocytes

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**We have taken advantage of the sequence relationships among the bone morphogenetic proteins (BMPs) to identify the mouse *Bmp15* and human *BMP15* genes. The 392-amino acid prepropeptides encoded by these BMP genes exhibit significant homology to each other, although the 70% identity observed between the 125-amino acid mature peptides is considerably lower than that seen in comparisons of other mouse and human orthologs. Both genes share a common structural organization and encode mature peptides that lack the cysteine residue normally involved in the formation of a covalent dimer. In addition, mouse *Bmp15* and human *BMP15* map to conserved syntenic regions on the X chromosome. We demonstrate, through a combination of Northern blot and *in situ* hybridization analyses, that mouse *Bmp15* is expressed specifically in the oocyte beginning at the one-layer primary follicle stage and continuing through ovulation. Interestingly, BMP-15 is most closely related to and shares a coincident expression pattern with the mouse growth/differentiation factor 9 (GDF-9) gene that is essential for female fertility. Our findings will be important for defining the role of BMP-15 in follicular development. (Molecular Endocrinology 12: 1809–1817, 1998)**

## INTRODUCTION

The transforming growth factor-$\beta$ (TGF$\beta$) superfamily, which includes the bone morphogenetic proteins (BMPs) and the growth/differentiation factors (GDFs), is the largest family of extracellular signaling proteins yet described. Homology-based cloning approaches have resulted in the identification of TGF$\beta$-like factors in such diverse species as *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Xenopus laevis* and have extended the number of mammalian members to more than 30 (1–3). These molecules, synthesized as prepropeptides and processed into dimeric proteins, are structurally related in their mature, carboxy-terminal region. Members of the BMP subfamily have been shown to be involved in a wide array of cellular processes during embryonic development. For example, BMP-2 and BMP-4 are required for cellular differentiation in early embryogenesis (4, 5), BMP-5 is required for the formation of specific skeletal structures (6), BMP-7 is necessary for normal eye and kidney development (7), and BMP-8a and BMP-8b are essential factors for spermatogenesis (8, 9). BMP subfamily members have also been shown to affect adult tissue repair. Animal models have demonstrated the ability of several BMPs to induce the formation of bone and cartilage (10, 11) and tendon/ligament (12). Identification of new members in this gene family is important for continued characterization of the key factors involved in mammalian development and physiology.

Reproductive development and function are complex processes involving both genetically determined and physiological events. Discerning the role that each of these growth factors plays *in vivo* would lead to a better understanding of the complex and sex-specific physiology of the mammalian reproductive system. Several members of the TGF$\beta$ superfamily, such as the inhibins and activins (13), Müllerian inhibiting substance (14), BMP-8a (8),

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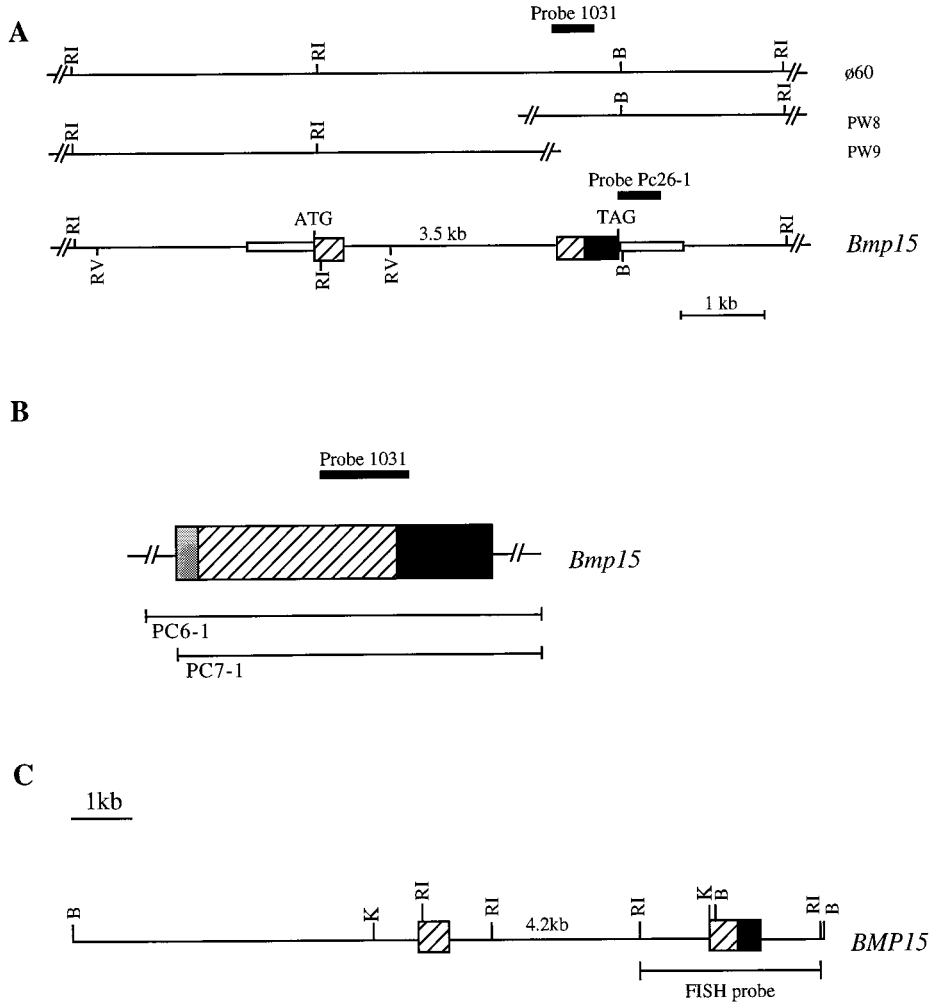


