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Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone

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ABSTRACT **Characterization of the polypeptides present in bone-inductive protein extracts from bovine bone has led to the cloning of seven regulatory molecules, six of which are distantly related to transforming growth factor β. The three human bone morphogenetic proteins (BMPs) we describe herein, BMP-5, BMP-6, and BMP-7, show extensive sequence similarity to BMP-2, a molecule that by itself is sufficient to induce *de novo* bone formation *in vivo*. The additive or synergistic contribution of these BMP-2-related molecules to the osteogenic activity associated with demineralized bone is strongly implicated by the presence of these growth factors in the most active fractions of highly purified bone extract.**

Protein extracts of demineralized bone contain an activity known as bone morphogenetic protein (BMP) (1, 2). We have extensively purified this bone-inductive activity from bovine bone (3) utilizing an *in vivo* assay system for ectopic cartilage and bone formation (4, 5). Initial peptide sequence information derived from this highly purified extract enabled the cloning of four polypeptides, BMP-1, BMP-2, BMP-3, and BMP-4. Due to the increasing size of the BMP family of molecules described in this paper, we refer to the molecule previously called BMP-2A simply as BMP-2 and rename BMP-2B as BMP-4. Preliminary studies with these recombinant (r) human (h) molecules indicated that they all had some cartilage-forming activity in the *in vivo* assay system (6). Subsequent analysis has demonstrated that rhBMP-2 by itself is sufficient to initiate the developmental cascade resulting in chondrogenesis and osteogenesis *in vivo* (7). Further biochemical characterization of the proteins present in active BMP preparations indicated that additional molecules related to BMP-2 were present. As part of an effort to clearly define the roles of all these molecules in cartilage and bone induction, we have attempted to identify cDNA clones corresponding to each protein in the bovine (b) BMP mixture.

In the present study we report the isolation of cDNA clones encoding human BMP-5, BMP-6, and BMP-7.* These proteins represent three members of the transforming growth factor β (TGF-β) family of growth and differentiation factors and are most closely related to the bone-inductive molecule rhBMP-2.

MATERIALS AND METHODS

Protein Purification and Peptide Sequence Analysis. Bovine bone extract, which was purified as in ref. 3, yielded the tryptic peptide sequences STPAQDVSR, NQEAL, and XANVXEN. The peptide HELYVSF was obtained from similar material that underwent further purification as follows: active fractions from the C_4 reverse-phase column were dialyzed against 6 M urea/25 mM diethanolamine, pH 8.6, loaded on a Mono Q 5 column, and fractionated with a linear gradient to 6 M urea/25 mM diethanolamine/0.5 M NaCl, pH 8.6. Active fractions were pooled, adjusted to 0.1% SDS, dialyzed, concentrated, and subjected to SDS/PAGE under nonreducing conditions. The 28- to 30-kDa region of the gel was excised; protein was electroeluted, reduced, alkylated, and submitted to SDS/PAGE. The resulting 14- to 20-kDa region was excised; protein was electroeluted, digested with trypsin, and sequenced as described (3).

RNA Preparation. RNA was isolated from the human osteosarcoma cell line U-2 OS by a modification of the Nonidet P-40 lysis method (8). Bovine bone RNA was prepared from primary cultures of fetal long bone (9). Enrichment of polyadenylylated RNA was obtained by oligo(dT)-cellulose chromatography.

cDNA Library Construction. Oligo(dT)- and specific oligonucleotide-primed cDNA synthesis from polyadenylylated RNA were performed as described (10) except that *Eco*RI restriction site methylation and subsequent redigestion steps were eliminated by using *Eco*RI adapters rather than linkers. The oligonucleotides GGAATCCAAGGCAGAATGTG and GCGAGCAATGGAGGATCCAG, designed on the basis of hBMP-6 and hBMP-7 3′ untranslated sequence, were utilized to prime first-strand cDNA synthesis from the U-2 OS mRNA template. Bovine bone cDNA was inserted into the vector λgt10 and all U-2 OS cDNA libraries were constructed in λZAP. Human placental and human fetal brain cDNA libraries were purchased from Stratagene (catalog numbers, 936203 and 936206, respectively).

