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IDENTIFICATION OF NOVEL PROTEINS SYNTHESIZED IN BONE CELLS BY ANTIBODY SCREENING OF A cDNA EXPRESSION LIBRARY

Elka Nutt, Chris Dunwiddie, John W. Jacobs and Ellen Simpson

Department of Biological Chemistry,
Merck Sharp & Dohme Research Laboratories,
West Point, PA 19486

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SUMMARY: Novel proteins synthesize predominantly in bone have been identified by antibody screening of bone cell cDNA expression libraries. Two unique cDNAs were identified whose structures do not match any known nucleic acid or protein sequence in the NIH computer bank. The first cDNA clone, BP-I, encoded a mRNA of 2300 bases in size which was expressed at high levels in 17/2.8 rat osteosarcoma cells, rat calvarial bone cells and placenta. A second clone, BP-II, encoded a mRNA of 1500 bases which was expressed at high levels in 17/2.8 osteosarcoma cells and in salivary gland. Expression of both mRNAs in osteosarcoma cells was modulated by the calciotropic hormone, vitamin D. Southern blot analyses indicated that the two cDNAs represented distinct, single copy genes in the rat genome. These novel gene products may serve as potential new markers to study bone turnover in metabolic bone disease.

The organic component of bone is approximately 90% insoluble Type I collagen with the remaining 10% representing a mixture of noncollagenous proteins (1). These proteins include both bone-specific proteins, as well as proteins found in serum which are concentrated on the bone matrix (2). The isolation and characterization of a number of these proteins have been achieved, including bone gla protein or osteocalcin, osteonectin, osteopontin, and sialoprotein (reviewed in reference 1). In large part, the functions of these noncollagenous bone proteins are unknown, although antibodies to a number of them are available and have been utilized to examine the extent to which these proteins serve as markers of bone turnover. Considerable interest still exists in identifying new bone-specific proteins as potential clinical markers for bone turnover and as tools to study the development of the osteoblast phenotype.

In the present study, we have utilized a molecular biology approach to identify novel mRNAs which are expressed predominantly in bone. Recombinant DNA libraries were screened with antibodies prepared against

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partially-purified bone matrix proteins and utilized to identify unique cDNA clones that reacted with the antibodies. DNA sequence analyses indicated that certain cDNA clones identified in the screening procedure coded for, heretofore, unidentified bone proteins. Hybridization analyses indicated that these cloned cDNAs encoded mRNAs which are expressed at high level in bone and can be regulated by the calciotropic hormone, vitamin D. The proteins we have identified may serve as potential new protein markers for bone turnover.

MATERIALS AND METHODS

cDNA Library Screening - Messenger RNA was prepared from a rat osteosarcoma cell line (3) and utilized to construct a cDNA expression library in the bacteriophage vector lambda GT11 (4). The amplified library contained 7.8×10^5 independent clones with an average insert size of 1.05 kb. The amplified library was plated on E. coli 1090 strain and induction of protein synthesis was accomplished with isopropyl beta-D-thioglactopyranoside (ITPG). Expressed antigens were immobilized on nitrocellulose filters and screened with an antiserum raised to partially purified bone matrix proteins. ^{125}I-goat-anti-rabbit antibody was used as the secondary antibody to visualize positive clones. Bacteriophage plaques showing a positive signal by autoradioagraphy were cored, replated and rescreened to plaque homogeneity. Recombinant cDNAs selected for further analysis were subcloned into M-13 vectors for dideoxy-sequencing (5).

Northern Blot Analysis - Intact RNA was isolated from a variety of rat tissues as well as two rat osteosarcoma cell lines, ROS 25/1 and ROS 17/28, according to the procedure of Chirgwin (3). Poly A RNA was prepared from total RNA from 17/28 cells by oligo-(dT)-cellulose chromatography (6). Twenty micrograms of total RNA (as determined by optical density at 260 nM) or 3 ug of poly-A RNA were loaded per lane and then fractionated on 1.2% agarose/2.2M formaldehyde gels containing 0.5 ug/ml ethidium bromide. The RNAs were then transferred to nitrocellulose according to the procedure of Thomas (7). The relative amounts of RNA were determined by densitometric analysis of hybrid images utilizing a Hoefer GS 300 transmittance scanning densitometer. As positive controls and for quantitation purposes, filters were subsequently rehybridized with a radiolabelled actin cDNA probe.

