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STRUCTURE AND EXPRESSION OF OSTEONECTIN mRNA IN HUMAN TISSUE

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A cDNA encoding osteonectin was isolated from a human bone cell cDNA library and used to examine osteonectin protein structure, mRNA structure and expression in human tissue. The deduced protein sequence shows complete identity with a recently isolated placental form and extensive homology to mouse and bovine counterparts. The protein is rich in cysteine residues, which are conserved between species except for cys 194 which is only present in the bovine. In the human, osteonectin mRNA is of two sizes, 2.3 and 3.0 kb, the former being dominant in all tissues studied. Human mRNA was detected in the Ewing sarcoma and in non-bone cell and tissue sources. The potential folded structure of osteonectin mRNA was estimated, based on computer predictions, and indicates the presence of a bulge at the 5′ end of the message which includes the start of translation. Southern analysis of human genomic DNA using radiolabeled osteonectin cDNA as probe demonstrates a simple banding pattern confirming earlier studies that the osteonectin gene is present in one copy per haploid human genome.

KEYWORDS: Osteonectin, non-collagenous bone protein, sparc, BM-40, culture shock protein

INTRODUCTION

Osteonectin is a phosphorylated glycoprotein (Mr = 32,000 d) which is one of the most abundant non-collagenous proteins in developing bone.[1,2] Immunohistochemical analysis using monospecific antibodies[1,11] to osteonectin showed that it is localized to preosteoblasts, osteoblasts, newly deposited osteoid mineralized matrix and osteocytes.[3,4] These data coupled with observations that osteonectin facilitated deposition of Ca^{2+}, and hydroxyapatite to Type I collagen *in vitro*[1] have led to speculation that the protein may be involved in modulating mineralization *in vivo*. DNA sequence analysis of a bovine bone osteonectin cDNA indicated it was nearly identical to a protein called sparc (secreted protein, acidic, rich in cysteine) which is enriched in the parietal endoderm of the yolk sac of the mouse during early development.[5] Osteonectin is also homologous to BM-40, a protein produced by the murine Engelbreth-Holm-Swarm (EHS) basement membrane tumor.[6] Amino terminal sequence of a "culture shock protein" produced by bovine endothelial cells in culture[7] suggests that it, too, is identical to osteonectin.[5] These and

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other data showing osteonectin localization to the expanding decidua of the placenta indicate that the protein is predominant in tissues which are remodeling or undergoing profound changes in tissue architecture.[8]

To determine whether the osteonectin produced by non-mineralizing tissue is identical in primary structure to the bone form, we have isolated a human bone osteonectin cDNA and compared its sequence to a cDNA isolated from human placenta.[9] In this report we describe the characterization of our human bone osteonectin cDNA, as well as its utilization to examine the expression of human osteonectin in a variety of normal and transformed human tissues. A potential structure for the 5′ end of human mRNA is presented as well as a preliminary analysis of the human genome encoding this protein.

MATERIALS AND METHODS

Bone Cell cDNA Library Construction

Total RNA was isolated from cultures of human bone cells[10] and poly (A+) RNA obtained by affinity chromatography using oligo(dt)-celluose (Pharmacia). Approximately 20 μg of poly (A+) RNA was used to construct a lambda zap cDNA library (Stratagene Cloning Systems). Briefly, cDNA was treated with Eco RI methylase and modified at its termini to contain Eco RI restriction sites. After removal of excess linkers, cDNA was ligated to a lambda Zap^R vector that had previously been restricted with Eco RI and dephosphorylated. cDNA was packaged using a Giga-Pack Plus (Stratagene) and used to infect BB4 strain *E. Coli*. Approximately 2.1×10^6 phage were obtained of which 97% were recombinants. Plaques were screened by *in situ* localization using anti-osteonectin antisera as first antibody and HRP-conjugated anti-rabbit antibody for detection as previously described.[11] One clone called Hon 164 also hybridized to a 5′ 0.2 kb fragment of a previously isolated bovine osteonectin cDNA[11] and was purified. Insert cDNA from Hon 164 was obtained by an automated excision process (Stratagene) which directly converts cDNA from a lambda phage vector to a plasmid vector. Restriction analysis of the purified recombinant plasmid revealed a single Eco RI fragment of 501 bp which appeared to encode the amino-terminal portion of the osteonectin molecule. This fragment was used to rescreen the same bone cDNA Zap^R library and a second clone was obtained which was presumed to be full length based on its total insert size of 2.3 kb. This latter clone has been called Hon 2.

