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MURINE OSTEOGENIC PROTEIN (OP-1): HIGH LEVELS OF mRNA IN KIDNEY

Engin Özkaynak, Patrick N. J. Schnegelsberg and Hermann Oppermann

Creative BioMolecules, Inc., 35 South Street, Hopkinton, Massachusetts 01748

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SUMMARY: The murine OP-1 gene (EMBL accession No. X56906) encoding the homolog of human osteogenic protein-1 was isolated from cDNA and genomic libraries using human OP-1 cDNA as probe. The deduced murine OP-1 amino acid sequence revealed 11 amino acids changes, three of them in the mature protein. Murine OP-1 probes were used for analysis of OP-1 mRNA in mouse embryo and organ tissues. Northern blot hybridization revealed multiple mRNA species: the major species of 2.2 kb, minor species of 1.8 and 2.4 kb and a large 4 kb species, which may represent alternative splices. Tissue specific expression was studied in brain, lung, heart, liver, spleen, kidney, adrenal and bladder tissue. Maximal levels of OP-1 mRNA were found in kidney which may explain the phenomenon of epithelial osteogenesis, first described by Huggins in 1931 using epithelium from the urinary tract. Moreover, our data suggest that kidneys may be the main site of OP-1 synthesis, even though it is distant from its physiological site of action, skeletal bone.

Members of the TGF-ß superfamily play important roles in regulating differentiation during development. For example, the Drosophila decapentaplegic gene product (DPP) is required for dorsal-ventral axis formation (1); activins induce mesoderm and anterior structure formation (2, 3); Müllerian inhibiting substance (MIS) may be required for male sex development (4, 5); growth/differentiation factor-1 (GDF-1) has been implicated in nerve development and maintenance (6); other morphogenetic proteins (BMP-2, -3, -4 and OP-1) induce bone formation (7, 8, 9, 10).

In 1931 Huggins (11) demonstrated that the epithelium of the urinary tract was capable of inducing new bone formation, by surgical transplantation of urinary epithelium to parietal fascia and he termed this *epithelial osteogenesis.* In 1965 Urist demonstrated *endochondral bone formation* which resulted from implantation of demineralized bone (12). The latter observation suggested the existence of an osteogenic protein and for decades thereafter, bovine diaphyseal bone became the source for enriched preparations of osteogenic protein (10, 12, 13, 14). Recently, the human OP-1 cDNA and its partial genomic locus were cloned from genomic and various cDNA libraries using a consensus probe (15). Human recombinant OP-1, expressed in mammalian cells, was shown to induce new bone

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formation in vivo (16). Like other members of the TGF-ß superfamily OP-1 is produced as a precursor, glycosylated and secreted as a dimer. Mature OP-1 is cleaved at a maturation site following a sequence with the pattern of RXXR (Fig. 1) (17, 18).

The TGF-ß superfamily includes subfamilies of highly related genes. Previously, we described a consensus probe for the 7-cysteine domain which we used for the cloning of OP-1, BMP-2, BMP-3 and BMP-4 (15). Because these and other related genes (19, 20) cross hybridize with one another due to their conserved 7-cysteine domains, the OP-1 specific probes were carefully selected for the present study of OP-1 mRNA expression.

MATERIALS AND METHODS

Library screening: The murine OP-1 gene was isolated using human OP-1 cDNA as probe, labeled with ^{32}P (21). A 17.5 day mouse embryo library (Clontech #ML1029a, Palo Alto, CA) and a mouse teratocarcinoma PCC4 library (Stratagene #936301, La Jolla, CA) were screened in order to obtain the complete sequence of murine OP-1. Since both libraries yielded only partial-length clones, the missing portion of the OP-1 gene was cloned from a mouse genomic library (Clontech #ML1030j). cDNA clones were sequenced using the method of Sanger (22).

