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Analysis of Differential Gene Expression in Rat Tibia After an Osteogenic Stimulus In Vivo: Mechanical Loading Regulates Osteopontin and Myeloperoxidase

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Abstract The skeleton has the ability to alter its mass, geometry, and strength in response to mechanical stress. In order to elucidate the molecular mechanisms underlying this phenomenon, differential display reverse transcriptase-polymerase chain reaction (DDRT-PCR) was used to analyze gene expression in endocortical bone of mature female rats. Female Sprague-Dawley rats, approximately 8 months old, received either a sham or bending load using a four-point loading apparatus on the right tibia. RNA was collected at 1 h and 24 h after load was applied, reverse-transcribed into cDNA, and used in DDRT-PCR. Parallel display of samples from sham and loaded bones on a sequencing gel showed several regulated bands. Further analysis of seven of these bands allowed us to isolate two genes that are regulated in response to a loading stimulus. Nucleotide analysis showed that one of the differentially expressed bands shares 99% sequence identity with rat osteopontin (OPN), a noncollagenous bone matrix protein. Northern blot analysis confirms that OPN mRNA expression is increased by nearly 4-fold, at 6 h and 24 h after loading. The second band shares 90% homology with mouse myeloperoxidase (MPO), a bactericidal enzyme found primarily in neutrophils and monocytes. Semiquantitative PCR confirms that MPO expression is decreased 4- to 10-fold, at 1 h and 24 h after loading. Tissue distribution analysis confirmed MPO expression in bone but not in other tissues examined. In vitro analysis showed that MPO expression was not detectable in total RNA from UMR 106 osteoblastic cells or in confluent primary cultures of osteoblasts derived from either rat primary spongiosa or diaphyseal marrow. Database analysis suggests that MPO is expressed by osteocytes. These findings reinforce the association of OPN expression to bone turnover and describes for the first time, decreased expression of MPO during load-induced bone formation. These results suggest a role for both OPN and MPO expression in bone cell function. J. Cell. Biochem. 68:355–365, 1998.

Key words: mechanical loading; gene expression; osteopontin; myeloperoxidase; rats; differential display

Although the anatomical form of the skeleton is inherited, the skeleton is a dynamic tissue that is able to sense and adapt to the mechanical stresses of gravity and exercise by altering its mass, geometry, and strength [Felts and Spurrell, 1966; Frost, 1964]. Several approaches and in vivo models have been developed to artificially increase mechanical stress, which increase bone formation and bone strength [Burr et al., 1989; Chow et al., 1993; Churches and Howlett, 1982; Goodship et al., 1979; Lanyon et al., 1982; O'Connor et al., 1982; Rubin and Lanyon, 1985; Turner et al., 1994].

The cellular and molecular mechanisms underlying this mechanical adaptation are not well understood; however, preliminary attempts to characterize these responses at the molecular level have been made. The osteogenic response to strains of physiological magnitude in vivo appears to be associated with changes in gene expression. To date, a few candidate genes

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have been identified following mechanical stimulation of bone in vivo. These include c-*fos*, insulin-like growth factor-I (IGF-I), glutamate/aspartate transporter, osteocalcin, osteopontin, and transforming growth factor-β1 (TGFβ1) [Inaoka et al., 1995; Lean et al., 1995, 1996; Mason et al., 1997; Raab-Cullen et al., 1994; Sun et al., 1995]. To understand the molecular processes fully and to identify other regulatory and controlling genes, expressed in response to mechanical loading, further molecular characterization is necessary. An approach suitable for uncovering regulated genes is differential display reverse transcriptase-polymerase chain reaction (DDRT-PCR), originally described by [Liang and Pardee, 1992]. This PCR-based technique has been used successfully for novel gene discovery in various tissues, including bone [Mason et al., 1997; Noel et al., 1995; Wang et al., 1996]. DDRT-PCR selectively amplifies subsets of the total cellular pool of mRNA (cDNA), which are displayed on a sequencing gel. Differentially regulated bands are subsequently isolated, cloned, sequenced, and identified.

The aim of the present study was to apply DDRT-PCR to analyze load-induced differential gene expression during the initiation of the anabolic response in tibia of mature female rats. Mechanical loading was applied using the rat tibia loading model as described previously [Turner et al., 1994]. We have isolated two genes, osteopontin (OPN) and myeloperoxidase (MPO), that are regulated in response to a loading stimulus on cortical bone.

MATERIALS AND METHODS

Animals

Female, 8-month-old Sprague Dawley rats, purchased from Harlan Laboratories (Indianapolis, IN), were housed with a 12-h light–dark cycle and given water and standard rat chow ad libitum. Animal protocols used in these experiments were approved by Indiana University Animal Care and Use Committee.

