Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6004–6008, July 1993
Biochemistry

Drosophila transforming growth factor β superfamily proteins induce endochondral bone formation in mammals

(decapentaplegic protein/60A protein/bone morphogenetic proteins/osteogenic protein 1/bone induction)

T. K. SAMPATH*†, K. E. RASHKA†, J. S. DOCTOR‡, R. F. TUCKER*, AND F. M. HOFFMANN‡

*Creative BioMolecules, Inc., Hopkinton, MA 01748; and ‡McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706

Communicated by Elizabeth D. Hay, March 22, 1993

ABSTRACT **Both decapentaplegic (dpp) protein and 60A protein have been implicated in pattern formation during *Drosophila melanogaster* embryogenesis. Within the C-terminal domain, dpp and 60A are similar to human bone morphogenetic protein 2 (75% identity) and human osteogenic protein 1 (70% identity), respectively. Both recombinant human bone morphogenetic protein 2 and recombinant human osteogenic protein 1 have been shown to induce bone formation *in vivo* and to restore large diaphyseal segmental defects in various animal models. We examined whether the *Drosophila* proteins, dpp and 60A, have the capacity to induce bone formation in mammals by using the rat subcutaneous bone induction model. Highly purified recombinant dpp and 60A induced the formation of cartilage, bone, and bone marrow in mammals, as determined by histological observations and by measurements of the specific activity of alkaline phosphatase and calcium content of the implants, thereby demonstrating that related proteins from phylogenetically distant species are capable of inducing bone formation in mammals when placed in sites where progenitor cells are available.**

Embryonic bone development begins with migration of mesenchymal cells to a predetermined site where they either condense, proliferate, and differentiate directly into bone-forming cells or pass through an intermediate cartilage stage before they are replaced with bone. In adult life, bone has a remarkable potential to repair itself upon fracture through a process that recapitulates embryonic bone development. Urist (1) and Reddi and Huggins (2) have shown that the cellular events involved in embryonic bone development are reproduced in predictable intervals in subcutaneous implants of demineralized bone matrix in rats. By employing a reconstitution assay in the rat subcutaneous bone induction model (3, 4) and molecular cloning approaches, several osteogenic proteins (OPs), also called bone morphogenetic proteins [BMPs; BMP-2 through BMP-6, OP-1 (also called BMP-7), and OP-2] have been identified (5–8). The predicted amino acid sequences of these proteins indicate that they are all members of the transforming growth factor β (TGF-β) superfamily, sharing a high degree of homology within the C-terminal seven-cysteine domain (9).

The TGF-β superfamily members are signaling molecules thought to be responsible for specific morphogenic events during development (9, 10). For example, increasing concentrations of *Xenopus* activins can cause animal cap cells to differentiate into various cell types (11) while BMP-4 (closely related to BMP-2) can instruct a ventral posterior positional cell fate on developing mesoderm in the *Xenopus* blastula (12, 13). In the mouse, localized expression of BMPs has been reported in skin, heart, nervous system, and developing limbs (14). A recent study demonstrates that mutation of BMP-5 causes subtle defects in skeletal structures in the mouse (15). In *Drosophila*, the decapentaplegic (dpp) protein specifies dorsal cell fate in the developing embryo and is also involved in the regulation of homeotic gene expression in gut morphogenesis and proximal–distal appendage development in the adult fly (16–19). The developmental function of the *Drosophila* 60A is presently not known, although it is expressed throughout early embryonic development (20, 21).

A comparison of amino acid sequences within the conserved seven-cysteine domain (TGF-β domain) indicates that dpp is more closely related to BMP-2/4 (75% identity), and 60A is more closely related to BMP-5/6 and OP-1 (BMP-7) (70% identity). Both recombinant human BMP-2 and recombinant human OP-1 have been shown individually to induce bone formation in the rat subcutaneous model and to restore large diaphyseal segmental defects in animal models (22–26). The amino acid sequence similarity of dpp and 60A to human BMPs suggests that they might have the ability to induce *in vivo* bone formation in mammals. In the present study, we demonstrate that the recombinant mature disulfide-linked homodimers of dpp and 60A induce the formation and differentiation of endochondral bone in the rat subcutaneous bone induction model.

