Characterization of a cDNA for Chicken Osteopontin: Expression during Bone Development, Osteoblast Differentiation, and Tissue Distribution†,‡

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ABSTRACT: The chicken bone phosphoprotein (~66-kDa BPP) is a major noncollagenous component of bone and is the major phosphoprotein synthesized by cultured chicken embryo osteoblasts [Gotoh, Y., Gerstenfeld, L. C., & Glimcher, M. J. (1990) *Eur. J. Biochem. 87*, 49–58]. A cDNA clone for this protein was isolated from an expression library made from embryonic chicken bone mRNA. The complete primary protein sequence of 264 amino acids was deduced from the cDNA sequence inclusive of a 16 amino acid signal peptide sequence and terminated by 4 in-frame stop sequences. A sequence alignment indicated an approximate 35% overall similarity in protein sequence between the avian ~66-kDa BPP and the mammalian protein osteopontin, while at the nucleotide level 60% similarity was observed. Features of this sequence which showed the greatest similarity to mammalian osteopontin included a region in which seven of nine consecutive residues are aspartic acid, a recognition sequence for integrin-mediated cell binding (-Arg-Gly-Asp), and four possible recognition sequences for phosphorylation by casein kinase II. Hybridization analysis indicated a message of 1.5 kb found predominantly in bone and kidney. The mRNA was inducible in phorbol ester treated primary cultures of chondrocytes which show no expression under normal growth conditions. A temporal induction was seen during osteoblastic differentiation both in vivo and in vitro, thus suggesting that regulation of the ~66-kDa BPP is under transcriptional control during osteoblast development. In summary, both the protein's primary structure and its biological features suggest that it is the avian homologue to mammalian protein osteopontin.

Phosphorylated extracellular matrix proteins are thought to play an integral role in the processes of the in vivo formation of vertebrate mineralized tissues (Glimcher, 1985; Veis, 1985). They have been isolated from the extracellular matrices of bones from a large number of species including bovine (Franzen & Heinegard, 1985), rat (Prince et al., 1987), human (Fisher et al., 1986), and porcine (Kubota et al., 1989). Common characteristics of these proteins are their extremely acidic nature and the presence of *O*-phosphoserine (Ser-P), *O*-phosphothreonine (Thr-P), and N-linked and O-linked glycosylation. At least three genetically distinct, highly phosphorylated, extracellular matrix proteins have been demonstrated in rat bones (Oldberg et al., 1986, 1988; Prince et at., 1987; Gorski et al., 1988), while homologues or similar proteins to two of the three rat phosphoproteins have been shown to be present in bovine (Franzen & Heinegard, 1985), human (Fisher et al., 1986), and chicken (Gotoh et al., 1990) bones. The most extensively characterized member of this class of noncollagenous bone proteins, which was independently isolated from different sources by several research groups, has been separately named osteopontin (OPN) (Oldberg et al., 1986), secreted phosphoprotein (SppI) (Senger & Perruzzi, 1985), 2ar (Smith & Dehhardt, 1987), 44-kDa phosphoprotein (Prince et al., 1987), and bone sialoprotein I (BspI) (Fisher et al., 1987; Young et al., 1990). The complete protein sequences deduced from cDNA analysis are available for the individual rat (Oldberg et al., 1986), mouse (Craig et al., 1988), human (Kiefer et al., 1989), and porcine (Wrana et al., 1990) osteopontins.

Osteopontin was independently identified as one of the primary gene products inducible by phorbol 12-myristate 13-acetate (PMA) in epidermal cell lines, showing new transcription within 3–6 h after treatment with PMA (Smith & Denhardt, 1987; Craig et al., 1989). It has also been shown to be a marker to all forms of metastatic carcinomas and sarcomas (Senger et al., 1988). Growth factors including TGF and FGF (Noda et al., 1988; Nomura et al., 1988; Kubota et nal., 1989; Rodan et al., 1989) and the steroid 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] (Prince & Butler, 1987; Yoon et al., 1987; Noda et al., 1988; Oldberg et al., 1989) have been shown to induce this gene. In contrast, down-regulation by human parathyroid hormone (Noda et al., 1988) and dexamethasone (Yoon et al., 1988; Oldberg et al., 1989) was noted in cultured osteosarcoma cell lines. Both whole embryo immunolocalization (Mark et al., 1988a,b) and in vitro hybridization analysis (Nomura et al., 1988) have shown this protein to be predominantly expressed in bone. However, lower levels of expression were seen in the kidney, decidua, and placenta.

A ~66-kDa phosphoprotein was subsequently isolated from mature chicken bone (Gotoh et al., 1990) which had several osteopontin-like features. Light microscopy immunolocalization of whole chick embryos revealed its presence primarily in the developing bones and in the kidney (Bruder et al., 1990). In addition to the presence of Thr-P and Ser-P residues, amino acid analysis indicated high contents of aspartic acid and glutamic acid residues, while carbohydrate analysis revealed the presence of neuraminic acid which for similar proteins in other species has led to their other name: bone sialic acid protein I. Partial microsequence analysis of tryptic peptides for the ~66-kDa phosphoprotein indicated the presence of a

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nine amino acid RGDS integrin cell binding sequence identical with that found in rat osteopontin (Oldberg et al., 1986). However, a comparison of the remainder of the tryptic fragments (~25% of total protein) with the sequence of mammalian osteopontins indicated no other apparent homology.

In defining the processes of bone formation, Gerstenfeld et al. (1987, 1988, 1990) had developed an in vitro model system utilizing cultured chicken osteoblasts. These cells were found to exhibit a temporal sequence of expression of the various phenotypic markers which characterize the differentiated osteoblast state. Over a 30-day growth period, 50–100-fold increases in alkaline phosphatase enzyme activity and osteocalcin synthesis were observed which accompanied progressive mineralization and the accumulation of type I collagen as 60–75% of total cell layer protein. The expression of the ~66-kDa phosphoprotein during the differentiation of cultured osteoblasts was recently reported (Gerstenfeld et al., 1990). The ~66-kDa phosphoprotein was determined to be temporally expressed concurrent with other phenotypic markers of osteoblastic differentiation and, through immunolocalization techniques, spatially related to mineralization both in vitro and in vivo (Gerstenfeld et al., 1990; McKee et al., 1990).