Loading

Bone formation in vivo was induced by mechanical loads applied to the rat tibia, using the loading model as previously described [Turner et al., 1994]. Briefly, the apparatus bends the tibia so that the lateral surface is in compression and the medial surface is in tension. Sham loads were applied at the same magnitude as bending forces, but the loading pads were arranged so that they squeezed the leg without creating significant bending moment. Loading was applied as a sine wave at a magnitude of 65 N at 2 Hz for 3 min. Sixty-eight rats were divided into three groups of 12 each, for the analysis of gene expression, and a group of 32 were assigned for bone histomorphometry. Each group was divided into two subgroups that received either bending or sham loading on their right tibiae. Groups for the study of gene expression were sacrificed at 1, 6, and 24 h after loading. The tibial diaphyses were removed, cleaned of soft tissue and total RNA was extracted. Differential gene expression was analyzed by DDRT-PCR at 1 h and 24 h after load. For bone histomorphometry, fluorochrome bone labels were given to the last group of rats at 4 and 11 days after the mechanical loading bout and sacrificed on day 15. After the rats were sacrificed, both tibiae were removed, fixed for 48 h in 10% neutral buffered formalin, and embedded in methyl methacrylate (K-Plast, Delaware Diamond Knives, Wilmington, DE). Transverse sections, 50 μm thick, were cut through the tibial midshaft in the region of maximum bending moments (about 6 mm proximal to the tibiofibular junction) using a diamond wire saw (Histo-Saw, Delaware Diamond Knives). From the fluorescent bone labels, bone formation rate (BFR/BS), double-labeled surface (dL.S) and mineral apposition rate (MAR) were measured on the endocortical bone surface using a Bioquant semiautomatic digitizing system (R&M Biometrics, Nashville, TN) attached to a Nikon Optiphot fluorescence microscope.

RNA Isolation and cDNA Synthesis

The tibiae were resected, and all connective tissue, including periosteum, was removed by scraping. Diaphyseal segments from the right tibia (Fig. 1) were snap-frozen and pooled into sham and bending loading groups for each time point after the mechanical loading bout. Segments were pooled to increase the amount of total RNA extracted. Total RNA was extracted by homogenization in Ultraspec-II (Biotecx Laboratories, Houston, TX) using a Polytron LS 10-35 (Kinematica, Cincinnati, OH), as recommended by the manufacturer. Isolated RNA was quantitated using spectrophotometry by measuring the absorbance at 260 nm with the 260/280-nm ratio calculated to ensure the ab-

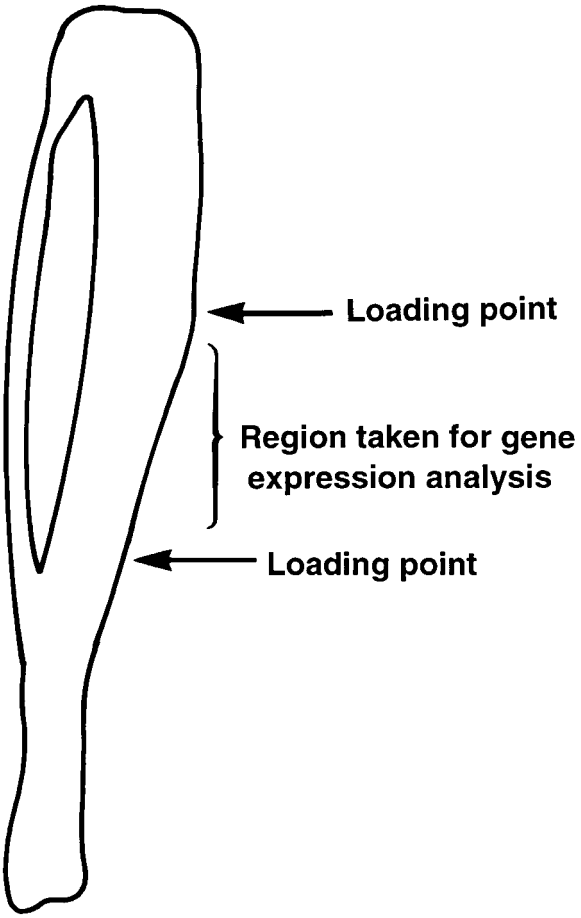

Fig. 1. Rat tibia showing the location from which bone samples were taken for gene expression analysis. The designated loading points are the two contact points between the tibia and upper loading pads. Sections for gene expression analysis were resected as indicated in the schematic diagram.

