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Transcriptionally Active Nuclei Isolated From Intact Bone Reflect Modified Levels of Gene Expression in Skeletal Development and Pathology

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Abstract Transcriptional regulation of gene expression in vivo in bone, associated with normal development or skeletal disorders, to date, has not been studied. We report the successful isolation of nuclei that are transcriptionally active from normal and osteopetrotic rat bone. Transcription rates of cell growth and bone-related genes (including histone H4, *c-fos, c-jun,* TGFβ1, β_2 macroglobulin, collagen, fibronectin, osteocalcin, osteopontin, and tartrate resistent acid phosphatase) change as a function of calvarial development from birth to 6 weeks and are selectively modified in osteopetrotic animals. Additionally, nuclei isolated from intact bone yield promoter binding factors. Bone nuclei, which transcribe faithfully and contain the normal complement of nuclear protein factors, offer a powerful approach for investigating in vivo gene regulation in skeletal development and pathology. © 1994 Wiley-Liss, Inc.

Key words: rat bone transcription, rat bone transcription factors, osteopetrotic bone transcription, osteocalcin transcription, collagen transcription

Central to understanding bone development and metabolic bone diseases is the delineation of mechanisms mediating the regulation of cell growth and tissue-related gene expression. While transcriptional and posttranscriptional mechanisms have been analyzed in several in vitro bone cell systems [Oldberg et al., 1989; Leboy et al., 1991; Gronowitcz et al., 1991; Shalhoub et al., 1992; Subramaniam et al., 1992] to date, only posttranscriptional mechanisms have been examined in bone in vivo [Yoon et al., 1987; Chen et al., 1992; Turner et al., 1992; Shalhoub et al., 1991, in press]. The limitation has been the mineralized nature of bone tissue, which does not lend itself readily to nuclear isolation procedures currently employed for soft tissues and cells in culture. Initial bone cell isolations employing enzymatic (e.g., collagenase/trypsin) digestion of the mineralized extracellular matrix using neonatal rat calvaria [Bellows et al., 1986; Aronow et al., 1990; Owen et al., 1990] or trabecular bone chips [Robey and Turner, 1985; Turner et al., 1992] are lengthy and call into question whether ongoing transcription at the end of the procedure is a valid measure of transcription at the time the tissue was obtained. While much is to be gained by investigating the various isolated osteoblast or organ culture systems, bone cells in situ are influenced by a variety of hormones and growth factors acting in concert, a situation that cannot yet be mimicked in vitro. Thus, an understanding of physiologic control of bone biology mandates that gene regulation at the level of transcription be examined in vivo.

The goal of the present study has been to develop a simple approach to isolate nuclei from bone tissue in order to address questions related to control of gene expression. We demonstrate that transcriptionally active nuclei can be isolated from young rat calvaria at different ages, and from long bones of 2-week-old normal and osteopetrotic (*ia*) rats. We report that transcription rates of genes encoding proteins expressed in bone are modified during calvarial development and when comparing mutant (*ia*) to normal rat long bone. We also report that nuclei isolated from intact bone yield promoter binding factors that interact in a sequence-specific manner with basal regulatory elements of a bone-specific gene, osteocalcin.

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METHODS

The animals of *ia* stock were obtained from our colonies at the University of Massachusetts Medical School bred to maintain this mutation. Male mutants (*ia*/*ia*) are fertile and were bred to heterozygous (*ia*/+) females. Thus, all normal animals from these breedings were heterozygotes. Homozygous mutants are referred to as *ia* rats and were distinguished from normal littermates by the failure of eruption of incisors on or after the tenth postnatal day. Animals were maintained and used in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals, prepared by the Institute on Laboratory Animal Resources, National Research Council (Department of Health and Human Services Publication NIH 86-23, 1985) and guidelines of the Animal Care Committee of the University of Massachusetts Medical School.

