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Bone Morphogenetic Protein Inhibits Differentiation and Affects Expression of Helix-Loop-Helix Regulatory Molecules in Myoblastic Cells

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Abstract Bone morphogenetic protein (BMP) reproducibly induces chondrogenesis and osteogenesis when implanted into skeletal muscle. The exact identity of the cell that responds to BMP is not known. Furthermore, controversy exists regarding the possibility that myoblastic cells may transdifferentiate to chondrocytes and osteoblasts under the influence of BMP. We have therefore, undertaken studies on the effects of BMP on differentiation in L6 and C2C12 cells, two rodent myoblastic cell lines. To gain insights into the mechanisms of action of BMP, we have studied the effects of BMP on the levels of expression of the four known myogenic determination genes: myogenin, Myo D, herculin, and myf-5. BMP inhibited myogenesis in myoblastic cells. Convincing evidence of transdifferentiation of myoblasts to chondrocytes or osteoblasts was not seen. BMP inhibited the expression of all four myogenic determination genes.

Key words: bone morphogenetic protein, helix-loop-helix molecules, myogenin, Myo D, herculin, myf-5

Bone morphogenetic protein (BMP) is a demineralized extract of noncollagenous bone matrix proteins which, when implanted into skeletal muscle, induces formation of ectopic cartilage and bone [Urist, 1965]. Numerous other tissues respond to the implantation of BMP with new bone formation but only bone itself has a higher proportion of implants that respond [Urist et al., 1969]. The exact identity of the cell type capable of responding to BMP in skeletal muscle, and in other tissues, has not been established. Certainly, skeletal muscle, as well as other tissues, contains undifferentiated mesenchymal cells. In vivo experiments by Buring using rats united in parabiosis suggested that the osteoclastic cells of ectopic bone were derived from circulating monocytic cells whereas the precursors of chondrocytes and osteoblasts were "perivascular connective tissue cells" [Buring, 1975]. In vitro studies using clonal W-20-17 mouse bone marrow stromal cells have shown that recombinant human BMP-2 (rhBMP-2) induced the expression of several osteoblastic markers including alkaline phosphatase (ALP), osteocalcin (OC), and the generation of cAMP in response to PTH in these cells [Thies et al., 1992]. In a number of other pluripotential mesodermal cell lines rhBMP has been demonstrated to induce the osteogenic phenotype. Treatment with rhBMP-2 has been shown to inhibit spontaneous myotube formation and enhance expression of osteoblastic markers in rat ROB-C26 cells [Yamaguchi et al., 1991]. In C3H10T1/2 mouse fibroblastic cells, treatment with rhBMP-2 resulted in an increase in cellular colonies exhibiting the adipocytic, chondrocytic, and osteoblastic phenotypes [Wang et al., 1992]. Other studies, however, have raised the possibility that myoblastic cells may be induced to form osteoblastic cells. Nathanson et al. have described a chondrogenic and osteogenic response of embryonic rat and chick myoblasts to culture on demineralized bone matrix [Nathanson et al., 1978; Nathanson and Hay, 1980a,b]. Results from in vitro studies using clonal L6 rat myoblasts suggested that chondrogenesis occurred after 30 days of

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culture on demineralized bone [Bettex-Galland and Wiesmann, 1986]. In the same cell line, treatment with rhBMP-2 inhibited spontaneous myotube formation in culture [Yamaguchi et al., 1991]. BMP in one form or another, therefore, appears to inhibit myogenesis and to promote chondrogenesis and osteogenesis in undifferentiated mesodermal cells and may induce osteogenesis in differentiated myoblastic cells.

Much progress has been made recently in understanding the molecular mechanisms underlying the process of mesodermal cell differentiation. The greatest amount of information currently available pertains to myogenesis and results, for the most part, from the cloning of four distinct determination genes, the enforced expression of which results in myogenic conversion of permissive cell types. These molecules, Myo D [Davis et al., 1987], myogenin [Wright et al., 1989], myf-5 [Braun et al., 1989], and herculin [Rhodes and Konieczny, 1989; Miner and Wold, 1990] belong to the helix-loop-helix (h-l-h) family of transcription factors. H-L-H regulatory molecules have also been demonstrated to play a role in the regulation of cytodifferentiation in other mesodermal cell types including osteoblastic [Murray et al., 1992] and hematopoietic cells [Kreider et al., 1992]. A coherent model has been promulgated to explain the regulation of myocyte cytodifferentiation in terms of the expression and interaction of h-l-h regulatory molecules [Weintraub et al., 1991]. H-L-H molecules function by forming heterodimers that bind to DNA and activate transcription of that set of genes which constitutes the differentiated phenotype. In the case of myogenesis, it has been proposed that the myogenic determination genes interact with positive modulating members of the family such as E12 [Weintraub et al., 1991; Murray et al., 1992]. Homodimers of the myogenic genes alone are thought to be ineffective in activation of transcription. Negative h-l-h regulators, such as Id, also exist and may function by binding to and titrating out either the positive modulators or the myogenic gene products [Weintraub et al., 1991]. Both E12 and Id are expressed in osteoblastic cells as is the h-l-h molecule mTwi which may also function as a negative modulator of osteoblastic differentiation [Murray et al., 1992].