In order to further characterize this protein and provide definitive sequence analysis for comparison to the mammalian noncollagenous bone phosphoproteins, a cDNA clone has been isolated and sequenced as described herein. The characterization of this clone and its use in determining the primary structure of the chicken ~66-kDa phosphoprotein, in vivo tissue distribution, in vitro and in vivo expression during osteoblast differentiation, and its expression in response to phorbol ester treatment are presented.

Materials and Methods

Isolation of RNA and cDNA Library Construction. Specific tissues were isolated from 18-day embryonic chicks and quick-frozen in liquid nitrogen, and the RNA was extracted as described by Gerstenfeld et al. (1983). RNA from cultured cells was similarly isolated except the cells were directly placed in proteinase K digestion buffer. Five milligrams of a mixture of 18-day tibia and calvaria bone RNA was twice selected by oligo(dT)–cellulose chromatography (Tate et al., 1983) to yield ~50 μg of polyadenylated RNA. This poly(A)-containing mRNA was used to prepare an oligo(dT)-primed cDNA library cloned into the *Eco*RI site of the λgt11 expression vector by using the method of Gubler and Hoffman (1983) (Applied Biotechnology, Cambridge, MA).

Screening and Isolation of Positive Clones. Approximately 100000 clones of the library were expressed in *Escherichia coli* Y1090 and subsequently screened with a 1:500 dilution of polyclonal antibody raised to the chicken bone ~66-kDa phosphoprotein. The production and characterization of this antibody were as described in Gerstenfeld et al. (1990), and the antibody was preabsorbed before use with a lysate of Y1090 *E. coli*. The expression library screening protocol of Young and Davis (1983), as detailed in Mierendorf et al. (1987), was utilized by employing an alkaline phosphatase conjugated goat anti-rabbit antibody (Sigma Chemical Co., St. Louis, MO) as the secondary antibody. Three clones retained their positive nature through a tertiary screening. Their clonal purity was verified through rescreening for 100% positive plaques, and a phage stock was prepared for storage (Maniatis et al., 1983). Large-scale purification of λDNA corresponding to each positive clone was performed using *E. coli* Y1090 as the host bacteria (Maniatis et al., 1983). The insert cDNA was released from the phage cDNA by *Eco*RI treatment, purified by preparative agarose gel electrophoresis, and subcloned into the *Eco*RI site of pBS (Stratagene, La Jolla, CA). Transformation of the recombinant plasmids was then performed into *E. coli* DH5α (Hanahan, 1983). CsCl gradient purification of plasmid DNA was performed according to Maniatis et al. (1983).

DNA Sequence Analysis. DNA sequence determination was by the method of Sanger et al., (1977) and carried out with Sequenase (U.S. Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions. Oligonucleotide sequencing primers were obtained either from Stratagene Corp. (primers SK and KS to flanking pBS sites) or from Operon Technologies (Alameda, CA), and synthesized according to internal cDNA sequences derived from initial sequencing reactions from the flanking cloning sites (Figure 1). Alignment of DNA sequences was performed with the ALIGN sequence alignment program (Scientific & Educational Software, State Line, PA) utilizing an algorithm developed by Hirschberg (1975) and Myers and Miller (1988). Software default values were used for the mismatch penalty (2), open gap penalty (4), and extend gap penalty (1) for all alignments.

Hybridization Analyses. For Northern blot analyses, RNA was denatured before use and electrophoresed through agarose gels containing 2.2 M formaldehyde according to the procedure of Lehrach et al. (1977). The RNA was transferred to a Zeta-Probe nylon membrane and hybridized according to the manufacturer's instructions (Bio-Rad, Richmond, CA). Random primer generated ^{32}P-labeled probe was prepared as described by Feinberg and Vogelstein (1984) from the *Eco*RI insert from pMMPP2. For slot blot analyses, denatured RNA was applied to nitrocellulose (Schleicher & Schuell, Keene, NH) and hybridized as detailed by Gerstenfeld et al. (1988). DNA standard (purified *Eco*RI–pMMPP2 insert) was denatured in 0.4 N NaOH for 15 min and neutralized with HCl before application to the filter. Blots were exposed to Kodak XAR-5 film for autoradiography and were quantitated by using an LKB Ultrascan II laser densitometer. Nondenaturing DNA gel electrophoresis on 1% agarose was carried out as described by Maniatis et al. (1983). DNA was blotted onto nitrocellulose, and hybridization was carried out as described by Southern (1975).

Cell Culture. Cultured chick embryonic osteoblasts and chondrocytes were prepared by the method of Gerstenfeld et al. (1987) and Finer et al. (1985), respectively. For experiments in which mRNAs were isolated during mineralization of the osteoblast cultures in vitro, the schedule of culture feeding and the time points of analysis were as previously described (Gerstenfeld et al., 1987, 1988, 1990). For experiments in which mRNAs were isolated from phorbol 12-myristate 13-acetate (PMA)-treated chondrocytes, the quantity and the schedule of PMA treatment were as previously described (Finer et al., 1985; Gerstenfeld et al., 1985).

