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Osteonectin mRNA: distribution in normal and transformed cells

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ABSTRACT

Overlapping cDNA clones encoding bovine osteonectin were isolated from a λgt11 expression library constructed from bovine bone cell mRNA. The longest clone, λOn 17 (insert size 2.0 kb) was studied in detail. The clone was shown to encode osteonectin by hybrid select translation experiments and by DNA sequence analysis. Northern analysis of bone cell RNA showed the length of the osteonectin mRNA to be 2.0 kb. Osteonectin message was found in bone but not in soft tissue (liver and brain) preparations consistent with the distribution of the protein in these tissues. On the other hand, osteonectin message was observed in tendon, a tissue in which little or no osteonectin protein is found in vivo. Hybridization of osteonectin cDNA was detected in cells from a number of species including human, rat, mouse and chick. The level of osteonectin mRNA was drastically decreased in chick embryo fibroblasts transformed by Rous sarcoma virus.

INTRODUCTION

Osteonectin is a major non-collagenous protein of bone. It is a phosphorylated glycoprotein with an apparent molecular weight of 38,000 on SDS gels (1). While Type I collagen is the major protein component of the bone matrix (>90% of protein content), it is widely distributed in non-mineralizing connective tissues such as skin, vasculature and tendon (2). Osteonectin, on the other hand, is localized predominantly to the osteogenic compartments of bone (3) and is an avid binding protein for calcium, hydroxyapatite and collagen (3,4). It also promotes the deposition of calcium phosphate mineral onto type I collagen in vitro (3). Thus it has been proposed that osteonectin may play a role in structural bone mineralization in vivo (3).

The regulation of osteonectin expression appears to be complex. Cell free translation of osteonectin mRNA produces a protein approximately 6,000-9,000 M_r larger than that extracted from intact tissue (5,6) suggesting that the secreted protein is extensively modified. In addition, despite observations that this protein is unique to bone tissue,

fibroblasts (e.g., from porcine periodontal ligament) produce detectable levels of newly synthesized osteonectin when cultured in vitro (7). Thus, it is possible that osteonectin production is elicited in these cells only when cultured in vitro or that osteonectin is synthesized in vivo in non-mineralizing tissues but is then rapidly cleared by proteolytic degradation.

In order to elucidate some of the apparent complexities of osteonectin gene expression, we have constructed osteonectin cDNA clones. Our experimental approach was to construct a cDNA library using bovine bone cell mRNA as template, and to insert the cDNA into the expression vector, λgt11. Recombinant clones were screened using specific polyclonal antisera directed against bovine osteonectin. In this report we describe the isolation and identification of a cDNA derived from a bovine osteonectin mRNA. The osteonectin cDNA has been used to demonstrate osteonectin mRNA variance in fetal tissues, different species and with viral transformation.

MATERIALS AND METHODS

Bone Cell Culture

Cultures of bovine bone cells (8) were obtained by isolating sub-periosteal scrapings from long bones of bovine fetuses (3 to 5 months gestational age). The scrapings were minced into small fragments (approximately 1 x 1mm) by placing them in a sterile glass conical tube (Pearce Reactivial) and cutting the slices with spring-handled straight microdissecting scissors. The minced bone pieces were washed extensively with nutrient medium containing no serum to remove blood components. The minced subperiosteal bone was then placed in 150mm petri dishes (Falcon) and fed with nutrient medium ("complete" medium) containing Dulbecco's Modified Eagle's medium (Biofluids), 10% heat-inactivated fetal bovine serum (Gibco), 2 mM glutamine (Gibco), 100 units/ml penicillin and 100 µg/ml of streptomycin sulfate (Gibco), 2.5 mM β-glycerol phosphate, and 25 µg/ml of ascorbic acid. Cells grew from the bone fragments and reached confluency in approximately three weeks.

Poly(A)+ RNA Isolation and cDNA Synthesis

Total cellular RNA was extracted from bovine bone cells using a guanidine-HCl extraction (9). Poly(A)+ RNA was isolated by affinity chromatography using oligo (dT)-cellulose (Type III, Collaborative Research). For cDNA construction, approximately 10 µg of poly (A)+ RNA was

used to construct cDNA modified at its termini to contain Eco R1 linkers (PL Biochemical) (10,11). Approximately 3.0 µg of Eco R1 digested λgt11 were added to the cDNA and ligated by incubation in 25 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.2 mM spermidine, 0.5 mM ATP and 5 units T_4 DNA ligase (NEB) for 48h at 12°C. When recombinant cDNA was packaged into phage particles in vitro (Amersham) approximately 20 x 10^6 phage particles were obtained. About 30% of the phage contained cDNA insert.