**Fig. 1.** Genomic and cDNA Representations of Mouse *Bmp15* and Human *BMP15*

In each case, *diagonally shaded boxes* indicate prepropeptide-encoding domains and *black boxes* indicate mature-encoding domains. A, Shown on the *bottom*, the structure of the mouse *Bmp15* gene as determined by overlapping phage clones and 5′-RACE. The entire protein-coding sequence is contained within two exons, separated by a 3.5-kb intron. 5′- and 3′-UTR sequences (*open boxes*) indicate the limits of the exons. Probe Pc26–1 is the region used to map the mouse *Bmp15* gene. B, Schematic representation of mouse *Bmp15* coding sequence and two representative clones isolated from the mouse ovary cDNA library. The signal peptide-encoding sequence is shaded. Probe 1031, which includes a portion of the propeptide- and mature-encoding domains, is shown. C, Structure of the human *BMP15* gene. The entire protein-coding sequence is contained within two exons (shown with *boxes*), separated by a 4.2-kb intron. A 3-kb *Eco*RI fragment encoding part of the propeptide domain and the entire mature domain was used for chromosomal localization by FISH. B, *Bam*HI; RI, *Eco*RI; RV, *Eco*RV; K, *Kpn*I.

BMP-8b (9), and GDF-9 (15, 16), are implicated as important regulatory factors in mammalian reproduction. For example, we have previously generated mice lacking this oocyte-specific growth factor using embryonic stem cell technology (16) and found that absence of GDF-9 results in an early block in folliculogenesis at the one-layer primary follicle stage leading to infertility. These studies have defined GDF-9 as the first oocyte-derived growth factor required for somatic cell function *in vivo*.

In the present study, we report the identification of new genes, which we have named bone morphogenetic protein 15 (*BMP15* in humans, *Bmp15* in mice).[1] Using the sequence relationships of previously characterized BMPs and a degenerate PCR approach, we first identified the mouse gene. To identify the human sequence, low-stringency hybridization with a probe derived from a mouse genomic clone was necessary because the two sequences were unusually divergent. Chromosomal mapping of mouse *Bmp15* and human *BMP15* has

[1] The mouse *Bmp15* cDNA and human *BMP15* gene sequences in this manuscript have been deposited in GENBANK under the accession numbers AF082348, AF082349, and AF082350.

identified them as X-linked genes. *In situ* hybridization in the mouse has demonstrated that *Bmp15* is expressed exclusively in the oocyte soon after primordial follicles are recruited, and expression is maintained until after ovulation. Thus, BMP-15 is the second oocyte-derived growth factor of the TGF$\beta$ superfamily that may be critical for ovarian function.

## RESULTS

### Identification of the Mouse *Bmp15* Gene and cDNA

To identify novel BMP genes, we adopted a PCR approach based on conserved amino acid sequences of the BMP/Vg-1/DPP subgroup of the TGF$\beta$ superfamily (12). Two consensus mature peptide sequences, WQ/NDWIV/IA and NHAIV/LQT, were used to design degenerate oligonucleotide primers. Based on the relative location of these consensus sequences in other BMPs, we predicted that 128-bp products would be generated by PCR. All products of this size range were subcloned and analyzed for novel BMP-like sequences. A subclone produced by degenerate PCR from mouse genomic DNA, designated mPC-3, contained such a novel sequence. Oligonucleotides derived from this sequence were used to screen a mouse strain 129SvEv genomic library and resulted in the identification of a recombinant phage clone, ø60, which contains sequences identical to that of mPC-3 (Fig. 1A). DNA sequence analysis of ø60 indicated that it contained a segment of a novel gene, which we named mouse *Bmp15*. Based on homology with other BMP subfamily members, this sequence identifies a single exon that encodes the complete mature domain and part of the propeptide region of BMP-15. A portion of ø60 that contained 508 bp of mouse *Bmp15* coding sequence was designated probe 1031 (Fig. 1, A and B).

Based on Northern blot analysis results (see below), we screened a mouse ovary cDNA library with probe 1031 and identified the complete coding sequence of mouse *Bmp15*. Several positively hybridizing clones were identified; two of these, designated PC6–1 and PC7–1, were characterized by DNA sequence analysis. These cDNA clones defined an open reading frame of 1176 bp that encoded a 392-amino acid BMP-15 prepropeptide. The transcriptional start site of the mouse *Bmp15* mRNA was determined by rapid amplification of cDNA ends (5′-RACE) (Fig. 1A). The 5′-untranslated region (UTR) and 3′-UTR are 0.64 kb and 1.6 kb, respectively.