Isolation of Nuclei From Whole Long Bone

The periosteum and marrow were removed and the bone shaft frozen in liquid nitrogen until nuclei were isolated. Nuclei were isolated from bone which was reduced to powder at −180°C in liquid nitrogen in a Bessman tissue pulverizer (Fisher Scientific, Pittsburgh, PA). The bone powder was transferred to 3 ml of a buffer containing 40% glycerol, 5 mM $MgCl_2$, 50 mM Tris, pH 8, and 0.1 mM EDTA, pH 8, at 4°C. The suspension was then passed through a Sweeny filter (Millipore, Bedford, MA) attached to a syringe to separate released cells and nuclei from the mineralized extracellular matrix. Complete cellular lysis was achieved by dounce homogenization. The nuclear suspension was clear of mineral and extracellular matrix at this stage, and the nuclei were intact as confirmed by light microscopy. The nuclei were then recovered by diluting the suspension with 1 volume of the above buffer without glycerol and centrifugation at 400*g* at 4°C for 5 min in a swinging bucket rotor. The yield was 2×10^7 nuclei per long bone tibiae from one 2-week-old rat.

Transcription in Nuclei From Intact Calvarial Bone

Sprague-Dawley rats, obtained from Charles River (Wilmington, MA), were killed by carbon dioxide asphyxiation. Calvaria were excised from normal animals at birth and at 2, 4, and 6 weeks, stripped of their periostea, and frozen in liquid nitrogen. The nuclear pellets obtained from calvaria as described above were resuspended in glycerol buffer and the nuclei counted using a hemacytometer. Nuclear run-on transcription assays were performed according to Greenberg and Ziff [1984]. The yield of nuclei was 1–2 × 10^6 nuclei per calvarium. Each reaction contained 1×10^6 nuclei, 100 μCi of (γ-^{32}P)UTP, and cold nucleotides. Plasmid DNAs containing 2 μg of the gene sequences of interest were immobilized onto Zetaprobe membranes. Plasmids included those containing coding region sequences for human histone H4, pF0002 [Pauli et al., 1989], rat type I collagen [Genovese et al., 1984], rat fibronectin [Schwarzbauer et al., 1983], rat osteocalcin, pOC3.4 [Lian et al., 1989], rat alkaline phosphatase, pRAP54 [Noda et al., 1987], rat osteopontin [Oldberg et al., 1986], human ribosomal LS6 [Wilson et al., 1978], mouse transforming growth beta-1 [Derynck et al., 1986], carbonic anhydrase II (CA II) [Montgomery et al., 1987], human tartrate resistent acid phosphatase (TRAP) [Ketcham et al., 1989], mouse *c-fos* [Miller et al., 1984], and *c-jun* [Angel et al., 1988]. The membranes were hybridized to calvarial transcripts (2.8×10^5 dpm) in a hybridization volume of 2 ml for each filter at 65°C for 3 days and then washed 5 times with 2 × SSC/0.1% SDS for 20 min each wash. They were then exposed to Kodak XAR-5 X-ray film (Kodak) between two Cronex Lightning Plus intensifying screens at −70°C for 10 days. The autoradiograms were quantitated using laser densitometry (LKB 2400 Gel Scan XL). Values are expressed relative to ribosomal RNA after normalizing to pUC19; that is, each densitometric value for each gene was divided by the densitometric value (from a lighter exposure) of ribosomal RNA at that age.

Transcription in Nuclei From Normal and *ia* Intact Rat Long Bone

Nuclei were isolated from long bone tibiae and nuclear run-on reactions performed as described above, except that each nuclear runon transcription reaction contained 10^7 nuclei and each filter was hybridized to 10^6 dpm of RNA transcripts. The filters were exposed for 7 days at −70°C as described above. Values were normalized to 28S RNA and pUC19. The use of 10^6 nuclei for nuclear runon assays with nuclei isolated from calvarae and 10^7 nuclei with nuclei isolated from long bone was dependent on availability of tissue.