Results

Isolation of cDNA Clones for the Major Bone Phosphoprotein. Approximately 50000 λgt11 phage from an 18-day embryonic chick bone library were initially screened with a polyclonal antibody raised to the major chick bone phosphoprotein. This resulted in the isolation of three positive clones, designated MMPP2, MMPP5, and MMPP13 with insert sizes of 1.1 kb, undetermined, and 0.8 kb, respectively. MMPP2 was found to cross-hybridize with MMPP13 but not MMPP5 by Southern blot analysis. Restriction enzyme analysis of both MMPP2 and MMPP13 revealed common *Sal*I and *Pst* fragments, and the 1.1-kb insert of MMPP2 was subcloned into the *Eco*RI site of pBS (designated pMMPP2), used for sequence analysis and as a hybridization probe. MMPP5 and

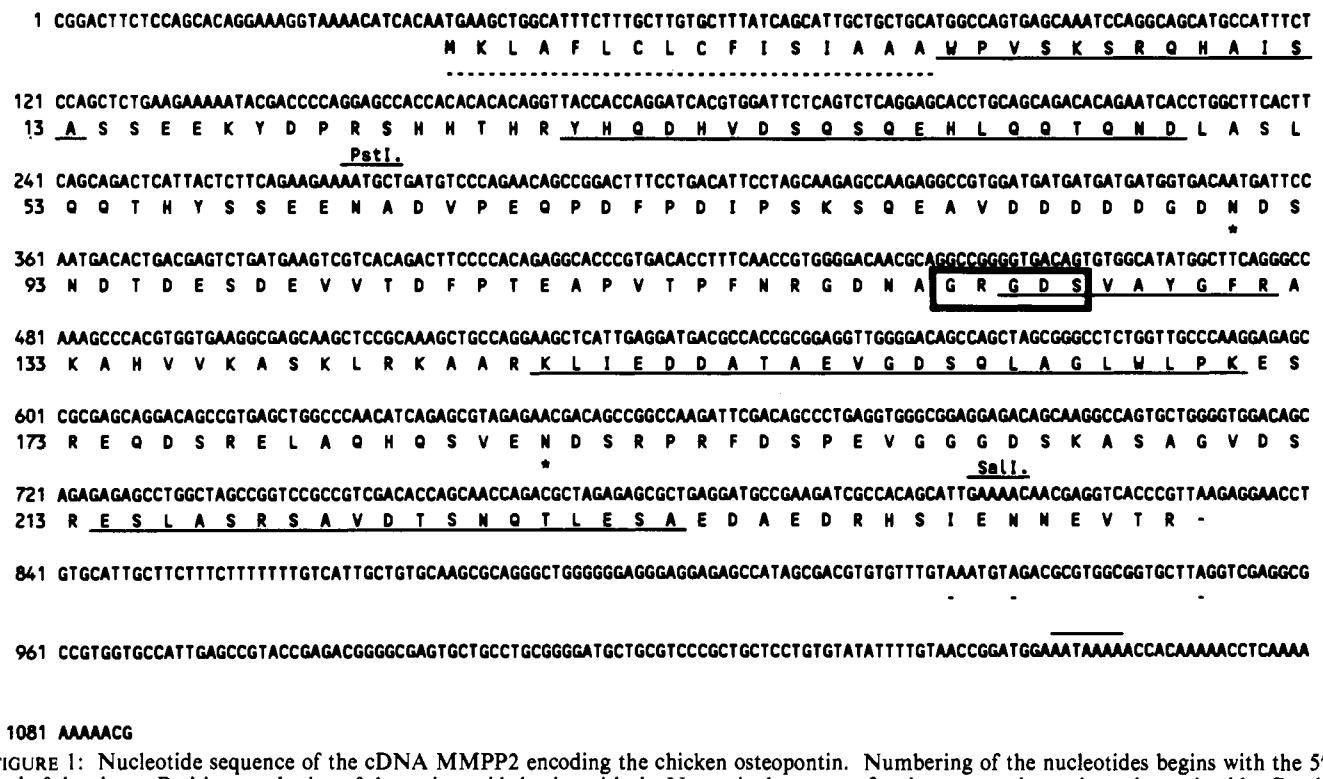

FIGURE 1: Nucleotide sequence of the cDNA MMPP2 encoding the chicken osteopontin. Numbering of the nucleotides begins with the 5′ end of the clone. Positive numbering of the amino acids begins with the N-terminal sequence for the processed protein as determined by Gotoh et al. (1990) and confirmed here in accordance with the predicted cleavage site of the signal peptide sequence (dashed underline). Amino acid sequences previously obtained by protein microsequencing techniques (Gotoh et al., 1990) are underlined. Note that all previously determined tryptic peptides are preceded by arginine. Sequences of interest include the poly(Asp)-rich region beginning at amino acid 83 and a GRGDS cell binding sequence beginning at amino acid 122. Possible sites of an N-linked glycosylation (NXS) are noted by asterisks. *Sal*I and *Pst*I restriction sites were predicted by sequence analysis and were confirmed by digestion of pMMPP2 with each enzyme (not shown). The apparent stop sequence is indicated by a dash as are subsequent in-frame stop signals. A potential polyadenylation site (AATAAA) is overlined.

MMPP13 were not further characterized.

Sequence Analysis of pMMPP2. Initial sequencing of pMMPP2 was performed by using the primers KS and SK which complement flanking regions to the *Eco*RI site of pBS. Internal oligonucleotide sequencing primers were synthesized as internal sequences were obtained. Their designation, sequence location, and indication of 5′–3′ orientation are as follows: KS1(343–359), KS2(6–22), KS3(687–703), SK1-(819–803), SK2(46–30), SK3(475–459), SK4(1054–1038), SK5(598–582), and SK6(384–368). The sequence of the 1087 bp cDNA was confirmed by sequence analysis of overlapping regions from both strands and by comparison to known protein sequence (Figure 1). Prior determination of the N-terminal amino acid sequence of this protein (Gotoh et al., 1990) allowed the assignment of the 16 amino acid signal peptide sequence as shown (von Heijne, 1983; Gierasch, 1989), beginning with methionine and preceded by a translation initiation sequence. An open reading frame of 264 amino acids is followed by a series of 4 in-frame stop codons, as well as an AATAAA polyadenylation signal (Birnstiel et al., 1987; Nevins, 1983), indicating that the cDNA was inclusive of the total coding sequence for this protein. Previous microsequence analysis of purified tryptic fragments of the protein (Gotoh et al., 1990) yielded a number of continuous in-frame sequences (underlined in Figure 1), all of which were identified and confirmed by the translation of the cDNA sequence represented here. In addition, the amino acid composition data of the deduced sequence were found to show good correspondence to those obtained earlier for protein purified from either embryonic chicken bone or chick osteoblast cultures as analyzed by amino acid analysis. The identity of this clone was thus confirmed as that of the 66-kDa phosphoprotein, and an apparent molecular mass of 27 378 daltons was predicted for the processed, unmodified 66-kDa chicken bone phosphoprotein.