cDNA Library Screening. Approximately 400,000 recombinants of the bovine bone cDNA library were screened with a 384-fold-degenerate 17-base oligonucleotide probe consisting of four pools: GTRCTYGANATRCANTC, GTRCTYGANATRCANAG, GTRCTYAAYATRCANTC, and GTRCTYAAYATRCANAG under tetramethylammonium chloride hybridization conditions (11, 12). Duplicate nitrocellulose replicas of all oligonucleotide positive clones were hybridized to the insert of bBMP-5 in standard hybridization buffer [SHB = 5× SSC/5× Denhardt's solution/0.1% SDS/denatured salmon sperm DNA (100 μg/ml)] at 65°C (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin). One set of filters was washed with 2× SSC/0.1% SDS and the other set, with 0.2× SSC/0.1% SDS, both at 65°C.

Duplicate nitrocellulose replicas of 750,000 recombinants from an oligo(dT)-primed U-2 OS/λZAP cDNA library were hybridized to a 493-base-pair (bp) *Sma* I restriction fragment of bBMP-6, which was ^{32}P-labeled by nick-translation. Hybridization was performed in SHB at 65°C and filters were washed under the dual stringency conditions described

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Abbreviations: TGF-β, transforming growth factor β; BMP, bone morphogenetic protein; r, recombinant; h, human; b, bovine.

*The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M38693 (hBMP-5), M38694 (hBMP-6), and M38695 (hBMP-7)].

above, with the exception that 1× SSC was substituted for 2× SSC.

Approximately 500,000 recombinants each of specific oligonucleotide primer-extended hBMP-6 and hBMP-7 cDNA libraries were screened with the oligonucleotides CAGAGTCGTAATCGC and GATCTCGCGCTGCAT, respectively. Hybridizations were done at 42°C in SHB and washes at 42°C with 5× SSC/0.1% SDS.

Approximately 1×10^6 recombinants each from a human placental and a human fetal brain cDNA library were screened with the oligonucleotide TCGGGCTTCCTGTACCGGCGGCTCAAGACGCAGGAGAAGCGGGAGATGCA. Hybridization was performed in SHB at 65°C and filters were washed at 65°C with 0.2× SSC/0.1% SDS.

Genomic Library Screening. Genomic hBMP-6 clones were obtained by screening 1×10^6 recombinants of a human genomic library (10) with the same oligonucleotide used to probe the human placental and human fetal brain cDNA libraries. bBMP-7 clones were isolated by screening 1×10^6 recombinants of a bovine liver genomic library (6) with a nick-translated 251-bp *Pst* I–*Stu* I fragment of a hBMP-7 cDNA clone. Hybridization and wash conditions were identical to those described above.

DNA Sequence Analysis. All DNA sequence analysis was performed by the dideoxynucleotide chain-termination method.

RESULTS AND DISCUSSION

bBMP-5 and bBMP-6. Amino acid sequence analysis of peptides derived from tryptic digestion of highly purified fractions of bovine bone extracts generated the additional sequences HELYVSF, STPAQDVSR, NQEALR, and XANVXEN. These tryptic peptides were not present in the derived amino acid sequences of bBMP-1, bBMP-2, bBMP-3, or bBMP-4. The sequence HELYVSF was used to design a probe consisting of a degenerate pool of 17-base oligonucleotides, which was used to screen a bovine bone cDNA library. Multiple recombinants hybridized to the oligonucleotide probe. DNA sequence analysis identified a recombinant that encoded the desired tryptic sequence and the protein encoded by this cDNA clone was named BMP-5. The insert of this clone was hybridized to duplicate nitrocellulose replicas of the remaining oligonucleotide-positive clones. One recombinant was found to hybridize to the bBMP-5 probe under reduced-stringency wash conditions only. The derived amino acid sequence of this cDNA clone encoded a distinct polypeptide that also contained the tryptic peptide sequence HELYVSF. Furthermore, this polypeptide contained another tryptic peptide sequence (STPAQDVSR) present in the highly purified preparation of bone-inductive extract. This protein was named BMP-6. Comparison of the BMP-5 and BMP-6 amino acid sequences to the other BMPs indicated a strong sequence similarity to BMP-2 and BMP-4.