DNA Sequencing

Plasmid Hon 164 was restricted with Eco RI, subcloned into a M13mp18 vector[12] previously restricted with Eco RI, and sequenced by the dideoxy-chain termination method.[13] Because the longer Hon 2 contained an internal Eco RI site, double stranded plasmid was sequenced over this junction using a synthetic oligonucleotide with the sequence 5′CCTTGCCTGGACTCTGAGC 3′ devised from the sequence of the 3′ end of the first 500 bp sequenced. cDNA (mRNA) sequences were analyzed by the programs of the University of Wisconsin Genetics Computer Group (UWGCG) using the programs called FOLD and SQUIGGLES.[14]

Northern Analysis

Total RNA was isolated from: cultured human bone, gingiva, periodontal ligament and skin cells; decidua from the expanding placenta at 8–12 weeks of gestation, two separate Ewing Sarcoma Tumors[15] and from cultured fetal bovine bone, intact bovine cartilage and cultured porcine cartilage. 3.5 ug of total RNA was electrophoresed in 1.2% formaldehyde agarose gels and transferred to nitrocellulose using previously described protocols.[11] The insert cDNA from the clone Hon 164 was nick-translated using [P32]-labeled dCTP (NEN cat#013H) to a specific activity of $1 \times 10^8/\mu g$ DNA and hybridized to the filter bound RNA. Hybridization was carried out at 41°C. The filters were washed and autoradiographed as described.[11]

Southern Analysis

High molecular weight DNA was isolated from bovine and human liver and 10 ug digested with the enzymes Sst I, Xba I, Hind III, Bam HI and Eco RI. Fragmented DNA was transferred to nitrocellulose by the method of Southern[17] and hybridized to a $\simeq$500 bp DNA fragment from clone Hon 164 which had previously been labelled with [P32] by nick translation (Amersham) using [P32] alpha-dCTP. Filters were hybridized, washed and autoradiographed as previously described.[11]

RESULTS AND DISCUSSION

Sequence and Identification of the Osteonectin cDNA

Our initial isolate Hon 164 (501 bp) was sequenced in its entirety and shown to contain a long open reading frame starting at base 21 from the 5′ end of the cDNA and extending to the end of the clone (see Fig. 1) When the sequence was compared to the determined protein sequence for human placental osteonectin,[9] complete identity was observed. These cDNA-derived bone (this paper) and placenta[9] sequences differ from a previously published[29] bone protein-derived osteonectin sequence in two positions (Fig. 2). These differences most probably result from protein sequencing errors.

The complete human osteonectin protein sequence was compared to that of bovine[11] and mouse[5] and shows a high degree of conservation between species (Fig. 3). The greatest divergence was observed for the amino terminus of the mouse protein located specifically between amino acid residues 5–23 (Fig. 3). It should be noted however, that while considerable divergence exists in this region, the character of the amino acids does not change substantially with the substituted amino acids being primarily acidic in nature. Analysis of bovine genomic DNA corresponding to this sequence indicates this region is confined to a sequence hypervariable third exon.[18] The number and position of cysteines in the molecule is also highly conserved with the exception of cysteine 194 which is only present in the cow. Structurally, the molecule is divided into distinct domains. The amino-terminus is enriched in acidic amino acids and is presumed to contain the binding site for hydroxyapatite.[19] There are two cysteine-rich domains which are divided by a hydrophillic domain. Interestingly, there are two potential EF hand consensus sequences which have been shown to be involved in Ca^{+2} binding in other systems.[20] In this regard,