MATERIALS AND METHODS

Expression and Purification of *Drosophila* dpp and 60A Proteins. *Drosophila* dpp and 60A proteins were produced using the *Drosophila* S2 cell expression system (21, 27). In brief, the full-length cDNA clone encoding either dpp or 60A protein was incorporated into an expression plasmid that contained the metallothionein promotor and leader. The expression plasmid DNA was cotransfected with a selectable dihydrofolate reductase gene. The *dpp* gene product was produced as a processed mature disulfide-linked dimer that was secreted into the medium and, subsequently, one-half of the dpp protein bound to the tissue culture plate. The dpp protein that bound to the plates was extracted with 250 mM $CaCl_2$/0.1% octyl β-glucoside/20 mM Mes, pH 7.2 (plate wash). The 60A protein was also produced as a processed mature disulfide-linked dimer, which was secreted into the medium.

The dpp and 60A proteins were purified from the plate wash and medium, respectively, using two chromatographic steps: S-Sepharose (Pharmacia) and reverse-phase HPLC (C_{18} Vydac). A typical purification utilized 50 ml of plate wash or medium containing 12.5% (vol/vol) fetal calf serum. The plate wash or medium was diluted with 2 vol of 9 M urea/20 mM Mes, pH 6.5, and applied to a 10- to 20-ml S-Sepharose column equilibrated with 6 M urea/20 mM Mes, pH 6.5/50 mM NaCl. After washing with the equilibration buffer, step elution of the bound protein was accomplished

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BMP, bone morphogenetic protein; OP, osteogenic protein; TGF-β, transforming growth factor β; dpp, decapentaplegic.

†To whom reprint requests should be addressed.