Probe 1031 was used to rescreen the mouse genomic library and identified several overlapping recombinant phage clones including PW-8 and PW-9 (Fig. 1A). Restriction endonuclease digestion, Southern blot hybridization, and DNA sequence analysis of

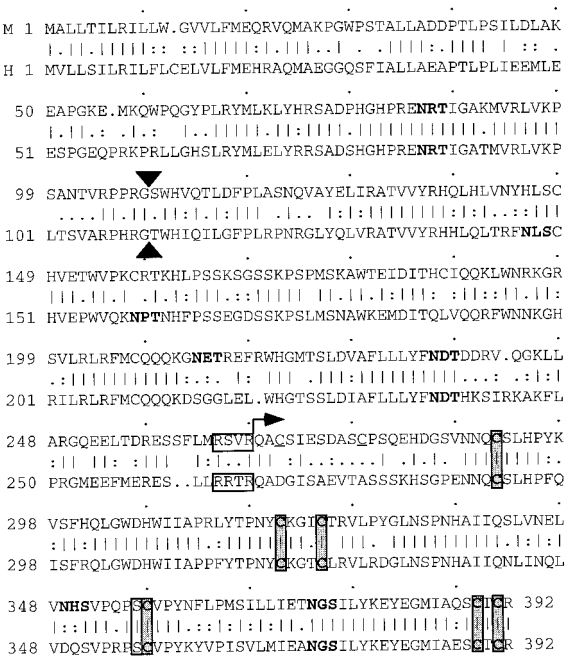


**Fig. 2.** Deduced Amino Acid Sequence of Mouse (M, *top*) and Human (H, *bottom*) BMP-15

The proteolytic processing sites are *boxed*, and the *arrow* indicates the predicted start of the mature peptides. The *triangles* indicate the positions of the single intron in the corresponding genomic DNA sequences. Potential N-linked glycosylation sites are in *bold*. Cysteine residues characteristic of TGF$\beta$ superfamily members are *boxed and shaded*; the cysteine residue thought to be involved in intermolecular bonding is replaced by a serine residue and is *boxed*. Two additional cysteines present in the N-terminal region of the mouse BMP-15 mature peptide are *underlined*. Note also the sequence divergence in the mature peptide before the first conserved cysteine.

PW-8, PW-9, and ø60 were used to determine the structure of the mouse *Bmp15* gene. Two exons, separated by a 3.5-kb intron, encode the entire mouse BMP-15 protein. The first exon encodes a 17-amino acid signal peptide (as predicted with the aid of the GeneWorks computer program, Oxford Molecular Groups, Inc., Oxford, England) and part of the propeptide domain. The second exon encodes the remaining propeptide region and the entire predicted 125-amino acid mature domain (Fig. 1, A and B).

### Identification of the Human *BMP15* Gene

A portion of the ø60 mouse clone was used to screen a human genomic library under reduced stringency conditions. Two hybridizing clones, JLDc1 and JLDc19, were characterized by DNA sequence analysis. Clone JLDc19 contained the complete coding sequence of human *BMP15*. The genomic structure of human *BMP15* was determined by restriction endonuclease digestion, Southern blot hybridization, and DNA sequence analysis.

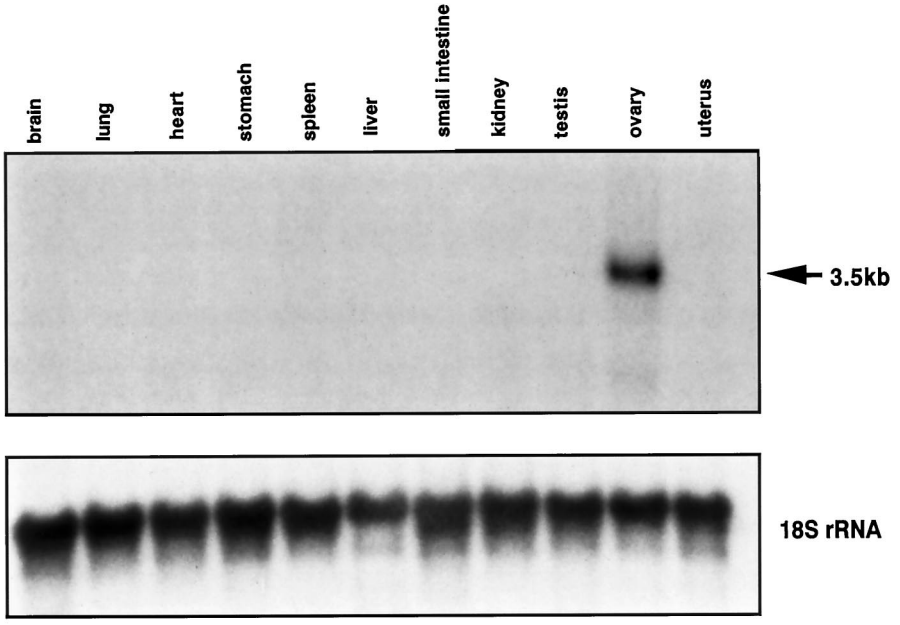


**Fig. 3.** Northern Blot Analysis of Mouse Total RNA from Various Tissues

A mouse *Bmp15* probe, generated from coding sequence, detected a signal of approximately 3.5 kb in RNA derived from ovary but no other tissue (*top*). 18S rRNA (*bottom*) was used as a control for RNA loading.