In order to more precisely define the effect of BMP on proliferating and differentiating myoblastic cells, we have undertaken a series of studies on the regulation of h-l-h gene expression in two myoblastic cell lines by BMP and present our results here.

METHODS

Bone Morphogenetic Protein

BMP was prepared as described in detail previously [Urist et al., 1983, 1987, 1991). Briefly, protein was extracted from bone in 4.0 M guanidine hydrochloride (GuHCl) and BMP separated from high molecular weight proteins by gel filtration. BMP was then recovered by differential precipitation under associative conditions in 0.25 to 0.5 M GuHCl. Water soluble BMP was then obtained by limited acid hydrolysis with 3.0 N citric acid. This material was lyophilized and stored at −20°C until it was used.

Cell Culture

L6 rat myoblastic cells [Yaffe, 1968] and C2C12 mouse myoblastic cells [Yaffe and Saxel, 1977; Blau et al., 1985] were obtained from American Type Culture Collection (Rockville, MD). Cells were routinely cultured in Dulbecco's modification of Eagle's medium (DMEM) (Mediatech, Washington, D.C.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 2.5 mM glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin in a humid atmosphere of 5% CO_2 and 95% air at 37°C. Media were changed three times weekly and cells were subcultured weekly. Cells used in experiments had been subcultured not more than three times. Muscle differentiation medium consisted of DMEM supplemented with glutamine, antibiotics, and 2% horse serum (HyClone Laboratories). The usual muscle differentiation protocol entailed culturing the cells in growth media to near confluence, then replacing the media with differentiation media for a period of 4 days. When BMP was added to the media, a stock solution of 0.25 mg/ml was made by dissolving 2.5 mg of BMP (fraction S) in 10 ml of DMEM and heating briefly at 37°C. This was then added to the media at a final concentration of 5 μg/ml. To limit proteolysis all serum was heat inactivated at 55°C for 30 min prior to use.

Immunocytochemistry

Immunocytochemical detection of myosin heavy chain (MHC) was used as a marker for myocyte differentiation. MHC was detected us-

ing monoclonal antibody MF-20 [Bader et al., 1982] and a peroxidase-linked biotin-avidin mediated method and commercial reagents (Vector Laboratories, Burlingame, CA). Photomicroscopy was performed with a Zeiss Axiovert 35 microscope equipped with a Nikon MF2 35 mm camera.

Alkaline Phosphatase Activity

ALP activity was assayed in cells treated with BMP and control cells as described in detail previously [Murray et al., 1992] following the method of Bessy et al. [Bessy et al., 1946]. At the end of the experimental period, the media were removed and the cells washed with phosphate buffered saline (PBS). Following release by trypsinization the cells were pelleted by centrifugation, resuspended in 1.0 ml of TXM buffer (10 mM Tris (pH 7.4), 1 mM $MgCl_2$, 20 μM $ZnCl_2$, and 0.02% (v/v) Triton X-100) and sonicated for 30 s. The sonicate was centrifuged and ALP activity was determined spectrophotometrically at 410 nm by measuring release of p-nitrophenol from p-nitrophenylphosphate. Total protein was assayed for each culture by using the dye-binding method of Bradford [Bradford, 1976].

Glycosaminoglycan Synthesis

Glycosaminoglycan (GAG) synthesis was assessed in L6 and C2C12 cells following 1 and 2 weeks of treatment with BMP. GAG synthesis was determined as described in detail elsewhere [Murray and Gorski, manuscript in preparation]. Cells were plated at a density of 1×10^5 cells/T-25 flask and cultured overnight in order to allow attachment. The cells were then cultured in growth media with and without BMP. At the time of the assay, media containing 1 uCi/ml of ^{35}S (sulfate, 25 Ci/mg) (Amersham, Arlington Heights, IL) was applied to the cells and the culture was continued for another 16 h. Following the incubation, the cells were released by trypsinization, an aliquot was removed for cell counting, the cells were pelleted by centrifugation and then resuspended in 1 ml of buffer [0.1 M NaH_2PO_4 (pH 6.5), 55 mM EDTA]. Papain (Worthington Biochemical Corp., Freehold, NJ) was activated by incubation at 22°C for 30 min at a concentration of 2 mg/ml in papain dilution buffer (0.1 M EDTA, 0.6 M β-mercaptoethanol, 0.5 M cysteine HCl, 0.1 M NaH_2PO_4 [pH 6.2]), then 1 ml of the cell suspension and 1 ml of the papain solution were combined and incubated at 55°C overnight. The next day another 0.5 ml of the papain solution was added and the incubation at 55°C continued overnight. A 0.25 ml aliquot of the final suspension was removed for assay. To this suspension 0.05 ml of a 5 mg/ml solution of chondroitin sulfate carrier was added. Then 0.3 ml of precipitation solution (0.2% cetyltrimethylammonium bromide (CTAB), 0.1 M NaCl) was added and the suspension cooled at 4°C for 2 h to allow precipitation of the GAGs. The samples were collected on GF/C filters and washed five times with a solution of 50 mM EDTA (pH 6.0), 0.2% CTAB. The filters were allowed to dry overnight and the radioactivity was determined by liquid scintillation counting after vigorous vortexing in 20 ml of scintillation fluid.

