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Structure and sequence of the mouse *Bmp6* gene

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The transforming growth factor- β (TGF-β) superfamily consists of structurally related, secreted disulfide-bonded dimers that modulate cellular growth and differentiation. Many of these factors have been shown to inhibit the proliferation or promote apoptosis of various cell types, to induce expression of various extracellular matrix proteins, and to modulate cell differentiation along particular lineages (reviewed in Derynck 1994; Kingsley 1994; Roberts and Sporn 1990). The largest group of related factors within this superfamily, which has been referred to as the decapentaplegic/vg-1-related proteins (DVR; Lyons et al. 1991), comprises the bone morphogenetic proteins (BMPs), *Xenopus* vegetal-1(vg-1), nodal, dorsalin, at least five factors referred to as growth-differentiation factors (GDFs), and three *Drosophila* homologs, decapentaplegic (dpp), 60A, and screw. Within this family, the largest subgroup consists of BMPs-5, -6 (also known as Vgr-1), -7 (also known as osteogenic protein-1, or OP-1), -8a (OP-2), and -8b (OP-3), all of which are homologous to the ancestral *Drosophila* protein 60A.

Inactivation of the *Bmp5* and *Bmp7* genes has provided preliminary insights into the developmental role of these proteins, with distinctive phenotypes of the mutant mice (Kingsley et al. 1992; Luo et al. 1995). We have begun to characterize the function of BMP-6 as a prototype factor of this subgroup. This cDNA was originally isolated from a murine embryonic cDNA library by screening under low stringency with a *Xenopus* vg-1 cDNA probe, and the corresponding protein was named Vgr-1 (Lyons et al. 1989a). cDNAs for the human and bovine homologs of Vgr-1 were subsequently isolated and were named BMP-6 (Celeste et al. 1990), although no bone morphogenetic activity was originally reported for this protein. Extensive in situ hybridization and immunohistochemical analyses have localized BMP-6 mRNA and protein expression in the central nervous system, suprabasal layer of the epidermis, and hypertrophic cartilage (Gitelman et al. 1994; Jones et al 1991; Lyons et al. 1989b; Wall et al. 1993). We have overexpressed this factor in CHO cells and have shown that, when these cells are introduced subcutaneously into nude, athymic mice, the secreted BMP-6 protein induces ectopic cartilage and bone in a pattern that recapitulates endochondral bone formation (Gitelman et al. 1994). We have also overexpressed BMP-6 within a pluripotent mesenchymal cell line and have shown that the protein acts as an autocrine factor that induces osteoblastic differentiation in vitro (Gitelman et al. 1995).

The *Bmp6* gene has been mapped previously to murine Chromosome (Chr) 13, 2.2 ± 1.5 cM distal to the Friend MuLV integrations site (Dickinson et al. 1990), and the human gene has been mapped to a related region on Chr 6p (Hahn et al. 1992). To further understand the mechanisms of transcriptional regulation of the endogenous *Bmp6* gene and to manipulate its expression in vivo, we have isolated the mouse *Bmp6* gene and characterized its structure and sequence.

A 129/Sv murine genomic library in pCOS-EMBL3 (gift of A. Frischauf) and a mouse genomic library in λCharon 4A were screened with the mouse Vgr-1 cDNA under high stringency hybridization conditions. Two overlapping cosmids and five phage were purified through subsequent rounds of screening. The clones were mapped, and restriction fragments hybridizing to the Vgr-1 cDNA were then subcloned into pUC119 or pBluescript (Stratagene, La Jolla, Calif.) for sequencing. We sequenced the coding regions of the *Bmp6* gene, as well as all intron-exon boundaries and 5′ and 3′ untranslated regions. The exon sequences corresponded to those previously reported for the BMP-6 cDNA (Gitelman et al. 1994). The gene consists of seven coding exons, all flanked by canonical intron/exon splicing sequences (see GenBank Accession #U73515–U75320).

The gene structures for a few members of the TGF-β superfamily are known. The genomic organization of *Bmp6* most closely resembles that of the genes for *Bmp7*/OP-1 and *Bmp8*/OP-2, which belong to the same subfamily as *Bmp6* (Fig. 1) (Ozkaynak et al. 1992). Both the *Bmp7* and *Bmp8a* genes contain seven exons, and comparison of the sequences and intron-exon boundaries with those of *Bmp6* reveals a conservation of identical intron-exon structure with boundaries at corresponding positions. These results suggest that the other members of this subgroup may have similar gene organizations and support their evolution from a common ancestral gene. The genes for the three TGF-β isoforms also contain seven exons (Derynck et al. 1987, 1988) (Fig. 1). However, closer inspection reveals that their coding sequences are distributed differently among the exons when compared with the *Bmp6, Bmp7,* and *Bmp8a* genes than among those of the TGF-β isoforms: the corresponding exons are of different sizes, and the mature protein is encoded by three full exons and a portion of a fourth in the *Bmp6, Bmp7,* and *Bmp8a* genes, as opposed to two full exons and a portion of a third for TGF-β.