The 1176-bp coding region is contained within two exons separated by 4.2 kb of intervening sequence: the first exon encodes a predicted secretory leader sequence of 17 amino acids followed by 97 amino acids of the propeptide domain; the second exon encodes the remaining 158 amino acids of the propeptide domain and the entire predicted 125-amino acid mature domain (Fig. 1C).

### Comparison of Mouse and Human BMP-15

Both mouse and human BMP-15 are encoded by two exons separated by a single intron. Cleavage at the predicted proteolytic processing sites (21, 22), Arg-Ser-Val-Arg in mouse or Arg-Arg-Thr-Arg in human BMP-15, produces 125-amino acid peptides. The prepropeptides (392 amino acids) exhibit an overall identity of 63% (76% at the nucleotide level), while the amino acid identities in the mature domain and propeptide domains are 77% (81% nucleotide identity) and 60% (74% nucleotide identity), respectively (Fig. 2). There are five potential N-linked glycosylation sites in the mouse and human BMP-15 proteins. Three of the sites are spatially conserved between the two species. Both mouse and human BMP-15 contain only six out of the seven cysteine residues observed in the vast majority of proteins in the TGF$\beta$ superfamily. Interestingly, the mouse and human gene products differ in that mouse BMP-15 has two additional cysteines upstream from the first conserved cysteine, a spatial pattern more characteristic of TGF$\beta$s and activins.

### Northern Blot Analysis of Mouse *Bmp15*

Many members of the BMP subfamily demonstrate widespread expression in the developing embryo and adult. To define the mRNA expression pattern of *Bmp15*, Northern blot analysis was performed using total RNA from multiple adult mouse tissues. As shown in Fig. 3, the *Bmp15* probe detected a single transcript of approximately 3.5 kb in mouse ovarian RNA. All other tissues examined were negative.

### *In Situ* Hybridization Analysis of Mouse *Bmp15*

*In situ* hybridization analysis on mouse ovaries revealed that *Bmp15* expression was confined to the oocyte (Fig. 4). *Bmp15* is not expressed in oocytes of primordial (type 2) or small type 3a follicles (23) but is first detected in oocytes of intermediate-size type 3a follicles (23) and all type 3b follicles (*i.e.* $>$20 granulosa cells surrounding the oocyte in largest cross-section) and is continually expressed in all oocytes of later stage follicles and 0.5-day postovulatory oocytes (Fig. 4). The spatio-temporal pattern of *Bmp15* expression is identical to that of *Gdf9* (Ref. 15 and data not shown).

### Chromosomal Mapping of Mouse *Bmp15* and Human *BMP15*

We used the Jackson Laboratories Backcross Panel to map the mouse *Bmp15* gene. As shown in Fig. 5A, mouse *Bmp15* maps adjacent to the centromere on the X-chromosome and cosegregates with *DXMit26*