Plasmids

Specific antisense riboprobes were generated for each species of RNA examined. Technical information for each plasmid that was used is shown in Table I.

Soluble Hybridization/RNase Protection Studies

Steady-state levels of RNA expression were determined by means of soluble hybridization/

TABLE I. Description of the Plasmids Used to Generate Riboprobes for the Described Studies

Gene product	Plasmid name	Species	Enzyme to linearize	Polymerase to transcribe	Probe length (bp)	Protected fragment length (bp)	Reference
MCK*	pBlue EP	Mouse	Hind III	T7	400	240	Bushkin et al., 1985
γ-actin	pSp6-4 γ-actin	Human	Hinf I	Sp6	110	65	Miner and Wold, 1991
Myo D	pMyo DE	Mouse	Dde I	T7	300	237	Davis et al., 1987
myf-5	pH352	Human	Bss HI	T3	350	242	Braun et al., 1989
Myogenin	pSRmyo 8	Rat	Sty I	T7	238	183	Wright et al., 1989
Herculin	pSa1 Rec	Mouse	Xho I	T7	365	270	Miner and Wold, 1990

*MCK = muscle creatine kinase.

RNase protection assays. This type of assay was employed because it is more sensitive and specific than other approaches, such as Northern blotting. RNA was extracted from treated and control cells cultured in 150 mm dishes using the acid guanidium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. Cells were lysed in 4 ml of a solution containing 4 M guanidinium thiocyanate, 0.5% sodium N-laurylsarcosine, 25 mM sodium citrate (pH 7.0), and 0.1 M β-mercaptoethanol. To this suspension, 0.4 ml of 2 M sodium acetate (pH 4.0) was added followed by 4 ml of water-saturated phenol and 0.8 ml of chloroform/isoamyl alcohol (49:1). After incubation on ice for 15 min the suspension was centrifuged at 10,000*g* for 20 min. The aqueous phase was removed to a fresh tube and the RNA precipitated with isopropyl alcohol. The precipitated RNA was washed with 0.5 ml of 70% ethanol and resuspended in 0.4 ml of TE buffer (10 mM Tris (pH 7.4), 1 mM EDTA). Residual DNA was digested by adding 1 unit of RNase-free DNase I (Promega, Madison, WI) and incubating at 37°C for 15 min. The solution was then extracted with an equal volume of phenol/chloroform (1:1) and the aqueous phase was removed and stored at −20°C until it was used.

Antisense riboprobes were generated using the specific plasmid for each assay. Probes were labeled with ^{32}P-UTP (800 Ci/mmol, 40 mCi/ml) (Amersham Corp., Arlington Heights, IL) to a specific activity of 2 labeled UTP residues per protected probe fragment. For each sample, 10 μg of RNA was hybridized overnight with 5×10^9 molecules of probe at 50°C in 30 μl of hybridization buffer (80% formamide, 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA). RNase digestion was accomplished in 0.3 ml of digestion buffer [10 mM Tris (pH 7.5), 5 mM EDTA, 300 mM NaCl] containing 300 U of ribonuclease T1 (United States Biochemical, Cleveland, OH) and 120 ng of ribonuclease A (Sigma Chemical Co., St. Louis, MO) at 30°C for 60 min. Following the RNase digestion, 0.2 ml of a solution containing 1.35% SDS, 0.3 mg/ml Proteinase K