Isolation of Protein Factors From Nuclei of Intact Bone

All procedures were performed at 4°C according to Dignam et al. [1983] with modifications. The nuclear pellets obtained above were resuspended in protein extraction buffer (20 mM Hepes, pH 7.9, 8 mM Tris, pH 7.9, 30% glycerol, 600 mM KCl, 1 mM EDTA) with fresh protease inhibitors (10 μg/ml trypsin inhibitor 70 μg/ml TPCK (L-1-Cloro-3-[4-tosylamido]-4-phenyl-2-butanone), 500 μM PMSF (phenylmethanesulfonyl fluoride), 1 μg/ml pepstatin, 0.75 mM spermidine, 0.5 mM DTT (dithiothreitol), 0.15 mM spermine, and 0.5 μg/ml leupeptin). Proteins were solubilized by homogenization (10 strokes in a 7 ml homogenizer) and extraction for 30 min with the aid of a magnetic stir bar. The resulting cloudy protein extract was centrifuged for 30 min at 4°C in a JA-20 rotor at 15,400*g* and dialyzed at 4°C against 20 mM Hepes, pH 7.9, 8 mM Tris, pH 7.9, 30% glycerol, 100 mM KCl, 1 mM EDTA, pH 8 (molecular weight cutoff 2 Kd). The dialysis buffer was changed after 1 h, and the protein extract was then centrifuged at 16,000*g* for 5 min in an Eppendorf microfuge (Madison, WI). The supernatant was divided into 20 μl aliquots, quick frozen in liquid nitrogen, and stored at −70°C. Protein quantitation was performed according to Bradford [1976]. The yield of protein obtained from long bone nuclei of one 2-week-old rat is approximately 40 μg, and from one 2-week-old calvaria approximately 6 μg. Experiments with the latter are not presented here. Each binding reaction contained approximately 5 μg of nuclear proteins. Probe labelling, binding reactions, and the gel mobility electrophoresis conditions were performed according to Markose et al. [1990], using the rat osteocalcin (OC) box (−99 to −76) as probe.

RESULTS AND DISCUSSION

Developmental Transcription of Cell Growth and Phenotypic Genes During Osteoblast Differentiation in Bone Nuclei

During calvarial development, osteoblasts, bone forming cells, secrete several proteins associated with biosynthesis and organization of the bone extracellular matrix (ECM) (e.g., type I collagen, accounting for 90% of the ECM protein, and fibronectin) and synthesize several proteins associated with mineralization that occurs during maturation of the bone cell phenotype (e.g., alkaline phosphatase, osteopontin, and osteocalcin). Alkaline phosphatase is an enzyme expressed on the plasma membrane early in osteoblast differentiation and contributes to rendering the ECM competent for mineralization. The ordered deposition of mineral crystals occurs within and between collagen fibrils. Osteocalcin, a bone-specific protein, and osteopontin are markers of the mature osteoblast phenotype that are maximally expressed, and have been localized, at the mineralization front in bone during ECM mineralization [Owen et al., 1990; Mark et al., 1987].

Figure 1 shows that cells encased in a mineralized matrix in adult rat bone can be released by a procedure which involves initial pulverization of frozen bone at −180°C, transfer to a buffer at 4°C, and filtration of the bone powder suspension. After complete cellular lysis by dounce homogenization and centrifugation, nuclei are used directly for transcription assays or protein factor isolation.

Figure 2 shows the transcription rates for genes encoding bone cell growth and these differ-

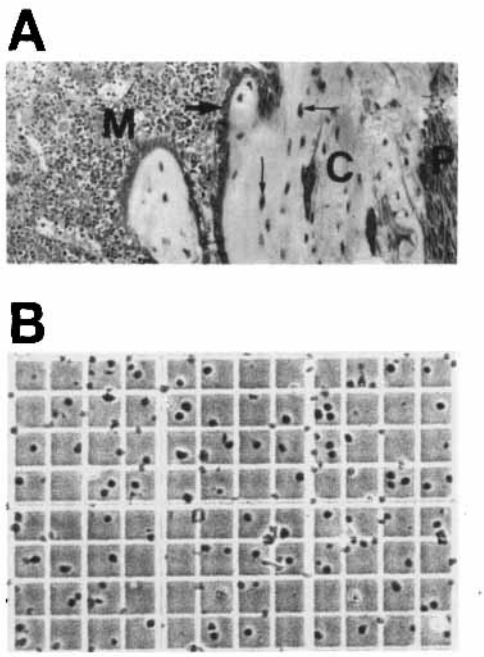


Fig. 1. Isolation of nuclei from whole bone. **A:** Section of 15-day-old rat bone (tibial diaphysis-glutaraldehyde fixation, demineralized and resin-embedded) showing the cortex (C) with osteocytes (small arrows), a periosteum (P) externally, and marrow cavity (M) internally. A layer of osteoblasts (large arrow) lines the marrow cavity surface of the bone. The periosteum and marrow were removed and the bone shaft frozen in liquid mitrogen until nuclei were isolated. ×100. **B:** Nuclei prepared from long bone as described in Methods. ×320.