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                              .                       .                       .                       .                       .                       .
  1  CCA CTA AGG GTT CCC AGC ACC ATG AGG GCC TGG ATC TTC TTT CTC CTT TGC CTG GCC GGG   60
                                 Met Arg Ala Trp Ile Phe Phe Leu Leu Cys Leu Ala Gly
                                                                     10
                                ⇓
                              .                       .                       .                       .                       .                       .
 61  AGG GCC TTG GCA GCC CCT CAG CAA GAA GCC CTG CCT GAT GAG ACA GAG GTG GTG GAA GAA  120
     Arg Ala Leu Ala Ala Pro Gln Gln Glu Ala Leu Pro Asp Glu Thr Glu Val Val Glu Glu
                              20                                      30
                              .                       .                       .                       .                       .                       .
121  ACT GTG GCA GAG GTG ACT GAG GTA TCT GTG GGA GCT AAT CCT GTC CAG GTG GAA GTA GGA  180
     Thr Val Ala Glu Val Thr Glu Val Ser Val Gly Ala Asn Pro Val Gln Val Glu Val Gly
                              40                                      50
                              .                       .                       .                       .                       .                       .
181  GAA TTT GAT GAT GGT GCA GAG GAA ACC GAA GAG GAG GTG GTG GCG GAA AAT CCC TGC CAG  240
     Glu Phe Asp Asp Gly Ala Glu Glu Thr Glu Glu Glu Val Val Ala Glu Asn Pro Cys Gln
                              60                                      70
                              .                       .                       .                       .                       .                       .
241  AAC CAC CAC TGC AAA CAC GGC AAG GTG TGC GAG CTG GAT GAG AAC AAC ACC CCC ATG TGC  300
     Asn His His Cys Lys His Gly Lys Val Cys Glu Leu Asp Glu Asn Asn Thr Pro Met Cys
                              80                                      90
                              .                       .                       .                       .                       .                       .
301  GTG TGC CAG GAC CCC ACC AGC TGC CCA GCC CCC ATT GGC GAG TTT GAG AAG GTG TGC AGC  360
     Val Cys Gln Asp Pro Thr Ser Cys Pro Ala Pro Ile Gly Glu Phe Glu Lys Val Cys Ser
                             100                                     110
                              .                       .                       .                       .                       .                       .
361  AAT GAC AAC AAG ACC TTC GAC TCT TCC TGC CAC TTC TTT GCC ACA AAG TGC ACC CTG GAG  420
     Asn Asp Asn Lys Thr Phe Asp Ser Ser Cys His Phe Phe Ala Thr Lys Cys Thr Leu Glu
                             120                                     130
                              .                       .                       .                       .                       .                       .
421  GGC ACC AAG AAG GGC CAC AAG CTC CAC CTG GAC TAC ATC GGG CCT TGC AAA TAC ATC CCC  480
     Gly Thr Lys Lys Gly His Lys Leu His Leu Asp Tyr Ile Gly Pro Cys Lys Tyr Ile Pro
                             140                                     150
```

FIGURE 1 Human osteonectin cDNA Sequence and Deduced Protein Sequence. The DNA sequence is numbered on the top line and the amino acid sequence below on the bottom line. Dots above the DNA sequence are spaced every 10 bases. The amino terminus of the mature protein is indicated by an arrow and the termination codon indicated by an asterisk.

FIGURE 1 Continued

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481  CCT TGC CTG GAC TCT GAG CTG ACT GAA TTC CCC CTG CGC ATG CGG GAC TGG CTC AAG AAC  540
     Pro Cys Leu Asp Ser Glu Leu Thr Glu Phe Pro Leu Arg Met Arg Asp Trp Leu Lys Asn
                              160                                 170

541  GTC CTG GTC ACC CTG TAT GAG AGG GAT GAG GAC AAC AAC CTT CTG ACT GAG AAG CAG AAG  600
     Val Leu Val Thr Leu Tyr Glu Arg Asp Glu Asp Asn Asn Leu Leu Thr Glu Lys Gln Lys
                              180                                 190

601  CTG CGG GTG AAG AAG ATC CAT GAG AAT GAG AAG CGC CTG GAG GCA GGA GAC CAC CCC GTG  660
     Leu Arg Val Lys Lys Ile His Glu Asn Glu Lys Arg Leu Glu Ala Gly Asp His Pro Val
                              200                                 210

661  GAG CTG CTG GCC CGG GAC TTC GAG AAG AAC TAT AAC ATG TAC ATC TTC CCT GTA CAC TGG  720
     Glu Leu Leu Ala Arg Asp Phe Glu Lys Asn Tyr Asn Met Tyr Ile Phe Pro Val His Trp
                              220                                 230

721  CAG TTC GGC CAG CTG GAC CAG CAC CCC ATT GAC GGG TAC CTC TCC CAC ACC GAG CTG GCT  780
     Gln Phe Gly Gln Leu Asp Gln His Pro Ile Asp Gly Tyr Leu Ser His Thr Glu Leu Ala
                              240                                 250

781  CCA CTG CGT GCT CCC CTC ATC CCC ATG GAG CAT TGC ACC ACC CGC TTT TTC GAG ACC TGT  840
     Pro Leu Arg Ala Pro Leu Ile Pro Met Glu His Cys Thr Thr Arg Phe Phe Glu Thr Cys
                              260                                 270

841  GAC CTG GAC AAT GAC AAG TAC ATC GCC CTG GAT GAG TGG GCC GGC TGC TTC GGC ATC AAG  900
     Asp Leu Asp Asn Asp Lys Tyr Ile Ala Leu Asp Glu Trp Ala Gly Cys Phe Gly Ile Lys
                              280                                 290

901  CAG AAG GAT ATC GAC AAG GAT CTT GTG ATC TAA    933
     Gln Lys Asp Ile Asp Lys Asp Leu Val Ile  *
                              300
```

```
                                                  ↓↓
1  MRAWIFFLLCLAGRALAAPQQEALPDETEVVEETVAEVTEVSVGANPVQVEVGEFDDGAE  60

                    APQQEALPDETEVVEETVAEVTEVPTGANPVQVEVG
```