hBMP-5, hBMP-6, and hBMP-7. To identify human mRNA sources for BMP-5 and BMP-6, Northern blot analysis was performed with mRNA from numerous human cell lines. A 464-bp fragment from the bBMP-5 cDNA and a 154-bp fragment of the bBMP-6 cDNA, derived from a region of minimal sequence similarity to BMP-5, were used as probes to distinguish the two mRNAs. This analysis demonstrated that the human osteosarcoma cell line U-2 OS produced low levels of BMP-6 mRNA and >10-fold higher levels of BMP-5 mRNA. The BMP-6-specific probe detects two mRNA species of ≈4.3 kilobases (kb) and 2.6 kb in size. A duplicate Northern blot hybridized to the BMP-5 probe identifies a major transcript of 2.8 kb and several minor species electrophoresing at ≈5.0 kb, 3.7 kb, 2.1 kb, and 1.6 kb. Interpretation of these results is complicated by the fact that the bBMP-5 probe was derived from a region of the cDNA sequence that would be expected to cross-hybridize with other highly related molecules (see below). Therefore, the minor bands observed could represent alternatively spliced BMP-5 mRNAs or transcripts of other related genes.

A 493-bp fragment of the bBMP-6 cDNA was chosen as a probe to screen a U-2 OS cDNA library based on its predicted ability to detect hBMP-5 and hBMP-6 cDNA clones under appropriate hybridization conditions. Two classes of cDNAs were detected based on hybridization signal intensity. DNA sequence analysis of several members of the weakly hybridizing class indicated that they encoded the entire hBMP-5. The hBMP-5 cDNA contains an open reading frame of 1362 bp, predicting a primary translation product of 454 amino acids commencing with a hydrophobic leader sequence characteristic of secreted proteins. Restriction enzyme and DNA sequence analyses of several members of the strongly hybridizing class demonstrated the existence of two distinct subgroups of cDNAs. One subgroup encodes the hBMP-6 protein, and the other subgroup encodes a closely related polypeptide designated BMP-7. The hBMP-7 cDNA sequence encodes the peptide sequence NQEALR, which was also present in the purified bovine extract. None of the oligo(dT)-primed cDNAs for hBMP-6 or hBMP-7 were determined to be full length; the full-length cDNA sequence of hBMP-7 was obtained by isolation of recombinants from a specific oligonucleotide primer-extended U-2 OS cDNA library. The hBMP-7 cDNAs contain an open reading frame of 1293 bp, indicating a 431-amino acid hBMP-7 protein. The presumed initiator methionine for BMP-7 is also followed by a region of hydrophobic amino acids characteristic of secreted proteins.

Isolation of clones encoding the amino-terminal region of hBMP-6 proved to be more difficult. Due to the low abundance of BMP-6 mRNA in the U-2 OS cell line, we decided to explore other tissue sources. A long oligonucleotide probe derived from the most 5′ sequence of a primer-extended hBMP-6 cDNA clone was utilized to screen human placental and brain cDNA libraries. Full-length hBMP-6 clones were isolated from both libraries. The presumed initiator methionine is preceded by termination codons in all three reading frames and defines an open reading frame of 1539 bp that encodes the 513-amino acid hBMP-6. The overall hydrophobicity of the hBMP-6 secretory leader sequence is diminished by the unusual presence of six proline residues between amino acid residues 24 and 32 of the primary translation product. The same oligonucleotide probe used to identify these cDNAs was employed to isolate human genomic clones. DNA sequence analysis of the corresponding region of these genomic clones substantiated the 5′ sequence obtained from the cDNAs.

The amino acid sequences of hBMP-5, hBMP-6, and hBMP-7 are shown in Fig. 1. The derived amino acid sequences indicate that all three proteins contain a sequence identical to the bovine tryptic fragment (HELYVSF) used to design the original oligonucleotide probes. The human sequences corresponding to the other tryptic peptides (STPAQDVSR and NQEALR) from purified bovine bone extract found in BMP-6 and BMP-7 are underlined in Fig. 1. hBMP-7 also contains the peptide sequence MANVAEN, which presumably corresponds to the bovine tryptic peptide XANVXEN. Preliminary analysis of bovine genomic clones has confirmed the presence of the NQEALR sequence in bBMP-7 (unpublished data). These data clearly indicate that BMP-6 and BMP-7 are present in the highly purified preparation of bovine bone-inductive extract.