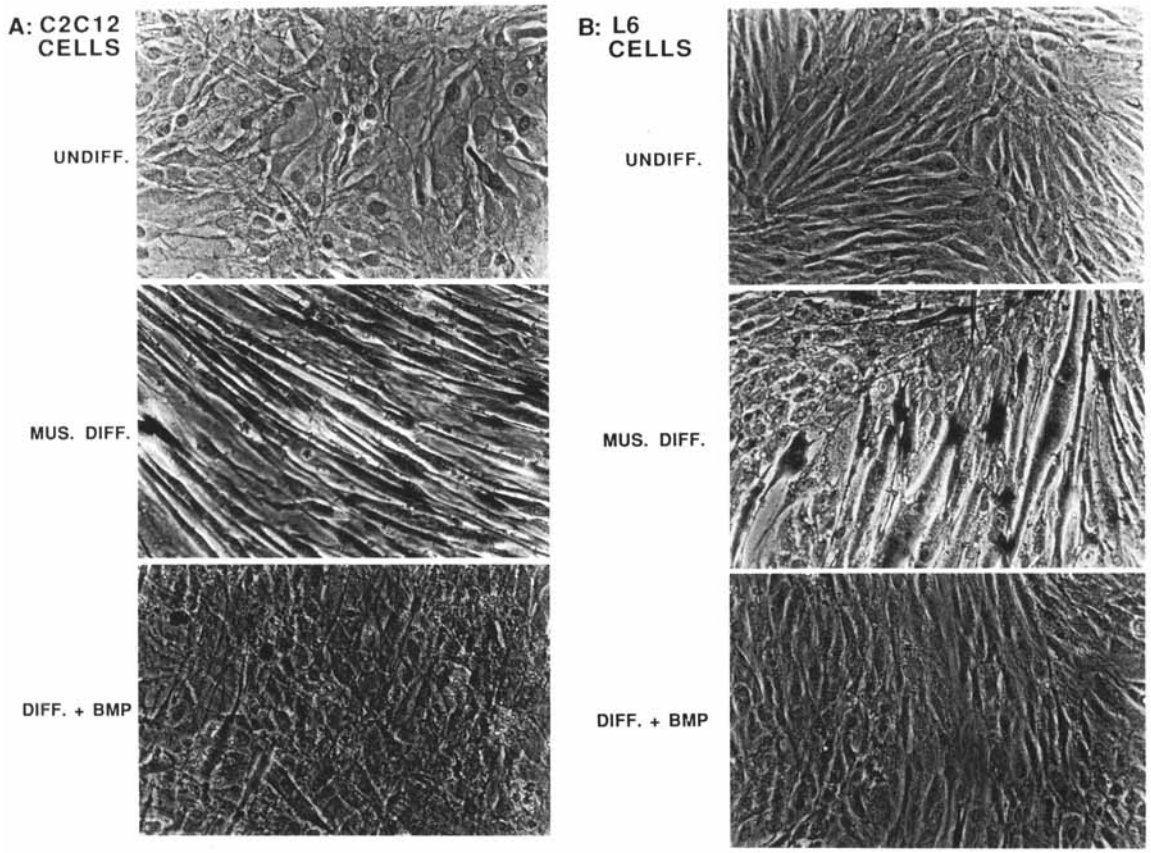

Fig. 1. Photomicrographs of C2C12 mouse myoblastic cells (**A**) and L6 rat myoblastic cells (**B**) cultured under nondifferentiating conditions, differentiating conditions, and differentiating conditions with the addition of BMP. Immunocytochemically stained for myosin heavy chain. Original magnification: ×320.

(Boehringer Mannheim Biochemicals, Indianapolis, IN), and 300 mM NaCl was added and the solution incubated at 37°C for 15 min. The solution was extracted with an equal volume of phenol/chloroform (1:1) and the RNA precipitated from the aqueous phase. The RNA products were separated by electrophoresis through an 8% acrylamide gel and subjected to autoradiography using X-OMAT AR film (Eastman Kodak Co., Rochester, NY).

Statistical Methods

Numerical analysis of data consisting of means of continuous variables was done with Student's *t*-test when there was one dependent variable and by ANOVA when there was more than one dependent variable. Analyses were performed on an Apple Macintosh IIsi computer using Statview 512+ software (BrainPower, Agoura Hills, CA).

RESULTS

Effect of BMP on Morphology and Myosin Heavy Chain Expression in Differentiating Myoblasts

The effects of treatment with BMP on the morphology and expression of myosin heavy chain (MHC) protein in differentiating C2C12 mouse and L6 rat myoblastic cells are shown in Figure 1. The undifferentiated C2C12 cells displayed the characteristic stellate morphology with no significant expression of MHC. Among the cells subjected to the differentiation protocol, a high proportion fused into thin, elongated multinucleated myotubes which showed high levels of MHC expression. However, the cells that were subjected to the differentiation protocol in the presence of BMP did not form myotubes and did not express appreciable levels of MHC. Similarly, undifferentiated L6 cells were stellate in shape and fused into large, plump multinucleated myotubes which displayed high levels of MHC immunoreactivity when subjected to the differentiation protocol. As in the previous case, addition of BMP to the differentiation media prevented both fusion into myotubes and expression of high levels of MHC.