FIGURE 2 Determined and Deduced Protein Sequence of Osteonectin. Numbering starts with the start of translation. First line is the deduced sequence from bone cell cDNA data; second line is the previously determined bone osteonectin sequence of Fisher *et al.*[29] Arrows designate differences between the two sequences.

Engel *et al.*[21] have detected a major conformational transition (a 35% increase in alpha helicity) induced after cooperative Ca^{+2} binding. The precise function of osteonectin in bone can only be speculated at this point but probably involves activities which require both Ca^{+2} and hydroxyapatite binding.

Osteonectin mRNA Expression

To determine the nature of osteonectin mRNA and its distribution we used radiolabelled osteonectin cDNA to probe Northerns containing human RNA from various normal and transformed cells. There are two sizes of osteonectin message in human tissue with lengths of 2.3 and 3.0 kb (Fig. 4). The larger species of message was consistently less abundant in all tissues examined. By comparison, bovine material completely lacks a larger species. In the pig, the 2.2 kb species of osteonectin mRNA predominates, but a faint band is seen in cartilage material that is larger than 3.0 kb. By analogy to the placental osteonectin/SPARC message, the larger species of mRNA probably arises from alternative poly A+ attachment site utilization.[9]

Quantitatively, large amounts of osteonectin mRNA were observed in cultured adult bone, gingiva, periodontal ligament and fetal skin cells. These levels do not correlate to the amount of protein present in the intact animal.[2,22] These observations may in part be due to a "culture shock" response; bovine endothelial cells which normally do not make osteonectin/SPARC express the protein when placed in culture and increase this expression with prolonged and repeated passage of cells in culture.[7]

In placental tissue, osteonectin mRNA was predominant in the expanding decidua during early pregnancy (Fig. 4). This observation confirms earlier work by Wewer *et al.*,[8] who, using bovine cDNA and *in situ* hybridization, found that the expression of osteonectin in decidua was localized to an intermediate-sized layer of cells. In contrast, the mRNA was absent in precursor stromal cells as well as in more mature larger cells.[8] Thus, in this developmental system, the expression of osteonectin is tightly controlled, both temporally and spatially. One goal of our laboratory is to try to determine the basis for this regulation at the genomic level.

Osteonectin mRNA expression was also examined in Ewing sarcoma from two different patients. This tumor is the second most common tumor of bone primarily affecting children and young adults.[16] Interestingly, osteonectin mRNA was evident in both bone tumor samples but not in a neuroepithelioma, a tumor line postulated to be related to the Ewing Sarcoma. Large amounts of osteonectin mRNA have also been demonstrated in cultured osteogenic sarcoma cells by Swaroop *et al.*[9] who used a human osteonectin/SPARC cDNA isolated from placenta as hybridization probe. These and other studies indicate that osteonectin message is predominant in bone and, in certain non-mineralized tissues

