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Purification and characterization of other distinct bone-inducing factors

(bone morphogenetic protein/bone formation)

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ABSTRACT **We purified a factor that induces bone formation >300,000-fold from guanidinium chloride extracts of demineralized bone. Fifty nanograms of highly purified protein was active in an *in vivo* cartilage and bone-formation assay. The activity resided in a single gel band, corresponding to a molecular mass of ≈30 kDa, which yielded proteins of 30, 18, and 16 kDa on reduction. The partial amino acid sequence obtained from these proteins confirmed our identification of specific factors that induce new bone formation *in vivo*.**

Bone is a complex tissue that undergoes constant remodeling in response to changing physical demands. The signals that control resorption and formation, whether from humoral or localized growth and differentiation factors, extracellular matrix, or other presently unknown controls, require much further study. One approach to studying bone development is use of *in vivo* ectopic bone formation—the best characterized model of which is induction by demineralized bone implanted intramuscularly or subcutaneously. During this sequence of events (*i*) mesenchymal cells are seen to migrate into the implant, proliferate after several days, and condense in regions. (*ii*) Chondroblasts, believed to be derived from the early-appearing mesenchymal cells, form a cartilaginous template in the area of presumptive bone. (*iii*) At 10–14 days, the cartilage hypertrophies, and the cartilage extracellular matrix is vascularized by hematopoietic and endothelial cells. (*iv*) The cartilage is gradually removed and replaced by bone, and at the end of 21 days an ossicle of bone, complete with marrow, has been formed. This response is localized to the implant itself. The morphological but not temporal developmental sequence is the same as seen in embryonic endochondral bone formation and adult fracture repair (1–3).

This induction of the natural sequence of bone formation immediately suggested potential application for human therapeutics and for developmental studies. Thus began the search for a factor, or factors, named bone morphogenetic protein (BMP) by Urist (1), that could induce bone formation. BMP was characterized as an activity tightly bound to the matrix of demineralized bone and extractable by denaturing solvents (4). Implantation of protein itself was sufficient to induce bone, but reconstitution of the factor with a collagenous matrix (5, 6) or synthetic matrices (7, 8) enhanced sensitivity of the assay. Although purification and characterization have been hampered by the cumbersome *in vivo* assay, numerous reports have described osteoinductive factors (8–10). Additionally, many other growth factors, namely fibroblastic growth factor, platelet-derived growth factor, transforming growth factors β1 and β2, insulin-like growth factors I and II, and bone-derived growth factor, have been implicated in bone development by their presence in bone and their effect on cartilage and bone cells *in vitro*, although no direct osteoinductive role has yet been identified *in vivo* (for reviews, see refs. 11 and 12). We used the rat ectopic bone formation assay to further analyze BMP activity from bovine bone. We report here the purification of a discrete BMP, as distinguished by physical characteristics and extremely high specific activity.

MATERIALS AND METHODS

Materials. Ground bovine bone (20-120 mesh) was obtained from American Biomaterials (Plainsboro, N.J.) and N-Glycanase was obtained from Genzyme. DE-52 cellulose and CM-cellulose were obtained from Whatman; the hydroxyapatite was purchased from LKB; all other resins were obtained from Pharmacia. All chemicals were reagent grade except for the urea and guanidinium chloride, which were ultrapure.

Methods. *Biological assay.* The ectopic bone formation assay was done as described (5) with the following modifications. The protein to be assayed was equilibrated in a volatile solvent, generally 0.1% trifluoroacetic acid (CF_3COOH), and then mixed with 20 mg of demineralized, guanidinium chloride-extracted rat bone matrix. The material was frozen and lyophilized, and the powder was enclosed in no. 5 gelatin capsules. The capsules were implanted subcutaneously in the abdominal thoracic area of 21- to 49-day male Long–Evans rats and routinely removed at 7 days. Samples were processed for histological analysis, with 1-μm glycolmethacrylate sections stained with Von Kossa and acid fuschin or toluidine blue. Sections from implants were scored on a scale of 0–5 for the presence of new cartilage and bone. A cartilage score (c) of 5 indicates that >50% of the section was cartilage, a score of 4 indicates 40–50% cartilage, and a score of 3 indicates 30–40%, etc. A +/− indicates <5% cartilage, and the score by itself is not considered significant. (Scoring of illustrative samples is shown in Fig. 1.)

