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GENOMIC ORGANIZATION AND CHROMOSOMAL LOCATION OF THE MOUSE TYPE I BMP-2/4 RECEPTOR

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We have characterized the structure of a mouse bone morphogenetic protein (BMP) type I receptor gene that can bind both BMP-2 and BMP-4. The mouse BMP-2/4 receptor gene is encoded by 11 exons and spans approximately 38-kb. Most of the intron/exon boundaries are not conserved compared to the kinase domain of the related, activin type II receptor. In addition, whereas the activin type II receptor gene contains large introns (>40 kb), the largest intron of the BMP-2/4 receptor gene is only 6.4-kb. The BMP-2/4 receptor gene (*Bmpr*) was mapped to mouse chromosome 14. *Bmpr* is closely linked to *Rbp3* in the region containing *pugnose*, a mutation that alters bone development. Knowledge of the genomic structure of *Bmpr* provides important information to create *Bmpr*-deficient mice.

Bone morphogenetic proteins (BMP) are members of the TGF-β gene super-family (1). This subfamily of TGF-β related ligands currently consists of 7 members (BMP-2 through BMP-8). BMPs were originally discovered for their ability to cause bone differentiation (2). However, recent studies suggest that several BMPs play critical roles during embryogenesis especially in dorso-ventral and/or anterior-posterior axis formation in several organisms (3). In *Xenopus laevis*, BMP-4 can act as a posterior-ventralizing factor in animal cap explants (4, 5), whereas activin acts as an inducer for dorsal mesodermal tissues (6, 7). The dorsalizing signal provided by activin can be overridden by BMP-4 (4, 5). In *Droshophila melanogaster*, mutations in

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decapentaplegic (dpp), which is believed to be a BMP homologue, cause dorso-ventral patterning abnormalities (8), further supporting a likely role for BMPs in vertebrate pattern formation.

Recently, type I and type II receptors for TGF-β family ligands have been cloned and shown to share conserved Ser/Thr kinase domains (9-15). In *Caenorhabditis elegans*, *Daf-4* encodes a type II receptor that can bind both human BMP-2 and BMP-4 (16). Mutations in *Daf-4* inhibit dauer larva formation (17). In *Drosophila*, *Saxophone* and *thick veins* have recently been cloned and shown to encode type I receptors for dpp (18-21). These findings suggest that BMP signaling is critical for invertebrate embryonic development, indicating that BMP signaling may also be important during vertebrate embryogenesis.

Recently, a cDNA (TFR-11) was isolated from mouse MC3T3-E1 cells by reverse transcription-PCR using degenerate oligonucleotide primers located in the conserved regions of the Ser/Thr kinase domain of the TGF-β and activin receptors (22). TFR-11 encodes a type I membrane bound Ser/Thr kinase receptor that binds both BMP-2 and BMP-4 but not TGF-β or activin A when expressed in COS cells. In addition, a mutant form of this type I BMP-2/4 receptor acts in a dominant negative manner to induce the formation of a secondary axis in *Xenopus* embryos suggesting that inhibition of BMP signaling by this receptor can alter the fate of embryonic cells towards a more dorsal identity (22). A human homologue of TFR-11, ALK-3 has recently been reported (23) and the same mouse gene has also been independently isolated (24).

We used the mouse TFR-11 cDNA to isolate phage clones that encompass the mouse type I BMP-2/4 receptor gene (*Bmpr*). The intron/exon structure of *Bmpr* was determined and compared with the structure of other TGF-β family receptors. The chromosomal location in the mouse was also determined. The structure of the type I BMP-2/4 receptor gene provides important information for the generation *Bmpr* mutant mice by gene targeting in mouse embryonic stem (ES) cells to examine the function of BMPs during mammalian development.

Materials and Methods

Screening of genomic library: A 129SvE mouse genomic DNA library was constructed using the vector lambda DASH II (Stratagene). 1.6 x 10^6 recombinant λ plaques were screened with the full length TFR-11 cDNA (22). Hybridization was performed at 42°C overnight in 50% formamide, 5X SSC (0.75M NaCl, 75 mM sodium citrate), 100 μg/ml salmon sperm DNA, 0.1% SDS and 10% dextran sulfate, and the filters were washed in 0.1X SSC/0.1% SDS for 30 minutes at 65°C prior to autoradiography. Twenty-two positive λ clones were identified upon initial screening of the genomic library. These clones were purified to homogeneity by secondary and tertiary screening using the TFR-11 cDNA.