Primary Structural Analysis with Comparisons to Mammalian Bone Phosphoproteins. As integrin cell binding sequence of GRGDS (boxed in Figure 1) and an upstream sequence, seven of nine consecutive residues of which were aspartic acid, showed conservation with the mammalian bone phosphoprotein osteopontin (Butler, 1989; Oldberg et al., 1986; Craig et al., 1988; Miyazaki et al., 1989; Kiefer et al., 1989; M. F. Young, personal communication). In addition, the mammalian proteins have a large population of S-X-E sequences and at least one N-X-S sequence which are potential O- and N-linked glycosylation sites (Hughes, 1973). The chicken protein is found to contain 14 S-X-E tripeptides utilizing 44% of all serine and 61% of all glutamic acid residues, respectively. Two N-X-S sequences (both NDS) are designated in Figure 1. Surprisingly, a second RGD sequence occurs in tandem, 5′ from the boxed GRGDS domain, that was not seen in the mammalian osteopontin. A comparison of the nucleotide and amino acid sequences of the chicken osteopontin to those of human, rat, porcine, and mouse osteopontin revealed approximately 60% nucleotide sequence similarity and 35% amino acid similarity to those of mammals (Table I). Interestingly, the amino-terminal "halves" of the proteins were found to coalign to a higher degree than the carboxyl terminus (Table I). A summary comparison of the primary structures for the regions of highest sequence conservation between the proteins from the different species is seen in Figure 2. Those regions included the 16 amino acids of the signal peptide, the poly(Asp) domain, the integrin receptor recognition sequence, and 4 possible recognition sites at which casein kinase II phosphorylation can take place (Edelman et al., 1987).

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CHICKEN -16    MKLAFLCLCFISIAAA/WPVSKSRQHAISASSEEK     18
HUMAN   -16    MRIAVICFCLLGITCA/IPV----KQADSGSSEEK     14
PORCINE -16    MRIAVIAFCLWGFASA/LPV----KQTNSGSSEEK     14
RAT     -16    MRLAVVCLCLFGLASC/LPV----KVAEFGSSEEK     14
MOUSE   -16    MRLAVICFCLFGIASS/LPV----KVTDSGSSEEK     14
CONSENSUS      MrlAvicfCl-giasa/lPV----k-a-sgSSEEK

CHICKEN  44    QTQNDLASLQQTHYSSEENADVPEQPDFPDIPSKSQEAV---DDDDDGDNDS    92
HUMAN    34    QKQNLLA--PQNAVSSEETND----FKQETLPSKSNESHDHMDDMDDEDDDD    79
PORCINE  34    QKQTFLA--PQNTISSEETDD----FKQETLPSKSNESPEQTDDVDDDDDED    79
RAT      34    QKQNLLA--PQNSVSSEETDD----FKQETLPSNWNESHDHMDDDDDDDDDG    79
MOUSE    34    QKQNLLA--PQNAVSSEEKDD----FKQETLPSNSNESHDHMDDDDDDDDDD    79
CONSENSUS      QkQnlLA--pQn-vSSEEtdD----fkqetlPSksnEshdhmDDdDDdDdDd

CHICKEN   96    DESDEVVTDFPTEAPV----TPFN-RGD-NAGRGDSVAYGFR    131
HUMAN    111    DESDELVTDFPTDLPATEVFTPVVPTVDTYDGRGDSVVYGLR    152
PORCINE  107    DESDELVTDFPTDTPATDV-TPAVPTGDPNDGRGDSVVYGLR    147
RAT      100    DESDESFT-ASTQADV---LTPIAPTVDVPDGRGDSLAYGLR    137
MOUSE    100    DESDETVT-ASTQADT---FTPIVPTVDVPNGRGDSLAYGLR    137
CONSENSUS       DESDE-vTdfpT-ap-----TP-vptvD--dGRGDSvaYGlR
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FIGURE 2: Comparison of conserved regions of the cDNA deduced amino acid sequence of the chicken osteopontin (Figure 1) with human osteopontin (Kiefer et al., 1989), rat osteopontin (Oldberg et al., 1986), mouse 2ar (Craig et al., 1989), and porcine secreted phosphoprotein (Wrana et al., 1989). Positive numbering begins at the putative signal peptide cleavage sites designated by a slash. The consensus sequence is presented wherein upper case letters represent amino acids found in all five sequences and lower case letters represent amino acids found in three or four of the five sequences. Note that the sequences presented are contained entirely within the N-terminal portion of the molecules as compared for homology in Table I.