Southern Blot Analysis - High molecular weight genomic DNA extracted from rat kidney was digested with various restriction endonucleases, fractionated on 0.7% agarose gels and transfersed to nitrocellulose according to the procedure of Southern (8). Hybridizations were done at 43°C for 16-20 hours with the addition of nick-translated ^{32}P-labeled probe (2.5×10^6 cpm/ml). The blots were initially washed three times in 2xSSC (20xSSC is 3M NaCl/0.3M sodium citrate/0.1% sodium dodecyl sulfate/0.1% sodium pyrophosphate) at room temperature. The blots were then washed twice at 50°C in 0.4xSSC/0.1% sodium dodecyl sulfate/0.1% sodium pyrophosphate. The filters were then sealed in plastic bags and exposed to x-ray film.

RESULTS AND DISCUSSION

As the antisera which was utilized in these studies was raised to a heterogeneous mixture of bone matrix proteins a number of distinct clones were identified. Based on a preliminary screen utilizing Northern blot analyses, two cDNA clones were identified that hybridize with mRNAs, that, based on their size, appeared to code for, heretofore, uncharacterized bone cell mRNAs (data not shown). These two cDNA clones were designated bone proteins I and

TABLE I

NUCLEOTIDE SEQUENCE AND DEDUCED AMINO ACID SEQUENCE OF BP-I AND BP-II cDNAs

BP-I

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                                          10
   Gly Ala Ala His Ser Leu Pro Thr Ser Val Pro Ala Ala Leu Leu Leu Leu Asp Tyr
5'-GGA GCA GCC CAC TCC CTT CCT ACC TCG GTT CCT GCA GCG CTA CTG CTC CTG GAT TAC

 20                                       30
Pro Pro Asp Arg Ile Ser Leu Phe Leu His Asn Asn Glu Val Tyr His Glu Pro His
CCG CCG GAC AGG ATC TCT CTT TTC CTT CAC AAC AAC GAG GTG TAC CAC GAG CCT CAC

     40                                   50
Ile Ala Asp Ala Gly His Ser Pro Gly Pro Leu Leu Ser Cys Lys Ala Ser Gly Ala
ATT GCA GAT GCT GGC CAC AGT CCA GGA CCA CTT CTC AGC TGT AAA GCT AGT GGG GCC

         60                                       70
Arg Gly Gly Leu Ser Ser Gly Glu Ala Arg Asp Met Ala Met Asp Ser Cys Arg Gln
AGA GGA GGC CTG AGC TCA GGG GAG GCC AGG GAC ATG GCC ATG GAT AGC TGT CGG CAG

             80
Asn Pro Ser Val
AAC CCG AGT GTG-3'
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BP-II

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                                          10
   Gln Pro Ser Asn Val His Ser Gly Ile Phe Leu Pro Asp Gln Gln Gln Asp His Val
5'-CAG CCG AGC AAC GTT CAC TCC GGC ATT TTT CTT CCA GAC CAA CAG CAG GAT CAT GTC

 20                                       30
Leu Ile Ala Phe Asp Phe Thr Ala Leu Ser Arg Arg Ile Thr Thr Asp Ala Asp Leu
CTT ATC GCC TTT GAT TTC ACC GCT CTA TCG AGA CGG ATC ACT ACT GAC GCA GAT CTC

     40                                       50
Gly Ile Leu Pro Asp Lys Ser Ser Gln Arg Phe Glu Ala Gly Phe His Ile His Leu
GGC ATT TTA CCG GAC AAA TCG TCC CAG CGT TTC GAA GCG GGA TTT CAC ATA CAT CTG

         60                                       70
Arg Leu Ser Ser Ala Ala Tyr Ser Ser Gly His Ser Val Arg Cys Val Asn Pro Gln
AGG CTT TCC AGC GCT GCG TAT AGC TCG GGT CAT AGC GTG CGA TGC GTC AAT CCA CAG

             80
Ser Leu Tyr Ile Glu His Asp
TCG CTC TAC ATT GAA CAT GAT-3'
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II (BP-I, BP-II) and both strands of their DNA were subjected to dideoxy sequence analysis. The DNA sequence and deduced protein sequence of BP-I and BP-II is shown in Table 1. The amino acid sequence shown is that protein sequence which is in-frame with the B-galactosidase gene of the lambda GT-11 vector and which would be expressed by the bacteriophage and recognized by the bone-matrix antibodies utilized in the library screening procedure. It should be stressed that the sequence shown in Figure 1 does not represent the complete protein structure of BP-I and II, but does represent the structure of