Within a separate subgroup of the BMP family, *Bmp2* and *Bmp4* are closely related to each other and are in turn related to the *dpp* gene in *Drosophila.* The *Bmp2* gene consists of 3 exons, two of which encode the precursor protein (Feng et al. 1994). Similarly, the *Bmp4* gene also has two coding exons with intron-exon boundaries at identical corresponding positions as in the *Bmp2* gene (Feng et al. 1995). In contrast to *Bmp2,* the *Bmp4* gene has three noncoding exons; two of these are first exons for part of the 5′-noncoding region, and their incorporation into the mRNA de-

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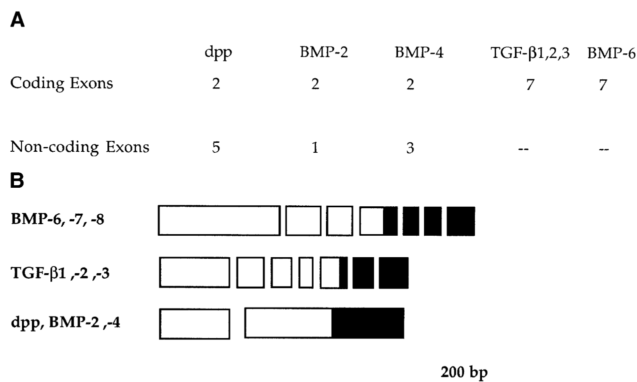

Fig. 1. A. Comparison of gene structures of the different TGF-β superfamily members: *dpp, Bmp2, Bmp4, Tgf*β*1,* -β*2,* -β*3, Bmp7, Bmp8a,* and now *Bmp6.* Noncoding exons refer to possible exons from the 5′ flanking DNA which have been shown to serve as alternate promoters. **B.** Coding exons for some members of the TGF-β superfamily, grouped according to both structural and sequence homology. Exons encoding the precursor region are designated with open boxes, and the mature region is shown by black boxes. 5′ and 3′ untranslated regions and introns are not represented.

pends on the differential transcriptional initiation from one of the two *Bmp4* promoters (Feng et al. 1995). The *Bmp2* and *Bmp4* genes bear close similarities to the structure of the *Drosophila dpp* gene, which contains two coding exons at positions corresponding to the intron-exon structures of the *Bmp2* and *Bmp4* genes (St. Johnston et al. 1990). In addition, the *dpp* gene has several alternate noncoding 5′ exons, and these alternate promoters are regulated in both a developmental and tissue-specific manner. Comparison of the *Bmp6* gene structure with those of the *Bmp2, Bmp4,* and *dpp* genes reveals little similarity in the localization of the intron-exon structures, even though the deduced BMP-6 protein sequence has homology to these members of the BMP group. The implications of these findings for the evolution of these two subgroups of genes within the TGF-β superfamily are unclear at this time, and may be clarified upon characterization of the gene structure of the *Drosophila 60A* gene, which is homologous to the genes for *Bmp5, Bmp6, Bmp7,* and *Bmp8.*

We have examined the 1730 base pairs of 5′ flanking sequence of the *Bmp6* gene for possible transcription factor binding sites, and some putative sites are shown in Fig. 2. In the mouse *Bmp4* gene, a COUP-TFI response element precedes one of the transcriptional initiation sites, and COUP-TFI functions as a silencer of *Bmp4* expression in fetal rat calvarial osteoblasts (Feng et al. 1995). In addition, some potential binding sites for homeobox transcription factors are noted in the 5′ flanking region. These findings suggest a connection between developmentally regulated transcription factors and *Bmp4* expression. Preliminary functional analysis of the *Bmp2* gene has shown that a putative homeobox-like factor may be important in enhancing gene transcription, suggesting that developmentally controlled transcription factors might also determine the spatial and temporal expression of the *Bmp2* gene (S.E. Harris, personal communication). *Bmp6* expression might also be modulated by a developmentally regulated transcription factor. One potential site for such regulation is through the putative bicoid binding site (Fig. 2). Another possible site is through the basic helix loop helix (bHLH) transcription factor MyoD, since a MyoD binding site can be found in the *Bmp6* promoter sequence (Fig. 2). Thus, a close correlation might exist between MyoD expression, or a related basic helix loop helix transcription factor, and *Bmp6* expression in differentiating mesenchymal cells. Further analysis of *Bmp6* transcription will be required to determine the elements central to its unique developmental and tissue-specific patterns of expression.

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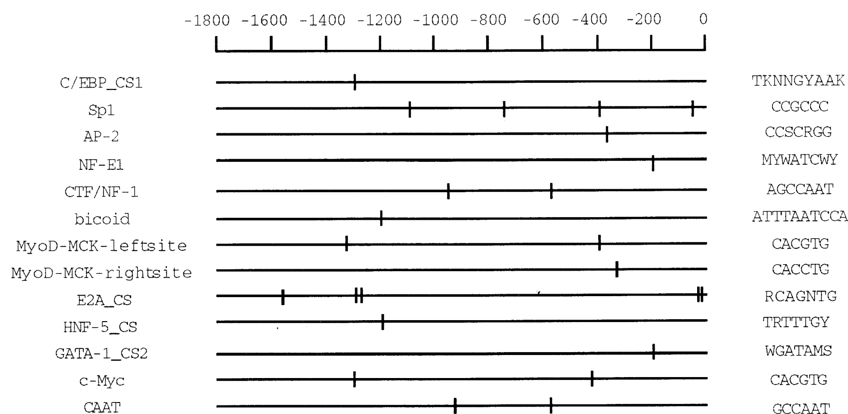

Fig. 2. Schematic diagram of location of transcription factor binding sites. The numbering across the top of the figure refers to nucleotide distance from the transcriptional initiation codon. The putative transcription factor binding sites are shown vertically in the column on the left, and the consensus binding site sequences are shown on the right-hand column. The abbreviations in these sequences are: N, any of the four nucleotides; R, A or G; Y, C or T; M, A or C; K, G or T; W, A or T; S, C or G.

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