Effect of BMP on Alkaline Phosphatase Activity and Glycosaminoglycan Synthesis in Myoblasts

Alkaline phosphatase (ALP) activity and glycosaminoglycan (GAG) synthesis were used as markers for osteoblastic and chondrocytic phenotypes in order to determine whether myoblastic cells had acquired the characteristics of either of these cell types. The results of treatment with BMP for 1 and 2 weeks on ALP activity in both myoblastic cell lines are shown in Figure 2A. In general, ALP activity was higher in L6 cells than in C2C12 cells. Numerical analysis by ANOVA indicated that in the case of L6 cells both treatment with BMP and time in culture

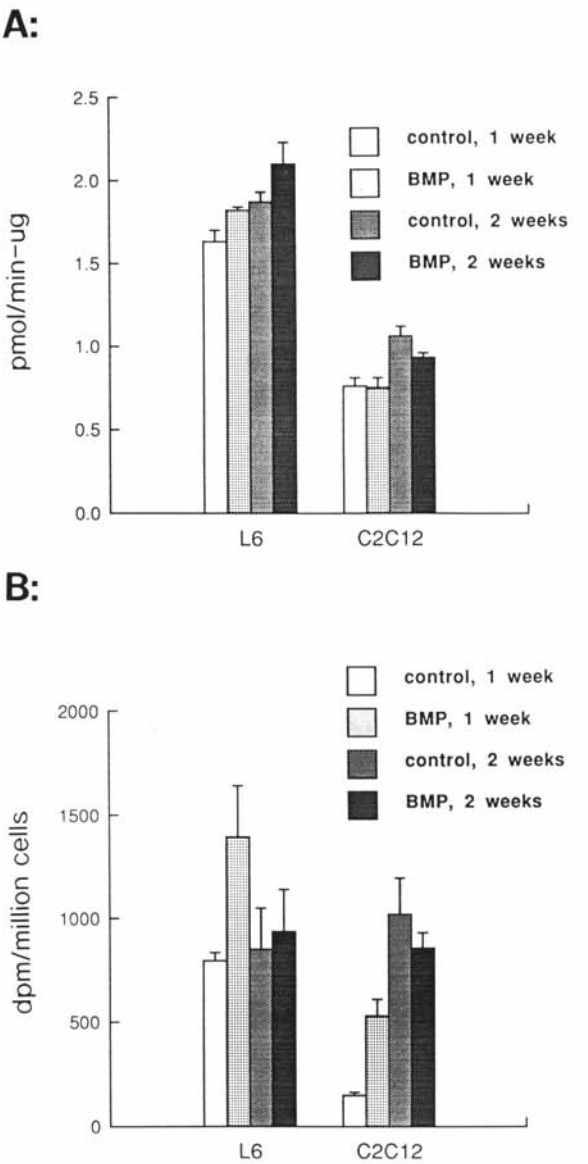

Fig. 2. ALP activity (**A**) and GAG synthesis (**B**) in C2C12 mouse and L6 rat myoblastic cells cultured for 1 and 2 weeks in growth media or growth media plus BMP. Data expressed as mean ± SEM (n = 3). Statistical analysis by ANOVA. A: C2C12 cells, treatment with BMP, P = not significant; time in culture, $P < 0.01$. L6 cells, treatment with BMP, $P < 0.05$; time in culture, $P < 0.05$. B: C2C12 cells, treatment with BMP, P = not significant; time in culture, $P < 0.05$. L6 cells, treatment with BMP, P = not significant; time in culture, P = not significant.