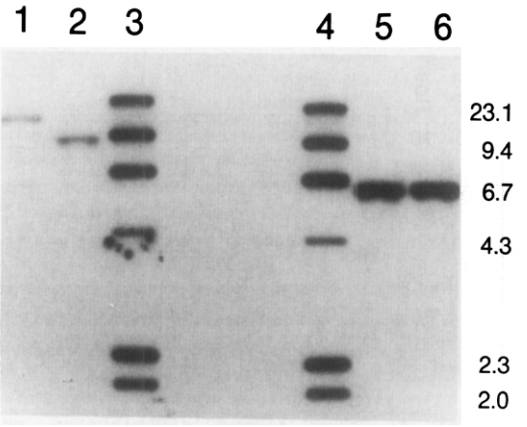

Figure 1. Southern blot analysis of BP-II (Lanes 1-3) and BP-II (Lanes 4-6) cDNA clones. High molecular weight rat DNA (15 ug) was digested with a number of restriction enzymes and hybridized with radiolabelled BP-I and BP-II cDNAs. Shown here are two such experiments: Lanes 1 and 5, double enzyme digestions with EcoRl and BamHl, Lanes 2 and 6, double enzyme digestions with EcoRl and HindIII; Lanes 3 and 4, phage lambda DNA molecular weight markers. Size, in base pairs of MW markers is listed on the right.

the expressed epitope recognized by the antibodies. Computer searches of all six possible reading frames of the complete DNA sequences indicated that none of the reading frames encoded any known proteins listed in the NIH computer bank (Intelligenetics Program).

To establish whether the two mRNAs were products of distinct genes, Southern blot analysis was performed with DNA extracted from rat kidney. As shown in Figure 1, 15 ug of rat DNA was digested with different restriction enzymes, fractionated by agarose gel electrophoresis and hybridized to nick-translated BP-I and BP-II cDNAs. The data from these hybridization experiments indicate that the cDNA probes recognize distinct genes that, most likely, represent single copy genes in the rat genome.

The two cDNAs were then utilized in mRNA/DNA hybridization assays to examine 1) the size of the mRNAs which encoded these proteins, 2) the tissue distribution of expression of these mRNAs and, 3) the regulation of accumulation of these mRNAs by $1,25(OH)_2$ vitamin D_3, a known regulator of bone protein biosynthesis. Figure 2 shows a Northern blot hybridization experiment with RNA prepared from a number of rat tissues. Radiolabelled BP-I cDNA hybridizes intensely to a mRNA of 2200 bases in the osteoblast-like, osteosarcoma cells (Lane 8, 9, 10). Hybridization can also be seen in RNA prepared from rat calvarial bone tissue, although the signal was not as intense. The only other tissue which showed significant hybridization to the BP-I probe was placenta, and minor amounts of hybridization was seen in brain, lung and salivary gland. It should be stressed that equivalent amounts (20 ug) of total RNA were loaded in each lane and subsequent control experiments utilizing a radiolabelled actin cDNA probe indicated equivalent quantities of

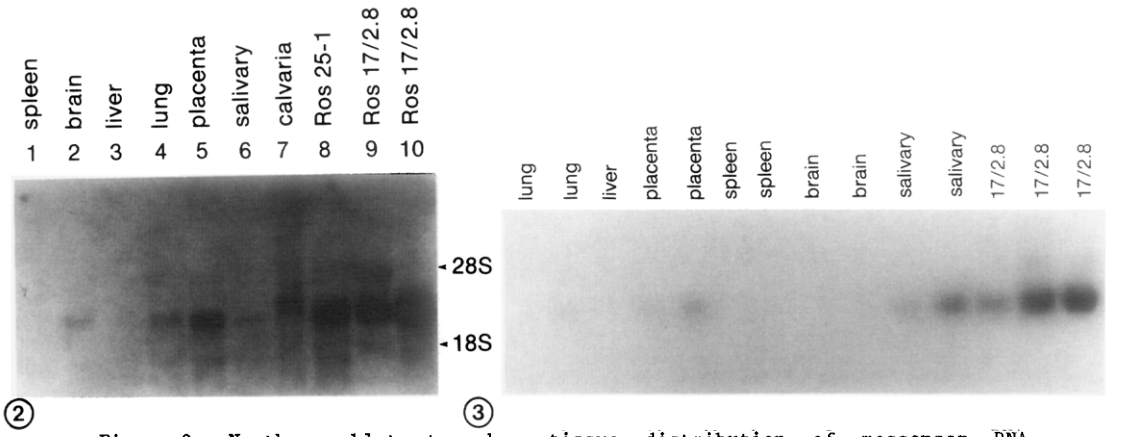

Figure 2. Northern blot to show tissue distribution of messenger RNA hybridizing to BP-I cDNA. Source of RNA is shown at the top of the figure. Amount of RNA loaded in each lane is as follows: Lanes 1-9, 20 ug RNA; Lane 10, 40 ug.