After the submission of this manuscript, Özkaynak *et al.* (14) reported the identification of human genomic clones encoding BMP-3, BMP-4, and OP-1 by utilizing a synthetic gene probe designed on the basis of sequence conservation between *dpp* (15) and Vg1 (16). Nucleotide and derived amino

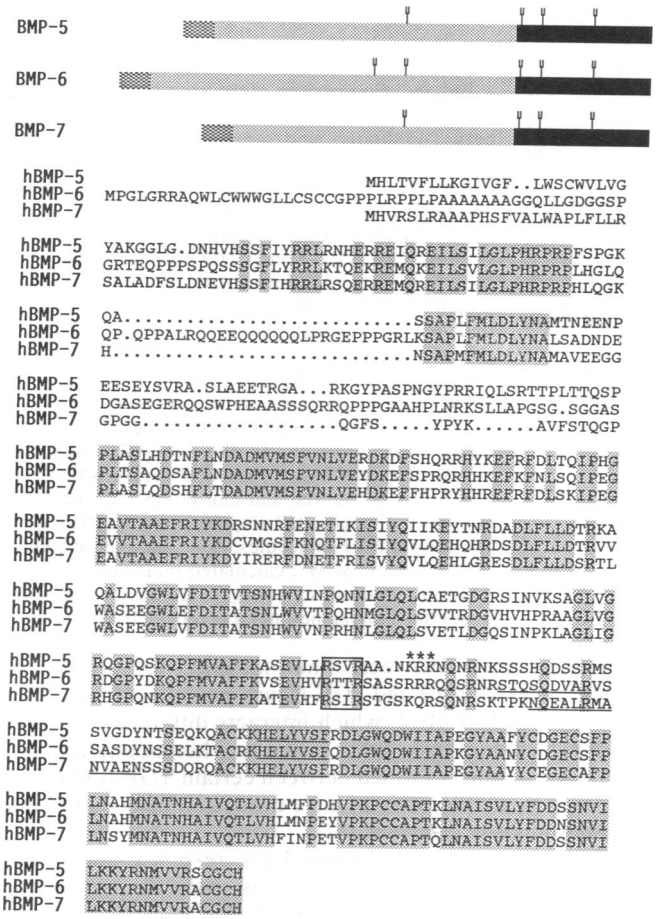

FIG. 1. Alignment of BMP-5, BMP-6, and BMP-7 amino acid sequences as derived from human cDNA clones. This alignment was generated with the help of the CLUSTAL multiple-sequence alignment program. The human homologues of tryptic peptide sequences obtained from bovine bone extracts are underlined. The predicted protease-recognition sequences used to define the amino termini of the mature regions of the BMPs are boxed. The location of the proposed protease cleavage recognition sequence of Vgr-1 (13) and OP-1 (14) is indicated with asterisks. Residues that are identical in all three proteins are shaded. The schematic diagram above depicts the domain structure of the three proteins; including the predicted leader sequence (dark stippling), the propeptide (light stippling), and the mature region (solid area). Potential N-linked glycosylation sites are indicated ⍱.

acid sequence comparisons indicate that OP-1 and hBMP-7 are identical polypeptides. Although the identification of OP-1 resulted from the isolation of a *dpp*/Vg1-related gene from a human genomic library, the presence of bBMP-7 in our most highly purified fractions of bovine bone-inducing activity supports the involvement of BMP-7 (OP-1) in bone induction.