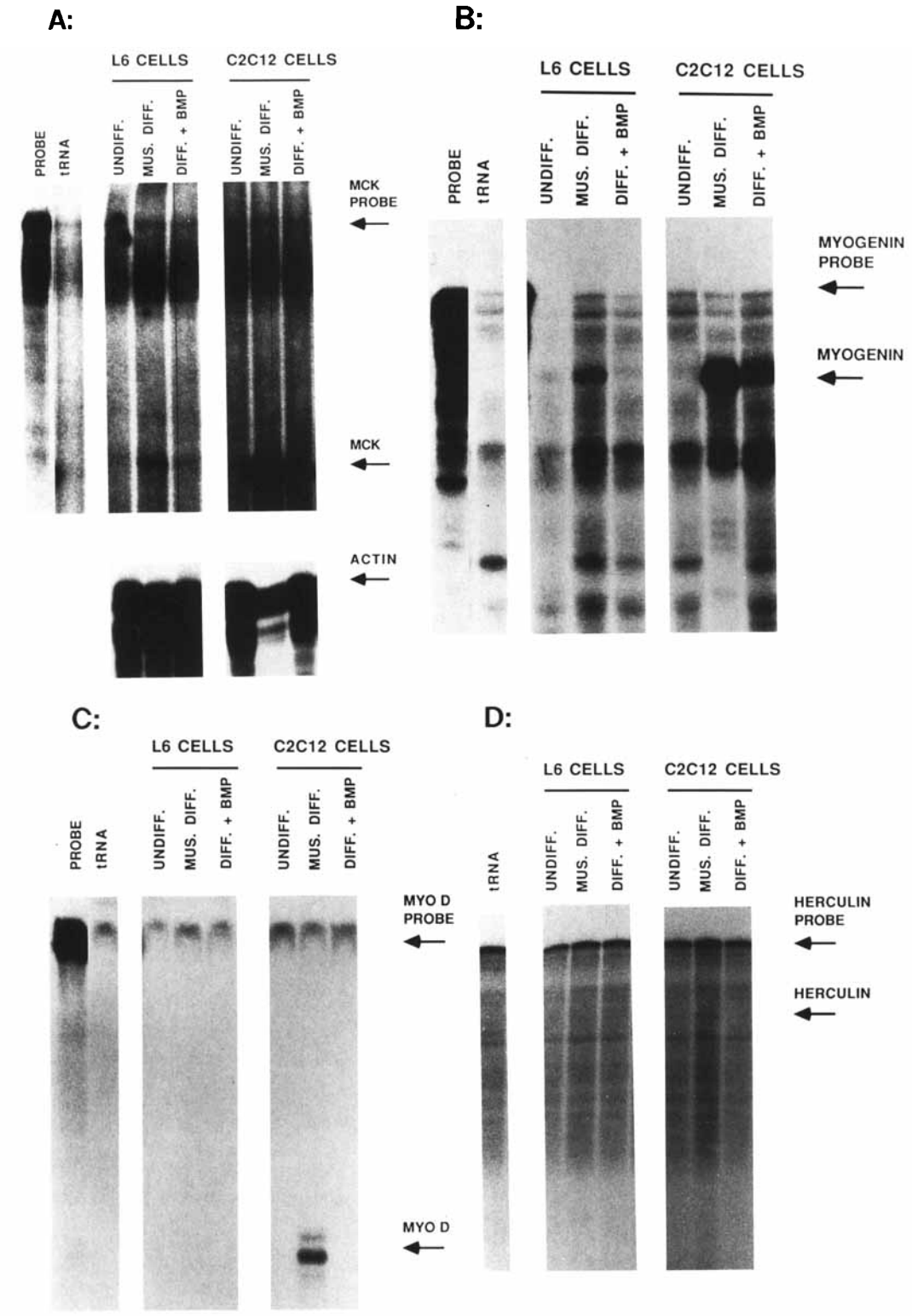

Fig. 3. Autoradiograms of RNase protection studies to detect specific RNA for MCK and γ-actin (**A**), myogenin (**B**), Myo-D (**C**), herculin (**D**), and myf-5 (**E**) in L6 rat and C2C12 mouse myoblastic cells cultured *under nondifferentiating conditions*, differentiating conditions, or differentiating conditions with BMP. For each study 10 μg of RNA was assayed. The upper arrow indicates undigested probe whereas the lower arrow indicates the protected fragment.

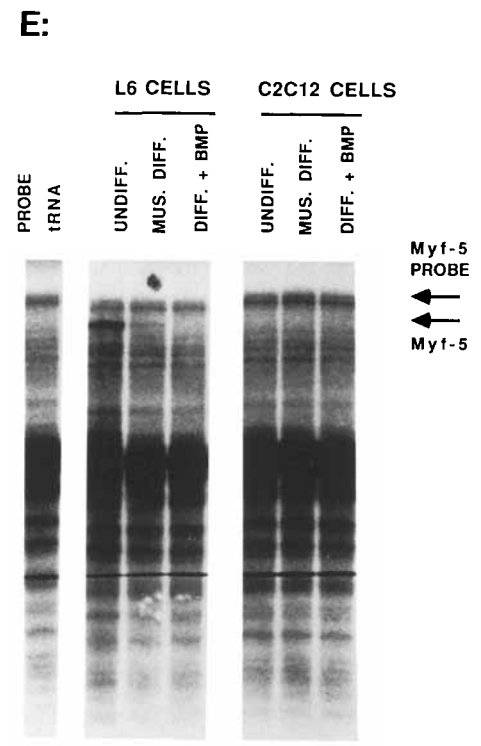

Figure 3 (*continued*).

had modest positive effects on levels of ALP activity. In the case of C2C12 cells, time in culture but not treatment with BMP had a positive effect. These effects were statistically significant but the degree of increase, which was less than 50% in all cases, was minimal. Figure 2B shows the effects of treatment with BMP on GAG synthesis at the same time points. In L6 cells neither time in culture nor treatment with BMP had a significant effect on levels of GAG synthesis when analyzed by ANOVA. In the case of C2C12 cells, time in culture had a statistically significant but numerically modest effect on levels of GAG synthesis.

Effect of BMP on Muscle Creatine Kinase RNA Expression in Differentiating Myoblasts

In order to demonstrate that the differentiation protocol was effective in modulating RNA expression of molecules associated with myoblastic cytodifferentiation, RNA specific for muscle creatine kinase (MCK) was assayed in both cell lines. These results are shown in Figure 3A. The RNA that was assayed is the same as that used in the experiments for all of the subsequent figures. Therefore, to document that the relative amount of RNA for each treatment group was equivalent, γ-actin was also assayed. These results are shown in the lower portion of Figure 3A. MCK was not expressed at high levels in either undifferentiated L6 myoblasts or C2C12 myoblasts. Subjection of the cells to differentiation conditions resulted in a dramatic increase in the level of expression of MCK RNA, to a greater extent in C2C12 cells than L6 cells. When BMP was added to the differentiation media the degree of MCK expression was markedly inhibited. The expression of γ-actin is very similar for all of the groups, though γ-actin mRNA levels in the differentiation protocol treatment group for C2C12 cells may be underrepresented (or possibly reflect a spurious signal for this particular sample).