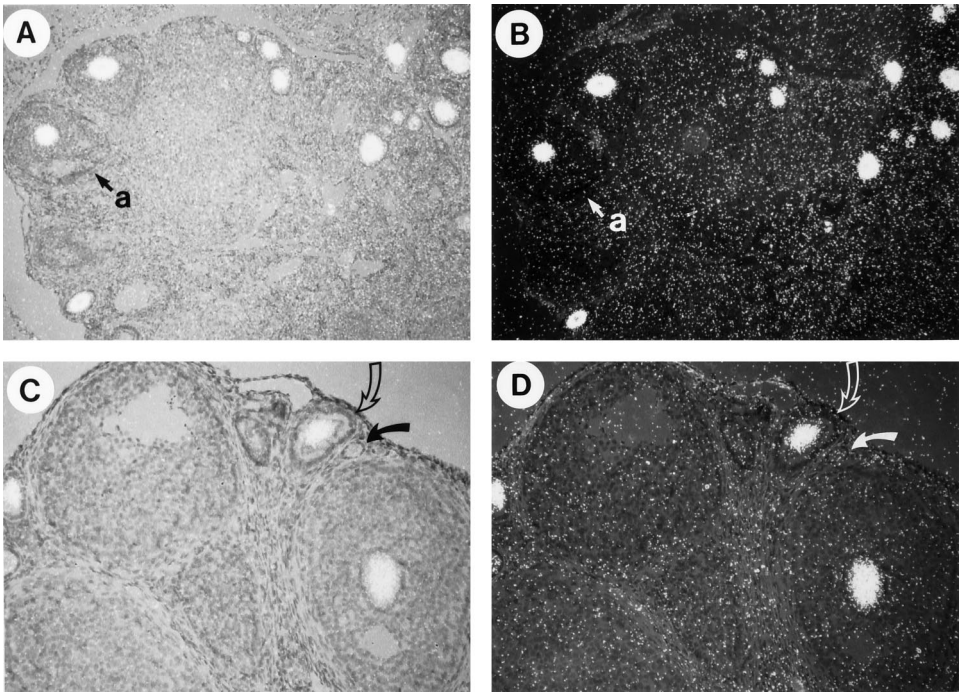


**Fig. 4.** *In Situ* Hybridization Analysis of *Bmp15* mRNA in Mouse Ovaries

Brightfield/darkfield (A and C) or darkfield (B and D) analysis of *Bmp15* mRNA in adult wild-type ovaries. Similar to *Gdf9* (Ref. 15 and our unpublished data), *Bmp15* is absent in primordial and small type 3a follicles (*solid arrow*, C and D) but is expressed in oocytes of larger type 3a, type 3b, and type 4 follicles (*open arrow*, C and D) and oocytes of all later stage follicles including antral (a) follicles (A and B).

and *Fsc1*. To confirm that the mouse *Bmp15* and human *BMP15* genes were orthologs, we performed fluorescence *in situ* hybridization (FISH) analysis to localize human *BMP15* to Xp11.2 (Fig. 5B). This chromosomal region in humans demonstrates conserved synteny with the chromosomal position of mouse *Bmp15* (24).

## DISCUSSION

We used degenerate PCR and reduced stringency hybridization conditions to identify a new member of the TGF$\beta$ superfamily of extracellular signaling proteins, BMP-15. The relatively low amino acid sequence identity between mouse and human BMP-15 originally led us to believe that the two sequences represented distinct genes. Similar sequence comparisons between mouse and human versions of other BMP-related genes typically exhibit identity of greater than 95%. For example, human GDF-9 and mouse GDF-9 are 96% identical (see Fig. 6), while human and mouse BMP-4 are absolutely conserved. Data from extensive genomic screening and Southern blot analysis suggest that the mouse *Bmp15* and human *BMP15* genes described here are the closest sequences to each other. DNA sequence analysis of the mouse *Bmp15* and human *BMP15* genomic clones revealed a highly conserved gene structure. Both genes possess a single intron at exactly the same relative location with respect to the coding sequence and predict primary translation products of the same length, 392 amino acids. Chromosomal mapping of these genes to conserved syntenic regions on the X-chromosome confirms that mouse and human BMP-15 are orthologs (25). Interestingly, there are no known mouse or human mutations to date that map to these regions.

The spatially conserved pattern of seven cysteine residues has served as a defining criteria of the TGF$\beta$ superfamily, although the discovery of additional members has introduced some variations to that motif. Several members, including the prototype protein TGF$\beta$, possess extra cysteines at the amino-terminal end of the mature protein. Probably more structurally relevant, however, are a few members (26, 27) that lack the cysteine residue shown to be responsible for interchain disulfide bond formation (28–30). Both mouse and human BMP-15, as well as their close relative GDF-9 (27), are missing this structurally important cysteine residue. The three-dimensional structure of TGF$\beta$ family members lacking the dimer cysteine has not been determined, but it is likely that their active forms are noncovalently associated dimers. Size exclusion chromatography of recombinant human GDF-9 and GDF-3/Vgr-2 produced in CHO cell expression systems indicated an apparent mol wt of