Screening of the Bovine cDNA Library

The bone cell cDNA library was screened by antibody recognition of an osteonectin-β-galactosidase fusion protein, as described by Young and Davis (12,13) with the following modifications. E. coli Y1090 cells infected with recombinant λgt11 phage were plated at a density of 20,000 plaques/plate on twenty 150 mm LB plates and incubated at 42°C for 5 h. Nitrocellulose filters, previously soaked with 20 mM IPTG and air dried, were placed on the plates and incubated for 16 hours at 37°C. Filters were removed and blocked for one h in a TBS solution (20 mM Tris-HCl pH 7.5, 500 mM NaCl) containing 3% BSA. Rabbit polyclonal (3) or mouse monoclonal anti-osteonectin antibodies were then added at a 1:2000 dilution and incubated at 25°C for 16 h. Filters were then washed with TBS, and incubated with 1:1000 dilution of HRP-conjugated goat anti-rabbit or sheep anti-mouse antibody (BioRad) for 2 h. Filters were again washed with TBS and developed with 4-chloro-1-naphthol(20). Positive plaques were identified, amplified, and rescreened to purity as described by Benton and Davis (15). To estimate the amount of osteonectin-β-galactosidase fusion protein produced by recombinants, known quantities of osteonectin were dotted on nitrocellulose filter paper and developed with the test filters.

Northern Analysis

Total RNA was extracted from cultured bovine bone cells (8) intact fetal bovine brain, liver and tendon, rat Swarm chondrosarcoma (21), rat osteosarcoma 17/2 cells (22) and cultured mouse, human, and chick fibroblasts (9). A 3.5 µg aliquot of total RNA was electrophoresed in 1.2% formaldehyde agarose gels and then transferred to nitrocellulose (11). Eco R1-restriction fragments were then labeled by nick translation (Amersham) with [^{32}P] deoxynucleotides to a specific activity of 10^8 cpm/µg and hybridized to filter-bound RNA. Hybridization was carried out at 41°C in a mixture of 40% formamide (Fluka), 0.1g/ml dextran sulphate, 5X SSC(75 mM sodium citrate, 750 mM sodium chloride), 10 mM Tris-HCl (pH 7.5), 4X

Denhart's solution (14) and 0.1 mg/ml denatured salmon sperm DNA. The filters were washed and autoradiographed as described (18).

Cell Free Translation and Immunoprecipitation

Cell free translation in a rabbit reticulocyte lysate system (nuclease treated, NEN,) was carried out in the presence of [^{35}S] methionine (∿1000Ci/mmol, NEN) at 37° for 1 h. Translation was terminated by the addition of a five fold excess of 125 mM Tris-HCl pH 6.8, 5.0% SDS, 20% glycerol, 0.1 mM β-mercaptoethanol and 0.02% bromophenol blue (gel sample buffer) or 0.1 M Tris-HCl (pH 7.2), 0.15 M NaCl, 1% triton-X-100, 1% deoxycholate, 0.1% SDS and 1.0% trasylol for immunoprecipitation. The latter mix was centrifuged at 15,000g to remove debris. Polyclonal rabbit antiserum against bovine osteonectin was coupled to protein A Sepharose (Boehringer Mannheim) and incubated with the supernatant at 4°C for 18 h with constant agitation. Antigen-antibody coupled beads were rinsed three times in a solution of cold PBS (pH 7.4), 0.5% Triton X-100 and 0.1% BSA. Antigen and antibody were released from beads by boiling in gel sample buffer. Protein bands were detected by fluorography at -70°C for 1-7 days.