Preparation of RNA and Northern blot analysis: Mice, strain CD-1, were obtained from Charles River Laboratories, Kingston, MI. Total RNA from mouse embryos and post-natal animals was prepared using the acid guanidine thiocyanate-phenol-chloroform method (23). The RNA was dissolved in TES buffer (10 mM Tris-HCl, 1 mM EDTA, 0.1% SDS, pH 7.5) and treated with Proteinase K (approx. 1.5 mg per g tissue sample) at 45°C for 1 hr. Poly(A)$^+$ RNA selection on oligo(dT)-cellulose (Type 7, Pharmacia LKB Biotechnology Inc., Piscataway, NJ) was done in a batch procedure by mixing 0.1 g oligo(dT)-cellulose with 11 ml RNA solution (from 1 g tissue) in TES buffer and 0.5 M NaCl). Thereafter the oligo(dT)-cellulose was washed in binding buffer (0.5 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5), and with 0.3 M buffer (0.3 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and poly(A)$^+$ RNA was eluted by water. Poly(A)$^+$ RNA (5 or 15 µg/lane) was fractionated on 1 or 1.2% agarose-formaldehyde gels (24). 1 µl of 400 µg/ml ethidium bromide was added to each sample prior to heat denaturation (25). Following electrophoresis, the gels were photographed and the RNA was blotted overnight onto Nytran membranes (Schleicher & Schuell Inc., Keene, NH) with 10 x SSC. The membranes were baked at 80°C for 30-60 min. and irradiated with UV light (1 mW/cm^2 for 25 sec.). The Northern hybridization conditions were as previously described (15). For re-use, the filters were deprobed in 1 mM Tris-HCl, 1 mM EDTA, 0.1% SDS, pH 7.5, at 90-95°C and exposed to film to assure complete removal of previous hybridization signals.

RESULTS AND DISCUSSION

Isolation of the mouse OP-1 homolog We used the cloned human OP-1 cDNA as probe to screen murine cDNA libraries. Two cDNA libraries, 17.5 day mouse embryo and PCC4 mouse teratoma, yielded partial-length cDNA clones of murine OP-1, the longest one lacking 165 nucleotides from the coding region. A mouse genomic library provided a 0.46

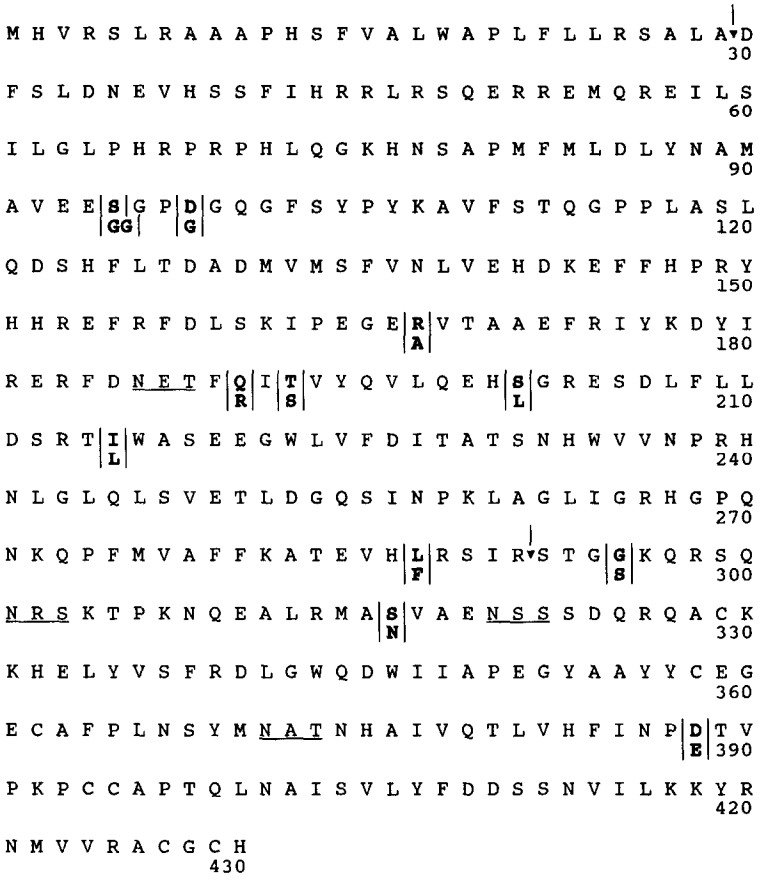

FIG. 1. Amino acid sequence of murine pre-pro OP-1. The variant amino acids of human OP-1 are shown below the murine sequence. The vertical arrow between positions 29 and 30 marks the predicted signal peptidase cleavage site and the arrow between positions 291 and 292 indicates the deduced cleavage site required for murine OP-1 maturation (the cleavage site for human OP-1 maturation has been determined by N-terminal sequence analysis of the recombinantly expressed protein, data not shown). The underlined amino acids 186-188, 301-303, 320-322 and 371-373 indicate the four potential N-glycosylation sites.

kb genomic PstI fragment encoding the start codon and first 120 amino acids. Alignment with human OP-1 showed that it included 103 untranslated nucleotides upstream of the first ATG, overlapping the longest cDNA clone (mouse embryo library) by 202 nucleotides.