Comparison of the BMPs to TGF-β-Like Molecules. Comparison of the published sequence of murine Vgr-1 (13) with that of hBMP-6 reveals a high degree of amino acid sequence identity (>91%), which leads us to conclude that Vgr-1 represents the murine homologue of BMP-6. The Vgr-1 sequence was identified by cross-hybridization to a cDNA encoding a portion of the *Xenopus* protein Vg1 (16). Alignment of the polypeptide encoded by the Vgr-1 cDNA to that encoded by the hBMP-6 cDNA shows hBMP-6 to contain an additional 75 amino acids at the amino terminus. This difference may represent a species variation, an alternatively spliced mRNA, or an incomplete Vgr-1 cDNA. This final possibility is supported by the lack of a hydrophobic secretory leader sequence after the presumed initiator methionine of Vgr-1, the difficulty we experienced in attempting to isolate a full-length hBMP-6 cDNA and the fact that the 5′ sequence of Vgr-1 was derived from a single clone. The hBMP-6 sequence we present was confirmed from cDNA clones derived from two mRNA sources and genomic DNA. The 5′ sequences of the Vgr-1 and hBMP-6 cDNAs have an extremely high G+C content: Vgr-1 is 72% G+C in the first 150 bp of coding sequence; hBMP-6 is 83%. This feature may result in an unusual secondary structure in the respective mRNAs that could interfere with their complete reverse transcription. Another unusual characteristic of the amino-terminal region of the Vgr-1 and BMP-6 is the presence of amino acid repeats. Vgr-1 contains a stretch of 10 consecutive glutamine residues, whereas BMP-6 has 8 out of 10 glutamine residues at its corresponding location (positions 109–118), in addition to seven consecutive alanines at positions 33–39 (see Fig. 1).

Similar to the other members of the TGF-β family, BMP-5, BMP-6, and BMP-7 are predicted to be synthesized as precursor molecules (17). The carboxyl-terminal portions of the precursor polypeptides constitute the mature part of these proteins (Fig. 1). This region contains all the tryptic peptide sequences identified from the analysis of natural BMPs and the seven cysteine residues that are conserved in all members of the TGF-β supergene family. By analogy to Vgr-1 (13), cleavage of the precursor may occur at a tribasic peptide sequence (KRK or RRR) present at homologous positions in BMP-5 and BMP-6; BMP-7 (OP-1), however, contains the sequence KQR at the corresponding position and it has been suggested that it would be cleaved at this site or after the sequence RSK located three residues in the carboxyl-terminal direction (14). However, the presence of a di- or tribasic amino acid sequence is not an absolute requirement for proteolytic processing, as a number of prohormones are known to be processed after single arginines that conform to a consensus cleavage sequence RXXR (18). At least two members of the TGF-β superfamily, Müllerian inhibiting substance (19) and *Drosophila* dpp (20), are processed in agreement with this pattern. It is interesting to note that the proteolytic cleavage sites of several other family members (i.e., TGF-β1, TGF-β2, TGF-β3, inhibin α, inhibin β_A, BMP-2 and BMP-4), which are defined by multibasic amino acid sequences, all conform to the RXXR consensus sequence (7, 21–27). Since BMP-5, BMP-6, and BMP-7 contain a conserved RXXR sequence, we predict that these molecules are proteolytically processed at this site, as indicated in Fig. 1. By assuming that cleavage occurs at this location, mature BMP-5 would consist of 138 amino acids, and BMP-6 and BMP-7 would be 139 amino acids long with a calculated molecular mass of ≈15.6 kDa (Fig. 2). In comparison, the calculated molecular masses of the mature BMP-2 and BMP-4 are ≈13 kDa and mature BMP-3 is ≈14.5 kDa. The mature forms of BMP-5, -6, and -7 each contain three potential N-linked glycosylation sites whereas mature BMP-4 contains two and BMP-2 and BMP-3 have only one site each (Fig. 2). The heterogeneous 16-kDa and 18-kDa bands observed upon SDS/PAGE analysis after reduction of the purified bBMP 30-kDa dimer (3) are likely to consist of glycosylated forms of at least BMP-2, -3, -6, and -7. The predicted isoelectric points of all the BMPs are basic (Fig. 2), consistent with the demonstration that BMP activity can be eluted from the basic region of an isoelectric focusing gel (3).