Purification. The initial extraction steps have been described (13, 14), the purification scheme is summarized in Table 1, and buffer conditions are detailed here. The dialyzed guanidinium chloride extract from 20 kg of bone was passed over a 3-liter DE-52 column equilibrated in 50 mM Tris/0.1 M NaCl/6 M urea, pH 7.2. The unbound fraction was then adsorbed to a 2-liter CM-cellulose column in 50 mM NaAc/50 mM NaCl/6 M urea, pH 4.6. After extensive washing, the activity was removed by elution with 50 mM NaAc/0.25 M NaCl/6 M urea, pH 4.6. This fraction was applied to a 500-ml hydroxyapatite column in 80 mM potassium phosphate/6 M urea, pH 6.0, and dissociated from the column in 100 mM potassium phosphate/6 M urea, pH 7.4. The active material was absorbed to a 100-ml heparin-Sepharose column in 50 mM potassium phosphate/0.15 M NaCl/6 M urea, pH 7.4,

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Abbreviation: BMP, bone morphogenetic protein.

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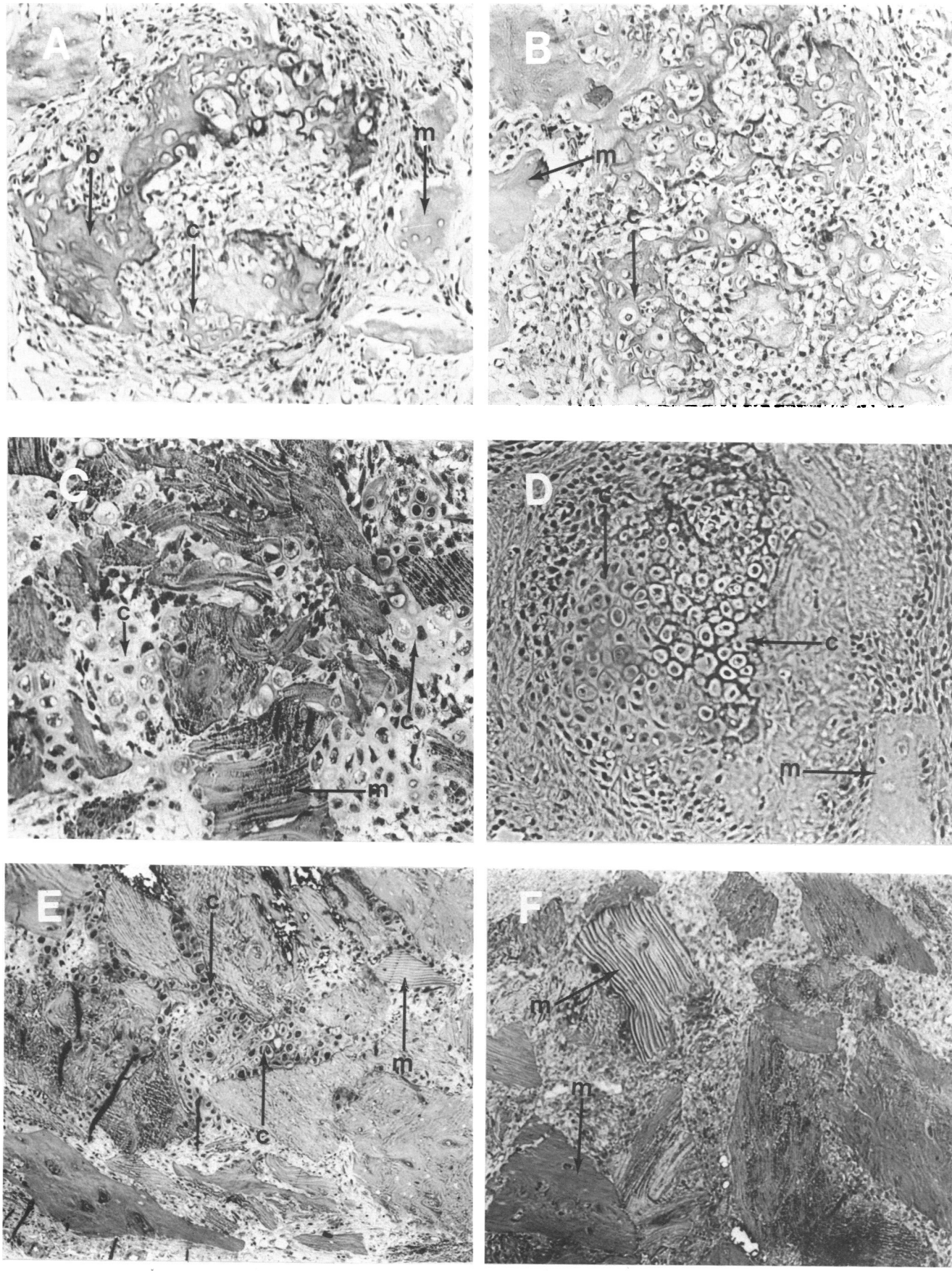