Hybrid Selected Translation

A sterile one-hole punch was used to obtain discs from a sheet of ABM Transa-bind filter paper (Schleicher & Schuell). These filters were activated in a glass test tube (Corex) containing a freshly prepared solution of 12N HCl and 10 mg/ml $NaNO_2$ for 30 min at 4°C with occasional agitation. The filters were then rinsed three times in cold sterile distilled water and twice in 0.2 M sodium acetate (pH 4.0) at room temperature. cDNA insert from clone λOn 17 (see Results) was strand separated by boiling for 90 seconds, frozen at -70°C and thawed on ice for 2.5 h. An 800 ng aliquot was spotted onto the activated filter previously placed in an Eppendorf tube (1.5ml volume). The filters were allowed to air dry for 2 h, then, tubes sealed, further drying was accomplished at room temperature overnight. The filters were then washed three times in sterile distilled water and cut into 1 mm squares on a sheet of parafilm and placed into fresh 1.5 ml Eppendorf tubes. They were again washed three times in 0.4 M NaOH for ten min at 37°C followed by three washes in sterile distilled water. The filters were pre-hybridized for one h at 42°C in solution A containing 60% formamide, 10 mM PIPES (pH 6.4), 0.4M NaCl and 20 mg/ml yeast tRNA (Boehringer Mannheim). Filters were mock eluted by the addition of 99 µl of formamide and 1 µl of 1 M Tris (pH 7.5) followed by incubation for one h at 65°C. They were then washed in 1 mM EDTA for two

min. After removal of supernatant, filters were again prehybridized for 30 min at 42°C in solution A. Finally, 75 µl of an mRNA preparation (0.25 µg/ml total RNA, 60% formamide, 10 mM PIPES and 0.4 M NaCl) was added and allowed to hybridize at 42°C overnight. The hybridization solution was removed and the filters washed ten times in 0.5xSSC, 0.5% SDS at 60°C and once with 2 mM EDTA (pH 8.0) at room temperature. Specifically hybridized mRNA was eluted by boiling for 90 sec in 1 mM EDTA (pH 7.5) and immediately placed at -70°C. After freezing, the filters were thawed on ice. The supernatant containing the hybrid selected mRNA was transferred to a fresh Eppendorf tube containing carrier calf liver tRNA (Boehringer Mannheim) and ethanol precipitated at -70°C. The precipitate was lyophilized and resuspended in 2.2 µl of sterile distilled water, translated in a cell free system and immunoprecipitated as described above.

DNA Sequencing and Gene Copy Number Analysis

The cDNA from clone λOn 17 was sequenced according to the method of Sanger (28). For gene copy analysis, 10 µg of bovine liver genomic DNA was digested to exhaustion by addition of excess restriction enzymes and separated by electrophoresis in 0.8% agarose gels in E buffer (29). DNA from an osteonectin genomic clone was obtained by screening a library of bovine genomic fragments (gift from D. Capon, Genentech). One genomic clone, called λOg 6 (containing ∿90% of the osteonectin coding region) was digested with the same restriction enzymes and analyzed in parallel with the total genomic DNA by Southern blot. Filters were hybridized with a [^{32}P] labeled 0.3 kb insert from λOn 17 (see fig. 2), washed to remove unbound probe and exposed to x-ray film for autoradiography.

RESULTS

A bovine bone cell cDNA library was screened with a polyclonal antibody against bovine osteonectin. Approximately 20-30% of the 400,000 phage screened contained insert DNA. After several rounds of purification, 20 clones remained strongly positive. By dividing the number of positive clones by the total number of recombinants screened and then correcting for the probability of the cDNA to be inserted in the correct reading frame (1/6 times), it was estimated that at least 0.1% of the total bone cell mRNA encoded osteonectin.

Figure 1 shows the immunostaining pattern of one of the positive clones, λOn 17, using both polyclonal and monoclonal antiosteonectin antiserum. Immunostaining known quantities of osteonectin showed this

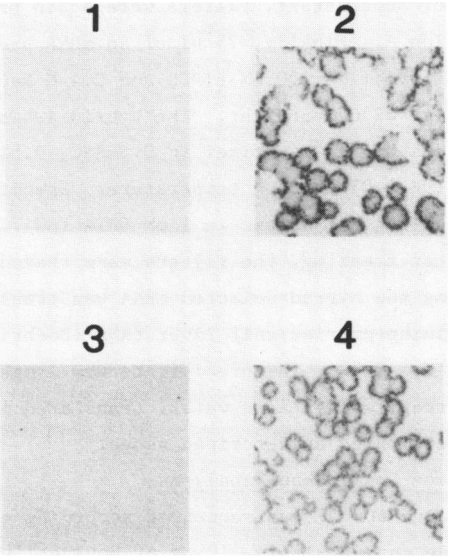

Fig. 1. Immunological screening of an osteonectin recombinant. Plaque purified wild type λgtll phage (panels 1 and 3) and recombinent λ On 17 phage (panels 2 and 4) were allowed to infect Y1090 cells at comparable titer and then transferred to nitrocellulose filters. Filters were incubated first with either polyclonal (top panels 1 and 2) or monoclonal (bottom panels 3 and 4) anti-osteonectin antiserum and then exposed to second antibody conjugated to HRP (19).