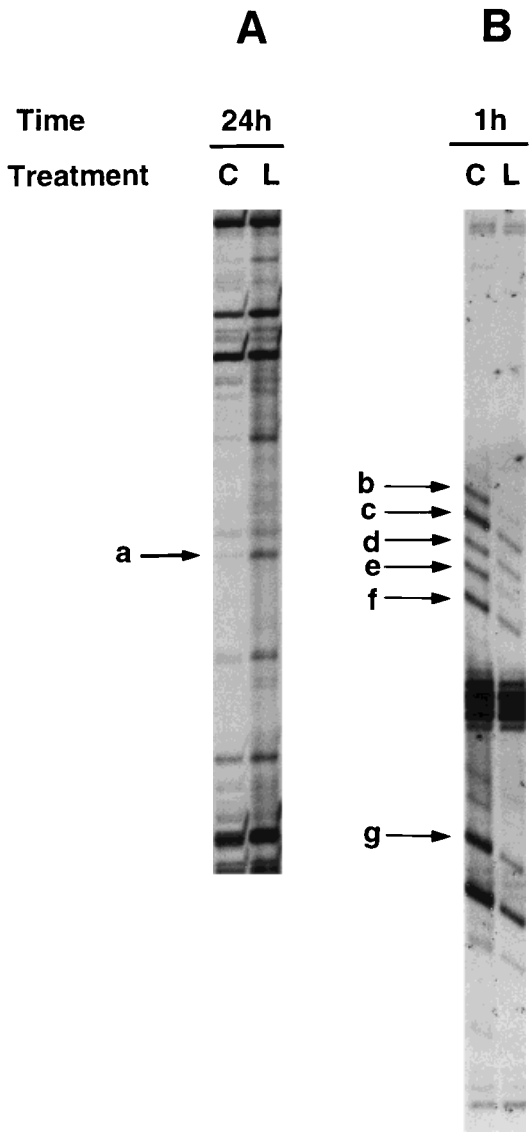

Fig. 2. Differential display using RNA from mechanical loaded (L) and sham control (C) tibia. PCR amplification of cDNA was performed using primer sets as described in Materials and Methods. PCR reactions were run on a 6% TBE/urea sequencing gel. *Arrows,* one band upregulated by loading (a) and 5 bands downregulated by loading (b, c, d, e, f, g).

sence of protein contaminati on. To remove contaminating DNA from the RNA preparation, samples were incubated with RNase-free DNase I (Boerhinger Mannheim, Indianapolis, IN) for 15 min at room temperature and then extracted with phenol/chloroform. First-strand cDNA was synthesized from 4 µg of total RNA by oligo-dT priming, using the Superscript Preamplification kit (Gibco/BRL, Gaithersburg, MD) in a final volume of 40 µl.

PCR and Differential Display

To amplify differentially expressed bands from cDNA, arbitrary primer sets were chosen. The upstream primer for the first set was 5′ CAG GCC CTT C 3′ (Fig. 2A) and the upstream primer for the second set was 5′ TGC TCT GCC C 3′ (Fig. 2B). The downstream primer used for both sets was 5′ TTT TTT TTT TTC C 3′. Using cDNA diluted 1:35, duplicate PCR reactions were assembled, with a Biomek 2000 (Beckman Instruments, Fullerton, CA) to a final concentration of 10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 2.0 mM dNTP, 1 µCi ^{33}P (Amersham, Arlington Heights, IL), and 1 unit AmpliTaq polymerase (Perkin-Elmer, Foster City, CA) in a final volume of 21 µl. Reactions were then subjected to the following PCR conditions: 1 cycle of 94°C for 2 min; 39 cycles of 94°C for 15 s, 40°C for 2 min, 72°C for 1 min; 1 cycle of 72°C

for 5 min. Subsequently, PCR products were separated on a 6% TBE/urea sequencing gel (Sequagel, National Diagnostics, Atlanta, GA) for 3 h at 1,700 V. Gels were dried and exposed to X-OMAT X-ray film (Eastman Kodak, Rochester, NY).

Reamplification, Cloning and Sequencing of cDNA

To characterize differential display products, bands of interest were excised from the gel, boiled for 5 min in distilled H_2O, and purified over a Centricon 50 column (Amicon, Beverly, MA). Samples were then reamplified to confirm the size and specificity of the primer sets used in the display. Reamplified bands were ligated in pCR2.1 TA cloning vector and transformed into INVαF′ cells (Invitrogen, San Diego, CA). For each clone, ten colonies were picked, amplified in LB broth, and the plasmid isolated (Wizard Plus, Promega, Madison, WI). Clones that contained inserts were submitted for automated cycle sequencing (Lilly DNA Technology Group, Indianapolis, IN). All sequences were analyzed using BLAST and FASTA against GenBank, EMBL and Bodymap databases (http://www.imcb.osaka-u.ac.jp/bodymap) to determine sequence identity and tissue distribution.

Generation of Probes for Northern Analysis

To generate radioactive probes for Northern blot analysis, either the inserts from the clones of interest were released from the plasmid by restriction digest or PCR amplification. DNA, 25 ng, of DNA was labeled by the random primer method (Gibco/BRL, Gaithersburg, MD) using α-^{32}P-dCTP (Amersham). Free nucleotides were removed by centrifugation through a Centricon-50 column (Amicon).

Northern Blot Analysis

Total RNA (25 µg) was electrophoresed through a 1.2% agaorse/formaldehyde gel for 3 h at 90 V and then transferred to Hybond N nylon membrane (Amersham) overnight in 10× SSC. RNA was cross-linked to the membrane using an ultraviolet (UV) cross-linker (Stratalinker, Stratagene, San Diego, CA), and prehybridization and hybridization were carried out in Rapid-Hyb hybridization solution (Amersham) as recommended by the manufacturer before adding 10^6 cpm/ml of denatured probe. After hybridization, the membranes were washed twice in 2× SSC/0.1% sodium dodecyl sulfate (SDS) at 65°C for 30 min and exposed to autoradiographic film (Hyperfilm, Amersham). The resulting autoradiograms were quantitated by scanning laser densitometry. As a control, the membranes were reprobed with cDNA for 18S ribosomal RNA. Data were quantitated as densitometric units and values were normalized to signal obtained with the 18S ribosomal band. The experiments were repeated 2–4 times for each time point to confirm findings.