Comparison of murine and human OP-1 amino acid sequences Figure 1 shows the amino acid sequence of murine pre-pro OP-1, as predicted from the combined genomic and cDNA information. Variant amino acid residues of human OP-1 are shown for comparison. The mature region of mouse OP-1, downstream of the RXXR maturation site, differs from human OP-1 in 3 out of 139 amino acids (98% identity). In the 7-cysteine domain, excluding region between the maturation site and the first cysteine, only one amino acid difference is

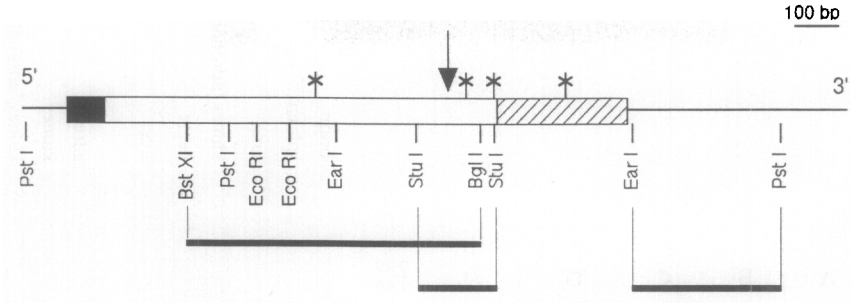

FIG. 2. Line diagram of the 1.9 kb OP-1 DNA. The solid box indicates the putative signal peptide and the hatched box corresponds to the TGF-ß-like domain that contains the seven cysteine residues. Asterisks indicate the potential N-glycosylation sites. The arrow marks the location of the cleavage site for OP-1 maturation. Three solid bars below the diagram indicate the OP-1 specific fragments used in making ^{32}P-labeled probes (0.68 kb BstXI - BglI fragment, 0.20 kb StuI - StuI fragment and 0.34 kb EarI - PstI non-coding fragment).

observed. In the pro-region, in an area of increased variability among related morphogenetic proteins, mouse OP-1 lacks one amino acid.

OP-1 specific probes for Northern hybridizations OP-1 mRNA expression was analysed in 17 day mouse embryos and 3 day post-natal mice by sequentially hybridizing filters with various probes. Probes from regions other than the highly conserved 7-cysteine domain of OP-1 were used, to avoid cross-hybridization of closely related genes (e.g. Vgr-1 message); for example, a probe from the region immediately upstream of the 7-cysteine domain was selected because it is highly variable among members of the TGF-ß superfamily. Figure 2 shows the fragments of OP-1, used as probes in the Northern hybridizations.

Multiple OP-1 mRNA transcripts Hybridization with a probe that covers approximately two thirds of the pro-region (the 0.68 kb BstXI-BglI fragment), reveals a 4 kb message and 3 messages at 1.8 kb, 2.2 kb and 2.4 kb (Fig. 3 B and D, and Fig. 4). Although the size of the Vgr-1 specific message is close to the 4 kb OP-1 species (Fig. 3, Panel C) the OP-1 specific 4 kb mRNA is somewhat larger. To further rule out cross-hybridization with a non-OP-1 message, we probed with the 0.2 kb StuI-StuI fragment which represents the gene specific sequences immediately upstream of the 7-cysteine domain (see above). This probe gave a hybridization pattern similar to the one shown in Fig. 3, Panel B (data not shown). A third probe, the 0.34 kb EarI-PstI fragment containing 3' untranslated sequences, also confirmed the pattern (Fig. 3, Panel D). Thus, we have observed four OP-1 specific messages with three distinct probes.

The 4 kb OP-1 message The appearance of a new 4 kb OP-1 mRNA species was initially interpreted as cross hybridization of OP-1 probe with Vgr-1 mRNA, which is approximately this size (Fig. 3, Panel C, and ref. 19). However, the 4 kb message was detected with three