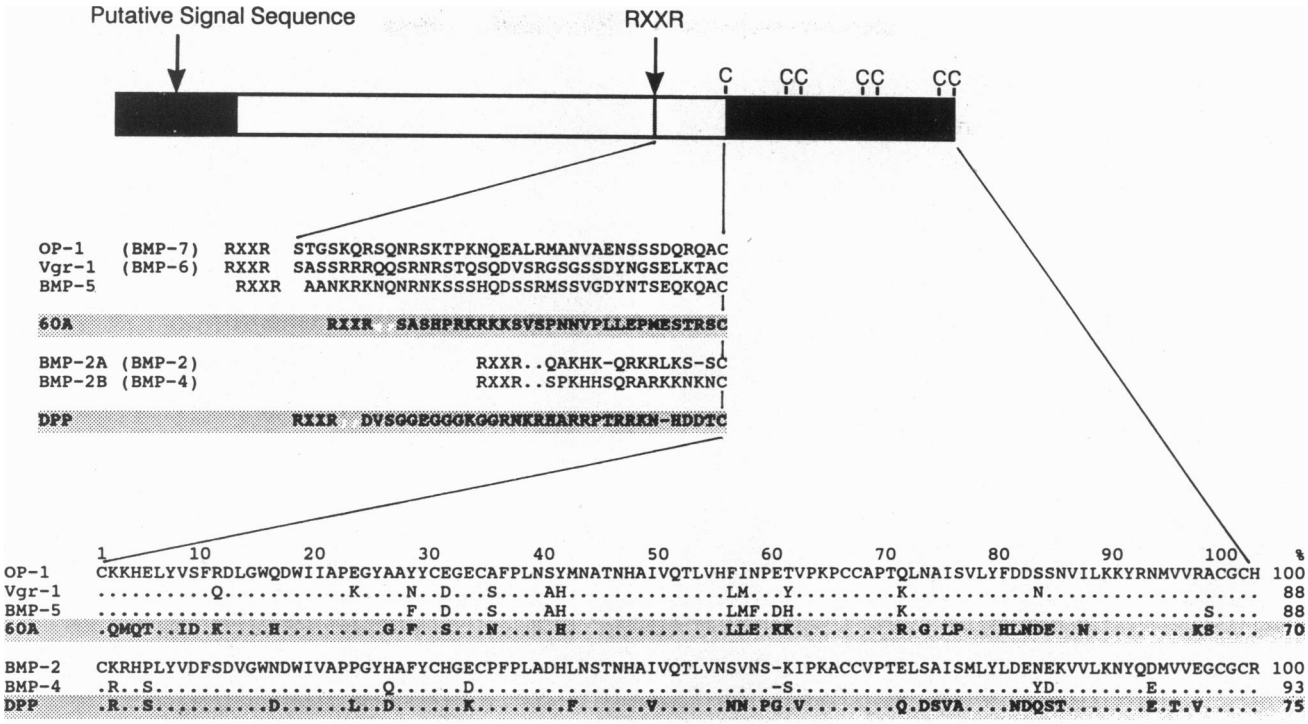

FIG. 1. Amino acid alignment of the mature *Drosophila* dpp and 60A proteins with related OPs and BMPs. Comparison of amino acid sequences within the conserved seven-cysteine domain (TGF-β domain) indicates that dpp is more closely related to BMP-2/4 (75% identity) and 60A is more closely related to BMP-5/6 and OP-1 (BMP-7) (70% identity). The degree of sequence similarity between dpp and 60A is considerably less (53%). The N-terminal extensions that precede the TGF-β domain, however, show considerably more evolutionary divergence among these proteins.

with the same buffer containing 100 mM NaCl, followed by 500 mM NaCl. The 500 mM NaCl fraction containing the active protein was sequentially dialyzed against water and 30% (vol/vol) acetonitrile/0.1% trifluoroacetic acid before being subjected to C_{18} reverse-phase HPLC as described (23). The fractions containing dpp or 60A, as determined by Western blot analysis (using affinity-purified dpp and 60A polyclonal antibodies, respectively) and by Coomassie blue staining, were pooled. The purity and concentrations of dpp and 60A proteins used for the evaluation of bone-forming activity were estimated by scanning at 580 nm the Coomassie blue-stained protein bands separated by SDS/PAGE (see Fig. 2). The concentration of dpp and 60A proteins was determined by comparison to a standard curve generated using known amounts of bovine serum albumin. A comparison of the absorbance by the bovine serum albumin band and a band containing known amount of standard OP-1 (previously quantitated by amino acid analysis) suggested that this approach is feasible for this family of proteins.

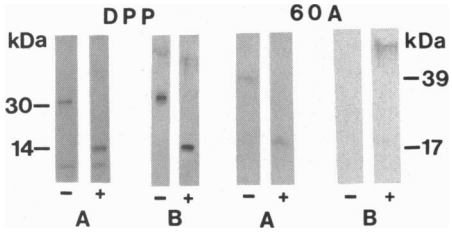

FIG. 2. Purification and characterization of recombinant *Drosophila* dpp and 60A proteins. Lanes: A, Coomassie blue staining of intact (−) and reduced (+) forms of dpp and 60A; B, Western blots using BMP-2-specific antiserum (for dpp) and OP-1-specific antisera (for 60A). Note BMP-2 antiserum is able to detect both intact and reduced forms of dpp, whereas OP-1 antiserum detects only the reduced form of 60A.

***In Vivo* Assay of Bone-Forming Activity.** Highly purified *Drosophila* dpp or 60A protein (see Fig. 2) or recombinant human OP-1 (23) was reconstituted with demineralized guanidine hydrochloride-extracted rat collagen carrier by the 50% acetonitrile/0.1% trifluoroacetic acid lyophilization method (23) and implanted in a subcutaneous site in the thorax region of 28- to 35-day-old male Long–Evans rats. In brief, 25 mg of demineralized 4 M guanidine hydrochloride-extracted rat collagenous bone matrix (rat collagen carrier) was added to various concentrations of protein dissolved in 200 μl of 50% acetonitrile/0.1% trifluoroacetic acid that was then vortex-mixed and, subsequently, lyophilized. Rat collagen carrier alone was the negative control. The day of implantation was designated as day 0 of the assay. Implants were removed on days 7, 12, and 21 for histological evaluation. Bone-forming activity in the day 12 implants was monitored by the specific activity of alkaline phosphatase or calcium content of the implant (2). Values are the average of four to six implants from two or three rats. For histological examination, implants were fixed in Bouin's solution, embedded in JB4 plastic medium,

Table 1. Bone-inducing activity by recombinant *Drosophila* dpp and 60A proteins

Protein	Protein concentration, ng per implant	Alkaline phosphatase, units/mg of protein	Calcium content, μg/mg of tissue	Histology
dpp	—	0.06	ND	−
	480	1.43	ND	+++
	1440	1.48	ND	+++
60A	—	ND	1.95	−
	400	ND	9.75	++
	800	ND	15.20	+++
	1600	ND	19.60	+++

−, Absence of bone formation; ++, moderate bone formation; +++, extensive bone formation; ND, not determined.