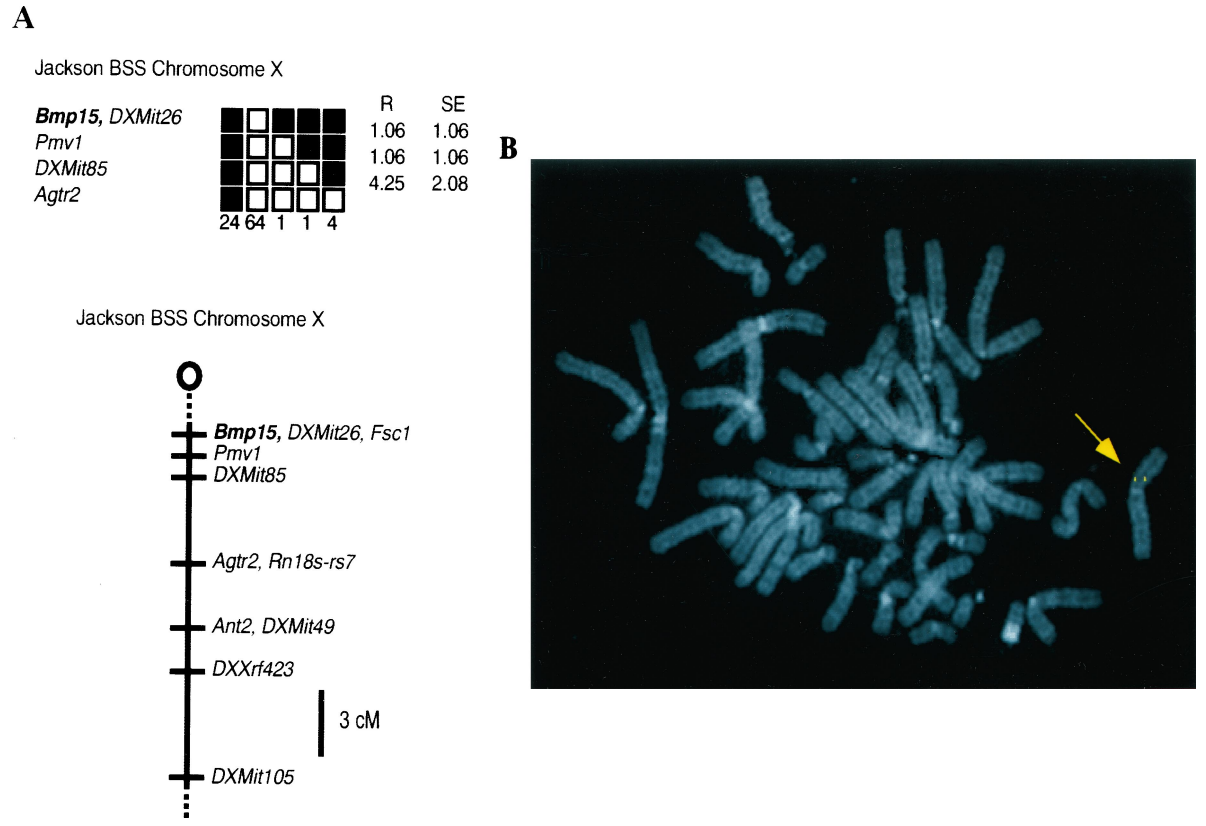


**Fig. 5.** Chromosomal Localization of Mouse *Bmp15* and Human *BMP15* Genes

A, *Bmp15* maps to the proximal region of the mouse X chromosome. Using the Jackson Laboratory interspecific backcross panels (C57BL/6JEi × SPRET/Ei)F1 × SPRET/Ei), the mouse *Bmp15* locus was placed adjacent to the centromere on the X chromosome. The segregation of *Bmp15* and flanking genes in 94 backcross mice typed for all loci are shown at the *top* of the figure. *Black and white boxes* indicate C57BL/6JEi and SPRET/Ei alleles, respectively. *Numbers at the bottom* of each column represent the number of backcross progeny that has inherited a recombinant or nonrecombinant chromosome from the (C57BL/6JEi × SPRET/Ei)F1 parent. Note that one mouse has placed *Bmp15* and *DXMit26* proximal to *Pmv1* and the more distal markers. A partial linkage map of the proximal X chromosome is shown below. B, *BMP15* maps to the human X chromosome by FISH. The *arrow* indicates the hybridization signals of twin dots on the human X chromosome. Based on analysis of metaphase chromosomes isolated from a male, the human *BMP15* gene was localized to Xp11.2.

approximately twice that of monomeric controls that were produced in *Escherichia coli* (N. Wolfman and P. Lowden, personal communication). Furthermore, increasing amounts of chaotrope (guanidine, urea) did not alter the apparent mol wt, indicating a highly stable association of the two monomeric subunits (N. Wolfman and P. Lowden, personal communication). Noncovalent association may allow for the formation of heterodimeric proteins in tissue settings in which more than one 6-cysteine partner is produced.

Several members of the TGF$\beta$ superfamily have been demonstrated to exist naturally as heterodimers and in some cases have different activities than their homodimeric counterparts (13, 31). The inhibins, which inhibit FSH release from the pituitary (32), have not been shown to exist in homodimeric form but, rather, they are examples of functional heterodimers of two distinct TGF$\beta$ superfamily members, inhibin $\alpha$ and activin $\beta$A or $\beta$B. Recombinant heterodimers between the BMP-2/4 subgroup and the BMP-5/6/7 subgroup have also been shown to exhibit a significant increase in biological activity when compared with homodimers assayed in the same system (33–35). With these BMPs, increased levels of homodimers can match the activity of heterodimers in these systems.

Female infertility in GDF-9-deficient mice is the result of arrested follicular development at the primary follicle (type 3B) stage. This block in folliculogenesis approximates the onset of expression of *Gdf9* and *Bmp15,* which are both detected in oocytes of one-layer primary (type 3A) follicles and remain highly expressed in the oocyte throughout the course of follicular maturation and ovulation. Interestingly, GDF-9-deficient mice continue to synthesize *Bmp15* mRNA (our unpublished data). Thus, based on their coincident expression pattern, one potential explanation for the lack of phenotypic rescue by BMP-15 of the GDF-9-deficient mice is that BMP-15 and GDF-9 are capable of forming heterodimers, and it is the heterodimeric form, BMP-15/GDF-9, which supports follicular maturation. Alternatively, BMP-15 and GDF-9 may only form homodimers with distinct biological functions.