Semiquantitative RT-PCR

To generate cDNA for PCR, 5 µg of total RNA from each treatment sample was reverse-transcribed in a total volume of 50 µl, using the Superscript Preamplification kit (Gibco/BRL). A control tube containing no reverse transcriptase, was included to detect the presence of genomic DNA contamination. PCR reactions were assembled using equivalent amounts of cDNA from each sample and cycled 15–20 times under the following conditions: initial denaturation at 95°C for 1 min; amplification at 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and final extension at 72°C for 5 min. After amplification, the PCR reactions were electrophoresed through a 1% agarose gel at 90 V for 1 h. Gels were denatured in 0.5 M NaOH/1.5 M NaCl for 30 min, neutralized in 1.0 M Tris–HCl/1.5 M NaCl, and equilibrated in 10× SSC for 10 min prior to transfer. DNA was transferred to a Nytran-plus membrane (Schleicher and Schuell, Keene, NH) in 10× SSC overnight. DNA was cross-linked to the membrane using a UV cross-linker (Stratalinker, Stratagene) and then prehybridized at 42°C for 30 min in hybridization solution (Ambion) before a denatured random-labeled probe was added (prepared as described above) to the hybridization solution, at a concentration of 10^6 cpm/ml. The probe was allowed to hybridize overnight at 42°C. Blots were washed at room temperature in 2× SSC/0.1% SDS for 1 h and then exposed to Hyperfilm X-ray film (Amersham) or a storage phosphor cassette (Molecular Dynamics, Sunnyvale, CA). Data was quantitated by scanning laser densitometry or on a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA).

Multi-tissue RNA analysis

To determine the distribution of the MPO transcript, we probed polyA+ RNA from rat tissues, using multiple tissue northern (MTN) blots (Clontech Laboratories, Palo Alto, CA).

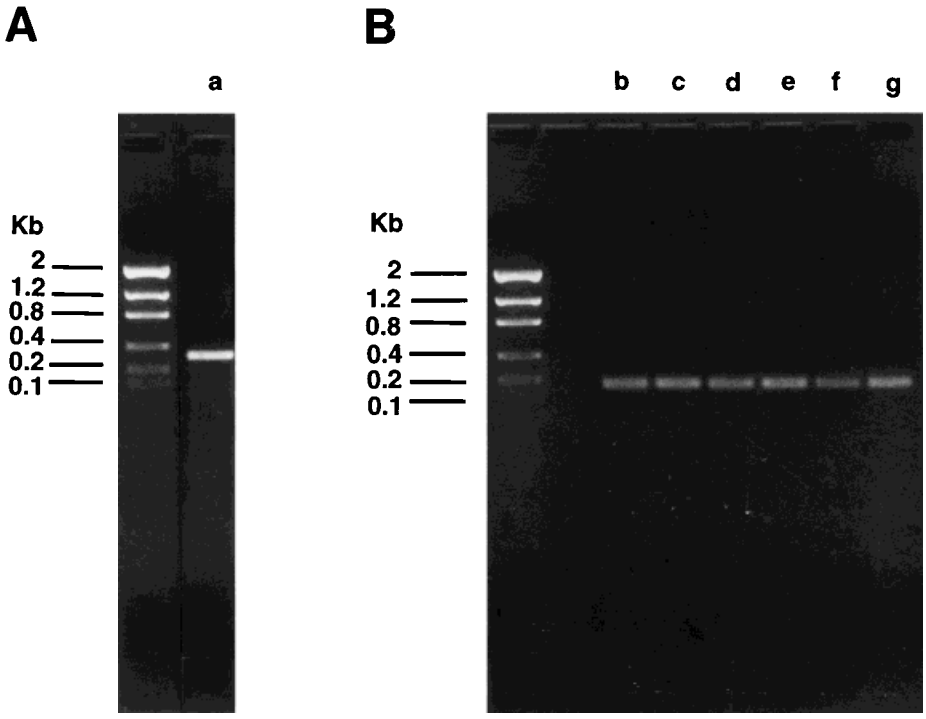

Fig. 3. Excision and re-amplification of load regulated bands. Band a, upregulated by loading (**A**) and bands (b, c, d, e, f, g) (**B**) downregulated by loading were excised from the gels in Fig. 2, eluted and re-amplified with the same primer combination used in the DDRT-PCR and separated on a 1.2% agarose gel.

The multiple tissue blot contained 2 µg of polyA+ RNA from heart, kidney, spleen, lung, liver, skeletal muscle, and testis. The blots were analyzed by hybridization with radiolabeled probes as described for Northern blot analysis.