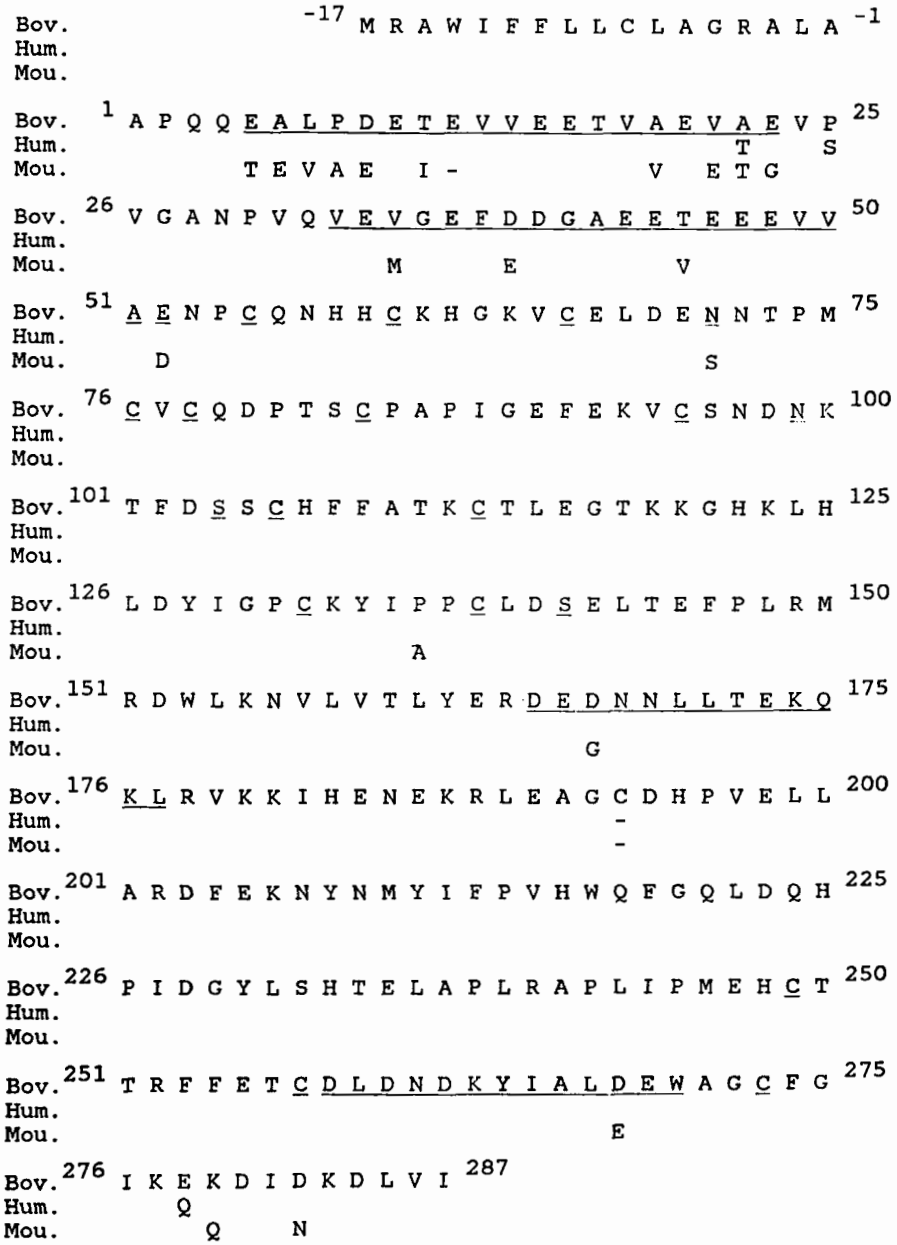

FIGURE 3 *Comparison of Osteonectin Protein Sequence in Three Species*. The deduced signal peptide is shown from amino acid −17 to −1. The first two underscored sequences show potential Ca^{+2} and/or hydroxyapatite binding domains. Each cysteine in the mature protein is underscored as well as potential N-glycosylation sites. The serine underscored in position 141 has been shown to be a site of phosphorylation and the underscored amino acids at positions 166–178 and 259–271 are sequences homologous to the Ca^{+2} binding EF hand consensus sequences. Only divergent amino acids are shown for the human and mouse sequences. Dashes have been inserted for optimal alignment.