Figure 3. Northern blot to show tissue distribution of messenger RNA hybridizing to BP-II cDNA. Source of RNA is listed at top of the figure. Amounts of total RNA loaded from left to right are: lung, 10 and 20 ug; liver, 10 ug; placenta, 10 and 20 ug; spleen, 10 and 20 ug; brain 10 and 20 ug; salivary, 10 and 20 ug; 17/2.8 bone cells, 10, 20 and 20 ug.

RNA were analyzed in each tissue (data not shown). Figure 3 shows the same type of experiment, but utilizing radiolabelled BP-II cDNA as the probe. Again, the most significant hybridization was to RNA prepared from bone cells (17/2.8 osteosarcoma cells) with lesser amounts of hybridization to RNA prepared from salivary, placenta and lung. The predicted size of this mRNA is 1800 bases.

Further studies were then undertaken to examine whether steady state levels of the two mRNAs showed hormonal regulation by $1,25(OH)_2$ vitamin D, a known regulator of bone cell protein biosynthesis. Rat osteosarcoma cells (17/2.8) were grown in the absence or presence of 10^{-9}M vitamin D for 36 hours. As can be seen in Figure 4A, vitamin D upregulated the levels of mRNA encoding BP-I related mRNA (Lanes 3 and 4). Densitometric analysis of the hybrid images visualized by autoradiography indicated a 2.5 fold enhancement by vitamin D of BP-I mRNA. In contrast, vitamin D downregulated the levels of mRNA encoding the BP-II mRNA as shown in Figure 4B, with densitometric analysis of the hybrid images showing a two-fold reduction in the relative levels of this RNA ("+" lanes, Figure 4B). Again, subsequent rehybridization with actin probes showed similar quantities of RNA were analyzed in each experiment (data not shown). Thus, in these initial and limited studies, steady state levels of both BP-I and BP-II mRNAs are regulated in an inverse fashion in bone cells by the calcium-regulating hormone, vitamin D. This finding adds further support to our hypothesis that BP-I and BP-II may have some role related to bone cell function.

In summary, we have used a molecular biology approach to identify novel proteins expressed in bone. By antibody screening of a cDNA expression

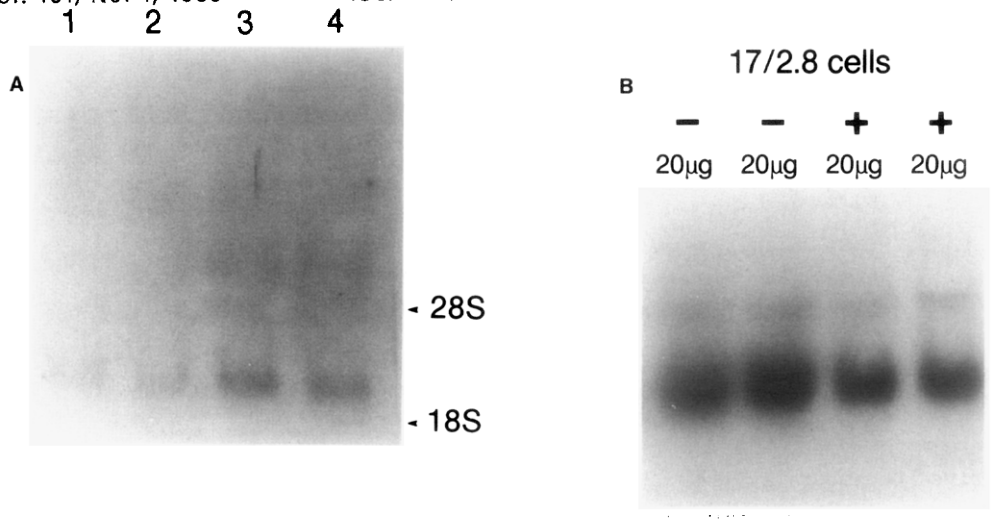

Figure 4. Treatment of bone cells with $1,25(OH)_2D_3$ and examination of steady state levels of BP-I and BP-II mRNAs. Cells were grown in 10^{-9}M Vitamin D_3 for 36 hours before messenger RNA was prepared and subjected to Northern blot analysis. A) BP-I mRNA: Lanes 1 and 2, 20 ug total RNA from untreated cells; lanes 3 and 4, 20 ug total RNA from vitamin D-treated cells. B) BP-II mRNA: Lanes labelled "-", 20 ug of total RNA from untreated cells; lanes labelled "+", 20 ug RNA from vitamin D-treated cells.

library prepared from bone cell mRNA, unique gene products were identified. The expression of these novel products is seen predominantly but, not exclusively, in bone. The protein structures predicted by these cDNA clones can now be used to prepare synthetic peptides, and used to study the potential use of these proteins as markers of protein turnover in metabolic bone disease and to study the development of the osteoblast phenotype in cultured cells.

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