RESULTS

RNA isolated from loaded and sham control tibia at 1 h and 24 h after mechanical loading were differentially displayed, using primer sets as described under Materials and Methods (Fig. 2). Parallel display of samples from sham and loaded bones on a sequencing gel showed several regulated bands, seven of which (bands a–g) were randomly picked and analyzed. Loading induced differential expression of band a at 24 h after treatment (Fig. 2A), and repressed bands b, c, d, e, f, and g, at 1 h (Fig. 2B). Individual bands were excised from the gel and reamplified by PCR with the primers used in the display. Separation of the PCR reactions on an agarose gel confirmed the size of the product and specificity of the primers. All bands amplified a single PCR product (Fig. 3). The size of band a product was approximately 380 bp (Fig. 3A), and bands b, c, d, e, f, and g yielded an approximately 160-bp product (Fig. 3B). The PCR products were then individually cloned and sequenced. Sequence analysis, using BLAST against the GenBank database is shown in Figure 4. Band a shared 99% sequence identity with a region of rat OPN that mapped to the end of the coding region and the 3′ UTR of OPN (Fig. 4A). The other bands b, c, d, e, f, and g were found to have identical sequence and shared 90% homology with mouse MPO 3′ UTR (Fig. 4B).

To confirm differential regulation of OPN, Northern blot analysis was performed using RNA isolated at 6 h and 24 h after mechanical loading. OPN mRNA expression was increased by nearly 3- to 4-fold at 6 h and 24 h after loading (Fig. 5). In contrast to OPN, MPO mRNA expression was detectable only after prolonged exposure (data not shown). Therefore, we confirmed differential regulation of MPO by more sensitive semiquantitative RT-PCR. As shown in Figure 6, semiquantitative RT-PCR analysis showed the expression of MPO decreased 10-fold within 1 h of mechanical loading. By 24 h after loading, MPO expression was still 4- to 5-fold below sham control levels.

Tissue distribution analysis (Fig. 7) showed that, except in bone (metaphysis), MPO expres-

A

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Band a:     7 GTCAGCCACGAGAGCACTGAGCAGTCGGATGCGATCGATATGCCGAGAAGCCGGATGCAATCGATAGTGCAGAGCGGT 84
              | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Rat OPN:  818 GCCAGCCACGAGAGCACTGAGCAGTCGGATGCGATCGATATGCCGAGAAGCCGGATGCAATCGATAGTGCAGAGCGGT 896
(M99252)
           86 CGGATGCTATCGACAGTCAGGCGAGTTCCAAAGCCAGCCTGGAACATCAGAGCCACGAGTTTCACAGCCATGAGG 160
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
          897 CGGATGCTATCGACAGTCAGGCGAGTTCCAAAGCCAGCCTGGAACATCAGAGCCACGAGTTTCACAGCCATGAGG 971

          161 GTACAAGCTAGTCCTAGACCCTAAGAGTAAGGAAGATGATAGGTATCTGAAATTCCGCAT 219
              | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
          972 GGACAAGCTAGTCCTAGACCCTAAGAGTAAGGAAGATGATAGGTATCTGAAATTCCGCAT 1029

          220 TTCTCATGAATTAGAGAGTTCATCTTCTGAGGTCAATTAAAGAAGAGGCAAAACCACAGT 279
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
         1030 TTCTCATGAATTAGAGAGTTCATCTTCTGAGGTCAATTAAAGAAGAGGCAAAACCACAGT 1089

          280 TCCTTACTTTGGCTTAAATAAAAACAAAAAGTAAATTCCAACAAGCAGGAATACTAACTGCTGTTT 346
              |||||||||||   || ||  ||||||||||||||||||||||||||||||||||||||||||   ||
         1090 TCCTTACTTTGCTTTTAAATAAAACAAAAAGTAAATTCCAACAAGCAGGAATACTAACTGCTTGTT 1154
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B

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Bands b-g:      8 CGCCCTTCCTAAACTGAATCTGGCTTCCTGGAAGGAGACCTAGAGGCCGGGAGTCTGCCC 67
                  | ||||||||||||||||| ||| |||||||||||||||||||||||  ||||||||||
Mouse MPO:   2619 CACCCTTCCTAAACTGAACCTGACTTCCTGGAAGGAGACCTAGAGGTTGGGAGTCTGCCG 2678
(X15313)
               68 GGAGAGGAACGGAGGCCATTGGCCTTTTTTGTGCTATCTGTACCTGAGGTTTACACTAATAAA 130
                  ||||| | |||||| |  | | | |  |  ||||||| |||||||||||||| ||||||||||
             2679 GGAGAAGGACGGAGCCATTGGCCTTCCT--GTGCTATTTGTACCTGAGGTTCACACTAATAAA 2739
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Fig. 4. **A:** Partial nucleotide sequence of band a showing 99% homology to a region of rat OPN that mapped to the end of the coding region and the 3′ UTR of OPN. **B:** Partial nucleotide sequence of bands b–g showing 90% homology to the 3′ UTR of MPO. Plasmids containing inserts of bands a–g were prepared and sequenced. The BLAST and FASTA programs were used to search GenBank and EMBL data bases to determine sequence identity.