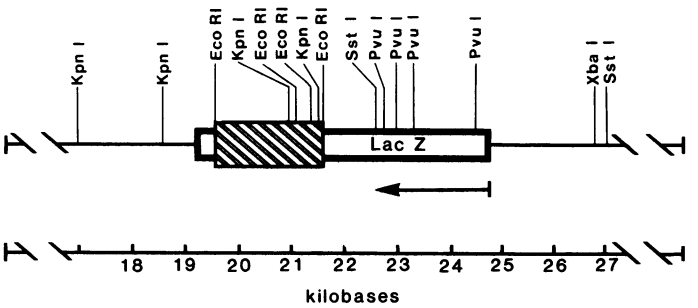

Fig. 2. Restriction map of λOn 17 constructed from a series of single and double enzyme digests of the recombinant clone. The numbers below the map refer to kilobases from the end of the long arm of the phage. The hatched line delineates the region encoding osteonectin and the open bar indicates the regions encoding the β-galactosidase gene. The arrow shows the direction of transcription of the β-galactosidase-osteonectin mRNA.

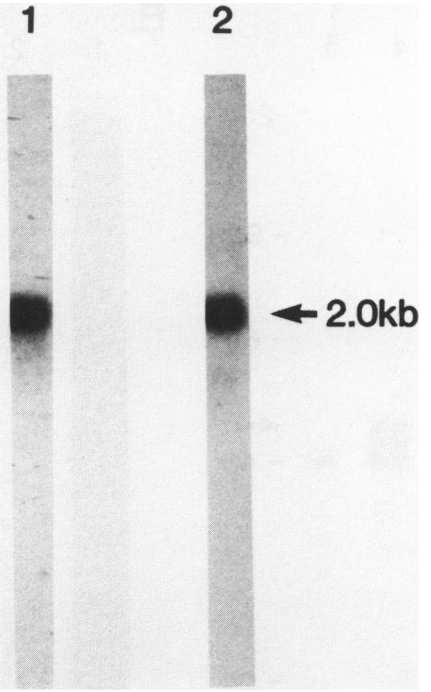

Fig. 3. Northern hybridization. Total RNA was extracted from cultured bovine bone cells as described in Methods. RNA was electrophoresed in 1.2% agarose gels and transferred to nitrocellulose. The filter was hybridized to ^{32}P-labeled 1.5 kb (lane 1) and 0.3 kb (lane 2) inserts from λOn 17 (see Fig. 2). The length of hybridized RNA species was determined by comparison with known sizes of λ DNA digested with Hind III.

detection system sufficiently sensitive to detect as little as 1.0 nanogram of antigen. The positive clones were estimated to produce 2.5-5 ng of recombinant osteonectin (data not shown).

After amplification and purification of the phage, four representative positive clones were analyzed by Eco Rl digestion. Analysis by acrylamide gel electrophoresis as well as by Southern blotting indicated that the clones shared extensive overlap and ranged in total insert size from 1.5 to 2.0kb in length (data not shown). Only the largest clone, λOn 17 was analyzed further. A restriction map, constructed by single and double restriction enzyme digestion of the recombinant phage DNA, is shown in Fig. 2.