λ genomic clone mapping and DNA sequencing analysis: DNA was isolated from 8 of the TFR-11 hybridizing λ clones and digested with EcoRI, HindIII or XbaI and analyzed by gel electrophoresis to generate a physical map. The DNA was transferred onto Hybond-N+ nylon membrane (Amersham) and probed with portions of the TFR-11 cDNA to determine the orientation of the gene and approximate position of the exons. Fragments that hybridized with these probes were subcloned into pBlueScript II KS(-) (Stratagene) for DNA sequence analysis. All sequencing was performed by the DNA Sequencing Core Facility at the M. D. Anderson Cancer Center using an automated fluorescent DNA sequencer (ABI). Genomic sequences were compared with the TFR-11 cDNA sequence using the GCG computer analysis program (Genetics Computer Group, Madison, WI).

Interspecific backcross mapping: Interspecific backcross progeny were generated by mating (C57BL/6J x *M. spretus*) F1 females and C57BL/6J males as described (25). A total of 205 N2 mice were used to map the *Bmpr* locus (see text for details). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, Southern blot transfer and hybridization were performed

essentially as described (26). All blots were prepared with Zetabind nylon membrane (AMF-Cuno). A 2292 bp EcoRI fragment of TFR-11 cDNA was used as a probe and washing was done to a final stringency of 0.6X SSCP, 0.1% SDS, 65°C. Fragments of 6.6, 5.6, 4.8, 4.2, 3.5 and 2.6-kb were detected in *Kpn*I digested C57BL/6J and fragments of 9.4, 6.0, 5.6, 4.8, 4.2, 3.4 and 2.4-kb were detected in *Kpn*I digested *M. spretus* DNA. The presence or absence of the 9.4 and 6.0 kb *M. spretus*-specific *Kpn*I fragments, that cosegregated, was followed in backcross mice.

A description of the probes and RFLPs for the loci linked to *Bmpr* including plasminogen activator, urokinase (*Plau*) and SP-A pulmonary surfactant protein (*Sftp1*) has been reported previously (27). One locus not previously reported is retinal binding protein, interstitial (*Rbp3*). The probe was a ~2.2 kb *Eco*RI fragment of human cDNA that detected fragments of 4.4, 1.9, 1.6 and 0.9 kb in *Sac*I digested C57BL/6J DNA and 5.4, 1.9 and 1.5 kb in *M spretus* DNA. Recombination distances were calculated as described (28) 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

Isolation and characterization of the mouse BMP-2/4 receptor gene

A mouse 129/SvE genomic library was screened with the 2.3-kb mouse *Bmpr* cDNA (22). Twenty-two recombinant λ clones were initially isolated and 8 of these were used to construct a restriction enzyme map of the *Bmpr* gene. Five overlapping genomic clones that span the entire *Bmpr* gene are shown in Figure 1. Restriction enzyme mapping, Southern blot analysis using specific regions of the cDNA as probe, and DNA sequencing revealed that the *Bmpr* gene consists of 11 exons that are distributed relatively evenly over 38.2-kb (Fig. 1).

All 11 exons of the mouse *Bmpr* gene and their intron/exon boundaries were sequenced and the nucleotide positions of the introns relative to the cDNA sequence are shown in Figure 2. Exons 2-10 vary in size from 97 bp to 298 bp and the protein coding regions of the first (exon 1) and last (exon 11) exons are 67 bp and 123 bp, respectively. These are consistent with the observation that the size of coding exons usually do not exceed 300 bp (29). The sequences of the intron/exon boundaries are shown in Figure 3. The size of the introns varied from 85 bp (intron 10) to 6.4-kb (introns 7 and 8). All of the introns begin with GT and end with AG consistent with the donor/acceptor splice rule. Moreover, most of the nucleotides around the boundaries were consistent with the consensus splicing acceptor and donor sequence in rodents (30).