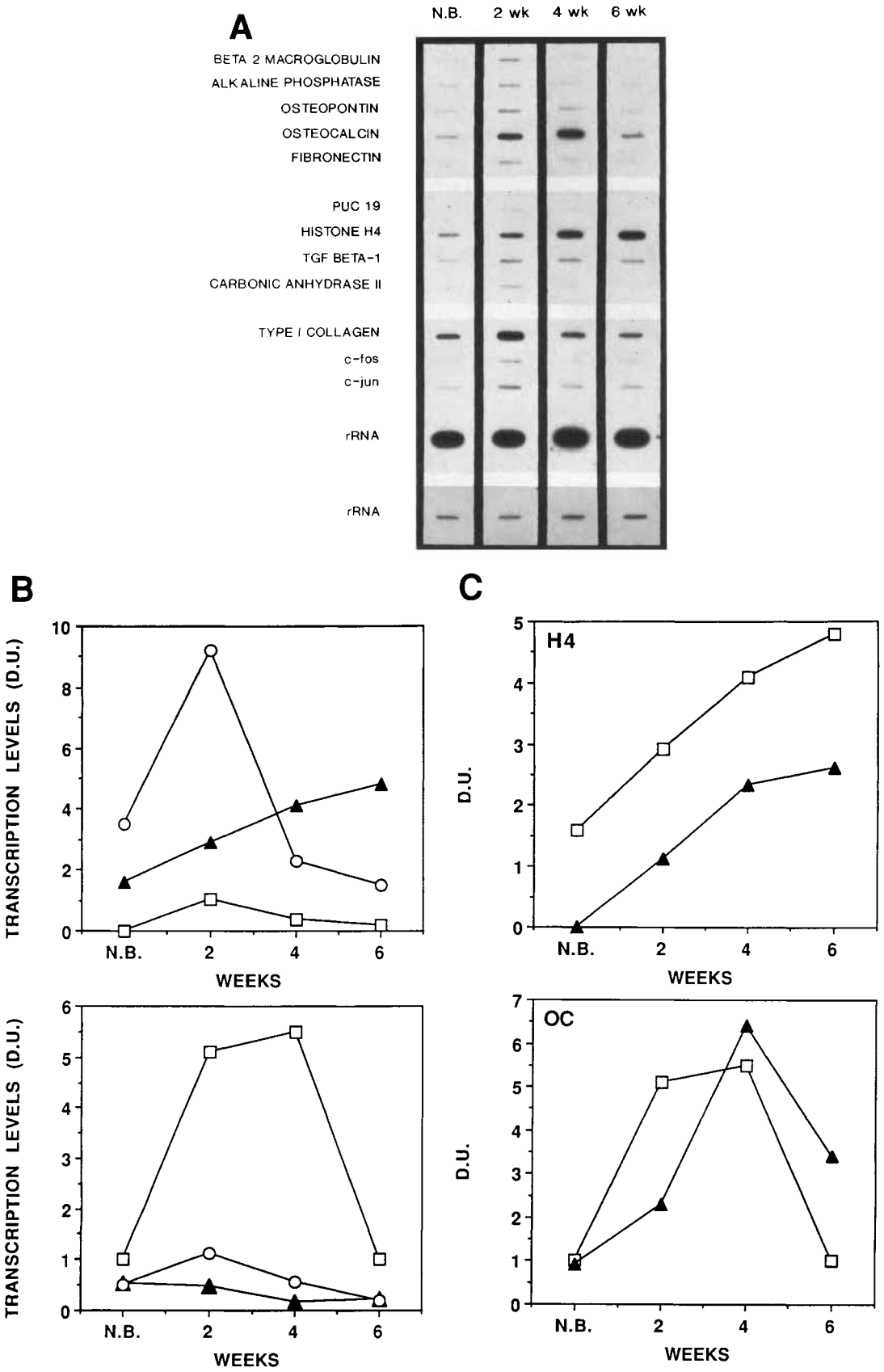
entiation-associated proteins, which are known to be expressed in the calvaria of normal rats as a function of age [Lian et al., 1982; Shalhoub et al., 1991; Chen et al., 1992; Turner et al., 1992]. During calvarial development transcription rates peak in the newborn for alkaline phosphatase, at 2 weeks for Type I collagen, fibronectin, and osteopontin, and at 4 weeks for osteocalcin. Transcription rates for a proliferation-related gene, histone H4, rise from birth to 6 weeks of age reflecting proliferation of bone cells with growth.