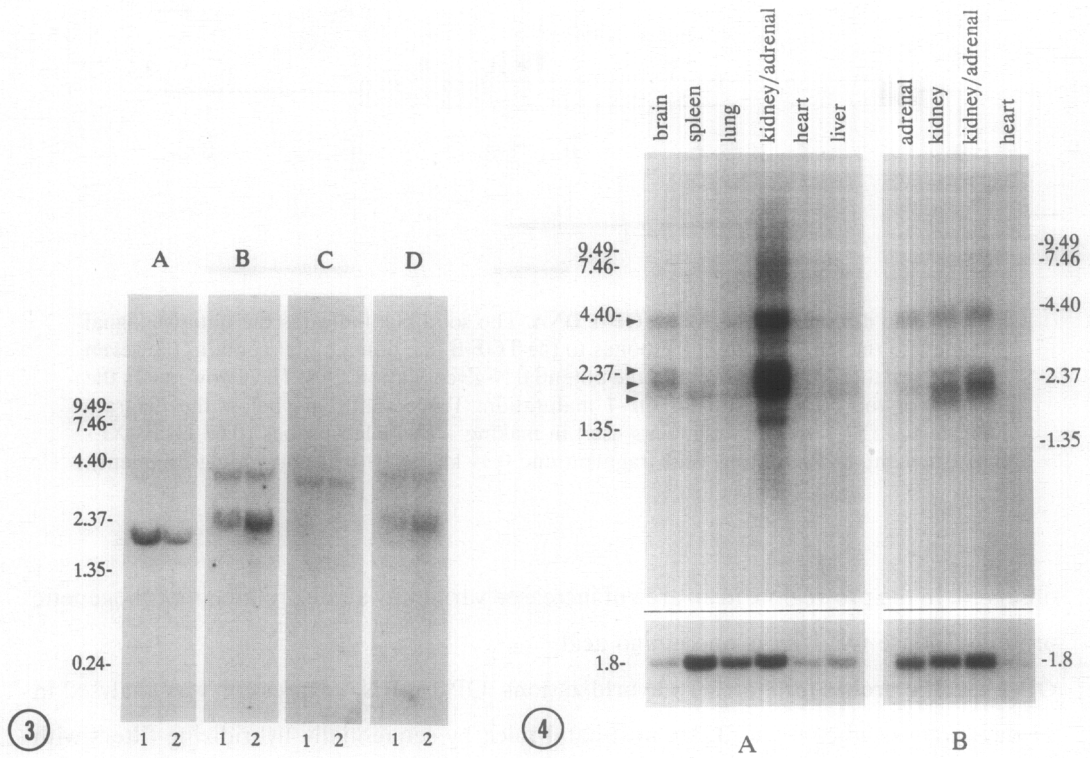

FIG. 3. The expression of OP-1, Vgr-1 and EF-Tu (a control) mRNA in 17 day embryos and 3 day post-natal mice. Equal amounts (15 μg) of poly(A)$^+$ RNA were loaded onto each lane, electrophoresed on a 1% agarose-formaldehyde gel, blotted and hybridized. 0.24 - 9.49 kb RNA ladder (BRL Inc.) was used as size standard. The same filter was used for sequential hybridizations with labeled DNA probes specific for OP-1 (Panels B and D), Vgr-1 (Panel C), and EF-Tu (Panel A). **Panel A:** the EF-Tu specific probe (a control) was the 0.4 kb HindIII-SacI fragment (part of the coding region), the SacI site used belonged to the vector; **Panel B:** the OP-1 specific probe was the 0.68 kb BstXI-BglI fragment (two thirds of the pro region and upstream sequences of the mature domain, not including any sequences from the 7-cysteine domain); **Panel C:** the Vgr-1 specific probe was the 0.26 kb PvuII-SacI fragment (part of the pro-region and upstream sequences of the mature domain, including the first cysteine). **Panel D:** the OP-1 (3' flanking) specific probe was the 0.34 kb EarI-PstI fragment (3' untranslated sequences immediately next to the gene).

FIG. 4. Expression of OP-1 mRNA in different mouse tissues. The tissues to be used for RNA preparation were obtained from two week old mice (Panel A) or 5 week old mice (Panel B), with the exception of poly(A)$^+$ RNA which was obtained from kidney/adrenal gland of two week old mice. Equal amounts of poly(A)$^+$ RNA (15 μg for Panel A and 5 μg for Panel B) were loaded into each well. After electrophoresis (1.2% agarose-formaldehyde gels), RNA was hybridized to the OP-1 specific 3' flanking probe described in the legend of Fig. 3 (Panel D). The 0.24-9.5 kb RNA ladder was used as size standard. The arrowheads indicate the OP-1 specific messages. The lower section of Panels A and B show the hybridization pattern obtained with the EF-Tu specific probe (a control).