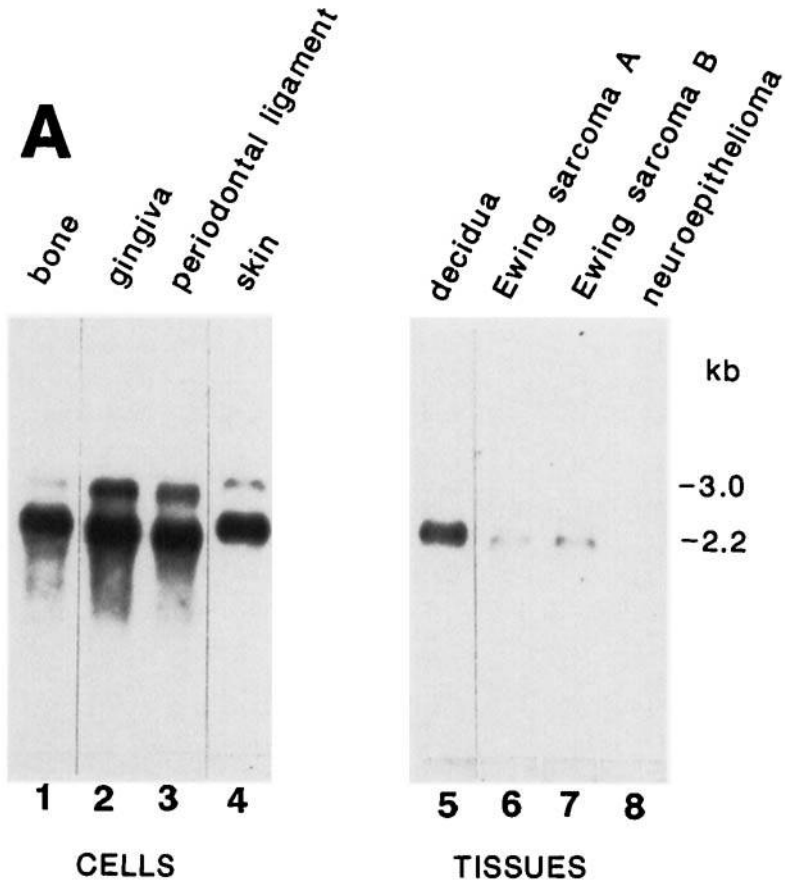

FIGURE 4 *Northern Analysis*. Ten micrograms of total RNA was analyzed by northern blotting as described in Materials and Methods. A: Osteonectin mRNA distribution in cultured cells and tissues. Lane 1, RNA from cultured adult human bone cells[10]; lane 2 and 3, gingival and periodontal ligament fibroblasts from a single patient; lane 4, RNA from the human fetal skin cell line 1106; lane 5 is RNA from human placental decudua isolated between 8 and 12 weeks of gestation[8]; lane 6 and 7 show the individual RNA's from a Ewing sarcoma tumor isolated from two separate patients and lane 8 shows RNA extracted from a human neuroepithelioma tumor. B: Osteonectin message sizes in three different species. Lane 1 is RNA from cultured adult bone cells; lane 2, RNA from cultured bovine bone cells; lane 3 RNA from adult bovine articular cartilage, and lane 4, RNA from adult porcine laryngeal cartilage that had been cultured for 2–3 days prior to RNA extraction.

during early development,[23,24] in neoplasia[8,9,11] and in certain specialized tissues like the expanding decidua of the placenta.[8] Lacking in this analysis, however, are complementary, comprehensive osteonectin protein localization data. Osteonectin protein localization has recently been analyzed in selected human skeletal and non-skeletal tissues.[3,4] In these studies, monospecific antibodies to human osteonectin were used to show distinct localization of osteonectin in osteoblast progenitor cells, active osteoblasts, young osteocytes and in the osteoid seam of new lamellar bone. High levels of osteonectin were also observed in "walled in" osteocytes of woven bone trabeculae. Little expression however was observed in quiescent osteocytes and inactive bone lining cells. Osteonectin protein has also been detected in platelets where it has been shown to be secreted during acti-