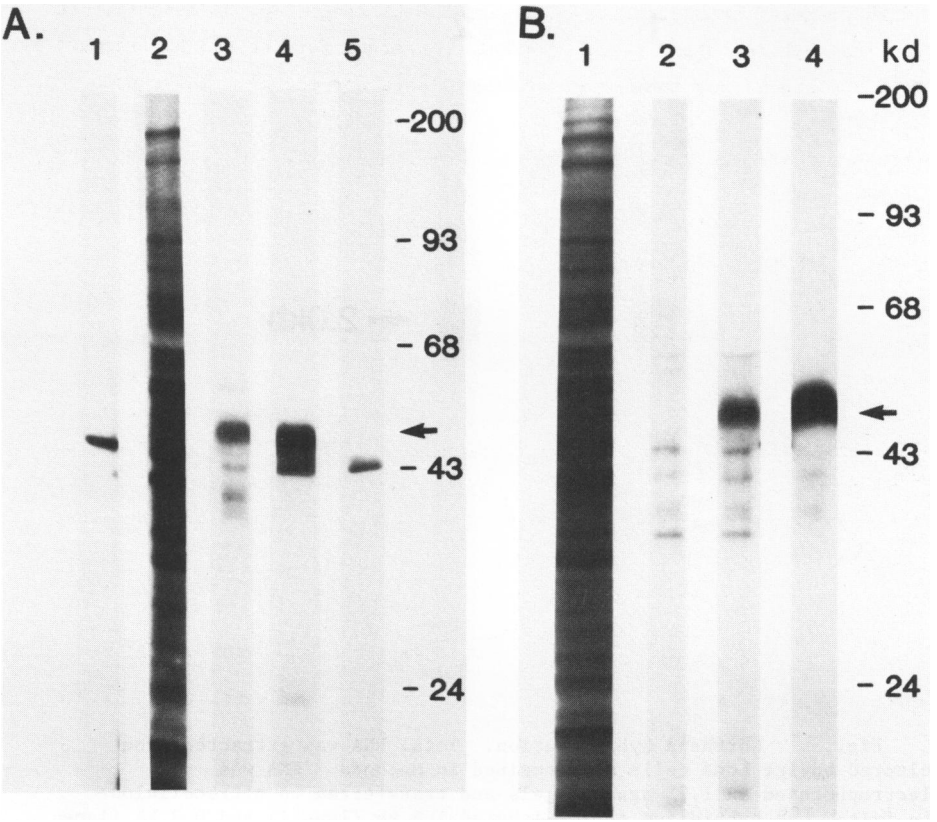

Fig. 4. Hybrid selected translation. A 1.5 kb Eco RI fragment from λOn 17 was immobilized on diazobenzyloxymethylcellulose paper and hybridized to total RNA from bone cells. Hybrid selected mRNA was thermally eluted, translated in a rabbit reticulocyte lysate and electrophoresed in 10% SDS-polyacrylamide gels. A) Total translation products: in the absence of RNA (lane 1); from total bone cell RNA (lane 2); from total bone cell RNA after immunoprecipitation with antiosteonectin antiserum (lane 3); from mRNA hybrid-selected with clone λOn 17 (lane 4); and from hybrid-selected nitrocellulose paper alone (lane 5). B) Total translation of bone cell mRNA: prior to immunoprecipitation (lane 1); after immunoprecipitation with non-immune rabbit serum (lane 2); after immunoprecipitation with anti-osteonectin antiserum (lane 3); after initial hybrid selection with λOn 17 followed by immunoprecipitation with anti-osteonectin antiserum (lane 4). Arrow points to migration of in vitro translated osteonectin.

Size Analysis of Osteonectin mRNA

To determine the size of mRNA coding for osteonectin, the three insert Eco Rl fragments (see Fig. 2) were radiolabeled with [α-^{32}P] and used as probes for hybridization to total RNA from cultured bovine bone cells. The

1.5 kb fragment hybridized to a message 2.0 kb long (Fig, 3, lane 1). An identical hybridization pattern was also observed for the 0.3 kb fragment (Fig. 3, lane 2) and the 0.2 kb fragment (data not shown). These data suggest that all three fragments contain sequences complementary to the osteonectin message and that the longest recombinant (λOn 17, insert size 2.0 kb) contains what may be a full length reverse transcript of osteonectin mRNA.

Osteonectin cDNA Identification

In order to determine the identity of the cDNA a hybrid selected translation experiment was performed. Total RNA from bovine bone cells was hybridized to the 1.5 kb fragment of λOn 17 which had previously been immobilized on DBM paper. Specifically bound mRNA was then thermally eluted and translated in a rabbit reticulocyte lysate in the presence of [^{35}S] methionine. Fig. 4 demonstrates that after SDS-polyacrylamide gel electrophoresis, the selected translation product is easily distinguished from the endogenous protein synthesized by the lysate system (Fig. 4a, lanes 1,4,5). The mobility of this selected protein is the same as the mobility of osteonectin immunoprecipitated by anti-osteonectin antiserum from cell free translation products of total bone cell RNA (Fig. 4a, lanes 3,4). Fig. 4b, lane 4, illustrates that this selected translation product was immunoprecipitated by anti-osteonectin antibodies.