FIG. 1. Cartilage and bone induced by BMP. m, Matrix particles, c, new cartilage, and b, new bone. (*A*) About 10 units of 1000-fold-purified BMP was reconstituted as described; histological scoring of this implant was cartilage (C) +2, bone (B) +3. (See text.) (×65.) (*B*) Activity induced by 3 units of 1000-fold-purified BMP; histological scoring of C+3, B+/−. (×65.) (*C*) Activity induced by 0.2 μg of reverse-phase-purified BMP (1.8 μg of protein); scoring of C+4. (×130.) (*D*) Activity induced by 0.07 μg of BMP as in *C*; scoring of C+2. (×130.) (*E*) Activity induced by 0.02 μg of BMP as in *C*; scoring of C+1. (×65.) (*F*) Implantation of matrix alone subjected to the reconstitution procedure with 0.1% CF_3COOH. (×65.)

Table 1. Summary of purification of BMP activity

Fractionation step	Protein, mg	BMP, units
1 Guanidinium chloride extract	30,000	ND
2 DE-52	13,000	ND
3 CM-cellulose	5,300	660
4 Hydroxyapatite	530	ND
5 Heparin–Sepharose	29	ND
6 Superose	7	200
7 Mono S	≈1	150
8 Reverse phase	≈0.02	120

ND, not determined.

and desorbed with 50 mM potassium phosphate/0.7 M NaCl/6 M urea, pH 7.4. Gel filtration was performed on the heparin-Sepharose-bound protein on Superose 6 and 12 HR 10/30 columns connected in series equilibrated in 4 M guanidinium chloride/20 mM Tris, pH 7.2. Active fractions had a relative migration corresponding to an approximate molecular mass of 30 kDa. Active material was then fractionated on a Mono S column (loading 25 mg of protein per ml of resin) in 50 mM NaoAc/6 M urea, pH 4.6, developed with a gradient from 0 M to 1.0 M NaCl. The active fractions were acidified to pH 3.0 with CF_3COOH and applied to a 0.46 × 25 cm Vydac C_4 column in 0.1% CF_3COOH, and the column was developed with a gradient to 90% acetonitrile/0.1% CF_3COOH.

Analytical methods. Protein was iodinated by the chloramine-T method (15), and molecular mass and isoelectric point analysis were done in the Laemmli system (16) and a Triton X-100/urea system (17). For analysis of BMP activity from these gel systems, samples were heated to 37°C for 15 min in sample buffer. Protein was eluted from the crushed gel slices in 50 mM Tris/0.1% $NaDodSO_4$, pH 7.8. The supernatant was acidified with 10% CF_3COOH to pH 3 and desalted on a 0.46 × 5 cm Vydac C_4 column developed with a gradient of 0.1% CF_3COOH to 90% acetonitrile/0.1% CF_3COOH. The fractions predicted to contain BMP (34–38% acetonitrile) were pooled, and appropriate amounts were assayed as described above. Protein was estimated by the method of Bradford (18) or by absorbance at 280 nm.