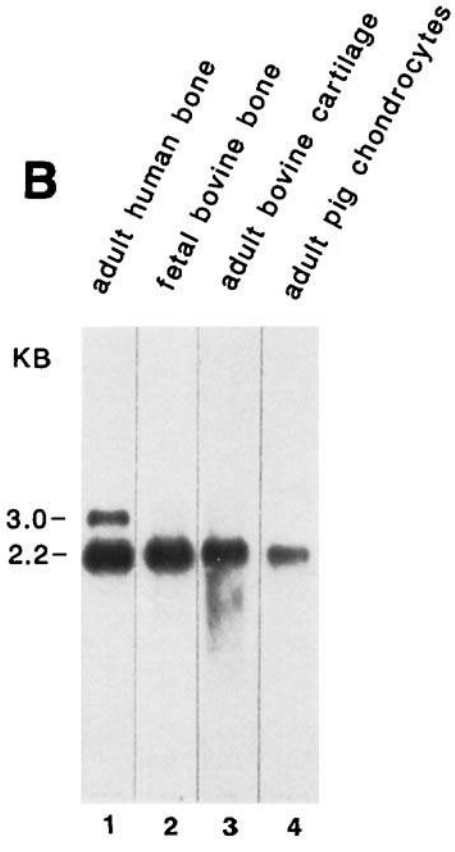

FIGURE 4B

vation.[30] It is not clear whether the function(s) of osteonectin is (are) the same in mineralizing and non-mineralizing tissues. Considering the observation that osteonectin undergoes changes in conformation upon binding Ca^{+2} ions[21] it is conceivable that the microenvironment of the protein may be crucial to its activities. Thus, osteonectin in bone may take on additional and distinct roles from that in other tissues.

Osteonectin Message Structure

To understand the possible basis for the translational regulation of osteonectin expression we studied its potential mRNA secondary structure using a computer program designed to predict RNA folding patterns. The predicted structures are based on minimum free energy and published values for stacking and destabilizing energies.[14] The analysis program, called FOLD is the most efficient and accurate method available to predict RNA conformation. In our analysis, we paid close attention to the 5′ end of the message (corresponding to the amino terminus of the protein) because of its alleged role in regulating translation.[26] Figure 5 shows a plot of a fold analysis for the first 100 bases of the osteonectin mRNA. The sequences of the bone CDNA were identical to the placental cDNA in this sequence. The data was transferred to a second program called SQUIGGLES[25]

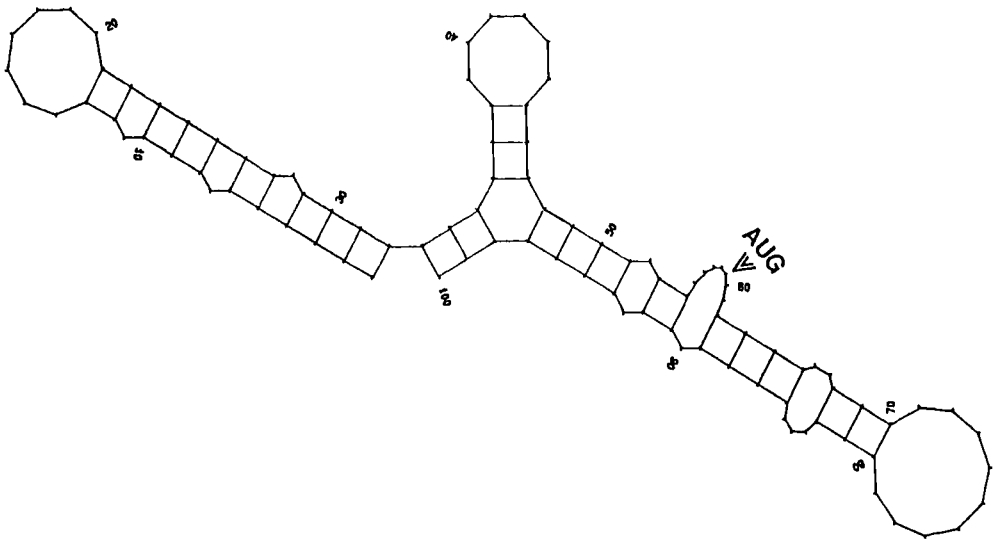

FIGURE 5 Predicted Conformation of Human Osteonectin mRNA. The first 100 bases of the human osteonectin mRNA were analyzed by the computer program FOLD (25). This optimum folded structure is depicted graphically using the program SQUIGGLES (25). Bases are numbered in 10 unit increments and a bulge loop is indicated at positions 54–62 showing its relationship to the start codon AUG. Extended sequences (−28 to −59) were derived from the human osteonectin/SPARC sequence of Swaroop *et al.*[9]

which converts the analysis files to graphic representation. Although this prediction clearly has limitations [for example, the structure reported is one of a family of structures with the same or nearly the same energy[25]] we detected a "bulge loop"[14] at bases 54–62, a sequence which contains the signal for the start of translation (AUG). It is interesting to speculate that this "bulge loop" at the 5′ end of the osteonectin message facilitates access to the ribosome or to associated protein elements to enhance osteonectin translation.