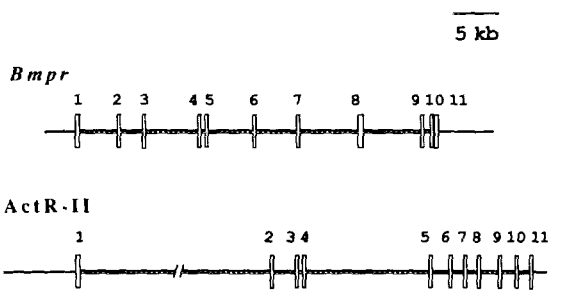

Fig. 1. Structure of the mouse type I BMP-2/4 receptor gene. The structure of the ActR-II gene is shown for comparison (31). Exons are indicated by numbered open boxes. The thick line connecting each exon represents introns.

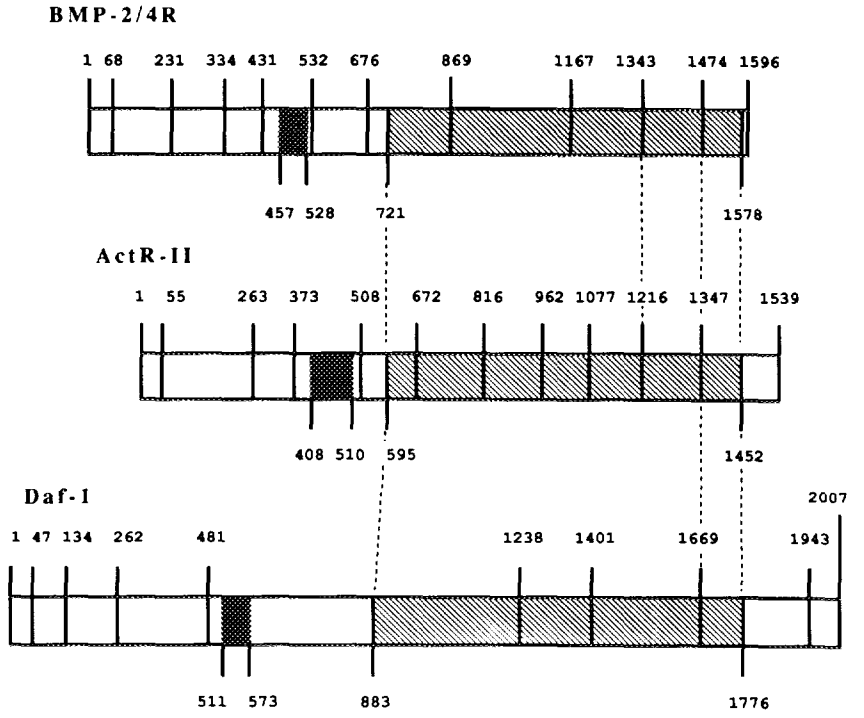

Fig. 2. Location of introns for *Bmpr* and other membrane bound Ser/Thr kinases. The first nucleotide of each exon is shown. For *Bmpr*, nucleotide 1 is the beginning of the translated protein and 1596 is the last nucleotide of the translated sequence. The first and the last nucleotides of the transmembrane (solid box) and kinase (hatched box) domains for each gene are also shown. The location of introns of the mouse activin receptor type II (31) and *C. elegans Daf-1* (32) are shown for comparison.

The structure of *Bmpr* was compared to the related mouse activin type II receptor gene (Fig. 2). The number of exons for *Bmpr* is the same as that of the mouse activin type II receptor gene (31). However, several differences were noted. The entire signal peptide of *Bmpr* is encoded in exon 1 (57 bp) whereas the sequence encoding the signal peptide of the mouse activin receptor type II gene is split between exons 1 (55 nt) and 2 (2 nt). Although the positions of intron 3 and 4 are identical between the activin type II and type IIB receptor genes (11, 31), these positions are divergent in comparison with *Bmpr*. In *Bmpr*, the transmembrane region is encoded in exon 5, whereas that of the activin receptor type II gene is encoded in exon 4. The *Bmpr* gene has 4 intron/exon junctions in the putative Ser/Thr kinase domain, whereas the mouse activin receptor type II has 6, and only two of these are conserved (positions 1343 and 1474 of *Bmpr*) between the two receptor genes. The *C. elegans Daf-1* gene has 3 intron/exon junctions in its Ser/Thr kinase domain and one (position 1669 of *Daf-1*) is conserved with *Bmpr* (32).