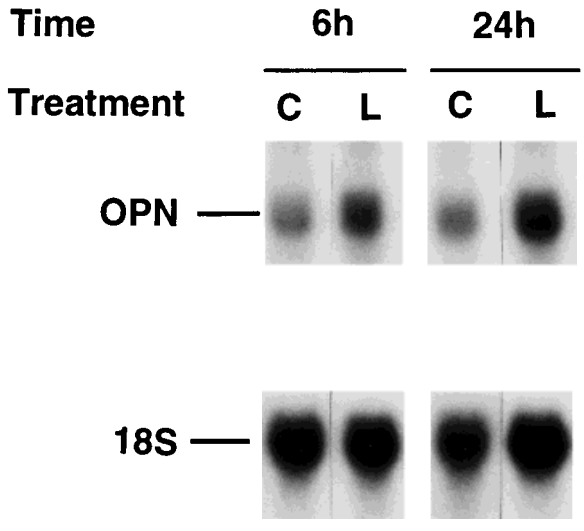

Fig. 5. Representative Northern blot reconfirming the effect of mechanical loading on expression of OPN. Total RNA was isolated from rat tibia (pooled, n = 6/group) following mechanical load (L) or sham application (C) at indicated times and analyzed by Northern blot hybridization as described under Materials and Methods. 18S was re-hybridized as a control for RNA quantification.

sion was not detectable in poly A+ RNA from rat heart, kidney, spleen, lung, liver, skeletal muscle, or testis. Additionally, MPO expression was not detectable in total RNA from UMR 106 osteoblastic cells, in confluent primary cultures of osteoblasts derived from either rat femur primary spongiosa [Onyia et al., 1997] or the diaphyseal marrow [Owen, 1988]. Tissue distribution analysis of MPO by electronic database searching identified expression in neutrophils, monocytes [Klebanoff, 1990; Zaki et al., 1990] and in an osteocyte library made from human reamed cancellous bone (http://www.imcb.osaka-u.ac.jp/bodymap/gallery/bone.html).

To demonstrate that the differentially regulated genes identified in this study were associated with a concomitant increase in bone formation parameters, histomorphometric measurements as well as mineral apposition rates and bone formation rates were determined. All parameters of bone formation measured histomorphometrically were increased by applied bending loads on the rat tibia (Table I). Double-labeled surface and the mineral apposition rate were increased about 2-fold ($P < 0.0001$) and the bone formation rate was increased more than 4-fold ($P < 0.0001$) by mechanical bending. Sham loading had no significant effect on bone formation.

DISCUSSION

Previously, we and others demonstrated mechanical loading induced bone formation in vivo [Burr et al., 1989; Chow et al., 1993; Churches

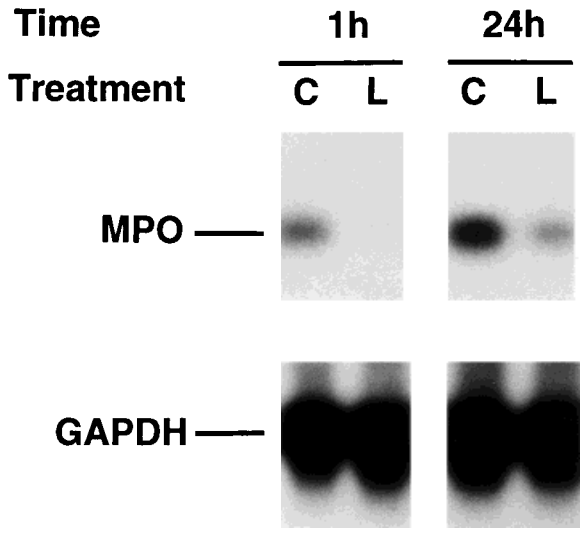

Fig. 6. Reconfirmation of the effect of mechanical loading on expression of MPO. Total RNA was isolated from rat tibia (pooled, n = 6/group) following mechanical load (L) or sham application (C) at indicated times and analyzed by semiquantitative PCR-Southern blotting as described under Materials and Methods. GAPDH was also analyzed as a control for RNA quantification. Autoradiograms of the blot are shown after hybridization with probes for band b or mouse MPO (top) and GAPDH (bottom). These blots represent one of three independent experiments.

and Howlett, 1982; Goodship et al., 1979; Lanyon et al., 1982; O'Connor et al., 1982; Rubin and Lanyon, 1985; Turner et al., 1994]. In the present study, we investigated putative molecular mediators of bone formation by identifying and cloning genes whose expression was altered during the initiation of the anabolic (bone formation) response of rat bone to mechanical loading. We identified rat OPN to be upregulated by loading and a second gene, with homology to mouse MPO, to be downregulated by loading. Given the high level of homology to mouse MPO, this sequence represents rat MPO or a very closely related gene. Mammalian MPO gene expression has been extensively studied in human and murine cells and explains the absence of sequences for rat MPO in GenBank or EMBL databases. The regulated expression of OPN and MPO genes was further confirmed using either Northern blotting or semiquantitative RT-PCR, or both. The expression of these genes preceded observations of loading-induced bone formation, indicating that OPN and MPO are produced during the osteogenic response of bone to mechanical loading. We suggest that this timely regulated expression of OPN and MPO is consistent with a fundamental role of these genes in loading-induced bone formation.