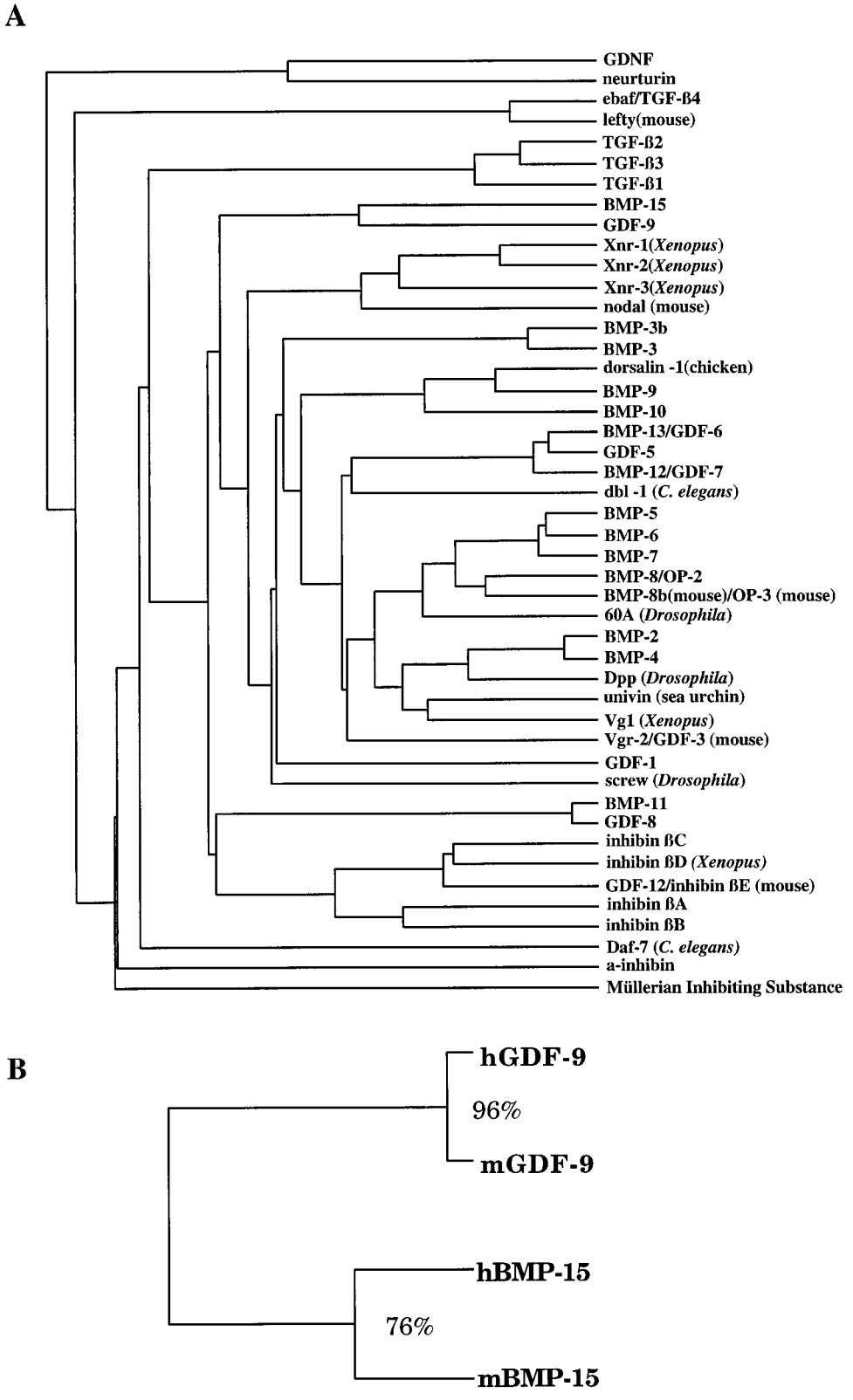


**Fig. 6.** Dendogram of the TGF-$\beta$ Superfamily and Comparisons of BMP-15 and GDF-9

A, The relationship of BMP15 to other members of the TGF$\beta$ superfamily. The figure was generated using the cysteine-rich COOH-terminal polypeptides of the TGF$\beta$ superfamily members, which were aligned using the PILEUP program (Genetics Computer Group, Madison, WI). B, The BMP-15/GDF-9 subfamily. Percent amino acid identities are indicated and were generated as described above.

Ongoing studies to generate *Bmp15* knockout mice may elucidate whether BMP-15 and GDF-9 form active heterodimers *in vivo* and will help us to further define their roles in reproductive physiology.

## MATERIALS AND METHODS

### Degenerate PCR

The oligonucleotides 5′-gcggatccTGG(C/A)ANGA(C/T)TGGAT(A/C/T)(G/A)TNGCN and 5′-gctctagaGT(C/T)TGNA(C/T)NATNGC(A/G)TG(A/G)TT-3′ were used for PCR on mouse genomic DNA to amplify BMP-related sequences as previously described (12). The *lowercase letters* include recognition sequences for the restriction endonucleases *Bam*HI and *Xba*I, respectively, which were added to facilitate the subcloning of the expected 128-bp PCR products into pGEM-3 (Promega, Madison, WI).