Comparison of these transcription rates for all of the genes shows parallel modulations in mRNA levels in rat calvaria during development, shown in this study (Fig. 2C), and as previously reported in this laboratory and elsewhere [Yoon et al., 1987; Shalhoub et al., 1991; Chen et al., 1992]. Figure 2C shows, as examples, the parallel changes observed in histone H4 and osteocalcin, demonstrating that a strong transcriptional component is associated with gene regulation during calvarial growth.

Aberrant Transcription of Developmentally Expressed Genes in Nuclei Isolated From Osteopetrotic Bone

Osteopetrosis is a metabolic bone disease characterized by increased skeletal mass due to impairment of the development and/or function of osteoclasts [Osier and Marks, 1992]. In a previous study we reported that mRNA levels for several proteins expressed in osteopetrotic mutant (*ia*) long bone and calvaria differed from the levels found in bone of normal littermates [Shalhoub et al., 1991, in press]. To explore the possible contribution of transcriptional regulation to these biological differences, transcription rates of proteins expressed in normal and mutant bone were examined. Isolation of nuclei from the 2-week *ia* rat long bone diaphysis showed that transcription rates for histone H4, type I collagen, osteopontin, and osteocalcin are lower than in normal littermates, whereas transcription rates for fibronectin are higher and alkaline phosphatase rates are not changed (Fig. 3).

Table I shows a comparison of the mutant-to-normal ratios for transcription and mRNA levels from 2-week-old *ia* rats and their normal littermates. Mutant/normal ratios for type I collagen, alkaline phosphatase and osteocalcin (0.34, 1.18, and 0.47, respectively) parallel previous [Shalhoub et al., 1991] mRNA results (0.21, 0.76, and 0.42, respectively), whereas mutant/normal ratios for histone H4, fibronectin, and osteopontin (0.55, 3.30, and 0.26, respectively) differ from previous [Shalhoub et al., 1991] mRNA findings (1.12, 0.97, and 2.79, respectively). Thus, type I collagen, alkaline phosphatase, and osteocalcin appear to be regulated at the transcriptional level. In the mutant diaphysis osteocalcin and type I collagen protein levels were also significantly decreased from control [Shalhoub et al., in press; Lian and Marks, 1990], consistent with the decrease in both transcription and mRNA levels of similar magnitude. This indicates that in both mutant and normal animals osteocalcin and type I collagen remain coordinately regulated at 2 weeks. Fibronectin transcription rates, but not mRNA levels in the mutant diaphysis, are elevated as compared to normal. The excess transcripts could be turning over more rapidly in the mutant, and, hence, this would be an example of a protein whose expression is controlled at the posttranscriptional level. In contrast, transcripts for histone H4 and osteopontin are probably stabilized in the mutant, and in the case of osteopontin this is probably the mechanism by which elevated levels of osteopontin protein accumulate in the mutant bone matrix [Shalhoub et al., in press]. Thus, some genes are controlled by a strong transcriptional component while other genes are controlled posttranscriptionally as a function of disease. Transcription rates for two genes related to osteoclast activity, carbonic anhydrase II (CA II) and tartrate resistant acid phosphatase (TRAP), were detected at low levels, most likely reflecting the paucity of these cells in the diaphysis of normal bone. In *ia* rat bone osteoclasts contain higher levels of TRAP activity compared to normal, because these cells can synthesize but not release lysosomal enzymes [Marks, 1973]. Normalization of data to pUC 19 and 28S ribosomal RNA reveals that the level of TRAP transcripts is similar between *ia* and normal rat long bone (Fig. 3, Table I). Because osteoclast numbers are higher in mutant bone, this indicates that fewer transcripts are being made per cell in *ia* rat bone. The threefold elevation in carbonic anhydrase II transcription (Fig. 3, Table I) may also relate to the threefold elevation in osteoclast numbers seen in the bone of this mutation [Miller and Marks, 1982]. Thus, the presence of osteoclast-related gene transcription in nuclei isolated from bone reflects the cellular composition of the tissue. It is not known whether these aberrations are the result of one


A
N.B.
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PUC 19
HISTONE H4
TGF BETA-1
CARBONIC ANHYDRASE II
TYPE I COLLAGEN
c-fos
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Figure 2.