Chromosomal location of the mouse BMP-2/4 receptor

The chromosomal location of *Bmpr* in the mouse was determined by interspecific backcross analysis using progeny derived from matings of [(C57BL/6J x *Mus spretus*)F1 X C57BL/6J] mice. This interspecific backcross mapping panel has been typed for over 1400 loci that are well

EXON	(SIZE)	EXON 3'	INTRON	(SIZE)	EXON 5'
Exon 1	(>208 bp)	CAA G Gln G	gtaaattcatacttg...intron 1	(3.8 kb)...catttttttaatacag	GG CAG ly Gln
Exon 2	(163 bp)	TGC AT Cys Il	gtaagtatttggtcc...intron 2	(2.8 kb)...gttttctctcaaaaag	A ACT e Thr
Exon 3	(103 bp)	TGC AAG Cys Lys	gtgaggacgaatttg...intron 3	(5.9 kb)...cattatttttcttaaag	GAT TCA Asp Ser
Exon 4	(97 bp)	ATA G Ile G	gtaggtcagcccaga...intron 4	(154 bp)...ctttggtcctagatag	GT CCG ly Pro
Exon 5	(100 bp)	TAT AA Tyr Ly	gtaagattcttattt...intron 5	(4.8 kb)...tccctgatttttaaag	G CAT s His
Exon 6	(145 bp)	TTA TTG Leu Leu	gtaagctgaaagcat...intron 6	(4.8 kb)...ctttgttttctgtatag	GTT CAG Val Gln
Exon 7	(193 bp)	CTT G Leu G	gtgagtttcttacgc...intron 7	(6.4 kb)...actcattaactaaacag	GT TTT ly Phe
Exon 8	(298 bp)	AAC AG Asn Se	gtaaatggttctttg...intron 8	(6.4 kb)...tcctccccctgtcttag	T GAT : Asp
Exon 9	(176 bp)	GGA G Gly G	gtaggaatttgaaaa...intron 9	(0.8 kb)...tttctcactcccagcag	GA ATC ly Ile
Exon 10	(131 bp)	GAT GAA Asp Glu	gtaagtggagccaa...intron 10	(85 b)...tgcaatgttctctttag	TGT CTT Cys Leu
Exon 11	(>678 bp)				

Fig. 3. Intron/exon boundaries of the *Bmpr* gene. Nucleotide sequence for each intron/exon boundary and size of each exon and intron are shown. The consensus donor sequence is (C/A)AG/GTUAGT and the consensus acceptor sequence is YYYYYYYYYYNCAG/G (30).

distributed among all the autosomes as well as the X chromosomes (25). 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 *Bmpr* cDNA probe. The 9.4 kb and 6.0 kb *M. spretus Kpn*I RFLPs (see Materials and Methods) were used to follow the segregation of the *Bmpr* locus in backcross mice. The mapping results indicated that *Bmpr* is located in the proximal region of mouse chromosome 14 linked to *Plau*, *Rbp3* and *Sftp1*. Although 148 mice were analyzed for every marker and are shown in the segregation analysis (Fig. 4), up to 185 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* - 15/156 - *Rbp3* - 0/160 - *Bmpr* - 3/185 - *Sftp1*. The recombination frequencies [expressed as genetic distances in centiMorgans (cM) $\pm$ the standard error] are - *Plau* - 9.6 $\pm$ 2.4 - [*Rbp3*, *Bmpr*] - 1.6 $\pm$ 0.9 - *Sftp1*. No recombinations were detected between *Rbp3* and *Bmpr* in 160 animals typed in common suggesting that the two loci are within 1.9 cM of each other (upper 95% confidence limit).