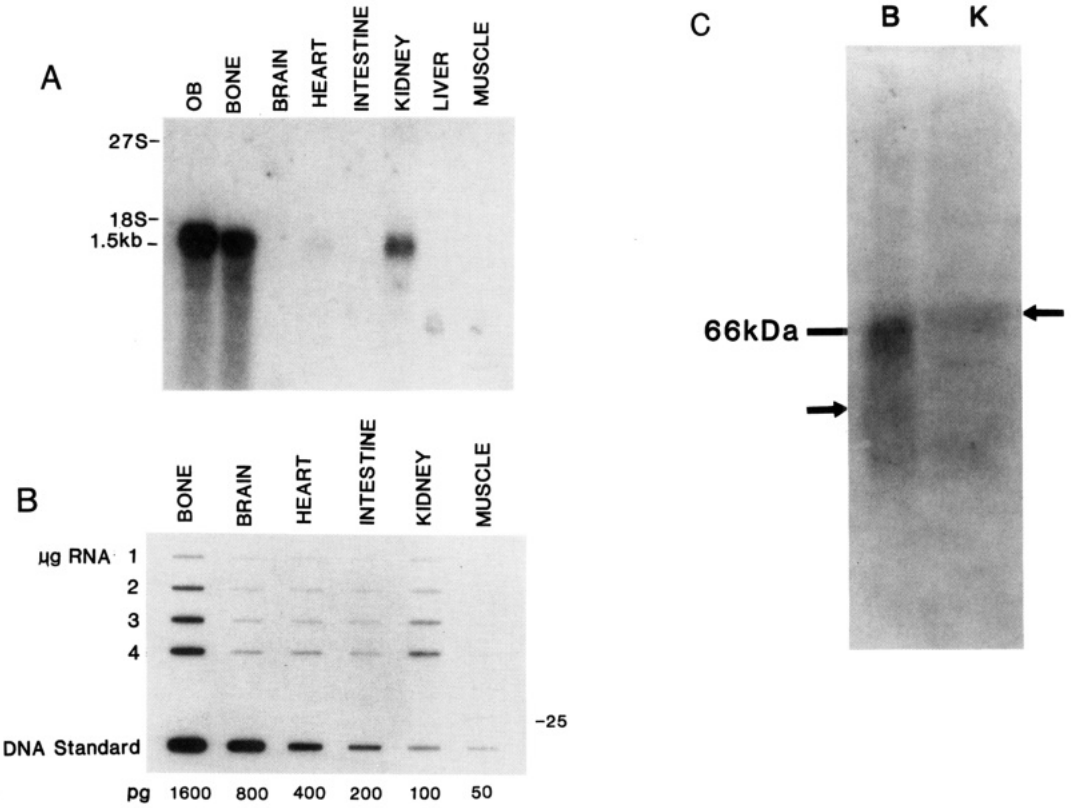

FIGURE 3: Tissue distribution, quantification, and mRNA size of the chicken osteopontin. (Panel A) Northern blot analysis of 16 µg of total RNA from various chicken tissues and from 23-day chick osteoblasts. The RNA was separated electrophoretically, followed by Northern blot analysis using MMPP2 as the hybridization probe. Using RNA size markers (New England Biolabs, Beverly, MA) including 27S and 18S chick rRNA, the mRNA for the major chick bone phosphoprotein was accurately sized to be 1.5 kb. (Panel B) Relative quantification of the mRNA levels in the various tissues was carried out by slot blot analysis of the same RNA as seen in panel A. Quantity of RNA and tissue source applied to the filter are denoted in the figure. Amounts of DNA standard (in picograms of purified MMPP2 insert) are also shown. With the assumption that approximately 1% of total RNA is mRNA with an average message size of 1.5 kb, densitometric analysis of this blot, utilizing the DNA standard as shown and correcting for its size and double-stranded characteristic, indicates that the message for the major chicken bone phosphoprotein comprises approximately 0.4% of the total message in chick bone and 0.1% in chick kidney on a mass average basis. (Panel C) Western blot analysis of total protein from chick tissues. The source of protein loaded is noted in the figure. The protein purification and blotting procedures are described in Gerstenfeld et al. (1990). Note the presence of an apparent higher molecular mass isoform in the kidney sample while the bone sample shows a lower molecular mass form previously shown to be a specific proteolytic product seen only in bone (Gotoh et al., 1990a).

Chicken Osteopontin mRNA Size and Tissue Distribution. RNAs from 23-day cultured chicken osteoblasts and several embryonic chicken tissues were analyzed by Northern blot analysis for the presence of the 66-kDa phosphoprotein mRNA (Figure 3, panel A). The cDNA hybridized to a single band centered at 1.5 kb in bone, cultured osteoblasts, and kidney. This is the same tissue distribution pattern and similar mRNA size as seen for both rat and mouse osteopontin (Yoon et al.,

Table I: Comparison of Nucleotide and Amino Acid Sequences of Chicken and Mammalian Osteopontins[a]

cDNA	nucleotide	amino acid	N-terminal	C-terminal
human	59% 68–1012	39% -16–298	43% -16–152	34% 153–298
rat	58% 80–1033	33% -16–301	39% -16–137	27% 138–301
mouse	57% 1–885	34% -16–278	40% -16–137	23% 138–278
porcine	57% 98–1010	35% -16–303	44% -16–147	26% 148–287

[a] Values are expressed relative to the chicken protein. Alignments were performed for the nucleotide and amino acid sequence data presented here for the chicken osteopontin and for human (Kiefer et al., 1989), rat (Oldberg et al., 1986), mouse (Craig et al., 1989), and porcine (Wrana et al., 1989) osteopontins. The region of nucleotide 37–831 and amino acid -16–248 comparisons spans the beginning of the coding region to the first stop sites. Separate alignments were performed on the N-terminal portions and the C-terminal portions of the molecules which are denoted in the table. For means of comparison, the human sequence contains 73%, 71%, and 71% homology to the porcine, rat, and mouse, respectively, and 64%, 65%, and 59% homology at the protein level while the rat and mouse exhibit sequence homologies of 83% and 85% relative to their rat nucleotide and amino acid sequences, respectively.