Effect of BMP on Expression of RNA of Myogenic Determination Genes in Differentiating Myoblasts

RNA specific for each of the four known myogenic determination genes was assayed for both cell lines under the same conditions as described above. Figure 3B shows the results for myogenin. Very low levels of myogenin RNA were detected in both undifferentiated L6 cells and C2C12 cells. When the cells were put through the differentiation protocol the level of myogenin RNA expression increased greatly, more so in C2C12 cells than in L6 cells. Treatment with BMP in conjunction with the differentiation regime resulted in marked attenuation of the increase in myogenin RNA expression in both cell lines. Lower levels of myogenin expression were seen in L6 cells and treatment with BMP resulted in almost complete suppression.

The results of the assay for Myo D-specific RNA are shown in Figure 3C. Myo D RNA expression was not detected in L6 rat myoblastic cells with the probe that we employed which is directed against the 3′ untranslated region of the mouse Myo D molecule. In C2C12 mouse myoblastic cells Myo D RNA expression was observed at very low but detectable levels in undifferentiated cells. There was, however, a high level of expression of Myo D RNA in differentiating C2C12 cells. The expression of Myo D RNA in association with culture under differentiating conditions was completely abolished by the addition of BMP to the differentiation regimen.

Figure 3D shows the results of the assay for herculin RNA. Herculin was not detected in L6 cells with the probe that we used. Herculin RNA expression was not seen with nondifferentiating

C2C12 cells but very low levels of expression were detected in differentiating C2C12 myoblastic cells. This expression was inhibited by the addition of BMP to the differentiation media.

The results of the experiments pertaining to the expression of myf-5 are shown in Figure 3E. In L6 cells myf-5 was expressed at relatively high levels in undifferentiated cells. Expression diminished somewhat upon differentiation and could not be detected in BMP-treated cells. Myf-5 RNA was not detected in the C2C12 samples used in this study but was detected in samples derived from cells subjected to a more prolonged differentiation protocol (data not shown).

DISCUSSION

Disagreement exists regarding the nature of the cells in muscle which are capable of responding to BMP by forming new cartilage and bone. Specifically, it is debated whether cartilage and bone arise from muscle-associated pluripotent undifferentiated mesenchymal cells or from myoblasts. Certainly cell lines exist which represent mesodermal cells which can be manipulated so as to produce differentiated cells of several mesodermal phenotypes including myocytes, adipocytes, chondrocytes, and osteocytes. [Taylor and Jones, 1979; Grigoriadis et al., 1988; Yamaguchi and Kahn, 1991]. Furthermore, Katagiri et al. reported that rBMP-2 induced an osteoblastic phenotype in C3H10T1/2 cells as manifested by a 7-fold increase in ALP activity and a 13-fold increase in cAMP generation in response to PTH [Katagiri et al., 1990]. Wang et al. have reported an increased number of cell colonies that showed the chondrocytic, osteocytic, and adipocytic phenotype when C3H10T1/2 cells were treated with rBMP-2 [Wang et al., 1992]. On the other hand, several groups have reported chondrogenesis and osteogenesis from muscle cells in response to native or recombinant BMP. Okada has reviewed the process of "transdifferentiation" [Okada, 1991]. As applied to chondrogenesis and osteogenesis from muscle cells transdifferentiation would entail the degeneration of myotubes, the dedifferentiation of myoblasts into pluripotent fibroblasts, and the recruitment of "genuine" fibroblasts and dedifferentiated myoblasts and induction of these cells into cartilage and bone precursor cells. Nathanson et al. presented an extensive body of data in support of this hypothesis [Nathanson et al., 1978; Nathanson and Hay, 1980a,b]. They reported that dissociated muscle cells or muscle fragments from embryonic rat muscle as well as cloned myoblasts from embryonic chicken muscle formed cartilage when cultured on bone matrix [Nathanson et al., 1978]. It is unclear, however, how undifferentiated cells, such as myomesial cells, were excluded from their cultures. In a subsequent series of studies, embryonic rat muscle cells cultured on bone matrix were studied with regards to their ultrastructural characteristics by means of electron microscopy after culture on bone matrix for various periods of time [Nathanson and Hay, 1980a,b]. Thus, while they were not observing the same cells they did conclude that there was a progression in the morphology from that of myoblast to that of fibroblast and ultimately chondrocyte.