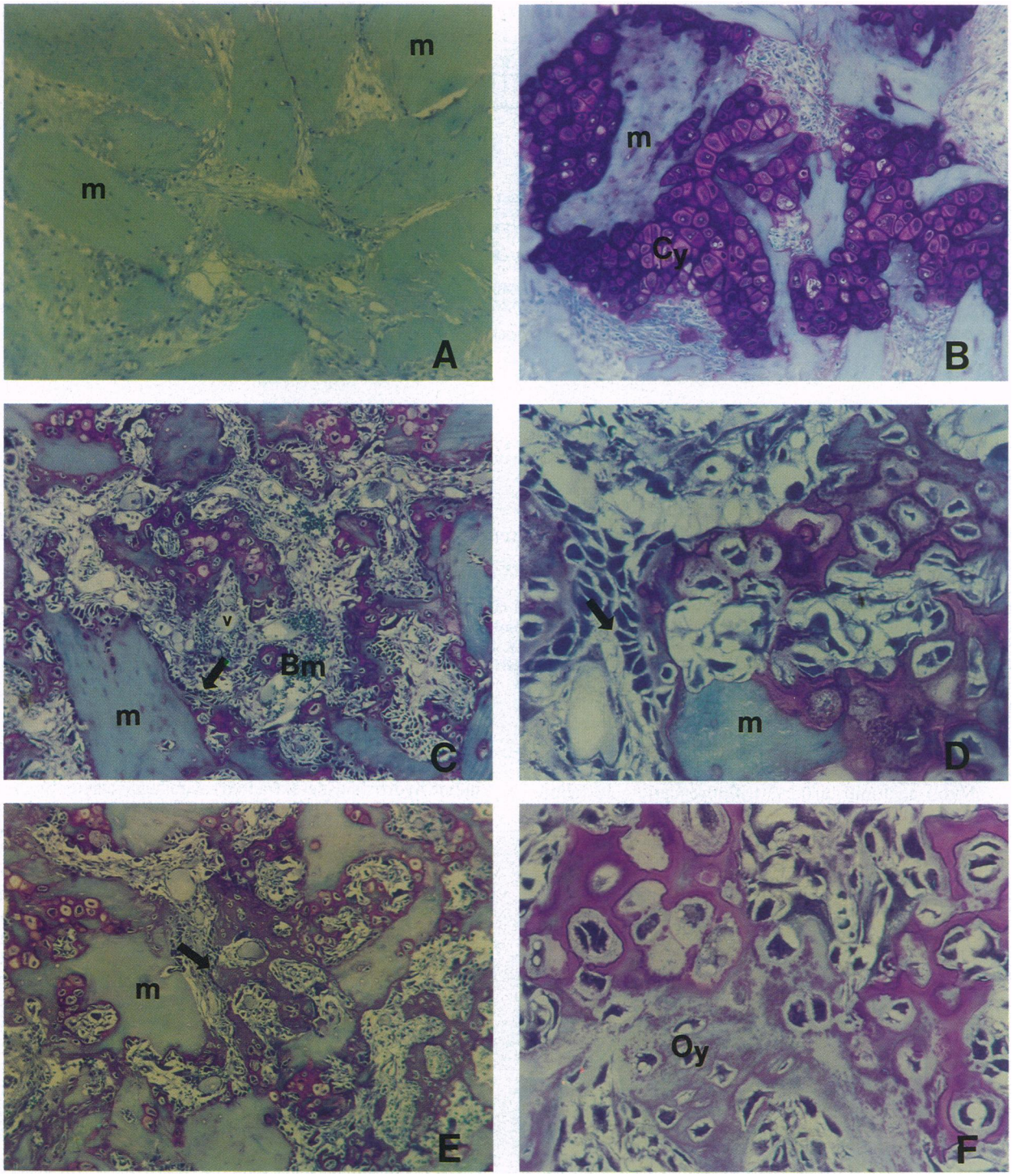

FIG. 3. Photomicrographs of histological sections of subcutaneous implants obtained from rats. (*A*) Negative control (day 12), guanidine hydrochloride-extracted rat demineralized bone matrix (m) (rat carrier). Note the absence of new bone formation. The implant consists of bone matrix and surrounding mesenchyme. (×160.) (*B*) Rat carrier (25 mg) reconstituted with 1 μg of recombinant *Drosophila* dpp (day 7). Evidence of chondrogenesis is seen. Newly formed cartilage cells, chondroblasts, and chondrocytes (Cy) are seen in close contact with the rat carrier matrix (m). A similar response was also seen in the *Drosophila* 60A-containing implants (data not shown). (×180.) (*C* and *D*) Rat carrier (25 mg) reconstituted with 2 μg of recombinant *Drosophila* dpp (day 12). Note evidence of endochondral bone formation (e.g., cartilage calcification, hypertrophy of chondrocytes, vascular invasion, and the onset of new bone formation). Arrows indicate the osteoblasts in close proximity with the vascular endothelium (v). Signs of remodeling are already apparent as shown by the presence of multinucleated osteoclasts. Also, there are early signs of bone marrow recruitment (Bm) in the newly formed ossicles. (*C*, ×220; *D*, ×290.) (*E* and *F*) Rat carrier (25 mg) reconstituted with 1.2 μg of *Drosophila* 60A protein (day 12). Note evidence of endochondral bone formation. The newly formed bone matrix deposited by osteoblasts is extensively mineralized and filled with numerous osteocytes (Oy). Signs of chondrolysis and bone remodeling are evident. (*E*, ×180; *F*, ×290.)

cut into 1-μm sections, and stained with toluidine blue (American HistoLab, Gaithersburg, MD).