OPN is a phosphorylated sialoprotein present in the mineralized bone matrix. It is ubiquitously expressed throughout the body, but is especially abundant in bone, kidney and epithelial tissue. OPN contains an RGD (Arg-Gly-Asp) sequence that binds to certain integrins and is involved in cell attachment and signaling [Miyauchi et al., 1991]. While the precise role of OPN in bone turnover is unclear, OPN expression has been localized at sites of bone formation as well as at sites of bone resorption [Chen et al., 1993; Dodds et al., 1995; Ikeda et al., 1992; McKee et al., 1993; McKee and Nanci, 1996; Merry et al., 1993; Yamate et al., 1997]. Specifically, OPN protein has been demonstrated in cement lines, mineralized bone matrix during endochondral and intramembranous ossification, cartilage matrix, and osteoclastic resorption lacunae. Our results, demonstrating that OPN is expressed under conditions in which bone formation is stimulated by mechanical loading, are in agreement with previous studies. Others have reported that OPN mRNA is increased in cultured osteoblasts following mechanical strain [Harter et al., 1995; Klein-Nulend et al., 1997; Kubota et al., 1993; Owan et al., in press]. [Raab-Cullen et al., 1994] reported that OPN mRNA was at first decreased, then increased 12 h after loading in the rat tibial periosteum using a loading system similar to that used in this study. Inaoka et al. [1995] showed a marginal increase (less than or equal to 2-fold) in OPN mRNA as early as 1 h after loading of rat caudal vertebrae. We found that OPN mRNA is increased at 6 h and 24 h after loading and precedes loading-induced bone formation. Taken together, these results suggest that OPN expression is sensitive to dynamic loading.

The rapid and sustained downregulation of MPO following mechanical loading suggests that MPO is also sensitive to dynamic loading. In DDRT-PCR, multiple downregulated bands corresponding to MPO were identified and provided additional confirmation of differential regulation of MPO. Sequence analysis revealed that bands b–g differed in length by 2–10 bp. We suggest this resulted from staggered priming of the downstream primer (5′ TTT TTT TTT TTC C 3′) in the polyA+ tail region of MPO. MPO is a lysosomal heme protein found in neutrophils and monocytes that plays a role in the bactericidal function of mature polymorphonuclear leukocytes [Klebanoff, 1990; Zaki et al., 1990]. MPO is thought to play a role in the

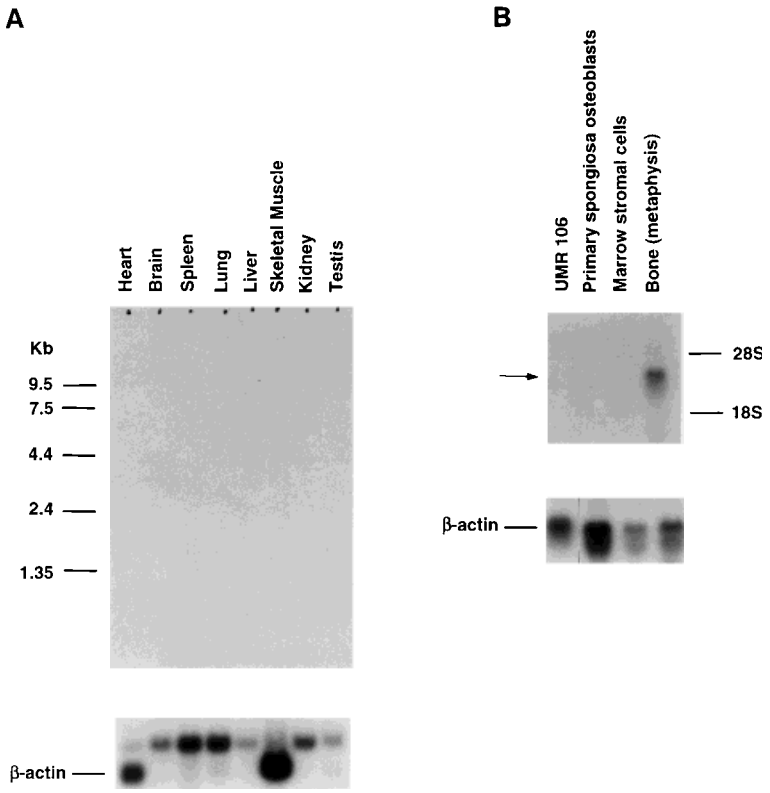

Fig. 7. Tissue distribution of MPO in polyA+ RNA from rat tissues. **A:** Northern blot showing expression of MPO was not detectable in heart, kidney, spleen, lung, liver, skeletal muscle, or testis. **B:** Using the same probe and identical conditions, expression was detected in a blot of total RNA extracted from bone (metaphysis) but not in total RNA from in vitro osteoblast-like cells, UMR 106, rat primary spongiosa osteoblast, and rat marrow stromal cells.