Amino acid sequencing. Nonreduced BMP was resolved by preparative $NaDodSO_4$/PAGE, reduced, and alkylated *in situ* by exposure to a combination of 2-mercaptoethanol and 4-vinylpyridine vapors, and then fixed in methanol/acetic acid/water. The fixed gel slice was rinsed in water and neutralized by immersion in a small volume of 0.1 M ammonium bicarbonate solution; finally, the protein was digested from the gel with tosylphenylalanine chloromethyl ketone (TPCK)-treated trypsin (1% by weight). Individual tryptic fragments were isolated after fractionation on a Vydac C_4 column developed in 0.1% CF_3COOH to 0.1% CF_3COOH/95% acetonitrile and sequenced on a model 470A gas-phase sequencer (Applied Biosystems).

RESULTS

Assay. We modified the *in vivo* bone formation assay (5) to increase its sensitivity, shorten its duration, and measure the activity. (*i*) Reconstitution of soluble protein in volatile solvents with inactive bone matrix by lyophilization has proven to be the most reproducible and sensitive procedure for sample preparation—particularly when assaying very low amounts of protein. (*ii*) We determined that the time course for the morphological development in these samples is accelerated compared with that previously described (2, 3, 19), and we were able to remove implants after 7 days instead of the 12–21 days previously required (9, 10). The time-course studies also showed that cartilage formation at 7 days correlated with bone formation at 10–14 days, regardless of whether crude or the most highly purified protein was implanted. (*iii*) We attempted to measure activity by using dose–response studies, where activity was seen within an ≈20-fold range. Each implant was histologically evaluated for the appearance of cartilage and bone, which was scored on a scale of 1–5, as illustrated in Fig. 1. One unit of activity is defined as the minimum amount of BMP required to produce cartilage formation of +2 at an ectopic site 7 days after implantation. Interestingly, we found that increasing dosages accelerate the rate of development in a limited manner, so that at 7 days, 1–2 units of BMP show moderate amount of cartilage (Fig. 1*B*), whereas 5–10 units of the same material show bone as well as cartilage (Fig. 1*A*). Note that the response is the same whether crude or highly purified bovine BMP had been implanted.

Purification. To define a factor that induces the *in vivo* formation of cartilage and bone, we purified BMP from bovine bone, a readily available source. A typical purification starting from 10 kg of bone is summarized in Table 1. Subsequent batches of 10 kg were pooled at intermediate fractionation steps to improve final recovery. Fractionation of BMP revealed several interesting physical properties. Insolubility of the factor or other proteins that copurify with BMP required that all but the last fractionation step be done in the presence of 6 M urea or 4 M guanidinium chloride; however, the activity was extremely stable even under these denaturing conditions. BMP activity binds to heparin-Sepharose, and its complete elution by 0.7 M NaCl shows heparin-binding affinity similar to that of platelet-derived growth factor and much lower than that exhibited by acidic or basic fibroblastic growth factor (20), although the inclusion of urea in the buffers may affect the affinity. BMP has an estimated molecular mass of 30 kDa as determined by gel filtration in guanidinium chloride (Fig. 2*A*). At the next step, Mono S fractionation, two widely separated peaks of BMP with similar activity were sometimes seen (Fig. 2*B*). The physical basis of this heterogeneity was not determined, and only the first peak, containing most of the units, was used for the next step. After the final chromatographic step of reverse-phase HPLC (Fig. 2*C*), the protein had a specific activity of 150 ng of protein per unit, and an overall purification of 300,000-fold relative to the starting guanidinium chloride extract was achieved. The yield of protein, estimated by absorbance at 280 nm, was ≈20 μg per 10 kg of bone; recovery of activity was generally 10–20% based on assay of material purified on CM-cellulose, which removed inhibitory or inflammatory material (5).