Production of Antibodies. The cDNA clones that encode the mature C-terminal region of the human OP-1 gene (8) (aa 293–431, ≈14 kDa) and of the human BMP-2 gene (5) (aa 282–396, ≈13 kDa) were expressed as fusion proteins, respectively, in *Escherichia coli* (22). The OP-1 or BMP-2 fusion proteins, which were produced intracellularly as in-

clusion bodies, were solubilized and cleaved using mild acid to release the leader peptide. After purification, the human OP-1 or human BMP-2 polypeptides were used to raise polyclonal antibodies in rabbits. Antisera were tested for reactivity to intact and reduced bovine OP preparations (highly purified bovine OP preparations were found to be composed of dimers of OP-1 and BMP-2) (22) by Western blot analyses (data not shown). Antibodies to the *Drosophila* 60A protein were prepared against fusion proteins and used for Western blot analysis, as described (21). Antibodies specific to the C-terminal portion of dpp were generated against a glutathione *S*-transferase fusion protein containing the C-terminal 132 aa of dpp. The fusion protein was solubilized from inclusion bodies, purified by SDS/PAGE, and injected into rabbits by methods identical to those used for the generation of antisera against 60A (21).

Analytical Methods. Protein fractions were characterized by SDS/PAGE on 15% mini gels (0.5 mm thick) with a 3% stacking gel (22). Samples dissolved in Laemmli sample buffer were heated in boiling water for 3 min with or without dithiothreitol (100 mM) prior to electrophoresis. For Western blot analysis, samples subjected to SDS/PAGE were transferred to Immobilon membranes (Millipore) and incubated with specific rabbit antisera and, subsequently, with goat anti-rabbit immunoglobulin-linked peroxidase. Amino acid sequence analysis was performed using an Applied Biosystems protein/peptide sequencer, as described (22).