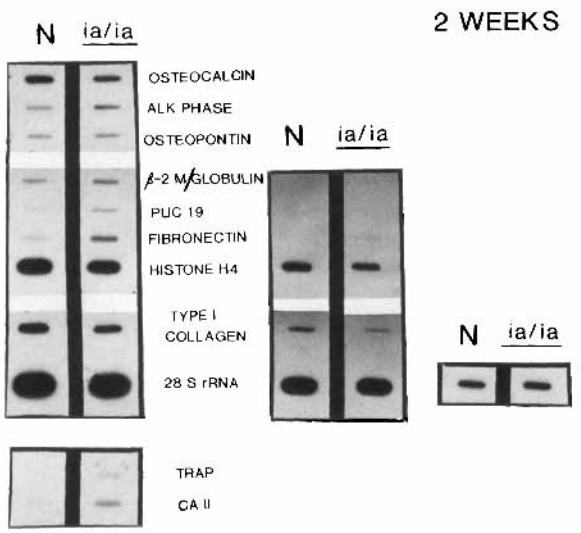

Fig. 3. *Transcription in nuclei isolated from intact long bone* from osteopetrotic (*ia*) rats and their normal littermates at 2 weeks of age. The nuclear pellets obtained from long bone were processed as described in Methods. Each nuclear runon transcription reaction contained 10^7 nuclei and each filter was hybridized to 10^6 dpm of RNA transcripts from long bone. Values were normalized to 28S RNA and pUC19 as described in the legend for Figure 2. N represents normal littermate.

or more defects in the regulation of a gene(s). How this defect(s) affects known, or as yet undefined, mechanisms of gene regulation can yield insight into both the disease process and normal functioning of bone cells in vivo.

Nuclei Isolated From Bone Provide Transcription Factors Which Interact With a Tissue-Specific Promoter Element

Nuclei isolated from bone contain the normal complement of intact nuclear transcription factors. This is shown by sequence-specific interactions with regulatory elements of the osteocalcin gene promoter. Figure 4 presents gel shift banding patterns obtained when an oligonucleotide representing the osteocalcin box (OC box), a primary regulatory domain of the osteocalcin gene containing a CCAAT motif, was used as a probe [Markose et al., 1990]. Nuclear extracts were obtained from normal and *ia* rat long bone [Dignam et al., 1983]. Representative preparations from either normal or *ia* rat bone show similar protein-DNA interactions. Competition with a 100-fold excess of cold oligonucleotides confirmed the specificity of protein-DNA binding. Three prominent bands of nuclear protein extracts from the bone tissue of 14-day-old animals are similar to those obtained from nuclear extracts isolated from 14-day primary normal diploid rat osteoblasts, but higher molecular weight complexes predominate in osteosarcoma ROS 17/2.8 cells in culture (Fig. 4).

TABLE I. Comparison of Transcription and mRNA Levels in the Long Bone of Normal and *ia* Osteopetrotic Rats Expressed as Mutant/Normal Ratios

	RNA	Transcription
Histone H4	1.12[a]	0.55
Type I collagen	0.21[a]	0.34
Fibronectin	0.97	3.30
Alkaline phosphatase	0.76	1.18
Osteopontin	2.79[a]	0.26
Osteocalcin	0.42[a]	0.47
Carbonic anhydrase II	[b]	3.20
Tartrate resistent acid phosphatase	[b]	1.00

[a]Taken from Shalhoub et al. [in press].
[b]Not assayed.