1987; Hogan et al., 1987). For comparison, the following mRNA sizes have been reported for various osteopontins and osteopontin-like proteins: rat osteopontin, 1.4 kb (Oldberg et al., 1986) and 1.5 kb (Yoon et al., 1987); rat SppI, 1.5 kb (Kubota et al., 1989); mouse 2ar, 1.6 kb (Smith & Denhardt, 1987; Craig et al., 1988; Nomura et al., 1988); mouse osteopontin-like protein, 1.4 kb (Miyazaki et al., 1989); and human osteopontin, 1.8 kb (M. F. Young, personal communication).

Slot blot analysis (Figure 3, panel B) confirms the tissue distribution observed by Northern analysis and provides for the relative quantitation of mRNA levels. Total hybridizable message for osteopontin in 18-day embryonic bone is 10-fold more abundant over apparent background hybridization to RNA from the same day of embryonic development for heart, brain, intestine, and muscle. Significant hybridization to kidney RNA is also noted and occurs at 3-fold levels over background. Initial estimates of mRNA abundance on a mass average basis (Figure 3) indicate that approximately 0.4% of the total mRNA in chicken bones are transcripts for osteopontin. This may be compared to an estimate that total phosphoproteins comprise 9% (w/w) of the total noncollagenous component which is between 15 and 30% for the total protein of bone from human, bovine, and rat (Triffitt, 1987; Glimcher, 1984). The relative abundance of this mRNA also shows a favorable comparison with the levels of relative synthesis from cultured primary osteoblast, estimated to comprise ~1.6% of total synthesis (Gotoh et al., 1990).

In order to test whether the mRNA was utilized in both bone and kidney, total protein extracts were electrophoresed, and the presence of the ~66-kDa phosphoprotein was examined by Western blot analysis (Figure 3, panel C). While the protein is found in both bone and kidney, it is interesting to note that the kidney isoform of the protein has a slightly larger molecular mass. In contrast, the bone form of the protein shows a second molecular mass form at ~40 kDa which was previously shown to be the probable result of proteolysis of the protein in the extracellular matrix of bone (Gotoh et al., 1990).

Expression of Osteopontin during Osteoblast Differentiation and in Response to Phorbol Ester Treatment. Steady-state mRNA levels for osteopontin were examined to determine if this gene was controlled during bone development and mineralization. There was a progressive increase either during bone development in vivo or during osteoblast differentiation in vitro (Figure 4). As can be seen from these results, either before day 12 in vitro or at early developmental periods in ovo

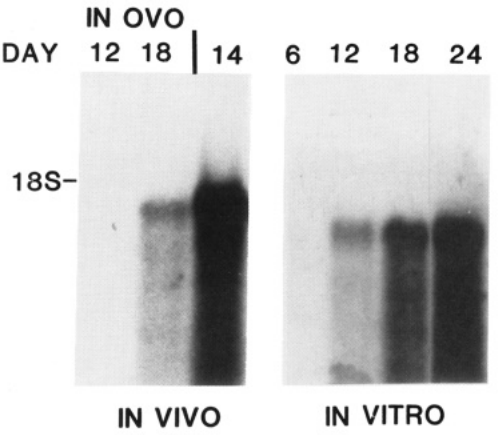

FIGURE 4: Expression of osteopontin during differentiation in vitro and during bone development in vivo. Four micrograms of total RNA was loaded per bone. For the in vivo samples, total mRNA was prepared from whole tibia bones, and 12 and 18 days in ovo and 2 weeks posthatch were examined. For the in vitro samples, total mRNA was prepared from cultures of osteoblasts on 6, 12, 18, and 24 days. The nature of samples is denoted in the figure. Autoradiographic exposure was for 2 days with an intensifying screen.

(day 12) only very low levels of mRNA were observed. Quantitative measurement of the mRNA levels by slot blot analysis demonstrated a 17-fold increase in mRNA levels from day 12 in ovo to 14 days posthatch. The levels in cultured osteoblasts, however, were 5 times higher on day 6 in culture than those observed in vivo at day 12, and showed only a 5-fold increase over the time course of osteoblast growth in culture. Comparing absolute levels, however, osteoblasts reproducibly showed 50–100% higher mRNA quantities than seen for the tissue in vivo. These results were consistent with both previous measurements of synthesis and protein accumulation measured both for osteoblasts in culture or during bone development in vivo (Gerstenfeld et al., 1990; McKee et al., 1990) and would suggest that the expression and accumulation of the chicken osteopontin were controlled at a pretranslational level. These results also suggest that the cultured osteoblasts may express higher levels of this mRNA than those observed in vivo. Synthesis and mRNA levels for the 66-kDa osteopontin protein were consistent with a restricted expression to only differentiated osteoblasts, and were temporally concurrent with the development of extracellular matrix mineralization.

The ability of phorbol 12 myristate 13-acetate to induce the expression of chicken osteopontin was examined, since in mammalian cells it was shown to induce osteopontin expression (Smith & Denhardt, 1987) (Figure 5). In these experiments, sternal chondrocytes were treated with PMA. This cell type was selected since in previous experiments it had been demonstrated that there was no detectable protein in hyaline cartilage and chondrocytes isolated from tissues that remain as permanent cartilage during development (Gerstenfeld et al., 1990; Bruder et al., 1990). As can be seen in this experiment, PMA clearly stimulated the expression of osteopontin mRNAs, which were detectable by 48 h after PMA treatment. Steady-state mRNA levels after 96 h are comparable to those seen in osteoblast or bone in vivo. In comparison, α1 type II collagen mRNAs showed a progressive decrease in expression as previously demonstrated (Finer et al., 1985).