Recombinant λOn 17 DNA was partially sequenced and the predicted amino acid sequence compared to that previously reported for the N terminus of the native adult bovine osteonectin protein (4). In addition, we compared this derived sequence to the N terminal amino acid sequence of dissociatively extracted, fetal bovine (1,3) osteonectin. Fig. 5 shows that complete agreement was obtained for all described amino acids in the

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
cDNA-derived osteonectin	Ala	Pro	Gln	Gln	Glu	Ala	Leu	Pro	Asp	Glu	Thr	Glu	Val	Val	Glu	Glu	Thr	Val	Ala
	GCC	CCT	CAA	CAG	GAA	GCC	TTG	CCT	GAT	GAG	ACA	GAA	GTG	GTG	GAA	GAA	ACC	GTG	GCC
Fetal bovine osteonectin	Ala	Pro	Gln	X	Glu	Ala	Leu	Pro	Asp	Glu	X	Glu	Val	Val	Glu	Glu	X	Val	Ala
Adult bovine osteonectin	X	Pro	Glx	X	Glu	Ala	Leu	Pro	Asp	Glx	Glu	Glx	Val	Glu	Glu	Glu	Leu	Val	Ala

Fig. 5. DNA sequence of the first 19 residues of the NH_2-terminus of bovine osteonectin. A 0.2 kb Eco RI DNA from λOn 17 was subcloned into M 13 mp 19 RF (BRL) and sequenced by the method of Sanger (28). The derived amino acid sequence of osteonectin is shown above the nucleotide sequence. The corresponding observed amino acid sequences for the adult and fetal forms of bovine osteonectin are shown below.

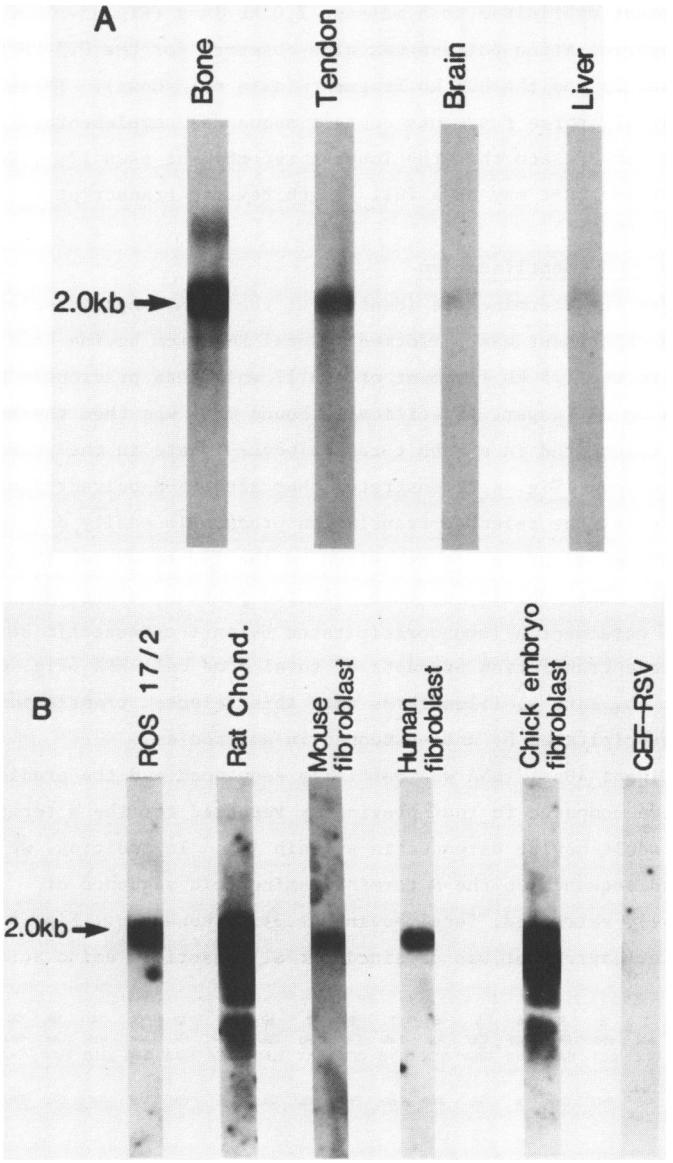

Fig. 6. Northern hybridization. Total RNA was extracted from a variety of cells and tissues and 3.5 µg aliquots electrophoresed in 1.2% agarose gels and transferred to nitrocellulose. The filters were hybridized simultaneously to the 1.5 kb insert from λOn 17 previously labeled with ^{32}P. Approximately 1.5 x 10^7 cpm were used for hybridization. Filters were washed and autoradiographed. The autoradiogram in panel A was exposed for 2 h while that for panel B was exposed for 24 h.