TABLE I. Bone Formation Indices (mean ± SEM) on the Endocortical Surface of the Rat Tibia as a Result of Four-Point Bending and Sham Loading†

Group	dL.S (%)	MAR (μm/day)	BFR/BS ($\mu m^3/\mu m^2/yr$)
Bending			
Right	39.7 ± 3.5*	2.06 ± 0.12*	309 ± 34*
Left	15.2 ± 2.2*	1.15 ± 0.10*	70 ± 11*
Sham			
Right	13.4 ± 1.7**	1.27 ± 0.12**	70 ± 12**
Left	11.5 ± 2.2**	1.24 ± 0.12**	58 ± 13**

†Histomorphometric parameters measured were double-labeled surface (dL.S), mineral apposition rate (MAR), and bone formation rate (BFR/BS). Means are presented with standard errors in parentheses. The notation after the brackets represents comparison between the right and left tibia (paired t-test).
*$P < 0.0001$.
**NS, not significant.

antimicrobial activity of mature neutrophils, by catalyzing the formation of hypochlorous acid (HOCl) from hydrogen peroxide and chloride anions (H_2O_2 and Cl^-). The resultant damaging reactive oxygen species (ROS) are able to kill microorganisms. Also, ROS may modify and/or degrade matrix molecules such as proteoglycans [Anderson et al., 1997; Klebanoff et al., 1993; Lindvall and Rydell, 1994; Malle et al., 1997] and are thought to be responsible for some of the tissue damage seen in myocardial ischemia, asthma, respiratory distress syndromes, nephrosclerosis, glomerulosclerosis, rheumatoid arthritis, and other inflammatory conditions [Ciuffetti et al., 1992; Johnson et al., 1988, 1994; Klebanoff, 1980; Van Dyke and Van Dyke, 1986; Weiss, 1989]. This enzyme is also present in bone marrow cells and is well estab-

lished as a marker for myeloid cell differentiation [Austin et al., 1996; Crisan et al., 1996; Friedman et al., 1991; Koeffler et al., 1985; Lubbert et al., 1991; Sagoh and Yamada, 1988; Tobler et al., 1988]. Studies have described a decrease in MPO mRNA levels as myeloid cells differentiate into mature neutrophils, raising speculation that MPO is not merely a marker but a potentially important enzyme during differentiation [Lee et al., 1994].

Our results suggest MPO is expressed in bone. However, the precise identity of the cells expressing MPO in bone still remains to be experimentally determined. We failed to detect MPO expression in in vitro cultures of UMR 106 osteoblast cells or in primary cultures of osteoblasts derived from the primary spongiosa [Onyia et al., 1997] and diaphyseal marrow stroma [Owen, 1988]. Also, expression of MPO was not detected in a human osteoblast library (http://www.imcb.osaka-u.ac.jp/bodymap/gallery/osteoblast.html). These results suggest that MPO may not be expressed in osteoblasts. As demonstrated by electronic database analysis, MPO expression has been reported in an osteocyte-enriched library made from human reamed cancellous bone (http://www.imcb.osaka-u.ac.jp/bodymap/gallery/bone.html) and could be the responding cell expressing MPO. Our results confirmed MPO expression in the metaphysis, a site enriched with cancellous bone. To our knowledge, this is the first demonstration of a mechanical loading-induced decrease in MPO expression and suggests the exciting possibility that decreased MPO expression in osteocytes may be one of the events influencing loading-induced bone formation.

The activity of MPO has been shown to be altered by some important regulators of bone balance. These alterations could have functional consequence on the role of MPO in bone. For example, there have been several reports demonstrating altered MPO activity in neutrophils following exhaustive exercise in humans [Suzuki et al., 1996]. Zuckerman and Bryan [1996] demonstrated that raloxifene, a selective estrogen receptor modulator (SERM) with beneficial effects in bone, inhibited tyrosyl radical formation catalyzed by MPO in murine peritoneal neutrophils. Since oxygen radicals and oxidatively modified proteins seem to participate in degenerative vascular and inflammatory diseases, oxidants generated by the MPO system could activate proteinases such as gelatinase and collagenase [Peppin and Weiss, 1986; Weiss et al., 1985] and modify bone matrix. We speculate that the oxidative activity of MPO might be a leading event both in normal and in diseased bone.

In summary, using DDRT-PCR, we have identified OPN and MPO to be regulated during mechanical loading. These results represent evidence for a role of OPN and MPO in the osteogenic response of bone to mechanical loading. Based on the findings in this study, we suggest that bone responds to mechanical stimulation with immediate and prolonged suppression of MPO activity and a delayed-early induction of OPN. Future studies would be required to understand how mechanical stimuli affect the expression of these genes in bone.

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