Discussion

In previous experiments, the ~66-kDa chicken bone phosphoprotein was shown to be one of the major secretory proteins synthesized by differentiated osteoblasts comprising

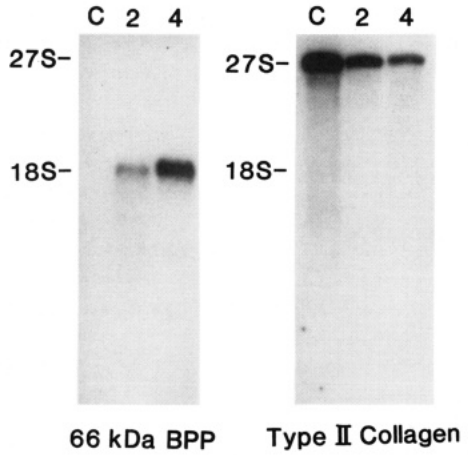

FIGURE 5: Expression of osteopontin in response to phorbol 12 myristate 13-acetate (PMA) treatment. One microgram of total RNA was loaded per lane. An identical set of samples was assayed by using α1 (type II) collagen as the hybridization probe to compare the loss of differentiated gene expression of the chondrocytes with the induction of osteopontin. Days after PMA treatment are denoted in the figure. Controls are 4-day cultures. Autoradiography exposure was for 2 days with an intensifying screen.

about 1.6% of the cells' total protein synthesis. Its DEAE-Sephacel elution characteristics, glycosylated nature, and amino acid composition (Gotoh et al., 1990a,b) were quite similar to those of the mammalian bone phosphoprotein osteopontin (Fisher et al., 1987; Franzen & Heinegard, 1985; Prince et al., 1987; Kubota et al., 1989); however, protein sequence analysis of five different tryptic peptides failed to show sequence similarity to osteopontin with the exception of a nine amino acid sequence that showed some sequence similarity to the Arg-Gly-Asp cell binding site (Gotoh et al., 1990). Subsequent studies of the expression and immunological localization of the chicken bone phosphoprotein during embryological development provided further evidence that the chicken protein was similar to mammalian osteopontin, based on its restricted expression in bone and kidney and its progressive appearance during bone formation and mineralization (Gerstenfeld et al., 1990; McKee et al., 1990; Bruder et al., 1990; Yoon et al., 1987; Nomura et al., 1988; Mark et al., 1988a,b).

In the present report, a full-length cDNA clone, corresponding to the chicken ~66-kDa bone phosphoprotein, has been isolated and characterized. These data confirm the previous protein sequence derived from the tryptic peptides (Gotoh et al., 1990), and upon analysis of the complete sequence, the protein was found to contain several osteopontin-like features. These data suggest that it may be the chicken homologue to those proteins found in rat, mouse, human, and pig. A greater sequence similarity was observed when comparing the amino halves of the mammalian osteopontin to chicken than the carboxyl halves of these proteins. This difference was primarily due to the addition of 38–53 residues at the carboxyl ends of the mammalian proteins. In this context, it is interesting to note that a comparison of the rat sequence to the other mammalian forms identified an additional 23 amino acids [233–255 in Oldberg et al. (1986)] that were not present in any of the other mammalian forms of this protein. The chicken protein produced a continuity in the sequence alignment with all of the other mammalian sequences up to the point of the insertion seen in the rat sequence. These data would suggest that the longer mammalian genes may have arisen by the addition of exon(s) at the carboxyl end of the gene or that the chicken genes lost these exons after diverging from mammals during evolution 180–230 million years ago. Two alternative possibilities to explain these differences are that multiple forms of mRNAs of varying lengths are produced by alternative exon splicing or that there are multiple genes coding for a closely related gene family within a given specie. These latter explanations, although not able to be completely ruled out, seem unlikely, since only one mRNA size was observed for this gene in various tissues examined and several different developmental stages. Southern blot analysis of the chicken gene under stringent hybridization conditions also demonstrated only a single set of restriction bands, consistent with internal restriction sites mapped within the cDNA. Comparable data for the restriction analyses of the mouse gene also led these investigators to conclude that only a single gene was present (Craig et al., 1989) in this species. Definitive proof or disproof of these alternatives awaits complete sequence analysis of these genes and less stringent hybridization analysis of the genome to determine if multiple forms of an osteopontin gene family exist.

Protein domains of the osteopontins from the various species which show the greatest conservation include the following: a GRGDS sequence known to be the recognition site for integrin-mediated cell binding; the aspartic acid rich region that varies in length from 7 to 10 consecutive aspartic acid residues, which can form part of a Ca^+ binding "E-F hand-like domain"; numerous S-X-E sites which can be the attachment points for O-linked glycosylation; at least 1 N-X-S site representing a recognition site for N-linked glycosylation; 4 substrate recognition sequences for phosphorylation of serine or threonine that would be recognized by casein kinase II (Edelman et al., 1987); and the signal peptide that directs endoplasmic reticulum membrane translocation.

Numerous research groups have focused on the role of the RGD sequence, and it was originally suggested that this protein promoted the attachment and cell spreading of osteoblasts during bone development, thereby providing a bridge between the mineral surface and osteoblasts. In vitro experiments for the rat protein (Oldberg et al., 1986; Somerman et al., 1987) and subsequent studies for the chicken osteopontin (Gotoh et al., 1990b) also demonstrated that these proteins would promote in vitro cell attachment and spreading in tissue culture of both osteosarcoma cell lines and normal periodontal fibroblasts. In subsequent experiments of the ultrastructural localization of the chicken osteopontin either in developing bone in vivo or in the mineralizing extracellular matrix of osteoblasts in vitro, this protein was clearly shown to be associated primarily with areas of mineralization well removed by several micrometers from the surface of the cells. These results suggested that any cell surface association of the chicken osteopontin in situ was probably only transient in nature (Gerstenfeld et al., 1900; McKee et al., 1990). More recently, a second suggestion put forward as to the role of the RGD sequence was that it might direct osteoclast attachment during mineral resorption by osteoclasts (Reinholt et al., 1990). Nevertheless, independent of its function, it shows a very high degree of conservation in widely divergent species during evolution and has retained its biological function of promoting cell attachment and spreading in vitro.