The BMP Protein Family. BMP-5, BMP-6, and BMP-7 represent three members of the BMP subfamily of TGF-β-like molecules, which includes the proteins BMP-2, BMP-3, and BMP-4. Comparative amino acid sequence analysis of the mature portions of the BMPs has allowed us to further divide these molecules into three distinct groups (Fig. 3). The members of the first group, BMP-2 and BMP-4, are very closely related (86% amino acid identity) to one another but

A

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hBMP-2                                QAKHKQRKRLKSSCKRHPLYVDFSDVGWNDWIVAPPGYHAFYCHGE
hBMP-3                             SQTLQFDEQTLKKARRKQWIEPRNCARRYLKVDFADIGWSEWIISPKSFDAYYCSGA
hBMP-4                                SPKHHSQRARKKNKNCRRHSLYVDFSDVGWNDWIVAPPGYQAFYCHGD
hBMP-5    AANKRKNQNRNKSSSHQDSSRMSSVGDYNTSEQKQACKKHELYVSFRDLGWQDWIIAPEGYAAFYCDGE
hBMP-6    SASSRRRQQSRNRSTQSQDVARVSSASDYNSSELKTACRKHELYVSFQDLGWQDWIIAPKGYAANYCDGE
hBMP-7    STGSKQRSQNRSKTPKNQEALRMANVAENSSSDQRQACKKHELYVSFRDLGWQDWIIAPEGYAAYYCEGE

hBMP-2    CPFPLADHLNSTNHAIVQTLVNSVNSK..IPKACCVPTELSAISMLYLDENEKVVLKNYQDMVVEGCGCR
hBMP-3    CQFPMPKSLKPSNHATIQSIVRAVGVVPGIPEPCCVPEKMSSLSILFFDENKNVVLKVYPNMTVESCACR
hBMP-4    CPFPLADHLNSTNHAIVQTLVNSVNSS..IPKACCVPTELSAISMLYLDEYDKVVLKNYQEMVVEGCGCR
hBMP-5    CSFPLNAHMNATNHAIVQTLVHLMFPDH.VPKPCCAPTKLNAISVLYFDDSSNVILKKYRNMVVRSCGCH
hBMP-6    CSFPLNAHMNATNHAIVQTLVHLMNPEY.VPKPCCAPTKLNAISVLYFDDNSNVILKKYRNMVVRACGCH
hBMP-7    CAFPLNSYMNATNHAIVQTLVHFINPET.VPKPCCAPTQLNAISVLYFDDSSNVILKKYRNMVVRACGCH
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B

	A.A.	M.W.	N-GLYCOS	pI
BMP-2	114	12.9	1	7.9
BMP-3	127	14.5	1	8.5
BMP-4	116	13.1	2	7.7
BMP-5	138	15.6	3	8.3
BMP-6	139	15.7	3	8.6
BMP-7	139	15.7	3	8.1

FIG. 2. Comparison of the amino acid sequence and physical properties of the BMP family members. (*A*) Amino acid sequence alignment of the mature region of the BMPs. Amino termini were defined by the following criteria: BMP-2, direct amino-terminal sequence analysis of CHO-produced rhBMP-2; BMP-3, multiple forms of bovine bone-derived BMP-3 and CHO-produced rhBMP-3 have been detected (data not shown); BMP-4, predicted from the corresponding position in BMP-2 and further substantiated by the report of direct amino-terminal sequencing of recombinant hBMP-4 (previously BMP-2B) (27); BMP-5, BMP-6, and BMP-7, predicted based on the presence of a single RXXR sequence present in corresponding positions in all three proteins. In the schematic diagram below, full solid boxes represent residues that are absolutely conserved and half boxes indicate conservative changes. (*B*) Physical characteristics of the mature BMP molecules. A.A., number of amino acid residues; M.W., calculated molecular mass in kDa; pI, isoelectric point; N-Glycos, number of potential asparagine-linked glycosylation sites.