### Genomic Library Screening

The oligonucleotides 5′-TCCTCGTCTCTATACCCCAAATTACTGTAAGGAATCTGT-3′ and 5′-ATCTGTACTCGGGTATTACCCTATGGTCTCAATTCACCC-3′ were kinased with [$\gamma^{32}$P]-ATP and hybridized to duplicate nitrocellulose replicas of 500,000 recombinants of a mouse genomic library (no. 946309, Stratagene, La Jolla, CA) in standard hybridization buffer (SHB = 5×SSC/5× Denhardt's solution/0.1% SDS/100 μg denatured salmon sperm DNA) at 60 C overnight. The filters were washed extensively with 5× SSC/0.1% SDS at 60 C and subjected to autoradiography for 2 days at −80 C with intensifying screens. An additional 600,000 recombinants of this same mouse genomic library were screened. Duplicate nitrocellulose filters were hybridized to a DNA fragment of genomic clone ø60 corresponding to nucleotides 1139–1418 of the mouse *Bmp15* sequence, hereafter designated probe 1031. This probe was random primed with [α-$^{32}$P]dCTP and hybridized to nitrocellulose filters in Church's solution [0.5 M sodium phosphate buffer (pH 5.2)/7.5% SDS] at 63 C. Filters were washed with 0.1× Church's solution and exposed overnight at −80 C. Approximately 1,000,000 recombinants of a human genomic library (Stratagene no. 945203) were screened with [α-$^{32}$P]dCTP random-primed mouse probe 1031 at 60 C in SHB overnight. Duplicate nitrocellulose filters were washed with 2× SSC/0.1% SDS at 60 C and exposed for 2 days at −80 C with intensifying screens.

### mRNA Isolation and cDNA Library Construction

Total RNA was extracted from various tissues of adult Swiss Webster mice or C57BL/6/129SvEv hybrid mice using RNA STAT-60 (Leedo Medical Laboratories, Houston, TX) as described by the manufacturer. Poly $(A)^+$ RNA was purified using Oligotex-d7 beads (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. Mouse ovarian mRNA was used to construct a directional (5′-*Eco*RI to 3′-*Xho*I), oligo (dT)-primed cDNA library in the bacteriophage vector λZAP Express using the λZAP Express cDNA cloning kit (Stratagene).

### Northern Blot Hybridization

Total RNA (12 μg) derived from multiple mouse tissues was electrophoresed on a 1.2% agarose/7.6% formaldehyde gel and transferred to Hybond-N (Amersham, Arlington Heights, IL) nylon membrane. Probe 1031 was random primed with [α-$^{32}$P]dCTP. The membrane was hybridized, washed, and subjected to autoradiography as described (17). An 18S ribosomal RNA cDNA probe was used for the loading control.

### cDNA Library Screening and 5′-RACE

Approximately 400,000 clones of the mouse ovarian cDNA library were hybridized to [α-$^{32}$P]dCTP random-primed probe 1031 in Church's solution at 63 C. Filters were washed with 0.1× Church's solution and exposed overnight at −80 C. 5′-RACE PCR was performed using the Marathon cDNA amplification kit (CLONTECH, Palo Alto, CA) under conditions described by the manufacturer. The oligonucleotides used for the amplification were: 5′-GGAAAGTCCAGGGTCTGTACATGCCA-3′ and 5′-CCATTGCTTCATCTCTCCTTGCCA-3′.

### *In Situ* Hybridization

*In situ* hybridization was performed as described previously (18). In brief, freshly dissected ovaries from C57Bl/6/129SvEv hybrid mice were fixed in 4% paraformaldehyde-PBS overnight, processed, embedded in paraffin, and cut to a 5 μm thickness. Mouse *Bmp15* probe 1031 antisense and sense strands were generated by labeling with [α-$^{35}$S]UTP using Riboprobe T7/SP6 Combination System (Promega). Hybridization was carried out at 50–55 C with $5 \times 10^6$ cpm of each riboprobe per slide for 16 h in 50% deionized formamide/0.3 M NaCl/20 mM Tris-HCl (pH 8.0)/5 mM EDTA/10 mM $NaPO_4$ (pH 8.0)/10% Dextran sulfate/1× Denhardt's/0.5 mg/ml yeast RNA. High-stringency washes were carried out in 2× SSC/50% formamide and 0.1× SSC at 65 C. Dehydrated sections were dipped in NTB-2 emulsion (Eastman Kodak, Rochester, NY) and exposed for 4–7 days at 4 C. After the slides were developed and fixed, they were stained with hematoxylin and mounted for photography.

### Chromosomal Mapping

Chromosomal localization of the mouse *Bmp15* gene was performed using the Jackson Laboratory Interspecific backcross DNA panel (19) using Pc26–1 probe located in the 3′-UTR of the mouse *Bmp15* gene. A 3-kb *Eco*RI fragment from a human *BMP15* genomic clone containing a portion of exon 2 and some intron sequence was used to chromosomally map the human *BMP15* gene by FISH as described (20). FISH was performed by Dr. Antonio Baldini and the FISH core in the Department of Molecular and Human Genetics at Baylor College of Medicine.

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