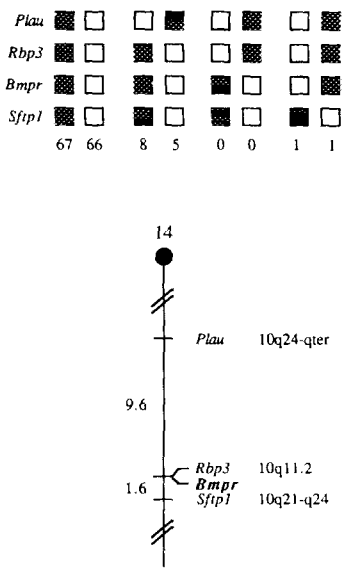

Fig. 4. *Bmpr* maps in the proximal region of mouse chromosome 14. *Bmpr* was placed on mouse chromosome 14 by interspecific backcross analysis. The segregation patterns of *Bmpr* and flanking genes in 148 backcross animals that were typed for all loci are shown at the top of the figure. For individual pair of loci, more than 148 animals were typed. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J x *M. spretus*)F1 parent. The shaded boxes represent the presence of a C57BL/6J allele and open boxes represent the presence of a *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 *Bmpr* 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). *Plau*, plasminogen activator, urokinase; *Rbp3*, retinal binding protein, interstitial; *Sftp1*, pulmonary surfactant protein.

Discussion

We have determined the genomic structure of the mouse BMP-2/4 type I receptor which is encoded by 11 exons and spans 38 kb. The mouse activin type II receptor is also encoded by 11 exons but the genomic structure of this related receptor is quit diverse (31). Only four of the eleven intron/exon junctions between these two genes are conserved. In addition, while the introns of *Bmpr* are between 85 bp and 6.4-kb in size, the range of the intron sizes of the mouse activin type II receptor gene is quite large (152 bp to >40-kb). From its amino acid sequence and calculated molecular weight, *Bmpr* is believed to encode a type I receptor. The *Daf-1* gene is also believed to be a type I receptor because it has a characteristic Ser/Gly rich domain in the intracellular juxtamembrane region (32). Since the genomic structure of *Bmpr* is completely different from that of *Daf-1* (32), it is likely that the difference in genomic structure between *Bmpr* and the mouse activin type II receptor is not due to the difference of the class of receptor but to their ligands. This

idea is supported by the recent findings that at least four of the intron/exon junctions in the kinase domain of the human activin type I receptor gene are similar to those of the mouse activin type II receptor (33). Characterization of the genomic structure of *Daf-4*, which is believed to be a BMP-2/4 type II receptor homologue in *C. elegans* is required to address this question. Beyond the fundamental information provided here about the structure of the *Bmpr* gene, these results provide important information necessary for designing gene targeting vectors to disrupt *Bmpr* in mouse embryonic stem cells to generate *Bmpr* mutant mice.

We have compared our interspecific map of chromosome 14 with a composite mouse linkage map that reports the map location of any uncloned mutations (compiled by M. T. Davidson, T. H. Roderick, A. L. Hillyard, and D. P. Doolittle, provided by GBASE, a computerized database maintained at the Jackson Laboratory, Bar Harbor, ME). *Bmpr* mapped in a region of the composite map that contains several mouse mutations, including *pugnose* (*pn*). *Pugnose* is a recessive mutation associated with skeletal abnormalities, notably craniofacial defects (34). Unfortunately, *pn* appears to be extinct. The relationship between *pn* and *Bmpr* should become apparent when *Bmpr* mutant mice become available. The proximal region of mouse chromosome 14 shares a region of homology with human chromosome 10q (summarized in Fig. 4). In particular, *Rbp3* has been placed on human 10q11.2. The tight linkage between *Rbp3* and *Bmpr* in mouse suggests that *Bmpr* will reside on 10q, as well.

Bmpr expression is detected in long bones but also in most adult mouse tissues (23, 24, Y. Mishina *et al*, unpublished data), suggesting that BMP-2/4 signals may not only be important for long bone formation but also the development of other organs. *Bmpr* expression is detected in the mouse as early as embryonic day 8.5, which is prior to cartilage and bone formation (Y. Mishina, J. Rivera, and R. Behringer, unpublished data). This implies that a signal transduced from this receptor may play an important role during embryogenesis prior to bone formation and organogenesis. The biological significance of this receptor during embryogenesis and organogenesis awaits the generation of *Bmpr* mutant mice.

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