their sequences differ significantly from those of BMP-3, -5, -6, and -7. BMP-5, BMP-6, and BMP-7 exhibit 71–80% identity to each other, thereby defining the second group. Of all the TGF-β-like BMPs, BMP-3 is the most distinct and by itself forms the third group. BMP-5, -6, and -7 also share a high degree of identity in the propeptide region (52–64%). The propeptides of BMP-2 and -4 are 57% identical whereas BMP-3 is only 16–21% identical to BMP-2, -4, -5, -6, and -7 in the propeptide region (Fig. 3). There is evidence that the propeptide region of other TGF-β family members may be essential for correct processing of the mature molecules and may be involved in forming latent but activatable complexes (28, 29). It is possible that the sequence similarity detected in this region may play some role in controlling heterodimer formation between various BMPs. Heterodimer formation is known to occur between other members of the TGF-β superfamily. TGF-β1 and TGF-β2 are capable of forming the heterodimer TGF-β1.2, which interacts differently with the TGF-β receptors than either of the two homodimeric forms (30). Heterodimers formed between certain TGF-β-like molecules (e.g, inhibin/activin) have even been demonstrated to exhibit opposite activities (31).

The BMPs and Bone Induction. Dose–response studies with rhBMP-2 suggest that although this molecule by itself is capable of inducing bone formation *in vivo*, ≈10-fold more rhBMP-2 is necessary to achieve the same level of bone-inductive activity observed with natural bBMP (7). At present we cannot exclude the possibility that rhBMP-2 is processed by Chinese hamster ovary (CHO) cells into a form that is less active than the native molecule. However, the pres-

A

	PROPEPTIDE					MATURE				
	BMP-3	BMP-4	BMP-5	BMP-6	BMP-7	BMP-3	BMP-4	BMP-5	BMP-6	BMP-7
BMP-2	21	57	26	30	27	45	86	60	57	55
BMP-3		19	16	18	17		43	37	43	37
BMP-4			24	27	27			54	55	56
BMP-5				52	64				80	75
BMP-6					60					71

B

	IDENTITY WITHIN GROUP		IDENTITY TO BMP-2	
	PRO	MATURE	PRO	MATURE
BMP-2/4	57	86	79	93
BMP-5/6/7	59	75	28	57
BMP-3	-	-	21	45

FIG. 3. (*A*) Pairwise amino acid sequence comparisons of the propeptide and mature regions of the BMPs. The numbers represent percent amino acid identity. (*B*) Table highlighting the propeptide and mature relationships within groups and the relationship of each group to BMP-2. The numbers represent the average percent amino acid identity for each group.

ence of other BMP-2-related gene products in highly purified bBMP suggests the possibility that multiple regulatory signals interact to produce BMP activity (32).

Highly purified preparations of osteogenin (BMP-3) have been demonstrated to induce bone formation in an *in vivo* system similar to the one used in our studies (33). Although these experiments were performed with nonrecombinant material, they imply that other related molecules may have the same activity as BMP-2 or may somehow potentiate its effect in this assay system. A separate analysis of bovine osteogenic protein preparations has revealed that it is comprised of bBMP-2 and OP-1 (bBMP-7) (34). Examination of subfractionated samples of this osteogenic protein preparation suggests that homodimers of these two molecules are the predominant species; however, the existence of heterodimers cannot be ruled out.

The other BMPs could augment the osteogenic capability of BMP-2, either through interaction with the same target cell or by acting on completely different cell populations during the complex process of bone formation. Alternatively, heterodimeric molecules could form that have different activities than the corresponding homodimers. Further biochemical characterization of the most active fractions from bovine bone extract will allow us to determine the subunit compositions and ratio of the BMPs present. In addition, the expression and production of each of the recombinant BMPs will enable us to evaluate the activity of each molecule alone and in combinations.

The identification of hBMP-6 in placenta and brain cDNA libraries, OP-1 (hBMP-7) in placenta and several other cDNA libraries, and the multitissue expression patterns of murine Vgr-1 and murine BMP-2 (35) indicate that the BMP family of proteins are likely to have other growth and differentiation activities unrelated to bone induction. Our molecular probes can be used to determine the patterns of BMP gene expression in skeletal development and in the regeneration of bone after fracture. These types of approaches should allow definition of the roles of the BMPs in bone growth and repair, aiding in the development of therapeutics targeted toward the induction and control of cartilage and bone formation in a variety of clinical settings.

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