The Human Osteonectin Genome

To determine the nature of the osteonectin gene in the human genome we fractionated human genomic DNA by restriction enzyme digestion and analyzed it by Southern blotting. Human genomic DNA was fragmented by several infrequent cutting (6 base recognition site) restriction endonucleases and probed with a 501 bp cDNA fragment that encoded the first amino terminal half of the osteonectin protein. A simple pattern of hybridization was noted (see Fig. 6). In the bovine, the pattern is similarly simple but different in the length of most of the hybridizing fragments. These results indicate that while the bovine and human genes show clear species variation, both patterns of hybridization imply the presence of one copy of the osteonectin gene per haploid genome. These results confirm and extend those of Swaroop *et al.*[9] who have localized the osteonectin gene to chromosome 5q31–5q33. It is interesting to note that using the same 501 bp fragment of osteonectin cDNA described above, Schwartz *et al.*[27] detected two restriction fragment length polymorphisms (RFLP's) in the osteonectin gene. Hinc II and Taq I polymorphisms were observed in 18 and 26 unrelated individuals respectively. For each polymorphism,

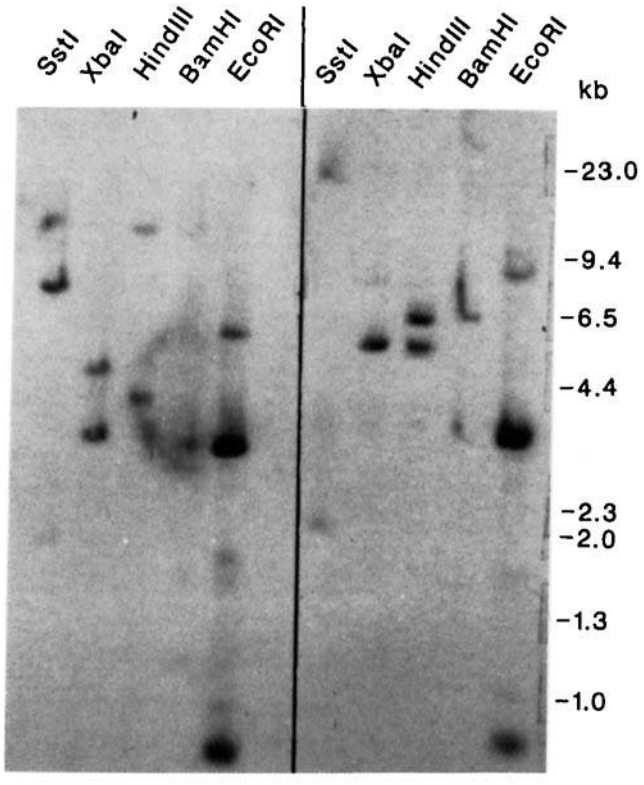

FIGURE 6 *Southern Analysis.* Approximately 10 μg of high molecular weight DNA isolated from bovine and human liver was digested with various restriction endonucleases and electrophoresed in 0.8% agarose gels. Separated DNA fragments were transferred to nitrocellulose and hybridized to a [p32] labeled 501 bp fragment of osteonectin cDNA as described in Materials and Methods.

in 2 kindreds of 8 the mode of inheritance was autosomal dominance. This study provides an important basis for the investigation of inherited diseases in which osteonectin expression is variable, e.g., Osteogenesis Imperfecta.[28,31]

CONCLUSIONS

Osteonectin/SPARC occurs as a single copy gene per haploid human genome. Its cDNA is identical in both bone (this paper) and placental[9] tissue. The protein is found at a very

high level in bone and is present in bone-related tumors. In non-bone tissue, osteonectin expression appears consistent with remodeling or growth-related processes. At present the physiological role(s) of osteonectin remain unknown, but appear related to its abilities to bind both Ca^{+2} and hydroxyapatite with high affinity. We speculate that the protein has both cell and matrix related roles in the tissues in which its expression is most highly regulated.

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