The most highly purified BMP was analyzed by PAGE. A nonreducing $NaDodSO_4$ gel of radioiodonated active fractions from the C_4 column (Fig. 2*C*) is shown in Fig. 3*A*; the activity, which peaks in fraction 40, correlates with the 30-kDa protein. As described above, gel filtration experiments also indicated a molecular mass of ≈30 kDa, but the molecular mass of the activity in $NaDodSO_4$/PAGE was needed to confirm the 30-kDa species as the active protein. About 3 μg of protein from the final purification step of a similar BMP preparation was separated on a 15% $NaDodSO_4$ gel, and activity in gel slices was determined as described. As shown in Fig. 3*B*, the activity corresponded to the protein species at 30 kDa, and no activity was seen in any other portion of the gel. Because the 30-kDa protein was approximately one-third of the total protein as determined by silver stain (Fig. 3*B*), the estimate for one unit of BMP activity was revised to ≈50 ng. In a similar experiment, the isoelectric point of BMP was determined to be ≈8.8 in a urea/Triton X-100 electrophoretic system (data not shown).

When the 30-kDa gel-purified BMP was reduced, components of 30, 18, and 16 kDa were seen in $NaDodSO_4$/PAGE analysis (Fig. 4). Based on the radioiodinated material, all three peptides appear equal in quantity. However, because tyrosine content is not known, no conclusions on stoichiometry or on subunit composition can be drawn. More impor-

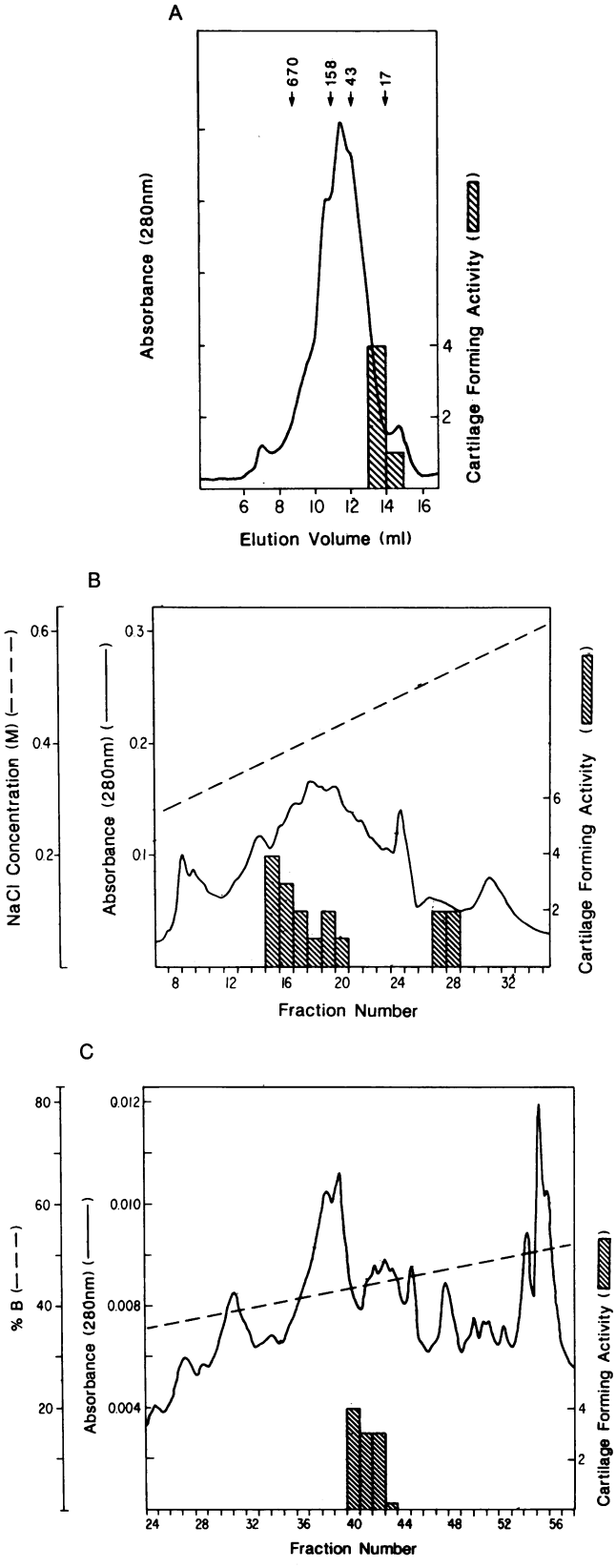

FIG. 2. Chromatography profiles of the last three steps in BMP purification. (*A*) Gel filtration of BMP on Superose 6 and 12 columns connected in series. Elution of Bio-Rad gel filtration markers is shown. BMP emerges at a position corresponding to ≈29 kDa. (*B*) Fraction of BMP on the Mono S column; two peaks of activity are seen. Only the earlier eluting BMP is subsequently purified. (*C*) Fractionation of BMP on a C_4 reverse-phase column. Buffer A is 0.1% CF_3COOH, and buffer B is 0.1% CF_3COOH/90% acetonitrile. Iodination of aliquots from this step is shown in Fig. 3.