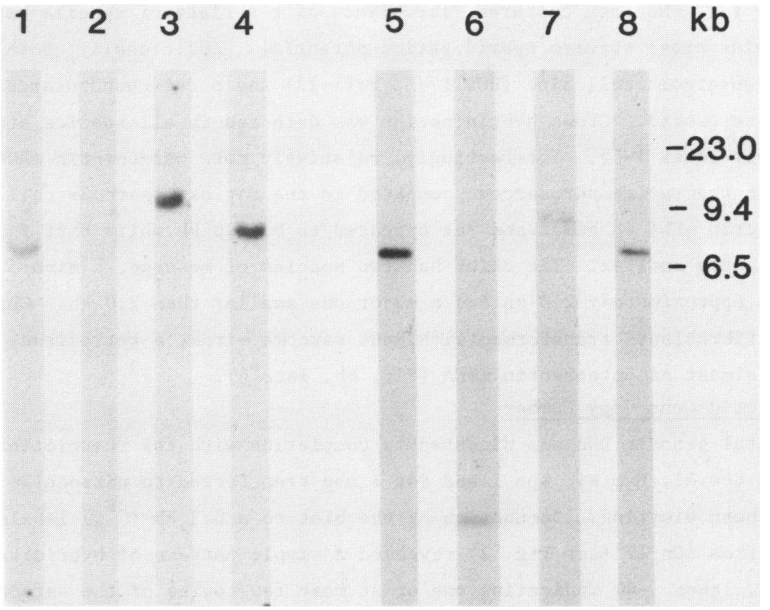

Fig. 7. Osteonectin gene copy analysis. Approximately 10 µg of genomic DNA was digested with Eco Rl (lane 1), Bam HI (lane 2), Kpn I (lane 3) and Sst I (lane 4) and electrophoresed in 0.8% agarose gels. Approximately 500 ng of purified phage DNA from λOg 6 (see methods) was digested with Eco Rl (lane 5), Bam HI (lane 6), Kpn I (lane 7) and Sst I (lane 8), electrophoresed in parallel with the total genomic DNA and transferred to nitrocellulose by Southern blotting. Filter bound DNA was hybridized to a 0.3 kb fragment of λOn 17 previously labeled with P^{32} and autoradiographed.

first 10 and in 6 of 9 of the remaining residues in this sequence. Of the three residues which showed discrepancy, the two observed amino acid sequences were different from each other and for one of these, our cDNA-derived sequence (from a fetal bone cell library) agreed with the fetal rather than the adult protein sequence (Fig. 5).

Tissue Specificity, Cross Species Hybridization and Transformation Sensitivity of Osteonectin mRNA

RNA from a variety of bovine tissues was extracted and analyzed by Northern blot. Substantial hybridization of radiolabeled osteonectin cDNA to osteonectin message was observed in bone cells (Fig. 6a, lane 1) compared to liver or brain tissue (Fig. 6a, lane 3,4). An intermediate level of osteonectin mRNA was detected in tendon (Fig. 6a, lane 2). Because osteonectin has been shown to be synthesized by fibroblasts in

culture (7), RNA from cultured fibroblasts of a variety of species was used to examine cross species hybridization potential. Additionally, both the rat osteosarcoma cell line (ROS 17/2, ref. 22) and a rat chondrosarcoma (21) were tested. Cross hybridization was detected in all species studied (Fig. 6b, lanes 1-5). Interestingly, relatively more osteonectin mRNA was found in the rat chondrosarcoma compared to the rat osteosarcoma cell line. Osteonectin mRNA in human and rat appeared to be 2.0 kb while that in mouse was slightly smaller. The chick had two species of message, a minor species approximately 2.0 kb and a major one smaller than 2.0 kb. Chick embryo fibroblasts transformed with Rous sarcoma virus, a retrovirus, showed almost no osteonectin mRNA (Fig. 6b, lane 6).