Myoblastic cell lines, especially L6 rat cells, have been studied in relation to chondrogenesis and osteogenesis. Schubert and Lacorbiere reported that treatment of L6 cells with dibutyryl cAMP prevented fusion of cells into myotubes and increased collagen synthesis by 50% and GAG synthesis by 70% (with a shift from hyaluronic acid to chondroitin sulfate) over a period of 1 to 3 days [Schubert and Lacorbiere, 1976]. This was interpreted as demonstrating a phenotypic transformation of myoblasts to chondrocytes. Thompson et al. reported that culture of L6 cells with demineralized bone matrix in agarose did not increase cartilage-specific proteoglycan synthesis [Thompson et al., 1985]. The most forceful data, however, is that of Bettex-Galland and Wiesmann who reported histologic chondrogenesis using L6 cells cultured on demineralized bone matrix [Bettex-Galland and Wiesmann, 1987]. In addition, in both pluripotent C26 cells and in L6 myoblastic cells, rBMP-2 has been shown to inhibit myotube formation [Yamaguchi and Kahn, 1991].

Our studies clearly show that soluble BMP strongly inhibits myogenic cytodifferentiation in the two cell lines examined as manifested morphologically by inhibition of fusion into myotubes and also by decreased myosin heavy chain immunoreactivity and decreased MCK. We were able to demonstrate a modest increase in ALP activity following treatment with BMP in L6 cells but not in C2C12 cells. However, ALP activity increased as a function of time in culture in both cell lines. Treatment with BMP did not increase total GAG synthesis in either cell line though time in culture was associated with an increase in total GAG synthesis in C2C12 cells. The small changes in these biochemical parameters that were observed are unlikely to be biologically significant. We have not been

successful in attempts to demonstrate chondrogenesis through culture of either cell line on demineralized bone matrix (data not shown). Thus, we did not demonstrate any consistent findings that would indicate that these cell lines exhibited osteoblastic conversion. The reasons for the disparities among the reported experiments, including our own, are not clear but may pertain to the form, composition, and dose of BMP used as well as the duration of the treatment and the exact culture conditions.

In the case of either pluripotent undifferentiated mesenchymal cells or transdifferentiated myoblastic cells, inhibition of the myogenic program will occur in conjunction with activation of the osteogenic program. While Chen and Jones have shown that enforced expression of Myo D in osteoblastic cells resulted in cells that displayed characteristics of both phenotypes, this is not the case in fully developed tissues [Chen and Jones, 1990]. That experiment did indicate, however, that the agents that control the expression of one phenotype over another will do so by regulating the levels of expression of effector molecules such as Myo D. We have, therefore, undertaken a series of experiments to determine the effect of BMP on the expression of known myogenic determination genes.

We detected low levels of expression of myogenin in undifferentiated L6 and C2C12 cells. Miner and Wold have previously reported low levels of myogenin expression in proliferating C2C12 cells and attributed it to spontaneously differentiating cells [Miner and Wold, 1991]. Myogenin expression increased dramatically with differentiation. This increase was inhibited by BMP. Myo D expression was not detected in L6 cells and at only very low levels in undifferentiated C2C12 cells. The failure to detect Myo D in the rat L6 cells may result from our use of a probe directed against the 3′ untranslated region of the mouse Myo D molecule. This region of the RNA molecule is susceptible to great variation and, therefore, detection with a nonhomologous probe from another species would be unlikely in a protection assay. Davis et al. have reported expression of Myo D in proliferating C2C12 cells [Davis et al., 1987]. In our studies the expression of Myo D in undifferentiated cells relative to differentiated cells was much lower than that reported by Davis et al. This may be related to our use of more fully differentiated cells whereas Davis et al. compared "growing" and "confluent" cells. In any case, Myo D expression was greatly increased in association with differentiation and this increase was inhibited by BMP. We did not detect herculin in L6 cells or in undifferentiated C2C12 cells. This molecule is detected frequently in muscle tissue but uncommonly in cell lines [Miner and Wold, 1991]. Our failure to detect herculin in L6 rat cells is probably related to using a probe directed against the mouse molecule since others have reported detecting low levels of expression of MRF4 (the rat homologue of herculin) in L6 cells [Rhodes and Konieczny, 1989]. As previously reported by others, we detected herculin in differentiated but not undifferentiated C2C12 cells [Miner and Wold, 1991]. This expression in association with differentiation was prevented by BMP. Expression of myf-5 RNA was detected in L6 but not C2C12 cells and, in the case of L6 cells, was reduced in differentiated cells relative to undifferentiated cells as has been described previously [Miner and Wold, 1991]. Treatment with BMP inhibited myf-5 expression in differentiating L6 cells. In summary, BMP inhibited myogenic conversion of myoblastic cells and inhibited the usual differentiation-associated increase in the expression of h-l-h determination genes that are felt to be the final effectors of cytodifferentiation in myoblastic cells.

In conclusion, BMP was shown to inhibit myogenic conversion in two rodent myoblastic cell lines. We could find no convincing evidence to indicate that myoblasts had transdifferentiated to chondrocytic or osteoblastic cells under the influence of BMP. The inhibition of myogenesis in myoblastic cells was correlated with a suppression in the expression of the myogenic determination genes.

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