different OP-1 specific probes, including one specific to the 3' untranslated region, and moreover it was separated from Vgr-1 message on the basis of size. Most likely, therefore, the 4 kb mRNA (and the three species of 1.8 kb, 2.2 kb and 2.4 kb) results from alternative

splicing of OP-1 transcripts. Recently, Lee (6) reported that the mRNA for murine and human GDF-1, 3 kb in size, is bicistronic and contains an open reading frame of about 1000 nucleotides located upstream from the GDF-1 coding sequences. (Also, a shorter GDF-1 specific mRNA species was detected at an early developmental stage, which may represent an alternatively spliced message.) Hence, by analogy to GDF-1, one must consider the possibility that the 4 kb OP-1 mRNA could also represent a bicistronic mRNA. The 4 kb message is a minor species in kidney, yet it is more prominent in adrenal tissue (see below).

Tissue specific expression of OP-1 mRNA Previously we found human OP-1 cDNA in several different cDNA libraries (placenta, hippocampus, calvaria). Furthermore, OP-1 mRNA was also seen in the human osteosarcoma cell line U2-OS (15). To compare the level of OP-1 expression in different tissues, poly(A)$^+$ RNA was prepared from brain, spleen, lung, kidney and adrenal gland, heart, and liver of 13 day post-natal mice. The RNA was analyzed on Northern blots by hybridization to various OP-1 probes (Fig. 4). Equal amounts of mRNA, as judged by optical density, A_{260}, were fractionated on agarose-formaldehyde gels. Ethidium bromide staining of the gels revealed some residual ribosomal RNA in addition to the mRNA and provided another assurance against mRNA degradation and quantitative or qualitative variation. As control for mRNA recovery, we probed for EF-Tu (translational elongation factor) mRNA (assuming uniform expression of EF-Tu in most tissues). A great variation in the level of OP-1 expression was observed in spleen, lung, kidney and adrenal tissues whereas EF-Tu mRNA levels appeared relatively constant in these tissues (Fig. 4, Panel A). By far the highest level of OP-1 mRNA was found in the kidneys. Uniformly lower levels of EF-Tu mRNA were found in brain, heart and liver (Fig. 4, Panel A). Additional analysis of OP-1 mRNA showed the presence of significant amounts of OP-1 mRNA in the bladder (data not shown). In summary, next to kidneys, bladder and adrenal tissue, brain contained the highest levels of OP-1 mRNA, whereas heart and liver did not give a detectable signal. This conforms with our earlier finding of human OP-1 cDNA in a hippocampus cDNA library but not in a fetal liver cDNA library (15).

Tissue specific patterns of different OP-1 mRNA species The OP-1 mRNA patterns display qualitative changes in the various tissues. Of the four messages found in brain, the 2.2 kb message is most abundant whereas in lung and spleen the 1.8 kb message predominates. Interestingly, levels of the 1.8-2.4 kb OP-1 mRNA are approximately two times higher in 3 day post-natal mice than in 17 day embryos, perhaps reflecting phases in bone and/or kidney development (see below). mRNA was also prepared from carefully separated renal and adrenal tissues of 5 week old mice. Northern blot analysis (Figure 4, Panel B) revealed that the high levels of 2.2 kb mRNA were derived from renal tissue whereas the 4 kb mRNA was more prominent in adrenal tissue. Attempts to clone the 4 kb message are underway.

Epithelial osteogenesis and kidney as principal site of OP-1 expression The detection of of OP-1 message primarily in the kidneys but also in bladder links OP-1 expression specifically with the urinary tract. Interestingly, the related Vgr-1 is also expressed at significant levels in kidneys although its main site of expression is lung (19, 18). The finding of OP-1 mRNA in kidney and bladder is particularly exciting since Huggins, as early as 1931 (11, 26), demonstrated the osteogenic potential of urinary tract epithelium. As he restated later (27): *"The surgical approximation of bladder epithelium with fascias of the trunk or limb evoked large amounts of bone which was evident in 10-12 days"*. The osteogenic activity of our recombinant OP-1 (16) and the mRNA data presented here suggest that OP-1 may be the osteoinductive factor responsible for the phenomenon of epithelial osteogenesis. The question remains as to whether the synthesis of the osteogenic protein in the kidney relates to bone metabolism or suggests other biological roles for OP-1, such as maintenance of renal tissue. Perhaps OP-1 made in the kidneys exerts its biological effects in an endocrine manner. Finally, in view of the presence of OP-1 mRNA in the brain, it remains possible that OP-1 may also have roles unrelated to bone induction.

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