The second aspect of the chicken osteopontin which demonstrated very high conservation were four sites in which several serine or threonine residues are followed by two acidic amino acids (the known phosphorylation sites for casein kinase II) (Edelman et al., 1987), and in chicken bone organ cultures, preliminary analysis indicates that the kinase is similar to casein kinase II (Takagaki & Glimcher, 1990). The third

aspect of these proteins which are conserved is the poly(aspartic acid) domain. The conserved characteristics of these proteins, their highly phosphorylated nature, and their high content of acidic amino acids appear to strongly influence the secondary structure of these proteins. The essential nature of the phosphorylated residues and the carboxylic residues of the aspartic acid domain to the secondary structure of the chicken osteopontin is indicated by biophysical studies of the proteins' conformation in the presence and absence of Ca^{2+} and in an unphosphorylated and phosphorylated state. These conclusions are based on CD, FT-IR, and fluorescent studies of the tryptophans in the presence and absence of Ca^{2+}. In these studies, definitive interactions were demonstrated between the modified phosphate side groups and both the carbonyls of the peptide backbone and carboxylic side groups of protein with Ca^{2+} ions. In the dephosphorylated form, the protein also showed a very different FT-IR spectrum both in the absence and in the presence of Ca^{2+} when compared to phosphorylated protein (Renugopalakrishna et al., unpublished results).

The final aspects of the studies presented here relate to the expression of the chicken osteopontin during normal embryological development and in the presence of PMA. The chicken osteopontin follows both the same temporal pattern and tissue distribution as seen for the mammalian osteopontins. Similarly, the gene appears to be regulated during osteoblast differentiation and shows a restricted expression when the tissue begins to mineralize (Yoon et al., 1987; Nomura et al., 1988). The chicken osteopontin also demonstrated induction by PMA, however, unlike the previous experiment (Smith & Denhardt, 1987), in which epidermal cell lines had been tested, primary cell cultures of normal diploid cells were examined. It is interesting to note that the osteopontin mRNA is seen after only 3 h in the presence of PMA (unpublished data), unlike the previous genes examined for PMA-treated chondrocytes such as β-actin, fibronectin, and type I collagen which only showed increased steady-state mRNA levels after 2–4 days (Finer et al., 1985; Gerstenfeld et al., 1985). These results therefore indicate that this gene's activation is one of the primary responses to PMA treatment. These results further suggest that both the mammalian and chicken genes must contain common regulatory elements that are activated during normal bone development or in the presence of PMA.

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Metabolic Origin of Urinary 3-Hydroxy Dicarboxylic Acids†

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ABSTRACT: 3-Hydroxy dicarboxylic acids with chain lengths ranging from 6 to 14 carbons are excreted in human urine. The urinary excretion of these acids is increased in conditions of increased mobilization of fatty acids or inhibited fatty acid oxidation. Similar urinary profiles of 3-hydroxy dicarboxylic acids were also observed in fasting rats. The metabolic genesis of these urinary 3-hydroxy dicarboxylic acids was investigated in vitro with rat liver postmitochondrial and mitochondrial fractions. 3-Hydroxy monocarboxylic acids ranging from 3-hydroxyhexanoic acid to 3-hydroxyhexadecanoic acid were synthesized. In the rat liver postmitochondrial fraction fortified with NADPH, these 3-hydroxy fatty acids with carbon chains equal to or longer than 10 were oxidized to ($\omega - 1$)- and ω-hydroxy metabolites as well as to the corresponding 3-hydroxy dicarboxylic acids. 3-Hydroxyhexanoic (3OHMC6) and 3-hydroxyoctanoic (3OHMC8) acids were not metabolized. Upon the addition of mitochondria together with ATP, CoA, carnitine, and $MgCl_2$, the 3-hydroxy dicarboxylic acids were converted to 3-hydroxyoctanedioic, *trans*-2-hexenedioic, suberic, and adipic acids. In the urine of children with elevated 3-hydroxy dicarboxylic acid levels, 3OHMC6, 3OHMC8, 3-hydroxydecanoic, 3,10-dihydroxydecanoic, 3,9-dihydroxydecanoic, and 3,11-dihydroxydodecanoic acids were identified. On the basis of these data, we propose that the urinary 3-hydroxy dicarboxylic acids are derived from the ω-oxidation of 3-hydroxy fatty acids and the subsequent β-oxidation of longer chain 3-hydroxy dicarboxylic acids. These urinary 3-hydroxy dicarboxylic acids are not derived from the β-oxidation of unsubstituted dicarboxylic acids.

The 3-hydroxy dicarboxylic acids, which include saturated and unsaturated homologues with chain lengths of 6, 8, 10, 12, and 14, are excreted in human urine (Greter et al., 1980; Svendsen et al., 1985; Tserng et al., 1989). The excretion of these acids increases with increased mobilization or inhibited oxidation of fatty acids, such as fasting and nonketotic dicarboxylic aciduria of various etiologies. Recently, a new disorder of nonketotic dicarboxylic aciduria characterized by a high urinary excretion of 3-hydroxy dicarboxylic acids was described (Tserng et al., 1989; Riudor et al., 1986; Poll-The et al., 1986; Kelley & Morton, 1988; Hagenfeldt et al., 1990). These data are consistent with a possible defect of 3-hydroxyacyl-CoA dehydrogenase in the patients. An understanding of the biogenesis of these acids will be useful for the investigation of disordered fatty acid metabolism.

In the urine of patients with nonketotic and ketotic dicarboxylic aciduria, the excretion of medium-chain dicarboxylic acids is increased in addition to that of 3-hydroxy dicarboxylic acids. Since the coexistence of dicarboxylic and 3-hydroxy dicarboxylic acids in the urine and a good correlation between the concentration of these two classes of acids were observed, Greter et al. (1980) proposed that 3-hydroxy dicarboxylic acids were produced from the β-oxidation of the corresponding dicarboxylic acids through dehydrogenation and hydroxylation. This metabolic route seems possible in light of the demonstrated β-oxidation of medium-chain dicarboxylic

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