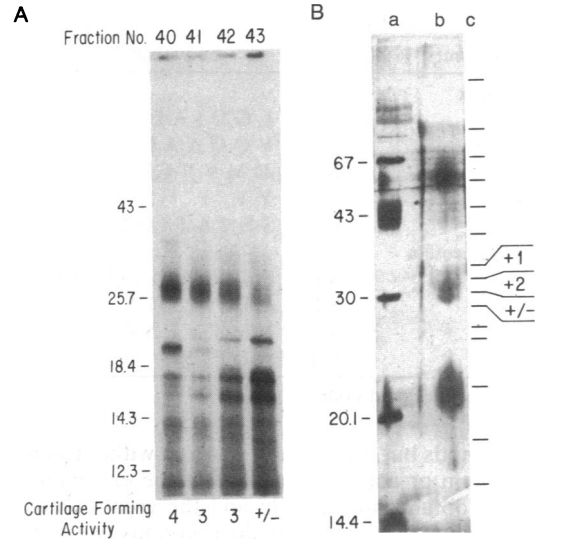

FIG. 3. Molecular mass analysis of BMP. (*A*) $NaDodSO_4$/PAGE analysis of ^{125}I-labeled fractions from the C_4 column fractionation shown in Fig. 2*C*. Fraction number is at top, and activity from 50-μl assays is indicated at bottom. (*B*) Molecular mass determination of BMP. Active reverse-phase purified protein was analyzed on a 15% gel. Gel slices to be analyzed were removed and the gel was silver stained. Lanes: b, 0.1 μg of BMP; c, 3.0 μg of BMP; and a, molecular mass markers in kDa. Gel slices were removed as noted, and activity was determined.

tantly, this analysis indicates that BMP activity might reside in one or more of these peptides.

All BMPs appear to be glycosylated: digestion of components of the 30-kDa BMP gel band with N-Glycanase reduced the observed molecular mass of each component by 3, 2.5, and 2.5 kDa, respectively (Fig. 4). About half of the highly purified BMP activity bound to a Con A–Sepharose column with Triton X-100 and without urea and was removed from the column with α-methyl-D-mannoside (data not shown). These results are consistent with the presence of an α-mannose containing asparagine-linked complex carbohydrate on BMP.

Although the reduced individual peptides of the 30-kDa BMP are easily resolved on $NaDodSO_4$/PAGE, treatment of BMP with reducing agents results in loss of activity (21, data not shown); thus the active component could not be directly analyzed from a reducing $NaDodSO_4$ gel. We attempted to further identify the active BMP species by trying to separate the components of the nonreduced 30-kDa material by

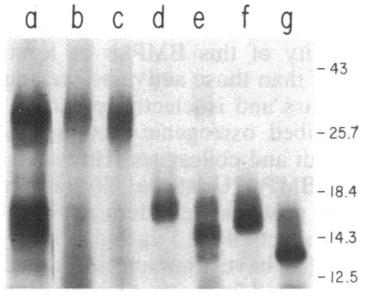

FIG. 4. Subunit composition of BMP. The 30-kDa ^{125}I-labeled BMP was purified from a 15% $NaDodSO_4$ gel and analyzed. Lanes: a, BMP, reduced and alkylated. Components are seen at 30, 18, and 16 kDa, respectively; b, 30-kDa component, reduced and alkylated; c, same 30-kDa component digested with 0.02 unit of N-Glycanase (Genzyme); d, 18-kDa component, reduced and alkylated; e, same 18-kDa component digested with 0.02 unit of N-Glycanase; f, 16-kDa component, reduced and alkylated; and g, same 16-kDa component digested with 0.02 unit of N-Glycanase.