Osteonectin Gene Copy Number

Total genomic DNA was digested to completion with the restriction enzymes Eco Rl, Bam HI, Kpn I and Sst I and transferred to nitrocellulose by Southern blotting. Incubation of the blot to a 0.3 kb (^{32}P) labeled insert from λOn 17 (see Fig. 2) revealed a simple pattern of hybridization (Fig. 7, lanes 1-4) indicating one or at most few copies of the osteonectin gene per haploid genome. To substantiate the copy number of the osteonectin gene, a purified clone encoding the gene was isolated and analyzed by restriction digest as described above. The hybridization pattern of the genomic isolate was virtually identical to the total genomic blot (Fig. 7, lanes 5-8) suggesting there is one copy of the osteonectin gene within the bovine genome.

DISCUSSION

A cDNA library was constructed from bovine bone cell mRNA and screened using a polyclonal antiserum known to recognize the unmodified cell free translation product of osteonectin. Osteonectin cDNA clones were characterized by restriction map and northern analysis and definitively identified by hybrid selected translation and DNA sequence analysis. Northern analysis using RNA from bovine bone cells prepared both as described above and as collagenase-digested explant cultures (19) indicated the size of the osteonectin message to be approximately 2.0 kb.

Osteonectin is enriched in bone (3), but known to be synthesized by some non-bone cells in culture (7). Thus, it was of interest to examine the localization of osteonectin mRNA in the various tissues studied. Northern analysis of RNA from fetal bovine brain and liver showed no appreciable levels of osteonectin mRNA, consistent with the distribution of

osteonectin in these tissues. It should be noted however that with prolonged exposure of the x-ray film, relatively small but clearly detectable levels of hybridization were detected in brain and liver. Because osteonectin message has been detected in various fibroblast preparations, we believe the minor amount of hybridization with these tissues may not reflect constitutive synthesis of osteonectin mRNA by all liver or brain cells, but more likely arose from fibroblastic cells in our total tissue preparations.

Compared to liver and brain, fetal tendon showed substantial hybridization despite observations that the protein is not detected in this tissue (3). However, preliminary pulse label experiments using intact tendon tissue indicate that some osteonectin is synthesized by the tissue (unpublished observations). Thus, in tendon, regulation of osteonectin expression probably occurs post-translationally, possibly at the level of processing or degradation of the protein. Similar phenomena may well occur in fibroblasts derived from other tissues which do not accumulate osteonectin *in vivo*, but when cultured *in vitro* synthesize substantial quantities (7) of the protein.

Osteonectin message was detected by our bovine probe in all species examined, suggesting that the structure of this protein is highly conserved. The size of osteonectin message was identical in calf, rat and human but smaller in mouse and chick. Two sizes of message were observed in chick fibroblasts. Because gene copy experiments indicate there is one copy of the osteonectin gene per haploid genome, it is probable that this heterogeneity may arise from utilization of multiple polyadenylation sites as demonstrated for another structural bone gene, the α2 (I) collagen gene (23,30).

Comparative analysis of RNA from a rat osteosarcoma cell line (ROS 17/2) and rat chondrosarcoma showed more osteonectin mRNA in the cartilage tumor. This anomaly may be related to the fact that these RNAs were derived from transformed cells. In both fibroblasts (24,26,27) and chondrocytes (25,31,32), the expression of extracellular matrix proteins is known to be sensitive to transformation. Because of the aforementioned anomalous results with the transformed ROS/17.2 and chondrosarcoma cells and because osteonectin is a predominant matrix protein, we decided to determine whether the expression of osteonectin was directly sensitive to transformation. Osteonectin mRNA levels were not detected in chick embryo fibroblasts infected with the Rous sarcoma virus. This dramatic decrease

upon transformation (Figure 6b) is similar to that previously reported for fibronectin (26) and collagen (27) in this cell. The mechanism of this decrease is not known but likely arises from expression of the v-src gene as previously demonstrated for fibronectin and type I collagen (27).

The abbreviations used are: kb, kilobases; bp, base pairs; PIPES, Piperazine-N, N'-bis[2-ethanesulfonic acid]; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; BSA, bovine serum albumin; HRP, horse radish peroxidase; LB, Luria broth; IPTG, isopropyl-β-D-thiogalactopyranoside.

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