RESULTS AND DISCUSSION

Full-length *Drosophila* dpp and 60A cDNA clones expressed in insect cells yielded correctly processed mature disulfide-linked dimeric protein. The recombinant proteins were purified from either extracts of the proteins adhering to the tissue culture plate (dpp) or from the conditioned culture medium (60A). As is the case with other members of the TGF-β superfamily, the *dpp* and *60A* gene products are synthesized as precursors that are approximately three times larger than the processed mature disulfide-linked dimeric proteins. The purity of the mature dpp and 60A proteins was confirmed by N-terminal amino acid sequence analyses, Coomassie blue staining, and Western blot analyses after SDS/PAGE under nonreducing and reducing conditions (see Fig. 2). The Coomassie-stained reduced dpp protein (16 kDa) and 60A protein (18 kDa) excised from the Immobilon membrane were used for N-terminal amino acid sequence analysis. The N termini of the purified proteins (DVSGGEGGGKGG, for dpp, and XAXHPRKRKKSV, for 60A) corresponded to the sequences of the predicted proteolytic processing sites of the mature dimeric proteins (Fig. 1). Western blot analyses of purified dpp and 60A proteins showed they reacted specifically with their respective antisera (data not shown). In addition, examination of cross-reactivity by Western blots demonstrated that both intact and reduced dpp reacted with human BMP-2 antisera with a similar intensity. 60A protein, however, reacted with human OP-1 antisera weakly under reducing conditions and did not react under nonreducing conditions (Fig. 2).

Evaluation of the bone-forming activity of recombinant *Drosophila* dpp protein and 60A protein in subcutaneous rat implants harvested on days 7, 12, and 21 indicated that both induce bone formation (via cartilage as intermediate tissue), which is subsequently remodeled and filled with functional bone marrow elements (Table 1 and Fig. 3). The sequence of cellular events is comparable to that exhibited by demineralized bone matrix implants. Both dpp and 60A proteins induced cartilage formation, determined by histology of the day 7 implants (Fig. 3*B*). Bone formation induced by dpp protein or 60A protein was associated with cartilage hypertrophy, cartilage calcification, and vascular invasion. Day 12 implants containing 1–2 μg of either dpp or 60A showed bone remodeling and early signs of bone marrow recruitment (Fig. 3 *C* and *E*). Bone-forming activity by dpp protein and 60A protein was also demonstrated by determining the specific activity of alkaline phosphatase or calcium content of day 12 implants (Table 1). Evaluation of day 18–21 implants showed evidence of further remodeling and formation of ossicles filled with bone marrow elements (data not shown). In the absence of dpp or 60A protein, the collagen carrier implants recruited mesenchymal cells and did not show any sign of cartilage or bone formation (Fig. 3*A*). Bone-inducing activity by dpp protein and 60A protein is reproducible and is exhibited only by the column fractions that contained either protein. The degree of response was dependent on the dose of dpp or 60A protein contained in the implants. Since extensive dose curves were not performed at various time intervals, we are unable to directly compare the specific bone-inducing activities of the dpp protein or 60A protein relative to those of OP-1 or BMP-2. However, the present study shows that the concentrations of dpp or 60A protein required to induce bone formation are within the dose ranges that have been reported for recombinant human BMP-2 and recombinant human OP-1 proteins (23, 24).

These insect proteins produced by insect cells were biologically active in mammals, as shown by their ability to initiate the same cellular and developmental responses as the related mammalian protein. The specific responses to the insect proteins were probably dictated by the microenvironment at the implant site and the developmental potential of the responding cells. Whereas bone formation was stimulated by either dpp protein or 60A protein in the present study, it is possible that other tissues will have specific, but different, responses to various BMPs. It is worth noting that the major sites for synthesis of OP-1 and BMP-4 mRNA are the kidney and lung, respectively (8). Recently, OP-1 was shown to induce the neural cell adhesion molecule in a neuroblastoma–glioma hybrid cell line (28). Neural cell adhesion molecule has been shown to play a fundamental role in the development and regeneration of the nervous system. It will be of interest to determine whether specific OPs/BMPs are sufficient to induce tissue regeneration in other tissues. The understanding of how tissues respond to the BMPs, the signal transduction processes caused by receptor activation, and the phenotypic change in different tissues will provide additional insight into the role of BMPs in tissue formation, regeneration, and repair. The functional homology of the ligands investigated in the present study suggests that BMP-mediated instruction of pluripotent cells to pursue specific developmental fates has been well conserved during evolution.

We thank M. Terranova, K. Anderson, and K. White for excellent technical assistance, and John E. Smart and Charles Cohen for reviewing the manuscript.