Table 2: Sequence of tryptic fragments from BMP

Fragments	Sequence
BMP mix	
1	A A F L G D I A L D E E D L G
2	A F Q V Q Q A A D L
3	N Y Q D M V V E G
4	F D A Y Y
5	L K P S N ? A T I Q S I V E
16-kDa protein	
6	S L K P S N H A T I Q S ? V
7	S F D A Y Y C S G A
8	V Y P N M T V E S C A
9	V D F A D I ? W

One-letter amino acid code is used.

various methods but were unable to do so without significant loss of protein or activity. Because of the enormous effort required to obtain pure protein and the great complexities involved in characterizing individual proteins, we decided to use amino acid sequence information to obtain cDNA clones for each protein and then characterize each protein on the recombinant level. To ensure the optimum amount of amino acid sequence, the 30-kDa nonreduced-BMP gel band was digested with trypsin, and the tryptic peptides were separated by reverse-phase chromatography. The sequences obtained from individual peptides are shown in Table 2. Additional sequence was obtained from another batch of material that was substantially enriched in the 16-kDa protein and is also included in Table 2.

DISCUSSION

Using a highly specific and sensitive bone-formation assay, we purified a BMP from guanidine extracts of demineralized bone >300,000-fold. As little as 50 ng of the factor can induce the formation of cartilage, although the effective amount of BMP delivered to the site is not known. The activity has been characterized as a basic protein of nonreduced molecular mass of ≈30 kDa, as determined by elution from gels. Gel-purified BMP is composed of three proteins of molecular mass 30, 18, and 16 kDa; because all attempts to separate the components resulted in inactivation, the exact identity of the active material was not determined. The requirements for bone induction in this system may be as simple as the 30-kDa protein alone or as complex as the 30-kDa protein in combination with an 18- and 16-kDa heterodimer.

The high specific activity and the physical properties suggest that the BMP we isolated is a growth factor composed of unusual proteins. Despite differences that might be expected because of the matrix used in the *in vivo* assay system, the specific activity of this BMP is at least an order of magnitude greater than those activities previously reported. The molecular mass and isoelectric point are also distinct from other described osteogenic factors. The osteogenin described by Reddi and colleagues (10) was a protein of 22 kDa, whereas the BMP of Urist *et al.* (9) was an acidic protein of 18.5 kDa; both proteins were derived from bovine bone, and they had *in vivo* activity at 5 μg and 1–5 mg, respectively. Takaoka *et al.* (8) have characterized a 22-kDa bone-inductive factor isolated from a murine osteosarcoma with an unknown specific activity. In addition, a survey of physical properties of other growth factors indicates similarity of BMP only to platelet-derived growth factor; both are basic 30-kDa proteins composed of two disulfide-linked subunits (22). Our experiments with human or porcine platelet-derived growth factor confirm the lack of *in vivo* cartilage-forming activity previously described (10). Also, none of the tryptic peptides isolated from BMP showed any identity to amino acid sequence in the National Biomedical Research Foundation data base.

Because of these results, we were convinced that the criteria of purity, potency, and specificity for another growth factor had been fulfilled by our 30-kDa protein. Because the amount of BMP is so limited, we used sequence information obtained from a mixture of the three proteins to clone the respective genes for further characterization of the individual activities of recombinant factors. Human cDNAs for each of these three proteins have been shown to encode rare proteins (J.M.W., V.R., A. J. Celeste, L. M. Mitsock, M. J. Whitters, R. W. Kriz, R.M.H. and E.A.W., unpublished work). Interestingly, the genes for the 18- and 16-kDa proteins are related, both being members of the inhibin/transforming growth factor type β gene family. More importantly, the recombinant proteins are individually active in cartilage induction, and each of the three factors presumably contributes to the complex process of bone formation.

Examination of BMP activity has been so far restricted to the bone-induction system, and the effect of BMP has been studied only in the very localized space of the implant, whether consisting of protein alone or protein in combination with a matrix. It will be very interesting to discover other *in vitro* and *in vivo* activities and specificities for these factors, and these results should help elucidate the regulation of cartilage and bone growth and differentiation.

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