CONCLUSIONS AND PROSPECTS

In summary, this is the first report of gene transcription in bone nuclei isolated from intact tissue during development and in skeletal disease. Moreover, we have demonstrated that this approach permits direct determination of cell growth and bone-related gene transcription. The ability to obtain sequence-specific promoter binding factors from bone nuclei is the basis for investigating transcriptional regulatory mechanisms in vivo. This approach provides the opportunity to compare in vivo the regulation of gene expression during bone development, remodelling, and pathology in bones of different developmental origin (intramembranous-calvarial and endochondral-long bone) and in different ana-

Fig. 2. **A:** Transcription in nuclei isolated from intact calvarial bone as a function of age. Sprague-Dawley rats, obtained from Charles River (Wilmington, MA) were killed by carbon dioxide asphyxiation. Calvaria were excised from normal animals at birth (N.B.) and at 2, 4, and 6 weeks. Nuclear runon transcription assays were performed according to Greenberg and Ziff [1984]. **B:** Quantitation of the parameters represented in the experiment shown in A. Values are expressed in relation to ribosomal RNA after normalizing to pUC19; that is, each densitometric value for each gene was divided by the densitometric value (from a lighter exposure) of ribosomal RNA at that age. Upper panel: Open squares, fibronectin; closed triangles, histone H4; open circles, type I collagen. Lower panel: Open squares, osteocalcin; closed triangles, alkaline phosphatase; open circles, osteopontin. Transcription levels are expressed in densitometric units (D.U.). **C:** Comparison of transcription and mRNA levels of osteocalcin and H4 as a function of age. Upper panel: histone H4. Lower panel: osteocalcin. Open squares, transcription; closed triangles, mRNA; both expressed in densitometric units (D.U.).

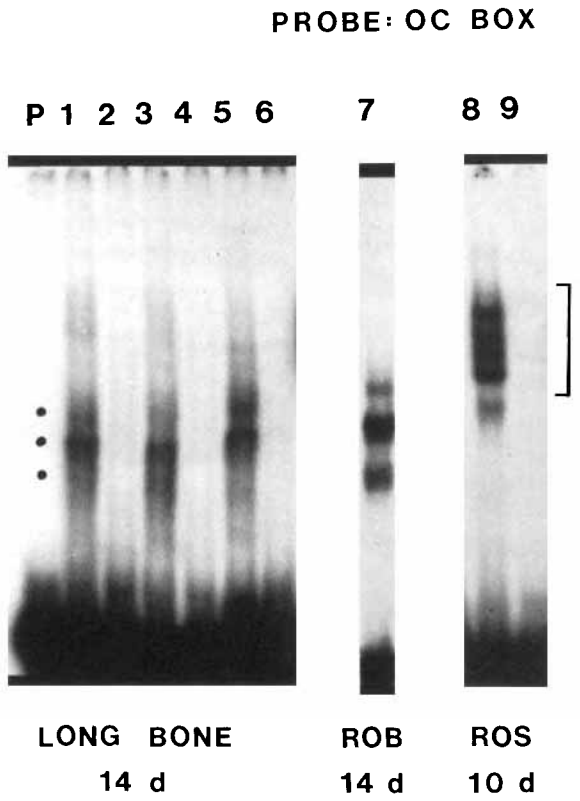

Fig. 4. Sequence-specific protein-DNA interactions in the promoter of the osteocalcin gene using factors isolated from nuclei of intact bone. All procedures were performed at 4°C according to Dignam et al. [1983] with modifications. Probe labelling, binding reactions, and the gel mobility electrophoresis conditions were performed according to Markose et al. [1990] using the rat osteocalcin (OC) box (−99 to −76) as probe (P). **Lanes 1,3,5:** Gel shift binding patterns obtained with OC box oligonucleotides and factors isolated from normal (lane 1) and *ia* (lanes 3,5) rat long bone nuclei. **Lanes 7,8:** Binding patterns obtained when factors are isolated from primary diploid rat osteoblasts (ROB) and rat osteosarcoma (ROS 17/2.8) cells grown in culture for the indicated times. **Lanes 2,4,6,9:** Competition with 100-fold excess of cold oligonucleotides. Three prominent bands in lanes 1, 3, 5, and 7 are identified by dots in the left margin, and the higher molecular weight complexes in lane 8 are bracketed.

tomical sites (cortical and trabecular bone, the latter affected most by metabolic bone diseases). The applications of this methodology include characterization of gene regulation during fracture repair, or assessing pathogenesis and treatment of inherited or acquired skeletal disorders including osteopetrosis and osteoporosis.

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