1. Urist, M. R. (1965) *Science* **150,** 893–899.
2. Reddi, A. H. & Huggins, C. B. (1972) *Proc. Natl. Acad. Sci. USA* **69,** 1601–1605.
3. Sampath, T. K. & Reddi, A. H. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7599–7603.
4. Sampath, T. K. & Reddi, A. H. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 6591–6595.
5. Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kritz, R. W., Hewick, R. M. & Wang, E. A. (1988) *Science* **242,** 1528–1534.
6. Özkaynak, E., Rueger, D. C., Drier, E. A., Corbett, C., Ridge, R. J., Sampath, T. K. & Oppermann, H. (1990) *EMBO J.* **9,** 2085–2093.
7. Celeste, A. J., Iannazzi, J. A., Taylor, R. C., Hewick, R. M., Rosen, V., Wang, E. A. & Wozney, J. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9843–9847.
8. Özkaynak, E., Schnegelberg, P. N. J., Jin, D. F., Clifford,

G. M., Warren, F. D., Drier, E. A. & Oppermann, H. (1992) *J. Biol. Chem.* **267**, 25220–25227.
9. Massague, J. (1990) *Annu. Rev. Cell Biol.* **6**, 597–641.
10. Lyons, K. M., Jones, C. M. & Hogan, B. L. M. (1991) *Trends Genet.* **7**, 408–412.
11. Green, J. B. A. & Smith, J. C. (1990) *Nature (London)* **347**, 391–394.
12. Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. E. & Hogan, B. L. M. (1992) *Development* **115**, 639–647.
13. Dale, L., Howes, G., Price, B. M. & Smith, J. C. (1992) *Development* **115**, 639–647.
14. Jones, C. M., Lyons, K. M. & Hogan, B. L. M. (1991) *Development* **111**, 531–542.
15. Kingsley, D. M., Bland, A. E., Grubber, J. M., Marker, P. C., Russell, L. B., Copeland, N. G. & Jenkins, N. A. (1992) *Cell* **71**, 399–410.
16. Panganiban, G. E. F., Reuter, R., Scott, M. P. & Hoffmann, F. M. (1990) *Development* **110**, 1041–1050.
17. Ferguson, E. L. & Anderson, K. V. (1992) *Cell* **71**, 451–461.
18. Irish, V. F. & Gelbert, W. M. (1987) *Genes Dev.* **1**, 868–879.
19. Padgett, R. W., St. Johnston, R. D. & Gelbart, W. M. (1987) *Nature (London)* **325**, 81–84.
20. Wharton, K. A., Thomsen, G. H. & Gelbart, W. M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9214–9218.
21. Doctor, J. S., Jackson, K. E., Rashka, M., Visalli, M. & Hoffmann, F. M. (1992) *Dev. Biol.* **151**, 491–505.
22. Sampath, T. K., Coughlin, J. E., Whetstone, R. M., Banach, D., Corbett, C., Ridge, R. J., Özkaynak, E., Oppermann, H. & Rueger, D. C. (1990) *J. Biol. Chem.* **265**, 13198–13205.
23. Sampath, T. K., Maliakal, J. C., Hauschka, P. V., Jones, W. K., Sasak, H., Tucker, R. F., White, K. H., Coughlin, J. E., Tucker, M. M., Pang, R. H. L., Corbett, C., Özkaynak, E., Oppermann, H. & Rueger, D. C. (1992) *J. Biol. Chem.* **267**, 20352–20362.
24. Wang, E. A., Rosen, V., D'Alessandro, J. S., Baudy, M., Cordes, P., Harada, T., Israel, D. I., Hewick, R., Kerns, K. M., LaPan, P., Luxenberg, J. M., McQuaid, D., Moutsatsos, I. K., Nove, J. & Wozney, J. M. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 2220–2224.
25. Yasko, A. W., Lane, J. M., Fellinger, E. J., Rose, V., Wozney, J. M. & Wang, E. A. (1992) *J. Bone Jt. Surg. Am. Vol.* **74**, 659–670.
26. Cook, S. D., Baffes, G. C., Wolfe, M. W., Sampath, T. K. & Rueger, D. C. (1993) *J. Bone Jt. Surg. Am.*, in press.
27. Panganiban, G. E. F., Rashka, K. E., Neitzel, M. D. & Hoffmann, F. M. (1990) *Mol. Cell. Biol.* **10**, 2669–2677.
28. Perides, G., Safran, R. M., Rueger, D. C. & Charness, M. E. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 10326–10330.